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# Microbiological approaches to reduce the sulphite addition in oenology

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# Approcci microbiologici per la riduzione dei solfiti in enologia

**Coordinatore :** Ch.mo Prof. Viviana Corich

**Supervisore** :Ch.mo Prof. Viviana Corich

**Dottoranda** : Chiara Nadai

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# ABSTRACT

Sulphite is widely used in winemaking for its antimicrobial and antioxidant properties, although its toxic effect on human health is proven. For this reason strategies for reducing chemical preservatives in winemaking is strongly demanded. Wine yeasts can cope with $SO_2$ by different systems, such as acetaldehyde and S-amino acids production or $SO_2$ export.

In this study a first screening of $SO_2$ resistance and on plate production of $SO_2$ and $H_2S$ have been performed for autochthonous strains, isolated in Veneto vineyards to be used as starter of fermentation in the production of Prosecco di Valdobbiadene DOCG and DOC Piave wines, compared to commercial strains.

Then the oenological characteristics of 11 *S. cerevisiae* strains of have been evaluated. These strains are 4 autochthonous strains isolated during local selection projects in DOCG Conegliano - Valdobbiadene and DOC Piave areas, together with 6 commercial strains, which genome have been recently sequenced, and relative informations are available in the principal genomic databases, and the laboratory strain S288c, the first one that has been sequenced. Main technology and quality characters have been evaluated to determine the suitability of strains for winemaking process. In particular have been studied the production of ethanol and glycerol, the glucose consumption at 2 and 7 days, the production of hydrogen sulphide, acetaldehyde and sulphur dioxide and the resistance to various concentrations of free sulphur dioxide in synthetic must. Sulphite response in yeast has been investigated in order to elucidate factors that affect sulphite production during vinification. Moreover acetaldehyde, another compound produced by yeast, linked with sulphite metabolism or detoxification, has been analysed since it affects wine quality.

Genetic characteristics identified after genome sequencing of 4 autochthonous strains (2 from Prosecco area and 2 from Raboso area), such as oenological SNPs, strain-specific genes and important translocations, have been analyzed in Real-time PCR for a large number of autochthonous strains.

Then the behaviour towards sulphite of 4 wine yeasts has been investigated, and transcriptome analysis during fermentation has been performed by means of next generation sequencing. For all strains, fermentation rate was monitored together with sulphite production in synthetic must supplemented with different doses of $SO_2$ (0 mg/l and 25 mg/l).

Finally, a selection of reference genes for Real-time PCR has been made, and a set of genes suitable for such conditions has been identified.

Results point out the importance of verifying strain attitudes towards sulphite at different sulphite concentrations. This study tries to clarify the complex regulative mechanisms of sulphites during fermentation, thus giving new guidelines for critic control of these fermentation parameters in order to maximize effect of sulphite added thus limiting the dose employed during vinification.

# RIASSUNTO

I solfiti sono ampiamente utilizzati nella vinificazione per le loro proprietà antimicrobiche e antiossidanti, sebbene il loro effetto tossico sulla salute umana sia dimostrato. Per questo motivo le strategie di riduzione dei conservanti chimici nel processo di vinificazione è fortemente richiesto. I lieviti possono rispondere alla presenza di $SO_2$ con sistemi diversi, come la produzione di acetaldeide e ammino acidi solforati o l'esporto di $SO_2$.

In questo studio è stato fatto un primo screening sulla resistenza alla $SO_2$ e sulla produzione di $SO_2$ e $H_2S$ in piastra da parte di ceppi autoctoni, isolati nei vigneti del Veneto per essere utilizzato come starter di fermentazione nella produzione di Prosecco di Valdobbiadene DOCG e Vini del Piave DOC, confrontati con dei ceppi commerciali.

Inoltre sono state valutate le caratteristiche enologiche di 11 ceppi di *S. cerevisiae*. I ceppi in esame sono 4 ceppi autoctoni isolati durante i progetti di selezione locale nelle aree Conegliano - Valdobbiadene DOCG e Piave DOC, insieme con 6 ceppi commerciali, il cui genoma è stato recentemente sequenziato, e le informazioni relative sono disponibili nelle banche dati genomiche principali, e il ceppo di laboratorio S288c , il primo che è stato sequenziato. I principali caratteri tecnologici e di qualità sono stati valutati per determinare l'idoneità dei ceppi alla vinificazione. In particolare, sono state studiate la produzione di etanolo e glicerolo, il consumo di glucosio a 2 e 7 giorni, la produzione di idrogeno solforato, acetaldeide e biossido di zolfo e la resistenza a varie concentrazioni di biossido di zolfo libero in mosto sintetico. La risposta ai solfiti nel lievito è stata studiata al fine di chiarire i fattori che influenzano la produzione dei solfiti durante la vinificazione. Inoltre l'acetaldeide, un altro composto prodotto da lievito, collegato con il metabolismo solfito o disintossicazione, è stata analizzata in quanto influisce sulla qualità del vino.

Con il sequenziamento del genoma di 4 ceppi autoctoni (2 da zona del Prosecco e 2 dalla zona Raboso) è stato possibile individuare delle caratteristiche genetiche, come ad esempio SNPs enologiche, geni ceppo-specifici e traslocazioni importanti, che sono stati analizzati in Real-time PCR per un gran numero di ceppi autoctoni .

Inoltre il comportamento di 4 lieviti enologici nei confronti dei solfiti è stato studiato, ed è stata effettuata l'analisi del trascrittoma durante la fermentazione per mezzo di next generation sequencing. Per tutti i ceppi la velocità di

fermentazione è stata monitorata, insieme alla produzione di $SO_2$, in mosto sintetico con differenti dosi di $SO_2$ (0 mg / l e 25 mg / l).
Infine, è stata fatta una selezione di geni reference da usare in Real-time PCR, e una serie di geni adatti a queste condizioni è stato identificato.
I risultati sottolineano l'importanza di verificare l'atteggiamento del ceppo nei confronti dei solfiti a diverse concentrazioni di $SO_2$. Questo studio cerca di chiarire i complessi meccanismi di regolazione dei solfiti durante la fermentazione, dando così nuove linee guida per il controllo critico di questi parametri di fermentazione, al fine di massimizzare l'effetto dei solfiti aggiunto limitando in tal modo la dose impiegata durante la vinificazione.

# 1. Introduction

Sulphur dioxide has been used as a preservative at least since the nineteenth century in winemaking. It is arduous to determine precisely when sulphur dioxide was employed for the first time. Pasteur (1866) mentions the use of other kinds of preservatives in ancient times, as described by the Greek and Roman Geoponic authors who recommended the use of pitch, herbs and resin. Pasteur (1866) himself suggested heating as an appropriate means to prevent microbial growth as well as burning sulphur in barrels to prevent spoilage. Just a few years later, Ladrey (1871) prescribed the burning of sulphur in barrels and described its transformation into sulphur dioxide in wine and its role in stopping alcoholic fermentation in an operation called “mutage”. This French word was coined from the observation that upon dissolution of sulphurous gas in a fermenting must, the wine becomes still or mute. Since then, the antiseptic properties of sulphur dioxide have been clearly demonstrated and the concentrations used today for wine production are strictly defined by the International Organisation of Vine and Wine and the respective national regulation of the producing countries. However, if the antiseptic activity of sulphur dioxide has been observed two centuries ago, its impact on the yeast cell has only been described over the past 40 years. Moreover, the cellular and molecular mechanisms of resistance to sulphur dioxide were only initially investigated around the turn of the millennium and mostly in Saccharomyces cerevisiae and every new published study reveals a further level of complexity and strain dependency of sulphite detoxification. Additionally, the different ways of cellular detoxification have most often been regarded as individual mechanisms independent from each other. However, systems biology techniques now allow researchers to assess the effectiveness of these mechanisms in a more holistic fashion (Divol et al. 2012).

## 1.1 Sulphur dioxide properties

Its many properties make $SO_2$ an indispensable aid in winemaking. Perhaps some wines could be made in total or near-total absence of $SO_2$ but it would certainly be presumptuous to claim that all of the wines produced in the various wineries throughout the world could be made in this manner. It must also be taken into account that yeasts produce small quantities of $SO_2$ during fermentation. In general, the amount formed is rarely more than 10 mg/l, but in

certain cases it can exceed 30 mg/l. Consequently, the total absence of sulphur dioxide in wine is rare, even in the absence of sulphating (Ribèreau-Gayon et al. 2006).

Its principal properties are as follows:

1. Antiseptic: it inhibits the development of microorganisms. It has a greater activity on bacteria than on yeasts. At low concentrations, the inhibition is transitory. High concentrations destroy a percentage of the microbial population. The effectiveness of a given concentration is increased by lowering the initial population, by filtration for example. During storage, $SO_2$ hinders the development of all types of microorganisms (yeasts, lactic bacteria, and, to a lesser extent, acetic bacteria), preventing yeast haze formation, secondary fermentation of sweet white wines, Brettanomyces contamination and the subsequent formation of ethyl-phenols, the development of mycodermic yeast (flor), and various types of bacteria spoilage.

2. Antioxidant: in the presence of catalyzers, it binds with dissolved oxygen according to the following reaction:

$$SO_2 + 1/2\ O_2 \rightarrow SO_3$$

This reaction is slow. It protects wines from chemical oxidations, but it has no effect on enzymatic oxidations, which are very quick. $SO_2$ protects wine from an excessively intense oxidation of its phenolic compounds and certain elements of its aroma. It prevents madeirization. It also contributes to the establishment of a sufficiently low oxidation–reduction potential, favoring wine aroma and taste development during storage and aging.

3. Antioxidasic: it instantaneously inhibits the functioning of oxidation enzymes (tyrosinase, laccase) and can ensure their destruction over time. Before fermentation, $SO_2$ protects musts from oxidation by this mechanism. It also helps to avoid oxidasic casse in white and red wines made from rotten grapes.

4. Binding ethanal and other similar products, it protects wine aromas and makes the flat character disappear. Adding $SO_2$ to wine raises a number of issues. Excessive doses must be avoided, above all for health reasons, but also because of their impact on aroma. High doses neutralize aroma, while even larger amounts produce characteristic aroma defects, e.g. a smell of wet wool that rapidly becomes suffocating and irritating, together with a burning sensation on the aftertaste. However, an insufficient concentration does not ensure the total stability of the wine. Excessive oxidation or microbial development can compromise its presentation and quality. It is not easy to calculate the precise

quantities required, because of the complex chemical equilibrium of this molecule in wine. It exists in different forms that possess different properties in media of different composition.

Today, wines are subject to EU legislation, which has gradually reduced the permitted level to 160 mg/l for most red wines and 210 mg/l for the majority of white wines. Higher doses may only be used in wines with very high sugar content. For white wines (excluding special wines) the average concentration is 105 mg/l; for red wines it is 75 mg/l.

Today, especially for health reasons, the possibility of further reducing the authorized concentrations in different kinds of wines is sought after. Such an approach consists of optimizing the conditions and perfecting the methods of using this product. This supposes more in-depth knowledge of the chemical properties of the sulphur dioxide molecule and its oenological role. Substitute products can also be considered. Due to the various effects of sulphur dioxide in wine, the existence of another substance performing the same roles without the disadvantages seems very unlikely, but, the existence of adjuvants, complementing the effect of $SO_2$ in some of its properties, is perfectly conceivable. Oenological research has always been preoccupied by the quest for such a product or substitution process. In conclusion, sulphur dioxide permits the storage of many types of wine known, today that would not exist without its protection. In particular, it permits extended barrel maturation and bottle aging. In view of its involvement in a wide variety of chemical reactions, it is not easy to determine the optimum dose to obtain all the benefits of $SO_2$ without any of its unfortunate side-effects. The adjustment should be made within plus or minus 10 mg/l (Ribèreau-Gayon et al. 2006).

### 1.2 Cellular uptake of $SO_2$

Once sulphur dioxide is added to wine or any aqueous solution, in any of the commonly used forms, it dissociates into three molecular species namely molecular $SO_2$ ($SO_2 \cdot H_2O$), bisulphite ($HSO_3^-$) and sulphite ($SO_3^{2-}$) as illustrated:

$$SO_2 + H_2O \leftrightarrow SO_2 \cdot H_2O$$

$$SO_2 \cdot H_2O \leftrightarrow HSO3^- + H^+$$

$$HSO3^- \leftrightarrow SO_3^{2-} + H^+$$

The chemical equilibrium between each species is dependent on the pH of the wine.

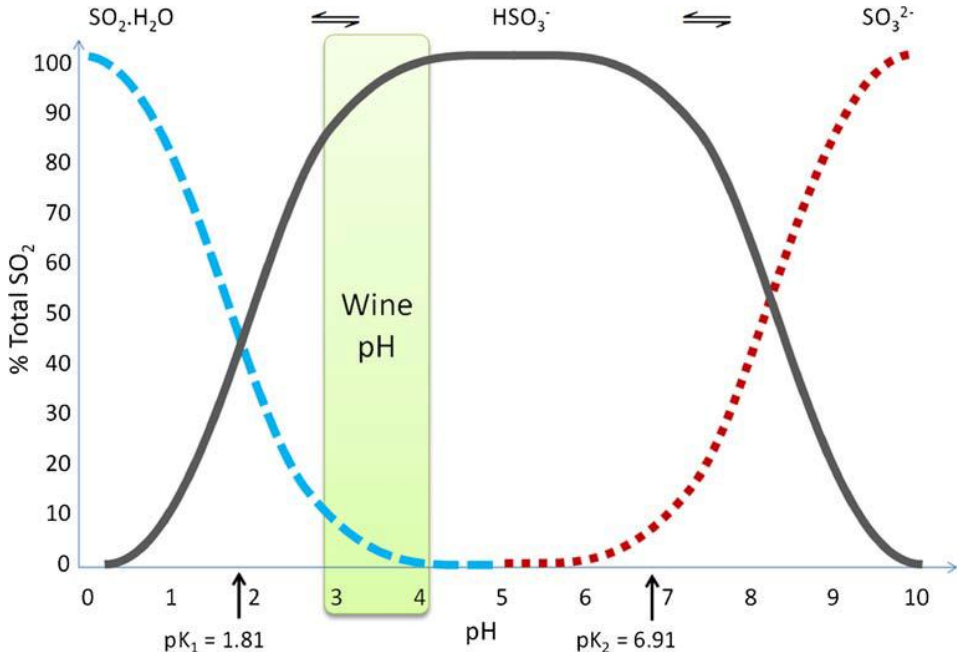


**Figure 1.1** $SO_2$ species in aqueous solution and their representation in percentage of total $SO_2$ throughout the pH range. $pK_1$ and $pK_2$ are the dissociation constants. The effective pH range of wine is highlighted.

As seen in Fig. 1.1, molecular $SO_2$ is most prevalent from pH 0 to 2 ($pK_1$=1,81), the bisulphite anion from pH 2 to 7 ($pK_2$=6,91) and sulphite from pH 7 to 10. In general, the pH of wines varies between 3 and 4, and therefore the dominant $SO_2$ species in wine is the bisulphite anion $HSO_3^-$.

$SO_2$ is a strong reducing agent and antioxidant. When sufficient $SO_2$ is added to the wine, it can prevent the oxidation of compounds such as anthocyanins in red wines and reduces the effects of browning in white wines caused by oxidative enzymes. However when added to wine, excess $SO_2$ can cause bleaching of anthocyanins resulting in a loss of colour in red wine (Bakker et al. 1998).

$SO_2$ behaviour in wine is however not as simple as depicted in Fig. 1.1. $SO_2$ interacts with various compounds which annul its properties. $HSO_3^-$ and $SO_3^{2-}$ are indeed highly reactive and can bind many of the compounds present in wine. It is therefore said that $SO_2$ exists in “free” and “bound” forms. The “free” species is the portion of $HSO_3^-$ and $SO_3^{2-}$ which is not already bound to compounds such as acetaldehyde, anthocyanins and organic acids present in the wine (Burroughs 1975). The concentration of free $SO_2$ present in wine is critical as it is the only form of $SO_2$ which is available to bind the compounds which would otherwise oxidise important flavour and colour compounds in the wine.

Free $SO_2$ was shown to be the form inhibiting the growth of microorganisms in general (Ripper 1892) and of yeast in particular (Ingram 1948). A study on the impact of pH on the antimicrobial activity of $SO_2$ demonstrated that $SO_2$ was inactive at neutral pH (Rahn and Conn 1944). Furthermore, it was also shown that molecular $SO_2$ was 100 to 500 times more active than bisulphite ion (Rehm and Wittmann 1962; Rehm andWittmann 1963). In fact, molecular $SO_2$ as part of the free $SO_2$ form was later shown to have antiseptic properties (Macris and Markakis 1974). The latter authors studied the uptake of $SO_2$ into the yeast cell. They showed that $SO_2$ uptake depends on the external $SO_2$ concentration and follows a kinetics similar to that of a Lineweaver–Burk enzymatic reaction. They also noticed an important effect of temperature (with an optimum at 50°C) and pH (acidic pHs facilitate $SO_2$ uptake). According to them, it is the molecular form that enters into the cell. As molecular $SO_2$ has no charge, it passes easily through microbial cell membranes by simple diffusion (Stratford and Rose 1986). Once inside the cell, approximate intracellular pH 5,5–6,5 (Imai and Ohno 1995), the molecule rapidly dissociates into bisulphite and sulphite anions. This decreases the intracellular molecular $SO_2$ concentration allowing more molecular $SO_2$ to enter the cell by diffusion. From these observations, it was concluded that $SO_2$ uptake is a passive phenomenon (facilitated diffusion through the cell membrane) (Stratford and Rose 1986) or an active transport (Macris and Markakis 1974; Pilkington and Rose 1988). However more recently, it was clearly demonstrated that the uptake of the bisulphite anion occurs via a carrier-mediated proton symport when sulphite is provided as the sole source of sulphur (Park and Bakalinsky 2004). It was found that the sulphate transporters Sul1p and Sul2p were not required for sulphite transport. Moreover, this transport is ATP-dependent in order to allow for the restoration of the intracellular pH. $SO_2$ uptake was found to be linear within the first 50 s and saturable thereafter when the external $SO_2$ concentration reaches 3 mM.

As molecular $SO_2$ is the active antiseptic species of $SO_2$ in wine, it is essential for winemakers to know its concentration. Unfortunately, only the free- and total-$SO_2$ concentrations can be determined but that of molecular $SO_2$ can be calculated using the Henderson–Hasselbalch equation. If one disregards the extremely low concentration of $SO_3^{2-}$ present at wine pH, the molecular $SO_2$ concentration in an aqueous solution buffered at wine pH is:

$$[\text{molecular } SO_2] = [HSO_3^-] / 10^{pH-pK1}$$

However, as explained above, $SO_2$ reacts with a number of chemical compounds in wine and molecular $SO_2$ can only be considered as a small fraction of free-$SO_2$ and not of total $SO_2$. The equation therefore becomes:

$$[\text{molecular } SO_2] = [\text{free } SO_2] / 1+10^{pH-pK1}$$

Even considering the limited variability of the pH range in wine, the concentration of molecular $SO_2$ varies greatly.

### 1.3 Toxic impact of SO2 on the yeast cell

Regardless of the mode of $SO_2$ transport, once inside the cell, bisulphite is the dominant species of $SO_2$ present, because of the intracellular pH. It therefore becomes the main antimicrobial reactive species.

The impact of sulphur dioxide on the wine yeast cell has been studied for several decades. It has mostly been studied in *S. cerevisiae*. The first studies demonstrated that sulphur dioxide had an impact on cell growth, sporulation and recovery after exposure (Baldwin 1951). It was also shown early on that the resistance of yeast cells was not the same at different growth phases. Cells were indeed shown to be more resistant in the exponential phase compared with late stationary phase (Ventre 1934). It has been shown that yeast cultures exposed to $SO_2$ can only tolerate it for a short period before being irreversibly damaged (Schimz 1980). This period of tolerance increases when the cells reach the late stationary phase. Moreover, high temperatures enhance $SO_2$ antimicrobial activity. Death subsequent to $SO_2$ exposure was shown to have three main causes (Anacleto and van Uden 1982): (a) damage to the membrane because of $SO_2$ binding to specific receptors, (b) leakage of intracellular metabolites and (c) drop in intracellular ATP concentration due to the hydrolysis of ATP by a membrane-bound ATPase (Freese et al. 1973; Schimz and Holzer 1979; Schimz 1980; Prakash et al. 1986).

$SO_2$ indeed inhibits microbial growth by interfering with intracellular processes. $SO_2$ is a highly reactive molecule and it binds to many metabolites and enzymes in the cell. The influx of $SO_2$ into an eukaryotic cell results in the immediate inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a critical enzyme in the glycolysis pathway (Hinze and Holzer 1986). This enzyme is responsible for the conversion of glyceraldehyde 3-phosphate to D-glycerate-1,3-bisphosphate in a two-step catalysis. The inhibition of GAPDH results in the

subsequent stalling of glycolysis and the reduction of ATP produced as well as NADH regeneration.

As reported by Maier et al. (1986), removal of sulphite from the cell by the addition of acetaldehyde to the medium resulted in ATP returning to 70% of its original level. Due to sulphites high affinity for acetaldehyde, the formation of ethanol is inhibited and the subsequent NADH regeneration does not occur. Other enzymes such as ATPase, alcohol dehydrogenase and $NAD^+$-glutamate dehydrogenase are inhibited by sulphite but it is not known if the inhibition is reversible (Maier et al. 1986). The expression of *ALD6* (encoding an acetaldehyde dehydrogenase) has also been shown to be altered by the presence of sulphite (Aranda et al. 2006). The latter authors showed in the same study that the expression of *MET16* (encoding a 3′-phosphoadenylylsulfate (PAPS) reductase, an enzyme involved in the sulphate metabolism pathway) is also strongly reduced by $SO_2$.

Besides the inhibition of key metabolic enzymes, sulphite also binds to proteins (destroying the disulphide bridge), coenzymes ($NAD^+$ and $FAD^+$) and co-factors such as the vitamins thiamine and menadione (Carmack et al. 1950). It has also been shown that sulphite can cause DNA point mutations by changing A/T to C/G (Mukai et al. 1970; Pagano and Zeiger 1987; Pagano et al. 1990; Meng and Zhang 1992).

$SO_2$ can also bind various metabolites: glucose, dihydroxyacetone-phosphate, pyruvate, acetaldehyde, oxaloacetic acid and α-ketoglutaric acid (Rankine and Pocock 1969), thereby preventing their further use as substrates for metabolic pathways.

As stated previously, sulphite has a major negative effect on the energy metabolism in eukaryotes. The increased stress placed on the cell could ultimately lead to cell death. In order to prevent this fatal outcome, yeasts have developed an array of defence mechanisms that will be described in the following sections.

### 1.4 Resistance to $SO_2$ in wine yeasts

Resistance to $SO_2$ was shown early to be a polygenic trait. It was indeed demonstrated by selective hybridisation and observation of sulphur resistance over a few generations that sulphur resistance was an inheritable and dominant trait as well as under the control of multiple genes (Thornton 1982). It was later

confirmed that $SO_2$ resistance is genetically inherited, even in the absence of $SO_2$ (Beech and Thomas 1985). In 1994, further genetic analysis led to the conclusion that one gene was responsible for $SO_2$ resistance and four genes for $SO_2$ sensitivity (Xu et al. 1994). The dominance of resistance trait was confirmed by spore hybridisation experiments (Casalone et al. 1992). A possible induction of $SO_2$ resistance was nevertheless envisaged by some authors (Romano and Tini 1975).

Resistance to sulphur dioxide is often accompanied by resistance to other fungicides such as sorbic acid and benzoic acid (Steels et al. 2000). It was hypothesised that yeast cell possessed an energy-dependent membrane-bound pump able to efflux these fungicides outside of the cell (Warth 1977).

Various assays have been optimised in the 1980s to test for $SO_2$ resistance/tolerance, from plate assays (Pilkington and Rose 1988) to liquid assays (Uzuka et al. 1985). Nevertheless, the literature remains poor in data related to the resistance of different yeast species, but yeasts tolerance to $SO_2$ is usually described as highly variable. It varies not only between species but also between strains. *Zygosaccharomyces bailii* has been described as a highly tolerant species (Warth 1977; Thomas and Davenport 1985; Warth 1985; Pilkington and Rose 1988; Divol et al. 2006), as well as *Schizosaccharomyces pombe* (Stratford et al. 1987). On the contrary, *Kloeckera apiculata* and *Hansenula anomala* were shown to be highly sensitive to $SO_2$ (Warth 1985). Strains of *S. cerevisiae* have been shown to be fairly tolerant to $SO_2$ in general when compared with other yeast species but display highly diverse $SO_2$ tolerance (Divol et al. 2006; Nardi et al. 2010). It was reported that in a spontaneous wine fermentation, 50 mg/l $SO_2$ in general is sufficient to inhibit most of the non-*Saccharomyces* yeasts found in grape juice except *Candida* spp. and selected *S. cerevisiae* while the addition of 20 mg/l inhibited only some of the non-*Saccharomyces* yeasts (Henick-Kling et al. 1998).

### 1.5 Cellular and molecular response of yeast cells exposed to sulphur dioxide

*S. cerevisiae* has been used as a model organism to determine the yeast response to $SO_2$ (Park and Bakalinsky 2000). Bacteria, yeast and mammalian cells have been shown to have four cellular responses to the presence of $SO_2$ in its environment: (1) sulphur reduction (Yoshimoto and Sato 1968; Kobayashi and Yoshimoto 1982), (2) sulphur oxidation (Heimberg et al. 1953; Beck-Speier et al.

1985; Kappler and Dahl 2001; Friedrich et al. 2005; Feng et al. 2007; Hellborg and Piskur 2009), (3) acetaldehyde production (Stratford et al. 1987) and (4) glutathione sulphitolysis (Mannervik et al. 1974; Kåtgedal et al. 1986), as well as one molecular response, the active efflux of $SO_2$ by the sulphite transporter Ssu1p (Park and Bakalinsky 2000). Figure 1.2 summarises the abovementioned responses to $SO_2$.

### 1.5.1 Reduction of sulphur dioxide

Sulphur is a crucial element in yeasts as it is used in the synthesis of sulphur-containing amino acids such as methionine, S-adenosylmethionine and cysteine (Thomas and Surdin-Kerjan 1997). The sulphur amino acid biosynthesis (SAAB) pathway plays a crucial role in the active transport of sulphate ($SO_4^{2-}$) into the cell by the membrane bound transporter protein Sul1p/ Sul2p. The genes *MET3*, *MET14*, *MET16* and *MET5/ MET10* code for the catalytic enzymes ATP sulphurylase, adenylyl-sulphate kinase, PAPS reductase and the two subunits of sulphite reductase respectively. The Met3p and Met14p each requires one ATP, Met16p one NADPH and Met5p/Met10p complex three NADPH molecules for the catalysis of $SO_4^{2-}$ to $S^{2-}$ as illustrated in Fig.1.2.

The available sulphide ($S^{2-}$) can be used in the synthesis of sulphur containing amino acids adenosine, methionine and cysteine as well as being excreted as $H_2S$. As seen in Fig. 1.2, $HSO_3^-$ is an intermediary in the SAAB and can be viewed as a potential sink for excess $HSO_3^-$ which has entered the cell.

Moreover, the concentration of these amino acids downregulates the SAAB pathway (Aranda et al. 2006). The concentration of methionine in particular seems to play an important role in the activation of the SAAB pathway but also in the resistance to $SO_2$. The latter authors indeed demonstrated that a higher concentration of methionine diminishes resistance to $SO_2$. They showed that in a strain very sensitive to $SO_2$, an irregular sulphur metabolism occurred. This demonstrated the important role of reduction in $SO_2$ detoxification. More surprisingly, the concentration of adenine enhances resistance to $SO_2$. Aranda et al. (2006) showed that the presence of sulphur or methionine alters negatively the expression of *ADE4*, a gene encoding an enzyme involved in the metabolism of adenine. The authors suggested that an unknown protein or metabolite somehow linked to the purine synthetic pathway was involved in sulphite

detoxification, as adenine nucleotides are necessary for sulphate metabolism (Thomas and Surdin-Kerjan 1997).

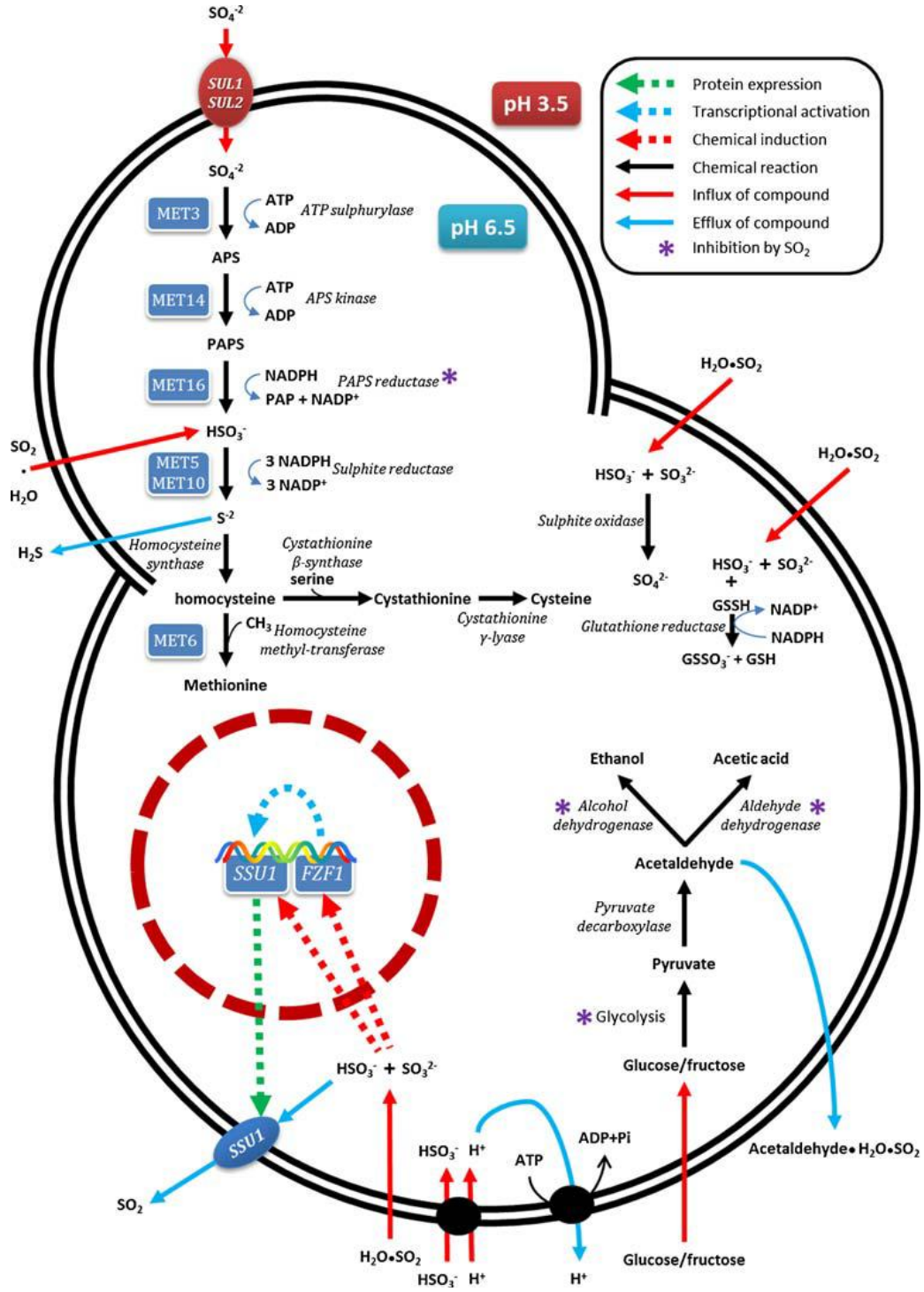


**Figure 1.2** A summary of the sulphate assimilation pathway and the cellular and molecular responses of *S.cerevisiae* to the presence of $SO_2$. (SAAB sulphur amino acid biosynthesis, SR sulphur reduction)

### 1.5.2 Oxidation

The oxidation of $SO_2$ in eukaryotes in general is poorly researched but it has been extensively researched in bacteria, especially thiobacilli (Charles and Suzuki 1966; Suzuki and Silver 1966; Silver and Lundgren 1968). There is no known sulphite oxidase in *S. cerevisiae* (Beck-Speier et al. 1985; Xu et al. 1994) but an enzymatic complex from *Rhodoturula*, which possesses thiosulfate as well as sulphur oxidising activities, was characterized (Kurek 1985). Sulphite oxidation was also inferred from mammalian liver extracts (Heimberg et al. 1953). In fact, the presence of sulphite oxidase seems to be more common in higher eukaryotes than in yeast. In plants, sulphite oxidase activity could be used as a means to detoxify cells from sulphites and prevent sulphitolysis (Hänsch et al. 2007). It is not known whether other yeast species possess a sulphite oxidase and to which extent such enzyme could play a role in sulphite detoxification.

### 1.5.3 Production of acetaldehyde

The role of acetaldehyde in winemaking is very important as it can contribute both positively and negatively to the wine aroma profile, where at low levels (e.g. below 100 mg/l) it can contribute to the complexity of red wine bouquet but at high levels imparts a pungent sherry, nutty and bruised apple off-flavour (Bartowsky and Pretorius 2009). Acetaldehyde is a highly volatile and reactive compound and binds to many compounds in the wine such as amino acids, proteins and $SO_2$.

Acetaldehyde is an intermediate metabolite that is produced in numerous metabolic pathways in mammals, bacteria and yeast. In yeast, it is considered a leakage product and is most prevalent during the decarboxylation of pyruvate by pyruvate decarboxylase, during anaerobic fermentation with ethanol or acetic acid as the end products. It is also biologically toxic at high levels and can form covalent bonds with DNA and cause DNA point mutations (Wang et al. 2000).

Its greatest impact in the wine fermentation is its strong affinity for unbound $SO_2$ where one mole of acetaldehyde binds one mole of $SO_2$, and hence reduces the sulphite stress on any bacteria and yeast present during the fermentation. It therefore plays a critical role in the $SO_2$-binding power of wine. The increase in the concentrations of acetaldehyde and other $SO_2$-binding compounds such as pyruvic acid and α-ketoglutaric acid produced and excreted by yeasts in the presence of $SO_2$ has already been observed for more than 60 years (Peynaud

and Lafourcade 1952; Ribéreau- Gayon and Peynaud 1960; Weeks 1969). This was later confirmed once again as a general trait for $SO_2$-resistant strains of *S. cerevisiae*, even in the absence of $SO_2$ (Stratford et al. 1987; Casalone et al. 1992). The same phenomenon was also observed during cidermaking (Herrero et al. 2003). $SO_2$ has direct inhibitory effects on many enzymes in energy metabolism pathways. A direct result of this is that the flux of intermediary metabolites changes drastically. Increasing the level of $SO_2$ in the growth media has been shown to result in the increased production and subsequent leakage of acetaldehyde by *S. cerevisiae* into the extracellular environment (Casalone et al. 1992; Divol et al. 2006). The increase in acetaldehyde production during yeast fermentation in the presence of $SO_2$ has been reported several times and previously reviewed (Liu and Pilone 2000).

This increase, although minimal, in extracellular acetaldehyde will immediately bind to any free $SO_2$. The removal of this portion of free $SO_2$ from the extracellular environment will subsequently reduce the molecular $SO_2$ stress on the cell. In fact, the overproduction of acetaldehyde when the cells are in the presence of $SO_2$ could be due to two complementary factors: the inhibition of the aldehyde dehydrogenase leading to acetaldehyde not being converted to ethanol and the binding of acetaldehyde to $SO_2$ resulting in a reduced amount to be metabolised into ethanol (Frivik and Ebeler 2003). Whether this is a predetermined stress response or that it is only a side-effect of the enzymatic inhibition caused by $SO_2$ stress, is yet to be determined.

### 1.5.4 Sulphitolysis

As $SO_2$ is a reducing agent and high $SO_2$ resistance has been positively correlated with high glutathione concentration and glutathione reductase activity (Kåtgedal et al. 1986; Casalone et al. 1989), it was also hypothesized that glutathione could be involved in SO2 detoxification via a reaction called sulphitolysis (Mannervik et al. 1974; Kåtgedal et al. 1986):

$$GSSG + HSO_3^- \leftrightarrow GSSO_3^- + GSH$$

In the above equation, GSSG represents the oxidized form of glutathione (e.g. glutathione disulphide), GSH the reduced form and $GSSO_3^-$ glutathione-S-sulphonate. This reaction is catalysed by a glutathione reductase, such as that encoded by the *GLR1* gene in *S. cerevisiae*. However, it was shown that exposure to $SO_2$ did not result in an increase in GSH (Casalone et al. 1992) and

that a decrease in GSH concentration was not sufficient to explain $SO_2$ sensitivity (Xu et al. 1994). There are therefore doubts that sulphitolysis would play a major role in $SO_2$ detoxification.

### 1.5.5 Intracellular accumulation and active efflux

The cellular mechanisms of detoxification reviewed above are definite assets for the cell upon exposure to $SO_2$, sulphite reduction and acetaldehyde production certainly being the most prevalent mechanisms. Nevertheless, Park and Bakalinsky (2000) noted that they could not fully explain the differences observed between strains with regards to $SO_2$ resistance. Results obtained earlier for instance showed that defects in methionine and cysteine metabolism did not result in enhanced $SO_2$ resistance, making $SO_2$ reduction a non-essential feature in combatting $SO_2$.

From the genetic inheritance observed by different authors (as mentioned above), some authors concluded that specific genes must be involved. The *FZF1* gene of *S. cerevisiae* was initially identified as being responsible for sulphite resistance (Casalone et al. 1994). This gene was later characterised as a five zinc finger protein (Avram and Bakalinsky 1996). The latter authors showed that upon overexpression on a multicopy plasmid, *FZF1* conferred resistance to $SO_2$ in sensitive mutants, especially those in which the *GRR1* gene had been deleted. *GRR1*, also referred to as *CAT80, COT2, SSU2* or *SDC1*, is an F-box protein component of the Skp–Cullin–F-box containing complex ubiquitin-ligase complex (or SCF complex). It plays multiple roles in the cell such as carbon catabolite repression (Bailey and Woodword 1984; Flick and Johnston 1991; Erickson and Johnston 1994), glucose-dependent divalent cation transport (Conklin et al. 1993), high-affinity glucose transport (Vallier et al. 1994), morphogenesis (Kim et al. 1994) and G1 cyclin turnover (Barral et al. 1995). *Δgrr1* deletion mutants are deficient in hydrogen sulphide formation. Their sensitivity to $SO_2$ has been noted regardless of the sugar used as carbon source. It is believed that *GRR1* would be indirectly involved in $SO_2$ resistance, which would be a consequence of deficiency in glucose uptake. Following this diminished glucose uptake, the rate of acetaldehyde production is negatively affected. This would ultimately impact on $SO_2$ resistance as described above. It was also hypothesised that an altered glucose uptake would also impact on energy production and therefore on all energy-dependent processes such as $SO_2$ efflux (Avram and Bakalinsky 1996).

*FZF1* was later characterised as the transcription factor of *SSU1*, by physically interacting on *SSU1* promoter in the region [−455; −378] (Avram et al. 1999). The first zinc finger of Fzf1p as well as the 11 N-terminal amino acids have been shown to be essential to ensure the binding of Fzf1p to the *SSU1* promoter. *SSU1* is a transmembrane protein located on chromosome XVI. It was shown that ***Ssu1Δ*** deletion mutants accumulate more $SO_2$ intracellularly (Avram and Bakalinsky 1997; Park and Bakalinsky 2004). This observation led to the conclusion that Ssu1p was a sulphite pump, able to efflux excess $SO_2$ outside the cell. This transporter does not seem to exhibit an ATP binding site. $SO_2$ resistant strains possess a specific allele of *SSU1* called *SSU1-R*, which has been seen to be 97 % identical to *SSU1*. However, a high level of polymorphism is observed between strains (Aa et al. 2006). A strong heterozygosity has been observed for the translocated *SSU1* locus (i.e. on chromosome XVI) (Nardi et al. 2010) with numerous strain-dependent nucleotide polymorphisms (Aa et al. 2006). However, there is an absence of heterozygosity for the untranslocated locus (e.g. on chromosome VIII) (Aa et al. 2006; Liti et al. 2009; Nardi et al. 2010). As wine strains of *S. cerevisiae* exhibit different degrees of ploidy and different levels of heterozygosity, the number of *SSU1* and *SSU1-R* could potentially explain the diverse range of resistance observed between strains.

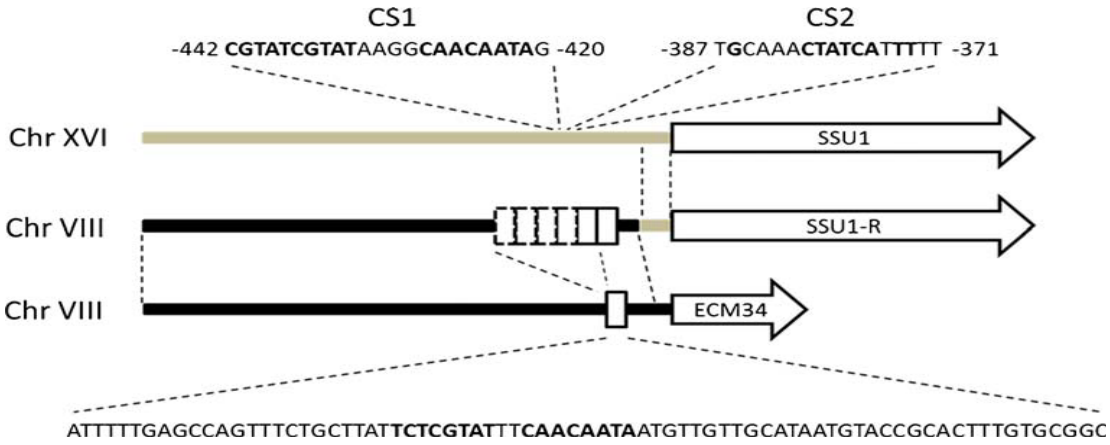


**Figure 1.3** Schematic representation of *ECM34*, *SSU1*, and *SSU1-R* promoters showing the translocations that occurred between different sections of chromosomes in some strains of *S. cerevisiae*. *CS1* conserved sequence described by Avram et al. (1999); the sequence protected by Fzf1p is written out, and the tandem region is mentioned in bold. The 76-bp repeat sequence is also written out and the region similar to the tandem region of the *SSU1* promoter is mentioned in bold. *CS2* conserved sequence described by Sarver and DeRisi (2005); bold letters show the nucleotides common to the promoter of all four genes activated under nitrosative stress.

The main mutations are in fact located upstream of the gene in its promoter region and have been shown to originate from a translocation from chromosome VIII (Goto- Yamamoto et al. 1998; Perez-Ortin et al. 2002). As a result, the promoter of *SSU1-R* has been nearly fully replaced by that of *ECM34* (Fig. 1.3). Using Northern blot, it was demonstrated that *SSU1* and *SSU1-R* expressions are constitutive, but that of *SSU1-R* is much higher than that of *SSU1* (Goto-Yamamoto et al. 1998). According to Avram et al. (1999), *SSU1-R* is more active than *SSU1* because of the presence of more binding sites for Fzf1p. The latter authors showed using a DNaseI protection assay that Fzf1p protects the region [−442; −420] which contains one 9-bp tandem repeat in *SSU1* promoter and two 8-bp tandem repeats in that of *SSU1-R*. It was also shown that the promoter of *SSU1-R* exhibits between two and six 76-bp repeats (Yuasa et al. 2004). It was also suggested that the number of repeats influences the expression of *SSU1* (Yuasa et al. 2005) and it was shown that the number of repeats increases in wine yeasts that display stronger $SO_2$ resistance. These authors also detected the presence of a low oxygen response element within the 76-bp repeat sequence of *SSU1-R*, but not in that of *SSU1*. This correlated with the observation of increased *SSU1-R* expression in microaerobic conditions. This aspect must nevertheless be confirmed. Finally, they also mentioned a mutation in the second nucleotide of the 76-bp repeat sequence differs between *SSU1* and *SSU1-R*, the former exhibiting a cystidine and the latter an adenine. They demonstrated that overexpression of *FZF1* increases the expression of *SSU1* but not that of *SSU1-R*. The reason for this is not fully understood. Nitrosative stress has also been shown to activate *SSU1* expression, via the induction of *FZF1* (Sarver and DeRisi 2005). The latter authors found that *FZF1* was required for the induction of the 4 genes activated under nitrosative stress via the binding on a conserved region (CS2) common to the promoter of all 4 genes, distinct from the previous conserved region (CS1) described previously (Avram et al. 1999) (Fig. 1.3). Consequently, it is now speculated that *SSU1* could therefore also be involved in nitric oxide-derived metabolites efflux (Sarver and DeRisi 2005).

Sulphite itself has been shown not to affect the expression of *SSU1* or *SSU1-R* (Yuasa et al. 2005; Aranda et al. 2006; Park and Hwang 2008). However, more recently, it was shown by using quantitative real-time PCR that the level of expression of *SSU1* increased progressively during alcoholic fermentation (Nardi et al. 2010). The level of expression of *FZF1* increased in both strains (that

possessing *SSU1* and that possessing *SSU1-R*) and correlated well with the increase in *SSU1* expression but not with that of *SSU1-R*. These latter authors could not attribute the increase in *SSU1* expression by a general alteration of sulphur metabolism. *SSU1* expression level was shown to be strain-dependent (Divol et al. 2006; Nardi et al. 2010). However, the presence of sulphites only increased *SSU1* expression but not that of *SSU1-R* (Nardi et al. 2010). Additionally, the strain possessing *SSU1* displayed a better growth in the presence of sulphites when the strain was pre-adapted to sulphites, unlike the strain possessing *SSU1-R*. The existence of a post-transcriptional factors affecting Ssu1p activity was suggested (Nardi et al. 2010), as the strongest expression of *SSU1* does not necessarily translate into the strongest resistance to $SO_2$.

Finally, it must be noted that the strains possessing *SSU1-R* were all isolated from the wine environment (Perez-Ortin et al. 2002). This indicates a possible adaptation to life in a sulphur containing habitat.

### 1.5.6 Global view of sulphite resistance at cellular level

All the mechanisms involved in $SO_2$ detoxification described above have long been regarded as independent of one another. However, with the emergence of systems biology techniques and especially global transcriptome analysis, recent findings show that all these mechanisms are induced at the same time and that some links between them can sometimes be established.

A transcriptome analysis was performed following exposure to $SO_2$ (Park and Hwang 2008). The results showed that the expression of 21 genes is induced with most of them being involved in sugar metabolism. This could be attributed to a resistance mechanism of the cells. Amongst the genes showing a clear induction, *PDC1* was identified. This gene encodes a pyruvate decarboxylase. *TDH3*, encoding a glyceraldehyde-3-phosphate dehydrogenase was shown to be the most strongly down-regulated gene together with *ADH1* encoding an alcohol dehydrogenase. This correlates well with previous findings regarding the decreased activity of these enzymes (Maier et al. 1986). Moreover, the induction of *PDC1* and inhibition of *ADH1* would lead to an accumulation of acetaldehyde, as previously reported by many authors following exposure to $SO_2$. Furthermore, the genes involved in sulphite detoxification via active expulsion (e.g. *FZF1* and *SSU1*), reduction (e.g. *MET1, MET5, MET8* and *MET10*) and sulphitolysis of

glutathione (*GLR1*) were also shown to be induced together with the genes involved in sulphite. Induction of *FZF1* and *SSU1* was previously noticed (Aranda et al. 2006) as well as the sulphitolysis of glutathione (Thomas et al. 1992). However, only the activation of *MET14* and *MET16* was observed by Northern blot analysis upon exposure to $SO_2$ (Donalies and Stahl 2002) but was not confirmed in this genome-wide analysis conducted by microarray hybridisation. *MET16* was also reported as being repressed in the presence of $SO_2$ in sulphite resistant strains (Aranda et al. 2006) and therefore this involvement of $SO_2$ in the expression of the *MET14* and *MET16* genes needs to be clarified as data found in literature are contradictory.

Finally, the level of expression of 37 genes decreased with the majority being involved in transcription, protein biosynthesis and cell growth. The down-regulation of these genes correlates well with the growth arrest observed upon exposure to $SO_2$ as a means for the cell to save energy.

Two studies, conducted by the same research group, mention a link between acetaldehyde concentration and the SAAB pathway and therefore $SO_2$, as intermediate of this pathway (Aranda and del Olmo 2004; Aranda et al. 2006). According to these authors, acetaldehyde induces the expression of genes involved in the SAAB pathway, leading to a stronger production of $SO_2$ by the cell. However, as acetaldehyde binds $SO_2$, it also contributes to detoxification and *MET16* is down-regulated in the presence of sulphite. $SO_2$ also inhibits the acetaldehyde dehydrogenase, leading to an accumulation of acetaldehyde. Moreover, methionine, one of the end-products of sulphur amino acid biosynthesis pathway has been shown to repress *SSU1-R* especially in the presence of sulphite, but not *SSU1* (Aranda et al. 2006). These confusing data highlight the complexity for the cell to deal with acetaldehyde and $SO_2$ when exposed to high concentrations, two normal compounds of general metabolic pathways that become toxic at high levels. The resistance to $SO_2$ being strain dependent, different strains might also react differently to the same concentration of $SO_2$ and explain these somewhat contradicting results.

As reported above, $SO_2$ resistance relies upon a complex array of mechanisms aiming directly (e.g. sulphite efflux) or indirectly (e.g. mainly sulphite reduction and acetaldehyde production) at eliminating $SO_2$ and therefore a potential fatal risk for the cell. However, the level of $SO_2$ resistance varies greatly between species and between strains, which seems directly linked to genome rearrangements in the latter case. Moreover, during winemaking, $SO_2$ is usually

added at the end of the fermentation at a level that is believed as inhibitory for microorganisms. Despite this, winemakers are sometimes confronted with the development of spoilage yeasts during wine ageing: *Brettanomyces bruxellensis* in dry red wines and *Zygosaccharomyces* spp., *Sch. pombe*, *Saccharomycodes ludwigii* and even specific strains of *S. cerevisiae* in sweet wines. Amazingly, most of the spoiled wines appear sterile when using classical microbiology techniques for detection of microorganisms. In the past decade, some authors have therefore demonstrated that yeast, like bacteria, can enter a VBNC state allowing them to survive in harsh conditions (Del Mar et al. 2000; Mills et al. 2002; Bleve et al. 2003; Divol and Lonvaud-Funel 2005; du Toit et al. 2005; Barata et al. 2008; Agnolucci et al. 2010; Serpaggi et al. 2012). It has recently been hypothesised that large amounts of $SO_2$ trigger the entry into this state, probably when the cells are no longer able to eliminate the intracellular $SO_2$ by means of the mechanisms described above.

## 1.6 Project outline

The aim of the project is to study the genetic bases of sulphite metabolism in autochthonous yeasts of *Saccharomyces cerevisiae* and to verify how this function can be influenced by other factors.

A first screening of $SO_2$ resistance and on plate production of $SO_2$ and $H_2S$ have been performed for autochthonous strains, isolated in Veneto vineyards to be used as starter of fermentation in the production of Prosecco di Valdobbiadene DOCG and DOC Piave wines. Afterwards a phenotypic characterization have been made in a selected number of strains, those whose genome has recently been sequenced. Oenological properties have been tested and the main phenotypic characters have been defined.

The genome sequencing allowed to identifiy some genetic characteristics, such as oenological SNPs, strain-specific genes and important translocations, that have been analyzed in Real-time PCR for a large number of autochthonous strains.

In this study the behaviour towards sulphite of *Saccharomyces cerevisiae* strains, whose genome has been sequenced, has been evaluated first in small laboratory scale and than, for selected strains, in controlled bioreactors.

RNA-seq of four strains was performed on RNA extracted during fermentation process under winemaking conditions in controlled bioreactors, collecting samples growth in synthetic wine media supplemented with 0 or 25 mg/l of $SO_2$, to investigate the molecular adaptation of wine yeasts in presence of high sugar content, low pH, and high ethanol concentration during mid-exponential phase.

Finally, for better understanding yeast behaviour and metabolism under sulphite stress condition, a selection of reference genes for Real-time PCR has been made, and a set of genes suitable for such conditions has been identified.

# 2. Characterization of sulphite production and sulphite resistance in *Saccharomyces cerevisiae* isolated from vineyard

## 2.1 Introduction

Sulphite is a widely used preservative in foods and beverages for its antimicrobial and antioxidant properties, although its toxic effect on human health is largely proven. Thus, reduction of sulphite use in wine processing is considered a primary objective in oenology.

During fermentation, yeasts usually produce a certain quantity of $SO_2$ by themselves, depending on strain and fermentation conditions. Most of wine starters now available on the market have been selected for their high sulphite resistance and are currently used for fermentation of sulphited musts. Lowering $SO_2$ amounts would allow the use of alternative strains more adapted to such condition such as autochthonous yeasts, isolated from the vineyard.

### 2.1.1 Ecology of wine yeasts

Yeasts are widespread in nature and are found in soils, on the surface of vegetables and in the digestive tract of animals. Wind and insects disseminate them. They are distributed irregularly on the surface of the grape vine; found in small quantities on leaves, the stem and unripe grapes, they colonize the grape skin during maturation. Observations under the scanning electron microscope have identified the location of yeasts on the grape. They are rarely found on the bloom, but multiply preferentially on exudates released from microlesions in zones situated around the stomatal apparatus. *Botrytis cinerea* and lactic acid bacteria spores also develop on the proximity of these peristomatic fractures. The number of yeasts on the grape berry, just before harvest, is between 103 ad 105, depending on the geographical situation of the vineyard, climatic conditions during maturation, the sanitary state of the harvest, and pesticide treatments applied to the vine (Ribereau-Gayon P. *et al.* 2006). Quantitative results available on this subject, anyway, are few. After the harvest, transport and crushing the crop, the number of cells capable of forming colonies on an agar medium generally attains 106 cells /ml of must. The number of yeast species significantly present on the grape is limited. Strictly oxidative metabolism yeasts, which belong to the genus *Rhodotorula* and a few alcohol sensitive species, are

essentially found there. Among the latter, the apiculated species (*Kloekera apiculata* and his sporiferous form *Hanseniasporauvarum*) are the most common. They comprise up to 99% of the yeasts isolated from certain grape samples. The following genera are associated with winemaking environment and they can be found but in lesser proportions: *Candida*, *Cryptococcus*, *Debaryomyces*;*Kluyveromyces*, *Metschnikowia Pichia*, *Saccharomycodes, Schizosaccharomyces* and *Brettanomyces* (and his sexual equivalent *Dekkera*) (Pretorius, 2000).

All the researches, that deal with this subject, confirm the extreme rarity of *S. cerevisiae* on grapes. Yet these yeasts are not totally absent. Their existence cannot be proven by spreading out diluted samples of must on a solid medium prepared in aseptic conditions but their presence on grapes can be proven by analyzing the spontaneous fermentative microflora of grape samples placed in sterile bags, then aseptically crushed and vinified in the laboratory in absence of contaminations.

#### 2.1.1.1 Origin of wine yeasts

The fermentation of grape must is a complex ecological and biochemical process involving the sequential development of microbial species, as affected by particolar environment. The process includes the interaction of fungi, yeasts, lactic acid bacteria, acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms (Pretorius, 2000). Of all these, yeasts are the heart of biochemical interaction with the must derived from the varieties of *V. Vinifera* and other grape species.

Although the non-*Saccharomyces* yeasts are the predominant species as the grape, they grow well in the early stage of fermentation, but are subsequently replaced during the following steges by *Saccharomyces* yeasts, which are more tolerant to ethanol (Fleet and Heard, 1993). So, though many genera and species of yeasts are found in the musts, the genus *Saccharomyces* and mainly the specie *Saccharomyces cerevisiae* is the one responsible for alcoholic fermentation (Pretorius, 2000). The origins of non-*Saccharomyces* are grape skin and winery equipments (Fleet, 1993). However, the origin of *S. cerevisiae* is controversial; although the most significant finding was that it is practically absent from grapes and vineyard soils (Martini, 1993), some authors propose that this species is a “natural” organism present on plant fruits (Mortimer *et al.* 1999;

Sniegowski *et al.* 2002). Others argue that there is an “artificial” origin and this species came from the hybridization of other *Saccharomyces* and then selected in a man-made environment (Martini, 1993), this model would be supported by the fact that *S. cerevisiae* has been found only in areas close to human civilization. Finally, some authors postulate that this species is a domesticated microorganism originating from its closest relative *Saccharomyces paradoxus*, a wild species found all around the world associated with insects, tree exudates and fermenting plant extracts. The occurrence of *S. cerevisiae* in the vineyard would be the consequence of back transportation from cellars by insects (Naumov, 1996).

Moreover, there is still a lack of agreement concerning the contribution to spontaneous fermentations of *S. cerevisiae* originating from the vineyard comparing to that originating from the winery. On one hand, spontaneous alcoholic fermentation is possible in sterilized vessels (Lopez *et al.* 2002) or in a newly built winery where *S. cerevisiae* has never been introduced (Beltran*et al.* 2002). On the other hand, as mentioned before, although it has been found on damaged berries (Mortimer *et al.* 1999) wild *S. cerevisiae* is extremely rare on intact grapes (Sabate *et al.* 2002) whereas it can be found colonizing the winery equipment (Beltran *et al.* 2002; Sangorrin *et al.* 2002; Vaughan-Martini et al. 1995): some strains are even found in the winery over several years (Beltran *et al.* 2002; Frezier and Dubourdieu, 1992; Rosini, 1984).

#### 2.1.1.2. Use of selected yeasts for oenological purposes

Originally, all wine was made by taking advantage of natural microflora for spontaneous fermentation; no deliberate inoculation was made to start the process. All the various yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural fermentations. A breakthrough was made in 1880 when Hansen, working at the Carlsberg winery in Denmark, isolated a pure culture derived from a single yeast cell and, in 1890,Muller-Thurgau from Geisenheim introduced the concept of inoculating wine fermentations with pure yeast starter cultures (Pretorius, 2000). In 1965, the first two commercial dried yeasts (ADWY) strains were produced for a large Californian winery (Fleet and Heard, 1993). These two strains, “Montrachet” and “Pasteur Champagne”, were offered worldwide as all purpose yeasts. The inoculation of selected pure yeast cultures into must is nowadays a

common enological practice established since the 1970s, in order to produce wine with desirable organoleptic characteristics and to guarantee the homogeneity of successive vintages. Today, several yeast-manufacturing companies market a wide variety of dehydrated cultures of various *S. cerevisiae* strains, and most of worldwide wine production relies on the use of such commercial starter yeasts.

In the past 30 years, strains of *S. cerevisiae* have been selected for their enological properties and are used as starters in winemaking processes. Yet these strains involved in fermentation play an important rule in determining the characteristics of the final product, in terms of high alcohol percentage and absence of undesirable compounds (Perez-Coello *et al.* 1999) while the diversity of native *S. cerevisiae* strains present in spontaneous fermentations contribute to the chemical composition and sensory qualities of the resulting wine (Lurton *et al.* 1995). Moreover, several studies support the hypothesis that active dried yeasts reduce the variability of strains that appear in spontaneous fermentations (Beltran *et al.* 2002; Fleet, 2003) and, possibly, the complexity of the resulting wine. For these reasons, winemakers looking for original flavours prefer spontaneous fermentation with indigenous yeasts. For the same reasons, lots of recent selection projects for new wine strains focus on ecotypical strains, trying to preserve biodiversity in selected areas and at the same time to guarantee optimal fermentation performances.

The recent discovery that an overabundance of living cells of *S. cerevisiae* is present in every kind winery is providing wine technologists with a large reservoir of strain diversity as a new source of locally selected starters for wine-making. Since *S.cerevisiae* populations, isolated from vineyard and wineries, endowed with enological properties wholly comparable to those of commercial starters, autochthonous starters may prevent excessive standardization engendered by the presence of only few active dry commercial starters in the international market (Martini, 2003).

#### 2.1.1.3. Genomic characteristics of wine yeast

Industrial *S. cerevisiae* strains are highly specialized organisms, which have evolved to utilize their full potential in the different environments or ecological niches that have been provided by human activity. This selection process can be described as “domestication” and can be responsible of the special genetic

characteristics of industrial strains (Querol *et al.* 2003). *S. Cerevisiae* has a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns. Haploid strains contain approximately 12-13 megabases of nuclear DNA, distributed along 16 linear chromosomes whose size vary from 250 to 2000 kb (Barre *et al.* 1992). In contrast to most *S.cerevisiae* strains used in the laboratory, which are either haploid or diploid and have a constant chromosome electrophoretic profile, wine yeast strains are mainly diploid, aneuploid, or polyploid, homotallic and highly heterozygous, and show a high level of chromosome length polymorphism. Moreover, wine yeast strain seem not to remain genetically uniform (reviewed in Pretorius -Pretorius, 2000- and in Querol *et al. 2003*). Their exacerbated capacity to reorganize its genome by chromosomal rearrangements, such as Ty-promoted chromosomal translocations (Longo and Vézinhet 1993.; Rachidi *et al.* 1999), mitotic crossing over(Aguilera *et al.* 2000)and gene conversion (Puig *et al.* 2000) promotes a faster adaptation to environmental changes than spontaneous mutations, which occur at comparatively very low rates. In particular, the ploidy of wine yeasts may confer advantages to adapt to variable external environments and increase the dosage of some genes important for fermentation (Bakalinsky et al. 1990; Salmon, 1997). The illegitimate recombination mediated by Ty elements and subtelomeric repeated sequences has several practical consequences: sporulation ability is very variable (between 0 and 75% ascus formation on a sporulation medium) and spore viability is also highly variable, ranging from 0 to 98% (Barre *et al*. 1993; Còdon *et al*. 1995).The meiotic segregants from wine strains diploidize with high frequency, indicating ahigh frequency of homotallism. Heterozygosity has been observed in both homothallic and heterothallic wine strains. In addition, the possibility of adaptive gross genomic changes occurring during laboratory growth conditions has been demonstrated by Hughes et al. (Hughes, Marton *et al.* 2000; Hughes, Roberts *et al.* 2000):those authors showed in multiple cases that the deletion of a single gene strongly favors the acquisition of a whole chromosome or a chromosome segment containing a compensatory copy of a close homolog of the deleted gene.

### 2.2 Selection of yeasts in oenology: commercial and autochthonous wine strains

Spontaneous fermentation, uncontrollable by man, is not longer used by anyone thanks to Hansen who introduced, in the beer fermentation, the practice of purity

fermentation, selecting and using pure cultures of yeasts. It was, however, Müller-Thurgau in 1890 to introduce the concept of selected yeast in the wine industry. These technological selected yeasts are able to conduct fermentation of musts and wines with predictable and projectable results. “Selected yeast or starter” means a strain characterized by physiological, biochemical and oenological properties optimized in relation to the technological requirements of fermentation processes in purity.

Up to half of the XX century, the use of yeast as a starter in winemaking was implemented in the countries new wine producers, such as Australia, USA, New Zealand and South Africa. In countries traditional producer of wine, selected cultures were instead primarily used to correct defects in fermentation and / or to activate refermentation. The selected starter cultures were maintained in collection, usually by research organizations, and it was difficult to use a starter for operators in the cellar that had little knowledge of microbiology. Under the pressure of bread industry finally, in the second half of the XX century, began the industrial production of microbial starters for winemaking in the form of compressed yeast. This preparation, while avoiding the multiplication phase, had the disadvantage of being easily perishable due to the high humidity content (70%), which effectively reduced its commercial diffusion.

To remedy this, in 1965 in California were proposed and commercialized the first two wine starters in form of active dry yeast (ADY). This new type of preparation, thanks to a high viability (50%), the long shelf-life due to reduced humidity content (4-8%) and to the system of vacuum packaging, have enabled the widespread use of ADY. In Italy, the rapid spread of the use of selected yeasts began in 1978 after the law that authorized the use (DM October 10, 1977). The species to be selected are those of the group of *Saccharomyces* (Zambonelli C. et al., 2000).

The first yeasts (defined precisely technological yeasts) were selected with the aim of enhancing the technological characteristics (fermentative vigour, alcohol tolerance), in order to obtain products without defects. Today yeasts of the latest generation are selected on the basis of characteristics that could improve the quality of wines through the expression of precursors already present in musts and the production of secondary metabolites (higher alcohols, esters, ketones, aldehyde) (Vincenzini et al., 2005).

Potentially, the use of selected yeasts may present some disadvantages not negligible, because of all the cultures commercialized of ADY are relatively few

those actually used by winemakers in the world. This could lead to a standardization of microbial agent with the result of obtaining a reduction of the biodiversity of wine yeasts associated with the environment of the cellar. The worst prospect, however, seems the loss of biodiversity in the vineyard, in fact after the harvest in the vineyard is found approximately 73% of commercial yeasts that are in the cellar, spreaded for the 94% by machines for grape harvesting in a radius ranging from 10 to 200 m from the cellar (Valero et al., 2005). In addition, starters available on the market, despite having characters certainly important in enology, are not always able to fully develop the flavours and aromas typical of a wine (Pretorius, 2000).

To overcome these problems, both microbiologists and winemakers, believe it is appropriate to introduce the use of autochthonous starter.

During the past few years there has been a noticeable increase in the demand for autochthonous wine yeasts to be used as fermentation starters. They are indigenous strains isolated from natural grapevine environments that are supposed to be the performers of spontaneous fermentations in the winemaking areas of origin, thus they can be selected for improving the *terroir* of local wines. The requirements for these yeasts are the ability to dominate during the fermentation process, and enhance, at the same time, the sensory characteristics of wines originating from different grapevine cultivars. In fact while commercial yeasts enable rapid and reliable fermentations reducing the risk of stuck and sluggish processes, they are ineffective in exalting the sensory properties of regional wines losing their typical *terroir* character.

### 2.2.1. Selection of ecotypical yeast strains

The main critics of the practice of guided fermentations (using starter cultures) dislike the fact that the commercial wine strains, despite being numerous, possess very ordinary characteristics. Commercial yeast strains produce wines with average qualities and do not enhance the aromatic traits that characterise many yeasts isolated from specific geographical areas. Studies on the improvement and the selection of wine yeasts to overcome this problem have recently been carried out.

In the last few years, there has been an increasing use of new local selected yeasts for controlled must fermentation in countries with a winemaking tradition. Though there are commercial yeasts to accomplish must fermentation, the use of

local selected yeasts is believed to be much more effective. Local yeasts are presumed to be more competitive because they are better acclimated to the environmental conditions. Therefore, they would be better able to dominate the fermentation and become the most important biological agent responsible for the vinification. Selection of the appropriate local yeasts assures the maintenance of the typical sensory properties of the wines produced in any given region. Strains of *S. cerevisiae* can be isolated from vineyards and wine fermentations, and selected to be used as commercial starter cultures. It is now believed that strains of *S. cerevisiae* indigenous to vineyards and wineries tend to be homozygous for most of the genes by a process known as `genome renewal' (Mortimer *et al.* 1994). This process would eliminate the recessive lethal or deleterious genes that adversely affect yeast fitness (e.g. slower growth, lower fermentation rate, reduced spore viability, etc.). Genome renewal could also be responsible for the replacement of the parental heterozygous strains by the new homozygous diploids bearing new recessive alleles that increase fitness. The practical implications of genome renewal and yeast population dynamics in the vineyards and wineries (and even within yeast starter cultures) are far reaching, whether winemakers rely on spontaneous fermentation of grape juice or whether they inoculate grape must with selected wine yeast strains. Although dramatic improvements in most characteristics cannot be expected, intra-strain selection has been used for decades to obtain improved wine yeast strains and is still, up to date, one of the most utilized selection strategies.

The selection of wine yeasts for oenological use is traditionally carried out on the basis of their technological and quality-linked phenotypic characteristics. For this purpose different methodologies were designed.

#### 2.2.1.1. Screening methods based on fitness traits

The technological characteristics required to wine strains may vary, depending on the musts and on the winemaking techniques used. However, some of these characteristics, like high fermentation vigour and ethanol production as well as low $H_2S$ and acetic acid formation, are of particular interest for the selection of any kind of starter strain (Giudici and Zambonelli, 1992).

Recently a two-step procedure was proposed: a pre-selection based on resistance to$SO_2$, killer activity, growth at high temperature and low foam production, followed by a selection based on volatile acidity, ethanol production,

and residual sugars. Another methodology based on phenotypic characteristics is carried on following four consecutive steps: (1) fermenting capacity of the strains (2) formation of volatile acidity, resistance to $SO_2$, production of $H_2S$, flocculation capacity and adherence to glass; (3) autolytic capacity of the yeast; (4) foaming properties of the autolysates obtained (Martinez et al. 2001).

The oenological traits can be evaluated by carrying out small-scale fermentations in synthetic media and eventually in grape juice.

To assess both fermentation efficiency and fermentation vigour, weight loss due to $CO_2$ formation during fermentation is usually followed: in particular Castelli in 1954 proposed micro fermentations in grape must enriched in glucose to a final content of 30% (excess of sugar) in flasks stoppered with sulphuric acid-containing valves (in order to avoid water loss), performed at 25°C. Some years later, Ciani and Rosini (1990) proposed micro fermentations performed on pastorized grape must where yeast cultures were pre-incubated in grape must for 48 h. Alternatively, micro fermentations can be performed on synthetic must as described by Bely et al., (1990). In any case, fermentation efficiency (the uppermost concentration of ethanol obtainable) is calculated from weight loss at the end of fermentation (when no variations in weight are observed for two consecutive days). Fermentation rate is expressed as grams of $CO_2$ developed in 24 h, calculated as the average of a 3-day measurement period and followed during fermentation. Fermentation vigour is normally expressed as g of $CO_2$ produced in the first 48 hours following the inoculation of the must. The same of fermentation conditions (better if in untreated natural must) can be used to test sulphur dioxide resistance: after pasteurization, the must is split in two: $SO_2$ as potassium metabisulphite is added (usually to a final concentration of 100 and/or150 mg/L) to one aliquot. Both Flasks are inoculated and incubated at 25°C. After 2 and 7 days the weight loss caused by $CO_2$ production is determined, sulphite resistance is obtained by comparison with flasks where no $SO_2$ is added. $SO_2$ determination at the end of fermentation in un-sulphited must is also important: ability to produce $SO_2$ by sulphate reduction is widespread among *S. Cerevisiae* natural isolate, and no strains completely unable to produce this anhydride have been ever described. Since production levels of some particular strains are astonishing (up to 200-300 mg/L and up to 500 mg/L if sulphite are previously added to must), this character should be considered during strain selection, and $SO_2$ production lower than 25-30 mg/L is recommended. (Zambonelli 2003).

#### 2.2.1.2. Screening methods based on quality traits

Some of these characters can be studied using Petri dishes containing the suitable growing medium. Hydrogen sulphide production is evaluable on ABY or BiGGY agar at 25°C for 48 h. The screening medium is inoculated with a small quantity of yeast biomass, and, after incubation, the colour of the growing colony (white, pale hazel, hazel, dark hazel, black) is observed: the darker the colony appears the higher is the $H_2S$ quantity on BiGGY agar. Analogously, acetic acid production can be evaluated on calcium carbonate agar at 25°C during a period of 7 days incubation: the presence of an halo around the colony indicates strains producing high quantities of acetic acid, which causes dissolution of calcium carbonate salt on the plate. The acetic acid production is a stable character (Romano et al. 1998) but it is influenced by the must composition thus a quantification of acetic acid production during fermentation is also desirable. At the end of micro fermentations (usually performed for fermentation efficiency or fermentation vigour determination), also some other endpoint products and by-products such as ethanol, acetic acid, succinic acid, glycerol, acetaldehyde, malic acid can be determined by standard chemical analysis, HPLC or enzymatic kits. Finally, the presence of several glycosidic enzymes and the quantification of their activity in oenological indigenous yeasts has been introduced as a test, in order to select strains that contribute to enhance the primary aroma of the regional grapevine.

Yeast strains can be screened to determine the presence of β-glucosidase and glycosidase activities. The most popular screening test for β-glucosidase activity is carried out on agar plates with arbutin as substrate: yeast isolates that possess the proper enzyme are able to hydrolyse the substrate and a dark brown halo develops in the agar medium. Glycosidase activities can be determined by using the appropriate 4-methylumbelliferyl glycoside as substrate, as described by Manzanares et al. (1999). The presence of the enzymatic activity is then visualized as a fluorescent halo surrounding yeast growth after plate exposure to UV light. Alternatively, the same 4-MUG substrates can be used to perform the test in liquid growing media (Fia et al. 2005).

## 2.3. Selection of autochthonous wine yeasts isolated from vineyards in Veneto region

Italy has a rich wine heritage of hundreds of varieties, 340 of whom were enrolled in the National Catalogue and can be cultivated. Many of these varieties have had a spread almost non-existent, others have become famous and now form the basis of Italian viticulture. Among them we can mention the Glera (better known as Prosecco) which has a prominent position among the white grapes grown in Italy (Calò et al. 2000). Minor nationally important, but covering an important role at the local level is the Raboso Piave, typical red grape native, with evidence that since 1600 it was present in the Treviso area. The Conegliano Valdobbiadene Prosecco Superiore DOCG wine has a straw yellow colour, a characteristic vinous fragrance with a slight fruity aroma particularly in sweet types, a pleasantly bitter taste in the dry type and fruity in the sweet type. The Raboso Piave DOC has an intense ruby red colour, and the impact of flavour is sour, dry, austere, fruity and tannic. The acidity of natural origin on one hand gives freshness and finesse, on the other hand makes the product particularly aggressive and harsh.

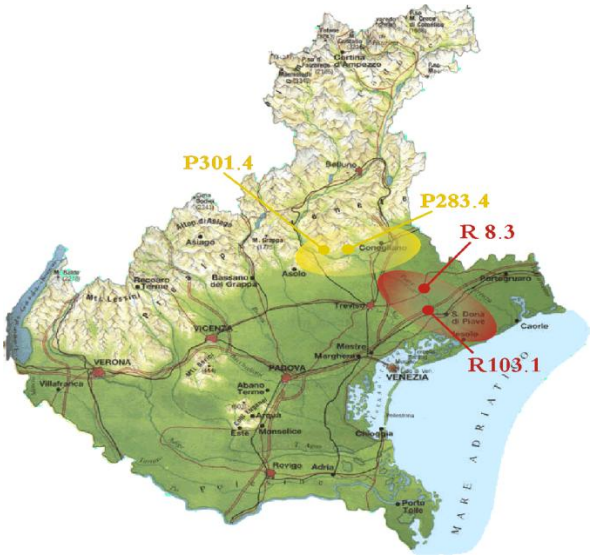


**Figure 2.1** Sampling areas of “Conegliano-Valdobbiadene Prosecco Superiore DOCG” (yellow) and “Raboso DOC Piave” (red) vineyards in Veneto region.

During the last years the microbiology research group of Prof. V. Corich in the Department of Agricultural Biotechnology of University of Padua isolated approximately 600 yeast strains fermenting grapes, collected from grapes in the vineyards of the “Conegliano-Valdobbiadene Prosecco Superiore DOCG” District and of the “Raboso DOC Piave” District in Veneto region (Fig.2.1). After isolation yeasts were identified and technologically characterized.

To obtain a strain-specific characterization of the isolates identified as *S. sensu stricto*, a method proposed by several authors was chosen (Querol et al. 1996,

Lopez 2001). This method uses mitochondrial DNA restriction fragment length polymorphisms analysis (mtDNA-RFLP) by enzymatic digestion of total DNA. The method is simple and yields results within 2 days. This technique has successfully been used by other authors to characterize strains of other yeast species (Martinez et al. 1995; Romano et al. 1996; Guillamon et al. 1997).

This is the most commonly genetic tool used for characterizing the *S. sensu stricto* group, in particular by usig the *HinfI* restriction enzyme (Lopez et al. 2001, Schuller et al. 2004). Restriction profiles obtained were compared by the GelComparII (Applied Maths) software that allows, by a matrix construction, to calculate the similarity level between strains and to draw it in a dendrogram.

The mtDNA-RFLP analysis evidenced the presence of 37 different profiles for Prosecco and 130 for Raboso, which are considered as different strains, from the analysis of all the 600 isolates.

## 2.4 Materials and methods

### 2.4.1 Yeasts

We investigated sulphite response, together with production of $SO_2$ and $H_2S$, in a collection of 167 autochthonous wine yeasts isolated from Veneto areas (Conegliano Valdobbiadene Prosecco Superiore DOCG and Raboso Piave DOC), and their performance have been compared with those of 52 commercial wine starters.

### 2.4.2 Culture media and growth condition

**Media**

YM solid agar medium
3 g/l yeast extract (Oxoid);
3 g/l malt extract (Oxoid);
5 g/l vegetatone peptone (DIFCO);
10 g/l glucose (PROLABO)
16 g/l Bacto Agar (DIFCO).
Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)
10 g/l yeast extract (OXOID)
20 g/l vegetatone peptone (DIFCO)
20 g/l glucose (PROLABO)
Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Fucsine Agar medium
3 g/l yeast extract (Oxoid);
3 g/l malt extract (Oxoid);
5 g/l vegetatone peptone (DIFCO);
10 g/l glucose (PROLABO)
0,002 g/l Fucsine (SIGMA)
16 g/l Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Biggy Agar medium (Oxoid)

1 g/l yeast extract
10 g/l glycin
10 g/l glucose
3 g/l sulphite ammonium
5 g/l bismuth ammonium citrate
16 g/l Bacto Agar
pH 6.8

Suspend 42g in 1 liter of distilled water and bring gently to the boil to dissolve the agar. Allow to cool to 50-55°C. Mix gently to disperse the flocculent precipitate and pour into sterile Petri dishes. Do not autoclave the medium.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

| | |
|---|---|
| 0,1 g/l | $CaCl_2$ |
| 0,1 g/l | NaCl |
| 1 g/l | $KH_2PO_4$ |
| 0,5 g/l | $MgSO_4 \cdot 7H_2O$ |
| 3 g/l | tartaric acid |

Micronutrients

| | |
|---|---|
| 0,2 mg/l | $NaMoO_4 \cdot 2H_2O$ |
| 0,4 mg/l | $ZnSO_4 \cdot 7H_2O$ |
| 0,5 mg/l | $H_3BO_3$ |
| 0,04 mg/l | $CuSO_4 \cdot 5H_2O$ |
| 0,1 mg/l | KI |
| 0,4 mg/l | $FeCl_3 \cdot 6H_2O$ |
| 0,4 mg/l | $MnSO_4 \cdot H_2O$ |

Vitamins

| | |
|---|---|
| 400 µg/l | pyridoxinehydrochloride |
| 400 µg/l | thiaminehydrochloride |
| 2000 µg/l | Inositol |

| | |
|---|---|
| 20 µg/l | Biotin |
| 400 µg/l | Calciumpantothenate |
| 400 µg/l | Nicotinic acid amide |
| 200 µg/l | P-amino-benzoic acid |

Variable components

| | |
|---|---|
| 0,3 g/l | $(NH_4)_2SO_4$ |
| 0,3 g/l | $(NH_4)_2HPO_4$ |
| 200 g/l | Glucose |
| 0,2 g/l | Hydrolyzed Casein |

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

**Growth conditions**

The yeast strains were grown at 25 ° C, the liquid cultures, for fermentation inoculum, were subjected to agitation of 130 oscillations per minute.

## 2.5 Results and discussion

The objective of the first phase of this work was to identify strains with different ability to produce hydrogen sulphide and sulphur dioxide, for subsequently study the metabolism in fermentation conditions. For this purpose have been performed a series of rapid tests for the evaluation of in plate production of $H_2S$ and $SO_2$, using the growth media Biggy Agar and Fucsine agar, of 219 yeast strains: 52 commercial strains, 37 strains isolated during the selection of yeasts in the area Prosecco DOCG and 130 strains isolated during a similar selection of yeasts in the Raboso Piave DOC.

### 2.5.1 Sulphuric acid production

Sulphuric acid ($H_2S$) production was tested on Biggy agar medium (Bismuth Sulphite Glucose Glycine Yeast) (Oxoid), based on the formulation developed by Nickerson.

In a study of sulphite reduction by yeasts, the ability of many yeasts to reduce a bismuthyl hydroxy polysulphite was noted. Growth on an acidic or neutral medium containing bismuth sulphite produced black colonies because of the extra-cellular reduction of the bismuth sulphite, to bismuth sulphide. Colonies turn brown with an intensity proportional to the amount of sulphur-containing substances produced.

The chromatic scales used for result consideration is: white colour no $H_2S$ production, beige colour low production, brown colour medium production, dark colour high production.

**Figure 2.2** Yeasts growth on Biggy agar medium

The colour assumed by the yeast colonies after growth on Biggy agar showed that most of the autochthonous strains are characterized by a modest production of $H_2S$ (Fig. 2.3 a), which determines a light brown coloration, whereas the commercial strains are equally distributed in all classes. About 9% are dark

brown, a situation that has never been occurred among yeasts of the two native collections.

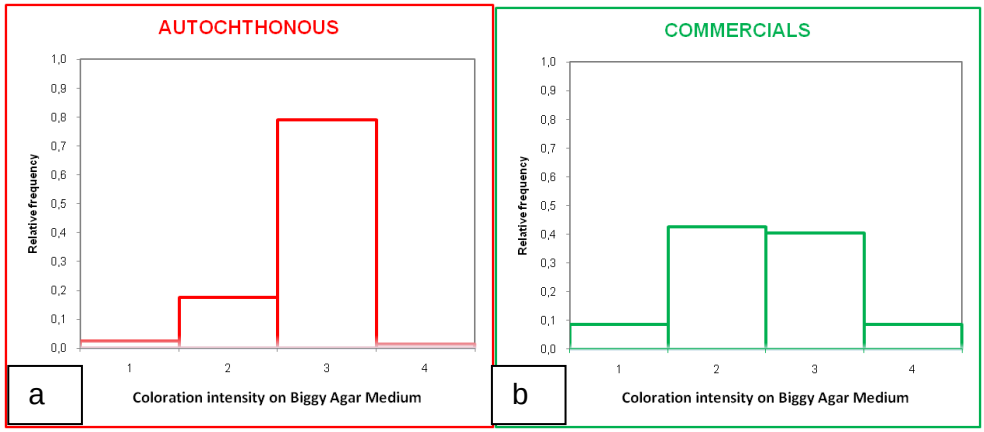


**Figure 2.3** Relative frequency of coloration intensity on Biggy agar medium of a. Autochthonous yeasts and b. Commercial yeasts.
Yeasts are grouped as Not producing (1) low (2) medium (3) and high (4) producer.

An important percentage of commercial strains (8% versus 3% in Prosecco and Raboso), are characterized by colonies of white colour, and seems not to produce $H_2S$. This situation usually occurs when the key enzyme does not work: the sulphite reductase, which converts sulphite ion to sulphide, then incorporated for the synthesis of sulphur amino acids. In these cases the sulphite ion is not transformed and exits the cell in the form of sulphur dioxide. It can be assumed, therefore, that these strains are characterized by production of high amounts of endogenous sulphur dioxide and therefore not very suitable for use in the cellar (Zambonelli, 2003).

### 2.5.2 Sulphur dioxide production

Fucsine agar medium (Caridi et al. 1999) was used to evaluate $SO_2$ production, that is revealed by the intensity of the pink coloration of the colonies.
The medium contains as an indicator the basic fucsine which tends to concentrates more inside the cells than in the growth medium outside; once inside the eventual $SO_2$ produced by the yeast combining with the fucsine (magenta) leads to the formation of a colourless compound.
The chromatic scales used for result consideration is: dark pink colour low $SO_2$ production, pink colour medium production, light pink colour high production, white colour very high production. (Fig.2.4)

**Figure 2.4** Yeasts growth on Fucsine agar medium.

Concerning $SO_2$ production all the strains analyzed were grouped into two core classes (medium producers or high producers, the colony colour pink or light pink respectively). Despite the poor variability in the data, it is possible to clearly see a difference between the distributions of natural isolates and commercial strains (Fig.5). The autochthonous yeasts are mostly medium producers of $SO_2$ (90% of the strains), while commercial strains are distributed almost equally between medium producers (51%) and high producers (49%).

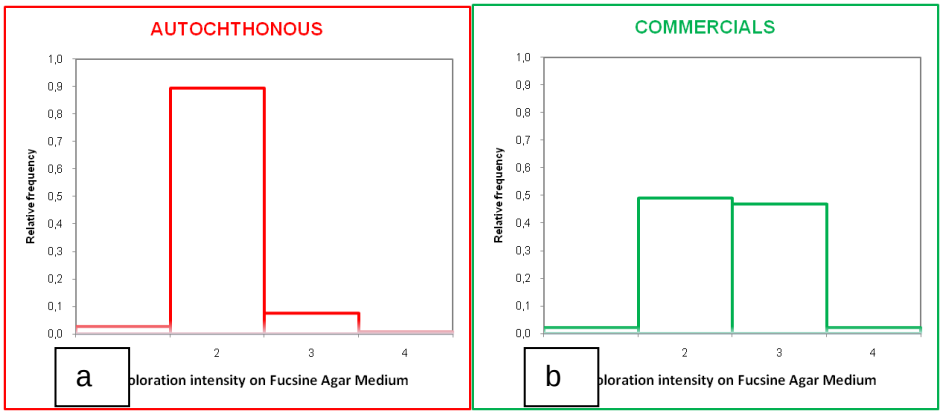


**Figure 2.5** Relative frequency of coloration intensity on Fucsine agar medium of a. Autochthonous yeasts and b. Commercial yeasts.
Yeasts are grouped as low (1) medium (2) high (3) and very high (4) producer.

The presence, in the group of commercial yeasts, of strains producing high doses of $SO_2$ it is in line with what was observed for the production of $H_2S$ (presence of many non-producing strains, probably able to produce more $SO_2$).

### 2.5.3 Sulphite resistance

Sulphite tolerance was studied by means of yeast growth measurement (optical density, OD) after 48h in Delfini synthetic must at different $SO_2$ doses (50 mg/l and 100 mg/l). Threshold for resistance was set at 0,1 $OD_{600}$.

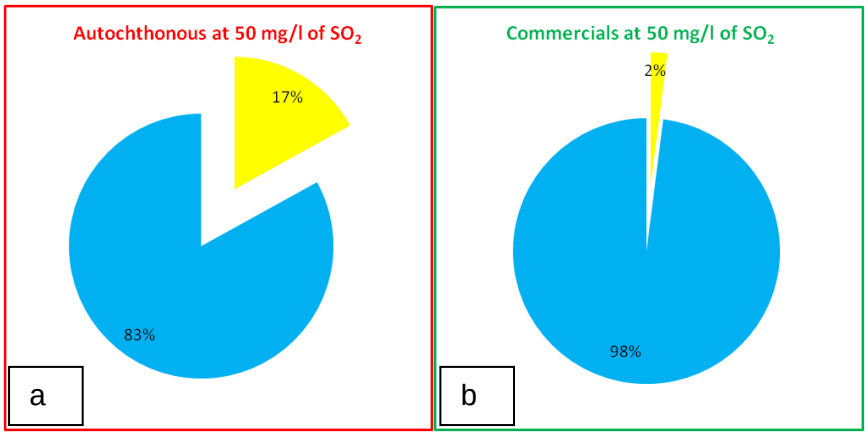


**Figure 2.6** Frequency of sensitive (■) and resistant (■) strains in autochthonous (a) and commercial (b) populations growth in the presence of 50 mg/l $SO_2$.

At 50 mg/l of $SO_2$ both populations show a good sulphite resistance, even if the difference between commercial and autochthonous isolates is remarkable: commercial strains show higher values than autochthonous yeast populations (98% vs 83%, respectively).

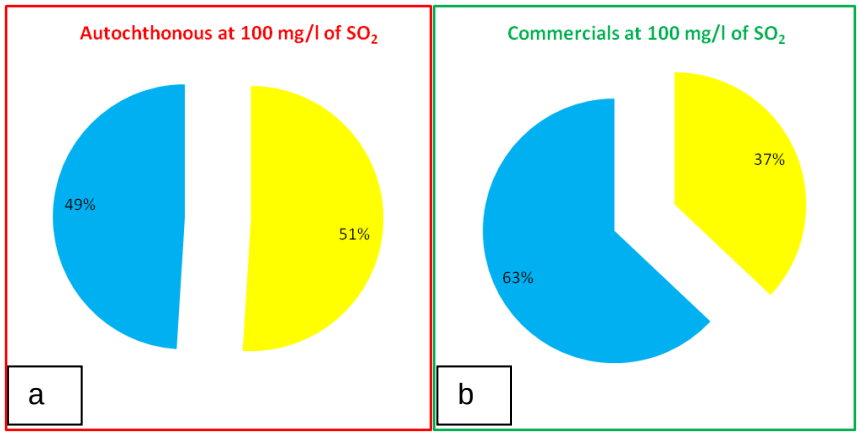


**Figure 2.7** Frequency of sensitive (■) and resistant (■) strains in autochthonous (a) and commercial (b) populations growth in the presence of 100 mg/l $SO_2$.

At 100 mg/l of $SO_2$ the percentage of resistant isolates decreases in both populations (49% for autochthonous and 63% for commercials) and the difference between commercial and autochthonous strains tolerance drops down.

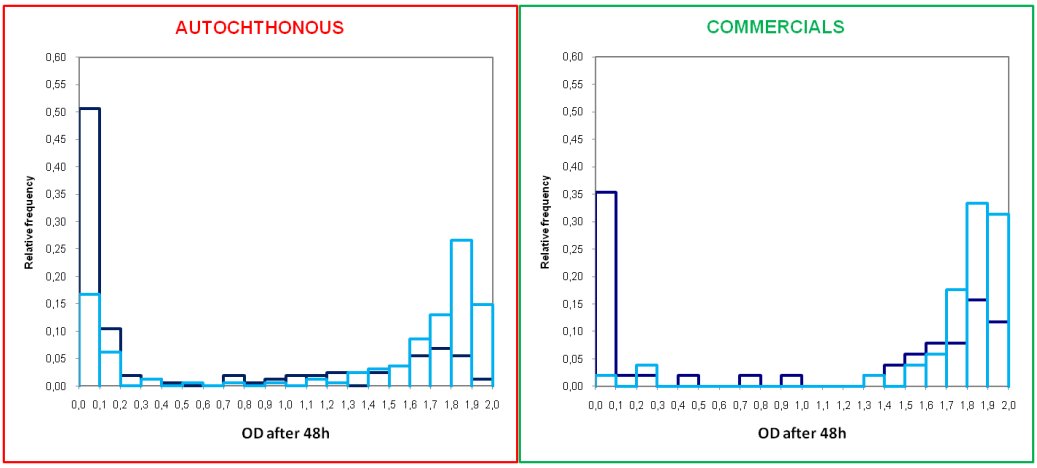


**Figure 2.8** Relative frequency distribution calculated on the OD values of 48h yeast cultures with 50 (■) and 100 (■) mg/l of $SO_2$ added.

Moreover, when 50 mg/l of $SO_2$ is added, 41 % of the autochthonous strains and 67 % of the commercial yeasts show an OD value comparable to the one obtained in the absence of $SO_2$ (≥1,8), suggesting complete tolerance to this concentration. Nevertheless only 7% of autochthonous strains and 29% of commercial yeasts exhibit complete tolerance to the concentration 100 mg/l (Fig. 2.8).

## 2.7 Conclusions

Concerning sulphur compounds production, autochthonous and commercial yeasts showed a certain variability in $SO_2$ and $H_2S$ production, ranging from low to high, but autochthonous yeasts grouped mostly in the class of medium producers of both compounds, while commercial strains are equally distributed between low and high producers. Sulphite tolerance is higher in commercial yeasts, but autochthonous isolates show a good sulphite resistance too.

These preliminary observations constitute the starting point for the identification of new strains to be use for vinification under low sulphite conditions. In terms of $H_2S$ and $SO_2$ production, as well as $SO_2$ resistance, autochthonous yeasts show a wider range of phenotypes compared to the commercial wine starters. Therefore this novel selected yeasts could be a more suitable pool of strains for the identification of the best performing starters in low sulphite wine production.

The vineyard is the best place for yeast selection, because of the presence of low sulphite producer (consequently less resistant). Lowering $SO_2$ amounts would allow the use of autochthonous strains, more adapted to such condition, and preferred by oenologists because exalting the sensory properties of regional wines and their typical *terroir* character.

# 3. Genomic features of yeasts with sequenced genome

## 3.1 Introduction

The yeast genome is quite small and highly packed, with about 6000 genes distributed over 16 chromosomes. *S. cerevisiae* also has two small cytoplasmic genomes: mitochondrial DNA and 2μ plasmid. The nuclear genome structure is intimately linked to yeast genetic properties, which reciprocally influence its life style. The first strain sequenced, S288c, is a commonly used laboratory strain that was obtained in 1950s by mating a strain isolated from a rotten fig (EM93) with a commercial strain (Mortimer and Johnston, 1986). While experimental condition may have left a significant footprint on the evolution of S288c (Gu et al., 2005), since 1996 its genome sequence has been the only reference sequence available for *S. cerevisiae*. Today the genomes of several other yeast strains have been sequenced, including that of RM11-1a, a haploid derivative of a natural vineyard isolate (www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae/Home.html), the clinical isolateYJM789 (Wei et al., 2007), and the diploid, heterozygous wine yeast strain EC1118 widely used as starter in the wine industry (Novo et al., 2009). The sequence divergence between these strains and the reference has been estimated at 0.5-1%, similar to that between humans and chimpanzees.

### 3.1.1 Genetic Characteristics

*S. cerevisiae* strains are mostly diploid in natural condition and display vegetative reproduction through multi-polar budding. Under specific nutritional condition cells may sporulate to form four haploid spores of different mating types, a or α. One peculiarity of wine strains is that many are homotallic, and descendants of these haploid spores mate with their own progeny to form a diploid. Homotallism is frequent in wine yeast, with about 70% of strains known to be homotallic (Mortimer, 2000), but the ecological significance of this property remains unclear. Upon sporulation and the self-mating of homothallic spores, homozygous diploids are generated. This process makes it possible to eliminate recessives mutation deleterious for the strains or to ensure that recessive mutation increasing strain fitness are expressed. Genome renewal is therefore likely to play a role in adaptation of yeasts to stressful wine environment. Little is known about the

sexual activity of yeasts in wine environments. The frequency at each yeasts sporulate and mate in such environment is unknown. The ability of wine yeast to sporulate is highly heterogeneous and varies from 0% to 100% on laboratory media. Early genetic studies on wine yeasts indicated that most strains were diploid though some were polyploid or aneuploid (Bakalinsky and Snow, 1990). An estimation of DNA content of a large set of commercial "fermentation" strains recently showed that most of this strains had a DNA content close to 2n (Bradbury et al., 2006). Unlike other industrial yeasts (baker's yeast and brewing yeast strains), which have ploidy levels exceeding 2n, most of the *S. cerevisiae* strains used in wine-making seem to be diploid. *S. cerevisiae* has a small (75 kb), circular mitochondrial DNA genome that encodes a small set of proteins involved principally in respiration. Mitochondrial DNA is not essential for yeast survival but it was observed that the ethanol resistance can depend on it and that the ethanol tolerance of a laboratory strain could be enhanced by introducing mitochondria from a flor yeast (Ibeas and Jimenez, 1997).

### 3.1.2 Chromosomal Rearrangements and SNPs

The existence of gross chromosomal rearrangements, such as translocations, deletions and insertion, was rapidly suspected based on the high level of chromosome polymorphism found in wine yeasts. Analysis of wine yeast chromosomes by Pulsed Field Gel Electrophoresis (PFGE) demonstrates major chromosome length polymorphism between wine yeast strains. Such variation in chromosome size clearly resulted from gross chromosomal rearrangements (GCR). Recombination between repeated Ty elements interspersed throughout the genome is shown to be a major cause of chromosomal translocation (Rachidi et al., 1999). Other types of repeated sequences may also serve as substrates for ectopic recombinations leading to chromosomal rearrangements (Carro et al., 2003). Some gene copy-number changes are specific to wine yeasts and have been identified as a possible wine yeast signature (Dunn et al., 2005). The differences between wine strains are moderated and mostly concern genes encoding membrane transporters. The gene amplified in wine yeasts are mostly located at the end of chromosomes confirming the plasticity of sub-telomeric regions and their role in adaptation to industrial environments (Louis, 1995). The effects on yeast fitness of most of these rearrangements remain unclear, although no differences in fermentation properties are found between different

structural variants (Longo and Vezinhet, 1993). The best studied case of contribution to adaptation is that of a translocation between chromosome VIII and XVI, which has a direct impact on sulfite resistance (Perez-Ortin et al., 2002).
With their small and compact *S. cerevisiae* and hemiascomycetes represent a powerful model for comparative genomics and studies of genome evolution. As a result, more than 18 hemiascomycetes species are either completely or partially sequenced. The availability of the sequence data has presented an unprecedented opportunity to evaluate DNA sequence variation and genome evolution in a phylum spanning a broad evolutionary range. This wealth of data on interspecific sequence differences stands in contrast to our limited knowledge of sequence variation within *S. cerevisiae*. Several work recently tried to cover this gap of understanding (Liti et al., 2009, Schacherer et al., 2009).

### 3.1.3 Finishing and gene prediction

The process of finishing a genome is aimed to move it from a draft stage, the result of sequencing and initial assembly, to a complete genome. This process is very challenging and time consuming but indispensable because only with a small number of scaffolds and gaps in the assembly it is possible to reach a good level genomic and SNPs comparison. Furthermore only a complete genome sequence allows a reliable gene finding and annotation.
The gene prediction, or annotation, is the problem of identifying stretches of sequence (genes) in genomic DNA that are biologically functional, and to define their internal structure. Existing approaches to solve this problem fall into two groups with respect to the technique they utilize: intrinsic or ab initio methods and extrinsic or similarity-based ones. The first class uses only the information contained in the input genomic sequence: it searches for typical patterns that generally characterize coding boundaries, and other signals inside and outside gene regions. The second type applies the information coming from external sources as EST, proteins, or other known references.
As the entire genomes of many different species are sequenced, a promising direction in current research on gene finding is a comparative genomics approach. This is based on the principle that the forces of natural selection cause genes and other functional elements to undergo mutation at a slower rate than the rest of the genome, since mutations in functional elements are more likely to negatively impact the organism than mutations elsewhere. Genes can thus be

detected by comparing the genomes of related species to detect this evolutionary pressure for conservation. This approach was first applied to the mouse and human genomes, using programs such as SLAM, SGP and Twinscan/N-SCAN. Comparative gene finding can also be used to project high quality annotations from one genome to another. Notable examples include Projector, GeneWise and GeneMapper (Birney and Durbin, 2000).

### 3.2. From genotype to phenotype

The correlation between different phenotypes with importance in enology and specific molecular patterns would simplify the characterization of the indigenous yeast populations in wine yeast selection programs Recently, a close correlation between molecular polymorphism and specific phenotypic traits was reported in non-*Saccharomyces* wild yeast strains (Rodriguez et al., 2004). However, the results obtained from genotype–phenotype relationships studies in wild wine *S. cerevisiae* populations are controversial (Nadal et al., 1996, Comi et al., 2000). In these studies, the degree of correlation was estimated taking into account the total number of isolates as a whole. In these studies, the degree of correlation was estimated taking into account the total number of isolates as a whole. In other works, when this statistical method is applied very low correlation coefficients are obtained. The use of more powerful statistical tools as the Generalized Procrustes Analysis (GPA) for the simultaneous analysis of molecular and physiological traits (Gower, 1975) allow to weigh the relationships for each isolate in particular, denoting a better degree of agreement between molecular and physiological data for most of the population analysed. Application of the GPA in studies on the genetic and/or phenotypic variability in the microbiological field evidence the possibility to quantify the relationship between molecular and phenotypic characteristics in wine yeasts (Lopes et al., 2006).

The NCBI Genome Project Database reports 46 genome sequencing projects on different strains of *S. cerevisiae*. Only the genome of *S. cerevisiae* S288c is completed, among the other projects, 27 genomes are assembled with coverage depths varying from 2.6 to 20x and 18 are still in progress. The sequenced strains include lab, pathogenic, baking, wine, natural fermentation, sake, probiotic and plant isolates. Most of the sequencing projects leaded to the comparison of the genomes of different strains to correlate genomic traits to specific phenotypes and to infer phylogenetic relationships and evolutionary histories. Analysis of

closely related strains have been performed too, for example genome of six commercial strains of *S. cerevisiae* used in wine fermentation and brewing were compared to find characteristics typical of these industrial classes of yeast (Borneman et al., 2011).

Regularly updated information concerning the genomic and functional analysis of yeasts is available on a number of extensive databases. These include the Génolevures project web site (Souciet, 2011), the Stanford Genome Database (SGD), the Munich information Center for Protein Sequences Comprehensive yeast Genome database (MIPS CYGD) and the Yeast Proteome Database (YPD).

Furthermore genome-wide transcriptional profiling has important applications in evolutionary biology for assaying the extent of heterozygosity for alleles showing quantitative variation in gene expression in natural populations. These studies have, in turn, stimulated renewed interest in the interactions among metabolic pathways and the control of metabolic flux. Most experiments thus far have dealt with comparisons of patterns of gene expression of organisms with the same genotype grown under different conditions or at different stages of the cell cycle. Genetic variability of wine yeasts has been demonstrated using various analysis tools at the molecular level (Schuller et al. 2004). The aCGH analysis has established that major differences between laboratory strains of *S. cerevisiae* are found in subtelomeric regions (Winzeler et al., 2003) and that *S. cerevisiae* wine strains show a gene copy number variation that differentiate them from laboratory strains and strains of clinical origin. Differences were found in genes related to the fermentative process such as membrane transporters, ethanol metabolism and metal resistance (Dunn et al., 2005, Carreto et al., 2008). With the objective of studying genomic and phenotypic changes between similar yeasts isolated from different origins, several genomic and phenotypic comparison of strains has been carried out. Various kinetic and fermentative parameters were evaluated and significant phenotypic differences were detected between strains, some of which may be explained by differences at the genomic level.

### 3.3 Next generation sequencing technology

In the last decade the incredible development of high-throughput and low-cost sequencing platforms have allowed to increase rapidly the number of sequenced genomes and stimulates the creation of new protocols to use these technologies

to study other aspects of the cell, such as transcriptional profiles, chromatin structures, non-conding RNAs. In fact, Next Generation Sequencing (NGS) technologies have a great impact both at economical and at research level, with increasing of data production and cost reduction. This new kind of techniques allow the sequencing of thousands of genomes from humans to microbes and they open entirely new areas of biological inquiry, including the investigation of ancient genomes, of human disease, the characterization of ecological diversity, and the identification of unknown etiological agents. The application field could be divided into three main arguments: genomic tasks (genome assembly, SNPs and structural variations), transcriptome analysis (gene prediction and annotation, alternative splicing discovering) and epigenetic problems.

Three commercial platforms are currently well established on the market, the Roche 454 Genome Sequencer, the Illumina Genome Analyzer, and the Life Technologies SOLiD System, but other technologies are also available or under development. All these high-throughput sequencing systems use new sequencing chemistries replacing Sanger's technology and do not require electrophoresis and individual amplification of the templates. They are based on the parallelization of the sequencing process to produce thousands of sequences at once and lower costs and time required for DNA sequencing (Zhou et al., 2010).

Before the coming of these technologies, big consortiums of laboratories were required to sequence just one genome. Today, on the contrary, also small labs can cope with sequencing projects. Thanks to these powerful technologies it is now possible to sequence lots of genomes and get several information by the comparison of them. As said, the sequencing of yeast strains used in winemaking, can be a powerful approach to identify the still unknown genes involved in fermentation and development of typical aroma. Moreover the transcriptional profile (complete set of transcripts in a cell for a specific physiological condition) of a strain, can be used to identify the differentially expressed genes with respect to other strains and to see how differences in the genome are mirrored by gene expression, and more generally by the phenotype.

### 3.3.1 Phylogenetic Relationship

During its long history of association with human activity, the genomic makeup of the yeast *S. cerevisiae* is thought to have been shaped through the action of

multiple independent rounds of wild yeast domestication combined with thousands of generations of artificial selection. As the evolutionary constraints that were applied to the *S. cerevisiae* genome during these domestication events were ultimately dependent on the desired function of the yeast (e.g baking, brewing, wine or bioethanol production), this multitude of selective schemes have produced large numbers of *S. cerevisiae* strains, with highly specialized phenotypes that suit specific applications (Querol et al., 2003, Fay et al., 2005). As a result, the study of industrial strains of *S. cerevisiae* provides an excellent model of how reproductive isolation and divergent selective pressures can shape the genomic content of a species There have been several attempts to characterize the genomes of industrial strains of *S. cerevisiae* which have uncovered differences that included single nucleotide polymorphisms (SNPs), strain-specific ORFs and localized variations in genomic copy number. However, the type and scope of genomic variation documented by these studies were limited either by technology constraints (e.g CGH arrays relying on the laboratory strain as a ‘‘reference’’ genome), or by the resources required for the production of high quality genomic assemblies which has limited the scope and number of whole-genome sequences available for comparison. In addition, to limit genomic complexity to a manageable level, previously published whole-genome sequencing studies on industrial strains used haploid representations of diploid, and often heterozygous, commercial and environmental strains (Liti et al., 2009, Borneman et al., 2008, Doniger et al., 2008, Argueso et al., 2009).

## 3.4 Materials and methods

### 3.4.1 Strains

In this work 213 autochthonous strains of *Saccharomices cerevisiae* isolated from vineyards located in the DOCG Prosecco Conegliano-Valdobbiadene, DOC Piave and DOCG Lison-Pramaggiore areas were considered. The strains were obtained after single fermentation of bunches of Glera (ex Prosecco) variety, Raboso and Tocai Italico varieties.

The survey was also conducted on 10 commercial strains, coming from wine, and 2 laboratory strains.

All the strains considered are reported in table 3.1

| NAME | ORIGIN |
|---|---|
| EC1118 | Wine, EUROPE (sequenced) |
| FR95 | Wine, EUROPE |
| MYC611 | Wine, EUROPE |
| CRU31 | Wine, EUROPE |
| P444 | Wine, EUROPE |
| QA23 | Wine, EUROPE (sequenced) |
| GY | Wine, EUROPE |
| 71B | Wine, EUROPE (sequenced) |
| CRIO SP | Wine, EUROPE (sequenced) |
| VRB | Wine, EUROPE |
| S288C | Laboratory, USA (sequenced) |
| Σ1278b | Laboratory, USA (sequenced) |
| B125.1 | Autochthonous, Glera bunches |
| B169.12 | Autochthonous, Glera bunches |
| B173.2 | Autochthonous, Glera bunches |
| B173.4 | Autochthonous, Glera bunches |
| B197.1 | Autochthonous, Glera bunches |
| B217.2 | Autochthonous, Glera bunches |
| B223.8 | Autochthonous, Glera bunches |
| P138.4 | Autochthonous, Glera bunches |
| P148.1 | Autochthonous, Glera bunches |
| P158.4 | Autochthonous, Glera bunches |
| P173.3 | Autochthonous, Glera bunches |
| P227.11 | Autochthonous, Glera bunches |
| P234.15 | Autochthonous, Glera bunches |
| P234.5 | Autochthonous, Glera bunches |
| P254.1 | Autochthonous, Glera bunches |
| P254.10 | Autochthonous, Glera bunches |
| P254.16 | Autochthonous, Glera bunches |
| P283.4 | Autochthonous, Glera bunches |
| P293.1 | Autochthonous, Glera bunches |
| P293.8 | Autochthonous, Glera bunches |
| P301.16 | Autochthonous, Glera bunches |
| P301.3 | Autochthonous, Glera bunches |
| P301.4 | Autochthonous, Glera bunches |
| P304.1 | Autochthonous, Glera bunches |
| P304.11 | Autochthonous, Glera bunches |
| P304.13 | Autochthonous, Glera bunches |

| | |
|---|---|
| P304.2 | Autochthonous, Glera bunches |
| P304.3 | Autochthonous, Glera bunches |
| P304.4 | Autochthonous, Glera bunches |
| P304.5 | Autochthonous, Glera bunches |
| P304.6 | Autochthonous, Glera bunches |
| S41 | Autochthonous, Glera bunches |
| S46 | Autochthonous, Glera bunches |
| S47 | Autochthonous, Glera bunches |
| R100.1 | Autochthonous, Raboso bunches |
| R101.1 | Autochthonous, Raboso bunches |
| R101.2 | Autochthonous, Raboso bunches |
| R101.3 | Autochthonous, Raboso bunches |
| R101.4 | Autochthonous, Raboso bunches |
| R101.5 | Autochthonous, Raboso bunches |
| R102.1 | Autochthonous, Raboso bunches |
| R102.2 | Autochthonous, Raboso bunches |
| R102.3 | Autochthonous, Raboso bunches |
| R103.1 | Autochthonous, Raboso bunches |
| R103.3 | Autochthonous, Raboso bunches |
| R103.4 | Autochthonous, Raboso bunches |
| R103.5 | Autochthonous, Raboso bunches |
| R104.2 | Autochthonous, Raboso bunches |
| R104.4 | Autochthonous, Raboso bunches |
| R104.5 | Autochthonous, Raboso bunches |
| R105.2 | Autochthonous, Raboso bunches |
| R105.3 | Autochthonous, Raboso bunches |
| R105.5 | Autochthonous, Raboso bunches |
| R106.2 | Autochthonous, Raboso bunches |
| R106.3 | Autochthonous, Raboso bunches |
| R106.5 | Autochthonous, Raboso bunches |
| R107.1 | Autochthonous, Raboso bunches |
| R107.3 | Autochthonous, Raboso bunches |
| R107.4 | Autochthonous, Raboso bunches |
| R107.5 | Autochthonous, Raboso bunches |
| R109.1 | Autochthonous, Raboso bunches |
| R11.1 | Autochthonous, Raboso bunches |
| R11.3 | Autochthonous, Raboso bunches |
| R110.1 | Autochthonous, Raboso bunches |
| R110.4 | Autochthonous, Raboso bunches |
| R111.1 | Autochthonous, Raboso bunches |
| R111.2 | Autochthonous, Raboso bunches |
| R111.5 | Autochthonous, Raboso bunches |
| R113.2 | Autochthonous, Raboso bunches |
| R113.3 | Autochthonous, Raboso bunches |
| R114.2 | Autochthonous, Raboso bunches |
| R115.2 | Autochthonous, Raboso bunches |
| R115.3 | Autochthonous, Raboso bunches |
| R115.5 | Autochthonous, Raboso bunches |
| R116.1 | Autochthonous, Raboso bunches |
| R116.3 | Autochthonous, Raboso bunches |
| R116.5 | Autochthonous, Raboso bunches |
| R117.1 | Autochthonous, Raboso bunches |
| R117.2 | Autochthonous, Raboso bunches |
| R117.5 | Autochthonous, Raboso bunches |
| R119.1 | Autochthonous, Raboso bunches |
| R119.2 | Autochthonous, Raboso bunches |
| R119.3 | Autochthonous, Raboso bunches |
| R119.5 | Autochthonous, Raboso bunches |
| R12.1 | Autochthonous, Raboso bunches |
| R12.2 | Autochthonous, Raboso bunches |

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|---|---|
| R12.3 | Autochthonous, Raboso bunches |
| R120.1 | Autochthonous, Raboso bunches |
| R120.2 | Autochthonous, Raboso bunches |
| R126.1 | Autochthonous, Raboso bunches |
| R127.4 | Autochthonous, Raboso bunches |
| R128.1 | Autochthonous, Raboso bunches |
| R130.1 | Autochthonous, Raboso bunches |
| R130.2 | Autochthonous, Raboso bunches |
| R130.3 | Autochthonous, Raboso bunches |
| R130.4 | Autochthonous, Raboso bunches |
| R131.2 | Autochthonous, Raboso bunches |
| R131.3 | Autochthonous, Raboso bunches |
| R131.4 | Autochthonous, Raboso bunches |
| R132.3 | Autochthonous, Raboso bunches |
| R132.5 | Autochthonous, Raboso bunches |
| R133.3 | Autochthonous, Raboso bunches |
| R133.4 | Autochthonous, Raboso bunches |
| R133.5 | Autochthonous, Raboso bunches |
| R135.2 | Autochthonous, Raboso bunches |
| R135.3 | Autochthonous, Raboso bunches |
| R136.3 | Autochthonous, Raboso bunches |
| R136.5 | Autochthonous, Raboso bunches |
| R137.2 | Autochthonous, Raboso bunches |
| R138.4 | Autochthonous, Raboso bunches |
| R138.5 | Autochthonous, Raboso bunches |
| R139.2 | Autochthonous, Raboso bunches |
| R139.3 | Autochthonous, Raboso bunches |
| R139.4 | Autochthonous, Raboso bunches |
| R14.1 | Autochthonous, Raboso bunches |
| R14.2 | Autochthonous, Raboso bunches |
| R14.3 | Autochthonous, Raboso bunches |
| R14.4 | Autochthonous, Raboso bunches |
| R14.5 | Autochthonous, Raboso bunches |
| R14.6 | Autochthonous, Raboso bunches |
| R14.7 | Autochthonous, Raboso bunches |
| R143.1 | Autochthonous, Raboso bunches |
| R143.2 | Autochthonous, Raboso bunches |
| R144.1 | Autochthonous, Raboso bunches |
| R144.3 | Autochthonous, Raboso bunches |
| R146.1 | Autochthonous, Raboso bunches |
| R146.2 | Autochthonous, Raboso bunches |
| R146.3 | Autochthonous, Raboso bunches |
| R146.4 | Autochthonous, Raboso bunches |
| R146.5 | Autochthonous, Raboso bunches |
| R149.1 | Autochthonous, Raboso bunches |
| R15.1 | Autochthonous, Raboso bunches |
| R15.2 | Autochthonous, Raboso bunches |
| R15.3 | Autochthonous, Raboso bunches |
| R15.4 | Autochthonous, Raboso bunches |
| R15.5 | Autochthonous, Raboso bunches |
| R15.6 | Autochthonous, Raboso bunches |
| R15.7 | Autochthonous, Raboso bunches |
| R150.1 | Autochthonous, Raboso bunches |
| R150.2 | Autochthonous, Raboso bunches |
| R150.3 | Autochthonous, Raboso bunches |
| R150.4 | Autochthonous, Raboso bunches |
| R150.5 | Autochthonous, Raboso bunches |
| R151.1 | Autochthonous, Raboso bunches |
| R151.2 | Autochthonous, Raboso bunches |
| R152.1 | Autochthonous, Raboso bunches |

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|---|---|
| R152.2 | Autochthonous, Raboso bunches |
| R152.4 | Autochthonous, Raboso bunches |
| R152.5 | Autochthonous, Raboso bunches |
| R153.1 | Autochthonous, Raboso bunches |
| R153.2 | Autochthonous, Raboso bunches |
| R153.4 | Autochthonous, Raboso bunches |
| R153.5 | Autochthonous, Raboso bunches |
| R154.1 | Autochthonous, Raboso bunches |
| R154.3 | Autochthonous, Raboso bunches |
| R154.4 | Autochthonous, Raboso bunches |
| R155.1 | Autochthonous, Raboso bunches |
| R155.2 | Autochthonous, Raboso bunches |
| R155.3 | Autochthonous, Raboso bunches |
| R155.4 | Autochthonous, Raboso bunches |
| R157.1 | Autochthonous, Raboso bunches |
| R157.2 | Autochthonous, Raboso bunches |
| R157.3 | Autochthonous, Raboso bunches |
| R157.4 | Autochthonous, Raboso bunches |
| R16.1 | Autochthonous, Raboso bunches |
| R16.2 | Autochthonous, Raboso bunches |
| R17.1 | Autochthonous, Raboso bunches |
| R17.2 | Autochthonous, Raboso bunches |
| R31.1 | Autochthonous, Raboso bunches |
| R31.2 | Autochthonous, Raboso bunches |
| R31.3 | Autochthonous, Raboso bunches |
| R31.4 | Autochthonous, Raboso bunches |
| R31.5 | Autochthonous, Raboso bunches |
| R31.6 | Autochthonous, Raboso bunches |
| R32.1 | Autochthonous, Raboso bunches |
| R32.2 | Autochthonous, Raboso bunches |
| R35.1 | Autochthonous, Raboso bunches |
| R35.2 | Autochthonous, Raboso bunches |
| R35.4 | Autochthonous, Raboso bunches |
| R35.5 | Autochthonous, Raboso bunches |
| R5.2 | Autochthonous, Raboso bunches |
| R6.1 | Autochthonous, Raboso bunches |
| R6.7 | Autochthonous, Raboso bunches |
| R7.1 | Autochthonous, Raboso bunches |
| R7.2 | Autochthonous, Raboso bunches |
| R8.2 | Autochthonous, Raboso bunches |
| R8.3 | Autochthonous, Raboso bunches |
| R8.5 | Autochthonous, Raboso bunches |
| R8.6 | Autochthonous, Raboso bunches |
| R8.7 | Autochthonous, Raboso bunches |
| T113B.1 | Autochthonous, Tocai Italico bunches |
| T21.1 | Autochthonous, Tocai Italico bunches |
| T23.1 | Autochthonous, Tocai Italico bunches |
| T306.11 | Autochthonous, Tocai Italico bunches |
| T314.1 | Autochthonous, Tocai Italico bunches |
| T317.2 | Autochthonous, Tocai Italico bunches |
| T411.1 | Autochthonous, Tocai Italico bunches |
| T411.10 | Autochthonous, Tocai Italico bunches |
| T415.1 | Autochthonous, Tocai Italico bunches |
| T424.1 | Autochthonous, Tocai Italico bunches |
| T522.13 | Autochthonous, Tocai Italico bunches |
| T525.1 | Autochthonous, Tocai Italico bunches |
| T602.3 | Autochthonous, Tocai Italico bunches |
| T603.2 | Autochthonous, Tocai Italico bunches |
| T604.3 | Autochthonous, Tocai Italico bunches |
| T605.3 | Autochthonous, Tocai Italico bunches |

| | |
|---|---|
| T605.5 | Autochthonous, Tocai Italico bunches |
| T605.7 | Autochthonous, Tocai Italico bunches |
| T606.3 | Autochthonous, Tocai Italico bunches |
| T606.4 | Autochthonous, Tocai Italico bunches |
| T606.8 | Autochthonous, Tocai Italico bunches |
| T611.4 | Autochthonous, Tocai Italico bunches |
| T9.1 | Autochthonous, Tocai Italico bunches |

**Table 3.1** Strains tested in this study

### 3.4.2 DNA isolation

Yeast cells were cultivated in 10 ml YPD medium (36 h, 25° C, 150 rpm) and genomic DNA was isolated by E.Z.N.A® yeast DNA kit (OMEGA Bio-Tech, USA).

### 3.4.3 Real-time analyses performed to verify translocations

Real-Time PCR was carried out on a CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells).

Reactions were prepared in a total volume of 15 µl containing:

| | |
|---|---|
| Primer F (MWG) | 0,4 µM |
| Primer R (MWG) | 0,4 µM |
| RNase,/DNase-free water | 0,1 µl |
| SsoFast EvaGreen Supermix (Bio- Rad) | 1X |
| DNA | 5 µl (approximately 10 ng) |

**Table 3.2** PCR master mix composition

Primers utilized are reported below (table 3.3).

| Name | Sequence (5'-3') |
|---|---|
| chr16_A_F | AGAACCGTGCTGCTCGTAAG |
| chr16_B_R | GCAAGCGATAGCAAACATGA |
| chr8_C_R | CATGGCAGCTAGAACCATCA |
| | |
| chr15_A_F | GCCGTATACCGTTGCTCATT |
| chr15_B_R | CAAGGTTTACCCTGCGCTAA |
| chr16_C_R | ACCAGCGGAATGATATCCAG |

**Table 3.3** Primers for amplification

The cycle conditions were set as follows:

initial template denaturation at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 sec, and combined primer annealing/elongation at 57°C for 40 sec.

The amount of fluorescence for each sample, given by the incorporation of EvaGreen into dsDNA, was measured at the end of each cycle and analysed via CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). Melting curves of PCR amplicons were obtained with temperatures ranging from 65°C to 95°C. Data acquisition was performed for every 0.2°C increase in temperature, with a 5 sec step.

### 3.4.5 Real-time analyses performed on strain-specific genes and high resolution melting analyses on SNPs.

Real-Time PCR was carried out on a CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells).

Reactions were prepared in a total volume of 15 µl containing:

| | |
|---|---|
| Primer F (MWG) | 0,4 µM |
| Primer R (MWG) | 0,4 µM |
| RNase,/DNase-free water | 0,1 µl |
| SsoFast EvaGreen Supermix (Bio- Rad) | 1X |
| DNA | 5 µl (approximately 10 ng) |

**Table 3.4** PCR master mix composition

Primers utilized are reported below (table 3.5).

| Name | Sequence (5'-3') |
|---|---|
| YHR162W F | GGATACGGAATGGCGACTCT |
| YHR162W R | GCGTTTATCTGCCCGTAGT |
| YDL168W F | ATGCTTTGGAAGCCTGTCAT |
| YDL168W R | CAAAAGCAGAGCCTTTCCAC |
| P301_O30021 F | CTTACCCGAGTCACCACGTT |
| P301_O30021 R | GTAAACAAGTGCCCGACGAT |
| R008_O14131 F | GAAACTTAATCGGCCCACAA |
| R008_O14131 R | TACCTGCCCTCCAATCTCTG |

**Table 3.5** Primers for amplification

The cycle conditions were set as follows:
initial template denaturation at 98°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 2 s, and combined primer annealing/elongation at 60°C (57°C for YDL168W primers) for 10 s.
The amount of fluorescence for each sample, given by the incorporation of EvaGreen into dsDNA, was measured at the end of each cycle and analysed via CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). Melting curves of PCR amplicons were obtained with temperatures ranging from 65°C to 95°C. Data acquisition was performed for every 0.2°C increase in temperature, with a 10 s step.
Results have been analysed by High-Resolution Melting analysis software (Bio-Rad Laboratories, Inc.), which automatically clusters the samples according to their melting profiles and assigns a confidence score to each sample. The confidence level threshold for a sample to be included in a cluster was 98.0%.

## 3.5 Results and discussions

### 3.5.1 Single nucleotide variations in enological strains

For SNPs analysis 18 *S. cerevisiae* strains have been selected among those with the best assembly quality in order to simplify the alignment process. The aim was to classify the 4 autochthonous strains in comparison with other yeasts having different geographical location, ecology or associated with different fermentation technologies, with no interest in a global population structure analysis since this is already been done (Liti et al., 2009, Schacherer et al., 2009, Legras et al., 2007). Strains selected comprise 11 wine strains having different origin (commercial and wild type –ecotypical- isolates) P283.4, P301.4, R8.3, R103.1, EC1118, AWRI1796, RM11, QA23, VL3, VIN13 and AWRI1631, two strains involved in beer fermentation, FosterO and FosterB, one used in Sake production, Kyokay7, one used for bioethanol production, a clinical isolate, YJM789, and two laboratory strains, S288c and Σ1278b. Polymorphisms were identified after genome alignment using MAUVE software for a total of 368408 SNPs. Pairwise SNPs difference in alignments were determined using dedicated PERL script and were used to determine a neighbour-joining tree using Phylip package. Heterozygous positions in the genome of diploid and tetraploid strains (Borneman et al., 2011) were also taken into consideration as SNPs differences. It is clear from the phylogenetic tree obtained considering the number of SNPs a measure of strain relatedness (data not shown) that ecotypical strains clustered in the same lineage with all other wine strains independently from their geographic origin. In fact they were grouped together with EC1118 strain isolated from Champagne fermentations, AWRI1631 deriving from N96 (related to EC1118), RM11 collected from a California vineyard and QA23 selected in Portugal. Strains derived from other technological environments (beer, laboratory, sake, pathogens) are more distantly related to oenological strains.

Furthermore SNPs identified in oenological strains have been analyzed and all bases present in oenological strains have been compared to all other strains. 306 positions have been found that are conserved in all oenological strains, but diverged in at least one of the other strains. Despite these position could be conserved because large part of wine yeasts are members of a single well-defined subpopulation and probably derive from a single (or a very small number) of domestication events (Schacherer et al., 2009, Legras et al., 2007), it is not possible to exclude that these are related to the function of some genes with a

significant role in oenological environments. In order to gain a better understand of this point, these data have been analyzed using SNPeff software (http://snpeff.sourceforge.net) in order to classify SNPs respect to their effect on protein-coding genes (synonimous and non-synonimous changes, changes in upstream and downstream regions). From the comparison with the reference strain we detected 74 non-synonymous amino acids changes (NSC) localized on 52 genes, 126 synonymous changes (SC) on 75 genes and the remaining SNPs localized in intergenic regions. Both SNPs determining SC and NSC tend to greatly affect chr 10.

Then it has been determined the conservation level of aminoacids (AA) modified by NSC in oenological strains by checking protein alignment among seven *Saccharomyces yeasts* (*S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevii, S. bayanus, S. castelli, S. kluyveri*) in the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). It has been found that 15 of these AA positions were highly conserved and this suggested that protein modification could have a functional role. Two of these genes were particularly interesting: YDL168W and YHR162W.

| Gene | Description |
|---|---|
| YDL168W | Bifunctional alcohol dehydrogenase and formaldehyde dehydrogenase; formaldehyde dehydrogenase activity is glutathione-dependent; functions in formaldehyde detoxification and formation of long chain and complex alcohols, regulated by Hog1p-Sko1p; protein abundance increases in response to DNA replication stress |
| YHR162W | Highly conserved subunit of the mitochondrial pyruvate carrier; a mitochondrial inner membrane complex comprised of Fmp37p/Mpc1p and either Mpc2p or Fmp43p/Mpc3p mediates mitochondrial pyruvate uptake; more highly expressed in glucose-containing minimal medium than in lactate-containing medium |

**Table 3.6** Genes of interest.

The first one (YDL168W) encodes a bifunctional enzyme containing both alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase activities involved in formaldehyde detoxification and formation of long chain and complex alcohols. This bifunctional enzyme is involved in aminoacid catabolism by the production of fusel alcohols during fermentation (Vaughan-Martini et al.,

1993) which contributes to the flavour and aroma of yeast-fermented foods and beverages (Alexandre et al., 2001).

The second gene (YHR162W) encodes Mpc2p, a subunit of the mitochondrial pyruvate transporter highly conserved in eukaryotes. The G>A change identified in oenological strains was responsible for the predicted Gly117Ser substitution in the C-terminal portion of the protein. Pyruvate is a key molecule involved in ethanol, amino acids and acetate production during alcoholic fermentation and is also the precursor of many important sensory constituents of alcoholic beverages, such as diacetyl. This compound , which has a butter-like flavour, is a critical off-flavour produced during fermentation and it derives from pyruvate. It was demonstrated that the selection of strains having increased mitochondrial pyruvate transport reduces off-flavours in alcoholic beverages (Horie et al., 2010), but the gene/s responsible for this phenotypic character is yet to be determined exactly.

Using the high resolution melting module (HRM) it has been examined the frequency of the two SNPs identified in YHR162W (Figure 3.1) and YDL168W (Figure 3.3) genes in the 213 autochthonous yeasts, and have been considered also ten commercial strains and two laboratory strains (controls). Results obtained from genome sequencing have been confirmed.

Figure 3.2 shows that a very high fraction of the 213 vineyard strains, about 83% (177/213), gave the same SNP calling for SNP on YHR162W than the vineyard strains sequenced (cluster 1, red lines in fig. 3.1). A smaller fraction, about 16% (34/213) gave the same SNP calling for SNP on YHR162W than the laboratory strains (cluster 2, green lines in fig. 3.1). Another small cluster, number 3 (orange line in fig. 3.1), contains 2 Prosecco strains, P148.1 and B217.12, with a different genotype. In commercial strains the result obtained using HRM is even more extreme than for autochthonous, with 9/10 of the strains giving the same SNP calling for SNP on YHR162W than the vineyard strains sequenced, and 1 commercial strain, 71B, clustering alone in a different cluster (cluster 4, blue line in fig. 3.1).

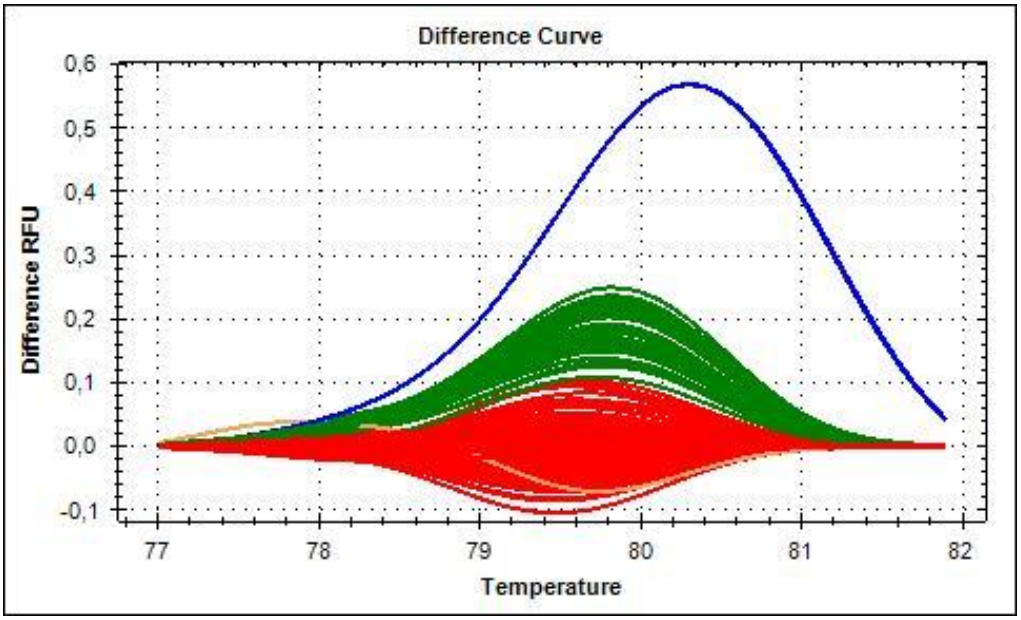



**Fig. 3.1** Normalized difference curve for different genotypes for SNP on YHR162W.

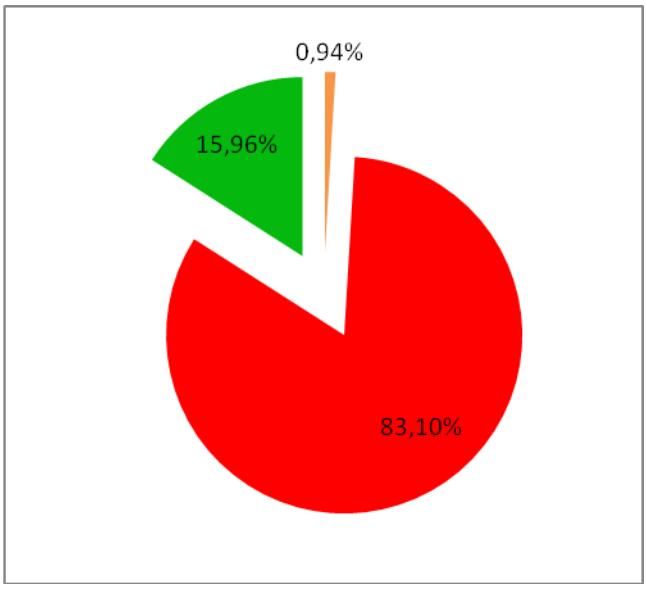



**Fig. 3.2** Frequency of autochthonous strains in cluster 1 (■) cluster 2 (■) and cluster 3 (■) for SNP on YHR162W.

Figure 3.4 shows that a very high fraction of the 213 vineyard strains, about 83% (177/213), gave the same SNP calling for SNP on YDL168W than the vineyard strains sequenced (cluster 1, red lines in fig. 3.3). A smaller fraction, about 10% (21/213) gave the same SNP calling for SNP on YDL168W than the laboratory strains (cluster 2, green lines in fig. 3.3). Cluster 3 (blue line in fig. 3.3) contains 2 strains isolated in Prosecco area and 6 strains isolated in Raboso area. In cluster

4 grouped 2 Raboso strains and in cluster 5 grouped 2 Tocai strains. Three other small cluster (6, 7 and 8) contain just 1 strain.

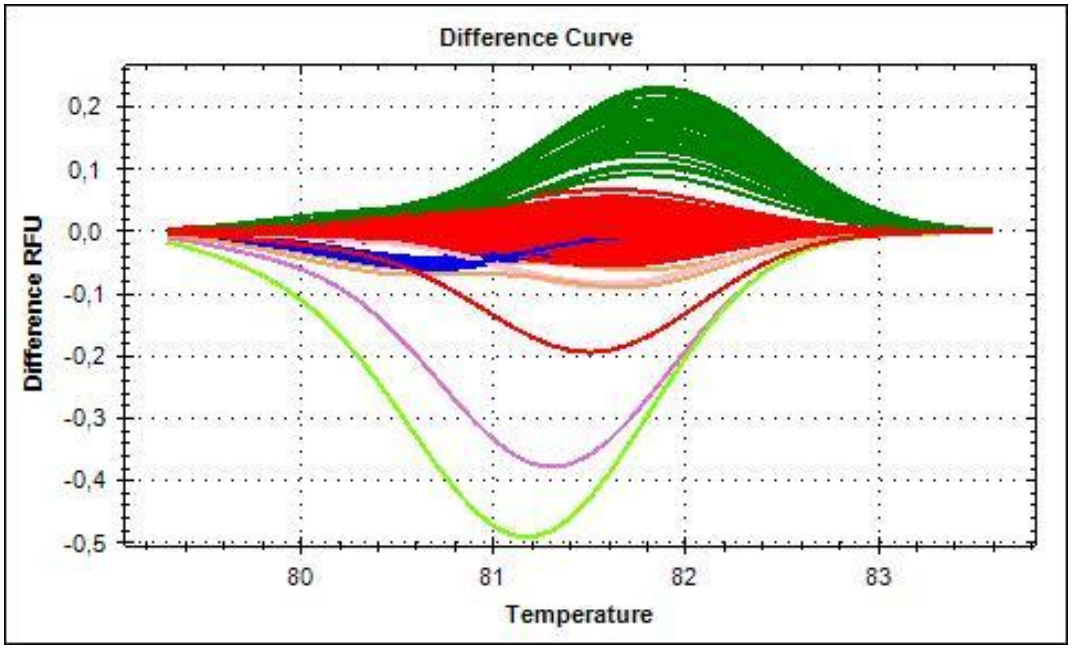



**Fig. 3.3** Normalized difference curve for different genotypes for SNP on YDL168W.

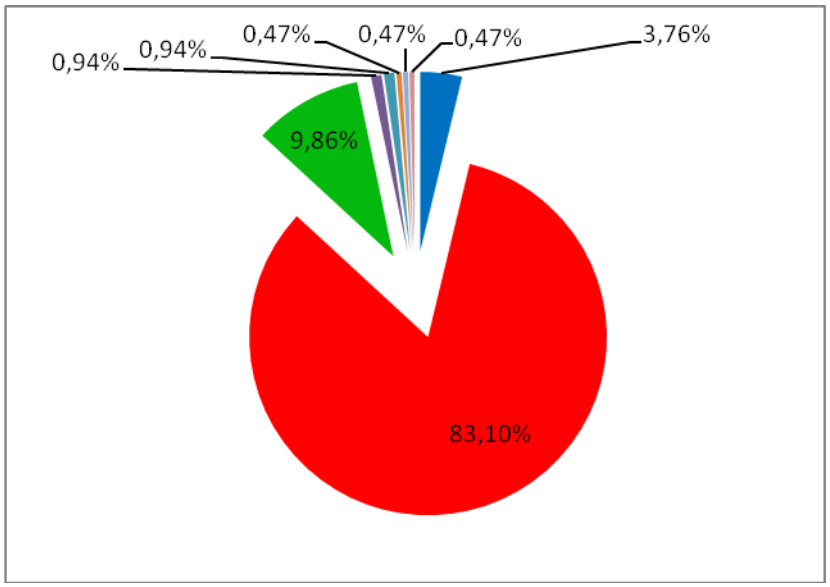



**Fig. 3.4** Frequency of autochthonous strains in cluster 1 (■) cluster 2 (■) cluster 3 (■) cluster 4 (■) cluster 5 (■) cluster 6 (■) cluster 7 (■) cluster 8 (■) for SNP on YDL168W.

The large number of cluster is probably due to the fact that, in addition to the G>A mutation, there are other less frequent mutation in the amplified region; moreover it is possible that some strains have the mutation in heterozygosis, so the genotype curve is different and they cluster separately.

In commercial strains the result obtained using HRM is even more extreme than for autochthonous, with 10/10 of the strains giving the same SNP calling for SNP on YHR162W than the vineyard strains.

### 3.5.2 Gene finding and annotation

The four sequenced genomes were annotated using the program RATT (Otto et al.,2011) which transfers orthologous genes from S288c to the four vineyard strains, while genome analysis with GeneMark.hmm was performed to identify strain-specific genes (Payne et al., 2008, Pizarro et al., 2008).

From the annotated 6607 ORFs of S288c genome, RATT transferred from 5580 to 5722 features in the different strains. Manual verification of the results obtained led to the removal of some dubious ORFs and to the identification of some genes derived from the fusion of adjacent ORFs in S288c. From genome alignments, 17 regions larger than 3 kbp, specific of the vineyard strains and absent in S288c, were identified. Not all these regions were present in all the four strains sequenced and some of these are slightly different in size. In seven of these strain specific regions, putative protein-coding genes were found and subsequently annotated through blast search. In these regions, gene finding revealed the presence of protein coding genes absent in S288c reference strain, named “strain-specific”. Besides the core gene set present in all the *S. cerevisiae* strains, a complementary specific set of ORFs characterize single strains (Mortimer, 2000, Goffeau et al., 1996, Dunn et al., 2012). 33 strain-specific ORFs have been identified; most of them has been annotated while some remain with unknown function. Two of these “strain-specific genes” are of particular interest: the allantoate transporter (R008_O14131) and the putative fructose symporter (P301_O30021).

| Gene | Description |
|---|---|
| R008_O14131 | Putative allantoate permease (low similarity with *S. cerevisiae* AWRI796) |
| P301_O30021 | Putative fructose symporter (low similarity with *L. thermotolerans*) |

**Table 3.7** “Strain-specific genes” of interest.

In *S. cerevisiae* the gene responsible for allantoate and ureidosuccinate transport is DAL5. It plays a role in the utilization of dipeptides as a nitrogen source (Cai et al., 2007), an important character in the fermentation of poor nitrogen media, like must. This character could be also relevant for yeast to develop in other environments such as the grape bunch surface or the bark of trees. A gene expression study suggests that R008_O4131 is subjected to nitrogen catabolite repression process (NCR), because its expression increases more than 29 times at 45 g/l when the concentration of poor nitrogen sources like allantoin become prevailing (data not published).

P301_O30021 has a high similarity to the gene encoding the fructose transporter Fsy1p in EC1118 strain. It was reported that this gene derives from a lateral gene transfer event and was found in other winemaking strains (Giudici et al., 2005), but it was possible to find this gene only in EC1118 and in P301.4. A gene expression study revealed a very similar behaviour of P301_O30021 in P301.4 and in EC1118, with a more than two fold increase in the second part of the fermentation. This confirms the importance of fructose utilization at the end of the fermentation process.

The presence of allantoate permease and fructose transporter genes have been verified by real time PCR in 213 *S.cerevisiae* autochthonous strains, included the four sequenced vineyard strains. Also 10 commercial and two laboratory strains have been considered.

Real-time PCR analysis revealed that fructose transporter was present in 50 out of 213 vineyard isolates, nearly 23,5% of the autochthonous strains (Figure 3.5a). Fructose transporter was identified in 7 out of 10 of the commercial strains (70%), suggesting that this gene seems to be positively selected in oenological strains (Figure 3.5b).

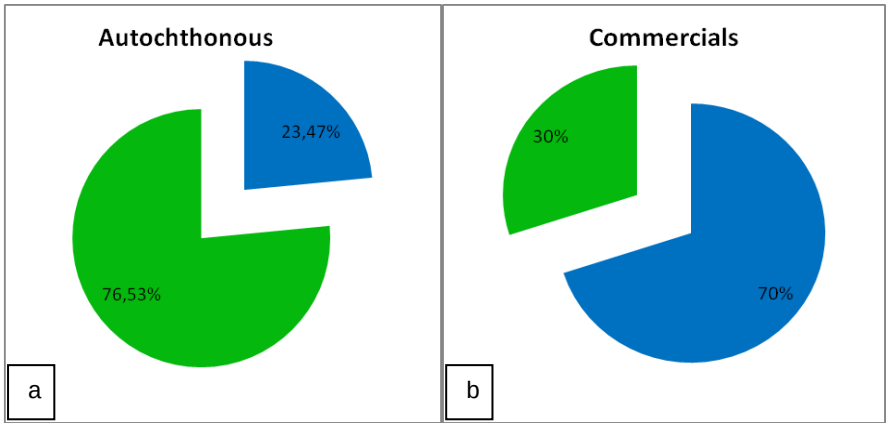



**Figure 3.5** Frequency of autochthonous (a) and commercial (b) strains owning (■) and not owning (■) fructose transporter.

In greater detail, autochthonous strains show different trends: fructose transporter was present in 42 out of 156 Raboso isolates, nearly 27% (Figure 3.6a), in 7 out of 34 Prosecco isolates, nearly 21% (Figure 3.6b) and in 1 out of 23 Tocai isolates, nearly 4% (Figure 3.6c). This result seems to confirm that Tocai isolates are genetically different from Prosecco and Raboso strains, as previously seen (data not published, manuscript preparing).

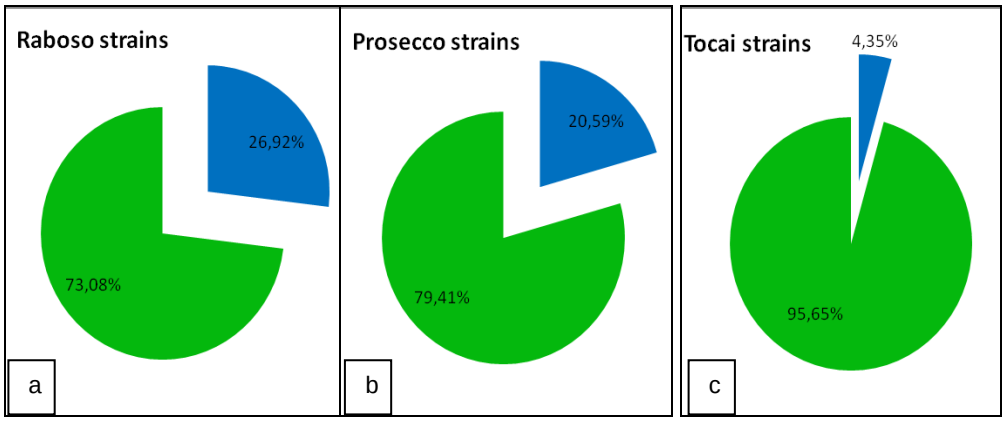



**Figure 3.6** Frequency of Raboso (a), Prosecco (b) and Tocai (c) strains owning (■) and not owning (■) fructose transporter.

Allantoate permease was present in 22 out of 213 vineyard isolates, nearly 10% of the autochthonous strains (Figure 3.7). Allantoate permease was identified in none of the commercial strains, suggesting that this gene is not positively selected in the commercial strains.

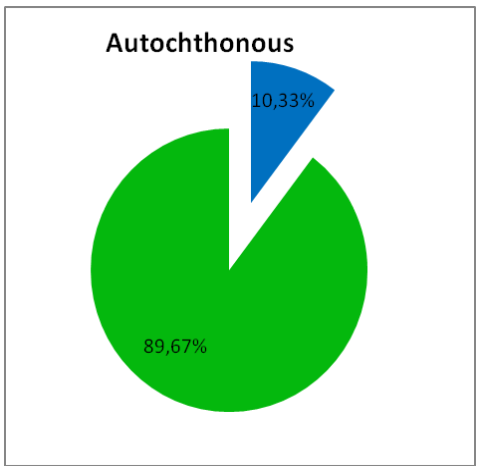



**Figure 3.7** Frequency of autochthonous strains owning (■) and not owning (■) allantoate transporter.
Commercial strains not shown because none have the allantoate transporter.

In greater detail, autochthonous strains show different trends: fructose transporter was present in 20 out of 156 Raboso isolates, nearly 13% (Figure 3.8a) and in 2 out of 34 Prosecco isolates, nearly 6% (Figure 3.8b). Allantoate permease was identified in none of the Tocai isolates.

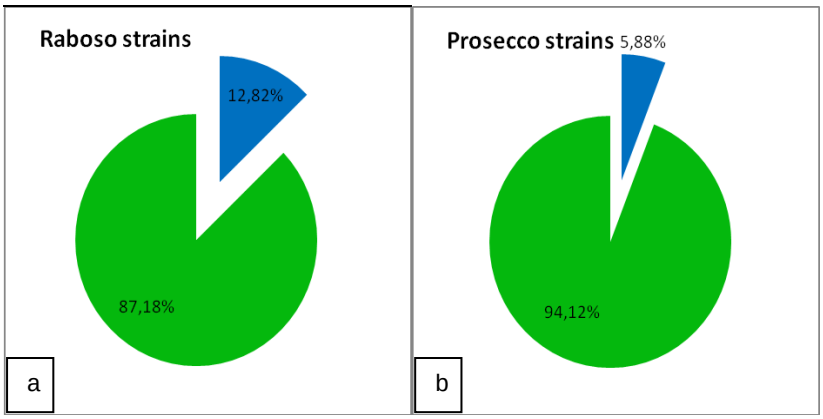



**Figure 3.8** Frequency of Raboso (a) and Prosecco (b) strains owning (■) and not owning (■) allantoate transporter.
Tocai strains not shown because none have the allantoate transporter.

It can be assessed that these genes are not rare in the vineyard yeast population, while only the fructose transporter seems to be positively selected in the commercial strains.

### 3.5.3 Genome finishing and structural variations

The genomes of 4 autochthonous strains, isolated during local selection projects from vineyards in Veneto areas Conegliano Valdobbiadene Prosecco Superiore DOCG, and Raboso Piave DOC, named P283.4, P301.4, R8.3 and R103.1, were sequenced and high quality assemblies, with an average coverage of approximately 17X, were obtained. A finishing process has been performed based on a bioinformatics strategy to obtain on average 2.5 scaffolds per chromosome that greatly improved gene finding, annotation and SNPs distribution analysis.
The genomes of the four vineyard strains along with EC1118 and S288c were aligned using the program Mauve, and the alignment was analyzed thank to the viewer tool. From the manual inspection of the alignment have been identified 9

translocations having size equal or higher than 10 kbp. Four of these translocations were identified (even though not exactly identical) in all the genomes of the vineyard yeasts, while other five were strain-specific. Among the structural variations the well-known translocation that involves SSU1 (YPL092W) was identified. This gene encodes a plasma membrane sulphite pump whose overexpression determines an increased sulphur dioxide resistance. In R8.3, R103.1 and P301.4 the gene is localized close to the translocation VIII-XVI, already identified in some oenological strains. The strain P283.4 carries a new translocation involving chromosome XV and chromosome XVI and positioning SSU1 in close proximity to ADH1 gene (YOL086C). Specific primers have been produced for two selected regions (table 3.6) and the presence of these rearrangements have been successfully tested by Real Time PCR.

| | **TRANSLOCATION 15-16** | | | | |
|---|---|---|---|---|---|
| **Strain** | **Forward** | **Reverse** | **Expected positive amplification** | **No amplification expected** | **validation** |
| EC1118 | A designed on chr 15 | B designed on chr 15 | AB | AC | no translocation |
| P283.4 | | C designed on chr 16 (scaffold27) | AC | AB | translocation confirmed |
| | **TRANSLOCATION 16-8** | | | | |
| **Strain** | **Forward** | **Reverse** | **Expected positive amplification** | **No amplification expected** | **validation** |
| S288c | A designed on chr 16 | B designed on chr 16 | AB | AC | no translocation |
| R8.3 | | C designed on chr 8 (scaffold13) | AC | | translocation confirmed |

**Table 3.8** Schematic representation of PCR used to verify the 2 selected translocations.

Three specific primers have been designed for each translocation: one forward and one reverse designed on the same chromosome and another reverse designed on the other chromosome. Real time PCR have been performed to verify the presence of translocations between chromosomes in the 213 autochthonous strains. First of all the amplification has been performed with the primers pair forward and reverse on the same chromosome, to verify, in case of amplification, in which strains the translocation was not present (Fig. 3.9 a and b).

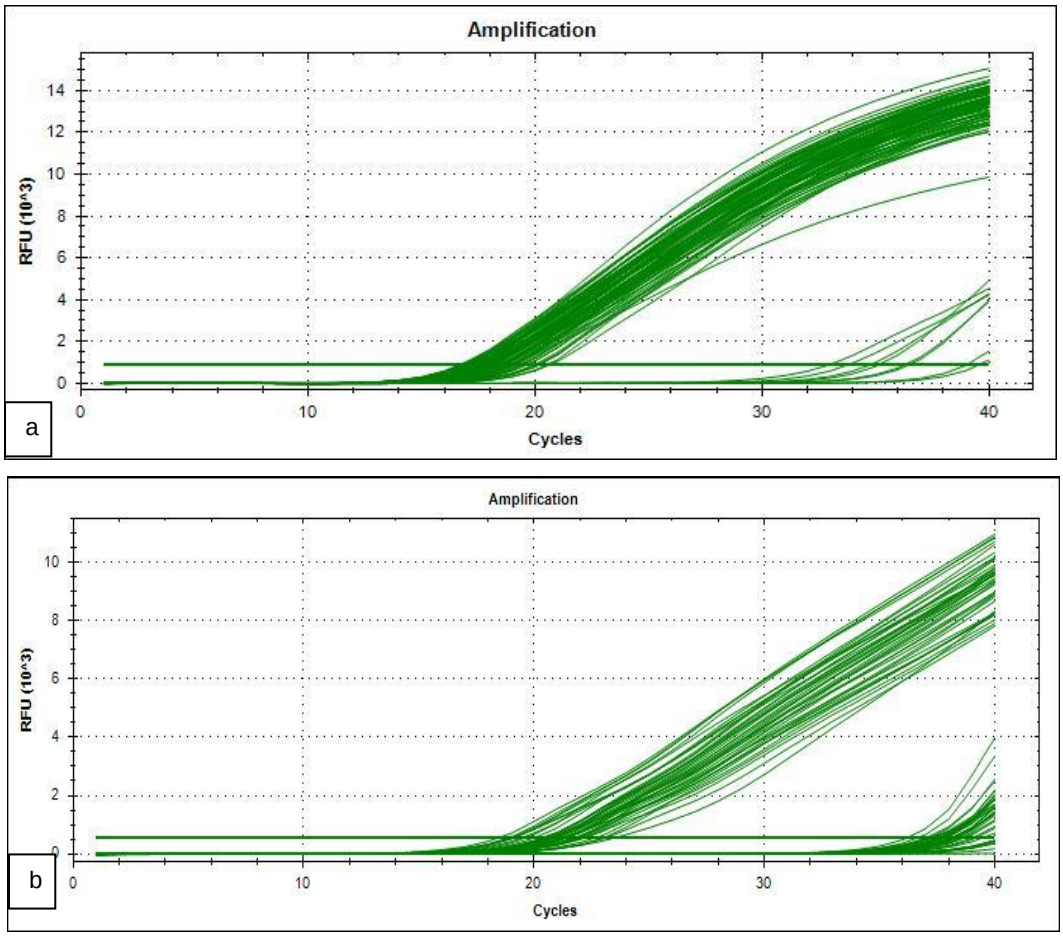



**Figure 3.9** Example of amplification curves for (a) translocation XV-XVI (primer A and B) and (b) translocation XVI-VIII (primer A and B).

After this first screening, another real time PCR have been performed with strains not amplifying (or amplifying later on) with the first pair of primers. It is possible that some strains have a small amount of fluorescence because of the long time of primer annealing/elongation and the strong specificity of the Eva Green polymerase, but, given the difference in cycles with the other strains, they can be considered not amplifying.

The second real time PCR has been performed with the primers pair forward A and reverse C, annealing on two different chromosome, so that the translocation between them can be identified. In this case an amplification means that in the strain the two chromosomes are translocated.

For the translocation between chromosome XV and XVI five strains have been analyzed, R16.2, T606.8, B217.2, P148.1, R153.4. As a positive control for amplification has been used the strain P283.4, and as a negative control the strain EC1118. Amplification curves are shown in Fig. 3.10: blue line is P283.4;

red line is R16.2; orange line is T606.8; green line is B217.2; light blue line is P148.1; yellow line is EC1118; pink line is R153.4.

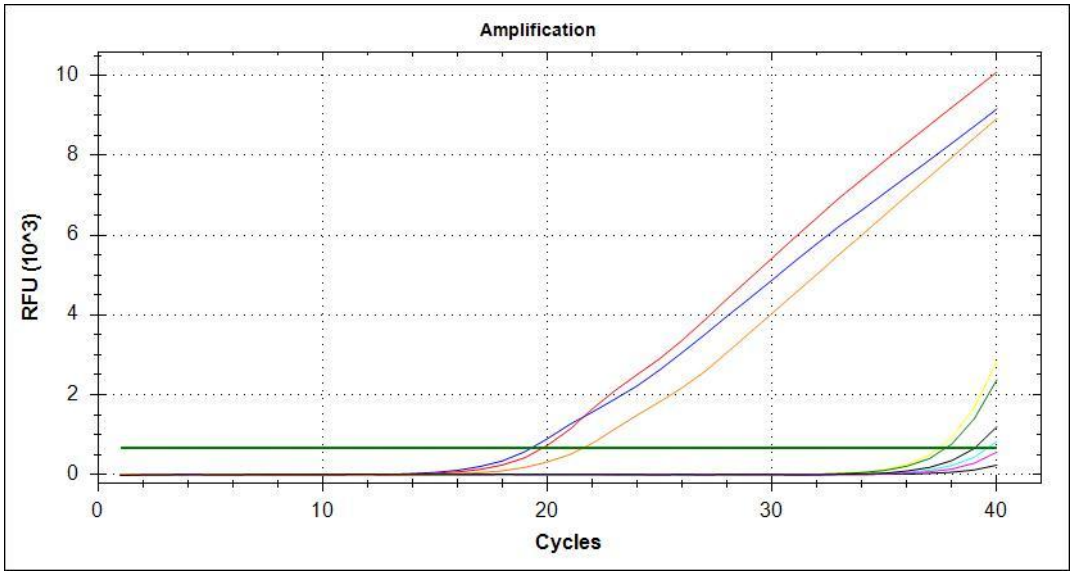



**Figure 3.10** Amplification curve of strains with possible translocation XV - XVI (primer A and C).

Strains R16.2 and T606.8, together with the positive control P283.4, amplify, so they have chromosomes XV and XVI translocated.

Regarding the translocation between chromosome XVI and VIII 114 autochthonous strains could have the translocation and must be analyzed; furthermore the analysis must be extended to the commercial strains (analysis still in progress).

## 3.6 Conclusions

Comparison of the strains belonging to the wine\European group with some others derived from different environments revealed the presence of 306 SNPs characterizing enological strains. These positions are identical in enological strains and differ in all the other strains considered. The genes harbouring these SNPs have been further investigated and results suggest their importance in the adaptation to the enological environment. Some of these SNPs led to amino acid changes in highly conserved proteins regions, in particular two of these genes encode proteins involved in aminoacids catabolism, in the pyruvate transport and in the biosynthesis of higher alcohols that have a strong impact on wine aroma.

The frequency of the two SNPs identified in YDL168W and YHR162W genes have been exanimate in 213 autochthonous yeasts, together with ten commercial strains and two laboratory strains. Results obtained from genome sequencing have been confirmed, and these 2 SNPs are very common in vineyard and commercial strains.

It has been found that nearly 10% of the vineyard strains isolated harbours the allantoate transporter gene, giving them the ability to use less-attractive nitrogen sources that become prevalent in the second part of the fermentation process. The gene encoding the fructose transporter is even more frequent (nearly 23%) in vineyard yeast population and was also frequently identified in the commercial strains examined. Ability to use fructose could confer an evolutionary advantage because, in ripen grapes, the concentration of fructose and glucose are similar. In the first part of the fermentation, yeast uses preferentially the “more attractive” nitrogen and carbon sources, while in the second part it uses “less attractive” compounds.

Regarding translocations analysis are still in progress, and must be extended to commercial strains. A preliminary analysis suggests that the new translocations XV-XVI it’s not common in vineyard strains (only 3 out of 213 strains), while the well known translocation XVI-VIII seems very frequent in the vineyard strains (114/213 isolates could have the translocation), but this result must be confirmed with further PCR analysis.

# 4. Phenotypic characterization of yeasts with sequenced genome

## 4.1 Introduction

Wine technologists gathered the basic properties required for the definition of a ‘‘selected *S. cerevisiae* strain for winemaking’’ in two categories (Reed G and Chan SL. 1979): (1) primary or fitness traits, defined as those strictly associated with the formation of ethylalcohol by fermentation, and (2) secondary or quality traits, defined as those related to the production of compounds that affect other parameters, such as the body of a wine, the higher alcohols complex (bouquet), and the appearance of undesirable off-flavours. Main primary and secondary traits are summarized in table 4.1, where some further traits, more specific and functional to the type of desire wine, are also listed (Pretorius 2000).

| **Fitness traits** | |
|---|---|
| **Fermentation properties** | **Technological properties** |
| Rapid initiation of fermentation | High genetic stability |
| High fermentation efficiency | High sulphite tolerance |
| High fermentation rate | Low sulphite binding activity |
| High ethanol tolerance | Low foam formation |
| High osmotolerance | Flocculation properties |
| Low temperature optimum | Copper resistance |
| Moderate biomass production | Resistance to desiccation |
| | Killer properties |
| | Proteolytic activity |
| | Low nitrogen demand |
| **Quality traits** | |
| **Flavour characteristics** | **Metabolic properties with health implications** |
| Low volatile acidity production | Low sulphite formation |
| Moderate higher alcohol production | Low biogenic amine formation |
| Low sulphite/DMS/thiol formation | Low ethyl carbamate (urea) potential |
| Liberation of glycosylated flavour precursors | |
| No phenolic off-flavours production | |
| High glycerol production | |
| Modified esterase activity | |
| Enhanced autolysis | |
| Hydrolytic activity | |

**Table 4.1** Main desirable characteristics of wine yeast.

Some of the requirements listed in Table 4.1 are complex and difficult to define genetically without a better understanding of the involved biochemistry and physiology. To date, no wine yeast present on the market has all the characteristics listed, and it is well established that wine yeasts have different behaviour concerning their winemaking abilities. Although this phenomenon can be ascribed to fermentation conditions that are hardly reproducible, the major source of variation can be attributed to the genetic constitution of the wine yeasts (Pretorius 2000).

### 4.1.1 Fitness traits

The technological traits influence the efficiency of the fermentation process. *S. cerevisiae* strains generally possess the technological characteristics required to perform an efficient fermentation. The determination of these traits is, however, necessary, since most of these characteristics are strain specifics.

#### 4.1.1.1 Main fermentation properties

The rate of fermentation and the amount of alcohol produced per unit of sugar during the transformation of grape must into wine is of considerable commercial importance. The fermentation efficiency is intended as the uppermost concentration of ethanol obtainable by fermentation from an excess of sugar. The fermentation rate (vigour)is the measure of the ability of a starter to bring the fermentative process to a fast completion. It is normally represented as grams of $CO_2$ developed in 24 h, calculated as the average of a 3-day measurement period (Martini 2003). During wine yeast glycolysis, one molecule of glucose or fructose yields two molecules each of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapour (Boulton et al. 1996). The ethanol production and fermentation rate are closely linked to ethanol tolerance: in fact while ethyl alcohol is the major desired metabolic product of grape juice fermentation, it is also a potent chemical stress factor that is often the underlying cause of sluggish or stuck fermentations. Apart from the inhibitory effect of excessive sugar content on yeast growth and vinification fermentation, the production of excessive amounts of ethanol, coming from harvest of over-ripe grapes, is known to inhibit yeast growth rate, viability and fermentation capacity:

cell growth stops at relatively low ethanol concentrations, and fermentation stops at relatively higher levels. Decreases in the rate of ethanol production are related to decreases in viable cell count. Cell growth inhibition by ethanol is non competitive and has been described as either a linear or an exponential function of ethanol concentration(Boulton et al. 1996; Benitez et al. 1996).Generally, sugar catabolism and fermentation proceed at a rate greater than desired, and are usually controlled by lowering the fermentation temperature (Fleet, 1993).Occasionally, wine fermentation ceases prematurely or proceeds too slowly. The commercial implications of sluggish or incomplete wine fermentations are usually attributed to inefficient utilization of fermenter space and wine spoilage resulting from the low rate of protective carbon dioxide evolution and high residual sugar content. Conversely, financial losses through `runaway' wine fermentations arise from the fact that fermentor space is reduced because of foaming and volatile aroma compounds are lost by entrainment with the evolving carbon dioxide. Thus, yeast behaviours towards temperature are also very important in wine making control: a wide range of growth temperatures is suitable for wine strains, and fermentation efficiency should not swiftly decrease as small temperature changes happen. Optimal performance of wine yeasts in white wine fermentations, conducted at cooler temperatures(10±15°C) so as to minimize the loss of aromatic volatiles, and red wine fermentations, performed at higher temperatures (18±30°C) to enhance extraction of anthocyanin pigments, is therefore of critical importance to wine quality and cost effectiveness(Henschke, 1997).

#### 4.1.1.2. Main technological properties

Several antimicrobial compounds, as well as ethanol, can interfere with yeast fermentation activity. Some of these compounds are usually added to fermentation tanks, as sulphite dioxide; other ones are found in grape must coming from agrochemical treatments as copper and pesticides; finally antimicrobial killer toxinsare produced by some yeasts and are lethal to other sensitive ones. Sulphur dioxide is widely used in enology for its antioxidant activity and as antimicrobial agent towards yeast, acetic and lactic acid bacteria in general. Moreover, *Saccharomyces* is the most resistant yeast among wine-related species, so $SO_2$ addiction selects this microorganism inhibiting apiculated ethanol-sensitive species; thus tolerance to sulphite forms the basis of selective

implantation of active dried wine yeast starter cultures into grape must. $SO_2$ addiction, anyway, can affect differently fermentation kinetics and although *S. cerevisiae* tolerates higher levels of sulphite than most unwanted yeasts and bacteria, excessive SO2 dosages may cause sluggish or stuck fermentations (Boulton et al. 1996) Wine yeasts strains vary widely in their resistance to sulphite, and the underlying mechanism of tolerance as well as the genetic basis for resistance are still unclear. Within the *Saccharomyces* species, resistant strains are quite frequent (around 30%) and they can develop in the presence of 150 ppm of $SO_2$, while more sensitive strains are inhibited at concentrations such as 100 ppm that mainly causes a prolongation of lag phase.

Wide application of copper-containing fungal pesticides (copper oxychloride) to control downy mildew (*Plasmopara viticola*) and, to a lesser extent, dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*) could lead to copper residues in musts that may cause lagging fermentation and affect wine quality detrimentally. This phenomenon recently increased due to the diffusion of the organic and integrated cultivations, where copper is widely used to reduce or eliminate the need of other chemical treatments. *S. cerevisiae* species exhibits a significant variability in copper resistance and the acquisition of this trait seems to be the result of an environmental adaptation (Romano, 2005).Several copper uptake, efflux and chelation strategies have been developed by yeasts to control copper ion homeostasis. In particular, copper sensitive strains do not change the metal concentration in wine, whereas resistant strains sensibly reduce this element accumulating copper inside the cell. Killer toxins are proteins produced by some yeasts that are lethal to sensitive wine yeast strains. The killers themselves, however, are immune to these mycovirus associated toxins. It remains controversial whether the growth and zymocidal activity of some wild killer yeasts have the potential to delay the onset of fermentation, cause sluggish or stuck fermentations and produce wines with increased levels of acetaldehyde, lactic acid, acetic acid and other undesirable sensory qualities. An unfortunate consequence of ignorance regarding the role of killer yeasts in wine fermentations is that some winemakers use co-cultures to inoculate fermentations, one strain being a killer and the other a sensitive strain. The advantage of using killer or neutral wine yeasts should therefore not be underestimated (Pretorius 2000).

### 4.1.2. Quality traits

The quality of wine is the outcome of complex chemosensory interactions that are difficult to predict because of the influences of many variables. The chemical composition of wine is the foundation of both sensory response and wholesomeness, and it is determined by many factors. These include the grape variety, the geographical and viticultural conditions of grape cultivation, the microbial ecology of the grape and fermentation processes, and winemaking practices (Owens and Noble, 1997).

Microorganisms have a prominent role in determining the chemical composition of wine. They affect the quality of the grape prior to harvest and, during fermentation, they metabolise grape sugars and other components into ethanol, carbon dioxide and hundreds of secondary end-products that, collectively, contribute to the subtlety and individuality of wine character (Nykanen L. 1986).

#### 4.1.2.1. Flavour characteristics

Alcoholic beverages contain mainly saturated, straight chain fatty acids. The volatile acid content of wine usually lies between 400 and 1000 mg/L, normally more than 90% of volatile acid consists of acetic acid. Although acetic and lactic acid bacteria can be associated with high levels of short chain fatty acid, acetic, propanoic and butanoic acids are by-products of alcoholic fermentation (Ribereau-Gayon et al. 2000). Fermentation purity is expressed as of the ratio between volatile acidity (as g acetic acid/L) and ethanol (% volume) produced at the end of the fermentation process. High values of this ratio denote the ability to form few undesirable by-products in the course of fermentation. Wines cannot be commercialized if volatile acidity exceeds one tenth of the ethanol content.

Another fermentation by-product affecting wine quality is glycerol. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol. Due to its non-volatile nature, glycerol has no direct impact on the aromatic characteristics of wine. However, this triol imparts certain other sensory qualities; it has a slightly sweet taste, and owing to its viscous nature, also contributes to the smoothness, consistency and overall body of wine. Wine yeast strains producing a consistent amount of glycerol would therefore be of considerable value in improving the organoleptic quality of wine. Among other yeast metabolites, the formation of sulphite and sulphide by wine strains greatly affects

the quality of wine. Sulphur is essential for yeast growth and *S. cerevisiae* can use sulphate, sulphite and elemental sulphur as sole sources. Unlike sulphur dioxide ($SO_2$), which when properly used, has some beneficial effects, hydrogen sulphide ($H_2S$) is one of the most undesirable yeast metabolite, since it causes, above threshold levels of 50-80 g/l, an off-favour reminiscent of rotten eggs (Snow R. 1983). Even though the compositional variability of musts (i.e., the precursors of bouquet molecules variably distributed within grape varieties) is considered the main source of organoleptic specificity, today the wine technologists re-evaluate the role of yeast metabolism (strain-related by-products of fermentation) in the formation of bouquet and aroma. In fact, the growth, by means of alcoholic fermentation as energy source, is the best way for yeasts to make a contribution to wine flavour, as well (Henschke, 1997).This phenomenon is carried out by several mechanisms that involves the degrading of grape juice constituents and the production of a great amount of different compounds: mainly ethanol and other solvents that help to extract flavour components from grape solids, hundreds of secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds) that contribute considerably to wine aroma and the products of autolytic activity that characterizes the stationary phase of yeast growth. Moreover a great variety of exoenzymes are normally produced by these microorganisms that can transform neutral grape compounds into flavour active molecules(Nykanen L. 1986). These reactions, especially the production of secondary metabolites, vary with the species and strain of yeast. Tables comparing the diversity of metabolite production by different yeasts may be found in Fleet, Lema et al. 1996, Romano 1997, Heard 1999, and Lambrechts and Pretorius. Thus, the uniqueness and individuality of the flavour contribution by yeasts depends on the species and strains operating the fermentation.

#### 4.1.2.2. Metabolic properties that influence wine safety

Today, it is generally accepted that moderate wine drinking can be socially beneficial, and that it can be effective in the management of stress and reducing the risk of coronary heart disease. In the selection and improvement projects concerning wine yeast strains, it is therefore of the utmost importance to focus on these health aspects and to obtain yeasts that may reduce the risks and enhance the benefits. Likewise, research in several laboratories around the world is directed towards the elimination of suspected carcinogenic compounds in wine,

such as ethyl carbamate, and asthmatic chemical preservatives, such as sulphites. It might even be possible to develop wine yeasts that could increase the levels of phenolic and antioxidative substances (e.g. resveratrol) associated with the so-called `French paradox', in which, despite the high dietary fat intake of the cheese loving population of southern France, the death rate from coronary heart disease is significantly lower than the one found in industrialized countries (Pretorius, 2000).

### 4.2 Yeast sequencing

In 1996, the budding yeast *S.cerevisiae* became the first eukaryotic organism to have its genome completely sequenced, thanks to a worldwide collaboration involving more than 600 scientists in Europe, North America and Japan (Goffeau et al. 1996). The sequence of 12,068 kilobases defines 5885 potential protein-encoding genes, and provides information about the higher order organization of yeast's 16 chromosomes and allows some insight into their evolutionary history. The strain sequenced, S288c, is a commonly used laboratory strain that was obtained in the 1950s, by mating a strain isolated from a rotten fig (EM93) with a commercial strain (Mortimer and Johnston 1986). As a non-oenological strain it has been selected for rapid and consistent growth in nutrient rich laboratory media, but it’s unable to grow in the low pH and high osmolarity of most grape juices and therefore cannot be used to make wine (Borneman et al., 2011). The sequence is conserved in the public database SGD (*Saccharomyces* Genome Database) and it is considered the reference strain for yeasts sequencing.

Today the genomes of several other *S. cerevisiae* strains have been sequenced, including a lot of wine strains, previously selected for the ability to grow and function under the concerted influences of a multitude of environmental stressors, which include low pH, poor nutrient availability, high ethanol concentrations and fluctuating temperatures (Borneman et al., 2008). In particular the diploid, heterozygous wine yeast strainEC1118, widely used as a starter in the wine industry.

In 2005-2006 the Broad Institute published the sequence assembly and results of annotation of a wine yeast, RM11-1a. This is a haploid derivative of Bb32, a natural isolate collected by Robert Mortimer from a California vineyard. It has high spore viability (80-90%) when crossed with different lab strains. Strains of both mating types and with a number of auxotrophic markers are available. RM11

has been subject of extensive phenotypic characterization, including growth under a wide range of conditions and gene expression profiling.

In 2007 a Chinese group sequenced the genome of *Saccharomyces cerevisiae* strain YJM789, which was derived from a yeast isolated from the lung of an AIDS patient with pneumonia. The strain is used for studies of fungal infections and quantitative genetics because of its extensive phenotypic differences to the laboratory reference strain S288c, including growth at high temperature and deadly virulence in mouse models (Wei et al., 2007).

In 2008 the Australian Wine Research Institute (AWRI, Adelaide) sequenced the genome of a wine yeast, AWRI1631. This haploid *S. cerevisiae* strain is descended from the diploid industrial wine strain N96 (Anchor Yeast, South Africa), similar to the strain known in the trade as EC1118. It has retained the robust fermentation kinetics of its parent while producing wine with a composition and flavour profile that is also equivalent to N96. However, due to its stable haploid genome, it is far easier to manipulate genetically. AWRI1631 sequence has been compared with both the laboratory strain S288c and the human pathogenic isolate YJM789. AWRI1631 was found to be substantially different from S288c and YJM789, especially at the level of single-nucleotide polymorphisms, and there were major differences in the arrangement and number of Ty elements between the strains, as well as several regions of DNA that were specific to AWRI1631 and that were predicted to encode proteins that are unique to this industrial strain (Borneman et al., 2008).

In 2009 a French group sequenced the complete genome of the diploid commercial wine yeasts EC1118. Lalvin EC1118, also known as “Prise de mousse,” is a *S. cerevisiae* wine strain isolated in Champagne (France) and deposited in the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, France) (Novo et al., 2009). This strain is one of the most frequently used fermentation starters worldwide and has been extensively studied as a model wine yeast (Rossignol et al., 2003; Varela et al., 2005). It is a strongly competitive strain, able to ferment at low temperature and with an excellent alcohol tolerance.

The comparison with S288c shows present in S288c but missing from EC1118 and genes present in EC1118 but missing from S288c. Moreover EC1118 possess 3 unique large regions, 2 of which were subtelomeric and the other identified as a 17-kb insertion into chromosome XIV. These regions encompass 34 newly genes involved in key wine fermentation functions, 20 of which were

found to encode proteins potentially involved in the metabolism and transport of sugar or nitrogen. The existence of these genes unique to EC1118 suggests the loss of these genes from other *S. cerevisiae* strains or their acquisition from non–*Saccharomyces* donors, in particular *Zygosaccharomyces* (Novo et al., 2009).

In 2009 the genome of a *S. cerevisiae* strain used in bioethanol production has been sequenced. This strain, JAY291, is a haploid derivative of the strain PE-2, a heterothallic diploid naturally adapted to the sugar cane fermentation process used in Brazil (Argueso et al., 2009).

In 2011 the Australian Wine Research Institute (AWRI, Adelaide) produced the whole-genome assemblies of 6 commercial strains of *S. Cerevisiae,* four commercial wine strains, AWRI796, QA23, VIN13 and VL3 and two brewing strains used for the production of ales, FostersO and FostersB (Borneman et al., 2011).

In the same year a Japanese group published the whole-genome sequencing of a sake yeast, Kyokai no. 7, a diploid *S. cerevisiae* strain commonly used for sake brewery (Akao et al., 2011)

Moreover, our research group, in collaboration with the Functional genomics group, Dr. Campanaro (Department of Biology, University of Padova), complete the genome sequences of 4 wine yeasts, isolated during local selection projects from vineyards in Veneto areas (Conegliano Valdobbiadene Prosecco Superiore DOCG and Raboso Piave DOC), named P283.4, P301.4, R8.3 and R103.1.

## 4.3 Materials and methods

### 4.3.1 Yeasts

In this study we investigated

Commercial wine yeasts

| | Producer | Strain name | Species |
|---|---|---|---|
| 1 | AWRI | AWRI 1631 | S. cerevisiae |
| 2 | MAURIVIN | AWRI 796 | S. cerevisiae |
| 3 | LALLEMAND | EC 1118 | S. cerevisiae |
| 4 | LALLEMAND | QA23 | S. cerevisiae |
| 5 | LAFFORT | ZYMAFLORE VL3 | S. cerevisiae |
| 6 | ANCHOR (EVER) MAURVIN | VIN13 | S. cerevisiae |
| 7 | CBS collection strain | S288c | S. cerevisiae |

Autochthonous wine yeasts

isolated from vineyards in Veneto areas (Conegliano Valdobbiadene Prosecco Superiore DOCG and Raboso Piave DOC)

Prosecco collection: P283.4 and P301.4

Raboso collection: R8.3 and R103.1

### 4.3.2 Culture media and growth condition

**Media**

YM solid agar medium

3 g/l yeast extract (Oxoid);

3 g/l malt extract (Oxoid);

5 g/l vegetatone peptone (DIFCO);

10 g/l glucose (PROLABO)

16 g/l Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g/l yeast extract (OXOID)

20 g/l vegetatone peptone (DIFCO)

20 g/l glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Fucsine Agar medium

3 g/l yeast extract (Oxoid);

3 g/l malt extract (Oxoid);

5 g/l vegetatone peptone (DIFCO);

10 g/l glucose (PROLABO)

0,002 g/l Fucsine (SIGMA)

16 g/l Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Biggy Agar medium (Oxoid)

1 g/l yeast extract

10 g/l glycin

10 g/l glucose

3 g/l sulphite ammonium

5 g/l bismuth ammonium citrate

16 g/l Bacto Agar

pH 6.8

Suspend 42g in 1 liter of distilled water and bring gently to the boil to dissolve the agar. Allow to cool to 50-55°C. Mix gently to disperse the flocculent precipitate and pour into sterile Petri dishes. Do not autoclave the medium.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

| | |
|---|---|
| 0,1 g/l | $CaCl_2$ |
| 0,1 g/l | NaCl |
| 1 g/l | $KH_2PO_4$ |
| 0,5 g/l | $MgSO_4 \cdot 7H_2O$ |
| 3 g/l | tartaric acid |

Micronutrients

| | |
|---|---|
| 0,2 mg/l | $NaMoO_4 \cdot 2H_2O$ |

| | |
|---|---|
| 0,4 mg/l | $ZnSO_4 \cdot 7H_2O$ |
| 0,5 mg/l | $H_3BO_3$ |
| 0,04 mg/l | $CuSO_4 \cdot 5H_2O$ |
| 0,1 mg/l | KI |
| 0,4 mg/l | $FeCl_3 \cdot 6H_2O$ |
| 0,4 mg/l | $MnSO_4 \cdot H_2O$ |

Vitamins

| | |
|---|---|
| 400 µg/l | pyridoxinehydrochloride |
| 400 µg/l | thiaminehydrochloride |
| 2000 µg/l | Inositol |
| 20 µg/l | Biotin |
| 400 µg/l | Calciumpantothenate |
| 400 µg/l | Nicotinic acid amide |
| 200 µg/l | P-amino-benzoic acid |

Variable components

| | |
|---|---|
| 0,3 g/l | $(NH_4)_2SO_4$ |
| 0,3 g/l | $(NH_4)_2HPO_4$ |
| 200 g/l | Glucose |
| 0,2 g/l | Hydrolyzed Casein |

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

**Growth conditions**
The yeast strains were grown at 25 ° C, the liquid cultures, for fermentation inoculum, were subjected to agitation of 130 oscillations per minute.

### 4.3.3 Fermentation surveys on Synthetic Nutrient Medium (NSM) (Delfini, 1995)

#### 4.3.3.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate10ml of YPD liquid medium. The tubes were left in incubation for 30 hours at25 °C, moved to obtain a culture on stationary phase (approximately$10^7$-$10^8$cells/ml) measured by spectrophotometry ($OD_{600}$ between 5 and 8).

#### 4.3.3.2 Test preparation

Based on the OD of the respective pre-inoculation, for each strain the culture volumes to obtained a final $OD_{600}$ of 0.5 (approximately $10^5$cells/ml) in 100 ml of medium at the beginning of fermentation, were calculated.
Each strain was inoculated in a 100 ml-Erlenmeyer flask sealed with silicon cap and supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use the synthetic must than the natural, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily growth monitoring operations.
The medium was sterilized by autoclaving at 100 ° C for 5 minutes.
Alcoholic fermentation development was controlled by measuring the weight loss daily from the beginning to the end of fermentation process. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

### 4.3.4 Ethanol production

It is interesting to evaluate the maximum alcohol content that a yeast can produce in optimal conditions of development and in the presence of 300 g/l of sugar. For this test synthetic must have prepared modifying MNS media recipe, increasing glucose content (300 g/l), tartaric acid (to 6 g/L), malic acid (6 g/l), hydrolyzed casein (1 g/l), ammonium sulphate and ammonium phosphate (both 0.9g/l). The medium was aliquoted into 100 ml flasks and pasteurized at 100°C for 5 minutes. The procedure and condition were previously described by Delfini (Delfini, 1995). Yeasts were grown in 100 ml of YPD at 25 ° C for 12 h and inoculated to normalize the final OD for all strains and replicas. Then the flasks were incubated at a constant temperature of 25°C and glucose fermented was determined by the measurement of flasks weight loss every 12 h with a precision balance (Gibertini EU-7500DR C), with a sensitivity of 0.01g. The amount of ethanol produced at the end of fermentation was determined with HPLC by

measuring the amount of residual sugar and using the conversion factor for sugar/alcohol of 0.61 (Delfini, 1995).

### 4.3.5 Chemical analysis on fermented must

Total and free sulphur dioxide were quantified at the end of synthetic must fermentation using iodometric titration.
Samples of synthetic must fermented by the different strains were analyzed with HPLC technique to verify the exact amount of residual glucose and glycerol. Components separation was carried out using a Waters 1525 binary HPLC pump with an Aminex ion exclusion column to HPX_87H 300 mm x 7.8 mm. A Waters 2414 Refractive Index Detector was set at 600nm wavelength for the determination of ethanol, glycerol and glucose, while for the detection of the peaks related to organic acids we used a Waters 2487 Dual Absorbance detector set at 210nm wavelength. A calibration has been done for each individual compound and it was used to calculate the corresponding g/L in each sample.
Acetaldehyde enzymatic determination was carried out using the kit R-BIOPHARM purchased by Roche. The chemical reaction used is:
Acetaldehyde + $NAD^+$ + $H_2O$ -> Acetic Acid + NADH + $H^+$
The determination of acetaldehyde is controlled by measuring the amount of NADH produced at OD340nm.

### 4.3.6 Statistical analysis

The comparative statistical analysis between the various groups of samples was conducted using the software XLSTAT, vers.7.5.2, using simple analysis of variance (one-way ANOVA), followed by the Tukey test as "post-hoc" tests. The analysis was conducted by comparing the averages of three independent replications and differences were considered statistically significant for p-value less than 0.05.

## 4.4 Results and discussions

### 4.4.1 Fermentative performance and technological strains characterization in synthetic must

#### 4.4.1.1 Fermentation kinetics and ethanol production

To evaluate the fermentative performance of the yeasts, they were inoculated in synthetic must (Delfini, 1995) under conditions that simulate oenological setting. Each strain was inoculated at a concentration of about $1*10^6$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use synthetic must with respect to natural juice for preliminary physiological assessments, is to standardize growth conditions and to facilitate significantly daily growth monitoring operations.

For each strain the fermentation test was set up in triplicate. The flasks were kept at a temperature of 25 ° C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation.

Figure 4.1 shows the cumulated $CO_2$ produced over time. Each value is obtained from the average of individual weight loss measured for three replicates. In these conditions, all strains have completed the fermentation in a comparable time (about 20 days).

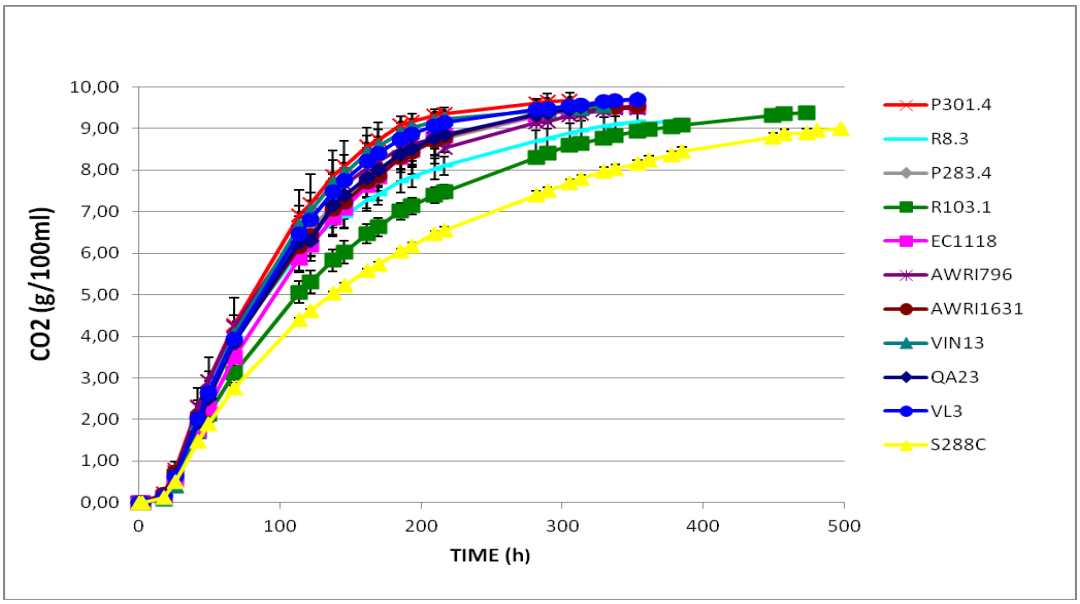



**Figure 4.1** Fermentation kinetics of selected yeasts in synthetic must.

Observing fermentation kinetics (Figure 4.1), it is possible to see that trends are very similar for almost all strains, except S288c, expected since it is not a wine yeast, and yeast native R103.1, showing a fermentation kinetics good, although slower. A slightly better fermentation kinetics is observed for R8.3. The remaining strains, isolates from Glera and commercial strains, show the best kinetics, ending earlier the fermentation. The 2 faster strains are one autochthonous yeast for the production of Prosecco, P301.4, and the commercial yeast VIN13.
Observing ethanol production at the end of fermentation (Figure 4.2), almost all strains tested complete the alcoholic fermentation, developing approximately 12% of alcohol and consuming all the sugar available. Strains resulted slower in fermentation are also those that reach the lower alcohol content, in particular the laboratory strain S288c.

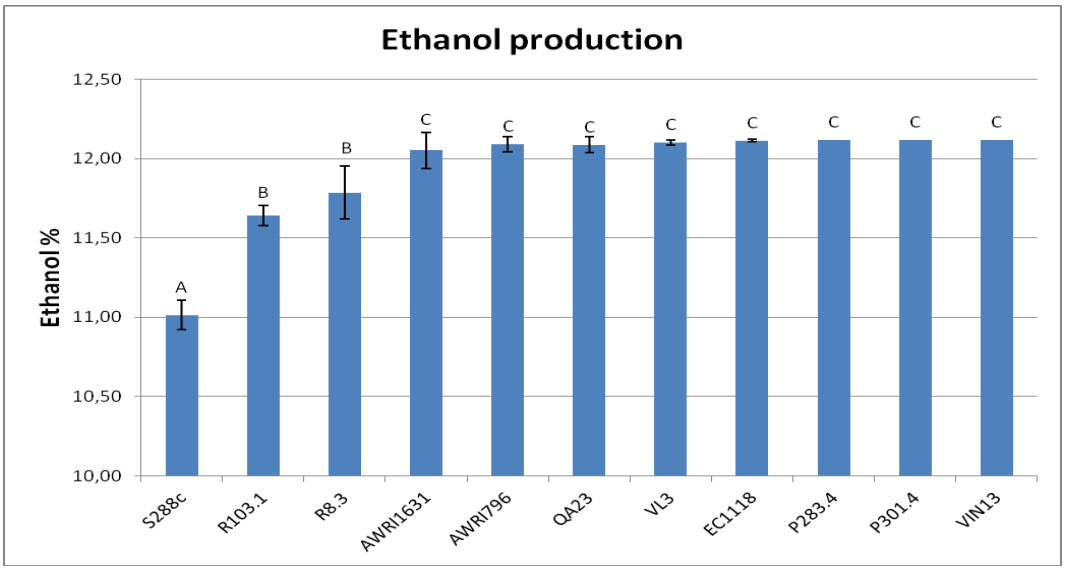



**Figure 4.2** Ethanol production at the end of the fermentation in syntethic must.

### 4.4.1.2 Fermentative vigour

The fermentative vigour, corresponding to the quickness of a strain to start and close the fermentative process, was evaluated. It was estimated by measuring flasks weight loss after 2 days from the start of the fermentative process. Considering the fermentative vigour, calculated as grams of glucose consumed after 2 days by the formula: sugar metabolized = weight losses *2,118 (Delfini, 1995), strains consume glucose in a range between 4 and 6.2 g/100 ml of sugar consumed (figure 4.3). The fermentative vigour is influenced by the adaptation ability of the strain to the oenological environment, and then by the duration of the lag phase.

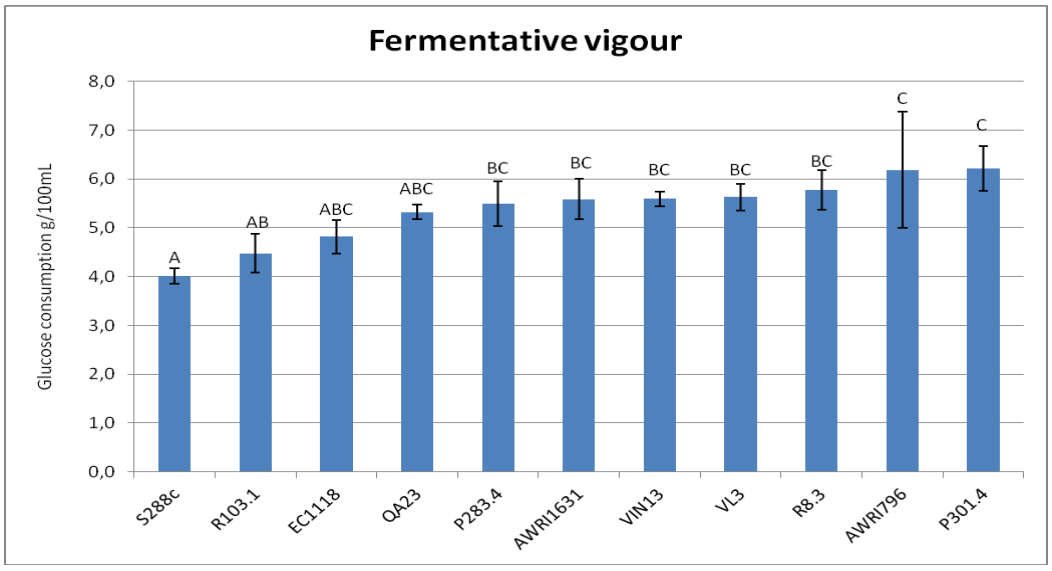



**Figure 4.3** Glucose consumption after 2 days in synthetic must.

The lower value, corresponding to 4 g/100 ml of glucose consumed, is associated with the not oenological strain S288c. This strain, together with the second slower strain, the autochthonous R103.1, which consumes 4.5 g/100 ml of sugar, differs statistically from the two most vigorous strains.

The highest fermentation vigour, around 6.2 g/100 ml of glucose consumed, is associated with the commercial strain AWRI796 ad with the autochthonous Prosecco strain P301.4. The other strains studied are included in a single group rather homogeneous, in which is also present EC1118, often considered as a reference in the commercial yeast.

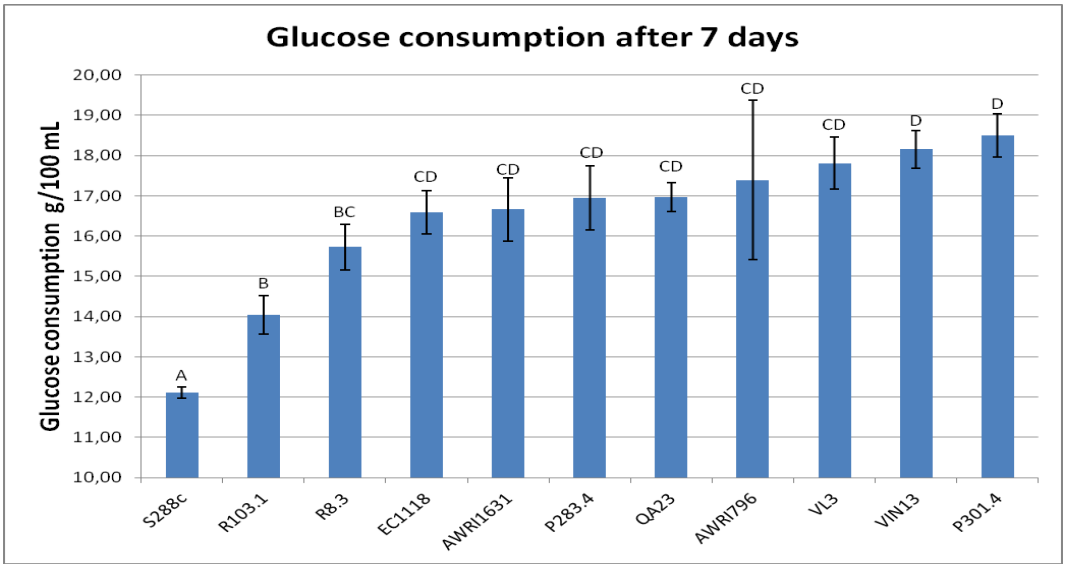



**Figure 4.4** Glucose consumption after 7 days of fermentation.

Moreover glucose consumption after 7 days of fermentation was analysed (figure 4.4). Values, as expected, are higher than after 2 days and between 12 and 18.5 g/100 ml of sugar consumed.
Results obtained after 7 days confirm both those observed at 48 hours and those relating to fermentation kinetics. Also in this case slower strains are S288c and R103.1, while the most vigorous are VIN13 and P301.4.

#### 4.4.1.3 Glycerol production

Glycerol is the most important chemical compound in wine, after water and ethanol, and the first of the secondary compounds of the alcoholic fermentation. Its content in wine is variable and fluctuates in a range between 1-12 g/l. A good production of glycerol is desirable because it gives structure and roundness, and plays an important role in defining the flavour and bouquet of the wine. The greater variability of production of glycerol is determined by the species of yeast. Generally *S. cerevisaie* produces wines with higher amounts of glycerol, about 7-8 g/l (Vincenzini et al. 2005). Glycerol is produced by yeasts at the beginning of the fermentation, in response to high sugars concentrations, for surviving osmotic stress. This compound is produced during the glyceropyruvic fermentation. (Ribèreau-Gayon et al., 2007). The low production of glycerol is associated with the low production of alcohol.
The values of glycerol product at the end of fermentation, obtained after HPLC analysis, are reported in Figure 4.5, and vary between 7 g/l and 14 g/l.

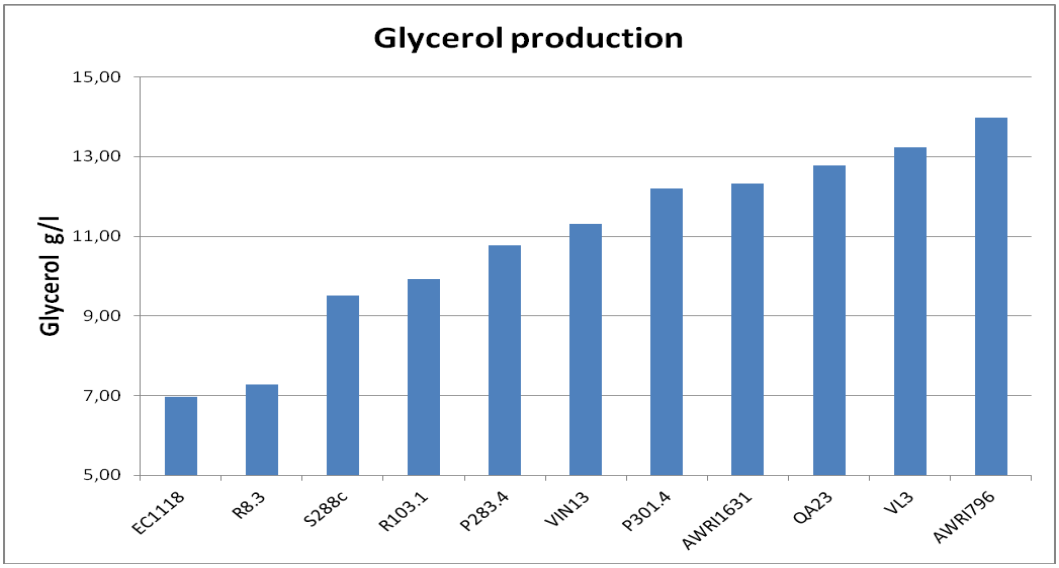



**Figure 4.5** Glycerol production at the end of fermentation.

In this analysis strains that produce a low amount of glycerol are EC1118, R8.3, S288c and R103.1, with a production between 7 g/l and 10 g/l, a value that is still higher than the average of *S. cerevisiae* documented in literature.
The strain AWRI796, with high fermentative vigour, is the highest producer of glycerol, about 14 g/l. It can therefore be concluded that the yeasts which produce more glycerol are those more adapted to sugars, and for this reason they grow faster.

### 4.4.2 Fermentative power

Another important character is the fermentative power, which is the maximum ethanol amount produced by yeasts during the fermentation of a must with an excess of sugar. To evaluate this feature the fermentation was performed in synthetic must with sugar concentration of 300g/l and with a greater availability of nitrogen useful for the metabolism of yeasts (Delfini, 1995). In fact, literature data report that the majority of strains belonging to the *S. cerevisiae* species isolated in nature exhibits an excellent ability to produce ethanol that normally reaches 14-15%v/v (Vincenzini et al., 2005).
The high sugar concentration used for this test imposes a significant stress to the yeast for two main reasons: the first is the high osmotic pressure, that leads to the production of higher amounts of glycerol and ethanol, and the second is given by the toxicity of ethanol that is produced in large quantities. Furthermore, at a concentration so high, the stationary phase, which starts after the production of about 2-3 ° alcohol, is much longer than under standard conditions, and then the stress is higher and the ethanol toxicity is more evident.
Even in this test for each strain the fermentation test was set up in triplicate. The flasks were kept at a temperature of 25 ° C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation.
Figure 4.6 illustrates the cumulated $CO_2$ produced over time. Each value is obtained from the average of individual weight loss measured for three replicates.

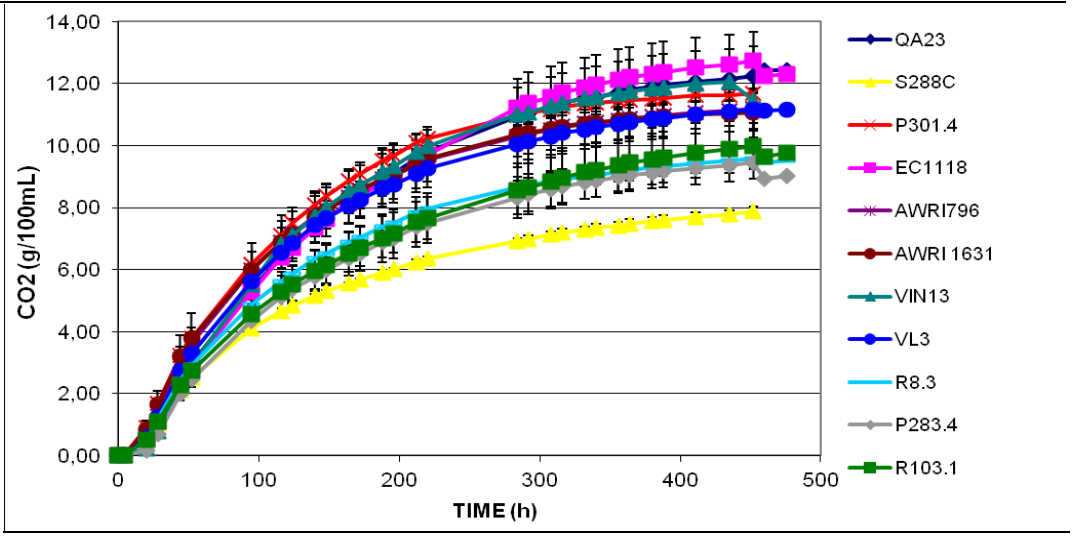



**Figure 4.6** Fermentation kinetics in synthetic must with 300g/l of glucose.

S288c confirmed to be a yeast with low oenological skills, showing a slower kinetics and consuming the least amount of sugar. Even R103.1 and R8.3, are strongly influenced by the high amount of sugar. Surprisingly P283.4, a good fermenter in standard conditions, shows in this case a bad kinetic, similar to the two previous strains. It is interesting to note the behaviour of the strain EC1118, with the best kinetics in the presence of 300 g/l of glucose, confirming to work better at high sugar concentrations and with a high resistance to ethanol.

The amounts of ethanol produced, ranging between 12.4% vol. and 16.5% vol., are shown in Figure 4.7, and show that not all the strains consumed all the glucose present in the must, but they reached high values of ethanol produced.

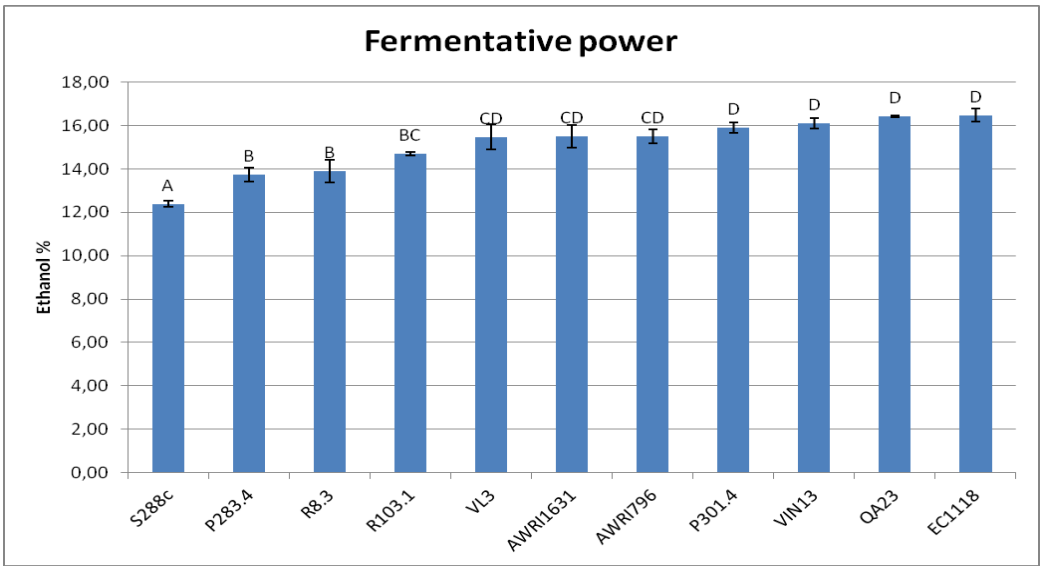



**Figure 4.7** Ethanol production in synthetic must with 300g/l of glucose.

This data confirms earlier observations deduced from the fermentation kinetics in must containing high amounts of sugar. In particular, S288c, with a production of ethanol of 12.4% vol., it's significantly different from the others. Strains R8.3, R103.1 and P283.4 produce an middle amount of ethanol, ranging from 13.8% vol. to 14.8% vol. The other strains are extremely tolerant to alcohol, with ethanol production around 16% vol.
The commercial strain VIN13 and the autochthonous strain P301.4 demonstrate to be two very versatile strains and extremely clever, with very fast fermentation kinetics under standard conditions and an excellent value for alcohol tolerance.

### 4.4.3 Sulphite metabolism

Sulphur is a very important element for the growth of yeast; it is involved particularly in the synthesis of sulfur amino acids, methionine and cysteine, that are essential for the structural conformation of proteins. Yeasts do not use organic sources of sulphur, but use the sulphate ion and organic it in sulphur amino acids. The formation of $H_2S$ or $SO_2$ is strongly linked to the activity of the enzyme sulphite reductase: if the enzyme is very active most of sulphur will be in the form of sulphide and the production of hydrogen sulphide is higher, if sulphite reductase is little active, the majority of sulphur will be in the form of sulphite ion with consequent high production of sulphur dioxide.
Sulphite is widely used in winemaking for its antimicrobial, antioxidasic and antioxidant properties. Furthermore, yeasts usually produce low-to-medium $SO_2$ amounts, depending on their genetic features and on fermentation conditions.
Wine yeasts can cope with $SO_2$ by different systems, such as: acetaldehyde production (that binds to the $SO_2$ inactivating it), production of glutathione, sulphite uptake and reduction or $SO_2$ export from the cell via a membrane transporter dedicated (SSU1 pump).

#### 4.4.3.1 Hydrogen sulphide and sulphur dioxide production

Sulphuric acid ($H_2S$) production was tested on Biggy agar medium (Bismuth Sulphite Glucose Glycine Yeast) (Oxoid), based on the formulation developed by Nickerson.The chromatic scales used for result consideration is: (1) white colour no $H_2S$ production, (2) beige colour low production, (3) brown colour medium production, (4) dark colour high production.

Fucsine agar medium (Caridi *et al*. 1999) was used to evaluate $SO_2$ production, that is revealed by the intensity of the pink coloration of the colonies. The chromatic scales used for result consideration is: (1) dark pink colour low $SO_2$ production, (2) pink colour medium production, (3) light pink colour high production, (4) white colour very high production.

| | Production | |
|---|---|---|
| **STRAIN** | **$H_2S$** | **$SO_2$** |
| EC1118 | 3 | 3 |
| AWRI1631 | 2 | 1 |
| VIN13 | 3 | 3 |
| AWRI796 | 2 | 2 |
| QA23 | 2 | 3 |
| VL3 | 3 | 2 |
| S288c | 4 | 1 |
| P301.4 | 3 | 2 |
| R8.3 | 3 | 3 |
| P283.4 | 3 | 2 |
| R103.1 | 2 | 2 |

**Table 4.2** Hydrogen sulphide and sulphur dioxide production on plate.

Concerning hydrogen sulphide production 6 strains EC1118, VIN13, VL3, P301.4, P283.4 and R8.3 are medium producers, while 4 AWRI1631, AWRI796, QA23 and R103.1 produce low concentrations of $H_2S$. The laboratory strain S288c is a high producer of this compound, logical as this is a laboratory strain not adapted to the oenological environment. The two autochthonous strains of Raboso R103.1 and R8.3, showing previously less marked oenological characteristics, in this case fall within the range of commercial wine strains, producing medium-low hydrogen sulphide.

The ability to produce sulphur dioxide does not appear in connection with the oenological characters previously described. Anyway all three categories of production are represented: 4 strains (EC1118, VIN13, QA23 and R8.3) are high producers, 5 strains (AWRI796, VL3, P301.4, P283.4 and R103.1) are medium producers, and 2 strains (AWRI1631 and S288c) are low producers.

This “on plate” methodology used allows to obtain only indicative data on the potential production of the two compounds, because it not reproduces the wine-making conditions. Results confirm that the production of sulphur compounds is a strain-specific character.

#### 4.4.3.2 Sulphite tolerance

The tolerance to sulphur dioxide is the ability to keep unchanged or high enough the speed fermentation in the presence of selective doses of $SO_2$. The antiseptic effect of sulphur dioxide added to the must eliminates microbes and not-oenological yeasts present in the must, but results in the delayed start of the alcoholic fermentation by wine yeasts. High doses can extend the lag phase, fermentation slow down and may lead stop, and this is deleterious in winemaking. Yeasts belonging to the species *Saccharomyces cerevisiae* have $SO_2$ detoxification systems more efficient respects to other species of yeasts, and this guarantee a lag phase not too prolonged.

Sulphite tolerance was studied by means of yeast growth measurement (optical density, OD) after 48h in Delfini synthetic must at different $SO_2$ doses (50 mg/l and 100 mg/l). Threshold for tolerance was set at 0,1 $OD_{600}$. For each strain the test has been set up in duplicate, and tubes were maintained at a temperature of 25 ° C. Concentrations in this test are rather high compared to those used in oenological situation; in addition the antiseptic effect of $SO_2$ in syntethic must is much more prominent as it lacks many components capable of seize it, such as tannins, residues on bunch's skin or lipids, normally present in the natural must.

OD values of the cultures are shown in figure 4.8, measured after 24 hours from inoculum, and in figure 4.9, measured after 48 hours. The concentration of 0 mg/l of $SO_2$ represents a negative control, to demonstrate that yeasts, in the absence of sulphur dioxide, grow regularly and that the slowdown in the growth is certainly due to the presence of $SO_2$.

Observing $SO_2$ tolerance after 24 hours, shown in Figure 3.8, the strain AWRI796, the highest producer of glycerol, is the most sensitive, and it's already inhibited at a concentration of 50 mg/l. Even the strain P301.4, an excellent fermenter, shows a great slowdown. The laboratory strain S288c evidenced a reduction in growth at a concentration of 50 mg/l, more evident at 100 mg/l. The other strains tolerate the concentration of 50 mg/l, while at 100 mg/l the sensitivity increases significantly, as expected, and only the strains VL3, plus VIN13 and R8.3, even if slightly, resist.

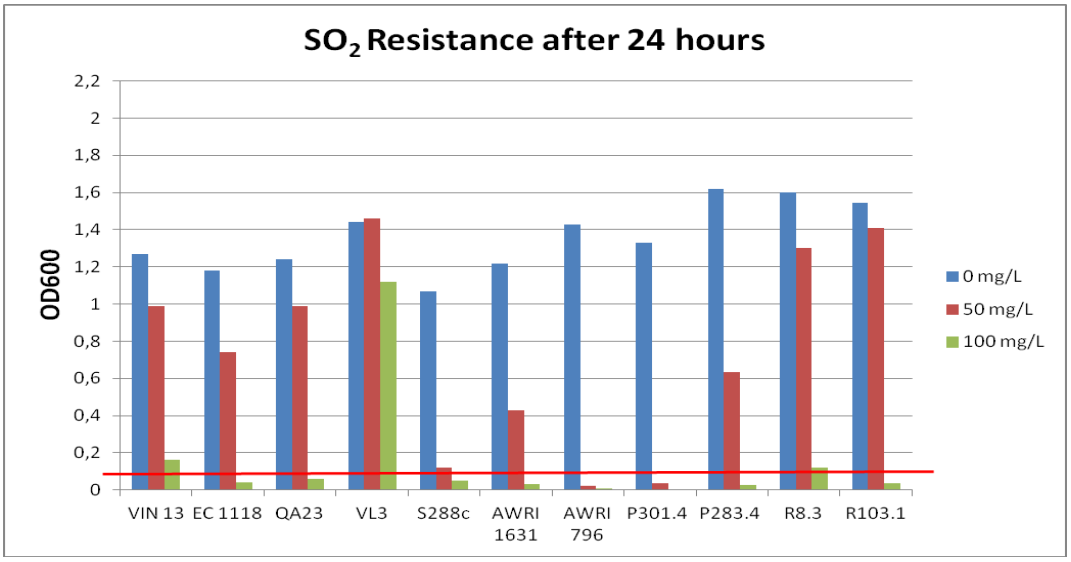



**Figure 4.8** Effect of different doses of $SO_2$ on yeasts growth after 24 hours.

Tolerance after 48 hours, shown in Fig 4.9, confirms the high sensitivity of the strain AWRI796, inhibited by both concentrations of $SO_2$ added. Instead strains S288C and P301.4, showing some difficulty after 24 hours, have a good growth at 50 mg/l of $SO_2$ added. The other strains confirm their resistance to the concentration of 50 mg/l of $SO_2$ added, while at 100 mg/l they confirmed their sensitivity, except strains VIN13, VL3, R8.3 and R103.1, very resistant.

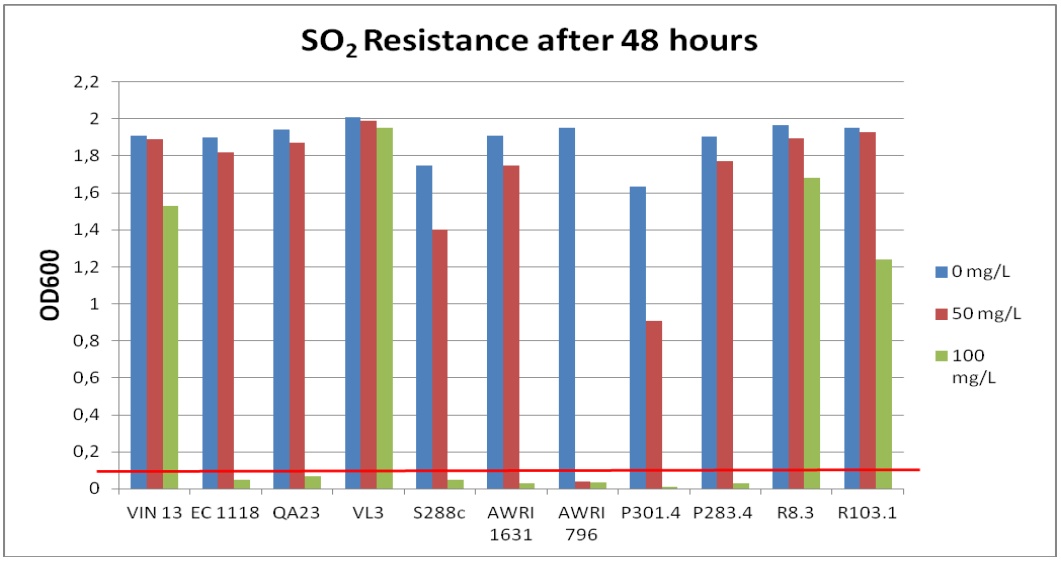



**Figure 4.9** Effect of different doses of $SO_2$ on yeasts growth after 48 hours.

It is interesting to note that autochthonous strains of Raboso R8.3 and R103.1, not considered great fermernters, nor in the presence of 200 g/l of sugar nor of

300 g/l, show a limited growth slowdown. The two commercial strains VIN13 and especially VL3 appear the most resistant.

#### 4.4.3.3 Acetaldehyde production

Acetaldehyde is a normal product in alcoholic fermentation and its content in wine can vary considerably, from 10 mg/l to over 300 mg/l. The evaluation of its content is used as an indicator of how much oxidated is a wine. A high level of acetaldehyde is undesirable because it is associated with the smell of rowan, which remove freshness and vivacity of the wine and covers the fruity scent. In addition, acetaldehyde combines easily with sulphur dioxide to form acetaldehyde combined, and therefore decreases antiseptic and antioxidant effects of sulphur dioxide. The greater variability of acetaldehyde content is determined by the species of yeast. The main producers are strains belonging to the species *S. cerevisiae*, considered relatively higher producers, from 50 to 120 mg/l of acetaldehyde (Vincenzini et al., 2005).

To evaluate the production of acetaldehyde at the end of fermentation, yeasts were inoculated in synthetic must (Delfini, 1995). Each strain was inoculated at a concentration of about $1*10^6$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). For each strain the fermentation test was set up in triplicate. The flasks were kept at a temperature of 25 ° C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation. After fermentation, the fermented must was used to determine the production of acetaldehyde by enzymatic kit. This determination is very delicate because acetaldehyde has an extremely low boiling point and is therefore very difficult to quantify with precision. For this reason, data obtained from this analysis show a standard deviation rather high and give only an indication of the production of this compound.

Values for the production of acetaldehyde are reported in Figure 4.10 and vary between 17.8 and 38.6 mg/l.

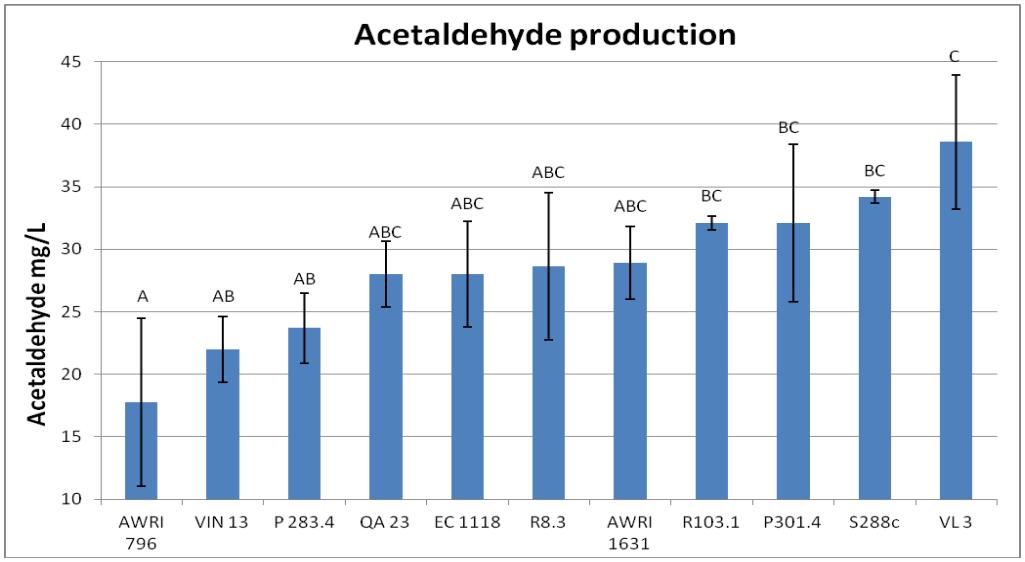



**Figure 4.10** Acetaldehyde production.

The strain AWRI796 is the lowest producer of acetaldehyde, and the value associated with this strain is statistically significant if compared with R103.1, P301.4, S288c and VL3.

Considering previously data it can be concluded that the strain AWRI796 does not possess adequate mechanisms to tolerate sulphur dioxide in the experimental conditions. On the other hand, the strain VIN13, while producing concentrations of acetaldehyde comparable with AWRI796 (the difference is not statistically significant), is very resistant, suggesting that the mechanism of resistance does not include the production of acetaldehyde, but an alternative way. A quite opposite situation is observed for strain VL3, for which the mechanism of resistance to $SO_2$ seems to be a high production of acetaldehyde. The laboratory strain S288c is a good producer of acetaldehyde.

#### 4.4.3.4 Sulphur dioxide production

The production of sulphur dioxide during fermentation, in the absence of exogenous sulphites, it's a character highly variable among strains in oenological yeasts. Most of the strains belonging to the species *S. cerevisiae* produces quantities of $SO_2$ ranging between 10-30 mg/l (Vincenzini et al., 2005).

Each strain was inoculated at a concentration of about $1*10^6$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). For each strain the fermentation test was set up in triplicate. Flasks were kept at a temperature of

25°C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation. After fermentation, the fermented product was used to determine, by iodometric titration, the production of free and total sulphur dioxide (the latter excludes the combined fraction with acetaldehyde).

Values of free $SO_2$ production are shown in Figure 4.11 and those of total $SO_2$ production in Figure 4.12.

The value of free $SO_2$ varies between 4.5 and 8 mg/l, a very low value, difficult to determine by titration. Based on this data there are no particularly interesting differences between the strains.

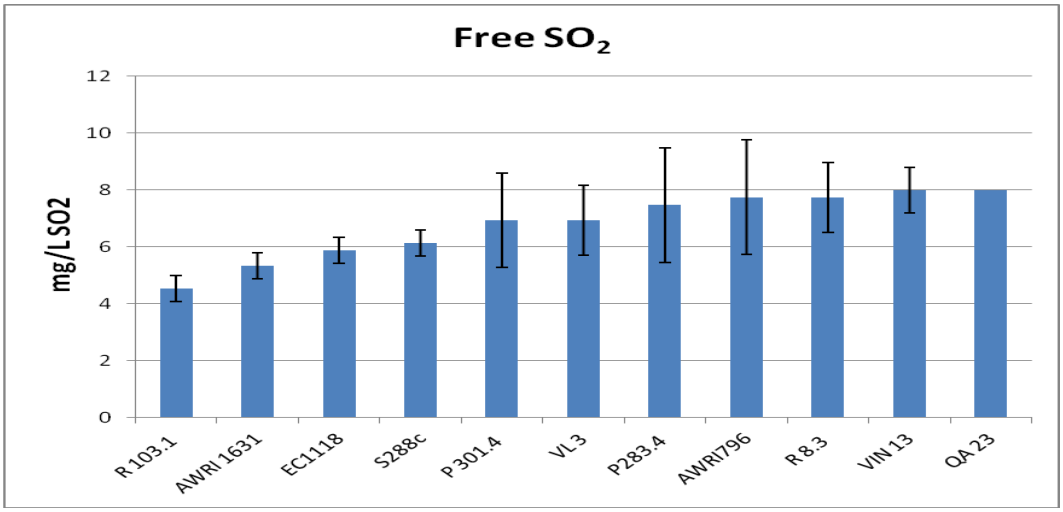



**Figure 4.11** Free $SO_2$ at the end of fermentation process using small-scale method.

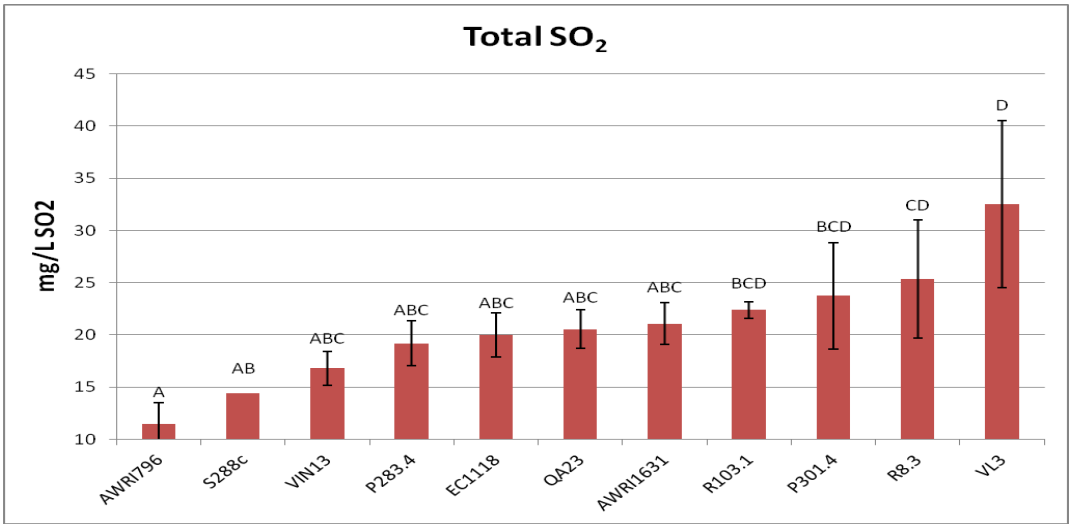



**Figure 4.12** Total $SO_2$ at the end of fermentation process using small-scale method.

Values of total $SO_2$ produced at the end of fermentation (Fig.4.12) show that all strains have produced sulphur dioxide, in a range from 11.5 mg/l to 32.5 mg/l. All these values are in the typical range of *S. cerevisiae*. Moreover the production of $SO_2$ is different in relation to the strains.

The strain AWRI796 is located in the group of low producers, as for acetaldehyde production: this strain, producing low concentrations of $SO_2$, does not need to buffer the toxic effect with acetaldehyde. On the contrary, the strain VL3 ranks among high producers: as a producer of high acetaldehyde, it uses this molecule to neutralize sulphite products. In general there is a modulation on the production of acetaldehyde when the total $SO_2$ produced vary, that appears to be strain-specific.

Therefore it can be assumed that the increase in production of acetaldehyde is used as a defense mechanism by $SO_2$ only in some strains. It's important to note, however, that this is only a hypothesis because, to properly correlate the production of $SO_2$ and acetaldehyde, is necessary to have data on the different stages of fermentation and confirm at what time the two compounds are produced by the yeast.

## 4.5 Conclusions

A physiological characterization have been carried out for 4 autochthonous yeasts (P301.4, P283.4, and R103.1 R8.3) and 6 commercial yeasts from Europe and South Africa (EC1118, AWRI796, AWRI1631, QA23, VL3, VIN13) together with the reference strain S288C, whose genome sequences are available.

Considering fermentative performance in standard conditions it's possible to assess that strains VIN13 and P301.4 have the best fermentation kinetics and the best fermentation vigour, so the strain isolated in DOCG Prosecco has interesting oenological characteristics. The laboratory strain S288c and R103.1 are the slower and less vigorous, and both produce low concentrations of glycerol. The strain EC1118, considered the French oenological yeast for excellence, is unsatisfying, because of the mediocre fermentation kinetics and the low fermentation vigour.

Considering fermentation kinetics in the presence of an excess of sugars the strain EC1118 has kinetics and shows a strong ability to work well at high concentrations of sugar, because of its excellent fermentative power. Strains poor fermenters under standard conditions (S288c, R103.1 and R8.3 in part) also possess a low fermentative power. Surprisingly the strain P283.4, good fementer in standard conditions, reveals a low fermentative power, so it is not suitable to ferment musts with high concentrations of sugars.

An important consideration must be made for the commercial yeast VIN13 and the autochthonous strain P301.4. These strains are very versatile and extremely capable, with fermentation kinetics very fast in standard conditions and an excellent fermentative power when fermenting high concentrations of sugars.

Considering the metabolism of sulphur compounds it's possible to assess that the commercial strain VL3 is the most resistant to sulphites. Strains VIN13 R8.3 and R103.1 reveal an excellent resistance. The strain more sensitive is AWRI796, and it produces the least amount of acetaldehyde and sulphur dioxide. These results indicate that there is a direct relationship between the performance of fermentation and the characteristics of sulphite tolerance. Strains with good fermentation kinetics may be the least resistant to sulphites and vice versa.

It was observed a direct relationship between the production of $SO_2$ and acetaldehyde. Greater is the production of sulphites by the strain, the greater is the amount of acetaldehyde produced. This result indicates that acetaldehyde

production is a way primarily used by yeasts to limit the effect of toxicity produced by endogenous sulphites.

Moreover the strain VIN13, while producing low concentrations of acetaldehyde, comparable with the most sensitive strain AWRI796, is very resistant, suggesting that the mechanism of resistance does not include the production of acetaldehyde, but an alternative way (for example the use of the pump SSU1 for the extrusion of sulphites). Situation quite opposite to that observed for the strain VL3, in which the mechanism of $SO_2$ tolerance seems to be a high production of acetaldehyde.

# 5. Effects of $SO_2$ on yeast metabolism and changes in the transcriptional profiles

## 5. 1 Introduction

RNA-Seq is a recently developed approach to transcriptome profiling that uses deep-sequencing technologies. Studies using this method have already altered our view of the extent and complexity of eukaryotic transcriptomes. RNA-Seq also provides a far more precise measurement of levels of transcripts and their isoforms than other methods.

### 5.1.1 RNA Sequencing

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns and other post-transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions (Wang et al., 2009).
The SOLiD™ 3 platform, developed by Applied Biosystems, allows an enormous throughput (more than 20 Gb) but it produces short sequences (400 million sequences 50 bp long). The high number of sequences produced and the possibility to align them on the reference genome using specific algorithms (Campagna et al., 2009) allows both the identification of the absolute expression level of the transcripts and the determination of their structure (Nagalakshmi et al., 2008). Concerning oenological yeasts, only few published researches use the novel genomic approach (nobody cDNA sequencing for trascriptome analysis).
This method allows the identification of the 3' and 5'-ends of the transcripts, the study of intron/exon boundaries and analysis of genes that are difficult to identify using bioinformatics (such as for example small RNAs). These sequencing strategies are imposing a new standard in gene expression projects, in fact the dynamic range is higher than in microarray experiments allowing the analysis of genes expressed at very different levels. Moreover, gene expression is no more

limited by oligos that are restricted to specific genomic regions such as in microarray experiments but is unbiased and directed to all the transcripts at a single base resolution. Recently developed genomic techniques allowed to carry out the precise mapping of both Mendelian and quantitative traits (QTL). In these projects the conventional breeding of haploid parental strains and phenotypical analysis of segregants are coupled with genomes sequencing to correlate the presence of DNA polymorphic sequences (SNPs) to phenotypic characters. All these methods can map the traits with a resolution ranging from 6 to 64 kb but the bulk segregant analysis seems faster and more cost-effective (Brauer et al., 2006). This peculiar use of the modern sequencing methods is particularly effective when complex phenotypic traits.

### 5.1.2 Gene expression studies of yeasts in the presence of sulphites

It has been report (Aranda et al., 2006) that sulphite resistance depends on sulphur and adenine metabolism. The amount of adenine and methionine in a chemically defined growth medium modulates sulphite resistance of wine yeasts. Mutations in the adenine biosynthetic pathway or the presence of adenine in a synthetic minimal culture medium increase sulphite resistance. The concentration of methionine in particular seems to play an important role in the activation of the sulphur amino acids pathway, but also in the resistance to $SO_2$. Indeed a higher concentration of methionine diminishes resistance to $SO_2$. In a strain very sensitive to $SO_2$, an irregular sulphur metabolism occurred. This demonstrated the important role of reduction in $SO_2$ detoxification. The concentration of methionine, adenine, and sulphite in a synthetic grape must influences the progress of fermentation and at the transcriptional level the expression of genes involved in sulphur (*MET16*), adenine (*ADE4*), and acetaldehyde (*ALD6*) metabolism. Sulphite alters the pattern of expression of all these genes. This fact indicates that the response to this stress is complex and involves several metabolic pathways. *MET16* in particular was reported as being repressed in the presence of $SO_2$ in sulphite resistant strains. Sulphite itself has been shown not to affect the expression of *SSU1* or *SSU1-R.*

A transcriptome analysis was performed following exposure to $SO_2$ by Park and Hwang (2008). The results showed that the expression of 21 genes is induced with most of them being involved in sugar metabolism. This could be attributed to a resistance mechanism of the cells. Amongst the genes showing a clear

induction, *PDC1* was identified. This gene encodes a pyruvate decarboxylase. *TDH3*, encoding a glyceraldehyde-3-phosphate dehydrogenase was shown to be the most strongly down-regulated gene together with *ADH1* encoding an alcohol dehydrogenase.

## 5.2 Matherials and methods

### 5.2.1 Yeasts

In this study we investigated

Commercial wine yeasts

| | Producer | Strain name | Species |
|---|---|---|---|
| 1 | AWRI | AWRI 1631 | S. cerevisiae |
| 2 | MAURIVIN | AWRI 796 | S. cerevisiae |
| 3 | LALLEMAND | EC 1118 | S. cerevisiae |
| 4 | LALLEMAND | QA23 | S. cerevisiae |
| 5 | LAFFORT | ZYMAFLORE VL3 | S. cerevisiae |
| 6 | ANCHOR (EVER) MAURVIN | VIN13 | S. cerevisiae |
| 7 | CBS collection strain | S288c | S. cerevisiae |

Autochthonous wine yeasts

isolated from vineyards in Veneto areas (Conegliano Valdobbiadene Prosecco Superiore DOCG and Raboso Piave DOC)

Prosecco collection: P283.4 and P301.4

Raboso collection: R8.3 and R103.1

### 5.2.2 Culture media

**Media**

YM solid agar medium

3 g/l yeast extract (Oxoid);

3 g/l malt extract (Oxoid);

5 g/l vegetatone peptone (DIFCO);

10 g/l glucose (PROLABO)

16 g/l Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g/l yeast extract (OXOID)

20 g/l vegetatone peptone (DIFCO)

5 g/l glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

| | |
|---|---|
| 0,1 g/l | $CaCl_2$ |
| 0,1 g/l | NaCl |
| 1 g/l | $KH_2PO_4$ |
| 0,5 g/l | $MgSO_4 \cdot 7H_2O$ |
| 3 g/l | tartaric acid |

Micronutrients

| | |
|---|---|
| 0,2 mg/l | $NaMoO_4 \cdot 2H_2O$ |
| 0,4 mg/l | $ZnSO_4 \cdot 7H_2O$ |
| 0,5 mg/l | $H_3BO_3$ |
| 0,04 mg/l | $CuSO_4 \cdot 5H_2O$ |
| 0,1 mg/l | KI |
| 0,4 mg/l | $FeCl_3 \cdot 6H_2O$ |
| 0,4 mg/l | $MnSO_4 \cdot H_2O$ |

Vitamins

| | |
|---|---|
| 400 µg/l | pyridoxinehydrochloride |
| 400 µg/l | thiaminehydrochloride |
| 2000 µg/l | Inositol |
| 20 µg/l | Biotin |
| 400 µg/l | Calciumpantothenate |
| 400 µg/l | Nicotinic acid amide |
| 200 µg/l | P-amino-benzoic acid |

Variable components

| | |
|---|---|
| 0,3 g/l | $(NH_4)_2SO_4$ |
| 0,3 g/l | $(NH_4)_2HPO_4$ |
| 200 g/l | Glucose |
| 0,2 g/l | Hydrolyzed Casein |

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

MS300 (synthetic must) 1l

| | |
|---|---|
| Macroelements | |
| 200 g | glucose |
| 0,155 g | $CaCl_2 \cdot 2H_2O$ |
| 0,2 g | NaCl |
| 0,75 g | $KH_2PO_4$ |
| 0,25 g | $MgSO_4 \cdot 7H2O$ |
| 0,5 g | $K_2SO_4$ |
| 0,46 g | $(NH_4)Cl$ |
| 6 g | malic acid |
| 6 g | citric acid |
| Microelements | |
| 4 mg | $MnSO_4 \cdot H_2O$ |
| 4 mg | $ZnSO_4 \cdot 7\ H_2O$ |
| 1 mg | $CuSO_4 \cdot 5H_2O$ |
| 1 mg | KI |
| 0,4 mg | $CoCl_2$ |
| 1 mg | $H_3BO_3$ |
| 1 mg | $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ |
| Vitamins | |
| 20 mg | Myo-inositol |
| 2 mg | Nicotinic acid |
| 1,5 mg | Calcium Panthotenate |
| 0,25 mg | Thiamine hydrochloride |
| 0,25m g | Pyridoxine hydrochloride |
| 0,003 mg | Biotin |
| Aminoacids | |
| 3,70 g | leucine |
| 5,80 g | threonine |
| 1,40 g | glycine |
| 38,60 g | glutamine |

| | |
|---|---|
| 11,10 g | alanine |
| 3,40 g | valine |
| 2,40 g | methionine |
| 2,90 g | phenyl alanine |
| 6,00 g | serine |
| 2,50 g | histidine |
| 1,30 g | lysine |
| 1,00 g | cysteine |
| 46,80 g | proline |
| 1,40 g | tyrosine |
| 13,70 g | tryptophan |
| 2,50 g | isoleucine |
| 3,40g | aspartic acid |
| 9,20g | glutamic acid |
| 28,60g | arginine |

Final pH 3.2

Prepare the aminoacids in a 1 litre aqueous solution and use 13,09 ml per litre of must. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2.

### 5.2.3 Fermentation surveys on Synthetic Nutrient Medium (NSM) (Delfini, 1995)

#### 5.2.3.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate 10ml of YPD liquid medium. The tubes were left in incubation for 30 hours at 25 °C, moved to obtain a culture on stationary phase (approximately$10^7$-$10^8$cells/ml) measured by spectrophotometry ($OD_{600}$ between 5 and 8).

#### 5.2.3.2 Test preparation

Based on the OD of the respective pre-inoculation, for each strain the culture volumes to obtained a final $OD_{600}$ of 0.5 (approximately $10^5$cells/ml) in 100 ml of medium at the beginning of fermentation, were calculated.

Each strain was inoculated in a 100 ml-Erlenmeyer flask sealed with silicon cap and supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use the synthetic must than the natural, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily growth monitoring operations.
The medium was sterilized by autoclaving at 100 ° C for 5 minutes.
Alcoholic fermentation development was controlled by measuring the weight loss daily from the beginning to the end of fermentation process. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

### 5.2.4 Chemical analysis on fermented must

Total and free sulphur dioxide were quantified at the end of synthetic must fermentation using iodometric titration.
Acetaldehyde enzymatic determination was carried out using the kit R-BIOPHARM purchased by Roche. The chemical reaction used is:
Acetaldehyde + $NAD^+$ + $H_2O$ -> Acetic Acid + NADH + $H^+$
The determination of acetaldehyde is controlled by measuring the amount of NADH produced at OD340nm.

### 5.2.5 Statistical analysis

The comparative statistical analysis between the various groups of samples was conducted using the software XLSTAT, vers.7.5.2, using simple analysis of variance (one-way ANOVA), followed by the Tukey test as "post-hoc" tests. The analysis was conducted by comparing the averages of three independent replications and differences were considered statistically significant for p-value less than 0.05.

### 5.2.6 Fermentation in Controlled Bioreactors

#### 5.2.6.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate 10ml of YPD liquid medium. The tubes were left in incubation for 24 hours at 25 °C, in agitation to obtain a culture on stationary phase

(approximately$10^7$-$10^8$cells/ml). 500µl of these cultures were used to inoculate 100ml of TPD liquid medium.

#### 5.2.6.2 Fermentation preparation

Yeast cultures were grown at 25 °C in agitation for 18 hours. Each culture have been centrifuged and the pellet was resuspended into the volume of synthetic must MS300 required to obtain an $OD_{600}$ of 0.5 of the 1:10 diluted solution ($5x10^6$ cells ml). 100 ml of this preinoculum have been add to 900 ml of MS300, a synthetic medium that mimics the composition of a white wine must.
Fermentation was performed at 25°C in 1 l bioreactors (Multifors, Infors HT) constantly monitoring the temperature and the $CO^2$ flux in a range of 1-20 ml/min (red-y mod. GSM-A95A-BN00).
The fermentations have been performed for each strain with no $SO_2$ added and with $SO_2$ added at a final concentration of 50 mg/l.

### 5.2.7 Cellular pellet sampling

Samples have been taken at specific times points during the fermentation. The first samples were taken after 30 minutes from the inoculum, then after 2 hours from inoculum, at the beginning of the fermentation when the $CO_2$ produced was nearly 6 g/l and at the middle fermentation stage when the $CO_2$ produced was nearly 45 g/l. Yeast cells were immediately centrifuged, washed with water and the pellet was immediately frozen by immersion in EtOH previously refrigerated at -80°C in order to maintain unaltered the transcriptional profile.

### 5.2.8 Total RNA extraction

The total RNA has been extracted from each sample using the PureLink® RNA Mini Kit (Ambion) that combines cell disruption, phenol extraction and RNA purification. All water used in the following procedures was treated overnight with diethylpyrocarbonate (DEPC) 0.1% v/v or dimethylpyrocarbonate (DMPC) 0.1% v/v and autoclaved before use to remove RNase. All disposable plastic-equipment used was RNase free guaranteed. Cells were resuspended in 400 µl TRIzol® Reagent (Invitrogen Life Technologies) and broken by vortexing for 4 min with 300 µl glass beads. The total volume was adjusted to 1 ml with Trizol solution. Extraction have been performed as explained by the protocol of the kit:

after a 5 min incubation at room temperature, 200µl chloroform was added to separate the aqueous and the organic phase with a brief agitation. After a 3 min incubation at room temperature the solution was centrifuged at 12000 × g for 15 min and the aqueous phase was recovered (nearly 600 µl). The RNA was precipitated by addition of an equal volume of 70% ethanol, the tube was vortexed to mix and the sample was transferred to the spin cartridge and centrifuged at 12000 × g for 15 sec at room temperature. Discarded the flow-through 700µl wash buffer I was added and centrifuged at 12000 × g for 15 sec and the spin cartridge was placed into a new collection tube. 500 µl wash buffer II with ethanol was added to the spin cartridge, centrifuged at 12000 × g for 15 sec and the flow-through was discarded (2 times). The spin cartridge was centrifuged 2 min at 12000 × g to dry the membrane with bound RNA. The spin cartridge was placed into a recovery tube, 35-50 µl RNase free water was added to the center of the spin cartridge, incubated for 1 min and centrifuged at 12000 × g for 2 min to elute the RNA from the membrane into the recovery tube. The elution step was repeated twice. The quality and the quantity of the purified total RNA samples were measured and 4µg of each replica for each strain were pooled together and freeze-dried. The three replicates for each strain should ensure the minimization of random fluctuation in gene expression due to external conditions.

### 5.2.9 RNA quantification and gel electrophoresis

RNA concentration was determined by spectrophotometric analysis in water: optical density at 260 and 280 nm was measured in UV transparent cuvettes. RNA concentration in the initial sample was calculated as follows:

RNA conc (ng/µl) = ODunits x 40 x dil. factor

The OD ratio 260/280 was also measured.

Samples containing 4-5 µg of RNA were resuspended in denaturating loading dye (formamide 30%, formaldehyde 10%, commercial loading dye 15% (Fermentas International Inc.) containing fycoll, bromophenol blue and xylene-cianol blue) heated at 65°C for 10 minutes and then run on 1.5% agarose gels under denaturing conditions (2% formaldehyde, 20 mM MOPS, 5 mM Na acetate, 1 mM EDTA, pH 7.0). An RNA ladder (0.3–7.4 kb, Fermentas International Inc.) was used as a molecular weight standard and bands were visualized by UV trans-illuminator after Ethidium bromide staining. Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY, USA).

### 5.2.10 rRNA Subtraction

The total RNA extracted from cells includes the complete collection of all transcribed elements of the genome, comprising mRNAs, rRNAs, and regulatory RNA molecules such as microRNAs and short interfering RNAs, snRNAs, and other RNA transcripts of yet unknown function. Large rRNAs constitutes 90-95% RNA species in total RNA so to sequence the transcriptome it is important to eliminate as much as possible rRNA molecules because being so numerous most of the reads produced would be sequences of these molecules. mRNA enrichment using polyA-selection methods is the most common approach used to eliminate rRNA and collect mRNA molecules, but this technique do not enrich the complete transcriptome because most of the regulatory RNA molecules do not have the polyA sequence so they can't be present in the samples. To get the complete set of transcribed RNA molecule, we chose a different approach. The RiboMinusTM Transcriptome Isolation Kit (Invitrogen) was used to selectively remove large rRNAs (18S and 26S in yeast) from total RNA. More than the 98% of rRNA molecules should be removed using this approach, and all the other kinds of RNA should remain in the enriched fraction. Large rRNAs depletion have been performed as suggested by the RiboMinusTM Transcriptome Isolation Kit protocol. RiboMinusTM Probes labeled with a biotin tag plus the hybridization buffer were added to the samples of purified RNA. The probes selectively bind rRNA molecules in solution. Then streptavidin coated magnetic beads are added to bind the biotin tags of the probes molecules. Using a magnet is then possible to separate the beads and everything bound to them and collect only the aqueous solution containing the total RNA without the contaminating large rRNA molecules. RNA samples are then purified and concentrated using silica-based membrane columns (RiboMinus Concentration Module from Invitrogen).

### 5.2.11 SOLiD Libraries preparation

The RNA obtained was used to prepare the libraries using the SOLiD Whole Transcriptome Analysis Kit protocol. RNA was initially chemically fragmented adding the RNaseIII enzyme plus the provided 10X buffer and incubating the reactions at 37°C for 10 minutes. Fragmented RNA was then purified and concentrated using silica-based membrane columns (RiboMinus Concentration Module from Invitrogen). Yield and size distribution of the fragmented RNA was assessed using the Qubit Fluorometer (Invitrogen) and the Agilent 2100

Bioanalyzer. The optimal fragment sizes range is from 35 to 500 nucleotides, and the average size should be 100–200 nt.
Reverse transcription of the RNA to cDNA require the ligation of specific adapters to the RNA molecules. This step was performed adding to the fragmented RNA the Adaptor Mix, the provided buffers and the Ligation Enzyme and incubating the reaction overnight at 16°C. Then reverse transcription was performed adding dNTPs, the reverse transcriptase and its buffer and incubating at 42°C for 30 minutes.
The cDNA was then purified using MinElute PCR Purification columns (Qiagen). cDNA samples were run on pre-casted polyacrylamide gels to separate cDNA molecules with respect to the size. Regions of the gel containing 100–200 nt cDNA molecules were excised and saved. The cDNA from gel slices was amplified by PCR using specific primers binding the adapters. Couples of primers with different barcode sequences in one of the primer have been used for the different samples. The barcode, once sequenced, allows to assign the reads to the correct sample. The DNA obtained was then purified and its yield and size distribution was assessed again using the Agilent 2100 Bioanalyzer, NanoDrop and Qubit Fluorometer. It was important to know the concentration of each sample because they were then pooled together and the same amount of DNA should be taken from each sample to balance them and to obtain a similar number of reads for each condition and strain under analysis. Once having pooled together the right quantity of each sample, the obtained solution underwent the emulsion PCR step.

#### 5.2.11.1 Emulsion PCR and beads enrichment

Emulsion PCR is a crucial step that allows to create beads covered by several DNA copies obtained through the amplification of the same single DNA molecule. It is important that each bead contains single strand copies obtained only from one DNA molecule and that all the obtained beads have DNA bound to them, for this reason it is important to balance accurately the number of beads and DNA molecules in the emulsion PCR.
The aqueous phase is prepared adding to the sample of pooled DNA all the elements provided and required to accomplish the PCR. Two kinds of primers are used, they specifically bind the DNA sequences of the primers used in the amplification step. Primer P2 is present only in the solution prepared for the

PCR, primers P1 are provided in the solution but they are also bound to the magnetic beads. The magnetic beads covered by P1, are added to the aqueous solution and then this solution is dispensed into the oil phase, and the mixture is emulsified by the ULTRATURRAX device. This instrument mixes the two phases to create small droplets of water separated by the oil. Each drop represents a micro reactor and the system is calibrated to obtain droplets containing a DNA molecule a bead and the PCR reagents. The emulsion is then dispensed in 96 well plates and amplification performed in a thermalcycler. At the end of the PCR beads are recovered and enriched. Beads enrichment allows recover only those beads which present correctly amplified DNA on themselves and discard nude and poorly DNA containing beads. This procedure uses polystyrene beads covered by single-stranded P2 adaptors to capture template beads covered by molecules of DNA. Only the beads collected from this step can be used for sequencing. The last step before sequencing run is the modification of 3'-ends. In order to prepare the P2-enriched beads for deposition and binding to the surface of the sequencing device, a dUTP is added to the 3′-end of the P2 templates using a terminal transferase reaction.

### 5.2.12 Sequencing with the SOLiD system

Once 3'-ends modification is accomplished beads are ready for sequencing run. Each bead is covered by several copies of the same molecule of DNA having the structure shown in figure 2.5. The extremity having the sequence of the P1 primer is bound to the bead, the other end has the sequence of the P2 primer and is used for the binding to the surface of the sequencing device. The central part of the molecule contains the target DNA sequence, an internal adaptor and the barcode.

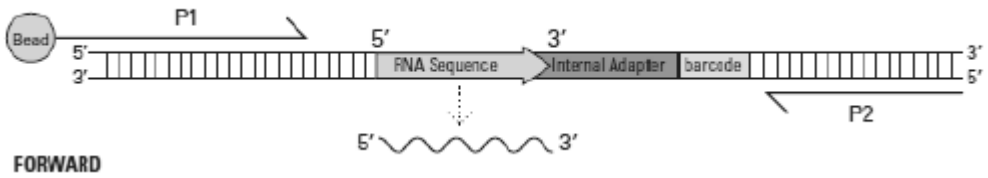



**Figure 5.1** Structure of the molecules of DNA bound to the beads. The target sequence is flanked by the adapter P1 that during the sequencing is bound by the primer to start each round of ligations. On the other end of the molecule there is the barcode which is

sequenced to know to which sample the sequence belong. Barcode is sequenced using the same mechanism used for the target, but ligation cycles start using primers binding the adapter P2.

An important step useful to verify the quality of the library before the sequencing run is the WFA (Work Flow Analysis). It is a quality control which is similar to the sequencing run but it uses only a small fraction of the sample to evaluate beads quality and polyclonal degree. For example, during this step the P2:P1 ratio is calculated to predict the number of optimal constructs (if the P2 adaptor is not present the DNA molecule bound to the bead it is not integer), and depending on the data from this run it is possible to predict how many beads we are going to deposit. After this procedure, the sequencing run is performed. SOLiD system is based on the sequencing-by-ligation technology (Shendure et al., 2005). A primer is hybridized to the adapter sequence within the library template. Then a set of oligonucleotide octamers each labeled with a specific fluorophore among 4 colours, are added. In these octamers, the first and second bases are characterized by one of four fluorescent labels at the end of the octamer. Only the octamers complementary to the sequence of the DNA can bind the DNA molecule and only the octamers binding with the first two bases the two positions after the primer can be ligated to the primer molecule. At this point the fluorescence from the label is detected and bases 1 and 2 in the sequence are thus determined. The ligated octamer oligonucleotides are cleaved off after the fifth base, removing the fluorescent label, then hybridization and ligation cycles are repeated Progressive rounds of octamer ligation enable sequencing of every five bases. Then the extension product is removed and the other round of ligation cycles are performed, starting from a different position in the DNA template. After five rounds the sequence is completely determinate (Zhou et al., 2010). Reads obtained from the sequencing run are encoded in “Colour Space”, each base position is described by two colours and, knowing the identity of the first position (inside the adapter sequence) and using particular rules, it is possible to convert colours into base calls. For some applications sequences are used with the “colour space” coding because this facilitates reads alignment and the identification of true differences (SNPs) and sequencing errors. The SOLiD™ 3 System should generates approximately 300 * $10^6$ reads (30-50 Gbp) per run with reads that

are 50 bases long (Zhou et al., 2010). With the current version of the sequencing system it is not possible to produce longer sequences because for every cycle the background noise increases and the quality of the fluorophore detection and of the sequence decrease.

### 5.2.13 Hierarchical Clustering using TMEV

TIGR MultiExperiment Viewer (TMEV), one member of the suite of microarray data analysis programs is an application that allows the visualization of gene expression data (RNA-seq or microarrays) and the identification of genes and expression patterns of interest (Saeed et al., 2006).

TMEV is composed by several modules, useful to perform different types of analysis in the same work session. Each program implemented in TMEV has a dialog window where the user can insert the parameters of interest.

MEV can interpret different file formats, including the MultiExperiment Viewer format (.mev), the TIGR ArrayViewer format (.tav), the TDMS file format (Tab Delimited, Multiple Sample format), the Affymetrix file format, and GenePix fileformat (.gpr). In my analysis the input file, a TDMS file, contains a matrix of log2 ratio expression values for each gene (rows) in each strain or condition examined (columns). log2 ratio expression values were calculated considering absolute expression values (number of uniquely mapped reads in the coding region of each gene identified) respect to the average value of each gene in all strains and conditions considered in gene expression experiments.

log2 (Ni/Niav)

“Ni” is the number of reads for the gene “i” in one strain and in one of the two conditions analyzed, while “Niav” is the average number of reads of the gene “i” calculated considering all strains (in which the genes is present) and conditions. To perform an unsupervised cluster analysis I used the HCL (Hierarchical Clustering) module of TMEV, an agglomerative algorithm that arranges genes and strains according to similarity in the gene expression pattern. The object of a hierarchical clustering is to compute a dendrogram that assembles all elements into a single tree. For any set of “n” genes, an upper-diagonal similarity matrix is computed, which contains similarity scores for all pairs of genes. The matrix is scanned to identify the highest value

(representing the most similar pair of genes). A node is created joining these two genes, and a gene expression profile is computed for the node by averaging observation for the joined elements. The similarity matrix is updated with this new node replacing the two joined elements, and the process is repeated “n-1” times until only a single element remains.
Agglomerative algorithms begin with each element as a separate cluster and merge them into larger clusters. An important step in any clustering process is to select the method to measure the distance between two clusters, which will determine how the similarity of two elements is calculated. This will influence the clustering, as some elements may be close to one another according to one distance and further away according to another. TMEV allows to calculate the distance with different approaches, in this study I chose the Euclidean distance method. Another parameter to set is the “Linkage Method” that indicates the approach used for determining cluster-to-cluster distances, when constructing the hierarchical tree. I used the "average linkage" method as a measure of cluster-to-cluster distance. The cluster analysis visualization of TMEV consists of colored rectangles, representing genes expression values. Each column represents all the genes from a single experiment, and each row represents the expression of a gene across all experiments.
The default color scheme used to represent expression level is red/green (red for overexpression, green for underexpression); black rectangles are not-differentially expressed genes and green those that do not have assigned value (NA). In the upper and left part of the graph is reported the dendogram structure that represents the correlation between genes (or experiments).

### 5.2.13 Gene Ontology

Genes significantly differentially expressed in oenological strains with respect to the reference S288c have been selected and Gene Ontology categories significantly enriched in these genes were identified using the YeastMine tool (http://yeastmine.yeastgenome.org /yeastmine/begin.do). This program takes as input the two lists of genes: the total set and those with a characteristic of interest, in this case the differential gene expression, and it use the Gene Ontology database to identify biological processes, molecular functions and cellular components typical of the genes on the lists provided. This program

automaticlly classify all the input genes in biological categories simplifying the subsequent biological data interpretation. Genes belonging to categories which are over-represented, are identified thanks to statistical test performed by the program. Output files with statistics on each gene and on the identify classes are produced (Zeeberg et al., 2003, Lopes et al. 2006).

## 5.3 Results and discussion

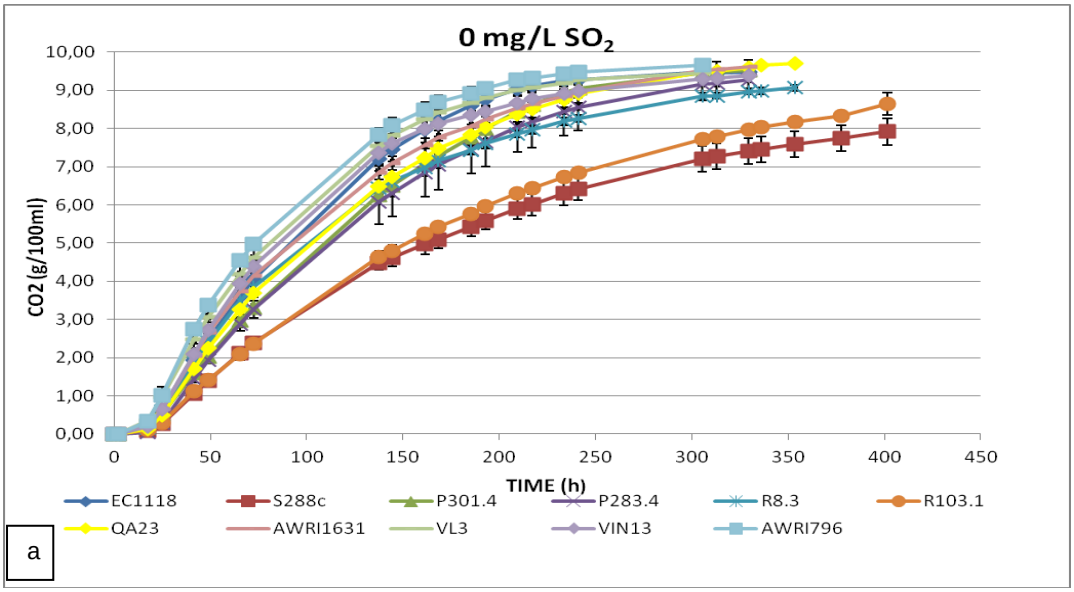
### 5.3.1 Evaluation of fermentation kinetics in the presence of different concentrations of sulphites

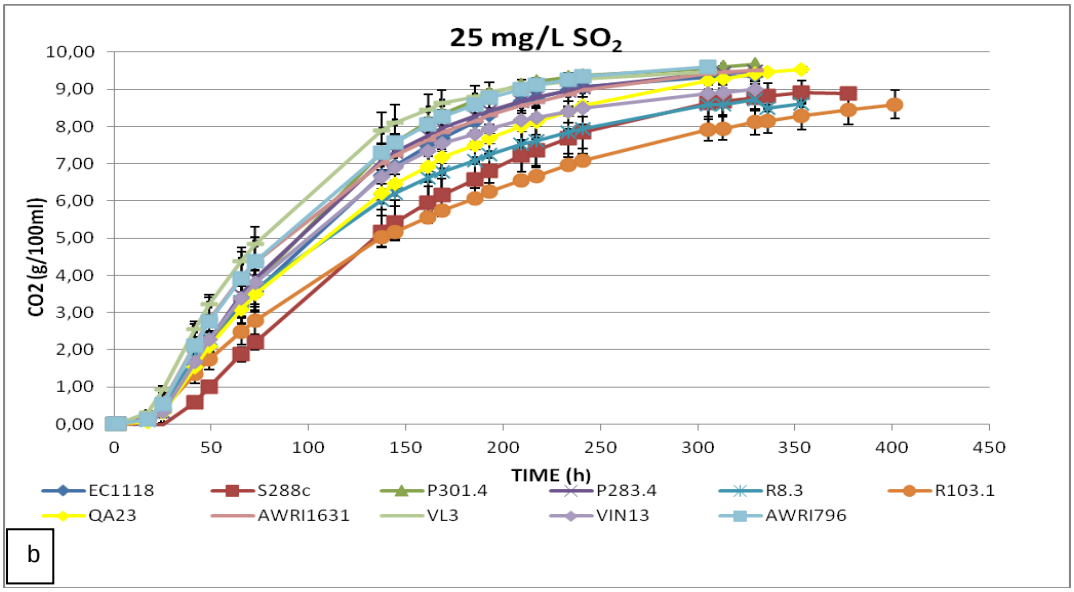
To evaluate the fermentative performance of the yeasts in the presence of different concentrations of sulphite, they were inoculated in Delfini synthetic must (Delfini, 1995) supplemented with 0, 25 or 50 mg/l of $SO_2$, under conditions that simulate oenological setting. These $SO_2$ concentrations represent those used in vinification, but they are very limited.

Strains analyzed, all belonging to the species *S. cerevisiae*, are 4 autochthonous strains P301.4, P283.4, and R8.3 R103.1, isolated from the vineyard in Raboso and Prosecco area, 6 commercial strains EC1118, AWRI796, AWRI1631, QA23, VL3, and VIN13, and the reference laboratory strain S288c (whose genome was the first to be sequenced in 1996).

Each strain was inoculated at a concentration of about $1*10^6$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use synthetic must for preliminary physiological assessments, is to standardize growth conditions and to facilitate significantly daily growth monitoring operations. For each strain the fermentation test was set up in triplicate. The flasks were kept at a temperature of 25° C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation.

Figure 5.1 shows the cumulated $CO_2$ produced over time. Each value is obtained from the average of individual weight loss measured for three replicates. In these conditions, all strains have completed the fermentation in a comparable time (about 20 days).


0 mg/L SO2
CO2 (g/100ml)
TIME (h)
EC1118
S288c
P301.4
P283.4
R8.3
R103.1
QA23
AWRI1631
VL3
VIN13
AWRI796
a


25 mg/L SO2
CO2 (g/100ml)
TIME (h)
EC1118
S288c
P301.4
P283.4
R8.3
R103.1
QA23
AWRI1631
VL3
VIN13
AWRI796
b

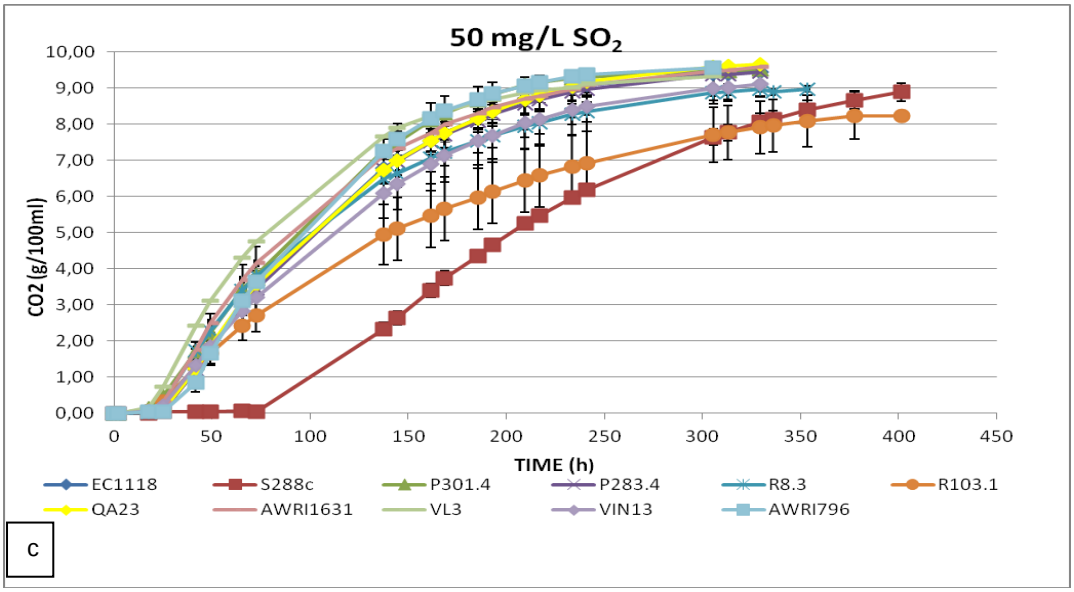

50 mg/L SO2
CO2 (g/100ml)
TIME (h)
EC1118
S288c
P301.4
P283.4
R8.3
R103.1
QA23
AWRI1631
VL3
VIN13
AWRI796
c

**Figure 5.2** Fermentation kinetics with 0 (a), 25 (b) and 50 (c) mg/l of SO2 added.

Observing fermentation kinetics shown in Fig 5.2a, relative to fermentations in the absence of $SO_2$, it is evident that most of the strains have a good kinetics, ending the fermentation in 13 to 17 days, even if showing peculiarities related to the strain. Differently strains S288c and R103.1 show slower kinetics, and leave a sugar residue, respectively about 3 g/100ml and 2 g/100ml, at the end of the trial.
Figure 5.2b shows fermentation kinetics in the presence of 25 mg/l of $SO_2$ added. In this case it can be observed that, in general, the lag time is longer for all strains, and this is reflected on the general speed of fermentation. In particular, the lag phase is more prolonged for strain S288c, for which the effect translates into a faster kinetics (as reported in literature). In this case the lag phase is more evident, but then the speed increases and the strain is able to end the fermentation (unlike at 0 mg/l of $SO_2$).
Figure 5.2c shows fermentation kinetics in the presence of 50 mg/l of $SO_2$ added. In this case the lag phase is generally more pronounced for all strains; in the group of good fermenters, at the beginning of the fermentation, there are strains that are affected by the antiseptic. For the strain S288c, the lag phase is even more prolonged, but then the sulphur dioxide has a positive effect on the kinetics and the speed increases, leading, also in this case, the strain to end the fermentation. A similar positive effect on the kinetics is also seen for the strain AWRI796, that initially has a lag phase longer than in the other conditions, but then velocity increases and the strain ends very well the fermentation, comparably to the other conditions.
Comparing the fermentation curves of the 11 yeast strains in 100-ml synthetic must supplemented with 25 mg/l and 50 mg/l, four different behaviours towards sulphites were highlighted (Fig.5.3). Eight strains, showing good fermentation performances without $SO_2$, were not affected by $SO_2$ in the tested condition (e.g. EC1118 in Fig.5.3); R103.1 showing a slower fermentation rate was not affected by $SO_2$, as well; the fast fermenting strain AWRI796 showed a prolonged lag phase, followed by a good recovery of the fermentation rate in the latter phases when $SO_2$ is present; finally the lab strain S288c was strongly delayed at the beginning of fermentation.
It infers that there isn't a direct relationship between the ability of fermentation and the tolerance to sulphites, and that the two features are separate traits, even

if in a situation of sensitivity to sulphites, for strains more scarce fermenters is more clear the improvement of the fermentation kinetics.

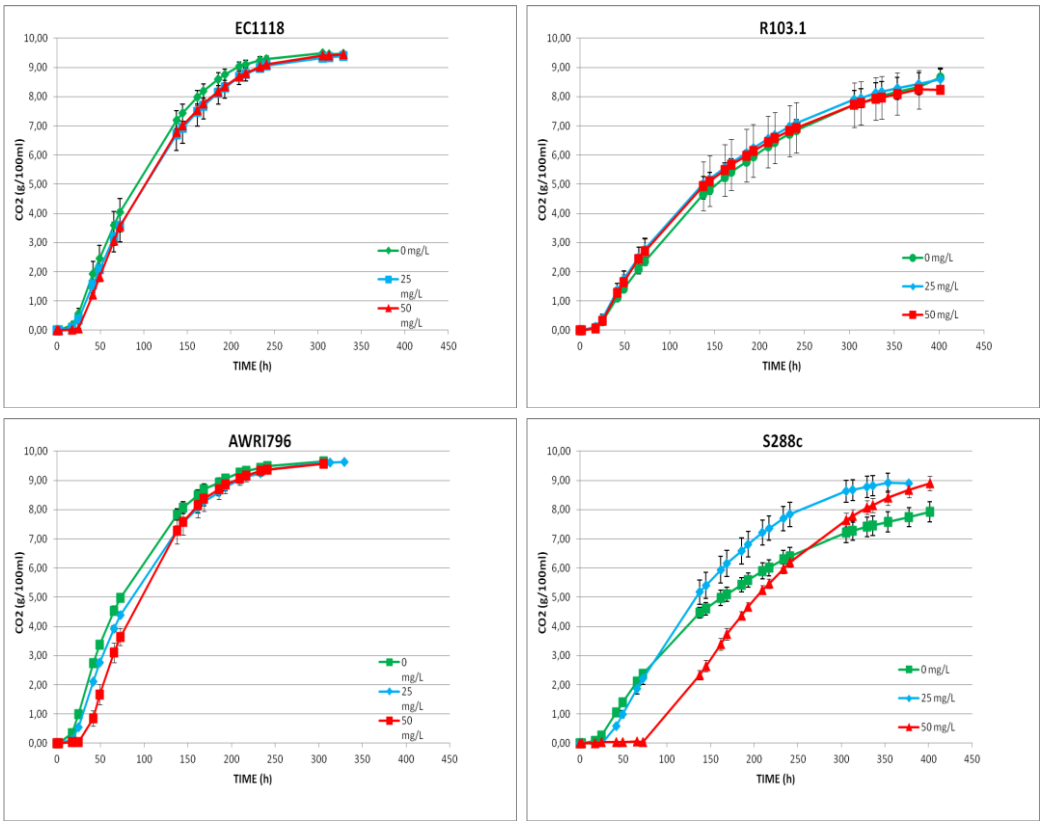



**Figure 5.3** Fermentation kinetics of yeasts with different behaviour towards sulphites.

### 5.3.1.1 Fermentative vigour

The fermentative vigour, corresponding to the quickness of a strain to start and close the fermentative process, even in the presence of antiseptics at legally doses and at temperatures ranging between 20° C and 30° C (Vincenzini et al. , 2005) was evaluated. It is expressed as the amount of glucose consumed in 100 ml of synthetic must after two days of fermentation, and it was estimated by measuring flasks weight loss after 2 days from the start of the fermentative process. Commonly, wine yeasts of the genus *Saccharomyces* are more vigorous, in particular those belonging to the species *S. cerevisiae* (Vincenzini et al., 2005). The fermentative vigour is influenced by the adaptation ability to the oenological environment of the strain and therefore by the duration of the lag phase. In particular a greater adaptive capacity will determine the reduction of the lag phase and then a greater fermentative vigour.

Considering the fermentative vigour, calculated as grams of glucose consumed after 2 days by the formula: sugar metabolized = weight losses *2,118 (Delfini, 1995), it can be observed that, in most cases, at different concentrations of sulphites added, fermentative vigours do not show significant differences (figure 5.4).

Strains S288c and AWRI796 instead lower the fermentative vigour, because the longer lag phase is longer, as already observed in fermentation kinetics. Even strains VIN13, QA23 and P283.4 shows a decline in the fermentative vigour, but lower than the previous two yeasts, in fact it has no effect on fermentation kinetics.

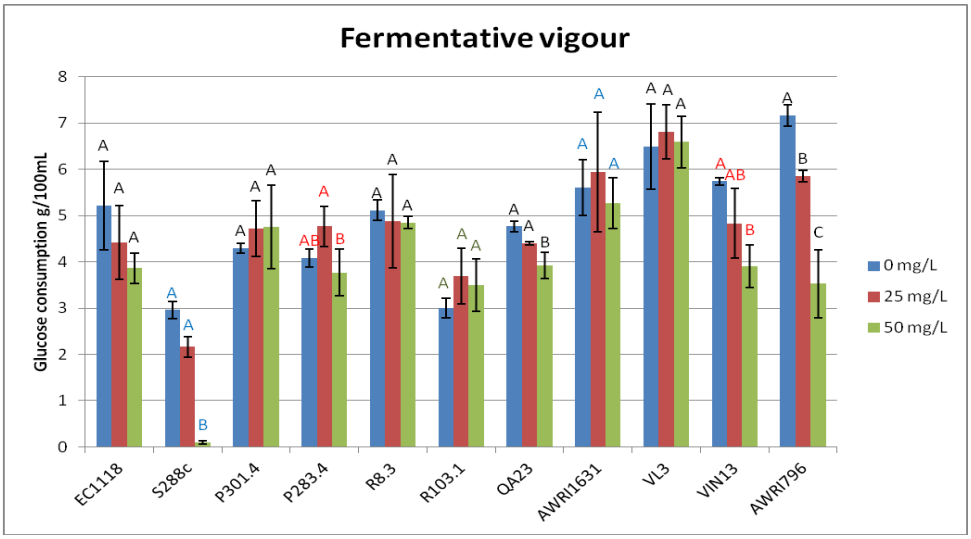



**Figure 5.4** Glucose consumption after 2 days of fermentation in synthetic must.

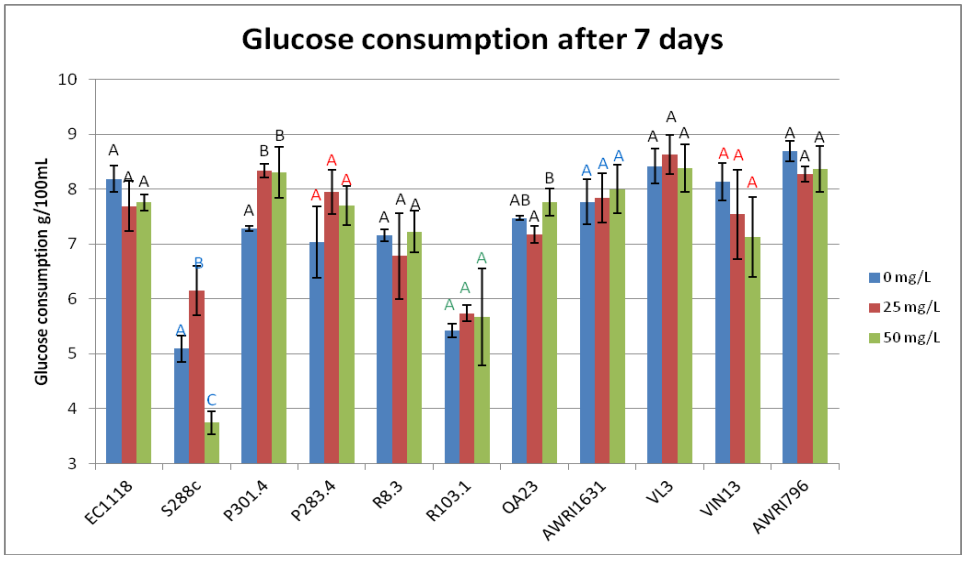



**Figure 5.5** Glucose consumption after 7 days of fermentation in synthetic must.

Moreover glucose consumption after 7 days of fermentation was analysed (figure 5.5). In most cases is not observed a significant difference between different conditions. For the strain S288c glucose consumption is greater when 25 mg/l of $SO_2$ has been added, thereby indicating that the fermentation in this case is faster. Strain QA23 increased glucose consumption, even if only little, at 50 mg/l of $SO_2$ added. Strains AWRI796, VIN13 and P283.4 show no differences.

#### 5.3.1.2 Total sulphur dioxide production

The production of sulphur dioxide during fermentation, in the absence of exogenous sulphites, is known as a strain-specific character, very variable in oenological yeasts. Most of the strains belonging to the species *S. cerevisiae* produces quantities ranging between 10-30 mg/l (Vincenzini et al., 2005).
In Fig 5.6 it can be observed that, in the absence of sulphites, strains largest producers of sulphur dioxide are P301.4, P283.4, R8.3 and VL3 (production between 30 mg/l and 40 mg/l), while strains producing low quantities of $SO_2$ are AWRI796 and S288c (production respectively of 15,5 mg/l and 17,6 mg/l).
When 25 mg/l of $SO_2$ are added (fig. 5.6a), strains AWRI796 and S288c end the fermentation without changing the value of $SO_2$ added, indicating that during fermentation they have reduced considerably the production of endogenous $SO_2$. Strains major producers of $SO_2$ when no sulphites are added are also those that increase more the value of $SO_2$ produced at the end of fermentation. P301.4 increases the production of about 20 mg/l, R8.3 of about 18 mg/l and VL3 of approximately 19 mg/l. However, it is necessary to emphasize that when 25 mg/l of $SO_2$ are added they greatly reduce their production (approximately 51-61%).

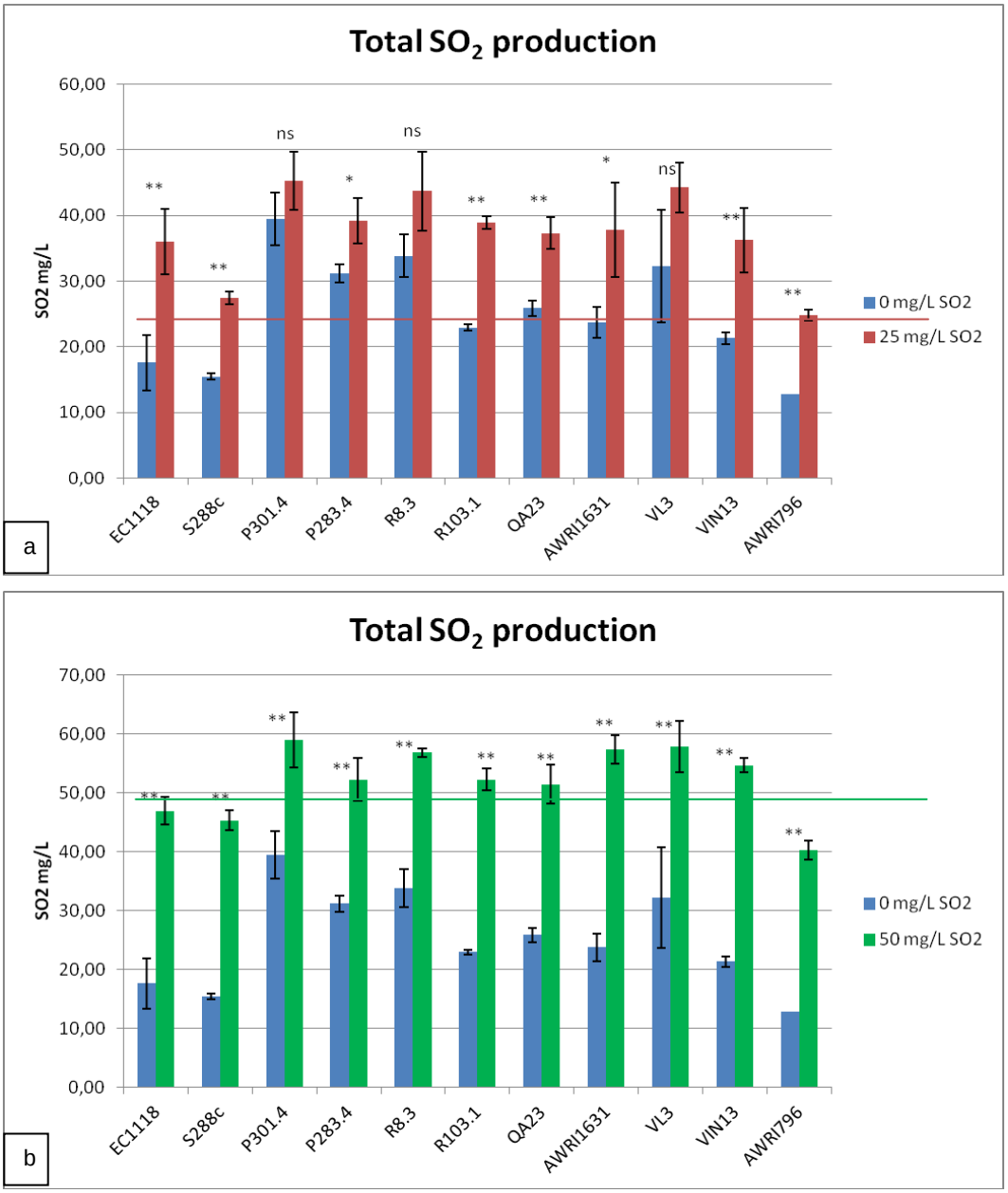



**Figure 5.6** Comparison of total $SO_2$ production at the end of fermentation when (a) 0 mg/l and 25 mg/l of $SO_2$ are added and (b) 0 mg/l and 50 mg/l of $SO_2$ are added.

When 50 mg/l of $SO_2$ are added (fig. 5.6b), it can be observed that the strain AWRI796 lowers the concentration of sulphur dioxide of 10 mg/l, indicating that for its metabolism it uses only $SO_2$ added. Most of the strains instead increase a little bit this value (about 1-2 mg/l). Strains major producers are once again those that increase more the value of $SO_2$ produced at the end of fermentation: P301.4 increases $SO_2$ of about 9 mg/l, VL3 about 8 mg/l and R8.3 and AWRI1631 about 7 mg/l. Also in this case these strains further reduce their production (approximately 5-30%).

#### 5.3.1.3 Acetaldehyde production

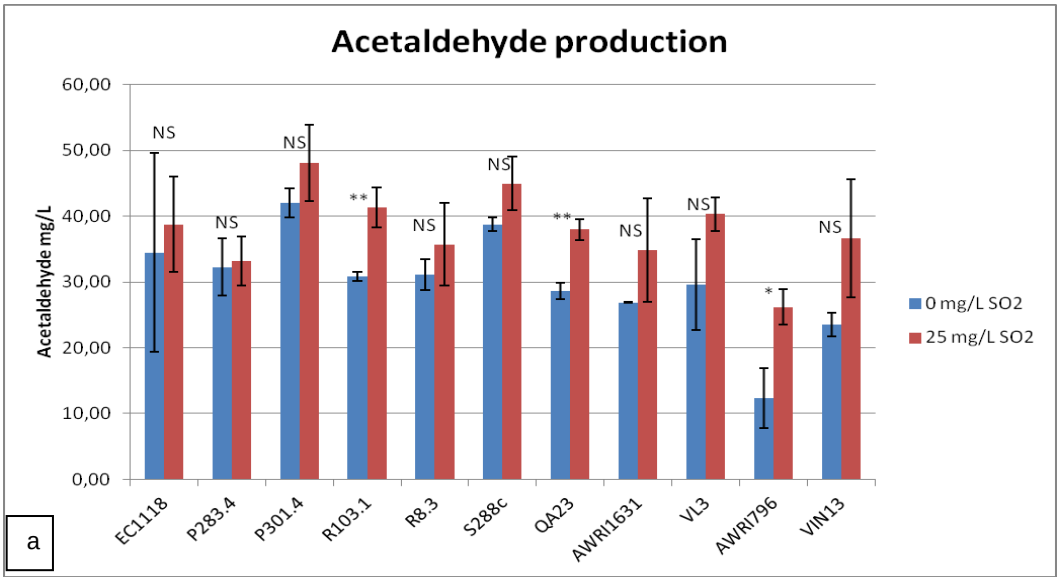
Acetaldehyde is a normal product in alcoholic fermentation and its content in wine can vary considerably, from 10 mg/l to over 300 mg/l. The evaluation of its content is used as an indicator of how much oxidated is a wine. A high level of acetaldehyde is undesirable because it is associated with the smell of rowan, which remove freshness and vivacity of the wine and covers the fruity scent. In addition, acetaldehyde combines easily with sulphur dioxide to form acetaldehyde combined, and therefore decreases antiseptic and antioxidant effects of sulphur dioxide. The greater variability of acetaldehyde content is determined by the species of yeast. The main producers are strains belonging to the species *S. cerevisiae*, considered relatively higher producers, from 50 to 120 mg/l of acetaldehyde (Vincenzini et al., 2005).

Acetaldehyde is produced by *S. cerevisiae* as a mechanism of resistance to sulphites: indeed, it combines easily with sulphur dioxide and therefore decreases its antiseptic, antioxidant and antimicrobial effect.

The main factor that determines the greater variability of acetaldehyde content is the species of yeast. The main producers are strains belonging to the species *S. cerevisiae*, considered relatively high producers of acetaldehyde, from 50 to 120 mg/l (Vincenzini et al., 2005).

The production of acetaldehyde at the end of fermentation in the presence of different concentration of sulphites added was evaluated. After fermentation, the fermented must was used to determine the production of acetaldehyde by enzymatic kit. This determination is very delicate because acetaldehyde has an extremely low boiling point and is therefore very difficult to quantify with precision. For this reason, data obtained from this analysis show a standard deviation rather high and give only an indication of the production of this compound.

The production of acetaldehyde during fermentation, in the absence of added sulphites, is strain specific (Fig. 5.7). The strain producing less acetaldehyde is AWRI796, that in other investigations has been found to be the less resistant to $SO_2$. Strains S288c and P301.4 are the major producers (production respectively of 48 mg/l and 45 mg/l). It is interesting to note that P301.4, resulting one of the most resistant, unlike S288c, suggesting that in P301.4 are present other mechanisms of sulphite tolerance (probably SSU1 pump).



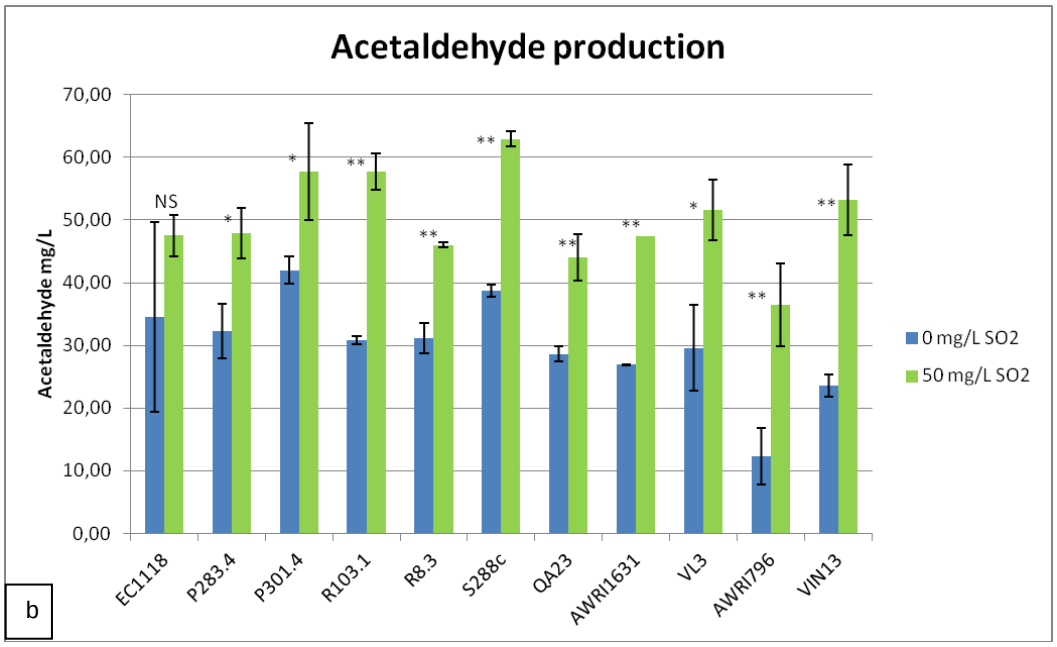




**Figure 5.7** Comparison of acetaldehyde production at the end of fermentation when (a) 0 mg/l and 25 mg/l of $SO_2$ are added and (b) 0 mg/l and 50 mg/l of $SO_2$ are added.

When 25 mg/l of $SO_2$ are added (fig. 5.7a) most of the strains do not show a significant increase in the production of acetaldehyde than in the condition with no sulphites added. Strains with significant difference are R103.1, which increases its production of approximately 30%, QA23 that also increases the production of approximately 30% and AWRI796 that increases the production of approximately 112%. There seems to be a relationship between resistance to sulphite and acetaldehyde production.

On the contrary, when 50 mg/l of $SO_2$ are added (fig. 5.7a) most of the strains show significant differences in the production of acetaldehyde. For almost all strains the increase in production varies between 37% and 87%. In this group there are strains AWRI1631 and VL3, which increase both the production of approximately 75%. The strain VL3 interestingly was the most resistant of tested strains in previous experiments, while AWRI1631 tolerance is on average, as well as the strain P301.4, which is characterized by an increase of only 37%. So it can be inferred that the production of acetaldehyde can have a different significance (higher or lower) in the mechanism of resistance to sulphites.
For two strains the production increase is higher: AWRI796 increased the production of 195% and VIN13 of 125%. It is interesting to note that AWRI796, despite the increase, is still the most sensitive to sulphur dioxide and the production of acetaldehyde at 50 mg/l of $SO_2$ added is 36.5 mg/l. VIN13 instead grouped among the most resistant and the value of acetaldehyde produced in this condition is 53 mg/l. It seems therefore that for VIN13 the prevalent mechanism of resistance to sulphites is the production of acetaldehyde.

### 5.3.2 Strains selection and fermentations in MS300 synthetic must

Considering all data collected from the phenotypic characterization of yeasts with sequenced genome (chapter 4) and the evaluation of their behaviour in the presence of different concentration of sulphites the 4 most interesting yeasts (VL3, AWRI796, R8.3 and EC1118) have been selected to perform a global analysis of gene expression with SOLiD technology.
The commercial strain VL3 shows good performances during fermentation, it is the most resistant to sulphites, the largest producer of $SO_2$ and glycerol, and its mechanism of resistance to $SO_2$ seems to be a high production of acetaldehyde. In the presence of added sulphites it increments the value of $SO_2$ produced at the end of fermentation, although it progressively reduces the production.
The autochthonous strain R8.3 shows good performances during fermentation, is among the most resistant to sulphites, it is a great producer of $SO_2$ and low producer of glycerol, and in the presence of sulphites it produces medium quantities of acetaldehyde.
The French oenological strain EC1118 is considered the reference strain, used in many experiments, it has an excellent fermentative power, shows good

performances during fermentation and on average tolerance to sulphites, it is a low producer of glycerol and it is not affected by the presence of added sulphites. Strain AWRI796 instead is characterized by good fermentation rate but is very sensitive to sulphites, even if it's positively influenced by low concentrations of added sulphites, ending the fermentation better than in the absence of sulphites. It produces the smallest amounts of acetaldehyde and sulphur dioxide, and it is the largest producer of glycerol. In the presence of added sulphites it shows a longer lag phase, but despite this the fermentation kinetics becomes faster, it improves its performance and ends the fermentation reducing the value of $SO_2$ added, indicating that during fermentation it has greatly reduced the production of $SO_2$ and has metabolized the endogenous one. Furthermore in the presence of added sulphites it significantly increases the production of acetaldehyde, but still remains the most sensitive to sulphites, and it has been chosen on purpose as "negative" control.

Alcoholic fermentation in MS300 synthetic must supplemented with different doses of $SO_2$ (0 mg/l and 50 mg/l) in 1l controlled bioreactors, under strict anaerobiosis conditions, was monitored.

After about 2 days from inoculum the fermentation was not started yet, then the trials were suspended to evaluate the dose of $SO_2$ to add, because in this situation 50 mg/l of $SO_2$ seems a growth limiting doses, maybe due to the different composition of MS300 must, that compared to Delfini must does not contain yeast extract, so it combines less sulphur dioxide, leaving in the must more free $SO_2$.

To evaluate the fermentative performance of the 4 selected strains in the presence of different concentrations of sulphite they were inoculated in MS300 synthetic must supplemented with 0, 25 or 50 mg/l of $SO_2$. Each strain was inoculated at a concentration of about $1*10^6$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must. For each strain the fermentation test was set up in triplicate. The flasks were kept at a temperature of 25° C until the end of fermentation. The performance was followed by daily monitoring of the decrease in weight of the flasks, due to the loss of $CO_2$ produced in fermentation. The growth was observed at 24 and 48 hours by recording the decrease in weight. These two time intervals have been chosen because a lag phase not longer than maximum 48 hours is considered acceptable in oenological conditions to start a regular alcoholic fermentation.

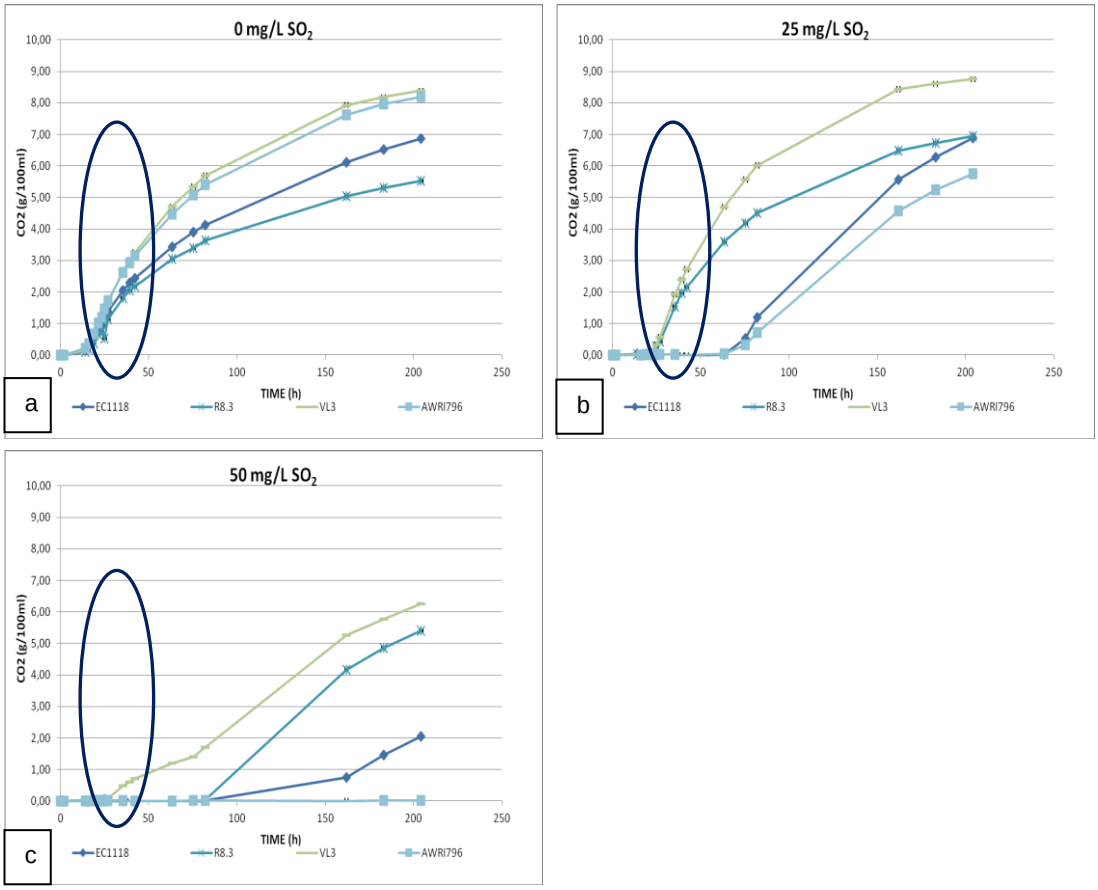



**Figure 5.8** Fermentation kinetics in MS300 with 0 (a), 25 (b) and 50 (c) mg/l of $SO_2$.

It was observed (fig. 5.8) that strains respond in a different way to different concentrations. In particular, the concentration of 50 mg/l of $SO_2$ was strongly inhibitory for the strain AWRI796, while at 25 mg/l of $SO_2$ the discrimination among strains resistant and sensitive to $SO_2$ added was obtained, and this concentration was chosen for new trials in bioreactors.

The fermentation behaviour of the 4 selected yeasts was monitored in 1 l bioreactors by means of $CO_2$ flux measurement, together with sulphite production, in MS300 synthetic must supplemented with 25mg/l $SO_2$. The fermentations in synthetic must with no $SO_2$ added was used as control. Sulphite titration in the medium has been carried out following the standard iodometric method. Finally, for better understanding sulphite metabolism in such conditions, it has been performed a transcription profiling analysis of these yeasts using the next-generation sequencing platform SOLiD.

Samples were taken from bioreactors at the beginning of the fermentation during early exponential phase (arrows in Fig. 5.9) and the $CO_2$ produced in the synthetic must reached 6 g/l/hour. Samples were taken also along the whole

process: after 2 hours from inoculum, in the stationary phase (45 g/l), when cells have passed the peak of high production of $CO_2$ but undergo ethanol stress, and the end of fermentation, to complete all chemical analyses and, if needed, for real time PCR confirmation of RNA-seq results. Figure 5.9 highlights the main differences between selected strains during fermentation with no $SO_2$ added and with 25mg/l of $SO_2$. VL3 is slightly affected by $SO_2$, and it ends the fermentation faster if sulphites are present in the must. R8.3 and EC1118 show good performances during fermentation, but they are delayed when $SO_2$ is added. AWRI796 is strongly affected by $SO_2$, but it concludes the fermentation more than one day before R8.3 and EC1118. Furthermore it displays a high peak of production of $CO_2$ and a sudden closure of the fermentative process.

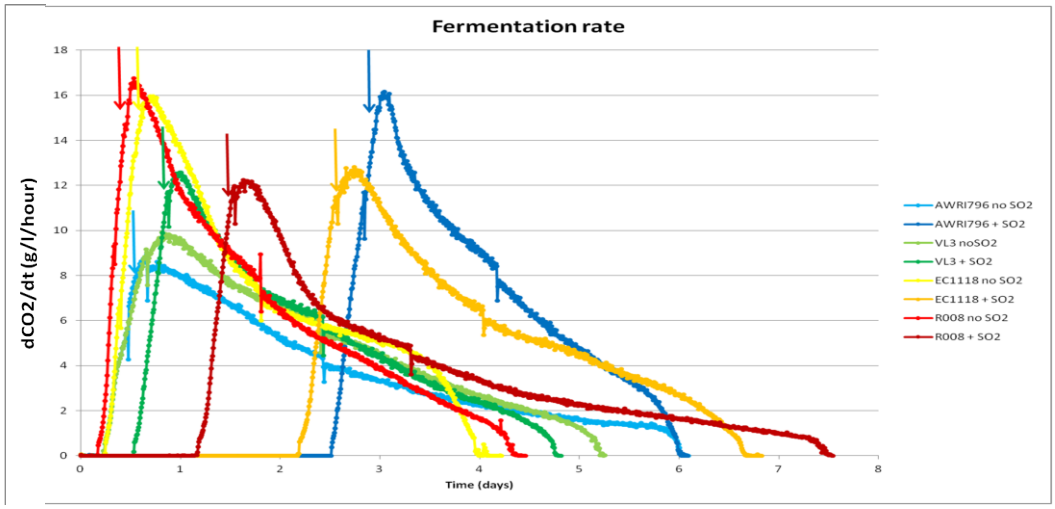



**Figure 5.9** Fermentation kinetics of the four selected strains with 0 and 25 mg/l of $SO_2$ added.

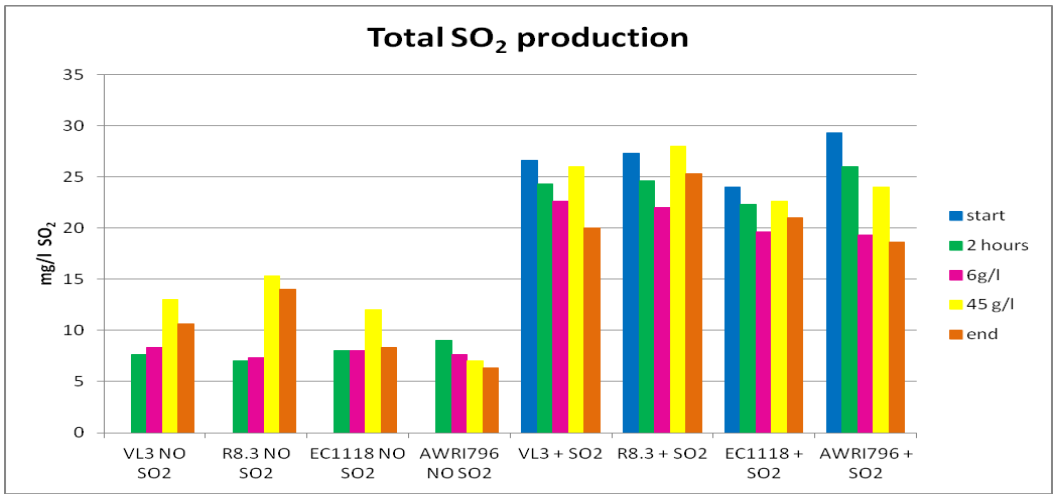



**Figure 5.10** Total $SO_2$ during the fermentation process when 0 and 25 mg/l of $SO_2$ are added.

In Figure 5.10 is reported the accumulation of sulphur dioxide. In the absence of added suphites the accumulation of $SO_2$ for all strains begins after the fermentation peak, at the entrance of the stationary phase. We can therefore assume that the accumulation of sulphur dioxide is linked to the growth slowing of yeast in the stationary phase, and then to a less need to synthesize amino acid, including sulphur amino acids. In the presence of 25 mg/l of $SO_2$ at the beginning of the fermentation it can be observed a trend towards the consumption of added sulphur dioxide, which decreases in concentration. Even in this case the accumulation begins after the fermentation peak.

Total RNA has been extracted for each sample. All RNA samples were examined as to their concentration, purity and integrity. Based on absorbance ratio at 260/280 nm and at 230/260 nm, all samples were pure, free from protein and organic pollutants derived from RNA extraction. Overall sample integrity was confirmed by denaturing formaldehyde agarose gel electrophoresis, showing sharp and intense 18S and 28S ribosomal RNA bands with absence of smears.

The three replicates of each condition were pooled and the pool of total RNA was subtracted ribosomal RNA using the kit. Samples (after quantification and quality control of RNA) were sequenced using the SOLiD sequencer of the CRIBI Biotechnology Centre and output files have been elaborated in collaboration with the Functional genomics group, Dr. Campanaro (Department of Biology, University of Padova).

### 5.3.3 RNA-seq results

RNA was extracted from each sample. Approximately 95% the total RNA is constituted by large rRNA molecules. It is important to eliminate them before the sequencing because being so most of the reads produced would be sequences of these abundant transcripts. rRNAs were subtracted from the samples using a specific kit that should remove 98% of the rRNA molecules. This means that after the subtraction of the rRNA, at least half of the molecules of the sample will be rRNA. After rRNA subtraction the quality and quantity of the samples were measured. Figure 5.11a and 5.11b shows the RNA profiles of two samples calculated by the bioanalyzer (Agilent). Molecules of RNA have lengths varying from 50 to some thousands of nucleotides. Length distribution shows that the RNA is integer because most of the molecules are longer than 500 nucleotides. The two higher peaks correspond to molecules representing residual rRNAs 18S

and 26S and these profiles show that after subtraction the rRNA contamination is still high, so their presence will be probably mirrored by the RNAseq results.

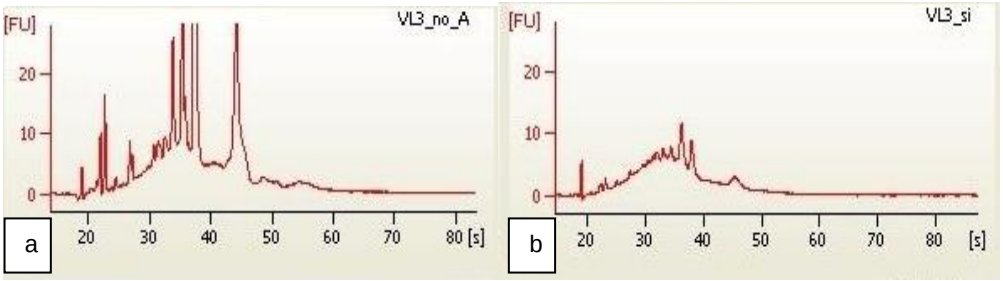



**Figure 5.11** RNA profiles of two samples calculated by the bioanalyzer (Agilent). Length distribution shows that the RNA is integer and that contaminating rRNAs are still present after subtraction. In the sample b the subtraction was more efficient than the sample of figure a, in fact peaks are lower and the amount of total RNA molecules is greater.

RNA-seq was performed using the SOLiD sequencer of the CRIBI Biotechnology Centre. Reads were aligned to the corresponding genomes using the software PASS (Campagna et al., 2009). PASS filters further on the reads and keeps only the high quality ones. It then uses these reads to perform the alignment. Not all the reads are successfully aligned by PASS, and this reduced the number of reads. Among the aligned reads, a fraction of them are uniquely aligned, others align in more than one position. Reads uniquely aligned are the target of the analysis because they are those that can be used to calculate the expression profile of each sequence. Reads mapping in more than one position represent those mapping in repetitive regions of the genome such as those coding for rRNAs and other repetitive elements. Eliminating as much rRNA as possible from the sample of total RNA is important to avoid to obtain lots of reads mapping on repeated regions at the expense of the uniquely mapped reads that are the more useful to create expression profiles. Table 5.1 clearly demonstrate that due to the high number of filters imposed during the different steps of reads detection and alignment, it is important to start from a high number of beads to be sure to get lots of uniquely mapped reads.

| Strain | Reads aligned by PASS | Unique read | % Unique | % Unique/ Aligned reads |
|---|---|---|---|---|
| **AWRI no $SO_2$** | 9705469 | 9458926 | 95% | 97% |
| **AWRI + $SO_2$** | 11430142 | 11036887 | 93% | 97% |
| **EC1118 no $SO_2$** | 28751608 | 15599019 | 45% | 54% |
| **EC1118 + $SO_2$** | 14199525 | 10632363 | 57% | 75% |
| **R8 no $SO_2$** | 19179784 | 18116495 | 62% | 94% |
| **R8 + $SO_2$** | 9374631 | 9289089 | 98% | 99% |
| **VL3 no $SO_2$** | 16553791 | 16273939 | 97% | 98% |
| **VL3 $SO_2$** | 18365544 | 18024467 | 96% | 98% |

**Table 5.1** Statistics from the SOLiD sequencing run and alignment of the obtained reads to the corresponding genomes performed by PASS. The table shows how for each subsequent step of the analysis reads are filtered to get only uniquely mapped reads that can be used to calculate the expression profile of the genomes.

### 5.3.4 GO Classes Enriched

First have been considered those genes differentially expressed in the 4 comparison: strain R8.3 with and without $SO_2$ added, strain EC1118 with and without $SO_2$ added, strain VL3 with and without $SO_2$ added, strain AWRI796 with and without $SO_2$ added.

A total of 61 genes has been identified, and GO terms enriched for genes differentially expressed between the 4 comparison are reported together with the p-value calculated using the Hypergeometric distribution. Holm-Bonferroni multiple test corrections have been also performed to take into account the number of tests being carried out and to correct the p-values accordingly. Results are shown below (table 5.2).

| Gene Ontology Enrichment | |
|---|---|
| **GO term** | **description** |
| GO:0002181 | cytoplasmic translation |
| GO:0031118 | rRNA pseudouridine synthesis |
| GO:0042254 | ribosome biogenesis |
| GO:0000154 | rRNA modification |
| GO:0001522 | pseudouridine synthesis |
| GO:0022613 | ribonucleoprotein complex biogenesis |

**Table 5.2** Table reporting GO categories and pathway tools enriched

The GO analysis suggest a strong expression variation of ribosome biogenesis (subclasses ribonucleoprotein complex biogenesis, rRNA pseudouridine synthesis and rRNA modification) due to the response to sulphite stress.

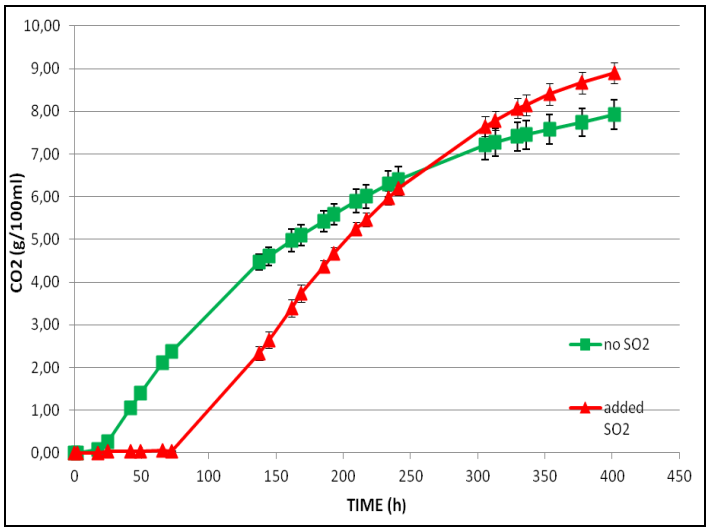



**Figure 5.12** Example of different fermentation kinectics in the absence (■) or in the presence (■) of $SO_2$.

It's known that sulphur dioxide has a stimulating effect when used at low concentrations on sensitive strains (an example is reported ion figure 5.12). After an initial delay of the fermentation start due to a lag phase that lasts longer than in absence of sulphite the start, the fermentation speed accelerates, and the sugar transformation ends more rapidly. Our results on transcritomic profiles suggest an explanation of this peculiar behavior: by means of increasing ribosome numbers and activity, yeast implements nitrogen consumption, recovering the fermentation kinetics, and closes the fermentation more rapidly.

Hierarchical clustering of these 61 differentially expressed genes was performed with TMEV. Values lower than the mean are coloured in green, values upper than the mean are coloured in red (Figure 5.13).

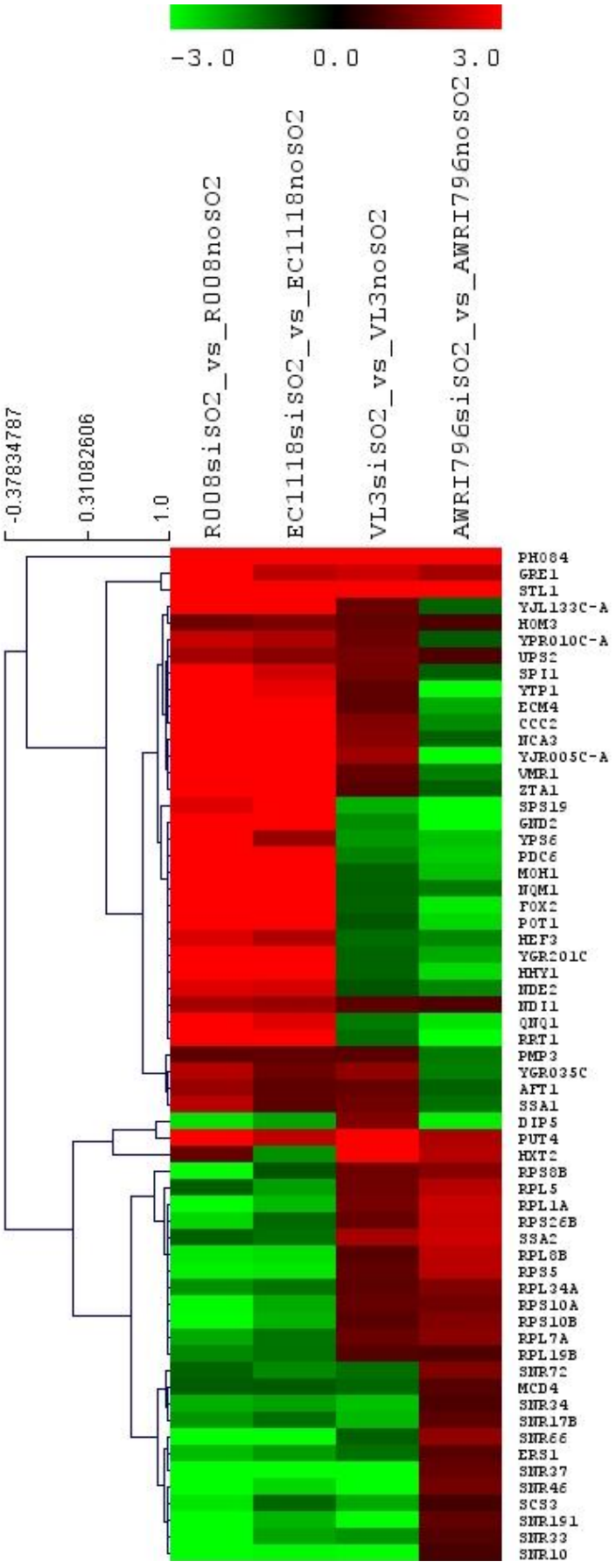



**Figure 5.13** Gene clusters obtained using TMEV software.

Considering the level of expression (fig. 5.13) our results suggest that there is a strong correlation between the fermentation behaviour and the overexpression of ribosomal biosinthesis genes. In fact strains AWRI796 and VL3 (partially) showed and over expression of the genes involved in ribosome biogenesis. Considering the fermentation curve, AWRI796 in the presence of $SO_2$ showed a higher peak (intended as maximal value of fermentation rate) than the one obtained during the growth without sulphites (fig.5.9). Despite the very long lag phase, in presence of sulphite this strain showed a recovery of the fermentation kinetics that allowed to close the fermentation simultanuesly in both condition. Even VL3, that showed a peak slightly higher in the presence of $SO_2$, could recover the fermentation kinetics, ending sugar transformation earlier.

On the contrary in strains R8.3 and EC1118 genes involved in ribosome biogenesis are underexpressed. Both strains showed a lower peaks (fig. 5.9) in presence of sulphites, did not recover the fermentation kinetics, indeed they ended the fermentation later.

After the subtraction of the 61 gene differentially expressed in all the conditions, for each strain a comparison between transcriptome profiles obtained in presence of sulphite and in controll condition (no $SO_2$ added) was performed.

Strain R8.3 showed the higher level of variability since, in presence of sulphite, 63 genes have been down-regulated and 151 up-regulated.

| **Gene Ontology Enrichment** | **A** |
|---|---|
| **Go term** | **Description** |
| GO:0006412 | translation |
| GO:0010467 | gene expression |
| GO:0006414 | translational elongation |
| GO:0002181 | cytoplasmic translation |
| GO:0044267 | cellular protein metabolic process |
| GO:0034645 | cellular macromolecule biosynthetic process |
| GO:0019538 | protein metabolic process |
| GO:0009059 | macromolecule biosynthetic process |
| GO:0042254 | ribosome biogenesis |
| GO:0044249 | cellular biosynthetic process |
| GO:1901576 | organic substance biosynthetic process |
| GO:0009058 | biosynthetic process |
| GO:0022613 | ribonucleoprotein complex biogenesis |
| GO:0044260 | cellular macromolecule metabolic process |

| | |
|---|---|
| GO:0043170 | macromolecule metabolic process |
| GO:0006364 | rRNA processing |
| GO:0016072 | rRNA metabolic process |

| **Gene Ontology Enrichment** | **B** |
|---|---|
| **GO term** | **description** |
| GO:0006536 | glutamate metabolic process |
| GO:0006950 | response to stress |
| GO:0019953 | sexual reproduction |
| GO:0031135 | negative regulation of conjugation |
| GO:0032502 | developmental process |
| GO:0050896 | response to stimulus |
| GO:0032989 | cellular component morphogenesis |
| GO:0044281 | small molecule metabolic process |
| GO:0035445 | borate transmembrane transport |
| GO:0009056 | catabolic process |
| GO:0005984 | disaccharide metabolic process |
| GO:0043335 | protein unfolding |
| GO:0022900 | electron transport chain |
| GO:0005975 | carbohydrate metabolic process |
| GO:0006091 | generation of precursor metabolites and Energy |
| GO:0072524 | pyridine-containing compound metabolic process |
| GO:0010876 | lipid localization |
| GO:0006811 | ion transport |
| GO:0009311 | oligosaccharide metabolic process |
| GO:0016052 | carbohydrate catabolic process |
| GO:0043650 | dicarboxylic acid biosynthetic process |
| GO:0033540 | fatty acid beta-oxidation using acyl-CoA oxidase |
| GO:0009064 | glutamine family amino acid metabolic process |
| GO:0044262 | cellular carbohydrate metabolic process |
| GO:0055085 | transmembrane tran sport |
| GO:0072526 | pyridine-containing compound catabolic process |
| GO:0006013 | mannose metabolic process |
| GO:0042026 | protein refolding |
| GO:0018298 | protein-chromophore linkare |
| GO:0044275 | cellular carbohydrate catabolic process |
| GO:0032862 | activation of Rho GTPase activity |
| GO:0046034 | ATP metabolic process |
| GO:0009117 | nucleotide metabolic process |
| GO:0006753 | nucleoside phosphate metabolic process |
| GO:0031505 | fungal-type cell wall organization |
| GO:0034727 | piecemeal microautophagy of nucleus |
| GO:0070941 | eisosome assembly |
| GO:0043648 | dicarboxylic acid metabolic process |

| | |
|---|---|
| GO:0042221 | response to chemical stimulus |
| GO:0042542 | response to hydrogen peroxide |
| GO:0046356 | acetyl-CoA catabolic process |
| GO:0015980 | energy derivation by oxidation of organic compounds |
| GO:0000719 | photoreactive repair |
| GO:0006200 | ATP catabolic process |
| GO:0006101 | citrate metabolic process |
| GO:0072350 | tricarboxylic acid metabolic process |

**Table 5.3** Table reporting GO categories and pathway tools enriched for (a) underexpressed genes and (b) overexpressed genes of R8.3

Considering GO analysis (Tab 5.3) the underexpressed genes revealed to be involved mainly in protein synthesis such as biosynthesis of tRNA (cellular protein metabolism), ribosomal protein (cellular macromolecule metabolic process, protein metabolic process) ribosomal subunit (ribosome biogenesis). On the contrary the up-regulated genes seems to be involved in different metabolic pathways. Interestingly, the gene clusters that are generally linked to the stationary phase entrance seems to be active during exponential growth. Genes involved in alternative carbon source fermentation such as isomaltase and maltase (carbohydrate metabolic process single-organism, single-organism carbohydrate metabolic process, maltose metabolic process) are prematurely expressed along with autophagy-specific protein coding genes (response to stimulus). Moreover glutamate dehydrogenase isoform (*GDH3*) and other genes involved in glutamate biosynthetic process reported as encoded during nonfermentable or limiting carbon sources are present. Genes involved in heat shock stress response, general stress response (among them *HSP70* family and *HSP104*), oxidative stress response (*CTT* and *GSH1*) generally induced by the presence of ethanol are expressed (Rossignol et. al. 2003). Genes involved in sporulation (cell morphogenesis, regulation of cell morphogenesis, sexual reproduction) are expressed as well, pointing out the stressing effect of added $SO_2$.

Strain EC1118 showed 35 down regulated and 39 up regulated genes. Although the number of differentially expressed genes is lower the involved genes pathways are similar (Tab 5.4) confirming the same genetic response to sulphite than R8.3.

| **Gene Ontology Enrichment** | **A** |
|---|---|
| **GO term** | **description** |
| GO:0006414 | translational elongation |
| GO:0006412 | translation |
| GO:0034645 | cellular macromolecule biosynthetic process |
| GO:0009059 | macromolecule biosynthetic process |
| GO:0044267 | cellular protein metabolic process |
| GO:0010467 | gene expression |
| **Gene Ontology Enrichment** | **B** |
| **GO term** | **description** |
| GO:0000003 | reproduction |
| GO:0005984 | disaccharide metabolic process |
| GO:0008645 | hexose transport |
| GO:0045229 | external encapsulating structure organization |
| GO:0007127 | meiosis I |
| GO:0006591 | ornithine metabolic process |
| GO:0070086 | ubiquitin-dependent endocytosis |
| GO:0009311 | oligosaccharide metabolic process |
| GO:0016052 | carbohydrate catabolic process |
| GO:0044262 | cellular carbohydrate metabolic process |
| GO:0006526 | arginine biosynthetic process |
| GO:0009064 | glutamine family amino acid metabolic process |
| GO:0044275 | cellular carbohydrate catabolic process |
| GO:0006592 | ornithine biosynthetic process |
| GO:0000730 | DNA recombinase assembly |
| GO:0071852 | fungal-type cell wall organization or biogenesis |

**Table 5.4** Table reporting GO categories and pathway tools enriched for (a) underexpressed genes and (b) overexpressed genes of EC1118.

The transcriptome profiles faced for VL3 and AWRI796 are completely different. Excluding the 61 genes differentially expressed in all the condition, the first strain showed only 8 and 3 genes over and under expressed, while AWRI796 21 and 17, respectively (Tab 5.5 and 5.6).

Considering GO result for AWRI796, the main difference is the overexpression of *SSU1* pump, the $SO_2$ transporter and the down-regualtion of the sporulation genss (regulation of mating-type specific transcription) indicating that in both strains no stress response is active.

| **Gene Ontology Enrichment** | **A** |
|---|---|
| **GO term** | **description** |
| GO:0006414 | translational elongation |
| GO:0010467 | gene expression |
| GO:0000395 | mRNA 5'-splice site recognition |
| GO:0045041 | protein import into mitochondrial intermembrane space |
| GO:0045292 | mRNA cis splicing, via spliceosome |
| GO:0006412 | translation |
| GO:0031118 | rRNA pseudouridine synthesis |
| **Gene Ontology Enrichment** | **B** |
| **GO term** | **description** |
| GO: | carbohydrate transport |

**Table 5.5** Table reporting GO categories and pathway tools enriched for (a) underexpressed genes and (b) overexpressed genes of VL3.

| **Gene Ontology Enrichment** | **A** |
|---|---|
| **GO term** | **description** |
| GO:0015942 | formate metabolic process |
| GO:0007535 | donor selection |
| GO:0007532 | regulation of mating-type specific transcription, DNA-dependent |
| GO:0072329 | monocarboxylic acid catabolic process |
| GO:0007533 | mating type switching |
| GO:0007530 | sex determination |
| GO:0007531 | mating type determination |
| GO:0045165 | cell fate commitment |
| GO:0016054 | organic acid catabolic process |
| GO:0046395 | carboxylic acid catabolic process |
| **Gene Ontology Enrichment** | **B** |
| **GO term** | **description** |
| GO:0000316 | sulfite transport |
| GO:0015886 | heme transport |
| GO:0006122 | mitochondrial electron transport |
| GO:1901678 | iron coordination entity transport |
| GO:0007129 | synapsis |
| GO:0051181 | cofactor transport |
| GO:0072348 | sulfur compound transport |

| | |
|---|---|
| GO:0015918 | sterol transport |
| GO:0015698 | inorganic anion transport |
| GO:0042773 | ATP synthesis coupled electron transport |
| GO:0042775 | mitochondrial ATP synthesis coupled electron transport |
| GO:0006119 | oxidative phosphorylation |
| GO:0070192 | chromosome organization involved in meiosis |

**Table 5.6** Table reporting GO categories and pathway tools enriched for (a) underexpressed genes and (b) overexpressed genes of AWRI796.

Hierarchical clustering of selected genes involved in sulfate uptake and metabolism was performed with TMEV (Fig 5.14). The two strains R8.3 and EC1118 showed a similar expression pattern where most of the genes are overexpressed. The expression of two of the few down-regulated genes (*THR1* and *HOM2*) is repressed in response to amino acid starvation. VL3 and AWRI796 showed generally no drammatic change in gene expression profile, but two genes. In VL3 *SUL1* expression strongly decrease. This gene is responsible for an high affinity sulfate permease. In *S. cerevisiae* the sulfate uptake is mediated by specific sulfate transporters Sul1p and Sul2p, which control the concentration of endogenous activated sulfate intermediates. *SUL2* is strongly overexpress in R8.3 and EC1118, while in VL3 no change in expression level was observed.

Concerning gene expression level of *SSU1* pump, considered one on the main responsible for $SO_2$ resistance in wine yeast (Avram and Bakalinsky, 1997) increased in R8.3 and EC1118 even if the basal gene expression is different for the two strains (R8.3 without $SO_2$ 1211 RKPM, R8.3 with $SO_2$ 2623,39 RKPM; EC1118 without $SO_2$ 426,02 RKPM ,EC1118 with $SO_2$ 952,03 RKPM). No expression differences have been detected in VL3 that showed a comparable expression level of EC1118 when the latter is growth presence of sulphite (VL3 without $SO_2$ 727,51 RKPM, VL3 with $SO_2$ 712,28 RKPM). Althoght a strong *SSU1* induction has been detected in AWRI796 the gene expression level is the lowerest found (VL3 without $SO_2$ 12,79 RKPM, VL3 with $SO_2$ 606,77 RKPM). (RKPM means reads per kilobase of sequence per million reads).

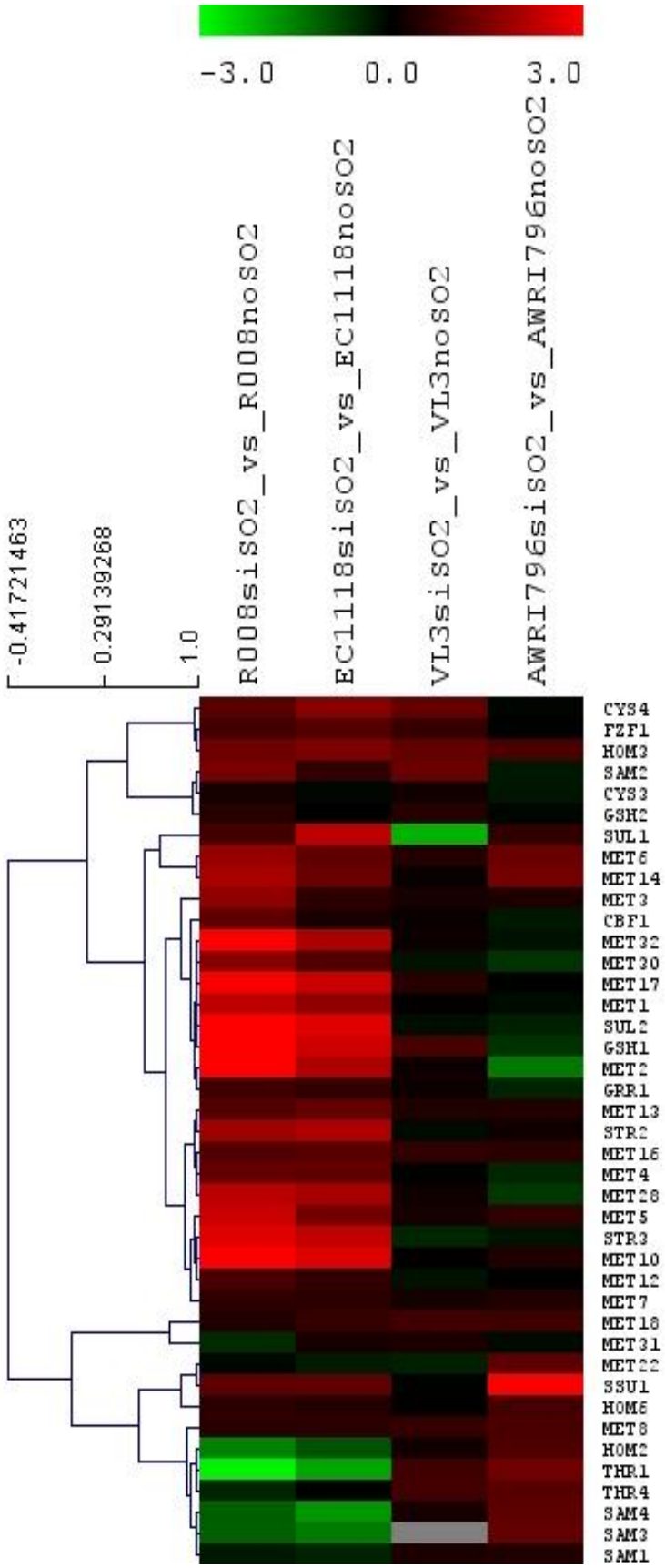



**Figure 5.14** Gene clusters obtained using TMEV software for genes involved in sulphite metabolism.

**Conclusions**

Concerning $SO_2$ production, a strain-dependent behaviour was confirmed when sulphite was not added. The high $SO_2$ producer yeasts reduced their production of 51-61% when 25mg/l of $SO_2$ was present and 5-30% when 50mg/l of $SO_2$ was present. Acetaldehyde production was shown to be strain dependent, as well, and clearly induced by $SO_2$ presence in the must.

These results point out the importance of verifying strain behaviour towards sulphite when wine yeasts are used to ferment sulphited must. Indeed, in this study it has been found that $SO_2$ production is strain-dependent, but not related to fermentation performances and regulated by yeasts on the basis of the initial sulphite concentration. Thus, wines with the same final sulphite content can be obtained starting from different quantity of $SO_2$ added. This study suggests that the choice of yeast strain for vinification can be a tool to limit the sulphite concentration used in winemaking. The on-going comparative analysis of yeast transcriptomes is contributing stronghly to clarify the different yeast behaviour in presence of added sulphite. In particular our results suggest that independent genetic mechanisms are involved in the lag phase lasting (linked to sulphate resistance trait) and in increasing the fermentation rate. Among the yeast analysed the strain (R8.3) that showed the lower sulphate impact on lag phase had the higher level of *SSU1* gene expression. EC1118 and VL3 showed similar level of *SSU1* expression (even if in VL3 the gene is not induced), but the latter is resistant to higher $SO_2$ concentration probably due to the strong down-regulation of the *SLU1* gene, the high affinity sulfate permease strongly induced in EC1118. The very low level of *SSU1* gene expression along with the down regulation of many genes of the sulfur amino acid biosynthetic pathway in AWRI796 determine the prolong lag phase that is peculiar of this strain.

Our result suggest the presence of sulphite stressing condition demonstrated by the expression of different stress response pathways that are not involved in determining the lag-phase lasting (correlated to sulphate resistance). Two strains (VL3 and AWRI796) although characterized by different $SO_2$ resistance level revealed to be not stress by sulphate presence that seems to enhance the ribosomal biosynthesis .

On the contrary R8.3 and EC1118 showing a strong and moderate $SO_2$ resistance respectively appear to be dramatic influenced during fermentation by sulphite and a premature stress resistance genes induction is observed, this causing a notably fermentation rate reduction.

# 6. Selection of reference genes for quantitative real-time PCR studies in *Saccharomyces cerevisiae* during alcoholic fermentation in presence of sulphite

## 6.1 Introduction

Real-time PCR has become the *de facto* standard for mRNA gene expression analysis of a limited number of genes. Given its large dynamic range of linear quantification, high speed, sensitivity (low template input required) and resolution (small differences can be measured), this method is perfectly suited for validation of microarray expression screening results on an independent and larger sample panel, and for studies of a selected number of candidate genes or pathway constituents in an experimental setup (biopsies, treated cell cultures or any other sample collection). More recently, real-time PCR has also entered the high throughput gene expression analysis field based on 384-well block thermal cyclers and newer platforms that allow parallel gene expression analysis of even higher number of genes and samples, depending on platform and configuration (Vandesompele et al., 2009).

It is important to realize that any measured variation in gene expression between subjects is caused by two sources. On one hand, there's the true biological variation, explaining the phenotype or underlying the phenomenon under investigation. On the other hand, there are several confounding factors resulting in non-specific variation, including but not limited to template input quantity and quality, yields of the extraction process and the enzymatic reactions (reverse transcription and polymerase chain reaction amplification).

One of the major difficulties in obtaining reliable expression patterns is the removal of this experimentally induced non-biological variation from the true biological variation. This can be done through normalization by controlling as many of the confounding variables as possible.

### 6.1.1 Reference genes as golden standard for normalization

Problems associated with the use of real-time PCR are linked to the variability associated with the various steps of the experimental procedure, and could lead to severe misinterpretation of the results: different amounts and quality of starting material, variable enzymatic efficiencies (i.e. efficiency of retrotranscription from RNA to cDNA, and PCR efficiency) or differences between tissues or cells in

overall transcriptional activity (Vandesompele et al., 2002, Ginzinger, 2002, Spinsanti et al., 2006).

There are several strategies to remove experimentally induced variation, each with their own advantages and considerations (Huggett et al., 2005). While most of these methods cannot completely reduce all sources of variation, it has been shown to be very important to try to control all the sources of variation along the entire workflow of PCR based gene expression analysis. If each step is not meticulously standardized, variation can and will be introduced in results that cannot be eliminated by applying the final normalization (Stahlberg et al., 2004). It is thus recommended to ensure similar sample size for extraction of RNA and to standardize the amount of RNA for DNase treatment and reverse transcription into cDNA.

Among several strategies proposed (Ginzinger, 2002, Hugget et al., 2005), reference genes are commonly accepted and frequently used to normalize qRT-PCR and to reduce possible errors generated in the quantification of gene expression. In this normalization strategy, internal controls are subjected to the same conditions as genes of interest and their expression is measured by qRT-PCR. The reference genes were expressed in the cells, and their mRNAs are present during sampling, nucleic acid extraction, storage, and any enzymatic processes such as DNase treatment and reverse transcription. The success of this procedure is highly dependent on the choice of the appropriate reference genes (Spinsanti et al., 2006).

Although many studies using qRT-PCR have relied upon only one endogenous control (Radonic et al., 2004, Suzuki et al., 2000), to date the use of a single reference gene appears to be insufficient, and normalization by multiple controls is required (Vandesompele et al., 2002, Pfaffl et al., 2004). A suitable reference gene should be constitutively expressed in the tissues or cells under investigation regardless of the experimental perturbation; exposed to the same experimental protocol of the gene of interest (GOI), should present stable expression levels.

However growing evidence indicates the absence of a single universal reference gene, which may be independent of all kind of experimental conditions. If the expression of the reference gene is altered by the experimental conditions or by external factors, such as contamination, and is affected by a large variation, the noise of the assay is increased and detection of small changes becomes unfeasible, producing results that may be entirely incorrect (Dheda et al., 2005). Since the normalization of real- time RT-qPCR data using a non-validated single

reference gene may engender misleading conclusions, in recent years, the calculation of a normalization factor based on the geometric average of validated multiple reference genes was suggested to discard possible outliers and differences in the abundance of different genes (Vandesompele et al., 2002, Cankour-Cetinkaya et al., 2012).

Several works (Bemeur et al., 2004, Selvey et al., 2001, Lee et al., 2005) prove how some of the most commonly used reference genes cannot always be considered as reliable controls and/or they show different behaviour in various tissues, emphasizing the importance of preliminary evaluation studies, aimed at identifying the most stable reference genes for each single experiments (Spinsanti et al., 2006). Some reference genes (those encoding for Act-B, GAPDH, HPRT1 and 18S ribosomal RNA) have been used as reference for many years in Northern blots, RNase protection tests and conventional quantitative PCR (qPCR), but more recently a number of reports demonstrate that they can vary extensively and are unsuitable for normalization purpose due to large measurement error (Huggett et al., 2005, Dheda et al., 2004, Bemeur et al., 2004). The general approach in normalization using multiple genes is the selection of reference genes among candidate genes, which have been commonly used for normalization. However a group of genes selected among the commonly used reference genes may not be a suitable reference gene set for each experimental condition. It is necessary to validate the suitability of reference genes under specific experimental conditions and to determinate a candidate reference gene set among novel genes instead of commonly used ones, which is suitable for use under the conditions of interest (Cankour-Cetinkaya et al., 2012).

A reasonably successful approach in the compilation of a candidate reference gene set is to select genes from a genome-wide background. The second key point is the determination of the most stable genes among the candidates under selected conditions. Several software tools were developed for the verification of the suitability of the candidate reference genes such as geNorm, NormFinder and Bestkeeper (Vandesompele et al., 2002, Pfaffl et al., 2004, Andersen et al., 2004).

In *Saccharomyces cerevisiae*, studies have focused on validation of reference genes under a particular physiological condition, such as glucose stimulation or dehydration (Stahlberg et al. 2008; Vaudano et al. 2011). Teste et al. (2009) validated a set of reference genes suitable for *S. cerevisiae* growing in a

synthetic minimal medium with 2% (w/v) glucose or galactose and pH 5.0. However, there is no established set of reference genes suitable for normalizing expression data of *S. cerevisiae* during alcoholic fermentation in sulphited condition, such as production of wine, under conditions characterized by low pH, high sugar concentration (120–250 g/l) and steadily increasing ethanol concentration. Vaudano et al. (2011) identified a set of reference genes suitable for normalization of RT-qPCR expression data in *S. cerevisiae* during alcoholic fermentation, but not in conditions with $SO_2$ added to the must.

## 6.2 Materials and methods

### 6.2.1 Yeasts

In this study we used 4 autochthonous strains isolated from vineyards in Veneto areas (Conegliano Valdobbiadene Prosecco Superiore DOCG and Raboso Piave DOC) P283.4 and P301.4 from Prosecco collection and R8.3 and R103.1 from Raboso collection, together with the commercial strain EC1118, product as dried powder by Lallemand SA (Toulouse, France) for the enological market.

### 6.2.2 Culture media

**Media**

YM solid agar medium

3 g/l yeast extract (Oxoid);
3 g/l malt extract (Oxoid);
5 g/l vegetatone peptone (DIFCO);
10 g/l glucose (PROLABO)
16 g/l Bacto Agar (DIFCO).
Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g/l yeast extract (OXOID)
20 g/l vegetatone peptone (DIFCO)
5 g/l glucose (PROLABO)
Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

MS300 (synthetic must) 1l

Macroelements

| | |
|---|---|
| 200 g | glucose |
| 0,155 g | $CaCl_2 \cdot 2H_2O$ |
| 0,2 g | NaCl |
| 0,75 g | $KH_2PO_4$ |
| 0,25 g | $MgSO_4 \cdot 7H2O$ |

| | |
|---|---|
| 0,5 g | $K_2SO_4$ |
| 0,46 g | $(NH_4)Cl$ |
| 6 g | malic acid |
| 6 g | citric acid |
| Microelements | |
| 4 mg | $MnSO_4 \cdot H_2O$ |
| 4 mg | $ZnSO_4 \cdot 7\ H_2O$ |
| 1 mg | $CuSO_4 \cdot 5H_2O$ |
| 1 mg | KI |
| 0,4 mg | $CoCl_2$ |
| 1 mg | $H_3BO_3$ |
| 1 mg | $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ |
| Vitamins | |
| 20 mg | Myo-inositol |
| 2 mg | Nicotinic acid |
| 1,5 mg | Calcium Panthotenate |
| 0,25 mg | Thiamine hydrochloride |
| 0,25m g | Pyridoxine hydrochloride |
| 0,003 mg | Biotin |
| Aminoacids | |
| 3,70 g | leucine |
| 5,80 g | threonine |
| 1,40 g | glycine |
| 38,60 g | glutamine |
| 11,10 g | alanine |
| 3,40 g | valine |
| 2,40 g | methionine |
| 2,90 g | phenyl alanine |
| 6,00 g | serine |
| 2,50 g | histidine |
| 1,30 g | lysine |
| 1,00 g | cysteine |
| 46,80 g | proline |
| 1,40 g | tyrosine |
| 13,70 g | tryptophan |
| 2,50 g | isoleucine |

3,40g aspartic acid
9,20g glutamic acid
28,60g arginine
Final pH 3.2
Prepare the aminoacids in a 1 litre aqueous solution and use 13,09 ml per litre of must. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2.

### 6.2.3 Fermentation in Controlled Bioreactors

#### 6.2.3.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate 10ml of YPD liquid medium. The tubes were left in incubation for 24 hours at 25 °C, in agitation to obtain a culture on stationary phase (approximately$10^7$-$10^8$cells/ml). 500µl of these cultures were used to inoculate 100ml of TPD liquid medium.

#### 6.2.3.2 Fermentation preparation

Yeast cultures were grown at 25 °C in agitation for 18 hours. Each culture have been centrifuged and the pellet was resuspended into the volume of synthetic must MS300 required to obtain an $OD_{600}$ of 0.5 of the 1:10 diluted solution ($5x10^6$ cells ml). 100 ml of this preinoculum have been add to 900 ml of MS300, a synthetic medium that mimics the composition of a white wine must.
Fermentation was performed at 25°C in 1 l bioreactors (Multifors, Infors HT) constantly monitoring the temperature and the $CO^2$ flux in a range of 1-20 ml/min (red-y mod. GSM-A95A-BN00).
The fermentations have been performed for each strain with no $SO_2$ added and with $SO_2$ added at a final concentration of 50 mg/l.

### 6.2.4 Cellular pellet sampling

Samples have been taken at specific times points during the fermentation. The first samples were taken after 30 minutes from the inoculum, then after 2 hours from inoculum, at the beginning of the fermentation when the $CO_2$ produced was nearly 6 g/l and at the middle fermentation stage when the $CO_2$ produced was

nearly 45 g/l. Yeast cells were immediately centrifuged, washed with water and the pellet was immediately frozen by immersion in EtOH previously refrigerated at -80°C in order to maintain unaltered the transcriptional profile.

### 6.2.5 Total RNA extraction

The total RNA has been extracted from each sample using the PureLink® RNA Mini Kit (Ambion) that combines cell disruption, phenol extraction and RNA purification. All water used in the following procedures was treated overnight with diethylpyrocarbonate (DEPC) 0.1% v/v or dimethylpyrocarbonate (DMPC) 0.1% v/v and autoclaved before use to remove RNase. All disposable plastic-equipment used was RNase free guaranteed. Cells were resuspended in 400 µl TRIzol® Reagent (Invitrogen Life Technologies) and broken by vortexing for 4 min with 300 µl glass beads. The total volume was adjusted to 1 ml with Trizol solution. Extraction have been performed as explained by the protocol of the kit: after a 5 min incubation at room temperature, 200µl chloroform was added to separate the aqueous and the organic phase with a brief agitation. After a 3 min incubation at room temperature the solution was centrifuged at 12000 × g for 15 min and the aqueous phase was recovered (nearly 600 µl). The RNA was precipitated by addition of an equal volume of 70% ethanol, the tube was vortexed to mix and the sample was transferred to the spin cartridge and centrifuged at 12000 × g for 15 sec at room temperature. Discarded the flow-through 700µl wash buffer I was added and centrifuged at 12000 × g for 15 sec and the spin cartridge was placed into a new collection tube. 500 µl wash buffer II with ethanol was added to the spin cartridge, centrifuged at 12000 × g for 15 sec and the flow-through was discarded (2 times). The spin cartridge was centrifuged 2 min at 12000 × g to dry the membrane with bound RNA. The spin cartridge was placed into a recovery tube, 35-50 µl RNase free water was added to the center of the spin cartridge, incubated for 1 min and centrifuged at 12000 × g for 2 min to elute the RNA from the membrane into the recovery tube. The elution step was repeated twice. The RNA was conserved at -80° C.

### 6.2.6 RNA quantification and gel electrophoresis

RNA concentration was determined by spectrophotometric analysis in water: optical density at 260 and 280 nm was measured in UV transparent cuvettes. RNA concentration in the initial sample was calculated as follows:

RNA conc (ng/µl) = ODunits x 40 x dil. factor
The OD ratio 260/280 was also measured.
Samples containing 4-5 µg of RNA were resuspended in denaturating loading dye (formamide 30%, formaldehyde 10%, commercial loading dye 15% (Fermentas International Inc.) containing fycoll, bromophenol blue and xylene-cianol blue) heated at 65°C for 10 minutes and then run on 1.5% agarose gels under denaturing conditions (2% formaldehyde, 20 mM MOPS, 5 mM Na acetate, 1 mM EDTA, pH 7.0). An RNA ladder (0.3–7.4 kb, Fermentas International Inc.) was used as a molecular weight standard and bands were visualized by UV trans-illuminator after Ethidium bromide staining. Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY, USA).

### 6.2.7 Reverse transcription (RT-PCR)

#### 6.2.7.1. DNAse treatment

To obtain DNA-free RNA the total RNA previously extracted was treated as follows:
Total RNA 1µg
10X Reaction buffer with $MgCl_2$ (Fermentas) 1µl
DNAse I, RNAse-free (Fermentas) 1µl (1U)
DMPC-treated water to 10µl
After 30 min incubation at 37° C, add 1 µl 50mM EDTA (Fermentas) and incubate at 65° C for 10 min to inactivate DNAse. The template can be used for reverse transcriptase.

#### 6.2.7.2 Synthesis of cDNAs for PCR amplification

cDNA were synthesized using RevertAid M-MuLV Reverse Transcriptase (200 u/µl) (Fermentas) using poliT(16) primers (MWG-biotech, HPSF purified).
Each reactions were assembled as follows:
Total RNA DNAse-free 11µl
Random Primers (0.5µg/µl, Promega) 0,4 µl
Oligo(dT) Primer (0.5µg/µl, MWG) 1 µl
Nuclease-Free Water 0,6 µl
(incubation at 65°C for 10 minutes)
RevertAid 5X Reaction Buffer (Fermentas) 4µl

dNTP mix for RNA (Promega) 2µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl) (Fermentas) 1µl
final volume 20µl
The reactions were incubated at room temperature (22–25°C) for 10 minutes and at 42°C for 2 hours. Afterwards for enzyme inactivation tubes were incubated at 70°C for 15 minutes. Each step was performed with PTC200thermal cycler (MJ Research Inc.)

### 6.2.8 Polymerase Chain Reaction and gel electrophoresis

To check quality control of cDNAs a PCR reaction was performed in a PTC200 thermal cycler (MJ Research Inc.). Reagents for the amplification reactions were added as follows:
Primers 50 µM 0,2 µl (each)
dNTPs 1,25 mM 4µl
GoTaq® DNA Polymerase (5u/µl) (Promega) 0,1 µl
GoTaq reaction buffer (Promega) 5µl
Nuclease free water 13,5µl
cDNA (dil. 1:10) 2µl
final volume 25µl
Amplification of the gene APE2 was performed on cDNAs both for checking the reverse-transcription efficiency and for excluding genomic DNA contamination.
Primer Sequence (5'-3')
APE2 F TGCGCATCAATGTAATGTGGAAGCAGAGTA
APE2 R TGAAATCAGGTTCCACGGTTAAATCGTAGTGT
The thermal protocol was designed as follows:
Cycle1 (1x) 95°C 3'
Cycle2 (35x) 95°C 15''
60°C 30''
72°C 1'
Cycle3 (1x) 72°C 5’
4°C for ever
Amplified samples were run on 1.5% agarose gel containing 1X GelRedTM Nucleic Acid Gel Stain (Biotium). Run was performed on horizontal electrophoresis apparatus with TBE 0,5x as running buffer (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) and the bands were visualized by UV trans-

illumination. Digital images were acquired with EDAS290 capturing system (Kodak).

### 6.2.9 Real-time analyses

Real-Time PCR was carried out on a CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells). A ready to use master-mix containing a fast proof-reading Polymerase, dNTPs, stabilizers, $MgCl_2$ and EvaGreen dye was used according to the manufacturer's instructions (Bio-Rad).

Reactions were prepared in a total volume of 15 µl containing:

Primer F 5µM (MWG) 1,2 µl

Primer R 5µM (MWG) 1,2 µl

RNase,/DNase-free water 0,1 µl

SsoFast EvaGreen Supermix 2X (Bio- Rad) 7,5 µl

cDNA 5 µl

The cycle conditions were set as follows:

initial template denaturation at 98°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 2 sec, and combined primer annealing/elongation at 60°C for 10 sec.

The amount of fluorescence for each sample, given by the incorporation of EvaGreen into dsDNA, was measured at the end of each cycle and analysed via CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). Melting curves of PCR amplicons were obtained with temperatures ranging from 65°C to 95°C. Data acquisition was performed for every 0.2°C increase in temperature, with a 2 sec step.

## 6.3 Results and discussion

### 6.3.1 Determination of the reference gene candidates

In order to determine the reference candidates displaying stable expression throughout time course experiments, genes were selected from those reported in literature (Teste et al, 2009, Schmitt et al., 2005, Nardi et al., 2010) and from a transcriptome data set provided from the Functional genomics group, Dr. Campanaro (Department of Biology, University of Padova).
Combining the 2 sources we selected 7 new potential reference genes based on the stability of their expression during fermentation in non sulphited must (data not shown) and 8 genes traditionally used as reference genes in expression studies, for a total 15 genes listed in Table 6.1.

| Gene | Function | Molecular function (SGD) | Biological process |
|---|---|---|---|
| **ACT1** | Actin, structural protein involved in cell polarization, endocytosis, and other cytoskeletal functions | structural constituent of cytoskeleton | Cell polarization, endocytosis and other cytoskeletal functions |
| **FBA1** | Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; locates to mitochondrial outer surface upon oxidative stress | fructose-bisphosphate aldolase activity | gluconeogenesis; glycolysis |
| **ALG9** | Mannosyltransferase, involved in N-linked glycosylation; catalyzes the transfer of mannose from Dol-P-Man to lipid-linked oligosaccharides; mutation of the human ortholog causes type 1 | mannosyltransferase activity | dolichol-linked oligosaccharide biosynthetic process; protein glycosylation |

| | | | |
|---|---|---|---|
| | congenital disorders of glycosylation | | |
| **PDA1** | E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex, catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA; phosphorylated; regulated by glucose | pyruvate dehydrogenase (acetyl-transferring) activity | acetyl-CoA biosynthetic process from pyruvate |
| **TAF10** | Subunit (145 kDa) of TFIID and SAGA complexes, involved in RNA polymerase II transcription initiation and in chromatin modification | chromatin binding; protein complex scaffold; RNA pol II transcription factor activity | Transcription initiation and chromatin modification |
| **TFC1** | One of six subunits of the RNA polymerase III transcription initiation factor complex (TFIIIC); part of the TauA globular domain of TFIIIC that binds DNA at the BoxA promoter sites of tRNA and similar genes; human homolog is TFIIIC-63 | RNA pol III transcription factor activity | Transcription initiation on Pol III promoter |
| **UBC6** | Ubiquitin-conjugating enzyme involved in ER-associated protein degradation; located at the cytosolic side of the ER membrane; tail region contains a transmembrane segment at the C-terminus; substrate of the ubiquitin-proteasome pathway | Ubiquitin-protein ligase activity | ER-associated protein catabolic process |
| FAS2 | Alpha subunit of fatty acid synthetase, which catalyzes the synthesis of long-chain saturated | Fatty Acid Synthetase activity | fatty acid biosynthetic process |

| | | | |
|---|---|---|---|
| | fatty acids; contains the acyl-carrier protein domain and beta-ketoacyl reductase, beta-ketoacyl synthase and self-pantetheinylation activities | | |
| **PMA1** | Plasma membrane H+-ATPase, pumps protons out of the cell; major regulator of cytoplasmic pH and plasma membrane potential; P2-type ATPase; Hsp30p plays a role in Pma1p regulation; interactions with Std1p appear to propagate [GAR+] | hydrogen-exporting ATPase activity, phosphorylative mechanism | proton transport; regulation of pH; transmembrane transport |
| LYS14 | Transcriptional activator involved in regulation of genes of the lysine biosynthesis pathway; requires 2-aminoadipate semialdehyde as co-inducer | RNA pol II core promoter transcription factor activity (positive regulation) | positive regulation of lysine biosynthetic process and of transcription from RNA pol II prom. |
| DED1 | ATP-dependent DEAD (Asp-Glu-Ala-Asp)-box RNA helicase, required for translation initiation of all yeast mRNAs; mutations in human DEAD-box DBY are a frequent cause of male infertility | RNA strand annealing activity; ATP-dependent RNA helicase activity | translational initiation |
| ENO1 | Enolase I, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction | phosphopyruvate hydratase activity | gluconeogenesis; glycolysis; regulation of vacuole fusion, non-autophagic |

| | | | |
|---|---|---|---|
| | during gluconeogenesis; expression is repressed in response to glucose | | |
| PFK1 | Alpha subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes | 6-phosphofructokinase activity | glycolysis |
| YRB1 | Ran GTPase binding protein; involved in nuclear protein import and RNA export, ubiquitin-mediated protein degradation during the cell cycle; shuttles between the nucleus and cytoplasm; is essential; homolog of human RanBP1 | Ran GTPase binding | RNA export and protein import into nucleus; ubiquitin-dependent protein catabolic process |
| ITR1 | Myo-inositol transporter with strong similarity to the minor myo-inositol transporter Itr2p, member of the sugar transporter superfamily; expression is repressed by inositol and choline via Opi1p and derepressed via Ino2p and Ino4p | myo-inositol transmembrane transporter activity | myo-inositol transport ; transmembrane transport |

**Table 6.1.** List of candidate reference genes: genes in bold are from literature, the other from transcriptome data set.

### 6.3.2 Design of primers

PCR primers for real-time assays were designed on the determined nucleotide sequences of new reference gene using Primer-BLAST, an online tool developed at NCBI to generate candidate primer pairs that are specific to the input PCR template. It uses Primer3 to generate the candidate primer pairs for a given template sequence and then submits them to BLAST search against user-selected database. The blast results are then automatically analyzed to avoid primer pairs that can cause amplification of targets other than the input template. Special attention was given to primer length (15-25 bp), annealing temperature (58°C – 62°C), base composition, 3'-end stability and amplicon size (80-200 bp). For genes selected from literature primers sequences were taken from relative papers.

All the primers were synthesized by MWG-Biotech (HPSF purified) and are listed below:

| Gene | Forward Primer Sequence [5'-->3'] | Reverse Primer Sequence [5'-->3'] | Amplicon Length |
|---|---|---|---|
| ACT1 | ATTATATGTTTAGAGGTTGCTGCTTTGG | CAATTCGTTGTAGAAGGTATGATGCC | 285 bp |
| ALG9 | CACGGATAGTGGCTTTGGTGAACAATTAC | TATGATTATCTGGCAGCAGGAAAGAACTTGGG | 156 bp |
| DED1 | TGGCTGAACTGAGCGAACAAGTGC | AAGAAGCTGCCACCGCCACG | 169 bp |
| ENO1 | TGCACGCTGTTAAGAACGTCAACGA | CAGCGGCAGCTCTGGAAGCA | 183 bp |
| FAS2 | AGGGTGCTGCTGGTGCATGG | ACACGGCTCTGACACCGTCG | 165 bp |
| FBA1 | GGTTTGTACGCTGGTGACATCGC | CCGGAACCACCGTGGAAGACCA | 125 bp |
| ITR1 | CGCAATCAAATGTTGGTGATGCCG | CGCTAGCGGGAGCCCTCTGTA | 129 bp |
| LYS14 | GCTAGAGCGGGATCTTTAGGTGGC | GCTCTGAAGTAGTGGGATGACCTGC | 148 bp |
| PDA1 | ATTTGCCCGTCGTGTTTTGCTGTG | TATGCTGAATCTCGTCTCTAGTTCTGTAGG | 285 bp |
| PFK1 | GAGGTTGATGCTTCTGGGTTCCGT | TGTGGCGGTTTCGTTGGTGTCG | 138 bp |
| PMA1 | GCCTGCTAAGACTTACGATGACGC | TTCACCGGCGGCAACTGGAC | 139 bp |
| TAF10 | ATATTCCAGGATCAGGTCTTCCGTAGC | GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG | 141 bp |
| TFC1 | GCTGGCACTCATATCTTATCGTTTCACAATGG | GAACCTGCTGTCAATACCGCCTGGAG | 223 bp |
| UBC6 | GATACTTGGAATCCTGGCTGGTCTGTCTC | AAAGGGTCTTCTGTTTCATCACCTGTATTTGC | 272 bp |
| YRB1 | ATTCGATGCCGATGCCAAGGAATG | AGTGAAGGCTTCTGCTTCACCTTCT | 235 bp |

**Table 6.2** Details of primers and amplicons for each of the 15 evaluated genes.

For each different pair of primers, efficiency of RT-PCR (E), slope values and correlation coefficients ($R^2$) were determined, using serial 1:5 dilutions of

template cDNA, on CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc). Efficiency was considered adequate when ranging from 90% to 110%, $R^2$ was considered acceptable when greater than 0,98-0,99.

### 6.3.3 Fermentation trials

Alcoholic fermentation in a synthetic must, containing 200 g/l of glucose and 0 or 50 mg/l $SO_2$, under strict anaerobiosis conditions in bioreactors was monitored. The fermentation profile for the 4 autochthonous strains (P301.4, P283.4, R8.3 and R103.1) and the commercial strain EC1118 was determined (fig. 6.1). Usually fermentation rate (dCO2/dt) reaches its maximal value around 12h, before entering stationary phase, and gradually declines thereafter until the end when sugar reserves are exhausted. Samples were taken from bioreactors along the whole process: the first after 30 minutes from the inoculum, the second after 2 hours from inoculum, the third at the beginning of the fermentation, when the cumulated $CO_2$ produced in the synthetic must reached 6 g/l, and the last at 45 g/l of $CO_2$ produced. These concentrations are not reached contemporaneously by the different strains, because the amount of $CO_2$ produced depends on the rapidity of the specific strain and on the presence of $SO_2$. In winemaking, those strains that are able to complete the fermentation quickly and thus consuming all the glucose and releasing $CO_2$ in solution in shorter times, and that are not affected by $SO_2$ are preferred. Fig. 6.1 highlights the main differences between selected strains during fermentation with no $SO_2$ added and with 50mg/l $SO_2$.
Total RNA has been extracted for each sample. All RNA samples were examined as to their concentration, purity and integrity. Based on absorbance ratio at 260/280 nm and at 230/260 nm, all samples were pure, free from protein and organic pollutants derived from RNA extraction. Overall sample integrity was confirmed by denaturing formaldehyde agarose gel electrophoresis, showing sharp and intense 18S and 28S ribosomal RNA bands with absence of smears.

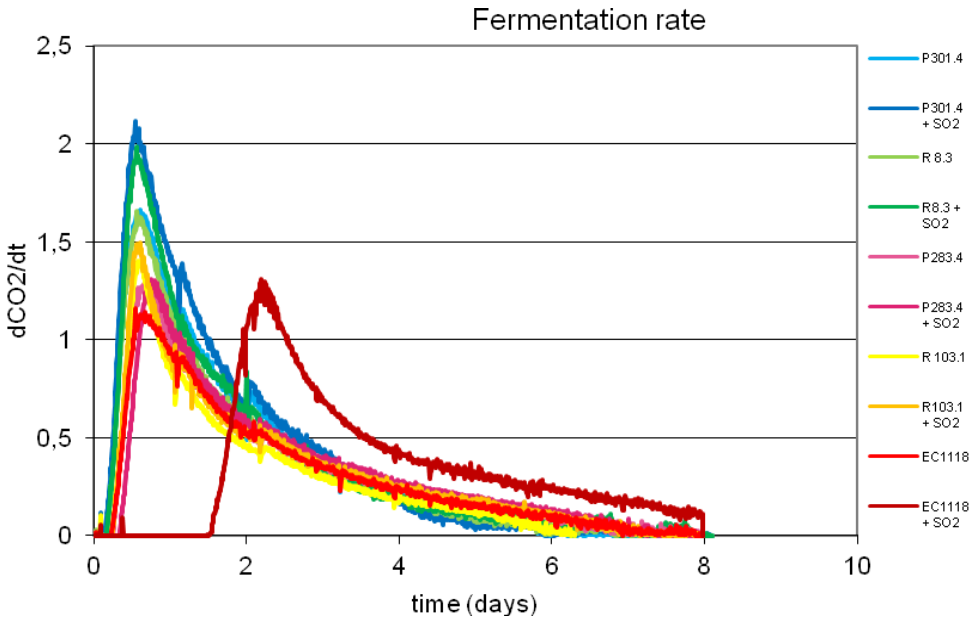



**Figure 6.1** Fermentation kinetics of the four natural strains plus the commercial one. $CO_2$ per hour produced is displayed.

A total of 40 different samples has been collected: 5 strains, P301.4, P283.4, R8.3, R103.1 and EC1118, for 2 conditions, 0 and 50 mg/l $SO_2$ added, for 4 samplings, after 30 min and 2 hours from inoculum, at the beginning of fermentation and at the middle-stage of fermentation.

### 6.3.4 Real-time PCR amplification of reference genes

Real-Time PCR was performed on a CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc.) as previously described. A total of 40 samples was analyzed, each sample twice, and a no-template control for each primers pair was included in all real-time plates, and for each gene was used one plate.

Baseline and threshold values were automatically determined for all plates using the CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). In order to ensure comparability between data obtained from different experimental plates, the threshold value has been subsequently manually set to the value corresponding to the arithmetic mean between the automatically determined thresholds annotated previously; then all data have been reanalyzed (Spinsanti et al., 2006). The data obtained have been converted into correct input files, according to the

requirements of the software, and analysed using geNorm, NormFinder and BestKeeper VBA applets.

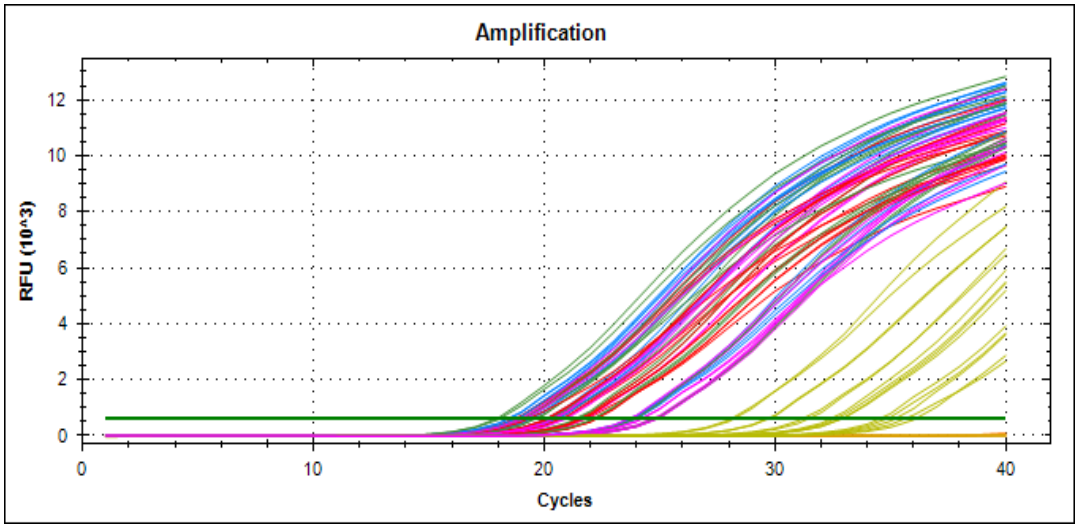



**Figure 6.2** Example of amplification curves.

Amplification curves for EC1118 samples were not acceptable (fig. 6.2, yellow lines), even if the condition was the same for each sample. Samples start amplifying very late or, sometimes, do not amplify.
For this reason 8 samples relative to EC1118 conditions (samples collected after 30 min and 2 hours from inoculum, at the beginning of fermentation and at the middle-stage of fermentation in the conditions 0 and 50 mg/l $SO_2$ added) were excluded from the analysis.

### 6.3.5 Data analysis

The data obtained for each sample, except those relative to the strain EC1118, and each reference were analysed using three different VBA applets, geNorm (Vandesompele et al., 2002), implemented in qBase, a flexible and open source program for qPCR data management and analysis (Hellemans et al., 2007), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004).
While reference genes have the intrinsic capacity to capture all non-biological variation and as such constitute the best normalizers, a major problem is that there is substantial evidence in the literature that most of the commonly used reference genes are regulated under some circumstances. It is thus of utmost importance to validate every single experimental situation whether a candidate reference gene is suitable for normalization (Vandesompele et al., 2009). The

implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization is recently demonstrated by Dheda et al. (2005). If unrecognized, unexpected changes in reference gene expression can result in erroneous conclusions about real biological effects. In addition, this type of change often remains unnoticed because most experiments only include a single reference gene.

#### 6.3.5.1 geNorm

Vandesompele et al. (2002) were the first to quantify the errors associated with the use of a single (non-validated) reference gene, to develop a method to select the most stably expressed reference genes, and to propose the use of multiple reference genes for calculation of a reliable normalization factor. To evaluate the presumed constant expression level of the tested candidate reference genes, a robust and assumption-free quality parameter was developed based on raw non-normalized expression levels. The underlying principle is that the expression ratio of two proper reference genes should be constant across samples. For each reference gene, the pairwise variation with all other reference genes is calculated as the standard deviation of the logarithmic transformed expression ratios, followed by the calculation of a reference gene stability value (M value) as the average pairwise variation of a particular reference gene with all other tested candidate reference genes (Vandesompele et al., 2009).

To manage the large number of calculations, the authors have written a freely available Visual Basic Application for Microsoft Excel (geNorm) that automatically calculates the expression stability values for any number of candidate reference genes in a set of samples. The software employs an algorithm to rank the candidate reference genes according to their expression stability by a repeated process of stepwise exclusion of the worst scoring reference gene. The authors outlined also a strategy to determine the minimal number of reference genes for accurate normalization, by variation analysis of normalization factors calculated for an increasing number of reference genes. It turned out that three stable genes sufficed for samples with relatively low expression variation (homogeneous samples), but that other tissues or cell types required a fourth or fifth reference gene to deal with the observed expression variation (Vandesompele et al., 2009).

Selected reference genes were ranked according to the determined control gene-stability measure (M, average pair-wise variation of a particular gene with all

other control genes), from the most stable (lowest M values) to the least stable (highest M values): *YRB1 – TAF10 – ALG9 – FBA1 – LYS14 – UBC6 – PFK1 – TFC1 – PDA1 – ITR1 – ACT1 – PMA1 – DED1 – FAS2 – ENO1* (Table 6.3; Figure 6.3b). All studied genes reach a high expression stability with low M values, below the default limit of M = 1.5 (Vandesompele et al., 2002). Interestingly, the expression of the gene *ACT1*, widely used as a reference gene in many studies, appears to be less stable than other genes in this conditions. Additionally, the assessment of the normalization factor allows the identification of the optimal number of control genes. The geNorm software suggests that an accurate normalization factor of qRT-PCR data can be calculated by using the four most stably expressed genes, but it is not possible to use less than 4 reference genes. As shown in Figure 6.3a, the addition of further reference genes will not significantly affect the reliability of the determined normalization factor, yielding a V4/5 value (pair-wise variation between two sequential normalization factors) of 0.130, the first value lower than the default cut-off value of 0.15.

| Reference Target | M value | CV |
|---|---|---|
| YRB1 | 0,458 | 0,615 |
| TAF10 | 0,499 | 0,822 |
| ALG9 | 0,524 | 0,406 |
| FBA1 | 0,601 | 0,426 |
| LYS14 | 0,662 | 0,803 |
| UBC6 | 0,709 | 0,504 |
| PFK1 | 0,773 | 0,609 |
| TFC1 | 0,842 | 0,307 |
| PDA1 | 0,896 | 0,507 |
| ITR1 | 0,962 | 0,46 |
| ACT1 | 1,022 | 0,524 |
| PMA1 | 1,073 | 0,644 |
| DED1 | 1,126 | 0,625 |
| FAS2 | 1,187 | 0,802 |
| ENO1 | 1,368 | 1,253 |

**Table 6.3** Candidate reference genes for normalization of qRTPCR ranked according to their expression stability (calculated as the average M value after stepwise exclusion of worst scoring genes) by the geNorm VBA applet.

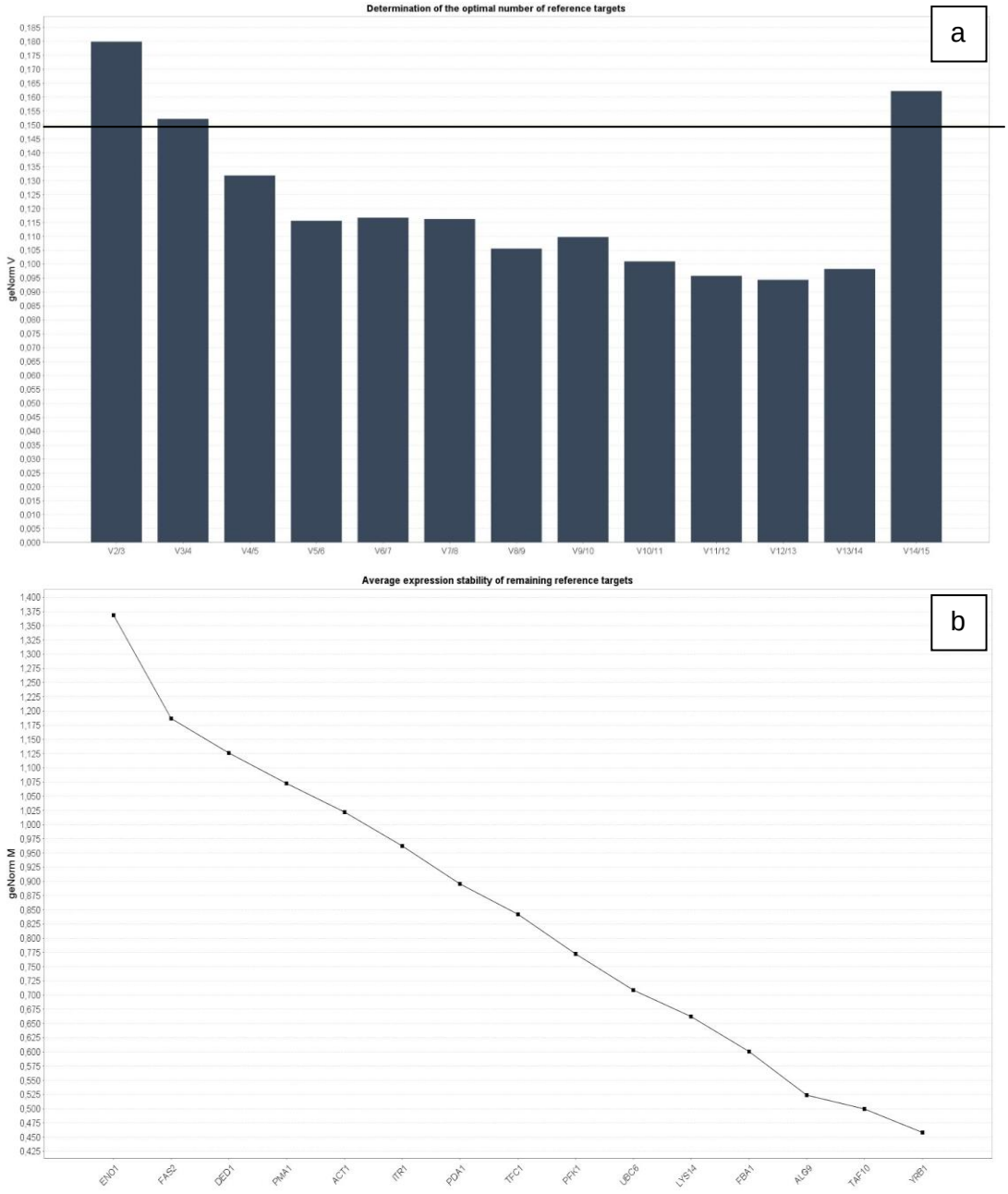



**Figure 6.3** geNorm output charts. (a) determination of the optimal number of control genes for normalization calculated on the basis of the pair-wise variation (V) analysis; V values under 0.15 threshold line indicate no need to include further HKG for calculation of a reliable normalization factor; (b) average expression stability measure (M) of control genes during stepwise exclusion of the least stable control genes.

According to the geNorm stability rank of the reference genes studied, the four gene to include in the calculation of a reliable normalization factor should be *YRB1, TAF10, ALG9* and *FBA1* (Table 6.3; Figure 6.3b).

### 6.3.5.2 NormFinder

NormFinder (freely available at http://www.mdl.dk/ publicationsnormfinder.htm) is another Excel applet based on an algorithm for identifying the optimal normalization gene(s) among a set of candidates. It ranks the candidate genes according to their mRNA expression stability value in a given sample set and a given experimental design (Andersen et al., 2004; Spinsanti et al., 2006). The input data format of this software is identical to that described for geNorm except for the transposition of the genes on the Y-axis and the samples on the X-axis. This approach combines the intra-group and inter-group expression variation to a stability value that enables the ranking of genes by mRNA expression stability. The most obvious advantage of NormFinder is that it examines the stability of each single candidate gene independently and not in relation to the other genes, as geNorm does (Andersen et al., 2004). This is important in the light of our limited knowledge regarding co-regulation. Moreover, NormFinder also tests for combinations of genes that may compensate for each other's fluctuations. This is helpful in situations where none of the candidate reference gene transcripts is found to be stably expressed (Schirman-Hildesheim et al., 2005).

The results of the NormFinder analysis applied to our data are shown in Table 6.4. In this ranking the best gene is *ALG9*, followed by *TFC1, FBA1* and *UBC6*, occupying the highest positions, while *TAF10*, second in geNorm ranking, is defined as one of the least reliable controls.

| Gene name | Stability value |
|---|---|
| ALG9 | 0,309 |
| TFC1 | 0,316 |
| FBA1 | 0,373 |
| UBC6 | 0,455 |
| PFK1 | 0,473 |
| YRB1 | 0,554 |
| ITR1 | 0,568 |
| PDA1 | 0,603 |
| LYS14 | 0,603 |
| ACT1 | 0,677 |
| TAF10 | 0,724 |
| DED1 | 0,788 |
| FAS2 | 0,841 |
| PMA1 | 0,864 |
| ENO1 | 1,686 |

**Table 6.4** Candidate reference genes for normalization of qRTPCR listed according to their expression stability calculated by the NormFinder VBA applet.

#### 6.3.5.3 BestKeeper

BestKeeper is an Excel-based tool determining the "optimal" HKGs by using a pair-wise correlation analysis of all pairs of candidate genes, and calculating the geometric mean of the "best" suited ones. It was developed by Pfaffl et al. (2004) and has many feature similarities with the previously discussed geNorm program. The main differences are that BestKeeper uses Ct values (instead of relative quantities) as input and employs a different measure of expression stability. The founding principle for identification of stably expressed reference genes is that proper reference genes should display a similar expression pattern. Hence, their expression levels should be highly correlated. As such, BestKeeper calculates a Pearson correlation coefficient for each candidate reference gene pair, along with the probability that the correlation is significant. All highly correlated (and putatively stably expressed) reference genes are then combined into an index value (i.e. normalization factor), by calculating the geometric mean. Then, correlation between each candidate reference gene and the index is calculated, describing the relation between the index and the contributing reference genes by the correlation coefficient, coefficient of determination ($r^2$) and the p-value. One unique feature of this software is that in addition to reference gene analysis, genes of interest can also be analyzed, using the same method. This identifies highly correlated genes, as well as genes that behave similarly to the reference genes, and may be included in the calculation of the normalizing index. In conclusion, the BestKeeper software allows pairwise correlation analysis for up to ten candidate reference genes, ten genes of interest, and 100 biological samples. In addition, a sample integrity value is calculated, allowing removal of spurious data (Vandesompele et al., 2009).

With this software it is possible to analyze not more than 10 reference genes together, so the first 10 for geNorm have been selected for the analysis. For this reason, the BestKeeper analysis should be considered only indicative. The 10 control genes tested in this analysis correlate well one with another, and also if compared with the BestKeeper index (Table 6.5). The best correlation between the reference genes and the BestKeeper index is obtained for *ALG9* (r = 0.985), followed by *FBA1, YRB1* and *UBC6* (Table 6.5).

| BestKeeper vs. | TAF 10 | LYS 14 | ALG 9 | ITR 1 | FBA 1 |
|---|---|---|---|---|---|
| coeff. of corr. [r] | 0,913 | 0,879 | 0,985 | 0,756 | 0,953 |
| p-value | 0,001 | 0,001 | 0,001 | 0,001 | 0,001 |

| BestKeeper vs. | UBC 6 | TFC 1 | PDA 1 | YRB 1 | PFK 1 |
|---|---|---|---|---|---|
| coeff. of corr. [r] | 0,918 | 0,869 | 0,824 | 0,947 | 0,860 |
| p-value | 0,001 | 0,001 | 0,001 | 0,001 | 0,001 |

**Table 6.5** Results from BestKeeper correlation analysis.

The different position of gene *TAF10* in geNorm and NormFinder ranking could be due to a correlation with the gene *YRB1* (first in geNorm ranking and sixth in NormFinder ranking). The inclusion of two correlated genes in the list of candidates may lead to false positive results due to the similarity in their expression profiles. For this reason the gene *TAF10* has been excluded and a new analysis has been performed.

#### 6.3.5.4 Data analysis without TAF10 gene

Selected reference genes, except *TAF10*, were ranked according to the determined control gene-stability measure (M, average pair-wise variation of a particular gene with all other control genes), from the most stable (lowest M values) to the least stable (highest M values): *ALG9 – YRB1 – FBA1 – UBC6 – LYS14 – PFK1 – TFC1 – PDA1 – ITR1 – ACT1 – PMA1 – DED1 – FAS2 – ENO1* (Table 6.6; Figure 6.4b). All studied genes reach a high expression stability with low M values, below the default limit of M = 1.5 (Vandesompele et al., 2002). Also in this case the geNorm software suggests that an accurate normalization factor of qRT-PCR data can be calculated by using the 4 most stably expressed genes. As shown in Figure 6.4a, the addition of further reference genes will not significantly affect the reliability of the determined normalization factor, yielding a V4/5 value (pair-wise variation between two sequential normalization factors) of 0.130, the first value lower than the default cut-off value of 0.15.

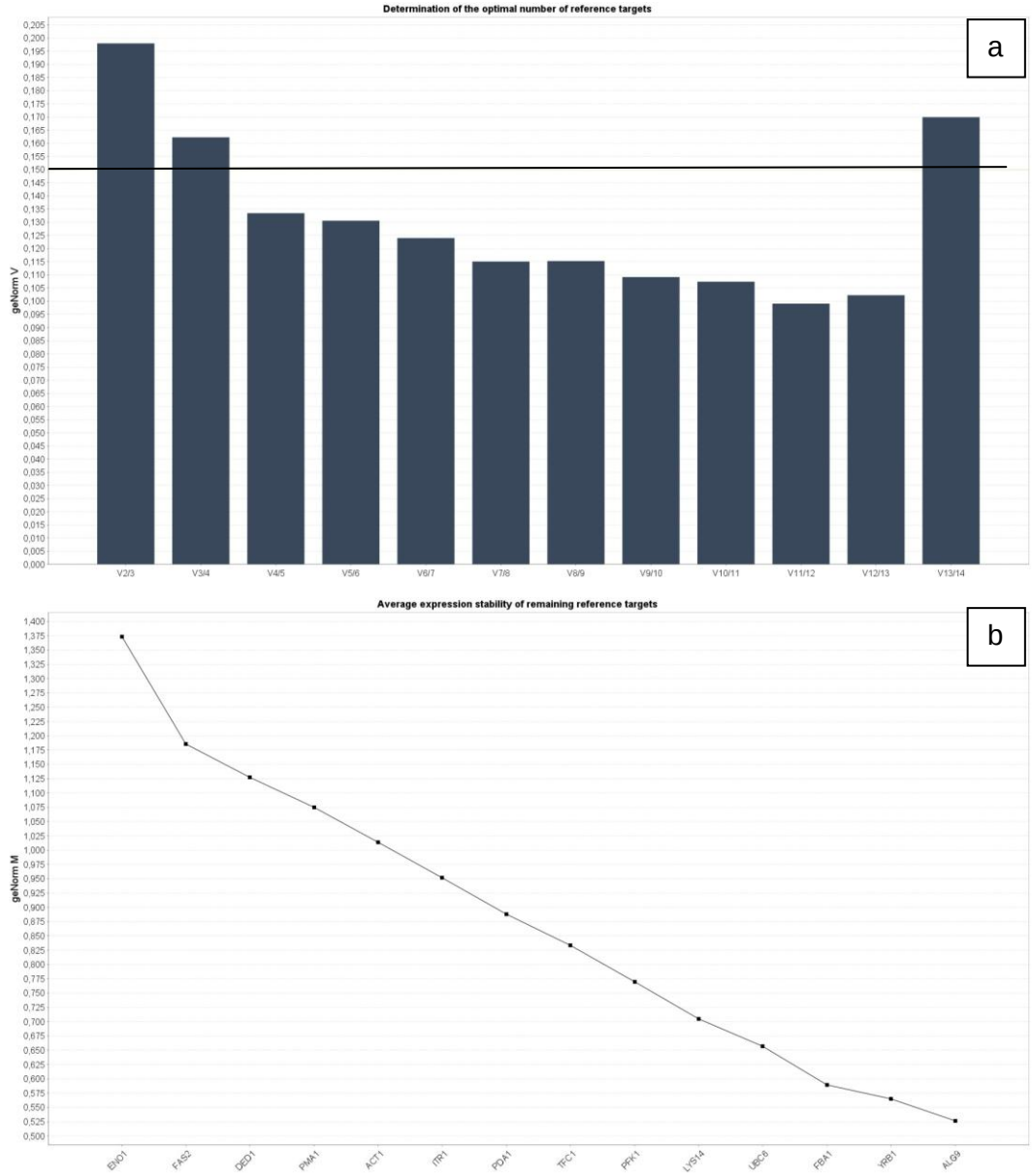



**Figure 6.4** geNorm output charts. (a) determination of the optimal number of control genes for normalization calculated on the basis of the pair-wise variation (V) analysis; V values under 0.15 threshold line indicate no need to include further HKG for calculation of a reliable normalization factor; (b) average expression stability measure (M) of control genes during stepwise exclusion of the least stable control genes.

| Reference Target | M value | CV |
|---|---|---|
| ALG9 | 0,527 | 0,469 |
| YRB1 | 0,565 | 0,679 |
| FBA1 | 0,589 | 0,475 |
| UBC6 | 0,657 | 0,527 |
| LYS14 | 0,705 | 0,855 |
| PFK1 | 0,77 | 0,631 |
| TFC1 | 0,833 | 0,288 |
| PDA1 | 0,888 | 0,532 |
| ITR1 | 0,952 | 0,43 |
| ACT1 | 1,014 | 0,535 |
| PMA1 | 1,075 | 0,664 |
| DED1 | 1,127 | 0,629 |
| FAS2 | 1,186 | 0,768 |
| ENO1 | 1,373 | 1,227 |

**Table 6.6** Candidate reference genes for normalization of qRTPCR ranked according to their expression stability (calculated as the average M value after stepwise exclusion of worst scoring genes) by the geNorm VBA applet.

According to the geNorm stability rank of the reference genes studied, the four gene to include in the calculation of a reliable normalization factor should be *ALG9, YRB1, FBA1* and *UBC6* (Table 6.6; Figure 6.4b).

The results of the NormFinder analysis applied to our data are shown in Table 6.7. In this ranking the best gene is *TFC1*, followed by *ALG9, FBA1* and *UBC6*.

| Gene name | Stability value |
|---|---|
| TFC1 | 0,280 |
| ALG9 | 0,363 |
| FBA1 | 0,416 |
| UBC6 | 0,458 |
| PFK1 | 0,489 |
| ITR1 | 0,534 |
| PDA1 | 0,608 |
| YRB1 | 0,610 |
| LYS14 | 0,644 |
| ACT1 | 0,672 |
| DED1 | 0,769 |
| FAS2 | 0,804 |
| PMA1 | 0,896 |
| ENO1 | 1,648 |

**Table 6.7** Candidate reference genes for normalization of qRTPCR listed according to their expression stability calculated by the NormFinder VBA applet.

The first 10 control genes for geNorm and NormFinder have been tested in BestKeeper analysis and correlate well one with another, and also if compared with the BestKeeper index (Table 6.8). The best correlation between the reference genes and the BestKeeper index is obtained for *ALG9* (r = 0.973), followed by *FBA1, YRB1* and *ACT1* (Table 6.8).

| BestKeeper vs. | ACT1 | LYS 14 | ALG 9 | ITR 1 | FBA 1 |
|---|---|---|---|---|---|
| coeff. of corr. [r] | 0,916 | 0,846 | 0,973 | 0,805 | 0,942 |
| p-value | 0,001 | 0,001 | 0,001 | 0,001 | 0,001 |

| BestKeeper vs. | UBC 6 | TFC 1 | PDA 1 | YRB 1 | PFK 1 |
|---|---|---|---|---|---|
| coeff. of corr. [r] | 0,905 | 0,908 | 0,838 | 0,920 | 0,856 |
| p-value | 0,001 | 0,001 | 0,001 | 0,001 | 0,001 |

**Table 6.8** Results from BestKeeper correlation analysis.

Each software suggest that *ALG9, FBA1* and *UBC6* are the best reference genes for this condition. geNorm recommend the use of 4 reference genes, so the fourth gene can be chosen among *PFK1* (fifth in NormFinder and sixth in GeNorm), *YRB1* (second in GeNorm and eighth in NormFinder) or *TFC1* (first in NormFinder and seventh in GeNorm).

## 6.4 Conclusions

This work constitutes a great effort for the selection of optimal control genes in qRT-PCR studies designed for the assessment of *S. cerevisiae* during alcoholic fermentation in sulphited condition.

The three softwares tested (geNorm, NormFinder and BestKeeper), based on different algorithms and analytical procedures, produced comparable results. From this study it can be conclude that *ALG9, FBA1* and *UBC6*, together with one among *PFK1, YRB1* or *TFC1*, are the most reliable reference genes of this set and their use is strongly recommend in future qRT-PCR studies on *S. cerevisiae*.

On the other hand, *ACT1, PMA1, DED1* and *FAS2* show unstable expression patterns and are always classified as the least reliable control genes of this group.

Moreover gene *TAF10* seems to be correlated with the gene *YRB1*, and cannot be considered a suitable reference genes.

# 7. CONCLUSIONS

A first screening of $SO_2$ resistance and on plate production of $SO_2$ and $H_2S$ have been performed for autochthonous strains, isolated in Veneto vineyards to be used as starter of fermentation in the production of Prosecco di Valdobbiadene DOCG and DOC Piave wines, compared to commercial strains.

Concerning sulphur compounds production, strains showed a certain variability in $SO_2$ and $H_2S$ production, ranging from low to high, in particular autochthonous yeasts grouped mostly in the class of medium producers of $SO_2$, while commercial strains are high producers. Autochthonous yeasts produced less $SO_2$ and are less resistant to $SO_2$, while commercial yeasts produced more $SO_2$ and are more resistant to high doses of $SO_2$ added.

This suggests that vineyard is the best place for yeast selection, because of the presence of low sulphite producer (consequently less resistant). Nowadays are used $SO_2$ doses ever lower, and this would allow the use of autochthonous strains, low sulphite producer and preferred by oenologists because exalting the sensory properties of regional wines and their typical *terroir* character.

The genome sequencing of 4 autochthonous strains (2 from Prosecco area and 2 from Raboso area) allowed to identify some genetic characteristics, such as oenological SNPs, strain-specific genes and important translocations, that have been analyzed in Real-time PCR for a large number of autochthonous strains.

Comparison of the strains belonging to the wine\European group with some others derived from different environments revealed the presence of 306 SNPs characterizing oenological strains. These positions are identical in oenological strains and differ in all the other strains considered. The genes harbouring these SNPs have been further investigated and results suggest their importance in the adaptation to the oenological environment. Some of these SNPs led to amino acid changes in highly conserved proteins regions, in particular two of these genes encode proteins involved in amino acids catabolism, in the pyruvate transport and in the biosynthesis of higher alcohols that have a strong impact on wine aroma. The frequency of the two SNPs identified in YDL168W and YHR162W genes have been exanimate in 213 autochthonous yeasts, together with ten commercial strains and two laboratory strains. Results obtained from genome sequencing have been confirmed, and these 2 SNPs are very common in vineyard and commercial strains.

It has been found that nearly 10% of the vineyard strains isolated harbours the allantoate transporter gene, giving them the ability to use less-attractive nitrogen sources that become prevalent in the second part of the fermentation process. The gene encoding the fructose transporter is even more frequent (nearly 23%) in vineyard yeast population and was also frequently identified in the commercial strains examined. Ability to use fructose could confer an evolutionary advantage because, in ripen grapes, the concentration of fructose and glucose are similar. In the first part of the fermentation, yeast uses preferentially the “more attractive” nitrogen and carbon sources, while in the second part it uses “less attractive” compounds.

Regarding translocations analysis are still in progress, and must be extended to commercial strains. A preliminary analysis suggests that the new translocations XV-XVI it’s not common in vineyard strains (only 3 out of 213 strains), while the well known translocation XVI-VIII seems very frequent in the vineyard strains (114/213 isolates could have the translocation), but this result must be confirmed with further PCR analysis.

Afterwards a phenotypic characterization have been made in a selected number of strains, those whose genome has recently been sequenced. Oenological properties have been tested and the main phenotypic characters have been defined. This characterization have been carried out for 4 autochthonous yeasts (P301.4, P283.4, and R103.1 R8.3) and 6 commercial yeasts from Europe and South Africa (EC1118, AWRI796, AWRI1631, QA23, VL3, VIN13) together with the reference strain S288C, whose genome sequences are available.

Considering fermentative performance in standard conditions it was possible to assess that strains VIN13 and P301.4 have the best fermentation kinetics and the best fermentation vigour, so the strain isolated in DOCG Prosecco has interesting oenological characteristics. The laboratory strain S288c and R103.1 are the slower and less vigorous, and both produce low concentrations of glycerol. The strain EC1118, considered the French oenological yeast for excellence, is unsatisfying, because of the mediocre fermentation kinetics and the low fermentation vigour.

Considering fermentation kinetics in the presence of an excess of sugars the strain EC1118 has kinetics and shows a strong ability to work well at high concentrations of sugar, because of its excellent fermentative power. Strains poor fermenters under standard conditions (S288c, R103.1 and R8.3 in part) also possess a low fermentative power. Surprisingly the strain P283.4, good fementer

in standard conditions, reveals a low fermentative power, so it is not suitable to ferment musts with high concentrations of sugars.

An important consideration must be made for the commercial yeast VIN13 and the autochthonous strain P301.4. These strains are very versatile and extremely capable, with fermentation kinetics very fast in standard conditions and an excellent fermentative power when fermenting high concentrations of sugars.

Considering the metabolism of sulphur compounds it's possible to assess that the commercial strain VL3 is the most resistant to sulphites. Strains VIN13 R8.3 and R103.1 reveal an excellent resistance. The strain more sensitive is AWRI796, and it produces the least amount of acetaldehyde and sulphur dioxide. These results indicate that there is a direct relationship between the performance of fermentation and the characteristics of sulphite tolerance. Strains with good fermentation kinetics may be the least resistant to sulphites and vice versa.

It was observed a direct relationship between the production of $SO_2$ and acetaldehyde. Greater is the production of sulphites by the strain, the greater is the amount of acetaldehyde produced. This result indicates that acetaldehyde production is a way primarily used by yeasts to limit the effect of toxicity produced by endogenous sulphites.

Moreover the strain VIN13, while producing low concentrations of acetaldehyde, comparable with the most sensitive strain AWRI796, is very resistant, suggesting that the mechanism of resistance does not include the production of acetaldehyde, but an alternative way (for example the use of the pump *SSU1* for the extrusion of sulphites). Situation quite opposite to that observed for the strain VL3, in which the mechanism of $SO_2$ tolerance seems to be a high production of acetaldehyde.

In this study the behaviour towards sulphite of *Saccharomyces cerevisiae* strains, whose genome has been sequenced, has been evaluated, first in small laboratory scale and then, for selected strains, in controlled bioreactors.

Concerning $SO_2$ production, a strain-dependent behaviour was confirmed when sulphite was not added. The high $SO_2$ producer yeasts reduced their production of 51-61% when 25mg/l of $SO_2$ was present and 5-30% when 50mg/l of $SO_2$ was present. Acetaldehyde production was shown to be strain dependent, as well, and clearly induced by $SO_2$ presence in the must.

These results point out the importance of verifying strain behaviour towards sulphite when wine yeasts are used to ferment sulphited must. Indeed, in this study it has been found that $SO_2$ production is strain-dependent, but not related

to fermentation performances and regulated by yeasts on the basis of the initial sulphite concentration. Thus, wines with the same final sulphite content can be obtained starting from different quantity of $SO_2$ added. This study suggests that the choice of yeast strain for vinification can be a tool to limit the sulphite concentration used in winemaking. The on-going comparative analysis of yeast transcriptomes is contributing stronghly to clarify the different yeast behaviour in presence of added sulphite. In particular our results suggest that independent genetic mechanisms are involved in the lag phase lasting (linked to sulphate resistance trait) and in increasing the fermentation rate. Among the yeast analysed the strain (R8.3) that showed the lower sulphate impact on lag phase had the higher level of *SSU1* gene expression. EC1118 and VL3 showed similar level of *SSU1* expression (even if in VL3 the gene is not induced), but the latter is resistant to higher $SO_2$ concentration probably due to the strong down-regulation of the *SLU1* gene, the high affinity sulfate permease strongly induced in EC1118. The very low level of *SSU1* gene expression along with the down regulation of many genes of the sulfur amino acid biosynthetic pathway in AWRI796 determine the prolong lag phase that is peculiar of this strain.

Our result suggest the presence of sulphite stressing condition demonstrated by the expression of different stress response pathways that are not involved in determining the lag-phase lasting (correlated to sulphate resistance). Two strains (VL3 and AWRI796) although characterized by different $SO_2$ resistance level revealed to be not stress by sulphate presence that seems to enhance the ribosomal biosynthesis .

On the contrary R8.3 and EC1118 showing a strong and moderate $SO_2$ resistance respectively appear to be dramatic influenced during fermentation by sulphite and a premature stress resistance genes induction is observed, this causing a notably fermentation rate reduction.

Finally, for better understanding yeast behaviour and metabolism under sulphite stress condition, a selection of reference genes for Real-time PCR has been made, and a set of genes suitable for such conditions has been identified. From this study it can be conclude that *ALG9, FBA1* and *UBC6*, together with one among *PFK1, YRB1* or *TFC1*, are the most reliable reference genes of this set and their use is strongly recommend in future qRT-PCR studies on *S. cerevisiae*. On the other hand, *ACT1, PMA1, DED1* and *FAS2* show unstable expression patterns and are always classified as the least reliable control genes of this group.

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