ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF WINE YEAST SPECIES FROM GRAPES OF THREE DIFFERENT VINEYARDS IN TURKEY

by

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ABSTRACT

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Wine production has been carried out by humanity for thousands of years. Besides grape, the second most important ingredient is yeast. Yeasts that involve in fermentation are basically denoted as *Saccharomyces* and *non-Saccharomyces* types. Discrimination and quantification of these yeast species play a crucial role in production of wine regarding its quality, taste, etc. In this study, yeast species from grapes that were collected from Adana, Tekirdağ and Urla regions were isolated. Selective media (ESA and Lysine) were used to biochemically distinguish yeasts. For molecular level, Internal Transcribed Spacer (ITS) region containing 5.8S rDNA gene was amplified by PCR for every isolates. The sequencing results were run by ClustalW and BLAST tools for identification of yeast species. Restriction digestion was utilized as a mean of comparison between species. For morphological differentiation, microscopic analysis was carried out. Biolog system was attained for a physiological point of view. To monitor the growth rate of species, growth curves were drawn by growing the species in YPD media. Additionally, Sulfur resistances of species are calculated by comparison with growth in sulfur containing and not containing YPD media. For the last step, lyophilisation of *Saccharomyces* species was done to transport the species to Kuscular Village. The conclusion of this study was the successful characterization of whole natural yeast flora of the vineyards and specific selection of *Saccharomyces* species for large scale wine production.

ÖZET

TÜRKİYE'NİN ÜÇ ÜZÜM BAĞINDAN TOPLANAN ÜZÜMLERDEN
ŞARAP MAYASI İZOLASYONU, TANILANMASI
VE KARAKTERİZASYONU

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Anahtar Kelimeler: Fermentasyon, Şarap, Maya, ITS bölgesi

Şarap üretimi insanlık tarafından binlerce yıldır süregelmektedir. Üzümden sonra şarap üretiminin en önemli ikinci malzemesi şüphesiz mayadır. Fermentastonda görev alan mayalar en temel olarak *Saccharomyces* ve *Saccharomyces-olmayan* şeklinde ikiye ayrılır. Şarabın kalitesi ve tadı gibi özellikleri göz önüne alınırsa, bu mayaların ayrımı ve miktarı şarap üretiminde büyük önem taşımaktadır. Bu çalışmada, Adana, Tekirdağ ve Urla yörelerinden gelen üzümlerden maya türleri izole edildi. Seçici ortamlar (ESA ve Lysine) kullanılarak bıyokimya düzeyinde ayrım sağlandı. Moleküler seviye ayrımı için ise Internal Transcribed Spacer (ITS) bölgesinde bulunan 5.8S rDNA geni PZR ile çoğaltıldı. Sekanslama sonuçları BLAST ve ClustalW araçları yardımı ile tanımlandı. Restriksiyon enzimleri sayesinde türler arasında bir karşılaştırma yapıldı. Morfolojik karşılaştırma adına ise mikroskop görüntüleri elde edildi. Biolog sistemi ise fizyolojik karşılaştırma için kullanıldı. Türlerin büyüme hızlarını görüntülemek için YPD ortamında her türün büyüme eğrileri çizildi. Buna ek olarak da türlerin sülfür dayanıklıkları, sülfür içeren ve içermeyen YPD ortamındaki büyümeleri karşılaştırılarak yapıldı. En son aşama olarak da *Saccharomyces* türleri, Kuşçular köyüne taşınması ve ondan sonra da büyük oranlarda şarap üretiminde denenmesi için liyofilize edildi. Bütün bu çalışmanın sonucunda 3 üzüm bağının bütün doğal maya florası karakterize edildi ve büyük oranlarda şarap üretimlerinde kullanılmak üzere *Saccharomyces* türleri spesifik olarak seçildi.

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ABBREVIATIONS

µl	Microliter
BUY agar	Biolog Universal agar
ESA	Ethanol sulfite agar
g	Gram
kg	Kilogram
ITS	Internal Transcribed Spacer
L	Liter
LM	Lysine medium
Min	Minute
ml	Milliliter
mtDNA	Mitochondrial DNA
OD	Optical density
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal RNA

1 INTRODUCTION

1.1 Grapevine and wine origin

The oldest recorded information about wine dates back to 5500 BC. The earliest known residues come from the early-mid fifth millennium B.C. –Hajji Firuz Tepe, in the northern Zagros Mountains of Iran (McGovern, Glusker, Exner, & Voigt, 1996). Additionally, evidence from Neolithic pottery from Georgia indicates that contemporaneous wine production was spread all over the region. Former examples of fermented beverages have been searched out, and they have been produced from rice, fruit and honey. Intrinsically, this kind of drinks were being produced in China even before 7000 BC (Garnier, Richardin, Cheynier, & Regert, 2003).

The gathering of ancient information about wine is related to wine residues identification techniques. The presence of wine residues is usually identified by the presence of tartaric acid. And also, identification of red wine is made by the presence of syringic acid, an alkaline breakdown product of malvidin-3-glycoside (Guasch-Jané, Andrés-Lacueva, Jáuregui, & Lamuela-Raventós, 2006).

According to literature, winemaking was discovered or, at least evolved, in southern Caucasia (present, this area covers northwestern Turkey, northern Iraq, Azerbaijan, and Georgia). According to history, domestication of the wine grape (*Vitis vinifera*) came from in the same area. Grapevine domestication also may have occurred independently in Spain (Núñez & Walker, 1989).

Even though grapes easily ferment indigenously, owing to the prevalence of fermentable sugars, the wine yeasts within (*Saccharomyces cerevisiae*) are not the major, indigenous member of the grape flora. The natural habitat of the ancestral strains

of *S.cerevisiae* appears to be the bark and sap exudates of oak trees. The fortuitous overlap in the distribution of the progenitors of both *S.cerevisiae* and *V. vinifera* with the northern spread of agriculture into Anatolia may have fostered the discovery of winemaking, as well as its subsequent development and spread. It may not be pure coincidence that most major yeast-fermented beverages and foods (wine, beer, and bread) have their origins in the Near East (Phaff, 1986).

Kloeckera apiculata and various *Candida spp.* are the other yeasts indigenous to grapes and they can readily initiate fermentation. However, they rarely finalize fermentation because of their vulnerability to alcohol accumulation and limited fermentative metabolism. On the other hand, beer with its lower alcohol content may have initially been fermented by yeasts other than *S.cerevisiae* (Esteve-Zarzoso, 1998).

Unlike the major cereal crops of the Near East (wheat and barley), cultivated grapes develop an extensive yeast population by maturity, although rarely including the wine yeast (*Saccharomyces cerevisiae*). Piled unattended for several days, grape cells begin to self-ferment as oxygen becomes limiting. When the berries rupture, juice from the fruit is rapidly colonized by the yeast flora. These continue the conversion of fruit sugars into alcohol (ethanol). Unless *S. cerevisiae* is present to continue the fermentation, the process usually ceases before all the sugars are converted to alcohol. Unlike native yeast populations, *S. cerevisiae* can completely metabolize fermentable sugars. During winemaking, the fermentation of grape juice into wine is efficiently facilitated if the fruit is first crushed. Crushing releases and mixes the juice with yeasts on the grape skins (and associated equipment). Although yeast fermentation is more rapid in contact with slight amounts of oxygen, continued exposure to air favors the growth of a wide range of yeasts and bacteria. The latter can quickly turn the nascent wine into vinegar. Although unacceptable as a beverage, the vinegar produced this way was probably valuable in its own way. As a source of acetic acid, vinegar expedited pottery production and the preservation (pickling) of perishable foods (Linda F. Bisson, 2005; Blackwell, 2001).

Grapes were the only fruits that can store carbohydrates predominantly in the form of soluble sugars which were gathered by the ancient man. So, in this manner, the major caloric source in grapes is in a form readily metabolized by wine yeasts. The rapid and extensive production of ethanol by *S. cerevisiae* quickly limits the growth of most bacteria and other yeasts in grape juice. Consequently, wine yeasts generate

conditions that rapidly give them almost exclusive access to grape nutrients (McBryde, Gardner, de Barros Lopes, & Jiranek, 2006).

Another unique property of grapes concerns the acids they contain. The major one found in mature grapes is tartaric acid. This acid occurs in small quantities in the vegetative parts of some other plants, but rarely in fruit. Because tartaric acid is metabolized by few microbes, wine remains sufficiently acidic to limit the growth of most bacteria and fungi. In addition, the acidity gives wine much of its fresh taste. The combined action of grape acidity and the accumulation of ethanol suppress the growth and metabolism of most potential wine-spoilage organisms. This property is enhanced in the absence of air (oxygen). For ancient man, the result of grape fermentation was the transformation of a perishable, periodically available fruit, into a relatively stable beverage with novel and potentially intoxicating properties (Jackson, 2008).

1.2 Commercial importance of grapes and wine

1.2.1 Global grape production

From its origins, grape production has been developed into being the world's most important fresh fruit crop. Worldwide grape production in 2007 was about 67 million metric tons. Although, this seems a huge amount of production when roughly compared with the production of oranges, bananas, and apples. According to the International Organization of vine and wine 2007 statistics, production of grapes is decreased in some leading countries because of unfavorable global climatic conditions as seen in Figure 1 and 2 (OIV, 2007).

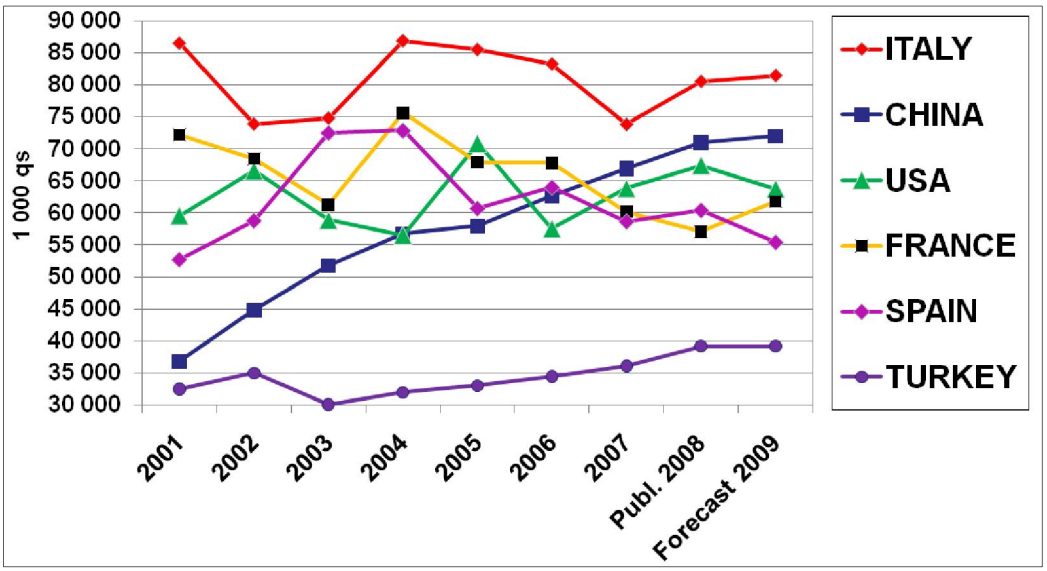

Figure 1 Recent developments of the leading countries grapes production (OIV, 2007)

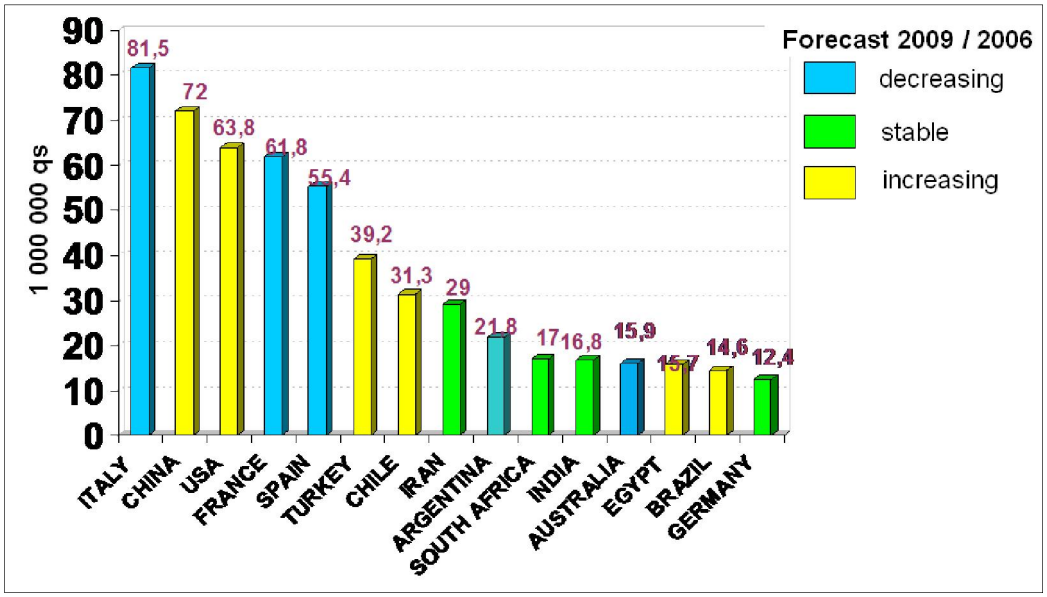

Figure 2 Global grapes production of 15 leading countries (OIV, 2007)

Grape production is largely restricted to climatic regions similar to those of the indigenous range of *Vitis vinifera*. This zone approximates the area 10°C to 20 ºC of annual isotherms (Figure 3). Grape culture is further largely restricted to regions characterized by Mediterranean-type climates. Extension into cooler, warmer, or moister environs is possible when local conditions modify the climate or viticultural practice compensates for less than ideal conditions. Commercial production even occurs in subtropical regions, where severe pruning stimulates nearly year-round vine growth (Jackson, 2008; Mortimer & Polsinelli, 1999).

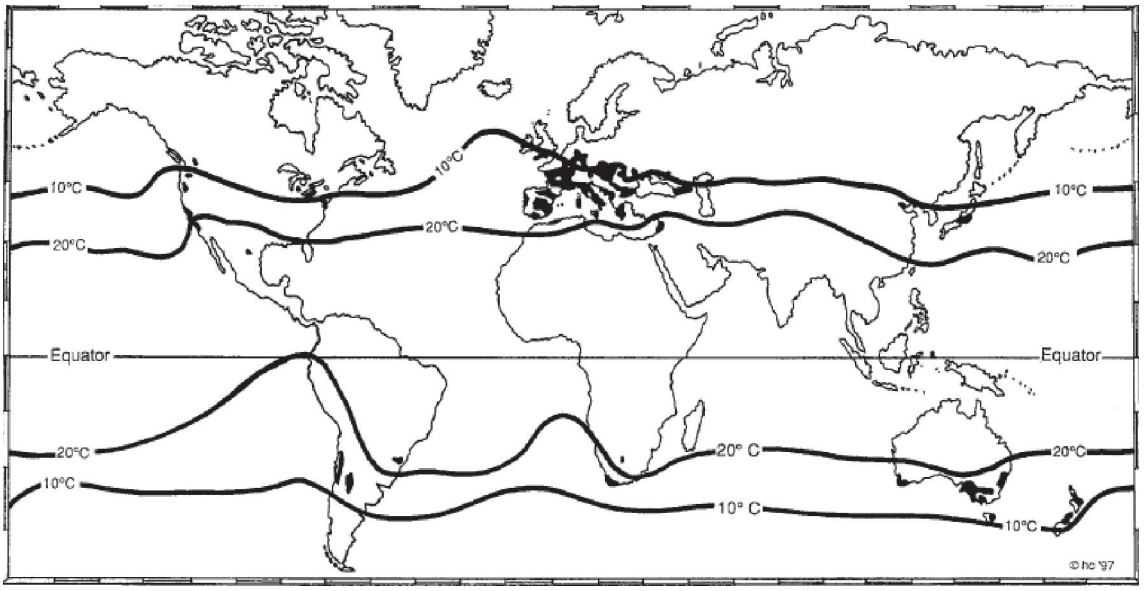

Figure 3 Association between the major viticultural regions of the world, with the 10 and 20 ˚C annual isotherms (Jackson, 2008)

1.2.2 Surface area of vineyards worldwide

The area planted under grapevines in 2007 is estimated at about 7.7 million hectares, down from a maximum of 10.2 million in the late 1970s (Figure 3 and 4). After the period of sustained growth which continued until the late 1970s, global vineyard acreage started to decline as a result of EU vine pull schemes and extensive vine pulls in the former Soviet Union (OIV, 2007).

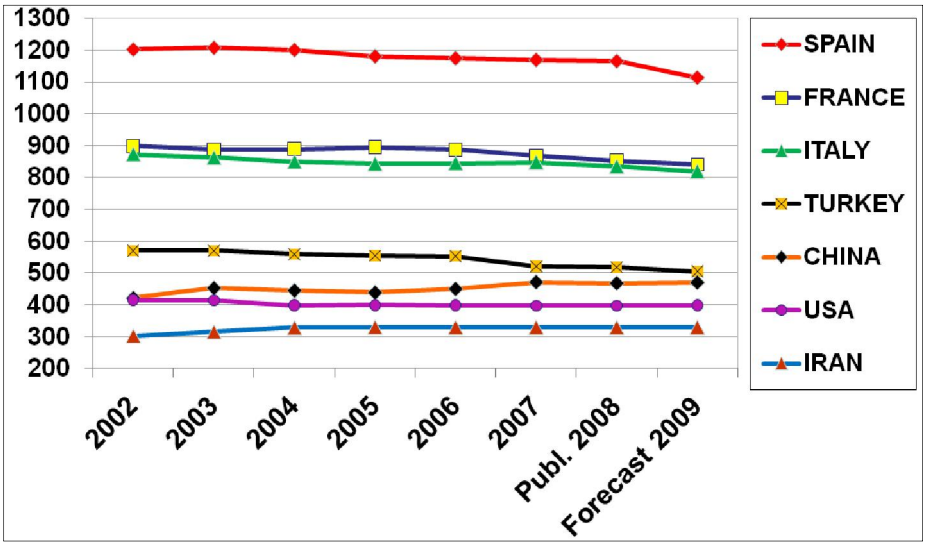

Figure 4 Recent developments of the leading vineyards (OIV, 2007)

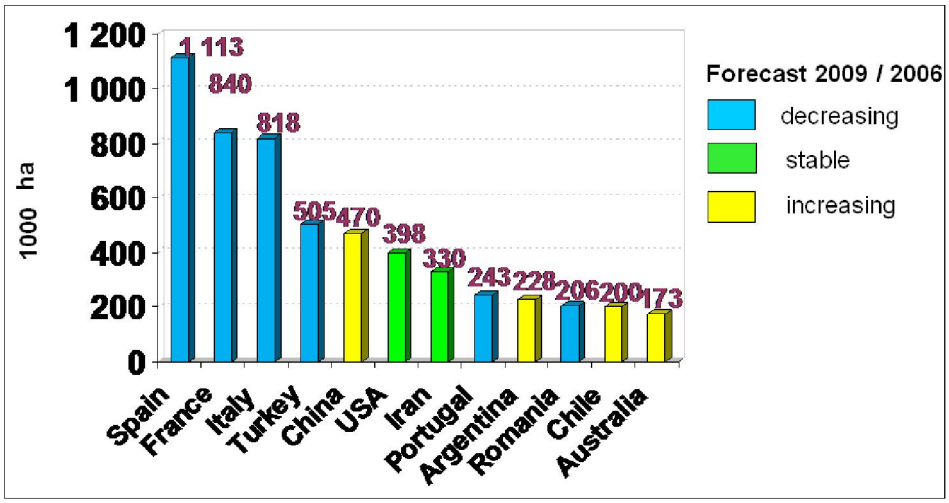

Figure 5 Areas planted in vines of the 12 leading countries (OIV, 2007)

1.2.3 Global wine production and consumption

Approximately 66% of the grape production gets fermented into wine, 18.7 % is consumed as a fresh fruit crop, and the remaining 7.7% is dried for raisins. The use varies from country to country, often depending on the physical, political or religious (wine prohibition) dictates of the region (Figure 6).

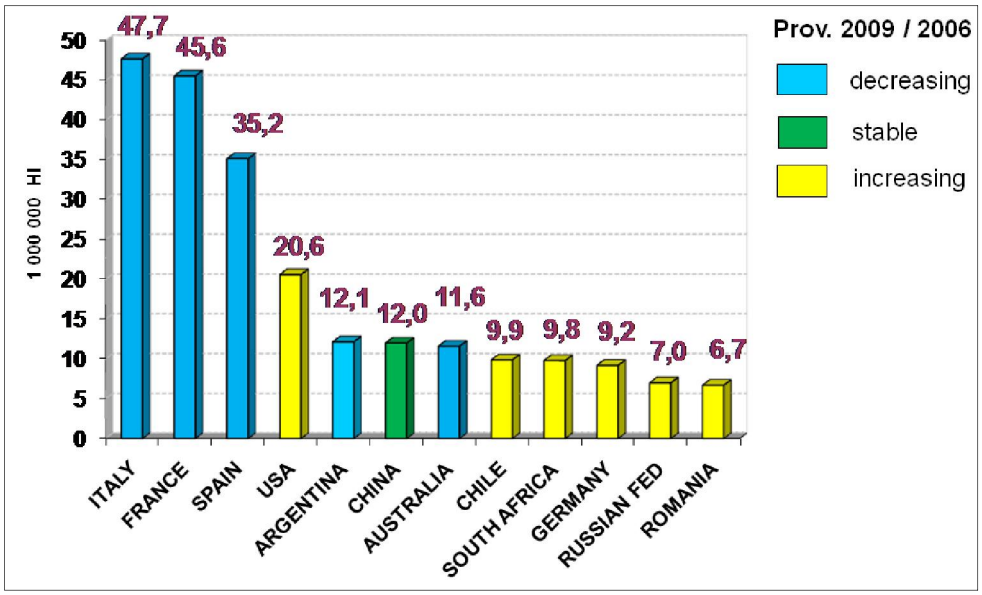

Figure 6 Production of wine of the 12 leading countries (OIV, 2007)

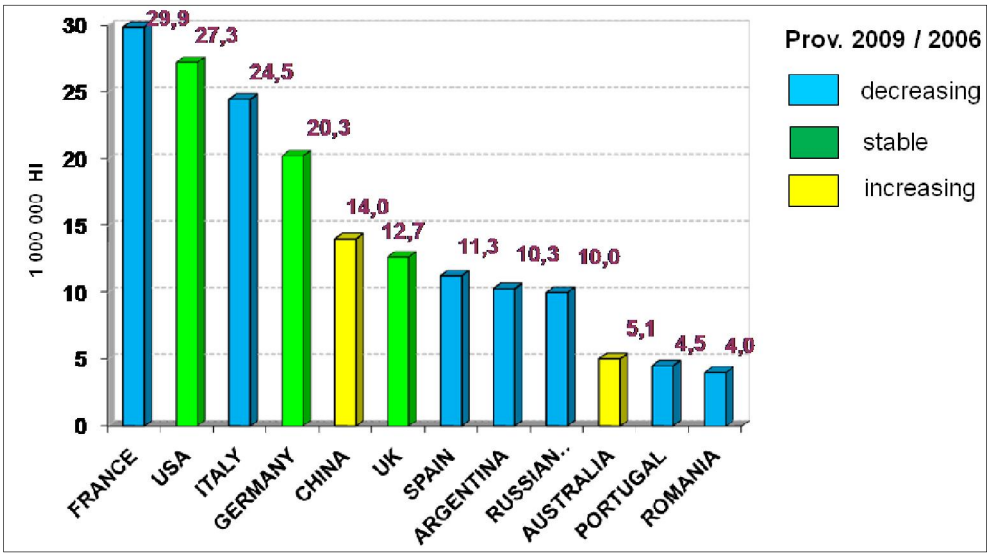

Figure 7 Consumption of wine of the 12 leading countries (OIV, 2007)

From the beginning of the 1980s to the mid-1990s, world wine consumption lagged. As was the case for production, it was during this period that the trend started to reverse, as may now be affirmed with ten years of hindsight. World consumption stopped falling and slowly started to rise as shown in the Figure 7 (OIV, 2007).

1.2.4 Health-related aspects of wine consumption

Until the 1900s, wine was used in the treatment of humans to ease the pain (Sutter, 1964). It was also a very important solvent for medications. One of the most widely documented benefits can be related to cardiovascular diseases. Moreover wine can help the decline of undesirable influences of stress, can enhance appetite, sociability, and self-esteem (Baum-Baicker, 1985), and also according to some researches, wine is the only alcoholic beverage associated with positive social expectations (Lindman & Lang, 1986).

A healthy balance in favor of low and high density lipoproteins in blood plasma as a benefit of wine consumption is now well known (Kinsella, 1993). On the other hand, wine consumption is also associated with toxication and other alcohol-related problems as in the Figure 8 (Reginald G. Smart, 1999).

In addition to revealing the potential benefits of wine consumption, researchers are also beginning to investigate the occasionally unpleasant consequences of moderate wine use. For instance, the induction of headaches by red wine has been correlated with insufficient production of platelet phenolsulphotransferase. Also, headache prevention has been associated with the prior use of acetylsalicylic acid and other prostaglandin synthesis inhibitors (Kaufman, 1992).

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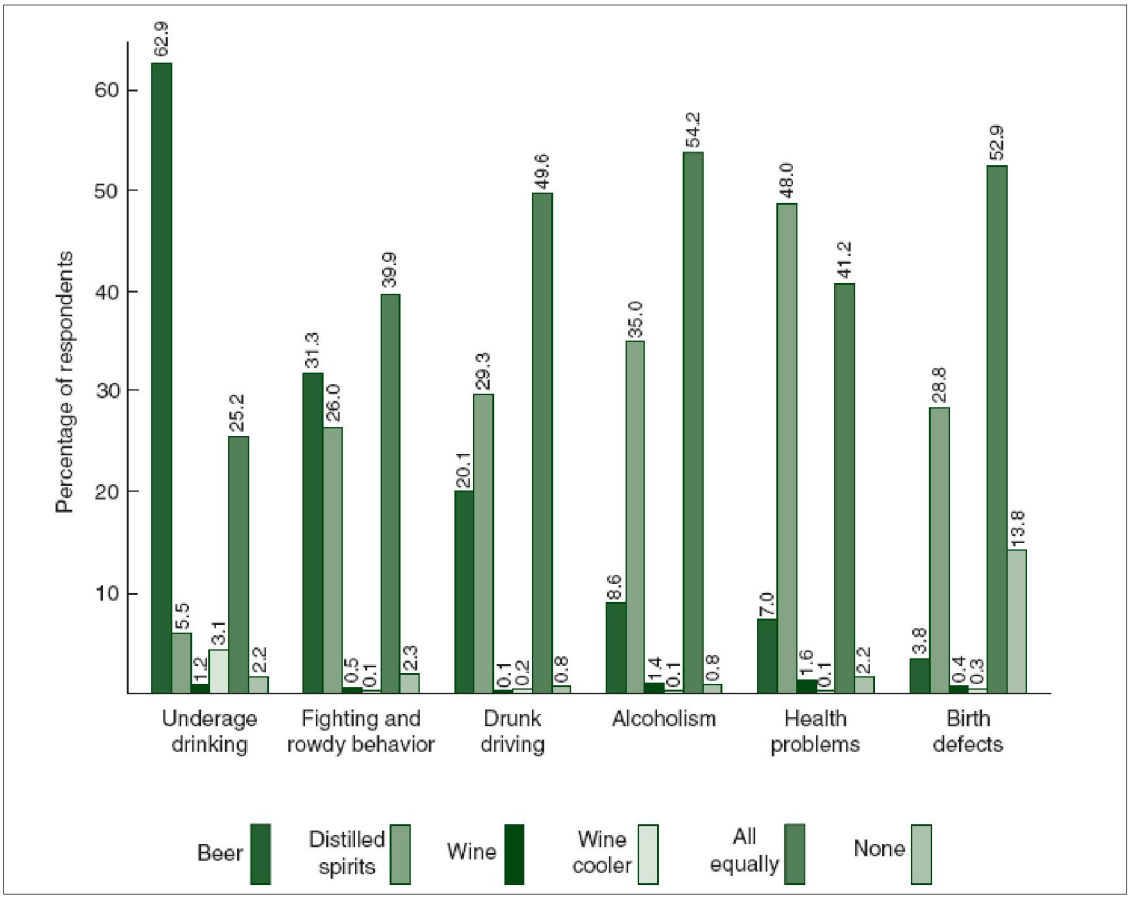


Figure 8 Comparison of the perception of adverse consequences associated with the consumption of different beverages containing alcohol (Hugh Klein, 1990)

1.3 Yeasts related with wine

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi and can be defined as unicellular fungi, either ascomycetous or basidiomycetous, that have vegetative states which predominantly reproduce by budding or fission and which do not form their sexual states within or on a fruiting body (Kurtzman & Phaff, 1987).

1.3.1 Methods in yeast Taxonomy

According to primary studies, yeasts were classified by their morphological characteristics of vegetative cells and spores. In addition to these two criteria, physiological characteristics were added after a while for adequate identification of

unknown yeasts. Nominately, previous yeast identification criteria included morphology of the vegetative cell, including size as well as shape, morphology, and mode of formation of the spores, if any, characteristics of the colony, surface growth on liquid medium, ability to grow on nitrite or nitrate as sole source of nitrogen, and ability to ferment and/or assimilate six sugars; glucose, galactose, maltose, sucrose, lactose, raffinose, and, implicitly, melibiose (the new yeast species were introduced by using more than 30 sole carbon sources). Consequently, the inadequacy of these rather limited criteria is followed by the emergence of molecular taxonomy (Blackwell, 2001).

1.3.2 Molecular Taxonomy

The first methods investigated were reassociation of RNA and DNA (determination of the degree of reassociation of RNA of one species with DNA from another), and the determination of the GC content of both genomic and mitochondrial DNA. GC content was generally determined from the "melting point" of genomic DNA and the differences indicated that the species were not identical, however, the same GC content gave no indication whatever of possible relationships or similarity (Kurtzman & Phaff, 1987).

In order to obtain more adequate and reliable results, the sequences of the ribosomal RNAs (rRNA) and ribosomal DNAs (rDNA) is being investigated as additional taxonomic criteria. These highly conserved sequences allow the determination of evolutionary distance between yeast species. Both methods are based on fragmentation of the rRNA or rDNA with restriction enzymes and separation of the fragments by gel electrophoresis for comparison. The patterns of repeated sequences are characteristic and can serve as a fingerprint for initial identification, and the DNA can be isolated for further investigation. When libraries of electrophoretic patterns of restriction digests of genomic DNA of known yeast species are available, tentative identifications of unknown isolates of yeasts may be possible directly (Gueho, Kurtzman, & Peterson, 1990).

Other related methods which have been developed for use in determining taxonomic relationships include differentiation by staining with dyes, restriction analysis of mitochondrial DNA, fermentation and assimilation patterns, sequence

variation in large subunits of ribosomal RNA, DNA hybridization, and separation of yeast chromosome by pulsed field gel electrophoresis (Spencer & Spencer, 1997).

Current taxonomies recognize 100 genera comprising more than 700 species, of which approximately 20 are relevant to winemaking. Yeast genera, with those non-*Saccharomyces* yeasts relevant to winemaking indicated in bold type, are listed in Table 1 (N.P. Jolly, 2006).

Table 1 A list of yeast genera (N.P. Jolly, 2006)

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Teleomorphic heterobasidio-mycetous genera (Basidiomycotina)	Ananorphic heterobasidio-mycetous genera (Basidiomycotina)
Ascomycotina	*Aciculoconidium*	*Agaricostilbum*	*Bensingtonia*
Ascoidea	*Arxula*	*Bulleromyces*	*Bullera*
Babjevia	*Blastobotrys*	*Chionosphaera*	***Cryptococcus***
Cephaloascu	*Botryozyma*	*Cystofilobasidium*	*Fellomyces*
Citeromyces	***Brettanomyces***	*Erythrobasidium*	*Hyalodendron*
Clavispora	***Candida***	*Fibulobasidium*	*Itersonilia*
Coccidiascus	*Geotrichum*	*Filobasidiella*	*Kockovaella*
Cyniclomyces	***Kloeckera***	*Filobasidium*	*Kurtzmanomyces*
Debaryomyces	*Lalaria*	*Holtermannia*	*Malassezia*
Dekkera	*Myxozyma*	*Leucosporidium*	*Moniliella*
Dipodascopsis	*Oosporidium*	*Mrakia*	*Phaffia*
Dipodascus	*Saitoella*	*Rhodosporidium*	*Pseudozyma*
Endomyces	*Schizoblastosporion*	*Sirobasidium*	*Reniforma*
Eremothecium	*Sympodiomyces*	*Sporidiobolus*	***Rhodotorula***
Galactomyces	*Trigonopsis*	*Sterigmatosporidium*	*Sporobolomyces*
Hanseniaspora		*Tilletiaria*	*Sterigmatomyces*
Issatchenkia		*Tremella*	*Sympodiomycopsis*
Kluyveromyces		*Trimorphomyces*	*Tilletiopsis*
Lipomyces		*Xanthophyllomyces*	*Trichosporon*
Lodderomyces			*Trichosporonoides*
Metschnikowia			*Tsuchiyaea*
Nadsonia			
Pachysolen			
Pichia			
Protomyces			
Saccharomyces			
Saccharomycodes			
Saccharomycopsis			
Saturnispora			
Schizosaccharomyces			
Sporopachydermia			
Stephanoascus			
Torulaspora			
Wickerhamia			
Wickerhamiella			
Williopsis			
Yarrowia			
Zygoascus			
Zygosaccharomyces			

1.3.3 Wine yeasts

1.3.3.1 The *Saccharomyces* group

Saccharomyces group is the most closely studied organism. *S.cerevisiae* and its close relatives have long been used by humans for bread making, brewing, and similar purposes. So far, it is the best understood and thoroughly studied of the yeast species; also it has a great industrial value. For instance, the gene for any desired protein of pharmaceutical or industrial interest can be cloned and expressed in yeast (Spencer & Spencer, 1997).

1.3.3.2 The genus *Zygosaccharomyces*

Members of the genus *Zygosaccharomyces* sporulate after conjugation of two haploid strains of opposite mating types. Two of the spores are found in one of the conjugating parents and two in the other, giving the ascus a dumb- bell shape. Yeasts in this group are included highly osmotolerant species, growing on 60% glucose-yeast extract agar. They are also spoilage yeasts and grow readily in fruit juices and fruit drinks (Spencer & Spencer, 1997).

1.3.3.3 The genera *Pichia* and *Hansenula*

Pichia and Hansenula are also osmotolerant yeast genera. *Pichia* has high tolerance for high concentrations of NaCl and produces high yields of xylitol (from xylose) and heptitols (Kurtzman & Phaff, 1987).

1.3.3.4 The genus *Torulaspora*

The genus *Torulaspora* is characterized by small, round cells and the production of round ascospores. Some of the species are osmotolerant.

In some countries *Torulaspora delbrueckii* has been used as a baker's yeast; its osmotolerance makes it useful for raising sweet breads and pastries. It's main

disadvantage as a baker's yeast is the small size of its cells, which makes recovery of the biomass during production more difficult (Spencer & Spencer, 1997).

1.4 Fermentation process

1.4.1 The yeast ecology of fermentation

International competition in the wine market, consumer demands for new styles of wines and increasing concerns about the environmental consequences of wine production are providing new challenges for innovation in wine fermentation technology (Linda F. Bisson, Waterhouse, Ebeler, Walker, & Lapsley, 2002).

Identification of yeast species that conduct the alcoholic fermentation and kinetics of their growth throughout this fermentation are essential steps in understanding how yeasts impact wine quality and how new styles of wines can be developed. The diversity of yeasts species arising from the grape berry and the winery environment have been known for a long time. Moreover, the information about non-*Saccharomyces* species' tasks during the alcoholic fermentation is well obtained. Many of these non-Saccharomyces species such as *Hanseniaspora*, *Candida*, *Pichia*, and *Metschnikowia* are exploited for the initiation of spontaneous alcoholic fermentation of the juice. However, they are very immediately overtaken by the growth of *S.cerevisiae* that dominates the mid to final stages of the process; most often being the only species found in the fermenting juice (Beltran, et al., 2002).

Based on early ecological studies, *S.cerevisiae* and *Saccharomyces bayanus* was considered as the main yeasts that complete the alcoholic fermentation; making them available for development of starter culture technology around them (G. H. Fleet, 2008).

Previous studies on quantitative growth of individual yeast species throughout juice fermentation demonstrated that *non-Saccharomyces* species commonly achieved maximum populations of 10^7 CFU mL^{-1}or more in the early stages of fermentation before they died off. From this result, the amount of biomass was adequate to impact on the chemical composition of the wine. Besides, under certain circumstances, such as low temperature fermentation, some *non-Saccharomyces* species did not die off and remained until the end of fermentation with *S.cerevisiae* (Heard & Fleet, 1988).

Previous experiments show that these indigenous *non-Saccharomyces* yeasts also grew in the case of inoculated fermentations with *S.cerevisiae*. It is now known that non-*Saccharomyces* species contribute to the overall kinetics of yeast growth during both spontaneous and *S.cerevisiae*-inoculated wine fermentations (Egli, Edinger, Mitrakul, & Henick-Kling, 1998; Granchi, Bosco, Messini, & Vincenzini, 1999; K. Zott, et al., 2010; Katharina Zott, Miot-Sertier, Claisse, Lonvaud-Funel, & Masneuf-Pomarede, 2008).

Wine fermentations, whether spontaneous or inoculated, are ecologically complex and do not only involve the growth of a succession of non-*Saccharomyces* and *Saccharomyces* species but also involve the consecutive development of strains within each species. Such complexity presents a challenge to conducting controlled fermentations with particular yeast cultures designed to impose a special character or style on the final product. In such cases, predictable, dominant growth of the inoculated strain or a mixture of strains would be required. Many factors such as grape juice composition, pesticide residues, sulfur dioxide addition, concentration of dissolved oxygen, ethanol accumulation and temperature affect the kinetics of yeast growth during wine fermentations, but little is known regarding how these factors might affect the dominance and succession of individual species and strains within the total population (Linda F. Bisson, 1999; G. H. Fleet, 2003; Katharina Zott, et al., 2008).

It is generally considered that the succession of strains and species throughout fermentation is generally determined by their different susceptibilities to increasing concentration of ethanol; the non-*Saccharomyces* species dying off earlier in the process because they are more sensitive to ethanol than *S.cerevisiae* (Mills, Johannsen, & Cocolin, 2002).

In addition to ethanol, other phenomena such as temperature of fermentation, dissolved oxygen content, killer factors, quorum-sensing molecules and spatial density influences are known to affect the competitive interaction between yeast species and strains in wine fermentations (G. H. Fleet, 2003; Holm Hansen, Nissen, Sommer, Nielsen, & Arneborg, 2001; Yap, de Barros Lopes, Langridge, & Henschke, 2000).

1.4.2 Spontaneous Fermentation

Grape must is a nonsterile substrate that contains several types of microorganisms, and in particular, there may be growth of various yeasts that can ferment the substrate. As a consequence, natural fermentation is carried out through a sequence of different yeast species. There is a sequential use of substrate: initially, apiculate yeasts (*Hanseniaspora/Kloeckera*) are abundant, although after 3-4 days, they are replaced by *Saccharomyces cerevisiae* (Mortimer & Polsinelli, 1999).

In addition, during the various stages of fermentation, it is possible to isolate other yeast genera, such as *Candida, Pichia, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Kluyveromyces,* and *Metschnikowia* (Raspor, Milek, Polanc, Smole Mozina, & Cadez, 2006; K. Zott, et al., 2010).

The growth of non-*Saccharomyces* species belonging to the genera *Kloeckera/Hanseniaspora* and *Candida* is generally limited to the first few days of fermentation, because of their weak ethanol tolerance. However, quantitative studies on grape juice fermentation have shown that *Kloeckera apiculata* and *Candida stellata* can survive at significant levels during fermentation, and for longer periods than thought previously (G. H. Fleet, Lafon-Lafourcade, S., Ribéreau-Gayon, P.,, 1984).

The presence and permanence of these non-*Saccharomyces* yeasts throughout fermentation is influenced by several physicochemical and microbiological factors. For instance, *K. apiculata* and *C. stellata* have increased tolerance to ethanol at lower temperatures (10–15℃). This behavior has also been confirmed in mixed cultures using *K. apiculata* and *S. cerevisiae* (Erten, 2002).

Recent studies have highlighted the important role of oxygen concentration in the survival of some *non-Saccharomyces* yeast during fermentation, such as T*orulaspora delbrueckii* and *Kluyveromyces thermotolerans*. Moreover, it has been shown that cell–cell interactions are involved in inhibition of these two non-*Saccharomyces* species. Thus, in the presence of high concentrations of viable cells of *S. cerevisiae* the growth of *T. delbrueckii* and *K. thermotolerans* is inhibited (Holm Hansen, et al., 2001).

1.4.3 Inoculated Fermentations

The use of selected starter cultures of *S. cerevisiae* can play an important role in the suppression of wild yeasts. Inoculated cultures of *Saccharomyces* are expected to suppress either indigenous non-*Saccharomyces* species & *Saccharomyces* strains or to dominate the fermentation. Moreover, the use of antiseptic agents, such as SO_2, to which most of the non-*Saccharomyces* yeasts are scarcely resistant, should guarantee the dominance of the inoculated strains (Ciani, Beco, & Comitini, 2006).

With the commercial availability of active dry cultures of S. *cerevisiae*, the inoculation of grape must has become more appealing and convenient. As such, the use of selected yeast cultures is widespread in both the new wine-producing countries, such as the United States, South Africa and Australia, and in the more traditional wine-producing countries, such as Italy, Germany and France. In this context, extensive use of starter cultures in all winemaking areas around the world represents an important advance in wine biotechnology. Nevertheless, the generalized use of selected starter cultures is a simplification of microbial fermentation communities that promotes the standardization of the analytical and sensory properties of wines (Toro & Vazquez, 2002).

1.4.4 Controlled fermentations with mixed strains of yeasts

Inoculated fermentation with single starter culture is mentioned above. However, some of these species are limited in their ability to completely ferment the grape juice sugars and in their ability to produce sufficient concentrations of ethanol. Some may grow too slow in comparison with other indigenous yeasts. Nevertheless, they have other properties of oenological relevance that would be worth exploiting. For example, some *Hanseniaspora/Kloeckera* species may produce more appealing mixtures of flavor volatiles, and higher amounts of glycosidases and proteases than *Saccharomyces* species. *C. stellata* gives increased levels of glycerol. *Kluyveromyces thermotolerans* gives increased levels of lactic acid. *Torulaspora delbrueckii* produces less acetic acid and *Schizosaccharomyces* species decrease wine acidity through malic acid metabolism (Capece, Fiore, Maraz, & Romano, 2005; Ciani, et al., 2006; Zironi, Romano, Suzzi, Battistutta, & Comi, 1993).

Conducting wine fermentations by controlled inoculation of mixtures of different yeast starter cultures is already known but, it now attracts greater interest because of its potential of introducing characteristics into wine and because winemakers have a more thorough knowledge of the ecology and biochemistry of wine fermentation and how to manage this process.

The mixtures of non-Saccharomyces species that grow interactively with *S. cerevisiae* in comparison with monocultures of the respective yeasts are shown in Table 2. Growth profiles are generally reported, along with glucose and fructose utilization, and the production of key metabolites such as ethanol, acetic acid, glycerol, ethyl acetate and, in some cases, various higher alcohols, higher acids and other esters. Essentially, these studies confirm that non-*Saccharomyces* yeasts grow in sequential patterns similar to those observed for spontaneous wine fermentations, but conditions such as temperature, sulphur dioxide addition, inoculum levels and time of inoculation can be manipulated to enhance the extent of their survival and contribution to the overall fermentation. Inoculating ethanol-sensitive or slow-growing non-*Saccharomyces* yeasts into the grape juice several days before inoculating *S. cerevisiae* (sequential inoculation) is one strategy for enhancing their contribution to the fermentation (Erten, 2002; Moreira, Mendes, Hogg, & Vasconcelos, 2005; Zironi, et al., 1993).

Table 2 Wine fermentation inoculated with defined mixtures of yeast species

Yeast species inoculated	Objective of study
S. cerevisiae, *K. apiculata*, *Candida* spp.	Ecological interactions
S. cerevisiae, *T. delbrueckii*, *K. thermotolerans*	Ecological interactions
S. cerevisiae, *H. uvarum*, *K. thermotolerans*, *T. delbrueckii*	Ecological interactions
S. cerevisiae, *Hanseniaspora* spp.	Ecological interactions
S. cerevisiae, *K. apiculata*	Ecological interactions
S. cerevisiae, *K. apiculata*, *T. delbrueckii*	Flavour modulation
S. cerevisiae, *T. delbrueckii*	Flavour modulation
S.cerevisiae, *H. guilliermondii*, *K.apiculata*	Flavour modulation
S.cerevisiae, *H. uvarum*, *K. apiculata*	Flavour modulation
S. cerevisiae, *C. stellata*	Flavour modulation
S. cerevisiae, *D. vanriji*	Flavour modulation
S. cerevisiae, *K. apiculata*, *C. pulcherrima*	Flavour modulation
S. cerevisiae, *P. fermentans*	Flavour modulation
S. cerevisiae, *P. anomala*	Flavour modulation
S. cerevisiae, *Hanseniaspora* spp.	Flavour modulation
S. cerevisiae, *K. thermotolerans*	Acid (lactic) enhancement
S. cerevisiae, *S. pombe*	Deacidification (malic acid)
S. cerevisiae, *C. cantarellii*	Glycerol enhancement
S. cerevisiae, *C. stellata*	Glycerol enhancement
S. cerevisiae, *T. delbrueckii*	Decrease volatile acidity
S. cerevisiae, *S. bayanus* (three strain mixtures)	Metabolic and flavour interactions
S. cerevisiae, *C. colliculosa*	Ecological interactions
S. cerevisiae, *C. stellata*	Flavour modulation
S. cerevisiae, *K. apiculata*	
S. cerevisiae, *C. pulcherrima*	

Wines made out of mixed cultures gave a combination of volatile aroma metabolites different from that obtained by blending to gather monocultures wines made with the same yeast strains. Thus, with respect to production of flavor volatiles in wine, the metabolic interactions of yeasts during mixed culture could be quite complex and difficult to predict. The ultimate evaluation of such fermentations should be based on sensory testing.

The impact of non-*Saccharomyces* yeasts in mixed culture with *S. cerevisiae* can be more definitive when specific wine properties are targeted, such as decreasing malic acid concentrations using *Schizosaccharomyces* species or using *Torulaspora delbrueckii* to prevent volatile acidity production in sweet wine fermentations. Sequential inoculation of *S. pombe* before *S. cerevisiae* appears to be necessary for a successful deacidification but, unfortunately, this yeast can give off-flavors to the wine. Possibly, a programme of selection of yeasts could avoid these problems and for future development of wine fermentation technology, these fundamental studies will help to

produce well controlled sensory evaluations of wine flavor and color (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008).

1.4.5 The role of non-*Saccharomyces* yeasts in must fermentation

Earlier studies considered non-*Saccharomyces* yeasts as 'wild' yeasts or 'spoilage' yeasts, because they were often isolated from stuck or sluggish fermentations, or from wines with anomalous analytical and sensorial profiles (Munoz & Ingledew, 1989).

Pure culture fermentations with non-*Saccharomyces* wine yeasts have shown several negative metabolite and fermentation characteristic that generally exclude their use as starter cultures. The most important spoilage metabolites produced by non-*Saccharomyces* wine yeasts are acetic acid, acetaldehyde, acetoin and ethyl acetate (Ciani, et al., 2006).

Moreover, most of the non-*Saccharomyces* wine-related species show limited fermentation aptitudes, such as low fermentation power (the maximum amount of ethanol in the presence of an excess of sugar) and rate, and a low SO_2 resistance. However, in mixed fermentations such as natural fermentations, some negative enological characteristic of non-*Saccharomyces* yeasts may not be expressed or be modified by *S. cerevisiae* cultures. In this context, following the investigations of the last decades on the quantitative presence and persistence of non-*Saccharomyces* wine yeasts during fermentation, several studies have been carried out to determine their oenological properties and their possible roles in winemaking (Egli, et al., 1998; Henick, Edinger, Daniel, & Monk, 1998; Romano, Fiore, Paraggio, Caruso, & Capece, 2003; Romano & Suzzi, 1996).

Experimental evidence has highlighted the positive role of non-*Saccharomyces* yeasts in the analytical composition of wine. Some non-*Saccharomyces* yeast species can improve the fermentation behavior of yeast starter cultures and the analytical composition of wine, or lead to a more complex aroma (G. H. Fleet, 2003).

Consequently, during recent years, there has been a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking and today more attention is being paid to the ecology of fermenting yeasts, to better understand the impact of non-*Saccharomyces* strains on the chemistry and sensory properties of wine. In this context, the enzymatic

activities of non-*Saccharomyces* wine yeasts are seen to influence the wine profile (Heard & Fleet, 1985).

Investigations of poly-galacturonase and β-D-xylosidase production by non-*Saccharomyces* yeasts involved in wine making showed that these activities are widely dispersed in these yeasts and can be used to enhance wine quality (Fernandez-Espinar, Lopez, Ramon, Bartra, & Querol, 2001).

Another biocatalytic activity widely associated with non-*Saccharomyces* wine yeasts is β-glucosidase activity. β-Glucosidase hydrolyses terpenyl-glycosides, and can enhance the wine aroma. In contrast to grape glucosidase, β-glucosidase produced by yeast is not inhibited by glucose, and it is involved in the release of terpenols during fermentation. This β-glucosidase activity has been found in several yeast species associated with winemaking, especially among the non-*Saccharomyces* species (Martinez-Rodriguez, Polo, & Carrascosa, 2001). The diffusion of this activity among non-*Saccharomyces* wine yeasts has confirmed the role of these yeasts in enhancing wine aroma (Manzanares, Ramón, & Querol, 1999).

In addition to the enzymatic activities of non-*Saccharomyces* wine yeasts, other specific properties of wine making have been evaluated to improve our knowledge of the metabolic characteristics, and to test the intraspecific variability of these wine yeasts. Non-*Saccharomyces* strains can be selected on the basis of their ability to produce favorable metabolites that contribute to the definition of the final bouquet of a wine. 38 yeast strains screened which is belonging to the *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* genera for acetate ester formation. Here, they identified *Hanseniaspora osmophila* as a good candidate for mixed cultures, due to its glucophilic nature, the ability to produce acetaldehyde within a range compatible for wine and acetate ester production, in particular of 2-phenylethyl acetate. A rapid method to evaluate wine-yeast performance based on the ability of a yeast species to produce levels of metabolites that contribute towards improving wine quality has been proposed (Romano, Fiore, et al., 2003; Viana, Gil, Genovés, Vallés, & Manzanares, 2008).

In particular, through determination of 2, 3-butanedioland acetoin stereoisomers, these compounds have been demonstrated to be characteristic for *S. cerevisiae* and *K. apiculata* yeast species. *S. cerevisiae* is a higher producer of 2,3-butanediol in comparison with *K. apiculata*. In literature, it is seen that the role of H. g*uilliermondii* and *Hanseniaspora uvarum* in pure and mixed starter cultures with *S.*

cerevisiae help with production of heavy sulphur compounds and esters. The results highlight that these apiculate yeasts enhance the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds (Moreira, et al., 2005; Romano, Granchi, et al., 2003).

1.4.6 Fermentation options

Microbial fermentations can be conducted as either batch processes or continuous processes. Almost all wines are produced by batch fermentation, which means that the juice is placed in a vessel and the entire batch is kept there until fermentation is completed, usually takes for 5-10 days (Jackson, 2008).

For the batch fermentation, there are two options existing in wine production: spontaneous (natural) fermentation or inoculated (starter culture) fermentation.
Spontaneous fermentations can give high-quality wines with a unique regional character that provides differentiation and added commercial value in a very competitive market. Unfortunately, reliance on 'natural' brings diminished predictability of the process, such as stuck or slow fermentations, and inconsistencies in wine quality. Even so, most of the wine production particularly in European countries is commercially produced by this process (Pretorius, 2000).

Starter culture fermentations offer the advantage of a more predictable and rapid process, giving wines with greater consistency in quality. And so, they are well suited for producing mass market wines by giving a commercial availability of dried concentrates of selected yeast strains (Manzano, et al., 2006). Usually, technological expertise is needed for success with these fermentations.

As commercial preparations, there are lots of *S.cerevisiae* and *S.bayanus* strains available, but starter culture wines may be lacking in flavor complexity and ordinary in character. To avoid this situation, unconventional strains of starter culture yeasts are selected and fermentations are conducted with controlled mixtures of yeast species and strains (Linda F. Bisson, 2005; Lilly, Lambrechts, & Pretorius, 2000; Pretorius, 2000).

1.4.7 The facts that effect the initial yeast population in winemaking

The population density and diversity of indigenous yeasts on grape berries are intricately linked to numerous factors, such as berry maturity, grape variety, geographic location, climatic condition, fungicide application, vineyard age, and viticultural practices (Chavan, et al., 2009; Combina, et al., 2005; Martini, 1996; Raspor, et al., 2006).

1.4.8 Criteria for selecting and developing new strains of wine yeasts

Criteria for selecting and developing new strains of wine yeasts can be grouped under three main headings as mentioned below:

1. Properties that affect the performance of the fermentation process,
2. Properties that determine wine quality and character and
3. Properties associated with the commercial production of wine yeasts.

For the first criteria, rapid, active and complete fermentation of grape juice sugars to high ethanol concentrations (> 8% v/v) are essential requirements of wine yeasts. The yeast should be tolerant of the concentrations of sulfur dioxide added to the juice as an antioxidant and antimicrobial, exhibit uniform dispersion and mixing throughout the fermenting juice, produce minimal foam and sediment quickly from the wine at the end of fermentation. These processing properties should be well expressed at low temperatures (e.g. 15℃) for white wine fermentations and at higher temperatures (e.g. 25℃) for red wine fermentations. It is important that the yeast does not give slow, sluggish or stuck fermentations (Linda F. Bisson, 1999; Ciani & Comitini, 2010; Pretorius, 2000; Pretorius & Bauer, 2002). Otherwise, with respect to wine quality and character, selected yeasts flavor metabolites such as, acetic acid, ethyl acetate, hydrogen sulphide and, sulphur dioxide never reach undesirable amounts during fermentation. They should not affect wine color or its tannic character unfavorably (Table 3, 4) (Linda F. Bisson, 2005; Swiegers & Pretorius, 2005).

Table 3 Technological characteristics to be considered in the selection of wine yeast strains (S. RAINIERI, 2000).

Ethanol tolerance
Fermentation vigour
Resistance to SO2
Type of growth in liquid media
Dispersed cells
Aggregates cells
Flocculence
Foam formation
Film formation
Sedimentation speed
Growth at high and low temperatures
Presence of killer factor

Table 4 Qualitative characteristics to be considered in the selection of wine yeast strains(S. RAINIERI, 2000)

Fermentation by-products
Glycerol
Succinic acid
Acetic acid
Acetaldehyde
n-Propanol
Iso-butanol
Isoamyl alcohol
β-Phenylethanol
Production of sulfuric compounds
H_2S
SO_2
Action on malic acid
Enzymatic activity
β-Glucosidas
Proteolytic enzymes

Looking from the commercial aspects as a wine producer, the yeast should be facilitated to large-scale cultivation on relatively inexpensive substrates such as molasses. For further steps, it needs to be tolerant of the stresses of drying, packaging, storage and, finally, rehydration and reactivation by the winemaker (Soubeyrand, Julien, & Sablayrolles, 2006).

However, wine consumers' demands have changed in recent years and now there are requests more distinctive and with specific styles, including those with healthier appeal such as, less ethanol, increased antioxidant levels, etc. For these purposes, properties to give these qualities are different from those of the past and yeast selection and development process should be designed according to the criteria listed below:

1. Improved fermentation performance (e.g. yeasts with greater efficiency in sugar and nitrogen utilization, increased ethanol tolerance, decreased foam production).
2. Improved process efficiency (e.g. yeasts with greater production of extracellular enzymes such as proteases, glucanases and pectinases to facilitate wine clarification; yeasts with altered surface properties to enhance cell sedimentation, floatation and flor formation, as needed; and yeasts that conduct combined alcoholic-malolactic fermentations).
3. Improved control of wine spoilage microorganisms (e.g. yeasts producing lysozyme, bacteriocins and sulphur dioxide that restrict spoilage bacteria).
4. Improved wine wholesomeness (e.g. yeasts that give less ethanol, decreased formation of ethyl carbamate and biogenic amines, increased production of resveratrol and antioxidants).
5. Improved wine sensory quality (e.g. yeasts that give increased release of grape terpenoids and volatile thiols, increased glycerol and desirable esters, increased or decreased acidity and optimized impact on grape phenolics) (Linda F. Bisson, 2005; Linda F. Bisson, et al., 2002; Verstrepen, Chambers, & Pretorius, 2006).

1.5 Identification of the isolated yeast strains from grapes

1.5.1 Sources of new wine yeasts

During the past 50–75 years, wine production has been transformed into a modern, industrialized process, largely based on the activities of only two yeast species: *S.cerevisiae* and *S. bayanus*. Future developments will continue to be based on innovation with these species, but opportunities for innovation using other species of yeasts cannot be overlooked. As mentioned already, various species of *Hanseniaspora*, *Candida*, *Kluyveromyces* and *Pichia* play significant roles in the early stages of most wine fermentations, and there is increasing interest in more strategic exploitation of these species as novel starter cultures (Ciani & Maccarelli, 1998; S. RAINIERI, 2000).

Their limitations with regard to ethanol tolerance may not be a hurdle in the production of wines with lower, final ethanol contents. Various species of *Zygosaccharomyces*, *Saccharomycodes* and *Schizosaccharomyces* are strong fermenters and are ethanol tolerant. Although they are generally considered as spoilage yeasts, there is no reason to doubt that a good programme of selection and evaluation within these yeasts would not discover strains with desirable winemaking properties (Zironi, et al., 1993).

It needs to be recalled that not all strains of *S.cerevisiae* produce acceptable wines, and that a systematic process of selection and evaluation is needed to obtain desirable strains. Consequently, in searching for and developing new yeasts, the wine industry of the future must look beyond *Saccharomyces* species. In addition, it must look beyond grapes and give broader consideration to other fruits as the starting raw material. With such vision, many new yeasts and wine products await discovery. Essentially, there are two strategies for obtaining new strains of wine yeasts for development as commercial starter cultures:

1. isolation from natural sources and
2. genetic improvement of natural isolates.

Once a prospective isolate has been obtained, it is screened in laboratory trials for essential oenological criteria as mentioned already. Isolates meeting acceptable criteria are then used in micro-scale wine fermentations and the resulting wines are then subjected to sensory evaluation. Strains giving good fermentation criteria and acceptable-quality wines under these conditions are then selected for further development as starter culture preparations (Cappello, Bleve, Grieco, Dellaglio, & Zacheo, 2004).

1.5.2 Natural sources

Generally, wine yeasts for starter culture development have been sourced from two ecological habitats, namely, the vineyard (primarily the grapes) and spontaneous or natural fermentations that have given wines of acceptable or unique quality. As mentioned above, yeasts are part of the natural microbial communities of grapes. Understandably, therefore, grapes are always considered a potential source of new wine yeasts. There is an attraction that unique strains of yeasts will be associated with particular grape varieties in specific geographical locations and, through this association, they could introduce significant diversity and regional character or 'terroir' into the winemaking process (Martinez, Cosgaya, Vasquez, Gac, & Ganga, 2007; Raspor, et al., 2006; Valero, Cambon, Schuller, Casal, & Dequin, 2007).

The yeast species and populations evolve as the grape berry matures on the vine and are influenced by climatic conditions such as temperature and rainfall, application of agrichemicals and physical damage by wind, hail and attack by insects, birds and animals. The predominant semi-fermentative and fermentative yeasts isolated from grapes at the time of maturity for winemaking are mostly species of *Hanseniaspora* (*Kloeckera*), *Candida*, *Metschnikowia*, *Pichia* and *Kluyveromyces*, although the data are not always consistent. If the berries are over-ripe, become damaged or are infected with filamentous fungi(mould), the yeast populations tend to be higher and include a greater incidence of fermentative species such as those of *Saccharomyces*, *Zygosaccharomyces*, *Saccharomycodes* and *Zygoascus* (Combina, et al., 2005; Martini, 1996).

It is difficult to isolate *Saccharomyces* species from mature, undamaged grapes by direct culture on agar media, but they are frequently found by enrichment culture methods, suggesting their presence in very low numbers. Grape berries that are aseptically harvested from vines and crushed will eventually ferment and strains of *S.*

cerevisiae and *S. bayanus* are easily isolated from the fully fermented must (Mercado, Dalcero, Masuelli, & Combina, 2007; Valero, et al., 2007).

Strains of *Saccharomyces paradoxus*, capable of producing wine, have also been isolated from grapes. However, recovery of *Saccharomyces* species from such ferments is not always consistent and can be determined by many factors that are likely to affect the occurrence and survival of yeasts on the grape surface, such as amount of rainfall, temperature and applications of agrichemicals. It was observed that the frequency of isolation of *Saccharomyces* species from aseptically harvested and crushed grapes can be significantly increased by removing the skin and allowing the juice to ferment. Possibly, such modifications give slow initial numbers of *Saccharomyces* a better chance to compete with the higher populations of other species. As mentioned above, damaged grape berries are more likely to yield *Saccharomyces* species than non damaged grapes. Based on molecular analyses, using pulsed field gel electrophoresis and restriction fragment length polymorphism of mtDNA, grape isolates of *S. cerevisiae* exhibit substantial genomic diversity, because many different strains have been obtained from grapes within the one vineyard or geographical region. In some cases, particular strains have been unique to one location, leading to the notion of a yeast 'terroir' (Raspor, et al., 2006; Vezinhet, Hallet, Valade, & Poulard, 1992).

Clearly, the grape itself is a primary source of the yeasts that occur in the juice and it is logical to conclude that any *Saccharomyces* strains from this source would be prominent in the final fermentation. However, processing of the juice and its transfer to fermentation tanks contributes to addition of microbial communities. These communities originate as contamination from the surfaces of winery equipment and are widely considered to be 'residential' flora that have built up in the winery over time, through a process of adaptation and selection, despite cleaning and sanitation operations. These floras are dominated by fermenting ethanol-tolerant yeast species such as *S. cerevisiae* and *S. bayanus* because of the selective conditions presented by the properties of fermenting grape juice (Mercado, et al., 2007; Santamaría, Garijo, López, Tenorio, & Rosa Gutiérrez, 2005).

Presumably, the *Saccharomyces* flora in the winery originally came from grapes and evolved with time. The source of *Saccharomyces* yeasts on the grapes is still a mystery, but contamination from insects in the vineyard is thought to be a likely possibility (Mortimer & Polsinelli, 1999).

1.5.3 New PCR based methods for yeast identification

Traditionally, yeasts are identified by morphological and physiological criteria, but these methods are generally laborious and time consuming. Moreover, they sometimes provide doubtful identification, because of the influence of culture conditions on yeast physiological characteristics. Genetic markers, DNA karyotyping, and PCR (DNA Polymeric Chain Reaction) amplification now provide direct, highly specific methods for identifying and following single strains through the course of fermentation, even when cell numbers are very low. These methods allow investigate or to enumerate the effectiveness of starter strains as well as the presence and possible contributions of other strains.

In recent years, especially two molecular techniques, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequence analyses of the ribosomal DNA (rDNA) region including 5.8S internal transcribed spacer (ITS) region, have proved to be useful for the rapid identification of wine yeast species. Additional techniques are also applied on wine yeasts (Table 5) (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Heras-Vázquez, & Rodríguez-Vico, 2004; Katharina Zott, et al., 2008). The latter methods have proven to be useful for the differentiation of wine yeasts at species level (Guillamon, Sabate, Barrio, Cano, & Querol, 1998).

Table 5 Molecular methods for wine yeast strain differentiation (Pretorius, 2000)

Method	Description
Electrophoretic karyotyping (chromosome fingerprinting)	Whole yeast chromosomes are separated electrophoretically using pulse- field techniques.
Restriction enzyme analysis	Total, ribosomal or mitochondrial DNA is digested with restriction endo-nucleases and specific fragments are detected. RFLP-mtDNA, RFLP-ITS/5.8S
RAPD- PCR	Amplification of DNA with random primers, fragment length polymorphism
r-DNA sequence analysis	ITS region sequence analysis

1.5.3.1 ITS region

Recently, PCR–RFLP of the rDNA internal transcribed spacer (ITS) region has been described as a valuable tool for the identification of several yeast species. Indeed, the ITS region, including the conserved gene coding for the 5·8 rRNA and the two flanking non-coding and variable internal transcribed spacers as seen in the Figure 9, ITS1 and ITS2, shows a high interspecific size variability but a low intraspecific polymorphism. Moreover, the highly conserved sequences of rRNA genes flanking the ITS region allow the use of universal primers for fungi (Guillamón, Sabaté, Barrio, Cano, & Querol, 1998).

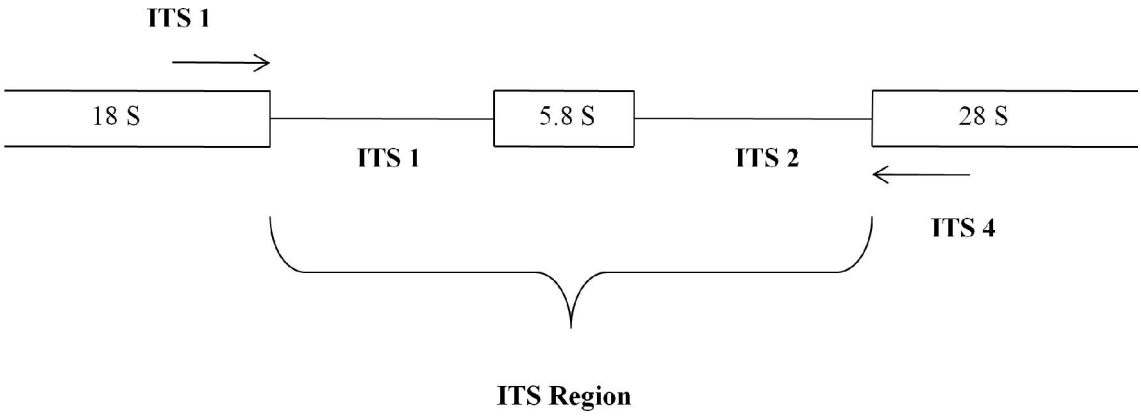


Figure 9 Organization of the ITS (Internal transcribed spacer) region. Arrows indicate orientation and approximate position of primer sites.

Therefore, when different yeast species are present simultaneously, as occurring during wine fermentations, PCR-based ITS region analysis seems to be safely applicable, as resulting amplicons show species-specific molecular sizes.

1.5.4 Biolog system for identification of the isolated yeasts

The MicroLog System is an easy- to use yet advanced tool for identifying and characterizing microorganisms. The combined databases include over 1,900 species of aerobic bacteria, anaerobic bacteria fungi and yeasts. They contain almost all of the significant species encountered in diverse practices of microbiology, including pharmaceutical, biotechnology, cosmetic, and medical device companies; veterinary and clinical medicine; agriculture and environmental science; food processing, spoilage, and safety; reference laboratories; industrial microbiology; and research and education.

1.5.4.1 Functionality of the system

Biolog's innovative, patented technology uses microbe's ability to use particular carbon sources to produce a unique pattern or ''fingerprint'' for that microbe. As a microorganism begins to use the carbon sources in certain wells of the MicroPlate, it respires (Praphailong, Van Gestel, Fleet, & Heard, 1997). The result obtained is a

pattern of colored wells on the MicroPlate that is characteristic for a microorganism Assimilation or growth is detected by the turbidity of the well (Truu, et al., 1999).

A yeast pattern is readable either visually or by a fiber optic reading instrument like the MicroStation Reader. This reader is required to read a yeast or fungal pattern. The fingerprint data is fed into MicroLog software, which searches its extensive databases and makes identification in seconds (Praphailong, et al., 1997).

1.5.4.2 The identification process

Microbial identification involves five basic steps as shown in Figure 10. These steps apply to all identifications. A small number of species have peculiarities that may require an extra step or special handling techniques.

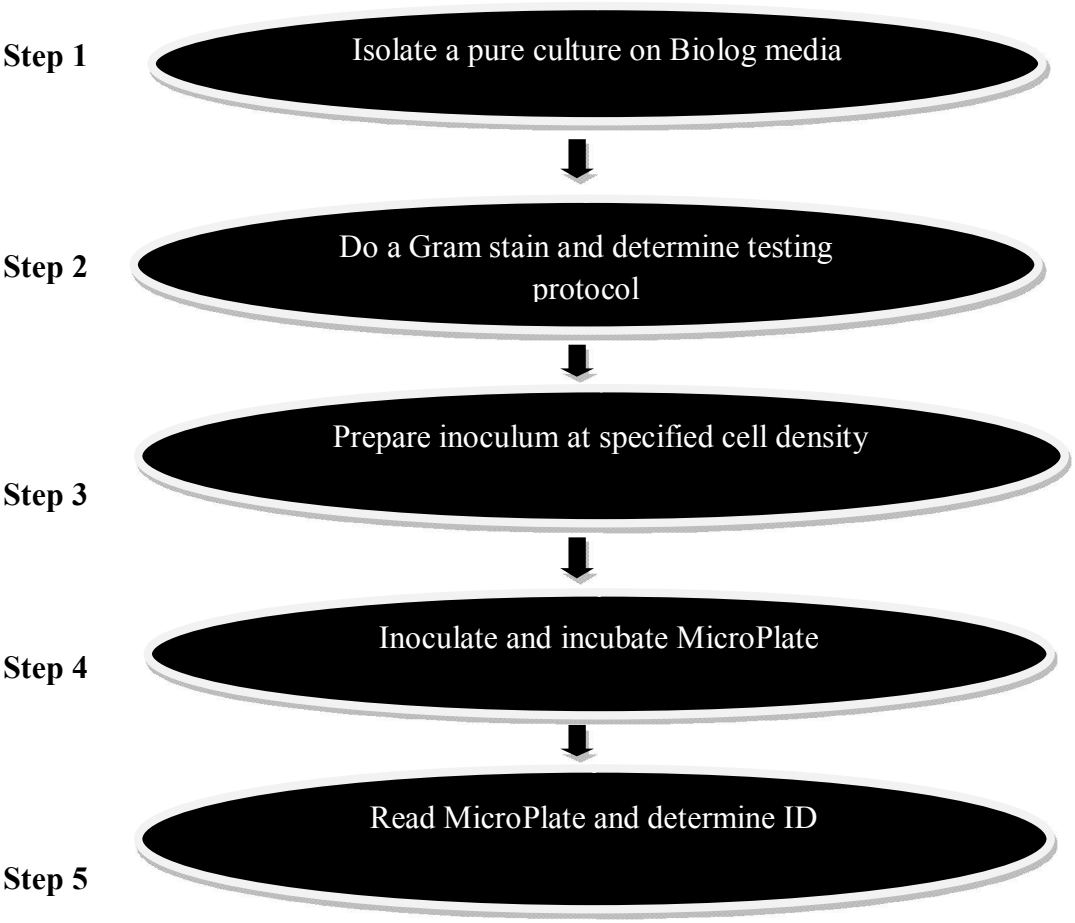


Figure 10 The microlog microbe identification process

1.5.5 Selective media for isolated yeasts

1.5.5.1 Lysine Medium (LM)

This medium is selective for yeasts other than *Saccharomyces* strains, which grow only very slowly or not at all in media with lysine as the sole nitrogen source .LM is used to monitor the presence of non-*Saccharomyces* species effectively, since it is a medium with L-Lysine as the sole nitrogen source and Saccharomyces spp. are unable to grow on this medium (van der Aa Kühle & Jespersen, 1998).

1.5.5.2 Ethanol Sulfite Agar (ESA)

This medium is selective for *Saccharomyces* strains. ESA medium is used to detect the native populations of *Saccharomyces* species, because non-*Saccharomyces* yeasts have lower tolerance of ethanol and sulfur dioxide (Kish, Sharf, & Margalith, 1983).

1.5.5.3 Wallerstein Laboratory Medium (WL)

This is useful for the wine industry to quantify and identify wine microorganisms, since it can discriminate between the yeast genus and species by colony morphology and color (Li, et al., 2010; Pallmann, et al., 2001)

1.6 Genetic improvement of wine yeasts

Through genetic improvement and metabolic engineering technologies, it is now possible to develop wine yeasts with a vast array of specific functionalities as mentioned in Table 3 and 4 (e.g. strain with enhanced glycerol production; strain with bacteriocin production;). However, it is important to be ensured that any genetic manipulation does not adversely affect its basic winemaking properties.

1. Mutagenesis.
2. Spheroplast fusion.
3. Intraspecific and interspecific hybridization.
4. Transformation and recombinant DNA techniques.
5. Adaptive evolution.
6. Systems biology and functional genomics.

Although, yeast mating and hybridization methods were used to develop strains of *S.cerevisiae* with improved properties, (e.g. flocculation, less hydrogen sulphide production earlier) recombinant DNA techniques overtook their place since their prices and convenient results; for instance, wine strains of *S.cerevisiae* that give enhanced release of volatile thiols and decreased ethyl carbamate production (Linda F. Bisson, 2005; Pretorius, 2000; Pretorius & Bauer, 2002; D. Schuller, Valero, Dequin, & Casal, 2004; Verstrepen, et al., 2006).

Unfortunately, consumer and government concerns about the public health and environmental safety of microbial strains engineered by recombination prevent the commercial usage of these yeasts, so far, only one recombinant strain of wine yeast has received approval for commercial use which is a strain of *S.cerevisiae* constructed to contain a malate-permease gene from the yeast, *Schizosaccharomyces pombe*, and the malolactic gene from the bacterium, *Oenococcus oeni*. This strain offers the advantage of improved process efficiency by eliminating the need for bacterial malolactic fermentation that is usually conducted after alcoholic fermentation (Husnik, et al., 2006; Dorit Schuller & Casal, 2005).

Hybridization, adaptive evolution, and systems biology are now used for the development of a new generation of wine yeasts. Inter- and intraspecies hybrids within strains of *Saccharomyces* (e.g. *S. cerevisiae* X *S. bayanus* and *S. cerevisiae* X *S. kudriazevii*) have been isolated from spontaneous fermentations and similar hybrids, now commercially available, have been produced by mating yeasts under laboratory conditions. Hybrids between *S. cerevisiae* and other species within *Saccharomyces* are also available (e.g. *S. cerevisiae* X *S.cariocanus*, *S. cerevisiae* X *S. paradoxus* and *S. cerevisiae* X *S.mikatae*). Hybridization expands the tolerance of some strains to the stresses of winemaking such as temperature of fermentation and ethanol concentration and increases the pool of strains available to enhance diversity in wine flavor (L. F.

Bisson, Karpel, Ramakrishnan, & Joseph, 2007; Gonzalez, Martinez-Rodriguez, & Carrascosa, 2003; González, Barrio, Gafner, & Querol, 2006).

Adaptive evolution is another aspect of selecting strains with oenological performance and flavor profiles matched to a particular winemaking need. In this case, yeasts are continuously and repeatedly cultured under a defined combination of conditions from which strains that have specifically adapted to these conditions can be isolated (McBryde, et al., 2006).

Systems biology exploits knowledge of the total genome and bioinformatics methods to select and develop new strains of wine yeasts with very specific functionalities and criteria, as determined by production, consumer and environmental demands. Because genomic information about wine yeasts is still very limited, this approach is at a conceptual stage of development and practical outcomes are yet to be realized (Borneman, Chambers, & Pretorius, 2007).

2 MATERIALS AND METHODS

2.1 Grape sampling

Grape samples were collected during harvest in 2010 vintage (between end of August and September). Grapes were sampled from three locations in Turkey as shown in Table 6.

Table 6 Locations of grape varieties from which yeasts were isolated.

	Vineyard Locations		
	Adana (Saimbeyli)*	**Urla (Kuşçular village)****	**Tekirdağ*****
Grape Varieties	K	MA 1	
	CK	MA 2	
	M	Y	
	MU		
	KA		
	KB		

*The varieties of the grapes taken to the lab are known

** Urla grapes are taken from 3 different vineyards and first two vineyards are special property and their varieties are unknown but the last vineyard's grapes are mixture of foreign grape varieties.

***Neither locations nor varieties are known for these grapes.

From each selected vineyard, only healthy and undamaged grapes were aseptically and randomly collected in plastic bags immediately before harvest. Around 1-2 kg of each sample were transported in cold boxes to the laboratory and analyzed within 24h of harvest from the vineyard.

2.2 Yeast enumeration and isolation

For each sample, two different methods were applied for the isolation of whole yeast flora.

1. Method: Streaking

Single grapes were aseptically separated from a single bunch and streaked from a berry on YPD agar (Duchefa) plate as seen in the pictures below.

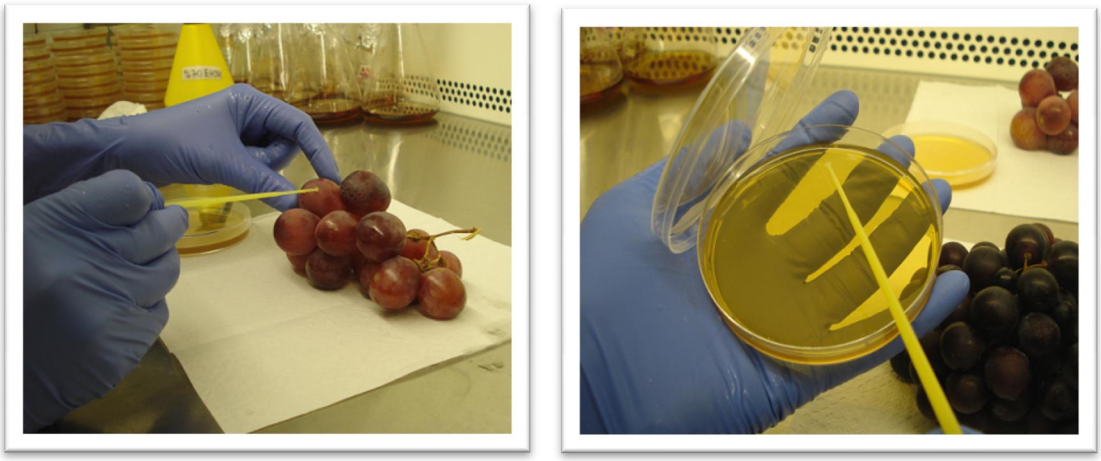


Figure 11 Representation of streaking method from one berry.

2. Method: Crushing and spreading

The content of each bag was aseptically crushed and homogenized by using sterile crucible (Figure 12). The homogenized samples were serially diluted with the sterile physiological saline (0.85%NaCl). For the enumeration of yeasts, 0.1 ml of each dilution was spread in triplicate on two different media: a non- selective YPD – agar medium (Duchefa) and Wallerstein Laboratory nutrient agar (WL; Fluka Analytical). The WL medium is useful for the wine industry to quantify and identify wine microorganisms, since it can discriminate between the yeast genus and species by colony morphology and color.

Figure 12 Representation of crushing the bunch of grapes

All plates were incubated at 28°C for 2-3 days. Based on colony morphology and color, different colonies were selected on WL medium. Selected colonies from WL medium and YPD–agar medium were purified by repetitive streaking on YPD-agar plates and then stored at 4°C for future identification.

2.2.1 Microvinifications

2.2.1.1 Pre-fermentation process

All the grape samples were pressed and crushed under sterile conditions and then filtered to pick up must using Nalgene filter as shown in Figure 13.

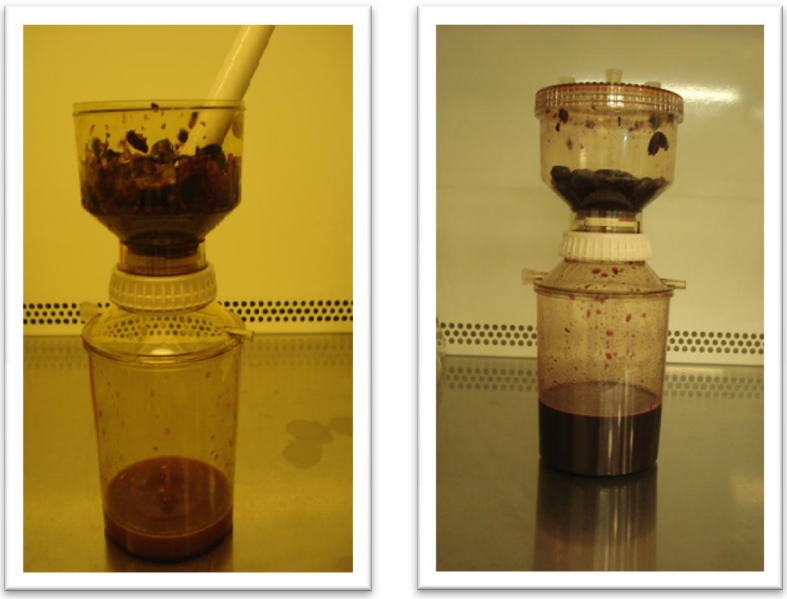


Figure 13 Pre-fermentation process: crushing and filtering

For the controlled experiments, 2 different conditions were prepared for whole varieties before the incubation period:

1. Must
2. Must& SO_2

Must and seeds were distributed to 100ml's sterile flasks and then Argon gas was sprayed into the flask before closing the fermentation caps to remove O_2 in the flasks.

2.2.1.2 Fermentation process

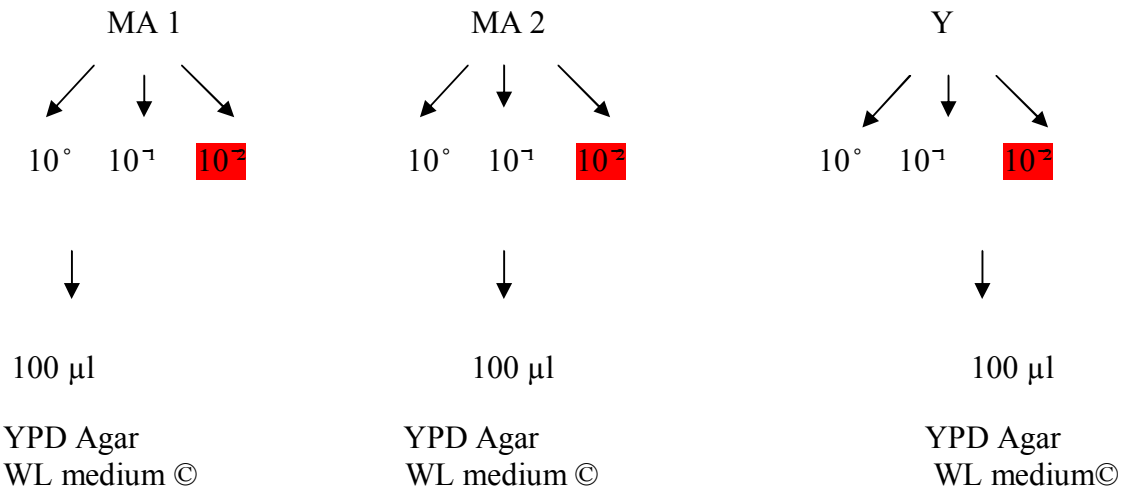
400µl SO_2 from 5% stock solution was added into previously determined flasks and fermentation process were carried out under the given conditions.

Table 7 Micro-fermentation conditions

Temperature	Shaking rate	Time
18C °	65 rpm	10 days

2.2.1.3 Post-Fermentation analysis

From 8th to last day of fermentation, 1-2 ml must samples were taken by an injector from all grape varieties and conditions. Taken samples were serially diluted with sterile physiological saline (0.85%NaCl) and spread on WL medium and YPD-agar medium as shown below.



2.2.1.4 Brix measurements and pH

Brix technique is a measurement of the amount of sugar in a liquid. Brix level is measured by hydrometer or refractometer to determine the maturity of grapes and sugar concentration that is converted to alcohol during the fermentation. As Brix numbers decrease, the amount of alcohol in the liquid increases (Boulton, 1998). For the Brix measurements of the must taken from Urla, samples were taken the day of 0, 4, 8 (100µl each) were measured with refractometer to determine the used sugar concentration during fermentation. Also acidity of the must samples was measured with pH meter.

2.2.2 Selective media (Drop Assay)

For the further selection of isolated pure yeasts, drop test assay was applied on two different media: Lysine medium (LM; Oxoid) and ethanol sulfite agar (ESA;

containing 1% yeast extract, 2% bacteriological peptone, 2% dextrose, 12% ethanol, 0.015% sodium metabisulfite and 2% agar).

After the elimination of moldy plates, all isolated yeasts that were grown on WL medium and YPD-agar medium, were incubated in liquid YPD medium for 24 h and 5μl of them were spotted in serial dilutions on LM and ESA agar plates.

2.3 Yeast identification

After morphological, physiological and microscopic characterization and selection of total isolates, a total of 85 yeast isolates from different varieties were submitted to identification using molecular methods. A commercial yeast strain for wine production, Zymaflore F15 which is a *S. cerevisae* strain, had been used as control for the experiments described below:

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2.3.1 rDNA gene amplification and primers

The ITS1, 5.8S and ITS2 regions of rDNA gene were amplified by PCR using the Primer ITS1 and ITS4 (in Table 7) and both primers were commercially synthesized in Integrated DNA Technologies.

Table 8 Primers for amplification of ITS region

Primer name	Sequence	GC content	Tm
ITS1 (forward)	5' – TCC GTA GGT GAA CCT GCG G – 3'	63.1 %	59.5 ° C
ITS4 (reverse)	5' – TCC TCC GCT TAT TGA TAT GC – 3'	45 %	52.1 ° C

2.3.2 Colony PCR

Selected colonies were used as PCR templates and 20µl of PCR reaction was performed by using ITS1 and ITS4 primers. Each reaction contained one yeast colony as a template, 1X PCR buffer without $MgCl_2$, 0.25mM dNTP mix, 0.5 pmol of each primer and 1 unit of Taq DNA polymerase (Fermentas). The PCR reaction was performed on a thermo cycler. To obtain the optimum PCR product, gradient PCR was applied from 53°C to 58°C and then from the most efficient Tm temperature PCR reactions were carried out as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturing at 95°C for 1 min; annealing at 55, 5°C for 2 min; an extension at 72°C for 2 min; and a final extension step of 10 min at 72°C. First, the concentration and the quality of isolated products were checked by Nanodrop spectrophotometry. Then amplified products were analyzed on 0.8% (w/v) agarose gels at 100V constant voltage for 1 h. Electrophoresis gels were stained with ethidium bromide (0.5µg/ml) and photographed under UV light. A 100-bp DNA ladder marker (Fermentas) was used as size standard.

2.3.3 Gel extraction

From the positive results of gel electrophoresis, gel extractions were done with the gel extraction kit (QIAGEN) according to manufacturer's instructions.

2.3.4 5.8S-ITS rDNA sequence analysis

After gel electrophoresis, 68 yeast isolates were determined and sequence analysis of 5.8S ITS rDNA region PCR products were commercially provided by Refgen using ITS1 and ITS4 primers.

The ITS1-5.8S-ITS2 sequences obtained were compared with sequences available in GenBank database available at the National Center for biotechnology Information (NCBI) using the basic local alignment search tool (BLAST). Sequences with 90% nucleotide identity or higher in the 5.8S-ITS rDNA region were considered to represent the same species. In addition, sequence alignments were performed with type

strains using ClustalW to obtain the percentage identity and phylogenetic trees were estimated according to these values.

2.3.5 Digestion screening of amplified DNA

The ITS/ 5.8S rDNA gene amplicons were digested with the restriction endonucleases CfoI and HaeIII (Fermentas) for 2 hours at 37C° in 30 µl volume according to the supplier's instructions. The restriction profiles of amplified products were screened by 2% agarose gel electrophoresis that was done at 100 mV and for 80 min. The sizes of the DNA fragments were estimated by comparing them to negative and positive controls as wells as in between each other.

2.4 Microscopic analysis

5 µl of samples were placed on microscope slides. After closure with cover slide, edges were shut with fingernail polish.

Olympus BX60 fluorescence microscope was used to visualize the cells 60X and 100X magnification were chosen for clearance. The machine's photograph apparatus was used for obtaining visualization.

2.5 Biolog System

Isolated yeasts were subcultured onto plates of Biolog Universal Yeast Agar (BUY) (Biolog inc.) and incubated 25°C for 1-2 days. The inocculum was prepared by suspending cells from plates in sterile distilled water to give 44-51% transmittance (%T) with the Biolog turbidimeter shown in Figure 14.

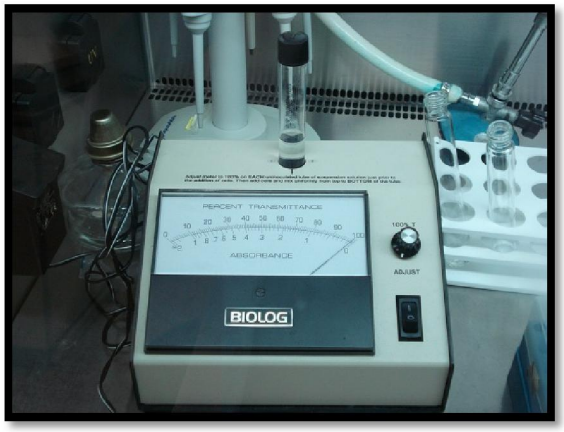


Figure 14 Turbidimeter (Biolog)

Inocculum (100µl) was dispensed into each well of a Biolog yeast (YT) microplate. The inoculated microplate was incubated at 25°C for 24 h, results were recorded by the Microplate reader and processed for identification by the Microlog software. Microplates were also read at 590 nm after 48 to 72 h, until a sufficient pattern is formed.

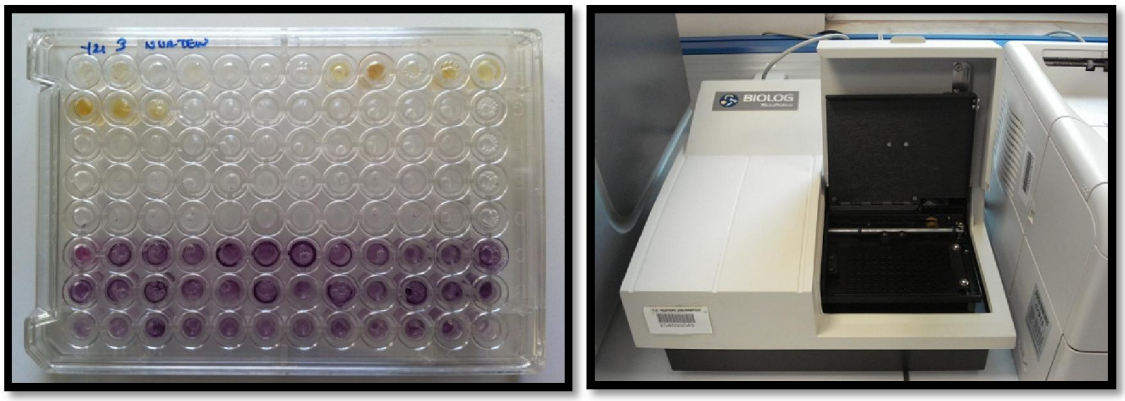


Figure 15 Microplate & microplate reader (Biolog)

2.6 Growth curve

Single colonies were taken and placed into 5 ml of liquid YPD media. Cells were grown o\n at 28^{o}C, 250 rpm. Following day, after OD600 measurement by spectrophotometer (BIORAD) 500 µl of o\n grown culture was transferred to fresh 5 ml YPD culture. OD600 measurement was done every two hours by varying dilutions. The results were plotted to a line graph by Sigma Plot.

2.7 Sulphur Resistance

Single colonies were taken and placed into 5 ml of liquid YPD media that contained 10 µl of SO_2 (5%). Cells were grown at 28°C, 250 rpm o/n. Following day, OD600 measurements were done by 1/100 dilution.

2.8 Preparation of glycerol stock

Glycerol stocks of yeasts were prepared in 15% sterile glycerol and stored at –80°C.

2.9 Pelleting the yeast cells and lyophilisation

For the initial culture, one single colony was taken from an YPD agar plate and put into 50 ml liquid YPD medium, incubated at 30°C at 250 rpm orbital shaker for 24 h. Before the batch culture, OD 600 of the cells was measured to adjust the amount of cell added to fresh media. The fresh cultures (500 ml) were inoculated with cells from overnight cultures and incubated overnight at 30°C at 250 rpm orbital shaker. After incubation period, OD 600 measurements of overnight cultures were done and cell concentrations of total culture were calculated according to the BioNumbers program of Harvard University (http://bionumbers.hms.harvard.edu/). To lyophilize easier, grown cultures were centrifuged at +4°C at 7000rpm (Sorvall SLA 3000) for 20 min to get rid of liquid media. After centrifuge, pellets were resolved and collected into falcon tubes in order to settle them into reservoir of the lyophilisator. Before the lyophilisation process, net weight of the cells was calculated. After lyophilisation, dry cells were resuspended into liquid YPD and streaked on ESA agar plate in order to be sure whether if the cells were viable or not.

3 RESULTS

3.1 Must sample analysis for Urla samples

According to literature, *Saccharomyces* sp. yeasts are present on grapes and winery equipments. Due to the extremely low occurrence, isolation of *Saccharomyces sp.* by direct plating from healthy undamaged grapes is a hardly difficult task. For this reason, microvinifications were done from the collected grapes in order to isolate *Saccharomyces sp.* after fermentation process.

3.1.1 Brix Measurements and PH

The day of 0, 4^{th} and 8^{th} of the fermentation, must samples were taken with injector to analyze the quality of the fermented must and also isolate *Saccharomyces sp.* which could not isolate at the beginning of the fermentation because of their very little amount on grape berries.

Refractometer was used to measure the glucose concentration per liter and the day of 0 measurements showed that the total glucose concentration of each grape must, in other words they were showed the fermentable sugar concentration. Throughout the fermentation, initial concentration of sugar was decreased as shown in the Table 9. Also, for a reliable fermentation, O_2 amount which was present in the fermentation flask was removed as possible. In the case of O_2 in the flask, fermentation would not be succeeded, because yeasts present in the must, would be used O_2 for degredating the glucose instead of fermenting it.

Table 9 Brix measurements and PH values of must samples, throughout the fermentation process

	PH	**Brix%(20)**		
	0	**0**	4th	8 th
MA 1 must	3.40	28.0	22.3	21.0
MA 1 must&SO_2	-	-	22.0	22.0
MA2 must	3.41	16.0	9.2	8.0
MA 2 must&SO_2	-	-	9.0	7.0
Y must	4.13	27.0	23.0	20.9
Y must& SO_2	-	-	21.2	15

As seen in Table 9, two different conditions were prepared and one involved SO_2 addition of SO_2 was to control the unwanted organisms during the fermentation.

With all taken samples, serial decimal dilutions were done and spread on WL and YPD agar media which were mentioned in section 2.2.1.3. From the samples taken day 4, only a few *Saccharomyces sp.* colonies were detected but the last taken samples were given the best results and from the 10^{-2} dilutions, single colonies were seen which are considered to be *Saccharomyces sp* as shown in Fig. 16.

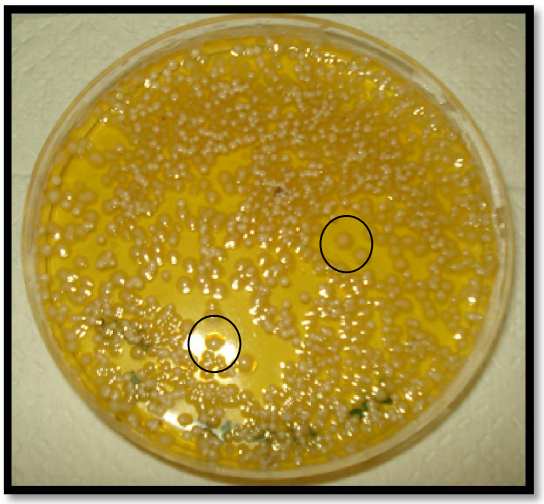


Figure 16 Creamy, white single colonies

3.2 Yeast isolation from WL and YPD agar plates

For the isolation of whole yeasts flora on grapes that were belonged to the different grape varieties, were treated as described in Section 2.2. After the incubation period, all plates were analyzed and different colonies were observed. The representative isolates were purified by repetitive streaking on YPD agar. Unfortunately, to obtain pure culture process was taken a long time, because the microflora of grape surface does not only consists of yeast species, it also consists of filamentous fungi and different bacteria species.

First, mouldy plates (in Fig.17) were eliminated and the single yeast colonies which were present in between the fungi colonies were isolated regarding the morphology of colonies.

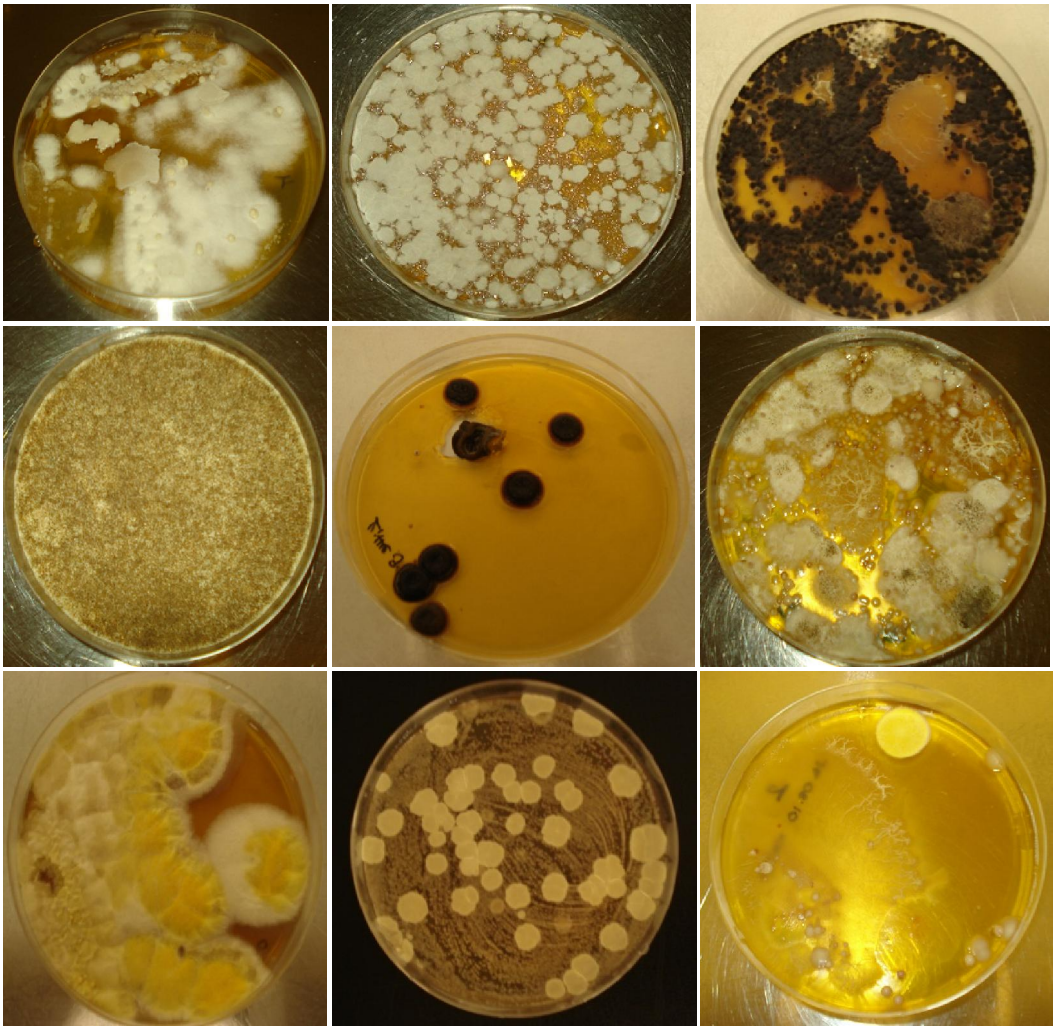


Figure 17 Some examples of mouldy plates

After the elimination of mouldy samples, colonies were identified with different morphology and color from YPD agar and especially WL agar. WL medium contains Bromocresol green which acts as PH indicator. As shown in Figure 18, the various colony morphologies and colors were easily determined and the yeast genus and species were discriminated.

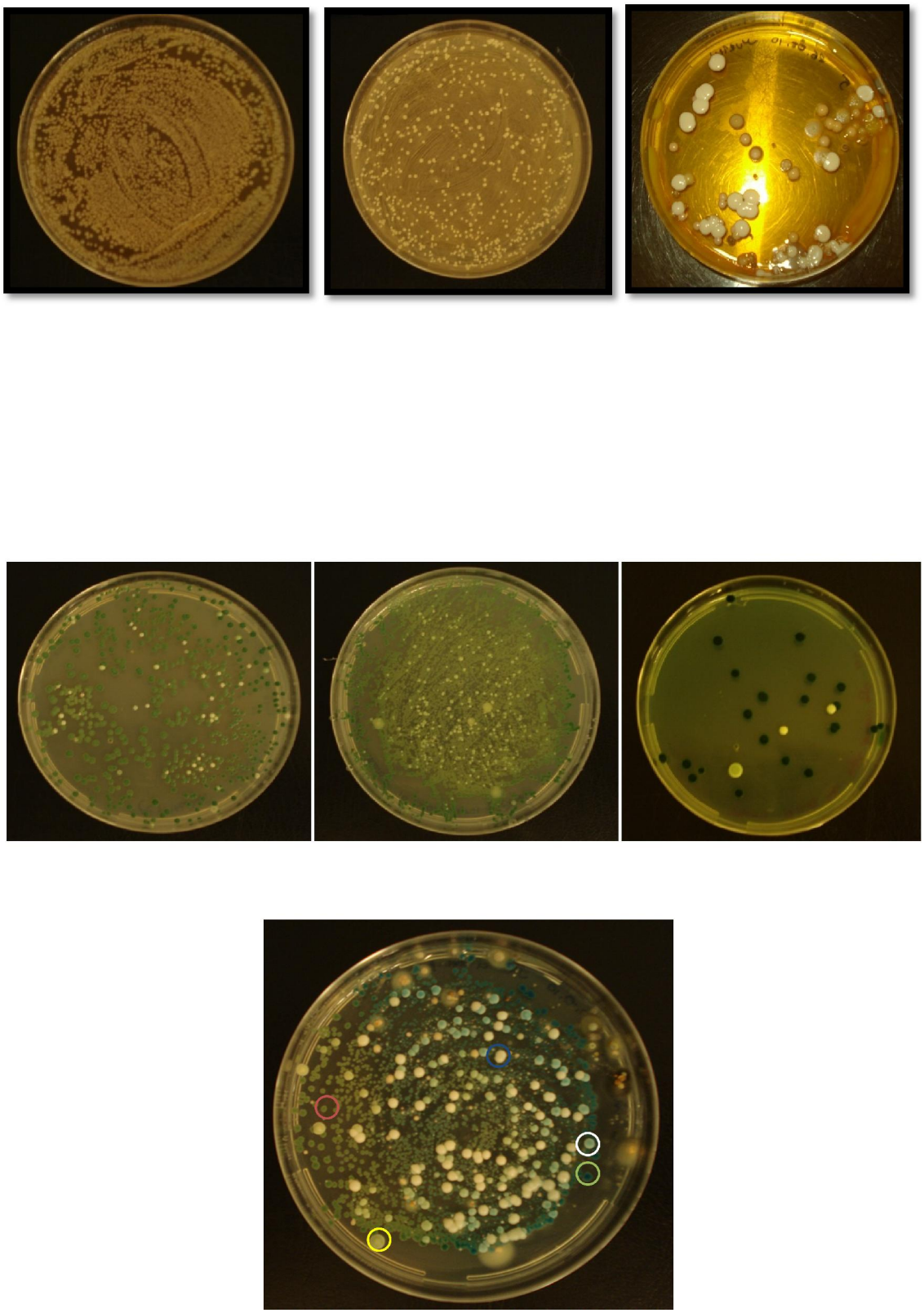


Figure 19 Growth on WL nutrient agar. Different colonies were labelled with different colored circles.

3.3 Selective media

After determining all the possible yeast colonies, identification step started by using two different selective media ESA and Lysine. Isolates were spotted on ESA and Lysine plates, as described in section 2.2.2, so the non-*Saccharomyces* and *Saccharomyces* yeasts could be discriminated basically.

Drop assay result examples are shown in Figure 20 and overall results are summarized in Table 10.

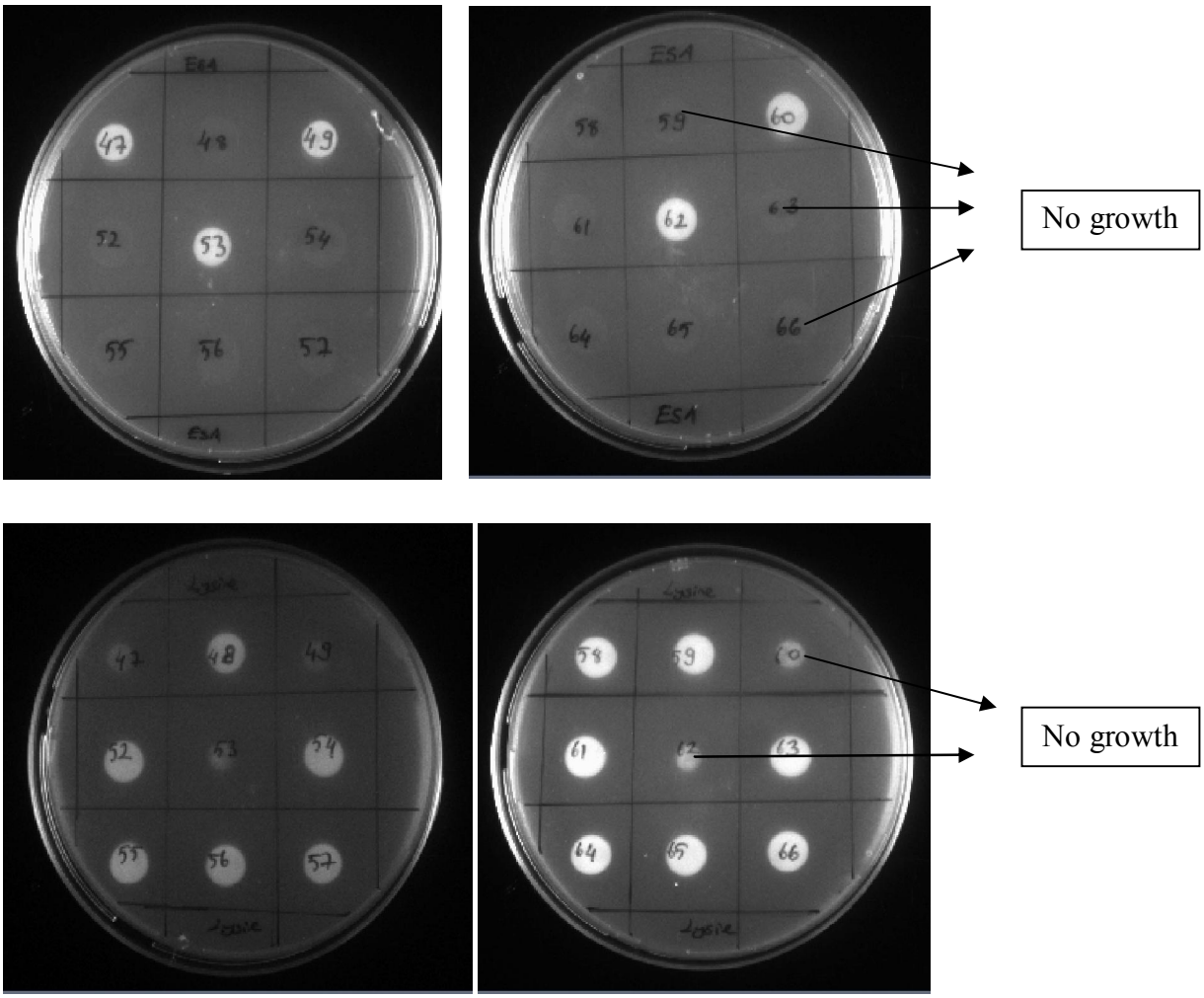


Figure 20 Growth on Lysine and ESA plates

Table 10 Existence of colonies on defined media plates

Isolate #	ESA	Lysine	Isolate #	ESA	Lysine	Isolate #	ESA	Lysine
1	+	-	24	-	+	47	+	-
2	+	-	25	-	+	48	-	+
3	+	-	26	-	+	49	+	-
4	-	+	27	-	+	50	-	+
5	-	+	28	-	+	51	-	+
6	-	+	29	-	+	52	-	+
7	-	+	30	-	+	53	+	-
8	-	+	31	-	+	54	-	+
9	-	+	32	-	+	55	-	+
10	-	+	33	-	+	56	-	+
11	0	0*	34	-	+	57	-	+
12	-	+	35	-	+	58	-	+
13	-	+	36	-	+	59	-	+
14	-	+	37	-	+	60	+	-
15	0	0	38	-	+	61	-	+
16	-	+	39	-	+	62	+	-
17	-	+	40	-	+	63	-	+
18	-	+	41	-	+	64	-	+
19	0	0	42	-	+	65	-	+
20	0	0	43	-	+	66	-	+
21	-	+	44	-	+	67	-	+
22	-	+	45	-	+	68	-	+
23	-	+	46	-	+			

*The “0” labels indicate no growth for both media.

3.4 Yeast identification

3.4.1 Colony PCR results of isolates

The ITS region of isolated wine yeast species was successfully amplified with ITS1 - ITS4 primers as described in Section 2.3.2 After electrophoresis in agarose gel (0.8 %), the PCR amplification products showed difference in size depending on the yeast species.

To get a grip of the efficiency of colony PCR for yeasts, increasing amounts of commercial *Saccharomyces* cerevisiae was placed in PCR tubes and efficiency is as seen in Figure 21 :

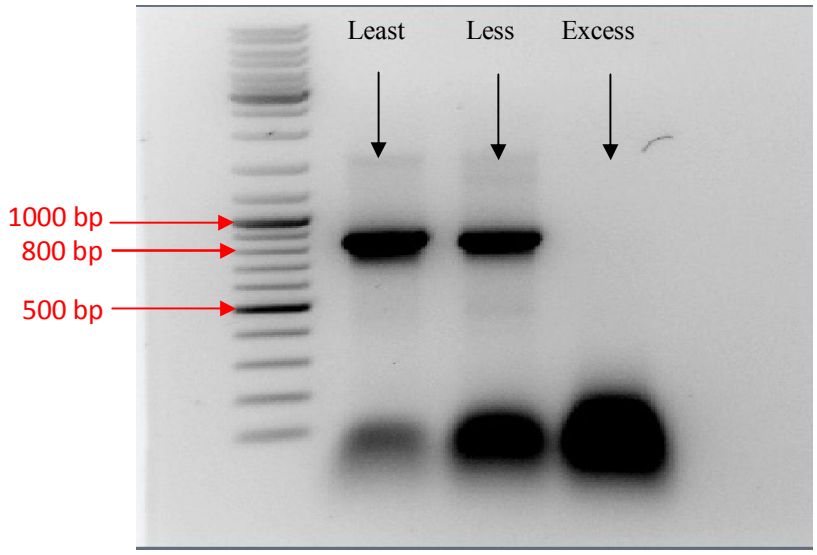


Figure 21 Efficiency test of colony PCR. Colony amount taken increases from left to right.

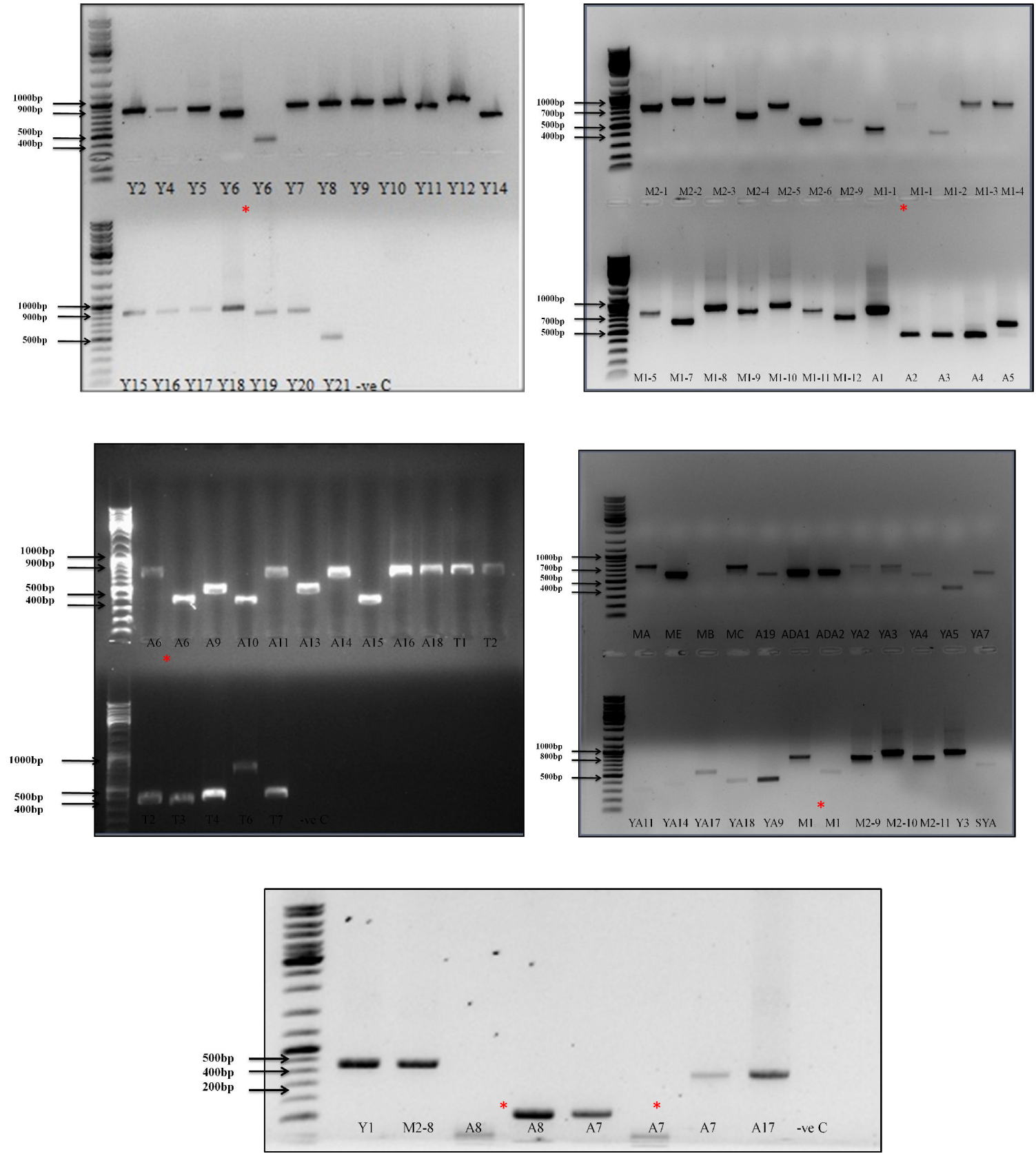


Figure 22 Colony PCR results of ITS regions after gel extraction

*The samples that have the same name origin from the same plate. Within the plate, by morphological comparison, it was observed that there was more than 1 type of colony, so both samples were amplified and after gel extraction the samples were named according to their band place on agarose gel as top, middle and bottom.

Out of 92 samples, the ones that differ in ITS region lengths and the ones that could be isolated from agarose gel are numerated (a total of 68 samples), as told in Section 2.3.4. The numeration can be seen in Table 11.

Table 11 Enumeration of isolates whose ITS regions were successfully amplified

Analysis no	Sample name	Analysis no	Sample name	Analysis no	Sample name
1	Y1	24	A1	47	M1 – 8
2	Y5	25	A2	48	M1 – 9
3	Y3	26	A5	49	M1 – 10
4	Y6 top band	27	A6 top	50	M1 – 11
5	Y6 bottom band	28	A6 bottom	51	M1 – 12
6	Y11	29	A8 top	52	M2 – 1
7	Y14	30	A8 bottom	53	M2 – 2
8	Y19	31	A9	54	M2 – 4
9	Y21	32	A10	55	M2 – 5
10	YA3	33	A11	56	M2 – 6
11	YA4	34	A13	57	M2 – 7 top
12	YA5	35	A15	58	M2 – 7 middle
13	YA7	36	A17	59	M2 – 7 bottom
14	YA11	37	A19	60	M2 – 8
15	YA14	38	ADA 1	61	M2 – 9
16	YA17	39	M1- 1 top	62	M2 – 10
17	YA 19	40	M1 – 1 bottom	63	M2 – 11
18	T1	41	M1 – 2	64	MA
19	T2 top	42	M1 – 3	65	MB
20	T3	43	M1 – 4	66	MC
21	T4	44	M1 – 6 top	67	ME
22	T6	45	M1 – 6 bottom	68	MF
23	T7	46	M1 – 7		

3.4.2 BLAST analysis of isolates

The sequence information of ITS regions was obtained from Refgen Company. With the help of the BLAST tool of NCBI, ITS sequences were identified and the sequence alignments yielded species and strain identifications of isolates. The identification results can be seen in Table 12.

Table 12 Identification of yeast isolates by 5.8S rDNA gene sequence analysis with Blast

Sample no	Description	Query coverage	E- value	% Identity
1	Saccharomyces cerevisiae, strain MUCL 51208	95%	3,00E-139	90%
2	Saccharomyces cerevisiae ,strain W24 18S	99%	2,00E-122	87%
3	Saccharomyces cerevisiae, strain W24 18S	93%	9,00E-120	90%
4	Hanseniaspora guilliermondii isolate NCL 122	85%	0.0	94%
5	Metschnikowia pulcherrima	93%	8,00E-160	97%
6	Hanseniaspora guilliermondii isolate NCL 122	92%	1,00E-112	100%
7	Pichia anomala ITS1 (partial), strain WM 2194	76%	0.0	99%
8	Hanseniaspora guilliermondii isolate NCL 122	94%	5,00E-69	100%
9	Metschnikowia fructicola isolate AP47	94%	3,00E-82	96%
10	Hanseniaspora guilliermondii isolate NCL 122	98%	1,00E-75	99%
12	Metschnikowia pulcherrima strain M320	96%	5,00E-121	98%
13	Rhodotorula mucilaginosa	96%	0.0	98%
14	Hanseniaspora guilliermondii	84%	7,00E-60	84%
16	Cryptococcus sp. 197B1	97%	2,00E-67	97%
17	Metschnikowia pulcherrima strain UMY14	88%	5,00E-59	97%
18	Pichia sporocuriosa isolate G5 18S	98%	4,00E-142	98%
19	Issatchenkia terricola	99%	0.0	98%
20	Metschnikowia pulcherrima strain BIO126	95%	4E-158	97%
21	Issatchenkia orientalis, strain H7S6K11	77%	0.0	99%
22	Metschnikowia pulcherrima strain BIO126	85%	1,00E-168	98%
23	Issatchenkia orientalis isolate NN2573	79%	0.0	99%
24	Metschnikowia pulcherrima	81%	7,00E-161	97%
25	Metschnikowia pulcherrima	73%	4,00E-154	96%
26	Hanseniaspora uvarum	93%	0.0	99%
27	Issatchenkia terricola	63%	0.0	98%
28	Metschnikowia pulcherrima	86%	3,00E-139	94%
29	Issatchenkia terricola	63%	0.0	99%
30	Hanseniaspora uvarum	90%	7,00E-68	96%
31	Issatchenkia terricola	74%	0.0	98%
32	Hanseniaspora opuntiae, strain H4S1K8	98%	3,00E-71	98%
33	Hanseniaspora opuntiae, strain H4S1K8	97%	6,00E-73	99%

Sample no	Description	Query coverage	E- value	% Identity
34	Pichia sporocuriosa isolate G5	81%	0.0	96%
35	Metschnikowia pulcherrima	81%	1,00E-159	97%
36	Hanseniaspora opuntiae, strain H4S1K8	96%	4,00E-111	99%
37	Metschnikowia pulcherrima	49%	5,00E-165	97%
38	Debaryomyces hansenii strain NJ147	98%	0.0	99%
39	Hanseniaspora guilliermondii isolate NCL 122	56%	9,00E-49	90%
40	Issatchenkia terricola	72%	0.0	99%
41	Metschnikowia chrysoperlae strain ATCC MYA-4304	89%	5,00E-121	98%
42	Hanseniaspora guilliermondii strain ZY3	92%	8E-83	97%
43	Hanseniaspora opuntiae, strain H2S2K5	97%	2E-89	97%
44	Hanseniaspora guilliermondii strain ZY3	94%	3E-92	100%
45	unknown			
46	Zygoascus meyerae strain UOA/HCPF 12067	89%	8E-51	99%
47	Saccharomyces cerevisiae, strain MUCL 51208	95%	2,00E-84	100%
48	Hanseniaspora guilliermondii isolate NCL 122	98%	2,00E-53	93%
49	Saccharomyces cerevisiae, strain MUCL 51208	95%	4,00E-85	100%
50	Kluyveromyces marxianus strain CHY1612	96%	6,00E-138	100%
51	Zygoascus hellenicus var. hellenicus strain CBS 6360	84%	1E-49	99%
52	Hanseniaspora guilliermondii	79%	0.0	98%
53	Saccharomyces cerevisiae, strain MUCL 51208	98%	0.0	87%
54	Pichia guilliermondii strain M29	76%	0.0	98%
55	Hanseniaspora guilliermondii strain ZY3	96%	0.0	97%
56	Issatchenkia orientalis isolate NN2573	74%	0.0	100%
57	Hanseniaspora guilliermondii isolate ZY7	93%	3,00E-72	96%
58	Pichia kudriavzevii strain RCEF4907	78%	0.0	99%
59	Metschnikowia pulcherrima	96%	1,00E-123	93%
60	Saccharomyces cerevisiae, strain MUCL 51208	97%	0.0	99%
61	Hanseniaspora guilliermondii	89%	3,00E-40	92%
62	Saccharomyces cerevisiae strain KDLYS901	95%	1,00E-86	90%
63	Kluyveromyces marxianus strain CHY1612	98%	0.0	99%
64	Debaryomyces hansenii strain ATCC 60978	98%	0.0	99%
65	Debaryomyces sp. BEA-2010 isolate A3	95%	1E-90	98%
67	Pichia guilliermondii strain ylx-1	71%	0.0	99%
68	Hanseniaspora guilliermondii	70%	0.0	98%

3.4.3 ClustalW2 Tool Results of isolates

After identification of isolates by BLAST tool, the overall alignment of sequences was carried out with ClustalW2 tool in EMBL official site. The tagging of isolates was done according to the data obtained from BLAST results, as shown in Figure 23.

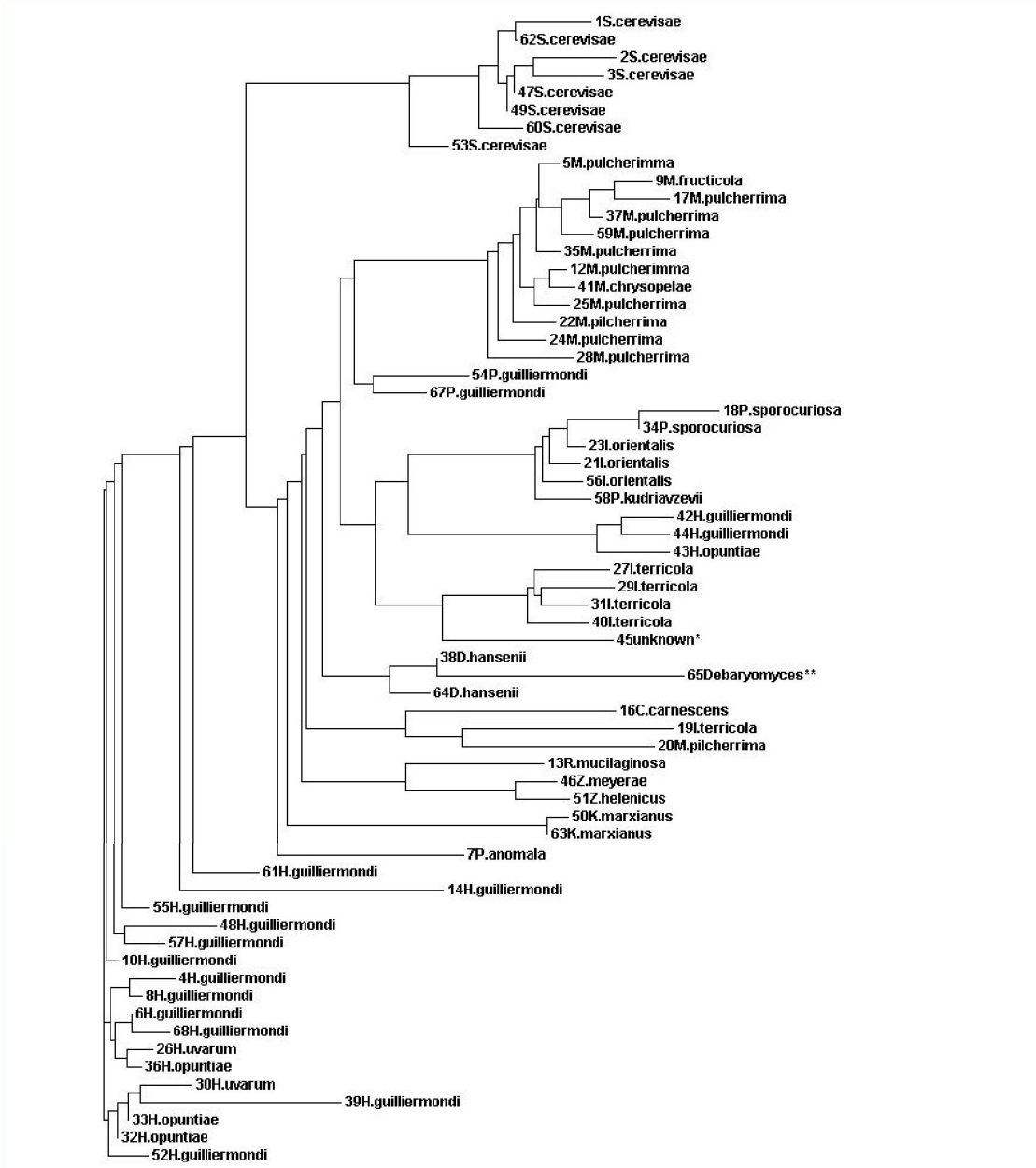
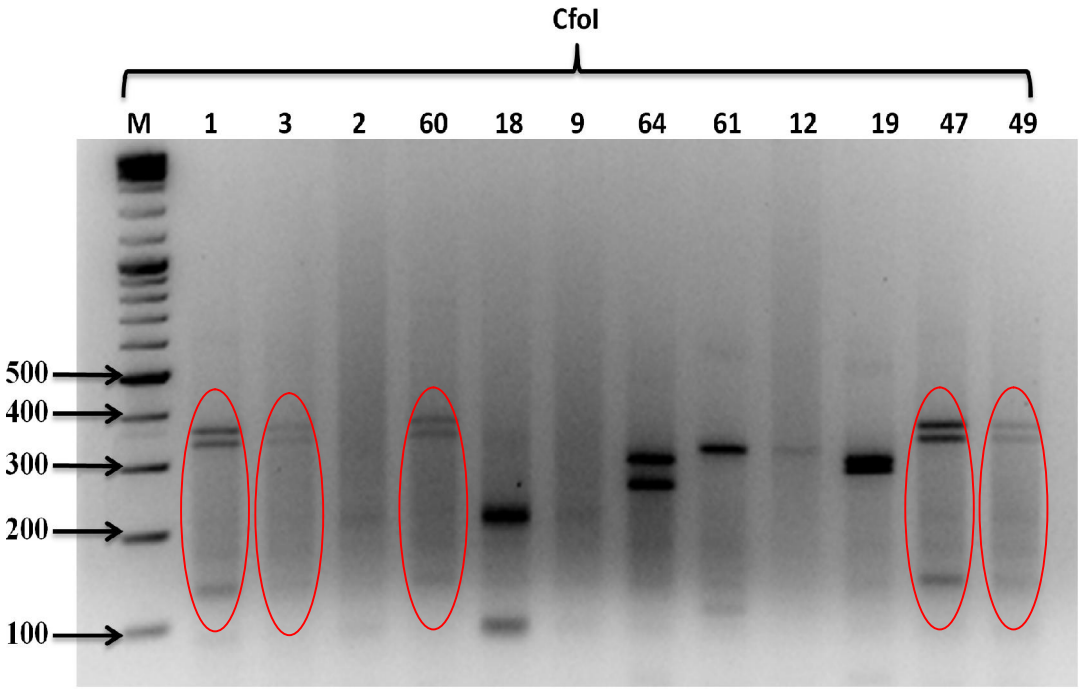
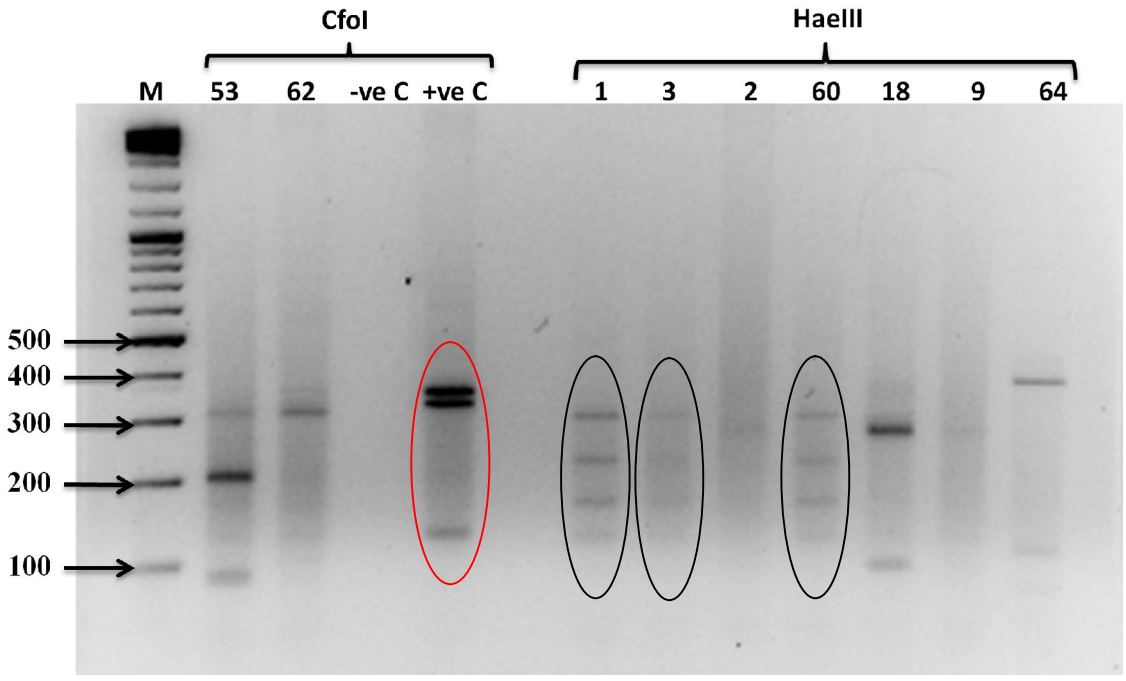


Figure 23 Multiple Alignment Tree obtained by ClustalW2 Tool

3.4.4 Digestion Screening of Isolates via Endonucleases

Amplified ITS regions were digested with CfoI, HaeIII enzymes, as described in section 2.3.5. The digested amplicons can be seen in Figure 24 below:





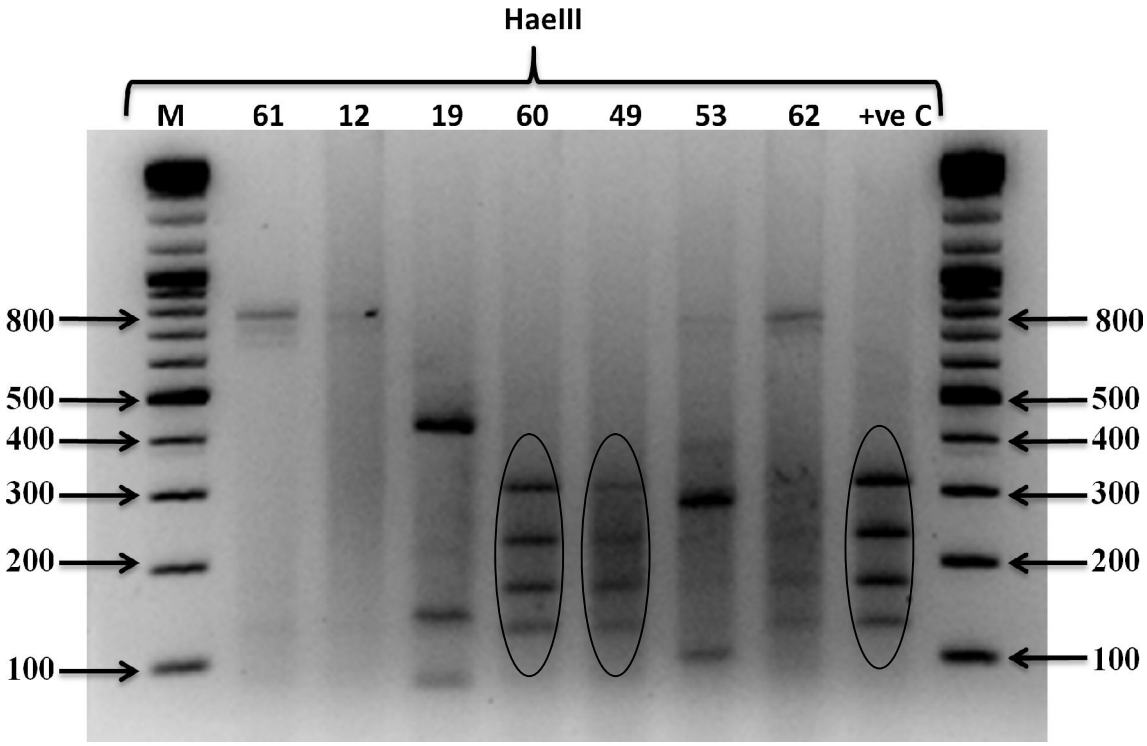
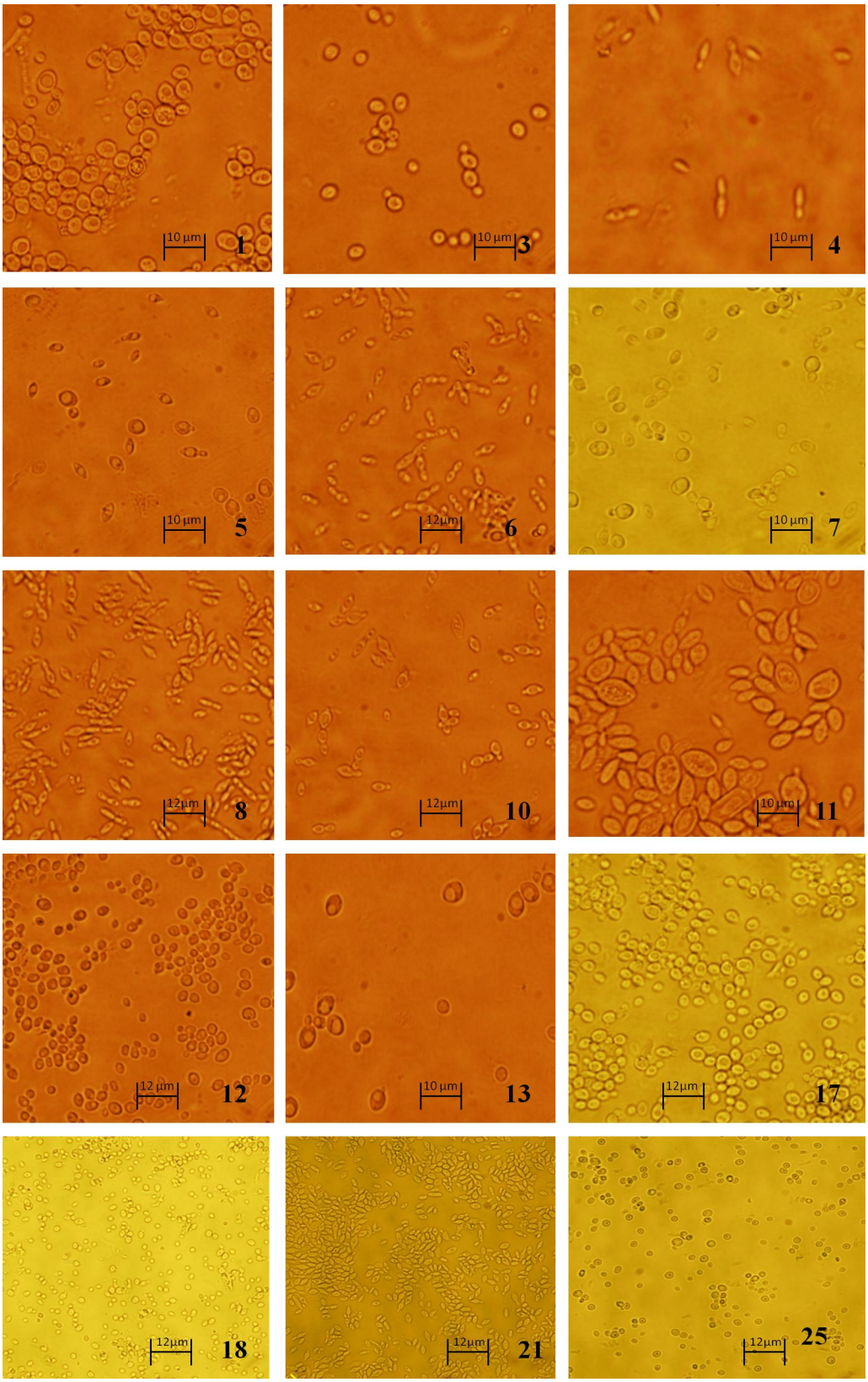


Figure 24 Digestion Screening of Isolates with the enzymes CfoI and HaeIII. "-ve C" denotes the digestion that contained no DNA and "+ve C" denotes the digestion of commercial yeast ITS region.

3.5 Microscope Analysis

The microscope visualizations were obtained as told in section 2.4. *Saccharomyces* spp. images are numbered accordingly. *Debaryomyces*, *Pichia* and *Metschnikowia* genus isolates were observed to be very familiar, so the images are also situated in the Figure 25. Additionally, *Hanseniaspora*, *Rhodotorula*, *Isaatchenkia*, *Zygoascus* genera are visualized and shown in Figure 25.


10 µm
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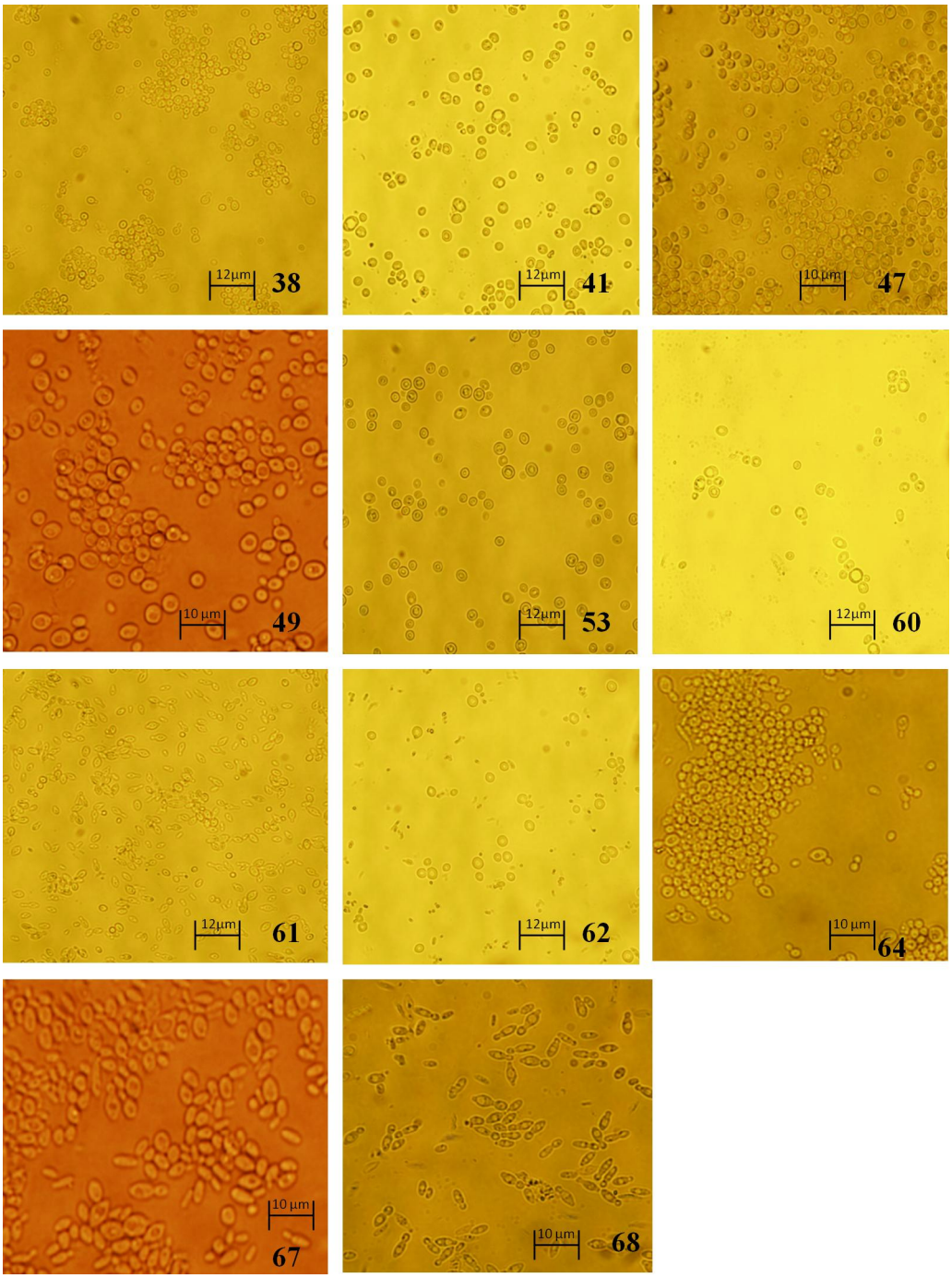
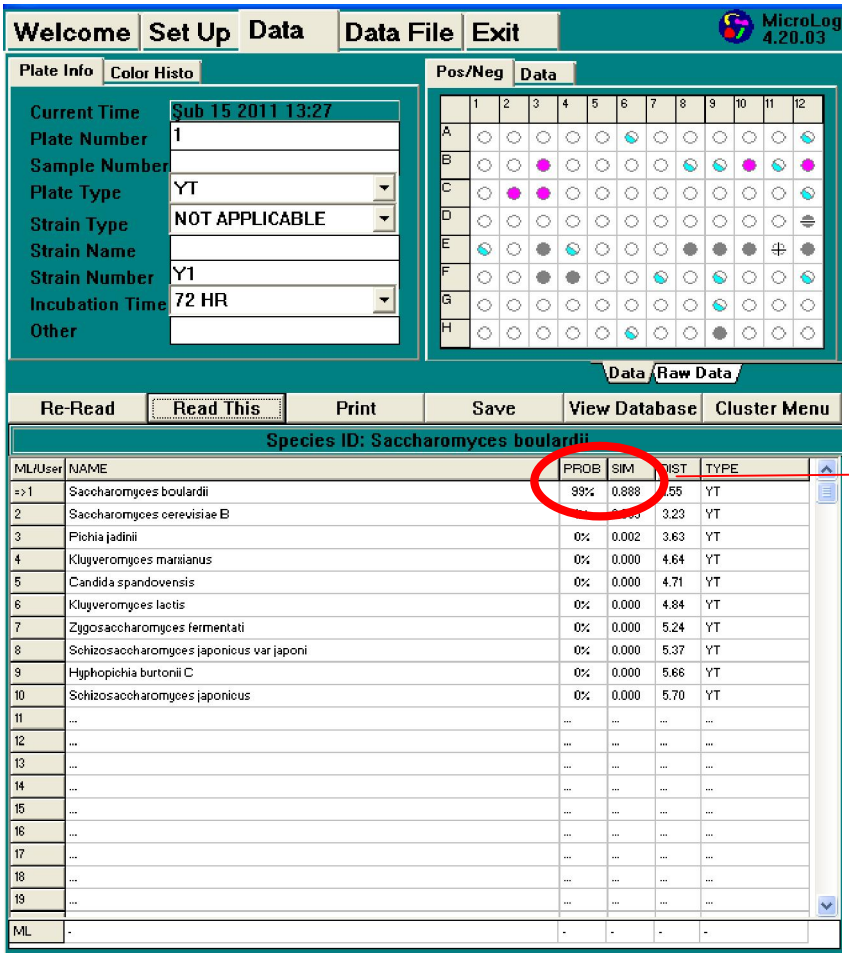


Figure 25 Microscopic visualizations of isolates

3.6 Biolog system

The Biolog system requirements are followed as described in section 2.5. The primary run for Biolog system contained 28 isolates; all *Saccharomyces* sp and one sample from each genera. Unfortunately, the machine got broken down and the second trial could only contain 8 samples. Identification of species as the result of Biolog system can be found in Table 14 and an example of the reading of the machine can be seen in Figure 26. The microplate is depicted as in the higher left side of the window. A1-D1 wells are negative controls. The other cells and the writings on define the comparison of data read with the negative controls; purple color defines false positive results, "-"and "+" sign indicates the decreases and increases in the 590 nm absorbance values respectively.



High probability & similarity values

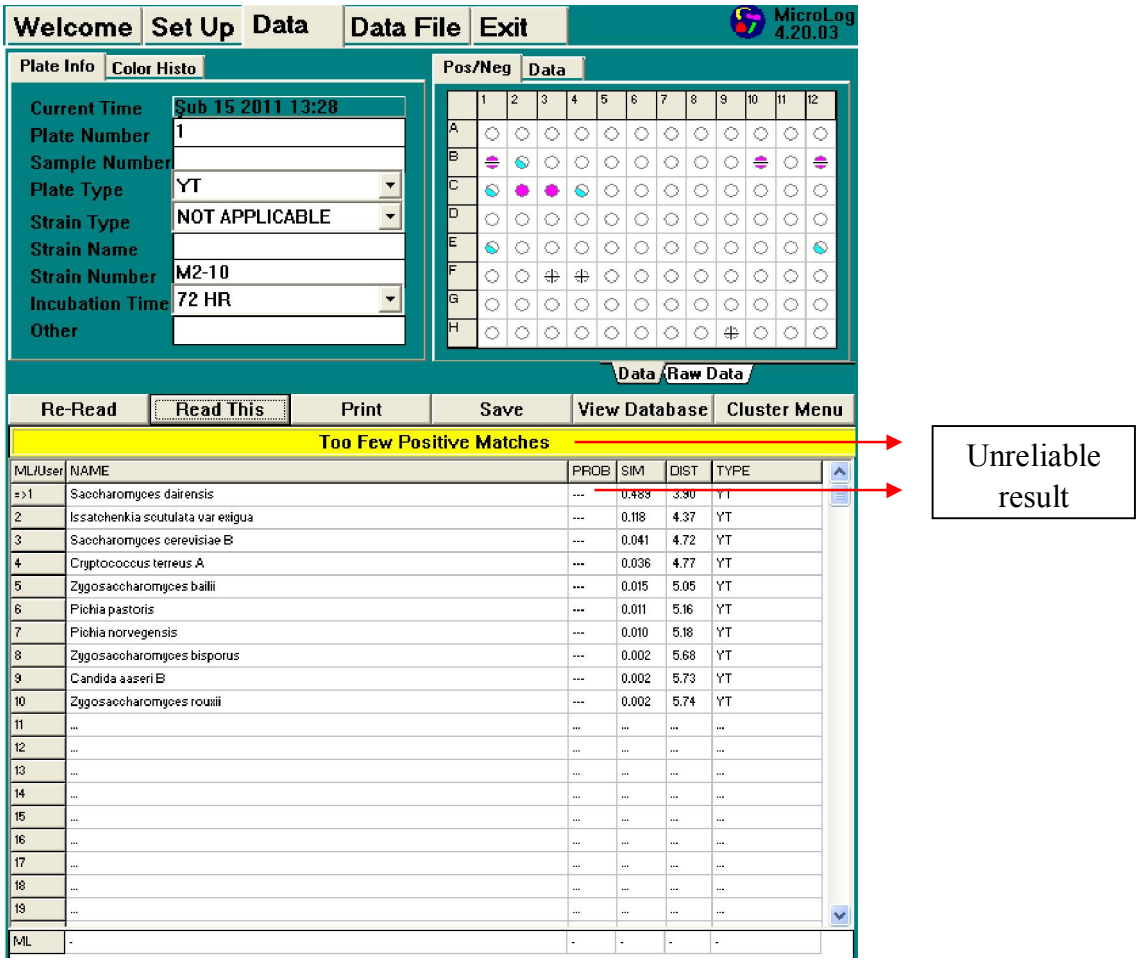


Figure 26 Example of reading results of Microplates

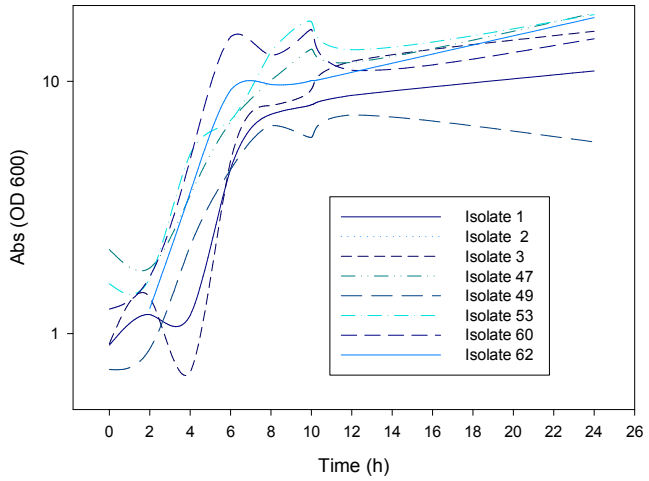
Table 13 Biolog Microplate Reading Summary

	Reading Results			
Microplate ID	**Genus**	**Species**	**Percentage**	**Similarity**
Y1 (1)	Saccharomyces	boulardi	99	0,88
Y3 (3)	Saccharomyces	cerevisae	98	0,656
Y5 (2)	Saccharomyces	capsularis	1	0,001
M1-8 (47)	Saccharomyces	cerevisae	n/a	0,373
M1-10 (49)	Saccharomyces	cerevisae	3	0,01
M2-2 (53)	Zygosaccharomyces	rouxii	1	0,003
M2-8 (60)	Saccharomyces	clariensis	86	0,721
M2-10 (62)	Saccharomyces	clariensis	n/a	0,489

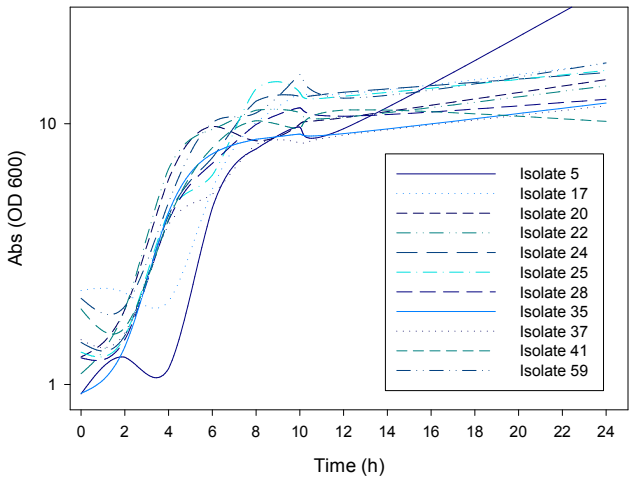
3.7 Growth Curves

Growths of isolates were obtained as detailed in section 2.6. The curves were drawn by Sigma Plot software; the y-axis is drawn in logarithmic scale. The clustering of isolates was done according to the genera they belong. Plots can be seen in Figure 27.

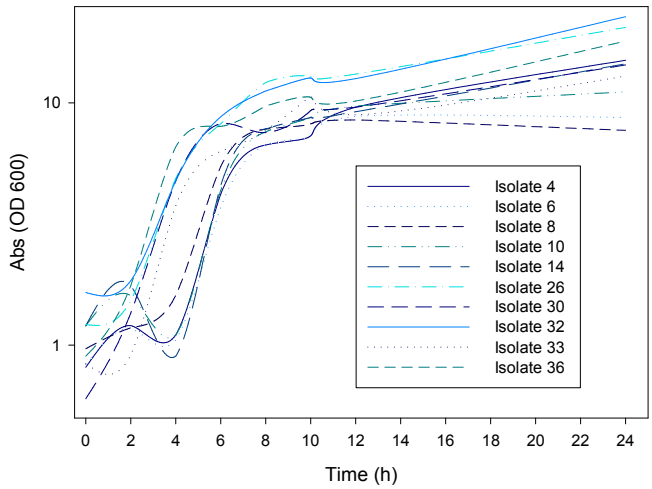
Saccharomyces sp. Growth Curve



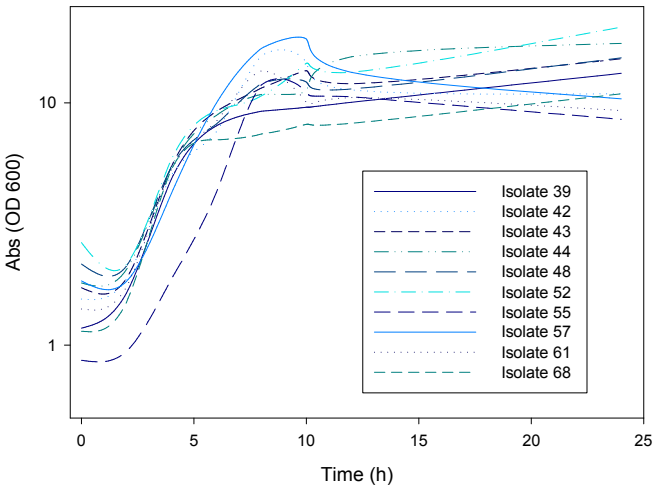
Metschnikowia sp. Growth Curve



Hanseniaspora sp. Growth Curve (Pt. 1)



Hanseniaspora sp. Growth Curve (Pt. 2)



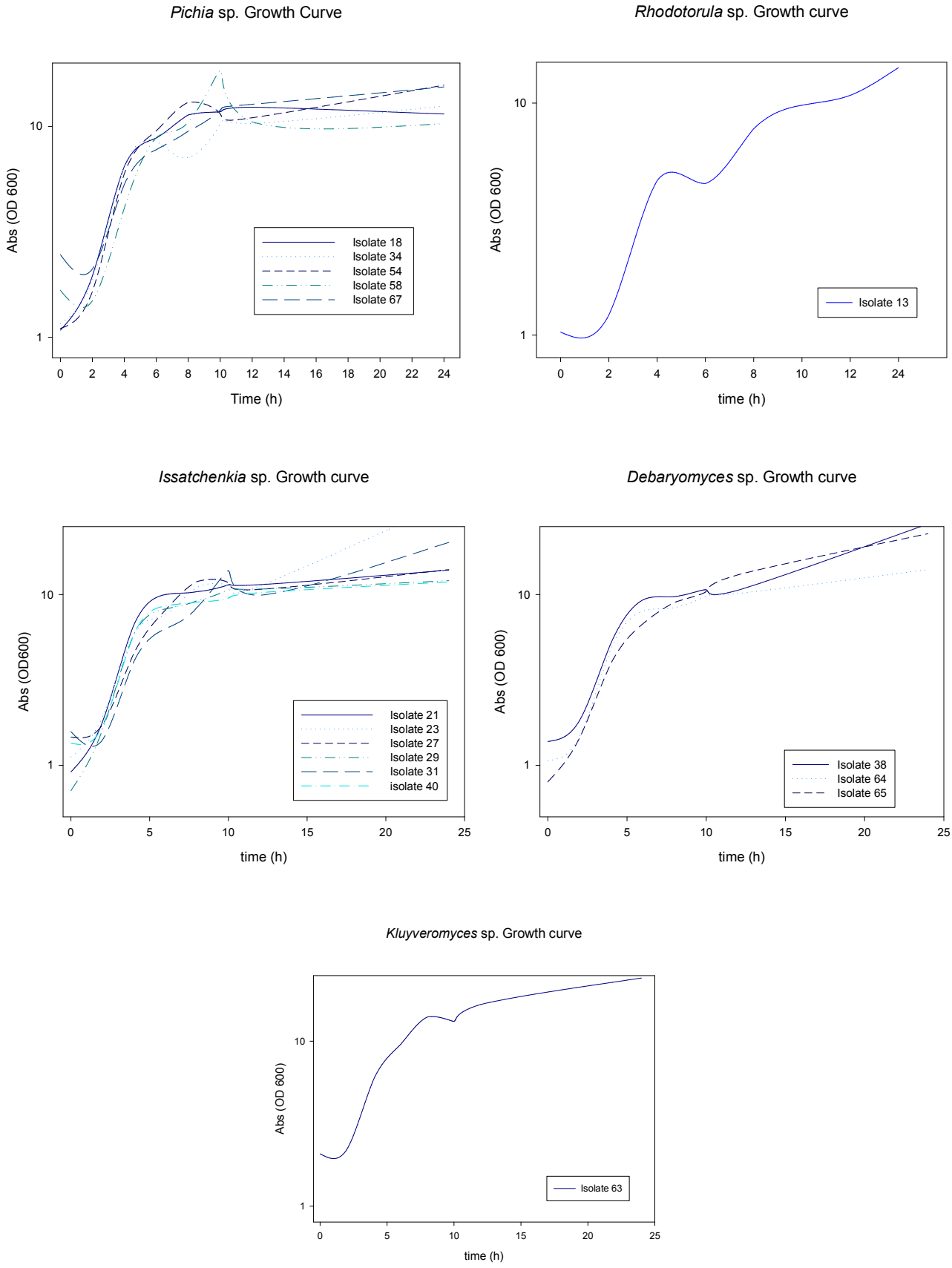


Figure 27 Growth Curve plots of isolates

3.8 Sulfur Resistance

Growth conditions of isolates for sulfur resistance are shown in section 2.7. Sulfur resistance was quantified as the following: OD 600 values of isolates grown in sulfur containing YPD media were divided by the OD 600 values of isolates that were grown in plain YPD media. The values obtained were multiplied by 100 to get a grasp of growth ratio between sulfur containing and plain media. The percentages can be found in the Table 15 below:

Table 14 Sulfur resistance of isolates

Sample #	YPD + SO2	YPD	%
1	13,5	9	150,0
2	0	0	0,0
3	12,5	9,1	137,4
4	2,15	8,1	26,5
5	6,2	9,2	67,4
6	2,1	8,3	25,3
7	6,15	11,1	55,4
8	2,35	9,66	24,3
9	9,6	18,4	52,2
10	0,7	12	5,8
11	3,5	12,9	27,1
12	5,95	14,4	41,3
13	6,1	10,3	59,2
14	1,8	12	15,0
15	4,3	5,64	76,2
16	0	0	0,0
17	6	22,92	26,2
18	8,65	10,8	80,1
19	3,45	9	38,3
20	9,1	12,75	71,4
21	0	0	0
22	8,95	11	81,4
23	6,6	11,2	58,9
24	9,4	14,5	64,8
25	8,55	13,3	64,3
26	2,7	12,15	22,2
27	4,75	14,65	32,4
28	8,2	12,65	64,8
29	3,3	7,1	46,5
30	1,75	6	29,2
31	3,85	15,75	24,4
32	1,2	16,5	7,3
33	1,3	8,4	15,5
34	5,6	11,65	48,1

Sample #	YPD + SO2	YPD	%
35	6,25	9,25	67,57
36	2,45	9	27,22
37	9,75	14,85	65,66
38	0	0	0
39	2,65	11,75	22,55
40	7,45	13,5	55,19
41	7,75	19,5	39,74
42	2,1	15,5	13,55
43	2,95	17,25	17,10
44	3,4	18,05	18,84
45	9,1	9	101,11
46	0	0	0
47	19	21,55	88,17
48	1,15	21,6	5,32
49	16,05	7,2	222,92
50	0	0	0
51	0	0	0
52	1,65	26,5	6,23
53	12,35	15,75	78,41
54	2,95	11	26,82
55	1,45	8,65	16,76
56	0,5	0,4	125,00
57	2,9	18,4	15,76
58	4,3	16,7	25,75
59	1,15	21,4	5,37
60	15,05	12,5	120,40
61	1,65	14,1	11,70
62	14,65	8,6	170,35
63	7,05	20,75	33,98
64	12,2	10,65	114,55
65	5,05	8	63,13
66	0	0	0
67	6,1	24,65	24,75
68	9,5	11,4	83,33

3.9 Lyophilisation

Lyophilisation of *Saccharomyces* species were accomplished according to Section 2.9. The plates and lyophilized cell powders are photographed, as seen in Figure 27.

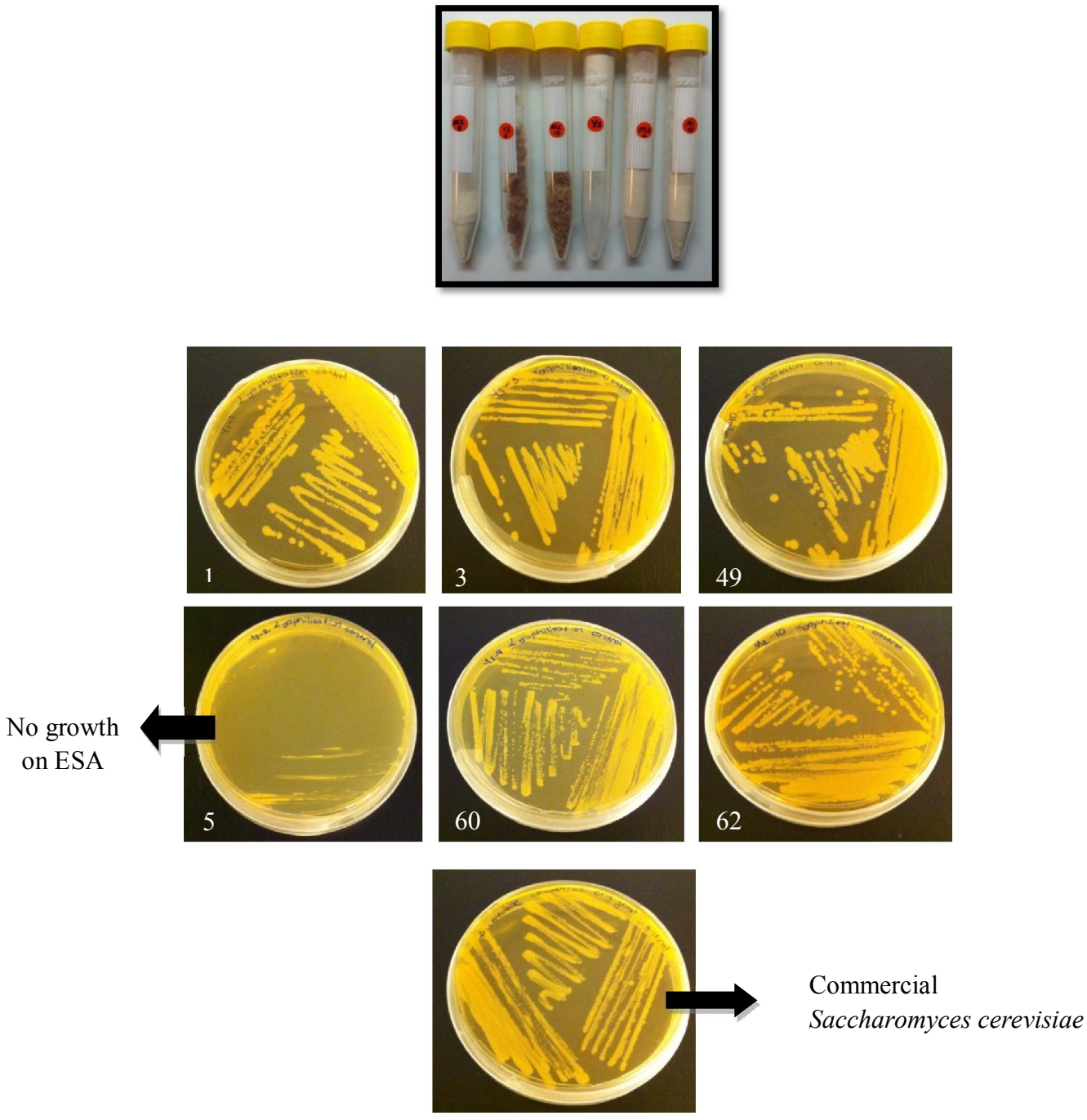


Figure 28 Pictures of Lyophilized *Saccharomyces* and their spreading controls

4 DISCUSSION

Studies enlisted in literature show that gaining information about yeast species that involve in alcohol fermentation should be ensured to have an idea on how yeasts affect the wine quality and how new styles of wines can be directed. This information can be found out by techniques such as growth curves, ITS regional analysis, sulfur resistance, selective media, etc. In this study, grapes from Tekirdağ, Urla and Adana were taken to the lab. Microorganisms were isolated by spreading and streaking techniques (Figure 11&12). Because grapes contain lactic acid bacteria, filamentous fungi and can contaminate the plates so easily (Raspor, et al., 2006), moldy and bacteria containing plates were eliminated (Figure 17). Three different regions were chosen for grape retrieval, but especially the grapes obtained from 2 vineyards of Urla were locally specific (Kuscular Village); there is no foreign grape varieties like Cabernet or Sangiovese for MA1 and MA2 varieties. This specificity makes this grape variety special, thus also the yeasts flora that would be obtained by these varieties. Additionally, there is a specific intention to produce boutique wine named after the region, so instead of using commercially available *S. cerevisae* strains, yeast strains that are specific to the region are desired to involve in alcohol fermentation. Obtaining *Saccharomyces* species is quite a hard process, which is eased by alcohol fermentation for only yeast strains can grow in fermentating medium (Henick, et al., 1998; Raspor, et al., 2006). Solely for this reason, microvinification experiments were conducted just for the grapes coming from this region (Figure 13). In literature, it is stated that between the pH range of 3.00 and 4.2 and Brix percentage of 20, alcohol fermentation can successfully take place: lower acidity triggers acetic acid bacteria and turns must into vinegar; decreasing amount of sugar ensures the fermentation process (Di Maro, Ercolini, & Coppola, 2007; Li, et al., 2010) .

As the next step of the study, all microorganisms from grapes were isolated via spreading or streaking onto WL and YPD media plates to ensure isolation of different yeast species. The property of selecting different yeast species of WL medium is highly utilized fir this step (Pallmann, et al., 2001). By using the color differentiation of the

colonies, different species were spread to YPD plates (for many times) in order to obtain single colonies (Figure 18 & 19). For further selection of colonies, ESA and Lysine media were attained: ESA media for the property of containing ethanol and sodium metabisulfide to select *Saccharomyces* species; Lysine media for the property of containing Lysine as the sole nitrogen source where *Saccharomyces* species cannot grow (Figure 20). As can be seen in Table 10, species that can grow in ESA medium cannot survive in Lysine medium and vice versa (Here, for simplicity, the samples that were sent to ITS region sequencing are listed). This result can be regarded as a very basic and primitive discrimination between *Saccharomyces* and non-*Saccharomyces* species (from here on, isolated species will be denoted as isolates).

Besides morphological and biochemical selections, molecular approach had been needed. Having high interspesific size variability and a low intraspecific polymorphism, ITS region can be used to finely characterize molecular distance between species. Thus, ITS regions of each isolates were intended to be amplified by PCR. Initially, isolation of genomic DNA of isolates was experimented, but the low efficiency and quality of DNA and the excess amount of isolates could not yield a fine result (data not shown). Then on, colony PCR was experimented directly to the isolates. The template (colony) amount was experimented for the first step with the commercially available *S. cerevisae* (Figure 21), and then taking the smallest amount was observed to be enough. Inevitably, ITS regions of some isolates could not be amplified; but the amplified regions fit to the literature lengths; between 400 and 1000bp (Guillamon, et al., 1998) (Figure 22). To be sure that the amplified fragments belong to ITS region, the amplicons were run on agarose gel and extracted via gel extraction kits. The reason of using gel extraction instead of PCR purification was to confirm the amplification of singular region instead of getting a smear or multiple amplicons. There were a total of 93 amplicons. The ones having very similar lengths and the ones that weren't observed to be clear were discarded. After the elimination, a total of 68 samples were enumerated (Table 11) and sent for sequencing to Refgen Company. Every sequence data that has been sent back was uploaded to BLAST tool in EMBL web site. The matches that have the lowest E-value, longest query coverage and highest identity were chosen; but especially the estimates that have probability lower than 90% cannot be trusted as a perfect match. According to the results, 10 genera of yeast (*Saccharomyces, Metschnikowia, Hanseniaspora, Pichia, Debaryomyces, Issatchenkia, Zygoascus, Kluyveromyces, Rhodotorula and Cryptococcus*) and 20 species (Table 12) were identified. According

to overall results, *Hanseniaspora* genus has been observed the most, which is an expected result according to the literature; other non-*Saccharomyces* genera also fit to the floral fraction (Ciani, Comitini, Mannazzu, & Domizio, 2009). A comparison between the regions that the grapes were collected can be done: Tekirdağ samples possessed generally *Isaatchenkia* and *Metschnikowia* genera; Adana samples carried *Metschnikowia* (the most) and *Hanseniaspora* genera but also had *Debaryomyces* and *Pichia*; Kuscular Village's foreign variety grapes contained dominantly genera of *Saccharomyces, Metschnikowia* and *Hanseniaspora*, and only singular *Rhodotorula, Cryptoccocus* and *Pichia* genera; Kuscular Village's local natural flora contained all the genera listed in the Table 12, but dominantly contained *Hanseniaspora* genus. The BLAST results also confirm the ESA and Lysine media results. BLAST results are also enhanced by a ClustalW tree obtained from ClusalW2 tool in EMBL webpage. Some of the branches in ClustalW tree don't belong where they should have been; maybe because of UV exposure during gel extraction or maybe because of sequencing errors. An additional comparison of ITS regions can be fulfilled by restriction digestion. A particular pattern is observed when amplified ITS region of commercially available *S. cerevisae* is digested via restriction enzymes. The comparison of this pattern with the digestion patterns of isolates' ITS regions can give a clue of whether the isolate is *S. cerevisae* or not. Thus, a digestion screening had been done with the restriction enzymes CfoI and HaeIII. The isolates 1, 3, 60, 47, 49 fit to the pattern observed by digestion of commercial yeast perfect for both enzymes. The isolate 2 and 53 are expected to show a similar pattern to commercial yeast which is not observable. The BLAST similarity scores for these isolates are low, so it is not right to trust the results completely. Additionally, the digestion pattern is not clear for isolate 2; instead there is a smear of DNA in the lane. The Biolog results also support the results; isolate 53 is estimated to be *Zygosaccharomyces* and the percentage and score for isolate 2 are really low. The other isolates that were digested, 18 (*Pichia sporacriosa*), 9 (*Metschnikowia fructicola*), 64 (*Debaryomyces hansenii*), 61 (*Hanseniaspora guillermondii*), 12 (*Metschnikowia pulcherima*), and 19 (*Isaatchenkia terricola*) (according to the BLAST results) do not fir to the pattern of commercial *S. cerevisae*.

Direct visualization of isolates was managed by microscopic analysis. A special fluorescent microscope was used and all isolates were visualized. Because species under the same genus looks almost the same under the microscope, a sample from every genus had been photographed (Figure 25). The magnification was done according to the

clearance and A thorough comparison is not efficient without any probes or dyes under the microscope; so all the isolates cannot be differentiated by just the photographs. *S cerevisae* has been studied for a long time, so the microscopic images of the species are established. With this information, at least comparison of the genera with *S. cerevisae* can be done. Accordingly, *Debaryomyces, Pichia* and *Metschnikowia* genera look highly similar to *Saccharomyces*.

Carbon utilization of yeast species varies for different carbon sources; every yeast species cannot utilize every carbon sourc. If different species are exposed to many different carbon sources, the utilization levels create a certain pattern. When this pattern is quantified, comparisons can be easily done. The Biolog system, as described in section 2.5, compares the pattern of carbon source utilization by a huge database provided by the company and yields a percentage of likeliness to a species. Initially, 27 samples, containing all the *S. cerevisae* strains and all the other species were taken to Yeditepe University for Biolog experiment. Unfortunately, the software of the machine locked itself and the readings of the samples could not be done and retrieved. 8 pre-bought Biolog Microplates were left, so every *S. cerevisae* species were taken for readings. All readings yielded similarity to *Saccharomyces* species (Figure 26). The plates are read at 72. hour of planting, so according to the user's manual, measurements that contain similarity scores greater than 0.5 are countable, thus measurements involving isolates 1, 3, 60 and 62 are reliable. The other measurements can't be relied on both regarding the percentages and the similarity scores. The main reason for this inconvenience is the malfunctions in the Microlog Reader. Most probably, the system could not recover from the damage. Nevertheless, the results are a success regarding the genus compatibility to the findings of BLAST tool.

During fermentation, the growth rate of microorganisms and their dominance over other species affect the process, so monitoring their growth becomes essential. The best known way to do so is to form a growth curve. Growth curves for each and every one of 68 samples were carried out. Plots of samples that belong to the same genera were plotted on the same graph (Figure 27). Stationary and death phases of the samples are not experienced within 24 hour period, as found in literature (Tofalo, et al., 2009). Observation of log phase was decided to be enough. The shapes and slopes of the curves don't seem to differ extensively, as can be seen in Figure 29 below:

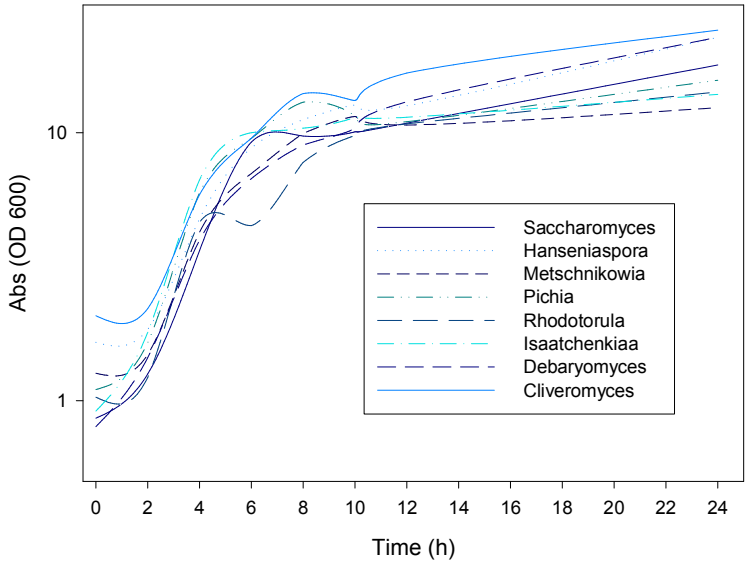


Figure 29 Comparison of genera growth curves

Among the genera, the extensive growth is observed between the 2. and 8. hours. The difference of the endpoints of curves can be denoted as insignificant because the measurements are done in small scales. All results of curves could be more accurate and reliable if the growth process would be held in large fermentors where the samples wouldn't be disturbed for every measurement time and where contamination danger would be less. Also, the growth curves could have been more reliable if different media and carbon sources were to be used. Additionally, the genera *Zygoascus* and *Cryptoccocus* could not be grown in YPD medium. Even after 24 hours, there was no significant growth for both genera.

Production of sulfites naturally occurs during fermentation process as a side effect of yeast metabolism. Also, the existence (or addition) of sulfites inhibit the growth of undesirable bacteria and yeast. Sulfur resistance levels of yeasts should be known for optimized fermentation (Egli, et al., 1998; Henick, et al., 1998). For this reason, the resistance of isolates against sulfur (SO_2) was measured (Table 15). The percentage was calculated by dividing OD 600 value of isolates grown in sulfur containing media to OD 600 value of plain YPD media and multiplying it by 100. The genus *Saccharomyces* resisted and even grew better with the addition of sulfur, and the other non-*Saccharomyces* genera could not: *S. cerevisae* species grew 50-80 % more in sulfur. As in the Table 15, especially the genus *Hanseniaspora* could not grow more

than 30%. The genera *Isaatchenkia* and *Debaryomyces* also resisted fine to sulfur addition.

Like stated above, wine production from Kuscular Village specific grape varieties was intended. To optimize fermentation, as stated above for many times, *Saccharomyces* species are desired to be taken to Kuscular Village. Isolates 1, 3, 47, 49, and 62 are the *Saccharomyces cerevisiae* that were isolated from this particular village vineyard. According to the drop assay results and morphological appearance, isolates 53 and 60 were observed as *Saccharomyces cerevisiae*. Unfortunately, in the light of the further identification steps, isolate 53 is a non-*Saccharomyces* yeast and isolate 60 is a different species of *Saccharomyces* genera. Lyophilisation was done for these isolates for transportation to the village as dried yeast (Figure 28). Streaking of these lyophilized yeasts onto ESA plates showed that the isolates are still intact and can be used as wine yeasts. Even though the isolates 53 and 60 were not determined *S. cerevisae* by the end of experiments, the isolates were additionally lyophilized and streaked on ESA. As seen in Figure 28, isolate 53 did not grow on ESA after lyophilisation; being a non-*Saccharomyces* species and in contrast to this, isolate 60 was grown on ESA being a *Saccharomyces* species.

5 CONCLUSION

For the sake of the fermentation step of wine production; the knowledge of natural flora growing on grapes should be well established for the fact that the natural flora is divergent caused by the differences in climate, soil characteristics, winery equipment, etc. These differences change the taste, the texture and the quality of the wine produced. Studies regarding the subject are being held by many groups around the world, but data concerning Turkish vineyards are not sufficient. In this study, natural floras on three different vineyard grapes were isolated. The species were basically differentiated by selective media (ESA – Lysine). ITS regions of all isolates were amplified by PCR, sequenced and compared by BLAST and ClustalW tools. The data is also used to identify the isolates' species. Visualization of cells was managed by using a fluorescent microscope. The confirmation of *Saccharomyces* species identification was done by Biolog system. Growth curves were drawn in order to determine the growth rates of isolates individually. Sulfur resistance differences were monitored for each isolates to determine the isolate feasibilities for sulfur addition step of wine making. As a last step, *Saccharomyces* species were grown in large cultures, lyophilized to ease the process of transport to vineyards. Survival strengths of lyophilized samples were tested by spreading onto ESA agar plates and observation of colony formations.

The initial task to be done as a future prospect can be the further characterization of isolates (especially *Saccharomyces* species), the capacity of isolates to produce secondary products of fermentation, such as higher alcohols, acetaldehyde, ethyl acetate, etc. should be determined. The methods to be used can be gas chromatography or HPLC, etc. After the determination of best alcohol yielding potentials, samples can be sent to Kuşçular Village and mass production can be tested. If the tests become successful, the samples can be commercially used as initial cultures. Also, the samples can be used to produce boutique wine specific to Kuşçular Village vineyard. Another aspect for further studies can be the formation of mixed cultures; the mixtures of *Saccharomyces* and non *Saccharomyces* can involve in differing ratios to create different tastes and textures. More microplates can be bought to double check the results of Biolog machine; for each and every species.

6 REFERENCES

Baum-Baicker, C. (1985). The psychological benefits of moderate alcohol consumption: A review of the literature. *Drug and Alcohol Dependence, 15*(4), 305-322.

Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., et al. (2002). Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. *Systematic and Applied Microbiology, 25*(2), 287-293.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed Torulaspora delbrueckii-Saccharomyces cerevisiae culture on high-sugar fermentation. *International Journal of Food Microbiology, 122*(3), 312-320.

Bisson, L. F. (1999). Stuck and Sluggish Fermentations. *Am. J. Enol. Vitic., 50*(1), 107-119.

Bisson, L. F. (2005). The Biotechnology of Wine Yeast. *Food Biotechnology, 18*(1), 63 - 96.

Bisson, L. F., Karpel, J. E., Ramakrishnan, V., & Joseph, L. (2007). Functional genomics of wine yeast *Saccharomyces cerevisiae*. *Adv Food Nutr Res, 53*, 65-121.

Bisson, L. F., Waterhouse, A. L., Ebeler, S. E., Walker, M. A., & Lapsley, J. T. (2002). The present and future of the international wine industry. *Nature, 418*(6898), 696-699.

Blackwell, M. (2001). The Yeasts, A Taxonomic Study, fourth edition, by C.P. Kurtzman and J.W. Fell. *Mycopathologia, 149*(3), 157-158.

Borneman, A. R., Chambers, P. J., & Pretorius, I. S. (2007). Yeast systems biology: modelling the winemaker's art. *Trends in Biotechnology, 25*(8), 349-355.

Boulton, R. B., Singleton, V.L., Bisson, L. F. (Ed.). (1998). *Principles and Practices of Winemaking*: Springer US.

Capece, A., Fiore, C., Maraz, A., & Romano, P. (2005). Molecular and technological approaches to evaluate strain biodiversity in Hanseniaspora uvarum of wine origin. *Journal of Applied Microbiology, 98*(1), 136-144.

Cappello, M. S., Bleve, G., Grieco, F., Dellaglio, F., & Zacheo, G. (2004). Characterization of Saccharomyces cerevisiae strains isolated from must of grape grown in experimental vineyard. *Journal of Applied Microbiology, 97*(6), 1274-1280.

Chavan, P., Mane, S., Kulkarni, G., Shaikh, S., Ghormade, V., Nerkar, D. P., et al. (2009). Natural yeast flora of different varieties of grapes used for wine making in India. *Food Microbiology, 26*(8), 801-808.

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology, 108*(2), 239-245.

Ciani, M., & Comitini, F. (2010). Non-*Saccharomyces*; wine yeasts have a promising role in biotechnological approaches to winemaking. *Annals of Microbiology*, 1-8.

Ciani, M., Comitini, F., Mannazzu, I., & Domizio, P. (2009). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Microbiol Lett*, 123-133.

Ciani, M., & Maccarelli, F. (1998). Oenological properties of non-Saccharomyces yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14*(2), 199-203.

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Heras-Vázquez, F. J. L., & Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology, 21*(2), 149-155.

Combina, M., Mercado, L., Borgo, P., Elia, A., Jofré, V., Ganga, A., et al. (2005). Yeasts associated to Malbec grape berries from Mendoza, Argentina. *Journal of Applied Microbiology, 98*(5), 1055-1061.

Di Maro, E., Ercolini, D., & Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *International Journal of Food Microbiology, 117*(2), 201-210.

Egli, C. M., Edinger, W. D., Mitrakul, C. M., & Henick-Kling, T. (1998). Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *Journal of Applied Microbiology, 85*(5), 779-789.

Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of Kloeckera apiculata and Saccharomyces cerevisiae; associated with winemaking in mixed cultures. *World Journal of Microbiology and Biotechnology, 18*(4), 377-382.

Esteve-Zarzoso, B., Manzanares, P., Ramon, D., Querol, A. (1998). The role of non-*Saccharomyces* yeasts in industrial winemaking. *Internatl Microbiol, 1*, 143-148.

Fernandez-Espinar, M. T., Lopez, V., Ramon, D., Bartra, E., & Querol, A. (2001). Study of the authenticity of commercial wine yeast strains by molecular techniques. *Int J Food Microbiol, 70*(1-2), 1-10.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int J Food Microbiol, 86*(1-2), 11-22.

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Research, 8*(7), 979-995.

Fleet, G. H., Lafon-Lafourcade, S., Ribéreau-Gayon, P.,. (1984). Evolution of Yeasts and Lactic Acid Bacteria During Fermentationand Storage of Bordeaux Wines.

Garnier, N., Richardin, P., Cheynier, V., & Regert, M. (2003). Characterization of thermally assisted hydrolysis and methylation products of polyphenols from modern and archaeological vine derivatives using gas chromatography-mass spectrometry. *Analytica Chimica Acta, 493*(2), 137-157.

Gonzalez, R., Martinez-Rodriguez, A. J., & Carrascosa, A. V. (2003). Yeast autolytic mutants potentially useful for sparkling wine production. *Int J Food Microbiol, 84*(1), 21-26.

González, S. S., Barrio, E., Gafner, J., & Querol, A. (2006). Natural hybrids from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces kudriavzevii* in wine fermentations. *FEMS Yeast Research, 6*(8), 1221-1234.

Granchi, L., Bosco, M., Messini, A., & Vincenzini, M. (1999). Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J Appl Microbiol, 87*(6), 949-956.

Guasch-Jané, M. R., Andrés-Lacueva, C., Jáuregui, O., & Lamuela-Raventós, R. M. (2006). The origin of the ancient Egyptian drink Shedeh revealed using LC/MS/MS. *Journal of Archaeological Science, 33*(1), 98-101.

Gueho, E., Kurtzman, C., & Peterson, S. (1990). Phylogenetic Relationships among Species of Sterigmatomyces and Fellomyces as Determined from Partial rRNA Sequences. *Internatıonal Journal of Systematic Bacteriology*.

Guillamon, J. M., Sabate, J., Barrio, E., Cano, J., & Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch Microbiol, 169*(5), 387-392.

Guillamón, J. M., Sabaté, J., Barrio, E., Cano, J., & Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Archives of Microbiology, 169*(5), 387-392.

Heard, G. M., & Fleet, G. H. (1985). Growth of Natural Yeast Flora during the Fermentation of Inoculated Wines. *Applied and environmental microbiology, 50*(3), 727-728.

Heard, G. M., & Fleet, G. H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Microbiology, 65*(1), 23-28.

Henick, K., Edinger, Daniel, & Monk. (1998). Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *Journal of Applied Microbiology, 84*(5), 865-876.

Holm Hansen, E., Nissen, P., Sommer, P., Nielsen, J., & Arneborg, N. (2001). The effect of oxygen on the survival of non-Saccharomyces yeasts during mixed culture fermentations of grape juice with Saccharomyces cerevisiae. *Journal of Applied Microbiology, 91*(3), 541-547.

Hugh Klein, D. J. P. (1990). Perceived Consequences Associated With the Use of Beer, Wine, Distilled Spirits, and Wine Coolers.

Husnik, J. I., Volschenk, H., Bauer, J., Colavizza, D., Luo, Z., & van Vuuren, H. J. (2006). Metabolic engineering of malolactic wine yeast. *Metab Eng, 8*(4), 315-323.

Kaufman, H. S. (1992). The red wine headache and prostaglandin synthetase inhibitors: a blind controlled study. *Journal of Wine Research, 3*(1), 43 - 46.

Kinsella, J. E., Frankel, E., German, J. B., and Kanner, J. (1993). Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technol*, 85-89.

Kish, S., Sharf, R., & Margalith, P. (1983). A note on a selective medium for wine yeasts. *Journal of Applied Microbiology, 55*(1), 177-179.

Kurtzman, C., & Phaff, H. (Eds.). (1987). *The Yeasts* (2nd ed. Vol. 1).

Li, S. S., Cheng, C., Li, Z., Chen, J. Y., Yan, B., Han, B. Z., et al. (2010). Yeast species associated with wine grapes in China. *Int J Food Microbiol, 138*(1-2), 85-90.

Lilly, M., Lambrechts, M. G., & Pretorius, I. S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Appl Environ Microbiol, 66*(2), 744-753.

Lindman, R., & Lang, A. R. (1986). Anticipated Effects of Alcohol Consumption as a Function of Beverage Type: A Cross-Cultural Replication. *International Journal of Psychology, 21*(1), 671 - 678.

Manzanares, P., Ramón, D., & Querol, A. (1999). Screening of non-Saccharomyces wine yeasts for the production of [beta]--xylosidase activity. *International Journal of Food Microbiology, 46*(2), 105-112.

Manzano, M., Medrala, D., Giusto, C., Bartolomeoli, I., Urso, R., & Comi, G. (2006). Classical and molecular analyses to characterize commercial dry yeasts used in wine fermentations. *Journal of Applied Microbiology, 100*(3), 599-607.

Martinez-Rodriguez, A. J., Polo, M. C., & Carrascosa, A. V. (2001). Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. *Int J Food Microbiol, 71*(1), 45-51.

Martinez, C., Cosgaya, P., Vasquez, C., Gac, S., & Ganga, A. (2007). High degree of correlation between molecular polymorphism and geographic origin of wine yeast strains. *J Appl Microbiol, 103*(6), 2185-2195.

Martini, C., Scorzetti. (1996). Direct Enumeration and Isolation of Wine Yeasts from Grape Surfaces.

McBryde, C., Gardner, J. M., de Barros Lopes, M., & Jiranek, V. (2006). Generation of Novel Wine Yeast Strains by Adaptive Evolution. *Am. J. Enol. Vitic., 57*(4), 423-430.

McGovern, P. E., Glusker, D. L., Exner, L. J., & Voigt, M. M. (1996). Neolithic resinated wine. *Nature, 381*(6582), 480-481.

Mercado, L., Dalcero, A., Masuelli, R., & Combina, M. (2007). Diversity of Saccharomyces strains on grapes and winery surfaces: Analysis of their contribution to fermentative flora of Malbec wine from Mendoza (Argentina) during two consecutive years. *Food Microbiology, 24*(4), 403-412.

Mills, D. A., Johannsen, E. A., & Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl Environ Microbiol, 68*(10), 4884-4893.

Moreira, N., Mendes, F., Hogg, T., & Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *International Journal of Food Microbiology, 103*(3), 285-294.

Mortimer, R., & Polsinelli, M. (1999). On the origins of wine yeast. *Res Microbiol, 150*(3), 199-204.

Munoz, E., & Ingledew, W. M. (1989). Effect of yeast hulls on stuck and sluggish wine fermentations: importance of the lipid component. *Appl Environ Microbiol, 55*(6), 1560-1564.

N.P. Jolly, O. P. H. A. a. I. S. P. (2006). The Role and Use of Non-Saccharomyces Yeasts in Wine Production. *S Afr J Enol Vitic*.

Núñez, D. R., & Walker, M. J. (1989). A review of palaeobotanical findings of early Vitis in the mediterranean and of the origins of cultivated grape-vines, with special reference to new pointers to prehistoric exploitation in the western mediterranean. *Review of Palaeobotany and Palynology, 61*(3-4), 205-237.

OIV. (2007). Annual report. from http://www.oiv.org

Pallmann, C. L., Brown, J. A., Olineka, T. L., Cocolin, L., Mills, D. A., & Bisson, L. F. (2001). Use of WL Medium to Profile Native Flora Fermentations. *Am. J. Enol. Vitic., 52*(3), 198-203.

Phaff, H. (1986). Ecology of yeasts with actual and potential value in biotechnology. *Microbial Ecology, 12*(1), 31-42.

Praphailong, W., Van Gestel, M., Fleet, G. H., & Heard, G. M. (1997). Evaluation of the Biolog system for the identification of food and beverage yeasts. *Letters in Applied Microbiology, 24*(6), 455-459.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast, 16*(8), 675-729.

Pretorius, I. S., & Bauer, F. F. (2002). Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends Biotechnol, 20*(10), 426-432.

Raspor, P., Milek, D. M., Polanc, J., Smole Mozina, S., & Cadez, N. (2006). Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. *International Journal of Food Microbiology, 109*(1-2), 97-102.

Reginald G. Smart, G. W. (1999). Heavy drinking and problems among wine drinkers. *Journal of Studies on Alcohol and Drugs*.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., & Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int J Food Microbiol, 86*(1-2), 169-180.

Romano, P., Granchi, L., Caruso, M., Borra, G., Palla, G., Fiore, C., et al. (2003). The species-specific ratios of 2,3-butanediol and acetoin isomers as a tool to evaluate wine yeast performance. *Int J Food Microbiol, 86*(1-2), 163-168.

Romano, P., & Suzzi, G. (1996). Origin and Production of Acetoin during Wine Yeast Fermentation. *Appl Environ Microbiol, 62*(2), 309-315.

S. Rainieri, I. S. P. (2000). Selection and improvement of wine yeasts. *Annals of Microbiology, 50*, 15-31.

Santamaría, P., Garijo, P., López, R., Tenorio, C., & Rosa Gutiérrez, A. (2005). Analysis of yeast population during spontaneous alcoholic fermentation: Effect of the age of the cellar and the practice of inoculation. *International Journal of Food Microbiology, 103*(1), 49-56.

Schuller, D., & Casal, M. (2005). The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. *Applied Microbiology and Biotechnology, 68*(3), 292-304.

Schuller, D., Valero, E., Dequin, S., & Casal, M. (2004). Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol Lett, 231*(1), 19-26.

Soubeyrand, V., Julien, A., & Sablayrolles, J.-M. (2006). Rehydration Protocols for Active Dry Wine Yeasts and the Search for Early Indicators of Yeast Activity. *Am. J. Enol. Vitic., 57*(4), 474-480.

Spencer, J. F. T., & Spencer, D. M. (Eds.). (1997). *Yeasts in Natural and Artificial Habitats*.

Sutter, R. A. (1964). A History of Wine as Therapy. *Journal of Occupational and Environmental Medicine, 6*(5), 235-236.

Swiegers, J. H., & Pretorius, I. S. (2005). Yeast modulation of wine flavor. *Adv Appl Microbiol, 57*, 131-175.

Tofalo, R., Chaves-López, C., Di Fabio, F., Schirone, M., Felis, G. E., Torriani, S., et al. (2009). Molecular identification and osmotolerant profile of wine yeasts that ferment a high sugar grape must. *International Journal of Food Microbiology, 130*(3), 179-187.

Toro, M. E., & Vazquez, F. (2002). Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. *World Journal of Microbiology and Biotechnology, 18*(4), 351-358.

Truu, J., Talpsep, E., Heinaru, E., Stottmeister, U., Wand, H., & Heinaru, A. (1999). Comparison of API 20NE and Biolog GN identification systems assessed by techniques of multivariate analyses. *Journal of Microbiological Methods, 36*(3), 193-201.

Valero, E., Cambon, B., Schuller, D., Casal, M., & Dequin, S. (2007). Biodiversity of Saccharomyces yeast strains from grape berries of wine-producing areas using starter commercial yeasts. *FEMS Yeast Res, 7*(2), 317-329.

Van der Aa Kühle, A., & Jespersen, L. (1998). Detection and identification of wild yeasts in lager breweries. *International Journal of Food Microbiology, 43*(3), 205-213.

Verstrepen, K., Chambers, P., & Pretorius, I. (2006). The Development of Superior Yeast Strains for the Food and Beverage Industries: Challenges, Opportunities and Potential Benefits. In A. Querol & G. Fleet (Eds.), *Yeasts in Food and Beverages* (pp. 399-444): Springer Berlin Heidelberg.

Vezinhet, F., Hallet, J.-N., Valade, M., & Poulard, A. (1992). Ecological Survey of Wine Yeast Strains by Molecular Methods of Identification. *Am. J. Enol. Vitic., 43*(1), 83-86.

Viana, F., Gil, J. V., Genovés, S., Vallés, S., & Manzanares, P. (2008). Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology, 25*(6), 778-785.

Yap, N. A., de Barros Lopes, M., Langridge, P., & Henschke, P. A. (2000). The incidence of killer activity of non-*Saccharomyces* yeasts towards indigenous yeast species of grape must: potential application in wine fermentation. *J Appl Microbiol, 89*(3), 381-389.

Zironi, R., Romano, P., Suzzi, G., Battistutta, F., & Comi, G. (1993). Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnology Letters, 15*(3), 235-238.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., & Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiology, 27*(5), 559-567.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., & Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-Saccharomyces yeasts during the early stages in winemaking. *International Journal of Food Microbiology, 125*(2), 197-203.

APPENDIX A

Sequencing Results of ITS Regions of Isolates

>1
ATCGGGTTATCTACCTGATTTGAGGTCAAACTTTAAGAACATTGTTCGCCTAGACGCTCTCTT
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AAAACGACCGTACTTTGCATTATACCTCAGGCCCGCAAAAAAACCTCTCTTTGGAAAAAAAA
CATCCAAGGAAAAGGCCAGCATTTTCAAGTTAACTCCAAAGAGTATCACTCATTACCAAAAA
AAAGGTTTGAGAAGGAAAGGCCGCTCAAACGGGCTTGCCCCCGGAAATACCAAGGGCCCCA
AGGGGGTTTCAAAGTTCCGTTGATTCTCGAAATTTGGCATTTCACATAACGTATCGAATTTCG
CGGCGTTCTTCTCCATGGCAAAAC

>2
GGGGTCTCCCTACCTGATTTGAGGTCAAACTTTAAGAACATTGTTCGCCTAGACGCTCTCTTC
TTATCGATAACGTTCCAATACGCTCAGTATAAAAAAAGATTAGCCGCAGTTGGTAAAACCTA
AAACGACCGTACTTTGCATTATACCTCAGGCCCGCAAAAAAACCTCTCTTTGAAAAAAAAAC
ATCCAAGGAAAGGGCCAGCAATTTCAGGTTAATTCCAAAAAGAATCATTCACAACCAAACG
AAAGGTTGAAAAAGGAAAGGACGCCCAAACAGGCTGGCCCCCGGAAATACCAGGGGGGGC
ATGGGGGGTTCAAAAATCCAAGGATTCACGAAATCCGCCAATTCCCTTTACGTTTGGCTTTT
CGCGGCGTCTTCCATCAATGCAAGACCCA

>3
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ACGACCGTACTTTGCATTATACCTCAGGCCCGCAAAAAAACCTCTCTTTGGAAAAAAAACAT
CCAATGAAAAGGCCAGAAATTTCAAGTTAACTCAAAAGAGAATCACTCCCTACCAAACGAA
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GGGGCGTTCAAAAATCCAAGGATCCCCGGAATTTGGCATTCCACTTCA

>4
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GCGTCTCCAAAGAAGCTAAGTGTTGAATTAAAAAAGATTGAAACAATCTTCAATTTCAAGCT
AACCCTGAGTATCGCCCACAACCAAAAAATAAAAAATTATCTTTTGAGAAGGAAATGACGC
TCAAACAGGCATGCCCTTGAGAATGCTCAAGGGCGCAATGTGCGTTCAAAAATTCAATGATT
CACGAGTATCTGCAATTCACATTACTTATCGCAATTCGCTACGTTCTTCATCGATGCGAGAAC
CAAGAGATCCGTTGTTGAAAGTTTTAAATTATTTTAAAATTTCCGTTACGAAAGTTGGTTTAG
TTTTAAAAATTATTTTCAAATAAAAAATAATTTGTTTTTTTTTTTTGGCCGGGGCCCCTTTCCA
TTCAAGCAAAAAAAAATAAAAATTTAAGTAAAAAATTTTCAAATGGACGCATACCCTTTCAC
TGGGAAAACCAGCACCCCCCCTTCAAGGGATTTACAATTTAAAAACGCGGCGCCCTTT

>5
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TTACGTATCGCAATTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAA
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TTATTAATGATCCTTCCGCAGGTTCACCTACGGAAGACTTTTTAA

>6
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AACAGCGTCTCCAAAGAAGCTAAGTGTTGAATTAAAAAAGATTGAAACAATCTTCAATTTCA
AGCTAACCCTGAGTATCGCCCACAACCAAAAAATAAAAAATTATCAATCAACAAGAC
>7
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TTCTAAACCTGCCTAGCTGATATAACGAGTTGGAAGAACCTAATACATTATTTCAGAAAGAC
TGCTTATTAGTACACTCTTGCTAAGTCAATATTTCAAGTTAACCCTTGACAGAGTATCACTCA
ATACCAAACCCGAAGGTTTGAGAGAGAAATGACGCTCAAACAGGCATACCCTCTGGAATAC
CAGAGGGTGCAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCACAATACG
TATCGCATTTCGCTGCGTTCTTCATCGTTGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTG
AAGATTTTAATTTTTGTTAAAAATTTTCATGACTATTGGTTAAAGGTTTTAACATTAAAAAAA
AAGTGTTTGGACCTTTGGGCCGAAACCCGGGTTCCCCCCCCGAGGGTTAGTTAAAATATTTA
GGGCCGGGTTGGGAAAGGTTAATCAAGGGGTTTTATTCCGCGGGGGGGGAAAACCCCCTGA
ACCCTCCCCAACGCCCCCCGGAGGG

>8
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GGTACATTACCATTTCCCTTGTAAAGTAAAACGAATAAATTCATAAATACATCACAGCGAGA
ACAGCGTCTCCAAAGAAGCTAAGTGTTGA

>9
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TTAGGCCGAAGCAGGACCAAACCGGAGGTTTGAGAGTAAATATCGCTCACCCACGCATGCC
CTGGGGAATACCCCGGGGCGCAATGTGCGTTCAAAGATTTAATGATTTTCGTCTGCAAGTTT
ATTACGTATCGC

>10
TCGGGGGAATCCTACCTGATTTGAGGTCAAACTTGATGAATATTAAAAGCAACCCTTTGCCT
AAGGTACATTACCATTTCCCTTGTAAAGTAAAACGAATAAATTCATAAATACATCACAGCGA
GAACAGCGTCTCCAAAGAAGCTAAGTGTTGAATTAAAAA

>12
TGGGCAAATCTTACGACGGTGAGGAAAGATGGGGCTAAAACTTATTCTAGCGCCGTTGATAT
TAGGCCGAAGCAGGACCAAACCGGAGGTTTGAGAGTAAATATCGCTCACCCACGCATGCCC
TGGGGAATACCCCGGGGCGCAATGTGCGTTCAAAGATTCAATGATTCACGTCTGCAAGTCAT
ATTACGTATCGCAATTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAA
AGTTTTTTAAT

>13
TGGGGGTGGTCTTCCCTGATTTGAGACTAATCTTAAAATGTAGACATTCTGATTAGAAGCTTC
CTTTAACCCAACCCGGCTCTAATCCGAAGACTAGAATTCCTCAGCGAATAGTCTATTACGCC
AAGTCAATCCGAAGTTCGATTGCGGATGCTAATGCATTACGAACGAGCTAGACCGTAAAGG
CCAGCAGCGCTCAGAAACCAAACACCTCTTCAATCATTAAGAAAGAGGAGGGTTGAAGTAT
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APPENDIX B

Carbon Sources Placed on Biolog Microwell Plates

YT MicroPlate™

A1 Water	**A2** Acetic Acid	**A3** Formic Acid	**A4** Propionic Acid	**A5** Succinic Acid	**A6** Succinic Acid Mono-Methyl Ester	**A7** L-Aspartic Acid	**A8** L-Glutamic Acid	**A9** L-Proline	**A10** D-Gluconic Acid	**A11** Dextrin	**A12** Inulin
B1 D-Cellobiose	**B2** Gentiobiose	**B3** Maltose	**B4** Maltotriose	**B5** D-Melezitose	**B6** D-Melibiose	**B7** Palatinose	**B8** D-Raffinose	**B9** Stachyose	**B10** Sucrose	**B11** D-Trehalose	**B12** Turanose
C1 N-Acetyl-D-Glucosamine	**C2** α-D-Glucose	**C3** D-Galactose	**C4** D-Psicose	**C5** L-Sorbose	**C6** Salicin	**C7** D-Mannitol	**C8** D-Sorbitol	**C9** D-Arabitol	**C10** Xylitol	**C11** Glycerol	**C12** Tween 80
D1 Water	**D2** Fumaric Acid	**D3** L-Malic Acid	**D4** Succinic Acid Mono-Methyl Ester	**D5** Bromosuccinic Acid	**D6** L-Glutamic Acid	**D7** γ-Aminobutyric Acid	**D8** α-Ketoglutaric Acid	**D9** 2 -Keto-D-Gluconic Acid	**D10** D-Gluconic Acid	**D11** Dextrin	**D12** Inulin
E1 D-Cellobiose	**E2** Gentiobiose	**E3** Maltose	**E4** Maltotriose	**E5** D-Melezitose	**E6** D-Melibiose	**E7** Palatinose	**E8** D-Raffinose	**E9** Stachyose	**E10** Sucrose	**E11** D-Trehalose	**E12** Turanose
F1 N-Acetyl-D -Glucosamine	**F2** D-Glucosamine	**F3** α-D-Glucose	**F4** D -Galactose	**F5** D-Psicose	**F6** L- Rhamnose	**F7** L-Sorbose	**F8** α-Methyl-D-Glucoside	**F9** β-Methyl-D-Glucoside	**F10** Amygdalin	**F11** Arbutin	**F12** Salicin
G1 Maltitol	**G2** D-Mannitol	**G3** D-Sorbitol	**G4** Adonitol	**G5** D-Arabitol	**G6** Xylitol	**G7** i-Erythritol	**G8** Glycerol	**G9** Tween 80	**G10** L-Arabinose	**G11** D-Arabinose	**G12** D-Ribose
H1 D-Xylose	**H2** Succinic Acid Mono-Methyl Ester plus D-Xylose	**H3** N-Acetyl-L-Glutamic Acid plus D-Xylose	**H4** Quinic Acid plus D-Xylose	**H5** D-Glucuronic Acid plus D-Xylose	**H6** Dextrin plus D-Xylose	**H7** α-D-Lactose plus D-Xylose	**H8** D-Melibiose plus D-Xylose	**H9** D-Galactose plus D-Xylose	**H10** m-Inositol plus D-Xylose	**H11** 1,2-Propanediol plus D-Xylose	**H12** Acetoin plus D-Xylose

Oxidation Tests Assimilation Tests

APPENDIX C

Chemicals and kit list

Name of Chemical	Supplier Company	Catalog Number
6X Loading Dye	Fermentas	R0611
Agar	Biolab	15080055
Agarose	peg GOLD	208153
Bacteriological Peptone	Duchefa	006264.02
BUY Agar	Biolog	70005
Dextrose	Amresco	293813220
DNA Ladder Mix	Fermentas	43869
dNTP mix	Fermentas	65110
EcoRI	Fermentas	47977
EDTA	Applichem	4Q006413
Ethanol	Sigma-Aldrich	32221
Ethidium Bromide	Applichem	9N008724
Glycerol	Duchefa	005442.02
HaeIII	Fermentas	47282
Liquid Nitrogen	Linde	
Lysine Medium	Oxoid	CM0191
Master Mix PCR	Qiagen	130170263
MspI	Fermentas	47560
NaCl	Applichem	A2942.0500
NaOH	Riedel de Haen	63130
SO2		
Sodium Metabisulfide	Sigma-Aldrich	31448
Taq Polymerase	Fermentas	48773
WL Medium	Fluka	17222
Yeast Extract	Applichem	4W11265
YPD Agar	Duchefa	p05479.02
YPD Broth	Duchefa	p06443.01

Name Of Kits	Supplier Company	Catalog Number
QIAquick Gel Extraction Kit	QIAGEN	28706
Biolog System Microplate	Biolog	1005

APPENDIX D

Equipments

Autoclave:	Hirayama, Hiclave HV-100, JAPAN
	Nuve, OT 032, TURKEY
Centrifuge:	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Beckman Coultier ™ MicrofugeR 18 Centrifuge, USA
	Sorvall RC5C plus, USA
Deep-freeze:	-80^{o}C, Thermo Electron Corporation, USA
	-20^{o}C, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA

Fluorescence microscope:	OLYMPUS, BX-60, JAPAN
Gel documentatiton:	UVITEC, UVIdoc Gel Documentation System,UK
	BIO-RAD, UV-Transilluminator 2000, USA
Heating block:	Bioblock Scientific, FRANCE
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life Sciences AUSTRIA
Ice machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
	Nuve EN 120, TURKEY
Laminar flow:	Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY
Lyophilisator	Christ Alpha 1-2 LD plus, UK
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY

Micropipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP Multical$_R$, GERMANY
Power Supply:	Wealtec, Elite 300, USA
	Biogen, AELEX, USA
Refrigerator:	+4^o, Bosch, TURKEY
Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA
	GFL, Shaker 3011, USA
	Innova™ 4330, New Brunswick Sci., USA
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	NanoDrop, ND-1000, USA
Thermocycler:	PE Applied biosystems, GeneAmp PCR System 9700,
	USA MJ Research, PTC-100, USA
	TECHNE, TC 512, UK
Turbidimeter:	Biolog #3531, Hayward CA, USA
Turbidity Standard:	Biolog #3415, Hayward CA, USA
Vacuum System:	Thermo