Chapter 10
Genetic Improvement of Wine Yeasts

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1 Introduction

For many years, wine biotechnology was developed in the absence of genetic improvement of the microorganisms responsible for wine fermentation. Nevertheless, unconscious domestication seems to have played a relevant role on shaping wine yeast strains as we currently find them (Pérez-Ortín et al. 2002; Querol et al. 2003; Legras et al. 2007; Warringer et al. 2011; Sicard and Legras 2011). Indeed, compared to other biotechnological industries, including industrial food production like dairy or beer, also the use of starter cultures became generalized relatively. In this context, genetic improvement was not even considered.

By the early 90's, both the use of wine yeast starter cultures and genetic engineering of microorganisms were well stablished late (Gonzalez et al. 2011). Some researchers in the field considered that natural genetic variation found in wild

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P. Romano et al. (eds.), *Yeasts in the Production of Wine*,
https://doi.org/10.1007/978-1-4939-9782-4_10

isolates of wine yeasts, that was the basis for improvement and innovation in wine biotechnology until that moment, could benefit from genetic engineering tools (Pérez-González et al. 1993; Dequin and Barre 1994; Laing and Pretorius 1993; González-Candelas et al. 1995). These tools would allow combining features not ordinarily found in the same strains; or jumping the species barrier, by incorporating mostly extracellular hydrolytic enzymes, as well as malolactic fermentation capabilities, to the genome of wine yeasts. However, only two genetically engineered wine yeasts reached the market (Husnik et al. 2006; Coulon et al. 2006). These strains were positively evaluated by FDA and Health Canada (Cebollero et al. 2007). Given the restricted marketing area, the market share of these strains is probably very low, but no official data are available. Negative public perception, the cost of putting a new genetically modified organism (GMO) in the European market as well as other countries, and the poor support by OIV (Organisation Internationale de la vigne et du vin), are surely among the main reasons for the lack of success of these recombinant wine yeasts in most wine producing countries.

During the XXI century, most researchers in the field have turned to more conventional genetic improvement tools, which had been the basis for microbial biotechnology in other sectors ever since the 50's. In first instance, these involved mostly random mutagenesis (Rous et al. 1983; Gonzalez et al. 2003; Cebollero et al. 2005; McBryde et al. 2006; Cordente et al. 2009; Quirós et al. 2010; González Ramos et al. 2010), as well as intra an interspecific hybridization (Kishimoto 1994; Marullo et al. 2006; Bellon et al. 2011; Thornton 1985; Pérez-Través et al. 2015). One important advantage of strains developed by the use of these techniques is they are free of the prejudices (and regulatory restrictions) surrounding the use of GMOs in the food industry. At the same time, the fact that these tools, especially interspecific hybridization, induce a much higher genetic variability than genetic engineering constitutes both a challenge and an opportunity. Indeed, sometimes, these random methods, relying on phenotypic selection, allow attaining targets almost unreachable by a rational design. Nevertheless, the challenge is sometime designing the right phenotypic test to screen the yeast populations obtained by random mutagenesis or genetic hybridization. Probably one of the most powerful tool for genetic improvement recently incorporated to our panoply is adaptive laboratory evolution (McBryde et al. 2006; Tilloy et al. 2014; Novo et al. 2014; Cadière et al. 2011; Kutyna et al. 2012). It also benefits from a rational design, especially when technological targets cannot be directly selected for. Anyway, the revival of all these genetic tools in wine biotechnology is already releasing strains to the market that are not direct natural isolates.

Nowadays, Systems Biology and NGS technologies are providing new tools to boost the potential of non-GMO genetic improvement of wine yeasts. Metabolic modelling provides new ways to design experimental evolution conditions, targeting features that might not be easily selectable in a direct way. High throughput yeast phenotyping and automatic culture management allow performing quick analyses of the strains derived from in vitro hybridization, as well as running multiple experimental evolution runs in parallel, so increasing the odds of success. Moreover, NGS analyses of these new strains can help redesigning the parameters for new

experimental evolution of hybrid selection experiments. Finally, synthetic biology and genome edition based on CRISPR are providing new alternatives for the genetic improvement of wine yeasts (Lee et al. 2016; Pretorius 2017). However, these later techniques will also face difficulties to get the market, at least in European and other wine producing countries, because despite they can be tuned to avoid the introduction of DNA from other species in wine yeasts, they will still fall under the definition of GMOs of the European regulations, according to a recent opinion of the European Commission (Kupferschmidt 2018).

2 Targets for Genetic Improvement of Wine Yeasts

S. cerevisiae has two important roles for the development of human well-being, on the one hand it has been in charge for thousands of years of producing some of our most important foods (wine, bread and partly beer), on the other hand, more recently, it is one of the model organisms for research (Botstein and Fink 2011). Being a model organism and having such an extended biotechnological use, gives it a unique status. This has allowed many of the tools developed as a model organism to reach the biotechnology field to improve different areas of the yeast fermentative process.

One of the areas of improvement has been the fermentation process itself, increasing the ability of *S. cerevisiae* to assimilate sugars or nitrogen sources, its fermentation rate or its performance at low temperatures. Different tools have been applied to improve the fermentation kinetics of the yeast *S. cerevisiae*, random mutagenesis targeting nitrogen assimilation and fermentation performance (Salmon and Barre 1998), experimental evolution more recently for similar purposes (Novo et al. 2014) or genetic hybridization (Romano et al. 1985). Hybrid strains of a *S. cerevisiae* with a second more cryotolerant parental species like *S. kudriavzevii* or *S. uvarum* have also been shown as a natural approximation to improve fermentation performance at lower temperatures. Hybrids of these species can be isolated from fermentation in areas with cold environments (González et al. 2006).

Another main target of improvement in wine is the aroma composition and complexity. In this sense, almost all the different approaches discussed in this chapter have been used for this purpose. Some of these examples are, for the release of aroma precursors, the development of recombinant yeast strains with the ability to express pectin degrading enzymes that increase varietal aroma compounds (González-Candelas et al. 1995; Manzanares et al. 2003). Another target for genetic engineering has been changing the levels of yeast metabolites for aromatic purposes (Swiegers et al. 2007). Hybrid strains have also been reported to improve secondary aromas (Steensels et al. 2014), experimental evolution trials have achieved the same goal (Cadière et al. 2011).

Like aroma compounds, the ethanol content of the wine plays an important role in the final product. The recent increase of this metabolite mainly for global climate warming, but also for consumer trends looking for more mature and fully body

wines, is also an objective for improvement. Different experimental approaches have been conducted in order to reduce the final concentration of this metabolite. Usually by changing the yeast metabolic carbon flux towards the production of other metabolites through genetic engineering (Remize et al. 2000) or experimental evolution (Tilloy et al. 2014).

Mannoproteins contribute to the wine quality by the stabilization of the final product or retention of aroma compounds among others (Waters 1994; Núñez et al. 2006). For this reason, its over-production has been achieved by the construction of genetically improved strains (Gonzalez-Ramos et al. 2008, 2009) or by random mutagenesis (Gonzalez-Ramos et al. 2010).

Another interesting trait with biotechnological character is flocculation. Flocculation is a well-studied mechanism of *S. cerevisiae* that is mainly under the control of the FLO family of genes, although it has also been shown to be environmentally dependent (Govender et al. 2010). The up-regulation of certain genes inside the FLO family leads to an increase in cell aggregation (Verstrepen and Klis 2006). This is an interesting feature that helps to avoid problems with the clarification of the wine, once the alcoholic fermentation has finished, removing easily the yeast cells (Pretorius and Bauer 2002; Soares 2011). Several works have succeeded in the construction of yeast strains using genetic engineering that increase their flocculation phenotype (Verstrepen et al. 2001; Wang et al. 2008).

Other traits that have been targets for improvement are the reduction of volatile acidity, accomplish by random mutagenesis (Cordente et al. 2013) and also using the hybridization of wine yeasts as approximation (Bellon et al. 2011, 2013); the process of malolactic fermentation performed by lactic acid bacteria after the alcoholic fermentation has finished, has been genetically engineering in a recombinant yeast strain (Husnik et al. 2007); reduction of foam production by yeast hybridization processes (Eschenbruch et al. 1982); or the increase in SO_2 tolerance by intraspecific breeding (Thornton 1982).

3 Genetic Engineering Based on Standard Homologous Integration

Starting early in the 1990s, pioneers in genetic engineering of wine yeasts took advantage of all the tools already developed and available for laboratory strains of *S. cerevisiae*. In most cases, genetic improvement involved the introduction of new enzymatic activities (Pérez-González et al. 1993; González-Candelas et al. 1995; Sánchez-Torres et al. 1996; Ganga et al. 1999; Volschenk et al. 2001), or enhancement of existing ones (Michnick et al. 1997). Gene disruption was also a target in many cases, (Cambon et al. 2006; Tabera et al. 2006; Gonzalez Ramos et al. 2009) as well as promoter substitution to change the expression pattern (Cardona et al. 2007; Govender et al. 2010), or the expression of dominant defective alleles (Cebollero et al. 2005). The scope of this section is genetic engineering tools

actually employed on *S. cerevisiae* wine yeast strains with applied purposes, either the modified yeast strains were finally commercialized or not. It is not intended as a general review of genetic modification on this model species. Indeed, despite almost all techniques developed for laboratory yeast strains have the potential to be useful on wine yeast improvement, there are several genetic and physiological features of wine yeast strains that differentiate them from model strains and become a hurdle for the transfer of some genetic tools. Some of the most relevant features to consider in this context are prototrophy, homothallism, and diploidy or aneuploidy (Bisson 2004; Novo et al. 2009). Technologies used for the construction of recombinant wine yeast strains have evolved over time in response to the genetic tools becoming available for yeast research (in any context). However, the choices were not only driven by technical considerations. Researchers took also decisions based on their perception of public opinion on GMOs in foods, and to what was expected for easy approval by health and food authorities; despite only two strains have gone through an official approval process, and this only in very few countries (Grossmann et al. 2011). A summary of the features of these two strains is shown in Table 10.1.

The main features of the genetic modification systems that must be taken in consideration are the way to make DNA go through the different cell layers up to the nucleus, integrative or replicative nature of the transformation vector, selection markers, and elements required to drive gene expression (i.e. promoter and terminator) and protein secretion. Concerning ways to introduce transforming DNA into wine yeast cells, fortunately, systems already working for laboratory strains use to work reasonably well for most wine yeast strains. From the first trials, lithium acetate transformation was shown to be effective (Pérez-González et al. 1993), but electroporation has also been extensively used (Husnik et al. 2006).

Replicative vectors were used on early times for the construction of some strains. Those were based on the 2 μm replication origin or on yeast chromosomal replication origins (episomal vectors) (Pérez-González et al. 1993; Volschenk et al. 2001). In the late case, CEN sequences (centromeric vectors) are also included in order to

Table 10.1 Main features of the two only wine yeast strains that have ever been commercially available

Commercial name	ML01	ECMo01
Genetic background	S92	UCD522
Expressed genes	Malate permease from *Schizosaccharomyces pombe*	*DUR1,2* from *Saccharomyces cerevisiae*
	Malic enzyme from *Oenococcus oeni*	
Control sequences	*PGK1* promoter and terminator sequences	*PGK1* promoter and terminator sequences
Tranformation marker	Phleomycin resistance (by co-transformation; cured from final strain)	Phleomycin resistance (by co-transformation; cured from final strain)
Integration locus	*URA3*	*URA3*

help mitotic heritability (Cebollero and Gonzalez 2004). However, in order to improve genetic stability of the engineered strains, integration in the genome, usually by site-directed recombination, has usually been the method of choice. Genetic stability is not only a requirement for a true industrial usefulness of the improved yeast strains, but also a key feature for commercial GMOs. Often integration sites are targeted by including in the same plasmid the construction of interest together with sequences homologous to the target site. These plasmids are usually linearized by restriction enzyme digestion in this homologous sequence before yeast transformation (Volschenk et al. 2001; Swiegers et al. 2007). Alternatively, site directed integration can be attained with linear DNA fragments on which the construction is flanked by sequences homologous to those flanking a genomic region to be replaced. This is usually done for total or partial gene deletion (Gonzalez-Ramos et al. 2009; Cuello et al. 2017). These homologous flanking sequences can be part of a genetic construction carried on a plasmid vector or be introduced by PCR amplification as 5′ extensions of the amplification primers, similar to the construction of the whole genome yeast knockout collection (Baudin et al. 1993; Giaever et al. 2002). Homologous flanking sequences can also be used for promoter replacement strategies (Cardona et al. 2007; Jiménez-Martí et al. 2009; Govender et al. 2010).

Since, for any transformation procedure, the number of viable non-transformed cells recovered is highly in excess over transformed ones, the use of selection markers during transformation is unavoidable. For many years, auxotrophic selection markers have been the most popular alternative for laboratory yeast strains. These strains are typically auxotroph for two or three nutrients among a number of amino acids or uridine (Pronk 2002). The defective alleles are also very well known in each case, so that transformants can be easily selected by incorporating a functional copy of the cognate gene in the transforming vector or DNA fragment. However, wine yeast strains are typically phototrophs, precluding the direct use of auxotrophic selection markers. Puig et al. (1998) addressed this issue by engineering a *URA3* auxotrophic wine yeast strain, by using a geneticin resistance selection and marker-rescue strategy. The resulting strain (T73–4), has been used by them, as well as other research groups, for developing multiple recombinant strains with an "industrial" background, using the *URA3* marker, but extrapolating this strategy to other genetic backgrounds would require performing all the construction steps for each of these strains. Alternatively, it was found to be relatively easy to select for induced or spontaneous auxotrophic variants, in the case of genetic markers that can be selected for both positively and negatively, like *URA3*, *LYS2*, or *MET15* among others (Boeke et al. 1984; Ito-Harashima and McCusker 2004; Cost and Boeke 1996; Hashimoto et al. 2005). Notwithstanding, most recombinant wine yeasts have been developed using dominant selection markers based on the resistance to drugs and chemicals. The main advantage of these markers is they do not depend on a previous genetic defect of the host strain, although basal tolerance might be a limitation in some cases. Some examples of dominant markers employed in this field include cycloheximide, sulfometuron, sulphite, p-fluorophenyl-alanine, based on *S. cerevisiae* mutant or overexpressed alleles; or geneticin (G-418), phleomycin,

nourseothricin, hygromycin, deriving from bacterial antibiotic resistance genes (Pérez-González et al. 1993; Casey et al. 1988; Goldstein and McCusker 1999; Cebollero and Gonzalez 2004; Coulon et al. 2006; López-Malo et al. 2014; Lilly et al. 2000; Swiegers et al. 2007). An important disadvantage of most of these dominant markers is they rely on the use of antibiotics, which constitutes a handicap for the commercial application of recombinant yeast strains. In order to clear the way to the market, researchers have used a co-transformation strategy (Husnik et al. 2006; Coulon et al. 2006). The approach is based on transforming yeast with of linear, integrative DNA fragment, carrying the construction of interest, together with a replicative plasmid carrying an antibiotic resistance marker. A good percentage of antibiotic resistant cells usually has also integrated the construction. Finally, the plasmid vector cured by culturing the transformed strain under non-selective conditions (Fig. 10.1).

Most recombinant wine yeast strains express heterologous or own genes (usually coding for intra- or extracellular enzymes), under the control of different *S. cerevisiae* promoters. Constitutive promoters are often preferred, but not all promoters considered as constitutive under standard laboratory growth conditions can be taken as such for winemaking conditions (Puig et al. 1996). It must be considered that most of the sugar consumption during wine fermentation usually takes place after the whole of yeast biomass was already produced. For example, the *ACT1* promoter (from the actin encoding gene) was used in some of the original constructs (Pérez-González et al. 1993). Its expression is associated to cell growth, so expression of these constructs is restricted to the beginning of the fermentation process, since most of the sugar is transformed into alcohol after yeast growth arrest. The promoters of

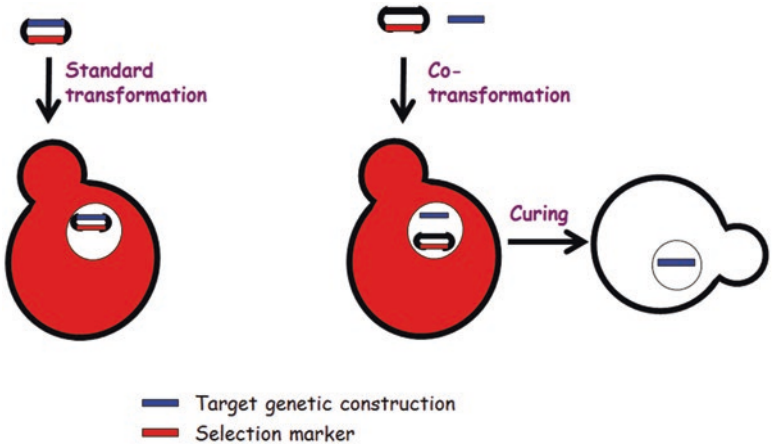

Fig. 10.1 Schematic representation of standard transformation and co-transformation of wine yeasts; the later allowing to easily curing the recombinant strain of auxiliary sequences and phenotypes not required in the final strain (e.g. antibiotic resistance). This is attained by sub-culturing the co-transformed strain in the absence of selective pressure. The target genetic construction is carried in a linear DNA fragment and stably integrated by homologous recombination

TDH3 (for glyceraldehyde-3-P-dehydrogenase), *ADH1* (alcohol dehydrogenase), and *PGK1* (phoshopglycerate-kinase) were among the most popular choices (Malherbe et al. 2003; Vilanova et al. 2000; Ehsani et al. 2009; Cambon et al. 2006. Efficient heterologous expression also requires the use of suitable transcription termination and polyadenylation sequences. Often, but not always, these sequences are derived from the same gene as the promoter used in that construction.

Extracellular enzymes derived from other yeast or fungal species do not usually require an exchange of signal peptides in order to be secreted by wine yeasts (Pérez-González et al. 1993; Sánchez-Torres et al. 1996; Ganga et al. 1999). Otherwise, mating pheromone α-factor signal peptide is considered a good choice to drive protein secretion in case extracellular expression is required (Laing and Pretorius 1993; Malherbe et al. 2003).

In some instances, the genetic improvement relies on the loss-of-function of a given gene, rather than on the acquisition of a new activity (Gonzalez-Ramos et al. 2009). Gene disruption on wine yeast strain might also aim to the characterization of the oenological impact of the genes involved. The fact that most wine yeast strains are at least 2n in terms of cell DNA content, meaning at least two copies of each chromosome are present, constitutes an additional challenge for the development of wine yeast recombinant strains by gene disruption. This has been addressed by several ways, including obtaining haploid derivatives, developing homozygous strains, marker rescue, or use of multiple selection markers. Haploid derivatives can be obtained from strains which are naturally heterologous for the HO *locus* (HO/ho), or by disruption of one copy of HO (Walker et al. 2003). Sporulation of these strains gives raise to both stable haploids and stable diploid strains in equal proportions. Homothallic strains can be sporulated, after a first gene disruption event, so that half of the haploid spores will become double disrupted after spontaneous diploidization. However, since most of these strains are heterozygous to some degree (sometimes highly heterozygous) there is a risk of obtaining strains that phenotypically differ to a significant extent from the parent strain, beyond the intended gene disruption. To avoid this, a homozygous strain can be obtained in the first instance. A confirmation of the phenotypic similarity with the original strain, including behaviour under wine fermentation conditions is advised, both for haploid and homozygous derivatives of industrial wine strains (Mangado et al. 2018). Using these strains homozygous deleted strains, isogenic to the parent strains can be obtained by first generating a hemizygous strain, deleted for one allele, followed by sporulation and spontaneous diploidization of isolated spores (Curiel et al. 2016). One half of the single spore clones obtained are homozygous for the intended deletion.

For gene deletion based genetic improvement, the availability of the whole genome YKO collection constitutes an interesting advantage (Giaever et al. 2002). Each strain from such collection already carries a copy of the cassette, flanked by the homologous upstream and downstream region of one of the *S. cerevisiae* ORFs. This construct can be "transplanted" to wine yeast be using genomic DNA from the appropriate strain as template for PCR amplification with primers upstream and downstream the target gene. This PCR fragment can then be used to transform wine yeast for geneticin resistance (Curiel et al. 2016; Salvadó et al. 2016).

4 Inter and Intraspecific Hybridization

Cross breeding has been a classic methodology to obtain individuals that have the best characteristics of their parents. This technique has been used with great success in the breeding of animals and plants. In yeast, intra-specific hybridization has also been widely used to introduce interesting characteristics from a strain into a second preferred strain. There are numerous examples where this type of approach has been successful in *S. cerevisiae* strains. In the oenology context the approach has been used to improve aroma production (Shinohara et al. 1994; Dufour et al. 2013; Steensels et al. 2014), to enhance fermentation performance coupled with low H_2S production (Romano et al. 1985), to reduce foam production (Romano et al. 1985) or to increase temperature tolerance (Marullo et al. 2009).

Perhaps more interesting than intra-specific hybridization are the phenomenons of inter-species hybridization in industrial applications among the *Saccharomyces* genus (Fig. 10.2). One of the most well studied cases occurs in the brewing industry where the species responsible for most of the fermentation is *Saccharomyces pastorianus*, a hybrid between *S. cerevisiae* and the recently described *Saccharomyces eubayanus* (Libkind et al. 2011; Gibson and Liti 2015). Also in the brewing industry, mostly found as contaminants is easy to find S. bayanus hybrid strains (*S. eubayunas* × *S. uvarum*) (Libkind et al. 2011; Nguyen et al. 2011). On the contrary, in the wine making industry, the major species responsible for the

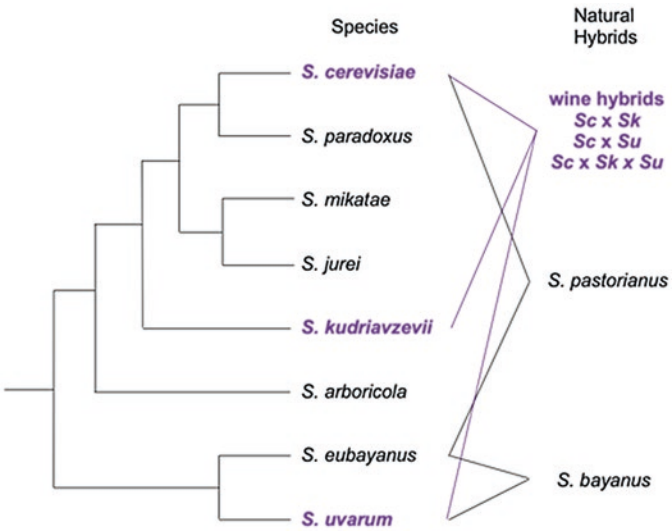

Fig. 10.2 Schematic cladogram of the phylogenetic relationship of the different *Saccharomyces* species, their recognized hybrids and the hybrids that can be isolated from oenological environments. Cladogram topography from Boynton and Greig, (2014; Fig. 1). The recently novel yeast species *Saccharomyces jurei* described by Naseeb et al. 2017 is included

fermentation is not a hybrid, but *Saccharomyces* cerevisiae. This does not mean that we can not find hybrids in an oenological context, the first reported case was a hybrid between *S. cerevisiae* × *S. uvarum* (*S. bayanus* var. *uvarum*) found in Italian wines (Masneuf-Pomarède et al. 1998). Since then hybrid strains have been isolated in wine making environments across Europe, North America, South Africa, Australia and New Zealand, involving *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* and in one occasion a triple hybrid (Barros Lopes et al. 2002; Belloch et al. 2009; Bradbury et al. 2006; Dunn et al. 2012; Erny et al. 2012; González et al. 2006, 2008; Heinrich 2006; Lopandic et al. 2007; Naumova et al. 2005; Peris et al. 2012a, b, c). These hybrids have been mostly isolated from low-temperature fermentations, providing the first clue for the success of these hybridization processes, both S. kudriavzevii and S. uvarum are better suited to this condition than *S. cerevisiae* (González et al. 2006; Belloch et al. 2008; Salvadó et al. 2011; Sampaio and Gonçalves 2008) (Fig. 10.2).

The ability of these hybrid strains to grow at lower temperatures than *S. cerevisiae* decreases the probabilities of halted or sluggish fermentations, a common problem under these conditions (Bisson 1999). Therefore, they are an interesting choice when conducting this kind of fermentations that have being shown to improve the wine aromatic profile (Boulton et al. 2013). Hybrid strains have also been shown to provide other advantages that the increase in performance at low temperature. They produce wines with higher glycerol content, reduced acetic acid production and lower ethanol concentration (González et al. 2006; Lopandic et al. 2007; Tronchoni et al. 2009; Masneuf-Pomarède et al. 2010; Paget et al. 2014).

These attractive features have promoted the interest of different research groups to create artificial hybrids in an attempt to increase the phenotypic variability of the yeast strains used to different biotechnological applications. *Saccharomyces* species share the same number of chromosomes (16), and have similar morphologic characteristics (Duina et al. 2014). They are defined as species by the biological concept and, therefore being postzygotically isolated. This allows the establishment of hybrid strains that typically will have alloploid genomes (usually being the combination of two diploid genomes). The main mechanisms to create these hybrid strains are: (a) spore to spore mating; (b) protoplast fusion (Curran and Bugeja 1996); (c) mass-mating (Nakazawa et al. 1999) and (d) rare-mating (Spencer and Spencer 1996) and its variant methodology HyPr (hybrid production), which involves the use of a set of plasmids converting the rare-mating into a frequent-mating event (Alexander et al. 2016; Peris et al. 2017). The different investigations that have being conducted in the study of artificial hybrids has also shown that these strains are unstable. Since hybrids have postzygotic barriers, they have to propagate by asexual reproduction. During this increase in number, the hybrid genome will be stabilized, usually by the loss of chromosomes from one of the parental species (Pfliegler et al. 2014; Steensels et al. 2014). An example of a hybrid with genome instability is the industrial wine yeast strain Vin7 (*S. cerevisiae* × *S. kudriavzevii*), one of the first commercial strains to be sequenced (Borneman et al. 2012). This strain can loss its copy of the

S. kudriavzevii chromosome III. The mitochondrial genome can also influence the stability of the hybrid genome. *S. cerevisiae* × *S. kudriavzevii* hybrid strains had a higher *S. kudriavzevii* contribution when possessing a *S. kudriavzevii* mitochondrial genome instead of the *S. cerevisiae* version (Peris et al. 2012c). Therefore, in order to use these hybrid strains, it is important to develop protocols for its genome stabilization, that could allow them to be commercially available. In this sense, it has been shown that adaptive evolution experiments may be a way to achieve this. Under the selection pressure of choice, this methodology stabilizes the genome of these hybrids and improves the key traits that were affected by hybridization (Pérez-Través et al. 2014a, b; Lopandic et al. 2016; Peris et al. 2017; Krogerus et al. 2018).

The combination of these two techniques, artificial hybrids breeding plus adaptive evolution, is one of the most promising tools to increase yeast variability for different industrial processes. Since these two methodologies are defined by the European Union as GM-free organisms, these strains can be commercialized and used by in the different industrial food processes.

5 Genetic Improvement by Random Mutagenesis

Random mutagenesis represents a classical mutagenesis approach useful to increase the rate of appearance of genetic mutations in a large population of cells. The introduction of genetic variability in a cell can be exploited for basic research, for the elucidation of protein structure-function relationships, and industrial sector, for proteins modification to improve or alter their characteristics thus generating improved phenotypes. In the latter context, the resulting mutant cells undergo to a higher general acceptance and possibility of commercialisation in comparison to genetically engineering products. The technique is simple and any prior knowledge about the genetic background of the investigated system is not mandatory, thus allowing for the unbiased discovery of novel or beneficial mutations. However, once mutants are generated, the problem then becomes how to isolate them from the treated population. It is clearly impractical to examine millions of microorganisms individually. Therefore, the isolation of mutants relies on a good screening system, better if direct, from which it is possible to fish out the desired mutant; usually, samples are analysed in cultural medium designed to allow the growth of the desired mutants only.

The induction of random mutations can be caused by agents that damage the DNA, called mutagens, that are of three main types: mutagenic chemicals, radiations and heat. When these agents are deliberately used on living cells, we talk about "*in vivo* mutagenesis"; alternatively, the mutagenic agent can be applied directly on purified DNA that has to be transferred into the living cell before to screen for the desired mutations (in vitro mutagenesis). Common mutagens are toxic compounds that can alter the chemical structure of the nucleotides in DNA. For example, EMS

(ethyl methane sulfonate) is widely used to mutagenize growing cells by introducing an ethyl group to bases in DNA; this event changes the shape and base-pairing properties of nucletides. Nitrite is used to mutate purified DNA because it converts amino groups to hydroxyl groups and, thus, converting the base cytosine to uracil. Radiations, such as high frequency electromagnetic radiation, ultraviolet radiation (UV light), X-rays and gamma rays (γ-rays), cause a direct damage on DNA. X-rays and γ-rays can react with water and other molecules to generate ions and free radicals, mainly hydroxyl radicals, or to interact directly with DNA; about 70% of the DNA damage is caused by hydroxyl radicals while the other 30% of the radiation damage is due to direct interaction of X-rays and γ-rays with DNA itself. X-rays tend to produce multiple mutations and often yield rearrangements of the DNA, such as deletions, inversions and translocations (Clark 2005). Ultraviolet radiation works within a wavelength from 100 to 400 nm. It acts directly on the DNA by forming dimers between two neighbouring pyrimidine bases that cross-react with each other. Thymine dimers are particularly frequent and at their level the DNA polymerase leaves a single-stranded region that needs repairing. The repair process can provoke the insertion of incorrect nucleotides in the synthesized strand, resulting in mutations.

Few studies on the use of random mutagenesis in wine yeasts are available in literature. They mainly refer to the genetic improvement of wine quality features that are linked to carbon, nitrogen and sulphur metabolism of wine yeasts, such as the production of acetic acid (Cordente et al. 2009), the nitrogen assimilation under oenological conditions (Salmon and Barre 1998) and the release of hydrogen sulphide (Cordente et al. 2013) in *S. cerevisiae*, and to secondary characteristics of wine like the generation of mutants overproducing mannoproteins (González Ramos et al. 2010; Quirós et al. 2010) or showing accelerated autolysis (Gonzalez et al. 2003; Nunez et al. 2005).

In the case of secondary characteristics an interesting example is the release of mannoprotein that can occur during the alcoholic fermentation of grape must in wine. These structural components of the cell have been recognized to impart beneficial properties of wine in terms of protein and tartaric stability, increasing perceptions of body and roundness, reducing astringency, retaining aromatic molecules and helping in the maintenance of sparkling wines. The increase of the release of mannoproteins represents an example of synergy between classical and genetic engineering approaches for the genetic improvement of wine yeast. Since mannoprotein overproduction cannot be directly selected, an overproducing mutant strain was first obtained by brute-force approach (González Ramos et al. 2010). In parallel, researchers identified *KNR4* inactivation as a potential genetic target for improvement, and found that strains deleted in some other interesting genes were killer-nine-resistant just as *KNR4* (Gonzalez-Ramos et al. 2009). This led to the development of a more efficient mannoprotein overproducing mutant selection scheme (Quirós et al. 2010).

6 Experimental Evolution of Wine Yeasts

Adaptive, directed or experimental evolution are all terms referred to engineering yeast strains in laboratory conditions by using the intrinsic mechanisms of adaptation of these microorganisms. The continuous growth for many cell divisions under a stress condition will select for individuals with improved fitness due to genomic changes (Kawecki et al. 2012). *S. cerevisiae* was the model organism when experimental evolution was proposed by Francis and Hansche (1972, 1973). The experimental setup was firstly though in a chemostat using continues culture but it can also be done in batch cultures keeping the conditions of choice for many generations or cell divisions. The probability of success in an experimental evolution assay varies depending on the starting population (Elena and Lenski 2003). For industrial purposes, usually, this population comes from an isogenic preferred strain to be improved under a given condition but, it can also be a population with genomic diversity. The starting variability will increase the chances of obtaining the desired phenotype. For this reason, experimental evolution is usually coupled to a first technique that increases the genomic variability of the starting population. This can be random mutagenesis, genetic engineering, hybridization events or genome shuffling of a heterogenic population by mass-mating. During the adaptation to the trait of evolution yeast cells can undergo different genomic changes. These changes can be at the genomic level with changes in the ploidy level, at chromosome level with chromosome copy number variation (CCNV), at gen level with changes in the number of copies of specific genes and also small changes at nucleotide level (base insertions, deletions or substitutions) (Dunham et al. 2002; Mangado et al. 2018).

In recent years this methodology has acquired great attention thanks to the last advances in sequencing techniques that allow to better understand which have been the different mechanisms that have taken place during the evolution experiment (Burke et al. 2014). The cheapening of the sequencing techniques allows not only to sequence several isolated clones at the end of the experiment but also to do it in several points for the whole population. This has allowed us to have a better follow-up of the different beneficial mutations that appear during the evolution and how they are fixed in the population. The technique has been improved in resolution, it has been showed that by tagging the whole population, each of the different beneficial mutations that arise in the population can be observed and not only those present in high frequency (Cvijović et al. 2018). Also, it has been improved in efficacy, mass scaled evolution experiments are platforms were millions of yeast clones undergo evolution independently and its performance can be followed by image analysis. This allows to cover a larger spectrum of the genomic landscape of evolution, identifying the mutations that occur in lower frequency but still contribute to increase the performance of the population under a certain stress (Zackrisson et al. 2016).

It must be taken into account that once the conditions of the evolution experiment have finished the evolved population could be unstable and lose its recently acquired fitness for a certain condition. It has been shown that some genomic

changes like aneuploidies can be beneficial under certain stress but to reduce fitness under normal conditions (Yona et al. 2012; Chang et al. 2013). Zhu et al. (2018) showed that even when no significant difference in growth can be observed in evolved aneuploid cells compared to the parental strain under regular conditions, changes in the number of chromosomes promote oxidative stress. This will drive the cell to lose again these aneuploidies back to regular ploidy levels.

In an oenological context, experimental evolution has been used to improve sugar consumption rates by growing yeast in wine-like fermentation conditions (McBryde et al. 2006), improved fermentation kinetics by cultivating in the presence of ethanol (Novo et al. 2014). There are also interesting examples where the growing conditions affect indirectly the metabolic target of study. Some of these examples are the reduction of ethanol yields by growing in gluconate as unique carbon source (Cadière et al. 2011) or in a hyperosmotic media (Tilloy et al. 2014). *Oenococcus oeni* has also being subjected to directed evolution in a multi-stressors environment in order to improve malolactic fermentation of the strain with satisfactory results (Jiang et al. 2018). It has been also used to understand the underlying mechanism behind the evolution of wine yeast strains under oenological conditions, showing that genomic changes occur at different genomic levels to accomplish the adaptation to a wine-like environment (Mangado et al. 2018). Although evolution experiments related to the wine industry usually involved one unique strain, Morrison-Whittle et al. (2018) recently showed how the co-evolution of *Candida glabrata* and *Pichia kudriavzevii* had a significant impact of on the production of metabolites that affect the flavour and aroma of experimental wines.

An important aspect of experimental evolution is that the microorganism resulting from this technique can be easily reach experiment the market, as in the case of Cadière et al. (2011), since they are considered non-GM organisms.

7 Genome Editing by the CRISPR/Cas9 Approach

Genome editing refers to a set of recent technologies able to modify DNA in a very precise way using programmable nucleases, including Zinc Finger Nucleases (ZFNs), TALENs (transcription-activator-like effector nucleases) and CRISPR (Clustered Regularly Interspected Short Palindromic Repeats) – Cas9 (CRISPR-associated protein 9) RNA-guided endonucleases (RGENs). These are useful in genome editing as they have specific endonuclease activity to a target sequence. ZFNs are proteins with a modular structure; each module, about 30 amino acids, with ββα structure, recognizes a single codon in an active site, where a zinc atom is present. Currently, there are modules for all 64 existing codons; thus, it is possible to form a protein with the desired modules with codons and selected sequences. Similar are the TALENs, endonucleases extracted from *Xanthomonas* with a modular and assembling structure; a domain of 33–35 amino acids recognizes a single base pair. More versatile for this reason than the ZFN but, however, limited by the necessity that the first recognized nucleotide is a thymine (Gaj et al. 2013). CRISPR/

Cas RGENs are considered more precise and versatile than the first two technologies that target specificity is determined by the modification of their DNA-binding domains. Moreover, being able to recognize the nucleotide sequence to be cut thanks to an association with a guide RNA (gRNA), the system "CRISPR/Cas9" can be customized by replacing the guide RNAs, making the system much more affordable and scalable (Kim 2016).

In general, technologies for genome editing exploit both the action of a nuclease to generate double strand breakings (DSBs) in the DNA at a desired point and the DNA repair mechanisms of the cell to insert a specific mutation at the cutting site. When the DNA is damaged on both strands with a DBS, the cell can rely on two repair methods: (i) non-homologous recombination (NHEJ – non-homologous end-joining), consisting of simple ligation of the two ends broken by the cut; (ii) homologous recombination (HR – homologous recombination) that allows the repair of the damaged genome site on the basis of a homologous fragment at the two ends where the cut occurred. While the NHEJ event can cause point mutations, or the insertion or deletion of some nucleotides, the manipulation of the nucleotide sequence at the cutting site by the HR takes place with the insertion in the cell of a 'donor' sequence, a fragment of double-stranded DNA that can provoke two types of changes (Kim 2016; Gratz et al. 2013; Mahfouz et al. 2014): (i) point mutations or non-sense mutations to induce an amino acid change or to insert a stop codon. In this case, the donor will be almost identical to the sequence in the proximity of the cutting site except for some nucleotides; (ii) insertion of a heterologous gene for its expression in a new organism. The donor will bring the gene sequence flanked by homologous sequences adjacent to the cutting site in order to trigger the recombination.

CRISPR elements were first discovered in *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance (Mojica et al. 1993; Mojica et al. 2005) even though repeated sequences with similar structure were already described in *Escherichia coli* (Ishino et al. 1987). They are also present in many other eubacteria and their role is to provide resistance against invading exogenous DNA such as that of bacteriophage or conjugative plasmids (Barrangou et al. 2007; Hryhorowicz et al. 2017; Ishino et al. 1987; Lander 2016). Foreign invading genetic material that is incorporated between CRISPR is transcribed and processed into CRISPR RNAs (crRNAs) (including both foreign and CRISPR repeat DNA). The crRNAs hybridize with transactivating CRISPR RNAs (tracrRNAs) and the resulting crRNA/tracrRNA complex acts as a guide for the endonuclease Cas, which cleaves invading nucleic acid sequences (Brouns 2012; DiCarlo et al. 2013).

The main elements of the CRISPR/Cas system are a bacterial CRISPR-associated protein nuclease (Cas) and a short guide of RNA. The type II CRISPR system is the most widely studied system and it exploits the Cas9 nuclease enzyme from *Streptococcus pyogenes*. The Cas9 contains two distinct endonuclease domains, a HNH domain and a RuvC-like domain, that independently cleave both stands at the target site to generate a DSBs. As far the RNA, it has alternately been referred to as a guide RNA (gRNA), a single-guide RNA (sgRNA) or a chimeric RNA (chiRNA). In the simplest form of the type II CRISPR system, Cas9 is guided by the gRNA to

a cleavage site; this latter is a specific DNA *locus* composed by 20 nucleotides and a protospacer adjacent motif (PAM). The PAM consists of a NGG sequence located at the 3′ end of the target sequence. The gRNA-Cas9 complex generates DSBs immediately before the PAM site on the target DNA (Ryan and Cate 2014) in the nuclease domains RuvC and HNH (Mahfouz et al. 2014). Finally, the DSBs in the chromosomal DNA are repaired with knockouts/deletions or knock-ins/insertion by NHEJ and HR (Gratz et al. 2013) (Fig. 10.3).

In 2012, the CRISPR/Cas9 system was used for the first time as a 'molecular machine'; this study shows that the engineering of the gRNA complex can guide the Cas9 to cut at a specific DNA sequence provided by an adjacent PAM sequence (Jinek et al. 2012). However, the attribution of the discovery of the CRISPR/Cas approach as a new technique for genome editing remains contested. Following the

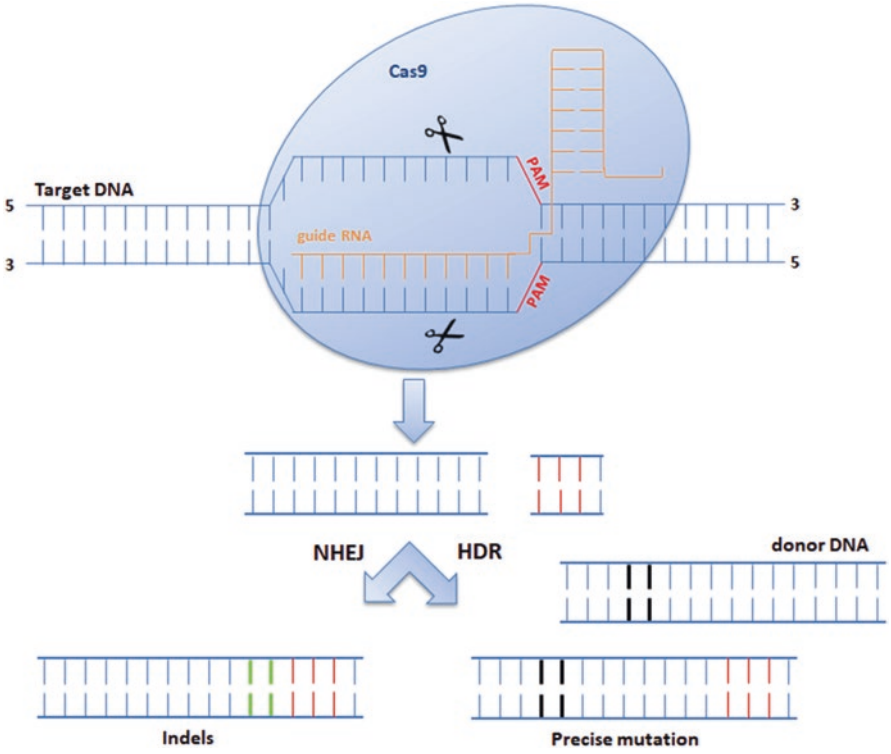

Fig. 10.3 Schematic representation of the CRISPR/Cas9 system. The Cas9 nuclease (blue circle) interacts with a guide RNA (orange lines) and the complex is directed to the target DNA at a specific locus adjacent to a PAM site (red lines). The presence of a PAM site adjacent to the targeted sequence (blue line, 3′–5′ direction) allow the cleavage at the *locus*. Double strand breaks (DSBs) between the third and the fourth bases 5′ to the PAM site on both strands of DNA are formed. After the endonuclease cleaves the DNA can be repaired by non-homologous end joining (NHEJ) or homologous recombination (Homology Directed Repair, HDR). Donor DNA: foreign DNA with flanking homologous regions to the target *locus*; indel: insertion/deletion

first publication, the CRISPR/Cas9 system has found wide use in various fields: the Cas9 has been modified and adapted to various organisms, including eukaryotes, thanks to the optimization of the codon usage. For example, a Cas9 firstly optimized for humans has been applied in the genome editing in yeast (DiCarlo et al. 2013). From 2015 the CRISPR/Cpf1 system is also applied. The Cpf1 nuclease works like the Cas9 but with some differences, offering different advantages: it recognizes a rich PAM (and therefore different from the NGG), is driven only by a crRNA and it cuts the two strands of DNA generating sticky ends (Zetsche et al. 2015; Verwaal et al. 2018). Transcriptional regulation, via the use of a nuclease deficient ("dead") Cas9 (dCas9) has been developed for repression of gene expression of endogenous genes (Gilbert et al. 2013; Jensen et al. 2017; Deaner and Alper 2017).

CRISPR/Cas9 is exploited today in the biotechnologies of every field, from the application on bacteria to that on eukaryotic cells: it is used on food-borne microorganisms, for the production of biofuels and other molecules of industrial interest, on fungi, on plants for genetic improvement of functional characters and to increase resistance to pathogens, on animals both for food and for disease carriers, as well as on some mosquitos, and finally on human cells and on humans, especially in the medical field, for the development of innovative gene therapies (Ledford 2015; Wang et al. 2016; Gorter de Vries et al. 2017; Fraczek et al. 2018; Alexander 2018). However, applications of CRISPR/Cas9 gene editing technology in *S. cerevisiae* are continuing developed (Giersch and Finnigan 2017).

The first application of the CRISPR/Cas9 system in yeast was reported in 2013 (DiCarlo et al. 2013). The Cas9 and the gRNA were expressed in the cell by two different plasmids, each with a different selectable marker. The cell was also co-transformed with a linear donor-DNA bringing the modified sequence for homologous recombination. A couple of year later, a single plasmid was developed, the pCRCT, which included the information for the Cas9, one or more guide RNAs and the corresponding donor-DNA (Bao et al. 2015). This last approach represents a great advantage in metabolic engineering studies; indeed, using a single Cas9 nuclease it is possible to modify multiple genes in the presence of several gRNAs, each carrying a target gene, and of the donors with the modified sequences to be inserted (Wang et al. 2016). There are at least four methods to modify multiple genes simultaneously using the CRISPR/Cas9 system (Stovicek et al. 2017): (1) construction of plasmids containing up to two gRNA expression cassettes with as many selectable markers; each cassette is first individually cloned into a plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t and then fused thanks to the Gibbson assembly (Mans et al. 2015); (2) HI-CRISPR system, involves the use of a single plasmid, the aforementioned pCRCT, containing the sequence of Cas9, plus the gRNA organized as an array in 'interspaced' crRNA and the respective donors (Bao et al. 2015); (3) co-transformation of cells, already equipped with Cas9, with multiple plasmids each with a different gRNA and a different selectable gene (Horwitz et al. 2015); (4) addition to a plasmid of a USER (Uracil Specific Excision Reaction) sequence which allow to include in itself tandem cassettes of gRNAs (Jakočiūnas et al. 2015).

The wine industry could particularly gain an advantage from this engineering system (Pretorius 2017); indeed, molecular studies should help understanding the

contribution of *Saccharomyces* and non-*Saccharomyces* species to several wine features such as those linked to wine quality and safety (i.e. aroma and off-flavours compounds, ethanol and glycerol production, sulphur dioxide resistance, toxic compound formation, etc.). Recently, Vigentini and co-authors successfully established the CRISPR/Cas9 system in two commercial starter yeasts of *S. cerevisiae* (EC1118, AWRI796), modifying the *CAN1* gene encoding for an arginine permease, in order to generate strains with reduced urea production (Vigentini et al. 2017). This can be useful to limit the formation of ethylcarbamate in wine, a carcinogenic compound in a number of mammalian species. In this study, the yeast strains carrying the *can1* mutation failed to produce urea in oenological conditions suggesting that the genetic modification could impaired the arginine metabolism with a consequent potential decrease in ethylcarbamate production. In the same year (de Trindade et al. 2017), a polygenic analysis (pooled-segregant whole-genome sequence Analysis) was combined with CRISPR/Cas9-mediated allele exchange approach in order to identify novel *S. cerevisiae* genes affecting the production of phenylethyl acetate (2-PEAc), a desirable flavor compound of major importance in alcoholic beverages imparting rose- and honey-like aromas. In particular, *FAS2* gene and a mutant allele of *TOR1* gene were found to be responsible for high 2-PEAc.

Thus, winemakers might benefit by the application of this new approach to yeasts and to grapes as well, enabling better understanding of the connections between wine features and wine yeast genetics. Nevertheless, the recent interpretation of genome editing in the EU GMO legislation by the ECJ (European Court of Justice) seems to close the door the CRISPR/Cas approach. Indeed, the court deliberates that genome editing fall under the techniques to obtain OGMs; this means that all genome edited organisms will have to comply with all provisions of the EU GMO legislation. However, the ECJ in its ruling does not consider all the risk-related issues opening the road toward possible criticisms. This decision could negatively affect the innovative research and development involving modern genetic engineering techniques such as CRISPR in Europe. Against the tide seems to be the Swedish Board of Agriculture (SBA) regarding the modified plants; indeed, SBA has recently debated that plants modified using CRISPR/Cas9 where DNA only has been deleted (and no exogenous DNA is inserted), should not be regulated as GMOs. This has opened up the possibility of producing such plants as “normal crops” (i.e. without the supervision of any authority) in Sweden.

8 Conclusion

An extensive repertoire of molecular tools is available for the genetic engineering of *S. cerevisiae* wine strains and some of them are working properly in non-*Saccharomyces* yeasts as well. In wine field, genetically modified yeasts can contribute to improve the winemaking process overall and the final characteristics of wines in terms of quality and complexity. Targets of these modifications are indeed several and they include traits of the primary and secondary metabolism of yeasts.

The success stories reporting the use of modified yeasts in the production of wines are numerous but sometimes the real exploitation of these microorganisms is limited. In fact, two different classes of microorganisms can be obtained with the molecular tools discussed in this chapter, non-GM and GM organisms. From a technical point of view, if the genetic background of the investigated yeasts is known, there are several advantages in using molecular strategies that edit specific genes because the desired modification can be easily achieved. Unfortunately, these approaches normally generate GMOs with all public concerns that can arise from their use in food production. On the other hand, non-GMOs are better accepted by consumers and they can be quickly introduced on the market. Among the promising techniques generating GM-free organisms, artificial hybrids breeding coupled with experimental evolution is of course a tangible chance for wine industry.

Modern viticulture and oenology could benefit by the CRISPR/Cas9 approach. Aside from the molecular advantage of producing quick genome changes by using a unique gene-editing approach, the CRISPR/Cas9 system has the potential to become soon the gold standard technique for the production of novel microorganisms suitable for the food industry. However, the scientific community needs actions to bring the EU regulatory framework in line with our current scientific understanding and with international developments.

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