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|---|---|
| Title | Stress resistance during the lag phase of wine fermentation and development of optimized yeasts |
| Authors | Ferreira, David |
| Publication date | 2017 |
| Original Citation | Ferreira, D. 2017. Stress resistance during the lag phase of wine fermentation and development of optimized yeasts. PhD Thesis, University College Cork. |
| Type of publication | Doctoral thesis |
| Rights | © 2017, David Ferreira. - http://creativecommons.org/licenses/by-nc-nd/3.0/ |
| Download date | 2026-11-15 03:37:42 |
| Item downloaded from | https://hdl.handle.net/10468/6786 |



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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE MONTPELLIER SUPAGRO

**En Sciences pour l'Ingénieur – Biotechnologie et Microbiologie**

**École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau**
**Portée par l'Université de Montpellier**

**Unité mixte de recherche Sciences pour l'œnologie**

**En cotutelle avec University College Cork – Microbiology Department**

# STRESS RESISTANCE DURING THE LAG PHASE OF WINE FERMENTATION AND DEVELOPMENT OF OPTIMIZED YEASTS

**Présentée par David FERREIRA**
**Le 18 Décembre 2017**

**Sous la direction de Sylvie DEQUIN et John MORRISSEY**

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# JOINT DOCTORAL AGREEMENT

In the scope of the European Commission Framework 7 – funded Marie-Curie Initial Training Network YEASTCELL, EU grant agreement number 606795, the present thesis was performed under a joint doctoral agreement (JDA) between the Institut National D'études Supérieurs Agronomiques de Montpellier (Montpellier SupAgro) and University College Cork (UCC), Ireland.

David Ferreira spent a total of 3 years at Montpellier SupAgro under the supervision of Dr Sylvie Dequin and a total of 6 months at UCC under the supervision of Dr John Morrissey.

Under the terms of the JDA, both (Montpellier SupAgro) and UCC will award a Doctoral Degree (PhD Diploma) for this thesis. This is a "Double Doctorate" and each Diploma will attest to the fact that this is a joint award for a single thesis.

# ACKNOWLEDGEMENTS

This thesis was conducted at UMR 1083 Sciences pour l'oenologie at INRA of Montpellier under the scope of the Marie Curie Initial Training Network YEASTCELL project. It was performed in direct collaboration with the company Lallemand SAS and the University College Cork (UCC). This way I thank UMR-SPO (in particular Jean-Marie Sablayrolles), Lallemand SAS and UCC for giving me the opportunity to conduct this thesis and providing all the support and conditions required for its success.

I would like to thank José M. Guillamón and Benoit Divol, examiners of this thesis, for showing interest in our work and accept evaluating it. In the same way, I thank all the members of the jury for their contribution and presence in my thesis defense.

Embarking in the journey that is doing a PhD was one of the most rewarding and valuable experiences I have done. Professionally and personally. While pursuing it I was able to acquire unique knowledge, technical skills and scientific thinking that make me today a better scientist. Throughout this adventure, I encountered many different people that made this experience as rich as humanly gratifying, leaving, in different ways, their mark in this thesis.

To Sylvie Dequin, my thesis director, thank you for your excellent guidance, scientific rigor, wise advices, the long discussions, for always pushing me to go one step further and aim for the highest level, for your honesty, kindness and comprehension throughout these 3.5 years. It was a true pleasure and honor to work with one of the best in the field.

To Virginie Galeote, my co-supervisor, thank you for your close mentoring and always precious advices, for passing me your bench knowledge and experience, for standing up for me and our goals even when things did not look good, for your patience and understanding during this thesis. I take with me all your teachings.

To Anne Ortiz-Julien, from Lallemand SAS, thank you for trusting me this mission, for your constant support and help in our decisions, for your humanity, generosity and in

particular for your enthusiasm and positiveness. Every meeting and talk we had were a boost of motivation.

To John Morrissey, my thesis co-director, thank you for your support throughout this thesis, your always pertinent opinions and scientific input, for your kindness and generosity as well as for providing the opportunity to pursue my thesis at UCC while having me in your laboratory. As a coordinator of YEASTCELL, thank you also for the excellent and incessant work in managing this project, organizing our fruitful meetings and activities, making sure we all had the right conditions to achieve our goals and mostly, for making our network a space of trust, productivity and good conviviality. The (much) success of YEASTCELL has undoubtedly your mark.

To all, I particularly thank you for your efforts, availability and perseverance in the final stretch of this thesis that contributed to get the most of our work. I am truly grateful.

I specially thank Jean-Luc Legras for his valuable scientific input, the extensive discussions, his patience, readiness and kindness in always helping us with our strategies. To Isabelle Sanchez, thank you for your support in the statistical part, plans design and availability in answering my questions. To Thibault Nidelet, I thank him for his precious help, particularly in showing me that R is our friend and that it can be that easy. To Frédéric Bigey, I also thank him for his availability and help in analyzing our ALE genomic data. To Sandrine Mallet, thank you for your support in our work towards the end, allowing us to move forward at a faster pace. Thank you Catherine Tesnière for your help in getting in touch with the MRI. To Stéphane Guezenec thank you for taking our strains out of the collection at the "last moment". To Christophe Duperray, thank you for your availability and professionalism in doing our cell sorting.

I thank all UMR-SPO, and in particular the microbiology team, for receiving and integrating me so well in the group, for making that workplace so pleasant to share and for the assistance in moments of need. Not less important I thank all of you for the moments of joy, conviviality and good spirit always present in the coffee breaks, evenings at the beach, Christmas lunches and marathons. Thank you Christian Picou for your help in "robot critical moments" (hope we can soon go for that bike ride), Marc Perez for the help in robot data treatment (we still have to do that Portuguese duet), Erick Casalta for his incredible energy (thank you for being Corsican), to Faiza Macna and Pascale Brial

for their outstanding good mood (I am really expecting those feathers), to my office colleagues, Amandine Deroite and Camille Duc for the comradeship, the rehearsals, Koh-lanta nights and the good moments (ok, after we eat that poutine, I will leave you "la baballe"). To all the others, Martine Pradal, Thèrése, Audrey Bloom, Carole Camarasa, Bruno Blondin, Peggy Rigou, Jessica Noble, Jean-Roch Mouret (Portuguese will never be cold and we know why), Vincent Farines, Delphine Sicard, Nicolas de Royer, Stéphanie Rollero, Thomas Ochando, Pauline Seguinot and Alexandre Chamas, thank you for showing such good hospitality and making our team so enjoyable to work with.

To Julie, Lucas, Stéphanie Carrillo, Laurent thank you for the good moments of conviviality. To Stéphanie Roi thank you for your support throughout most of this thesis.

I would like to thank the Lallemand Inc. team at Montreal, Canada, for receiving and integrating me so well, making my first professional and personal experience on the other side of the Atlantic so pleasant. In particular, thank you Caroline Wilde for making sure everything went smoothly as well as for your scientific input. Also to Jeyadarshan Jeyabalaratnam thank you for the good spirit and teaching me how to handle those fermenters (that Sri Lanka food is still the spiciest I ever ate). To the Lallemand SAS team at Blagnac, France, thank you for making me feel "at home" from the first moment, it was a true pleasure to work among you and to participate in the gathering Fridays. A special thanks to Céline Raynal and Benjamin Leroux for their guidance, efforts and kindness in helping me with our experiments.

I also thank my dear colleagues and friends from Lab 441 at the UCC. Javier Varela (qué pasa tío?) Noemi Montini (your whale watching style is now the best), Valentina Sforza (so so much for your help), José Madeira Junior (yeah yeah é nois) and Fabrizia Tittarelli thank you for the excellent atmosphere, comradeship, laughs, "random Tuesdays" and good mood that made Cork such a nice experience.

I thank all the YEASTCELL members, from ESRs to PIs to collaborators, for their scientific contributions, networking, advices, constructive criticism as well as for the relaxing moments of good conviviality. You all made this PhD experience much more

rich, thrilling and profitable. A special thanks to Francesca Doonan and Lucy Taylor for their constant support and readiness in making sure everything went well with the project.

Coming to Montpellier was such a good experience in part due to great and close friends I made there. I thank my good friend Matthias Eder (sweeet), with whom I shared this adventure since day one, for always being there, ready and humorous. May those gaming nights prevail across time. To Anna Vallverdú-Queralt, Hervé Romary, Guillaume Billerach, Chloé Béssiere, Clément Agret, Souhir Marsit, Meriem Essoussi, Noemie LaFeuille and Stéphanie David and "the gang" thank you for all the good moments, the randonnées, the dinners at home, the nights out, the laughs, the serious and not so serious discussions, the corrections on my French, the coffees and mostly your complicity, it has been more than a pleasure.

To my Portuguese friends who even two countries (or more) away managed to, as always, be present and show their immense support, a variable independent of time and distance. To Unas (por motivos), Mafaldinha (a Doris de sempre), Ani (no fim mas contou tanto), Luís, Maria, Adriano, Anabela, Joana, Cláudia, Ba e Frederico (oh Márcio), thank you for your constant encouragements, even with "once a year" reunions. You are gold.

To Jacqueline, my trustworthy bicycle for never letting me down under the sun, rain and wind.

To my family, my safe harbor in the ups and downs of this life, for always being there and cheering for me in one more adventure. In particular to my parents for their unconditional support, for sharing my dreams and incentivate me to chase them with joy and confidence no matter where that takes us.

My sincere,

Obrigado

“The good life, as I conceive it, is a happy life.
I do not mean that if you are good you will be happy.
I mean that if you are happy you will be good.”

Bertrand Russell

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# LIST OF ABBREVIATIONS

## General abbreviations

| | |
|---|---|
| ADY | Active dry yeast |
| ALE | Adaptive laboratory evolution |
| ATP | Adenosine triphosphate |
| CFU | Colony-forming unit |
| CNV | Copy Number Variation |
| DNA | Deoxyribonucleic acid |
| DW | Dry weight |
| ESR | Environmental stress response |
| FACS | Fluorescence-activated cell sorting |
| GCMS | Gas chromatography mass spectrometry |
| HGT | Horizontal gene transfer |
| HPLC | High Performance Liquid Chromatography |
| INDEL | Insertion/deletion of base pairs in the genome |
| LOD | Logarithm of odds |
| mRNA | Messenger RNA |
| PCA | Principal component analysis |
| PCR | Polymerase Chain Reaction |
| PDC | Pyruvate decarboxylase |
| PDH | Pyruvate dehydrogenase |
| QTL | Quantitative Trait Locus |
| RNA | Ribonucleic acid |
| SM | Synthetic must |
| SNP | Single Nucleotide Polymorphisme |
| $V_{max}$ | Maximal $CO_2$ release rate |
| x-QTL | Extreme QTL |
| YAN | Yeast assimilable nitrogen |
| YPD | Yeast Extract, Peptone, Dextrose |

**Molecules and compounds**

| | |
|---|---|
| $CO_2$ | Carbon dioxide |
| EDTA | Ethylenediaminetetraacetic acid |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2S$ | Hydrogen sulfide |
| KCl | Potassium chloride |
| NaCl | Sodium chloride |
| $SO_2$ | Sulfur dioxide |

**Measurement units**

| | |
|---|---|
| % | Percentage |
| (v/v) | Volume/volume |
| °C | Degrees Celsius |
| bp, kbp | Base pair, kilo base pair |
| g, mg, µg | Gram, milligram, microgram |
| h, min, s | Hour, minute, second |
| L, mL, µL | Liter, mililiter, microliter |
| M, mM, µM | Molar, milimolar, micromolar |
| mol, mmol, µmol | Mole, millimole, micromole |
| nm | Nanometer |
| O.D. | Optical density |
| p-value | Probability of the null hypothesis being true |
| rpm | revolutions per minute |

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# COMMUNICATIONS ASSOCIATED TO THIS WORK

**Peer reviewed publications:**

Ferreira D., Galeote V., Sanchez I. *et al*. Yeast multistress resistance and lag-phase characterisation during wine fermentation. *FEMS Yeast Res* 2017 ; **17**, DOI : 10.1093/femsyr/fox051.

**Oral presentations:**

- Ferreira D., Galeote V., Sanchez I., Legras JL., Ortiz-Julien A., Dequin S.: Stress resistance during wine fermentation and development of optimized wine yeasts at 33rd International Specialized Symposium on Yeasts, Cork, Ireland, 26th – 29th June 2017.

- Ferreira D., Galeote V., Sanchez I., Legras JL., Ortiz-Julien A., Dequin S.: Stress resistance during wine fermentation and development of optimized wine yeasts at Journée de l'École Doctorale GAIA, filière APAB, Montpellier, France, 14th June 2016.

- Ferreira D., Galeote V., Sanchez I., Legras JL., Ortiz-Julien A., Dequin S.: Stress resistance during wine fermentation and development of optimized wine yeast at YEASTCELL Summer School 2016, 2015 and 2014 editions.

- Ferreira D., Galeote V., Sanchez I., Legras JL., Ortiz-Julien A., Dequin S.: Stress resistance during wine fermentation and development of optimized wine yeast **(**elevator pitch and full presentation) at 31st International Specialized Symposium on Yeasts, Vipava, Slovenia, 9th – 12th October, 2014.

**Poster presentations:**

- Ferreira D., Magalhães F., Eder M.: The YEASTCELL project (in the scope of YEASTCELL's outreach program) at European's Researchers Night, Helsinki, Finland, 30th September 2016.

- Ferreira D., Galeote V., Ortiz-Julien A., Dequin S.: Yeast multi-stress resistance and lag phase duration in wine fermentation at 6th Conference on Physiology of Yeast and Filamentous Fungi, Lisbon, Portugal, 11th – 14th July 2016.

- Ferreira D., Galeote V., Ortiz-Julien A., Dequin S.: Yeast multi-stress resistance and lag phase duration in wine fermentation at 32nd International Specialized Symposium on Yeasts, Perugia, Italy, 13th – 17th July 2015.

- Ferreira D., Galeote V., Ortiz-Julien A., Dequin S.: Yeast multi-stress resistance and lag phase duration in wine fermentation at Journée de l'École Doctorale GAIA, filière APAB, Montpellier, France, 19th June 2015.

**Awards**

**2017 -** Best oral presentation prize at the congress 33rd International Specialized Symposium on Yeasts. Cork.

**2016** - Best oral presentation prize at Journée de l'École Doctorale GAIA, filière APAB, Montpellier.

**2015 -** 1st prize for best beer ("Dom Beerignon") at International Campus Perle Brewing Competition (in the scope of YEASTCELL outreach program), Hamburg.

**2014 -** Best elevator pitch at the congress 31st International Specialized Symposium on Yeasts, Vipava.

# GENERAL INTRODUCTION

Wine is today one the most influent beverages in our society. Going from its presence in many cultures to its economic role worldwide, its importance can be easily perceived. Naturally, since the first fermentations thousands of years ago, the goal has always been to obtain the best wines while having the most efficient fermentative process. Many biotechnological advances have been achieved in that sense. Today, from the grape harvest to the opening of a wine bottle, the winemaking process has been greatly deconstructed and is mastered at many different levels. However, winemaking is yet to be fully controlled and perfected. Different steps are critical and decisive in the success of a wine fermentation and demand the highest attention. This is the case for grape must inoculation. Whereby selected wine yeasts are deliberately inoculated into the must. This widespread practice guarantees a higher confidence in the final product quality and properties as well as an increased reliability in the fermentative process. The critical point comes with the fact that this is a harsh process for wine yeast.

Commonly, selected wine yeasts are previously propagated at industrial scale and dried in order to extrude most of their water content, stop their metabolic activity and extend their shelf-life. Despite being globally positive, the desiccation process comes at a (stressful) price that forces yeasts to undergo major cellular and metabolic rearrangements. After proper rehydration, the stressful scenario continues with the must inoculation. Far from being the optimal medium for yeast growth, different stress factors are already present when yeasts get in contact with the must. Osmotic stress appears as an inescapable stress factor. The sugars accumulated by the grapes during ripening are present at high concentrations (180 g/L – 260 g/L) in the must and will cause major need for quick osmo-adaptation. The undergoing changes in climatic conditions (more sunlight/year) cause these concentrations to rise even more, thereby resulting in higher osmotic stresses. $SO_2$ appears as another stress factor. For centuries, this compound has been used in winemaking due to its antiseptic and antioxidant properties. Its presence and impact have induced modifications in the yeast genomic structure by positively selecting a particular translocations that confers a fitness advantage when in contact with $SO_2$. Low vitamins also constitute another source of stress particularly in the case of thiamine. The presence of natural flora leads to a quick depletion of the vitamins naturally present in the must, some of them fundamental for growth of the selected wine yeast. Without them, yeast growth and fermentation capacity is often suboptimal. In the particular case of white wine fermentations, the lower temperatures (10ºC – 20ºC) at which these are performed

also represent a major challenge to yeast that is far from its 28ºC optimal growth temperature. Ultimately, this also demands major physiological changes and adaptation from the yeast that delays growth. Finally, low lipid content can also be a source of stress. White wines are typically clarified which entails the removal of some important molecules such as lipids. Their lower concentration can compromise yeast stress response and membrane maintenance as well as fermentative performance.

Overall, this represents a stressful scenario at the moment of inoculation that pushes yeast's resilience to the limit. If generally yeasts are still capable of bypassing such adversities and performing well, often this is not the case. The main consequence of such stress factors can be the extension of yeast's lag phase. Lag phase can be defined as the period where yeast is adapting its metabolism to a new environment before initiating growth. The presence of such stress factors in exacerbated levels provoke the need for extended periods of adaptation by yeast. As a consequence, fermentation can become sluggish or stuck if yeasts cannot properly adapt, begin fermentation and reach its maximum performance. Simultaneously, the natural microbiome present in the must has a chance to develop and to confer unwanted properties in the final wine. Ultimately, this can lead to wine spoilage, extra costs and be time consuming.

When searching for a solution for such problems it becomes clear that the stress factors present cannot be eliminated. Each one of them is intrinsic to the fermentation itself or required for developing a proper fermentation process. Although controllable to some extent, their presence will always have potential to cause the problems described above. An alternative is then to better understand and improve yeast's behavior at this particular moment and obtain a more reliable control of fermentation course. In different occasions yeast has proven to be part of the solution to complex problematics, particularly in winemaking context, such as lower ethanol production, improved aroma production or reduction in off-flavor release.

In line with this philosophy this thesis is as a study developed in the sense of better understanding the factors modulating yeast lag phase in oenological conditions, including yeast strain diversity, while trying to provide solutions that can be applied on the field. For that purpose, this thesis was divided into 3 main parts and a complementary study.

In a first part, we wanted to have a better overview of wine yeasts' lag phase diversity. To this day, very few studies have been conducted to specifically characterize lag phase response variety, particularly among wine yeasts. To achieve this objective, we selected a diverse set of commercial wine yeast strains that covered different species, strains and fermentative profiles. We then designed a factorial plan that allowed to not only to characterize their lag phase but also determine in a multi-stress context what would be the impact of each stress factor present in lag phase.

In the second part of this thesis, we became interested in improving the lag phase of commercial wine yeast strains. A major constraint is the lack of knowledge on the molecular determinants of lag phase duration. In the last decade, evolutionary engineering emerged as a good approach, which allows to improve a specific phenotype without the prior need of in-depth understanding. Different evolutionary approaches in the winemaking context using yeasts have been successful and proven their value such as in improving yeast's aromatic profile or reducing ethanol production. Aside from the applied side of obtaining a marketable wine yeast strain, we also became interested in identifying the genetic modifications at the basis of the evolved phenotype, with the aim to generate more knowledge about lag phase.

The third chapter consists on the use of a unique QTL approach specifically designed to study lag phase. The phenotype shows an elevated degree of complexity that is impossible to study with classical approaches (*e.g.* breeding). Highly diverse yeast recombinant populations were generated to select the fastest budding cells, and bulk sequencing analysis was used to unveil important alleles connected to lag phase modulation. This approach allowed to obtain unique genetic and molecular information otherwise virtually impossible to obtain. Additionally, a valorization of such knowledge can be obtained by introducing positive alleles, by non Genetically Modified Organisms means, in commercial wine yeast strains.

A fourth chapter ends this thesis. In the scope of YEASTCELL's secondments, at UCC, Cork, we focused not specifically on lag phase but rather on the overall potential of *Kluyveromyces marxianus* as non-conventional yeast in oenological conditions. *K. marxianus* performance and organoleptic contribution have been so far poorly

characterized which led us to test its benefits when sequential and co-inoculation with *S. cerevisiae.*

# RÉSUMÉ FRANÇAIS

L'histoire du vin remonte à 7000 ans av. J.-C., époque à laquelle il a commencé à être produit dans les régions du Caucase et de la Mésopotamie. Au cours des siècles, les pratiques de vinification se sont progressivement répandues à travers le monde, et sont aujourd'hui inscrites dans presque toutes les civilisations et cultures. L'influence et l'impact économique du vin ont atteint une importance considérable dans le monde entier, ce qui a stimulé la recherche continue d'amélioration et d'innovation dans ce domaine. Ces avancées ont conduit à une maîtrise accrue des procédés de vinification, à une plus grande diversité et à une meilleure qualité des produits.

Le processus de vinification correspond à la transformation anaérobie des sucres (principalement le glucose et le fructose) en éthanol, dioxyde de carbone ($CO_2$) et de nombreux sous-produits. La levure *Saccharomyces cerevisiae* est le principal agent de la fermentation œnologique. Au fil des années, l'utilisation inconsciente par l'homme de *S. cerevisiae* dans le processus de vinification depuis des millénaires a abouti à la sélection de souches adaptées a ce processus. Dans les années 70-80, des travaux de sélection et d'amélioration des souches ont émergé, et la recherche de souches supérieures, améliorées pour différents traits d'intérêt œnologique se poursuit aujourd'hui. Depuis la vendange jusqu'à l'ouverture d'une bouteille de vin, un travail complexe doit être effectué impliquant de nombreuses étapes. La possibilité, de nos jours, de déguster les meilleurs vins jamais produits est sans doute dû à l'alliance des savoirs traditionnels et scientifiques réunis au fil des siècles de vinification.

Une problématique actuelle d'intérêt en œnologie est le moment de l'inoculation du moût de raisin et du début de la fermentation. En effet, les pratiques de vinification les plus répandues à ce jour consistent à inoculer dans le moût de raisin une levure sélectionnée. Cela garantit une fiabilité accrue dans le processus de fermentation et une meilleure qualité du produit final. Les levures de vin sélectionnées sont préalablement propagées à l'échelle industrielle et séchées afin d'extruder la plus grande partie de leur teneur en eau, de minimiser leur activité métabolique et ainsi augmenter leur durée de conservation. Le processus de dessiccation est une étape stressante pour la levure, qui l'oblige à réaliser des ajustements cellulaires et métaboliques majeurs. Par la suite, même si les levures sèches actives (LSA) sont correctement réhydratées, elles sont encore confrontées à des difficultés considérables lorsqu'elles sont inoculées. En effet, différents facteurs de stress sont présents lorsque la levure entre en contact avec le moût de raisin.

Le stress osmotique est un facteur de stress important, du fait de la présence de sucres, qui ont été accumulés par les raisins pendant la maturation, à des concentrations élevées (180-280 g/L) dans le moût. De plus, les conditions climatiques actuelles (plus de lumière solaire/année) entraînent une augmentation encore plus forte des teneurs en sucre, générant un stress osmotique d'intensité plus élevée. Le $SO_2$ est un autre facteur de stress. Depuis des siècles, ce composé est utilisé dans la vinification en raison de ses propriétés antiseptiques et anti-oxydantes. Sa présence et son impact ont entrainé la sélection de souches de levures possédant une translocation entre les chromosomes XVI et VIII, conduisant à une augmentation de l'expression du gène *SSU1* (responsable de l'export des sulfites) et qui confère un avantage aux levures la possédant en présence de $SO_2$. De faibles quantités de vitamines, en particulier de la thiamine, sont également source de stress. La présence de la flore naturelle conduit à un appauvrissement rapide des vitamines naturellement présentes dans le moût, dont certaines sont essentielles pour la croissance de la levure de vin sélectionnée. Sans ces vitamines, la croissance de la levure et sa capacité fermentaire sont souvent sous-optimales. Dans le cas des vinifications en blanc, les températures basses (10ºC- 20ºC) représentent également un défi majeur pour la levure, qui est loin de sa température optimale de croissance (28ºC). Enfin, une faible teneur en lipides peut également être une source de stress. Les vins blancs sont généralement clarifiés, ce qui implique l'élimination de nutriments importants comme les lipides. Leur faible concentration peut compromettre la réponse au stress de la levure ainsi que ses performances fermentaires. Ainsi, ces facteurs de stress peuvent entrainer une extension de la phase de latence lors de l'inoculation de la levure dans le mout de raisin. Durant cette phase, la levure adapte son métabolisme à un nouvel environnement avant d'amorcer sa croissance. La présence de  stress entraine des périodes d'adaptation plus longues et peut conduire à des fermentations lentes, voire des arrêts de fermentation. Ceci a pour conséquence une augmentation du risque de développement de la flore naturellement présente dans le moût, pouvant entrainer une altération du vin et des coûts supplémentaires pour les professionnels de la filière.

L'objectif de cette thèse est d'étudier et d'améliorer la phase de latence de la levure œnologique dans des conditions de stress multiples lors de la fermentation du moût de raisin. Pour atteindre cet objectif, nous avons dans un premier temps étudié la diversité de la phase de latence parmi différentes souches de levures commerciales et évalué

l'impact de différents stress présents en début de fermentation sur la durée de phase de latence de ces souches. Puis deux axes parallèles et complémentaires ont été développés. En premier lieu, nous avons mis au point une approche d'évolution expérimentale visant à développer des souches améliorées présentant une phase de latence plus courte. Nous avons également cherché à identifier les modifications génétiques à l'origine de cette amélioration. D'autre part, en raison de la complexité de ce trait, nous avons conçu et mis en œuvre une approche originale de cartographie QTL (quantitative trait loci) pour déterminer les allèles ayant une influence majeure dans la phase de latence. Les connaissances générées pourront permettre une valorisation industrielle ultérieure. Enfin, la dernière partie de ce travail a consisté à évaluer les potentialités d'utilisation de la levure *Kluyveromyces marxianus* en culture mixte avec *S. cerevisiae* en fermentation alcoolique. En effet, depuis ces dernières années, l'utilisation de levures non conventionnelles, en cultures mixtes avec *S. cerevisiae* connait un regain d'intérêt, notamment dans l'optique d'obtenir des vins plus diversifiés sur le plan organoleptique. Ainsi l'intérêt de levures non *S. cerevisiae*, d'hybrides *Saccharomyces* inter-espèces et de levures non *Saccharomyces* est de plus en plus étudié pour la vinification. *Kluyveromyces marxianus* est une levure non-conventionnelle qui a été très peu exploitée pour ses capacités fermentaires en conditions œnologiques, même si dans d'autres contextes biotechnologiques cette levure a montré un très bon potentiel

Dans le chapitre 1, nous avons étudié 10 souches de levures commerciales (6 souches *S. cerevisiae*, 2 hybrides *S. cerevisiae* x *Saccharomyces kudriavzevii*, 1 souche *Torulaspora delbrueckii* et 1 souche *Saccharomyces uvarum*). Une première analyse génétique des souches *S. cerevisiae* a montré que celles-ci était phylogénétiquement très diversifiées, représentant ainsi un bon point de départ pour caractériser leur phase de latence. En établissant un plan factoriel, nous avons évalué l'effet individuel et combiné des principaux facteurs de stress présents au début de la fermentation alcoolique (température, stress osmotique, $SO_2$, présence de faible concentration de thiamine et de phytostérols), ce qui a révélé une grande diversité de durée de phase de latence entre ces souches, celle-ci variant de 10h à 70h.

L'étude de l'impact individuel des facteurs de stress a révélé que la température et le stress osmotique étaient les deux facteurs les plus importants. En moyenne, la température est responsable d'une augmentation de la durée de phase de latence de près de 24h entre

28ºC et 16ºC. Le stress osmotique affecte également de façon marquée le temps de latence de toutes les souches. Le $SO_2$ apparait ensuite comme un facteur affectant significativement la phase de latence de 6 souches sur 10 à 28ºC et de 4 souches sur 10 à 16ºC. Nous avons supposé que cet effet de température était lié au fait que le pouvoir de combinaison du $SO_2$ augmentait à des températures plus basses et que, par conséquent, la fraction active de cette molécule était diminuée. Nous avons également mis en évidence que la translocation $XVI^{VIII}$ est assez fréquente parmi les souches étudiées (6 sur 8) au contraire de la translocation $XVI^{XV}$ (absente des 8 souches) plus récemment découverte, qui permet également une augmentation de l'expression du gène *SSU1*. En ce qui concerne les phytostérols et la thiamine, leur impact est apparu ponctuel et spécifique de la souche, ce qui souligne leur importance mais de manière souche-dépendante. Enfin, aucune interaction transversale entre les facteurs à toutes les souches n'a été détectée, ce qui montre que dans les conditions particulières de multi-stress propres à l'inoculation du vin, chaque facteur de stress a une influence individuelle.

L'objectif du chapitre 2 était de mener une approche d'ingénierie évolutive (ALE pour Adaptive Laboratory Evolution) visant à développer des souches ayant une durée plus courte de phase de latence en conditions œnologiques. Dans ce cadre, deux expériences d'évolution ont été menées en triplicata, mimant des conditions de fermentation en vin rouge et en vin blanc. Pour la première, le stress osmotique, la présence de $SO_2$ et une faible concentration en thiamine ont été choisis comme trois principaux facteurs de stress. Pour la seconde, une température basse, la présence de $SO_2$ et une faible quantité de phytostérols ont été considérés comme les plus représentatifs. Le choix des souches a reposé sur l'étude menée au chapitre 1, en prenant en compte 3 critères : présence de la translocation $XVI^{VIII}$ pour éviter une évolution trop orientée sur la résistance au $SO_2$, une phase de latence lente, et des critères commerciaux définis par la société Lallemand SAS, en lien avec une éventuelle commercialisation future. Ainsi, les souches L7 et L1 ont été sélectionnées et des expériences d'évolutions dans des conditions de vin rouge et blanc ont été menées. Nous n'avons pas pu obtenir de souche améliorée pour la phase de latence dans les conditions en blanc ; nous avons donc centré nos efforts sur les expériences d'évolution menées avec la souche L7 en conditions de vinification en rouge.

Les expériences menées avec cette souche ont permis d'obtenir des populations évoluées en parallel (R1, R2 et R3) ayant une phase de latence réduite d'environ 4h sur

moût synthétique. De manière intéressante, ce phénotype a été obtenu pour deux populations (R2 et R3) après 150-200 générations, alors qu'aucune évolution n'a pu être observée pour la population R1 même après 500 générations dans ces conditions. Six souches issues des populations R2 et R3 ont ensuite été isolées sur la base de leur phénotype "phase de latence réduite" et leur génome a été séquencé. Les données de séquençage ont révélé une perte générale d'hétérozygotie (LOH) dans le chromosome 9, commune à 5 des 6 clones. Bien que les LOH ait été décrits comme un mécanisme d'évolution, le fait que la souche R3C ne présentait aucun LOH mais provient de la même population que R3F et R3I, suggère que le phénotype phase de latence réduite est lié à une modification génétique en dehors de cette région, puisque les 3 clones présentent une duration de phase de latence équivalent. Les informations sur les mutations de novo contribuant potentiellement au phénotype amélioré font émerger des candidats impliqués dans l'intégrité et la maintenance de la paroi cellulaire. Le fait que R2 et R3 possèdent des mutations dans des gènes différents mais impliqués dans la maintenance de la paroi renforce un rôle possible de la paroi cellulaire dans la résistance au stress présent en début de fermentation alcoolique.

Les travaux menés en parallèle et décrits au chapitre 3 nous ont permis d'aller plus loin dans la caractérisation des bases génétiques de la phase de latence, un phénotype complexe qui résulte d'une combinaison de différents mécanismes cellulaires. Ainsi, avons développé une approche d'analyse de ségrégants en « bulk » (BSA pour Bulk Segregant Analysis), à partir de croisements issus de 4 souches parentales, afin d'étudier les bases génétiques et moléculaires mises en jeu au cours de la phase de latence en condition de fermentation alcoolique. L'avantage de cette approche par rapport à une approche traditionnelle de cartographie QTL est qu'il n'y a pas besoin de génotyper et phénotyper individuellement des centaines de segrégants. De plus, l'originalité de notre approche a reposé sur la combinaison d'une approche multi-parentale, de la propagation industrielle et séchage des populations issues de ces croisements, et d'une étape de cytométrie en flux afin de sélectionner les cellules bourgeonnantes. L'addition dans notre approche des étapes de propagation et de séchage des levures, qui influence certainement la phase de latence et qui sont typiques de toute production industrielle de levure de vin, nous a permis de mimer au plus près les conditions réelles de vinification.

L'objectif principal de ce chapitre était d'identifier les QTL (et donc les allèles) contrôlant la phase de latence dans un contexte de vinification. En plus de générer de nouvelles connaissances académiques, nous pouvons envisager, à terme, une valorisation industrielle de ces connaissances, basée sur le transfert des allèles identifiés à d'autres levures de vin commercialisées par des techniques non-OGM de façon à améliorer leurs performances.

Nous avons ainsi sélectionné, sur la base de la caractérisation de souches réalisée au chapitre 1, 4 souches possédant des phases de latences contrastées, deux avec des phases de latence réduite et deux avec des phases de latence plus longue. Afin de faciliter l'isolement des premières cellules bourgeonnantes, nous avons marqué la protéine Clb3, une cycline de type B présente au début du cycle cellulaire (phase S) et dégradée en fin de phase G2, avec la GFP à son extrémité C-terminale. Les 4 souches marquées ont ensuite été croisées de manière à obtenir 6 populations recombinantes. Ces populations ont alors été propagées et séchées dans des conditions industrielles afin d'obtenir des levures sèches actives (LSA). Puis, les premières cellules bourgeonnantes issues de 5 populations ont été sélectionnées à l'aide d'un Fluorescence-Activated Cell Sorting (FACS) après inoculation directe des LSA dans un moût synthétique mimant un moût de raisin. Enfin, les populations triées ont été séquencées et nous avons comparé les fréquences alléliques entre ces populations séchées et la population de départ propagée, non séchée et non triée.

Cette stratégie nous a permis de détecter 18 régions avec des différences de fréquences alléliques, incluant 3 QTLs détectés dans au moins deux populations. Afin de faciliter la détection des gènes impliqués dans le phénotype, nous avons caractérisé les Single Nucleotide Polymorphism (SNP) présents et leur implication majeure. En nous focalisant sur les régions codantes et les mutations non-synonymes, nous avons identifié 9 gènes codant pour des fonctions biologiques pouvant jouer un rôle dans la modulation de la phase de latence. Ces gènes codent pour des protéines impliquées dans la croissance cellulaire et le bourgeonnement des cellules, dans l'activation de la réponse au stress ainsi que dans le transport des sucres. Ainsi, tous les gènes identifiés par notre approche présentent des fonctions biologiques qui peuvent influencer directement la phase de latence de différentes manières. Le fait que l'enrichissement allélique sur la population sélectionnée soit principalement observé sur les croisements impliquant les souches les plus performantes (phase de latence plus courte) ne fait que renforcer le fait que ces allèles

peuvent jouer un rôle important dans le phénotype. L'étape suivante consistera, pour chaque gène candidat, à construire des souches hémizygotes à partir des souches parentales, différant par la présence de chaque allèle. Cela permettra de valider l'impact de ces variants sur la phase de latence, et d'envisager par la suite une valorisation industrielle en transférant ces allèles dans une souche de levure de vin déjà commercialisée par hybridation assistée par marqueurs.

Enfin, dans le chapitre 4, nous avons évalué les potentialités d'utilisation de cultures mixtes *K. marxianus* et *S. cerevisiae* en fermentation œnologique. Dans ce but, le profil fermentaire de deux souches de *K. marxanius*, K1 and K4, une souche de laboratoire et une souche industrielle respectivement, a été étudié ainsi que la meilleure stratégie de fermentation mixte avec *S. cerevisiae*. De plus, nous avons évalué les principales contributions aromatiques de la souche K1 en co-inoculations avec *S. cerevisiae* en conditions œnologiques. Dans un premier temps, l'étude de la croissance cellulaire en fermentation individuelle sur milieu synthétique mimant un moût naturel a montré que la souche K1 présente une croissance inférieure à la souche L1 de *S. cerevisiae* et à la souche K4. Cependant, en inoculation séquentielle, lorsque L1 est ajouté à la fermentation 12h après l'ensemencement avec K1, K1 représente entre 52% et 66% de la population totale alors que K4 prend le dessus conduisant à une fermentation complète et à l'élimination de la souche L1. Le fait que cette souche ait été sélectionnée pour une utilisation industrielle peut justifier sa bonne capacité fermentaire du fait très certainement d'une meilleure résistance aux conditions stressantes du milieu. Bien que cette souche possède des capacités inattendues et que cela ouvre de nouvelles perspectives pour son utilisation en fermentation individuelle, dans le cadre de notre étude, nous avons décidé de poursuivre notre travail uniquement avec la souche K1. Ainsi, des co-inoculation avec les souches K1 et L1 ont été réalisées, montrant que la souche L1 est plus performante avec un taux entre 70% et 55% à 24h et 48h de fermentation respectivement et une cinétique fermentaire ralentie. Cependant, la co-inoculation comme l'inoculation séquentielle, avec 12h de différence, ne compromet pas la fermentation. Du côté des apports aromatiques, K1 produit à la fois des arômes positifs et négatifs dans les inoculations pures alors qu'en inoculation séquentielle la présence de composés particuliers uniques sont produit par *K. marxianus* révélant ainsi des interactions et contributions positives de K1 en fermentation alcoolique. Les potentialités de *K. marxianus* ouvrent de ce fait des perspectives; de

nouvelles études et une caractérisation plus approfondie des conditions optimales de culture seront nécessaires pour évaluer l'intérêt d'une utilisation de cette levure comme espèce non-conventionnelle en conditions œnologiques.

# BIBLIOGRAPHIC INTRODUCTION

## 1. General considerations about yeast

Yeasts are ubiquitous eukaryotic unicellular microorganisms and are part of the fungi kingdom which, includes other multi-cellular organisms such as mushrooms and molds. Natural yeast strains can be found in many different environments such as plants, soil from all regions around the world, fruit skin, insects, gastrointestinal tracts or even in aquatic environments (Gadanho *et al.* 2003). The successful colonization of such wide range of subtracts is intimately related to their metabolic and physiological adaptability to highly variable environments. In 1860 Louis Pasteur was the first to observe and describe yeast, establishing a positive correlation between yeast's presence and the fermentation process (Pasteur 1860). Isolation of yeast strains only occurred years later in 1938 when Emil Mrak isolated yeast from rotten figs in California, USA. Later on, Robert Mortimer crossed that fig isolated strain with strains obtained by other researchers and obtained a new strain called S288C. This *S. cerevisiae* strain became then a reference and until the 2000´s was the main strain used by many laboratories around the world for yeast and genetic research (Mortimer and Johnston 1986). Today more than 149 genera and 1500 species have been described (Kurtzman *et al.* 2010). Most of them are commensal being therefore innocuous to humans however some, such as *Candida albicans*, can also be opportunistic pathogens. One of the most studied genera is *Saccharomyces* where 8 different species can be found: *S. cerevisiae*, *S. eubayanus*, *S. kudriavzevii*, and *S. uvarum* often associated with anthropic activities, and *S. arboricola*, *S. jurei*, *S. mikatae*, and *S. paradoxus*, which are commonly found in natural environments. Hybrids such as *S. bayanus* (*S. cerevisiae* × *S. eubayanus* × *S. uvarum*) and *S. pastorianus* (*S. cerevisiae* × *S. eubayanus*) can also often be found in anthropic niches, particularly those linked to beverage fermentations (Figure N.1).

### 1.1 Why a model organism?

The combination of several convenient features found in yeast justifies the great interest and extensive study of this microorganism over the years.

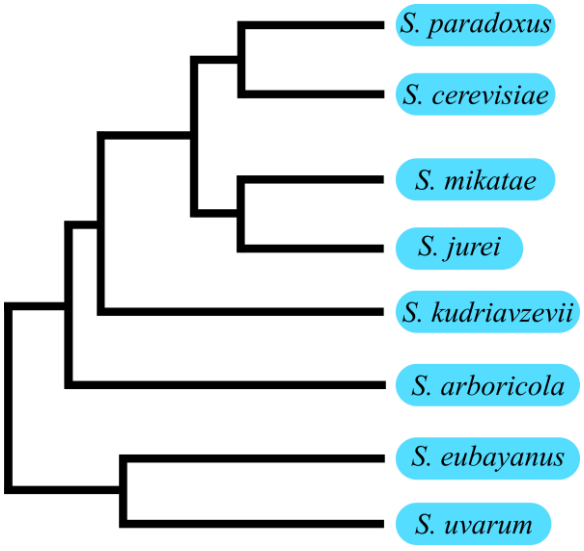



**Figure N.1. Phylogeny of the genera *Saccharomyces*;** adapted from (Dujon and Louis 2017).

"Baker's yeast" is relatively small (often 3-6 µm), most often non-pathogenic, easy to manipulate, economical to maintain and has a fast growth rate. In rich medium (YPD), 90 minutes are usually enough for a yeast population to double (Sherman 2002). Being unicellular and eukaryotic provides a perfect tool for cell biology studies and in more recent years the integration of more complex fields such as genomics, transcriptomics, proteomics, metabolomics and even synthetic biology. Throughout evolution the same basic cell functions and regulations have been kept across Eukarya. By using yeast as a model organism, valuable extrapolations are possible in the study of high eukaryotes such as humans where many yeast genes and proteins have homologs (Kachroo *et al.* 2015). This was certainly one of the main reasons for the yeast strain S288C being the first eukaryotic genome to be fully sequenced more than 2 decades ago (Goffeau *et al.* 1996). 16 chromosomes, 6200 genes and 12068 kilo base pairs (kpb) are some of the features that characterize the strain's genome. The small genome, and the ease of genetic manipulation, make yeast one of the first choices for eukaryotic research in cell biology and genetics. Today, the genome sequence and respective annotation of S288C and many other *S. cerevisiae* strains are available on the website http://www.yeastgenome.org/, Saccharomyces Genome Database - SGD (Cherry *et al.* 1998).

### 1.2 Life cycle

Yeast can exist in different ploidy levels. Diploids are the most commonly found in nature and industry, however triploidy or aneuploidy (abnormal small or greater chromosome number than the wild type) can also occur (Bakalinsky and Snow 1990). Typically yeast reproduces asexually by mitotic division either by fission (symmetrically) or budding (asymmetrically) (Herskowitz 1988). This method of proliferation is clearly predominant over sexual reproduction. On average, 1000 mitotic divisions for *S. cerevisiae* occur before one meiotic cycle takes place (Tsai *et al.* 2008). Nevertheless, under specific conditions, *e.g.* lack of nutrients, diploids can also sporulate. A meiosis takes place and yields an ascus containing 4 haploid spores, each one resulting from a unique recombination of the parental genome. When the conditions are favorable again, the spores are freed and germinate to haploid cells that have now a specific mating type, **a** or α, controlled by the gene *MAT* (**MATa**/MATα). When put in contact, haploids of opposite mating types are capable of mating by cell and nuclear fusion, resulting in a diploid cell (Herskowitz 1988).

Yeast can be classified either as heterothallic or homothallic. The latter are capable of changing their mating type when dividing mitotically. This means that progeny with both mating types can arise so mother and daughter cells can mate right after mitotic division. This process of mating-type interconversion is controlled by the gene *HO*. It codes for an endonuclease that will intervene at the *MAT* locus and ultimately define the mating type at each division (Herskowitz 1988). When a defective allele is present (*ho*), yeasts will be heterothallic as they cannot change their mating type. This way they can only mate with an independent haploid of opposite type. This makes these haploids genetically more stable which is often sought in yeast molecular and genetics research.

### 1.3 Yeast under biotechnological influence

Yeast and mankind have been tightly connected in biotechnological processes for several millennia now. The most ancient evidence of yeast associated to fermented beverages has been found in pottery and bronze vessels in China dating as far as 7000

B.C. (McGovern *et al.* 2004). Since then, many traditional applications were born. Aside from winemaking, brewing, sake brewing, production of distilled beverages and baking are some examples. The principle always remained the same: use yeast to transform foodstuffs, extend their shelf life and add new organoleptic properties to a final product. Without realizing it, man accelerated the yeast selection process for different fields and application, taking advantage of the existing diversity and natural yeast adaptability. As more knowledge was gathered and technological breakthroughs accompanied it, not only were these traditional applications greatly improved (still are) but also new applications appeared where yeast potential can be explored. For a few decades, yeast has been used as an alternative to fossil fuels (*e.g.* bioethanol), for the production of proteins of interest, medical treatments such as vaccines or insulin, alimentary aromas and additives and animal nutrition. Lallemand Inc. (Canada), Associated British Foods plc (U.K.), Chr. Hansen Holding A/S (Denmark), Lesaffre Group (France) and Alltech (USA) are some of the current major companies well established in the yeast and specialty yeasts market. It is estimated that this market will reach EUR 4.2 billion value in less than 5 years. It becomes clear that yeast has earned an important role in our society at different levels, culturally, scientifically and economically as a valuable biotechnological alternative.

## 2. The oenological fermentation

Wine appreciation and influence in our society has been present for a very long time now. This becomes evident when we realize that most of civilizations across the globe have included to some extent wine and wine fermentation in their culture, customs and traditions. Aside from cultural, wine clearly assumes an economic role as well. Recent values disclosed by International Organization of Vine and Wine (OIV) for the year 2015 showed that there is a total of 7.5 million vineyard hectares, 274 million hectoliters being produced (the top 3 being Italy, France and Spain) and a total of 240 million hectoliters consumed worldwide. This is translated to a global market value around EUR 28.3 billion. To achieve this overview, mankind and oenological fermentation have come a long way over several millennia. Since the first records of wine fermentation back in 5400 B.C. (McGovern *et al.* 1996), much knowledge has been gathered resulting in a process that today is more and more thoroughly understood. Technological improvements

accompanied this knowledge allowing a fine control and optimization at each different step. Although these advances help realize the full potential of the oenological fermentation, the same basic fermentation principles still apply when it comes to going from the grapes to the wine bottle on the table.

### 2.1 The wine must

Any oenological fermentation starts with the grape harvest and pressing from which a wine must can be obtained. The characteristics of each must will be highly dependent on several factors. Vine variety, the meteorological conditions during that year (sunlight, water, temperature…), the soil and the wine style that will be performed (red or white) all greatly contribute to a must's composition. Although these factors can vary to some extent, some must conditions can be considered common to every must: high sugar concentration (180-260 g/L equimolar glucose and fructose), low pH (2.9-4.2), added sulfites (40-80 mg/L total) and limiting amounts of assimilable nitrogen (60 – 500 mg/L), lipids and vitamins (Bely *et al.* 1990a). Oxygen is present at the beginning of the fermentation (both in air and liquid phase) but it is quickly consumed making the fermentation mostly an anaerobic process. If some of these parameters are unbalanced, stuck or sluggish fermentations can occur and the final wine properties be affected. For research purposes, grape must can be replaced by synthetic must which apart from having a similar composition to natural must, allows one to precisely control every parameter of the must (Bely *et al.* 1990b).

### 2.2 The fermentation itself

In a simple form, wine fermentation could be defined as the conversion of sugar into ethanol and $CO_2$ under anaerobic conditions:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_6O$$

This fermentation process can occur spontaneously and the microbiome naturally present in the grapes and cellar is often very diverse (*Saccharomyces*, *Hanseniaspora*, *Rhodoturula*, *Candida*, *Debaryomyces*, *Torulaspora*, *Dekkera*, *Pichia*, *Schizosaccharomyces*…) (Pretorius 2000, Jolly et al., 2014). Once the must is obtained, flora multiplication quickly starts followed by the fermentation. When the ethanol concentration rises, only *S. cerevisiae* is able to resist and ends up taking over performing most of the fermentation. This method was actually used for most part of wine fermentation history but it presents important drawbacks. With such microbial variation in natural populations and the fermentation process the final wine features (*e.g.* organoleptic properties) become much less predictable. In fact, quite often spoilage yeast such as *Brettanomyces* are present and can add undesired horsy and barnyard flavors while suppressing the pleasant fruity and floral wine aromas (Romano *et al.* 2008).

Spontaneous fermentations started to be abandoned in the 1980's and were gradually replaced by fermentations with a controlled inoculation (Pretorius 2000a). This implies deliberately adding a selected wine yeast before the fermentation start, usually at a concentration close to $1x10^6$ cells/mL. Today, 80% of wine fermentations are conducted with added yeast inocula and more than 200 wine yeast strains are available on the market (Pretorius 2000b; Sablayrolles 2009). These are usually sold as active dry yeasts (ADY) and produced/distributed by commercial companies. ADY are always selected on the basis of their good fermentative capacity, positive contributions to final wine properties, must characteristics and the type of fermentation. The process itself usually takes several weeks depending on the conditions in which the fermentation occurs (*e.g.* temperature). Nevertheless, one can always identify 5 main stages during fermentation: 1) lag phase; 2) exponential phase; 3) transition phase; 4) stationary phase; 5) end of fermentation. 1) typically corresponds to the period where yeast is adapting its metabolism to the new environmental conditions *i.e.* the wine must (Pérez-Torrado *et al.* 2002). This phase can last from several hours to few days when conditions are not optimal. The main factors that influence its duration are the yeast strain, inoculation load, must characteristics and fermentation conditions such as temperature or presence of inhibitors (*e.g.* $SO_2$). During this phase, very low metabolic activity occurs and $CO_2$ and ethanol production is almost null (Figure N.2). Throughout this phase, the must composition globally remains the same with the exception of thiamine.

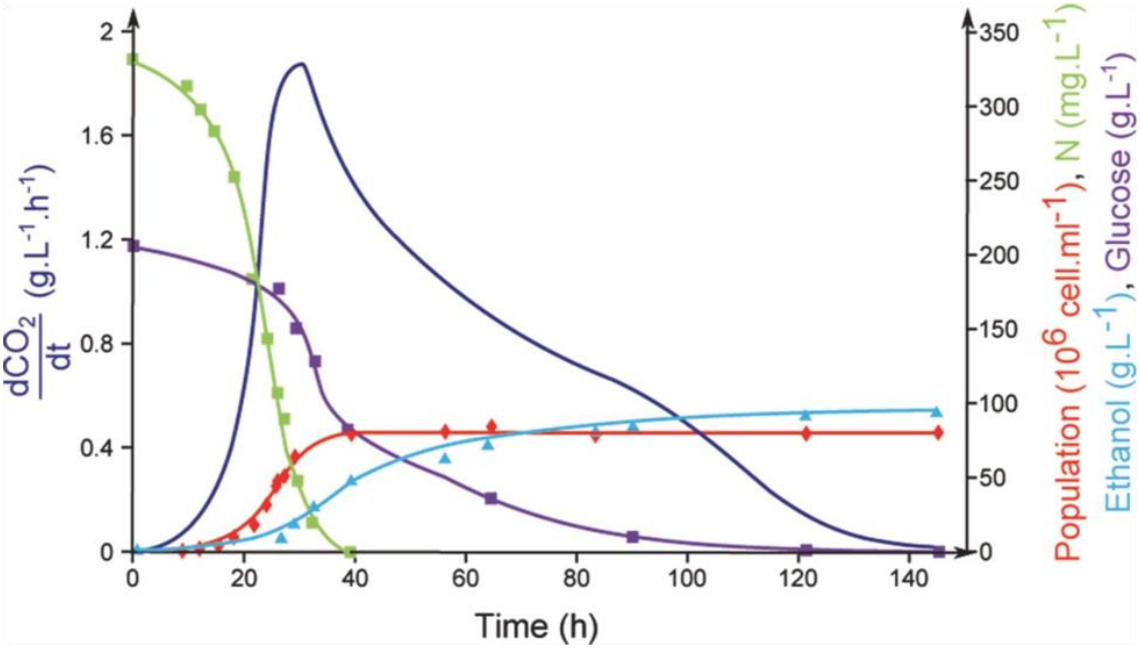



**Figure N.2. Representative dynamics of main fermentation parameters during anaerobic wine fermentation by yeast.** The different fermentation phases are connected to the availability of nutrients and the accumulation of metabolites. Dark blue: fermentation rate expressed as $CO_2$ release; light blue: ethanol concentration; red: yeast cell number; green: nitrogen concentration; purple: concentration of sugars; from Marsit and Dequin (2015).

This vitamin tends to be quickly consumed, often by other microorganisms other than *S. cerevisiae* (Bataillon *et al.* 1996). 2) is the stage where yeast growth increases exponentially. During this stage, the fermentative activity is optimal and can be measured by the rate of $CO_2$ released over time ($dCO_2/dt$) which eventually reaches its maximum ($V_{max}$) by the end of this phase (Figure N.2). $CO_2$ produced stays dissolved in the must until saturation (around 1.5 g/L) and then gets released as a gas. 3) at this stage, growth rate decreases as one or more nutrients become limiting (Figure N.2). Typically nitrogen is the first nutrient to get exhausted (Bely *et al.* 1990b). Nevertheless, other nutrients can also become limiting such as vitamins or lipids (particularly in white wine fermentations due to clarification). Yeast population maximum is reached during this stage and can vary from 50 to 250 x $10^6$ cells/mL. This value mainly depends on the concentration of the limiting nutrient. In parallel with growth, yeast metabolic activity also decreases. 4) is the phase where most of the sugars will be transformed into ethanol and $CO_2$. At this point cells are no longer dividing but remain viable and actively fermenting (Figure N.2). Due to the increasing stress factors, such as ethanol accumulation, the fermentative rate and

$CO_2$ release progressively diminishes due to an inhibition of the hexose transporters (Salmon *et al.* 1993). Transcriptomic data have provided evidence for major metabolic changes when entering in this phase, particularly to deal with the high stress level that develops (Rossignol *et al.* 2003). 5) is reached when all sugars present are consumed. $CO_2$ release stops and the fermentation rate equals zero (Figure N.2). With all nutrients exhausted, yeast viability drastically drops close to zero. The ethanol final concentration will now have reached a 9 to 17 % (v/v) and also contributes to cell death.

## 2.3 Wine yeast

### 2.3.1 Diversity and characterization

*S. cerevisiae* is considered as the wine yeast *par excellence*. Its unmatched qualities and resistance to harsh conditions during wine fermentation make it the perfect microorganism for the purpose. Additionally, the wine yeast group contains a wide genetic and phenotypic diversity that has been more and more explored. Different studies and methods have been applied to better characterize both of these components as well as better understand the evolutionary history of the species within the oenological context.

#### Phenotypic variation

Phenotypic simple traits have been used since the first yeast observations to characterize and distinguish species. Since then, the tendency has been to extend these parameters to more complex phenotypic traits allowing a deeper characterization and to correlate them to ecological niches and functions. Spor *et al.* 2008, 2009 did a phenotypic characterization focused on life-style parameters such as maximal population, cell size, and growth rate submitting 12 different industrial strains to different fermentative contexts. With the results obtained, they classified strains either as “grasshoppers” or “ants”. The former characterize themselves by generating a smaller population with larger cells that rapidly consume glucose whereas the latter reach a bigger population size but have smaller cells and consume glucose at a slower rate. Later on, it was also

demonstrated that maximal fermentation rate was correlated with the final population size (Albertin *et al.* 2011).

Metabolism is another phenotypic parameter that can be used to characterize yeast. Camarasa *et al.* 2011 conducted a study in which 72 different strains with a very diverse origin were characterized under oenological conditions. By analyzing several oenological parameters (*e.g.* total $CO_2$ released, fermentation rate) and metabolites (glycerol, acetate, succinate and eight volatile organoleptic compounds), important metabolic differences were detected. Strains isolated from environments with low-sugar concentration (laboratory, oak, soil) had a slow fermentation rate, produced little biomass, high amounts of acetate and low amounts of isoamyl acetate. Conversely, strains isolated from sugar-rich environments (fruit, vineyards) exhibited overall the opposite fermentative profile showing a metabolic profile much more adapted for fermentation. Within this latter set, it was still possible to distinguish commercial wine from vineyard strains. The former had shorter fermentation time, less variation within the group and presented a more appellative metabolic and oenological profile (Camarasa *et al.* 2011). This reflects human selection for technological traits. Different metabolic profiles can also critically determine the performance and survival when submitted to environmental stresses or compounds. Ethanol stress, osmotic stress, copper and sulfite resistance are only some examples of contexts where yeast must adapt its metabolism and in oenological conditions this can determine the fermentation success or not (Park and Bakalinsky 2000; Fay *et al.* 2004; Ding *et al.* 2009; Li *et al.* 2009; Teixeira *et al.* 2009). Finally, in recent years, much improvements have been made the prediction of metabolic fluxes such as in the central carbon metabolism (Nidelet *et al.* 2016) or of the redox balance for metabolite production (Celton *et al.* 2012). This will allow a deeper understanding and better control of wine fermentation depending on the must characteristics and conditions being used.

**Genetic variation**

Genetic variation is at the basis of much of the diversity that can be later observed phenotypically in almost, if not all, cell parameters. A great part of this variation comes from spontaneous mutations that naturally occur overtime. It has been estimated at two different *loci* (*URA3* and *CAN1)* that these mutations could occur at rates such as 3.8 -

6.4 x $10^{-10}$/bp/generation (Lang and Murray 2008). These values are not constant over the genome as essential functions and specific regions of the genome, such as telomeres, tend to be preserved within species and even within the same clade (*e.g.* humans and yeasts share the same and homologous genes for cell cycle regulation). On the other hand, genomic regions called "mutation hotspots" like tandem repeats and subtelomeric regions (Gemayel *et al.* 2010) are likely to suffer mutations 10–100,000 times more than in other parts of the genome. Nevertheless, not every mutation leads to a positive change in phenotype. In fact, most are neutral or even deleterious, the latter are often masked by heterozygosity when in diploid strains. However, several genetic events are in agreement to be the main promoters of genetic variation.

Single-nucleotide polymorphism (SNP) is the most common mechanism. A SNP is defined as a variation in a single nucleotide that occurs at a specific position in the genome. At the moment of DNA replication, errors are prone to happen, resulting in the modification of a nucleotide, when synthetizing a new strand. Depending on where the SNP occurs this can lead to different consequences. If within a coding sequence this can lead to a modification in a protein due to changes in amino acid sequence. The variant gene can be then considered as an allele. SNPs can also occur in non-coding regions which is less likely to have a practical consequence. However, if it is located in a promoter region, it can affect gene transcription and consequently protein translation. *S. cerevisiae* strains with the same origin have a SNP variation frequency of 1 SNP/kpb while up to 6 SNP/kpb are found for strains that are genetically distant (Fay and Benavides 2005; Liti *et al.* 2009; Schacherer *et al.* 2009; Dequin and Casaregola 2011).

Another known mechanism is the insertion or deletion of a nucleotide (Indels). One to several thousand nucleotides can be added or deleted by error during DNA replication as with SNPs. If it is located in a coding region, unless the length is a multiple of 3, it will cause a frameshift mutation completely modifying the amino acid sequence that will usually go on until a stop codon appears. Indels are less likely to happen than SNPs. For example, the laboratory strain S288C and the wine strain EC1118 differ by 5000 indels and by 46000 SNPs (Novo *et al.* 2009).

Copy number variations (CNV) happen when entire sections of the genome are deleted or repeated. They are considered to be structural variations. Often they can be a consequence of an adaptation to new environments or when the population is submitted to stress factors (Adams *et al.* 1992; Dunham *et al.* 2002). Ultimately, having several

copies of the same gene can increase its expression and consequently the protein content level.

Chromosomal rearrangements represent major structural changes. Different events can take place such as deletions, duplications, inversions and translocations. The main cause for these is a breakage of the DNA double helices at two different locations, followed by a reintegration of this new fragment in a different manner. Naturally, this causes a change in the chromosome sequence and gene order (Giffiths *et al.* 1999). Duplications are frequently found in the telomeric and subtelomeric regions and involve transporter genes and drug resistance (Dequin and Casaregola 2011). A good example of rearrangement in oenological strains is a reciprocal translocation between the left arm of chromosome 16 and the left telomere of chromosome 8 (Pérez-Ortín *et al.* 2002). The practical consequence of such translocation is the exchange of the promoter of the gene *SSU1* (coding for a sulfite exporter) with the promoter of the gene *ECM34*. This considerably increases the expression of *SSU1*, which in turn increases yeast's sulfite resistance (Pérez-Ortín *et al.* 2002). As sulfite has been used for hundreds of years in winemaking, this translocation has been retained in most of the wine yeast strains and increased their performance (more on this later).

Horizontal gene transfer (HGT) can be defined as the transfer and integration of stable genetic material between different species (Doolittle 1999). This mechanism has long been considered as rare in yeast however with genome sequencing advances, new evidence reveals its importance, for instance, in industrial contexts. Genetic analysis of the wine strain EC1118 showed strong evidence of the HGT of 3 DNA regions containing 34 genes, which encode proteins potentially involved in key functions in wine fermentation (Novo *et al.* 2009). For instance, it was suggested that the gene *FSY1*, coding for a high-affinity fructose/$H^+$ symporter, could confer an advantage during wine fermentation by enabling yeast to better use fructose present at the end of the fermentation (Galeote *et al.* 2010). More recently, Marsit *et al.* 2015 demonstrated an HGT between *Torulaspora microellipsoides* and *S. cerevisiae* concerning two duplicated *FOT* genes. These genes allow yeast to use more diverse and higher number of oligopeptides as a source of nitrogen (a limiting nutrient in must), conferring an important competitive advantage when in oenological conditions. Finally, a recent population genomics study performed at INRA, SPO revealed that the acquisition of specific genes by introgression or horizontal transfer is frequent in the *S. cerevisiae* population (Legras et al, submitted).

Several methods and techniques have been developed to better detect these types of events and characterize yeast. These methods can also help tracing back important genetic events as the ones described above. Ultimately, they allow identification of important moments of differentiation and selection in yeast history and reconstruct part of the course of yeast evolution. Examples of such techniques are the use of microsatellites, amplified fragment length polymorphisms (AFLP) and highly conserved independent genes (Azumi and Goto-Yamamoto 2001; Hennequin *et al.* 2001; Fay and Benavides 2005; Liti *et al.* 2006; Legras *et al.* 2007). These methods are based on the detection of specific molecular markers that arise from the genetic polymorphism that naturally exists (or not) between different strains. Legras *et al.* 2007 gathered the largest set used for a microsatellite analysis, 651 *S. cerevisiae* strains, with diverse origins (China, Japan, Central Europe, South Africa, USA, Brazil) and contexts (sake, winemaking, brewing, baking, rum, distillery). Ultimately, it was demonstrated that more than the geographical origin, the ecological niche plays a major role in genetic background divergence or convergence. Other methods include the study of the complete genome by means of the tiling microarrays technique (Schacherer *et al.* 2007, 2009) and low coverage sequencing (as low as 1 to 4 fold) of strains from different origins (Liti *et al.* 2009). These helped to confirm the considerable impact that domestication has on yeast diversity distribution.

Strains isolated from nature tend to be grouped depending on their origin, however, strains under anthropomorphic influence tend to be grouped by function or ecological niche (Liti *et al.* 2009). This is the case of oenological yeast strains. It has been demonstrated that these form a very specific phylogenetic group with an unique origin while keeping low variation (Legras *et al.* 2007; Liti *et al.* 2009; Schacherer *et al.* 2009). Very likely, they would have been domesticated in Mesopotamia (Legras *et al.* 2007). Based on tartaric acid detection, archeologists found evidence of the first wine production traced back to 5400 B.C. in a Neolithic village in Iran's northern Zargos mountains (McGovern *et al.* 1996). From there, different migration routes could have led to the expansion of the population to Europe, passing through the Mediterranean to southern countries such as France, Spain and Italy, as well by the Danube valley (Sicard and Legras 2011). Oenological strains are one good example, however, it is likely that it could be extended to the different yeast strains used by man in biotechnological processes that they all derived from an ancestral (and wild) population, differentiating themselves later through domestication events (Fay and Benavides 2005). This ultimately means that, even

if unconsciously, man has been shaping yeast‘s populations, diversity and evolution through the millennia (Legras et al, 2007; Liti *et al.* 2009; Stefanini and Dapporto 2012).

### 2.4 Metabolism

*S. cerevisiae* is metabolically classified as a facultative-anaerobe. Although it naturally respires, under anaerobic conditions such as wine fermentation, it is capable of converting sugars into ethanol and $CO_2$ as a means to obtain the energy necessary to thrive. Depending on type and concentration of sugars and/or oxygen availability it can display a fully respiratory metabolism, fermentative metabolism or even a mix of both. Two main known effects can help explain this adaptability. The Pasteur effect: in the presence of low oxygen, yeast growth is inhibited and glucose consumption is promoted, resulting in the production of ethanol and $CO_2$. Although the glycolysis rate increases considerably, the energy production efficiency is much lower when compared to respiration (2 moles of ATP vs 32 moles of ATP per mole of glucose respectively) (Lagunas *et al.* 1981, 1986). The Crabtree effect: under high external glucose concentrations, respiration is repressed and fermentation promoted, even in an aerobic scenario where yeast would be expected to produce biomass. It is believed that this phenomenon appeared as a competition mechanism to outrun other species by relying on the antiseptic properties of ethanol. Although *S. cerevisiae* is typically Crabtree-positive, other yeast species can be Crabtree negative, not being able to use such mechanism (Swanson and Clifton 1948; De Deken 1966; Pronk, Steensma and Van Dijken 1996; Pfeiffer and Morley 2014).

#### 2.4.1 Carbon metabolism during wine fermentation

During wine fermentation, sugar (equimolar mix of glucose and fructose, both hexoses) is transported into the cell by several membrane permeases that are part of a hexose transporter family (*HXT*). Throughout the fermentation, these are differentially expressed depending on their affinity to hexoses and sugar concentration in the environment (Perez *et al.* 2005). By the end of the fermentation, 92% of the hexoses will have been converted to ethanol and $CO_2$. The remaining carbon is converted to biomass

(2%) and other by-products with an important contribute for the wine organoleptic balance. Some of these include glycerol, acetic acid, pyruvic acid, succinic acid, higher alcohols and esters.

Alcohol is formed by alcohol dehydrogenase (*ADH1*) from acetaldehyde. The alcohol yield (relative to the sugar consumed) is around $0.47 gg^{-1}$ and among *S. cerevisiae* strains does not vary much (Camarasa *et al.* 2011). Its final concentration is directly related to the initial sugar concentration in the must. As in many other beverages, alcohol in wine fermentation is expressed as a percentage. 1% (v/v) alcohol represents 7.89 g of ethanol and comes from 16.8 g of sugar consumed.

Glycerol is formed from the reduction of dihydroxyacetone phosphate (DHAP) into glycerol 3-phosphate (G3P) by the NADH-dependent glycerol 3-phosphate dehydrogenase (Gpd) encoded by the genes *GPD1* and *GPD2*. G3P is then converted to glycerol by glycerol 3-phosphate phosphatase (Gpp) encoded by the genes *GPP1* and *GPP2*, liberating one inorganic phosphate (Albertyn et al., 1994). Glycerol plays several important roles both in yeast survival and wine properties. For yeast, it plays a crucial role in keeping the redox balance under an anaerobic environment. In such conditions, NADH will accumulate in excess. Glycerol synthesis allows its reoxidation into $NAD^+$ and this way maintains redox homeostasis (van Dijken and Scheffers 1986). It has been demonstrated that without a functional *GPD1* and *GPD2* (Ansell *et al.* 1997) or *GPP1* and *GPP2* (Norbeck *et al.* 1996) yeast cannot survive in anaerobic conditions. Another important role of glycerol is osmoprotection. When submitted to osmotic stress, glycerol becomes *S. cerevisiae*'s main compatible solute and is responsible for maintaining cells optimal turgor, hydration and ultimately viability. As with redox balance, genes related to glycerol synthesis such as *GPD1*, *GPP1* and *GPP2* are induced by osmotic stress (van Dijken and Scheffers 1986; Eriksson *et al.* 1995; Overkamp *et al.* 2002). Another role of glycerol in yeast survival includes being connected to phospholipid biosynthesis (glycerol 3-phosphate is a precursor) thus contributing to ethanol, oxidative and thawing stress resistance (Hohmann 2002; Hallsworth *et al.* 2003). Finally, glycerol can also be used as a carbon source. In fermentation conditions *GUT1* and *GUT2* genes are repressed. However, when in aerobic conditions, and in contact with non-fermentable compounds such as glycerol or ethanol, the genes are activated and the respective proteins are capable of degrading glycerol (Larsson *et al.* 1998). Concerning glycerol's organoleptic contribution to wine, it does not generally affect the aroma profile since, in most wines,

it is found in concentrations ranging 5 to 9 g/L (Scanes *et al.* 1998), which is below the sensorial detection threshold. Instead, its influence is related to a stronger wine body and better mellowness in mouth.

Acetic acid is synthetized through the PDH (pyruvate dehydrogenase) by-pass, being the unique cytosolic acetyl-CoA provider for lipids and sterols synthesis (Flikweert *et al.* 1996; Pronk *et al.* 1996). Here, the enzymes pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and acetyl-CoA synthase convert pyruvate into acetyl-CoA. Acetic acid is the main acid responsible for wine's volatile acidity. This is desirable until a certain threshold (0.8 g/L) after which it contributes a vinegar aroma (unpleasant). In normal circumstances, acetic acid reaches concentrations of 0.2-0.5 g/L, mostly or entirely achieved during exponential phase.

Succinate is a product in the tricarboxylic acid cycle (TCA or Krebs Cycle). Two branches can lead to its production, the oxidative and the reductive one, although the last is the most active when in oenological conditions (Camarasa *et al.* 2003). Succinate is considered a positive contributor to the wine's organoleptic properties, being responsible for some bitterness, freshness and acidity. Its concentration usually varies from 0.5 to 2 g/L (Whiting 1976).

### 2.4.2 Nitrogen metabolism

Nitrogen is a vital element in wine fermentation particularly for yeast growth and fermentation rate. In must, typically it can be found in concentrations ranging from 60 to 400 mg/L (Bely *et al.* 1990a; Bell and Henschke 2005) in different forms, namely peptides (25 – 40%), amino acids (25-30%), proteins (5-10%) and ammonium ions (3-10%) (Ribéreau-Gayon *et al.* 2006). To assimilate these different sources, yeast possesses a wide variety of transporters such as transmembrane transporters and permeases. These have different affinities for nitrogenous molecules, making their assimilation sequential/preferential, particularly in the case of the amino acids (Magasanik and Kaiser 2002). Although nitrogen requirements vary among wine yeast strains, 120 mg/L is considered the minimum required for a proper fermentation. Below that concentration, a nitrogen supplementation is advised, usually done with diammonium phosphate (DAP) (Bely *et al.* 1990b; Palma *et al.* 2012). Without supplementation, there is a high risk of

reducing fermentation rate, growth (biomass), and delaying fermentation completion. Ultimately this can lead to undesired sluggish or stuck fermentations (Bely *et al.* 1990a, 1990b; Martínez-Moreno *et al.* 2012).

## 3. Improving yeast traits

### 3.1 Why?

A wine fermentation is the biotechnological process that needs to occur to obtain the desired wine. Throughout the years, winemakers have been successfully ameliorating it at many different levels. Despite this, the recent decades have been particularly challenging, bringing new and more demanding issues that quickly need to be addressed. 1) climate change: any agricultural activity is dependent on climate and vineyards are very sensitive to it. Some vine cultivars can cope with variations up to 10ºC, others, however, have a tolerance as low as 2ºC (Santisi 2011). This will probably force winemakers to move their vines poleward in the near future. Aside being unpractical and very costly it will most likely change the quality and properties of many grape cultivars that we currently appreciate. In the meantime, with the extra heat and sun, grapes are already accumulating more sugar than usual. Winemakers in some regions such as Alsace are already forced to shorten the growing season by shifting their harvest to some weeks/months earlier (Mozell and Thach 2014). One of the main consequences of extra sugar is the corresponding increase in the final ethanol content in wine, which can alter and spoil the wine's organoleptic properties. 2) Health security: more and more consumers are looking after their health and want wines with less alcohol. Excessive alcohol contributes to important diseases such as heart attacks, obesity and even addiction. Consumers currently want to appreciate a glass of wine without the need to overly worry about their health. Another parallel factor is the stricter and stricter laws against driving under alcohol influence. Wines with lower alcohol content assure a lower probability of legal problems and more importantly of putting life at risk. These points only reinforce the need to combat the problems of climate change in the winemaking context. 3) Innovation and diversity: on top of the high quality, wine consumers more and

more search for a wider range of product diversity. This can be achieved by having wines with stronger aromatic profile or new combinations of organoleptic properties. 4) Process efficiency: as with any other biotechnological process, maximum optimization and efficiency are sought when producing wine. Fermentation duration, energy consumption, equipment usage and limiting the chances of failed fermentation are some of the parameters that can often present additional challenges and represent a field of interest.

For all these ongoing challenges, and many others, surely different solutions can be found. However, wine fermentation process can only be adapted or modified to a limited extent. The main steps and constraints associated have to remain the same and cannot simply be bypassed or the process would lose its authenticity and reliability affecting the final product. Moreover, tight alimentary and safety regulations also reduce the scope to maneuver for modifications and improvements. One possible solution to overcome this is modifying and improving the factor that is tightly connected to every fermentation parameter: the yeast. In the past thirty years much research has been conducted in this sense and yeast has proven to be a valuable asset in finding proper solutions (Dequin 2001; Pretorius and Bauer 2002; Pretorius *et al.* 2012). In fact, for the issues mentioned above, yeast might be the best asset to find proper solutions. Sugar in grapes cannot be altered, however, the fraction that yeast converts to ethanol can be reduced and redirected to other metabolites (*e.g.* glycerol) helping reduce the alcohol content in wine (Kutyna *et al.* 2010; Tilloy *et al.* 2014). New strains have been obtained with a strong aromatic profile (Cadière *et al.* 2011) or with a reduced production of off-flavors compounds such as hydrogen sulfide ($H_2S$) or sulfur dioxide ($SO_2$), considerably improving the wine's organoleptic profile (Noble *et al.* 2015). Finally, improved strains with a higher stress resistance (desiccation, osmotic, ethanol, nutrient starvation…), faster growth or fermentation rate can greatly improve the fermentation process efficiency. This helps reducing fermentation time and avoid the risk of extra costs caused by stuck or sluggish fermentations.

### 3.2 How?

When considering improving yeast, many different techniques are available. The choice of which one to use relies on the available resources, drawbacks and advantages but as importantly, the goal aimed for. The first deliberate strain improvements were based on yeast selection from natural diversity. Isolating a strain that has been demonstrated to perform well under a specific environment is a good way of making sure the desired phenotype is obtained. Evidence from recent population genomics studies point to yeast diversity being immense, most yet to be discovered and explored (Liti *et al.* 2009; Wang *et al.* 2012). Although this diversity can be used in different ways, industrial requirements usually present natural yeasts *per se* with major obstacles for one reason or another to overcome, thus limiting their use. Another pioneer method is mutagenesis carried out by gamma and X-ray or mutagenic compounds such as methylnitronitrosoguanidine (MNG) or Ethyl methanesulfonate (EMS) (Steensels *et al.* 2014). Although simple to perform, this approach delivers quite random results and requires massive clone screening, which can be unpractical depending on the phenotype being tested (Cebollero *et al.* 2007). Additionally, even if a positive mutant is found, it is likely that other phenotypes are affected due to random mutagenesis. Improvement by sexual mating is also among the first techniques used. It is a good method to mix specific genomes of interest and generate new phenotypes. It can be done intra or even interspecies as it was shown by Libkind *et al.* 2011 for *S. pastorianus*, which is an interspecific hybrid between *S. cerevisiae* and *S. eubayanus* that would have "accidently" been formed in fermentation tanks of lager breweries and be more efficient fermenting at low temperatures.

In wine making, yeast mating has been used for improving wine organoleptic properties such as lower $SO_2$ production and reduced foaming (Eschenbruch *et al.* 1982) as well as practical features like flocculation (Thornton 1985). Despite this, a major drawback is that wine yeast strains are particularly difficult to mate due to the low spore viability and homothallism typical of this group (Pretorius 2000b). With the development of better genetic/molecular biology tools, the 1970's and 1980's opened a wide range of new possibilities with genetic engineering. One could improve virtually any phenotype as long as having the proper knowledge for it. Many approaches were based on the principles of recombinant DNA technology that allowed the development new yeast

strains for industrial purposes (Dequin 2001). This pushed research even further and consequently much genetic and metabolic knowledge was generated.

However, the practical and industrial applications of such advances soon hit a wall. Almost as soon as the genetic engineering era was born, public concerns about the safety of the so-called genetically modified organisms (GMO) were raised. The lack of background and studies to assess their impact on food safety, public health and environment led to the creation of strict regulations and legislation during the 1990's. Since then, several European Regulations (*e.g.* EC258/97, EC1829/2003, 65/2004) have been issued to regulate every aspect of GMO use in the EU (Pérez-Torrado et al, 2015). Around the world, each country or region has its own policies regulated by different internal institutions (*e.g.* "European Food Safety Authority" in Europe, "Food and Drug Administration" in USA). Their guidelines for cultivation and importation can range from completely prohibited to freely allowed. This makes GMO a complex subject to deal with at the international level (Pérez-Torrado *et al.* 2015). In oenology, different strains were genetically modified, for instance, to obtain better aromatic profiles (Pérez-González *et al.* 1993; Ganga *et al.* 1999; Manzanares *et al.* 2003) or to overproduce glycerol and reduce ethanol yield (Michnick *et al.* 1997; Remize *et al.* 1999). However their use in the EU and other countries is far from simple: long and costly administrative procedures, international and local regulations (*e.g.* International Organization of Vine and Wine – OIV and "appellation d'origine" respectively), consumer distrust and the desire to keep the process within traditional boundaries point to a future in the wine industry without GMO (Cebollero *et al.* 2007). Due to all the GMO restrictions, in the last few decades new effective non-GMO alternatives for strain improvement have been developed and improved. Two of the most popular and successful techniques are the use of Quantative Trait Locus (QTL) mapping approaches and Evolutionary engineering. In the next sections, their principles and applications will be detailed.

## 3.3 Evolutionary Engineering

### 3.3.1 What is evolution?

Talking about evolution is referring to one of the most important mechanisms to which every life form that exists, or has existed, is submitted and ultimately results from. In short, evolution can be defined as the change in genetic and heritable characteristics of a given population over successive generations. This concept has been behind species definition as in the creation of new species (speciation), change within species (anagenesis) and loss of species (extinction). After traveling around the world collecting much data, Charles Darwin published the book *On the Origin of Species* mid-19$^{th}$ century where he explains his theory of evolution based on natural selection. To this day, aside from minor revisions, this theory is the one broadly accepted in the scientific community to best explain evolution. The theory bases itself on three main principles: 1) genetic variation occurs among individuals of the same species in a given population leading to phenotypic variations as in morphology, physiology and behavior traits. 2) in each specific environment, different traits confer different survival rates and different reproduction chances, yielding a differential fitness. 3) Upon natural selection, advantageous traits can be passed from generation to generation in a stable heritability. If these principles are combined, the progeny of the fittest (best adapted) in a given environment will gradually replace the members of a population and take over. Nevertheless, genetic diversity never stops being generated and submitted to natural selection. In the case of a sudden environment change, this is one of the main mechanisms on which species relies to keep thriving and avoid extinction. How can one go from these principles to an improved wine yeast is what will be discussed in the next sections.

### 3.3.2 The principles

Evolutionary engineering, also designated as adaptive, directed or experimental evolution, is the process by which the evolution principles are applied to a population in a known and controlled environment in order to test a hypothesis and aim for particular

outcome. As in Darwin's evolution theory, the process is based on the assumption that genetic variation is constantly generated in a given population allowing the differentiation of individuals and their consequent selection (Dragosits and Mattanovich 2013). This variation comes from mutations that occur at a determined rate and can be accumulated over time. If submitted to specific constraints, individuals initially not optimally adapted may evolve and gain a higher fitness when positive mutations accumulate. As the fittest, these individuals will be able to better utilize the available resources, grow faster and multiply faster/in higher number. This can be used as a measure of evolution or higher fitness when comparing the capacity/rate of individuals successfully reproducing within a specific environment (Elena and Lenski 2003). The natural course is then for their progeny to gradually replace the individuals of the population and take over. Evolved individuals can be newly submitted to the evolutionary conditions and compared with the parental strain, separately or in competition in order to evaluate the relative improvement of the phenotype (Elena and Lenski 2003).

### 3.3.3 Evolving a microbial population

Multiple evolutionary engineering experiments have been performed with different organisms such as *Drosophila* (Rose *et al.* 1992), domestic mouse (Dohm *et al.* 1994; Dumke *et al.* 2001), the unicellular algae Chlamydomonas (Colegrave *et al.* 2002), *Pseudomonas fluorescens* (Rainey and Travisano 1998), *Escherichia coli* (Lenski and Travisano 1994) and *S. cerevisiae* (Paquin and Adams 1983). Although the principles stay the same, evolutionary experiments performed with unicellular microorganisms differ in some aspects from those with multicellular ones. For the former, evolution is measured at the population level rather than at the individual one. They also present some advantages such as a high number of individuals within the same population, being easy to maintain/grow and a fast generation time. Microbial evolutionary engineering approaches are typically done in one of two ways: serial cultivation (batch) or continuous cultivation (chemostat). Both are equally valid but may lead to evolved strains with different phenotypes. Their selection will depend on the experimental conditions and objectives. With the former, the principle is to aliquot the culture into new fresh medium at regular intervals. Often performed in shake flasks, due to manipulation easiness and

economic maintenance this method allows several parallel cultures. On the downside, batch cultures are prone to some uncontrolled fluctuations in population density, growth rate or dissolved oxygen (Dragosits and Mattanovich 2013). Serial batch propagation usually selects for microorganisms with shorter lag phase or higher growth rate but certain regimes might also allow the selection of higher biomass formation or a better survival after nutrient depletion (Cadière *et al.* 2011; Tilloy *et al.* 2014; Bachmann *et al.* 2015; Rabbers *et al.* 2015). As for continuous cultivations, bioreactor vessels are typically used. Here, all experimental parameters such as medium influx rate, temperature, oxygenation and pH are continuously monitored leading to constant growth rates and populations densities. Continuous cultures favor selection for higher substrate affinity (Bachmann *et al.* 2015). The major disadvantages are the much higher costs and a limitation in parallel experiments, depending on how many chemostats are available (Dragosits and Mattanovich 2013).

### 3.3.4 The possible outcomes

Although evolutionary engineering is based on principles that have driven evolution across eras, these approaches are characterized by a degree of empiricism (Pérez-Torrado *et al.* 2015). Contrary to other approaches, an evolutionary engineering process can be performed with little or no knowledge while still achieving a successful outcome. A proper initial phenotypic characterization under the selective pressures being tested is usually enough. During the experiment, the mutations and therefore the diversity generated, are completely random and cannot be predicted or controlled (Luria and Delbrück 1943). In fact, if two or more identical but independent populations are submitted to the same evolutionary experiment, different outcomes can occur: 1) one or more populations do not evolve into a higher fitness. This may indicate that selective pressures need to be reviewed as they might not be suited or strong enough. 2) populations will present an identical higher fitness. In this case, they may or may not be genetically identical. If not, it means that the same solution (evolved phenotype) was obtained with different mutations. 3) One or more populations present different phenotypes, including a higher fitness or not. Again, this may indicate the selective pressures need to be reviewed. Their optimization plays a key role in the selection power and obtaining a

positive result (*i.e.* improved fitness). In case of 2), the analysis of its outcomes has a high potential to generate new knowledge. By studying the different outcomes and correlating them to the obtained phenotypes, one can gather important knowledge. Not only specific mutations that led to a particular phenotype can be identified but also which different genes and pathways regulate the phenotype of interest.

Finally, in the case of a positive evolution, the search for the presence of trade-offs is advised. A trade-off occurs when a particular phenotypic trait gets improved at the expense of one or more other phenotypic traits. This becomes particularly important for strains with an industrial purpose. This is illustrated by a study by Wenger *et al.* 2011 who successfully evolved *S. cerevisiae* for a higher fitness in anaerobic glucose limited media. However, when in aerobic, carbon rich environments, the evolved clones performed less well than their ancestor resulting from a trade-off. Similarly, yeast cells evolved for efficient galactose consumption (Hong *et al.* 2011) presented trade-offs when grown on glucose as a carbon source. In opposition to trade-offs, there is the cross-protection, also verified in yeast. As an example, Çakar *et al.* 2009 evolved *S. cerevisiae* for higher cobalt resistance and later verified a parallel higher resistance to other metal ions and thermal and oxidative stress.

Deleterious mutations frequency increase their probability with time (*i.e.* number of generations). This means finding a good compromise between reaching the best fitness possible and the time during which the population is submitted to the evolutionary conditions. A concept related to trade-off is hitchhiking. Here, mutations (neutral or deleterious) increase their frequency in a population by being linked to a positive mutation that is being selected. To note that a different concept from evolution is plasticity. The last refers to the versatility of a genome to adapt to different environments versus the former that concerns the selection of inherited positive genetic modifications.

### 3.3.5 Evolutionary engineering applied to yeast

As a non-GM technique, evolutionary engineering is today a very popular approach to improve microorganisms. It started to be widely used more than a decade ago (Portnoy *et al.* 2011) and since then yeast has been the focus of many studies within this scope. When it comes to the strain choice, haploid strains have the advantage of evolving faster and

making it easier to later on detect which mutations occurred (Gerstein *et al.* 2011). However diploid strains have the ability to more often buffer deleterious mutations in opposition to haploids where they become lethal more easily. This way diploid strains tend to be more stable and robust throughout the process.

Main subjects investigated are often related to improving substrate utilization such as lactose (Guimarães *et al.* 2008) or xylose and arabinose from lignocellulosic feedstocks (Wouter Wisselink *et al.* 2009; Garcia Sanchez *et al.* 2010) as well as resistance to several stress factors like freezing/thawing, oxidative, osmotic, temperature and ethanol stress (Çakar *et al.* 2005, 2012; Wei *et al.* 2007; Dinh *et al.* 2008; Ekberg *et al.* 2013). Different laboratory strains have been used in such approaches to generate knowledge about these and other phenotypes. Nevertheless, using industrial strains can also be of major interest. While still generating academic knowledge, their phenotype and therefore performance, can be improved (Çakar *et al.* 2012; Steensels *et al.* 2014; Bachmann *et al.* 2015). To achieve it, evolutionary engineering should occur in conditions as close as possible to the actual conditions in which the strain will later perform. By doing so, yeasts will not only face the selective pressure chosen for evolution but also all the remaining constraints/stress factors naturally present (Çakar *et al.* 2012). For instance, during brewing fermentations, yeasts need to cope with stress factors as diverse as low pH and temperature, nutrient deprivation, ethanol, osmotic and oxidative stress (Gibson *et al.* 2007). A similar scenario is also valid for oenological conditions where yeasts need to adapt to several stress factors naturally present during wine fermentation (Querol *et al.* 2003).

While using a long-term batch culturing on gluconate (a carbon source poorly assimilated by *S. cerevisiae*), Cadière *et al.* 2011 evolved a commercial wine yeast strain obtaining interesting results. Evolved clones presented a carbon flux through the pentose phosphate pathway increased by 6% when compared to the parental strains. This also resulted in a higher fermentation rate, lower levels of acetate production and increased production of aroma compounds. As the process was carried out at laboratory scale but in realistic (oenological) conditions, the same phenotypic improvements were verified when the evolved strain was used in pilot-scale trials (Cadière *et al.* 2012). It was identified that the evolved strain produced higher levels of phenyl ethanol, isobutanol, isoamyl alcohol, ethtyl acetate, isoamyl acetate and ethyl esters (Mouret *et al.* 2014). Other authors were able to obtain a stable wine yeast strain with slightly enhanced

glycerol production. By employing sulfite as a selective agent in an alkaline pH, Kutyna *et al.* 2012 obtained evolved clones with an increase of 41% in glycerol production, which can have a benefic impact in wine organoleptic properties. To reduce final ethanol content in wine, Tilloy *et al.* 2014 submitted a wine strain to hyperosmotic stress for 200 generations, which yielded evolved clones that survived better under osmotic stress, glucose starvation and produced markedly more glycerol, but also succinate and 2,3-butanediol. The approach was then complemented with an intra-strain breeding strategy that further increased the glycerol yield and reduced ethanol production in wine by up to 1.3% (v/v). Recently, López-Malo *et al.* 2015 performed an evolutionary process aiming for a higher performance for low temperature fermentations (12ºC). It was discovered that inositol and mannoproteins limitation were responsible for an evolution towards shorter fermentation times and a higher final population. After genome sequencing, it was discovered that a SNP in the gene *GAA1*, fundamental in inositol and mannoprotein synthesis, was at the basis of the improvement.

As in some of these works, after confirming a positive evolution, it is of interest to understand which genetic variations occurred during the evolutionary process and how the evolved strains differ from the parental one. Approaches to achieve this include genome microarray hybridization and direct DNA sequencing (Dunham *et al.* 2002; Hong *et al.* 2011; López-Malo *et al.* 2015).

### 3.4 Quantitative Trait Locus

The latest advances in genome sequencing techniques together with the associated diminishing costs have generated much genetic and molecular knowledge in recent years. With the GMO restrictions prevailing, new alternatives to make the link between that knowledge and practical outcomes have also been widely developed. Quantitative Trait Locus or QTL mapping is one of the most popular approaches being used in that sense due to its accuracy and great potential for academic and practical outcomes. The principles and applications of QTLs will be discussed in the next sections.

### 3.4.1 Fundamentals of QTL mapping

QTL is an approach used to establish a link between a region of the genome (locus) and a phenotypic variation (the quantitative trait). This approach is particularly powerful when the variation of a trait is controlled by different loci scattered along the genome. This is the case for most quantitative (continuous) phenotypes in opposition to qualitative (discrete) ones. Aside identifying the loci (and therefore the genes) implicated in a particular phenotype, it also allows evaluation of the impact of different alleles of the same gene. The first study using the principles of QTL was done almost 100 years ago (Sax 1923). At the time, Sax performed a genetic analysis correlating the size of beans with the color of pigmentation. Shortly after, the concept was applied to agriculture and since then has been widely used in many different organisms such as *Drosophila melanogaster* (Mackay 2004), *Arabidopsis thaliana* (Shindo *et al.* 2007), crops (Würschum 2012) and in yeast (Steinmetz *et al.* 2002).

### 3.4.2 The setup of a yeast QTL approach

A classical QTL approach is based on the progeny of two parental strains. These strains have their genome sequenced and are phenotyped for the trait of interest. Selecting the correct parents is one fundamental step for a successful approach. Both need to be capable of reproducing sexually and, optimally, have a contrasting phenotype for the trait being studied. Once crossed, the diploid sporulates and a progeny (F1) of haploid segregants is generated. Each segregant has now a unique recombination of the parental genomes generated during meiosis. Often, F1 is used to generate a F2 generation. This greatly increases the recombination rate which in turn will later increase the resolution and accuracy of the QTL mapping. A high number of individuals is also preferable. Aside increasing the genomic diversity (unique recombination) within the population analyzed, the linkage analysis between genotype and phenotype is statistically reinforced. This is perfectly illustrated with the example of the work of Demogines *et al.* 2008 and Ehrenreich *et al.* 2010. The former used 123 segregants to test 4-Nitroquinoline 1-oxide (4-NQO) sensitivity and found one single locus related to that phenotype. The latter used

a much higher number of segregants ($10^7$) and identified thirteen additional loci that showed a significant linkage to 4-NQO sensitivity.

### 3.4.3 Genotyping

The QTL mapping analysis is based on the distribution of the allelic variation. The key relies in identifying molecular markers that correlate a genomic region to a phenotype and simultaneously reveal the parental origin of such region. Molecular markers are defined as polymorphic DNA sequences that can vary from one single nucleotide to several thousands. The most common molecular markers used are SNPs or INDELs. Different methods exist to detect these markers and perform the genotyping (Swinnen et al 2012). An example is the high-density oligonucleotide arrays first described by Winzeler *et al.* 1998. Here, the genomic DNA of strains is hybridized with high-density oligonucleotide arrays. Depending on the hybridization differences (and therefore the sequence), a fluorescent signal is emitted with different intensities that can be quantified. Although many probes (oligonucleotides) need to be synthesized, hybridization differences can be used as markers. QTLs for different phenotypes have been identified using this technique such as for high temperature growth (Sinha *et al.* 2008), sporulation efficiency (Deutschbauer and Davis 2005) and cell morphology (Nogami *et al.* 2007). Another method used is the whole genome re-sequencing using next-generation sequencing technology (Metzker 2010). Initially expensive, its gradual popularization has made it one of the first choices for genotyping due to its sequencing volume capacity, accuracy and availability of the whole genome (and therefore all markers). The principle is to physically separate short single DNA molecules that will endure up to millions of sequencing reactions. These will constitute partially overlapping sequences called reads that can be assembled *de novo* but are more commonly aligned to a known reference (Metzker 2010). This is done for parental strains (control pool) as well as for the selected one. The final step is to align the reads against one of the parental sequences and statistically analyze the difference in frequency of each molecular marker between the control and selected pool. Ehrenreich *et al.* 2010 used this method to study a large progeny ($10^7$) set submitted to 17 diverse chemical agents. They discovered that the resistance to those was genetically variable, going from one major locus for some traits to 20 loci for

others. Parts *et al.* 2011 used a similar approach to identify loci correlated to high temperature growth. By crossing an oak tree bark strain tolerant to high temperature and palm wine strain not tolerant, they produced a F12 segregant progeny (highly recombinant). Ultimately, this allowed a high resolution in QTL identification that yielded new genes not identified so far as relevant for the trait. Finally, Bulk Segregants-Analysis (BSA) is also another approach (Michelmore et al, 1991). Segregants that present the same phenotype are pooled together (usually the trait of interest *vs* absent or poorly expressed) and both bulks are sequenced. By analyzing the allelic frequency, important gene variants will be detected unveiling genes that play a role in the phenotype of interest. Independently of the genotyping technique, the goal is to generate a genetic map of each chromosome based on the molecular markers. The more these are numerous and homogenous along the genome the more accurate will be the coverage and the QTL identification.

### 3.4.4 Phenotyping

Parents and segregants have to be phenotyped for the traits of interest (one or several). The use of enough replicates to validate the phenotypic data is crucial for several reasons: 1) assure that the experimental variability is low and not biasing measurements. 2) verify that the phenotype being measured is not influenced by other environmental conditions. If so, few of its variation will be genetically explained and therefore low probability to identify QTLs associated. 3) reinforce the phenotypic data collected and certify a good reproducibility. The type of phenotype distribution can give the first information about how the phenotype is controlled. The most common distribution found is the Gaussian which typically results from a trait controlled by different genes. Depending on the parents' positioning, it is possible to assess the type of contribution the alleles are giving and how they interact. Another possible distribution is the bimodal which means that measurements will consist of two distinct groups. This is explained when a particular allele has a major contribution to the phenotype. Bimodal distribution is less frequent as most phenotypes tend to be complex and be under polygenic control.

### 3.4.5 Statistical and linkage analysis

Once the genotyping and phenotyping are finalised, it can be determined if the markers found have a link or not to the phenotype of interest. For that, different statistical methods can be used to assess a significant linkage or not. Interval-mapping, based on genetic maps and distances between markers, is an alternative considered more powerful than the previous by including extra genetic and structural information (Broman 2001). When dealing with bulks, the analysis will focus on imbalanced allele proportions within the bulks. Alleles that appear detached, both in the "strong" and "weak" pool should be directly related to the phenotype.

### 3.4.6 QTL dissection and identifying candidate genes

Once the QTLs are identified, each one will delimit a particular region of the genome giving a certain probability of an impacting allelic variation being located there. This region can go from few hundreds to thousands of thousands of Kbs. The more precise the QTL peak is, the smaller it will be. The challenge is to analyze each region and search for the gene or genes that, according to their biological function, are likely to play a role on the trait of interest. However, one always needs to keep an open mind when dissecting QTLs. Genes, first perceived as completely unrelated to the phenotype, might actually play a role in the trait of interest if well located within the peak. This is why QTLs have been major drivers of "unrelated" genes discoveries. Databases with the annotation of the genes in these regions are available, as well as their biological function. Other information that helps in the dissection is gained by analyzing the parental sequence of that region to determine which parent contributed the alleles. Once a candidate gene (or several) is chosen, it has to be validated experimentally. This is usually done by performing a Reciprocal Hemizygosity Analysis (RHA) or allelic replacement. In short, two strains will be phenotyped while having the same exact genetic background with the exception of an allelic variation of the candidate gene. Once the candidate gene is validated, it is possible to identify the DNA polymorphism that is behind the phenotype modulation (Steinmetz *et al.* 2002). Ultimately, the identified allele can be then introduced into other

yeast strains by breeding using the allelic variant(s) as marker(s). This is particularly interesting and valuable for industrial yeast strains where there are less constraints due to GMO regulations.

### 3.4.7 QTL applied to wine yeast

An increasing number of studies are being done focusing on yeast applications. Naturally, wine yeasts have been the focus of several researchers trying to unveil new information on phenotypes of interest for winemaking. As a consequence, many new genetic variants have been discovered contributing to a large catalogue of alleles that modulate complex traits. Ultimately, these alleles represent an important genetic source of potential yeast improvement within a winemaking context (Legras *et al.* 2017).

With that they also corroborate the academic and practical added-value that a QTL approach can bring. Marullo *et al.* 2007 was one of the first to do so by studying acetic acid production during wine fermentation. Based on 108 segregants and more than 2000 markers identified, a particular allele of the gene *ASP1* (coding for an asparaginase) was found relevant for the phenotype. Based on a single SNP, this allele was responsible for canceling the respective enzyme activity, which has an impact on nitrogen assimilation and fermentation rate. Ambroset *et al.* 2011 also found interesting results in the same sense, however this time related to an allele of the gene *ABZ1*, implicated in the biosynthesis of p-aminobenzoate, after using S288C and a haploid derivative of the industrial wine yeast EC1118 as parental strains. Results pointed to a possible defective *ABZ1* allele common to laboratory strains. Using the same crossing, Steyer *et al.* 2012 found an additional phenotype associated to defective *ABZ1*, namely a lower production of some metabolites including 2-phenyl ethanol. Other QTLs related to production of aromas such as ethyl esters and nerolidol were also identified to be related to *PLB2* and *PDR8* respectively. Directly related to lag phase, Zimmer *et al.* 2014 found a QTL that identified a chromosomal translocation positively affecting lag phase duration in wine fermentation. This translocation, between chromosomes XVI and XV, concerned the promoter region of *ADH1* and the gene *SSU1* coding for a sulfite pump. As with translocation $XVI^{VIII}$, strains possessing this newly identified translocation had an advantage in resisting $SO_2$ stress, particularly during the lag phase. As with the

translocation $XVI^{VIII}$, it is thought to be a result of domestication, however a more recent event due to a lower level of dissemination among wine yeast. In another domain, Noble *et al.* 2015 crossed two industrial wine strains, one high sulfite producer, and the other low. By using 60 segregants issued from this hybrid, the QTLs obtained identified *MET2* and *SKP2* as responsible for differences found in sulfite production (undesired) during stationary phase. When focusing on nitrogen metabolism, Cubillos *et al.* 2017 successfully identified a series of QTLs by using a highly recombinant population resulting from a strategy of intercross between 4 parents. The approach allowed the identification of several genes contributing to nitrogen metabolism such as *CPS1*, *LYP1*, *ALP1*, and *ASI2*. These would contribute to variations in nitrogen consumption by adding small phenotypic effects rather than drastic impacts. Finally, a recent QTL approach was performed to elucidate low temperature adaptation during wine fermentation (García-Ríos *et al.* 2017). By using two industrial *S. cerevisiae* strains with an opposite performance at low temperature, the authors used a bulk segregant strategy on F13 containing 300 segregants from that crossing. Results showed a particular role of some subtelomeric regions that need to be further sequenced to reveal the specific alleles that re responsible for the phenotype.

## 4. Non-conventional yeasts for oenological applications

### 4.1 The context for non-conventional yeast

*S. cerevisiae* is the first choice for most biotechnological processes involving yeast. Its robustness, reliability and capacity to perform well under many different and stressful scenarios have pushed the use of the species much further both in research and in applications. Nevertheless, biotechnological processes often face bottlenecks and new alternatives are required. One possible solution was partially covered in the previous section (3), which is improving the strains already available. However, in the recent decades, a solution that is as new as old has resurfaced with more and more interest: exploiting yeast natural diversity. Here, non-conventional yeasts definitely play an important role. Until recently, they were regarded as under-performers as well as hard to

work with, both academic and industrially, due to the lack knowledge and data available. The new technological advances and tools, some developed for *S. cerevisiae* in the first place, have made the discovery, characterization and improvement of these yeasts more accessible. Their current applications are as diverse as high titer lipid production (*Yarrowia lipolytica*), improved sugar alcohol production (*Pichia anomala*), xylose and cellobiose fermentation for ethanol production (*Hansenula polymorpha*), production of biopharmaceutical compounds of interest (*Komagataella pastoris*) and carotenoid production (*Phaffia rhodozyma*) (Ryabova *et al.* 2003; Vogl *et al.* 2013; Blazeck *et al.* 2014; Mannazzu *et al.* 2015; Zhang *et al.* 2015)**.** The current tendency is to explore this potential, particularly in applications where *S. cerevisiae* can be outperformed or a unique contribution can be added.

### 4.2 Wine fermentation can be more than *S. cerevisiae*

Wine fermentation, as a biotechnological process that relies on yeast, can also benefit from the exploitation of non-conventional yeasts. For most of the oenology history, *S. cerevisiae* was the only desired species to perform wine fermentation. The natural flora of a must contains a wide diversity of species just prior to fermentation but these were always unwelcome due to the potential of fermentation and wine spoilage (Padilla *et al.* 2016). However, despite the advantages of using a pure starter culture that delivers an efficient fermentation process, final wines can lack a certain organoleptic complexity, only achieved when other yeasts have the chance to give their contribution (Jolly *et al.* 2014; Padilla *et al.* 2016). Adding to a current demand by consumers for a more unique and pleasurable sensory experience, wine yeast research and industry had to rethink this paradigm.

In the last decades, different non-conventional yeasts have been used to add value to wine fermentation. Some of those include *Torulaspora*, *Candida*, *Hanseniaspora*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Lachancea* and *Pichia* species (Jolly *et al.* 2014). Such species are frequently poor fermenters and not capable of performing a full fermentation without *S cerevisiae* addition. When present, however, their contribution can be quite important. Aromatic compounds such as terpenoids, esters, higher alcohols, glycerol, acetaldehyde and succinic acid, as well as improvement in color stability or

reduction of the final ethanol concentration are some of the advantages these yeasts can provide (Benito *et al.* 2011; Jolly *et al.* 2014; Gamero *et al.* 2016; Varela 2016a; Mestre Furlani *et al.* 2017).

One of the best ways to take advantage of this is by using mixed starter cultures or sequential inoculations. In this way, the advantages of selected non-conventional yeasts are achieved, while the risk of fermentation process failing is still low. Comitini *et al.* 2011 selected four non-*Saccharomyces* wine strains to test under oenological conditions. Among them, a *Lachancea thermotolerans* strain showed interesting results when combined with *S. cerevisiae*. The volatile acidity and pH were considerably reduced together with an increase in glycerol and 2-phenyethanol content. Due to climate change and higher temperatures that tend to increase musts' pH (Mira de Orduña 2010), the increase of the total acidity of wines is very desirable nowadays. In this case, *L. thermotolerans* could be used as an acidifying agent and positively contribute to wine stability. Dashko *et al.* 2015 found a positive contribution of *Kazachstania gamospora* and *Zygosaccharomyces kombuchaensis* to the production of Ribolla Gialla wine. In a sequential inoculation with an industrial *S. cerevisiae* (first at laboratory scale then at a pilot one), the authors verified a considerable increase in the production of esters and higher alcohols such as 2-phenylethyl acetate, ethyl propionate and 2-phenylethyl alcohol. Ultimately, this increased the aromatic complexity of such wines, which were otherwise quite plain. Anfang *et al.* 2009 also found interesting results, this time concerning thiols production. These are responsible for fruity aromas such as tropical, passion fruit and guava-like nuances, very appreciated in wine. In co-fermentation with *S. cerevisiae* for Sauvignon blanc production, a strain of *Pichia kluyveri* produced 3-mercaptohexyl acetate (3MHA) at concentrations much higher than the detection threshold. Finally, rather than an impact during fermentation itself, non-conventional yeast can also positively contribute once wine fermentation is over. Belda *et al.* 2016 discovered that *Torulaspora delbrueckii* can release high quantities of mannoproteins during wine ageing on lees, up to values even higher than a *S. cerevisiae* overproducer strain. Mannoproteins are important contributors for the mouthfeel, roundness, aromatic prevalence and protein and tartare instability, all features that can greatly improve a wine's quality (Vidal *et al.* 2004; Chalier *et al.* 2007; Gonzalez-Ramos *et al.* 2008).

Although many parameters require a fine-tuning in these mixed cultures (*e.g.* temperature, moment and rate of inoculation, type of fermentation), there is no doubt that

overall, non-conventional yeasts can give a precious and positive contribution to wine fermentation. On the other hand, these always need to be closely surveilled when in association with *S. cerevisiae* as they can also be a source of undesired defects. These include excessive production of acetic acid, ethyl acetate, diacetyl, sulfites, higher alcohols among other compounds that if not controlled, can lead to off-flavors and negative organoleptic properties (Loureiro and Malfeito-Ferreira 2003; Jackson 2008a).

### 4.3 Is *Kluyveromyces marxianus* an adequate non-conventional yeast for wine fermentation?

*K. marxianus* is a non-conventional yeast within the *Saccharomycetales* order. The main features of the species include rapid growth rates (faster than *S. cerevisiae* in rich yeast media), thermotolerance (up to 52ºC), lactose utilization (absent in *S. cerevisiae*), a good tolerance to inhibitory molecules and a diverse and flexible physiology (Fonseca *et al.* 2008; Varela *et al.* 2017). Such features have naturally attracted attention for both academic and industrial purposes, which together with the sister species *K. lactis*, has promoted their study, particularly in the last decade (Lane and Morrissey 2010; Jeong *et al.* 2012; Johnson 2013; Varela *et al.* 2017). As a consequence, *K. marxianus* has today a diverse set of biotechnological applications.

A primary application is the production of native enzymes (β-galactosidases, inulinase and pectinases) as well as heterologous proteins at interesting yields (Fleer 1992; Romanos *et al.* 1992; Lane and Morrissey 2010). These can be then extracted and purified for further use in other biotechnological processes. Another important application is bioethanol production. Growing interest has been dedicated to the fermentation of non-food crops and crop wastes. These materials are mainly composed of cellulosic material, which not all yeasts (including *S. cerevisiae*) are capable of naturally utilizing. It has been shown that *K. marxianus* can be very effective fermenting cellulosic material at high temperatures (Yanase *et al.* 2010) to the point where the saccharification step could be combined with the fermentation one, yielding a process with one single step (Hong *et al.* 2007). Another substrate the species is capable of fermenting is whey permeate from dairy wastes by being able to import and use lactose as a primary source of carbon (Guimarães *et al.* 2010).

However, in an oenological context, the most attractive feature of *K. marxianus* is the capacity to produce particular aromatic compounds such as 2-phenylethanol (2-PE), ethyl acetate and other acetate esters, which can provide fruity and floral fragrances (Wittmann *et al.* 2002; Löser *et al.* 2014; Morrissey *et al.* 2015; Varela *et al.* 2017). To date, very few or no work has been done in strict oenological conditions assessing *K. marxianus* organoleptic contribution. Its ability to produce considerable amount of these compounds in different contexts has, however, been studied. After starting with a diverse set of species, Etschmann, Sell and Schrader 2003 discovered that *K. marxianus* was the one capable of producing the higher amounts of 2-PE when using molasses as a matrix. López-Alvarez *et al.* 2012 isolated a natural *K. marxianus* strain from agave and then compared with a *S. cerevisiae* strain for agave fermentation and tequila production. Results showed higher concentrations of particular esters and higher alcohols such as 2-hydroxyethyl propionate, 2-phenylethyl acetate and ethyl dodecanoate as well as other volatile aromas like 2,3-butanedione and isoamyl alcohol. Additionally, fermentations with *K. marxianus* strain yielded higher alcohol content indicating a better efficiency in fermenting the available sugars. Garavaglia *et al.* 2007 tested the production of 2-PE in grape must while trying to optimize different parameters (pH, temperature and L-phenylalanine (L-PHE), the precursor of 2-PE). The results showed that grape must can be a good matrix to maximize 2-PE production. The authors were able to achieve titers of 0.62 g of 2-PE/ g L-PHE, which is close to the maximum theoretical yield of 0.75 (w/w) when at 37ºC, pH 7.0 and 3 g/L of initial L-PHE. Although the typical wine fermentations conditions differ from these, the fact that grape must was a good matrix for 2-PE production opens the possibility for aromatic contributions from *K, marxianus*. Finally, Kourkoutas *et al.* 2004 used immobilized and free cells of a *K. marxianus* strain that showed positive organoleptic contributions when fermenting grape must at high temperature. Although not all sugars were consumed, no off-flavors where detected and an overall pleasant aromatic profile was obtained, suggesting a possible application for wine blending. All these works point to a potential of *K. marxianus* in oenological conditions that so far has been poorly exploited. The fact that the species carries the GRAS (US – Generally Regarded As Safe) and QPS (European Food Safety Authority – Qualified Presumption of Safety) labelling makes it a perfect non-conventional yeast candidate to be applied in the food and beverage industry.

## 5. Yeast stress response and the lag phase importance

### 5.1 Generalities about stress response

Independently of the species, yeast is naturally equipped with an arsenal of different mechanisms and strategies to cope with stressful conditions. This is fundamental for their survival in natural habitats. Although these can highly diverse, scarce nutrients, temperature and pH oscillations, osmotic stress, toxic and inhibitory compounds, as well as competition with other microorganisms are some of the common stress factors yeast often has to face. In anthropogenic environments, such as biofuel production, baking or winemaking, yeast has adapted to respond to the specific stress factors of those environments in order to keep the best yields and quality products.

When facing one or several stress factors, the yeast response can be mainly divided in two stages: early stage response and late stage response. The first is based on mechanisms that guarantee yeast survival and quickly repair damages affecting vital functions. Once this is assured, a late stage response takes over and yeast's metabolism is shaped to the best setting possible. This will optimize yeast growth and vitality in those particular conditions. This dual response is translated into differential enzymatic activity as well as gene expression. Schade *et al.* 2004 observed these transient changes in gene expression when studying the yeast response to cold shock (30ºC to 10ºC). The authors identified an early cold response (ECR) occurring in the first 2h of the cold shock whereas a late cold response (LCR) was observed between 12h and 60h. For the ECR, genes induced such as *OLE1* were mainly associated to lipid and amino acid metabolism, transport and transcription. LCR on the other hand, included overexpression of genes directly related to stress response such as *HSP104*, *HSP12* (heat shock proteins), *GPD1* (osmotic stress), *TPS1, TPS2* (trehalose metabolism), *GPH1* and *GDB1* (glycogen metabolism). Gasch *et al.* 2000 used the DNA microarray technique to study the yeast genomic expression variation when facing a wide range of different environmental changes. These included osmotic and oxidative stress, different temperature shocks and nutrient starvation. Although in a shorter time-window (thoroughly followed for 60 minutes), the authors were also able to observe transient changes in gene expression. Immediately after stress

exposure, cells responded with rapid and dramatic alterations in gene expression, specifically related to that stress factor but also with responses (often called ESR - environmental stress response) common to other stress factors. For each stress factor tested a clear "emergency" stress response strategy was found demonstrating a transient gene expression. Another conclusion is that the timing and duration of these transient changes were dependent on the severity of the stress. The more severe, the more delay there is in the response, which in turn also tends to be stronger and more sustained.

The transcription factors encoded by *MSN2*/*MSN4* are the main regulators of this general response (Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996). Msn2p/Msn4p zinc finger proteins bind as a heterodimer to a specific nucleotide sequence (CCCCT functional in both orientations) required for the gene activation. This sequence, called STRE (for STress Responsive Element) is located in the promotor region of numerous genes related to stress (Kobayashi and McEntee 1993; Marchler *et al.* 1993). In the absence of stimulus (*i.e.* stress), both transcription factors are phosphorylated by protein kinase A (PKA) (in the presence of cAMP) which prevents their migration from the cytoplasm to the nucleus (Görner *et al.* 1998). However when facing a stress factor, PKA activity is reduced, Msn2p and Msn4p are hyper-phosphorylated causing their migration to the nucleus where they activate a particular global stress response (Garreau *et al.* 2000).

Among the genes controlled by *MSN2*/*MSN4* are the catalase gene *CTT1* (oxidative stress), the trehalose synthesis genes *TPS1* and *TPS2*, heat-shock proteins genes *HSP12* and *HSP104* and the DNA damage responsive gene *DDR2* (Marchler *et al.* 1993; Schüller *et al.* 1994; Ruis and Schüller 1995; Varela *et al.* 1995; Winderickx *et al.* 1996). Although these remain a few examples and many other genes are under influence of *MSN2*/*MSN4* (Boy-marcotte *et al.* 1998), other transcriptional factors are known to play a role, this time not in a general stress response but rather in specific stress responses. It is the case of *YAP1*, *YAP2* and *SKNP7*, transcriptional factors responsible for the regulation of genes related to oxidative and toxic compounds stress such as the thioredoxin gene *TRX2*, the glutathione synthase gene *GSH2* and the cadmium-factor-encoding gene *YCF1* (Bauer and Pretorius 2000).

Aside from these transcriptional factors, other changes in gene expression and metabolism adaptation can occur to specifically cope with particular stress factors. In a winemaking context, Brion *et al.* 2013 found important linkages related to multi-stress

response showing that mechanisms such as glucose sensing, nitrogen and vitamin metabolism as well as cell detoxification and export systems play a major role in modulating cell survival and can help explaining some of the phenotypic variations observed in this regard.

### 5.2 The stress factors present at the beginning of a fermentation

A wine fermentation is far from being ideal conditions for yeast to proliferate. The hostile composition of a wine must and the harsh conditions in which wine fermentation usually occurs add up for a stressful scenario. This can be verified from the very beginning of the fermentation where several stress factors are already present when inoculating the yeast. In the next section, we will go through these and analyze the main facts known about each:

#### 5.2.1 Active Dried Yeast: production, use and stresses associated

Since its first appearance around half a century ago (Fugelsang and Edwards 2007), almost every yeast-based beverage and food industry has steadily expanded the use of Active Dried Yeast (ADY). Wine producers are no exception and ADY has become a first choice in yeast starters, where there currently exists a high diversity of wine strains commercially available (Soubeyrand *et al.* 2006; Rodríguez-Porrata *et al.* 2008). Besides being relatively economical and easy to use, ADY brought important improvements over traditional yeast culture preparations (*e.g.* liquid cultures). Despite this, the different steps of ADY production are themselves quite stressful and their impact on the yeast's later performance during wine fermentation should not be neglected.

**Propagation:** Any ADY process starts with yeast propagation to achieve a considerable number of cells. In a consecutive batch and fed-batch process accompanied by a proper scale-up, yeast is cultured in way to favor respiration rather than fermentation assuring that most energy consumed goes for biomass production (Attfield 1997; Bauer and Pretorius 2000). During batch, osmotic stress is present due to the concentrated sugars

present in molasses, a common media for propagation. In a scaled-down propagation, Pérez-torrado *et al.* 2005 used the expression of specific stress genes as markers to monitor the stress response of two wine yeast strains during this process. High expression of *HSP12*, *TRX2* and *GPD1* was detected confirming a general stress response, oxidative and osmotic stress respectively. During fed-batch, abundant nutrients are present but glucose is kept low (by using diluted molasses) while keeping a good aeration (Attfield 1997; Bauer and Pretorius 2000). This promotes respiratory metabolism and a consequent optimal biomass production, however oxidative stress becomes very evident (Pérez-Torrado *et al.* 2009). By using the microarray hybridization technique, Gómez-Pastor *et al.* 2010 studied the gene expression variation throughout the propagation process. After shifting to respiration, cells showed a strong induction of genes tightly correlated to oxidative stress such as *TRX2*, *PRX1*, *GRX1*, *GRX2*, *OXR1*, *GLO1* and *GLO2*, implicated in thioredoxin and glutathione-glutaredoxin antioxidant systems. This oxidative stress response was further supported by a matching proteomic profile.

Towards the end of propagation, producers are advised to promote a maturation step. Here, residual glucose is consumed and cells are briefly exposed to particular stressful conditions (such as low oxygen and nitrogen sources) that will activate yeast stress responses. Ultimately, carbohydrate reserves (*e.g.* glycogen and trehalose), lipids reserves and other intracellular metabolites are intracellularly accumulated, increasing yeast's survival rate during the remaining process (Van Dijck *et al.* 1995; Bauer and Pretorius 2000). Once properly propagated, cells are separated from the medium by being washed (causing an additional hypo-osmotic stress), concentrated by centrifugation, vacuum filtration and pressing, yielding a dense cream with reduced water content (Bauer and Pretorius 2000).

**Drying:** at this point, yeast is usually mixed with food-grade additives (emulsifying agents and anti-oxidants) used to protect yeast during the harsh desiccation. Next, the cream is extruded into strands and finally dried with warm air-drying (Attfield 1997). The yeast is then vacuum packed and can be distributed and sold to winemakers. The desiccation process has been studied in several occasions from a stress point of view (França *et al.* 2005; Singh *et al.* 2005). Main dehydration consequences reported are changes in cell volume and membrane polarization leading to an increased permeability and cell death (Simonin *et al.* 2007), protein loss of function due to conformational changes, sometimes irreversibly (Prestrelski *et al.* 1993) and a considerable level of

oxidative stress mainly by the accumulation of reactive oxygen species (ROS) and heat that lead to protein denaturation, membrane disruption and damage to nucleic acids (França *et al.* 2007). Different works have studied yeast's oxidative stress response during desiccation and showed a clear increase in trehalose, glutathione content and its oxidized form (GSSG), activation of thioredoxin and glutathione-glutaredoxin antioxidant systems as well as the expression of genes connected to the oxidative response such as *GSH1*, *TRR1*, *GRX5* (Pereira *et al.* 2003; França *et al.* 2007; Garre *et al.* 2010).

Despite the stressful process of desiccation, yeast cells should be capable of keeping a good viability and extended shelf-life. It is in fact a fundamental criterion for wine yeast selection independently of their good fermentative properties. A yeast that cannot endure propagation and drying cannot be commercialized and sold to winemakers, at least at an industrial scale. If resistant enough, their survival period can go as far as a few years with only a 0.5%-1% monthly loss of fermentation activity. This is explained by the very low or non-existent metabolic activity due to the 5-8% of water left within the cells (Ribéreau-Gayon *et al.* 2006; Soubeyrand *et al.* 2006; Fugelsang and Edwards 2007; Redón *et al.* 2008). To maintain this extended shelf-life, rigorous storage conditions must be followed. ADY packages must be kept at low temperature (4ºC) and unopened until its usage to avoid any exposure to oxygen and moisture (usually achieved with vacuum packaging) or otherwise it can lead to reduced viability and a later consequent poor fermentation (Redón *et al.* 2008).

**Rehydration:** Before using ADY and inoculating it into the wine must, a rehydration process is typically performed. This procedure is a critical step since it can greatly affect yeast fermentation capability, viability and overall performance. If not done properly it can lead to later sluggish or stuck fermentations (Soubeyrand *et al.* 2006; Krieger-Weber 2009). Despite protocol variations, most recommend a rehydration in water or in a 1:1 diluted grape juice at 37-40ºC for 15 to 20 minutes (Fugelsang and Edwards 2007). During this process, cells experience considerable osmotic variations that lead to changes in cell volume and plasma membrane integrity and function. If these variations are too extreme they can lead to an excessive membrane permeability and ultimately cell death (Simonin *et al.* 2007). It has been shown that a rehydration in the presence of inactive yeast preparations (which contains sterols and other nutrients) is beneficial to cell recovery and stabilization. In the rehydration process, yeasts need to mobilize lipid reserves to repair the plasma membrane and sterols play an important role in this subject

as an extra lipid source that can be incorporated in the plasma membrane by reactivated cells (Krieger-Weber 2009).

A study of gene expression during this stage showed that genes involved in general stress response, oxidative and nitrogen metabolism and respiration are considerably repressed (Rossignol *et al.* 2006). On the other hand, acid stress response genes as well as glycolytic genes were strongly up-regulated, probably as a response to cope with cells' energetic needs in such critical moment. After achieving an active growing population, the culture is now ready to be inoculated.

### 5.2.2 Temperature

Temperature is one of the most determinant parameters in a wine fermentation. Its influence is transversal to the whole fermentation process. A variation as small as one or two degrees Celsius can impact yeast's performance, fermentation kinetics and duration as well as the final organoleptic properties of the wine (Ribéreau-Gayon *et al.* 2006). The temperature choice in a wine fermentation is mainly determined by the kind of fermentation being performed and the control of specific parameters. Nevertheless, two major fermentation types can be established: red wine and white wine fermentation. The first are typically started at 24ºC/25ºC and let freely range between 24ºC-28ºC throughout the fermentation. Cooling systems are used to ensure that temperature does not excessively increase due to the fermentative activity and energy released. In some cases these cooling systems can be dismissed and fermentation can reach 30ºC (Ribéreau-Gayon *et al.* 2006; Jackson 2008b). Yeast's optimal temperature can vary depending on species and strains but typically *S. cerevisiae* is the most efficient at 24ºC-30ºC range. This makes the fermentation temperature of red wine ideal and therefore the level of stress due to temperature in these is minor.

White wines fermentation on the other hand is typically carried out at lower temperatures. The most common temperature used is 16ºC-18ºC however it can range from 20ºC down to 10ºC (Ribéreau-Gayon *et al.* 2006; Jackson 2008b). One of the main advantages of such low temperatures is to promote the production and accumulation of particular aromatic compounds that otherwise have a tendency to not be retained due to their volatility or simply not be synthetized (Torija *et al.* 2003). A common example of

these aromatic molecules are some esters such as ethyl hexonoate, isoamyl, isobutyl and 2-phenethyl acetate responsible for floral and fruity aromas of pear and banana. Additionally, the production of off-flavor compounds such as sulfur molecules (*e.g.* Hydrogen sulfide - $H_2S$) and higher alcohols has a tendency to be lower (Ribéreau-Gayon *et al.* 2006).

As expected, these lower temperatures have a great impact on yeast metabolism and are an important source of stress. An immediate consequence is a deceleration on yeast's sugars uptake rate which affects multiplication, fermentation kinetics and viability (Llauradó *et al.* 2002; Beltran *et al.* 2008). In more severe cases, too low a temperature can lead to stuck or sluggish fermentation due to yeast incapacity to adapt and thrive (Bisson 1999). One of the main cellular components affected by low temperature stress is the plasma membrane. Its fluidity and permeability are greatly altered and yeast must have the capacity to quickly change its lipid composition accordingly. Increasing unsaturated fatty-acyl or the medium fatty acids are two possible strategies to counteract low temperatures and membrane disruption (Beltran *et al.* 2008). Oxidative stress has also been linked to low temperatures. Zhang *et al.* 2003 submitted yeast cells to a drastic temperature downshift (from 30ºC to 10ºC) and verified the induction of genes encoding enzymes typically involved in the oxidative stress response, such as *SOD1* (encoding Cu–Zn superoxide dismutase), *CTT1* (encoding catalase T) and *GSH1* (encoding γ-glutamylcysteine synthetase). The increased expression and activity of these enzymes was found to be caused by increased generation of $H_2O_2$ after the temperature downshift (probably generated from ROS increase as well) proving that a higher oxidative stress is generated at lower temperatures. Additionally, mutants lacking the genes previously mentioned showed a decreased viability.

One of the strategies that yeast uses to counteract temperature impact is the production of trehalose. This non-reducing disaccharide composed of two α (1,1)-linked glucose molecules (Francois and Parrou 2001) has been reported to be directly involved in resistance to several stresses such as alcohol desiccation, high temperatures, osmotic and oxidative stress, freezing and nutrient starvation (Singer and Lindquist 1998; Silljé *et al.* 1999; Pretorius 2000b; Francois and Parrou 2001; Ding *et al.* 2009). In parallel with freezing tolerance, it has been shown in several occasions that trehalose also plays an important role in cold tolerance. Aside from its accumulation within cells, the expression of genes directly related to trehalose metabolism such as *TPS1* and *TPS2* (trehalose-6-

phosphate synthases) and *TSL1* (regulatory subunit of trehalose-6-phosphate synthase-phosphatase complex) is greatly induced under cold shock/stress (Francois and Parrou 2001; Sahara *et al.* 2002; Kandror *et al.* 2004; Schade *et al.* 2004; Al-Fageeh and Smales 2006). Trehalose acts by stabilizing proteins, preventing denaturation and aggregation, as well as stabilizing and neutralizing membrane damage caused cold or other stress factors (Singer and Lindquist 1998; Pretorius 2000b; Walker and Van Dijck 2006).

Finally, other than trehalose-related, expression of other stress genes have been documented to vary in consequence of low temperatures reinforcing the level and diversity of stress this parameter can cause. *MSN2*/*MSN4* (transcriptional activators of different stress responses), *HSP104*, *HSP12* (heat shock proteins, general stress response), *TTR1* (glutaredoxin), *PRX1* thioredoxin), *GTT2* (glutathione transferase) connected to oxidative stress, *GLG1* (glycogen synthesis initiator), *GSY1* (glycogen synthase) connected to glycogen metabolism and *INO1* (inositol-3-phosphate synthase) and *OLE1* (fatty acid desaturase) connected to phospholipid synthesis are examples of genes whose expression is induced to cope with low and near-freezing temperatures (Sahara *et al.* 2002; Kandror *et al.* 2004; Aguilera *et al.* 2007).

### 5.2.3 Osmotic stress

At the beginning of a wine fermentation different solutes and molecules are present at high concentrations. Among the most concentrated compounds in wine must are the sugars accumulated during grape maturation, mainly glucose and fructose. Although concentrations can greatly vary, 200 g/L – 280 g/L are commonly found with a tendency to increase due to climate changes and temperature rising. Such concentrations represent a major source of osmotic pressure variation. This occurs because the concentration differential of a particular compound, here sugars, between the intracellular (low osmolarity) and extracellular (high osmolarity) media is large. Under this scenario, cells experience hyper-osmotic stress that needs to be quickly counteracted. The main physiological consequences are changes in cell size and shape, diminished turgor, reduced water availability as well as membrane disruption (Bauer and Pretorius 2000; Pratt *et al.* 2003; Tamás and Hohmann 2003; Hohmann 2015). If not effectively addressed, it can lead to cell death. One of the main strategies yeast finds is to accumulate

compatible solutes that allow the reestablishment of the correct osmotic pressure and cell volume. A compatible solute can defined as an osmolyte that can be transported or synthesized by the cell up to high concentration while being metabolically inert (Bauer and Pretorius 2000). Different compatible solutes such as amino acids, trehalose, potassium ions and alcohols can be used as osmolytes by yeast however glycerol is commonly accepted as the main one used by *S. cerevisiae*.

The osmotic stress response, including glycerol synthesis, is mostly based on a serial activation of signaling cascades. This is the case of the high osmolarity glycerol (HOG) mitogen-activated kinase (MAP) pathway, the main mechanism used by the yeast to cope with osmotic stress (Mager and Siderius 2002; Saito and Posas 2012). Its activation starts with Sln1p and Sho1p, two membrane proteins capable of sensing osmotic stress (Maeda *et al.* 1995; Posas *et al.* 1996). These are part of two independent branches but both converge to Pbs2p that by phosphorylation activates the MAP kinase Hog1p and promote its nuclear translocation and accumulation (Saito and Posas 2012; Hohmann 2015). This all occurs very quickly as Hog1p phosphorylation can be detected one minute after stress stimulation (Brewster *et al.* 1993). Once inside the nucleus, the protein will activate and regulate the expression of different genes that lead to an osmotic response to reestablish the correct volume and turgor. Among these, it is included *GDP1*, coding for a NAD-dependent glycerol-3-phosphate dehydrogenase and *GPP2* coding for Glycerol-1-phosphate phosphohydrolase, both directly involved in glycerol biosynthesis. 30 min after osmotic stress, glycerol reaches intermediate concentrations capable of reestablishing good osmotic homeostasis (Klipp *et al.* 2005).

Aside from glycerol biosynthesis, Hog1p also participates in other osmotic stress responses. It is responsible for activating some transcription factors such as *SMP1*, *SKO1* and *MSN2*/*MSN4* which are then responsible for regulating genes directly implicated in the general stress response and DNA replication (Tamás and Hohmann 2003). Another mechanism in which Hog1p seems to be implicated, is in the regulation of the cell cycle. When submitted to osmotic stress, yeast induces a rapid and transient delay in the cell cycle capable of arresting it at the different checkpoints. This allows cells to properly develop a stress response before resuming the cell cycle progression (Clotet and Posas 2007; Yaakov *et al.* 2009).

Finally, another very quick and effective mechanism is the inactivation of Fps1p. Its regulation is not completely clear but it is thought to be independent of HOG pathway

(Luyten *et al.* 1995). Fps1p is a aquaglyceroporin, a transmembrane channel responsible for mediating glycerol export. Upon hyper-osmotic stress, Fps1p is rapidly closed in order to accumulate glycerol intracellularly (Tamás *et al.* 1999). Fps1p constitutively open mutants fail to accumulate glycerol. This greatly limits growth under high osmolality and leads to an overproduction of glycerol in an attempt to compensate the glycerol loss (Hohmann *et al.* 2007; Ahmadpour *et al.* 2014).

### 5.2.4 Sulfur dioxide ($SO_2$)

$SO_2$ is a chemical compound that has been associated with winemaking for many centuries. Burning sulfur was used by the ancient Egyptians, Greeks and Romans inside empty wine vessels to keep them fresh and free from vinegar smell (Jackson 2008c). Since then, the presence of $SO_2$ was known to benefit wine preservation and in the ninetieth century it started to be deliberately added in wine must, either as a liquefied gas or in salt from as potassium metabisulfite ($K_2S_2O_5$) (Ribéreau-Gayon *et al.* 2006).

The way how $SO_2$ positively contributes to winemaking is by acting as an anti-oxidant and anti-microbial agent. For the first, $SO_2$ can combine with $O_2$, avoiding excessive oxidation of phenolic and aromatic compounds. This ultimately helps preserving the good organoleptic properties of the wine. For the second, it is capable of inhibiting and even destroying a good percentage of certain microbial populations when at higher concentrations. In wineries, it can be used as a sanitizer to maintain the equipment and spaces clean. In wine, it is responsible for keeping undesirable bacteria and spoilage yeast such as *Brettanomyces* at low levels, avoiding further wine spoilage (Ribéreau-Gayon *et al.* 2006; Jackson 2008c).

The action of $SO_2$ starts with the penetration into the cell by diffusion. Once in the cytoplasm, the higher pH provokes the dissociation of $SO_2$ and consequent reaction with essential biological molecules such as enzymes, coenzymes and vitamins. Growth is then arrested and eventually cell death occurs (Romano and Suzzi 1993). To better understand $SO_2$ dynamics, it is important to know that when in wine it can exist in a free and bound form and their sum is referred to as total $SO_2$. The bound forms exist because $SO_2$ naturally combines with many molecules present in wine, particularly those with carbonyl groups. These combinations can be reversible or irreversible if highly stable. Examples

of such molecules include acetaldehyde, ketonic acids, anthocyanins, some sugars and derivatives (Ribéreau-Gayon *et al.* 2006). When combinations are maximized, the remaining $SO_2$ exists then as free $SO_2$ divided in different forms, namely bisulfite ions ($HSO_3^-$), free sulfite ions ($SO_3^{2-}$) and molecular $SO_2$ (as dissolved gas). This last fraction is the actual responsible for the anti-microbial activity previously mentioned. The equilibrium between these free forms is highly dependent on pH and temperature. The higher the pH and/or the lower the temperatures, the more the equilibria shifts towards the ion forms. The lower the pH and/or the higher the temperature the more molecular $SO_2$ there will be and therefore the stronger its action for the same amount of total $SO_2$. This way particular attention to these parameters is needed when adding $SO_2$ (Ribéreau-Gayon *et al.* 2006; Jackson 2008c). At pH between 3.2 and 3.4, 5 g/hL is a common concentration of total $SO_2$ used, aiming for values around 0.8 – 1.5 mg/L of molecular $SO_2$, considered enough to inhibit growth of most wild yeast and bacteria. In fact, $SO_2$ use is tightly surveilled and legally regulated in winemaking due to health risks associated if consumed in high quantities. In the US, the upper limit of total $SO_2$ allowed in wine is 350 mg/L whereas in the EU it is 210 mg/L for white and rosé wines and 160 mg/L for red wines. Moreover, at low concentrations $SO_2$ is mostly undetectable but at free $SO_2$ concentrations over 50 mg/L unpleasant aromas are likely to develop. This should also further motivate winemakers to moderate its use (Ribéreau-Gayon *et al.* 2006; Jackson 2008c).

From a biotechnological point of view, yeast resistance to $SO_2$ is of great interest. Even though wine yeasts tend to globally be resistant to $SO_2$, its influence on yeast's performance is still significant and a source of stress. To cope with that, yeast has different mechanisms that help overcome the presence of $SO_2$. A very well-known example is the case of gene *SSU1* that codes for a plasma membrane sulfite pump, responsible for preventing sulfite accumulation within the cell (Park and Bakalinsky 2000). Together with the gene *FZF1*, a positive regulator of *SSU1* transcription, they constitute one of the main mechanisms in yeast to deal with sulfite (Casalone *et al.* 1992; Avram *et al.* 1999).

In the case of some wine yeast, they found a way to push further the advantages of such mechanism. Pérez-Ortín *et al.* 2002 discovered a reciprocal translocation between the chromosomes XVI and VIII involving *SSU1*. As in S288C, the gene is naturally located in chromosome XVI. However, strains possessing this translocation have an allele of *SSU1* (*SSU1* - *R*) in chromosome VIII (Goto-Yamamoto *et al.* 1998). The promoter of

*SSU1 – R* contains up to 76-bp enhancer tandem repeats coming from the promoter of *ECM34*, a highly expressed protein (Figure N.3). As a consequence, *SSU1 – R* is expressed at higher levels and cells are capable of exporting sulfite at higher rates and therefore have a higher resistance to $SO_2$ (Goto-Yamamoto *et al.* 1998).

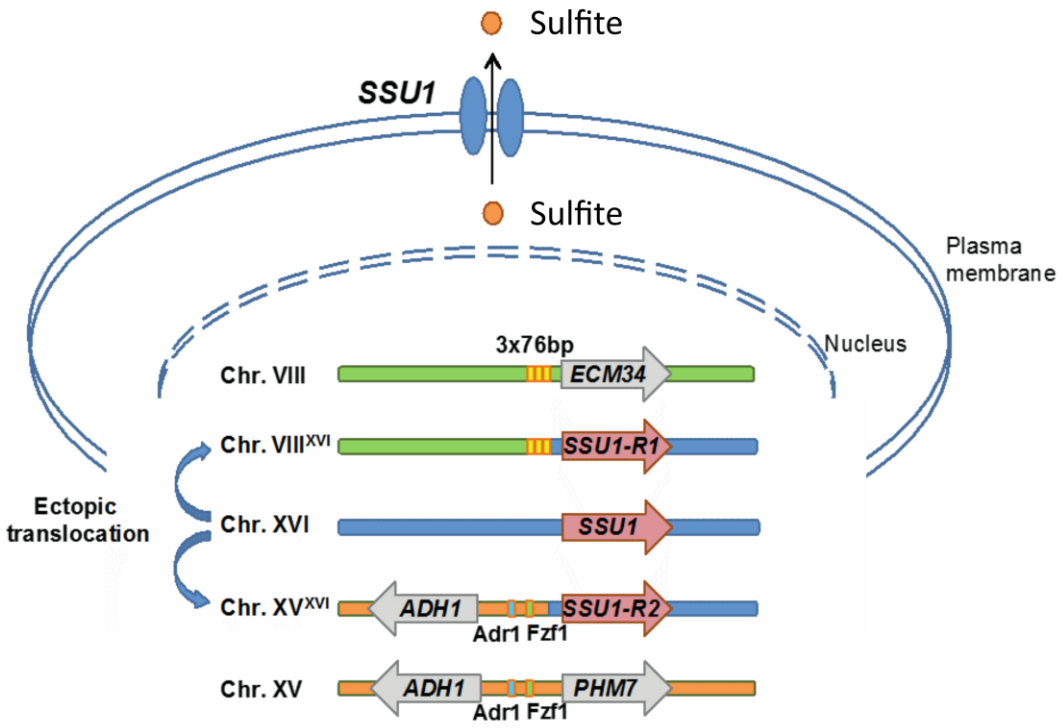



**Figure N.3. Mechanisms of sulfite resistance through reciprocal translocations.** Adapted from Marsit and Dequin 2015.

Interestingly, most wine yeasts possess this translocation, which combined with the ancient use of $SO_2$ in winemaking, suggests an adaptive evolution combined with an unconscious selection by man (Pérez-Ortín *et al.* 2002; Yuasa *et al.* 2004; review in Marsit and Dequin 2015). More recently, Zimmer *et al.* 2014 discovered a new translocation involving the gene *SSU1* when studying the lag phase. This time it is between chromosomes XVI and XV and involves the promoter region of *ADH1*, conferring higher sulfite resistance in the first hours of fermentation (Figure N.3).

Contrary to the translocation $XVI^{VIII}$, this one is thought to be much less disseminated among wine yeasts suggesting to be a much more recent event (Zimmer *et al.* 2014).

Aside from *SSU1*, other mechanisms of $SO_2$ resistance by yeast include producing and exporting acetaldehyde to the extracellular environment. As external molecular $SO_2$ combines with acetaldehyde, its fraction diminishes while innocuous bound-$SO_2$ forms (Pilkington and Rose 1988; Liu and Pilone 2000) reducing this way the stress imposed by sulfite. Another possibility is to upregulate sulfite reduction system and metabolism. The $SO_2$ that enters in the cells can be reduced to $H_2S$ by means of sulfite reductases (such as *MET10* and *MET5*). From here, yeast can either excrete $H_2S$ to the external media or use it in the synthesis of sulfur-containing amino-acids (Thomas and Surdin-Kerjan 1997). Additionally, it has also been shown that *MET2* and *SKP2* are responsible for regulating the sulfur amino acid synthesis pathway and this way modulate the sulfite/sulfide produced (Noble *et al.* 2015). Finally, it is important to mention that even if $SO_2$ is not used at all during a wine fermentation, sulfur compounds are still likely to be found in the final wine. This is because yeasts are themselves sulfite producers although in small quantities. Frequently, less than 10 mg/L are produced however it can go up to 100 mg/L (Eschenbruch and Bonish 1976; Ribéreau-Gayon *et al.* 2006). If produced at high concentrations, $H_2S$ can be responsible for the aroma of rotten eggs being as well the precursor of other compounds with undesired organoleptic properties (Swiegers *et al.* 2005). The production of sulfur compounds by yeast has been shown to be highly strain-dependent (Kumar *et al.* 2010) and commercial wine yeast strains have received particular attention in order to gather more knowledge regarding this trait (Noble *et al.* 2015).

### 5.2.5 Low lipids

Sterols and unsaturated fatty acids are fundamental constituents of yeast plasma membrane and play an important role in the presence of unfavorable conditions (*e.g.* ethanol, low temperatures and oxygen deprivation). They participate in modifications of the membrane lipid composition as a form of adaptation and survival to adverse conditions and ultimately will influence membrane integrity, cell viability and fermentative activity (Alexandre *et al.* 1994; Beltran *et al.* 2008; Mannazzu *et al.* 2008).

These lipids cannot be produced *de novo* by yeast if the wine fermentation occurs in strict anaerobic conditions and require therefore the presence of oxygen to be synthesized (Andreasen and Stier 1953). Consequently, yeasts are highly dependent on the lipid content present in grape must to assure their proper survival. Their absorption allows for higher levels of viability, particularly towards the end of fermentation (Luparia *et al.* 2004), allowing yeast to compensate to some extent the lack of oxygen. One of the main types of sterols assimilated by yeast are phytosterols, present in grape berry skins and mainly divided in β-sitosterol, campesterol and stigmasterol. β-sitosterol represents more than 70% of total phytosterols (Le fur *et al.* 1994).

This subject becomes particularly important for white and rosé wines that are routinely clarified before fermentation. Clarification has the goal of improving wine final quality and organoleptic properties. Some of the main advantages of such process are to reduce turbidity (undesired), help preserve positive fermentation aromas while reducing the occurrence of off-flavors (*e.g.* hydrogen sulfide) and prevent excessive oxidation (Ribéreau-Gayon *et al.* 2006; Jackson 2008b). Different methods can be used to clarify a grape must. Natural settling is often combined with further addition of clarifying agents such as bentonite, potassium caseinate, tannins, pectinases and charcoal. These protect must from oxidation and help in the flocculation of sediments and unstable proteins. Alternatively, centrifugation, filtration and carbon dioxide or nitrogen flotation can also be used as clarifying methods (Ribéreau-Gayon *et al.* 2006; Jackson 2008b).

In the cases where clarification is excessive, it can be responsible for removing more than 90% of fatty acids and sterol content (Bertrand and Miele 1984; Delfini *et al.* 1993). This can have several negative consequences during fermentation, such as diminishing yeast viability, promoting excessive acetic acid production and provoking sluggish or stuck fermentation. Different strategies can be used to compensate an excessive clarification such as the adding oxygen during fermentation (Ortiz-Julien *et al.* 2000), which promotes the synthesis of ergosterol, or the addition of lees as an external source of lipid nutrients (Casalta *et al.* 2013).

### 5.2.6 Low thiamine

Vitamins are essential nutrients required for yeast growth and vitality. Grape musts contain a wide range of different vitamins however thiamine (vitamin $B_1$) deserves more detailed attention due to its importance and particular kinetics during wine fermentation. Its main role during fermentation is acting as a cofactor in numerous enzymatic reactions, particularly in the decarboxylation of pyruvate (Ribéreau-Gayon *et al.* 2006). From a technological point of view, thiamine also participates in the decarboxylation of ketonic acids and prevents their excessive accumulation. As these combine with free $SO_2$, if thiamine is scarce it means that a greater portion of free $SO_2$ will combine with ketonic acids (bound form) and the anti-microbial and anti-oxidant action will be under the desired levels (Ribéreau-Gayon *et al.* 2006). Depending on grape varieties, agricultural practices and grape processing, a must can contain 150 – 750 µg/L of thiamine (Bataillon *et al.* 1996).

At the beginning of the fermentation, the existing thiamine is quickly consumed by yeast becoming depleted within 6h after fermentation start (Ough *et al.* 1989; Bataillon *et al.* 1996). However, contaminant flora that might exist in must is also capable of quickly consuming thiamine, sometimes at a higher rate than *S. cerevisiae*. This means that at the moment of inoculation, the vitamin levels might already be very low. Additionally, thiamine can also suffer a slow destruction (McCourt *et al.* 2006). All this generates a fierce competition for the vitamin with the ultimate consequence that it becomes a limiting factor. In fact, low thiamine concentrations can be the cause of sluggish or stuck fermentations which of course is not desired (Ough *et al.* 1989; Bataillon *et al.* 1996).

In thiamine-deficient musts, thiamine addition is permitted (*e.g.* in the EU up to 50 mg/hL) and commonly performed in cellars. With such addition, not only can regular fermentation kinetics be restored but also yeast viability increased by 30% (Bataillon *et al.* 1996; Ribéreau-Gayon *et al.* 2006). Aside from importing, yeast is also capable of synthesizing thiamine. In the presence of high thiamine concentrations, the activity of all biosynthetic enzymes is kept low as well as the uptake (Kawasaki *et al.* 1990). When the vitamin is depleted, it can be sensed by Thi3p and together with Thi2p and Pdc2p, the family of genes (*THI*) responsible for thiamine biosynthesis are then activated (Nosaka *et al.* 2005)**.** Although thiamine synthesis is a possibility, the concentrations which yeast is

capable of producing are often not sufficient to compensate its scarcity in the wine must. Moreover, the synthesis of thiamine comes as a relatively energy-costly process which makes uptake the main and preferred solution (Hohmann and Meacock 1998).

### 5.3 A stressful lag phase

As mentioned in a previous section (2.2), lag phase is the period right after inoculation where yeast is adapting to a new environment. During this phase, growth and fermentative activity are arrested, which is the reason why we observe a lag in yeast multiplication and fermentation start (Lewis *et al.* 1993; Brejning and Jespersen 2002). A detailed study in gene expression upon must inoculation showed that different cellular and metabolic modifications occur during this period (Rossignol *et al.* 2006) Overall, genes involved in oxidative metabolism, nitrogen utilization, acid stress response and glycolysis were downregulating whereas genes related to biosynthetic functions were up-regulated, showing a clear adaptation of the cells to the new environment (Rossignol *et al.* 2006). The length of this lag period is highly dependent of many factors, the most important being the must's characteristics and the fermentation conditions. In the last sections, we detailed the context and consequences of the most typical and impacting stress factors present at the moment of inoculation. Their presence can be common to all fermentation or conversely associated with a type of fermentation. White wine fermentations are typically clarified and run at lower temperatures making these stresses more associated to this type of fermentation Red wine fermentation on the other hand can reach higher sugar concentrations and their thiamine can be consumed at a faster rate. As common features, $SO_2$ is widely used in both types of fermentation, and propagation and drying are used for commercial wine yeast independently of the strain or fermentation intended. One of the main practical consequences of the presence of such stress factors is favoring extended lag phases. When too intense, these stress factors make yeast take longer to adapt and it is difficult to achieve a proper fermentation start. Frequently, this can lead to a stuck or sluggish fermentation compromising the viability of the process. Additionally, since the inoculated yeast takes longer to multiply, this leaves space for other indigenous flora to develop and take part in the fermentation outside the desired limits. All together, these facts can lead to negative consequences at the different levels.

The fermentation process can be compromised and stops being reliable, predictable and efficient. The final quality of the wine can also change or be jeopardized as new variables occurred during the fermentation process. Ultimately, this potentially represents time losses combined with extra financial costs due to wine spoilage and pointless equipment use. Currently, different strategies can be used to reduce the risk of extended lag phases, however it remains a fermentation step with associated risks that are not fully mastered. With this current problem in hand, we believe that yeast inoculation and fermentation start require a deeper study of the parameters and conditions modulating them, with particular focus on the yeast response. Simultaneously, we also believe this is a stage of wine fermentation that can still be improved and ultimately contribute to an even more reliable fermentation process. With this vision in mind, we defined the objectives for our work.

## 6. Thesis objective

The goal of this thesis was to characterize and improve the wine yeast lag phase under alcoholic wine fermentation while focusing on a multi-stress context typical for this stage. In order to achieve this goal, this work was structured into three main parts that are complementary and contributed differently for the main objectives.

1) Characterization of wine yeast lag phase and stress response diversity as well as assessment of the impact of different stress factors, individually and in combination. Few studies have focused on wine fermentation lag phase and multi-stress resistance. By starting with a diverse set of industrial wine yeasts provided by the company Lallemand SAS, we aimed to shed new light on this subject while also performing an optimized strain choice for the following approaches.

2) Apply the principles of evolutionary engineering and obtain an evolved industrial wine yeast strain with a faster fermentation start, meaning a shorter lag phase. By using a non-GMO approach, the strain(s) obtained could later integrate industrial production as well as be commercialized for wine fermentation. Concomitantly, the study of the genetic modifications at the basis of the evolution was expected to contribute to more knowledge and information on lag phase duration.

3) Obtain a deeper understanding of the genetic basis and molecular mechanisms that modulate stress resistance and lag phase. For that purpose, a specific experimental design of QTL mapping, combining a diallele – like design with bulk segregant analysis as well as a flow cytometry to select budding cells, was built to reveal the complex basis of lag phase regulation. Knowledge generated could be further used in other projects for industrial wine strains improvement.

In the scope of the Marie-Curie project, an additional objective yielded a fourth part in this thesis where the potential of the non-conventional yeast *K. marxianus* for wine fermentation is globally assessed.

# MATERIAL AND METHODS

## 1. Yeast strains

### 1.1 Yeast strains used in this work

Yeast strains used in this work are summarized in Table N.1. Commercial wine yeast strains were used in chapter 1, 4 and as a starting point for chapter 2 and 3. An additional laboratory strain was used in chapter 4.

**Table N.1. Strains used in this work.**

| Strain | Source | Species | Genotype |
|---|---|---|---|
| **L1** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **L3** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **L4** | Lallemand SAS | *S. cerevisiae/kudriavzevii* | *MATa/MATα* |
| **L5** | Lallemand SAS | *T. delbrueckii* | *MATa/MATα* |
| **L6** | Lallemand SAS | *S. cerevisiae/kudriavzevii* | *MATa/MATα* |
| **L7** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **L8** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **L9** | Lallemand SAS | *S. uvarum* | *MATa/MATα* |
| **L10** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **L11** | Lallemand SAS | *S. cerevisiae* | *MATa/MATα* |
| **K4** | Lallemand SAS | *K. marxianus* | *MATa/MATα* |
| **K1** | UCC | *K. marxianus* | *MATa/MATα* |

### 1.2 Yeast strains generated during this work

Different yeast strains (all *S. cerevisiae*) as well as recombinant populations were generated in the scope of our work, namely for chapter 2 and 3. These are summarized in Table N.2 and 3.

All strains were stocked at -80ºC in YPD with 20% (v/v) glycerol.

**Table N.2. Strains generated during this work.**

| Strain | Source | Genotype |
|---|---|---|
| **L1aGFP** | Transformation L1 | *MATa, HO::nat1, CLB3-* GFP |
| **L7αGFP** | Transformation L7 | *MATα, HO::blast, CLB3-* GFP |
| **L8αGFP** | Transformation L8 | *MATα, HO::hph, CLB3-* GFP |
| **L8aGFP** | Transformation L8 | *MATa, HO::hph, CLB3-* GFP |
| **L11aGFP** | Transformation L11 | *MATa, HO::ble, CLB3-* GFP |
| **L11αGFP** | Transformation L11 | *MATa, HO::ble, CLB3-* GFP |
| **D1.7** | Hybrid L1aGFP x L7αGFP | *MATa/MATα, HO::nat1/HO::blast, CLB3*-GFP |
| **D1.11** | Hybrid L1aGFP x L11αGFP | *MATa/MATα, HO::nat1/HO::ble, CLB3*-GFP |
| **D7.8** | Hybrid L7αGFP α x L8aGFP | *MATa/MATα, HO::blast/HO::hph, CLB3*-GFP |
| **D7.11** | Hybrid L7αGFP x L11aGFP | *MATa/MATα, HO::blast/HO::hph, CLB3*-GFP |
| **D8.1** | Hybrid L8αGFP x L1aGFP | *MATa/MATα, HO::hph/HO::nat1, CLB3*-GFP |
| **D8.11** | Hybrid L8αGFP x L11aGFP | *MATa/MATα, HO::hph/HO::ble, CLB3-* GFP |
| **R2A** | L7 evolution | *MATa/MATα* |
| **R2D** | L7 evolution | *MATa/MATα* |
| **R2E** | L7 evolution | *MATa/MATα* |
| **R3C** | L7 evolution | *MATa/MATα* |
| **R3F** | L7 evolution | *MATa/MATα* |
| **R3I** | L7 evolution | *MATa/MATα* |

**Table N.3. Populations generated during this work**

| Population | Source | Observations |
|---|---|---|
| **P1.7** | Diploid D1.7 | Propagated and dried |
| **P1.11** | Diploid D1.11 | Propagated and dried |
| **P7.8** | Diploid D7.8 | Propagated and dried |
| **P7.11** | Diploid D7.11 | Propagated and dried |
| **P8.1** | Diploid D8.1 | Propagated and dried |
| **P8.11** | Diploid D8.11 | Propagated and dried |

## 2. Plasmids and oligonucleotides

The plasmids and oligonucleotides used in this work are summarized in Table N.4 and 5 respectively.

**Table N.4. Plasmids used during this work.** All plasmids were propagated in bacteria *E.coli*.

| Plasmid | Characteristics | Source |
|---|---|---|
| **pSF-TEFI-TPI1-Blast** | Bacterial plasmid, KanR, Blast cassette | Sigma |
| **pAG25** | Bacterial plasmid, AmpR, nat1 cassette, loxP | (Goldstein and McCusker 1999) |
| **pAG32** | Bacterial plasmid, AmpR, hph cassette, loxP | (Goldstein and McCusker 1999) |
| **pUG66** | Bacterial plasmid, AmpR, ble cassette, loxP | (Güldener *et al.* 1996) |
| **pKT127** | Bacterial plasmid, AmpR, kan cassette | (Sheff and Thorn 2004) |
| **phS3** | Bacterial plasmid, AmpR, NAT cassette, *HO* | John McCusker (unpublished) |

**Table N.5. Primers used in this work.** Homologous sequences to Blast, nat1, hph, and ble of plasmids pSF-TEFI-TPI1-Blast, pAG25, pAG32, and pUG6 respectively are in bold. Homologous sequences to yEGFP of plasmid pKT127 are in italic.

| Primer | Sequence | Purpose |
|---|---|---|
| **HOdelFOR** | 5' ATGCTTTCTGAAAACACGAC TATTCTGATGGCTAACGGTG **TTCGTACGC TGCAGGTCGACC** 3' | Obtaining the different antibiotic cassettes from the different plasmids |
| **HOdelREV** | 5' TTAGCAGATGCGCGCACCTG CGTTGTTACCACAACTCTT **GCATAGGCCACTAGTGGATCTG** 3' | Obtaining the different antibiotic cassettes from the different plasmids |
| **HOdelVerifFOR** | 5' TGTTGAAGCATGATGAAGCG 3' | Confirming antibiotic cassette insertion (up/downstream of *HO*) |
| **HOdelVerifREV** | 5' TGAAACAAATCAGTGCCGGT 3' | Confirming antibiotic cassette insertion (up/downstream of *HO*) |
| **Phleo1** | 5' AAGCCCAACCTTTCATAGA 3' | Confirming *HO*::ble insertion |
| **Phleo2** | 5' ATCTCAAGCTGGAGTTCTTC 3' | Confirming *HO*::ble insertion |
| **ClonNAT1** | 5' CATCCAGTGCCTCGATGGCC 3' | Confirming *HO*::nat1 insertion |

(Continuation of Table N.5).

| Primer | Sequence | Purpose |
|---|---|---|
| **ClonNAT2** | 5’ TCGAGGACAT CGAGGTCGCC 3’ | Confirming *HO*::nat1 insertion |
| **Hygro1** | 5’ GCAGCTATTTACCCGCAGGA 3’ | Confirming *HO*::hph insertion |
| **Hygro2** | 5’ TTCTGGAGGC CGTGGTTGGC 3’ | Confirming *HO*::hph insertion |
| **BlastChvF** | 5’ AGATCATCTCTGCTGTCCAC 3’ | Confirming *HO*::Blast insertion |
| **ECM34D** | 5’ TCGAACATCGAGCATGCA 3’ | Confirming of translocation $XVI^{VIII}$ presence |
| **SSU1R** | 5’ GACACCCATGACCATCAC 3’ | Confirming of translocation $XVI^{VIII}$ presence |
| **P760** | 5’ GCCCCTCCATGTTCTACTATT 3’ | Confirming of translocation $XVI^{VIII}$ /$XVI^{XV}$ presence |
| **p761** | 5’ AATGCAGGCGCAAAAAACTC 3’ | Confirming of translocation $XVI^{XV}$ presence |
| **P761** | 5’ GAGTTTTTTGCGCCTGCATT 3’ | Confirming of translocation $XVI^{XV}$ presence |
| **Mat1** | 5’ AGTCACATCAAGATCGTTTATGG 3’ | Confirming mating type |
| **Mat2** | 5’ GCACGGAATATGGGACTACTTCG 3’ | Confirming mating type |
| **Mat3a** | 5’ ACTCCACTTCAAGTAAGAGTTTG 3’ | Confirming mating type |
| **CLB3GFP_F** | 5’GTGGATAGCATTAGCTGAACACA GAGTAGAAAGATCTAAC*ATGTCTAA AGGTGAAGAATTA* 3’ | Obtaining GFP marker from pKT127 |
| **CLB3GFP_R** | 5’CTTTTTCCTTTGTTGATGCCATGT CTCGAGCTGAGGCTTT*TGGATGGCG GCGTTAGTATC* 3’ | Obtaining GFP marker from pKT127 |
| **CLB3_F** | 5’ CCGTTGGCCACCATTATTT 3’ | Confirming *CLB3*-GFP insertion |
| **CLB3_R** | 5’ CCATATGGATTGTGGATGCTT 3’ | Confirming *CLB3*-GFP insertion |
| **CLB3GFP_VR** | 5’ GGACGAGGCAAGCTAAACAG 3’ | Confirming *CLB3*-GFP insertion |

## 3. Media

### 3.1 Laboratory media

Standard rich medium YPD and sporulation medium GNA and SpoMA were used for yeast cultures. Their composition is summarized in Table N.6.

**Table N.6. Composition of the laboratory media**

| Media | Compound | Reference | Amount / L |
|---|---|---|---|
| **YPD** | Yeast extract<br>Peptone<br>Glucose<br>MilliQ water | BD 212750<br>BD 211677<br>Panreac 141134.0416 | 10 g<br>20 g<br>20 g<br>to 1 L |
| **GNA** | Yeast extract<br>Peptone<br>Glucose<br>MilliQ water | BD 212750<br>BD 211677<br>Panreac 141341.0416 | 10 g<br>5 g<br>100 g<br>to 1 L |
| **SpoMA** | Yeast extract<br>Glucose<br>Potassium acetate<br>Adenine<br>MilliQ water | BD 212750<br>Panreac 141341.0416<br>Sigma P-1147 | 1 g<br>0.5 g<br>10 g<br>20 mg |

Media were sterilized by autoclaving at 110ºC for 30 min at 1 bar. Liquid media were stored at room temperature whereas Petri dishes at 4ºC. When preparing solid media 20 g/L of agar were added just prior to autoclaving. For the preparation of solid media with antibiotic, ClonNAT (WernerBioAgentsGmbH, 96736-11-7) (200 µg/mL), Geneticin (G418, Sigma A-1720) (200), Hygromycin (Sigma H-7772) (200 µg/mL), Phleomycin (InvivoGen, 11006-33-0) (150 µg/mL) and Blasticidin (Sigma, 15205) (500 µg/mL) were added accordingly after YPD was autoclaved and at a temperature around 50ºC in order to avoid denaturation of the antibiotics.

## 3.2 Must

### 3.2.1 Synthetic must

Fermentations were carried out in synthetic must (SM) that simulates standard grape juice (Bely *et al.* 1990b) This medium is characterized by high equimolar concentrations of glucose and fructose, a limiting content of nitrogen (YAN) and an acid pH of 3.3. Ammonia nitrogen and amino acid nitrogen were added as assimilable nitrogen sources. A basal SM 250 mg/L of assimilable nitrogen was used during this thesis and its composition is summarized from Table N.7 to Table N.11. For the different experiments requiring different levels of stress, their respective compounds (sugar, anaerobic factors, thiamine and $SO_2$ were accordingly adjusted to the right concentration. (its different compounds concentrations adjusted accordingly the each experiment. NaOH 10M was used to adjust pH. SM was stored at -20 °C, steam sterilized for 10 min and cooled to the right temperature before use.

**Table N.7. Composition of micronutrient stock solution (sterilized by 0.22 µm filtration and stored at 4°C).**

| Compound | Chemical formula | Reference | Amount / L |
|---|---|---|---|
| **Manganese sulfate monohydrate** | $MnSO_4.H_2O$ | Sigma M-7634 | 4 g |
| **Zinc sulphate heptahydrate** | $ZnSO_4.H_2O$ | Sigma Z-4750 | 4 g |
| **Copper sulfate pentahydrate** | $CuSO_4.5H_2O$ | Sigma C7631 | 1 g |
| **Potassium iodide** | KI | Sigma P-4286 | 1 g |
| **Cobalt chloride hexahydrate** | $CoCl_2.6H_2O$ | Sigma C3169 | 0.4 g |
| **Boric acid** | $H_3BO_3$ | Sigma B-7660 | 1 g |
| **Ammonium heptamolybdate** | $(NH_4)_6Mo_7O_{24}$ | Sigma A-7302 | 1 g |
| **MilliQ water** | | | to 1 L |

**Table N.8. Composition of the basal synthetic must (SM) used for fermentations.**

| Compound | Chemical formula | Reference | Amount / L |
|---|---|---|---|
| **Glucose** | $C_6H_{12}O_6$ | Panreac 141134.0416 | Adjusted accordingly |
| **Fructose** | $C_6H_{12}O_6$ | Panreac 142728.0416 | Adjusted accordingly |
| **Malic acid (DL)** | $C_4H_6O_5$ | Sigma M-0875 | 6 g |
| **Citric acid** | $C_6H_8O_7$ | Sigma C-7129 | 6 g |
| **Potassium phosphate** | $KH_2PO_4$ | Sigma P-5379 | 0.75 g |
| **Potassium sulfate** | $K_2SO_4$ | Sigma P-0772 | 0.5 g |
| **Magnesium sulfate** | $MgSO_4.7H_2O$ | Sigma M-9397 | 0.25 g |
| **Calcium chloride** | $CaCl_2.2H_2O$ | Sigma C-7902 | 0.155 g |
| **Sodium chloride** | NaCl | Sigma S-9625 | 0.2 g |
| **Ammonium chloride** | $NH_4Cl$ | Sigma A-4514 | 0.27 g |
| **Iron chloride stock solution** | $FeCl_3$ | Sigma F-7134 | 0.02 g |
| **Amino acid stock solution** | - | - | 8.1 mL |
| **Micronutrient stock solution** | - | - | 1 mL |
| **Vitamins stock solution** | - | - | 10 mL |
| **Anaerobic Factor stock Solution** | - | - | Adjusted accordingly |
| **MilliQ water** | $H_2O$ | - | to 1 L |

**Table N.9. Composition of the anaerobic factors stock solution (stored at 4ºC).**

| Compound | Chemical formula | Reference | Amount |
|---|---|---|---|
| **β-sitosterol** | $C_{29}H_{50}O$ | Sigma 85451 | 0.5 g |
| **Tween 80** | - | Sigma P-1754 | 50 mL |
| **Absolute ethanol** | $CH_3CH_2OH$ | - | to 100 mL |

**Table N.10. Composition of the amino acid stock solution. (stored at -20ºC).**

| Compound | Nº of atomes N assimilable | Reference | Amount / L |
|---|---|---|---|
| **Aspartic acid** | 2 | Sigma A-9256 | 3.4 g |
| **Glutamic acid** | 1 | Sigma G-1251 | 9.2 g |
| **Alanine** | 1 | Sigma A-7627 | 11.1 g |
| **Arginine** | 3 | Sigma A-5006 | 28.6 g |
| **Cysteine** | 1 | Sigma C-7352 | 1 g |
| **Glutamine** | 2 | Sigma G-3126 | 38.6 g |
| **Glycine** | 1 | Sigma G-7126 | 1.4 g |
| **Histidine** | 2 | Sigma H-8000 | 2.5 g |
| **Isoleucine** | 1 | Sigma I-2752 | 2.5 g |
| **Leucine** | 1 | Sigma L-8000 | 3.7 g |
| **Lysine** | 2 | Sigma L-5501 | 1.3 g |
| **Methionine** | 1 | Sigma M-9625 | 2.4 g |
| **Phenylalanine** | 1 | Sigma P-2126 | 2.9 g |
| **Proline** | 0 | Sigma P-0380 | 46.8 g |
| **Serine** | 1 | Sigma S-4500 | 6 g |
| **Threonine** | 1 | Sigma T-8625 | 5.8 g |
| **Tryptophan** | 2 | Sigma T-0254 | 13.7 g |
| **Tyrosine** | 1 | Sigma T-3754 | 1.4 g |
| **Valine** | 1 | Sigma V-0500 | 3.4 g |
| **MilliQ water** | | | to 1 L |

**Table N.11. Composition of the vitamin stock solution (stored at -20ºC).**

| Compound | Reference | Amount |
|---|---|---|
| **Myo-inositol** | Sigma I-5125 | 2 g |
| **Calcium pantothenate** | Sigma P-2250 | 0.15 g |
| **Thiamine hydrochloride** | Sigma T-4625 | Adjusted accordingly |
| **Nicotinic acid** | Sigma N-4126 | 0.2 g |
| **Pyridoxine** | Sigma P-5669 | 0.025 g |
| **Biotin** | Sigma B-4501 | 0.3 mg |
| **MilliQ water** | | to 1 L |

### 3.2.2 Grape must

Fermentations carried out in grape must, using a Merlot 2016 previously flash pasteurized. This grape must in particular contained 240 g/L sugar, 90 mg/L of assimilable nitrogen (supplemented with YAN – Fermaid E at 1/3 of fermentation at 30 g/hL). $SO_2$ was added just prior to fermentation in a way that 1.2 mg/L molecular $SO_2$ final concentration was achieved.

## 4. Determination of the cellular population and viability

### 4.1 Cell quantification

Cells were quantified for different purposes such as inoculation of a precise cellular concentration in a given media or for population following during fermentation. For that, 2 main methods were used: 1) spectrophotometry. The cellular concentration of a given suspension was estimated using a spectrophotometer (Uviline 9400, Secoman) by measuring the O.D. at 600 nm. Suspensions being measured were always diluted before measurement in such a way to achieve an O.D. not superior to 0.5 and stay within the linear range of the instrument. 2) cell counting. The number of cells was determined using an electronic particle counter (Beckman Coulter, model ZB-2). In order to stay within the linear range, cellular suspensions were diluted in isoton to a concentration between 2.5 x $10^3$ and 5 x $10^4$ in cells/mL. Dilutions were ten submitted to an ultrasound system (Branson Sonifier 250) for 30 s to eliminate any cellular agglomerates. By passing between 2 electrodes separated by 100 µm, cells were detected and counted.

### 4.2 Drop tests

Drop tests were used to screen cell growth under different stress factors. Solid YPD in petri dishes was used as base medium for the purpose and accordingly supplemented for each stress factor. For ethanol stress, the correct volume was added to melted YPD at

45ºC (just prior to solidification to avoid ethanol evaporation) in a way to obtain petri dishes with 10%, 12.5% and 20% (v/v). For temperature stress, regular YPD was incubated at 15ºC, 37ºC and 46ºC. For oxidative stress, $H_2O_2$ was added to melted YPD at 45ºC (just prior to solidification to avoid $H_2O_2$ evaporation) in a way to obtain petri dishes with 5 mM, 6.5 mM and 8 mM. For citric acid stress, concentrated YPD (10x) and agar (2x) were prepared and adjusted to pH 3. Stock solution of citric acid was also adjusted to pH 3. All compounds were mixed and adjusted to a final concentration of 1x with autoclaved distilled water, also adjusted to pH 3. For cell cultures, strains were pre-cultured in 5 mL YPD at 28ºC overnight, newly refreshed in 20 mL YPD at an initial O.D. of 0.1 and incubated a 28ºC. After 8h, cells were washed in sterile water (2x), suspended at 1 x $10^7$ cells/mL and then serially diluted from $10^{-1}$ to $10^{-5}$ in a total volume of 200 µL of sterile water, using sterile microplates as a support. Serial dilutions of cells were then spotted onto YPD petri dishes. A control petri dish (regular YPD) was incubated at 28ºC. All Petri dishes were incubated for 48h at 28ºC (except temperature drop tests). Additionally, 15ºC petri dishes were left for 96h whereas 12.5% and 20% ethanol ones were left first 48h at 28ºC and 5 additional days at room temperature (21ºC).

### 4.3 Viability and proportion determination

In fermentations where two different species were inoculated (*S. cerevisiae* and *K. marxianus*) their viability for propagation determination was performed. For the purpose, an aliquot of the cell suspension was taken, 12h, 18h, 24h and 48h. Cells then were centrifuged (4000 rpm for 5 min), washed in sterile water (2x) and serially diluted in sterile water to $10^{-5}$. Using this dilution, 100 µl were plated on YPD agar Petri-dishes and homogenously spread with sterile L-shaped spreaders. Each sample was spread in two petri dishes. One was incubated at 30ºC whereas the second at 42ºC, for 48h. At 30ºC both strains are able to grow however at 42ºC only *K. marxianus* is able to grow, hence the differentiation. By counting the colony-forming units (CFU), the proportion of each species was determinated.

## 5. Implementation of fermentations and monitoring of fermentation kinetics

Fermentations were carried out using 4 different methods, detailed below. For all, a pre-culture was carried out in 15 mL falcon tubes containing 5 mL of YPD left overnight at 28ºC with agitation (180 rpm). Cells were then centrifuged (4000 rpm for 10 min), washed in sterile water and resuspended in the same SM as the one being used for the respective fermentations. Cells were then counted to determine the correct volume for a 1 x $10^6$ cells/mL inoculation. When $SO_2$ was used, a concentrated solution of Potassium metabisulfite ($K_2S_2O_5$) (Labosi - A4871451) was prepared (43.8 mg/L). Right before inoculation, the correct volume of this solution was added (*e.g.* 1 µl/mL for 0.8 mg/L molecular $SO_2$ at a pH of 3.3) to the SM suspension as well as to SM being inoculated.

### 5.1 Microplate fermentations

Greiner transparent 96-well flat microplates with cover were used and each well was filled with a final volume of 200 µl. Once inoculated these were put to agitate and read in the microplate reader TECAN M200 Infinite Pro for 280 cycles (≈ 48 h) if at 28°C and for 560 cycles (≈ 96 h) if at 16°C. Agitation program included an orbital agitation (8 min at 140 rpm) as well as a linear one (2 min at 300 rpm). Readings were done in a multi-reading well system using the circle filled scheme with 3x3 measure points.

### 5.2 Mini-fermenters and flasks (300 mL)

The fermentations were carried out in glass mini-fermenters of 300 mL, filled with 250 mL of SM. The fermenters were equipped with water-filled airlocks to maintain anaerobiosis for the major part of the fermentation. The fermentations are kept at a constant temperature of 28 °C under continuous magnetic stirring at 150 rpm within a rack specifically designed for the purpose. Fermentation progress following was based on $CO_2$ release and the linear correlation between glucose consumed and ethanol and $CO_2$ produced (1 g of glucose consumed results in 0.46 g of $CO_2$ released). $CO_2$ release was

determined by weighing each fermenter during the fermentation (Bely *et al.* 1990a). To facilitate the monitoring of the fermentation, a custom built robot (PlateButler) was used to automatically bring each fermenter to a scale every 60 min, weigh it and return it to the original rack. Alfis was used as software to calculate the $CO_2$ release rate (g/L/h), the total of $CO_2$ released (g/L) and store those information in a common data base that could be accessed at any moment to retrieve the data (alfis.supagro.inra.fr/).

For some fermentations, these were also conducted 300 mL flasks with manual weighing.

### 5.3 Standard laboratory fermenters (1.2 L)

Fermentations performed with 1.2-L fermenters rely on the same exact principles, techniques and acquisitions as those the previous section (3.2.1). Aside the volume (1 L of SM used in each fermenter), the difference relies on each fermenter being placed directly on top of a scale and having its weight measured at regular intervals of 20 min.

### 5.4 Fermentations with Alcoholic Fermentation Monitor (AFM)

Fermentations were run using the instrument Alcoholic Fermentation Monitors, AFM, (Applikon-Biotechnology). This instrument is capable of monitoring the $CO_2$ release during fermentation by using an internal $CO_2$ sensor. This way $CO_2$ release rate and total production could be determined over time. 500-mL flasks containing 400 mL of SM were used. Temperature and agitation were kept constant at 30ºC and 200 rpm respectively with the help of the heating/steering bar. This technique was particularly used in Chapter 4 when assessing *K. marxianus* suitability under oenological conditions. For sequential fermentations, *S. cerevisiae* L1 was added after 12h of *K. marxianus* inoculation. Both strains were inoculated at 0.1 O.D. For co-inoculation, both strains were simultaneously inoculated at 0.05 making a total inoculation at 0.1 O.D.

## 6. Methods and plans

### 6.1 Lag phase determination

Every time the microplate method was used (O.D. following) the method chosen to determine the lag-phase duration was based on a local regression. By tracing a tangent line that contains the inflection point (the point where the acceleration equals zero and the velocity is maximal) and by determining where this tangent crossed the 0.1 O.D. value, lag-phase duration could be exactly determined (Swinnen *et al.* 2004; Gagneur and Neudecker 2012).

When using fermenters (300 mL and 1.2 L) a threshold of 25 g h/L/$CO_2$ was defined as the end of the lag phase.

### 6.2 Factorial plan

In order to test different stress factors in the same experiment we followed a factorial plan (FP) test using the microplate methodology. With a FP design not only the interaction between parameters can be studied but also the individual linear effects of each parameter (Montgomery 1991; Droesbeke *et al.* 1997).

The plan is based on the additive model $Y = \beta_0 + \beta_{1x1} + \beta_{2x2} + \beta_{3x3} + \beta_{4x4} + \beta_{12x1x2} + \beta_{13x1x3} + \beta_{14x1x4} + \beta_{23x2x3} + \beta_{24x2x4} + \beta_{34x3x4} + \varepsilon$. The parameter being measured (Y) is equal to an average value ($\beta_0$) modulated by the addition of the impact (unknown) of each factor, by the addition of the interactions between 2 factors and by an experimental error ($\varepsilon$) unknown. A summary of the experimental conditions used in the plan is shown in Table 12. Later, a Student's t-test was applied in order to verify the significance level of the impact of each stress factor (and possible interactions) on the lag-phase duration.

In the test, we included five stress factors. For each stress factor two values were considered, one non-stressful and another stressful (in bold): temperature – **16ºC** and 28ºC; osmotic stress – 200 g $L^{-1}$ and **280 g $L^{-1}$**; SO2 – 0 mg $L^{-1}$ and **0.8 g $L^{-1}$**; phytosterols – **1 g $L^{-1}$** and 5 g $L^{-1}$; thiamine – **25 µg $L^{-1}$** and 250 µg $L^{-1}$. With five stress factors to consider, we decided to exclude temperature as a factor due to its major impact and in order to simplify further result interpretation. With this, sixteen different experiments

were defined (Table N.12) and run at 16ºC and 28ºC separately. For reproducibility verification a particular experiment (Exp17) was repeated with each microplate, meaning five times by temperature. For each FP test, a p-value of 0.05 was considered statistically significant. In addition, the normality of residual distributions and homogeneity of variance were studied by standard diagnostic graphics; no violation of the assumptions were detected. For data analysis, statistical treatment and graphic representation the R software version 3.3.3 was used.

**Table N.12. Conditions used in the factorial plan.** The set of conditions was done at 28°C and at 16°C. Experiment 17 was repeated 5 times as measure of reproducibility and here each stress factor has an average level regarding their extremes in the plan.

| Experiment | Sugar (g/L) | $SO_2$ (mg/L) | Phytosterols (mg/L) | Thiamine (µl L) |
|---|---|---|---|---|
| **Exp1** | 200 | 0 | 1 | 25 |
| **Exp2** | 280 | 0 | 1 | 25 |
| **Exp3** | 200 | 0.8 | 1 | 25 |
| **Exp4** | 280 | 0.8 | 1 | 25 |
| **Exp5** | 200 | 0 | 5 | 25 |
| **Exp6** | 280 | 0 | 5 | 25 |
| **Exp7** | 200 | 0.8 | 5 | 25 |
| **Exp8** | 280 | 0.8 | 5 | 25 |
| **Exp9** | 200 | 0 | 1 | 250 |
| **Exp10** | 280 | 0 | 1 | 250 |
| **Exp11** | 200 | 0.8 | 1 | 250 |
| **Exp12** | 280 | 0.8 | 1 | 250 |
| **Exp13** | 200 | 0 | 5 | 250 |
| **Exp14** | 280 | 0 | 5 | 250 |
| **Exp15** | 200 | 0.8 | 5 | 250 |
| **Exp16** | 280 | 0.8 | 5 | 250 |
| **Exp17a** | 240 | 0.4 | 3 | 138 |
| **Exp17b** | 240 | 0.4 | 3 | 138 |
| **Exp17c** | 240 | 0.4 | 3 | 138 |
| **Exp17d** | 240 | 0.4 | 3 | 138 |

### 4.3 Central composite plan

In order to determine the influence of sugar (osmotic stress) and $SO_2$ presence in lag phase we used a central composite design to build a model describing the simple and quadratic effects as well as the interactions between those two factors. In this experimental design, we used the following levels to have a rotatable design (Table N.13):

**Table N.13. Summary of the different stress levels of osmotic stress (sugar) and $SO_2$. used in synthetic musts for the central composite plan.**

| **Factors** | **Levels** | | | | |
|---|---|---|---|---|---|
| | Lowest -1.41 | Low -1 | Center 0 | High +1 | Highest +1.41 |
| Sugar (mg/L) | 270 | 280 | 300 | 320 | 330 |
| $SO_2$ (mg/L) | 0.7 | 0.8 | 1 | 1.2 | 1.3 |

A total of 12 experiments were carried out. The effect of the two independent variables on lag phase (Y) was modeled with a polynomial response surface:

$$Y= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_{12} + \beta_{11} X_{11} + \beta_{22} X_{22} + \varepsilon$$

where $X_1$ and $X_2$ represent the coded values of initial sugar and initial $SO_2$ content respectively, Y is the predicted response, $\beta_0$ is the intercept term, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the quadratic coefficient, and $\beta_{ij}$ is the interaction coefficient and $\varepsilon$ are independent N (0, $\sigma^2$) error terms.

In addition, the normality of residual distributions and homogeneity of variance was studied with standard diagnostic graphs; no violation of the assumptions was detected.

The accuracy and general ability of each polynomial model described above was evaluated by a lack of fit test, the Fisher test and the adjusted coefficient of determination adjR2. Statistical analysis was performed with R software, version 3.1.3 (Team 2015) and the rsm library (Lenth 2009).

## 7. Adaptive laboratory evolution experiments

An adaptive laboratory evolution (ALE) based on a long-term serial transfer procedure in SM was conducted in sense of obtaining strains with a shorter lag phase under oenological conditions. As selective pressures, we selected the stress factors that are typically present at the beginning of a red wine fermentation meaning osmotic stress, $SO_2$ and low thiamine. The staring SM contained 300 g/L of sugar (150 g/L of glucose and 150 g/L of fructose), 0.8 mg/L molecular $SO_2$, and thiamine at 25 µg/L (added from a concentrated solution at 250 mg/L sterile filtered), considered a low level of thiamine. To increase our chances of success, our selective pressures were progressively increased during the ALE experiment, namely for osmotic stress and $SO_2$. In the case of thiamine, 1) we determined in Chapter 1 that its impact was among the least significant. 2) considered that a further reduction in its concentration would lead to nearly its absence from the SM, not representative of realistic conditions. A resume of the progressive increase in the selective pressures is in Table N.14.

**Table N.14. Summary of osmotic and $SO_2$ augmentations across ALE experiment and at which generations they were performed.**

| Generation | Stress level |
|---|---|
| **0 - 200** | 300 g/L sugar; 0.8 mg/L $SO_2$; 25 µg/L thiamine |
| **200 - 300** | 320 g/L sugar; 1.0 mg/L $SO_2$; 25 µg/L thiamine |
| **300 - 500** | 340 g/L sugar; 1.2 mg/L $SO_2$; 25 µg/L thiamine |

The experiment was performed using 13 mL double-capped tubes containing 5 mL of SM. For the first inoculation, yeast was cultured overnight at 28ºC in 5 mL of YPD, washed in sterile water, resuspended in the SM and inoculated at 0.1 O.D. Tubes were then incubated at 28ºC with agitation (225 rpm). From there, every 12h the cell suspension had its O.D. measured and an aliquot was used to inoculate fresh SM in such a way that the initial O.D. was 0.1. The selection of this timing (12h) was the result of a delicate balance between the purpose of our experiment and the principles of an evolutionary engineering. Our purpose was to obtain a reduction in lag phase time. After lag phase, cells readily enter in the exponential phase. By selecting cells specifically during lag

phase, we made sure cells being re-inoculated could only evolve for this stage and not for traits that could confer an advantage during exponential phase (*e.g.* faster growth rate). On the other hand, during a strict lag phase, no cell multiplication, and therefore no DNA replication, is occurring which is fundamental for positive mutations accumulation. The only way to overcome this was to re-inoculate at a moment where a minimum of generations occurred. We determined then that in the starting SM, at 12h, 4 – 6 generations occurred. We considered this a good compromise between the constraints stressed above. Additionally, 12h allowed a good practicability in managing the moments of re-inoculation during the same day.

The experiment was conducted using 3 independent replicates. This allowed to later assess if different evolutionary strategies could be used to achieve the same evolution. Every 50 generations, 2 ml samples of the evolving population were taken and stored at -80°C in 20% glycerol.

## 8. Molecular biology techniques

### 8.1 DNA extraction

Yeast genomic DNA for sequencing was extracted using the MasterPure Yeast DNA Purification Kit (epicentre). To extract the DNA, around 1 x $10^9$ cells were recovered from an overnight pre-culture in 15 mL YPD at 28ºC with agitation. Cell were centrifuged at 2000 rpm for 5 min, resuspended in 1 mL of sterile water and transferred to a sterile 2 mL microcentrifuge tube. This was centrifuged at 13000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 600 µL of Yeast Cell Lysis Solution. Then, 100 µL of RNase A solution (Qiagen, 2 mg/mL) was added to the tube, the suspension was vortexed for 2 min and incubated at 37 °C for 1h30. The suspension was further incubated at 65ºC for 15min then immediately placed on ice for 5 min. Next, 300 µL of MPC Protein Precipitation Reagent were added and the mixture was vortexed for 2 min. Cellular debris was pelleted by centrifugation at 13000 rpm for 10 min. The supernatant was transferred to a clean microcentrifuge tube, 1 mL of isopropanol were added and the mixture was mixed by inversion (5 times). This was left incubating on ice

for 10 min then the DNA was pelleted by centrifugation at 13000 rpm for 10 min and the supernatant was removed by pipetting. The pellet containing the DNA was washed with 0.5 mL of 70% ethanol and centrifuged at 13000 rpm for 3 min. Then, the ethanol was carefully removed by pipetting and with the tubes put in a SpeedVac in order to eliminate any residual ethanol. Finally, the DNA was suspended in 100 µL of TE buffer and stored at -20 °C.

Yeast genomic DNA for further analysis was extracted according to the protocol of Lööke *et al.* 2011. One yeast colony was picked from an agar plate and suspended in 100 µL lysis buffer (200 mM LiOAC, 1% SDS). The solution was incubated for 15 min at 70 °C, 300 µL of 96% ethanol were added and the mixture was vortexed. The cell debris was spinned down in a microcentrifuge for 5 min at 13000 rpm and the pellet was subsequently washed with 70% ethanol. The pellet was spinned down in a microcentrifuge for 5 min at 13000 rpm, the ethanol was removed by pipetting and remaining ethanol was evaporated using a vacuum centrifuge. Then, the pellet was dissolved in 100 µL milliQ water and the cell debris was spinned down in a microcentrifuge for 15 s at 13000 rpm.

### 8.2 DNA amplification by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) allows the amplification of a defined DNA strand, *e.g.* a genomic region. For this thesis, PCR was used for the construction of transformation cassettes, verification of performed transformations, verification of translocations $XVI^{VIII}$ and $XVI^{XV}$ presence and mating type confirmation. The PCRs were carried out in a thermocycler with a heating lid (T3 thermocycler, Biometra or Mastercycler pro, Eppendorf). Two different polymerase enzymes were used for the PCR, the high fidelity KAPA HiFi Polymerase (KapaBiosystems) and the low fidelity Taq Polymerase (Thermo Scientific). The compositions of the PCR mixes are given in Table N. 15.

The PCR programs were adapted to the chosen enzyme, to the hybridization temperature of the used primers and to the size of the fragment to be amplified. In general, the programs consist of an initial denaturation phase at 95 °C for 5 min, 30 - 35 cycles

including a denaturation phase between 95 °C and 98 °C for 20 sec to 1 min, a hybridization phase between 52ºC and 70ºC for 20 to 30 sec and an elongation phase at 72 °C for 30 sec to 5 min. The PCR program ends with a terminal elongation phase at 72 °C for 5 to 10 min. All primers were synthesized by MWG (Eurofins MWG Operon).

**Table N. 15. Reaction mix for the PCR using Taq polymerase (left) and Kapa HiFi polymerase (right).**

| Compound | Quantity | Volume (µL) | Compound | Quantity | Volume (µL) |
|---|---|---|---|---|---|
| **Buffer KCl 10×** | 1/10 | 2.5 | **Buffer KAPA HiFi 5×** | 1/5 | 10 |
| **dNTPs 10 mM** | 200 µM | 0.5 | **dNTPs 10 mM** | 200 µM | 1.5 |
| **Primer fw (10 µM)** | 0.4 µM | 1 | **Primer fw (10 µM)** | 0.5 µM | 1.5 |
| **Primer rv (10 µM)** | 0.4 µM | 1 | **Primer rv (10 µM)** | 0.5 µM | 1.5 |
| **Taq polymerase** | 0.035 U/µL | 0.25 | **Kapa polymerase** | 1 U/µL | 1 |
| **DNA (25ng/µL)** | 50 ng | 2 | **DNA (25ng/µL)** | 50 ng | 2 |
| **$MgCL_2$ 25 mM** | 2 mM | 2.5 | | | |
| **MilliQ water** | | to 25 | **MilliQ water** | | to 25 |

### 8.3 DNA purification

DNA samples obtained by PCR were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). One volume of sample was mixed with 2 volumes of Buffer NTI and 700 µL were loaded into a NucleoSpin Gel and PCR Clean-up Column placed into a collection tube (2 mL). The mixture was centrifuged in a microcentrifuge at 13000 rpm for 30 s, the flow-through was discarded and the column was placed back into the collection tube. Then, 700 µL of Buffer NT3 were added to the column. The mixture was centrifuged at 13000 rpm for 30 s, the flow-through was again discarded and the column was placed back into the collection tube. In order to remove Buffer NT3, the mixture was centrifuged at 13000 rpm for 1 min. Then, the column was placed into a new 1.5 mL microcentrifuge tube. 15-30 µL of 70 °C warm Buffer NE were added and the tubes were incubated at room temperature for 1 min. Subsequently, the tubes were

centrifuged at 13000 rpm for 1 min to eluate the DNA solution. The elution step was repeated to increase the DNA recovery rate.

### 8.4 Determination of DNA quantity and quality

After DNA extraction or PCR, the quality of the DNA was measured by spectrophotometry with a Nanodrop device (Nanodrop 1000, Thermo Scientific). The spectrophotometer gives certain wavelength signal ratios, which allows the detection of possible contaminations of RNA (260/280 nm higher than 2) and phenol (260/230 nm higher than 1.8).

DNA quantity was determined by fluorometric assay with the Quantus Fluorometer (Promega), using the QuantiFluor dsDNA system (Promega). First the QuantiFluor dsDNA Dye was diluted in 1:400 TE buffer to make the dye working solution. Then 1 µL of unknown DNA sample was added to 200 µL dye working solution into a 0.5 mL PCR tube. The samples were incubated at room temperature for 5 min and the fluorescence of the samples, indicating the DNA concentration, was measured.

### 8.5 Agarose gel electrophoresis

The validation of a PCR was performed by agarose gel electrophoresis, which allows the visualization of an amplification product and the determination of its size. The agarose gel consists of 0.8% agarose dissolved in 1 × TBE buffer (90 mM boric acid, 2 mM EDTA, 90 mM pH 8 Tris), with the addition of 0.2 µg/mL ethidium bromide. The PCR products are supplemented with a loading buffer, containing glycerol to increases the density of the sample and bromophenol blue to visualize its migration. A size marker (1 kb or 100 bp) is run along with each gel and allows the size determinations of the PCR products by comparing the migration distances. Gel electrophoresis is carried out in an electrophoresis device by submerging the gel in TBE buffer and applying a voltage of 120 V for 30 - 40 min. After migration, the ethidium bromide, which intercalates with the

DNA, allows the visualization of DNA by fluorescence under U.V. light. The gel is photographed subsequently (Kodak Gel Logic 100 Imaging system).

### 8.6 Yeast transformation

Yeast transformation was carried out according to the lithium acetate transformation method described by Güldener et al. 1996. Cells to transform are grown overnight in 20 mL of YPD at 28ºC. The O.D. of the culture is determined and 50 mL of YPD are inoculated to an OD of 0.5 at 28ºC. The suspensions are grown until the exponential phase is reached, which corresponds to an O.D. of 2.0 – 2.2, to ensure maximal transformation efficiency. The cells are recovered by centrifugation (3000 rpm for 5 min), washed with 40 mL of TRIS buffer (10 mM Tris HCl, pH 7.5), resuspended in 50 mL of lithium acetate buffer (0.1 M LiAc in 10 mM Tris HCl, pH 7.5) and incubated for 40 min at room temperature under gentle shaking. After centrifugation (3000 rpm for 5 min), the cells are resuspended in 2.2 mL of lithium acetate buffer. In a 13 mL double-capped tube, 100 µL of the cell suspension are mixed with 10 µL of salmon sperm DNA (denatured at 100 °C for 20 min and cooled in ice) and 1 – 3 µg of the transformation cassette. A second tube without the addition of transforming DNA is prepared as a negative control. After incubation for 10 min at room temperature, 300 µL PEG4000 (Sigma 95904) solution (50 % w/v in TRIS buffer, sterile filtered) are added. The mixture is incubated for 10 min at room temperature. Subsequently a thermal shock is applied by submersion of the tubes in a water bath of 42 °C for 15 min. The thermal shock stimulates DNA uptake into the cells. Afterwards, the mixture is centrifuged (1400 rpm for 3 min), and the cell pallet is resuspended in 0.5 mL of YPD and incubated overnight at 28ºC. The cells are then centrifuged (1400 rpm for 3 min), resuspended in TRIS buffer and spread on selective solid medium in two concentrations (1/10 and 9/10).The plates are incubated at 28ºC for 3 to 5 days and grown colonies are isolated and verified by external and overlapping PCR.

### 8.8 Microsatellite characterization

Microsatellite characterization allowed to determine how phylogenetically distant *S. cerevisiae* strains were this way have a measure of how genetically different they were. We plotted our strains with a considerable number of other strains coming from very different origins. For the purpose, 12 loci described by Legras *et al.* 2007 were used. Two multiplex of six primers pairs corresponding to loci C5, C3, C8, C11, C9, SCYOR267c and YKL172w, ScAAT1, C4, SCAAT5, C6, YPL009c, were amplified using the QIAGEN multiplex (polymerase chain reaction) PCR kit according to the manufacturer's instructions. PCRs were run in a final volume of 12.5 µL containing 10–250 ng yeast DNA. Amplification was carried out using an Eppendorf Mastercycler prothermalcycler (Eppendorf, Hamburg, Germany) with a three-phase temperature program: phase one, 1 cycle: 95°C for 15 min; phase two, 34 cycles: 94°C for 30s, 57°C for 2 min, 72°C for 1 min; phase three, 1 cycle: 60°C for 30 min. PCR products were sized for 12 microsatellite loci on a capillary DNA sequencer (ABI 310) using the polyacrylamide Pop4 and the size standards HD400ROX. For rare fragments larger than 400 nucleotides, three DNA fragments of 420, 450 and 485 bp amplified from phage M13 were added to the sample. Before the analysis, the PCR amplicons were first diluted 50 fold and then 1 µL of the dilution was added to 18.75 µL of formamide (Applied Biosystem) and 0.25 µL of HD400ROX size marker, and the mixture was denaturated at 92°C for 3 min. Allele distribution into classes was carried out using Geneious 9.1.6 (Biomatters, Auckland , NZ).

## 9. Obtaining segregant populations

### 9.1 Obtaining heterothallic antibiotic resistant strains

Selected diploid strains for the QTL approach were transformed with an antibiotic resistance cassette. This made possible the later haploid and diploid selection, necessary to recover the desired strains. This transformation was specifically done in a way to inactivate the gene *HO*, resulting in later heterothallic haploids, only capable of sexually

reproducing with another independent haploid. Cassettes using for such transformation included ClonNAT, blasticidin, hygromycin, and phleomycin, cassettes and were integrated using the short-flanking homology PCR technique (Schiestl and Gietz 1989). The clonNAT, blasticidin, hygromycin, and phleomycin cassette were PCR amplified from pAG25, p8F, pAG32 and pUG66 plasmids respectively.

The PCR fragments were used to transform strains using the lithium acetate method described by Güldener et al. 1996. The deletions were verified by PCR analysis of total DNA isolated from antibiotics-resistant transformants, using primers flanking the *HO* regions as well as primers within the cassettes.

### 9.2 Sporulation and asci dissection

Selected diploids, previously transformed with respective antibiotic resistance cassette, were inoculated in 15 mL of rich media GNA, incubated at 28ºC overnight. Cells were then centrifuged (1500 rpm for 5 min) and resuspended in poor media SpoMa. This step was repeated once in order to eliminate any residual GNA. The cell suspension was newly centrifuged and half of the pellet resuspended in 20 mL of SpoMa. This suspension was left at room temperature with slight gentle agitation until asci could be observed in the microscope (5 to 10 days). When sporulation sufficiently took place, asci were digested with a solution of 5% Lyticase (from Arthrobacter luteus, crude, Sigma) for 8 min resulting in a deterioration of the walls. 2 - 5 µL of cell suspension were spread on a seed strip at the side of the micromanipulation plate (thinner YPD). The dissection and isolation of yeast asci was carried out using an MSM300 micromanipulator (Singer Instruments, UK). The micromanipulation system is equipped with a needle that manually and individually retrieves the different spores of the same ascus and positions them on a grid, which serves as a visual reference. Spore germination was stimulated by incubation for 48 to 72 h at 28ºC on YPD plates containing the appropriate of each respective antibiotic (200 µg/ ClonNAT mL; 200 µg/mL Hygromycin; 150 µg/mL Phleomycin; 500 µg/mL Blasticidin). Each haploid strain was then confirmed to still have the translocation $XVI^{VIII}$ by PCR as well as a similar lag phase profile of the respective parental strains.

### 9.3 Reversing mating types

In order to cross all haploids between them, which corresponds to 6 independent crossings in total, we changed the mating type of two strains, L8α and L11a. This required a diploidization step, obtained by transforming the haploids with the plasmid phS3 (Güldener *et al.* 1996) using the lithium acetate method (Schiestl and Gietz 1989). This plasmid contains the gene *HO* and once integrated, it restores the same functions as a genomic *HO*. Cells were then cultivated and became diploid after few generations. The diploidy was confirmed by PCR with primers Mat1, Mat2 and Mat3, by observation of two bands simultaneously present. Plasmid loss was promoted by cultivating cells in fresh media for 24h with several reinoculations. After plasmid loss confirmation (no growth on ClonNAT), a new sporulation process was initiated as previously described, in order to select haploids with an opposite mating type of the initial haploid, in this case L8α *MATα* and L11a *MATa*. Several haploids were tested their mating type was confirmed by PCR. Haploids L8a and L11α were selected, possessing an opposite mating type.

### 9.4 Construction of *S. cerevisiae CLB3*-GFP reporter strains

Haploid strains had their mating type determined both by PCR (***MATa***/*MATα* amplification) and yeast crossing, by putting each haploid together with one of two reference strains of known mating type (**a** or α). Next, GFP reporter constructs targeted for integration into *CLB3* gene were generated in the 6 haploids strains using the short-flanking homology PCR technique (Güldener et al. 1996). The plasmid pKT127 containing a reporter cassette with the *GFP* gene and the kanamycin resistance marker was used as the PCR template to generate KANMX4-GFP integration cassettes. The primers used for this amplification contained homolog tails for integration in the C-terminal region of the gene *CLB3*. The integrations were verified by PCR analysis of total DNA isolated from geneticin-resistant transformants, using primers flanking the *CLB3* regions (CLB3_F/CLB3_R) as well as within the cassette (CLB3GFP_VR). After this process we were able to obtained 6 haploids GFP marked.

### 9.4 Crossing the haploids

For crossing, each pair of haploids were pre-cultivated (separately) and had 10 µL of their cellular suspension deposited on YPD Petri dish in an overlapping fashion. Cells were left incubating for 6h at 28ºC. By microscopic observation crosses were confirmed by identifying typical mating forms (similar to clovers). Diploids were then selected by striking these in solid YPD containing the respective double antibiotics. After 48h at 28ºC, hybrids were isolated and confirmed by PCR, checking the presence of both resistance cassettes by using the combination of the 3 PCR previously used for each cassette. In total, 6 diploids were obtained.

### 9.5 Promoting crossing over to obtain highly recombinant populations

In order to promote crossing-over and recombination, the previous diploids obtained were newly submitted to a sporulation process. This would later on increase the resolution QTL mapping and facilitate the identification of relevant peaks. The sporulation suspension was submitted to a killing ether-zymolyase treatment, a procedure for ascospore isolation from cultures containing unsporulated vegetative cells (Bahalul et al. 2010). 500 µL of sporulation suspension and 500 µL of diethyl ether were mixed and left incubating for 20 min at room temperature. Cells were washed four times with sterile water to completely eliminate ether trace elements. Cells were then resuspended in 900 µl of sterile water and 100 µL of zymolyase solution 20 T (200 U/mL) were added for ascus digestion. The mixture was incubated 20 min at 30ºC and vortexed for 1 min. In order to stabilize the spores population these were diploidized by incubation for 6h in YPD at 28ºC and inoculated in solid YPD overnight at 28ºC. This allowed us to obtain 6 individual highly recombinant populations containing segregants with unique allelic combinations.

### 10. Flow cytometry and cell sorting

Flow cytometry was used to confirm and follow GFP signal as well as to perform the cell sorting using FACS.

As GFP was associated with a cyclin (Clb3p) expressed from the S–phase to $G_2$-phase, once in lag phase, we were able to detect the exact moment when cells entered multiplication and left lag phase. Additionally, we used the marker Vybrant DyeCycle Ruby which allows to detect the amount of DNA present within the cell, meaning distinguishing between **n** and **2n**, without the need of fixing the cells.

For the purpose, 1.25 mg of the dried yeast preparation obtained for each segregant population was directly inoculated in SM media at 28°C with moderate agitation. 20 min before lag phase exit moment, 0.5 mL of cell suspension at 5 x $10^5$ cells/mL had 1 µL of Vybrant DyeCycle Ruby stain (Thermo Fisher) added to a final concentration of 5 µM. The suspension was incubated at 28ºC for 15 min, protected from light. Without washing, cells were ran in a flow cytometer cytometer using the band pass filter FL1 for GFP detection (488 nm excitation, emission collected at 533/30 nm) as well as FL4 for Vybrant DyeCycle detection (640 excitation, emission collected at 675/25 nm).

For signal following the flow cytometer Accuri C6 (BD Biosciences) was used. For cell sorting FACS Aria (Becton-Dickinson, Mountain View, CA) was used instead. The speed of sorting was 5,000 events/sec and were acquired, stored and analyzed using the FACS Diva software (Becton-Dickinson, Mountain View, CA). Gating for sorting was adjusted to control population (0h of fermentation, immediately after inoculation) where cells where not dividing and therefore no GFP was being expressed, eliminating therefore natural fluorescence. 0.3% of positive events were admitted in the gatings. Sorted cells were recovered in strict sterile conditions directly into a 15 mL falcon tube containing 5 mL YPD. These were then left to grow overnight.

## 11. Propagation and drying

To be closer to the realistic conditions in which any commercial wine yeast is produced, we included in our experiments an industrial propagation and drying process performed at Lallemand Inc (Montreal, Canada) and Lallemand SAS (Blagnac, France).

### 11.1 Propagation

Yeast propagation aims for a considerable increase in biomass within controlled and optimized conditions. For the purpose a serial scale up of batch cultures are performed in order to produce sufficient biomass for the subsequent fed-batch steps. From a fresh Petri dish yeast is inoculated in 25 mL of laboratory media for 48h at 28ºC. An aliquot is then used for another batch fermentation this time using 1.4 L of diluted molasses, incubated 24h at 28ºC. Next, 2 fed-batch cultures, the first called seed, the second commercial, are performed in 25 L fermenters. During fed-batch fermentation different parameters are strictly controlled. These include molasses and nitrogen feeding rate, aeration, temperature, pH, yeast growth and ethanol. These are optimized in such a way that just enough sugar and nutrients are fed to the yeast to maximize multiplication and minimize production of ethanol. This process is computer-controlled and recipes may vary depending on yeast being propagated. The seed, performed in molasses for 24h, is usually split and used as an inoculum to produce 3 to 4 commercial fed-batch propagations, also performed in molasses, during 16h.

### 11.2 Drying

Once a commercial fed-batch is finished, yeast produced undergoes several steps that gradually remove water from the biomass until an active dried yeast is obtained. Yeast was firstly separated from the molasses by washing and centrifugation. The resulting cream (18-20% DW) was then vacuum-filtered and pressed yielding a cake yeast (30-32% DW). The cake was finally mixed with an emulsifier to help in rehydration, extruded and dried leading to 100 g of active dry yeast with ≈ 95% DW.

## 12. Genomic analysis

Each population of sorted cells were cultivated on 10 mL YPD media at 28°C, and DNA was extracted with kit MasterPure Yeast DNA purification kit (Epicentre) according to manufacturer's instructions.

Genomic DNA samples were submitted to the sequencing platform Genotoul (Toulouse, France) and sequenced using the Illumina technology (paired end, 2 x 150 bp, HiSeq 3000) at a sequencing depth of 20-100 fold.

Library preparation and sequencing were performed at GetPlage plateform Toulouse. DNA was cheered and library prepared using a PCR free kit (Illumina TruSeq Nano), and sequencing was performed in a Illumina HiSeq3000 sequencer from the two paired extremities of DNA fragments (2 x150bp reads).

### 12.1 Genomic data treatment and QTL peak analysis

Reads obtained from sequencing were processed for quality using Trimmomatic (v 0.32.1) under Galaxy environment. Then reads were mapped on S288C genome sequence with Bowtie (v2.2.6 for Galaxy). Resulting alignment files were then genotyped simultaneously using the Mpileup v2.1.3 and bcftools view v1.40 modules of samtools for Galaxy. From the final multi individual variant file, the sequencing depth of each allele which reflects the allelic frequencies in the population was extracted using customs perl scripts. Variant position between each parent were filtered, and only position, in which less than 10 reads for the minor allele for one parent (and more than 50 for the other parent) were kept for QTL analysis. The software multipool (Edwards and Gifford 2012) was used using the contrast method, assuming a minimum segregant population of 10000 individuals. The outcome was the determination across the genome of significant allelic frequency differences between both populations, designated as LOD scores. The higher this score (defined on a logarithmic scale) the higher are the chances of the frequency differences found being strictly correlated to phenotype studied. To increase

our selection power, we decided to only consider peaks with LOD scores superior to 10. In these regions, we retained only positions with LOD values differing less than 1 from the maximum peak value.

### 12.2 Genomic data treatment and identification of positive mutations

We used the Genome Analysis Toolkit (GATK) version 2.7 for making SNP and indel calls, following "Best Practice Variant Detection with GATK version 4, for release 2.0" available online (McKenna *et al.* 2010). Briefly, the workflow is divided into four sequential steps: initial mapping, refinement of the initial reads, multi-sample indel and SNP calling, and finally variant quality score recalibration. First, reads were aligned to the S288C reference genome (release number R64-1-1, downloaded from SGD) using BWA version 0.6.2 (Li and Durbin 2009) resulting in aligned reads in a BAM file format. Second, optical and PCR duplicates were removed using MarkDuplicate from the Picard Tools version 1.84 (http://picard.sourceforge.net). Reads around indels were realigned to increase indel call accuracy using IndelRealigner (GATK). Base quality was recalibrated using BaseRecalibrator/PrintReads (GATK). After recalibration, the recalibrated base quality scores in the output BAM file were closer to their actual probability of mismatching the reference genome, and were subsequently more accurate. Moreover, the recalibration tool attempted to correct for variation in quality with machine cycle and sequence context. At the end of this step we got the analysis-ready reads. Third, we performed SNP and indel discovery across all evolved and ancestral sequences simultaneously using UnifiedGenotyper (GATK). Fourth, we used Variant Quality Score Recalibration (VQSR) to build an adaptive error model using known variant sites and then applied this model to estimate the probability that each variant in the callset is a true genetic variant or a machine/alignment artifact. This step was performed with the VariantRecalibrator / ApplyRecalibration tools of GATK, using an unpublished dataset of known SNP and indels obtained from 86 available genomes to train the model. Variants from each project were merged using GATK. This genotyping pipeline provided us a final multi-sample VCF file containing 502,296 SNP and 32,173 Indels discovered across samples to which were associated a genotyping quality for each strain. For the loss of

heterozygosity assessment Custom perl scripts were used to compute average shifted histogram (averaging 10 windows of 10,000 bp) of variant frequency across the genome. A custom R script was used to plot the variant density, differentiating homozygous from heterozygous genotypes. Finally, for genetic variations and coding impact we used a custom perl script to extract variant positions where at least one genotype differed among the experimentally evolved isolates. SnpEff version 3.3h (Cingolani *et al.* 2012a, 2012b) was then used to predict variant effect at these selected positions (see http://snpeff.sourceforge.net for details).

**13. Extraction, identification and quantification of volatile compounds**

Assessment of volatiles produced during fermentation on SM was carried out by the Teagasc Moorepark analytical facility.

1 ml of sample was added to a 20 ml screw capped SPME vial and equilibrated to 40ºC for 10 mins with pulsed agitation of 5 sec at 500 rpm. Samples were analysed in triplicate. Sample introduction was accomplished using a Shimadzu AOC 5000 Autosampler. A single 50/30 um CarboxenTM/divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used. The SPME fiber was exposed to the headspace above the samples for 20 min at depth of 1 cm at 40°C. The fiber was retracted and injected into the GC inlet and desorbed for 2 min at 250ºC. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60m x 0.32mm x 1.8µm) column using a split/splitless injector in splitless mode. A merlin microseal was used as the septum. The temperature of the column oven was set at 40ºC, held for 5 min, increased at 5ºC/min to 230ºC then increased at 15°C/min to 260°C, yielding at total GC run time of 50 min. The carrier gas was helium held at a constant flow of 1.2ml/min. The detector was a Shimadzu TQ8030 mass spectrometer detector, ran in single quad mode. The ion source temperature was 220 C and the interface temperature was set at 260ºC. The MS mode was electronic ionization (70v) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, a commercial flavour and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound using Kovats index. Retention indices were

matched against peer reviewed publications where possible to confirm compound identification. Spectral deconvolution was also performed to confirm identification of compounds using AMDIS. Batch processing of samples was carried out using MetaMS (Wehrens *et al.* 2014). MetaMS is an open-source pipeline for GC-MS-based untargeted metabolomics. An auto-tune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. A set of external standards was ran at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection was performing within specification.

# RESULTS

# CHAPTER 1

## YEAST MULTI-STRESS RESISTANCE AND LAG PHASE CHARACTERIZATION DURING WINE FERMENTATION

**Chapter's context**

Wine making includes undoubtedly several stages which progress directly dictates the success or failure of a wine fermentation. Today some are very well mastered whereas others still require improvement and better understanding. A particular example of the latter, and to which we decided to focus our attention, is the lag phase of a wine fermentation. Much is known about lag phase *sensu lato*, a period where typically a microorganism adapts to a new environment before being able to multiply and start using the resources present. Although many different works have studied lag phase, their number gets considerably reduced if we specifically look for the context of wine fermentation and the use of industrial wine yeast strains. Limited physiological and molecular information is today available that can provide a complete insight in this matter. With this in mind we decided to perform a global phenotypic and genotypic characterization of features that we considered relevant for lag phase trait. For that, a set of 10 industrial wine yeast strains was provided, and currently commercialized, by the company Lallemand SAS. To obtain a broader overview on lag phase response we included in this set strains of different species, with different origins and diverse types of fermentation to which they are usually intended for. We believe our work is an additional step towards a deeper knowledge and characterization of wine yeast during a wine fermentation start. Ultimately, it may contribute to a better mastering of the lag phase and help prevent stuck/sluggish fermentations, wine spoilage and extra costs associated.

FEMS Yeast Research, 17, 2017, fox051

doi: 10.1093/femsyr/fox051
Advance Access Publication Date: 10 July 2017
Research Article

RESEARCH ARTICLE

# Yeast multistress resistance and lag-phase characterisation during wine fermentation

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**One sentence summary:** New insights on conventional and non-conventional yeast's lag phase during alcoholic wine fermentation focusing on genetic and multistress resistance to environmental factors typically present at fermentation start.

**Editor:** John Morrissey

## ABSTRACT

*Saccharomyces cerevisiae* has been used to perform wine fermentation for several millennia due to its endurance and unmatched qualities. Nevertheless, at the moment of inoculation, wine yeasts must cope with specific stress factors that still challenge wine makers by slowing down or compromising the fermentation process. To better assess the role of genetic and environmental factors that govern multistress resistance during the wine fermentation lag phase, we used a factorial plan to characterise the individual and combined impact of relevant stress factors on eight *S. cerevisiae* and two non-*S. cerevisiae* wine yeast strains that are currently commercialised. The *S. cerevisiae* strains are very genetically diverse, belonging to the wine and flor groups, and frequently contain a previously described $XVI^{VIII}$ translocation that confers increased resistance to sulphites. We found that low temperature and osmotic stress substantially affected all strains, promoting considerably extended lag phases. $SO_2$ addition had a partially temperature-dependent effect, whereas low phytosterol and thiamine concentrations impacted the lag phase in a strain-dependent manner. No major synergic effects of multistress were detected. The diversity of lag-phase durations and stress responses observed among wine strains offer new insights to better control this critical step of fermentation.

**Keywords:** yeast; wine fermentation; lag phase; multistress resistance; environmental factors; non-conventional yeast

## INTRODUCTION

*Saccharomyces cerevisiae* is the yeast species that is most commonly used for winemaking. Among *S. cerevisiae*, wine yeasts form a particular genetic cluster (Fay and Benavides 2005; Legras *et al.* 2007; Liti *et al.* 2009). Flor yeasts, used in sherry wine fermentation and typically forming a biofilm on wine surfaces, also form a distinct cluster but one that is very close to that of wine (Legras, Erny and Charpentier 2014; Coi *et al.* 2017). In recent decades, man has been trying to improve the performance and desired features of wine yeast by using different approaches such as genetic engineering, adaptive evolution, quantitative trait locus mapping and marker-assisted hybridisation (Dequin 2001; Chambers and Pretorius 2010; Steensels *et al.* 2014). In recent years, the use of other *Saccharomyces* species or of mixed cultures of *S. cerevisiae* and non-*Saccharomyces* yeasts has attracted a growing interest in efforts to improve the complexity and enhance the particular and specific characteristics of wines (Romano *et al.* 2003; Ciani, Beco and Comitini 2006; Comitini *et al.* 2011). Although generally less efficient than *S. cerevisiae*, some non-*Saccharomyces* strains such as *Torulaspora delbrueckii*

**Received:** 6 May 2017; **Accepted:** 6 July 2017
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can bring unique and appealing features to the winemaking process such as specific aromas or reduced ethanol production (Varela 2016), while *Saccharomyces* species such *S. uvarum* and *S. kudriavzevii* or interspecies hybrids have the possibility to enable fermentation at lower temperatures (Gonzalez *et al.* 2007). These results have created a recent demand for the exploitation of 'non-conventional yeasts' that can add appealing values to the final product and/or the fermentation process in winemaking.

However, despite such great achievements during recent decades, the process of winemaking is far from fully optimised. A particular issue that still challenges winemakers is the moment of must inoculation and the start of fermentation. Due to its particular characteristics, grape must is a stressful and inhospitable medium that is far from the optimal growth conditions of yeast. The usually high sugar concentration (180–280 g $L^{-1}$ equimolar glucose and fructose), low pH, addition of $SO_2$ (40 to 100 mg $L^{-1}$) as an antimicrobial agent, presence of other microorganisms, limiting amount of nutrients and low temperatures at which the fermentation might occur (especially in the case of white wines) add up to a very stressful inoculation scenario (Bauer and Pretorius 2000; Ribéreau-Gayon *et al.* 2006; Arroyo-López *et al.* 2009; Medina *et al.* 2012; Matallana and Aranda 2016; Nadai *et al.* 2016). Common issues caused by these conditions include delays in fermentation starts, the appearance of undesired and uncontrolled flora and, later, sluggish/stuck fermentation. Ultimately, these setbacks can cause extra expenses, time wasting and final product spoilage.

The capacity of yeast to successfully overcome all these stress factors that are present in the grape must play a very important role in the start of wine fermentation, which is directly correlated to the yeast's lag phase. The lag phase is typically a period of adjustment in which a given microbial population adapts to a new medium before it starts growing exponentially (Medawar, Strehaiano and Délia 2003; Swinnen *et al.* 2004). Although several papers have addressed the molecular changes that occur at this growth stage (Brejning and Jespersen 2002; Brejning, Jespersen and Arneborg 2003; Rossignol *et al.* 2006; Gutiérrez *et al.* 2013), very few have addressed the role of stress factors in yeast's lag phase. Recently, Zimmer *et al.* (2014), using a quantitative trait locus (QTL) mapping approach, discovered a new translocation ($XVI^{XV}$) involving the gene *SSU1* (coding for a sulphite exporter) and the promoter region of *ADH1*; this translocation confers higher sulphite resistance in the first hours of fermentation to the strains that have it and is thought to be a recent event, contrary to the $XVI^{VIII}$ translocation that is widely disseminated among wine yeasts, resulting in higher expression of *SSU1* and greater resistance to sulphites (Pérez-Ortín *et al.* 2002).

The objective of this work is to shed new light on wine fermentation lag-phase modulation and yeast stress resistance during this particular stage. Here, we focused on understanding the role of environmental factors (i.e. stress factors) that are present in wine must at the moment of inoculation and the extent of their impact on the lag phase. Concomitantly, we also investigated the yeast stress response diversity by using 10 different currently commercialised yeast strains (six *S. cerevisiae*, two *S. cerevisiae* × *S. kudriavzevii* hybrids and two non-*S. cerevisiae*). We analysed the combined effects of the main stress factors at the beginning of a wine fermentation (temperature, osmotic stress, $SO_2$ presence and low levels of phytosterols and thiamine) in conditions that mimic wine fermentation conditions. The obtained knowledge will contribute to a better understanding of the behaviour of wine yeast strains during the wine fermentation lag phase. Ultimately, this step forward might be used to improve winemaking by reducing the risks of failed fermentation starts, decreasing expenses and increasing the reliability of the fermentation process.

## MATERIAL AND METHODS

### Strains and precultures

All wine yeast strains were provided by the company Lallemand SAS and are currently commercialised for winemaking. In total, 10 different strains were used including six *Saccharomyces cerevisiae* (L1, L3, L7, L8, L10 and L11) two *S. cerevisiae* × *S. kudriavzevii* hybrids (L4 and L6), one *Saccharomyces uvarum* (L9) and one *Torulaspora delbrueckii* (L5). Precultures were carried out in 15-ml falcon tubes containing 5 ml of YPD (1% Bacto yeast extract, 2% bactopeptone and 2% glucose) that were left overnight at 28°C with moderate agitation (180 rpm).

### DNA extraction, PCR and microsatellite characterisation

DNA extraction was based on the protocol reported by Löoke, Kristjuahan and Kristjuhan (2011). A colony of each strain was picked from a petri dish culture and resuspended in 100 $\mu$l of solution (200 mM LiOAc and 1% SDS). This cell suspension was incubated for 5 min at 70°C, and 300 $\mu$l of absolute ethanol (96%–100%) was then added. After vortexing the sample and centrifuging it at 15 000 × g for 3 min, the resulting pellet was washed with 70% ethanol. The ethanol was completely evaporated, and the pellet was dissolved in 100 $\mu$l of standard TE (10 mM Tris and 1 mM EDTA, pH 8.0). Finally, the cell debris was spun down for 15 s.

The presence or absence of the chromosomal translocations that were previously described to be involved in $SO_2$ resistance was investigated using PCR. Sigma-Aldrich Taq DNA polymerase enzyme was used, and the manufacturer's procedures were followed. The final concentration of each component in each PCR reaction was the following: 1x PCR buffer, 200 $\mu$M dNTP mix, 0.5 $\mu$M forward (Fw) and reverse (Rv) primers, 0.05 Taq units $\mu L^{-1}$ and 200 pg $\mu L^{-1}$ of DNA template in a 25 $\mu$l final volume. The primers targetting each chromosome were the following: chromosome 8, Fw—TCGAACATCGAGCATGCA, Rv—CCATATTTGTGATGATATCG; chromosome 15, Fw—ACCTATCGAGTCTCCCAC, Rv—GACACCCATGACCATCAC; and chromosome 16, Fw—AAAGAAGTTGCATGCGCCTA, Rv—GCCCCTCCATGTTCTACTATT. The PCR amplification conditions were (i) 95°C for 10 min, (ii) 94°C for 1 min, (iii) 51°C for 2 min, (iv) 72°C for 2 min and (v) 72°C for 10 min. Steps ii–iv were repeated 30 times.

For the microsatellite characterisation, 12 loci that were described elsewhere (Legras *et al.* 2007) were used. Two multiplex reaction using six primers pairs that corresponded to loci C5, C3, C8, C11, C9, SCYOR267c, YKL172w, ScAAT1, C4, SCAAT5, C6 and YPL009c were performed using the QIAGEN multiplex (polymerase chain reaction) PCR kit according to the manufacturer's instructions. PCRs were run in a final volume of 12.5 $\mu$L and contained 10–250 ng of yeast DNA. Amplification was carried out using an Eppendorf Mastercycler pro thermal cycler (Eppendorf, Hamburg, Germany) with a three-phase temperature programme: phase 1, 1 cycle: 95°C for 15 min; phase 2, 34 cycles: 94°C for 30 s, 57°C for 2 min and 72°C for 1 min; and phase 3, 1 cycle: 60°C for 30 min. PCR products were sized for 12 microsatellite loci on a capillary DNA sequencer (ABI 310) using POP-4 polymer and the size standards HD400ROX. For rare fragments that were

**Table 1.** Conditions used in the factorial plan.

| Experiment | Sugar ($g\,L^{-1}$) | $SO_2$ ($mg\,L^{-1}$) | Phytosterols ($mg\,L^{-1}$) | Thiamine ($\mu L\,L^{-1}$) |
|---|---|---|---|---|
| Exp1 | 200 | 0 | 1 | 25 |
| Exp2 | 280 | 0 | 1 | 25 |
| Exp3 | 200 | 0.8 | 1 | 25 |
| Exp4 | 280 | 0.8 | 1 | 25 |
| Exp5 | 200 | 0 | 5 | 25 |
| Exp6 | 280 | 0 | 5 | 25 |
| Exp7 | 200 | 0.8 | 5 | 25 |
| Exp8 | 280 | 0.8 | 5 | 25 |
| Exp9 | 200 | 0 | 1 | 250 |
| Exp10 | 280 | 0 | 1 | 250 |
| Exp11 | 200 | 0.8 | 1 | 250 |
| Exp12 | 280 | 0.8 | 1 | 250 |
| Exp13 | 200 | 0 | 5 | 250 |
| Exp14 | 280 | 0 | 5 | 250 |
| Exp15 | 200 | 0.8 | 5 | 250 |
| Exp16 | 280 | 0.8 | 5 | 250 |
| Exp17a | 240 | 0.4 | 3 | 138 |
| Exp17b | 240 | 0.4 | 3 | 138 |
| Exp17c | 240 | 0.4 | 3 | 138 |
| Exp17d | 240 | 0.4 | 3 | 138 |
| Exp17e | 240 | 0.4 | 3 | 138 |

The set of conditions was done at 28°C and at 16°C. Experiment 17 was repeated five times as measure of reproducibility and here each stress factor has an average level regarding their extremes in the plan.

larger than 400 nucleotides, three DNA fragments of 420, 450 and 485 bp that were amplified from phage M13 were added to the sample. Before the analysis, the PCR amplicons were first diluted 50-fold, and then 1 $\mu$L of the dilution was added to 18.75 $\mu$L of formamide (Applied Biosystems) and 0.25 $\mu$L of HD400ROX size marker; the mixture was then denaturated at 92°C for 3 min. Allele distribution into classes was carried out using Geneious 9.1.6 (Biomatters, Auckland, New Zealand).

## Experimental design and determining the lag-phase duration

The method chosen to determine the lag-phase duration is based on a local regression (Fig. S1, Supporting Information). By tracing a tangent line that contains the inflection point (the point where the acceleration equals zero and the velocity is maximal) and by determining where this tangent line reaches an OD value of 0.1, the lag-phase duration could be exactly determined (Swinnen *et al.* 2004; Gagneur and Neudecker 2012).

To test different stress factors in the same experiment, we followed a factorial plan (FP) test. With an FP design, not only the interaction between parameters but also the individual linear effects of each parameter can be studied (Montgomery 1991; Droesbeke *et al.* 1997).

The plan is based on the additive model $y = B0 + B1 \times 1 + B2 \times 2 + B3 \times 3 + B4 \times 4 + B12 \times 1 \times 2 + B13 \times 1 \times 3 + B14 \times 1 \times 4 + B23 \times 2 \times 3 + B24 \times 2 \times 4 + B34 \times 3 \times 4 + PtC + E$. The parameter being measured (y) is equal to an average value (B0) that is modulated by the addition of the impact (unknown) of each factor, by the addition of the interactions between two factors, by the addition of a central point (PtC) for reproducibility assessment and by an experimental error (E) unknown. A summary of the experimental conditions that were used in this plan is shown in Table 1. Later, a Student's t-test was applied in order to verify the significance level of the impact of each stress factor (and their possible interactions) on the lag-phase duration.

In the test, we included five stress factors. For each stress factor, two values were considered. One value was moderately stressful, and another (in bold) was highly stressful; these factors and their values were temperature – **16** and 28°C, osmotic stress – 200 and **280 g L$^{-1}$**, $SO_2$ – 0 and **0.8 g L$^{-1}$**, phytosterols – **1** and 5 g L$^{-1}$ and thiamine – **25** and 250 $\mu$g L$^{-1}$. With five stress factors to consider, we decided to exclude temperature as a factor (due to its high impact) in order to simplify the interpretation of results. With this arrangement, 16 different experiments were defined (Table 1) and run separately at 16°C and 28°C. For reproducibility, a particular experiment (Exp17) was repeated five times (for each temperature) with each microplate (represented in the model as the factor PtC). For each strain, it was verified that PtC did not have a significant impact ($P$-value > 0.05), meaning that there were no significant differences between the curves of Exp17 and therefore the experiments were reproducible. For each stress factor, a $P$-value < 0.05 was considered to indicate that the influence of the factor was statistically significant. In addition, the normality of residual distributions and the homogeneity of variance were studied by standard diagnostic graphics; no violation of the assumptions was detected. Data analysis was performed with a statistical treatment and graphically represented using the R software version 3.3.3.

## Fermentations

Fermentations were performed on synthetic must (SM), which mimics a natural grape must, as described by Bely, Sablayrolles and Barre (1990). The SM base that was used in this work contained 6 g L$^{-1}$ of malic acid, 6 g L$^{-1}$ of citric acid, salts (0.75 g L$^{-1}$ $KH_2PO_4$, 0.5 g L$^{-1}$ $K_2SO_4$, 0.25 g L$^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.155 g L$^{-1}$ $CaC_{12}{\cdot}2H_2O$ and 0.2 g L$^{-1}$ NaCl), trace elements (4 mg L$^{-1}$ $MnSO_4{\cdot}H_2O$, 4 mg L$^{-1}$ $ZnSO_4{\cdot}7H_2O$, 1 mg L$^{-1}$ $CuSO_4{\cdot}5H_2O$, 0.4 mg L$^{-1}$ $CoCl_2{\cdot}6H_2O$, 1 mg L$^{-1}$ $H_3BO_3$ and 1 mg L$^{-1}$ $(NH_4)_6Mo_7O_{24}$) and 10 mg L$^{-1}$ of vitamin stock solution (composed of 20 mg L$^{-1}$ myo-inositol, 1.5 mg L$^{-1}$ calcium pantothenate, 2 mg L$^{-1}$ nicotinic acid, 0.25 mg L$^{-1}$ pyridoxine and 0.003 mg L$^{-1}$ biotin).

The nitrogen source was composed of 0.27 g L$^{-1}$ $NH_4Cl$ and 7.7 ml of amino acid stock solution (composed of 1.4 g L$^{-1}$ tyrosine, 13.7 g L$^{-1}$ tryptophan, 2.5 g L$^{-1}$ isoleucine, 3.4 g L$^{-1}$ aspartate, 9.2 g L$^{-1}$ glutamate, 28.6 g L$^{-1}$ arginine, 3.7 g L$^{-1}$ leucine, 5.8 g L$^{-1}$ threonine, 1.4 g L$^{-1}$ glycine, 38.6 g L$^{-1}$ glutamine, 11.1 g L$^{-1}$ alanine, 3.4 g L$^{-1}$ valine, 2.4 g L$^{-1}$ methionine, 2.9 g L$^{-1}$ phenylalanine, 6 g L$^{-1}$ serine, 2.5 g L$^{-1}$ histidine, 1.3 g L$^{-1}$ lysine, 1 g L$^{-1}$ cysteine and 46.8 g L$^{-1}$ proline) summing to a total of 250 mg L$^{-1}$ of assimilable nitrogen. The pH of the SM was adjusted to 3.3 with 10 M NaOH.

Depending on the conditions that were defined by the FP, each stress factor was accordingly adjusted when added to the SM. For osmotic stress, 200 or 280 g L$^{-1}$ of equimolar glucose and fructose was used. For $SO_2$, a concentrated solution of potassium metabisulfite ($K_2S_2O_5$) (Labosi—A4871451) was prepared (43.8 mg L$^{-1}$). Right before inoculation, 1 $\mu$l mL$^{-1}$ was added to the must in such a way that a final concentration of 0.8 mg L$^{-1}$ molecular $SO_2$ would be obtained for a pH of 3.3. For experiments with 0 mg L$^{-1}$ of $SO_2$, the same volume of sterile water was added instead. For phytosterols (85 451, Sigma Aldrich), two different concentrations were used: 1 and 8 mg L$^{-1}$. The stock solution was composed of 5 g L$^{-1}$ phytosterols in Tween 80 and ethanol (1:1, v/v). For thiamine (Sigma T4625), a concentrated solution (250 mg L$^{-1}$) in sterile distilled water was prepared, and the correct volume was added to obtain the desired final concentration

(25 or 250 $\mu g\,L^{-1}$). Finally, the SM was steam sterilised for 10 min and cooled to the correct temperature.

All fermentations were performed using the microplate methodology based on monitoring the OD during the fermentation.

In a preliminary step, we validated this methodology by comparing fermentations in microplates to those performed in 1.2-L fermenters, in conditions that were proven to accurately reproduce wine fermentations (Sablayrolles, Barre and Grenier 1987). We used for this approach to validate the standard SM medium (200 $g\,L^{-1}$ sugars, 0 $mg\,L^{-1}$ $SO_2$, 5 $mg\,L^{-1}$ phytosterols and 250 $\mu g\,L^{-1}$ thiamine) at 28°C. Fermenters were filled with 1 L of SM and agitated with a constant magnetic stirring at 150 rpm. Instead of monitoring OD, the amount of $CO_2$ that was released was estimated using an automatic measurement of the weight loss of the fermenter every 20 min. The rate of $CO_2$ production was calculated by polynomial smoothing of the amount of $CO_2$ released (Sablayrolles, Barre and Grenier 1987). For the microplates, a total of 200 $\mu l$ of SM were used in each well. After cell counting, precultures were washed in sterile water and resuspended in the respective SM. This process was done in such a way that when inoculating, a final concentration of $1 \times 10^6$ cells $mL^{-1}$ would be obtained. Greiner transparent 96-well flat microplates with covers were used in this work. Once inoculated, these were agitated and read in a TECAN M200 Infinite Pro microplate reader for 280 cycles (≈48 h) at 28°C or for 560 cycles (≈ 96 h) at 16°C. The agitation programme included an orbital agitation (8 min at 140 rpm) as well as a linear one (2 min at 300 rpm). Readings were done in a multireader system that used the filled circle scheme with $3 \times 3$ measured points. For the purposes of comparison, we defined a suitable threshold for to determine the end of the lag phase (microplates at 0.1 OD and 1.2-L fermenters at 0. 25 $g\,h^{-1}\,L^{-1}$ $CO_2$) and compared results.

## RESULTS

### Genetic diversity

We first investigated the genetic diversity of the eight *Saccharomyces* strains that were used in this study based on Bruvo's genetic distance (Bruvo *et al.* 2004) and a phylogenetic tree (Fig. 1). We amplified specific *Saccharomyces cerevisiae* microsatellites and plotted the results of our strains together with those of other strains that had been previously characterised (Legras *et al.* 2007). This analysis showed that the strains were widely and evenly distributed, revealing a high genetic diversity among our set. Concomitantly, we observed that this distribution occurs within two specific clusters that contain wine and flor yeast strains.

We then investigated the presence of translocations that are known to confer $SO_2$ resistance (Pérez-Ortín *et al.* 2002; Zimmer *et al.* 2014). Indeed, the presence of these translocations could play a fundamental role during wine fermentation lag phase, as $SO_2$ is frequently used in musts and wines as a preservative and an antioxidant and may represent an important source of stress. We showed that among the *S. cerevisiae* and hybrid strains, two did not have any previously identified translocations that are involved in sulphite resistance, and six strains were either homozygous or heterozygous for the translocation $XVI^{VIII}$ (Table 2). In contrast, translocation $XVI^{XV}$, which was recently described (Zimmer *et al.* 2014), was not detected in any of the strains.

### Lag phase: a tale of many stress factors

All the experiments to determine the lag-phase duration were performed using a microplate method that we first validated by comparing its results with those of wine fermentation in 1.2-L fermenters (see material and methods). Lag-phase durations globally matched and strains' relative positions were found to be similar in the two experiments (data not shown). Thus, we decided to use the microplate-based conditions to execute the FP. Ultimately, this methodology allowed more practicability as well as considerable savings in cost and time.

We first studied the diversity of the lag-phase duration among wine strains during alcoholic wine fermentation. As stress resistance plays a major role during the lag phase, we focused our study on the impact of a variety of stress factors (temperature, osmotic stress, $SO_2$, lack of phytosterols and thiamine) that are present at the moment of inoculation.

To test such combinations in the same experiment, we designed an FP that was based on microplate fermentations (Montgomery 1991; Droesbeke *et al.* 1997). In short, the plan sets the specific combinations of stress factors that are required to evaluate the impact of each stress factor individually as well as their synergistic (two-by-two) influences (Table 1).

An overview of the performance of all strains is shown in Fig. 2. Here, all experiments were considered, so the overall performance of each strain in its lag phase is illustrated. We observed important variations that depended on the strain and the conditions, as the lag phase lasted from under 10 h up to almost 70 h. This difference underlines the considerable impact that stress factors have on lag-phase modulation. The *S. cerevisiae* strains L11, L3 and L8 were the fastest, with the shortest median lag phases, 12.5, 12.7 and 13.7 h, respectively. Although these strains were all *S. cerevisiae*, other strains of this species, L1 (16 h), L7 (18 h) and L10 (19.8 h) also appeared among the moderately paced/slowest strains. The hybrids L4 (14.6 h) and L6 (15.2 h) performed moderately, while *S. uvarum* L9 (17.5 h) and *Torulaspora delbrueckii* L5 (18.5 h) appeared to be among the slowest. Overall, if we consider the wine and flor clusters, despite the narrow sampling, the wine cluster seems to have a tendency to exhibit shorter lag phases (Fig. S2).

### Temperature

Dramatic differences were found when analysing the effect of temperature on the duration of the lag phase (Fig. 3). When fermenting at 28°C, the median lag phase took 8.6 h. The fastest best strain was L3, which was closely followed by L11 and L8, all with a lag phase lasting ∼7.1 h. In contrast, the slowest strains were L10 (11.6 h), L5 (11 h) and L7 (9.8 h). When shifting to 16°C, the median lag phase took 32.3 h, an almost 4-fold increase. Here, L3 and L11 remained the best (26.4 h) followed by L6 (28.7 h). L10 (38.7 h), L7 (36.4 h) and L9 (34.5 h) were identified as the slowest. Due to such considerable differences between the two sets of temperatures and to simplify our FP, we decided to determine the significance of the impact (Student's *t*-test) of the remaining stress factors separately and to perform two independent FP experiments. An overall heat map (Fig. S3, Supporting Information) for both temperature sets will be discussed in the next sections.

### Osmotic stress

Osmotic stress was clearly the factor with the greatest impact after temperature (Fig. 4). With no exceptions, all strains were significantly affected ($P \leq 0.001$ and $P \leq 0.01$) by an increase in sugar concentration (Fig. S3, Supporting Information). At 28°C and 200 $g\,L^{-1}$ sugar, lag phases took ∼7 h and increased by 4 h when at 280 $g\,L^{-1}$ sugar. The equivalent fermentations at 16°C showed roughly a 25-h lag phase at 200 $g\,L^{-1}$ sugar, with an

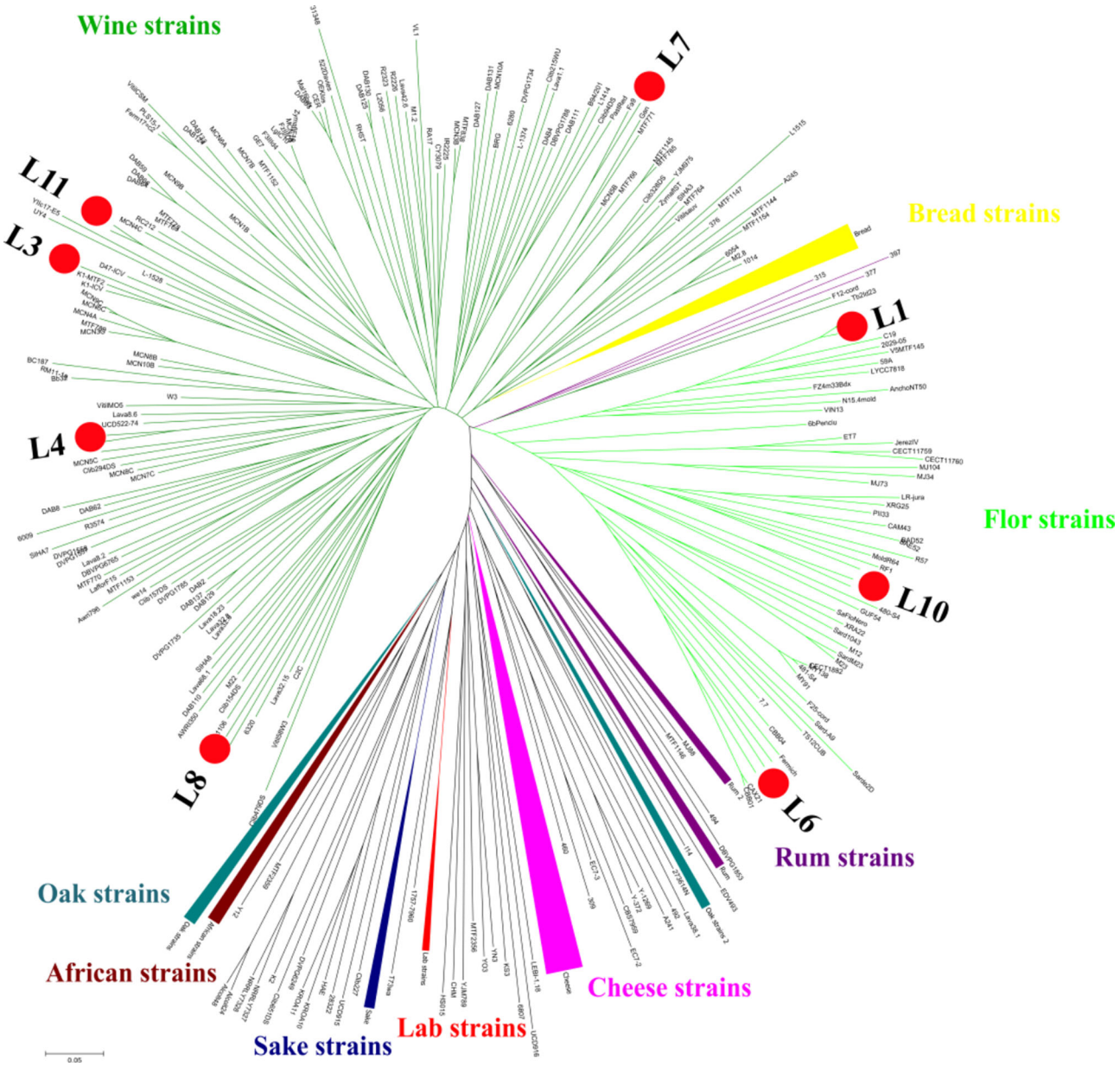



**Figure 1.** Phylogenetic tree. Neighbour-joining tree-based Bruvo genetic distance estimated from the amplification of 12 *S. cerevisiae* microsatellite loci. Red dots represent the position of our initial set of strains relative to yeast strains that have already been characterised and have many different origins. Branches and group names are coloured according to the substrate from which strains have been isolated. Colour code: green—wine; yellow—bread; light green—flor; purple—rum; pink—cheese; red—laboratory; dark blue—sake; dark red—African; and light blue—oak. Red dots represent the strains of our set.

increase of 15 h or more at 280 g $L^{-1}$ sugar (Fig. 4). Specifically, L3, L8 and L11 were consistently the fastest strains, independent of temperature or sugar concentration. At 16°C and 280 g $L^{-1}$ sugar, L1 and L5 (≈36 h) appeared among the fastest strains, but at 200 g $L^{-1}$ sugar these strains, together with L10, were among the slowest (27–33 h). Under the remaining conditions, L5, L7, L9 and L10 were always among the slowest strains, revealing an overall difficulty in starting the fermentation when facing different stress levels of temperature and osmotic pressure.

## $SO_2$

For this stress factor, the impact differed depending on the temperature. At 28°C, strains L1, L3, L5, L7, L8 and L11 were significantly affected (≤0.001 and ≤0.05), while at 16°C, only L5, L8, L9 and L11 were affected (Fig. S3). Among these, L5, the *T. delbrueckii* strain, was the most affected at both temperatures and showed a particular high sensitivity to $SO_2$. Comparing samples with 0 and 0.8 mg $L^{-1}$ of $SO_2$, the strain showed a delay in the median lag phase of 5 and 8.4 h at 28°C and 16°C, respectively (Fig. S4, Supporting Information). After L5, the most impacted strains were L7 (1.8 h delay) and L1 (1.1 h delay) at 28°C and L11 (8.3 h delay) and L9 (6.6 h delay) at 16°C. The fastest were L1, L3, L8 and L11. Interestingly, despite always being a slow strain, L10 showed one of the highest levels of resistance to $SO_2$ by not having its lag phase affected, particularly at 28°C (0 h delay). The fact that strains such as L1, L3 and L11 showed sensitivities only at 28°C (Fig. S3) suggested an additive effect between the two stress factors. As *SSU1* translocations have been shown to increase $SO_2$ resistance, we analysed the relationship between lag-phase

**Table 2.** Strain species and presence of translocations involved in $SO_2$ resistance.

| Strain | Species | Translocation |
|---|---|---|
| L1 | *S. cerevisiae* | Het $XVI^{VIII}$ + $VIII^{XVI}$ |
| L3 | *S. cerevisiae* | Hom $XVI^{VIII}$ + $VIII^{XVI}$ |
| L4 | *S. cerevisiae/kudriavzevii* | X |
| L5 | *T. delbrueckii* | NA |
| L6 | *S. cerevisiae/kudriavzevii* | Het $XVI^{VIII}$ + $VIII^{XVI}$ |
| L7 | *S. cerevisiae* | Het $XVI^{VIII}$ + $VIII^{XVI}$ |
| L8 | *S. cerevisiae* | Hom $XVI^{VIII}$ + $VIII^{XVI}$ |
| L9 | *S. uvarum* | NA |
| L10 | *S. cerevisiae* | X |
| L11 | *S. cerevisiae* | Het $XVI^{VIII}$ + $VIII^{XVI}$ |

Hom $XVI^{VIII}$ + $VIII^{XVI}$: Homozygous for the translocation '16/8'; Het $XVI^{VIII}$ + $VIII^{XVI}$: Heterozygous for the translocation '16/8'; X: no translocation detected; NA: not attributed.

duration and temperature, $SO_2$ stress and translocation presence. In our results, no particular correlation was found between these factors (Fig. 5). Strains that did not have the translocation were slightly affected by $SO_2$ stress (L10 and L4). However, strains that did have the translocation were either not (L6 and L8), slightly (L1 and L3) or considerably (L11 and L7) affected by $SO_2$ stress.

## Phytosterols

Overall, this stress factor triggered smaller variations than the previous ones. When in the presence of 5 mg $L^{-1}$ phytosterols, median lag phases varied between 12.5 and 19.9 h (Fig. S5, Supporting Information), whereas lag phases were between 13 and 20.9 h at 1 mg $L^{-1}$ phytosterols. At both concentrations (at both temperatures), the fastest strains were L3, L11 and L8 (≈13 h) and the slowest were L9, L5 and L10 (≈19 h). At 28°C, L4, L6 and L7 were significantly ($P < 0.05$ and $P < 0.01$) affected by low levels of phytosterols (Fig. S3). However, for L6 and L7, lower phytosterol levels (1 mg $L^{-1}$) represented a reduction in the lag-phase

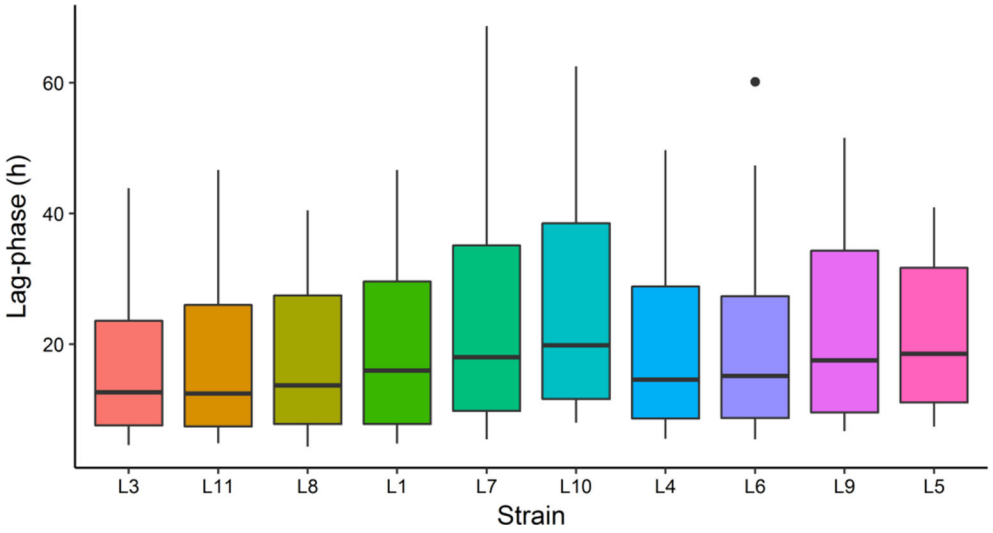



**Figure 2.** Lag phase of strains. Boxplot of the performance of strains in the lag phase. Here, all stress factors were considered (temperature, osmotic stress, $SO_2$, phytosterols and thiamine).

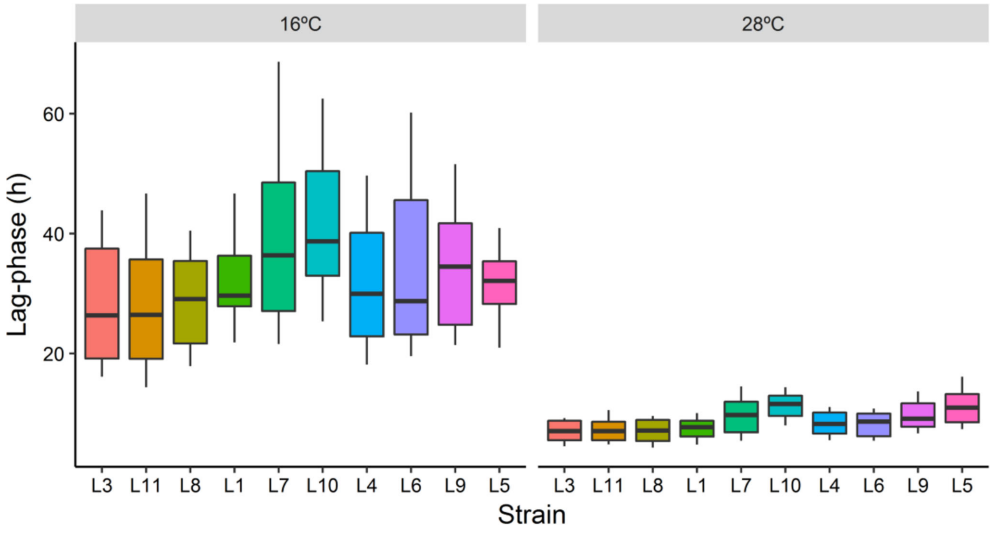



**Figure 3.** Temperature impact. Boxplot comparing lag phases at both temperatures (16°C and 28°C).

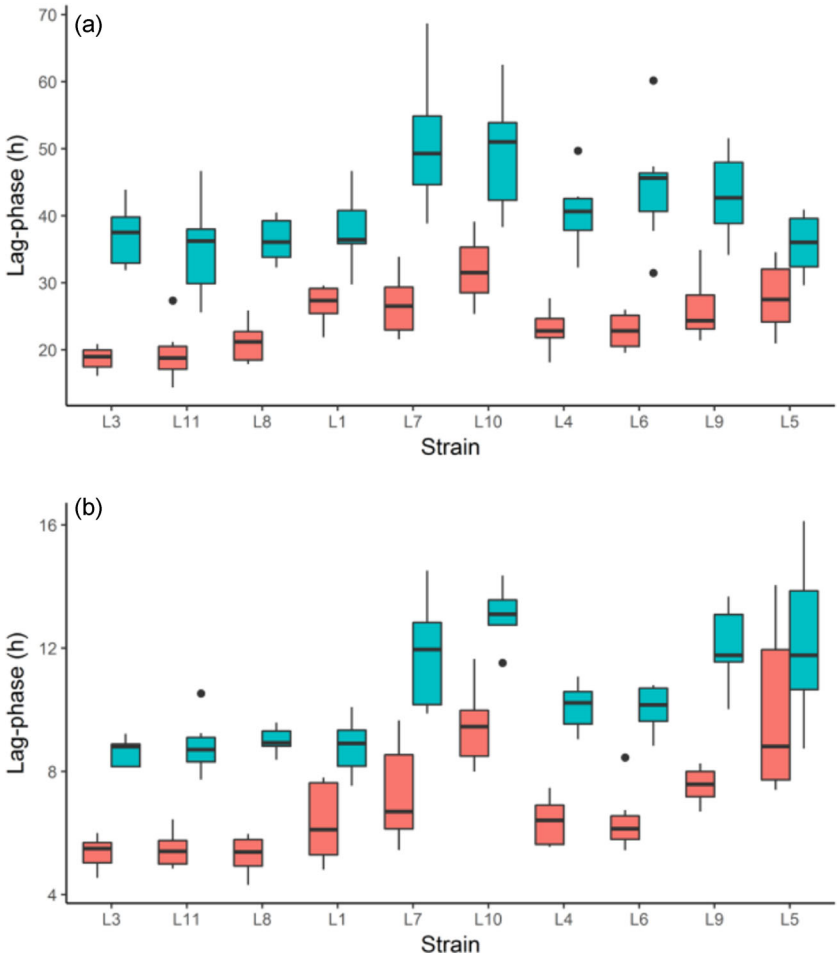



**Figure 4.** Osmotic stress impact. Boxplot of osmotic stress impact on lag phase at **(a)** 16 and **(b)** 28°C. Red bars represent musts with a concentration of 200 g $L^{-1}$ sugar, which is common in real musts, whereas blue bars indicate 280 g $L^{-1}$ sugar, a much higher osmotic stress level.

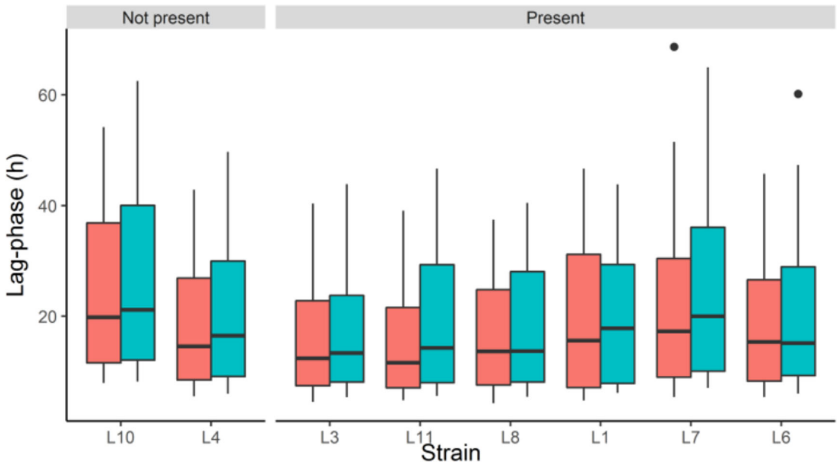



**Figure 5.** $SO_2$-resistance translocation impact. Boxplot of $SO_2$-stress impact on lag phase considering all conditions. The division 'Not present'/'Present' stands for strains without and with the translocation, respectively. Red bars represent fermentations with 0 mg $L^{-1}$ $SO_2$, blue bars represent 0.8 mg $L^{-1}$ $SO_2$.

duration of 1.6 h (Fig. S5b). Even if not significantly affected, the same tendency could be observed for L10 (1.4 h shorter). At 16°C, no significant impact was detected, and when facing low phytosterol levels, the previously mentioned ranking remained the same (Fig. S5a).

## Thiamine

Overall, L3, L11 and L8 were the fastest strains, and L5, L7 and L10 were the slowest, but when looking specifically at each temperature, low levels of thiamine had effects on only a few strains. At 28°C, only two strains, L5 and L9 ($P < 0.05$), were significantly

impacted (Fig. S3). For L5, lower levels of thiamine promoted longer lag phases (1.9 h delay), but for L9, the opposite occurred (1 h shorter). This observation is reinforced in the 16°C fermentations. L4 and L5 were significantly affected by low levels of thiamine (7.1 and 4.3 h delays, respectively), whereas L9 showed lag phases that were 8.2 h faster (Fig. S6, Supporting Information). All strains that were affected were non-*S. cerevisiae* strains (Fig. S3), raising the possibility of differential regulation of thiamine processing.

### Stress factor interactions

After analysing all stress factors per se, we were interested in searching for possible interactions and synergic effects that could exist between stress factors, excepting temperature. As shown in Fig. S3, only few significant interactions were detected. Interaction between low levels of thiamine and phytosterols was observed for several strains (for L9 at 28°C and for L8, L10 and L11 at 16°C). In addition, L11 was additionally affected at 16°C by interactions between $SO_2$ and phytosterols and $SO_2$ and thiamine.

## DISCUSSION

A first objective of this study was to analyse the diversity of lag-phase durations among different wine yeast strains. We used a set of diverse strains, including *Saccharomyces cerevisiae* strains that were evenly distributed among the wine and flor clusters and thus represented a wide genetic variability. Despite the narrow sampling, *S. cerevisiae* wine strains (L3, L4, L8 and L11) showed an overall higher performance regarding lag-phase duration (i.e. shorter lag phase) than *S. cerevisiae* flor strains (L1, L6 and L10). This relationship might be related to the trend of flor strains to thrive at the end of some wine fermentations and in the presence of traces of sugars to produce a particular type of wine (sherry wine) that confers a particular oenological profile (Legras *et al.* 2007, 2016; Coi *et al.* 2017).

Regarding the species, the fastest strains were *S. cerevisiae*. This species is known for typically being able to quickly start fermentations and dominate wild yeast and bacteria that might be present in the hostile must conditions (Bisson 1999; Bauer and Pretorius 2000). As expected, *S. cerevisiae* × *S. kudriavzevii* hybrids (L4 and L6) and non-*S. cerevisiae* strains (L9 and L5), despite their potential for unique contributions (Belda *et al.* 2014; Liu *et al.* 2016), showed longer lag phases.

When focusing the attention on the stress factor influences, temperature and osmotic stress were by far the most impactful factors, creating the most difficulties for the start of fermentation. The negative effect of low temperature on growth rate and cell multiplication has been well documented (Bisson 1999; Llauradó *et al.* 2002; Beltran *et al.* 2008). We could verify this as well for the lag phase, which showed a delay of dozens of hours when fermenting at temperatures as low as 16°C. At 28°C, it was not surprising that *S. cerevisiae* strains were the fastest to exit lag phase (Matallana and Aranda 2016) but, at the lower temperature (16°C), hybrids (due to the *S. kudriavzevii* genome counterparts) and *S. uvarum* strains were expected to show cryotolerance and therefore have an advantage in the fermentation start (Massoutier *et al.* 1998; Gonzalez *et al.* 2007; Belloch *et al.* 2008; Tronchoni *et al.* 2009; Masneuf-Pomarède *et al.* 2010). However, only one hybrid (L6) appeared among the fastest strains with a shorter lag phase. The other hybrid and the *S. uvarum* strain presented medium or even long lag phases and did not show a particular advantage in colder fermentations.

Like temperature, osmotic stress had a very high impact on the lag phase in all strains. When exposed to concentrations as high as 280 g $L^{-1}$, a high/extreme osmotic level occasionally found in red musts (Matallana and Aranda 2016), all strains were significantly affected. Diminished turgor pressure, low water availability, changes in membrane permeability and delays in stress response are some of the negative consequences that yeast has to cope with and that explain the considerable delay and major variations in the lag-phase duration (Attfield 1997; Pratt, Bryce and Stewart 2003; Tamás and Hohmann 2003; Babazadeh *et al.* 2013; Miermont *et al.* 2013). Although *S. cerevisiae* were the fastest strains to exit lag phase, in some cases they were also the strains that were most affected by osmotic stress. To illustrate this relationship, when shifting from 200 to 280 g $L^{-1}$ sugar at 16°C, L3, L8 and L11 were among the most affected strains with lag-phase delays from 15 to 18.5 h. In contrast, L5 and L1 (which, at 200 g $L^{-1}$, were among the slowest) presented a delay of only ≈9 h. Thus, although L3, L8 and L11 always remained the fastest strains, at 280 g $L^{-1}$, L5 and L1 also appear within the fast cohort, indicating that the impact of osmotic stress was less severe for these strains. This result clearly demonstrates that besides the environmental factors, other strain features can play a major role in lag-phase modulation and lead to different phenotypes under the same conditions.

In regard to $SO_2$, the fact that six out of eight strains (*S. cerevisiae* and hybrids) possessed the translocation $XVI^{VIII}$ (Table 2) is in agreement with what was found by Goto-Yamamoto *et al.* (1998) and Pérez-Ortín *et al.* (2002). The ancient and recurrent practice of using $SO_2$ as an antioxidant and an antimicrobial agent created a selective pressure that over time positively retained the translocation $XVI^{VIII}$ and the allele *SSU1-R* that is responsible for a greater $SO_2$ resistance. This history caused the translocation to currently be widely disseminated among oenological strains. On the other hand, Zimmer *et al.* (2014) recently discovered a new translocation, $XVI^{XV}$, which is a much more recent event and therefore not yet highly disseminated among oenological strains; this fact was also verified in our set. Intriguingly, we did not find a correlation between the presence of the $XVI^{VIII}$ translocation and the $SO_2$ impact in our study. This result suggests that the advantage that is conferred by these translocations is not effective at such an early stage as the fermentation lag phase. Nadai *et al.* (2016) also obtained similar results for a particular strain (R008) that despite having the translocation $XVI^{VIII}$ only showed intermediate sulphite resistance. Additionally, strains without the translocation were both drastically affected (AWR1796) or not affected at all (VL3) by a moderate concentration of $SO_2$. Rather than the translocation presence itself, these and other authors attributed the $SO_2$ resistance to a high basal level of gene expression of *SSU1*, specific cell wall proteins, enzymes involved in lipid biosynthesis and enzymes directly involved in $SO_2$ assimilation and efflux (Divol, Miot-Sertier and Lonvaud-Funel 2006; Nadai *et al.* 2016). The differences found between fermentations at 16°C and 28°C can be explained by greater ability to bind $SO_2$ (and therefore less active $SO_2$) at low temperatures than at higher temperatures (Ribéreau-Gayon *et al.* 2006). This difference explains the significantly lower impact at 16°C found in our results. Nevertheless, L5 (*Torulaspora delbrueckii*) was particularly affected by $SO_2$ at both temperatures, which is in agreement with the high sensitivity of *T. delbrueckii* that was reported in previous studies (Ciani, Beco and Comitini 2006; Xufre *et al.* 2006; Comitini *et al.* 2011; Renault, Albertin and Bely 2013; Belda *et al.* 2014).

Among the stress factors studied here, the impact of low initial concentrations of phytosterols and thiamine was rather

faint and more specific to each strain. Although phytosterols have been reported to be an important exogenous source of lipids that have a positive effect on yeast growth, fermentation rate and viability (Daum *et al.* 1998; Luparia *et al.* 2004; Ochando *et al.* 2016), they seem to not be crucial overall for the fermentation start. In fact, different authors have pointed to the important contributions of lipids towards the end of fermentation (Luparia *et al.* 2004). In the same way, we found only little impact from the low concentration of thiamine. This vitamin was shown to improve yeast growth and fermentative rates (Bataillon *et al.* 1996; Bisson 1999; Ribéreau-Gayon *et al.* 2006; Sablayrolles 2009; Wolak *et al.* 2014) and to play a role in resistance to oxidative, osmotic and thermal stresses in the first hours of growth (Wolak *et al.* 2014). We found higher impacts at 16°C than at 28°C, which could be explained by a reduced metabolism rate at low temperature, which would likely reduce thiamine uptake and ultimately cause a significant impact on lag-phase duration (Iwashima, Nishino and Nose 1973). It is known that low levels of thiamine promote accumulation of ketonic acids that then combine with $SO_2$ at higher rates, reducing its efficiency (Ribéreau-Gayon *et al.* 2006). In our results, no particular effect on the lag-phase duration due to the combination of low thiamine and the presence of $SO_2$ was observed since only one strain (L11) was significantly affected by this combination.

Finally, as different synergic effects such as ethanol/high temperature or ethanol/acetic acid (Aldiguier *et al.* 2004; Gibson *et al.* 2007; Borrull, Poblet and Rozès 2015) have been reported to impact yeast during wine fermentation, we searched for the presence of synergic interactions in our data set. Very few interactions were detected, and only one strain, L11, was significantly affected by half of the possible interactions ($SO_2$/phytosterols, $SO_2$/thiamine and phytosterols/thiamine). Thus, each stress factor that was tested in this work caused most of its impact on the lag phase by itself, while the simultaneous presence of several factors did not cause any further difficulties.

In conclusion, we show in this study that there is a high diversity in lag-phase duration in different wine yeast species and strains. *Saccharomyces cerevisiae* strains showed an overall better performance than non-*S. cerevisiae* strains did in terms of wine fermentation lag phase when facing different stress factors. Contrary to expectation, *S. cerevisiae* × *S. kudriavzevii* hybrids with potential cryotolerance only occasionally showed an advantage at 16°C. Temperature and osmotic stress had a high impact on lag phase, independent of strain or species. On the other hand, $SO_2$, phytosterols and thiamine, while also playing an important role in lag-phase modulation, have greater strain-dependent effects. No strict correlation was observed between the presence of chromosomal translocations that are involved in sulphite resistance and the impact of $SO_2$ addition on lag-phase duration, suggesting that other genetic variations contribute to the diversity of this trait.

These data offer new insights to better control this critical step of fermentation. The diversity of lag-phase duration and response to different stresses that was observed among our set of strains emphasises the importance of choosing the most suitable strain for a faster fermentation start, depending on must composition or winemaking conditions. These data also provide a unique framework for strain improvement using adaptive evolution or QTL mapping to elucidate the genetic and molecular basis behind lag-phase modulation and stress resistance.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

## ACKNOWLEDGEMENTS

The authors would like to thank Dr Thibault Nidelet for his valuable scientific input and Dr John Morrissey for his critical reading of the manuscript.

## FUNDING

This work was supported by the YEASTCELL Marie-Curie ITN project (REA Grant No. 606795) under the EU's Seventh Framework Programme for Research (FP7).

***Conflict of interest.*** None declared.

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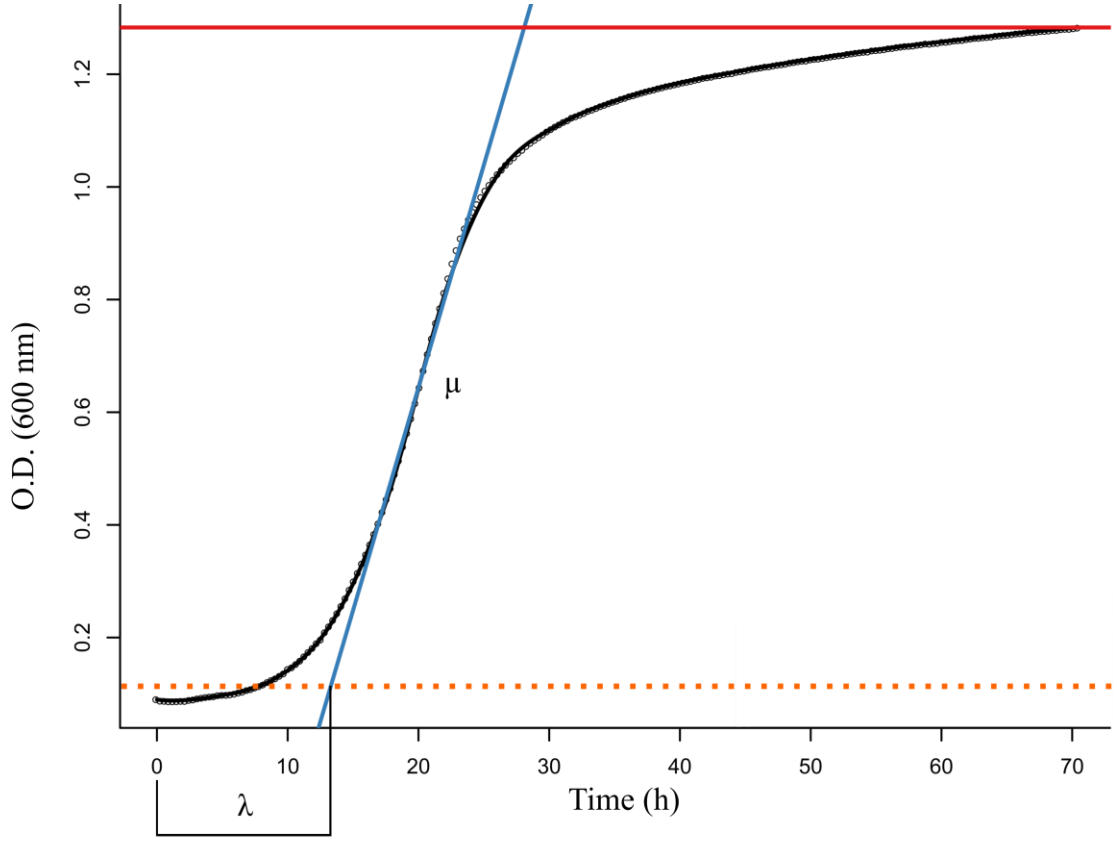
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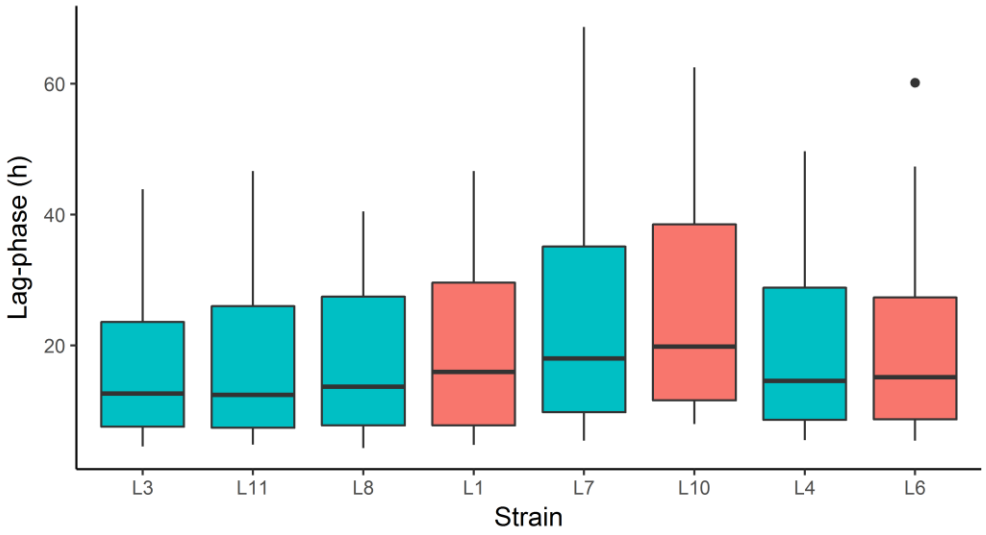
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# Supplementary data

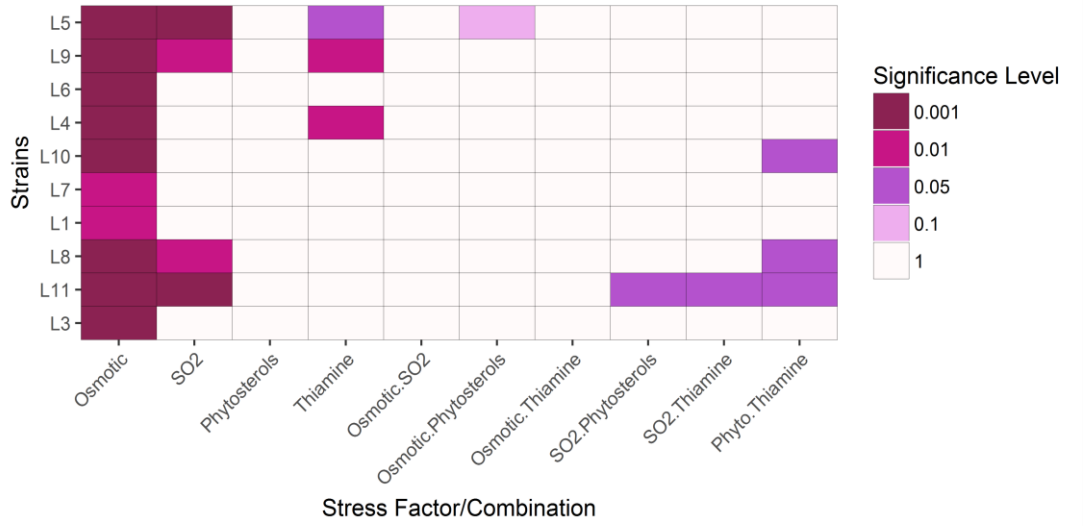



**Supplementary Figure 1. Example of a raw growth curve for lag phase determination.** Growth curve of strain L9 in the conditions of Exp14. The graph displays the O.D. (black line) monitored by using the microplate method. The period of the lag phase (λ) was calculated using a local linear regression based on the maximal growth rate (blue line - μ), its intersection with an O.D. value of 0.1 (dashed orange line) and the maximum growth (red line).

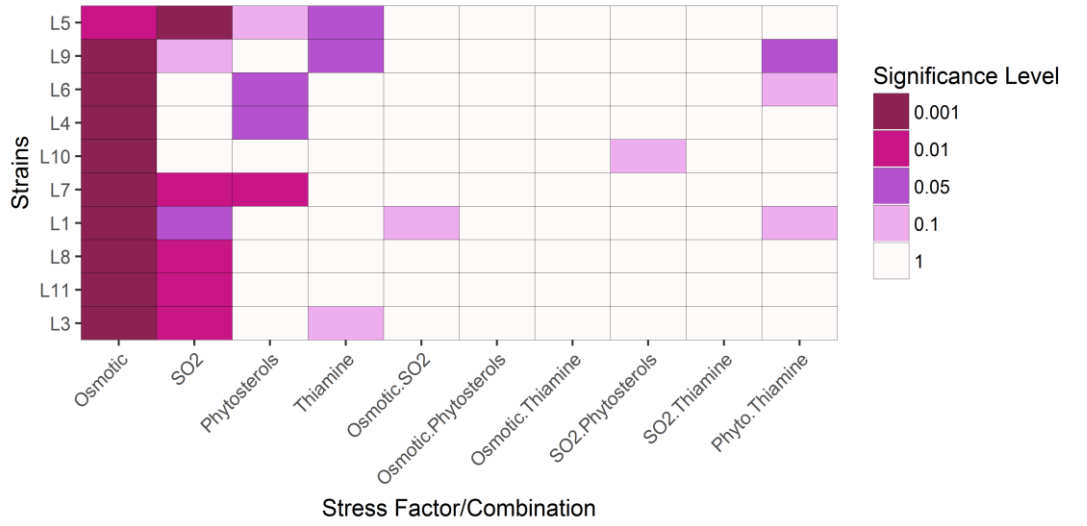



**Supplementary Figure 2. Yeast cluster lag phase comparison.** Boxplot of the lag phases of strains that belong to the wine cluster (blue) versus those that belong to the flor cluster (red), based on our set (see Figure 1).

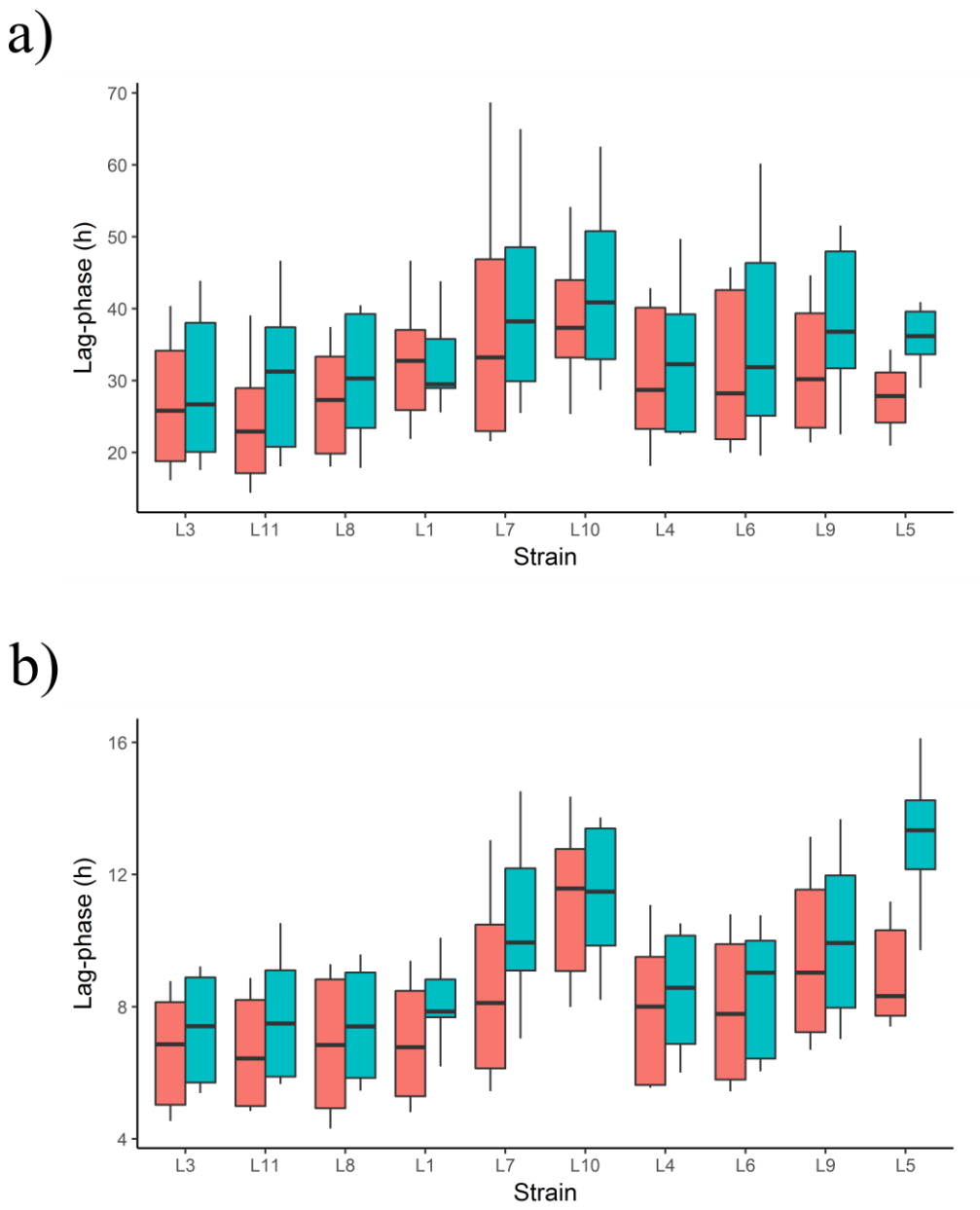
a)



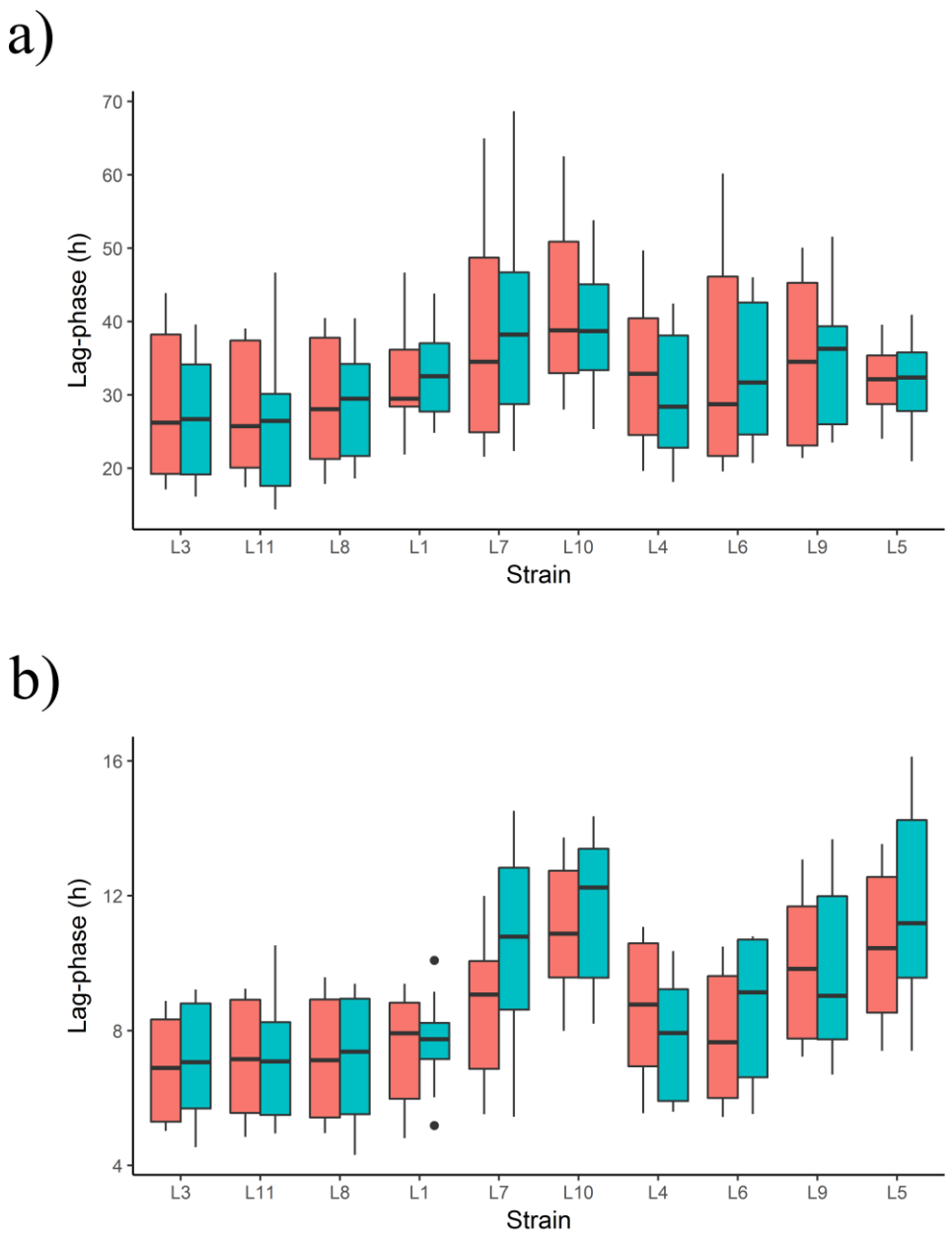

b)




**Supplementary Figure 3. Heatmap of stress factor impact at 16 (a) and 28°C (b).** The significant impact level of each stress factor at 16 and 28°C was determined by a Student's t-test. The colours of dark purple, purple, violet, light violet and white correspond to 0.001, 0.01, 0.05, 0.1 and 1, respectively. For values from 0.001 to 0.05, factors were considered to have a significant impact, 0.1 indicates a tendency, and a 1 indicates that the factor has no effect at all.

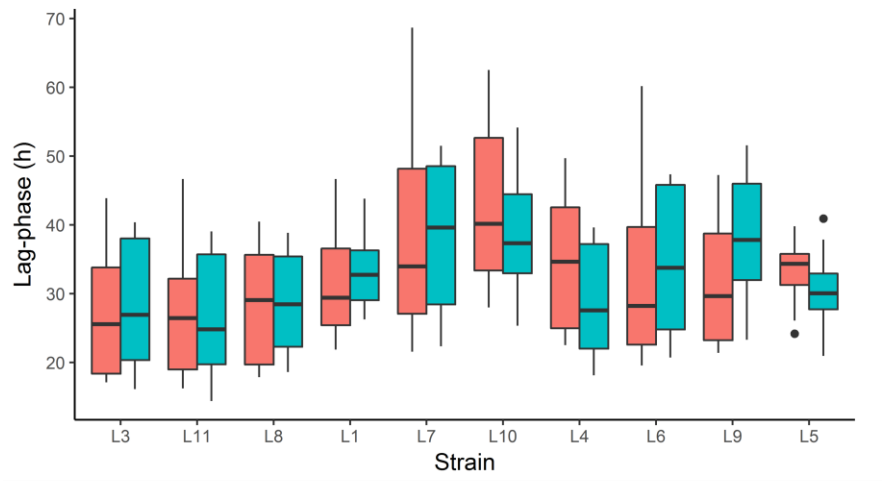



**Supplementary Figure 4. $SO_2$-stress impact.** Boxplot of $SO_2$-stress impact on lag phase at a) 16 and b) 28°C. Red bars represent musts with no $SO_2$ added, whereas blue bars represent a final concentration of 0.8 mg $L^{-1}$ molecular $SO_2$.

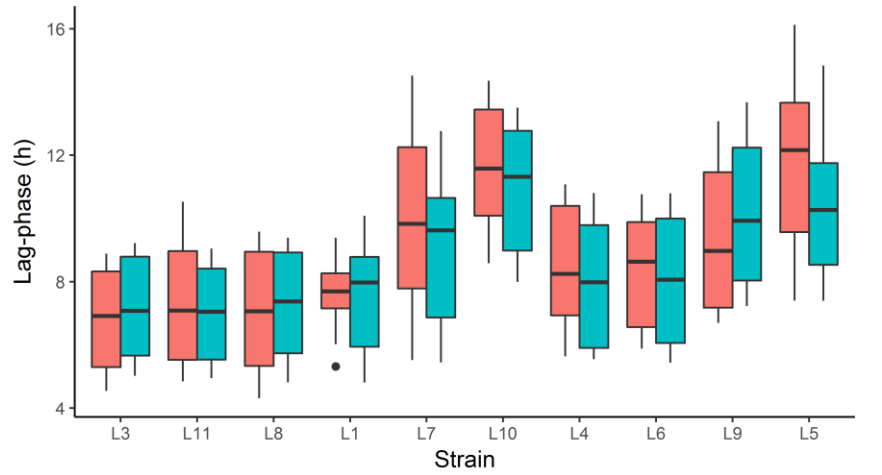



**Supplementary Figure 5. Phytosterol stress impact.** Boxplot of phytosterol stress impact on lag phase at a) 16 and b) 28°C . Blue bars represent musts with 5 mg $L^{-1}$ phytosterol concentration. Red bars represent musts with 1 mg $L^{-1}$ phytosterol concentration.

a)




b)




**Supplementary Figure 6. Thiamine stress impact.** Boxplot of thiamine-stress impact on lag phase at a) 16 and b) 28°C. Blue bars represent musts with a regular thiamine concentration (250 µg $L^{-1}$), whereas red bars represent musts with a low thiamine concentration (25 µg $L^{-1}$).

# CHAPTER 2

## EVOLUTIONARY ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR IMPROVED STRESS RESISTANCE AND REDUCED LAG PHASE DURING WINE FERMENTATION

**Chapter's context**

The information obtained in chapter 1 clearly showed that different strains have different lag phase profiles. Aside from the species/strain dependency, the stress factors typically present at the beginning of the fermentation showed to greatly influence this phenotype. It was then implied that, from a stress resistance point of view, some of the strains tested could be improved, which consequently would lead to a faster fermentation start. To do so, an option would be to genetically engineer these wine yeast strains. However, the restrictions imposed to food and beverages industry in recent years controlling the use of GMOs greatly limit the tools and approaches that can be used to improve wine yeast. On the other hand, in the last decades several techniques that can bypass this limitation have emerged and become popular due to their great success, particularly in yeast applications.

Evolutionary engineering appears among these techniques as an excellent alternative that has proven its value in many different studies. By mimicking the desired biotechnological context and applying appropriate selective pressures, countless phenotypic traits can be improved. This, together with a commercial interest, pushed us to perform an evolutionary engineering approach aiming for a reduced lag phase and an increased stress resistance. Based on the results generated in chapter 1, we selected suitable strains for a lag phase improvement which in the end led us to two final strains with improved lag phase. In this chapter, we present the results that led to their generation and the characterization of the evolved profile. Aside from the industrial outcomes, we were also interested in studying more in depth the genetic determinants of the lag phase modulation. To our knowledge, no evolutionary engineering approaches have been applied to lag phase under oenological conditions. In this sense, we designed the approach in such a way that genetic and molecular information could be obtained by whole genome sequencing of parental and evolved strains. The comparative genomic approach is expected to help us to identify the genetic variations responsible for the evolved phenotype. Combined with the information generated in chapter 3, these data will be useful for a better understanding of the molecular mechanisms involved in the regulation of the lag phase duration.

# EVOLUTIONARY ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR IMPROVED STRESS RESISTANCE AND REDUCED LAG PHASE DURING WINE FERMENTATION

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## Abstract

Wine fermentation onset remains a critical step yet to be fully understood and controlled. The presence of several stress factors in wine must and fermentation conditions are often at the basis of extended lag phases, which in turn compromise fermentation reliability and wine quality. We used an evolutionary engineering approach to improve wine yeast lag phase under oenological conditions, particularly in red wine fermentations. Osmotic stress, $SO_2$ and low thiamine were used as selective pressures typically present at the beginning of fermentation. A successful evolution was obtained, yielding evolved strains with reduced lag phases when inoculated in synthetic must. After verifying the maintenance of important features, selected evolved strains were submitted to industrial propagation and drying, and were used in grape must fermentation. Lag phase reduction was shown and the improved strains additionally displayed a higher $V_{max}$ and a reduction in total fermentation time. Whole genome sequencing of parental and evolved strains revealed several genetic variations including mutations affecting genes connected to cell wall integrity and cell cycle regulation, likely to be involved in the evolved phenotype.

**Introduction**

Following the inoculation of a microorganism into a new medium, a delay is typically observed until growth starts. This period, commonly referred to as lag phase, occurs due to metabolic adjustments required for a proper adaptation to the new environmental conditions, before thriving (Lewis *et al.* 1993; Brejning and Jespersen 2002). This way, the duration of a lag phase is directly related to the media composition. At a laboratory scale, different authors studied the impact of different stress factors such as ethanol (Vriesekoop and Pamment 2005), copper (Dönmez and Aksu 1999) or weak acid preservatives (Neves *et al.* 1994) present in very different industrial contexts. All were able to determine that lag phase extension was greatly influenced by the presence as well as intensity of those stress factors. In a classic wine fermentation inoculation, Rossignol *et al.* 2006 studied in detail the gene expression variation upon inoculation. Results showed a shift from respiratory to fermentative metabolism due to the presence of high glucose concentrations, which repressed the oxidative metabolism and gluconeogenesis genes while activating the hexose transporters (*HXT*). Other up and down gene regulations included those related to stress, glycolysis, nitrogen metabolism and protein and nucleic acid synthesis. These metabolic shifts are part of cell adaptation and take variable time to occur leading to a lag. In winemaking, a considerable part of adaptation goes to the stress factors that are already present at inoculation time. The sugar accumulated during grape ripening can reach 180 - 280 g/L. This induces a high osmotic stress that requires major physiological and cellular adaptation such as the production of glycerol as a compatible solute (Pérez-Torrado *et al.* 2002; Pratt *et al.* 2003; Tamás and Hohmann 2003). If not addressed quickly enough, the loss of turgor and water availability can lead to cell death. Inhibitory compounds can also be present in grape juice. $SO_2$ is often used as an antimicrobial and antioxidant to control the development of undesired microorganisms and simultaneously preserve wine properties (Ribéreau-Gayon *et al.* 2006). Despite the overall positive contribution, sulfite has a negative impact on metabolic and enzymatic activities fundamental for yeast thriving (Romano and Suzzi 1993). It has been shown that, due to the systematic use of $SO_2$ in wine fermentation, a particular translocation between chromosome XVI and VIII is today highly disseminated among wine yeast (Pérez-Ortín *et al.* 2002). This translocation results in a higher expression of the gene *SSU1*, coding for a sulfite exporter, which is controlled in the translocated form by the promotor region of the highly expressed gene *ECM34*. As a

consequence, strains possessing such translocation have a higher capacity to expel $SO_2$ and therefore higher resistance to $SO_2$ (Goto-Yamamoto *et al.* 1998). A similar but much less disseminated translocation, $XVI^{XV}$, was recently found in some commercial wine yeast strains (Zimmer *et al.* 2014). Even with $SO_2$ addition, the natural microbiome can still be a source of stress due to competition for resources. This is particularly true for thiamine, an important vitamin for yeast, quickly consumed by different microorganisms during the first hours of fermentation (Ough *et al.* 1989; Bataillon *et al.* 1996). In its absence, fermentation rate as well as viability can be greatly affected (Bataillon et al. 1996). Overall, all these environmental factors come together to create a stressful and typical inoculation scenario in red wine fermentations, far from the optimal conditions for yeast development. Even though *S. cerevisiae* excels in wine fermentation, due to these stress factors extended lag phases occur more often than the desired. Ultimately they can be responsible for sluggish and stuck fermentations, wine spoilage and extra costs.

To address this problem different strategies can be used. Genetic engineering has proven to be an excellent tool to shape yeast phenotypes in different domains and be capable of delivering viable solutions for yeast improvement (Dequin 2001; Schuller and Casal 2005; Donalies *et al.* 2008). However, the current panorama of food and beverages industries where GMOs are tightly regulated and poorly accepted in many countries, limits the use of such approaches. Evolutionary engineering, also denominated adaptive laboratory evolution (ALE), has proven to be an excellent alternative to achieve improved phenotypes. Main advantages of such approach are the relative short time required to obtain an evolved strain, the empirical set up where no deep knowledge behind the phenotype of interest is needed and most importantly, the obtainment of a non-GMO microorganism that can be later used and commercialized in industry. ALE has been successfully used to obtain evolved strains with higher resistance to cobalt (Çakar *et al.* 2009), use of mixed sources of energy (Wouter Wisselink *et al.* 2009), anaerobic growth on xylose (Sonderegger and Sauer 2003) and improved maltotriose consumption in brewing context (Brickwedde *et al.* 2017). In a winemaking context, ALE approaches were also successfully conducted for obtaining strains with improvement of aromatic profile (Cadière *et al.* 2011), higher glycerol production (Kutyna *et al.* 2012) and reduction of ethanol yield (Tilloy *et al.* 2014).

In this study, we developed an ALE approach under oenological conditions for lag phase time reduction. For this purpose, we submitted a commercial wine yeast strain to a multi-stress context typical of grape juice for 500 generations. Evolved population and strains presenting a shorter lag phase were identified. Next, strains isolated from evolved populations were characterized for maintenance of important phenotypic traits. In this scope an industrial propagation and drying were also included. Finally, fermentations using grape must (Merlot) were performed in order to explore the relevance of the evolved phenotype under realistic conditions of wine fermentation. Whole genome sequencing of 6 evolved strains was also performed to reveal the mutations and genetic variations selected during the evolutionary process.

## Material and methods

### Yeast strain and growth conditions

The *S. cerevisiae* wine yeast strain L7 was used as the parental strain. L7 and evolved strains were propagated in rich YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) and maintained on YPD plates (2% agar) at 4°C or stored at -80°C in 20% glycerol.

### Adaptive evolution experiments

Adaptive evolution was based on a long-term serial transfer procedure in synthetic must (SM) mimicking a standard grape juice and using osmotic stress, $SO_2$ and low thiamine as selective pressures. MS medium was prepared as described by Bely, Sablayrolles and Barre 1990. The SM base used in this work contained 300 g/L of sugar (150 g/L of glucose and 150 g/L of fructose) further adjusted during the evolutionary process, 6 g/L of malic acid, 6 g/L of citric acid, salts (0.75 g/L $KH_2PO_4$, 0.5 g/L $K_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.155 g/L $CaC_{12} \cdot 2H_2O$, 0.2 g/L NaCl, trace elements (4 mg/L $MnSO_4 \cdot H_2O$, 4 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $CuSO_4 \cdot 5H_2O$, 0,4 mg/L $CoC_{12} \cdot 6H_2O$, 1 mg/L $H_3BO_3$, 1 mg/L $(NH_4)_6Mo_7O_{24}$ and 10 mg/L of vitamin stock solution (composed as

follows: 20 mg/L myo-inositol, 1.5 mg/L calcium pantothenate, 2 mg/L nicotinic acid, 0.25 mg/L pyridoxine, 0.003 mg/L biotin).

The nitrogen source was composed of 0.27 g/L of $NH_4Cl$ and 7.7 mL of aminoacids stock solution (composed as follow: 1.4 g/L tyrosine, 13.7 g/L tryptophan, 2.5 g/L isoleucine, 3.4 g/L aspartate, 9.2 g/L glutamate, 28.6 g/L arginine, 3.7 g/L leucine, 5.8 g/L threonine, 1.4 g/L glycine, 38.6 g/L glutamine, 11.1 g/L alanine, 3.4 g/L valine, 2.4 g/L methionine, 2.9 g/L phenylalanine, 6 g/L serine, 2.5 g/L histidine, 1.3 g/L lysine, 1 g/L cysteine and 46.8 g/L proline) adding up to a total of 250 mg/L of yeast assimilable nitrogen. The pH of the SM was adjusted to 3.3 with NaOH 10 M. For $SO_2$, a concentrated solution of potassium metabisulfite ($K_2S_2O_5$) was prepared (43.8 mg/L). Immediately prior to inoculation, an aliquot of this solution was added accordingly to the final molecular $SO_2$ concentration desired considering a pH of 3.3. For thiamine, a concentrated solution (250 mg/L) in sterile distilled water was prepared and the correct volume added for a final concentration of 25 µg/L. SM was supplemented with 5 mg/L of phytosterols from a stock solution with 5 g/L phytosterols in Tween 80 and ethanol (1:1, v/v). Finally, the SM was steam sterilized for 10 min and cooled to the correct temperature.

The strain L7 was cultured overnight at 28°C in 5 ml of YPD and the resulting cell suspension inoculated in capped tubes (13 ml), each containing 5 ml of SM. The experiment was carried out in triplicate (R1, R2, R3). The cultures were incubated at 28°C under agitation at 225 rpm and re-inoculated every 12h, point where 4-6 generations occurred. At that moment, the optical density (O.D.) of the culture was measured (600 nm) and an aliquot was used to inoculate fresh medium at an O.D. of 0.1. Such serial transfers were repeated for 500 generations. Every 50 generations, 2 ml samples of the evolving population were taken and stored at -80°C in 20% glycerol. Osmotic stress and $SO_2$ stress were punctually increased and accordingly adjusted at generations 200 and 300 (Table II.1) in order to increase the selective pressure power.

**Table II.1. Summary of osmotic and $SO_2$ increments across ALE experiment and at which generations they were performed.**

| Generation | Stress level |
|---|---|
| **0 - 200** | 300 g/L sugar; 0.8 mg/L $SO_2$; 25 µg/L thiamine |
| **200 - 300** | 320 g/L sugar; 1.0 mg/L $SO_2$; 25 µg/L thiamine |
| **300 - 500** | 340 g/L sugar; 1.2 mg/L $SO_2$; 25 µg/L thiamine |

**Wine fermentation**

1) Laboratory scale

1.1) Microplates: a total of 200 µl of the final SM used in adaptive evolution experiments were deposited in each well of Greiner transparent 96 wells flat microplates with cover. Each well was inoculated at 1 x $10^6$ cells/mL. Once inoculated these were put to agitate and read in the microplate reader TECAN M200 Infinite Pro for 280 cycles (≈ 48h) at 28°C. Agitation program included an orbital agitation (8 min at 140 rpm) as well as a linear one (2 min at 300 rpm). Readings were done in a multi-reading well system using the circle filled scheme with 3x3 measure points. The method chosen to determine the lag-phase duration is based on a local regression and was previously validated by Ferreira *et al.* 2017. By tracing a tangent line that contains the inflection point (the point where the acceleration equals zero and the velocity is maximal) and by determining where this tangent crosses the 0.1 O.D. value, lag-phase duration could be exactly determined (Swinnen *et al.* 2004; Gagneur and Neudecker 2012). Experiments were done in duplicate. With microplates we also used a central composite design to build a model describing the simple and quadratic effects as well as the interactions between sugar (osmotic stress) and $SO_2$ and their impact on lag phase duration. In this experimental design, we used the following levels to have a rotatable design:

**Table II.2. Summary of the different stress levels of osmotic stress (sugar) and $SO_2$ used in synthetic musts for the central composite plan.**

| **Factors** | **Levels** | | | | |
|---|---|---|---|---|---|
| | Lowest -1.41 | Low -1 | Center 0 | High +1 | Highest +1.41 |
| Sugar (mg/L) | 270 | 280 | 300 | 320 | 330 |
| $SO_2$ (mg/L) | 0.7 | 0.8 | 1 | 1.2 | 1.3 |

A total of 12 experiments were carried out. The effect of the two independent variables on lag phase (Y) was modeled with a polynomial response surface:

$$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{12}X_{12} + \beta_{11}X_{11} + \beta_{22}X_{22} + \varepsilon$$

where X1 and X2 represent the coded values of initial sugar and initial $SO_2$ content respectively, Y is the predicted response, $\beta_0$ is the intercept term, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the quadratic coefficient, and $\beta_{ij}$ is the interaction coefficient and $\varepsilon$ are independent N (0, $\sigma^2$) error terms.
In addition, the normality of residual distributions and homogeneity of variance was studied with standard diagnostic graphs; no violation of the assumptions was detected.

The accuracy and general ability of each polynomial model described above was evaluated by a lack of fit test, the Fisher test and the adjusted coefficient of determination adjR2. Statistical analysis was performed with R software, version 3.1.3 (Team 2015) and the rsm library (Lenth 2009).

1.2) 1-L fermenters: fermentations were performed in 1.2 L fermenters containing 1 L of the last SM used in ALE or grape must Merlot 2016 previously flash pasteurized. This grape must contained 240 g/L sugar, 90 mg/L of assimilable nitrogen (supplemented at 1/3 of fermentation with Fermaid E at 30 g/hL). $SO_2$ was added just prior to fermentation in a way that 1.2 mg/L molecular $SO_2$ final concentration was achieved. ADY strains were rehydrated (as recommended 30 min at 37ºC) and inoculated at 25 g/hL. Temperature and agitation were kept constant at 28ºC and 350 rpm respectively. Fermentation kinetics were monitored by calculation of the amount of $CO_2$ released determined by automatic weighing of the fermenters at fixed intervals. All fermentation experiments, except for the fermentation on grape must, were performed in duplicate.

**Drop tests**

Solid YPD in Petri dishes was used as base medium and accordingly supplemented for each stress factor. Ethanol: the correct volume was added to melted YPD at 45ºC (just prior to solidification to avoid ethanol evaporation) in a way to obtain petri dishes with 10%, 12.5% and 20% (v/v). Temperature: regular YPD was incubated in at 15ºC, 37ºC and 46ºC. $H_2O_2$: the correct volume was added to melted YPD at 45ºC (just prior to solidification to avoid $H_2O_2$ evaporation) in a way to obtain Petri dishes with 5 mM, 6.5 mM and 8 mM. Citric acid: concentrated YPD (10x) and agar (2x) were prepared and adjusted to pH 3. Stock solution of citric acid was also adjusted to pH 3. All compounds

were mixed and adjusted to a final concentration of 1x with autoclaved distilled water, also adjusted to pH 3. Cells were washed with sterile water, suspended at 1 x $10^7$ cells/mL and then serially diluted from $10^{-1}$ to $10^{-5}$ in sterile water. Serial dilutions of cells were spotted onto YPD plates. A control plate was incubated at 28ºC. All plates were incubated for 48h at 28ºC (except temperature drop tests). Additionally, 15ºC petri dishes were left for 96h whereas 12.5% and 20% ethanol ones were left first 48h at 28ºC and 5 additional days at room temperature (21ºC). All experiments were done in triplicate.

**Propagation and drying**

Evolved strains were propagated and dried as in a regular industrial process. For the propagation, yeast underwent a scale-up of batch cultures followed by fed-batch cultures. First yeast was inoculated in 25 mL of laboratory media for 48h at 28ºC. Next, a new batch culture was performed, this time in 1.4 L of diluted molasses for 24h at 28ºC. The biomass obtained here was used to inoculate a 25 L reactor for fed-batch culture in diluted molasses. During this process several parameters such as nitrogen and molasses feeding, temperature, pH and aeration were computer-controlled to optimize biomasse production. After separation from molasses (washing and centrifugation) a yeast cream with 20% dry weight (DW) was obtained. The cream was further vaccum-filtered and pressed yielding a cake yeast (32% DW) that was extruded and dried. In the end, around 100 g of active dry yeast with 95% DW were obtained.

**Genotyping**

The genomic DNA of parental and evolved strains was extracted with the MasterPure Yeast DNA Purification kit (Epicentre) according to manufacturer's instructions. DNA samples were processed to generate libraries of short 400-bp inserts. After passing quality control, the libraries were sequenced using the Illumina technique by an Illumina HiSeq 3000 at INRA's genomic platform of Toulouse. Sequencing from both ends generated paired-end reads of 2 x 100 bp, resulting in a theoretical sequencing depth of 100X. Trimmomatic version 0.322 (Bolger *et al.* 2014) was used for cleaning and trimming low quality regions and adapters in Illumina data. We used the Genome Analysis Toolkit (GATK) version 2.7 for making SNP and indel calls, following "Best Practice Variant Detection with GATK version 4, for release 2.0" available online (McKenna *et al.* 2010).

Briefly, the workflow is divided into four sequential steps: initial mapping, refinement of the initial reads, multi-sample indel and SNP calling, and finally variant quality score recalibration. First, reads were aligned to the S288C reference genome (release number R64-1-1, downloaded from SGD) using BWA version 0.6.2 (Li and Durbin 2009) resulting in aligned reads in a BAM file format. Second, optical and PCR duplicates were removed using MarkDuplicate from the Picard Tools version 1.84 (http://picard.sourceforge.net). Reads around indels were realigned to increase indel call accuracy using IndelRealigner (GATK). Base quality was recalibrated using BaseRecalibrator/PrintReads (GATK). After recalibration, the recalibrated base quality scores in the output BAM file were closer to their actual probability of mismatching the reference genome, and were subsequently more accurate. Moreover, the recalibration tool attempted to correct for variation in quality with machine cycle and sequence context. At the end of this step we got the analysis-ready reads. Third, we performed SNP and indel discovery across all evolved and ancestral sequences simultaneously using UnifiedGenotyper (GATK). Fourth, we used Variant Quality Score Recalibration (VQSR) to build an adaptive error model using known variant sites and then applied this model to estimate the probability that each variant in the callset is a true genetic variant or a machine/alignment artifact. This step was performed with the VariantRecalibrator / ApplyRecalibration tools of GATK, using an unpublished dataset of known SNP and indels obtained from 86 available genomes to train the model. Variants from each project were merged using GATK. This genotyping pipeline provided us a final multi-sample VCF file containing 502,296 SNP and 32,173 Indels discovered across samples to which were associated a genotyping quality for each strain. For the loss of heterozygosity assessment Custom perl scripts were used to compute average shifted histogram (averaging 10 windows of 10,000 bp) of variant frequency across the genome. A custom R script was used to plot the variant density, differentiating homozygous from heterozygous genotypes. Finally, for genetic variations and coding impact we used a custom perl script to extract variant positions where at least one genotype differed among the experimentally evolved isolates. SnpEff version 3.3h (Cingolani *et al.* 2012a, 2012b) was then used to predict variant effect at these selected positions (see http://snpeff.sourceforge.net for details).

## Results

### Evolutionary engineering applied to wine yeast lag phase

An evolutionary engineering approach was conducted on the *S. cerevisiae* wine yeast strain L7, focusing on lag phase improvement under oenological conditions. Stress factors typically present at that stage in red wine fermentations were picked as selective pressures: high osmotic stress, $SO_2$ and low thiamine and were accordingly settled in synthetic must for the evolutionary process. In a previous study on the characterization of lag phase in similar conditions, L7 ranked 8$^{th}$ on 10 strains in its overall lag phase duration (Ferreira *et al.* 2017). When looking specifically at osmotic and $SO_2$ stress, this strain showed to be significantly affected in both cases (p-value $\leq 0.01$) and was always among the strains with the longest lag phase. This showed that the strain had room for improvement and therefore potential to be evolved. Thiamine on the other hand did not have a significant effect on strain L7 (Ferreira *et al*, 2017) however it was still included, since it is a typical stress of the fermentation start.

In order to determine the optimal conditions for the evolution experiment, we first investigated the respective weight of each of the two main stress factors, osmotic stress and $SO_2$ using a central composite design (Figure II.1). The reliability of the fitted model was good, as illustrated by a non-significant lack of fit test at a 0.05 threshold, a significant Fisher test at a 0.05 threshold and an adjusted R2 of 84% (which means the model explains 84% of the variability in data). The estimated model was: $Y=22.97 + 2.35 X_1 + 0.86 X_2 – 0.7X_1^2 + 0.26X_2^2$. Osmotic stress individual impact was more important than that of $SO_2$ being responsible for a 10h delay *versus* 2h caused by $SO_2$. We found a good equilibrium by selecting 300 g/L of sugar and 0.8 mg/L of $SO_2$. With this combination, a considerable stress level is being applied (and therefore a delay in lag phase) however still leaving room for strain's improvement (at least 5h). Such concentrations also left the possibility of a later stress level increase. Based on these data, three independent parallel ALE were started in synthetic must with high osmotic stress (300 g/L sugar), $SO_2$ (0.8 mg/L) and low thiamine (25 µg/L) using the strain L7, which was serially re-inoculated every 12h. In order to increase our chances of success we

gradually increased the stress level of our selective pressures along the process (see material and methods).

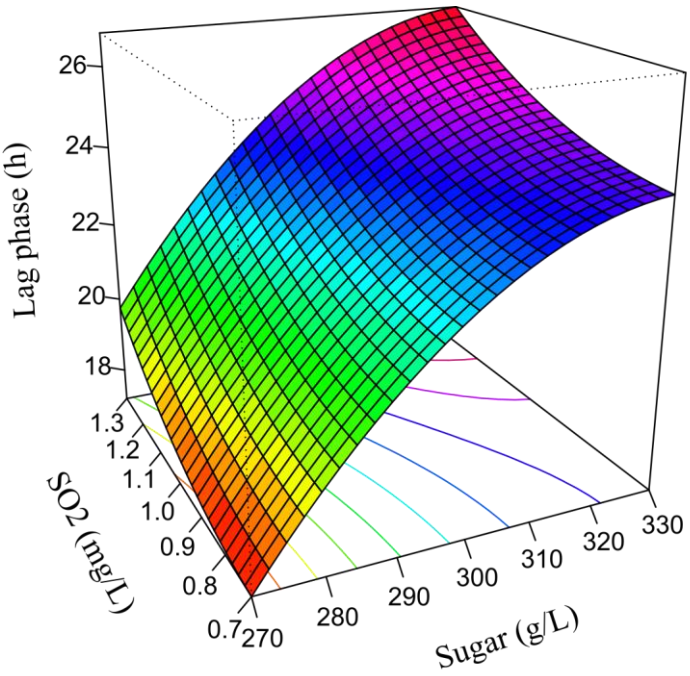



**Figure II.1. Response surface of osmotic and $SO_2$ stress and their lag phase modulation based on a central composite design.**

The ALE was run for 63 days, corresponding to 500 generations, and the three populations obtained were characterized during wine fermentation in 1.2-L fermenters in the last conditions of the evolution experiment (Figure II.2).

We observed that L7 evolved population R1 had a lag phase marginally different from the parental strain L7 (25h – 27h) while R2 and R3 showed a marked shorter lag phase of 18h and 23h respectively (Figure II.2), indicating a positive evolution.

We therefore analyzed the dynamic of the R2 and R3 experiments in the same final conditions of the ALE (Figure II.3). For both R2 and R3, a marked evolution occurred at the $100^{th}$ generation (Figure II.3). Both populations showed a reduction in lag phase of 3.9h and 3h for R2 and R3 respectively. The decrease in lag phase duration culminated at 200 generations with a respective decrease of 4.2h and 4.1h. From there, R2 had a loss in the improved phenotype at the $300^{th}$ generation which was partially recovered until

generation 500. Interestingly, R3 followed a very similar pattern with an increase in lag phase at the 300$^{th}$ generation and progressive recovery until the 500$^{th}$ generation.

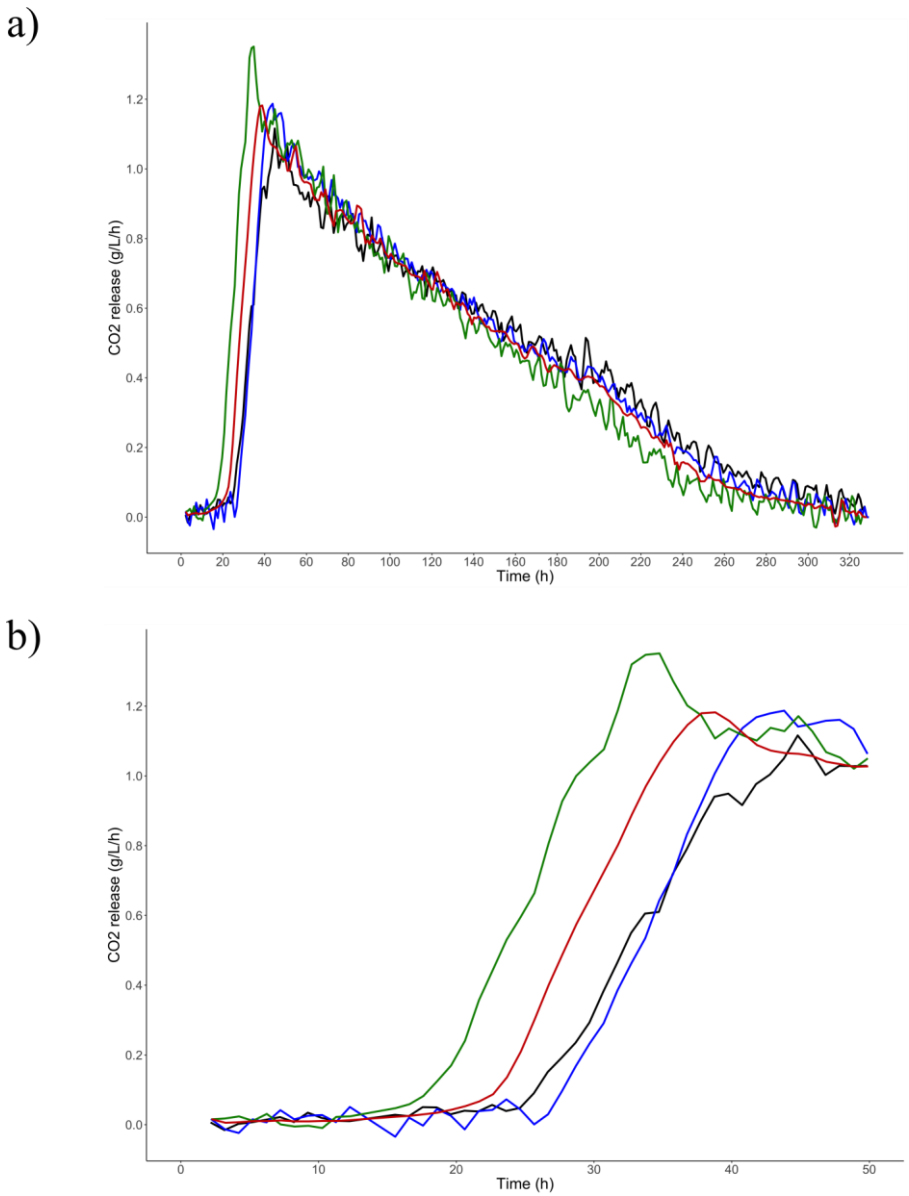



**Figure II.2. Fermentation kinetics of parental and evolved populations.** L7 (black) and evolved populations R1 (blue), R2 (green) and R3 (red) after 500 generations in 1.2 L fermenters in the last condition of the ALE experiment (340 g/L sugar, 1.2 mg/L $SO_2$, 25 µg/L thiamine) a) overall fermentation kinetics; b) zoom on the lag phase showing a positive evolution for populations R2 and R3. A representative of two duplicated experiment is shown.

Considering these results, the generation 200$^{th}$ was further studied for both R2 and R3.

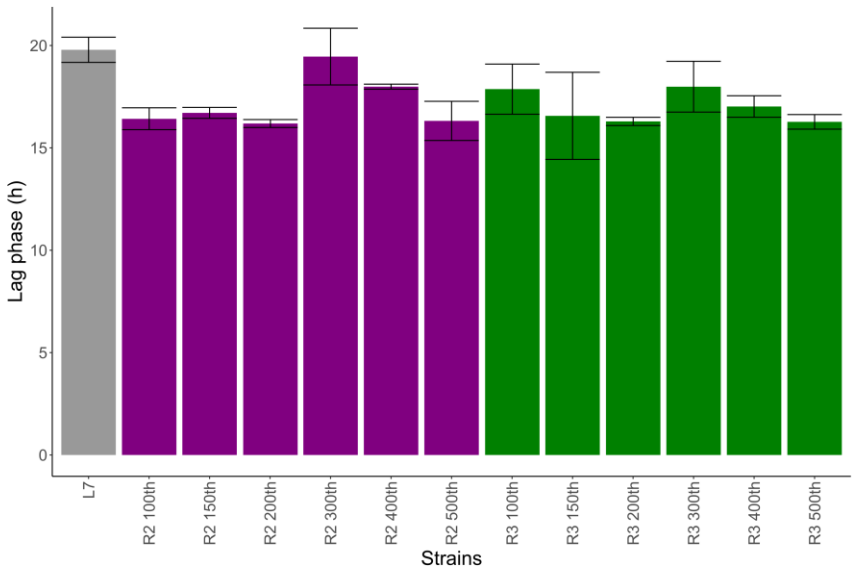



**Figure II.3. Bar-plot of R2 and R3 lag phase populations screening.** Performed using synthetic must in the final conditions of the ALE experiment (340 g/L sugar, 1.2 mg/L $SO_2$, 25 µg/L thiamine) with the microplate method. Grey – L7; purple – R2 populations; green – R3 populations. Experiments were performed in duplicate.

## Selection and phenotypic characterization of evolved strains

Due to the natural accumulation of mutations across generations, the population obtained after 200 generations could be non-homogenous. Consequently, the evolved phenotype could reflect the characteristics of a mixed population. We therefore isolated single colonies from each evolution experiment and the lag phase duration of 7 (R2) and 8 (R3) evolved strains was determined (Figure II.4).

Both evolved populations and respective strains presented a shorter lag phase than parental strain that took 20h to exit lag phase. R2 evolved strains showed lag phases with values close to R2 population, indicating that the positive phenotype was frequent across the population. Small variations between the evolved strains were observed, with lag phases ranging from 15.8h (R2A) to 18.1h (R2C) in comparison with 16.4h for R2 population. The same pattern was observed for R3, with a comparable lag phase of all evolved clones (16.7h – 18.6h) to the R3 population (17.8h) with small variations. R2 population showed to be slightly faster than R3 population (1.4h) and this trend was also verified for the isolated strains. We selected and studied in more detail 3 evolved strains

from each population, R2A, R2D, R2E and R3C, R3F, R3I, among those which presented the fastest lag phase.

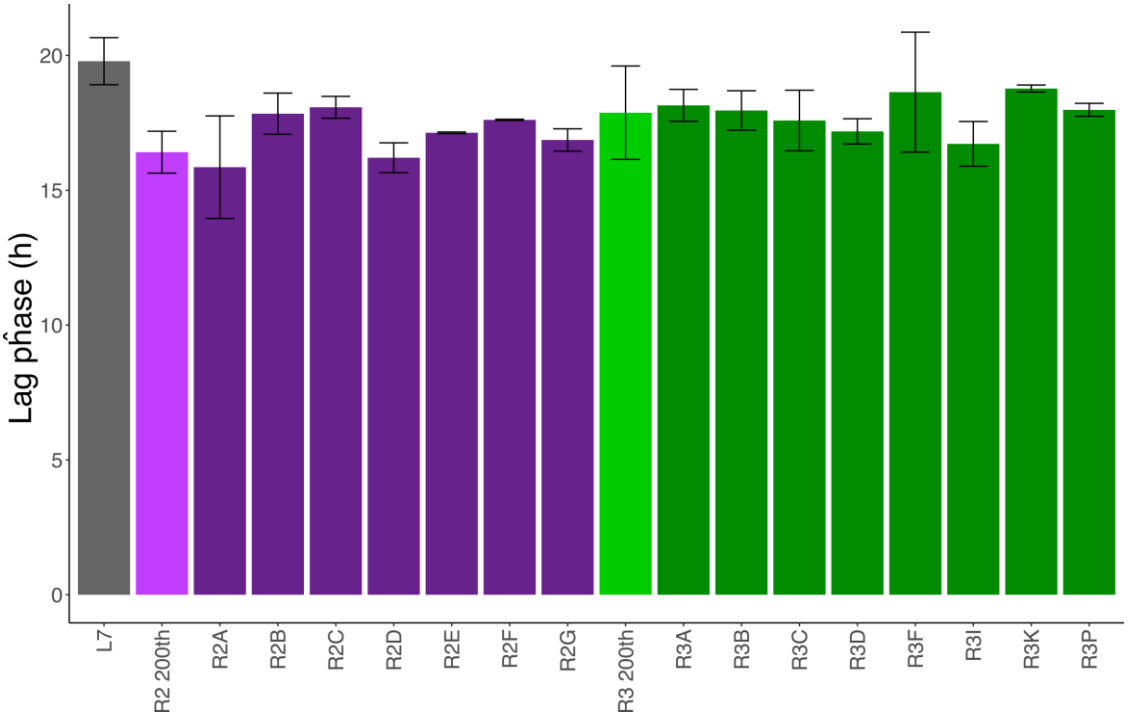



**Figure II.4. Bar-plot of the lag phase of evolved clones.** Determination in synthetic must in the final conditions of the ALE experiment (340 g/L sugar, 1.2 mg/L $SO_2$, 25 µg/L thiamine). Samples included L7 (dark grey), R2 and R3 population at the 200$^{th}$ generation (light purple and light green respectively) as well as different clones isolated from these population (purple and green respectively) based on the microplate method and O.D. following. Experiments were duplicated.

Stress resistance is an important requirement of commercial wine yeasts, which must endure very diverse stresses at different steps of the process (biomass production, drying, fermentation). As a preliminary study, we investigated the resistance of the 6 evolved strains to stresses which could be indicative of those found in winemaking: KCl (osmotic stress during propagation and fermentation), $H_2O_2$ (oxidative stress during propagation and drying), acetic, citric and lactic acid (low pH in wine must), temperature (highly variable) and ethanol (main stress factor at the end of fermentation). Compared to L7, differences in growth were detected for the evolved strains on ethanol, temperature, $H_2O_2$ and citric acid stress resistance (Figure II.5). Overall, the strain R2A was particularly affected in different stress conditions. At low temperature (15ºC), parental and evolved strains grew equally; however when shifting to 37ºC, strain R2A showed reduced growth. At an extreme temperature of 46ºC no cells grew at all. A similar pattern was verified for

citric acid resistance. When at 7%, all clones were able to grow normally with the exception of R2A that did not grow from the third dilution ($10^4$ cells). When checking for oxidative stress, all clones grew well at 5 mM and 6 mM of $H_2O_2$ with the exception of R2A that either showed great difficulties to grow or did not grow at all. The parental strain L7 also showed some difficulties at 6 mM $H_2O_2$ from the third dilution ($10^4$). This indicates a slight higher oxidative stress resistance of the evolved strains, particularly R2D, R3F, R2E and R3I. Finally, when testing for ethanol resistance, cells were left to grow for 48h at 28ºC as with all the other conditions. With 12.5% and 20% of ethanol no growth was recorded for any of the strains. After one week at room temperature (20ºC), growth occurred, and revealed deficiencies for R2A and R3F. Strains R2E and R3I show the greater resistance to ethanol and were even more resistant that the parental strain at 20%. Based on these phenotypes together with lag phase profile we selected two strains, one of each population, R2E and R3I. In terms of lag phase, R2A was the best among the R2 isolated strains (Figure II.3) however the major growth difficulties under different stress factors made us eliminate it as a viable choice. R2E and R2D were close in terms of lag phase but R2E showed to be more ethanol resistant (Figure II.5). As for R3 evolved strains, R3I had the fastest lag phase as well as an indication of higher ethanol resistance. Additionally, R3C and R3F showed a slightly lower resistance to oxidative and ethanol stress respectively.

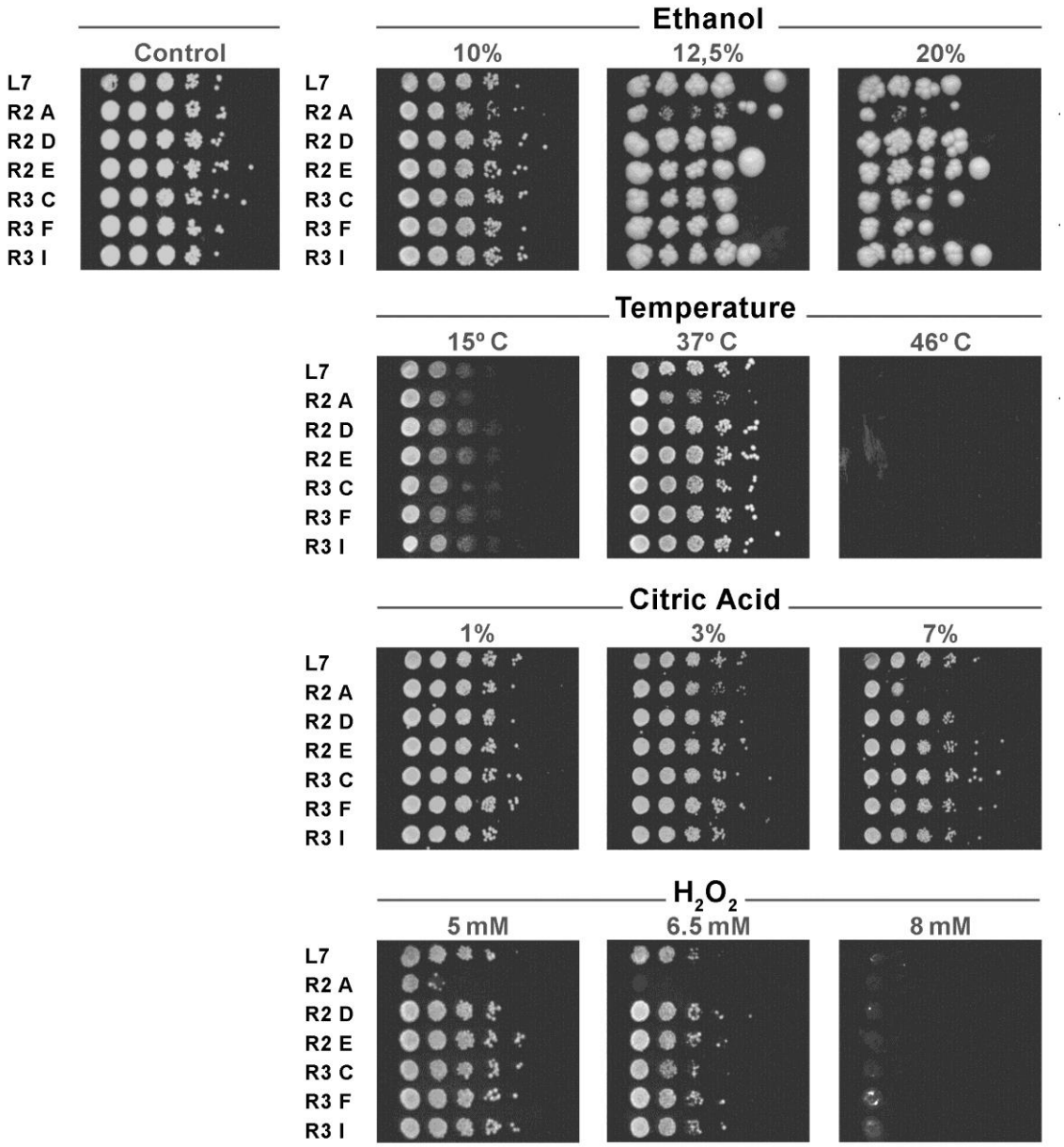



**Figure II.5. Drop tests of L7 and selected evolved strains under different stress factors.** After cultivation, serial dilutions (10-fold) were inoculated in drop form and the plates were incubated for 48h at 28ºC, except for the conditions 15ºC (96h) and 12.5% and 20% ethanol (1 week at room temperature). Experiments were done in triplicate.

## Industrial context and performance

To test the relevance of the two selected evolved strains for wine making, we submitted the selected evolved strains to an even more realistic context. For that, the two evolved strains were first propagated and dried as in typical industrial ADY production in 30 L fermenters. At the end of the process both showed good indicators by achieving standard parameters such as a high-count cell count ($1.6 \times 10^{10}$), fast sugar consumption (clinitest

< 2h) and low water content in the ADY form (< 5%). The two clones were then tested in 1 L fermentations (1 L) with real red wine must instead of synthetic must. For the first set of fermentation a Merlot 2016 grape must was used to represent a realistic media for a red wine fermentation. An overall fermentative profile and respective zoom on the lag phase are shown in Figure II.6.

Both evolved strains showed a faster fermentation start followed by a steeper fermentative rate that culminates on a $V_{max}$ at 1 g/L/h of $CO_2$ release rate against 0.84 g/L/h for L7. This was then translated into a faster fermentation time where both R2E and R3I finished fermentation in 165h versus 195h of L7. Zooming on the lag phase (Figure II.6 b) shows that L7 has a lag phase around 3h and 2h hours longer than R2E and R3I respectively. This showed that the strains managed to preserve their evolved phenotype in real grape must.

**Whole genome sequencing of evolved strains**

Parental strain and 6 evolved strains (R2A, R2D, R2E, R3C, R3F, and R3I) were sequenced in order to identify the genetic modifications at the basis of the improved phenotype. The first major difference detected was a loss of heterozygosity (LOH) in two chromosomes (Figure II.7).

5 out of the 6 evolved clones (exception for R3C) presented LOH in chromosome IX. This consistently ranged from 0 kbp to 280 kbp. Conversely, only R2A presented an additional LOH zone, this time in chromosome XII, going from position 480 kpb to almost the end of the chromosome at 1.05 Mpb. Outside of these regions we searched for *de novo* mutations common to the 3 strains of each population. To further increase our sorting power, for each population we focused on those lying in coding regions with an impact determined by SnpEff as moderate (*e.g.* codon insertion) and high (*e.g.* nonsense and frameshift mutations) (Tables S1, S2 and S3, supplementary information). In total 47 and 82 variant positions between BC and R2 and R3 respectively were identified (Table S1 and S2, Supplementary data). As expected in evolving diploid populations, the vast majority of these were heterozygous polymorphisms as only 1 and 7 were homozygous for R2 and R3 respectively.

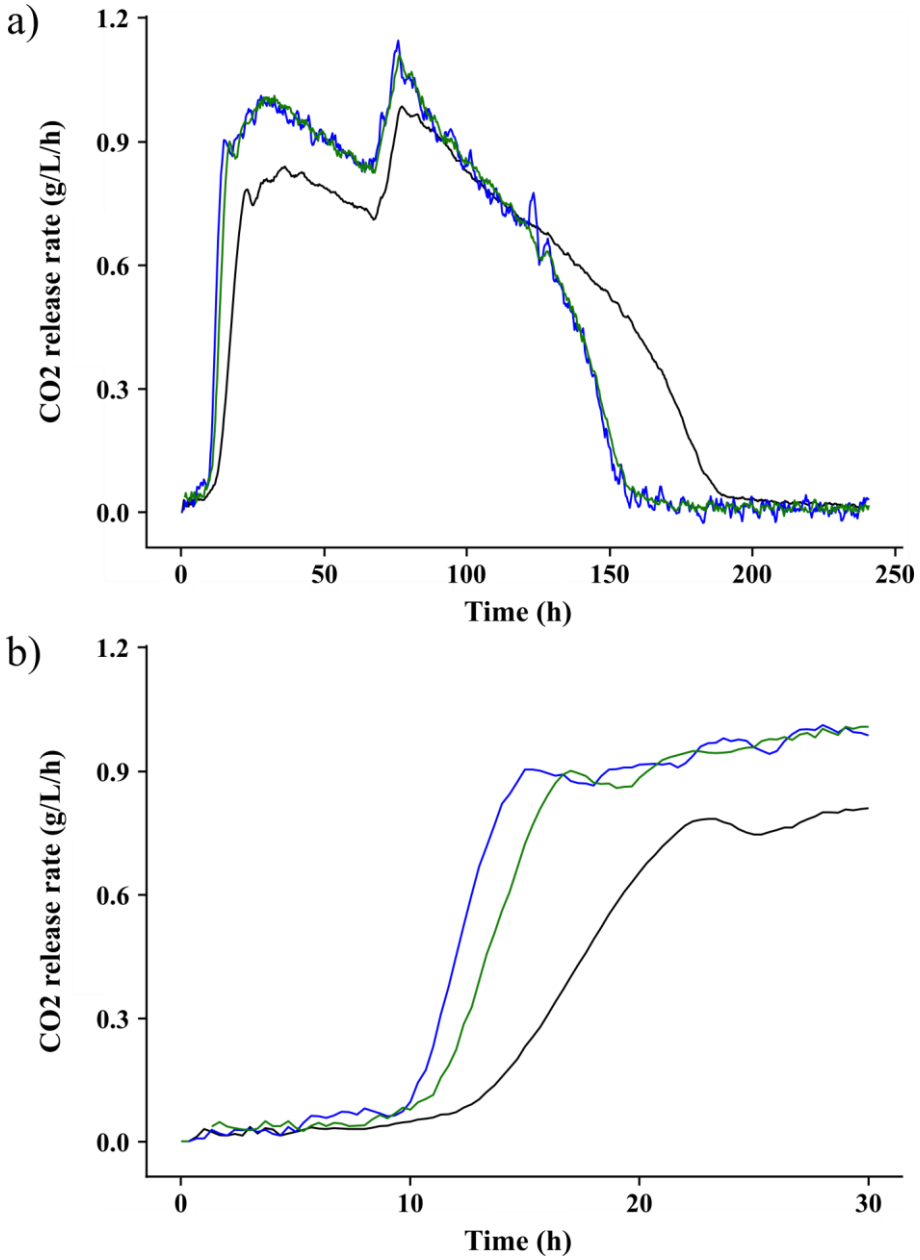



**Figure II.6. $CO_2$ release rate in 1 L fermenters using a real red wine must.** As the level of assimilable nitrogen was low (90 mg/L), a nitrogen supplementation at around 1/3 of fermentation (60h) was made using FermaidE. In a) overall fermentation kinetics; b) zoom on the lag phase moment showing a shorter lag phase for evolved clones R2E (blue) and R3I (green) in comparison with L7 (black).

After excluding synonymous SNPs, we focused on polymorphisms whose associated gene, based on their known function, may have a contribution to our evolved phenotype (Table S3, Supplementary data).

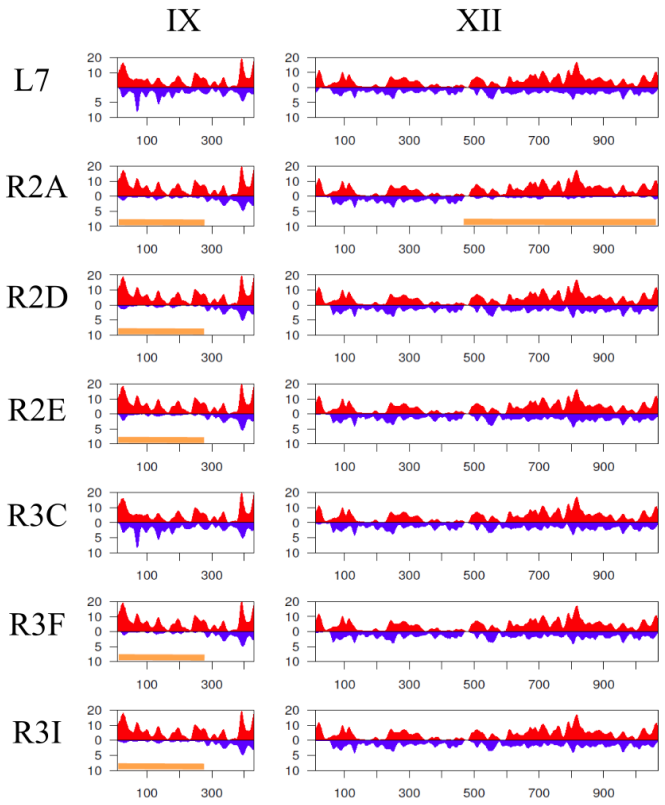



**Figure II.7. LOH detection in evolved clones.** Plots of the frequency of variant positions (SNP or Indel) over 1000 bp were used to detect of LOH on the chromosomes of evolved yeasts. Frequency was computed by the average shifted histogram method, averaging 10 windows of 10000 bp. The frequency of the homozygous variants (SNP or Indels) is plotted in red, whereas that of the heterozygous in blue. Orange bars highlight LOH regions.

This means that different strategies and pathways could be used to achieve the same lag phase improvement. However, among the mutated genes, several have a function related to the cell wall. R2 presented mutations in 4 particular genes, *TIR3*, *DAN4*, *DSE2* and *SLT2* directly involved in cell wall regulation. The first two code for a cell mannoprotein expressed exclusively under anaerobic conditions (Abramova *et al.* 2001). For *TIR3* a codon insertion occurred towards the end of the protein (position 212 in 269) whereas *DAN4* had a non-synonymous codon switch (polar amino acid into non-polar one) mid protein (position 523 in 1161). *DSE2* has similarities to glucanases, and is responsible for degrading the cell wall of daughter cells causing their separation from the mother cell (Colman-Lerner *et al.* 2001). In the evolved strains a codon insertion at position 184 in total of 325 was detected. Finally, *SLT2* is a serine/threonine MAP kinase that, among other functions, is involved in regulating maintenance of cell wall integrity as well as cell cycle progression (de Nobel *et al.* 2000). When looking at R3, two

particular genes stood out, *AZF1* and *CWP2*. The former is a zinc-finger transcription factor implicated in the activation of genes involved in the maintenance of cell wall integrity and genes involved in growth and carbon metabolism when in glucose (Slattery *et al.* 2006). Different mutations occurred with a codon insertion and modification at position 371 (in a total of 914), a codon insertion at 372 and 373 and finally another codon insertion and modification at position 373. The latter, *CWP2*, is a major cell wall mannoprotein which plays a role in its stabilization as well as in the resistance to low pH (Van der Vaart *et al.* 1995). It had a codon deletion on position 41 in a total of 92 in a tandem repeat area. Overall the fact that in both populations mutations occurred in genes connected to cell wall and its maintenance points to a specific role of the cell wall in the lag phase, most likely connected stress resistance and cell cycle progression.

**Discussion**

In this study, we used an adaptive laboratory evolution (ALE) approach to reduce lag phase duration under oenological conditions. For the purpose, selective pressures were defined as typical stress factors present at that stage of fermentation, particularly for red wine fermentations. Osmotic stress, $SO_2$ and low thiamine levels were defined accordingly to be realistic and sufficiently harsh for a stressful inoculation scenario capable of pushing adaptation and evolution. Yeast cultures were serially transferred during 500 generations with punctual increments in stress intensity.

By applying such strategy, we were able to obtain evolved strains with a shorter lag phase. The use of particular stress factors as selective pressures that conducted to a successful ALE reinforces the importance of stress resistance in modulating this phenotype. At the end of the experiment, from three independent experiments, two yielded a positive evolution (with different degrees of improvement) whereas one did not present an evolution at all. This illustrates the stochasticity of mutations accumulation that can occur during ALE. Woods *et al.* 2006, using *Escherichia coli* in 12 identical and parallel ALE experiments, showed that different random mutations can be fixed in different populations. As consequence, the final outcome of an evolutionary process can be altered, which underlines the interest of performing several experiments in parallel.

Evolved strains were isolated from the generation that presented the shortest lag phase while trying to keep a low/medium number of generation to limit the accumulation of

unrelated and undesired mutations. Even so, clone R2A presented particular growth deficiencies when submitted to different stress factors such as ethanol, oxidative, citric acid and temperature stress. Considering its lag phase similar to the remaining evolved strains, this suggests a fitness trade-off which is not rare in ALE approaches (Dragosits and Mattanovich 2013). They occur when organisms adaptively evolve a particular trait in a new environment while other trait(s) get their function diminished when in alternative environments. Wenger *et al.* 2011 reported the adaptation of yeast strains to aerobic carbon-limited conditions that, when back to aerobic carbon-rich conditions, showed an underperformance inexistent before. Whole-genome sequencing of the mutants showed that the determinant mutations were located in genes responsible for glucose sensing, signaling, and transport. In a stress resistance context, Çakar *et al.* 2009 found a fitness trade-off when evolving *S. cerevisiae* for cobalt resistance. Similarly to our results, the adapted strains presented sensitivity to other stress factors such as ethanol and hydrogen peroxide. These losses of fitness can be due to the adaptive mutations themselves but also to the accumulation of other mutations (e.g. deleterious mutations) along the evolutionary process. Although we did not further investigate this, the fact that R2A was the only strain to present such deficiencies while having a similar oenological behavior to the remaining evolved strains, makes the last hypothesis more likely.

The lag phase of all evolved strains, although non-homogenous (frequent in ALE experiments), was markedly improved when compared to L7. Combined with the drop tests results, R2E and R3I were selected as the best evolved strains to move forward for an industrial propagation and drying. Including such processes was fundamental in our characterization, as both have been extensively reported as highly stressful and with potential impact on yeast's later performance (Pérez-torrado *et al.* 2005; Singh *et al.* 2005; França *et al.* 2007; Gómez-Pastor *et al.* 2010). The fact that no issues were detected assured that no loss of fitness occurred for this stage. Additionally, this also ensured that, if desired, the strains would be suitable for later industrial production.

When assessed in grape must, the dried evolved strains showed reduced lag phase, confirming the phenotype identified in synthetic must. Surprisingly, the evolved strains also exhibited a higher $V_{max}$ and shorter fermentation duration, which could be an interesting trait for winemaking. The higher stress resistance acquired during lag phase seems therefore to also play a role in fermentation rate. This comes in line with the fact

that a higher stress resistance promotes fermentations with more vigor and higher reliability (Ivorra *et al.* 1999).

The phenotypic differences observed may be due to the fact that, although SM closely mimics real musts, grape musts have a more complex matrix, with additional compounds (*e.g* tannins, type of nitrogen sources) which might contribute to some differences in the phenotypes assessed in synthetic must. The composition of grape musts is highly variable (i.e. for sugar, nitrogen and micronutrient levels). Thus, in complement of the promising results obtained in Merlot, further evaluation of the evolved strains on a variety of different grape musts will be required in order to better evaluate the performances of these strains for winemaking.

The whole-genome sequencing of 6 evolved strains gave us some clues to elucidate the genetic basis of the improved phenotype. LOH has been frequently observed during experimental evolution of diploid, heterozygous strains (Tilloy et al, in preparation). While all strains presented equivalent lag phases profiles, R3C did not possess any LOH region, which suggests that either LOH is not the main determinant of the evolved phenotype, or, if these regions would be impacting, particularly the one on chromosome IX, R3C alone would had found other strategy to reduce the lag phase. Coming from the same population as R3F and R3I this was very unlikely. On the other hand, R2A presented an extra LOH region on chromosome XII that no other strain displayed. Considering its unique profile on the drop tests, we can hypothesize that this particular LOH might have impacted its resistance to other stress factors, by affecting a common stress response mechanism. Regarding *de novo* mutations, we found that they impact different genes implicated in cell wall, such as *TIR3* and *DAN4*, which are part of the *DAN*/*TIR* cell wall mannoproteins family. These genes are characterized by having variable number tandem repeats (often rich in serine and threonine) considered as microsatellites that can be used as a genetic markers (Marinangeli *et al.* 2004; Bowen *et al.* 2005; Bradbury *et al.* 2006). These mannoproteins are known to be repressed in aerobic contexts and conversely required for anaerobic growth (Abramova *et al.* 2001; Tai *et al.* 2005; Wu *et al.* 2006). Although their functions are not fully understood (partially due to the redundancy between the genes of the same family), several studies have reported a role in stress response, including cold shock, hydrostatic pressure and chemical compounds (Abramova *et al.* 2001; Wu *et al.* 2006; Abe 2007; Zhang *et al.* 2016). In fact, their polymorphic alleles rich in serine/threonine have been pointed as directly related to stress

response in the cell wall, being the stress the main driver of their selection/generation (Bowen *et al.* 2005). As one of the main constituents of the cell wall (40%), these mannoproteins play a major role in cell wall permeability and integrity. At the light of their structural role and importance in stress resistance, we can hypothesize that the genetic modifications in *TIR3* and *DAN4* in the evolved strains could have brought an increased resistance to the stress factors at the beginning of the fermentation as well as to ethanol that develops during fermentation. *DSE2*, another impacted gene implicated in cell wall maintenance, codes for a daughter cell-specific protein similar to glucanases that degrades the cell wall from the daughter side, leading to the separation of daughter and mother cell (Colman-Lerner *et al.* 2001). Interestingly, when this gene is deleted, yeast cells become more resistant to low pH, particulary to acetic acid (Kawahata *et al.* 2006; Suzuki *et al.* 2013). Considering that wine fermentation occurs in an acidic context, mutations on this gene could potentially lead to a higher stress resistance that in turn would improve yeasts' performance. *SLT2* is mitogen-activated protein (MAP) kinase which is considered the main signaling pathway implicated in proper cell wall construction and preventing cell lysis under a variety of stressful scenarios (de Nobel *et al.* 2000; Fuchs and Mylonakis 2009). Its activation is often associated with polarized growth (budding) and cell wall stress, which, among other consequences, leads to a massive deposit of chitin in the lateral walls of both mother and daughter cell in order to maintain cell wall integrity (Zarzov *et al.* 1996; Klis *et al.* 2006). This helps protecting the cell which at this stage experiences the greatest wall stress and is the most vulnerable to lysis. Stress factors that activate this pathway include heat stress, hypo-osmotic stress, actin cytoskeleton depolarization, cell wall-stressing agents such as cell wall-degrading enzymes or caffeine and interestingly acetic acid at low pH (Levin 2005; Mollapour *et al.* 2009). Transporting this to our ALE, we can hypothesize that the mutation in *SLT2* lead to a faster or more intense cell wall protection, particularly at the budding stage, in turn leading to a faster fermentation start. *AZF1* is a zinc-finger transcription factor involved in transcriptional activation of different genes that depends on the carbon source. Its role in cell wall is only detected when in non-glucose sources, such as glycerol-lactate, where genes required for cell wall maintenance are activated (Slattery *et al.* 2006). When in glucose, the gene is responsible for activating carbon and energy metabolism genes. More interesting in our context is its function in regulating the transcription of *CLN3*, a $G_1$ cyclin responsible for cell cycle progression. *AZF1* specifically binds to *CLN3* by means

of a $A_2GA_5$ sequence that leads to its expression. A positive mutation in this zinc domain could possibly lead to a higher affinity to *CLN3* leading then to a faster cell cycle. Finally, *CWP2* codes for a mannoprotein of the outer cell wall and is also connected to the cell cycle (Caro *et al.* 1998). *CWP2* mRNA reaches its peak at $G_2$, coincident with the higher number of buds. Deletion of this gene causes increased sensibility to calco-fluor white, congo red and zymolyase, revealing an important role in stabilizing the cell wall structure. Nadai *et al.* 2016 observed a higher $SO_2$ stress resistance due to increased expression levels of *CWP2*. The authors hypothesized that by increasing the cell wall stability, the toxicity of $SO_2$ could be reduced and its effects less pronounced. Considering our ALE conditions where $SO_2$ was fairly present, this could be an important phenotype to further confirm.

Overall, the genes identified by comparative genome analysis are all connected to two main biological functions, cell wall and cell cycle. As the cell cycle is directly connected to cell multiplication, an improvement in this function can certainly lead to a faster fermentation start with cells dividing more efficiently. From a stress resistance point of view, increased cell wall stability can provide a faster adaptation, not only to the stress factors present at inoculation but also to those that develop along fermentation. All together, these improved functions could explain the improved lag phase as well as the higher $V_{max}$ and faster fermentation time observed on grape must

This study demonstrates that it is possible to successfully apply an ALE approach to improve the resistance of commercial wine yeast strains the multistress conditions of wine fermentation, particularly during the lag phase. By sequencing the evolved strains we were able to shed new light on lag phase regulation, which points to an important role of the cell wall and cell cycle regulation. Additionally, the evolved strains generated in this study offer promising perspectives as starter strains with a faster fermentation start.

# Supplementary data

**Table II.S1. List of variant positions between L7 strain and R2 strains, and predicted effect of the SNP from the snpEFF analysis.** Alleles are reported for each strain (L7, R2A, R2D, R2E, R3c, R3F and R3I). Predicted protein modification is indicated with single letter of amino acid from reference S288C followed by the position number of the aa change and by the amino acid substituted. 0 represents an identical allele to S288C. 1, 2 and 3 different alleles. ./. not determined.

| *Chr* | *Gene* | *Gene* | *Gene start* | *Gene end* | *Muation position* | *Ref* | *Alt* | *snpEFF* | *L7* | *R2A* | *R2D* | *R2E* | *R3C* | *R3F* | *R3I* | *Protein modification* | *Protein lenght* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *I* | YAL005C | SSA1 | 139503 | 141431 | 139562 | C | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | P624A | 642 |
| *I* | YAL005C | SSA1 | 139503 | 141431 | 139565 | T | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | F623V | 642 |
| *II* | YBR108W | AIM3 | 454822 | 457665 | 455272 | A | G | NON_SYNONYMOUS_CODING | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | M151V | 947 |
| *II* | YBR108W | AIM3 | 454822 | 457665 | 455280 | caa | cCACaa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/2 | 1/2 | 1/2 | 0/3 | 1/2 | 1/1 | Q154PQ | 947 |
| *II* | YBR108W | AIM3 | 454822 | 457665 | 455280 | - | TATAATAGTATCCAC | CODON_INSERTION | 1/1 | 1/2 | 1/2 | 1/2 | 0/3 | 1/2 | 1/1 | -154YNSIH | 947 |
| *II* | YBR108W | AIM3 | 454822 | 457665 | 455280 | - | TATCAC | CODON_INSERTION | 1/1 | 1/2 | 1/2 | 1/2 | 0/3 | 1/2 | 1/1 | -154YH | 947 |
| *III* | YCR061W | | 225563 | 227458 | 225671 | A | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | N37D | 631 |
| *IV* | YDR505C | PSP1 | 1456695 | 1459220 | 1458171 | caa | caCAACAGCAACAACAGa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/2 | 0/1 | 0/1 | Q348HNSNNR | 841 |
| *IV* | YDR505C | PSP1 | 1456695 | 1459220 | 1458171 | caa | caCAACAGa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/2 | 0/1 | 0/1 | Q348HNR | 841 |
| *IV* | YDR505C | PSP1 | 1456695 | 1459220 | 1458174 | caa | caCAGCAACAACAGa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 0/2 | 0/2 | 0/2 | 0/1 | 0/2 | 0/1 | Q348HSNNR | 841 |
| *IV* | YDR505C | PSP1 | 1456695 | 1459220 | 1458174 | caa | caCAGa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 0/2 | 0/2 | 0/2 | 0/1 | 0/2 | 0/1 | Q348HR | 841 |
| *V* | YER133W | GLC7 | 432495 | 433958 | 433180 | | ATTTTTTTTTTTTTTT | SPLICE_SITE_ACCEPTOR | 1/1 | 0/1 | 0/1 | 0/1 | 1/1 | 1/1 | 1/1 | | |
| *VI* | YFL024C | EPL1 | 87847 | 90345 | 87975 | caagca | caa | CODON_DELETION | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | QA789Q | 832 |
| *VI* | YFL010W-A | AUA1 | 114990 | 115274 | 115159 | cag | caCCGTCAAATg | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | 1/2 | 0/1 | Q57HRQM | 94 |
| *VI* | YFL010W-A | AUA1 | 114990 | 115274 | 115159 | cag | caTCAAATg | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | 1/2 | 0/1 | Q57HQM | 94 |
| *VI* | YFL010C | WWM1 | 115108 | 115743 | 115159 | gct | gATTTGACGGct | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | 1/2 | 0/1 | A195DLTA | 211 |
| *VI* | YFL010C | WWM1 | 115108 | 115743 | 115159 | gct | gATTTGAct | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | 1/2 | 0/1 | A195DLT | 211 |
| *VI* | YFR026C | ULI1 | 205748 | 206257 | 205939 | G | A | NON_SYNONYMOUS_CODING | ./. | 1/1 | 1/1 | 1/1 | 0/1 | 1/1 | 0/1 | G107S | 169 |
| *VII* | YGL122C | NAB2 | 278946 | 280523 | 280128 | cct | cCATct | CODON_CHANGE_PLUS_CODON_INSERTION | ./. | 1/2 | 1/2 | 1/2 | 1/2 | 1/1 | 1/1 | P132PS | 525 |
| *VII* | YGL122C | NAB2 | 278946 | 280523 | 280128 | - | CA | FRAME_SHIFT | ./. | 1/2 | 1/2 | 1/2 | 1/2 | 1/1 | 1/1 | -132? | 525 |
| *VIII* | YHR030C | SLT2 | 168890 | 170344 | 169196 | cagcag | - | CODON_DELETION | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | QQ380- | 484 |
| *VIII* | YHR143W | DSE2 | 385510 | 386487 | 386059 | - | CTCAACATCATCCTCATCAT | FRAME_SHIFT | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | -184LNIILI? | 325 |
| *VIII* | YHR173C | | 450815 | 451153 | 451132 | - | CATATA | FRAME_SHIFT | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | -5 | 112 |
| *IX* | YIL011W | TIR3 | 333727 | 334536 | 334354 | - | GCT | CODON_INSERTION | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | -212A | 269 |
| *IX* | YIR016W | | 382628 | 383425 | 382839 | gat | gaCCTATCCAAGGCTCTAGAt | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | D72DLSKALD | 265 |
| *X* | YJL123C | MTC1 | 188004 | 189440 | 189029 | - | AAGAAGAA | FRAME_SHIFT | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | -137KK? | 478 |
| *X* | YJL032W | | 386348 | 386662 | 386348 | - | ATATATATGTGTTT,AT | FRAME_SHIFT | 0/1 | 1/2 | 1/2 | 1/2 | 0/1 | 0/1 | 0/1 | -1IY? | 104 |
| *X* | YJL032W | | 386348 | 386662 | 386348 | - | ATATATGT | FRAME_SHIFT | 0/1 | 1/2 | 1/2 | 1/2 | 0/1 | 0/1 | 0/1 | -1 | 104 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *X* | YJL005W | CYR1 | 425157 | 431237 | 426683 | - | AAGAACGAC | CODON_INSERTION | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | -510KND | 2026 |
| *X* | YJR151C | DAN4 | 712255 | 715740 | 714174 | A | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | S523G | 1161 |
| *XI* | YKL032C | IXR1 | 380065 | 381858 | 381505 | caacaacaacaacaa | - | CODON_DELETION | 1/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | QQQQQ113- | 597 |
| *XI* | YKL032C | IXR1 | 380065 | 381858 | 381523 | cag | cTACTTCGCAGTCag | CODON_CHANGE_PLUS_CODON_INSERTION | 2/3 | 1/2 | 1/2 | 1/2 | 1/3 | 1/2 | 1/2 | Q112LLRSQ | 597 |
| *XI* | YKL032C | IXR1 | 380065 | 381858 | 381523 | - | ACAGTAT | FRAME_SHIFT | 2/3 | 1/2 | 1/2 | 1/2 | 1/3 | 1/2 | 1/2 | -112TV? | 597 |
| *XI* | YKL032C | IXR1 | 380065 | 381858 | 381523 | - | CAGTC | FRAME_SHIFT | 2/3 | 1/2 | 1/2 | 1/2 | 1/3 | 1/2 | 1/2 | -112Q? | 597 |
| *XII* | YLL008W | DRS1 | 131729 | 133987 | 132258 | - | GAGGAAGAGGA | FRAME_SHIFT | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | -178EEE? | 752 |
| *XII* | YLL008W | DRS1 | 131729 | 133987 | 132260 | aag | aGGGAAGag | CODON_CHANGE_PLUS_CODON_INSERTION | ./. | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | K178REE | 752 |
| *XIII* | YML103C | NUP188 | 62582 | 67549 | 65933 | caa | cGAATGGaa | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | 0/1 | Q539RME | 1655 |
| *XIII* | YMR175W-A | | 611314 | 611508 | 611331 | - | A | FRAME_SHIFT | ./. | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | 0/0 | -7 | 64 |
| *XIII* | YMR306C-A | | 886183 | 886572 | 886518 | ttttat | ttt | CODON_DELETION | 1/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | 1/1 | FY17F | 129 |
| *XIV* | YNL065W | AQR1 | 503724 | 505484 | 503833 | gaa | gaTGATAAAGTTGATCCa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | E37DDKVDP | 586 |
| *XIV* | YNL065W | AQR1 | 503724 | 505484 | 503833 | - | TGAT | FRAME_SHIFT | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | -37*? | 586 |
| *XIV* | YNL065W | AQR1 | 503724 | 505484 | 503833 | - | TG | FRAME_SHIFT | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | -37? | 586 |
| *XV* | YOR373W | NUD1 | 1036834 | 1039389 | 1037514 | A | C,T | NON_SYNONYMOUS_CODING | 1/1 | 1/2 | 1/2 | 1/2 | 1/1 | 1/1 | 1/2 | K227N | 851 |
| *XV* | YOR373W | NUD1 | 1036834 | 1039389 | 1037514 | A | C,T | NON_SYNONYMOUS_CODING | 1/1 | 1/2 | 1/2 | 1/2 | 1/1 | 1/1 | 1/2 | K227N | 851 |
| *XVI* | YPL089C | RLM1 | 379120 | 381150 | 379432 | aac | aaAACAACAATc | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | N570KTTI | 676 |
| *XVI* | YPL032C | SVL3 | 488887 | 491364 | 490095 | T | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | 0/0 | S424A | 825 |
| *XVI* | YPR195C | | 927965 | 928294 | 928107 | G | A | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/1 | 0/0 | 0/1 | 0/1 | C63Y | 109 |

**Table II.S2. List of variant positions between L7 strain and R3 strains, and predicted effect of the SNP from the snpEFF analysis.** Alleles are reported for each strain (L7, R2A, R2D, R2E, R3c, R3F and R3I). Predicted protein modification is indicated with single letter of amino acid from reference S288C followed by the position number of the aa change and by the amino acid substituted. 0 represents an identical allele to S288C. 1, 2 and 3 different alleles. ./. not determined.

| Chr | Gene | Gene | Gene start | Gene end | Mutation position | Ref | Alt | snpEFF | L7 | R2A | R2D | R2E | R3C | R3F | R3I | Protein modification | Protein lenght |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| II | YBL081W | | 71866 | 72972 | 72395 | t | aaCAACAACAACAACAAt | CODON_CHANGE_PLUS_CODON_INSERTION | 2/2 | 1/2 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | N182NNNNNN | 368 |
| II | YBL081W | | 71866 | 72972 | 72395 | agc | agTTTTCAc | CODON_CHANGE_PLUS_CODON_INSERTION | 2/2 | 1/2 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | S177SFH | 368 |
| II | YBR013C | | 265492 | 265881 | 265492 | - | TAACATGAACA | FRAME_SHIFT | 2/2 | ./. | 1/1 | 1/2 | 1/1 | 1/1 | 1/1 | -130? | 129 |
| II | YBR013C | | 265492 | 265881 | 265492 | - | TA | FRAME_SHIFT | 2/2 | ./. | 1/1 | 1/2 | 1/1 | 1/1 | 1/1 | -130? | 129 |
| II | YBR108W | AIM3 | 454822 | 457665 | 455657 | cct | ccCGCACAACCACAt | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | P279PAQPH | 947 |
| II | YBR108W | AIM3 | 454822 | 457665 | 456730 | - | TCGCAA | CODON_INSERTION | 1/1 | 1/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | -638SQ | 947 |
| II | YBR272C | HSM3 | 746361 | 747803 | 746372 | - | CAATA | FRAME_SHIFT | ./. | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | -477Q? | 480 |
| III | YCR076C | FUB1 | 249293 | 250045 | 249324 | - | TTATTTA | FRAME_SHIFT | 0/0 | 1/1 | ./. | 0/1 | 1/1 | 1/1 | 1/1 | -241LF? | 250 |
| IV | YDR299W | BFR2 | 1059627 | 1061231 | 1059940 | gag | gaAGAGGAAGAAGGAGAg | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | E106EEEEGE | 534 |
| IV | YDR443C | SSN2 | 1345676 | 1349938 | 1346530 | cagcag | - | CODON_DELETION | 1/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | QQ1133- | 1420 |
| IV | YDR504C | SPG3 | 1456311 | 1456694 | 1456592 | ttg | ttTTTTTTg | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | L33FFL | 127 |
| IV | YDR504C | SPG3 | 1456311 | 1456694 | 1456592 | - | TT | FRAME_SHIFT | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | -33? | 127 |
| V | YEL070W | DSF1 | 19589 | 21097 | 19788 | tGg | tAg | STOP_GAINED | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | W67* | 502 |
| VI | YFL021C-A | | 95761 | 96615 | 96024 | tct | tTATATAct | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | S193LYT | 284 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96024 | - | TATATA | CODON_INSERTION | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | -25YI | 510 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96024 | - | TATA | FRAME_SHIFT | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | -25Y? | 510 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96034 | - | GA | FRAME_SHIFT | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | -24? | 510 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96034 | - | TATAGATA | FRAME_SHIFT | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | -25YR? | 510 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96038 | - | GATATATA | FRAME_SHIFT | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | -26DI? | 510 |
| VI | YFL021W | GAT1 | 95966 | 97498 | 96038 | - | TATA | FRAME_SHIFT | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | -29Y? | 510 |
| VI | YFL013W-A | | 107799 | 108602 | 107819 | tgt | tATGTATgt | CODON_CHANGE_PLUS_CODON_INSERTION | 1/2 | 1/2 | 1/2 | 1/1 | 1/3 | 1/3 | 1/3 | C8YVC | 267 |
| VI | YFL013W-A | | 107799 | 108602 | 107819 | - | ATGTATGTAT | FRAME_SHIFT | 1/2 | 1/2 | 1/2 | 1/1 | 1/3 | 1/3 | 1/3 | -8MYV? | 267 |
| VI | YFL013W-A | | 107799 | 108602 | 107819 | - | AT | FRAME_SHIFT | 1/2 | 1/2 | 1/2 | 1/1 | 1/3 | 1/3 | 1/3 | -8? | 267 |
| VI | YFR019W | FAB1 | 184502 | 191338 | 186254 | aat | aGTGCCAGCGat | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | N585SASD | 2278 |
| VII | YGL255W | ZRT1 | 20978 | 22108 | 21523 | gaC | gaG | NON_SYNONYMOUS_CODING | ./. | 0/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | D182E | 376 |
| VII | YGL081W | | 357377 | 358339 | 358135 | gaa | gaGGAa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | E254EE | 320 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *VII* | YGR070W | ROM1 | 627806 | 631273 | 628218 | - | ACTATTATCATTC | FRAME_SHIFT | 1/1 | 2/2 | 0/2 | 1/1 | 1/2 | 1/2 | 1/2 | -139TIII? | 1155 |
| *VII* | YGR070W | ROM1 | 627806 | 631273 | 628218 | - | ACTATTC | FRAME_SHIFT | 1/1 | 2/2 | 0/2 | 1/1 | 1/2 | 1/2 | 1/2 | -139TI? | 1155 |
| *VII* | YGR070W | ROM1 | 627806 | 631273 | 628221 | - | ACTATTATCATTT | FRAME_SHIFT | 1/1 | 1/3 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | -139TIII? | 1155 |
| *VII* | YGR070W | ROM1 | 627806 | 631273 | 628221 | - | ACTATTA | FRAME_SHIFT | 1/1 | 1/3 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | -139TI? | 1155 |
| *VII* | YGR070W | ROM1 | 627806 | 631273 | 628221 | - | ACTA | FRAME_SHIFT | 1/1 | 1/3 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | -139T? | 1155 |
| *IX* | YIL123W | SIM1 | 128151 | 129581 | 128717 | tcctcctct | tct | CODON_CHANGE_PLUS_CODON_DELETION | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | SSS193S | 476 |
| *IX* | YIL123W | SIM1 | 128151 | 129581 | 128717 | tcctct | tct | CODON_CHANGE_PLUS_CODON_DELETION | 1/1 | 1/2 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | SS194S | 476 |
| *IX* | YIL119C | RPI1 | 136654 | 137877 | 137138 | aac | aaAATc | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | N243KI | 407 |
| *IX* | YIL119C | RPI1 | 136654 | 137877 | 137138 | - | A | FRAME_SHIFT | 1/1 | 1/1 | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | -246? | 407 |
| *IX* | YIR019C | FLO11 | 389572 | 393675 | 390789 | G | A | NON_SYNONYMOUS_CODING | ./. | 1/1 | 0/1 | 1/1 | 1/1 | 1/1 | 1/1 | V963I | 1367 |
| *IX* | YIR019C | FLO11 | 389572 | 393675 | 391184 | G | C | NON_SYNONYMOUS_CODING | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | S831T | 1367 |
| *X* | YJL123C | MTC1 | 188004 | 189440 | 189048 | gaggag | - | CODON_DELETION | 1/1 | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | EE129- | 478 |
| *X* | YJL020C | BBC1 | 398937 | 402410 | 400271 | G | A | NON_SYNONYMOUS_CODING | 1/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | P714S | 1157 |
| *X* | YJL019W | MPS3 | 402897 | 404945 | 404068 | cag | cTGCag | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | Q392LQ | 682 |
| *X* | YJR161C | COS5 | 742848 | 743999 | 743395 | A | T | NON_SYNONYMOUS_CODING | 0/0 | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 | N202I | 383 |
| *XI* | YKL139W | CTK1 | 182958 | 184544 | 184501 | - | AAT | CODON_INSERTION | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | -524N | 528 |
| *XI* | YKL139W | CTK1 | 182958 | 184544 | 184501 | - | TTAAATAATAATAATAATAATAAT | CODON_INSERTION | 1/1 | 1/1 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | -521LNNNNNNN | 528 |
| *XI* | YKL096W-A | CWP2 | 259253 | 259531 | 259363 | accaccgaagctaccaccactgct | - | CODON_DELETION | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | TTEATTTA41- | 92 |
| *XII* | YLL013C | PUF3 | 122075 | 124714 | 123487 | cag | caCAAg | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Q403HK | 879 |
| *XII* | YLR055C | SPT8 | 251272 | 253080 | 252778 | gct | gcGGCt | CODON_CHANGE_PLUS_CODON_INSERTION | 1/3 | 1/1 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | A100AA | 602 |
| *XII* | YLR055C | SPT8 | 251272 | 253080 | 252778 | - | GGCTCTC | FRAME_SHIFT | 1/3 | 1/1 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | -101GS? | 602 |
| *XII* | YLR055C | SPT8 | 251272 | 253080 | 252778 | - | GGCTGCTCTC | FRAME_SHIFT | 1/3 | 1/1 | 1/2 | 1/3 | 1/2 | 1/2 | 1/2 | -101GCS? | 602 |
| *XII* | YLR255C | | 645600 | 645953 | 645790 | aga | agAGAGGGa | CODON_INSERTION | 1/1 | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | R52REG | 117 |
| *XII* | YLR255C | | 645600 | 645953 | 645795 | aga | aGGGGAAga | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | R53RGR | 117 |
| *XII* | YLR342W-A | | 815810 | 815983 | 815914 | - | AA | FRAME_SHIFT | ./. | 1/1 | 1/1 | 0/1 | 1/1 | 1/1 | 1/1 | -36? | 57 |
| *XII* | YLR396C | VPS33 | 910235 | 912310 | 911157 | C | T | NON_SYNONYMOUS_CODING | 0/0 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | T385I | 691 |
| *XIII* | YML074C | FPR3 | 120089 | 121324 | 120601 | gaagaagaacaa | gaa | CODON_DELETION | 1/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | EEEQ238E | 411 |
| *XIII* | YMR008C | PLB1 | 280590 | 282584 | 280734 | - | CTG | CODON_INSERTION | ./. | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | -616L | 664 |
| *XIII* | YMR008C | PLB1 | 280590 | 282584 | 280739 | gcc | gcCGTc | CODON_INSERTION | ./. | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | A615AV | 664 |
| *XIII* | YMR173W-A | | 608897 | 610081 | 609648 | T | C | NON_SYNONYMOUS_CODING | 1/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | M251T | 394 |
| *XIII* | YMR173W-A | | 608897 | 610081 | 609651 | C | T | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 | A252V | 394 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| XIII | YMR173W-A | | 608897 | 610081 | 609660 | T | C | NON_SYNONYMOUS_CODING | ./. | 0/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | M255T | 394 |
| XIV | YNL208W | | 254418 | 255017 | 254872 | A | G | NON_SYNONYMOUS_CODING | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | E152G | 199 |
| XIV | YNL152W | INN1 | 346313 | 347542 | 347518 | aga | aAGAga | CODON_CHANGE_PLUS_CODON_INSERTION | 0/0 | 0/1 | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | R403KR | 409 |
| XIV | YNL065W | AQR1 | 503724 | 505484 | 503833 | gaa | gaTGATAAAGTTGATCCa | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | E37DDKVDP | 586 |
| XIV | YNL065W | AQR1 | 503724 | 505484 | 503833 | - | TGAT | FRAME_SHIFT | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | -37*? | 586 |
| XIV | YNL065W | AQR1 | 503724 | 505484 | 503833 | - | TG | FRAME_SHIFT | 1/1 | 1/3 | 1/3 | 1/3 | 1/2 | 1/2 | 1/2 | -37? | 586 |
| XIV | YNL019C | | 598376 | 599230 | 598429 | C | T | NON_SYNONYMOUS_CODING | 1/1 | 1/1 | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 | R268W | 284 |
| XV | YOL144W | NOP8 | 53098 | 54552 | 53991 | - | CCAGG | FRAME_SHIFT | ./. | 0/2 | 0/2 | 0/1 | 0/1 | 0/1 | 0/1 | -299P? | 484 |
| XV | YOL144W | NOP8 | 53098 | 54552 | 53991 | - | GAAAACCCATCCGAGG | FRAME_SHIFT | ./. | 0/2 | 0/2 | 0/1 | 0/1 | 0/1 | 0/1 | -299ENPSE? | 484 |
| XV | YOL144W | NOP8 | 53098 | 54552 | 53996 | - | AAGGAAAACCCATCCGAGGT | FRAME_SHIFT | 1/1 | 0/1 | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | -300KENPSE? | 484 |
| XV | YOL144W | NOP8 | 53098 | 54552 | 53996 | - | AAGT | FRAME_SHIFT | 1/1 | 0/1 | 1/2 | 0/1 | 0/1 | 0/1 | 0/1 | -300K? | 484 |
| XV | YOL051W | GAL11 | 234940 | 238185 | 236228 | - | GGCAC | FRAME_SHIFT | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | -430G? | 1081 |
| XV | YOR053W | | 427817 | 428158 | 427932 | A | G | NON_SYNONYMOUS_CODING | 1/1 | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | H39R | 113 |
| XV | YOR082C | | 479296 | 479637 | 479422 | gttttgttttgt | gtt | CODON_DELETION | 1/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | VLFC68V | 113 |
| XV | YOR113W | AZF1 | 534075 | 536819 | 535183 | aac | aCCAac | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | N371TN | 914 |
| XV | YOR113W | AZF1 | 534075 | 536819 | 535185 | - | TAC | CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | -372Y | 914 |
| XV | YOR113W | AZF1 | 534075 | 536819 | 535188 | - | CAC | CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | -373H | 914 |
| XV | YOR113W | AZF1 | 534075 | 536819 | 535189 | aat | aCCAat | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | N373TN | 914 |
| XV | YOR113W | AZF1 | 534075 | 536819 | 535191 | aat | aaCCAt | CODON_CHANGE_PLUS_CODON_INSERTION | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | N373NH | 914 |
| XV | YOR372C | NDD1 | 1034807 | 1036471 | 1036171 | cag | caGAACAAg | CODON_INSERTION | 0/1 | 0/1 | 0/1 | 0/1 | 0/2 | 0/2 | 0/2 | Q100QNK | 554 |
| XV | YOR372C | NDD1 | 1034807 | 1036471 | 1036171 | - | AACAA | FRAME_SHIFT | 0/1 | 0/1 | 0/1 | 0/1 | 0/2 | 0/2 | 0/2 | -100N? | 554 |
| XVI | YPL277C | | 15405 | 16868 | 16786 | G | A | NON_SYNONYMOUS_CODING | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | G28E | 487 |
| XVI | YPL229W | | 117067 | 117687 | 117201 | G | C | NON_SYNONYMOUS_CODING | 1/1 | 1/1 | 1/2 | 1/1 | 1/2 | 1/2 | 1/2 | Q45H | 206 |
| XVI | YPL025C | | 502473 | 503030 | 502840 | - | TC | FRAME_SHIFT | ./. | 0/1 | ./. | 1/1 | 1/1 | 1/1 | 1/1 | -63 | 185 |
| XVI | YPR042C | PUF2 | 650435 | 653662 | 650490 | aatagt | aat | CODON_DELETION | 1/1 | 0/1 | 0/1 | 1/1 | 0/1 | 0/1 | 0/1 | NS1056N | 1075 |

**Table II.S3. List of selected candidate genes and protein modification resulting from *de novo* mutations.** Alleles are reported for each strain (L7, R2A, R2D, R2E, R3c, R3F and R3I). Predicted protein modification is indicated with single letter of amino acid from reference S288C followed by the position number of the aa change and by the amino acid substituted. 0 represents an identical allele to S288C. 1, 2 and 3 different alleles. ./. not determined.

| *Chr* | *Gene* | *Gene* | *Protein modification* | *Protein lenght* | *L7* | *R2A* | *R2D* | *R2E* | *R3C* | *R3F* | *R3I* | *Descritpion / Function* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *VIII* | YHR030C | SLT2 | QQ380- | 484 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | Serine/threonine MAP kinase; coordinates expression of all 19S regulatory particle assembly-chaperones (RACs) to control proteasome abundance; |
| *VIII* | YHR143W | DSE2 | -184LNIILI | 325 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 | Daughter cell-specific secreted protein with similarity to glucanases; degrades cell wall from the daughter side causing daughter to separate from mother; expression is repressed by cAMP |
| *IX* | YIL011W | TIR3 | -212A | 269 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | Cell wall mannoprotein; member of Srp1p/Tip1p family of serine-alanine-rich proteins; expressed under anaerobic conditions and required for anaerobic growth; TIR3 has a paralog, TIR2, that arose from the whole genome duplication |
| *X* | YJR151C | DAN 4 | S523G | 1161 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/0 | Cell wall mannoprotein; has similarity to Tir1p, Tir2p, Tir3p, and Tir4p; expressed under anaerobic conditions, completely repressed during aerobic growth |
| *XI* | YKL096W -A | CWP 2 | TTEATTTA 41- | 92 | 0/0 | 0/0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Covalently linked cell wall mannoprotein; major constituent of the cell wall; plays a role in stabilizing the cell wall; involved in low pH resistance; precursor is GPI-anchored |
| *XV* | YOR113W | AZF1 | N371TN | 914 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Zinc-finger transcription factor; involved in diauxic shift; in the presence of glucose, activates transcription of genes involved in growth and carbon metabolism; in nonfermentable carbon sources, activates transcription of genes involved in maintenance of cell wall integrity; relocalizes to the cytosol in response to hypoxia |
| *XV* | YOR113W | AZF1 | -372Y | 914 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Zinc-finger transcription factor; involved in diauxic shift; in the presence of glucose, activates transcription of genes involved in growth and carbon metabolism; in nonfermentable carbon sources, activates transcription of genes involved in maintenance of cell wall integrity; relocalizes to the cytosol in response to hypoxia |
| *XV* | YOR113W | AZF1 | -373H | 914 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Zinc-finger transcription factor; involved in diauxic shift; in the presence of glucose, activates transcription of genes involved in growth and carbon metabolism; in nonfermentable carbon sources, activates transcription of genes involved in maintenance of cell wall integrity; relocalizes to the cytosol in response to hypoxia |
| *XV* | YOR113W | AZF1 | N373TN | 914 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Zinc-finger transcription factor; involved in diauxic shift; in the presence of glucose, activates transcription of genes involved in growth and carbon metabolism; in nonfermentable carbon sources, activates transcription of genes involved in maintenance of cell wall integrity; relocalizes to the cytosol in response to hypoxia |
| *XV* | YOR113W | AZF1 | N373NH | 914 | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | Zinc-finger transcription factor; involved in diauxic shift; in the presence of glucose, activates transcription of genes involved in growth and carbon metabolism; in nonfermentable carbon sources, activates transcription of genes involved in maintenance of cell wall integrity; relocalizes to the cytosol in response to hypoxia |

**Acknowledgments**

The authors thank Céline Raynal and Benjamin Leroux from Lallemand SAS for their valuable contribution in propagating, drying and fermentation following. Also to Thibault Nidelet from UMR-SPO for his input during the redaction of this manuscript.

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# CHAPTER 3

## QTL MAPPING OF LAG PHASE MODULATION IN WINEMAKING CONDITIONS WITH INTERCROSS YEAST POPULATION

**Chapter's context**

In this chapter, our main objective was to investigate the genetic determinants controlling lag phase duration. As outlined in the bibliography section, the phenotype of lag phase duration results from many different cellular mechanisms that contribute to yeast adaptation, growth and survival, already complex on their own. It became clear that studying this phenotype at an in depth level could not be done by using classical approaches. This way, in parallel with chapter 2, we went further in lag phase characterization and set up a unique strategy based on a combination of QTL mapping and flow cytometry to select budding cells, which is described in this chapter.

Its originality comes from the use of a diallele – like design between 4 parental strains combined with bulk segregant analysis (BSA), with an industrial propagation and drying, typical of any wine yeast production and a cell sorting of first budding cells using FACS technique. This set-up allowed the detection of QTLs specific to a cross or present in all crosses and to determine if each QTL corresponded to the presence of deleterious or beneficial alleles specific to one parent. The choice of parental strains was based on the characterization performed in chapter 1. The main goal of this chapter was then to identify QTLs (and therefore alleles) with high importance in lag phase duration when in a winemaking context. Aside from generating novel academic knowledge, an industrial valorization of this knowledge was also expected in the long term by transferring the identified alleles to other commercialized wine yeast strains by non-GMO techniques and improve their performance.

# QTL MAPPING OF LAG PHASE MODULATION IN WINEMAKING CONDITIONS WITH INTERCROSS YEAST POPULATION

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## Abstract

The yeast *S. cerevisiae* is the main actor of wine fermentation and its use as starter culture is widespread in winemaking. In this process, lag phase has a particular importance fermentation's success but represents a poorly understood stage of fermentation. Here we present a specific experimental design of QTL mapping to decipher the genetic basis of yeast lag phase in oenological conditions. We first developed a strategy based on *CLB3*-GFP lineages enabling cell sorting for first budding cells and thus selection for lag phase emergence. This approach was applied to a diallele – like crossing of 4 commercial wine yeast strains with contrasting genetic and phenotypic profiles to generate 6 highly diverse populations. These were propagated and dried as in real wine yeast production and used to obtain 6 pools containing the very first dividing cells. After whole genome sequencing of these pools of cells, the comparison of allelic frequencies in the populations obtained before and after sorting revealed several QTLs explaining the differences between parental strains. Several genes involved in budding and cell cycle, glucose sensing, osmotic stress and cell integrity potentially implicated in lag phase modulation as well as alleles that could confer a major advantage in fermentation start in the winemaking context were identified in these QTLs.

**Introduction**

Alcoholic wine fermentation is one the most ancient biotechnological applications conducted by mankind, having in its core the yeast *S. cerevisiae* (McGovern *et al.* 1996). In the recent decades, quantitative trait locus (QTL) mapping has surfaced as an ideal solution to decipher the genetic basis of complex traits that can be quantified. In the winemaking context, this technique has been successfully used to study different phenotypes such as acetic acid production in correlation with fermentation rate (Marullo *et al.* 2007), aromatic contributions (Steyer *et al.* 2012), fermentation rate (Ambroset et al, 2012), sulfite production as a main off-flavor (Noble *et al.* 2015), nitrogen metabolism (Brice *et al.* 2014, Cubillos *et al.* 2017) and adaptation to low temperatures (García-Ríos *et al.* 2017).

Since the first appearance of the QTL concept, different strategies have emerged presenting different advantages (Liti and Louis 2012). The conventional QTL mapping approach relies on bi-parental populations and on the individual genotyping of each segregant obtained (Marullo *et al.* 2006). This can represent a laborious genetic and phenotypic characterization and for this reason, bulk segregant analysis (BSA) gained popularity in recent years since its discovery (Michelmore *et al.* 1991). BSA relies on the comparison of pools of segregants that display opposite phenotypes. These two bulks have a random distribution of alleles in all loci of the genome except for loci that are associated with the measured phenotypic variation. By sequencing the two bulks, relevant QTLs can be identified (Wenger *et al.* 2010). Increased sensitivity in QTL mapping analysis has been achieved with approaches like X-QTL (extreme QTL) or iQTLs (intercross-selection QTLs) that have been developped during the last decade (Liti and Louis 2012). The first relies on the use of extremely large segregating populations (Ehrenreich *et al.* 2010) whereas the second on the use of several intercross lines that further reduce linkage between nearby loci and allow determining QTLs down to single gene precision (Parts *et al.* 2011; Cubillos *et al.* 2013).

Lag phase is among such complex traits but so far has received little attention. By definition, a lag phase corresponds to the period of adaptation where a microorganism, after inoculation in a new media, is re-adjusting its metabolism before growth can start (Lewis *et al.* 1993; Brejning and Jespersen 2002). Its complexity can be easily foresee when considering that various processes such as the cell cycle, glucose and nitrogen

metabolism or stress resistance have a direct influence on lag phase (Neves *et al.* 1994; Ma *et al.* 1997; Dönmez and Aksu 1999; Vriesekoop and Pamment 2005; Singh *et al.* 2006). The lag phase in winemaking gains a particular interest since it can dictate the success of a fermentation. The wine must is a considerably stressful media and upon inoculation yeast has to cope with several stress factors present that can compromise growth. High sugar content (osmotic stress), inhibitors compounds (such as $SO_2$), lack of vitamins, low lipids and low temperatures particularly for white wines, are some of the stress factors present at this stage and that can be responsible for extended lag phases (Bataillon *et al.* 1996; Ribéreau-Gayon *et al.* 2006; Jackson 2008b; Ferreira *et al.* 2017).

In this context, Pérez-Ortín *et al.* 2002 showed that the recurring practice of using $SO_2$ in winemaking led to the selection of a translocation between chromosome XVI and VIII. The gene *SSU1*, coding for a sulfite exporter and originally located in chromosome XVI, becomes highly expressed after being translocated to chromosome VIII due to a modification of its promotor. As a consequence, higher levels of Ssu1p allow a higher sulfite expelling rate (Goto-Yamamoto *et al.* 1998). Ultimately, this provides a higher stress resistance to this compound, reason why this particular translocation became highly disseminated among wine yeast strains. A similar translocation, this time involving chromosomes XVI and XV, was identified by QTL mapping during alcoholic fermentation of grape juice with an impact in lag phase duration (Zimmer *et al.* 2014). However, it is thought to be a much more recent event due to its lower dissemination among wine yeast. Aside the stress factors present in wine must, the fundamental steps of propagation and drying of any commercial wine yeast also play a role in the lag phase. Even with proper rehydration, at the moment of inoculation cells are vulnerable and are still readjusting their metabolic and cellular homeostasis Thus, this means that to a certain extent the lag phase response can partially be a consequence of such processes. Overall, lag phase is yet to be fully understood. When not controlled, it is one of the main causes of development of undesired microbiome, stuck/sluggish fermentations, wine spoilage, and important economic losses for the wine industry.

In this study, we used a strategy combining a cell sorting based on FACS (fluorescence-activated cell sorting) and detection of cell cycle activation together with a diallele-like cross design and bulk segregant analysis (BSA) (Ehrenreich *et al.* 2010; Liti and Louis 2012) for studying lag phase under oenological conditions. Four different

commercial wine yeast strains with contrasting lag phase, were GFP labelled for the gene *CLB3* encoding a cyclin of the cell cycle and intercrossed, leading to 6 recombined populations. By including an industrial propagation and drying process step followed by inoculation in synthetic must, the cell sorting was performed in a realistic oenological context. The measurement of allelic frequencies by pool sequencing of the different population enabled the successful identification of several QTLs, and revealed the likely importance of genes that are involved in budding and cell cycle, glucose sensing, osmotic stress and cell integrity.

## Materials and Methods

### Yeast strains and media

*S. cerevisiae* strains L1, L7, L8 and L11 are diploid commercial wine strains that were provided by the company Lallemand SAS. Based on them, different strains were constructed for the purpose of this study, summarized in Table III.1.

Yeast strains were pre-cultivated in Yeast Peptone Dextrose (YPD) (2% glucose, 1% yeast extract, 2% peptone) and maintained on YPD plates at 4ºC or stored at -80ºC in 20% glycerol. For solid media 20 g/L of agar were added before autoclaving. Media were sterilized by autoclave at 121ºC for 15 min at 1 bar.

For sporulation, GNA (1% Bacto yeast extract, 0.5% Bacto peptone, 10% glucose) and SpoMA (0.1% Bacto yeast extract, 0.05% glucose, 1% potassium acetate, 0.002% adenine) media were used. When asci were observed, these were deposited on YPD micromanipulation solid medium (thiner) dissected and spores isolated.

Synthetic must (SM) mimicking a standard grape juice was used for the fermentations. SM medium was prepared as described by Bely *et al.* 1990. The SM used in this work contained 260 g/L of sugar (130 g/L of glucose and 130 g/L of fructose), 6 g/L of malic acid, 6 g/L of citric acid, salts (0.75 g/L $KH_2PO_4$, 0.5 g/L $K_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.155 g/L $CaC_{12} \cdot 2H_2O$, 0.2 g/L NaCl, trace elements (4 mg/L $MnSO_4 \cdot H_2O$, 4 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $CuSO_4 \cdot 5H_2O$, 0,4 mg/L $CoC_{12} \cdot 6H_2O$, 1 mg/L $H_3BO_3$, 1 mg/L $(NH_4)_6Mo_7O_{24}$ and 10 mg/L of vitamin stock solution (composed as follows: 20 mg/L

myo-inositol, 1.5 mg/L calcium pantothenate, 0.223 mg/L thiamine hydrochloride, 2 mg/L nicotinic acid, 0.25 mg/L pyridoxine, 0.003 mg/L biotin).

**Table III.1. Strains used and constructed in this study.**

| Strain | Source | Genotype |
|---|---|---|
| **L1** | Lallemand SAS | *S. cerevisiae, MATa/MATα* |
| **L7** | Lallemand SAS | *S. cerevisiae, MATa/MATα* |
| **L8** | Lallemand SAS | *S. cerevisiae, MATa/MATα* |
| **L11** | Lallemand SAS | *S. cerevisiae, MATa/MATα* |
| **L1aGFP** | Transformation L1 | *MATa, HO::nat1, CLB3*- GFP |
| **L7αGFP** | Transformation L7 | *MATα, HO::blast, CLB3*- GFP |
| **L8αGFP** | Transformation L8 | MATα, HO::hph, CLB3- GFP |
| **L8aGFP** | Transformation L8 | *MATa, HO::hph, CLB3*- GFP |
| **L11aGFP** | Transformation L11 | *MATa, HO::ble, CLB3*- GFP |
| **L11αGFP** | Transformation L11 | *MATa, HO::ble, CLB3*- GFP |
| **D1.7** | Hybrid L1aGFP x L7αGFP | *MATa/MATα, HO::nat1/HO::blast, CLB3*-GFP |
| **D1.11** | Hybrid L1aGFP x L11αGFP | *MATa/MATα, HO::nat1/HO::ble, CLB3*-GFP |
| **D7.8** | Hybrid L7αGFP α x L8aGFP | *MATa/MATα, HO::blast/HO::hph, CLB3*-GFP |
| **D7.11** | Hybrid L7αGFP x L11aGFP | *MATa/MATα, HO::blast/HO::hph, CLB3*-GFP |
| **D8.1** | Hybrid L8αGFP x L1aGFP | *MATa/MATα, HO::hph/HO::nat1, CLB3*-GFP |
| **D8.11** | Hybrid L8αGFP x L11aGFP | *MATa/MATα, HO::hph/HO::ble, CLB3*-GFP |

The nitrogen source was composed of 0.27 g/L of $NH_4Cl$ and 7.7 mL of aminoacids stock solution (composed as follow: 1.4 g/L tyrosine, 13.7 g/L tryptophan, 2.5 g/L isoleucine, 3.4 g/L aspartate, 9.2 g/L glutamate, 28.6 g/L arginine, 3.7 g/L leucine, 5.8 g/L threonine, 1.4 g/L glycine, 38.6 g/L glutamine, 11.1 g/L alanine, 3.4 g/L valine, 2.4 g/L methionine, 2.9 g/L phenylalanine, 6 g/L serine, 2.5 g/L histidine, 1.3 g/L lysine, 1 g/L cysteine and 46.8 g/L proline) adding up to a total of 250 mg/L of assimilable nitrogen. The pH of the SM was adjusted to 3.3 with NaOH 10 M. For $SO_2$, a concentrated solution of potassium metabisulfite ($K_2S_2O_5$) was prepared (43.8 mg/L). Right before inoculation, an aliquot of this solution was added accordingly to the final molecular $SO_2$ concentration desired considering a pH of 3.3. SM was supplemented with 5 mg/L of phytosterols from a stock solution with 5 g/L phytosterols in Tween 80 and ethanol (1:1,

v/v). Finally, the SM was steam sterilized for 10 min and cooled to the correct temperature.

Fermentations were carried out in microplates and 300-mL fermenters. For the microplates, a total of 200 µl of SM were deposited in each well of Greiner transparent 96 wells flat microplates with cover. Each well was inoculated at 1 x $10^6$ cells/mL. Once inoculated these were put to agitate and read in the microplate reader TECAN M200 Infinite Pro for 280 cycles (≈ 48 h) at 28°C. Agitation program included an orbital agitation (8 min at 140 rpm) as well as a linear one (2 min at 300 rpm). Readings were done in a multi-reading system using the circle filled scheme with 3x3 measure points. The method chosen to determine the lag-phase duration is based on a local regression as previously described by Ferreira *et al.* 2017. By tracing a tangent line that contains the inflection point (the point where the acceleration equals zero and the velocity is maximal) and by determining where this tangent crosses the 0.1 O.D. value, lag-phase duration could be exactly determined (Swinnen *et al.* 2004; Gagneur and Neudecker 2012). Experiments were done in duplicate.

For the 300-mL fermenters, 250 mL of SM was used, inoculated with 1 x $10^6$ cells/mL incubated at 28°C with continuous stirring (350 rpm). Temperature and agitation were kept constant at 28ºC and 350 rpm respectively. Fermentation kinetics were monitored by calculation of the amount of $CO_2$ released determined by automatic weighing of the fermenters at fixed intervals. All fermentation experiments were performed in duplicate.

**Haploid strains construction**

Deletion of the *HO* gene was performed to create isogenic haploid strains, in L1, L7, L8 and L11 strains by inserting clonNAT, blasticidin, hygromycin, and phleomycin, cassettes respectively, using the short-flanking homology PCR technique (Güldener *et al.* 1996). These selection cassettes were PCR amplified from pAG25, pSF-TEFI-TPI1-Blast, pAG32 and pUG66 plasmids respectively using the primers listed in Table III.2.

The PCR fragments were used to transform strains using the lithium acetate method (Schiestl and Gietz 1989). The deletions were verified by PCR analysis of total

DNA isolated from antibiotics-resistant transformants, using primers flanking the *HO* regions as well as primers within the cassettes (Table III.3).

**Table III.2. List of plasmids used in this work.** All plasmids were propagated in bacteria *E.coli*.

| Plasmid | Characteristics | Source |
|---|---|---|
| **pSF-TEFI-TPI1-Blast** | Bacterial plasmid, KanR, Blast cassette | Sigma |
| **pAG25** | Bacterial plasmid, AmpR, nat1 cassette, loxP | (Goldstein and McCusker 1999) |
| **pAG32** | Bacterial plasmid, AmpR, hph cassette, loxP | (Goldstein and McCusker 1999) |
| **pUG66** | Bacterial plasmid, AmpR, ble cassette, loxP | (Güldener *et al.* 1996) |
| **pKT127** | Bacterial plasmid, AmpR, kan cassette | (Sheff and Thorn 2004) |
| **phS3** | Bacterial plasmid, AmpR, NAT cassette, *HO* | John McCusker (unpublished) |

By combining them, we performed 3 different PCR for each construction that confirmed a correct insertion. Positive transformants were pre-cultivated in 10 ml of GNA medium overnight at 28◦C at 450 rpm. Then, 200 µl of cultures were inoculated in 20 ml SpoMA and incubated for 2 weeks at room temperature (RT) with slight agitation. Asci were digested with a solution of 5% Lyticase (from Arthrobacter luteus, crude, Sigma) for 8 min and spores were separated using a micromanipulator (MSM300 Singer Instrument). Spores carrying the *HO* gene deletion were selected on YPD plates containing the appropriate concentration of each antibiotic (200 µg/ ClonNAT mL; 200 µg/mL Hygromycin; 150 µg/mL Phleomycin; 500 µg/mL Blasticidin). Strains obtained were genotyped for mating type by PCR using primers listed in Table III.3.

**Table III.3. Primers used in this work.** Homologous sequences to Blast, nat1, hph, and ble of plasmids pSF-TEFI-TPI1-Blast, pAG25, pAG32, and pUG6 respectively are in bold. Homologous sequences to yEGFP of plasmid pKT127 are in italic.

| Primer | Sequence | Purpose |
|---|---|---|
| **HOdelFOR** | 5' ATGCTTTCTGAAAACACGAC TATTCTGATGGCTAACGGTG **TTCGTACGC TGCAGGTCGACC** 3' | Obtaining the different antibiotic cassettes from the different plasmids |
| **HOdelREV** | 5' TTAGCAGATGCGCGCACCTG CGTTGTTACCACAACTCTT **GCATAGGCCACTAGTGGATCTG** 3' | Amplification of antibiotic cassettes from the different plasmids |
| **HOdelVerifFOR** | 5' TGTTGAAGCATGATGAAGCG 3' | Confirmation insertion of antibiotic cassette (up/downstream of *HO*) |
| **HOdelVerifREV** | 5' TGAAACAAATCAGTGCCGGT 3' | Confirmation insertion of antibiotic cassette (up/downstream of *HO*) |
| **Phleo1** | 5' AAGCCCAACCTTTCATAGA 3' | Confirmation *HO*::ble insertion |
| **Phleo2** | 5' ATCTCAAGCTGGAGTTCTTC 3' | Confirmation *HO*::ble insertion |
| **ClonNAT1** | 5' CATCCAGTGCCTCGATGGCC 3' | Confirmation *HO*::nat1 insertion |
| **ClonNAT2** | 5' TCGAGGACAT CGAGGTCGCC 3' | Confirmation *HO*::nat1 insertion |
| **Hygro1** | 5' GCAGCTATTTACCCGCAGGA 3' | Confirmation *HO*::hph insertion |
| **Hygro2** | 5' TTCTGGAGGC CGTGGTTGGC 3' | Confirmation *HO*::hph insertion |
| **BlastChvF** | 5' AGATCATCTCTGCTGTCCAC 3' | Confirmation *HO*::Blast insertion |
| **ECM34D** | 5' TCGAACATCGAGCATGCA 3' | Detection $XVI^{VIII}$ translocation |
| **SSU1R** | 5' GACACCCATGACCATCAC 3' | Confirmation of Detection $XVI^{VIII}$ translocation |
| **P760** | 5' GCCCCTCCATGTTCTACTATT 3' | Detection $XVI^{VIII}$ /$XVI^{XV}$ translocation |
| **p761** | 5' AATGCAGGCGCAAAAAACTC 3' | Detection $XVI^{XV}$ translocation |
| **P761** | 5' GAGTTTTTTGCGCCTGCATT 3' | Detection $XVI^{XV}$ translocation |
| **Mat1** | 5' AGTCACATCAAGATCGTTTATGG 3' | Confirming mating type |
| **Mat2** | 5' GCACGGAATATGGGACTACTTCG 3' | Confirming mating type |
| **Mat3a** | 5' ACTCCACTTCAAGTAAGAGTTTG 3' | Confirming mating type |

(Continuation of Table III.3).

| Primer | Sequence | Purpose |
|---|---|---|
| **CLB3GFP_F** | 5'GTGGATAGCATTAGCTGAACACAG AGTAGAAAGATCTAAC*ATGTCTAAAG GTGAAGAATTA* 3' | Amplification GFP marker from pKT127 |
| **CLB3GFP_R** | 5'CTTTTTCCTTTGTTGATGCCATGTCT CGAGCTGAGGCTTT*TGGATGGCGGCG TTAGTATC* 3' | Amplification GFP marker from pKT127 |
| **CLB3_F** | 5' CCGTTGGCCACCATTATTT 3' | Confirmation *CLB3*-GFP insertion |
| **CLB3_R** | 5' CCATATGGATTGTGGATGCTT 3' | Confirmation *CLB3*-GFP insertion |
| **CLB3GFP_VR** | 5' GGACGAGGCAAGCTAAACAG 3' | Confirmation *CLB3*-GFP insertion |

Each haploid strain was then confirmed to still have the translocation $XVI^{VIII}$ by PCR as well as the same lag phase profile of the respective parental strain (Figure S1, supplementary information). The genotype of the final strains is reported in Table III.1.

**Reversing mating types**

In order to cross all haploids between them, which corresponds to 6 independent crossings in total, we changed the mating type of L8α and L11a. This required a diploidization step, obtained by transforming the haploids with the plasmid phS3 (Güldener *et al.* 1996) using the lithium acetate method (Schiestl and Gietz 1989). This plasmid contains the gene *HO* and once integrated, it restores the same functions as a genomic *HO*. Cells were then cultivated and became diploid after few generations. The diploidy was confirmed by PCR with primers Mat1, Mat2 and Mat3 (see Table III.3), by observation of two bands simultaneously present. Plasmid loss was promoted by cultivating cells in fresh medium for consecutive 24h several times. After plasmid loss confirmation (no growth on ClonNAT), a new sporulation process was initiated as previously described, in order to select haploids with an opposite mating type of the initial haploid, in this case L8α *MATα* and L11a *MATa*. Several haploids were tested their

mating type was confirmed by PCR. Haploids L8a and L11α were selected, possessing an opposite mating type.

**Construction of *S. cerevisiae CLB3*-GFP reporter strains**

GFP reporter constructs targeted for integration into *CLB3* gene were generated in the 6 haploids strains using the short-flanking homology PCR technique (Güldener *et al.* 1996). The plasmid pKT127 containing a reporter cassette with the GFP gene and the kanamycin resistance marker was used as the PCR template to generate KANMX4-GFP integration cassettes. The primers used for this amplification contained homolog tails for integration in the C-terminal region of the gene *CLB3* (Table III.3). The integrations were verified by PCR analysis of total DNA isolated from geneticin-resistant transformants, using primers flanking the *CLB3* regions (CLB3_F/CLB3_R) as well a within the cassette (CLB3GFP_VR) (Table III.3).

**Generating crosses and segregant populations**

Each pair of haploids were crossed separately through mass mating (Figure III.1). For this purpose, haploids were pre-cultivated in YPD and 10 µl of each pair of haploids were deposited in solid YPD in the same spot. Cells were left incubating for 6h at 28ºC. By microscopic observation, crosses were confirmed by identifying typical mating forms (similar to clovers). Diploids were then selected by streaking these in solid YPD containing the respective 2 antibiotics. After 48h at 28ºC, hybrids were isolated and confirmed by PCR, checking the presence of both resistance cassettes by using the combination of the 3 PCR previously used for each cassette.

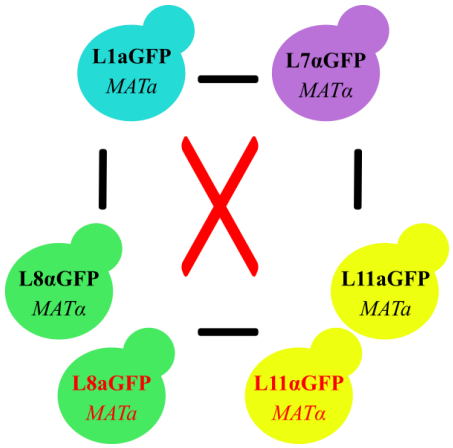



**Figure III.1. Schematic representation of all crosses performed.** L1aGFP, L7αGFP, L8αGFP and L11aGFP correspond to the original haploids in terms of mating type. After GFP marking black lines show which crosses could be performed. After mating type change an GFP marking, the reaming crossings with L8aGFP and L11αGFP could be performed (red lines).

Thereafter, another round of sporulation was performed to increase the recombination and crossing-over. Each diploid was sporulated individually as described before. The sporulation suspension was then submitted to a killing ether-zymolyase treatment, a procedure for ascospore isolation from cultures containing unsporulated vegetative cells (Bahalul *et al.* 2010). 500 µl of sporulation suspension and 500 µl of diethyl ether were mixed and left incubating for 20 min at RT. Cells were then washed four times with sterile water to completely eliminate ether trace elements. Cells were then resuspended in 900 µl of sterile water and 100 µl of zymolyase solution 20 T (200 U/ml) were added for ascus digestion. The mixture was incubated 20 min at 30ºC and vortexed for 1 min. In order to stabilize the spores population these were diploidized by incubation for 6h in YPD at 28ºC and inoculated in solid YPD overnight at 28ºC. The populations were named P1.7; P1.11; P7.8; P7.11; P8.1 and P8.11.

## Propagation and drying

Evolved strains were propagated and dried as in a regular industrial process. For the propagation, yeast underwent a scale-up of batch cultures followed by fed-batch cultures. First yeast was inoculated in 25 mL of laboratory media for 48h at 28ºC. Next, a new batch culture was performed, this time in 1.4 L of diluted molasses for 24h at 28ºC. The

biomass obtained here was used to inoculate a 25 L reactor for fed-batch culture in diluted molasses. During this process several parameters such as nitrogen and molasses feeding, temperature, pH and aeration were computer-controlled to optimize biomasse production. After separation from molasses (washing and centrifugation) a yeast cream with 20% dry weight (DW) was obtained. The cream was further vaccum-filtered and pressed yielding a cake yeast (32% DW) that was extruded and dried. In the end, around 100 g of active dry yeast with 95% DW were obtained.

**Flow cytometry**

1.25 mg of the dried yeast preparation obtained for each segregant population was directly inoculated in SM media at 28°C. After a specific time of incubation (more on this later), 0.5 mL of cell suspension at 5 x $10^5$ cells/mL had 1 µL of Vybrant DyeCycle Ruby stain (Thermo Fisher) added to a final concentration of 5 µM. The suspension was incubated at 28ºC for 15 min, protected from light. This marker allows the detection of the amount of DNA present within the cells, meaning distinguishing between **n** and **2n**, without the need of fixing the cells. Without washing, samples sorted with a FACS Aria (Becton-Dickinson, Mountain View, CA). Logarithmic GFP fluorescence was detected through a 530/30 nm band pass filter (FL1). The speed of sorting was 5,000 events/sec and cells were acquired, stored and analyzed using the FACS Diva software (Becton-Dickinson, Mountain View, CA). Gating for sorting was adjusted to control population (0h of fermentation, immediately after inoculation) where cells were not dividing and therefore no GFP was being expressed, eliminating therefore background fluorescence. 0.3% of positive events were admitted in the gatings. Sorted cells were recovered in strict sterile conditions directly into a 15 mL falcon tube containing 5 mL YPD. These were then incubated overnight.

**QTL analysis**

Each population of sorted cells were cultivated on 10 ml YPD media at 28°C, and DNA was extracted with kit MasterPure Yeast DNA purification kit (Epicentre) according to manufacturer's instructions.

Library preparation and sequencing were performed at GetPlage plateform Toulouse. DNA was cheered and library prepared using a PCR free kit (Illumina TruSeq Nano), and sequencing was performed in a Illumina HiSeq3000 sequencer from the two paired extremities of DNA fragments (2 x150bp reads). Reads were processed for quality using Trimmomatic (v 0.32.1) under Galaxy environment. Then reads were mapped on S288C genome sequence with Bowtie (v2.2.6 for Galaxy). Resulting alignment files were then genotyped simultaneously using the Mpileup v2.1.3 and bcftools view v1.40 modules of samtools for Galaxy. From the final multi individual variant file, the sequencing depth of each allele which reflects the allelic frequencies in the population was extracted using customs perl scripts. Variant position between each parent were filtered, and only position, in which less than 10 reads for the minor allele for one parent (and more than 50 for the other parent) were kept for QTL analysis. The software multipool (Edwards and Gifford 2012) was used using the contrast method, assuming a minimum segregant population of 10000 individuals. The outcome was the determination across the genome of significant allelic frequency differences between both populations, designated as LOD scores. The higher this score (defined on a logarithmic scale) the higher the chances of the frequency differences found being strictly correlated to phenotype studied. To increase our selection power, we decided to only consider peaks with LOD scores superior to 10. In these regions, we retained only positions with LOD values differing less than 1 from the maximum peak value.

**Results**

A QTL strategy based on the BSA method was performed to study lag phase modulation under oenological conditions. For the purpose, different steps and techniques were brought together in a unique experimental setup, specifically designed for this phenotype (Figure III.2).

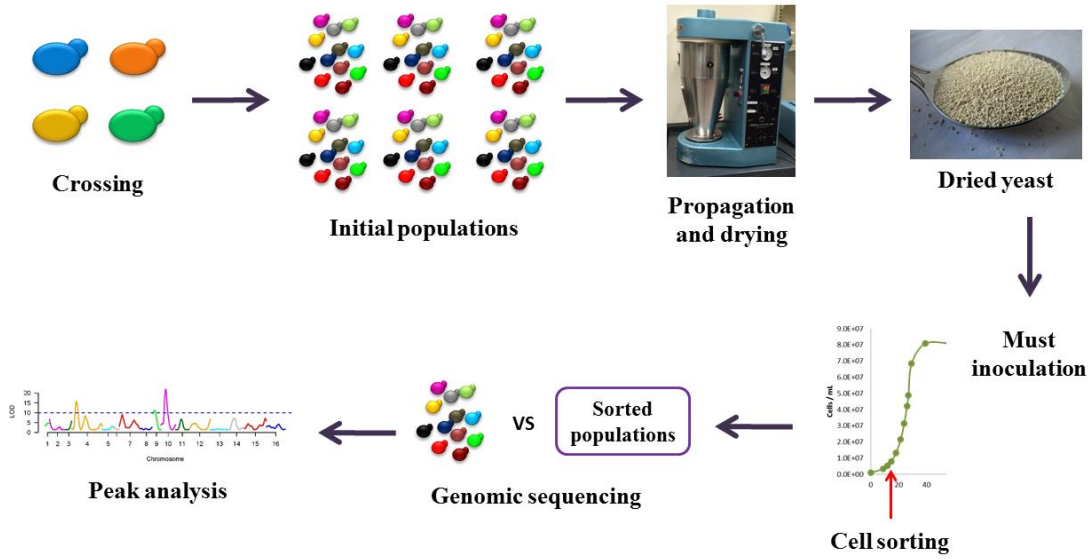



**Figure III.2. Overall roadmap of the QTL strategy**. Highlight on its main steps meaning the cross between the haploids constructed, the populations resulting from their respective diploids, propagation and drying, must inoculation and cell sorting by FACS, genome sequencing and QTL peak analysis.

## Parental strain choice and tagged recombinant populations preparation

Four strains with different abilities to overcome stress associated to the onset of growth and wine making were selected using the data of Ferreira *et al.* 2017. These different *S. cerevisiae* strains presented all the translocation $XVI^{VIII}$ associated to sulfite resistance (Pérez-Ortín *et al.* 2002) and contrasted phenotypes concerning lag phase duration. Strains L1 and L7 appeared among the slowest strains to start growth with a global lag phase of 16h and 18h respectively contrasting with L8 and L11, the fastest, with a global lag phase of 13.7h and 12.5h respectively.

In order to detect the start of cell growth, we decided to follow the level of the protein *CLB3*, which codes for a B-type cyclin that accumulates during the S-phase of the cell cycle and responsible for its progression until the $G_2$ phase (Figure III.3). To this end, for each parental strain, the reporter GFP was fused to the C-terminal of the cyclin Clb3.

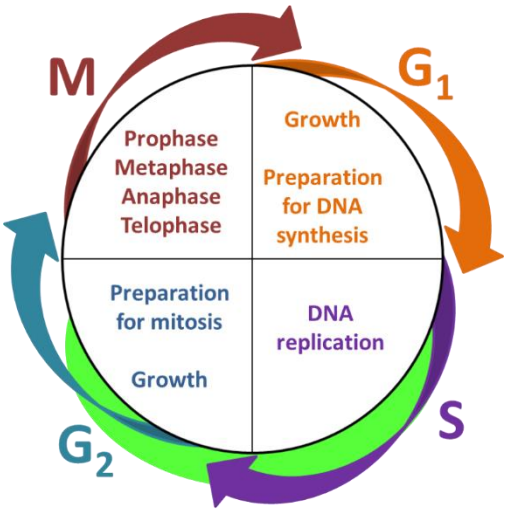



**Figure III.3. Schematic representation of the cell cycle with the different phases.** Green highlight shows when was *CLB3*-expressed during the cell cycle.

These 4 parental lineages were crossed in a diallele-like design, sporulated and mass mated, generating 6 independent recombinant populations (P1.7; P1.11; P7.8; P7.11; P8.1 and P8.11, see material and methods). These recombinant populations were propagated and dried following an industrial procedure making the process as realistic as possible. At the end of the process, these populations were verified, which revealed that population P8.1 did not correspond to the initial parental cross. This population was thus eliminated from the analyses.

## Cell sorting of the first budding cells

In order to detect and to select the very first cells beginning to bud, we determined, in a preliminary experiment, the exact moment when cells started to grow in synthetic must for each recombinant population. Differences in lag phases were observed for the different pools (Figure III.4). The lag phase of each recombinant population varies according to the respective parents. Population P8.11 (parents with the shortest lag phases) was the first to grow. On the other hand, population P1.7 (parents with the longest lag phases) was the last population to recover growth. The remaining populations (P1.11, P7.8 and P7.11) combining a parent with long and short lag-phase, presented an intermediate growth curve, very close to the best population. This indicates that the allelic

contribution (from a fast parent) can partially overcome the defects of slower parents, which should occur in the case of recessive alleles.

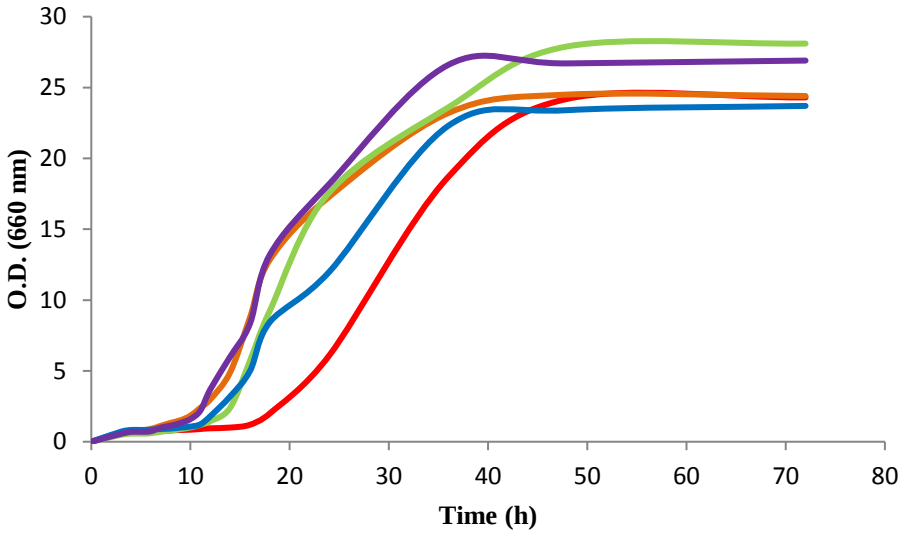



**Figure III.4. O.D. following of the different populations for lag phase time**. Determination using the microplate method and synthetic must (260 g/L sugar, 0.8 mg/L molecular $SO_2$). Color code: red – population P1.7; orange – population P1.11; green – population P7.8; blue – population P7.11; purple – population P8.11 Experiment performed in duplicate.

On the other hand, the recombinant population, P1.7, obtained from the slowest parents present a clear slower growth, suggesting either that these two parents share the same less performing alleles or that these strains carry co-dominant less performing alleles. Nevertheless, since these populations are composed of a mixture of recombinant cells, the results observed represent an average of the abilities of the cells inside this population, not showing its inner variance.

The detection of the first budding cells by following the *Clb3*-Gfp protein fusion along growth phase using flow cytometry provided more information (Figure S2). Control used for each population was the population itself right after rehydration (0h into fermentation) in a synthetic must where no cell cycle was occurring. After inoculation, no GFP signal

was detected in the first hours for all populations. As expected, cells were adapting to the new conditions before multiplying and therefore in lag phase which lasted a different time for almost all populations (Figure S2). Eventually, a GFP signal was detected indicating the start of cell cycle activation and multiplication. After this, the signal became progressively more intense as more and more cells started multiplying. At 48h all populations stopped showing a GFP signal, getting back to values equivalent to the control (0h) as they entered in stationary phase (Figure III.4, Figure S2), which indicates a degradation of the Clb3 protein.

To get further insight into the cell cycle progress, we also attempted the combination of the GFP signal with a second marker, the Vybrant DyeCycle that fluoresces in the red wavelength. This particular dye stains the cells for their DNA content allowing, in theory, the distinction of **n** cells from **2n** cells which could increase our sorting power. However, in our case, we could not observe such distinction (Figure III.5) as our populations presented a similar Vybrant signal across lag phase and upon exponential phase start.. This way, the sorting was performed focusing exclusively on the GFP signal.

Figure III.5 illustrates the cell sorting results for two populations. Gating at 0h was adapted according to each population in order to eliminate basal fluorescence. As soon as a GFP signal was detected, the first budding cells (indicated by a green arrow) where sorted (Figure III.5). We further confirmed the relevance of using *CLB3*-GFP construct for cells sorting by observing that the cells sorted had a bigger sizer as well as a higher complexity, in agreement with budding. In total, 1 x $10^5$ cells for each population were recovered and propagated for biomass enrichment.

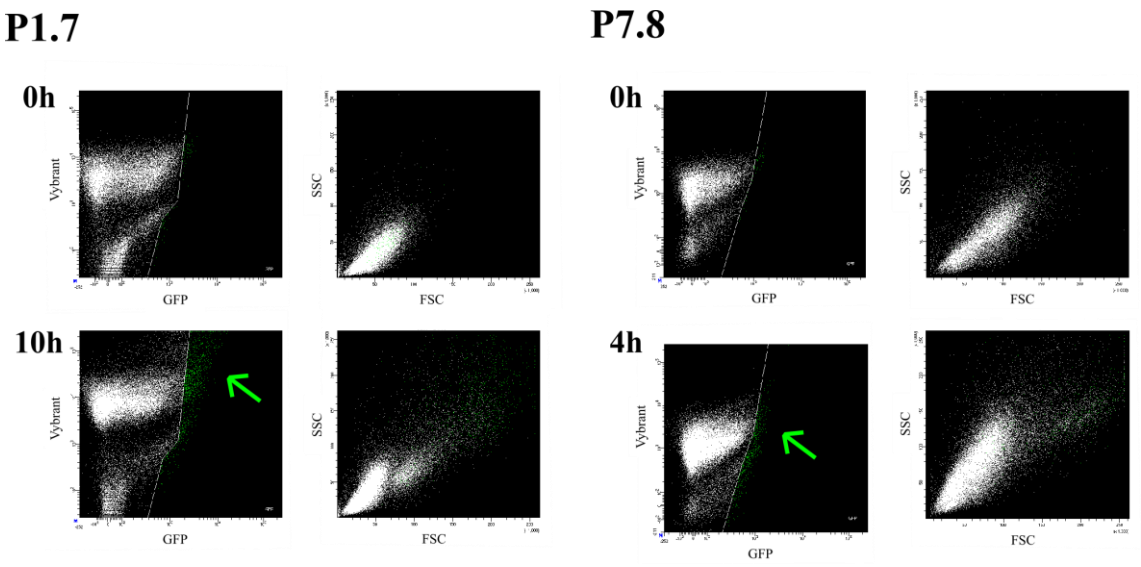



**Figure III.5. Example of the cell sorting performed for populations P1.7 and P7.8.** Graphics on the left of each time point combine GFP (x-axis) and Vybrant (y-axis) signal. Green fluorescence was used to define a proper gating at 0h. Graphics on the right of each time point combine cell size (FSC, x-axis) and granularity (SSC, y-axis) helping visualizing cells being sorted. Green dots represent cells sorted expressing GFP (indicated by green arrows). White dots represent cell not sorted and not expressing a GFP signal.

## QTL identification

Each recombinant population of sorted cell was compared to the pool of cells that had followed the industrial propagation process, before the drying step. The latter contained a high genetic diversity among the pool. By specifically sorting the cells that exited first the lag phase, we expected to have an allelic enrichment corresponding to an advantageous genotype for such conditions, meaning exit lag phase under oenological conditions.

The sequencing of haploid parental strains, of populations before drying and sorted populations revealed between 9303 and 17218 SNPs per cross depending on the set of strains being considered. The analysis of allelic frequencies with Multipool enabled us to detect 18 QTLs with LOD score values higher than 10 in the different crosses (Figure III.6). After analyzing each peak, 9 peaks with a LOD score ranging from 11.9 to 67 based on the distributions of allelic frequencies in the peaks were selected for further investigations. Three regions were shared by two QTL crosses (populations P1.7, P1.11 and P7.11) while the remaining three QTL were found in single QTL set ups (two in population P7.8 and one in P7.11). The observation of the allelic frequencies at the regions of these peaks showed that every time a short lag phase parent was present, the

allele retained in the pool of sorted cells came from them. This was the case for populations P1.11, P7.8 and P7.11. Interestingly, for population P8.11 the peak with the highest LOD was only 6.3 (Figure III.6), suggesting that QTL with less important effect might exist in this cross. On the other hand, in each population where parent L7αGFP (long lag phase) was implicated, an abrupt drop in allelic frequency at the beginning of chromosome X for the pool of sorted cell and for the control was observed. This indicates the presence of a highly deleterious allele in the genome of parent L7αGFP which had been purged along the industrial multiplication.

In order to facilitate the detection of the genes involved in the trait, the variant file was further analyzed using the SnpEff software, which allowed the characterization of the SNP's obtained and their major implications (Cingolani *et al.* 2012b). With this, we attempted to narrow down the number of candidate genes by focusing on coding regions. In these QTL, we also restrained the analysis to genes distant no more from 10 kb of the maximum value of the peak. Among the six different regions (of which one was shared by 3 populations, Figure III.6), a total set of 56 genes was identified based on these criteria. We further narrowed down the candidates to those that carried non-synonymous polymorphisms (down to 28) and with a biological function potentially impacting lag phase (down to 16). We ended with a final candidate list of 9 genes, summarized in Table III.4. Their implication ranged from budding/cellular proliferation, stress response activation and sugar sensing/transport.

**Table III.4. Summary table of the final peaks identified, the populations where those were generated, as well as the chromosome and position of each peak.** Best candidate genes are listed together with the parental strain who enriched the sorted population with a positive allele and led to the QTL detection.

| QTL | Pop. | Chr | Peak value | Position (+/-1 LOD) | Candidates | Allelic enrich. |
|---|---|---|---|---|---|---|
| **P1.7/P7.11a** | P1.7<br>P7.11 | IV<br>IV | 14.5<br>27.4 | 204750-258750<br>215500-264000 | *CYK3*, *NUP84*, *RGT2* | L1aGFP<br>L11aGFP |
| **P1.7/P7.11b** | P1.7<br>P7.11 | X<br>X | 22.3<br>11.9 | 214750-257250<br>211000-265250 | *BCK1*, *SAP185* | L1aGFP<br>L11aGFP |
| **P1.11/P7.11** | P1.11<br>P7.11 | XV<br>XV | 67<br>27.5 | 477500-524000<br>473250-523500 | *RAS1* | L11αGFP<br>L11aGFP |
| **P7.11** | P7.11 | IV | 17.1 | 1058000-1180750 | *HXT3* | L11aGFP |
| **P7.8a** | P7.8 | X | 58.9 | 171750-195250 | *PBS2* | L8aGFP |
| **P7.8b** | P7.8 | XV | 19 | 1027000-1075000 | *NUD1* | L8aGFP |

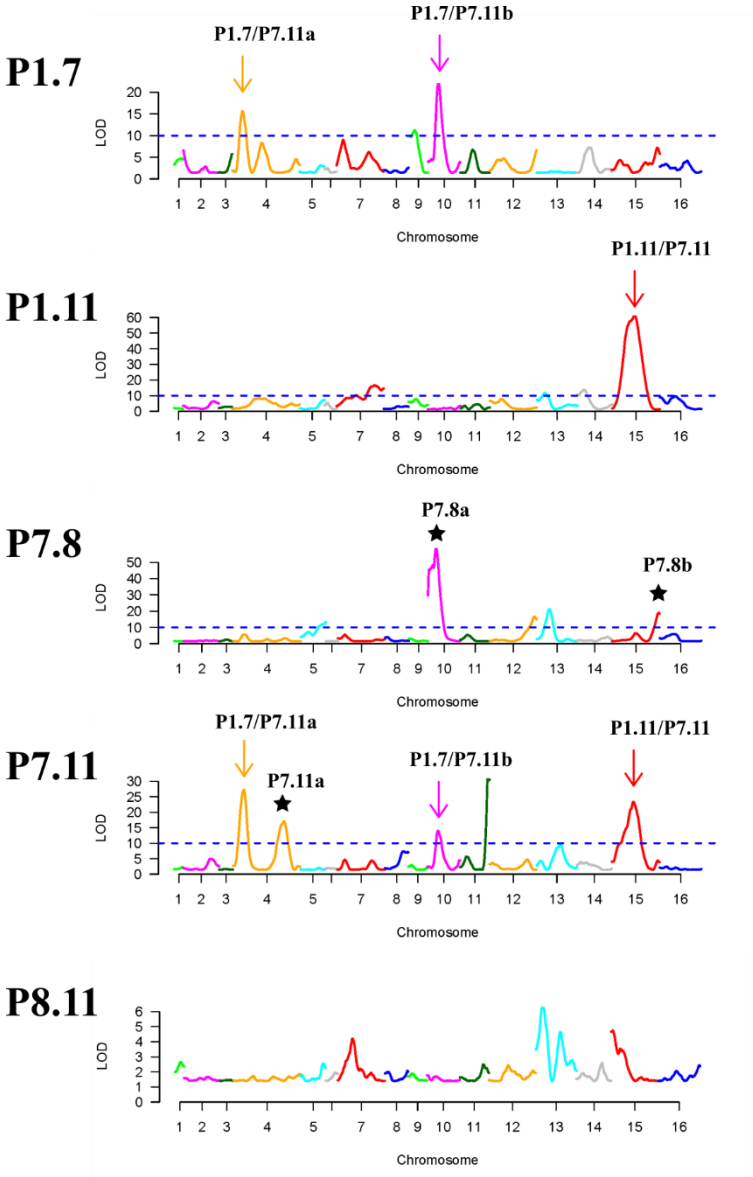



**Figure III.6. QTL mapping of lag phase under oenological conditions using 5 highly diverse populations, selected for their lag phase.** Chromosomes are displayed on the X-axis each with a distinctive color. LOD scores are presented by the Y-axis. The threshold of 10 chosen for significance is defined by a blue dashed line. Each peak corresponds to the nominal P-value of linkage between a marker located in the x position and a faster lag phase duration. Shared QTLs are pointed with an arrow whereas single QTLs are pointed with a start.

**Discussion**

Here, we used a QTL mapping approach to investigate the modulation of lag phase under oenological conditions. For the purpose, a design with four diverse wine *S. cerevisiae* strains tagged at *CLB3* with GFP was used to generate six recombinant populations. The inclusion of a propagation and drying process helped mimicking the realistic conditions in which wine yeast are produced and later inoculated. For each population, we then recovered a large progeny enriched for alleles that conferred a short lag phase when in must fermentation. To do so, we sorted the very first budding cells with the help of FACS and using the *CLB3*-GFP tagging. Finally, after sequencing sorted and control populations, we performed a comparison of allelic frequency along the genome in order to determine which alleles contributed for the positive (and extreme) phenotype.

Traditional QTL mapping approaches typically rely on two-parent crosses, which, although functional, can be limiting in terms of genetic diversity and later QTL resolution power (Deutschbauer and Davis 2005; Demogines *et al.* 2008). The first advantage offered by the use of four strains in our study is the information it provides on the generality of the QTL found. The use of strains with contrasting lag phase performances enables to obtain recombined population with variable differences between parental strains. Another advantage offered by our set up (crosses 2 by 2 kept independently) was the possibility to detect the improvement provided by each allele when the QTL was shared between several crosses, and thus further confirmation of its importance. An extremely positive allele given by one parental strain should be enriched in the respective 3 populations and appear as a common QTL. On the other hand, if substantially disadvantageous, (leading to long lag phases) an allele would therefore be counter-selected in every pool. In addition, we used a mass mating approach in order to obtain large populations and to perform bulk segregant analysis such as achieved for X-QTL (Ehrenreich *et al.* 2012; Cubillos *et al.* 2017).

For 3 different populations (P1.7, P1.11 and P7.11) a QTL was detected in the same region showing the strategy applicability and the importance of the respective alleles. In

this case the alleles preserved in the selected pool were consistently the ones from parent L11 (Table III.4). The fact that the allele of the strain with short lag phase parent prevailed over the alternative one is in agreement with the superior phenotype of the original strain (Ferreira *et al.* 2017). In addition, we also detected a deleterious allele in the genome of parent L7αGFP, confirming the interest of these multiple crosses. In this case, since we did not observe a displacement of the frequencies but rather their drop in both control and selected pools, this indicates that this allele was probably chased away during the industrial propagation step.

Finally, the fact that population P8.11 did not present detectable peaks when applying our criteria (≥ 10 LOD score) is in agreement with both parental strains having short lag phase profile and an equivalent allelic background in what concerns this phenotype. Nevertheless, a peak of LOD 6.3 is observed in this crosses centered around *CMP2* (a stress-response transcription factor), suggesting that allelic differences with less pronounced effects exist in this crosses. This led to less marked differences in the progeny that would require a more intense sorting (*i.e.* with a second-round sorting step) to be selected for.

When dissecting the peaks, we found 3 overall biological functions where all genes could fit. Cell growth and budding, glucose sensing and transport and cell integrity and stress. The peaks we considered more important were the common ones. Here, the peak P1.7/P7.11a comprises 3 genes, *CYK3*, *NUP84* and *RGT2*. *CYK3* is a SH3-domain protein located in the bud neck and cytokinetic actin ring. Its main roles are related to cytokinesis, promoting the acting ring constriction, septation and subsequent cell separation (Korinek *et al.* 2000; Meitinger *et al.* 2010). Null mutants are deficient in cytokinesis, present an aberrant mother-bud neck as well as a slower growth (Korinek *et al.* 2000). Interestingly, it has been shown that a high copy *CYK3* can also suppress to some extent the growth defects of different mutants (*inn1*Δ, *chs2-AA*Δ) whose cytokinesis and cell cycle could be compromised (Nishihama *et al.* 2009; Oh *et al.* 2012). *NUP84* is a subunit of a nuclear pore complex that is responsible for mRNA export, nuclear envelop structure and DNA repair (Su *et al.* 2015). It has been demonstrated that *NUP84* null mutants exhibit hypersensitivity to DNA damaging agents like Methyl methanesulfonate (MMS), bleomycin and ionizing radiation (Bennett *et al.* 2001; Deutschbauer and Davis 2005;

Loeillet *et al.* 2005) and lose the ability in repairing subtelomeric breaks (Therizols *et al.* 2006). Ultimately, it ensures the necessary fidelity of either DNA replication or repair which is fundamental in case of stress and DNA breakage. Last gene within the peak, *RGT2*, codes for a transmembrane low affinity glucose sensor that upon glucose stimulus generates an intracellular glucose signal (Johnston 1999; Rolland *et al.* 2002). Among other functions, it is responsible for the maximal activation of the low glucose affinity transporter *HXT1*, which play an important role in conditions where extracellular glucose concentration is high (Ozcan *et al.* 1996). The peak P1.7/P7.11a revealed 2 genes, *BCK1* and *SAP185*. The first codes for an upstream kinase of MAPK module (Lee and Levin 1992). Upon activation by Pkc1p, the cascade follows into the activation of Mkk1p, Mkk2p and Slt2 which in turn will culminate in the regulation of genes related to integral cell wall proteins and proteins involved in cell was biogenesis (Jung and Levin 1999). Additionally, it has also been shown that strains lacking a functional Bck1p are particularly sensitive at low pH (Claret *et al.* 2005). *SAP185* codes for a regulatory subunit part of a family of similar proteins (SAP) that controls Sit4p (Luke *et al.* 1996; Rohde *et al.* 2004). The last is responsible for promoting progression through $G_1$ via regulation of $G_1$ cyclin production (Sutton *et al.* 1991; Fernandez-Sarabia *et al.* 1992). Interestingly, Sit4p also plays a role in glycogen metabolism and its accumulation (Posas *et al.* 1991; Jablonka *et al.* 2006; Ruiz *et al.* 2011). The last shared peak, P1.11/P7.11, revealed a single gene, *RAS1*, which codes for a small GTP-binding protein, sister with *RAS2*. Both proteins have an influence in growth by regulating the activity of cAMP levels, important in cell cycle and accumulation of storage carbohydrates (*e.g.* glycogen and trehalose) (Broach 1991; Thevelein and De Winde 1999). Interestingly, overexpression of *RAS1* has no particular effect on life span however its deletion increases it by 30% (Sun *et al.* 1994).

On the single QTLs, P7.11a yielded the gene *HXT3*, a low glucose affinity transporter (Reifenberger *et al.* 1997). It gains a particular importance in the first part of wine fermentations, being one the main transporters of sugar into the cell (Luyten *et al.* 2002; Varela *et al.* 2005). Considering the peak position, *HXT3* seems like the most plausible gene to consider due to our high extracellular glucose content context. However, *HXT7*, another glucose transporter but with high affinity (important in low glucose contexts) is 10 kb next to *HXT3* and was recently demonstrated to have an impact in growth and lag phase both in low and high glucose (Ziv *et al.* 2017) making this gene a serious candidate

as well. The peak P7.8a also harbored one significant gene, *PBS2*, coding for a MAP kinase kinase of the HOG signaling pathway (O'Rourke *et al.* 2002). In turn, this will activate important cellular and metabolic stress responses, particularly under osmotic stress (Albertyn *et al.* 1994). Finally, the last peak, P7.8b, included the gene *NUD1*. It codes for a component of the spindle pole body outer plaque (Wigge 1998; Adams and Kilmartin 1999), which plays a main role in mitotic exit regulation (Gruneberg *et al.* 2000), being therefore important during vegetative growth. Additionally, it has also been reported to play a role in the regulation of sporulation and meiosis (Gordon *et al.* 2006).

As described above, all genes identified by our approach present biological functions that can directly impact lag phase in different ways. The fact that the allelic enrichment on the selected population was mainly provided by the most performing strains (shorter lag phase) reinforces that these alleles can play an important role in the phenotype. The next step will be to demonstrate the role and contribution of these alleles to the lag type phenotype using reciprocal hemizygosity analysis (RHA) or allelic swap.

Overall, these data provide a very valuable framework for shedding new light on the lag phenotype that so far has been poorly studied. Additionally, after experimental confirmation of the importance of the alleles identified in this study, it will be possible to develop improved wine yeast strains with reduced lag phase by hybridization and marker assisted selection. It will be particularly interesting in this context to evaluate the performance of these strains after rehydration of active dry cells, but also in a process of direct inoculation, which would provide a valuable simplicity of use in the cellar.

# Supplementary information

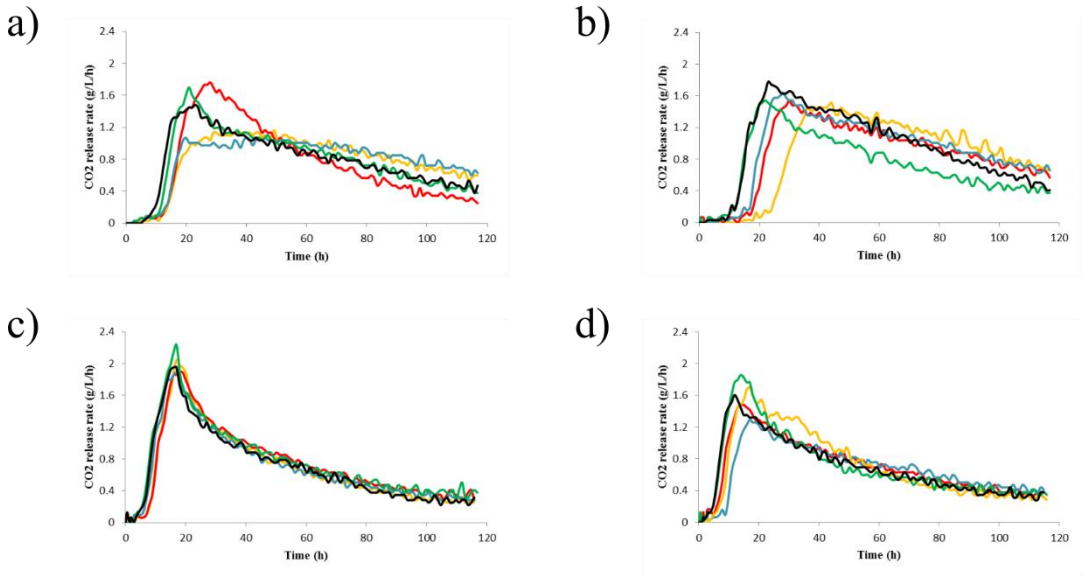



**Figure III.S1. Fermentation kinetics of haploids and their respective parental strains.** Fermentations were performed in 300 ml fermenters using the SM. a) L1 screening; b) L7 screening; c) L8 screening; d) L11 screening. Color code: black – parental strain; green – selected haploid with the most similar lag phase to the parental strain; yellow, red and blue – clones with a lag phase too different from the parental strain and therefore not selected.

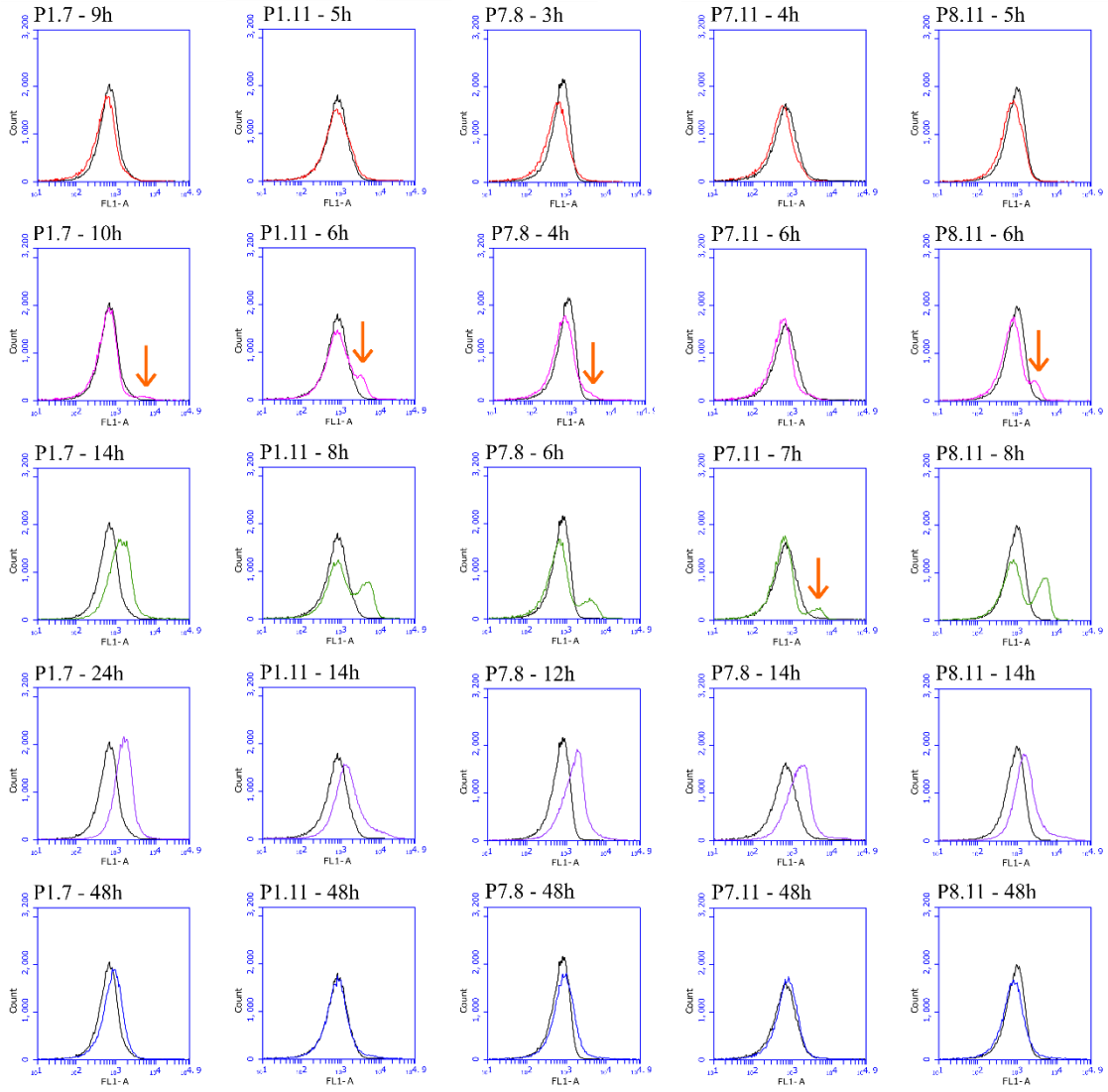



**Figure III.S2. Histograms of GFP signal following over time using the flow cytometry technique.** Each population had a different lag phase duration, moment at which the first signal and therefore first budding cells were detected, indicated by the orange arrow (P1.7 – 10h; P1.11 – 6h; P7.8 – 4h; P7.11 – 7h; P8.11 – 6h). Basal fluorescence (black line) was determined for each population at 0h just after inoculation. Prior to lag phase ending and at 48h, cultivated cells have similar fluorescence to the control.

## Acknowledgements

The authors thank Catherine Tesnière from UMR-SPO and Christophe Duperray from Plateau D'imagerie MRI-DBS for their assistance and contribution for the cell sorting. Also to Caroline Wilde and Jeyadarshan Jeyabalaratnam from Lallemand Inc. for their help in performing the propagation and drying of the populations.

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# CHAPTER 4

## AN EVALUATION OF THE POTENTIAL OF THE NON-CONVENTIONAL YEAST *K. MARXIANUS* IN WINE FERMENTATION

**Chapter's context**

In the scope of the YEASTCELL project and the joint doctoral agreement established for this thesis, a secondment was conducted at UCC under the supervision of Dr John Morrissey. There, *Kluyveromyces marxianus* is the main focus of the research, with applications for both beverage and industrial biotechnology under investigation. In recent years, much novel knowledge has been gathered concerning the metabolism and applications of this yeast but its potential in a wine fermentation context has been poorly assessed. This contrasts with the current demand for new and improved organoleptic properties in wine to which non-conventional yeasts more generally have been contributing significantly. Considering this, we decided to do a first characterization of potential of *K. marxianus* under oenological conditions, this time not focusing on the lag phase but rather on the overall fermentation. As a respiro-fermentative yeast, its capability of performing a full fermentation is reduced, which is the reason why we approached this subject under a co-inoculation with *S. cerevisiae* perspective. The main objective of this chapter was not to perform a full characterization of the fermentative profile of *K. marxianus* but rather to conduct a preliminary study that will point the direction for future deeper studies.

# AN EVALUATION OF THE POTENTIAL OF THE NON-CONVENTIONAL YEAST K. MARXIANUS IN WINE FERMENTATION

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## Abstract

In the last few years, there has been an increasing interest in using non-conventional yeast in mixed cultures with *S. cerevisiae* for improving the organoleptic properties of wines. In this context, *Kluyveromyces marxianus* appears as one non-conventional yeast whose potential in wine fermentation has been poorly characterized despite evidence of a positive aromatic profile. In the present work, we evaluated the relevance of using *K. marxianus* for wine fermentations in mixed inoculation with *S. cerevisiae*. A sequential inoculation was determined as the best modality to achieve an intermediate fermentative kinetics allowing the contribution of this non-conventional yeast while assuring completion of the fermentation. Using this system, we assessed the overall aromatic profile and contribution of *K. marxianus* to volatile aromas.

**Introduction**

*S. cerevisiae* is the yeast of first choice to perform a wine fermentation due to its unmatched performance and reliable aromatic contributions. However, in the recent decades an increasing demand for new and improved organoleptic properties has surfaced and put winemakers on the search for solutions to this challenge. One possibility is to go beyond *S. cerevisiae* and exploit yeast's natural diversity. Different non-conventional yeast such as *Torulaspora, Candida, Hanseniaspora, Zygosaccharomyces, Schizosaccharomyces, Lachancea* and *Pichia* species have been used to give new and unique contributions to wine (Jolly *et al.* 2014). Most can produce specific aromatic compounds including terpenoids, esters, higher alcohols, glycerol, acetaldehyde among other organoleptic contributions that can positively change the classical wine profile (Benito *et al.* 2011; Jolly *et al.* 2014; Gamero *et al.* 2016; Varela 2016a; Mestre Furlani *et al.* 2017). Since these species are usually poor fermenters, a good compromise to take advantage of their positive contributions and still have a reliable fermentative process is to use mixed starter cultures or sequential inoculations with *S. cerevisisae* strains. Different studies have been conducted in this sense and proven that the concept is viable and yields positive results (Anfang *et al.* 2009; Comitini *et al.* 2011; Dashko *et al.* 2015).

To date, *Kluyveromyces marxianus* is a non-conventional yeast that has been poorly exploited for its organoleptic properties, although it is commonly found in spontaneously fermented dairy products. Due to its inherent traits such as rapid growth, thermolerance (up to 52ºC) and lactose utilization this yeast has been used in the production of native enzymes (β-galactosidases, inulinase and pectinases), fermentation of whey permeate and cellulosic material at high temperatures (Guimarães *et al.* 2010; Lane and Morrissey 2010; Yanase *et al.* 2010). There are indications that *K. marxianus* can harbor a positive organoleptic profile. It has been shown that the species is able to produce 2-Phenylethanol (2-PE), along with ethyl acetate and other acetate esters, which can provide fruity and floral fragrances (Wittmann *et al.* 2002; Löser *et al.* 2014; Morrissey *et al.* 2015; Varela *et al.* 2017). Although there have been a few studies that tested *K. marxianus* in an oenological context (Kourkoutas *et al.* 2004; Garavaglia *et al.* 2007), none has completely focused on determining the suitability and behavior of the species under classical wine fermentation conditions.

With this in mind, we present here an evaluation of the potential of *K. marxianus* as a yeast for wine fermentation. For this purpose, we evaluated the fermentative profile of

two *K. marxianus* strains and assessed the best inoculation strategy when sequentially or co-inoculating with a *S. cerevisiae* strain. Additionally, we also determined the main aromatic contribution of one *K. marxianus* strain when inoculated in those different modalities, always comparing with a well know *S. cerevisiae* wine yeast strain.

## Material and methods

### Yeast used

Strains used in this work included a laboratory *K. marxianus* strain K1 (UCC collection) as well as two industrial strains, *K. marxianus* strain K4 and *S. cerevisiae* strains L1, both provided by the company Lallemand SAS. Strains were maintained on YPD plates at 4ºC or stored at -80ºC in 20% glycerol.

### Media

### Yeast culture media

Strains were routinely cultured in standard Yeast Extract Peptone Dextrose (YPD) with 1% bacto yeast extract, 2% bactopeptone, 2 glucose). For solid media 20 g/L of agar were added before autoclaving. The medium was sterilized by autoclaving at 121ºC for 15 min at 1 bar.

### Synthetic must

Fermentation experiments were carried out in synthetic must (SM), which mimics a standard grape juice. SM was prepared as described by Bely *et al.* 1990b with the following modifications: 5 mg/L phytosterols and 0.8 mg/L molecular $SO_2$. SM was stored at -20ºC. Before use, it was steam sterilized for 10 min and cooled to the right temperature.

**Yeast cultivation**

**Pre-cultures and cultures**

Pre-cultures were performed by inoculating a single yeast colony into 5 ml of YPD, left overnight (30ºC, 200 rpm) and refreshed in 20 mL YPD with an initial 0.1 O.D. After 8h, cultures were washed in miliQ steril water used for inoculation.

**Fermentations**

**Flasks**

Fermentations in flasks (250 mL) were performed using 200 mL of SM. $SO_2$ was added just prior to inoculation to a final concentration of 0.8 mg/L of molecular $SO_2$ at pH 3.3. Cells previously washed, were resuspended in the same SM and inoculated to a final 0.1 O.D. For co-inoculation and sequential fermentations, *K. marxianus* strains were inoculated first and either after 12h or 18h, *S. cerevisiae* strain L1 was inoculated in the same fashion. Flasks were kept at a constant agitation of 200 rpm at 30ºC for the duration of the fermentation. Over time, O.D. measurements were performed (UVmini – 1240, Shimadzu) at 600 nm after proper dilution.

**AFM**

Alcoholic fermentations were run using Alcoholic Fermentation Monitors, AFM, (Applikon-Biotechnology) with 500-mL flasks containing 400 mL of SM. $SO_2$ was added just prior to inoculation. Cells previously washed, were resuspended in the same SM and inoculated to a final OD of 0.1. For sequential fermentations, *S. cerevisiae* L1 was added after 12h of *K. marxianus* inoculation. For co-inoculation, both strains were inoculated at 0.05 making a total inoculation at 0.1 O.D. as for the other experiments. Temperature and agitation was kept constant at 30ºC and 200 rpm respectively with the help of the heating/steering bar. The $CO_2$ release rate was followed by the internal AFM $CO_2$ sensor over time.

**Viability assessment**

Samples for strain viability/proportion assessment at 30ºC and 42ºC were taken at 24h and 48h from both flasks and AFM fermentations. At 30ºC both strains can grow however only *K. marxianus* is able to grow at 42ºC, allowing this way the determination of the

species' proportions. Serial dilutions to $10^{-5}$ were done using sterile milliQ $H_2O$. 100 µl of the final dilution were plated on YPD agar petri-dishes and homogenously spread with sterile L-shaped spreaders before incubating at 30ºC and 42ºC for 48h as appropriate. Each condition was done in triplicate and colony-forming units (CGU) were calculated by counting colonies and multiplying by the dilution factor.

**Extraction, identification and quantification of volatile compounds**

Assessment of volatiles produced during fermentation on SM was carried out by the Teagasc Moorepark analytical facility.

1 ml of sample was added to a 20 ml screw capped SPME vial and equilibrated to 40ºC for 10 mins with pulsed agitation of 5 sec at 500 rpm. Samples were analysed in triplicate. Sample introduction was accomplished using a Shimadzu AOC 5000 Autosampler. A single 50/30 µm CarboxenTM/divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used. The SPME fiber was exposed to the headspace above the samples for 20 min at depth of 1 cm at 40°C. The fiber was retracted and injected into the GC inlet and desorbed for 2 min at 250ºC. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60m x 0.32mm x 1.8µm) column using a split/splitless injector in splitless mode. A merlin microseal was used as the septum. The temperature of the column oven was set at 40ºC, held for 5 min, increased at 5ºC/min to 230ºC then increased at 15°C/min to 260°C, yielding at total GC run time of 50 min. The carrier gas was helium held at a constant flow of 1.2ml/min. The detector was a Shimadzu TQ8030 mass spectrometer detector, ran in single quad mode. The ion source temperature was 220 C and the interface temperature was set at 260ºC. The MS mode was electronic ionization (70v) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, a commercial flavour and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound using Kovats index. Retention indices were matched against peer reviewed publications where possible to confirm compound identification. Spectral deconvolution was also performed to confirm identification of compounds using AMDIS. Batch processing of samples was carried out using MetaMS (Wehrens *et al.* 2014). MetaMS is an open-source pipeline for GC-MS-based untargeted

metabolomics. An auto-tune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. A set of external standards was ran at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection was performing within specification.

## Results

### Fermentation in flasks

Based on a previous screening for enzymatic activity and overall stress resistance (data not shown), two *K. marxianus* strains were selected, K1 and K4, a laboratory and industrial strain respectively. Together with L1 (an industrial *S. cerevisiae* wine yeast strain), these were individually submitted to flask fermentation of wine must with O.D. followed until stabilization (Figure IV.1). The synthetic must used represented standard conditions commonly found in winemaking. As expected, L1 (*S. cerevisiae*) performed best, reaching a peak of 40 O.D. after 56h and keeping the highest values across the fermentation. Impressively, one of the *K. marxianus* strains, K4 equally followed L1 until 14h. At this point, the growth rate of K4 slightly slowed down hence the difference that can be observed after 17h (Figure IV.1). Nevertheless, K4 reached similar O.D. values around 32h-48h, where it reached its peak (35 O.D). The *K. marxianus* strain K1 behaved quite differently. It also closely followed the other two strains with exponential growth until 17h (~13 O.D.) but growth stagnated for ~14h at around 14 O.D. Between 32h and 39h, there was another increase in growth until 63h followed by another stabilization (Figure IV.1). After 63h, all strains suffered an O.D. decrease indicating cell death until further stabilization. We then became interested in the behavior of these *K. marxianus* strains in mixed culture. We first tested a sequential inoculation, inoculating first the *K. marxianus* strains and then *S. cerevisiae* L1 at 12h or 18h. The proportion of cells at different time points revealed very different profiles for each *K. marxianus* strain. With *K. marxianus* K1, when inoculating *S. cerevisiae* L1 at 12h, the latter was able to establish and achieve proportions of 34% to 48% in the population (Table IV.1). This was not the case when inoculating *S. cerevisiae* L1 at 18h, where in the first 24h 19% of the population was *S. cerevisiae* L1 decreasing to 8% at 48h.

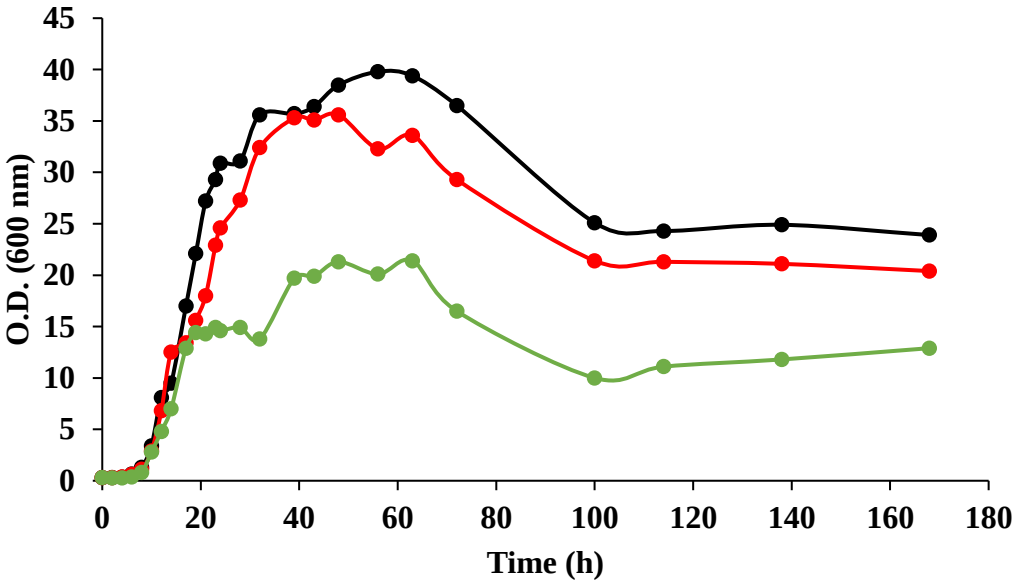



**Figure IV.1.** Growth of *S. cerevisiae* and *K. marxianus* strains in synthetic wine must. **Individual fermentations were performed in flasks at 30ºC. Color code: black – *S. cerevisiae* L1; red – *K. marxianus* K4; green *K. marxianus* K1. The results are representative of duplicates performed.**

In contrast, with *K. marxianus* K4 a balanced population as not achieved in a sequential inoculation experiment (Table IV.1). Both at 12h and 18h of *S. cerevisiae* L1 inoculation, this latter could not get established in the population at any time. (Table IV.1). The strong growth of *K. marxianus* K4, as was apparent in Figure IV.1, meant that *S. cerevisiae* L1 was unable to establish and compete. With this, the decision was made to focus on *K. marxianus* K1. When *K. marxianus* K1 and *S. cerevisiae* L1 were co-inoculated simultaneously, the result was a dominance of L1 throughout the fermentation, ranging from 55% to 71%.

**Table IV.1. Summary of *S. cerevisiae* and *K. marxianus* proportions in mixed cultures.** *K. marxianus* strains and *S. cerevisiae* strain were tested in mixed cultures and the proportion of each species was accessed by platting and incubation at differential temperatures. For sequential inoculation, 12h or 18h were tested as different moments for *S. cervisiae* L1 inoculation. Experiments were performed in triplicate.

| **Sequential** | **12h** | **24h** | **48h** | **18h** | **24h** | **48h** |
|---|---|---|---|---|---|---|
| **K1 proportion** | 100% | 52% | 66% | 100% | 81% | 92% |
| **L1 proportion** | 0% | 48% | 34% | 0% | 19% | 8% |
| **K4 proportion** | 100% | 97% | 96% | 100% | 100% | 100% |
| **L1 proportion** | 0% | 3% | 4% | 0% | 0% | 0% |
| **Co-inoculation** | | | | | | |
| **K1 proportion** | - | 29% | 45% | - | - | - |
| **L1 proportion** | - | 71% | 55% | - | - | - |

### 3.4 Fermentation profile in AFM

Knowing both 12h sequential inoculation and co-inoculation with *K. marxianus* K1 was successful, we took the proof of concept further and ran these fermentations using the AFM instrument, which allowed monitoring of ethanol production (Figure IV.2). As was observed when monitoring growth (Figure IV.1), *S. cerevisiae* L1 was the most efficient fermenter, reaching a high $CO_2$ release rate (10.2 mL/min) at 16h and concluding the fermentation by 65h (Figure IV.2, black line). Fermentation with *K. marxianus* K1 alone had a very different profile (Figure IV.2, green line). Maximum $CO_2$ release only reached 1.7 mL/min (15h). From there, a sluggish fermentation developed, taking too long for a realistic fermentation. As for mixed inoculations, fermentation following of the co-inoculation of *K. marxianus* K1 and *S. cerevisiae L1* (Figure IV.2, purple line) behaved very similarly to *S. cerevisiae* L1 alone. A slightly longer phase (2h) and a slightly lower $V_{max}$ resulted in a longer final fermentation time (78h vs 65h). The sequential inoculation where *S. cerevisiae* L1 was inoculated 12h after *K. marxianus* K1 (Figure IV.2, blue line) yielded an interesting fermentative profile, also with a longer lag phase (3h) when comparing to *S. cerevisiae* L1 and a much lower $V_{max}$ (≈6 mL/min at 25h). Nevertheless, the fermentation carried on and was finished in 90h.

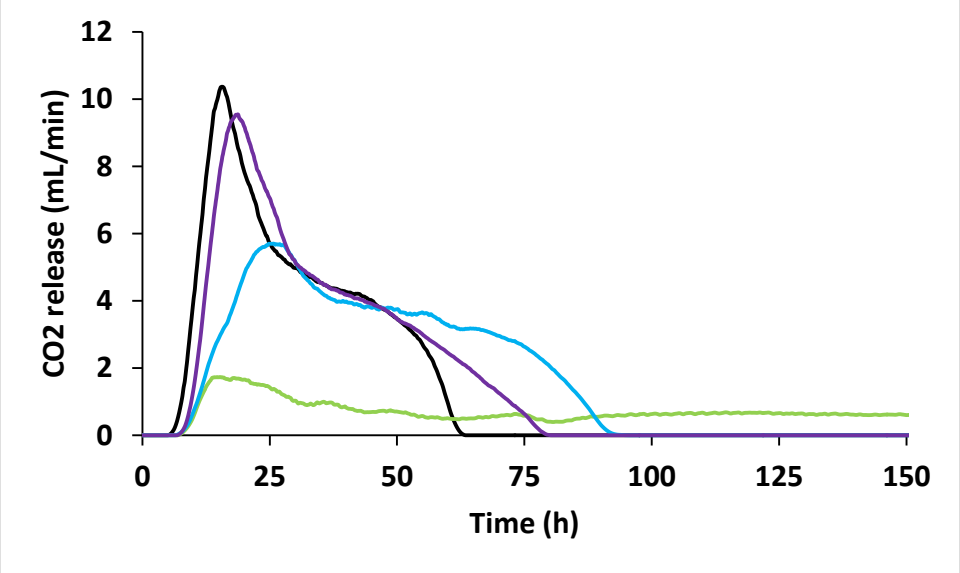



**Figure IV.2. AFM fermentation with $CO_2$ release rate following showing fermentation kinetics of individual and mixed inoculations.** Color code: black – *S. cerevisiae* L1; purple – co-inoculation *K. marxianus* K1 and *S. cerevisiae* L1; blue – Sequential inoculation of *K. marxianus* K1 and *S. cerevisiae* L1 at 12h; green – *K. marxianus* K1. The results are representative of duplicates performed.

**Aromatic profile**

By analyzing the previous fermentation kinetics, we could see that the sequential inoculation of *K. marxianus* K1 with *S. cerevisiae* L1 was the one that allowed the *K. marxianus* strain to have the most significant contribution. With this in mind, we decided to investigate whether there were aromatic contributions from *K. marxianus*. The synthetic must recovered at the end of fermentations (*S. cerevisiae* L1, *K. marxianus* K1 and sequential inoculation of *K. marxiaus* K1 with *S. cerevisiae* L1) was analyzed by gas chromatography mass spectrometry (GC-MS).

A chromatogram elucidating the main compounds produced by each type of inoculation can be seen in Figure IV.3. A first assessment of the main compounds produced in all treatments indicates that the diversity produced in the sequential inoculation is similar to that produced by *S. cerevisiae* L1. When looking at *K. marxianus* K1, a lower diversity of compounds can be observed and those produced are already produced by *S. cerevisiae* L1. This pointed to a minor contribution of *K. marxianus* K1 but the compounds shown here are at high concentrations, which can be misleading as other compounds are also present at lower, but significant levels. A zoom in the chromatograms revealed in fact other compounds that although in lower concentrations, still appear in considerable amounts, potentially impacting the aromatic composition of the wines (data not shown).

To try to understand to which extent these represented a true variation between the fermentations and impacted their composition, we performed a principal component analysis (Figure IV.4A). The immediate and obvious conclusion is that there is clear separation between the three conditions, demonstrating that there is a unique volatile profile in the sequential inoculation. When plotting the individual treatments, we can observe a clear association of each type of inoculation and a distribution along dimension 2 (explains 24.3% of variation). *K. marxianus* K1 appears on the left side clearly in opposition to *S. cerevisiae* L1. Furthermore, the fact that Seq. appear in the middle of this axis shows that these fermentations result from an average of the two strains variations. Concerning dimension 1 (explains 58.9% of the variation), *S. cerevisiae* L1 and *K. marxianus* K1 appear both as equivalent in the upper part of the axis, however there seems to be an additive effect as Seq. fermentations which appear on the inferior part of the axis.

When transposing this distribution in terms of variables, meaning the aromatic compounds responsible for individuals distribution, new insights are possible (Figure IV.4B). The right side of the PCA plot appears populated with many different compounds with high contributions (purple and red) coming from *S. cerevisiae* L1. However, on the left side of dimension 2, few compounds appear as significant. Nonanal, ethyl acetate, isobutyrate and propionate and 1-butanol appear as pleasant aromas ranging from whiskey, orange flower, rose, fruity, grape, and pineapple notes.

However, compounds such as 3-methylbutanoic acid and 3-(methylthio)-1-propanol, which are known for cheesy, sulfurous and onion scents also appear to be produced by *K. marxianus* K1 and are normally considered unpleasant. As for the Seq. fermentations, specific compounds are also at the origin of its separation on the vertical axis. Butyrolactone, 3-(methylthio) propyl acetate, 2-methyl-1-propanol appear as the most variant in these fermentations. Overall, these aromatic compounds represent a mixture of caramellic, spicy, cinnamon and winey notes that can also be pleasant as organoleptic properties.

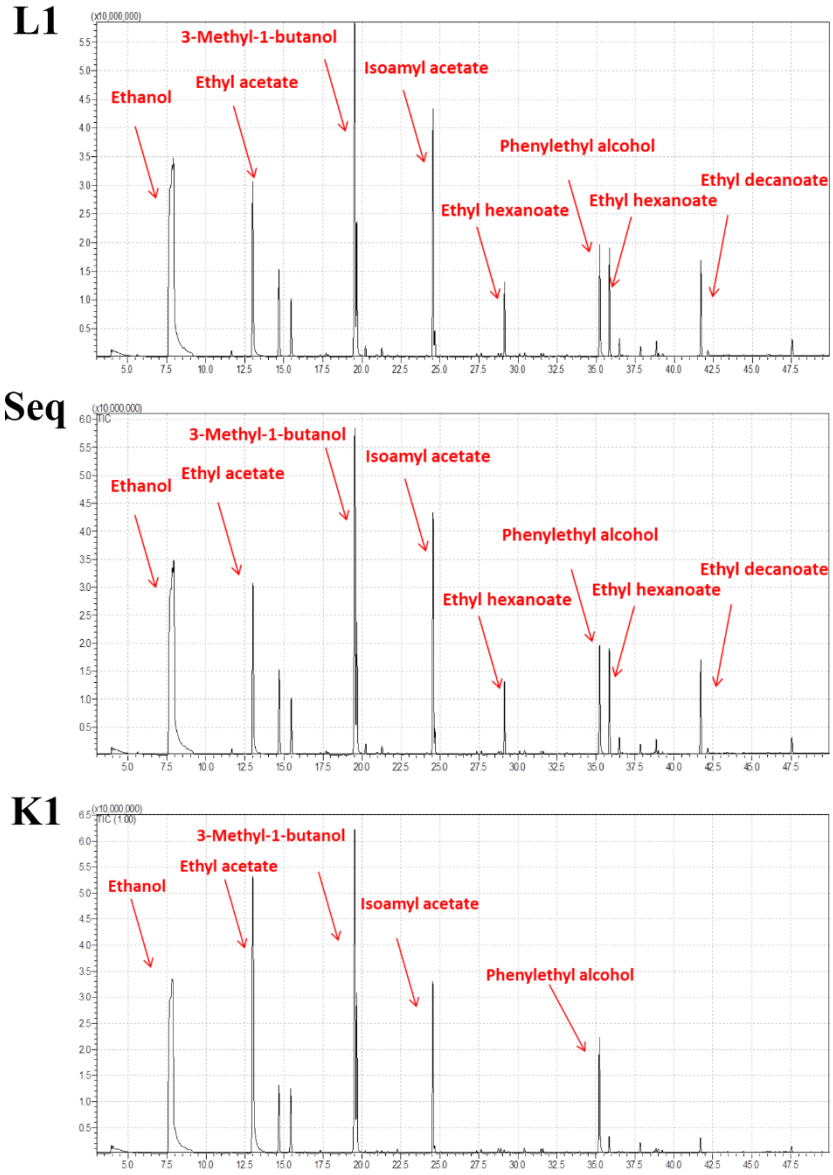



**Figure IV.3. Chromatograms showing the main compounds produce by each inoculation type.**

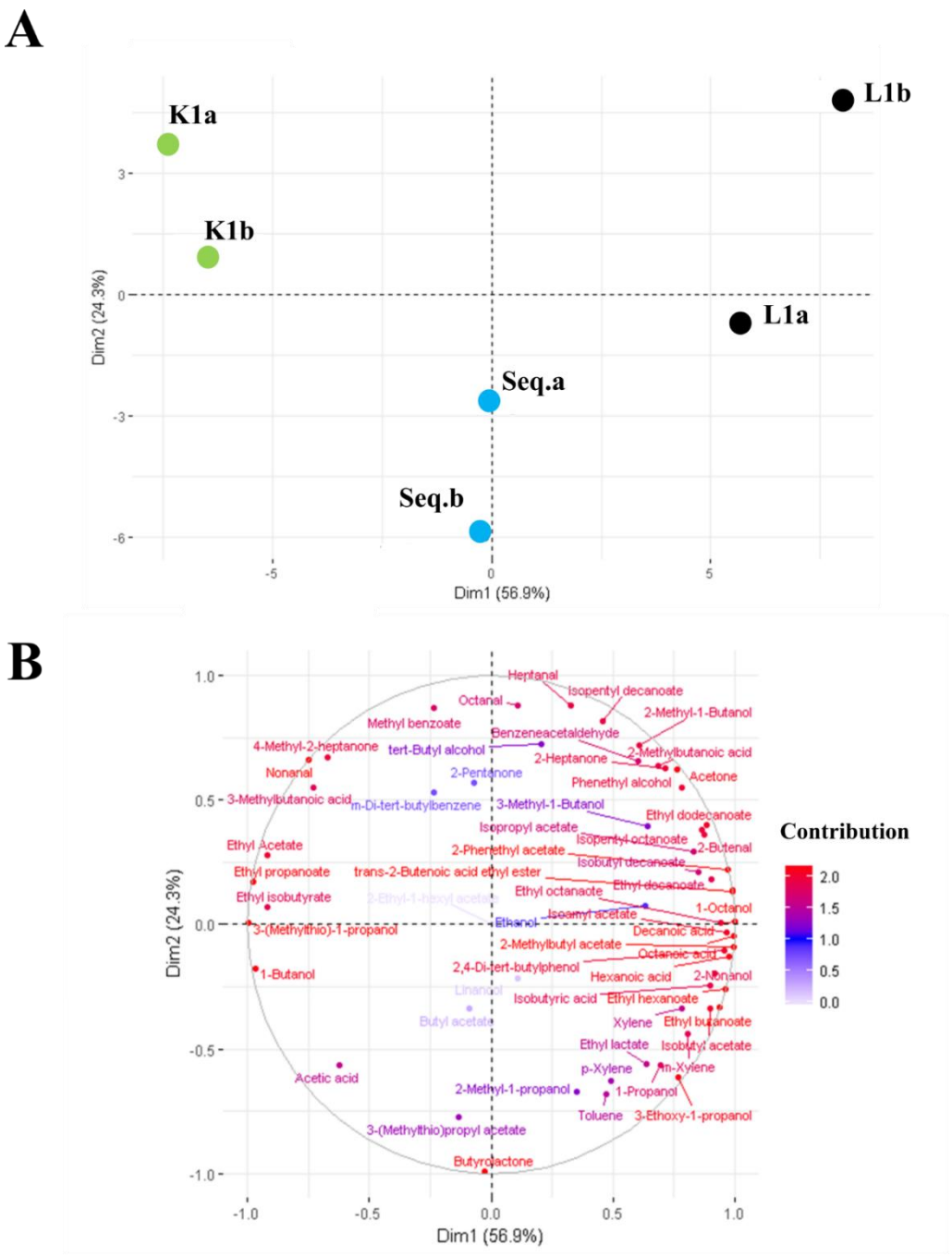



**Figure IV.4. Principal component analysis (PCA) combining the final aromatic composition of the musts concerning *S. cerevisiae* L1, *K. marxianus* K1 and sequential inoculation fermentations.** The top PCA (A) is based on the individual's variation. Duplicates are shown as a and b. "K1" and "L1" correspond to the respective strains. "Seq." corresponds to the sequential inoculation. Bottom PCA (B) is based on variables analysis meaning each compound detected in the must. Significance of their contribution is in function of their position to the outer circle (the closer to it the more significant). A color code helps visualizing this significance (faded – insignificant; blue – moderate contribution; red highly significant contribution).

**Discussion**

This work marks another important step in assessing the potential of *K. marxianus* under oenological conditions. After confirming the inability of this yeast species to properly conduct a full wine fermentation, we assessed the best mixed culture process to allow the *K. marxianus* contribution to develop. When analyzing the aromatic profiles of the individual fermentations, particular compounds appeared as unique to *K. marxianus* and to a mixed fermentation with *S. cerevisiae*.

In this study, we started by testing two different *K. marxianus* strains, K1 and K4, in comparison with a well-known wine *S. cerevisiae* strain, L1. When individually inoculated, *K. marxianus* K1 presented a considerable inferior growth in synthetic wine must than *S. cerevisiae* L1. Since *K. marxianus* is a non-*Saccharomyces* yeast, this was expected as these are widely known for an inferior fermentative capacity (Jolly *et al.* 2014). When checking its behavior in sequential inoculation, however, there was a good balance of *S. cerevisiae* and *K. marxianus* viable cells until 48h, showing the concept of a sequential inoculation for fermentation was a genuine possibility (Gobbi *et al.* 2013). In contrast, *K. marxianus* K4 showed growth equivalent to *S. cerevisiae* L1 in pure culture in synthetic must, not having particular difficulties in conducting the fermentation. In fact, growth was so good that when inoculation of *S. cerevisiae* L1 was delayed to 12h (sequential inoculation), *S. cerevisiae* was not able to establish and only *K. marxianus* was recovered after 48h. In another type of fermentation (agave), it has been demonstrated that *K. marxianus* can produce high amounts of ethanol revealing a good fermentative capacity (López-Alvarez *et al.* 2012). Additionally, the fact that this strain has been industrially selected might justify the superior profile and resistance to difficult conditions. Nevertheless, this kind of profile was still unexpected. This could open a new line of study to access the potential of this strain as a solo fermenting yeast. The different behavior of the two *K. marxianus* strains in our study, K1 and K4, as well as the varying reports in the literature indicate that there is considerable variability in the capacity of *K. marxianus* to carry out alcoholic fermentation and broad generalizations on this topic should be considered with caution.

In the scope of our study, we decided to proceed only with *K. marxianus* K1, since the preliminary experiments suggested the possibility of designing an experimental system to allow the contribution of both *S. cerevisiae* and *K. marxianus* to be assessed.

AFM fermentations allowed a more detailed fermentative profile of our fermentations. *S. cerevisiae* L1, as before, remained the best performing strain. *K. marxianus* pure culture showed to be sluggish (fermentations were stopped after more than 200h as no differences were observed) but this was not surprising as non-*Saccharomyces* yeast can show particular difficulties in carrying out a full fermentation (Andorrà *et al.* 2012). Co-inoculation showed a fermentation profile that provided evidence of an influence of *K. marxianus*. A slightly longer lag phase and lower $V_{max}$ translated into a longer fermentation time. The effect of co-inoculation in wine must of *S. cerevisiae* with varied non-conventional yeast has been assessed in different studies. Andorrà *et al.* 2010 showed that while the presence of non-*Saccharomyces* strains slowed the fermentation rate, maximum total yeast populations were quickly reached, which seems to also be our case. Gobbi *et al.* 2013 using *Lachancea (Kluyveromyces) thermotolerans* and *S. cerevisiae* as an inoculation mixture also found similar results with the co-inoculation reaching lower values of $CO_2$ production as well as a longer time to consume all sugars. In both studies, *S. cerevisiae* strains ended up dominating, particularly towards the end of the fermentation. In our experiments, the impact of *K. marxianus* was even more pronounced in the sequential inoculation when inoculation of *S. cerevisiae* was delayed for 12h. This delay allowed *K. marxianus* K1 establish, although the initial fermentation was slow due to to the inferior fermentative capacity of *K. marxianus* K1. Nevertheless, fermentations were able to be carried out using this sequential inoculation system, which was not always the case in previous studies (Contreras *et al.* 2014).

Since we wanted to assess the potential of a *K. marxianus* aromatic contribution, we selected the sequential inoculation as the best compromise between allowing *K. marxianus* development and achieving a complete fermentation as this reflects what would be required in realistic conditions. Previous studies have confirmed the aromatic potential of non-conventional yeasts in wine fermentation (Comitini *et al.* 2011; Andorrà *et al.* 2012; Sadoudi *et al.* 2012; Gobbi *et al.* 2013). Concerning *K. marxianus* and aromatic profiles, different studies have demonstrated that the species is capable of producing positive and pleasant compounds such as 2-phenylethanol, ethyl acetate, 2,3-butanedione, isoamyl alcohol, 2-phenylethyl acetate and ethyl dodecanoate among other esters and higher alcohols (Wittmann *et al.* 2002; López-Alvarez *et al.* 2012; Löser *et al.* 2014; Morrissey *et al.* 2015; Varela *et al.* 2017). In this study, the pure culture of *S.*

*cerevisiae* clearly showed the highest diversity of aromatic compounds. Nevertheless, *K. marxianus* K1 in pure culture also showed a particular profile and equivalent aromatic compounds with positive notes such as isobutyrate and propionate responsible for fruity and fresh scents were evident. It must be stated however, that these appeared among few less positive aromatic compounds that are usually undesired in wine. Interestingly, all these contributions did not surface when in sequential inoculation with *S. cerevisiae* L1. Here, different compounds appeared as distinct from the respective pure fermentations. Thus, rather than simply being a direct "sum of the parts", there seems to be interactions between the two yeasts that led to a particular aromatic profile. These interactions have been pointed as the cause for either a more intense aromatic profile or for the creation of different combination of scents that in pure cultures would not be obtained (Comitini *et al.* 2011; Andorrà *et al.* 2012; Ciani *et al.* 2016). As some of these new compounds have positive aromatic notes, this leaves room to consider *K. marxianus,* represented here by *K. marxianus* K1, as potential non-conventional yeast for generating improved, or at least, unique, aromatic profiles in wine fermentation. There are potential negatives as well. For example, acetic acid production was also detected as partially explaining sequential inoculation variance. It has been reported previously that increased acetic acid production can be associated with fermentations by different species in synthetic musts (Beltran *et al.* 2008; Andorrà *et al.* 2012). This would be a concern as high concentrations of this compound are undesirable in wine but its true production levels would need to be assessed with fermentations in more realistic conditions, *e.g.* natural must.

From the fermentative profile to the aromatic contributions, many different parameters can influence a mixed inoculated fermentation. This already starts with the strain choice, which as we demonstrated can lead to different fermentative profiles and therefore different impacts on the final wine (Sadoudi *et al.* 2012; Contreras *et al.* 2014). Additionally, inoculum rate, timing of inoculation, type of mixture inoculation (co-inoculation or sequential), interactions between yeasts, and temperature are all factors that can significantly affect how the fermentation develops and the final product obtained (Comitini *et al.* 2011; Andorrà *et al.* 2012; Gobbi *et al.* 2013; Ciani *et al.* 2016). Rather than trying to optimize such parameters, our objective was to solely assess the relevance of using *K. marxianus* as a non-conventional yeast in oenological conditions. We showed that both sequential or co-inoculation with *K. marxianus* K1 yielded good fermentative

profiles that do not compromise the fermentation. On the aromatic contribution aspect, *K. marxianus* K1 produced both positive and negative aromas when in pure inoculations but this changed when in sequential inoculation where positive interactions and contributions to the fermentation were observed. In conclusion, this study established that *K. marxianus* does have potential as a yeast that can contribute with a new dimension to wine fermentation. This was only a first study, however, and it would be necessary to explore this further, considering strain selection and oenological conditions in much more detail.

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# GENERAL CONCLUSIONS AND PERSPECTIVES

The objective of the present thesis was to study and improve wine yeast lag phase in multi-stress conditions during wine fermentation. To achieve this objective we first studied the lag phase diversity and modulation using a set of 10 yeast strains used in winemaking to then move into two parallel and complementary axis. One axis consisted in obtaining an improved strain with a shorter lag phase while determining the genetic modifications leading to such improvement. We found in evolutionary engineering the best approach to provide those outcomes while preserving the non-GMO status of the yeasts used. The other axis concerned achieving a deeper knowledge about the molecular and genetic basis regulating lag phase. Due to the complexity of this trait we constructed an original approach based on a 4-parent intercross QTL mapping strategy combined to cell sorting of propagated population dried in industrial conditions to select the first budding cells. This strategy, specifically designed to unveil alleles with a major influence in lag phase, allowed to take into account a combination of stress factors including propagation, drying and inoculation, that can modulate the lag phase. Additionally, this knowledge had the potential for a later industrial valorization.

Lag phase is a well-known state of any microbial population required for proper adaptation to a new media (Lewis *et al.* 1993; Brejning and Jespersen 2002). In the winemaking context, the molecular changes and gene expression occurring at this stage have even been detailed (Rossignol *et al.* 2006). However, so far, no study had specifically addressed the diversity of lag phase among wine yeast strains as well as the factors regulating this diversity. In particular, little was also known about the relative impact of the environmental factors typically present at a fermentation start and their interaction in a multi-stress context. Chapter 1 came together to help answering these questions while providing fundamental information for the further work intended.

We first determined that the set of strains selected was phylogenetically diverse providing then a good starting point for lag phase characterization. By designing a factorial plan we could evaluate the individual and combined effect of different stress factors. The strain diversity was further confirmed when, overall, lag phase ranged from 10h to 70h.

When dissecting each stress factor we concluded that temperature and osmotic stress were the two most impacting factors. On average, temperature was responsible for an increment of almost 24h when going from 28ºC to 16ºC. Osmotic stress on its turn significantly impacted all strains ($p \leq 0.001$ and $p \leq 0.01$ for osmotic stress) considerably increasing lag phase time as well. In addition to the impact of these stress factors well established (Llauradó *et al.* 2002; Tamás and Hohmann 2003; Beltran *et al.* 2008), we further confirmed that in such multi-stress context, they are two of the stress factors that can promote extended lag phases the most and therefore should be closely monitored when in real fermentation. $SO_2$ came next significantly ($p \leq 0.001$ and $p \leq 0.05$), impacting 6 out of 10 strains when at 28ºC and 4 out of 10 at 16ºC. We hypothesized that this temperature effect was certainly related to $SO_2$ binding power being increased at lower temperatures and therefore the active portion is diminished (Ribéreau-Gayon *et al.* 2006). In agreement with Pérez-Ortín *et al.* 2002 and Zimmer *et al.* 2014 we did find the presence of the translocation $XVI^{VIII}$ quite disseminated among our strains (6 out of 8) but not the translocation $XVI^{XV}$ (0 out of 8). However, contrary to described by Goto-Yamamoto *et al.* (1998), the translocation presence was not consistent in providing an advantage under $SO_2$ stress. Strains that did not have the translocation were slightly impacted (L10 and L4) whereas strains that did were both not affected (L6 and L8) and affected (L1, L3, L7, and L11) which made us hypothesize that rather than the translocation itself, it is the basal *SSU1* transcription that mainly contributes for the resistance (Nadai *et al.* 2016). In this focus, the strain L5 (*T. delbrueckii*) showed a particular sensitivity to $SO_2$. With regard to low phytosterols and thiamine, their impact was punctual and strain specific which corroborates their importance but in a more strain dependent manner.

From a strain point a view, it was curious to observe that the strains consistently with a shorter lag phase, L3, L8 and L11, were sometimes amongst the most impacted by the different stress factors. On the opposite, strains like L5 and L1 under osmotic stress for instance, appeared among the slowest strains but the least affected. This clearly showed

that despite stress factors playing an important role in lag phase modulation, other intrinsic features independent of stress resistance regulate this phenotype. From a species point of view *S. cerevisiae* strains showed to be, overall, the most performant as expected under the particular stressful conditions of winemaking (Matallana and Aranda 2016). For the two hybrids and *S. uvarum* we were expecting a particular advantage under low temperature conditions (Tronchoni *et al.* 2009; Masneuf-Pomarède *et al.* 2010), however only one hybrid, L6, showed cryophilic advantages.

Finally, no particular impacting interactions between factors and transversal to all strains were detected showing that in the particular multi-stress scenario of wine must inoculation, each stress factor has its major influence on its own.

Although the sampling was limited to 10 strains, chapter one shed new light into a subject that had been poorly exploited so far. Aside from the diversity of lag phase profile, we also determined the weight of what we thought being the most important factors influencing lag phase. These included not only the stress factors themselves but also intrinsic yeast features such as $SO_2$ resistance translocation, genetic background and strain dependency. Additionally, we also gathered important information that allowed us to move on into two parallel approaches, chapter 2 and 3, an evolutionary engineering and QTL approach respectively.

For chapter 2, the goal was to perform an evolutionary engineering approach (also called ALE) aiming for a reduction in lag phase duration when in oenological conditions. Since stress factors are one of main constraints negatively modulating lag phase, we decided to use as selective pressures the same stress factors studied in chapter 1, representative of red wine fermentation. Osmotic stress, $SO_2$ and thiamine were selected as the three main stress factors. Our strain choice mainly relied on 3 criteria: presence of translocation $XVI^{VIII}$ to avoid an evolution too directed on $SO_2$ resistance; a slow lag phase (determined in chapter 1) so more easily an improvement could be achieved; and commercial interest by the company Lallemand SAS, since in the case of a successful evolution, a possible commercialization would be considered. Strain L7 was selected for an ALE in red wine conditions. As an outcome, an evolution in lag phase reduction was obtained with a maximum of 4h lag phase reduction when in synthetic must. Interestingly,

this phenotype was obtained for two (R2 and R3) out three populations where the other exhibited null evolution (R1). This clearly shows that identical but independent ALE can lead to different phenotypes, most likely due to the accumulation of different mutations in different alleles. This phenotype was obtained at the 100$^{th}$ generation and improved until the 200$^{th}$ generation even though the experiment was conducted for 500 generations. From 200 to 500 generations no bigger improvement than the one already obtained was observed. This is in agreement with many ALE approaches where the most important mutations for the phenotype of interest occur at the beginning of the adaptation. From there, the tendency is the accumulation of additional mutations that usually have little or no effect in improving even further the evolved phenotype. In fact, as these accumulate randomly in the genome the tendency is to modify (more often negatively) other phenotypes unrelated to the ALE objective. This was exemplified with R2A in our strain screening when performing the drop tests. This strain clearly showed deficiencies concerning other phenotypes most likely due to the posteriori accumulation of genetic modifications after lag phase evolution.

From an applied point of view, we did preliminary assays where we first propagated and dried the evolved strains to ensure their endurance to these process. Next, we used a natural grape must to perform fermentations and we found a reduction in lag phase duration of 3h of the same magnitude order than in synthetic must (4h). On the other hand, a higher Vmax as well as shorter fermentation time were observed in grape must but not in synthetic must. Differences between real and synthetic musts' matrix as well as different stress levels may have contributed to mask/reveal the differences found in these phenotypes. Further conditions and matrixes should be tested to characterize in detail the potential of these strains in various winemaking conditions.

Sequencing data revealed a general loss of heterozygosity (LOH) in chromosome 9, common to 5 out of 6 clones. Although LOH has been reported as a mechanisms of evolution, the fact that R3C did not present any LOH but came from the same population as R3F and R3I, points to a genetic modification outside of this region, since all 3 clones presented equivalent lag phase profile. Information about *de novo* mutations potentially

contributing to the improved phenotype point to candidates mostly responsible for cell wall integrity and maintenance. The fact that R2 and R3 presented both candidates implicated in cell wall reinforces the possible cell wall role.

In chapter 3 we developed a bulked segregant analysis (BSA) approach in order to study the genetic and molecular basis behind lag phase modulation. The BSA approach was first described by Michelmore et al, 1991 and the biggest advantage over a traditional QTL mapping approach is that there is no need for genotyping and phenotyping each of dozens or hundreds of individuals in a segregating population. More recently, this technique has been taken further by using intercrosses between several parental strains and increasing QTL mapping resolution (Cubillos *et al.* 2017; Roberts *et al.* 2017).

The novelty in our work relied on combining this type of approach, multi-parent BSA, together with an industrial propagation and drying, followed by a cell sorting based on FACS. This technique is not only highly precise in sorting the cells with the selected criteria but also allows collecting a high number of cells that correspond to those very same criteria. In our case, this allowed the isolation of the very first cells that ended lag phase and started multiplying, containing a specific allelic enrichment that led to that phenotype. Having included the propagation and drying in our approach made us be even closer to the realistic conditions in which a lag phase takes place. Being processes with major consequences in yeast cellular architecture and metabolism, their impact is certainly present during lag phase. This can be easily supported by the fact that yeast manufacturers strongly advise following a proper rehydration protocol before inoculation to avoid defective fermentation starts. The rehydration period is in fact what allows yeast to recover from the stressful drying process (Fugelsang and Edwards 2007). Its efficiency will be directly dependent on how the drying was performed but also in yeasts natural resilience, quite strain dependent, all factors that will influence lag phase profile (Soubeyrand *et al.* 2006; Krieger-Weber 2009).

As with chapter 2, our strain choice relied on information obtained in chapter 1. Among the criteria followed, the most important was the selection of strains with contrasting phenotypes, meaning lag phase durations. This way we ensured that alleles responsible for the extremes would be present in our pool. The way how we engendered

their identification had to do with our decision of keeping each population separated as well as their analysis rather than pooling all in one bulk. If one of the strains would contribute with an extremely positive allele for shorter lag phase, we would expect that this would be retained in every sorted population independently of the allele provided by the second parent. On the contrary, an unfavorable allele would always be "chased away" independently of the population. The peaks we obtained were shared by 2 different populations (and never by 3) which revealed that no "super" allele existed but nevertheless allow to apply this strategy. Of the 3 common peaks, 2 shared by populations P1.7 and P7.11 and 1 by populations P1.11 and P7.11, we could see that alleles coming from parent L7αGFP were never retained. This is in agreement with the inferior performance that the strain always showed. On the other hand, every time the parent L11aGFP or L11αGFP was present it was always retained over L7αGFP and L1aGFP, also in agreement with a faster lag phase that the strain always showed. Ultimately, this helped validating our design as well as the alleles revealed by the QTL peaks. As for the single QTLs, we decided to consider them, particularly those with a highest LOD score as their contribution might as well be considerable. Like in the shared ones, the allele of the most performing parent, L11aGFP or L8aGFP was kept over a least performing one, L7αGFP.

The genes identified in these peaks are in agreement with our premise that lag phase is a complex trait influenced by different biological functions. Cell growth and budding, glucose sensing and transport and cell integrity and stress are all functions that we were able to identify and understandably influence lag phase. When establishing a parallel between results obtained in chapter 2 and 3, it was interesting to verify that some of these functions were shared between the two approaches. The genes identified in chapter 2, such as *DSE2* and *SLT2*, were mainly connected to cell wall integrity and maintenance. On chapter 3, *BCK1* appeared as an important gene regulating cell wall biogenesis and maintenance (Jung and Levin 1999), further reinforcing the important role that this cellular component has in lag phase. Cell cycle, directly related to multiplication, was also commonly found between the two chapters and revealed by genes such *AZF1* and *RAS2* which can provide an important indication on where focusing further research in this matter. Finally, glucose sensing and transport are likely to also play a major role in lag phase. Recently, Ziv *et al.* 2017 identified *HXT7* as a high affinity glucose with an impact in growth and lag phase, both in low and high glucose. It is interesting to see that

in chapter 3 we also identified glucose transporters, namely *HXT3* and *RGT2*, directly responsible for activating *HXT1* (Ozcan *et al.* 1996). Both of these are low affinity glucose transports which would be in agreement with lag phase context. For further conclusions, the validation and extent of these functions and genes associated has now to be determined.

In Chapter 4, we evaluated the potential of using *K. marxianus* and *S. cerevisiae* in mixed cultures under oenological conditons. For this, the fermentative profile of two *K. marxanius* strains, K1 and K4, a laboratory strain and an industrial strain respectively, was studied in mixed fermentations *S. cerevisiae*. The fact that both strains showed very different growth and fermentative profiles indicates a wide strain diversity within the species, that can certainly be exploited. *K. marxianus* K4 showed a high growth rate (almost as high as *S. cerevisiae*) which in turn made the use of mixed cultures impractical by precluding *S. cerevisiae* strain to establish during fermentation. On the other hand, *K. marxianus* K1 revealed a behavior more typical of non-conventional yeasts by presenting a less important growth and leading to sluggish fermentations. However, when mixed cultures, sequential and co-inoculation, *S. cerevisiae* strain was able to grow and ferment while *K. marxianus* K1 remained present, up to 66% after 48h of sequential inoculation. When interested in evaluating the aromatic profile of this particular strain, we found out that both positive and negative aromas were produced in pure inoculations. This was different when in sequential inoculations, where rather positive and unique aromas surfaced as significant. This indicated a possible interaction between both strains leading to this aromatic profile, not possible by solo fermentations of both strains. As a first proof of concept in using *K. marxianus* as a non-conventional yeast under oenological conditions, this chapter opened promising perspectives for a more in depth characterization of *K. marxianus* in mixed cultures.

The work conducted during this thesis represents a valuable addition to the current knowledge on lag phase in winemaking context, a subject that so far had not been extensively addressed. The information that resulted from it opens a wide range of new questions and possibilities to be investigated. We successfully characterized the lag phase

of a set of strains when submitted to the most typical stress factors present at that moment. This set was composed of 10 strains. For a more complete overview of lag phase response a wider range of wine yeast strains could be used. Not only this information will help better understanding lag phase modulation but also yeast producers and wine makers will know, beforehand, how the strain they are selecting will behave. This behavior is of course, always dependent on the conditions where the yeast is inoculated. In our work we tested the stress factors we considered the most impacting, many times pushed to extreme stress levels. However, a wider range of stress levels in their different combinations would be interesting to test and assess this phenotype evolution across different stress levels. Moreover, the impact of additional stress factors that we did not focused on, such as pH or interaction with natural flora, could also be integrated in new studies for a more complete information. Such experiments will certainly require a high throughput method to test numerous stress combinations, however, the work developed with microplates during this thesis showed that it can be used as a reliable method to study lag phase.

As an immediate future perspective, there is the phenotypic validation of the alleles identified both by the ALE and QTL approaches. This will be done by obtaining hemizygous strains for each allele and characterize their lag phase. With a proper validation of these alleles, new perspectives will open in terms of industrial applications. By means of non-GMO methods (*e.g.* back-crossing) these alleles can be introduced in already commercialized wine yeast strains and improve their lag phase. Virtually, this could considerably reduce the problems of stuck/sluggish fermentations due to extended lag phases and ultimately reduce wine spoilage and extra costs within this context.

In addition to many other successful ALEs in a winemaking context, we showed once more that very positive outcomes can be obtained when evolving wine yeast strains, in this case under the lag phase problematic. Stress factors showed to play a major role in this evolution which opens the possibility to try different combinations of those and further improve lag phase. Additionally, other stress factors could be used to improve yeast performance this time at later stages of the fermentation such as the stationary phase with the appearance of ethanol and nutrient starvation. Finally, the evolve strain, R2E,

was propagated and dried in a scaled-up process and is currently being tested in experimental wine cellars giving very positive indications. If confirmed its suitability, the strain will likely hit the market in a near future.

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**Abstract**: *Saccharomyces cerevisiae* has been used for millennia to perform wine fermentation due to its endurance and unmatched qualities and is nowadays widely used as wine yeast starter. Nevertheless, at the moment of inoculation, wine yeasts must cope with specific stress factors that can compromise the fermentation start. The objective of this work was to elucidate the metabolic and molecular bases of multi-stress resistance during wine fermentation lag phase. We first characterized a set of commercialized wine yeast strains by focusing on stress factors typically found at this stage in red wines and in white wines. Temperature and osmotic stress had a drastic impact in lag phase for all strains whereas $SO_2$, low lipids and thiamine had a more strain dependent effect. Based on these data, we developed two parallel approaches. Using an evolutionary engineering approach where selective pressures typically present in lag phase were applied, we obtained evolved strains with a shorter lag phase in winemaking conditions. Whole genome sequencing allowed to identify several *de novo* mutations potentially involved in the evolved phenotype. In parallel, a QTL mapping approach was conducted, combining an intercross strategy, industrial propagation and drying of the progeny populations and selection of the first budding cells by FACS. Both strategies allowed the identification of several allelic variants involved in cell wall, glucose transport, cell cycle and stress resistance, as important in lag phase phenotype. Overall, these results provide a deeper knowledge of the diversity and the genetic bases of yeast adaptation to wine fermentation lag phase and a framework for improving yeast lag phase. Additionally, we showed that *K. marxianus* has potential for mixed cultures and positive aromatic contributions under oenological conditions, opening new possibilities for further studies.

**Title**: Stress resistance during the lag phase of wine fermentation and development of optimized yeasts

**Keywords**: Wine fermentation, yeast, lag phase, multi-stress resistance, QTL, adaptive evolution, *K. marxianus*

**Résumé** : *Saccharomyces cerevisiae*, utilisée depuis des millénaires pour la fermentation du vin du fait de son endurance et de ses qualités inégalables, est de nos jours largement utilisée pour inoculer les mouts de raisin. Néanmoins, lors de l'inoculation, les souches œnologiques doivent faire face à des stress spécifiques qui peuvent compromettre le début de la fermentation. L'objectif de ce travail est d'élucider les bases métaboliques et moléculaires de la résistance multi-stress pendant la phase de latence en conditions œnologiques. Nous avons tout d'abord caractérisé un ensemble de levures œnologiques en mettant l'accent sur des facteurs de stress caractéristiques des vins rouges et des vins blancs. La température et le stress osmotique affectent fortement cette phase pour toutes les souches, alors que le $SO_2$, les lipides et la thiamine ont un effet souche-dépendant. Ces données ont servi de base à deux approches parallèles. Une approche d'évolution expérimentale a permis, en appliquant des pressions sélectives caractéristiques de la phase de latence, de sélectionner des souches évoluées présentant une phase de latence plus courte. Plusieurs mutations *de novo* potentiellement impliquées dans le phénotype évolué ont été identifiées par séquençage de leur génome. En parallèle, une approche QTL combinant des croisements inter-souches, une étape de propagation industrielle et séchage des descendants, et la sélection de cellules bourgeonnantes par FACS a été développée. Ces deux stratégies ont permis d'identifier plusieurs variants alléliques impliqués dans la paroi cellulaire, le transport du glucose, le cycle cellulaire et la résistance au stress, jouant un rôle potentiellement important pendant la phase de latence. L'ensemble de ces résultats apporte de nouvelles connaissances sur la diversité et les bases génétiques de l'adaptation des levures à la phase de latence œnologique et offre un cadre d'amélioration des propriétés des souches. De plus, nous avons montré que *K. marxianus* a un potentiel pour des cultures mixtes et des contributions aromatiques positives en conditions œnologiques, ouvrant de nouvelles possibilités pour des études ultérieures.

**Titre** : Résistance au stress lors de la phase de latence en fermentation œnologique et développement de levures optimisées

**Mots clés** : Fermentation œnologique, levure, phase de latence, résistance multi-stress, QTL, évolution adaptative, *K. marxianus*