Reply to the Objections by the African Centre for Biosafety with Regards to the Commercialization of the Malolactic Wine Yeast ML01 in South Africa

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The bacterial malolactic fermentation process is unreliable despite the availability of commercial starter cultures of *Oenococcus oeni*. South African wineries, like other wineries around the world, experience problems with this secondary fermentation and sluggish and stuck malolactic fermentations often lead to spoilage of wines and the production of biogenic amines. Research on the genetic construction of ML01 was initiated in South Africa and was funded by the South African wine industry for five years. Funding over the next nine years for this project was obtained from the National Science and Engineering Council in Canada and a French yeast company.

ML01 construction

In their objection the African Centre for Biosafety claims that "A **shuttle vector** containing an integration cassette with genes encoding malate permease from *S. pombe*, malolactic enzyme from *O. oeni*, regulatory genes and integration sequences, directing homologous recombination at an **unspecified chromosomal locus** was used to modify *S. cerevisiae* strain S92." The construction of the malolactic cassette is shown in Figures 5-11 in the Notification. A schematic representation of the **linear cassette** is shown in Figure 12 in the Notification and in Figure 1 in Husnik et al. (2006). The exact nucleic composition of the linear integration cassette is detailed in Table 3 and the sequence of the cassette is given in Fasta format in Appendix 7. We did not transform the wine yeast S92 with a shuttle vector containing the malolactic cassette as was claimed by the African Centre for Biosafety. We used a fully characterized and sequenced linear integration cassette consisting of the "malolactic cassette", which is the fragment to be integrated, flanked by homologous sequences of the *S. cerevisiae URA3* locus. The claim that the cassette was integrated into "an unspecified chromosomal locus" is also false. The *URA3* flanking sequences directed integration into one of the *URA3* loci. The malolactic cassette was successfully integrated into a *URA3* locus in the parental strain S92 and scientific evidence was provided in Husnik et al., 2006. These false claims by the African Centre for Biosafety are the same as those of Cummins (2005) who did not read our publication or Notifications to the FDA, and Health Canada and Environment Canada.

Elimination of bacteria from wine making

It is indeed true, as stated by the African Centre for Biosafety, that malolactic bacteria metabolize amino acids in grape musts. The result is the production of biogenic amines that are neurotoxins that cause headaches and other allergenic symptoms such as, migraines, hypotension, oedema, palpitations, flushing, vomiting, diarrhea and hypertension (Wantle et al., 1994; Santos, 1996; Soufleros et al., 1998). Furthermore, alcohol, anti-depressive drugs and other biogenic amines such as cadaverine and putrescine enhance the toxic effect of histamine, tyramine and phenylethylamine (ten Brink et al., 1990; Straub et al., 1995). Biogenic amines are also linked to carcinogenesis and histamine, putrescine, spermidine and spermine can induce cell transformation and

tumour pathogenesis (Medina et al., 1999; Pryme et al., 2001; Wallace et al., 2001). It can hardly be argued that these reactions are beneficial for consumers.

The African Centre for Biosafety further states that "Sensory analyses have shown that many other reactions may change the aromas and make malolactic fermentation beneficial, but what these are are not yet known." On the one hand they claim that MLF is beneficial as shown by sensory analyses and then they state exactly the opposite …. "these beneficial reactions are not yet known". They furthermore claim that skilled wine makers can avoid creating the head-ache causing amines without sacrificing flavour; the reference they provide is from a newspaper, the Sacramento Bee. I have recently attended the International Wine Microbiology Symposium that was held in Yosemity in California. The presence of bioamines in wine is a major and increasing concern for wineries and consumers around the world. Data presented at the Symposium indicated that ca. 50% of 400 wines tested in the Bordeaux region contained lactic acid bacteria that contained the enzyme to decarboxylate naturally occurring amino acids into bioamines. Furthermore, 60% of a large number of wines produced in California contained bioamines. It must be stressed that it is not only wines from these two regions that contain bioamines, the presence of bioamines in wines that have undergone the bacterial malolactic fermentation is a general phenomenon in wines produced in all countries; especially wines that have undergone problematic bacterial malolactic fermentations. It is not possible to fully prevent the production of bioamines in wines without eliminating the presence of lactic acid bacteria. The only way to accomplish this is to use the ML01 yeast.

Persistence of yeast cells

The claim that "In yeasts which have transgenes, such chromosomal rearrangements may result in unexpected toxicity" is unsubstantiated. DNA cannot be toxic and Cummins (2005) provides no explanation for this comment. It is important to note that living cells, including wine yeasts, have mobile genetic elements called transposons (Lewin, 1990). These *Ty* elements insert themselves into different loci in the yeast genome at a frequency of 10^{-7} to 10^{-8}. They can cause deletions or inversions that damages the chromosome and these recombination events in wine yeasts may create novel open reading frames (ORF's) that encode for proteins that have not been characterized or it may disrupt ORF's and prevent expression of certain genes. Recombination events in "natural" wine yeasts are thus ongoing processes and it is unpredictable in contrast with the integration event in ML01 that was targeted and fully characterized at the level of the genome, transcriptome, proteome and metabolome. If a controlled and fully characterized recombination event in wine yeasts (such as in ML01) is unacceptable to wineries, regulatory bodies and the public, the use of all "natural" wine yeasts with ongoing, uncontrolled and uncharacterized recombination events, should be totally unacceptable.

The argument that small amounts of DNA and proteins from ML01 may persist in wine is not relevant since **no DNA or proteins foreign to the wine making process were introduced into the malolactic wine yeast ML01**. Wines produced by bacterial malolactic fermentation may also contain small amounts of DNA including the *mleA* gene encoding the malolactic enzyme; the protein could also be present. The same argument can be made for the malate permease genes from *O. oeni* and *S. pombe* which

are present in wine. It should be noted that is unlikely that the malate transport protein (from *O. oeni*, *S. pombe* or ML01) that contains many transmembrane domains, will be present in wine since this protein is hydrophobic and will not remain in solution.

I am not sure what is meant at the bottom of p. 4 by "genetic *characteristics* of the yeast in the abandoned winery persisted for over ninety years". Did yeast cells survive that were used 90 years ago, was only DNA from these yeasts found or did they detect yeast hulls? Were these yeasts fingerprinted 90 years ago to enable one to come to the conclusion that it was the same yeast or was a yeast contaminant accidentally introduced a day before the tests were done? The same arguments apply for yeast had been used in winemaking at least as far back as 3150 BC. Yeast cells cannot survive that long without nutrients; we have found that cells survive for a maximum of three years in wine without any fresh nutrients (unpublished). We have indeed considered that the ML01 yeast, same as other wine yeasts and the parental strain S92, might become resident in a winery. It is for this very reason that we tested what number of ML01 yeast cells is required to conduct the malolactic fermentation. From Figure 17 in the Notification it is clear that no malolactic fermentation occurred when less than 1% of ML01 yeast was present in the inoculum at the start of fermentation. Even without washing and cleaning the fermentation tanks in which ML01 was previously used, it will be impossible to reach a population of 10^6 cells/ml of ML01 under the worst circumstances. Apart from the malolactic fermentation which is conducted by ML01 only when high cell numbers are present, ML01 is identical to the parental strain S92 and resident ML01 cells in a winery is therefore of no concern.

What is GRAS?

The African Centre for Biosafety goes to great lengths to discredit the US FDA and states that the FDA did not examine our Notification. This is indeed not the case. We had a face to face meeting with the FDA committee comprising scientists from different disciplines; they raised several questions on our Notification. We also had a written request for clarification of data from the FDA. Furthermore, the African Centre for Biosafety conveniently ignores the fact that ML01, in addition to the FDA, was approved by both Health Canada (safety for human consumption) and Environment Canada (safe use in the environment). These two Canadian regulatory bodies are two of the strictest regulatory bodies in the world. We are not asking that ML01 be exempt from full and complete scrutiny for biosafety in South Africa. Indeed, we are willing to fully cooperate with South African regulatory authorities. However, we trust that scientific facts, and not misconstrued facts or irrational emotional arguments, will be used to evaluate the malolactic yeast ML01.

Yeasts are regarded as processing agents in the USA, Canada and also in Europe. According to European regulations, labeling is required only when the final product contains more than 0.9% of the GM product. Even in unfiltered wines, yeast will not comprise close to 0.9% of the wine. An analogous situation that has been fully accepted by consumers around the world is the use of the genetically engineered enzyme, chymosin, which is used for the production of most of the world's cheeses. This enzyme is produced by a genetically engineered yeast strain; the enzyme remains in the GM cheese produced in Europe, South Africa and other countries around the world. The enzyme is also seen as a processing aid and none of the GM cheeses produced with this

enzyme requires labeling. Consumers or the African Centre for Biodiversity do not seem to have a problem with GM cheese.

I fully understand the position of the South African wine industry, the Australian wine industry and the California Wine Institute. I was involved in a two-day workshop organized by the California Wine Institute on the use of genetically engineered yeasts and grape vines before they made a decision how to handle this situation. This workshop was attended by many prominent scientists (not only from the wine industry). The use of ML01 featured prominently. I believe it is prudent for the South African wine industry to state that they do not support the use of ML01 at this stage because of possible consumer resistance. However, the presence of bioamines and ethyl carbamate (a carcinogen in alcoholic beverages and food) is of great concern to consumers and the wine industry. Switzerland is the first country to ban the sale of wines that contain bioamines that exceed a certain concentration. I have had a delegation from Germany in my office that consulted with me on the use of ML01 to prevent the formation of bioamines in wine. More countries are bound to follow Switzerland's example and ban the sale of wines containing bioamines since many consumers get sick when they ingest food or alcoholic beverages contaminated with bioamines; this places an extra financial burden on health providers and governments in all countries. Scientists, food producers and wineries have a responsibility to ensure that foods and alcoholic beverages consumed by the public do not contain ANY compounds that could have a detrimental effect of the health of consumers. The ML01 yeast will provide significant benefits to consumers who are sensitive to bioamines and will prevent spoilage of wines that undergo sluggish or stuck bacterial malolactic fermentations; efficient malolactic fermentation by ML01 will prevent spoilage of wines by other microorganisms which will have significant financial benefits to wineries.

Main Findings

I have addressed most of the comments of the African Centre for Biosafety in my reply so far. Two issues raised by the African Centre for Biosafety under Main Findings remain to be discussed:

2. The African Centre for Biosafety suggest that "The lack of a bacterial inoculum might impact on the flavour and development of the wine and the use of ML01 might alter the taste of the final product". We have done extensive studies on the influence of ML01 on the sensory and physicochemical properties of wine. Sensory analyses were done a by an independent panel of trained judges under leadership of Dr. M. Cliff, a University of California (Davis) trained sensory specialist. For the record I would like to state that I was not a member of this panel. The main descriptive attributes that are associated with wine produced by ML01 are highest quality, fruity taste, sweetness (perceived because of a lack of acidity when compared to other wines) and body, whereas, dark color and high acidity are attributes of wine produced with S92 without a MLF. Wines produced by S92 (control yeast) with a bacterial malolactic fermentation were judged to be more acidic, less sweet, have less body, less fruity taste, and lower in quality than wines produced by ML01. Mean sensory attributes of these Chardonnay wines tasted after four months of aging were similar to those obtained after four years of aging. A manuscript on the "Functional analyses of the malolactic wine yeast ML01" (Husnik et al, 2007) has been accepted for publication and will be published in the next

issue of the American Journal of Enology and Viticulture. I will forward this manuscript to the Directorate Genetic Resources in South Africa as soon as it becomes available. Most important, however, is the fact that commercial wineries that have used ML01 are extremely happy with the yeast and have used it repeatedly over a period of three years.

7. The African Centre for Biosafety implies that the FDA endorsement of ML01 was subject to industry and political pressures. On one hand they state that the wine industry in California does not support use of the ML01 yeast and now they state that the industry pressurized the FDA into endorsing the yeast. We submitted the Notification to the FDA without informing the wine industry or politicians. This claim by the African Centre for Biosafety is completely unsubstantiated and shows their bias towards the FDA.

Conclusions

The African Centre for Biosafety misinterpreted scientific data that we have published in the Notification and in our recent paper (Husnik et al., 2006). Furthermore, they have justified many of their standpoints by liberally using quotes from a newspaper and from opponents of genetically engineered products on the internet that were not peer reviewed. At least one individual, (Cummins, 2005) wrote his report without reading the Notification or our publication. His report had many factual errors which I have conveyed to him; he even confused the malolactic yeast with the malo ethanolic yeast.

The argument that small amounts of DNA and proteins from ML01 may persist in wine is not relevant since no DNA or proteins foreign to the wine making process were introduced into the malolactic wine yeast ML01. Wines produced by the bacterial malolactic fermentation may also contain small amounts of DNA including the *mleA* gene encoding the maloactic enzyme; the protein could also be present. The same argument can be made for the malate permease genes from *O. oeni* and *S. pombe* which are present in wine.

Furthermore, the interpretation of the African Centre for Biosafety of "data" on the so called "survival of yeasts" is scientifically unacceptable as I have indicated in my comments. It is impossible for anybody to show that yeasts survived that long without knowing which yeast was originally used.

I therefore reject the comments by the African Centre for Biosafety and suggest that they re-examine the scientific facts and not come to the wrong conclusions based on reports in newspapers and on the internet written by individuals who oppose GM technology without even examining the facts.

The malolactic yeast ML01 has been fully tested and no other genetically enhanced cells has been tested to the same extent; this includes the genetically engineered yeast that is used to produce chymosin that is used for the manufacture of most GM cheeses in the world, also in South Africa. My request is that the Registrar examines the scientific facts that have been presented in our Notification; if you require further information I will gladly provide it.

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