Characterisation of the wine meta-metabolome: linking aroma profiles to yeast genotype

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requirements for the degree of

Doctor of Philosophy

Jade Haggerty

The University of Adelaide

School of Agriculture, Food and Wine

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Abstract

This thesis outlines the development of a high throughput headspace solid phase micro-extraction gas chromatography mass spectrometry (HS-SPME GC-MS) method to analyse aroma compounds in white wine which are considered important to the overall aroma of the 'fermentation bouquet'. Important aroma compounds were determined as those which have previously been included in 'fermentation bouquet' aroma studies in white wine and have odour activity values (OAV) greater than 1; compounds which are found naturally at concentrations higher than their odour perception thresholds. The designed method was then used to create aroma profiles of fermentations formed with a *Saccharomyces cerevisiae (S. cerevisiae)* overexpression library to explore the relationship between the overexpressed genes and the aroma profile of the wine. The overexpression library includes approximately 1500 plasmids contained in an *Escherichia coli (E. coli)* host which required extraction and purification prior to transformation into a wine strain of *S. cerevisiae*. Multivariate analysis of the resulting datasets narrowed down the search field from ca. 1500 clones to 87 clones. After further analyses, a relationship between the genes overexpressed in the yeast and the aroma profile displayed was discovered, leading to a hypothesis for future research.

Chapter 1 of this thesis contains an introduction to the current literature relating to the formation and importance of aroma compounds in white wine and previous research in the area of metabolomics in regards to aroma compounds in wine. Chapter 1 provides the background knowledge to the research and provides context for the findings.

Chapter 2 details the research into the development of the HS-SPME GC-MS method required for this thesis. This chapter describes the proposal of a novel scoring system for choosing the correct

SPME fibre for volatile studies in young white wines. This scoring system is based on the coefficients of determination of the linear curve associated with standard curves formed in 10% ethanol solutions. Using this new method, two out of the five fibres studied were determined as the best for use in the development of a high throughput method to be applied in larger wine studies. The best fibres were chosen utilising the proposed novel scoring system to rate their overall ability to extract the compounds of interest while taking into consideration peak symmetry and sensitivity. This chapter was published in the *Australian Journal of Grape and Wine Research* in 2014.

Chapter 3 continues on from Chapter 2 and describes the final selection of the best fibre for use in studies looking at the 'fermentation bouquet' aroma compounds found in white wines. The selection of the 65 µm PDMS/DVB fibre was made after optimising the analytical parameters used in typical HS-SPME GC-MS methods with regards to the compounds of interest. This fibre was then used in the development and validation of a semi-quantitative method to use in high throughput analyses of 'fermentation bouquet' aroma compounds in white wines. The method was validated through a thorough examination of standard curves formed in three different media; a bag-in-box white wine, CDGJM-Leu fermentation using the parental strain to be used in the final overexpression screen with a blank plasmid (isoC9d ΔLeu + pGP564), and in 10 % ethanol model wine. The results showed that only one internal standard was needed for consistent results and that there were limited differences in the line of best fit seen for each aroma compound in the different media analysed. This chapter was published in the *Australian Journal of Grape and Wine Research* in 2015.

Chapter 4 follows the formation of the important aroma compounds in the 'fermentation bouquet' in white wine throughout the entire fermentation timeline in a CDGJM-Leu medium using the isoC9d ΔLeu + pGP564 yeast. This chapter outlines the similarities and differences of the progression of

the aroma compounds within the CDGJM-Leu as compared to previous real wine and other model media (MS300) studies. The results indicate that fermentations using CDGJM-Leu media using the isoC9d ΔLeu + pGP564 yeast show similar trends in the formation of aroma compounds as a conventional ferment, or a ferment with MS300, with the exception of the compounds related to the biosynthesis of leucine, which fluctuated in concentration until the end of fermentation. This chapter was accepted for publication in the *American Journal of Enology and Viticulture* in late 2015.

Chapter 5 describes the major research undertaking of this project. Specifically, the preparation and testing of the overexpression library of ca. 1500 clones, which was screened along with 20 commercially available yeast (Laffort) for their aroma profile. The library and commercial strains were set up as five replicates. The fermentations were followed according to fermentation progress of the parental strain and a set of replicates sacrificially sampled at the designated time-point, for analysis of total sugars (glucose and fructose) via enzymatic methods and then frozen until aroma analysis. The final sampling was performed at four days after the parental strain had finished fermentation. Only fermentations which were dry at each specific time-point were analysed for their aroma profiles. The results of the screen showed 51% of the library finished fermentation within the allocated time period and that of these 737 clones and 19 Laffort yeasts, we were able to show that 92 clones differed to the rest of the library with respect to their aroma profile. Of these interesting clones, 87 were overexpression clones and 5 were commercial yeasts. It was also hypothesised that for a yeast to retain its plasmid throughout the experimental fermentations, the LEU2 marker is not sufficient and a faster growth rate would increase the rate of plasmid rejection, hence more cells will die due to a lack of nutrients. For the plasmid to be retained, either a beneficial gene, or a gene which when overexpressed decreased vegetative growth also needed to be present. These findings will be beneficial for future studies using or creating overexpression libraries for fermentation studies.

Chapter 6 details the synthesis of deuterated analogues of the aroma compounds studied which could be used as internal standards in future quantitative experiments. This synthesis chapter also details a new green method for the synthesis of ethyl esters and acetates. This method is particularly useful as it describes the purification of each compound to the point where they are pure enough for use as internal standards, which is difficult to achieve due to the low boiling point and high volatility of the compounds within this study.

Chapter 7 completes the thesis by giving an overall summary of the important aspects of this study and its potential impacts on the wine industry. This chapter also proposes future directions and research studies to follow on from this comprehensive work.

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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Publications

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Conferences/ Presentations

Crush Conference, 28th-30th September 2011, Adelaide

Presented a talk entitled: “The biggest fermentation exo-metabolomic study to date”

University of Adelaide School of Agriculture, Food and Wine Postgraduate Symposium, 5th-6th October 2011, Adelaide

Presented a talk entitled: “Developement of a new HS-SPME GC-MS method to be used in quantification of the most important 38 aroma compounds”

3-Minute Thesis Competition, University of Adelaide, School of Agriculture, Food and Wine, Department of Wine Science and Business, Department Round, 2010, Adelaide

Presented a talk entitled: “Characterisation of the wine meta-metabolome: linking sensory attributes to yeast genotype”

3-Minute Thesis Competition, University of Adelaide, School of Agriculture, Food and Wine, Department of Wine Science and Business, Department and School Round, 2011, Adelaide

Presented a talk entitled: “Journey to the Holy Grail of Wine Aroma”

9th Annual Conference of the Metabolomics Society, 1st-4th July 2013, Glasgow

Presented a poster entitled: “Characterisation of the wine metabolome: linking sensory attributes to genotype”

Presentation to Funding Bodies, 17th June 2013, Laffort Oenology, Bordeaux

Presented a talk entitled: “Characterisation of the wine metabolome: linking sensory attributes to genotype”

Supervisory Panel

Prof. Dennis Taylor

School of Agriculture, Food and Wine

The University of Adelaide

Prof. Vladimir Jiranek

School of Agriculture, Food and Wine

The University of Adelaide

Dr. Paul Bowyer

School of Agriculture, Food and Wine

The University of Adelaide

Dr. Tertius Van Der Westhuisen

Laffort Australia

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Abbreviations

2,3-butOH	2,3-butandiol
2,3-butone	2,3-butanedione
2MBacid	2-methylbutanoic acid
2MBOH	2-methylbutanol
2MBA	2-methylbutyl acetate
IBacid	2-methylpropanoic acid
IBOH	2-methylpropanol
IBA	2-methylpropyl acetate
2PE	2-phenylethanol
2PEA	2-phenylethyl acetate
3MBacid	3-methylbutanoic acid
3MBOH	3-methylbutanol
3MBA	3-methylbutyl acetate
Acetal	acetaldehyde
AA	acetic acid

Acetoin	acetoin
ca.	approximately
Benz	benzyl alcohol
Bacid	butanoic acid
BOH	butanol
CW	Carbowax©
CAR	carboxen
CDGJM	chemically defined grape juice medium
CDGJM-Leu	chemically defined grape juice medium without leucine
Decacid	decanoic acid
°C	degrees celsius
DVB	divinylbenzene
eV	electron volts
EIB	ethyl 2-methyl propanoate
E2MB	ethyl 2-methylbutanoate
E3MB	ethyl 3-methylbutanoate
EA	ethyl acetate

EB	ethyl butanoate
ED	ethyl decanoate
EDod	ethyl dodecanoate
EH	ethyl hexanoate
EL	ethyl lactate
EO	ethyl octanoate
EP	ethyl propanoate
GC-MS	gas chromatography mass spectrometry
g	grams
HS	head space
Hacid	hexanoic acid
HOH	hexanol
HA	hexyl acetate
hr	hours
L	litres
MetOH	Methionol (3-(methylthio)propanol)
µg	micrograms

mg	micrograms
µL	microlitres
mL	millilitres
mmol	millimole
Min-DO	minimal dropout media
min	minutes
M	molar
mol	mole
nm	nanometre
POH	n-propanol
Octacid	octanoic acid
OAV	odour activity value
OD_{600}	optical density at wavelength of 600nm
PDMS	polydimethylsiloxane
Pacid	propanoic acid
rpm	rotations per minute
SIM	selected ion monitoring

SPME	solid-phase microextraction
SIDA	stable isotope dilution assay

Literature review

This literature review covers the literature mainly up to September 2009, which was the first 6 months of candidature. Literature which was published after this date has been reviewed in the introductions of each chapter.

The History of Wine in Australia

It has been said that the first wine in Australia was made in 1795 from grapes grown in Rose Hill Vineyard (Beeston, 2001), however it was not until 1803 when the French prisoners arrived, that the wine industry started to begin, albeit unsuccessfully due to the lack of knowledge of the climate. It was in 1822 that Gregory Blaxland sent a shipment of wine to London for a review, where it received a Silver medal. In 1827 he tried again and won a gold medal and became the first commercial winemaker of New South Wales (Beeston, 2001). New South Wales stayed the leading state in winemaking up until 1850, when South Australia seized the title for about 20 years before New South Wales regaining the title once again (Henzell, 2007). During the time of 1920-1940, South Australia was producing about three quarters of Australia's wine, after the removal of inter-state trade restrictions (Henzell, 2007). The wine industry in Australia continued to grow and in the financial year of 2012-2013, South Australia held the lead of being the biggest producer of wine in Australia for the past 4 years, producing almost half of the country's total wine each year (Statistics, 2013).

'New World' versus 'Old world' winemaking styles

The process of winemaking has been occurring for many thousands of years. The winemaking process is comprised of what is known as the 'Old world' and 'New world' styles of winemaking (Birnbaum et al., 2000). 'Old world' winemaking is the process of winemaking that has been taking place in the European and Mediterranean basins for thousands of years, whereas the 'New world' style is wine made in countries which were settled by Europeans over the past 500 years, hence being considered new. The 'Old world' winemaking style is considered, by those who retain this style of production, to rely on the natural side of the process, making use of the *terroir*, the type of grapes (Birnbaum et al., 2000) and wild ferments (Goode, 2005). Wild ferments are a form of

winemaking where the yeast used for the fermentation is not deliberately added; the yeast used in these fermentations are those which are naturally found on the grapes (Goode, 2005). The New world winemaking styles tend to rely on new technologies and science to enhance their wines. This style also tends to follow the process whereby commercially cultured yeasts are added and chosen for specific fermentation properties (Goode, 2005). The competition between the Old and New world styles has grown over the years forcing the Old world regions to try and retain the history of the winemaking 'art' through trade marking winemaking styles such as that of 'Champagne' (Campbell and Guibert, 2007). This difference in wild fermentation versus the addition of cultured yeasts also leads on to the argument about what factors influence a wine's quality the most.

Wine Quality

There is debate on what is the most important factor governing the quality of wine. Multiple factors are reported to be important to the quality of wine. The quality of a wine can be categorised and graded on certain criteria, including the sensory attributes such as colour and intensity, aroma, sweetness, acidity, mouthfeel, and the body of the wine (Schamel, 2000). Each of these qualities can be varied through different winemaking practices. An extensive amount of research has been occurring over the past few decades to understand the science behind each of these criteria to hopefully gain a better understanding of the winemaking practices that will improve the quality of a wine. These practices being investigated range from the processes in the vineyard all the way to the bottling of the completed wine. This particular thesis will be focussing on the wine quality criteria of aroma, since it is one of the first qualities of the wine that a consumer will encounter.

Wine aroma

In regards to the quality of the aroma of the wine, authors are split on whether it is the yeast used in the fermentation which is most important (Son et al., 2009) or the variety/composition of the grapes

being used. In any case, it seems clear that it's a combination of these two aspects that are important to the quality of the aroma of the final wine (Vilanova et al., 2007, Ugliano and Moio, 2008). The composition of the grapes contributes to the "varietal" aromas in the wine and the yeast contributes to the "fermentative" aromas (Rapp, 1998). With fruity aromatic wines being very popular today comprising a significant proportion of the market (LAFFORT, 2007), research into aroma compounds has become an important aspect of wine science. Metabolomics is a new emerging field and will be very useful in the wine sciences for determining the biological reason behind the formation of the chemicals associated with the aroma and other qualities of a wine.

Definition of Metabolomics

To understand a new field of science, a new language must be developed. Some words have a different meaning when used in common usage compared with scientific usage. Although the definitions of most scientific terms are common, there are some grey areas regarding the specific meaning of particular words (Oldiges et al., 2007). Thus, definitions of words frequently used in metabolomics must be provided. To commence, a definition of the suffix "-omics" must be established. According to the Oxford dictionary of Biochemistry and Molecular Biology, "-omics" is "the rigorous, systematic analyses of the -omes" (Attwood et al., 2006). Initially, the term "genome" was generated by Hans Winkler in 1920 by combining the words, gene and chromosome (Oldiges et al., 2007). In more recent times, the suffix "-omes" has been changed to define "an inclusive class of cellular constituents (e.g. metabolome, proteome, transcriptome) by analogy with genome" (Attwood et al., 2006).

For the purpose of this paper, we will be focussing on metabolomics, which is a relatively new technique being used in the life sciences. Due to genome sequencing becoming easier and more accessible, research into "-omics" has boomed. Metabolomic research is increasing with the

possibility of uses seemingly endless. With the use of all of the "-omics" techniques, it is becoming easier to elucidate the functions of specific genes and to identify new genes involved in specific metabolic pathways. Metabolomics is allowing the detection of many new and known compounds in fast, simple and effective ways. At present there are limited papers which have independently generated lists of wine compounds using nuclear magnetic resonance (NMR) or mass spectroscopy (MS) (Skogerson et al., 2009). Recently, one study was able to separate over 40 different sugars and derivatives using small sample volumes of 50 µL with minimal work-up (Villas-Bôas et al., 2006), which has not been achieved before. This highlights that the use of metabolomic techniques may provide the methodology required for this to occur, thus quantifying all the known compounds as well as identifying any new compounds. However, many metabolomic techniques such as footprinting and fingerprinting (Table 1) may determine a profile of the wine without associating compounds to each peak in the profile; therefore in these circumstances, when new compounds are uncovered, techniques such as NMR and MS are required to identify the compounds (Fiehn, 2002).

The sensitivity of the method needed for analysis depends on which outcomes are required. There are different methods used in metabolomics, all of which have their uses within the field. As there are disagreements within the field on the definitions of each of these analyses (Kitteringham et al., 2009), this study will use the definitions described in Table 1.

Since metabolites can be defined as small molecular weight molecules present inside and/or outside a cell (Attwood et al., 2006), metabolomic studies can be classed into two distinct areas: the intrametabolome and the exometabolome. The intrametabolome consists of the metabolites found inside a cell, and the exometabolome is the combination of metabolites that a cell either excretes into the surroundings or fails to take up (Kell et al., 2005). The wine meta-metabolome is

the entirety of compounds found in wine, having originated from grapes, additives, yeast or bacteria. As a result of the multitude of inputs and influencing parameters, this meta-metabolome is essentially what gives each wine its distinctive characteristics.

Table 1: Description and definitions of different techniques used in metabolomics.

Technique	*Description*
Single compound analysis	The analysis of one predefined metabolite, where the analysis of the metabolome has been optimised for the compound (Trethewey, 2004).
Targeted analysis	Similar to a single compound analysis where the compounds being analysed are predefined, however a targeted analysis identifies multiple compounds (Trethewey, 2004). Targeted analysis is a term used in metabolomics, where the compounds being analysed are of a single class (Trethewey, 2004).
Metabolic profiling	Profiling of a specific set of predefined compounds or metabolites (Fiehn, 2002, Dunn and Ellis, 2005), forming a high complexity overview of the metabolites present (Trethewey, 2004, Dunn and Ellis, 2005). Usually examines a set of metabolites from a specific metabolic pathway (Dunn and Ellis, 2005), where multiple classes of compounds are investigated and the metabolites are identified. Generally, the sensitivity of the analytical technique has been optimised for these compounds prior to detection (Dunn and Ellis, 2005).
Metabolic footprinting	Non-targeted metabolomics of the exometabolome (Allen et al., 2003) which is more general than the previously explained techniques. Metabolic footprinting creates very complex profiles (Kell et al., 2005) of metabolites from the exometabolome (Villas-Bôas et al., 2006) using high-throughput methods. Within this technique, each of the metabolites found in the profile are identified (Kell et al., 2005).
Metabolic fingerprinting	Non-targeted metabolomics of the endometabolome (Raamsdonk et al., 2001) similar to metabolic footprinting. Allows the formation of very complex profiles (Trethewey, 2004, Dunn and Ellis, 2005) of the metabolites of multiple samples using high-throughput methods (Kell et al., 2005). The data are found quickly (Fiehn, 2002) and the identification of the metabolites is not necessarily always needed (Dunn and Ellis, 2005). The data are then run through a statistical program to find clustering between spectra to find similarities or differences between the organism, species or strain (Fiehn, 2002, Kell et al., 2005). This technique is not very sensitive and is used for fast identification or classification of different organisms or species.
Metabonomics	A term used for metabolic analysis in the medical industry. Metabonomics is the comparison of metabolite levels from an individual before and after being overcome by disease or changes due to therapeutic treatments (Dunn and Ellis, 2005, Constantinou et al., 2005).

Flavour compounds formed by *Saccharomyces cerevisiae*

One of the major contributors to the variation in the wine meta-metabolome is the yeast used in fermentation. The composition of a wine produced using one strain of yeast will have marked differences to that produced using another strain. Many compounds are produced in wine as by-products of the fermentation process. These include volatile compounds contributing to the flavour and aroma of the wine, and non-volatile compounds which contribute to the flavour, colour or mouthfeel of the wine (Figure 1). This thesis will be focussing on the volatile compounds found in the wine, which are produced by yeast, with emphasis on the most important aroma compounds. Although the genes involved in the pathways producing these aroma compounds are known, there are 1134 protein-coding genes in *S. cerevisiae* which no published data on the molecular or biological functions of these proteins (Christie et al., 2009). Therefore, it is possible that there are other proteins involved in these metabolic pathways, which have previously gone undetected.

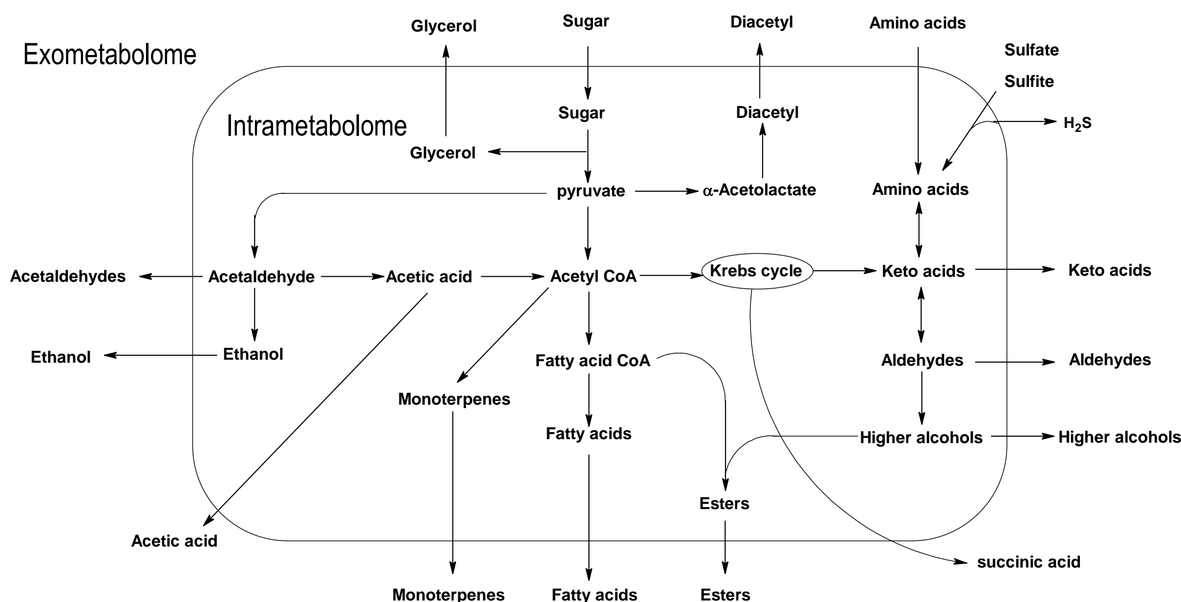

Figure 1: Overview of the metabolic pathways resulting in flavour-active compounds found in wine due to fermentation (adapted from (Swiegers et al., 2005))

In a recent study reconstructing the *S. cerevisiae* genome, it was shown that there are 1175 metabolic reactions taking place in yeast. Of the 584 metabolites formed, 121 are of the extracellular type (Förster et al., 2003). It was also found that there are fewer open reading frames (ORF) than there are reactions, thus implying that the isomerases and transferases are less specific in *S. cerevisiae* than in other organisms (Förster et al., 2003). These results suggest that alterations of genes involved in the formation of isomerases and transferases will in fact affect more than one pathway, creating a snowballing effect from one slight change. Thus, it could be hypothesised that overexpression of one key gene will give an increase in multiple beneficial aroma compounds. Studies in gene manipulation are becoming increasingly popular in wine science, even though usage of genetically modified yeast in winemaking is not approved in many countries. The results obtained from experiments such as this can, however, provide more information into the importance of specific genes and thereby guide research into non-recombinant strain selection and optimisation strategies.

The major metabolic pathway in fermentation is the breakdown of glucose into useable products and energy. This pathway, glycolysis or the Embden-Meyerhof pathway, stops at the formation of pyruvic acid, (Figure 2). Glycolysis is the major starting point for many of the flavour compounds found in wine (Swiegers et al., 2005). There are numerous steps involved in glycolysis thus providing many points for the alteration of catalysing enzymes to produce the greatest effect in one or several flavour compounds. However, past studies in model media have shown that overexpression of any single glycolytic gene will not affect the overall flux of the reaction (Schaaff et al., 1989). Although, it is possible that overexpression of more than one glycolytic gene, or a single non-glycolytic gene, may have an effect on the final concentration of products formed (Schaaff et al., 1989). From this backbone pathway, several other pathways arise; the simple pathways include formation of ethanol, glycerol, acetic acid and lactic acid (Figure 2) and the formation of acetoin and

2,3-butanediol (González et al., 2000), Figure 3. It is then from acetic acid, or acyl-CoA, that the other major pathways involved in creating the other flavour compounds arise (Figure 1).

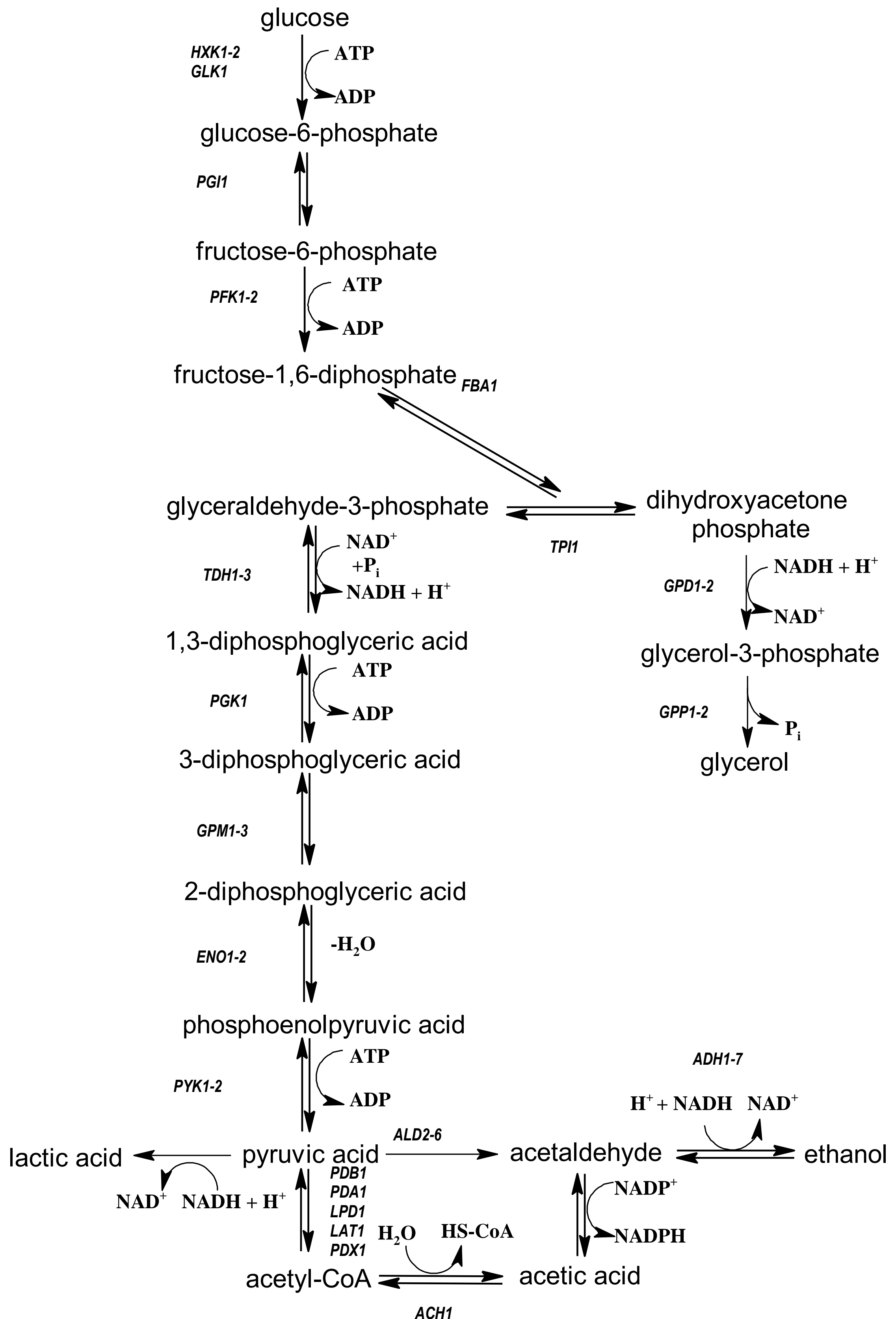

Figure 2: Pathway for glycolysis in *Saccharomyces cerevisiae* (Todar, 2008, Elliot and Elliot, 2005, Modig et al., 2002) where the genes involved in the reactions are shown in italics (Salusjarvi et al., 2008).

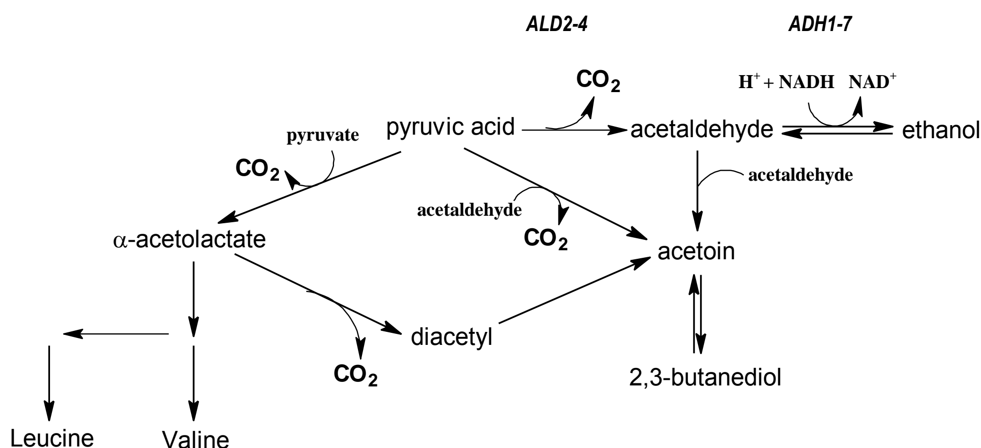

Figure 3 : Pathway for the formation of acetoin and 2,3-butanediol (González et al., 2000).

Although amino acid synthesis can occur *in vivo*, many amino acids are taken up by the cell as a source of nitrogen, thus creating a separate pathway from glycolysis. The breakdown of amino acids via the Ehrlich pathway (Hazelwood et al., 2008) (Figure 4) is responsible for aroma compounds such as higher alcohols (Sentheshanmuganathan, 1960, Hazelwood et al., 2008). The higher alcohols are formed through deamination by 2-oxogluterate forming the α-keto acid, then carboxylation occurs to form the aldehyde which can then be oxidised or reduced to form the carboxylic acid or the higher alcohol respectively (Sentheshanmuganathan, 1960, Hazelwood et al., 2008). Until 2006, the only genes in this pathway that had been well characterised were *BAT1* and *BAT2* (Schoondermark-Stolk et al., 2006). Since 2006, only *ARO10*, *AAD10* and *AAD14* have been examined in more detail in terms of the production of aroma compounds in wine (Vuralhan et al., 2005, Rossouw et al., 2008).

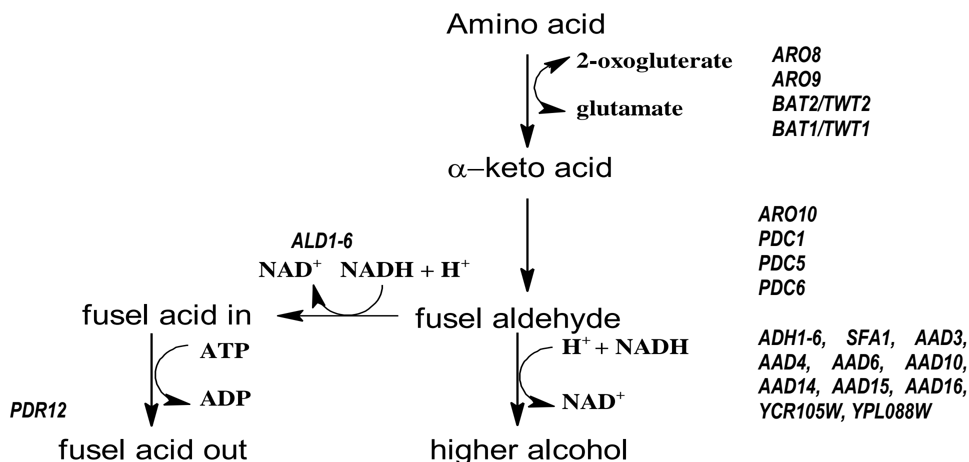

Figure 4: The Ehrlich pathway: formation of fusel acids and higher alcohols in *Saccharomyces cerevisiae* (Hazelwood et al., 2008).

Determining important fermentation aroma compounds

The important aroma compounds are considered those with an odour activity value (OAV) greater than 1 (Table 2). The OAV is the concentration of a compound found in the wine divided by the odour detection threshold (cited in Patton, 1964). The odour detection threshold can be determined in wine, water, an ethanol solution, or other media and is defined as the concentration of the compound in a specific medium where 50 % of the population are able to perceive the aroma by smell. These values can change depending on the medium in which they were determined, as the solvent polarity or aroma of other compounds can mask or enhance the intended compound's aroma. As a rule of thumb, if the OAV is greater than 1, then the aroma will be perceived, however if the OAV is considerably greater than 1, then the aroma may become overpowering and thus have a negative impact on the bouquet of the wine.

Table 2: Aroma compounds formed by *Saccharomyces cerevisiae* with OAV's greater than 1.

Compound	Aroma description	Odour threshold (μg/L)
Ethyl esters		
ethyl acetate	pineapple, fruity, solvent	7500
ethyl 2-methylpropanoate	sweet, rubber	15
ethyl 2-methylbutanoate	apple	18, 1
ethyl 3-methylbutanoate	fruit	3
ethyl butanoate	apple	20
ethyl hexanoate	apple peel, fruit	14, 5
ethyl octanoate	fruit, fat	2
ethyl decanoate	grape	200
ethyl lactate	fruit	14
ethyl dodecanoate	soapy, estery	-
ethyl propanoate	fruity	1800
Acetates		
3-methylbutyl acetate	banana	30
2-methylbutyl acetate	fruit	160
2-phenylethyl acetate	rose, honey, tobacco	250
hexyl acetate	fruit, herb	670
2-methylpropyl acetate	fruit, apple banana	1.6
Acids		
2-methylpropanoic acid	rancid, butter, cheese	2300
3-methylbutanoic acid	sweat, acid, rancid	33.4
acetic acid	sour	200000
2-methylbutanoic acid	cheese, sweaty	1500
butanoic acid	rancid, cheese, sweat	173
hexanoic acid	sweat	420
octanoic acid	sweat, cheese	500
decanoic acid	rancid, fat	1000, 8100
Alcohols		
benzyl alcohol	sweet, flower	900000
butanol	wine, fusel, spiritous	150000
2-methyl propanol	wine, solvent, bitter	40000
3-methyl butanol	whisky, malt, burnt	30000
2-methyl butanol	wine, onion	65000
2-phenyl ethanol	honey, spice, rose, lilac	14000, 10000
methionol	sweet potato	1000
n-propanol	alcohol, pungent	306000
2,3-butandiol	fruity	150000
hexanol	resin, flower, green	8000
Miscellaneous		
2,3-butanedione	butter	100

Aroma descriptors are from Flavornet (Acree and Arn, 2004), odour thresholds determined in ca. 10% ethanol (Francis and Newton 2005; Ferreira et al., 2000; Guth, 1997; Peinado et al., 2006; Peinado et al., 2004; Salo, 1970; Siebert et al., 2005).

Current Wine Metabolomics studies

Research into the metabolomics of wine had been occurring for many years before the term metabolomics was defined. Since many of the aroma compounds found in a wine arise as by-products of fermentation (Swiegers et al., 2005), any experiments examining the changes in aroma compounds in wine produced under different conditions can reasonably be considered as metabolomics. Previous studies have looked at viticultural effects on the aroma of wine, for example the grape variety (González et al., 2007), as well as oenological effects, including the strain of yeast used (Rojas et al., 2003, González et al., 2007), temperatures of fermentation (González et al., 2007, Molina et al., 2007), aeration (Rojas et al., 2001, Quilter et al., 2003), use of additives (Quilter et al., 2003) and nitrogen availability (Miller et al., 2007). The use of metabolomics will enable more of the aroma compounds to be discovered or quantified as well as helping pinpoint their origin. Therefore, with further research the ability to alter the sensory properties of a wine will be possible via the vineyard and/or winery (Skogerson et al., 2009). Processes which increase or decrease the quantities of beneficial aroma compounds in wine could be altered to optimise sensory impact (Skogerson et al., 2009).

The winemaking process can change the concentrations of certain aroma compounds in a wine compared with those concentrations found in the grapes, therefore some wines from the same batch of grapes can produce different aroma compounds depending on the winemaking procedures undertaken (Esti and Tamborra, 2006). Conversely, some compounds can remain the same throughout the winemaking process, thus providing a marker for the type of grape used in the wine (Esti and Tamborra, 2006, Piñeiro et al., 2006), including terpeniol, linalool and geraniol (Piñeiro et al., 2006). Terpene concentrations can stay constant throughout the fermentation process, however, they can be increased by maceration of solids and the release of glycosylated precursors and are decreased by heat and oxidation (Piñeiro et al., 2006). Cold soaking increases the amount

of terpenes and other compounds in the grapes (Piñeiro et al., 2006), however the extent to which the phenomenon occurs depends on the grapes being used.

In terms of the usage of the word “metabolomics”, and the use of multivariate analyses such as principle component analysis (PCA), partial least squares (PLS) and analysis of variance (ANOVA), there is little information on metabolic studies on the aroma profile of wines. Currently, the main metabolomic studies in wine tend to use amino acids, malic, succinic, citric and tartaric acids, and glycerol as discriminating compounds (Son et al., 2008, Soufleros et al., 2003, Skogerson et al., 2009). One example is a study on Greek wines, where wines with low concentrations of primary amino acids had glutamic acid, lysine and alanine as the major amino acids present (Soufleros et al., 2003). However, in wines with high concentrations of primary amino acids, the major amino acids included arginine and γ-amino butyric acid (Soufleros et al., 2003). In wines made from aromatic grapes such as Muscats, it has been shown that the major amino acids found in the resulting wine are arginine and γ-amino butyric acid (Soufleros et al., 2003). These studies do not give much information about the aroma of a wine, whereas metabolomics of grape varieties using these compounds will provide some insight into the expected aroma profile of the resulting wine. A high amino acid-producing grape should produce a wine with a high concentration of aroma compounds (Miller et al., 2007), since many amino acids are precursors for aroma compounds (Hazelwood et al., 2008).

Differentiation between grape varieties has been performed in various studies with the aid of multivariate analysis (Son et al., 2009). Differentiation between four grape varieties was found using metabolomics and PCA, however none of the compounds used in discrimination were aroma compounds (Son et al., 2009). Metabolic fingerprinting of different grape varieties sampled from different vintages and soil types has also shown that discrimination between vintages is possible

using soluble sugars, organic acids and amino acids (Pereira et al., 2006). On the other hand, discrimination between soils in which the grapes were grown was not possible (Pereira et al., 2006). These results coincided with results from Cabrita *et al.* showing that climatic effects are more important in determining the metabolites in grapes than the soil type (Pereira et al., 2006, Cabrita et al., 2007). A study looking at wine quality and product origin, distinguishing between Korean Campbell early; Australian, French and Californian Cabernet Sauvignon; and Australian Shiraz grapes was possible using multivariate analysis PCA and partial least squares-discriminant analysis (PLS-DA) (Son et al., 2008), showing how wines are able to be grouped according to region and grape variety.

Summing up these results, it is obvious that the wines produced in different vintages from the same vineyard and grape variety can show different aroma profiles due to many aroma compounds being formed from amino acids, which in turn will vary in concentration from vintage to vintage. This hypothesis is supported by results from a study where it was shown that the vintage of grapes, rather than the region or variety, is more important to the free aroma compounds found in wine (Cabrita et al., 2007), mimicking the results obtained from grape variety discrimination studies (Pereira et al., 2006, Cabrita et al., 2007).

One aroma-related metabolomic study was able to determine the aroma compounds which give Lychee and Gewürztraminer wine their similarity to canned lychees (Ong and Acree, 1999). The compounds determined by OAV as the main odorants were *cis*-rose oxide, linalool, 2-phenylethanol, geraniol and ethyl hexanoate (Ong and Acree, 1999). Two studies that stand out in metabolomic research illustrate the possibility of using predictive models to distinguish the “body” or viscous mouthfeel of a wine (Skogerson et al., 2009, Rochfort et al., 2010). With further research it could be possible to perform metabolomic footprinting with multivariate analysis to determine the

sensory components of a wine instead of using expensive, time consuming sensory tests (Skogerson et al., 2009, Rochfort et al., 2010). The Rossouw *et al.* study showed that there is a negative correlation between tartrate concentration and the perceived wine body. Tartrate has never previously been noted as having an influence on wine body (Skogerson et al., 2009). These results prove that metabolomics will help the industry determine new compounds associated with wine quality, which have previously gone undetected.

Effect of the medium

It is very important to interpret the data found in various studies according to the medium being used. The medium in which fermentation takes place can greatly affect the final products in the resulting wine. It has been shown that a wine produced from a grape juice medium and that produced in a model medium will not produce the same compounds even though they are produced in the same manner (Boulton, 1998). In a study determining the breakdown of esters under different storage conditions, it was seen that the rate of decay and the breakdown trends differed greatly between the model system and the real system (Forrester, 2009). These results showed that the complexity of the total compounds in the medium affect the behavior of the targeted compounds. Therefore, fermentation studies performed in model media may show significantly different results compared with real wine media. For aroma studies, real wine systems should be used to discover how yeast will behave in the winery. However, as explained earlier, it has been shown that the vintage greatly affects the composition of the grapes used in winemaking (Pereira et al., 2006), therefore, even if using grapes from the same vineyard over a period of years, the wine will be compositionally different and hence the study cannot be reproduced. This is where use of a model medium is beneficial, as the results obtained are reproducible.

Two different model media have been designed which produce similar results to a real wine system. Henschke and Jiranek, defined the general composition of grape juice and formulated a chemically-defined grape juice medium (CDGJM) (Henschke and Jiranek, 1993), Table 3. CDGJM has been utilized in multiple studies and is a good representation of a real wine system (Gardner et al., 2005, Jiranek et al., 1995, Harris et al., 2008). The use of the CDGJM improves experimental reproducibility, through nullifying the change in composition of grapes due to different vintages. Rossouw et al. defined another model medium, MS300 (Table 3), which also showed results similar to real wine systems (Rossouw and Bauer, 2009). Comparing the gene expression shown in MS300 and a real wine, it was seen that the transcripts present did not change to a significant degree between the two media (Rossouw and Bauer, 2009). The main differences occurred for transport activities, although the metabolic gene expressions were not affected greatly (Rossouw and Bauer, 2009). Thus, research initially performed in a model medium appears to be a good indicator of the outcome in the real wine, but the extent to which these results will hold true depends on the concentration of the compounds initially present in the fermentation medium. Therefore, secondary experiments in real juice should be carried out to confirm the results obtained in the model system. Better still, the use of a real wine system in a full scale winery fermentation would be advantageous to confirm results found in CDGJM or MS300, as explained by Pena-Castillo *et. al.* (Pena-Castillo and Hughes, 2007):

“Achieving a full catalogue of yeast gene functions may require a greater focus on the life of yeast outside the laboratory”

Table 3: Composition of model media for MS300 (Rossouw and Bauer, 2009) and CDGJM (Henschke and Jiranek, 1993). (Assimible nitrogen was calculated as total nitrogen content excluding that from proline)

Component	MS300	CDGJM	Component	MS300	CDGJM (mg/L)
pH (NaOH)	3.3	3.2-3.5	**Vitamins**	(mg/L)	(mg/L)
			Myo-inositol	20	100
Sugars	(g/L)	(g/L)	Nicotinic acid	2	2
Glucose	125	100	Calcium pantothenate	1.5	1
Fructose	125	100	Thiamine HCl	0.25	0.5
			Pyrixodine HCl	0.25	2
Amino acids	(mg/L)	(mg/L)	Biotin	0.003	0.125
Alanine	145.3	100	p-amino benzoic acid	-	0.2
Arginine	374.4	750	Riboflavin	-	0.2
Asparagine	-	150	Folic acid	-	0.2
Aspartic acid	44.5	350			
Cysteine	13.1	-	**Mineral salts**	(mg/L)	(mg/L)
Glutamic acid	120.4	500	K_2SO_4	500	-
Glutamine	505.3	200	NaCl	200	-
Glycine	18.3	50	$MnSO_4.H_2O$	4	-
Histidine	32.7	150	$ZnSO_4$	4	-
Isoleucine	32.7	200	$CuSO_4.5H_2O$	1	-
Leucine	48.4	300	KI	1	-
Lysine	17.0	250	$CoCl_2.6H_2O$	0.4	-
Methionine	31.4	150	KH_2PO_4	750	1140
Phenylalanine	37.9	150	$MgSO_4.7H_2O$	250	1230
Proline	612.6	500	$CaCl_2.2H_2O$	155	440
Serine	78.5	400	H_3BO_3	1	0.0057
Threonine	759.3	350	$NaMoO_4.2H_2O$	1	0.0242
Tryptophan	179.3	100	$Co(NO_3)_2.6H_2O$	-	0.0291
Tyrosine	18.3	20	$CuCl_2$	-	0.0136
Valine	44.5	200	$FeCl_2$	-	0.0320
Total amino acids	3113.9	4870	$ZnCl_2$	-	0.1355
Assimilable nitrogen (mg N/L)	419	723	KIO_3	-	0.0108
Ammonium chloride	460	100	K tartrate	-	5000
Total Assimilable Nitrogen (mg N/L)	537	749	L-Malic acid	-	3000
			Citric acid	-	200
			$MnCl_2.4H_2O$	-	0.1982

Yeast overexpression studies

Overexpression libraries are a new field for wine *S. cerevisiae* and, to our knowledge, no large-scale screens of overexpression libraries in wine have been previously performed. In many earlier reports, individual genes or groups of genes have been overexpressed and the aroma profiles studied (Table 4). The problem with these studies is that many examined the effects of overexpression of a gene on a few key compounds. However, the fermentations were produced in different media and the overexpression of the genes was performed with different yeasts. As a result of these variations, it is hard to compare the results and explain any unexpected results.

Table 4: A sample of overexpression studies performed on wine yeast to identify changes in aroma compounds.

Reaction/protein classification	Gene	Media	Results
AMINO ACID METABOLISM			
Amino acid→α-keto acid	BAT2/TWT2	Beer	Expression levels were higher than *bat1*(Saerens et al., 2008)
		Model	No correlation between expected results and actual results (Rossouw et al., 2008)
		Wine	Increase in total esters but decrease in isoamyl acetate; increases in some higher alcohols and decrease in others (Lilly et al., 2006)
	BAT1/TWT1	Beer	Correlation between *bat1* expression levels and higher alcohol end concentration (Saerens et al., 2008)
		Model	Increase in higher alcohols and acids (Rossouw et al., 2008)
		Wine	Overall decrease in esters but an increase in isoamyl acetate; increase in some higher alcohol and decrease in others (Lilly et al., 2006)
α-keto acid→fusel aldehyde	ARO10	Model	When grown on glucose medium no overexpression was seen; when grown with ethanol as the carbon source, overexpression was seen showing a decrease in the α-keto acids. (Vuralhan et al., 2005)
Fusel aldehyde→higher alcohol	AAD10	Model	Increase in ethyl esters and higher acids and alcohols; thus possible relation in production of higher alcohols and esters (Rossouw et al., 2008)
	AAD14	Model	Increase in ethyl esters and higher acids and alcohols; thus possible relation in production of higher alcohols and esters (Rossouw et al., 2008)
GLYCOLYSIS			

Dihydroxyacetone phosphate →glycerol-3-phosphate	GPD1	Model	Deletion of *ald6* decreases acetate levels, and overexpression of *gpd1* increases glycerol production; high levels of acetoin, butanediol and acetaldehyde; decreased levels of ethanol (Cambon et al., 2006)
Acetaldehyde→ethanol	ADH1	Beer	Highly expressed gene as expected due to involvement in ethanol production (Saerens et al., 2008)
OTHER			
Acetyl CoA + Higher Alcohol→acetate ester	ATF1	Beer	Positive correlation between ***expression levels*** and concentration of higher acetate esters (Saerens et al., 2008)
		Model	Significantly increases the concentrations of ethyl acetate and isoamyl acetate and all acetate esters studied and significantly decreases the concentration of isoamyl alcohol and other alcohols studied (Verstrepen et al., 2003)
		Wine	Increase in acetate esters and some ethyl esters; decrease in alcohols (Lilly et al., 2000)
	ATF2	Beer	Positive correlation between ***expression levels*** and concentration of higher acetate esters (Saerens et al., 2008)
		Model	Increases the concentrations of ethyl acetate and isoamyl acetate and all acetate esters studied and other alcohols studied (Verstrepen et al., 2003)
Acyl-CoA+ ethanol→ethyl ester	EHT1	Beer	Negative correlation between expression levels and ethyl octanoate and decanoate; has both esterase and biosynthesis activity, thus the hydrolytic activity is seen as most predominant *in vivo* (Saerens et al., 2008)
	EEB1	Beer	Most important genes of the two for ethyl ester production; no correlation with expression and *eeb1*, therefore shows that this gene is not the only one involved in final concentrations of the ethyl esters(Saerens et al., 2008)
Uptakes leucine, isoleucine and valine	BAP2	Beer	Higher final expression levels than bat genes, thus uptake of amino acid is important during fermentation (Saerens et al., 2008)
Isoamyl acetate esterase	IAH1	Beer	No correlation between expression and acetate esters (Saerens et al., 2008)
Mitochondrial branched chain Amino acid biosynthesiser	ILV5	Beer	Saw no correlation under the conditions analysed (Saerens et al., 2008)
Carnitine acetyltransferase	CAT2-mit	Model	Reduces the concentration of esters i.e. Ethyl acetate and isoamyl acetate (Cordente et al., 2007)
	CAT2-cyt	Model	Reduces the concentration of esters i.e. Ethyl acetate and isoamyl acetate (Cordente et al., 2007)
Putative Acetyltransferase	YMR210W	Model	No change in aroma compounds studies (Rossouw et al., 2008)
Acetyl CoA Synthetase	ACS1	Model	Increase in acetate esters and butanol and butyric acid; decrease in ethyl caprate (Rossouw et al., 2008)
	TRX2	Wine	Increases in certain acetate esters concentrations,

			excluding ethyl acetate and a drop in concentration of isoamyl alcohol (Gomez-Pastor et al., 2010)
	EXG/BGL1	Wine	Increase in terpenes and alcohols (Gil et al., 2005)
Catalyses the first step in amino Acid synthesis	ILV5	Beer	Decrease in pyruvate and acetate; increase in isobutanol, amylalcohol and isoamyl acetate i.e. The compounds formed from isoleucine/valine (Omura, 2008)

Rossouw et al. have performed many transcriptomic and metabolomic studies over recent years. In one particular study, five yeast strains' transcript expressions during fermentation were analysed and aroma profiling was performed (Rossouw et al., 2008). Using multivariate analyses, multiple genes were considered significant to the change in the aroma profile, however, only five of these genes were analysed further. The overexpression of five genes was analysed and compared to wildtype VIN13 fermented in an MS300 synthetic medium. The increase or decrease in concentrations of aroma compounds is shown in (Figure 4), however the extent of change is not depicted. Comparison of the changes in concentrations of aroma compounds in the overexpression strains and wildtype VIN13 showed a close relationship between expected results, from multivariate analyses, and the real results, obtained from transcriptomics and exo-metabolomic studies (Rossouw et al., 2008). Of the five genes studied, only ACS1 was seen to be less significantly correlated with the expected results, however 8 out of 13 compounds did vary in the expected direction of change, just not to the extent predicted (Rossouw et al., 2008). The Rossouw *et. al.* study is the opposite of what will be investigated in this thesis, where metabolic profiling of an overexpression library will occur and the multivariate results will be used to predict the aroma profiles formed from currently available strains of yeast.

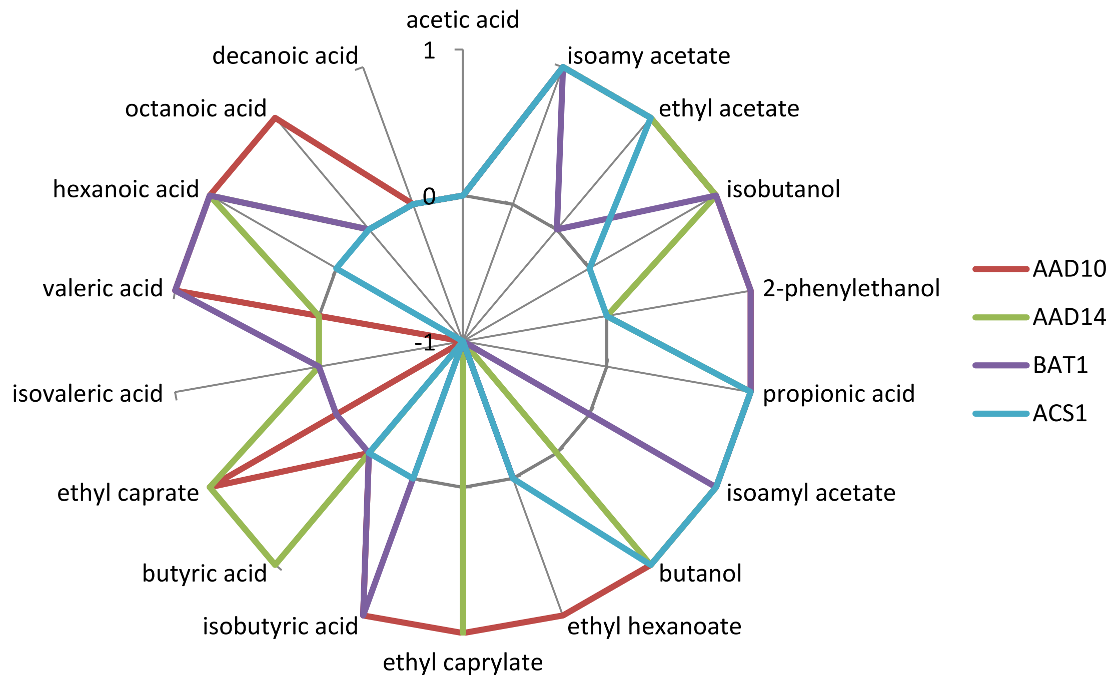

Figure 4: Comparison of the concentrations of aroma compounds in overexpression yeast compared with VIN13, where -1 represents a significant decrease in concentration and 1 represents a significant increase in concentration. (adapted from (Rossouw et al., 2008)).

Analytical methods

For high throughput studies, especially a screen of over 1500 samples, the analytical method needs to be as fast as possible and therefore automated steps are very beneficial. The steps involved in an analytical method include the extraction of the compounds, sample preparation, injection of the sample into a gas chromatogram, the running of the chromatographic method and the analysis of the data. The extraction of the sample can be performed through one of a few different methods:

- Liquid liquid extraction (LLE)
- Solid phase micro extraction head space or immersion (SPME)
- Solid phase extraction (SPE)
- Stir bar sorptive extraction (SBSE)

Environmentally friendly chemistry is becoming more of a focus over past years. Tobiszewski mentions the acronym of Green Analytical Chemistry (GAC) in a recent article, giving focus to environmentally friendly analytical chemistry and how the sample preparation step can be the most environmentally detrimental part of the process (Tobiszewski and Namieśnik, 2012, Tobiszewski et al., 2009). Liquid-liquid extraction is one of the analytical sample preparations, which can use large amounts of solvent. This process also requires a large sample size and is a timely process. Performing LLE on upwards of 1500 samples would require large ferment volumes, which is impractical for the size of the screen and would require a sizeable amount of solvents, which also goes against the new trend in GAC.

Of the aformentioned four methods, SPME is the most automated method. The advantages of SPME include automated extraction and desorption into the GC injection port. The only non-

automated part of the method is the sample preparation and the data analysis. SPME is quite often used in analysis of aroma compounds in wine (Bonino et al., 2003, De Calle García et al., 1998, Díaz-Maroto et al., 2004, Risticevic et al., 2010, Siebert et al., 2005, Valduga et al., 2010, Vas et al., 1998). SPME was designed in 1990 by Professor Janusz Pawliszyn (Arthur and Pawliszyn, 1990) to allow for automation of the similar technique SPE (Arthur and Pawliszyn, 1990). Both SPE and SPME are based on similar concepts of adsorbing the desired compounds onto a solid phase and then desorbing the compounds either by means of thermal desorption or being washed off by a solvent. The benefits of these methods is that there can be a lack for the need of solvents and there is less time required in the preparation of samples (Arthur and Pawliszyn, 1990). The advantage of SPME over SPE is that the adsobent is a fused silica fibre whereas the SPE cartridges are made from plastic which can interfere with the analytes being analysed (Arthur and Pawliszyn, 1990); an SPME fibre can be desorbed in the inlet of a GC using a autosampler; there is less sample preparation time. Originally the fibre was made of fused silica and placed inside a normal syringe, replacing the metal plunger wire (Arthur and Pawliszyn, 1990). These days the fibres have evolved to be more specific for different techniques, being made of a fused silica fibre coated with one/ or a combination of the many different coatings available, which is bonded to a metal plunger and the fibre protected by a flexible metal sleeve. Specific fibre holders are also available to allow for the use of SPME in auto samplers.

The chromatographic method for a screen also needs to be as fast as possible, however there must be a compromise between the speed of the method as well as the resolution of the compounds to be analysed. To be most accurate, the best method would be as short as possible with resolution of all compounds, however it may not be possible, or feasible to do this.

Solid Phase Micro-Extraction (SPME)

SPME is a good analytical tool; however the fibres being used degrade over time. Rebiere et al. (2010) discussed a method that they used which involved the use of four different internal standards; two of which were used for analytical standards and all four used to determine the degradation of the fibre. The internal standards were used to form a Shewhart chart based on the methods described in the Miller and Miller publication (2005).

There are two different types of SPME fibres that can be purchased. These are fibres which adsorb the analytes of interest and those which absorb these analytes as cited in (Luks-Betlej et al., 2001). The two different types of fibres have their advantages and disadvantages. The adsorbent type consists of a crystalline structure which allows the adsorption of the sample as cited in (Luks-Betlej et al., 2001). The absorbent type is a liquid phase, which allows for absorption of the analytes. The adsorbent phases can only adsorb a certain amount of molecules and thus there is a limiting effect to this type of phase. Displacement of weaker affinity compounds can take place by the compounds with higher affinities which usually occurs with higher concentration samples or with longer extraction times (Risticevic et al., 2010). The advantage of these types of coatings is that they can be more specific for certain types of compounds and more sensitive to the compounds of interest. These solid-phase coatings can be used in preference to the absorption type coatings as the extraction conditions can be manipulated to find the optimum conditions for the desired compounds. They also allow for the ability to use less concentrated samples and shorter extraction times (Risticevic et al., 2010).

Summary of research aims

The aroma of a wine is very important to the overall quality of a wine and research into the fermentation bouquet is of great interest in the industry today. This project aims to link the sensory characteristics of a wine to the genotype of the yeast used in fermentation. Using an overexpression library containing 1500 overexpression clones, we were able to simplify the ambitious task of quantifying 38 of the most important aroma compounds formed in the fermentation bouquet.

In 2005 Seibert et al designed a very useful method for the analysis of fermentation aroma compounds. The problem now is that the fibre they used is now no longer commercially available. Therefore to be able to quantify the 38 aroma compounds using SPME, it was first necessary to determine the best commercially available fibre for these experiments. Currently, there are five different SPME fibres available, which are recommended for analysis of low molecular weight aroma compounds. These fibres are the:

- white – 85 µm polyacrylate (PA)
- Blue – 65 µm divinylbenzene/ Polydimethylsiloxane (DVB/PDMS)
- Grey - 50/30 µm divinylbenzene/carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS)
- Black - Carboxen/Polydimethylsiloxane (CAR/PDMS)
- Red – 100 µm Polydimethylsiloxane

Due to the fact that the original fibre was no longer available a new fibre needed to be established for the optimum extraction of the desired aroma compounds and therefore a new method was needed to be developed and validated. This thesis details this newly developed method and equation for choosing a fibre for volatile analysis, along with a detailed study on the currently

available fibres recommended for these studies, which may be used to assist future researchers. The conditions for the best fibre were then optimised and a high throughput Headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS) method for the analysis of 38 aroma compounds was developed and validated.

The overexpression library to be used in these experiments was encoded in a 1500 E-coli plasmid library, in a 2 micron-based *LEU2* vector. The only restriction with this library is the *Leu2*Δ mutation required in the transformant strain and the need for a fermentation media lacking leucine. This is a point which needs to be justified with the expression of compounds such as isoamyl alcohol and isoamyl acetate whose precursors are Leucine. This explains the differences in aroma compounds produced by the Leu-yeast strain and the parental strain in various juice media and chemically defined grape juice media. This library was also contained in an E-coli host and thus preparation of the library into a wine yeast had to occur as well as thorough testing of the transformed library.

Since a model media was used for this study, confirmation of the point during fermentation when the production of aroma compounds occurs in a chemically defined grape juice media (CDGJM) was discovered. This was then compared to previous literature, which was conducted with real juice and another chemically defined media fermentations.

Once the method was validated and the library was ready for use, the major aim of the study could be conducted. High throughput fermentations of an overexpression library and a range of commercially available yeasts were sampled over a period of 5 time-points during fermentation. Analysis of the resulting fermentations using the newly designed high throughput analytical method for differences in the 38 aroma compounds was the achieved. The results were then statistically

analysed to discover a new phenomenon, which may be helpful for future research with yeast overexpression libraries.

Synthesis of the deuterated compounds which could be used as potential internal standards was also performed. However, whilst these did not end up being used in this investigation due to the final discovery and time restrictions, they are included here in the final chapter for future researchers.

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A comparative study on the sensitivity of different solid-phase micro extraction (SPME) fibre coatings for the analysis of fermentation bouquet compounds

There are numerous techniques currently employed within the analytical chemistry field to quantify and characterise compounds found in wine. One of the most beneficial techniques used for the analysis of aroma compounds found in wines is to use gas chromatography mass spectrometry (GC-MS). However, there are also many different techniques employed to extract these aroma compounds from the wine prior to analysis using the GC-MS. A common practise since the 1990s is to use solid-phase micro-extraction (SPME) for extraction. This technique is very powerful as it can be fully automated after the sample preparation step and is therefore widely used in high throughput experiments. SPME uses a silica fibre coated in an adsorbent or absorbent compound in various combinations, ratios and sizes. Due to the sudden popularity of this technique, there are a number of different fibres currently commercially available. Moreover, there have also been some previously widely used fibres that have been discontinued and new fibres developed to replace them. Consequently, analytical methods exploiting these new fibres are often being updated.

This chapter compares five different commercially available SPME fibres, which have been highly utilised for the analysis of volatile compounds in wine as demonstrated by numerous publications within the literature. The five fibres were thoroughly evaluated and then narrowed down to the two best fibres based on a novel scoring system developed herein and to be used when selecting a fibre for use in wine studies. We were able to recommend two fibres as being the best for the analysis of a large library of fermentation bouquet compounds due to their overall ability to extract the compounds of interest as well as taking into consideration peak symmetry and sensitivity. This chapter was published in the *Australian Journal of Grape and Wine Research* in 2014.

A comparative study on the sensitivity of different solid-phase micro extraction (SPME) fibre coatings for the analysis of fermentation bouquet compounds

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Author Contributors:

Name of principle author (PhD Candidate): Jade Haggerty

Contribution to the paper: Designed and performed experiments, analysed data and interpreted data, and drafted/constructed manuscript.

Name of Co-Author: Paul Bowyer

Contribution to the paper: Oversaw the experimental design and aided in the drafting and construction of the manuscript.

Name of Co-Author: Vladimir Jiranek

Contribution to the paper: Oversaw the experimental design and aided in the drafting and construction of the manuscript.

Name of Co-Author: Dennis Taylor

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments, aided in the drafting and construction of the manuscript and submitted the manuscript as corresponding author.

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Optimisation and validation of a high-throughput semi-quantitative solid-phase microextraction method for analysis of fermentation aroma compounds in metabolomic screening studies of wines

Chapter two of the thesis detailed a comparative study on the sensitivity of solid-phase microextraction fibre coatings for the analysis of fermentation bouquet compounds. This study allowed us to narrow down the possible fibre selection to two solid-phase microextraction (SPME) fibres which performed the best when quantifying a large set of aroma compounds. This chapter details the next step in developing a robust metabolomic screening method for volatile fermentation bouquet compounds, which was to optimise and validate the analytical parameters associated with the SPME method. The best fibre was chosen based on observed sensitivity and overall ability to extract the compounds of interest.

The best fibre was observed to be the 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre and the semi-quantitative, high throughput, headspace solid-phase micro-extraction gas chromatography mass spectrometry (HS-SPME GC-MS) method needed to be designed and validated. Initially two internal standards (IS) were used to create standard curves in three different wine media. The two internal standards were 1-octanol and methyl nonanoate. At the end of the study it was found that the majority of the 34 volatile aroma standards being analysed for had best coefficients of determination for their standard curves with 1-octanol. The few which showed the best correlation to methyl nonanoate also portrayed useable curves with only slightly lower coefficients of determination when compared with 1-octanol. Therefore it was decided that a single IS would suffice for a high throughput screening method. The method was validated in three different media; bag-in-box white wine, a model wine and a fermentation in chemically defined grape juice media lacking leucine (CDGJM-Leu) using the strain of yeast to be used in the final metabolic screen (Chapter 5). The three media showed very similar standard curves for each compound of interest; showing repeatability of results in media with similar, but not exact, matrices. This result means that slight variations in the final fermentations produced by the overexpression library fermentations within the screening study, will not greatly affect the overall outcome and that the validity of this method for a high throughput screening method is justified. The optimised method described herein provides adequate results for rapid comparison screening of white wines, allowing for the narrowing down of results and data sets before undertaking more quantitative studies, therefore reducing time and costs associated with large high-throughput metabolomic

studies of wines. This chapter was published in the *Australian Journal of Grape and Wine Research* in 2016.

Optimisation and validation of a high-throughput semi-quantitative solid-phase microextraction method for analysis of fermentation aroma compounds in metabolomic screening studies of wines

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Author Contributors:

Name of principle author (PhD Candidate): Jade Haggerty

Contribution to the paper: Designed and performed experiments, analysed data and interpreted data, and drafted/constructed manuscript.

Name of Co-Author: Paul Bowyer

Contribution to the paper: Oversaw the experimental design and aided in the drafting and construction of the manuscript.

Name of Co-Author: Vladimir Jiranek

Contribution to the paper: Oversaw the experimental design and aided in the drafting and construction of the manuscript.

Name of Co-Author: Dennis Taylor

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments, aided in the drafting and construction of the manuscript and submitted the manuscript as corresponding author.

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Monitoring Volatile Aroma Compounds During Fermentation in Chemically Defined Grape Juice Medium

Model media are frequently used to study wine fermentation outcomes as they are considered an acceptable and reproducible representation of real juices. Identifying and quantifying the progression of the aroma compounds formed during fermentation is of importance for studies aimed at tailoring winemaking outcomes. The progression of the formation of a range of important aroma compounds in the 'fermentation bouquet' within real wine and model media (MS300) has been previously studied, however to our knowledge the progression of these compounds within a chemically defined grape juice media (CDGJM) has never been studied or in media lacking certain amino acids. Leucine is an essential amino acid, which in the past has always been added to model media to mimic a real juice solution. The first major aspect of this work was to evaluate the progression of a library of 34 common aroma compounds formed during fermentation using a leucine-requiring wine strain derivative of *S. cerevisiae* bearing the overexpression library platform plasmid and grown in a CDGJM-Leu media. The second important aspect was to show that under the conditions within this study that the progression of all of the compounds of interest would behave in a similar manner to previously reported studies in other model media and in real juice fermentations. Duplicate fermentations were conducted in CDGJM-Leu using the isoC9d ΔLeu + pGP564 yeast. Daily samples were taken from each fermentation in duplicate, giving a total of 4 samples to be analysed by headspace solid-phase micro-extraction gas chromatography mass spectrometry (HS-SPME GC-MS). After careful analysis of the literature and comparison with the data found herein, it was discovered that fermentations using CDGJM-Leu using the isoC9d ΔLeu + pGP564 yeast will show similar trends in the formation of aroma compounds as in a conventional ferment, or a ferment in MS300. These results are with the exception of the compounds relating to the biosynthesis and metabolism of leucine.

The findings herein will be very useful for future research, since model media are very important to use in laboratory wine studies due to the ability to reproduce results across many seasons and years. It is also important to note that our findings confirm the likely utility of this system for evaluating the importance of overexpression of specific genes in aroma compound production This chapter was accepted for publication in the *American Journal of Enology and Viticulture* in late 2015.

Monitoring volatile aroma compounds during fermentation in a chemically defined grape juice medium deficient in leucine

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Author Contributors:

Name of principle author (PhD Candidate): Jade Haggerty

Contribution to the paper: Designed and performed experiments, analysed data and interpreted data, and drafted/constructed manuscript.

Name of Co-Author: Dennis Taylor

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments and aided in the drafting and construction of the manuscript.

Name of Co-Author: Vladimir Jiranek

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments, aided in the drafting and construction of the manuscript and submitted the manuscript as corresponding author.

Monitoring Volatile Aroma Compounds During Fermentation in a Chemically Defined Grape Juice Medium Deficient in Leucine

Jade J. Haggerty[1], Dennis K. Taylor[1], Vladimir Jiranek[1]*

Author affiliations

[1] School of Agriculture, Food & Wine, The University of Adelaide, Waite campus, PMB 1, Glen Osmond, 5064, Australia.

*__Corresponding author__

Prof Vladimir Jiranek, email vladimir.jiranek@adelaide.edu.au

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Short version of title

Monitoring Aroma Compounds During Fermentation

21 **Abstract:**

22 Model media are frequently used to study wine fermentation outcomes as they are considered
23 an acceptable and reproducible representation of real juices. Identifying and quantifying the
24 progression of the aroma compounds formed during fermentation is of importance for studies
25 aimed at tailoring winemaking outcomes. Overexpression libraries of the yeast
26 *Saccharomyces cerevisiae* can have utility in determining the impact of the overexpression of
27 specific genes. Recently, an *S. cerevisiae* overexpression library has been reported that
28 encompasses a plasmid-borne construct, which utilizes a leucine selectable marker to
29 differentiate between yeast that contain the plasmid and those that do not. As such these
30 experiments with this library require a leucine deficient fermentation medium. Aroma
31 progression studies have been performed in real juice and in an MS300 model media,
32 however to date none have been performed in chemically defined grape juice media
33 (CDGJM) or in media lacking certain amino acids. This study reports on the progression of a
34 library of 34 oenologically relevant aroma compounds formed during fermentation using a
35 leucine-requiring wine strain derivative of *S. cerevisiae* bearing the overexpression library
36 platform plasmid and grown in a CDGJM-Leu media. The results indicate that the production
37 and accumulation of all 34 aroma compounds followed similar progression trends to that
38 found in previous studies with MS300 and juice exploiting wild type yeast. This is with the
39 exception of the compounds associated with pathways connected to the biosynthesis and
40 metabolism of leucine. The findings confirm the likely utility of this system for evaluating
41 the importance of overexpression of specific genes in aroma compound production.

42

43 **Keywords:** *Saccharomyces cerevisiae*; CDGJM-Leu; aroma compounds; fermentation;
44 leucine; overexpression library

45

46 **Introduction**

47 Given that the aroma of a wine is very important to the perceived overall quality, numerous
48 studies have focused on ways of improving the fermentation bouquet; the aroma compounds
49 formed by the yeast during fermentation. The use of model media for these experiments can
50 improve experimental reproducibility and maximize the clarity of the data, through nullifying
51 the known change in grape composition over different vintages (Pereira et al. 2006), grape
52 varieties (Son et al. 2009, Son et al. 2008), location and terroir (Son et al. 2008). One
53 suggested way to increase the accumulation of aroma compounds in a wine is to overexpress
54 yeast genes that are involved in specific metabolic pathways and analyze the aroma
55 compounds formed as a result. Jones et al. (2008) developed an overexpression library for *S.*
56 *cerevisiae* comprising some 1,500 plasmid-borne fragments each with 3-5 genes, which as a
57 tiled arrangement span the entire genome. The benefit of this library is that the yeast genes
58 are under the control of their native promoters as opposed to the *GAL1/10* promotor driven
59 constructs of previous libraries (Sopko et al. 2006). The latter fusion constructs are
60 unsuitable for wine-like fermentation conditions since the high glucose environment
61 represses the *GAL1/10* promoter. As well as avoiding this issue, the tiled library (Jones et al.
62 2008) can be introduced into any strain background, wine derivatives being preferred. The
63 recipient strain must however be deficient in leucine biosynthesis to accommodate plasmid
64 retention via a *LEU2* selectable marker. This is necessary since *LEU2* is the gene encoding
65 one of the four key enzymes in the metabolic pathway for leucine biosynthesis catalyzing
66 stepwise formation of α-ketoisocaproate from α-ketoisovalerate. Use of the library also

67 requires a leucine-deficient medium to be used. Since grape juices often contain leucine,
68 (Bell and Henschke 2005), defined media from which leucine can be explicitly excluded, are
69 a useful alternative.
70
71 Studies on the progression of aroma compounds during fermentation have been performed in
72 real juices (Callejón et al. 2012, Mallouchos et al. 2002, Vianna and Ebeler 2001, Zhang et
73 al. 2011) and in the model media MS300 (Molina et al. 2007). Both model media (MS300
74 and chemically defined grape juice media (CDGJM)) have been extensively utilized to mimic
75 real juice fermentations and usually display excellent agreement with results found from real
76 juice ferments in studies analyzing amino acid utilization and yeast kinetics (Gardner et al.
77 2005, Harris et al. 2008, Henschke and Jiranek 1993, Jiranek et al. 1995, Molina et al. 2007,
78 Rossouw and Bauer 2009, Govender et al. 2011, Kondratiene et al. 2003, Martínez et al.
79 2014, Varela et al. 2012, Walker et al. 2014). Surprisingly, there have been no detailed
80 reports on the evolution of aroma compounds formed or consumed during fermentation in the
81 CDGJM or in media lacking certain amino acids. This study reports for the first time on the
82 progression of the production and accumulation of 34 aroma compounds during fermentation
83 in a defined medium (CDGJM-Leu; i.e. lacking leucine). The strain used was a wine strain
84 derivative bearing the platform plasmid of the tiled array overexpression library (Jones et al.
85 2008). Findings are compared with those previously reported for wine and MS300 media.
86 Although these two media both mimic fermentations and results in grape juices, their
87 composition is fairly different, most notably the CDGJM contains ca. 60% more total amino
88 acids and the MS300 utilizes assimilable nitrogen instead to make up the difference in
89 nitrogen availability (Supplemental Table 1). It should be noted that the differences in the
90 availability of nitrogen in each media could ultimately effect the production of aroma

91 compounds formed. The findings reported here demonstrate that the CDGJM, wine strain
92 derivative and tiled-array plasmid combination represents a viable platform for discovery of
93 genes impacting on yeast aroma compound production under wine-relevant conditions.
94
95
96 **Materials and Methods**
97 **Media**
98 Minimal drop-out Leucine (MinDO-Leu) and CDGJM-Leu and starter media were prepared
99 using modified versions of previously reported methods (McBryde et al. 2006, Sherman
100 2002) as outlined previously (Haggerty et al. 2015).
101
102 **Chemical standards**
103 Aroma compounds were chosen based on their being produced by yeast and that their odor
104 activity values (OAV, the ratio of the concentration to the odor threshold) were greater than
105 1; Supplemental Table 2). Standards for all compounds were prepared from high purity
106 stocks from: Aldrich (Milwaukee, WI, USA) (2-phenylethyl acetate (99%), ethyl decanoate
107 (≥ 99%), hexyl acetate (99%), ethyl octanoate (≥ 99%), ethyl hexanoate (≥ 99%), 2-methyl
108 butyl acetate (99%), ethyl 2-methylbutanoate (99%), ethyl 3-methylbutanoate (99%), ethyl
109 butanoate (99%), 2-methyl butanol (≥ 99%), methionol (≥ 98%), ethyl 2-methylpropanoate
110 (99%), ethyl acetate (99.9%), propanol (≥ 99.5%), 3-methylbutyric acid (99%), butyric acid
111 (≥ 99%), hexanoic acid (≥ 99.5%), hexanol (≥ 99%), ethyl dodecanoate (≥ 98%), ethyl
112 propanoate (99%)); Sigma (St. Louis, MO, USA) (2-phenyl ethanol (≥ 98%), 2-methylpropyl
113 acetate (99%), 3-methylbutyl acetate (≥ 99%), octanoic acid (≥ 99%)); Unilab
114 (Mandaluyong City, Philippines) (1-octanol (≥ 95%)); Fluka (Buchs, SG, Switzerland) (3-

115 methylbutyl acetate (≥ 99.7%), 2-methylpropanoic acid (99.5%)); and Chemsupply (Gilman,
116 SA, Australia) ethyl 3-methylbutanoate (≥ 99.7%), 3-methylbutanol (≥ 99.8%), 2,3-butandiol
117 (mixture of racemic and *meso* forms) (≥ 99.0%), 2,3-butanedione (≥ 99.0%), decanoic acid
118 (≥ 99.5%) 2-methylpropanol (≥ 99.5%), 2-methylpropanoic acid (≥ 99.5%), acetic acid (AR
119 grade)).
120
121 **Fermentation conditions**
122 The background strain iso-C9DΔLEU (Walker et al. 2003) used in this study is a derivative
123 of the commercial wine yeast L2056 (Lallemand). The strain was transformed (Gietz et al.
124 1992) with the platform vector, pGP564, from the overexpression library reported by Jones et
125 al. (2008). The iso-C9DΔLEU+pGP564 was plated onto MinDO-Leu agar media from
126 glycerol stock and incubated overnight at 28 °C. A single colony of iso-C9DΔLEU+pGP564
127 was placed in 50 mL MinDO-Leu and grown overnight at 28°C with shaking at 120 rpm.
128 Cell counts were performed and 19.5 mL of the culture were spun down at 5,000 rpm for 5
129 minutes, resuspended in ~2 mL CDGJM-Leu starter and added to 198 mL CDGJM-Leu
130 starter media to give an inoculation rate of 5 x 10^6 cells/mL. This new suspension was left to
131 grow overnight at 28 °C with shaking at 120 rpm. The starter culture was used to inoculate
132 the CDGJM-Leu at a rate of 5 x 10^6 cells/mL in the same manner as the starter medium was
133 inoculated. Starter medium (99 mL) was spun down at 5,000 rpm for 5 minutes. The
134 supernatant was removed and the pellet resuspended in 1 mL CDGJM-Leu and added to 2 L
135 of CDGJM-Leu in 2 L Schott bottles equipped with a bung and a fermentation lock. The
136 ferments were kept at 28 °C with shaking: 100 rpm for the first 10 days and then 110 rpm
137 until fermentation was complete. Progress of fermentation was followed by refractive index
138 (Brix) until ~7 Brix, after which reducing sugars were measured using the Benedict's test

139 until ferments were dry. Samples (10 mL) were collected daily, a portion of which was spun
140 down at 5,000 rpm for 5 minutes and approximately 7 mL of supernatant was filtered (0.22
141 μm) into glass vials subsequently sealed with aluminum screw cap closures with minimal
142 headspace. The accumulated samples were stored at 5 °C until fermentation was complete.
143

144 **Sample preparation and quality assurance**

145 Duplicate samples of each fermentation sample (i.e. 4 replicates per time point; two samples
146 from each of two fermentations) were taken following the general SPME procedure outlined
147 previously (Haggerty et al. 2015). Quality assurance was performed as set out in a previous
148 study (Haggerty et al. 2015), specifically, a minicurve was created at the beginning of the
149 chromatographic run to ensure the GCMS was working correctly. Duplicate samples of a
150 concentrated standard solution were also run at the start, middle and end of the run to ensure
151 there was no variation between analyzes.
152

153 **Instrumentation and parameters**

154 The samples were analyzed via GC-MS with HS-SPME extraction using a 65 μm
155 divinylbenzene/polydimethylsiloxane (DVB/PDMS) SPME fibre using a Gerstel MPS2
156 twister auto-sampler. The extraction conditions employed were optimized in a previous study
157 (Haggerty et al. 2015), whereby the samples were allowed to incubate at 50 °C for 10
158 minutes while agitating at 250 rpm to reach equilibrium. The volatile compounds were then
159 extracted with the SPME fibre at a depth of 21 mm for 10 minutes at 50 °C and then
160 desorbed in the GC-MS inlet at 250 °C at a depth of 54 mm for a total of 10 minutes to allow
161 baking of the fibre. Chromatographic separation was performed using an Agilent 6890 gas
162 chromatogram equipped with a 5973N mass spectrometer, with the instrumental parameters

163 employed for identification of all aroma compounds as set out in the optimized procedure
164 reported previously (Haggerty et al. 2015).
165
166 **Results and Discussion**
167 This study sought to assess the potential of using an experimental system incorporating a
168 derivative of a wine strain (iso-C9DΔLEU), the Jones et al. (2008) overexpression library
169 platform (pGP564) and a defined medium (CDGJM-Leu) as a basis for studying the
170 evolution of selected aroma compounds during fermentation. The plasmid-bearing strain
171 (iso-C9DΔLEU+pGP564) exhibited an acceptable pattern of growth and fermentation in
172 CDGJM-Leu. Specifically, in preliminary work fermentation kinetics of iso-C9D and iso-
173 C9DΔLEU+pGP564 agreed well (Supplemental Figure 1), and in the main study there was
174 no biological contamination of the control culture and the fermentation kinetics of the
175 replicates showed a high degree of agreement (Supplemental Figure 2). Accumulation of a
176 library of 34 aroma compounds commonly found in wine (Supplemental Table 2) was
177 monitored during fermentations in CDGJM-Leu. The averaged results for the replicate
178 ferments after analysis in duplicate are displayed in Figures 1-4, with the compounds being
179 grouped as alcohols (Figure 1), acetate esters (Figure 2), ethyl esters (Figure 3), fatty acids
180 and 2,3-butandione (Figure 4). A few compounds that co-eluted during analysis, but which
181 were able to be qualitatively differentiated, were analyzed together. These co-eluting
182 compounds included the isomers, 2-methylbutyl acetate and 3-methylbutyl acetate, 2-
183 methylbutanoic acid and 3-methylbutanoic acid, and 2-methylbutanol and 3-methylbutanol,
184 as well as ethyl dodecanoate and hexanoic acid. The extent of scattering of points along a
185 trend line was small for most compounds except in cases where leucine utilization is
186 important, *vide infra*.

187

188 The *S. cerevisiae* strain iso-C9DΔLEU+pGP564 is a *LEU2* deletant with leucine prototrophy
189 being restored by the inclusion of the pGP564 plasmid, which in the Jones et al. (2008)
190 overexpression library contains sections of the yeast genome to be overexpressed. In this
191 study the plasmid was empty (i.e. contained no yeast genomic sequence within the insert
192 junction). To favor retention of the plasmid, leucine was excluded from the CDGJM. Since
193 many aroma compounds are formed by the metabolism of amino acids, the exclusion of the
194 precursor leucine was expected to affect the final aroma profile of the fermentations,
195 including compounds such as 3-methyl butanol, 3-methyl butanoic acid, 3-methyl butyl
196 acetate and ethyl 3-methyl butanoate (Hazelwood et al. 2008). The fact that these compounds
197 were formed in our experimental system demonstrates that the complementation of the
198 plasmid is effective and the yeast are making sufficient leucine not only for metabolic
199 requirements but also to produce aroma compounds. It was also expected that the hexyl
200 derived compounds, hexyl acetate, ethyl hexanoate and hexanoic acid would either not be
201 present or be seen in very small quantities due to results seen in previous experiments (data
202 not shown), since the medium does not contain hexanol and that the yeast will only
203 synthesize a limited amount of hexanol *in vivo*. Even though these compounds were seen in
204 low quantities, for most they still followed similar trends to other previously published
205 works.

206

207 Comparison of the progression of aroma compound formation with that seen in studies with
208 real juices indicates that the majority of trends were similar for the alcohols (Callejón et al.
209 2012, Mallouchos et al. 2002, Rapp and Mandery 1986, Stashenko et al. 1992), ethyl esters
210 (in a high temperature ferment; Callejón et al. 2012, Mallouchos et al. 2002, Vianna and

211 Ebeler 2001, Zhang et al. 2011) and the acids (Bardi et al. 1999, Trinh et al. 2012, Zhang et
212 al. 2011), except for the compounds associated with the biosynthetic pathway of leucine. All
213 of the alcohols, except 2,3-butandiol, benzyl alcohol and hexanol showed significant
214 formation during the first 4 days or so of fermentation (Figure 1), roughly coincident with the
215 exponential phase of growth (data not shown).
216
217 The trend in formation of acetate esters (Figure 2) differed to that observed in other studies
218 (Callejón et al. 2012, Stashenko, et al. 1992). The only acetate ester analyzed herein that was
219 an exception and followed previously reported trends (e.g. Callejón et al. 2012) was 2-phenyl
220 ethyl acetate, which accumulated rapidly in the first few days of fermentation and reached a
221 steady state at day 4. The remaining acetate esters varied around a more or less constant
222 average value. Being formed from the metabolism of leucine (Supplemental Figure 3), 3-
223 methylbutyl acetate was not expected to follow similar trends to those seen in previous
224 studies, as the precursor compound (leucine) was excluded from the CDGJM. In fact it was
225 found that the combined accumulation of both 2- and 3-methylbutyl acetate did not follow
226 the exponential growth trend as shown by others (Callejón et al. 2012, Stashenko et al. 1992).
227 This observation could suggest yeast are preferentially diverting the key intermediates α-
228 ketoisovalerate and α-ketoisocaproate to leucine synthesis for cell growth (Supplemental
229 Figure 2) rather than the formation of aroma compounds 2-methylpropyl acetate and 3-
230 methylbutyl acetate (analysed together with 2-methylbutyl acetate). During fermentation the
231 genes involved in biosynthesis of amino acids are usually down-regulated after the
232 exponential growth phase and therefore the expression of *LEU2* is usually low at the end of
233 fermentation (Molina et al. 2007, Rossignol et al. 2003). In this experiment, leucine was
234 absent from the medium and would need to be synthesized if not for biomass then to permit

235 protein (re)synthesis by the stationary phase culture. The assimilable nitrogen added to the
236 medium (750 mg N/L) would suffice for biomass formation (Jiranek et al. 1995) with
237 residual amounts fueling the synthesis of required amino acids, particularly leucine.
238
239 It has been shown that fermentations carried out at a higher temperatures show a more
240 constant rate of accumulation of ethyl esters throughout fermentation (Mallouchos et al.
241 2002). The same was observed here for most of the targeted ethyl esters (Figure 3) except for
242 ethyl acetate and ethyl dodecanoate (analyzed together with hexanoic acid), which
243 reached their maximum concentration within the first 7 days of fermentation
244 spanning the exponential phase of the yeast growth and then changed little. Amounts of
245 ethyl hexanoate and ethyl decanoate, once peaked were also largely stable, similar to
246 previous studies in grape juices (Callejón et al. 2012, Mallouchos et al. 2002, Vianna and
247 Ebeler 2001, Zhang et al. 2011).
248
249 Analysis of the accumulation of the acids throughout fermentation has not been previously
250 studied in great detail in different media. Acetic acid, decanoic acid and octanoic acid are the
251 main acids previously studied (Bardi et al. 1999, Trinh et al. 2012, Zhang et al. 2011), the
252 findings for which (Figure 4) show similar trends to the current study. The other acids
253 monitored herein showed a progressive accumulation over the course of fermentation, albeit
254 at different rates.
255
256 In relation to the similarity of the CDGJM-Leu and the MS300 model media, the trends in
257 the accumulation of aroma compounds are very similar. One study employing MS300 at
258 fermentation temperatures of 15 °C and 28 °C, representing a white wine and a red wine

259 fermentation, respectively, showed similar trends at each temperature with the exception of
260 the acids (Molina et al. 2007). At 15 °C the acids portrayed more of a steady state after 40%
261 sugar consumption, whereas the higher temperature fermentation showed higher peak
262 concentrations of the acids before decreasing over the latter stages of fermentation (Molina et
263 al. 2007). The trend seen in the higher temperature fermentation was also observed in this
264 study (also carried out at 28 °C) for octanoic acid and decanoic acid (Figure 4). The alcohols
265 in both studies followed similar trends as did the accumulation of acetates described herein.
266

267 **Conclusion**

268 Overall, CDGJM has previously been shown to yield similar fermentation performance to
269 that of the MS300 model media and grape juice fermentations; however it has not yet been
270 shown to provide similar aroma progression trends or aroma profiles. This study has shown
271 that fermentations in a CDGJM-Leu with a *S. cerevisiae* Δ*LEU* strain containing a
272 complementing plasmid will show similar trends in the formation of aroma compounds as a
273 conventional ferment, or a ferment in MS300, with the exception of the compounds related to
274 the biosynthesis of leucine. The aroma compounds whose accumulation was affected by
275 employing CDGJM-Leu as the fermentation medium included 2,3-butandiol, ethyl
276 propanoate, ethyl butanoate, 2-methylbutyl acetate, 2-methylpropyl acetate and 3-
277 methylbutyl acetate. Nevertheless a suite of nearly 30 enologically relevant aroma
278 compounds could be seen to be produce under these defined conditions, thereby providing a
279 platform for discovery of genes important to the kinetics and extent of their production.
280 Screens for genes impacting other relevant attributes might also be revealed using this
281 system.
282

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378 **Figure Legends**

379

380 **Figure 1:** Formation of alcohols during fermentation by iso-C9DΔLEU+pGP564 in
381 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

382

383 **Figure 2:** Progression of acetate esters during fermentation by iso-C9DΔLEU+pGP564 in
384 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

385

386 **Figure 3:** Formation of ethyl esters during fermentation by iso-C9DΔLEU+pGP564 in
387 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

388

389 **Figure 4:** Formation of fatty acids and 2,3-butandione during fermentation by iso-
390 C9DΔLEU+pGP564 in CDGJM-Leu. Values are the average of duplicate fermentations
391 analyzed in duplicate.

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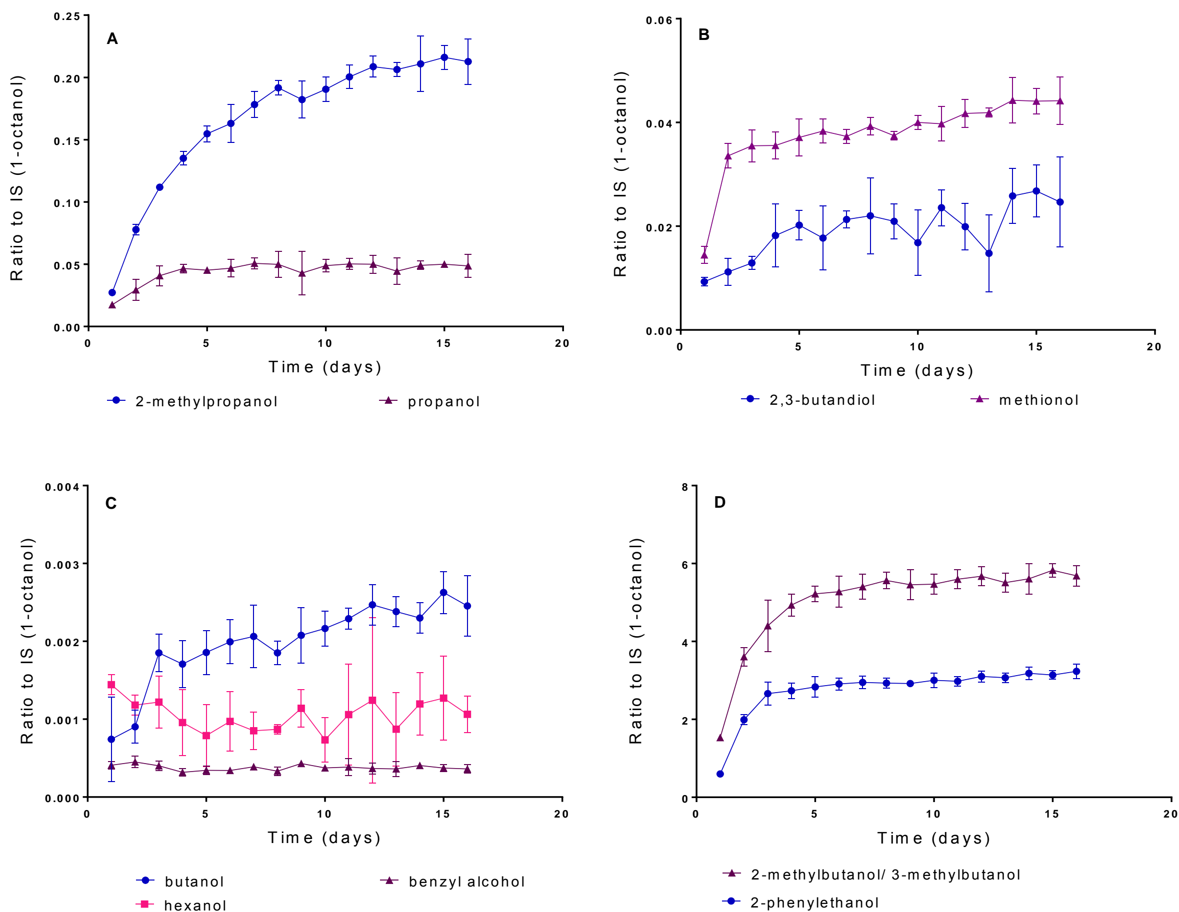

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404 **Figure 1:** Formation of alcohols during fermentation by iso-C9DΔLEU+pGP564 in
405 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

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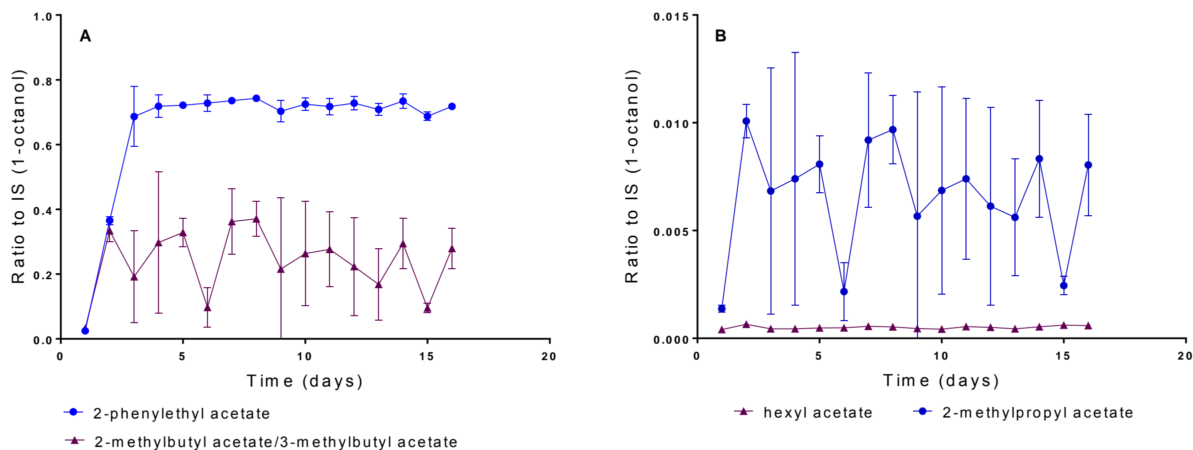

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416 **Figure 2:** Progression of acetate esters during fermentation by iso-C9DΔLEU+pGP564 in
417 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

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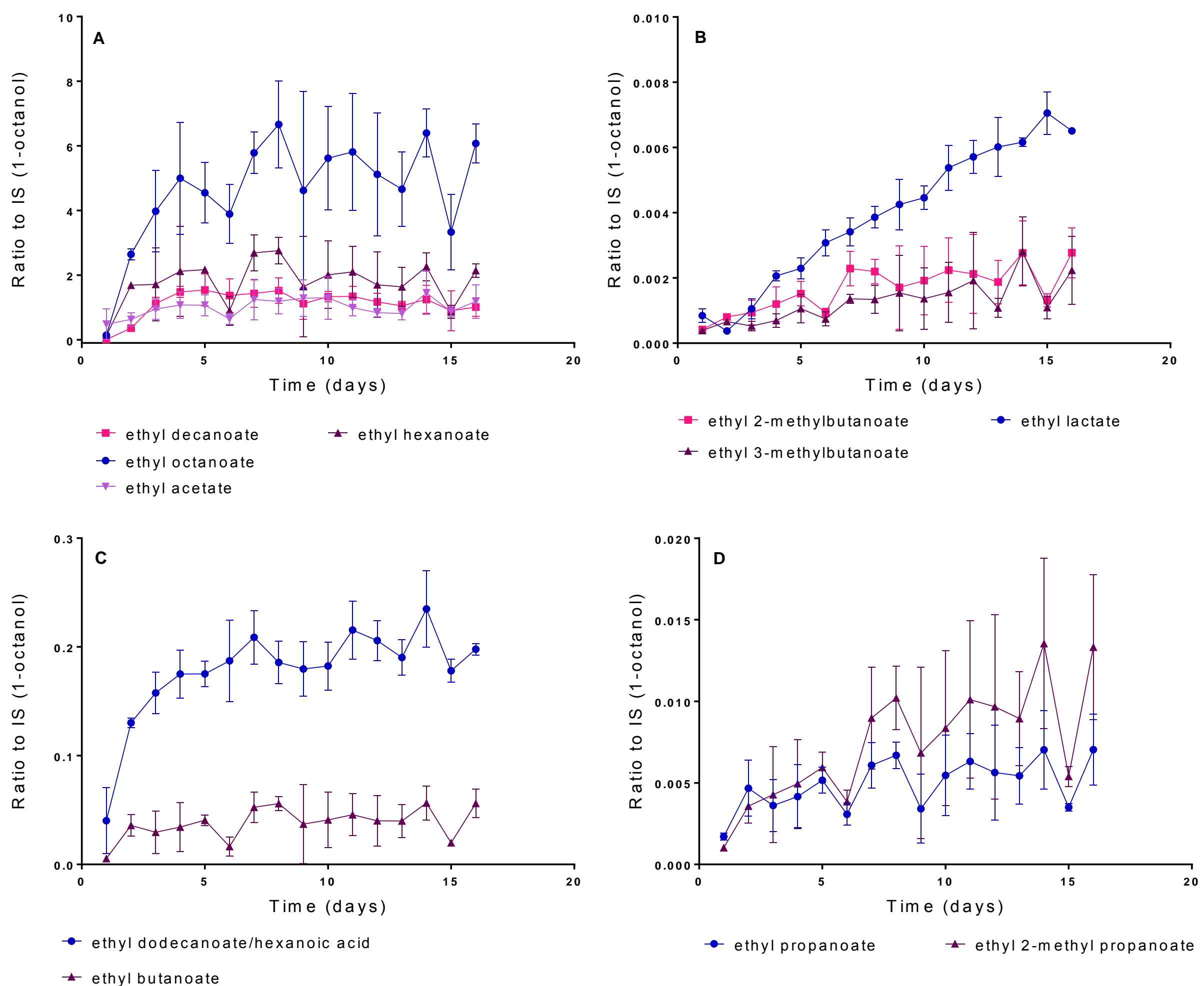

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436 **Figure 3:** Formation of ethyl esters during fermentation by iso-C9DΔLEU+pGP564 in
437 CDGJM-Leu. Values are the average of duplicate fermentations analyzed in duplicate.

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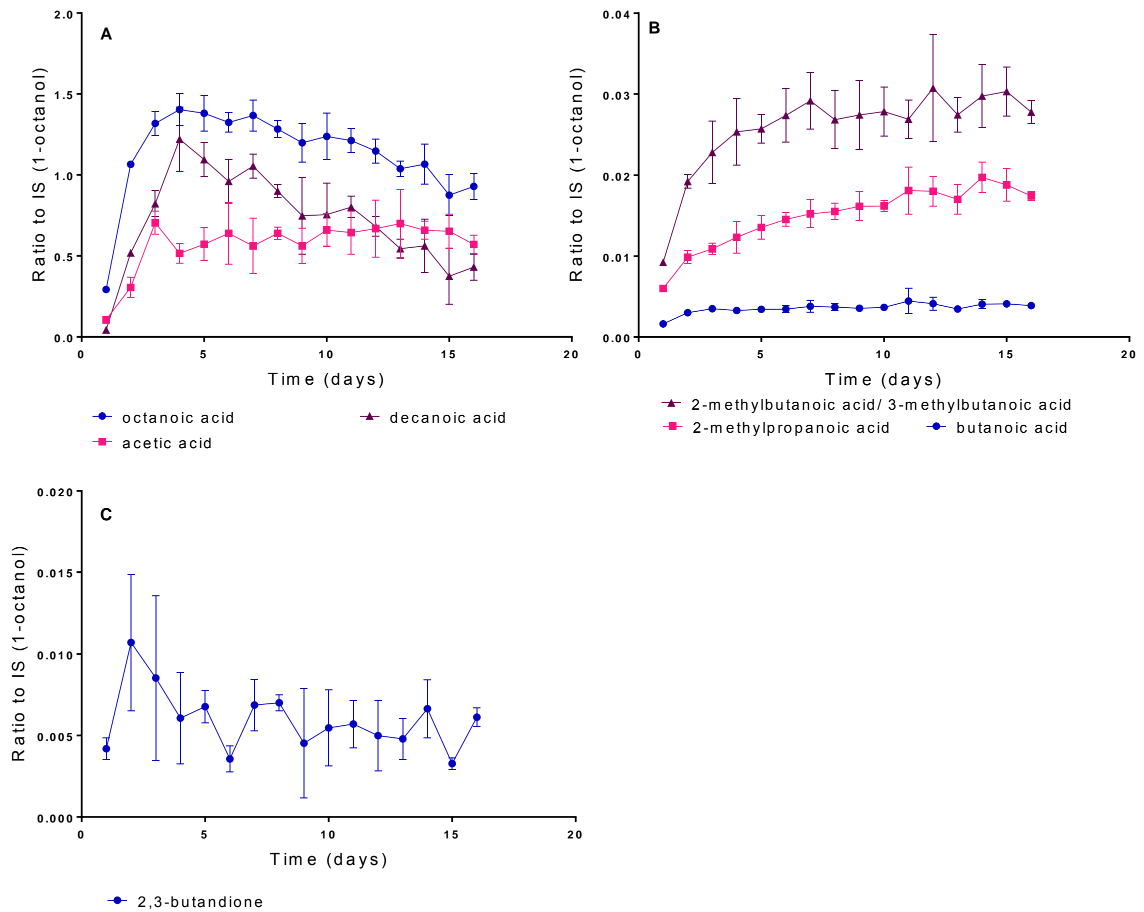

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445 **Figure 4:** Formation of fatty acids and 2,3-butandione during fermentation by iso-
446 C9DΔLEU+pGP564 in CDGJM-Leu. Values are the average of duplicate fermentations
447 analyzed in duplicate.

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Supporting Information

MONITORING VOLATILE AROMA COMPOUNDS DURING FERMENTATION IN CHEMICALLY DEFINED GRAPE JUICE MEDIA DEFICIENT IN LEUCINE (CDGJM-LEU)

J. HAGGERTY, D. K. TAYLOR and V. JIRANEK

Table of Contents

1. **Supplementary Tables S1-S2**
2. **Supplementary Figures S1-S2**

Supplementary Table 1. Aroma compounds formed by *Saccharomyces cerevisiae* with OAVs* greater than 1.

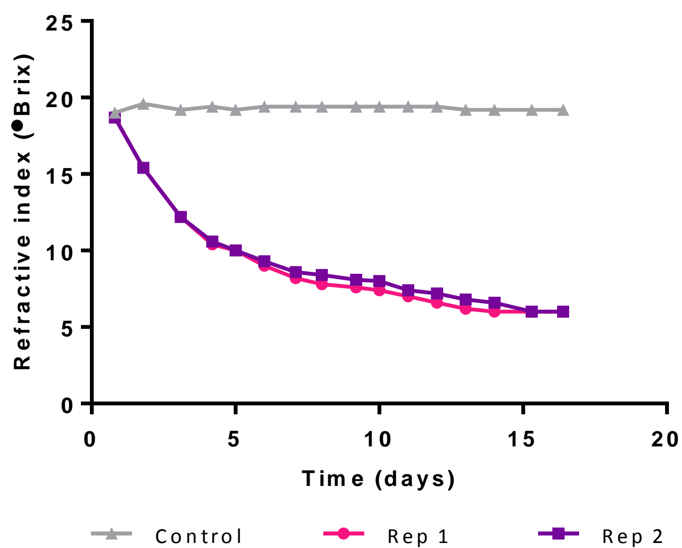
Compound	Aroma description	Odour threshold (μg/L)
Ethyl esters		
ethyl acetate	pineapple, fruity, solvent	7,500
ethyl 2-methylpropanoate	sweet, rubber	15
ethyl 2-methylbutanoate	apple	18/ 1
ethyl 3-methylbutanoate	fruit	3
ethyl butanoate	apple	20
ethyl hexanoate	apple peel, fruit	14/ 5
ethyl octanoate	fruit, fat	2
ethyl decanoate	grape	200
ethyl lactate	fruit	14
ethyl dodecanoate	soapy, estery	-
ethyl propanoate	fruity	1,800
Acetates		
3-methylbutyl acetate	banana	30
2-methylbutyl acetate	fruit	160
2-phenylethyl acetate	rose, honey, tobacco	250
hexyl acetate	fruit, herb	670
2-methylpropyl acetate	fruit, apple banana	1.6
Acids		
2-methylpropanoic acid	rancid, butter, cheese	2,300
3-methylbutanoic acid	sweat, acid, rancid	33.4
acetic acid	sour	200,000
2-methylbutanoic acid	cheese, sweaty	1,500
butanoic acid	rancid, cheese, sweat	173
hexanoic acid	sweat	420
octanoic acid	sweat, cheese	500
decanoic acid	rancid, fat	1,000/ 8,100
Alcohols		
benzyl alcohol	sweet, flower	900,000
butanol	Wine, fusel, spiritous	150,000
2-methyl propanol	wine, solvent, bitter	40,000
3-methyl butanol	whisky, malt, burnt	30,000
2-methyl butanol	wine, onion	65,000
2-phenyl ethanol	honey, spice, rose, lilac	14,000/ 10,000
methionol	sweet potato	1,000
n-propanol	alcohol, pungent	306,000
2,3-butandiol	fruity	150,000
hexanol	resin, flower, green	8,000
Miscellaneous		
2,3-butanedione	butter	100

* OAVs, Odour activity values.

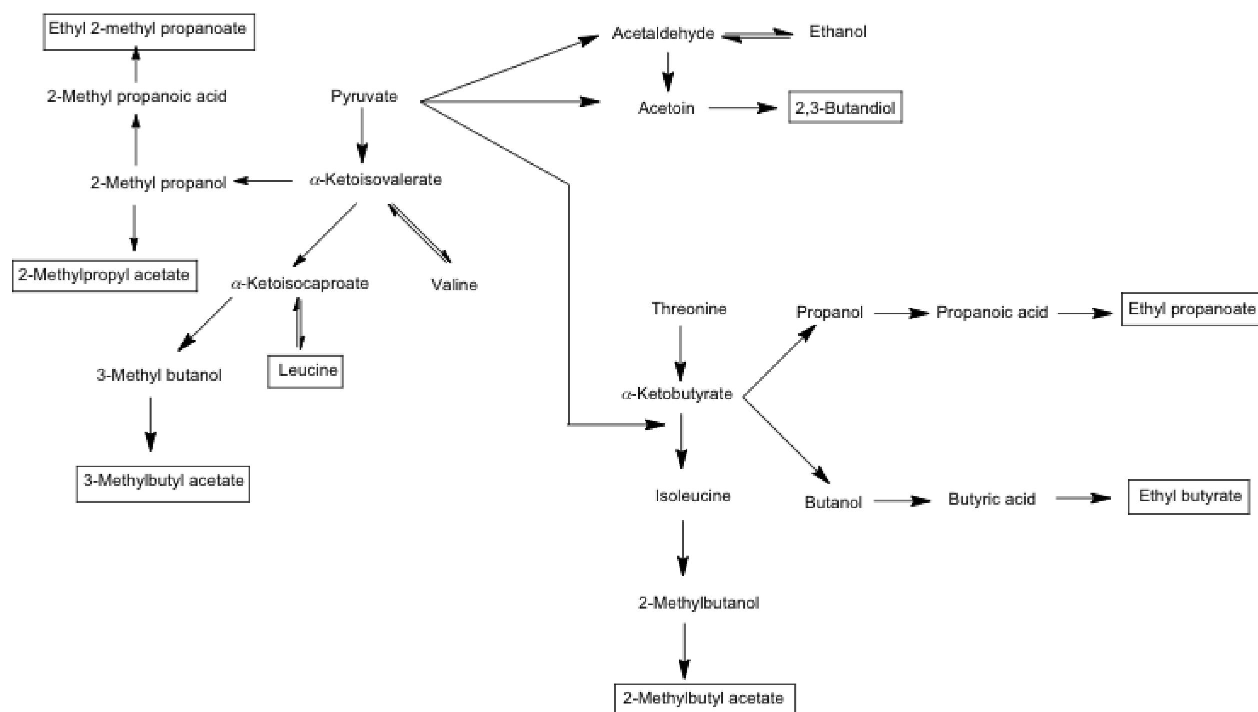
Aroma descriptors are from Flavornet (Acree and Arn, 2004), odour thresholds determined in ca. 10% ethanol (Francis and Newton 2005, Ferreira et al. 2000, Guth 1997, Peinado et al. 2006, Peinado et al. 2004, Salo 1970, Siebert et al. 2005).

Supplementary Table 2: Composition of model media for MS300 (Rossouw and Bauer 2009) and CDGJM (Henschke and Jiranek 1993). (Assimible nitrogen was calculated as total nitrogen content excluding that from proline)

Component	MS300	CDGJM	Component	MS300	CDGJM (mg/L)
pH (NaOH)	3.3	3.2-3.5	**Vitamins**	(mg/L)	(mg/L)
			Myo-inositol	20	100
Sugars	(g/L)	(g/L)	Nicotinic acid	2	2
Glucose	125	100	Calcium pantothenate	1.5	1
Fructose	125	100	Thiamine HCl	0.25	0.5
			Pyrixodine HCl	0.25	2
Amino acids	(mg/L)	(mg/L)	Biotin	0.003	0.125
Alanine	145.3	100	p-amino benzoic acid	-	0.2
Arginine	374.4	750	Riboflavin	-	0.2
Asparagine	-	150	Folic acid	-	0.2
Aspartic acid	44.5	350			
Cysteine	13.1	-	**Mineral salts**	(mg/L)	(mg/L)
Glutamic acid	120.4	500	K_2SO_4	500	-
Glutamine	505.3	200	NaCl	200	-
Glycine	18.3	50	$MnSO_4.H_2O$	4	-
Histidine	32.7	150	$ZnSO_4$	4	-
Isoleucine	32.7	200	$CuSO_4.5H_2O$	1	-
Leucine	48.4	300	KI	1	-
Lysine	17.0	250	$CoCl_2.6H_2O$	0.4	-
Methionine	31.4	150	KH_2PO_4	750	1140
Phenylalanine	37.9	150	$MgSO_4.7H_2O$	250	1230
Proline	612.6	500	$CaCl_2.2H_2O$	155	440
Serine	78.5	400	H_3BO_3	1	0.0057
Threonine	759.3	350	$NaMoO_4.2H_2O$	1	0.0242
Tryptophan	179.3	100	$Co(NO_3)_2.6H_2O$	-	0.0291
Tyrosine	18.3	20	$CuCl_2$	-	0.0136
Valine	44.5	200	$FeCl_2$	-	0.0320
Total amino acids	3113.9	4870	$ZnCl_2$	-	0.1355
Assimilable nitrogen (mg N/L)	419	723	KIO_3	-	0.0108
Ammonium chloride	460	100	K tartrate	-	5000
Total Assimilable Nitrogen (mg N/L)	537	749	L-Malic acid	-	3000
			Citric acid	-	200
			$MnCl_2.4H_2O$	-	0.1982



Supplementary Figure 1: Fermentation curve for replicate 1, replicate 2 of strain C9DΔLEU+pGP564 and uninoculated control as measured by refractrometry in °Brix.



Supplementary Figure 2: A simplified version of the pathways involved in the production of the aroma compounds that showed a significant difference in final concentration when one fermentation was left under fermentation conditions for an extra 2 days after completion so that removal of the replicates coincided (Baichwal et al. 1983, Bollon 1974, González et al. 2000, Guymon et al. 1961, Hazelwood et al. 2008, Ingraham et al. 1961).

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Characterisation of the wine metabolome: linking sensory attributes to genotype

To be able to further untangle the relationship between the aroma compounds found in wine and the genotype of the yeast used for fermentation is of importance to the wine industry and future research studies on yeast and wine. Many different research groups have utilised deletion libraries with regards to yeast metabolomics, but never an overexpression library. Recently a new *Saccharomyces cerevisiae* (*S. cerevisiae*) overexpression library has been produced and is now commercially available. This library is available encoded in overexpression plasmids where the overexpressed portion of the genome contains 3-5 genes and is encoded by endogenous promoters. This library contains ca. 1500 clones and covers over 97% of the *S. cerevisiae* genome. The only limitation to this library is that it contains a LEU2 marker, requiring the use of a *ΔLEU* yeast strain and a media lacking leucine for the plasmids to be retained.

This chapter explains the preparation and testing of the overexpression library in a full overexpression screen. The fermentations screen was set up to include 5 time-points of the overexpression library along with 20 commercially available yeasts which were provided by LAFFORT. The fermentations were followed by the parental strain fermentations and at each allocated time-point one replicate was removed from the fermentation trials. At each time-point enzymatic analysis of total sugars (glucose and fructose) was performed and then the time-point was frozen for latter aroma analysis. The experiment was considered complete at 4 days after the parental strain had finished fermentation. Only fermentations which were considered dry (≤ 4.5 g/L total sugar) were analysed for their aroma profiles. A total of 51% of the library finished fermentation within the allocated time period. From the 737 clones + 19 Laffort yeast that finished fermentation, we were able to determine 92 clones which showed a variation in their aroma profile when compared to the rest of the library and were considered 'interesting clones'. Eighty seven of these interesting clones were overexpression clones and 5 were commercial yeasts. The final outcome of this study hypothesised that for yeast to retain its plasmid, throughout the fermentation experimental conditions, the LEU2 marker is not sufficient and a faster growth rate will increase the rate of plasmid rejection, hence more cells will die due to a lack of nutrients. For the plasmid to be retained, either a beneficial gene, or a gene which when overexpressed decreases vegetative growth needs to be present with 78 % of the interesting clones meeting these criteria; which is a significant percentage with regards to a biological study. These results are beneficial for future studies using or creating overexpression libraries for fermentation studies. This chapter is to be submitted to the *Journal of Applied microbiology and biotechnology* in 2016.

Fermentation screen of a *Saccharomyces cerevisiae* overexpression library: linking aroma profiles to genotype

Publication Status: To be submitted

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Author Contributors:

Name of principle author (PhD Candidate): Jade Haggerty

Contribution to the paper: Designed and performed experiments, analysed data and interpreted data, and drafted/constructed manuscript.

Name of Co-Author: Dennis Taylor

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments and aided in the drafting and construction of the manuscript.

Name of Co-Author: Vladimir Jiranek

Contribution to the paper: Oversaw the experimental design, supervised the practical experiments, aided in the drafting and construction of the manuscript and submitted the manuscript as corresponding author.

1 **Fermentation screen of a *Saccharomyces cerevisiae* overexpression library: linking aroma**
2 **profiles to genotype**

3 Jade J. Haggerty[1], Dennis K. Taylor[1], Vladimir Jiranek[1]*

4

5 **Author affiliations**

6 [1] Department of Wine and Food Science, The University of Adelaide, Waite campus, PMB 1, Glen
7 Osmond, 5064, Australia.

8

9 ***Corresponding author**

10 Prof Vladimir Jiranek, email vladimir.jiranek@adelaide.edu.au

11

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17 Dr. Michelle Walker and Dr. Joanna Sundstrom for their ongoing support and ideas; and Dr.
18 Tommaso Liccioli for help preparing the fermentation screen.

19

Abstract:

Aims: Aroma analysis of a yeast overexpression library was used to link genes to aroma compound production. To remove any bias, the only predetermination was the aroma compounds analysed, being chosen as those typically found above their odour detection values in wine.

Methods and Results: A plasmid-borne overexpression library was transformed into a wine yeast derivative and fermentations performed in deep 96-well plates on ~1,500 clones and some commercial reference strains. The aroma bouquet was determined in the ferments using GC-MS.

Conclusions: Only 51 % of the library completed fermentation. Aroma profiling and statistical analysis (PCA) highlighted 87 clones for further investigation based on their enhanced aroma compound production. Plasmid retention varied across the clones. It is proposed that plasmids containing either a beneficial gene or a gene whose overexpression decreased vegetative growth were more likely to be retained.

Significance and Impact of Study: The metabolic pathways of *Saccharomyces cerevisiae*, including those yielding sensorially important compounds are well known. However, such studies are usually only carried out on a predetermined set of aroma compounds with the genes already known to be linked to a specific metabolic pathway. This study seeks to remove such biases by using a genome-wide approach and quantitation of volatiles likely to occur in oenologically significant amounts.

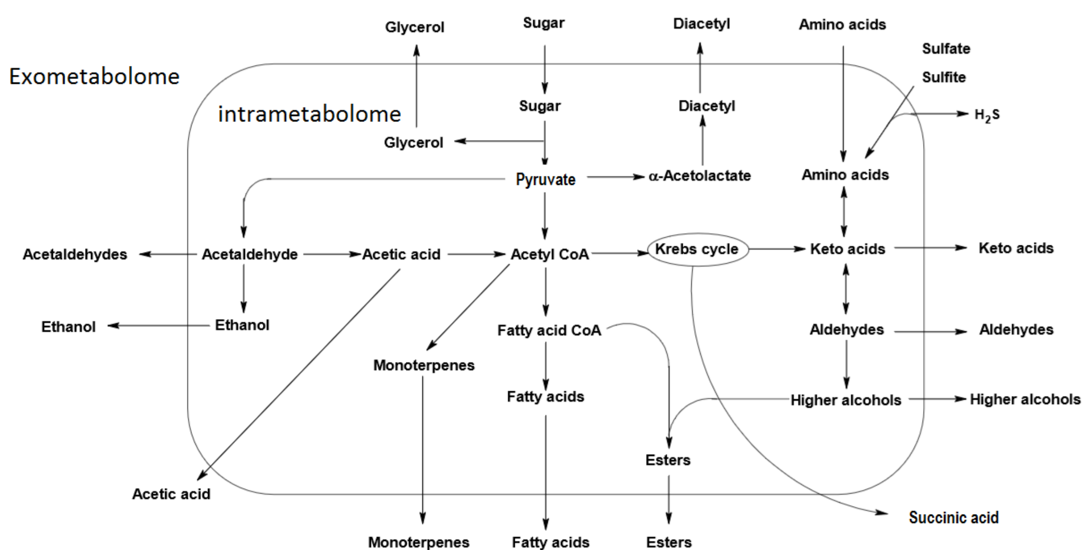
Highlights:

- Screening of fermentations by a ~1,500 clone overexpression library in a wine derivative strain of *Saccharomyces cerevisiae*
- Potential link between vegetative growth and plasmid retention

42 ***Keywords:*** overexpression, principle component analysis (PCA), metabolomics, *Saccharomyces*
43 *cerevisiae*, plasmids, aroma compounds, high-throughput screen

44 **1. Introduction**

45 One of the major contributors to the variation in the wine meta-metabolome is the yeast used in
46 fermentation. The composition of a wine produced using one strain of yeast can have marked
47 differences to that produced using another strain. Many compounds are produced in wine as
48 by-products of the fermentation process. These include volatile compounds contributing to the
49 flavour and aroma of the wine, and non-volatile compounds which contribute to the taste, colour or
50 mouth-feel of the wine (Figure 1). This paper focuses on the volatile compounds found in wine that
51 are produced by yeast, with emphasis on those produced at or above their aroma threshold.
52 Although the genes involved in the pathways producing these aroma compounds are known, there
53 are 1134 protein-coding genes in *S. cerevisiae* for which there is no published data on the
54 molecular or biological functions of their proteins [1]. Therefore, it is possible that there are other
55 proteins involved in these and related metabolic pathways, which have previously gone undetected.



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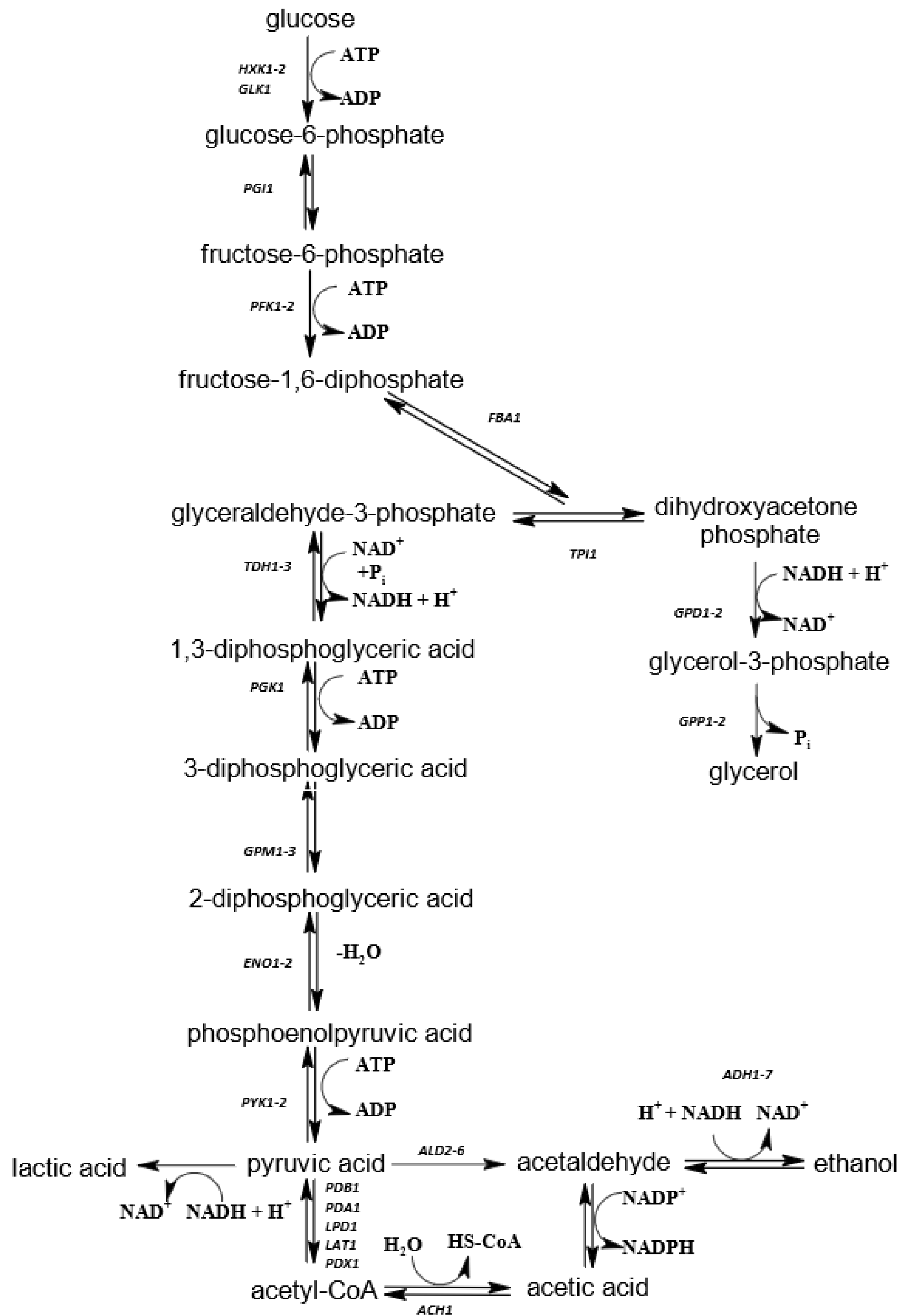
57 Figure 1: Overview of the metabolic pathways resulting in flavour-active compounds found in wine
58 due to fermentation (adapted from [3]).

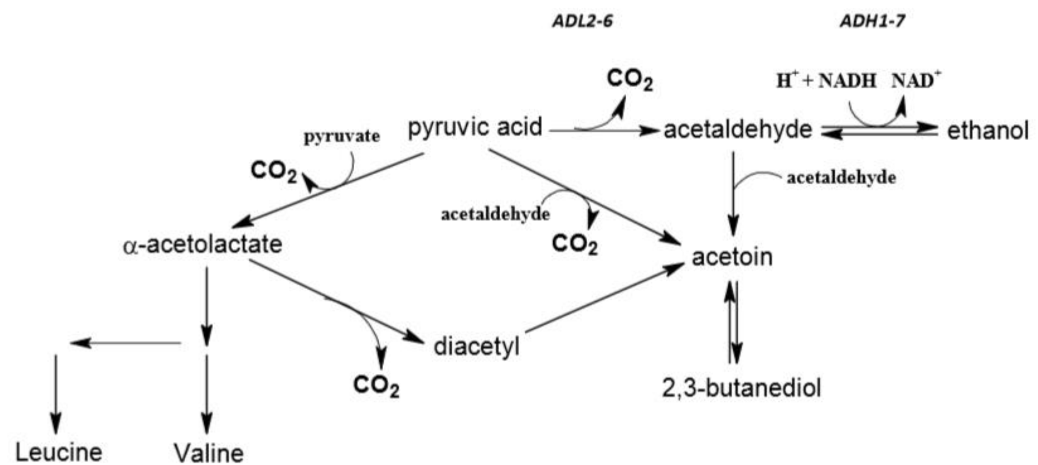
59 In a study reconstructing the *S. cerevisiae* genome, it was shown that there are 1175 metabolic
60 reactions taking place in yeast. Of the 584 metabolites formed, 121 are of the extracellular type [2].
61 It was also found that there are fewer open reading frames (ORF) than there are reactions, thus
62 implying that the isomerases and transferases are less specific in *S. cerevisiae* than in other
63 organisms [2]. These observations suggest that alterations of genes involved in the formation of
64 isomerases and transferases will in fact affect more than one pathway, creating a snowballing
65 effect from one small change. Thus, it could be hypothesised that overexpression of one key gene
66 will give an increase in multiple desirable aroma compounds. Studies in gene manipulation are
67 numerous in wine science, even though usage of genetically modified yeast in wine making is not
68 possible in many countries. The results obtained from such experiments can, however, provide
69 more information into the importance of specific genes and thereby guide research into non-
70 recombinant strain selection and optimisation strategies.

71 The major metabolic pathway in fermentation is the breakdown of glucose into useable products
72 and energy. This pathway, glycolysis or the Embden-Meyerhof pathway, stops at the formation of
73 pyruvic acid (Figure 2). Glycolysis is the major starting point for many of the flavour compounds
74 found in wine, as described by Swiegers et al. [3]. The numerous steps involved in glycolysis
75 should provide many opportunities to alter the catalysing enzymes and produce a marked effect in
76 one or several flavour compounds. However, past studies in model media have shown that
77 overexpression of any single glycolytic gene does not markedly affect the overall flux of the reaction
78 [4], although, it is possible that overexpression of more than one glycolytic gene, or a single non-
79 glycolytic gene, may have an effect on the final concentration of products formed. From this
80 backbone pathway, several other pathways arise; the simple pathways include formation of
81 ethanol, glycerol, acetic acid and lactic acid (Figure 2) and the formation of acetoin and
82 2,3-butanediol [5] (Figure 3). It is then from acetic acid, or acyl-CoA, that the other major pathways
83 involved in creating other flavour compounds arise (Figure 1).



84

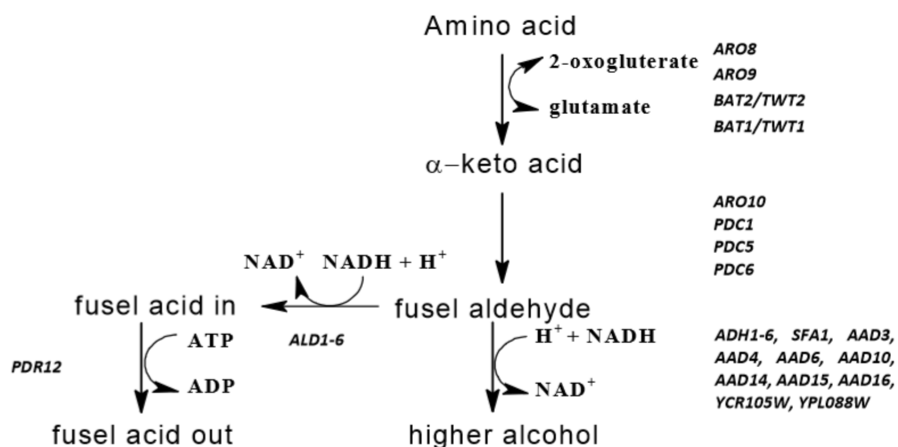
85 Figure 2: Pathway for glycolysis and selected fates of the end product, pyruvate, in *Saccharomyces*
86 *cerevisiae* [6-8]. The genes involved in the reactions are shown in italics [9].



87

88 Figure 3 : Pathway for the formation of acetoin and 2,3-butanediol [5].

89 Although amino acid synthesis can occur *in vivo*, many amino acids are taken up by the cell as a
90 source of nitrogen, thus creating a separate pathway achieve glycolysis for aroma compound
91 formation. The breakdown of amino acids via the Ehrlich pathway [10] (Figure 4) is responsible for
92 aroma compounds such as higher alcohols [10, 11]. The higher alcohols are formed through
93 deamination by 2-oxoglutarate forming the *α*-keto acid, followed by carboxylation to form the
94 aldehyde, which can then be oxidised or reduced to form the carboxylic acid or the higher alcohol,
95 respectively [10, 11]. Until 2006, the only genes in this pathway that had been well characterised
96 were *BAT1* and *BAT*2 [12]. Since then, only *ARO10, AAD10* and *AAD14* have been examined in
97 more detail in terms of the production of aroma compounds in wine [13, 14].



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99 Figure 4: The Ehrlich pathway: formation of fusel acids and higher alcohols in *Saccharomyces*
100 *cerevisiae* [10]

101 Fruity, floral wines are very popular in today's market and therefore research into the origin of the
102 aroma compound responsible has the potential to benefit the industry. In general the important
103 aroma compounds are considered those with an odour activity value (OAV) greater than 1. The
104 OAV is the concentration of a compound found in the wine divided by the odour detection threshold
105 [cited in 15]. The important aroma compounds found in a white wine were compiled previously [16]
106 and these compounds were targeted in this study in a fermentation screen using the
107 overexpression library from Jones and co-workers [17]. The object was to determine which genes,
108 when overexpressed were linked to changes in concentrations of aroma compounds of sensory
109 importance in wine.

110 **2. Materials and methods**

111 2.1 Chemical standards

112 The standards for the target aroma compounds that were quantified in this study have been
113 described previously [16]. In addition, octanol was used as an internal standard.

114

2.2 Media

2.2.1 Kanamycin + LB low salt solution

A 1 L stock solution of low salt Lysogeny broth (LB) medium was formed by dissolving bacto tryptone (10 g), yeast extract (5 g) and sodium chloride (5 g) in ~400 mL of RO water and then diluting with RO water up to 1 L. The media was adjusted to pH 7.5 using 1 M sodium hydroxide, divided into 500 mL aliquots and autoclaved. To each aliquot of this stock was added 0.5 mL of a kanamycin (KAN) stock, made by dissolving kanamycin (1 g) in deionised water (20 mL) and sterile filtered (0.22 µm).

2.2.2 Chemically defined grape juice medium –Leu (CDGJM-Leu), Minimal drop-out –Leu (MinDO–Leu) and starter medium

A CDGJM-Leu solution, minimal drop-out –Leu and starter media were prepared as described previously [18].

2.3 Preparation of the overexpression library

2.3.1 Preparation of plasmids

The 96-well plates containing the library of *E. coli* bearing the overexpression plasmids were removed from storage and aliquots of each well promptly transferred as a scraping made with a sterile 200 µL pipette into individual 12 ml tubes containing 10 mL of LB low salt + KAN solution. The tightly capped tubes were shaken at 140 rpm at 37 ˚C overnight. Plasmids were extracted from each of the resulting *E. coli* cultures using the Wizard plasmid mini-prep system (Promega, America, 2009). Purification of the plasmids was confirmed using electrophoresis (1% agarose, TAE) and quantified by Nanodrop™ analysis (Thermo Scientific). The purified plasmids were then re-set into 96-well plates.

138 *2.3.2 Transformation of plasmids into* S. cerevisiae

139 Each of the purified plasmids were transformed into the isoC9d ΔLeu2 *S. cerevisiae* yeast using the
140 entire 96-well plates via a modified protocol [19]. The plasmids that were not able to be transformed
141 under these conditions were then transformed individually using the standard, unmodified
142 transformation method.

143 2.4 Testing for retention of plasmids under different fermentation conditions

144 Various fermentation conditions were evaluated prior to performing the screen to maximise the
145 retention of the plasmids during the screen (See *Supplementary Information*).

146 2.5 Preparation of commercial yeast

147 The Laffort yeast were prepared following the manufacturer's instructions. Approximately 1 g of the
148 active dried wine yeast (Laffort) (1.5 mL) was resuspended in 10 mL of deionised water and held at
149 28°C for ~20 min. The suspension was then diluted to 20 mL and left for 15 min before streaking
150 out 5 µL onto a YPD agar plate and incubation at 28 °C overnight. The next day the plates were
151 check for contamination, a mixed culture swab transferred from each plate into 10 mL of YPD and
152 incubated at 28°C overnight. Each culture was made into a glycerol stock using sterile 80% glycerol
153 solution (47 µl) and cell culture (203 µL) and stored at -80 °C in a 96-well plate format. NB: MRS 2
154 was made separately and was not frozen before use in the screen.

155 2.6 Screening fermentations:

156 *2.6.1 Sample preparation*

157 The isoC9d ΔLeu2 strain including a blank LEU2 complementation plasmid (isoC9d ΔLeu2 +
158 pGP564) was plated onto MinDO-Leu agar from a glycerol stock and incubated overnight at 28 °C.
159 A single colony of isoC9d ΔLeu2 + pGP564 was grown overnight in 50 mL Min DO-Leu at 28 °C
160 while shaking at 120 rpm. Cell counts were performed on the culture and 19.5 mL spun down at

161 5,000 rpm for 5 minutes, resuspend in ~2 mL CDGJM-Leu starter and added to 198 mL CDGJM-
162 Leu Starter medium to give an inoculation rate of 5 x 10^6 cells/mL. This new suspension was grown
163 as above, used to inoculate the CDGJM-Leu at a rate of 5 x 10^6 cells/mL and incubated as above.
164 Starter media (2 x 49.5 mL) was spun down (5,000 rpm, 5 min). The supernatant was removed and
165 each pellet resuspended in CDGJM-Leu and added to 2 L of CDGJM-Leu in 2 L Schott bottles
166 equipped with a bung and an airlock. The ferments were kept at 28 °C with shaking at 100 rpm for
167 the first 10 days and then increased to 110 rpm until fermentation was complete. The parental
168 fermentations were followed by refractive index (°Brix) until reaching ~7 Brix when reducing sugars
169 were measured using the Benedict's test until dry. When required, residual glucose and fructose
170 content was accurately determined from the supernatant by an enzymatic method [18] adapted for
171 96-well plates.

172 *2.6.2 Screen layout*

173 The final screen was performed as micro-fermentations in 1.8 mL volumes of CDGJM-Leu with
174 inoculations prepared by a Corbett liquid-handling robot, (Supplementary information, Figure
175 S2(C)). Plates were stacked with 'stackers' between each plate to ensure paralleled conditions
176 (Supplementary information, Figure S2(A)). These stacks were then placed in closed tubs with wet
177 paper towels to limit evaporation (Supplementary information, Figure S2(B)). The 21 plates were
178 replicated 5 times for sacrificial sampling at time-points defined as 6 days prior to the expected
179 completion of fermentation by the parental strain and then every 2 days until 2 days after the
180 parental strain completed fermentation. At each time-point residual glucose and fructose content
181 was determined from the supernatant by an enzymatic method [19] adapted for 96-well plates and
182 then the remainder closed with an aluminium foil seal and stored at -20 °C until aroma analysis.
183 Fermentations were followed using the parental strains on a daily basis using the same method as
184 per the whole plate analysis [20].

185

186 2.7 Aroma compound analysis:

187 Prior to analysis the frozen fermentation plates were removed from the freezer and allowed to thaw
188 at room temperature. Only fermentations which were considered complete (less than 4.5 g/L total
189 sugar) were analysed for changes in the selected aroma compounds. Each 'dry' fermentation was
190 analysed following the general SPME procedure and run under the validated headspace solid-
191 phase microextraction method coupled with gas chromatography mass spectrometry (HS-SPME
192 GC-MS) as set out in a previous paper [21].

193 Quality assurance (QA) was carried out in the same manner as described elsewhere [21]. Each QA
194 run was set prior to using a new fibre with a mini curve containing dilutions 1, 3, 5, 7, 9 and 11 in
195 duplicate. Prior to each 24 hour run a blank sample containing deionised water and salt was run in
196 duplicate as well as duplicate samples of dilutions 3. Samples of dilution 3 were repeated at the
197 middle and end of the run.

198 After quantifying the aroma compounds present, the data was analysed using statistical software
199 (SIMCA) to highlight important clones for further analysis in subsequent experiments.

200 **3. Results and Discussion**

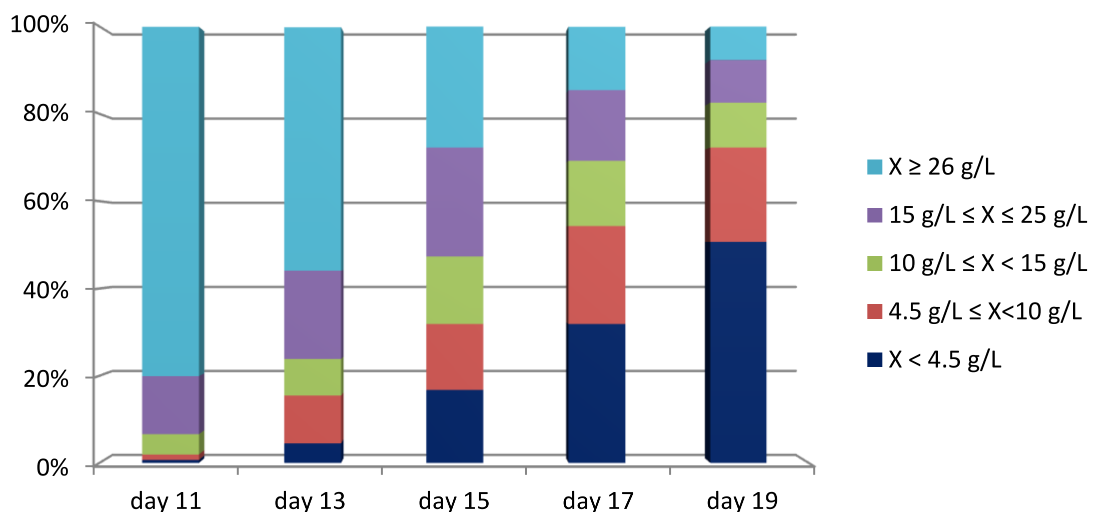
201 3.1 Overexpression library preparation

202 After plasmid extraction and purification, electrophoresis confirmed single bands corresponding to
203 the expected plasmid size in each sample preparation (data not shown). All the purified plasmid
204 preparations were transformed into the isoC9d ΔLeu2 strain. For the few transformations that were
205 unsuccessful, repeat attempts were made with altered parameters (e.g. plasmid concentration,
206 incubation time or full-scale method). In this way all plasmids were successfully transformed into
207 the wine yeast background (data not shown).

208

209 3.2 Fermentation screen

210 Fermentation progress by the parental strain was followed by sugar analysis through the screen
211 revealing an expected fermentation, similar to that seen in the trial experiments and a similar linear
212 relationship for Brix plotted against the total sugars (Figure S3). The time points for analysis of the
213 entire library were set at 11, 13, 15, 17 and 19 days of fermentation, with a set of plates being
214 removed on each day and frozen for subsequent determination of residual sugar. The distribution of
215 the individual clones into groups according to their residual sugar gives an indication of the
216 progress of fermentation (Figure 5). The parental strain finished fermentation on day 16, which was
217 slightly later than expected. Overall, only 51 % of the clones completed fermentation (<4.5 g/L)
218 during the duration of the experiment. A total of 17% of the clones finished fermentation before the
219 parental strain and about 8% of the fermentations had stuck.



220

221 Figure 5: Progression of fermentation shown as percentage of total library (X) within certain total
222 sugar brackets at days 11, 13, 15, 17 and 19 of fermentation; where (■) < 4.5 g/L; (■)
223 4.5 g/L ≤ X <10 g/L; (■) 10 g/L ≤ X <15 g/L; (■) 15 g/L ≤ X ≤ 25 g/L; (■) X ≥ 26 g/L.

224

225 3.3 Aroma compound analysis

226 Aroma analysis was performed on all wells that were considered dry and the area under the
227 individual peaks was used as a semi-quantitative measure of the amount of aroma compounds
228 present. Prior testing of the storage conditions (data not shown) indicated there to be no/limited
229 change in the aroma compounds during storage of the fermentation samples prior to analysis and
230 that any differences seen were in fact due to the clones used in the study.

231 Principle component analysis (PCA) was performed on the results of the aroma analyses acquired
232 by GC-MS. The Hotelling's plot revealed 6% of the clones lay above the 95% confidence interval
233 giving a total of 47/756 clones of potential interest (Figure 6). According to the distance to the
234 model plot (DModX) 9% of the clones lay above the 95% confidence interval giving a total of
235 70/756 possible interesting clones (Figure 7). Some of the clones overlap in the Hotelling's model
236 and the DModX, giving a total of 92 overall outliers, of which 87 were overexpression clones and 5
237 were commercial yeast. These results show importance in 12% of clones which completed
238 fermentation or 0.06 % of the initial clones.

239

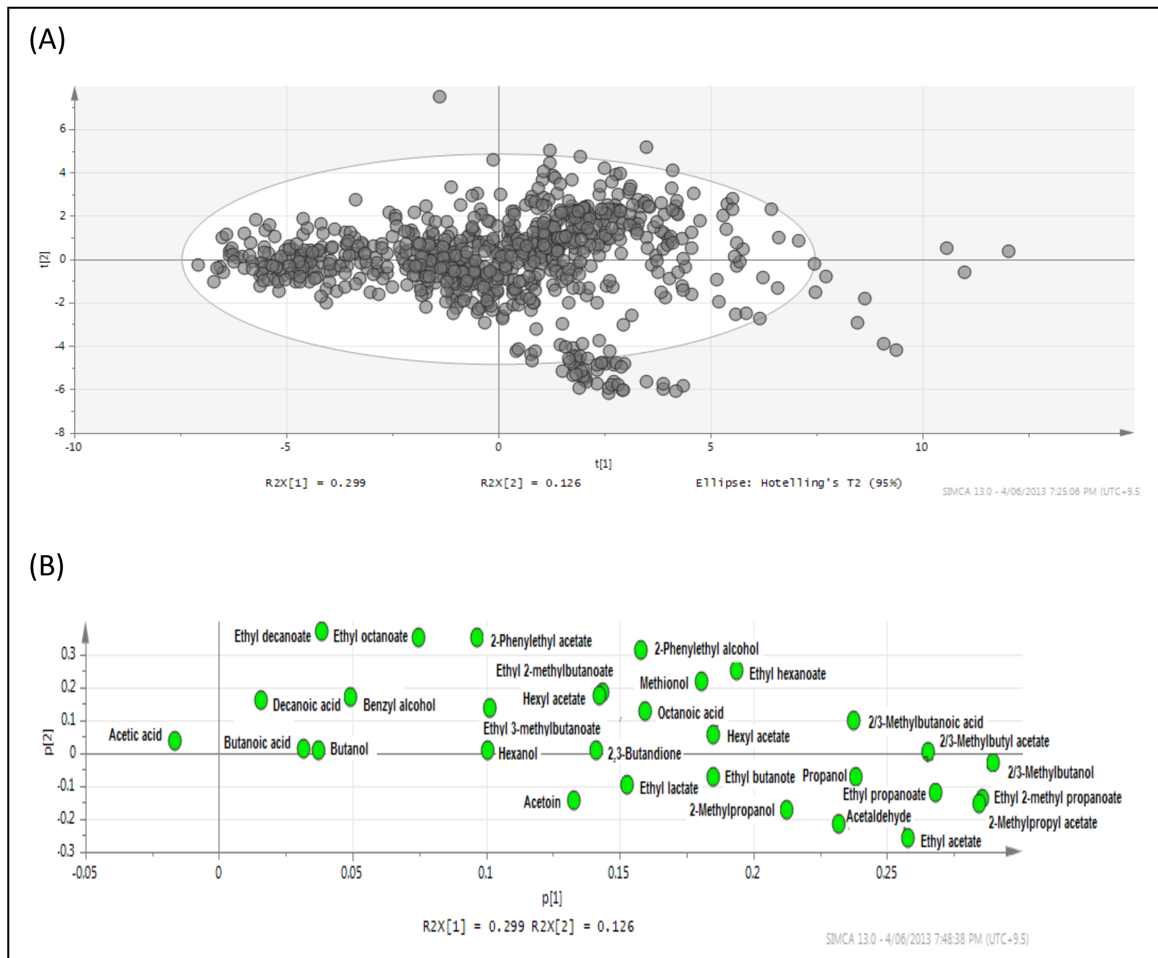


Figure 6: (A) Principal Component Analysis (PCA) plot using a Hotelling's model at the 95% confidence interval to detect potentially interesting clones. (B) Loading's plot showing the influence that each aroma compound has on the model.

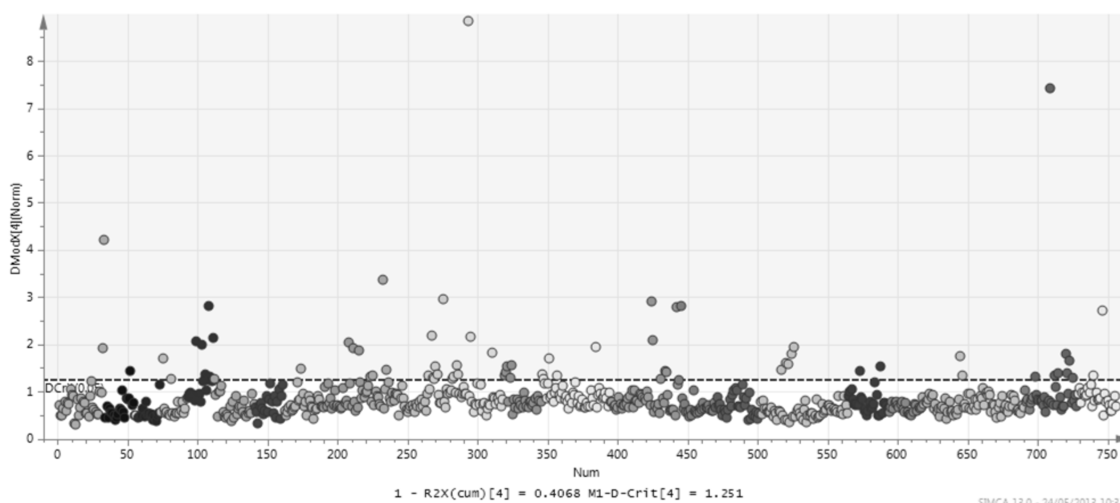
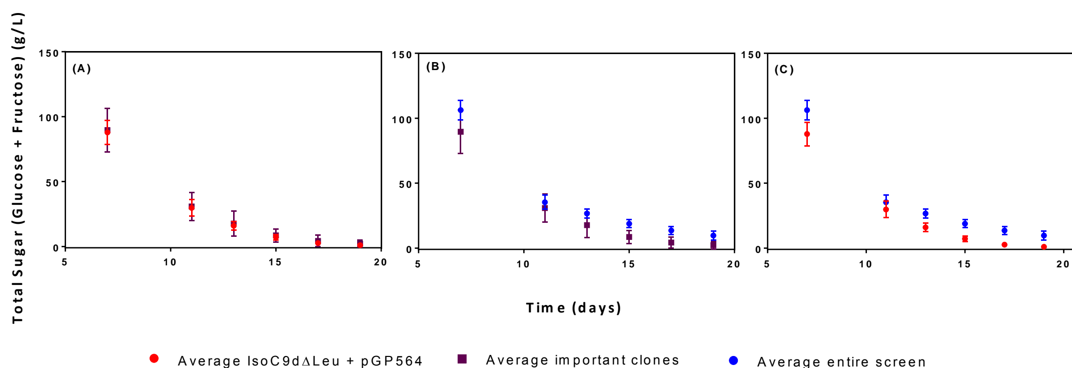


Figure 7: Distance to the model (DModX) plot showing potentially interesting clones (those above the line at 1.251)

The aroma profiles of the 87 clones were compared by classes including acids, alcohols, ethyl esters, acetate esters or miscellaneous. The comparison of the average of each class for the entire screen showed that 9/87 or 10 % of the selected clones had increased concentrations of all classes of compounds and 5/87 or 6 % had an overall decrease in concentration in all classes of compounds (Table S3). When analyzing the change, (greater or less than the entire library average for each class) it was seen that clones bearing some of the same genes in their over-expression constructs showed similar changes (Table S3-S4). It was also noted that 78 % of the plasmids in the 87 selected clones contain at least one gene that has previously been used in overexpression experiments and showed a decrease in vegetative growth rates (Table S4) [22]. This result supports the hypothesis put forward in the *Supplementary Information* 'Testing of library and fermentation conditions' that plasmid retention is enhanced where the retained plasmid additionally imparts a beneficial quality, else the plasmid is likely to be rejected. It is conceivable that possession of the *LEU2* selectable marker alone is not sufficient for the cell to retain the plasmid once there is an insert in the plasmid, and is also dependent on cell growth rate; where a faster growth rate enables the cell to remove the plasmid more often [23]. However, Zhang [24] proposed that increasing the G2/M phase of the cell cycle would decrease plasmid loss and mutations therein. It is possible then that in this study, plasmids containing an important gene or whose overexpression decreased vegetative growth were being preferentially retained.

279 3.4 Analysis of fermentation curves

280 Analysis of the fermentation curves formed during the overexpression screen were performed using
281 three average fermentation curves. The three fermentation curves were generated using the
282 average of the total sugar present on each day of analysis; for the entire overexpression screen, for
283 the clones of interest and for the 21 replicates of the blank isoC9d ΔLeu2 + pGP564 used within the
284 experiment. When comparing the fermentation curves it is clear that the important clones on
285 average followed almost the same fermentation curve as the blank isoC9d ΔLeu2 + pGP564, but
286 had a greater standard deviation (Figure 8A). Comparison of the average of the entire screen and
287 the blank isoC9d ΔLeu2 + pGP564 shows significantly different fermentation curves, where the
288 entire library shows a slower rate of fermentation (Figure 8B). This slower fermentation rate is
289 expected, since only half of the screen finished fermentation within the allocated time-frame. When
290 comparing the entire screen with the clones of interest there were similarities in fermentation
291 curves, but only due to overlapping standard deviations (Figure 8C). In previous experiments (data
292 not shown), similar fermentation curves were seen between the blank isoC9d ΔLeu2 + pGP564 and
293 the parental isoC9d strain. This in turn suggests that the important clones in general followed
294 similar fermentation curves to the parental strain, and that in general the entire library followed a
295 significantly different fermentation curve to the parental strain and some similarity with the
296 'important clones'.



297

298 **Figure 8:** Comparisons between the fermentation curves. (A) the average of the important clones
299 (■) and the average of 21 blank isoC9dΔLeu + pGP564 ferments (•); (B) the average of the
300 important clones (■) and the average of the entire fermentation screen (•) and (C) the average of
301 21 blank isoC9dΔLeu + pGP564 ferments (•) and the average of the entire fermentation screen (•).

302

303 **4. Conclusions:**

304 Overall, this project follows on from a previous study [21], using the entire Jones *et al.* [13]
305 overexpression library. The results herein show that the entire overexpression library [13] is able to
306 be transformed into a wine yeast background strain and utilised in fermentation studies using a
307 chemically defined medium lacking leucine to find potentially interesting genes for improved aroma
308 compound formation. The results of the screen showed that 51% of the library finished fermentation
309 within the allocated time period and that of these 737 clones + 19 Laffort yeast, we were able to
310 identify 92 yeast which differed from the rest of the library with respect to their aroma profile. Of
311 these interesting yeast, 87 were overexpression clones and 5 were commercial yeast. These
312 interesting yeast should be used in further experiments to confirm their phenotype and to provide a
313 more accurate quantification of the aroma compounds formed at the end of fermentation. In this
314 study it was also hypothesised that for yeast to retain their plasmid when bearing an
315 overexpression insert, the *LEU2* marker was not itself sufficient and that a faster growth rate would
316 increase the rate of plasmid rejection. For the plasmid to be retained, either a beneficial insert
317 gene, or a gene which when overexpressed decreased vegetative growth needed to be present.
318 These results are beneficial for future studies using or creating overexpression libraries for
319 fermentation studies.

320 **Notes.** The authors declare no competing financial interest.

321

322 **5. References**

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1 # Supporting Information

2

3 ## Fermentation screen of a *Saccharomyces cerevisiae* overexpression library:

4 ## linking aroma profiles to genotype

5

6 **J. HAGGERTY, D. K. TAYLOR and V. JIRANEK**

7

8 **Table of Contents**

9 1. **Testing of Overexpression library**
10 2. **Supplementary Tables S1-S5**
11 3. **Supplementary Figures S1-S11**

12

Testing of library and fermentation conditions prior to screening

1. Methods

1.1 Test for retention of plasmids in different starter media

Transferal of yeast from glycerol stocks to minimal drop-out was performed whereby the entire glycerol stock plate was thawed, the cells resuspended and 2 µL of the cell suspension transferred into 200 µL minimal DO-Leu (20 g/L sugar), as well as a back-up Min DO-Leu agar plate, using a multichannel pipette. In this test, three clones were chosen from plate 00R; well A11 containing isoC9d ∆Leu2 + pGP564, well A2 containing isoC9d ∆Leu2 + plasmid 1 (strain 1) and well G9 containing isoC9d ∆Leu2 + plasmid 2 (strain 2). Inoculated plates were incubated for 2 days to ensure sufficient growth of all strains. Then, cultures were grown overnight in one of three starter media at an inoculation rate of 5 x 10^6 cells/mL. The starter media tested were Minimal DO-Leu, with 100 g/L of sugar as glucose and Tween 80 and ergosterol; a ½ minimal DO-Leu + ½ CDGJM-Leu with 100 g/L sugar; and a CDGJM-Leu starter with 100 g/L sugar. After overnight growth ca. 200 cells from each medium were spread plated in duplicate on both YPD, Minimal DO-Leu agar and CDGJM-Leu agar incubated overnight at 28 °C

1.2 Test for retention of plasmids during fermentation in 2.0 mL deep-well plates

Trial fermentations were set up using isoC9d and five variants of isoC9d ∆Leu2 bearing plasmids, including; isoC9d ∆Leu + pGP564, Plate 00R clone E6, Plate 00R clone G9, Plate 00R clone D10 and Plate 00R clone G3. Four of the strains were the same as those used in previous retention trials, and two were new strains. These two new strains were chosen from plate 00R which had already been opened and carried plasmids which were not detrimental to yeast growth.

Fermentations were set up in 100 mL volumes in a conical flask set-up at an inoculation rate of 5 x 10^6 cells/mL. The retention of the plasmids was performed in the same manner as set out previously,

37 Supplementary information Section 1.1, using only the CDGJM (20 g/L) agar plates. Testing was
38 performed before fermentation on the third day of fermentation and then after completion of
39 fermentation.

40 ### 1.3 Test for maximum fermentation volume able to be used in 2.0 mL deep-well plates

41 Two different sets of plates were made with different fermentation volumes. Thus aliquots of 1200
42 µL (dispensed by a Corbett liquid handling robot) or 600 µL (dispensed by hand) were inoculated
43 with the starter culture at a rate of 2.5 x 10^6 cells/mL using a the liquid handling robot. Each row in
44 each plate was inoculated with isoC9d or isoC9d ∆Leu + pGP564 in alternate rows. Column 11 was
45 left with only medium and column 12 was left blank for sugar testing, as shown in Figure S1.

46 The plates were covered with Breathe-Easy™ toppers and stored on wet paper towel in plastic tubs
47 and covered with cling wrap.

48 On days 3, 4, 5, 7 and 8, one plate of each volume was removed, spun down and the supernatant
49 frozen until residual glucose and fructose content were determined by an enzymatic method [1]
50 adapted for 96-well plates.

51 ### 1.4 Test for fermentations in 2.4 mL deep-well plates

52 The fermentations were performed in a similar manner as the 2.0 mL trial. The 2.4 mL plates were
53 set up with 1.8 mL volume ferments with an inoculation rate of ca. 2 x 10^6 cells/ mL in a horizontal
54 'half-half' plate set-up. Half of the plate was inoculated from a single colony and half from a smear
55 growth culture. The fermentations were followed by total sugar and degree brix using different
56 replicate plates until day 6, 8 and 9, where the whole plate was analysed for total sugar. Residual
57 glucose and fructose content was determined from supernatant by an enzymatic method [1] adapted
58 for 96-well plates.

59

60 1.5 Test for retention of plasmids during fermentation

61 Trial fermentations were set up using isoC9d and five variants of isoC9d ∆Leu2 bearing plasmids,
62 including; isoC9d ∆Leu + pGP564, Plate 00R clone E6, Plate 00R clone G9, Plate 00R clone D10
63 and Plate 00R clone G3. Four of the strains were the same as those used in previous retention trials,
64 and two were new strains. These two new strains were chosen from plate 00R which had already
65 been opened and carried plasmids which were not detrimental to yeast growth.

66 Fermentations were set up in 100 mL volumes in a conical flask set-up with an inoculation rate of 5
67 x 10^6 cells/ mL. The retention of the plasmids was performed in the same manner as set out
68 previously, *Supplementary information* Section 1, using only the CDGJM (20 g/L) agar plates. Testing
69 was performed before fermentation on the third day of fermentation and then after completion of
70 fermentation.

71 **2. Results and discussion**

72 2.1 Plasmid retention during starter culture preparation

73 Variations of the starter media to be used including Minimal DO-Leu with 100 g/L of glucose including
74 Tween 80 and ergosterol; a ½ minimal DO-Leu + ½ CDGJM–Leu with 100 g/L sugar; and a CDGJM-
75 Leu starter with 100 g/L sugar were tested for highest retention of plasmids. The inclusion of the
76 parental strain, iso-C9d, along with the clones containing plasmids with and without inserts was used
77 to see whether differences observed were due to the loss of plasmid. Appropriately diluted cultures
78 were applied to a CDGJM-Leu agar to determine the overall extent of plasmid retention according to
79 the starter media being used and the type of selective agar being used in plating. Although there was
80 little difference between the retention of the plasmids with each type of starter medium (Table S1), a
81 marginally higher retention was seen with the 50:50 and the chemically defined grape juice (CDGJ)
82 starter medium. For ease of preparation, the CDGJ starter was used in subsequent experiments.
83 Insert Table S1 here

84 Overall, it was seen that there was an influence from the strain, the starter and the plating medium
85 used. However a marked consistency of outcome was seen for a given strain across the media
86 combinations tested, suggesting that the strain was the more dominant variable. Low levels of
87 recovery of all strains when cultured in either Min DO-Leu or 50:50 medium indicate that growth and
88 viability were poor when starter cultures were prepared in these media. Interestingly the best
89 combination of starter and plating conditions was seen when cultures were grown in the more
90 comprehensive CDGJM-Leu but plated onto Min DO-Leu. Even though there was a drop in colony
91 count for the parental strain, there was a similar drop in the colony count for the G9 clone, which
92 indicates that even the parental strain struggled to grow on the selective medium agar plates. For the
93 isoC9DΔLeu2, isoC9DΔLeu2isoC9DΔLeu2 isoC9DΔLeu2 + pGP564, and the E6 clone there was a
94 greater drop in colony count than the parental strain isoC9d and the G9 clone. This implies that the
95 cells tended to retain their plasmid if there was a benefit derived from the overexpressed gene and
96 that the *LEU2* selective marker was not enough for the cell to retain the plasmid. Another possible
97 reason for differences in the loss of plasmid could be the rate of growth of the cells, where faster
98 growth provided more opportunities for plasmid loss [2]. This can be explained by the fact that
99 previous overexpression experiments have shown that one of the genes within the G9 clone, *SNT1*,
100 showed decreased vegetative growth [3, 4]. This seems counterintuitive since the G9 clone grew well
101 in all media; however this observation of growing well may be due to the fact that the plasmid is kept
102 rather than there being a faster growth rate. The E6 clone appeared to grow slower than the other
103 clones and overexpression experiments previously performed on genes within this clone do not
104 indicate any effect on the growth of the cell due to its overexpression [4]. This implies that the clone
105 grew quickly and stopped replicating the plasmid, after which the cells died due to not being able to
106 synthesise leucine in a leucine deficient media.

107

108 2.2 Testing the retention of plasmids during fermentation

109 The cultures were tested for retention of the plasmids before fermentation, on the third day of
110 fermentation and then after completion of fermentation. The results showed similar trends for
111 retention of the plasmid for each clone used (Table S2). There were trends with regards to the strain
112 and the extent of growth in the various media. Both the parental strain and clone from plate 00R G9
113 showed similarity in the colony count on both YPD and selective agar. It was also seen that the other
114 clones previously tested again showed poor plasmid retention. Clones from plate 00R G3 and D10
115 were chosen for inclusion due to their possession of genes deemed important for fermentation; the
116 G3 clone includes *ADH7* whose product facilitates conversion of acetaldehyde to ethanol [4] and the
117 D10 clone includes *YPI1* encoding a protein conveying ionic stress resistance [4]. Also noteworthy,
118 loss of plasmids was increased during the preparation of the starter cultures and retention was
119 increased as fermentation progressed with cells retaining viability at the end of fermentation having
120 high percentage retention of plasmids (Table S2). These results suggest most plasmid loss occurred
121 during the preparation of the starter cultures. This implies that expression of *LEU2* is not enough to
122 ensure plasmid retention by the cell. As shown in this experiment, the beneficial genes chosen did
123 not increase plasmid retention during growth, as expected, therefore the notion outlined in section
124 2.1 in the supplementary information is supported, i.e. that differences in the loss of plasmid could
125 be due to the rate of growth of the cells, where a faster growth rate enabled the cell to remove the
126 plasmid more often.

127 Insert Table S2 here

128 2.3 Evaluation of fermentations performed in 2 mL deep-well plates

129 Deep-well plate fermentations have previously been performed in this laboratory in a maximum of
130 600 µL volumes in 2 mL well plates. For optimal aroma compound analysis, a volume of 1.5 mL of
131 sample wine was needed. Therefore two volumes were trialled: 600 µL and 1200 µL per well. The

coefficients of determination for the standard curves used to analyse the sugar content in both trialled volumes show good accuracy for predicting the sugars in the fermentation with values being greater than 0.99 (data not shown).

The total residual sugars were determined for each volume trial and each strain, A11 (isoC9d + pGP564) and isoC9d. The average was then taken for each row and then each strain per trial. These results showed a gradual decrease in sugars during fermentation and that there was limited variation introduced according to well position in the plate (data not shown). Overall, by day 8 the 1200 µL ferments had completed and the 600 µL ferments followed closely with outlying samples finished within the next day. It was also noted that the parental strain completed fermentation sooner than isoC9d + pGP564 in both volume trials. These results indicate that removing the plates at day 8 allowed the parental strains to complete, but that some clones would not be finished. To allow for the eventuality that fermentations would finish at different times, replicate fermentations were prepared so that sampling could occur at several points in relation to the performance of the parent strains e.g. 4 and 2 days prior to parent completion, the same day as parental completion, and 2 and 4 days after the parents completed.

It has been shown in two different experiments [5, 6] that aroma compounds are formed during the exponential phase of yeast growth during fermentation. From this point the compounds attain a peak and depending on the compound, either exponential decay occurs, or a steady state maximum is retained. This implies that if a fermentation is not stopped as soon as it is complete, there should be little change in aroma compound concentration if only left for a short period of time.

2.4 Evaluation of fermentations performed in 2.4 mL deep-well plates:

The 2.4 mL deep-well plate trials were followed daily by °Brix and by total sugar determinations (data not shown), revealing a similar trend to that seen in the 2.0 mL plate trial.

155 Due to the decision to stop fermentations at several time points bracketed around the completion
156 time of the parent strain, a curve relating Brix to residual sugar was prepared to enable fermentation
157 progress to be estimated and kinetics and thereby harvest times to be predicted from Brix readings
158 (Figure S3).

159

160 **References for Supplementary information**

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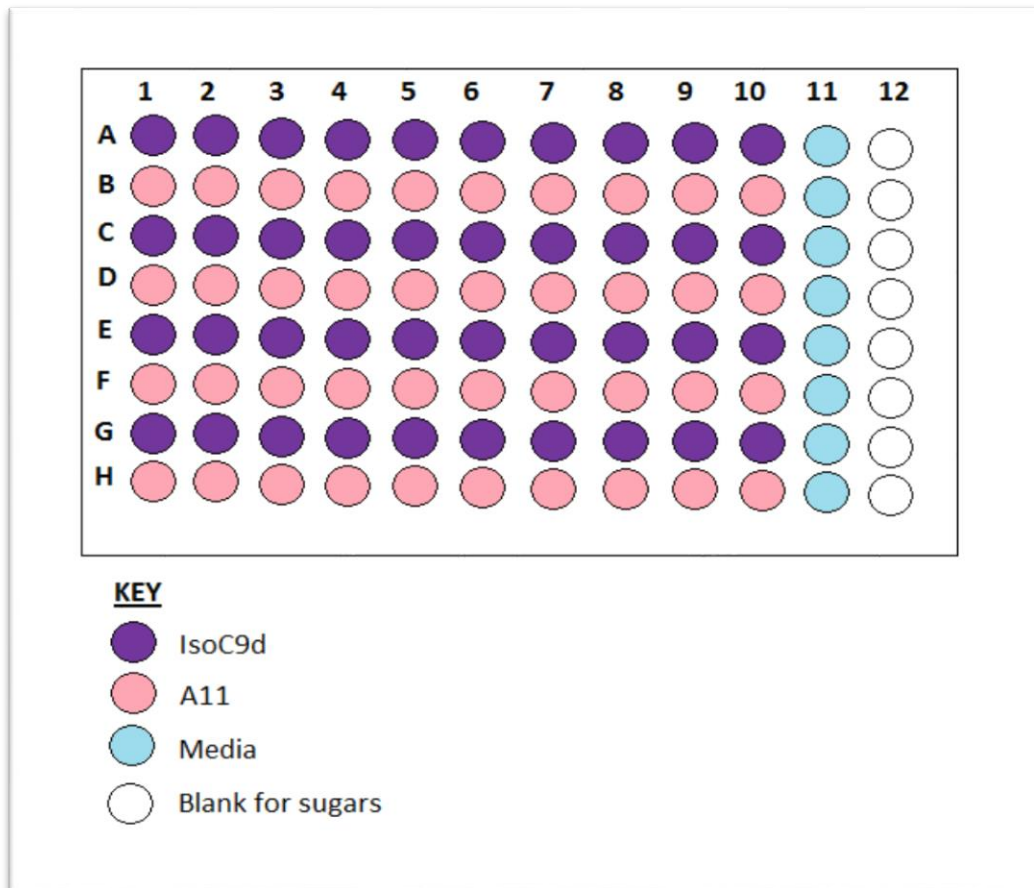
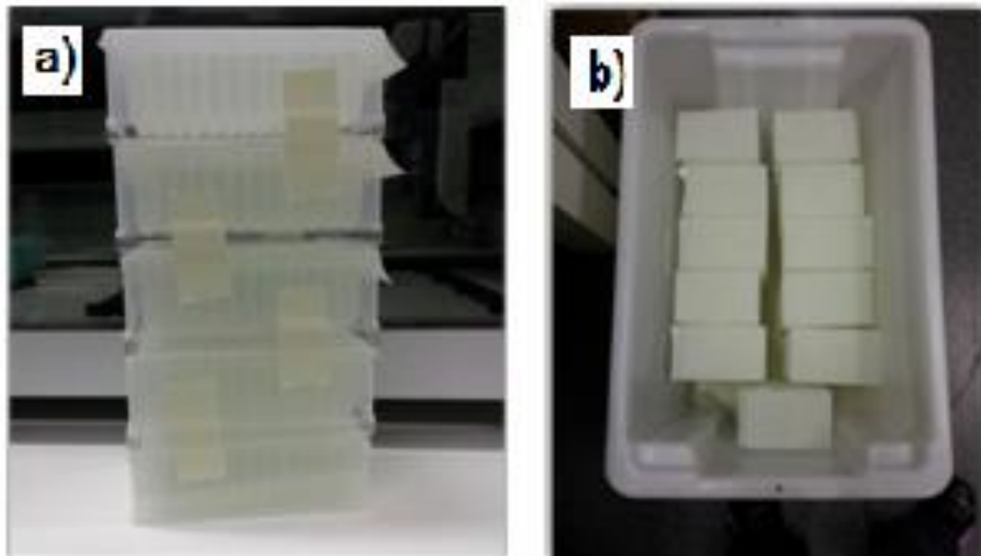


174

175 Figure S1: plate set-up for trial fermentations



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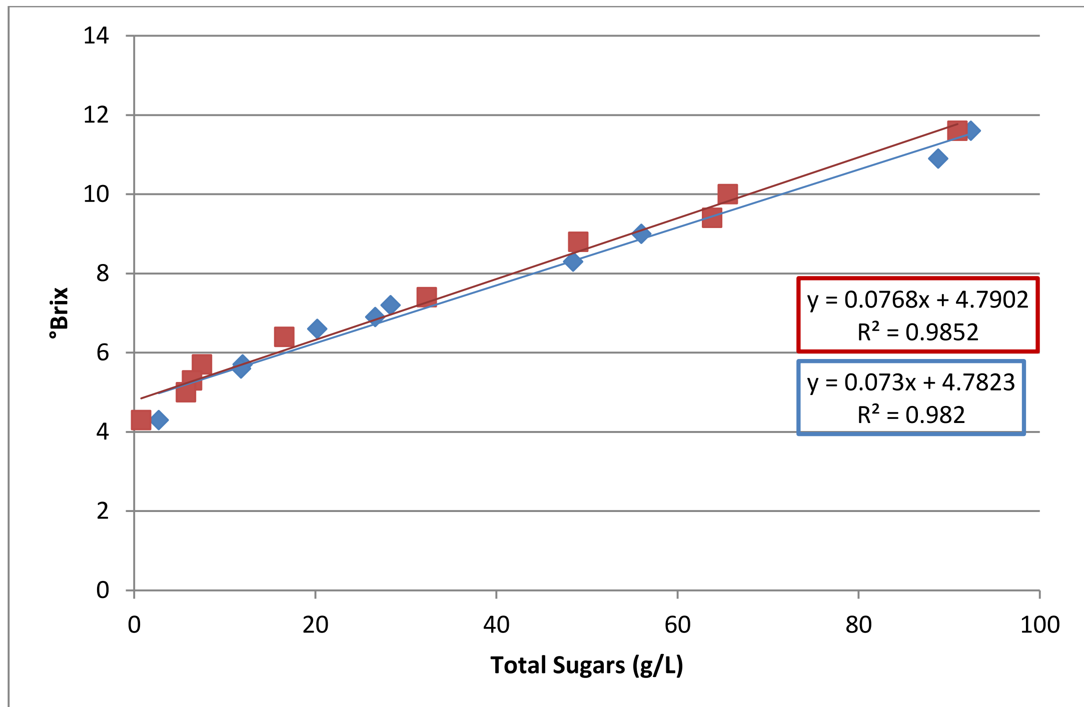
177 Figure S2: (a) stacks of fermentations in deep-well plates with stacker gaps. (b) inside one of the
178 tubs where fermentation took place



179

180 Figure S3: Linear relationship for °Brix vs total sugars for approximating time-points for removal of
181 plates in the screen from 2.4 mL deep-well fermentation. (■) well E1 single colony (■) well A1 smear.

182

183 Table S1: Number of colonies recovered upon duplicate plating of ca. 200 cells onto either YPD
184 agar or one of two different selective media. Plasmid retention is expressed as a
185 percentage of the number of colonies found on the appropriate selective medium
186 compared to YPD.

Strain	Starter Medium	YPD	Min DO-Leu		CDGJM-Leu	
		Average number of colonies	Average number of colonies	Average number of colonies compared to YPD (%)	Average number of colonies	Average number of colonies compared to YPD (%)
A11	CDGJM-Leu	199	128	64%	115	58%
	Min DO-leu	96.5	57.5	60%		
	50:50	174	111	64%		
G9	CDGJM-Leu	176.5	154	87%	143.5	81%
	Min DO-leu	84	71	85%		
	50:51	121.5	104	86%		
IsoC9d	CDGJM-Leu	240.5	197.5	82%	187.5	78%
	Min DO-leu	117	88	75%		
	50:50	186.5	154	83%		
E6	CDGJM-Leu	227	75	33%	93	41%
	Min DO-leu	132	33	25%		
	50:50	173	66.5	38%		

187

188

189 Table S2: Percentage of colonies recovered on selective media compared to YPD upon plating of
190 ca. 200 cells in duplicate onto CDGJM-Leu selective media at different stages of fermentation.

Strains	% Growth on CDGJM-Leu 20 g/L sugar compared to YPD		
	Before fermentation	After 3 nights growth	After end of fermentation
A11	42%	74%	69%
G9	111%	102%	129%
IsoC9D	97%	82%	118%
E6	18%	39%	43%
G3	40%	58%	89%
D10	45%	51%	82%

191

192

193 Table S3: Comparison of the summation of the ratio to the internal standard of each category of
194 aroma compounds for each clone compared to the average of each category of the entire
195 screen

		summation of each aroma compound's ratio to the internal standard				
		acids alcohol ethyl acetate misc				
Average of entire screen		0.33	12.10	4.93	0.94	0.18
Day analysed	Plate	acids	alcohol	ethyl	acetate	misc
Day 10	00A_B4	0.48	18.21	5.79	1.62	0.36
Day 10	00A_C2	0.44	17.09	4.76	1.72	0.35
Day 10	00A_C4	0.47	19.29	7.96	1.86	0.37
Day 10	00A_D3	0.65	17.61	5.17	1.71	0.31
Day 10	00A_E5	0.42	16.62	6.06	1.52	0.51
Day 4	00B_C2	0.45	8.97	5.04	0.96	0.07
Day 12	00B_D3	0.29	14.27	4.74	1.37	0.37
Day 12	00B_E10	0.23	18.60	5.09	1.64	0.62
Day 12	00B_F4	0.21	15.41	4.76	1.48	0.24
Day 12	00B_F6	0.21	13.42	4.49	1.39	0.39
Day 12	00B_G5	0.27	15.25	4.56	1.59	0.41
Day 12	00B_G9	0.26	17.83	4.90	1.79	0.42
Day 4	00B_H10	0.31	6.40	2.39	0.53	0.21
Day 10	00C_D4	0.44	14.52	6.45	1.33	0.17
Day 10	00C_E9	0.42	14.06	4.83	1.07	0.36
Day 26	00D_A10	0.26	11.84	4.58	0.85	0.30
Day 26	00D_B5	0.25	13.11	5.69	1.03	0.25
Day 12	00E_D10	0.45	14.90	5.35	1.21	0.23
Day 5	00E_H6	0.51	13.34	7.32	1.19	0.25
Day 12	00E_H8	0.31	16.27	6.31	1.32	0.40
Day 12	00F_A10	0.28	15.41	4.83	1.33	0.25
Day 12	00F_D5	0.32	16.64	5.94	1.72	0.27
Day 12	00F_E5	0.23	16.53	5.54	1.35	0.22
Day 12	00F_F4	0.34	17.39	8.52	1.74	0.30
Day 5	00F_F6	0.49	15.84	3.52	1.27	0.34
Day 21	00G_A2	0.33	11.27	3.65	0.96	0.21
Day 13	00G_B2	0.43	12.77	2.51	1.61	0.15
Day 21	00G_B7	0.27	12.44	4.11	1.15	0.16
Day 13	00G_C2	0.39	11.00	3.10	1.60	0.30
Day 21	00G_C5	0.26	12.64	4.79	1.10	0.17
Day 21	00G_F8	0.27	12.46	4.52	1.19	0.17
Day 21	00G_G4	0.27	13.40	6.65	1.19	0.26
Day 13	00G_G7	0.38	11.42	3.02	1.79	0.32
Day 14	00H_A1	0.43	10.85	4.06	1.27	0.14
Day 14	00H_A5	0.40	11.54	3.75	1.37	0.16
Day 14	00H_B5	0.39	11.37	3.27	1.54	0.14
Day 5	00H_C5	0.42	13.23	4.48	1.04	0.28
Day 14	00H_C9	0.39	11.17	3.29	1.60	0.14
Day 14	00H_D2	0.41	10.98	3.11	1.53	0.13
Day 5	00H_D5	0.50	15.08	4.39	1.37	0.23
Day 5	00H_E5	0.26	12.78	3.99	1.09	0.22
Day 5	00H_F4	0.48	15.40	5.41	1.24	0.18
Day 14	00H_G2	0.36	11.85	2.86	1.62	0.38
Day 14	00H_H7	0.37	13.13	3.74	1.80	0.36
Day 14	00I_A2	0.43	12.84	3.87	1.77	0.42
Day 14	00I_E3	0.36	11.85	2.84	1.59	0.24
Day 14	00I_E5	0.36	12.05	3.29	1.69	0.29
Day 23	00I_G8	0.32	13.80	5.30	0.78	0.12
Day 14	00I_H2	0.37	12.62	3.66	1.78	0.28
Day 6	00J_A2	0.69	13.51	7.15	1.24	0.25

		summation of each aroma compound's ratio to the internal standard				
		acids alcohol ethyl acetate misc				
Average of entire screen		0.33	12.10	4.93	0.94	0.18
Day analysed	Plate	acids	alcohol	ethyl	acetate	misc
Day 14	00J_A3	0.37	11.89	3.34	1.67	0.36
Day 14	00J_A9	0.36	11.62	2.80	1.65	0.29
Day 6	00J_B4	0.59	12.98	9.09	1.23	0.11
Day 23	00K_A5	0.25	12.46	3.94	0.81	0.44
Day 13	00K_A6	0.41	11.26	3.27	1.71	0.35
Day 13	00K_C6	0.39	11.21	3.22	1.54	0.25
Day 13	00K_E5	0.40	12.75	3.75	1.69	0.37
Day 13	00K_E6	0.44	12.52	3.88	1.79	0.41
Day 16	00M_E2	0.64	14.36	3.65	0.89	0.18
Day 15	00N_A2	0.59	12.95	3.43	0.57	0.09
Day 15	00N_B1	0.58	12.76	5.84	0.56	0.09
Day 15	00N_C10	0.68	14.47	5.88	0.86	0.24
Day 8	00N_C5	0.69	11.83	4.02	0.82	0.15
Day 15	00O_B4	0.66	14.50	6.34	0.95	0.14
Day 17	00Q_B6	0.46	14.50	7.44	0.93	0.20
Day 17	00Q_D5	0.40	16.27	6.25	0.98	0.32
Day 17	00Q_F5	0.27	16.89	7.35	1.09	0.34
Day 17	00Q_H7	0.26	15.03	6.04	0.95	0.25
Day 18	00R_A2	0.46	13.35	7.27	0.74	0.12
Day 18	**00R_A5**	**0.38**	**13.40**	**4.39**	**0.80**	**0.11**
Day 18	00R_C5	0.34	14.85	7.96	0.67	0.20
Day 18	00R_E9	0.27	14.86	7.13	1.13	0.25
Day 18	00R_F3	0.30	13.67	4.77	1.01	0.20
Day 18	00R_H7	0.41	16.31	9.18	1.16	0.57
Day 19	00S_A5	0.32	11.53	6.44	0.71	0.10
Day 29	00T_A1	0.21	8.78	3.17	0.44	0.08
Day 29	00T_E1	0.22	9.57	4.88	0.53	0.13
Day 29	00T_E7	0.27	10.42	5.14	0.69	0.25
Day 29	00T_F1	0.39	9.48	1.34	0.53	0.24
Day 29	00T_G10	0.25	10.04	3.46	0.66	0.25
Day 29	00T_H1	0.25	10.02	4.74	0.58	0.23
Day 29	00T_H3	0.28	9.78	3.23	0.51	0.16
Day 29	00T_H5	0.21	10.85	2.78	0.60	0.28
Day 9	00U_A1	0.53	12.25	6.45	0.86	0.19
Day 30	00U_A8	0.34	11.56	**10.80**	0.48	0.13
Day 9	00U_C1	0.60	12.67	6.85	0.93	0.35
Day 30	00U_C5	0.23	11.37	9.18	0.73	0.16
Day 9	00U_G1	0.88	10.65	5.07	0.83	0.12
Day 2	00U_G2	0.41	5.28	2.04	0.44	0.06
Day 2	00U_G5	0.26	7.42	2.78	0.55	0.16
Day 1	00U_H10	0.08	5.52	0.70	0.33	0.10
Day 1	00U_H9	0.40	5.75	3.20	0.65	0.05

197 Table S4: Genes included in the plasmid included in the 'interesting clones'; (■) genes which are involved in the cell cycle, (■) genes which when
198 overexpressed show a decrease in vegetative growth rates, (■) genes which are duplicated in another 'important' clone, (■) genes which are known
199 to be involved in the formation of aroma compounds, (■) genes which could possibly be important in the formation of aroma compounds, (**BOLD**)
200 genes which look like they show a direct impact on specific results seen in differences in aroma compounds, (■) clones which showed a dramatic
201 overall increase in production of all categories of aroma compounds.

202

Obs ID (Primary)	CHROMOSOME	Genes included in plasmid								
00A_B4	I	[YAR064W]&	YAR066W	YAR068W	YAR069C	YAR070C	PHO11	IMD1	[YAR075W]	
00A_C2	I	[FRT2]&	YAL027W	YAL026C-A	DRS2	MAK16	[LTE1]&			
00A_C4	I	YAR060C	YAR061W	YAR062W	YAR064W	YAR066W	YAR068W	YAR069C	YAR070C	PHO11
00A_D3	I	[YAR009C]*	YAR010C	tA(UGC)A	BUD14	ADE1	KIN3	[CDC15]&		
00A_E5	II	[YBL086C]	BOI1	CDC27	YBL083C	[RHK1]&				
00B_C2	II	[SLI15]*	ICS2	AMN1	YBR159W	CDC28	CSH1	TOS1	YSY6	[DEM1]*
00B_D3	II	[CDC47]&	COS111	YBR204C	KTR3	YBR206W	FTH1	[DUR1%2C2]&		
00B_E10	III	[RAD18]&	SED4	ATG15	CPR4	IMG2	[YCR072C]&			
00B_F4	II	[YBR238C]*	YBR239C	THI2	YBR241C	YBR242W	ALG7	GPX2	[ISW1]&	
00B_F6	III	[YCL069W]	YCL068C	HMLALPHA2	HMLALPHA1	YCL065W	CHA1	[VAC17]*		
00B_G5	II	[SSH1]*	YBR284W	YBR285W	APE3	ZSP1	APM3	[SNF5]*		
00B_G9	III	[BPH1]&	SNT1	FEN1	RRP43	RBK1	[PHO87]&			
00B_H10	III	[PAT1]*	YCR079W	SRB8	YCR081C-A	AHC2	TRX3	[TUP1]&		
00C_D4	IV	[PHO2]*	QRI2	QRI7	QRI1	CDC2	DUN1	[ARR4]&		
00C_E9	IV	[PET100]&	TFB5	VPS41	**PDC2**	STN1	[RRP8]*			
00D_A10	IV	RPS18A	YHP1	PPN1	TSA2	GUK1	[YDR455C]&	[NHX1]*		
00D_B5	IV	[YDR307W]&	SRB7	GIC2	SUM1	[TFB1]*				
00E_D10	VI	[YFL054C]*	DAK2	YFL052W	YFL051C	[ALR2]&				
00E_H6	V	[SLX8]*	RPL23B	SHO1	AVT6	YER119C-A	SCS2	YER121W	GLO3	[YCK3]*
00E_H8	V	[DNF1]&	BCK2	CCA1	RPH1	ADK2	[RAD3]*			
00F_A10	VII	[YGL006W-A]	PMC1	COG7	RPN14	CDH1	ERP6	[ERG26]&		
00F_D5	VII	[SUT1]&	YIP5	YGL160W	YGL159W	tL(CAA)G1	[RCK1]*			
00F_E5	VII	[RCK1]	YGL157W	AMS1	CDC43	LYS5	[PEX14]*	[YGL152C]&		

00F_F4	VII	[TOS3]									
00F_F6	VII	[ABC1]	tW(CCA)G1	YGL118C	YGL117W	CDC20	SNF4	[YGL114W]*			
00G_A2	VII	[YGR122W]	YGR122C-A	tR(UCU)G3	PPT1	tI(AAU)G	ASN2	[YGR125W]*			
00G_B2	VII	[ASN2]&	YGR125W	YGR126W	YGR127W	UTP8	SYF2	YGR130C			
00G_B7	VII	[SLH1]&	YGR271C-A	YGR272C	YGR273C	TAF1	RTT102	[RNH70]&			
00G_C2	VII	[YGR131W]	PHB1	PEX4	[CAF130]*						
00G_C5	VII	[CCH1]&	tG(GCC)G2	CRM1	YGR219W	[MRPL9]					
00G_F8	VIII	[GOS1]*	[YHL030W-A]	ECM29	YHL029C	WSC4	[RIM101]*				
00G_G4	VII	[YGR198W]	PMT6	ELP2	YGR201C	PCT1	YGR203W	[ADE3]*			
00G_G7	VII	YGR287C	MAL13	MAL11	YGR290W	YGR291C	MAL12	YGR293C	YGR294W		
00H_A1	VIII	[KIC1]	SBE22	GRE3	YPT35	[TRR2]*					
00H_A5	IX	**[VTH1]&**	**YIL172C**	**YIL171W-A**	**YIL171W**	**YIL170W**					
00H_B5	IX	**[VTH1]&**	**YIL172C**	**YIL171W-A**	**YIL171W**	**YIL170W**	**YIL169C**				
00H_C5	IX	**YIL169C**	YIL168W	YIL167W	YIL166C	**YIL165C**	**NIT1**	**YIL163C**	**[SUC2]***		
00H_C9	IX	[NOT3]*	PRM2	CST6	CKA1	[CAP2]	[BCY1]&				
00H_D2	VIII	[SKG6]*	PEX28	YHR151C	SPO12	SPO16	[RTT107]*				
00H_D5	IX	**YIL165C**	**NIT1**	**YIL163C**	SUC2	**YIL161W**	**POT1**	**[BNR1]***			
00H_E5	IX	[SUC2]&	**YIL161W**	**POT1**	**[BNR1]***						
00H_F4	VIII	[YHR212C]*	YHR212W-A	YHR213W	YHR213W-A	YHR213W-B	YHR214W	YHR214W-A	[YHR214C-B]&		
00H_G2	VIII	[SOL3]&	DNA2	[PRP8]&							
00H_H7	IX	[AVT7]	YIL087C	YIL086C	KTR7	SDS3	YIL083C	[YIL082W-A]*	YIL082W	[YIL080W]*	
00I_A2	X	[MRS3]	YJL132W	YJL131C	[URA2]&						
00I_E3	X	[KHA1]*	TOK1	HPR5	GWT1	[DPB11]&					
00I_E5	X	[NUP192]*	tD(GUC)J3	tR(UCU)J2	YJL038C	YJL037W	tV(AAC)J	SNX4	TAD2	[KAR2]*	
00I_G8	X	[TOR1]&	YAE1	RFC2	HAM1	LIA1	YJR071W	NPA3	OPI3	MOG1	HOC1
00I_H2	X	[UTP10]*	PRM10	YJL107C	IME2	SET4					
00J_A2	XI	[SNU114]	EBP2	YKL171W	MRPL38	YKL169C	[KKQ8]&				
00J_A3	XI	[APL2]*	37529	YKL133C	RMA1	YKL131W	SHE2	[MYO3]&			

00J_A9	XI	[YSR3]*	[DYN1]&								
00J_B4	XI	[GFA1]*	LAP4	YKL102C	HSL1	YKL100C	YKL100W-A	UTP11	YKL098W	YKL097C	YKL096C-B
00K_A5	XII	[MDN1]*	REX3	snR6							
00K_A6	XII	[SLX4]&	TIS11	YLR137W	NHA1	SLS1		RRN5	PUT1	tD(GUC)L1	[YLR143W]*
00K_C6	XII	[SPE4]*	SMD3	PEP3	YLR149C	YLR149C-A	STM1	[PCD1]	[YLR152C]&		
00K_E5	XII	[CLF1]*	YLR118C	SRN2	YPS1	YLR120W-A	YPS3	YLR122C	YLR123C	YLR124W	YLR125W
00K_E6	XII	[RDN25-1]*	YLR154W-B	TAR1	RDN58-1	YLR154W-E	YLR154W-F	RDN18-1	RDN5-1	RDN25-2	YLR154C-G
00M_E2	XIII	[YMR206W]&	HFA1	ERG12	YMR209C	[YMR210W]*					
00N_A2	XIV	[TCB2]&	YNL086W	MKT1	END3	[SAL1]*					
00N_B1	XIV	YNL114C	RPC19	DBP2	CYB5	NOP15	YNL109W	YNL108C	[YAF9]		
00N_C10	XV	[IRA2]&	REX4	YOL079W	AVO1	ATP19	BRX1	MDM20	[YOL075C]&		
00N_C5	XIV	[ACC1]*	MAS6	YNR018W	ARE2	YNR020C	[YNR021W]*				
00O_B4	XV	[RPO31]*	RPT5	YOR118W	RIO1	GCY1	YOR121C	PFY1	LEO1	[UBP2]&	
00Q_B6	I	[CYS3]&	SWC3	MDM10	SPO7	FUN14	ERP2	tP(UGG)A	SSA1	YAL004W	EFB1
00Q_D5	VIII	[CBP2]*	YHL037C	MUP3	VMR1	YHL034W-A	SBP1	[RPL8A]	[GUT1]&		
00Q_F5	XII	[ERG3]&	YLR057W	SHM2	REX2	FRS1	RPL22A	BUD28	YLR063W	[YLR064W]*	
00Q_H7	IV	[COS7]&	YDL247W-A	MPH2	SOR2						
00R_A2	II	[YBR094W]&	RXT2	YBR096W	VPS15	[MMS4]*					
00R_A5	**IV**	**[ARO1]&**	**YDR128W**	**SAC6**	**FIN1**	**[YDR131C]&**					
00R_C5	IV	YCF1	VPS61	RGP1	[HPR1]*						
00R_E9	VI	[FRS2]*	YFL021C-A	GAT1	PAU5	YFL019C	tP(UGG)F	**LPD1**	SMX2	GNA1	MDJ1
00R_F3	III	[YCR100C]*	YCR101C	YCR102C	YCR102W-A	PAU3	**ADH7**	[RDS1]*			
00R_H7	IV	[tL(CAA)D]	GIN4	GNP1	YDR509W	SMT3	YDR510C-A	ACN9	EMI1	TTR1	[YDR514C]&
00S_A5	IX	[YVH1]*	DAL1	DAL4	DAL2	DCG1	YIR030W-A	DAL7	[DAL3]		
00T_A1	XII	[CDC25]*	YLR311C	YLR312C	MRPL15	SPH1	YLR312C-B	[CDC3]			
00T_E1	XII	[NMA1]&	REC102	CHS5	JIP3	MID2	**tD(GUC)L2**	**snR61**	**snR55**	**snR57**	**RPS25B**
00T_E7	XV	[YOL037C]*	[YOL036W]&	YOL035C	snR50	SMC5	MSE1	YOL032W	SIL1	[GAS5]*	

00T_F1	XII	tD(GUC)L2	snR61	snR55	snR57	RPS25B	YLR334C	tE(UUC)L	NUP2	SGD1	VRP1
00T_G10	XV	[YOR389W]&	YOR390W	HSP33	YOR392W	ERR1	YOR394W	YOR394C-A	[YOR396W]*		
00T_H1	XII	[RPL26A]	YLR345W	YLR346C	[KAP95]&						
00T_H3	XIII	[RPL15B]*	YMR122C	YMR122W-A	PKR1	YMR124W	[STO1]*				
00T_H5	XIV	RIA1	YNL162W-A	RPL42A	CBK1						
00U_A1	II	[POL12]*	STU1	RIB1	HEK2	SHE1	PET9	YBL029C-A	YBL029W	YBL028C	[RPL19B]*
00U_A8	II	[NPL4]*	[SEC66]	SMY2	UMP1	YBR174C	SWD3	ECM31	EHT1	YBR178W	[FZO1]&
00U_C1	IV	[UBA2]	tG(CCC)D	YDR391C	SPT3	SHE9	RPT3	[SXM1]*			
00U_C5	VI	[YFL066C]*	YFL065C	YFL064C	YFL063W	COS4	DDI2	SNO3	SNZ3	[THI5]*	
00U_G1	**SAR MB10**	No Information, Commercially available yeast (Laffort)									
00U_G2	**SAR MB09**	No Information, Commercially available yeast (Laffort)									
00U_G5	**F33**	No Information, Commercially available yeast (Laffort)									
00U_H10	**ALPHA**	No Information, Commercially available yeast (Laffort)									
00U_H9	**DELTA**	No Information, Commercially available yeast (Laffort)									

Synthesis of deuterated analogues of important aroma compounds in the 'fermentation bouquet' of white wines

Most quantitative analytical studies using gas chromatography mass spectrometry (GC-MS) employ deuterated internal standards (IS). Often in thorough quantitative studies one IS per compound is utilised, where a deuterated analogue of each compound being analysed for is used for a more accurate quantitative relationship between the IS and the compound being evaluated. Because the deuterated IS has the same properties as the non-deuterated version, both should act in the same manner throughout sample preparation, extraction and throughout analysis. Since this is a very expensive and time-consuming way of analysing compounds, it is not feasible or justifiable to carry out a high throughput screen using such a method. This type of method should be used once the initial screen has been narrowed down to a select few samples that require further, more detailed analysis to understand in more detail why the few samples are so important to ones research findings.

This chapter describes the synthesis of the non-commercially available deuterated analogues of the important aroma compounds in the 'fermentation bouquet' library, which were being analysed for within this thesis. Moreover, given the current trend about utilising environmentally friendly chemical syntheses, this synthesis chapter also details a new 'green method' for the synthesis of ethyl esters and acetates using microwave technology, which dramatically reduces the need for the utilisation of large amounts of chemical solvents as is needed in traditional chemical syntheses. This chapter is particularly useful as it describes the purification of each compound to the point where they are pure enough for use as internal standards, which is often difficult to achieve due to the low boiling points and high volatilities of these important aroma compounds. Unfortunately, these compounds were not used within this thesis due to the final outcomes of the metabolic screen, however this chapter is important to note due to the creation of a new simple synthetic method as well as detailing the method of the purification for each deuterated aroma internal standard.

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Introduction:

Chromatography is one of the major techniques used in analytical chemistry to separate and quantify compounds of interest in various matrices. In the wine science arena, either liquid chromatography (LC) [1-4] or gas chromatography (GC) [5-12], are the preferred modes of chromatographic utilisation along with coupling with various different detectors to aid in substrate identification and quantification. With respect to analysing volatile aroma compounds in wines, GC is the most beneficial technique to be employed. The two most prominent detectors for a gas chromatograph are a flame ionisation detector (FID) or a mass spectrometer detector (MS). For accuracy and when using any analytical instrument, an internal standard often needs to be used to nullify any experimental errors. When using a GC-MS for quantitative analysis there are different types of internal standards (IS) that can be employed, including category/characteristic internal standards or identical deuterated standards for all compounds that need to be analysed for and quantified [11]. It should also be highlighted that the accurate internal standard needs to be of relative purity (≥ 95 %) or at least of a known purity so that an equilibrium comparison can be formed [13].

The characteristic/category technique is often used when trying to decrease the time of analysis or when running a semi-quantitative method. There can be one or more internal standards added to the samples of interest which have been chosen as they have similar characteristics to those compounds being analysed for and will act in a similar manner throughout the analysis. One example using this technique is a study where 30 compounds in wine were analysed using a flame ionisation detector and classed into five groups according to their behaviours during extraction; acetaldehyde, diacetyl, acetoin (3-hydroxy butanone), fusel alcohols and their acetates, and fatty acids and their ethyl esters. The four internal standards that were chosen, after experimentation,

27 were 2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol due to their
28 retention times and similar polarities to those compounds from each of the four groups (14).

29 The technique of using deuterated internal standards is known as Stable isotope dilution assay
30 (SIDA) and is ideal for quantitative analysis studies. The deuterated standard has identical
31 properties to the substrate being analysed for and therefore both the internal standard and
32 substrate being analysed should behave in the same manner throughout the analysis, thus
33 avoiding effects of sample preparation for example. The deuterated standards do however differ in
34 mass from their protio analogues and as such are readily identifiable by MS even if other
35 compounds co-elute at the same retention time.

36 For the purpose of this thesis a number of authentic aroma compounds, to be used in method
37 development, and their corresponding deuterated compounds were either acquired commercially or
38 were synthesised for use as standards based on methods developed by Siebert et al. (2005) or
39 Rowan et al. (1996) [11, 15]. This chapter describes the synthesis of the deuterated isotopes of the
40 aroma compounds that are not commercially available and that were needed for use as internal
41 standards.

42 Conducting environmentally friendly chemistry has become more of a focus over the past decade.
43 Tobiszewski mentions the acronym of Green Analytical Chemistry (GAC) in a recent article, giving
44 focus to environmentally friendly analytical chemistry and how the sample preparation step can be
45 the most environmentally detrimental part of the process [16, 17]. Not only is green chemistry being
46 expanded within the analytical world of chemistry, but has now moved into the synthetic chemistry
47 arena also. The CEM corporation has tended to this new trend by creating a new microwave
48 system (Discover Microwave Reactor) to enable synthetic chemists to undertake chemical

49 reactions which in the past "...took hours, or even days, to complete can now be performed in
50 minutes with better yields and cleaner chemistries."[18]

51 Keeping this idea of green chemistry in mind, a new method utilising microwaves was developed to
52 synthesise the deuterated esters needed within this thesis. This new method proved to be very fast
53 and environmentally friendly, with most losses in yield occurring due to the volatility of the
54 compounds during work-up and purification.

55 MATERIALS AND METHODS

56 Commercially available standards and reagents

57 All standard samples were acquired at high purity levels from Aldrich (Milwaukee, WI, USA) (2-
58 phenylethyl acetate (99%), ethyl decanoate (≥ 99%), hexyl acetate (99%), ethyl octanoate (≥ 99%),
59 ethyl hexanoate (≥ 99%), 2-methyl butyl acetate (99%)), ethyl isobutyrate (99%), ethyl 2-
60 methylbutyrate (99%), ethyl butyrate (99%), 2-methyl butanol (≥ 99%), Methionol (≥ 98%), ethyl
61 hexanoate (≥ 99%), isovaleric acid (99%), butyric acid (≥ 99%), hexanoic acid (≥ 99.5%), 1-
62 hexanol (≥ 99%), ethyl dodecanoate (≥ 98%), ethyl propanoate (99%), Sigma (St. Louis, MO, USA)
63 (2-phenyl ethanol, isobutyl acetate (99%), isoamyl acetate (≥ 99%), *n*-hexane (≥ 97%), octanoic
64 acid (≥ 99%)), Unilab (Mandaluyong City, Philippines) (1-octanol (≥ 95%)), Fluka (Buchs, SG,
65 Switzerland) (isopentyl acetate (≥ 99.7%)) and Chemsupply (Gilman, SA, Australia) (diethyl ether
66 (≥ 98%), ethanol (≥ 95%), ethyl isovalerate (≥ 99.7%), isoamyl alcohol (≥ 99.8%), 2,3-butandiol
67 (mixture of racemic and meso forms) (≥ 99.0%), 2,3-butanedione (≥ 99.0%), acetoin (mixture of
68 monomer and dimer) (≥ 97.0%), acetaldehyde (≥ 99.5%), decanoic acid (≥ 99.5%) isobutanol (≥
69 99.5%), isobutyric acid (≥ 99.5%)).

70 All commercially available deuterated standards were acquired at high purity levels from C/D/N
71 isotopes through SciVac Pty. Ltd. (Hornsbury, NSW, Australia). Acetic acid-d_4 99.5% atom D,
72 butyric acid-d_7 98% atom D, propanoic acid-d_6 98 % atom D, hexanoic acid-d_{11} 98% atom D,
73 octanoic acid-d_{15} 98% atom D, decanoic acid-d_{19} 98% atom D, benzyl alcohol-d_7 99% atom D, *n*-
74 propanol-d_7 98% atom D, methanol-d_4 99.8% atom D, 2,3-butandiol-d_8 98% atom D (mixture of
75 stereoisomers), 2,3-butanedione-d_6 98% atom D, acetoin, acetaldehyde-d_4 99% atom D, hexanol-
76 d_{13} 98% atom D.

77 **SYNTHESIS**

78 **General procedure for the synthesis of ethyl esters utilising microwaves.**

79 The general procedure to prepare the labelled ethyl esters required the use of deuterated ethanol-
80 d_6 and the corresponding non-labelled pure carboxylic acid, Scheme 1. The solventless reaction
81 exploited Dowex 50W as an acid catalyst in a 1:1 w/w ratio with the acid. Ethanol-d_6 (1.5 equiv.)
82 was employed for the esterification reaction which was conducted in a Discovery microwave with a
83 temperature program as follows: stirring speed: high, temperature: 100˚C, Time: 2 hours, power
84 max: off. The reaction mixture was allowed to cool to room temperature and then the solution was
85 decanted from the Dowex 50W and the residue washed with ether (5 x 10 mL). The ethereal
86 solution was washed with sodium bicarbonate (50 mL) [for acids with C ≥ 10 sodium hydroxide was
87 employed instead of sodium bicarbonate] and then washed with water and brine. The ethereal layer
88 was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. For esters
89 with low boiling points, concentration under reduced pressure was not used and kugelrohr
90 distillation was employed at temperatures relevant to the compound of interest.

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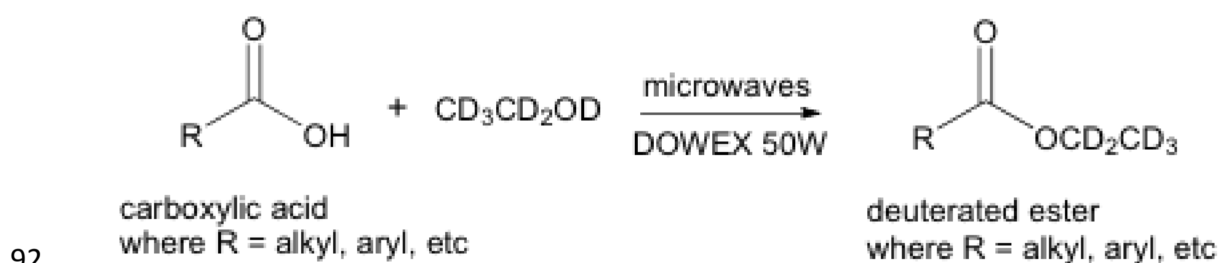
92 

93 **Scheme 1**. General procedure for the synthesis for ethyl esters utilising microwaves.

94 ***Ethyl isobutyrate-d_5***

95 Ethyl isobutyrate-d_5 was synthesised using the general method highlighted above for ethyl esters
96 employing Dowex 50W (1.096 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.13 mL,
97 12.18 mmol), affording ethyl isobutyrate-d_5 in moderate yield (0.66 g, 45%).

98 ***Ethyl isovalerate-d_5***

99 Ethyl isovalerate-d_5 was synthesised using the general method highlighted above for ethyl esters
100 employing Dowex 50W (1.259 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.33 mL,
101 12.24 mmol), affording ethyl isovalerate-d_5 in moderate yield (1.050 g, 63%).

102 ***Ethyl 2-methylbutyrate-d_5***

103 Ethyl 2-methylbutyrate-d_5 was synthesised using the general method highlighted above for ethyl
104 esters employing Dowex 50W (1.267 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.33
105 mL, 12.19 mmol), affording ethyl 2-methylbutyrate-d_5 in high yield (1.295 g, 79% yield).

106 ***Ethyl butyrate-d$_5$***

107 Ethyl butyrate-d_5 was synthesised using the general method highlighted above for ethyl esters
108 employing Dowex 50W (1.077 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.13 mL,
109 10.10 mmol), affording ethyl butyrate-d_5 in low yield (0.246 g, 20%).

110 ***Ethyl hexanoate-d$_5$***

111 Ethyl hexanoate-d_5 was synthesised using the general method highlighted above for ethyl esters
112 employing Dowex 50W (1.420 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.53 mL,
113 12.21 mmol), affording giving ethyl hexanoate-d_5 in high yield (1.482 g, 81%).

114 ***Ethyl octanoate-d$_5$***

115 Ethyl octanoate-d_5 was synthesised using the general method highlighted above for ethyl esters
116 employing Dowex 50W (1.76 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (1.93 mL,
117 12.18 mmol), affording ethyl octanoate-d_5 in good yield (1.552 g, 72%).

118 ***Ethyl decanoate-d$_5$***

119 Ethyl decanoate-d_5 was synthesised using the general method highlighted above for ethyl esters
120 employing Dowex 50W (2.121 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (2.351 g,
121 13.65 mmol), affording ethyl decanoate-d_5 in low yield (1.016 g, 36%).

122 ***Ethyl propanoate-d_5***

123 Ethyl propanoate-d_5 was synthesised using the general method highlighted above for ethyl esters
124 employing Dowex 50W (0.907 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (0.913 mL,
125 12.2 mmol), affording ethyl propanoate-d_5 in low yield (0.204 g, 16%).

126 ***Ethyl dodecanoate-d_5***

127 Ethyl dodecanoate-d_5 was synthesised using the general method highlighted above for ethyl esters
128 employing Dowex 50W (2.44 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (2.456 g,
129 12.26 mmol), affording ethyl dodecanoate-d_5 in moderate yield (1.271 g, 44%).

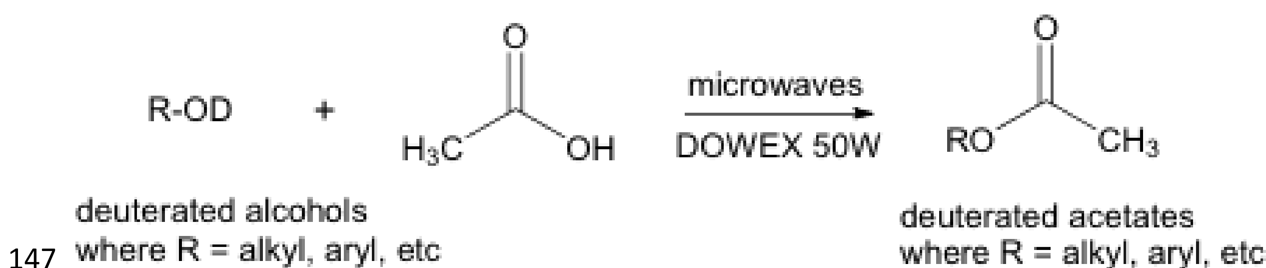
130 ***Ethyl lactate-d_5***

131 Ethyl lactate-d_5 was synthesised using the general method highlighted above for ethyl esters
132 employing Dowex 50W (0.907 g), ethanol-d_6 (1.0 mL, 19.19 mmol) and isobutyric acid (0.913 mL,
133 12.2 mmol), affording ethyl lactate-d_5 (0.2 g). Kugelrohr distillation resulted in substantial product
134 losses due to its volatility.

135 **General procedure for the synthesis of acetates utilising microwaves.**

136 The general procedure to prepare the labelled acetates required the use of the deuterated alcohols
137 and acetic acid, Scheme 2. The solventless reaction exploited Dowex 50W as an acid catalyst in a
138 1:1 w/w ratio with the acid. Acetic acid (2-3 equiv.) was employed for the esterification reaction
139 which was conducted in a Discovery microwave with a temperature program as follows: stirring
140 speed: high, temperature: 100˚C, Time: 2 hours, power max: off. The reaction was allowed to cool

141 to room temperature and then the solution was decanted from the Dowex 50W and the residue
142 washed with ether (5 x 10 mL). The ethereal solution was washed with sodium bicarbonate (50 mL)
143 followed by water and then brine. The ethereal layer was dried over anhydrous $MgSO_4$, filtered and
144 concentrated under reduced pressure. Excess ether was removed via distillation under atmospheric
145 pressure. For acetates with low boiling points, concentration under reduced pressure was not used
146 and kugelrohr distillation was employed at temperatures relevant to the compound of interest.

147 

148 **Scheme 2.** General procedure for the synthesis for acetate utilising microwaves.

149 ***Hexyl acetate-d_{13}***

150 Hexyl acetate-d_{13} was synthesised using the general method highlighted above for acetates
151 employing Dowex 50W (0.369 g), hexanol-d_{13} (0.3 mL, 2.17 mmol) and acetic acid (0.35 mL, 3.415
152 mmol), affording ethyl lactate-d_5 in excellent yield (100%).

153 ***2-Phenylethyl acetate-d_7***

154 2-Phenylethyl acetate-d_7 was synthesised using the general method highlighted above for acetates
155 employing Dowex 50W (0.71 g), 2-phenylethanol-d_7 (0.5 g, 3.87 mmol) and acetic acid (0.71 g).
156 After standard workup the product was further purified by column chromatography using 10% ethyl

157 acetate : hexane as the mobile phase, affording 2-phenylethyl acetate-d_7 in moderate yield (0.38 g,
158 57%).

159 ***2-Methylbutyl acetate-d_5***

160 2-methylbutyl acetate-d_5 was synthesised using the general method highlighted above for acetates
161 employing Dowex 50W (0.498 g), 2-methylbutanol-d_5 (0.250 g, 2.62 mmol) and acetic acid (0.478
162 g, 7.96 mmol), affording 2-methylbutyl acetate-d_5 in good yield (0.282 g, 79%).

163 ***Isoamyl acetate-d_9***

164 Isoamyl acetate-d_9 was synthesised using the general method highlighted above for acetates
165 employing Dowex 50W (0.386 g), isoamyl alcohol-d_9 (0.207 g, 2.12 mmol) and acetic acid (0.386 g,
166 6.43 mmol), affording isoamyl acetate-d_9 in moderate yield (184 mg, 62%).

167 ***Isobutyl acetate-d_9***

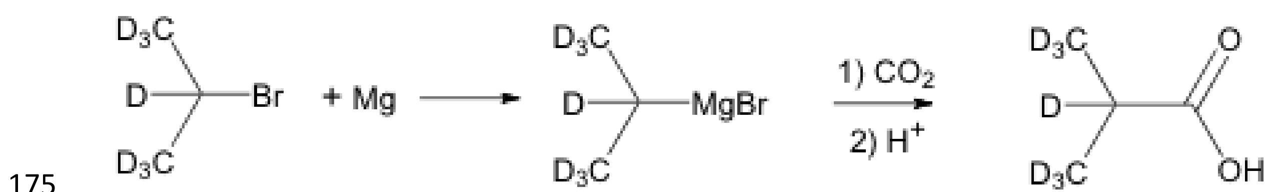
168 Isobutyl acetate-d_9 was synthesised using the general method highlighted above for acetates
169 employing Dowex 50W (0.262 g), isobutanol-d_9 (0.12 g, 1.34 mmol) and acetic acid (0.263 g, 4.37
170 mmol), affording ethyl lactate-d_5 in low yield (54.9 mg, 33% yield).

171

172 **Procedures for the synthesis of the required carboxylic acids**

173 ***Isobutyric acid-d_7***

174 Prepared according to that outlined in Scheme 3 utilising a Grignard carboxylation procedure.

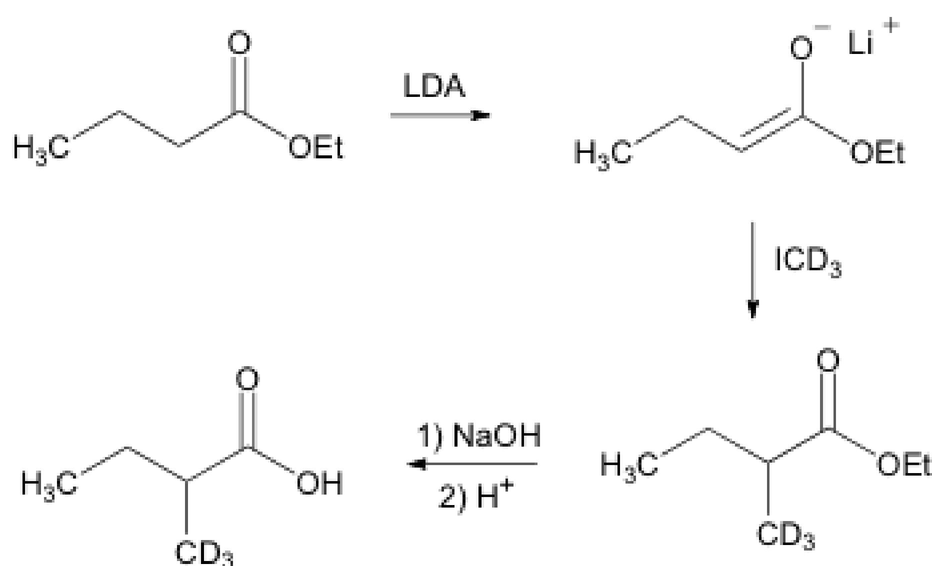
175 

176 **Scheme 3.** Synthesis of Isobutyric acid-*d_7*.

177 To a solution of magnesium turnings (1.22 g), diethylether (100 mL) and a few crystals of iodine
178 under nitrogen was added dropwise 2-bromopropane-*d_7* (5 g, 38 mmol) with stirring over 20
179 minutes. The mixture was then heated under reflux for 3 hours before being allowed to cool to
180 ambient temperature. The mixture was then cooled to 0 ^{o}C and dry ice added carefully so as to
181 maintain the temperature below 15 ^{o}C. Additions of dry ice were carried out every 15 minutes over
182 a 3 hour period. The reaction was quenched with water and stirred until homogeneous. An aqueous
183 solution of 1M hydrochloric acid was added until all magnesium turnings were dissolved and the
184 solution was at pH 1. The product was extracted with ether (5 x 20 mL) and the ethereal layer dried
185 over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was
186 purified by kugelrohr distillation at 160˚C/760 mmHg to afford isobutyric acid-*d_7* (2.63 g, 73%) as an
187 oil.

188 ***2-Methyl butyric acid-d_7***

189 Prepared according to that outlined in Scheme 4 following an alkylation protocol with lithium
190 diisopropylamide (LDA).



191

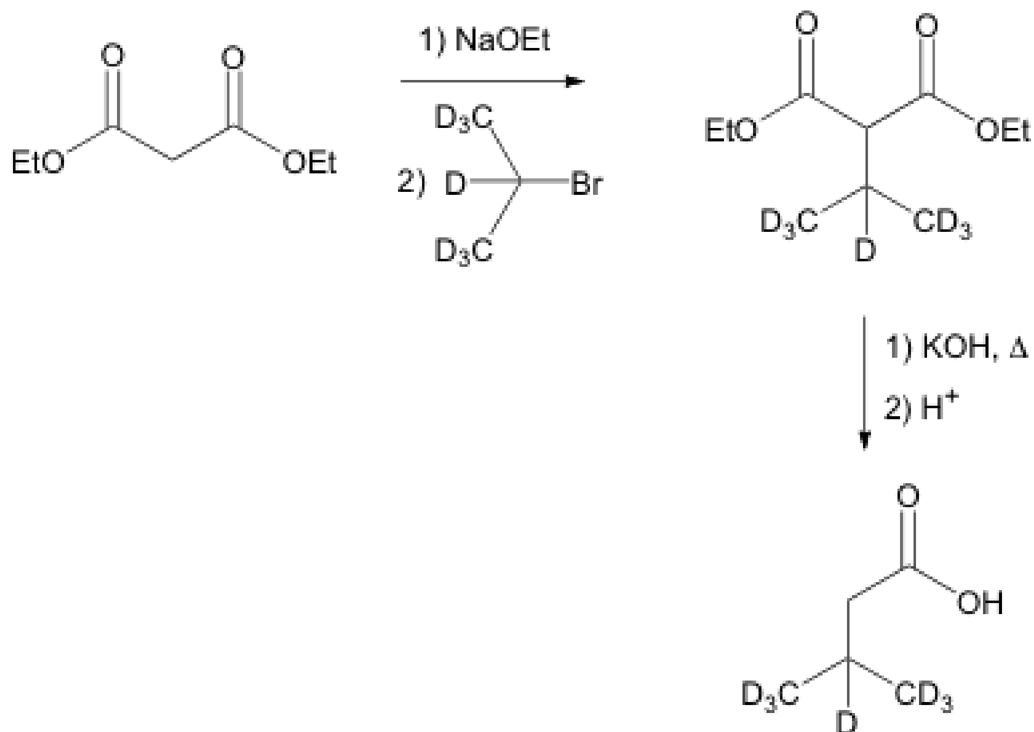
192 **Scheme 4.** Synthesis of 2-methylbutyric acid-d_7.

193 To a solution of diisopropylamine (4.94 g, 35 mmol) in dry THF (175 mL) was added *n*-butyllithium
194 (2.3 M, 14.99 mL) dropwise at -73˚C with stirring under a dry nitrogen atmosphere. The solution
195 was further stirred for 5 min before the dropwise addition of ethyl butyrate (4.23 mL, 31.5 mmol).
196 The mixture was stirred for an additional 30 min before the addition of iodomethane-d_3 (2.40 mL,
197 37.8 mmol) as a single aliquot. The mixture was allowed to warm to ambient temperature and 1M
198 hydrochloric acid (175 mL) was added. The mixture was then extracted with diethylether (5 x 175
199 mL) and the combined organic layers washed with water (175 mL) and brine (175 mL), dried over
200 anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was then
201 purified via Kugelrohr distillation at 120°C-133°C to give ethyl 2-methylbutyrate-d_3 in high yield
202 (3.41 g, 81%). Hydrolysis of the ethyl 2-methylbutanoate (2.605 g, 19.5 mmol) was accomplished in

203 10% sodium hydroxide solution (134 mL) at room temperature over 65 hours. The mixture was
204 extracted with 2M hydrochloric acid (210 mL) and then extracted with dichloromethane (5 x 100
205 mL). The combined dichloromethane extracts were dried over anhydrous $MgSO_4$, filtered and
206 concentrated under reduced pressure to furnish 2-methylbutyric acid-d_7 in moderate yield (1.223 g,
207 60 %).

208 ***Isovaleric acid-d_7***

209 Prepared according to that outlined in Scheme 5.



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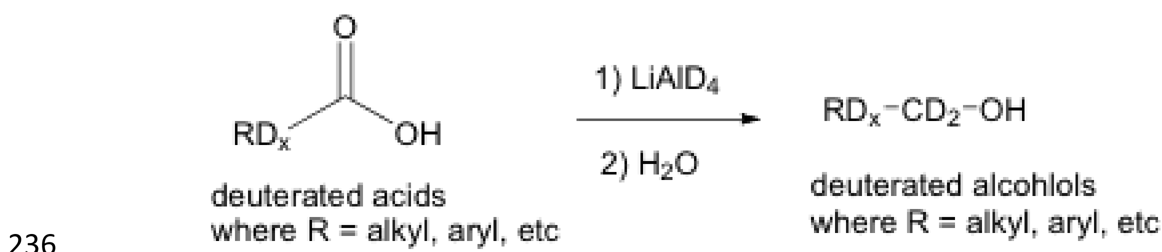
211 **Scheme 5.** Scheme for the synthesis of Isovaleric acid-d_7.

212 A solution of sodium ethoxide was prepared by dissolving sodium metal (1.116 g) in ethanol (20
213 mL) and heating at ~45°C for 30 minutes under an inert atmosphere. Diethyl malonate (6.98 mL,
214 45.98 mmol) was then added dropwise to the sodium ethoxide and the mixture stirred at ~45°C for

215 40 minutes. 2-Bromopropane-d_7 (5 g, 38 mmol) was then added dropwise and the mixture heated
216 under reflux for 3 hours. The mixture was then cooled to room temperature and brine (25 mL) and
217 water (25 mL) added, before acidifying with an aqueous solution of 1M hydrochloric acid (5 mL).
218 The mixture was extracted with diethylether (6 x 25 mL), dried over anhydrous $MgSO_4$, filtered and
219 concentrated under reduced pressure to furnish crude diethyl isopropylmalonate-d_7 (9.73 g). A
220 solution of potassium hydroxide (8.55 g, 152.4 mmol) in water (30 mL) was prepared at 70°C and
221 the diethyl isopropyl malonate-d_7 (7.14 g, 34.14 mmol) was added to the solution along with THF (2
222 mL) dropwise. The mixture was then heated under reflux for 2 hours and then allowed to attain
223 room temperature. The ethanol produced during the reaction was removed under reduced pressure
224 and the resulting mixture cooled to 0°C before being acidified with an aqueous solution of 5 M
225 sulphuric acid (5 mL). The mixture was then heated under reflux for 3 hours, cooled to room
226 temperature and extracted with diethylether (6 x 210 mL). The combined ethereal layers were dried
227 over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to give the crude
228 isovaleric acid-d_7 (6.8005 g). A portion of the crude product was further purified by washing with
229 sodium bicarbonate (x5). The basic washings were then neutralised with sulphuric acid and
230 extracted with diethylether (3 x 50 mL). The ethereal extracts were dried over anhydrous $MgSO_4$,
231 filtered and concentrated under reduced pressure. Final purification by kugelrohr distillation at room
232 temperature and 15 torr afforded pure isovaleric acid-d_7 in 27% yield.

233 **Procedures for the synthesis of the required alcohols**

234 A number of the requisite deuterated alcohols were prepared via reduction of their corresponding
235 carboxylic acids, Scheme 6.

236 

237 **Scheme 6.** General scheme for the preparation of the deuterated alcohols from deuterated acids.

238 ***Isoamyl alcohol-d*$_9$**

239 To a solution of lithium aluminium deuteride (0.627 g, 14.93 mmol) in dry diethylether (20 mL) with
240 stirring under nitrogen was added dropwise a solution of isovaleric acid-d_7 (1.458 g, 13.48 mmol)
241 dissolved in dry diethylether (6 mL). The mixture was heated under reflux for 2.5 hours and then
242 allowed to attain ambient temperature. The reaction was quenched with a saturated solution of
243 sodium sulfate until the solution turned white. The mixture was added to diethylether (150 mL) and
244 filtered through a celite pad under reduced pressure. The eluent was then concentrated under
245 reduced pressure to afford crude isoamyl alcohol-d_9 (4.01 g), which was further purified via
246 kugelrohr distillation at 40°C and 154 torr to afford the pure product in 27% yield.

247 ***2-Methylbutanol-d*$_5$**

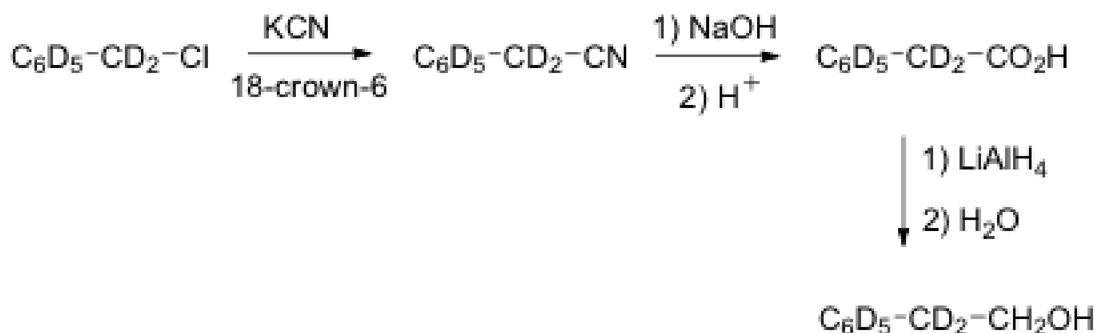
248 2-Methylbutanol-d_9 was prepared in the same manner as the isoamyl alcohol-d_9 above using lithium
249 aluminium deuteride (0.4792 g, 11.41 mmol) and 2-methylbutyric acid-d_3 (0.606 g, 5.77 mmol). Due
250 to the low boiling point of the 2-methylbutanol, the volatiles were carefully removed using kugelrohr
251 distillation at 60˚C under atmospheric pressure. The crude 2-methylbutanol-d_5 (0.632 g) was then
252 purified by kugelrohr distillation at 160°C under atmospheric pressure and then re-concentrated at
253 60°C under atmospheric pressure furnishing pure 2-methylbutanol-d_5 (0.3615 g, 67 % yield).

254 ***Isobutanol-d_9***

255 Isobutanol-d_9 was prepared in the same manner as the isoamyl alcohol-d_9 above using lithium
256 aluminium deuteride (0.919 g, 21.89 mmol) and isobutyric acid-d_7 (0.974 g, 10.24 mmol). Due to
257 the low boiling point of the isobutanol-d_9, the volatiles were carefully removed using kugelrohr
258 distillation at 60˚C under atmospheric pressure. The crude Isobutanol-d_9 (0.323 g) was then purified
259 by kugelrohr distillation at 120°C under atmospheric pressure and then re-concentrated at 60°C
260 under atmospheric pressure furnishing pure isobutanol-d_9 (0.14 g, 16 % yield).

261 ***2-Phenyl ethanol-d_7***

262 The preparation of 2-phenyl ethanol-d_7 began with the conversion of benzyl chloride-d_7 into the
263 corresponding carboxylic acid followed by reduction, Scheme 7.



264

265 **Scheme 7.** Synthetic procedure for the formation of 2-phenyl ethanol-d_9.

266 Dry acetonitrile (15 mL) was added to benzyl chloride-d_7 (3.56 g, 26.6 mmol), 18-crown-6 (0.51 g)
267 and potassium cyanide (1.79 g, 27.5 mmol). The mixture was stirred under nitrogen overnight after
268 which time dichloromethane (200 mL) was added to the solution, and the organics washed with
269 water (2 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced

270 pressure furnishing benzyl cyanide-d_7 in a quantitative yield. Benzyl cyanide-d_7 (3.30 g, 26.6 mmol)
271 was then heated under reflux for 2 hours in the presence of 10 % aqueous sodium hydroxide (40
272 mL). The mixture was acidified with concentrated hydrochloric acid to pH 1 and the mixture
273 extracted with diethylether (3 x 50 mL). The ethereal extracts were dried over anhydrous $MgSO_4$,
274 filtered and concentrated under reduced pressure to afford 2-phenyl acetic acid-d_7 in a quantitative
275 yield.

276 To a solution of lithium aluminium hydride (0.52 g) in diethylether (22 mL) was added dropwise a
277 solution of 2-phenyl acetic acid-d_7 (3.32 g, 26.6 mmol) in diethylether (64 mL) under a nitrogen
278 atmosphere. The mixture was heated under reflux for 2 hours and then quenched with saturated
279 sodium sulfate. Diethylether (150 mL) was then added and the mixture filtered through a celite pad.
280 The eluent was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure
281 affording crude desired product (2.55 g, 79%) which was further purified via kugelrohr distillation at
282 107°C at 15 torr to furnish pure 2-phenyl ethanol-d_7 in low yield (1.14 g, 33%).

283 RESULTS AND DISCUSSION

284 General microwave method for the preparation of the esters and acetates

285 The method developed here for the synthesis of the esters and acetates employed neat reactants
286 and Dowex 50W as an acid catalyst in the presence of microwaves. The transformations proved to
287 be fast and reliable and could be conducted on large scale. This method can also be considered
288 green when compared to conventional methods of esterification as the latter methods rely on the
289 use of harmful solvents to conduct the reactions in and also require solvents for the work-up
290 procedures. Indeed our syntheses reported within this chapter only employed a small amount of
291 solvent during the work-up stages of the reactions. The yields ranged from as low as 16% to as

high as 100%, Table 1. The first major reason for the reduction in observed yield is due to the natural volatility of some of the ester substrates. Clearly, conducting the necessary purification distillations at low temperatures on volatile substances will result in the loss of material. Yields appeared to improve for the higher boiling substrates. For example, hexyl acetate has a boiling point of 168-170°C and was isolated in a yield of 100%, compared to isobutyl acetate, which has a boiling point of 117-118°C and was isolated in a yield of only 33 % yield, Table 1. Another potential cause for yield reduction was that during the work-up stages it was necessary to wash the esters/acetates in solution with cold solutions of bases to remove any unreacted carboxylic acid starting material. Naturally, this may cause some competing hydrolysis, thus resulting in yield reductions.

Table 1. Yields of purified deuterated standards after purification.

Ethyl esters	**% yield**	**Acids**	**% yield**	**Acetate esters**	**% yield**
ethyl butyrate-d_5	20%	2-methyl butyrate-d_3	40%	2-phenyl ethyl acetate-d_7	57 %
ethyl hexanoate-d_5	81%	isobutyric acid-d_7	37%	hexyl acetate-d_{13}	100 %
ethyl isobutyrate-d_5	45%	isovaleric acid-d_7	27%	2-methylbutyl acetate-d_5	79 %
ethyl propanoate-d_5	16%			isobutyl acetate-d_9	33 %
ethyl isovalerate-d_5	63%	**Alcohols**		isoamyl acetate-d_9	62 %
ethyl 2-methylbutyrate-d_5	79%	2-methyl butanol-d_5	40%		
ethyl decanoate-d_5	36%	isobutanol-d_9	16%		
ethyl dodecanoate-d_5	44%	isoamyl alcohol-d_9	18%		
ethyl octanoate-d_5	72%	2-phenyl ethanol-d_7	33%		
ethyl lactate-d_5	-				

Synthesis of the requisite acids and alcohols

The synthesis of the acids and alcohols involved multistep sequences along with often smaller scale reactions due to the expense of the deuterated starting materials. Again losses occurred during the purification processes due to substrate volatility and the small quantities being used. Consequently, whilst all syntheses were successful, final yields were only low to moderate.

309 **CONCLUSIONS**

310 Overall, a new simple and considerably green synthetic method for the formation of esters and
311 acetates from neat starting materials was developed employing a microwave reactor and Dowex
312 50W as a catalyst. All requisite deuterated compounds were synthesised and purified successfully
313 with the exception of ethyl lactate, which was lost during purification due to its volatility. Whilst it
314 turned out that these deuterated standards were not needed in the studies reported within this
315 thesis they are now available to be used as internal standards for further studies requiring more
316 accurate analytical analysis than using non-deuterated internal standards.

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367

Thesis conclusions and future directions

This thesis follows the sequential steps required to perform a screen of fermentations utilising an overexpression library of *S. cerevisiae* encoded in ca. 1,500 plasmids. Each plasmid was transformed into the isoC9d *ΔLeu S. cerevisiae* wine yeast. The screen performed herein analysed important aroma compounds found within the 'fermentation bouquet'. The steps required prior to the screen being undertaken are outlined in Chapters 1, 2, 3 and 4. Before performing the fermentation screen, the literature relating to the formation of aroma compounds in white wine and previous research in the area of metabolomics in regards to aroma compounds in wine was researched thoroughly. The information gathered is outlined in Chapter 1 and provides the necessary background to the research within this thesis as well as its implications. Initially the aroma compounds in white wine which are considered important to the overall aroma of the fermentation bouquet were determined. This process was carried out by researching previous studies which analysed fermentation bouquet aroma compounds of white wine. From these, the important aroma compounds were determined as those with odour activity values (OAV) greater than 1; compounds which are found naturally at concentrations higher than their odour perception thresholds.

The next step was to develop a high throughput HS-SPME GC-MS method to analyse the important aroma compounds formed during the fermentation screen. The first objective of this method development was to determine the optimal SPME fibre for use in the planned experimental system. Chapter 2 describes the identification of two optimal fibres, from an initial group of 5 recommended by the supplier, and proposes a novel scoring system for choosing the correct SPME fibre for volatile studies in young white wine. This novel scoring system is based on the coefficients of determination of the linearity associated with standard curves created using standard compounds in 10% ethanol solutions. The 5 recommended fibres analysed were ranked according

to their score using the novel scoring system. Out of the 5 recommended fibres, two were finally chosen as the best fibres for the analysis of the compounds of interest. The two fibres chosen scored within the top 3 fibres and overall provided the best sensitivity and ability to extract the compounds of interest, along with the best symmetry of the resulting chromatographic peaks.

Since two optimal fibres were discovered for use in the development of the high throughput HS-SPME GC-MS method, it was important to determine the best of the two. This process was described in Chapter 3, whereby the analytical parameters used in typical HS-SPME GC-MS methods were optimised with regards to the compounds of interest, thereby highlighting the 65 µm PDMS/DVB fibre as the better fibre. Accordingly, this fibre was then used in the development and validation of a semi-quantitative method to use in high throughput analyses of fermentation bouquet aroma compounds in white wine. Validation of said method was performed using various matrices similar to that which would be used in the final overexpression library screen. Standard curves were formed in three different media; a bag-in-box white wine, CDGJM-Leu fermentation using the parental strain to be used in the final overexpression screen with a blank plasmid (isoC9d ΔLeu + pGP564), and in 10 % ethanol model wine. The results showed that only one internal standard was necessary for use within a high-throughput method and that each aroma compound displayed a similar line of best fit in the three different media analysed.

As the fermentation screen was to be performed in a CDGJM-Leu with the overexpression library, it was imperative to understand the formation trends of the important aroma compounds. Chapter 4 describes the formation of the important aroma compounds in the fermentation bouquet of white wine throughout fermentation in a CDGJM-Leu media using the isoC9d ΔLeu + pGP564 yeast. This chapter compared the trends seen herein with previous real wine and other model media (MS300) studies. The results concluded that fermentations using CDGJM-Leu media using the isoC9d ΔLeu + pGP564 yeast will show similar trends in the formation of aroma compounds as a conventional

ferment, or a ferment with MS300, with the exception of the compounds relating to the biosynthesis of leucine, which fluctuate until the end of fermentation.

The final overexpression screen was then performed after preparation and testing of the overexpression library, as described in Chapter 5. The screen included five time-points for fermentations utilising the ca. 1500 clone *S. cerevisiae* overexpression library as well as 20 commercially available yeast provided by Laffort. During the allocated time period for fermentation to take place, it was seen that only 51% of the library finished fermentation, comprising 737 clones and 19 Laffort yeast. Only those fermentations which were considered dry were analysed using the newly developed high throughput HS-SPME GC-MS method to create aroma profiles. After multivariate analysis was performed with the aroma profiles for the 756 completed fermentations, it was possible to detect 92 fermentations which differed to the rest of the library with respect to their aroma profile. Of these interesting fermentations, 87 were overexpression clones and 5 were commercial yeast. When comparing the average of each class of aroma compounds, for the entire screen and the clones of interest, it was seen that 10 % of the interesting clones had increased concentrations of all classes of compounds and 6 % had an overall decrease in concentration in all classes of compounds. It was also noted that 78 % of the plasmids in the 87 interesting clones contained at least one gene that has previously been used in overexpression experiments and showed a decrease in vegetative growth rates.

The conclusions of this final screen provided a hypothesis, which can be researched further in the future. This hypothesis can be used as a backbone for future research studies and as forethought for any researcher wanting to perform similar studies in the future. The hypothesis proposed is as follows;

> *For yeast to retain its plasmid throughout experimental fermentation conditions the LEU2 marker alone is not sufficient and a faster growth rate will increase the rate of plasmid*

rejection, hence more cells will die due to a lack of nutrients. For the plasmid to be retained, either a beneficial gene, or a gene which when overexpressed decreases vegetative growth needs to be present.

From the results of this thesis, there are bountiful opportunities for future research to evolve from the proposed hypothesis, the newly developed high throughput method and from the demonstration that large fermentation screens are possible in small scale fermentations. The following is a list of a few such future directions and studies:

Testing the proposed hypothesis

Proving or following on from the above hypothesis would entail repeating fermentations with the 87 interesting clones as well as other clones which did not complete fermentation or were not considered less important. Throughout these fermentations, plasmid retention during fermentation would be determined to indicate the rate of plasmid loss. After determining this, transcriptomic and proteomic studies would be beneficial in determining whether the genes included in the plasmid are being expressed. It would also be beneficial to compare clones, including an overexpressed gene which is beneficial when overexpressed as well as clones including genes which when overexpressed decrease the vegetative growth rate, and comparing these to clones only containing the blank plasmid.

1. **Future directions if the hypothesis is correct**

If the hypothesis is proven correct, then a new plasmid backbone including a gene whose overexpression decreases vegetative growth rate could be constructed. After creating a new plasmid backbone, the entire screening process could be performed again after splicing out the inserts in the current library and inserting them into the more beneficial plasmid backbone. Such a new construct would encourage plasmid retention and therefore provide a clear identification of

genes which are involved the production of aroma compounds which have previously been overlooked.

2. Future directions if the hypothesis is disproven

a. Part of the original thesis plan was to continue narrowing down the search field, where upon a fully quantitative stable isotope dilution assay (SIDA) HS-SPME GC-MS method was to be formed. This method was then going to be used to analyse replicate fermentations of the interesting clones, to provide more comprehensive data to be analysed using multivariate techniques. This proposed experiment could narrow down the search field even further. Chapter 6 covers the synthesis of the non-commercially available deuterated standards which were to be used in the formation of a SIDA method. This thesis follows the aroma profiles formed in a chemically defined media, which is not always transferable to industry, therefore repeating aroma profile testing in a real grape juice media would be beneficial for industry knowledge.

b. The important clones which were discovered could be included in larger scale fermentations in a grape juice medium to be used in sensory testing. After finding the overall chemical change in aroma profiles it would be beneficial to know whether these chemical changes can be noted in sensory studies, Figure 1. After this, a consumer preference study could be conducted to find the clone responsible for the most preferred aroma profile.

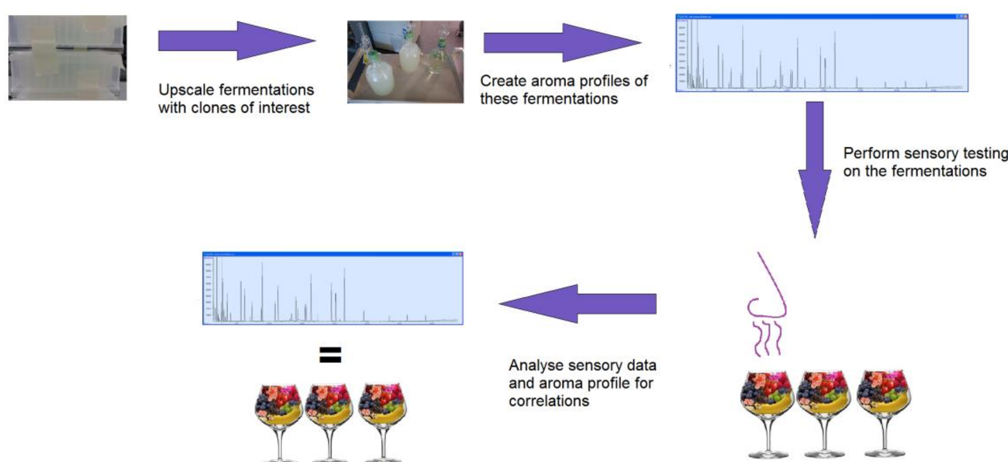
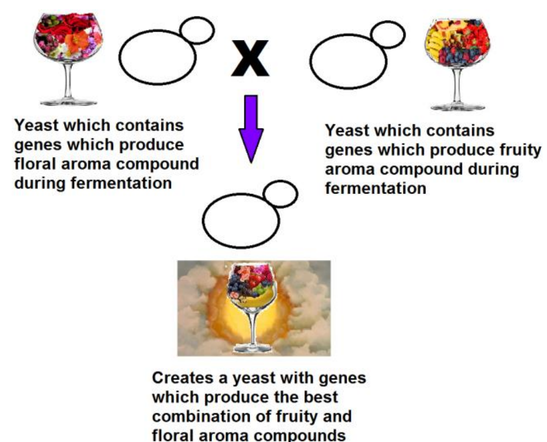


Figure 1: Flow chart for performing a sensory trial

c. The specific gene responsible for these aroma profile changes could be identified by creating overexpression clones including only one of the genes included in the interesting plasmid. Fermentations with these constructs would be analysed for aroma production to discover genes which could be linked to the formation of aroma compounds, which have previously been overlooked.

d. After discovering the gene/s required for changing the aroma profile, a cross-bred *S. cerevisiae* could be constructed for commercial use. This would entail DNA profiling of various yeast to find those containing an overexpression mutation of the genes discovered to be responsible for aroma profile changes and cross-breeding those yeast which have differing beneficial qualities, Figure 2.

Figure 2: crossbreeding of two yeast containing genes proven to create beneficial aroma compounds during fermentation.



Overall this study has laid the background to future research into linking S. *cerevisiae* genetics to their corresponding aroma profiles formed in white wines. This is beneficial for predicting the aroma profile of a white wine from a yeast's genome and can lead to the production of yeast which produce more aromatically appealing white wines. This study has also provided knowledge and methodology for future fermentation screens using small scale fermentations to save time and funds. The high-throughput screening method for aroma compounds will be useful not only for yeast metabolomic studies, but also for screening different wine varieties. The possibilities for future research that lead on from this thesis are numerous and not only limited to the wine industry.