Yeast Strain Construction Using CRISPR/Cas9: A Tool for Research and Strain Optimisation

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B.Sc (Hons)

A thesis submitted for the degree of

Doctor of Philosophy.

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March 2021

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Abstract

Saccharomyces cerevisiae is well known as the workhorse of many fermented beverage industries underpinned by its strong capability to create alcohol and sensory metabolites through the fermentation of sugars. Whilst *S. cerevisiae* is generally reliable, there is a drive to improve characteristics of available strains. The production of new and improved strains has been reliant on time-consuming techniques considered non-recombinant, in order to avoid their designation as genetically modified organisms (GMOs). While genetic engineering presents a more targeted and reliable approach, in many jurisdictions the use of such techniques is restricted. The recent emergence of CRISPR/Cas9, however, may change this, with some countries classifying CRISPR-edited strains as non-GMO when used in certain circumstances.

This study investigated the use of CRISPR/Cas9 to generate improved strains of the commercial *Saccharomyces* wine strain, Lalvin EC1118. The effects of previously reported gene disruptions, as well as novel QTLs and SNPs derived from other yeast backgrounds were used as case studies. This study also evaluated the potential of generating gene disruptions via the 'error prone' non-homologous end joining (NHEJ) repair pathway.

First, CRISPR/Cas9 was used to produce a disruption mutation in *ECM33*, by introducing a premature stop codon (Gly61stop). Previously, deletion of the *ECM33* open reading frame lead to decreased fermentation duration in a haploid wine yeast derivative. In the present study this mutation disrupted gene function and improved fermentation performance as before. However, homozygous disruption in EC1118, lead to the discovery of another novel phenotype, cellular aggregation, which was masked by the typical flocculation of previously used haploid yeasts.

Second, novel QTLs and SNPs were introduced, a method considered non-GMO in the United States and Japan. Two different phenotypes were chosen, slower growth associated with a loss of function QTL in *SER1* derived from a sake yeast (Ser1p G78R), and proline accumulation by mutations identified from a chemical mutagenesis screening of baking yeast (Pro1p I150T, P247S and E415K). In co-inoculated fermentations, the slowed growth phenotype allowed for non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* or *Lachancea thermotolerans*) to begin fermentation, with the slow growing *S. cerevisiae* eventually completing fermentation. An additional use of a slow growing *S. cerevisiae* was identified, *i.e.*, sequestering sulfite, enabling the growth of SO_2 sensitive *Lachancea thermotolerans* in mixed culture in sulfured juice. Proline accumulation in yeast is associated with resistance to baking related stresses but the effects in wine yeast are unknown. EC1118 with these mutations similarly accumulated more proline, although there was no obvious improved tolerance towards ethanol or SO_2, with minor resistance to osmotic stress observed.

Finally, NHEJ was investigated with a view to developing non-GMO mutants relevant for Australian regulations. Targeting of the *CAN1* gene (encoding arginine permease) to reduce urea production resulted in two different frame-shift mutants. In synthetic grape must with arginine as sole nitrogen source, the mutants grew slower and reduced urea as expected. However, in Chardonnay juice, the mutants behaved as the unmodified EC1118.

This research demonstrates the applicability of CRISPR/Cas9 to industrial yeast strain modification and highlights the unbound possibilities for mutant strain production.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Tom Lan

Signature

Date: 30/3/21

Acknowledgements

First and foremost, I would like to express my sincere thanks to my principal supervisor Professor Vladimir Jiranek. Thanks for providing me with the opportunity to do this project and your guidance through it, be it in the experimental design, interpretation of results, offering advice and helping with writing. To my co-supervisor, Dr Michelle Walker, thank you so much for your help with everything, be it helping in the lab, providing support and encouragement, reading through numerous drafts and the occasional catchup over a cuppa. Without both of you I do not know where this project would have gone.

I would also like to extend my thanks to everyone in the Wine Microbiology Lab. Thank you all for being a lovely and fun bunch to work with and for putting up with me over the years. Special thanks must go to Nick van Holst, the man with all the answers. Thanks for putting up with all my questions and helping in the lab, as well as your willingness to catch up over a beer and for the near weekly lunches.

All of this work would not have been possible with out the help of many people around the world. I would like to thank Dr Paul Boss, Emily Nicholson and Sue Maffei from the CSIRO for assistance with the wine volatile compound analysis, Dr Gwen Mayo from Adelaide Microscopy for assistance with the confocal microscopy, Professor Hiroshi Takagi from NAIST for assisting with the amino acid measurements and Dr Tom Ellis and Dr Will Shaw for providing their CRISPR/Cas9 plasmids.

Last, but certainly not least, I would like to thank all my friends and family. Thank you all so much for your help, support, and encouragement through what feels like a lifetime of doing this doctorate. You are all wonderful people.

Chapter 1: Literature Review

“Genome engineering and editing in *Saccharomyces* yeast used in fermented beverage production: Now and tomorrow”

Preface

As part of candidature a literature review is written in the first 6 months (August 2016) to produce a background of the research area and establish the aims of the thesis. At this time CRISPR/Cas9 was relatively new with regard to producing industry relevant strains, with minimal published research focusing on strain research and development for industrial application. As such, the focus of this research was to adapt the CRISPR/Cas9 technology used in fundamental research (with laboratory yeast) and apply them to an industrial setting, i.e. new strains for wine production. As the research regarding the implementation of CRISPR/Cas9 in yeast strain improvement has evolved during the subsequent 3.5 years of candidate, the original literature has been updated to reflect this.

This review highlights the role of CRISPR/Cas9 in yeast research and development in key fermented beverage industries, the current legislative regulations regarding the use of GMOs for beverage production and discusses future directions of research in this field. The development of CRISPR-edited wine yeast being the goal of this thesis. The review is a critique of the published literature up until November 2020, with specific regard to the use of CRISPR/Cas9 in research related to fermented beverages.

Introduction

Yeast are responsible for the production of many beverages consumed globally, such as beer (ales and lager) and wine (including rice and sake). *Saccharomyces cerevisiae* is the primary yeast used in alcoholic beverage production, but not the only fermentative yeast used. *Saccharomyces pastorianus*, an interspecific hybrid of *S. cerevisiae* and *S. eubayanus*, is associated with lager beers. Non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii* and *Lachancea thermotolerans* are emergent yeasts in winemaking (Gobbi *et al*., 2013, Renault *et al*., 2009, van Breda *et al*., 2013) and brewing (Canonico *et al*., 2016, Domizio *et al*., 2016, Zdaniewicz *et al*., 2020), where they are used in co-fermentations to provide additional complexity to these beverages.

These fermentations rely on the successful implantation of yeast starter cultures into a sugar rich medium (grape must, wort, etc), and timely conversion of hexose sugars to ethanol and carbon dioxide, together with a plethora of yeast-derived metabolites, characteristic of the beverage. Overall fermentation efficiency relies on the ability of the inoculum to adapt to stress factors such as in winemaking: initial sugar concentration, low pH and increasing ethanol content and nutrient limitation with fermentation progression (Bisson 1999, Monteiro and Bisson 1991). Paradoxically, possession of these traits does not necessarily correlate to a superior product, as the production of aroma-related compounds may both negatively and positively influence the final sensory outcomes. As the quality of the beverage is paramount, most studies have targeted the metabolic pathways linked to aroma compound formation. For instance, work has centred on the reduction of "off flavours" such as dimethyl trisulfide in sake (Ikeda *et al*., 2018) or diacetyl in beer (Gibson *et al*., 2018, Kusunoki and Ogata 2012, Wang *et al*., 2008) or to increase positive aroma compounds such as isoamyl acetate (a Ginjo flavour

component in sake (Asano *et al*., 1999, Hirosawa *et al*., 2004)) or 2-phenylethanol and 2-phenylethyl acetate (rose-petal aroma) in wine (Cordente *et al*., 2018). In many of these industrial processes, there is a drive to find and create new yeast strains either more adapted to and therefore more efficient in their respective environments, or capable of producing new and interesting flavours and aromas in the final product.

Recent strain improvement has been reliant on non-recombinant paradigms such as bioprospecting of "wild" *Saccharomyces spp.* and non-*Saccharomyces* isolates with desirable and/or novel attributes (reviewed for beer by Cubillos *et al*., 2019, wine by Alperstein *et al*., 2020 and sake by Abe *et al*., 2019). Empirical approaches to generating yeast with desirable phenotypes include chemical mutagenesis (Abe *et al*., 2019, Cordente *et al*., 2009, Long *et al*., 2018, Ohashi *et al*., 2020). More recently strategies include evolutionary engineering (Gibson *et al*., 2018, Kayacan *et al*., 2020, McBryde *et al*., 2006, Mezzetti *et al*., 2017) and marker assisted breeding (Dufour *et al*., 2013, Jubany *et al*., 2008, Marullo *et al*., 2009; Marullo *et al*., 2007). However, these methods can be laborious, involving timely screening to select desirable strains. An alternative is to use recombinant DNA technologies, targeting genes of interest to achieve a desired phenotype, thereby removing the randomness and unpredictability of success associated with these non-recombinant strategies.

Recombinant technology in laboratory *S. cerevisiae* is commonplace, with the techniques applicable in industrial yeasts for studying and improving fermentation properties. These techniques can either introduce targeted gene deletions, using dominant selection markers (e.g. antibiotic resistance) or recessive (auxotrophic) markers, or produce new/improved metabolic enzymes via plasmid expression systems (Steensels *et al*., 2014). Whilst these methods may play a critical role in advancing our understanding of yeast metabolism, depending on geographical location, they may or may not be

commercially viable due to legislation and guidelines limiting their application or requiring mandatory labelling as GMO (reviewed in Hanlon and Sewalt 2020). Some countries such as the United States have relaxed guidelines, allowing for commercial sale and use of GM strains, such as Lallemand's Sourvisiae®; a *S. cerevisiae* strain capable of producing high levels of lactic acid for sour beer production. Conversely, the European Union tends to have a stricter approach with the use and labelling of foods produced with GMOs. The use of genome editing with programmable nucleases is being considered as an alternative to these 'first-generation' recombinant approaches. Yeasts modified by these 'second-generation' editing techniques in some instances, require minimal regulation in countries such as the United States, whilst in Australia legislation regulates their use dependent upon the mode of construction.

When discussing genome editing with programmable nucleases there are 3 main systems that have been developed, zinc finger nucleases (ZFNs) (Kim *et al*., 1996), transcription activator-like effector nucleases (TALENs) (Cermak *et al*., 2011) and clustered regularly interspaced short palindromic repeats (CRISPR) coupled with a CRISPR associated nuclease (Cas) (CRISPR/Cas) (Jinek *et al*., 2012). Of these methods, the Nobel Prize winning CRISPR/Cas9 is generally the most utilised due to its simplicity, high specificity and high efficiency. Briefly, CRISPR/Cas9 utilises a variable RNA sequence to guide a nuclease to a genomic location and cause a double stranded break (DSB) in the DNA (Jinek *et al*., 2012). When this is applied to genomic DNA *in vivo*, genomic variation can occur by one of host organism's DNA repair mechanisms, namely non-homologous end joining (NHEJ), where random insertions or deletions (indels) can occur, or homology directed repair (HDR), where specific DNA with homologous sequences can be incorporated (Cong *et al*., 2013). CRISPR/Cas9 allows for

multiplexing, or the simultaneous targeting and modification of multiple loci, which is useful in terms of altering gene pathways (Cong *et al*., 2013).

The last decade has seen the development of techniques for the use of CRISPR/Cas9 predominantly in *S. cerevisiae*, and to a lesser extent in non-*Saccharomyces* yeasts. CRISPR/Cas9 systems in *S. cerevisiae* are based on HDR, the dominant repair mechanism, with alternative of NHEJ being an extremely rare event (DiCarlo *et al*., 2013). NHEJ-based methods offer potential in strain construction should regulatory bodies require it. In Australia, due to amendments to key legislation, the *Gene Technology Regulations 2001* (Compilation No. 9), organisms developed with NHEJ can avoid GMO classification on the proviso that no net exogenous DNA is introduced, e.g. plasmids. This relaxing of legislation provides an opportunity to turn around public sentiment regarding genetically modified yeasts in the beverage industry.

With CRISPR/Cas9 well understood in laboratory *S. cerevisiae*, researchers have applied the technique to industrial yeasts, where *S. cerevisiae* is the dominant species for application in wine, brewing and rice wine production. A diversity of phenotypic outcomes (**Table 1**) include increasing pleasant floral aromas in wine making (Cordente *et al*., 2018), decreasing unpleasant phenyl off flavours (POF) in brewing (Gorter de Vries *et al*., 2017), and eliminating foaming in sake production (Ohnuki *et al*., 2019). Whilst researchers have looked at numerous mutations, the potential targets of CRISPR/Cas9 are limitless in terms of strain improvement, whether in enhancing aromatic properties or fermentation performance, furthering our understanding of yeast metabolism, or ultimately, providing new 'fit for purpose' strains for industry.

This review will explore the application of recombinant DNA technology in the fermented beverage industries, with emphasis on the currently approved recombinant

strains and regulations applied to their use. Discussion on promising new genome editing technologies, which could address some of the regulatory limitations, will focus on CRISPR/Cas9 - a hot topic in many biological fields. Review of the uses of CRISPR/Cas9 in fermented beverage related research will examine potential new targets in relation to strain development and their future use in industry.

Table 1: Industry relevant yeast strains modified by CRISPR/Cas9. Superscript denotes yeast species other than *Saccharomyces cerevisiae*

Target	Mutation	Purpose	Parental Strains	Reference
Brewing Yeast				
URA3	*ura3Δ*	Proof of concept	IMX585[a]	Gorter de Vries *et al.*, (2017)
ILV6 (*S. eubayanus* sub-genome)	*ilv6Δ*	Proof of concept	IMX1187[a] CBS1483[a]	Gorter de Vries *et al.*, (2017)
ATF1/ATF2 (*S. eubayanus* sub-genome)	*atf1Δ atf2Δ*	Proof of concept	CBS1483[a] Weihenstephan 34/70[a]	Gorter de Vries *et al.*, (2017)
FDC1	460C>T	Lowered 4-vinyl guaiacol	S288C, BE002, BE014, BE020, BE074, WL022[b], WL024[c], H1[c], H2[c]	Mertens *et al.*, (2019)
SGA1	*sga1Δ::ScTEF1*$_{pr}$*-SeMALT1-ScCYC1*$_{ter}$*::ScTDH3*$_{pr}$*-ScMAL12-ScADH1*$_{ter}$	Confirming function of putative *S. eubayanus* maltose transporters	IMZ616	Brickwedde *et al.*, (2018)
SGA1	*sga1Δ::ScTEF1*$_{pr}$*-SeMALT2-ScCYC1*$_{ter}$*::ScTDH3*$_{pr}$*-ScMAL12-ScADH1*$_{ter}$	Confirming function of putative *S. eubayanus* maltose transporters	IMZ616	Brickwedde *et al.*, (2018)
SGA1	*sga1Δ::ScTEF1*$_{pr}$*-SeMALT3-ScCYC1*$_{ter}$*::ScTDH3*$_{pr}$*-ScMAL12-ScADH1*$_{ter}$	Confirming function of putative *S. eubayanus* maltose transporters	IMZ616	Brickwedde *et al.*, (2018)
SGA1	*sga1Δ::ScTEF1*$_{pr}$*-AGT1-ScCYC1*$_{ter}$*::ScTDH3*$_{pr}$*-ScMAL12-ScADH1*$_{ter}$	Positive control for maltose transport	IMZ616	Brickwedde *et al.*, (2018)

SeMALT1	*SemalT1Δ/ SemalT1Δ*	Systemic deletion for malt utilisation analysis	CBS12357^{T}/FM 1318[b]	Brickwedde *et al*., (2018)
SeMALT2/T4	*SemalT2Δ/ SemalT2Δ, SemalT4Δ/ SemalT4Δ*	Systemic deletion for malt utilisation analysis	CBS12357^{T}/FM 1318[b]	Brickwedde *et al*., (2018)
SeMALT3	*SemalT3Δ/ SemalT3Δ*	Systemic deletion for malt utilisation analysis	CBS12357^{T}/FM 1318[b]	Brickwedde *et al*., (2018)
ADE2	Various	Assessing gene combinations for geraniol and linalool production	Laboratory and industrial (WLP001)	Denby *et al*., (2018)
Rice Wine Yeast				
YPR196W (*MAL73*)	*ypr196wΔ*	Assessing gene function, maltose utilisation deficient	K1801M	Ohdate *et al*., (2018)
AWA1	*awaΔ/awaΔ*	Reduced foaming during fermentation	K6, K7, K9, K10	Ohnuki *et al*., (2019)
HO	*hoΔ::PKG1p-DUR3-PKG1t*	Overexpression of DUR3. Reduced ethyl carbamate production	Na^{DUR1,2}-c, Nα^{DUR1,2}-c	Wu *et al*., (2020)
Wine Yeast				
CAN1	*CAN1 G70** (-GGC- > -TAG-)	Lowered urea	EC1118, AWRI 796	Vigentini *et al*., (2017)
TYR1	550G>A, 591G>T or 629C>T	Increased floral aroma compounds	AWRI 1631	Cordente *et al*., (2018)
ARO4	64G>T, 497A>G or 584C>Y	Increased floral aroma compounds	AWRI 1631	Cordente *et al*., (2018)
GPD1	*FBA1p-GPD1*	Increased glycerol production	AWRI 1631	van Wyk *et al*., (2020)
ATF1	*TEF1p-ATF1*	Increased acetate esters, decreased acetic acid	AWRI 1631	van Wyk *et al*., (2020)
URA3	*ura3Δ::natMX*	Auxotrophy, proof of concept	AWRI2804(UCD2041)[d]	Varela *et al*., (2020)
ADE1	*ade1Δ::natMX*	Auxotrophy, proof of concept	AWRI2804(UCD2041)[d]	Varela *et al*., (2020)
SSU1	*ssu1Δ::natMX*	Sulfite sensitivity	AWRI2804(UCD2041)[d]	Varela *et al*., (2020)
URA3	*ura3Δ::natMX / ura3Δ::kanMX*	Auxotrophy, proof of concept	AWRI1613[d]	Varela *et al*., (2020)

[a] *Saccharomyces pastorianus*

[b] *Saccharomyces eubayanus*

[c] *Saccharomyces cerevisiae x Saccharomyces eubayanus* interspecific hybrid

[d] *Brettanomyces bruxellensis*

First-generation recombinant technology used in strain improvement

Genetic engineering in yeast is nothing new, with pioneering experiments dating back some 40 years. Early recombinant techniques in *S. cerevisiae* involved the transformation of plasmids (Hinnen *et al*., 1978) or integrative homologous DNA (Orr-Weaver *et al*., 1981). These methods are now considerably improved and still used to today. Importantly, the use of homologous recombination to introduce selectable markers such as *kanMX* for stable gene deletions (Wach *et al*., 1994) has advanced our understanding of yeast gene function through the production of large-scale yeast deletion libraries in both laboratory (Giaever *et al*., 2002; Ryan *et al*., 2012) and wine yeast strains (Peter *et al*., 2018). Together with gene deletions, the introduction of new genes or genes with altered expression provide a means of exploring and improving yeast phenotypes. This relies on either plasmid expression or genomic integration. In industrial yeast research, stable genomic integration is the method of choice to counteract the need for selective conditions to stably maintain plasmid. DNA integration in yeast can take place using integrative shuttle vectors (Sikorski and Hieter 1989), using homologous integration with selective markers, as previously mentioned, or marker-free methods such as *delitto perfetto* (Stuckey *et al*., 2011). Many of these genetic engineering tools have been used in industrial yeast strains to investigate and improve phenotypes related to fermentation efficiency, sensorial quality, health-related quality and microbial stability (reviewed in Steensels *et al*., 2014). With the wealth of knowledge on yeast genetic modification and mutations positively affecting yeast fermentation, it is not surprising there are yeasts approved for industrial use.

Commercial GM yeast in the fermented beverage industries

The United States (US) and Canada benefit from their looser labelling requirements around the use of GM yeasts as processing aids in the production of fermented beverages, as food produced with GM processing aids are not required to have a 'GMO-derived' label. In these countries, there are two approved genetically modified winemaking yeast strains on the market, ML01 and ECMo01. The recombinant ML01 strain has a cassette integrated into the *URA3* locus, which contains the structural *mae1* gene for the malate transporter from *Schizosaccharomyces pombe* and *mleA* encoding the malolactic enzyme from *Oenococcus oeni*, both constitutively expressed from the yeast phosphoglycerate kinase 1 (*PGK1*) promoter (Husnik et al. 2006). This modification to ML01 allows the yeast to efficiently uptake and metabolise malic acid akin to a malolactic fermentation, a process that does not usually occur in *S. cerevisiae*, but rather results from the metabolism of *O. oeni* or *Lactobacillus spp.* in wine. ECMo01 is a strain modified to reduce urea concentrations, a precursor of ethyl carbamate, a known potential human carcinogen. An additional copy of *DUR1,2*, encoding urea amidolyase, is integrated in the *URA3* locus, with constitutive expression via a *PGK1* promoter (Coulon *et al*., 2006). In 2019, Lallemand marketed a genetically modified (bio-engineered) brewer's yeast, Sourvisiae® in the US. Whilst the origin of the lactate dehydrogenase used in the modification is not stated other than from a food organism, FDA granted GRAS (generally regarded as safe) status (GRAS Notice No. GRN 000841) in March 2019 to Mascoma LLC, a Lallemand research and development centre, for a lactate dehydrogenase producing yeast for sour beer production. This suggests it is a codon-optimised lactate dehydrogenase from *Rhizopus oryzae*, a GRAS organism, expressed by *ADH1* promoter and *PDC1* terminator sequences.

In the United Kingdom, the government has also approved the use of two genetically modified yeasts, a baking yeast, and a brewing yeast. The brewing yeast involved the introduction of glucoamylase, *STA2*, to degrade starch to fermentable sugars (Hammond 1995), similar to the ability of *S. cerevisiae* var. *diastaticus* (Tamaki 1978). The baking yeast was reported to have additional copies of maltose permease and maltase genes to allow for the efficient uptake and utilisation of maltose, with the goal of faster CO_2 evolution and therefore quicker bread rising (Aldhous 1990). As "no genetic material has been added to the yeast from another species" (Aldhous 1990), the strain would be regarded today as a 'self-cloning' yeast (Akada 2002), where net changes to the genome do not involve any foreign sequences derived from other species.

In Japan, self-cloning yeast have avoided regulation altogether, with the guidelines revised in 2001 (Akada 2002), allowing for potentially a large number of yeast to be used for commercial wine, beer and sake production. Indeed many yeast have already been improved using self-cloning mutations (reviewed in Fischer *et al*., 2013). In brewing, self-cloning yeasts have been produced to increase glutathione content (Wang *et al*., 2008, Wang *et al*., 2007, Wang *et al*., 2009), or enhance flavour stability through the expression of a superoxide dismutase fusion protein (Wu *et al*., 2016). Other attributes include increased acetate esters (Dong *et al*., 2019), improved foam stability by proteinase A disruption (Wang *et al*., 2007, Wu *et al*., 2016), introduction of flocculation to non-flocculant yeast (Ishida-Fujii *et al*., 1998) and reducing negative sensory properties (Iijima and Ogata 2010, Kusunoki and Ogata 2012, Wang *et al*., 2008). Likewise, many rice wine (sake) yeast are enhanced by self-cloning techniques and include strains which increase intracellular proline (Takagi *et al*., 2007), or reduce the carcinogen ethyl carbamate (Dahabieh *et al*., 2010, Li *et al*., 2015). Aroma profiles have been targeted by increasing levels of positive aroma compounds, ethyl caproate (Aritomi

et al., 2004) and isoamyl acetate (Hirosawa *et al*., 2004) associated with 'fruity pineapple' and 'banana' aroma, respectively, and reducing the negative aroma compound, dimethyl trisulfide, responsible for 'asparagus, cooked *Brassica*' aromas (Ikeda *et al*., 2018). In wine yeast, self-cloning is an underutilised resource when modifying specific phenotypes or traits, with only a handful of examples - none of which have been adopted by industry. These include enhancing varietal aroma characteristics in Sauvignon Blanc wines (Holt *et al*., 2012), increasing glycerol and glutathione concentrations to improve wine mouthfeel and flavour stability (Hao *et al*., 2012) and lowering acetic acid production (Cordente *et al*., 2013) since levels above 0.7 g L^{-1} are considered detrimental to wine quality.

Interestingly, the two other large jurisdictions, Australia and the European Union (EU), do not have any listed GM yeasts approved by the current regulatory bodies, with the majority of registered organisms being crops such as canola, cotton and maize (European Commission GMO Register (https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm) and the OGTR (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ir-1). The absence of GM yeast in these regions is most likely due to tighter regulations around the definition (and classification) of a GMO, the use of GMOs in food production and product labelling requirements. Interestingly, labelling requirements for Australia and the EU are similar to the US and Canada in relation to GM processing aids, with the key difference being that no DNA from the GMO used can be detectable in the final product. However, with the introduction of new genome editing technologies through programmable nucleases this may change. Whilst the EU regards gene editing as a GM technique, recent changes to Australian legislation may allow for its use in limited circumstances where the modified organisms are not required to have the GMO classification. The use of such technologies has also been discussed in Japan and the

USA, where organisms derived from gene editing will not be regarded as GMOs provided the mutations introduced are also possible through conventional breeding methods (reviewed in Hanlon and Sewalt 2020).

Precise genetic engineering through programmable nucleases

Programmable nucleases are comprised of clustered regularly short palindromic repeats (CRISPR) coupled with a CRISPR associated nuclease (Cas) (CRISPR/Cas) (Jinek *et al*., 2012), zinc finger nucleases (ZFNs) (Kim *et al*., 1996) and transcription activator-like effector nucleases (TALENs) (Cermak *et al*., 2011). These technologies utilise highly specific targeting of nucleases to loci in the DNA, each of which use different methods of targeting (Kim and Kim 2014). Where ZFNs and TALENs rely on the modification of protein binding motifs for DNA targeting, CRISPR/Cas9 uses a complimentary RNA targeting approach. Of these techniques, the Nobel Prize winning CRISPR/Cas9 (https://www.nobelprize.org/prizes/chemistry/2020/press-release/) is the newest and holds the most promise due to its simplicity, high specificity, and high efficiency.

CRISPR/Cas9 mediated genome editing

CRISPR/Cas9 is a highly specific and efficient RNA-guided DNA nuclease system, which has the potential to create double stranded DNA breaks *in vivo.* The CRISPR/Cas system was initially found to be an "immune system" in *Streptococcus thermophilus*, where different short DNA sequences (protospacers) flanked by a repeated sequence, in combination with a CRISPR-associated (Cas) enzyme appeared to induce protection against bacteriophage (Barrangou *et al*., 2007). The protospacer regions of the

CRISPR/Cas system were key to resistance, and consisted of small DNA sequences originating from the phage genome. The processed RNA of these protospacers including a downstream repeated region (crisprRNA (crRNA)) interacts with the trans-activating crRNA (tracrRNA) to form a dual RNA structure that is capable of interacting with the Cas9 nuclease. The resulting complex guides the Cas9 protein (enzyme) to the specific protospacer DNA sequence directly upstream from a 3 base pair motif recognised by Cas9, termed the protospacer adjacent motif or PAM site, which results in the double-stranded cleavage of the target DNA (Jinek *et al*., 2012) (**Figure 1**). The authors simplified the process by creating a chimera of crRNA and tracerRNA, which achieved the same outcome and demonstrated the programmability of the system, effectively targeting and cleaving the plasmid DNA at the targeted sites.

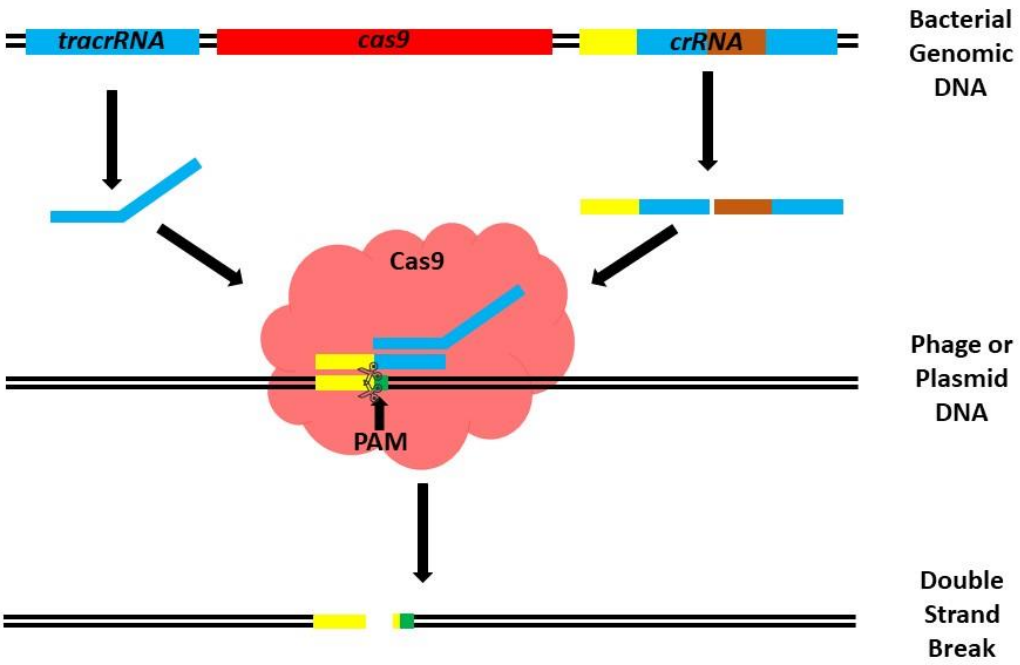

Figure 1: Bacterial CRISPR/Cas9 defence against foreign phage or plasmid DNA resulting in a double-stranded break (DSB) and inactivation of the invading DNA.

Adapted from Kim and Kim (2014). The PAM site (5'-NGG-3', where "N" is any nucleobase followed by two guanine (G) nucleobases) is in green.

Jinek and co-workers (2012) speculated at the use of CRISPR/Cas9 as a programmable genome editing tool, which was validated by Cong and co-workers (2013). The authors demonstrated the use of the *Streptococcus pyogenes* CRISPR/Cas9 system in human and mouse cells to create double stranded breaks (DSBs) at specific locations within the DNA. Furthermore, the DSBs were repairable by one of the host cell's DNA repair systems via non-homologous end joining (NHEJ) or homology directed repair (HDR).

The CRISPR/Cas9 system has proven versatile, with its adoption for use in other model organisms, including zebrafish (*Danio rerio*; Hwang *et al.* 2013), *Drosophila melanogaster* (Gratz *et al*., 2014) and yeast (*Saccharomyces cerevisiae*; DiCarlo *et al*., 2013). CRISPR/Cas9 mediated genome editing systems for fungi (including ascomycetous yeasts) are relatively new and have yet to reach their full potential as research tools and in construction of 'industry ready' strains.

DNA repair from DSBs

In its simplest form, the CRISPR/Cas9 system exploits two host repair mechanisms, non-homologous end joining (NHEJ), or homology directed repair (HDR) (**Figure 2**). For simple deletions, NHEJ is the key mechanism. NHEJ occurs in the absence of a homologous DNA template to guide repair, giving rise to either no alteration or base pairs added or removed at the cleavage site. The resultant frameshift mutation can cause loss of gene function when targeted at or near the start of the open reading frame (ORF; DiCarlo *et al*., 2013). Alternatively, HDR requires a homologous template

to repair the cleaved DNA strand; in nature, this is the sister chromatid in a diploid yeast cell.

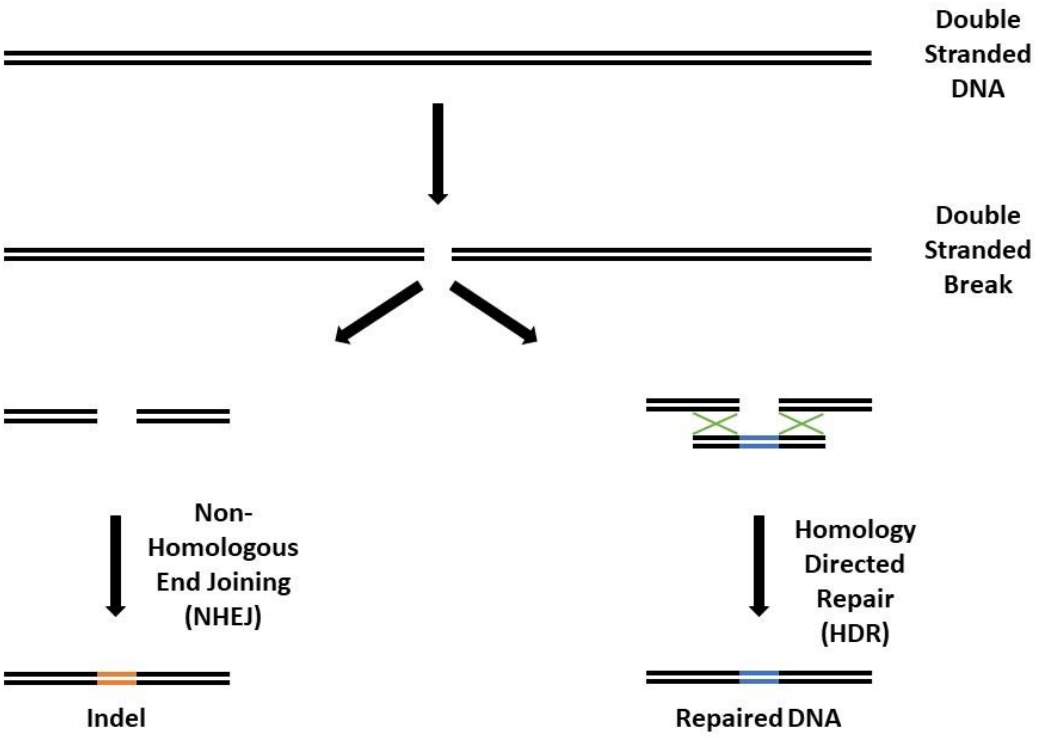

Figure 2: The DSB repair mechanism of eukaryotes. When a double stranded break (DSB) occurs in the chromosome, the damage is repaired in the absence or presence of a repair template (which typically is the sister chromatid in diploid organisms). Lack of a repair template leads to non-homologous end joining (NHEJ), where insertion or deletion of nucleotides can occur, potentially leading to frame shift mutations. The presence of a repair template leads to homology directed-repair (HDR), where the homologous DNA acts as a template at the DSB. Adapted from Alberts *et al*. (2007).

CRISPR/Cas9 mediated genome editing in *S. cerevisiae*

Genetic modification in *S. cerevisiae* generally relies on HDR, whether by traditional recombinant technology (e.g., single-step gene replacement; Rothstein 1989) or the emergent CRISPR/Cas9 editing technologies (DiCarlo *et al*., 2013). The HDR mechanism allows nucleotide sequence alterations *in vivo* in the presence of a homologous repair template (with the desired changes) either as double or single stranded DNA or the repair template cloned in the CRISPR/Cas9 expression plasmid.

CRISPR/Cas9 editing in *S. cerevisiae*, is typically reliant on a plasmid-based system, consisting of a vector from which *S. pyogenes* Cas9 is expressed, flanked by a nuclear localisation signal (NLS), a 20 bp genomic targeting sequence (located 5' of a PAM recognition sequence (NGG for Cas9) in the yeast). A cRNA/tracerRNA chimera sequence is directly 3' to form a synthetic guide RNA (sgRNA). For plasmid selection and maintenance, there is a selectable marker (usually an antibiotic resistance gene or auxotrophic marker) (Bao *et al*., 2015, DiCarlo *et al*., 2013).

The initial application of this technology simply involved inducing double strand breaks to delete genes, analysing targeting sequence efficiency and recombination efficiency when coupled with homologous DNA to introduce SNPs to restore gene function (DiCarlo *et al*., 2013). Modifications included small changes such as SNPs resulting in frameshifts, premature stop codons, insertions and deletions (DiCarlo *et al*., 2013, Mans *et al*., 2015). The ability to make precise modifications has led to a plethora of paradigms ranging from simultaneous deletion of multiple gene functions (multiplex genome engineering; Cong *et al*., 2013), and single vector multiplex engineering with integrated homologous repair sequences (homology integrated CRISPR/Cas9 (HI-CRISPR; Bao *et al*., 2015). Other applications include gene mapping without the need for genetic crossing (Sadhu *et al*., 2016), heterologous expression of gene pathways (Tsai

et al., 2015), chromosome splitting (Sasano *et al*., 2016) and deletion of large DNA fragments (Hao *et al*., 2016); all of which can be achieved without integrating selectable marker genes.

Multiplex Genome Engineering

The CRISPR/Cas9 system has been used to simultaneously target multiple genes simply by having multiple sgRNA sequences on the expression plasmid. This model parallels the bacterial immune system, where multiple spacer RNAs are present and used to target multiple invasive DNA sequences (Barrangou *et al*., 2007), and as such can be result in multiple concurrent gene deletions (Cong *et al*., 2013). An advancement in the method (termed homology-integrated CRISPR/Cas9 (HI-CRISPR; Bao *et al*., 2015), included extra 'homologous repair' sequences (100 base pairs) 5' of the sgRNA sequence in the expression plasmid. This allowed the plasmid itself to be the homologous repair template, enabling multiple site cleavage coupled with specific repair of DSBs by HDR. As co transformation of the expression vector is reliant only on one double-stranded repair template, and not multiple ones, the recombination mechanism is simplified, with close to 100% integration of the template, in this case for three genes, when sufficient maturation time is given in the medium used for plasmid selection. This method is useful as a single step, multi-gene optimisation by incorporation of previously identified non-synonymous SNPs at multiple specific genomic locations, or a combination of quantitative trait loci (QTL) incorporated with gene deletions previously identified to be beneficial.

CRISPR/Cas9 Genome editing in non-*Saccharomyces* yeasts

Recent interest in the application of CRISPR/Cas9 in non-conventional yeast has led to research into the use of 'broad host range' plasmid systems. Juergens and

coworkers (2018) were the first to demonstrate this kind of system, successfully modifying four yeasts belonging to two genera (*Kluyveromyces lactis, K. marxianus, Ogataea polymorpha* and *O. parapolymorph*). These species are of particular interest for their roles in the production of biofuels (Kata *et al*., 2016, Kurylenko *et al*., 2014, Siso 1996), heterologous proteins (Gellissen 2000), biomass, flavour and fragrance compounds (Morrissey *et al*., 2015). Similarly, CRISPR/Cas9 based genome editing systems have been achieved for other nonconventional yeasts; *Scheffersomyces stipitis* (Cao *et al*., 2017), *Yarrowia lipolytica* (Schwartz *et al*., 2016) and *Pichia pastoris* (Weninger *et al*., 2016), as well as different *Candida* species (Lombardi *et al*., 2019). This is of particular interest given the recent move towards the use of non-*Saccharomyces* in alcoholic beverage production for the purposes of reducing ethanol content or improving the aroma-sensory profile of alcoholic drinks. Species of interest include *Torulaspora delbrueckii, Lachancea thermotolerans, Brettanomyces bruxellensis* and others, including members of the *Saccharomyces sensu stricto* and *S. sensu lato* families. Varela and co-workers (2020) have made progress towards this, developing an expression-free CRISPR/Cas9 system to modify *B. bruxellensis*. The authors assembled crRNA, tracrRNA and Cas9 into a ribonucleoprotein complex *in vitro*, for co-transformation with deletion cassettes for two targeted genes (*ADE1* or *URA3*). This differs from the *in vivo* expression of the ribonucleoprotein complex components using plasmids as is common with other species. Heterozygous (diploid) deletion strains were generated in the first transformation, with homozygous deletions needing an additional transformation. The authors validated the industrial application of the method, in relation to the aetiology of sulfite tolerance of *B. bruxellensis* as a spoilage organism. Ssu1 mutants with the *SSU1* gene replaced using NatMX (*ssu1::NatMX*) showed sensitivity to sulfite, with inhibition at 0.09 mg L^{-1} molecular SO_2 compared to the wild type, which

grew at 0.24 mg L^{-1} mSO_2. Methods such as these may facilitate mutation of strains resilient to classical homologous recombination-based transformation strategies, or production of NHEJ mutants in other ascomycetes. This may impart stricter levels of physical containment due to their poor characterisation and the regulatory use of modified non-standard laboratory organisms, as in Australia with the *Gene Technology Regulations 2001*. The use of CRISPR/Cas9 in fermentative non-*Saccharomyces* yeasts will undoubtedly be an area of continued research focus.

With our current understanding of *S. cerevisiae* and a number of genome editing tools available, research and development has progressed in relation to the improvement and understanding of fermentation across rice wine, beer and wine fermentations. Yeasts derived from CRISPR/Cas9 have the potential to be used in industry, in line with legislation such as in the US and Australia (reviewed in Hanlon and Sewalt 2020). The US has opted to deregulate gene-edited crops if the introduced mutations could be bred conventionally, and by default, to other organisms similarly constructed. An early, unregulated use of CRISPR/Cas9 in the US, was the removal of one of the polyphenol oxidase genes to decrease browning in the white button mushroom, *Agaricus bisporus*, a trait in mushrooms that is negatively perceived by consumers (Waltz 2016).

CRISPR/Cas9 in brewing yeast research

When producing beer, the different styles can depend on the species of yeast used, with beers being primarily ales or lagers, produced with either *S. cerevisiae* or *S. pastorianus*, respectively. Both yeasts are modifiable through CRISPR/Cas9. Gorter de Vries and co-workers (2017) demonstrated the effective targeting of genes in the hybrid *S. pastorianus* using a plasmid-based system; with HDR gene knockouts in *URA3* and *ILV6* in the *S. eubayanus* sub-genome. Furthermore, they simultaneously knocked out the

S. eubayanus ATF1 and *ATF2* loci without affecting the *S. cerevisiae* sub-genome, demonstrating a proof of concept in these hybrids. In improving hybrid lager yeasts, Mertens and co-workers (2019) focussed on reducing yeast-derived phenol off flavour (POF) concentrations. The authors introduced a premature stop codon in the ferulic acid decarboxylase (*FDC1*) found naturally in POF- domesticated ale yeasts into a number of POF+ *S. cerevisiae*, *S. eubayanus* and *S. cerevisiae* x *S. eubayanus* hybrid strains. Edited strains lowered production of 4-vinyl guaiacol, a generally unwanted metabolite, which results in clove-like sensory characteristics, to below sensory thresholds without altering fermentation kinetics or production of other yeast metabolites (Mertens *et al*., 2019). CRISPR/Cas9 has been used to functionally characterise the *S. eubayanus* maltose utilisation genes. Through Oxford Nanopore MinION sequencing of *S. eubayanaus* strain CBS 12357^{T}, Brickwedde and co-workers (2018) identified potential maltose transporter genes (*seMALT(1-4)*). To validate, a maltose-transport-deficient *S. cerevisiae* strain (constructed by CRISPR/Cas9; Marques *et al*., 2018) was used to overexpress *seMALT(1-4)*. Positive growth on maltose validated the function of the *S. eubayanus* genes. Further investigation through CRISPR/Cas9 mediated knockouts in CBS 12357^{T} identified *seMALT2* and *seMALT4* as being responsible for maltose transport.

For the production of ales, CRISPR/Cas9 has also been used to improve *S. cerevisiae* strains, with Denby and co-workers (2018) producing a yeast capable of making favourable hop (*Humulus lupulus*) derived monoterpenes, linalool and geraniol. This strain was developed by introduction of a mutant farnesyl pyrophosphate synthase which results in increased concentrations of the precursor compound geranyl pyrophosphate, overexpression of a truncated HMG-CoA reductase lacking an inhibitory regulatory domain, a truncated linalool synthase from mint (*Mentha citrata*) and geraniol synthase from basil (*Ocimum basilicum*). Sensory analysis of beers brewed with the

mutant yeast increased “hoppy” sensory characteristics compared to the parental strain (WLP001 California Ale Yeast®, White Labs) when brewing a classic American Ale.

CRISPR/Cas9 in rice wine yeast research

The production of sake and other rice wines also relies on *S. cerevisiae* as the fermentative organism, with CRISPR/Cas9 featuring in yeast research and strain development. Sake yeast are poor maltose utilisers, so this is a targeted attribute. Ohdate and co-workers (2018) identified an EMS mutant with enhanced maltose utilisation, due to a single base pair insertion in *YPR196W*, a putative maltose-responsive transcription factor, which resulted in a frameshift in the coding sequence and restored gene function. The ancestral strain, Kyokai No. 7, has a truncated gene, due to a single base pair deletion resulting in poor activity. Knockout of *YPR196W* in the EMS mutant via CRISPR/Cas9 confirmed the functionality of the mutated gene, with no growth evident on media with maltose as the sole carbon source. Reverse genetics confirmed the mutation as causal for maltose utilisation, with plasmid-mediated transfer of the gene into other sake strains enabling growth on maltose. New phenotypes such as reduced foaming during fermentation are possible through CRISPR/Cas9 editing. As the foam produced by sake yeast can take up a large proportion of the fermentation vessel, non-foaming strains are desirable in terms of space efficiency during fermentation. Ohnuki and co-workers (2019) used CRISPR/Cas9 to completely delete the *AWA1* open reading frame, a gene coding for a GPI-anchored protein responsible for foam formation in sake brewing.

The production of Chinese rice wine has also benefited from CRISPR/Cas9 technology, specifically in ameliorating carcinogenic ethyl carbamate levels. Wu and co-workers (2020) used CRISPR/Cas9 to overexpress the urea transporter *DUR3* in a strain previously modified to overexpress the urea amidolyase *DUR1,2*, reporting a reduction in both urea (92.0%) and ethyl carbamate (58.5%). Beyond *Saccharomyces* in rice wine

production, *Aspergillus spp.* filamentous fungi (referred to as koji in Japanese) perform the important role of saccharification of starches in the rice, creating a fermentable medium for the yeast. Two such koji species are *Aspergillus oryzae* (used in sake and fermented foods such as miso) and *Aspergillus luchuensis* (in distilled rice wine beverages shochu and awamori). CRISPR/Cas9 systems have been developed for both species, *A. oryzae* (Katayama *et al*., 2016) and *A. luchuensis* (Kadooka *et al*., 2020).

CRISPR/Cas9 in wine yeast research

In the field of winemaking, CRISPR/Cas9 enabled the evaluation of a diverse assortment of mutations in *S. cerevisiae*, investigating various aspects of winemaking from production of the unwanted by-product, ethyl carbamate, to improving the sensory characteristics of the wine produced. As previously mentioned with Chinese rice wine, the production of ethyl carbamate is undesirable and therefore a target for strain improvement. Whilst these modifications relate to wine yeast, they are transferable to other industrial yeasts that can ferment an arginine rich medium to produce urea, a product of arginine catabolism (Middelhoven 1964) and precursor to ethyl carbamate production (Monteiro *et al*., 1989). Different strategies have targeted urea degradation as a means of reducing ethyl carbamate concentrations. Whilst Wu and co-workers (2020) targeted *DUR1,2* and *DUR3*, Vigentini and co-workers (2017) chose a different gene, the arginine permease encoding *CAN1*. Early stop codons (G70*) were introduced into the *CAN1* ORF by CRISPR/Cas9 in diploid wine yeast strains EC1118 (Lallemand) and AWRI 796 (Maurivin). No urea was detectable during red and white wine fermentations. Urea was significantly reduced in a synthetic grape must with arginine as the sole nitrogen source (AWRI 796 by 18.5% and EC1118 by 35.5%) when compared to the unmodified

strains, albeit, yeast growth and fermentation rates were affected. Additionally, Chin and co-workers (2016) demonstrated yet another method for ethyl carbamate reduction in laboratory strain CEN.PK2-1D by the introduction of an early stop codon (Q26*) or deleting the entire ORF of the arginase structural gene, *CAR1*. Resulting strains demonstrated a significant reduction in both urea (64.3% and 68.7%, respectively) and ethyl carbamate (60.5% and 67.3%, respectively) production without affecting fermentation performance in YPD.

Improving safety is but one facet enhanced by CRISPR/Cas9 in wine yeast. Another is the improvement of sensory characteristics in wine by modulation of genes responsible for the production of compounds that can contribute either positively or negatively to wine. Cordente and co-workers (2018) demonstrated the use of CRISPR/Cas9 to increase levels of desirable aroma compounds 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA), which are responsible for pleasant floral rose aromas. Mutants in AWRI 796 were initially isolated after EMS mutagenesis and subsequent growth on the toxic phenylalanine analogues *p*-fluoro-DL-phenylalanine and *o*-fluoro-DL-phenylalanine. These analogues allow for positive selection of decreased feedback inhibition of phenylalanine production (Fukuda *et al*., 1991a, Fukuda *et al*., 1991b). The strains exhibiting desirable sensory characteristics were sequenced and novel SNPs were identified in prephenate dehydrogenase, *TYR1*(550G>A, 591G>T and 629C>T) and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, *ARO4*(64G>T, 497A>G and 584C>Y). When 3 SNPs from each gene were individually introduced into haploid wine yeast AWRI 1631 with CRISPR/Cas9, 2-PE was significantly increased in all strains, with *TYR1*(550G>A) and *TYR1*(591G>T) mutants producing 2-PEA over the sensory threshold of 0.25 mg L^{-1} (Swiegers *et al*. 2005), an approximate 100-fold increase. Likewise, all of the mutations significantly increased concentrations of 2-PE in

comparison to AWRI 1631, which already produced 2-PE over the sensory threshold of 10 mg L^{-1}, with the largest increase (53-fold) resulting from *TYR1*(550G>A). Other alleles have been identified by QTL analysis, which result in increased concentrations of 2-PEA. Trindade de Carvalho and co-workers (2017) identified novel mutations in the Belgian ale yeast WLP575 *FAS2* (A57T, S565N, A1136T, V1624I and S1800N), which were correlated to increased 2-PEA concentrations. Introduction of the *FAS2* allele and a correction for an early stop codon in *TOR1*(E216*) into a haploid derivative of bioethanol strain, Ethanol Red, increased 2-PEA concentrations (3.5 mg L^{-1}) compared to the parent strain (1.9 mg L^{-1}); both of which are already over sensory threshold (0.25 mg L^{-1}; Swiegers *et al*., 2005).

Conversely, there are aroma compounds that impart negative "off" sensory properties in wine that can affect wine quality. Acetic acid, a by-product of glycolysis, which is responsible for vinegar-like volatile acidity in wine, is often an unwanted consequence of gene modification to increase glycerol production (Lopes *et al*., 2000). van Wyk and co-workers (2020) have been able to modify the pathway to allow for increased glycerol and acetate esters whilst reducing acetic acid. To increase glycerol production, glycerol-3-phosphate dehydrogenase (*GPD1*) was overexpressed through the introduction of the *FBA1* promoter sequence, acetate esters were increased, and acetic acid decreased through overexpression of alcohol acetyltransferase 1 (*ATF1*) using the *TEF1* promoter sequence. Glycerol and acetate esters (ethyl acetate, isoamyl acetate, isobutyl acetate, phenethyl acetate and hexyl acetate) were both shown to significantly increase with their respective mutations and a strain containing a combination of both mutations. Both glycerol and acetate esters can positively enhance wine through improved mouthfeel and fruity sensory characteristics, respectively. Increased acetic acid (0.43 ± 0.02 g L^{-1}) from *GDP1* overexpression was complimented by that of *ATF1*, which

reduced acetic acid concentrations (0.26 ± 0.01 g L^{-1}) to near wild type levels (0.21 ± 0.00 g L^{-1}). None of the levels exceeded the 0.70 g L^{-1} sensory threshold of acetic acid (Corison et al. 1979) and thus are likely to have little impact.

Whilst the use of CRISPR/Cas9 in yeast for the production of fermented beverages is limited, there is a plethora of gene targets transferable to wine yeast with gene editing. These include gene modifications previously identified in 'experimental' haploid strains, or beneficial or interesting mutations from other industrial strains (biofuel, sake, brewing) to examine their effect in a winemaking context.

To the future: potential new targets for improved yeasts

The sequencing of the first eukaryotic genome - *S. cerevisiae* (Goffeau *et al*., 1996) - was transformative for yeast researchers, enabling a rapid transition from classical yeast genetics to deciphering of yeast cell biology, through the progressive implementation of more developed DNA technologies over the last two decades. Our advanced knowledge and understanding of *S. cerevisiae* has proven advantageous when looking to improve yeast strains, based on genetic differences. If we examine what characteristics are desirable for introduction, consensus would be to improve the final product (e.g., enhanced production of aroma compounds), or improve yeast productivity (e.g., fermentation speed or ability to ferment in nutritionally inadequate or stressful environments). Several of these phenotypes have already been addressed by the previously mentioned self-cloning techniques, however, these single mutations could be easily adapted into other strains in a quick single-step manner rather than using time consuming self-cloning techniques, whilst multiplexing allows for the concurrent introduction of multiple beneficial alleles.

The desired outcome when improving yeast productivity is a cost saving, by reducing the active time in the tank thereby reducing the burden on monitoring fermentations and allowing for faster turnover of product. This comes with having a yeast that can consistently and reliably complete fermentation under stressful conditions with minimal intervention, or by lowering the nutritional requirements of yeast such that fermentation can finish with reduced addition of nutrients such as assimilable nitrogen.

Gene deletions and disruptions are one of the simplest modifications to improve yeast productivity. Our group recently reported on a novel function of *ECM33*, encoding for a GPI-anchored protein, related to fermentation, with *ecm33Δ* mutants in a haploid wine yeast, C911D, having significantly reduced fermentation duration (Zhang *et al*., (2018). *ECM33* is poorly characterised and is involved in glucose uptake (Umekawa *et al*., 2017), n-glycosylation and mannoprotein anchoring (Pardo *et al*., 2004). Furthermore, 15 gene deletions were identified in a library screen as beneficial to low nitrogen fermentations, the genes acting as mating pheromones, and having amino acid metabolism and ubiquitin-related functions (Peter *et al*., 2018). Equivalent disruption mutations can be easily introduced into yeast by CRISPR/Cas9, with minimal DNA sequence alteration, such as an early stop codon (TAG, TAA), giving rise to an unstable truncated protein, as in Vigentini and co-workers (2017).

Improving nitrogen utilisation would also be beneficial in many fermentation environments, especially winemaking. Proline is the most abundant but essentially non-assimilated amino acid in beer wort and grape juice (Bell and Henschke 2005), because of the requirement for molecular oxygen to be present (Duteurtre *et al*., 1971, Ingledew *et al*., 1987). As such, this amino acid could be key to increasing the nitrogen pool available to yeast through improved utilisation. To date, many novel single nucleotide polymorphisms (SNPs) have been identified in genes either responsible for proline

catabolism directly or its regulation in *S. cerevisiae*, allowing for improved proline utilisation. In work from our group, Long and co-workers (2018) created a strain with increased proline utilisation. Sequencing of key proline utilisation genes identified mutations in proline oxidase, *PUT1*(-55 G>T, 260 G>A and 580 C>G) and proline permease, *PUT4*(465 W>T). Proline utilisation in *S. pastorianus* (used for lager brewing) has been improved through modification of Put4p n-terminal lysine residues thought to be sites for ubiquitination to arginine (K9R, K34R, K35R, K60R, K68R, K71R, K93R, K105R and K107R; Omura *et al*., 2005). This increased steady state Put4p levels and allowed for increased uptake of proline.

Another option is to target regulators of nitrogen catabolite repression. Disruption of transcription factors involved in nitrogen catabolite repression may also be useful in improving proline utilisation. Salmon and Barre (1998) demonstrated a knockout in *URE2*, a transcriptional regulator of the nitrogen catabolite transcriptional activator *GLN3*, resulting in improved fermentation kinetics and proline assimilation under fermentation, especially when small amounts of dissolved oxygen (as low as 6 mg L^{-1}) were added to fermentations after the growth phase. Another positive regulator of genes repressed by nitrogen catabolite repression is *GAT1*. Zhao and co-workers (2016) identified nuclear localisation signals in the transcription factor Gat1p, which in the presence of preferred nitrogen sources are phosphorylated, resulting in the Gat1p being excluded from the nucleus. Gat1p is a transcription factor for genes subject to nitrogen catabolite repression, thus, phosphorylation of these residues downregulates genes responsible for utilisation of non-preferred nitrogen sources. Two Gat1p modifications (S360A and S361A) increased the transcription of nitrogen catabolite repressed genes and so improved utilisation of non-preferred nitrogen sources including proline.

Whilst it is a possible source for additional nitrogen, proline is also known to be important in response to stressful environments in many organisms including bacteria, plants and yeast, where it is produced in response to oxidative, osmotic, heat-shock and freeze-thaw stress (Ando *et al*., 2007, Grothe *et al*., 1986, Liang *et al*., 2014, Sasano *et al*., 2012). Several amino acid variants identified in yeast strains result in the accumulation of proline, with the potential to minimise fermentation related stresses. Mutations identified to cause proline accumulation include amino acid substitutions in Pro1p (I150T, D154N, P247S and E415K); a gamma-glutamyl kinase utilised in the synthesis of proline (Sasano *et al*., 2012, Takagi *et al*., 2005, Tsolmonbaatar *et al*., 2016). Alternatively, the 'loss of function' substitution (A401E) in ubiquitin ligase Rsp5p that facilitates the degradation of the general amino acid permease Gap1p was effective (Hoshikawa *et al*., 2003). These mutations result in over-accumulation of proline, with improved viability in the presence of environmental stresses. Furthermore, we have identified novel mutations in the *PUT4* encoded proline specific permease, which allow for constitutive expression under nitrogen catabolite repressive conditions (Poole *et al*., 2009). Two mutations, (-160T>C) in the promoter, and protein (S605A) resulted in increased proline uptake and subsequent improvements in response to high temperature and osmolarity related stresses.

Sensitivity to sulfur dioxide (SO_2) in wine yeast is another parameter worth further consideration, as potassium metabisulfite is a common additive to prevent spoilage by the indigenous microbiota in grape must. Many studies have shown that increased expression of *SSU1*, a plasma membrane sulfite pump, increases resistance to sulfur dioxide (Avram and Bakalinsky 1997, Goto-Yamamoto *et al*., 1998, Park and Bakalinsky 2000, Yuasa *et al*., 2005, Zimmer *et al*., 2014). Increased *SSU1* expression is correlated by QTL analysis with translocation of *SSU1* to the promoter region of either

EMC34 (dubbed *SSU1-R*; Perez-Ortin *et al*., 2002) or *ADH1* (Peltier *et al*., 2018, Zimmer *et al*., 2014). CRISPR/Cas9 would allow for the replication of these translocations to validate their corresponding phenotypes as well as for introducing sulfite resistance to existing commercial strains. Increased expression due to these translocations has also been correlated to an increase in lag phase (Zimmer *et al*., 2014). Whilst this may not sound like an ideal phenotype, Albertin and co-workers (2017) demonstrated its use as a novel co-inoculation method with other non-*Saccharomyces* yeast, resulting in a fruitier more complex wine. Other novel slow growing phenotypes that are potentially useful as *S. cerevisiae* starter cultures in mixed fermentations include *SER1*. Reiner and co-workers (2006) in their investigation of sterol uptake, noted that a *SER1* knockout mutant had a slower growing phenotype under anaerobic conditions. Jung and co-workers (2018) have also identified a naturally occurring *SER1* 'loss of function' QTL in a sake yeast related to chronological aging. CRISPR/Cas9 would enable this variant to be tested for industry use as a non-GM yeast, under Japanese or US regulations. Increasing *SSU1* expression is demonstrated to have the added benefit of reducing fermentation off flavours such as hydrogen sulfide (rotten egg), 3-methyl-2-buten-1-thiol (skunky, coffee, lightstruck characteristic) and 2-mercapto-3-methyl-1-butanol (onion) (Iijima and Ogata 2010). Hydrogen sulfide reduction is also achievable through mutations in other genes involved in synthesis of sulfur amino acids or their precursors, including *MET5/10* (Cordente *et al*., 2009), *MET2* (Huang *et al*., 2014) and *TUM1* (Huang *et al*., 2016). Although the presence of hydrogen sulfide may appear to be undesirable, its production early in fermentation has been proposed to be beneficial for the production of 3-mercapto-hexanol (3-MH), a sulfur-based compound that contributes tropical fruit aromas (Harsch *et al*., 2013). In work from our group, Huang and co-workers (2016) identified a novel pathway of H_2S production from cysteine through *TUM1* encoding the rhodanese domain sulfur

transferase. Furthermore, *TUM1* overexpression resulted in a large burst of H_2S at the beginning of fermentation, which in the presence of (*E*)-2-hexenal resulted in increased thiols 3-MH and 3-mercaptohexyl acetate (3-MHA) in chemically defined grape juice (Huang 2017). *TUM1* is a worthy target for CRISPR/Cas9 to produce wine with enhanced fruity characteristics. This may also be beneficial for beer and sake production dependant on the concentrations of (*E*)-2-hexanal present in their respective pre-fermented products.

Whilst there are many gene targets as demonstrated above, the method of their introduction in relation to the regional jurisdiction rulings are key factors in their overall acceptance in industry. As mentioned before, in countries such Japan or the US, organisms where mutations are introduced by gene editing are not regulated as GMOs provided they are also transferable as part of a breeding program. This definition presents an opportunity to introduce and validate naturally occurring QTLs in relation to improved phenotypes via gene editing with CRISPR/Cas9. In Australia, the edited strain is only classed as a non-GMO if no template DNA is introduced. This would make NHEJ-based systems an ideal choice specifically in the construction of gene knockouts, via frameshift mutations created by NHEJ associated indels. Whilst there are no current reports on non-GMO CRISPR/Cas9 derived strains in use, it will be interesting to see what the future holds for the fermented beverage industries.

Conclusion

Saccharomyces spp. yeast are largely important for many fermented beverages produced worldwide. For decades, there has been a drive to understand and improve the genetics of yeast for these processes. Many early techniques involved introducing new DNA through homologous recombination using plasmids or DNA cassettes with bacterial

antibiotic resistance genes in order to select for transformed cells. Fast forward to today and some of these techniques have been adapted to produce industry approved genetically modified yeasts for beverage production.

Countries such as the US, Japan and the United Kingdom all have approved genetically modified yeast, the majority of which were produced using self-cloning techniques. Other jurisdictions like Australia and the European Union are not as fortunate, having no approved GM yeasts available, likely due to stricter regulations. Newer genome editing techniques, in particular CRISPR/Cas9, show promise in the ability to create highly specific mutations efficiently through HDR or to create mutations at a specific site without a net introduction of new DNA, relying on the host organism's NHEJ repair mechanisms. The adoption of these technologies is dependent upon sovereign legislation in countries such as the US, Japan and Australia. Australia has taken a conservative approach, which allows NHEJ-derived mutations to be classed as non-GMO, provided all exogenous DNA are removed from the organism (e.g. out-breeding). Public education is paramount to acceptance, with these types of gene editing more akin to 'targeted' rather than 'random' mutagenesis. Japan and the US have taken a more relaxed approach, allowing the use of genome editing providing the mutations introduced occur in nature. With the growing amount of QTL data on fermentation phenotypes, this may be an appropriate alternative to breeding yeasts to transfer these phenotypes.

Worldwide the adoption of this technology has the potential to improve fermented beverage production, be it through performance-enhanced yeast, or yeasts that contribute positively to the end-product beverages though lessened faults or increased (and new interesting) sensorial compound production. Like with other genome engineering techniques, the incorporated changes need to be justifiable. As the end-product is intended for human consumption the introduced mutations need to be proven safe. Opting

for a targeted mutagenesis approach or introducing mutations that would be possible through breeding should be no cause for alarm in these instances. With some hope and optimism these techniques will be adopted, and we will see new GRAS yeast that have some very interesting attributes and from the basis of new product development in the beverage industry.

Project aims and objectives

This literature review summarises recent advancements using CRISPR/Cas9 technology within the fermented beverage industries and provides an outline of potential future research. Based on these future recommendations related to wine yeasts, the aims of my research are:

1. to use CRISPR/Cas9 to disrupt fermentation relevant genes to improve wine fermentation in a diploid yeast background (Chapter 2). This extends past research undertaken in a haploid wine yeast background, which is not representative of commercial wine yeasts, which are typically diploid.
2. to investigate new 'tailored-made' wine yeast strains with novel phenotypes generated by CRISPR/Cas9 using Homology Directed Repair (HDR) to integrate the mutations. These 'self-cloned' yeast have the potential for industrial uptake in wine regions where 'self-cloning' is permitted as a means of strain improvement. Two phenotypes were investigated: a) a slow growth mutation for novel co-inoculation methods (Chapter 3), and b) proline accumulation for stress resistance (Chapter 4).
3. to investigate the generation of 'non-GMO' yeast by CRISPR/Cas9-induced Non-Homologous End Joining (NHEJ) repair i.e., without a DNA repair template.

NHEJ edited yeasts are of potential industrial applicability, as the modifications comply with current Australian legislation on Gene Technology (Chapter 5).

Chapter 2

"Disruption of *ECM33* in diploid wine yeast EC1118: Cell morphology and aggregation and their influence on fermentation performance"

This chapter is written as a manuscript for FEMS Yeast Research

(Submitted for publication*)

ID NO: FEMSYR-20-11-0169

***Post thesis submission manuscript has been accepted for publication**

Statement of Authorship

Title of Paper	Disruption of ECM33 in diploid wine yeast EC1118: Cell morphology and aggregation and their influence on fermentation performance
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	FEMS Yeast Research

Principal Author

Name of Principal Author (Candidate)	Tom Lang
Contribution to the Paper	- Conceptualisation - Laboratory work/Analysis - Writing
Overall percentage (%)	80%
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Disruption of *ECM33* in diploid wine yeast EC1118: Cell morphology and aggregation and their influence on fermentation performance

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One sentence summary: Gene-editing was used to disrupt the *ECM33* gene in a wine yeast strain, confirming the enhanced fermentation performance of this mutation, previously only shown in a haploid, laboratory strain.

Key words: CRISPR/Cas9, *Saccharomyces cerevisiae*, *ECM33*, fermentation

Abstract

Interest in how yeast adapt and proliferate in grape juice to undergo fermentation is long-standing. Research has often focused on identifying genes and biological pathways behind this process. Mutations that improve fermentation, such as making it quicker, are not only desirable *per se* but are a resource in yeast breeding. Traditionally, their characterisation has been confined to haploid or laboratory yeast given the ease of their genetic manipulation, even though they do not necessarily share the fermentative behaviour of typically diploid industrial strains. *ECM33*, for example, was first associated with improved fermentation upon its deletion in the haploid wine yeast, C911D, but remained uncharacterised in a diploid industrial strain in a fermentation setting. We report on the homozygous disruption of *ECM33* in Lalvin EC1118 using CRISPR/Cas9. EC1118 *ecm33* completed fermentation 20% and 13% sooner under limiting and sufficient nitrogen availability, respectively. Cellular aggregation in the diploid mutant was previously unidentified in C911D *ecm33*Δ, as haploid yeast naturally aggregate. The novel phenotype led to premature settling; the yeast behaving similarly to EC1118 in wine-like semi-static fermentations. The study draws attention to phenotypes being condition dependent, highlighting the need to characterise and verify fermentation efficiency mutations in industrial yeast.

Introduction:

The discovery of CRISPR/Cas9 as a highly efficient and easily programmable genome editing tool (Cong *et al.,* 2013) has paved the way for innovation within biological sciences, with its potential ability to be used in any organism. In fermented beverage production, this technique has been proven to be useful in both the commonly used industrial yeast, *Saccharomyces cerevisiae*, and lager yeast, *S. pastorianus*, an interspecific hybrid between *S. cerevisiae* and another yeast species, *S. eubayanus*. However, its use in the investigation and improvement of commercial beverage fermentation yeasts to date is limited, as reflected in the literature with only a handful of research articles. Examples of CRISPR/Cas9 gene editing include the production of non-foaming sake yeasts (Ohnuki *et al.*, 2019), reduced phenolic off-flavours in lager yeast, *S. pastorianus* (Mertens *et al.,* 2019) and urea (Vigentini *et al.,* 2017) and ethyl carbamate production in wine yeast (Chin *et al.,* 2016). Other modified wine yeasts include those with increased floral aroma compounds (Cordente *et al.,* 2018), glycerol and acetate esters (van Wyk *et al.*, 2020). Besides the previously mentioned phenotypes utilising CRISPR/Cas9, attention has also focused on understanding fermentation relevant characteristics such as fermentation duration (Zhang *et al.,* 2018) and nitrogen source utilisation (Zhao *et al.*, 2016, Peter *et al.*, 2018) by the use of traditional methods.

Prior to the advent of CRISPR/Cas9, research into fermentation relevant characteristics was reliant on techniques such as directed evolution (McBryde *et al.*, 2006, Liccioli, 2011), and mutagenesis either separately (Cordente *et al.*, 2013, Long *et al.*, 2018), or in combination (Liccioli, 2011), quantitative trait loci (QTL) analysis (Steyer *et al.,* 2012, Zimmer *et al.,* 2014), sporulation (De Vero *et al.,* 2011), as well as marker assisted gene deletions (Huang *et al.,* 2016, Zhang *et al.,* 2018). All of the above paradigms have drawbacks, for example directed evolution and mutagenesis are not gene-

targeted approaches, and sporulation is not readily amenable to industrial strains as they are typically poor spore producers. Many marker assisted gene deletion studies utilise haploid yeast, which are not representative of diploid industrial strains, and as such introduce more complexity due to ploidy effects. Whilst it is possible to achieve diploid gene deletion through multiple marker genes or marker recycling this makes the process more time consuming.

With access to simple and highly efficient genome engineering techniques such as CRISPR/Cas9, we can introduce mutations easily into diploid yeast across both alleles simultaneously. Of interest is *ECM33* (ExtraCellular Mutant), a GPI-anchored protein of unknown function except for its role in *N*-glycosylation and mannoprotein anchoring (Pardo *et al.*, 2004). The mis-assembly of mannoproteins in the *ecm33*Δ mutant leads to an abnormal distribution in the cell, and mannoprotein secretion into the culture medium (Pardo *et al.*, 2004). Disruption of *ECM33* is also associated with altered morphological features, which can be correlated with cell wall integrity, such as increased cell size (Jorgensen *et al.*, 2002), and growth sensitivity to calcofluor-white (Lussier *et al.*, 1997, Pardo *et al.*, 2004, Zhang *et al.*, 2018), congo red (Zhang *et al.*, 2018), hygromycin B (Lussier *et al.*, 1997, Pardo *et al.*, 2004), K1 killer toxin (Lussier *et al.*, 1997) and caffeine (Pardo *et al.*, 2004). Deletion of *ECM33* results in increased uptake of glucose in nutrient rich YEPD (Umekawa *et al.*, 2017), and increased consumption of glucose and fructose (i.e., reduced fermentation duration) during fermentation in both synthetic and natural grape juice independent of nitrogen status (Zhang *et al.*, 2018).

Whether these earlier reports can be applied to diploid commercial wine yeast needs to be evaluated, as they were reliant on haploid derivatives of commercial wine yeast, or laboratory strains. Whilst haploid genotypes by their very nature, are homozygous and easy to modify, they differ from diploid genotypes such as industrial

yeasts. To generate homozygous mutations in a diploid strain more time-consuming methods such as meiotic segregation, marker recycling or multiple selective markers would be necessary unless both alleles can be mutated simultaneously as through the use of CRISPR/Cas9.

In this study we sought to characterise the disruption of *ECM33* in the diploid wine *S. cerevisiae* strain Lalvin EC1118 (Lallemand), through the introduction of a premature stop codon via CRISPR/Cas9 gene editing. Whilst the EC1118 *ecm33* mutant also had shortened fermentation duration, similar to that described by Zhang *et al.* (2018) with a haploid homozygous *ecm33*Δ mutant, the phenotype in the diploid mutant was dependent upon nitrogen content. Furthermore, we report on a novel cellular aggregation phenotype and discuss its impact on fermentation duration in laboratory scale (100 mL) chemically defined grape juice fermentations. This research furthers our understanding of the functions of *S. cerevisiae ECM33*, whilst demonstrating how experimental design and genotype, specifically ploidy, can influence phenotype outcome.

Materials and Methods:

Microbial strains, media and plasmids

Saccharomyces cerevisiae wine strain Lalvin EC1118 was the genetic background for strain construction (**Table 1**). Yeast strains were routinely grown in YEPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) at 28 °C. Media was solidified with 20 g L^{-1} agar. *Escherichia coli* NEB® 5-Alpha (C2987I, New England Biolabs) was used for plasmid propagation. *E. coli* transformation was according to manufacturer's instructions. Bacteria were grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride), supplemented with kanamycin (50 mg L^{-1})

or ampicillin (100 mg L^{-1}) for plasmid selection. Plasmids were extracted and purified with the Wizard® Plus SV Minipreps DNA Purification System (Promega).

Construction of non-sense mutation (Gly61stop) in *ECM33* using CRISPR/Cas9

The EC1118 *ecm33* mutant was generated using CRISPR/Cas9 by a two-plasmid method described in Shaw *et al.* (2019) and the Ellis lab website (https://benchling.com/pub/ellis-crispr-tools). This method utilises co-transformation of a linearised pWS082 derivative (in this study pWS-ECM33) containing a cloned sgRNA sequence for expression, and linearised pWS173 for Cas9 expression and G418 resistance (**Table 2**). Linearised fragments are gap-repaired inside the yeast to form a single plasmid containing both Cas9 and sgRNA expression required for CRISPR/Cas9 genome editing.

sgRNA expression plasmid (pWS-ECM33) construction

The sgRNA target for *ECM33* (5'-GTTGGTAACTTGACCATCAC-3') was designed using the Benchling platform (benchling.com). This target was selected based on the guide sequence having the highest on-target score, with an off-target score of 100 within an exon sequence. The S288C genome is the default genome used for sequence analysis. As S288C is a laboratory yeast, candidate sequences including the 3' NGG protospacer sequence were analysed for homology to the wine yeast EC1118 genome (taxid:643680) using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Overlapping primers, sgECM33 F and sgECM33 R (**Table 3**), were created containing this guide sequence for cloning into pWS082 by Golden Gate cloning (Engler *et al.*, 2008). Primer design and cloning were according to the method in Shaw *et al.* (2019). The final cloning reaction was transformed into NEB 5-Alpha, plated on solid LB

medium containing 100 mg L^{-1} ampicillin and incubated overnight at 37 °C. As pWS082 contains a GFP cassette in the sgRNA cloning site, non-fluorescent colonies are assumed positive integrants. Plates were viewed under UV light (365 nm; transilluminator), however GFP containing colonies are also visibly green to the naked eye. Non-fluorescent colonies were selected and grown in 10 mL LB medium containing 100 mg L^{-1} ampicillin at 37 °C with shaking (200 rpm). Overnight cultures were prepared using Wizard® Plus SV Minipreps DNA Purification System (Promega) prior to Sanger sequencing (Australian Genome Research Facility, Australia) with primer pWS082 seq (**Table 3**) to confirm correct integration of sgRNA sequences.

Donor DNA construction

Donor DNA was constructed by amplification of overlapping 60 bp primers, ECM33-HDR F and ECM33-HDR R, with the mutated sequence (GGT>TAA; **Table** 3) corresponding to the desired Ecm33p (Gly61stop) sequence, flanked by 49 bp 5' and 47 bp 3' of homology. Primers were amplified with Velocity DNA Polymerase (BIO-21098; Bioline) in a standard 50 µL reaction using 8 µL of each primer (100 µM), 0.5 µL of 100 mM dNTP mix (BIO-39028; Bioline), 10 µL of supplied 5x HIFI buffer, 1 µL of polymerase (2 U) and Milli-Q® water to 50 µL. Standard cycling conditions were followed; initial denaturation (98 °C; 2 min), followed by 30 cycles of denaturation (98 °C; 30 sec), annealing (57 °C; 30 sec) and extension (72 °C; 10 sec) and a final extension step (72 °C; 4 min). Donor DNA was semi-quantified by gel electrophoresis using DNA molecular weight markers (Hyperladder™ 50 bp (BIO-33054); Bioline).

Plasmid digestion

Plasmids pWS-ECM33 and pWS173 (**Table 2**) were linearised prior to co-transformation. pWS173 was digested with BsmBI (New England Biolabs) and the largest restriction fragment (10.4 kb) containing Cas9 and KanMX expression constructs was isolated and purified using Wizard® SV Gel and PCR Cleanup System (Promega). Immediately prior to transformation, 200 ng of pWS-ECM33 were linearised in a 10 µL reaction with EcoRV-HF. The entire 10 µL reaction was used in the transformation reaction without purification.

Transformation of CRISPR plasmid components

Yeast transformation was undertaken using the lithium acetate method (Gietz & Schiestl, 2007). An overnight culture was diluted 1 in 100 in 100 mL YEPD and grown for 4–5 h until an OD_{600} of 0.4–0.5. Cells were pelleted and washed twice in 0.1 M lithium acetate and resuspended to a final volume of 500 µL in 0.1 M lithium acetate. For each transformation, 50 µL of cells were pelleted prior to resuspension with 54 µL DNA mixture (100 ng linearised pWS173, 200 ng digested pWS-ECM33 and ~5 µg of donor DNA), 10 µL salmon sperm DNA (10 mg mL^{-1}), 36 µL 1 M lithium acetate, and 260 µL 50% w/v polyethylene glycol 4000. The reaction was incubated at 30 °C for 30 min, prior to heat shock (42 °C, 30 min). Following centrifugation (20,000 x *g*, 1 min), the supernatant was discarded and the cells resuspended in 1 mL YEPD for recovery at 30 °C for 2.5 hours. The cells were pelleted by centrifugation and resuspended in 200 µL sterile Milli-Q® water prior to plating onto YEPD agar containing G418 and incubation (30 °C, 48 h).

Confirmation of yeast mutants

Single G418 resistant colonies were picked and grown 72 h in YEPD (without antibiotic) to enable the yeast cells to be 'cured' of the plasmids. The cultures were single-colony purified on YEPD agar. Single colonies were replica plated onto YEPD agar with and without G418 to confirm plasmid loss. Isolates lacking the plasmids were G418 sensitive and unable to grow in the presence of antibiotic. Genomic DNA was isolated from these strains using phenol/chloroform and glass beads (Adams *et al.*, 1998), and used as template DNA for PCR amplification of *ECM33* using Velocity DNA Polymerase (Bioline) with primers ECM33 A and ECM33 D (**Table 3**). PCR reactions were prepared following the NEB Enzymatic PCR Cleanup protocol using Shrimp Alkaline Phosphatase and Exonuclease 1 (https://www.neb.com/-/media/nebus/files/application-notes/enzymatic-pcr-cleanup-exo_sap-app-note.pdf?la=en&rev=57a419a3155043c89d7365eda33b2ae9). Sanger sequencing was performed with primer ECM33 seq to confirm correct donor DNA integration (Australian Genome Research Facility, Australia). DNA sequence analysis was by Clustal Omega (Sievers *et al.*, 2011) (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Laboratory scale (100 mL) fermentations: evaluation in Chemically Defined Grape Juice Medium (CDGJM)

Fermentations were conducted using a robotic fermentation platform to allow for automated sampling (Peter *et al.*, 2018). Fermentation performance was evaluated in CDGJM (115 g L^{-1} glucose, 115 g L^{-1} fructose) supplemented with a mixture of amino acids and ammonium chloride as the Yeast Assimilable Nitrogen (YAN) (Jiranek *et al.*, 1995). Two concentrations were chosen, 90 mg YAN L^{-1} and 250 mg YAN L^{-1}, which

in terms of fermentation performance are considered low and sufficient amounts (Jiranek *et al.*, 1995; Mendes-Ferreira *et al.*, 2004). Starter cultures were prepared from a single yeast colony grown overnight (28 °C; 180 rpm) in a 1:1 ratio of YEPD and CDGJM. Yeast counts were quantified by hemocytometer, washed and resuspended in CDGJM (as per fermentation) prior to inoculation at 5 x 10^6 cells mL^{-1}. Fermentations (100 mL) were conducted in triplicate at 23 °C (350 rpm). Fermentation progress was monitored by weight loss, with Clinitest® (Bayer) tablets being used to determine completion (< 2.5 g L^{-1} total sugar) following a minimal change in weight loss over a 24 h period.

Static fermentations followed the same setup, with stirring once daily for 5 minutes to resuspend the cultures prior to sampling. Fermentation endpoints were determined using Clinitest® (Bayer) tablets.

Fluorescent and confocal microscopy, and cell size determination

To assess chitin and mannoprotein localisation, cells were grown for 48 h in CDGJM containing 250 mg YAN L^{-1} and stained with either Calcofluor White (chitin) or FITC-ConA (mannoproteins) (Okada & Ohya, 2016) prior to fluorescence microscopy (ECLIPSE 50i/DS-2MBW, Nikon, Japan) or confocal microscopy (A1R/DS-Ri1, Nikon, Japan) using a UV-2A filter. Images were taken and manipulated using NIS-Elements Basic Research (fluorescent microscopy) or Advanced Research (confocal microscopy) software (Nikon, Japan). For confocal images, a Z-series was acquired prior to visualising maximum intensity projections. Cell size and cell volume ($V=^4/_3\ \pi\ r^3$; where r is the radius) were determined for each strain, from 100 randomly chosen cells using the measure function in NIS-Elements Basic Research software.

Yeast settling assay

To assess the settling ability of yeast mutants, fermentations were performed in CDGJM with adequate nitrogen (as previously mentioned) in 250 mL bottles. Fermentations were progressed for 72 h until yeast reached stationary phase, where bottles were static overnight. Overnight static cultures were photographed side-by-side using a Nikon D3400 for comparison of settling phenotypes.

Statistical methods

All data were statistically analysed using GraphPad Prism 8.0.0 software. Statistics of fermentation completion times was performed using the Unpaired t-Test function at the 95% confidence level.

Results

High efficiency introduction of *ECM33* G61stop mutation by CRISPR/Cas9

To introduce a loss of function in *ECM33*, an early stop codon was introduced into the *ECM33* open reading frame at glycine 61 (181GGT>TAA) according to the Ellis Laboratory CRISPR/Cas9 protocol (https://benchling.com/pub/ellis-crispr-tools). An early stop codon was introduced at glycine 61 to create a 60 amino acid non-functional protein in comparison to the 429 amino acid Ecm33p in wild-type EC1118. This mutation was designed such that the PAM site required for Cas9 activity was removed to prevent further double stranded breaks.

Plasmid pWS173 (Addgene plasmid #90960) was used for G418 selection, as a dominant antibiotic selectable marker in prototrophic strains, to avoid additional genome

modification, as for auxotrophic selection. To confirm correct integration of sequences and assess CRISPR/Cas9 efficiency, the mutation site within the *ECM33* ORF of 7 random transformants and wild type EC1118 were PCR amplified and Sanger sequenced. Sequencing revealed that 6 out of 7 random colonies contained a correct integration of the nonsense mutation across both alleles when compared to wild type EC1118 (**Figure 1)**. Further characterisation of the *ECM33* disruption mutant was undertaken using isolate t1, referred to as EC1118 *ecm33*.

Disruption of *ECM33* in EC1118 results in improved fermentation efficiency in low and adequate nitrogen conditions

Disruption of *ECM33* in wine yeast derivative C911D has previously been shown by our laboratory to improve fermentation performance in CDGJM under limiting (55 mg YAN L^{-1}) and sufficient nitrogen (441 - 450 mg YAN L^{-1}) conditions (Zhang *et al.*, 2018). The *ecm33*Δ mutant outperformed C911D by 33% under both conditions, although the fermentation under limiting YAN was severely protracted, as to be expected (Mendes-Ferreira *et al.*, 2004).

The *ECM33* disruption mutant (EC1118 *ecm33*), generated by CRISPR/Cas9 gene editing of the diploid EC1118, was initially evaluated for fermentation duration in CDGJM. However, the sugar and nitrogen content were altered to better reflect white juice contents, i.e., 230 g L^{-1} sugar supplemented with either limiting (90 mg YAN L^{-1}) or sufficient (250 mg YAN L^{-1}) nitrogen. EC1118 *ecm33* demonstrated a higher rate of weight loss and completed fermentation significantly faster in both limiting and sufficient nitrogen conditions ($P = 0.019$ and 0.013 respectively; **Figure 2A** and **B**). In the presence of sufficient nitrogen, the mean time to fermentation completion for EC1118 *ecm33* was

194 ± 9.2 hours compared to 223 ± 7.5 hours in EC1118, which corresponds to a 13% reduction in duration. Fermentation improvement was more evident under limiting nitrogen conditions, where the mean completion time was 350 ± 13 hours for EC1118 *ecm33* compared to 437 ± 29 hours for EC1118 – representing a 20% reduction in fermentation duration. These results differed from our earlier work (Zhang *et al.* 2018), wherein a decrease in fermentation duration of approximately 30% was seen across both high and low nitrogen conditions in CDGJM. Whilst this disparity may reflect differences in genetic background (EC1118 *vs* L2056), ploidy (haploid *vs* diploid) as well as temperature and nitrogen conditions representative of a white wine-like fermentation, our results suggest that the fermentation phenotype of the *ecm33* mutant may be related to nitrogen utilisation efficiency.

Disruption of *ECM33* results in increased chitin distribution, enlarged cells and cellular aggregation in the EC1118 background

EC1118 and EC1118 *ecm33* were evaluated by fluorescence microscopy for chitin (Calcofluor white) and mannoprotein (FITC-ConA) distribution. In contrast to early electron microscopy work where the distribution of mannoproteins was affected by disruption of *ECM33* (Pardo *et al.*, 2004), there were no differences between EC1118 and EC1118 *ecm33* (**Figures 3A and B)**. Distribution of chitin was, however, consistent with other reports of *ECM33* disrupted yeasts (Zhang *et al.*, 2018). EC1118 *ecm33* showed an increased distribution of chitin across the cell wall as well as in the bud scars (**Figure 4B**), whereas in the wild-type (EC1118), chitin was predominantly localised at bud scars with the remainder of the cell having minimal fluorescence (**Figure 4A**). In addition to chitin distribution, *ECM33* disrupted cells also demonstrated an enlarged cell

and aggregation phenotype. To assess cell size, 100 cells of each strain were measured; average size and standard deviation was calculated and converted to cell volume, with cell shape assumed as spherical. EC1118 *ecm33* cells were significantly larger (145.12 ± 1.18 μm^3) in comparison to those of EC1118 (86.16 ± 0.43 μm^3; **Table 4**). The cells of EC1118 *ecm33* were aggregated, with small clusters of cells seemingly joined at either the cell wall or bud scars (**Figure 4B**), whereas EC1118 cells occurred individually or as doublets (mother-daughter cells) joined during budding (**Figure 4A**).

To further investigate the chitin distribution and aggregation phenotypes, EC1118 and EC1118 *ecm33*, and C911D and C911D *ecm33*Δ (Zhang *et al*., 2018) were examined by confocal microscopy after staining with Calcofluor white (**Figures 5A–D**). In both instances, the *ecm33* mutant cells showed increased fluorescence across their cell walls (**Figures 5B and D**) when compared to their respective parental strains (**Figures 5A and C**). Again, these results indicate an increased distribution of chitin across the cell surface in *ecm33*. In the parental strains, Calcofluor White was almost exclusively localised to the bud scars with minimal staining around the remainder of the cell wall. Our findings confirm previous results with C911D and C911D *ecm33*Δ when grown in YEPD and stained with the azo dye, Congo Red to visualise chitin (Zhang *et al.*, 2018). Typical of diploid yeast, the cells in EC1118 were single cells, with no obvious aggregation (**Figure 5a**). Cellular aggregation in the C911D *ecm33*Δ mutant was not a distinguishing phenotype, as the parental strain, being haploid, aggregated into small clumps of single cells.

Cellular aggregation as a result of *ECM33* disruption results in a loss of fermentation efficiency in semi-static conditions

To investigate the winemaking consequences of the aggregation phenotype, laboratory-scale fermentations were undertaken in CDGJM with low or adequate YAN (as previously described), but in semi-static conditions. Fermentations were agitated only once per day for sampling, thereby simulating industrial fermentations such as for white wines that are typically static, or red wines that are agitated intermittently during cap management. Endpoint analysis of fermentations revealed no significant difference in duration between EC1118 and the *ecm33* disruption mutant under both nitrogen conditions (**Figure 6**). Under these largely static conditions, mean fermentation duration was significantly extended ($P < 0.05$) for both strains under adequate nitrogen conditions (EC1118, +95 h; EC1118 *ecm33*, + 144 h) and for EC1118 *ecm33* under limiting nitrogen conditions (+58.3 h). EC1118 under limiting nitrogen condition showed a mean decrease in fermentation time of 45.3 h, however, this was not significantly different ($P = 0.11$).

Cellular aggregation of *ECM33* disrupted yeasts results in enhanced settling

To more directly link increased premature settling of yeast to slowed fermentation kinetics, cultures were grown in CDGJM (230 g L^{-1} sugar, 250 mg YAN L^{-1}) with agitation until stationary phase (~72 h), prior to being left to settle overnight. In both genetic backgrounds, *ecm33* showed clearer fermentation media with larger yeast sediments on the flask bottom, which was indicative of increased settling, compared to the respective parental strains (**Figure 7**). Ploidy influences on culture settling (Hope & Dunham, 2014) were noted, with the haploid, C911D, settling more than the diploid, EC1118, because of flocculation which was independent of *ECM33* disruption. In

contrast, the diploid cells (EC1118) represented a homogenous suspension, displaying little to no settling in the fermentation medium.

Discussion

Evaluation of the cellular impact of gene disruptants is a well-tested means of determining gene function. This information in turn may be valuable to create targeted improvement in *Saccharomyces cerevisiae* strains, making them 'fit-for-purpose'. This study focuses on *ECM33* (*YBR078W*), which encodes a glycosyl-phosphatidylinositol (GPI)-anchored cell surface protein; the specific function of which remains poorly defined. To date, research has focused on mutants in the laboratory strains S288C (Breslow *et al.*, 2008) and BY4741 (García *et al.*, 2015), with contradictory findings. For example, a competitive fitness outcome was dependent upon the allelic (homozygous (Δ/Δ) (Brown *et al.*, 2006), heterozygous (WT/Δ) (Deutschbauer *et al.*, 2005), and hypomorphic (DamP) (Breslow *et al.*, 2008)) mutation. Very few of the phenotypes studied have been related to fermentation, with the exception of Zhang *et al.* (2018), where the authors reported that deletion of the open-reading frame of the *ECM33* (*YBR078W*) gene in a haploid wine yeast improved fermentation performance in wine and wine-like fermentations. To date, Ecm33p, has been associated with cell wall integrity, mannoprotein assembly and distribution, and protein *N*-glycosylation (Pardo *et al.*, 2004), as well as glucose uptake and target of rapamycin complex 1 (TORC1) signalling (Umekawa *et al.*, 2017). The relevance of these biological processes in relation to overall fermentative capacity is unclear. Whilst *ECM33* is of particular interest not only in fundamental but applied research, further characterisation of the gene is warranted in order to verify causality in relation to improved fermentation phenotypes, and the possible targeting of this gene in strain improvement programs.

In this study, we utilised the CRISPR/Cas9 system to further define the morphological changes and fermentation effects of the disruption of *ECM33* in the diploid commercial wine yeast, EC1118. Previously, CRISPR/Cas9 was used in wine yeasts to lower ethyl carbamate production (Vigentini *et al.*, 2017), increase floral aroma compound concentrations (Cordente *et al.*, 2018) and simultaneously increase glycerol and acetate ester production (van Wyk *et al.*, 2020). We have previously shown haploid wine yeast lacking Ecm33p were better able to consume glucose and fructose during fermentation in a chemically defined grape juice medium and a juice (Zhang *et al.*, 2018). As such, we were interested to study this gene deletion in the sequenced (Novo *et al.*, 2009) commercial wine strain, Lalvin EC1118, because haploid and diploid yeasts are known to behave differently, especially in industrial fermentations such as commercial wine production. We introduced a non-sense mutation in *ECM33*, giving rise to a premature stop codon (Gly61stop (+181 GGT>TAA) to give a truncated protein with only 60 amino acid residues (14%) of the native protein. This substitution was predicted to be deleterious to the protein structure using PROVEAN software (Choi & Chan, 2015) and MutPred-LOF http://mutpredlof.cs.indiana.edu/ (Stenson *et al.*, 2017). The unstable truncated protein lacks the C-terminal hydrophobic region found in GPI-anchored proteins required for the GPI Transamidase Complex activity at the endoplasmic reticulum lumen, resulting in attachment of the GPI-anchor moiety needed to anchor Ecm33p to the plasma membrane to function (Fraering *et al.*, 2001, Galian *et al.*, 2012).

The phenotypic characteristics associated with the disruption of *ECM33* in EC1118 via the introduction of a premature stop codon, were consistent with previous research. Morphologically, we observed a significant increase in mean cell size (calculated as cell volume), which was comparable to the haploid deletion mutants (from EuroScarf) used by Jorgensen *et al.* (2002). There was also an increase in chitin

distribution across the cell wall, rather than at the bud scars (Figures 4A, 4B, 5A and B), similar to the *ecm33*Δ mutants generated by Zhang *et al.* (2018) and Pardo *et al.* (2004) in haploid wine and laboratory strains, respectively.

Homozygous deletion of *ECM33* in the haploid wine strain, C911D, was also associated with more efficient sugar catabolism under wine-like fermentation conditions, which was independent of nitrogen availability (Zhang *et al.*, 2018). The disruption of *ECM33* in EC1118 also resulted in shortened fermentation duration, however, the reduction (as a percentage of the wild-type) reflected the initial nitrogen content. Fermentation timing was reduced by ~30% for EC1118 *ecm33* under N limitation (90 mg YAN L^{-1}; **Figure 2B**), compared to 13% when supplied with sufficient nitrogen (250 mg YAN L^{-1}; **Figure 2A**). The occurrence of this may be due to effects of ploidy (haploid vs diploid) and differing strain backgrounds. EC1118 is described as having a fast fermentation rate, and low N requirement (https://www.lallemandbrewing.com/en/uni

ted-states/product-details/lalvin-ec-1118/), whereas L2056 (the ancestral strain of C911D in Zhang *et al.* (2018)) has a moderate fermentation rate and nitrogen requirement (https://www.lallemandwine.com/en/eastern-countries/products/catalogue/wine-yeasts/3

1/lalvin-l-2056/). The smaller reduction in fermentation duration under low N conditions could be related to the low N requirement and inherent, recognised superior performance of EC1118, although this needs to be more extensively tested.

The fact that mannoprotein distribution in the EC1118 *ecm33* mutant was unchanged, thereby diverging from the findings of Pardo *et al.* (2004), could relate to that groups using the haploid laboratory strain FY1679, isogenic to S288C, as the background. The authors alluded to a defective assembly of mannoproteins on the cell wall on the basis of transmission electron microscopy data, which reveals the mannoprotein outer

layer to be very thin or completely absent in many regions of the cell surface. These differences may reflect the lack of sensitivity and resolution by fluorescence microscopy to efficiently show these changes in EC1118 *ecm33*, if in fact they were present. We also observed the formation of small aggregates of cells, a previously unreported phenotype in relation to *ecm33* mutants in haploid genotypes. These mutants appear to form aggregates by lack of dissociation post-budding, which is similar to the phenomenon observed in snowflake yeast (Ratcliff *et al.*, 2012). Snowflake phenotypes have been observed in adaptively evolved yeasts (Ratcliff *et al.*, 2012, Oud *et al.*, 2013, Ratcliff *et al.*, 2013, Kayacan *et al.*, 2020), as well as strains with *ACE2 (*King & Butler, 1998, Voth *et al.*, 2005, Oud *et al.*, 2013) and/or *CBK1 (*Voth *et al.*, 2005) deletions, which are key genes involved in bud separation.

Yeast exhibiting the snowflake phenotype also exhibit an enhanced settling phenotype (Voth *et al.*, 2005, Ratcliff *et al.*, 2012, Oud *et al.*, 2013, Ratcliff *et al.*, 2013, Kayacan *et al.*, 2020). We observed this in EC1118 *ecm33* when stationary phase wine-like fermentations were left to settle overnight. The fermentation impact of this phenotype was also explored in 100 mL fermentations, which were mixed once per day, as might occur in red wine fermentations. Any beneficial phenotype previously seen to arise from the mutant under agitation was lost, with the fermentation duration showing no statistical significance between strains under static conditions in CDGJM containing sufficient (250 mg YAN L^{-1}) or limiting nitrogen (90 mg YAN L^{-1}). This outcome nullifies some commercial benefits of this mutant, should these gene modifications be approved, unless agitation of commercial fermentations is an option. The cost effectiveness of more rapid fermentations vs energy costs for agitation will no doubt vary from winery to winery.

Whilst mutants created in this manner would also result in regulation as a genetically modified organism (GMO), there is still potential for its use in some

jurisdictions. In the United States and Canada for example, legislation allows for exclusion from GMO labelling as yeast is regarded a processing aide. In Australia and the European Union on the other hand, this would require no detectable DNA from the GMO in the end product (Hanlon & Sewalt, 2020), a difficult feat to achieve.

To conclude, we have successfully created an *ECM33* disruption mutant in a diploid wine yeast that exhibits previously identified beneficial phenotypes in agitated fermentations. We have also identified a novel link to yeast aggregation and sedimentation, which to the best of our knowledge has not been previously reported. Due to the sedimentation phenotype of the *ecm33* strain, it appears agitation will be required to realise the full benefit of its accelerated fermentation kinetics. The phenotypic differences seen for *ecm33* across haploid lab strains and diploid commercial strains, highlight the importance of confirming phenotypic characterisation in the appropriate strain background.

Acknowledgements

The authors would like to thank Nick van Holst for his technical assistance, Dr Gwenda Mayo from Adelaide Microscopy for training and technical assistance with and use of the confocal microscope and Tom Ellis and William Shaw from Imperial College London for providing plasmids pWS173 and pWS082.

Funding

This project was supported by funding from Wine Australia [UA1803_2.1] and The Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number IC170100008), which is funded by the Australian Government with additional support from Wine Australia and industry

partners. Wine Australia invests in and manages research, development and extension on behalf of Australia's winegrowers and winemakers and the Australian Government. Tom Lang is a recipient of a University of Adelaide Faculty of Sciences Divisional Scholarship and Wine Australia Supplementary Scholarship (AGW Ph1603).

The University of Adelaide is a member of the Wine Innovation Cluster (http://www/thewaite.org/waite-partners/wine-innovation-cluster/).

Conflicts of Interest

The authors declare there are no conflicts of interest regarding the study.

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Tables

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	**Genotype**	**Source**
EC1118	Lalvin EC1118	Lallemand, France
EC1118 *ecm33*	EC1118 *ECM33* Gly61stop (181GGT>TAA)	This study
C911D	L2056 derivative; *MATα, Δho*	Walker *et al.* (2003)
C911D *ecm33*Δ	*MATα, Δho, ecm33*Δ*::KanMX*	Zhang *et al.* (2018)

Table 2. Plasmids used in this study

Plasmid	**Description**	**Source**
pWS082	sgRNA cloning vector	Shaw *et al.* (2019). Addgene plasmid* #90516
pWS173	Cas9 linear co-transformation vector	Addgene plasmid* #90960
pWS-ECM33	sgRNA vector targeting ECM33	This study

*https://www.addgene.org/

Table 3. DNA oligonucleotide primers used in this study.

Primer	**Sequence (5'-3')**	**Source**
sgECM33 F	GACTTTGTTGGTAACTTGACCATCAC	This study
sgECM33 R	AAACGTGATGGTCAAGTTACCAACAA	This study
pWS082 seq	GTCATCTGGAGGTCCTGTGTTC	This study
ECM33-HDR F	GGACAAAATCTCCGGTTGTAGTACCATTGTTGG TAACTTGACCATCACCTAAGACTTGGG	This study
ECM33-HDR R	GAACCATCAATCTCTTGGATACTAGCCAAAGCA GCGGAACCCAAGTCTTAGGTGATGGTC	This study
ECM33 A	AGCCGGTATAAATATTCAATGTCAA	SGDP
ECM33 D	AGAAGAGCAGTAAAGATGGCAGTTA	SGDP
ECM33 seq	GGATTAGGGCGAGTAATGAA	This study

*SGDP; *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group /yeast_deletion_project/deletions3.html).
All primers were purchased from Sigma-Aldrich, cartridge purified and supplied at 100 µM in water.
** Underline denotes GGT>TAA base pair change

Table 4. Calculated mean volumes (μm^3) from a sample of 100 randomly measured cells

	Mean	Minimum	Maximum
EC1118	86.16 ± 0.43	21.88	209.60
EC1118 *ecm33*	145.12 ± 1.18	19.86	414.39

Figures

A

```
wt      GTTGGTAACTTGACCATCACCGGTGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t7      GTTGGTAACTTGACCATCACCGGTGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t1      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t2      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t3      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t4      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t5      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
t6      GTTGGTAACTTGACCATCACCTAAGACTTGGGTTCCGCTGCTTTGGCTAGT    51
        ********************   ***************************
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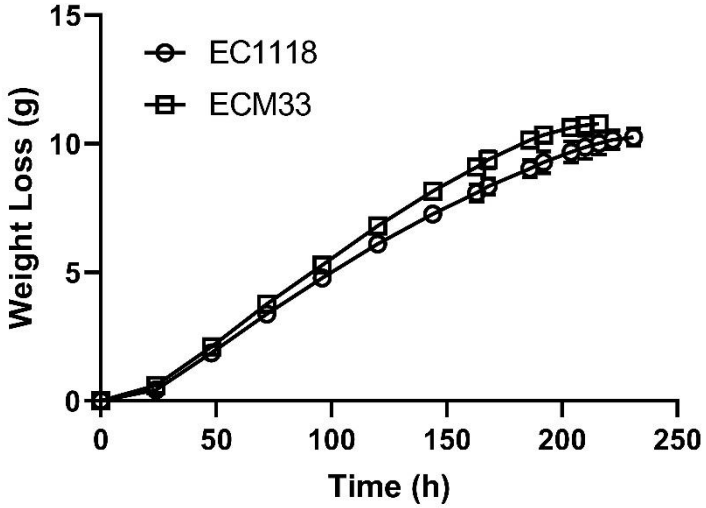
B

```
wt      VGNLTITGDLGSAALAS   17
t7      VGNLTITGDLGSAALAS   17
t1      VGNLTIT*DLGSAALAS   16
t2      VGNLTIT*DLGSAALAS   16
t3      VGNLTIT*DLGSAALAS   16
t4      VGNLTIT*DLGSAALAS   16
t5      VGNLTIT*DLGSAALAS   16
t6      VGNLTIT*DLGSAALAS   16
        ******* *********
```

Figure 1: Confirmation of introduced premature stop codon by Sanger sequencing of EC1118 and 7 randomly selected transformants

DNA sequence alignment (Clustal Omega) (A) showing introduced early stop codons (bold) and original PAM sequence (underlined). Amino acid alignment (Clustal Omega) (B) showing introduced translation stop site.

A



B

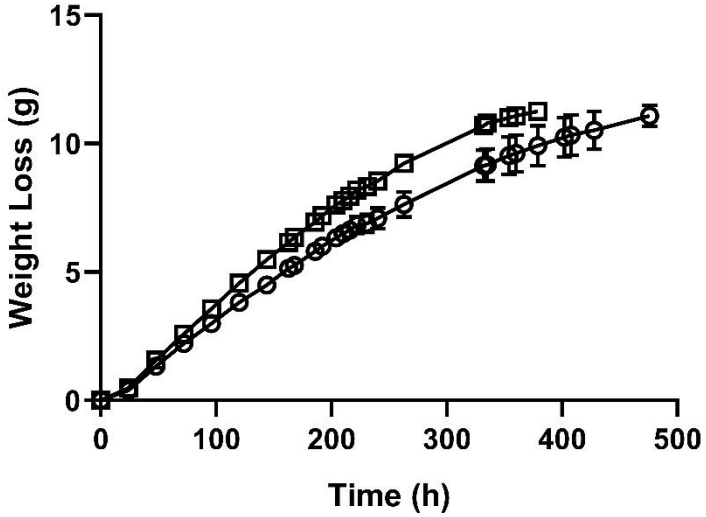


Figure 2: Fermentation performance of EC1118 and EC1118 *ecm33*

Fermentations were conducted in CDGJM with 230 g L^{-1} sugar and variable nitrogen. (A) 250 mg L^{-1} YAN or (B) 90 mg YAN L^{-1}. Fermentation progress was recorded as cumulative weight loss. EC1118 (wild type) (○) and EC1118 *ecm33* (□). Values represent triplicate fermentations ± standard deviation (as error bars).

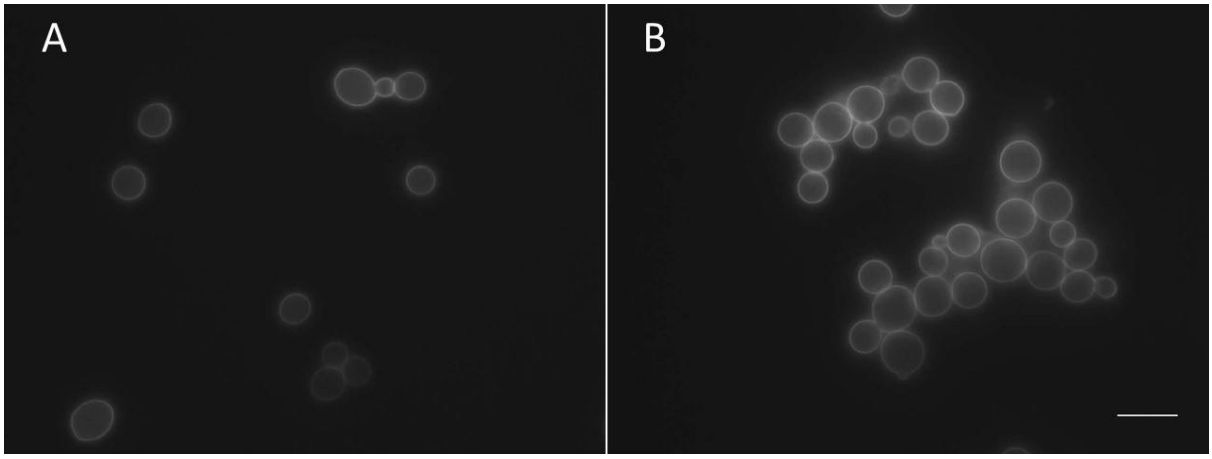


Figure 3: FITC-ConA fluorescent microscopy

FITC-ConA fluorescent microscopy of EC1118 (A) and EC1118 *ecm33* (B) from 48h fermentations in CDGJM with 250 mg YAN L^{-1}. Scale bar indicates 10 µm.

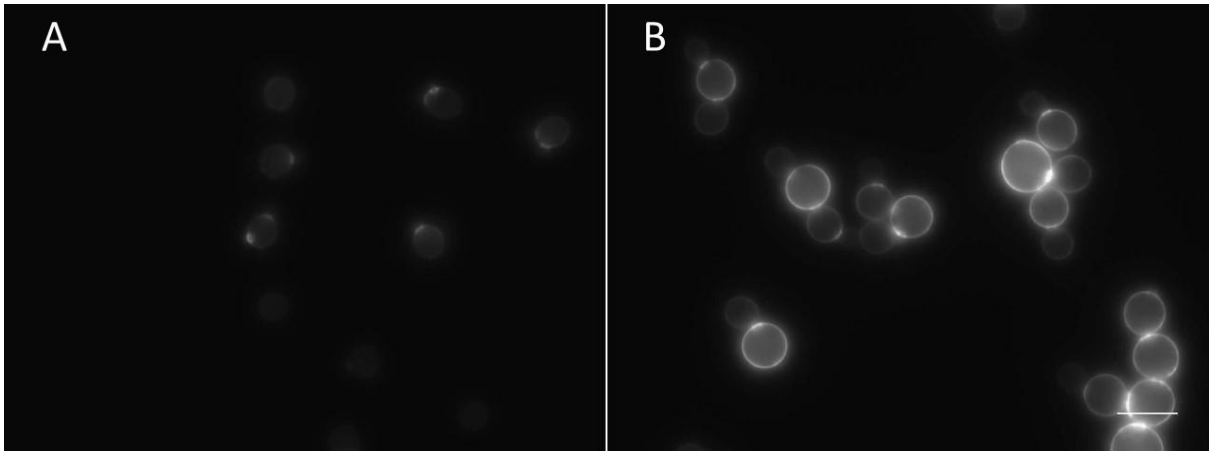


Figure 4: Calcofluor White fluorescent microscopy

Calcofluor White fluorescent microscopy of EC1118 (A) and EC1118 *ecm33* (B) from 48h fermentations in CDGJM with 250 mg YAN L^{-1}. Scale bar indicates 10 µm.

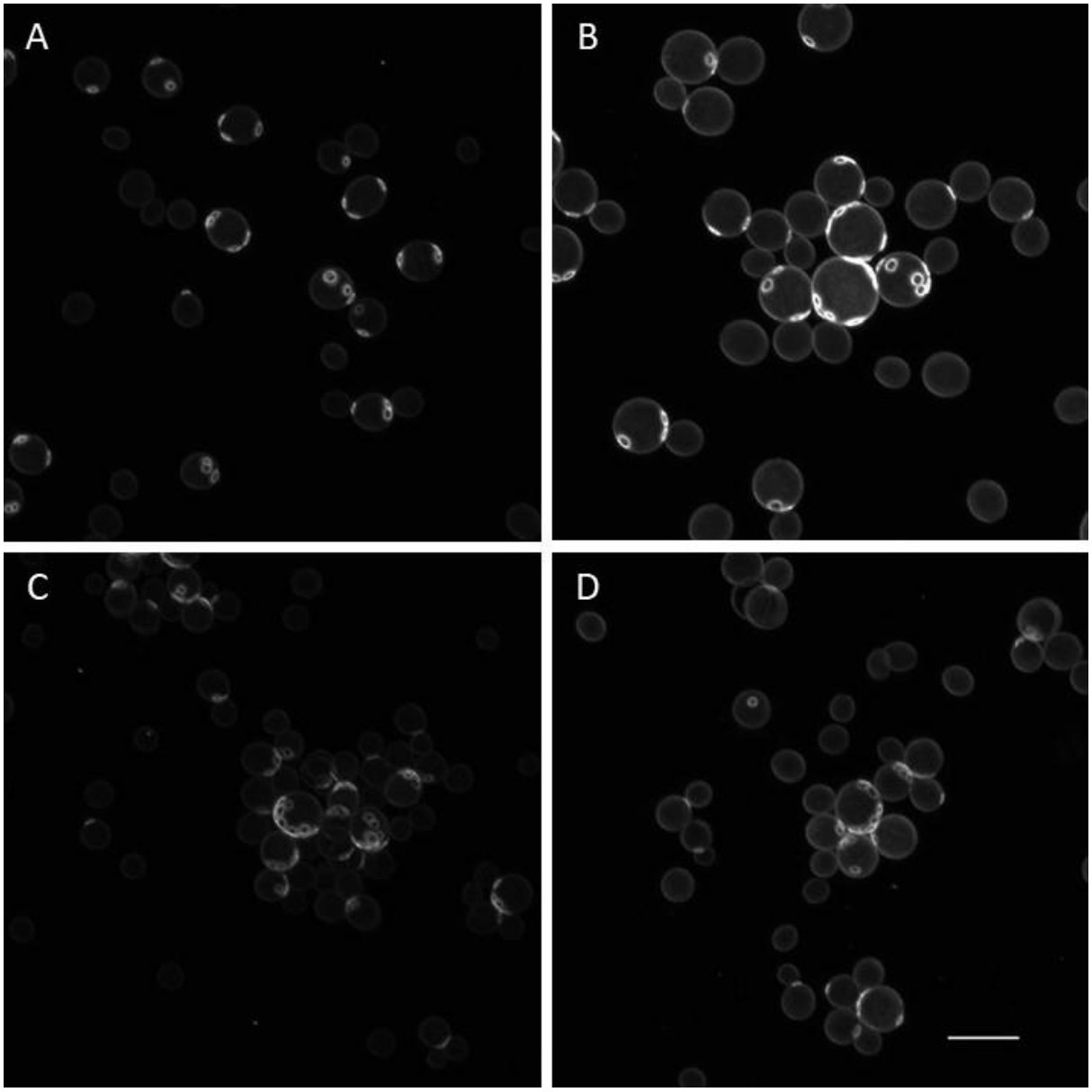
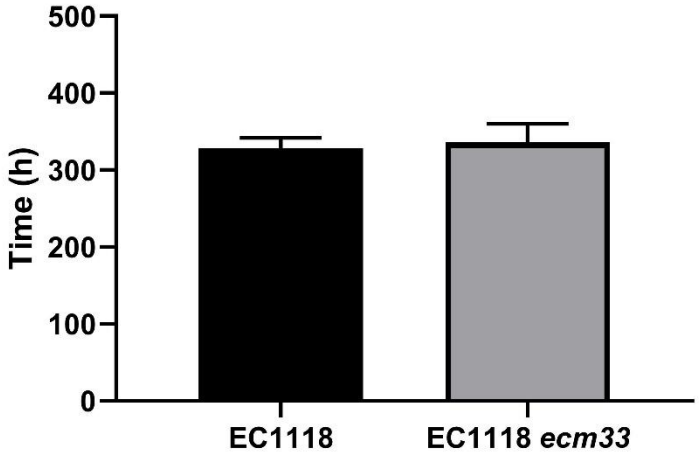


Figure 5: Calcofluor White confocal microscopy

Calcofluor White confocal microscopy for EC1118 (A), EC1118 *ecm33* (B), C911D (C) and C911D *ecm33*Δ (D) from 48h fermentations in CDGJM with 250 mg YAN L^{-1}. Scale bar indicates 10 µm.

A



B

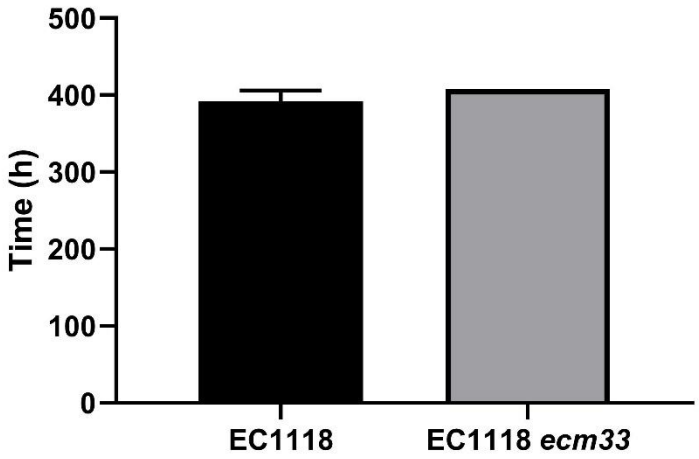


Figure 6: Fermentation performance of EC1118 and EC1118 *ecm33* under semi-static conditions

Average time to fermentation completion under semi static conditions in CDGJM containing A: sufficient (250 mg L^{-1}) or B: limiting (90 mg L^{-1}) YAN.

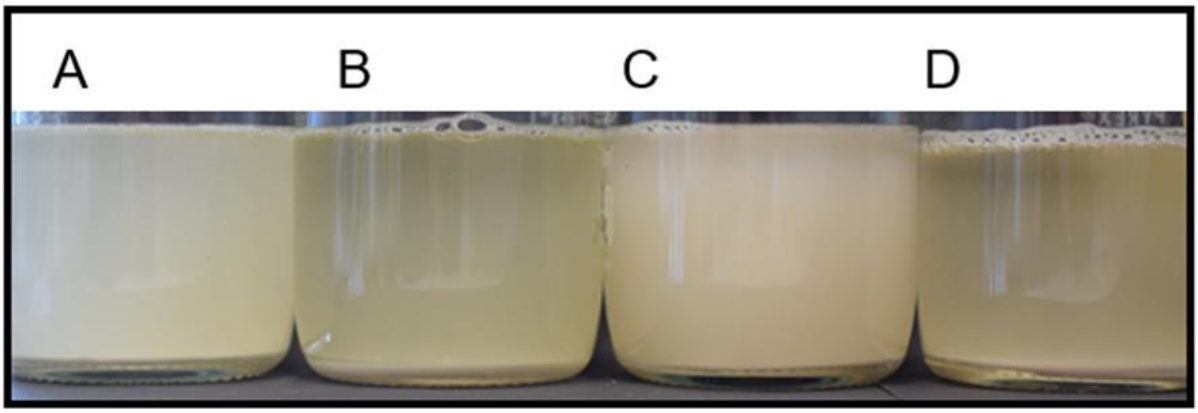


Figure 7: Settling assay of *Ecm33* mutant strains in CDGJM.

CDGJM was fermented with shaking for 72 h to stationary growth phase prior to being left to settle overnight to assess the settling ability of yeasts. Left to right; C911D (haploid, wild type), C911D *ecm33*Δ, EC1118 (diploid, wild type) and EC1118 *ecm33*.

Chapter 3

"Effect of 'loss of function' mutation in *SER1* in wine yeast: Fermentation characteristics in co-inoculation with non-*Saccharomyces*"

This chapter is written as a manuscript for OENO One

Statement of Authorship

Title of Paper	Effect of 'loss of function' mutation in SER1 in wine yeast: Fermentation characteristics in co-inoculation with non-Saccharomyces
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Tom Lang
Contribution to the Paper	- Conceptualisation - Laboratory work/Analysis - Writing
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Effect of 'loss of function' mutation in *SER1* in wine yeast: Fermentation characteristics in co-inoculation with non-*Saccharomyces*

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Abstract

In wine fermentation, improved wine complexity and sensorial properties can arise from the use of non-*Saccharomyces* yeast. Generally lower in alcohol tolerance, such strains often can't finish fermentation, therefore requiring a second inoculation of the more traditional *Saccharomyces cerevisiae*, usually added on day 3. This sequential approach affords non-*Saccharomyces* time to make an impact before being overtaken by *S. cerevisiae*. But two inoculations are inconvenient, therefore the identification of a slow growing *S. cerevisiae* strain that can be used in a single co-inoculation with the non-*Saccharomyces* yeast is highly attractive.

In this study we investigated the use of the naturally occurring 'loss of function' *SER1* variant, identified in a sake yeast, for the purposes of co-inoculated wine fermentations. The *SER1-232*(G>C; G78R) change was introduced into the commonly used wine strain, EC1118 via CRISPR/Cas9 editing. The *SER1-232*(G>C; G78R) mutant grown in a chemically defined grape juice medium had slower growth, longer fermentation duration and increased acetic acid, succinic acid, and glycerol concentrations in the wine. Simultaneous inoculation of the slower-growing mutant with a *Metschnikowia pulcherrima* or *Lachancea thermotolerans* strain in sterile Sauvignon

Blanc juice resulted in differences in sensorial compounds, most likely derived from the presence of non-*Saccharomyces* yeasts during fermentation. The *SER1* mutation in EC1118 led to completion of fermentation with *M. pulcherrima* MP2, and in fact improved the viability of the non-*Saccharomyces* yeast in contrast to when fermented as a monoculture. The *SER1-232*(G>C; G78R) mutant also promoted the growth of the SO_2 sensitive *L. thermotolerans* strain, Viniflora® Concerto™ in a high SO_2 juice, and its subsequent dominance during fermentation. In co-fermentations with wild-type EC1118, the Concerto™ population was substantially reduced with no significant changes in wine properties. This research adds to our understanding of the use of a novel slow growing *S. cerevisiae* yeast in wine fermentations co-inoculated with non-*Saccharomyces* strains.

Keywords: *Saccharomyces cerevisiae*, *SER1*, CRISPR/Cas9, co-inoculation, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, SO_2

Introduction

A simplistic view of winemaking involves the biochemical transformation of hexose sugars present in grape must to ethanol and carbon dioxide by *Saccharomyces cerevisiae* and/or other *Saccharomycetaceae*. *S. cerevisiae* is generally the dominant fermentative species, as it is able to withstand fermentation related stresses (especially ethanol) as well as inhibit other species, such that fermentation completes in a timely manner (Albergaria & Arneborg, 2016). As such, it is common practise for grape must to be inoculated with commercial monocultures to ensure fermentation reliability, although wines created in this way can lack sensorial complexity (Padilla *et al.*, 2016). Conversely, wine made by "wild" fermentation, making use of the native microflora present on grapes, can provide a complexity not achieved through monoculture (Reviewed in Belda *et al.* 2017). Whilst these un-inoculated or 'spontaneous/wild' fermentations can be risky because of potential microbial failure if incorrectly managed, between 3-6% of wines produced in Australia are made by this means, in particular, within the premium wine sector (The Australian Wine Research Institute, 2019). A more recent and reliable alternative is the use of selected non-*Saccharomyces* strains co-cultured with *S. cerevisiae* allowing for increased complexity and mouthfeel (attributes associated with wine quality) and consistent fermentation reliability (Padilla *et al.*, 2016). The use of these techniques is reflected in the increasing availability of commercial non-*Saccharomyces* starter cultures (including *Metschnikowia spp*, *Lachancea thermotolerans*, and *Torulaspora delbrueckii*) either as single strains or mixed with other non-*Saccharomyces* and/or *S. cerevisiae*.

Mixed fermentations involving *S. cerevisiae* and non-*Saccharomyces* yeast are generally performed using two different inoculation regimes: co-inoculation or sequential inoculation. Co-inoculation involves the simultaneous inoculation of more than one

yeast, whereas sequential inoculation involves an inoculation of the non-*Saccharomyces* species first, followed by *S. cerevisiae* some days later to enable fermentation completion (Padilla *et al.*, 2016). Co-inoculation benefits wine characteristics such as acidification by *Lachancea thermotolerans* (Comitini *et al.*, 2011; Gobbi *et al.*, 2013; Kapsopoulou *et al.*, 2007), decreased acetic acid (Comitini *et al.*, 2011; Zohre & Erten, 2002), increased acetate esters (Varela *et al.*, 2017; Zohre & Erten, 2002) and lower alcohol contents by *Meschinokowia pulcherrima* (Varela *et al.*, 2017). Whilst both methods can improve wine sensory complexity, sequential inoculation is generally favoured due to the additional control over *S. cerevisiae* populations, through timing and inoculation rate, allowing expression of non-*Saccharomyces* characteristics (Vilela, 2020).

Recent advancements in co-inoculation strategies include the use of slow growing *S. cerevisiae* strains, to allow for greater non-*Saccharomyces* abundance. Albertin *et al.* (2017) demonstrated the use of a long lag phase yeast strain in co-fermentation with five non-*Saccharomyces* yeast, *Hanseniaspora uvarum*, *Candida zemplinina*, *Metschnikowia* spp., *Torulaspora delbrueckii*, and *Pichia kluyveri*. That study utilised yeast with a long lag phase associated with the presence of sulfite, a phenotype observed with a translocation of the sulfite pump, *SSU1*. The wines resulting from these co-fermentations exhibited increased complexity and fruitiness, because of the increased presence of the non-*Saccharomyces* yeast. Whilst this is one example of a novel inoculation phenotype, other yeast mutants that exhibit a long lag phase or slow growth independent of the presence of sulfite would also be of merit.

We are particularly interested in the disruption of *SER1*, encoding 3-phosphoserine aminotransferase, because of its slow growth phenotype, which might be appropriate for co-inoculation purposes. The *ser1Δ* deletion was first associated with the slowing of initial growth in a heme deficient laboratory strain (Reiner *et al.*, 2006). *SER1*

disruption was later reported as a naturally occurring 'loss of function' variant, Ser1p (G78R) in sake yeast, resulting in reduced growth rate and biomass production (Jung *et al.* (2018). The introduction of this naturally occurring mutation into wine *Saccharomyces* genotypes using "self-cloning" techniques has commercial potential as the modified strains would be classified as non-recombinant in jurisdictions such as Japan and the US (reviewed in Hanlon and Sewalt 2020).

In this study we introduce the Ser1p (G78R) variant into the commonly used wine strain EC1118 by CRISPR/Cas9 gene editing and assess its fermentation capabilities in monoculture and co-culture with either *Lachancea thermotolerans* or *Metschnikowia pulcherrima*, as a preliminary to determining its potential applicability as a wine starter culture.

Materials and methods

1. Microbial strains and media

All *Saccharomyces cerevisiae* strains used in this study were derived from the wine strain Lalvin EC1118 (Table 1). *Metschnikowia pulcherrima* MP2 (Hranilovic *et al.*, 2020) and *Lachancea thermotolerans* Concerto™ (Chr. Hansen) were used in co-inoculation experiments (Table 1). All yeast strains were routinely grown in YEPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) at 28 °C. When appropriate, media were solidified by the addition of 20 g L^{-1} agar. *Escherichia coli* NEB® 5-Alpha (C2987I, New England Biolabs) was used for plasmid transformation and storage. Transformation reactions were performed according to the manufacturer's instructions with appropriate antibiotic selection for plasmids. *E. coli* were routinely grown in LB medium (10 g L^{-1}

tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride), supplemented with kanamycin (50 mg L^{-1}) or ampicillin (100 mg L^{-1}) depending on plasmid selection. Wizard® Plus SV Minipreps DNA Purification System (Promega) was used to isolate plasmids. Plasmids and oligonucleotides are described in Table 2.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
EC1118	Lalvin EC1118 (*SER1*/*SER1*) wt	Lallemand, France
SER1(G78R)	EC1118 *SER1*-232 (G>C; G78R) homozygous allele	This Study
Concerto™	*L. thermotolerans* Viniflora Concerto™	Chr. Hansen, Denmark
MP2	*M. pulcherrima* MP2	Hranilovic *et al.* (2020)

Table 2. Plasmids and oligonucleotides used in this study

Plasmid	**Description**	**Source**
pWS082	sgRNA cloning vector (Amp^R)	Shaw *et al.* (2019) Addgene plasmid* #90516
pWS173	Cas9 linear co-transformation vector (Kan^R, 2 µ vector)	Addgene plasmid* #90960
pWS-SER1	sgRNA vector targeting *SER1* (Amp^R)	This study
Oligonucleotides	**Sequence (5' – 3')**	**Source**
sgSER1 F	gactttGAAGTGTTCTACTTGCAAGG	This Study
sgSER1 D	aaacCCTTGCAAGTAGAACACTTCaa	This Study
pWS082 seq	GTCATCTGGAGGTCCTGTGTTC	
SER1-HDR F	ATCGAACTGCTAAATATTCCTGACAC TCATGAAGTGTTCTACTTGCAA**CGT**G GTGGCAC	This Study

SER1-HDR R	CAGCTGCCAAATTAGTAGCAACGGAAGAAAAACCAGTAGTGCCACC**ACG**TTGCAAGTAGA	This Study
SER1 A	CAAAAGAAAAGCCATAATAAGGACA	SGDP**
SER1 D	AGATAGTTCAGTCTCACCCACATTC	SGDP**
SER1 seq	AATGCCTACACCAGTTTTGC	This Study

**https://www.addgene.org/*

***SGDP; Saccharomyces Genome Deletion Project (http://www-sequence.stanford.edu/group /yeast_deletion_project/deletions3.html).*

Lowercase font represents nucleotides to reconstruct the BsmBI site in the CRISPR/Cas9 sgRNA in the plasmid pWS082.

Bold font denotes GGT>CGT codon change for SER1(G78R) construction whilst the underlined font represents overlapping sequence in construction of double stranded DNA mutation templates.

2. Yeast genome editing by CRISPR/Cas9

The *SER1*-232 (G>C; G78R) mutation, referred herewith as *SER1*(G78R) was generated by CRISPR/Cas9 according to Shaw *et al.* (2019) and https://benchling.com/pub/ellis-crispr-tools. sgRNA sequences and ligation oligonucleotides (sgSER1 F and sgSER1 R; **Table 2**) for pWS082 were designed using Benchling (benchling.com). sgRNA sequences were designed such that the desired sequence to introduce the G78R codon change (GGT>CGT) in *SER1* would also disrupt the CRISPR/Cas9 guide sequence, preventing further Cas9 nuclease activity. Guide sequences were confirmed to match the EC1118 genome (taxid:643680) using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). sgRNA sequences were cloned into pWS082 using the previously designed oligonucleotides by Golden Gate assembly (Engler *et al.*, 2008) according to Shaw *et al.* (2019). Ligation reactions were transformed into NEB® 5-Alpha cells (C2987I, New England Biolabs) using standard protocol and subsequently plated onto LB medium with 100 mg L^{-1} ampicillin and incubated at 37 °C. Selection for non-fluorescent colonies was achieved by visualisation with UV light (365

nm). Non-fluorescent colonies were inoculated into 10 mL LB medium with 100 mg L^{-1} ampicillin and incubated at 37 °C with shaking (200 rpm). Overnight cultures were used for plasmid purification by Wizard® Plus SV Minipreps DNA Purification System (Promega). To confirm correct integration of sgRNA sequences, plasmids were Sanger sequenced with primer pWS082 seq (**Table 2**; Australian Genome Research Facility, Australia).

Homology directed repair (HDR) templates were constructed by PCR using overlapping oligonucleotides SER1-HDR F and SER1-HDR R (**Table 2**). PCR reactions followed a standard 50 µL reaction using Velocity DNA Polymerase (BIO-21098; Bioline) with some modifications. No template DNA was used, instead 8 µL of each overlapping oligo (100 µM) was used. Standard cycling conditions for Velocity DNA Polymerase were used, with an annealing temperature of 57 °C and an extension time of 10 sec. The amplified HDR templates were semi-quantified by gel electrophoresis using DNA molecular weight markers (Hyperladder™ 50 bp (BIO-33054); Bioline).

A standard lithium acetate transformation protocol was used to introduce CRISPR/Cas9 components into EC1118 to create the *SER1*(G78R) mutant. An overnight culture (1 mL) was inoculated into 100 mL of fresh YEPD and grown at 28 °C with shaking (140 rpm) to an OD_{600} of 0.4-0.5. Cells were pelleted, twice washed and resuspended in 0.1 M lithium acetate to a final volume of 500 µL. Cell suspension (50 µL) was pelleted and resuspended with the DNA components (100 ng of BsmBI digested and purified pWS173 plasmid, 200 ng of EcoRV-HF digested and un-purified pWS-SER1 plasmid, ~ 5 µg of HDR template DNA) and sterile ultrapure water) totalling 54 µL. Post resuspension, the other transformation components were added (10 µL salmon sperm DNA (10 mg mL^{-1}), 36 µL lithium acetate (1 M) and 260 µL polyethylene glycol 4000 (50% w/v). Reactions were statically incubated at 30 °C for 30 min, followed by 42

°C for 30 min. Following centrifugation (20,000 x *g*, 1 min) cells were resuspended in 1 mL YEPD and incubated at 30 °C for 2.5 h. Cells were then pelleted and resuspended in 200 µL of sterile ultrapure water before plating onto YEPD agar containing 100 mg L^{-1} G418 and incubation at 30 °C for 2 days.

G418 resistant colonies were grown in non-selective YEPD at 28 °C with shaking (140 rpm) for 72 h to facilitate plasmid loss. Cultures were streaked onto YEPD agar to select for single colonies, which were then replica plated on YEPD with and without 100 mg mL^{-1} G418 to determine plasmid loss. Genomic DNA was isolated from yeast colonies no longer conferring G418 resistance, by the phenol/chloroform glass bead method (Adams *et al.*, 1998). Isolated genomic DNA was subsequently used for amplification of the *SER1* gene using primers SER1 A and SER1 D (**Table 2**) using Velocity DNA Polymerase. Reactions were purified using Wizard® SV Gel and PCR Cleanup System (Promega) and Sanger sequenced (Australian Genome Research Facility, Australia) using primer SER1 seq (**Table 2**) to confirm correct integration of the HDR template.

3. Evaluation of the *SER1*(G78R) mutant via fermentation of Chemically Defined Grape Juice Medium

Fermentations were performed in Chemically Defined Grape Juice Medium (CDGJM) containing 200 g L^{-1} sugar (100 g L^{-1} glucose, 100 g L^{-1} fructose) and 450 mg Yeast Assimilable Nitrogen (YAN) L^{-1} (Jiranek *et al.*, 1995). Single colony of each of EC1118 and *SER1*(G78R) were each inoculated into 100 mL of 1:1 YEPD and CDGJM as a starter medium and grown overnight at 28 °C with shaking (140 rpm). Initial yeast counts were quantified by flow cytometry (Guava® easyCyte™, Luminex) using

propidium iodide (PI). Cells were diluted in phosphate buffered saline with 10 µg mL^{-1} PI prior to analysis and counting of 5000 events per sample. PI staining was monitored using the yellow bandpass filter (583/26 nm) in combination with the blue laser (488 nm). Cells were washed and resuspended in CDGJM prior to inoculation at 5 x 10^6 viable cells mL^{-1}. Fermentations were conducted in triplicate at 23 °C with shaking (140 rpm). Regular samples were used to assess viablility by flow cytometry (as described above) and residual glucose, fructose, and total sugar (D-fructose/D-glucose Assay Kit, Megazyme). Fermentations were considered complete when total residual sugars were less than 2.5 g L^{-1}. End-point acetic acid, succinic acid, and glycerol were determined by HPLC according to Gardner *et al.* (2005) with modification. An Agilent 1100 system (Agilent Technologies, USA) was utilized with detection by refractive index detector (G1362A; Agilent Technologies, USA) for glycerol and diode-array detector (G1315A/B; Agilent Technologies, USA) for lactic acid, succinic acid and acetic acid.

4. Co-inoculated fermentation in Sauvignon Blanc juice

Fermentations were undertaken in filter sterilized Sauvignon Blanc juice (19 ° Brix, pH 3.4, 4 g L^{-1} malic acid, 27 mg free SO_2 L^{-1}, 40 mg total SO_2 L^{-1} and 250 mg YAN L^{-1}) and inoculated with a 1:1 or 1:9 ratio of *S. cerevisiae* (EC1118 or *SER1*(G78R) and non-*Saccharomyces* (MP2 or Concerto™). The total number of cells inoculated was 1 x 10^7 cells mL^{-1}. Monocultures of each strain were also undertaken, using 1 x 10^7 cells mL^{-1} as inoculum. For starter cultures, a single colony of each yeast was inoculated separately into 100 mL of 1:1 YEPD and Sauvignon Blanc juice, and grown overnight at 28 °C with shaking (140 rpm). Yeast culture density was quantified by flow cytometry, and cells washed and resuspended in Sauvignon Blanc juice prior to inoculation to 5 x 10^6 viable cells mL^{-1}. Fermentations were conducted in triplicate at 17 °C with shaking

(140 rpm) and sampled regularly for determination of total cell numbers by plating on WL agar (Oxoid) to differentiate the *Saccharomyces* (cream-coloured colonies) and non-*Saccharomyces* (green-coloured colonies). Fermentations were considered dry when the total residual sugar was below 2.5 g L^{-1} (D-fructose/D-glucose Assay Kit, Megazyme). End-point acetic acid, succinic acid, lactic acid and glycerol were determined by HPLC as described above. Endpoint acetate esters (propyl acetate, isobutyl acetate, isoamyl acetate, *cis*-3-hexenyl acetate, 2-phenylethyl acetate and ethyl acetate) were determined by Gas Chromatography-Mass Spectroscopy (GC-MS; Lin *et al.* (2020)).

5. SO_2 resistance of Concerto™ in co-inoculated fermentations

To further evaluate the sensitivity of *L. thermotolerans* Concerto™ to added sulfite, fermentations were undertaken in Chardonnay juice (22 °Brix, 249 mg YAN L^{-1}, 3.3 pH, 16 mg L^{-1} free SO_2 and 34.4 mg L^{-1} total SO_2). Yeast strains were grown overnight from a single colony in 100 mL of 1:1 YEPD and Chardonnay juice. Yeast cultures were quantified (Guava® easyCyte™, Luminex), washed and resuspended in Chardonnay juice prior to inoculation at 1 x 10^7 viable cells mL^{-1} in a 1:1 ratio. Fermentations were incubated at 17 °C with shaking (140 rpm) for 17 h with regular sampling for determination of the total cell number on YEPD and lysine agar (Fowell, 1965) to differentiate *L. thermotolerans*.

6. Statistical analysis

Data was analysed using GraphPad Prism 9.0.0 software, with datasets compared with the Unpaired t-Test or One-way Analysis of Variance (ANOVA) with multiple comparisons (Tukey's test) at the 99% confidence level. Samples from Sauvignon Blanc

fermentations were further analysed using a Principal Components Analysis (PCA) using the statistically significant HPLC and GC-MS data at $p < 0.01$.

Results

1. Introduction of the *SER1*(G78R) variant into EC1118 via CRISPR/Cas9

S*ER1*(G78R) is a naturally occurring 'loss of function' variant identified in a sake yeast strain by QTL analysis, which is associated with increased chronological age (Jung *et al.*, 2018). The associated glycine to arginine substitution was introduced into the wine yeast EC1118 as a non-synonymous mutation at nucleotide position +232 (G>C i.e., GGG>CGG) by homology directed repair using CRISPR/Cas9 (Shaw *et al.*, 2019). Both the *SER1*(G78R) naturally occurring 'loss of function' variant (Jung *et al.*, 2018) and a *SER1* deletion in a *ser1Δ* heme deficient yeast (Reiner *et al.*, 2006) have reduced growth. Whilst serine biosynthesis via glycolysis is disrupted, the mutants are not auxotrophs as serine can be produced from glycine via gluconeogenesis (Melcher & Entian, 1992). The reduced growth phenotype may be beneficial in wine co-inoculations, since the increased numbers (and viability) of non-*Saccharomyces* in the later stages of fermentation, may be reflected in terms of wine complexity and novelty from aroma compounds derived from these yeasts.

The fermentation characteristics of *SER1*(G78R) were compared to wild type EC1118 in 100 mL fermentations of CDGJM. The mutant exhibited a slower initial growth (**Figure 1A**), similar to that observed in *ser1Δ* heme deficient strains (Reiner *et al.*, 2006) and in other strains containing the *SER1*(G78R) mutation (Jung *et al.*, 2018). EC1118 took 48 h to reach stationary growth phase, whereas *SER1*(G78R) took 144 h, achieved a reduced viable population (**Figure 1A**) and took an extra 120 h to complete

fermentation (i.e., 288 h *vs* 168 h) (**Figure 1B**). Analysis of fermentation relevant metabolites by HPLC showed significantly higher acetic acid (1.61 g L^{-1} *vs* 1.90 g L^{-1}), lactic acid (0.00 g L^{-1} *vs* 0.07 g L^{-1}), succinic acid (0.98 g L^{-1} *vs* 1.32 g L^{-1}), glycerol (4.30 g L^{-1} *vs* 4.62 g L^{-1}) and ethanol (100.20 g L^{-1} *vs* 102.10 g L^{-1}; **Table 3**). Increases in succinic acid, acetic acid and glycerol were expected as they have previously been noted in some instances with *ser1Δ* strains (Chidi *et al.*, 2016). Whilst significant increases in lactic acid and ethanol were also observed, the changes were small and likely negligible. The acetic acid concentrations in both strains would be sensorially significant, as they are both over the sensory threshold (1.19 g L^{-1} for white wines; Corison *et al.* 1979) and contributed to a harsh 'vinegar' smell of the fermentations.

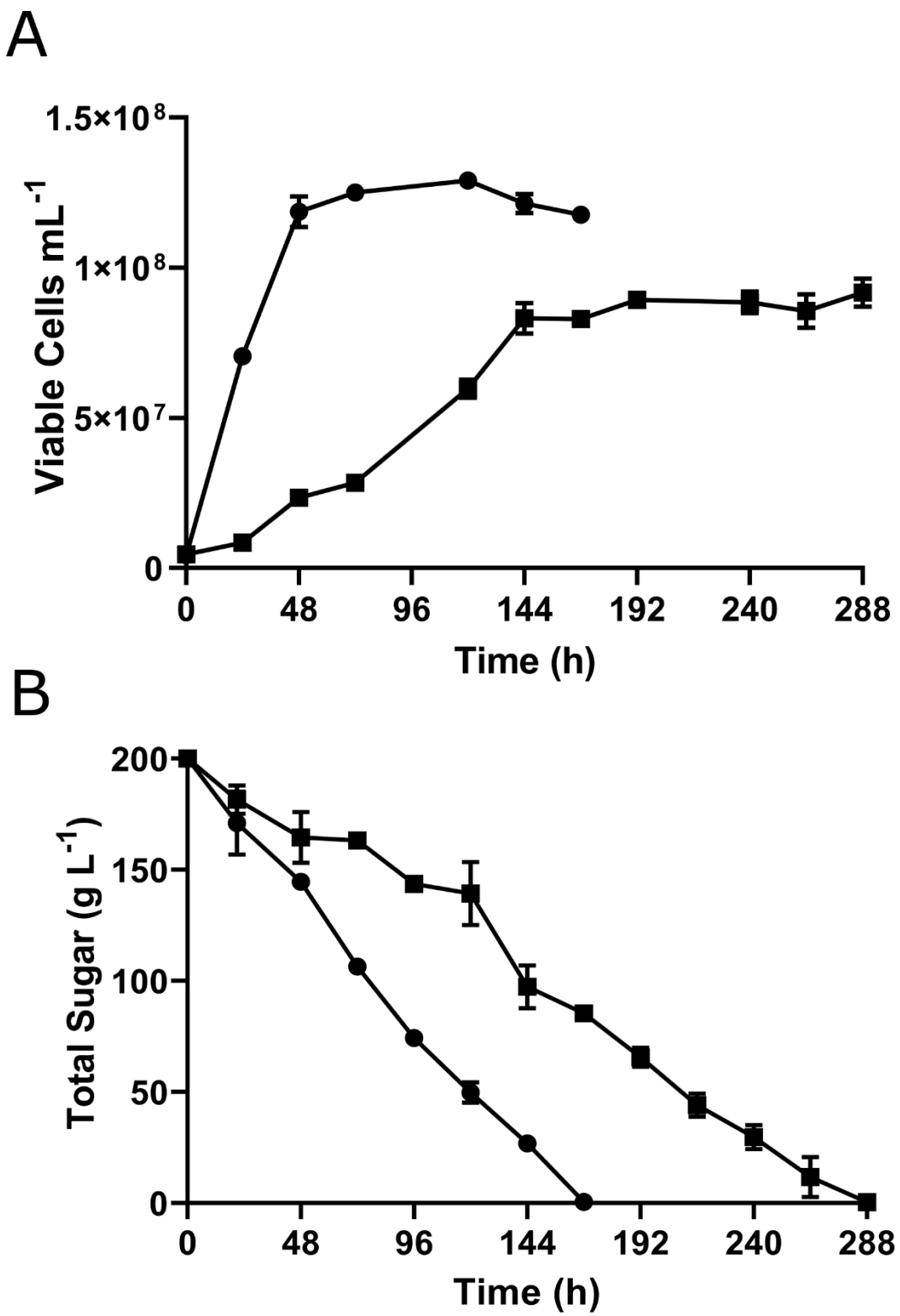

Figure 1. Evaluation of fermentation performance and viability of EC1118 and *SER1*(G78R) grown in CDGJM

Fermentations were conducted in 100 mL of CDGJM containing 200 g L^{-1} total sugars and 450 mg YAN L^{-1} (n = 3). Viable cell counts (A) and total sugars (B) were determined by flow cytometry and enzymatic analysis, respectively, for EC1118 (○) and SER1(G78R) *(□). Error bars denote standard deviation from the mean.*

Table 3. Fermentation duration (h) and metabolite content (g L^{-1}) of 'wines' produced by EC1118 and *SER1*(G78R) when fermenting in CDGJM

	Duration	Acetic Acid	Lactic Acid	Malic Acid	Succinic Acid	Glycerol	Ethanol
EC1118	168 ± 0.00	1.61 ± 0.03	0.00 ± 0.00	3.25 ± 0.03	0.98 ± 0.01	4.30 ± 0.03	100.20 ± 0.49
SER1(G78R)	288 ± 0.00*	1.90 ± 0.03*	0.07 ± 0.00*	3.20 ± 0.02	1.32 ± 0.04*	4.62 ± 0.03*	102.10 ± 0.25*

**Denotes significant difference (Unpaired t-test; $p < 0.01$)*

2. Co-inoculated fermentations in Sauvignon Blanc juice: Fermentation performance and metabolite contribution

Yeast with slowed initial growth have been shown to be potentially useful in co-inoculation experiments (Albertin *et al.*, 2017). To investigate the effects of *SER1*(G78R) in co-inoculated winemaking, 100 mL fermentations were undertaken in Sauvignon Blanc juice with both monocultures and *S. cerevisiae*/non-*Saccharomyces* co-inoculated cultures. Both monocultures of EC1118 and *SER1*(G78R) completed fermentation, whereas MP2 was sluggish with 54.46 ± 5.01 g L^{-1} residual sugar when the other fermentations were concluded (**Supplementary Table 1**). This was likely due to a reduction in viable cells present in the fermentation after 264 h when the ethanol reached 55.68 g L^{-1} (5.5 % (w/v)) (**Figure 2A, Supplementary Table 1**). Concerto™ monocultures largely failed to consume sugars (**Supplementary Table 1**) with the yeast dying 24 h post-inoculation (**Figure 2A**). These results support the recommendation of the manufacturer that Concerto™ is used in un-sulfured grape juice, as the yeast is sulfite (SO_2) sensitive at 40 mg total SO_2 L^{-1}, which is within the range (20-50 mg L^{-1}), ordinarily considered non-inhibitory to non-*Saccharomyces* (Henick *et al.*, 1998). As with CDGJM, the fermentation duration of *SER1*(G78R) was significantly extended (+216 h; **Table 4**) and had a lower population density compared to EC1118 (**Figure 2A**).

All co-inoculated fermentations completed fermentation. EC1118 quickly dominated the fermentations when co-inoculated with MP2, successfully outcompeting *Metschnikowia* regardless of the inoculation regime (**Figure 2B**), with the fermentations having the same duration to the EC1118 monoculture (**Table 4, Figure 2A**). The population dynamics of EC1118 and Concerto™ mimicked that of MP2, where EC1118 rapidly dominated the fermentation resulting in no detectable Concerto™ cells by WL plating after 72 h (**Figure 2C**).

Interestingly, the slower growing *SER1*(G78R) mutant permitted greater populations of non-*Saccharomyces* in co-culture than EC1118. This was evident in *SER1*(G78R) fermentations co-inoculated with MP2 (**Figure 2D**). Thus, while EC1118 dominated the culture early on and no colonies of MP2 were noted under either inoculation regime (**Figure 2B**), this was not the case for the mutant (**Figure 2D**). The maximum cell number reflected the amount inoculated for the given inoculation ratio, MP2 CFUs declined after 168 h, eventually dropping to zero at the end of fermentation. The presence of *SER1*(G78R) allowed the MP2 fermentations to achieve dryness after 408 h (**Figure 2D**), in contrast to the MP2 monoculture. The duration of the mixed fermentation was identical to *SER1*(G78R) as a monoculture (**Figure 2A**). In both cases, the *Metschnikowia* population declined with increasing ethanol content as fermentation progressed (**Figure 2A and 2D**).

The growth behaviour of the Concerto™ *Lachancea* in monoculture and co-culture with *SER1*(G78R) was surprisingly different, with the presence of the slow-growing mutant enabling growth of the SO_2-sensitive *Lachancea* at SO_2 conditions that were lethal in the Concerto™ monoculture (**Figure 2A**). This led to Concerto™ dominating the co-inoculated fermentation in numbers greater than observed with EC1118 (**Figures 2C** and **2E**), and an earlier completion of fermentation than the *SER1*(G78R) monoculture (i.e., 240 h *vs* 408 h; **Table 4**). These results allude to a synergistic interaction between the two yeasts when in co-culture in sulfured Sauvignon Blanc juice, the basis of which is unclear. Whilst the fermentation duration was extended in the co-inoculations with the *SER1*(G78R) mutant compared to the EC1118 counterpart, the presence of Concerto™ and to a lesser extent, MP2, would be anticipated to influence the chemical composition of the resultant wines.

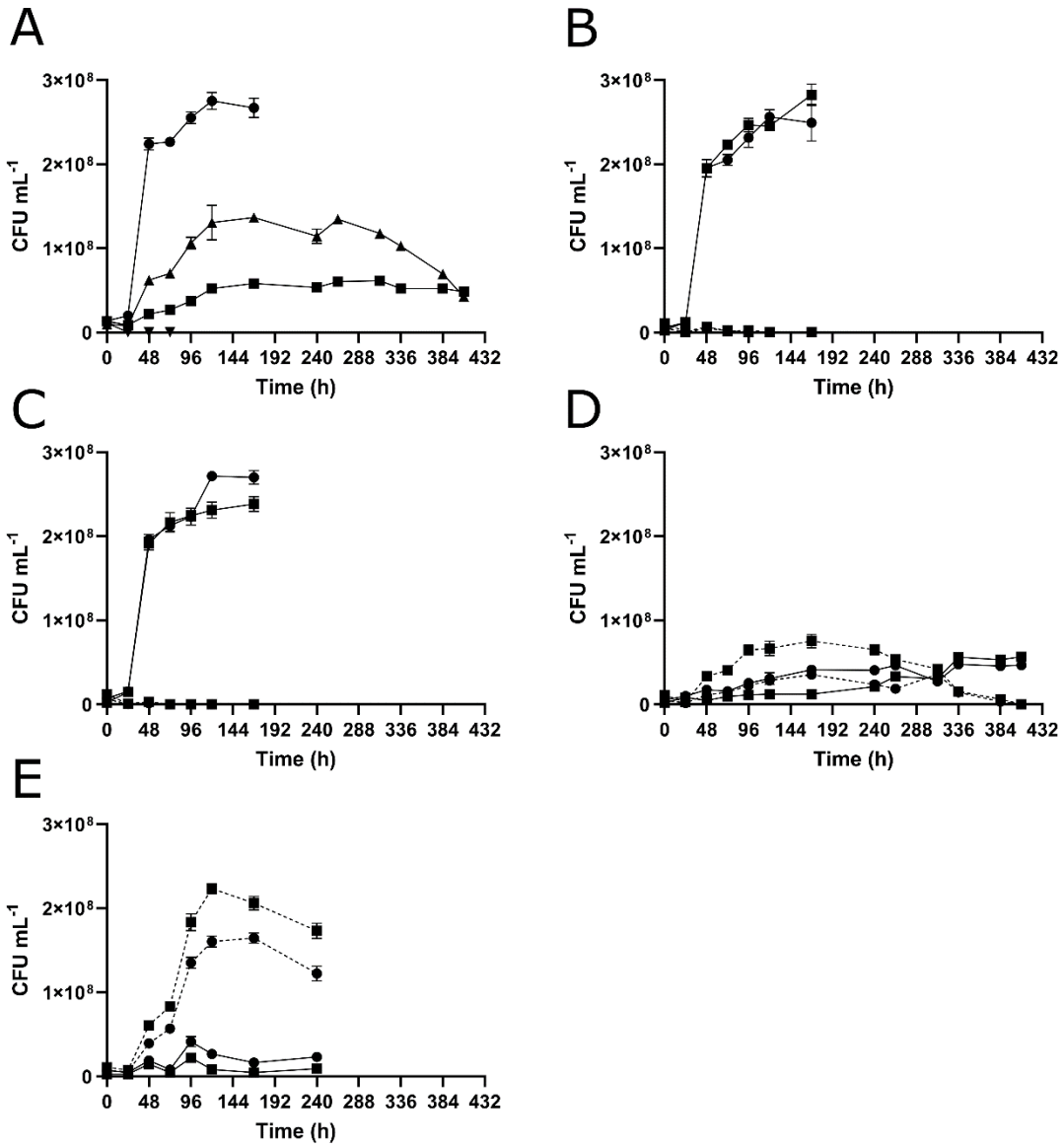

Figure 2. Viable yeast counts from Sauvignon Blanc juice fermentations

Viable yeast counts from WL agar plating of either monocultures (A) of EC1118 (○), SER1(G78R) *(□), MP2 (△) or Concerto™ (▽) or co-inoculated cultures of (B) EC1118 with MP2, (C) EC1118 with Concerto™, (D) SER1*(G78R) *with MP2 or (E) SER1*(G78R) *with Concerto™. Solid lines in co-inoculated fermentations denote* S. cerevisiae *populations, whereas dashed lines denote non-*Saccharomyces *populations. Square (□) markers in co-inoculated fermentations correspond to a 1:9 inoculation ratio, whereas circles (○) correspond to a 1:1 inoculation ratio.*

HPLC analysis of the completed wines was undertaken to assess the contributions of organic acids, glycerol, and ethanol by the mutant and non-*Saccharomyces* co-inoculated fermentations. Lactic acid was specifically quantified since *L. thermotolerans* is reported to produce increased amounts of the acid in a strain-dependent manner (Hranilovic *et al.*, 2020; Vaquero *et al.*, 2020). Interestingly, there were no significant differences in lactic acid concentration between EC1118 or *SER1*(G78R) and Concerto™ in the monocultures and co-inoculated fermentations (**Table 4**). Furthermore, no significant differences were observed for any of the quantified metabolites in the wines produced by EC1118 as monoculture and co-inoculated fermentations (**Table 4**). This can likely be attributed to the dominance of EC1118 in these fermentations (**Figure 2A**).

Comparison of the monocultures of the parent EC1118 and the *SER1*(G78R) mutant alluded to the effect of the 'loss of function' mutation on glycolysis during alcoholic fermentation (**Supplementary Figure 1**). The glycolysis pathway at 3-phosphoglycerate feeds directly into serine biosynthesis with the *SER1* encoded phosphoserine transaminase catalysing a bi-directional reaction between 3-phosphooxypyruvate and 3-phospho-L-serine. Disruption of *SER1* is postulated to result in a feed-back by the accumulation of precursors such that the glycolytic flux favours acetic acid production. This notion is corroborated by the large increase in acetic acid in wines produced by the *SER1*(G78R) mutant compared to EC1118 (1.56 g L^{-1} *vs* 0.09 g L^{-1}; **Table 4**). Such concentrations may be considered a fault, as they exceed the odour threshold (1.19 g L^{-1} for white wines; Corison *et al.*, 1979), contributing to an unpleasant 'vinegar-like' aroma as seen in CDGJM. In contrast, the marginal change in succinic acid (3.85 g L^{-1} (EC1118) *vs* 3.55 g L^{-1} (*SER1*(G78R)); **Table 4**), indicated the flux through the tricarboxylic acid cycle was not greatly affected by the mutation. The 29.5% increase in glycerol by the mutant (8.59 g L^{-1} *vs* 6.63 g L^{-1}; **Table 4**), also alluded to the

modulation of glycolysis between 3-phosphoglycerate and glyceraldehyde-3-phosphate because of the *SER1* disruption (**Supplementary Figure 1**). This difference would likely alter the perception of sweetness but not viscosity (Noble & Bursick, 1984), as detectable changes are noted at 5.2 g L^{-1} and 25 g L^{-1}, respectively. The classification of the strain as genetically modified in Australia, prevented sensory analysis because of containment regulations.

The slow growth of *SER1*(G78R) enabled non-*Saccharomyces* to persist in the co-inoculated fermentations, such that significant differences in the wine metabolites were seen between the monoculture and most co-fermentations. Acetic acid and glycerol yields reflected the non-*Saccharomyces* co-inoculated with *SER1*(G78R) and the inoculation regime (**Table 4**). Co-fermentation with *Metschnikowia* (MP2) resulted in 11% more glycerol when inoculated at a 9-fold higher rate than the *Saccharomyces*. This was associated with a 73% decrease in acetic acid (0.41 vs 1.56 g L^{-1}) and a 2% decrease in ethanol (-2.58 g L^{-1}) compared to the *SER1*(G78R) monoculture (**Table 4**). These findings mirror earlier observations (Hranilovic *et al.*, 2020), where MP2 also increased glycerol and decreased ethanol and acetic acid in monocultures and sequentially inoculated fermentations. Similarly, Sadoudi *et al.* (2017) also demonstrated decreased acetic acid (40 %) and increased glycerol (12 %) in a sequential culture of *S. cerevisiae*/*M. pulcherrima* (inoculated 1:10) compared to the pure *S. cerevisiae* culture.

Table 4. Fermentation duration (h) and metabolite content (g L^{-1}) of monoculture and co-inoculated fermentations in Sauvignon Blanc juice

Strain(s)	Duration	Acetic Acid	Lactic Acid	Malic Acid	Succinic Acid	Glycerol	Ethanol
EC1118 (monoculture)	168 ± 0.00^{c}	0.21 ± 0.07^{de}	0.69 ± 0.02^{a}	3.97 ± 0.01^{a}	3.85 ± 0.01^{b}	6.63 ± 0.03^{e}	86.29 ± 0.11^{a}
EC1118 + MP2 (1:1)	168 ± 0.00^{c}	0.24 ± 0.02^{de}	0.70 ± 0.15^{a}	3.96 ± 0.02^{a}	3.79 ± 0.03^{b}	6.65 ± 0.06^{e}	85.95 ± 0.45^{a}
EC1118 + MP2 (1:9)	168 ± 0.00^{c}	0.12 ± 0.11^{e}	0.77 ± 0.04^{a}	4.05 ± 0.02^{a}	3.75 ± 0.04^{b}	6.69 ± 0.01^{e}	86.13 ± 0.34^{a}
EC1118 + Concerto™ (1:1)	168 ± 0.00^{c}	0.26 ± 0.02^{de}	0.68 ± 0.05^{a}	3.91 ± 0.02^{a}	3.81 ± 0.03^{b}	6.63 ± 0.00^{e}	86.22 ± 0.05^{a}
EC1118 + Concerto™ (1:9)	168 ± 0.00^{c}	0.22 ± 0.03^{de}	0.72 ± 0.02^{a}	3.92 ± 0.03^{a}	3.81 ± 0.03^{b}	6.61 ± 0.01^{e}	86.37 ± 0.15^{a}
SER1(G78R) (monoculture)	408 ± 0.00^{a}	1.56 ± 0.11^{a}	0.55 ± 0.09^{a}	4.12 ± 0.16^{a}	3.55 ± 0.02^{c}	8.59 ± 0.04^{c}	85.87 ± 0.14^{a}
SER1(G78R) + MP2 (1:1)	408 ± 0.00^{a}	1.28 ± 0.03^{b}	0.74 ± 0.06^{a}	4.06 ± 0.06^{a}	3.48 ± 0.02^{c}	8.83 ± 0.03^{b}	85.66 ± 0.06^{a}
SER1(G78R) + MP2 (1:9)	408 ± 0.00^{a}	0.41 ± 0.10^{cd}	0.72 ± 0.12^{a}	3.83 ± 0.11^{a}	3.15 ± 0.08^{d}	9.60 ± 0.07^{a}	83.29 ± 0.38^{b}
SER1(G78R) + Concerto™ (1:1)	240 ± 0.00^{b}	0.62 ± 0.02^{c}	0.58 ± 0.16^{a}	3.31 ± 0.19^{b}	3.79 ± 0.02^{b}	7.36 ± 0.12^{d}	86.22 ± 1.25^{a}
SER1(G78R) + Concerto™ (1:9)	240 ± 0.00^{b}	0.39 ± 0.01^{d}	0.58 ± 0.01^{a}	3.08 ± 0.04^{b}	4.05 ± 0.01^{a}	7.22 ± 0.05^{d}	86.50 ±0.60^{a}

[a] Letters denote significant difference between values in columns (Tukey's HSD test; $p < 0.01$)

**Monocultures of Concerto™ and MP2 did not finish fermentation, having 166.20 ± 5.26 g L^{-1} and 54.46 ± 5.01 g L^{-1} residual sugar, respectively. Refer to Supplementary Table 1.*

Co-fermentations with Concerto™ saw *L. thermotolerans* dominate, which resulted in less glycerol than the *SER1*(G78R) monoculture but higher than EC1118; with the lowest concentrations measured in the high (1:9) inoculum ratio (**Table 4**). These findings allude to Concerto™ being a high producer of glycerol, the amounts produced in the co-fermentations (7.22–7.35 g L^{-1}) similar to the manufacturer's specifications of 5–8 g L^{-1} (https://www.gusmerwine.com/wp-content/uploads/sites/7/2015/07/Chr-Hansen-Viniflora -CONCERTO-PDS.pdf).

Strain specific differences were observed for succinic acid between the two non-*Saccharomyces* and different inoculation ratios (*Saccharomyces*:non-*Saccharomyces*). Co-inoculation of *SER1*(G78R) with MP2, gave significantly lower succinic acid at the higher (1:9) ratio compared to the *SER1*(G78R) monoculture (3.15 g L^{-1} vs 3.55 g L^{-1}; **Table 4**). The small reduction in succinic acid at the 1:1 co-inoculation ratio with MP2 was not statistically significant (3.48 g L^{-1} vs 3.55 g L^{-1}). These findings differ from Hranilovic *et al.* (2020), who reported MP2 produced either high or comparable amounts of succinic acid to that of EC1118 grown in CDGJM or grape juice, respectively. Co-inoculation of *SER1*(G78R) with Concerto™ resulted in increased succinic acid. When inoculated equally, succinic acid (3.79 g L^{-1}) was similar to the EC1118 fermentations (3.81 g L^{-1}), whilst the higher inoculum of Concerto™ (1:9) resulted in a 6.3% increase (4.05 g L^{-1}). These concentrations are considerably higher than in the commercial white wines surveyed by Coulter *et al.* (2004) (0.1–1.6 g L^{-1}; mean 0.6 g L^{-1}). Whilst increased succinic acid in wine can be identifiable as a salty sour taste (Coulter *et al.*, 2004), sensory analysis was not be undertaken here due to regulatory restrictions.

Malic acid degradation by wine yeast is a useful attribute, providing an alternative to the malolactic fermentation undertaken by *Oenococcus oeni* to modulate wine acidity and aroma (Du Plessis *et al.*, 2017). Malic acid was quantified to check for degradation

by non-*Saccharomyces* as opposed EC1118, which has minimal impact (Redzepovic *et al.*, 2003). As expected, malic acid in the EC1118-dominated fermentations was not reduced from the initial 4 g L^{-1} in the Sauvignon Blanc juice (**Table 4**). Residual malic acid was comparable across *SER1*(G78R) in monoculture and when inoculated 1:1 with MP2. However, under high inoculation (1:9), MP2 was in sufficient numbers to reduce L-malic acid by ~5% (to 3.83 ± 0.11 g $L^{-`1}$; **Figure 2D, Table 4**). Co-inoculation of *SER1*(G78R) with Concerto™ at 1:1 reduced malic acid by 20% (to 3.31 ± 0.19 g L^{-1}), and by 25% (to 3.08 ± 0.04 g L^{-1}) when inoculated at 1:9 (**Table 4**). These results agree with reports of *L. thermotolerans* degrading L-malic acid in a strain-dependant manner, with our range (0 to ~26 % degradation) matching that reported in *S. cerevisiae* (reviewed in Benito 2018).

Acetate esters result from esterification of ethanol or higher alcohols (from Ehrlich degradation of amino acids; reviewed in Belda *et al.* 2017) with acetyl-CoA and are important contributors to wine aroma. Importantly, acetic acid was increased by *SER1*(G78R), and MP2 is reported to increase acetate ester production (Hranilovic *et al.*, 2020). End-point ester analysis revealed several differences. Monocultures of EC1118 and *SER1*(G78R) differed significantly in propyl acetate (pear-like), being 8.64 µg L^{-1} for *SER1*(G78R) compared to 2.66 µg L^{-1} for EC1118 (**Table 5**), but these concentrations are well below the sensory threshold of 4,740 µg L^{-1} in white wines (Miller, 2019). No significant differences were found between the EC1118 mono- and co-inoculated fermentations, dominated by EC1118, which is considered 'neutral' in relation to its aroma contribution to wine (https://www.lallemandbrewing.com/en/united-states/product-details/lalvin-ec-1118/).

Both propyl acetate and ethyl acetate increased to the greatest extent in the *SER1*(G78R)/MP2 co-inoculated fermentations at the 1:9 ratio (1.7-fold and 3.7-fold

increase, respectively; **Table 5**) but contributors to fruity aromas are only evident above the sensory threshold of 7,500 µg L^{-1} (Guth (1997). In excess of 100 mg L^{-1} ethyl acetate is considered a fault, having a 'nail polish remover' scent (Sumby *et al.*, 2010). Comparison of *SER1*(G78R) with the MP2 co-culture, at 1:1 inoculation, showed no other significant differences. Higher inoculation of MP2 to the *Saccharomyces* mutant, also increased all other esters, except *cis*-3-hexenyl acetate. Isobutyl acetate (up 5.9x to 14 µg L^{-1} *vs* the *SER1*(G78R) monoculture; **Table 5**) has a sensory threshold of 1.6 µg L^{-1}, imparting a fruity, apple, banana-like scent (Haggerty, 2016). Isoamyl acetate, increased 3.5-fold (to 76 µg L^{-1}; **Table 5**), thus above the 30 µg L^{-1} sensory threshold of this 'banana-like' acetate ester (Guth, 1997). A ~2.5-fold increase in 2-pheneylethyl acetate remained below the sensory threshold of 250 µg L^{-1} for this rose, honey, tobacco-like aroma compound (Guth, 1997). No samples in this study exceeded this threshold. Overall, these results demonstrate the increase of acetate esters as previously described for fermentations utilising *M. pulcherrima* (Hranilovic *et al.*, 2020).

Table 5. Acetate ester content (μg L^{-1}) of monoculture and co-inoculated fermentations in Sauvignon Blanc juice

Strain(s)	Propyl Acetate	Isobutyl Acetate	Isoamyl Acetate	*cis*-3-Hexenyl Acetate	2-Phenylethyl Acetate	Ethyl Acetate
EC1118 (Monoculture)	2.66 ± 0.21^{d}	1.58 ± 0.12^{cd}	35.88 ± 7.17^{b}	56.39 ± 2.05^{a}	17.51 ± 1.36^{b}	2184 ± 177.0^{c}
EC1118 + MP2 (1:1)	2.65 ± 0.22^{d}	1.51 ± 0.11^{d}	35.38 ± 10.60^{b}	60.95 ± 3.77^{a}	19.59 ± 0.95^{b}	2238 ± 230.1^{c}
EC1118 + MP2 (1:9)	2.97 ± 0.52^{d}	1.65 ± 0.23^{cd}	36.23 ± 15.98^{b}	53.86 ± 11.66^{a}	20.53 ± 4.06^{b}	2996 ± 419.2^{bc}
EC1118 + Concerto™ (1:1)	2.76 ± 0.12^{d}	1.59 ± 0.04^{cd}	26.9 ± 8.41^{b}	56.76 ± 3.09^{a}	19.74 ± 1.24^{b}	2432 ± 142.1^{bc}
EC1118 + Concerto™ (1:9)	2.88 ± 0.71^{d}	1.71 ± 0.55^{cd}	36.22 ± 8.96^{b}	53.39 ± 0.99^{a}	18.74 ± 0.91^{b}	2228 ± 334.1^{c}
SER1(G78R) (Monoculture)	8.64 ± 1.17^{c}	2.37 ± 0.32^{bcd}	21.84 ± 2.86^{b}	63.18 ± 9.45^{a}	18.64 ± 1.58^{b}	2363 ± 189.2^{bc}
SER1(G78R) + MP (1:1)	11.29 ± 1.34^{b}	3.15 ± 0.40^{b}	21.98 ± 5.76^{b}	54.36 ± 11.30^{a}	22.03 ± 2.10^{b}	3598 ± 519.6^{b}
SER1(G78R) + MP2 (1:9)	14.85 ± 0.46^{a}	14.06 ± 0.32^{a}	75.86 ± 22.13^{a}	60.97 ± 5.62^{a}	46.33 ± 2.38^{a}	8801 ± 526.9^{a}
SER1(G78R) + Concerto™ (1:1)	4.42 ± 0.18^{d}	2.75 ± 0.16^{bc}	15.21 ± 7.37^{b}	8.96 ± 0.81^{b}	2.31 ± 0.21^{c}	3342 ± 135.6^{bc}
SER1(G78R) + Concerto™ (1:9)	3.87 ± 0.50^{d}	3.28 ± 0.59^{b}	15.93 ± 5.66^{b}	5.54 ± 1.03^{b}	1.90 ± 0.19^{c}	3275 ± 631.1^{bc}

[a] Letters denote significant difference between values in the same column (Tukey's HSD test ($p < 0.01$)

**Monocultures of Concerto™ and MP2 did not finish fermentation; data available in supplementary Table1*

In the Concerto™ fermentations there were either no significant differences or significantly reduced acetate esters with the exception of isobutyl acetate, which was significantly increased under high inoculum when compared to EC1118 fermentations (**Table 5**). This agrees with Hranilovic *et al.* (2018), who previously identified Concerto™ as having increased isobutyl acetate production. Isobutyl acetate exceeded the sensory threshold of 1.6 µg L^{-1}, with 2.75 µg L^{-1} and 3.28 µg L^{-1} measured for the 1:1 and 1:9 inoculated fermentations, respectively (**Table 5**). Whilst we (Hranilovic *et al.*, 2018) also showed significant increases in other acetate esters not observed here, the overall mean values for acetate esters were still higher in Concerto™/*SER1*(G78R) co-inoculated fermentations (**Table 5**). *cis*-3-hexenyl acetate and 2-phenylethyl acetate were reduced in both co-inoculation regimes when compared to *SER1*(G78R). *cis*-3-hexenyl acetate (fruity, green tea aroma) does not have a reported sensory threshold in wine and so the influence of this compound in wine is unclear.

Principal Component Analysis (PCA) was performed with the HPLC and GC-MS data to look at the relationship between the fermentation regime and wine metabolites (**Figure 3**). Four distinct groups were identified within the samples. The first group (top right) was comprised solely of fermentations containing EC1118, both monoculture and co-inoculated. Clustering is likely due to lack of significant differences of sensory compounds analysed (**Table 4 and 5**). This is not surprising considering the predominant concentrations of EC1118 in these fermentations (**Figure 2A, 2B and 2C**). For the *SER1*(G78R) fermentations co-inoculated with MP2 there were two clusters. The second (far left) was comprised of the 1:9 co-inoculation ratio of *SER1*(G78R) and MP2. This group was distinguished from others by the high production of acetate esters (propyl, isobutyl, isoamyl, 2-phenylethyl and ethyl acetates) and glycerol. The third group (centre left) was comprised of *SER1*(G78R)/MP2 1:1 co-inoculations as well as *SER1*(G78R)

monoculture fermentations and is largely influenced by acetic acid production - a phenotype associated with *SER1*(G78R) fermentations. Final wine volatile compound composition was dependent upon the inoculation regime, with the higher inoculation ratio (having greater initial MP2 cell density; **Figure 2D**) resulting in largely different fermentations, compared to when MP2 was inoculated at a lower density, which resulted in fermentations more similar to the *SER1*(G78R) monocultures. The final group (bottom right) was comprised of all fermentations involving *SER1*(G78R)/Concerto™ co-inoculations; the Concerto™ monoculture being non-viable. The resultant wines reflect the dominance of the Concerto™ population in both co-fermentations, and thereby the characteristics of this non-*Saccharomyces* (**Figure 2E**). These fermentations were typically characterised by lower production of some acetate esters, in particular, *cis*-3-hexenyl acetate and 2-phenylethyl acetate.

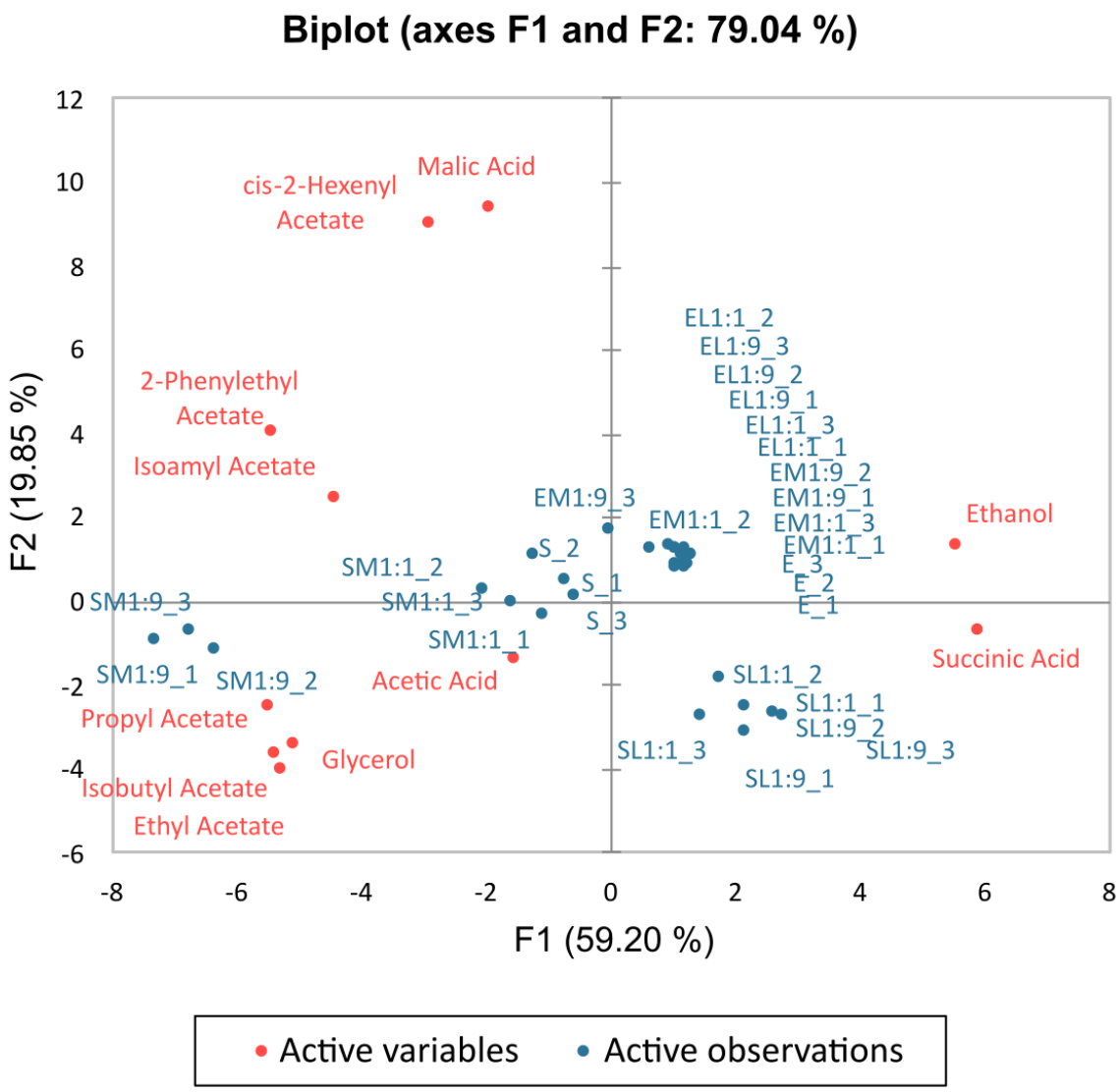

Figure 3. Principal component analysis of acetate esters, organic acids, and glycerol for individual fermentations in Sauvignon Blanc wine fermentations.

Red represents loadings of compounds analysed where blue represents the PC scores of individual fermentations. Samples suffixed with E = EC1118 monoculture, S = SER1(G78R) *monoculture, EM = EC1118/MP2 co-inoculation, EL = EC1118/Concerto™ co-inoculation, SM = SER1*(G78R)*/MP2 co-inoculation and SL – SER1*(G78R)*/Concerto™ co-inoculation. 1:1 and 1:9 denote the inoculation ratio used.*

3. *S. cerevisiae* / Concerto™ co-inoculated fermentations in Chardonnay juice confirm alleviation of SO_2 sensitivity

The SO_2 sensitivity of Concerto™ as influenced by the presence of *Saccharomyces* in co-culture was further investigated in fermentations conducted in a Chardonnay juice containing 34.4 mg L^{-1} total SO_2. The aim was to determine whether the experimental results could be repeated under similar sulfite concentrations independent of white juice variety. Plating on lysine media revealed the extended presence of viable *L. thermotolerans* in co-inoculation with either EC1118 or *SER1*(G78R**)**, but that this was otherwise lost after 24 h in a monoculture (**Figure 4A**). In the 72 h time-point in the Sauvignon Blanc fermentation, Concerto™ reached 5.7 x 10^7 CFU mL^{-1} when co-inoculated with *SER1*(G78R), whereas no *L. thermotolerans* colonies were seen when co-inoculated with EC1118. These results imply that the SO_2 resistance effect is due to the presence of *S. cerevisiae* biomass in general. To test this, an equivalent experiment was undertaken using heat-inactivated EC1118 or *SER1*(G78R) cells. In this instance, however, all fermentations lost viability after 24 h (data not shown), indicating that the SO_2 resistance phenotype experienced by Concerto™ when co-inoculated with *S. cerevisiae* strains requires biologically functional yeast rather than biomass alone.

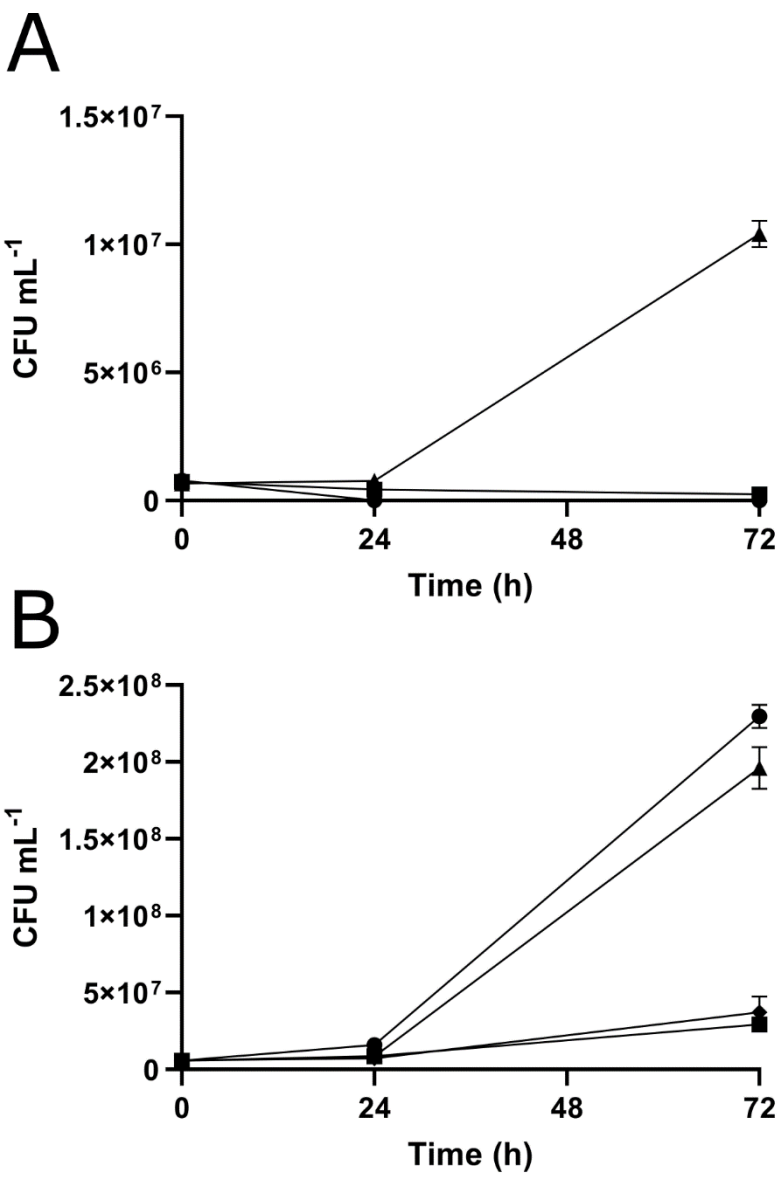

Figure 4: Viable cell densities of Concerto™ or *S. cerevisiae* after 72 h in Chardonnay juice.

Fermentations (100 mL) were conducted in Chardonnay juice containing 34.4 mg L^{-1} total SO_2. Concerto ™ was present as a (A) monoculture (○) or co-inoculated 1:1 with EC1118 (□) or SERI(G78R) (△) with a cell density determined as CFU mL^{-1} by lysine plating. S. cerevisiae *population counts (B) were determined by YEPD plating, for EC1118 (○) and SER1(G78R) (□) monocultures, or Concerto ™ cultures co-inoculated 1:1 with EC1118 (△) or SER1(G78R) (◊). In mixed culture fermentations, values from lysine plating was subtracted from total values determined by YEPD plaiting.*

Discussion

In winemaking a mixed fermentation by *S. cerevisiae* and non-*Saccharomyces* yeast is a common method for the introduction of complexity to wines. Typically, the yeasts are added sequentially, with the non-*Saccharomyces* inoculated first, with the aim of altering the aroma profile (and reducing ethanol), and the ethanol-tolerant *Saccharomyces* is added a few days later, to finish the fermentation. As *Saccharomyces* is quickly implanted and outcompetes the non-*Saccharomyces*, research has focused on how to maximise the non-*Saccharomyces* population and persistence during fermentation, in particular, when yeasts are co-inoculated rather than sequential. One approach is to extend the lag phase - the period before exponential growth - where the yeast adapt but do not divide. One example of this is by Albertin *et al.* (2017) who used classical genetics (backcrossing) to identify *S. cerevisiae* strains with long lag phases, and which were associated with sulfite pump (*SSU1*) translocations and subsequent regulatory changes. These strains could be co-inoculated with five different non-*Saccharomyces* species to allow improved wine fruitiness and complexity (Albertin *et al.*, 2017). An alternative option is the use of gene mutations that result in protracted growth, such as *SER1* mutants. *SER1* codes for 3-phosphoserine aminotransferase, an important enzyme in the production of both serine and glycine from glycolysis. The lack of *SER1* in a heme deficient yeast strain has previously been shown to negatively impact the initial growth phase of *S. cerevisiae* compared to other mutants under the same conditions (Reiner *et al.*, 2006). The lack of heme production prevents oxygen use, mimicking anaerobic conditions in relation to sterol synthesis. This may be similar to the "self-anaerobic" conditions experienced in winemaking, whereby CO_2 produced during the catabolism of high concentrations of sugar during fermentation, results in an essentially anaerobic environment. For industry applicability, the use of a naturally

occurring *SER1* mutation would be a more desirable, as its introduction would be classified as "self-cloning", thereby allowing such strains to be used in industry in countries that permit it. One such *SER1* 'loss of function' variant has been identified by Jung *et al.* (2018), *SER1*(G78R). This mutation is a naturally occurring variant identified in a sake yeast strain in a QTL study investigating chronological aging (Jung *et al.*, 2018). Parental strains (lacking *SER1*(G78R) allele) with the introduced *SER1*(G78R) allele exhibited slowed growth rate and lower biomass production when grown in YNB based media containing either glucose (0.5 %, 2 % or 10 %) or galactose (2 %) as the carbon source (Jung *et al.*, 2018). This phenotype may be beneficial for similar co-inoculation strategies.

In this study we assessed the effect of *SER1*(G78R) in alcoholic fermentation, when inoculated simultaneously with two non-*Saccharomyces* species commonly used in winemaking. *SER1*(G78R) was introduced into the widely used wine yeast, Lalvin EC1118, via CRISPR/Cas9 and confirmed to exhibit a slow growth phenotype in a chemically defined grape juice medium (**Figure 1**). The *SER1*(G78R) mutant took days longer to get reach stationary phase and exhibited lower total cell number in comparison to EC1118 (**Figure 1A**), both of which contributed to fermentation duration being extended by 120 h (**Figure 1B**). By using un-sulfured CDGJM, the phenotype was demonstrated to be SO_2 independent, unlike previously reported slow growth (*SSU1)* mutants (Albertin *et al.*, 2017). *SER1*(G78R)'s natural occurrence ensures a "self-cloning" designation for strains into which it is introduced meaning it would not be regulated as a genetically modified organism in countries such as Japan and the USA (Hanlon & Sewalt, 2020).

Previous metabolic analysis of *ser1Δ* in a laboratory yeast background (BY4742) in a synthetic must medium showed increased concentrations of succinic, acetic and

pyruvic acid concentrations throughout fermentation (Chidi *et al.*, 2016). In the same study glycerol concentration was slightly increased in the *ser1Δ* mutant fermentations, however concentrations were not significantly different to the BY4742. Interestingly, with regard to acetic acid production, Jung *et al.* (2018) noted a significant decrease in acetic acid in *SER1*(G78R) strains, converse to Chidi *et al.* (2016). Our initial phenotypic characterisation of the *SER1*(G78R) mutant in the EC1118 background showed similarities to Chidi *et al.* (2016), with significant increases in succinic acid, acetic acid, and glycerol when fermented in CDGJM (**Table 3**). Similarly, in Sauvignon Blanc fermentations, significantly higher concentrations of acetic acid and glycerol were noted, but with less succinic acid (**Table 4**). Acetic acid is known for its vinegar-like aroma, whilst succinic acid imparts a lingering salty bitter taste (Coulter *et al.*, 2004), such that increased amounts of these compounds in wines are considered a fault. In contrast, glycerol has the potential to improve wine sensory properties, with small increases contributing to perceived sweetness (> 5.2 g L^{-1}) in white wines (Noble & Bursick, 1984). Whilst the amount produced by the mutant and EC1118 in CDGJM was below this 'sweetspot', the glycerol content of the Sauvignon Blanc fermentations exceeded this concentration. Acetate esters were also quantified in Sauvignon Blanc EC1118 and *SER1*(G78R) fermentations, with significant differences only observed for propyl acetate, which was still lower than the sensory threshold and so unlikely to contribute to any sensorial differences in the wines. Whilst the *SER1*(G78R) monocultures are not useful *per se*, when co-inoculated with non-*Saccharomyces* yeast some of these potential negative phenotypes may be minimised through greater expression of non-*Saccharomyces* attributes in the resultant wines.

The *SER1*(G78R) mutant was evaluated against EC1118 in co-inoculated fermentations with (i) *L. thermotolerans* strain Concerto™ and (ii) *M. pulcherrima* strain

MP2 in a Sauvignon Blanc juice. *L. thermotolerans* yeast produce lactic acid (Benito *et al.*, 2015; Gibson *et al.*, 2018; Jolly *et al.*, 2014; Kapsopoulou *et al.*, 2007; Morata *et al.*, 2018), a trait whose exploitation has proven beneficial for the bioacidification of low acid Merlot wine to improve sensory properties (Hranilovic *et al.*, 2021). *M. pulcherrima* on the other hand is characterised by its ability to produce high concentrations of some acetate esters and lower alcohol content during wine fermentations (Hranilovic *et al.*, 2020; Varela *et al.*, 2016).

With co-inoculation regimes such as these, it would be expected that wild-type EC1118 would quickly dominate fermentation and the *SER1*(G78R) mutant to exhibit slowed growth allowing in a larger and more persistent population of non-*Saccharomyces* yeast. These expectations were confirmed experimentally in this study (**Figure 2**). When co-inoculated with *SER1*(G78R), Concerto™ showed *S. cerevisiae*-like growth, dominating the fermentation. *SER1*(G78R) also supported a large population density of MP2 under both inoculation ratios before a decline in MP2 in the later stages, and *SER1*(G78R) finishing the fermentation. This pattern was similar to the sequential fermentation reported by Contreras *et al.* (2014), where the *M. pulcherrima* CFUs declined, and a subsequent inoculation with an *S. cerevisiae* strain after 50% sugar consumption allowed for fermentation completion. The causality in decline most likely reflects the low ethanol tolerance of *M. pulcherrima* (3–5 %), with only a few tolerating up to 9% (Barbosa *et al.*, 2018).

The relative abundance of EC1118 in the co-inoculated fermentations was reflected in the lack of significant variation in the metabolite profiles of the resultant wines (**Tables 4** and **5**). This scenario typifies co-inoculated fermentations and is one reason why winemakers rely on a sequential inoculation regime (Vilela, 2020) to ensure expression of non-*Saccharomyces* attributes to alter wine sensory profiles (Dutraive *et*

al., 2019; Englezos *et al.*, 2018; Hranilovic *et al.*, 2020; Lin *et al.*, 2020; Lu *et al.*, 2017). *SER1*(G78R) co-inoculated fermentations on the other hand resulted in largely greater concentrations of non-*Saccharomyces* yeasts, which is reflected in the concentrations of key metabolites (**Table 4**) and volatile compounds analysed (**Table 5**).

Avoiding the risk of high acetic acid and volatile acidity is often tackled through mixed fermentation involving *L. thermotolerans* (Benito *et al.*, 2015; Benito, 2018; Comitini *et al.*, 2011) or *M. pulcherrima* (Hranilovic *et al.*, 2020; Varela *et al.*, 2016). Similar findings were observed in the fermentations co-inoculated with *SER1*(G78R), whereby the high amounts of acetic acid produced by *SER1*(G78R) (**Supplementary Figure 1**) were decreased through the uptake and metabolism of hexose by low acetic acid producing non-*Saccharomyces* yeasts (**Table 4**). As such, concentrations of acetic acid were close to the legal limits (> 1.5 g L^{-1} in Australia) in fermentations using *SER1*(G78R) in monoculture and with MP2 (1:1), but were significantly reduced through high inoculation rates of non-*Saccharomyces* (9:1).

The other organic acids of interest in terms of wine acidity were lactic acid and malic acid. Malic acid in wine is generally known for its harsh acidic quality and is decarboxylated to lactic acid by lactic acid bacteria such as *Oenococcus oeni* or *Lactobacillus* species to improve sensorial properties and improve wine stability (Bartowsky, 2014). Some yeast species, such as *Schizosaccharomyces pombe*, are known to completely degrade malic acid as they contain genes coding for malate permease and malic acid decarboxylase (Benito *et al.*, 2016). Malic acid degradation is also associated with *L. thermotolerans* strains (Gobbi *et al.*, 2013; Hranilovic *et al.*, 2018; Kapsopoulou *et al.*, 2005; Whitener *et al.*, 2017), as observed here too, although only *L. thermotolerans* dominated fermentations reduced malic acid by up to ~25 %. Interestingly, this was not reflected in an increase in lactic acid, a phenotype typically associated with *L.*

thermotolernas yeast, however, Concerto™ is considered a poor producer of lactic acid (Vaquero *et al.*, 2020).

Glycerol and ethanol modulation using non-*Saccharomyces* yeast has been extensively researched with the view to producing 'reduced alcohol' wines. Both species used in this study are able to increase glycerol (Contreras *et al.*, 2014; Gobbi *et al.*, 2013; Hranilovic *et al.*, 2020; Kapsopoulou *et al.*, 2007) and decrease ethanol content (Contreras *et al.*, 2014; Hranilovic *et al.*, 2021; Hranilovic *et al.*, 2020; Hranilovic *et al.*, 2018; Varela *et al.*, 2016). Glycerol production was improved when MP2 was co-cultured with *SER1*(G78R) – whilst the influence of Concerto™ was more complicated, producing less glycerol than the *SER1*(G78R) monoculture, but still significantly higher than all EC1118 fermentations (**Table 4**). *SER1*(G78R) can still be regarded as a superior glycerol producer compared to EC1118 but was dependent upon its abundance in co-fermentations (**Figure 2E**). Increased glycerol production in *S. cerevisiae* yeast occurs with a concurrent increase in acetic acid (Eglinton *et al.*, 2002; Remize *et al.*, 1999; van Wyk *et al.*, 2020), needed to balance the NAD^+/NADH ratio which trends towards NAD^+ by glycerol biosynthesis. An increase in acetic acid and glycerol was observed in the *SER1*(G78R) monoculture, but not in the non-*Saccharomyces* co-inoculated cultures, alluding to Concerto™ and MP2 as a way to address redox balance. Previously, Sadoudi *et al.* (2017) demonstrated an increase in glycerol and decrease in acetic acid in *M. pulcherrima/S. cerevisiae* sequential fermentations, with the presence of *M. pulcherrima* altering the expression of genes involved in acetic acid biosynthesis in *S. cerevisiae*. This could explain the reduction of acetic acid in the co-inoculated fermentations in this study. Another plausible explanation is that MP2-derived acetic acid is reduced through the production of acetate esters, a phenomenon observed in studies involving *M. pulcherrima* (Binati *et al.*, 2020; Contreras *et al.*, 2014; Hranilovic *et al.*, 2020; Sadoudi *et al.*, 2012;

Varela *et al.*, 2016). van Wyk *et al.* (2020) demonstrated that overexpression of alcohol acetyltransferase (*ATF1*) in *S. cerevisiae* strains that accumulate both glycerol and acetic acid due to NAD-dependent glycerol-3-phosphate dehydrogenase (*GPD1*) overexpression, resulted in lower acetic acid and higher acetate ester concentrations. Whilst outside the scope of this paper, it would be interesting to assess *ATF1* and *GPD1* transcript levels in *M. pulcherrima* strains to determine if this is also the case.

Many authors have noted specific increases in acetate esters in mixed fermentations involving *M. pulcherrima*. In this study, five esters (ethyl acetate, isoamyl acetate, 2-phenylethyl acetate, isobutyl acetate, *cis*-3-hexenyl acetate) were assessed based on prior reports of their increase with *M. pulcherrima* inclusion in fermentation (Contreras *et al.*, 2014; Hranilovic *et al.*, 2020; Sadoudi *et al.*, 2012; Varela *et al.*, 2016). Propyl acetate was also analysed. In most instances, there were statistically significant increases in acetate esters with the higher MP2 inoculations correlating to higher acetate esters (**Table 5**).

Hranilovic *et al.* (2018) also demonstrated an increase in acetate esters by Concerto™ when sequentially inoculated with *S. cerevisiae* in Shiraz fermentations. Specifically, significant increases were observed in ethyl acetate, ethyl phenylacetate, isoamyl acetate and isobutyl acetate. In this study, whilst higher mean concentrations of propyl acetate, isobutyl acetate were observed, and ethyl acetate and isobutyl acetate significantly increased at the higher inoculation ratio, isoamyl acetate, *cis*-3-hexenyl acetate and 2-phenylethyl acetate were reduced (**Table 5**). Differences between studies may relate to wine type (red *vs* white) and variety (Shiraz *vs* Sauvignon Blanc). Hranilovic *et al.* (2018) further reported on vintage-dependent variability in acetate esters, in particular isoamyl acetate, which in one juice co-inoculated with Concerto™ had significantly higher concentrations compared to *S. cerevisiae*, but the inverse in a

subsequent vintage. Such trends highlight the importance of juice composition in winemaking outcome.

Arguably, the most interesting observation of the present study was the alleviation of SO_2 sensitivity associated with Concerto™ in sulfured Sauvignon Blanc and Chardonnay juice. The inability of the monocultures to thrive in these juices agrees with the manufacturer's (Chr. Hansen) recommendation for the strain to be used in unsulfured grape juice as in organic winemaking (https://www.gusmerwine.com/wp-content/uploads/sites/7/2015/07/Chr-Hansen-Viniflora-CONCERTO-PDS.pdf). The results with the *SER1*(G78R) co-fermentations are therefore promising, as they allow the use of SO_2 sensitive non-*Saccharomyces* yeasts, which might offer other interesting oenological properties, in otherwise lethal concentrations of SO_2. Similar findings were made for the EC1118 co-inoculated cultures when plating on selective lysine media (**Figure 4A**), although the Concerto™ concentrations were reduced. Considering this phenomenon is observed with both wild-type and mutant *S. cerevisiae* strains, it may be implied that other slow growing yeast (e.g., Albertin *et al.*, 2017) possess this ability. Further research would be of benefit to both understanding this phenotype and developing novel strains for the use in co-inoculation and SO_2 sequestration.

Conclusion

The transfer of a novel naturally occurring *SER1* variant (*SER1*(G78R)) into the wine yeast EC1118 through CRISPR/Cas9 results in a slower growth phenotype. This mutation may also have the added advantage over previously reported slow growing yeasts, as SO_2 is not needed to elicit slow growth, thereby giving it broader applicability. This phenotype benefited novel mixed cultures where the mutant was co-inoculated with

non-*Saccharomyces* as it allowed for greater expression of positive sensory effects imparted by the non-*Saccharomyces*. Furthermore, this strain can be used with non-*Saccharomyces* strains that have low sulfite tolerance, thereby permitting the standard wine making practice of using SO_2 to prevent oxidation and microbial spoilage. Further evaluation is required of this strain, in combination with other non-*Saccharomyces*, inoculation ratios, etc. to determine whether it would be suited for use as a 'mixed' starter culture comprised of multiple yeast species. The construction of this strain is an example of 'self-cloning' and as such is likely to be permitted for use in countries such as Japan and USA where regulations are less stringent than in Europe and Australia. To date, Australia's 'clean green image' in winemaking and regulation of GMOs prevents the use of genetically modified yeast and bacteria, apart from variants isolated from the vineyard and point mutations generated by mutagenesis.

Acknowledgements

The authors would like to thank Nick van Holst for his technical assistance and Tom Ellis and William Shaw from Imperial College London for providing plasmids pWS173 and pWS082. We also thank Emily Nicholson and Sue Maffei at CSIRO for their assistance with the GC-MS analysis of the wine volatile compounds.

Funding

This project was supported by funding from Wine Australia [UA1803_2.1] and The Australian Research Council Training Centre for Innovative Wine Production

(www.ARCwinecentre.org.au; project number IC170100008), which is funded by the Australian Government with additional support from Wine Australia and industry partners. Wine Australia invests in and manages research, development and extension on behalf of Australia's winegrowers and winemakers and the Australian Government. Tom Lang is a recipient of a University of Adelaide Faculty of Sciences Divisional Scholarship and Wine Australia Supplementary Scholarship (AGW Ph1603). The University of Adelaide and CSIRO are members of the Wine Innovation Cluster (http://www/thewaite.org/waite-partners/wine-innovation-cluster/).

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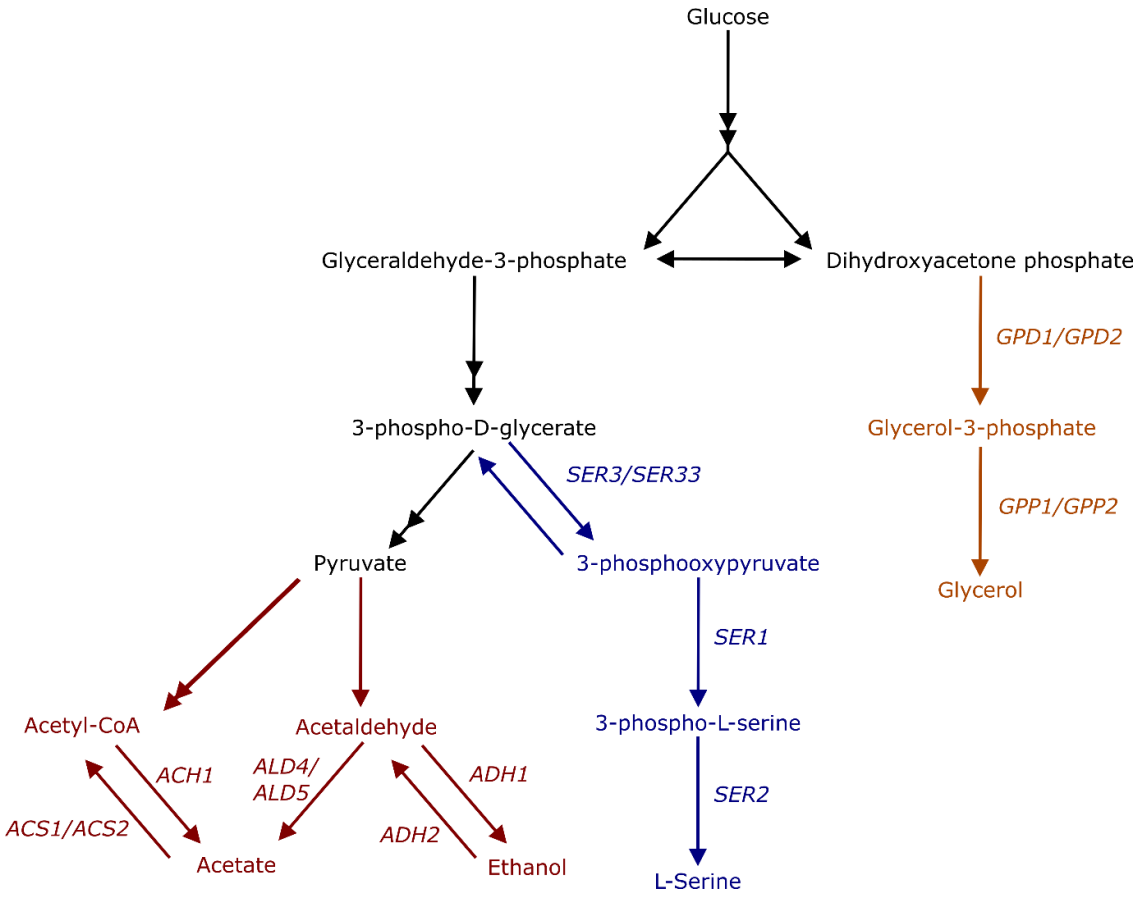
Supplementary Table 1. Residual sugar and metabolite analysis of incomplete monoculture MP2 and Concerto™ fermentations in Sauvignon blanc juice

Strain(s)	Residual Sugar (g L^{-1})	Acetic Acid (g L^{-1})	Lactic Acid (g L^{-1})	Malic Acid (g L^{-1})	Succinic Acid (g L^{-1})	Glycerol (g L^{-1})	Ethanol (g L^{-1})
MP2	54.46 ± 5.01	0.00 ± 0.00	0.04 ± 0.08	3.95 ± 0.03	1.85 ± 0.10	6.09 ± 0.16	55.68 ± 2.05
Concerto™	166.20 ± 5.26	0.02 ± 0.03	0.08 ± 0.14	3.53 ± 0.28	2.95 ± 0.18	0.55 ± 0.50	5.99 ± 5.08

Supplementary Table 2. Acetate ester analysis of incomplete monoculture MP2 and Concerto™ fermentations in Sauvignon blanc juice

Strain(s)	Propyl Acetate (µg L^{-1})	Isobutyl Acetate (µg L^{-1})	Isoamyl Acetate (µg L^{-1})	*cis*-3-Hexenyl Acetate (µg L^{-1})	2-phenylethyl Acetate (µg L^{-1})	Ethyl Acetate (µg L^{-1})
MP2	0.98 ± 0.19	2.68 ± 0.03	10.58 ± 0.50	0.73 ± 0.06	0.41 ± 0.15	2363.00 ± 111.70
Concerto™	0.50 ± 0.17	0.43 ± 0.37	0.49 ± 0.18	1.66 ± 0.46	0.03 ± 0.01	383.10 ± 117.00



Supplementary Figure 1. Glycolysis and Serine biosynthesis in *Saccharomyces cerevisiae*. Colours highlight glycerol (orange), serine (blue), and acetate and ethanol (maroon) biosynthesis from glycolysis. Adapted from the *Saccharomyces* Genome Database's YeastPathways resource (https://pathway.yeastgenome.org/)

Chapter 4

“Proline accumulation in the wine yeast EC1118: Evaluating responses to fermentation related stresses”

Introduction

The production of wine involves the conversion of grape sugars to ethanol by yeast, typically, *Saccharomyces cerevisiae*. During wine fermentation yeast can experience stress from extremes of these compounds. Exposure to stress begins at inoculation when yeast are exposed to high sugar concentrations, resulting in osmotic stress. High solute concentrations outside of cells result in intracellular water passively diffusing into the outside medium by osmosis. In response to this, the high osmolarity glycerol (HOG) pathway is rapidly activated resulting in the production of glycerol (reviewed by Hohmann, 2015). The glycerol produced acts as an osmolyte, balancing solute concentrations inside of cells, thereby minimising water loss. In response to osmotic stress caused by high sugar concentration, increases in other potential osmolytes, trehalose (Kaeberlein *et al.*, 2002) and glycogen (Gomar-Alba *et al.*, 2015), have also been observed.

The consumption of grape sugars through glycolysis sees a parallel increase in ethanol, often to concentrations that are detrimental to yeast (Kubota *et al.*, 2004, Henderson *et al.*, 2013). High ethanol concentrations inhibit yeast through impaired membrane fluidity as well as compromised transport of ions, sugars, and nitrogen sources, thereby affecting cell viability and growth (reviewed by Elis *et al.*, 2019). In the presence of high ethanol concentrations yeast produce reactive oxygen species (ROS; Costa *et al.*, 1997), resulting in oxidative stress. ROS produced include hydrogen peroxide, superoxide anions and hydroxyl radicals (Jamieson, 1998), which at high concentrations can result in protein and lipid degradation (Wolff *et al.*, 1986), and DNA mutagenesis (Storz *et al.*, 1987). ROS are degraded via catalase and superoxide dismutase, which convert hydrogen peroxide to water and oxygen, and superoxide anions

to oxygen or hydrogen peroxide, respectively, or are removed by glutathione, acting as a free radical scavenger during the oxidative stress response (Jamieson, 1998).

Whilst metabolites such as glycerol and trehalose act as stress protectants, the accumulation of proline may also represent a beneficial stress response mechanism in certain scenarios (Eardley & Timson, 2020). In plants, proline is known for its abiotic stress response properties, accumulating during osmotic stress (low water or high salinity) or exposure to low temperatures, heavy metals or UV radiation (reviewed by Hayat *et al.*, 2012). The high proline content of grape juice (Ough, 1968, Long *et al.*, 2012) makes proline of particular interest in terms of strain improvement as a stress protectant and alternative nitrogen source during fermentation. However, proline usage by *Saccharomyces cerevisiae* is limited because of nitrogen catabolite repression and inhibition of proline transport at the transcriptional and post-translational level, respectively, in the initial stages of fermentation (Forsberg & Ljungdahl, 2001). Once preferred nitrogen sources are consumed proline uptake is possible, however, anaerobic conditions prevent proline metabolism, as proline oxidase (*PUT1*) requires oxygen (Duteurtre *et al.*, 1971). Previous work from our group identified novel mutations in *PUT4* and the corresponding promoter sequence that enabled proline uptake under nitrogen catabolite repressive conditions (Poole *et al.*, 2009). The mutant yeast harbouring a combination of a Put4p(S605A) variant with a promoter mutation (-160T>C) had increased tolerance when exposed to high temperatures (45 °C) and osmotic shock (1.1 M NaCl).

Proline-mediated stress tolerance is also possible through the over-accumulation of proline via the proline biosynthetic pathway. This approach involves desensitising Pro1p (gamma-glutamyl kinase, catalysing the first step of proline biosynthesis from glutamate; **Figure 1A**), to feedback inhibition through novel missense mutations in the

PRO1 open reading frame (Takagi *et al.*, 2007, Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016, Murakami *et al.*, 2020). The resultant biosynthetic accumulation of proline is effective against ethanol stress as in sake brewing (Takagi et al., 2007) and freeze-thaw and osmotic stress as in baking (Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016). In sake brewing, Takagi *et al.* (2007) demonstrated that only moderate proline levels were required, and that ethanol resistance was not further improved in extremely high proline producing yeast. Whilst the *PRO1* mutations relate to sake and baking yeast backgrounds, their aetiology in relation to proline biosynthesis and accumulation is unknown in wine yeast and hence the subject of this study.

This chapter reports on the investigation of the *PRO1* homozygous mutations (I150T, P247S or E415K) in Lalvin EC1118 (Lallemand), a wine strain anecdotally regarded as the 'workhorse' of commercial red and white wine production (The Australian Wine Research Institute, 2019). The effect of the cytoplasmic localisation of Put2p (delta-1-pyrroline-5-carboxylate dehydrogenase) on proline accumulation was also investigated, through removal of the first 16 amino acids required for mitochondrial targeting (Martin *et al.*, 2003). The intermediate in both proline biosynthesis and degradation, Δ^1-pyrroline-5-carbxylate (P5C), is toxic to cells (Nomura & Takagi, 2004). This study proposed that the potential toxic accumulation of P5C resulting from excess Pro1p activity could be mitigated by re-routing P5C degradation from the mitochondrion to the cytosol to produce glutamate (**Figure 1A–1C**). Interestingly, tolerance to sorbitol-induced osmotic stress is improved in some mutants, whilst increased sensitivity or no effect was observed in the presence of ethanol or sulfur dioxide. These findings allude to proline acting an osmolyte when intracellular levels are moderately increased.

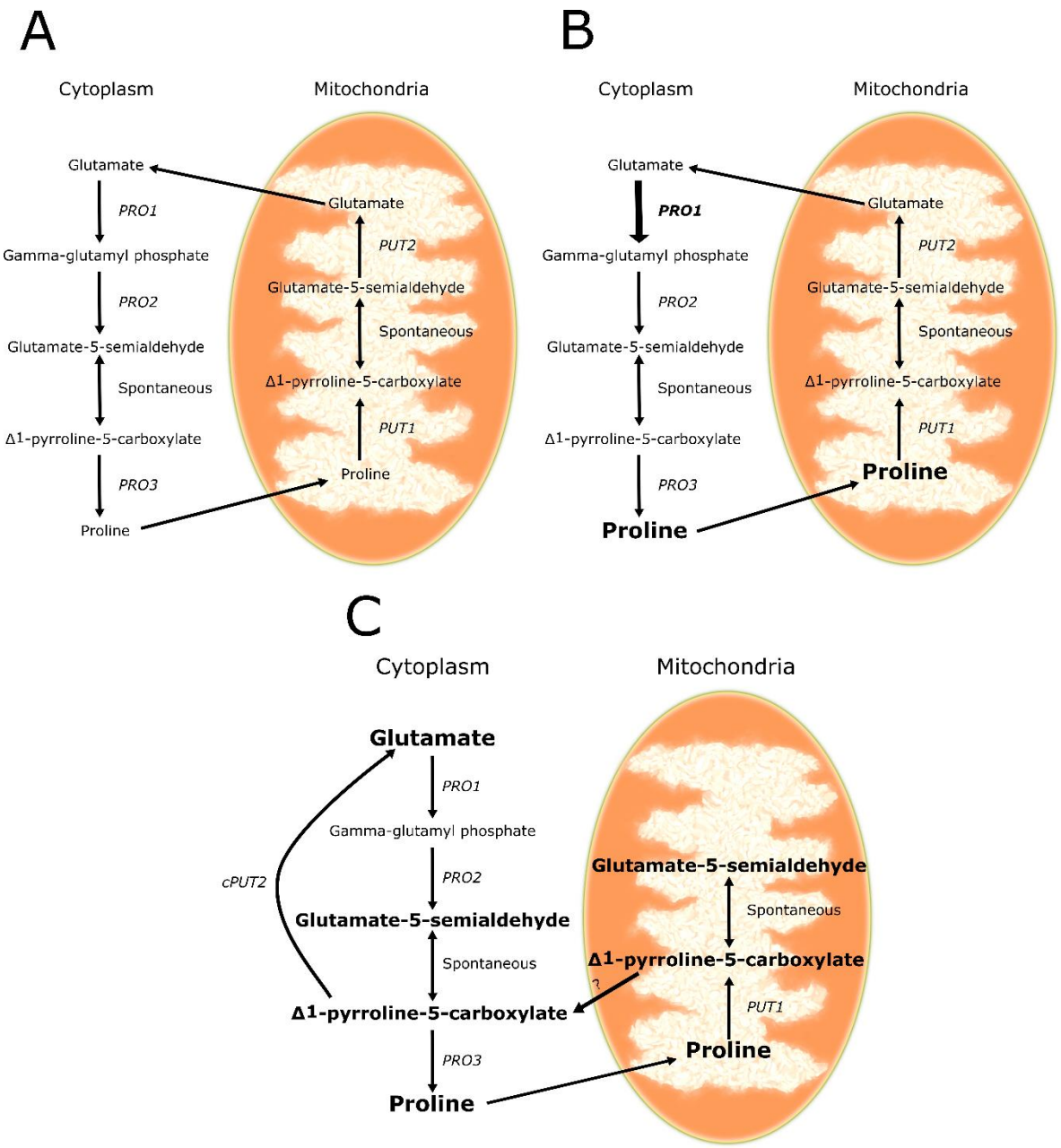


Figure 1. Proposed proline biosynthetic/degradation pathways of *S. cerevisiae* used in this study.

Proline biosynthesis in yeast from glutamate occurs via the use of 3 key enzymes; gamma-glutamyl kinase (*PRO1*), gamma-glutamyl phosphate reductase (*PRO2*) and Δ^1-pyrroline-5-carboxylate reductase (*PRO3*). Degradation of proline to glutamate occurs in the mitochondria; using the key enzymes; proline oxidase (*PUT1*) and Δ^1-pyrroline-5-carboxylate dehydrogenase (*PUT2*). **Figure 1** depicts wild-type (A), expected *PRO1* mutant (B) and the modified cytoplasmic *PUT2* (c*PUT2*) pathway (C) used in this study.

Compounds in bold are expected to increase. For *PRO1* mutations (B) used in this study increases in proline content have been identified (Tsolmonbaatar *et al.*, 2016). c*PUT2* mutations (C) are expected to increase glutamate from the cytosolic P5C pool (Martin *et al.*, 2003). Homozygous integration of c*PUT2* will result in a *Δput2* phenotype as mitochondrial localisation will not be possible. *Δput2* strains are known to exhibit increased proline and P5C content (Nomura & Takagi, 2004). ? denotes an unknown method of Δ^1-pyrroline-5-carboxylate translocation to the cytoplasm (Nomura & Takagi, 2004).

Materials and Methods:

Microbial strains, media and plasmids

All yeast mutants were derived from the *Saccharomyces cerevisiae* wine strain Lalvin EC1118 (**Table 1**). For plasmid transformation and propagation, *Escherichia coli* NEB® 5-Alpha (C2987I, New England Biolabs) was used. Yeasts were routinely cultured in YEPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) at 28 °C and *E. coli* were grown at 37 °C in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride), supplemented with kanamycin (50 mg L^{-1}) or ampicillin (100 mg L^{-1}) for plasmid propagation as required. Yeast selection medium was based on synthetic defined (SD) medium (2 % glucose, 1.7 % yeast nitrogen base without ammonium sulfate or amino acids; Difco Laboratories) with nitrogen either as 0.5 % allantoin (SD-N + AL) or 0.5 % ammonium sulfate (SD-N + AS; Tsolmonbaatar *et al.,* 2016). SD medium pH was adjusted to 3.5 to make it similar to winemaking conditions. For plates, the medium was solidified using 20 g L^{-1} agar. Plasmids used in this study (**Table 2**) were extracted from *E. coli* using Wizard® Plus SV Minipreps DNA Purification System (Promega).

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	**Genotype**	**Source**
EC1118	Lalvin EC1118 (WT), diploid	Lallemand, France
PRO1(I150T)	*PRO1*(I150T)/*PRO1*(I150T) (447ATC>ACC)	This study
PRO1(P247S)	*PRO1*(P247S)/*PRO1*(P247S) (738CCT>TCT)	This study
PRO1(E415K)	*PRO1*(E415K)/*PRO1*(E415K) (1242GAA>AAA; 1269G>A)	This study
c*PUT2*	*PUT2*(delL2:F16)/*PUT2*(delL2:F16)*	This study
PRO1(I150T) c*PUT2*	*PRO1*(I150T)/*PRO1*(I150T), *PUT2*(delL2:F16)/*PUT2*(delL2:F16)*	This study
PRO1(P247S) *cPUT2*	*PRO1*(P247S)/*PRO1*(P247S), *PUT2*(delL2:F16)/*PUT2*(delL2:F16)*	This study
PRO1(E415K) *cPUT2*	*PRO1*(E415K)/*PRO1*(E415K), *PUT2*(delL2:F16)/*PUT2*(delL2:F16)*	This study

*delL2:F16 refers to complete deletion of codons from the leucine codon at position 2 to the phenylalanine codon at position 16.

Table 2. Plasmids used in this study

Plasmid	**Description**	**Source**
pWS082	sgRNA cloning vector	Shaw *et al.* (2019). Addgene plasmid* #90516
pWS173	Cas9 linear co-transformation vector	Addgene plasmid* #90960
pWS-PRO1 I150T	sgRNA vector targeting *PRO1*(I150T)	This study
pWS-PRO1 P247S	sgRNA vector targeting PRO1(P247S)	This study
pWS-PRO1 E415K	sgRNA vector targeting PRO1(E415K)	This study
pWS-PUT2	sgRNA vector targeting *PUT2* (delL2 :F16)	This study

*https://www.addgene.org/

Construction of yeast mutants by CRISPR/Cas9

All yeast mutants were generated using the method described in Shaw *et al.* (2019) and on the website https://benchling.com/pub/ellis-crispr-tools. Briefly, sgRNA target sequences and ligation primers for pWS082 (**Table 3**) were designed using Benchling (benchling.com). sgRNA sequences were designed such that repair templates would disrupt sgRNA or PAM sequences. The designed sequences, including the 3' NGG PAM sequences, were analysed for homology against the EC1118 genome (taxid:643680) using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as Benchling software uses the S288C genome by default. The ligation oligonucleotide primers were cloned into pWS082 via Golden Gate assembly (Engler *et al.*, 2008) as instructed in Shaw *et al.* (2019). Resulting ligations were transformed into NEB® 5-Alpha (C2987I, New England Biolabs) according to the manufacturer's protocol and plated onto solid LB medium containing 100 mg L^{-1} ampicillin and incubated overnight at 37 °C. Plates were visualised using UV light (365 nm) to select for non-fluorescent transformed colonies. Non-fluorescent isolates were grown overnight in 10 mL LB medium containing 100 mg L^{-1} ampicillin at 37 °C with shaking (200 rpm) and plasmids isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Plasmids were sequenced with primer pWS082seq (**Table 3**; Australian Genome Research Facility, Australia) to confirm the correct sgRNA sequences.

Table 3. Oligonucleotide primers used in this study

Primer	**Sequence (5'-3')**	**Source**
sgRNA cloning		
pWS-PRO1 I150T F	GACTTTTCTGTTAGAGAAATCAAATT	This study
pWS-PRO1 I150T R	AAACAATTTGATTTCTCTAACAGAAA	This study

pWS-PRO1 P247S F	GACTTTAGCGATACACCTGCGAATAT	This study
pWS-PRO1 P247S R	AAACATATTCGCAGGTGTATCGCTAA	This study
pWS-PRO1 E415K F	GACTTTTGTCGCTCATAGAGAAAATT	This study
pWS-PRO1 E415K R	AAACAATTTTCTCTATGAGCGACAAA	This study
pWS-PUT2 F	GACTTTCAGGAATTATGCTATCAGCA	This study
pWS-PUT2 R	AAACTGCTGATAGCATAATTCCTGAA	This study
HDR template synthesis		
I150T HDR F	GGGCGTTATTCCCATTGTGAATGAAAACGACACACTATCTGTTAGAGAAACCAAATTTGG	This study
I150T HDR R	TAAAGCAGAAGTAATTGCTGATAAAGTGTCATTGTCACCAAATTTGGTTTCTCTAACAGA	This study
P247S HDR F	AACGAATGCCGGTGTTCATACGTTGATCATGAAAAGCGATACATCTGCGAATATAGGTAG	This study
P247S HDR R	CATCGTCAAGTTCTAGAGTTTGCATATACTCGACAATTCTACCTATATTCGCAGATGTAT	This study
E415K HDR F	AGGTTTGCACAGTGACCAAATCGAAGAGGAATTGGGCTATAATGACAGCAAATATGTCGC	This study
E415K HDR R	TCGTTTCAACGAGGTGGGAATGCTAAATTTTCTCTATGAGCGACATATTTGCTGTCATTA	This study
PUT2 HDR F	AATATAATCTATATTGTATAGAAGGCCAATTCAAATTCACAGGAATTATGTCACAACTGGGAC	This study
PUT2 HDR R	ACAGGTTCATTTCTTATGTGCTTGGGGGGCTTGATGTGTCCCAGTTGTGACATAATTCCTGTG	This study
Gene specific amplification		
PRO1 F	CATCAGCGAACTAATGCTTTCTC	This study
PRO1 R	GTTAAGAACACTAATGTGGATTAGG	This study
PUT2 A	AGAAACACACTCATCTATGACAGCA	SGDP
PUT2 D	TCTTTAAGCTTACGAATGGACGTAT	SGDP
Sequencing		
I150T seq	GCAATTTGATCAACGTATCGC	This study

P247S seq	GATCTTAGTTGTCCCAGATCTC	This study
E415K seq	GCACTTTCCATGAGTTAGAATG	This study
PUT2 seq	AATGCTTATCTACGCCATCG	This study
pWS082 seq	GTCATCTGGAGGTCCTGTGTTC	This study

*SGDP; *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group /yeast_deletion_project/deletions3.html).
All primers were purchased from Sigma-Aldrich, cartridge purified and supplied at 100 µM in water.

Repair template DNA was constructed by PCR amplification of overlapping primers including desired mutant sequences (**Table 3**) in a standard 50 µL reaction using Velocity DNA Polymerase (BIO-21098; Bioline) with modifications. Each 50 µL reaction consisted of 8 µL of each primer (100 µM), 0.5 µL of 100 mM dNTP mix (BIO-39028; Bioline), 10 µL of supplied 5x HIFI buffer, 1 µL of polymerase (2 U) with the remaining volume as ultrapure water. Recommended cycling conditions were followed; 98 °C for 2 min (initial denaturation) followed by 30 cycles of 98 °C for 30 sec (denaturation), 57 °C for 30 sec (annealing) and 72 °C for 10 sec (extension), and a final 72 °C for 4 min extension. Sizing and quantification of the amplified repair template DNA was by electrophoresis and comparison to DNA molecular weight markers (Hyperladder™ 50 bp (BIO-33054); Bioline).

The *PRO1*(I150T, P247S or E415K) mutations were individually introduced into EC1118 using the standard lithium acetate protocol (Gietz & Schiestl, 2007). An overnight culture was diluted 1 in 100 in 100 mL YEPD and grown for 4–5 h until an OD_{600} of 0.4–0.5. Cells were pelleted, washed twice and resuspended in 0.1 M lithium acetate to a final volume of 500 µL. Each transformation reaction used 50 µL of cells, which were pelleted prior to resuspension with 100 ng of BsmBI linearised (purified)

pWS173 plasmid, 10 µL of EcoRV-HF digested (but not purified) sgRNA plasmid (200 ng), ~ 5 µg of repair template DNA and sterile ultrapure water to 54 µL. The remaining volume of the transformation reaction consisted of 10 µL salmon sperm DNA (10 mg mL^{-1}), 36 µL 1 M lithium acetate, and 260 µL 50% w/v polyethylene glycol 4000. The reactions were incubated without shaking (30 °C, 30 min followed by 42 °C, 30 min). Following centrifugation (20,000 x *g*, 1 min), the heat-shocked cells were resuspended in 1 mL YEPD and incubated at 30 °C for 150 min. The transformed cells were pelleted and resuspended in 200 µL of sterile ultrapure water before plating on YEPD agar containing 100 mg L^{-1} G418 and incubation at 30 °C for 2 days.

To confirm mutants, G418 resistant colonies were grown for 72 h in non-selective YEPD liquid to facilitate plasmid loss. Serially diluted cultures were plated onto YEPD agar for single colony purification. Individual colonies were then replica plated onto YEPD (master plate) and YEPD with G418 (selection) to determine plasmid loss. Genomic DNA was isolated from G418 sensitive colonies via phenol/chloroform glass bead method (Adams *et al.*, 1998). Isolated genomic DNA was used as template for *PRO1* gene amplification with primers PRO1 A and PRO1 D (**Table 3**) using Velocity DNA Polymerase (Bioline). PCR reactions were purified with the Wizard® SV Gel and PCR Cleanup System (Promega) and Sanger sequenced (Australian Genome Research Facility, Australia) using primer PRO1 seq (**Table 3**).

For mutants containing *PUT2* lacking the mitochondrial localisation signal sequence (L2-F16), an additional round of transformation was undertaken, with constructs made in an analogous manner using pWS-PUT2 (**Table 2**) and *PUT2* specific oligo pairs pWS-PUT2 F and pWS-PUT2 R and PUT2 HDR F and PUT2 HDR R. Sequence confirmation was with primer PUT2 seq (**Table 3**).

Determination of AZC resistance of modified yeasts

To demonstrate proline biosynthesis and accumulation, the toxic non-protein homolog of proline, azetidine-2-carboxylic acid (AZC) was used (Tsolmonbaatar *et al.*, 2016). In wild type yeast, AZC is taken up and incorporated into protein, and is detrimental to cell viability. Proline accumulating mutants incorporate proline instead of AZC, thus are viable. Single colonies were grown overnight in 10 mL YEPD at 30 °C with shaking. Overnight cultures were serially diluted with sterile ultrapure water. 5 µL of each dilution (10^{-1} to 10^{-4}) were spot plated onto solid SD-N + AL medium (Tsolmonbaatar *et al.*, 2016) containing 5 mg mL^{-1} AZC (Sigma-Aldrich). Plates were incubated at 30 °C for 5 days before being photographed.

Determination of intracellular proline content

Yeast were pre-cultured overnight in 10 mL YEPD at 28 °C with shaking (140 rpm) and used to inoculate 10 mL SD-N + AL or SD-N + AS with 5 x 10^6 cells mL^{-1} and grown again under the same conditions. 1 mL of culture was harvested by centrifugation (20,000 x *g*, 1 min) and washed twice prior to resuspension in 500 µL ultrapure water. Resuspended cells were boiled at 100 °C for 20 min to extract amino acids. Supernatants were shipped at ambient temperature from The University of Adelaide (Adelaide, Australia) to the Nara Institute of Science and Technology (Ikoma, Japan) for analysis. Proline, glutamate, arginine and ornithine were quantified from the supernatant using an amino acid analyser (AminoTac JLC-500/V; JEOL, Tokyo, Japan). Amino acid concentrations were expressed as a percentage of yeast dry weight or mass per cell (fg $cell^{-1}$).

Stress tolerance of mutant strains

Yeast were precultured in 10 mL YEPD medium overnight at 28 °C with shaking (140 rpm). The cultures were washed once in PBS prior to resuspension in the appropriate culture medium and used to inoculate 2 mL of SD-N + AL or SD-N + AS medium containing either ethanol (13% v/v), 80 mg L^{-1} potassium metabisulfite (30 mg free SO_2 L^{-1}, 0.6 mg L^{-1} molecular SO_2) or sorbitol (2 M) with 5 x 10^6 cells mL^{-1} in deep-well 96-well plates. Each treatment was done in triplicate per strain. Plates were sealed with Breathe-Easy® plate seals (Diversified Biotech) and incubated without shaking at 28 °C overnight. Cultures were resuspended and then assessed by flow cytometry (Guava® easyCyte™, Merck Millipore). Cells were diluted in phosphate buffered saline containing 5 µg mL^{-1} propidium iodide (PI) such that 500 events per µL was not exceeded. 5,000 events were counted per analysis. PI excitation was achieved by the blue laser (488 nm) and emission monitored by yellow bandpass filter (583/26 nm). Data for live (PI negative), damaged/dead (PI stained) and total cell numbers were acquired and subsequently analysed using InCyte software version 3.3 (Merck Millipore). The values were used to determine total cell number per mL and viability.

Statistical analysis

GraphPad Prism 9.0.0 software (www.graphpad.com) or XLSTAT 2021.1.1 software (https://www.xlstat.com/) was used for data analysis. All graphs were created using GraphPad Prism. For all datasets one-way ANOVA with multiple comparisons (Tukey's HSD test) was used to determine statistical significance ($p < 0.05$) using XLSTAT.

Results and discussion:

The yeast *Saccharomyces cerevisiae* is used across many applications for food and beverage production, with each industrial use exposing the yeast to various stresses. This has led to researchers investigating novel methods for improving responses to these stresses. One such focus has been on the overproduction of proline to protect from baking (Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016) and sake brewing related stresses (Takagi *et al.*, 2005, Takagi *et al.*, 2007, Murakami *et al.*, 2020). Proline overproduction as a means of providing stress protection has not been studied in a winemaking yeast background, and as such forms the basis of this study.

Novel proline accumulating yeast mutants have been developed and investigated in both a sake and bread making background. In sake brewing yeasts, increased accumulation of proline improved the response to ethanol, when directly inoculated into SD medium containing 9 % (Takagi *et al.*, 2005, Takagi *et al.*, 2007) or 18 % ethanol (Takagi *et al.*, 2005). In baker's yeast, responses to both osmotic and freeze-thaw stresses were enhanced through proline accumulation (Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016). Research in wine yeast has focused on increasing the availability of nitrogen in the fermentation. In winemaking, concentrations of yeast assimilable nitrogen (YAN) required for fermentation completion generally range between 120 and 140 mg N L^{-1} (Bisson, 1999). However, concentrations present in grape must can range between 60 and 2400 mg N L^{-1} (Henschke & Jiranek, 1993), so there is potential for YAN concentrations to be below the required amounts. Whilst supplementation of ammonium in the form of diammonium phosphate is common practice to improve YAN concentrations, the improved uptake and use of poorly (proline; Salmon & Barre, 1998, Poole *et al.*, 2009, Long *et al.*, 2018) or incompletely (arginine; Martin *et al.*, 2003) assimilated nitrogen sources is of interest. Previously, our group showed constitutive expression of *PUT4*

increased proline uptake, resulting in improved resistance to high temperatures (45 °C) and osmolarity (1.1 M sodium chloride; Poole *et al.*, 2009). However, to the best of my knowledge no one has looked at the effects of increased proline synthesis and accumulation in wine yeast in relation to fermentation-associated stresses.

Construction of EC1118 mutant strains

The novel codon changes in *PRO1* shown to result in proline overproduction (Sekine *et al.*, 2007, Tsolmonbaatar *et al.*, 2016) were introduced into the diploid wine yeast strain EC1118 via CRISPR/Cas9 editing. The first modifications sought to incorporate the amino acid changes known for increased proline accumulation (Pro1p I150T (447 ATC>ACC), P247S (738 CCT>TCT) or E415K (1242 GAA>AAA). As there were no sgRNA sequences (including the PAM site) that directly overlapped the E415 codon, an additional silent mutation (1269 G>A) was introduced in the PAM site (1268 TGG>TAG) to prevent further CRISPR/Cas9 activity (**Table 1**). The modified strains are hereafter referred to as *PRO1*(I150T), *PRO1*(P247S) and *PRO1*(E415K), corresponding to their *PRO1* mutations. Tsolmonbaatar *et al.* (2016) showed the mutations exhibited a range of proline increases from low (E415K), to moderate (P247S) to high (I150T). Tsolmonbaatar *et al.* (2016) demonstrated these mutations improve the response to freeze-thaw stress, whilst the I150T Pro1p variant also improved tolerance to high sugar concentrations (combined 27.8 % sucrose and 2.78 % maltose) in liquid culture medium after short term (1 h) exposure.

The biosynthesis of proline occurs in the cytoplasm, where its subsequent degradation to glutamate occurs in the mitochondria (**Figure 1A**). Both reactions involve Δ^1-pyrroline-5-carboxylate (P5C) as either a substrate (biosynthesis) or a product

(degradation). Excess P5C is toxic to the cell through the inhibition of mitochondrial respiration and increased reactive oxygen species through the production of superoxide anions (Nishimura *et al.*, 2012). A build-up of P5C, is hypothesised to be potentially detrimental to yeast health. Put2p normally converts P5C to glutamate in the mitochondria as part of proline metabolism (**Figure 1A**), however, cytoplasmic Put2p localisation may boost cell viability and proline production if P5C concentration is inadvertently raised due to increased Pro1p activity. Excessive P5C may potentially overburden the native Pro3p (delta 1-pyrroline-5-carboxylate reductase, which catalyses the last step in proline biosynthesis, converting P5C to proline; Tomenchok & Brandriss, 1987) and as such P5C toxicity may occur. Due to the efficiency of CRISPR/Cas9, the introduction of the cytoplasmic Put2p (c*PUT2*) mutation is homozygous, and as such no Put2p would be present in the mitochondrion to complete glutamate synthesis from P5C (**Figure 1C)**. Nomura & Takagi (2004) speculated on the presence of P5C in the cytoplasm through active transport from the mitochondria, or P5C-induced mitochondrial permeability and leakage into the cytosol. This would allow for cPut2p to complete the final step in glutamate biosynthesis from proline (**Figure 1C**). Furthermore, EC1118 has been identified as having the *N*-acetyltransferase gene, *MPR1*, which is usually associated with Σ1278b derived strains, but it has also been found in wine yeast derivative strains RM11-1a and AWRI 1631 (Novo *et al.*, 2009). *MPR1* is involved in ethanol tolerance and resistance to oxidative stress (Du & Takagi, 2007). Mpr1p is also reported to reduce oxidative stress in relation to overproduction of P5C in *put2* disrupted cells and acetylate P5C (or glutamate-gamma-semialdehyde, its equilibrium product; Nomura and Takagi 2004). As such, mechanisms may exist that minimise effects associated with strains lacking mitochondrially localised Put2p. To investigate this, a mutation in *PUT2* was also introduced, removing the mitochondrial translocation signal that allows for cytoplasmic

activity and P5C reduction (Martin *et al.*, 2003). Codons 2 through 16, *PUT2* (L2-F16), were removed via CRISPR/Cas9 editing in EC1118 and some of the Pro1p variants (**Table 1**) during a second round of transformations. The modified strains containing this mutation were suffixed with c*PUT2*, denoting a cytoplasmic variant.

AZC resistance and amino acid content of *PRO1* and *PUT2* mutants

The initial screen for increased proline production in the CRISPR-generated mutants was by plating on medium containing the toxic proline analogue, azetidine-2-carboxylate (AZC). Resistance to AZC has been used as a screen for proline accumulating mutants (Sekine *et al.*, 2007, Kaino *et al.*, 2008, Tsolmonbaatar *et al.*, 2016, Ohashi *et al.*, 2020) and proline transport deficient mutants (Andreasson *et al.*, 2004, Mat Nanyan *et al.*, 2019). AZC is taken up via the proline transporters, Put4p, Gap1p, Agp1p and Gnp1p (Andreasson *et al.*, 2004). In wild type yeast, the intracellular proline pool is limited, leading to incorporation of AZC into protein, which is detrimental to the cell. In contrast, when proline is overproduced and accumulates in the cell, its incorporation into protein enables growth in the presence of the AZC. The wild type, EC1118 and the mutants were plated on synthetic defined medium using allantoin as the sole nitrogen source (SD-N + AL) to remove the influence of nitrogen catabolite repression. Replica plating in the presence and absence of AZC demonstrated that all the mutants except for *PRO1*(E415K) were AZC resistant (**Figures 2A and 2B**). *PRO1*(E415K) exhibited similar growth to the wild type EC1118 (**Figure 2A**). The c*PUT2* mutation also exhibited AZC resistance in both EC1118 and *PRO1*(E415K) backgrounds; the yeast biomass indicative of sufficient proline being accumulated to overcome AZC toxicity.

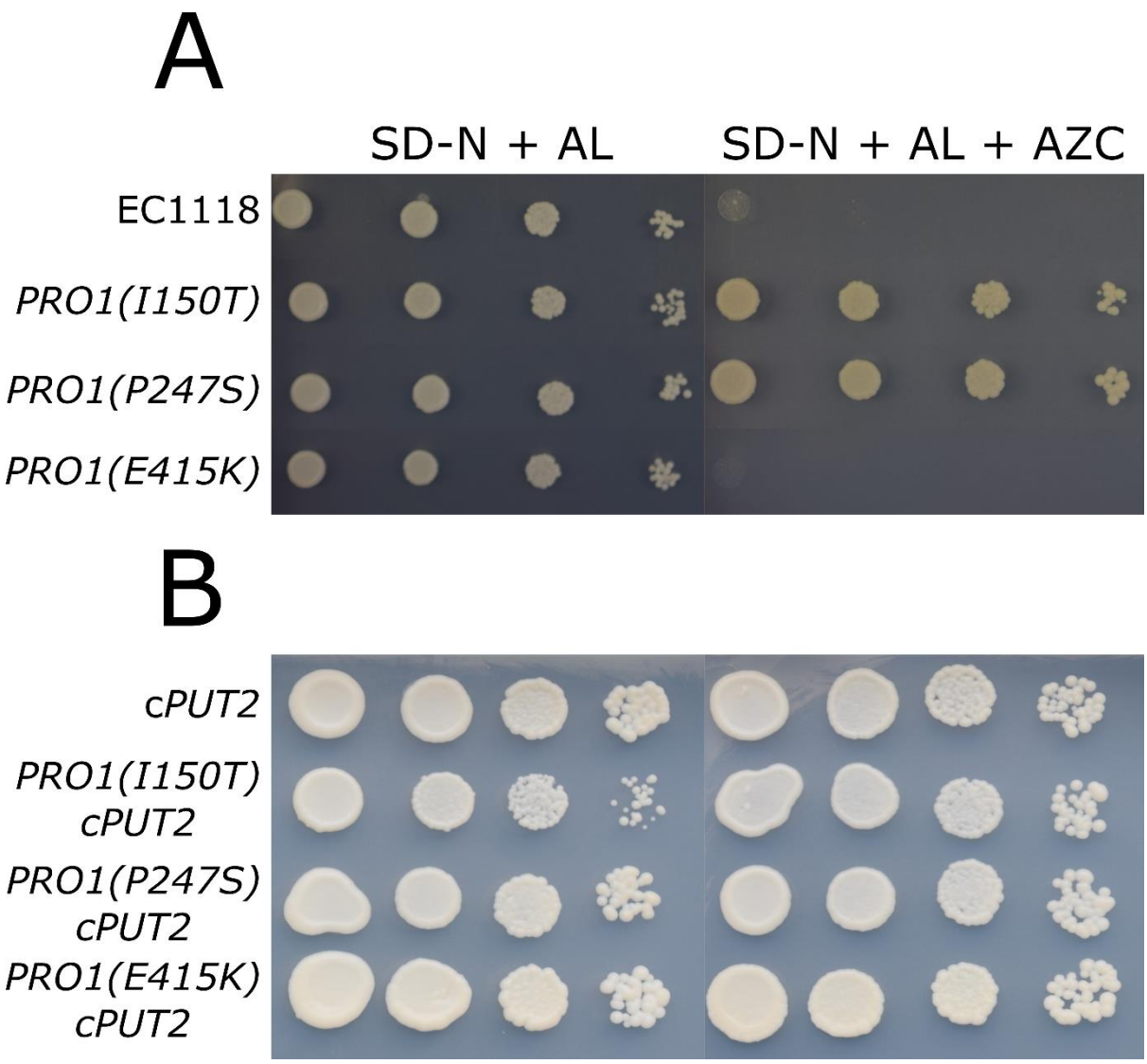


Figure 2. Biosynthetic accumulation of proline as indicated by resistance to the toxic non-protein homologue of proline, azetidine-2-carboxylic acid (AZC)

Serially diluted cultures of EC1118 and the *PRO1* (A) and *PUT2* (B) mutant derivatives grown on Synthetic Dextrose medium supplemented with allantoin as the nitrogen source (SD-N + AL) with 5 mg L^{-1} azetidine-2-carboxylic acid (AZC) to indicate proline accumulation. Strains were grown overnight in YEPD prior to spot plating (5 µL) of serial dilutions (10^{-1} to 10^{-4}; left to right) onto SD-N + AL medium with or without AZC. Colonies appear larger in panel B, this reflects differences in spot plating proximity and photography rather than actual colony size as plating occurred on separate occasions.

The intracellular amino acid contents of the mutants were analysed in terms of proline, arginine, ornithine, and glutamate content and compared to EC1118. Primarily proline and glutamate were of interest, being the desired product or the result of P5C amelioration. Cells were grown on both repressive (ammonium sulfate) and non-repressive (allantoin) nitrogen sources to demonstrate any differences due to nitrogen catabolite repression (NCR) as amino acid transport and metabolism are subject to this regulatory mechanism (Cooper *et al.*, 1992, Forsberg & Ljungdahl, 2001, Beltran *et al.*, 2004). Tsolmonbaatar *et al.* (2016) demonstrated a range of proline accumulation phenotypes associated with *PRO1* mutations, which result in high (*PRO1*(I150T)), moderate (*PRO1*(P247S)) and low (*PRO1*(E415K)) over-accumulation. Similar observations were noted when the amino acid content was normalised to dry cell weight, with all strains exhibiting significantly higher proline content across both media, except for *PRO1*(E415K) grown in allantoin-based media (**Appendix 1**). This mirrors the findings for AZC resistance on SD-N + AL, where EC1118 and *PRO1*(E415K) showed similar low growth (**Figure 2A).**

Whilst normalising the amino acid content to dry cell weight may provide insight into amino acid accumulation, discrepancies may occur due to the presence of dead and lysed cells. As such, the amino acid contents were related to viable cell number (determined by flow cytometry as PI negative events) to ensure accurate reporting of amino acid content (**Figure 3**). When normalised in this manner, mean values of the mutant strains were higher than EC1118 in both allantoin and ammonium sulfate-based media, exhibiting the same production hierarchy (**Figure 3A and 3B**). In both allantoin and ammonium sulfate media EC1118 exhibited the lowest mean proline concentrations (76.72 fg cell^{-1} and 43.52 fg cell^{-1}, respectively) and *PRO1*(I150T) c*PUT2* the highest (675.30 fg cell^{-1} and 372.90 fg cell^{-1}, respectively). However, in terms of statistical

significance, significant increases were only noted with *PRO1*(I150T), *PRO1*(I150T) c*PUT2* and *PRO1*(P247S) c*PUT2* when grown in ammonium sulfate. This differed from the allantoin-based cultures, where significant increases were observed in all strains except *PRO1*(E415K) and c*PUT2*. This highlights the differences in normalisation of the amino acid content data depending upon the denominator (cell weight or viable cell number). Given amino acid analysis uses actively growing yeast (which are washed, pelleted and boiled to release the contents), it is more sensible to normalise the data based on viable (PI negative) cell numbers, and as such, all amino acid contents will be expressed this way.

Interestingly, when cells are grown in SD-N + AL significant increases in proline content are observed in *PRO1*, *cPUT2* double mutants compared to their non-*PUT2* counter parts. Namely, *PRO1*(I150T) c*PUT2* (675.30 fg cell^{-1}) *vs PRO1*(I150T) (568.3 fg cell^{-1}), *PRO1*(P247S) c*PUT2* (500.60 fg cell^{-1}) *vs PRO1*(P247S) (284.80 fg cell^{-1}), and *PRO1*(E415K) c*PUT2* (244.10 fg cell^{-1}) *vs PRO1*(E415K) (130.30 fg cell^{-1}). The homozygosity of the c*PUT2* allele should not allow for wild-type mitochondrial localisation of Put2p at all, thus the mutant's behaviour is likely displaying the '*PUT2* deletion-like' proline accumulation phenotype as described by Nomura & Takagi (2004). In both instances, the mutant yeast would lack Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p) in the mitochondrion. Proline production was increased in cells grown in SD-N + AL compared to SD-N + AS (**Figures 3A and 3B**). The difference between the media most likely reflects the differing nitrogen content of ammonium sulfate and allantoin, with the latter containing ~40% more nitrogen on a per gram basis.

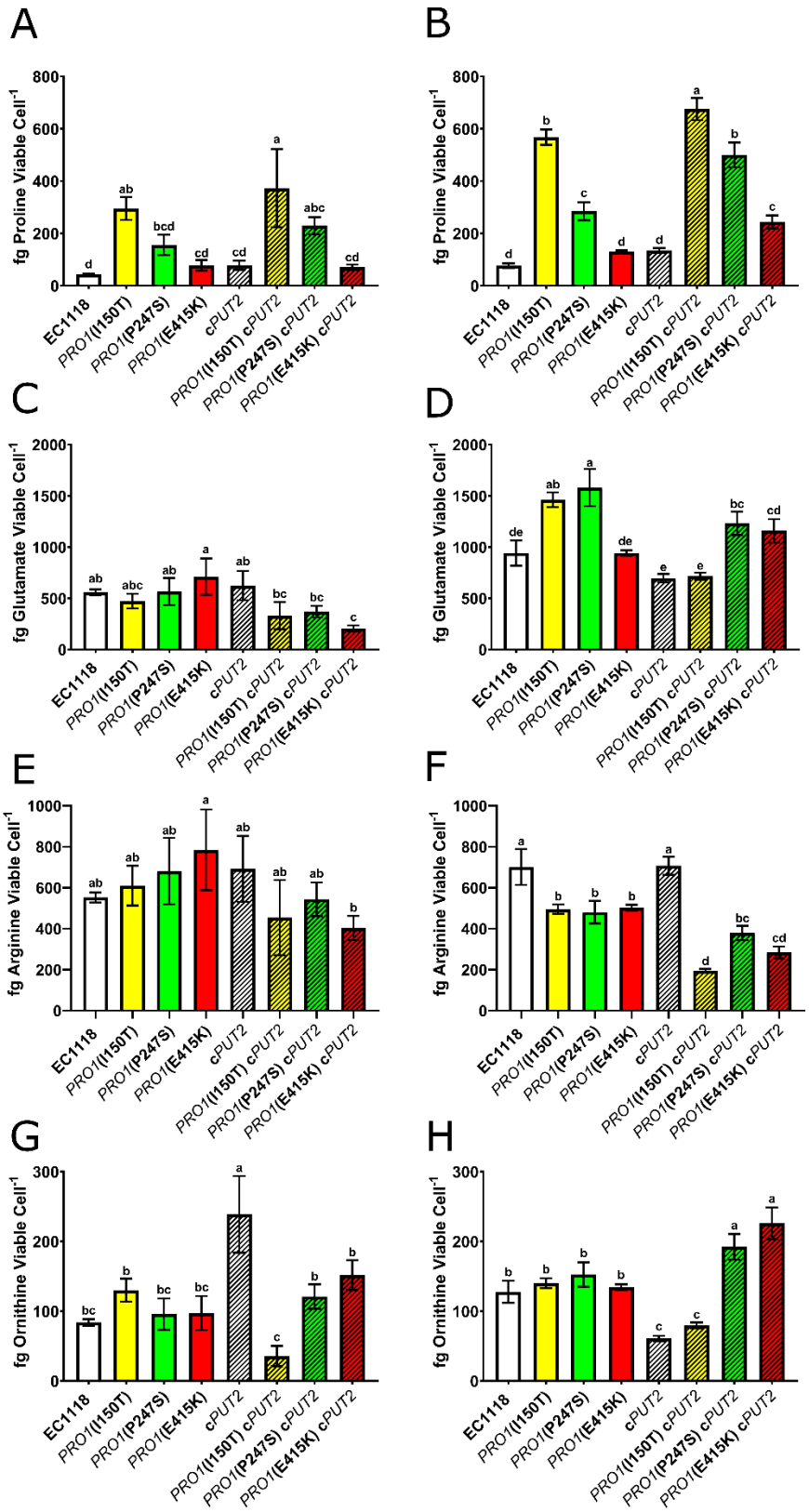


Figure 3. Quantification of the intracellular amino acid content in wine yeast mutants.

EC1118 (WT) and the mutant derivatives were grown overnight in Synthetic Dextrose medium without nitrogen (SD-N medium) supplemented with either ammonium sulfate (A, C, E, G) or allantoin (B, D, F, H) as the sole nitrogen source. Proline was quantified using an amino acid analyser, with 500 µL of cell extract (representing a 2-fold concentrate from a 1 mL culture). Proline content was expressed as mass of amino acid per viable cell (fg Amino acid Viable Cell^{-1}). Different letters denote significant differences between samples (Tukey's HSD test; $p < 0.05$).

Differences in glutamate production were also noted between the two nitrogen sources. Cells grown in the presence of ammonium sulfate showed significantly lower levels of glutamate only in *PRO1*(E415K) c*PUT2* when compared to EC1118 or its non-c*PUT2* equivalent *PRO1*(E415K) (**Figure 3C**). Lower mean (but not significant) concentrations of glutamate were also observed in *PRO1*(P247S) c*PUT2* and *PRO1*(I150T) c*PUT2* (**Figure 3C**). These observations may reflect the *c*PUT2 mutation, such that the mutant lacking a wild-type Put2p is incapable of completing proline degradation in the mitochondrion (**Figure 1B**). This hypothesis would be consistent with Nomura & Takagi (2004), who demonstrated *Δput2* strains accumulate both proline and the intermediate P5C, which cannot be converted to glutamate, resulting in its build-up. All other strains under these conditions exhibited no difference when compared to EC1118 or their wild-type *PUT2* counterparts (**Figure 3C**). Another possibility may be the transcriptional regulation of *PUT2*. Under these favourable nitrogen conditions, it would be expected that *PUT2* is subject to nitrogen catabolite repression resulting from Ure2p activity, however, *PUT2* is also upregulated by Put3p (ter Schure *et al.*, 2000). Put3p is a transcriptional activator of the *PUT* family genes, which is produced when proline is present in cells (Huang & Brandriss, 2000, ter Schure *et al.*, 2000). Whilst *PUT3* is expressed due to proline presence, its activity is reliant on a nitrogen source

quality-dependent phosphorylation. Huang & Brandriss (2000) demonstrated low *PUT2* expression in the presence of 0.2 % ammonium sulfate. Similar would be expected here, as evidenced with strains *PRO1*(I150T), *PRO1*(P247S) and *PRO1*(E415K) when compared to their c*PUT2* equivalents, which had lower mean glutamate due to their *Δput2*-like mutation. In SD-N + AL, *PUT2* repression should be alleviated, with higher expression leading to increased glutamate levels from proline degradation. This was observed in strains *PRO1*(I150T) and *PRO1*(P247S), which also exhibited increased proline production (**Figure 3D**). *PRO1*(E415K), which exhibited similar proline amounts to EC1118 also showed similar concentrations of glutamate (**Figure 3D**). For strains containing the c*PUT2* mutation, however, there is no discernible pattern of glutamate concentration. Interestingly, the lowest (c*PUT2*) and highest (*PRO1*(I150T) c*PUT2*) proline accumulating *PUT2* mutant strains had similar glutamate contents, whilst the c*PUT2* strains (*PRO1*(P247S) c*PUT2* and *PRO1*(E415K) c*PUT2*) had higher mean concentrations (**Figure 3D**). Considering that the c*PUT2* strains lack wild-type Put2p activity to complete degradation in the mitochondria, it would be expected that glutamate would be reduced in these strains, especially in *PRO1*(P247S) c*PUT2*, a high proline producing strain. However, the cytoplasmic function of c*PUT2* may allow for glutamate to be produced from cytoplasmic P5C (**Figure 1C**). Glutamate itself is also used as a precursor in many reactions to produce other amino acids, with many intermediates in these reactions. This would make it difficult to pinpoint these differences in glutamate in the EC1118 mutants.

Whilst not directly involved in the enzymatic functions of mutants in this study, arginine and ornithine, intermediates in proline biosynthesis, were also investigated. Ornithine is required to produce citrulline, an arginine precursor (Jauniaux *et al.*, 1978), but is also a product of arginine degradation and subsequent proline biosynthesis

(Brandriss & Magasanik, 1980). The production of proline from ornithine is dependent on Car2p (ornithine transaminase), a reaction independent of Pro1p. As such, it would be expected that mutants that accumulate proline from the novel *PRO1* mutations would have less of these intermediates due to increased glutamate consumption for proline biosynthesis. When yeast were grown in SD-N + AS, this is not the case. No significant difference in arginine levels were observed in any *PRO1* mutant strains when compared to EC1118 or c*PUT2* (**Figure 3E**). These findings may be explained in the context of arginine metabolism. The first step in arginine degradation is catalysed by Car1p (arginase). *CAR1* is regulated by NCR (ter Schure *et al.*, 2000), and as such would be downregulated in these conditions, minimising arginine catabolism. This in combination with arginine biosynthetic genes such as *ARG1* (arginosuccinate synthetase) and *ARG3* (ornithine carbamoyltransferase) being repressed by the presence of arginine (Crabeel *et al.*, 1988), would result in arginine production to the point of biosynthetic repression without subsequent catabolism. When grown in non-repressive SD-N + AL medium, however, arginine concentrations were more consistent with what is expected, with all strains containing a *PRO1* mutation exhibiting lower arginine concentrations (**Figure 3F**).

Changes in ornithine concentrations become more difficult to predict due to ornithine's involvement in multiple reactions. Under repressive nitrogen conditions it would be expected that ornithine concentrations from arginine biosynthesis would be minimised due to *CAR1* repression by NCR. Ornithine can also be utilised to produce arginine and proline. Despite the complexity in this pathway, for all non-c*PUT2* strains no significant differences in ornithine concentrations were noted under repressive (**Figure 3G**) and non-repressive (**Figure 3H**) conditions. A similar pattern of ornithine production was observed in *PRO1* mutants that also contain the c*PUT2* mutation

(**Figures 3G and 3H**). However, a largely higher concentration of ornithine was observed in the c*PUT2* strain under repressive nitrogen conditions (**Figure 3G**) compared to non-repressive (**Figure 3H**). It would be expected that *PUT2* expression under these conditions would be minimal due to NCR and as such it is difficult to ascertain as to why this increase occurs.

Assessing proline accumulating mutants for wine fermentation stress resistance

To investigate the applicability of these proline accumulating strains, they were subjected to wine fermentation related stresses. Yeast were exposed to either ethanol (13 % v/v), high osmolarity (2 M sorbitol to mimic hexose sugars) or sulfur dioxide (30 mg L^{-1} free SO_2 (0.6 mg L^{-1} molecular SO_2) provided as 80 mg L^{-1} potassium metabisulfite) to mimic the temporal stresses during fermentation. The assays were again conducted under repressive (ammonium sulfate) and non-repressive (allantoin) nitrogen conditions. Yeast were also grown in the absence of stressors as a control to determine the effect of proline accumulation on cell growth, as Maggio *et al.* (2002) demonstrated an inverse correlation between proline accumulation and cell growth. Total and live cell numbers of the mutants were negatively affected independent of proline concentration, with cell viability minimally affected in most instances under both repressive and non-repressive media (**Table 4 and 5**). When grown with ammonium sulfate as the nitrogen source, all mutant strains except *PRO1*(P247S) had significantly lower live cell concentrations and significantly lower total cell concentrations except for *PRO1*(P247S) and c*PUT2*. However, in both instances EC1118 had the highest mean live (3.94×10^7 cells mL^{-1}) and total (4.87×10^7 cells mL^{-1}) cell concentrations. Cells grown in allantoin as the nitrogen source gave similar results, except that all mutants had significantly lower live cell

numbers. Significantly lower total cell numbers are also observed in all mutants excluding *PRO1*(I150T) and *PRO1*(P247S) (**Table 5**). Growth on allantoin (**Table 5**) yielded lower viable and total cell numbers compared to cells grown on ammonium sulfate (**Table 4**). A possible explanation for this may arise from the energy demands of the ATP-dependant hydrolysis of urea (Milne *et al.*, 2015), a product of allantoin degradation by the bi-functional Dur1,2p or urea amidolyase. As all nitrogen present in allantoin is eventually incorporated into urea, this energy dependent step is required for allantoin to be degraded to ammonium for its use as a sole nitrogen source. This additional energy usage may be detrimental to yeast biomass formation.

Table 4. Live and total cell counts (x 10^6 cells mL^{-1}) of yeast exposed to fermentation related stresses (30 mg free SO_2 L^{-1}, 13 % ethanol and 2 M sorbitol). AS denotes SD-N + AS as the base medium. Values are the mean ± standard deviation of 3 replicates. Different letters denote significant differences between samples in a given column (Tukey's HSD test; $p < 0.05$).

	AS			AS + SO2			AS + Ethanol			AS + Sorbitol		
	Live	Total	%	Live	Total	%	Live	Total	%	Live	Total	%
EC1118	39.42 ± 4.48 a	48.72 ± 5.31 a	81.30 ± 6.61 a	35.66 ± 0.29 a	42.16 ± 2.29 ab	84.72 ± 4.01 a	3.10 ± 0.17 a	4.47 ± 0.27 a	69.43 ± 1.90 a	16.84 ± 0.99 cd	18.93 ± 1.07 bc	88.92 ± 0.35 b
PRO1(I150T)	27.11 ± 1.93 bc	31.87 ± 1.91 bcd	85.07 ± 2.46 a	26.60 ± 2.47 ab	33.44 ± 3.68 abc	79.79 ± 6.68 a	1.21 ± 0.12 cd	2.47 ± 0.06 cd	49.00 ± 3.50 de	15.33 ± 0.47 de	18.67 ± 0.50 cd	82.17 ± 0.33 d
PRO1(P247S)	32.65 ± 2.91 ab	38.61 ± 3.53 ab	84.72 ± 5.94 a	31.92 ± 3.35 ab	40.02 ± 3.33 abc	79.92 ± 7.99 a	1.47 ± 0.17 c	2.58 ± 0.13 c	56.65 ± 4.34 bcd	18.17 ± 1.29 bcd	20.88 ± 1.37 bc	86.99 ± 0.56 c
PRO1(E415K)	29.76 ± 2.56 b	35.36 ± 2.80 bc	84.16 ± 2.96 a	31.01 ± 5.03 ab	35.83 ± 5.23 abc	86.43 ± 1.59 a	1.22 ± 0.26 cd	2.02 ± 0.27 def	59.98 ± 5.58 abc	20.32 ± 1.56 ab	21.69 ± 1.45 abc	93.66 ± 0.89 a
cPUT2	29.13 ± 1.04 b	38.21 ± 5.32 ab	76.95 ± 7.77 a	34.02 ± 7.11 a	45.06 ± 7.52 a	75.18 ± 3.03 a	2.29 ± 0.29 b	3.54 ± 0.21 b	64.55 ± 4.24 ab	9.31 ± 0.41 f	10.90 ± 0.49 e	85.44 ± 0.98 c
PRO1(I150T) *cPUT2*	19.03 ± 3.37 c	23.91 ± 3.74 d	79.55 ± 5.20 a	22.48 ± 2.18 b	27.72 ± 2.81 c	81.11 ± 0.91 a	0.74 ± 0.09 de	1.71 ± 0.06 f	43.07 ± 3.73 e	12.55 ± 0.31 e	15.76 ± 0.29 d	79.61 ± 0.71 e
PRO1(P247S) *cPUT2*	25.63 ± 0.95 bc	31.18 ± 1.05 bcd	82.26 ± 4.60 a	30.05 ± 4.38 ab	34.20 ± 5.10 abc	87.9 ± 2.02 a	0.89 ± 0.09 de	1.76 ± 0.06 ef	50.59 ± 3.38 cde	19.66 ± 0.77 abc	21.86 ± 0.95 ab	89.95 ± 0.58 b
PRO1(E415K) *cPUT2*	20.19 ± 4.96 c	25.23 ± 5.18 cd	79.66 ± 3.84 a	25.02 ± 1.91 ab	30.67 ± 3.54 bc	81.64 ± 5.80 a	0.59 ± 0.08 e	2.21 ± 0.13 cde	26.79 ± 2.10 f	22.17 ± 1.53 a	24.62 ± 1.70 a	90.06 ± 0.69 b

Table 5. Live and total cell counts (x 10^6 cells mL^{-1}) of yeast exposed to fermentation related stresses (30 mg free SO_2 L^{-1}, 13 % ethanol and 2 M sorbitol). AL denotes SD-N + AL as the base medium. Values are the mean ± standard deviation of 3 replicates. Different letters denote significant differences between samples in a given column (Tukey's HSD test; $p < 0.05$).

	AL			AL + SO2			AL + Ethanol			AL + Sorbitol		
	Live	Total	%	Live	Total	%	Live	Total	%	Live	Total	%
EC1118	25.41 ± 1.40 a	29.32± 2.75 a	86.87 ± 7.73 a	28.57 ± 2.15 a	36.35 ± 7.65 a	80.03 ± 10.45[a]	3.04 ± 0.37 a	3.71 ± 0.40 a	81.86 ± 1.85 a	9.89 ± 0.17 bc	10.40 ± 0.16 bc	95.05 ± 0.66 a
PRO1(I150T)	19.95 ± 1.62 b	23.06 ± 3.16 ab	87.03 ± 5.80 a	21.01 ± 1.92 b	25.47 ± 0.87 abcd	82.39 ± 4.73 a	1.65 ± 0.05 d	2.39 ± 0.08 de	69.20 ± 0.38 d	10.40 ± 0.30 b	11.50 ± 0.38 b	90.39 ± 0.41 c
PRO1(P247S)	17.87 ± 3.37 b	25.21 ± 6.32 ab	71.65 ± 4.62 b	22.32 ± 1.25 b	28.82 ± 2.47 ab	77.72 ± 5.76 a	2.38 ± 0.26 b	3.06 ± 0.25 bc	77.72 ± 2.27 abc	9.27 ± 0.40 bcd	9.94 ± 0.43 c	93.34 ± 0.10 b
PRO1(E415K)	9.71 ± 1.09 d	12.34 ± 0.45 cd	78.51 ± 5.84 ab	13.31 ± 1.48 de	16.46 ± 1.05 cd	80.94 ± 7.76 a	2.15 ± 0.13 bc	2.67 ± 0.19 cd	80.48 ± 1.23 ab	8.99 ± 0.35 cd	9.42 ± 0.35 c	95.46 ± 0.29 a
cPUT2	15.96 ± 1.30 bc	20.42 ± 3.24 bc	78.88 ± 6.73 ab	20.01 ± 0.96 bc	26.33 ± 1.07 abc	76.01 ± 1.50 a	2.54 ± 0.05 b	3.41 ± 0.07 ab	74.46 ± 1.14 c	5.68 ± 0.32 e	6.12 ± 0.33 d	92.75 ± 0.54 b
PRO1(I150T) *cPUT2*	12.21 ± 0.89 cd	14.02 ± 0.44 cd	87.02 ± 4.03 a	15.58 ± 2.35 cd	20.40 ± 7.54 bcd	80.16 ± 15.30[a]	1.33 ± 0.01 d	2.07 ± 0.07 e	64.30 ± 2.22 e	8.24 ± 0.31 d	9.22 ± 0.33 c	89.38 ± 0.16 c
PRO1(P247S) *cPUT2*	9.76 ± 0.48 d	11.61 ± 0.88 d	84.17 ± 2.94 ab	12.43 ± 0.53 de	15.44 ± 2.66 cd	81.76 ± 11.10[a]	1.74 ± 0.10 cd	2.27 ± 0.12 de	76.71 ± 0.83 bc	9.77 ± 0.62 bc	10.18 ± 0.69 c	95.95 ± 0.61 a
PRO1(E415K) *cPUT2*	9.65 ± 1.11 d	11.99 ± 2.40 cd	81.32 ± 6.35 ab	10.48 ± 0.61 e	14.43 ± 2.68 d	73.54 ± 7.81 a	1.32 ± 0.04 d	2.71 ± 0.09 cd	49.04 ± 1.40 f	18.36 ± 0.54 a	19.13 ± 0.66 a	95.99 ± 0.58 a

The addition of SO_2, a common wine additive, as a stressor did not highlight in any significant improvement in any mutant strains. Whilst there were no significant differences in cell viability in either nitrogen condition, live and total cell numbers were affected (**Table 4 and 5**). When grown in SD-N + AS no significant differences in live and total cells were observed in comparison to EC1118, apart from *PRO1*(I150T) c*PUT2*, which was significantly lower in both instances (**Table 4**). When grown in SD-N + AL, live and total cell concentrations were more adversely affected. Under these conditions, all mutants exhibited significantly lower viable cell numbers compared to that of EC1118. Similar observations were made for total cell number, except for *PRO1*(I150T), *PRO1*(P247S) and c*PUT2*. EC1118 had the highest mean and total cell numbers (**Table 5**). Similarities between mutants grown with and without SO_2 may suggest that SO_2 concentrations in this study were not significant enough to elicit a stress. Considering the results here were as seen in SD-N medium lacking stressors, these mutations would appear not to confer any protection from SO_2 related stress when compared to wild-type EC1118. EC1118 is described as having intermediate SO_2 tolerance (Nadai *et al.*, 2016), and as such may not be affected by the SO_2 concentrations used in this study. Rankine (1989) recommends free SO_2 concentrations between 30 and 50 mg L^{-1} for white table wines of pH 3.50 post fermentation. As such, concentrations used in this study are representative of winemaking conditions. A future consideration might be to screen EC1118 across a higher range of free SO_2 concentrations to determine at what concentration stress occurs.

Ethanol toxicity was also examined as yeast must adapt to increasing ethanol concentrations, as a product of sugar catabolism during fermentation. Previous studies have identified proline accumulation as an effective protectant to ethanol (Takagi *et al.*, 2005, Takagi *et al.*, 2007). However, in this study exposure to 13 % (v/v) ethanol was

detrimental to all mutant strains. Both live and total cell counts were significantly lower in both nitrogen conditions for all mutant strains, except for c*PUT2* (SD-N + AL: total counts) (**Table 5**). This was especially evident with *PRO1*(E415K) c*PUT2*, having the lowest live cell counts when grown in both nitrogen sources (**Table 4 and 5**). *PRO1*(E415K) c*PUT2* live cell numbers were 81 % lower than EC1118 in SD-N + AS (3.1×10^6 *vs* 5.9×10^5 viable cells mL^{-1}) and 57 % lower than EC1118 in SD-N + Al (3.04×10^6 *vs* 1.32×10^6 viable cells mL^{-1}). In most instances, viability was also significantly reduced. In repressive nitrogen conditions, *PRO1*(E415K) and c*PUT2* were the only strains that did not exhibit significantly lower viability (**Table 4**). Under these nitrogen conditions, both strains showed no difference in proline accumulation, having only marginally higher mean proline values compared to EC1118 (**Figure 3A**). Whilst proline accumulation was similar when the two mutations were combined (*PRO1*(E415K) c*PUT2***; Figure 3A**), the resulting strain had the lowest viability in both repressive and non-repressive nitrogen conditions when exposed to 13 % ethanol (26.76 ± 2.10 % and 49.04 ± 1.40 %, respectively). The inclusion of the c*PUT2* mutation in other strains also resulted in lower live cell numbers and viabilities compared to their non-c*PUT2* counterparts. The c*PUT2* mutants are envisaged to behave similarly to *Δput2,* whereby the lack of a mitochondrial enzyme results in the accumulation of the intermediate, P5C. Given that *Δput2* strains are reported to have increased levels of ROS (Nomura & Takagi, 2004) and this phenomenon is also experienced upon ethanol exposure (Costa *et al.*, 1997), it is probable that the additive accumulation of ROS through these mechanisms would negatively affect cell growth and viability. Assessing the ROS accumulation in these strains would be an interesting facet to further investigate, but is beyond the time frame of this PhD candidature. As ethanol affected all mutant strains, it would also be interesting to also measure ROS species in the non-c*PUT2* strains

as EC1118 is described by the manufacturer (Lallemand) as having an ethanol tolerance of up to 18% (v/v). The homozygous nature of these mutations may also contribute to inadequate resistance to ethanol. The accumulation of proline in sake yeast strains containing *PRO1* alleles *PRO1*(D154N) (Takagi *et al.*, 2005) - an allele not investigated in this study - and *PRO1*(I150T) (Takagi *et al.*, 2007), led to increased viability in media containing 9 % and 18 % ethanol. Takagi *et al.* (2007) demonstrated a gene dosage effect, where strains heterozygous for the *PRO1*(I150T) mutation in a *Δput1* background showed superior tolerance to 18 % ethanol, demonstrating a haploproficiency phenotype. A strain homozygous for the same mutation, however, exhibited reduced viability when compared to the wild type. As the mutant strains in this study are homozygous for their respective *PRO1* alleles due to the efficiency of CRISPR/Cas9, this a probable cause of the reduction of viability. The genetic variation between EC1118 and sake yeast (Borneman *et al.*, 2011), most likely would also influence mutant phenotype in relation to ethanol tolerance, although it is difficult to ascertain why these *PRO1* alleles negatively impact ethanol resistance rather than improve it. In these instances, the accumulation of proline does not confer any benefit to yeast protection against ethanol or SO_2 stresses.

The influence of the *PRO1* and c*PUT2* mutations to osmotic stress was also examined as proline accumulation is known to alleviate osmotic stress from sorbitol and sodium chloride. Using plate-based methods, proline accumulating yeast mutants are resistant to 2.5 M sorbitol (Sekine *et al.*, 2007) and 1 M NaCl (Takagi *et al.*, 1997). In liquid culture, proline accumulation has also been associated with resistance to prolonged exposure (2 h) to high sucrose concentrations (27.8 %; Sasano *et al.*, 2012). Interestingly, deletion of *PRO1* has also been attributed to resistance to 0.8 M NaCl (Butcher & Schreiber, 2004), most likely due to proline biosynthesis being still possible from arginine and ornithine, but not glutamate. However, the authors did not measure proline

content, and as such a direct correlation between proline biosynthesis and osmotic stress tolerance cannot be made. In a preliminary comparison of *PRO1*(I150T) and *PRO1*(P247S) to EC1118 exposed to 1.1 M NaCl, *PRO1*(P247S) had similar viability whilst *PRO1*(I150T) showed sensitivity, having significantly lower viability (**Appendix 2**). These findings allude to increased proline biosynthesis having a negative effect in terms of protection against NaCl as an osmotic stressor. Sodium chloride was replaced in later experiments with 2M sorbitol (364.34 g L^{-1}), a six-carbon polyol structurally related to glucose. Under these conditions, some of the mutants appear to offer an advantage to the yeast in terms of increased viable cell numbers. *PRO1*(E415K) c*PUT2* had significantly higher live and total cell counts in both nitrogen sources (**Table 4 and 5)**, whilst for *PRO1*(P247S) c*PUT2* and *PRO1*(E415K), the increase in viable cell number was confined to SD-N + AS (**Table 4**). Significantly lower viabilities were observed in *PRO1*(I150T), *PRO1*(P247S), c*PUT2* and *PRO1*(I150T) c*PUT2* grown in SD-N + AL (**Table 5**). The differences were small, with the largest reduction in viability observed by *PRO1*(I150T) *cPUT2* (-5.66 %). Similar observations were made for SD-N + AS medium, with *PRO1*(E415K) having significantly higher viability (+4.74 %; **Table 4**). The results were unexpected, as high proline mutations such as *PRO1*(I150T) improve resistance to osmotic stress (Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016). Differences may partly be explained by either choice of fermentation medium (YEPD or liquid fermentation medium *vs* SD-N + AS and SD-N + AL) or choice of osmolyte (sucrose *vs* sorbitol). Differences in results between this study and previous reports are most likely a reflection of the differing nutrient composition, in particular nitrogen, as evidenced in this study. YEPD, which was used in the earlier studies, is highly complex compared to ammonium (as the sulfate salt) and allantoin, a cyclic N compound imidazolidine-2,4-dione. The nitrogen in YEPD consists of yeast and animal-derived proteins, peptides,

amino acids of variable composition and nitrogen content, which provides yeast with more than sufficient nutrients for growth. Further variability could arise from the two different sugar sources – the disaccharide, sucrose (consisting of glucose and fructose) and sorbitol, a sugar alcohol or polyol. Both are routinely used as individual stressors in growth sensitivity assays. Whilst these are valid considerations, the genetic background of the yeast is the critical parameter, as the genomes of sake, bread and wine yeast are highly divergent, with the genetic distance between the strains related to their ecological niche (Borneman *et al.*, 2011, Sicard & Legras, 2011, Gallone *et al.*, 2018) as well as within a niche-type, for example wine yeast (Borneman *et al.*, 2016). Such divergence has implications for the nuances of cell metabolism and regulation with respect to adaptability to environmental stresses.

From this study, there appear to be no clear correlations between proline accumulation and the ability to withstand fermentation related stresses (SO_2, ethanol and hyperosmolarity). Takagi *et al.* (2007) reported on a gene dosage effect on the amount of proline produced as a result of a single mutation (*PRO1*(I150T) and strain tolerance to 18% ethanol. The authors demonstrated the heterozygous strain, having slightly less proline, was more resistant than the homozygous strain. In this PhD study, three separate *PRO1* mutations were examined in relation to proline content and stress tolerance as single mutations or in combination with the cPut2p variant. The mutant alleles were homozygous in the seven constructed strains. Modification of EC1118 to enhance proline accumulation through biosynthesis, did not result in a discernible pattern of stress tolerance. For example, both c*PUT2* (low proline) and *PRO1*(I150T) c*PUT2* (high proline) exhibited poor viability (**Table 4 and 5**). Whilst cell number and viability were dependent upon the mutation(s) and stress condition, overall, the proline-accumulating

mutants were no more resistant to ethanol nor SO_2 than EC1118. Proline as an osmolyte, was only protective against osmotic stress.

It is difficult to ascertain whether there are other mechanisms at play, for example, cytotoxicity from the intermediates (P5C) or proline itself, or whether excessive proline biosynthesis causes a metabolic load within the cell, leading to poor growth and viability outcome. As such, the use of an already stress resistant strain such as EC1118 may not have been an appropriate choice for further improvement. The interpretation of the experimental data is further hindered by the compartmentalisation of the proline-degrading enzyme, Put2p. Put2p, is normally located in the mitochondrion, as part of the PUT (Proline UTilisation) pathway, to convert proline to glutamate. In this instance, it was directed to the cytoplasm to prevent P5C accumulation there (**Figure 1C**). Whilst the findings are variable, they are still useful in terms of better understanding proline metabolism and fermentation related stress.

The modulation of proline metabolism is still a useful target for wine strain improvement, specifically, accumulation of proline resulting from uptake of this highly abundant amino acid in grape must (Huang & Ough, 1989). Proline uptake is mediated through the high-affinity proline permease, Put4p, as well as the amino acid permeases, Gap1p, Agp1p and Gnp1p (Andreasson *et al.*, 2004). In the case of Put4p, Poole *et al.* (2009) demonstrated a mutant to be insensitive to nitrogen catabolite repression. Plasmid expression of a combination of the underlying Put4p(S605A) variant with a promoter mutation (-160T>C) increased proline accumulation and resistance to high temperature (45 °C) and osmotic stress (1.1 M NaCl). The authors did not examine these mutations in the presence of high ethanol or SO_2 conditions.

Whilst increased proline uptake would be of merit in winemaking, as it could decrease the amount of nitrogen supplementation needed to ensure fermentation reliability, little is known about the impact on the wine aroma. Fermentation in high proline media has been linked to increased ethyl acetate production in both lager (*Saccharomyces pastorianus*) and ale (*S. cerevisiae*) brewing strains (Procopio *et al.*, 2013). As such, further research into the blending of both enhanced proline uptake and biosynthesis mutations may be useful as it is not known how these mutations may work in tandem, however, these suggestions are outside the scope of this research.

Whilst the *PRO1* and *PUT2* mutations in this study are not useful in a winemaking context, they may be of potential use in terms of using the yeast lees post fermentation in industrial processes such as beer brewing to produce nutrient enriched products for functional food and dietary supplement production (Podpora *et al.*, 2015, Rakowska *et al.*, 2017). Yeast lees from industrial fermentations in some countries are used as a food ingredient such as sake kasu in Japan, or Vegemite in Australia. Yeast with increased amino acid production could in turn enhance amino acid concentrations in these foods and thus act as a food-based alternative to amino acid supplementation. This has been suggested by Ohashi *et al.* (2020) who reported on a sake yeast mutant with enhanced ornithine production. Increased ornithine concentrations were observed in both the sake beverage, and the spent lees. Considering these strains would be classed as 'self-cloned' this would potentially allow for their use for this purpose in some countries.

Conclusions:

This study looked at the effects of proline overproduction in a wine yeast background derived from specific non-synonymous mutations integrated into the genome via CRISPR/Cas9. In the presence of ethanol and SO_2 based stresses, proline

accumulation had no effect or a negative one on cell numbers or viability, depending upon the mutation and stress. However, low-level proline overproduction improved tolerance to sorbitol-induced osmotic stress. Whilst these mutants may not be appropriate for wine production, these findings build on our current understanding of how proline acts as a stress response molecule in *S. cerevisiae* in different industrial yeasts and fermentation settings.

Chapter 5

"Non-Homologous End Joining Repair (NHEJ) as a mean to construct disruption mutants of arginine permease (*CAN1*) in wine *Saccharomyces cerevisiae* with CRISPR/Cas9"

This chapter is written as a manuscript for FEMS Yeast Research

Statement of Authorship

Title of Paper	Non-Homologous End Joining Repair (NHEJ) as a mean to construct disruption mutants of arginine permease (CAN1) in wine Saccharomyces cerevisiae with CRISPR/Cas9
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Tom Lang
Contribution to the Paper	- Conceptualisation - Laboratory work/Analysis - Writing
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 29/3/21

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Non-Homologous End Joining Repair (NHEJ) as a means to construct disruption mutants of arginine permease (*CAN1*) in wine *Saccharomyces cerevisiae* with CRISPR/Cas9

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Abbreviations:

NHEJ, Non-homologous End Joining; *CAN1*, Arginine Permease

Abstract

The use of genetically modified yeast for commercial beverage production is becoming more prominent especially where legislation permits introduction of exogenous genes from different species, or generation of strains by 'self-cloning' methods using DNA from the same or similar species. In Australia, stricter regulations on these organisms prevent their use in industry. However, recent amendments to the *Gene Technology Regulations (2001)* allow for modification of organisms without the introduction of net foreign DNA, as in the case with the non-homologous end joining (NHEJ) DNA repair pathway in combination with CRISPR/Cas9. We report on the use of CRISPR/Cas9 without a repair template to target and disrupt the function of arginine permease (Can1p). We successfully isolated two variants of *CAN1* (encoding for arginine permease) in the diploid wine yeast EC1118 where deletion of a single nucleotide results in a frameshift and introduction of an early stop codon (Ile71Stop) in both instances. Protein dysfunction was confirmed by the ability to grow on the toxic analogue, canavanine, and reduced production of urea during fermentation on arginine as nitrogen source. This research is a step towards engineering "non-GMO" yeast strains suitable for wine production under the new Australian regulations.

Introduction

There is a strong drive to produce new yeast strains with improved fermentation characteristics for the production of wine. Several non-recombinant methods have been used, which rely on phenotypic changes associated with DNA variation. These generally involve quantitative trait loci (QTL) identification for marker assisted breeding (Marullo *et al.*, 2007, Jubany *et al.*, 2008, Marullo *et al.*, 2009, Dufour *et al.*, 2013), evolutionary engineering (McBryde *et al.*, 2006, Mezzetti *et al.*, 2017, Gibson *et al.*, 2018, Kayacan

et al., 2020) or mutagenesis (Cordente *et al.*, 2009, Long *et al.*, 2018, Abe *et al.*, 2019, Ohashi *et al.*, 2020).

Whilst useful in producing 'industry ready' strains, they are non-specific and time-consuming, and rely on large-scale screening of candidates to identify improved phenotypes arising from beneficial mutations or nucleotide polymorphisms. The more targeted approach of gene technology has come about because the whole genome sequencing of *Saccharomyces cerevisiae* (Goffeau *et al.*, 1996) enriched the understanding of yeast physiology and metabolism (and the associated gene pathways). Specific mutations introduced into target genes allow for modified strains with predetermined outcomes.

To date, two genetically modified strains are available commercially in the United States, which are prohibited in Europe and Australia because of tight restrictions on the use of GMOs in the production of food and beverages. ML01, a yeast modified to undergo malolactic fermentation, is a product of heterologous expression of the *mae1* malate transporter from *Schizosaccharomyces pombe* and the *mleA* malolactic enzyme from *O. oeni* (Husnik *et al.*, 2006). ECMo01 targets urea production in order to lower levels of the carcinogen, ethyl carbamate. Urea is reduced through constitutive expression of an additional copy of *DUR1,2*, encoding urea amidolyase (Coulon *et al.*, 2006). The emergence of new genome editing technologies, in particular CRISPR/Cas9, with its specificity, efficiency, and ability to target mutations with no net DNA introduced, may be pivotal in addressing these restrictions.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR Associated) is an RNA-guided DNA nuclease system derived from an adaptive immune system found in the bacteria *Streptococcus pyogenes* (Jinek *et al.*, 2012). The

CRISPR component codes for an RNA molecule containing a 20 bp guide sequence, which interacts with the Cas9 nuclease, guiding it to a complementary sequence directly 5' of a PAM (Protospacer adjacent motif) sequence (5' NGG 3') to enable excision. This nuclease system has been adapted as a genome editing technique in many organisms including human cells (Cong *et al.*, 2013), *Drosophila melanogaster* (Gratz *et al.*, 2014), zebrafish (*Danio rerio*; Hwang *et al.* 2013) and *Saccharomyces cerevisiae* (DiCarlo *et al.*, 2013).

Genome editing relies on two predominant DNA repair mechanisms in the host; non-homologous end joining (NHEJ) or homology directed repair (HDR). In *S. cerevisiae*, the more precise HDR is favoured, where DNA damage is repaired with a DNA template homologous to the region requiring repair. Recent research on improving wine yeasts with HDR-mediated CRISPR/Cas9 has focused on generating yeasts with gene knockouts or specific overexpressed genes to decrease urea production (Vigentini *et al.*, 2017) or improve sensory characteristics (Cordente *et al.*, 2018, van Wyk *et al.*, 2020). To date, no studies have reported the use of the error prone NHEJ in wine yeast to create mutant strains with desirable characteristics.

Australia has recently amended legislation, with the *Gene Technology Regulations 2001* (Compilation No. 9) (https://www.legislation.gov.au/Details/F2020C00957) changing the definition of a genetically modified organism (GMO). These amendments allow for an organism to not be classified as a GMO when produced using site-directed nucleases if no nucleic acid is introduced to guide genome repair, therefore allowing NHEJ derived mutants. Thus, whilst NHEJ repair is a rare event in *S. cerevisiae* (DiCarlo *et al.*, 2013), CRISPR-edited strains produced in this manner may be more readily acceptable from a consumer and regulatory perspective.

DiCarlo et al. (2013) demonstrated the extremely low efficiency of NHEJ mutations in *CAN1* in a laboratory strain of *S. cerevisiae*. *CAN1* is important in wine yeast as it codes for a high affinity arginine permease (Can1p), which mediates arginine import into cells, where arginine catabolism can result in increased production of urea, a precursor to the carcinogenic compound ethyl carbamate (Jiao *et al.*, 2014). Vigentini et al. (2017) used a guide sequence reported by DiCarlo et al. (2013) to disrupt *CAN1* by introduction of a premature stop codon (via HDR) in wine yeasts EC1118 and AWRI796.

In this study, we newly report on two arginine permease (Can1p) mutants of EC1118 produced by NHEJ-mediated CRISPR /Cas9 editing. The *can1* mutants significantly reduce urea production during alcoholic fermentation on arginine-containing chemically defined grape juice medium, thereby validating this approach to precisely tailoring industrial strains such that they could be designated non-GMO.

Materials and Methods

Microbial strains, media and plasmids

Strain modifications used a clonal isolate of *Saccharomyces cerevisiae* Lalvin EC1118. For yeast growth, strains were cultured in YEPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) at 28 °C. When required, media were solidified with 20 g L^{-1} agar. For plasmid propagation and selection of recombinant clones, *Escherichia coli* NEB® 5-Alpha (C2987I, New England Biolabs) was used and grown on LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) supplemented with Carbenicillin (100 mg L^{-1}). Plasmid purification used the Wizard® Plus SV Minipreps DNA Purification System (Promega).

Construction of *CAN1*-targeting plasmid

Mutant strains were generated using the pUDP002 plasmid method described in Juergens *et al.* (2018). Briefly, a *CAN1* targeting sgRNA sequence was introduced into pUDP002 by Golden Gate assembly of the *CAN1* sgRNA gBlock (Integrated DNA Technologies) using the identical reagent setup to Juergens *et al.* (2018). Thermal cycler conditions followed the NEB Golden Gate Assembly protocol with 30 cycles of 42 °C for 5 min followed by 16 °C for 5 min, with a final incubation of 60 °C for 5 min. The resulting reaction was transformed into NEB® 5-alpha Competent *E. coli* (C2987I) according to the manufacturer's protocol, plated onto solid LB medium containing 100 mg L^{-1} carbenicillin and grown overnight at 37 °C. Plasmids were isolated from single colonies using Wizard® SV Minipreps DNA Purification Systems (Promega) and Sanger sequenced (Australian Genome Research Facility, Australia) with pUDP-seq primer (**Table 1**). Correct sequence integration was confirmed using Geneious (8.1.9) software.

Transformation of EC1118

Yeast transformation was undertaken using the lithium acetate method (Gietz & Schiestl, 2007). As the frequency of mutants arising from NHEJ would be extremely low, 30 identical transformation reactions were performed as described. An overnight culture was diluted 1 in 100 in 100 mL YEPD and grown for 4 – 5 hours until an OD_{600} of 0.4 – 0.5. Cells were pelleted and both twice washed and resuspended to a final volume of 500 µL of 0.1 M lithium acetate. For each transformation, 50 µL of cell suspension was pelleted and resuspended in 54 µL of sterile ultrapure water (containing 100 ng pUDP-CAN1), 36 µL 1 M lithium acetate, and 260 µL 50% w/v polyethylene glycol 4000. Cells

were incubated at 30 °C for 30 min, prior to heat shock (42 °C, 30 min). Following centrifugation (20,000 x *g*, 1 min), the supernatant was discarded, and the cells resuspended in 1 mL YEPD to recover for 2.5 hours at 30 °C. The cells were pelleted by centrifugation and resuspended in 200 µL sterile ultrapure water prior to plating onto YEPD agar containing hygromycin (200 mg L^{-1}) and incubation (30 °C, 48 h).

Confirmation of mutant strains

Hygromycin-resistant yeast colonies were grown for 72 h in liquid YEPD medium to allow for plasmid loss. 72 h cultures were streaked onto solid YEPD media for single colony isolation, followed by replica-plating on YEPD (with and without antibiotic) to select for plasmid loss. Genomic DNA was extracted from hygromycin-sensitive isolates using phenol/chloroform and glass beads (Adams *et al.*, 1998). The genomic DNA provided the template for PCR amplification of *CAN1* using Velocity DNA Polymerase (Bioline) and CAN1 A and CAN1 D primers (**Table 1**). The purified PCR products (Wizard® SV Gel and PCR Clean-up System (Promega)) were sequenced in both directions using CAN1 seq F or CAN1 seq R primers (**Table 1**; Australian Genome Research Facility). Sequence comparison using Geneious (8.1.9) software confirmed a frameshift in the targeted *CAN1* region (nucleotide position). Mutant protein sequences were analysed by PROVEAN (Choi & Chan, 2015) to confirm deleterious effects. To confirm canavanine resistance, strains of interest were grown overnight in 10 mL liquid YEPD, serially diluted and spot-plated (5 µL) onto solid synthetic dextrose medium (Kaiser *et al.*, 1994) without arginine (SD -Arg) and supplemented with 60 µg mL^{-1} L-canavanine. Plates were grown at room temperature for 4 days.

Urea production during fermentation

Urea production was evaluated in yeast mutants fermented in (i) Chemically Defined Grape Juice Medium (CDGJM; Jiranek *et al.*, 1995) containing 230 g L^{-1} total sugars (115 g L^{-1} glucose, 115 g L^{-1} fructose) with 2 g L^{-1} arginine as the sole nitrogen source, and (ii) sterile-filtered (0.22 µm) Chardonnay juice containing 249 mg N L^{-1} YAN (170 mg N L^{-1} Primary Amino Nitrogen, 79 mg N L^{-1} ammonium), with an additional 2 g L^{-1} L-arginine (or 643 mg L^{-1} YAN). Strains were grown overnight in 50% fermentation medium and 50% YEPD at 28 °C, washed in sterile ultrapure water, and resuspended in the appropriate medium prior to inoculation at 5 x 10^6 cells mL^{-1}. Fermentations in CDGJM were conducted at 23 °C, as a middle point between red and white wine fermentation temperatures, whilst Chardonnay fermentations were held at 17 °C. Fermentation progress was monitored as weight loss due to CO_2 release with endpoint sugars tested with Aimtab™ Reducing Substances Tablets (Rowe Scientific). Endpoint urea was determined by spectrophotometry (Tecan, Mannedorf, Switzerland; M200 Infinite) using an L-Arginine-Urea-Ammonia Assay Kit (K-LARGE; Megazyme).

Statistical analysis

All statistics were analysed using GraphPad Prism 9.0.0 software. Statistics of fermentation completion and urea concentrations were performed using the one-way ANOVA function with multiple comparisons to EC1118 (Dunnett's test) at the 99% confidence interval.

Results

Disruption of *CAN1* by NHEJ using CRISPR/Cas9

In an attempt to create *CAN1* disruption mutants by CRISPR/Cas9 and NHEJ, the single plasmid 'broad host range' system pUDP002 was used (Juergens *et al.*, 2018). The sgRNA targeting *CAN1* (5'- GATACGTTCTCTATGGAGGA -3') has previously been used in the haploid laboratory strain BY4733 (DiCarlo *et al.,* 2013) and diploid wine strains EC1118 and AWRI796 (Vigentini *et al.*, 2017). Thirty identical transformation reactions (totalling 1.2 x 10^8 cells transformed with 3 µg of plasmid) were undertaken because of the low efficiency of NHEJ in *S. cerevisiae*. Each transformation represented 4 x 10^6 cells transformed with 100 ng of plasmid DNA. Six hygromycin-resistant transformants (TANG1 to TANG6) were isolated.

Sequencing of the *CAN1* ORF revealed two isolates had identical sequence to wild type EC1118 (TANG3 and TANG4). The others either had a frameshift mutation within the guide RNA sequence corresponding to amino acid 67 (TANG6) or 69 (TANG1, TANG2 and TANG5) (**Figure 1**). The latter are likely clonal isolates amplified during recovery in YEPD, as they originate from the same transformation plate. Both frameshifts in the mutants led to premature termination at position 71, and protein truncation (**Figure 1**). Analysis of the resulting proteins by PROVEAN identified both genomic variants as deleterious. The identification of two different sequences in the NHEJ isolates represented a mutation frequency of 1.7 x 10^{-7}, which is lower than the 7.03 x 10^{-4} reported by DiCarlo *et al.* (2013). Differences in methodology, however, are likely to account for the discrepancies between the studies. DiCarlo *et al.* (2013) used galactose induction of Cas9 post transformation for site cleavage. This differs from our

approach that utilised the pUDP002 system, where Cas9 is constitutively expressed by the *TEF1* promoter. Differences in calculations (number of pre-transformed cells *vs* number of pre-induced cells) or the strain genetic background (diploid EC1118 *vs* haploid BY4733) may also contribute towards these differing efficiencies.

Growth assays with the toxic and structurally similar amino acid L-canavanine in place of L-arginine, demonstrated TANG1 and TANG6 were dysfunctional for Can1p-mediated arginine transport (**Figure 2**). The mutants grew on synthetic dextrose lacking L-arginine (SD –arg) supplemented with 60 µg mL^{-1} L-canavanine whilst the parent, EC1118, having a functional arginine permease, failed to grow after 4 days.

Urea determination and fermentation efficiency of *can1* mutants in Chardonnay juice and CDGJM

The fermentation properties of EC1118 (wild type) and the mutants TANG1 and TANG6 (representing both *CAN1* genotypes) were evaluated in 100 mL fermentations, with urea measured at the end. Fermentations occurred in filter-sterilised Chardonnay juice enriched with an additional 2 g L^{-1} L-arginine or CDGJM with 2 g L^{-1} L-arginine as the sole nitrogen source. In Chardonnay juice, there were no differences in fermentation progress (as weight loss), fermentation duration or urea production. Throughout fermentation, all strains exhibited similar weight loss patterns (**Figure 3**) and completed fermentation in 168 h (**Table 2**). Enzymatic determination of urea showed no detectable urea in the wine produced by any strains (**Table 2**).

In CDGJM with L-arginine as the sole nitrogen source, differences in weight loss and endpoint urea were observed. Wild-type EC1118 exhibited the fastest weight loss, completing fermentation after 168 h, while TANG1 and TANG6 showed significantly

retarded fermentation performance, completing after 264 h and 192 h, respectively (**Figure 4**). In the *can1* strains, arginine uptake would be reliant on lower affinity transporters since arginine permease (Can1p) the high affinity arginine transporter, is non-functional. These transporters include Alp1p (Regenberg *et al.*, 1999) and Gap1p (Grenson *et al.*, 1970). The slower growing mutants also showed markedly lower urea production. TANG1 and TANG6 produced about 4 mg L^{-1}, in comparison to 25 mg L^{-1} produced by EC1118. Interestingly, these concentrations are much lower than other reports i.e., 120 mg L^{-1} for EC1118 and 80 mg L^{-1} for the *can1*) Vigentini *et al.* (2017). While differences in methodologies may be responsible, the desired effect was observed nonetheless.

Discussion

The drive to produce new and improved yeast strains for fermentation has relied on older, non-recombinant techniques to yield "non-GMO" strains. These include the use of hybridisation to produce the Lallemand strain Cross Evolution® (https://catalogapp.lallemandwine.com/uploads/yeasts/docs/1f3b34a4d7880e11cd42390814d3d8a9c2f2244d.pdf), interspecific hybridisation to produce AB Biotek strains AWRI Paragon (AWRI 1501) and AWRI Zevii (AWRI 1503; Bellon *et al.* (2011)) or marker-assisted breeding to produce Laffort strains Zymaflore® FX10, CX9, X5, XPURE, RX60 and X16 (https://laffort.com/en/focus-en/selection-des-levures-le-breeding-assiste-par-marqueur-qtl/). There are two recombinant wine yeast approved for use in the United States and Canada. The first, ML01, is capable of malolactic fermentation (Husnik *et al.*, 2006), while the second, ECMo01, efficiently utilises urea through constitutive expression of an additional *DUR1,2*, encoding urea amidolyase, to

reduce ethyl carbamate in wine (Coulon *et al.*, 2006). Whilst these strains may have some benefits in the USA and Canada, many countries have tight regulations around their use. Newer, more precise genome editing techniques including programmable nucleases such as CRISPR/Cas9 are attractive alternatives to these techniques for strain improvement. Countries such as the USA, Japan and Australia have already expressed their stance on these by altering their legislation. Both Japan and the USA allow for organisms to be edited using HDR, provided the modification would be possible in nature, whereas Australia will only allow for NHEJ derived mutations to go unregulated (Hanlon & Sewalt, 2021). As such this study is an important proof-of-concept.

Early CRISPR/Cas9 experiments in laboratory *S. cerevisiae* strains demonstrated the high efficiency of HDR and low efficiency and lethality of NHEJ when repairing double-stranded breaks (DSBs) in the DNA (DiCarlo *et al.*, 2013, Akhmetov *et al.*, 2018). These findings are the likely reason behind the current literature on CRISPR/Cas9 in yeasts being confined to HDR-based methods (Vigentini *et al.*, 2017, Cordente *et al.*, 2018, van Wyk *et al.*, 2020). To the best of our knowledge, there is no reported use of NHEJ in any industrially relevant *S. cerevisiae* strains. Therefore, we aimed to demonstrate the feasibility of NHEJ repair of CRISPR/Cas9-derived DSBs in wine *S. cerevisiae* strains, as a means of producing "non-GMO" strains, which may be acceptable to the global industry.

In this study, we evaluated the use of CRISPR/Cas9 to introduce NHEJ based mutations in *CAN1* in the widely used wine yeast, EC1118. This strain is popular in wine making due to its claimed high alcohol tolerance, low nitrogen demand, fast fermentation and low hydrogen sulfide production (https://catalogapp.lallemandwine.com/uploads/yeasts/docs/d6ed47e6f0cc6c2f988123728759d189b2576ce6.pdf). *CAN1* was chosen as a gene target due to its applicability to

winemaking and prior reporting of CRISPR/Cas9 in wine (Vigentini *et al.*, 2017) and laboratory yeast (DiCarlo *et al.*, 2013) backgrounds. *CAN1* encodes a plasma membrane arginine specific permease responsible for efficient uptake of arginine. Disruption of *CAN1* by CRISPR/Cas9 in a wine yeast background has previously been shown to reduce urea production, a precursor to ethyl carbamate (Vigentini *et al.*, 2017). In this study, we used the *CAN1* targeting sequence reported by Vigentini *et al.* (2017) and DiCarlo *et al.* (2013) but used a different approach to the earlier publications. A single plasmid system (pUDP002; Juergens *et al.* 2018) was used whereby the Cas9 protein and ribozyme were cloned together; the plasmid transformed in the absence of a repair template. Multi-plasmid systems were central to previous *CAN1* disruptions, which either used laboratory yeast strains (DiCarlo *et al.*, 2013) or HDR to introduce mutations (DiCarlo *et al.*, 2013, Vigentini *et al.*, 2017). Both these scenarios running against the objective of producing non-GMO yeast by NHEJ.

The inefficiency of NHEJ in *Saccharomyces* (Akhmetov *et al.*, 2018) was confirmed with the recovery of only six yeast colonies after transformation of the CRISPR/Cas9 components without an HDR repair template. The two frame shift mutations resulting from a single nucleotide deletion, corresponding to a change at amino acid 67 or 69, produce a truncated protein of 70 amino acids. The likely deleterious nature of both genotypes was suggested from a previously reported premature stop codon at position 70 (i.e., a protein one amino acid shorter than our mutants; Vigentini *et al.* 2017) and structural analysis of amino acid sequences by PROVEAN. Having identical *CAN1* sequences, TANG1, TANG2 and TANG5 represent likely clonal enhancement from culturing of a single transformant. Factoring this in, a mutation efficiency of 1.7×10^{-7} can be proposed based on transformants per total cells (1.2×10^{8}) and the mutants identified (2 variants). This value largely differs from the 0.07% reported by DiCarlo *et*

al. (2013) for the same gRNA sequence. It should be noted that that group used a haploid laboratory yeast and genome editing was undertaken post galactose induction of Cas9. As such, calculation was based on post-induction conditions and not post-transformation conditions, as used in this study. Whilst an inducible CRISPR/Cas9 system would favour NHEJ-mediated editing to introduce simple mutations, key to both systems is a positive phenotype selection as a screen for the correct mutation. Other examples include molybdate resistance (for enhanced glutathione production; Mezzetti *et al.* 2014, or lowered hydrogen sulfide (H_2S) production; De Vero *et al.* 2011), chromate resistance (for lowered H_2S production; De Vero *et al.* 2011), *p*-fluoro-DL-phenylalanine or *o*-fluoro-DL-phenylalanine resistance (for increased 2-phenylethanol and 2-phenylethyl acetate production; Cordente *et al.* 2018), azetidine-2-carboxylate resistance (for increase proline production; Tsolmonbaatar *et al.*, 2016, Murakami *et al.*, 2020, Ohashi *et al.*, 2020) and methylamine resistance (for increased proline uptake; Long *et al.* 2018).

We have demonstrated the ability to create NHEJ-based mutants in an industrial *S. cerevisiae* strain, which introduced frameshift mutations that should be industry applicable. It is well known that arginine is the major source of urea arising from yeast metabolism (Monteiro *et al.*, 1989), with increased urea production observed in yeasts with a higher capability to degrade arginine. Increased arginine in the fermentation medium increases urea excretion and decreases re-absorption (An & Ough, 1993), thus grape must with higher arginine may be at risk of excessive urea production. In tackling this risk, a disruption of *CAN1* is perceived to work in two ways; firstly, by slowing arginine uptake through a reliance on the general amino acid permease, *GAP1*, and a lowly expressed arginine permease, *ALP1*, for its uptake (Ljungdahl & Daignan-Fornier, 2012). Secondly, *can1* strains have suppressed arginine consumption (Zhang *et al.*, 2016) and decreased expression of arginase, *CAR1* (Zhang & Hu, 2018), slowing urea

production and allowing its catabolism by *DUR1,2* (Genbauffe & Cooper, 1986) to CO_2 and ammonium before excretion into the wine milieu to reacts with ethanol to form ethyl carbamate (Ough *et al.*, 1988, Monteiro *et al.*, 1989).

Disruption of the *CAN1*-encoded arginine permease is reported to lower urea production in commercial wine strains, EC1118 and AWRI 796, in arginine-enriched red or white grape juice, or a synthetic must with L-arginine as the sole nitrogen source (Vigentini *et al.* 2017). The authors also observed slower fermentation by the *can1* mutants compared to the parental strains. These findings are similar to our observations when fermenting synthetic must with arginine as the sole nitrogen source. *Can1* mutants, TANG1 and TANG6, exhibited slower weight loss (**Figure 4**) and lowered urea concentrations (**Table 2**) compared to the wild type EC1118. Interestingly, TANG6 did not show a similar weight loss pattern as TANG1, instead exhibiting an intermediate phenotype with an initially slow fermentation (similar to TANG1), but not slowing further post-96 h as does TANG1. In this way, TANG6 completed fermentation only 24 h after EC1118 and 72 h ahead of TANG1. One potential explanation for this could be the presence of other mutations derived from off-targeting of Cas9, although these are rare events. Through whole genome sequencing, Ohnuki *et al.* (2019) revealed the possibility and rarity of this event through a single off-target mutation when targeting *AWA1* in the industrial sake strain, K7. In order to explain the greater fermentation speed of TANG6, the loci of mis-targeting would need to be linked to fermentation performance, perhaps fermentation-essential genes constituting the Fermentome (Walker *et al.* 2014). Further investigation of TANG6 including whole genome sequencing is warranted to verify this phenomenon.

Conversely, we did not observe the same fermentation behaviour to Vigentini *et al.* (2017) when fermenting Chardonnay juice enriched with 2 g L^{-1} L-arginine. Strains

TANG1 and TANG6 showed identical weight loss and lack of urea production as wild type EC1118. This was unexpected, as Vigentini *et al.* (2017) demonstrated in both red (Cabernet Sauvignon) and white (Chardonnay) that fermentations slowed. There were, however, differences in the experimental setup including scale (100 *vs* 250 mL), temperature (17 vs 20 °C) and different juice sources. Whilst fermentation volume is not likely to result in differences in urea production, differences in temperature and juice composition may do. Previously, Ough *et al.* (1991) investigated the effects of temperature differences (20 *vs* 15.5 °C) on urea production in different yeast in 11 different grape juices from 10 different wineries comprised of either Sauvignon Blanc, Pinot Noir, Chardonnay or Gewurztraminer. In a strain dependant manner, the temperature difference affected the production of urea, in some instances reducing it by ~50% in the cooler fermentations. Furthermore, juice composition also appears to play a role in urea production. In the study, Ough *et al.* (1991) demonstrated that two Sauvignon Blanc juices of similar amino acid makeup, pH and °Brix, resulted in vastly different urea production: one strain producing ~91 % less urea across the juices in the same conditions. Ough *et al.* (1991) also showed that at very high amino acid contents, arginine metabolism was delayed and therefore urea excretion was lowered. The variable nitrogen composition in grape juice very likely alters the expression of arginine catabolism genes through nitrogen catabolite repression, but whilst interesting, this topic is beyond the scope of this paper.

To conclude, we have demonstrated the use of a CRISPR/Cas9 system to create a gene disruption in *CAN1* by the error-prone NHEJ repair system in an industrial wine yeast strain. Whilst there seemed to be no phenotypic variance between mutant and wild type strains when fermenting an arginine-enriched Chardonnay juice, there were observable differences when fermenting a synthetic must with arginine as the sole

nitrogen source. Mutants exhibited lower end-point urea concentrations at the cost of a slower fermentation. It may be that modulation of urea production in a winemaking setting (i.e., juice) is not reliably altered by single gene modifications, and further work is warranted to better understand the function of Can1p in relation to nitrogen metabolism and urea production. Nonetheless, the findings from this study are valuable as a positive step towards the generation of CRISPR-edited strains with a 'non-GMO' classification, as is the case in Australia.

Acknowledgements

The authors wish to the Nick van Holst for his technical assistance and Associate Professor Paul Grbin for providing the Chardonnay juice. We thank Dr Jean-Marc Daran for the gift of plasmid pUDP002 (Addgene plasmid #103872; http://n2t.net/addgene:103872; RRID: Addgene_103872).

Funding

This project was supported by funding from Wine Australia (UA1803_2.1) to MW. Wine Australia invests in and manages research, development and extension on behalf of Australia's winegrowers and winemakers and the Australian Government. VJ is partly supported by the Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number IC170100008), which is funded by the Australian Government with additional support from Wine Australia and industry partners. TL is a recipient of a University of Adelaide Faculty of Sciences Divisional Scholarship and Wine Australia Supplementary Scholarship (AGW Ph1603).

The University of Adelaide is a member of the Wine Innovation Cluster (http://www/thewaite.org/waite-partners/wine-innovation-cluster/).

Conflicts of Interest

The authors declare there are no conflicts of interest regarding the study.

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Table 1. Oligo nucleotide and gBlock sequences used in this study.

Primer	**Sequence (5' – 3')**
pUDP CAN1 sgRNA gBlock	GGTCTCGCAAA**CGTATC**CTGATGAGTCCGTGAGGACGAAA CGAGTAAGCTCGTCGATACGTTCTCTATGGAGGAGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC AACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGC ATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATG CTTCGGCATGGCGAATGGGACACAGCGAGACC
pUDP002 Seq	GACGGTAGGTATTGATTGTAATTCT
CAN1 A	CTATCAATGAAAATTTCGAGGAAGA
CAN1 D	GACGTGAAGATAACGAAAAATGAGT
CAN1 seq F	GAAGACGCCGACATAGAGGAG
CAN1 seq R	GGATGAATGTAGCCATTTCACCC

Bold font denotes variable region of the hammerhead ribozyme sequence derived from the reverse complement of the first six nucleotides of the underlined guide RNA sequence.

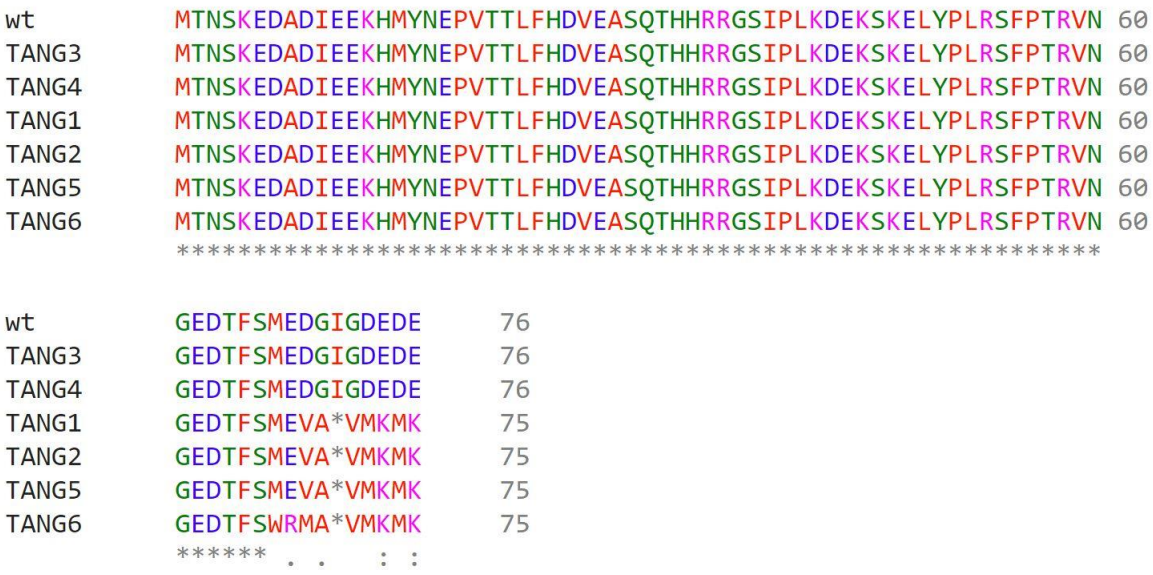


Figure 1. **Clustal Omega sequence alignment of partial amino acid sequences of *CAN1* in hygromycin-resistant isolates.**
TANG1, TANG2 and TANG 5 show frame shift mutations at amino acid 69 and TANG6 a frame shift at amino acid 67. Both frameshift mutations result in an early stop codon at position 71 (*) and result in a truncated protein of 70 amino acids.

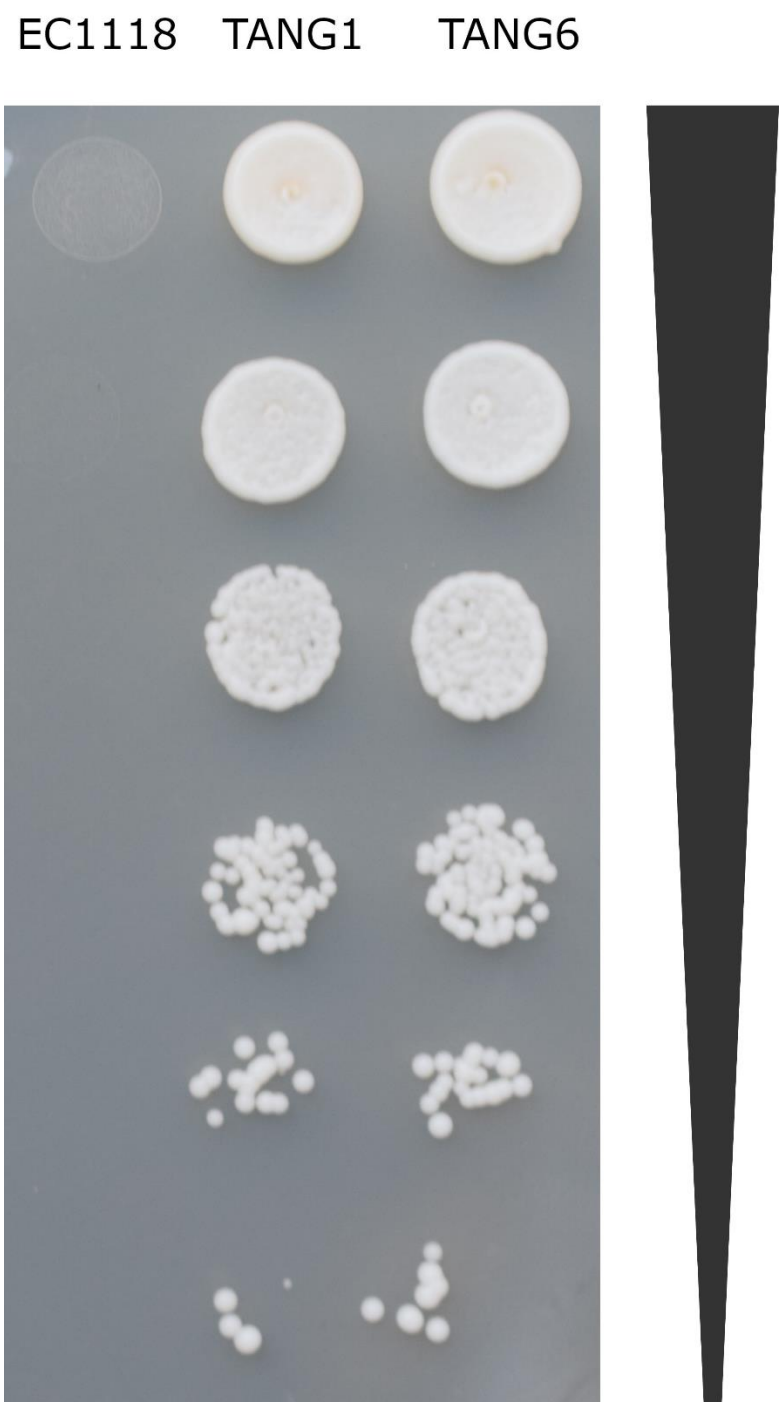


Figure 2. Spot plating for canavanine resistance of wild type EC1118, TANG1 and TANG6.

Overnight YEPD cultures of wild type EC1118 and mutant strains, TANG1 and TANG6, representing both mutant genotypes were serially diluted and 5 µL were plated onto SD -Arg with 60 µg mL^{-1} L-canavanine. Images reveal colony growth after overnight incubation.

Table 2. Fermentation duration and urea production of EC1118 and strains representing the two *CAN1* mutant genotypes.

	Synthetic Juice		Chardonnay	
Strain	**Duration (h)**	**Urea (mg L^{-1})**	**Duration (h)**	**Urea (mg L^{-1})**
EC1118	168	24.6 ± 4.6	168	nd
TANG1	264	3.7 ± 0.5	168	nd
TANG6	192	4.1 ± 0.9	168	nd

Fermentation duration relates to the time taken to achieve 0 g L^{-1} sugar (i.e. a negative Aimtab test) after one day of minimal weight change. Urea was determined at fermentation endpoint a Megazyme L-Arginine-Urea-Ammonia Assay Kit. Urea concentrations lower than 20 mg L^{-1} were regarded as not detectable (nd) and outside the detection limitations of the enzymatic kit.

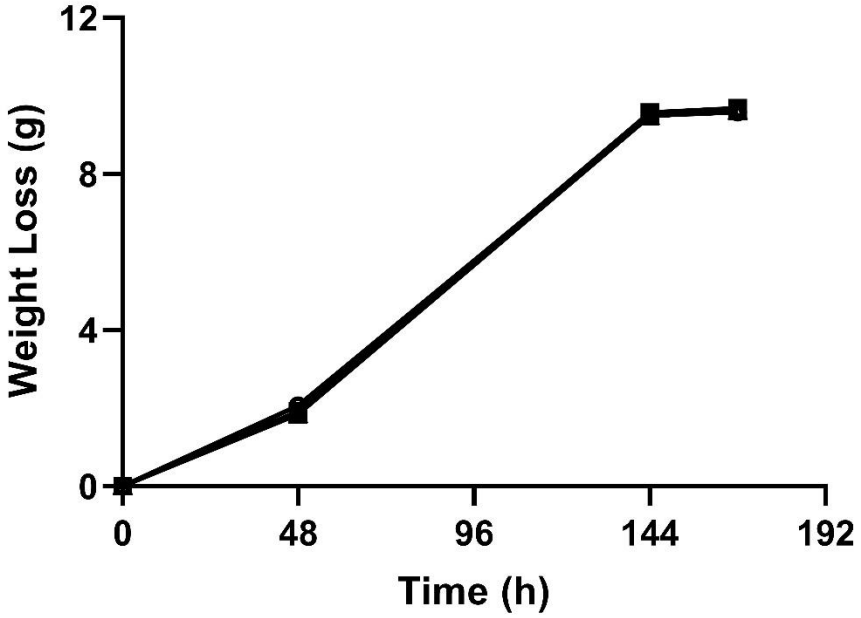


Figure 3. Fermentation performance of wild type EC1118 and *CAN1* mutants TANG1 and TANG6 in Chardonnay juice enriched with arginine.

Fermentations were conducted in filter sterilised Chardonnay juice enriched with 2 g L^{-1} L-arginine. Fermentation progress was monitored by cumulative weight loss (g) for strains EC1118 (○), TANG1 (□) and TANG6 (△). Values represent triplicate fermentations ± standard deviation (as error bars).

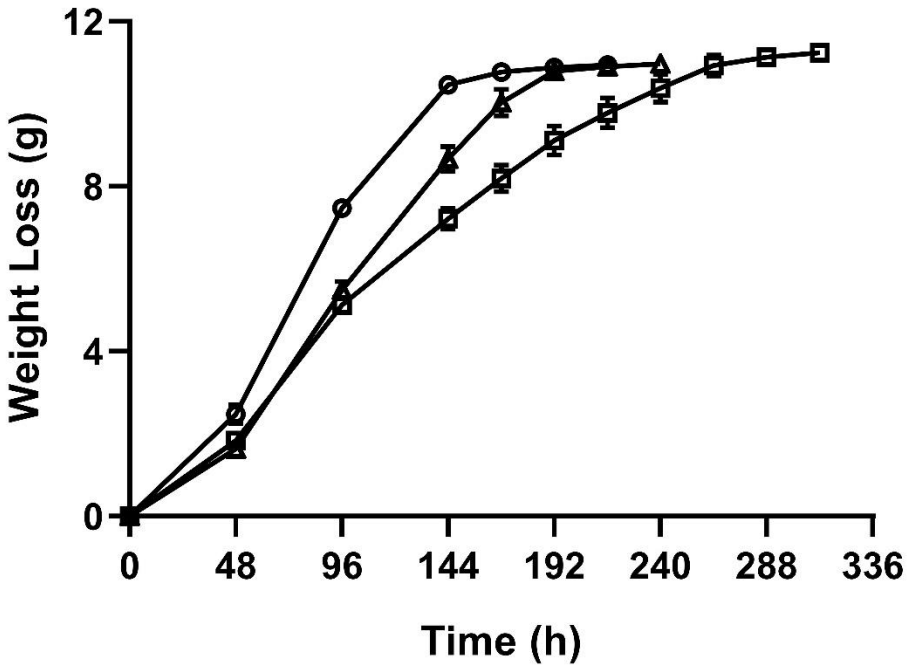


Figure 4. Fermentation performance of wild type EC1118 and *CAN1* mutants TANG1 and TANG6 in synthetic grape juice with arginine as the sole nitrogen source.

Fermentations were conducted in synthetic grape juice with 2 g L^{-1} L-arginine as the sole nitrogen source. Fermentation progress was monitored by cumulative weight loss (g) for strains EC1118 (○), TANG1 (□) and TANG6 (△). Values represent triplicate fermentations ± standard deviation (as error bars).

Chapter 6

General Conclusions and Future Directions

General conclusions and future directions

The discovery of the genome editing technique, CRISPR/Cas9 in the last decade has been a 'game-changer' in biotechnological industries where yeast are considered 'cell factories' in the production of high value products. CRISPR/Cas9 has the potential to revolutionise strain development in the beverage industries. CRISPR/Cas9 differs from conventional recombinant technology in one key respect - it allows for simplified homozygous integration of specific DNA changes in diploid industrial yeast strains (Stovicek *et al.*, 2015). CRISPR/Cas9 editing has great appeal for strain construction, as it allows for the introduction of gene disruptions and other targeted mutations of interest (such as SNPs and QTLs), without the need for genomic integration of a marker gene; the yeast retaining only the gene modification and not the 'intermediary' exogenous DNA.

The focus of this research was to demonstrate the use of CRISPR/Cas9 to develop wine yeast strains with improved fermentation characteristics, investigate novel wine yeast phenotypes associated with QTLs linked to sake and bread making, and develop mutant yeasts that would avoid the definition of GMO in countries such as Australia. Specifically, this research looked at a) adapting research on *ECM33* disruption to diploid wine yeast to improve fermentation performance, b) introducing novel QTL mutations in *SER1* and *PRO1* to investigate yeast with novel phenotypes and c) modifying yeast without a CRISPR/Cas9 DNA repair template, to avoid GMO classification in Australia. The latter mutation was generated by non-homologous end joining (NHEJ) repair. CRISPR/Cas9 was successfully implemented to introduce all the above mutations in the diploid wine strain, Lalvin EC1118.

The potential implementation of these mutants by industry, is dependent upon the regional jurisdiction, in terms of the gene technology used to modify the yeast but also the industrial application. Of mutants developed in this thesis, all except EC1118 *ecm33* (Chapter 2) may have industry applicability. Strains with mutations derived from naturally occurring sources (e.g. QTL analysis; *SER1*(G78R), or chemical mutagenesis screens; *PRO1*(I150T), *PRO1*(P247S) and *PRO1*(E415K)) are considered as "self-cloned", as only DNA from the same or similar species is introduced. The use of these strains should be permitted in countries such as the USA and Japan, where they are not regulated as GMOs (Hanlon & Sewalt, 2021). Whilst these strains would not avoid GMO status in Australia (the impetus for this study), recent changes to legislation (*Gene Technology Regulations 2001*, Compilation No. 9) may allow for strains produced with no net introduction of DNA (e.g. cured of plasmids and no repair templates used) to be employed. This legislative amendment provides the basis for the final research chapter, where CRISPR/Cas9 mediated NHEJ repair was successfully used to disrupt *CAN1* encoding the arginine permease.

While this study has focused on the use of CRISPR/Cas9 for *Saccharomyces cerevisiae* strain production, future research in the field of gene edited yeasts for beverage production has the potential to broaden these horizons. In many areas, the use of non-*Saccharomyces* yeast has been gaining interest, with mixed culture fermentations used as a means of altering beverage composition because of the differences in metabolism between *S. cerevisiae* and other yeasts. Whilst the different non-*Saccharomyces* genera individually exhibit a high degree of heterozygosity and phenotypic-genetic diversity (e.g., Hranilovic *et al.*, 2017), their genetics and cell physiology in relation to fermentation are not well understood. Furthermore, few genomic and genetic tools are available in relation to strain development. Researchers are now looking at opportunities

to manipulate these yeasts as *de novo* genome sequences become available and transformation systems are developed. CRISPR/Cas9 protocols specific for non-*Saccharomyces* but based on current knowledge in *S. cerevisiae* would be of benefit, especially in the improvement of fermentation phenotypes such as ethanol or sulfite tolerance, which can be low for many of these species. Current genetic manipulation with CRISPR/Cas9 is reliant on broad host plasmids such as pUDP002 (Juergens *et al.*, 2018) used in Chapter 5, which are suitable for ascomycete yeasts, which must be readily transformable in numbers such that mutations are identifiable through Sanger sequencing, as many genes are not associated with selectable phenotypes. Although CRISPR/Cas9 editing in *S. cerevisiae* is highly efficient when a DNA template is used to repair double stranded DNA breaks via homologous recombination, the resultant strains are considered genetically modified under Australian legislation. Whilst HDR is the major repair mechanism in *S. cerevisiae* there is a NHEJ repair mechanism similar but not identical to mammals and filamentous fungi (Dudásová *et al.*, 2004), which allows gene editing with CRISPR/Cas9 in the absence of a repair template, but the efficiency is very low (this work, DiCarlo *et al.* 2013). In contrast, NHEJ is the major repair pathway for double stranded DNA breaks in non-*Saccharomyces* and filamentous fungi (Álvarez-Escribano *et al.*, 2019). NHEJ offers great promise in terms of the construction of non-GMO strains as defined by the current legislation in Australia (*Gene Technology Regulations 2001* (Compilation No. 9)). This could further be improved by the use of non-plasmid methods such as those used by Varela *et al.* (2020), where CRISPR/Cas9 ribonuclear protein complexes were used to modify *Brettanomyces bruxellensis*. If this were adapted to other non-*Saccharomyces* yeasts and mutations were to occur by NHEJ, the need for a GMO intermediate strain (having plasmids present) would be eliminated. This approach may also circumvent the legal requirement for higher physical containment laboratory

facilities (e.g., PC2) by the Australian Office of the Gene Technology Regulator (http://www.ogtr.gov.au/) regarding genetically engineered yeasts other than *S. cerevisiae*.

This method of producing GMOs is not limited to yeasts but could also apply to bacteria used in brewing and winemaking. *Oenococcus oeni*, the principal bacteria used in secondary malolactic fermentation in winemaking is a prime target. This bacterium has been highly sequenced, with genomic analysis revealing that genetic diversity is dependent upon the ecological niche e.g., wine (Bartowsky, 2017), cider and kombucha (Lorentzen *et al.*, 2019). Recombinant DNA technology is in its infancy, with few reports of successful transformation systems (Beltramo *et al.*, 2004, Assad-García *et al.*, 2008, Betteridge *et al.*, 2015). While *O. oeni* are difficult to transform, an expression-free CRISPR/Cas9 system may provide a suitable and effective alternative to the current plasmid expression systems available for gene function studies and ultimately strain development, which currently rely on bioprospecting, adaptive evolution and mutagenesis (reviewed in Betteridge *et al.*, 2015, Virdis *et al.*, 2020).

Lactobacillus plantarum is another wine bacterium receiving interest in terms of malolactic fermentation (Brizuela *et al.*, 2019, Krieger-Weber *et al.*, 2020). Unlike *O. oeni*, CRISPR/Cas9 gene editing protocols have been developed for *L. plantarum* (Huang *et al.*, 2019, Leenay *et al.*, 2019, Myrbråten *et al.*, 2019, Zhou *et al.*, 2019). CRISPR/Cas9 in *L. plantarum* has been effective in introducing small mutations (e.g., silent mutations) and whole gene deletions by HDR, however, effectiveness appears to vary in a strain dependent manner (Leenay *et al.*, 2019). The use of a NHEJ-based method has not been demonstrated in *L. plantarum*, however, CRISPR/Cas9-induced DSBs have resulted in lethality (Huang *et al.*, 2019). *L. plantarum* appears similar to *S. cerevisiae* in this sense,

thus HDR-based methods may be more appropriate. This would still allow for gene deletions and the introduction of other mutations (e.g., SNPs and QTLs), which still may have commercial value in countries that allow the use of 'self-cloned' organisms.

As already mentioned, the CRISPR/Cas9 system can be considered a 'game changer' not only in how fundamental studies are undertaken, but also how industrially relevant organisms are produced. This is well reflected in the abundance of literature published in the last decade (especially since the start of candidature in 2016) within the fermented beverages research alone (see Chapter 1). This doctorate not only adds to our knowledge of *S. cerevisiae* physiology and gene function but also has generated strains with potential applications in the beverage industry, depending on regional legislation.

ECM33 disruption as a novel factor in cellular aggregation

ECM33 was originally identified as a candidate gene effecting fermentation, as a deletion, in a research effort to better understand the mechanisms behind nitrogen efficiency and fermentation (Zhang *et al.*, 2018). The authors produced a haploid wine yeast with superior fermentation performance under nitrogen-limited conditions. Whilst the present study in a diploid wine yeast, EC1118, corroborates Zhang's research under laboratory conditions with agitated fermentations, an additional, unreported microscopic phenotype was also observed, cellular aggregation. This phenotype negates the positive fermentation performance effect when fermentations are undertaken statically, as is more likely in industry. Whilst in many circumstances this may prevent the use of this mutation commercially, there may be applicability in fermentations (e.g. wine or beer) where the infrastructure allows for agitation. It remains to be seen how these yeast behave in larger industrial fermentations, but this is beyond the scope of this project, certainly while

legislation in Australia restricts such work with a GMO. From a yeast physiology standpoint, however, more research is warranted in order to understand how these cells form small aggregates. In Chapter 2, the yeast were suggested to behave similarly to snowflake yeast, where the cells do not dissociate after budding (Ratcliff *et al.*, 2012, Oud *et al.*, 2013, Ratcliff *et al.*, 2013, Kayacan *et al.*, 2020). It may be of interest to observe these cells by inverted light microscopy with chambered slides to allow visualisation of cellular aggregates without disrupting their structure and also monitoring their growth in real time to assess aggregate formation.

SER1 **disruption for slow-growing yeast for use in *S. cerevisiae*/non-*Saccharomyces* co-inoculated fermentations**

Chapter 3 describes the introduction of a naturally occurring *SER1* disruption allele (*SER1*(G78R)) in *S. cerevisiae* wine strain EC1118, which resulted in slowed initial growth of the mutants, corroborating previous studies (Reiner *et al.*, 2006, Jung *et al.*, 2018). The mutant was subsequently evaluated in separate co-inoculated fermentations with *Lachancea thermotolerans* (Concerto™) or *Metschinokowia pulcherrima* (MP2) strain. The slow growth of *S. cerevisiae* associated with the (*SER1*(G78R)) mutation resulted in 2 beneficial phenotypes. Firstly, it allowed for the expression of non-*Saccharomyces* characteristics in wines, and the subsequent completion of fermentation when the non-*Saccharomyces* populations began to decline (as with *M. pulcherrima* MP2). This is of interest to winemakers as an alternative blended *S. cerevisiae* and non-*Saccharomyces* yeast starter culture (*cf.* Viniflora® Melody ™), which can be used as a single inoculum. The second beneficial phenotype was the apparent sequesteration of SO_2 from the grape juice, allowing for the growth of the sulfite sensitive strain of *L.*

thermotolerans, Concerto™. These properties would be advantageous in scenarios where non-*Saccharomyces* yeasts cannot be used, such as when sulfite is routinely added to prevent spoilage of grape juice. Having tested a single sulfite sensitive yeast strain, it would be appropriate to further screen additional non-*Saccharomyces* strains and species that could be used in conjunction with the EC1118 (*SER1*(G78R)) mutant. Further experiments are required to assess how these strains behave in larger scale fermentations in an industrial setting. Whilst this is impossible to do in Australia due to the legislative limitation on culture size of GMOs, it may be possible in other countries where self-cloning yeast are considered non-GMO due to the mutation being naturally occurring. An alternative would be to use marker-assisted breeding to introduce the mutation from the original sake strain where it was identified through a QTL study (Jung *et al.*, 2018). This would then eliminate the GMO classification and allow for these larger scale experiments, but also commercialisation in countries where GMO yeast are tightly regulated.

Enhancing proline accumulation in wine yeast negatively impacts wine-related stress resistance except for osmotolerance

The introduction of *PRO1* and *PUT2* mutations into EC1118 both singularly and in tandem, resulted in various intracellular proline amounts. The accumulation of proline from biosynthesis has been identified as a factor positively affecting stress response to baking (Sekine *et al.*, 2007, Kaino *et al.*, 2008, Sasano *et al.*, 2012, Tsolmonbaatar *et al.*, 2016) and sake brewing (Takagi *et al.*, 2005, Takagi *et al.*, 2007) stresses. However, this was not evident in a wine yeast background, where proline accumulation resulted in lowered growth without stressors, but also sensitivity to SO_2, ethanol and osmotic stress

in most instances. One strain, *PRO1*(E415K) c*PUT2*, exhibited enhanced resistance to high osmolarity but was the more sensitive to high ethanol. Although these strains do not appear to increase their tolerance to individual fermentation-related stresses, further experiments are required to assess their performance in grape juice, and how increased proline content may affect the sensory outcomes of the wine. Alternatively, the use of these strains as a food supplement because of their nutrient (proline) enriched biomass warrants investigation. Normally this would entail their growth under sterile conditions akin to brewing (boiled wort), but it would still be useful to see whether they could be used for wine production, with the proline content in the yeast lees sufficient to warrant re-processing rather than disposal. This would add value in terms of a premium by-product as well as reduced financial and environmental costs associated with disposal. Such nutritionally enhanced yeast biomass could be sold as a culinary ingredient such as sake kasu (spent rice and yeast residues) used in Japan or be processed into yeast extract, which is used in many food products and is the primary ingredient in Vegemite - an Australian icon at breakfast (vegemite on toast).

CRISPR/Cas9 as a tool to create non-GM yeast mutants by NHEJ in accordance with Australian legislation

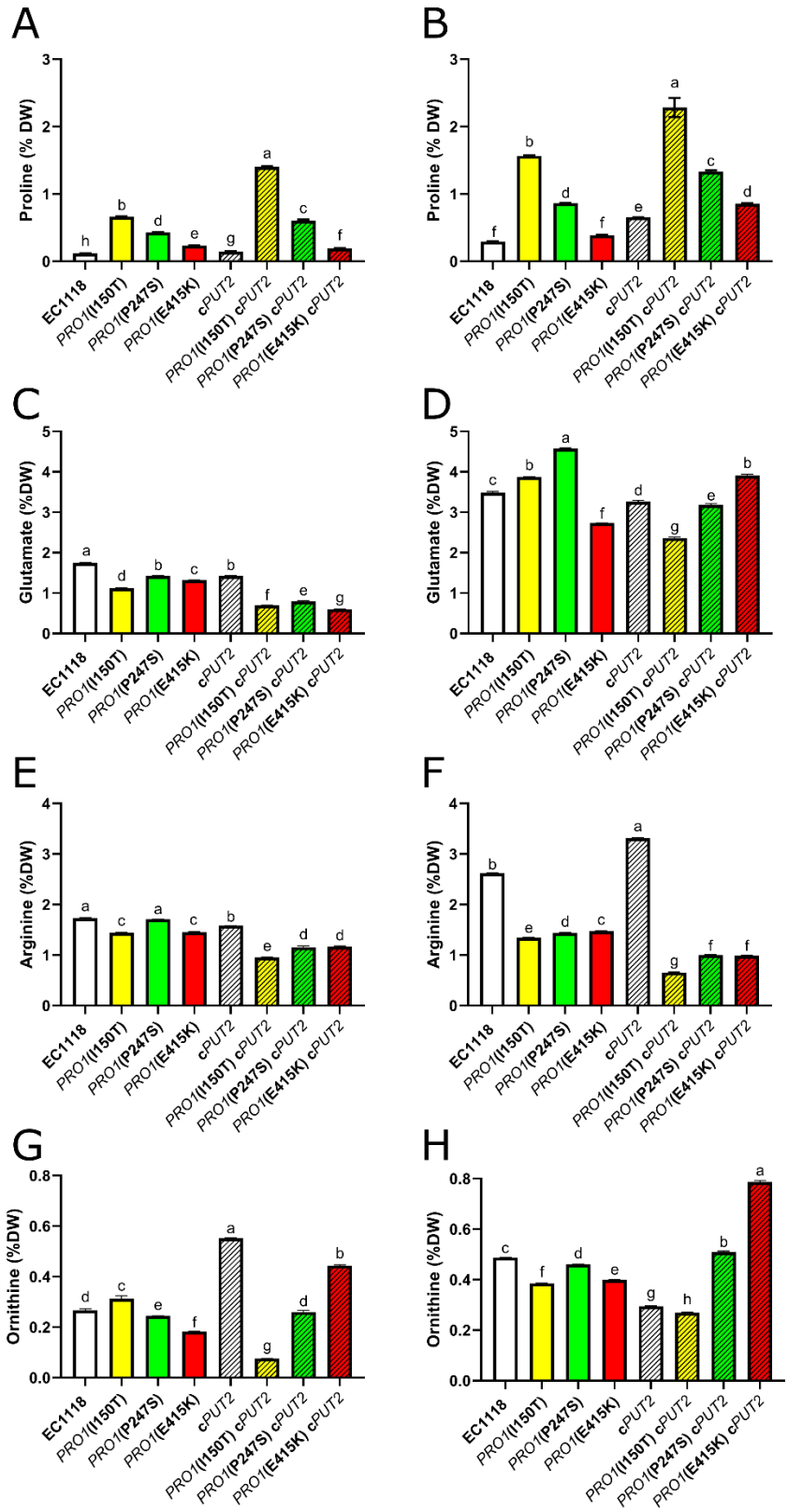
With changes to gene technology legislation in Australia, there is great potential for CRISPR/Cas9 in non-GM yeast production using methods reliant on NHEJ rather than homology directed repair (HDR) to introduce loss-of-function mutations through the insertion or removal of nucleotides during the repair of the DSB. Chapter 5 describes the construction of NHEJ-edited mutants of EC1118, targeting *CAN1*. A broad host-range plasmid targeting *CAN1* was used to mediate the editing in *S. cerevisiae*, although the

original plasmid was developed to edit non-*Saccharomyces* yeasts (Juergens *et al.*, 2018). Two different NHEJ-derived mutants (TANG1 and TANG6) were identified out of six transformants originating from 1.2 x 10^8 cells transformed with the plasmid. TANG1 and TANG6 were able to produce lower levels of urea (a precursor to carcinogenic ethyl carbamate) from arginine. Whilst NHEJ repair is an inefficient method of genome modification in *S. cerevisiae*, which preferentially uses HDR, it may be more applicable to non-*Saccharomyces* yeasts. The use of broad-host plasmids such as pUDP002 (Juergens *et al.*, 2018), are highly applicable, since the CRISPR/Cas9 machinery and drug resistance markers are strongly expressed under the *TEF1* promoter sequences (from *Eremothecium gossypii* (*Ashbya gossypii*) and *Arxula adeninivorans* (*Blastobotrys adeninivorans*)), which are recognised by other *Saccharomycetes* yeasts (Terentiev *et al.*, 2004, Steinborn *et al.*, 2006, Juergens *et al.*, 2018). The broad-host applicability of the system offers great potential for wine non-*Saccharomyces*, with the proviso that transformation methods exist, genome sequences are available and gene orthologs have similar phenotypes to their *S. cerevisiae* equivalents. Assessment of the applicability of CRISPR/Cas9 in different non-*Saccharomyces* species may simply be screened by targeting adenine biosynthesis and looking for the development of red *ADE2*-mutant colonies, as demonstrated by Juergens *et al.* (2018) when using this plasmid in *Kluyveromyces* and *Ogataea* yeasts. Alternatively, non-plasmid methods could be used such as that used by Varela *et al.* (2020) to modify yeast with no developed transformation or expression systems, or to avoid the introduction of any DNA at all. The development of CRISPR/Cas9 systems in non-*Saccharomyces* yeast would have a two-fold effect. Firstly, it would provide a valuable tool for research to improve our fundamental understanding of these yeasts. Secondly, it would allow us to use these tools to create and improve new strains, similar to that done with *S. cerevisiae* for decades.

Final concluding remarks

This thesis demonstrates the applicability of CRISPR/Cas9 for wine yeast research and industrial strain development. It demonstrates the continuation of research identifying mutations with potential relevance to the wine industry. It also highlights a mechanism for evaluating in winemaking naturally occurring mutations or those developed in other industrial yeasts, with the ultimate goal of developing 'tailored' and 'fit-for-purpose' yeasts, which are readily acceptable and taken up by industry. This is particularly relevant with genetically engineered yeasts produced via CRISPR/Cas9 and NHEJ, where the mode of modification can be considered as alluring as chemical mutagenesis and QTL-assisted breeding programs in terms of strain improvement. All in all, this thesis adds to the ever-growing pool of CRISPR/Cas9 based research and demonstrates is applicability for industry-based strain production

Appendices



Appendix 1. Quantification of the intracellular amino acid content in wine yeast mutants.

EC1118 (WT) and the mutant derivatives were grown overnight in synthetic dextrose without nitrogen (SD-N medium) supplemented with either ammonium sulfate (A, C, E and G) or allantoin (B, D, F, and H) as the sole nitrogen source. Proline measured using an amino acid analyser, with 500 µL of cell extract (representing a 2-fold concentrate from a 1 mL culture). Proline content was expressed as a percentage of cell dry weight. Letters denote significant differences between samples (Tukey's HSD test; $p < 0.05$).

Appendix 2. Live and total cell counts (x 10^5 cells mL^{-1}) and viability (%) of yeast exposed to sodium chloride related osmotic stress. Values are the mean of triplicates ± standard deviation of the mean.

Strain	**Viable cells**	**Total cells**	**Viability (%)**
EC1118	14.54 ± 0.13	22.23 ± 0.25	65.39 ± 0.67
PRO1(I150T)	9.68 ± 0.06	20.75 ± 0.22	46.64 ± 0.33*
PRO1(P247S)	16.12 ± 9.80	28.03 ± 12.82	55.16 ± 7.81

***** denotes significant difference compared to EC1118 (Dunnett's Test, $p < 0.05$)

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