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ORIGINAL ARTICLE

Genetically modified wine yeasts and risk assessment studies covering different steps within the wine making process

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Abstract The use of gene technology to modify the genome of wine yeasts belonging to the species *Saccharomyces cerevisiae* began in the early 1990s. From a purely scientific point of view, many yeast constructs [genetically modified organisms (GMO)] have been made so far, covering more or less all stages of the wine making process in which microorganisms or commercial enzymes play a key role. The range of theoretical applications is summarised in this report. So far, only two wine-producing countries worldwide allow the use of engineered wine yeasts; the changing situation in Germany regarding consumers' attitudes towards gene technology, and foodstuffs thus produced, will be outlined here. Experiments at the Geisenheim Research Center have highlighted the essential stages of the wine making process where yeasts are involved by using engineered wine yeasts in comparison with non-engineered yeast strains. Greenhouse simulations revealed the persistence of genetically modified (gm) yeasts when these were used as fertilizers, as vintners do with yeast lees after the fermentation process. Furthermore, the persistence of engineered yeast was also monitored in fermentations, after bottling, and after biological treatment of winery waste water. It turned out that engineered wine yeast strains behave like non-engineered wine yeasts. They also persist in the winery interior and installations as well as becoming part of the yeast flora on grape vines in a vineyard with annual fluctuations in the composition of the yeast populations.

Keywords *Saccharomyces cerevisiae* · Alcoholic fermentation · Wine making · Genetic engineering · Risk assessment

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Introduction

Techniques for beer, wine and bread production have been in use for thousands of years, although the microbiological features underlying these processes have begun to be understood only relatively recently, namely in the second half of the nineteenth century. The identification of yeasts as "wine makers" and the fundamental aspects of alcoholic fermentation are tightly linked with the name Louis Pasteur and a couple of other European chemists and biologists of that era (for reviews on this history, see Barnett 1998, 2000; Barnett and Lichtenthaler 2001). It is likely that most modern wines have little in common with the wines produced by the Georgians, Egyptians or Romans. Through increased knowledge of yeast physiology and yeast genetics, wine quality has improved continuously since Pasteur's findings. Choice of wine yeast strains was quickly realised to have a major impact on wine quality and different

flavour expressions (Kunkee and Amerine 1970), which has led to extensive yeast selection programs all over the world (for review, see Fleet 1993).

In addition, researchers have attempted to improve and combine positive attributes mediated by different yeast strains by sexual breeding. As is also the case with other microorganisms, *Saccharomyces cerevisiae* yeasts formerly exhibited two main limitations to the application of this technique: the lack of genetic markers, and the complex genomic structure of industrial wine yeasts (Bakalinsky and Snow 1990). Nevertheless some examples of genetic improvement after mating, sporulation and isolation of single spore derivatives do exist (Ramirez et al. 1999).

Among other investigated methods, e.g. hybridisation, rare-mating and spheroplast fusion, hybridisation is still the procedure most often applied to combine genetic material of yeast strains from the same species or species very closely related to *Saccharomyces cerevisiae* (Pretorius and van der Westhuizen 1991; Querol and Ramon 1996).

However, these classical methods lack the precision and capability of gene cloning. Since the first successful transformation of yeast in 1978, genetic engineering procedures have improved continuously, with a wide variety of integrating, autonomously replicating or other specialised vectors now being available (Pretorius et al. 2003). The wine sector now has access to the large number of engineered wine yeasts that have been constructed so far. In 2007, Cebollero et al. asked: "transgenic wine yeast technology comes of age: is it time for transgenic wine?"

Table 1 lists some examples of genetically modified (gm) yeasts and the corresponding steps within the wine making process where these strains might be used for practical applications. This list also highlights the fact that not only distinct biochemical reactions can be enhanced or diminished but also new properties can be introduced into the yeast metabolism (e.g. lysozyme production).

Even physical treatments of grape must or wine, such as bentonite fining, can be replaced by the use of engineered yeasts.

Schuller and Casal (2005) carried out a comprehensive survey of publications dealing with gm yeasts used for wine production. Dequin (2001), Akada (2002) and Donalies et al. (2008) enlarged such overviews by describing the abilities of engineered yeasts also in the brewing, baking and sake-producing industries. In addition, recombinant *Saccharomyces cerevisiae* strains were also described for bioethanol production as well as for production of fine and bulk chemicals (Nevoigt 2008).

Despite the availability of a broad range of gm yeasts, only two strains are permitted for use in the United States and Canada (Table 2).

Strains ML01 and strain ECMo01 share the property of being advertised as being able to improve the healthiness of wine consumption as they significantly reduce the risk of the production of biogenic amines (allergenic metabolites of lactic acid bacteria) as well as the generation of ethyl carbamate (a mutagenic and possibly also carcinogenic substance).

The wine making process and wine itself are regarded as part of the cultural heritage of mankind's civilisation, which makes it difficult to introduce new technologies into wine production. When it comes to using gene technology for food production, many consumers are worried about eating gm food. While a consumer survey in Germany in 1994 (Menrad and Blind 1996) showed that nearly 80% completely rejected the use of gene technology, a more detailed opinion poll within the population became available in 2002, when 80% accepted gene technology as a useful tool to combat severe illness (Allensbach 2002). However, in 2006 the rejection quota for gm food was still 75% (GfK-Marktforschung 2007).

Besides public opinion, the use of engineered organisms and the resulting products is widely regulated in the European Union (EU), the United States and most other countries in the world. In fact, there are some differences in the worldwide regulations concerning the investigations of possible side effects of gm organisms when used under non-contained conditions, e.g. in agriculture. The implementation of international regulations should cover not only risk assessment studies before decisions can be made regarding allowing the marketing of GMOs, but also identity preservation systems, which are claimed to be established to preserve and guarantee the purity of non-GMO food (Varzakas et al. 2007).

Risk assessment studies concerning gm wine yeasts within the wine making process are still rather rare. Schoeman et al. (2009) used sand columns and sand cells to demonstrate the behaviour of yeasts in a nature simulating scenario. It was shown that the gm yeast strain formed a biofilm and became part of the microbial population in the sand but never dominated the yeast flora.

To broaden our knowledge of gm yeast behaviour during the wine making process, we investigated the potential impact of these yeasts on natural yeast flora in a vineyard, the impact on winery flora, and the behaviour of gm yeasts during waste water treatment and also in bottled wines, simulating the marketing of unfiltered wines when it comes to bottling. Figure 1 illustrates areas of winemaking investigated in the scope of this project.

Materials and methods

Strains and culture conditions

All *Saccharomyces cerevisiae* strains used in this study are listed in Table 3.

Table 1 Potential benefits of genetically modified (gm) wine yeasts in different wine making steps

Wine characteristic	Enhancement	Benefit	Reference
Wine aroma			
Fermentation bouquet	Enhancement of esterase activity (*ATF1*)	More fruitiness	Lilly et al. 2000
Varietal aroma; red wine colour	Enhancement of glycosides splitting hydrolases	More varietal character	Perez-Gonzalez et al. 1993; Ganga et al. 1999; Louw et al. 2006; van Rensburg et al. 2007; Gil and Vallés 2001
	Enhancement of S-linalool synthase	*de novo* production of aromatic terpenes	Herrero et al. 2008
Taste	Enhancement of GPD1-enzyme; enhancement of H_2O-NADH oxidase	More glycerol, extract and full-body; reduced alcohol production	Michnick et al. 1997; Heux et al. 2006
	Malate degradation; lactate production;	Balanced acidity	Volschenk et al. 1997,2001; Dequin et al. 1999
	Enhancement of yeast autolysis	Higher complexity and maturity	Cebollero et al. 2009
Must/wine treatment			
Grape must	Enhancement of pectinase activity; enhancement of protease activity	Improved filtration alternative for bentonite	Gonzalez-Candelas et al. 1995
Young wine	Enhancement of glucanase activity	Improved filtration	Perez-Gonzalez et al. 1993
Wine and human health			
Wine hygiene	Production of bacteria inhibiting proteins	Lower SO_2-requirement	Schoeman et al. 1999
Health promotion	Enhancement of existing enzymes and/or introduction of external enzymes	Hydrolyses of bound resveratrol; formation of various vitamins and unsaturated fatty acids	Gonzalez-Candelas et al. 2000; Pretorius 2000 (review)

All strains were cultured on YEPD (agar) (1% yeast extract, 2% glucose, 2% peptone adjusted to pH 6.5) or malt medium (3% malt, adjusted to pH 6.5). In case of plates 2% agar was added.

Detection of gm yeast

Phenotypic characterisation

It was possible to detect the S.c. VIN13-*lka*1 strain by growing on Phadebas medium plates. 6.7 g DIFCO™ Yeast Nitrogen Base (w/o amino acids and ammonium sulfate), 1 g glucose, 20 Phadebas pills and 20 g agar were solved in 1 l distilled water at pH 6.5 to 7 to cast medium plates. *S. cerevisiae* strains were inoculated on these plates, and bright, clear halos around genetically modified VIN13-*lka*1 strains were detected 48 h later (Fig. 4).

Strain S.c. VIN13-*end*1 was detected via growth on CMC medium plates. To prepare this medium, 10 g yeast extract, 20 g glucose, 20 g peptone, 3.0 g viscose carboxymethyl cellulose and 20 g agar were solved in 1 l distilled water at pH 6.5 to 7. *S. cerevisiae* strains were cultivated for 48 h on these plates. Thereafter, the Petri dishes were washed with TE buffer (150 mM Tris, 20 mM EDTA, pH 7) and a Congo Red solution (0.1%) was applied for 10–15 min to show bright, clear halos around the colonies in case of gm yeast strains.

Genotypic detection

Genotypic detection of different gm *S. cerevisiae* strains was done by PCR (Fig. 3; Table 4). Standardised PCR conditions were used: 1 μl template DNA, 0.2 mmol/l dNTPs, 1 μl of each primer (100 pmol/μl), 2.5 mmol/l MgCl, 0.5 U *Taq* DNA

Table 2 Authorised wine yeasts

Country	Yeast strain	Date of authorisation	Property
United States	ML 01	30 June 2003	Malolactic fermentation
	ECMo01	6 January 2006	Degradation of urea
Canada	ML 01	12 July 2006	Malolactic fermentation
	ECMo01	16 November 2006	Degradation of urea

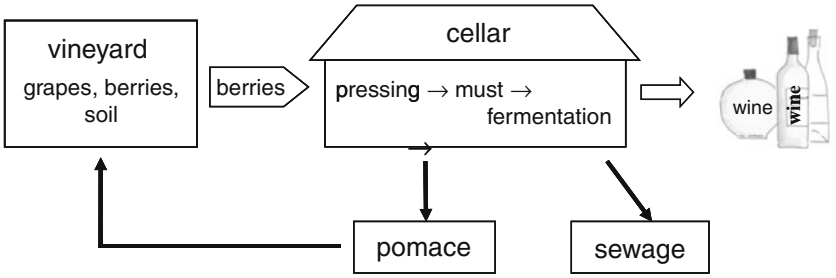

Fig. 1 Areas of winemaking investigated in the scope of the project: *I* vineyard (grapes, pomace, organic waste)—simulations in S1 greenhouse and S1 laboratory; *II* cellar techniques—simulations in S1 laboratory; *III* detection inside the filled endproduct—experiments in S1 laboratory; *IV* behaviour inside sewage plants—simulation in S1 laboratory

polymerase (Fermentas, St. Leon-Rot, Germany) and the PCR buffer supplied by the manufacturer in a final volume of 50 µl. The primers listed in Table 4 were used for detection of the gm yeast strains. For detection of S.c. VIN13-*end*1, the following program was used: initial denaturation for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C, and a final extension for 5 min at 72°C.

S.c. VIN13-*lka*1 was detected using the following cycler conditions: initial denaturation at 94°C for 3 min, then 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension for 5 min at 72°C.

S92 ML01 was detected with the following cycler conditions: initial denaturation at 94°C for 3 min, then 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension for 5 min at 72°C.

DNA extraction

Yeast isolates were cultured in 8 ml liquid YEPD medium (1% yeast extract, 2% glucose and 2% peptone adjusted to pH 6.5) for 48 h. Samples (1.5 ml) were taken and the yeast cells were harvested by centrifugation for 5 min at 5,000 rpm at 4°C. The pellet was resuspended in 200 µl yeast denaturation buffer (10 mmol/l Tris-HCl, 10 mmol/l EDTA, 100 mmol/l NaCl, 1% SDS and 2% Triton X-100). Glass-beads (0.3 g, 0.5 mm) and 200 µl phenol-chloroform-isoamylalcohol (25:24:1) were added for mechanical disruption of the cells by vortexing for 1 min. Then, 200 µl TE buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA) was added and cell debris were separated and sedimented by centrifugation for 5 min at 12,000 rpm and 4°C. The upper phase was transferred to a new tube and DNA was precipitated by adding 0.7 volume isopropanol. The tube was centrifuged again for 10 min at 12,000 rpm and 4°C. The DNA was washed with 70% ethanol, dried and dissolved in 50 µl TE buffer containing 2 U RNase.

DNA extraction from sewage sludge

For detection of gm yeast in sewage sludge (Fig. 1) or other difficult environmental samples based on DNA, the following effective extraction method was applied.

A 300–500 µl sample of sewage sludge was placed in an Eppendorf tube and centrifuged for 1 min at 6,000 rpm. The pellet was resuspended in 1 ml wash solution [50 mM/l Tris-HCl, 25 mM/l EDTA, 0.1% SDS, 0.1% polyvinylpyrrolidon (PVP), pH 7.7] and again centrifuged for 1 min at 6,000 rpm. The supernatant was discarded and the pellet resuspended in 35 µl lysis buffer (50 mM/l Tris-HCl, 25 mM/l EDTA, 3% SDS, 1.2% PVP, pH 8.0) before heating in a microwave (600–700 Watt) for 1.5 min; 400 µl extraction solution preheated to 65°C was then added. DNA was extracted with a standard protocol (Sambrook et al. 1989) using phenol chloroform

Table 3 *Saccharomyces cerevisiae* strains used in this study

Strain	Description	Reference
S.c. VIN13	Commercially available wine yeast strain	Anchor Yeast, SA (http://www.anchor.co.za)
S.c. VIN13-*lka*1	Genetically modified strain based on VIN13 Contains a gene coding for an α-amylase of *Lipomyces kononkoae*	Gundllapalli Moses et al. 2002
S.c. VIN13-*end*1	Genetically modified strain based on VIN13 Contains genes coding for an endo-1,4-glucanase of *Butyrivibrio fibrisolvens* and an endo-β-xylanase of *Aspergillus niger*	Strauss 2003
S.c. S92- ML01	Genetically modified strain based on S92 Contains genes coding for a malate permease of *Schizosaccharomyces pombe* and a malolactic enzyme of *Oenococcus oeni*	Husnik et al. 2006

Table 4 Primers used to detect gm yeast in this study

Primer	Sequence (5′-3′)	Annealing temperature [°C]
END1/2	ATTACTGAACTGAAATATTAA	45
END1for	ACTAAAGTTTCTGCCGCTGGCG	45
LKA1-5′	ATGTTGCTGATCAACTTTTTCATCGCT	50
LKA1-3′	TCTCTACATGGAGCAGATTCCAGAGCC	50
ML01 for	GTTGTAATGTGACCAATGAG	53
ML01 rev	CTCTTTATATTTACATGCTAAAAATGG	53
ML01-Mae1	CCCACACCATCCACTCGCC	56
ML01-mleA	CCCCAGTCTCCAATCCC	56
PGK1-Prom2	CCGAATCGTGTGACAACAA	56

extraction, isopropanol precipitation and washing with 70% ethanol. The DNA was dissolved in 50 µl TE buffer.

Yeast species identification

PCR amplification and RFLP analysis of the rDNA-5.8 S-ITS region

To identify isolated yeasts, the 5.8 S-ITS region was amplified in an Eppendorf Mastercycler epgradient apparatus (Eppendorf, Hamburg, Germany) using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) previously described (White et al. 1990). The PCR conditions were: 1 µl template DNA, 0.2 mmol/l dNTPs, 1 µl of each primer, 2.5 mmol/l MgCl, 0.5 U *Taq* DNA Polymerase (Fermentas) and the PCR buffer supplied by the manufacturer in a final volume of 50 µl. Cycler conditions were: initial denaturation for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 2 min at 52°C and 2 min at 72°C, and a final extension for 8 min at 72°C. PCR products were checked on 1.5% agarose gels and staining with ethidium bromide for 5 min.

The amplified PCR products were digested with the restriction enzymes *Hin6*I (isoschizomer to *Cfo*I), *BsuR*I (isoschizomer to *Hae*III) and *Hinf*I (Fermentas). Restriction digestion was performed according to the supplier's instructions. RFLP analyses were done after separation of the fragments by electrophoresis on a 2% agarose gel. A 100 bp DNA ladder (MassRuler, Fermentas) was used as a size standard.

Yeast species were identified according to the restriction patterns published by Guillamón et al. (1998), Esteve-Zarzoso et al. (1999), Granchi et al. (1999), Las Heras-Vazquez et al. (2003) and Sturm et al. (2005).

Identification by amplification of the D1/D2 domain and sequencing

Another way to identify isolated yeast species was amplification of the D1/D2 domain of the 26 S rDNA-

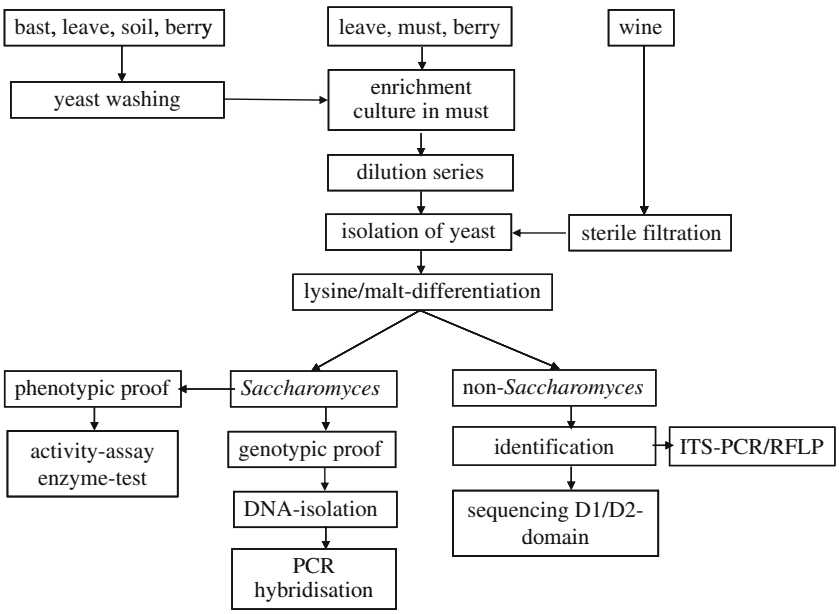

Fig. 2 Flow chart sampling and identification of *Saccharomyces*, non-*Saccharomyces* and genetically modified yeast (gmy)

Table 5 Occurrence of yeast species before and after dissemination of gm yeast in an artificial vineyard inside a S1-greenhouse. Samples were taken from each block and yeast species identified by D1/D2 domain sequencing or by RFLP analysis of the 5.8 S rDNA ITS region

Yeast	1st year Without release	2nd year Release of VIN 13 and GMY	3rd year Release of VIN 13 and GMY
(Aureobasidium pullullans)		X	X
Candida lambica	X	X	
Candida parapsilosis	X	X	X
Candida pulcherrima	X	X	X
Candida spp.	X	X	X
Cryptococcus spp.		X	X
Debaryomyces hansenii	X	X	
Hanseniaspora uvarum	X	X	
Kloeckera apiculata	X	X	
Kluyveromyces lactis	X		
Pichia anomala		X	X
Pichia guilliermondii	X	X	X
Pichia kluyveri	X	X	
Pichia spp.	X	X	X
Rhodutorula spp.	X	X	X
Saccharomyces cerevisiae	X	X	X
Saccharomycodes ludwgii	X		
Torulaspora delbrueckii			X
Yarrowia lipolytica	X	X	X

gene (Kurtzman and Robnett 1998). This domain was amplified in an Eppendorf Gradient thermocycler using the primers D1/D2 Forward (5′-GCATATCAATAAGCGG AGGAAAAG-3′) and D1/D2 Reverse (5′-GGTCCGTG TTTCAAGACG-3′). PCR conditions were as above. Cycler conditions were: initial denaturation for 2 min at 94°C, followed by 36 cycles of 1 min at 94°C, 2 min at 52°C and 2 min at 72°C, and a final extension for 10 min at 72°C. The PCR products were checked in the same way as before. After purification, the PCR product was sent to a commercial sequencing service (SRD, Oberursel, Germany).

Characterisation of *S. cerevisiae* on strain level

A method to distinguish between different strains of the species *S. cerevisiae* is the so-called δ-PCR described by Ness et al. (1993) and Legras and Karst (2003). δ-Sequences are parts of the TY1 elements existing in around 35 copies in the genome of *S. cerevisae*. Using the primers δ-12 (5′- TCAACAATGGAATCCCAAC-3′) and δ-2 (5′- GTGGATTTTTATTCCAAC-3′) it is possible to obtain specific patterns of DNA bands in an agarose gel for each isolated strain. Standardised PCR conditions were the same as before, with the only difference being the use of standardised amounts of DNA as template. Cycler conditions were as described by Legras and Karst, 2003: initial denaturation for 2 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 1 min at 43°C and 1 min at 72°C, and a final extension for 10 min at 72°C. Amplified bands were visualized on a 1.5% agarose gel after staining with ethidium bromide.

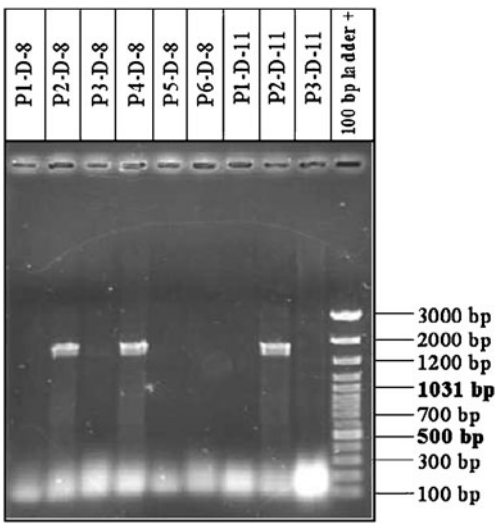

Fig. 3 Genotypical detection of the genetically modified S.c. VIN13-*lka*1 by PCR using the primers LKA1-3′ and LKA1-5′. A selection of isolates from block P4 of the artificial greenhouse vineyard was tested

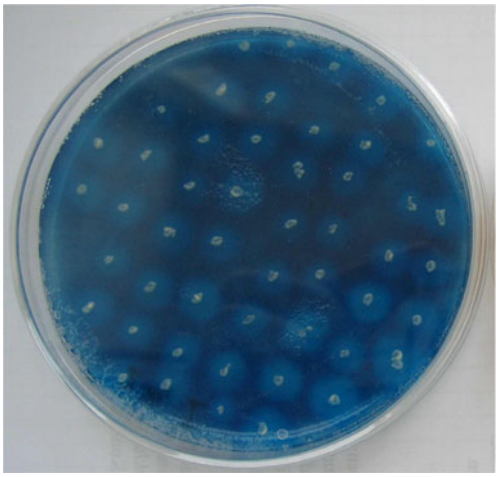

Fig. 4 Phenotypical detection of the gm yeast S.c.VIN13-*lka*1 on Phadebas medium. Yeast with α-amylase activity shows clear halos around the growing colonies

Differentiation of yeast isolates

Methods of sampling and identification of *Saccharomyces*, non-*Saccharomyces* and genetically modified yeast (gmy) are detailed in the following sections and summarised in Fig. 2

Saccharomyces vs. non-Saccharomyces

After isolation from plates, yeast strains were differentiated using several special media. Pre-identification of yeast belonging to the genus *Saccharomyces* was performed on lysine agar selective medium (0.67% Difco™ Yeast Nitrogen Base (w/o amino acids and ammonium sulfate), 0.089% L-lysine monohydrochloride, 5% glucose and 1.8% agar). Only yeast not belonging to the genus *Saccharomyces* could grow on this selective medium.

Selection of different non-Saccharomyces strains

To divide and select as many non-*Saccharomyces* species as possible, a yeast differentiation medium was used. Two solutions (solution 1: 24 g glucose, 2 g Difco™ Yeast Nitrogen Base (w/o amino acids and ammonium sulfate), 0.9 g ammonium sulfate, 0.05 g bromocresol green, 0.05 g methyl red, 6 g agar in 300 ml distilled water adjusted to pH 6.5) (solution 2: 3 g methionine, 3.6 g galactose, 9 g ammonium sulfate in 100 ml distilled water) were prepared and autoclaved separately and then mixed to cast plates. Different yeast species showed more specifiable morphology growing on this medium.

Effects of gm yeast in a vineyard

Experimental vineyard

To test the influence of gm yeast on the natural yeast flora and the distribution and survival rate of the gm yeast itself vineyard (Fig. 1), experiments were performed in a greenhouse under genetic safety level S1. The greenhouse was subdivided into four blocks. In each block, 20 *Vitis vinifera* "Riesling" root stocks were planted in flower pots. The root stocks were treated with fertilisers and plant pesticides over the whole experimental time according to standard practice in viticulture. The blocks were separated by curtains to avoid cross-contamination during the release of the test organisms. Typically 1.5 l of a solution

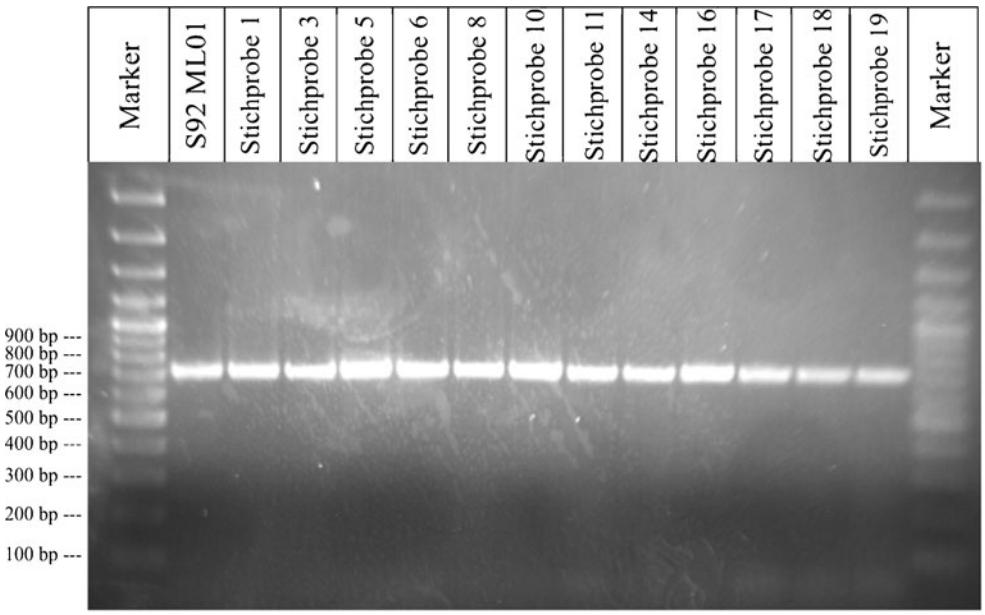

Fig. 5 Detection of the gm yeast S92 ML01 by PCR using the primers ML01 for and ML01 rev. In sample S92 ML01 DNA of this strain was used as template to be a reference. For all other samples DNA from randomly isolated strains of an enrichment culture of grapes from block P2 of the artificial greenhouse vineyard were used. S92 ML01 was liberated in this block

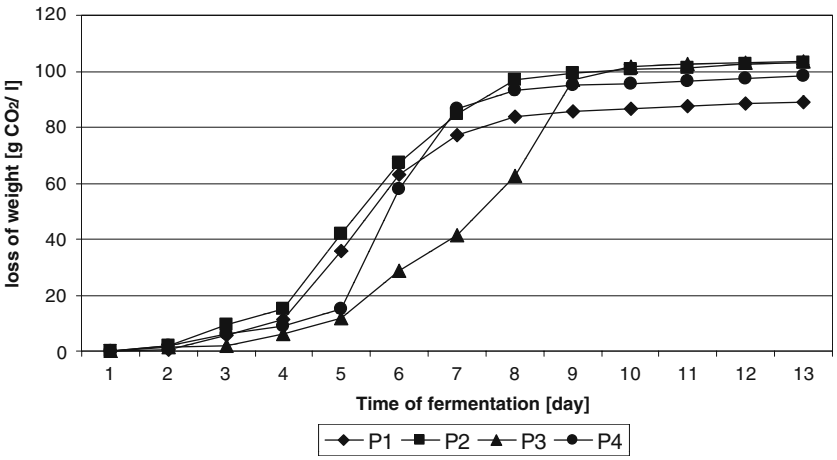

Fig. 6 Course of spontaneous fermentations with grape juice of grapes grown on vines in differently treated blocks of the S1-vineyard. In blocks P2 and P4, the gm yeasts S.c. VIN13-*lka*1 and S.c. VIN13-*end*1 were released. Block P1 was the control without any release and in block P3 the commercial wine yeast S.c. VIN13 was released also as a control

with $2x10^6$ cells/ml genetically modified yeast strains was released at several times. Block 1 was the control without any release of yeast strains. In block 2, the strains S.c. VIN13-*lka*1 and S.c.VIN13-*end*1 were released over several years. Strain S.c. S92 ML01 was released only in the final year of the experiment. Block 3 was also a control. Over the whole duration of the experiment, the commercial wine strain S.c. VIN13 was released at several time points. In block 4, the gm yeast S.c.VIN13-*lka*1 was applied at several time points.

Sampling of yeast

Different plant material, such as bark, leaves, berries and soil, were taken as samples to isolate yeasts and to detect gm yeast (Fig. 2). The material was washed with physiological NaCl solution (0.9% NaCl, 0.1% Triton X-100). These solutions were then diluted in decadic steps and 100 µl dilution was sprayed on malt or YEPD plates. After 2 days of incubation at 25°C, colonies were counted and single strains to be differentiated by different selective media (see also Differentiation of yeast) were isolated.

Enrichment cultures

Only small amounts of microorganisms existed in some samples. Enrichment cultures were necessary to isolate yeasts. For this purpose, 250 ml Erlenmeyer flasks were prepared with 200 ml grape juice and different sample material like berries, bark, leaf pieces or soil were added directly and incubated at room temperature for several days. Samples between 100 µl and 1 ml were taken to be sprayed on YEPD or malt plates after dilution for yeast isolation.

Microvinifications

For spontaneous fermentation experiments, grapes of the different greenhouse blocks were harvested under sterile

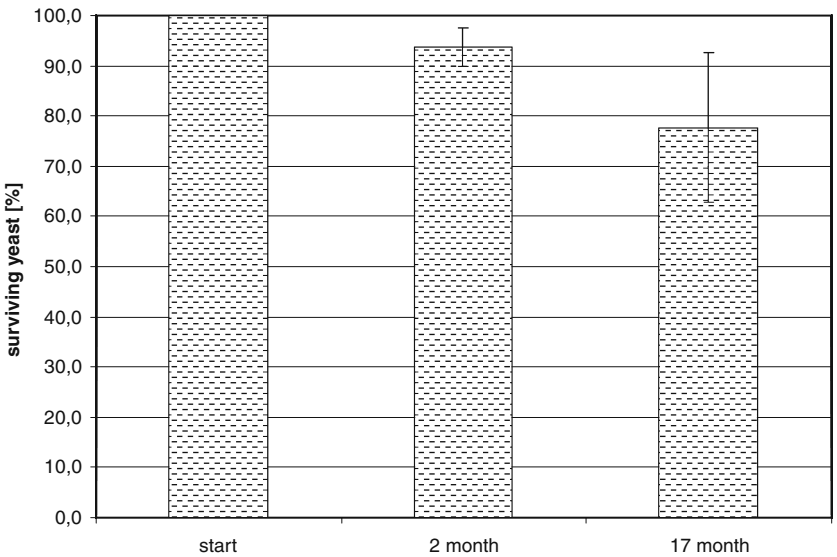

Fig. 7 Survival rates of S.c. VIN13-*lka*1 in bottled wines. Dry white wine was inoculated with defined amounts (between 200 and 2,000 yeast cells) of S.c. VIN13-*lka*1 and stored for periods of 2 and 17 months at cellar temperatures (15°C). The survival rate was tested by membrane filtration and phenotypical detection on Phadebas medium

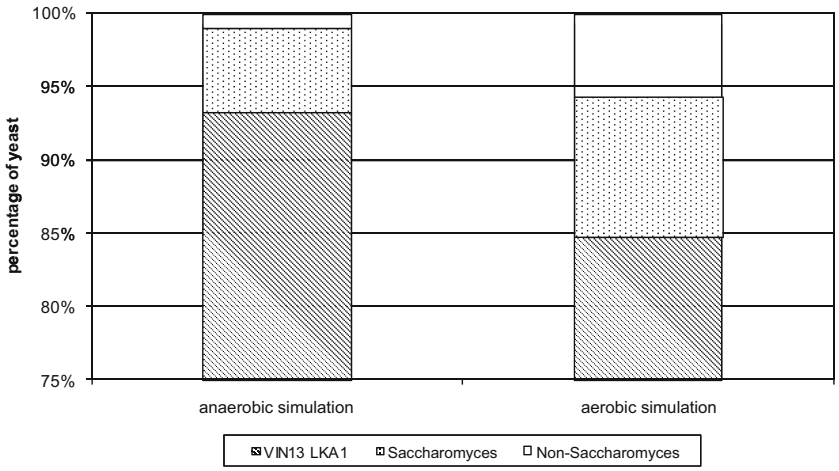

Fig. 8 Percentage distribution of yeast surviving aerobic and anaerobic simulations of a sewage plant treatment. In each simulation, 300 yeasts were isolated and tested phenotypically by growing on Phadebas medium and genotypically by PCR

conditions. Grapes were pressed in sterile plastic bags by hand and 150 or 200 ml settled must was placed in flasks for vinification at room temperature. Fermentation activity was shown by loss of CO_2 and weighing the flasks. Samples were taken to determine total cell counts and also to detect gm yeasts.

Survival of gm yeast in bottled wine

To test the survival rate of gm yeast in bottled wine (Fig. 1), bottles were spiked with S.c. VIN13-*lka*1. Defined amounts of cells of this yeast strain were added to the filled wine before closing the bottles with a screw cap. Filtrations were done directly, after 2 months and 17 months using a filtration system (Sartorius, Göttingen, Germany) and filters with pore sizes of 0.45 μm and 0.2 μm. These filters were incubated for 3–6 days at 25°C on malt medium and the amount of growing colonies was determined. Growing colonies were also checked for gm yeast.

Simulation of a sewage plant

To conduct experiments relevant to waste water, it was necessary to construct a model to simulate the environmental conditions of a local sewage plant under laboratory conditions at safety level 1. A sewage plant has two steps that have a strong influence on microbiology. In the first step, sewage passes through an aeration tank with aerobic conditions. The sewage sludge is then processed in a digestion tower under anaerobic conditions.

The sewage plant was simulated using sewage sludge from a local sewage plant (communal sewage plant Rüdesheim, Rüdesheim, Germany). The sludge was placed in a flask with a magnetic stirrer for aerobic simulation, and in an OxiTop IS12 system (WTW, Weilheim, Germany) to simulate anaerobic processing conditions considering the COD (chemical oxygen demand) of the respective sludge sample. In the simulations, 2.2×10^7 to 1×10^8 yeast cells/ml were inoculated and incubated for 5 days at 25°C. In a first experiment, the simulations were first treated with the commercial wine yeast S.c. VIN13, and later with the gm yeast S.c. VIN13-*lka*1. Because of the high rates of occurrence of bacteria during sampling, chloramphenicol (30 μg/ml final concentration) was used to reduce bacterial growth and to facilitate yeast isolation. Isolated yeast strains were differentiated and identified as described above.

Table 6 Presence of gm yeast (GMY1, GMY2) after dissemination in a vineyard in the year of release, and 1 and 2 years after release. Twenty "Riesling" rootstocks inside an S1 greenhouse were treated with gm yeasts as indicted. Detection of gm yeast strains (and amounts) are indicated

Treatment	1st year Release of GMY1, GMY2	2nd year No release	3rd year No release
P1 control	No gm yeasts detected	No gm yeasts detected	No gm yeasts detected
P2 GMY1, GMY2	GMY1, GMY2 (88%, 12%)	GMY2 (8.5%)	No gm yeasts detected
P3 S.c. VIN13	No gm yeasts detected	No gm yeasts detected	No gm yeasts detected
P4 GMY1	GMY1 (83%)	–	GMY2 (3%)

[a] 1.5 l with 2×10^6 yeast cells/ml was released

Results

Behaviour of gm yeasts in vineyard experiments

Effects on natural yeast flora in an artificial vineyard in the greenhouse

One aspect of this study was to test the possible influence of gm yeast on the natural yeast flora of a vineyard. Thus it was necessary to isolate yeasts in the artificial vineyard in the greenhouse before and after dissemination of the gm test microorganisms. In the 1st year of experiments, the status quo of encountered yeast species was determined using D1/D2 domain sequencing after isolating yeasts from several materials like soil, bark, leaves and berries at several time points. In the 2nd and 3rd year of the experiment, gm yeast strains and the commercial wine yeast strain S.c. VIN13 were released into different blocks of the greenhouse as described in Materials and methods. The occurrence of the natural yeast flora was tested as in the 1st year. Identification of yeast species only was then performed via analysis of RFLP patterns of the 5.8 S rRNA gene and internal transcribed spacer regions (Table 5).

Over the whole period of examination, several yeast species typical of the winemaking environment were detected, including members of the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Rhodutorula*, *Saccharomyces*, *Saccharomycodes* and *Torulaspora*. *Yarrowia lipolytica* was also detectable in samples from the greenhouse. Only minor fluctuations in species composition were observed. In the 1st year *Cryptococcus species*, *Pichia anomala* and *Torulaspora delbrueckii* were not detected. In the 2nd year, *Cryptococcus species* and *Pichia anomala* were identified but *Kluyveromyces lactis* and *Saccharomycodes ludwigii* were absent. *Aureobasidium pullulans*, a fungus growing in some growth stages like yeast, was also present.

In the 3rd year *Candida lambica*, *Debaryomyces hansenii*, *Hanseniaspora uvarum* and *Pichia kluyveri* were not detectable but *Torulaspora delbrueckii* was present. Over the 3 years of this experiment, a fluctuation range like that found in natural habitats was observed.

Detection of gm yeast strains in an artificial vineyard

Over a period of 3 years, the presence of gm yeasts disseminated in different blocks of the experimental vineyard inside a greenhouse (see Experimental vineyard in Materials and methods) was detected (Table 6). Release of yeast took place only in the 1st year. Block P1 was the control without yeast release. S.c. VIN13-*lka*1 (GMY1) and S.c. VIN13-*end*1 (GMY2) were sprayed in block P2. The commercial, genetically unmodified, wine yeast strain S.c. VIN13 was used in block P3 also as a control. The only gm yeast released in block P4 was S.c. VIN13-*lka*1. Yeasts were isolated and identified as gm yeasts at several time points over the 3-year study period using the protocol described above (see Materials and methods; Figs. 3, 4). During this period, no gm yeast strains were detected in blocks P1 and P3 (control and commercial wine yeast). Both liberated gm yeast strains were detected during the 1st year in block P2, with 88% of GMY1 and 12% GMY2 within all isolated *Saccharomyces* yeasts. The yeast strain S.c. VIN13-*lka*1 became non-detectable after winter and the resulting vegetation break after the 1st year. This yeast was also no longer present in the 3rd year. Only S.c. VIN13-*end*1 could be found over the whole 3-year experimental period in this block (8.5% 2nd year and 15% 3rd year). S.c. VIN13-*lka*1 was present in the 1st year in block P4 (83% of isolated *Saccharomyces*) but fell below detection levels in the 2nd year. In the 3rd year only the other gm yeast S.c. VIN13-*end*1 was isolated from sample material of this block (3% of isolated *Saccharomyces*). This finding was strong evidence of cross contamination.

This result illustrates that it is possible to survive in the environment of an artificial vineyard plus the four seasons of a year, but that survival depends on each single yeast strain and cannot be calculated a priori: one gm yeast strain was always more detectable than the other over the 3-year period.

Detection of the malolactic yeast ML01 in an artificial vineyard

Part of this project involved testing the possibility of identifying the genetically modified yeast strain S92 ML01. This strain has been granted official GRAS status in several countries and can be used for winemaking in Canada and the United States. The strain was released into block P2 in the same manner as the other gm yeasts used in this study. Isolation and detection procedures were as described above. For detection by PCR, the primers ML01 for and ML01 rev were used successfully. The results of one detection experiment are shown in Fig. 5 as an example. The presence of this gm yeast strain could be demonstrated over one vegetation period.

Genetically modified yeast in fermentations and wine

Influence of gm yeast on fermentation

Genetically modified yeasts could be detected in different amounts depending on the yeast strain on grapes from rootstocks growing in blocks after dissemination of gm yeasts in the artificial vineyard (see above). To prove that the gm yeast in this study could have an influence on

vinification, spontaneous fermentations were carried out with grape juice prepared from the four different blocks of the experimental vineyard. The resulting fermentation curves, based on fermentations done in triplicate, are shown as mean values in Fig. 6. In all four variants, a lag phase of about 2 days was apparent. From the 3rd day on, all fermentations showed fermentation activity.

The fermentations P1 and P2 showed the highest fermentation rates during the days 4–6. Variants of P4 were slightly weaker in activity during this time. P3 fermentations had the lowest fermentation rate over the first 8 days. Fermentation variants P2, P3 and P4 reached the same end fermentation level after 10 days. In contrast, the sugar level of P1 was lower at the end. No large differences in fermentation courses and activities were observed, indicating that there was no dominance of the gm yeast strains.

Survival of S.c. VIN13-lka1 in bottled wines

Because of the fact that about 30% of filled wines potentially have problems with contamination by microorganisms (Menke et al. 2007), it would be important to determine the status of gm yeasts in such situations. Bottles filled with a dry white wine (Riesling) were spiked with different amounts of S.c. VIN13-*lka*1 and stored for 2 and 17 months at cellar temperature to test the survival of the yeasts. The wines were then filtered to recover and determine the rate of surviving and gm yeast. Figure 7 presents the results from these stored bottles. After 2 months, an average of about 94% surviving yeasts was detected. All isolated yeasts were gm, as demonstrated by growth with clear halos on Phadebas medium. At 17 months after spiking and bottling of the wines, a survival rate of 77% could be observed. All isolated strains were attributed to strain S.c. VIN13-*lka*1 by growth test on Phadebas medium. These results show definitively that this gm strain can survive in bottled wines.

Genetically modified yeast in waste water

Survival of gm yeast in sewage simulations

This part of the study focussed on the possible risks of dissemination of gm yeast via the waste water of wine cellars. First, it was necessary to develop a simulation system to perform aerobic (aeration tank) and anaerobic (digestion tower) simulations of sewage plants in order to determine the appropriate conditions for this experiment.

For aerobic simulation, Erlenmeyer flasks and stirrers were used to mix the sewage sludge dilution and to bring in the required amount of oxygen. The best way to simulate the digestion tower was to use the OxiTop BSB system.

The first step was to test the survival of the yeast under sewage plant conditions. The hypothesis was that, although there are high concentrations of organic material in a sewage plant, the C sources are not right for yeast. The simulations were inoculated with defined amounts of the commercial wine yeast S.c. VIN13. The liberated yeast survived. About 80% of the *Saccharomyces* isolated by differentiation on malt and lysine and characterized by δ-PCR belonged to this strain (data not shown).

The experiments were then repeated using the gm yeast S.c. VIN13-*lka*1. Amounts of 1×10^8 to 2.5×10^8 cells were inoculated. After isolating and differentiation on lysine medium, about 99% and 93% of the yeasts were related to *Saccharomyces* in the anaerobic and aerobic simulations, respectively. About 92% of the yeast in the anaerobic simulation and about 85% of the yeast under aerobic conditions showed clear halos around the colonies in a growth test on Phadebas medium (see Fig. 8). These results demonstrate clearly that gm yeast strains could survive in sewage plants, and could be disseminated into the environment along with purified sewage plant effluent.

Discussion

The potential benefits of gm yeasts have been presented in various papers (Pretorius 2000), which have stressed the requirement for intensive studies on the possible risks that might result from using this type of yeast. Although the public perception of gm food, especially beverages, is gradually becoming more positive, overall acceptance of gm technology still tends to apply only to human health-related (pharmaceutical) products, i.e. for diagnosis and therapy of severe diseases. The commercialisation of two gm yeast strains in the United States and Canada since 2003 and 2006, in conjunction with differences in the legal requirement to demonstrate potential risks of gm products, especially between the US and EU, demands that more information about the long-term effects of usage of gm yeasts become available.

Schoeman and co-workers (2009) published one of the first studies about the behaviour of gm yeasts in a natural but artificially constructed habitat (sandy soil). Their results indicate clearly that, under these conditions, gm yeasts did not interfere with other microorganisms in such a way that they became dominant partners in these mini-cosms.

Over the last few years, we have elaborated test systems that demonstrate the behaviour of gm yeasts at different stages of the vinification process (vineyard, spontaneous alcoholic fermentation, cellar equipment and bottled wines, see Fig. 1).

The vineyard trials, where gm yeasts were not only used as fertilizers, as vintners do in spring to ensure nitrogen supply to the vines, but were actually sprayed onto leaves and grapes to generate a “worst case scenario”, clearly

showed that over years and vintages, gm yeasts did not dominate the endogenous yeast flora. However, on the other hand, they also did not disappear completely but became part of the natural population. Investigations on the spread of commercial yeast strains around wineries and in vineyards yielded the same results (Pretorius et al. 1999; Schuller et al. 2005). Spontaneous fermentations with musts from grapes formerly infected with gm yeasts showed the participation of the gm yeasts in the alcoholic fermentation but also no dominance.

Simulating treatments of gm yeasts containing winery effluents revealed the same behaviour for these yeasts as for all other yeasts: reduction of cell counts but no complete elimination.

Although the tested gm yeast strains behaved more or less like other non-engineered wine yeasts, these findings should be addressed with caution and not taken as a general rule. Careful experiments have to be made with each gm strain that is to be marketed, especially when it comes to yeast strains that combine several enzymatic activities, like pectinase, glucanase and protease activities, in their genome (van Rensburg et al. 1997). Such combinations would be useful for wine makers and facilitate the wine making process but potential side effects on grape vines or other plant material must be taken into consideration. In order to avoid the introduction of new, possibly plant pathogenic organisms, precisely conducted case-by-case studies should become a prerequisite in worldwide legislation governing the usage and commercialisation of gm wine yeasts.

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