ORIGINAL SUBMISSION

phyterra

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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
U.S.A.

RECEIVED
AUG 11 2010
Division of
Biotechnology and
GRAS Notice Review

August 9, 2010,

Re: GRAS Notice for modified yeast to reduce hydrogen sulfide in fermented foods and beverages

Dear Dr. Martin,

In accordance with provisions of Sec. 170.30 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination], Phyterra Yeast Inc hereby submits a notification that modified yeast to reduce hydrogen sulfide in fermented foods and beverages is GRAS and therefore is exempt from the premarket approval requirements of the Federal, Food, Drug and Cosmetic Act.

Enclosed are three copies of the GRAS report and one electronic copy.

Sincerely,

(b) (6)

Garth Greenham

President
Phyterra Yeast Inc.

GRAS Notification

For the Use of a Modified Yeast to reduce
Hydrogen Sulfide in Fermented Foods & Beverages

Prepared by

Phyterra Yeast Inc.

Charlottetown, Prince Edward Island, CANADA

Garth Greenham

President

Dated: January 7, 2010

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GRAS Notice

1 GRAS Exemption Claim

1.1 NAME AND ADDRESS OF THE NOTIFIER

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The research and development of a low hydrogen sulfide (H_2S) wine yeast strain was initiated in the United States of America by researchers at the University of California, Davis (PCT publication no. WO 2008/115759). The technology is based on the discovery of an allele of the *MET10* gene that was isolated from a wine yeast by Prof. Linda Bisson from University of California, Davis. This technology was then subsequently exclusively licensed to Phyterra Yeast Inc. for global commercialization. The development of a commercial low H_2S wine yeast strain was completed in the research laboratories of Phyterra Yeast Inc., Charlottetown, PEI, Canada.

1.2 COMMON OR USUAL NAME OF THE SUBSTANCE THAT IS THE SUBJECT OF THE GRAS EXEMPTION CLAIM

The subject of this notice is a wine yeast strain obtained from a commercial *Saccharomyces cerevisiae* wine strain. The commercial strain now carries an allele of the *MET10* gene obtained from another *S. cerevisiae* wine strain, UCD932. Swapping of the *S. cerevisiae* native *MET10* gene with the UCD932 *MET10* allele using recombinant DNA technology effectively reduces the formation of hydrogen sulfide in the wine. This transformation event involves extracting DNA originating from one *S. cerevisiae* wine strain, and then inserting it into another *S. cerevisiae* wine yeast strain. There is no foreign DNA involved in this self-cloned organism. This novel yeast strain is called P1Y0.

1.3 APPLICABLE CONDITIONS OF USE OF THE NOTIFIED SUBSTANCE

1.3.1 Purposes for which the substance is used

The production of volatile sulfur compounds such as H_2S during alcoholic fermentation is a problem that affects the brewing, winemaking and sake industries. Hydrogen sulfide is an undesirable by-product of the sulfate assimilation pathway formed in *S. cerevisiae* during fermentation (Figure 1). Production of H_2S by *S. cerevisiae* strains ranges from 0 ug/L to 290 ug/L, well above the human detection threshold of 11 ng/L (Amoore and Hautala, 1983). The undesirable characteristics of H_2S include introducing a rotten egg odor to the fermented beverage, which can render the product unsaleable, resulting in significant losses to the wine producer. It also has the potential to form mercaptans and thiols which will persist in the wine due to the low pH (Thoukis, 1962). Mercaptans and thiols, are not only dangerously reactive compounds, but they impart onion and canned vegetable aromas. The removal of these undesirable sulfur compounds is technically difficult and strips the wine of desirable flavor compounds.

These sensory characteristics are an extremely important issue to the wine producer. The current method in the wine industry to remove sulfides from the wine is to add copper in attempts to chelate the sulfur. Copper can lead to the catalysis of deleterious compositional changes as well as increase the amount of waste produced by wineries. The special treatments required and waste ultimately result in higher production costs for wineries and higher wine costs for the consumer. Moreover, the use of copper as a fining agent may lead to high residual copper levels in wine. The US Trade and Tax Bureau allows a residual copper level of 0.5 mg/l for wine (Federal Register, 2007). Winemakers who use copper to remove hydrogen sulfide must then take measures to reduce the copper levels in the wine. Given the potential adverse health effects associated with excessive copper ingestion, particularly neurological disorders such as Alzheimers, the World Health Organization has recommended dietary restrictions on consumption of this compound (WHO, 2004). The availability of a commercial yeast strain that does not produce H_2S as a by-product of fermentation, will eliminate the deleterious practice of copper treatment of wines.

As a result of the formation of H_2S by *S. cerevisiae* as a by-product of fermentation, Phyterra Yeast Inc. proposes the use of a self-cloned wine yeast strain of *S. cerevisiae* named P1Y0 to reduce H_2S formation in fermented beverages. The modified strain has its native *MET10* gene replaced with the 'low-H_2S' *MET10* allele from the *S. cerevisiae* wine yeast UCD932. The *MET10* allele swap results in minimal H_2S leakage from the cell (Figure 2). **Since the DNA fragment containing the 'low-H_2S' *MET10* allele is obtained from *S. cerevisiae*, P1Y0 is non-transgenic.**

1.3.2 Foods in which the substance is to be used

The P1Y0 active dry yeast is intended for use as starter culture for alcoholic beverage fermentation such as grape must, brewing wort and rice fermentations, in accordance with Good Manufacturing Practices (GMP). The following fermented products are known to have H_2S issues during manufacturing, and may benefit by using Phyterra P1Y0 proprietary yeast strain during production to reduce H2S levels:

- Red and white wine
- Champagne
- Sherry
- Sake & other rice wines
- Beer

The following products may also benefit from using the P1Y0 yeast strain in the fermentation process:

- Brandy & cognac
- Whiskey
- Rum
- Biofuels

1.3.3 Levels of use of the P1Y0 active dry yeast in grape must

To reduce the risk of H_2S production, we recommend that winemakers use approximately 0.2 gram of active dry yeast per litre of wine for grape must fermentation. The Bureau of Alcohol, Tobacco and Firearms (27 CFR 24.176) and the International Oenoligical Codex (2002), however, allow the winemaker to determine the appropriate level of yeast inoculation in wine.

If a mixture of P1Y0 strain and a H_2S producing yeast strain is used in a fermentation, H_2S reduction may not be carried out to optimal efficiency. In other words, mixing wine yeast strains are not likely to reduce H_2S to the lowest levels that might be obtained by using the Phyterra P1Y0 strain exclusively in the inoculation.

1.4 THE BASIS FOR THE GRAS DETERMINATION

Phyterra Yeast Inc. has determined that the yeast P1Y0 is 'Generally Regarded as Safe' (GRAS) for use as a starter culture in grape must and alcoholic beverage fermentations, through scientific analysis. Based on the scientific data, the company also concludes that P1Y0 can be considered GRAS for use as a starter culture in any alcoholic beverage fermentation. Phyterra also asserts that P1Y0 is substantially equivalent to the host strain from which it was derived, except for the enhancement of the sulfate assimilatory pathway. Specifically, the genetic insertions to enhance the efficiency of the sulfate assimilatory pathway to prevent formation of toxic hydrogen sulfide are all derived from *S. cerevisiae.* The modification amounts to only 3 nucleotide differences between the 'low H_2S' *MET10* allele and the native *MET10* gene of the transformed commercial strain. To place these 3 nucleotide differences into perspective - the fully sequenced *S. cerevisiae* reference strain S288C, has a haploid genome of 12,070,897 nucleotide bases. Moreover, one of these nucleotide differences does not result in an amino acid change (i.e. a silent mutation). Therefore, only two amino acids are altered, meaning the rest of the entire genome remains effectively unaltered in form or function. The precision of the genetic swap of the *MET10* alleles means there is a negligible chance of unintended effects in the enhanced organism.

1.5 STATEMENT THAT THE DATA AND INFORMATION THAT ARE THE BASIS FOR THE NOTIFIERS' GRAS DETERMINATION ARE AVAILABLE FOR THE FOOD AND DRUG ADMINISTRATION REVIEW AND COPYING

Phyterra Yeast Inc. undertakes that all data and information used in this GRAS determination for use of the P1Y0 yeast strain in production of alcoholic beverages will be made available for the FDA to review and copy at the following address:

Phyterra Yeast Inc.
Attention: Mr. Garth Greenham, President and COO
PO Box 21147
Charlottetown, PEI, C1A 9H6
Canada

Data will be sent to the FDA upon request.

2 Detailed information about the identity of the notified substance

2.1 HOST MICROORGANISM

The host yeast strain is an industrial wine strain of *Saccharomyces cerevisiae* subspecies *bayanus* called UCD2034 which has been commonly used in commercial wine production in the US.

2.1.1 History of use

The yeast *Saccharomyces cerevisiae* is found as a commensal organism on our bodies. It is in the air that we breathe and it grows naturally on many foods that we eat regularly. This yeast strain has been used in food production for over five thousand years. Ancient Egyptians, Hebrews and Greeks utilized yeast to produce fermented foods and beverages such as baked goods, wine and beer. Over 2.5 million tons of yeast are commercially produced each year worldwide, the largest volume of any microorganism.

Baker's yeast serves as a model organism for scientific research for numerous reasons. Yeast cells are structurally and genetically similar to human cells. They each contain about 6,000 genes, many of which are identical or similar to human genes. Over the last 20 years, researchers have found that yeast genes can be manipulated with relative ease and efficacy. As a result, most of what we know about the cell cycle and human cancer was discovered from research on yeast. Throughout time, the classification of *S. cerevisiae* has undergone many changes (a historical survey of the species is found in Lodder and Kreger-van-Rij, 1967; Lodder, 1970; Kregar-van Rij, 1984; Kurtzman and Fell, 1998).

Yeast are very convenient microorganisms to work with in small and large volumes. Yeast needs are very basic, their growth rates are high and predictable, and their proliferation can be controlled and scaled. The primary reason that yeast has become one of the most widely-studied and understood organism, is that *S. cerevisiae* are known to be generally non-pathogenic and safe.

2.1.2 Characteristics

The UCD2034 wine yeast strain is diploid and it sporulates. It is commonly used in industry to obtain wine active dry yeast. The production of UCD2034 vegetative cells during manufacturing occurs asexually through budding to obtain wine active dry yeast. After rehydration and during fermentation of must, reproduction also occurs asexually.

UCD2034 is a strong fermenter over a wide temperature range with good ethanol tolerance, and will readily ferment grape musts and fruit juice to dryness. It also has good tolerance to free sulphur dioxide with relatively low nitrogen demands. It is recommended for use in production of classic and premium red and white wines, sparkling white wines, cider, fruit wines and mead. UCD2034 is noted

for its low volatile acidity, low foaming and ability to ferment under stressful conditions.

UCD2034 is normally grown on cane and beet molasses under highly aerobic conditions, which assures better survival of live cells toward the end of the fermentation than yeasts grown in must under anaerobic conditions.

2.2 DONOR ORGANISMS

2.2.1 Taxonomy

Only *Saccharomyces cerevisiae* strain UCD932 contributed DNA to P1Y0, and therefore this enhanced strain of the yeast is 100% *Saccharomyces cerevisiae*. The history of use of this species is given in Section 2.1.1.

2.2.2 Genetic material from donor organisms

The *MET10* gene

The *MET10* gene was derived from *S. cerevisiae* strain UCD932 (chromosome VI). The entire sequence of the *MET10* gene from this strain has been described in the patent 'Compositions and Methods for Reducing H_2S Levels in Fermented Beverages' (PCT publication no. WO 2008/115759). The *MET10* gene from UCD932 was also sequenced by Phyterra Yeast Inc. (Figure 3) and the sequence in FASTA format is given in Appendix 1.

The *MET10* gene codes for subunit alpha of assimilatory sulfite reductase, which converts sulfite into sulfide. The length of the amino acid sequences is 1,035 and the molecular weight of this protein has been determined to be 114,827

2.3 THE MODIFIED MICROORGANISM

2.3.1 Final construct used in the integration strategy

2.3.1.1 Construction strategy

For the technology to be effective the native *MET10* gene must be completely removed from the genome of the final 'low-H_2S' commercial strain. Removal of the two copies of the native *MET10* gene is accomplished by knocking out the gene with markers, kanMX and hphMX, via homologous recombination with the non-coding upstream and downstream *MET10* flanking sequences. Subsequently, the markers are replaced with the 'low-H_2S' *MET10* allele of UCD932. A schematic of the method is given in Figure 4.

The kanMX and hphMX markers are antibiotic resistance markers that convey resistance to the antibiotics G418 and hygromycin, respectively. The use of such markers provides a direct screening method for 'knocked-out' *MET10*$_{\text{Native}}$ yeast cells and a negative selection screening method for 'knocked-in' *MET10*$_{\text{UCD932}}$ gene replacements.

The hphMX cassette was constructed by DNA synthesis (Mr. Gene GmbH, Regensburg, Germany). Figure 5 is a schematic of the hphMX cassette within the plasmid vector pMK (Mr. Gene GmbH, Regensburg, Germany) and without the vector pMK backbone. The linear cassette is released from the plasmid after digestion with the restriction endonuclease PmeI. Digestion with PmeI results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences. The sequence for the linear hphMX cassette, showing the location of the blunt-end restriction enzyme PmeI, is shown in Figure 6 and the sequence in FASTA format is given in Appendix 2.

The kanMX cassette was constructed by the PCR using a high fidelity, proof-reading DNA polymerase (KOD Hot Start DNA polymerase, Novagen) and the following primers 5′-TATGCGGCCGCATGGGTAAGGAAAAGACTCACG-3′ and 5′-CTGACTTAAGTTAGAAAAACTCATCGAGCATCAAATGA-3′, with built in restriction enzyme sites for NotI and AflII, respectively (underlined). The template for the PCR of the kanMX marker was a previously synthesized DNA cassette named 'LEU2_kanMX' (Mr. Gene GmbH, Regensburg, Germany) which harbors the kanMX marker. The kanMX PCR product was subsequently digested with the restriction enzymes NotI and AflII and purified. Concurrently, the plasmid hphMX pMK (Figure 5) was also digested with NotI and AflII, and the plasmid backbone including the *MET10* flanking sequences and the TEF promoter/terminator sequences were purified via gel extraction. Plasmid kanMX-pMK was obtained by subcloning the NotI/AflII digested kanMX PCR fragment into the linearized pMK vector containing the *MET10* flanking sequences and the TEF promoter/terminator sequences. Figure 7 is a schematic of the kanMX cassette and the cassette as it is located within the plasmid kanMX-pMK. The sequence for the linear kanMX cassette is given in FASTA format in Appendix 3.

The *MET10* integration sequence was constructed by PCR using a high fidelity, proof-reading DNA polymerase (KOD Hot start DNA polymerase, Novagen) and the following primers 5′-TTGAAACCGTGGCAAATGCCTACAG-3′ and 5′-CAAAACATGGGAGAAAGTGACACTTG-3′. The template was genomic DNA from UCD932. Figure 8 is a schematic of the *MET10*UCD932 integrating cassette and the sequence in FASTA format from forward to reverse primer is given in Appendix 4.

2.3.1.2 Detailed description of the final construct

The *MET10*UCD932 integration cassette is 4220 bp long. The sequence contains the *MET10* ORF (3108 bp) and flanking sequences of 514 bp and 598 bp, upstream and downstream of the ORF, respectively. The sequence for the *MET10*UCD932 integration cassette showing the location of the forward and reverse primers and the *MET10* ORF is shown in Figure 9 (see Appendix 4 for the sequence in FASTA format).

2.3.2 The transformation event

2.3.2.1 Genetic material used for the transformation method

The linear hphMX cassette excised from the vector hphMX-pMK, the linear the kanMX cassette excised from the vector kanMX-pMK and the *MET10*UCD932

integrating DNA synthesized by PCR were the only DNA sequences used in the transformation method.

2.3.2.2 Screening method for transformants

The first transformation was completed using the kanMX cassette and the cells were plated onto G418 containing media in order to select transformed cells. A single colony was chosen and streaked for purity and then re-transformed with the hphMX cassette and plated onto media containing hygromycin and G418. A pure colony was then streaked onto minimal media lacking the amino acid methionine. The lack of growth on media lacking methionine confirmed that both *MET10*^{Native} alleles were knocked out and that the strain was now auxotrophic for methionine. The *MET10* double knock-out yeast was then transformed with the *MET10*$^{\text{UCD932}}$ integrating cassette and plated onto minimal media lacking the amino acid methionine. Only *MET10*$^{\text{UCD932}}$ transformed cells were capable of forming colonies on such media and non-transformed cells did not grow. The colonies of *MET10*$^{\text{UCD932}}$ transformed cells were then screened for sensitivity to G418 and hygromycin. A colony that was sensitive to both G418 and hygromycin was selected for further studies. The lack of resistance to the antibiotics was indicative that both markers were replaced during the transformation with *MET10*$^{\text{UCD932}}$ allele. Functionality of the *MET10*$^{\text{UCD932}}$ was then assessed on BiGGY agar media. BiGGY is an abbreviation for Bismuth Glucose Glycine Yeast and is a commercially available differential media capable of detecting sulfide producing micro-organisms. Lack of sulfide production is indicated by white to light tan colonies and the presence of sulfide reduces the Bismuth Ammonium citrate causing the colonies to be brown to black in color. The *MET10*$^{\text{UCD932}}$ transformed yeast produced white to cream colored colonies indicating no H_2S was produced while the parental strain UCD2034 produced H_2S as indicated by dark tan to brown colonies. Figure 10 shows the colony growth and H_2S production of P1Y0 and UCD2034 on BiGGY agar.

2.3.3 Genetic characterization of the modified microorganism

2.3.3.1 The loss of the antibiotic resistance genes

Loss of the antibiotic resistance genes was confirmed by streaking P1Y0 onto G418 and hygromycin containing media and confirming sensitivity. Figure 11 clearly shows that P1Y0 is sensitive to each of those antibiotics like the parental strain UCD2034.

2.3.3.2 Genetic analysis of the *MET10*$^{\text{UCD932}}$ allele

Since both copies of the *MET10*^{Native} allele were replaced with two copies of the *MET10*$^{\text{UCD932}}$ allele, the stability of the cassette will not be affected by gene conversion or crossing over recombination events. There is no reason to suspect that the stability of the *MET10*$^{\text{UCD932}}$ allele would be any different than the *MET10*^{Native} allele.

2.3.4 DNA sequencing of the integrated locus

The *MET10* ORF and surrounding flanking sequences from the strain P1YO, the parental strain UCD2034 and the source of the 'low-H2S' *MET10* allele, strain UCD932 were sequenced. A multiple sequence alignment was completed comparing the obtained sequences for the *MET10* ORF and flanking sequences. No differences were found in the flanking sequences amongst the strains sequenced. There was also 100% sequence homology between the *MET10* ORF of P1Y0 and UCD932 (as expected) and three nucleotide differences were found in the parental strain UCD2034 (Appendix 5).

2.3.4.1 Differences found in the *MET10* ORF

Three differences were found in the *MET10* sequence when P1Y0 was compared to the parental strain UCD2034. We believe that these sequence variations were due to natural genetic polymorphisms and not the result of DNA synthesis error during the amplification steps involved in the construction of the cassette or sequencing errors. Our sequence results for the *MET10* ORF of UCD932 align perfectly with P1YO. Moreover, our sequence results of the *MET10* ORF of P1Y0/UCD932 align perfectly with the published sequence for UCD932 completed in another laboratory (Dr. Bisson, UC Davis:PCT publication no. WO 2008/115759). The differences found in the parental strain UCD2034 are also known to exist in other *MET10* ORF sequence data (PCT publication no. WO 2008/115759) and are therefore, highly likely to be natural polymorphisms and not sequencing errors.

The first difference is found at nucleotide position 404 of the *MET10* ORF (Appendix 5) where the parental strain, UCD2034, has a 'C' and the P1Y0/UCD932 sequence has an 'A'. The second difference is located at position 1278, where UCD2034 has a 'G' and P1Y0/UCD932 has an 'A'. The final difference is found at position 1985 and UCD2034 has a 'C' residue and P1Y0/UCD932 has an 'A'. The single nucleotide polymorphism (SNP) found at positions 404 and 1278 are common to a number of strains (PCT publication no. WO 2008/115759) and the SNP found at position 1985 is unique to the *MET10* ORF of UCD932 (and now the *MET10* ORF of P1YO). Only two of these SNPs result in amino acid changes since the SNP at nucleotide position 1278 results in a silent mutation.

The *MET10* ORF and flanking sequences were shown to contain only DNA from *Saccharomyces cerevisiae*. The sequence data also confirmed that the 'low-H_2S' allele from UCD932 was successfully integrated into the commercial strain P1Y0 with 100% homology. When comparing the *MET10* ORF sequences between P1Y0/UCD932 to UCD2034 sequences, 3 nucleotide differences were found. One of these SNPs results in a silent mutation, the other two involving the exchange of one amino acid for another. These results show that the *MET10* sequences of the P1Y0 strain are exactly the same as the original sequences isolated from the donor strain UCD932.

2.3.5 Absence of difference between genetic profiles of the transformed and the host strain

The genome of *Saccharomyces cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and has been used to fingerprint and differentiate between strains of *Saccharomyces cerevisiae*. Using PCR and primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' to amplify these δ sequences we have verified the genetic relationship between the host strain and P1Y0 (Figure 12). This technique is commonly employed to differentiate between industrial *Saccharomyces cerevisiae* strains.

We therefore conclude that the P1Y0 strain is not different than the UCD2034 parent strain, except for the three nucleotide differences. No major DNA reorganization event has occurred upon integration of the *MET10*UCD932 allele into the P1Y0 strain.

2.3.6 Method of manufacture of the P1Y0

No selective media needs to be used for the propagation of the P1Y0 yeast strain. The P1Y0 wine active dry yeast can therefore be manufactured in the exact manner as all active dry yeast used in bakery, brewing or winemaking, as described in the literature (*e.g.*, see Reed and Nagodawithana, 1991). A brief description of the manufacturing of P1Y0 active dry yeast is given in Figure 13.

3 Information on any self-limiting levels of use

Functional Technologies Corp. sees no reason why the inoculation rate should be different than the one recommended as indicated in Section 1.3.3.

4 Determination that the use of the wine active dry yeast obtained from the P1Y0 strain in the fermentation of grape must is exempt from the pre-market approval requirements of the act because such use is GRAS

4.1 SAFETY ASSESSMENT OF THE HOST STRAIN

The host yeast strain is an industrial wine strain of *Saccharomyces cerevisiae* subspecies *bayanus* called UCD2034 that has been commonly used in commercial wine production in the US.

S. cerevisiae is an organism that has an extensive history of safe use. It has been used for millennia in fermentation processes, such as bread leavening and wine or beer production. It is responsible for spontaneous fermentation of grape juice (Lodder and Kreger-van-Rij, 1967). The Bureau of Alcohol, Tobacco and Firearms rates yeast or yeast cultures grown in juice of the same kind of fruit (here grapes) as permitted material added in the production of natural wines (27 CRF 24.176).

S. cerevisiae is considered GRAS through its use in the brewing, baking and winemaking industry. Its genome has been sequenced, and it has been determined that the yeast is free of known pathogenicity traits. Genetically modified yeast strains such as the malolactic yeast (Wine Research Centre, UBC) and Phyterra's self-cloned ECMo01 strain have been previously granted GRAS status by the USFDA.

In the 27th report of The Scientific Committee for Human Food of the European Community the authors state that *Saccharomyces cerevisiae* has a safe history of use in food and belongs to a species that is known not to produce toxins. In addition, the Environment Protection Agency (EPA) has included *S. cerevisiae* as a recipient microorganism for exemptions from EPA review and expedited EPA review (40 CFR 725.420). This exemption was made because this species is found to have little adverse effects. They also determined that the introduction of genetic material would not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable.

The UCD2034 yeast strain belongs to the *Saccharomyces cerevisiae* species which have been used for more than 7,000 years by humans in fermented beverages. It can therefore be concluded that species will remain GRAS, even after genetic transformation, as long as no harmful DNA material is added to it.

4.2 SAFETY ASSESSMENT OF THE GENETIC MATERIAL USED TO CONSTRUCT THE MODIFIED ORGANISM

4.2.1 Foreign genetic material source and product

No foreign (non-*Saccharomyces*) genetic material is in the P1Y0 yeast strain.

4.2.2 Native genetic material source and product: *MET10* sequences.

4.2.2.1 The *MET10* gene

Source: *Saccharomyces cerevisiae*

The *MET10* gene was isolated from the wine strain UCD932 of *S. cerevisiae* as described in Section 2.2.2.

Product: subunit alpha of assimilatory sulfite reductase

When comparing the sequence of the 'low-H_2S' *MET10* allele in the P1Y0 strain and the native gene sequence in the *S. cerevisiae* host UCD2034, 3 changes were found. However, one of these 3 differences corresponds to a silent mutation. The remaining two nucleotide differences do correspond to a change in amino acids, nevertheless we can infer that these changes do not affect the overall protein structure since the 'low-H_2S' *MET10* allele is functional.

Therefore, to the best of our knowledge, the *MET10* gene product of the P1Y0 strain is similar to that of the parent UCD2034 *S. cerevisiae* strain.

4.2.3 Construction of the modified organism

4.2.3.1 Vector used as source of the integration cassette

The hphMX cassette was constructed by DNA synthesis (Mr. Gene GmbH, Regensburg, Germany). Figure 5 is a schematic of the hphMX cassette within the plasmid vector pMK (Mr. Gene GmbH, Regensburg, Germany) and without the vector pMK backbone. The kanMX cassette was constructed by the PCR using a high fidelity, proof-reading DNA polymerase (KOD Hot Start DNA polymerase, Novagen) and the following primers 5'-TATGCGGCCGCATGGGTAAGGAAAAGACTCACG-3' and 5'-CTGACTTAAGTTAGAAAAACTCATCGAGCATCAAATGA-3', with built in restriction enzyme sites for NotI and AflII, respectively (underlined). Plasmid kanMX-pMK was obtained by subcloning the NotI/AflII digested kanMX PCR fragment into the linearized pMK vector containing the *MET10* flanking sequences and the TEF promoter/terminator sequences. In both cases the linear cassette is released from the plasmid after digestion with the restriction endonuclease PmeI. Digestion with PmeI results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences.

The *MET10* integration sequence was constructed by PCR using a high fidelity, proof-reading DNA polymerase (KOD Hot start DNA polymerase, Novagen) and the following primers 5'-TTGAAACCGTGGCAAATGCCTACAG-3' and 5'-

CAAAACATGGGAGAAAGTGACACTTG-3′. The template was genomic DNA from UCD932. Figure 8 is a schematic of the *MET10*UCD932 cassette and the sequence in FASTA format from forward to reverse primer is given in Appendix 4.

4.2.3.2 Transformation of the host strain with the integration cassette

The linear hphMX cassette excised from the vector hphMX-pMK, the linear kanMX cassette excised from the vector kanMX-pMK and the *MET10*UCD932 integrating DNA synthesized by PCR were the only DNA sequences used in the transformation method.

The first transformation was completed using the kanMX cassette and the cells were plated onto G418 containing media in order to select transformed cells. A single colony was chosen and streaked for purity and then re-transformed with the hphMX cassette and plated onto media containing hygromycin and G418. A pure colony was then streaked onto minimal media lacking the amino acid methionine. The lack of growth on minimal media lacking methionine confirmed that both *MET10*Native alleles were knocked out and that the strain was now auxotrophic for methionine. The *MET10* double knock-out yeast was then transformed with the *MET10*UCD932 integrating cassette and plated onto minimal media lacking the amino acid methionine. Only *MET10*UCD932 transformed cells were capable of forming colonies on such media and non-transformed cells did not grow. The colonies of *MET10*UCD932 transformed cells were then screened for sensitivity to G418 and hygromycin. A colony that was sensitive to both G418 and hygromycin was selected for further studies. The lack of resistance to the antibiotics was indicative that both markers were replaced during the transformation with *MET10*UCD932 allele.

Standard microbiological methods were employed in order to verify that antibiotic markers had been eliminated from the P1Y0 strain. These methods relied on the fact that P1Y0 strain had lost its ability to grow on a G418 containing media and hygromycin containing media, indicating the absence of the markers kanMX and hphMX resistance genes (Figure 11).

The results show that the kanMX and hphMX markers are not present in the P1Y0 strain.

4.3 SAFETY ASSESSMENT OF THE MODIFIED ORGANISM

4.3.1 Characterization of the transformation event

The genome of *S. cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and has been used to fingerprint and differentiate between strains of *S. cerevisiae*. Using PCR and primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' to amplify these δ sequences we have verified the genetic relationship between the host strain and P1Y0 (Figure 12).

Loss of the antibiotic resistance genes was confirmed by streaking P1Y0 onto G418 and hygromycin containing media and confirming sensitivity. Figure 11

clearly shows that P1Y0 is sensitive to each of those antibiotics like the parental strain UCD2034.

DNA sequencing of the *MET10* ORF and surrounding flanking sequences from the strain P1YO, the parental strain UCD2034 and the source of the 'low-H2S' *MET10* allele, strain UCD932 were completed. No differences were found in the flanking sequences amongst the strains sequenced. There was also 100% sequence homology between the *MET10* ORF of P1Y0 and UCD932 (as expected) and three nucleotide differences found in the parental strain UCD2034 (Appendix 5).

The P1Y0 strain has been genetically characterized. The integration site contains no DNA sequences other than those present in the isolated *MET10*$_{UCD932}$ integration DNA. No antibiotic resistance sequences are present in the genetic material of strain P1Y0. Therefore, to the best of our knowledge, the P1Y0 strain is genetically substantially equivalent to the UCD2034 strain, except for the three nucleotide differences present in the 'low-H_2S' *MET10* allele.

4.3.2 Presence of three single nucleotide polymorphisms

Saccharomyces cerevisiae has been employed by humans in production of food and beverages for millenia. It is known that during this period, genetic changes have occurred, however, this yeast is still considered GRAS. We contend that the three single nucleotide polymorphisms (SNPs) present in the new *MET10* allele do not raise any safety concerns. The wine strain UCD2034 has never shown to release allergenic or toxic substances and the strain UCD932 (the source of the *MET10* allele) is also a wine yeast strain. The close sequence similarity of the two *MET10* alleles and the presence of multiple SNPs found in other *MET10* sequences from other strains (PCT publication no. WO 2008/115759) further supports this conclusion.

There is no reason to think that the P1Y0 strain will release toxic or allergenic substances as a result of the presence of three single nucleotide polymorphisms that were attained from another wine yeast.

4.3.2.1 Subunit alpha of assimilatory sulfite reductase

Only two amino acid differences are found to be present in the 'low-H2S' *MET10* allele as one of the SNPs corresponds to a silent mutation. The first variation is found at position 135 and corresponds in a change from threonine to asparagine (Appendix 6). Both threonine and asparagine are polar and carry a neutral charge, constituting a minor change. The second variation is found at position 662 and corresponds to a change from threonine to lysine and constitutes a change from neutral to a positively charged amino acid. This change also falls in an area of the protein considered to be the catalytic site and is the basis of the technology. Figure 14 clearly shows that the first amino acid variation falls outside of any known protein domains/motifs and the second variation occurs in an active part of the enzyme. The independent analysis completely supports the data published in the UC Davis patent (PCT publication no. WO 2008/115759). This change does not compromise the safety of the microorganism and will in fact

enhance the final product (i.e. wine, beer etc) by reducing H_2S concentrations and thereby negating the use of toxic copper sulfate to remove the contaminating H_2S.

With respect to the 'low-H_2S' MET10p, its fate in the final product would be similar to any other MET10p (as found in wild yeast or other inoculated commercial strains). During storage on lees, cell proteins normally undergo intracellular enzymatic degradation by yeast intracellular proteases and amino peptidases (see Section 4.4.2.1). In addition, bentonite treatment, which is common in white wines, will also remove protein fractions of the wine (see Dawes *et al*., 1994). **It is therefore reasonable to conclude that use of the P1Y0 strain will not result in additional MET10p presence in wines at the time they are consumed**.

4.3.3 Consequence of the genetic modification on the physiology of the P1Y0 strain

4.3.3.1 Overview of the function of the *MET10* gene

The *MET10* gene codes for the subunit alpha of assimilatory sulfite reductase. The subunit alpha of assimilatory sulfite reductase, converts sulfite into sulfide and is essential to methionine biosynthesis. This enzyme is 1035 amino acids long and 114,827 Da in weight.

4.3.3.2 Manufacture of active dry yeast during fed-batch aerobic cultures

Section 2.3.6 gave an overview of the method of manufacture of the P1Y0 strain in the form of wine active dry yeast. This method of manufacture is identical to that used for the current commercial manufacture of wine active dry yeast obtained with the host strain UCD2034.

4.3.3.3 CO_2 production of PY10 and UCD2034 strains during laboratory-scale vinifications

Vinification trials performed in the laboratory have shown that the UCD2034 and the P1Y0 strain have similar fermentation rates as illustrated by the CO_2 production during fermentation which is virtually identical to that of the parent strain (Figure 15).

Therefore, the presence of three SNPs does not give the P1Y0 strain any growth advantage, even though the organism is able to reduce the production of H_2S.

DNA sequencing studies have shown that the P1Y0 strain is genetically similar to the UCD2034 host strain, except for three nucleotide variations. This leads to the conclusion that strain P1Y0 can be regarded as substantially equivalent to the wild type strain UCD2034, with an enhanced capacity to prevent H_2S formation. Physiological studies carried out in different culture conditions indicate that no difference between the P1Y0 and UCD2034 strains can be seen, except for reduced H_2S production. The reduction of H_2S doesn't confer any significant growth advantage to the P1Y0 strain. Therefore, it can be concluded that the P1Y0 strain is substantially equivalent to the host strain UCD2034 except for the ability of P1Y0 to produce less H_2S during wine fermentations.

4.4 SAFETY ASSESSMENT OF THE PRODUCT DERIVED FROM THE MODIFIED ORGANISM

4.4.1 Changes in the winemaking procedures as a consequence of the P1Y0 fermentation

The use of the P1Y0 yeast strain leads to no change in winemaking procedures. Winemaking procedures for white and red wines are shown in figures 16 and 17, respectively, which could be employed using our yeast.

4.4.2 Changes in wine composition as a consequence of the P1Y0 fermentation

4.4.2.1 Yeast cells and release of yeast products during winemaking

After alcoholic fermentation, the viable yeast population starts to decrease in wine and yeast autolysis occurs. The wine is either immediately clarified or is kept on lees.

Wine clarification

Wine clarification is usually performed by letting the solid particles of the wine sediment by gravity and subsequent elimination of the sediments. During this process, a majority of the yeast cells is eliminated and the total yeast population falls to 10^3 or 10^4 cells/milliliter of wine (Ribéreau-Gayon *et al*., 1998b).

Alternatively, wine can be clarified by centrifugation. This process eliminates more than 99% of the yeast cells (Ribéreau-Gayon *et al*., 1998b). This clarification procedure is an obligatory step in the wine processing. However, it can be delayed by storage of the wine on lees.

Keeping the wine on lees

Some winemaking practices involve storage of the wine on lees. This facultative winemaking practice involves the conservation of the wine and yeast cells that have performed the alcoholic fermentation together, either in tanks or in barrels. The main objective of this step is the release of wine yeast constituents that influence the wine sensory properties. During storage on lees, cell proteins and

nucleic material first undergo an intracellular enzymatic degradation due to the liberation of yeast intracellular proteases, amino peptidases, nucleases and phosphatases (Fornairon-Bonnefond *et al*., 2001; Charpentier and Feuillat, 1993). The yeast cell gradually loses its hydrolyzed constituents by breakage of the cell wall. In the wine, the yeast constituents can be further degraded by proteases present in the extra cellular media. Autolysis of the yeast cells is thus accompanied by an increase of the wine in amino acids and peptides (Martinez-Rodriguez *et al*., 2000). Simultaneously, the protein content of the wine decreases as a result of the release of proteases and larger peptides are broken down, giving rise to smaller ones (Ari'lzumi *et al*., 1994; Martinez-Rodriguez *et al*., 2000).

Storing wine on P1Y0 lees will not result in a significant difference in the type of proteins that are released, as autolysed yeast cells liberate only small amounts of proteins (Martinez-Rodriguez *et al*., 2000). Rather it will be its hydrolysis products such as peptides and amino acids which will be found in wines stored on lees and with only two amino acid variations between the P1Y0 and UCD2034 *MET10* proteins – this difference is considered insignificant.

The cellular content of anautolyzing yeast is rich in nucleases and phosphatases. As a result of complete yeast autolysis, nucleic material will normally be found in wine as single nucleic bases or small nucleic base chains.

Wine stabilization

After clarification, wines are normally stabilized. The clarification process employed is dependent on whether the wine is red or white.

Bentonite treatment of white wines

In white wine, instable proteins may form a protein haze after bottling (Ribéreau-Gayon *et al*., 1998b). Stabilization of white wine requires the removal of these instable proteins. Elimination of these proteins is done by treating the wine with bentonite, a montmorillonite clay. Although only a certain part of proteins are responsible for formation of protein haze in wine, bentonite treatment removes equally all protein fractions of the wine (Dawes *et al*., 1994).

If the wines are not stored on lees, yeast proteins and yeast protein products will not normally be found in wines. If the wine are stored on lees, yeast proteins will be liberated, but mostly as hydrolyzed fractions. However, the intact proteins as well as the bigger polypeptides may be removed from wine upon bentonite treatment.

Hence, even if a small proportion of the 'low-H2S' MET10 protein could be present in wine, it and other larger hydrolysis products will be partially removed like all wine proteins by a bentonite treatment.

Red wine stabilization

Red wines are not subject to protein haze like white wines, because proteins associate with tannins and little remain freely in the wine (Ribéreau-Gayon *et al*., 1998a). The protein-tannins complexes are partially eliminated after clarification with gelatins or egg white albumin which both possess the ability to precipitate

colloidal structures (Ribéreau-Gayon et al., 1998b). Therefore, **if any 'low-H2S' MET10 protein is present in red wine, it will interact with the wine tannins and be partially removed upon clarification.**

The presence of the 'low-H_2S' MET10 protein should only occur if the wine has been stored on lees after alcoholic fermentation. However, clarification processes, which are obligatory winemaking practices, will remove part of the MET10 protein, as well as larger polypeptide fragments of this protein. Hence, only the smaller polypeptides and amino acids of these proteins will remain in the wine.

It should also be noted that products of the MET10 protein will also be found in wine as a result of fermentation by unmodified yeast. Hence essentially no difference in overall amino acid profiles or peptide profiles will be found in the final product (wine, beer etc).

Filtration

Filtration is an important step in wine clarification as it decreases the turbidity of the wine before bottling. This practice is not always used for red wines and some winemakers rely simply on clarification of the wine with gelatins or egg white albumin. However, the later procedure is rarely used and most wines are submitted to a filtration before being bottled.

Filtration of the wine can be done in several ways, but a perfect wine clarification will require several filtration steps.

Filtration on diatomaceous earth

This type of filtration is used for wines that have not been previously pre-filtered and is usually the first step in wine filtration. Different types of diatomaceous earth exist, some being more permeable than others. Filtration can remove a substantial proportion of wine yeast cells, the wine viable yeast cell count upon filtration being in the range of 10^3 cells/100 milliliters of wine (Ribéreau-Gayon *et al*., 1998b). After this type of filtration, the wine is either bottled immediately or submitted to another filtration step, usually involving cellulose filters or membrane filters.

Cellulose filters

This type of filtration is largely utilized just before bottling of the wine when the wine has been previously clarified either by gravity sedimentation, centrifugation, or diatomaceous earth filtration. Two types of filters exist, their use depend on whether a complete sterilization of the wine is wanted or just a fine clarification is needed. Clarification of wines using cellulose filter will allow the yeast cell count to drop to less than 50 cells/100 milliliters of wine (Ribéreau-Gayon *et al*., 1998b). More efficient filters will decrease this count to less than one cell / 100 milliliters of wine. A prerequisite for the efficiency of the filtration for microbial stability of the wine is the sterilization of the filter before use.

Membrane filters

Just before bottling, a membrane filtration of the wine can be performed. This type of filtration is particularly used when the wine is required to be sterile.

Membrane filters can have different porosity, spanning from 0.45 micrometers to 10 micrometers. For yeast cells to be removed from wine, a porosity of less than 1.2 micrometers is needed. Hence, when a complete removal of yeast cell sis required, these are the types of filters that should be used.

Standard and modern procedures

The modern and standard procedure consists in filtering wine in order to obtain a limpid solution having a very low NTU by turbidimetry, especially in the case of white wines and in order to obtain a wine as sterile as possible. Consequently, the modern wine procedure consists in filtering the wine with a porosity inferior to 1 micrometer, generally 0.45 micrometer for white wines and 0.65 micrometers for red wine. The filtration eliminates all live yeast cells or ghost cells.

Our data show that the only difference between the UCD2034 and the P1Y0 yeast is the presence of three SNPs, one of which leads to a silent mutation, one that causes an amino acid change found outside of any essential domains of the MET10p, and one that leads to an amino acid change that leads to the reduction of the H2S. Vinification trials showed that P1Y0 wines were not different in their global composition than control wines.

Depending on the clarification and filtration process, different proportions of P1Y0 yeast cells might be found in the final product. Since the 'low-H2S' MET10 allele was isolated from another wine yeast strain, the presence of a small proportion of P1Y0 cells in the final product should not pose a safety risk.

4.4.2.2 Formation of unwanted substances in wine

Hydrogen sulfide

Results shown in Figure 10 indicate that the P1Y0 strain produces significantly less H_2S than the parental UCD2034 strain.

Hence, the P1Y0 strain produces less H_2S than the parental strain, and could be used to help reduce the copper content of alcoholic beverages since copper sulfate is employed to remove this contaminate.

Other undesirable substances

The 'low-H2S' *MET10* allele present in the P1Y0 strain as the result of the genetic modification does not code for either toxic or allergenic proteins, nor proteins implicated in the formation of undesirable compounds.

These considerations lead us to conclude that allergenic or toxic risks related to the presence of this *MET10* allele within the new P1Y0 strain are negligible.

Therefore, to the best of our knowledge, the use of the P1Y0 strain in winemaking will not lead to the release or the enhancement of the presence of undesirable compounds in wine. Moreover, the undesirable compound, H2S is significantly reduced in wines produced using the P1Y0 strain, and copper concentrations will also be reduced by the reduced use of copper sulfate.

Figures

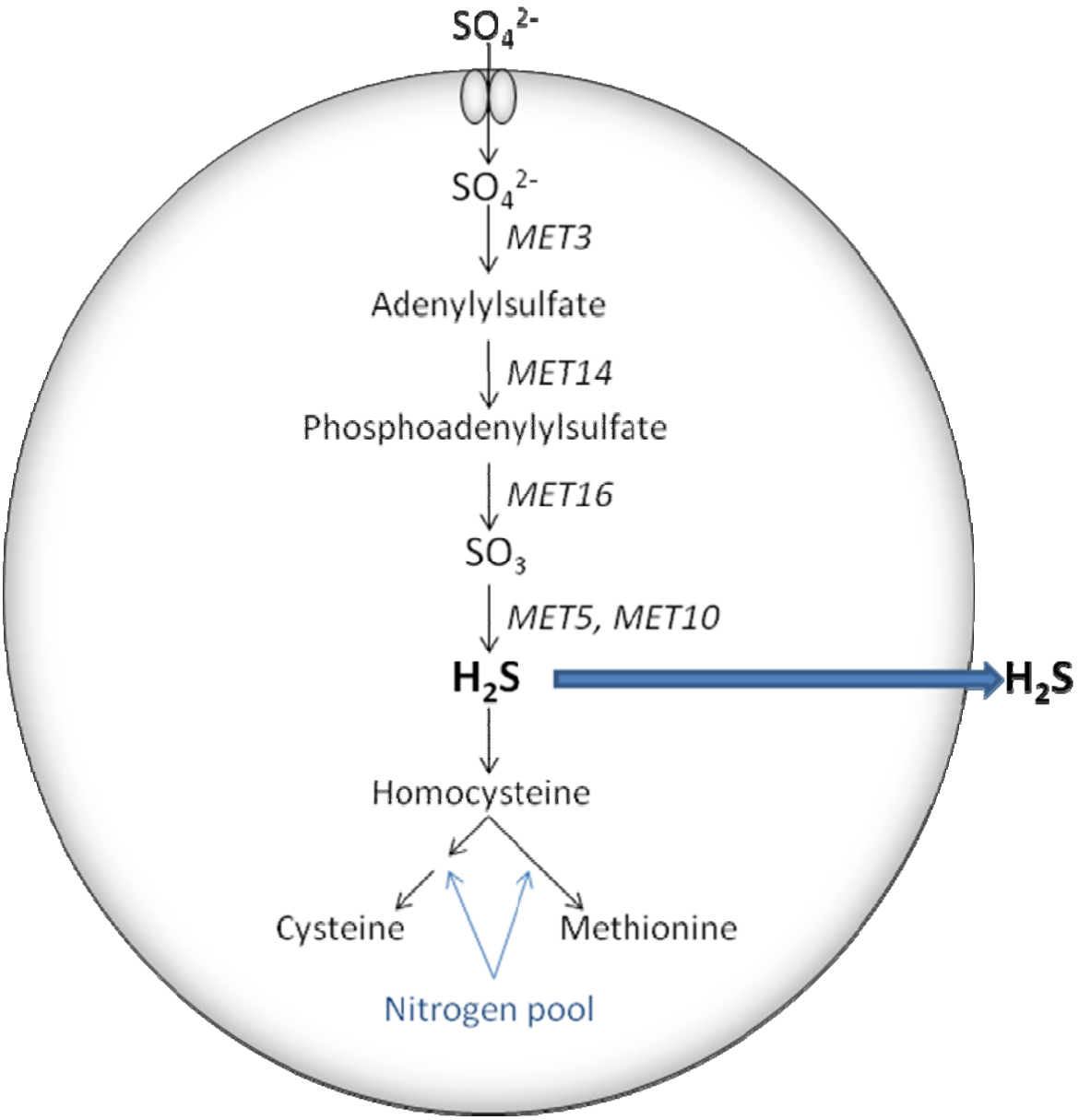


Figure 1: Sulfate assimilatory pathway and natural formation of H_2S by *Saccharomyces cerevisiae*.

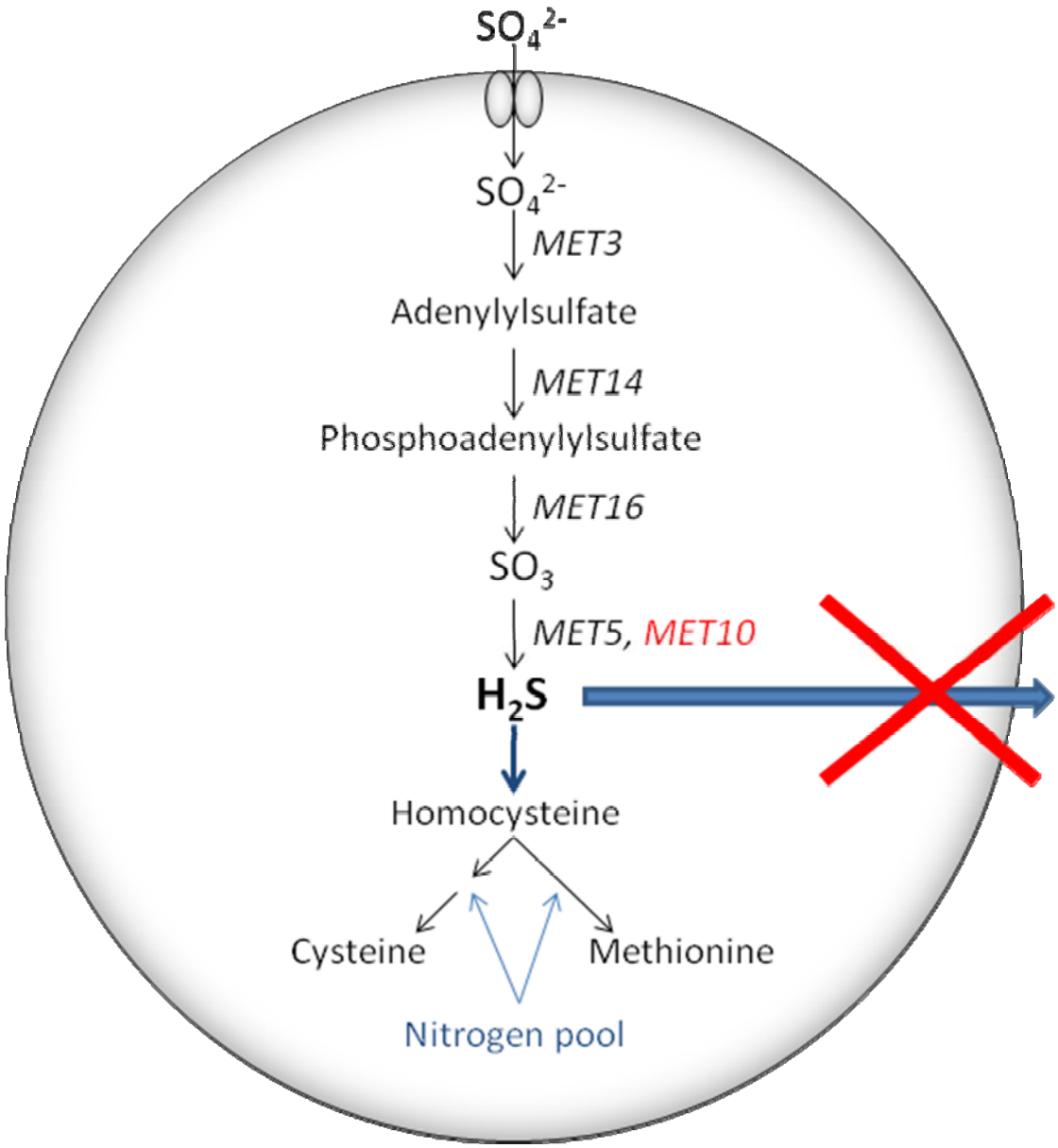


Figure 2: Sulfate assimilatory pathway and lack of H_2S formation in *Saccharomyces cerevisiae* strains harboring the 'low-H2S' *MET10* allele from UCD932.

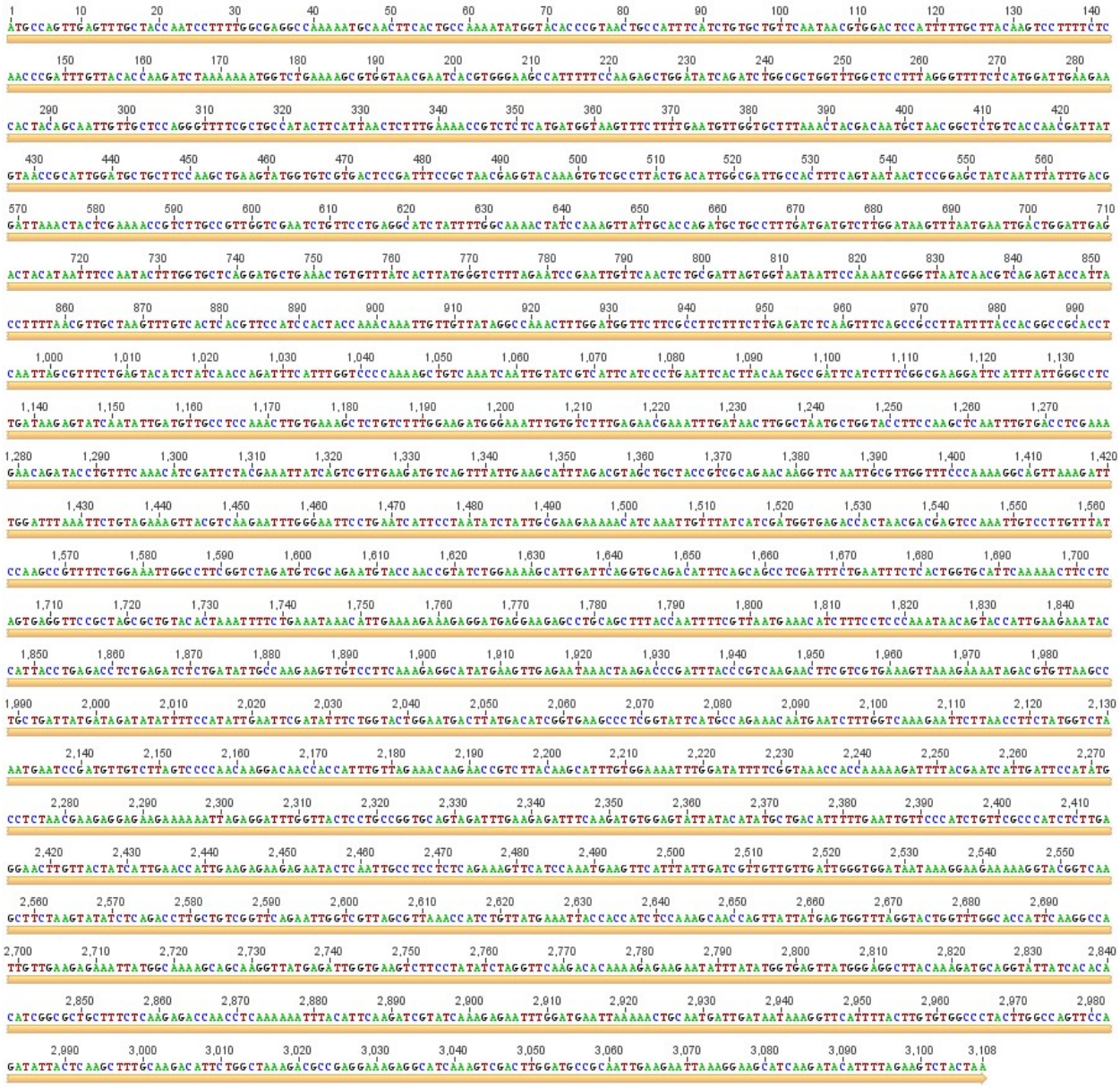


Figure 3: *MET10* sequence from UCD932.

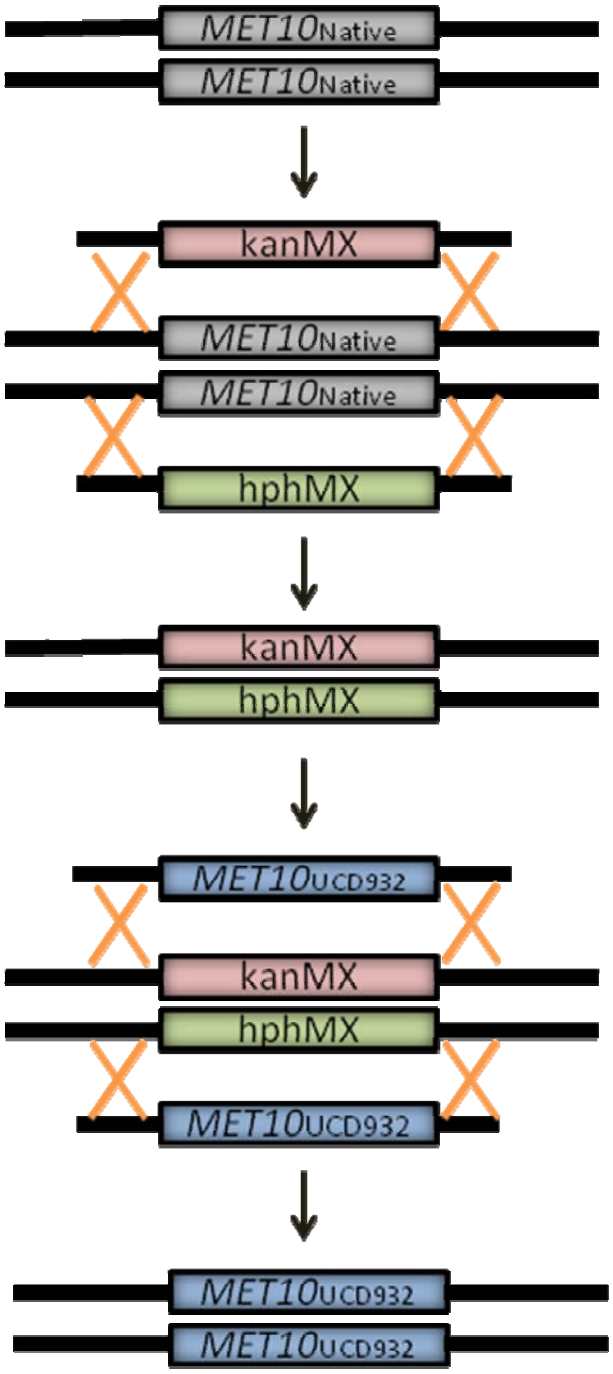
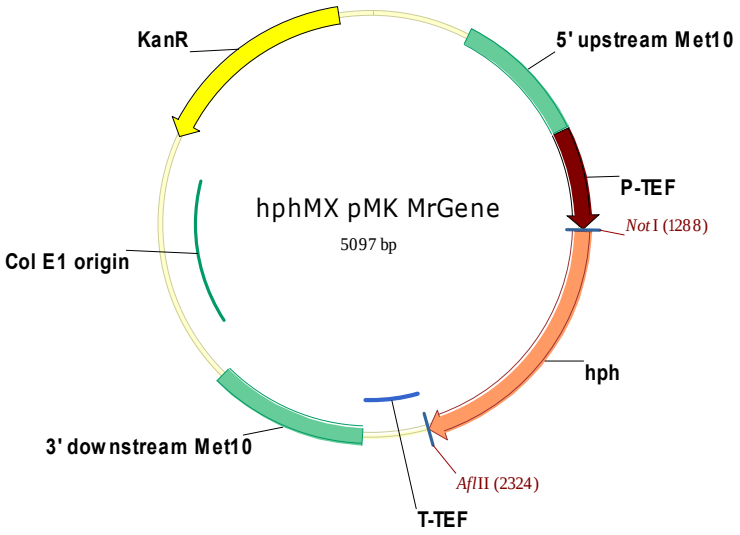


Figure 4: Flow diagram illustrating the strategy used to construct the commercial strain P1Y0.



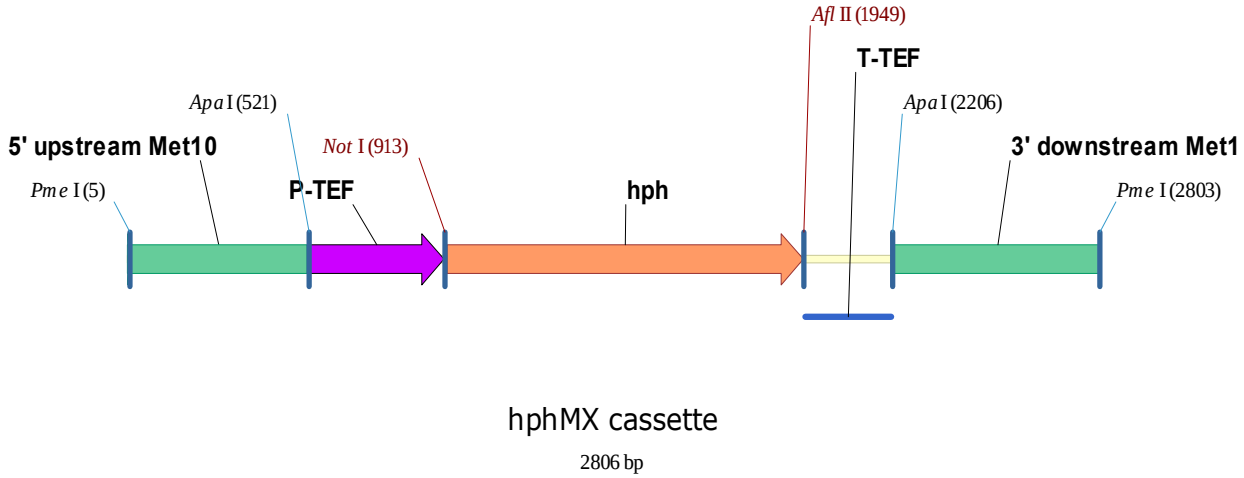
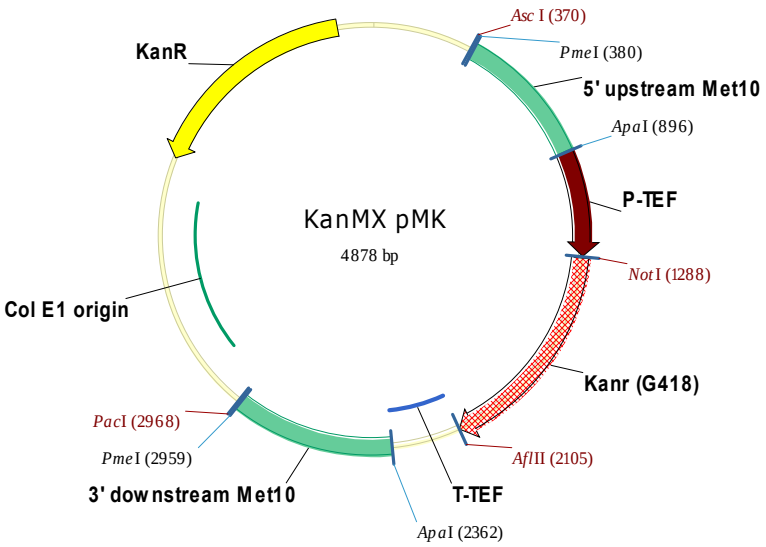


Figure 5: A schematic of the hphMX cassette within the plasmid vector pMK (Mr. Gene GmbH, Regensburg, Germany) and the linear cassette without the vector pMK backbone.

```
1         10        20        30        40        50        60        70        80        90        100       110       120       130       140       150
PmeI (5)
GTTTAAACCGTGGCAAATGCCTACAGGGCTTATGAATCAGCTACTTTGCGGTTTAACCCAGAGGGCAACAAGCTTTATATTCTCGACAGAAAGGGGACTTTCTTTGTGGAAGACTTTGCATATGGCTTGCCCCAATCTCGCGAAATCACC
          160       170       180       190       200       210       220       230       240       250       260       270       280       290       300

AAATGTAAGCAAATATTCCACAAATAATGCATCTAAATATATACGTATGTTTAAGGTTCTGGTATACAGGTATTAAAAGAAAACACTATCAACATTCCCAATAAGATATACCACACCACGTGAGCTTATAGAAGCACGTGACCACAATTC
          310       320       330       340       350       360       370       380       390       400       410       420       430       440       450

ACCCCACAGGTGTGGCTTTTTTGGTGCCGTAGAAAAGACTCATTCATGAATCGTCGGAAACCCATAGTCATCTTCGAGCAAAAGGTATATATAAGCAACAGAGGGCAGTAGTTCTCGAGACCACCATCTTTTGATTGGAAATAGTTTCGT
          460       470       480       490       500       510       520       530       540       550       560       570       580       590       600

TTAGATGGGGTGCACATAGTTTTTTTCAACTGCTTTTCCTCGAGGTCACCCAAATATACAACGAGGGGCCCCGATCTGTTTAGCTTGCCTCGTCCCCGCCGGGTCACCCGGCCAGCGACATGGAGGCCCAGAATACCCTCCTTGACAGTC
          610       620       630       640       650       660       670       680       690       700       710       720       730       740       750

TTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCATGTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCGGACCTGCGAGCAGG
          760       770       780       790       800       810       820       830       840       850       860       870       880       890       900

GAAACGCTCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAAC
          910       920       930       940       950       960       970       980       990       1,000     1,010     1,020     1,030     1,040     1,050

ATAAACAACCGCGGCCGCATGGGTAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGG
          1,060     1,070     1,080     1,090     1,100     1,110     1,120     1,130     1,140     1,150     1,160     1,170     1,180     1,190     1,200

CGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGC
          1,210     1,220     1,230     1,240     1,250     1,260     1,270     1,280     1,290     1,300     1,310     1,320     1,330     1,340     1,350

CGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAA
          1,360     1,370     1,380     1,390     1,400     1,410     1,420     1,430     1,440     1,450     1,460     1,470     1,480     1,490     1,500

TACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCAC
          1,510     1,520     1,530     1,540     1,550     1,560     1,570     1,580     1,590     1,600     1,610     1,620     1,630     1,640     1,650

CTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAG
          1,660     1,670     1,680     1,690     1,700     1,710     1,720     1,730     1,740     1,750     1,760     1,770     1,780     1,790     1,800

CAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGC
          1,810     1,820     1,830     1,840     1,850     1,860     1,870     1,880     1,890     1,900     1,910     1,920     1,930     1,940     1,950

GACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAACTT
          1,960     1,970     1,980     1,990     2,000     2,010     2,020     2,030     2,040     2,050     2,060     2,070     2,080     2,090     2,100

AAGTCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTGCCTCGACATCATCTGCCCAGATGCGAAGTTAAG
          2,110     2,120     2,130     2,140     2,150     2,160     2,170     2,180     2,190     2,200     2,210     2,220     2,230     2,240     2,250

TGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGTGTCGAAAACGGGGGGGCCCATTAATATAGTAATAAAAACTAAATATCTATTTATTGAACCTGT
          2,260     2,270     2,280     2,290     2,300     2,310     2,320     2,330     2,340     2,350     2,360     2,370     2,380     2,390     2,400

CTTGAACATTTCTATTTTTTTTTTACTTTTAGTTTTCTTCTATGCGCAAGCTTTTCTATTGGCTGCCAAATAGAAAATTATTGAAATATGATTACATTACAATATTTATTTGTCTTATGAAAACTAACCATCACATTATACTAACTACGG
          2,410     2,420     2,430     2,440     2,450     2,460     2,470     2,480     2,490     2,500     2,510     2,520     2,530     2,540     2,550

AGGTACCATCTTGAAATCTCGTCCTGAAGACCCTATTAGCATTGGCAAACATACCTTCTTTAAGCGAGACTACAATGAACTGAGATCCTTTAAACCTTGTTTTAATCAAATGACCTATATTTTGCGTATGACTTAAATCAAGAGCAGCAT
          2,560     2,570     2,580     2,590     2,600     2,610     2,620     2,630     2,640     2,650     2,660     2,670     2,680     2,690     2,700

CAACTTCATCCAAAATATACATAGGCGCTGGTCGAAATTGCAACAATGCCATAATCAATGACAAAGCAATCAACGACCTTTGCCCACCGGACAATTCAATCAAACTTTCCTTCCATATATTACCAAGCTTTACTTTAACCTCTAAACCCT
          2,710     2,720     2,730     2,740     2,750     2,760     2,770     2,780     2,790     2,800  2,806
                                                                                              PmeI (2,803)
GCGTTACGTCCTTACCTTCGCATGGAACCAATTTTGCAAATGAATTTGGTAAAAGGTCTGCAAAAATATTACCAAAATCAAGTGTCACTTTCTCCCATGTTTAAAC
```

Figure 6: The linear hphMX cassette with the PmeI restriction enzyme sites indicated.



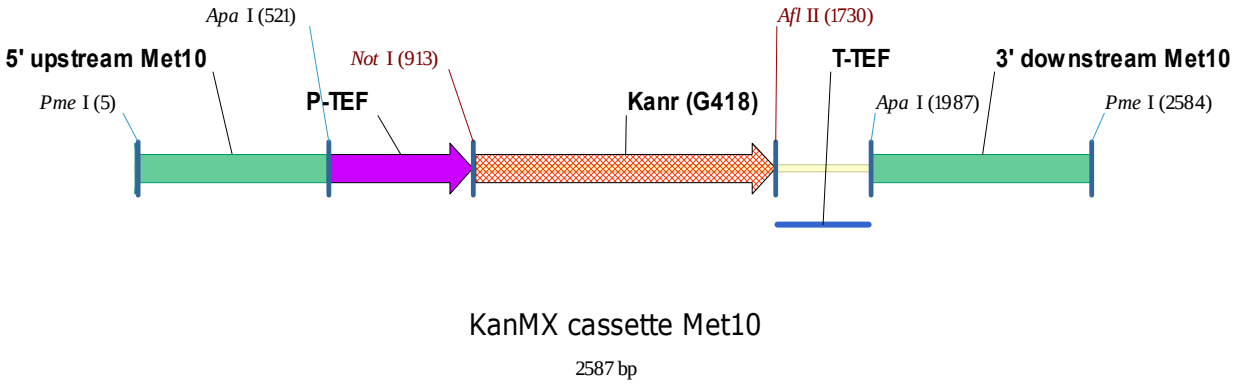


Figure 7: A schematic of the plasmid vector kanMX-pMK and the linear cassette without the vector plasmid backbone.

The kanMX marker was cloned into the NotI and AflII sites of the hphMX-pMK vector (Figure 5).

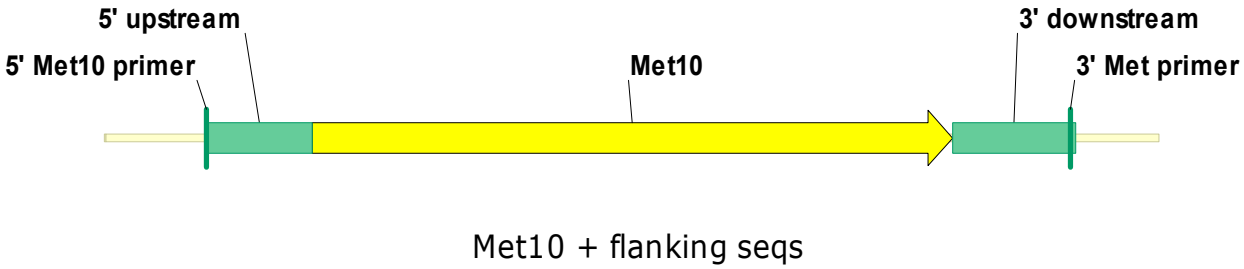


Figure 8: Schematic diagram of the *MET10*UCD932 integrating cassette.

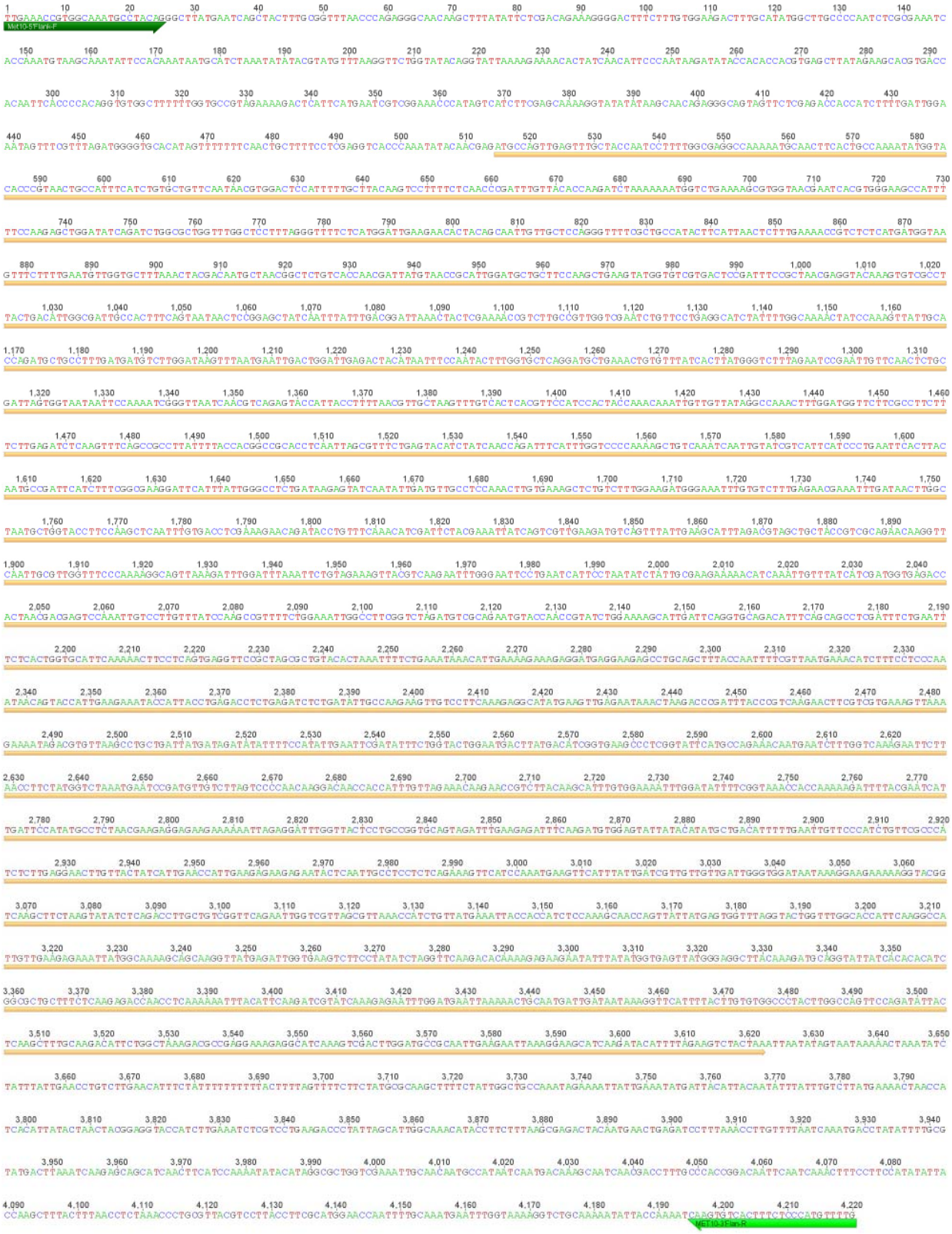


Figure 9: The *MET10*UCD932 integration cassette showing the forward and reverse primers (green arrows) and the *MET10* ORF (yellow arrow).

Figure 10: *S. cerevisiae* strain P1Y0 and the parental strain UCD2034 on BiGGY agar.

P1Y0 does not produce H_2S while UCD2034 produces H_2S.

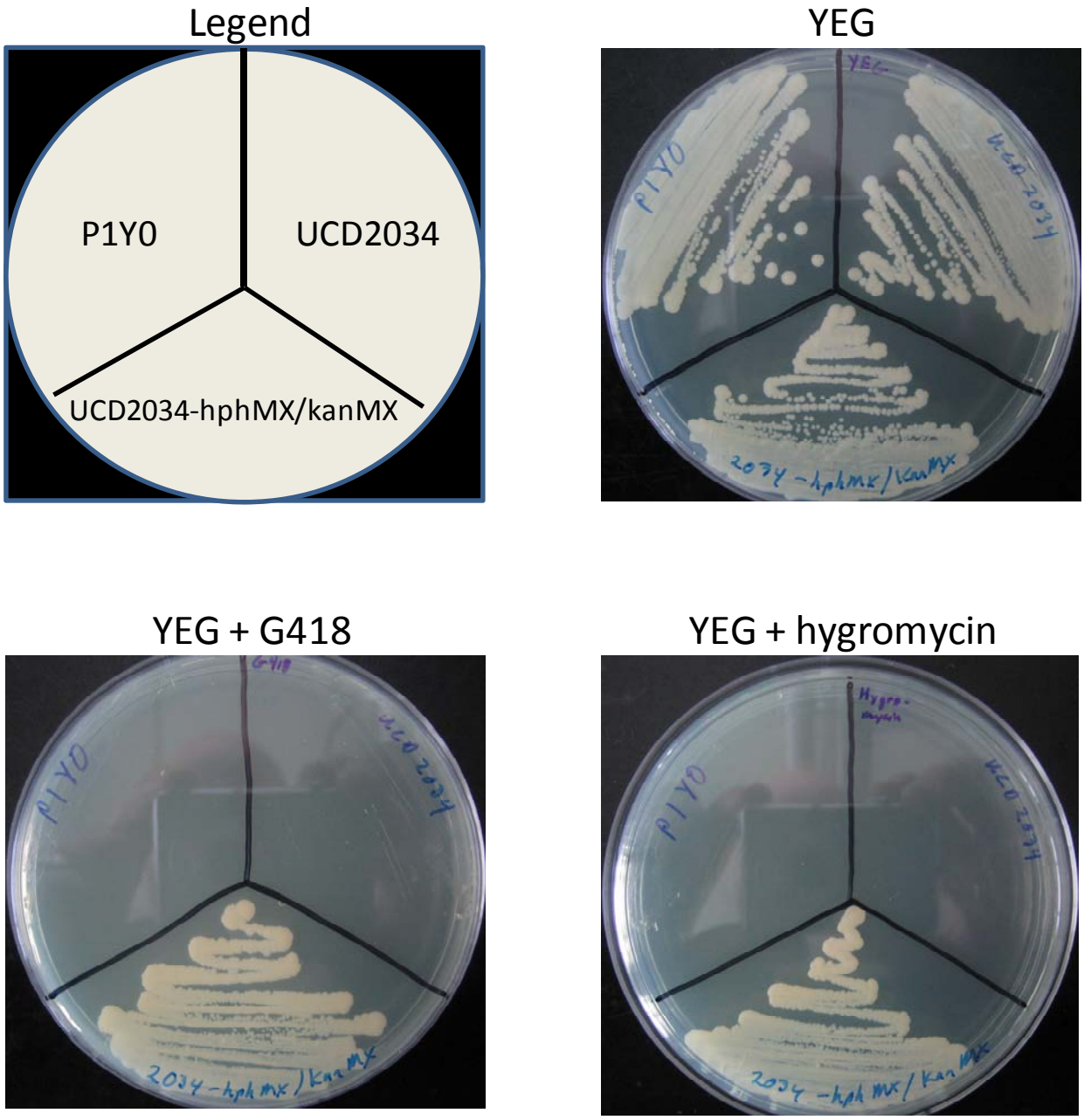


Figure 11: Confirmation of the loss of the kanMX and hphMX markers that convey resistance to the antibiotics G418 and hygromycin, respectively.

The base media is 10 g/l Yeast extract, 20 g/l of Glucose and 15 g/l of agar (YEG). The concentration of antibiotics was 500ug G418/L and 300 ug hygromycin/L. The double knock-out parental strain containing a kanMX and hphMX marker is designated as UCD2034-hphMX/kanMX.

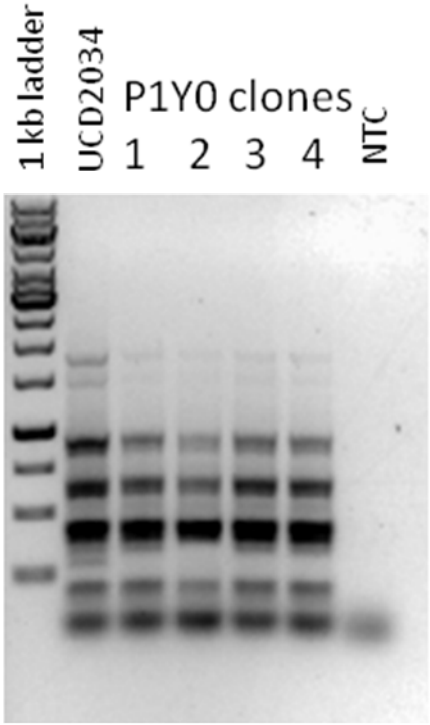


Figure 12: PCR of genetic patterns of the UCD2034 and P1Y0 yeast strains (four clones of P1Y0 are shown).

PCR was performed using primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' on genomic DNA isolated from UCD2034 and P1Y0 strains. This technique is described by Schuller *et al*. (2004).

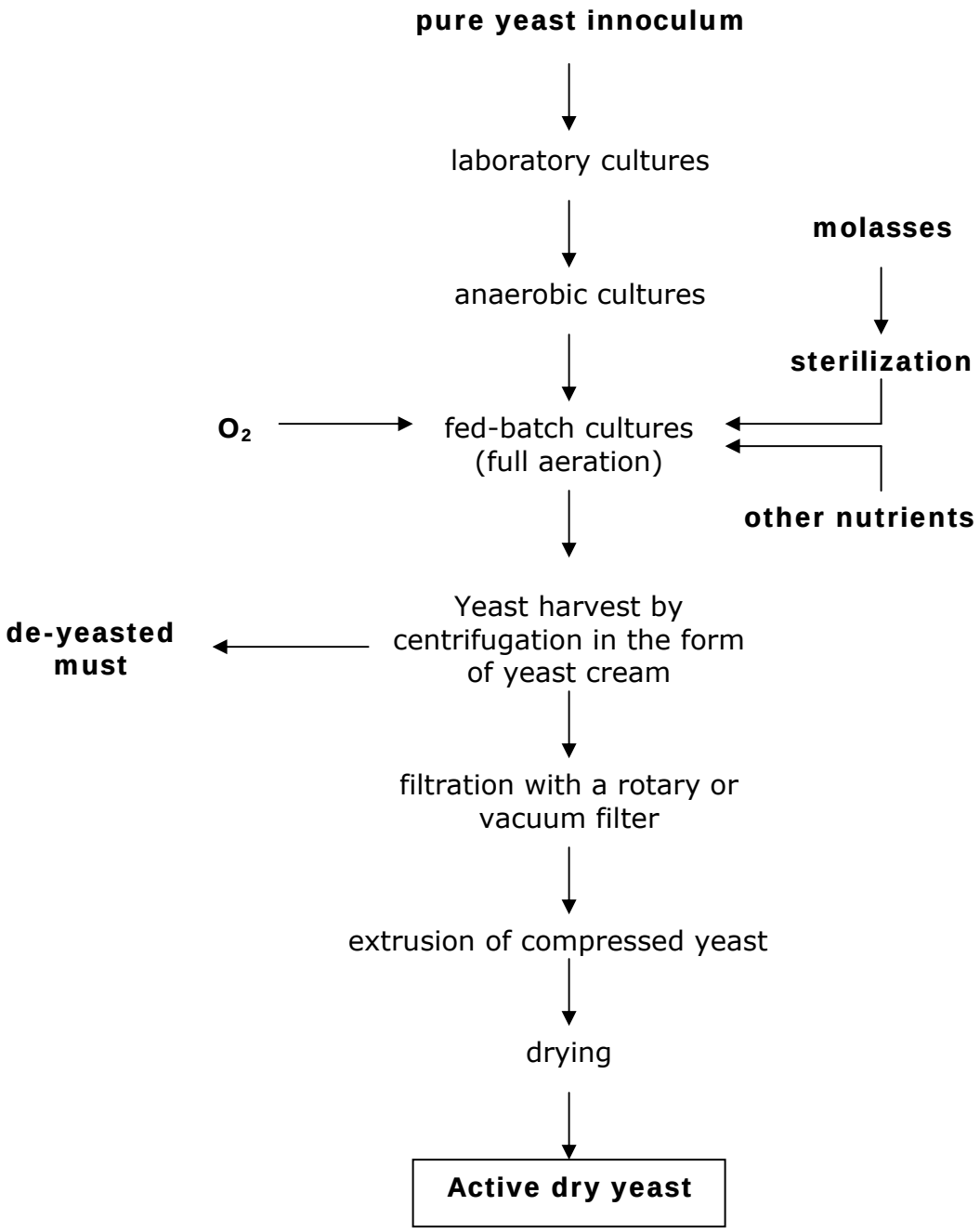


Figure 13: Flow diagram describing the manufacture of active dry yeast.

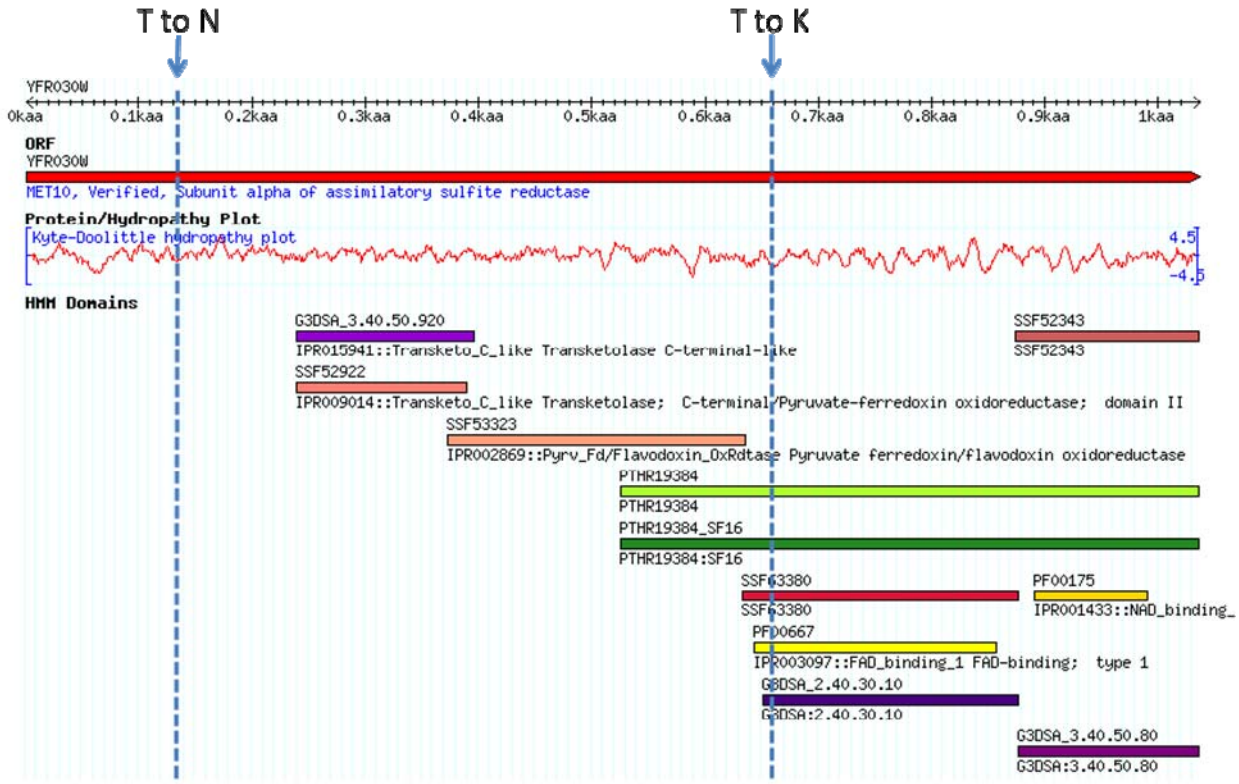


Figure 13: Domain analysis of the subunit alpha of assimilatory sulfite reductase showing the position of the amino acid variations between the parent strain UCD2034 and P1Y0/UCD932.

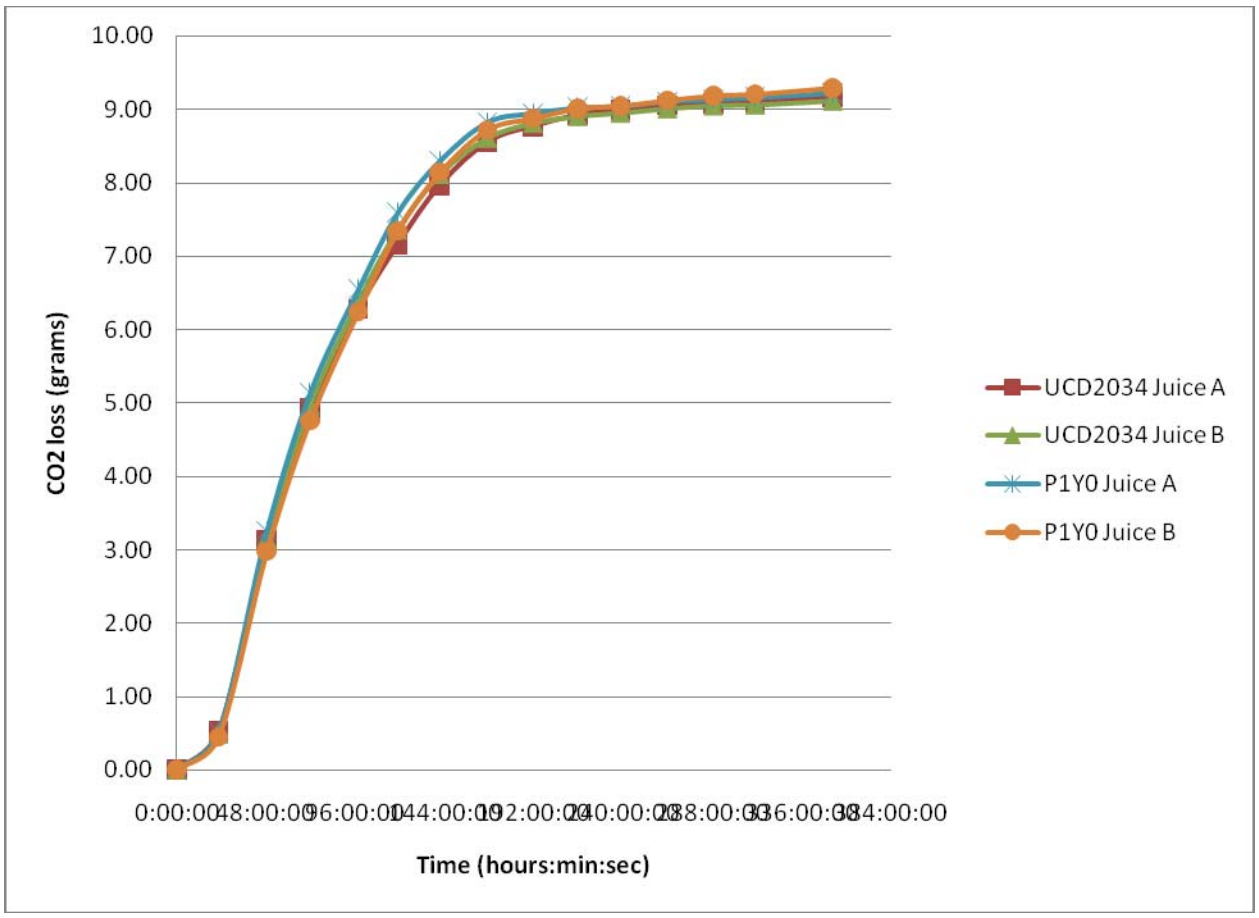


Figure 15: Carbon dioxide evolution in fermentations of UCD2034 and P1Y0 as measured as weight loss.

Sterile flasks capped with vapor locks containing 80 mL unfiltered Chardonnay grape juice were inoculated to $4x10^6$ cell/ mL with either UCD2034 or P1Y0. Fermentations were conducted at 20 °C. Fermentations were monitored daily for CO_2 evolution. The results are the average of two replicates per juice type.

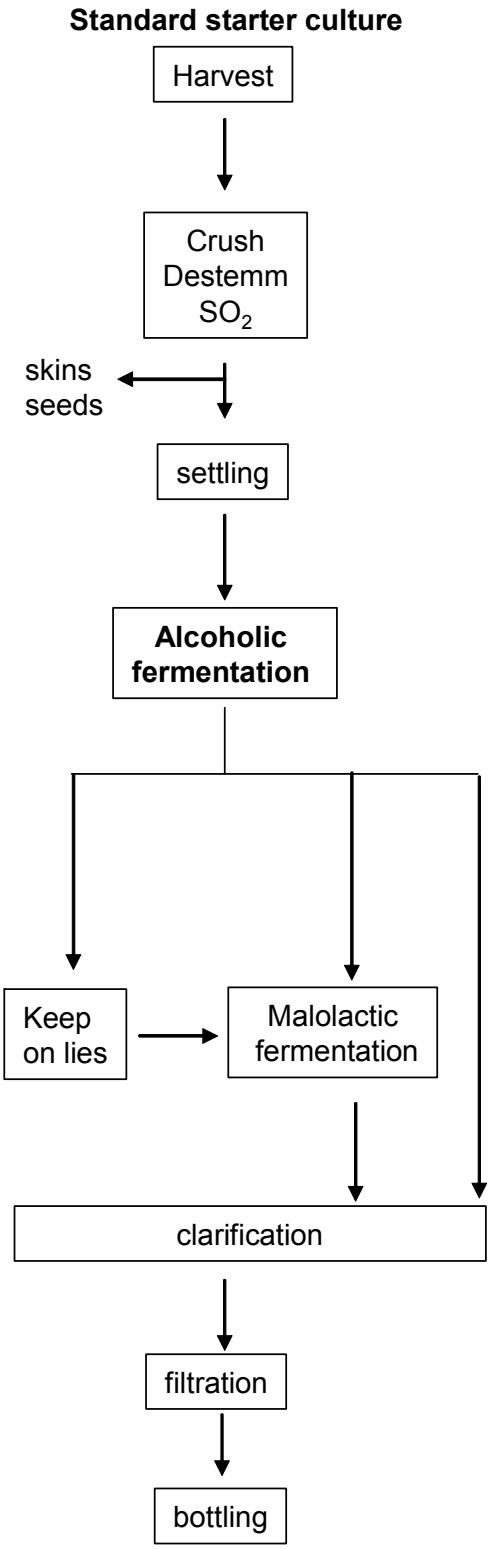


Figure 16: White winemaking practices and the use of a standard or P1Y0 yeast strain.

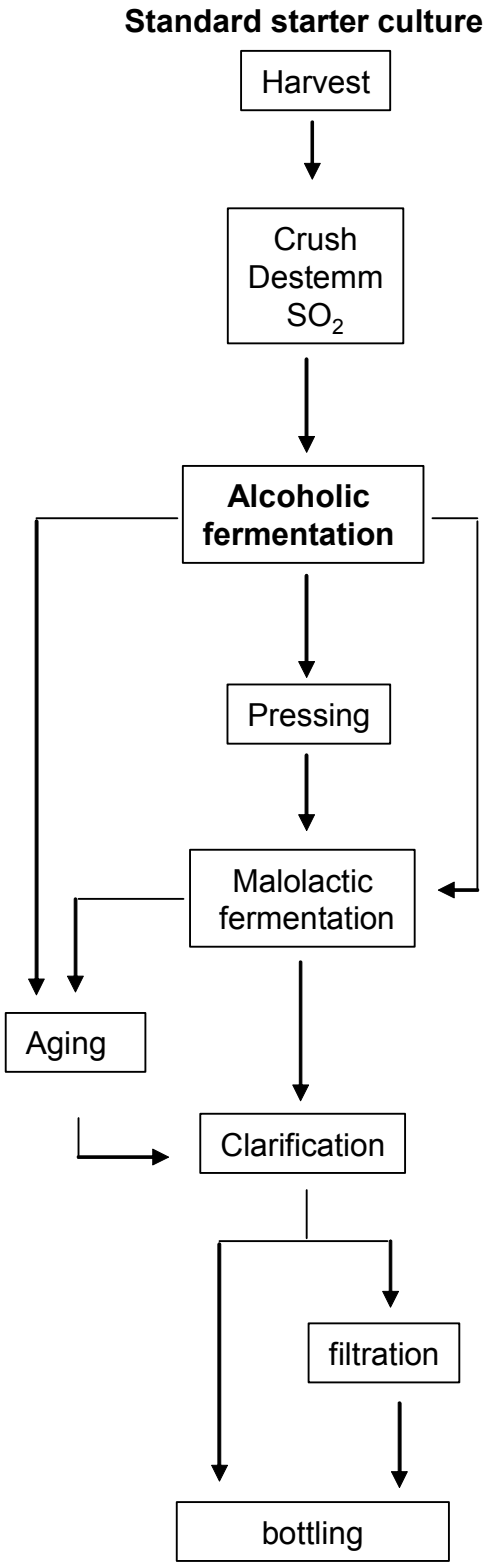


Figure 17: Red winemaking practices and the use of a standard or P1Y0 yeast strain.

Appendix 1: The *MET10* gene from UCD932 (FASTA format)

>
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TCCGCTAACGAGGTACAAAGTGTCGCCTTACTGACATTGGCGATTGCCACTTTCAGTAAT
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AGAGTACCATTACCTTTTAACGTTGCTAAGTTTGTCACTCACGTTCCATCCACTACCAAA
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Appendix 2: The linear hphMX cassette (FASTA format)

>
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Appendix 3: The linear kanMX cassette (FASTA format)

>
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TTTAAAC

Appendix 4: The *MET10*^{UCD932} integrating cassette (FASTA format)

>
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GAACTTCGTCGTGAAAGTTAAAGAAAATAGACGTGTTAAGCCTGCTGATTATGATAGATA
TATTTTCCATATTGAATTCGATATTTCTGGTACTGGAATGACTTATGACATCGGTGAAGC
CCTCGGTATTCATGCCAGAAACAATGAATCTTTGGTCAAAGAATTCTTAACCTTCTATGG
TCTAAATGAATCCGATGTTGTCTTAGTCCCCAACAAGGACAACCACCATTTGTTAGAAAC
AAGAACCGTCTTACAAGCATTTGTGGAAAATTTGGATATTTTCGGTAAACCACCAAAAAG
ATTTTACGAATCATTGATTCCATATGCCTCTAACGAAGAGGAGAAGAAAAAATTAGAGGA
TTTGGTTACTCCTGCCGGTGCAGTAGATTTGAAGAGATTTCAAGATGTGGAGTATTATAC
ATATGCTGACATTTTTGAATTGTTCCCATCTGTTCGCCCATCTCTTGAGGAACTTGTTAC
TATCATTGAACCATTGAAGAGAAGAGAATACTCAATTGCCTCCTCTCAGAAAGTTCATCC
AAATGAAGTTCATTTATTGATCGTTGTTGTTGATTGGGTGGATAATAAAGGAAGAAAAAG
GTACGGTCAAGCTTCTAAGTATATCTCAGACCTTGCTGTCGGTTCAGAATTGGTCGTTAG
CGTTAAACCATCTGTTATGAAATTACCACCATCTCCAAAGCAACCAGTTATTATGAGTGG
TTTAGGTACTGGTTTGGCACCATTCAAGGCCATTGTTGAAGAGAAATTATGGCAAAAGCA
GCAAGGTTATGAGATTGGTGAAGTCTTCCTATATCTAGGTTCAAGACACAAAAGAGAAGA
ATATTTATATGGTGAGTTATGGGAGGCTTACAAAGATGCAGGTATTATCACACACATCGG

CGCTGCTTTCTCAAGAGACCAACCTCAAAAAATTTACATTCAAGATCGTATCAAAGAGAA
TTTGGATGAATTAAAAACTGCAATGATTGATAATAAAGGTTCATTTTACTTGTGTGGCCC
TACTTGGCCAGTTCCAGATATTACTCAAGCTTTGCAAGACATTCTGGCTAAAGACGCCGA
GGAAAGAGGCATCAAAGTCGACTTGGATGCCGCAATTGAAGAATTAAAGGAAGCATCAAG
ATACATTTTAGAAGTCTACTAAATTAATATAGTAATAAAAACTAAATATCTATTTATTGA
ACCTGTCTTGAACATTTCTATTTTTTTTTTACTTTTAGTTTTCTTCTATGCGCAAGCTTT
TCTATTGGCTGCCAAATAGAAAATTATTGAAATATGATTACATTACAATATTTATTTGTC
TTATGAAAACTAACCATCACATTATACTAACTACGGAGGTACCATCTTGAAATCTCGTCC
TGAAGACCCTATTAGCATTGGCAAACATACCTTCTTTAAGCGAGACTACAATGAACTGAG
ATCCTTTAAACCTTGTTTTAATCAAATGACCTATATTTTGCGTATGACTTAAATCAAGAG
CAGCATCAACTTCATCCAAAATATACATAGGCGCTGGTCGAAATTGCAACAATGCCATAA
TCAATGACAAAGCAATCAACGACCTTTGCCCACCGGACAATTCAATCAAACTTTCCTTCC
ATATATTACCAAGCTTTACTTTAACCTCTAAACCCTGCGTTACGTCCTTACCTTCGCATG
GAACCAATTTTGCAAATGAATTTGGTAAAAGGTCTGCAAAAATATTACCAAAATCAAGTG
TCACTTTCTCCCATGTTTTG

Appendix 5: Optimal DNA alignment of the *MET10* ORF sequences of the *S. cerevisiae* strains UCD2034, UCD932 and P1Y0

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FILE: Multiple_Sequence_Alignment
MAXLENGTH: 3108
NAMES: UCD2034, UCD932, P1Y0

ORIGIN
UCD2034    ATGCCAGTTGAGTTTGCTACCAATCCTTTTGGCGAGGCCA     40
UCD932     ATGCCAGTTGAGTTTGCTACCAATCCTTTTGGCGAGGCCA     40
P1Y0       ATGCCAGTTGAGTTTGCTACCAATCCTTTTGGCGAGGCCA     40
Consensus  atgccagttgagtttgctaccaatccttttggcgaggcca

UCD2034    AAAATGCAACTTCACTGCCAAAATATGGTACACCCGTAAC     80
UCD932     AAAATGCAACTTCACTGCCAAAATATGGTACACCCGTAAC     80
P1Y0       AAAATGCAACTTCACTGCCAAAATATGGTACACCCGTAAC     80
Consensus  aaaatgcaacttcactgccaaaatatggtacacccgtaac

UCD2034    TGCCATTTCATCTGTGCTGTTCAATAACGTGGACTCCATT    120
UCD932     TGCCATTTCATCTGTGCTGTTCAATAACGTGGACTCCATT    120
P1Y0       TGCCATTTCATCTGTGCTGTTCAATAACGTGGACTCCATT    120
Consensus  tgccatttcatctgtgctgttcaataacgtggactccatt

UCD2034    TTTGCTTACAAGTCCTTTTCTCAACCCGATTTGTTACACC    160
UCD932     TTTGCTTACAAGTCCTTTTCTCAACCCGATTTGTTACACC    160
P1Y0       TTTGCTTACAAGTCCTTTTCTCAACCCGATTTGTTACACC    160
Consensus  tttgcttacaagtccttttctcaacccgatttgttacacc

UCD2034    AAGATCTAAAAAAATGGTCTGAAAAGCGTGGTAACGAATC    200
UCD932     AAGATCTAAAAAAATGGTCTGAAAAGCGTGGTAACGAATC    200
P1Y0       AAGATCTAAAAAAATGGTCTGAAAAGCGTGGTAACGAATC    200
Consensus  aagatctaaaaaaatggtctgaaaagcgtggtaacgaatc

UCD2034    ACGTGGGAAGCCATTTTTCCAAGAGCTGGATATCAGATCT    240
UCD932     ACGTGGGAAGCCATTTTTCCAAGAGCTGGATATCAGATCT    240
P1Y0       ACGTGGGAAGCCATTTTTCCAAGAGCTGGATATCAGATCT    240
Consensus  acgtgggaagccatttttccaagagctggatatcagatct

UCD2034    GGCGCTGGTTTGGCTCCTTTAGGGTTTTCTCATGGATTGA    280
UCD932     GGCGCTGGTTTGGCTCCTTTAGGGTTTTCTCATGGATTGA    280
P1Y0       GGCGCTGGTTTGGCTCCTTTAGGGTTTTCTCATGGATTGA    280
Consensus  ggcgctggtttggctcctttagggttttctcatggattga

UCD2034    AGAACACTACAGCAATTGTTGCTCCAGGGTTTTCGCTGCC    320
UCD932     AGAACACTACAGCAATTGTTGCTCCAGGGTTTTCGCTGCC    320
P1Y0       AGAACACTACAGCAATTGTTGCTCCAGGGTTTTCGCTGCC    320
Consensus  agaacactacagcaattgttgctccagggttttcgctgcc
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UCD2034    ATACTTCATTAACTCTTTGAAAACCGTCTCTCATGATGGT    360
UCD932     ATACTTCATTAACTCTTTGAAAACCGTCTCTCATGATGGT    360
P1Y0       ATACTTCATTAACTCTTTGAAAACCGTCTCTCATGATGGT    360
Consensus  atacttcattaactctttgaaaaccgtctctcatgatggt

UCD2034    AAGTTTCTTTTGAATGTTGGTGCTTTAAACTACGACAATG    400
UCD932     AAGTTTCTTTTGAATGTTGGTGCTTTAAACTACGACAATG    400
P1Y0       AAGTTTCTTTTGAATGTTGGTGCTTTAAACTACGACAATG    400
Consensus  aagtttcttttgaatgttggtgctttaaactacgacaatg

UCD2034    CTACCGGCTCTGTCACCAACGATTATGTAACCGCATTGGA    440
UCD932     CTAaCGGCTCTGTCACCAACGATTATGTAACCGCATTGGA    440
P1Y0       CTAaCGGCTCTGTCACCAACGATTATGTAACCGCATTGGA    440
Consensus  cta cggctctgtcaccaacgattatgtaaccgcattgga

UCD2034    TGCTGCTTCCAAGCTGAAGTATGGTGTCGTGACTCCGATT    480
UCD932     TGCTGCTTCCAAGCTGAAGTATGGTGTCGTGACTCCGATT    480
P1Y0       TGCTGCTTCCAAGCTGAAGTATGGTGTCGTGACTCCGATT    480
Consensus  tgctgcttccaagctgaagtatggtgtcgtgactccgatt

UCD2034    TCCGCTAACGAGGTACAAAGTGTCGCCTTACTGACATTGG    520
UCD932     TCCGCTAACGAGGTACAAAGTGTCGCCTTACTGACATTGG    520
P1Y0       TCCGCTAACGAGGTACAAAGTGTCGCCTTACTGACATTGG    520
Consensus  tccgctaacgaggtacaaagtgtcgccttactgacattgg

UCD2034    CGATTGCCACTTTCAGTAATAACTCCGGAGCTATCAATTT    560
UCD932     CGATTGCCACTTTCAGTAATAACTCCGGAGCTATCAATTT    560
P1Y0       CGATTGCCACTTTCAGTAATAACTCCGGAGCTATCAATTT    560
Consensus  cgattgccactttcagtaataactccggagctatcaattt

UCD2034    ATTTGACGGATTAAACTACTCGAAAACCGTCTTGCCGTTG    600
UCD932     ATTTGACGGATTAAACTACTCGAAAACCGTCTTGCCGTTG    600
P1Y0       ATTTGACGGATTAAACTACTCGAAAACCGTCTTGCCGTTG    600
Consensus  atttgacggattaaactactcgaaaaccgtcttgccgttg

UCD2034    GTCGAATCTGTTCCTGAGGCATCTATTTTGGCAAAACTAT    640
UCD932     GTCGAATCTGTTCCTGAGGCATCTATTTTGGCAAAACTAT    640
P1Y0       GTCGAATCTGTTCCTGAGGCATCTATTTTGGCAAAACTAT    640
Consensus  gtcgaatctgttcctgaggcatctattttggcaaaactat

UCD2034    CCAAAGTTATTGCACCAGATGCTGCCTTTGATGATGTCTT    680
UCD932     CCAAAGTTATTGCACCAGATGCTGCCTTTGATGATGTCTT    680
P1Y0       CCAAAGTTATTGCACCAGATGCTGCCTTTGATGATGTCTT    680
Consensus  ccaaagttattgcaccagatgctgcctttgatgatgtctt
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UCD2034    GGATAAGTTTAATGAATTGACTGGATTGAGACTACATAAT    720
UCD932     GGATAAGTTTAATGAATTGACTGGATTGAGACTACATAAT    720
P1Y0       GGATAAGTTTAATGAATTGACTGGATTGAGACTACATAAT    720
Consensus  ggataagtttaatgaattgactggattgagactacataat

UCD2034    TTCCAATACTTTGGTGCTCAGGATGCTGAAACTGTGTTTA    760
UCD932     TTCCAATACTTTGGTGCTCAGGATGCTGAAACTGTGTTTA    760
P1Y0       TTCCAATACTTTGGTGCTCAGGATGCTGAAACTGTGTTTA    760
Consensus  ttccaatactttggtgctcaggatgctgaaactgtgttta

UCD2034    TCACTTATGGGTCTTTAGAATCCGAATTGTTCAACTCTGC    800
UCD932     TCACTTATGGGTCTTTAGAATCCGAATTGTTCAACTCTGC    800
P1Y0       TCACTTATGGGTCTTTAGAATCCGAATTGTTCAACTCTGC    800
Consensus  tcacttatgggtctttagaatccgaattgttcaactctgc

UCD2034    GATTAGTGGTAATAATTCCAAAATCGGGTTAATCAACGTC    840
UCD932     GATTAGTGGTAATAATTCCAAAATCGGGTTAATCAACGTC    840
P1Y0       GATTAGTGGTAATAATTCCAAAATCGGGTTAATCAACGTC    840
Consensus  gattagtggtaataattccaaaatcgggttaatcaacgtc

UCD2034    AGAGTACCATTACCTTTTAACGTTGCTAAGTTTGTCACTC    880
UCD932     AGAGTACCATTACCTTTTAACGTTGCTAAGTTTGTCACTC    880
P1Y0       AGAGTACCATTACCTTTTAACGTTGCTAAGTTTGTCACTC    880
Consensus  agagtaccattaccttttaacgttgctaagtttgtcactc

UCD2034    ACGTTCCATCCACTACCAAACAAATTGTTGTTATAGGCCA    920
UCD932     ACGTTCCATCCACTACCAAACAAATTGTTGTTATAGGCCA    920
P1Y0       ACGTTCCATCCACTACCAAACAAATTGTTGTTATAGGCCA    920
Consensus  acgttccatccactaccaaacaaattgttgttataggcca

UCD2034    AACTTTGGATGGTTCTTCGCCTTCTTTCTTGAGATCTCAA    960
UCD932     AACTTTGGATGGTTCTTCGCCTTCTTTCTTGAGATCTCAA    960
P1Y0       AACTTTGGATGGTTCTTCGCCTTCTTTCTTGAGATCTCAA    960
Consensus  aactttggatggttcttcgccttctttcttgagatctcaa

UCD2034    GTTTCAGCCGCCTTATTTTACCACGGCCGCACCTCAATTA   1000
UCD932     GTTTCAGCCGCCTTATTTTACCACGGCCGCACCTCAATTA   1000
P1Y0       GTTTCAGCCGCCTTATTTTACCACGGCCGCACCTCAATTA   1000
Consensus  gtttcagccgccttattttaccacggccgcacctcaatta

UCD2034    GCGTTTCTGAGTACATCTATCAACCAGATTTCATTTGGTC   1040
UCD932     GCGTTTCTGAGTACATCTATCAACCAGATTTCATTTGGTC   1040
P1Y0       GCGTTTCTGAGTACATCTATCAACCAGATTTCATTTGGTC   1040
Consensus  gcgtttctgagtacatctatcaaccagatttcatttggtc
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UCD2034    CCCAAAAGCTGTCAAATCAATTGTATCGTCATTCATCCCT   1080
UCD932     CCCAAAAGCTGTCAAATCAATTGTATCGTCATTCATCCCT   1080
P1Y0       CCCAAAAGCTGTCAAATCAATTGTATCGTCATTCATCCCT   1080
Consensus  cccaaaagctgtcaaatcaattgtatcgtcattcatccct

UCD2034    GAATTCACTTACAATGCCGATTCATCTTTCGGCGAAGGAT   1120
UCD932     GAATTCACTTACAATGCCGATTCATCTTTCGGCGAAGGAT   1120
P1Y0       GAATTCACTTACAATGCCGATTCATCTTTCGGCGAAGGAT   1120
Consensus  gaattcacttacaatgccgattcatctttcggcgaaggat

UCD2034    TCATTTATTGGGCCTCTGATAAGAGTATCAATATTGATGT   1160
UCD932     TCATTTATTGGGCCTCTGATAAGAGTATCAATATTGATGT   1160
P1Y0       TCATTTATTGGGCCTCTGATAAGAGTATCAATATTGATGT   1160
Consensus  tcatttattgggcctctgataagagtatcaatattgatgt

UCD2034    TGCCTCCAAACTTGTGAAAGCTCTGTCTTTGGAAGATGGG   1200
UCD932     TGCCTCCAAACTTGTGAAAGCTCTGTCTTTGGAAGATGGG   1200
P1Y0       TGCCTCCAAACTTGTGAAAGCTCTGTCTTTGGAAGATGGG   1200
Consensus  tgcctccaaacttgtgaaagctctgtctttggaagatggg

UCD2034    AAATTTGTGTCTTTGAGAACGAAATTTGATAACTTGGCTA   1240
UCD932     AAATTTGTGTCTTTGAGAACGAAATTTGATAACTTGGCTA   1240
P1Y0       AAATTTGTGTCTTTGAGAACGAAATTTGATAACTTGGCTA   1240
Consensus  aaatttgtgtctttgagaacgaaatttgataacttggcta

UCD2034    ATGCTGGTACCTTCCAAGCTCAATTTGTGACCTCGAAGGA   1280
UCD932     ATGCTGGTACCTTCCAAGCTCAATTTGTGACCTCGAAaGA   1280
P1Y0       ATGCTGGTACCTTCCAAGCTCAATTTGTGACCTCGAAaGA   1280
Consensus  atgctggtaccttccaagctcaatttgtgacctcgaa ga

UCD2034    ACAGATACCTGTTTCAAACATCGATTCTACGAAATTATCA   1320
UCD932     ACAGATACCTGTTTCAAACATCGATTCTACGAAATTATCA   1320
P1Y0       ACAGATACCTGTTTCAAACATCGATTCTACGAAATTATCA   1320
Consensus  acagatacctgtttcaaacatcgattctacgaaattatca

UCD2034    GTCGTTGAAGATGTCAGTTTATTGAAGCATTTAGACGTAG   1360
UCD932     GTCGTTGAAGATGTCAGTTTATTGAAGCATTTAGACGTAG   1360
P1Y0       GTCGTTGAAGATGTCAGTTTATTGAAGCATTTAGACGTAG   1360
Consensus  gtcgttgaagatgtcagtttattgaagcatttagacgtag

UCD2034    CTGCTACCGTCGCAGAACAAGGTTCAATTGCGTTGGTTTC   1400
UCD932     CTGCTACCGTCGCAGAACAAGGTTCAATTGCGTTGGTTTC   1400
P1Y0       CTGCTACCGTCGCAGAACAAGGTTCAATTGCGTTGGTTTC   1400
Consensus  ctgctaccgtcgcagaacaaggttcaattgcgttggtttc
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UCD2034    CCAAAAGGCAGTTAAAGATTTGGATTTAAATTCTGTAGAA   1440
UCD932     CCAAAAGGCAGTTAAAGATTTGGATTTAAATTCTGTAGAA   1440
P1Y0       CCAAAAGGCAGTTAAAGATTTGGATTTAAATTCTGTAGAA   1440
Consensus  ccaaaaggcagttaaagatttggatttaaattctgtagaa

UCD2034    AGTTACGTCAAGAATTTGGGAATTCCTGAATCATTCCTAA   1480
UCD932     AGTTACGTCAAGAATTTGGGAATTCCTGAATCATTCCTAA   1480
P1Y0       AGTTACGTCAAGAATTTGGGAATTCCTGAATCATTCCTAA   1480
Consensus  agttacgtcaagaatttgggaattcctgaatcattcctaa

UCD2034    TATCTATTGCGAAGAAAAACATCAAATTGTTTATCATCGA   1520
UCD932     TATCTATTGCGAAGAAAAACATCAAATTGTTTATCATCGA   1520
P1Y0       TATCTATTGCGAAGAAAAACATCAAATTGTTTATCATCGA   1520
Consensus  tatctattgcgaagaaaaacatcaaattgtttatcatcga

UCD2034    TGGTGAGACCACTAACGACGAGTCCAAATTGTCCTTGTTT   1560
UCD932     TGGTGAGACCACTAACGACGAGTCCAAATTGTCCTTGTTT   1560
P1Y0       TGGTGAGACCACTAACGACGAGTCCAAATTGTCCTTGTTT   1560
Consensus  tggtgagaccactaacgacgagtccaaattgtccttgttt

UCD2034    ATCCAAGCCGTTTTCTGGAAATTGGCCTTCGGTCTAGATG   1600
UCD932     ATCCAAGCCGTTTTCTGGAAATTGGCCTTCGGTCTAGATG   1600
P1Y0       ATCCAAGCCGTTTTCTGGAAATTGGCCTTCGGTCTAGATG   1600
Consensus  atccaagccgttttctggaaattggccttcggtctagatg

UCD2034    TCGCAGAATGTACCAACCGTATCTGGAAAAGCATTGATTC   1640
UCD932     TCGCAGAATGTACCAACCGTATCTGGAAAAGCATTGATTC   1640
P1Y0       TCGCAGAATGTACCAACCGTATCTGGAAAAGCATTGATTC   1640
Consensus  tcgcagaatgtaccaaccgtatctggaaaagcattgattc

UCD2034    AGGTGCAGACATTTCAGCAGCCTCGATTTCTGAATTTCTC   1680
UCD932     AGGTGCAGACATTTCAGCAGCCTCGATTTCTGAATTTCTC   1680
P1Y0       AGGTGCAGACATTTCAGCAGCCTCGATTTCTGAATTTCTC   1680
Consensus  aggtgcagacatttcagcagcctcgatttctgaatttctc

UCD2034    ACTGGTGCATTCAAAAACTTCCTCAGTGAGGTTCCGCTAG   1720
UCD932     ACTGGTGCATTCAAAAACTTCCTCAGTGAGGTTCCGCTAG   1720
P1Y0       ACTGGTGCATTCAAAAACTTCCTCAGTGAGGTTCCGCTAG   1720
Consensus  actggtgcattcaaaaacttcctcagtgaggttccgctag

UCD2034    CGCTGTACACTAAATTTTCTGAAATAAACATTGAAAAGAA   1760
UCD932     CGCTGTACACTAAATTTTCTGAAATAAACATTGAAAAGAA   1760
P1Y0       CGCTGTACACTAAATTTTCTGAAATAAACATTGAAAAGAA   1760
Consensus  cgctgtacactaaattttctgaaataaacattgaaaagaa
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UCD2034    AGAGGATGAGGAAGAGCCTGCAGCTTTACCAATTTTCGTT   1800
UCD932     AGAGGATGAGGAAGAGCCTGCAGCTTTACCAATTTTCGTT   1800
P1Y0       AGAGGATGAGGAAGAGCCTGCAGCTTTACCAATTTTCGTT   1800
Consensus  agaggatgaggaagagcctgcagctttaccaattttcgtt

UCD2034    AATGAAACATCTTTCCTCCCAAATAACAGTACCATTGAAG   1840
UCD932     AATGAAACATCTTTCCTCCCAAATAACAGTACCATTGAAG   1840
P1Y0       AATGAAACATCTTTCCTCCCAAATAACAGTACCATTGAAG   1840
Consensus  aatgaaacatctttcctcccaaataacagtaccattgaag

UCD2034    AAATACCATTACCTGAGACCTCTGAGATCTCTGATATTGC   1880
UCD932     AAATACCATTACCTGAGACCTCTGAGATCTCTGATATTGC   1880
P1Y0       AAATACCATTACCTGAGACCTCTGAGATCTCTGATATTGC   1880
Consensus  aaataccattacctgagacctctgagatctctgatattgc

UCD2034    CAAGAAGTTGTCCTTCAAAGAGGCATATGAAGTTGAGAAT   1920
UCD932     CAAGAAGTTGTCCTTCAAAGAGGCATATGAAGTTGAGAAT   1920
P1Y0       CAAGAAGTTGTCCTTCAAAGAGGCATATGAAGTTGAGAAT   1920
Consensus  caagaagttgtccttcaaagaggcatatgaagttgagaat

UCD2034    AAACTAAGACCCGATTTACCCGTCAAGAACTTCGTCGTGA   1960
UCD932     AAACTAAGACCCGATTTACCCGTCAAGAACTTCGTCGTGA   1960
P1Y0       AAACTAAGACCCGATTTACCCGTCAAGAACTTCGTCGTGA   1960
Consensus  aaactaagacccgatttacccgtcaagaacttcgtcgtga

UCD2034    AAGTTAAAGAAAATAGACGTGTTACGCCTGCTGATTATGA   2000
UCD932     AAGTTAAAGAAAATAGACGTGTTAaGCCTGCTGATTATGA   2000
P1Y0       AAGTTAAAGAAAATAGACGTGTTAaGCCTGCTGATTATGA   2000
Consensus  aagttaaagaaaatagacgtgtta gcctgctgattatga

UCD2034    TAGATATATTTTCCATATTGAATTCGATATTTCTGGTACT   2040
UCD932     TAGATATATTTTCCATATTGAATTCGATATTTCTGGTACT   2040
P1Y0       TAGATATATTTTCCATATTGAATTCGATATTTCTGGTACT   2040
Consensus  tagatatattttccatattgaattcgatatttctggtact

UCD2034    GGAATGACTTATGACATCGGTGAAGCCCTCGGTATTCATG   2080
UCD932     GGAATGACTTATGACATCGGTGAAGCCCTCGGTATTCATG   2080
P1Y0       GGAATGACTTATGACATCGGTGAAGCCCTCGGTATTCATG   2080
Consensus  ggaatgacttatgacatcggtgaagccctcggtattcatg

UCD2034    CCAGAAACAATGAATCTTTGGTCAAAGAATTCTTAACCTT   2120
UCD932     CCAGAAACAATGAATCTTTGGTCAAAGAATTCTTAACCTT   2120
P1Y0       CCAGAAACAATGAATCTTTGGTCAAAGAATTCTTAACCTT   2120
Consensus  ccagaaacaatgaatctttggtcaaagaattcttaacctt
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UCD2034    CTATGGTCTAAATGAATCCGATGTTGTCTTAGTCCCCAAC   2160
UCD932     CTATGGTCTAAATGAATCCGATGTTGTCTTAGTCCCCAAC   2160
P1Y0       CTATGGTCTAAATGAATCCGATGTTGTCTTAGTCCCCAAC   2160
Consensus  ctatggtctaaatgaatccgatgttgtcttagtccccaac

UCD2034    AAGGACAACCACCATTTGTTAGAAACAAGAACCGTCTTAC   2200
UCD932     AAGGACAACCACCATTTGTTAGAAACAAGAACCGTCTTAC   2200
P1Y0       AAGGACAACCACCATTTGTTAGAAACAAGAACCGTCTTAC   2200
Consensus  aaggacaaccaccatttgttagaaacaagaaccgtcttac

UCD2034    AAGCATTTGTGGAAAATTTGGATATTTTCGGTAAACCACC   2240
UCD932     AAGCATTTGTGGAAAATTTGGATATTTTCGGTAAACCACC   2240
P1Y0       AAGCATTTGTGGAAAATTTGGATATTTTCGGTAAACCACC   2240
Consensus  aagcatttgtggaaaatttggatattttcggtaaaccacc

UCD2034    AAAAAGATTTTACGAATCATTGATTCCATATGCCTCTAAC   2280
UCD932     AAAAAGATTTTACGAATCATTGATTCCATATGCCTCTAAC   2280
P1Y0       AAAAAGATTTTACGAATCATTGATTCCATATGCCTCTAAC   2280
Consensus  aaaaagattttacgaatcattgattccatatgcctctaac

UCD2034    GAAGAGGAGAAGAAAAAATTAGAGGATTTGGTTACTCCTG   2320
UCD932     GAAGAGGAGAAGAAAAAATTAGAGGATTTGGTTACTCCTG   2320
P1Y0       GAAGAGGAGAAGAAAAAATTAGAGGATTTGGTTACTCCTG   2320
Consensus  gaagaggagaagaaaaaattagaggatttggttactcctg

UCD2034    CCGGTGCAGTAGATTTGAAGAGATTTCAAGATGTGGAGTA   2360
UCD932     CCGGTGCAGTAGATTTGAAGAGATTTCAAGATGTGGAGTA   2360
P1Y0       CCGGTGCAGTAGATTTGAAGAGATTTCAAGATGTGGAGTA   2360
Consensus  ccggtgcagtagatttgaagagatttcaagatgtggagta

UCD2034    TTATACATATGCTGACATTTTTGAATTGTTCCCATCTGTT   2400
UCD932     TTATACATATGCTGACATTTTTGAATTGTTCCCATCTGTT   2400
P1Y0       TTATACATATGCTGACATTTTTGAATTGTTCCCATCTGTT   2400
Consensus  ttatacatatgctgacatttttgaattgttcccatctgtt

UCD2034    CGCCCATCTCTTGAGGAACTTGTTACTATCATTGAACCAT   2440
UCD932     CGCCCATCTCTTGAGGAACTTGTTACTATCATTGAACCAT   2440
P1Y0       CGCCCATCTCTTGAGGAACTTGTTACTATCATTGAACCAT   2440
Consensus  cgcccatctcttgaggaacttgttactatcattgaaccat

UCD2034    TGAAGAGAAGAGAATACTCAATTGCCTCCTCTCAGAAAGT   2480
UCD932     TGAAGAGAAGAGAATACTCAATTGCCTCCTCTCAGAAAGT   2480
P1Y0       TGAAGAGAAGAGAATACTCAATTGCCTCCTCTCAGAAAGT   2480
Consensus  tgaagagaagagaatactcaattgcctcctctcagaaagt
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UCD2034    TCATCCAAATGAAGTTCATTTATTGATCGTTGTTGTTGAT   2520
UCD932     TCATCCAAATGAAGTTCATTTATTGATCGTTGTTGTTGAT   2520
P1Y0       TCATCCAAATGAAGTTCATTTATTGATCGTTGTTGTTGAT   2520
Consensus  tcatccaaatgaagttcatttattgatcgttgttgttgat

UCD2034    TGGGTGGATAATAAAGGAAGAAAAAGGTACGGTCAAGCTT   2560
UCD932     TGGGTGGATAATAAAGGAAGAAAAAGGTACGGTCAAGCTT   2560
P1Y0       TGGGTGGATAATAAAGGAAGAAAAAGGTACGGTCAAGCTT   2560
Consensus  tgggtggataataaaggaagaaaaaggtacggtcaagctt

UCD2034    CTAAGTATATCTCAGACCTTGCTGTCGGTTCAGAATTGGT   2600
UCD932     CTAAGTATATCTCAGACCTTGCTGTCGGTTCAGAATTGGT   2600
P1Y0       CTAAGTATATCTCAGACCTTGCTGTCGGTTCAGAATTGGT   2600
Consensus  ctaagtatatctcagaccttgctgtcggttcagaattggt

UCD2034    CGTTAGCGTTAAACCATCTGTTATGAAATTACCACCATCT   2640
UCD932     CGTTAGCGTTAAACCATCTGTTATGAAATTACCACCATCT   2640
P1Y0       CGTTAGCGTTAAACCATCTGTTATGAAATTACCACCATCT   2640
Consensus  cgttagcgttaaaccatctgttatgaaattaccaccatct

UCD2034    CCAAAGCAACCAGTTATTATGAGTGGTTTAGGTACTGGTT   2680
UCD932     CCAAAGCAACCAGTTATTATGAGTGGTTTAGGTACTGGTT   2680
P1Y0       CCAAAGCAACCAGTTATTATGAGTGGTTTAGGTACTGGTT   2680
Consensus  ccaaagcaaccagttattatgagtggtttaggtactggtt

UCD2034    TGGCACCATTCAAGGCCATTGTTGAAGAGAAATTATGGCA   2720
UCD932     TGGCACCATTCAAGGCCATTGTTGAAGAGAAATTATGGCA   2720
P1Y0       TGGCACCATTCAAGGCCATTGTTGAAGAGAAATTATGGCA   2720
Consensus  tggcaccattcaaggccattgttgaagagaaattatggca

UCD2034    AAAGCAGCAAGGTTATGAGATTGGTGAAGTCTTCCTATAT   2760
UCD932     AAAGCAGCAAGGTTATGAGATTGGTGAAGTCTTCCTATAT   2760
P1Y0       AAAGCAGCAAGGTTATGAGATTGGTGAAGTCTTCCTATAT   2760
Consensus  aaagcagcaaggttatgagattggtgaagtcttcctatat

UCD2034    CTAGGTTCAAGACACAAAAGAGAAGAATATTTATATGGTG   2800
UCD932     CTAGGTTCAAGACACAAAAGAGAAGAATATTTATATGGTG   2800
P1Y0       CTAGGTTCAAGACACAAAAGAGAAGAATATTTATATGGTG   2800
Consensus  ctaggttcaagacacaaaagagaagaatatttatatggtg

UCD2034    AGTTATGGGAGGCTTACAAAGATGCAGGTATTATCACACA   2840
UCD932     AGTTATGGGAGGCTTACAAAGATGCAGGTATTATCACACA   2840
P1Y0       AGTTATGGGAGGCTTACAAAGATGCAGGTATTATCACACA   2840
Consensus  agttatgggaggcttacaaagatgcaggtattatcacaca
```

```
UCD2034    CATCGGCGCTGCTTTCTCAAGAGACCAACCTCAAAAAATT   2880
UCD932     CATCGGCGCTGCTTTCTCAAGAGACCAACCTCAAAAAATT   2880
P1Y0       CATCGGCGCTGCTTTCTCAAGAGACCAACCTCAAAAAATT   2880
Consensus  catcggcgctgctttctcaagagaccaacctcaaaaaatt

UCD2034    TACATTCAAGATCGTATCAAAGAGAATTTGGATGAATTAA   2920
UCD932     TACATTCAAGATCGTATCAAAGAGAATTTGGATGAATTAA   2920
P1Y0       TACATTCAAGATCGTATCAAAGAGAATTTGGATGAATTAA   2920
Consensus  tacattcaagatcgtatcaaagagaatttggatgaattaa

UCD2034    AAACTGCAATGATTGATAATAAAGGTTCATTTTACTTGTG   2960
UCD932     AAACTGCAATGATTGATAATAAAGGTTCATTTTACTTGTG   2960
P1Y0       AAACTGCAATGATTGATAATAAAGGTTCATTTTACTTGTG   2960
Consensus  aaactgcaatgattgataataaaggttcattttacttgtg

UCD2034    TGGCCCTACTTGGCCAGTTCCAGATATTACTCAAGCTTTG   3000
UCD932     TGGCCCTACTTGGCCAGTTCCAGATATTACTCAAGCTTTG   3000
P1Y0       TGGCCCTACTTGGCCAGTTCCAGATATTACTCAAGCTTTG   3000
Consensus  tggccctacttggccagttccagatattactcaagctttg

UCD2034    CAAGACATTCTGGCTAAAGACGCCGAGGAAAGAGGCATCA   3040
UCD932     CAAGACATTCTGGCTAAAGACGCCGAGGAAAGAGGCATCA   3040
P1Y0       CAAGACATTCTGGCTAAAGACGCCGAGGAAAGAGGCATCA   3040
Consensus  caagacattctggctaaagacgccgaggaaagaggcatca

UCD2034    AAGTCGACTTGGATGCCGCAATTGAAGAATTAAAGGAAGC   3080
UCD932     AAGTCGACTTGGATGCCGCAATTGAAGAATTAAAGGAAGC   3080
P1Y0       AAGTCGACTTGGATGCCGCAATTGAAGAATTAAAGGAAGC   3080
Consensus  aagtcgacttggatgccgcaattgaagaattaaaggaagc

UCD2034    ATCAAGATACATTTTAGAAGTCTACTAA               3108
UCD932     ATCAAGATACATTTTAGAAGTCTACTAA               3108
P1Y0       ATCAAGATACATTTTAGAAGTCTACTAA               3108
Consensus  atcaagatacattttagaagtctactaa
```

Appendix 6: Amino acid alignment of the subunit alpha of assimilatory sulfite reductase of the *S. cerevisiae* strains UCD2034, UCD932 and P1Y0

```
PROJECT: Amino acid alignment
MAXLENGTH: 1035
NAMES: UCD2034, UCD932, P1Y0

ORIGIN
UCD2034    MPVEFATNPFGEAKNATSLPKYGTPVTAISSVLFNNVDSI     40
UCD932     MPVEFATNPFGEAKNATSLPKYGTPVTAISSVLFNNVDSI     40
P1Y0       MPVEFATNPFGEAKNATSLPKYGTPVTAISSVLFNNVDSI     40
Consensus  mpvefatnpfgeaknatslpkygtpvtaissvlfnnvdsi

UCD2034    FAYKSFSQPDLLHQDLKKWSEKRGNESRGKPFFQELDIRS     80
UCD932     FAYKSFSQPDLLHQDLKKWSEKRGNESRGKPFFQELDIRS     80
P1Y0       FAYKSFSQPDLLHQDLKKWSEKRGNESRGKPFFQELDIRS     80
Consensus  fayksfsqpdllhqdlkkwsekrgnesrgkpffqeldirs

UCD2034    GAGLAPLGFSHGLKNTTAIVAPGFSLPYFINSLKTVSHDG    120
UCD932     GAGLAPLGFSHGLKNTTAIVAPGFSLPYFINSLKTVSHDG    120
P1Y0       GAGLAPLGFSHGLKNTTAIVAPGFSLPYFINSLKTVSHDG    120
Consensus  gaglaplgfshglknttaivapgfslpyfinslktvshdg

UCD2034    KFLLNVGALNYDNATGSVTNDYVTALDAASKLKYGVVTPI    160
UCD932     KFLLNVGALNYDNAnGSVTNDYVTALDAASKLKYGVVTPI    160
P1Y0       KFLLNVGALNYDNAnGSVTNDYVTALDAASKLKYGVVTPI    160
Consensus  kfllnvgalnydna gsvtndyvtaldaasklkygvvtpi

UCD2034    SANEVQSVALLTLAIATFSNNSGAINLFDGLNYSKTVLPL    200
UCD932     SANEVQSVALLTLAIATFSNNSGAINLFDGLNYSKTVLPL    200
P1Y0       SANEVQSVALLTLAIATFSNNSGAINLFDGLNYSKTVLPL    200
Consensus  sanevqsvalltlaiatfsnnsgainlfdglnysktvlpl

UCD2034    VESVPEASILAKLSKVIAPDAAFDDVLDKFNELTGLRLHN    240
UCD932     VESVPEASILAKLSKVIAPDAAFDDVLDKFNELTGLRLHN    240
P1Y0       VESVPEASILAKLSKVIAPDAAFDDVLDKFNELTGLRLHN    240
Consensus  vesvpeasilaklskviapdaafddvldkfneltglrlhn

UCD2034    FQYFGAQDAETVFITYGSLESELFNSAISGNNSKIGLINV    280
UCD932     FQYFGAQDAETVFITYGSLESELFNSAISGNNSKIGLINV    280
P1Y0       FQYFGAQDAETVFITYGSLESELFNSAISGNNSKIGLINV    280
Consensus  fqyfgaqdaetvfitygsleselfnsaisgnnskiglinv

UCD2034    RVPLPFNVAKFVTHVPSTTKQIVVIGQTLDGSSPSFLRSQ    320
UCD932     RVPLPFNVAKFVTHVPSTTKQIVVIGQTLDGSSPSFLRSQ    320
P1Y0       RVPLPFNVAKFVTHVPSTTKQIVVIGQTLDGSSPSFLRSQ    320
Consensus  rvplpfnvakfvthvpsttkqivvigqtldgsspsflrsq
```

```
UCD2034    VSAALFYHGRTSISVSEYIYQPDFIWSPKAVKSIVSSFIP    360
UCD932     VSAALFYHGRTSISVSEYIYQPDFIWSPKAVKSIVSSFIP    360
P1Y0       VSAALFYHGRTSISVSEYIYQPDFIWSPKAVKSIVSSFIP    360
Consensus  vsaalfyhgrtsisvseyiyqpdfiwspkavksivssfip

UCD2034    EFTYNADSSFGEGFIYWASDKSINIDVASKLVKALSLEDG    400
UCD932     EFTYNADSSFGEGFIYWASDKSINIDVASKLVKALSLEDG    400
P1Y0       EFTYNADSSFGEGFIYWASDKSINIDVASKLVKALSLEDG    400
Consensus  eftynadssfgegfiywasdksinidvasklvkalsledg

UCD2034    KFVSLRTKFDNLANAGTFQAQFVTSKEQIPVSNIDSTKLS    440
UCD932     KFVSLRTKFDNLANAGTFQAQFVTSKEQIPVSNIDSTKLS    440
P1Y0       KFVSLRTKFDNLANAGTFQAQFVTSKEQIPVSNIDSTKLS    440
Consensus  kfvslrtkfdnlanagtfqaqfvtskeqipvsnidstkls

UCD2034    VVEDVSLLKHLDVAATVAEQGSIALVSQKAVKDLDLNSVE    480
UCD932     VVEDVSLLKHLDVAATVAEQGSIALVSQKAVKDLDLNSVE    480
P1Y0       VVEDVSLLKHLDVAATVAEQGSIALVSQKAVKDLDLNSVE    480
Consensus  vvedvsllkhldvaatvaeqgsialvsqkavkdldlnsve

UCD2034    SYVKNLGIPESFLISIAKKNIKLFIIDGETTNDESKLSLF    520
UCD932     SYVKNLGIPESFLISIAKKNIKLFIIDGETTNDESKLSLF    520
P1Y0       SYVKNLGIPESFLISIAKKNIKLFIIDGETTNDESKLSLF    520
Consensus  syvknlgipesflisiakkniklfiidgettndesklslf

UCD2034    IQAVFWKLAFGLDVAECTNRIWKSIDSGADISAASISEFL    560
UCD932     IQAVFWKLAFGLDVAECTNRIWKSIDSGADISAASISEFL    560
P1Y0       IQAVFWKLAFGLDVAECTNRIWKSIDSGADISAASISEFL    560
Consensus  iqavfwklafgldvaectnriwksidsgadisaasisefl

UCD2034    TGAFKNFLSEVPLALYTKFSEINIEKKEDEEEPAALPIFV    600
UCD932     TGAFKNFLSEVPLALYTKFSEINIEKKEDEEEPAALPIFV    600
P1Y0       TGAFKNFLSEVPLALYTKFSEINIEKKEDEEEPAALPIFV    600
Consensus  tgafknflsevplalytkfseiniekkedeeepaalpifv

UCD2034    NETSFLPNNSTIEEIPLPETSEISDIAKKLSFKEAYEVEN    640
UCD932     NETSFLPNNSTIEEIPLPETSEISDIAKKLSFKEAYEVEN    640
P1Y0       NETSFLPNNSTIEEIPLPETSEISDIAKKLSFKEAYEVEN    640
Consensus  netsflpnnstieeiplpetseisdiakklsfkeayeven

UCD2034    KLRPDLPVKNFVVKVKENRRVTPADYDRYIFHIEFDISGT    680
UCD932     KLRPDLPVKNFVVKVKENRRVkPADYDRYIFHIEFDISGT    680
P1Y0       KLRPDLPVKNFVVKVKENRRVkPADYDRYIFHIEFDISGT    680
Consensus  klrpdlpvknfvvkvkenrrv padydryifhiefdisgt
```

```
UCD2034    GMTYDIGEALGIHARNNESLVKEFLTFYGLNESDVVLVPN    720
UCD932     GMTYDIGEALGIHARNNESLVKEFLTFYGLNESDVVLVPN    720
P1Y0       GMTYDIGEALGIHARNNESLVKEFLTFYGLNESDVVLVPN    720
Consensus  gmtydigealgiharnneslvkefltfyglnesdvvlvpn

UCD2034    KDNHHLLETRTVLQAFVENLDIFGKPPKRFYESLIPYASN    760
UCD932     KDNHHLLETRTVLQAFVENLDIFGKPPKRFYESLIPYASN    760
P1Y0       KDNHHLLETRTVLQAFVENLDIFGKPPKRFYESLIPYASN    760
Consensus  kdnhhlletrtvlqafvenldifgkppkrfyeslipyasn

UCD2034    EEEKKKLEDLVTPAGAVDLKRFQDVEYYTYADIFELFPSV    800
UCD932     EEEKKKLEDLVTPAGAVDLKRFQDVEYYTYADIFELFPSV    800
P1Y0       EEEKKKLEDLVTPAGAVDLKRFQDVEYYTYADIFELFPSV    800
Consensus  eeekkkledlvtpagavdlkrfqdveyytyadifelfpsv

UCD2034    RPSLEELVTIIEPLKRREYSIASSQKVHPNEVHLLIVVVD    840
UCD932     RPSLEELVTIIEPLKRREYSIASSQKVHPNEVHLLIVVVD    840
P1Y0       RPSLEELVTIIEPLKRREYSIASSQKVHPNEVHLLIVVVD    840
Consensus  rpsleelvtiieplkrreysiassqkvhpnevhllivvvd

UCD2034    WVDNKGRKRYGQASKYISDLAVGSELVVSVKPSVMKLPPS    880
UCD932     WVDNKGRKRYGQASKYISDLAVGSELVVSVKPSVMKLPPS    880
P1Y0       WVDNKGRKRYGQASKYISDLAVGSELVVSVKPSVMKLPPS    880
Consensus  wvdnkgrkrygqaskyisdlavgselvvsvkpsvmklpps

UCD2034    PKQPVIMSGLGTGLAPFKAIVEEKLWQKQQGYEIGEVFLY    920
UCD932     PKQPVIMSGLGTGLAPFKAIVEEKLWQKQQGYEIGEVFLY    920
P1Y0       PKQPVIMSGLGTGLAPFKAIVEEKLWQKQQGYEIGEVFLY    920
Consensus  pkqpvimsglgtglapfkaiveeklwqkqqgyeigevfly

UCD2034    LGSRHKREEYLYGELWEAYKDAGIITHIGAAFSRDQPQKI    960
UCD932     LGSRHKREEYLYGELWEAYKDAGIITHIGAAFSRDQPQKI    960
P1Y0       LGSRHKREEYLYGELWEAYKDAGIITHIGAAFSRDQPQKI    960
Consensus  lgsrhkreeylygelweaykdagiithigaafsrdqpqki

UCD2034    YIQDRIKENLDELKTAMIDNKGSFYLCGPTWPVPDITQAL   1000
UCD932     YIQDRIKENLDELKTAMIDNKGSFYLCGPTWPVPDITQAL   1000
P1Y0       YIQDRIKENLDELKTAMIDNKGSFYLCGPTWPVPDITQAL   1000
Consensus  yiqdrikenldelktamidnkgsfylcgptwpvpditqal

UCD2034    QDILAKDAEERGIKVDLDAAIEELKEASRYILEVY        1035
UCD932     QDILAKDAEERGIKVDLDAAIEELKEASRYILEVY        1035
P1Y0       QDILAKDAEERGIKVDLDAAIEELKEASRYILEVY        1035
Consensus  qdilakdaeergikvdldaaieelkeasryilevy
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5 References

Ari'Izumi K., Suzuki Y., Kato I., Yagi Y., Otsuka K., Sato M. Winemaking from Koshu variety by the *sur lie* method : change in the content of nitrogen compounds. *Am J Enol Vitic.* 1994. **45 (3)**:312-318.

Amoore, J.E. and E. Hautala. 1983. Odor as an aid to chemical safety: Odor thresholds compared with threshold limit valuses and volatilities for 214 chemicals in air and water dilution. *J. Appl. Oxicol*. 3:272-290.

Bechet, J., M. Grenson and J.M. Waime. 1970. Mutations affecting the repressibility of arginine biosynthetic enzymes in *Saccharomyces cerevisiae*. *Eur. J. Biochem*. **12**: 31-39.

Charpentier C., Feuillat M. Yeast autolysis. In : *Wine Microbiology and Biotechnology.* Fleet G.H. (ed). Reading : Hardwood Academic. 1993. 225-242.

Cherry JM, Ball C, Weng S, Juvik G, Schmidt R, Adler C, Dunn B, Dwight S, Riles L, Mortimer RK, Botstein D (1997) Genetic and physical maps of Saccharomyces cerevisiae. *Nature.* **387 (6632 Suppl)**:67-73

Chisholm G, Cooper TG. 1982. Isolation and characterization of mutants that produce the allantoin-degrading enzymes constitutively in *Saccharomyces cerevisiae*. *Mol Cell Biol*. **Sep; 2(9):**1088-95.

Commission des Communautés Européennes. Rapports du comité scientifique de l'alimentation humaine (vingt-septième série). EUR **14181** FR. p 15-22.

Coulon, J. Ph.D. Thesis. 2001.

Crous J.M., Pretorius I.S., van Zyl W.H. Cloning and expression of an *Aspergillus kawachii* endo-1,4-□-xylanase gene in *Saccharomyces cerevisiae. Curr Genet*. 1995. **28**: 467-473.

Cunningham T.S., Cooper T.G. Expression of the *DAL80* gene, whose product is homologous to the GATA factors and is a negative regulator of multiple nitrogen catabolic genes in *Saccharomyces cerevisiae*, is sensitive to nitrogen catabolic repression. *Mol Cell Biol*. 1991. **11 (12)**: 6205-6215.

Dawes H., Boyes S., Keene J., Heatherbell D. Protein instability of wines : influence of protein isoelectric point. *Am J Enol Vitic*. 1994. **45 (3)**:319-325.

Dunn, B., R.P. Levine, G. Sherlock. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics*. 2005. Apr 16; **6 (1)**:53.

Fornairon-Bonnefond C., Camarasa C., Moutonnet M., Salmon J-M. New trends on yeast autolysis and wine ageing on lees : a bibliographic review. *Int J Wine Sci*. 2001. **36 (2)**:49-69.

Gatignol A., Dassain M., Tiraby G. Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on Phleomycin resistance. *Gene*. 1990. **91 (1)**:35-41.

Gietz R.D., Sugino A. New yeast *Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*. 1988. **74**:527-534.

Grenson, M., M. Mousset, J.M. Wiame and J. Bechet. 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a speicfic arginine-transporting system. *Biochim. Biophys. Acta.* **127**: 325-338.

Hill J.E., Myers A.M., Koerner T.J., Tzagoloff A. Yeast/*E.coli* shuttle vectors with multiple unique restriction sites. *Yeast*. 1986. **2**:163-197.

International Agency for Research on Cancer. 1974, 1982, 1987. www.iarc.fr.

Kreger-van-Rij N.J.W. (Ed). *The yeasts : a taxonomy study*. 3rd ed. Amsterdam : Elsevier Science Publishers B.V. 1984.1082p

Kurtzman C.P., Fell J.W. (Eds). *The yeasts. A taxonomic study*. 4th edition. Amsterdam : Elsevier Science B.V. 1998. 1055 pp.

Lilly M., Lambrechts M.G., Pretorius I.S. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wines and distillates. *Appl Environ Microbiol*. 2000. **66 (2)**:744-753

Lodder J. (Ed). *The yeasts : a taxonomic study*. 2nd ed. Amsterdam : North Holland Publishing Company. 1970. 1385 pp.

Lodder J., Kreger-van-Rij N.J.W. (Eds). *The yeasts : a taxonomic study*. 2nd ed. Amsterdam : North Holland Publishing Company. 1967. 713 pp.

Martinez-Rodriguez A.J., Polo M. Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *Am Chem Soc*. 2000. **48 (4)**: 1081-1085.

Messing J., Vieira J. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene*. 1982. **19 (3)**:269-276.

Mortimer R.K., Johnston J.R. Genealogy of principal strains of the yeast genetic stock center. *Genetics*. 1986. **113**: 35-43.

Office International de la vigne et du vin. Codex Œnologique International, Edition 2002. Paris, OIV.

Olson M.V., Dutchik J.E., Graham M.Y., Brodeur G.M., Helms C., Frank M., MacCollin M., Scheinman R., Frank T. Random-clone strategy for genomic restriction mapping in yeast. *Proc Natl Acad Sci USA*. 1986. **83**: 7826-7830.

Puig S., Perez-Ortin J.E. Expression levels and patterns of glycolytic yeast during wine fermentation. *System Appl Microbiol*. 2000a. **23**: 300-303.

Puig S., Perez-Ortin J.E. Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. *Yeast*. 2000b. **16**: 139-148.

Reed R., Nagodawithana T.W. *Yeast Technology*. 2nd edition. New York : Van Nostrand Reinhold. 1991. 454 pp.

Ribéreau-Gayon P., Dubourdieu D., Donèche B., Lonvaud A. Traité d'Œnologie 1. Microbiologie du vin. Vinifications. Paris : Dunod. 1998a. 617 pp.

Ribéreau-Gayon P., Glories Y., Maujean A., Dubourdieu D. Traité d'Œnologie 2. Chimie du vin. Stabilisation et traitements. Paris : Dunod. 1998b. 519pp.

Roon, R.J. and Levenburg, B. Urea Amidolyase. *J. Biol. Chem*. 1972. **247(13)**: 4107-4113.

Schuller D, Valero E, Dequin S, Casal M. Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol Lett*. 2004. **Feb 9; 231(1)**:19-26.

Short J.M., Fernandez J.M., Sorge J.A., Huse W.D. Lamba ZAP : a bacteriophage lamba expression vector with *in vivo* excision properties. *Nucleic Acids Res*. 1988. **16:** 7583-7600.

Subden, R.E., 1990. Wine yeast: Selection and modification. In: Panchal C.J. (Ed.), Yeast strain selection, Marcel Dekker Inc., NY, pp. 113-137.

The Regents of the University of California. Malolactic gene. EP0103399. March 21, 1984.

Thoukis, G. and L.A. Stern. 1962. A review and some studies of the effect of sulfur on the formation of oo-odors in wine. *Am. J. Enol. Vitic*. 13(3):133-140.

U.S. Bureau of Alcohol, Tobacco and Firearms. Code of Federal Regulations, Title 27, Parts 1 to 199.

U.S. Environmental Protection Agency. 40 CFR Parts 700, 720, 721, 723, and 725. Microbial products of biotechnology; Final regulation under the Toxic Substances Control Act, Final Rule. *Federal Register*. 1997. **62 (70):** 17909-17958.

U.S. Environmental Protection Agency. Final decision document : TSCA section 5 (H) (4) exemption for *Saccharomyces cerevisiae.* Unpublished.

Velculescu V.E., Zhang L., Zhou W., Vogelstein J., Basrai M.A., Bassett D.E., Hieter P., Vogelstein B., Kinzler K.W. Characterisation of the yeast transcriptrome. *Cell*. 1997. **88 (2):** 243-251.

Viera J. , Messing J. The pUC plasmids, an M13mp7- derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene*. 1982. **19**: 259-268.

Volschenk H., Viljoen M., Grobler J., Bauer F., Lonvaud-Funel A., Denayrolles M., Subden R.E., van Vuuren H.J.J. Malolactic fermentation in grape must by a genetically engineered strain of *Saccharomyces cerevisiae. Am J Enol Vitic*. 1997a. **48 (2):** 193-197.

Volschenk H., Viljoen M., Grobler J., Petzold B., Bauer F., Subden R.E., Young R.A., Lonvaud A., Denayrolles M., van Vuuren H.J.J. Engineering pathways for malate degradation in *Saccharomyces cerevisiae. Nature Biotech*. 1997b. **15:** 253-257.

SUBMISSION END

AM

Mosley, Sylvester

From: Suzanne Plamondon [splamondon@eastlink.ca]
Sent: Wednesday, September 08, 2010 4:17 PM
To: Mosley, Sylvester
Cc: John Husnik; Garth Greenham
Subject: Re: Question Regarding Your GRAS Notice

Attachments: image002.jpg; ATT00001..txt

image002.jpg (2 KB) ATT00001..txt (166 B)

Hello Dr. Mosley,

Thank-you for the update of the status of Phyterra's submission for GRAS for our hydrogen sulphide reducing technologies. To clarify your question regarding the end use of this exciting technology, it will be for alcoholic beverages only.

Thank-you.

Very kind regards,

On 2010-09-08, at 3:56 PM, Mosley, Sylvester wrote:

> Good afternoon Drs. Husnik and Plamondon,
>
> We are working on your acknowledgement letter for your submission (Saccharomyces cerevisiae strain P1Y0 with enhanced expression of gene MET10 from S. cerevisiae strain UCD932). We are seeking clarification on the intended food categories that the S. cerevisiae strain P1Y0 will be used in conjunction with. In your cover letter you state the following, "GRAS Notice for modified yeast to reduce hydrogen sulfide in fermented foods and beverages." However, on page 4, section 1.3.2 (Foods in which the substance is to be used) you only list alcoholic beverages. Please clarify.
>
> Thanks,
> Sylvester
> Sylvester L. Mosley, Ph.D.
> Consumer Safety Officer
> Division of Biotechnology and GRAS Notice Review
> Office of Food Additive Safety-CFSAN
> U.S. Food and Drug Administration
> 5100 Paint Branch Parkway (HFS-255)
> College Park, MD 20740-3835
> Phone: 301-436-1333
> Fax: 301-436-2965
> Email: Sylvester.Mosley@fda.hhs.gov
>
>

Suzanne Plamondon, DVM, MBA
Head Companion Animal Division

AM

From: John Husnik
To: Mosley, Sylvester;
cc: garth.greenham@phyterra.com; mike.horne@phyterra.com;
Subject: RE: GRN000350 Statement
Date: Thursday, February 03, 2011 11:11:05 AM

Hello Dr. Mosley,

Our low hydrogen sulfide technology can be applied to any yeast used in alcoholic fermentations. Our GRAS submission thoroughly discusses the use of our low hydrogen sulfide technology in winemaking and this can be paralleled for products such Champagne, fortified wines (i.e. Sherry), cider, rice wines (i.e. Sake), beer, base wines for Brandy/Cognac and the primary fermentations for distilled alcoholic beverages (i.e. whisky). For each of these various products the bio-conversion of sugar to alcohol by yeast is the same. The raw substrate for providing the sugar may be different (grain, fruit or vegetable) and some of the processing steps to develop the individual characteristics of the products may also differ; however, our technology is applied to the yeast that is completing the bio-conversion of sugar to alcohol without hydrogen sulfide being produced - as described in the submission.

Please do not hesitate to contact me for further information as required.

Finally, please note that Dr. Plamondon no longer works for Phyterra Yeast Inc.

Thank you,

John

John Husnik, Ph.D.
Senior Research Scientist
Phyterra Yeast Inc./Functional Technologies Corp.

Office: 902 892-1074
Lab: 902 566-3302
Cell: 902 314-7500

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From: Mosley, Sylvester [mailto:Sylvester.Mosley@fda.hhs.gov]