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REVIEW

Transgenic wine yeast technology comes of age: is it time for transgenic wine?

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Abstract *Saccharomyces cerevisiae* is the main yeast responsible for alcoholic fermentation of grape juice during wine making. This makes wine strains of this species perfect targets for the improvement of wine technology and quality. Progress in winemaking has been achieved through the use of selected yeast strains, as well as genetic improvement of wine yeast strains through the sexual and pararexual cycles, random mutagenesis and genetic engineering. Development of genetically engineered wine yeasts, their potential application, and factors affecting their commercial viability will be discussed in this review.

Keywords genetic engineering · GMO · wine biotechnology

Introduction

Winemaking is an ancient art allowing the conversion of grape juice (must) into wine. From a sensorial point of view, the predominant character of must is sweetness, while wine flavor is full of complexity and refinement. In addition, winemaking and wine consumption are charged with positive cultural and social connotations in several countries. For some, it also carries some of the negative image associated with alcohol consumption, even though the number of claims of health promoting activities for wine is ever-increasing (Cooper et al. 2004; Bianchini and Vainio 2003; Di Castelnuovo et al. 2002; Wu et al. 2001). Wine quality is the result of a complex network of interactions, established along the different steps in the winemaking process, from the agronomical management of grapevine culture to the conditions in which wine is finally served to the consumer (Lund and Bohlmann 2006; Fleet 1993). Most producing regions around the world are proud of a long historical tradition and the flavor of the centuries is currently one major source of perceived quality and added value for wine. Notwithstanding, the wines we now enjoy have probably little in common with those ancient Egyptians or Romans used to drink. This is the consequence of a continuous improvement in wine quality over the centuries, involving all the different steps in the production process. Nowadays the winemaking industry debates between tradition and technological evolution.

In this review we will focus on the fermentation step of winemaking and, more specifically, on the yeast used for alcoholic fermentation which has been one major source of improvement of wine

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technology during the second half of the last century. Development of genetically engineered wine yeasts, their potential application and factors affecting their commercial viability will be specifically discussed (additional information can be found in Pretorious 2000; Rainieri and Pretorious 2000).

Yeast and wine quality

The identification of yeast as being responsible for alcoholic fermentation of grape must dates to the works of Pasteur. The main species responsible for this process is *Saccharomyces cerevisiae* (Querol and Fleet 2006). Apart from alcoholic fermentation, which is the main chemical transformation (in quantitative terms) that takes place during winemaking, yeasts are also responsible of the production of a series of other small molecules like glycerol, acetate, succinate, pyruvate, and several esters, all of them contributing to the sensorial properties of the wine (Fleet 1993). In addition, yeast cells release cell constituents including proteins and polysaccharides, which also contribute to wine quality (Escot et al. 2001).

Choice of wine yeast strain is recognized as having a major influence in the quality of both still and sparkling wines (Kunkee and Amerine 1970; Querol and Ramon 1996; Martinez-Rodriguez et al. 2001). In practice this observation led to the use of selected yeast strains that are usually commercialized as active dry yeast. The particular strain used should be adapted to the type of wine that is to be produced. Selection criteria for wine yeast strains have been discussed in several reviews (see for example Fleet 1993). These criteria have evolved since the first commercialized wine yeast strains were used and were simply expected to ensure complete fermentation with rapid kinetics. Winemakers now look for more sophisticated properties, that influence wine quality, including production or release of primary and secondary aroma compounds, other sensory properties, tolerance to difficult fermentation conditions (due to chemical composition of musts (sugar content, SO_2, antifungal compounds) or to external factors like too high or to low temperatures), killer phenotype, chemical stability or technological properties.

Genetic improvement

The search for new yeast strains, in order to improve characteristics of wine, or to facilitate specific stages of the production process, has traditionally relied on the isolation and screening of new yeast strains from grape and wine samples, and this is indeed the origin of the vast majority of wine yeast strains currently on the market. Most of these strains are listed in the commercial catalogues as being especially useful for specific fermentation conditions, wine styles or grape varieties, among other claims. Even though the search for new natural strains, in order to improve or at least ensure wine quality keeps going in different wine producing regions around the world, it has become clear that, unless new selection criteria are introduced, the limits of this strategy have been attained for most of the traits previously selected for. This unstated conclusion has led wine microbiologists to look for alternative ways to exploit yeast natural genetic diversity or even to genetically manipulate yeast strains in order to improve specific properties.

Genetic improvement of wine yeast through the sexual cycle

Traditionally, genetic improvement of livestock or crops has been performed by sexual breeding. In contrast, genetic improvement of industrial microorganisms is rarely based on the sexual cycle, either because they lack it, it is difficult to manipulate or there are faster or cheaper alternatives. Wine yeasts are not an exception and technologies based on the sexual cycle of *S. cerevisiae* have been seldom used. The two main limitations for this technology are the lack of genetic markers and the genomic structure of industrial wine yeast (Bakalinsky and Snow 1990; Dunn et al. 2005) that limits sporulation efficiency as well as spore viability (Gimeno-Alcañiz and Matallana 2001). Nevertheless there are examples of genetic improvement of wine yeast strains by mating, sporulation and isolation of single-spore derivatives (Ramirez et al. 1999). On occasion this constitutes a first instrumental step in the genetic improvement procedure, for obtaining genetically more tractable strains from an industrial isolate (Bony et al. 1997).

In contrast, other authors have found that these single-spore derivatives can lose some of the industrially interesting traits of the mother strain, including fermentation performance (Gimeno-Alcañiz and Matallana 2001). Yeast mating has been used for the elimination of deleterious or undesirable properties like SO_2 formation or excess foaming (Eschenbruch et al. 1982) of wine yeast strains, as well as for introduction of desirable characters like flocculation (Thornton 1985).

Genetic improvement of wine yeast through parasexual hybridization

An alternative to the sexual cycle in industrially important microorganisms is parasexual hybridization, in the form of protoplast (or spheroplast) fusion, more pompously known as genome shuffling, especially when more than two parent strains are used (reviewed by Giudici et al. 2005). Protoplast fusion can be intraspecific (two strains from the same species) or interspecific (cells from two more or less distant species are fused). There is an increasing interest on this technology for the genetic improvement of wine yeast. Especially since several authors (de Barros Lopes et al. 2002; Masneuf et al. 1998; Gonzalez et al. 2006, in press) showed that several industrially important strains are the result of natural interspecific hybridization events. Similar to what happens for sexual hybridization, one barrier to be solved in order to apply parasexual hybridization to the genetic improvement of industrial yeasts is the lack of genetic markers. Several authors have addressed this problem by looking for spontaneous or induced mutants showing resistance to different antimicrobials (Yamazaki and Nonomura 1991, 1994; Ramirez et al. 1998). However, up to now, the main application of protoplast fusion to the genetic improvement of wine yeast has been transferring killer determinants to industrially interesting strains (Sulo and Michalcakova 1992; Seki et al. 1985).

Genetic improvement of wine yeast through random mutagenesis

Random mutagenesis with chemical or physical agents is perhaps the simplest way to genetically improve industrial microorganisms. It has been extensively used for antibiotic or enzyme producing microorganisms (Backus and Stauffer 1955). One of the main limitations to the usefulness of random mutagenesis in wine yeast strains comes again from their genomic structure (Bakalinsky and Snow 1990; Dunn et al. 2005), since most genes will be present in two or more copies, selecting recessive mutations is difficult.

Random mutagenesis is often performed by UV radiation but chemical agents, such as ethylmethane sulfonate, can also be used. The right dose of mutagen is a compromise between the chances of getting the desired phenotype and those of producing to many mutations in non-related genes that would compromise other properties of the strain, for example its fermentation performance. Because large numbers of mutants have to be screened, the task of selecting improved strains can be greatly simplified by designing appropriate screening criteria, preferably plate assays, that would predict the behavior of the mutants in winemaking conditions. There are only few examples of random mutagenesis applied to wine yeast improvement, these include a series of works by Alinkhanyan et al. cited by Snow (1983), and more recently improvement of the autolytic behavior of second fermentation sparkling wine strains (Gonzalez et al. 2003; Martinez-Rodriguez et al. 2004; Nuñez et al. 2005) or nitrogen assimilation and fermentation kinetics (Salmon and Barre 1998).

Genetic improvement of wine yeast through genetic engineering

In this section we will describe the development of wine yeast genetic engineering since its beginnings in the early 1990s. It must be stated that, despite the optimism we may have about the potential benefits of using GM yeasts, to our knowledge, there is only one single recombinant wine yeast so for approved for winemaking (see below). The actual impact of this strain on the winemaking market is still unknown. A common trend is that the concepts behind the genetic improvement strategy are usually tested in laboratory strains, before proceeding with the industrial strains. For the sake of simplicity and

concision, in this review we will focus mainly on results involving genuine industrial strains.

Requirements for efficient genetic engineering of wine yeasts

One of the main advantages of genetic engineering over the methodologies described above is the high level of control over the modifications to be introduced into the microorganism. In addition, genes from any biological species can be incorporated to the yeast genome. However, in contrast to random approaches, a deeper knowledge of the mechanisms, and specifically of the genes involved in the yeast properties to be improved is needed. This is probably why most of the applications of genetic engineering to wine yeast involve monogenic characters and usually consist in the expression of enzyme coding genes.

Several tools must be available for efficient genetic engineering of microorganisms, including tools for introducing foreign DNA into microbial cells and for the stabilization of transgenic DNA, selectable markers, and suitable promoters. All of them are easily available for laboratory *S. cerevisiae* strains, since they have been, for years, a model for biochemistry, genetics or molecular biology; and more recently for genomics, proteomics and metabolomics. Notwithstanding, there are important genomic and physiological differences between laboratory and industrial strains that usually preclude the direct application of these tools to wine strains.

Perez-Gonzalez et al. (1993) tested several methods for transformation of an industrial strain and concluded that the more suitable method was lithium acetate transformation (Gietz et al. 1992). Most recombinant wine strains constructed since that time have used variations of the lithium acetate procedure.

The construct, or vector, introduced must be able to replicate inside the transformed cell and be transmitted to progeny. Maintenance of the transgenic DNA in the recombinant strains can be achieved by autonomous replication or by genomic insertion of the transforming DNA. Autonomously replicating plasmids based on the replication origin of the natural 2-micron plasmid of *S. cerevisiae* were the vectors of choice for the construction of the first recombinant wine yeast strains. However, modification of gene expression through gene disruption requires insertion of the transforming DNA into a yeast chromosome. Insertion also makes recombinant strains more stable, avoiding the need of maintaining selective pressure during conservation and use of the new strains. In *S. cerevisiae* insertion is easily directed to specific *loci* by incorporating the cognate homologous sequence into the vector or construction of interest in order to promote homologous recombination between chromosomal and vector sequences (Klinner and Schafer 2004) The frequency of homologous recombination would depend on several factor, including the length of the homologous regions, topology of the transforming DNA (linear or circular), or the presence of double strand breaks in the region of homology.

In order to identify transformed cells in the background of non-transformed surviving cells resulting from any genetic transformation experiment, it is necessary to use selectable markers, that can be either recessive, semidominant or dominant. Recessive auxotrophic transformation markers are commonly used for the transformation of laboratory strains, *URA3* or *LEU2* being among the more popular. Constructions carrying one of these genes allow the survival of auxotrophic strains, lacking a functional copy of the cognate gene, in non-supplemented minimal medium. Unfortunately, because wine yeast strains are usually prototrophic (and auxotrophic mutants are not easily obtained) the use of these markers has been precluded for industrial strains. This view has been challenged by a recent work by Hashimoto et al. (2005) that were able to obtain auxotrophic mutants from industrial wine yeast strains by UV mutagenesis, these mutants would greatly facilitate genetic engineering by allowing the use of recessive selectable markers.

Dominant or semi-dominant selectable markers have the advantage that they can be used for the transformation of virtually any yeast strain. They confer resistance to different antibiotics or other growth inhibitors, and usually encode enzymatic activities catalyzing detoxification of the antimicrobial agent; or mutated versions of the biological target of the antimicrobial, being

the mutant version less sensitive than the normal one. Cycloheximide resistance, a semi-dominant marker encoding a mutant version of the ribosomal protein L29, previously used for the transformation of brewing yeasts (del Pozo et al. 1991), has been used very often since it was introduced to the field by Perez-Gonzalez et al. (1993). The second, more popular, selectable marker for genetic transformation of industrial wine yeast has been resistance to the antibiotic G418 (Jimenez and Davies 1980; Wach et al. 1994).

Because one of the objectives more frequently pursued is the production of a specific enzyme-encoding gene, an appropriate promoter has to be used, especially in the case of heterologous genes. *ACT1*, encoding actin, or *ALDH1*, encoding aldehyde dehydrogenase, have been employed for this purpose by several groups (Perez-Gonzalez et al. 1993; Bony et al. 1997). Puig et al. (1996) have identified other promoters whose expression pattern would be more suitable for the expression of foreign genes during winemaking. Depending on the specific objective, suitable promoters would have different requirements in terms of transcription rates or the moment of maximal expression (beginning, middle or end of alcoholic fermentation). Results from transcriptome analyses during alcoholic fermentation, could also allow the identification of appropriate promoters (Zuzuarregui et al. 2006; Varela et al. 2005; Hauser et al. 2001).

Addressing commercial and regulatory issues

The use Genetically Modified Organisms (GMO) for food applications is strictly regulated in the EU, USA and most other countries in the world. Apart from these general regulations concerning transgenic food, in order to be marketed as wine, new enological practices must be accepted by the International Organisation of Vine and Wine (OIV); and in order to be marketed under the name of a specific ''appellation d'origine'', this practice must also be recognized by the appropriate authorities, not forgetting the preferences of consumers.

These legal and commercial limitations have been taken into account by several research groups working in the field, so that considerable effort has been devoted to develop genetic modification procedures that fit present and forthcoming GMO regulations and are more acceptable for the consumers. This research has focused in two main objectives: reducing the amount of non-yeast DNA integrated in the modified strains, and avoiding the use of antibiotic-resistance markers; and obtaining genetically stable transformants in the absence of selective pressure by preferring integration of the modification into the yeast genome rather than using autonomously replicating plasmids. The strategy proposed by Puig et al. (1998), consisted in the construction of artificially auxotrophic wine yeast strains, through the interruption of both copies of URA3 by genetic engineering, following the same strategy used in large-scale projects of functional analysis of the yeast genome (Wach et al. 1994). In this strategy, the interruption cassette is directed to the locus of interest by incorporating into the construction sequences homologous to the promoter and terminator of the gene of interest. In a second step, the selection marker (kan^R, conferring G418 resistance to yeast) is eliminated by homologous recombination of two direct repeats, also present in the construction, flanking kan^R. This allows the use of the same selection marker and construction for the interruption of the other copies of the gene in diploid or aneuploid cells. At the end of the process, antibiotic-resistance genes or any other sequences of bacterial origin have been completely removed and the strain can be transformed by using *URA3* selection. The main limitation of this strategy is that the whole procedure has to be repeated for every new industrial strain to be transformed.

Other authors have tried to identify dominant selectable markers different from antibiotic resistance; this includes sulfometuron resistance and *p*-fluorophenylalanine (PFP) resistance (Petering et al. 1991; Cebollero and Gonzalez 2004). These are usually mutant alleles of yeast genes (Xie and Jimenez 1996) so that, with the aid of an appropriate design, the final recombinant strains can be free of any sequence derived from bacteria. The possibility of getting auxotrophic mutants of industrial strains, as mentioned above (Hashimoto et al. 2005) would open the way to the use of common recessive

markers for yeast transformation. Even though it has not been applied to wine yeast strains, it is worth mentioning here a system developed for the genetic modification of sake yeasts (Akada et al. 1999). This system allows introduction of small modifications, including point mutations, in a given gene of the host strain. Any other sequence used during the procedure are removed at the end. The modification is performed in two steps. The first one can make use of any transformation marker, and generates two truncated copies of the gene of interest, one of them carrying the desired mutation. In the second step recombination between the two copies is selected for, this eliminates all the sequences used in the construction and there is roughly the same probability of getting the original gene or the desired mutant.

Applications of genetic engineering to winemaking

The tools described above have been used to construct recombinant wine yeast strains for several purposes as summarized in Table 1 and Fig. 1. First, killer-sensitive strains were transformed to a killer phenotype by expressing killer toxin/immunity cDNA (Lee and Hassan 1988). Afterwards labelled wine strains were constructed by inserting a reporter gene that allowed studies of imposition of the inoculated strain over the natural yeast population (Petering et al. 1991).

The first modification of an industrial wine yeast strain addressing the improvement of wine quality was performed by Perez-Gonzalez et al. (1993), this recombinant strain expressed an endoglucanase from *Trichoderma longibrachiatum*, allowing the improvement of varietal white wine aroma. Improvement of varietal aroma was the aim in the construction of a whole series of recombinant wine yeast expressing heterologous genes encoding plant cell-wall hydrolytic enzymes (Ganga et al. 1999); or glycolytic enzymes allowing the release of aroma compounds from glycosilated precursors (Sanchez-Torres et al. 1996, 1998; Manzanares et al. 2003). Over-expression of heterologous or homologous pectinolytic enzymes has also been assayed as an alternative to direct addition of these enzymes in order to improve extraction, clarification and filtration steps during wine making (Fernandez-Gonzalez et al. 2005; Vilanova et al. 2000; Gonzalez-Candelas et al. 1995). Secondary aroma compounds of wine originate during fermentation, with important impacts of esters derived from yeast metabolism. Expression levels of alcohol acetyl transferases and esterases in industrial yeasts, have been the target of several groups trying to improve secondary aroma (Lilly et al. 2000). One modification that attracted much attention, even before it was implemented in real wine yeasts, was the construction of *S. cerevisiae* strains able to perform malolactic fermentation. Efficient malolactic fermentation by yeast required both the malate permease from *Schizosaccharomyces pombe* and the malolactic enzyme from either *Lactococcus lactis* (Bony et al. 1997) or *Oenococcus oeni* (Husnik et al. 2006). The last one is now approved for winemaking in two countries: USA and Republic of Moldova, and commercialized by Lesaffre as ML01. The opposite effect on wine acidity has been the goal pursued by Dequin and Barre (1994), by diverting carbon metabolism from alcoholic fermentation to lactate production. Alternatively, and more interesting from an applied point of view, carbon metabolism has been diverted to glycerol production (Michnick et al. 1997), or glycogen production (Perez-Torrado et al. 2002). However, in the first case, the unacceptable levels of acetate production forced a second modification of the recombinant strain, deletion of the *ALD6* gene (Remize et al. 2000; Eglinton et al. 2002). Finally, other modifications have been designed for specific winemaking styles, for example autolysis properties have been improved for yeast used for second fermentation of sparkling wines (Cebollero et al. 2005; Tabera et al. 2006).

Concluding remarks

As shown in Table 1 and Fig. 1, a number of recombinant wine yeast strains have been developed over the last 15 years. These yeasts have been engineered to improve different aspects of wine production and quality. Although most of these strains, especially the earliest ones, do not

Table 1 Different improvements in wine quality and technology addressed by the construction of recombinant wine yeasts

	Description	References
1	Enhanced resistance of wine yeast to nutritional stress during the industrial production of starters by increasing the glycogen synthase activity and eliminating glycogen phosphorylase activity.	Perez-Torrado et al. (2002).
2	Improved juice extraction, wine clarification and filtration by overexpressing genes encoding pectinolytic enzymes in wine yeast.	Gonzalez-Candelas et al. (1995); Fernandez-Gonzalez et al. (2005); Vilanova et al. (2000)
3	Over-expression the *GPD1* gene in wine yeast resulting in a substantial increase in glycerol production at the expense of ethanol in wine; and deletion of *ALD6* and *ALD4* genes in order to reduce acetate production.	Michnick et al. (1997); Remize et al. (2000); Eglinton et al. (2002)
4	Expression in wine yeast of the *Trichoderma longibrachiatum* β-(1,4)-endoglucanase gene, resulting in wines with an enhanced varietal aroma.	Perez-Gonzalez et al. (1993)
5	Expression in wine yeast of the α-L-arabinofuranosidase B gene from *Aspergillus niger* in order to enhance varietal aroma.	Sanchez-Torres et al. (1996)
6	Expression of a *Candida molischiana* β-glucosidase gene in wine yeast in order to enhance varietal aroma.	Sanchez-Torres et al. (1998)
7	Expression in wine yeast of the *Aspergillus nidulans* β-(1,4)-endoxylanase gene, resulting in wines with enhanced varietal aroma.	Ganga et al. (1999)
8	Expression in wine yeast of the *rhaA* gene from *Aspergillus aculeatus* encoding an alpha-L-rhamnosidase in order to enhance varietal aroma.	Manzanares et al. (2003)
9	Over-expression in wine yeast of the *ATF1* gene, encoding an acetyltransferase, to improve the secondary aroma profiles of wine.	Lilly et al. (2000)
10	Expression of a gene encoding a glycosyl-hydrolase to increase resveratrol content in wine.	Gonzalez-Candelas et al. (2000)
11	Expression in wine yeasts of the gene encoding the L (+)-lactate dehydrogenase from *Lactobacillus casei* for acidification of high-pH wines.	Dequin and Barre (1994)
12	Expression of heterologous malate permease and malolactic genes, for yeast strains performing malolactic fermentation.	Bony et al. 1997; Husnik et al. (2006)
13	Overexpression of *csc1-1* allele to accelerate yeast autolysis for accelerated aging of sparkling wines (Champagne or Cava).	Cebollero et al. (2005)
14	Construction of an autolytic yeast strain by deletion of the *BCY1* gene for accelerated aging of sparkling wines	Tabera et al. (2006)

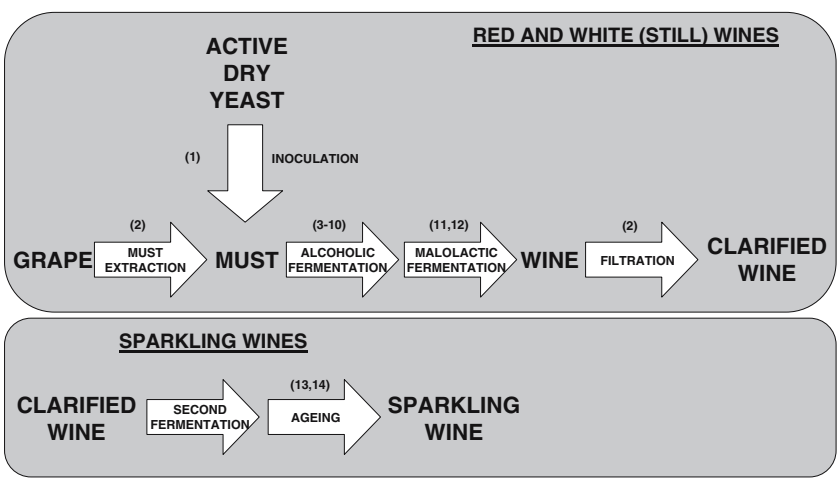

Fig. 1 Different steps of winemaking where potentially useful recombinant wine yeast strains have been constructed. See numbering in Table 1 for details

meet the requirements of genetic stability, and lack of bacterial DNA and antibiotic resistance markers; technologies to enable meeting these requirements are already available. However, the incorporation of new yeast strains to the enological industry still relies almost exclusively on natural genetic variability via the isolation and screening of natural strains. Apart from technical limitations, the reasons ''GMO wine'' does not reach consumers are essentially the same as those associated with getting GM foods to the market. These include long and costly administrative procedures, at least for the European Union, as well as consumer distrust, and activist opposition. In the case of wine additional problems arise from the specificities of the wine market, including international (OIV), national, and local (''appellation d'origine'') regulations. Finally, as mentioned in the introduction, ''tradition'' is an important strength for wine brands and production regions, and GMO technology constitutes a threat for the traditional image of the product. For all these reasons, the answer to the question, is it time for transgenic wine? would be, not yet. But an evolution is to be expected during the next few years, especially in New World countries. Obviously, the premium quality wine will continue to be made by ''traditional'' methods.

In the meantime, while waiting for the market to become more accepting of GM wines, strains and technologies keep improving, covering quality aspects related to different steps of the production process (Fig. 1). Aging of still wines constitutes one of the gaps still to be covered by yeast genetic engineering, but probably this will be covered in the near future by the construction of strains overproducing mannoproteins even though some of the modifications aiming to the improvement of sparkling wines (i.e. autolytic strains; Cebollero et al. 2005; Tabera et al. 2006) could eventually be useful for that purpose, since mannoproteins are among the major compounds released by yeast during autolysis.

One side effect of the bad press of GMOs is that some researchers are turning their sights to traditional genetics methodologies for genetically improving wine yeasts, which historically have not received much attention from the enological word. This includes a growing interest on random mutagenesis, as well as sexual and parasexual hybridization. In this context it is necessary remember that protoplast fusion is also considered as ''genetic modification'' under the GMO regulations in the EU.

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