Review

Next Generation Winemakers: Genetic Engineering in *Saccharomyces cerevisiae* for Trendy Challenges

Patricia Molina-Espeja

Department of Biocatalysis, Institute of Catalysis, CSIC, Cantoblanco, 28049 Madrid, Spain;
patricia.molina@icp.csic.es

check for updates

Abstract: The most famous yeast of all, *Saccharomyces cerevisiae*, has been used by humankind for at least 8000 years, to produce bread, beer and wine, even without knowing about its existence. Only in the last century we have been fully aware of the amazing power of this yeast not only for ancient uses but also for biotechnology purposes. In the last decades, wine culture has become and more demanding all over the world. By applying as powerful a biotechnological tool as genetic engineering in *S. cerevisiae*, new horizons appear to develop fresh, improved, or modified wine characteristics, properties, flavors, fragrances or production processes, to fulfill an increasingly sophisticated market that moves around 31.4 billion € per year.

Keywords: genetic engineering; *Saccharomyces cerevisiae*; wine

1. Introduction

Open the bottle, fill the glass (the appropriate glass, the appropriate amount), smoothly remove it to oxygenate, observe the tears, close your eyes, smell it, and then, and only then, taste it. The ritual of wine. Looks simple, but behind these sensations there is a myriad of components that are continuously being sought for perfection. In the center of it all. *Saccharomyces cerevisiae*. This yeast is mainly responsible of the process of winemaking. As far as we know, humankind has been using *S. cerevisiae* for around 8000 years [1,2] although obliviously, for the production of bread, beer and wine. All along this time there has been a domestication of the yeast [3,4], although, again, without even being aware of it. Winemakers have been selecting for *S. cerevisiae* strains to improve flavor, fragrances, better performance, etc. It was 130 years ago that Emil Hansen at the Carlsberg Laboratory isolated for the first time the yeast responsible for beer fermentation, and his lead was followed by wine production [5,6]. Since then, and by the hand of biotechnological developments, such a "domestication" has experienced an intended boost. Now we know the chemical reactions that transform the sugars in the must into ethanol and CO_2 by alcoholic fermentation, we know metabolic reactions that *S. cerevisiae* performs in the meanwhile, and how those affect the final product. We also know the genome of our favorite yeast: it was the first eukaryotic genome in to be fully sequenced in 1996 (strain S288C) [7], and keeps being updated at the *Saccharomyces* Genome Database (www.yeastgenome.org). Since then, sequencing efforts have been shedding light on the history of our yeast. For instance the sequencing of 38 *S. cerevisiae* strains from different sources (laboratory, pathogenic, baking, wine, food spoilage, natural fermentation, sake, probiotic and plant isolates) were analyzed, including in the study the strain S288C as reference [8]; 235,127 single-nucleotide polymorphisms (SNPs) and 14,051 insertions/deletions (indels) were disclosed, while five lineages were identified. More recently, the analysis of around 1000 natural *S. cerevisiae* isolates found 1,625,809 SNPs and 125,701 indels, most of them grouped in 26 clades [9]. All this knowledge is put to the service of an industry that produced 292 million hL and moved 31.4 billion € in 2018 worldwide, according to the International Organization of Vine and Wine (OIV, www.oiv.int). Although there is an economic stability in this market, there are variations in

the interest put on wine by different countries. For example, France is lowering the production and consumption of wine, while China is an emergent country in this field (Figure 1).
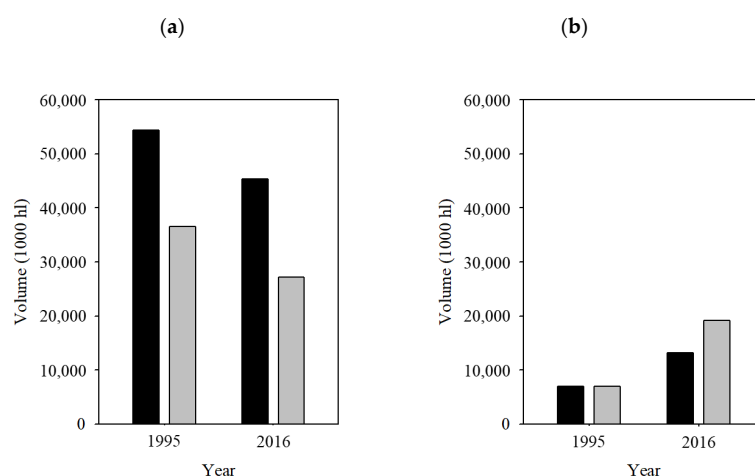
(a)　　　　　(b)

Figure 1. Differences from 1995 to 2016 in production and consumption of wine of two countries: (**a**) France is traditionally one of the largest wine producers and consumers of the world, and (**b**) China is an emergent country in this industry. Black bars: wine production; grey bars: wine consumption. Source: International Organization of Vine and Wine (OIV).

Biotechnology and *S. cerevisiae* walk hand in hand-in-hand numerous aspects of industry: from the use of the yeast as a factory to produce for, instance, insulin or bioethanol [10–14]; Its use to improve enzymes by directed evolution or rational design [15–17]; its relevance as a model organism in genetics, molecular biology and physiology [18,19]; or in synthetic biology, for instance, by the ambitious project Yeast 2.0 (www.syntheticyeast.org) [20], to name a few. In particular, the genome of the commercial strain EC1118 (Lalvin EC1118, also known as Premier Cuvee or Prise de Mousse) has been fully sequenced and is an essential player in the issue at hand, since it is commonly employed as a model for understanding the process and as a winemaking fermentation starter [21]. This review focuses on recent and relevant works in genetic engineering employing *S. cerevisiae* for the production of wine based on current traits to be improved, modified or added (Figure 2).
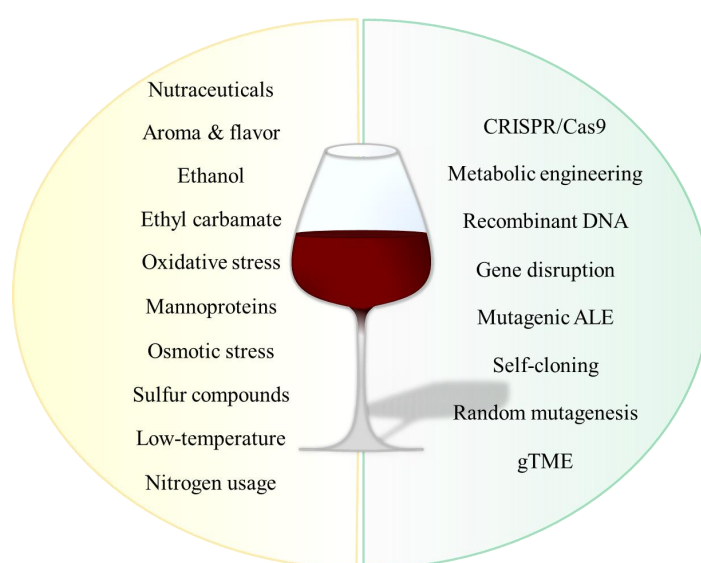

Figure 2. Genetic engineering of winemaking using *S. cerevisiae* as a tool and producer organism. On the left, the main traits targeted; on the right, the main genetic engineering techniques employed for this end.

2. Nutraceuticals

In past decades, several wine components have been targeted as beneficial for human health. Some of them are resveratrol, tyrosol, hydroxytyrosol, melatonin, serotonin, caffeic acid, tryptophol, glutathione or trehalose [22]. Here, focus is directed mainly to resveratrol and hydroxytyrosol, which show promising results.

2.1. Resveratrol

When stress appears due to fungal infection, UV radiation or wounds, molecules such as resveratrol (3,5,4′-trihydroxystilbene) are produced by grape berries, mainly in their skin [23]. To obtain red wine, the fermentation proceeds in the presence of skin, acquiring in the process values of up to 14.3 mg/L resveratrol [24]. In the case of white wine, 3- to 10-fold lower concentration of resveratrol is produced, since the fermentation occurs usually in the absence of the grape berry skin [25]. Beneficial effects of this polyphenol on health, even in micromolar amounts [26], include cardio-vascular protection, antioxidant and anti-inflammatory activity, anticancer properties, inhibition of platelet aggregation, lowering of blood glucose, protection against neurodegenerative diseases, obesity and osteoporosis, and also showed positive effects on fertility, lungs, visual, hepatic and renal systems [27]. It has been calculated that the intake of wine to reach therapeutic doses of resveratrol is, in the best case, 505 L per day [25]. To avoid such a hangover, approaches to increase the amount of this nutraceutical in wine have been tackled. For instance, resveratrol can be synthetized from malonyl-CoA and *p*-coumaroyl-CoA by the resveratrol synthase. Malonyl-CoA is already present in *S. cerevisiae* while *p*-coumaroyl-CoA can be formed from coumaric acid, also present in our yeast. However, this latter step needs the coenzyme-A ligase which is absent in *S. cerevisiae*. Thus, by inserting the coenzyme-A ligase gene from a hybrid poplar (4CL216) and the resveratrol synthase (RS) gene from grapewine (vst1) into yeast expression cassettes, and their expression in a laboratory strain of *S. cerevisiae*, resveratrol was successfully produced [28]. In another work, these genes (for 4CL, from *Arabidopsis thaliana* and RS from *Vitis vinifera*) were also included in the yeast strain EC1118, and in this case the culture media was supplemented with 1 mM *p*-coumaric acid. The outcome was a higher production of resveratrol when antibiotics (200 mg/L G418, 200 mg/L hygromycin) were added to the culture medium: 8.249 mg/L vs. 3.317 mg/L when absent [29].

In this line, the genes codifying phenylalanine ammonia lyase, cinnamic acid 4-hydroxylase, 4-coumarate: coenzyme-A ligase and stilbene synthase from different organisms were heterologously expressed in *S. cerevisiae* in order to fulfill and improve the metabolic pathway to resveratrol [30]. Since malonyl-CoA can be limiting, the GAL1 promoter (P_{GAL1}) was introduced instead of the native promoter of the acetyl-CoA carboxylase, resulting in an overexpression of the enzyme. All this, plus the supplementation with tyrosine, led to the production of 5.8 mg/L of resveratrol.

As an aside before continuing with our theme, it can be noted that throughout all the text, the format of genes and proteins can seem inconsistent. This is due to the different origins of the elements: the organisms of which genes and proteins are obtained belong to different groups that have different rules. For instance, regarding the format for yeast, the convention is to use upper case letters for genes and lower case letters for proteins. For bacteria, genes are written in lower case and proteins with initial upper case. Here, italics have been avoided for genes and proteins.

2.2. Hydroxytyrosol

The main source in diet of this phenylethylalcohol is extra virgin olive oil, followed by wine [31], where its concentration ranges from 0.28 to 9.6 mg/L [32]. Following the Ehrlich pathway, tyrosine is transformed into tyrosol during alcoholic fermentation by yeast [33], which is the hydroxytyrosol precursor. In this section the focus is directed to hydroxytyrosol since it seems to have stronger antioxidant power than tyrosol [34]. The higher amount of hydroxytyrosol reported in wine is 25 mg/L [35], while for tyrosol is 31.62 mg/L [36]. Possitive effects on health of hydroxytyrosol

have been listed as antioxidant, antiatherogenic and cardioprotective, osteoprotective, anticancer, antimicrobial and antiviral, neuroprotective, antidiabetic, lipid-regulating and antiobesity, as well as having beneficial results in inflammatory diseases, immune system, protective effect on UV-B in skin, and in renal hypoxia [34,37]. In sight of its potential, production of hydroxytyrosol has been promoted by introducing the hydroxylase HpaBC complex from *Escherichia coli* in *S. cerevisiae* [32]. The best results were 4.6 mg/L with supplementation of 1 mM of tyrosol while the control roughly reached 40 μg/L. A kind of "reverse" experiment consisted in expressing the genes codifying the ketoacid decarboxylase ARO10 and the alcohol dehydrogenase ADH6 from *S. cerevisiae* and overexpressing the native HpaBC complex in *E. coli* which led, together with media optimization, to a production of 647 mg/L of hydroxytyrosol from simple carbon sources [38]. Other studies have been centered in improving toluene monooxygenases (TMOs) by directed evolution and rational design to produce hydroxytyrosol via double hydroxylation of 2-phenylethanol [39]. Although this work is an example that uses *E. coli* as heterologous host, such approach could be transferred to *S. cerevisiae* wine strains, gaining in one step the production of the beverage and the nutraceutical. Other hydroxylative enzymes could follow this lead and be evolved to produce hydroxytyrosol from tyrosol such as cytochrome P450 monooxygenases or unspecific peroxygenases [40–42].

This interesting line of investigation on nutraceuticals can also take advantage of "waste" material from the winemaking process such as pomace or grape seeds, which accumulate a great amount of potential compounds with benefits for wellness [43]. Besides, tryptophol and tyrosol (together with 2-phenylethanol, amongst others) have been seen to be involved in quorum sensing in the yeast, which can open a useful line of investigation for improving wine fermentation, for instance, applying genetic engineering on the genes ARO8, ARO9, and ARO10, which are implied in the synthesis of these molecules [44,45].

3. Aroma and Flavor

In the course of yeasts performance towards wine, its action can influence the aroma composition (one of the most important characteristics of wines) [46] by de novo generation of primary or secondary metabolites, or by modifications such as the release of moieties of molecules whose "kidnapping" was hiding its scent [47]. We know how smell can influence flavor, and the best wine-tasters are capable of distinguish varieties and qualities of wine almost only with their nose. To innovate in this wine attribute, different strategies have been faced, such as has been recently reviewed [47]. Here, some interesting examples are depicted.

Aromatic thiols such as 3-mercaptohexan-1-ol (3MH) show grapefruit, passion fruit, and boxwood flavors, and can be produced along wine fermentation. *E. coli* tnaA gene codifies a tryptophanase with strong cysteine-β-lyase activity, which can produce 3MH from its non-volatile cysteinylated precursors Cys-3MH. This gene was cloned and overexpressed in a wine yeast strain under the control of the yeast constitutive promoter PGK1 (phosphoglycerate kinase I, P_{PGK1}). This modified strain was able to release up to 25-fold more 3MH under fermentation conditions than the control host strain [48]. In a similar approach, it was proved that the native Srt3p enzyme showed activity on Cys-3MH in fermentative wine processes. The commercial yeast strain VIN 13 was engineered to integrate an additional copy of the native STR3 *S. cerevisiae* gene under the control of the P_{PGK1}, in order to increase its expression [49]. The modified yeast strain released as a result 278 ng/L of 3MH in fermentation, an amount 27% higher than the control, while pH, glycerol, ethanol and organic acid production were preserved.

Another sensory molecule that has attracted interest is raspberry ketone [4-(4-hydroxyphenyl)butan-2-one]. This phenylpropanoid can be found in fruits, berries and vegetables, such as grapes, in quantities of 1–4 mg/kg. To generate this compound in *S. cerevisiae* with a dual purpose, to be a wine component or to industrially produce it (isolation and purification), a de novo pathway was generated [50]. The best combination and codon optimization for several genes (from different sources) was determined, and anaerobic/aerobic fermentations were carried out to prove

the fitness of the strategy. The genes codified coumarate-CoA ligase (4CL), benzalacetone synthase (BAS), phenylalanine ammonia lyase (PAL), and cinnamate-4-hydroxylase (C4H), which allow the production of the ketone from the aromatic amino acid precursors phenylalanine and tyrosine. After testing the strain in grape juice, 0.68 and 3.49 mg/L of raspberry ketone were produced under anaerobic and aerobic conditions, respectively.

Additionally, volatile compounds such as monoterpenes (C_{10} class of terpenes) can play a part on it, but *S. cerevisiae* lacks an efficient route to produce these molecules. For instance, linalool provides a sweet floral alcoholic note. By functionally expressing the S-linalool synthase from *Clarkia breweri* in our yeast, geranyl diphosphate (GDP, present in the yeast) can be converted into linalool in microvinification conditions at least at twice its minimum perception threshold, without affecting growth or alcohol tolerance [46]. In another approach, heterologous expression of a geraniol synthase (GES) in the *S. cerevisiae* wine strain T_{73}-4 in microvinification excreted ~0.75 mg/L geraniol de novo, an amount that surpasses 10-fold its olfactory threshold for perception [51]. Additionally citronellol, linalool, nerol, citronellyl acetate and geranyl acetate were detected as a further metabolization of geraniol of the yeast, with a final concentration of total monoterpenes concentration of ~1.56 mg/L, meaning a 230-fold increase. Monoterpenes have also been found to have antimicrobial, antiviral, anti-proliferative, antioxidative, anxiolytic, hypotensive or anti-inflammatory properties, having also an interest as nutraceuticals.

In some cases, acidic environments that are detrimental for yeast growth and lead to products with poorer taste, are required for the wine fermentation process. To try to solve this problem, *S. cerevisiae* yeast with higher tolerance to low pHs can be beneficial. To tackle this goal, atmospheric and room temperature plasma (ARTP) [52] to induce microbial mutation followed by high-throughput screening (HTS) and adaptive laboratory evolution (ALE) [53] were performed on *S. cerevisiae* ET008 for greengage plum wine production [54]. After 15 rounds of mutagenesis, five strains were selected which were in turn subjected to continuous cultivation with different pH values (3.0–2.5) for 90 days. The best survival rate at pH 2.5 was 95.5% which corresponded to ET008-c54, while for the parental it was only 9.73%. The main aromatic compounds were detected by GC-MS and the results indicated that the evolved strain ET008-c54 produced higher aromatic quality than the obtained in the parental; specifically, benzaldehyde is essential for the almond aroma in this type of wine, and its quantity in the new strain was twice that detected for the parental ET008.

A different approach to improve sensory qualities of wine is to carry out the fermentative process at low temperatures [55]. By culturing 27 commercial *S. cerevisiae* strains and introducing genetic variability by treatment with ethyl methanesulfonate, evolutionary engineering was performed [56]. After 200 generations at 12 °C, several strains reached twice the maximum OD (optical density) of the initial values and the full fermentative process was reduced from more than 720 h to 350 h. Metabolic changes detected as crucial for these variations were related to inositol and mannoprotein production.

The famous Clustered Regularly Interspaced Short Palindromic Repeats and associated protein nuclease system, CRISPR/Cas 9 in short, provides adaptive immunity against foreign nucleic acids to bacteria and archaea with the need of RNA-guided nuclease activity [57], and is broadly used as editing tool for researches, also in *S. cerevisiae* (Figure 3) [58–60]. The magnitude and relevance of this technique has been recognized with 2020's Nobel Prize in Chemistry to Emmanuelle Charpentiere and Jennifer A. Douda (not less important is the work of Francisco Mojica, who discovered the basis of CRISPR). In a mixed properties approach, CRISPR/Cas9 was employed to overexpress the alcohol acetyltransferase 1 (ATF1, whose proteic product participates in acetate ester production) in *S. cerevisiae* AWRI 1631 [61]. The original promoter was substituted by a stronger one, specifically P_{TEF1}. When fermentations were performed, the production of acetate ester (related to aroma) in the engineered strain spiked, mainly for isobutyl acetate with a ~15-fold increase. Since there is no foreign genetic material, the use of this strategy can ease the way to commercialize yeast for wine production with improved or ad hoc characteristics.
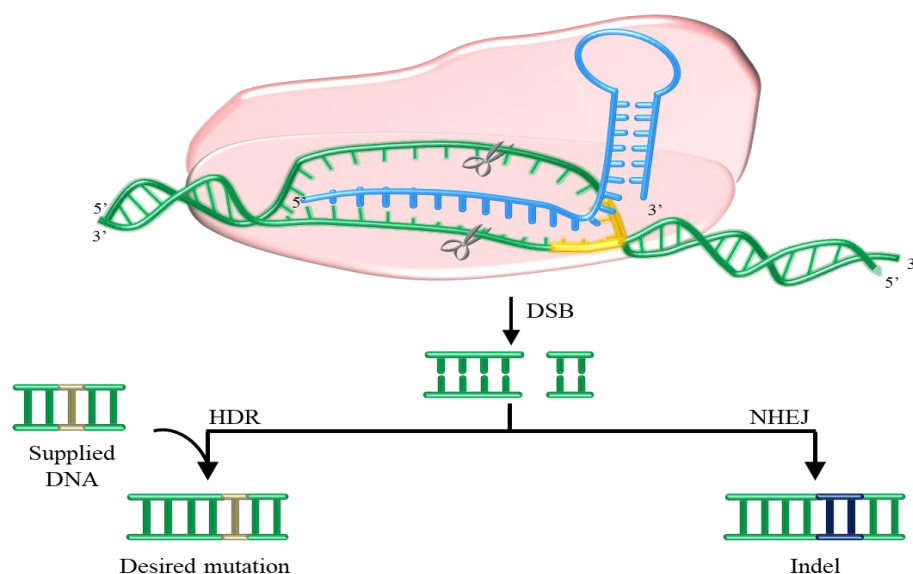
Figure 3. Scheme of the CRISPR/Cas9 editing mechanism. Cas9 protein (pink) and gRNA (light blue) get associated to the target DNA (green). By its nuclease activity, Cas9 produces a double-strand break (DSB) in the target sequence, guided by the PAM (protospacer adjacent motif, yellow). Then, the cell needs to repair the damage, by homologous direct repair (HDR) which can introduce controlled mutations by supplying a donor DNA, or by non-homologous end-joining (NHEJ), producing insertions or deletions (Indel).

Dzialo and co-workers [62] have reviewed the principal pathways and genes related to aroma compounds formation that are present in yeast, which can be a sort of guide to target future approaches in wine engineering.

4. Ethanol

The concentration of ethanol influences several aspects of wine, not only regarding the process itself, in relation with the tolerance of the yeast (inhibiting growth when reaches ~110 g/L at 30 °C) [63], but also with the volatility of aroma compounds [64]. Of course it is also a matter of consumer preferences: some like it with the usual graduation, some like it "light" to keep enjoying a glass of wine in a healthier way. Additionally, there has been an increase in alcohol concentration of wine of ~2% (*v/v*) during the last two decades [65], due to climate change and a trend to late harvest in order to obtain wines with proper aromatic and phenolic maturity [66]. A higher alcohol graduation also implies higher taxes and regulation issues. In this section, approaches to vary the concentration of ethanol in the final product are reviewed.

4.1. Lower Ethanol Content

In order to produce wine with a reduced percentage of ethanol, redirection of the carbon flux during fermentation to give rise to different molecules than ethanol has been a recurrent approach. For instance, by introduction of a lactate dehydrogenase in yeast, pyruvate can be converted into ethanol and lactate [67]. However, these strategies create a metabolic imbalance that can affect the final quality of the wine [68]. To correct this detrimental side effect, further genetic engineering can be planned, such as the deletion of ALD6, encoding aldehyde dehydrogenase, to decrease acetic acid production [69] and overexpression of BDH1, encoding 2,3-butanediol dehydrogenase, to diminish acetoin generation [70]. Another work joined overexpression of glyceraldehyde-3-phosphate dehydrogenase gene (GPD1) and deletion of ALD6 to give rise to a *S. cerevisiae* strain, AWRI2531, which carried out fermentation with 1.5% *v/v* less ethanol than its parent [71]. Again, however negative effects in the quality of the beverage were generated. To avoid it, transcriptomics, proteomics proteomics and metabolomics were taken

together so the main volatile metabolites and the genes implied responsible for the loss of good traits were identified. With this integrative approach gene remediation was achieved, and the results can be useful for future wine developments [72].

Another appropriate plan could be to divert the carbon flux to several metabolites. In a yeast engineering work, a NADH oxidase was expressed in *S. cerevisiae* so the NADH pool in the cell was reduced, which affected several redox reactions involved in various metabolic pathways [73]. The ethanol content was 15% lower than in the wild-type strain but its growth abilities were altered because of the accumulation of high levels of acetaldehyde. To solve this inconvenient, oxygen was supplied just during the stationary phase, with a 7% reduction in the ethanol content, but avoiding the negative effects in growth and fermentation [74].

A *S. cerevisiae* mutant strain named BY4743pdc2Δ519 was developed to reduce alcohol content in wine [75]. In this case, the performance of the pyruvate decarboxylase was modified by partial deletion of the gene PDC2, which codifies a transcription factor influencing its activity. The result was a reduction of 7.4% in the final ethanol content in lab scale-vinifications, while no negative effects were detected regarding residual sugar or volatile acidity. Moreover, when tested in a native and a commercial strain, the reduction achieved was 10% and 15%, respectively.

The reduction of the amount of glucose to be converted to ethanol in the fermentation process can be also taken as a strategy for reducing ethanol content. A way for this goal is to introduce in *S. cerevisiae* the gene encoding a glucose oxidase that leads to the production of H_2O_2 and gluconic acid [76]. This approach is limited for the low presence of oxygen demanded for the fermentation process and the fact that accumulation of gluconic acid is detrimental for the final quality of wine.

Other genes implied in trehalose biosynthesis, central glycolysis, the oxidative pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle were noted to be potential targets for genetic engineering with the aim to obtain lower alcohol wines [77].

4.2. Higher Ethanol Content

For those wines requiring a higher content of ethanol, yeasts with increased tolerance towards this compound are beneficial. For instance, Chinese rice wine can reach 20% (*v/v*) ethanol, which negatively affects growth and metabolism, having as a consequence the sluggishness of the culture. In an adaptive laboratory evolution experiment aided by chemical mutagenesis with ethyl methane sulfonate, *S. cerevisiae* G85 was cultivated in sequential batch growth for 240 generations [78]. G85X-8 showed a death rate of 41.3% while for the parent was 93.5%, and the ethanol concentration reached in the wine fermentation process was 130 g/L vs. 126.6 g/L achieved for the wild-type. This strain was also found to display better tolerance to osmotic stress and temperature without significant differences in the final product. This effect was attributed to a higher presence of oleic acid and linoleic acid and lower proportions of myristic acid, a higher ratio in the plasma membranes of C_{18}/C_{16} fatty acids, and a higher percentage of trehalose for the mutant G85X-8 compared with the wild-type.

In some cases, to achieve a determined phenotype in an organism, modification of one gene is not enough since multiple genes can be involved. Thus, by global transcription machinery engineering (gTME), transcription levels of several genes can be reprogrammed to obtain the desired traits [79]. In this sense, directed evolution of gTME has been used to increase ethanol tolerance in the *S. cerevisiae* wine strain Y01 [80]. SAGA (Spt-Ada-Gcn5 acetyltransferase) is a complement complex involved in transcription in *S. cerevisiae* which contains two TBP (TATA-box binding protein)-associated factors, SPT3 and SPT8 (suppressor of ty insertions) [81]. Mutations in SPT8 reduced cell vitality and increased cell death. A library was created by error prone PCR on SPT8 and screened with up to 16% ethanol. The best variant had an increased viability of 8.9%. Gene transcription analysis by real-time PCR revealed that SPT8 expression was 5.3-fold higher than in the original Y01.

5. Ethyl Carbamate

Ethyl carbamate (EC) is present in wines and since it was classified as probably carcinogenic for humans by the International Agency for Research on Cancer, reducing its content is rather convenient. Regulations worldwide have determined the maximum levels allowed in alcoholic beverages ranging from 30 μg/L in wine to 1000 μg/L in fruit spirits (European Food Safety Authority 2014:EN-578). Arginine is one of the most abundant amino acids in grape musts, and *S. cerevisiae* can transform it by an arginase, codified by CAR1, to ornithine and urea [82]. Then, urea can be metabolized by a yeast urea amidolyase encoded by the gene DUR1,2 [83] or it can chemically react with ethanol to form EC. Since arginine is the major source of EC (although not the only one), it is an interesting target for this goal. For instance, by overexpressing DUR1,2, a yeast with 89% less EC was developed [84]. In another recent engineering work, an arginase-deficient recombinant strain YZ22 was constructed by deleting CAR1 gene, which was able to reduce the formation of EC by 73.78% while its formation along storage was also slowed down, and no differences were detected in the growth and fermentation parameters with the parental strain [82].

Citrulline is a precursor of arginine, so its metabolism could also affect EC content. To determine if altering citrulline metabolism will reduce EC content, *S. cerevisiae* N85 strain was genetically engineered by disruption of ARG3 gene (its enzymatic product synthetize citrulline from carbamoyl phosphate and ornithine) or over-expression of ARG1/ARG4 genes (encoding two enzymes that transform citrulline in arginine) [85]. Unfortunately, the results in this case showed that the strategy followed is not appropriate to reduce the formation of EC during Chinese rice wine fermentation. We can take this case as an example of how intricate is the metabolism along the process and of the complexity of the task.

The CRISPR/Cas9 tool has also been exploited to reduce EC in wine by reducing urea in two yeast strains, EC1118 and AWRI796. CAN1 plasma membrane arginine permease is involved in the arginine degradation pathway that leads to urea production. This gene was eliminated by CRISPR/Cas9 and both strains showed a reduction in its capability to produce urea of 18.5% and 35.5%, respectively [86]. When wine production conditions were mimicked, again both engineered yeasts failed to produce urea while fermentation was completed after 8–12 days. The advantages of CRISPR/Cas9 were also used to further engineer a previously genetically manipulated yeast strain in which overexpression of DUR1,2 was carried out. DUR3 was also overexpressed giving rise to a yeast strain with a higher capacity to use urea, reducing its amount in 92% and avoiding in this way the formation of 58.5% EC [87].

The ALE strategy has also been used in order to diminish the amounts of urea in wine. *S. cerevisiae* XZ-11 was cultivated along 360 rounds of transfer with urea as the sole nitrogen source to apply selective pressure towards its utilization [88]. A selected strain was able to decrease the residual urea concentration to a 43.9% when using 0.6 g/L in the initial medium, and 47.9% when wine fermentation conditions were applied. For EC, the levels were 40.5% lower with this variant than those for the parental type, all of this without varying the volume percent of ethanol in the final product. The variant also showed high genetic stability after six generations. Sequence analysis revealed fast evolving genes such as AAT2, (transformation of α-ketoglutarate to glutamate), PRO1 and ARG2 (both involved in the conversion of glutamate to ornithine).

Can1p, Gap1p and Alp1p are amino acid permeases involved in arginine intake into the cells. In another engineering work, seven combinations of arginine permeases gene disruption were tested. When the mutant strains were grown in yeast nitrogen base plus the 20 amino acids, those with the disrupted CAN1 gene reduced by 80% the concentration of extracellular urea [89]. When the broth used was YPD, more similar to wine fermentation, the variant with disrupted CAN1 and GAP1 genes had a 68% lower extracellular urea. Moreover, since Gln3p is a nitrogen catabolite repressor (NCR) regulator acting in the activation of NCR-sensitive genes transcription [90], a truncated Gln3p was overexpressed in the different variants, and those with disrupted GAP1 + CAN1, and ALP1 + GAP1 + CAN1 reduced to a higher extent the presence of extracellular urea (67% and 32%, respectively).

6. Other Oenological Traits

6.1. Oxidative Stress

One of the molecules that helps to reduce the oxidative stress is glutathione (GSH), together with other molecules and enzymes (such as trehalose, catalase, superoxide dismutase, and glutathione reductase) [91], which lengthens the preservation of the precious wine properties. By performing random genetic recombination of 69 clones of the *S. cerevisiae* strain UMCC 855, a GSH high-producer variant was disclosed, which in turn showed a strong fermentative aptitude [92]. In a similar approach, two evolved strains were found that increased GSH presence in wine of by up to 100% compared with the parental strain [93].

Proteins like superoxide dismutases Sod1p and Sod2p, and Hsp12p play a part in preventing oxidative stress, so overexpression of the three genes SOD1, SOD2, and HSP12 was carried out in three *S. cerevisiae* strains, leading to variants with improved specific activity and higher levels of GSH peroxidase and reductase activities, together with a higher intracellular GSH content and lower peroxidized lipid concentration [94]. These strains developed the velum faster and it was of a higher quality, which can speed up metabolism and wine aging, lowering the period for wine maturation, an interesting improvement for reducing expenses.

6.2. Mannoproteins

Proteins with a high content in mannose-type sugars are known as mannoproteins (30% peptides, 70% sugar residues, of which 98% are mannose) and are found in yeast cell walls. When wines are aged in lees, components of the cells are incorporated to the product, for instance by autolysis, providing different qualities. Mannoproteins are of important influence in this fact, and have been proved to be implied in increasing the wine heat and cold stabilities, aid the growth of malolactic bacteria, influence aromatic contents of wine, have effects on organoleptic characteristics such as gustatory sensations of texture, roundness and mouthfeel, and are beneficial for the formation and stabilization of sparkling wine foams [95]. Since Knr4p is involved in coordinating the synthesis of the cell wall and the emergence of the bud, the effects of deletion of KNR4 in *S. cerevisiae* have been tested with regard to mannoprotein occurrence in wine making. An increase in the freed mannoproteins was observed in the strain EKD-13 (obtained from EC1118) [96,97]. Further analysis suggested that neither autolysis nor overexpression of mannoprotein codifying genes were responsible of this phenotype, but the inability of Knr4p to perform its coordination function leading to a failure in the anchoring of the mannoproteins to the cell wall [98].

6.3. Osmotic Stress

Some juices, such as icewine juice, can have up to 340 g/L of sugars [99], producing hyperosmotic stress in *S. cerevisiae* during wine fermentation. To fight it, yeasts can produce glycerol or take it from the extracellular environment by H^+/glycerol symporter Stl1p [100]. In order to determine Stl1p's effect in such situations, CRISPR/Cas9 was used in the commercial yeast K1-V1116 to eliminate the full open reading frame of its encoding gene [101]. When this modified yeast was cultured in icewine juice, the growth was slower and less biomass was achieved than in the case of its parental; additionally, elevated glycerol and acetic acid production was detected, showing how Stl1p is involved in osmotic stress and signaling this symporter as a target for improvement of strains in this conditions.

6.4. Sulfur Compounds

Sulfur dioxide, SO_2, is usually added to the wine fermentation process because of its antioxidant properties and in order to avoid the growth of unwanted microorganisms from the starter culture that can spoil the process [102]. This molecule dissociates into bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) once it enters a cell [103], and their negative effects in the yeast include depletion of ATP, damage of the plasma membrane, proteins, vitamins and coenzymes [104–106]. Legal levels of SO_2 are limited to up

to 400 mg/L, a concentration that some spoilage microorganisms can tolerate [107]. The sulfite efflux pump Ssu1 is known to have a key role in *S. cerevisiae* defense towards sulfite [108,109], and recently, the transcription factor Com2 was analyzed for this function. The *S. cerevisiae* strain BY4741 was used as a parental type to create a COM2 deleted mutant [107]. Transcriptomics and genome-wide phenotypic analyses were conducted that showed that Com2 controls the expression of more than 80% of the genes activated by SO_2 and the cell viability is compromised. These results suggest that protection against SO_2 includes the sulfate reduction pathway genes and most of the genes employed in lysine biosynthesis and arginine. This work also demonstrated that lysine supplementation can diminish toxic effects of SO_2 in wine yeast.

The CRISPR/Cas9 technique has also been applied to efficiently engineer uniquely targeted and multiple reciprocal translocations, in order to characterize the implication of structural variants in phenotypic diversity regarding different conditions and genetic background [110]. This work disclosed the fact that when a translocation found in wine isolates (SSU1/ECM34, from a recombination event between four base-pair micro-homology regions on chromosomes VIII and XVI) was reproduced in the laboratory strain BY4741, sulfite resistance was lower in the laboratory strain than for the wine isolate. Promoter repeats at the SSU1 gene were added to the engineered strain, showing an increased resistance, but still lower than that showed by wine isolates. This is an example of how tricky it can be to transfer conclusions between laboratory and wine yeast strains and conditions.

Regarding H_2S, its apparition during wine fermentation is due to *S. cerevisiae* metabolism, with the consequent unwanted off-flavors. In a highly successful work, mutations were introduced in MET10 and MET5 genes (encoding α- and ß-subunits of the sulfite reductase enzyme) in the commercial wine yeast PDM, resulting in a H_2S generation reduced up to 99% in synthetic and also in grape juice fermentations, without detecting other differences in chemical composition [111].

6.5. Low Temperature Fermentation

As it has seen above, carrying out the fermentation of the must at low temperature is a proper strategy to improve organoleptic properties of the final beverage, but this condition can also help the yeast when facing low pH, high osmotic pressure, or low nitrogen accessible in grape must [112]. The psychrotolerant *S. cerevisiae* AXAZ-1 strain has been used to investigate and develop intense aromatic wines [113,114], while it represents an interesting prospect for genomic analysis to elucidate the mechanisms responsible of such an advantageous capacity. In an effort to elucidate genetic determinants involved in good performance at low temperature, quantitative trait loci (QTLs) were mapped in the *S. cerevisiae* commercial strains P5 (Lalvin®ICVGRE) and P24 [115]. By crossing the two of them, populations of segregants were obtained in 13 generations, the results pointing at regions in chromosomes XIII, XV and XVI (subtelomeric regions) and in chromosome XIV as responsible for low temperature adaptation, opening new possibilities for genetic engineering. In another approach, QTL on chromosome I was spotted as related to the maximal fermentation rate at low temperature, and the FLO1 gene was found to be involved in this effect [116]. Additionally, genes involved in translation efficiency have been disclosed as responsible of limiting cold adaptation [112], while the study of knockouts in *S. cerevisiae* BY4743 pointed at AHP1, MUP1, and URM1 genes as implied in low temperature growth capacities [117].

6.6. Nitrogen Usage

Another essential target for the betterment of the final product and the efficiency of the process is the nitrogen consumption by *S. cerevisiae*. Although this element is present in the grape juice, it commonly acts as a limiting factor since not all nitrogen sources are well assimilated by the yeast, which causes low biomass and sluggish or stuck fermentative process [118]. In this regard, a term to have in mind is YAN (Yeast Assimilable Nitrogen), which is usually formed by amino acids (mainly glutamine, glutamate and asparagine) and ammonium [119]. The methods employed to avoid this handicap are the fertilization of the grapevine or the addition of nitrogen sources to the

must, both of them having negative consequences in the costs and the production of off-flavors and toxic compounds such as EC [119]. Biotechnology and genetic engineering, once again, come to the rescue in this matter. In selection experiments in which SGRP-4X population was employed (four strains of yeast lineages—North American, Sake, West African and Wine European—were outcrossed for 12 generations giving rise to the SGRP-4X population, ~10–100 million random segregants) the ECM38 allele from the North American strain was detected as the effector of the best growth rate when nitrogen-limited conditions were used [120]. The TORC1 pathway has been seen to act as the main mechanism in nitrogen-limiting adaptation (amongst other genetic elements), which situates the genes involved as interesting targets for modifications to this end [118,121–125].

7. GMOs and Non-GMOs

When taking in consideration the real commercial implementation of all these and other advances, a barrier arises: engineered yeasts are usually considered genetically modified organisms (GMO) and legal issues impede their use. To the best of our knowledge, so far only two strains have been allowed for commercial implementation [84,126], although they are not extendedly used. In the European Union, Regulation (EC) 1829/2003 sets the legislation on genetically modified food and feed.

The laboratory created yeast strains that are not subject to these specific and complicated regulations are those obtained by random mutagenesis, interspecific and intraspecific genetic hybridization, rare mating, or experimental evolution (ALE) [5]. In the case of the so-called self-cloned organisms (without heterologous DNA, e.g., promoter usage), yeast strains were allowed for use in beverage production in Japan [127], so this strategy can help the commercialization of modified strains [128].

An interesting alternative to explore genetic variability without creating GMOs is to take advantage of genes with an outstanding easiness to mutate spontaneously, such as the flocculins encoding FLO family. This behavior is due to their subtelomerical position and tandem repeats [129]. Flocculins are of interest for winemakers because they help in the clarification of wine, and act protectively when the conditions of the environment are harsh [130].

Additionally, public opinion on GMOs is crucial to the success of engineered yeasts, and it is far from being positive. Public opinion and legislation are intertwined and influence each other, so for the wine industry (and food and beverage industries in general) to benefit from all the efforts in the field, both should move forward, without compromising human nor Earth's safety.

8. Conclusions

There is an actual preference for quality rather than for amount, meaning that in the demanding for premium wines, less is more. Here, different wine traits that can be modulated by genetic engineering have been reviewed and exemplified (Figure 2), but this technology can be exploited for winemaking in a "virtually infinite process" to adapt the product to the changing times [92]. To this challenge, some other relevant biotechnological techniques (and, of course, non-biotechnological) are used, such as hybridization, but are not described here since they imply non-*S. cerevisiae* species, and have been reviewed elsewhere [5,131]. In todays globalized and 2.0 world, where news are spread at the speed of light, public opinion can determine waves of "dos and don'ts" and legislation is constantly being updated, it is preferable to conduct research for non-GMOs. Additionally, a vast effort is required to develop completely safe GMOs, not only for the sake of economic interests, but for the sake of progress. These organisms can help us in countless aspects of life, not all of them as hedonist as enjoying a glass of wine, but related to health, economy, poverty or environment.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McGovern, P.; Jalabadze, M.; Batiuk, S.; Callahan, M.P.; Smith, K.E.; Hall, G.R.; Kvavadze, E.; Maghradze, D.; Rusishvili, N.; Bouby, L.; et al. Early Neolithic wine of Georgia in the South Caucasus. *Proc. Nalt. Acad. Sci. USA* **2017**, *114*, E10309–E10318. [CrossRef] [PubMed]

2. Mortimer, R.K. Evolution and variation of the yeast (*Saccharomyces*) genome. *Genome Res.* **2000**, *10*, 403–409. [CrossRef] [PubMed]

3. Martini, A. Origin and domestication of the wine yeast *Saccharomyces cerevisiae*. *J. Wine Res.* **1993**, *4*, 165–176. [CrossRef]

4. Duan, S.F.; Han, P.J.; Wang, Q.M.; Liu, W.Q.; Shi, J.Y.; Li, K.; Zhang, X.L.; Bai, F.Y. The origin and adaptive evolution of domesticated populations of yeast from Far East Asia. *Nat. Commun.* **2018**, *9*, 2690. [CrossRef] [PubMed]

5. Gonzalez, R.; Tronchoni, J.; Quirós, M.; Morales, P. Genetic improvement and genetically modified microorganisms. In *Wine Safety, Consumer Preference, and Human Health*; Moreno-Arribas, M.V., Bartolomé Sualdea, B., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 71–96, ISBN 978-3-319-24512-6, ISBN (eBook) 978-3-319-24514-0. [CrossRef]

6. Wortmann, J. *Anwendung und Wirkung Reiner Hefen*; Verlagsbuchhandlung Paul Parey: Berlin, Germany, 1895.

7. Goffeau, A.; Barrell, B.G.; Bussey, H.; Davis, R.W.; Dujon, B.; Feldmann, H.; Galibert, F.; Hoheisel, J.D.; Jacq, C.; Johnston, M.; et al. Life with 6000 genes. *Science* **1996**, *274*, 546–567. [CrossRef]

8. Liti, G.; Carter, D.M.; Moses, A.M.; Warringer, J.; Parts, L.; James, S.A.; Davey, R.P.; Roberts, I.N.; Burt, A.; Koufopanou, V.; et al. Population genomics of domestic and wild yeasts. *Nature* **2009**, *458*, 337–341. [CrossRef]

9. Peter, J.; De Chiara, M.; Friedrich, A.; Yue, J.X.; Pflieger, D.; Bergström, A.; Sigwalt, A.; Barre, B.; Freel, K.; Llored, A.; et al. Genome evolution across 1011 *Saccharomyces cerevisiae* isolates. *Nature* **2018**, *556*, 339–344. [CrossRef]

10. Baeshen, N.A.; Baeshen, M.N.; Sheikh, A.; Bora, R.S.; Ahmed, M.M.M.; Ramadan, H.A.I.; Saini, K.S.; Redwan, E.M. Cell factories for insulin production. *Microb. Cell Fact.* **2014**, *13*, 141. [CrossRef]

11. Wang, G.; Huang, M.; Nielsen, J. Exploring the potential of *Saccharomyces cerevisiae* for biopharmaceutical protein production. *Curr. Opin. Biotechnol.* **2017**, *48*, 77–84. [CrossRef]

12. Hitzeman, R.A.; Chen, C.Y.; Hagie, F.E.; Lugovoy, J.M.; Singh, A. Yeast: An alternative organism for foreign protein production. In *Recombinant DNA Products: Insulin, Interferon and Growth Hormone*; Bollon, A.P., Ed.; CRC Press: Boca Raton, FL, USA, 2018; pp. 47–65, ISBN 978-1-315-89715-8, ISBN 978-1-351-07625-8. [CrossRef]

13. Ko, J.K.; Lee, S.M. Advances in cellulosic conversion to fuels: Engineering yeasts for cellulosic bioethanol and biodiesel production. *Curr. Opin. Biotechnol.* **2018**, *50*, 72–80. [CrossRef]

14. Favaro, L.; Jansen, T.; van Zyl, W.H. Exploring industrial and natural *Saccharomyces cerevisiae* strains for the bio-based economy from biomass: The case of bioethanol. *Crit. Rev. Biotechnol.* **2019**, *39*, 800–816. [CrossRef] [PubMed]

15. Morawski, B.; Quan, S.; Arnold, F.H. Functional expression and stabilization of horseradish peroxidase by directed evolution in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2001**, *76*, 99–107. [CrossRef] [PubMed]

16. Gonzalez-Perez, D.; Garcia-Ruiz, E.; Alcalde, M. *Saccharomyces cerevisiae* in directed evolution: An efficient tool to improve enzymes. *Bioengineered* **2012**, *3*, 1–6. [CrossRef] [PubMed]

17. Viña-Gonzalez, J.; Gonzalez-Perez, D.; Alcalde, M. Directed evolution method in *Saccharomyces cerevisiae*: Mutant Library creation and screening. *J. Vis. Exp.* **2016**, *110*, e53761. [CrossRef]

18. Fraschini, R. Divide precisely and proliferate safely: Lessons from budding yeast. *Front. Genet.* **2019**, *9*, 738. [CrossRef]

19. Kim, S.; Park, J.; Kim, T.; Lee, J.S. The functional study of human proteins using humanized yeast. *J. Microbiol.* **2020**, *58*, 343–349. [CrossRef]

20. Pretorius, I.S.; Boeke, J.D. Yeast 2.0-connecting the dots in the construction of the world's first functional synthetic eukaryotic genome. *FEMS Yeast Res.* **2018**, *18*, foy032. [CrossRef]

21. Novo, M.; Bigey, F.; Beyne, E.; Galeote, V.; Gavory, F.; Mallet, S.; Cambon, B.; Legras, J.L.; Wincker, P.; Casaregola, S.; et al. Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 16333–16338. [CrossRef]

22. Vilela, A. The importance of yeasts on fermentation quality and human health-promoting compounds. *Fermentation* **2019**, *5*, 46. [CrossRef]

23. Stervbo, U.; Vang, O.; Bonnesen, C. A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. *Food Chem.* **2007**, *101*, 449–457. [CrossRef]

24. Mukherjee, S.; Dudley, J.I.; Das, D.K. Dose-dependency of resveratrol in providing health benefits. *Dose Response* **2010**, *8*, 478–500. [CrossRef]

25. Weiskirchen, S.; Weiskirchen, R. Resveratrol: How much wine do you have to drink to stay healthy? *Adv. Nutr.* **2016**, *7*, 706–718. [CrossRef] [PubMed]

26. Pirola, L.; Frojdo, S. Resveratrol: One molecule, many targets. *IUBMB Life* **2008**, *60*, 323–332. [CrossRef] [PubMed]

27. Kuršvietienė, L.; Stanevičienė, I.; Mongirdienė, A.; Bernatonienė, J. Multiplicity of effects and health benefits of resveratrol. *Medicina* **2016**, *52*, 148–155. [CrossRef]

28. Becker, J.V.W.; Armstrong, G.O.; van der Merwe, M.J.; Lambrechts, M.G.; Vivier, M.A.; Pretorius, I.S. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res.* **2003**, *4*, 79–85. [CrossRef]

29. Sun, P.; Liang, J.L.; Kang, L.Z.; Huang, X.Y.; Huang, J.J.; Ye, Z.W.; Guo, L.Q.; Lin, J.F. Increased resveratrol production in wines using engineered wine strains *Saccharomyces cerevisiae* EC1118 and relaxed antibiotic or auxotrophic selection. *Biotechnol. Prog.* **2015**, *31*, 650–655. [CrossRef]

30. Shin, S.Y.; Jung, S.M.; Kim, M.D.; Han, N.S.; Seo, J.H. Production of resveratrol from tyrosine metabolically *engineered Saccharomyces cerevisiae*. *Enzyme Microb. Technol.* **2012**, *51*, 211–216. [CrossRef]

31. Rebollo-Romero, I.; Fernández-Cruz, E.; Carrasco-Galán, F.; Valero, E.; Cantos-Villar, E.; Cerezo, A.B.; Troncoso, A.M.; Carmen Garcia-Parrilla, M. Factors influencing the production of the antioxidant hydroxytyrosol during alcoholic fermentation: Yeast strain, initial tyrosine concentration and initial must. *LWT-Food Sci. Technol.* **2020**, *130*, 109631. [CrossRef]

32. Muñiz-Calvo, S.; Bisquert, R.; Puig, S.; Guillamón, J.M. Overproduction of hydroxytyrosol in *Saccharomyces cerevisiae* by heterologous overexpression of the *Escherichia coli* 4-hydroxyphenylacetate 3-monooxygenase. *Food Chem.* **2020**, *308*, 125646. [CrossRef]

33. Romboli, Y.; Mangani, S.; Buscioni, G.; Granchi, L.; Vincenzini, M. Effect of *Saccharomyces cerevisiae* and *Candida zemplinina* on quercetin, vitisin A and hydroxytyrosol contents in Sangiovese wines. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1137–1145. [CrossRef]

34. Karković Marković, A.; Torić, J.; Barbarić, M.; Jakobušić Brala, C. Hydroxytyrosol, Tyrosol and derivatives and their potential effects on human health. *Molecules* **2019**, *24*, 2001. [CrossRef]

35. Proestos, C.; Bakogiannis, A.; Psarianos, C.; Koutinas, A.A.; Kanellaki, M.; Komaitis, M. High performance liquid chromatography analysis of phenolic substances in Greek wines. *Food Control.* **2005**, *16*, 319–323. [CrossRef]

36. Minuti, L.; Pellegrino, R.M.; Tesei, I. Simple extraction method and gas chromatography-Mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine. *J. Chromatogr. A* **2006**, *1114*, 263–268. [CrossRef] [PubMed]

37. Fernández-Mar, M.I.; Mateos, R.; García-Parrilla, M.C.; Puertas, B.; Cantos-Pinar, E. Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonine: A review. *Food Chem.* **2012**, *130*, 797–813. [CrossRef]

38. Li, X.; Chen, Z.; Wu, Y.; Yan, Y.; Sun, X.; Yuan, Q. Establishing an artificial pathway for efficient biosynthesis of hydroxytyrosol. *ACS Synth. Biol.* **2018**, *7*, 647–654. [CrossRef]

39. Brouk, M.; Fishman, A. Protein engineering of toluene monooxygenases for synthesis of hydroxytyrosol. *Food Chem.* **2009**, *116*, 114–121. [CrossRef]

40. Molina-Espeja, P.; Cañellas, M.; Plou, F.J.; Hofrichter, M.; Lucas, F.; Guallar, V.; Alcalde, M. Synthesis of 1-naphthol by a natural peroxygenase engineered by directed evolution. *ChemBioChem* **2016**, *17*, 341–349. [CrossRef]

41. Boronat, A.; Mateus, J.; Soldevila-Domenech, N.; Guerra, M.; Rodríguez-Morató, J.; Varon, C.; Muñoz, D.; Barbosa, F.; Morales, J.C.; Gaedigk, A.; et al. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. *Free Radic. Biol. Med.* **2019**, *143*, 471–481. [CrossRef]

42. Li, Z.; Jiang, Y.; Peter Guengerich, F.; Ma, L.; Li, S.; Zhang, W. Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications. *J. Biol. Chem.* **2020**, *295*, 833–849. [CrossRef]

43. Georgiev, V.; Ananga, A.; Tsolova, V. Dietary supplements/nutraceuticals made from grapes and wines. In *Wine Safety, Consumer Preference, and Human Health*; Moreno-Arribas, M.V., Bartolomé Sualdea, B., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 201–227, ISBN 978-3-319-24512-6, ISBN (eBook) 978-3-319-24514-0. [CrossRef]

44. Avbelj, M.; Zupan, J.; Kranjc, L.; Raspor, P. Quorum-Sensing Kinetics in *Saccharomyces cerevisiae*: A Symphony of ARO Genes and Aromatic Alcohols. *J. Agric. Food Chem.* **2015**, *63*, 8544–8550. [CrossRef]

45. Avbelj, M.; Zupan, J.; Raspor, P. Quorum-sensing in yeast and its potential in wine making. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 7841–7852. [CrossRef] [PubMed]

46. Herrero, O.; Ramón, D.; Orejas, M. Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. *Metab. Eng.* **2008**, *10*, 78–86. [CrossRef] [PubMed]

47. van Wyk, N.; Grossmann, M.; Wendland, J.; von Wallbrunn, C.; Pretorius, I.S. The whiff of wine yeast innovation: Strategies for enhancing aroma production by yeast during wine fermentation. *J. Agric. Food Chem.* **2019**, *67*, 13496–13505. [CrossRef] [PubMed]

48. Swiegers, J.H.; Capone, D.L.; Pardon, K.H.; Elsey, G.M.; Sefton, M.A.; Francis, I.L.; Pretorius, I.S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* **2007**, *24*, 561–574. [CrossRef]

49. Holt, S.; Cordente, A.G.; Williams, S.J.; Capone, D.L.; Jitjaroen, W.; Menz, I.R.; Curtin, C.; Anderson, P.A. Engineering *Saccharomyces cerevisiae* to release 3-mercaptohexan-1-ol during fermentation through overexpression of an *S. cerevisiae* gene, STR3, for improvement of wine aroma. *Appl. Environ. Microbiol.* **2011**, *77*, 3626–3632. [CrossRef]

50. Lee, D.; Lloyd, N.D.R.; Pretorius, I.S.; Borneman, A.R. Heterologous production of raspberry ketone in the wine yeast *Saccharomyces cerevisiae* via pathway engineering and synthetic enzyme fusion. *Microb. Cell Fact.* **2016**, *15*, 49:2–49:7. [CrossRef]

51. Pardo, E.; Rico, J.; Gil, J.V.; Orejas, M. De novo production of six key grape aroma monoterpenes by a geraniol synthase-engineered *Saccharomyces cerevisiae* wine strain. *Microb. Cell Fact.* **2015**, *14*, 136:1–136:8. [CrossRef]

52. Zhang, X.; Zhang, X.F.; Li, H.P.; Wang, L.Y.; Zhang, C.; Xing, X.H.; Bao, C.Y. Atmospheric and room temperature plasma (ARTP) as a new powerful mutagenesis tool. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5387–5396. [CrossRef]

53. Dragosits, M.; Mattanovich, D. Adaptive laboratory evolution—Principles and applications for biotechnology. *Microb. Cell Fact.* **2013**, *12*, 64. [CrossRef]

54. Tian, T.; Wu, D.; Ng, C.T.; Yang, H.; Sun, J.; Liu, J.; Lu, J. A multiple-step strategy for screening *Saccharomyces cerevisiae* strains with improved acid tolerance and aroma profiles. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 3097–3107. [CrossRef]

55. Samoticha, J.; Wojdyło, A.; Chmielewska, J.; Nofer, J. Effect of different yeast strains and temperature of fermentation on basic enological parameters, polyphenols and volatile compounds of aurore white wine. *Foods* **2019**, *8*, 599. [CrossRef] [PubMed]

56. López-Malo, M.; García-Ríos, E.; Melgar, B.; Sanchez, M.R.; Dunham, M.J.; Guillamón, J.M. Evolutionary engineering of a wine yeast strain revealed a key role of inositol and mannoprotein metabolism during low temperature fermentation. *BMC Genom.* **2015**, *16*, 537. [CrossRef]

57. Wiedenheft, B.; Sternberg, S.H.; Doudna, J.A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* **2012**, *482*, 331–338. [CrossRef] [PubMed]

58. Mitsui, R.; Yamada, R.; Ogino, H. CRISPR system in the yeast *Saccharomyces cerevisiae* and its application in the bioproduction of useful chemicals. *World J. Microbiol. Biotechnol.* **2019**, *35*, 111. [CrossRef] [PubMed]

59. Adames, N.R.; Gallegos, J.E.; Peccoud, J. Yeast genetic interaction screens in the age of CRISPR/Cas. *Curr. Genet.* **2019**, *65*, 307–327. [CrossRef]

60. Zhao, J.; Fang, H.; Zhang, D. Expanding application of CRISPR-Cas9 system in microorganisms. *Synth. Syst. Biotechnol.* **2020**, *5*, 269–276. [CrossRef]

61. van Wyk, N.; Kroukamp, H.; Espinosa, M.I.; von Wallbrunn, C.; Wendland, J.; Pretorius, I.S. Blending wine yeast phenotypes with the aid of CRISPR DNA editing technologies. *Int. J. Food Microbiol.* **2020**, *324*, 108615:1–108615:7. [CrossRef]

62. Dzialo, M.C.; Park, R.; Steensels, J.; Lievens, B.; Verstrepen, K.J. Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiol. Rev.* **2017**, *41*, S95–S128. [CrossRef]

63. Luong, J.H. Kinetics of ethanol inhibition in alcohol fermentation. *Biotechnol. Bioeng.* **1985**, *27*, 280–285. [CrossRef]

64. Athes, V.; Pena y Lillo, M.; Bernard, C.; Perez-Correa, R.; Souchon, I. Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. *J. Agric. Food Chem.* **2004**, *52*, 2021–2027. [CrossRef]

65. Kutyna, D.R.; Varela, C.; Henschke, P.A.; Chambers, P.J.; Stanley, G.A. Microbiological approaches to lowering ethanol concentration in wine. *Trends Food Sci. Technol.* **2010**, *21*, 293–302. [CrossRef]

66. Rodrigues, A.J.; Raimbourg, T.; González, R.; Morales, P. Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration. *LWT-Food Sci. Technol.* **2016**, *65*, 1038–1043. [CrossRef]

67. Dequin, S.; Baptista, E.; Barre, P. Acidification of grape must by *Saccharomyces cerevisiae* wine yeast strains genetically engineered to produce lactic acid. *Am. J. Enol. Vitic.* **1999**, *50*, 45–50.

68. Goold, H.D.; Kroukamp, H.; Williams, T.C.; Paulsen, I.T.; Varela, C.; Pretorius, I.S. Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microb. Biotechnol.* **2017**, *10*, 264–278. [CrossRef] [PubMed]

69. Cambon, B.; Monteil, V.; Remize, F.; Camarasa, C.; Dequin, S. Effects of GPD1 overexpression in *Saccharomyces cerevisiae* commercial wine yeast strains lacking ALD6 genes. *Appl. Environ. Microbiol.* **2006**, *72*, 4688–4694. [CrossRef] [PubMed]

70. Ehsani, M.; Fernández, M.R.; Biosca, J.A.; Julien, A.; Dequin, S. Engineering of 2,3-butanediol dehydrogenase to reduce acetoin formation by glycerol-overproducing, low-alcohol *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2009**, *75*, 3196–3205. [CrossRef]

71. Varela, C.; Kutyn, D.R.; Solomon, M.R.; Black, C.A.; Borneman, A.; Henschke, P.A.; Pretorius, I.S.; Chambers, P.J. Evaluation of gene modification strategies for the development of low-alcohol-wine yeasts. *Appl. Environ. Microbiol.* **2012**, *78*, 6068–6077. [CrossRef]

72. Varela, C.; Schmidt, S.A.; Borneman, A.R.; Pang, C.N.I.; Krömerx, J.O.; Khan, A.; Song, X.; Hodson, M.P.; Solomon, M.; Mayr, C.M.; et al. Systems-based approaches enable identification of gene targets which improve the flavour profile of low-ethanol wine yeast strains. *Metab. Eng.* **2018**, *49*, 178–191. [CrossRef]

73. Heux, S.; Cachon, R.; Dequin, S. Cofactor engineering in *Saccharomyces cerevisiae*: Expression of a H_2O-forming NADH oxidase and impact on redox metabolism. *Metab. Eng.* **2006**, *8*, 303–314. [CrossRef]

74. Heux, S.; Sablayrolles, J.M.; Cachon, R.; Dequin, S. Engineering a *Saccharomyces cerevisiae* wine yeast that exhibits reduced ethanol production during fermentation under controlled microoxygenation conditions. *Appl. Environ. Microbiol.* **2006**, *72*, 5822–5828. [CrossRef]

75. Cuello, R.A.; Flores Montero, K.J.; Mercado, L.A.; Combina, M.; Ciklic, I.F. Construction of low-ethanol-wine yeasts through partial deletion of the *Saccharomyces cerevisiae* PDC2 gene. *AMB Express* **2017**, *7*, 67. [CrossRef] [PubMed]

76. Malherbe, D.F.; du Toit, M.; Cordero Otero, R.R.; van Rensburg, P.; Pretorius, I.S. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 502–511. [CrossRef] [PubMed]

77. Rossouw, D.; Heyns, E.H.; Setati, M.E.; Bosch, S.; Bauer, F.F. Adjustment of trehalose metabolism in wine *Saccharomyces cerevisiae* strains to modify ethanol yields. *Appl. Environ. Microbiol.* **2013**, *79*, 5197–5207. [CrossRef] [PubMed]

78. Chen, S.; Xu, Y. Adaptive evolution of *Saccharomyces cerevisiae* with enhanced ethanol tolerance for Chinese rice wine fermentation. *Appl. Biochem. Biotechnol.* **2014**, *173*, 1940–1954. [CrossRef]

79. Alper, H.; Stephanopoulos, G. Global transcription machinery engineering: A new approach for improving cellular phenotype. *Metab. Eng.* **2007**, *9*, 258–267. [CrossRef]

80. Xue, T.; Chen, D.; Su, Q.; Yuan, X.; Liu, K.; Huang, L.; Fang, J.; Chen, J.; He, W.; Chen, Y. Improved ethanol tolerance and production of *Saccharomyces cerevisiae* by global transcription machinery engineering via directed evolution of the SPT8 gene. *Food Biotechnol.* **2019**, *33*, 155–173. [CrossRef]

81. Srivastava, R.; Rai, K.M.; Pandey, B.; Singh, S.P.; Sawant, S.V. Spt-ada-gcn5acetyltransferase (SAGA) complex in plants: Genome wide identification evolutionary conservation and functional determination. *PLoS ONE* **2015**, *8*, e0134709. [CrossRef]

82. Guo, X.W.; Li, Y.Z.; Guo, J.; Wang, Q.; Huang, S.Y.; Chen, Y.F.; Du, L.P.; Xiao, D.G. Reduced production of ethyl carbamate for wine fermentation by deleting CAR1 in *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 671–679. [CrossRef]

83. Genbauffe, F.S.; Cooper, T.G. The urea amidolyase (DUR1,2) gene of *Saccharomyces cerevisiae*. *DNA Seq.* **1991**, *2*, 19–32. [CrossRef]

84. Coulon, J.; Husnik, J.I.; Inglis, D.L.; van der Merwe, G.K.; Lonvaud, A.; Erasmus, D.J.; van Vuuren, H.J.J. Metabolic engineering of *Saccharomyces cerevisiae* to minimize the production of ethyl carbamate in wine. *Am. J. Enol. Vitic.* **2006**, *57*, 113–124.

85. Wu, D.; Li, X.; Sun, J.; Cai, G.; Xie, G.; Lu, J. Effect of citrulline metabolism in *Saccharomyces cerevisiae* on the formation of ethyl carbamate during Chinese rice wine fermentation. *J. Inst. Brew.* **2018**, *124*, 77–84. [CrossRef]

86. Vigentini, I.; Gebbia, M.; Belotti, A.; Foschino, R.; Roth, F.P. CRISPR/Cas9 system as a valuable genome editing tool for wine yeasts with application to decrease urea production. *Front. Microbiol.* **2017**, *8*, 2194:1–2194:11. [CrossRef] [PubMed]

87. Wu, D.; Xie, W.; Li, X.; Cai, G.; Lu, J.; Xi, G. Metabolic engineering of *Saccharomyces cerevisiae* using the CRISPR/Cas9 system to minimize ethyl carbamate accumulation during Chinese rice wine fermentation. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 4435–4444. [CrossRef] [PubMed]

88. Zhang, W.; Cheng, Y.; Li, Y.; Du, G.; Xie, G.; Zou, H.; Zhou, J.; Chen, J. Adaptive Evolution relieves nitrogen catabolite repression and decreases urea accumulation in cultures of the Chinese rice wine yeast strain *Saccharomyces cerevisiae* XZ-11. *J. Agric. Food Chem.* **2018**, *66*, 9061–9069. [CrossRef] [PubMed]

89. Zhang, P.; Hu, X. Metabolic engineering of arginine permeases to reduce the formation of urea in *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* **2018**, *34*, 47:1–47:8. [CrossRef] [PubMed]

90. Feller, A.; Georis, I.; Tate, J.J.; Cooper, T.G.; Dubois, E. Alterations in the Ure2 αCap domain elicit different GATA factor responses to rapamycin treatment and nitrogen limitation. *J. Biol. Chem.* **2013**, *288*, 1841–1855. [CrossRef] [PubMed]

91. Torrellas, M.; Rozès, N.; Aranda, A.; Matallana, E. Basal catalase activity and high glutathione levels influence the performance of non-*Saccharomyces* active dry wine yeasts. *Food Microbiol.* **2020**, *92*, 103589. [CrossRef]

92. Bonciani, T.; De Vero, L.; Mezzetti, F.; Fay, J.C.; Giudici, P. A multi-phase approach to select new wine yeast strains with enhanced fermentative fitness and glutathione production. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2269–2278. [CrossRef]

93. Mezzetti, F.; de Vero, L.; Giudici, P. Evolved *Saccharomyces cerevisiae* wine strains with enhanced glutathione production obtained by an evolution-based strategy. *FEMS Yeast Res.* **2014**, *14*, 977–987. [CrossRef]

94. Fierro-Risco, J.; Rincón, A.M.; Benítez, T.; Codón, A.C. Overexpression of stress-related genes enhances cell viability and velum formation in Sherry wine yeasts. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6867–6881. [CrossRef]

95. Marangon, M.; Vegro, M.; Vincenzi, S.; Lomolino, G.; de Iseppi, A.; Curioni, A. A novel method for the quantification of white wine mannoproteins by a Competitive Indirect Enzyme-Linked Lectin Sorbent Assay (CI-ELLSA). *Molecules* **2018**, *23*, 3070. [CrossRef] [PubMed]

96. Gonzalez-Ramos, D.; Gonzalez, R. Genetic determinants of the release of mannoproteins of enological interest by *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **2006**, *54*, 9411–9416. [CrossRef] [PubMed]

97. Gonzalez-Ramos, D.; Cebollero, E.; Gonzalez, R. A recombinant *Saccharomyces cerevisiae* strain overproducing mannoproteins stabilizes wine against protein haze. *Appl. Environ. Microbiol.* **2008**, *74*, 5533–5540. [CrossRef] [PubMed]

98. Penacho, V.; Blondin, B.; Valero, E.; González, R. Flocculation and transcriptional adaptation to fermentation conditions in a recombinant wine yeast strain defective for KNR4/SMI1. *Biotechnol. Prog.* **2012**, *28*, 327–336. [CrossRef] [PubMed]

99. Inglis, D.L.; Pickering, G.P. Vintning on thin ice—The making of Canada's iconic dessert wine. In *The World of Niagara Wine*; Ripmeester, M., Mackintosh, P., Fullerton, C., Eds.; Wilfrid Laurier University Press: Waterloo, ON, Canada, 2013; pp. 229–248, ISBN 9781554584055.

100. Ferreira, C.; van Voorst, F.; Martins, A.; Neves, L.; Oliveira, R.; Kielland-Brandt, M.C.; Lucas, C.; Brandt, A. A member of the sugar transporter family, Stl1p is the glycerol/H$^+$ symporter in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **2005**, *16*, 2068–2076. [CrossRef] [PubMed]

101. Muysson, J.; Miller, L.; Allie, R.; Inglis, D.L. The use of CRISPR-Cas9 genome editing to determine the importance of glycerol uptake in wine yeast during Icewine fermentation. *Fermentation* **2019**, *5*, 93. [CrossRef]

102. Capece, A.; Pietrafesa, R.; Siesto, G.; Romano, P. Biotechnological approach based on selected *Saccharomyces cerevisiae* starters for reducing the use of sulfur dioxide in wine. *Microorganisms* **2020**, *8*, 738. [CrossRef] [PubMed]

103. Corte, L.; Roscini, L.; Zadra, C.; Antonielli, L.; Tancini, B.; Magini, A.; Emiliani, C.; Cardinali, G. Effect of pH on potassium metabisulphite biocidic activity against yeast and human cell cultures. *Food Chem.* **2012**, *134*, 1327–1336. [CrossRef]

104. Hinze, H.; Holzer, H. Analysis of the energy metabolism after incubation of *Saccharomyces cerevisiae* with sulfite or nitrite. *Arch. Microbiol.* **1986**, *145*, 27–31. [CrossRef]

105. Romano, P.; Suzzi, G. Sulphur dioxide and wine microorganisms. In *Wine Microbiology and Biotechnology*, 1st ed.; Fleet, G., Ed.; CRC Press: Boca Raton, FL, USA, 1993; pp. 373–393, ISBN 3718651327, ISBN 9783718651320.

106. Fugelsang, K.; Edwards, C. *Wine Microbiology*; Springer: Boston, MA, USA, 2007; ISBN 978-0-387-33341-0 (print), ISBN 978-0-387-33349-6 (online). [CrossRef]

107. Lage, P.; Sampaio-Marques, B.; Ludovico, P.; Mira, N.P.; Mendes-Ferreira, A. Transcriptomic and chemogenomic analyses unveil the essential role of Com2-regulon in response and tolerance of *Saccharomyces cerevisiae* to stress induced by sulfur dioxide. *Microb. Cell* **2019**, *6*, 509–523. [CrossRef]

108. Park, H.; Bakalinsky, A.T. SSU1 mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* **2000**, *16*, 881–888. [CrossRef]

109. García-Ríos, E.; Nuévalos, M.; Barrio, E.; Puig, S.; Guillamón, J.M. A new chromosomal rearrangement improves the adaptation of wine yeasts to sulfite. *Environ. Microbiol.* **2019**, *21*, 1771–1781. [CrossRef] [PubMed]

110. Fleiss, A.; O'Donnell, S.; Fournier, T.; Lu, W.; Agier, N.; Delmas, S.; Schacherer, J.; Fischer, G. Reshuffling yeast chromosomes with CRISPR/Cas9. *PLoS Genet.* **2019**, *15*, e1008332. [CrossRef] [PubMed]

111. Cordente, A.G.; Heinrich, A.; Pretorius, I.S.; Swiegers, J.D. Isolation of sulfite reductase variants of a commercial wine yeast with significantly reduced hydrogen sulfide production. *FEMS Yeast Res.* **2009**, *9*, 446–459. [CrossRef]

112. Salvadó, Z.; Ramos-Alonso, L.; Tronchoni, J.; Penacho, V.; García-Ríos, E.; Morales, P.; Gonzalez, R.; Guillamón, J.M. Genome-wide identification of genes involved in growth and fermentation activity at low temperature in *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2016**, *236*, 38–46. [CrossRef]

113. Ganatsios, V.; Terpou, A.; Gialleli, A.I.; Kanellaki, M.; Bekatorou, A.; Koutinas, A.A. A ready-to-use freeze-dried juice and immobilized yeast mixture for low temperature sour cherry (*Prunus cerasus*) wine making. *Food Bioprod. Process.* **2019**, *117*, 373–379. [CrossRef]

114. Terpou, A.; Ganatsios, V.; Kanellaki, M.; Koutinas, A.A. Entrapped psychrotolerant yeast cells within pine sawdust for low temperature wine making: Impact on wine quality. *Microorganisms* **2020**, *8*, 764. [CrossRef]

115. García-Ríos, E.; Morard, M.; Parts, L.; Liti, G.; Guillamón, J.M. The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genom.* **2017**, *18*, 159. [CrossRef]

116. Deed, R.C.; Fedrizzi, B.; Gardner, R.C. *Saccharomyces cerevisiae* FLO1 gene demonstrates genetic linkage to increased fermentation rate at low temperatures. *G3 (Bethesda)* **2017**, *7*, 1039–1048. [CrossRef]

117. García-Ríos, E.; Ramos-Alonso, L.; Guillamón, J.M. Correlation between low temperature adaptation and oxidative stress in *Saccharomyces cerevisiae*. *Front. Microbiol.* **2016**, *7*, 1199. [CrossRef] [PubMed]

118. Kessi-Pérez, E.I.; Molinet, J.; Martínez, C. Disentangling the genetic bases of *Saccharomyces cerevisiae* nitrogen consumption and adaptation to low nitrogen environments in wine fermentation. *Biol. Res.* **2020**, *53*, 2. [CrossRef] [PubMed]

119. Kessi-Pérez, E.I.; Molinet, J.; García, V.; Aguilera, O.; Cepeda, F.; López, M.A.; Sari, S.; Cuello, R.; Ciklic, I.; Rojo, M.C.; et al. Generation of a non-transgenic genetically improved yeast strain for wine production from nitrogen-deficient musts. *Microorganisms* **2020**, *8*, 1194. [CrossRef]

120. Kessi-Pérez, E.I.; Ponce, B.; Li, J.; Molinet, J.; Baeza, C.; Figueroa, D.; Bastías, C.; Gaete, M.; Liti, G.; Díaz-Barrera, A.; et al. Differential Gene Expression and Allele Frequency Changes Favour Adaptation

of a heterogeneous yeast population to nitrogen-limited fermentations. *Front. Microbiol.* **2020**, *11*, 1204. [CrossRef] [PubMed]

121. James, R. Broach. Nutritional control of growth and development in yeast. *Genetics* **2012**, *192*, 73–105. [CrossRef]

122. Tesnière, C.; Brice, C.; Blondin, B. Responses of *Saccharomyces cerevisiae* to nitrogen starvation in wine alcoholic fermentation. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7025–7034. [CrossRef]

123. Vallejo, B.; Matallana, E.; Aranda, A. *Saccharomyces cerevisiae* nutrient signaling pathways show an unexpected early activation pattern during winemaking. *Microb. Cell Fact.* **2020**, *19*, 124. [CrossRef]

124. Molinet, J.; Salinas, F.; Guillamón, J.M.; Martínez, C. GTR1 affects nitrogen consumption and TORC1 activity in *Saccharomyces cerevisiae* under fermentation conditions. *Front. Genet.* **2020**, *11*, 519. [CrossRef]

125. Vallejo, B.; Peltier, E.; Garrigós, V.; Matallana, E.; Marullo, P.; Aranda, A. Role of *Saccharomyces cerevisiae* nutrient signaling pathways during winemaking: A Phenomics Approach. *Front. Bioeng. Biotechnol.* **2020**, *8*, 853. [CrossRef]

126. Husnik, J.I.; Volschenk, H.; Bauer, J.; Colavizza, D.; Luo, Z.; van Vuuren, H.J. Metabolic engineering of malolactic wine yeast. *Metab. Eng.* **2006**, *8*, 315–323. [CrossRef]

127. Hirosawa, I.; Aritomi, K.; Hoshida, H.; Kashiwagi, S.; Nishizawa, Y.; Akada, R. Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 68–73. [CrossRef]

128. Fischer, S.; Procopio, S.; Becker, T. Self-cloning brewing yeast: A new dimension in beverage production. *Eur. Food Res. Technol.* **2013**, *237*, 851–863. [CrossRef]

129. Steensels, J.; Snoek, T.; Meersman, E.; Picca Nicolino, M.; Voordeckers, K.; Verstrepen, K.J. Improving industrial yeast strains: Exploiting natural and artificial diversity. *FEMS Microbiol. Rev.* **2014**, *38*, 947–995. [CrossRef] [PubMed]

130. Di Gianvito, P.; Tesnière, C.; Suzzi, G.; Blondin, B.; Tofalo, R. Different genetic responses to oenological conditions between a flocculent wine yeast and its FLO5 deleted strain: Insights from the transcriptome. *Food Res. Int.* **2018**, *114*, 178–186. [CrossRef] [PubMed]

131. Vigentini, I.; Gonzalez, R.; Tronchoni, J. Genetic improvement of wine yeasts. In *Yeasts in the Production of Wine*; Romano, P., Ciani, M., Fleet, G.H., Eds.; Springer: New York, NY, USA, 2019; pp. 315–342, ISBN 978-1-4939-9780-0, ISBN 978-1-4939-9782-4. [CrossRef]