New research, new yeast, new selection tool

Jessica Berlese-Noble, Céline Raynal, Anthony Silvano, Daniel Granes, Caroline Bonnefond, Anne Ortiz-Julien and Bruno Blondin **report on the development of a new wine yeast combining low SO_2, H_2S and acetaldehyde production with high fermentative performance using a new selection tool.**

SULFITES are widely used in oenology. However, there is a tendency to reduce their use and control their final amount.

Apart from being added to musts or wines, sulfites can also be produced by wine yeasts in varying amounts during alcoholic fermentation, which can lead to a delay of the onset of malolactic fermentation if desired, and to a lack of control of the final concentration of sulfites.

Furthermore, sulfites are precursors for the synthesis of sulfide, a highly undesirable by-product.

Although wine yeasts differ widely in their capacity to produce these sulfur compounds, the molecular basis of such differences is not known.

We undertook a genetic study to identify the molecular basis of these properties.

Using a quantitative genetic approach, two loci impacting SO_2 and H_2S production were mapped.

Dissecting the quantitative trait loci (QTLs) led to identifying two alleles of the genes MET2 and SKP2 responsible for the differences in sulfur-compound production between two wine yeast strains.

A functional validation demonstrated their involvement and highlighted the strength and the extent of their control over the phenotypes, as well as SO_2 and H_2S production.

The transfer of those alleles through breeding offers great opportunities for further wine yeast improvement, and a new wine yeast has already been selected using molecular marker-assisted selection.

AT A GLANCE:

- Sulfites are precursors for the synthesis of sulfide, a highly undesirable by-product.
- Although wine yeasts differ widely in their capacity to produce these sulfur compounds, the molecular basis of such differences is not known.
- Demand for new wine yeast enhanced for specific technological properties has steadily increased in recent years.
- Understanding the molecular basis of the phenotypic diversity of wine yeasts is a first step towards breeding strategies for improving the technological properties of yeasts.

INTRODUCTION

The demand for new wine yeast enhanced for specific technological properties has steadily increased in recent years.

The growing knowledge and the development of new technologies offer new opportunities for developing wine yeasts.

Clonal selection, genetic engineering and evolutionary selection can be implemented, but hybridisation is the most common technique utilised for such improvements.

Furthermore, breeding does not require the intervention of genetic manipulation and results in natural, non-genetically modified (non-GM) yeasts.

However, this method requires knowledge of the molecular basis of the properties of interest. The technique of quantitative trait locus (QTL) mapping has recently been expanded for those applications.

This method consists of, for example, identifying a link between one or several regions of the genome and a phenotypic variation in a microorganism.

Once this region has been identified, it is then possible to direct its transfer from one yeast strain to another to combine several properties of interest.

In this study, we tried to identify the region of the genome responsible for numerous oenological characteristics, particularly sulfite production.

Although sulfites are widely used in oenology for their numerous technological properties (e.g., their antimicrobial, antioxidant and antioxydasic properties), the current trend is to reduce their use and control their final amount.

High levels of sulfites in wines can lead to a negative sensory impact, can induce a delay at the onset of malolactic fermentation (MLF) and can be responsible for health concerns. Sulfites are also likely to be produced by wine yeasts in significant amounts during alcoholic fermentation (AF). Wine yeasts are able to produce from a few mg/L of sulfites to more than 90 mg/L, depending on the fermentation conditions and the yeast strain.

Sulfur dioxide is an intermediate metabolite in the sulfate assimilation pathway, leading to sulfur amino acid synthesis.

Under certain conditions, it may be synthesised in excess then excreted into the medium. Furthermore, sulfites are precursors for the synthesis of sulfide, a highly undesirable by-product.

Although the sulfate assimilation pathway has been widely studied, little is known about the parameters that influence sulfite production, and the molecular basis responsible for the differences between yeast strains has not yet been identified.

One of the most relevant SO_2 binding compounds is acetaldehyde, which has very high binding properties and is usually found in high concentrations in wines. Acetaldehyde typically accounts for 75 per cent of the bound SO_2 in white wines and 50 per cent in red wines.

Its reactivity and ability to bind with sulfites explain to a large extent why wines need varying amounts of SO_2 and why sulfur dioxide management is important in winery operations and to post-bottling stability.

The ability to control the production of acetaldehyde by wine yeast is, therefore, an important parameter to consider with sulfite content in wines.

The purpose of this study was to develop new oenological yeasts combining several phenotypes of interest, such as low SO_2/H_2S/acetaldehyde production and high fermentative properties. The original approach was the use of new genetic tools, leading to non-GM yeast.

STRATEGY FOR YEAST IMPROVEMENT: BREEDING ASSISTED BY MOLECULAR MARKERS

Two wine yeasts presenting complementary properties of interest were selected for this study. The JN10 strain is very robust, able to complete the fermentation in such harsh conditions as low/high temperatures and highly clarified must, while the JN17 strain has low nitrogen requirements, presents a balanced volatile compound profile, and produces low amounts of SO_2, acetaldehyde and H2S.

QTL mapping was undertaken to identify the regions of the JN17 yeast strain genome linked to the phenotypes of interest: low SO_2 and low H_2S.

Through a combination of phenotypic and genotypic datasets, we succeeded in pointing out a region of the XIV chromosome that is linked to the sulfite production.

In this region, we actually identified two genes of the sulfur metabolism pathway, MET2 and SKP2.

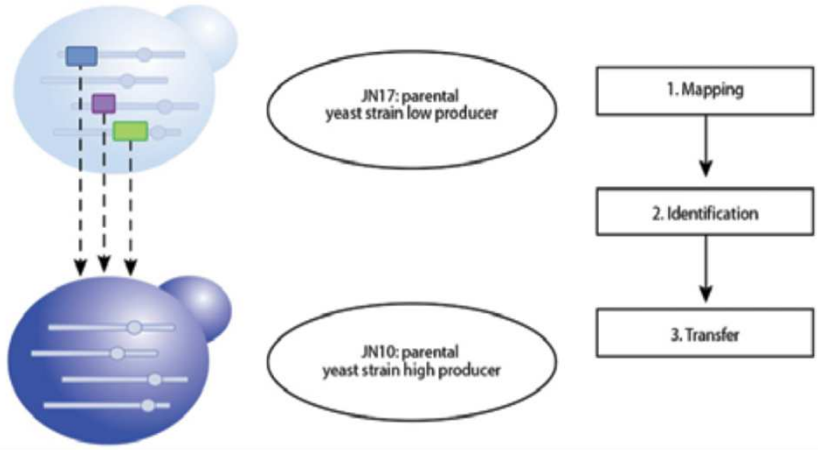


Figure 1. General strategy of directed breeding assisted by quantitative trait locus markers

Once the molecular markers linked to the properties of interest have been identified, it is possible to transfer them from one strain to the other through directed breeding.

In our study, we managed to transfer the low SO_2/H_2S property from the JN17 yeast strain to the JN10 yeast strain while retaining all the good oenological properties of the JN10 yeast strain. In parallel, we succeeded in transferring two other phenotypes of the JN17 parental strain (low acetaldehyde production and low nitrogen requirements).

Our intent was to achieve two main objectives:

- Transfer the phenotypes of interest from the JN17 yeast strain to the JN10 yeast strain by following the presence of molecular markers at each stage of hybridisation.
- Maintain the major part of the JN10 yeast strain genetic background by backcrossing each generated haploid hybrid with the JN10 parental strain.

Figure 2 explains the development of the new yeast strain through this backcrossing cycle strategy. After four cycles of breeding, more than 93 per cent of the JN10 yeast strain genome was restored, and we validated the transfer of all the targeted phenotypes coming from the JN17 yeast strain: low SO_2, low H2S, low acetaldehyde production and low nitrogen requirements.

From this fourth-generation hybrid, a new step of selection began in order to obtain the best strain combining the properties of this hybrid and satisfying such

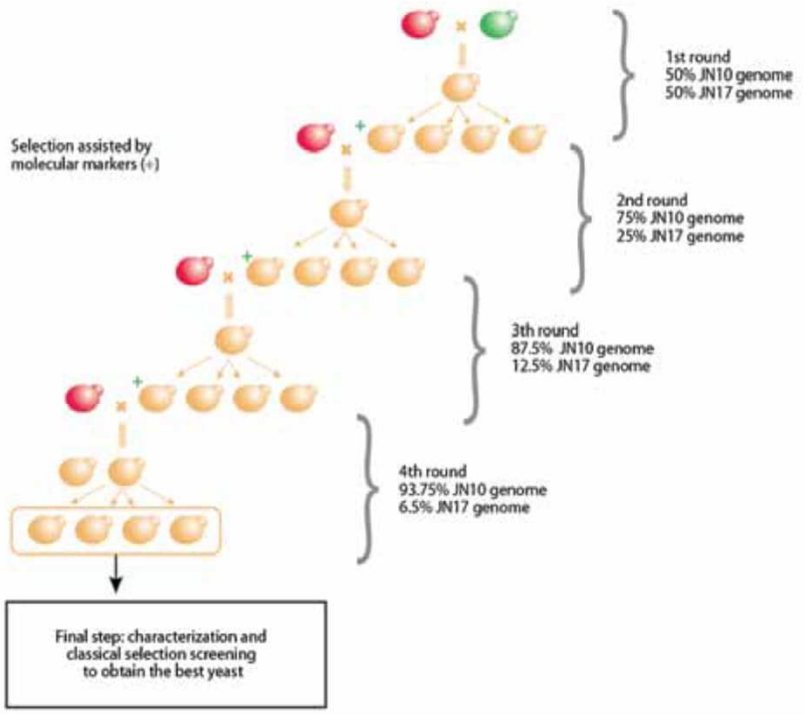


Figure 2. New yeast strain development through backcrossing cycles.

Anton Paar

Brix and Baume in Seconds

DMA 35

The **density meter DMA 35** provides unparalleled ease of use and complete transparency and traceability of the sample filling and measurement process.

- **Ideal tool for the Wine Industry:** Baumé, Brix, SG measurement, for juices and fermenting juices
- **User friendly:** Compact, lightweight design enabling one-hand measurement
- **Robust:** Rugged, leakproof housing design
- **Efficient:** Storage of up to 100 sample IDs for easy sample identification

The DMA 35 is an economic solution for general industrial and laboratory applications with constant or only occasionally changing measurement settings.

available at

MEP instruments
The right chemistry.

MEP Instruments Pty Ltd
Australia Tel 1300 720 485
New Zealand Tel 09 477 0620
www.mep.net.au/winelab

oenological behaviour as strong fermentative capacity, resistance to extreme temperatures, interesting sensory traits, etc.

LABORATORY CHARACTERISATION TO SELECT THE FINAL YEAST STRAIN

All strains issuing from the fourth cycle of backcrossing were thoroughly characterised at lab scale in different oenological conditions.

The goal was to validate the positive properties of the JN10 yeast strain remained, but also that generated yeasts showed an improvement in the phenotypes of interest.

One yeast (named Lalvin ICV oKay®) was selected and retained for the final characterisation steps.

The kinetics and the final wine analysis confirm the improved yeast obtained through breeding retained the positive traits of one of the parental yeast strains (JN10), including strong and reliable fermentation activity, with no off-flavour or volatile acidity issues.

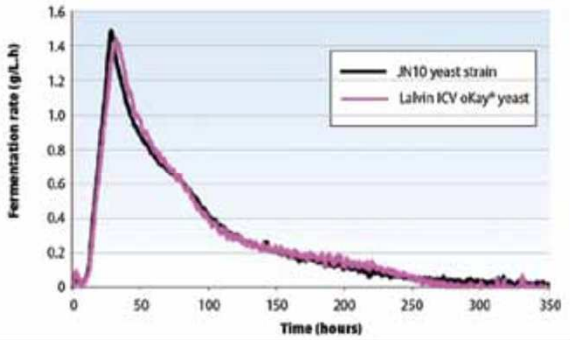


Figure 3. Fermentation kinetics performed at laboratory scale on a Chardonnay must fermented at 20°C in isothermal conditions.

PILOT-SCALE TRIALS WITH LALVIN ICV OKAY® YEAST

The trials were carried out at the INRA Experimental Unit of Pech-Rouge in France. The objective was to compare the Lalvin ICV oKay® yeast with a reference yeast.

Fermentations were performed on juices from three different grape varietals: Merlot, Syrah Rosé and Maccabeu.

The Merlot was obtained through the flash détente technique – the pressing of the treated grapes and racking under cold static conditions.

The Syrah Rosé and Maccabeu were pressed and racked after cold settling. The yeasts were rehydrated using Go-Ferm Protect® and inoculated at a rate of 25 g/hL.

When needed, nutrients were added in the form of organic and complex nutrients at the one-third point of AF.

The Merlot fermented at temperatures ranging from 22C to 25C. The Syrah Rosé and Maccabeu were fermented at 18C. MLF was carried out with Lalvin VP41® at 1 g/hL.

The fermentation kinetics were monitored as well as the SO_2, H2S and acetaldehyde production.

As shown in Figure 6, in each case the new yeast (Lalvin

	Total volatile acidity % vol	Residual sugar (g/L)	Volatile acidity H_2SO_4 g/L	Total acidity H_2SO_4 g/L
JN10 yeast strain	12.94	1.8	0.16	4.32
Lalvin ICV oKay® yeast	13.01	0.7	0.19	4.40

Table 1. Classic oenological parameters measured at the end of alcoholic fermentation (laboratory scale).

NOW IF YOU DON'T ANALYSE YOUR OWN WINE, YOU NEED TO BE ANALYSED

VINTESSENTIAL'S NEW SPECTROPHOTOMETERS.

You don't have to be a rocket scientist or have an astronomical budget to do your own wine analysis. With a new spectrophotometer, our award-winning wine test kits and our support, you can do all your essential testing in-house, saving time, money and frustration. Prices start from just $1600 + GST.

VINTESSENTIAL® LABORATORIES

THERE IS AN ART TO GOOD WINE. AND A SCIENCE.

Phone: 1300 30 2242 (Australia Wide)
Email: info@vintessential.com.au or visit vintessential.com.au

VIN_J000689

WINERY SANITATION chemical free

Gebhardt Turbo-Steamer

Ideal for chemical free sterilisation of oak barrels & cuves, stainless steel tanks, filters, bottling lines,...and more.

FEATURES

3 heat settings per unit.
Automatic error shut-off.
German made.

✓ USER FRIENDLY
✓ MOBILE
✓ MAINTENANCE FREE
✓ QUICK Generates instant and continuous steam in just 1-2 minutes

For further information, please contact Kauri

NZ Tel: 0800 KAURIWINE AUS Tel: 1800 127 611
NZ Fax: 04 910 7415 AUS Fax: 1800 127 609
Email: winery@kauri.co.nz Web: www.kauriwine.com

KAURI

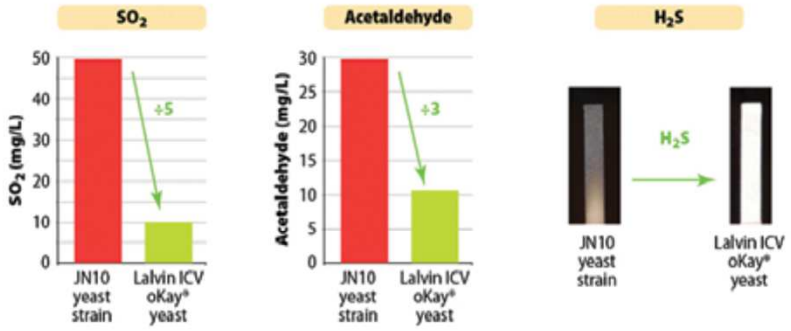


Figure 4. Validation of the transfer of phenotypes of interest coming from the JN17 yeast strain (laboratory scale).

	Merlot	Syrah Rosé	Maccabeu
Sugar (g/L)	254	190	211
Total acidity (H_2SO_4 g/L)	2.85	4.15	2.6
YAN (yeast assimilable nitrogen) (mg/L)	84	150	200
Initial free SO_2 (mg/L)	23	4	28
Initial total SO_2 (mg/L)	45	15	55
Turbidity (NTU)	110	60	60

Table 2. Must analysis of the three different varietals after sulfiting the juice.

ICV oKay®) performed better than the reference yeast. The lag phase was also shortened, showing good vitality and fermentation activity until the late stage of the fermentation, avoiding any risk of stuck or sluggish fermentation.

During AF, we measured the SO_2 in the wines and validated the non-production of SO_2 by the Lalvin ICV oKay® yeast compared to the reference yeast (figure 7).

SENSORY CHARACTERISATION

Four different comparative trials were carried out at the Institut Coopératif du Vin (ICV), France, experimental winery to get information on the sensory profile obtained from different grapes varieties and vinification strategies:

- A Merlot red must from the flash détente treatment followed by pressing and clarification through juice vacuum filtration.
- A Syrah rosé must from direct pressing with pectolytic enzymes and a 20-hour cold settling.
- A Grenache rosé must from a 4-hour skin maceration followed by pressing with pectolytic enzymes and a 20-hour cold settling.
- A Chardonnay must from direct pressing with pectolytic enzymes and a 20-hour cold settling.

Different yeasts from the ICV range were compared under the same AF conditions.

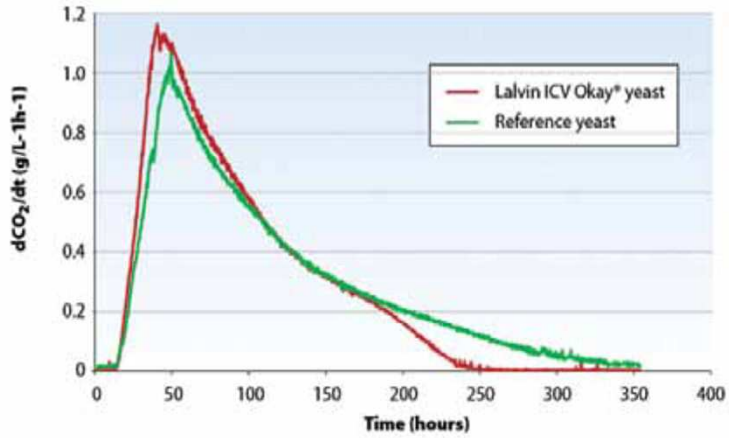
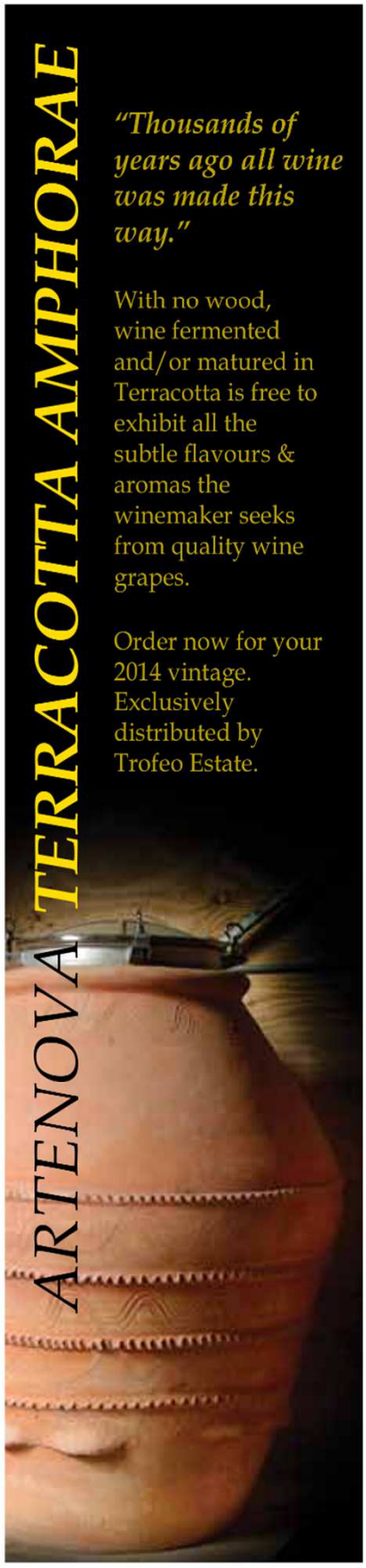


Figure 5. Example of fermentation kinetics on Maccabeu grapes comparing the Lalvin ICV oKay® yeast with the reference yeast.



TROFEO ESTATE

MORNINGTON PENINSULA DROMANA

Email ***info@trofeoestate.com*** *for more information*

www.trofeoestate.com

The yeasts were rehydrated using Go-Ferm Protect® and inoculated at a rate of 30 g/hL. When needed, nutrients were added in the form of organic and complex nutrients at the one-third point of AF.

The Merlot fermented at temperatures ranging from 18C to 22C. MLF was carried out with Lalvin Elios 1® at 1 g/hL inoculated after two rackings following the end of AF. The two rosés and the Chardonnay were fermented at 18C.

Kinetics were monitored and sensory characterisations were performed with the ICV quantitative descriptive sensory analysis (QDSA) method (Granès et al. 2010) and analysis of negative sulfur off-compounds (e.g., H2S, methanethiol and ethyl mercaptan) were done on wines, two months after bottling.

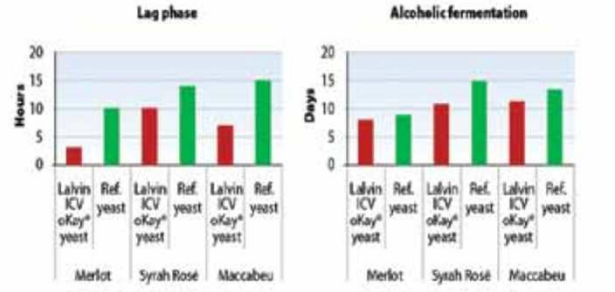


Figure 6. Key fermentation parameters in pilot trials comparing the Lalvin ICV oKay® yeast with the reference yeast.

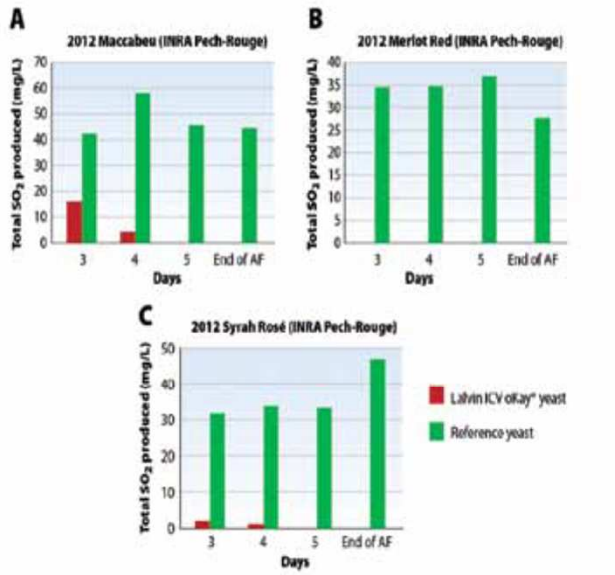


Figure 7. Total SO_2 production during alcoholic fermentation in three wines comparing Lalvin ICV oKay® yeast with the reference yeast.

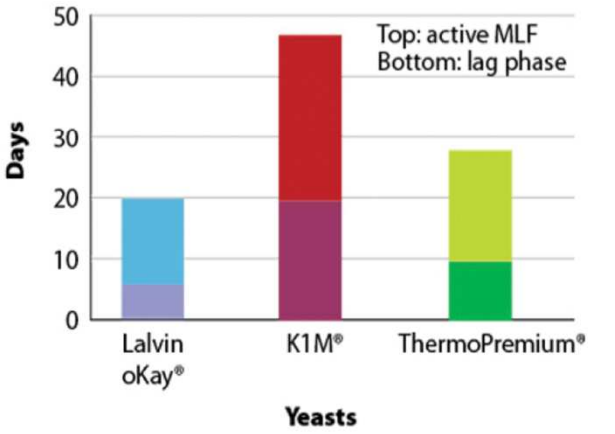


Figure 8. Duration of malolactic fermentation in a thermo-vinified Merlot

MALOLACTIC FERMENTATION

On the thermo-vinified Merlot, analysis of the malic acid concentration after inoculation with Lalvin Elios 1® was carried out three times per week and shows (figure 8) the interaction with the Lalvin ICV oKay® yeast was the most efficient.

5.2 Sensory profiles

All comparative trials showed a tendency for Lalvin ICV oKay® to:

- Produce fewer sulfur off-flavours.
- Show more aromatic complexity with a blend of amylic and fruity sensations.
- Have a positive balance between fore-mouth volume and acidity.
- Reduce bitterness.

Figures 9 and 10 illustrate the results of trials on the sensory profiles of Chardonnay and Grenache grapes.

ANALYSIS OF SULFUR OFF-COMPOUNDS

The sulfur off-compounds H2S, methanethiol and ethyl mercaptan were measured two months after bottling.

Taking into account the yeasts that had fermented, the grape variety and the must analysis, the statistical analysis shows the main factor impacting the levels of these molecules was the grape variety.

Nevertheless, Lalvin ICV oKay® yeast was found to be significantly different from all the others and produces fewer of the sulfur off-compounds analysed.

Then taking into account the threshold of these molecules, we converted their concentration into "odour units." Figure 11 illustrates some of the results obtained.

CONCLUSION

Understanding the molecular basis of the phenotypic diversity of wine yeasts is a first step towards the utilisation of breeding strategies for improving the technological properties of yeasts.

In this study, we were able to identify the molecular basis responsible for the phenotypic variation in the production of sulfites in wine yeasts.

A global approach, combining a physiological study, a transcriptomic analysis and QTL research was implemented. The study shows the combination of two genes, MET2 and SKP2, is responsible for the phenotypic variation observed between the two parental strains.

Furthermore, it has been demonstrated these genes are also involved in the phenotypic change of other characters of

	Chardonnay	Merlot	Syrah Rosé	Grenache Rosé
Sugar g/L	247.1	233.7	248.9	221.3
% Potential degree	14.56	13.89	14.67	13.03
Total acidity (H_2SO_4 g/L)	3.83	4.28	2.88	2.96
Volatile acidity (H_2SO_4 g/L)	0.07	0.07	0.08	0.08
Free SO_2 (mg/L)	< 10	< 10	< 10	< 10
Total SO_2 (mg/L)	< 15	< 15	< 15	< 15
pH	3.45	3.71	3.50	3.45
Malic acid (g/L)	2.77	1.48	2.8	1.28
Lactic acid (g/L)	< 0.1	< 0.1	0	-0.05
Tartaric acid (g/L)	4.79	3.75	2.63	5.09
DO280	-	94	-	-
Potassium (g/L)	1.9	2.5	1.13	1.36
Yeast-available nitrogen (YAN) (mg/L)	250	200	224	160
Turbidity (NTU)	60	< 50	67	62

Table 3. Must analysis just before yeast inoculation.

technological interest, such as the production of SO_2, H2S and acetaldehyde.

So far, genetic studies have focused on controlling the production of H2S, but the proposed solutions (i.e., reduced sulfite reductase activity) had the disadvantage of greatly increasing the production of SO_2 (Cordente et al. 2009, and Linderholm et al. 2008).

Subsequently with the QTL method, many other wine yeasts could be improved following the same procedure, as a significant number of oenological yeasts currently on the market have one or more of these defects.

Now, the Lalvin ICV oKay® yeast obtained with this method has demonstrated remarkable qualities.

All the trials with Lalvin ICV oKay® yeast conducted worldwide over the past two years have shown its production of SO_2, H2S and acetaldehyde is almost null, and the resulting wines can express their full aromatic potential while respecting the market demand for wines with low SO_2 content.

Contact: Jason Amos, Lallemand.
Phone: 61 8 8276 1200.
Email: jamos@lallemand.com.

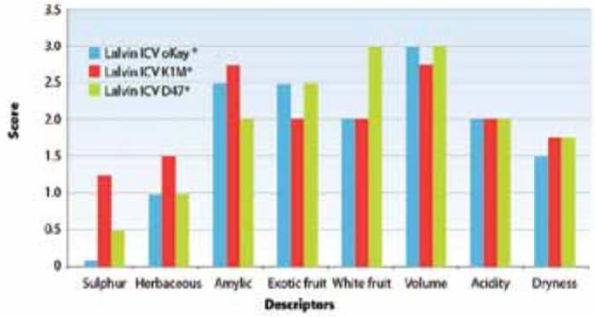


Figure 9. Sensory profile for 2012 Chardonnay fermented with three different yeasts (ICV R&D).

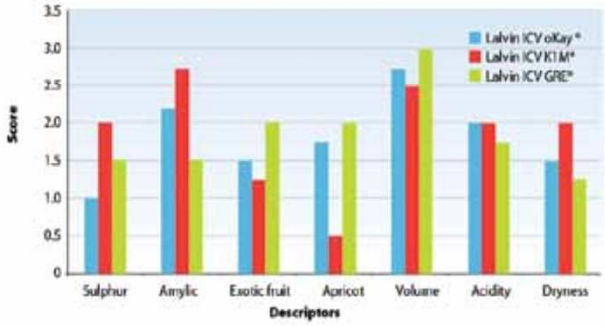


Figure 10. Sensory profile for 2012 Grenache Noir Rosé fermented with three different yeasts (ICV R&D).

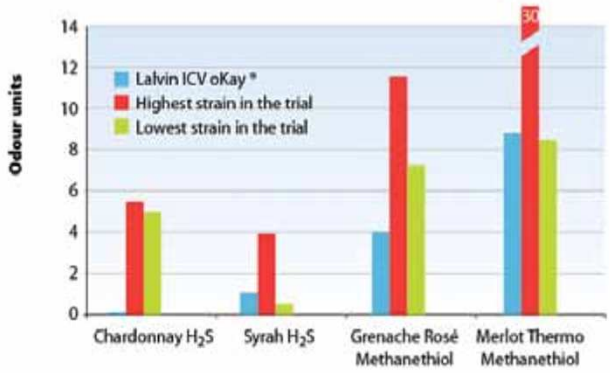


Figure 11. Comparison of dosages of sulfur off-compounds in trials of four different grapes (2012 ICV R&D).

Authors:

Jessica Berlese-Noble, Lallemand SAS, 19, rue des Briquetiers, 31702 Blagnac, France.

Céline Raynal, Lallemand SAS, 19, rue des Briquetiers, 31702 Blagnac, France.

Anthony Silvano, Lallemand SAS, 19, rue des Briquetiers, 31702 Blagnac, France.

Daniel Granes, Institut Coopératif du Vin, La Jasse de Maurin, 34970 Lattes, France.

Caroline Bonnefond, Institut Coopératif du Vin, La Jasse de Maurin, 34970 Lattes, France.

Anne Ortiz-Julien, Lallemand SAS, 19, rue des Briquetiers, 31702 Blagnac, France.

Bruno Blondin, Montpellier SupAgro, 2, Place Viala, 34060 Montpellier, France

References and Bibliography:

Ambroset, C., M. Petit, C. Brion, I. Sanchez, P. Delobel, C. Guérin, H. Chiapello, P. Nicolas, F. Bigey,

S. Dequin, and B. Blondin. 2011. Deciphering the Molecular Basis of Wine Yeast Fermentation Traits Using a

Combined Genetic and Genomic Approach. G3: Genes|Genomes|Genetics. 1(4):263-281.

Bely, M., J. M. Sablayrolles, and P. Barre. 1990. Description of Alcoholic Fermentation Kinetics: Its Variability

and Significance. Am. J. Enol. Vitic. 41(4):319-324.

Casalone, E., C. M. Colella, S. Daly, E. Gallori, L. Moriani, and M. Polsinelli. 1992. Mechanism of resistance to

sulfite in Saccharomyces cerevisiae. Current Genetics. 22(6): 435-440.

Cordente, A. G., A. Heinrich, I. S. Pretorius, and J. H. Swiegers. 2009. Isolation of sulfite reductase variants of a commercial wine yeast with significantly reduced hydrogen sulfide production. FEMS Yeast Research. 9(3):446-459.

Deutschbauer, A. M.,and R. W. Davis. 2005. Quantitative trait loci mapped to single-nucleotide resolution in

yeast. Nat Genet. 37(12):1333-1340.

Donalies, U. E., and U. Stahl. 2002. Increasing sulfite formation in Saccharomyces cerevisiae by overexpression of MET14 and SSU1. Yeast. 19(6):475-484.

Eschenbruch, R. 1974. Sulfite and Sulfide Formation during Winemaking – A Review. Am. J. Enol. Vitic.

25(3):157-161.

Eschenbruch, R., and P. Bonish. 1976. The influence of pH on sulfite formation by yeasts. Archives of Microbiology. 107(2):229-231.

Granès, D., L. Pic-Blayteron, J. Negrel, and C. Bonnefond. 2010. Method for a Common Language, Quantitative Descriptive Sensory Analysis. Practical Winery and Vineyard Journal. Sept./Oct. 2010.

Hansen, J., and M. C. Kielland-Brandt. 1996. Inactivation of MET2 in brewer's yeast increases the level of sulfite in beer. Journal of Biotechnology. 50(1):75-87.

Linderholm, A. L., C. L. Findleton, G. Kumar, Y. Hong, and L. F. Bisson. 2008. Identification of Genes Affecting Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Appl. Environ. Microbiol. 74(5):1418-1427.

Marullo, P., M. Aigle, M. Bely, I. Masneuf-Pomarede, P. Durrens, D. Dubourdieu, and G. Yvert. 2007. Single

QTL mapping and nucleotide-level resolution of a physiologic trait in wine Saccharomyces cerevisiae strains.

FEMS Yeast Research. 7(6):941-952.

Ribéreau-Gayon, P., D. Dubourdieu, and A. Lonvaud. 1998. L'emploi du dioxyde de soufre dans le traitement

des moûts et des vins. Traité d'oenologie. Dunod/Lavigne.

Rossignol, T., L. Dulau, A. Julien, and B. Blondin. 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast. 20(16):1369-1385.

Thomas, D., and Y. Surdin-Kerjan. 1997. Metabolism of sulfur amino acids in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 61(4):503-532.

Thornton, R. J. 1985. The Introduction of Flocculation into a Homothallic Wine Yeast. A Practical Example of the Modification of Winemaking Properties by the Use of Genetic Techniques. Am. J. Enol. Vitic. 36(1):47-49.

Wainwright, T. 1970. Hydrogen sulfide production by yeast under conditions of methionine, pantothenate or vitamin B6 deficiency. Journal of General Microbiology. 61(1):107-119.

Yoshida, S., J. Imoto, T. Minato, R. Oouchi, Y. Kamada, M. Tomita, T. Soga, and H. Yoshimoto. 2011. A novel

mechanism regulates H_2S and SO_2 production in Saccharomyces cerevisiae. Yeast. 28(2):109-121.