A SHIFT IN THE RIGHT DIRECTION - UNLOCKING GENETICALLY MODIFIED YEAST TO MAKE MORE CONSISTENT, HEALTHIER, AND MORE FLAVORFUL WINE

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ABSTRACT

The winemaking industry has made a strong stance of not using genetically modified organisms until consumer acceptance. Thus, the winemaking industry is lagging behind the viticulturists, and unable to adopt the benefits of this technology for their practices. Genetic modifications have been adopted in many other industries, therefore, many of the labelling laws have well been established - United States and Canada are the first countries that have allowed optional labelling on food products. Genetic modifications have been achieved through breeding techniques in the past, but recently CRISPR/Cas9 system has led to more precision and allows scientists to create GMOs with intent rather than chance. Some of the genetically modified yeast have been improved for fermentation kinetics, aroma/flavor enhancements, alcohol reduction, eliminating carcinogens, and introducing malolactic fermentation capabilities. Genetic modifications are a part of the future, it is time to harness the technology and learn how to put it to beneficial use before passing it down to further generations.

BIOGRAPHICAL SKETCH

Heather Rene Deulen holds a Bachelor of Science (*cum laude*) from Washington State University Vancouver in General Biology with a minor in Psychology and Molecular Biology conferred upon her in 2019. In 2020, Heather attended Cornell's Agriculture and Life Science department (CALS) for a Master of Professional Studies in Food Science. Heather is specializing in the field of Enology and Viticulture and her degree will be conferred at the end of December 2020. She has studied in Bordeaux, interned in Napa, and is now pursuing her dreams of co-owning a winery with her dear friend Yanan-Lu, whom she met while studying at Cornell University.

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CHAPTER 1. INTRODUCTION

Wine is not only a delectable beverage enjoyed in recent times, but it has also made its way through history, across cultures, and has been used for countless symbolic practices. Contents of white wine were discovered in King Tutakhamun's tomb; the royal families drank wine, and believed it was necessary for a good after-life (Guasch-Jane et al. 2006). Archaeologists have found evidence that suggests wine was stored in pottery jars in China as early as 7000 BCE for medical, social, and religious reasons (McGovern et al. 2004). The transparency of wine throughout history demonstrates how it has been cultivated as early as humanity, suggesting humanity and wine are inseparable.

For hundreds of years winemakers have been trialing fermentation practices, readily adopting favorable ones, and leaving less suitable practices in the past. Trial and error improved winemaking techniques, but it was not until the mid-1800's that the role of microbiology and its impact on wine began to be understood. Louis Pasteur, the father of microbiology, discovered that living yeast cells were the main actors that convert sucrose into ethanol (Pasteur, 1858). Fermentation became known as respiration without the presence of air, a vital process that *Saccharomyces cerevisiae* carries out in juice.

S. cerevisiae is one of the most well-studied eukaryotic organisms in the world. The first successful transformation of wine yeast was completed in 1978 where cells unable to make leucine were converted to cells that can make their own leucine by being induced to take-up extracellular DNA containing leucine synthesis genes (Hinnen, 1978). In 1996, *S. cerevisiae* was the first eukaryotic organism to have its entire genome sequenced and published (Goffeau et al. 1996). *S. cerevisiae* is one of the most useful eukaryotic organisms for biological research due to the ease of manipulating, editing, and stressing the cell. *S. cerevisiae* can undergo mitotic and

meiotic reproduction, which increases its potential for experiments. Scientists can control the sexual cycle in the laboratory making it one of the most well understood eukaryotic organisms, however, this does not always hold true for its life cycle in natural settings because of all the uncontrolled variables.

One of the greatest struggles in the new age of winemaking is meeting consumer demand with growing competition among winemakers. Consumers are seeking high quality wines that are also unique, putting pressure on the industry to revamp winemaking techniques. We are at a turning point in history with consumers demanding greater wine diversity, and we need more tools to meet these demands. One of the most recent and significant advancements was the use of dehydrated yeast. Uninoculated fermentations were conducted up to 1963, which mostly changed once active dry wine yeasts became commercially available for use (Reed & Chen, 1978). Dehydrated yeasts revolutionized the industry because winemakers could predict the outcome of fermentations and select yeasts based on certain flavors, aroma profiles, and fermentation capabilities.

Creating novel yeast strains that express certain traits can be accomplished in various ways, including classic breeding/hybridization, spheroplast fusion, and using modern biotechnology. Hybridization techniques are based on simple Mendelian genetics. Yeast cells are induced to undergo meiotic reproduction, turning into gametes (also called spores). Gametes of different strains can then merge to create a hybrid strain (Sipicki, 2008). Scientists will select cells that express certain traits and continue producing offspring hoping to establish a new strain expressing the desirable phenotypes. This will create a mosaic of the two parental genes (Sipiczki, 2008). It seems practical to mate yeast until desirable traits are achieved, however, there is always the risk of producing undesirable traits during recombination (Kishimoto, 1994).

Genetic hybridization is one of many technologies used to create new strains, however, technological advances claim to be able to create new strains with higher precision and accuracy.

Hybridization is not the first method humans utilized to select for certain traits for self-serving purposes. Crop domestication is defined as people artificially selecting plants for cultivation practices, taste, yield, and storage purposes (Chen et al. 2015). Over the years, people have selected the most superb crops and left those less desirable in the past. A portion of the farming industry have moved towards genetic engineering to create delicious products with fewer interventions (e.g., less water, herbicides, and pesticides). The winemaking industry has largely lagged behind the farming industry and it is time for the acceptance of genetically modified organisms (GMOs) to advance the field as a whole.

Genetic engineering is a process of manipulating an organism's DNA through the use of biotechnology. Biotechnology, and more specifically genetic modification, remains a controversial topic in modern day society, and is generally not accepted by the winemaking industry, and its consumers. The aim of this article is to shed light on how GMO yeast can benefit the winemaking industry and bridge the gap between consumer demand and growing winemaking competition. While questions over consumer acceptance remain, there is no question that GMO yeast can expand the winemakers' toolbox by introducing yeast that can reduce off-flavors, improve fermentation kinetics, create new aroma profiles, remove toxic compounds, reduce ethanol, reduce sulfur, increase survival behavior, and change the clumping behavior in wine.

CHAPTER 2. LABELING LAWS FOR GMOS IN THE WINE INDUSTRY

Genetic modifications (GM) are used to accelerate the rate of genetic adaptations in the face of the global warming crisis. Plants and animals are normally capable of adapting to a changing environment; however, humans are accelerating the rate of change to unsustainable levels. Due to the crisis, each country has created a set of guidelines and rules to establish what foods are acceptable for genetic modifications, and the proper way to inform the public. Below is a brief summary for GMO usage in prominent wine-producing countries.

United States

In 2016, the United States published Public Law 114-216 'National Bioengineered Food Disclosure Standard'. They defined GMOs as food that has recombinant DNA that would not be achieved through conventional breeding techniques or found in nature (National Bioengineered Food Disclosure Standard Act of 2016). Public Law 114-216 also states that animals who have eaten genetically modified food, food that does not have any detectable GM DNA in the final product, and food in which the primary or secondary ingredient is not GM, then it does not have to be labeled as a GMO. Per the FDA, the winemaking industry does not have to disclose if their juice was fermented with the aid of genetically modified yeast, so long as the cells are filtered out prior to bottling. Set forth by the pressures of non-GMO groups, food can bear a QR code for people to receive more information about their food (Bovay and Alston 2018).

Canada

Canada allows voluntary labeling because they have found the safety and nutrition levels of GMOs indistinguishable from their non-GM counterparts (Government of Canada, 2020). Although the United States and Canada have legalized the use of GM yeast, there is no official documentation of the frequency of use in both countries.

New Zealand

New Zealand has shown great deal of caution towards GMO's and currently has no commercial crops, fresh produce, or meat sold that has been genetically modified. They do however import GMOs into their country under strict regulations. In 1996, they passed the Hazardous Substances and New Organisms Act (HSNO) to help review their environmental legislation. Section 40 outlines the application and approval process to import GMOs into the country. Per regulations, the GMO must be identified, the project and experimental outline must be provided, and a detailed list of biological materials and foreign DNA material, along with a list of the adverse effects of the organism on the environment must be included (HSNO Act of 1996). An organism can be declined if it causes adverse effects on native species, causes diseases, becomes a vector for plants, animals, or human disease, causes adverse effects to natural habitats, causes a disruption in New Zealand's inherent genetic diversity, or causes adverse effects on human health. Over fifty GMOs have been approved for importation into New Zealand, including soybeans, potatoes, wheat, rice, canola, sugar beet, lucerne, safflower, corn, and cotton (Current GM Applications and Approvals, 2019). Despite New Zealand being one of the most rigorous countries to approve importation of GMOs, the winemaking industry has stated they would not accept GMOs even if they become legal. Although fifty GMOs have been declared safe and legal for farming in New Zealand, the wine industry has taken a strong stand against GM vines or GM yeast.

Australia

Australia proposes strict regulations for growing, importing, and selling GM items within the country. To date, genetically modified canola, cotton, and safflower are farmed in Australia.

The Record of GMO Dealings (Part 9, Division 6, the Act), outlines the safety of GMOs and keeps a record of applicable information for the public (Record of GMO Dealings). The Australian government requires testing similar to New Zealand to ensure the safety of the product for their consumers. The Gene Technology Act 2000 (which took effect on June 21st, 2001) is supported by the Record of GMO Dealings and is meant to help support the health and safety of their community. Although a select few GMOs have been adopted in Australia, it is clear that the winemaking industry will not be doing so. Per the Australian Wine Research Institute, Australia wants to gain consumer acceptance and understand the potential roles of each organism in the environment before they adopt them into the industry. They claim to not be anti-GMO, but in 2005, they stated that there would be no use of GMOs in the foreseeable future, and they have not budged since.

South Africa

South Africa passed the Genetically Modified Organisms Act of 1997 to outline the research, production, and marketing of GMOs. An Executive Council, Advisory Committee, and Registrar have been elected to oversee the use of GMOs. Any research on the use of a genetically modified organism, requires an application be submitted to the Registrar for approval. The Registrar reviews potential adverse effects from the engineered phenotype, the likelihood of adverse effects being realized in the future, a consequence evaluation, and an estimate of the overall risk (Roudik, 2014). Once the Registrar approves the application, it is sent to the Executive Council for final approval. South Africa currently farms maize, cotton and soya for herbicide and insect tolerance. The use of GMOs in the wine industry is strictly prohibited in South Africa. In 2006, the Registrar was sent an application for the use of ML01 yeast, which was regarded safe for use but was denied because of socioeconomic reasons and the opinion they

would denigrate their reputation by approving a GM yeast. Despite the fact that wine GMOs are not legalized for commercial use in South Africa, they have one of the leading research institutes for developing both non-GMO and GMO yeast strains at the Institute for Wine Biotechnology at Stellenbosch University.

European Union

The European Union (EU) has a huge interest in protecting their consumers and ensuring they are aware of the products they are purchasing. The *Treaty establishing the European Community* and its article 169(1) *Treat on the Functioning of the European Union* established their standpoint on consumer protection:

> In order to promote the interests of consumers and to ensure a high level of consumer protection, the Union shall contribute to protecting the health, safety and economic interests of consumers, as well as to promoting their right to information, education and to organize themselves in order to safeguard their interests (*Consolidated version of the Treaty Establishing the European Community,* 2007).

The EU protects consumers through a highly regulated labeling system (Du, 2014). Despite it being mandated by law for food to be labeled as GM, the EU still has a very limited market of GM foods -- this eliminates the choice for consumers and provides them with food of so-called "natural" origin. For instance, the EU has banned the use of Bt corn in society, even though it has been proven safe for use and it will not disrupt the ecological system (Agnes et al. 2010; Pellegrino et al. 2018).

Per the Regulation (EC) No 1830/20003, any products in the EU with more than 0.9% GM ingredients must be labeled as such (Berrie, 2011). If a GM yeast is used to ferment the

juice, it will be filtered out with a 0.45-micron filter, therefore, the final product would not have genetically modified organisms.

Country or Countries	**Labeling Rule**	**Threshold Level**
European Union	Mandatory	0.9%
Australia and New Zealand	Mandatory	1%
United States	Mandatory	0.9%
South Africa	Mandatory	Not Specified
Canada	Voluntary	5%
• Labeling exemptions not included		

Table 1. Labeling requirements by Country and Trade organization – data compiled from Gruère and Rao (2007).

CHAPTER 3. HOW TO CONSTRUCT A GMO

Genetic engineering can be accomplished in several different ways; however, the end goal is always the same: editing the genome to acquire a desired mutation. Many technologies have been developed to aid in genome manipulation. Transformation was the first technology developed to induce cells to take up extracellular DNA, which is simply forcing the cell to incorporate foreign DNA (Hinnen et al. 1978). In addition, PCR is used to amplify specific segments of DNA, restriction enzymes and the ligase enzyme allowed researchers to cut and paste DNA sequences, selectable plasmids can be used to transport genes into organisms, DNA synthesis allows researchers to custom design DNA sequences, and DNA sequencing technologies are used to directly read the genetic code. More recently site-specific nucleases have been employed to significantly boost the efficiency of genetic modification; the CRISPR/Cas9 system is a popular example of this technology. Combining these technologies with some of the cell's normal biology, including DNA repair mechanisms non-homologous end joining (NHEJ) or homology-drive recombination (HDR), allows scientists to efficiently modify genomes. Traditional breeding techniques (e.g., mating, hybridization, and spheroplast fusion) also achieve alterations of the genome, however, since the products of those techniques are not considered GMOs, they will not be discussed further in this manuscript.

The first step of constructing genetically modified yeast is selecting the correct gene of interest. The field of genetics has undergone extensive research to determine the roles of individual genes. Despite the fact scientists have just begun sequencing entire genomes, the first being S. *cerevisiae* in 1996, genome sequencing has radically expanded the toolbox for many molecular scientists. Genome engineering is only limited to the imagination of its users, which is why there is vast potential for practical applications.

Transformation is a process where a cell takes up extracellular DNA and incorporates it into its own genome. There are three ways that molecular geneticists can increase the likelihood of the transformation reaction: chemical-based, electricity based, and ballistic-based. Cells can be treated with chemicals to increase their competence (a.k.a. ability to take-up DNA); calcium and magnesium are typically used for *E. coli*, while lithium is used for *S. cerevisiae*. Another way to transform DNA into the cell is through electroporation. Electroporation causes small holes in the cell wall via electrical current, thus allowing passage for DNA. The ballistic-based technique uses a gene gun: small particles, often gold, are coated with DNA and quite literally shot at the cells. Some fraction of the remaining living cells will incorporate the DNA.

To confirm that cells have incorporated target DNA, molecular biologists often use selection. Similar to natural selection in evolution, where only the fittest survive, scientists create a hostile environment to all cells except those that incorporate the target DNA. This allows for "selection" of desired organisms. Molecular biologists will typically include a toxic drug resistance gene or a nutrient utilization gene along with any other target DNA. The cells are then placed in an environment with either the limiting nutrient or the toxic drug, and only transformed cells will survive.

One of the easiest ways to move DNA between microbial cells is to use plasmids. Plasmids are small, self-replicating, circular DNA. While plasmids are found in nature, and often contain genes related to pathogenicity, humans have engineered plasmids to be useful tools. Most plasmids used in yeast have several common DNA sequences for proper function, in addition to a copy of the gene of interest: an origin of replication allows the plasmid to be replicated in *E. coli*, while a separate replication element is used for yeast replication, a "polylinker" region with many restriction enzymes cut sites for cutting and pasting DNA, an

antibiotic resistance gene for selection in *E. coli*, and a nutrient utilization gene for selection in yeast. These self-replicating circles of DNA will be transformed into the yeast cell - allowing the cell to remain viable when placed into a selective growth media.

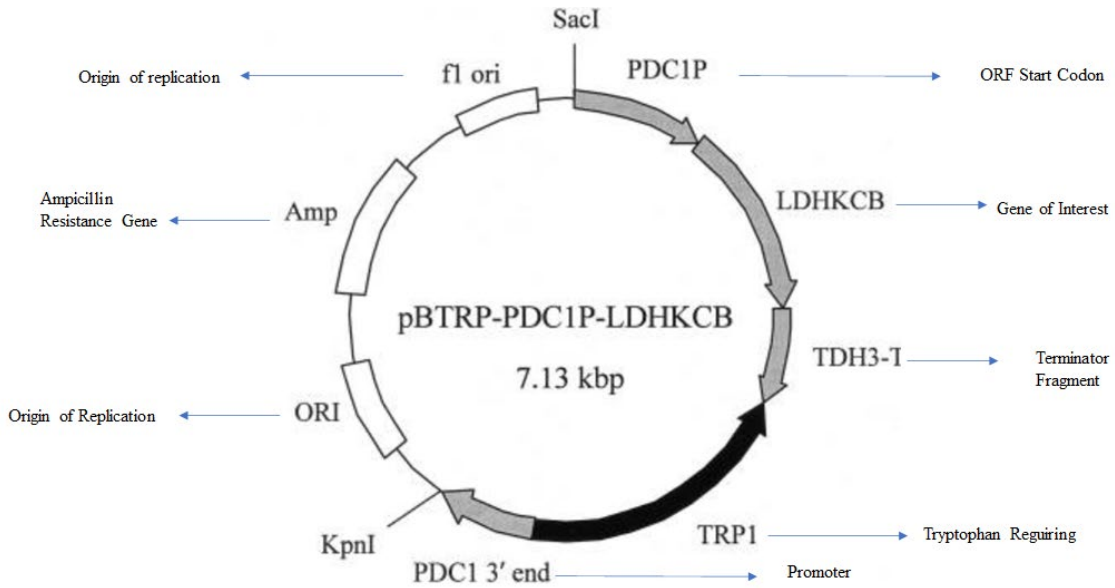

Figure 1. An example plasmid. This plasmid and plasmid map were constructed by Ishida and colleagues (2005). LDH codes for dehydrogenase desired to increase the production of L-lactic Acid. Two origins of replications, ampicillin resistance, tryptophan requiring, promoters, and terminators are all labeled on the plasmid. The f1 ori is the origin of replication for *E. coli*. Tryptophan is the auxotrophic marker for selection assays. PDC is a promoter to encourage replication.

Figure 1 is an example of a plasmid used to transform the LDHKCB gene (lactate dehydrogenase) into a yeast cell. The plasmid bears two selectable markers to ensure correct selection in yeast or *E. coli* (resistance to the antibiotic ampicillin for *E. coli* selection, and the *TRP1* gene confers ability to produce the amino acid tryptophan for yeast selection). Plasmids allow for overexpression or different expression of genes from the organism of interest, or new genes from another organism. The plasmid from Figure 1 used genes from another organism,

thus yeast cells containing this plasmid would be considered a genetically modified organism since it has foreign DNA.

For a cell to maintain exogenous DNA and pass it onto future generations, the gene of interest must result in a stable phenotype that will increase the fitness of the species (Parker, 2001). Transformation is a powerful tool in the field of genetic engineering and has led to the development of yeast strains which produce low levels of ethanol, decrease urea production, increase the cleavage of thiols and optimize fermentation kinetics (Cuello et al. 2017; Manzanares et al. 2003; Swiegers et al. 2007; Volschenk et al. 1997).

In addition to plasmids, scientists can also directly modify the chromosomal DNA of a living organism. The chromosome-based method uses multiple aspects of normal cell biology, including random or directed double-stranded breaks of the DNA, and also the main DNA repair mechanisms. NHEJ and HDR repair double-stranded breaks in DNA and can be used to create small nucleotide polymorphisms (SNPs), small insertions or deletions (indels), large insertions, or large deletions of DNA to alter the genome. NHEJ does not require a repair template DNA strand and is often considered mutagenic as the two ends of the double-stranded break are often trimmed back and then ligated together. NHEJ often results in indel mutations. HDR on the other hand, requires a repair template to guide DNA repair. The repair template DNA sequence ends must mimic the DNA sequences flanking the double-stranded break; therefore, the repair enzymes will be tricked into thinking that the template is native DNA and repair the double stranded break with the repair template. The repaired DNA will now contain any sequences engineering into the repair template.

To perform HDR engineering one must first PCR amplify the gene of interest and include flanking regions identical to the place in the genome where the DNA will be inserted. Then the template must be transformed into the cells. Proper integration into the chromosome will only occur in cells that have a damaged DNA in the exact spot the new DNA is expected to go. This process relies on the fraction of cells that have a double-stranded break in your region of interest, the fraction of those that are competent for transformation, and the fraction of those that are able to stably integrate the DNA into the chromosome, so the correctly engineered cells are fairly rare within a large population. Therefore, selection is often required after transformation to find a cell that expresses the desired mutations. One of the limitations within this approach is gambling on the frequency of cells with double-stranded breaks in your region of interest. Site-directed nuclease enzymes, like CRISPR/Cas9 are machines that cut the DNA at a specific, defined location, decreasing this gamble in the molecular biology world.

CRISPR is an acronym for clustered regularly interspaced short palindromic repeats and is a natural part of the bacterial immune system for defense against bacteriophages (viruses that only infect bacteria). The CRISPR system relies on a nuclease enzyme to cut viral DNA, and Cas9 is the version of that nuclease widely used in genetic engineering. Cas9 is a protein that constantly scans DNA and when it encounters target DNA, it can cut the genome. Jennifer A. Doudna and Emmanuelle Charpentier are the bright female minds that came up with the idea of using the CRISPR/Cas-9 protein to edit the genome and they were awarded the Nobel prize in October of 2020. CRISPR/Cas-9 is still a highly controversial topic since it is a recent innovation and there are not clear guidelines for the use of the technology.

Cas9 is a large protein that has endonuclease activity. A chimeric RNA molecule, known as gRNA, will direct the Cas9 protein to the correct base location based on its complementary

base sequences. Once the Cas9 registers the sequence, it will create a double stranded cut in the DNA (Figure 2). This allows scientists to alter the DNA in the desired location (Muyddon et al., 2019). Once the double stranded DNA break is generated, it can be repaired using the often-mutagenic process of NHEJ. If repair template DNA was also transformed into the cell, the break can be repaired with HDR.

CRISPR/Cas9 can be used to modify yeast in order to increase enzymes which release more aromatic compounds, decrease ethanol production, increase fermentation kinetics, eliminate carcinogens, improve stress tolerance, and much more (Chin et al., 2016; Manzanares et al., 2003; Swiegers et al., 2007, Vigentini et al., 2017; Volschenk et al., 1997). These improvements are going to have a vast impact on the winemaking industry by not only improved consistency, but also the overall quality of the wine produced. Aromatic complexity and balance achieved through alcohol are important factors when developing a perfect wine. One study sought to overexpress the *ATF1* gene and overexpress the *GPD1* gene in a single strain of *S. cerevisiae* (Wyk et al., 2020). *ATF1* encodes an enzyme that produces acetate esters, while *GPD1* encodes an enzyme that participates in glycerol synthesis and could reduce ethanol concentrations by using that carbon to produce glycerol. A yeast expressing both of these traits would complement that of a Riesling - especially from a warm region where the wine can begin to taste too hot from the high ethanol levels.

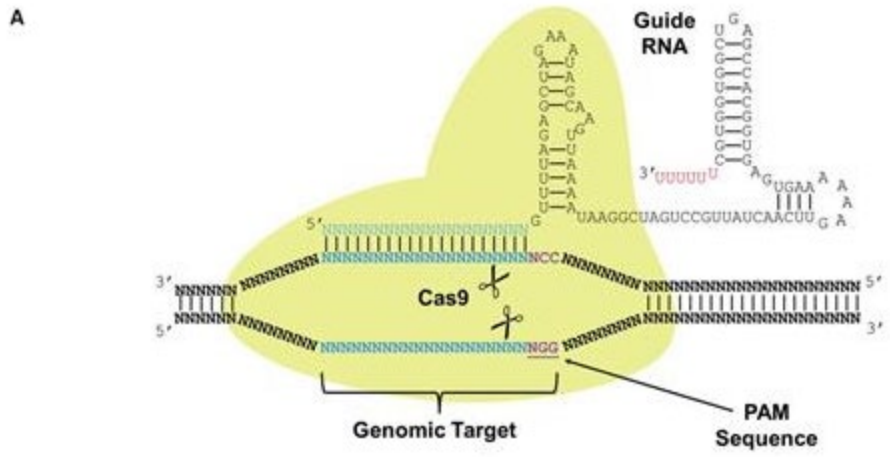

Figure 2. Illustration of CRISPR/Cas9 complex associated with target DNA. The Cas9 enzyme is depicted in yellow. The enzyme is directed to its target sequence by the depicted guide

Introducing mutations can occur by blasting cells with X-rays, UV light, or mutagenic chemicals to alter the DNA. Spheroplast fusion is a separate technique in which the cell wall is enzymatically removed, and two different cells are forced together through increasing their relative gravity. These techniques create random mutations in the genome, which is why it can take longer to create an organism that harbors the desired phenotype. Technically these techniques are not labelled as a GMO, since they do not use molecular techniques to modify the genome (National Bioengineered Food Disclosure Standard Act of 2016).

For decades, molecular biology researchers have studied the functions of many individual genes, both to understand the rules of life and to use those genes in biotechnological applications for the betterment of humankind. There are still many genes with completely unknown functions, and this work continues today. The era of GMOs and CRISPR/Cas9 technology has presented us with questions related to the safety of this technology, both for humans and for the environment. Anti-GMO lobbying groups have pushed back against the use of GMOs and promote fear among the public; encouraging people to believe genetic engineering is tampering with nature. Everything humans have done up to this point -infrastructure, technology, medical

interventions- defy nature in some way. Ignoring the use of genetic engineering is not going to restore 'nature'. With the right intent, it can be utilized to restore some of the damage humans have caused by reducing harmful waste we produce by living on this planet.

	Classified as a GMO	**Achieving Genome Modification**
Rare mating	No	Yes
Spheroplast Fusion	No	Yes
Hybridization	No	Yes
CRISPR/Cas9 Inserting foreign DNA	Yes	Yes
CRISPR/Cas9 to make single mutation	Maybe?	Yes
Transformation of Foreign DNA	Yes	Yes

Table 2. Various ways of altering the genome and the classification of GMO based on United States official government definitions (National Bioengineered Food Disclosure Standard Act of 2016).

CHAPTER 4. PRACTICAL APPLICATIONS OF GMO YEAST IN THE WINE INDUSTRY

Various targets for wine yeast genetic engineering include, but are not limited to, increasing aroma compounds, improving ethanol toxicity limits, improving fermentation's kinetics, reducing alcohol production, enhancement of enzymatic reactions, production of bacteria-inhibiting proteins, eliminating carcinogens, decreasing faulty aromas, and much more. Genetic engineering of *S. cerevisiae* began in the early 1990's, and even though only two GM yeast strains are legally available for use in the United States and Canada there are so many other genetically improved yeasts that have been developed based on the industry demands. Many scientists have collectively worked together to create novel new strains for the winemaking industry, but alas, those strains will have to wait on public acceptance.

GMO Target: Altering Aromatics

Despite public and industry disapproval of genetically modified wine yeast, various yeast strains have been developed to improve the ever-changing demands of consumers. The first genetically modified wine yeast was constructed in 1993, in which a beta-(1,4)-endoglucanase gene was transformed into a yeast cell to increase the fruity aromas through the action of the secreted endoglucanase enzyme (Perez-Gonzalez et al. 1993). This yeast was intended for white wines, such as Rieslings, Chardonnays, and other fruity varietals. Other genes have been targeted to increase overall aroma complexity such as glycoside hydrolyzing enzymes, aromatic thiol releasing enzymes, and increasing monoterpene concentrations (Manzanares et al. 2003; Perez-Gonzalez et al. 1993; Swiegers et al. 2007).

GMO Target: Lowering Alcohol

Due to the changing climate, many researchers have begun exploring techniques to lower alcohol levels in wine. Grapes are consistently reaching higher maturity levels year after year, making it difficult to meet consumer expectations. A plethora of genetic alterations have been explored to reduce alcohol concentration, including *GPD1/GPD2* overexpression, upregulated *PDC* genes, *ADH* mutants, *TPI1* deletions, *NOX* mutants, *FPS1* mutants, *GOX* mutants, and *HXT* mutants (Kutyna et al. 2010). Each of these genes play a role in carbon metabolism, making them potential ways to curtail rising alcohol levels by shunting carbon to other metabolic products. Another trend appreciated in today's culture is non-alcoholic beer and wine. Due to social reasons or health reasons, people can now enjoy traditionally alcoholic beverages without the alcohol by mutating *ADH1*, which encodes an enzyme that reduces acetaldehyde to ethanol (Lutstorf et al. 1968). Complete deletion of this gene leads to no alcohol production due to the immediate arrest of the mitochondrial electron transport chain (Drewke et al. 1990). More studies are needed in this field of research to determine the optimum way to make non-alcoholic wine.

GMO Target: Spoilage Control

Other targets for genetic modifications include production of antifungal and antibacterial compounds for better control over spoilage microbes. Although there is no formal definition for natural wines, there is a consumer and industry push for wines with minimal intervention. There is therefore potential value in engineered yeast strains that eliminate the risk of spoilage, and therefore can also reduce or eliminate use of other chemical preservatives. Not only would this strain be useful for wine production, but could also be potentially used in agriculture to protect crops from pathogens.

One antimicrobial protein of interest is chitinase, an enzyme that can hydrolyze chitin naturally present in fungal walls. Carstens and colleagues (2003) transformed the *CTS1-2* gene into *S. cerevisiae* and found that the hyphal tips of *B. cinerea* were degraded and the cellular components leaked from the cell walls leading to a significantly lower concentration of the fungi. Pediocin transcripts have also been explored for their bactericidal use in yeast (du Toit & Pretorius 2000). Pediocin is an antimicrobial peptide that inhibits *Listeria* and *Leuconostoc* growth (Schoeman et al. 1999). It was found to inhibit bacterial activity, however, further studies are necessary to determine the correct level of expression in yeast. Lysozyme addition has been studied to determine the efficacy of inhibiting lactic acid bacteria in wine (Gao et al. 2008; Luburdi, Benucci, and Esti 2014), however, only addition rates have been studied and thus this would be an excellent area of interest for genetically modified yeast. Overall, the use of GMO wine yeast for antifungal and antimicrobial activity is fairly understudied. More studies are necessary before practical applications will be safe for industry use.

GMO Target: Protein Haze

Protein haze is another problem frequently encountered in winemaking. Bentonite fining is one technique used in industry to bind to these proteins, which then settle to the bottom of the tank. The wine is then racked off the settled material. Whenever this process is completed a portion of the wine is lost to the lees and during the racking. *HPF* encodes for a mannoprotein that confers haze protection functionality (Brown et al. 2007). Transforming this gene into wine yeast can potentially eliminate the need for bentonite and decrease losses associated with bentonite fining.

GMO Target: Human Health

Genetically modified yeast can be altered for human health. For instance, EcMo01 produces less urea (which is a carcinogen) compared to other commercial strains through the overexpression of DUR1 and DUR2 (Coulon et al. 2006). DUR1 and DUR2 are proteins encoded for the intracellular enzyme urea amidolyase and when overexpressed in yeast, there is less carcinogens in wine. Antioxidants, like resveratrol, can also be overexpressed in wines (Becker et al. 2003). Resveratrol is naturally found in the grape skins of wines as a fungal defense mechanism; therefore, red wines typically have higher concentrations compared to white wines. Becker and colleagues (2003) wanted to create a novel yeast strain that could increase the levels in white wine for consumer health, which is why they created yeast that overexpressed resveratrol.

Target Gene	Activity in Yeast Cell	Effect on Wine	Reference
DUR1,2	Decreases urea production	Reduction of carcinogens	Coulon et al. 2006
mae1	Encodes a permease for malate	Completes MLF	Husnik et al. 2006
VST1	Synthesizes resveratrol	Increase of antioxidants	Becker et al., 2003
egl1	increases beta-(1,4)-endoglucanase activity, i.e., hydrolysis of glycosides	Increase in fruity aroma	Perez-Gonzalez et al. 1993
rhaA	Codes for glycosidase enzymes to increase liberation of monoterpenes	Increase in grape aroma	Manzanares et al. 2003
tnaA	encodes for a tryptophanase enzyme that has strong cysteine-β-lyase activity	Increase in volatile thiol compounds	Swiegers et al. 2007
PAL/TAL, C4H, BAL, 4CL	phenylalanine/tyrosine ammonia lyase, cinnamate-4-hydroxylase, coumarate-CoA ligase and benzalacetone synthase, respectively	Raspberry ketone production	Lee et al. 2016
ATF1	increases alcohol acetyltransferase activity	Increases fruity aroma stability post bottling	Lilly et al. 2000
GPD2	overexpression of leads to higher concentrations of glycerol and acetic acid	Increase in sweetness, lower alcohol levels, and acetic acid off-aromas	Lopes et al. 2000
FPS1	Facilitates in glycerol export/import, deregulation of this gene leads to less glycerol import	Lowers alcohol levels	Kutyna et al. 2010
GPD2 & ALD6	Overexpression of GDP2 and deletion of the aldehyde dehydrogenase enzyme	Lowers alcohol levels, eliminates acetic acid production, and increases sweetness levels	Eglinton et al. 2002
PDC2	Decreases the pyruvate decarboxylase isozyme activity	Reduces ethanol content	Cuello et al. 2017
ADH1	Eliminates the reduction of acetaldehyde to ethanol	Zero alcohol production	Drewke et al. 1990
RuBisCO	expression enable NADH	Improves growth rate and increases	Papapetridis

and PRK	reoxidation	ethanol yields	et al. 2018
CTS1-2	Overexpression leads to increased levels of chitinase - a potential antifungal	Significantly reduces the biomasse of hyphae	Carstens et al. 2003
pedA	Encode amino acids precursor of the PA-1 pediocin	Acts as an antimicrobial	Schoeman et al. 1999
HPF	Expression of HPF genes increases mannoproteins production	Decreases haziness	Brown et al. 2007
KNR3	Deletion leads to increased mannoprotein release	Decreases haziness	Gonzalez-Ramos et al. 2008
GAA1	Encodes a GPI transamidase comex, which is required for inositol synthesis	Allows yeast to ferment at lower temperatures	Lopez-Malo et al. 2015

Table 3. Genes edited in wine yeast for improvement of fermentation along with sensorial and health benefits. The genes along with their specific activity are outlined within the first two columns, the effect on wine and/or the microflora of the wine are outlined in the third column.

CHAPTER 5. CONCLUSION

In the late 19th century, the wine industry went through the phylloxera epidemic, which nearly destroyed all wine production in Europe (Banerjee et al. 2010). Luckily, it was discovered that American grape varieties were resistant to the disease and viticulturists were able to graft the European vines onto American root systems. Without this grafting system, *Vitis vinifera* would be scarce. Humans have been engineering life to our benefit for centuries through techniques like grafting, domestication, breeding, etc. Modern biotechnology is simply an advancement in the technology we must continue trying to solve the problems of humanity. Many GMOs are already widely used. Several vaccines are produced via genetically modified microbes, almost all medical insulin is produced by genetically modified yeasts and bacteria, medicines and many other compounds are also produced by microbes, genetically engineered crops are solving the hunger crisis by increasing yield, while also decreasing pesticide and herbicide use. New research has sought out GM yeast to convert sewage and waste into biodiesel production (Angerbauer et al. 2008). Genetic modification has already been applied to practically solve problems that have arisen in society without harming or sacrificing other people, animals, or the planet.

As technology advances, it is up to the users to apply it in a beneficial way. Many scientists have paved the groundwork to deal with global warming and rising alcohol levels through the use of genetically modified yeast (Cuello et al. 2017; Eglinton et al. 2002; Kutyna et al. 2010; Lutstorf et al. 1968). These yeasts will require less intervention from winemakers, which is a quality marker by consumers. The industry refuses to put GM wine yeast into practice because of negative consumer reaction, however, there is a lack of research indicating that consumers are unwilling to purchase, and drink wine made from GMOs. Wine is typically sterile

filtered prior to bottling to avoid secondary fermentations in the bottle. During this filtration, bacteria and yeast are eliminated from the final product, therefore, consumers would not be consuming GMOs.

Genetic modifications have not only been applied to yeast research, but practical applications have also been used to increase stress tolerance, disease resistance, pest control, and even environmental sustainability measures in vines (Vivier and Pretorius 2002). The harmony between genetically modified vines and yeast would lead to better quality wine, but more importantly, it would help the industry become more environmentally friendly. Every industry is making strides to become greener, which is necessary to ensure the survival of our planet and future generations. How GMs are applied to society is dependent on government regulations and consumer attitude. It is a time-sensitive issue, and the morality of this technology should be parsed out before it is passed onto another generation.

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