Chapter

An Overview on *Saccharomyces cerevisiae* Indigenous Strains Selection Methods

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Abstract

From the fundamental studies of Louis Pasteur in the XIX century to the current genomic analysis, the essential role of microorganisms in winemaking industry is well recognised. In the last decades, selected *Saccharomyces cerevisiae* strains with excellent fermentative behaviour have been widely commercialised in form of active dry yeasts. Currently, the production of organic and "natural" wines represents a new economically relevant trend in the wine sector. Based on this market demand, the use of industrial yeast starter could be perceived as non-organic practice and then, rejected. However, in order to preserve wines sensory quality, healthiness, and to avoid organoleptic defects given by undesirable microorganisms, the "yeast factor" (*S. cerevisiae* or non-*Saccharomyces*) cannot be ignored. The purpose of this chapter is to describe the methods of selection of wine yeasts focusing the attention on indigenous *S. cerevisiae* strains. In fact, the use of ecotypic yeasts may represent a good compromise between the needs of microbiologically controlled fermentation and a modern vision of wine as natural expression of its "terroir", also from the microbiological point of view.

Keywords: *Saccharomyces cerevisiae*, selection methods, ecotypic strains, terroir, wine organoleptic profile

1. Introduction

Microorganisms are of primary importance in the agri-food industry. The knowledge of the microbial metabolic processes, as well as their behaviour and their technological characteristics, are required for any transformation process aiming to obtain healthy and quality foodstuffs. Wine production is also based on this assumption.

In oenology, the availability of yeasts able to drive alcoholic fermentation (AF) process and bacteria that efficiently carry out malolactic fermentation is required. In fact, in the first phase of the wine production process the yeasts, mostly belonging to the genus *Saccharomyces*, transform glucose into ethanol and carbon dioxide through the primary metabolism of sugars. Subsequently, lactic acid bacteria (LAB), usually *Oenococcus oeni* or *Lactobacillus* spp., metabolise malate into lactate, thus reducing the wine acidity [1, 2] and avoiding microbiological alteration.

In the past, fermentation of fruit juice, like those of apple and pear to produce cider, grape to obtain wine, or grains to make beer and so on for any kind of alcoholic beverages, have carried out by indigenous and naturally occurring microorganisms present in the original "must" [3–5].

The first molecular evidence in a Chinese Neolithic village, dated back to 7000 BC, shows that the food processing activity has given rise, without awareness, to the evolution of the genus *Saccharomyces* with the formation of new species, probably by interspecies hybridization or polyploidization [3]. Referring to *Saccharomyces cerevisiae*, its genetic evolution, which is due to human manufacturing, reflects the spread of grapevine cultivation and led to the origin of numerous strains [4–6].

Since the discovery of fermented beverages, their production process has undergone many evolutions, but initially the role of the microorganisms was unknown. Only in a second moment the choice of the best microorganisms to be used in a specific production, and their genetic improvement, become a conscious option. Hence, a certain degree of genetic yeast improvement was implemented in response to the requirements of wine production processes [3]. In fact, the scientific community proposed to the industry the use of starter cultures, that could be defined as a microbial (bacteria, yeast, mould) preparation containing a large number of live cells or resting forms of at least one species/strain that once added to a raw material leads to the production of a fermented food by accelerating and driving the fermentation process. The starter culture could contain unavoidable residues of additives and culture media [7–10].

Regarding wine production, until 150 years ago, also the transformation of grape must into wine took place without knowing the biological agent driving the fermentation process. In the usual cellar practices, it was carried out the inoculation of the must with a small amount of matrix from a previous successful fermentation, that in wine production was called "pied de cuve" [9]. In 1864, the role of microorganisms in fermentation was discovered by Louis Pasteur thus paving the way to the modern microbiology. Further research developments, achieved through microbiology, ecology, biochemistry and recently, molecular biology, have elucidated the metabolisms and in particular the biochemical process of alcoholic fermentation (**Figure 1**), as well as the interactions among microbial communities involved in winemaking, the phylogenetic and taxonomy. Based on this knowledge, the key role of yeasts in determining the quality of wine is now universally accepted [1, 11–13].

These scientific achievements have made it possible to supply oenological products and starter cultures appropriate for the industry. In fact, beginning from the mid-1960, the production and use of *S. cerevisiae* strains in form active dry yeasts (ADY) has expanded from California (United States) to the rest of the world [11–14]. In the major wine producing countries France, Italy, Spain, USA, Australia and Sud-Africa the use of ADY has almost fully replaced the spontaneous fermentation, especially in large-scale productions [3, 11, 13].

The importance of the adoption of yeast starter inoculation mainly consists in provide a faster beginning of AF. This is a stable and reproducible wine making procedure and, at the same time, ensures the absence of defects due to unwanted microorganism contamination [3, 9, 11]. The genetic selection of commercial ADY by the industry is based on the identification of specific technological and physiological features (**Table 1**) [3, 11, 15, 16].

The discovery of DNA, together with the development of molecular techniques further contributed to the taxonomic classification and, in a more practical context, to the identification of useful and spoilage microbes [17].

This also allowed the development of genetic improvement programs aiming at increasing genetic variability using diverse techniques (e.g. intra- or inter-specific

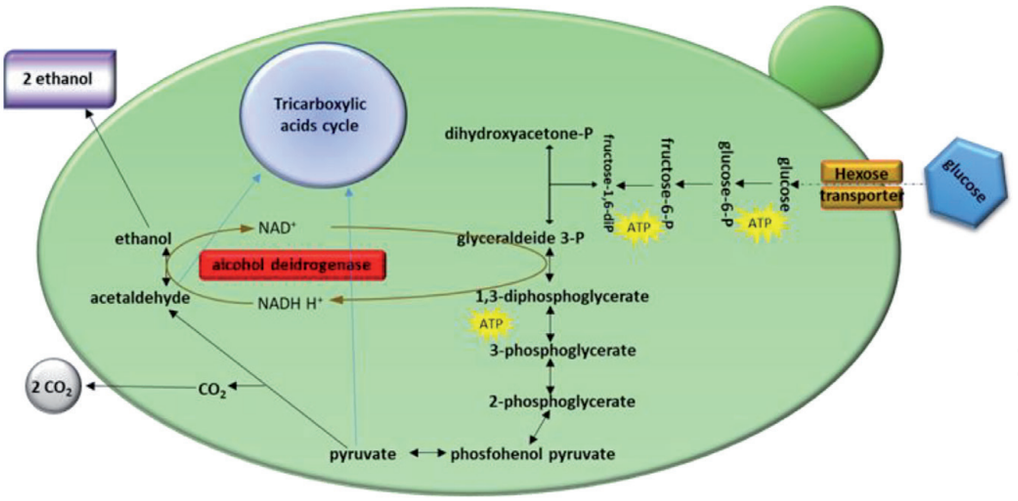

Figure 1.
Central metabolisms of alcoholic fermentation in yeasts.

Technological features	Desirable	Undesirable	Depending on process
Ethanol tolerance	x		
Complete fermentation of sugar	x		
Fermentation vigour	x		
Resistance to SO_2	x		
Type of growth in liquid media (Dispersed cells, Aggregates cells, Flocculence, Foam formation, Film formation, Sedimentation speed)			x
Growth at high and low temperature			x
Killer factor			x
Qualitative features			
Fermentation by-products (e.g Glycerol, 2-Phenyl ethyl acetate, Ethyl butanoate, Isoamyl alcohol, β-Phenylethanol)	x		
Volatile acidity, Sulphuric compounds (H_2S, SO_2)		x	
Enzymatic activity (e.g. β-Glucosidase, Esterase, Proteolytic enzymes, Carbon-sulphur lyase)			x
Ethyl carbamate precursor		x	
Effect on wine colour			x

Table 1.
General features to be considered in the selection of wine yeast.

hybridization) and by genetic engineering techniques, mainly focused on improving the yeast qualitative characteristics [18–20]. In the last decades, genetically modified yeast was also obtained by insertion of useful genetic determinants of different species in *S. cerevisiae* genome [18, 21, 22].

More recently, a new technology to engineer the genome of microorganisms, based on CRISPR/Cas9 system, has been developed. Vigentini et al. [23] applied this editing system in engineering of wine yeast to obtain genotypes with low production of urea through the deletion of DNA coding for arginine permease.

This character is important because urea represent a precursor of ethyl-carbamate (EC) which is considered probably carcinogenic to humans [23–26].

Despite these scientific developments, the current appreciation of local, natural and organic food and wines by consumers has led again to the exploitation of spontaneous fermentation [27]. In fact, organic producers and some consumers consider the use of industrial yeast starter as a non-organic or non-natural practice. Moreover, due to the use of the same commercial strain for various wine style in different winemaking geographical areas, a standardisation of wine sensory characteristics is possible and negatively considered. These criticisms are justified, but, on the other hand, a spontaneous fermentation has to deal with the risks of loss quality related to potential stuck, uncontrolled microorganism development, spoilage and off-flavour production. These problems are only partially addressed by technological strategies aimed at controlling the process [8, 9]. Another aspect to be considered is the wine safety: the uncontrolled development of unwanted microorganisms could lead to the production of toxic compounds, such as biogenic amine, ethyl carbamate or mycotoxins which could negatively impact on human health [8, 9, 28].

As reported by the International Organisation of Vine and Wine (OIV), from winemaking point of view, there is a constant requirement to improve the wine style to answer to the consumer's demand for natural products and to compete in the globalised market [29–31]. As in the past, even today the scientific answers to these new market demands can be found by moving to specific yeasts selection. Massive propagations of yeast isolated from their own vineyard in order to inoculate the must, is an alternative strategy for winegrowers that combines unique sensory attributes with safe fermentations. Furthermore, the exploitation of indigenous yeasts is emerging as a marketing plan in several wine regions because the wines are perceived with more complex taste and flavour [9, 32].

The research of wild strains of *S. cerevisiae* to be applied in wine production processes started in the late 1990s. Other studies on non-*Saccharomyces* genus are currently performed in many regions of the world [33, 34]. The research of new strains is based on the need of new genotypes coming from genetic variability. As previously mentioned, different yeast strains can develop different secondary metabolites profile, therefore providing distinct character to the wine [32, 35].

A strategy to find *Saccharomyces* spp. genetic variability is to search it in the natural biodiversity of microflora present in the vineyard. Sampling in cellars would not be very fruitful for this purpose, because cellar premises and equipment could be heavily contaminated by commercial starters [36–38].

Based on these ideas, the approach of propagation of the autochthonous yeasts for wine production encounters the consumer needs as well as the main winemakers' target: terroir-yeast in the production of more complex tasting wines with a certain stylistic distinction, while preserving quality [36–38].

The aim of this chapter is to describe the methods applied for the selection of wine yeasts particularly on the indigenous *S. cerevisiae*. The possibility of using autochthonous yeasts is an innovative approach that increases the link with the terroir and a wine stylistic distinction. Moreover, it allows to obtain greater communication and product differentiation in terms of marketing.

2. Selection program of indigenous *Saccharomyces cerevisiae* strains

Considering the oenological objectives described, the selection of indigenous yeasts must be planned and involves experiments aimed to isolate and propagate yeasts, and to test various oenological feature on laboratory and pilot scale (**Figure 2**).

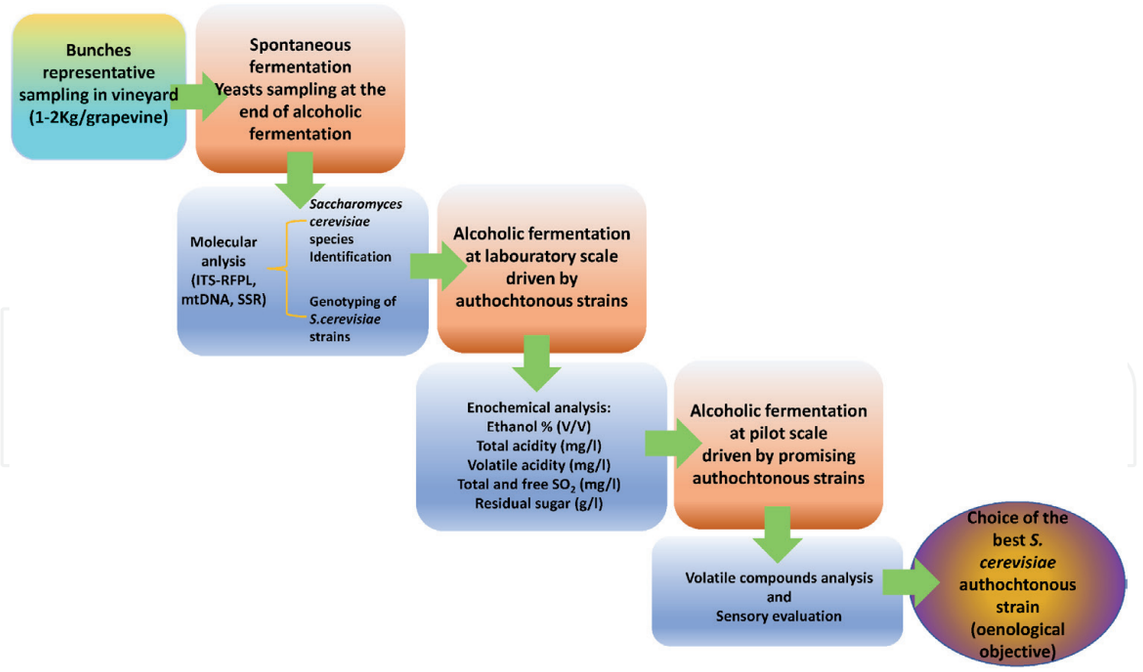

Figure 2.
Scheme of a selection process of indigenous S. cerevisiae *yeasts.*

2.1 Yeast sampling in vineyard

The vineyard soil would represent a reservoir of genetically different *Saccharomyces* spp. strains especially when the fruits are ripening and after the harvest. In fact, the increase of the number of fermentative yeasts during or near the harvest time has been recorded by molecular analysis, identification of culturable microorganisms and metagenomic approach [39, 40]. However, soil sampling at harvest time is not the optimal strategy for the isolation of wine yeast. The presence of *S. cerevisiae* in vineyard and at beginning of the fermentation process is sporadic [39–41]. In fact, yeasts belonging to the genus *Saccharomyces* spp. are not dominant on sound berries. The huge biodiversity of microflora living on bunch of grapes is related to insects and birds, that visit the ripe grapes [42]. *S. cerevisiae* strains are mainly detected during spontaneous fermentation when autochthonous grape yeasts and bacteria reduce their density due to the harsh environmental conditions represented by the high sugar content in must (realising a hypertonic living condition), and the increasing ethanol concentration in wine [32, 42]. To obtain an efficient selection of native yeasts, it is strongly recommended to start a spontaneous fermentation under controlled conditions [43, 44].

Several studies on spontaneous fermentations demonstrated the occurrence of an ecological succession with continuous shifts of the microbiota composition until the end of the process [42]. Due to the extreme condition of the must, especially high sugar concentration (250 g/l), low pH (3.5), nutrient availability and high osmotic pressure, the fermentative yeasts result to be more favoured compared to the species coming from the vineyard. *S. cerevisiae* is not dominant in this early step, but several fermentative yeasts such as *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Pichia* spp. and *Candida* spp. are detectable and carry on the alcoholic fermentation. The density of ethanol sensitive yeast species is reduced by the increase of alcohol concentration. *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *C. stellata*, *C. zemplinina*, *Lachancea thermotolerans* can resist at 6–8% of ethanol, while *S. cerevisiae* proliferate vigorously up to consuming all the sugar and can easily tolerate up to 15–16% (V/V) of alcohol. After three days from AF start the *S. cerevisiae* population is in exponential

growth phase (10^6–10^7 colony forming units/ml). In the final step of alcoholic fermentation, over 10% of alcohol, the process is dominated by several *S. cerevisiae* strains. This stage is the most profitable to isolate the fermentative microflora and collect a certain number of genotypes belonging to *S. cerevisiae* species [35, 41].

Performing the grape harvest at ripening time allows to obtain a good degree of yeast biodiversity representing an excellent starting point for the strain selection [32, 43]. The practice of experimental scheme of grape sampling may vary according to the vineyard feature and economic considerations. In optimal situation, the criteria that could be respected have been described by Setati et al. [41]. In detail, it's recommended to:

- Pay attention at any factor which can affect the microbiota community of the vineyard: climate conditions, microclimate (cooler and wetter area may contain a greater population of yeasts), geographical location, microbial vectors, vineyard management (conventional, integrated, organic or biodynamic farming), disease and pests, chemical and pesticides treatment, soil management, and so on [41, 45];

- Collect bunches in proximity of harvest, in order to take the highest *Saccharomyces* spp. biodiversity, also at subspecies level, due to presence of insect and birds at physiological ripeness stage [41];

- A good method to sample is based on the Theory of Sampling (TOS); where a two-dimensional yield is linearised into an elongated one-dimensional lot from which to extract samples at equidistant intervals [41].

As general principles, in the environment and in the vineyard agroecosystem too, yeast populations suffer from spatial and temporal fluctuation, so grape samples should be taken in several locations to gather a sufficient amount of *S. cerevisiae* strains that can be considered for the selection procedure [12, 37, 38]. It should be considered that damaged berries are a source of biodiversity for the sampling of fermentation yeasts [43].

Then, grape bunches should be placed in sterile bags avoiding the contamination with microorganisms unrelated to the sample, and transferred to the laboratory and processed as soon as possible according to the experimental protocol [41].

2.2 *S. cerevisiae* strains isolation

After the harvest of bunches, the spontaneous fermentation must be started, crushing the grapes. In order to avoid the contamination of the cultures, sterile conditions must be ensured by using sterilised or disposable equipment. In this step, di-ammonium phosphate (DAP) can be used as yeast nutrient and SO_2 in the form of potassium metabisulphite can be added to promote the dominance of *S. cerevisiae* strain respect to SO_2-sensitive non-*Saccharomyces*. Alternatively, the process could proceed without any addition of other nutrients or additive, except grape juice. The contact of must with berries skins is essential since the highest yeast concentration is in this compartment. Because of its resistance to osmotic pressure, tolerance to high sucrose concentration and to its efficient fermentation of sugar, *S. cerevisiae* is well adapted to the grape must [12, 42].

Due to the ethanol tolerance of *S. cerevisiae* and to the sensitivity of other yeast species, when the alcoholic fermentation is close to the end (ethanol more than 10% V/V), a sample of fermenting must-wine should be collected to isolate those yeasts that are driving the spontaneous process [12, 42]. Yeast isolation is performed by plating the collected samples on selective laboratory media in controlled conditions.

The dilution of fermenting must or wine at the end of AF is critical to evaluate a reasonable number of colonies in the solid artificial media. However, a compromise with the risk to lose biodiversity with the dilution procedure must be found, so that the sample should represent the yeast population in each vinification. Usually, the sample is diluted until 10^{-5} or 10^{-6} and aliquots of these suspensions are plated. Wallestein Laboratory (WL) agar solid media allowing to differentiate among yeast species on the basis of different colours of the colonies is usually used for yeast growth (**Figure 3**). The incubation temperature must be 24–26° C.

The genotypes loss during the isolation phase, is a problem to deal with during the selection procedure. As the different *S. cerevisiae* strains are morphologically indistinguishable, the colonies must be sampled randomly in plates with 250 colonies maximum. A total of 24–30 colonies for each plate must be sampled and analysed by molecular techniques for species assignation and strain differentiation [46]. Once the isolation and genetic identification phases have been completed, the strains are usually long term stored at −80° C in glycerol 50% V/V to preserve membrane integrity [32, 41, 47] and in slant with YEPD (Yeast Extract Peptone Dextrose) solid agar for short term conservation at 4°C. This procedure has been applied in several studies such as Capece et al. [43], Efstratios et al. [48], Viel et al. [49].

2.3 Genotyping: Molecular biology applied to yeast species identification and *S. cerevisiae* strain characterisation

One of the main goals in microbiology is to obtain a valid identification of microorganisms. Traditionally, before the application of molecular biology techniques, yeasts have been identified by morphological and physiological criteria. These methods are basically labor-intensive, time-consuming, and usually provide doubtful identifications. This is due to similar colony morphology, to the influence of culture conditions on yeast physiology and to the presence of different teleomorphic and anamorphic forms in the same species [50, 51].

The progress in molecular biology allowed to develop fast and efficient methods to identify both species and strains. Methods based on DNA technique,

Figure 3.
Some S. cerevisiae *colonies on Wallestein laboratory (WL) agar medium.*

some of these based on DNA Polymerase Chain Reaction (PCR) proved to be the most effective identification tool. Allozyme patterns, DNA–DNA hybridization, electrophoretic karyotyping, microsatellite analysis, nested-PCR, random amplified polymorphic DNA (RAPD) and mitochondrial DNA restriction analysis are the molecular biology techniques which first contributed to yeast identification [50–58]. As an example, electrophoretic karyotyping is based on the weight analysis of the yeast entire genome according to the species [52]. Other examples of molecular analysis are: insertion site polymorphism of delta elements, simple nucleotide polymorphism (SNP), amplified fragment length polymorphism (AFLP), intron splice sequence amplification, PCR of intron of mitochondrial genes, ribosomal DNA sequencing [12, 54, 57, 59, 60].

Moreover, the genome of *S. cerevisiae* S288C, a model organism in both cell biology and medicine, was entirely sequenced in 1996 and this reference DNA is at the base of the *Saccharomyces* Genome Database (SGD). This achievement facilitates the introduction of new molecular techniques [61, 62].

In this paragraph we will describe more in detail the most relevant techniques for the identification and characterisation of *S. cerevisiae*. RAPD is a PCR based technology in which DNA polymorphism is analysed by amplifying random DNA segments with single primers with an arbitrary nucleotide sequence. A single primer is used to anneal to the genomic DNA at different sites.

Quesada and Cenis in 1995 [53] and Baleiras Couto et al. in 1996 [54] used this method in the taxonomic identification of wine yeast strains both at genera and species level [53, 54]. In 2010, Capece et al. have used a RAPD-PCR with M13 primer to execute a fingerprint on 341 isolates obtaining 130 indigenous strains [43]. This technique can be applied both for interspecific and intraspecific characterisation [55]. The advantage of using RAPD is that it is rapid and easy to assay and there is no need of knowing the DNA sequence, but the main drawback is the low reproducibility.

In 1994, some authors focused the attention on mitochondrial DNA (mtDNA) for fast characterisation of *Saccharomyces sensu stricto complex* [49, 63]. The high polymorphism of this DNA can be highlighted after restriction enzymes digestion (endonucleases: *AluI, DdeI, HinfI, RsaI*). The resulting mtDNA band patterns is species-specific and allows the identification of *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, *S. pastorianus* species [63]. The mtDNA restriction analysis (RFLP-mtDNA) was also applied in many experimentations at strain level due to high degree of intraspecific heterogeneity [42, 47, 64].

For the identification at species level, the main used technique is based on the amplification of the rDNA Internal Transcribe Spacer (ITS) region and subsequent digestion with restriction enzymes. This is a specific type of RFLP also called Amplified Ribosomal DNA Restriction Analysis (ARDRA). The amplified target region includes the conserved gene coding for the 5.8 rRNA subunit and the two flanking non-coding and variable internal transcribed spacers named ITS1 and ITS2 [64, 65].

This method was described by Guillamón et al. in 1998 [64], Granchi et al. [50] and Esteve-Zarzoso et al. in 1999 [51] and is used in oenological yeast species identification still today [50, 51, 64, 65]. According to Guillamón et al. [64], the method is based on a first step of amplification targeting the nuclear rRNA gene region by using primers ITS1 and ITS4. This region includes the coding zone for the RNA ribosomal 5.8S and two non-coding regions at its ends (ITS1 and ITS2) (**Figure 4**). PCR products show a high length variation according to the different species leading to a preliminary discrimination among yeasts after agarose gel electrophoresis. The second step consists in PCR product digestion using three enzymes, endonucleases, *Hinf*I, *Cfo*I and *Hae*III. Each species shows a specific restriction pattern

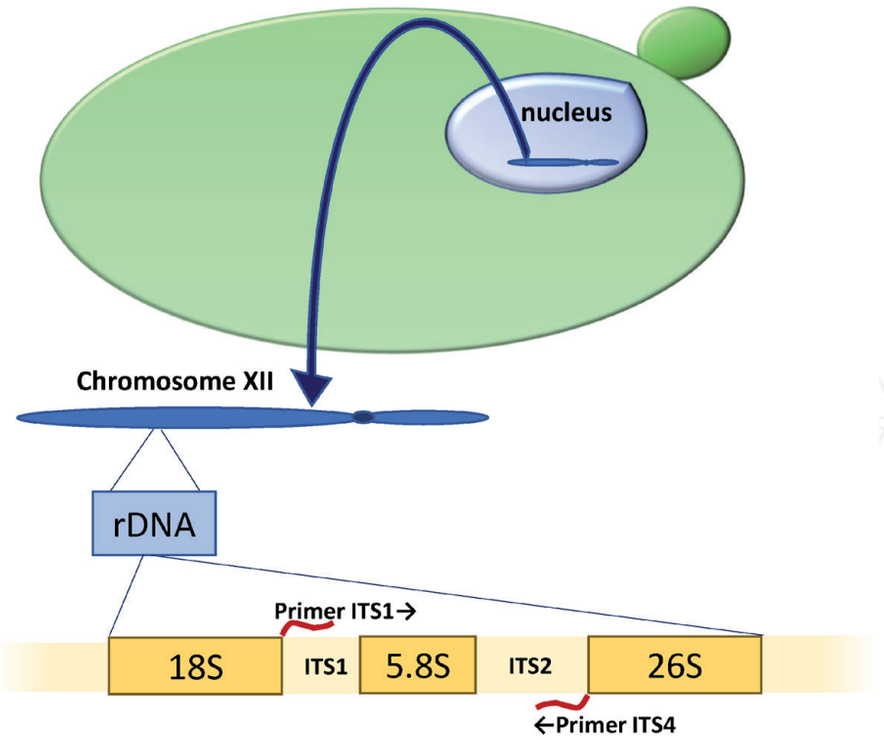

Figure 4.
Nuclear rRNA gene and region of DNA amplification through PCR using primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATA TGC-3′).

according to each endonuclease. So that a discrimination at species level is easily obtained. Thanks to this method it was possible to distinguish with confidence the presence for example of *Hanseniaspora uvarum*, *Candida stellata*, *C. vini*, *S. cerevisiae*, *S. paradoxus*, *S. bayanus*, etc. during spontaneous must fermentation [51, 64, 65]. Similar results have been obtained by Esteve-Zarzoso et al. [51] who analysed 243 different strains belonging to 132 different species, from the Spanish Type Culture Collection (CECT). In the experiment the amplicon digestion has carried out using *HinfI*, *CfoI* and *HaeIII* and other four endonucleases (*AluI*, *TaqI*, *DdeI* and *ScrFI*). This second set of endonouclease was necessary in some particular cases where more restriction patterns were required to get an efficient identification.

In general, this technique is highly reproducible and allows the discrimination of large number of samples.

Focusing on *S. cerevisiae* strain discrimination, inter-delta analysis and microsatellite polymorphism analysis represent useful and easy-to-use molecular tools. Inter-delta regions are some repetitive DNA sequences in *S. cerevisiae* genome, often associated with the transposon Ty1. These regions can be used for the genetic identification of *S. cerevisiae* strains thanks to their different number and location within the species by amplifying these regions with specific primers. Several authors studied inter-delta fingerprinting of *S. cerevisiae* strains and showed that PCR-amplification of DNA delta sequences is a reproducible, strain-specific and simple method that can be successfully applied to monitor strain population dynamics in wine fermentation [47, 66–68].

Microsatellite markers, based on Simple Sequence Repeats (SSRs) scattered throughout the genome [69–73], represent the "gold standard" for this discrimination. Microsatellites are short DNA motifs, 2–6 bases (e.g GATA, GACA, etc.), tandemly repeated five to fifty times (**Table 2**). Their sequence lengths are intra- and interspecific polymorphic across species [56, 69–73]. Moreover, SSRs are characterised by higher mutation rate than the rest of the genome, representing a formidable tool for the genetic differentiation of *S. cerevisiae* strains, as reported by

Locus	SSR Motif	Open Reading Frame Coordinates	Primer sequences (FW: forward; RV: reverse)
ScAAT2	TAA	YBL084c	FW:CAGTCTTATTGCCTTGAACGA **RV:GTCTCCATCCTCCAAACAGCC**
ScAAt3	TAA	YDR160w	FW:TGGGAGGAGGGAAATGGACAG **RV:TTCAGTTACCCGCACAATCTA**
C5	GT	VI-210250/210414	FW:TGACACAATAGCAATGGCCTTCA **RV:GCAAGCGACTAGAACAACAATCACA**
C3	CAA	YGL139w	FW:CTTTTTATTTACGAGCGGGCCAT **RV:AAATCTCATGCCTGTGAGGGGTAT**
C8	TAA	YGL014w	FW:CAGGTCGTTCTAACGTTGGTAAAATG **RV:GCTGTTGCTGTTGGTAGCATTACTGT**
C11	GT	X-518870/519072	FW:TTCCATCATAACCGTCTGGGATT **RV:TGCCTTTTTCTTAGATGGGCTTTC**
YKR072c	GAC	YKR072c	FW:AGATACAGAAGATAAGAACGAAAA **RV:TTATTGATGCTTATCTATTATACC**
SCYOR267c	TGT	YOR267c	FW:TACTAACGTCAACACTGCTGCCAA **RV:GGATCTACTTGCAGTATACGGG**
YKL172w	GAA	YKL172w	FW:CAGGACGCTACCGAAGCTCAAAAG **RV:ACTTTTGGCCAATTTCTCAAGAT**
ScAAT1	TTA	XIII-86902/87140	FW:AAGCGTAAGCAATGGTGTAGATACTT **RV:CAAGCCTCTTCAAGCATGACCTTT**
C4	TAA+ TAG	XV-110701/110935	FW:AGGAGAAAAATGCTGTTTATTCTGACC **RV:TTTTCCTCCGGGACGTGAAATA**

Locus	SSR Motif	Open Reading Frame Coordinates	Primer sequences (FW: forward; RV: reverse)
C9	TAA	YOR156c	FW:AAGGGTTCGTAAACATATAACTGGCA **RV:TATAAGGGAAAAGAGCACGATGGC**
ScAAT5	TAA	XVI-897051/8970210	FW:AGCATAATTGGAGGCAGTAAAGCA **RV:TCTCCGTCTTTTTTGTACTGCGTG**
C6	CA	XVI-485898/485996	FW:GTGGCATCATATCTGTCAATTTTATCAC **RV:CAATCAAGCAAAAGATCGGCCT**
YPL009c	CTT	YPL009c	FW:AACCCATTGACCTCGTTACTATCGT **RV:TTCGATGGCTCTGATAACTCCATTC**
SC8132X (YPL009C)	GAA	XVI-536776/536705	FW: GGTGACTCTAACGGCAGAGTGG **RV: GGATCTACTTGCAGTATACGGG**
SCPTSY7	TTA	XIII-86953/87057	FW: AAAAGCGTAAGCAATGGTGTAGAT **RV: AAATGATGCCAATATTGAAAAGGT**

Table 2.
Some simple sequence repeat motif and primers' origin and sequence for Saccharomyces cerevisiae *typing.*

several papers in last 20 years [46, 49, 56, 69–75]. Hence, they are optimal molecular markers for the strains typing due to their size polymorphism. In general, they are useful for fingerprinting, linkage studies and knowledge on population genetic structure [5, 56, 76].

In 2016, Börlin M. et al. [74] characterised the population structure of more than 653 isolates of *S. cerevisiae* from three French cellars located at less than 10 Km from each other. Using 15 microsatellites loci as molecular markers they observed 503 different genotypes. Hence, based on SSRs analysis and using specific indexes concerning the origin of the three populations it was possible to assess a certain degree of overlapping between genotypes from two of the three cellars and the existence of a local and stable cluster of strains which shared some ancestor over 20 years. The similar composition of the *S. cerevisiae* population structure is explained by a series of events that have repeated over the years. One of these is the proximity of the wineries, which leads to a certain uniformity of the population due to the action of yeast vectors (birds, fruit flies, bees and wasps). And on the other hand, the practice of "pied de cuve", which consists in the inoculation of must with an amount of already fermenting must from a cellar to another. They noted that the SSRs-based method is more robust and sensitive compared to the inter-delta analysis, Pulsed-field Gel Electrophoresis (PFGE) and mtDNA RFPL methods [74].

Rex et al. [76] in 2020 have validated a SSRs molecular markers method for *S. cerevisiae* strain differentiation through PCR-multiplex. The method is based on two multiplex sets of primers of different size targeting polymorphic loci and it was applied on nine well characterised commercial yeasts. A set combines the six primers: ScAAT2, ScAAt3, C5, SCYOR267c, C8, C11, resulting in six different patterns after PCR and gel electrophoresis. The other one combines six other primers: YKL172w, C4, C9, ScAAT5, C6, YPL009c, resulting in five different patterns after the same process. The validation was achieved through the comparison of fragment lengths obtained by capillary sequencing and agarose gel electrophoresis image. The procedure was repeated to characterised 50 strains of *S. cerevisiae* from five different spontaneous fermentations. Through SSRs markers, 21 different new strains were recognised and characterised for their diverse aromatic profile respectively [76].

The strain identification based on SSRs polymorphisms analysis with multiplex PCR application has been used for rapid and low budget procedure too [46]. As an example, Vaudano and Garcia-Moruno [46] performed the typing of 30 commercial wine strains. The discrimination was achieved by performing a multiplex PCR using primers designed on three highly polymorphic loci: SC8132X, YOR267C and SCPTSY7 and subsequent gel electrophoresis and band pattern analysis and comparison.

Then, this analysis was employed in a dominance study between two co-inoculated strain at different temperature of fermentation, 15°C and 20°C. This trial was finalised to control the ability of these *S. cerevisiae* strains in leading the fermentation process.

Methods such as the latter can be used for applicative purpose both in oenology and in wild yeasts selection. In particular, molecular marker supports the screening of the large number of yeasts isolated from natural fermentation [75, 76].

2.4 Phenotype evaluation: technological characterisation, analysis of volatile compounds and sensory evaluation

When different genotypes have been identified, the analysis of the phenotype represented by physiological tests and micro-vinification assay is the following stage of the procedure. The physiological tests are for example:

production of hydrogen sulphide, killer toxin synthesis, SO_2 sensitivity, nitrogen requirement [32, 77].

An interesting test consists in the *in vitro* evaluation of β-glucosidase activity. This enzyme is involved in hydrolysis of monoglucosides with the release of volatile compounds, such as benzenoid/phenylpropanoid, monoterpenes and norisoprenoides, that contribute to aromatic profile. However, β-glucosidase can affect the colour of red wine due to the lysis of anthocyanins compounds with colour alteration or loss; thus the yeast ability to modulate the anthocyanin's colour during AF must be considered in the case of red winemaking [78].

In micro-vinification, the resulting wine is then evaluated through chemical analysis of basic features and volatile compounds [45]. Then, the behaviour of the native strains selected was monitored on a pilot scale in comparison with a known yeast used as control.

An example of this pilot test has been performed in 2019 in Lebanon and aimed to identify the most efficient indigenous starter from three autochthonous *S. cerevisiae* strains previously selected during natural fermentation of Merwah wine (M.6.16, M.10.16, M.4.17). In this study, the fermentation kinetic was evaluated measuring the reduction of the density by using a hydrometer and the residual sugars were analysed by UV–visible spectrophotometry, the dominance of the strains was monitored with Inter-delta-PCR [34].

In any described cases the evaluation of technological characters (**Table 1**) at the end of AF for each indigenous strain considered was always performed, generally using official OIV methods, standards Methods (ISO) or a multiparameter analyser. The more relevant features to be considered are: fermentation trend, ethanol production (%V/V), total acidity (g/l tartaric acid equivalent), volatile acidity (g/l acetic acid equivalent), pH, free and total SO_2 (mg/l), residual sugar (g/l glucose + fructose). For the microbiological stability of wine is essential a residual sugar less than 2 g/l.

Concerning the volatile acidity, it is positive a low-producer yeast, 0.2–0.4 g/l in acetic acid. High producer strains of sulphur compounds are discarded in the selection. SO_2 tolerance is a positive selection criterion [79]. The killer factor is traditionally studied, but its relevance is controversial as it seems that under fermentation conditions it has no influence on sensitive yeast [80].

The evaluation of the phenotype concerns also the wine aromatic profile derived from the secondary metabolism of yeasts. The production of volatile compounds is also affected by the composition of must, in particular depending on the biochemical precursors derived from vine variety. For example, the release of the volatile thiol 4-mercapto-4-methylpentan-2-one (4MMP) from its grape-derived cysteine-bound precursor is carried out by enzymes that possess carbon-sulphur lyase activity and it dependents on yeast [15].

Some volatile compounds belong to the category of higher esters and higher alcohols are shown in **Table 3** [34, 43, 48, 81–88]. In wines, esters can be formed by two different processes: fermentative ones, that involve enzymatic esterification performed by yeast, and storage for long periods that leads to chemical esterification. These two processes can concur in the synthesis of the same ester. The concentration in wine ranges from 10 to 20 mg/l. Higher alcohols are produced by yeasts, both from sugars directly and from grape amino acids through the Ehrlich reaction. They are mostly of fermentative origin and can be found in wines in quantities ranging from 150 to 550 mg/l. The main fermentative higher alcohols, part of the so-called "Fusel oils", are isobutyl alcohol (2-methyl-propan-1-ol) and amyl alcohols (mixture of 2-methyl-butan-1-ol and 3-methyl-butan-1-ol). At concentration lower than 300 mg/l they participate in the aromatic complexity of the wine; at higher concentrations their penetrating odour masks the wine's aromatic finesse.

Volatile Compound	Aroma descriptor	Olfactory threshold	Concentration in Wine	References
Esters				
Ethyl acetate	Fruitiness, varnish	7.5 mg/l*	22.5–63.5 mg/l	Swiegers et al. 2005 [81]
Isoamyl acetate	Banana, pear	0.03 mg/l*	0.1–3.4 mg/l	Swiegers et al. 2005 [81]
Ethyl butanoate	Fruity	0.02 mg/l*	0.01–1.8 mg/l	Swiegers et al. 2005 [81]
Ethyl 3-hydroxybutyrate	Fruity, grapefruit, winy	—	—	
2-Phenyl ethyl acetate	Floral, rose, hyacinth, honey	0.25 mg/l*	0–18.5 mg/l	Swiegers et al. 2005 [81]
Methyl hexanoate	Pineapple	—	—	
Ethyl hexanoate (ethyl caproate)	Green apple, pineapple	0.05 mg/l*	0.03–3.4 mg/l	Swiegers et al. 2005 [81]
Ethyl 2-methylbutanoate	Strawberry	—	—	
Ethyl heptanoate	Grape	—	—	
Ethyl octanoate (ethyl caprylate)	Fruity, floral, wax	0.02 mg/l*	0.05–3.8 mg/l	Swiegers et al. 2005 [81]
Ethyl decanoate (ethyl caprate)	Fruity, apple, soap	0.2 mg/l**	0–2.1 mg/l	Swiegers et al. 2005 [81]
Ethyl dodecanoate (ethyl laurate)	Waxy	—	—	
Ethyl lactate	Buttery, butterscotch	—	—	
Higher alcohols				
Propanol	Alcoholic, pungent, harsh, fermented, weak fusel, musty, yeasty	500 mg/l***	9.0–68 mg/l	Swiegers et al. 2005 [81]
3-Methyl-1-pentanol	Fusel, cognac, wine, cocoa, green, fruity	—	—	
Butanol	Fusel, spiritous	150 mg/l*	0.5–8.5 mg/l	Swiegers et al. 2005 [81]
Isobutanol	Fusel, Ethereal, winey	40 mg/l*	9.0–174 mg/l	Swiegers et al. 2005 [81]
Isoamyl alcohol	Solvent, Varnish, nail polish, ripe fruit, harsh	30 mg/l*	6.0–490 mg/l	Swiegers et al. 2005 [81]
Amyl alcohol	Almond	—	—	
1-Hexanol	Mowed grass, herbaceous, green	4 mg/l***	0.3–12.0 mg/l	Swiegers et al. 2005 [81]
2,3-Butanediol	Fusel, cognac, wine, cocoa, green, fruity	—	—	
2-Phenylethanol	Dried rose, floral	10 mg/l*	4.0–197 mg/l	Swiegers et al. 2005 [81]

Volatile Compound	Aroma descriptor	Olfactory threshold	Concentration in Wine	References
Benzyl alcohol	Jasmine	—	—	
3-(Methylthio)-propanol (Methionol)	Cauliflower	1 mg/l[****]	0.17-2.4 mg/l	Ferreira et al. 2000 [82]
3-Mercapto-1-hexanol	Passion fruit, grapefruit,	$6*10^{-5}$ mg/l[*****]	0-1.28 * 10^{-2} mg/l	Tominaga et al. 1998 [83]

[*] *Aqueous solution 10% ethanol.*
[**] *Synthetic wine.*
[***] *Wine.*
[****] *Red wine.*
[*****] *Aqueous solution 12% ethanol.*

Table 3.
Some volatile compounds from S. cerevisiae *metabolism, respective odour descriptors, olfactory threshold and common concentration in wine.*

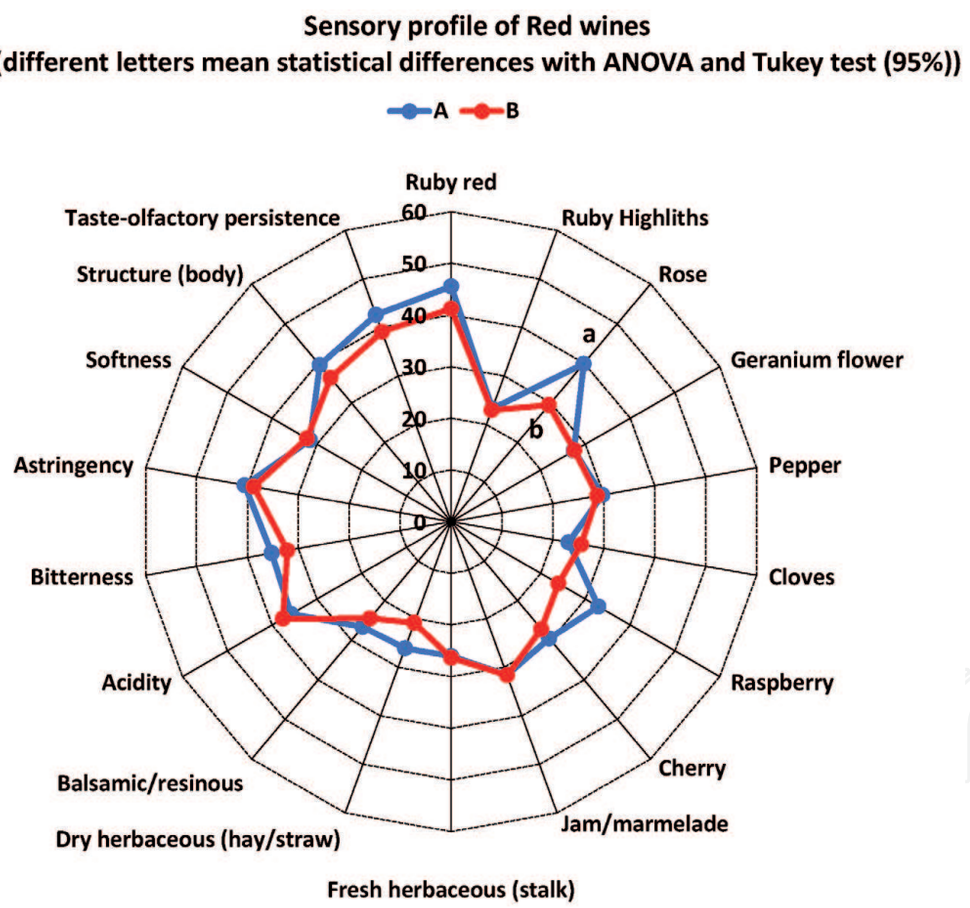

Figure 5.
Comparison of sensory profiles of two (A and B) red wines fermented with two different indigenous strains of S. cerevisiae.

Acetic esters of these alcohols, especially isoamyl acetate, have a banana fragrance that may play a positive role in the aroma of some young red wines (primeur or nouveau) [79].

Usually, the analysis of volatile is performed by gas chromatography equipped with Mass Spectrometer as detector (GC–MS) [43, 48, 81–88].

The last examination at the end of a pilot scale production is the sensory evaluation performed by a panel test. That consist in the personal evaluation of wine

descriptors fulfilled by a group of judges trained in the recognition of organoleptic features (appearance, odour, taste, texture) (ISO 1993). The panel, in short, quantifies the level of descriptors using an intensity scale as required by the ISO 2003 standard b. The sensory session must be performed in standard condition of the room, glasses, temperature, time, so that the environment does not affect the judges [34, 43, 48, 81–88]. An example of sensory analysis results is shown in **Figure 5**. This sensory examination could be useful to predict the consumer appreciation. At the end of this process, all the data obtained by every test must be statistically analysed. The strain or strains which show the best performance and which better meet the enologist's preferences, can be used in an industrial scale assay.

3. Conclusions

In winemaking, the role of yeast is fundamental for a good fermentation process. There is a high biodiversity among the *S. cerevisiae* strains which differently influences the fermentation and the final wine. The choice of the strain is extremely important for the quality and the organoleptic characteristic of wine.

In this chapter a workflow aimed to select indigenous *S. cerevisiae* strains as starter for AF has been described. The main steps are a good sampling in vineyard, the application of rapid but efficient molecular methods, the analysis of the technological features and the final sensory properties.

In consideration of the increasing appreciation by consumers of wines connoted by organoleptic complexity also linking with the territory of origin, the selection of indigenous *S. cerevisiae* strains represents a valid and safe scientific approach aimed at the production of wines with a typical character (terroir).

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Conflict of interest

The authors declare no conflict of interest.

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