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Genetically Modified Yeasts in Wine Biotechnology

*Cecilia Picazo, Víctor Garrigós, Emilia Matallana
and Agustín Aranda*

Abstract

Modern enology relies on the use of selected yeasts, both *Saccharomyces* and non-conventional, as starters to achieve reliable fermentations. That allows the selection of the right strain for each process and also the improvement of such strain, by traditional methods or approaches involving genetic manipulation. Genetic engineering allows deletion, overexpression and point mutation of endogenous yeast genes with known interesting features in winemaking and the introduction of foreign and novel activities. Besides, it is a powerful tool to understand the molecular mechanisms behind the desirable traits of a good wine strain, as those directed mutations reveal phenotypes of interest. The genetic editing technology called CRISPR-Cas9 allows a fast, easy and non-invasive manipulation of industrial strains that renders cells with no traces of foreign genetic material. Genetic manipulation of non-*Saccharomyces* wine yeasts has been less common, but those new technologies together with the increasing knowledge on the genome of such strains opens a promising field of yeast improvement.

Keywords: wine, *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts, genetically modified organisms, gene editing

1. Introduction

Wine production is a process that happening since the antiquity. For more than 7000 years there has been a continuing evolution in grape juice fermentation and wine production. Humans have used yeasts for wine production without any knowledge about them. Yeast cells were observed for the first time in a microscope in 1680 by Antoine Van Leeuwenhoek. Between 1850 and 1875, Louis Pasteur the role of yeast in alcoholic fermentation for the first time [1]. Grape juice fermentation is a complex microbiological process with a lot of microorganism interactions (yeast, bacteria, filamentous fungi) [2]. *Saccharomyces cerevisiae* has been identifying as the main microorganism responsible of the grape juice fermentation and the bacteria *Oenococcus oeni* as the one for malolactic fermentation that is important for some wines. But in the grape surface there are a lot of species of yeast, while *S. cerevisiae* is hardly found in the vineyard, although is a common resident in winery environments. Non-*Saccharomyces* yeasts contribute to the organoleptic complexity of wine, but are displaced by *Saccharomyces* species that are strong fermenters and are highly tolerant to ethanol [3]. Modern enology relies on the use of starters, generally in the form of active dry yeast (ADY). Select the yeast that you are going to use is

important to have a fast and complete fermentation, decrease lag phase and have a reproducible parameter in the final product [4]. Those starters have been isolated from many environments for their good performance, but they can be further improved by human action by different means.

In this chapter, we focused on the study of the improvement of wine yeast for wine production by recombinant technologies that produce Genetically Modified Organisms (GMOs). That allows a better understanding of the molecular processes relevant for wine yeasts too. We will describe the aspects that have been targeted for improvement, the new technologies of gene editing and synthetic biology and the potential use of these technologies on non-conventional yeasts.

2. Improving relevant winemaking aspects by genetic manipulation

The traditional ways of genetically manipulating yeast include gene deletion, gene overexpression under the control of heterologous promoters or the introduction of foreign genes [5]. The latter can be done using plasmids (both single or multicopy) or seeking a more stable chromosomal integration. Many different systems for modifying chromosome sequences inside cells have been created. A PCR-based gene targeting approach, that uses exogenous DNA introduced into the cell through various transformation methods, has become one of the most widely used. Selectable markers, sometimes involving antibiotic resistance are needed for validation and maintenance of integrated sequences. To eliminate those markers, scientists used a marker recycling approach that takes advantage of site-specific recombinase technologies. loxP-mediated Cre recombinase is a good example of this method [6]. Many characteristics of wine strains of *S. cerevisiae* can be improved by gene manipulation. We will focus on stress tolerance, nutrient optimization, sensory improvement and health enhancement.

2.1 Improving stress tolerance

S. cerevisiae must deal with different stress conditions, osmotic stress due to the high levels of sugars, oxidative stress, low nitrogen levels and high levels of ethanol among others. These stresses can produce problems in the wine fermentation process [7]. One way to solve these problems is to use engineering yeast strains that can grow better in these conditions.

The main component in the grape juice is monosaccharides (glucose + fructose) and their total concentration vary between 170 and 220 g/L [2] but can be up to 340 g/L. This extremes levels of sugars can inhibit yeast growth because of the osmotic pressure, that is called hyperosmotic stress. High Osmolarity Glycerol response (HOG) is the pathway that are regulated the response against osmotic stress, inducing the gene expression for glycerol production (*GPD1* and *GPD2*) and for glycerol uptake (*STL1*) [8]. Deletion of *Stl1* (glycerol symporter) has a slower growth in ice wine juice and elevate glycerol and acetic acid production, so these genes could be a target to improve these conditions [9].

Aerobic organisms depend on oxygen in cellular respiration but at high concentrations its oxidant power produces cytotoxic compounds called reactive oxygen species (ROS) that are unstable oxygen species with unpaired electrons that if they are not remove from the cell can damage macromolecules as DNA, proteins and lipids. It is during the active dry yeast production (ADY) where the yeast is in a higher oxidative stress condition. For example, oxidative stress-related genes (as thioredoxins, glutaredoxins and peroxiredoxins) are induced during this process [10]. Overexpression of the cytosolic thioredoxin 2 gene, *TRX2*, leads a wine yeast

increase biomass production [11]. This ADY process cause an internal oxidative stress and there are molecules and enzymes that helps to reduce the oxidative stress as glutathione (GSH), trehalose, catalase, superoxide dismutase and glutathione reductase [12]. For example, deletion of the main cytosolic peroxiredoxin, Tsa1, in the industrial wine yeast L2056 increase trehalose and glycogen accumulation playing a role in the regulation of metabolic reactions that are important for the final product [13]. Moreover, overexpression of superoxide dismutase 1 and 2 (*SOD1* and *SOD2*) and *HSP12* (a plasma membrane protein involved in maintaining membrane organization) genes improves cell formation and cell viability in three strains of Sherry flor yeast, and improve in the specific activities and higher levels of GSH peroxidase and glutathione reductase activities and higher intracellular concentrations of GSH and lower peroxidized lipid concentration [14]. Moreover, an indigenous strain of *S. cerevisiae* called RIA with the insertion thought homologs recombination of *ilv2Δ::GSH1-CUP1* improves glutathione production (19%) with the same fermentation capacity than the wild type [15].

At the end of the fermentation process, there are high levels of ethanol (11–14%). The toxicity of ethanol inhibited glucose and amino acid uptake because ethanol damage cell membranes [2, 16]. Overexpression of *TPS1* (synthase subunit of trehalase-6-P synthase/phosphatase complex) and deletion of *NTH1* (neutral trehalose) increase ethanol tolerance [17]. Besides, overexpression of *GSY2* (Glycogen synthase) and *NTH1* increased respectively glycogen and trehalose levels that are important for the fermentative capacity [18]. Global transcription machinery engineering (gTME) is a technique that alter key proteins to regulate the global transcriptome by error-prone polymerase chain reaction (epPCR) mutations. With this technique, a *SPT15* (TATA binding protein) mutagenesis strains was constructed with a higher ethanol tolerance [19] and it was found that the mutant of the *SPT8* (SAGA complex) gave 8.9% higher ethanol tolerance [20]. Direct evolution method was performed to engineer RNA polymerase II (RNAPII) subunit 7 (which plays a central role in mRNAs synthesis) in the yeast strain (M1) that improved ethanol titer and improved other stress as osmotolerance [21].

Some species of *Saccharomyces* genus have shown better adaptation at low temperatures than *cerevisiae*, which was the case of cryotolerant yeast *S. uvarum* and *S. kudriavzevii*. This better cold adaptation is because the higher amount of proteins related with translation (more ribosomes proteins in psychrotolerant strains) and the importance of the oxidative stress response in the adaptation of cold fermentation (mutants in *AHP1*, *MUP1* and *URM1* has a strongly impaired low-temperature growth) [22, 23]. Recently, Ying Su *et al* noticed that the hybrids low nitrogen-demanding cryotolerant *S. eubayanus* and *S. uvarum* conferred better fermentations rates under low temperature or low-nitrogen conditions [24].

2.2 Nutrient usage and fermentation performance

The right use of metabolites is key for a successful fermentation. One of the most important steps in the fermentation process is the hexose uptake. Overexpression of fructose/H⁺ symporter *FSY1* from *S. pastorianus* results in improve glucose and fructose uptake during wine fermentation [25]. Moreover, using a null hexose transporter mutant *HXT1* to *HXT7* of *S. cerevisiae* (KOY.TM6*P) and overexpression of chimeric *HXT1-HXT7* gene in this strain showed that there is a decreased ethanol production and increased biomass under high glucose concentration [8]. The first step of the glycolysis is depend on the role of cytosolic thioredoxins 1 and 2. The double mutant of these thioredoxins in the haploid wine yeast C9 (derived from commercial strain L2056) has a problem in the use of the sugars at the levels of the hexokinase 1 and 2 and in the glucokinase 1 that produce a slow fermentation [26].

One of the most important nutrients in the grape juice is the nitrogen and it could be a limiting nutrient for the growth of yeast because low levels of nitrogen can stop the fermentation when the sugars are still remained in the medium. *S. cerevisiae* cannot assimilate inorganic nitrogen nor polypeptides and proteins, so its grow depend on ammonium and free amino acids, called YAN (Yeast Assimilable Nitrogen). Concentrations below 140 mg/L of YAN in a normal sugar concentration, can produce negative effect in the fermentation process and nitrogen depletion irreversibly arrest hexose transport. One way to improve the nitrogen assimilation is through deletion of *URE2* repressor of alternative nitrogen sources as prolines. It controls the *PUT1*-encoded proline oxidase and *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase to create yeast that can efficiently assimilate the abundant supply of proline and arginine in grape juice [25, 27]. *MFA2* deletion (encoding mating factor- α) is another way to improve the fermentation efficiency under nitrogen limitation (75 mg/L). They used a deletion in the haploid wine yeast AWRI1631 under microvinification conditions [28]. Another work by Jin Zahng using a transposon library in wine yeast, selected five candidate genes to efficiently complete a model of oenological fermentation with limited nitrogen availability. They did the gene disruptions in the haploid wine yeast C911D where they found that the deletion of *ECM33* (GPI-anchored protein involved in efficient glucose uptake) resulted in the shortest fermentation (up to 31%) in grape juice and there were no differences in the nitrogen utilization, cell viability or biomass with the parental strain. This mutant has an up-regulation in the cell wall integrity regulated genes [29].

2.3 Increasing the quality of the wine

Understanding wine flavor compound composition is a key to improve the final product. Yeast metabolism during wine fermentation produce ethanol and secondary metabolites that are important for the wine. The generation of wine yeast able to produce wines with reduced ethanol concentrations while retaining harmonious balance between the level of alcohol, acidity, sweetness, and other sensory qualities has been the focus of extensive research. The main idea is to divert partially the carbon metabolism from the formation of ethanol to glycerol, but it is difficult to do it without a significant impact on wine quality, as acetic acid rises [30]. For example, overexpression of the main glycerol producing enzyme *GPD1* (NAD-dependent glycerol-3-phosphate dehydrogenase) together with the deletion of *ALD6* (aldehyde dehydrogenase) is able to decrease acetic acid production in the strain AWRI2531 and produce a fermentation with 15–20% less ethanol and more glycerol [31]. Reduction of 7.4% of ethanol without negative consequences was possible through the partial deletion of *PDC2* (transcription factor required for expression of the two isoforms of pyruvate decarboxylase *PDC1* and *PDC5*) [32]. Overexpression of *TPS1* (trehalose synthase gene) produce a 10% ethanol decrease [33]. NADH oxidase was expressed in *S. cerevisiae* so the NADH pool was reduce getting a 15% lower of ethanol but the redox reactions and grow was affected [34]. Decreases in ethanol levels was carry on by expression of *GOX1* (glucose oxidase gene) from *Aspergillus niger* [35]. Alternative, deletion of TORC1 pathway kinase *SCH9* in the haploid wine yeast C9, increase glycerol production during wine making conditions [36].

The most significant effect on the aroma of wine are acetate esters, ethyl acetate (fruity and tart aromas), 2-phenylethyl acetate (honey, rose) and isoamyl acetate (banana flavor) [37]. Increase these compounds in the wine is important to get a good final product. Overexpression of *ATF1* (alcohol acetyltransferase) got a significant increase in acetate ester production. Moreover, deletion of *ATF1* and *ATF2* abolished the formation of isoamylacetate but still produces ethyl acetate and

overexpression of esterase (*IAH1*) decrease significantly concentration of ethyl acetate and isoamyl acetate among others [38, 39].

Terpenoids or isoprenoids are naturally compounds which are involved in the fragrance and aroma of flowers and fruits. One way to improve the production of these positive compounds in the wine is using genes from species that produce this aroma. For example, using S-linalool synthase (*LIS*) from *Clarkia breweri* in *S. cerevisiae* produce a novo production of linalool in wine about 19 µg/L [40]. Through the expression of the *Ocimum basilicum* (sweet basil) geraniol synthase (*GES*) gene in the industrial wine yeast T73, Pardo *et al.* got an recombinant yeast which excreted geraniol de novo at an amount 750 µg/L that was further metabolized in other interested monoterpenoids and esters as citronellol, linalool, nerol, citronellyl acetate and geranyl acetate [41]. Expression and secretion of the *Aspergillus awamori* α-L-arabinofuranoside in combination with either β-glucosidase from *Saccharomycopsis fibuligera* or from *Aspergillus kawachii* in the industrial yeast VIN13 has higher concentrations of monoterpenoids and improve sensory characteristics [42].

Other volatile sulfur compound is hydrogen sulfur, H₂S, that has an undesirable 'sulfurous', 'rotten egg'-like off flavor even at low concentrations (1 µg/L) that it is a significant problem for the global wine industry. Reduced H₂S amount in the wine it is another improvement that can has beneficial effects for the wine. Specific site directed mutation in both *MET10* and *MET5* genes (α and β subunits of sulfite reductase enzyme) reduced by 50–99% the H₂S production depending on the strain [43]. Using the strain UCD932 a strain producing little or no detectable H₂S during wine fermentation was constructed and identified the allele of *MET10* (*MET10-932*) as a responsible. Replacing the *MET10* allele of high- H₂S producing strain with *MET10-932* prevented H₂S formation [44].

2.4 Improving human health

Yeast metabolism can be diverted to produce compounds that has specific influence in human health. This section will focus on two beneficial compounds for human health (resveratrol and hydroxytyrosol) and one potentially dangerous, ethyl carbamate.

Grape juice has a lot of polyphenols, one of them, resveratrol is a stress metabolite produced by *Vitis vinifera* grape vines and it is a potent antioxidant with multiple beneficial effects. Red wines contain a much higher resveratrol concentration than white wine, due to skin contact during fermentation [45]. In plants, resveratrol synthesis is from malonyl-CoA and *p*-coumaroyl-CoA by the resveratrol synthase. But in *S. cerevisiae* coenzyme -A ligase is absent, and it is necessary for the last steps of the resveratrol synthesis. In 2003, Becker *et al.* by co-expressing the coenzyme-A ligase gene (*4CL216*) from a hybrid polar and the grapevine resveratrol synthase gene (*vstl1*) resveratrol production was successfully for the first time. Introduction of 4 heterologous genes (phenylalanine ammonia lyase gene from *Rhodospiridium toruloides*, the cinnamic acid 4-hydroxylase and 4-coumarate coenzyme A ligase genes both from *Arabidopsis thaliana*, and the stilbene synthetase gene from *Arachis hypogaea*), overexpression of acetyl-CoA carboxylase gene (*ACC1*) and addition of tyrosine to the medium produced an increase in concentration of resveratrol up to 5.8 mg/L in *S. cerevisiae* laboratory W303-1A strain [46]. Moreover, two expression vector carrying 4-coumarate coenzyme A ligase gene (*4CL*) from *Arabidopsis thaliana* and resveratrol synthase gene (*RS*) from *Vitis vitifera* were introduced in the industrial yeast EC1118 [47]. This strain produced 8.25 mg/L of resveratrol. Indeed, resveratrol was produced with fed-batch fermentation directly from glucose (416.65 mg/L) and from ethanol (531.41 mg/L) [48]. With an optimization of the same strategy with the electron transfer to the cytochrome P450 monooxygenase,

800 mg/L of resveratrol was obtained [49]. Recently, a co-culture platform with two different species was used to produce 36 mg/L [50]. *Escherichia coli* excrete *p*-coumaric acid into the media and *S. cerevisiae* with an inactivation-resistant version of acetyl-CoA carboxylase (ACC1^{S659A,S1157A}) that modulate constitutively the expression of 4-coumarate-CoA ligase from *Arabidopsis thaliana* (4CL) and resveratrol synthase from *Vitis vinifera* (STS) to produce resveratrol.

Another polyphenol that has a strong antioxidant capacity is hydroxytyrosol (HT). It is found in extra virgin olive oil, less in wine (with a range between 0.28–9.6 mg/L). In yeast, tyrosol is synthesized from tyrosine through the well-established Ehrlich pathway. In bacteria, there are some ways to produce hydroxytyrosol using yeast genes. For example, co-expression of yeast *ARO8* and *ARO10* genes for an important accumulation of tyrosol when was added in the media. Moreover, co-expression of yeast *ARO10* and *ADH6* and the overexpression of the native aromatic hydroxylase complex HpaBC produce important amounts (647 mg/L) of HT in *E. coli* [51]. Recently, HT production (4 mg/L) was possible with the introduction of the *E. coli* hydroxylase HpaBC complex components (*hpaB* and *hpaC*) in laboratory BY4743 yeast strain and with the addition of tyrosol to the media [52].

Ethyl Carbamate (EC) is a toxic present in wines. During wine fermentation, *S. cerevisiae* metabolizes arginine (one of the major amino acid in grape juice) using arginase *CAR1* to ornithine and urea, but this urea is not fully metabolizing and is secreted. Urea degradation is an energy-dependent two-step process catalyzed by urea amidolyase (*DUR1*, 2 genes). The urea that is secreted by yeast to the media can react with the ethanol of the wine to form ethyl carbamate that is classified as probably carcinogenic for humans. With the overexpression of *DUR1* and *DUR2* under *PGK1* promoter, 89.1% less of EC was developed in Chardonnay wine [53]. Deletion of *CAR1* in the YZ22 strain blocked urea secretion and there is a reduction of EC production [54]. This fermentation results showed that the content of urea and EC in wine decreased by 77.89% and 73.78% respectively and no differences were detected in growth and fermentation parameters with the parental strain.

3. New technologies in genetic engineering

The traditional methods of genetic manipulation are time-consuming when dealing with industrial strains, as they usually have multiple copies for each gene. Gene editing by using the CRISPR-Cas9 technology is faster to cause multiple gene deletions and introducing punctual changes. New tools as genome editing and genome synthesis are building up a new era for the synthetic biology. Their application for yeasts of biotechnological interest will change the paradigm in the ways we approach the use of those microorganisms for a particular task, as their abilities can be tailored from the beginning to the end.

3.1 Wine yeasts genome editing by CRISPR-Cas9

Industrial yeast strains are usually diploid or polyploidy with a more complex genetic background than the well-studied haploid laboratory strains. Using a traditional PCR-based technique for the genetic manipulation of industrial strains is normally very time consuming, laborious and often even impossible [55]. In recent years, the development of an alternative genome editing approach, Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR–Cas9) system, can help to solve the problem [55, 56].

At first, CRISPR-Cas system was discovered to provide an immunological weapon for bacteria and archaea against the attack by viruses (bacteriophages) or

invading mobile genetic elements [57, 58]. The CRISPR system from *Streptococcus pyogenes* has been well characterized and it is still the most widely used in yeast genetic engineering. Two elements are necessary for the correct operation of the CRISPR-Cas system. The Cas9, a 160 kilodalton protein, is a RNA-mediated endonuclease that recognizes a 3-nucleotide protospacer adjacent motif (PAM), NGG (where N is any nucleotide, followed by two guanines (G)), and makes double-stranded breaks (DSBs) between the third and fourth nucleotides upstream to the PAM site. Another key component is a single guide RNA (sgRNA) that guides Cas9 to target sites. The sgRNA derives from a duplex of two RNA molecules: a CRISPR targeting RNA (crRNA), which is complementary to the target, and a trans-activating CRISPR RNA (tracrRNA). The first 20 base pairs at 5' end of crRNA binds to the complementary genomic target, and PAM site must be found immediately at 3' end of the desired locus in genome [59]. The sgRNA has a concrete secondary structure to recruit Cas9 to establish a functional complex. Following the guide of sgRNA, Cas9 target the genome specific sequence with PAM and cut double-strand DNA [60]. DSB must be repaired by cells via non-homologous end-joining (NHEJ) or homologous recombination (HR). Normally, NHEJ repair is considered to generate small nucleotide insertions or deletions, and HR is used for precise modifications with the existence of donor DNA (Figure 1).

CRISPR-Cas9 genome-editing technology was first applied in *S. cerevisiae* in 2013 [56]. Vigentini and co-authors successfully established the CRISPR-Cas9 system in commercial wine strains EC1118 and AWRI1796. In this study, *CAN1* gene encoding for an arginine permease was deleted, in order to generate strains with reduced urea production [61] (see above about EC). The resulting *can1Δ* mutants were characterized by decreased urea production (18 and 35.5% compared to EC1118 and AWRI1796, respectively) under micro-winemaking conditions, in Chardonnay and Cabernet Sauvignon grape musts. Recently, Wu et al. [62] use CRISPR-Cas9 system for over-expressing the *DUR3* gene in a previously engineered rice wine strain with *CAR1* gene disrupted and *DUR1,2* genes over-expressed [63]. *CAR1* encodes an arginase responsible for the arginine cleavage generating urea. Urea can be hydrolysed into NH_3 and CO_2 by urea amidolyase (encoded by *DUR1,2*), and *DUR3* encodes a transporter that transfers urea from the fermentation broth to yeast cells when nitrogen source is insufficient. A laboratory fermentation experiment of Chinese rice wine shows that the CRISPR-Cas9 engineered strain reduces urea and EC concentrations by 92% and 85%, respectively, compared with those of the original strain (N85).

In another work, a polygenic analysis combined with CRISPR-Cas9-mediated allele exchange reveals novel *S. cerevisiae* genes involved in the production of 2-phenylethyl acetate (PEA). PEA is a desirable flavor compound that provides alcoholic beverages a rose and honey aromas. With the mentioned approach, unique alleles of the *FAS2* gene (encodes de α subunit of fatty acid synthase) and a mutant allele of *TOR1* (growth regulator in response to nitrogen sources) were identified to be responsible for high PEA production. Then, using CRISPR-Cas9, wild type alleles were replaced with mutant ones in commercial wine strains. PEA production in these yeasts increased by 70% [64].

In a recent study, Walker and co-authors [65] used CRISPR-Cas9 system to introduce selected mutations in *SUL1* and *SUL2* genes in wine strains EC1118. These genes encoded two high-affinity sulfate transporters. Under nitrogen limitation, sulfate contributes to hydrogen sulfide (H_2S) production, a common wine fault with a rotten-egg odor. The introduced mutations affect protein-structure function of Sul1 and Sul2 and shown to reduce H_2S accumulation during fermentation in Riesling juice or a chemical defined grape juice.

Figure 1. Overview of the CRISPR-Cas9 system. The Cas9 interacts with sgRNA and form a complex. The Cas9-sgRNA complex binds to the target DNA sequence upstream of PAM site. The Cas9 protein cleaves DNA sequence complementary to the 20 bp guide sequence producing a double-strand break (DSB). After the nuclease cuts the DNA can be repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR).

In *S. cerevisiae*, glycerol is a key polyol that reduces osmotic stress and controls intracellular redox balance. Muysson et al. [66] established a CRISPR-Cas9-based genome-editing approach to investigate the Stl1p (a H⁺/glycerol symporter) role in ice wine fermentations. In this study, *STL1* gene was deleted in *S. cerevisiae* K1-V1116 strain. During ice wine fermentation, the *stl1*Δ mutant presents increased glycerol and acetic acid production compared to the original strain, suggesting that Stl1 plays an important role in these conditions. In a study carried out by van Wyk et al. [67], CRISPR-Cas9 system was used to increase glycerol and ester

production in the AWRI1631 wine yeast strain. First, two newly strains were created, one that overexpressed *GPD1* and the other that overexpressed *ATF2*. *GPD1* encodes a glycerol-phosphate dehydrogenase involved in glycerol formation; and *ATF2* encodes an alcohol acetyltransferase which promotes condensation between alcohols and acetyl-CoA resulting in more acetate esters produced, important for flavor in fermented beverages. Mating these engineered strains, the authors obtained a new strain that overexpressed *GPD1* and *ATF1* genes. Riesling wine from the resulting strain showed increased glycerol and acetate ester levels compared to the parental strain.

Vallejo and co-authors [68] described recently that nutrient signaling pathway genetic manipulation can be a good target of yeast performance improvement during winemaking. Using CRISPR-Cas9 system in commercial wine strain EC1118, *PDE2* gene encoding for a phosphodiesterase was deleted. Pde2 is a cAMP degrading enzyme whose deletion increases cAMP-dependent protein kinase A (PKA) activity. The resulting *pde2Δ* mutant showed increased fermentation speed compared to EC1118, in red grape juice. The results suggest that Pde2p inactivation is a way to increase fermentative performance.

3.2 Synthetic genome engineering

Synthetic biology seeks to standardize and modularize the design and engineering of organisms to achieve novel functions, or to construct genomes or even organisms from the ground up using rational laboratory procedures or automation [69]. Synthetic biology is regarded as the most exciting interdisciplinary science of the twenty-first century, with applications in yeast biotechnology and strain development, among other things. Given yeast's importance in the fermentation industry as well as its role as an experimental research model organism in the advancement of Synthetic Biology, the wine industry will be impacted by the outcomes of this field. Synthetic Biology techniques are already being applied to the production of better wine yeast strains [70, 71].

In 1996, the 14 Mb genome of a haploid laboratory strain (S288c) of *S. cerevisiae* was sequenced for the first time, revealing that its 16 chromosomes encode 6000 genes, of which 5000 are non-essential. In 2009, the first synthetic yeast genome project (Sc. 2.0 project) was launched to redesign and chemically synthesize a slightly modified version of the *S. cerevisiae* S288c strain genome. This project allows to find answers to a broad range of questions about fundamental properties of chromosomes, genome organization, gene content, RNA splicing mechanism, the role of small RNAs in yeast biology, the distinction between prokaryotes and eukaryotes, and genome structure and evolution [72]. The Sc2.0 genome was designed to contain specific base substitutions inside some of the ORFs to accommodate desirable enzyme recognition sites or deletions of undesirable enzyme recognition sites. All TAG stop codons were recoded to TAA to free up one codon for future inclusion of unusual amino acids; all repetitive and dispensable sequences were omitted; and all tRNA genes were relocated to a novel neochromosome.

In 2011, the first step toward building the ultimate yeast genome was taken with the construction of synthetic chromosome arms [73]. In 2014, *S. cerevisiae* became the first eukaryotic cell to be equipped with a fully functional synthetic chromosome, the chromosome 3 [74]. In 2017, six redesigned yeast chromosomes were completed [72]. In 2018, 16 natural chromosomes of *S. cerevisiae* were successfully fused into a single chromosome, like in prokaryotic cells, and the artificial *S. cerevisiae* still has normal cellular functions [75]. These works blur the lines between natural and artificial life, pointing to a near-future for custom-designed yeast to fulfill all the customers' needs.

Wine yeast strain development is well positioned to benefit from technological advances made with the genetic and genome engineering of non-wine strains of *S. cerevisiae*. For example, the first “synthetically engineered” wine yeast reveals a whiff of raspberries in an experimental Chardonnay wine. *S. cerevisiae* AWRI1631 wine strain was equipped with a biosynthetic pathway, including four separate enzymatic activities, to produce the highly desirable raspberry ketone (4-(4-hydroxyphenyl)butan-2-one) [76].

4. Genetic manipulation of non-*Saccharomyces* yeasts

Despite the increasing relevance of non-conventional yeast in modern enology, there are few examples and tools of genetic manipulation for those yeasts. The targets of modification are shared with *S. cerevisiae* strains and usually are devoted to an organoleptic improvement. Recently, Badura and co-authors [77] developed a tool for the genetic modification of *Hanseniaspora uvarum*. In the past, *Hanseniaspora* populations have been regarded to be spoilage yeasts due to some strains produce large quantities of acetaldehyde, acetic acid, and ethyl acetate. However, *Hanseniaspora* wine strains have oenological benefits such as lower final ethanol levels and higher acetate and ethyl ester concentrations. In this study, authors used a traditional PCR-based technique for the disruption of the *HuATF1*, which encodes a putative alcohol acetyltransferase involved in acetate ester formation. This approach introduces the first steps in the development of gene modification tools of this yeast.

Some *Kluyveromyces marxianus* strains are able to ferment sugars in high temperature environments (up to 45°C) including grape juice [78]. This yeast is also in some commercial preparations of yeast to contribute flavor complexity. In a study published in 2014, *K. marxianus* BY25569 strain was evolved and genetically engineered for overproduction of 2-phenylethanol (2-PE) from glucose [79]. 2-PE confers “rose” and “floral” scents, almost non-existent but interesting in winemaking. *Kluyveromyces lactis* is a kind of non-*Saccharomyces* yeast that aims to solve the problem of low total acid and high pH in wine, due to its high lactate production. In this direction, *K. lactis* was genetically modified by introducing a heterologous L-lactate dehydrogenase gene (LDH) and deleting pyruvate decarboxylase gene KIPDC1 and/or the pyruvate dehydrogenase (PDH) E1 subunit gene [80, 81]. With these modifications, the central carbon flux of *K. lactis* was diverged from the production of ethanol to enhance lactate production. *K. lactis* was also metabolically engineered for L-ascorbic acid (vitamin C) production [82]. On the palate, the wines with added ascorbic acid were perceived as less oxidized, less ripe and fresher. To achieve this aim, GDP mannose 3,5-epimerase (GME), GDP-L-galactose phosphorylase (VTC2), and L-galactose-1-phosphate phosphatase (VTC4) from *A. thaliana* were introduced in *K. lactis* CBS2359 strain.

Pichia pastoris has been described as one of the most popular and standard tools for the production of recombinant protein in molecular biology [83]. This fact can be exploited in the wine production field. For example, The EPG1–2 gene, which codes for an endopolygalacturonase in *K. marxianus* CECT1043, has been expressed in *P. pastoris* X33 strain [84]. The use of this endopolygalacturonase improves Albariño wine aroma, providing an increase of citric, balsamic, spicy and above all floral (violet and rose) aromas [85].

CRISPR-based genome-editing approaches have also been applied in many non-conventional yeasts. However, due to non-*Saccharomyces* species had been considered spoilage yeasts in wine fermentations, and CRISPR in wine yeasts still falls under the definition of GMOs of the European regulations, less progress has been

made in the field of fermented foods and beverages. Therefore, in non-conventional yeasts CRISPR-Cas9 system has been applied mainly in the production of biofuels, chemicals, nutraceuticals, enzymes or recombinant proteins [86, 87]. In *Pichia pastoris* (syn. *Komagataella phaffii*), CRISPR-Cas9 system has been applied to improve its efficiency for the production of high-value pharmaceuticals [88]; in *Ogataea polymorpha*, a thermotolerant methylotrophic yeast, for the production of bioethanol [89] or for the introduction of all the genes necessary for the biosynthesis of resveratrol [90]. For biofuels and chemicals production in *Issatchenkia orientalis* [91]; in *Kluyveromyces marxianus* for its use as cell factory [92, 93]; in *Kluyveromyces marxianus* for the production of recombinant proteins [94, 95], or for integrating a synthetic muconic acid pathway [96]; in *Schizosaccharomyces pombe* [97]; in *Candida* species for the production of xylonic acid and ethanol [98] or for biosynthesis of β -carotene and its derivatives [99]; and in *Yarrowia lipolytica* for the production of renewable chemicals and enzymes for fuel, feed, oleochemical, nutraceutical and pharmaceutical applications [100].

In a recent study, CRISPR-Cas9 system was applied in the AWRI2804 *Brettanomyces bruxellensis* strain [101]. This species has been described as the principal spoilage yeast in the winemaking industry. From the enological point of view, *B. bruxellensis* is known for its high resistance to ethanol and ability to survive in low-nutrient, low-pH conditions, allowing for long-term proliferation in winemaking processes [102]. Using CRISPR-Cas9 in combination with gene transformation cassettes tailored for *B. bruxellensis*, the authors were able to delete *SSU1* genes (conferring sulfite tolerance) and provide the means for targeted gene deletion in this species.

5. Conclusion

Coupling traditional molecular genetic techniques with, synthetic biology and genome edition based on CRISPR, can enable the rapid optimization of wine yeasts [70]. Even though the era of yeast synthetic biology began in *S. cerevisiae*, it is swiftly expanding to non-*Saccharomyces* yeasts. However, genetic engineering in these yeasts is more challenging and limited by a lack of sophisticated genome editing tools yet and an incomplete knowledge of their genomes, metabolism and cellular physiology [103].

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Cecilia Picazo, Víctor Garrigós, Emilia Matallana and Agustín Aranda*
Institute for Integrative Systems Biology, I2SysBio, University of Valencia-CSIC,
Paterna, Spain

*Address all correspondence to: agustin.aranda@csic.es

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