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Review

Homologous Recombination: A GRAS Yeast Genome Editing Tool

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Abstract: The fermentation industry is known to be very conservative, relying on traditional yeast management. Yet, in the modern fast-paced world, change comes about in facets such as climate change altering the quality and quantity of harvests, changes due to government regulations e.g., the use of pesticides or SO_2, the need to become more sustainable, and of course by changes in consumer preferences. As a silent companion of the fermentation industry, the wine yeast *Saccharomyces cerevisiae* has followed mankind through millennia, changing from a Kulturfolger, into a domesticated species for the production of bread, beer, and wine and further on into a platform strain for the production of biofuels, enzymes, flavors, or pharmaceuticals. This success story is based on the 'awesome power of yeast genetics'. Central to this is the very efficient homologous recombination (HR) machinery of *S. cerevisiae* that allows highly-specific genome edits. This microsurgery tool is so reliable that yeast has put a generally recognized as safe (GRAS) label onto itself and entrusted to itself the life-changing decision of mating type-switching. Later, yeast became its own genome editor, interpreted as domestication events, to adapt to harsh fermentation conditions. In biotechnology, yeast HR has been used with tremendous success over the last 40 years. Here we discuss several types of yeast genome edits then focus on HR and its inherent potential for evolving novel wine yeast strains and styles relevant for changing markets.

Keywords: homologous recombination; genome editing; wine yeast; lager yeast; fermentation; genetic engineering

1. The Yeast *Saccharomyces cerevisiae*—A Companion through the Ages

Fermented beverages have followed mankind through history and the domestication of barley around 10,000 BC was an indication that alcoholic beverages were an integral part of society [1,2]. In an archaeological investigation of ancient Egyptian pottery dated to approximately 3150 BC during the era of the Scorpion King I, it was found that these vessels were used for beverage fermentations [3]. This indicates that yeast, and particularly the strong fermenting yeast *Saccharomyces cerevisiae*, was part of human societies first as a *Kulturfolger* that then became domesticated through continuous use. Domestication events included in the case of a very popular wine yeast, EC1118, the introgression of a high-affinity fructose/H+ symporter from a closely-related species and horizontal gene transfers of DNA from a wine-spoilage organism, *Zygosaccharomyces bailii* and another yeast *Torulaspora microellipsoides*. These alterations provided this wine yeast with genes improving its nitrogen and carbon metabolism and stress responses to harsh fermentation conditions [4–6]. Other introgressions of a region containing *SUC2*, *HPF1*, and *AWA1* from *Saccharomyces paradoxus* were specifically found

in wine yeasts. These genes code for an invertase, a α-glucosidase, and a protein involved in haze formation, respectively [7].

Yet, yeasts used an array of genome edits to adapt to wine fermentation conditions. These include loss-of-function mutations, genomic rearrangements by reciprocal translocations, copy number variations, and changes in the level of gene expression. Loss-of-function was observed for the aquaporin genes, *AQY1* and *AQY2*, which encode channels that promote water transport across the cell membrane. This presents an adaptation to high-osmolarity conditions [8,9]. A chromosomal rearrangement occurred between chromosomes VIII and XVI. By crossing over within the promoter regions of the *ECM34* and *SSU1* genes an increase in the expression of the *SSU1*-sulphite exporter was achieved conferring an increased tolerance to high levels of sulphite and shortening the lag phase to the onset of fermentation [10–12]. Another rearrangement between chromosomes XV and XVI had the same effect [13].

Copy number variations and increase in expression were found for *CUP1*, encoding a copper-binding metallothionein [14,15]. These adaptations provide increased tolerance to $CuSO_4$, which has been used as a fungicide against powdery mildew since the 1880s [16]. *S. cerevisiae* has acquired foreign DNA encoding the oligopeptide transporters *FOT1-2* through horizontal gene transfer from *Torulaspora microellipsoides*, which enhanced wine yeast's ability to transport oligopeptides and provided a fitness advantage in grape must [6].

These natural genome edits allowed the adaptation of wine yeasts to the harsh fermentation conditions with high sugar but low nitrogen availability and the high alcohol levels at the end of fermentation. These routes of domestication events and genome edits in wine yeasts were uncontrolled, but interestingly enough the beneficial mutations selected by vintners and the vast majority of wine strains used today are derived from one cluster suggesting common origin [17,18].

That yeast shaped this human relationship with alcoholic beverages through the millennia was only rather recently discovered by Pasteur in 1863. The study of microorganisms and the analysis of successful vs. unsuccessful fermentations finally resulted in the generation of pure culture yeast strains. This was first achieved by Emil Christian Hansen working at the Carlsberg Laboratory [19]. He isolated *Saccharomyces carlsbergensis*, Unterhefe No.1, which was from then on used as a starter culture for beer fermentations. All lager yeasts such as *S. carlsbergensis* are hybrids of *S. cerevisiae* and *Saccharomyces eubayanus*. They belong to two groups, ~triploid strains of group I, including *S. carlsbergensis* and ~tetraploid strains of group II, including *S. pastorianus*, sharing a common origin [20,21]. Julius Wortmann, working at the Geisenheim Research Institute, inspired by Hansen's work, promoted the isolation and characterization of wine yeasts in the 1890s, some of which are still in use today for wine fermentations [22].

Wine making has changed dramatically in the last one hundred years. Yet, yeast strain improvements did not keep up this pace and were limited to the isolation of interesting strains, particularly of hybrid yeasts [23–27].

The discovery of a sexual cycle in *S. cerevisiae* was made in the 1930s [28]. Since then yeast genetics has advanced *S. cerevisiae* to a model eukaryote and the industry's number one workhorse [29,30]. The use of fairly unsophisticated genome edits via yeast breeding in advancing lager and wine yeast strains has recently emerged again [31–35]. Commercially, yeast breeding is an interesting technology since it allows the generation of non-GM yeast strains with novel contributions to wine fermentations [36]. The available commercial wine yeast strains are in need of refurbishment, they are "old, but not obsolete—not yet". They are basically derived from the Hansen and Wortmann period, i.e., as ancient as an old mechanical clock from 1890 (Figure 1). However, the application of current knowledge allows for much more precise and straightforward gene and genome edits. Homologous recombination provides one such clean tool for these purposes.

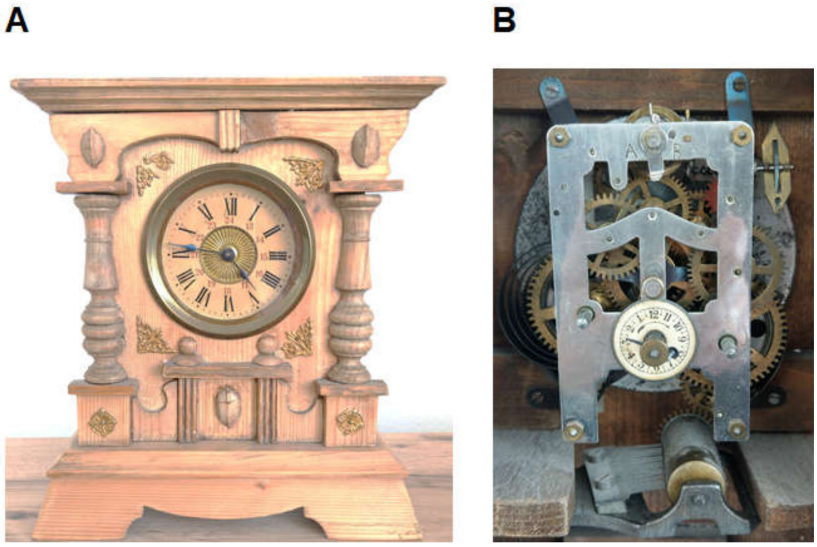

Figure 1. A clock from 1890. (**A**) Front view and (**B**) the intricate inner workings of the gears of a mechanical clock combined with a musical clock.

2. Homologous Recombination—A Trusted 'Copy + Paste' Genome Editing Tool

Prokaryotic and eukaryotic cells have several options to deal with DNA damage [37]. With double-strand breaks (DSBs), two highly conserved, i.e., evolutionarily ancient, systems—HR and non-homologous end-joining (NHEJ)—compete inside a cell to initiate their repair. Their basic features and molecular mechanisms are compared in Figure 2. DSBs can be caused by different exogenous or endogenous stimuli such as ultraviolet or ionizing radiations, genotoxic chemicals, or reactive oxygen species, or caused accidentally during replication [38]. Various facets of DNA repair processes evolved to maintain genome stability [39]. Failure to do so may result in cell death or in cell abnormalities that can lead to cancer [40,41]. DNA repair via HR is a highly accurate 'copy + paste' mechanism requiring a template, while NHEJ can stitch together DSBs without a template. This, however, makes NHEJ quite error-prone by placing high priority on repairing a DSB at any cost. The mechanism of choice in DSB repair may be species-dependent, vary with the type of lesion, or be regulated in a cell-cycle-specific manner [42–44].

Although DSBs are deleterious lesions threatening the survival of a cell, some physiological cell processes actually require programmed site-specific DSBs. One example found in *S. cerevisiae*, is Ho-endonuclease specific DNA cleavage at the *MAT* locus to initiate mating-type switching (Figure 3) [45]. Deletion of the *HO* gene, *HO* for homothallism, generates cells with stable mating type, i.e., heterothallic cells. This indicates that the DSB is required for mating-type switching [46,47]. Heterothallic strains, i.e., *ho* mutants, are preferred in the laboratory as they can be kept as stable haploid strains [48]. *S. cerevisiae* wild strains are generally diploid, as mother cells switch mating type and can then form zygotes with their daughter cells. Interestingly, only mother cells can initiate a mating type switch [49]. In daughter cells the *HO* gene is repressed [50]. To achieve mating type switching yeast cells actually play with fire: they introduce a potentially deadly DSB and trust gene conversion via HR to fix this. By doing so they gain the advantage of being able to form diploid cells that may sporulate and produce Dauerstadien under adverse environmental conditions. The evolution of this mechanism as such is proof that the process is GRAS, generally recognized as safe.

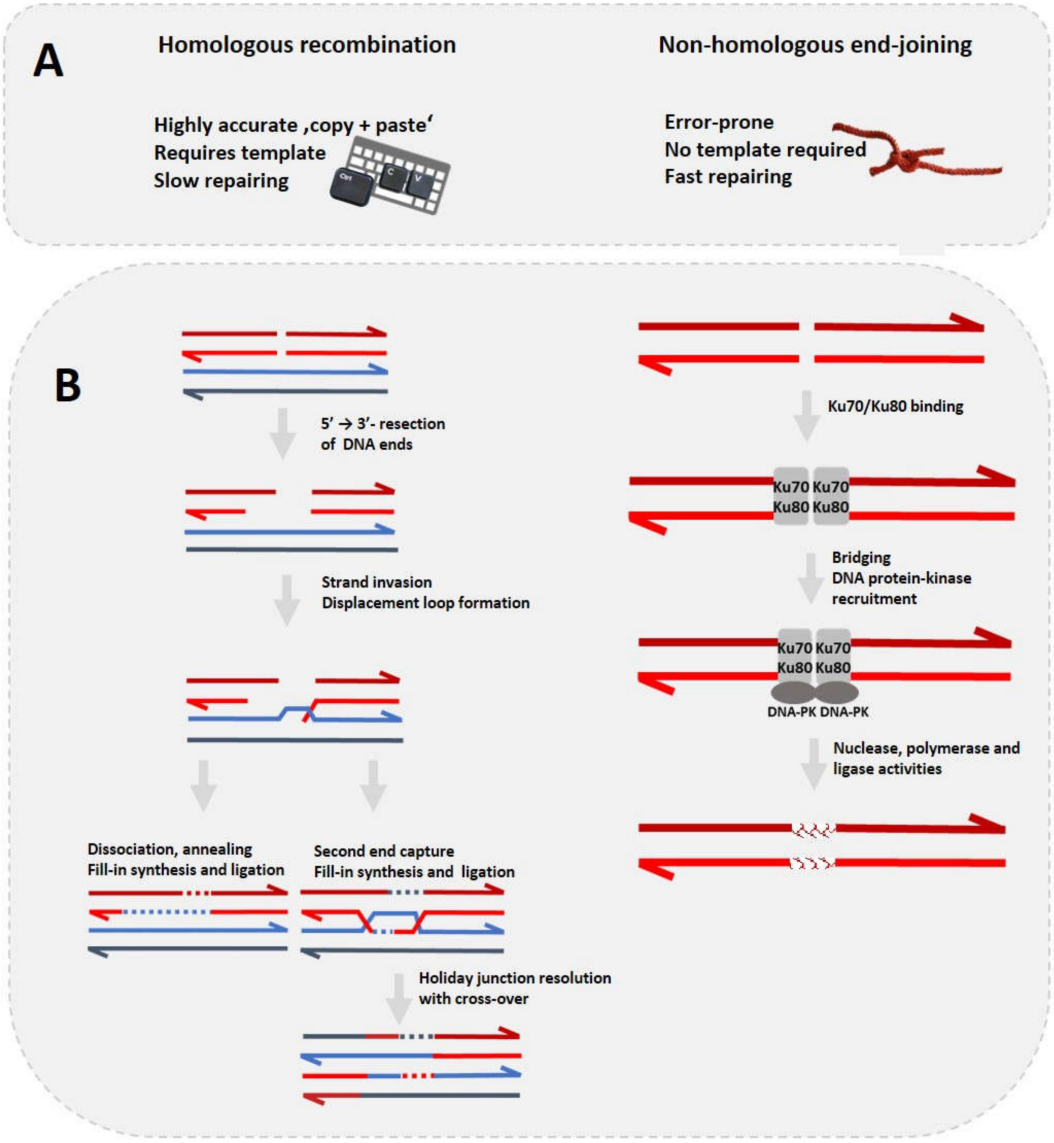

Figure 2. (**A**) Comparison of homologous recombination (HR) and non-homologous end-joining (NHEJ). On the occasion of a DNA double-strand break there are two possible repair mechanisms. HR (left panel) requires a template, takes several hours to complete but is highly accurate, and is comparable to the "copy + paste" computer command. NHEJ (right panel) does not require a template, is error-prone but faster, and can be associated with rope knotting. (**B**) Schematic overview of HR (left) and NHEJ (right) mechanisms. HR: after resection of DNA strands by exonuclease, Rad51 and other proteins form a nucleoprotein complex that searches for and invades a template strand to form a D-loop. The displacement loop can be resolved by synthesis-dependent strand annealing (bottom left), which does not result in cross-overs. Double-strand-break repair can resolve the Holiday junctions by either crossover (shown) or non-crossover. NHEJ: the Ku70/Ku80 protein dimer protects DNA ends from degradation/resection and recruits e.g., a DNA-dependent protein kinase catalytic subunit (DNA-PK). Sister chromatids are represented by two arrows, respectively, in red and blue. Light and dark color distinguish DNA strands. Arrows indicate 5′–3′ orientations of the DNA strands and dashed lines represent newly- synthetized DNA.

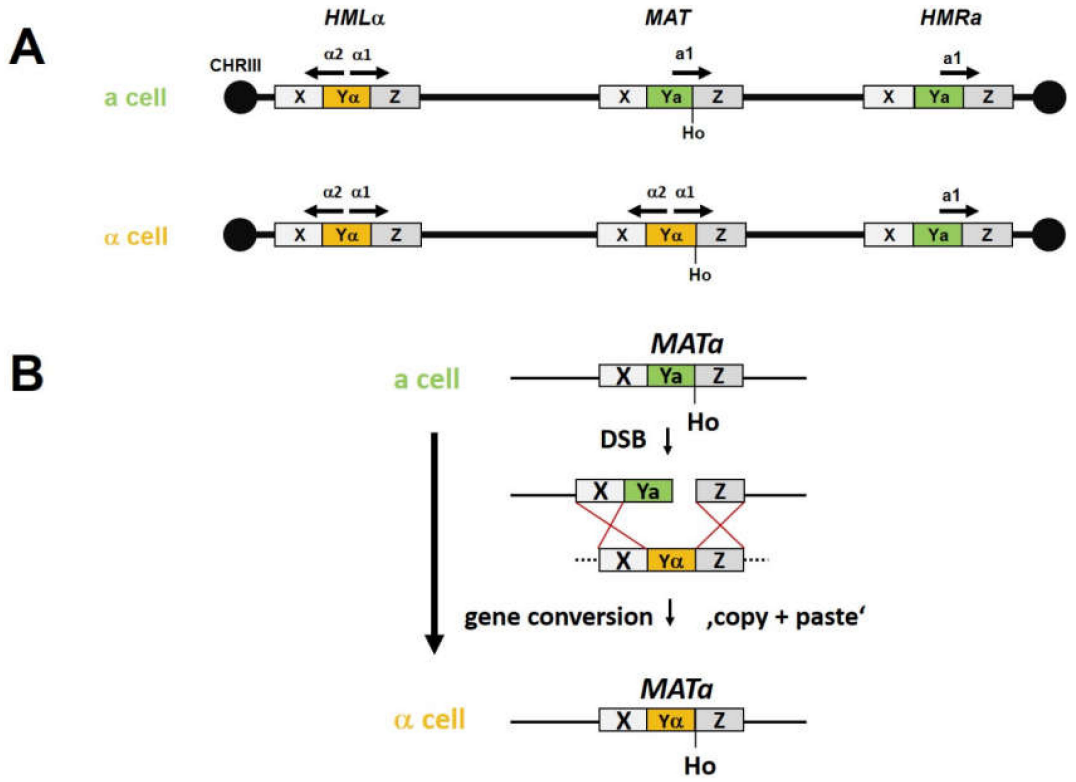

Figure 3. Mating-type switch in *Saccharomyces cerevisiae*. (**A**) Cell type in *S. cerevisiae* is determined by the *MAT* locus. In **a** cells there is the **a** allele at the *MAT* locus, while α cells possess *MAT*α. The *MAT* locus resides on CHRIII. At the telomeric ends of this chromosome there are silent cassettes of both mating types, *HML*α on the left and *HMR****a*** on the right. (**B**) Mating-type switching is initiated by the Ho endonuclease which cleaves at a specific site at the border of the Y-Z region generating a double-strand break (DSB). This triggers a gene conversion using, with high fidelity, the opposite silent mating type cassette as template for DSB repair resulting in a mating-type switch [51].

3. Progress in Yeast Genome Editing

Yeast itself is a part-time genome engineer when it re-edits its own genome to achieve a mating-type switch. However, yeast also uses this technology to upgrade itself to be able to survive harsh conditions such as sugar-rich environments of alcoholic beverage fermentations (see above). Once a method of yeast transformation was developed, man stepped in to engineer the yeast genome in various ways. The first genome edit was the gene conversion of a *leu2* strain that is auxotrophic for leucine back to leucine prototrophy by providing a *LEU2* gene that replaced the defunct allele by gene conversion [52]. This opened new opportunities to study yeast not only on the biochemical level but also on the genetic level. It resulted in several years of intense study of yeast genes. Yet, with the genome sequence of *S. cerevisiae* established in 1996, it became clear that for the majority of genes no functional studies had been done so far [53].

Mating-type switching uses flanking homology regions of about 700 bp [48]. Yet, remarkably, *S. cerevisiae* can perform these genome edits with a mere 35 bp of flanking homology region [54]. These short flanking homology regions can be incorporated in oligonucleotide primers so that with a single PCR reaction a disruption cassette can be produced (Figure 4). The efficiency of PCR-based gene targeting combined with a heterologous selectable marker gene, *kanMX*, was then used to establish the yeast gene deletion collection [54,55].

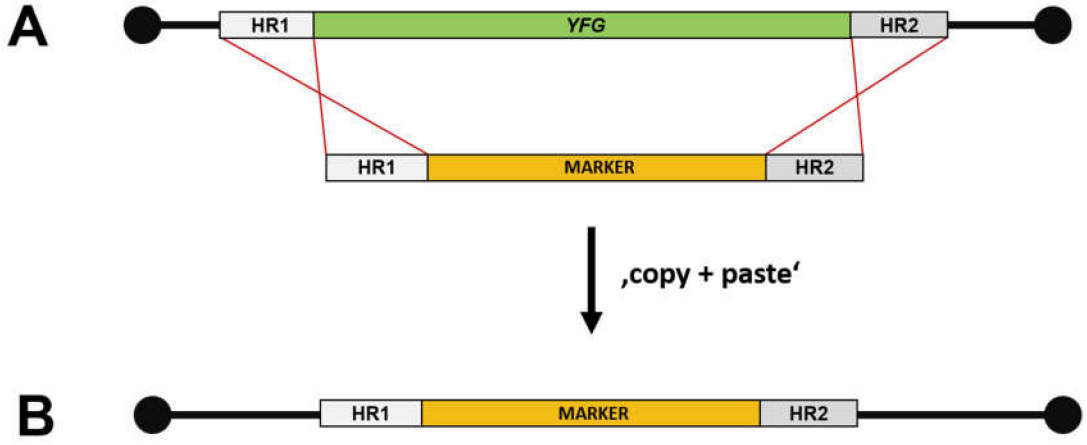

Figure 4. PCR-based gene targeting. (**A**) A disruption cassette with a selectable marker gene (MARKER) flanked by 5′- and 3′-homology regions (HR1, HR2) is obtained by PCR amplification and transformed into yeast cells. HR1 and HR2 direct recombination into the target locus of *YFG* ("your favorite gene"). (**B**) The marker cassette is now integrated into the locus.

Interestingly, in lager yeast hybrids of *S. cerevisiae* and *S. eubayanus*, PCR-based gene targeting could not be employed due to the low efficiency of HR. This seems contradictory but may be due to the polyploidy/aneuploidy of these strains. Overexpression of *RAD51*, which encodes a key role enzyme of the strand-invasion step or HR (see Figure 2), helped to improve this situation allowing genome edits of these strains [56]. The high in vivo recombination efficiency of *S. cerevisiae* is also used for in vivo cloning, which is advantageous over cloning via restriction endonucleases and ligation as it works seamlessly without the need to introduce foreign restriction sites to a sequence [57]. Yeast can help building synthetic constructs, e.g., promoter–gene fusions and plasmids and can even assemble complete bacterial genomes from overlapping fragments [58–60]. The limits of this yeast assembly machine have been probed and they are astonishing—in one experiment, 25 large DNA fragments of 17 kb to 26 kb in length were used to assemble the genome of *Mycoplasma genitalium* in *S. cerevisiae* [61,62]. This technology was also used to assemble the completely synthetic *S. cerevisiae* genome (the Yeast 2.0 or Sc2.0 project) [63].

To retrieve transformants with the desired genome edits selectable marker genes are used. This poses two problems—for multiple genome edits several marker genes are required and this is more problematic as these marker genes often code for antibiotic resistance that when left in the genome could contribute to the spread of these genes in the environment. To overcome this problem, marker-recycling methods have been developed. They are based either on non-inducible mitotic recombination between direct repeats or on inducible systems utilizing a recombinase [64]. The *URA3* gene is very useful as it serves as a selection marker to trace genome edits, but it can then be selected against using 5′-fluoroorotic acid. In this way mutant strains that have lost the selection marker via recombination or plasmid loss can be identified [65,66]. The Cre-loxP method is based on the phage P1-specific recombination system. loxP is a 34bp sequence recognized by a recombinase encoded by *cre* gene [67]. A marker cassette flanked by *loxP* sites is integrated in the yeast gene locus of interest. Then, the Cre recombinase is expressed in *S. cerevisiae* (e.g., under the control of the inducible *GAL1* promoter on a *CEN-ARS* plasmid). Recombination between *loxP* sites leads to the removal of the internal DNA sequence leaving one loxP site behind. Moreover, simultaneous multiple cassettes recombination can be performed [68].

The state-of-the-art technology is seamless genome editing that does not leave behind a genomic imprint at the changed locus. In this way only the desired edits are present in the final strain. A seamless technique that revolutionized the field of genetic engineering, is CRISPR/Cas9 (CRISPR = clustered regularly interspaced short palindromic repeats; Cas = CRISPR associated), which was first applied in *S. cerevisiae* in 2013 [69]. CRISPR is the basis of a prokaryotic adaptive immunity system protecting

from incoming phage DNA [70,71]. Cas9 is an endonuclease, which is guided to its target locus by a small RNA. This guide RNA is programmable, i.e., the target sequence can be engineered so that any target in any organism can be manipulated (Figure 5) [72,73]. CRISPR and HR complement one another in generating precise, seamless, and marker-free genome edits.

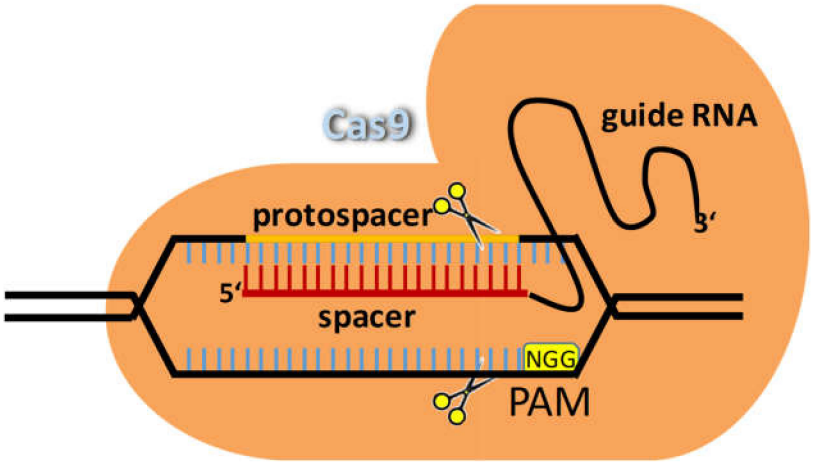

Figure 5. Genome editing via CRISPR/Cas9. Cas9 is a restriction endonuclease (depicted by the scissors) that introduces a DSB at a target sequence defined by the guide RNA. The guide RNA base pairs with the protospacer sequence and Cas9 cuts if the protospacer adjacent motif (PAM, here NGG required for the *Streptococcus pyogenes* Cas9) is present.

A profound advantage of CRISPR is the ability to multiplex genome edits. With the ability of Cas9 to interact with different guide RNAs, multiple targets can be attacked in a single cell [74,75]. Multiplexing of genome edits allows complex traits to be mapped, which was used, e.g., to decipher mutations that improve ethanol or glucose tolerance [76]. In this rapidly-developing field other endonucleases have also received attention, in particular Cpf1 (CRISPR from Prevotella and Francisella 1/Cas12a) which is smaller in size than Cas9, lacks a tracrRNA, and utilizes a T-rich PAM is a suitable alternative [77].

4. Genome Editing in the Beverage Industry

Key advantages of genome editing over random mutagenesis and selection are the full control over the genome edits and the minimal changes introduced into the genome leaving all other genomic regions unaltered. These key features should be much more appreciated and better communicated into the public domain. Strains to be used in the food and beverage sector need to be approved by regulatory bodies such as the Food and Drug Administration (FDA) in the US, the European Food Safety Authority (EFSA), and the OIV (International Organisation of Vine and Wine). However, beyond facing lengthy regulatory procedures in actually getting approval to use GM strains, there are general consumer distrust and negative opinions towards GM food and beverages. Reasonably, GM yeasts need to compete with regular yeasts in winemaking, and only if they produce a decisive benefit that finds customer approval will any strain and its derived products be successful. Thus, the genome engineers will need to provide this proof to make a convincing and marketable case [78].

There are two generally-different methods of yeast strain design, one that does not introduce foreign DNA and another that uses, e.g., foreign genes to alter metabolic pathways in yeast. The first generates genome edits without introducing any DNA or only introducing genetic material present in the genus *Saccharomyces*. The idea behind this approach is that these edits could, in principle, also occur naturally by breeding and introgression. This approach is commonly known as 'self-cloning' and acceptable in several countries i.e., these edits are not regarded as generating recombinant organisms [79]. Self-cloned strains include, amongst others: (i) sake and brewer's yeast strains overexpressing the *ATF1* gene, which encodes an alcohol acetyltransferase that improves ester formation and flavor production in yeast [80];

(ii) the construction of a brewer's yeast strain with the integration of a copper resistance gene (*CUP1*) into the *ILV2* locus, which increases the copper tolerance of this strain, while deletion of one allele of α-acetohydroxyacid synthase gene *ILV2* results in decreased diacetyl production [81]; (iii) an alternative approach to reduce diacetyl levels used the integration of an extra copy of *ILV5*, which acts downstream of *ILV2*, into a bottom fermenting lager yeast strain [82]; and (iv) the overexpression of *DUR1,2* or *DUR3* in sake and wine yeasts [83,84]. Dur1,2 is a urea aminolyase that converts urea into CO_2 and NH_3. Otherwise, urea may be converted into ethyl carbamate, which is carcinogenic. Dur3 encodes an uptake transporter of urea. A strain overexpressing this gene removes urea from the medium.

This shows that these edits were done on carefully-selected targets that generate beneficial yeast strains not found in nature [85].

The second approach in yeast genome editing also employs genes from other organisms. This is exemplified by the malolactic yeast strain ML01, which has gained FDA approval [86]. This yeast was designed to be able to carry out malolactic fermentation. Malolactic fermentation, i.e., the decarboxylation of malate into lactate, is a secondary fermentation carried out preferably by *Oenococcus oeni* which follows alcoholic fermentation. This reduces the acidity of many wines. Yet, the use of lactobacilli has several drawbacks, which could be ameliorated by using a wine yeast that takes over this process. To generate ML01 the open reading frames of the *O. oeni* malolactic gene *mleA* and the *Schizosaccharomyces pombe* malate transporter gene *mae1* were placed under control of the *S. cerevisiae PGK1* promoter and terminator regions and stably integrated into the *S. cerevisiae URA3* locus [86]. *PGK1* encodes a highly-expressed glycolytic 3-phosphoglycerate kinase. The ML01 strain can now simultaneously perform alcoholic and malolactic fermentations. However, even though this strain has gained approval by the FDA, it was not able to replace bacterial malolactic fermentation in the wine industry.

5. Genome Edits in Non-GM Yeasts

Due to customer refusal and legal issues in getting GM yeast approval the industry standard remains on non-GM yeasts. Conventional strain improvement strategies revert to the selection of novel *Saccharomyces* yeasts and use yeast breeding within *S. cerevisiae* strains but also between e.g., *S. cerevisiae* and *Saccharomyces kudriavzevii* to increase genetic diversity [87]. To introduce change mutagenesis and selection or evolutionary engineering strategies can be employed [88,89]. The use of non-conventional and non-fermentative yeasts has been suggested e.g., as flavor enhancers or for the reduction of ethanol content [90,91].

Several selection procedures have been developed over the years to improve yeast strains. To enhance flavor output via channelling amino acids through the Ehrlich pathway is of key importance to improve ester production in yeast [92]. Here the use of toxic fluoro-amino acids has been helpful in selecting yeasts that overproduce the corresponding amino acids by cancelling feedback inhibition. Using tri-fluoroleucine, naturally-occurring mutants of the *S. cerevisiae LEU4* gene can be obtained from a culture by simple selection in a non-GM fashion. These mutants show increased leucine biosynthesis, which will result in improved isoamyl acetate flavor production [93,94]. Similarly, mutants resistant to fluorophenlyalanine will be improved in 2-phenylethyl acetate production [95]. To date, these strains have not been commercialized. In addition, no study has been reported to undertake a comprehensive genetic analysis as to which of these mutants is the best flavor producer. To this end mutational analysis of a 70 bp region of the *LEU4* gene could be insightful [93].

This demonstrates that workarounds have been developed over the years to improve wine and beer yeasts without genetically modifying them. These efforts are often compared to ploughing a field with many men, horses, and plows instead of using tractors. Grinding these gears has resulted in the characterization and development of several target genes (Figure 6). We still, however, place a billion-euro industry at the hands of technology that dates back to the days of Hansen, Wortmann, and Winge. For nostalgia, the use of the 1890 clock shown in Figure 1 is nice. Yet, this particular clock,

due to its age and lack of maintenance, lags 10 min per hour behind real time and is certainly not comparable in accuracy and efficiency to an atomic clock [96].

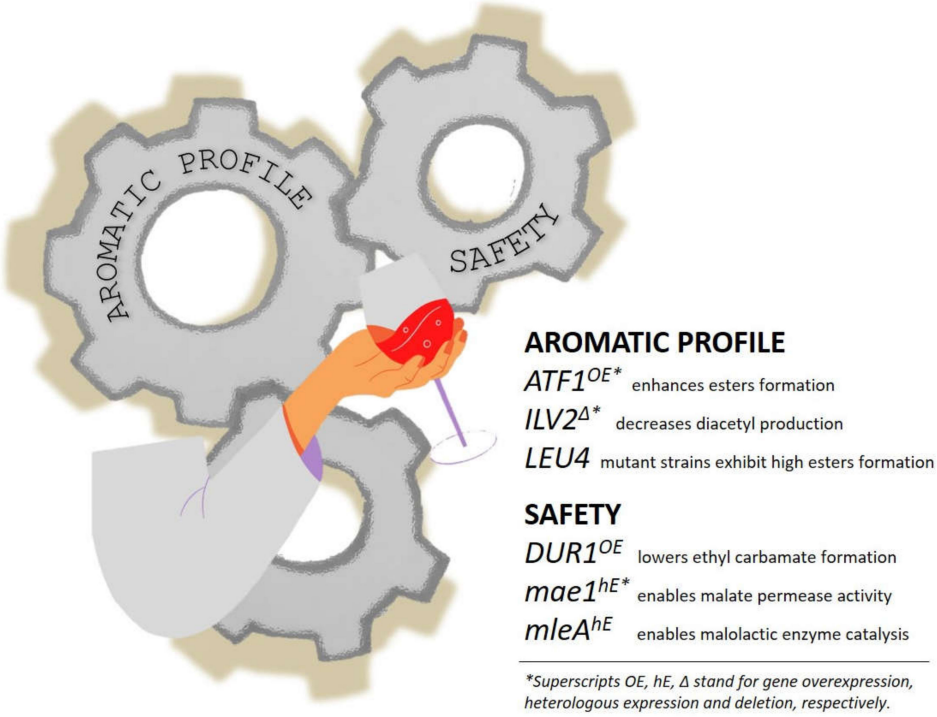

Figure 6. Grind the gears of wine and beer yeasts. The complexity of the aromatic profile can be improved i.e., by disrupting the α-acetohydroxyacid synthase gene *ILV2* with the decrease of diacetyl production ("butter flavour") or enhancing isoamyl acetate flavor production ("banana flavour") by overexpressing the alcohol-acetyl transferases encoded by *ATF1* or mutated *LEU4*, key-role enzymes in the leucine biosynthesis pathway. The overexpression of urea aminolyase encoded by *DUR1,2* decreases the production of urea and therefore of ethylcarbamate (urethane) that has discussed carcinogenic risk. The heterologous expression of *S. pombe mae1* and *O. oeni mleA* enables yeast to assimilate malate and convert malic acid into lactic acid, respectively, avoiding the necessity of supplementing bacteria to fermentative batches. For a comprehensive reading about genome edits in wine yeast see [97].

6. Genome Editing in the 21st Century

So why do we not employ a trusted technology and embrace the state-of-the-art technology available for yeast genome editing? There are two main issues that are of concern: (i) regulatory requirements on risk assessment including health and safety issues as well as the effect on the environment and (ii) verification of the genome edits.

Since *S. cerevisiae* is generally recognized as safe, all genome edits in yeast gain the same status. Yet, with each novel strain any impact on safety and potential effects to the environment and its biodiversity need to be assessed. Interestingly, for wine yeasts it was demonstrated that starter culture yeasts were only recovered near the wine cellars of the winery in which they were used and they did not persist and were lost in subsequent years [98]. If one wanted to implement additional strain-specific safety features that decrease the fitness of wine yeasts in natural environments, one could, e.g., remove from these strains the ability to mate and sporulate. The inclusion of signature barcodes could also be used for bio-tracking to trace strains and evaluate their fitness in different environments [99,100].

For the verification of genome edits whole genome sequencing has become the gold standard. This will provide comprehensive data on the intended and any potentially-unintended genome edits and thus secure an unprecedented level of knowledge of each strain. This level of accuracy has already been put to the test in the Sc2.0 synthetic yeast project. The aim of the project is to generate the first functional synthetic eukaryotic genome [63]. During the project complete synthetic chromosomes

have been assembled in a parental yeast strain. Defects in the fitness of a parent strain bearing one complete synthetic chromosome were rigorously hunted down to ill-designed edits or construction errors which upon their removal resulted in strains of wildtype-like fitness, thus demonstrating the power of modern genome editing and verification tools [101,102].

7. Conclusions

The very conservative alcoholic beverage sector certainly does not gain first-mover status for the introduction of state-of-the-art genome editing technology. However, HR combined with next-generation sequencing provides an important advance to the field. We can trust HR in producing yeasts for the beverage sector because we do not have to put all our trust into it; we can verify every single base pair change by genome sequencing. Thus, strains can be designed, generated, and verified to contain exactly those and only those changes that were intended. This provides for maximum safety and reliability, which should convince regulatory authorities such as EU and OIV to install regulations allowing the use of genome-edited yeasts for the benefit of advancing the beer and wine sector.

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