The assessment of the environmental impact of genetically modified wine yeast strains

F.F. BAUER[1], S. DEQUIN[3], I.S. PRETORIUS[1,2], H. SCHOEMAN[5], G. WOLFAARDT[5], M.B. SCHROEDER[4], M.K. GROSSMANN[4]

[1] Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Stellenbosch, ZA 7600, South Africa, e-mail: fb2@sun.ac.za
[2] Australian Wine Research Institute, Waite Road, Urrbrae, Adelaide, SA 5064, Australia, e-mail: sakkie.pretorius@awri.com.au
[3] Laboratory of Microbiology and Fermentation Technology, Institute for Wine Products, 2, place Viala, 34060 Montpellier, Cedex 2, France; e-mail: dequin@ensam.inra.fr
[4] The Geisenheim Research Institute, Department of Microbiology and Biochemistry, Von-Lade-Strasse 1, D-65366 Geisenheim, Germany, e-mail: manfred.grossmannn@fa-gm.de
[5] Institute for Wine Biotechnology, Department of Microbiology, Stellenbosch University, Stellenbosch, ZA 7600, South Africa.

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ABSTRACT

In recent years, considerable efforts have been made to improve strains of the wine yeast *Saccharomyces cerevisiae* through the use of modern biotechnological tools. The main targets for these wine yeast strain development programmes have been, and still are, the improvement of fermentation performance, processing efficiency, and wine sensory quality, as well as the development of new strains with reduced risks and enhanced benefits for health. Currently, numerous stable genetically modified wine yeast strains already exist in laboratories, while many others are being constructed. Genetically modified wine yeast strains have not, as yet, been used in the wine industry. This situation is mainly due to concerns relating to GMOs in food. Nevertheless, the arrival on the market of genetically modified wine yeast strains appears imminent and an urgent need to assess the potential risks that may be associated with the use of this new technology throughout the wine production chain exists. This presentation will focus on the existing data generated by current research projects regarding the assessment of such risks. More specifically, we will present data and projects on (i) the available detection methods to monitor genetically modified wine yeast strains through the overall wine production chain, (ii) the survival and dynamics of industrial wild type and (iii) genetically modified wine yeast strains in natural habitats and winemaking environments, (iv) the potential for natural gene transfer between yeast and the natural wine micro-flora, and (v) the analysis of possible side effects of genetic modifications on yeast strain and product quality. *(Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 514-528).*

ZUSAMMENFASSUNG

Umweltverträglichkeitsprüfung gentechnisch veränderter Weinhefestämme

In den letzten Jahren wurden beträchtliche Anstrengungen unternommen, um die Weinhefestämme Saccharomyces cerevisiae durch den Einsatz moderner biotechnischer Hilfsmittel zu verbessern. Die hauptsächlichen Ziele dieser Entwicklungsprogramme für Weinhefestämme waren und bleiben bis heute die Verbesserung der Fermentationsleistung, die Verarbeitungseffizienz und die sensorische Weinqualität, sowie die Entwicklung neuer Hefestämme mit geringeren Risiken und gesundheitsfördernden Eigenschaften. Zahlreiche stabile, gentechnisch veränderte Weinhefestämme existieren heute bereits in den Labors und viele andere befinden sich in der Entwicklung. Bisher wurden genetisch veränderte Weinhefestämme noch nicht in der industriellen Weinbereitung eingesetzt. Der Grund dafür liegt in Bedenken hinsichtlich des Einsatzes von GVOs in Nahrungsmitteln. Trotzdem steht die Markteinführung gentechnisch veränderter Weinhefestämme offensichtlich unmittelbar bevor und es besteht die dringende Notwendigkeit, die potentiellen Risiken, die mit dieser neuen Technologie innerhalb der gesamten Kette der Weinproduktion bestehen könnten, abzuschätzen. Dieser Beitrag konzentriert sich auf existierende Daten, die aus verschiedenen aktuellen Forschungsprojekten hinsichtlich diesbezüglicher Risikoabschätzung stammen. Insbesondere stellen wir Daten und Projekte bezüglich (i) der verfügbaren Erkennungsmethoden zur Kontrolle gentechnisch veränderter Weinhefestämme entlang der Kette der Weinproduktion, (ii) das Überleben und die Dynamik industrieller Wildtypen und (iii) gentechnisch veränderte Weinhefestämme im natürlichen Lebensraum und im Umfeld der Weinbereitung, (iv) das Potential natürlichen Gentransfers zwischen der Hefe und der natürlichen Weinmikroflora und (v) die Analyse möglicher Nebenwirkungen gentechnischer Veränderungen auf Hefestämme und die Qualität des Endprodukts vor. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

RÉSUMÉ

Evaluation des effets sur l'environnement des souches de levures du vin génétiquement modifiées

Des efforts considérables ont été réalisés pour améliorer des souches de levures du vin Saccharomyces cerevisiae à l'aide des outils modernes de la biotechnologie. L'objectif principal de ces programmes de développement des souches de levures a toujours été l'amélioration de la qualité de la fermentation, l'efficacité dans l'élaboration, l'amélioration de la qualité sensorielle du vin, ainsi que le développement de nouvelles souches avec des risques minimes et des bénéfices accrus pour la santé. Actuellement, de nombreuses souches de levures génétiquement modifiées existent en laboratoire, tandis que beaucoup d'autres sont en cours de développement. A ce jour, les souches de levures génétiquement modifiées n'ont pas été utilisées dans le secteur du vin. Ceci est dû, en grande partie, à des préoccupations vis-à-vis de l'utilisation des OGM dans les produits alimentaires. Cependant, l'arrivée sur le marché des souches de levures génétiquement modifiées semblerait être imminente et il existe un besoin urgent d'évaluer les risques potentiels associés à l'utilisation de cette nouvelle technologie dans l'élaboration du vin. Cette présentation montrera les données existantes issues des

projets de recherches actuels concernant l'évaluation de ces risques. Plus précisément, nous exposons des données et des projets concernant: (i) les méthodes de détection disponibles pour contrôler les souches de levures génétiquement modifiées pendant les différentes étapes de l'élaboration du vin, (ii) la survie et la dynamique du type sauvage industriel, (iii) les souches de levures génétiquement modifiées en milieu naturel et dans les milieux de l'élaboration du vin, (iv) le potentiel de transfert naturel de gènes entre les levures et la microflore naturelle du vin et (v) l'analyse des effets secondaires potentiels des modifications génétiques sur les souches de levures et la qualité du produit. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

Resumen

Evaluación del impacto medioambiental de las cepas de levaduras geneticamente modificadas

Se han realizado esfuerzos considerables para mejorar las cepas de levaduras del vino Saccharomyces cerevisiae utilizando las herramientas modernas de la biotecnología. El objetivo principal de estos programas de desarrollo de las cepas de levaduras ha sido siempre el de mejorar la calidad de la fermentación, aumentar la eficacia del proceso de elaboración, mejorar la calidad sensorial del vino, así como desarrollar nuevas cepas con una reducción de los riesgos y un mayor beneficio para la salud. Actualmente, numerosas cepas de levaduras genéticamente modificadas existen en laboratorio, mientras que muchas otras se están desarrollando. Hasta el día de hoy, las cepas de levaduras genéticamente modificadas no han sido utilizadas en el sector del vino. Esto se debe en gran parte a la preocupación respecto a la utilización de los OGM en los productos alimentarios. Sin embargo, la llegada al mercado de las cepas de levaduras genéticamente modificadas parece inminente y existe una necesidad urgente de evaluar los riesgos potenciales que podrían esta ligados a la utilización de esta nueva tecnología en la elaboración del vino. Esta presentación mostrará los datos existentes provenientes de los actuales proyectos de investigación relativos a la evaluación de estos riesgos. Más precisamente, exponemos los datos y los proyectos relativos: (i) los métodos de detección disponibles para controlar las cepas de levaduras genéticamente modificadas durante toda la cadena de producción del vino, (ii) la supervivencia y la dinámica de los tipos industriales, (iii) las cepas de levaduras genéticamente modificadas en medio natural y en los medios de la elaboración del vino, (iv) el potencial de transferencia natural de genes entre las levaduras y la microflora natural del vino, (v) el análisis de los posibles efectos secundarios de las modificaciones genéticas en la cepa de levadura y en la calidad del producto. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

Riassunto

La valutazione d'impatto ambientale di lieviti enologici geneticamente modificati

Negli ultimi anni, sono stati compiuti innumerevoli sforzi per perfezionare le varietà del lievito enologico Saccharomyces cerevisiae mediante l'uso di moderni strumenti biotecnologici. I principali obiettivi dei programmi per lo sviluppo dei lieviti sono stati, e sono tuttora, il miglioramento della qualità della fermentazione, l'efficacia del processo stesso, la qualità sensoriale del vino, nonché lo sviluppo di nuove varietà che presentino minori rischi e

maggiori benefici per la salute. Attualmente esistono già in laboratorio numerose varietà di lieviti stabili geneticamente modificati, mentre molti altri sono in via di realizzazione. I lieviti geneticamente modificati non sono stati usati, finora, nell'industria enologica. Tale situazione è dovuta principalmente alle preoccupazioni riguardanti gli OGM nei prodotti alimentari. Tuttavia, l'arrivo sul mercato di lieviti geneticamente modificati appare imminente, mentre c'è l'urgente necessità di valutare i rischi potenziali che possono essere legati all'uso di questa nuova tecnologia in tutta la catena produttiva del vino. Questa presentazione sarà incentrata sui dati esistenti ottenuti dagli attuali progetti di ricerca che riguardano la valutazione di tali rischi. Più specificatamente, verranno presentati dati e progetti su (i) i metodi di rilevamento disponibili per monitorare i lieviti geneticamente modificati in tutta la catena di produzione del vino, (ii) la sopravvivenza e la dinamica dei lieviti usati a livello industriale, sia naturali che (iii) geneticamente modificati, negli habitat naturali e negli ambienti di vinificazione, (iv) il potenziale del trasferimento del gene naturale dal lievito alla microflora naturale del vino, e (v) l'analisi dei possibili effetti collaterali di modificazioni genetiche sul lievito e sulla qualità del prodotto. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

INTRODUCTION

Wine fermentation used to be conducted by the microorganisms present on the grapes or the winery equipment, including yeast, fungi and bacteria. During these spontaneous fermentations, a succession of microorganisms dominates the early stages of the alcoholic fermentation, while yeasts, mainly of the species *Saccharomyces cerevisiae*, invariably dominate the latter stages of the process, when alcohol concentration is high. Today, spontaneous fermentations are still employed in some wineries, mainly by smaller producers of quality wines. However, most wine makers use commercially produced active dried wine yeast strains (ADWY) to inoculate grape must and kick-start the fermentation process. Inoculation presents several advantages that off-set the additional costs incurred when purchasing the yeast. The advantages include (i) the improved control of the fermentation process, (ii) the reduction of the risk of sluggish or stuck fermentations and of microbial contamination, and (iii) the choice of yeast strains that impart specific characteristics to the wine and achieve a desired outcome. No disadvantages have been associated with the use of ADWY, but for the observation that wines fermented by a single yeast strain may sometimes display reduced complexity when compared to those having undergone spontaneous fermentation.

Most commercial wine yeast strains available today have been bred and/or selected to display particular oenological characteristics, and yeast producers market their strains for specific applications and for specific styles of wines. These and other technological developments have without doubt contributed to an improvement in the average quality of wine, and have enhanced the ability of wine makers to control the fermentation process and to achieve specific outcomes. In this context, it is not surprising that continuous innovation is widely considered essential to maintain a competitive edge in the wine market. Innovation will be driven by several key technologies. Progress may still be made on improving the existing traditional viti- and vini-cultural practices. However, the probably biggest potential for

developing innovative solutions and for generating new products resides in the application of modern biotechnological tools, in particular genetic engineering.

The last two decades have seen a rapid increase in biotechnological know-how and in the development of modern molecular biology. The yeast *S. cerevisiae* has played an essential part in the development of these technologies, and a tremendous amount of knowledge has been accumulated about this organism. The potential for significant scientific and technological breakthroughs, based on the application of our accumulated know-how of yeast molecular biology to wine-related problems, promises therefore benefits for wine producers and consumers alike.

However, the application of modern biotechnology, particularly in agriculture and food production, remains controversial. Countless reports on the topic have been published, and the arguments for and against the use of genetic modification, be they of an ethical, economic, environmental or otherwise scientific nature, have been made extensively in public debates. Most of these discussions have not been conducted in a rational environment, and have frequently been hijacked by particular interests. This situation, combined with the sometimes irresponsible political handling of sensitive, public health-related issues and a silent scientific community, has created a negative public perception of GM-technology, particularly in European countries.

The future application of this technology in the wine industry therefore hinges on a scientifically sound evaluation of the safety and of the potential environmental and economic impact of genetically modified organisms. This evaluation requires the study of complex interactions and ecosystems, and needs to assess a large number of interrelated parameters. It therefore has to integrate multidisciplinary approaches, and include ecologists, microbiologists, geneticists, biochemists and other scientists. It is probably due to the intrinsic complexity of the topic that few studies have been conducted to holistically assess the environmental impacts of microbial GMOs (*Table I*).

Table I

Problems associated with the investigation of the ecological impact of introduced microorganisms

Conceptual	Inability to predict how potential impacts will be expressed within a complex ecosystem. Inability to define the level at which ecological impacts should be sought (micro- vs. macro-ecology) Absence of a framework of references for scientific interpretation of results (e.g. statistical vs. ecological significance) Impossibility to extrapolate data obtained in closed model systems to open natural systems
Technological	Monitoring techniques not adapted for specific purpose (I.e. sensitivity too high-too low) Techniques have only been assessed in model systems Quantitative data difficult to generate reliably

The research presented here describes several approaches that, when taken together, should provide us with a holistic view of the potential impact of GM yeast in a winery and associated environments. It is specifically focused on addressing several unresolved issues, including:

(i) Evaluating available detection methods for GM yeast.
(ii) Assessing the spreading of commercial wine yeast strains in the environment.
(iii) Comparing the behaviour of parental and genetically modified strains in model systems to assess whether GM strains may possess a selective advantage which could lead to their spreading.
(iv) Evaluating the probability of trans-genes spreading vertically to other yeast strains or horizontally to other species.
(v) Assessing the consequences of genetic modification on the modified yeast itself.

In the following sections, the current status of some research projects within each of these major topics will be described.

AVAILABLE METHODS FOR THE DETECTION OF GM YEAST

The available methods for the detection of genetically modified organisms in food were recently reviewed by Ahmed (2002). The most commonly used techniques are listed in *Table II*, and include Southern blotting and PCR analysis for the detection of transgenic DNA, and immunological tests for the detection of GM proteins. All of these methods require detailed *a priori* knowledge of the specific GMO to be monitored, in particular regarding the specific sequences used for the modification.

If no such information is available, it may be possible to investigate strains for the presence of those sequences that are most commonly used in genetic engineering, including selection markers (e.g. antibiotic resistance genes), particular promoters (sequences that regulate the expression of an inserted gene) or secretion signal sequences (if the GMO is suspected to produce a secreted protein), but modern strategies provide a wide, indeed virtually limitless variety of choices to biotechnologists, making such a search a rather hazardous undertaking.

PCR-based methods are highly sensitive, but high sensitivity also leads to an increased risk of generating false positive results. This problem is amplified when investigating the presence of a GMO or of GM-products in a complex food-product like wine, where the donor species of the investigated heterologous gene may also have been present during the production process. Indeed, many currently ongoing yeast improvement projects are using DNA isolated from organisms that are naturally present in wine.

Our data show that all techniques described in *Table II* can be used to trace GM yeast in a wine or vineyard environment. However, when complex populations have to be assessed, none of the techniques offered easily reproducible results. In addition, quantification of GM yeast within a mixed culture using quantitative DNA methods has proved extremely difficult. Furthermore, none of the currently available methods is suitable for standard analysis in a wine cellar environment.

Table II

Summary of methods that specifically detect recombinant DNA or its products in food-stuff (Adapted from Ahmed, 2002)

Parameter	Protein-based			DNA-based			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR and limiting dilution	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High	High
Duration	2 d	30-90 min	10 min	6 h’	1.5 d	2 d	1 d
Gives quantitative results	No	Yes	No	No	No	Yes	Yes
Suitable for field test	No	Yes	Yes	No	No	No	No
Employed mainly in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

THE SPREADING OF COMMERCIAL WINE YEAST STRAINS IN VINEYARDS

Commercially used yeast strains are released in large numbers and on an annual basis by most wine producers. These strains will in most cases not have originated from the area of release, and may therefore be expected to have an impact on the naturally occurring yeast micro-flora. This impact has to be carefully assessed, since the existing commercial wine yeast strains will be the target for genetic manipulations. It is absolutely necessary to know to what extend these existing commercial wine yeast strains survive and spread in nature and to what extend they influence the fermentations of the following year.

To study the effects of introduced new yeast varieties, be they genetically modified or not, we assessed the spread of commercially used wine yeast strains. We specifically investigated the potential spreading of the wine yeast strains most commonly used in South Africa and in the wine estates investigated, including VIN13, VIN7, WE228, N96, WE14, WE372, D254, CY3079, Bordeaux Red, EC1118, D47, DV10, L2056 and QA23. The yeast strains were isolated during the 1998 and 1999 harvesting seasons from vineyards on six wine farms in different climatological regions in the Western Cape. Six sampling sites, identified according to their relative position to the cellar, the dumping sites of grape skins, the topology of the area (including drainage lines etc) and the prevailing wind direction were selected on each farm. Due to the large number of yeasts isolated, a near-infrared spectroscopy (NIR) technique to identify the yeast isolates was developed. The results of the NIR were further verified by pulsed-field gel electrophoresis (CHEF).

The data confirmed previous reports (Van der Westhuizen *et al.*, 2000) indicating that *Saharomyces cerevisiae* is not present in large numbers in vineyards, since the vast majority of isolated yeast belonged to the genera *Kloeckera* and its anamorph *Hanseniaspora*, as well as *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Rhodotorula*. A total of 34 and 55 different "wild" *Saccharomyces* strains were identified after spontaneous fermentation of the grape must during the 1998 and 1999 seasons, respectively. Out of 1500 individual colonies screened, only eight (or less than 1%) were identified as commercial wine yeast strains, seven corresponding to VIN13, while one isolate was identified as N96. Both of these strains are widely used in the South-African wine industry. None of the other commercial strains could be detected at any of the sampling sites. All industrial *Sacchromyces cerevisiae* strains identified were found at sites closely associated with the winery dumping sites. Considering the position of the sampling sites, which were selected for the high probability of accidental dispersal of yeast from the dumping sites, and taking into account the large number of yeast isolated, the data suggest that commercial wine strains are not easily dispersed from the cellar to the vineyard. The data are in accordance with findings by other groups in France and Portugal that will also be presented at this meeting.

THE DETECTION AND MONITORING OF GENETICALLY MODIFIED YEAST STRAINS WITHIN MICROBIAL VINEYARD POPULATIONS

To monitor the behaviour of genetically modified yeast strains during wine fermentation and in the vineyard, and to compare the relative fitness of

these strains and of the corresponding parental strains, several trials have been conducted at the Wine Research Institute in Geisenheim, Germany, and at the Department of Microbiology and the Institute for Wine Biotechnology in Stellenbosch, South Africa. Since GM yeast may not be released into the environment, the studies were conducted in confined wine cellars and vineyards established in greenhouses.

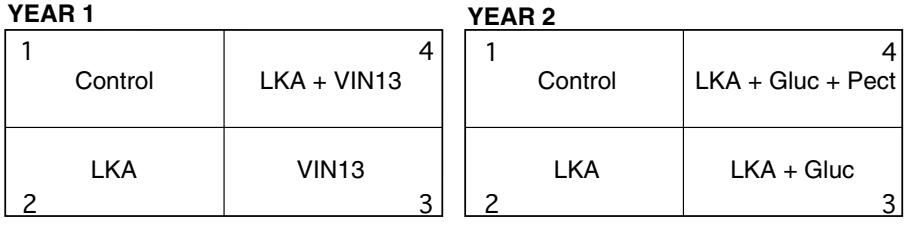
The greenhouse trial was started in 1999, using a newly established vineyard of one year old vines. In the first year of investigation, only the naturally occurring yeast population was assessed. The most prevalent yeast genera and species that were isolated from the berries, leaves, stems and the soil included *Rhodotorula*, *Saccharomycodes*, *Yarrowia lipolytica*, *Pichia guilliermondii*, *Metchnikowia pulcherima* and *Hanseniaspora*. To a lesser extent *Candida parapsilosis* and *Debaromyces hansenii* were also detected.

A well-known industrial wine yeast strain, *Saccharomyces cerevisiae* VIN13, was used as the parental strain. Several genes were transformed into this strain, either as single genes or in combination. These genes originated either from bacteria or non-*Saccharomyces* yeast, and included the *LKA1* gene, which encodes a raw starch degrading α-amylase, the end1 gene (encoding endo-β1,4-glucanase) and the *XYN4* gene (encoding xylanase), as well as the pelE (encoding pectate lyase) and peh1 (encoding polygalacturonase) genes. All genes are under the control of strong yeast promoters and terminators and have been integrated into the genome of VIN13, together with the yeast-derived resistance marker *SMR*-410 or the kanMX gene which confers resistance to geneticin.

Before the spraying of the yeasts, the greenhouse was divided into four individually contained blocks, each block consisting of 20 vines. In the first year of the trial, block 1 was left untouched, and no yeast strain was released, whereas the other three blocks were each treated with 1.5 l of a solution containing 2.5×10^6/ml of colony forming units (CFU) of pure yeast culture. Treatment was applied by directly spraying the yeast solution onto the vines. Block 2 was sprayed with the GM yeast alone, block 3 with the parental strain VIN13, whereas block 4 was sprayed with a 1:1 mixture of both strains (Figure 1).On a weekly basis, the yeast populations on the grapes, leaves, stem and soil in the different blocks were monitored. Although a high concentration of yeast was sprayed, few *S. cerevisiae* strains could be isolated

FIGURE 1

Arrangements of blocks in greenhouse trials. Control: No yeast sprayed. VIN13: Only parental strain sprayed. LKA: GM yeast with *LKA1* gene. Gluc: GM Yeast with glucanase-encoding genes. Pect: GM yeast with pectinase-encoding genes.



at any given time. The yeast population in the sprayed blocks was otherwise very similar to the one found on the control vines, indicating that the commercial or GM yeast did not affect the overall ecological balance of the micro-flora. Furthermore, no significant differences between the behaviour of the genetically modified and the parental strains could be detected.

Micro-vinifications were conducted from the grapes harvested from each block, and the numbers of *S. cerevisiae* cells present during the fermentations was monitored. In all cases, the must obtained from grapes harvested from the blocks that had been sprayed with commercial or GM yeast strains fermented significantly more efficiently than the must obtained from the control block, indicating the presence of increased numbers of *S. cerevisiae* strains on the grapes. However, no significant differences were observed between the blocks that had been sprayed with either the GM yeast or with the parental strain.

In the second year, the four blocks were again sprayed, but GM yeasts with activities that may provide a selective advantage over the parental strains were used (*Figure 1*). Indeed, the GM yeasts were designed to secrete significant amounts of polysaccharide-degrading enzymes, in particular glucanases and pectinases, which may provide some advantages to the strains when released on the vines. However, the same patterns as in the previous year were observed, and no significant differences were detected between the transformed and the parental strains, both with regard to their presence in the vineyard and to the cell numbers and fermentation efficiencies during spontaneous fermentations.

The data again suggest that the GM yeasts did not benefit from any specific advantage in terms of overall fitness when released in the vineyard, since the weekly sampling did not reveal any statistically significant differences between the three blocks that had been sprayed. During spontaneous fermentations of the grape musts derived from the different blocks, no significant difference in fermentation speed, efficiency or the number of colony forming units could be observed, and none of the different yeast was able to dominate other yeast strains in both the vineyard and during spontaneous fermentation (*Figure 2*).

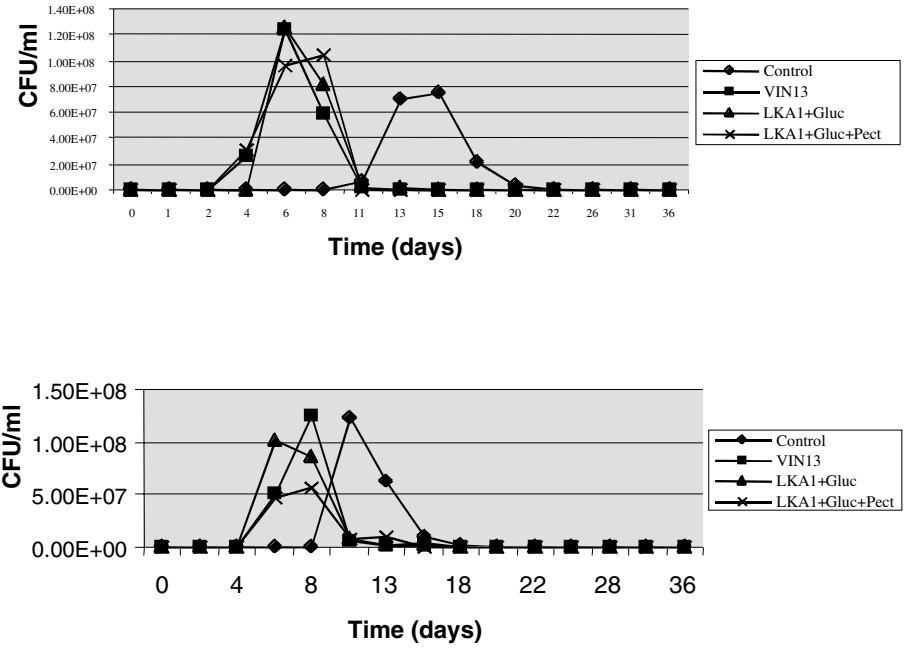
We are currently assessing the fermentation behaviour of the same strains when directly inoculated into grape must, either as single strains or as a mixture of GM and parental strains. These experiments will allow assessing the relative fitness of these strains when subjected to direct competition during wine fermentation.

THE ASSESSMENT OF VERTICAL AND HORIZONTAL GENE TRANSFER FROM WINE YEAST STRAINS TO OTHER STRAINS OR MICROORGANISMS

The assessment of potential risks associated with GMOs has to take into account the possibility that a specific character conferred through GM technology may be able to spread beyond the confines of the initially modified strain. Vertical gene transfer refers to the transmission of DNA through sexual reproduction. In the case of wine yeast strains, it would require sporulation of the strain, followed by mating of the spores with either each other or with spores of another *S. cerevisiae* strain that may have been present during the fermentation process. To our knowledge, no direct observations

FIGURE 2

Spontaneous fermentations of grapes harvested from the different blocks. The graphs represent two independently conducted experiments (A and B), and indicate the number of colony forming units observed during the fermentations. The data correlate well with fermentative activity (data not shown).



of sporulation in commercial wine yeast strains during wine fermentation have been published, and its occurrence will therefore have to be assessed during this study. Horizontal gene transfer, on the other hand, refers to the asexual transfer of DNA from one species to another, and has recently attracted wide-spread interest and media coverage. The analysis of entire genome sequences has revealed that most, if not all species, have been subjected to horizontal transfers in their evolutionary history, and that the process has probably played an essential role in evolution. Horizontal transfer of DNA has been observed between a number of microorganisms, both between two strains of the same species and between unrelated species. However, few if any data exist regarding its occurrence and possible mechanisms in eukaryotic organisms, but for the transfer of genes by viruses. Furthermore, no data are available regarding the possible occurrence of such transfers between microorganisms present during wine fermentation, and in particular between a wine yeast strain and either other yeast strains and species or bacteria.

Two projects are currently underway at the Institute for Wine Biotechnology (South Africa) to assess the probability of DNA transfer between two

wine yeast strains and between wine yeast strains and other microorganisms during wine fermentation. The first project more specifically assesses the probability of sexual DNA transfers, whereas a second project investigates the general probability of DNA transfer, particularly through non-sexual mechanisms.

Most industrial wine yeast strains are either diploid or polyploid. Sexual reproduction of these strains requires that cells sporulate, which results in the formation of four haploid or aneuploid ascospores. A single spore can multiply vegetatively until encountering a cell of the opposite mating type, which will lead to the fusion of the two cells. Sporulation and mating would be the most obvious and easiest way through which specific characters or genes, whether established through genetic modification or traditional methodologies, can be transferred from one strain to another. The presence or absence of sporulating cells in a culture is difficult or impossible to assess visually if the percentage of these cells is low, which will be the case during wine fermentation. To overcome this problem, we will fuse an Open Reading Frame (ORF) encoding a Green Fluorescent Protein (GFP) to the promoter of a strong sporulation-specific gene. Several such genes have been identified. A plasmid carrying a dominant selection marker combined with the above mentioned construct will be transformed into industrial wine yeast strains, and the transformed strains will be inoculated individually into experimental fermentors containing grape must. Each wine yeast strain of interest can be monitored, and the frequency of sporulation can be established. The influence of environmental factors on sporulation frequency can also be assessed.

To assess the occurrence of horizontal gene transfer, several industrial wine yeast strains with a well established genetic background and karyotype will be transformed with plasmid DNA carrying a single dominant selection marker, which confers resistance to either sulfomethurone (*SMR1*) or geneticin (kanMX). Plasmids will be both centromer (single copy) and 2-micron (multiple copy) based. Two transformed strains, each carrying a different marker, will be co-inoculated at variable initial ratios into grape must or specific laboratory media. After having completed micro-vinifications under various conditions, possible transfers of DNA will be assessed by isolating strains resistant to both compounds. Should such strains be found, they will be analysed genetically and karyotyped in order to assess whether the transfer was limited to plasmid DNA or involved the transfer of chromosomal fragments as well.

The experimental set up provides a framework to assess a "worst case scenario", since it uses conditions which might be optimal for horizontal DNA transfer to occur:

- Plasmid based genes are probably more likely to be transferred. Plasmids are small, autoreplicating elements and therefore are more likely to be both released from cells and to be taken up by another cell. The possibility of transfer will be further increased through the use of high copy number plasmids;
- Cells will be grown to high density, increasing contact between cells and therefore increasing the likelihood of transfer;
- Grape must fermentation results in high levels of ethanol which is known to increase membrane permeability, and should therefore increase the

possibility of DNA transfer. Laboratory media with high ethanol content will also be used during the experimental phase;
– Each assay will be monitored for a prolonged period of time, even after the end of fermentation. Numerous cells undergo autolysis during the late stages of fermentation which should lead to increased release of DNA and increased transfer probability.

The data will provide essential information regarding the assessment of risks associated with genetically modified yeast. Since the experiments are designed to improve the chances of transfer, a negative result would provide a strong indication that such a transfer is very unlikely to occur and that the spreading of characters that have been modified in GMO yeast is an unlikely event.

Assessing unforeseen consequences of genetic modification on the modified organism

Genetic engineering has a number of advantages over classical breeding and selection. In particular, it allows the specific modification of a single trait of a target organism, without changing the genetic background. In most cases, genetic engineering aims either to introduce new enzymatic activities or to change the metabolic flux through specific pathways by changing existing enzymatic activities. In both cases, the change will have some metabolic consequences, be they due to the demand for additional protein synthesis in the case of expressing new enzymes destined for secretion, the modification of metabolite concentrations or the presence of new, foreign metabolites.

It is likely that the changed parameters will be sensed by the complex regulatory mechanisms that exist within any living cell, and will lead to a specific molecular response. This response may result in some unforeseen, indirect consequences regarding the metabolic activity of the cell. In several cases of attempted metabolic engineering of wine yeast strains, such unforeseen consequences have been described, an example being the increased production of acetate in strains with increased levels of glycerol-synthesising enzymes. Modern biotechnological tools allow to systematically assess any biological process on a 'global' level, by analysing the entire transcriptome (all mRNAs present in a cell), and – in the near future – the proteome (all proteins) and metabolome (all metabolites). The transcriptome can be analysed by microarrays, which monitor the transcription of all protein-encoding genes present in the genome of yeast.

Several studies have already been conducted to compare the transcriptional regulation in parental and genetically modified yeast strains. By monitoring the transcriptome, possible unexpected side-effects of the genetic modification can be revealed and analysed. In Montpellier, the effects of the introduction of a gene that lead to the production of high levels of lactate by *S. cerevisiae* (Dequin *et al*., 1999) has been studied in some detail. This yeast has been transformed with a bacterial gene encoding lactate dehydrogenase, and produces up to 35 g/l of lactate. The high level of lactate suggests that the metabolism of this specific yeast strain has been profoundly modified, and the strain indeed displays several additional phenotypes, including reduced fermentative activity and numerous knock-on effects

are therefore expected. The data show that the modified strain responds to the increased levels of acid by modifying the expression of more than 100 genes. For the most, these genes belong to groups which are required to adjust or to maintain the internal pH of the cell or to protect the cell against environmental stress, including proton transporters and heat-shock proteins. Other genes whose expression has been modified are involved in general metabolism (glycolytic genes). Similar studies are underway in several laboratories, and will lead to the establishment of databases where all metabolic side effects of genetic modifications can be analysed. In the near future, it will be possible to fully assess the consequences of any genetic modification at all levels of cellular metabolism.

CONCLUSION

The projects described in this report are designed to generate a scientifically sound, holistic view of the potential effects of genetically modified yeasts in the wine industry. Most of these projects will have been finalised within the next two years, and the data will provide a framework for the assessment of individual strains by regulatory bodies, including the OIV and national governments.

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