Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations (Organisms) (NSNR[O]) of the *Canadian Environmental Protection Act*, *1999*
EAU-224: *Saccharomyces cerevisiae* strain ML01

This document has been prepared to explain the regulatory decision taken under Part 6 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) regarding the manufacture of *Saccharomyces cerevisiae* strain ML01 by Van Vuuren and Associates, for introduction anywhere in Canada.

Saccharomyces cerevisiae strain ML01 was notified pursuant to subsection 3(1) of the CEPA 1999 New Substances Notification Regulations (Organisms).

The New Substances Assessment and Control Bureau of Health Canada has assessed the information submitted by Van Vuuren and Associates, and other available scientific information to determine whether *S. cerevisiae* strain ML01 is *toxic*[1] or capable of becoming *toxic* as defined by section 64 of CEPA 1999.

Regulatory Decision:

Based on the hazard and exposure considerations, the risk assessment conducted by Health Canada concluded that *S. cerevisiae* strain ML01 is not considered to be *toxic* to the Canadian environment or human health as described in section 64 of the CEPA 1999. Therefore, manufacture of *S. cerevisiae* strain ML01 for introduction anywhere in Canada may proceed after February 14, 2006.

The evaluation does not include an assessment of human health risk in the occupational environment nor does it include an assessment of the potential exposure and risk to humans associated with the use of the organism in or as an item that falls under the purview of the *Food and Drugs Act*.

NSNR(O) Schedule:	1 (manufacture of micro-organisms for introduction anywhere in Canada).
Organism Identity:	*Saccharomyces cerevisiae* strain ML01
Notifier:	Van Vuuren and Associates, 20 Kelvin Grove Way (PO Box 715), Lions Bay, BC, V0N 2E0
Date of decision:	February 14, 2006
Proposed use:	Active dry yeast used to convert L-malate to L-lactate during commercial production of alcoholic beverages

[1] In accordance with section 64 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) a substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

Strain History/Genetic Modification:
Saccharomyces cerevisiae strain ML01 was derived from the naturally occurring *S. cerevisiae strain* S92 that was isolated from the Champagne region of France (LeSaffre Yeast Collection) and is commonly used in the wine industry. The purpose of creating *S. cerevisiae* strain ML01 is to allow for the conversion of L-malate to L-lactate during alcohol fermentation.

The identification of strain S92 was based on morphological characteristics, API20C AUX carbohydrate utilisation tests, protein quantification using iTRAQ analysis, and transcriptome analysis using Affymetrix GeneChip® Yeast Genome S98 Array.

S. cerevisiae ML01 was developed using polyethylene glycol (PEG) transformation to introduce a DNA expression cassette into *Saccharomyces cerevisiae* S92, via homologous recombination. The introduced sequence consists of the *mleA* and *maeI* genes both under the control of the *S. cerevisiae* PGK1 promoter and terminator sequences necessary for expression in yeast.

The *mleA* gene was isolated from *Oenococcus oeni* wine strain Lo 8413 (GenBank X82326) and encodes for a malolactic enzyme that converts L-malate to L-lactate. The *mae1* gene was isolated from *Schizosaccharamyces pombe* strain NCYC 1913 (GenBank U21002) and codes for the protein malate permease that allows malate to enter the wine yeast. *S. cerevisiae* AB972 (ATCC 204511) is the source of the PGK1 promoter and terminator sequences used in the construct. *S. cerevisiae* GC210 is the source of the URA 3 flanking regions (SGD S000747) used to integrate the construct by homologous recombination.

The host strain, *S. cerevisiae* S92, was transformed with a mixture of the integration cassette and plasmid pUT322 that carries a phleomycin resistance marker. Transformants were isolated based on their ability to grow on selective media containing phleomycin, followed by screening for L-lactate production using a colorimetric reaction method. Plasmid pUT332 was removed from ML01 by subculturing over several passages on a non-selective media and the absence of the plasmid was confirmed by hybridizing the yeast total DNA with the Tn5*Ble* and the *Amp*^{r} (present on pUT322) probes.

Strain ML01 was analysed to confirm the stable integration of the modifications. Pulsed field gel electrophoresis and subsequent hybridization were also used to verify that the chromosomal patterns of the host strain S92 and strain ML01 are identical and that no major sequence rearrangement had occurred during the integration of the malolactic cassette. Genetic stability was demonstrated over 100 generations in the absence of selective pressure.

HAZARD CONSIDERATIONS:
Environmental Hazard
S. cerevisiae is a saprophytic yeast that is widely distributed in nature. It has been isolated from sediments, soil, water, animals and plants under varying ecological conditions. The nutritional requirements, along with the ability to produce ascospores under starvation conditions, enhance its ability to survive in nature.

Despite its ubiquitous nature and wide use in the food and wine industries, reports of *S. cerevisiae* pathogenicity to insects, birds, fish, animals and plants in the available scientific literature are exceedingly rare. Only one case has been reported associating *S. cerevisiae* with chronic diarrhea in a dog [1]. The Canadian Food Inspection Agency, under the *Plant Protection Act*, recognizes that non-recombinant *Saccharomyces* spp. are not plant pests and do not require a plant protection permit for import into Canada [2]. Thus, *S. cerevisiae* is not generally considered a phytopathogen.

Since the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, the overall potential environmental impacts from the release of *S. cerevisiae* strain ML01 are not expected to be any different from other well-known *S. cerevisiae* strains commonly found in nature.

Human Health Hazard

S. cerevisiae is predominantly found in association with human activities, particularly the production of bread and alcoholic beverages. *S. cerevisiae* has been isolated from human intestinal flora and is regarded as an opportunistic pathogen with low virulence. The non-recombinant *S. cerevisiae* strain is recognized as a Risk Group 1 agent, by the Public Health Agency of Canada. Reported *S. cerevisiae* clinical infections in healthy populations appear to be rare. It has been implicated in diseases, particularly in individuals with predisposing conditions such as prolonged hospital stays, immunosuppression, broad-spectrum antibiotic therapy and prosthetic devices [3-6]. Exogenous infections such as vaginitis have been documented [7-9].

The principal virulence factor of yeasts is the secretion of phospholipases. Of a wide range of fungi assayed for phospholipase production, *S. cerevisiae* was found to have the lowest level of activity [10]. The ability to grow at elevated temperatures (up to 42°C) has also been shown to be an important factor associated with virulence in clinically isolated strains of *S. cerevisiae* [11].

The use of combination antifungal therapy is recommended for the treatment of *S. cerevisiae*-induced disease, as is prolonged therapy [12]. In the unlikely event of *S. cerevisiae* strain ML01 infection to humans, antifungal treatments are currently available. Amphotericin B is considered the treatment of choice for serious *S. cerevisiae* infections except where underlying conditions preclude its use [4], in which case prolonged treatment with azole antifungal agents (e.g., clotrimazole, fluconazole, itraconazole, voriconazole) has also been found effective [13,14]. Since the antibiotic resistance genes used in the transformation process were removed from the genome of the final strain construct, dissemination of those genes to the environment is not possible.

The notifier has shown that the parental strain S92 is closely related or identical to commercial strains traditionally used in winemaking. It is therefore expected that strain ML01 is unlikely to behave differently from the non-recombinant parental strain aside from its new malolactic fermentation trait. The likelihood of significant harm to human health is therefore expected to be low. The *S. cerevisiae* strain ML01 received the Generally Regarded as Safe (GRAS) affirmation from the US Food and Drug Administration in 2003 [15].

The notifier claims that routine checks of each yeast batch will ensure the absence of food-borne pathogens (*Salmonella* spp., *Staphylococcus aureus, Escherichia coli*, and *Clostridium perfringens*) thus the risk of microbial contaminants in the final exported product is negligible. Prior to shipping, the yeast will also be compared to a reference culture by genetic typing using Yeast Transposons TY elements.

EXPOSURE CONSIDERATIONS:
The notified micro-organism will be imported from Bio Springer located in Maison-Alfort Cedex, France. An estimated amount of 400 kg containing 1.2×10^{16} viable *S. cerevisiae* ML01 cells will be imported annually by Van Vuuren & Associates into Canada. The yeast will be shipped in 500 g vacuum sealed containers and sold to approximately 200 wineries in Ontario and British Columbia.

It is expected that the amount of *S. cerevisiae* strain ML01 in bottled wine will range from 0 to 10 cells/mL depending on the manufacturing process employed. Commonly, between 0.1 to 0.2 grams of active dry yeast is used to manufacture a litre of wine. However, clarification followed by filtration can substantially reduce the levels of yeast cells to less than 0.5 cfu/ml of wine while ensuring the absence of any additional urea amidolyase. Membrane filtration (1.2 µm porosity) would allow for complete removal of yeast cells.

As with naturally occurring *S. cerevisiae* wine strains, human exposure may occur via inhalation. The level of human exposure is expected to be comparable to those *S. cerevisiae* strains normally encountered during the manufacture of foods and beverages.

Exposure to the ML01 or to the newly introduced proteins either through disposal of unused wine or by wine consumption is considered significantly low since the processing procedures used in the winemaking will remove intact yeast cells, debris associated with the autolyzed yeast cells and proteins released during autolysis of yeast cells.

No special precautions are recommended for the storage of the ML01 yeast other than storing it for a period of less than two years at ambient temperature. Although no special disposal methods are required for unused portions of the yeasts, the notifier provided detailed procedures to disinfect all solid and liquid wastes. No contingency plans are in place in case of accidental release. The method for treatment and disposal of wastes containing spent yeast cells will vary depending on the size and location of the wineries. The liquid effluents can either be treated at a municipal wastewater treatment plant or onsite. Since the non-recombinant *S. cerevisiae* strain is recognized as a Risk Group 1 agent it requires minimal operational and physical containment requirements for a large scale process as described in the Public Health Agency of Canada's Laboratory Biosafety Guidelines [16].

Persistence and Dispersal
There is currently limited information on the ecological characteristics of ML01. Valero *et al.* performed a 3-year field study to track the spreading and survival of

industrial yeast strains in vineyards of North Portugal and South France [17]. Results show that commercial strains behave similarly to naturally occurring yeast strains.

The behaviour of genetically modified *S. cerevisiae* strains within microbial populations of a confined wine cellar and greenhouse vineyard has also been evaluated and no significant difference was found between modified strains and commercial yeast strains [18]. The introduction of strain ML01 is expected to have no significant effect on the ecological balance of vineyard associated flora.

A 48-day study conducted to compare the survivability of *S. cerevisiae* strain ML01 with that of strain S92 and four naturally occurring *Cryptococcus* strains in agricultural soil indicated that the *Saccharomyces* population decreased towards the end of the experiment compared to the other autochthonous yeasts. Furthermore, the recombinant strain showed no competitive advantage over the parental strain and the other soil yeasts.

Any notified strain released into the environment as a result of the large-scale manufacturing process can be dispersed by wind, by fauna existing at the wineries, or by run-off with surface water. Considering the notifier provided detailed procedures to disinfect all solid and liquid wastes provided by the notifier, it is expected that the dissemination of strain ML01 in the wineries and surrounding areas will be restricted to short distances and limited periods of time. It is anticipated that since the notified strain is adapted to well-defined media, it would be less competitive than the naturally occurring yeasts in soil.

References:

[1] Milner RJ, Picard J, and Tustin R. (1997). Chronic episodic diarrhoea associated with apparent intestinal colonization by the yeasts *Saccharomyces cerevisiae* and *Candida famata* in a German shepherd dog. JS Afr Vet Assoc. 68(4):147-9.

[2] CFIA. (2005). Organisms that do not require a plant protection permit to import. Plant Products Directorate. Plant Health Division. Export/Import Section. Canadian Food Inspection Agency. http://www.inspection.gc.ca/english/plaveg/oper/orglste.shtml [viewed on June 13, 2006].

[3] Anaissie E, Bodey GP, Kantarjian H, Ro J, Vartivarian SE, Hopfer R, Hoy J, and Rolston K. (1989). New spectrum of fungal infections in patients with cancer. Rev Infect Dis. 11:369-78.

[4] Aucott JN, Fayen J, Grossnicklas H, Morrissey A, Ledermann MM, and Salatta, R. (1990). Invasive infection with *Saccharomyces cerevisiae*: report of three cases and review. Rev Infect Dis. 12(3):406–411.

[5] Belet N, Dalgic N, Oncel S, Ciftci E, Ince E, Guriz H, Barlas M, and Dogru U. (2005). Catheter-related fungemia caused by *Saccharomyces cerevisiae* in a newborn. Pediatr Infect Dis J. 24(12):1125.

[6] Malgoire JY, Bertout S, Renaud F, Bastide JM, and Mallie M. (2005). Typing of *Saccharomyces cerevisae* clinical strains by using microsatellite sequence polymorphism. J Clin Microbiol. 43:1133-1137.

[7] Posteraro B, Sanguinetti M, d'Amore G, Masucci L, Morace G, and Fadda G. (1999). Molecular and Epidemiological Characterization of Vaginal *Saccharomyces cerevisiae* Isolates. J Clin Microbiol. 37(7):2230-2235.

[8] Saporiti AM, Gomez D, Levalle S, Galeano M, Davel G, Vivot W, and Rodero L. (2001) Vaginal candidiasis: etiology and sensitivity profile to antifungal agents in clinical use. Rev Argent Microbiol. 33: 217-222.

[9] Al-Hedaithy SS. (2002). Spectrum and protease production of yeasts causing vaginitis in Saudi Arabian women. Med Sci Monit. 8: CR498-501.

[10] BarrettBree K., Hayes Y, Wilson R.G., and Ryley J.F. (1985). A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J. Gen. Microbiol. 131(5):1217- 1221.

[11] McCusker, J.H., Clemons, K.V., Stevens, D.A., and Davis, R.W. (1994). *Saccharomyces cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42°C and form pseudohyphae. Infect. Immun. 62: 5447–5455.

[12] Tiballi RN, Spiegel JE, Zarins LT, and Kauffmann CA. (1995). *Saccharomyces cerevisiae* infections and antifungal susceptibility studies by colorimetric and broth macrodilution methods. Diagn Microbiol Infect Dis. 23:135–40.

[13] Sobel JD, Vazquez J, Lynch M, Meriwether C, and Zervos MJ. (1993). Vaginitis due to *Saccharomyces cerevisiae*: epidemiology, clinical aspects, and therapy. Clin Infect Dis. 16(1):93-99.

[14] Swinne, D., Watelle, M., Van der Flaes, M., and Nolard, N. (2004). In vitro activities of voriconazole (UK-109, 496), fluconazole, itraconazole and amphotericin B against 132 non-albicans bloodstream yeast isolates (CANARI study). Mycoses. 47(5-6):177-83.

[15] USFDA. (2003). Agency Response Letter: GRAS Notice No. GRN 000120. United States Food and Drug Administration. Center for Food Safety and Applied Nutrition. Office of Food Additive Safety. http://www.foodsafety.gov/~rdb/opa-g120.html

[16] PHAC (2004). Laboratory Biosafety Guidelines 3rd ed. Health Canada. Population and Public Health Branch. Center for Emergency Preparedness and Response. http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/pdf/lbg_2004_e.pdf

[17] Valero E, Schuller D, Cambon B, Casal M, and Dequin S. (2005). Dissemination and survival of commercial wine yeast in the vineyard: a large-scale, three-years study. FEMS Yeast Res. 5(10):959-69.

[18] Bauer F, Dequin S, Pretorius I, Shoeman H, Wolfaardt MB, Schroeder MB, and Grossmann MK. (2003). The assessment of the environmental impact of genetically modified wine yeast strains. Proceedings of the " Actes de 83ème Assemblée Générale de l'O.I.V".