

# Malolactic yeast ML01 – The Facts

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Most red wines and some white wines, notably Chardonnay, undergo a secondary bacterial malolactic fermentation (MLF) to decarboxylate malic acid (tart) in wine to lactic acid that has a softer mouthfeel. MLF also renders wine microbiologically stable. The bacterial MLF process is unreliable despite the availability of commercial starter cultures of *Oenococcus oeni*. Wineries around the world experience problems on a regular basis with this secondary fermentation and sluggish and stuck MLF's often lead to spoilage of wines and the production of biogenic amines. Malolactic bacteria and other lactic acid bacteria present in fermenting grape must produce toxic biogenic amines (see Lonvaud-Funel, 2001 for a review) such as histamine, cadaverine, phenylethylamine, putrescine and tyramine (Zee et al., 1983; Lehtonen, 1996, Lonvaud-Funel, 2001). These chemicals in wine have been shown to produce undesirable physiological effects in susceptible individuals; histamine causes headaches, and other allergenic symptoms such as, hypotension, palpitations, flushing, oedema, diarrhea, and vomiting (Wantle et al., 1994; Santos, 1996; Soufleros et al., 1998). Tyramine and phenylethylamine are associated with migraines and hypertension (Soufleros et al., 1998). Biogenic amines are also linked to carcinogenesis. Nitrosable secondary amines such as dimethylamine, piperidine, pyrrolidine, spermidine, spermine in wine can react with nitrous acid and its salts to form carcinogenic nitrosoamines (Santos, 1996). Histamine, putrescine, spermidine and spermine can induce cell transformation and tumour pathogenesis (Medina et al., 1999; Pryme et al., 2001; Wallace et al., 2001). Use of ML01 will allow wineries to add sulphite at a much earlier stage of fermentation (no more sulphite will be required than is usually applied) that will prevent the growth of malolactic and spoilage bacteria. Switzerland is the first country to ban the sale of wine containing bioamines that exceed a certain concentration. [Consumers will benefit directly since wines produced with ML01 will be free of allergenic bioamines and precursors to carcinogens produced by lactic acid bacteria.](#)

The genetic construction (7 years) and testing (7 years) of the malolactic yeast ML01 is described in Volschenk et al., 1997 ([PDF](#)), Husnik et al., 2006 ([PDF](#)) and Husnik et al., 2007 ([PDF](#)). The malolactic wine yeast contains no antibiotic resistance marker gene and analyses of the phenotype, genotype, transcriptome, proteome and metabolome have shown conclusively that ML01 is substantially equivalent to the parental strain Prise de Mousse S92. [Wineries will benefit from the use of ML01 since this yeast efficiently conducts the MLF, no spoilage of wine will occur due to stuck MLF, wines have lower volatile acidity \(acetic acid – experts opposed to ML01 have predicted an increase in volatile acidity without any data available to them\), colour properties of wine are improved, wine quality is higher, wines are more fruity and have an improved mouthfeel \(body\) \(see Husnik et al., 2007\).](#)

The malolactic yeast ML01 is the first genetically improved yeast to receive approval from the FDA and Health Canada and Environment Canada for commercial

application. A Notification has been submitted to the Registrar Genetic Resources in South Africa requesting permission for commercial application in South African wineries. Notifications are currently being prepared for all of the other major wine producing regions in the world.

A number of reports have been published on the internet by individuals who are opposed to the use of ML01 for the commercial production of wine. All of these publications contain faulty information, or deliberate misinformation. The African Center for Biosafety and Biowatch SA have objected to the use of ML01 for the production of commercial wines in South Africa. Their objections and our response to their objections are also provided in this document.

### **1. Dr. Joe Cummins**

Cummins wrote a report in Sustainable Agriculture Research and Education on the internet in which he describes the construction of ML01 and condemns the use of this yeast for winemaking <http://lists.ifas.ufl.edu/cgi-bin/wa.exe?A2=ind0512&L=sanet-mg&P=6101>

*Cummins stated that “The yeast ML01 was modified using a shuttle vector containing a chromosome integration cassette with genes for malolactic enzyme, malate transporter (permease), regulatory genes and a sequence directing homologous recombination at a chromosomal locus (not specified in the FDA report)”. We did not use a shuttle vector to transform the parental yeast strain as claimed by Cummins. A schematic representation of the linear cassette that was used is shown in Figure 1 in Husnik et al. (2006). Evidence that the malolactic cassette was integrated into the *URA3* locus of the parental yeast S92 is provided in Figure 3 (Husnik et al., 2006). No regulatory genes were present on the linear cassette as was stated by Cummins. He further stated that the plasmid bearing a selectable phleomycin marker gene is “unstable and frequently lost from the yeast cell”. Scientific evidence that the plasmid, used only for co-transformation and initial screening purposes, was indeed lost is provided in Figure 4 (Husnik et al., 2006).*

*In the latter part of paragraph 2 of his report, Cummins confuses the genetic construction of the malolactic yeast with the genetic construction of the malo-ethanolic yeast (Volschenk et al. 2001).*

*In paragraph 5 Cummins states, without providing an explanation, “Numerous translocations have been observed uniquely in wine yeasts and such chromosome rearrangements involving transgenes can lead to unexpected toxicity in the final product”. DNA cannot be toxic and Cummins 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.

*In paragraph 6 of his report, Cummins elaborates on the presence of yeast nucleic acid and proteins in wine. He is apparently also concerned about the presence of the malolactic enzyme and the malate permease protein in wine produced with ML01.* 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 it 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.

*In paragraph 7 Cummins states that the "genetic characteristics of the yeast in the abandoned winery persisted for over ninety years".* Did yeast cells survive that were used 90 years ago or was DNA from these yeasts found? Were these yeasts fingerprinted 90 years ago to enable Cummins to come to the conclusion that it was the same yeast? 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 5 (Husnik et al., 2007) 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.

*In a second and similar report on the internet (Institute of Science in Society - <http://www.i-sis.org.uk/GMwine.php>), Cummins repeats his faulty statements previously discussed in this document despite the fact that by now he had read our paper in which we described the genetic construction of ML01 (Husnik et al., 2006). In addition, he claims that "ML01, was found to be only **somewhat** substantially equivalent to the unmodified yeast, as a cytochrome p450 enzyme protein (?) appeared to have been*

*altered from the parental strain based on a comprehensive analysis of yeast cell proteins, and a number of codon changes were observed in the malolactic gene cassette, but those changes were not considered significant*". Cummins clearly misinterpreted our data as far as the lanosterol 14-demethylase cytochrome P450 is concerned. Cellular proteins in ML01 and S92 (parental strain) were identified and quantified by multidimensional liquid chromatography and tandem mass spectrometry. The concentration of only one protein, lanosterol 14-demethylase cytochrome P450, was shown to be different at a p-value < 0.05 across duplicate experiments. Lanosterol 14-demethylase cytochrome P450 had a weighted average ratio of 0.799 (using the S92 data as the denominator). The enzyme was not altered at all, the concentration of this enzyme in ML01 was slightly lower (0.799) than what was found in the parental yeast. It is important to note that the concentration of many proteins will differ among different wine yeast strains due to differences in ploidy. All tests conducted showed that ML01 was **substantially** equivalent to the parental strain S92 (Husnik et al., 2006) and not "somewhat" as claimed by Cummins. He also stated that *number of codon changes were observed in the malolactic gene cassette, but those changes were not considered significant*". The *PGK1* promoter, *PGK1* terminator and partial *URA3* flanking sequences do not code for proteins and therefore don't contain codons; only open reading frames coding for proteins (genes) have codons. There are two base pair changes present in the *mleA* gene in ML01 compared to the sequence of one published *mleA* gene. Several *mleA* gene sequences are now available (NCBI) and an alignment of all seven published sequences reveals that the "two unintended genetic changes" are in fact natural single nucleotide polymorphisms in the *mleA* gene. We have aligned the sequences of seven *mleA* genes from *O. oeni*; the genetic polymorphisms in question are highlighted in yellow. Other polymorphisms in the *mleA* gene in seven *O. oeni* strains are highlighted in red. Both glutamate and aspartate (nucleotide 1614) are thus present at this position in natural strains of *O. oeni* used by the wine industry (see reply to Biowatch SA).

## 2. Erica Martenson

Martenson discusses "The dangers of genetically modified wine yeast" on the Food Consumer and other web sites.  
[http://foodconsumer.org/7777/8888/Consumer\\_Affair\\_26/The\\_dangers\\_of\\_genetically\\_modified\\_wine\\_yeast.shtml](http://foodconsumer.org/7777/8888/Consumer_Affair_26/The_dangers_of_genetically_modified_wine_yeast.shtml)

*In her second paragraph Martenson states "This yeast is available only in North America where GMO's are unregulated."* This statement is simply wrong. The use of all genetically improved cells in Canada is subject to approval by Health Canada (health aspects) and Environment Canada (environmental safety). These two regulatory bodies are among the strictest regulatory bodies in the world. ML01 has been found safe for wine making and for release into the environment. Genetically engineered strains of *S. cerevisiae* have been exempted from EPA review in the USA due to the long and safe use of this yeast.

Her arguments why ML01 is "dangerous" is based on the faulty and biased arguments of Cummins that have been addressed in this document. She clearly did not

read any of our scientific papers or Notifications on ML01. The Australian wine industry that are opposed to the use of GMO's until other countries use the ML01 yeast, stated that "*What are the risks associated with using ML01? In terms of health risks there should be none. The two foreign genes incorporated into the wine yeast to make it MLF-competent come from organisms that are typically associated with foods and/or beverages. One comes from the yeast Schizosaccharomyces pombe, which is found in many alcoholic beverages, and the other comes from O. oeni, which is used routinely in the wine industry for MLF*". They further commented "*It would seem from balancing some of the more obvious risks and benefits associated with the use of ML01, that having access to this yeast might be a good thing for Australian winemakers*".

<http://www2.awri.com.au/infoservice/media/releases/nogogmo.asp>

The malolactic yeast ML01 is as safe but better characterized than agricultural crops obtained by traditional methods. Traditional plant improvement relies on (1) mating of two plants of the same species; (2) interspecies hybridization which relies on the transfer of "alien" genes between different but related species and (3) wide crosses between members of different genera. These wide crosses, which do not occur in nature, introduce many entirely new genes into crop plants. For example, Triticale represents an artificial hybrid of such wide crosses between wheat and rye (Miller and Conko, 2004). Furthermore, mutation of seeds or young plants by chemicals or radiation often kills most plants exposed to this treatment; some survive and are screened for required traits. All of these traditional treatments lead to genetic modification of the seed or plant; the final plant is not screened for unwanted mutations or recombination. The public and many of the self-appointed "experts" who oppose products of genetic engineering, ignore the fact that "plant breeding" produces uncharacterized genetic events which, is far less accurate and predictable than what can be achieved by recombinant methods in yeast. In the end, plant breeding and genetic engineering both entail genetic modification of the cell. Clearly, it is not the process but rather the products that should be carefully analysed.

Martenson also tries to discredit ML01 by stating that "*the pombe yeast is found in Africa and used to make beer*". The yeast *Schizosaccharomyces pombe* was indeed first isolated from beer in Africa but it has subsequently been isolated from wineries and it is now commercially available (as ProOenol) for deacidification of wine. She further states that "*a developer has an interest in getting its product to market as soon as possible, whether it has been proven safe or not*". It has taken us 14 years to develop and test the ML01 yeast before it was commercialized. One can hardly argue that this product was brought to the market "as soon as possible". No other genetically modified cell, obtained by traditional breeding or recombinant methods, has been characterized to the same extent as the malolactic wine yeast ML01. Furthermore, independent environmental studies conducted at the University of Stellenbosch have shown that the ML01 strain behaves in an identical fashion to the parental strain and it does not affect growth or survival of soil bacteria.

In her final paragraph Martenson states that "*In our society, we often talk about our rights and discuss very little our responsibilities – to our neighbors*". It is hardly responsible to indoctrinate and confuse the public on the internet without verifying facts.

If she has the interest of the public at heart, she should also ask the wineries that she has listed if their wines are free of bioamines and ethyl carbamate, a carcinogen found in food and alcoholic beverages. We are poisoning our bodies with chemicals present in food and alcoholic beverages that we consume on a daily basis. This is contributing to the fact that healthcare has become unsustainable in many countries. As a scientist I have made it my responsibility to use any safe technology, including genetic engineering, to rid alcoholic beverages and food of toxic compounds.

### **3. Objection to the use of ML01 in South Africa by Biowatch SA and Response to the Objection.**

The objections of Biowatch SA to the use of ML01 in commercial wineries in South Africa and our response to the objection can be found in the following document ([PDF](#)).

#### **4a. Objection to the use of ML01 in South Africa by The African Centre for Biosafety**

The objections of The African Centre for Biosafety to the use of ML01 in commercial wineries in South Africa is presented in the following document ([PDF](#)).

#### **4b. Response to the Objections of The African Centre for Biosafety ([PDF](#)).**

## **5. Literature cited**

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