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Ciclo XIX

MOLECULAR APPROACHES FOR THE INDIVIDUATION AND CHARACTERIZATION OF TECHNOLOGICAL AND QUALITY TRAITS IN MICROORGANISMS OF ENOLOGICAL INTEREST

Approcci molecolari
per l'individuazione e la definizione
di caratteri tecnologici e di qualità
nei microrganismi di interesse enologico

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ABSTRACT

The genus *Saccharomyces* includes microorganisms important for many technological applications, particularly in the industry of fermented foods. In the enological field, yeast strains currently used in wine fermentations (belonging to *Saccharomyces sensu stricto* group) were selected from natural spontaneous fermentations and are specifically adapted to the winery's environment. Some of their essential properties that are at the basis of strain selection are the capability of these yeasts to transform carbohydrates into alcohol by fermentation, with high transformation efficiency and remarkable tolerance to high alcohol levels. Yeasts belonging to the *S. sensu stricto* group are also adapted to grow in grape musts with high sugar content, low pH, limited nitrogen, lipids and vitamins concentrations and presence of added sulphites.

During the selection programs of wine yeasts, the first stages of the experimental work regard the collection of several hundred isolates that are screened with the aim to find those that possess important enological properties. In most of the cases, this equivalent to establish that these strains belong to the *Saccharomyces sensu stricto* complex. For that reason, the availability of a molecular test that could rapidly, ditely and easily identifies these enological yeast would be extremely useful. In the first part of this work the analysis of yeast ITS region on rDNA was proposed as screening method. Using a tailor-made profile database, more then 350 natural isolates collected from marks of Prosecco and Moscato grape were subjected to molecular identification. As the protocol needs several time consuming experimental steps (amplification and digestion) a new method was proposed. For this purpose an original pair of primers, designed within the variable D1/D2 region of the 26S subunit of ribosomal yeast RNA, was constructed. These generate an amplification fragment specific for the *Saccharomyces sensu stricto* species, while no signal was obtained from *Saccharomyces sensu lato* strains or from another 18 selected species commonly found in enological environments. A second pair of primers was also constructed, within the 18S rRNA gene, composed of perfectly conserved sequences common for all 42 yeast species examined, which generate a common band for all strains. This was used as a positive experimental control in multiplex PCR analysis. The new method, and also ITS analysis, allow a "genotypic characterization" of enological strains that is required to start a "technological characterization" for the definition of the enological traits. The innovative DNA-microarray technology that has become a standard tool for the analysis of genome-wide expression profiles, can be used to investigate, from a molecular point of view, the differences in the expression of technological and quality characters of enological yeast in laboratory and also winery conditions. For this purpose in the second part of the work, using microarray technology, an investigation of yeast metabolic shifts at transcriptional level in both laboratory and industrial conditions was faced up. Two commercial yeast strains widely used in wineries, Lallemand 71B and Lallemand EC1118, have been

compared during fermentation of both 1 l and 100 l natural white must. These strains have different behaviours and attitudes: 71B is known to be a strong producer of fermentative aromas, EC1118 is an efficient fermenter, quite neutral from an aromatic point of view. Comparing the two strains, the metabolic pathway of sulphured amino acids production displayed an higher expression level in 71B, together with the sulphite efflux responsible gene *SSU1*. Moreover, genes involved in the production of fermentative aromas, such as esters and higher alcohols, showed a slightly higher expression in 71B: all these evidences have been confirmed by Real-time PCR, another high throughput tool for expression analysis. The up-regulated genes during the scale-up experiment, on the other hand, seem to be linked to anaerobiosis stress response, probably due to small differences in fermentation conditions which have been sensed by yeast.

As final remark, this study tries to give a contribution for understanding the genetic basis of the differences that are found in fermentation performances of wine yeasts in winery conditions. Furthermore it may help in assessing the reproducibility of yeast behaviours during alcoholic fermentation, when a laboratory scale is used.

RIASSUNTO

Il genere *Saccharomyces* include microrganismi importanti per molti processi tecnologici, in particolare nell'industria alimentare e delle fermentazioni. In campo enologico, i ceppi di lievito generalmente utilizzati per la fermentazione del vino (appartenenti al gruppo dei *Saccharomyces sensu stricto*) sono stati selezionati nel tempo a partire da fermentazioni spontanee e sono ceppi specificamente adattati alle condizioni di cantina. Alcune caratteristiche essenziali, che sono alla base dei criteri per la loro stessa selezione, sono la capacità di trasformare efficientemente i carboidrati in alcol attraverso la fermentazione ed una notevole resistenza all'etanolo. I lieviti appartenenti al gruppo dei *S. sensu stricto* sono in grado di crescere in mosto d'uva, dove il livello di zuccheri è molto alto, il pH basso, azoto, lipidi e vitamine sono presenti in scarse quantità e spesso vengono aggiunti solfiti prima sella fermentazione. Nei programmi che prevedono la selezione di lieviti da utilizzare in ambiente enologico, nelle prime fasi sperimentali, vengono raccolti centinaia di isolati naturali che sono poi sottoposti a caratterizzazione con lo scopo di evidenziare quelli che posseggono importati caratteristiche tecnologiche. Nella maggior parte dei casi, ciò significa selezionare solo ceppi appartenenti alla categoria tassonomica *Saccharomyces sensu stricto.* Per questo motivo è estremamente utile disporre di un test genetico che permetta di distinguere rapidamente e in modo inequivocabile lieviti di potenziale interesse tecnologico. Nella prima parte di questo lavoro è stata verificata la possibilità di utilizzare l'analisi della regione ITS, contenuta nel DNA ribosomale, come metodo per determinare l'identità di isolati naturali. Dopo aver costruito un raccolta dei profili elettroforetici dei principali lieviti enologici, sono stati sottoposti ad identificazione, mediante confronto, 350 isolati naturali provenienti da vinacce di Moscato e Prosecco destinate alla produzione della Grappa. Questo metodo, ampiamente utilizzato in letteratura, richiede l'impiego di tempo per l'esecuzione che male si adatta alle già citate esigenze di un programma di selezione. Per questo motivo è stata proposta una nuova metodica la cui messa a punto ha previsto la produzione di una coppia di primers per amplificazione PCR, disegnata all'interno della regione variabile D1/D2 della subunità 26S dell'RNA ribosomiale del lievito, con la quale si ottiene un frammento di DNA specifico per le sette specie appartenenti al gruppo *Saccharomyces sensu stricto*, mentre non si osserva nessuna amplificazione nei ceppi *Saccharomyces sensu lato* né in altre 18 specie tra le più diffuse in ambiente enologico, saggiate come controlli. E' stata inoltre disegnata una seconda coppia di primers, nella regione di DNA codificante l'rRNA 18S, composta di sequenze perfettamente conservate nelle 42 specie di lieviti enologici esaminate: questa coppia genera un amplificato di circa 900 pb comune per tutti i ceppi ed è stata usata come controllo positivo di reazione per la messa a punto di un protocollo di *multiplex PCR*.

Il nuovo metodo proposto, insieme all'analisi ITS, permette di ottenere una "caratterizzazione su basi genetiche" dei ceppi enologici che sicuramente è richiesta

per affrontare, in una fase successive, la “caratterizzazione tecnologica” che ha lo scopo di valutare le proprietà enologiche possedute dai singoli ceppi. La tecnologia DNA-microarray che è ormai diventata uno strumento di riferimento per una analisi simultanea dell’intera espressione genica di un individuo, può essere utilizzata per valutare le differenze presenti tra ceppi enologici nell’espressione di caratteri tecnologici e di qualità. Per questo scopo, la seconda parte del lavoro sperimentale, ha riguardato l’investigazione, proprio mediante la tecnologia DNA-microarray, dei cambiamenti a livello trascrizionale che si verificano in due ceppi commerciali di lievito durante la vinificazione condotta in condizioni di laboratorio e su scala pilota in cantina. Sono stati scelti due lieviti comunemente utilizzati in enologia (71B e EC1118, Lallemand), analizzati e confrontati durante fermentazioni in volumi di 1 litro e 100 litri utilizzando mosto naturale bianco. Questi ceppi hanno attitudini e caratteristiche enologiche differenti: 71B è noto per essere un forte produttore di aromi fermentativi mentre EC1118 è dotato di notevole vigore fermentativo ma è più neutro dal punto di vista aromatico. Dal confronto dei due ceppi è emersa una sostanziale differenza nella regolazione della via metabolica di produzione degli aminoacidi solforati (maggiormente attiva nel ceppo 71B), come anche del gene responsabile per l’efflusso dei solfiti (*SSU1*). Inoltre è stato osservato che i geni coinvolti nella produzione di aromi secondari (esteri ed alcoli superiori) mostrano una sensibile differenza di espressione, seppur meno marcata che nei casi precedenti. Tutti i risultati ottenuti sono stati confermati tramite analisi in Real-time PCR, tecnica molecolari di ultima generazione ampiamente utilizzata per lo studio dell’espressione genica. Confrontando i due volumi impiegati per le vinificazione, i geni sovra-espressi sono risultati essere legati alla risposta allo stress, in particolare alle differenti condizioni di anaerobiosi che si sono verificate a causa delle dimensioni diverse delle masse da vinificare. Questa situazione ha prodotto minime difformità nelle condizioni di fermentazione che sono state percepite dal lievito.

I risultati ottenuti permettono di affermare che questo lavoro ha contribuito ad aumentare la comprensione delle basi genetiche che determinano le differenti caratteristiche di fermentazione associate ai lieviti enologici e a definire il livello di riproducibilità delle loro performance in condizioni di laboratorio e di cantina.

1. Introduction

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 10^3 ad 10^5, 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[213].

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 10^6 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 *Hanseniaspora uvarum*) 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*) [185].

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.

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 particular 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 [185]. 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 [68]. 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 [185].The origins of non-*Saccharomyces* are grape skin and winery equipments [69]. However, the origin of *S. cerevisiae* is controversial; although the most significant finding was that it is practically absent from grapes and vineyard soils [141], some authors propose that this species is a “natural” organism present on plant fruits [155, 237]. 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 [141], 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 [160].

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 [128] or in a newly built winery where *S. cerevisiae* has never been introduced [17]. On the other hand, as mentioned before, although it

has been found on damaged berries [155] wild *S. cerevisiae* is extremely rare on intact grapes [224] whereas it can be found colonizing the winery equipment [17, 229, 255]: some strains are even found in the winery over several years [17,73, 222].

1.1.2. Use of selected yeasts for enological 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 [185]. In 1965, the first two commercial dried yeasts (ADWY) strains were produced for a large Californian winery [68]. 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 [174] while the diversity of native *S. cerevisiae* strains present in spontaneous fermentations contribute to the chemical composition and sensory qualities of the resulting wine [130]. Moreover, several studies support the hypothesis that active dried yeasts reduce the variability of strains that appear in spontaneous fermentations [17, 71] 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 [142].

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 [192]. *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 [15]. 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 [185] and in Querol *et al.* [193]). Their exacerbated capacity to reorganize its genome by chromosomal rearrangements, such as Ty-promoted chromosomal translocations [127, 195], mitotic crossing over [2] and gene conversion [186] 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 [12, 226]. 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% [14, 37]. The meiotic segregants from wine strains diploidize with high frequency, indicating a high 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. [101, 102]: 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.

1.2. Targets for selection and improvement of wine yeasts

Wine technologists gathered the basic properties required for the definition of a ‘‘selected *S. cerevisiae* strain for wine making’’ in two categories [205]: (1) **primary or fitness traits**, defined as those strictly associated with the formation of ethyl alcohol 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-flavors. Main primary and secondary traits are summarized in table 1.1, where some further traits, more specific and functional to the type of desired wine, are also listed [185].

Table 1.1 Main desirable characteristics of wine yeast

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	

Some of the requirements listed in Table 1.1 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 [185].

1.2.1. 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.

1.2.1.1. 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 [142]. 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 [23].

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 noncompetitive and has been described as either a linear or an exponential function of ethanol concentration [23,19].

Generally, sugar catabolism and fermentation proceed at a rate greater than desired, and are usually controlled by lowering the **fermentation temperature** [68].

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 [98].

1.2.1.2. 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 toxins are 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 SO_2 dosages may cause sluggish or stuck fermentations [23]. 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 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 [220].

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 [248]. 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 [220]. Several copper uptake, efflux and chelation strategies have been developed by yeasts to control copper ion homeostasis [8]. 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 [24].

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 [236]. 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 [185].

1.2.2. 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 [171].
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 [118, 170].

1.2.2.1. 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 [97]. Altough acetic and latic acid bacteria can be associated with high levels of short chain fatty acid, acetic, propanoic and butanoic acids are by-products of alcoholic fermentation [212].
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 [142].
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 [230]. Wine yeast strains producing a consistent amount of glycerol would therefore be of considerable value in improving the organoleptic quality of wine [151, 209].
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 [238].

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 [142]. 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 [98]. 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 exo-enzymes are normally produced by these microorganisms that can transform neutral grape compounds into flavour active molecules [171, 118]. 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 [70], Lema *et al.* [121], Romano [217], Heard [95], and Lambrechts and Pretorius [118]. Thus, the uniqueness and individuality of the flavour contribution by yeasts depends on the species and strains operating the fermentation [68, 71].

1.2.2.2. 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 cheeseloving population of southern France, the death rate from coronary heart disease is significantly lower than the one found in industrialized countries [185].

1.3. Selection strategies for new strains of enological interest

Selection and genetic improvement of an organism is based on the ability to achieve a specific task or to do a precise function. In the case of wine yeasts, it is necessary that the selected strains have some basic traits combined with others more specific and functional to the type of wine desired (as summarised in Table 1.1).

The primary selection criteria applied to most strain development programs relate to the overall objective of achieving a better than 98% conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate and without the development of off-favours. The growth and fermentation properties of wine yeasts have, however, yet to be genetically defined. What makes the genetic definition of these attributes even more complex is the fact that lag phase, rate and efficiency of sugar conversion, resistance to inhibitory substances and total time of fermentation are strongly affected by the physiological condition of the yeast, as well as by the physicochemical and nutrient properties of grape must.

1.3.1. Clonal selection

The starting point for the genetic improvement of wine yeasts is always the isolation from grapes, grape musts and wines of a high number of yeast strains, which are then submitted to the analysis of their enological properties [86]. The process, named ‘clonal selection’, produces pure strain clones which can either meet or not all the desired traits for winemaking, but it allows the constitution of a biodiversity

background, which is very useful for successive selection steps or improvement programs.

The selection is generally carried out within the genus *Saccharomyces*. Yeast cultures are preferably isolated from grape juice or wine. S*accharomyces* strains growing in these substrates are, in fact, well adapted to the enological environment and can therefore ferment grape juice very efficiently. Nevertheless, *Saccharomyces* yeasts are scarcely present on grapes. Their isolation on solid media, may therefore not be suited, especially if a relevant number of cultures need to be collected. The use of an enrichment technique is generally preferred [40, 69, 72, 259, 235]. This method consists of creating the conditions that favour the growth of some microorganisms in a mixed population, and inhibit the growth of the rest. The high concentration of ethanol that accumulates in grape juice during fermentation is the main factor favouring the selection of *Saccharomyces* strains. Isolations are therefore carried out after the fermentation (or micro-fermentation) of grape juice. The resulting yeasts are then submitted to characterization steps.

1.3.1.1. Phenotypic characterization of yeasts

In the first step of a selection program a great number of isolates is subjected to different phenotypic tests with the aim of identifying and characterizing yeast strains and species. Taxonomists first delimitated the yeast species using morphological and physiological criteria. The first classifications were based on phenotypic differences between yeasts: cell shape and size, spore formation, cultural characters, fermentation and assimilation of different sugars, assimilation of nitrates, growth-factor needs, resistance to cyclo-heximide. Since then, many rapid, ready to use diagnostic kits have been also developed to determine yeast response to different physiological tests [213].

Due to the relatively limited amount of yeast species significantly present on grapes and in wine, most of these phenotypic tests can easily identify enological yeasts; some of them can be identified by simple observation of growing cells under the microscope. Small apiculated cells, having lemon-like shape, are typical of the species *Hanseniaspora uvarum* and its imperfect form *Kloekera apiculata*. *Saccharomycodes ludwigii* is characterized by apiculated cells of a larger size (10-20µm). Since most yeasts multiply by budding, the genus *Schizosaccharomyces* can

be recognized because of its typical vegetative reproduction by binary fission. Finally, the budding of *Candida stellata* produces star-shape cells.

According to Barnett *et al.* [13] the physiological characteristics listed in table 1.2 can be used to distinguish between the principal grape and wine yeasts.

These features can be studied individually setting up selective fermentation and growth tests, or in combined trials. On the basis of physiological tests the researchers Lafon-Lafourcade and Joyeux [116] and, in the same period, Cuinier and Levau [43] designed a ready to use kit (API 20 C system) for the identification of enological yeasts. It contains eight fermentation tests and ten concerning assimilation and resistance to cyclo-heximide. For a more complete identification, the API 50 CH system was developed, it contains 50 substrates for fermentation (under paraffin) and assimilation tests. Finally, Fleet and Heard in 1990 [67] proposed a system that uses the different tests listed in Barnett's work ([12], see table 1.2). Appling this new method, it was found that some of these characteristics (for example sugars fermentation profiles) vary within the species and are even unstable for a given strain under vegetative multiplication [212].

There is a considerable part of the current literature that uses the cell fatty-acyl composition as a means of yeast identification. This taxonomic tool [41] has been applied especially to identify wine spoilage yeasts [133] but also to characterize various species and strains [110, 247].

In general, during a selection program, the most used tests, among phenotype-based systems for distinguishing *Saccaromyces* species from other yeasts related to enological environment, are based on selective growth media and phenotypic evaluation of colony colour and morphology (i.e. on WL nutrient agar). They have the great advantage to be easy to perform and very cheap [32] but it was found that strains of *S. cerevisiae* can form colonies slightly different on these kind of media, and the morphological characteristics can be unstable under several multiplications. Thus this approach can not be considered decisive, since possible variations at strain level could lead to erroneous attributions. It is therefore currently accepted that phenotypic analyses are not sufficient to reach a trustworthy identification [112, 136].

1.3.1.2. Genetic identification of yeast species

The advent of molecular genetics has provided more reliable methods for taxonomic studies. The deeper understanding of the microbiology of the winemaking process is a consequence of the employment of such techniques in wine yeast characterization. The use of extensive ecological surveys of wine yeast strains from a number of different ecosystems [73, 261] has led to more suitable and better characterized strains for commercial use [119, 190, 260]. Monitoring of induced fermentations gave an understanding of the evolution of the entire microflora during this process, making clear that wine quality is a consequence of the dynamics and composition of the microorganisms involved in its production [191; 235]. More detailed surveys on the geographical distribution of wine yeast strains in entire areas became possible, while phylogenetic affinities and evolutionary scenarios were explored [156, 259]. This new knowledge, coupled with the possibility of correlating genetic patterns of strains with enologically useful characteristics [156], is based on the novel ability to look at the molecular structure of yeast strains, employing techniques such as restriction or amplification of specific or random polymorphic DNA regions [179].

Differentiation between the taxa since the advent of molecular biology is usually achieved by comparison of ribosomal RNA (rRNA) and its template ribosomal DNA (rDNA): this technique has been used extensively in recent years to assess both close and distant relationships among many kinds of organisms including yeast species identification [193]. Some of these methods are based on sequence analysis, primarily of the **26S rDNA D1/D2 domain** [111] and of the **18S subunit** [104]. However, concerning identification of yeast isolates, these techniques are impractical for the routine screenings of a large number of species since they were developed for species characterization.

For yeast identification at species level, in 1999 a rapid and easy method for routine yeast identification has been proposed [63], basing on PCR amplification and restriction analysis of the 5.8S rRNA gene and the **internal transcribed spacers** (ITS1 and ITS2); a restriction profile database has also been created and improved to allow identification of more than 300 yeast species. Using the same methodology, but amplifying a different region, i.e. 18S rRNA and ITS1, Dlauchy *et al.* [56] constructed a database of restriction fragment patterns of 128 species associated mainly with food and fermented drinks.

Recently, several works focused on species attribution of yeasts belonging to the *Saccharomyces* genus, which has undergone innumerable changes during the 150 years of his history [254], including the division in *sensu stricto* for species associated with alcoholic fermentation and *sensu lato* for all other species [250]. The species within the Saccharomyces sensu stricto group (*S. cerevisiae, S. bayanus, S. pastorianus, S. paradoxus, S. mikatae, S. kudriavzevii* and *S. cariocanus* according to Kurtzman and Robnett [112]), cannot be easily distinguished by classical microbiological methods, hence molecular techniques have recently been used for species assessment of these yeast strains.

In 1994 Hansen and Kielland-Brandt [93] proposed **MET2 gene PCR** amplification and restriction to differentiate *S. cerevisiae* from *S. bayanus*. This gene, encoding for the homoserine acetyl transferase, has different sequences in the two species. Then, this PCR-RFLP technique has been developed and adapted for rapid analyses (using only two restriction enzymes, and succeeding to identify *S. paradoxus* as well [144, 145]). De Barros Lopez *et al.* [45] developed an interesting technique using PCR primers based on the wide conserved **intron splicing sites (ISS),** generating amplification fragments of different lengths within the *Saccharomyces* species. This method, nevertheless, can be troubled by intra-specific polymorphism.

More recently, **Denaturing Gradient Gel Electrophoresis (DGGE)** and **Temperature Gradient Gel Electrophoresis (TGGE)** protocols [36, 136, 137] were developed for distinguishing species belonging to the *Saccharomyces sensu stricto* group. Both these methods take advantage from one or few nucleotides substitution in the rDNA sequences, which confere different mobility properties to amplification fragments.

1.3.1.3. Intra-specific differentiation of yeast strains

Saccharomyces cerevisiae species includes a large number of strains that show different technological properties. The need to discriminate between strains was already understood by Drawert and Bednar in 1983 [57]. The ability to identify different individuals is necessary in ecological studies of spontaneous and starter-guided fermentation, to select strains presenting the best enological qualities and to document the dominance of selected yeasts [25, 90]

In recent years, several methodologies of typing based on DNA polymorphisms have been developed which allowed discrimination among closely related yeast strains.

These techniques have enabled the population dynamics of *Saccharomyces cerevisiae* strains in vineyards or wineries to be studied [73, 191] as well as the control of industrially dried yeast production. They also proved extremely beneficial for yeast laboratories testing strains for their enological properties in order to optimise wild-strain isolates collections [120].

Chromosome separation by **pulsed field gel electrophoresis** (PFGE) revealed considerable variability in the chromosomal constitution of commercial yeast strains and turned out to be a useful method for yeast strain identification [91, 260]. As chromosome karyotyping may be too complex, laborious or time-consuming for the analysis of numerous yeast isolates, several other molecular methods of typing have been developed for this purpose.

Since the first application of **mitochondrial DNA restriction profiling** to brewing yeast [3], several improvements of this technique have been developed. Restriction fragment length polymorphism analysis (RFLP) of mitochondrial DNA was first applied to wine yeast [71] and then simplified [190, 191] to turn this protocol, a fast and easy method. Digestion of mtDNA with restriction enzymes like *Hinf*I or *Rsa*I is associated to a highest polymorphism, and was also used to study the authenticity of commercial wine yeast strains [65].

Several **PCR based methods** have been proposed to discriminate different strains of *S. cerevisiae.* Its genome contains repetitive DNA sequences, such as the *N* (regions) sequences that are frequently associated with the Ty1 transposon [27, 163]. Polymerase chain reaction profile analysis of these sequences (also known as interdelta) has a good level of discrimination for analyzing commercial strains, but, on the other hand, it seems to be less powerful when used to identify indigenous strains isolated from natural environments [119, 144]. In 2003, an extensive BLAST search allowed the optimization of the pair of primers used for **interdelta analysis**, resulting in highly polymorphic patterns. This improved PCR typing had a similar discriminatory power to pulsed field electrophoresis karyotyping [120].
More recently, a powerful **microsatellite-based technique** has been developed [96]: these short tandem repeats widely vary in length as a result of DNA replication errors. The use of specific primers pairs designed on microsatellites allows the

differentiation of yeast from various origins (worldwide different environments) through the analysis of seven loci. The potential of such a technique for enological strains is under evaluation; it is a promising tool, providing accurate and unequivocal results that can be even quantify as base pair number (or number of repeats) [234]. An **AFLP (Amplified Fragment Length Polimorphism**) based analysis of yeast strains, clustering beer, whisky, bakery, and sake yeasts, was also described [10]. However, this method (based on selective PCR amplification of fragments resulting from total DNA restriction) demands higher equipment and time investment.
Another polymerase chain reaction based techniques, **RAPD** (**randomly amplified polymorphic DNA)** were developed using random primers to obtain a strains specific PCR profile of wine yeast [88, 184]. RAPD PCR was firstly proposed for bacteria analysis and then widely used in microbiology, nevertheless discrimination of wine strains obtained with each primer set is not as accurate as what found for mtDNA restriction technique. Moreover the amplification, generally, has a low level of reproducibility.

1.3.2. 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 wine-making tradition. Though there are commercial yeasts to accomplish must fermentation, the use of local selected yeasts is believed to be much more effective [46, 149, 190]. 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 [207].

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' [154]. 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 [184].
The selection of wine yeasts for enological use is traditionally carried out on the basis of their technological and quality-linked phenotypic characteristics. For this purpose different methodologies were designed.

1.3.2.1. 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 [85].
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 [207]. 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 [140].

The enological 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 microfermentations in grape must enriched in glucose to a final content of 30% (eccess 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 [35] proposed microfermentations performed on pastorized grape must where yeast cultures were pre-incubated in grape must for 48 h. Alternatively, microfermentations can be performed on synthetic must as described by Bely *et al.,* [18]. 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 [142] 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/or 150 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 [272]. **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 (SO_2 production lower than 25-30 mg/L is recommended [273]).

1.3.2.2. 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 [166]. 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 [82]: 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 [218] but it is influenced by the must composition [47] thus a quantification of acetic acid production during fermentation is also desirable. Alternatively, paper impregnated with PbAcO to saturation point can be used to carry out the qualitative control of H_2S production during fermentations. At the end of microfermntations (usually performed for fermentation efficiency or fermentation vigour determination), also some other **end-point 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 enological 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 [221]: 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.* [134]. 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 [66].

1.3.3. Genetic improvement of wine yeasts

S. cerevisiae can be modified genetically using many techniques. Some methods alter narrow regions of a single chromosome, whereas other techniques are used to recombine or rearrange the entire genome (reviewed by Pretorius [185]). Techniques having the greatest potential in genetic programming of wine yeast strains besides the clonal selection are: mutant selection, hybridization, rare-mating, spheroplast fusion, gene cloning and transformation. The combined use of tetrad analysis, replica-plating, mutagenesis, hybridization and recombinant DNA methods have dramatically increased the genetic diversity that can be introduced into yeast cells.
The knowledge of the genetic nature of the desired trait (monogenic or polygenic) is essential to perform the appropriate choice among different genetic improvement approaches. Usually the most important enological traits, such as fermentative vigour, ethanol yield and tolerance or growth temperature profile, depend on a multitude of loci (QTLs), which are not well characterised, as they are broadly distributed throughout the whole genome. To make things worse, each locus is often responsible only for a small fraction of phenotypic variation. Therefore, in a context of low phenotype-genotype correlation, both rational approaches based on DNA technologies and random approaches based on mutagenesis meet serious obstacles. However, different “blind” strategies could be applied in order to obtain quickly strains with recombinant traits.

1.3.3.1. Yeast improvement by conventional genetics

Traditionally the genetic manipulation strategies of wine yeasts to produce better new strains exploits different strategies, which included the selection of natural and inducted mutants by sexual recombination methods [86, 270, 271]. Hybridisation of laboratory heterothallic strains was the first method used for yeast improvement. The wild strains are mostly homothallic and heterozygous [14]; for this reason conjugation by micromanipulator or mixing sporulated cultures is possible among germinating spores before autodiploidization. The sexual recombination can be performed with gametes obtained by single-spore cultures or with spores obtained directly from parental strains. The recombination among a small number of parental strains allows to collect a complex progeny, which is then submitted to selective processes. This

method is based on random events, and it is very similar to the new combinatorial approaches that were used for the determination of the optimal genetic configuration in industrial microbes [275].

To rationalize the latter strategy, the first requirement is to try to establish the importance of the genetic determinism of the enological parameters of yeast. Specifically, crosses and progeny analysis could theoretically be used to improve genotypes, thereby accumulating general and specific properties in a strain. The availability of relevant and reliable phenotypic tests to screen a large population of yeast strains in laboratory conditions is the prerequisite condition to appreciate the contribution of genetics in different characters [143].

In particular, hybridization can be carried out to support different methods depending on yeast strains characteristics. Intra-species hybridization (mating) involves the mating of haploids of opposite mating-types to yield a heterozygous diploid. Recombinant progeny are recovered by sporulating the diploid, collecting individual haploid ascospores and repeating the mating/sporulation cycle as required. Thus, in theory, crossbreeding can permit the selection of desirable characteristics and the elimination of undesirable ones [14]. Elimination or inclusion of a specific property could thus be achieved relatively quickly by hybridization, when the trait has simple genetic basis, for example it is coded by one or two genes [181]. Unfortunately, many desirable wine yeast characteristics are determined by several genes or are the result of numerous controlling system interacting each other. Wine yeast strains that fail to express a mating-type can be forced to mate (rare-mating) with haploid MATα and MATa strains. For instance, industrial strains that have a defective form of mtDNA, (respiratory-deficient mutants) can be force-mated with auxotrophic haploid strains having normal respiratory characteristics. Rare-mating is also used to introduce cytoplasmic genetic elements into wine yeasts without the transfer of nuclear genes from a non-wine yeast parent. This last method of strain improvement is called “cytoduction”. Cytoductants (or heteroplasmons) receive cytoplasmic contributions from both parents, but retain the nuclear integrity of only one of them [92].

1.3.3.2. Use of genetically modified strains in wine industry

S. cerevisiae is doubtless the most important commercial microorganism with GRAS (`generally regarded as safe') status. In recent years the completion of the yeast

genome project and the related available databases have offered an enormous amount of information concerning each of the 6,000 yeast genes and their protein functions, structures and interactions, making the application of the recombinant DNA technologies more feasible for industrial yeasts genetic engineering. *S. cerevisiae* was in fact also the first genetically modified organism (GMO), as distinguished from a genetically modified product, to be cleared for food use, as a baking and brewing strain [264]. The major advances in the fields of molecular genetics, physiology and biotechnology, made possible the construction of specialised commercial wine strains (GMY, genetically modified yeast), mainly by heterologous gene expression or by altered gene dosage (overexpression or deletion). The most important targets for strain improvement deal to enhancement of technological and quality characters, such as better fermentation performance, higher level of ethanol tolerance, better sugar utilisation and nitrogen assimilation, and higher organoleptic properties through the modification of sensorial characteristics. These aspects are summarised by several reviewers [21, 48, 49, 182, 183, 185].

The basic steps of gene cloning and transformation are: (i) identification of a target gene; isolation of the corresponding DNA fragment; (ii) identification and linearization, using specific restriction enzymes, of a suitable plasmid vector; (iii) joining of the DNA fragment containing the target gene to the linearized plasmid generating recombinant DNA molecules; (iv) insertion of the recombinant DNA molecules into host cells by transformation; and (v) screening of the transformed cells and selection of those containing the target gene. Recent advances in genomic technologies, including DNA microarray and gene sequencing, have improved the ability to identify the genes responsible for the desired traits. Nevertheless, only when one or few well-known genes encode the desired trait, the recombinant strain construction is relatively easy and feasible.

In general, all genetic materials involved in the construction of microorganisms used in the field of fermented food must derive from GRAS (generally regarded as safe) organisms that has been employed for a long time in food preparation, whereas the use of DNA sequences from species taxonomically closely related to pathogens should be avoided. Heterologous gene expression was used in most cases, being the genes of interest isolated from *Lactobacillus casei*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Pediococcus acidilactici*, *Schizosaccharomyces pombe*, hybrid poplar, grapevine, *Aspergillus* sp. or *Fusarium solani*. In other cases, the introduced

genes, such as ATF1, GPD1 or PGU1, derived by self-cloning from *S. cerevisiae* (reviewed by Schuller and Casal [233]).
Moreover, all these GMYs must follow the complex requirements specified by current legislation [231]. Up to date, beverage products made with recombinant yeasts have not yet been used commercially, in spite of the immense progress in genomic technologies. Finally, although very efficient, the low level of acceptance of this technology by consumers has limited its impact [197].

1.4. Study of yeast metabolism for improvement of wine strains

Many programs for the improvement of industrial properties of microorganisms used for the production of fermented food are limited by lack of knowledge of the inter related regulatory and metabolic processes that happen in the cell. In this field genomics and functional genomics can provide some solutions. Implementation of functional genomics programs on food microorganisms will enable various industrial objectives to be achieved in the near future: to determine side effects of genetic alterations on functionality in final products, to create desired pleiotropic effects by specific regulatory mutations, to predict and improve stress responses and to direct metabolic engineering efforts [176].
Nearly all organisms respond to changes in their environment by differentially transcribing genes that are important for cell fitness and survival. Wine yeasts have evolved mechanisms to sense and respond to environmental changes and thus maintain metabolic activities and cellular integrity [16]. A successful adaptation implies a metabolic reorganization in order to maintain cellular activity, so it also involves changes in gene expression profiles where a large number of genes are up- or down-regulated. The use of the new genome-wide analysis tools to track these changes can provide an insight view into the gene networks and control circuitry that are underlying the adaptive response.

1.4.1. New tools to study yeast metabolism

Among other high-throughput analytical methods for exploiting the information of genome sequences, the DNA-microarray technology has become a standard tool for the analysis of genome-wide expression profiles (reviewed by Perez-Ortin [175]). Its possibility, however, are not limited to the analysis of the mRNA content of the cells (transcriptome) but, as it is based on hybridization, any nucleic acid molecule, such as genomic DNA, plasmid DNA, genomic RNA (from virus) and any kind of non-messenger RNA or non maturated mRNA can be, in principle, analyzed.

Another powerful technique designed to gain a direct and quantitative measure of global gene expression has recently been utilized on wine yeast: the so-called SAGE, serial analysis of gene expression, enables to consider simultaneously thousands of expressed genes and a total quantification of each transcript [251,258]. SAGE is based mainly on two principles: representation of mRNA by short (9-10nt) tags and linkage of these tags for cloning. Unlike DNA microarrays, SAGE does not require prior knowledge of the genes to be analyzed, indeed SAGE allows efficient identification of novel transcripts [34]. This technique, anyway, is not designed to perform direct dual comparisons.

Finally, The development of “two-step” analysis methodologies (DNA microarrays and two-dimensional electrophoresis combined with subsequent identification and characterization by mass spectrometry) has allowed a detailed analysis of changes in both gene expression and protein levels during vinification [278].

Studies on laboratory strains of *S. cerevisiae* allowed the accumulation of a huge amount of information about genetics and physiology of this species. However, most of the laboratory strains derived from an exceedingly small number of progenitors, which have been crippled by successive mutations. In addition, laboratory media and growth conditions are completely different from those that wild type yeasts encounter in nature or in some commercial applications.

During the last few years, an important effort has been made to characterize gene expression profiles during vinification. In particular, DNA microarrays have been used to describe the transcriptome of industrial wine yeast studied in standard laboratory culture media, synthetic wine media and natural must [11, 62, 94, 138, 223].

1.4.1.1. Transcriptome analysis and microarray technology

The molecular bases of technological properties of wine yeast strains are still largely unknown. However, it is clearly possibly that these strains adaptability to the enological environment is dependent on specific expression profiles of their genomes. This should be the consequence of genetic differences with regard to other kinds of *S. cerevisiae* strains. As mentioned before, enological yeast strains have been selected for their ability to efficiently ferment grape juice sugars under rather stressing conditions. Stress conditions throughout the wine fermentation process, such as nutrient limitation, starvation, temperature variations and ethanol toxicity, affect the yeast's metabolism, eliciting metabolic response. Responses to stress situations include transcriptional and post transcriptional mechanisms [175].

The advent of DNA-microarray technology has made possible the analysis of global patterns of gene expression and revealed unexpected networks of coordinated regulation [266]. These studies have, in turn, stimulated renewed interest in the interactions among metabolic pathways and the control of metabolic flux [50]. Most experiments thus far have dealt with comparisons of gene expression patterns of organisms belonging to the same species grown under different conditions or at different stages of the cell cycle [101].

Studies on wine yeasts by means of DNA array analysis have been made with different approaches in relation to growth conditions. The use of standard growing cultures to study the behaviour of enological yeasts enables the comparison of specific metabolic and physiological features of natural isolates and commercial wine yeasts to the laboratory strains which have already been studied with many approaches, including DNA chips. Comparative analyses of gene expression between industrial and non-industrial strains and among different industrial strains could lead to the identification of genes involved in the fitness of the strains to industrial environments [reviewed in 175]. For instance, the variation of global gene expression levels in natural vineyard isolates has been shown unexpectedly high [31]. Although the use of laboratory culture conditions facilitates the analysis, it does not efficiently restore the natural environment of wine yeasts (acid pH, high sugar concentrations, variable nitrogen conditions). With the aim of understanding how yeast strains cope with their stressful environment, studies have been performed mimicking wine making conditions, using synthetic medium simulating a natural must

and anaerobiosis conditions [11, 223]: global analysis during wine fermentation has indicated changes in the expression of many genes throughout the process; particularly, the entry into stationary phase is followed by a general stress response.

1.4.2. Genome wide expression analysis in yeast cells coping with grape must fermentation

When dry active yeast cells are inoculated into the must they have to cope with a hyper-osmotic stress because of the high sugar concentration in this medium. As fermentation proceeds they are affected by the progressive nutrient limitation, depletion and ethanol toxicity. Depending on the winemaking process, other stress factors during the alcoholic fermentation stage can occur including increases of temperature (even if infrequent due to the modern control systems), cold stress, high CO_2 levels, high SO_2 concentrations and the presence of competing organisms.

1.4.2.1. Grape must composition and yeast nutritional requirements

Grape musts used for wine production usually contain 16-26% w/v of sugars (an equimolecular mixture of glucose and fructose); for the production of noble late harvest or ice wines, however, sugar concentration may be as high as 50%. Hence, a typical environment in which *S. cerevisiae* thrives is rich in sugar, moreover it is low in pH (pH 2.9-3.8) and nitrogen is most frequently the limiting nutrient for growth [23]. Many juices, however, contain ample amounts of essential macro- and micronutrients: under these conditions, populations generally enter stationary phase because of an attainment of maximal cell density rather than a limitation of any given nutrient.

The sugar content of grape juice, anyway, generates an osmoregulatory response in *S. cerevisiae* which have been well characterized both in winemaking [62] and standard growth conditions [139, 210]. *S. cerevisiae* adapts to increased osmotic stress by enhanced production of intracellular glycerol the main compatible solute to counter-balance the osmotic pressure [reviewed in 99, 163]. The transcriptional response for glycerol production is quick and transient [208]. Probably for this reason the expression of these genes (particularly the glycerol-P-dehydrogenases GPD1

and GPD2) is significantly lower a few hours later during vinification, when yeast cells have adapted to this adverse situation.
In addition, glycerol, glycogen and threalose futile cycles are enhanced to avoid an accelerated death due to glucose toxicity: the up-regulation of genes in these futile cycles acts as glycolytic safety valves under conditions of high sugar stress. When sugar concentration increases (up to 40 % w/v, as described by Erasmus *et al*.[62]), sugar induced osmotic stress greatly affects the yeast transcriptome, and the major responses include small molecule transport as well as carbohydrate, nucleotide, amino acid metabolisms, and a large number of protein with unknown function. When the yeast finds itself under severe sugar stress, control of carbon flux through the glycolytic and the pentose phosphate pathways might be more complex than what was previously thought. By shunting more glucose-6-phosphate and fructose-6-phosphate into the oxidative and non-oxidative branches of the pentose phosphate pathway, respectively, the yeast cell may prevent accumulation of fructose-1,6-bisphosphate in the glycolytic pathway and concomitant depletion of phosphate resulting in substrate-accelerated death.
Furthermore it has been shown that yeast, and not bacterial contaminants as previously thought, produces additional acetic acid during fermentation of grape musts with high sugar contents by up-regulating four the iso-genes encoding for aldehyde dehydrogenases, according to previous evidences that under conditions of stress, acetate formation plays an important role in maintaining the redox balance in yeast cells since they require NAD+ for this reaction to proceed [163].

Among all nutrients, nitrogen source has been accepted as the main growth limiting factor, since its deprivation imposes a nutritional stress on metabolic activities. Unlike grape sugars that are usually present in large excess to that needed for maximal yeast growth, as mentioned above, the total nitrogen content of grape juices ranges 40-fold (from 60 to 2400 mg/L) and can therefore be growth-limiting. Among all nutrients assimilated by yeast during wine fermentations, nitrogen is quantitatively second only to carbon. Carbon-nitrogen imbalances and, more specifically, deficiencies in the supply of assimilable nitrogenous compounds, remain the most common causes of poor fermentative performance and sluggish or stuck fermentations [105, 106]. Other problems related to the nitrogen composition of grape must include the formation of reduced-sulphur compounds, in particular

hydrogen sulphide, and the potential formation of ethyl carbamate from metabolically produced urea [97]. *S. cerevisiae* is incapable of adequately hydrolyzing grape proteins to supplement nitrogen-deficient musts, and relies therefore on the ammonium and amino acids present in the juice.

Since wine yeast strains vary widely in their nitrogen requirement, the regulation of nitrogen assimilation by yeast under fermentative conditions has been investigated by several works [reviewed in 175 and 138]. In general, different transcriptome studies have concluded that, in enological yeast strains, the genes involved in amino acid biosynthesis, as well as purine biosynthesis, generally showed high expression levels, indicating a high replicative activity. The growth of the yeast cultures in a high as well as in a low nitrogen source (arginine) concentration [11] allowed to determine how the expression of single or multiple genes (involved in the same or related metabolic pathway) varies between both experimental conditions. In high nitrogen conditions, usually at the start of the fermentation due to the initial high sugar concentration, the fermentative activity is high and the glycolytic enzymes show high expression levels (typical fermentative process with anaerobic pathways acting). However, in low nitrogen conditions there are genes clearly overexpressed, many of which are under glucose repression control. The expression of many ribosomal protein genes is also enhanced. In low nitrogen conditions an important fraction of glucose is always present in the medium. It might be suggested that the response to low nitrogen conditions can lead to a switch from fermentation to respiration pathway. This is reminiscent of the known 'Pasteur effect': inhibition of fermentation at low-nitrogen conditions by the presence of O_2. Although the Pasteur effect has been considered as irrelevant to laboratory yeast growth conditions [117], now it seems that it might be relevant to winemaking conditions. The known consequence of this situation is arrested fermentation. This explains at the level of gene expression the practice of adding supplementary nitrogen source to sluggish or stuck fermentations.

1.4.2.2. Stress factors and yeast response

Under stress conditions yeast cell survival depends on stress response mechanisms [64, 99]. These mechanisms involve sensor systems and signal transduction pathways which activate transcription factors. Consequently, significant transcription changes take place in yeast cells [11, 64, 83, 99]. Heat shock proteins increase

under several conditions (heat-shock, osmotic, ethanol and acetaldehyde stress for instance[4,6,178]).

The effect of the natural environment and the specific role of *S. cerevisiae* during a typical fermentative process, has caused this species to adapt to both anaerobic conditions and high ethanol concentrations, as well as, to acquire resistance to some usual winemaking procedures. These situations imply an important stress situation for growing cells, with the subsequent physiological yeast response, often reflected at gene expression level. Effectively, differences in the expression levels for genes related with these specific conditions and stresses have been found. Thus, in natural anaerobic fermentation, the expression levels for genes involved in the oxidative metabolism remain low [11].

As mentioned before, another stress condition to which wine yeast has adapted is the high ethanol concentration in the growth medium. Alterations in the membrane composition, such as fatty acids, sterols and phospholipids levels, are needed for the maintenance of the ethanol tolerance. The expected higher expression levels for genes encoding these membrane components were observed in conditions resembling those of a natural fermentation [11].

The analysis of gene expression by Rossignol *et al.* [223] has revealed the induction of many stress genes at the moment of entrance into stationary phase or immediately after. These authors consider the stationary phase as a stress condition which is likely amplified due to the ethanol accumulation. Sometimes the reason for entry into stationary phase is not clear, as it took place when neither sugar nor nitrogen was limiting. However, ethanol production could affect the assimilation of nitrogen compounds during vinification [153], or, due to its toxicity for several cellular processes, could provoke the transcriptional activation of several genes. The expression of stress response genes during vinification can help to understand differences in fermentative behaviour among strains. The ability to properly conduct wine fermentation may depend on a complicated equilibrium between the plethora of stress conditions to which yeast cells should respond, and the requirement of growth efficiency [276, 277].

1.4.3. Yeast metabolism and its importance to wine aroma

The flavour of wine is a sensory perception that varies with the individual, the context of the consumer experience and the chemical composition of the product. The final response is the outcome of complex chemosensory interactions that are difficult to predict because of the influences of many variables. Nevertheless, research on many fronts is gradually providing an understanding of these influences [246]. The chemical composition of wine is the foundation of the sensory response and 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 [39].

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 [118, 170].

Wine flavour is composed of a wide variety of compounds with different aromatic properties. It includes flavour compounds originating from the fruit (varietal flavour or primary aroma), compounds formed during operations of extraction and conditioning of must (pre-fermentative flavour), other molecules produced by yeasts and bacteria during alcoholic and malolactic fermentation (fermentative flavour, or secondary aroma) and compounds that appear during the ageing process (post-fermentative flavour) [reviewed in 23, 204, 232].

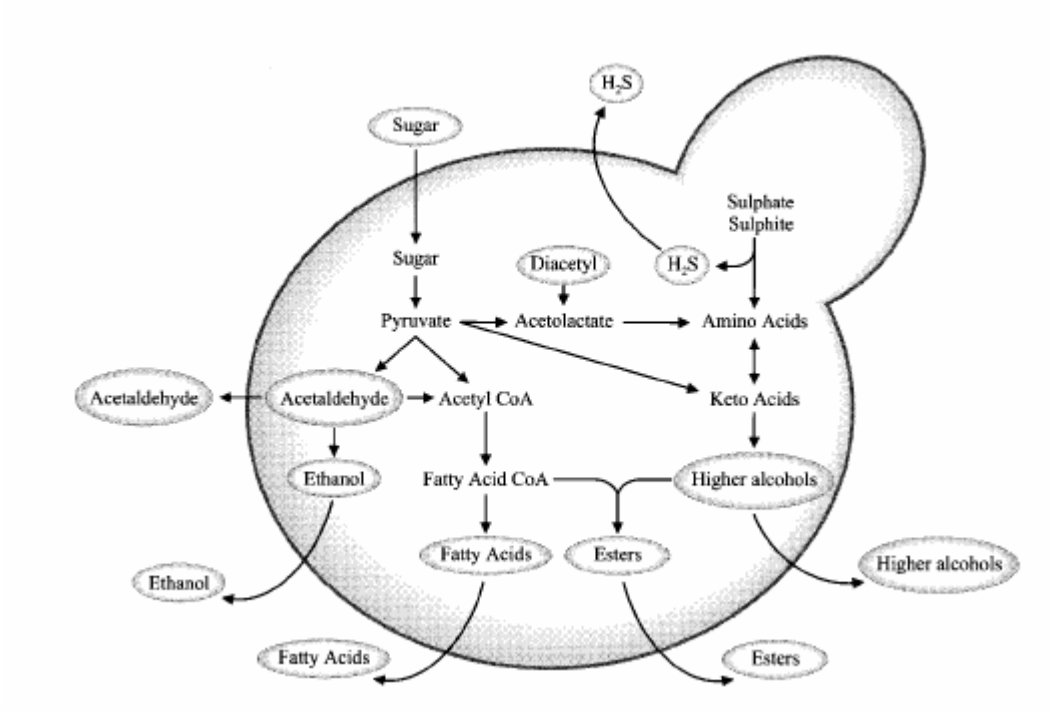


Fig.1 A schematic representation of derivation of flavour compounds from sugar, amino acids and sulphur metabolism by wine yeast [185].

1.4.3.1. Yeast contribution on varietal aroma

The varietal flavour of grapes is mainly determined by the accumulation of variable volatile secondary metabolites in *V. vinifera*. However, a high percentage of these metabolites occur as their respective, non-volatile O-glycosides. Several studies have shown that increased enzymatic hydrolysis of aroma precursors present in grape juice can liberate the aglycone to intensify the varietal character of wines [28]. For instance, terpenols such as geraniol and nerol can be released from terpenyl-glycosides by the grape-derived β-D-glycosidase activity present in muscat grape juice. However, grape glycosidases are unable to hydrolyze sugar conjugates of tertiary alcohols such as linalool [28]. Moreover, these grape enzyme activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine [97]. Thanks to these limiting characteristics of grape-derived glycosidases and the fact that certain processing steps during the clarification of must and wine profoundly reduce their activity, these endogenous enzymes of grapes have a minimal effect in enhancing varietal aroma during winemaking [28].

This has led to renewed interest in the more active β-glucosidases produced by wine yeasts: unlike the grape glycosidases, yeast β-glucosidases are not inhibited by

glucose, and the liberation of terpenols during fermentation can be ascribed to their action on the terpenyl-glycoside precursors [97]. By now, numerous works have shown that yeasts involved in vinification processes possess β-glucosidase activity, and that this activity is greater in non-*Saccharomyces* yeast strains than in *S. cerevisiae* [135, 148, 214, 221]. Among grape yeasts, some strains belonging to *Candida* spp. and *Debaryomyces* spp. are producers of extracellular β-glucosidase [148, 221], while in *Hanseniaspora* spp. and *Kloeckera* spp. the enzyme has been localized essentially within the cell [221, 252,253]. Also among wine-spoilage yeasts, some strains belonging to *Brettanomyces* spp., *Dekkera* spp., *Pichia* spp., and *Hansenula* spp. have been reported to produce β-glucosidase [135, 148, 221, 240]. In these yeasts, the repression of the enzyme biosynthesis was observed when glucose was used as carbon source [22, 33, 252]. Recently, some studies illustrated the impact that the use of non-*Saccharomyces* yeasts as co-starter culture can have on the sensory character of wines (for a review see Lambrechts and Pretorius [118]).
With regard to *S. cerevisiae*, β-glucosidase activity, present at rather low levels, was assayed on intact cells [33, 148, 221] and intracellularly [241]. Delcroix et al. [53] by measuring the activity of the enzymes in three different strains during fermentation of Moscato grapes. In Sauvignon fermentation, a non-terpenic variety, an increase in geraniol content has been described [58, 274]. Enzymatic extracts in *S. cerevisiae* have been shown to hydrolyze Muscat glycosides and liberate the corresponding terpenes. This activity was maintained even at high glucose concentrations [147]. None of the ORFs present in the *S. cerevisiae* genome sequence database can be included in the β-d-glucosidase families GH1 and GH3 [42]. However, there are three exo-1,3-β-glucanase activities encoded by the *EXG1* [257] and *EXG2* [161] genes that show β-d-glucosidase activity since they can use synthetic glucosides p-nitrophenyl- β-d-glucoside, as *EXG1* codes for two proteins, EXG1a and EXG1b, that determine most of the exo-glucanase activity present during vegetative growth, the latter shows the highest exo-1,3- β-glucanase activity [161]. These two enzymes are produced constitutively and are firstly secreted to the periplasmic space and afterwards released into the culture medium where they accumulate [201, 228]. In a recent study, it has been shown that volatiles such as 2-phenethyl alcohol, nerol or geraniol, that play an important role in fruity aroma formation, are increased by 2- to 4-fold in wines when strains over-expressing the *EXG1* gene are used to perform

vinification, whereas there was no increase in the release of linalool, one of the more abundant terpenes in grapes [84].

1.4.3.2. Fermentative flavours: esters and higher alcohols production

During fermentation, *S. cerevisiae* produces a range of minor but sensorially important volatile metabolites that gives wine its vinous character. These volatile metabolites, which are derived from the sugar and amino acid metabolism, include esters, carbonyls, volatile fatty acids, sulphur compounds and higher alcohols (for recent reviews, see Lambrechts & Pretorius [118]; Swiegers & Pretorius [243]; Swiegers *et al*. [244]).

In wines and brandies, the major products of yeast fermentation, esters and alcohols, contribute to a generic positive background flavour [167] whereas subtle combinations of trace components derived from the grapes usually elicit the characteristic aroma notes of these complex beverages [39].

It is generally believed that esters make the greatest contribution to the desirable fermentation bouquet of wine [203]. The characteristic fruity odours of fermentation bouquet are primarily due to a mixture of hexyl acetate, ethyl caprilate giving an apple-like aroma, isoamyl acetate giving a banana-like aroma and 2-phenylethyl acetate giving a fruity and flowery flavour with a honey note [203].

Ester production during alcoholic fermentation is closely related to the particular yeast strain involved [202, 239, 242] and with respect to acetate esters is widely believed to be dependent on the balance of ester synthesis by alcohol acetyltransferases and ester hydrolysis by ester-hydrolases [78, 79, 103]. The synthesis of acetate esters such as isoamyl acetate and ethyl acetate in *S. cerevisiae* is ascribed to at least three acetyltransferase activities, namely alcohol acetyltransferase (AAT), ethanol acetyltransferase (EAT), and iso-amyl acetyltransferase (IAT) [132, 152]. These acetyltransferases are sulfhydryl enzymes which react with acetyl coenzyme A (acetyl-CoA) and, depending on the degree of affinity, with various higher alcohols to produce esters. It has also been shown that these enzymatic activities are strongly repressed under aerobic conditions and by the addition of unsaturated fatty acids to a culture [77, 80, 81]. The best-known enzymes involved in ester synthesis are the so-called alcohol acetyltransferases (AATases; EC 2.3.1.84). In *Saccharomyces* these AATases are encoded by *ATF1*, the *ATF1* homologue *Lg-ATF1*, and *ATF2*, respectively. While *ATF1* and *ATF2* are present in

both *Saccharomyces cerevisiae* and *Saccharomyces bayanus* strains, *Lg-ATF1* is found only in *S. bayanus* strains (reviewed by Mason and Dufour [146]). Homology-based searches of the *S. cerevisiae* genome have not revealed other genes with high similarity to *ATF1* and/or *ATF2*. Atf1p seems to be responsible for the majority of all acetate esters: for most of them, including the important flavour component isoamyl acetate, deletion of the *ATF1* gene causes a reduction of 60 to 90% compared to the level in wild-type cells. Compared to *ATF1*, deletion of *ATF2* resulted in only minor (10 to 35%) decreases in the formation of most esters [77, 157, 249]. Atf1p and Atf2p seem to be the only enzymes involved in the synthesis of acetate esters from long-chain alcohols (C5 or longer).

In addition to the three known AATases, a two possible alcohol acyltransferase, Eht1p (ethanol hexanoyl transferase) and Eeb1p, have recently been described [225]. These ester synthases are involved in the bioformation of medium-chain (C6-C10) fatty acid esters: deletion of either one or both of these genes results in severely reduced medium-chain fatty acid ethyl ester production.

During alcoholic fermentation, *S. cerevisiae* produces higher alcohols that can influence the flavour of the end-product. The term ‘higher alcohol’ refers to alcohols that possess more than two carbon atoms and have a higher molecular weight and boiling point than ethanol. Higher alcohols, also known as fusel alcohols, are quantitatively the largest group of aroma compounds in many alcoholic beverages [5]. They are identified by a strong, pungent smell and taste and can have a significant effect on the sensorial quality and character of wine and brandy [180, 203, 243, 244]. Higher alcohols are aliphatic and aromatic [169]. The aliphatic alcohols include propanol, isobutanol, active amyl alcohol and isoamyl alcohol, while phenylethyl alcohol is considered to be one of the most important aromatic alcohols contributing to wine flavour. The sulphur-containing alcohols, for example methionol, might also have a strong influence on taste and flavour [118]. Isoamyl alcohol, active amyl alcohol and isobutanol are also known as branched-chain alcohols because they are the degradation products of the branched-chain amino acids, leucine, isoleucine and valine [118].

The higher alcohols produced by yeast can originate from the degradation of imported branched-chain amino acids (BCAA) or from endogenous biosynthesis. The BCAA uptake in *S. cerevisiae* is mediated by at least three transport systems, i.e. the

general amino acid permease Gap1p, the BCAA permease Bap2p, and one or more unknown permeases [55]. The amino acids are converted to their corresponding α-keto acids by transamination. This transamination reaction is catalysed by mitochondrial and cytosolic branched-chain amino acid transferases (BCAATases) encoded by the *BAT1* and *BAT2* genes, respectively [60, 61, 107]. Alternatively, these α-keto acids can be generated through the *de novo* synthesis pathway from glucose [53]. In a recent study, *BAT1* and *BAT2* genes have been constitutively over-expressed, showing their strong correlation with higher alcohols production levels. The most significant modifications were observed for isoamyl alcohol, isovaleric acid, isobutanol and isobutyric acid concentrations. Compounds that are found further downstream in the pathway of valine and isoleucine degradation, propionic acid and propanol, appeared largely unaffected by the modified expression levels of these genes [122].

1.5 Aim of this work

During the selection programs of wine yeasts, the first stages of the experimental work regard the collection of several hundred isolates that are screened with the aim to find those that possess important enological properties. In most of the cases, this equivalent to establish that these strains belong to the *Saccharomyces sensu stricto* complex. For that reason, the availability of a molecular test that could rapidly, definitely and easily identifies these enological yeast would be extremely useful. In the first part of this work the analysis of yeast ITS region on rDNA was proposed and tested as screening methods. Since the protocol needs several time consuming experimental steps (amplification and digestion) a new method based on rDNA sequence amplification was set up. For this purpose an original pair of primers was designed in order to generate an amplification fragment specific for the *Saccharomyces sensu stricto* species, and a second pair of primers was also constructed to generate a common band for all enological strains (to be used as a positive experimental control in multiplex PCR analysis). The proposal of this new method intends to allow a “genotypic characterization” of enological strains that is required to start a “technological characterization” for the definition of the enological traits. In this regard, the innovative DNA-microarray technology that has become a standard tool for the analysis of genome-wide expression profiles, can be used to investigate, from a molecular point of view, the differences in the expression of technological and quality characters of enological yeast in laboratory and also winery conditions. For this purpose in the second part of the work, using DNA-microarray technology, an investigation of yeast metabolic shifts at transcriptional level in both laboratory and industrial conditions was faced up; all microarray evidences have been confirmed by Real-time PCR, another high throughput tool for expression analysis. Two commercial yeast strains widely used in wineries, Lallemand 71B and Lallemand EC1118, have been compared during fermentation of both 1 l and 100 l natural white must. These strains have different behaviours and attitudes: 71B is known to be a strong producer of fermentative aromas, EC1118 is an efficient fermenter, quite neutral from an aromatic point of view. This study pursues the aim of understanding the genetic basis of the differences, in fermentation performances, among enological yeasts in winery conditions; furthermore it may help in assessing

the reproducibility of yeast behaviours during wine-making process, when a laboratory scale is used.

2. Setup of a rapid method for differentiating *Saccharomyces sensu stricto* strains in an enological environment

2.1. Introduction

The name *Saccharomyces* was proposed for bread and beer yeasts by Meyen in 1838 [150], but it was Reess in 1870 [206] who first defined the genus. The classification of *Saccharomyces* yeasts has always been problematic at species level; a number of classifications have been proposed over the years. Names of individual strains and species, as a consequence, have undergone several changes. The *Saccharomyces* genus includes two groups of species: *Saccharomyces sensu stricto* (originally designated by van der Walt [250] as the *Saccharomyces* species strictly associated with the fermentation industry) and *Saccharomyces sensu lato* [126] comprising species that are more distantly related to *S. cerevisiae* and are not characterized by the same fermentative performance. As additional species were successively discovered and assigned to *Saccharomyces* (in particular to *Saccharomyces sensu lato*), subgroups differing in morphology and physiology were recognized and finally Yarrow [267] separated some of them in the genera *Torulaspora* and *Zygosaccharomyces*, although species assignments were often difficult. With the introduction of nuclear-DNA reassociation techniques, Kurtzman and Robnett in 1998 have shown that previous species assignments among species of the genus *Saccharomyces* were often incorrect and proposed *Saccharomyces sensu stricto* group as composed of four species: *Saccharomyces bayanus*, *Saccharomyces cerevisiae*, *Saccharomyces paradoxus* and *Saccharomyces pastorianus*. This grouping, mainly based on DNA reassociation analysis and on some physiological characteristics seem to be artificial in the case of *S. bayanus.*

This species, in fact, contains strains that possess heterogeneous phenotypic and genotypic characteristics but several studies have recently demonstrated the existence of a homogeneous group of strains within the species *S. bayanus* [200, 187]. These strains were referred to as *S. uvarum* because they possess the phenotypic characteristics of strains originally described for the species *S. uvarum* by van der Walt in 1970 [164].

Up to date, the sensu stricto group includes seven species giving a vigorous alcoholic fermentation, six heterothallic biological species (*S. bayanus*, *S. cerevisiae*, *S. paradoxus S. kudriavzevii, S. cariocanus, S. mikatae)* and the hybrid

species S. pastorianus [158]. The seven species accepted are regarded as genetically isolated from one another on the basis of genetic crosses [158] as well as from molecular comparisons [112]. Although *S. cariocanus, S. cerevisiae* and *S. paradoxus* appear to be separate biological species from genetic crosses [158], they show relatively little gene sequence divergence [112].

The identification and classification of yeasts have traditionally been based on morphological, physiological and biochemical traits, as diffusely described in chapter one. The differentiation of taxa is usually achieved by comparison of morphological traits and physiological features [13; 108], which in some cases can lead to an incorrect classification of species or a false identification of strains. It is therefore currently accepted that phenotypic analyses are not sufficient to reach a trustworthy identification [112; 137] but the advent of molecular genetics has provided more reliable methods for taxonomic studies.

In the present study, two different purposes were subsequently pursued. Firstly, the aim was the molecular identification of natural yeast isolates collected from marks of Prosecco and Moscato grapes coming from the typical growing areas and used for distillation and Grappa production. To achieve this object, the method based on restriction profiles of Internal Transcribed Spacers region (proposed by Esteve-Zarzoso *et al.* [63]) was tested. As reference strains, a collection of enological species was used in order to construct a small database tailor-made to evaluate yeast biodiversity present in Moscato and Prosecco grape pomaces.

The second purpose was the designation and optimization of a rapid method for differentiating *Saccharomyces sensu stricto* strains from other yeast species in an enological environment. It can be particularly useful during selection programs of wine yeasts, when several hundred isolates are usually screened. During the first stages of selection in fact, a definite species designation is less relevant than the assessment of enologically important properties. In most cases, this is equivalent to establishing that strains are part of the *Saccharomyces sensu stricto* complex. As the initial number of isolates is normally very high, the availability of a test that could rapidly and easily detect target strains would be extremely useful.

2.2. Materials and methods

2.2.1. Yeast strains

The strains used in this study are listed in table 2.2.1. Type strains of all the currently accepted species of the genus *Saccharomyces* (seven *sensu stricto* and 17 *sensu lato*) reported by Kurtzman [115] are included. Another group of 18 non-*Saccharomyces* species that are related to the wine environment were also considered.

Species	Strain[a]	26S rDNA accession number	18S rDNA accession number
Saccharomyces sensu stricto			
Saccharomyces bayanus	NRRL Y-12624^T	**AY048156**	**AY046227**
Saccharomyces cariocanus	NRRL Y-27337^T	**AF398478**	**AY046224**
Saccharomyces cerevisiae	NRRL Y-12632^T	**AY048154**	**Z75578**
Saccharomyces kudriavzevii	NRRL Y-27339^T	**AF398480**	**AY046226**
Saccharomyces mikatae	NRRL Y-27341^T	**AF398479**	**AY046225**
Saccharomyces paradoxus	NRRL Y-17217^T	**AY048155**	**X97806**
Saccharomyces pastorianus	NRRL Y-27171^T	**AF113893**	**X97805**
Saccharomyces sensu lato[b]			
Saccharomyces (Kazachstania) barnettii	NRRL Y-27223^T	**AY048164**	**AY046242**
Saccharomyces (Kazachstania) bulderi	NRRL Y-27203^T	**AF398486**	**AY046241**
Saccharomyces (Naumovia) castellii	NRRL Y-12630^T	**AY048167**	**Z75577**
Saccharomyces (Naumovia) dairenensis	NRRL Y-12639^T	**AY048168**	**Z75579**
Saccharomyces (Kazachstania) exiguus	NRRL Y-12640^T	**AY048163**	**X98868**
Saccharomyces (Kazachstania) humaticus	IFO 10673^T	**AB040999**	**AB016513**
Saccharomyces (Lachancea) kluyveri	NRRL Y-12651^T	**U68552**	**Z75580**
Saccharomyces (Kazachstania) kunashirensis	NRRL Y-27209^T	**AJ279064**	**AY046235**
Saccharomyces (Kazachstania) martiniae	NRRL Y-409^T	**AF398481**	**AY046231**
Saccharomyces (Kazachstania) naganishii	IFO 10181^T	**AB088404**	**AB016512**
Saccharomyces (Kazachstania) rosinii	NRRL Y-17919^T	**AY048160**	**AY046232**
Saccharomyces (Kazachstania) servazzii	NRRL Y-12661^T	**AY048157**	**AY251643**
Saccharomyces (Kazachstania) spencerorum	NRRL Y-17920^T	**AY048162**	**X97807**
Saccharomyces (Kazachstania) transvaalensis	NRRL Y-17245^T	**AY007911**	**AY046230**
Saccharomyces (Kazachstania) turicensis	NRRL Y-27345^T	**AF398485**	**AY046240**
Saccharomyces (Kazachstania) unisporus	NRRL Y-1556^T	**AY048158**	**AY046228**
Saccharomyces (Kazachstania) yakushimaensis	IFO 1889^T	**AY007900**	**AB016514**
non-*Saccharomyces*			
Candida stellata	NRRL Y-1446^T	**U45730**	**AB018175**
Debaryomyces carsonii	NRRL YB-4275^T	**U45743**	**AB054260**
Dekkera anomala	NRRL Y-17522^T	**U84244**	**X83820**
Dekkera bruxellensis	NRRL Y-12961^T	**AF113890**	**X83814**
Hanseniaspora guillermondii	NRRL Y-1625^T	**U84230**	**AY046256**
Hanseniaspora uvarum	NRRL Y-1614^T	**U84229**	**AY046257**
Issatchenkia occidentalis	NRRL Y-7552 ^{T}	**U76348**	**AB053240**
Kluyveromyces lactis	NRRL Y-8279^T	**U94922**	**AY046264**
Kluyveromyces marxianus	NRRL Y-8281^T	**U94924**	**X89523**
Metchnikowia pulcherrima	NRRL Y-7111^T	**U45736**	**AB023473**
Pichia anomala	NRRL Y-366^T	**U74592**	**AB126679**
Pichia membranifaciens	NRRL Y-2026^T	**U75725**	**AB053233**
Rhodotorula bogoriensis	NRRL Y-12675^T	**AF189923**	**AF444536**
Saccharomycodes ludwigii	NRRL Y-12793^T	**U73601**	**AY046261**
Schizosaccharomyces japonicus	NRRL Y-1361^T	**U94943**	**AB000966**
Schizosaccharomyces pombe	NRRL Y-12796^T	**AY048171**	**AY046272**
Torulaspora delbrueckii	NRRL Y-866^T	**AJ508558**	**X98120**
Zygosaccharomyces bailii	NRRL Y-2227^T	**U72161**	**X91083**

Table 2.2.1. Strains used in this work and relative sequences utilized for primer construction

Moreover 365 isolates collected from marks of Prosecco and Moscato grapes were subjected to ITS analysis.

2.2.2. Growth conditions

All yeasts were routinely grown on YM agar medium (3 g L^{-1} yeast extract; 3 g L^{-1} malt extract; 5 g L^{-1} proteose peptone; 10 g L^{-1} glucose; 15 g L^{-1} agar) or Wallerstein Laboratory (WL) nutrient agar [89], at 25 ºC for 1 to 10 days, depending on the yeast species. For DNA extraction yeasts were grown on YM medium (3 g L^{-1} yeast extract; 3 g L^{-1} malt extract; 5 g L^{-1} proteose peptone; 10 g L^{-1} glucose), at 25ºC under agitation, for 18 to 72h depending on yeast species.

2.2.3. Sample preparation for DNA amplification

For routine analysis, PCR were performed on cell suspensions in water. Single yeast colonies (1-2 mm diameter) were picked up with a sterile toothpick from YM or WL plates and resuspended in 20 µl of sterile deionized water in 0.5 ml tubes. Two µl of the suspension were used for PCR amplification.

2.2.3.1. DNA extraction

Yeast DNA was extracted and purified using *MasterPure™ Yeast DNA Purification Kit* by EPICENTRE (Madison, WI, USA) according to manufacturer's instructions. Three hundreds µl of Yeast Cell Lysis Solution were added to each microcentrifuge tube containing the collected pellet. Cells were suspended by either vortex mixing or pipetting repeatedly using a 1 ml capacity pipet tip. Samples were incubate at 65ºC for 15 minutes, then placed on ice for 5 minutes. Hundred and fifty µl of MPC Protein Precipitation Reagent were added and samples were vortexed mix for 10 seconds. Cellular debris were pelleted by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm, the supernatant was transferred to a clean microcentrifuge tube and 500 µl of cold isopropanol were added. After a thorough mix by inversion, the DNA was precipitated by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm, the supernatant was removed and discarded. Washes of pellet containing the DNA were done using 0.5 ml of 70% ethanol. Afterwards ethanol was carefully removed and the DNA was suspended in 35 µl of TE Buffer and stored at -20ºC to 4ºC.

2.2.4. PCR assays and gel electrophoresisis

All the PCR reactions were performed in a PTC200 thermal cycler (MJ Research Inc., MA, USA). All the primers have been designed using Primer Select software (from DNAstar, Madison, WI, USA) and synthesized by MWG-Biotech (HPSF purified). A proof-reading Taq Polymerase, its buffer and dNTPs (Amersham Biosciences, Uppsala, Sweden) have been used for all the amplification reactions at the following concentrations:

Component	Final concentration
Primers	2 µM (each)
dNTPs	0,05 mM (each)
Taq polymerase	0,02 U/µl
Buffer	1X
DNA (25 ng/µl) or cell suspension	2µl

2.2.4.1. ITS amplification

Amplification of the ITS region was performed on yeast DNA or on yeast cells suspension. ITS primers are hereby reported:

Primer	**Sequence (5'-3')**	**Amplification on genomic DNA**	**Source**
ITS1	TCCGTAGGTGAACCTGCGG		
ITS4	TCCTCCGCTTATTGATATGC	470 to 940	White T.J. *et al*., 1990

The thermal protocol was designed as follows:

Cycle1 (1x)	95°C	5'30"
Cycle2 (40x)	95°C	15"
	55°C	1'
	72°C	2'
Cycle3 (1x)	72°C	5'
	4°C	∞

Amplified samples were run on 1% agarose gel containing 0,1 µg/ml of Ethidium bromide. 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, Rochester, NY, USA).

2.2.4.2. D1/D2 amplification

Amplification of the D1/D2 region was performed on yeast DNA or on yeast cells suspension. D1/D2 primers are hereby reported:

Primer	Sequence (5'-3')	Amplification on genomic DNA*	Source
NL-1	GCATATCAATAAGCGGAGGAAAAG		
NL-4	GGTCCGTGTTTCAAGACGG	560	Kurtzman and Robnett, 1998

*Relative to *S. cerevisiae* Y-12632T 18S rRNA gene sequence (GenBank accession no. Z75578).

The thermal protocol was designed as follows:

Cycle1 (1x)	95°C	5'30"
Cycle2 (40x)	95°C	15"
	55,5°C	1'
	72°C	2'
Cycle3 (1x)	72°C	5'
	4°C	∞

Amplified samples were run on 1.2 % agarose gel containing 0,1 µg/ml of Ethidium bromide. Electrophoresis and image acquisition was performed as described in par. 2.2.4.1.

2.2.4.3. 18S and "SAC18" amplification

Amplification of the 18S ribosomal DNA was performed on yeast DNA or on cell suspension. Two primer pairs for 18S amplification are hereby reported:

Primer	Sequence (5'-3')	Melting temp (°C)	Annealing temp (°C) Ta	Amplification on genomic DNA* (bp)
SAC18-F	CTGCGAATGGCTCATTAAATCAG	58.9	53	900
SAC18-R	CCCTAACTTTCGTTCTTGATTAATG	58.1		

*Relative to S. cerevisiae Y-12632T 18S rRNA gene sequence (GenBank accession no. Z75578).

The thermal protocol (for both primer pairs) was designed as follows:

Cycle1 (1x)	95°C	5'
Cycle2 (40x)	95°C	30"
	53°C	15"
	72°C	1'30"
Cycle3 (1x)	72°C	5'
	4°C	∞

Amplified samples were run on 1.2 % agarose gel containing 0,1 µg/ml of Ethidium bromide. Electrophoresis and image acquisition was performed as described in par. 2.2.4.1.

2.2.4.4. "SAC26" amplification

Amplification of the 26S ribosomal DNA was performed on yeast DNA or on yeast cells suspension. *Saccharomyces sensu stricto* specific primers for 26S ribosomal DNA amplification are hereby reported:

Primer	Sequence (5'-3')	Melting temp (°C)	Annealing temp (°C) Ta	Amplification on genomic DNA* (bp)
SAC26-F	GAGAGGGCAACTTTGGGRCCGT	64.9	58.1	471
SAC26-R	ACCATTATGCCAGCATCCTTGACTTAC	63.4		

*Relative to S. cerevisiae Y-12632T 18S rRNA gene sequence (GenBank accession no. Z75578).

The thermal protocol (for both primer pairs) was designed as follows:

Cycle1 (1x)	95°C	5'
Cycle2 (40x)	95°C	30”
	58°C	15”
	72°C	1’
Cycle3 (1x)	72°C	5’
	4°C	∞

Amplified samples were run on 1.2 % agarose gel containing 0,1 µg/ml of Ethidium bromide. Electrophoresis and image acquisition was performed as described in par. 2.2.4.1.

2.2.4.5. Multiplex PCR SAC18-SAC26

Multiplex PCR protocol was set up with primer pairs SAC18 and SAC26 (previously listed). The amplification reaction was performed with the following reagents concentrations:

Component	Final concentration
Primers SAC18F and R	2 µM (each)
Primers SAC26F and R	0.2 µM (each)
dNTPs	0,05 mM (each)
Taq polymerase	0,02 U/µl
Buffer	1X
DNA (25 ng/µl) or cell suspension	2µl

The thermal protocol was designed as follows:

Cycle1 (1x)	95°C	5'
Cycle2 (35x)	95°C	30”
	54°C	15”
	72°C	1’30”
Cycle3 (1x)	72°C	5’
	4°C	∞

Amplified samples were run on 1.2 % agarose gel containing 0,1 µg/ml of Ethidium bromide. Electrophoresis and image acquisition was performed as described in par. 2.2.4.1.

2.2.5. DNA digestion and gel electrophoresis

Restriction reactions were performed as described by Ausbel *et al*. [7] and Sambrock *et al*. [227] using 8µl of amplified DNA in a total volume of 20 µl. Restriction enzymes were purchased to Fermentas International Inc. (Canada).
Digested DNA samples were run on 1% to 1.5% agarose gel containing 0,1 µg/ml of Ethidium bromide. 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).

2.2.6. DNA preparation for sequencing

DNA sequencing (Sanger reaction with fluorescent nucleotides and capillary electrophoresis run and analysis) was performed by BMR-genomics (Padova, Italy).
PCR products to be sequenced were purified from primers and short polynucleotides by ExoSap™ Cleanup method (usb-United States Biochemical, USA) just adding 1µl of ExoSap enzyme to 5µl of PCR products and incubating at room temperature for 30 minutes. DNA was then quantified by gel electrophoresis using Kodak Edas 2.9 software which compares band intensities to those of a suitable weigh marker.

Then purified DNA was prepared for sequencing, following BMR-genomics instructions (available on www.bmr-genomics.it) as follows:

Component	Q.ty
PCR product (ExoSap purified)	10 to 20ng for 100bp of product length
Primer	3.2 pMol

The DNA mixture was dried under vacuum and sent for sequencing.

2.3. Results and discussion

2.3.1. Molecular identification of yeast species: construction of a restriction profile database

A large number of previously isolated yeasts, collected for the characterization of indigenous microflora of Prosecco and Moscato grape marks used for Grappa production, was considered for identification purpose. To characterize these yeasts, the method proposed by Esteve-Zarzoso *et al.* in 1999 based on amplification and restriction of Internal Transcribed Sequences of the rDNA cluster was used and an enological database of digestion profiles was developed. This method, at the same time, allows both to recognize the presence of *Saccharomyces* and to identify other genus and species of yeasts.

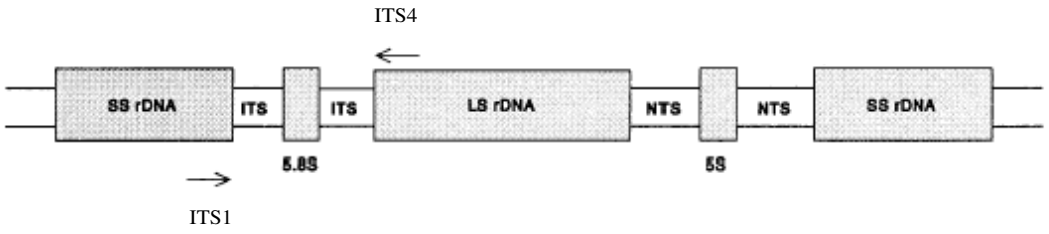


Fig 2.3.1 Ribosomal RNA coding region on yeast genome, modified by Baleiras Couto M. M. *et al* (1996).

2.3.1.1. ITS amplification and restriction results

ITS1 and ITS4 primers were used to amplify the region of the rDNA repeat unit that includes the 5.8S rRNA gene and the two non-coding regions so-called Internal Transcribed Spacers (ITS1 and ITS2) [265], amplified fragments were subjected to digestions [63] with the restriction enzymes *Hae*III and *Hinf*I. Figure 2.3.2 shows an example of restriction pattern after *Hae*III digestion. Table 2.3.1 shows the sizes of the PCR products and the fragments obtained using the restriction endonucleases *Hae*III and *Hinf*I from 36 species widely diffused in vineyard and winery environments. Some of these species, especially within the *Saccharomyces* genus, were not reported in the main reference work from Esteve-Zarzoso [63].

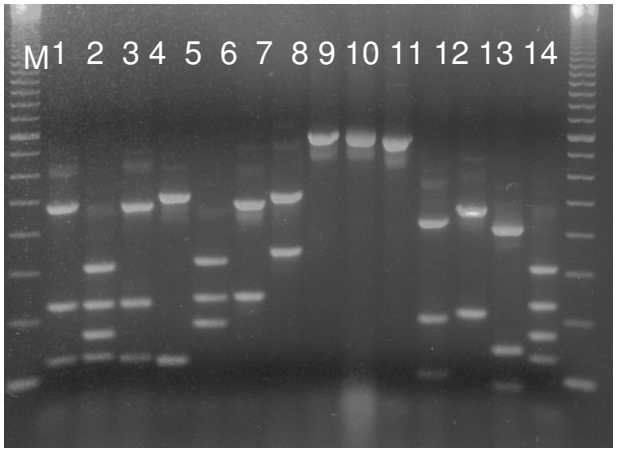


Fig.2.3.2 Example of *Hae*III restriction profiles of ITS region obtained from speacies belonging to *Saccharomyces sensu stricto* (4), *Saccharomyces sensu lato* (9) and non-*Saccharomyces* (1). M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) *S. mikatae*; (2) *S. paradoxus*; (3) *S. kudriavzwevii; (4): S. unisporus*; (5) *S. servazz* ;(6) *S. bulderi* ; (7) *S. transvaalensis*; (8) *S. dairenensis* ; (9) *S. castelli*; (10) *S. martiniae;* (11) *S. spencerorum*; *(12) S. kunashirensis*; (13) *Debaryomyces carlsonii*; (14) *S. cariocanus.*

	ITS	HaeIII Digestion				HinfI Digestion			
S. sensu stricto									
Saccharomyces cerevisiae	855	310	235	180	145	650	360	130	
S. paradoxus	855	310	235	180	145	650	360	130	
S. cariocanus	835	310	235	180	145	650	360	130	
S. pastorianus	835	500	255	155		650	360	130	
S. bayanus	835	500	255	155		650	360	130	
S. bayanus (uvarum)	835	500	255	155		650	360	130	
S. mikatae	830	480	240	140		650	360	130	
S. kudriavzevii	830	480	240	140		650	360	130	
S. sensu lato									
S. unisporus	740	515	140			670	380	360	
S. servazzi	740	330	250	200		640	370		
S. bulderi	710	490	255			310	250	140	
S. transvaalensis	810	515	355			375	350	135	
S. barnetti	740	475	215			350	300		
S. dairenensis	800	780				620	360	340	100
S. rosinii	740	500	215			380	345		
S .castelli	780	780				640	340	260	130
S. martiniae	755	755				390	370		
S. spencerorum	730	435	210	115		650	385	360	
S. kunashirensis	680	470	220			610	335		
Non *Saccharomyces*									
Pichia anomala	610	630				320			
P. membranifaciens	435	295	145			240	120		
Dekkera bruxellensis	500	380	120			250	220		
Dekkera anomala	540	230				370			
Torulaspora delbrueckii	780	780				395			
Zygosacharomyces bailii	795	700				340	235	170	
Kluveromyces marxianum	710	615				255	190		
Kluveromyces lactis	710	615				295	190	130	
Debaryomyces carlsonii	650	410	155			555	320		
Metschnikowia pulcherrima	400	360	295			200			
Candida stellata	470	470				255	240		
Hanseniaspora guilliermondii	750	740				350	200	170	
Hanseniaspora uvarum	750	740				350	200	170	
Saccharomycodes ludwigii	750	600	150			410	255		
Schizosaccharomyces pombe	940	940				440			
Schizosaccharomyces japonicus	655	655				300	200	135	
Rhodotorula bogoriensis	650	475	130			320	210	180	

Table 2.3.1 Sizes of PCR products and *Hae*III and *Hinf*I restriction fragments obtained from the analysis of 36 species most of them diffused in enological environment.

As expected, a wide polymorphism in ITS amplification fragment length (varying from 400 bp in *Candida* and *Metschnikowia* to 800 bp in *Saccharomyces)* was found.
Concerning the results from *Hae*III restictions, a good discrimination level within the *sensu stricto* group was found. This enzyme allowed the subdivision of yeast strains belonging to *sensu stricto* group into three sub-groups according to genetic similarity [111]: the first one (pink square in table 2.3.1) contains *S. cerevisiae*, *S. paradoxus* and *S. cariocanus*, the second (red square) groups *S. bayanus* and *S. pastorianus* while in the third one (blue square) are present *S. mikatae* e *S. kudriavzwevii*. Various patterns were found among *sensu lato* strains after ITS digestion with *Hae*III, whereas the same enzyme did not allow a good discrimination among non-*Saccharomyces* enological yeasts (in most cases the ITS fragment was not cut at all, or only one restriction site was recognized).
Opposite results were obtained using *Hinf*I. This enzyme, in fact, showed an high discrimination level among non-*Saccharomyces* yeasts analyzed (green square in table 2.3.1) giving easily differentiable profiles. Concerning the *sensu stricto* group, on the contrary, *Hinf*I restriction did not gave any polymorphism, showing a single restriction profile for all the seven species (liliac square in table 2.3.1).

2.3.1.2. Characterization of natural yeast isolates form marks

ITS amplification was performed on 365 yeast colonies previously isolated from Prosecco and Moscato grape marks coming from the typical growing areas and used for distillation and Grappa production. PCR products were digested with *Hae*III and *Hinf*I enzymes and restriction patterns were compared. Yeasts having identical restriction profiles after both digestions were clustered, 21 different groups were found. Three of these were clearly identified by comparing with type strain database: the *Saccharomyces cerevisiae/paradoxus/cariocanus* group, *Saccharomycodes ludwigii* and *Hanseniaspora uvarum/guillermondii*.
All the other groups, whose restriction profile was not found in the database, were named with letters from A to T. The D1/D2 region of two isolate for each group (including yeasts from the three already identified groups) were amplified and sequence analysis for species identification was performed. Sequences were submitted to BLAST researches (available at http://www.ncbi.nlm.nih.gov/BLAST) and identified by similarity. The BLAST output compared with ITS analysis of the type strain is reported in table 2.3.2.

D1/D2 sequence	ITS restriction profile
Groups of clear identification	
Saccharomyces cerevisiae	*S.cerevisiae/paradoxus/cariocanus*
Saccharomycodes ludwigii	*Saccharomycodes ludwigii*
Hanseniaspora guillermondii	*Hanseniaspora*
Other groups found in Moscato marks	
Metschnikowia pulcherrima	D
Metschnikowia pulcherrima	H
Metschnikowia pulcherrima	O
Lachancea meyersii	E
Lachancea meyersii	L
Lachancea meyersii	N
Tosrulaspora delbruekii	M
Other groups found in Prosecco marks	
Issatchenchia occidentalis	Q
Tosrulaspora delbruekii	A
Pichia galeiformis	B
Issatchenchia orientalis	C
Issatchenchia orientalis	F
Metschnikowia pulcherrima	T
Tosrulaspora delbruekii	P
Tosrulaspora delbruekii	G
Tosrulaspora delbruekii	I
Tosrulaspora delbruekii	R
Tosrulaspora delbruekii	S

Tab.2.3.2 D1/D2 sequence analysis performed on the 21 groups obtained from restriction pattern analysis.

By means of sequence analysis, only 8 enological species were found to correspond to all the 21 restriction profiles. Four different ITS groups were associated with *Metschnikowia pulcherrima* species, 3 with *Lachancea meyersii*, 2 with *Issatchenkia orientalis* and 6 with *Torulaspora delbruekii*. The ITS restriction method proved to be unambiguous and reliable in yeast discrimination, in particular it has been shown that none of the unknown groups contained *Saccharomyces* strains. For species assessment adding information by means of sequencing analysis was often necessary to obtain the taxonomical collocation. This need is due to the observation that strains belonging to the same species generally give more than one profile that usually in different from the one found for the corresponding type strain. This result shows that ITS analysis, in many case could be more efficient in characterizing yeast at strain or sub-species level than at species level.

2.3.2. Setup of a multiplex PCR for differentiating Saccharomyces sensu stricto strains from other yeast species in an enological environment

During selection program an early identification of yeast with relevant enological properties is extremely important. In most cases, this is equivalent to establishing that these strains are part of the *Saccharomyces sensu stricto* complex. The ITS method previously described allows *Saccharomyces sensu stricto* identification, but the protocol needs two experimental steps (amplification and digestion). For that reason it is not useful when a large number of isolates have to be analysed.

Thus a new method to rapidly differentiate *Saccharomyces sensu stricto* yeasts from all other species widely diffused in an enological environment was set up. In particular, a pair of primers, designed within the variable D1/D2 region of the 26S subunit of ribosomal yeast RNA, have been constructed. These generate an amplification fragment of 471 bp that is specific for the seven *Saccharomyces sensu stricto* species, while no signal was obtained for *Saccharomyces sensu lato* strains (17 species) or for another 18 selected species commonly found in enological environments. A second pair of primers was also need to be used as a positive experimental control in multiplex PCR analysis allowing a clear discrimination of yeasts belonging to *Saccharomyces sensu stricto* group by one-step amplification.

2.3.1.3. "SAC26" specific primers designation

In order to identify a suitable region for primer design, the D1/D2 rRNA gene sequences of the type strains of the seven *Saccharomyces* species constituting the *sensu stricto* complex were aligned using the CLUSTAL W program. Nucleotide sequences were obtained from GenBank and the corresponding accession numbers are reported in table 2.2.1. An accurate inspection of various alignments (figure 2.3.3) revealed the presence of two small highly conserved regions among the *sensu stricto* strains that are sufficiently different from the other species. The first, located from positions 92 to 113 of the S. cerevisiae type strain sequence, is conserved in all *sensu stricto* species except *Saccharomyces kudriavzevii*, which has an 'A' instead of a 'G' at position 109 and *Saccharomyces mikatae* that has three substitutions ('T' for 'C', 'C' for 'T' and 'A' for 'G' at positions 103, 106 and 108, respectively). The second sequence, perfectly conserved in all seven *sensu stricto* type strains, was found from

positions 536 to 562. Based on these *consensi*, primers SAC26F (22 nt) and SAC26R (27 nt) were designed. The corresponding regions of the *sensu lato* strains contain various mismatches (figure 2.3.2) that, although not numerous, are concentrated at the 3' ends of the primers. This characteristic is extremely important as it strongly affects primer functionality (Niemann et al., 1999). Primer sequence similarity was also checked on the 18 non-*Saccharomyces* yeasts reported in table 2.2.1, including some enologically negative species, and others commonly isolated in the vineyard or the winery (figure 2.3.2). As expected, considering the increasing taxonomic distance, the overall sequence similarity decreased, again as seen as a series of mismatches at the 3' primer ends.

CONSENSUS	GAGAGGGCAACTTTGGGRCCGT	GTAAGTCAAGGATGCTGGCATAATGGT
Saccharomyces sensu stricto		
S. bayanus	----------	----------
S. cariocanus	----------	----------
S. cerevisiae	----------	----------
S. kudriavzevii	----------A-----	----------
S. mikatae	----------T--C-A------	----------
S. paradoxus	----------	----------
S. pastorianus	----------	----------
Saccharomyces sensu lato		
S. barnettii	T-------G----------G-C	A-TC----------
S. bulderi	T-------G----------G-C	A-TC----------
S. castellii	AGAG--AT-CT--Gt----T-G	T-T----------
S. dairenensis	T------AT----------T-G	A-TC----------
S. exiguus	T-------G----------G-C	A-TC----------
S. humaticus	-TAGAA-GT-TC----AATG--	C-TT----------T----------
S. kluyveri	--AGAA-T----------T-A	T-TT----------T----------
S. kunashirensis	T------AT----------	A-TC----------
S. martiniae	T------AT----------	C-TT----------T----------
S. naganishi	T------AT---C---------	T-GC-C----------
S. rosinii	T------GT----------T--	T-TT----------T----------
S. servazii	T------AT----------	T-TT----------T----------
S. spencerorum	T------AT-----T----C--	T-T----------
S. transvaalensis	-TAGAA-GT-TC----AATG--	T-TT----------T----------
S. turicensis	T-------G----------G-C	A-TC----------
S. unisporus	T------AT----------	T-TT----------T----------
S. yukushimaensis	-TAGAA-GT-TC----AATG--	C-TT----------T----------
non-*Saccharomyces*		
Candida. stellata	A--TTA-G-TTC----A----A	n.a.
Dekkera anomala	----C--G-TAC-A-A--GA-G	TA-C-C----------G--C-AG
Dekkra bruxeellensis	----C--G-CAC-A-A--GGAG	TGTCAC----------G--C-AG
Debaryomyces. carsonii	A---A---T-------A-TT-G	T-TT-A--------T---------A--
Hanseniaspora guillermondii	T---ATTTGT-----ATTAG--	C-TC-G--------T----------
Hanseniaspora. uvarum	T---ATTTGT-----ATTAG--	C-TC-G--------T----------
Issatchenkia occidentalis	A-A--CT--GA----AA-T---	A-CT---TC----------C--C--C
Kluyveromyces. lactis	A---A----------TA--T-G	T-TT----------G-------
Kluyveromyces marxianus	A---A---G------TA--T-G	T-TT----------G-------
Metschnikowia pulcherrima	A----ATTTGGG-CC--C-G-C	-C--TCT----------G-------
Pichia anomala	A---T--T---C-----TTT-G	T-TT-A-T----------G-----A-
Pichia membranifaciens	T--GC--G-GTC--T-T-GA-C	T-CT---TC----------C--C--C
Rhodotorula bogoriensis	CGAGAA-TGTT--CC-C---AG	T-CG-CTT----------TGG-----C
Saccharomycodes ludwigii	A-----TT-CT--G-A---T-G	T-T-TG--------T----TT------
Schizosaccharomyces japonicus	A---A-CTGCT--GA-T-TT-C	C-TT----------A---------
Schizosaccharomyces pombe	A---A-CTGCT--GA-T-TA-A	TCGT-C----------A---------
Torulaspora delbrueckii	T---A--T----------T-G	T--C----------
Zygosaccharomyces bailii	T---A---G---C-----CT-G	T-T----------

Fig.2.3.3. Alignment of the D1/D2 26S rRNA gene sequence traits used for construction of primers SAC26F (left) and SAC26R (right). The top line reports the consensus. In the aligned sequences, the dashes correspond to nucleotide conservation. In the case of a mismatch, the letter indicates the substitution. Small letters indicate base insertions. Base deletions are located between two adjacent underlined nucleotides. In the presence of insertions or deletions, alignments to the consensus were performed starting from the 30 primer polymerizing end. NA, not available (the published sequence does not cover the zone of the consensus).

Using these primers for PCR amplifications, as expected, all *Saccharomyces sensu stricto* strains produced a single clear band of 471 bp, while no signal was detected for the other strains studied (figure 2.3.4).

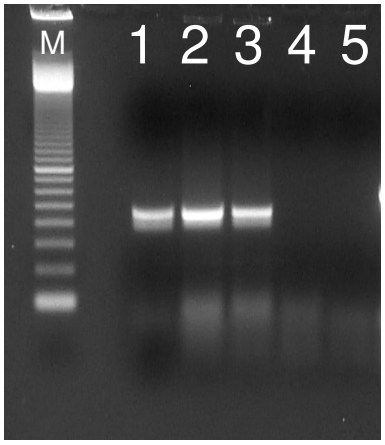


Fig.2.3.4. Gel electrophoresis of PCR amplification products of some strains reported in table 2.2.1. The band is generated by primers SAC26F and SAC26R. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) *Saccharomyces cerevisiae;* (2) *Saccharomyces kudriavzevii;* (3) *Saccharomyces mikatae;* (4) *Saccharomyces castellii,* (5) *Hansenispora uvarum*

2.3.1.4. Choice of a primer pair as amplification control and 18S primer design

In order to exclude the presence of false-negative results (i.e. lack of amplification due to experimental problems), it was deemed necessary to choose a pair of primers producing an amplification fragment for all strains studied as a positive control in the same PCR experiment. These sequences were initially searched within the most conserved rDNA regions.

For this purpose, the primer pair designed by Kurtzman and Robnett [111] for the amplification of D1/D2 region was tested in multiplex PCR with SAC26 primers. The former primers, when tested alone, generate a 560 bp amplification band but, when they are used in presence of SAC26 pair the D1/D2 band often disappeared (figure 2.3.5): this can be due to primer competition as the two amplicons are generated from the same rDNA region (as previously described SAC26 primers were designed within the D1/D2 region, as well).

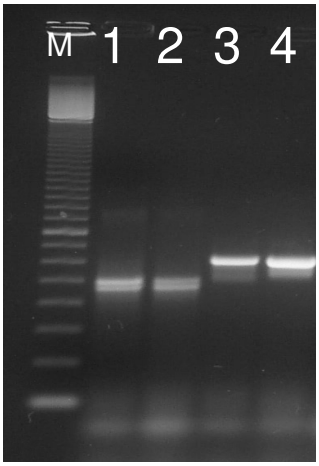


Fig.2.3.5. Gel electrophoresis of amplification products by multipex PCR some strains reported in table 2.2.1. The upper bands are generated by primers NL-1 and NL-4 and the lowers, the *sensu stricto*-specific ones are produced by SAC26F and SAC26R. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) *Saccharomyces cerevisiae;* (2) *Saccharomyces* bayanus*;* (3) *Saccharomyces castellii,* (4) *Hansenispora uvarum*

Successively, the primer pair that amplifies the Internally Transcribed Spacer (ITS) 1 region (White et al., 1990) used for ITS characterization (see par. 2.3.1) was also tested in multiplex PCR experiment with SAC26 primers.

This amplification fragment, although present in all yeasts, produced bands of variable lengths in different species (figure 2.3.6), making interpretation of results less immediate. Considering *Candida stellata* as an example, there is a single amplification band coming from the ITS region (lane 4 in figure 2.3.6), as expected, but its size is very close to the one obtained by SAC26 primers when *Saccharomyces sensu stricto* strains are amplified. In this case the analysis could lead to an ambiguous interpretation of the results.

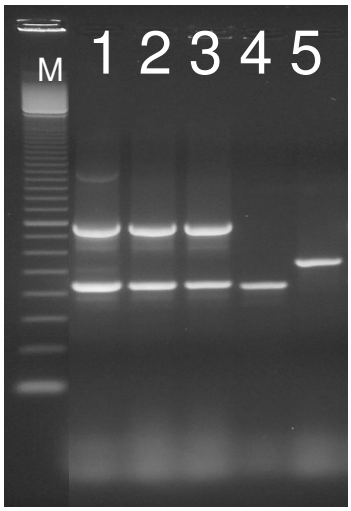


Fig.2.3.6 Gel electrophoresis of multipex PCR amplification products where the upper bands are generated by primers ITS1 and ITS4 and the lower by SAC26F and SAC26R. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) *Saccharomyces cerevisiae;* (2) *Saccharomyces kudriavzevii;* (3) *Saccharomyces mikatae;* (4) *Candida stellata* (5) *Pichia anomala*

Thus, for the construction of the primer pair the 18S rRNA gene, which has a very high conservation level [112] was considered. By CLUSTAL W alignments of the sequences of the 42 strains reported in table 2.2.1, two suitable, perfectly conserved, regions were found from positions 87 to 109 and 962 to 986 of the S. cerevisiae 18S rRNA gene sequence. Based on these *consensi*, primers SAC18F and SAC18R were designed (figure 2.3.7). Genomic DNA amplification of primers SAC18F and SAC18R produced a fragment ranging from 863 to 915 bp in all the strains tested, which could be well separated from the *sensu stricto*-specific band of 471 bp on 1.2% agarose gels.

```
Sch.pombe            AAGTATAAGCAATTTTGTACT-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 98
Sch.japonicus        AAGTATAAGCAACTT-GTACT-GTGAA-CTGCGAATGGCTCATTAAATCAGTTATCGTTT 116
K.lactis             AAGTATAAGCAATTT-ATACATGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 67
Group1               AAGTATAAGCAATTT-ATACA-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 118
Group2               --GTATAAGCAATTT-ATACA-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 56
Group3               AAGTATAAGCAATT--ATACA-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 114
P.membranifaciens    AAGTATAAGCATT---ATACG-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 116
M.pulcherrima        AAGTATAAACATCT--ATACA-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 96
C.stellata           AAGTATAAGCAATCT-ATACA-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTT 117
                       ****** **      ***   ***** ********************************
```

A

```
Sch.pombe            ACTGCGAAAGCATTT-GCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG 985
Sc.japonicus         ACTG-GAAAGCATTTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG 997
GroupB               ACTGCGAAAGCATTT-GTCAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG 961
K.lactis             ACTGCGAAAGCATTT-GNNAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG 987
GroupA               ACTGCGAAAGCATTT-GCCAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG 991
C.stellata           GCTGCGAAAGCGTTT-GCCAAGGACATCTTCATTAATCAAGAACGAAAGTTAGGGGATCG 953
                      *** ******* *** *  *****  * ********************************
```

B

Fig.2.3.7. Alignment of the 18s rRNA gene sequence traits used for construction of primers (in colour) SAC18F **(A)** and SAC18R **(B).** For clarity, yeasts having the same sequence in each trait are grouped. **Group1** includes *Saccharomyces bayanus, S. cariocanus, Saccharomyces cerevisiae, S. kudriavzevii, Saccharomyces paradoxus; S. pastorianus; Saccharomyces mikatae; S. barnetti; S. bulderi; S. servazii; S. transvaalensis; S. kluyveri; S. dairenensis; S. castelli; S. martiniae; S. rosinii; S. spencerorum; S. turicensis;, Hanseniaspora guilliermondii, Hanseniaspora uvarum, Kluyveromyces marxianus, Torulaspora delbrueckii, Zygosaccharomyces bailii, Saccharomycodes ludwigii, Pichia anomala, Rhodotorula bogoriensis, Issatchenkia occidentalis.* **Group2**: *Saccharomyces kunashirensis; S. unisporus;* **Group3**: *Dekkera anomala, Dekkera bruxellensis*
GroupA includes *Saccharomyces bayanus, S. cerevisiae, S. kudriavzevii, S. cariocanus; S. paradoxus; S. pastorianus; S. mikatae; S. barnetti; S. bulderi; S. servazii; S. transvaalensis; S. kluyveri; S. dairenensis; S. castelli; S. martiniae; S. rosinii; S. spencerorum; S. turicensis;, S. kunashirensis; S. unisporus; Kluyveromyces marxianus, Torulaspora delbrueckii, Zygosaccharomyces bailii, Pichia anomala, Pichia membranifaciens, Rhodotorula bogoriensis, Issatchenkia occidentalis* **GroupB** : *Hanseniaspora guillermondii; Hanseniaspora uvarum, Saccharomycodes ludwigii, Meschnikowia pulcherrim*a

2.3.1.5. Multiplex PCR protocol optimization

The multiplex PCR protocol was finally optimized for both temperature and primers concentrations.

Because of an higher efficiency of SAC26, in multiplex PCR with equimolar concentrations of all primers the 18S band was fainter than 26S one. In order to optimize multiplex PCR efficiency, different primer pair concentrations were tested. The optimal balance of 2 µM SAC18F and SAC18R versus 0.2 µM SAC26F and SAC26R (1:10 ratio), gave two amplification bands of similar intensity on agarose gels as shown in figure 2.3.8.

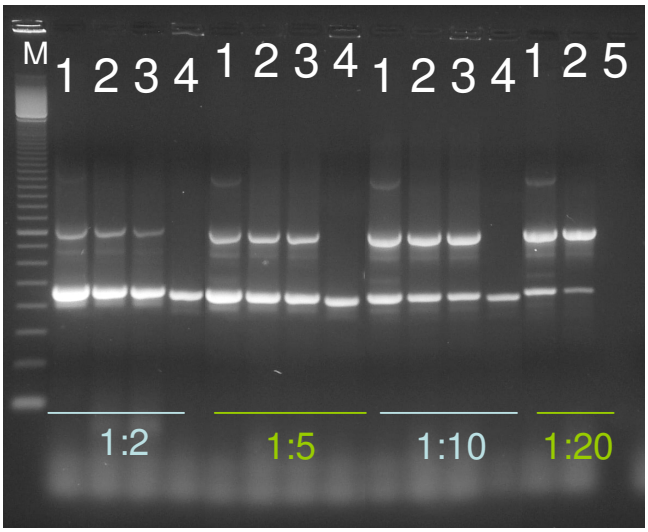


Fig.2.3.8. Gel electrophoresis of multipex PCR amplification products of some *Saccharomyces sensu stricto* strains at different primers concentrations. The upper band is generated by primers SAC18S and SAC18R and the lower one is produced by SAC26F and SAC26R. Ratios in the figure indicate SAC18(2µM):SAC26 concentrations proportion, except for lanes 4 where only SAC26 primers were present. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) *Saccharomyces cerevisiae;* (2) *Saccharomyces bayanus;* (3) *Saccharomyces pastorianus;* (4) *Sachharomyces cerevisiae* (no SAC18 primers) (5) Negative control.

Successively the annealing temperature of the multiplex PCR protocol was lowered to suit the needs of *S. mikatae*, which has three mismatches on the SAC26F primer sequence. It was found that 54 °C was a good compromise, allowing the proper detection of all *sensu stricto* strains, while still maintaining primer selectivity towards other yeasts (figure 2.3.9).

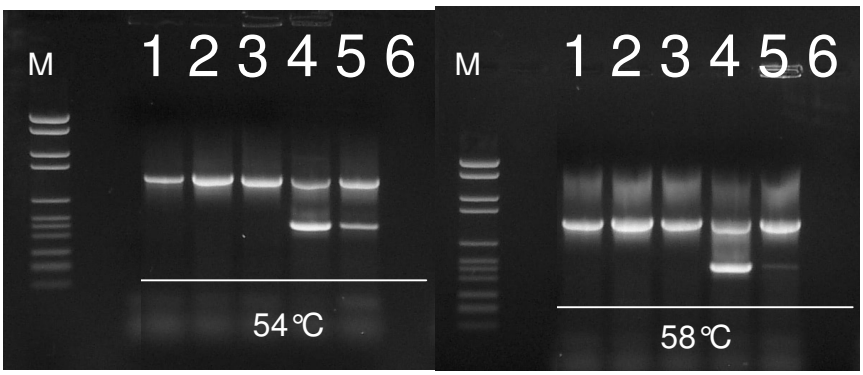


Fig.2.3.9. Gel electrophoresis of multipex PCR amplification products obtained from some of the strains listed in table 2.2.1 at different temperatures. The upper band is generated by primers SAC18S and SAC18R and the lower one is produced by SAC26F and SAC26R. M, molecular weight marker 'VI', Roche. Lanes: (1) *Hanseniaspora uvarum;* (2) *Saccharomyces castellii;* (3) *Saccharomyces barnetti;* (4) *Saccharomyces cerevisiae* (5) *Saccharomyces mikatae* (6) Negative control.

2.3.1.6. Results on enological yeasts

The multiplex PCR optimized protocol has been tested on all the yeast strains listed in table 2.2.1 (type strains of the principal yeast species diffused in an enological environment). The results confirm the selectivity of SAC26 primers in amplifying only *Saccharomyces sensu stricto* yeasts and the efficiency of SAC18 primers on all the strains tested (an example of the PCR amplification is shown in figure 2.3.10).

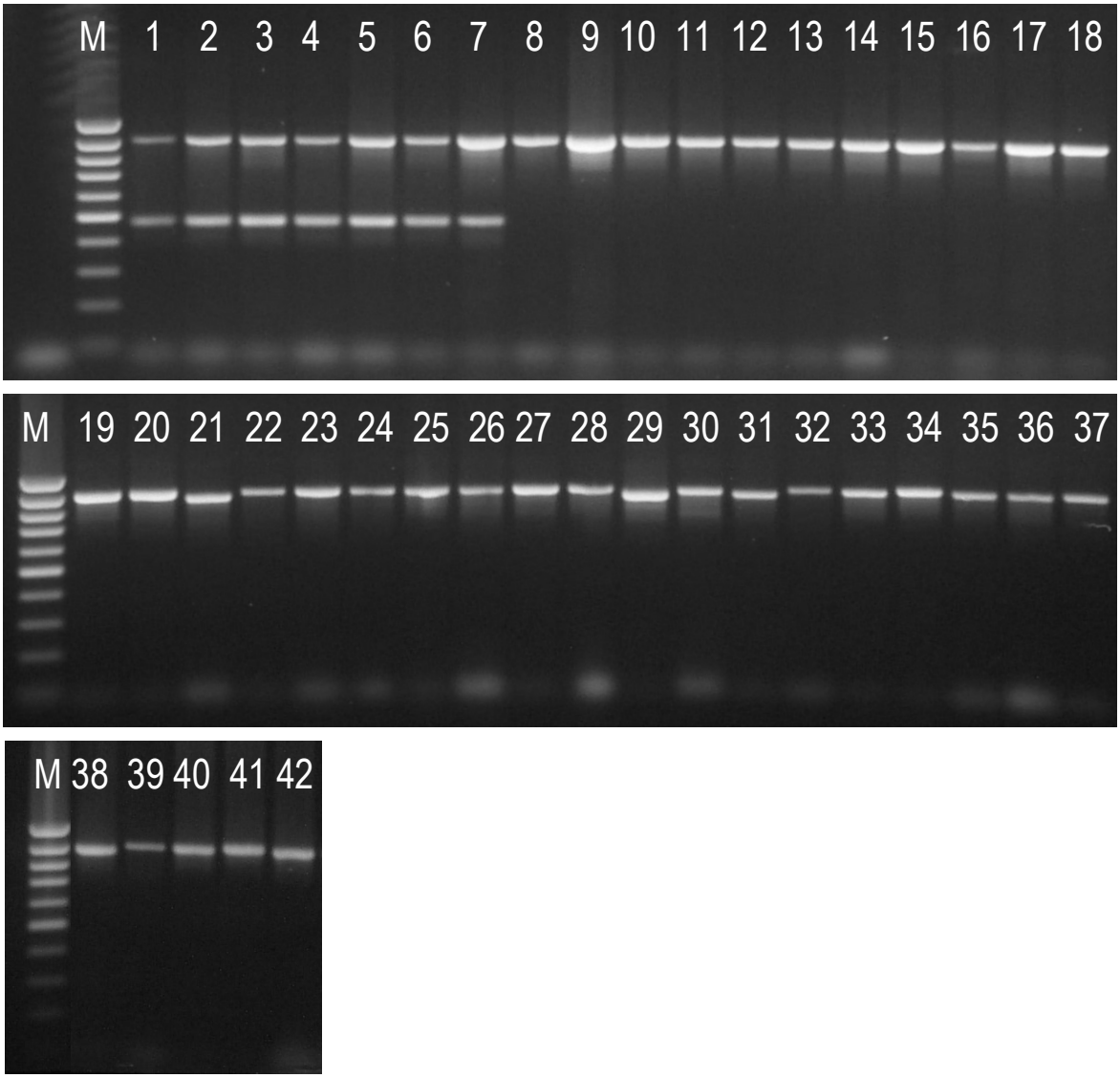


Fig.2.3.10. Gel electrophoresis of multipex PCR amplification products of the strains reported in table 1. The upper common band is generated by primers SAC18F and SAC18R and the lower, sensu stricto-specific one is produced by SAC26F and SAC26R. M, molecular weight marker 'GeneRulerTM 100-bp DNA ladder', Fermentas (the higher band is 1000 bp). Lanes: (1) *Saccharomyces bayanus*; (2) *Saccharomyces cariocanus*; (3) *Saccharomyces cerevisiae;* (4) *Saccharomyces kudriavzevii;* (5) *Saccharomyces paradoxus;* (6) *Saccharomyces pastorianus;* (7) *Saccharomyces mikatae;* (8) *Saccharomyces barnetti;* (9) *Saccharomyces bulderi;* (10) *Saccharomyces servazii*; (11) *Saccharomyces transvaalensis; (*12) *Saccharomyces unisporus;* (13) *Saccharomyces kluyveri;* (14) *Saccharomyces dairenensis;* (15) *Saccharomyces castelli;* (16) *Saccharomyces kunashirensis;* (17) *Saccharomyces martiniae;* (18) *Saccharomyces rosinii;* (19) *Saccharomyces spencerorum; (*20) *Saccharomyces turicensis;* (21) *Candida stellata;* (22) *Debaryomyces carsonii*; (23) *Dekkera anomala;* (24) *Dekkera bruxellensis;* (25) *Hanseniaspora guillermondii*; (26) *Hanseniaspora uvarum*; (27) *Kluyveromyces lactis*; (28) *Kluyveromyces marxianus*; (29) *Metchnikowia pulcherrima*; (30) *Pichia anomala*; (31) *Pichia membranifaciens*; (32) *Rhodotorula bogoriensis*; (33) *Schizosaccharomyces japonicus*; (34) *Schizosaccharomyces pombe*; (35) *Saccharomycodes ludwigii*; (36) *Torulaspora delbrueckii*; (37) *Zygosaccharomyces bailii*; (38) *Issatchenkia occidentalis*; (39) *Saccharomyces naganishii*; (40) *Saccharomyces unisporus*; (41) *Saccharomyces yakushimaensis*; (42) *Saccharomyces exiguus*.

The technique proposed has been successfully tested on hundreds of natural isolates during a yeast selection program in the “Prosecco” area and, in all positive cases, successive analyses have confirmed that all belong to the *Saccharomyces sensu stricto* complex (data not shown).
WL medium is routinely used for yeast colony isolation in enological studies [32]. On this medium, the majority of strains typically found in wine fermentations can be distinguished on the basis of colony colour and/or morphology. *Saccharomyces cerevisiae* and *Saccharomyces bayanus* can be distinguished from other species by having a colony colour from cream to green, a smooth and opaque surface of creamy consistency and a typically knob-like shape [172]. In our tests, all the colonies that corresponded to this description gave positive results when subjected to multiplex PCR analysis using the proposed primers. However, some colonies with different colour or morphologies also produced the 471-bp amplification band, indicating an affinity to sensu stricto strains as confirmed by 26S rRNA gene sequencing (data not shown).

These results confirm the capability of the proposed technique to identify strains that could be missed by conventional screening techniques. Once the *sensu stricto* attribution has been accomplished, a number of published methods are available for species determination, if necessary. As these are normally more laborious, or require more careful interpretation, they can be performed on a reduced number of strains.
Regardless of any past or future taxonomic variations, the procedure proposed in this work allows for a simple and rapid identification of technologically important strains of the *Saccharomyces sensu stricto* complex. Recent acquisitions on the genetics of the genus *Saccharomyces* evidenced that a considerable level of genomic rearrangement occurred between individuals of this genus, particularly within the *sensu stricto* group, leading to the creation of hybrids, as the type strain of *S. bayanus* NRRL Y-12624 ^{T}, generated by an interspecific mating between *S. cerevisiae* and *S. uvarum* [165] or the species *S. pastorianus*, which results to be the hybridization product of *S. cerevisiae* and *S. bayanus* [30]. It emerges therefore that this group of microorganisms should be better seen as a continuum of genome structures, rather than a cluster of separate species [44]. It has also to be added that, due to genetic rearrangements, different taxonomic attributions could be obtained by

analysing different genetic traits, such as the emblematic case of *S. uvarum*, that is still awaiting a proper taxonomic collocation [164].

These findings evidence how problematic the assignment of an isolate to a species could be within this genus, particularly among the *sensu stricto* members. Indeed, from an applicative point of view, it is much more important to be able to individuate strains with relevant technological properties, that are peculiar to all the *sensu stricto* group members, traditionally characterized by having strong fermentative capacity, high transformation efficiency and noteworthy alcohol tolerance.

3. Study of yeast gene expression in natural must fermentation

3.1. Introduction

Wine yeasts are subjected to stress conditions that change continuously during a dynamic process such as alcoholic fermentation, thus they have evolved mechanisms to sense and respond to environmental changes to maintain metabolic activity and cellular integrity [16]. Moreover, the molecular basis of industrial wine yeasts properties is largely unknown. This lack of knowledge limits the definition of specific targets for further genetic improvements in the selection of wine yeasts and for better understanding their behaviour in enological environment.

Among high-throughput analytical methods for exploiting the information of genome sequences, the DNA microarray technology has become a standard tool for the analysis of genome-wide expression profiles (reviewed by Perez-Ortin [175]). Due to utility of DNA chips to investigate how specific genes change their expression within biological processes, several attempts have been made on wine yeasts [11, 31, 62, 94, 223, 223a]. Thus, the knowledge of genetic features as well as the specific expression profiles in different growth conditions of the yeast strains can help us to understand more clearly the biological process of fermentation at molecular level.

Studies on wine yeasts by DNA array analysis have been made using different approaches in relation to growth conditions. The use of standard growing cultures to study the behaviour of enological yeasts enables the comparison of specific features to those of laboratory strains which have already been studied. However this kind of cultures does not efficiently restore the natural environment where wine yeasts are used to develop (such as acid pH, high sugar concentrations, variable nitrogen conditions). With the aim of understanding how yeast strains cope with the enological stressful environment, studies have been performed miming wine-making conditions, using synthetic medium simulating a natural must and anaerobiosis growth [223]. Strong transcriptional reprogramming have been found during alcoholic fermentation, due to changes in nutritional, environmental and physiological conditions. In this work yeast metabolic shifts at transcriptional level was investigated during wine production in industrial conditions, which deeply differ from fermentations performed at a lab scale level.

In this study, DNA-microarray technology has been used to follow the behaviours, during alcoholic fermentation, of two commercial yeast strains widely used in wineries, Lallemand 71B and Lallemand EC1118. These strains have different characteristics and attitudes. The former, 71B, is known to be a strong producer of fermentative aroma and is therefore used in musts lacking varietal flavours or for the production of *nouveau* wines. The latter, EC1118, is an efficient fermenter, widely used also for sparkling wines, but quite neutral as regards both varietal and fermentative aroma.

This experimentation tries to understand the genetic basis that can explain differences in the fermentation behaviours and performances of these well-known enological strains in winery and laboratory conditions. Furthermore it may help in assessing the reproducibility of yeast behaviours during wine-making process, when a laboratory scale is used.

For each strain, two different fermentation settings were carried out: laboratory (1 litre) and pilot (100 litres) scale trials were performed in natural white must. Fermentation kinetics and cell growth have been monitored during all the process and cell transcriptome have been analysed after the entrance to stationary phase .

Firstly, kinetic parameters were analysed to investigate differences between the strains and the “scale up” effect on each strain.

A cross comparison have been constructed to understand, using whole transcriptional board, whether differences in fermentation behaviours were due to volume (1L versus 100L) and/or to genotypic (strain 71B versus strain EC1118) effects. Because of the considerable costs of microarray experiments, mRNAs from fermenting yeasts were collected only once, immediately before entering the stationary phase (45 g/l of overall CO_2 produced). In order to validate the results of DNA-microarray analysis all the gene expression shifts found were confirmed by Real-time PCR.

3.2. Materials and methods

3.2.1. Strains

The strains used in this study are *S. cerevisiae* EC1118 and 71B, two commercial yeasts product as dried powder by Lallemand SA (Toulouse, France) for the enological market.

3.2.2. Fermentations

Four fermentation experiments (each one performed in two replicates) were carried out with natural white must "Viognier 2004", produced by the INRA station of Pech Rouge (France). Clarified must was homogenised and strongly deoxygenated by bubbling pure sterile argon (1L fermenters) or CO_2 (100L fermenters) for 20 min before inoculation. SO_2 was added at a final concentration of 50 mg/l, sugar content was 180 g/l, then the must was put in four glass fermenters (1.1L working volume) and four inox fermenters (100L volume).

Active dried yeasts were rehydrated in 50 g/L glucose at 37°C for 30 min and used to inoculate Viognier must at a concentration of 1 x 10^6 cells/ml. Fermentations were performed under isothermal conditions 20°C, in fermenters equipped with lockers to maintain anaerobiosis.

CO_2 production was automatically acquired every 20 minutes, the rate of CO_2 production was calculated using a polynomial smoothing.

The amount of CO_2 released was determined in 1L fermenters by automatic measurement of fermentor weight loss every 20 min [18]. The CO_2 production rate was calculated by polynomial smoothing of the last ten measurements of fermentor weight loss [18]. In 100L fermenters the CO_2 production rate was directly determined by a mass flow meter measuring the amount of CO_2 released per minute; then the total CO_2 production was calculated by integration.

The number of cells was determined by an electronic particle counter (Beckman Coulter, Fullerton, CA, USA).

3.2.3. Chemical determinations

3.2.4.1. Assimilable nitrogen determination

Assimilable nitrogen content was determined by formaldehyde titration [74].

One hundred ml of pre-filtered sample (in a 200-ml beaker) were neutralized to pH 8.0 using 1 N sodium hydroxide and pH meter. Because of sulfur dioxide presence, 10 ml of barium chloride 1N were added and the suspension was allowed to deposit for 15 minutes. Using a 200-ml volumetric flask the sample was brought to volume with deionized water and mixed. The solution was filtered through Whatman No. 1 filter paper, then a 100-ml aliquot was transferred into a beaker, stirred and calibrated with a pH/reference electrodes. The pH was re-adjusted to 8.0, if necessary. Twenty five ml of previously neutralized formaldehyde (pH 8.0) were then added to the aliquot, mixed, and the resulting solution was titrated to pH 8.0 using 0.10 N sodium hydroxide.

The concentration of assimilable nitrogen was calculated as follows:

mg N/L (NH^{4+} + amino nitrogen) = (ml of 0.1N NaOH titrated) x 28

3.2.4. RNA extraction

3.2.4.1. Total RNA extraction and 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.

Total RNA was extracted using Trizol Reagent™ (Invitrogen Life Technologies, Carlsbad, CA, USA). Samples containing approximately 10^9 cells were pelleted by centrifugation (5000 × *g* for 5 min), resuspended in 400 µl Trizol and broken by vortexing for 4 min with 300 µl glass beads. The total volume was adjusted to 4 ml with Trizol solution RNA extraction was performed following the protocol provided by the manufacturer: after a 5 min incubation at room temperature, 0.8 ml 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 15 000

× *g* for 15 min and the aqueous phase was recovered. The RNA was precipitated by addition of an equal volume of cold (–20 ◦C) isopropyl alcohol and centrifugation at 10 000 × *g* for 10 min, then resuspended in RNase free water.

A maximal amount of 100 µg of total RNA was purified from contaminants (DNA included) using the RNeasy kit™ (Qiagen, Hilden, Germany) following the "Cleanup protocol" in the manufacturer's instructions. The elution step was repeated twice with 35 to 50 µl of RNase free water.

3.2.4.2. RNA quantification and gel electrophoresis

RNA concentration was determined by spectrophotometric analysis (1:250) 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 250 (dil. factor).
The OD ratio 260/280 was also measured.

All the glass equipment used in the following procedures was treated overnight at 180°C (dry owen) before use to remove RNase.
Samples containing 4-5 µg of RNA were resuspended in denaturating loading dye (formamide 30%, formaldehyde 10%, commercial loading dye 15% (Fermentas International Inc., Canada) 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 weigh 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).

3.2.5. Reverse transcription (RT-PCR)

3.2.5.1. Synthesis of labeled probes for microarray hybridization

Fluorescently labelled probes were synthesized by reverse transcription using Cy®3-dUTP and Cy®5-dUTP (Amersham Bioscience AB, Uppsala, Sweden); all other

reagents were supplied by the Pronto cDNA labelling kit (Promega, Madison, WI, USA).
Two reverse-transcription reactions were assembled as follows:

Component	Cy®3	Cy®5
Total RNA or Total RNA Positive Control	10µg	10 µg
Random Primers (3µg/µl)	1µl	1µl
Oligo(dT) Primer (2µg/µl)	1µl	1µl
Nuclease-Free Water to a total volume of (incubation at 70°C for 10 minutes)	20µl	20µl
ChipShot™ 5X Reaction Buffer	8µl	8µl
$MgCl_2$ (25mM)	4.8µl	4.8µl
dNTP mix for Total RNA	2µl	3µl
Cy®3 dCTP (1mM)	1µl	–
Cy®5 dCTP (1mM)	–	1µl
ChipShot™ Reverse Transcriptase	3.2µl	3.2µl
Nuclease-Free Water	1µl	–
final volume	**40µl**	**40µl**

Each reaction was incubated at room temperature (22–25°C) for 10 minutes, then at 42°C for 2 hours, in both cases protected from light. Afterwards, 1.0µl RNase H and 0.35µl RNase Solution were added to each cDNA-synthesis reaction and incubated at 37°C for 15 minutes.

Probes were purified with the nucleotide removal column supplied by the kit according to manufacturer's instructions except for the elution volume that was 75 µl instead of 60 µl.

Probes were quantified by absorbance readings at 260 (for total DNA), 550 (for Cy®3) and 650 (for Cy®5) nm, directly, on undiluted cDNA in microcuvettes (containing max 70µl). The cDNA used for spectrophotometry were then recovered for the hybridization reaction.

3.2.5.2. Synthesis of non-labeled cDNAs for PCR amplification

Non-labelled cDNA were synthesized using SuperscriptII™ reverse transcriptase (Invitrogen Life Technologies) using poliT(16) primers (MWG-biotech, HPSF purified). Each reactions were assembled as follows:

Component	Vol/Q.ty
Total RNA	5µg
Random Primers (0.5µg/µl, Promega)	1µl
Oligo(dT) Primer (0.5µg/µl, MWG)	2µl
Nuclease-Free Water to a total volume of (incubation at 65°C for 10 minutes)	12µl
SuperscriptII™ 5X Reaction Buffer (invitrogen)	4µl
DTT (0.1M, Invitrogen)	2µl
dNTP mix for RNA (Promega)	1µl
SuperscriptII™ Reverse Transcriptase	1µl
final volume	20µl

The reactions were incubated at room temperature (22–25°C) for 2 minutes and at 42°C for 2 hours. Afterwards, 15 µl of sterile NaOH 0.1M were added to each cDNA-synthesis reaction, the tubes were incubated at 70°C for 15 minutes, then the neutralization was performed with 15 µl of HCl 0.1M.

3.2.6. Micro Arrays

3.2.6.1. Hybridization

Eurogentec (Belgium) microarrays slides, where each polymerase chain reaction products or oligonucleotides corresponding to 5,660 genes are spotted in twice, were used. The design of these slides is optimized to reduce cross-hybridization between related sequences [244].

All the hybridization materials, including soak and pre-soak solutions, hybridization solution and all washing solutions were provided by the Pronto!™ kit (Promega).

Slides were immersed in Pronto!™ Universal Pre-Soak Solution and incubated at 42°C for 20 minutes, then washed and transferred to pre-heated Universal Pre-

Hybridization Solution (42°C) and incubate for 15 minutes, washed with water, and dried with argon under pressure.

The two probes labeled with Cy3 and Cy5 were joined and concentrated under vacuum to 10 µl then 30 µl of Pronto!™ Universal Hybridization Solution and 2 µl of boiled salmon sperm (10 mg/ml) were added. This mixture was heated for 5 min at 95 °C protecting samples from light before transferring onto the surface of the printed side of the slide. Slides were covered with a coverslip and placed in a hybridization chamber (Corning, Madison, WI, USA) at 42°C for 14-20 hours.

3.2.6.2. Microarray washing and scanning

After hybridization the slides were immersed in Pronto!™ Wash Solution 1 at 42°C for 1–2 minutes until the cover glass felt from the slide then transferred to a fresh container of Wash Solution 1 at 42°C and incubated for 5 minutes. Afterwards, arrays were transferred to Pronto!™ Wash Solution 2 at room temperature (22–25°C) and incubated for 10 minutes and finally to Pronto!™ Wash Solution 3 at room temperature and incubated for 2 minutes.

The slides were dried with argon under pressure before scanning.

Two microarray experiments were performed for each stage with a swap of Cy3 and Cy5 between samples. The GenePix 4000B microarray scanner (Axon Instruments Inc., Union City, CA, USA) was used and images were analyzed using GenePix Pro software (Axon instruments).

3.2.6.3. Data analysis

Data were log2 transformed for analysis and graphical representation. Analysis of variance were performed for each experiments with the GeneANOVA software [54] available on www.genopole.cnrs.fr. It provides p-values that give the significance of the variation for each gene due to the considered factor (time of sampling) and that is used as an indicator of the strength of the evidence for differential expression. Genes with a p-value lower than 0.05 were considered to be significantly differentially expressed. Gene ontology online software (http://yeastgenome.org/termfinder) was used for cluster analysis by the gene ontology method. The cluster contents were analyzed for enrichment of functional categories using the FunSpec interpreter [219] available online at http://funspec.med.utoronto.ca.

3.2.7. Polymerase Chain Reaction and gel electrophoresis

All the PCR reactions were performed in a PTC200 thermal cycler (MJ Research Inc., MA, USA). All the primers were designed using Primer Select software (from DNAstar, Madison, WI, USA) and synthesized by MWG-Biotech (HPSF purified). A proof-reading Taq Polymerase, its buffer and dNTPs (Amersham Biosciences) were used for all the amplification reactions at the following concentrations:

Component	Final concentration
Primers	2 µM (each)
dNTPs	0,05 mM (each)
Taq polymerase	0,02 U/µl
Buffer	1X
DNA or cDNA	10-100 ng/µl

3.2.7.1. Quality control on cDNAs

Amplification of the gene *APE2* was performed on cDNAs both for checking the reverse-transcription efficiency and for excluding genomic DNA contamination. *APE2* primers are hereby reported:

Primer	Sequence (5'-3')	Melting temp (°C)	Annealing temp (°C)	Amplification on mRNA or cDNA (bp)	Amplification on genomic DNA (bp)
APE2fw	TGCGCATCAATGTAATGTGGAAGCAGAGTA	64.4	60		
APE2rv	TGAAATCAGGTTCCACGGTTAAATCGTAGTGT	64.6	60.3	221	603

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	∞

Amplified samples were run on 1.5% agarose gel containing 0,1 µg/ml of Ethidium bromide. 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).

3.2.7.2. SSU1 promoters amplification

Amplification of the promoters of the gene *SSU1* was performed on purified genomic DNA of both strains. *SSU1*-promoter primers are hereby reported:

Primer	Sequence (5'-3')	Melting temp (°C)	Annealing temp (°C) Ta	Amplification on genomic DNA (bp)
SSU1-16-fw	AGTGGTAAGCGTGGTGTCGGTAAGA	59.8	55.2	1050
SSU1-16-rev	AAGGGGTCAAACTGCCTCGTAAGA	59.1		
SSU1-8-fw	CAGAGATGGGGGTCACAGT	54.1	51.6	920
SSU1-8-rev	TTGCAGGATATGGGAAGC	55.6		

The thermal protocol was designed as follows:

Cycle1 (1x)	95°C	3'
Cycle2 (35x)	95°C	15"
	Ta(°C)	30"
	72°C	1'30"
Cycle3 (1x)	72°C	5'
	4°C	∞

Amplified samples were run on 1 % agarose gel containing 0,1 µg/ml of Ethidium bromide. Electrophoresis and image acquisition was performed as described in par. 3.2.7.1.

3.2.8. Real-Time PCR

3.2.8.1. Amplification

All the Real-Time PCR reactions were performed in an i-Q thermal cycler (MJ-BioRad, Hercules, CA, USA). A ready to use master-mix containing a proof-reading Taq Polymerase, reaction buffer, dNTPs and SybrGreen was used according to the manufacturer's instructions (Bio-Rad). Optimized reactions were performed in 0.5-ml MicroAmp optical plates (Bio-Rad), and each 25 µl reaction mixture contained the following:

Component	Final concentration
Primers	200 nM (each)
MasterMix 2X	1X
cDNA (various dilutions)	5 µl
Water to final volume of	25 µl

All the primers were designed using Primer Select software (DNAstar) and synthesized by MWG-Biotech (HPSF purified). Primers were constructed to amplify a 150bp fragment with an annealing temperature of 60°C; primers characteristics are summarized in the following table:

Primer	Sequence (5'-3')	Melting temp (°C)	Annealing temp (°C)	Amplification on cDNA (bp)
ACT1-Q-fw	AATGCAAACCGCTGCTCAATCTTCTTCA	65,4	60	142
ACT1-Q-rv	AATACCGGCAGATTCCAAACCCAAAACAG	66		
FBA1-Q_fw	CTCCATTGCTGCTGCTTTCGGTAACTGT	65,1	60	153
FBA1-Q_rv	GAACCACCGTGGAAGACCAAGAACAATG	65,1		
ATF1-Q_fw	TGGCTTAGGGTTCAATATACAAGGCTTCGTTC	65,4	60,1	153
ATF1-Q_rev	GAATATTTGGCATCGGGCTCCTCTAACTGAT	65,6		
ATF2-Q_fw	TACGCCTATCTAATCTCCTCATTCGACATTCC	64,1	60	149
ATF2-Q_rev	ATAAAATCAAGTTGTAGGACCCCCAGACCAAT	64,2		
EHT1-Q_fw	CGATCATCCTCCCACAGTCAAGAATCCAT	65,7	60,4	160
EHT1-Q_rev	GCTGGCCGCTTTATAATACTCCATAGCATTTG	65,6		
IAH1-Q_fw	TTCCGTACCAACGAGAACTTTGCCATTTATTC	66,0	60,3	157
IAH1-Q_rev	TTCCGGAAAAGTGCAGTCCATCTGTTAGC	65,7		
EEB1-Q-fw	GCAACGGATGATCCAGTTACAGGTGAAAAC	65,1	60	151
EEB1-Q_rev	CGGCAGCTTGCTTTGTTAACCAGGAAT	64,9		
MET10-Q_fw	GTACACCCGTAACTGCCATTTCATCTGTGC	65,3	60.3	148
MET10-Q_rev	AATGGCTTCCCACGTGATTCGTTACCA	65		
MET17-Q_fw	GCCAAGAGAACCCTGGTGACAATGCTC	64,2	59,8	141
MET17-Q_rev	GGAAACGGGAATAGACGTAACCTGGAACTTCT	64,1		
BAT1-Q_fw	CAAAACGAACCCGGTGTCTTGTCCAGAGT	65,2	60	137
BAT1-Q_rev	TGCCCTTGCAAAACAATGGTCATTCTACTTAG	65,1		
BAT2-Q_fw	TAAATGGGGTACCGGTAGTGTTAGACCTGAAC	65,3	60,2	152
BAT2-Q_rev	GCAAATTGTGGCTTGATTGGAGATAAGTCTAA	64,4		
SSU1-Q_fw	TTTGCGTTTGTTGGTCAATTCTATGCCTTTTA	65,5	60,2	151
SSU1-Q_rev	TCCACGCTTTCAATGCTGTTATACGGAGAA	65,8		

Each reaction was performed in triplicate, the thermal protocol was designed as follows (* indicates fluorescence acquisition at the end of this step):

Cycle1 (1x)	95°C	3'
Cycle2 (35x)	95°C	15"
	60°C	45" *
Cycle3 (1x)	72°C	5'
	4°C	∞

3.2.8.2. Data analysis and statistical validation

The threshold cycle (CT), described as the first PCR cycle where fluorescence level is higher then the background, was determined automatically by using sequence detector software iCycler IQ user (version 3.2; Bio-Rad). When threshold value was manually adjusted, i-Q user software was forced to recalculate threshold cycles.

The $2^{-\Delta\Delta CT}$ method to calculate relative changes in gene expression determined from real-time quantitative PCR experiments was chosen [124]. The fundamental equation from which the all the $2^{-\Delta\Delta CT}$ method was derived is:

$$X_n = X_0 \times (1 + E_X)^n,$$

where X_n is the number of target molecules at cycle n of the reaction, X_0 is the initial number of target molecules. EX is the efficiency of target amplification, and n is the number of cycles. For amplicons less than 150 bp and for properly optimized primers and Mg^{2+} concentrations, the amplification efficiency is assumed to be close to one; therefore, the amount of target, normalized to an endogenous reference (internal control gene), reaction is calculated as: amount of target = $2^{-\Delta\Delta CT}$.

During analysis of real-time PCR data, before calculating any expression ratio, a statistical validation was performed to assess significance of ΔCt variations where ΔCT is expressed as the difference in threshold cycles for target (X) and reference (R) (CT,X - CT,R).

In ΔCT calculation the variance estimated from the replicate CT values is carried through to the final calculation of relative quantities using standard propagation of error methods [124]. After ΔCt calculation, values of ΔCt for the same target coming from biological relicates of the same sample were averaged. To determine if differences between ΔCT in several samples were significant the t-Student test for means was applied:

$$t = (\overline{X}_A - \overline{X}_B) \cdot \sqrt{\frac{n_A \cdot n_B \cdot (n_A + n_B - 2)}{(n_A + n_B) \cdot (S^2_{X_A} + S^2_{X_B})}}$$

Where $\overline{X}_A$, $\overline{X}_B$ are mean values of ΔCt in the two samples, n_A, n_B the number of replicates, $S^2_{X_A}$, $S^2_{X_B}$ are variances.

After ΔCt validation, the ΔΔCt was calculated as follows:

$\Delta\Delta Ct = (\Delta Ct_A - \Delta Ct_B)$

where ΔCt_A is the difference in threshold cycles for target(X) and reference (R) genes in strain A and ΔCt_B is the difference for target(X) and reference (R) in strain B.

Finally, expression ratios between the two strains for the target gene (X) were calculated with the $2^{-\Delta\Delta CT}$ formula.

3.2.9. DNA extraction and preparation for sequencing

3.2.9.1. DNA extraction

Yeast DNA was extracted and purified using *MasterPure™ Yeast DNA Purification Kit* by EPICENTRE (Madison, WI, USA) according to manufacturer's instructions. Three hundreds µl of Yeast Cell Lysis Solution were added to each microcentrifuge tube containing the collected pellet. Cells were suspended by either vortex mixing or pipetting repeatedly using a 1 ml capacity pipet tip. Samples were incubate at 65°C for 15 minutes, then placed on ice for 5 minutes. Hundred and fifty µl of MPC Protein Precipitation Reagent were added and samples were vortexed mix for 10 seconds. Cellular debris were pelleted by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm, the supernatant was transferred to a clean microcentrifuge tube and 500 µl of cold isopropanol were added. After a thorough mix by inversion, the DNA was precipitated by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm, the supernatant was removed and discarded. Washes of pellet containing the DNA were done using 0.5 ml of 70% ethanol. Afterwards ethanol was carefully removed and the DNA was suspended in 35 µl of TE Buffer and stored at -20°C to 4°C.

3.2.9.2. DNA preparation for sequencing

DNA sequencing (Sanger reaction with fluorescent nucleotides and capillary electrophoresis run and analysis) was performed by BMR-genomics (Padova, Italy).

PCR products to be sequenced were purified from primers and short polynucleotides by ExoSap™ Cleanup method (usb-United States Biochemical, USA) just adding 1µl

of ExoSap enzyme to 5µl of PCR products and incubating at room temperature for 30 minutes. DNA was then quantified by gel electrophoresis using Kodak Edas 2.9 software which compares band intensities to those of a size ladder having a known concentration.

Then purified DNA was prepared for sequencing, following BMR-genomics instructions (available on www.bmr-genomics.it) hereby reported:

Component	Q.ty
PCR product (ExoSap purified)	10to20ng for 100bp of product length
Primer	3.2 pMol

The DNA mixture was dried under vacuum and sent for sequencing.

3.3. Experimental design

The experimental design for microarray assay was a balanced latin square, as shown in figure 3.3.1. Four experimental factors have been considered, the yeast strains (EC1118 and 71B) growing in two fermentation volumes (1L and 100L) and a cross comparison has been constructed to understand, using whole transcriptional board, whether differences in fermentation behaviours were due to volume (1L versus 100L) and/or to genotypic (strain 71B versus strain EC1118) effects. For each situation, two fermentations (independent biological replicates) were carried out and finally eight microarray hybridizations have been performed: four to compare one yeast in both volumes and four to compare the two yeasts in the same volume. Each cDNA sample have been labelled once in red (Cy5) and once in green (Cy3) to perform a dye swap.

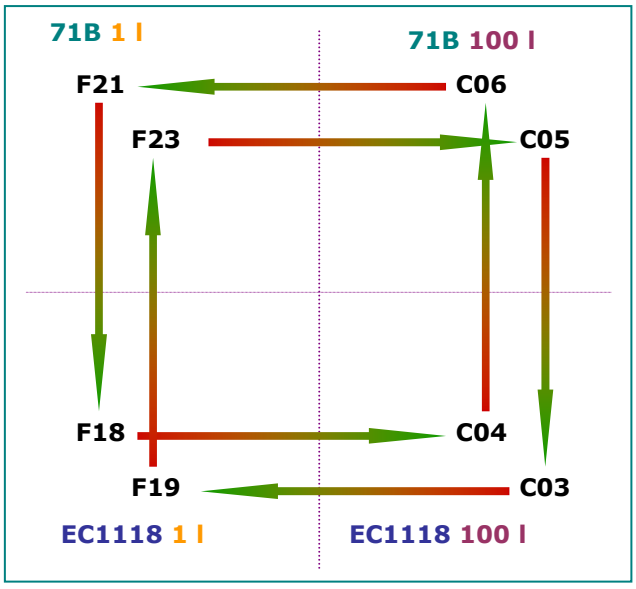


Fig. 3.3.1 Experimental design of microarray experiment. Each arrow represents one slide, the tip indicates green labelled sample (Cy3), the back indicates red labelled sample (Cy5).

3.4. Results and discussion

3.4.1. Fermentation kinetics

The gene expression of the commercial strains EC1118 and 71B throughout the alcoholic fermentation in a natural white must (Viognier 2004) containing 175 g/L of glucose under strict anaerobiosis conditions was monitored; the higher expected amount of released CO_2 was at 76 g/L of residual sugars. The fermentation profiles for both strains was determined, cell number was monitored throughout the process.

3.4.1.1. EC1118

The EC1118 strain growth curve in 1L fermenters (figure 3.4.1) shows an initial lag phase of around 20 hours, then the yeast entered in a quite slow exponential growth phase (60 hours) and about 40% of the sugars were fermented when the cells reached the stationary phase. The fermentation rate (dCO_2/dt) reached its maximal value (1,1 ± 0,1 g/L/h at 60h) before to entry the stationary phase and gradually declined thereafter until the end of the fermentation, when sugar reserves were exhausted and ethanol concentration had reached 10% (v/v). Final development of CO_2 did not reach the maximal expected value of 76 g/L, this indicated that the fermentation was not completely concluded (the process is considered to be off when the wine contains less than 2 g/l of residual sugar) leaving 8.5-9 g/l of residual sugars. The accumulation of ethanol followed the same time course, as well as the cumulative release of CO_2. Final population was around 60 millions of cells per ml.

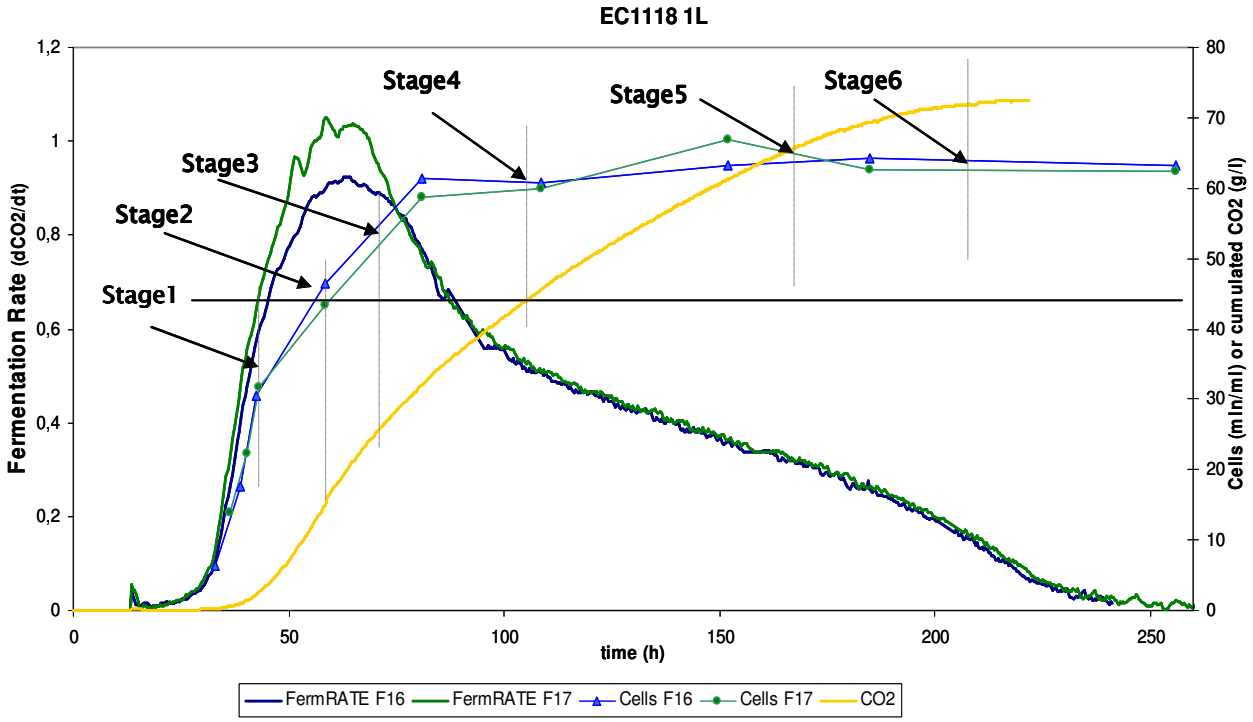


Fig.3.4.1 Fermentation kinetic of EC1118 yeast strain in 1L of Viognier must. The six samplings of the time-course for RNA extraction are indicated by arrows and dotted lines. F16 and F17 are two independent fermentations. Circles (or triangles) on growth curves represent cell count sampling points.

Comparing EC1118 fermentation rate in 1L and 100L (figure 3.4.2) the first outstanding evidence is that this strain seems to be really affected by the scale-up process: lag phase is shorter in 100-litres volume than in 1 litre but maximal speed is halved and then decreases slowly; total fermentation time increases from 250 h in 1liter to 350 hours in 100 litres.

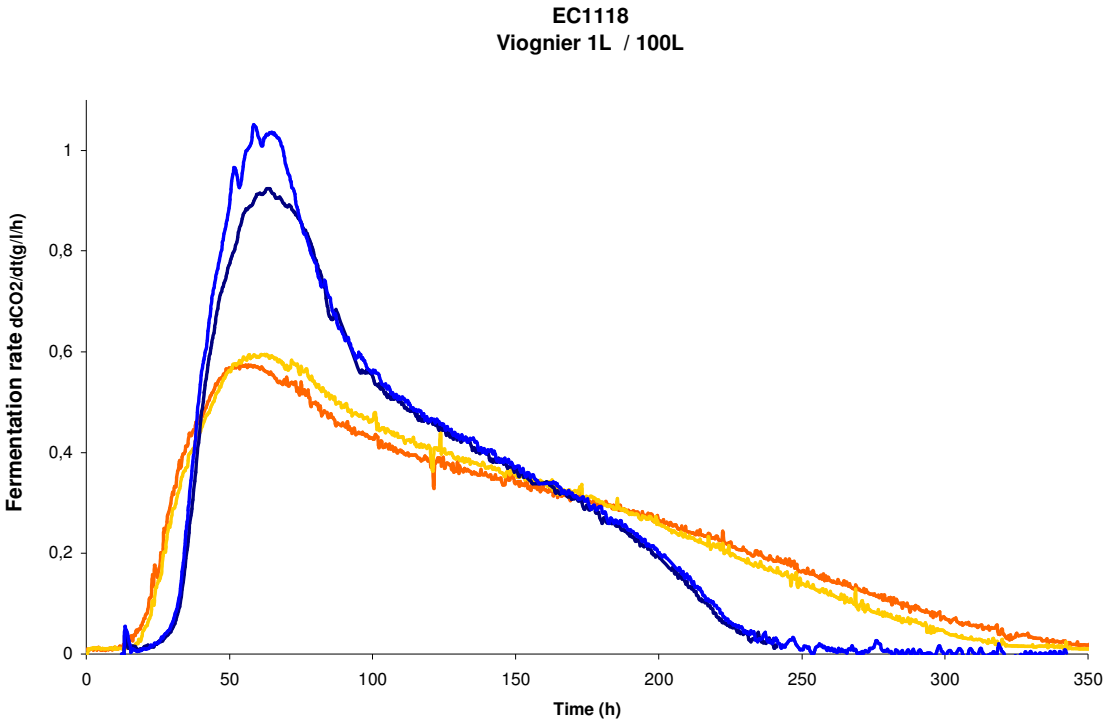


Fig.3.4.2 Fermentation kinetic of EC1118 yeast strain in 1L and 100L of Viognier must. Red curves represent fermentation rate obtained in two independent 100L fermenters, blue curves represent fermentation rate obtained in two independent 1L fermenters.

It is important to remark that either 10 days (250h) or 15 days (350h) fermentations are generally rejected in normal productive condition. Although 250h period is too long, it can be accepted if sugars are totally fermented and the last part of fermentation is not going slowly. In any case, the obtained fermentation time was unexpected from an efficient strain, such as EC1118. The long-lasting fermentation and the presence of residual sugars after the fermentation stopped can be explained by the absence, in these experiments, of some widely diffused enological treatments, such as the addition of an assimilable nitrogen source at the beginning of the process and brief oxygenations. Moreover, the high clarification level of the used Viognier must and sulphite addition could have negatively affected the yeast development.

3.4.1.2. 71B

The growth curve of 71B strain in 1L fermenters (fig.3.4.3) presented an initial lag phase longer than EC1118 (40 hours), then this strain entered in a shorter exponential growth phase (50 hours). The fermentation rate (dCO_2/dt) reached its maximal value (0,75 ± 0,08 g/l/h at 67h of fermentation) before to entry the stationary phase and gradually declined thereafter until the end of the fermentation, when

ethanol concentration had reached 10% (v/v). The maximal fermentation rate was lower than EC1118. Final CO_2 development did not reach the maximal expected value, indicating that, even in this case, the fermentation was not completely concluded (the final wine sugar content was 9-10 g/L after 260h). Final population was around 50 millions of cells per mL, a value lower than the one found for EC1118 strain (70 millions per mL).

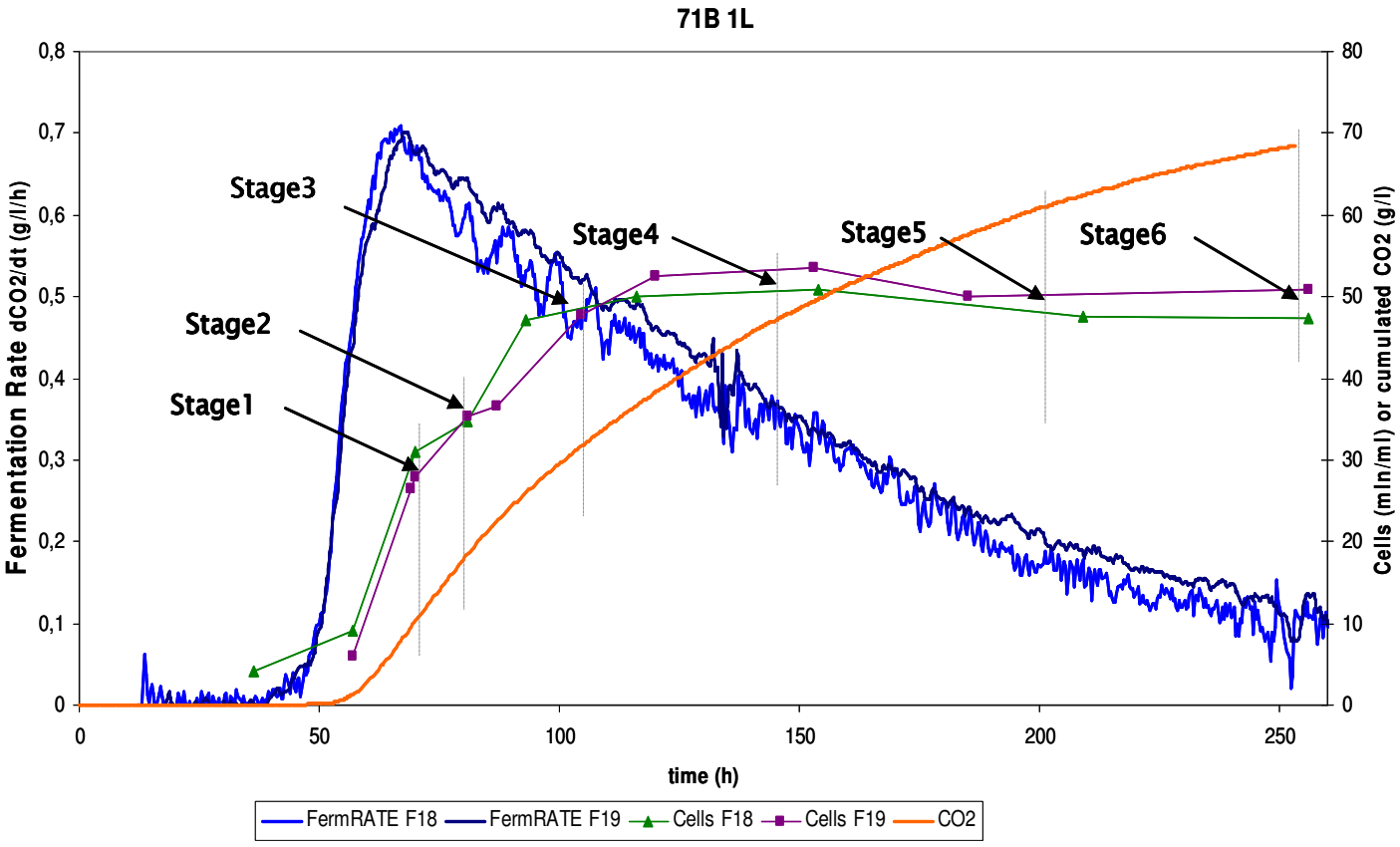


Fig.3.4.3 Fermentation kinetics of 71B yeast strain in 1l of Viognier must. The six samplings of the time-course for RNA extraction are indicated by arrows and dotted lines. F18 and F19 are two independent fermentations. Squares (or triangles) on growth curves represent cell count sampling points.

Comparing 71B fermentation rate curves obtained in 1L and 100-litres volume the strain show a similar behaviour (fig.3.4.4). There is a reduction in the length of the lag phase at 100 litres that may be due to a quicker adaptation to the larger volume (the dispersion and diffusion of the inoculum could not be efficiently managed when must volumes is considerable), but the maximal rate of fermentation and slope of the

fermentation rate curve are almost identical. Due to the shorter lag phase, in this case, total fermentation time decreases from 300 h (in 1liter volume) to 250 hours (in 100-litres volume).

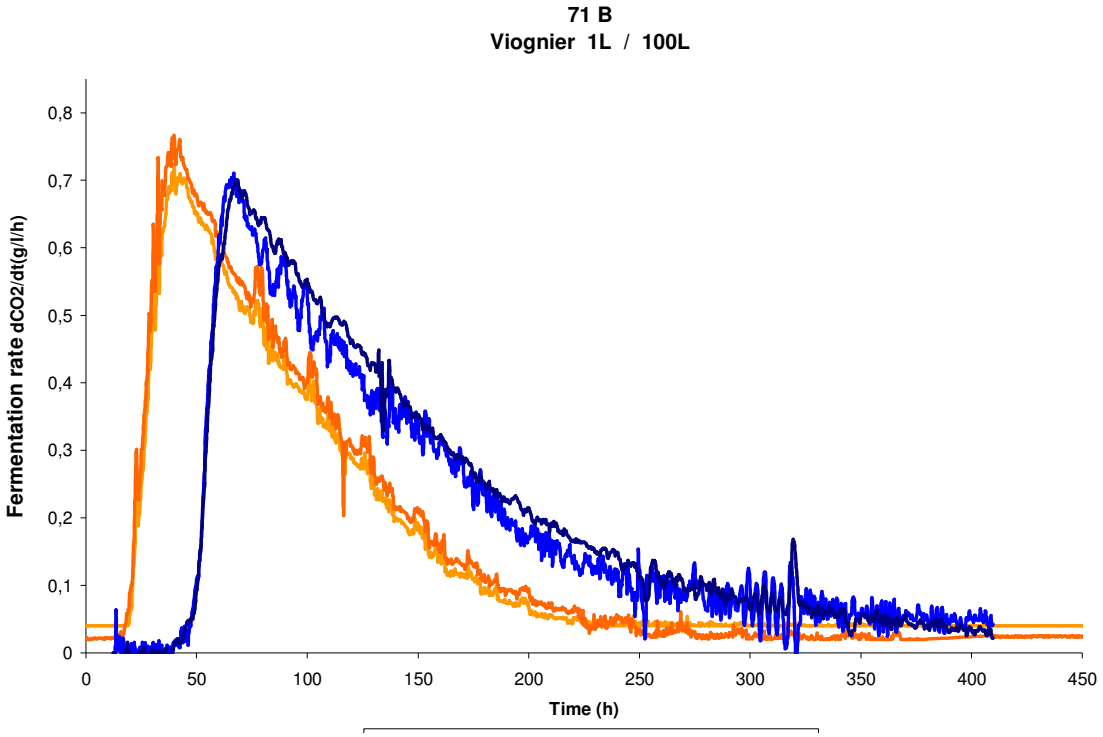


Fig.3.4.4 Fermentation kinetics of 71B yeast strain obtained in 1L and 100L of Viognier must. Red curves represent fermentation rate in two independent 100L fermenters, blue curves represent fermentation rate in two independent 1L fermenters.

Global gene expression profiles were examined for both strains when a particular amount of total CO_2 released was reached during the fermentation process: the use of this parameter instead of fermentation time for choosing the right sampling time allows a normalization of the fermentation kinetics, which is closely linked to the disappearance of the substrate from the medium. For this reason samples were collected at 45 g/l of total CO_2 released: at this point yeasts were entered the stationary phase recently, they were no longer proliferating and ethanol concentration was 6% (v/v). RNA was extracted and used to synthesize labelled cDNA for microarray gene expression profiling.

For further time-course analysis, concerning just the most interesting genes that outcome from DNA-microarray experiments, yeast cells were collected at six different

stages of the fermentation process (table 3.4.1). Sampling at stage 1, which corresponds to the start of the growth phase, was performed when the growth curve reached 30 millions of cells each ml. All the other samples were collected when the CO_2 production reached particular values during fermentation progress. In the first two stages the cells were actively growing, while the stage 3 is characterized by a slowing of the cell growth due to the recent enter into the stationary phase. At stages 4–6, the cells were no longer proliferating and ethanol concentration was increased.

Fermentation stage	**1**	**2**	**3**	**4**	**5**	**6**
CO_2 (g/L)	0	**15**	**30**	**45**	**60**	**70**
Ethanol (v/v)	0,0	2,2	4,1	6,0	7,9	9,1
EC1118 cells ($x10^6$/ml)	**30**	46,4	57,8	60,8	63,2	62,6
71B cells ($x10^6$/ml)	30	47,2	50,2	51,2	48,5	48,5

Tab.3.4.1 Fermentation parameters measured at each cell sampling for the time-course analysis. At stage 4 (in red) the sampling for the global gene expression analysis was performed.

3.4.2. Gene expression results

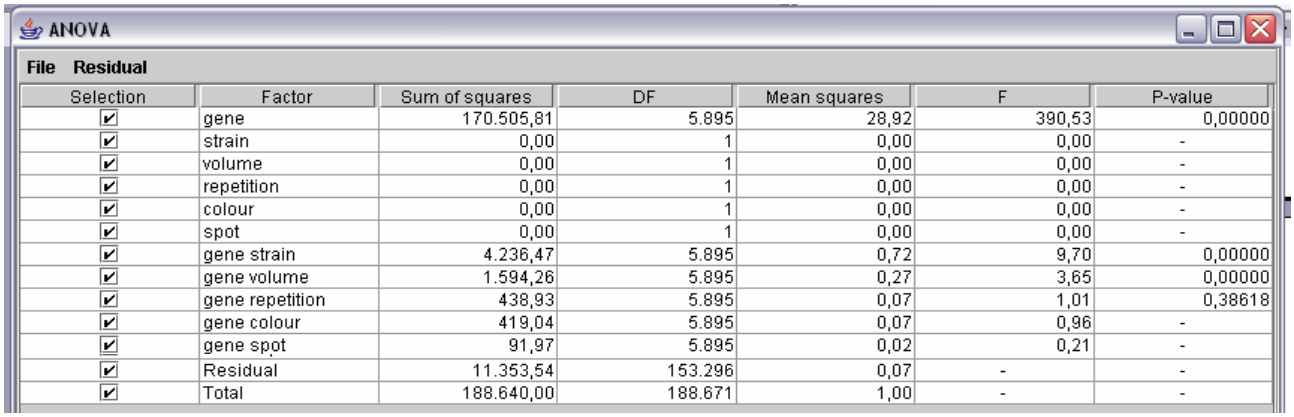
3.4.2.1. ANOVA

After microarray hybridization, slides scanning and data collection, statistical analysis on microarray data (intensities of red and green light for each spot and background intensities) was based on ANOVA. No normalization or background subtraction were done because of the ANOVA requisite concerning the total independence among data. For this reason the GeneScan output data set (fluorescence in arbitrary units) was directly submitted to the statistical analysis.

Firstly, the Principal Component Analysis on raw data from all arrays was carried on and showed that the most important variation sources were strain (4,8%) and volume (2,6%) dependents.

Successively the Latin square representing experimental design (see fig.3.3.1) was imported in GeneANOVA software to perform a global ANOVA analysis on the whole data set. Known sources of variation were set as follows: yeast strain, fermentation volume, biological replicate, fluorescent dye (colour) and spot (technical replicate on

the slide). For each one of the 5896 genes spotted in double on *S. cerevisiae* microarrays, data, coming from the 8 slides, were treated together. Fluorescence values were transformed to log2 intensities and for each array red and green data set were centre-reduced (assuming a gauss curve representing the 5896 genes, the mean value was set to zero); the ratios used in tables and graphics correspond to averaged log2 value after subtraction.


ANOVA

File Residual

Selection	Factor	Sum of squares	DF	Mean squares	F	P-value
☑	gene	170.505,81	5.895	28,92	390,53	0,00000
☑	strain	0,00	1	0,00	0,00	-
☑	volume	0,00	1	0,00	0,00	-
☑	repetition	0,00	1	0,00	0,00	-
☑	colour	0,00	1	0,00	0,00	-
☑	spot	0,00	1	0,00	0,00	-
☑	gene strain	4.236,47	5.895	0,72	9,70	0,00000
☑	gene volume	1.594,26	5.895	0,27	3,65	0,00000
☑	gene repetition	438,93	5.895	0,07	1,01	0,38618
☑	gene colour	419,04	5.895	0,07	0,96	-
☑	gene spot	91,97	5.895	0,02	0,21	-
☑	Residual	11.353,54	153.296	0,07	-	-
☑	Total	188.640,00	188.671	1,00	-	-

Fig. 3.4.5. Variation sources ranking by geneANOVA.

ANOVA provides p-values that give the significance of the variation for each gene due to the considered factor (strain or volume). It is used as an indicator of the strength of the evidence for differential expression. Genes with a p-value lower than 0.05 were considered to be significantly differentially expressed.

This analysis allowed to find out among all the 5,660 genes occurring on the array, the ones over or under-expressed by each strain or in small/large volume conditions.

3.4.2.2. Fermentation volume and anaerobiosis stress

Considering the “volume” effect, it appears to be generally linked to a small number of genes.

In table 3.4.2 are reported the genes involved: the over-expressed ones in 100-litres volume by both strains are listed on the left, the down regulated in 100-litres volume by both strains on the right. Genes were sorted by ANOVA with a cut-off on expression ratio (1lL: 100L or 100L:1L) higher that 1.75 and a filter on p-value lower than 0.05.

Gene	Ratio 100L : 1L		Gene	Ratio 100L : 1L	
	EC1118	71B		EC1118	71B
BSD2	1,95	1,65	ECM13	0,55	0,34
YDL010w	2,49	1,57	YDL037c	0,31	0,32
YJL149w	1,70	2,02	KRS1	0,56	0,52
YDR250c	1,96	1,24	YDR492w	0,41	0,33
YER187w	2,30	1,28	ERG28	0,49	0,49
YFR022w	2,23	1,16	FCY2	0,55	0,31
YGL046w	1,91	1,62	HSP12	0,48	0,32
TOS3	1,86	2,48	VHT1	0,49	0,39
PDE1	2,21	1,44	ERG1	0,50	0,37
MTL1	1,91	1,49	HXT4	0,50	0,53
SPS100	2,78	2,98	NCA3	0,48	0,53
YJL051w	3,18	1,31	YLL012w	0,43	0,35
SPG1	1,71	2,02	ACS2	0,41	0,38
YLR099w-a	1,89	1,37	YML093w	0,46	0,53
SCW10	2,13	1,76	ERG5	0,44	0,29
YIP3	1,88	1,69	ALD3	0,48	0,32
MET14	1,59	2,39	CYB5	0,31	0,34
YNL144c	1,98	1,32	DBP2	0,44	0,33
PLB3	2,35	1,89	ALD6	0,52	0,40
CSR2	1,92	2,11	ROX1	0,54	0,40
			GPH1	0,51	0,46

Tab. 3.4.2. Genes sorted by volume effect in both strains: expression ratio 100L:1L or 1L:100L >1.75 at least in one strain and p-val<0.05.

Unfortunately, most of the up-regulated genes in 100-litres volume (11/20) are associated with unknown function or biochemical process, and no correlation, among these 20 genes, is possible by gene clustering.

Gene clustering analysis on over-expressed genes in 1-litre volume condition, on the contrary, pointed out the up-regulation of the sterols and steroids biosynthetic pathway. This pathway is linked to anaerobiosis stress response and a difference in oxygen availability between the two sizes of fermenters could explicate this phenomenon (in the experimental practice, strong anaerobiosis was easier to manage in 1 -litre fermentations than in 100 litres).

Interestingly, there are some genes that were not sorted by the ANOVA, when the “volume” effect is considered, but, most properly, their expression was controlled by a change of volume. In one strain these genes were over (or under) expressed only

when they are coped with scale up effect, otherwise in the same condition they were repressed or activated by the other strain.

Gene	Ratio 100L : 1L		Gene	Ratio 100L : 1L	
	EC1118	71B		EC1118	71B
PAU7	0,56	2,48	UGA4	2,25	0,83
DAN3	0,38	2,60	DAL80	2,16	0,37
YCL026c-b	0,62	2,20	MEP2	2,61	0,28
PAU2	0,33	2,28	PUT4	2,27	0,37
YER181c	0,62	2,06	YOL128c	1,74	0,44
PAU5	0,27	2,40			
YGL261c	0,42	2,48			
YGR146c	0,67	3,51			
YGR294w	0,44	2,31			
YHL046c	0,61	2,54			
YIL176c	0,40	2,05			
YIR020w-a	0,60	1,93			
YIR041w	0,40	2,56			
RNR2	0,69	1,95			
PAU1	0,38	2,43			
YKL224c	0,18	2,47			
YLL025w	0,36	2,32			
YLL064c	0,40	2,74			
DAN2	0,38	2,01			
PAU4	0,31	2,26			
YMR325w	0,30	2,13			
PAU6	0,43	2,87			
RPS15	0,73	1,93			
YOL161c	0,31	3,87			

Tab. 3.4.3. Genes sorted by volume effect in each strains. Expression ratios (R) 100L:1L values are: R>1.75 in 71B and R<0 in EC1118 in the three columns on the left, R>1.75 in EC1118 and R<0 in 71B in the three columns on the right; p-value<0.05. In blue genes induced by anaerobiosis.

In the left part of table 3.4.3 are listed the genes up-regulate by 71B when the larger volume is considered, and down regulated by EC1118 in the same condition. As expected, most of these genes are stress responsive elements, or anaerobic responsive genes (indicated in blue in tab.3.4.3) belonging to *DAN/TIR* or *PAU* family or heat shock proteins, known for being highly expressed during alcoholic fermentation [1, 196]. The difference among the expression levels of these stress responsive genes in the two strains could help to explicate the great diversity in the fermentation kinetics previously described: EC1118 did not reach the same fermentation performance when the fermenter capacity raised up to pilot fermentation and seemed to be strongly affected by volume change in this experiment.

In general, the most part of known genes sorted by the “volume change” effect are in some way linked to anaerobiosis stress or general stress response, indicating different fermentation conditions between the two volumes or different strategy of yeast adaptation to environment. Some possible causes that can produce disparities in experimental conditions, moreover, were known *a priori* (but not eliminable because they depend on the enological equipment used to obtain de-oxygenation and to add sulphite). In any case, the basal metabolism of both yeast strains (energy, fermentation, transport, anabolic and catabolic pathways except for sterols synthesis) seems not to be strongly affected by the volume change, suggesting that scale up process does not cause any whole transcriptional reprogramming in yeast cells (at least at this stage of fermentation) and that gene expression studies on yeasts on small scale fermentations are well-representative of what happens in larger volume vinification.

3.4.2.3. Strain effect: an overview on genes expressed at higher in EC1118

Filtering microarray data set for “strain effect” provided a number of genes much larger than “volume” effect: 277 genes with different expression were globally found (sorted by ANOVA with cut-off ratio>1.75 and p-value<0,05, with the same parameters only 76 genes were found to be linked to volume change). Hundred and forty six out of 277 genes were expressed at higher level in EC1118 and 131 genes in 71B. This two groups of genes were sorted out by ANOVA analysis independently from volume effect.

Submitting the list of genes expressed at higher level in EC1118 (146 genes) to gene clustering some biological function categories came out, as listed in table 3.4.4.

EC1118 vs 71B ratio>1.75, pVal<0.05

Gene Ontology term	Cluster frequency	Genome frequency of use	P-value	Genes annotated to the term
ATP metabolism |	8 out of 146 genes, 5.4%	20 out of 7291 annotated genes, 0.2%	9,64E-09	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
nucleoside phosphate metabolism	8 out of 146 genes, 5.4%	20 out of 7291 annotated genes, 0.2%	9,64E-09	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
purine nucleoside triphosphate biosynthesis |	8 out of 146 genes, 5.4%	22 out of 7291 annotated genes, 0.3%	2,00E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
purine ribonucleoside triphosphate biosynthesis |	8 out of 146 genes, 5.4%	22 out of 7291 annotated genes, 0.3%	2,00E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
proton transport |	8 out of 146 genes, 5.4%	24 out of 7291 annotated genes, 0.3%	3,87E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
hydrogen transport |	8 out of 146 genes, 5.4%	24 out of 7291 annotated genes, 0.3%	3,87E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
ribonucleoside triphosphate biosynthesis |	8 out of 146 genes, 5.4%	24 out of 7291 annotated genes, 0.3%	3,87E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20
ribonucleoside triphosphate metabolism |	8 out of 146 genes, 5.4%	24 out of 7291 annotated genes, 0.3%	3,87E-08	ATP3, ATP16, INH1, ATP5, ATP7, ATP14, ATP18, ATP20

Tab. 3.4.4. Results of gene ontology clustering for genes higher expressed in EC1118.

The finding that seven *ATP* genes (encoding for 7 of the 23 subunits of the F1/F0 ATP synthase, a large enzyme complex required for ATP synthesis) and *INH1* (encoding for an inhibitor of ATP hydrolysis by F1/F0) were differently regulated in the two strains was quite astonishing since in fermentation conditions no aerobic respiration was supposed to exist. Therefore it is not easy to interpret these results. The only evidence is that no other genes associated with aerobic respiration were found to be more expressed in EC1118, this could suggest that ATP genes higher expression in this strain may be linked to ATP needs or to proton homeostasis and to maintain the membrane proton motive force in the mitochondria rather than to a respiration process *tout court.*

3.4.2.4. Strain effect: an overview on genes expressed at higher in 71B

Submitting the list of genes expressed at higher level in 71B than in EC1118 (131 genes) to gene clustering, the most important biological function categories found were those of amino acids metabolism (as listed in table 3.4.5).

71B vs EC1118 ratio>1.75 pVal<0.05

Gene Ontology term	Cluster frequency	Genome frequency of use	P-value	Genes annotated to the term
amino acid and derivative metabolism |	20 out of 131 genes, 15.2%	200 out of 7291 annotated genes, 2.7%	6,15E-10	HIS4, CIT2, ARO3, HOM3, STR3, MET28, URA2, MET3, ILV3, CPA2, MET14, MAE1, MET17, FMS1, MET2, MET22, CPA1, GRS2, ASN1, MET16
nitrogen compound metabolism |	21 out of 131 genes, 16.0%	238 out of 7291 annotated genes, 3.2%	2,00E-09	HIS4, CIT2, ARO3, HOM3, STR3, MET28, DAL7, URA2, MET3, ILV3, CPA2, MET14, MAE1, MET17, FMS1, MET2, MET22, CPA1, GRS2, ASN1, MET16
sulfur amino acid metabolism |	9 out of 131 genes, 6.8%	32 out of 7291 annotated genes, 0.4%	8,82E-09	HOM3, STR3, MET28, MET3, MET14, MET17, MET2, MET22, MET16
methionine metabolism |	8 out of 131 genes, 6.1%	23 out of 7291 annotated genes, 0.3%	1,20E-08	HOM3, STR3, MET3, MET14, MET17, MET2, MET22, MET16
sulfur metabolism |	11 out of 131 genes, 8.3%	59 out of 7291 annotated genes, 0.8%	1,26E-08	HOM3, MET10, STR3, MET28, GSH1, MET3, MET14, MET17, MET2, MET22, MET16
carboxylic acid metabolism |	20 out of 131 genes, 15.2%	303 out of 7291 annotated genes, 4.1%	5,41E-07	VID24, HIS4, CIT2, ARO3, HOM3, STR3, MET28, URA2, MET3, ILV3, CPA2, MET14, MAE1, MET17, MET2, MET22, CPA1, GRS2, ASN1, MET16
organic acid metabolism |	20 out of 131 genes, 15.2%	303 out of 7291 annotated genes, 4.1%	5,41E-07	VID24, HIS4, CIT2, ARO3, HOM3, STR3, MET28, URA2, MET3, ILV3, CPA2, MET14, MAE1, MET17, MET2, MET22, CPA1, GRS2, ASN1, MET16
sulfur utilization |	5 out of 131 genes, 3.8%	10 out of 7291 annotated genes, 0.1%	1,25E-06	MET10, MET3, MET14, MET22, MET16
sulfate assimilation |	5 out of 131 genes, 3.8%	10 out of 7291 annotated genes, 0.1%	1,25E-06	MET10, MET3, MET14, MET22, MET16
methionine biosynthesis |	3 out of 131 genes, 2.2%	5 out of 7291 annotated genes, 0.0%	0,00011	STR3, MET2, MET22
organic cation transport |	3 out of 131 genes, 2.2%	6 out of 7291 annotated genes, 0.0%	0,00018	MEP1, MEP2, MEP3
ammonium transport |	3 out of 131 genes, 2.2%	6 out of 7291 annotated genes, 0.0%	0,00018	MEP1, MEP2, MEP3

Tab. 3.4.5. Results of gene ontology clustering for genes higher expressed in 71B.

In particular, sulphur amino acids biosynthesis was up-regulated in 71B and part of branched amino acids anabolism seemed also to be induced. Moreover, sulphate

uptake and reduction pathway (which corresponds in part to the one for sulphured amino acids biosynthesis) was up-regulated. Three permeases involved in ammonium transport were also highly expressed. The sulphured amino acids metabolism will be analyzed and described in detail in paragraph 3.4.4.

3.4.3. Genes involved in fermentative aroma production and differentially expressed in 71B and EC1118

As mentioned in chapter 1, synthesis of acetate esters such as isoamyl acetate and ethyl acetate in *S. cerevisiae* is ascribed to acetyl-transferase activities; the best known enzymes (alcohol-O-acetyl transferases, AATases) are encoded by *ATF1* and *ATF2* genes [77]. In addition to these AATases, two possible alcohol acyl-transferase, Eht1p and Eeb1p, have recently been described [225]. Furthermore, the product of gene *IAH1*, with esterase activity, has been associated with hydrolysis of esters as isoamyl-acetate [79], contrasting part of the biosynthetic activity of previously described genes.

The higher alcohols produced by yeast, on the other hand, can originate from the degradation of imported branched-chain amino acids (BCAA) or from endogenous biosynthesis. The amino acids are converted to their corresponding α-keto acids by transamination. This transamination reaction is catalysed by mitochondrial and cytosolic branched-chain amino acid transferases (BCAATases) encoded by the *BAT1* and *BAT2* genes, respectively that, if constitutively over-expressed, show their strong correlation with higher alcohols production levels [61].

3.4.3.1. Microarray evidence

Since the 71B strain is widely known for being a strong producer of fermentative aromas, any difference in gene expression involving yeast flavours production was considered. First of all, expression ratios of these genes were extrapolated from microarray data set; as shown in table 3.4.6.

Gene	Ratio 71B : EC1118	p-Value
ATF1	1,22	0,04
ATF2	1,79	0,00
EHT1	1,27	0,02
(EEB1)YPL095c	1,47	0,06
IAH1	0,76	0,03
BAT1	2,14	0,05
BAT2	1,32	0,03

Tab. 3.4.6. Genes involved in fermentative aroma production and their expression ratios.

Among these genes, only *ATF2* would pass both the p-value and ratio cut-off previously decided for filtering over-expressed genes. Notwithstanding, the general expression trend of these genes seemed to be not random. Even if none of the genes showed strong differences in expression, together they might play a role (probably of minor importance) in the fermentative performance of 71B strain. As this evidence is really weak it do not allow any further speculation.
For this reason the expression level of these genes by real-time PCR was investigated, more deeply, to confirm or refute the hypothesis of a significant difference in general expression trend between the two strains.

3.4.3.2. Real-time PCR confirmation

ATF1, *ATF2*, *IAH1*, *EEB1* and *EHT1* gene expressions were confirmed by Real-time PCR. The assays for *BAT1* and *BAT2* genes are now in progress (at the moment primers have been designed and tested by qualitative PCR).

Real-Time PCR assay was carried on as described in materials and methods, comparing genes of interest (target genes) with two reference genes (*ACT1* and *FBA1*) chosen for their constant expression during fermentation (data not shown).The gene coding for the transduction elongation factor eEF1a was also tested, while genes involved in aerobic respiration could not be used due to the experimental conditions.
As the use of a single reference gene can lead to erroneous normalizations and data interpretation [26, 52], for each PCR plate both *FBA1* and *ACT1* were amplified as

reference genes. Target genes were separately analyzed and the expression compared with both: when the data coming from the two normalizations agreed, the analysis was carried on. Thus, only the comparison with *ACT1* gene is reported. In the near future, recently developed software, that allows the analysis with two or more reference simultaneously, will be available and better normalization of the data set will be obtained [100].

The quantification of the relative changes in gene expression using real-time PCR is based on a series of theoretical assumptions, that can be applied differently considering the amplification condition. For that reason testing the diverse options to choose the more suitable one is extremely important before starting the data interpretation. In this work the $2^{-\Delta\Delta CT}$ method to calculate relative changes in gene expression determined from real-time quantitative PCR experiments [124] was applied. For amplicons less than 150 bp and for optimized concentration of primers and $Mg2^{+}$, the amplification efficiency is assumed to be close to one; therefore, the amount of target, normalized to an endogenous reference (internal control gene) in the reaction is: amount of target = $2^{-\Delta\Delta CT}$ where $\Delta\Delta Ct = (\Delta Ct_A - \Delta Ct_B)$, ΔCt_A andΔ Ct_B are the differences in threshold cycles for target (X) and reference (R) genes in strain A and in strain B (as explained in par. 3.2.8.2). In any case, for a correct $\Delta\Delta CT$ calculation, the amplification efficiencies of the target and reference genes must be approximately equal. A sensitive method for assessing if two amplification have the same efficiency is to look at how ΔCt varies with template dilution: the curves must be parallel.

For these reasons, before starting each Real-time PCR analysis, calibration of the amplifications were carried out to check primers efficiency. Calibration curves for *ATF1*, *ATF2*, *IAH1*, *EEB1* and *EHT1* primer pairs and the two references *ACT1* and *FBA1* are shown in figure 3.4.6 (the template was a standard yeast cDNA and its dilutions).

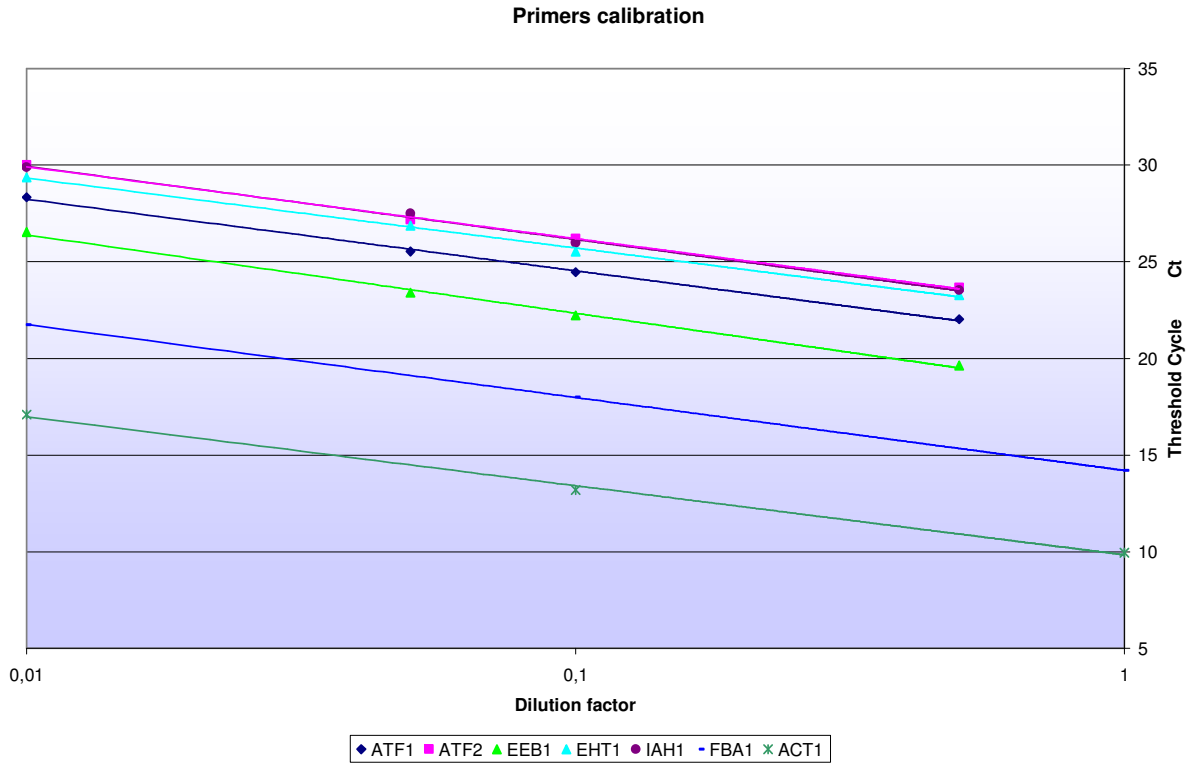


Fig. 3.4.6 Calibration curves for *ATF1*, *ATF2*, *IAH1*, *EEB1*, *EHT1*, *ACT1* and *FBA1* genes.

As the lanes showed parallel orientations (for equations, correlation coefficients of curves and efficiency values, see Appendix A), after primer calibration, the same RNA pools analyzed by microarrays were transcribed to cDNA and assayed by Real-Time PCR.

Before calculating the ratio between 71B and EC1118 expression level of the target genes from PCR data, a comparison was performed on raw data (threshold cycles (Ct) values normalized on Actin Ct) in order to validate statistic significance of any possible differences (fig. 3.4.7). A variation of one unit in threshold cycles corresponds to a 2-fold difference in expression; the lower is threshold cycle, the higher is expression.

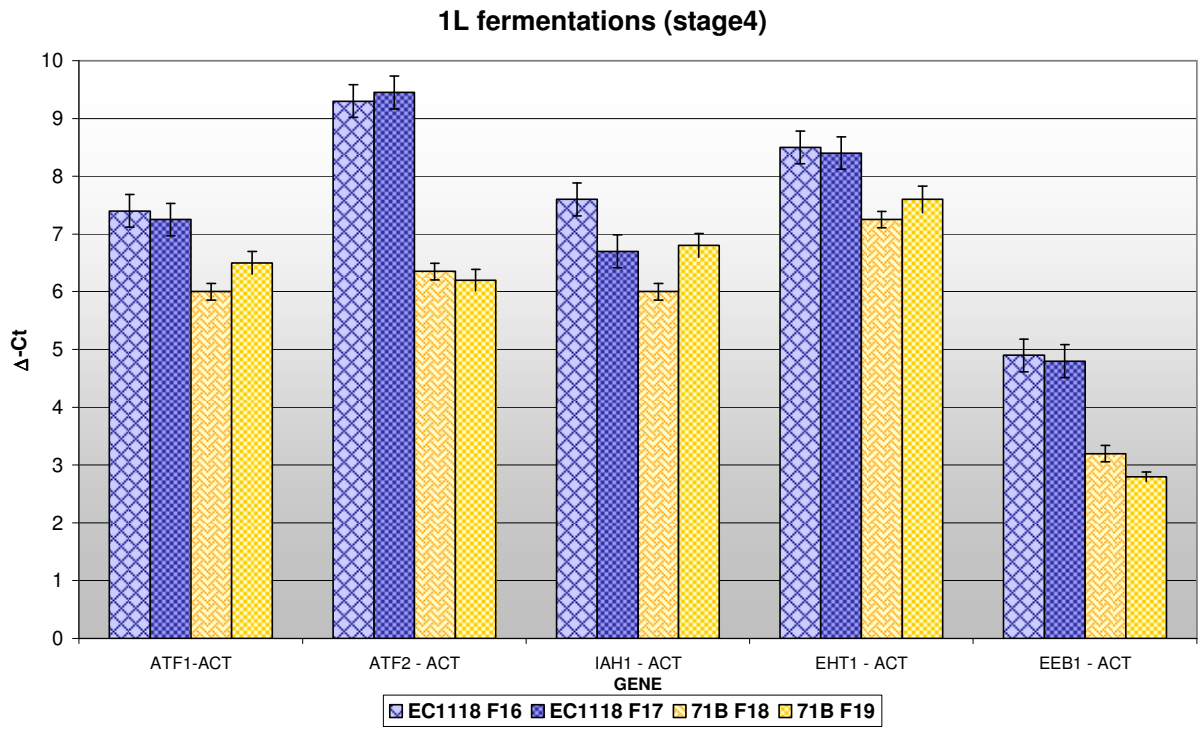


Fig. 3.4.7. Threshold cycles of *ATF1*, *ATF2*, *IAH*, *EHT1* and *EEB1* genes normalized on Actin.

In fig. 3.4.7 each bar results from subtraction of the average values of target gene Ct from the Actin gene *ACT1* Ct (three replicates of each biological sample were considered): when replicates are run on the same PCR amplification, the ΔCt calculation on averaged Ct values from the three replicates seem to be more accurate [124]. The standard deviation was calculated using the statistical application of the standard propagation of error. A complete table of threshold cycles and ΔCt data is available in Appendix A.

As first remark, it has been found that genes involved in esters formation seem to be barely expressed in yeast cells at stage 4 (45 g/l of overall produced CO_2, stationary phase) of alcoholic fermentation: they are from 3 to 10-fold less expressed than Actin (and from 2 to 9-folds lower than FBA, data not shown). Considering *ATF2* expression, as expected from microarray data, a clear difference between the two strains was observed, while some lower variations was noticed for the other genes. Thus, before calculating any expression ratio, a statistical validation was performed to assess significance of ΔCt differences found using the t Student test for means

comparison. In this case, t test validated difference between strains for *ATF1*, *ATF2*, *EHT1* and *EEB1* genes (p-value<0.05) and did not confirm significance for IAH data. The data used to compare altogether the gene expression of the two strains with the $2^{-\Delta\Delta CT}$ method came from two separate fermentations from which RNA preparations were made for each strain and carried through the analysis. Therefore, it made sense to treat each sample separately and averaged the results just before the $2^{-\Delta\Delta CT}$ calculation. Figure 3.4.8 presents the average expression ratio between 71B and EC1118 of the four genes studied (*IAH1* was omitted because of its lack of significance).

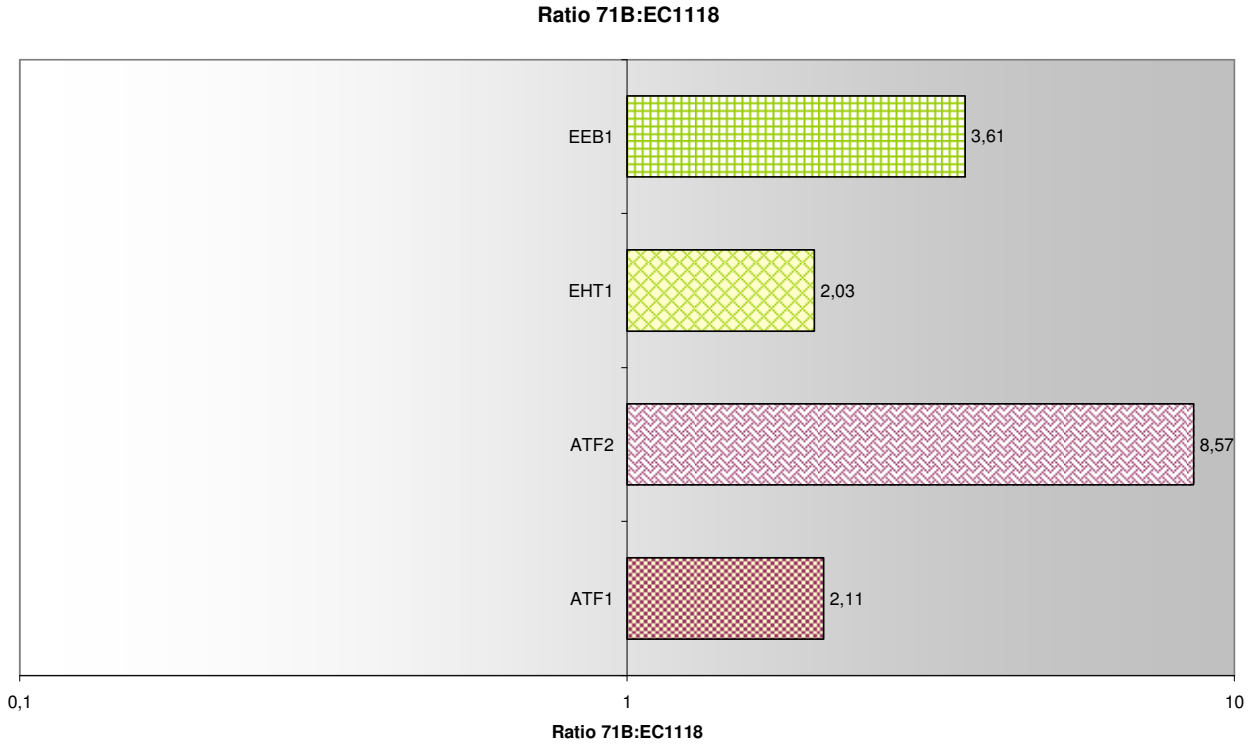


Fig. 3.4.8 Expression ratio of genes involved in esters production. Central value is set to 1 (identical expression in both strains).

All the genes were expressed at higher level in 71B than in EC1118 at stage 4 of fermentation (45 g/l of overall produced CO_2, stationary phase), this results confirm and validate microarray findings. Differences in expression were higher than those emerged from microarray analysis (at least 2-folds ratio instead of 1.2<R<1.7): this can be explained by the general low expression level of these genes, since microarray sensitivity decreases as fluorescence signal lowers.

3.4.3.3. Time-course expression analysis

The expression analysis of the five genes involved in ester formation and hydrolysis at three stages of fermentation (stage1: cells in exponential growth phase; stage 4: 45 g/l of overall produced CO_2; stage 6: cells in late stationary phase, cfr in tab.3.4.1).

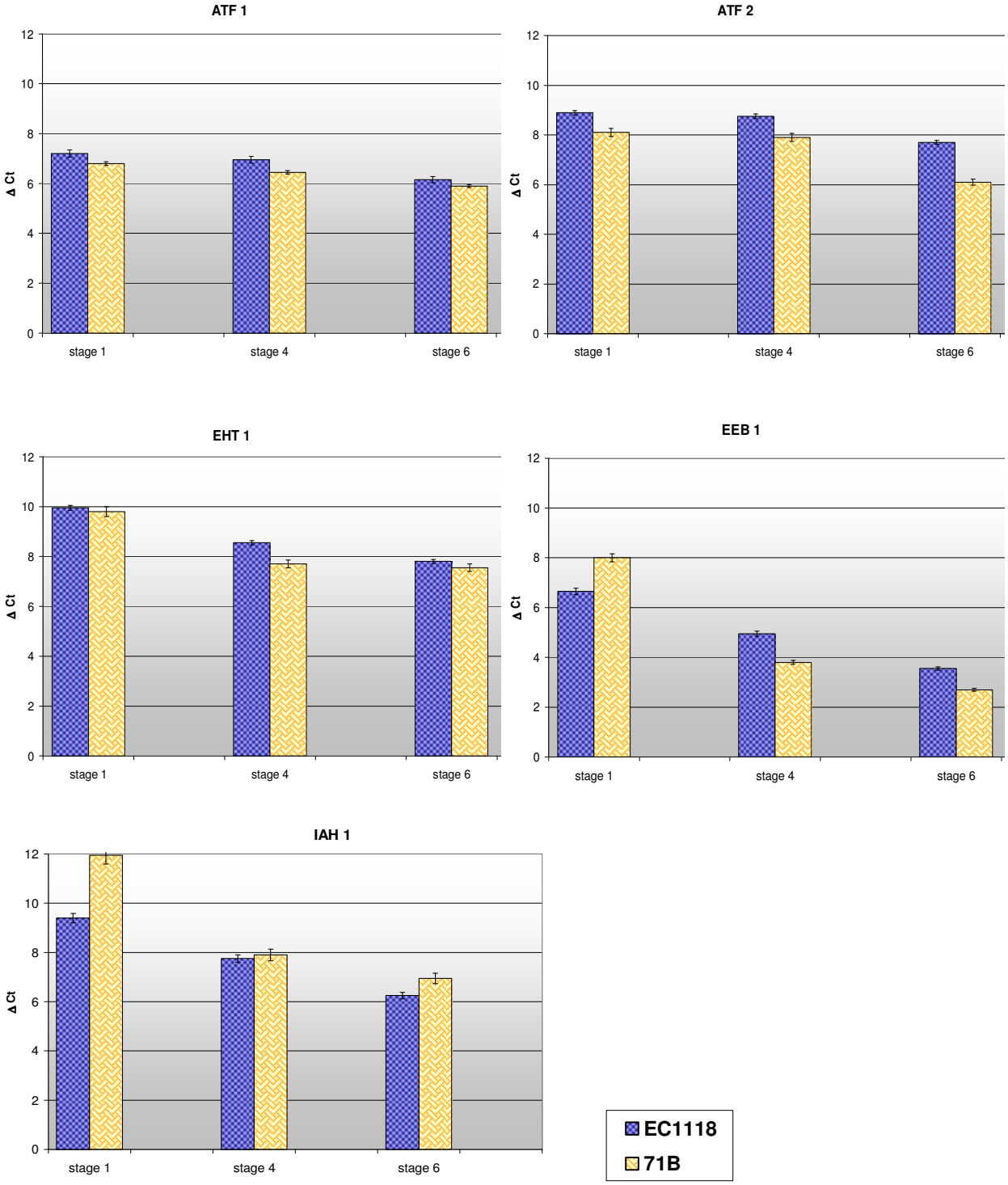


Fig. 3.4.9. Threshold cycles of *ATF1*, *ATF2*, *IAH*, *EHT1* and *EEB1* genes normalized on Actin at different stages of fermentation.

The statistical validation of ΔCt was performed as previously described; t-test in this case excluded *EHT1* at stage 1 and *IAH1* at stage 4 (as in the previous experiment). A complete table of Threshold cycles and ΔCt data is available in Appendix A.

An overall representation of *ATF1*, *ATF2*, *IAH*, *EHT1* and *EEB1* gene expression ratio (71B : EC1118) throughout fermentation is shown in figure 3.4.10.

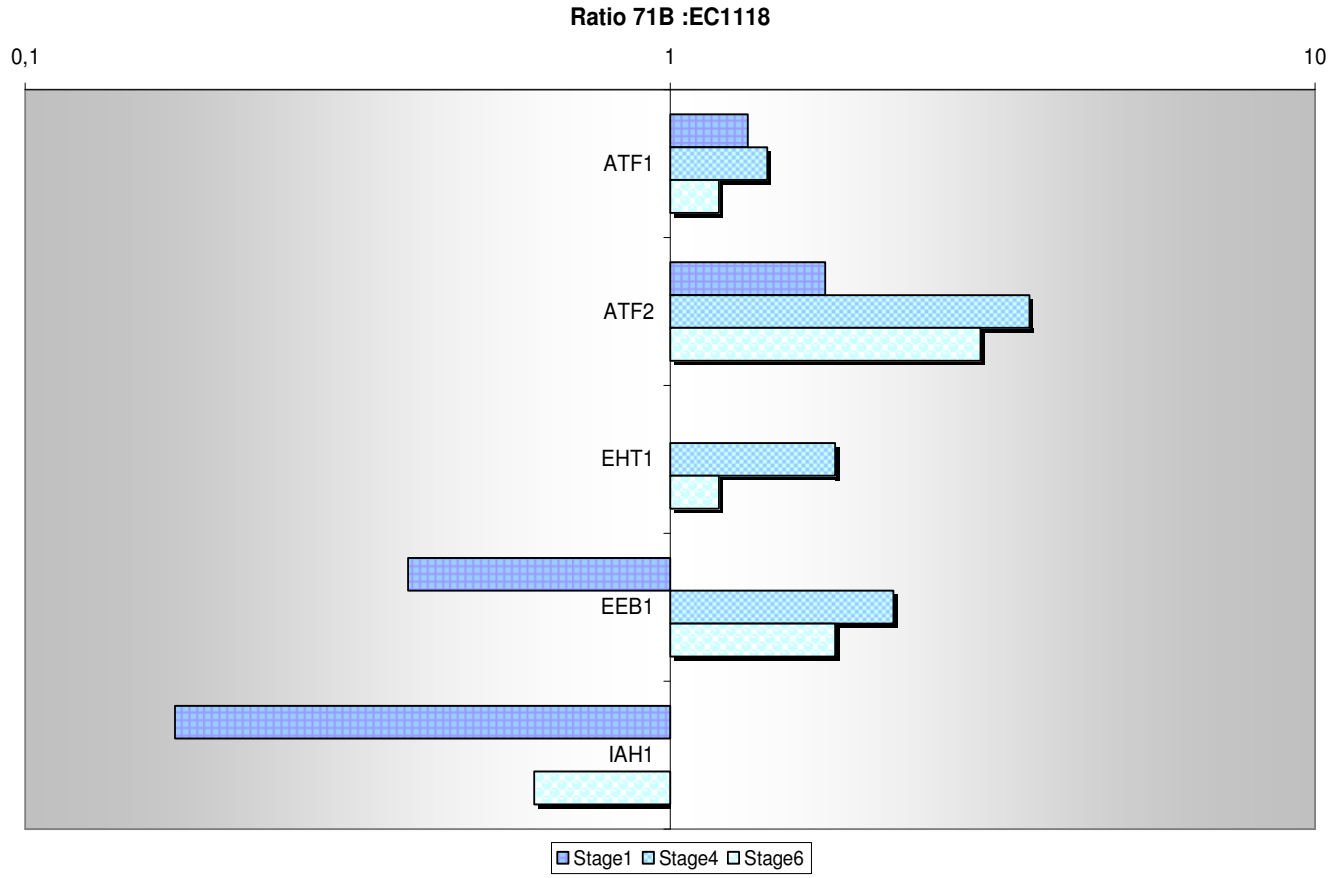


Fig. 3.4.10 Expression ratio of genes involved in esters production throughout fermentation. Central value is set to 1 (identical expression in both strains).

The five genes showed a dynamic expression profile during the fermentation progress.

The two ATF genes were expressed at higher level in 71B strain throughout the fermentation, and the ratio was higher during the stationary phase. *EHT1* showed no significant difference in expression between the two strains at stage 1 (active growth phase) but was expressed at higher level in 71B during the stationary phase. *IAH*1, the only esterase activity in the list, showed higher expression in EC1118 at both active growth phase and late stationary phase, it seems not to be over-expressed by

either strains at stage 4. *EEB1* was the only gene that is more expressed in one strain (EC1118) during growth phase but is less expressed at stationary phase.

This kind of comparison, nevertheless, does not take into account the absolute expression level of the genes. The general expression level of the esters-related genes in both strains is shown in figure3.4.11.

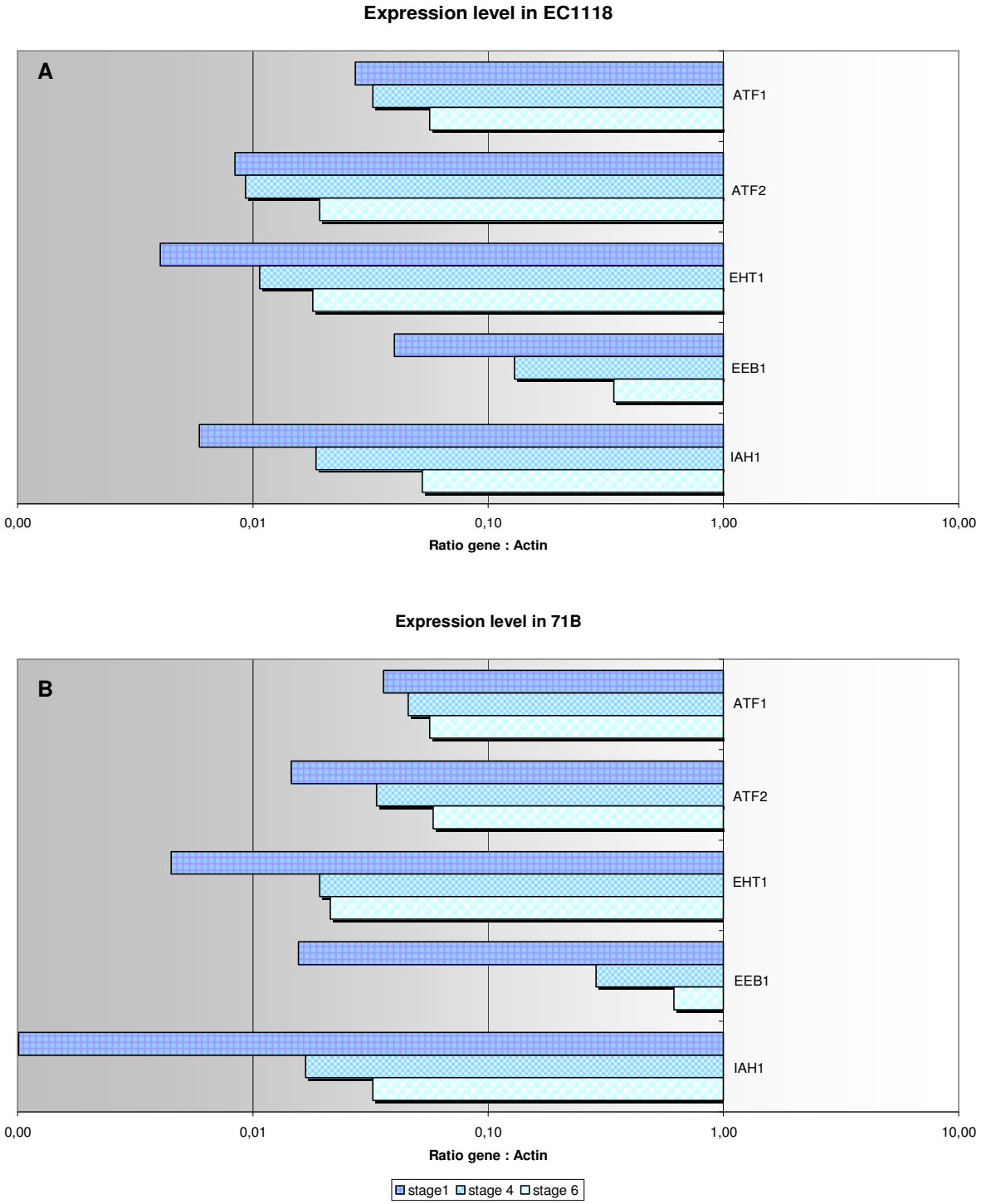


Fig.3.4.11. Expression level of *ATF1* , *ATF2*, *IAH*1, *EHT1* and *EEB1* genes compared with Actin in EC1118 (A) and 71B (B).

The expression trends of the five genes showed an outstanding induction at stationary and late stationary phase in both strains: this confirms the positive correlation between ester (and fermentative aroma) production and the progression of the alcoholic fermentation [71, 180]. The graphs show once again that all genes were barely expressed by yeast cells during alcoholic fermentation (compared to Actin), except for *EEB1* at stationary phase.

Further remarks can be express looking at these graphs more carefully, concerning the expression ratio between 71B and EC1118 (fig 3.4.10). The Alcohol-O-Acetyl-transferases Atf1p and Atf2p are responsible for acetate esters production; it is well known that Atf1p contributes more to this activity (i.e. deletions in *ATF1* sequence cause 60 to 90% reduction of isoamyl-acetate production, deletion in *ATF2* results in only 10 to 35% decrease, as shown by Verstepen *et al.* [249]). In the reported experiments, *ATF2* showed the highest 71B: EC1118 ratio, but it is remarkable that the expression level of *ATF1* was higher than *ATF2* and *ATF1* showed higher expression in 71B than in EC1118, as well.
Among Ethanol binding Acyl-transferses (Eht1p and Eeb1p), responsible for medium-chain fatty acid ethyl ester production, Eeb1p is known to be the most important [225]. In the reported analysis Eeb1p showed a low expression level during the active growth phase and then strongly increased when the strains enter the stationary phase; the gene was more expressed in EC1118 than 71B in the active growth phase where expression was generally low, but it was higher in 71B during the stationary phase when its induction reached the maximum level of expression.
Finally, the esterase *IAH1* was the lower expressed gene among the ones analysed, but showed a strong induction during the late stationary phase. EC1118 displayed an higher gene expression level in both early and late stages. Thus, the odd ratio found at stage 4 (the only one close to stage 1) could be explained by a different timing in gene induction between the two strains.
Nevertheless, ester hydrolysis, is not only ascribed to enzymatic activities during wine fermentation: chemical degradation acts at wine pH, thus, in most cases, long fermentation times can contribute significantly to reduce esters final content in wine.

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3.4.4. Genes involved in sulphured aminoacids biosynthesys differentially expressed in 71B and EC1118

By means of gene clustering performed on 71B microarray data, genes linked to sulphured amino acids metabolism sorted out as differently expressed.

3.4.4.1. Microarray evidence

In particular, fitting the microarray data with the enzyme specific function and position in their pathway, a clear up-regulation of the upper part of sulphate uptake/Methionine and Cystein biosinthethic pathway was pointed out, as shown in figure 3.4.12. Genes in violet squares are up-regulated (ratio and p-value are also reported) in 71B according to microarray data, whereas those in red squares are not over-expressed and/or not statistically significant.

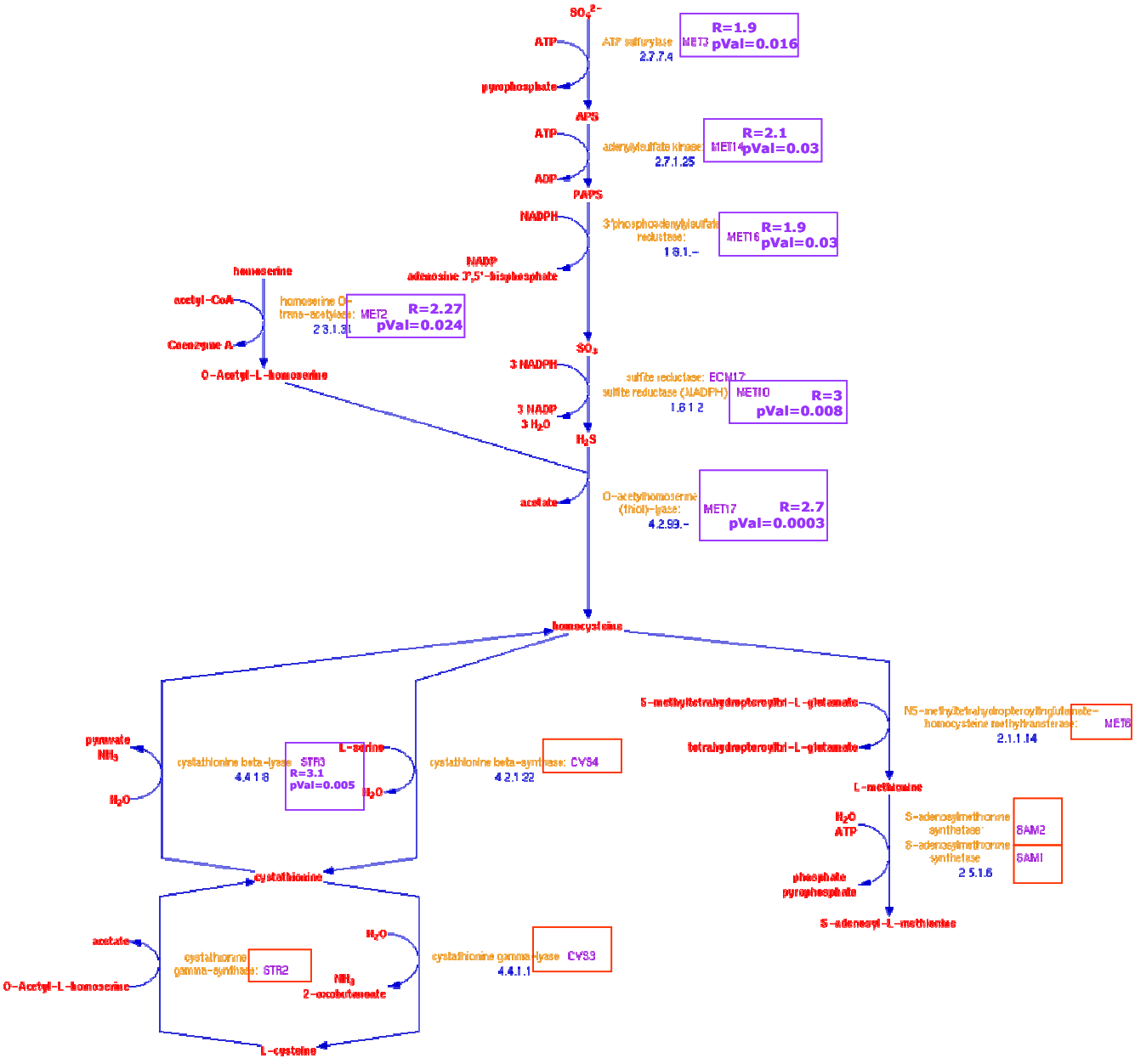


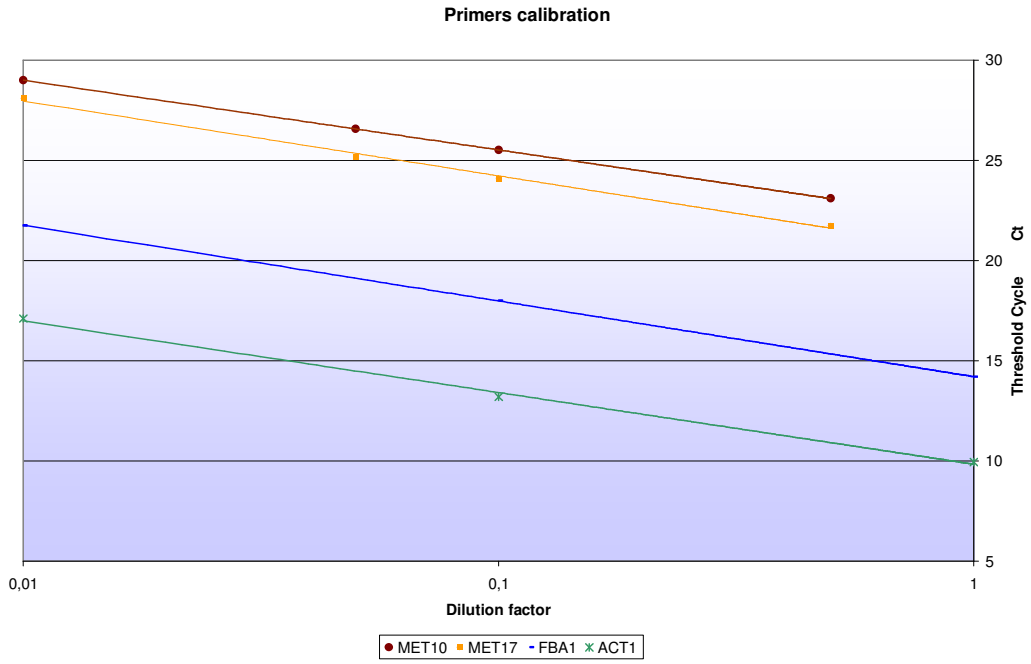
Fig. 3.4.12. Biosynthetic pathway of sulphured amino acids; in squares ratio values (71B : EC1118) and p-values from microarray data set associated with each validated gene .

3.4.4.2. Real-time PCR confirmation

Real-time PCR confirmation of sulphured amino acids biosynthesis up-regulation in 71B was performed on two genes chosen randomly among the possible targets: *MET10* and *MET17*.

Primers for Real-time amplification of *MET10* and *MET17* were firstly checked by calibration as previously explained (par. 3.4.3). Calibration curves, displaying parallel

trends, are shown in figure 3.4.13 (for equations and correlation coefficients of curves and efficiency values, see Appendix A).



A

Fig. 3.4.13 Calibration curves for *MET10*, *MET17*, *ACT1* and *FBA1* genes.

After primer calibration, the same cDNA pools analized for genes involved in aroma production were assayed for MET genes by Real-Time PCR.

Before calculating the ratio between 71B and EC1118 expression level of the target genes from PCR data, a comparison was performed on raw data (threshold cycles (Ct) values normalized on Actin Ct) in order to validate statistic significance of any possible differences (fig. 3.4.14).

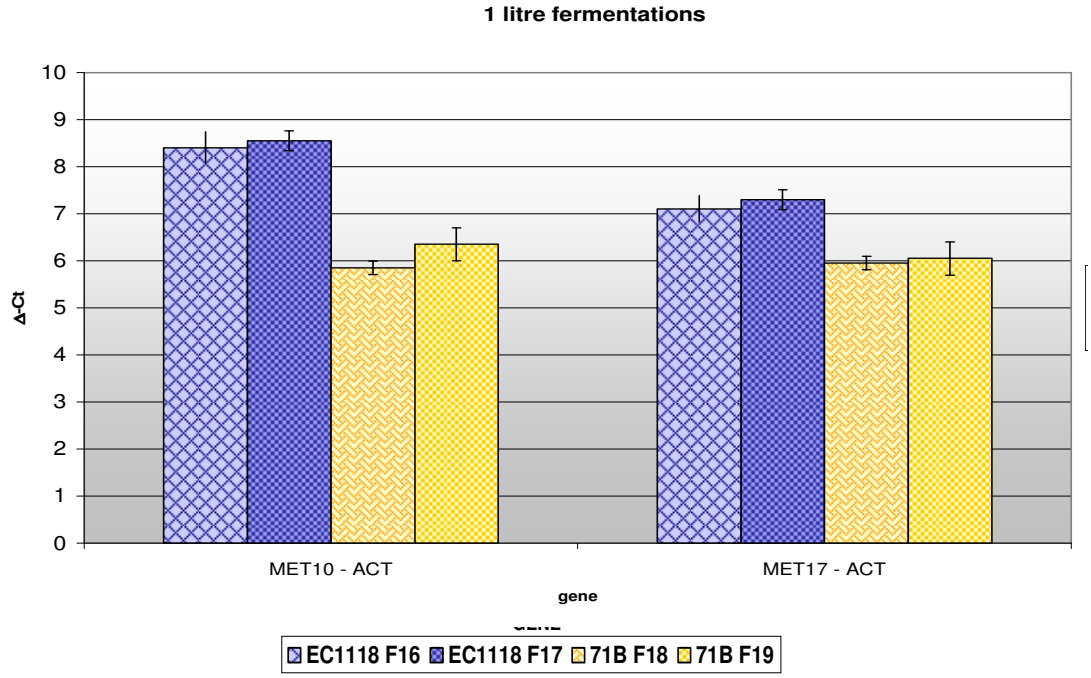


Fig. 3.4.14. Threshold cycles of *MET10* and *MET17* genes normalized on Actin.

A statistical validation was performed to assess significance of ΔCt differences (t-Student test for means comparison): t test validated difference between strains for *MET10* and *MET17* genes (p-value<0.05). A complete table of threshold cycles and ΔCt data is available in Appendix A.

Then, for comparing altogether the two strains with the $2^{-\Delta\Delta CT}$ method, the average expression ratio between 71B and EC1118 of the two genes was calculated.

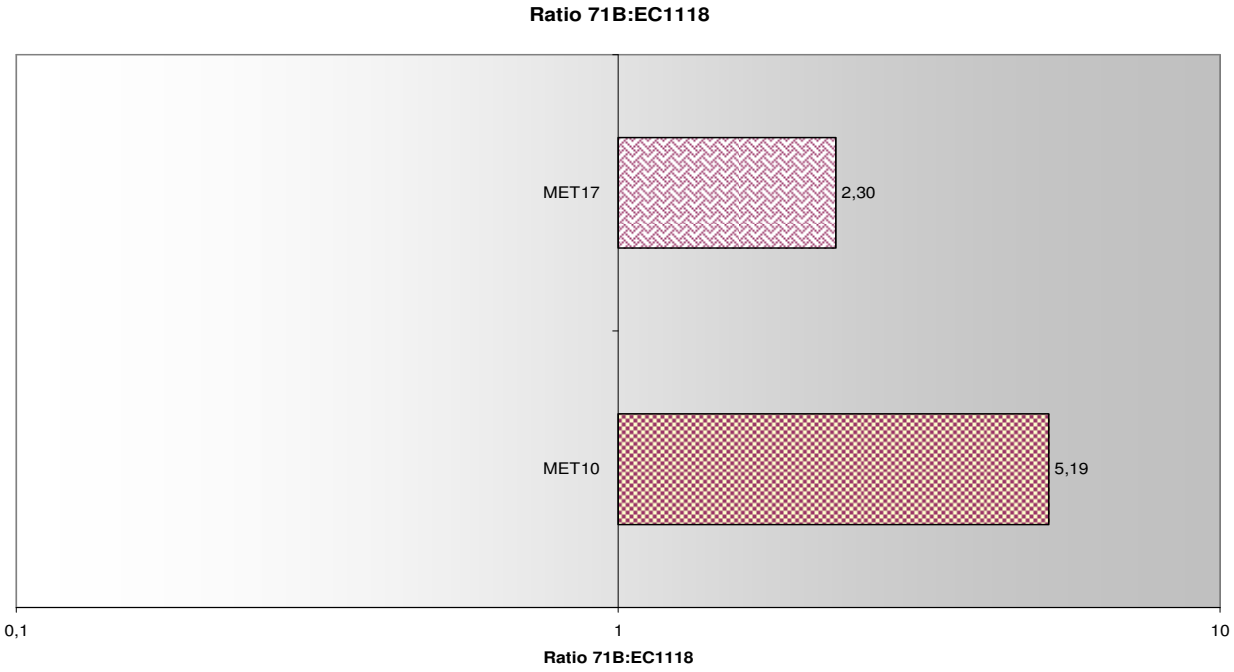


Fig. 3.4.15. Expression ratio of *MET10* and *MET17* genes. Central value is set to 1 (identical expression in both strains).

The expression ratio shown in figure3.4.15 confirmed microarrays expectations presenting MET genes more expressed in 71B than in EC1118, in particular MET 10. Before performing any further experiment, the assimilable nitrogen, present in the fermented must obtained from laboratory size and 100L volume fermentations, at stage 4, was measured, as the aminoacid biosynthetic pathways and in particular sulphured amino acids pathway can be regulated by nitrogen starvation. Assimilable nitrogen titration gave the same results for both strains (data not shown), thus excluding this kind of regulation as the cause of differential expressions. The used natural must had an initial concentration of 125 ± 5 mg/l of assimilable nitrogen; at stage 4, for both strains, the value dropped down to around 40mg/L (38-42.5); at stage 6 residual assimilable nitrogen was averagely 20 mg/L for both strains (19-21). After nitrogen titration, confirmation experiments continued with time-course expression analysis by Real-time PCR.

3.4.4.3. Time-course expression analysis

Four time-course samples for each strain were considered for Real-time PCR determinations: at stage 1 and 2 where the cells are in active growth phase, at stage 4, the early stationary phase, where also the DNA microarray data come from and at stage 6, the late stationary phase (cfr tab.3.4.1).

Comparison on ΔCt raw data (*MET10-ACT* and *MET17-ACT*) are graphed in figure 3.4.16.

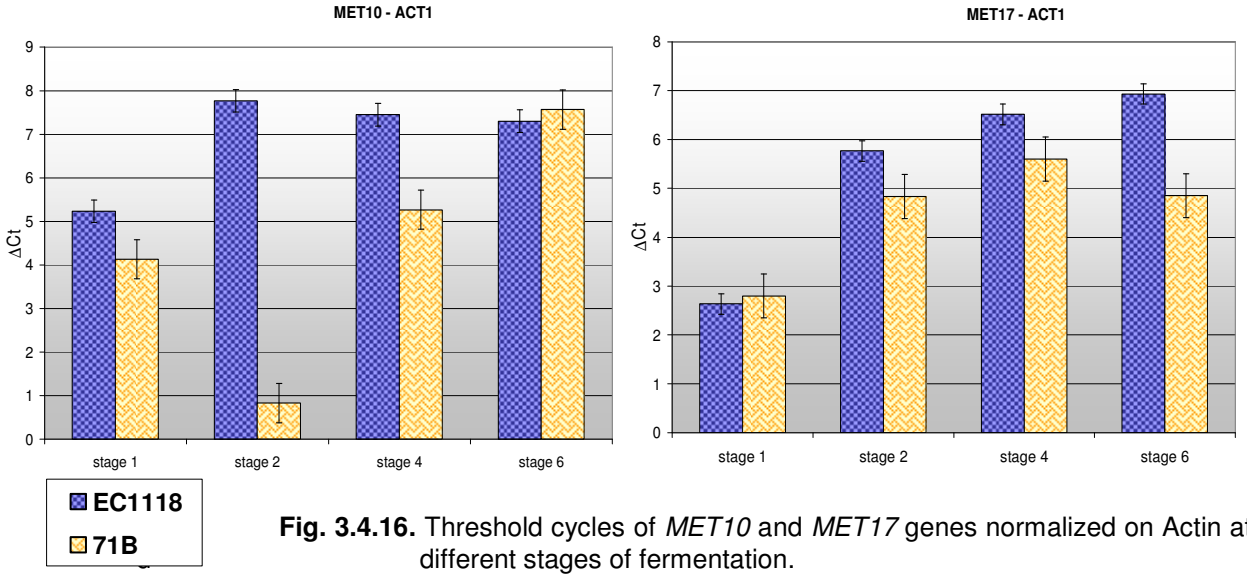


Fig. 3.4.16. Threshold cycles of *MET10* and *MET17* genes normalized on Actin at different stages of fermentation.

The statistical validation of ΔCt was performed as previously described; t-test in this case excluded *MET10* at stage 6 and *MET17* at stage 1. A complete table of Threshold cycles and ΔCt data is available in Appendix A. The previously described differences at stage 4 were confirmed. Both *MET10* and *MET17* genes were more expressed during the active growth phase than in the stationary phase; concerning *MET10*, the expression trend in the two strains was completely different: in 71B the highest expression level was reached at stage 2 with a strong up-regulation and then gradually decreased; in EC1118 the expression was stronger during the early active growth phase then assessed on lower and more stable levels until the end of the fermentation. For *MET17* gene there were not great differences in expression trends between two strains. More properly as the expression level lowered (from stage two for both strains), differences in the expression level between them took place.

Looking at expression ratio between the two strains (fig. 3.4.17), the higher expression of *MET10* and *MET17* in 71B was found in 71B, with an astonishing ratio

of 100 folds expression for *MET10* at stage 2 of fermentation. It is interesting to remark that significant differences in *MET10* expression are found from stage 1 to stage 4 whereas for *MET17* significant differences were located from stage 2 to 6.

Methionine biosynthesis pathway is feedback regulated: in presence of methionine the biosynthesis is repressed. The higher expression level of MET genes at the beginning of fermentation could indicate a low presence of methionine in the natural must and probably the MET10 induction in 71B strain at stage 2 could be due to a complete depletion of this aminoacid at that time (stage 2 corresponds to the end of the active growth phase) for 71B strain but not for EC1118.
After a rapid activation, genes seem to be no more induced, probably because methionine requirements were satisfied. Moreover, absence of available nitrogen represses MET genes: proceeding through the end of fermentation, both strains suffered of nitrogen starvation and this situation could be one of the causes for the gradual repression of MET genes.

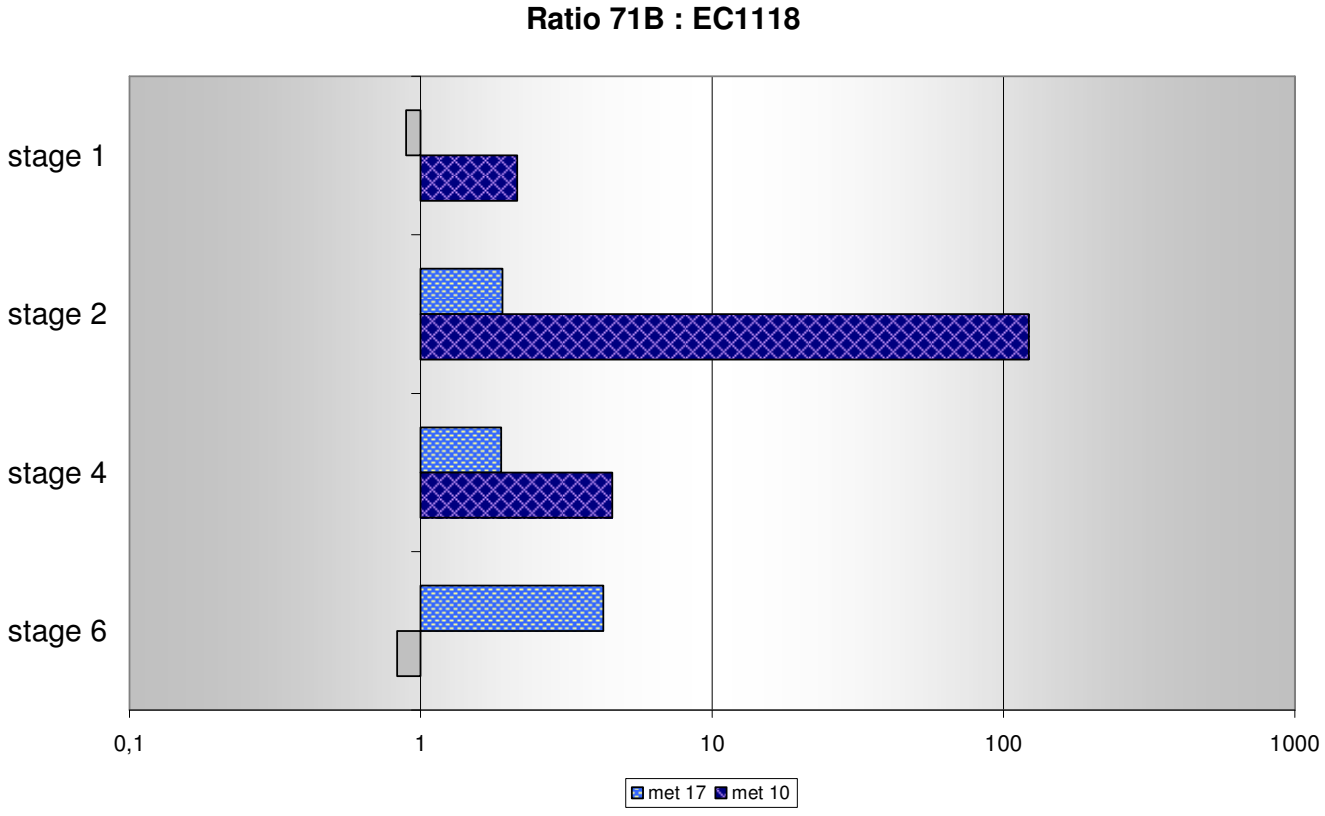


Fig. 3.4.17. Expression ratio of *MET10* and *MET17* genes throughout fermentation. Central value is set to 1 (identical expression in both strains), grey bars for not statistically significant data.

It is not easy to infer about differences in methionine and cysteine consumption and consequently in the biosynthesis between two strains fermenting in the same conditions. It is possible that the needed amount of methionine for cell growth is not the same. A better understanding of this behaviour will need further investigations to clear up the different utilization of these aminoacids.

3.4.5. Sulphite efflux system is highly expressed in 71B

In previous comparative study of transcriptomes, it has been found that *SSU1*, a gene involved in mediating sulphite efflux in *S. cerevisiae* and, hence, conferring sulfite resistance [173], showed a significantly higher expression in T73, a wine yeast strain, than in a laboratory strain also tested [94]. *SSU1* encodes a plasma membrane permease; mutations in *SSU1* cause sensitivity, whereas over-expression confers heightened resistance [87, 173], suggesting a role for *SSU1* in sulphite detoxification.

3.4.5.1. Microarray evidence

An interesting finding from microarrays data analysis concerns SSU1 as one of the most differently expressed gene, as following reported:

Gene	Ratio 71B : EC1118	p-Value
SSU1	4,93	0,0001

Since all the fermenters had the same quantity of added sulphite (50 mg/l), both causes and consequences of such differences in the gene expression could be interesting, thus Real-time confirmation and further analysis were performed on SSU1 gene.

3.4.5.2. Real-time PCR confirmation

Primers for Real-time amplification of SSU1 were firstly tested by calibration as previously explained (par. 3.4.3). Calibration curves displaying parallel trends are shown in figure 3.4.18 (for equations and correlation coefficients of curves and efficiency values, see Appendix A).

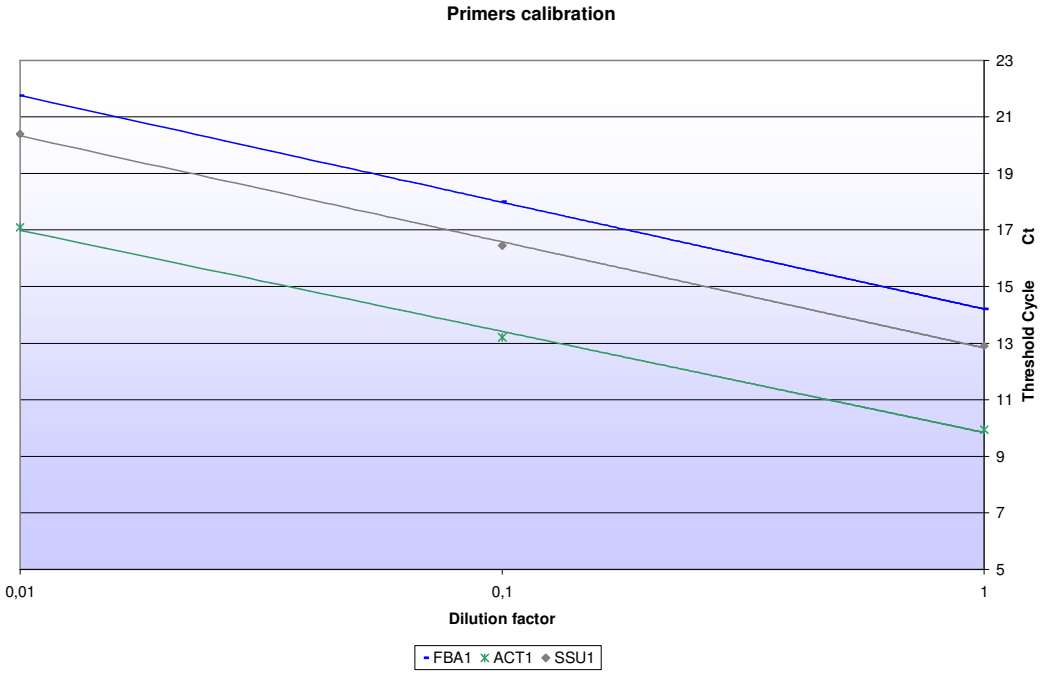


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Fig. 3.4.18 Calibration curves:*SSU1*, *ACT1* and *FBA1* genes.

After primer calibration, the same cDNA pools analyzed for genes involved in aroma production and methionine synthesis were assayed for the *SSU1* gene by Real-time PCR.

Before calculating the ratio between 71B and EC1118 expression level of target genes from PCR data, a comparison was performed on raw data (threshold cycles (Ct) values normalized on Actin Ct) in order to validate statistic significance of any possible differences (fig. 3.4.19). A statistical validation was performed to assess significance of ΔCt differences (t-Student test for means comparison): t test validated difference between strains for *SSU1*gene (p-value<0.05).

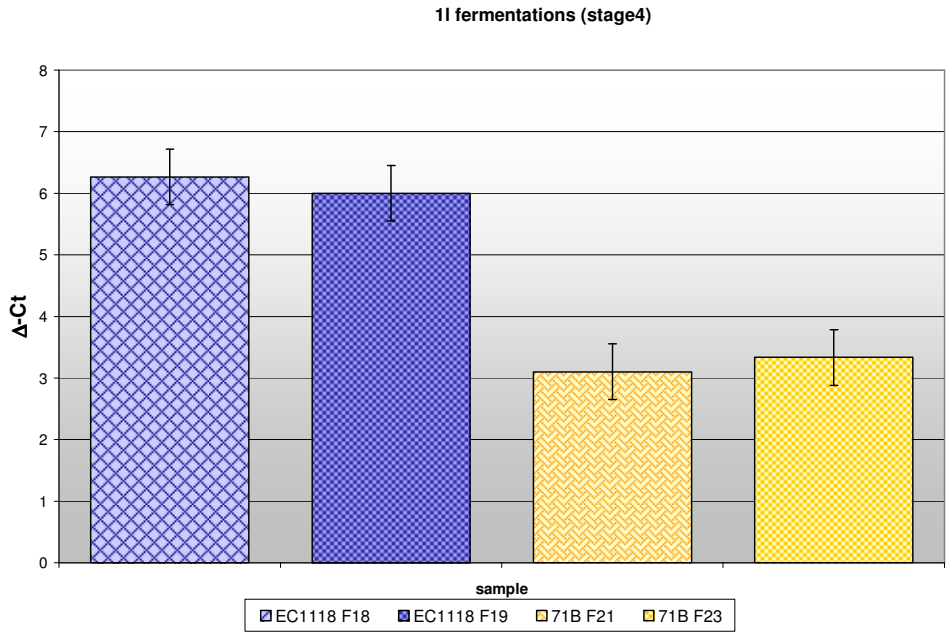

Fig. 3.4.19. Threshold cycles of *SSU1* normalized on Actin at stage 4.

The expression ratio of SSU1 (71B:Ec1118) found was 10.2. The microarray data are once again validated by Real-time PCR analysis.

3.4.5.3. Time-course expression analysis

Four time-course samples were considered for Real-time PCR determinations: at stage 1 and 2 where the cells are in active growth phase, at stage 4, the early stationary phase, where also the DNA microarray data come from, and at stage 6, the late stationary phase (cfr tab.3.4.1).

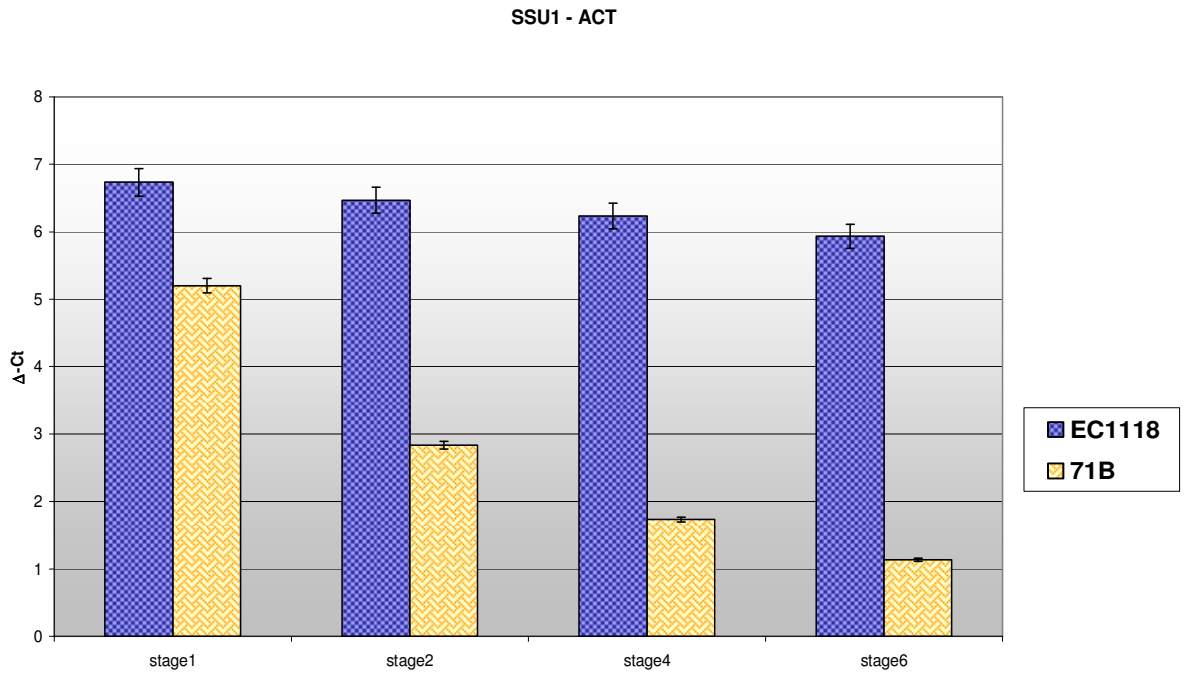

Fig. 3.4.20. Threshold cycles of SSU1 gene normalized on Actin at different stages of fermentation.

Comparison on ΔCt data (SSU1-ACT) are graphed in figure 3.4.20. It is really interesting to observe the expression trend of this gene in 71B: SSU1 seems to be activated during stationary phase. Previous studies on *SSU1* gene, in fact, proposed and demonstrated different activation mechanisms for *SSU1* [173, 269] such as a point mutation in its transcriptional activator *FZF1* or chromosome translocation and a consequent promoter activity change (cfr par. 3.4.5.4). In any case all these mechanisms are described as constitutive and there was no indication of any time course or signal-dependent transcriptional activation. In this work is reported the first evidence for a regulated *SSU1* gene expression. Actually Yuasa *et al* [268] suggested that *SSU1* was regulated by anaerobiosis, but this observation does not fit with the fermentation time course, as anaerobiosis occurs more rapidly .

Looking at expression ratio between the two strains (fig.3.4.21), the higher expression of *SSU1* in 71B is clear, at all fermentation samplings and increased at stationary phase.

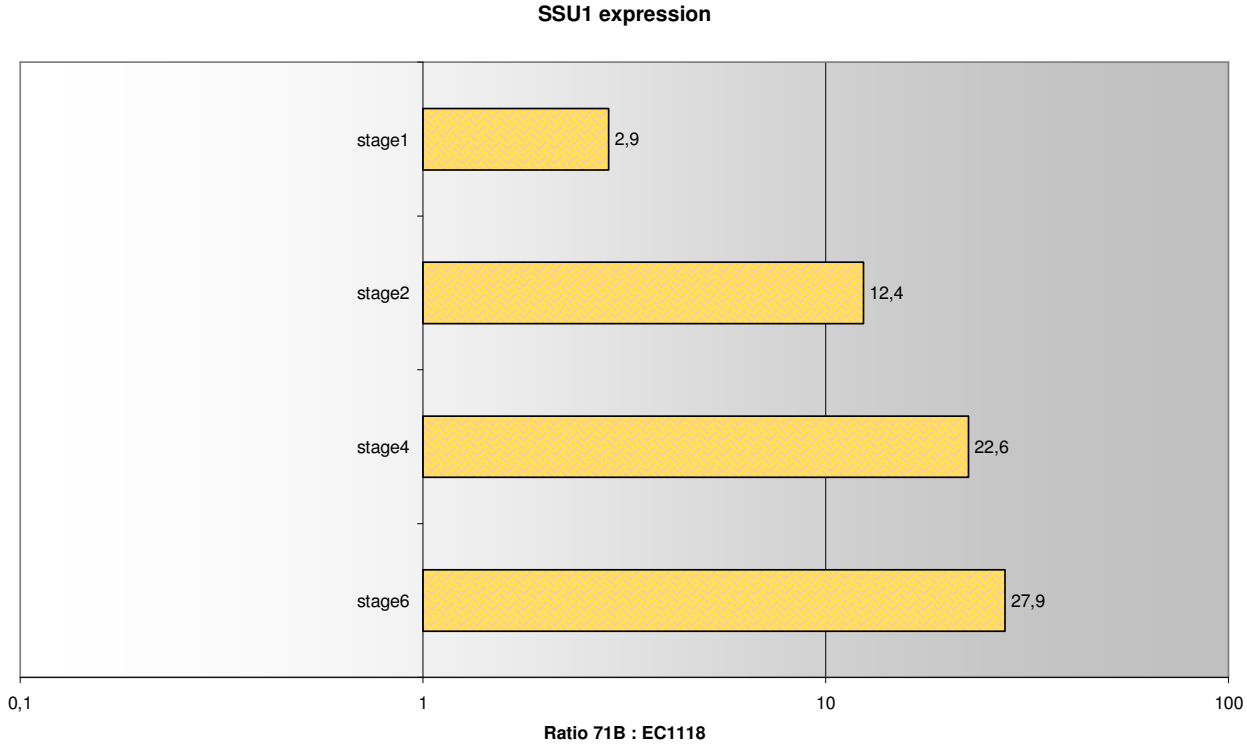

Fig. 3.4.21. Expression ratio of SSU1 gene throughout fermentation. Central value is set to 1 (identical expression in both strains).

3.4.5.4. *SSU1* gene position in the genome

In previous studies it has been found that, in contrast to the allele present in the laboratory strains, a highly sulphite-resistant wine strain exhibited a translocation involving the promoter region of the gene (*SSU1-R* allele), increasing the sulphite resistance [87]. The location of *SSU1-R* upstream region is separated from that of *SSU1*. *SSU1* lies on chromosome XVI, while *SSU1-R* lies on chromosome VIII and it contains up to six repeats of the 76 bp found in the upstream promoter of well-studied strain *ECM34*, as resumed in figure3.4.22. Perez-Ortin *et al* [176] found that the *SSU1-R* allele is the product of reciprocal translocation between chromosomes VIII and XVI.

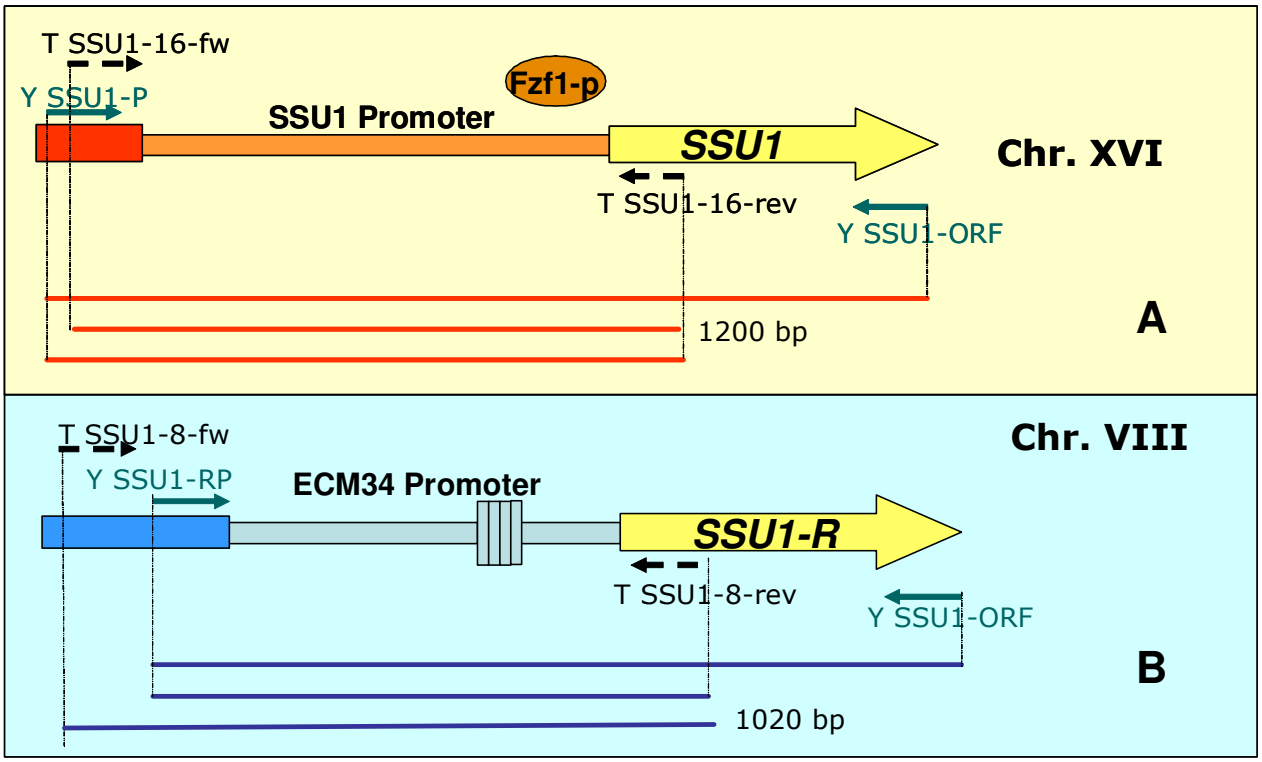

Fig. 3.4.22. Diagram representing two promoters associated with *SSU1* gene in wild type **(A)** and translocated **(B)** genotypes of S. cerevisiae. Primers used for PCR amplification are shown with black-dotted arrows; primers designed by Yuasa et al [269], useful for amplification of both promoter and coding sequence, are also shown with grey arrows.

In the present study, we designed PCR primers to explore the organization of this gene at the molecular level in both EC1118 and 78B strains. Primer pairs were designed for the amplification of SSU1 promoter on chromosome XVI (SSU1-16fw and rev) and of *SSU1*-R promoter on chromosome VIII (SSU1-8fw and rev). Primers sequences are listed in par.3.2.7.2, primers positions are shown in figure 3.4.22 (black dotted arrows).

PCR reactions on genomic DNA from EC1118 and 71B for amplifying *SSU1* and *SSU1-R* promoter regions were performed as described in materials and methods (cfr par. 3.2.7.2).

Gel electrophoresis results are shown in fig 3.4.23.

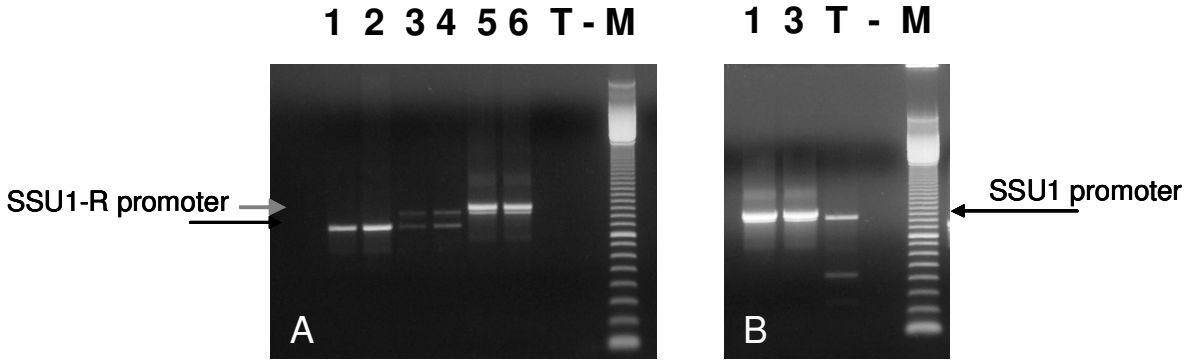

Fig. 3.4.23. **(A)** Gel electrophoresis of *SSU1-R* promoter region amplification on chromosome VIII. Primers used: SSU1-8-fw and SSU1-8-rev. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1&2) EC1118; (3&4) 71B; (5) L2056 (commercial wine strain of *S.cerevisiae*);(6) DV10 (commercial wine strain of *S.cerevisiae*); (T) *S.cerevisiae* type strain NRRLY-12632; - negative control. **(B)** Gel electrophoresis of SSU1 promoter region amplification on chromosome XVI. Primers used: SSU1-PR-fw and SSU1-PR-rev. M, molecular weight marker '100-bp DNA ladder', Amersham. Lanes: (1) EC1118; (3) 71B; (T): S.cerevisiae type strain NRRLY-12632; - negative control.

As expected [268], both strains have either the copy of *SSU1* on chromosome XVI (*SSU-1*) and the copy on chromosome VIII after translocation (*SSU1-R*), situation that is widespread among wine yeasts. EC1118 showed a *SSU1-R* promoter fragment whose length corresponds to 2 repetitions of the 76 bp *ECM34* enhancer sequence. Other commercial wine strains as DV10 or L2056 by Lallemand showed *SSU-R* promoter amplicons whose length corresponds to 4 repetitions of 76 bp sequence. Interestingly 71B showed both fragments. It seems that this strain has one *SSU1-R* copy containing 2 repetitions of the promoter enhancer sequence and one with 4. Since wine yeasts are usually diploid, it has already been shown that there are strains having 2 copies of *SSU-R*, but usually they have no copy of *SSU1* on chromosome XVI. From this findings, the outstanding feature of 71B strain is to own, very probably, two different copies of *SSU1-R* and one of *SSU1*.

3.4.5.5. Sequence of SSU1 promoters

All the amplification fragments shown in figure 3.4.23 have been sequenced from both edges (separate sequence reactions with forward and reverse primers) after electrophoretic separation. Resulting chromatograms were checked and sequences aligned to *SSU1* and *SSU1-R* promoter regions by CLUSTAL W [245].

Sequence alignments confirmed the *SSU1* promoter on chromosome XVI for both strains, as expected from literature. There were small differences in sequences, in particular 3 identical point mutations in both strains were found (at positions -114, -228 and -266 from ATG), two point mutations occurred only in EC1118 (at positions –465 and -468 from ATG) and other two exclusively in 71B (at positions -146 and -488 from ATG). For full alignments see Appendix B.

Part of the sequence comparisons (only the promoter regions that showed differences) obtained from chromosome VIII amplification are reported in figure 3.4.24 and 3.4.25.

A.

```
71B-high band   AGATGCATT-TCCAATTTGAGGCCAGTTTCTGAGGGTTCTCGTATTTCAACAATAATGTT 79
SSU1-R PROM     GCATGCATTGTCCAATTTGAGGCCAGTTTCTGAGGGTTCTCGTATTTCAACAATAATGTT 660
                  ******* **************************************************

71B-high band   GTTGCGTAATGTACCGCACTGTGTGCGGCATTTTTGAGCCAGTTTTTGCTTATTCTCGTA 139
SSU1-R PROM     GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 720
                ***** ************** ************************ **************

71B-high band   TTTCAACAATAATGTTGTTGCATAATGTTCCGCCCTTTGTGCGGCATTTTTGAGCCAGTT 199
SSU1-R PROM     TTTCAACAATAATGTTGTTGCATAATGTTCCGCCCTTTGTGCGGCATTTTTGAGCCAGTT 780
                ************************************************************

71B-high band   TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 259
SSU1-R PROM     TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 840
                ************************************************************

71B-high band   CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 319
SSU1-R PROM     CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 900
                ************************************************************

71B-high band   ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 379
SSU1-R PROM     ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 960
                ************************************************************
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B.

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SSU1-R PROM     GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 660
71B-low band    GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 232
                ************************************************************

SSU1-R PROM     GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 720
71B-low band    GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 292
                ************************************************************

SSU1-R PROM     TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTT 780
71B-low band    TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTT---------- 342
                ************************************************

SSU1-R PROM     TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 840
71B-low band    ------------------------------------------------------------

SSU1-R PROM     CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 900
71B-low band    ------------------------------------------------------------

SSU1-R PROM     ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 960
71B-low band    ----------------------ATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 380
                                       ************************************
```

Fig. 3.4.24 Sequence alignments of the two *SSU1-R* amplicons found in 71B with *SSU1-R* promoter of the strain Y-9 (genbank accession number AB002531). Colours show different repetitions of 76 bp *ECM34* enhancer region.

Figure 3.4.24 shows *SSU1-R* promoter fragments obtained in 71B strain: the shorter lacked two repetitions of the 76bp enhancer region from *ECM34* promoter, whereas the longer had four complete repetitions of this small sequence. The rest of the alignment (not shown, available in Appendix B) was completely identical for both queries (shorter and longer fragments). In EC1118, the single amplicon found corresponded to the 71B shorter fragment, having 2 complete repetitions of the 76 bp enhancer (as shown in figure 3.4.25).

```
SSU1-R PROM       GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 660
EC1118            GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 507
                  ************************************************************

SSU1-R PROM       GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 720
EC1118            GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 567
                  ************************************************************

SSU1-R PROM       TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTT 780
EC1118            TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTT---------- 617
                  ********************************************

SSU1-R PROM       TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 840
EC1118            ------------------------------------------------------------


SSU1-R PROM       CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 900
EC1118            ------------------------------------------------------------


SSU1-R PROM       ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 960
EC1118            ----------------------ATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 655
                                         **********************************
```

Fig. 3.4.25 Sequence alignment of the EC1118 SSU1-R amplification with SSU1-R promoter of the strain Y-9 (genbank accession number AB002531). Colours show different repetitions of 76 bp *ECM34* enhancer region.

To better understand the different regulation of *SSU1* gene in the two strains further experiments are needed. A deeper characterization of sulphite resistance of 71B in different musts will be performed: up to date the two strains showed the same sulphite resistance level at concentrations of 50 and 100 ppm, even if at higher concentrations results were less reproducible. In any case it is important to remark that enological yeasts have different systems for sulphite resistance, some of them are even independent from *SSU1* transport (i.e. enhanced acetaldehyde production). The *SSU1* activation can be due to different mechanisms, for example a point mutation in its *FZF1* regulator [173]. Gene sequencing of *FZF1* in both strains will be carried out in the next future.

4. Conclusions

Originally, all wine was made by taking advantage of natural microflora for spontaneous fermentation; no deliberate inoculation was made to start the process. A breakthrough was made in 1880 when Hansen, working at the Carlsberg brewery 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 [185]. In 1965, the first two commercial dried yeasts (ADWY) strains were produced for a large Californian winery [68]; these two strains were then 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 a steady product during the successive vintages. Today, several yeast-manufacturing companies market a wide variety of dehydrated cultures of *S. cerevisiae* strains, and most of worldwide wine production relies on the use of such commercial starter yeasts.

The main critics to the practice of guided fermentations (using starter cultures) dislike the fact that the commercial wine strains, despite being a considerable number, possess very ordinary characteristics: commercial yeast strains produce wines with average qualities and do not enhance the aromatic traits that characterise specific geographical areas [185]. Moreover, several studies support the hypothesis that active dried yeasts reduce the variability of strains that appear in spontaneous fermentations [17, 71] and, possibly, the complexity of the resulting wine.

The reason of these problems can be found in the criterions traditionally used for wine yeast selection: in the past 30 years, enological strains of *S. cerevisiae* have been selected for their technological traits such as fermentation performance, ethanol tolerance and absence of undesirable compounds [174]. As the *S. cerevisiae's* role in winemaking is now firmly established, there is an ever-growing demand for new specialised wine yeast strains. Studies on the improvement and the selection of new wine yeasts to overcome this problem have recently been carried out with the aim of proposing a “second generation” of starters that may prevent excessive standardization engendered by the presence of only few active dry commercial starters in the international market [142]. In addition to the primary role of wine yeast to catalyze the efficient and complete conversion of grape sugars to alcohol without the development of off-flavours, starter culture strains of *S. cerevisiae* must now possess a range of other properties, taking into account that strains involved in

fermentation play an important rule in determining the chemical composition and sensory qualities of the resulting wine [130]. The importance of these additional yeast characteristics differs with the type and style of wine to be made and the technical requirements of the winery [185].

The diversity of native *S. cerevisiae* strains present in spontaneous fermentations is known to largely contribute to the chemical composition of the wine by producing notable differences in the texture or mouth-feel and intensifying the varietal flavour [130]. In the last few years there has been an increasing use of new local selected yeasts for controlled must fermentation in countries with a wine-making tradition. Moreover, the use of local selected yeasts is believed to be much more effective [46, 149, 190] mainly exalting the sensory properties of the typical regional wines [207].

For these reasons, lots of recent selection projects for new wine strains focus on ecotypical autochthonous yeast, trying to preserve biodiversity in selected areas and at the same time to guarantee territory-linked characteristics in wine. Unfortunately most of these strains show fermentation performances that are lower than the ones found for traditional commercial yeasts. A better understanding of the molecular mechanisms involved in wine yeast metabolism during alcoholic fermentation, the knowledge of genetic features as well as specific expression profiles of yeast strains could help to understand more clearly the biological process of fermentation at molecular level and to find the suitable working condition to improve the fitness of these new native strains. During the last two decades a considerable knowledge of *S. cerevisiae* genetics and physiology has been generated as well as numerous genetics tools. Among them, implementation of functional genomics programs on wine yeasts, improving the knowledge at molecular level, will enable, after all, to better control the vinification and to refine wine-making practices during alcohol fermentations in order to maximize wine quality even when native strains are used [185].

On this context, the work proposed in the thesis tries to investigate the problems concerning the selection and the utilization of the new-born ecotypical autochthonous yeasts by two different approaches, on one hand, improving the isolation and identification methods that are at the basis of selection programs, on the other, trying to understand at molecular level their behaviour during alcoholic fermentation.

In the first part of this work, an innovative molecular test for characterizing new wine yeasts is proposed to facilitate and accelerate the initial phase of strain collection during clonal selection programs, when several hundred of isolates are screened to find those that possess important enological properties. Since in most of the cases this equivalent to establish if these strains belong to the *Saccharomyces sensu stricto* complex, the efforts were focused on the construction of a method that could rapidly, easily and unambiguously identify these enological yeasts.
For this purpose an original pair of primers, designed within the variable D1/D2 region of the 26S subunit of ribosomal yeast RNA, was constructed. These generate an amplification fragment specific for the *Saccharomyces sensu stricto* species, while no signal was obtained for *Saccharomyces sensu lato* strains or for another 18 selected species commonly found in enological environments. A second pair of primers was also constructed, within the 18S rRNA gene, composed of perfectly conserved sequences common for all 42 yeast species examined, which generate a common band for all strains. The more traditional ITS analysis was also tested to evaluate the possibility of applying this technique to a enological environment. The two methods allow a “genotypic characterization” of wine strains that is required to start a “technological characterization” for the definition of the enological traits.

With the aim of trying to understand differences between the behaviours of a strong fermenter yeast compared with those of a less vigorous strain able to enhance the sensory qualities of wine, the second part of the project was performed taking advantage from DNA-microarray technology, which allows whole genome expression profiling, measuring in a single assay the global transcriptional response. For this purpose an investigation of yeast metabolic shifts at transcriptional level in both laboratory and industrial conditions was faced up. The commercial strains widely used in wineries, Lallemand EC1118 and Lallemand 71B, were compared during fermentation: the former, 71B, is known to be a strong producer of fermentative aroma and is therefore used in musts lacking varietal flavours or for the production of *nouveau* wines. The latter, EC1118, is an efficient fermenter, widely used also for sparkling wines, but quite neutral as regards both varietal and fermentative aroma. For each strain, two different fermentation settings were carried out: laboratory (1 litre) and pilot (100 litres) scale trials were performed in natural white must.

The obtained results show that the up-regulated genes during the scale-up experiment seem to be linked to anaerobiosis stress response (for example *DAN/TIR* and *PAU* gene families), probably due to small differences in fermentation conditions which have been sensed by yeast. In general, the metabolic shifts caused by scale-up did not affect anabolic o catabolic pathways but seem to reflect a stress response. A slight difference in oxygen availability between the two sizes of fermenters could explicate this phenomenon (in the experimental practice, strong anaerobiosis was easier to manage in 1 litre fermentations than in 100 litres).

Comparing the two strains, the metabolic pathway of sulphured amino acids production displayed an higher expression in 71B, in particular during first stages of fermentation. Moreover, all identified genes known to be involved in production of fermentative aromas, such as esters and higher alcohols, showed a slightly higher expression in 71B and confirmed literature data of being more expressed in stationary and late-stationary phase than in active growth phase. Finally, the sulphite efflux responsible gene *SSU1* displayed a higher expression in 71B; in addition, in this strain, this gene seemed to be inducible during fermentation, increasing when the yeast reaches the stationary phase. Although the genetics of this permease was already known, in this work the first evidence for a regulated *SSU1* gene expression is reported. All these evidences have been confirmed by Real-time PCR, a high throughput tool for expression analysis. Moreover *SSU1* position in the genome of these yeasts was determined, since its expression level is affected by a promoter change when the frequent translocation between chromosomes VIII and XVI takes place [176, 268] changing the position of this gene.

These expression results open new fields of investigation. In fact fermentation performances of these strains were, as expected, really different, but microarray data (concerning early stationary phase) did not show differences in general regulation of pathways directly involved in alcoholic fermentation, amino acids uptake and consumption, even if this last aspect could be further investigated since sulphured amino acids biosynthesis, as mentioned before, resulted different between the two strains.

Moreover, 71B behaviours towards sulphite will be deeply analyzed through sulphite resistance tests and sulphite uptake determinations, since a link could exist between

the up regulation of both the detoxifying membrane pump *SSU1* and sulphite reductase which opens sulphured amino acid biosynthetic pathway.
Then, the unexpected finding that some *ATP* genes, encoding for 7 subunits of the ATP synthase, were up-regulated in the stronger fermenter EC1118, should be also confirmed to understand their meaning in fermentation conditions, where aerobic respiration is supposed to be null. Furthermore, in the near future, the link between expression findings on genes involved in aroma production and 71B behaviours will be further characterized by chemical analysis of aromatic compounds on the end-point products. This will enable to establish if the general trend of expression of these genes really corresponds to a tangible difference in aromatic compounds concentrations.

All these results try to better understand metabolism of those yeasts that are able to strongly affect sensorial properties of wine, even being weaker fermenters if compared with traditional industrial strains; this gives the basis for analysis of new strains selected with this innovative criterion.

5. References

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Appendix A

Real-Time PCR data

1.Genes involved in aroma production

Threshold cycles (Ct)

	FBA	ACT	ATF 1	ATF 2	IAH 1	EHT 1	EEB 1
F16	18,8	12,9	20,1	22,2	20,5	21,4	17,8
	18,8	12,8	20,2	22,3	20,7	21,3	17,8
	18,7	12,8	20,5	22,2	20,5	21,4	17,8
mean	18,77	12,83	20,27	22,23	20,57	21,37	17,80
st dev	0,06	0,06	0,21	0,06	0,12	0,06	0,00
cv %	0,31	0,45	1,03	0,26	0,56	0,27	0,00
F17	18,7	13,3	21	23,1	20,7	22	18,3
	18,9	13,4	20,6	23	20,5	22	18,4
	19,8	13,8	20,6	22,9	19,8	21,9	18,4
mean	19,13	13,50	20,73	23,00	20,33	21,97	18,37
st dev	0,59	0,26	0,23	0,10	0,47	0,06	0,06
cv %	3,06	1,96	1,11	0,43	2,32	0,26	0,31
F18	21,5	16	22	22,3	21,8	23,2	19,1
	21,6	16	22	22,1	22	23	19
	21,9	15,8	21,8	22,2	22	23,1	19,1
mean	21,67	15,93	21,93	22,20	21,93	23,10	19,07
st dev	0,21	0,12	0,12	0,10	0,12	0,10	0,06
cv %	0,96	0,72	0,53	0,45	0,53	0,43	0,30
F19	21,2	16,1	22,6	22,3	22,1	23,7	18,9
	21,4	16,2	22,4	22,3	22	23,4	19
	21,5	16,3	22,7	22,3	21,8	23,3	19
mean	21,37	16,20	22,57	22,30	21,97	23,47	18,97
st dev	0,15	0,10	0,15	0,00	0,15	0,21	0,06
cv %	0,71	0,62	0,68	0,00	0,70	0,89	0,30

ΔCt and statistics

		ATF1-ACT	ATF2-ACT	IAH1-ACT	EHT1-ACT	EEB1-ACT
F16	**mean**	7,43	9,40	7,73	8,53	4,97
	st dev	0,22	0,08	0,13	0,08	0,06
F17	**mean**	7,23	9,50	6,83	8,47	4,87
	st dev	0,35	0,28	0,54	0,27	0,27
EC1118	**mean**	7,33	9,45	7,28	8,50	4,92
F18	**mean**	6,00	6,27	6,00	7,17	3,13
	st dev	0,16	0,15	0,16	0,15	0,13
F19	**mean**	6,37	6,10	5,77	7,27	2,77
	st dev	0,18	0,10	0,18	0,23	0,12
71B	**mean**	6,18	6,18	5,88	7,22	2,95
	p-val t-test	0,05	0,003	0,18	0,004	0,045
71B-EC1118	**ΔΔCt**	-1,15	-3,27		-1,28	-1,97
	$2^{-\Delta\Delta Ct}$	2,11	8,57		2,03	3,61

2.Genes involved in aroma production, time course analysis

Threshold cycles (Ct)

	FBA	ACT	ATF 1	ATF 2	IAH 1	EHT 1	EEB 1
	16,80	11,90	19,20	20,90	21,50	22,00	18,60
EC1118-1	16,60	12,10	19,20	20,90	21,30	21,90	18,70
	16,60	12,00	19,00	21,00	21,40	21,90	18,90
mean	16,67	12,00	19,13	20,93	21,40	21,93	18,73
st dev	0,12	0,10	0,12	0,06	0,10	0,06	0,15
cv%	0,69	0,83	0,60	0,28	0,47	0,26	0,82
	24,90	19,70	26,80	28,40	27,60	28,60	24,70
EC1118-4	25,20	19,70	26,50	28,50	27,30	28,30	24,60
	25,00	19,60	26,80	28,60	27,40	28,40	24,60
mean	25,03	19,67	26,70	28,50	27,43	28,43	24,63
st dev	0,15	0,06	0,17	0,10	0,15	0,15	0,06
cv%	0,61	0,29	0,65	0,35	0,56	0,54	0,23
	22,50	16,80	22,00	24,30	22,60	24,70	20,40
EC1118-6	22,10	16,60	22,00	24,50	22,60	24,30	20,10
	21,90	16,80	21,80	24,30	22,80	24,60	20,20
mean	22,17	16,73	21,93	24,37	22,67	24,53	20,23
st dev	0,31	0,12	0,12	0,12	0,12	0,21	0,15
cv%	1,38	0,69	0,53	0,47	0,51	0,85	0,75

	FBA	ACT	ATF 1	ATF 2	IAH 1	EHT 1	EEB 1
	16,20	11,90	18,80	20,10	23,80	21,70	20,10
71B-2	16,10	12,10	N/A	20,20	24,10	21,90	19,90
	16,00	12,00	19,00	N/A	24,00	21,90	20,00
mean	16,10	12,00	18,90	20,15	23,97	21,83	20,00
st dev	0,10	0,10	0,14	0,07	0,15	0,12	0,10
cv%	0,62	0,83	0,75	0,35	0,64	0,53	0,50
	21,40	16,10	22,50	23,90	23,90	23,80	19,80
71B-4	21,40	16,00	22,50	24,00	24,00	23,70	19,90
	21,30	16,00	22,40	23,90	23,70	23,60	19,80
mean	21,37	16,03	22,47	23,93	23,87	23,70	19,83
st dev	0,06	0,06	0,06	0,06	0,15	0,10	0,06
cv%	0,27	0,36	0,26	0,24	0,64	0,42	0,29
	25,40	19,90	25,10	25,90	26,90	27,30	22,70
71B-6	25,20	19,90	24,60	26,10	26,80	27,60	22,50
	25,60	20,00	24,70	26,10	26,80	27,30	22,50
mean	25,40	19,93	24,80	26,03	26,83	27,40	22,57
st dev	0,20	0,06	0,26	0,12	0,06	0,17	0,12
cv%	0,79	0,29	1,07	0,44	0,22	0,63	0,51

ΔCt and statistics

		ATF1-ACT	ATF2-ACT	IAH1-ACT	EHT1-ACT	EEB1-ACT
EC1118-1	mean	7,13	8,93	9,40	9,93	6,73
	st dev	0,15	0,12	0,14	0,12	0,18
EC1118-4	mean	7,03	8,83	7,77	8,77	4,97
	st dev	0,18	0,12	0,16	0,16	0,08
EC1118-6	mean	5,20	7,63	5,93	7,80	3,50
	st dev	0,16	0,16	0,16	0,24	0,19

		ATF1-ACT	ATF2-ACT	IAH1-ACT	EHT1-ACT	EEB1-ACT
71B-1	mean	6,90	8,15	11,97	9,83	8,00
	st dev	0,17	0,12	0,18	0,15	0,14
71B-4	mean	6,43	7,90	7,83	7,67	3,80
	st dev	0,08	0,08	0,16	0,12	0,08
71B-6	mean	4,87	6,10	6,90	7,47	2,63
	st dev	0,27	0,13	0,08	0,18	0,13
stage1	p-val t-test	0,03	0,03	0,01	0,06	0,02
stage4	p-val t-test	0,03	0,01	0,08	0,02	0,01
stage6	p-val t-test	0,03	0,01	0,02	0,03	0,04
stage1	ΔΔCt	-0,23	-0,78	2,57		1,27
	$2^{-\Delta\Delta Ct}$	1,18	1,72	0,17		0,42
stage4	ΔΔCt	-0,60	-0,93		-1,10	-1,17
	$2^{-\Delta\Delta Ct}$	1,52	1,91		2,14	2,24
stage6	ΔΔCt	-0,33	-1,53	0,97	-0,33	-0,87
	$2^{-\Delta\Delta Ct}$	1,26	2,89	0,51	1,26	1,82

3.*MET10* and *MET17* genes

Threshold cycles (Ct)

	FBA	ACT	MET 10	MET 17
F16	20	14,1	23,1	21,3
	20,1	14,1	22,6	21,3
	20,3	14,6	22,4	21,6
mean	20,13	14,27	22,70	21,40
st dev	0,15	0,29	0,36	0,17
cv %	0,76	2,02	1,59	0,81
F17	20,2	14,9	23,5	22,2
	20,5	15	23,4	22
	20,4	14,7	23,2	22
mean	20,37	14,87	23,37	22,07
st dev	0,15	0,15	0,15	0,12
cv %	0,75	1,03	0,65	0,52
F18	21,6	16,4	22,2	22,2
	21,5	16,2	22,3	22,3
	21,6	16,2	22,1	22,3
mean	21,57	16,27	22,20	22,27
st dev	0,06	0,12	0,10	0,06
cv %	0,27	0,71	0,45	0,26
F19	20,4	14,6	21	20,8
	20,4	14,8	20,8	20,6
	20,2	14,7	21	20,6
mean	20,33	14,70	20,93	20,67
st dev	0,12	0,10	0,12	0,12
cv %	0,57	0,68	0,55	0,56

ΔCt and statistics

		MET10 - ACT	MET17 - ACT
F16	**mean**	8,43	7,13
	st dev	0,46	0,34
F17	**mean**	8,50	7,20
	st dev	0,22	0,19
EC1118	**mean**	8,47	7,17
F18	**mean**	5,93	6,00
	st dev	0,15	0,13
F19	**mean**	6,23	5,97
	st dev	0,15	0,15
71B	**mean**	6,08	5,98
	p-val t-test	0,032	0,005
71B-EC1118	**ΔΔCt**	-2,38	-1,18
	$2^{-\Delta\Delta Ct}$	5,19	2,3

4.*MET10* and *MET17* genes , time course analysis

Threshold cycles (Ct)

	FBA	ACT	MET10	MET17		FBA	ACT	MET10	MET17
	18,2	13,9	19,1	16,4		18,2	13,6	17,7	16,3
EC1118-1	18,1	13,6	18,9	16,4	71B-1	18	13,5	17,7	16,4
		13,7	18,9	16,3			13,5	17,6	16,3
mean	18,15	13,73	18,97	16,37	**mean**	18,10	13,53	17,67	16,33
st dev	0,07	0,15	0,12	0,06	**st dev**	0,14	0,06	0,06	0,06
cv%	0,39	1,11	0,61	0,35	**cv%**	0,78	0,43	0,33	0,35
	18,8	15	22,7	20,7		20,7	16,8	17,7	21,7
EC1118-2	18,9	14,9	22,8	20,8	71B-2	20,6	16,9	17,7	21,6
		14,9	22,6	20,6			16,8	17,6	21,7
mean	18,85	14,93	22,70	20,70	**mean**	20,65	16,83	17,67	21,67
st dev	0,07	0,06	0,10	0,10	**st dev**	0,07	0,06	0,06	0,06
cv%	0,38	0,39	0,44	0,48	**cv%**	0,34	0,34	0,33	0,27
	27,3	N/A	28,5	28,6		23,1	18	N/A	23,7
EC1118-4	27,4	21,1	28,3	28,6	71B-4	23,1	18	23,2	23,6
		21	28,7	28,5			18,1	23,4	23,6
mean	27,35	21,05	28,50	28,57	**mean**	23,10	18,03	23,30	23,63
st dev	0,07	0,07	0,20	0,06	**st dev**	0,00	0,06	0,14	0,06
cv%	0,26	0,34	0,70	0,20	**cv%**	0,00	0,32	0,61	0,24
	23,9	18,6	26	25,6		27,2	21,1	29,1	26,4
EC1118-6	23,8	18,6	25,9	25,5	71B-6	26,7	21,5	28,9	25,9
		18,6	25,8	25,5			21,3	28,6	N/A
mean	23,85	18,60	25,90	25,53	**mean**	26,95	21,30	28,87	26,15
st dev	0,07	0,00	0,10	0,06	**st dev**	0,35	0,20	0,25	0,35
cv%	0,30	0,00	0,39	0,23	**cv%**	1,31	0,94	0,87	1,35

ΔCt and statistics

		MET10-ACT	MET17-ACT
EC1118-1	mean	5,23	2,63
	st dev	0,12	0,12
EC1118-2	mean	7,77	5,77
	st dev	0,12	0,12
EC1118-4	mean	7,45	7,52
	st dev	0,21	0,09
EC1118-6	mean	7,30	6,93
	st dev	0,10	0,06
71B-1	mean	4,13	2,80
	st dev	0,08	0,08
71B-2	mean	0,83	4,83
	st dev	0,08	0,08
71B-4	mean	5,27	5,60
	st dev	0,15	0,08
71B-6	mean	7,57	4,85
	st dev	0,32	0,41
stage1	p-val t-test	0,01	0,28
stage2	p-val t-test	0,0003	0,01
stage4	p-val t-test	0,02	0,002
stage6	p-val t-test	0,43	0,02
stage1	ΔΔCt	-1,10	
	$2^{-\Delta\Delta Ct}$	2,14	
stage2	ΔΔCt	-6,93	-0,93
	$2^{-\Delta\Delta Ct}$	122,22	1,91
stage4	ΔΔCt	-2,18	-1,92
	$2^{-\Delta\Delta Ct}$	4,54	3,78
stage6	ΔΔCt		-2,08
	$2^{-\Delta\Delta Ct}$		4,24

5. *SSU1* gene

Threshold cycles (Ct)

	FBA	ACT	SSU1
F16	20	15,9	22,4
	20,1	16,1	22,2
	20,3	16,4	22,6
mean	20,13	16,13	22,40
st dev	0,15	0,25	0,20
cv %	0,76	1,56	0,89
F17	20,2	16,1	22,2
	20,5	16,1	21,9
	20,4	16	22,1
mean	20,37	16,07	22,07
st dev	0,15	0,06	0,15
cv %	0,75	0,36	0,69
F18	21,6	18,1	21,1
	21,5	18	21,5
	21,6	18,1	20,9
mean	21,57	18,07	21,17
st dev	0,06	0,06	0,31
cv %	0,27	0,32	1,44
F19	20,4	16,5	19,8
	20,4	16,3	19,6
	20,2	16,2	19,6
mean	20,33	16,33	19,67
st dev	0,12	0,15	0,12
cv %	0,57	0,94	0,59

ΔCt and statistics

		SSU1-ACT
F16	mean	6,27
	st dev	0,32
F17	mean	6,10
	st dev	0,16
EC1118	mean	6,18
F18	mean	3,10
	st dev	0,31
F19	mean	3,33
	st dev	0,19
71B	mean	3,22
	p-val t-test	0,004
71B-EC1118	ΔΔCt	-2,97
	$2^{-\Delta\Delta Ct}$	7,82

6. *SSU1* gene, time course analysis

Threshold cycles (Ct)

	FBA	ACT	SSU1		FBA	ACT	SSU1
	18,2	14,3	20,9		18,2	14	19,1
EC1118-1	18,1	14,3	20,9	71B-1	18	13,8	19
		14	21			13,7	19
mean	18,15	14,20	20,93	**mean**	18,10	13,83	19,03
st dev	0,07	0,17	0,06	**st dev**	0,14	0,15	0,06
cv%	0,39	1,22	0,28	**cv%**	0,78	1,10	0,30
	18,8	13,8	20,1		20,7	17,2	19,5
EC1118-2	18,9	13,7	20,2	71B-2	20,6	16,5	19,6
		13,6	20,2			16,5	19,6
mean	18,85	13,70	20,17	**mean**	20,65	16,73	19,57
st dev	0,07	0,10	0,06	**st dev**	0,07	0,40	0,06
cv%	0,38	0,73	0,29	**cv%**	0,34	2,42	0,30
	27,3	21,5	27,8		23,1	17,9	19,9
EC1118-4	27,4	21,6	27,9	71B-4	23,1	18,7	20,1
		21,6	27,7		23,00	18,1	19,9
mean	27,35	21,57	27,80	**mean**	23,07	18,23	19,97
st dev	0,07	0,06	0,10	**st dev**	0,06	0,42	0,12
cv%	0,26	0,27	0,36	**cv%**	0,25	2,28	0,58
	23,9	18,5	24,4		27,2	21,9	23,3
EC1118-6	23,8	18,4	24,4	71B-6	26,7	22	22,8
		18,5	24,4			21,8	23
mean	23,85	18,47	24,40	**mean**	26,95	21,90	23,03
st dev	0,07	0,06	0,00	**st dev**	0,35	0,10	0,25
cv%	0,30	0,31	0,00	**cv%**	1,31	0,46	1,09

ΔCt and statistics

		SSU1-ACT
EC1118-1	mean	6,73
	st dev	0,12
EC1118-2	mean	6,47
	st dev	0,12
EC1118-4	mean	6,23
	st dev	0,12
EC1118-6	mean	5,93
	st dev	0,06
71B-1	mean	5,20
	st dev	0,41
71B-2	mean	2,83
	st dev	0,41
71B-4	mean	1,73
	st dev	0,43
71B-6	mean	1,13
	st dev	0,27
stage1	p-val t-test	0,04
stage2	p-val t-test	0,009
stage4	p-val t-test	0,003
stage6	p-val t-test	0,002
stage1	ΔΔCt	-1,53
	$2^{-\Delta\Delta Ct}$	2,89
stage2	ΔΔCt	-3,63
	$2^{-\Delta\Delta Ct}$	12,41
stage4	ΔΔCt	-4,50
	$2^{-\Delta\Delta Ct}$	22,63
stage6	ΔΔCt	-4,80
	$2^{-\Delta\Delta Ct}$	27,86

7. Calibration curves

equations and correlation coefficients

ACT1: $y = -3{,}575x + 9{,}8417$ $R^2 = 0{,}9973$

FBA1: $y = -3{,}775x + 14{,}208$ $R^2 = 1$

ATF1: $y = -3{,}7031x + 20{,}831$ $R^2 = 0{,}9983$

ATF2: $y = -3{,}712x + 22{,}488$ $R^2 = 0{,}9983$

IAH1: $y = -3{,}7883x + 22{,}366$ $R^2 = 0{,}9963$

EEB1: $y = -3{,}8956x + 18{,}284$ $R^2 = 0{,}997$

EHT1: $y = -3{,}6159x + 22{,}098$ $R^2 = 0{,}9977$

MET10: $y = -3{,}795x + 21{,}208$ $R^2 = 1$

MET17: $y = -3{,}6822x + 20{,}079$ $R^2 = 0{,}9967$

SSU1: $y = -3{,}7343x + 16{,}392$ $R^2 = 0{,}9963$

Appendix B

Alignments

1.Chromosome XVI *SSU1* promoter – EC1118 sequences

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CLUSTAL W (1.83) multiple sequence alignment
SSU1 PROM refers to Genbank sequence YPL092W
EC1118-SSU1-16fw: sequence on EC1118 DNA obtained with SSU1-16fw primer.
EC1118-SSU1-16rev: sequence on EC1118 DNA obtained with SSU1-16rev primer.
A mutation
A mutated also in 71B
A not reliable in chromatogram


SSU1 PROM          ---------------------GCCAAGATGG---AAAGAAGAGAAAGAAATAGACATGCCA 37
EC1118-SSU1-16rev  TTNTANNTCNNNAAGNNCNNNNNACGATGGCCAAGATGNAGAGAGNNNNTAGACATGCCA 60
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          AGCAA-GGTGAATCTGATAGACACAATGCTGTTTCCTTATCAAAGCATTTATTCAGTGGT 96
EC1118-SSU1-16rev  AGCAAAGGTGAATCTGATAGACACAATGCTGTTTCCTTATCAAAGCATTTATTCAGTGGT 120
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          AAGCGTGGTGTCGGTAAGACAGATTTCCGTTGATTTCTCTACTTCTTATTCTTTCTTTTG 156
EC1118-SSU1-16rev  AAGCGTGGTGTCGGTAAGACAGATTTCCGTTGATTTCTCTACTTCTTATTCTTTCTTTTG 180
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          CATGTACTTTAAAAAAAA-TATGAATATAAAATGTGGATGTACGTACACGACATTCTAAT 215
EC1118-SSU1-16rev  CATGTACTTTAAAAAAAAATATGAATATAAAATGTGGATGTACGTACACGACATTCTAAT 240
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          CTTTTTGGGCTGGTAGGATTACCATTTACATTTGATTATCTCTCTCACTATTAGTGGTTT 275
EC1118-SSU1-16rev  CTTTTTGGGCTGGTAGGATTATCATTTACATTTGATTATCTCTCTCACTATTAGTGGTTT 300
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          CTTTTTCTTTCTTTCTCACTTTTTCTGTATCTTTTTTTTAAAAAAATTTTATTTAATCTG 335
EC1118-SSU1-16rev  CTTTTTCTTTCTTTCTCACTTTTTCTGTATCTTTTTTTTAAAAAAATTTTATTTAATCTG 360
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          TATAATAATAATAAACCGATTTAAATTATCCAACAAGCCGACCCCTCCATGTTCTACTAT 395
EC1118-SSU1-16rev  TATAATAATAATAAACCGATTTAAATTATCCAACAAGCCGGCCCCTCCATGTTCTACTAT 420
EC1118-SSU1-16fw   ------------------------------------------------------------


SSU1 PROM          TTTTTTGTATGTCACTGGATGTATACAAATAATTAAGCATGTGGAAAAAGAAGGGGTGGG 455
EC1118-SSU1-16rev  TTTTTTGTATGTCACTGGATGTATACAAATAATTAAGCATGTGGAAAAAGAAGGGGTGGG 480
EC1118-SSU1-16fw   -------------ACTGGATGTATACAAATAATTAAGCATGNGGAAAAAGAAGGGGTGGG 47
                               **************************** ******************


SSU1 PROM          ATAGCGTCAAGATGACACTTCTACTTTTTTGTGACACATCATCATGCAACCTATCGAGTC 515
EC1118-SSU1-16rev  ATAGCGTCAAGATGACACTTCTACTTTTTTGTGACACATCATCATGCAACCTATCGAGTC 540
EC1118-SSU1-16fw   ATAGCGTCAAGATGACACTTCTACTTTTTTGTGACACATCATCATGCAACCTATCGAGTC 107
                   ************************************************************


SSU1 PROM          TCCCACGAGGTTGACAAATAAGAAATTGTTATCGTTTTTGCAGTGTATCGTATAAGGCAA 575
EC1118-SSU1-16rev  TCCCACGAGGTTGACAAATAAGAAATTGTTATCGTTTTTGCAGCGTATCGTATAAGGCAA 600
EC1118-SSU1-16fw   TCCCACGAGGTTGACAAATAANAAATTGTTATCGTTTTTGCAGCGTATCGTATAANGCAA 167
                   ******************** ******************** *********** ****


SSU1 PROM          CAATAGCGATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTATCATTTTTTTTTT 635
EC1118-SSU1-16rev  CAATAGCGATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTATCATATTATTTTT 660
EC1118-SSU1-16fw   CAATAGCNATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTATCATATTATTTTT 227
                   ******* ****************************************** ** *****
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Appendix B

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SSU1 PROM           TTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAATCTTAAAAAACAG 695
EC1118-SSU1-16rev   TTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAATCTTAAAAAACAG 720
EC1118-SSU1-16fw    TTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAATCTTAAAAAACAG 287
                    ************************************************************

SSU1 PROM           AAGACTCATCGCCGTCTTTGGCACATTGGGGTTGTCTTTGACTTCTTTTGCTTATTTTTG 755
EC1118-SSU1-16rev   AAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTTTGCTTATTTTTG 780
EC1118-SSU1-16fw    AAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTTTGCTTATTTTTG 347
                    ********************************** ************************

SSU1 PROM           CTCACTTTGCTTATTTTACCTATTTAACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTG 815
EC1118-SSU1-16rev   CTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTG 840
EC1118-SSU1-16fw    CTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTG 407
                    ************ ********************************************

SSU1 PROM           TAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATATTGGCTGAACAAA 875
EC1118-SSU1-16rev   TAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATATTGGCTGAACAAA 900
EC1118-SSU1-16fw    TAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATATTGGCTGAACAAA 467
                    ************************************************************

SSU1 PROM           TTCTCCGCATTTAGACAACACACAAATTACAGCTTTCCCCTAGTAACGATTGTTGATTGA 935
EC1118-SSU1-16rev   TTCTCCACATTTAGACAACACACAAATTACAGCTTTCCCCTAGTAACGATTGTTGATTGA 960
EC1118-SSU1-16fw    TTCTCCACATTTAGACAACACACAAATTACAGCTTTCCCCTAGTTACGATTGTTGATTGA 527
                    ****** ********************************** ***************

SSU1 PROM           GCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGTACAAGA 995
EC1118-SSU1-16rev   GCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGTACAAGA 1020
EC1118-SSU1-16fw    GCTCAGACA-TACGCGCAATTTAAAA-CGTTTTATAGTGTAAGAGA-GACAAGTACAGGA 584
                    ********* ***************** ****************** ********** **

SSU1 PROM           AAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACCCCTTCATGTTTA 1055
EC1118-SSU1-16rev   AAAAAATGGT-GCCAATTGNNCNTNCNNNC------------------------------ 1049
EC1118-SSU1-16fw    AAAAAN-GGTTGCNATTGGNACTTGCTCTTACGANGCAGTTTGACCCCTCATGTTTGTGA 643
                    *****  *** ** * * *

SSU1 PROM           TGATGGTCATGGGTGTCGGCATTTCATCGAATATTCTATATAGCTTCCCATATCCTGCAA 1115
EC1118-SSU1-16rev   ------------------------------------------------------------
EC1118-SSU1-16fw    TGGTCATGGNGTCGNATTCATCGATATTTCNANNNANCTNNNNATCCTGCAGGGGCTAGA 703


SSU1 PROM           GGTGGCTAAGAATATGCTCCTACATCATGTTTGCTATCGCTTGCCTTATTTTCATTGCTG 1175
EC1118-SSU1-16rev   ------------------------------------------------------------
EC1118-SSU1-16fw    AANTGNNCTACNNNTGNTTGCNNTCNNNCTTTATTTNNTGGCTGGNANGGNCTTCANN-- 761
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2.Chromosome XVI *SSU1* promoter – 71B sequences

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CLUSTAL W (1.83) multiple sequence alignment
SSU1 PROM refers to Genbank sequence YPL092W
71B-SSU1-16fw: sequence on 71B DNA obtained with SSU1-16fw primer.
71B-SSU1-16rev: sequence on 71B DNA obtained with SSU1-16rev primer.
A mutation
A mutated also in EC1118
A not reliable in chromatogram


SSU1 PROM           GCCAAGATGGAAAGAAGAGAAAGAAATAGACATGCCAAGCAAGGTGAATCTGATAGACAC 60
71B-SSU1-16fw       ------------------------------------------------------------
71B-SSU1-16rev      ------------------------------------------------------------


SSU1 PROM           AATGCTGTTTCCTTATCAAAGCATTTATTCAGTGGTAAGCGTGGTGTCGGTAAGACAGAT 120
71B-SSU1-16fw       ------------------------------------------------------NNNNNA 6
71B-SSU1-16rev      --------------------------NNNNTTGNTAANNGCCNATGTCGTAAGACAGAT 33


SSU1 PROM           TTCCGTTGATTTCTCTACTTCTTATTCTTTCTTTTGCATGTACTTTAAAAAAAATATGAA 180
71B-SSU1-16fw       TTTCGTTGATTTCTCTACTTCTTATTCTTTCTTTTGCATGTACTTTAAAAAAAATATGAA 66
71B-SSU1-16rev      TTCCGTTGATT-CTCTACTTNT-ATTCTTTCTTTTGCATGTACTTTAAAAAAA-TATGAA 90
                    ** ******** ******** * ****************************** ******


SSU1 PROM           TATAAAATGTGGATGTACGTACACGACATTCTAATCTTTTTGGGCTGGTAGGATTACCAT 240
71B-SSU1-16fw       TATAAAATGTGGATGTACGTACACGACATTCTAATCTTTTTGGGCTGGTAGGATTATCAT 126
71B-SSU1-16rev      TATAAAATGTGGATGTACGTACACGACATTCTAATCTTTTTGGGCTGGTAGGATTATCAT 150
                    ************************************************************


SSU1 PROM           TTACATTTGATTATCTCTCTCACTATTAGTGGTTTCTTTTTCTTTCTTTCTCACTTTTTC 300
71B-SSU1-16fw       TTACATTTGATTATCTCTCTCACTATTAGTGGTTTCTTTTTCTTTCTTTCTCACTTTTTC 186
71B-SSU1-16rev      TTACATTTGATTATCTCTCTCACTATTAGTGGTTTCTTTTTCTTTCTTTCTCACTTTTTC 210
                    ************************************************************


SSU1 PROM           TGTATCTTTTTTTTAAAAAAATTTTATTTAATCTGTATAATAATAATAAACCGATTTAAA 360
71B-SSU1-16fw       TGTATCTTTTTTTTAAAAAAATTTTATTTAATCTGTATAATAATAATAAACCGATTTAAA 246
71B-SSU1-16rev      TGTATCTTTTTTTTAAAAAAATTTTATTTAATCTGTATAATAATAATAAACCGATTTAAA 270
                    ************************************************************


SSU1 PROM           TTATCCAACAAGCCGACCCCTCCATGTTCTACTATTTTTTTGTATGTCACTGGATGTATA 420
71B-SSU1-16fw       TTATCCAACAAGCCGGCCCCTCCATGTTCTACTATTTTTTTGTATGTCACTGGATGTATA 306
71B-SSU1-16rev      TTATCCAACAAGCCGGCCCCTCCATGTTTTACTATTTTTTTGTATGTCACTGGATGTATA 330
                    *************** ************ ******************************


SSU1 PROM           CAAATAATTAAGCATGTGGAAAAAGAAGGGGTGGGATAGCGTCAAGATGACACTTCTACT 480
71B-SSU1-16fw       CAAATAATTAAGCATGTGGAAAAAGAAGGGGTGGGATAGCGTCAAGATGACACTTCTACT 366
71B-SSU1-16rev      CAAATAATTAAGCATGTGGAAAAAGAAGGGGTGGGATAGCGTCAAGATGACACTTCTACT 390
                    ************************************************************


SSU1 PROM           TTTTTGTGACACATCATCATGCAACCTATCGAGTCTCCCACGAGGTTGACAAATAAGAAA 540
71B-SSU1-16fw       TTTTTGTGACACATCATCATGCAACCTATCGAGTCTCCCACGAGGTTGACAAATAAGAAA 426
71B-SSU1-16rev      TTTTTGTGACACATCATCATGCAACCTATCGAGTCTCCCACGAGGTTGACAAATAAGAAA 450
                    ************************************************************


SSU1 PROM           TTGTTATCGTTTTTGCAGTGTATCGTATAAGGCAACAATAGCGATGTCTCCCATCAATTG 600
71B-SSU1-16fw       TTGTTATCGTTTTTGCAGCGTATCGTATAAGGCAACAATAGCGATGTCTCCCATCAATTG 486
71B-SSU1-16rev      TTGTTATCGTTTTTGCAGCGTATCGTATAAGGCAACAATAGCGATGTCTCCCATCAATTG 510
                    ****************** ****************************************


SSU1 PROM           ACTGATAAATTCCTGCAAACTATCATTTTTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGC 660
71B-SSU1-16fw       ACTGATGAATTCCTGCAAACTATCATTTTTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGC 546
71B-SSU1-16rev      ACTGATGAATTCCTGCAAACTATCATATTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGC 570
                    ****** ****************** ********************************
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Appendix B

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SSU1 PROM           CGAAAATCTGAGAGTGCATGAATCTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACA 720
71B-SSU1-16fw       CGAAAATCTGAGAGGGCATGAATCTTAAAAAACAGAAAACTCATCGCCGTCTTTGGCACA 606
71B-SSU1-16rev      CGAAAATCTGAGAGTGCATGAATCTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACA 630
                    ************** ********************** **********************

SSU1 PROM           TTGGGGTTGTCTTTGACTTCTTTTGCTTATTTTTGCTCACTTTGCTTATTTTACCTATTT 780
71B-SSU1-16fw       TTGGGGTTGCCTTTGACTTCTTTTGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTT 666
71B-SSU1-16rev      TTGGGGTTGCCTTTGACTTCTTTTGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTT 690
                    ********* ************************************ ************

SSU1 PROM           AACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCT 840
71B-SSU1-16fw       AACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCT 726
71B-SSU1-16rev      AACTAGAGGTTCTAAAAGAATAGCCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCT 750
                    ************************************************************

SSU1 PROM           TGTTAAGTTGAAACTTGTGATATTGGCTGAACAAATTCTCCGCATTTAGACAACACACAA 900
71B-SSU1-16fw       TGTTAAGTGGAAACTTGTGATATTGGCTGAACAAATTCTCCACATTTAGACAACACACAA 786
71B-SSU1-16rev      TGTTAAGTGGAAACTTGTGATATTGGCTGAACAAATTCTCCACATTTAGACAACACACAA 810
                    ******** ******************************** ******************

SSU1 PROM           ATTACAGCTTTCCCCTAGTAACGATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAA 960
71B-SSU1-16fw       ATTACAGCTTTCCCCTAGTAACGATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAA 846
71B-SSU1-16rev      ATTACAGCTTTCCCCTAGTAACGATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAA 870
                    ************************************************************

SSU1 PROM           AACGTTTTATAGTGTAAGAGAAGACAAGTACAAGAAAAAAATGGTTGCCAATTGGGTACT 1020
71B-SSU1-16fw       AACGTTTTATAGTGTAAGAGAAGACAAGTACAAGAAAAAAATGGTTGCCAATTGGGTACT 906
71B-SSU1-16rev      AACGTTTTATAGTGTAAGAGAAGACAAGTACAAGAAAAAAATGGT-GCCAATTGN-TNCN 928
                    ******************************************** ********  * *

SSU1 PROM           TGCTCTTACGAGG---CAGTTTGACCCCTTCATGTTTATGATGGTCATGGGTGTCGGCAT 1077
71B-SSU1-16fw       TGCTCTTACGAGGGTANNTTTTAACCACACNATA-------------------------- 940
71B-SSU1-16rev      CNNNNN------------------------------------------------------ 934
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3.Chromosome XVI *SSU1* promoter – 71B and EC1118

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CLUSTAL W (1.83) multiple sequence alignment
SSU1 PROM refers to Genbank sequence YPL092W
EC1118-SSU1-16fw: sequence on EC1118 DNA obtained with SSU1-16fw primer.
EC1118-SSU1-16rev: sequence on EC1118 DNA obtained with SSU1-16rev primer.
71B-SSU1-16fw: sequence on 71B DNA obtained with SSU1-16fw primer.
71B-SSU1-16rev: sequence on 71B DNA obtained with SSU1-16rev primer.
A mutation in 71B
A mutated in EC1118
A not reliable in chromatogram


EC1118-SSU1-16fw    CGTATAANGCAACAATAGCNATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTAT 215
71B-SSU1-16fw       ----------------------TGTCTCCCATCAATTGACTGATGAATTCCTGCAAACTAT 39
EC1118-SSU1-16rev   CGTATAAGGCAACAATAGCGATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTAT 648
71B-SSU1-16rev      CGTATAAGGCAACAATAGCGATGTCTCCCATCAATTGACTGATGAATTCCTGCAAACTAT 660
SSU1 PROM           CGTATAAGGCAACAATAGCGATGTCTCCCATCAATTGACTGATAAATTCCTGCAAACTAT 623
                                      ********************** ***************

EC1118-SSU1-16fw    CATATTATTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAAT 275
71B-SSU1-16fw       CATTTTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAAT 99
EC1118-SSU1-16rev   CATATTATTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAAT 708
71B-SSU1-16rev      CATTTTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAAT 720
SSU1 PROM           CATTTTTTTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAATCTGAGAGTGCATGAAT 683
                    *** ** ************************************************** 

EC1118-SSU1-16fw    CTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTT 335
71B-SSU1-16fw       CTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTT 159
EC1118-SSU1-16rev   CTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTT 768
71B-SSU1-16rev      CTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACATTGGGGTTGCCTTTGACTTCTTT 780
SSU1 PROM           CTTAAAAAACAGAAGACTCATCGCCGTCTTTGGCACATTGGGGTTGTCTTTGACTTCTTT 743
                    ********************************************* ************

EC1118-SSU1-16fw    TGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAG 395
71B-SSU1-16fw       TGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAG 219
EC1118-SSU1-16rev   TGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAG 828
71B-SSU1-16rev      TGCTTATTTTTGCTCACTTTGCTTTTTTTACCTATTTAACTAGAGGTTCTAAAAGAATAG 840
SSU1 PROM           TGCTTATTTTTGCTCACTTTGCTTATTTTACCTATTTAACTAGAGGTTCTAAAAGAATAG 803
                    ********************** ************************************

EC1118-SSU1-16fw    CCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATAT 455
71B-SSU1-16fw       CCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTGGAAACTTGTGATAT 279
EC1118-SSU1-16rev   CCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATAT 888
71B-SSU1-16rev      CCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTGGAAACTTGTGATAT 900
SSU1 PROM           CCAACCAGCGTGTAGTTCTTATTTCTAATCCTTGTCTTGTTAAGTTGAAACTTGTGATAT 863
                    ********************************************* **************

EC1118-SSU1-16fw    TGGCTGAACAAATTCTCCACATTTAGACAACACACAAATTACAGCTTTCCCCTAGTTACG 515
71B-SSU1-16fw       GGGCTGAACAATTTCTCCACATTTAGACA-CACACAAATTACAGCTTTCCCCTAGTAACG 338
EC1118-SSU1-16rev   TGGCTGAACAAATTCTCCACATTTAGACAACACACAAATTACAGCTTTCCCCTAGTAACG 948
71B-SSU1-16rev      TGGCTGAACAAATTCTCCACATTTAGACAACACACAAATTACAGCTTTCCCCTAGTAACG 960
SSU1 PROM           TGGCTGAACAAATTCTCCGCATTTAGACAACACACAAATTACAGCTTTCCCCTAGTAACG 923
                     ********** ****** ********** ************************** ***

EC1118-SSU1-16fw    ATTGTTGATTGAGCTCAGACA-TACGCGCAATTTAAAA-CGTTTTATAGTGTAAGAGAGA 573
71B-SSU1-16fw       ATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAG 398
EC1118-SSU1-16rev   ATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAG 1008
71B-SSU1-16rev      ATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAG 1020
SSU1 PROM           ATTGTTGATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAG 983
                    ********************* **************** ******************

EC1118-SSU1-16fw    CAA-GTACAGGAAAAAAN-GGTTGCN-ATTGGNA-CTTGCT-CTTACGANGCAGTTTGAC 628
71B-SSU1-16fw       ACA-GTACAAGAAAAAAATGGTTGCCTATTGGNNACTTGCTTNTTACGAGGCAGTTCGAC 457
EC1118-SSU1-16rev   ACAAGTACAAGAAAAAAATGGT-GCCAATTGNNCNTNCNNNC------------------- 1049
71B-SSU1-16rev      ACAAGTACAAGAAAAAAATGGT-GCCCAATGGACCGTNNNNC------------------- 1061
SSU1 PROM           ACAAGTACAAGAAAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACC 1043
                      * ***** *******  *** **  * **
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4.Chromosome VIII *SSU1-R* promoter – 71B sequences

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CLUSTAL W (1.83) multiple sequence alignment

SSU1 PROM refers to Genbank sequence AB002531
71B-LB-SSU1Rfw: sequence on 71B ChrVIII low band obtained with SSU1-8fw primer.
71B-HB-SSU1Rfw: sequence on 71B ChrVIII high band obtained with SSU1-8fw primer.
71B-LB-SSU1Rrev: sequence on 71B ChrVIII low band obtained with SSU1-8rev primer.
71B-HB-SSU1Rrev: sequence on 71B ChrVIII high band obtained with SSU1-8rev primer.



SSU1-R PROM     GATCTGGAGATGAGAAGTAATGCAGCAACCAACATTAATGATTCTGGTAATAGTTCATTA 60
71B-LB-SSU1Rfw  ------------------------------------------------------------
71B-HB-SSU1Rfw  ------------------------------------------------------------


SSU1-R PROM     CACATCGAATTGGGCACTTATATTTTTAAAGCATTGGCCGTTTTCAGGAACTCTGTTGAC 120
71B-LB-SSU1Rfw  ------------------------------------------------------------
71B-HB-SSU1Rfw  ------------------------------------------------------------


SSU1-R PROM     AAGTACTGGGAGGATAAGTATCCAGAGATGGGGGTCACAGTTTGATTGGAAGGTATTTCT 180
71B-LB-SSU1Rfw  ----------------------------------------------NNNNNNNNNTNTN 13
71B-HB-SSU1Rfw  ------------------------------------------GNNNNNNNNGANTNTNNTN 19


SSU1-R PROM     AGGTTCTACTTTAGTTTTTT-ATAACGCATCCAGTACAAAGAAATGATATTGGTATTTGG 239
71B-LB-SSU1Rfw  TGGTTCTACTTTAGTTTTTT-ATAACGCATCCAGTACAAAGAAATGATATTGGTATTTGG 72
71B-HB-SSU1Rfw  GGGTTCTACTTTAGTTTTTTTATAACGCATCCAGTACAAAGAAATGATATTGGTATTTGG 79
                 ******************* ****************************************

SSU1-R PROM     AAAAGTCATTGAATATTCTTGAATAGATTATAAGCGAGCTTCCTTTCTGTATCAGGATAT 299
71B-LB-SSU1Rfw  AAAAGTCATTGAATATTCTTGAATAGATTATAAGCGAGCTTCCTTTCTGTATCAGGATAT 132
71B-HB-SSU1Rfw  AAAAGTCATTGAATATTCTTGAATAGATTATAAGCGAGCTTCCTTTCTGTATCAGGATAT 139
                ************************************************************

SSU1-R PROM     GTGGCTTAACTTTAAACTCGTATAATAAAAGTACTCGTATTTTTGGGGGATGTTTGCCTC 359
71B-LB-SSU1Rfw  GTGGCTTAACTTTAAACTCGTATAATAAAAGTACTCGTATTTTTGGGGGATGTTTGCCTC 192
71B-HB-SSU1Rfw  GTGGCTTAACTTTAAACTCGTATAATAAAAGTACTCGTATTTTTGGGGGATGTTTGCCTC 199
                ************************************************************

SSU1-R PROM     CGTAAATTTATAAATGGCAACAGGAACACTATTATAATGTAATGTTACAATATTACTTTT 419
71B-LB-SSU1Rfw  CGTAAATTTATAAATGGCAACAGGAACACTATTATAATGTAATGTTACAATATTACTTTT 252
71B-HB-SSU1Rfw  CGTAAATTTATAAATGGCAACAGGAACACTATTATAATGTAATGTTACAATATTACTTTT 259
                ************************************************************

SSU1-R PROM     ACTGTATAAGTTTAATGCTTTATAAAACTAGATGGCAGCTTCTAAGTTGTGGCTTGTCTC 479
71B-LB-SSU1Rfw  ACTGTATAAGTTTAATGCTTTATAAAACTAGATGGCAGCTTCTAAGTTGTGGCTTGTCTC 312
71B-HB-SSU1Rfw  ACTGTATAAGTTTAATGCTTTATAAAACTAGATGGCAGCTTCTAAGTTGTGGCTTGTCTC 319
                ************************************************************

SSU1-R PROM     GGAGGTTCAAGCTGTCCTCCAAGTTCAGCGCAGCGATTATTTGCAAATTGTCTACGTAAG 539
71B-LB-SSU1Rfw  GGAGGTTCAAGCTGTCCTCCAAGTTCAGCGCAGCGATTATTTGCAAATTGTCTACGTAAG 372
71B-HB-SSU1Rfw  GGAGGTTCAAGCTGTCCTCCAAGTTCAGCGCAGCGATTATTTGCAAATTGTCTACGTAAG 379
                ************************************************************

SSU1-R PROM     GACTATATAAACGTTTCGATGCTCTCTCTTCACATCTTCTGTGTCGAACCTCGAACATCG 599
71B-LB-SSU1Rfw  GACTATATAAACGTTTCGATGCTCTCTCTTCACATCTTCTGTGTCGAACCTCGAACATCG 432
71B-HB-SSU1Rfw  GACTATATAAACGTTTCGATGCTCTCTCTTCACATCTTCTGTGTCGAACCTCGAACATCG 439
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71B-HB-SSU1Rrev AGATGCATT-TCCAATTTGAGGCCAGTTTCTGAGGGTTCTCGTATTTCAACAATAATGTT 79
71B-LB-SSU1Rrev GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 232
SSU1-R PROM     GCATGCATTGTCCAATTTGAGGCCAGTTTCTGAGGGTTCTCGTATTTCAACAATAATGTT 660
                  ******* **** *** ****************************************

71B-HB-SSU1Rrev GTTGCGTAATGTACCGCACTGTGTGCGGCATTTTTGAGCCAGTTTTTGCTTATTCTCGTA 139
71B-LB-SSU1Rrev GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 292
SSU1-R PROM     GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 720
                ***** ************** ********************** **************

71B-HB-SSU1Rrev TTTCAACAATAATGTTGTTGCATAATGTTCCGCCCTTTGTGCGGCATTTTTGAGCCAGTT 199
71B-LB-SSU1Rrev TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTT---------- 342
SSU1-R PROM     TTTCAACAATAATGTTGTTGCATAATGTTCCGCCCTTTGTGCGGCATTTTTGAGCCAGTT 780
                ************************************************

71B-HB-SSU1Rrev TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 259
71B-LB-SSU1Rrev ------------------------------------------------------------
SSU1-R PROM     TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 840


71B-HB-SSU1Rrev CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 319
71B-LB-SSU1Rrev ------------------------------------------------------------
SSU1-R PROM     CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 900


71B-HB-SSU1Rrev ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 379
71B-LB-SSU1Rrev ----------------------ATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 380
SSU1-R PROM     ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 960
                                     **************************************

71B-HB-SSU1Rrev AAGAACTACAAGGAAGTTGTAACTACAAAAAATTACAGCTTTCCCCTAGTAACGATTGTT 439
71B-LB-SSU1Rrev AAGAACTACAAGGAAGTTGTAACTACAAAAAATTACAGCTTTCCCCTAGTAACGATTGTT 440
SSU1-R PROM     AAGAACTACAAGGAAGTTGTAACTACAAAAAATTACAGCTTTCCCCTAGTAACGATTGTT 1020
                ************************************************************

71B-HB-SSU1Rrev GATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGT 499
71B-LB-SSU1Rrev GATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGT 500
SSU1-R PROM     GATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGT 1080
                ************************************************************

71B-HB-SSU1Rrev ACAAGAAAAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACCCCTTCA 559
71B-LB-SSU1Rrev ACAAGAAAAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACCCCTTCA 560
SSU1-R PROM     ACAAGAAAAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACCCCTTCA 1140
                ************************************************************

71B-HB-SSU1Rrev TGTTTGTGATGGTCATGGGTGTCGGCATT-CATCNNNNCNC------------------- 599
71B-LB-SSU1Rrev TGTTTGTGATGGTCATGGGTGTCGGCAT--CATNNNTNCNCC----------------- 600
SSU1-R PROM     TGTTTGTGATGGTCATGGGTGTCGGCATTTCATCGAATATTCTATATAGCTTCCCATATC 1200
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5.Chromosome VIII *SSU1-R* promoter – EC1118 sequences

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CLUSTAL W (1.83) multiple sequence alignment

SSU1 PROM refers to Genbank sequence AB002531
EC1118-SSU1-8rev: sequence on EC1118 DNA obtained with SSU1-8rev primer.

SSU1-R PROM       GATCTGGAGATGAGAAGTAATGCAGCAACCAACATTAATGATTCTGGTAATAGTTCATTA 60
EC1118-SSU1-8rev  ------------------------------------------------------------


SSU1-R PROM       CACATCGAATTGGGCACTTATATTTTTAAAGCATTGGCCGTTTTCAGGAACTCTGTTGAC 120
EC1118-SSU1-8rev  ------------------------------------------------------------


SSU1-R PROM       AAGTACTGGGAGGATAAGTATCCAGAGATGGGGGTCACAGTTTGATTGGAAGGTATTTCT 180
EC1118-SSU1-8rev  ---------------------------------GGCACAGTTTGATTGGAAGGTATTTCT 27
                                                    * ************************

SSU1-R PROM       AGGTTCTACTTTAGTTTTTTATAACGCATCCAGTACAAAGAAATGATATTGGTATTTGGA 240
EC1118-SSU1-8rev  AGGTTCTACTTTAGTTTTTTATAACGCATCCAGTACAAAGAAATGATATTGGTATTTGGA 87
                  ************************************************************

SSU1-R PROM       AAAGTCATTGAATATTCTTGAATAGATTATAAGCGAGCTTCCTTTCTGTATCAGGATATG 300
EC1118-SSU1-8rev  AAAGTCATTGAATATTCTTGAATAGATTATAAGCGAGCTTCCTTTCTGTATCAGGATATG 147
                  ************************************************************

SSU1-R PROM       TGGCTTAACTTTAAACTCGTATAATAAAAGTACTCGTATTTTTGGGGGATGTTTGCCTCC 360
EC1118-SSU1-8rev  TGGCTTAACTTTAAACTCGTATAATAAAAGTACTCGTATTTTTGGGGGATGTTTGCCTCC 207
                  ************************************************************

SSU1-R PROM       GTAAATTTATAAATGGCAACAGGAACACTATTATAATGTAATGTTACAATATTACTTTTA 420
EC1118-SSU1-8rev  GTAAATTTATAAATGGCAACAGGAACACTATTATAATGTAATGTTACAATATTACTTTTA 267
                  ************************************************************

SSU1-R PROM       CTGTATAAGTTTAATGCTTTATAAAACTAGATGGCAGCTTCTAAGTTGTGGCTTGTCTCG 480
EC1118-SSU1-8rev  CTGTATAAGTTTAATGCTTTATAAAACTAGATGGCAGCTTCTAAGTTGTGGCTTGTCTCG 327
                  ************************************************************

SSU1-R PROM       GAGGTTCAAGCTGTCCTCCAAGTTCAGCGCAGCGATTATTTGCAAATTGTCTACGTAAGG 540
EC1118-SSU1-8rev  GAGGTTCAAGCTGTCCTCCAAGTTCAGCGCAGCGATTATTTGCAAATTGTCTACGTAAGG 387
                  ************************************************************

SSU1-R PROM       ACTATATAAACGTTTCGATGCTCTCTCTTCACATCTTCTGTGTCGAACCTCGAACATCGA 600
EC1118-SSU1-8rev  ACTATATAAACGTTTCGATGCTCTCTCTTCACATCTTCTGTGTCGAACCTCGAACATCGA 447
                  ************************************************************

SSU1-R PROM       GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 660
EC1118-SSU1-8rev  GCATGCATTGTCCATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTT 507
                  ************************************************************

SSU1-R PROM       GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 720
EC1118-SSU1-8rev  GTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTTTCTGCTTATTCTCGTA 567
                  ************************************************************

SSU1-R PROM       TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTTTGAGCCAGTT 780
EC1118-SSU1-8rev  TTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGGCATTTT---------- 617
                  **************************************************

SSU1-R PROM       TCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGTACCGCACTTTGTGCGG 840
EC1118-SSU1-8rev  ------------------------------------------------------------


SSU1-R PROM       CATTTTTGAGCCAGTTTCTGCTTATTCTCGTATTTCAACAATAATGTTGTTGCATAATGT 900
EC1118-SSU1-8rev  ------------------------------------------------------------
```

```
SSU1-R PROM       ACCGCACTTTGTGCGGCATTTTATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 960
EC1118-SSU1-8rev  ----------------------ATAGCTTACAGATACAGATGAATTTACGAGCTGTATAA 655
                                        ************************************

SSU1-R PROM       AAGAACTACAAGGAAGTTGTAACTACAAAAAATTACAGCTTTCCCCTAGTAACGATTGTT 1020
EC1118-SSU1-8rev  AAGAACTACAAGGAAGTTGTAACTACAAAAAATTACAGCTTTCCCCTAGTAACGATTGTT 715
                  ************************************************************

SSU1-R PROM       GATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGT 1080
EC1118-SSU1-8rev  GATTGAGCTCAGACAATACGCGCAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGT 775
                  ************************************************************

SSU1-R PROM       ACAAGAAAAAAATGGTTGCCAATTGGGTACTTGCTCTTACGAGGCAGTTTGACCCCTTCA 1140
EC1118-SSU1-8rev  ACAAGAAAAAAATGGT-GCCA---------------------------------------- 795
                  *************** ****

SSU1-R PROM       TGTTTGTGATGGTCATGGGTGTCGGCATTTCATCGAATATTCTATATAGCTTCCCATATC 1200
EC1118-SSU1-8rev  ------------------------------------------------------------
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