Rapid and high-resolution analysis of winemaking yeasts using MALDI-TOF MS

by

Junwen Zhang

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Abstract

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Winemaking is a biologically diverse and dynamic process in which the grape sugar is converted into ethanol, CO_2 and other aromatic compounds by yeasts. *Saccharomyces cerevisiae* is the main species used for wine production, whereas the contribution of non-*Saccharomyces* yeasts to the distinctiveness of wine was not acknowledged until the 1980s. The indigenous yeasts present in the vineyard mainly belong to non-*Saccharomyces* species, which can have an important impact on the final wine quality, especially where spontaneous fermentation practices are used. However, metabolic profiles of individual strains of both non-*Saccharomyces* and *Saccharomyces* species may differ significantly, and thus lead to different organoleptic properties that are important to increase the expression of *terroir* in the wine. In this sense, some of these yeast strains may be desirable to be isolated and used for further development of novel wine products. It is also important to identify spoilage yeasts that may contaminate wine with off-flavours. Both cases require the ability to identify yeast strains that contribute particular flavour profiles to the wine.

Recently, an emerging proteomic approach of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been successfully applied to identify yeast species relevant to winemaking. This technology has shown potential in the prediction of the utility of individual yeast strains in the production of different wine styles. Despite this interest, most work focuses on its capacity for clinical identification purposes, and the list of winemaking yeasts in current MALDI-TOF databases is not exhaustive. Furthermore, the predictive potential of this approach has not gone unchallenged. With this in mind, this study aims to further develop MALDI-TOF MS as a rapid and low-cost method for yeast identification and characterisation, as well as assess it as a tool to predict the suitability of individual yeast strains in the production of different wine styles.

Based on 14 type strains and 19 field isolates representing 21 yeast species, the efficiency of MALDI-TOF MS for wine yeasts identification was improved by comparing the dried-droplet (DM) and pre-mixing (PM) methods, as well as two mass ranges of *m/z* 2,000-20,000 and 500-4,000. With this improved protocol, MALDI-TOF MS was used to identify the yeast isolates recovered from the production of Pinot Noir wines that were spontaneously fermented in vineyard versus in winery by an organic wine producer in Waipara, New Zealand. The corresponding MALDI profiles were integrated into our in-house database stored in Software BioNumerics v 7.6. Meanwhile, 26S rRNA sequencing was used in conjunction with Restriction Fragment Length Polymorphism (RFLP) to cross-check the yeast identification results. Afterwards, eight *Saccharomyces* strains of diverse origin were examined to investigate the influence of growth conditions on MALDI-TOF spectra and to determine the best medium for the use of MALDI-TOF MS to predict wine yeast utility for different wine styles production, including the Pinot Noir grape juice, Chardonnay grape juice, synthetic grape juice, and laboratory-grade artificial culture media (YPD broth and agar). With the pre-selected culture media, YPD agar and YPD broth, a panel of 59 commercial yeasts including 47 wine yeasts and 12 brewing yeasts were then used to validate the predictive potential of MALDI-TOF profiling for individual yeast strains application. Dimensionality reduction techniques (DRTs) of PCA, MDS and UMAP were performed to analyse the data by using BioNumerics v 7.6 and the conda-forge packages for Python.

Compared to the routine DM method, PM improved the performance of MALDI-TOF MS on wine-associated yeast analysis and yielded well-defined identification results. This is the first known usage of low-mass range m/z 500-4,000 profiles in winemaking yeast characterisation; this mass range appears unsuitable for the identification at the species level, but may offer some advantages for infraspecific (*i.e.* strain) classification. This improved MALDI-TOF MS protocol was then successfully applied to indigenous yeast isolated from organically produced Pinot Noir wines for diversity analysis. Thirteen species belonging to eight genera (10 non-*Saccharomyces* and 3 *Saccharomyces* yeasts) were identified, with taxonomic diversity reducing as fermentation progressed. MALDI-TOF utility also confirmed the impact of differing production systems on yeast diversity and dynamics of spontaneous fermentation. Furthermore, the MALDI profiles appeared to reflect the impact of different fermentation environments and fermentation stages on individual yeast proteomics. In addition, the yeast cultivation conditions also showed a significant impact on MALDI-TOF profiles, with YPD agar being recommended for taxonomic studies, while YPD broth may offer an improved intra-subspecific differentiation by yielding more discriminatory peaks. MDS and UMAP analyses supported the potential of MALDI-TOF proteomics in predicting the utility of yeast strains in winemaking and brewing sectors, although further studies are necessary to more comprehensively investigate the possible commercial benefits.

Keywords: 26S rDNA sequencing, BioNumerics, dimensionality reduction techniques, identification, indigenous yeast, MALDI-TOF MS, MDS, New Zealand, PCA, PCR/RFLP, Pearson Correlation, Pinot Noir, proteome, spontaneous fermentation, strain differentiation, UMAP, UPGMA, Waipara, wine variety, wine yeast, yeast diversity

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Abbreviations

	Abbreviation	Full Name
A	**ACN**	Acetonitrile
	AFLP	Amplified Fragment Length Polymorphism
	AGJ	Autoclaved Grape Juice
B	**bp**	Base Pair
C	**CBS**	Centraalbureau voor Schimmelcultures (Dutch)
	cfu	Colony forming units
	CGH	Comparative Genome Hybridization
	CH	Chardonnay
	CHEF	Contour clamped Homogeneous Electric Field
	CoNS	coagulase-negative staphylococci
	CWT	Continuous Wavelet Transform
D	**DAN**	1,5-Diaminonaphthalene
	DGGE	Denaturing Gradient Gel Electrophoresis
	DHAP	2,5-Dihydroxyacetophenone
	DHB	2,5-dihydroxybenzoic acid
	DM	Dried-droplet Method
	DO	Designations of Origin
	DRTs	Dimensionality Reduction Techniques
	dNTPs	deoxynucleotide triphosphates
E	**EG**	Ethylguaiacol
	EP	Ethylphenol
	EtBr	Ethidium Bromide
F	**FIGE**	Field-Inversion Gel Electrophoresis
	FWHM	Full Width at Half Maximum
G	**GSH**	Glutathione
H	**HCCA**	α-cyano-4-hydroxycinnamic acid
	HCDC	Hydroxycinnamic acid decarboxylase
	HGJ	Heated Grape Juice
	HPA	3-hydroxypicolinic acid
	HPDE	High Density Polyethylene
	IGS	Intergenic Spacer
I	**ITS**	Internal Transcribed Spacer
	ISD	In-Source Decay
K	**kb**	Kilobase
M	**MALDI-TOF MS**	Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry
	MDS	Multidimensional scaling
	MLF	Malolactic Fermentation
	MLST	Multilocus Sequence Typing
N	**NCYC**	National Collection of Yeast Cultures
	NCBI	National Center for Biotechnology Information
	NTC	Non-template Control
	NTS	Non-Transcribed Spacer
O	**OFAGE**	Orthogonal-Field Alternation Gel Electrophoresis
P	**PACF**	Programmable Autonomously-Controlled Electrodes
	PCR	Polymerase Chain Reaction
	PCA	Principal Component Analysis
	PDM	*Prise de Mousse*
	PFGE	Pulse-Field Gel Electrophoreses

	PHOGE	Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis
	PM	Pre-mixing Method
	PN	Pinot Noir
	PTC	Positive-template Control
Q	**qRT-PCR**	quantitative Real-Time Polymerase Chain Reaction
	RAPD	Randomly Amplified Polymorphic DNA
R	**RBCA**	Rose Bengal Chloramphenicol Agar
	rDNA	ribosomal DNA
	RFLP	Restriction Fragment Length Polymorphism
	RGE	Rotating Gel Electrophoresis
S	**SA**	Sinapinic acid
	SGJ	Synthetic Grape Juice
	SME	Small-medium Enterprises
	SV1	Sample Vineyard – stage 1; same as SV2, SV3, SV4
	SW1	Sample Winery – stage 1; same as SW2, SW3, SW4
T	**TAFE**	Transverse-Alternating Field Gel Electrophoresis
	TFA	Trifluoroacetic acid
	TGGE	Temperature Gradient Gel Electrophoresis
	THAP	2,4,6-trihydroxyacetophenone
	TSA	Trypton Soya Agar
U	**UGJ**	Unautoclaved Grape Juice
	UMAP	Uniform Manifold Approximation and Projection
	UPGMA	Unweighted-Pair Group Method with Arithmetic mean
V	**VBNC**	Viable But Non-Culturable
W	**WL**	Wallerstein Laboratory
Y	**YMA**	Yeast Malt Agar
	YPD/YEPD	Yeast Peptone Dextrose
	YPG	Yeast Peptone Glucose

Chapter 1

Introduction

The changing nature of the wine industry globally is forcing wine producers to understand the demands for different markets better and provide superior and distinct wine styles accordingly. Wine is a highly complex fermented beverage as a result of interactions between grapes and a series of microorganisms, in which the species and strains of yeast play an important role (Vigentini et al. 2016). Furthermore, metabolic profiles of individual indigenous yeast strains may differ significantly, yielding diverse organoleptic profiles unique to the regional characteristics of wines, which may demonstrate a better expression of *terroir* (Knight et al. 2015). Extensively complex compounds are produced by yeasts, including ethanol, esters, higher alcohols, sulphur-containing compounds, and many others, which gives distinctive attributes to the wine (Swiegers et al. 2005).

Pinot Noir wine, the most widely planted red grape variety in New Zealand, is stylistically different by the aroma, in-mouth flavour, and mouthfeel attributes between the four main Pinot Noir production regions: Martinborough, Marlborough, Waipara, and Central Otago in New Zealand (Tomasino et al. 2013). In New World wine regions, exploiting indigenous yeast strains to modulate wine style is a re-emerging option as indigenous yeasts fermented wines are typically more complex by showing more varietal flavours and aroma (Varela et al. 2009). Genera *Hanseniaspora* and *Pichia* showed high production of ethyl acetate, isobutyl acetate and isoamyl acetate (Viana et al. 2008). The aroma enhancement of non-*Saccharomyces* yeasts is mainly contributed by various enzymes converting odourless precursors present in the grape must to odour-active volatile compounds such as β-damascenone and a range of desired esters (Liu et al. 2016).

The dominance of yeast species on grape berry is largely determined by the grape health status, the oxidative or weakly fermentative ascomycetous populations (*e.g. Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp., *Pichia* spp.) predominate on healthy berries, whereas the damaged berries increase the risk of spoilage yeasts (*e.g. Zygosaccharomyces* spp., *Torulaspora* spp. and *Dekkera* spp.) (Barata et al. 2012). In the wine industry, the contamination of spoilage yeast is a substantial threat producing unpleasant flavour and aroma, consequently, damaging the wine quality and causing significant economic losses. Some yeasts (mainly non-*Saccharomyces* yeast species) produce undesirable compounds at a high level, but interestingly, at lower levels they are considered to be beneficial for a particular wine style (Swiegers et al. 2005). Phenolic off-flavours-producing yeasts mostly belong to *Rhodotorula, Candida*, *Cryptococcus*, *Pichia*, *Hansenula* and *Brettanomyces* genera (Shinohara et al. 2000). For this reason, the ability to identify yeast strains that confer either positive or negative flavour profiles to wines is essential.

Yeast identification has often been conducted using classical methods based on morphology, biochemical characteristics, and more recently DNA-based techniques. Morphological observation and biochemical detection requires experienced technicians, with sometimes ambiguous results obtained, while DNA-based techniques (*e.g.* PCR-RFLP analysis, 26S rDNA sequencing, and 5.8S-ITS-RFLP technology) could get more accurate results but is expensive, with more stringent laboratory requirements as well (Baffi et al. 2010, Teixeira et al. 2015, Liu et al. 2016, Polizzotto et al. 2016, Vigentini et al. 2016, Mendoza et al. 2017).

The emerging proteomic method MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) has shown its rapidness in minutes (5.1 min) at low cost ($ 0.50 per sample) in yeast identification (Dhiman et al. 2011). The global wine industry is composed primarily of small-medium enterprises (Gilinsky Jr et al. 2016), which will profit from a highly cost-effective method. MALDI-TOF MS is a technique based on "soft ionization" where microbial cells are embedded in a suitable matrix that accentuates the ionisation potential of a laser, releasing highly charged cell components (notably peptides). These ions are separated as a function of their mass/charge ratio in an electric field and detected by Time-Of-Flight [TOF] spectrometry and finally presented as a mass spectrum. The microbial identification is achieved by the comparison of mass spectrum between an unknown yeast isolate and the known yeast species in a MALDI database.

Initially, MALDI-TOF MS served as a quick and cost-effective pathogen species identification tool for microbiological diagnosis, such as *Escherichia coli*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and coagulase-negative *staphylococci* (CoNS) (Anhalt and Fenselau 1975, Cain et al. 1994, Carbonnelle et al. 2007, Rajakaruna et al. 2009). Its introduction undoubtedly has revolutionised clinical microbiology greatly. Within the last decade, MALDI-TOF MS has been incorporated into the routine workflow of many medical microbiology laboratories (Sauget et al. 2017).

Until recently, this approach has been applied to identify a wide range of yeast species relevant to winemaking (Gutiérrez et al. 2017). Of more pertinence, Usbeck et al. (2014) presented the use of MALDI-TOF MS to predict yeast strain application in the production of different wine styles including Chardonnay, Beaujolais, and Champagne. Lauterbach et al. (2017) also classified 52 brewing *Saccharomyces* strains into several major beer types according to their MALDI profiles. These results indicate the potential of using MALDI-TOF fingerprints to predict the application of indigenous yeasts for producing specific wines and beers with particular organoleptic properties.

To the best of our knowledge, most of the studies on MALDI-TOF MS are limited to microbial identification, and there is still a huge gap (*e.g.* data mining) that needs to be filled to extend the utility of MALDI-TOF MS to contribute to the unknown strain application prediction as well as the indigenous yeast strain selection with desirable phenotypes. The list of winemaking yeasts is not

exhaustive to date, and several species including *Saccharomyces*, *Kluyveromyces,* and *Brettanomyces* spp. have not to our knowledge been examined. Moreover, the method is widely used in clinical microbiology, but for industrial purposes databases are far less developed. A wide range of important wine-associated yeasts like *Lachancea hermotolerans*, *Candida zemplinina* (*Starmerella bacillaris*) and *C. stellata* were not in the MALDI Biotyper database (Du Plessis et al. 2017).

Chapter 2

Literature Review

The grape cultivation history in New Zealand dates back to the first European settlers, but only relatively recently has New Zealand wine received worldwide recognition (Bicknell and MacDonald 2012). In the 1980s, Marlborough's exquisite Sauvignon Blanc put New Zealand on the international wine stage. In 2020, New Zealand wine export value hit a record-breaking NZ$2 billion milestone irrespective of the global COVID-19 coronavirus pandemic impact. According to the study of Carbone et al. (2021), France, Italy, and New Zealand export in the market for more sophisticated wines than the other 8 major exporter countries (*Old Wine World* including Germany, Portugal, and Spain, *New Wine World* including Argentina, Australia, Chile, South Africa, and the USA). The exporters' competitiveness largely relies on (i) preferences and income level, (ii) price (relative prices and nominal exchange rates), and (iii) wine quality (Cardebat and Figuet 2019, Carbone et al. 2021). To meet the evolving demands and increase product value in more segmented markets, wine product differentiation and quality features are important to wine producers and exporters (Carbone et al. 2021). Likewise, many consumers are always looking for something new in wine - the regional typicity and vintage variation (Charters and Pettigrew 2007).

Wine is a "differentiated experience" product (Bicknell and MacDonald 2012), and it is complex to assess the wine's organoleptic quality sensorially (*e.g.* sight, smell, taste, and touch) (Swiegers et al. 2005). Suggested by Charters and Pettigrew (2007), wine quality is a multi-dimensional concept including both extrinsic dimensions like grapes, winemaking, marketing elements (label, price, and reputation) and intrinsic dimensions like the organoleptic nature of the product (*e.g.* taste, balance, flavour intensity and complexity). Similarly, Bicknell and MacDonald (2012) proposed that the price of a bottle of wine depends on objective characteristics (grape variety, region of origin, and climatic conditions) and subjective sensory characteristics (complexity of the aroma and/or taste of the wine).

To enhance the market competitiveness, the concept of *terroir* has become a useful marketing tool to link essential wine quality attributes to the location of production (Overton and Murray 2014). The term of *terroir* originally developed for wine, linking the uniqueness and quality of products to the environment where they are produced, gives the consumers a "sense of place" (Vaudour et al. 2015), including (i) Physical environment (*e.g.* climate, slope, aspect, and topography); (ii) Biological factors (*e.g.* soils, grape variety, and microbiota); (iii) the amorphous historical and cultural aspects (Overton and Murray 2014, Belda et al. 2017). Wines from different countries or regions can be discriminated through their chemical composition and/or sensorial profiles (Vaudour et al. 2015). Marlborough of New Zealand has the reputation to produce premier Sauvignon Blanc, which is praised by wine

professionals and consumers for its distinctiveness (Parr et al. 2007). The soft brand image that "Marlborough" and "New Zealand" are particularly connected with unique Sauvignon Blanc wine style has been deeply rooted in the mind of consumers (Overton and Murray 2014). Bramley et al. (2020) indicated that Marlborough has a varied *terroir* far from a uniform area. Until now, there are 21 geographical indications (GIs) registered by groups and regions in New Zealand. The Geographical Indications (Wine and Spirits) Registration Act 2006 came into force in 2017, providing a regime for registering New Zealand place names (*e.g.* Marlborough, Hawke's Bay) as intellectual property to help the New Zealand wine industry protect their geographical brand in competitive overseas markets.

The role of microbial *terroir* aspect has been largely ignored mainly due to the limitation of technologies to look into the microbial community structure of the bacterial and yeast species of significance (Gilbert et al. 2014). The use of conventional microbial techniques and molecular methods confirmed the contribution of indigenous microbial species and strains to the wine specificities (Renouf et al. 2005, Renouf et al. 2006). Bokulich et al. (2014) alluded that the existence of non-random microbial *terroir* act as a determining factor in regional variation among wine grapes. The grapes and wines associated-microbial consortia was considered as a decisive factor influencing wine aroma and consumer's preferences by winemakers (Belda et al. 2017). More than 100 yeast species and millions of strains are well known to contribute to the microbial *terroir* (Carrau et al. 2020).

2.1 Wine yeast

Winemaking is a grape-based fermentation process where complex interactions occur between fungi, yeasts, and bacteria. The microbial consortium structure and diversity are influenced by practices in vineyard and winery, which plays a prominent role in determining the chemical composition of wine (Zarraonaindia et al. 2015, Bokulich et al. 2016), especially the dominant impact exerted by yeasts during alcoholic fermentation (Fleet 2003). Yeasts in the winemaking process include both *Saccharomyces* and non-*Saccharomyces* yeasts (Varela and Borneman 2017).

Among *Saccharomyces* spp., *S. cerevisiae* is the most important species responsible for fermenting grape sugars into alcohol and CO_2, for this reason, *S. cerevisiae* is usually simply referred to as the "wine yeast" (Jolly et al. 2014). The earliest fermented beverages involving the use of *S. cerevisiae* can be traced back in China as far as 7000 B.C.E. (McGovern et al. 2004). Non-*Saccharomyces* yeasts represent a heterogeneous yeast group that resides on the grape berry surface or winery equipment and dominates at the very early stage of fermentation, including more than 20 genera in both Ascomycota and Basidiomycota phyla (Padilla et al. 2016).

As wine fermentation progresses, the population of the non-*Saccharomyces* yeasts are gradually replaced by *Saccharomyces* spp. due to their inability to survive the harsh wine environments (*e.g.*

increased alcohol concentration, low pH values, and limited nutrition). At the final phase of wine fermentation, *S. cerevisiae* is the dominant species that can be isolated, while non-*Saccharomyces* yeasts only existed in appreciable population at mid-fermentation (Garofalo et al. 2016, Padilla et al. 2017, Varela and Borneman 2017). However, in some cases, few non-*Saccharomyces* species (*e.g. Starmerella bacillaris* and *Torulaspora delbrueckii*) with higher tolerance to a harsh environment could coexist longer with *S. cerevisiae* at the final stage of fermentation (Wang et al. 2016).

2.1.1 Commercial yeasts used in wine production

Spontaneous fermentation (fermenting activity triggered mainly by indigenous microbiota on grape berries) was standard in the wine industry until the middle of the 20th century (Borneman et al. 2016). Since the first commercial active dry yeasts started being released in 1965, hundreds of *S. cerevisiae* strains have been developed commercially (Borneman et al. 2016). Depending on the grape variety and desired wine styles, a diverse range of commercial wine yeast options is currently available on market, including both *Saccharomyces* and non-*Saccharomyces*.

***Saccharomyces* strains**

S. cerevisiae is extensively utilized for commercial wine fermentation. This practice has improved the microbiological management during winemaking and guaranteed a more consistent and reliable fermentation process (Marsit and Dequin 2015, Borneman et al. 2016). The ability of *S. cerevisiae* to outcompete other microbial species during wine fermentation has traditionally been ascribed to its strong fermentative power and high tolerance to harsh environments (*i.e.* high levels of ethanol and organic acids, low pH values, anaerobic conditions and depletion of certain nutrients) (Albergaria and Arneborg 2016). Two related properties of “Crabtree effect” and “make-accumulate-consume” strategy were then found in *S. cerevisiae* and its closely related species, the former is referred to their ability to produce and accumulate ethanol (even under aerobic conditions), while the latter strategy could confer these species competitive dominance by accumulating ethanol firstly to inhibit the other species, and consequently consuming ethanol (Marsit and Dequin 2015). Moreover, defensive strategies like cell-cell contact and production of killer-like toxins (*e.g.* antimicrobial peptides) were also recently revealed as mechanisms adopted by *S. cerevisiae* to combat non-*Saccharomyces* yeasts and lactic acid bacteria (Albergaria and Arneborg 2016). Nevertheless, these physiological features made *S. cerevisiae* the important species with positive traits like rapid fermentation completion and low risk of stuck fermentation and off-flavour production, and thus *S. cerevisiae* is the primary choice for commercial wine starters.

Inoculation of different commercial *S. cerevisiae* strains could result in very different profiles of metabolites associated with sensory properties in finished wines (Carrau et al. 2020), particularly the volatile aroma compounds produced by yeasts (Callejon et al. 2010). Twenty commercial *S. cerevisiae* strains exhibited high phenotypic diversity (*e.g.* fermentation fitness and metabolic traits) when

exposed to different biotechnologically relevant conditions (Barbosa et al. 2014). A study on Australian Shiraz reported that sensory attributes like colour, astringency and mouthfeel associated with anthocyanins, tannins and polysaccharides were greatly affected by inoculation of 10 different commercial *S. cerevisiae* strains (Bindon et al. 2019). Another study on sparkling wines showed that inoculation of different commercial *Saccharomyces* spp. yeasts (IOC FIZZTM, IOC DIVINETM, LEVULIA CRISTALTM, IOC 18-2007TM) had a key impact on the volatile aroma compounds and consequent sensory characteristics, of which ethyl octanoate and ethyl decanoate were representatives for all variants, defining their fruity (especially banana, apple) and floral notes (elderflower), as well as, a minor but important influence on physico-chemical parameters (*e.g.* density, total acidity, and residual sugar) (Cotea et al. 2021).

Apart from *S. cerevisiae*, closely related "*Saccharomyces sensu stricto*" species (*S. bayanus, S. cerevisiae*, *S. paradoxus*, *S. pastorianus, S. uvarum*, *S. arboricola*, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*) (Borovkova et al. 2020), between which natural introgression usually occurs (Pulvirenti et al. 2000), have also been commercialised for wine production, such as Vitilevure Pris Mouse (*S. bayanus*), Fermichamp 67 J (*S. bayanus*), Lalvin M69 (*S. bayanus*), I. Oenologique Champagne (*S. bayanus*), and Uvaferm UVA (*S. uvarum*) (Carrasco et al. 2001). Moreover, the hybrids of *Saccharomyces* yeasts often present more advantageous features over their parental species resulted from new combinations of genes. For example, the high level-glycerol producer Velluto Evolution™ (*S. cerevisiae* x *S. uvarum*) has a good tolerance of low temperatures (at 12˚C) that is inherited from cryotolerant species *S. uvarum* (Lallemand Inc.). According to the manufacturer, the AWRI Fusion (AB Biotek Inc.) is an interspecific hybrid *S. cerevisia*e x *S. cariocanus*, which can increase complexity of wine by improving mouthfeel and texture, and enhancing aromas (peach, nectarine, lemon zest, and floral notes for white wines; cherries, red berries, perfume, and crushed violets in red wines).

Non-*Saccharomyces* strains

Wines produced by *Saccharomyces* spp. are usually consideredless organoleptic complexity, and growing number of studies has approved that the co-inoculation of non-*Saccharomyces* species with commercial *S. cerevisiae* strains can increase aroma and flavour diversity (Medina et al. 2013, Maturano et al. 2015, Mas et al. 2016, Tristezza et al. 2016). The contribution of non-*Saccharomyces* yeasts to wine quality was not acknowledged until the 1980s (Fleet 2008). Several non-*Saccharomyces* yeasts species like *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii*, *P. kluyveri*, and *Schizosaccharomyces pombe* have also been successfully applied in commercial wine production (Lu et al. 2016, Padilla et al. 2016, Prior et al. 2019). For example, according to the manufacturers' instructions, LEVEL2 BIODIVA™ TD 291 (*T. delbrueckii*) was selected for its properties to enhance wine aromatic and mouthfeel complexity; IOC Gaïa™ is a *M. pulcherrima* strain with no fermenting power but a natural tool to protect wine from harmful microflora and reduce the use of SO_2 in

winemaking. The use of *L. thermotolerans* is increasing due to their properties in softening and improving the sensory quality, especially the unique quality in lowering the pH by production of lactic acid, such as LEVEL2 LAKTIA™ and Kt 421 Viniflora® CONCERTO™ (Vaquero et al. 2020). Moreover, the mixture of different yeasts for wine fermentation is also commercially available on the market, such as Viniflora® MELODY™ (20 % *T. delbrueckii*, 20 % *L. thermotolerans*, and 60 % *S. cerevisiae*) (Albergaria and Arneborg 2016).

Non-*Saccharomyces* yeast starters are generally used in combination with *S. cerevisiae*, the strategies of co-inoculating different non-*Saccharomyces* and *S. cerevisiae* yeasts could produce wines with more complex aroma and flavours due to the microbial interactions between the non-*Saccharomyces* and *S. cerevisiae* yeasts (Maturano et al. 2015). In the study conducted by Maturano et al. (2012), the mix of 1% *S. cerevisiae* and 99% *T. delbrueckii* was shown as an interesting starter culture, which produced the highest enzymes activities (pectinases, proteases, amylases or xylanases) in fermenting must compared to any other combinations among *S. cerevisiae*, *H. vinae* and *T. delbrueckii*. Sequential inoculation is more widely adopted than simultaneous inoculation, as it is more similar to spontaneous fermentation, allowing the non-*Saccharomyces* yeasts to proliferate at the start of the fermentation without the competition of *S. cerevisiae* (nutrient competition and physical cell-cell contact), and thus making more of a contribution to wine complexity (Lu et al. 2018, Petitgonnet et al. 2019). All in all, the use of non-*Saccharomyces* yeasts in commercial winemaking has become a trend as their metabolic heterogeneity not only overcomes certain shortcomings in most *S. cerevisiae*, but also allows the production of wines with distinctive characteristics regarding the sensorial, technological, and safety aspects (Belda et al. 2016).

2.1.2 Indigenous yeast

The initial microbial diversity of grape must can be affected by the grape variety, berry health condition, vintage, harvest methods, and viticultural and oenological practices, but 99.9% of yeasts are usually non-*Saccharomyces* species (Clavijo et al. 2010, Maturano et al. 2016, Carrau et al. 2020, Mateo et al. 2020). Yeast diversity in a Spanish vineyard demonstrated that *K. thermotolerans* and *H. uvarum* in the 2007 harvest were more frequently isolated compared to the 2006 vintage; and Cabernet Sauvignon fermentations showed greater yeast diversity than Merlot and Syrah (Clavijo et al. 2010). The organic farming system usually shows greater yeast biodiversity than conventional farming (Cordero-Bueso et al. 2011). Generally speaking, yeasts belonging to genus *Hanseniaspora* and *Metschnikowia* are the most abundant (Fleet 2008). *Hanseniaspora* spp. could take up 50-75% of the total yeast population on the grape berry surface, and the other species of genera *Candida*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula,* and *Torulaspora* are present in relatively lower amounts (Albergaria and Arneborg 2016, Garofalo et al. 2016, Drumonde-Neves et al. 2017). The main non-*Saccharomyces* yeasts related to winemaking are summarised in Table 2.1.

Table 2.1 Non-*Saccharomyces* yeast species found in winemaking (Liu et al. 2016, Varela 2016, Varela and Borneman 2017).

Genus	Species
Aureobasidium	*A. pullulans*
Brettanomyces	*B. bruxellensis*
Candida	*C. bentonensis / C. apicola / C. hellenica / C. azymoides (rare) / C. zemplinina*
Debaryomyces	*D. pseudopolymorphus / D. hansenii / D. vanrijiae*
Hanseniaspora	*H. guillermondii / H. osmophila / H. uvarum / H. vineae*
Hansenula	*H. anomala*
Issatchenkia	*I. orientalis*
Kluyveromyces	*K. gamospora / K. thermotolerans / K. marxianus*
Lachancea	*L. thermotolerans*
Metschnikowia	*M. pulcherrima*
Millerozyma	*M. guilliermondii*
Pichia	*P. caribbica / P. fermentans / P. kluyveri / P. kudriavzevii*
Rhodotorula	*R. mucilaginosa*
Saccharomycodes	*S. ludwigii*
Schizosaccharomyces	*S. pombe*
Starmerella	*St. bacillaris*
Torulaspora	*T. delbrueckii*
Wickerhamomyces	*W. anomalus*
Williopsis	*W. saturnus*
Zygosaccharomyces	*Z. bailii / Z. Florentina*

Population structure and genetic diversity of the *S. cerevisiae* wine group has been associated with the geographic location at global and regional scales, grape variety, and environmental conditions (Higgins et al. 2021), for example, the differential ability of copper resistance and sulphur dioxide production of wild *S. cerevisiae* strains isolated from different regions as well as the produced volatile aromatic compounds in final wine (Mauriello et al. 2009). Spontaneous fermentation favoured a high *S. cerevisiae* strain diversity in organic wineries, and their presence, especially the winery-specific strains, improved the wine complexity and differentiation (Castrillo et al. 2020). New Zealand *S. cerevisiae* is highly geographically structured at a local level, and this high diversity correlates with European colonists to New Zealand, followed by subsequent diversification and admixture and produced a distinct group of *S. cerevisiae* wine-group strains (Higgins et al. 2021). Yeast population and diversity investigation in the North Island of New Zealand depicted a region-specific sub-population and a reasonable level of gene flow of *S. cerevisiae* among different regions (Gayevskiy and Goddard 2012). Besides, its regionally genetically differentiated *S. cerevisiae* population was reported to be associated with different wine phenotypes in terms of the ferment performance and chemical profiles (Knight et al. 2015).

On the other hand, non-*Saccharomyces* strains are considered more restricted to local regional *terroirs* than *Saccharomyces* (Carrau et al. 2020). *St. bacillaris* isolates collected from different grape varieties but within the same Italian region were still showing little genetic distance (Englezos et al.

2015). In the study of Knight et al. (2020), there is no *S. cerevisiae* population differentiation observed between four investigated New Zealand vineyards in close proximity, alternatively, the regional characteristics of wines are more likely contributed to by non-*S. cerevisiae* species present during fermentation.

Contribution of non-*Saccharomyces* yeasts

Aroma and flavour compounds produced during fermentation make an important contribution to a pleasant wine sensory experience. Wine aroma is a unique mixture of volatile compounds derived from grapes (*varietal* aromas), secondary metabolites (*fermentative* aromas), and aging (*post-fermentative* aromas) (Swiegers et al. 2005), of which the *fermentative* aromas make up the largest proportion of the overall wine aroma composition (Polaskova et al. 2008). A complex "flavour phenotype" of more than 1300 volatile compounds can be produced by non-*Saccharomyces* yeasts (Tofalo et al. 2016), such as terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid, and succinic acid (Swiegers et al. 2005, Fleet 2008).

The significant contribution of non-*Saccharomyces* yeasts to wine organoleptic characteristics (Domizio et al. 2011, Tofalo et al. 2016, Canonico et al. 2018) depends on the quality and quantity of metabolites produced. The esters category (both ethyl and acetate esters) has the largest number of aroma compounds, which mainly contribute fruity aromas to the wine and their production is positively related to the generation of ethanol and higher alcohols (Boss et al. 2015). The medium-chain fatty acids are mainly produced by yeasts as intermediates in the biosynthesis of long-chain fatty acids. They can directly contribute to the wine flavour by enhancing fatty, rancid, fruity, or cheesy odours, and participate in the ethyl acetate formation (Liu et al. 2016). Enhanced yeast-derived acetaldehyde production was reported to contribute to form stable polymeric pigments that is important for colour stability in red wine (Escott et al. 2018). Furthermore, the effect of yeast metabolites on wine quality is concentration dependent. For example, a low level (approximately 50 mg/L) of ethyl acetate may increase the aroma complexity, but a high concentration (over threshold 150 mg/L) would contribute negative aroma normally described as the nail polish remover (Varela et al. 2016). An excessive concentration (over threshold 400 mg/L) of higher alcohols can result in a strong and pungent smell, whereas at low level (below 300 g/mL) they impart a pleasant character to the wine (Liu et al. 2016).

In general, the contribution of non-*Saccharomyces* yeasts is largely due to their capacity to produce a wide range of extracellular hydrolytic enzymes. About 70%-80% of the isolated non-*Saccharomyces* yeasts possessed one or more enzymes of biotechnological interests (Fernández et al. 2000, Ganga and Martinez 2004). Escribano et al. (2017) have reported thirteen enzymes related to wine aroma, colour, and clarity, which were secreted from ninety-seven non-*Saccharomyces* wine yeast strains belonging to ten different genera and species. The huge enzymatic potential of non-*Saccharomyces* yeasts exerts a substantial impact on wine aroma and flavour and facilitates greater expression of

grape varietal characters (Fleet 2008, Englezos et al. 2015). For example, *Metschnikowia* spp. exhibited remarkable aroma-related enzymatic activities (Binati et al. 2019). However, the secretion of each enzyme is not characteristic of a particular genus or species but strain-dependent (Ganga and Martinez 2004, Belda et al. 2016). It is therefore important to characterize each isolate for their extracellular enzyme secretion during the wine yeast starter selection process (Escribano et al. 2017). Table 2.2 lists some enzymes of oenological interest, in an order of their importance in winemaking, detected in non-*Saccharomyces* yeast species.

Although non-*Saccharomyces* yeasts would die off during the alcoholic fermentation process, their enzymes secreted at an early stage remain and function in wines (Maturano et al. 2012). The main underlying mechanisms of how enzymes influence the wine aroma can be summarised as: (i) some enzymes interact with odourless aroma precursors, which are glycosidically bounds (Hjelmeland and Ebeler 2015), or cysteine- and glutathione-conjugated (Capone et al. 2012), to produce volatile aroma compounds, *e.g.* terpenes and C13-norisoprenoids are released from their glycoside form (Girard et al. 2016) due to the cleavage of glycosidic bonds by β-glucosidase (Fernández et al. 2000, Escribano et al. 2017); (ii) some enzymes are secreted outside the cell membrane to break down complex compounds and polymers in their surroundings so as to provide the yeast with more energy and nutrients (Borren and Tian 2020); *e.g.* protease that liberates assimilable nutrient sources (*e.g.* amino acid and peptides) to prompt yeast growth as well as the production of aroma compounds (Maturano et al. 2015); (iii) certain enzymes directly catalyse the formation of primary and secondary aroma compounds in wine (Borren and Tian 2020), *e.g.* esterase catalyses the hydrolysis and synthesis of fruity esters (Escribano et al. 2017).

Apart from the contribution to wine aroma, certain aroma-related enzymes (*e.g.* carbohydrolases: pectinase, cellulase, xylanase, and glucanase) also play a role in wine colour and stability. The degradation of the structural polysaccharides by carbohydrolases can result in higher extraction of phenolic compounds, and therefore promote more polymeric pigments formation in aged red wine, leading to an improvement in colour intensity and stability (Escribano et al. 2017). The other applications of non-*Saccharomyces* yeasts were also reported in improving mouthfeel and body of wine due to the enhanced yeast polysaccharides released (Domizio et al. 2011, Domizio et al. 2014), controlling spoilage microflora (*e.g. P. anomala/K. wickerhamii*-secreted killer toxins against *Brettanomyces* spoilage yeasts (Comitini et al. 2004)), and reducing ethanol content (Quirós et al. 2014, Varela et al. 2016, Comitini et al. 2017, Escott et al. 2018). A recent study also demonstrated that some strains of *L. thermotolerans*, *Metschnikowia* spp. and *St. bacillaris* are strong producers of a powerful antioxidant, glutathione (GSH), and thus the use of their mixed-culture fermentation with *S. cerevisiae* was shown to be a promising strategy to lower inputs and ultimately obtain a healthier wine product with minimal requirement of SO_2 (Binati et al. 2021).

Table 2.2 Enzymes of oenological interest detected in different non-*Saccharomyces* yeasts.

Enzymes	**Species**	**Contribution**	**Reference**
β-Glucosidase	*A. pullulans, H. uvarum, H. opuntiae, H. osmophila, M. pulcherrim, M. viticola, St. bacillaris, Candida* spp.*, Cryptococcus* spp.*, L. thermotolerans*, *Z. bailii,T. delbrueckii, K. marxianus*, *Meyerozyma guilliermondii*, *W. anomalus, Rhodosporidium toruloides*	Wine aroma (Terpenes-varietal aroma)	(Fernández et al. 2000, Englezos et al. 2015, Belda et al. 2016, Escribano et al. 2017)
Protease	*H. uvarum, H. opuntiae, H. osmophila, P. membranifaciens, M. pulcherrima, St. bacillaris, Dekkera spp., Candida spp., T. delbruekki, W. anomalus, R. toruloides, A. pullulans, Metschnilowia* spp.,	Clarification and stabilization; avoid sluggish fermentation	(Fernández et al. 2000, Ganga and Martinez 2004, Englezos et al. 2015, Belda et al. 2016)
α-L-arabinofuranosidase	*Z. microellipsoides, M. guilliermondii, W. anomalus, R. toruloides, A. pullulans, Cryptococcus amylolentus, Metschnilowia* spp.,	Volatile terpenes increase	(Ganga and Martinez 2004, Belda et al. 2016)
β-lyase	*T. delbrueckii, M. guilliermondii, and K. marxianus*	Volatile thiols release	(Belda et al. 2016)
Esterase	*Candida* spp.*,* *Cryptococcus* spp.*, Debaryomyces hansenii, L. thermotolerans*, *M. pulcherrima, P. kluyveri*, *Sporodiobulus salmonicolor*, *T. delbrueckii*, *Williopsis pratensis, Z. bailii, St. bacillaris*	Wine aroma	(Englezos et al. 2015, Escribano et al. 2017)
Esterase-Lipase	*Candida* spp.*,* *Cryptococcus* spp.*, Debaryomyces hansenii, L. thermotolerans*, *M. pulcherrima, P. kluyveri*, *Sporodiobulus salmonicolor*, *T. delbrueckii*, *Williopsis pratensis, Z. bailii*	Wine aroma	(Escribano et al. 2017)
Pectinase	*Candida* spp.*,* *Cryptococcus* spp.*, Debaryomyces hansenii, L. thermotolerans*, *L. kluyveri, M. pulcherrima, P. kluyveri*, *Sporodiobulus salmonicolor*, *T. delbrueckii*, *Z. bailii*	Must clarification, colour, and aroma	(Fernández et al. 2000, Escribano et al. 2017, Binati et al. 2019)
Xylanase	*L. thermotolerans*	Wine aroma, colour, and fining	(Escribano et al. 2017)
Glucanase	*Cryptococcus* spp.*, Debaryomyces hansenii, L. thermotolerans*,	Wine aroma, colour, and fining	(Escribano et al. 2017)
Lipase	*L. thermotoleran, Sporodiobulus salmonicolor*	Wine aroma	(Escribano et al. 2017)
Cellulase	*A. pullulans, Z. cidri, Z. fermentati, Dekkera spp., M. pulcherrima, Candida spp., T. delbruekii, Z. microellipsoide,* *Cryptococcus* spp.*, Debaryomyces hansenii, L. thermotolerans*, *Z. bailii*	Wine aroma, colour, and fining	(Ganga and Martinez 2004, Belda et al. 2016, Escribano et al. 2017)

Polygalacturonase	*A. pullulans, P. membranifaciens, M. pulcherrima*, *Brettanomyces clausenii, P. anomala, K. thermotolerans, C. stellate, T. delbruekii, Metschnilowia* spp.	Wine clarification and filterability, colour and flavour compounds release	(Fernández et al. 2000, Belda et al. 2016)
Sulfite reductase	*H. uvarum, H. osmophila, H. opuntiae, T. delbruekii*	H_2S production	(Belda et al. 2016)

Indigenous yeast selection

It is becoming ever more important to select yeasts that are suitable for a particular style of wine, region, and even microclimate (Suárez-Lepe and Morata 2012). Traditionally, yeasts have been selected for their high fermentation efficiency, suitable fermentative kinetics at different temperatures, high improvement in sulphite tolerance, and killer activity, and low acetic acid production. However, the new selection criteria have evolved to be able to improve the technological properties (*e.g.* wine colour, aroma, structure, and body) and sensorial features of wines (Suárez-Lepe and Morata 2012, Escribano et al. 2017). On another note, from the perspective of consumers, the specific flavour was considered as a sub-dimension of good wine quality by Charters and Pettigrew (2007), and distinctiveness and complexity are commonly sought by professionals and high-involvement consumers.

Novel starter culture tailored to reflect the *terroir* and yeast biodiversity as reflected by spontaneous fermentation, undoubtedly, is a profitable strategy to assure the wine quality and preserve the typical wine characteristics of "*terroir*" (Settanni et al. 2012, Padilla et al. 2017, Capece et al. 2019). Wine yeast for starter culture development is mainly sourced from grapes in the vineyard and spontaneous fermentation (Fleet 2008), supported by the extensive microbial biodiversity (Diaz et al. 2013, Garofalo et al. 2016, Drumonde-Neves et al. 2017). Indigenous non-*Saccharomyces* yeast strains isolation has been implemented in different wine regions on various grape varieties (Settanni et al. 2012, Medina et al. 2013, Ilieva et al. 2016, Ilieva et al. 2017, Wang et al. 2019), and they are increasingly investigated as co-starters to augment the complexity and regionality of wine (Binati et al. 2019). Teixeira et al. (2015) revealed the diversity of wine aroma profiles generated by a variety of non-*Saccharomyces* yeast species isolated from the spontaneous fermenting must of Touriga Nacional (*H. uvarum* and *H. guilliermondii, P. kudriavzevii*, *P. terricola*, *P. manshurica*, *St. bacillaris*, *Zygoascus hellenicus*, *Z. bailii* and *Z. bisporus*). Rossouw and Bauer (2016) presented the most potential of vineyard non-*Saccharomyces* isolates for commercial application in sugar utilisation, low ethanol production, and improved aroma.

As mentioned above, the influence of yeast on wine characters is often species/strain dependent and grape variety dependent. The rate and extent of sugar utilisation, ethanol yield, and glycerol production varied significantly among different isolates within single species. Inoculation of non-*Saccharomyces* in the Sauvignon Blanc fermentation appeared to have a greater impact on ethanol production compared to the Pinotage fermentations (Rossouw and Bauer 2016). The wines produced by selected indigenous strains of *H. uvarum*, *M. pulcherrima*, *T. delbrueckii*, *St. bacillaris* and three different strains of *S. cerevisiae* inoculated sequentially were differentiated by sensory analysis based on the tasting panellists' appreciation (Padilla et al. 2017). Arguably, more work should be done to exploit the beneficial oenological traits of non-*Saccharomyces* isolates. Among the non-*Saccharomyces* species, strains of *Hanseniaspora* spp., *M. pulcherrima*, *L. thermotolerans*, *T.*

delbrueckii, *P. kluyveri,* and *St. bacillaris*, generally, are showing more unique oenological properties (Borren and Tian 2020).

Sequential inoculation with *H. vineae* followed by *S. cerevisiae* resulted in increased aroma and flavour diversity and palate length and body as well as a significant increase in fruit intensity (*i.e.* banana, pear, apple, citric fruits, and guava) (Medina et al. 2013). *Metschnikowia* spp. were reported to exhibit remarkable aroma-related enzymatic activities by Binati et al. (2019) who also reported the prized quality of species *L. thermotolerans* in reducing volatile acidity while by generation of lactic acid. Moreover, *M. pulcherrima* was considered to be a highly effective biocontrol yeast and a good alternative for spoilage microorganism control during wine fermentation due to the production of pigment pulcherrimin (Turkel and Ener 2009).

In addition to increased aroma complexity, non-*Saccharomyces* yeasts also play a key role in potential healthy wine production and contribute to the "low input winemaking" concept. Vineyard isolates of *H. uvarum*, *H. opuntiae*, *H. vinae*, *P. kudriavzevii* and *C. flavescens* all showed a substantial reduction (>1.5%) in the final ethanol content of the sequential fermentations with *S. cerevisiae* (Rossouw and Bauer 2016). Species *St. bacillaris* has been investigated extensively for its potential of low alcohol wine production (Lemos Junior et al. 2019) and low SO_2 input (Binati et al. 2021) with the increased glycerol production contributing to smoothness, sweetness, and complexity (Binati et al. 2019), as well as, its fructophilic nature that can avoid sluggish fermentation.

Furthermore, the selected indigenous yeast strains may be better adapted to local fermentation conditions (wine region and the grape must composition) than commercial strains (Esteve-Zarzoso et al. 2000, Liu et al. 2016, Padilla et al. 2017), therefore it is highly recommended to incorporate the indigenous yeasts in a mixed inoculum to achieve distinct wine styles. Table 2.3 shows some recent reports of the contribution of non-*Saccharomyces* yeasts to resulting wine.

Table 2.3 Effect of mixed inoculation of *S. cerevisiae* with different non-*Saccharomyces* yeasts on organoleptic properties of wine.

Non-Saccharomyces species	**A significant contribution to flavour compounds**	**References**
L. thermotolerans,	"+": Lactic acids; "-": Ethanol	(Binati et al. 2020, Binati et al. 2021)
Metschnikowia spp.	"+": Alcohols and esters; "-": Volatile phenols	
Torulaspora delbrueckii	"+": Glycerol, Pyruvic acid, Volatile thiols, polysaccharides, Acetic acid ethyl ester; "-": Acetic acid, Alcohol	(Belda et al. 2015, Belda et al. 2017)
Starmerella bacillaris	"+": Glycerol; "-": Acetaldehyde, Acetic acid, SO_2, Ethanol, Malic acid	(Englezos et al. 2015, Lemos Junior et al. 2019, Binati et al. 2020)
Starmerella bacillaris / *Hanseniaspora uvarum*	"+": Glycerol; "-": Biogenic amines	(Tofalo et al. 2016)
Hanseniaspora vinea	"+": Glycerol, Acetyl, Ethyl ester; "-": Alcohol, Fatty acid	(Medina et al. 2013)

Notes: "+" and "-" means the "increased" and "reduced" concentration of corresponding metabolites in mixed starter cultures in comparison to the wines fermented with a single starter culture of *S. cerevisiae* as the control.

Spoilage yeast

Winemaking occurs under non-sterile conditions, so the potential existence of some yeast species may show undesirable effects on wine quality. Spoilage yeasts are a substantial threat to the wine industry as they can produce unpleasant flavour and aroma, which has detrimental effects on wine quality and consequently causes significant economic losses. Typical spoilage includes the production of ethyl acetate by *Kloeckera/Hanseniaspora* spp. before fermentation, H_2S by *S. cerevisiae* during fermentation giving a "rotten egg" character, acetaldehyde by film-forming yeasts (typically *Candida* spp. and *Pichia* spp.) during bulk storage imparting a "bruised or rotten apples" smell, and volatile phenols by *B. bruxellensis* during storage or after bottling (Malfeito-Ferreira 2011). Accordingly, wine spoilage is usually recognized as film formation in stored wines, cloudiness and re-fermentation in bottled wines, and off-flavours at all winemaking phases (Loureiro and Malfeito-Ferreira 2003).

Of them, *B. bruxellensis* is the spoilage yeast of most concern in winemaking, of particular notoriety in high-quality red wines matured in costly oak barrels (Malfeito-Ferreira 2011). *Kloeckera/Hanseniaspora* spp. can be easily controlled by preventive measures like low temperature, addition of SO_2, and hygiene. The control of film-forming yeasts is mainly achieved through their weak tolerance to low oxygen tensions, which enhance the inhibitory effect of ethanol or preservatives to be used (Malfeito-Ferreira 2011). However, it is more difficult to control *B. bruxellensis* due to their strong capacity to survive with limited nutrients (Smith and Divol 2018) and oxygen, and even higher ethanol-resistance than *S. cerevisiae* (Renouf et al. 2007). Besides, at the

early stage of wine fermentation, *B. bruxellensis* is hard to be detected due to their initial low levels and slow-growing characteristics. Moreover, this species has long-term viability and can proliferate when the conditions become suitable (Renouf and Lonvaud-Funel 2007). Furthermore, certain strains belonging to *B. bruxellensis* have been reported to produce toxic substance of biogenic amines (Caruso et al. 2002, Granchi et al. 2005). During winemaking processes, alcoholic fermentation and aging in oak barrels, wines are more susceptible to the contamination of *B. bruxellensis*, which means the prevention and control strategies should be conducted at these stages (Renouf et al. 2007).

The volatile phenols (*Brett character*) produced by *B. bruxellensis* are mainly 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), which contributes unpleasant aromas typically described as "barnyard" or "horse sweat" (Pinto et al. 2020). The EP is usually formed during the enzymatic process of wine yeast and aging involving two enzymes of hydroxycinnamic acid decarboxylase (HCDC) and vinylphenol reductase (Šućur et al. 2016). Firstly, HCDC turns the hydroxycinnamic acids (*p*-coumaric and ferulic acids) in grape juice into vinylphenols, which are then reduced to ethyl derivatives (4-EP and 4-EG) by vinylphenol reductase (Šućur et al. 2016, Smith and Divol 2018, Tubía et al. 2018). These aroma characters can be considered either negative or positive depending on the concentration. For example, 4-EP is only considered as spoilage when it presents over the level of 620 µg/L. In contrast, at level below 400 µg/L it contributes favourably to the complexity of wine by imparting spices, leather, smoke, or game aromas (Loureiro and Malfeito-Ferreira 2003).

In general, addition of SO_2 (an antioxidant and antimicrobial substance) is a common practice and the most effective measure to prevent spoilage microorganisms proliferation (Šućur et al. 2016), the other chemical compounds used are sorbic acid and benzoic acids. With regard to physical operations, clarification can remove suspended microorganisms, fining agents target microorganisms during wine settling, while pre-bottling filtration is the most common procedure to achieve wine "sterilisation" during wine ageing (Malfeito-Ferreira 2011). Although heat treatments are rarely used in the wine industry, this technique is particularly appropriate for processing rotten grapes (Malfeito-Ferreira 2011). Another interesting strategy is the introduction of indigenous yeasts or bacteria, which can act not only against spoilage microorganisms, but also help retain the regional wine distinctiveness (Berbegal et al. 2017). Recently, there have been numerous novel approaches, such as high hydrostatic pressure, ultrasounds, pulsed electric fields, UV irradiation, microwaves, ozone and electrolysed water, novel finishing agents (Pinto et al. 2020).

2.1.3 Wine yeast identification

As discussed above, microbial diversity in the vineyard can have an impact on the efficacy and quality of the winemaking process. The potential risks of spoilage yeasts emphasize the importance of appropriate microbiological control and strict fermentation management throughout winemaking, while the recovery of individual strains of some indigenous yeasts present on grapes would be

desirable for the production of distinctive wine styles. In both cases, accurate yeast identification and differentiation methods are important to the wine industry.

Conventional methods

Conventional identification techniques are usually based on the morphological and physiological traits of different yeast species. To facilitate rapid and accurate identification, a large variety of commercial products have been introduced to fulfil phenotypic testing, including differential chromogenic isolation agar, species-specific direct enzymatic colour tests, direct immunological tests, and enhanced manual and automated biochemical and enzymatic panels (Freydiere et al. 2001).

Wallerstein Laboratory (WL) nutrient agar and Lysine agar are usually used for preliminary isolation (Esteve-Zarzoso et al. 2000, van Breda et al. 2013). Certain yeast species can be readily discriminated based on their specific colony morphology and colour on WL nutrient agar, whereas only non-*Saccharomyces* yeasts can grow on Lysine agar due to their susceptibility to lysine (Liu et al. 2016). Other selective media include Chromagar™ Candida that contains various chromogenic components showing different colours when different yeast species are growing on it (van Breda et al. 2013), and Rose Bengal Chloramphenicol Agar (RBCA) that are specific for yeasts and moulds growth and the grape berries from vineyards can be placed on RBCA directly (Diaz et al. 2013).

Colonies isolated can then be subjected to tests including sugar fermentation pattern determination (*e.g.* maltose, sucrose, and lactose), fermentation profile, auxotrophy profiles, and the ability to produce fertile hybrids (Rainieri et al. 1999, Ženišová et al. 2014, Tokpohozin et al. 2016). However, these methods are not accurate and reproducible due to the limitation of varying physiology of cells under different culture conditions and heterogeneous phenotypes (Mortimer and Polsinelli 1999, Fernández et al. 2000, Barata et al. 2012). Based on the morphology of vegetative cells and asci as well as the metabolic data, only 37% isolates from WL nutrient agar (68 strains out of 185 isolates) can be assigned to genus *Saccharomyces* (Esteve-Zarzoso et al. 2000). The work conducted by Tokpohozin et al. (2016) demonstrated that morphological and physiological (fermentation and assimilation) traits do not enable non-*Saccharomyces* yeasts discrimination. With the API 20 C AUX system, non-*Saccharomyces* yeasts isolated from Lysine medium can only be identified at genus level as *Candida, Dekkera, Hanseniaspora, Kluyveromyces, Torulaspora,* and *Zygosaccharomyces* (Esteve-Zarzoso et al. 2000).

Molecular methods

The transition from phenotypic identification of yeast to molecular identification began with the determination of the mol% guanine + cytosine (G+C) ratios of nuclear DNA, strains differing by 1-2 mol% are recognized as different species (Kurtzman 2006). Such quantitative assessment of genetic similarity was firstly achieved by nuclear DNA reassociation, however, this method is time-consuming and its genetic resolutions limited to closely related species (Kurtzman 2006). With the further

development of biological techniques, especially the invention of PCR technology (Kb 1987, Mullis and Faloona 1987), a large number of DNA-based techniques appeared and were applied in wine-associated microbial populations and diversity analysis.

Broadly speaking, identification techniques can be categorized into culture-independent and culture-dependent methods. Culture-dependent, by definition, is used for cultured microorganisms, whereas culture-independent methods are used to profile the whole population or to identify specific microbes in a mixed population directly without steps of cultivation and isolation (Ivey and Phister 2011). Compared to the culture-dependent techniques, these techniques allow the identification of viable but non-culturable (VBNC) cells and dead microorganisms (Albergaria and Arneborg 2016), which is also more rapid as the isolation and subsequent culture time is saved. Moreover, its ability to detect VBNC cells may be useful for the spoilage organisms monitoring, *e.g.* SO_2 in winemaking can induce *B. bruxellensis* into VBNC state (Capozzi et al. 2016).

Culture-independent techniques

Culture-independent techniques are typically applied to wine yeast community population and diversity analysis directly from the grapes or wine ferments, in which quantitative real-time PCR (qRT-PCR) (Zott et al. 2010, Diaz et al. 2013, Ženišová et al. 2014, Wang et al. 2015, Maturano et al. 2016, Padilla et al. 2017) and PCR-denaturing gradient gel electrophoresis (DGGE) are most widely used (Manzano et al. 2004, Perez-Martin et al. 2014, Wang et al. 2015, Maturano et al. 2016, Sha et al. 2018).

The difference of qRT-PCR from traditional PCR is the incorporation of a fluorescent dye; the fluorescence increases in PCR product after each PCR cycle. According to the manner of fluorescence link to DNA amplification, qRT-PCR can be classified as SYBR Green-based and probe-based, and in both assays the samples will be considered positive once the amount of fluorescence exceeds a predetermined threshold value (known as cross threshold or Ct value) (Ivey and Phister 2011), which will be compared with a standard curve to quantify the sample. The main limitation of qPCR is the limited specificity of the qPCR probes and the method efficiency, for example, it has been found to be more sensitive for detection of *H. uvarum,* but not for *St. bacillaris* (Padilla et al. 2017) .

PCR-DGGE was firstly applied to characterize the yeast diversity in a commercial sweet wine fermentation by Cocolin et al. (2001). Generally, yeast DNA was extracted directly from a fermenting must and used as a template for 26S rDNA gene amplification, followed by DGGE separation, the resulting bands being isolated and sequenced for strain identification (Cocolin et al. 2001). In brief, DGGE is based on the separation of DNA fragments that have the same length but differing nucleotide sequences, which will show varied electrophoretic mobility due to their different DNA melting characteristics in a polyacrylamide gel containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Kurtzman 2006). A related technique, PCR-temperature gradient

gel electrophoresis (TGGE), is similar but based on a linear temperature gradient (Barata et al. 2012). Generally, qRT-PCR is more appropriate for a specific microorganism, whereas the DGGE (or TGGE) is more suitable for microbial community profiling (Kioroglou et al. 2018).

Another approach worth mentioning is metagenomics, which is defined as the direct genetic analysis of the collective of genomes within an environmental sample (Thomas et al. 2012), which can be achieved via whole metagenome sequencing (higher resolution but a higher cost) or amplicon-based sequencing (lower resolution but cheaper) (Franzosa et al. 2015). Its development has been largely driven by next-generation sequencing advances. The contribution of high-throughput next-generation sequencing and metagenomics approaches to vineyard microbial ecology have been reviewed by Morgan et al. (2017). In the case of wine sample analysis, amplicon-based sequencing is more frequently used, which targets only one or few specific marker genes amplification; in contrast, whole metagenome sequencing is typically utilized for taxonomic and functional profiling of examined microbial community, which is performed on millions of random genomic fragments (Franzosa et al. 2015). To examine the richness and composition of grapevine associated-fungal communities and the geographical community structure among four major wine regions in New Zealand, Taylor et al. (2014) pyrosequenced the 26S rDNA gene regions, acquiring 200 taxa that are 10-fold more than previously recovered using culture-dependent based methods.

Culture-dependent techniques

The ribosomal DNA (rDNA) sequencing is the most frequently used method in wine yeast identification, particularly the reference method 26S rDNA sequencing (Kurtzman and Robnett 1998). In practice, it is more common to apply the sequencing of DNA extracted from the yeast isolates purified on a solid medium (Ivey and Phister 2011), and, thus, this method is classified under the culture-dependent category in this literature review. The 26S rDNA sequencing targets the D1/D2 domain of 26S ribosomal (primers NL1, NL4), and the resulting gene sequence (ca. 600 bp fragment) is then compared to a gene database (*e.g.* GenBank NCBI database) of known yeast species for identification. Generally, yeast strains within a species show no more than 0-3 nucleotide differences (0-0.5%), and separate species show 6 or more noncontinuous substitutions (1%) (Kurtzman 2006). This method is the primary choice as it has a long history in yeast identification and there is a well-established database for identification of a large number of yeast species (Ivey and Phister 2011).

Gene 5.8-ITS (Internal Transcribed Spacer) rDNA regions (primers ITS1 and ITS4) (Turkel and Ener 2009, Wieme et al. 2014, Liu et al. 2016, Raymond Eder et al. 2017, Guzzon et al. 2020) have also been widely used for wild yeast isolate identification, and these regions can provide somewhat finer taxonomic resolution than D1/D2 region. The 5.8S is a highly conserved region of ribosomal and ITSs are the variable zones, which combine with the same fragment that makes them a useful marker for closely related strain differentiation (Rainieri et al. 1999). Some other genes are also used for yeast identification, *e.g.* intergenic spacer (IGS) and non-transcribed spacer 2 (NTS2) of rRNA. In terms of

the sequencing techniques, the suitable primers that are effective for essentially all species and comprehensive database construction are necessary for accurate identification (Wang et al. 2015).

While single-gene sequencing is useful for rapid species identification in most cases, its resolution can be confused for closely related species and the common natural hybrids. This is particularly evident among the *Saccharomyces* complex (*S. bayanus, S. cerevisiae*, *S. paradoxus*, *S. pastorianus* and *S. uvarum*), *i.e.* the D2 domain rRNA sequence of *S. pastorianus* is identical to that of *S. bayanus* (Peterson and Kurtzman 1991). Multigene sequencing offers an insight into the diversity of *Saccharomyces* interspecies hybrids, which may extend to other fungal hybrids identification. Different combinations of genes *Seu*NTS2 (rDNA), *ScMAL31*, *MTY1,* and *SuMEL1* from *S. bayanus* CBS 380^{T} revealed the evidence of genomic makeup among the 33 strains examined (Nguyen and Boekhout 2017).

Additionally, other techniques used for wine yeast species identification/typing include multilocus sequence typing (MLST) and fingerprinting-based techniques such as randomly amplified polymorphic DNA (RAPD) (Binati et al. 2019), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphisms (RFLP) and karyotyping (Ivey and Phister 2011). These techniques are often used for stain differentiation, particularly the fingerprinting-based techniques (RAPD, AFLP, RFLP and karyotyping), creating a unique banding pattern by digesting or amplifying regions of genes, to provide rapid and less expensive alternatives without further sequencing requirements.

The MLST approach compares the sequence of 6-8 housekeeping genes in an organism (Ivey and Phister 2011), where variation accumulates relatively slowly and is selectively neutral (Tofalo et al. 2013). It is highly reliable for strain typing and useful for inferring phylogeny, but less sensitive for discriminating closely related yeast strains (Hart et al. 2019). In one study of 84 *S. cerevisiae* strains, MLST differentiated Asian strains from Lebanese and European commercial strains with a discriminatory power of 92.27%, but appeared less discriminatory on closely related wine yeasts than microsatellite genotyping (99.84%) and δ sequence typing (99.90%) (Ayoub et al. 2006). More discriminatory molecular techniques utilize different classes of DNA elements such as retrotransposons, mitochondrial-based microsatellites, thereby revealing genetic variation through increased genome coverage (Agarwal et al. 2008). Both δ sequence and microsatellite genotyping are PCR-based fingerprinting methods and are extensively used for *S. cerevisiae* typing. The δ sequence amplification targets the δ sequence regions flanking the 100 or so Ty1 retrotransposons, while the mini- and micro-satellites are repetitive regions found throughout the *S. cerevisiae* genome (Ivey and Phister 2011). The number of repeats is highly variable among individuals, therefore the amplicons acquired using the primer specific to the regions flanking the microsatellites will differ in length and can be analysed by polyacrylamide gel electrophoresis (Agarwal et al. 2008).

Molecular markers provide a direct measure of genetic diversity, however, the different molecular markers differ in terms of cost, speed, genomic abundance, locus specificity, reproducibility, labour, and degree of polymorphism (Costa et al. 2016). Restriction Fragment Length Polymorphism (RFLP) analysis was the first reported molecular marker technique in the detection of DNA polymorphism in 1980 (Agarwal et al. 2008). It requires a large quantity of high-quality genomic DNA and prior sequence information. In contrast, RAPD and AFLP are less complex, without the limitation of prior sequence knowledge. The RAPD method is simple and rapid, but lacking reproducibility due to the mismatch annealing, whereas AFLP is more reproducible and combines the advantages of RFLP by ligating primer-recognition sequences to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers (Agarwal et al. 2008). Therefore, the AFLP allows higher numbers of reproducible polymorphic bands with just a few primer combinations (Costa et al. 2016).

Of the three methods above, the RFLP is the most widely used for winemaking yeast typing, which is based on the restriction patterns (*e.g.* restriction enzyme *Hinf*I, *Alu*I) generated from the selected genomic regions with 5.8-ITS being a highly popular targeted region (Ganga and Martinez 2004, Maturano et al. 2012, Diaz et al. 2013, Padilla et al. 2017). The RFLP-5.8-ITS profiles of non-*Saccharomyces* yeasts (69 isolates of pronounced enzymatic activity and 11 isolates without any activities) produced different molecular profiles, typing revealed the possibility of intraspecific differences in *P. membranifaciens* as different molecular profiles had one or more restriction bands in common (Fernández et al. 2000). The 26S rDNA sequencing could not separate *S. uvarum* and *S. bayanus*, but these two species can be differentiated easily by comparing their RFLP-NTS2 profiles (Nguyen and Boekhout 2017, Zhang et al. 2021). The identification can also be achieved by comparing the size of the PCR products. The RFLP analysis of 5.8S-ITS gene using the endonucleases *Hinf*I, *HaeIII*, and *Hha*I produced 12 different restriction profiles with 8 of which being identified by comparing the molecular mass of the restriction enzyme products with those previously described (Clavijo et al. 2010).

Karyotyping, also known as pulsed-field gel electrophoresis (PFGE) typing, is based on chromosome length polymorphism that delimits strains according to specific karyotypes. PFGE using the concept of two alternating electric fields was firstly introduced in 1983 by Schwartz et al. (1983). It allows larger DNA molecules separation than conventional DNA electrophoresis whose separation capacity only up to 50 kilobase pairs (kb), whereas yeast chromosomes range from several hundred to several thousand kb (Zimmermann and Fournier 1996). Since then a variety of instruments have been developed to increase the size resolution of both large and small DNA molecules, *e.g.* Field-Inversion Gel Electrophoresis (FIGE), Transverse-Alternating Field Gel Electrophoresis (TAFE) (Longo and Vezinhet 1993, Schuller et al. 2004), Orthogonal-Field Alternation Gel Electrophoresis (OFAGE), Rotating Gel Electrophoresis (RGE), Programmable Autonomously-Controlled Electrodes (PACF),

Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis (PHOGE), and Contour-Clamped Homogeneous Electric Fields (CHEF) (Longo and Vezinhet 1993). Currently, the CHEF is the most widely used apparatus with resolution of molecules up to 7,000 kb (Basim and Basim 2001). The CHEF karyotyping is efficient for closely related *Saccharomyces* strains differentiation (*S. bayanus* from *S. cerevisiae* and *S. paradoxus*) (Pavlovic et al. 2014), as well as, for the *S. cerevisiae* hybrids (Hart et al. 2016).

Generally, the culture-independent methods provide a better overview of the true microbial community diversity and composition, as it is hard to detect the non-culturable cells by culture-dependent methods (Wang et al. 2015). Padilla et al. (2017) detected *Saccharomyces* spp. using qPCR, but no isolate from these species was recovered from the fresh juice. However, fewer numbers of minor yeast species were detected by culture-independent methods, because of the enrichment effect of the culture media using culture-dependent methods, as well as the used culture-independent technique biases like the detection limitation, and the preferential amplification caused by variable amplicon sizes of targeted regions (Perez-Martin et al. 2014, Wang et al. 2019).

However, culture-independent techniques are usually used in conjunction with culture-dependent techniques (Table 2.4) to compensate the low detection sensitivity of the latter (Taylor et al. 2014), as well as to confirm the viability of yeasts detected by culture-independent (Albergaria and Arneborg 2016), as only culturable yeast strains can be further exploited for their potential in winemaking. Furthermore, the broad taxonomic range of molecular tools for community analysis limits their resolution, often resulting in a lack of information at the species level.

Table 2.4 Culture-dependent and culture-independent techniques adopted for wine yeast identification/typing.

Culture-dependent	**Culture-independent**	**Reference**
PCR-RFLP-5.8-ITS; 26S rDNA (D1/D2) sequencing	High-throughput sequencing analysis (ITS3-KYO2 and ITS4)	(Wang et al. 2019)
PCR-AFLP-5.8-ITS; 26S rDNA (D1/D2) sequencing	-	(Vaudano et al. 2019)
Microsatellite multiplex PCR (*S. cerevisiae* typing)		
PCR-ITS-sequencing	PCR-DGGE/DGGE eluted bands sequencing	(Sha et al. 2018)
26S rDNA (D1/D2) sequencing, PCR-RFLP-5.8-ITS	Quantitative PCR (qPCR)	(Padilla et al. 2017)
δ sequence typing (*S. cerevisiae* typing)		
26S rDNA (D1/D2) sequencing	PCR-DGGE, qPCR	(Maturano et al. 2016)
26S rDNA (D1/D2) sequencing, RFLP-5.8-ITS	PCR-DGGE, qPCR, massive sequencing	(Wang et al. 2015)
PCR-RFLP-5.8-ITS	qPCR	(Diaz et al. 2013)

2.2 MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry (MALDI-TOF MS), a proteomic approach, has been widely used in clinical microbiology due to its ease of operation, high speed of analysis and low cost (Carbonnelle et al. 2007, Qian et al. 2008, Bader 2013, Posteraro et al. 2013, Sun 2015, Greco et al. 2018, Flores-Trevino et al. 2019). The first time that MALDI-TOF MS was applied to identification of yeasts from non-clinical origins was conducted by Vallejo et al. (2013) who reported its use in identification of *S. cerevisiae* isolates from a fermented beverage - chicha fermentation. By comparison with classical molecular methods, Blattel et al. (2013) suggested the potential of MALDI-TOF MS as a standard method for closely related *Saccharomyces* yeasts discrimination and classification.

Since then MALDI-TOF MS has been gradually integrated into wine yeasts identification (Usbeck et al. 2014, Gutiérrez et al. 2017, Kačániová et al. 2020) as well as the "winemakers' nightmare" spoilage yeasts from wine samples (Kántor and Kačániová 2015). Moreover, MALDI-TOF MS has been employed in yeast diversity analysis in winemaking (Moothoo-Padayachie et al. 2013, Usbeck et al. 2013, Usbeck et al. 2014, Kántor and Kačániová 2015, Kántor et al. 2016, Gutiérrez et al. 2017) and brewing environments (Wieme et al. 2014, Tokpohozin et al. 2016, Lauterbach et al. 2017). Although MALDI-TOF MS has only been recently applied to yeast species, its potential for species- and strain-analysis seems well-founded, like its ability for the typing of *S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii* and *H. uvarum* strains (Moothoo-Padayachie et al. 2013, Usbeck et al. 2014, Du Plessis et al. 2017).

2.2.1 Development of MALDI-TOF MS

In 1975, mass spectrometric methods were pioneered for microbial identification (Anhalt and Fenselau 1975), the characteristic mass spectra were acquired from a panel of routine pathogenic bacterial species. Due to the compositional differences in these cells the corresponding mass spectra were greatly related to the taxonomic relationships. In the 1980s, a new era of microbial identification came along with the introduction of "soft ionization" Matrix-Assisted Laser Desorption/Ionization (MALDI) (Karas et al. 1985, Karas et al. 1987) and the development of time-of-flight (TOF) mass spectrometry (MS) (Tanaka et al. 1988). In a pioneering study by Beavis and Chait (1989), MALDI-TOF MS was applied to high-mass quasimolecular ions (up to 116,000 Da) detection with a simple linear TOF analyser, leading to an improved mass resolution of approximately 500 (Full Width at Half Maximum (FWHM)) for proteins with molecular weight less than 20,000 Da (FWHM of the peak is used to define the resolution in a TOF analyser).

MALDI-TOF MS allows the ionization and vaporization of large non-volatile biomolecules without inducing ion fragmentation in a high sensitivity and efficiency with the ability to analyse molecules in femtomolar (10^{-15} mol/L) and attomolar (10^{-18} mol/L) concentration (Meyer et al. 2017). Since then,

it has rapidly evolved as a valuable tool for the detection and characterization of proteins/peptides, and extend to large glycopeptides, oligosaccharides, nucleotides, carbohydrates, and fatty acids, especially in mixtures and crude samples (Kaufmann 1995, Flamini and De Rosso 2006, van Belkum et al. 2012, Hajduk et al. 2016, Meyer et al. 2017). Also, this technique has a remarkably high tolerance towards contaminants like salts and buffers, because the co-crystallization process of matrix/analyte can separate the contaminants from the targeted samples (Kaufmann 1995). In 1994, MALDI-TOF MS was applied as a new methodology for bacterial identification based upon their water-soluble-protein profiles from the disrupted cells, with the principal advantages being the efficient and minimal sample preparation (Cain et al. 1994). In 1996, Holland et al. (1996) first reported successful bacterial chemotaxonomy by MALDI-TOF MS analysis on the whole cells.

2.2.2 Principles of MALDI-TOF MS in microbial identification

In practice, MALDI-TOF MS is a technique based on "soft ionization" where microbial cells are embedded in a suitable matrix (*e.g.* α-cyano-4-hydroxycinnamic acid (HCCA) or sinapinic acid (SA)) that extract and crystallise the native proteins and assist in their ionisation when exposed to a laser beam. Exposed into the high vacuum system in MALDI mass spectrometer, a laser pulse of a few nanoseconds is targeted to matrix/sample mixture, where the matrix absorbs energy from the laser and transfers it to the microbial samples, resulting in the desorption of embedded samples into the gas phase and followed by the ionization of the molecules (Posteraro et al. 2013).

Through an electrostatic field, the ions are accelerated to the same kinetic energy by an electrode within the spectrometer in the electric potential range of 20 kV, and travel down a "flight tube" - a field-free region of usually 1-2 m in length until they reach the detector. TOF instrument is used to separate the released ions mentioned above, which are moving at different velocities after a common start. Due to the vacuum within the spectrometer, eliminating the collisions of the ions with air molecules, separation depends only on the mass and charge of ions, which is proportional to *m/z* (z=1), as the MALDI process almost entirely produces single charged ions with relative lack of fragmentation (Kaufmann 1995, Reich 2013). The ions captured by the detector are processed and presented as a final mass spectrum, conveying the ion mass (*m/z*) on the x-axis, and the number of ions of a particular size that hit the detector (peak intensity) on the y-axis (Figure 2.1) (Giebel et al. 2010, Posteraro et al. 2013). As a result, a complex microbial cell is broken down into a simplified mass spectrum characterized by specific *m/z* and peak intensities. Each individual microorganism produces its own unique mass spectrum, whereby MALDI-TOF MS can achieve the microbial identification purpose.

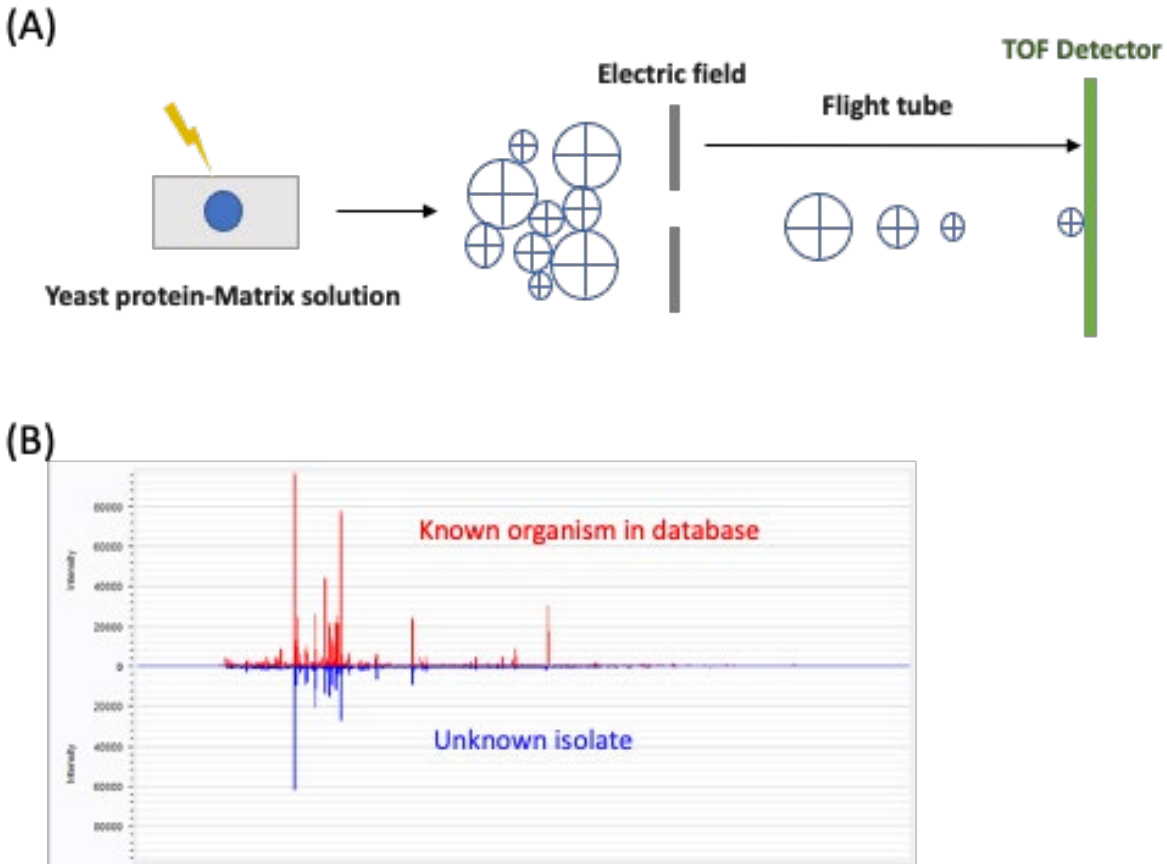


Figure 2.1 (A) Basic principle of MALDI-TOF MS; (B) Microbial identification achieved by matching with MALDI-TOF MS database (modified after Singhal et al. (2015)).

Microbial identification is achieved by the comparison of an unknown mass spectrum with the available mass spectra in database, therefore a comprehensive spectral database is important to the identification results (Wang et al. 2017, Huber et al. 2018). Currently, MALDI Biotyper (Bruker Daltonics, Germany) and Vitek MS (BioMérieux, France) are the major commercial MALDI-TOF MS systems used in the field of microbiology. Some other manufacturers include Andromas (Andromas SAS, France), Axima@Saramis (Shimadzu/AnagnosTec, Germany), MassARRAY (Sequenom, San Diego, CA) and Ibis PLEX ID (Abbott) (van Belkum et al. 2012, Vlek et al. 2014, Andrade et al. 2018). Regardless of the manufacturer, a MALDI-TOF MS instrument consists of three main units: (1) an ion source, (2) a mass analyser, and (3) a detector, in which the first two components define the capabilities of an MS instrument (Meyer et al. 2017). Various MALDI-TOF MS systems have comparable sensitivities and specificities. The key difference is that each platform has its own spectral database and algorithms for microbial identification (Cassagne et al. 2016). For example, Bruker Main Spectra Peak (MSP) analysis is achieved by the comparison of a newly recorded spectrum to the deposited MSPs of reference strains in the database using a pattern-matching approach, including peak positions, intensities and frequencies and reproducibility across the whole mass range peaks (van Belkum et al. 2012). In the case of the bioMérieux system, the SuperSpectra deposited in the database are constructed by the accumulation of replicate spectra obtained from reference strains and random clinical isolates grown under different conditions (van Belkum et al.

2012). In addition to the bioinformatics tool provided by MALDI instrument manufactures (*e.g.* Flexanalysis (Bruker Daltonik GmbH) and MALDI Biotyper (Bruker Daltonik GmbH)), the further advanced MALDI-TOF spectral analysis can be achieved by a variety of bioinformatics programs available at the market (Sauget et al. 2017), such as the commercial software BioNumerics (Applied Maths) used in our study (Vranckx et al. 2017).

Despite the accuracy of MALDI-TOF analysis, only those species/strains whose reference data are accessible can be identified correctly, however, most of the commercially available spectra are used for clinical microorganisms (Gutiérrez et al. 2017). Agustini et al. (2014) reported that 32.3% of strains were not identified in 845 environmental yeast strains due to the absence of related reference spectra. Expansion of in-house MALDI-TOF MS spectra greatly improved the performance for routine yeast species identification (Sogawa et al. 2012, Pavlovic et al. 2014, Cassagne et al. 2016). Additionally, it is unlikely to compare the obtained spectra directly among different laboratories, given the variations in instruments between manufacturers (Vlek et al. 2014, Huber et al. 2018), as well as the technicians (Wunschel et al. 2005, Oberle et al. 2016). From this point of view, it is necessary to build a database that matches to a particular laboratory or MALDI instrument (Williams et al. 2003).

2.2.3 MALDI-TOF MS for yeast identification

MALDI-TOF MS can be applied either into the whole-cell analysis directly or the crude cell protein extracts. Generally, direct cell analysis is sufficient for bacteria identification, but additional protein extraction is recommended for yeast identification due to the thick yeast cell wall structure (Arnold et al. 1999, Walker et al. 2002, Williams et al. 2003, Wilson et al. 2017, Huber et al. 2018). Thus, a general workflow for yeast identification usually includes cell cultivation, protein extraction, sample/matrix preparation, MALDI-TOF MS analysis, raw spectra pre-processing, and follow-up data analysis. Figure 2.2 describes the yeast identification workflow in our project.

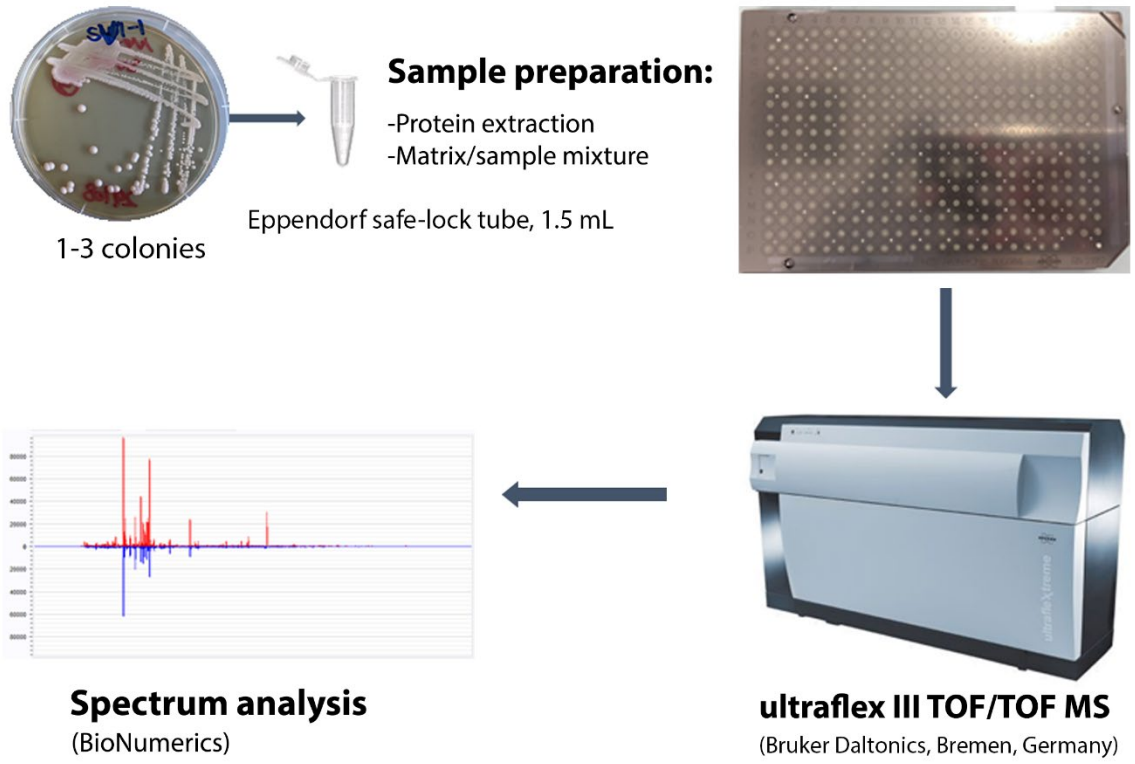


Figure 2.2 MALDI-TOF MS identification of yeast isolates in this study.

The most important concern for MALDI-TOF MS and other mass spectrometric methods is the reproducibility and stability of the acquired protein profiles. The MALDI instrument model used, instrument parameters (*e.g.* resolving capability, laser power and laser shot), sample preparation, and the microbial physiological state itself all could have impact on the final profile patterns (Anderson et al. 2012), therefore the identification accuracy. In the following text, the potential impact is described from aspects of *MALDI instrument parameters*, *microbial cultivation conditions* and *sample preparation*.

MALDI instrument parameters

There are a number of factors that need to be considered when setting up a MALDI instrument for analysis. Low resolving capability of the MALDI instrument and inappropriate mass calibration (internal and external calibration) can cause mass discrepancy at the same particular *m/z* (Wang et al. 1998). Insufficient or too much laser power can result in overall low peak intensities or broadened peaks, leading to a dramatic drop in resolution and signals that will degrade the spectrum quality greatly. Increased laser shots on the matrix-analyte crystalline surface can completely ionize a sample and reduce the background noise caused by variations in samples amount and distribution, therefore improve the reproducibility of mass spectra (Meyer et al. 2017). Another key parameter is the mass range selection, where the final mass spectra patterns are distinct as the different types of protein/peptides predominate specific mass range (Figure 2.3). For the microbial identification purpose, *m/z* 2, 000-20, 000 is generally used where the ribosomal and housekeeping proteins of

taxonomic relevance predominate, so this mass range would be expected to be both sequence-conserved regardless of cell physiology variation and of high interspecific diversity (Wunschel et al. 2005, Welker 2011, Wieme et al. 2014, Oberle et al. 2016). Moreover, their biochemical nature is readily ionized during the MALDI process and the matrix condition is favourable for their extraction as well (Ryzhov and Fenselau 2001, Meyer et al. 2017). In contrast, the capability of lower mass range (*e.g.*, m/z 500-2,500) for identification largely depends on the microorganisms actually studied (Welker 2011).

Figure 2.3 Type of particular proteins detected in certain mass ranges and two TOF modes within 0-200 kDa (modified after Welker (2011)).

Microbial cultivation conditions

Another challenge of MALDI-TOF MS stems from the highly dynamic nature of the proteome in living cells. Different protein expression levels of yeast, including surface structure proteins and intracellular proteins, are regulated according to their adaptability to the external environmental stresses (Sahara et al. 2002, Kolkman et al. 2005). During the winemaking process, the growth of yeast often encounters high concentration of grape sugar, and limited oxygen and nutrients (*e.g.* carbon and nitrogen). Enzymes involved in central carbon metabolism pathways showed a significant change in wild-type *S. cerevisiae* under glucose- or ethanol-limited conditions (Kolkman et al. 2005); glucose- and ammonia-deficiency induced 51 proteins upregulated in *S. cerevisiae*, respectively (Kolkman et al. 2006). Therefore, yeast cells under different growth conditions (*e.g.* growth media, pH, temperature and culture time) may produce distinct spectra profiles due to the changed cell physiology and composition that consequently cause the variations in generated protein/peptide ions.

Early publications demonstrated that the MALDI protein fingerprinting was capable of identifying an organism, independent of the varying culture conditions (culture media, pH, growth rate and temperature) (Valentine et al. 2005, Wunschel et al. 2005). Similar results were observed on spoilage

yeasts analysis (*Saccharomyces cerevisiae* var. *diastaticus*, *Wickerhamomyces anomalus* and *Debaryomyces hansenii*), core mass peaks remained constant but with differences of varying degrees under all tested conditions including environmental and physiological parameters (oxygen availability, nutrients components, cell density and growth phase) (Usbeck et al. 2013). Additionally, the influence was reported to be strain-dependent (Qian et al. 2008, Moothoo-Padayachie et al. 2013), thereby a prior examination of the cultivation conditions would be necessary.

Sample preparation

Sample preparation includes steps of protein extraction and matrix/sample spotting. The efficiency of protein extraction plays a very important role in determining the number of proteins desorbed and the S/N ratio; the matrix/sample step is the core of the MALDI procedure, promoting co-crystallization of matrix and analyte molecules, which is a decisive factor of the ionized molecules range. MALDI sample preparation should be optimized to suit specific applications.

There are a number of strategies for protein extraction, such as the use of high concentration of acids (trifluoroacetic acid, formic acid, nitric acid, and acetic acid) and enzyme cleavages (zymolyase), as well as the direct protein extraction using commercial yeast protein extraction reagents, ultrasonication, glass beads, and corona plasma discharge (Amiri-Eliasi and Fenselau 2001). Compared to physical and enzymatic treatments, the organic solvent (ethanol/formic acid) is the most frequently used for its easy-to-handle and high reproducibility in spectra generation (Usbeck et al. 2013). Under acidic conditions, ethanol fixation unfolds the cell surface macromolecules so that exposing medium-sized molecules to the matrix, or promoting the release of the cell membrane or cytosolic/ribosomal materials to the cell surface, placing them in contact with the matrix, allowing more efficient ionization and higher identification rates (Qian et al. 2008, Anderson et al. 2012).

The acquired protein extracts are then spotted on the MALDI targets with matrix solution. Varying the matrix/sample spotting methods would produce different spectral patterns, as the resulting film of the matrix/sample would present crystalline morphology of different degrees of heterogeneity (Toh-Boyo et al. 2012). Therefore, the resulting spectra differed in terms of the repeatability, reproducibility, resolution, signal strength, background intensity and detectability (Penno et al. 2009). The “dried-droplet” method is most commonly used in yeast identification, the analyte being spotted on the MALDI plate and allowed to air dry, then covered by the matrix solution (Williams et al. 2003). The other frequently used methods include 1) “sandwich” method, in which the matrix was added prior to the dried-droplet method (Gutiérrez et al. 2017, Mello et al. 2017); 2) two-layer method (the matrix solution is placed on a MALDI probe and allowed to dry to form a microcrystal layer, and followed by a mixture solution containing both the analyte and the matrix (Dai et al. 1999, Qian et al. 2008)); and 3) premixing method (equal amount of analyte and matrix solution are mixed thoroughly prior to spotting on the target). Other methods can be referred to fast evaporation,

vacuum drying, crushed- crystal, slow crystal growing, active film, pneumatic spray, and electrospray (Dai et al. 1999).

Apart from the spotting methods, the selection of organic solvent, matrix type, the concentration and pH of the final matrix solution are all crucial to the crystallization process (Williams et al. 2003). Generally, the matrix should be water-soluble, not too volatile, and chemically not aggressive (Kaufmann 1995). For different types of analytes, a suitable matrix should be considered cautiously, the commonly available matrix are listed in Table 2.5, namely 2,5-Dihydroxyacetophenone (DHAP), 2,5-dihydroxybenzoic acid (DHB), *trans*-3,5-dimethoxy-4-hydroxycinnamicacid (sinapinic acid, SA), α-cyano-4-hydroxycinnamic acid (HCCA), 2,4,6-trihydroxyacetophenone (THAP), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3-hydroxypicolinic acid (HPA), 1,5-Diaminonaphthalene (DAN). The matrix ions can be completely suppressed by sufficient sample protein, so a mass spectrum with solely analyte ions is more likely to generate (Knochenmuss and Zenobi 2003). In the case of matrix excess, laser tuning can be used to improve the detection sensitivity (Baumann et al. 2005).

Table 2.5 The specialized applications of some common matrix in MALDI-TOF MS (Bruker Guide to MALDI Sample Preparation – Instruction for Use, Revision E, 2015).

Matrix	Specialized applications
HCCA	Highly sensitive of peptides/proteins from 0.7 to 20 kDa;
2,5-DHB	A wide variety of peptides, proteins, polymers and carbohydrates, including phosphopeptides and glycoproteins;
2,5-DHAP	Proteins (8-100 kDa) and proteomic profiling studies and glycoproteins analysis due to its prevention to in-source decay (ISD) fragmentation;
SA	Large proteins (10-150 kDa) and some polar polymers; suitable for the generation of ISD spectra of intact proteins;
SDHB	90:10 mixture of 2,5-DHB and 2-Hydroxy-5-methoxybenzoic acid for very large proteins and glycoproteins; suitable for the generation of ISD spectra of intact proteins;
3-HPA	Mixed oligonucleotide samples (DNA/RNA) between 1and 30 kDa;
1,5-DAN	Peptides/proteins containing disulfide linkages in top-down sequencing of intact proteins (ISD; T^3);

In summary, standardized protocol development and strict control of the MALDI analysis procedure are needed to be considered in the application of MALDI-TOF MS in microbiology laboratories. To date, there is not a standard MALDI-TOF MS protocol available that works for all microbes. However, ethanol/formic acid extraction advised by Bruker Daltonics is usually favoured, followed by the dried-droplet spotting method, in which 1 µL of protein extract is applied onto the MALDI plate and left to dry before covered by 1 µL of matrix solution (10 mg/mL of HCCA freshly dissolved in 50% acetonitrile (ACN) and 2.5% trifluoroacetic acid (TFA)) (Welker 2011).

MALDI-TOF MS potential in wine yeast proteomics

It is difficult to predict gene products accurately from genomic data, and thus verification of a gene product by proteomic approaches is an important first step in 'annotating the genome'. Being

complementary to genomics, proteomics is defined as the large-scale analysis of proteins targeting on the gene products (Pandey and Mann 2000). Yeast strains with similar DNA profiles do not necessarily produce similar wine characteristics (Ilieva et al. 2017). There was no consistency found in intraspecies grouping by genotypic and phenotypic clusters (Binati et al. 2019). In contrast, the proteome is closer to the end-products of metabolites responsible for the wine characteristics. The strain-specific stress response (Trabalzini et al. 2003) and the proteomic evolution in yeast adaptation to stressful winemaking conditions may correlate better to their unique properties of different oenological strains (Szopinska et al. 2016).

There is also evidence to show that proteome and metabolite profiling can be correlated either positively or negatively under certain conditions (Lafaye et al. 2005). The protein profiles acquired using MALDI-TOF MS should be the *real-time* state of the cell at the given moment. In this regard, as a novel proteomic approach, in addition to its well-known capacity on yeast identification at species-level, MALDI-TOF MS may be able to monitor the subtle proteomic evolution of yeast cells over the winemaking process and differentiate the indigenous yeast(s) at strain level, although the ability of MALDI-TOF MS for wine *S. cerevisiae* biotyping seems to be conflicted in the study of Usbeck et al. (2014) and Gutiérrez et al. (2017). Table 2.6 lists the recent MALDI-TOF analysis employed in wine associated yeasts characterization.

Of interest is the work of Usbeck et al. (2014) who demonstrated the potential of MALDI-TOF MS in predicting the utility of individual yeast strains for different wine styles including Chardonnay, Sauvignon blanc, Beaujolais and Champagne, which will be a promising tool in new strain selection compared to traditional laborious strain selection procedures. A recent study on brewing yeasts (Lauterbach et al. 2018) also suggested that MALDI-TOF MS provided a prediction of application potential to different beer styles (*e.g.* Ale, Lager, Kölsch, Wheat beer) for which they are currently used.

Table 2.6 MALDI-TOF MS applications in winemaking yeast.

Yeast (s)	Culture condition	Sample preparation		Software	Instrument	Objective	Reference
		Protein extraction	Matrix/sample				
Yeasts in grape, new wine "federweisser" and unfiltered wine samples.	3-5 days, 25℃, trypton soya agar (TSA, Oxioid)	ethanol/formic acid	HCCA, dried-droplet	Biotyper Realtime Classification 3.1 with BC-specific software	Microflex LT/SH (Bruker Daltonics) using Flex Control 3.4	Identification	(Kačániová et al. 2020)
S. cerevisiae; non-*Saccharomyces* strains	20-24 h, 28℃, yeast malt agar (YMA)	ethanol/formic acid	HCCA, dried-droplet, 394 wells Anchorchip plates	FlexAnalysis Version 2.4 (Bruker Daltonics); CWT-based algorithm implemented in R using the MassSpecWavelet library	Ultraflex II LIFT (Bruker Daltonics) using Flex Control 2.4	Identification	(Gutiérrez et al. 2017)
Yeast diversity in wine "federweisser"	Malt extract agar, Wort agar, wild yeast medium, 5 days, 25℃	ethanol/formic acid	HCCA, dried-droplet, a polished MALDI target plate	Real-time Classification software by used database "Taxonomy" (Bruker Daltonics, Germany)	Microflex LT/SH (Bruker Daltonics) using flex Control software	Identification	(Kántor et al. 2016)
S. cerevisiae strains/hybrids and commercial wine yeast	20 h, 30℃, YPG broth, rotary shaker at 180 rpm	ethanol/formic acid	HCCA, dried-droplet, MTP 384 polished	Biotyper; Bruker Daltonik	Bruker UltrafleXtreme MALDI-TOF/TOF MS	Biotyping	(Hart et al. 2016)

			steel target plate				
S. cerevisiae	20 h, 30℃, YPG broth, a rotary shaker at 180 rpm	ethanol/formic acid	HCCA, dried-droplet, a MALDI polished steel target	Biotyper 3.0	Microflex LT (Bruker Daltonik) using Flex Control 3.3	Differentiation	(Usbeck et al. 2014)
Environmental yeasts from *Vitisvinifera*L. grapes	24-48 h, 25℃, must agar	ethanol/formic acid	HCCA, dried-droplet, a 96-well stainless steel MALDI target plate	FlexAnalysis, Biotyper 3.0 (Bruker Daltonik)	Microflex (Bruker Daltonik)	Identification	(Agustini et al. 2014)
S. cerevisiae	48 h, 30℃, YEPD agar	ethanol/formic acid	HCCA, dried-droplet, a ground-steel MALDI target plate	Biotyper 3.0	AutoFlex III Smartbeam (Bruker, Germany) using Flex Control 3.0	Biotyping	(Moothoo-Padayachie et al. 2013)
S. cerevisiae var. *diastaticus*, *Wickerhamomyces anomalus*, *Debaryomyces hansenii*	20 h, 30℃, YPG broth, rotary shaker at 180 rpm	ethanol/formic acid	HCCA, dried-droplet, a MALDI polished steel target	Biotyper 3.0	Microflex LT (Bruker Daltonik) using Flex Control 3.3	Differentiation	(Usbeck et al. 2013)

Note: CWT: Continuous Wavelet Transform.

2.3 Aim and Objectives

Therefore, this study aims to optimise the method of MALDI-TOF MS as an innovative tool in the wine industry, offering the small-medium enterprises (SME) the possibility of in-house yeast identification with rapidity and cost–effectiveness, as well as strain prediction for different wine styles, and ultimately to increase the competitive edge of New Zealand wine products in the international market.

Objectives:

To achieve the aim, the objectives are as follows:

(1) to optimize a method for MALDI-TOF analysis of wine-associated yeasts and corresponding MALDI spectra database for use in New Zealand;

(2) to apply this optimized MALDI-TOF MS method to elucidate the extensive diversity of yeast species in wild ferments using an organic vineyard in the Waipara area of Canterbury as a case study, with results cross-validated by molecular techniques 26S rDNA sequencing and PCR/RFLP analysis as relevant;

(3) to evaluate the impact of growth conditions on yeast MALDI-TOF spectra, and the potential impact on analyses to predict yeast strain suitability for a given wine style;

(4) to assess the ability of MALDI-TOF MS in yeast strain application prediction using commercial wine yeast strains and a few brewing yeast strains.

2.4 Hypothesis

Based on the previously quoted literature, I wish to propose the following hypotheses:

(1) MALDI-TOF profiles of yeast strains will vary when cultured in different media, but a set of core peak classes should remain stable;

(2) MALDI-TOF MS analyses can provide useful insights into taxonomic and wine-variety relationships useful for winemakers;

(3) MALDI-TOF profiles of different yeast strains within the same species will present minor variation in peak position or intensity, and this variation is related to the isolation source.

2.5 Thesis structure

This thesis consists of eight chapters (Figure 2.4). C**hapter 1** gives a brief introduction of this study. Current **Chapter 2** provides an extensive background for this study from aspects of 1) wine yeast and 2) the overview of MALDI-TOF MS technique, as well as the aims to be achieved from **Chapters 4** to **7**. **Chapter 3** summarizes the main materials and methods used in this study.

Chapter 4 describes the optimized MALDI-TOF analysis method based on the previous study; with the optimized method and established spectra database, **Chapter 5** provides the practical application of MALDI-TOF MS in analysing indigenous yeast diversity. Due to the influence of culture media on yielded MALDI profile patterns, **Chapter 6** investigates the best growth conditions for yeast identification purposes and extends to **Chapter 7** where the predictive potential of MALDI-TOF analysis for commercial wine and beer-making yeast strains application was assessed.

Final **Chapter 8** concludes the overall outcome of this study and further perspectives were envisioned.

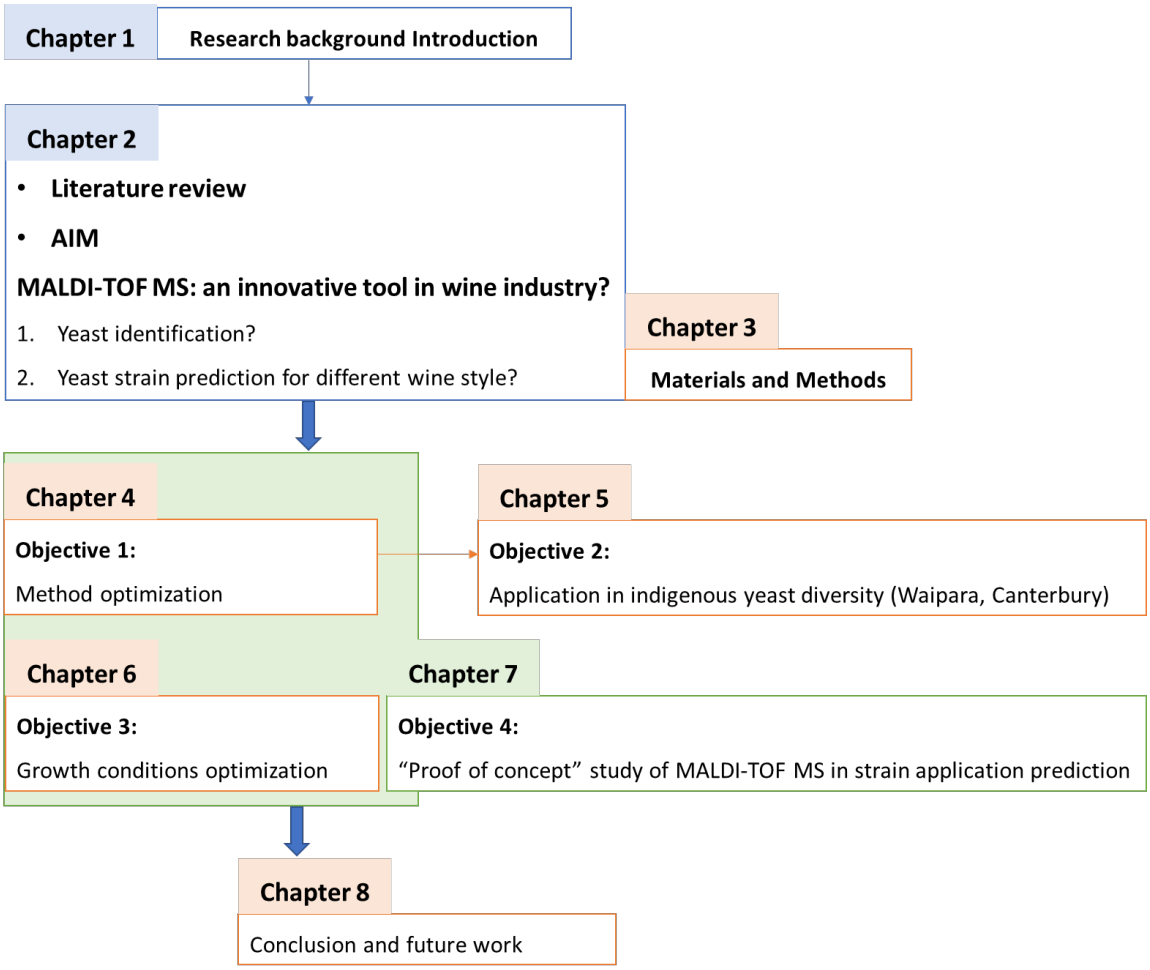


Figure 2.4 Flow chart of the thesis structure.

Chapter 3

Materials and Methods

3.1 Yeast strains and growth conditions

3.1.1 Reference strains

Fourteen type strains (*Brettanomyces anomalus* NCYC 615, *B. bruxellensis* NCYC 370^T, *B. naardenensis* NCYC 924, *Hanseniaspora uvarum* NCYC 2739, *Kluyveromyces dobzhanskii* NCYC 538^T, *K. lactis* NCYC 416^T, *K. wickerhamii* NCYC 546^T, *Saccharomyces bayanus* NCYC 2578^T, *S. cerevisiae* NCYC 505^T, *S. paradoxus* NCYC 700^T, *S. pastorianus* NCYC 396^T, *Torulaspora delbrueckii* NCYC 2629^T, *Zygosaccharomyces bailii* NCYC 1416^T and *Z. rouxii* NCYC 568^T) were purchased from NCYC (National Collection of Yeast Cultures); T refers to *Type Strain*.

3.1.2 Commercial strains

A collection of 47 commercial wine yeast strains and 12 brewing yeast strains used in this study are listed in Table 3.1, of which 8 wine strains Fermicru_ROSE, AWRI_Fusion, Lalvin DV10, Fermicru 4F9, Lalvin EC 1118, Lalvin QA 23, IOC 18-2007, Maurivin PDM are associated with the *Prise de Mousse* (PDM) collection of Champagne production (Dunn et al. 2012, Borneman et al. 2016).

Table 3.1 47 commercial wine yeast strains and 12 commercial brewing yeast strains used in this work (wine strains were kindly provided by Lincoln University Winery, and brewing strains were purchased from BREWSHOP), the genetic background listed on the right list were obtained from their manufacture instructions.

Commercial strains	**Genetic background**
Wine strains	
AWRI Fusion	*S. cerevisiae* x *S. cariocanus*
Cepage Cabernet	*S. cerevisiae*
Cepage Chardonnay	*S. cerevisiae* -Strain n° LW05
Collection CepagePinot	*S. cerevisiae*
Cross Evolution	*S. cerevisiae*
Enartisferm Aroma White	*S. cerevisiae*
Enoferm AMH™	*S. cerevisiae*
Enoferm M1	*S. cerevisiae*
Fermi champ	*S. cerevisiae*
Fermicru 4F9	*S. cerevisiae* -Strain n° 4F9
Fermicru AR2	*S. cerevisiae* -Strain n° L0122
Fermicru Rose	*S. cerevisiae* -Strain n°LW10

Fermicru XL	*S. cerevisiae* -Strain n° CECTA 11947
IOC 18-2007	*S. cerevisiae*
Lalvin C	*S. cerevisiae*
Lalvin CLOS	*S. cerevisiae*
Lalvin CY 3079	*S. cerevisiae*
Lalvin DV10	*S. cerevisiae*
Lalvin EC1118	*S. cerevisiae*
Lalvin ICV D47	*S. cerevisiae*
Lalvin OKAY	*S. cerevisiae*
Lalvin RC212	*S. cerevisiae*
Lalvin Rhone 2226	*S. cerevisiae*
LalvinQA 23	*S. cerevisiae*
Levuline BRG	*S. cerevisiae*
Maurivin AWRI 350	*S. cerevisiae*
Maurivin PDM	*S. cerevisiae*
Premium Chardonnay	*S. cerevisiae*
PREMIUM®PROTIOL	*S. cerevisiae*
Renaissance Allegro	*S. cerevisiae*
Renaissance Andante	*S. cerevisiae*
Renaissance Brio (Brioso)	*S. cerevisiae*
Renaissance Maestoso	*S. cerevisiae*
Renaissance Vivace	*S. cerevisiae*
Rennaissance Ossia	*S. cerevisiae*
Safoeno™ CK	*S. cerevisiae*
Sauvignon L3	*S. cerevisiae*
UCD522	*S. cerevisiae*
Velluto Evolution™	*S. cerevisiae*/*uvarum* (hybrid)
Viniflora Jazz	*S. cerevisiae*
Viniflora® PRELUDE™	*Torulaspora delbrueckii*
Viniflora®CONCERTO™	*Lachancea thermotolerans*
Vitilevure Syrah	*S. cerevisiae*
Zymaflore VL1	*S. cerevisiae*
Zymaflore VL3	*S. cerevisiae*
Zymaflore X5	*S. cerevisiae*
Zymaflore® X16	*S. cerevisiae*
Brewing strains	
BRY-97 American West Coast Yeast	*S. cerevisiae*
LalBrew KÖln	*S. cerevisiae*
Belle Saison	*S. cerevisiae* var. *diastaticus*
Mangrove Jack's New World Strong Ale Yeast	*S. cerevisiae*
Philly Sour	*Lachancea spp.*
LalBrew Verdant IPA	*S. cerevisiae*
Mangrove Jack's Californian Lager Yeast	*S. cerevisiae*
Mangrove Jack's Bohemian Lager Yeast	*S. cerevisiae*
Saflager S-23 Yeast	*S. cerevisiae*

Mangrove Jack's Bavarian Wheat Yeast	*S. cerevisiae*
Mangrove Jack's Belgian Wit Yeast	*S. cerevisiae*
Safbrew WB-06 Wheat Yeast	*S. cerevisiae var. diastaticus*

3.1.3 Wine samples and yeast isolation

Pinot Noir grapes (clone: 115) sourced from the Greystone Block 5 vineyard were harvested on 22th March 2018. Spontaneous fermentation was carried out in 1.5 tons of tanks made of high density polyethylene (HDPE) which were placed (A) in the winery (an indoor environment) or (B) in the vineyard (an outdoor environment without temperature control) respectively. Pinot Noir grape juice ferment samples from each of the winery and vineyard environments were collected at four key different stages of fermentation, namely: (i) ~1 °Brix dropped; (ii) 6-8 °Brix dropped; (iii) half of °Brix dropped; and (iv) at the end of fermentation (Table 3.2).

Table 3.2 Pinot Noir ferments sampled from four key stages of fermentation in Winery and Vineyard wine production systems. Isolates were recovered from 500ml aliquots of each sample. The number of isolates examined at each stage is given in square brackets. Isolate selection was based on careful and systematic screening (see the following for details).

	S1 (beginning of fermentation)	**S2 (6-8 ˚ Brix dropped)**	**S3 (half of ˚ Brix dropped)**	**S4 (end of fermentation)**
Winery	SW1 [**34**] (morning, 27/03/2018)	SW2 [**33**] (afternoon, 28/03/2018)	SW3 [**19**] (morning, 29/03/2018)	SW4 [**20**] (12/04/2018)
Vineyard	SV1 [**48**] (morning, 26/03/2018)	SV2 [**39**] (afternoon, 26/03/2018)	SV3 [**20**] (morning, 27/03/2018)	SV4 [**22**] (11/04/2018)

Pinot noir grape juice ferment sample of 500 mL from each stage was collected in 750 mL wine bottles and quickly (within 90 min) transported on ice to the microbiology laboratory at Lincoln University, where 50 mL aliquots was centrifuged (3,000 x g, 10 min, 4°C) (Heraeus Multifuge X3R, Thermo Scientific) and the pellet was then resuspended in YPD-30% (v/v) glycerol medium to facilitate storage of viable microorganisms at -80°C. For yeast isolation, 1 mL of each defrosted Pinot Noir grape juice ferment sample from each fermentation system (*i.e.* winery and vineyard) was taken, and a ten-fold serial dilution series prepared (in 0.1% peptone water) from each. Then, 100 µL of each dilution was spread on YPD agar (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) with 0.1 g/L of chloramphenicol (Sigma) and 0.25 g/L of sodium propionate (Sigma) and incubated at 28 °C for 2-3 days. Experiments were performed in triplicate. Hence, each sample had been cultured on 30 agar media plates for microbial analysis and comparison. Total yeast colonies and the percentage of each species were counted using plates with 30-300 colonies, the results were given

as cfu/mL (colony-forming units per millilitre). 3-5 colonies of each morphologically-classified isolate (*i.e.* colonies classified by their colour, shine, shape, edge and size) were selected and re-streaked on the same fresh media 3-4 times. Care was taken to ensure all morphologically distinct types were represented at each stage by careful examination of cultures from each dilution, including replicates. All isolates were subjected to MALDI-TOF MS analysis, and stored in 30% (v/v) glycerol stock at -80 °C.

3.1.4 Growth conditions

Culture media

Liquid media used in this work included YPD broth (Difco, Fort Richard Laboratories) (pH 6.5), Laboratory YPD broth (Yeast Extract (10 g/L)(Oxoid LP0021, ThermoFisher Scientific), Peptone (20 g/L)(Oxoid CM0509, ThermoFisher Scientific), D(+)-Glucose (20 g/L)(BDH AnalaR, ThermoFisher Scientific), Agar (15 g/L)(Oxoid LP0011, ThermoFisher Scientific), pH 6.5), synthetic grape juice (SGJ), Pinot Noir (PN) and Chardonnay (CH) grape juice.

Synthetic Grape Juice was made as described by Oro et al. (2014), and comprised solution A (110 g/L glucose, 110 g/L fructose, 10 mg/L ergosterol and1 ml/L Tween80), solution B (6 g/L tartaric acid, 3 g/L malic acid and 0.5 g/L citric acid), and solution C (1.7 g / L yeast nitrogen base with amino acids, 0.2 g $CaCl_2$, 2 g/L casamino acids, 0.8 g/L arginine-HCl, 1 g/L proline and 0.1 g/L tryptophan) were separately sterilized at 121°C for 20 min and mixed in a laminar flow cabinet aseptically, in which solution B and C were adjusted to pH 3.5 using KOH and HCl, respectively. The final pH and ˚Brix was 3.5 and 19.

Pinot Noir (harvested in 2019) and Chardonnay (harvested in 2020) grapes were collected from the Lincoln University vineyard and stored at -20 °C. Pinot Noir grape juice was prepared according to Barbosa et al. (2018) with minor modifications. Briefly, grape juice was obtained by pressing Pinot Noir grapes in sterilized filter bags (Stomacher® lab system 400 classic, Seward). After homogenization, juice was transferred to 50 mL tubes and centrifuged at 4,700 x g for 30 min (Heraeus™ Multifuge™ X3 Centrifuge, ThermoFisher Scientific), the supernatant was collected and split into three lots for further treatments. Chardonnay grape juice was processed and obtained directly from the Lincoln University winery and stored frozen. After thawing at room temperature, the juice was also split into three lots for the following treatments: i) Autoclaved Grape Juice (AGJ) - sterilized at 121 °C for 15 min, ii) Heated Grape Juice (HGJ) – heated in a water bath at 70 °C for 10 min to eliminate background yeast then immediately cooled and stored at -20 °C, iii) Unautoclaved Grape Juice (UGJ) without further treatment and stored at -20 °C.

Thereafter, the eight selected yeast strains (*S. bayanus* NCYC 2578 ^{T}, *S. cerevisiae* NCYC 505 ^{T}, *S. paradoxus* NCYC 700 ^{T}, *S. pastorianus* NCYC 396 ^{T}, Lalvin RC 212, Lalvin QA23, Lalvin ICV D47, *S. cerevisiae* v128) were cultured on each of 10 culture media, *i.e.* YPD agar (Difco, Fort Richard Laboratories), YPD broth (Difco, Fort Richard Laboratories), Laboratory YPD broth, SGJ, PN-(AGJ, HGJ, UGJ) and CH-(AGJ, HGJ, UGJ), from which microbial growth was subjected to MALDI-TOF analysis.

Three treatments of PN juices had the same initial pH and ˚Brix at 3.8 and 23, but the three treatments of CH juices showed different ˚Brix with AGJ at 24, HGJ over 32, and UGJ at 21, respectively. The pH was measured at 3.0 for all three treatments of CH juices. The pH and ˚Brix were measured by an HI 9025 microcomputer pH meter (Hanna instruments) and a refractometer (Bellingham + Stanley).

Growth conditions

Commercial strains were activated by rehydrating directly in 15 mL YPD broth (Difco, Fort Richard Laboratories) followed by an anaerobic incubation overnight at 28 ˚C in a 50-mL tube covered tightly without agitation.

Yeast strains were cultured on YPD agar (Difco, Fort Richard Laboratories) for 3 days at 28 °C on 3 different days to obtain 3 biological replicates. A single colony was picked to inoculate in 15 mL YPD broth (Difco, Fort Richard Laboratories) and subcultured twice prior to inoculation to the different liquid media used. Subsequently, 15 mL of nine fresh liquid growth media were inoculated with 4% (v/v) (approx. $OD_{(600nm)}$ 0.05) of the overnight culture and incubated as described above for 48 h to monitor the growth. Growth curves of the eight strains in each of the nine liquid media above were recorded using optical density $OD_{(600nm)}$ (SmartSpec^{TM} 3000 Spectrophotometer, BIO-RAD) at various time points (8, 12, 14, 16, 18, 20, 22, 24, 36, 38, and 48 h), with corresponding media as the blank. Calibration curves for each strain were made to determine the relation between the $OD_{(600nm)}$ value and corresponding cell number by the hemacytometer. All experiments were conducted in triplicate.

3.2 MALDI-TOF MS

3.2.1 Sample preparation

Optimization of the MALDI-TOF MS procedure

Optimization was achieved by comparing two matrix/sample methods and mass ranges (m/z 2,000-20,000 and low (m/z 500-4,000)), using 33 yeast strains grown on YPD agar (Difco, Fort Richard Laboratories), including 14 type strains and 19 yeast isolates representing seven species (*Candida*

californica, *Metschnikowia pulcherrima*, *Pichia membranifaciens*, *P. terricola*, *P. kluyveri*, *H. uvarum*, and *Starmerella bacillaris*).

The first represented a proposed standard "dried-droplet" method (hereafter DM) (Gutiérrez et al. 2017) with minor modifications, described below. Here, 1 µL of protein extract (compared with 2 µL used by Gutiérrez et al. (2017)) was applied onto the MALDI ground steel target plate (MTP 384, Bruker Daltonics®) and allowed to dry. Thereafter, 1 µL HCCA matrix solution (10 mg/mL in 75% ACN and 2.5% TFA) was immediately overlaid and dried at room temperature. The second method we describe as a pre-mixing method (PM), in which 8 µL of each protein extract and HCCA matrix solution (10 mg/mL in 75% ACN and 2.5% TFA) were mixed well and 1 µL of this mixture was deposited onto the target plate (MTP 384, Bruker Daltonics®) till dry.

YPD agar

On YPD agar, 1-3 colonies were picked using a sterile 200 µL pipette tip and emulsified into 300 µL deionized water. Afterwards, 900 µL absolute ethanol was added and vortexed for 1 min. After centrifugation (13, 400 rpm, 12,100 x g, 4min) (Eppendorf AG, Minispin 5452), the pellet was kept and air-dried in laminar-flow hood.

Liquid media

Strains grown in liquid media was collected at 24 h and prepared according to Usbeck et al. (2013). In order to obtain sufficient quantities of yeast cells for MALDI analysis, briefly, 900 µL culture of YPD broth and 1.5 mL culture of the other seven grape juice-based media at 24 h were transferred into a 1.5 mL tube (Safe-Lock, Eppendorf) and centrifuged at 12,100 x g for 4 min (Eppendorf AG, Minispin 5452). The supernatant was discarded, and the pellet was i) washed with 900 µL sterilized deionized water three times or ii) kept unwashed to verify the influence of the wash step on the final spectra. Subsequently, the pellet was resuspended into 300 µL deionized water, and vortexed for 1 min with 900 µL absolute ethanol. After centrifugation (12,100 x g, 4min), the pellet was air-dried in a laminar-flow hood and stored at -20 °C prior to protein extraction.

Protein extraction and matrix/sample preparation

To extract proteins, 50 µL of 70% formic acid (v/v) was added to each yeast pellet and mixed thoroughly by vortexing for 1 min, then 50 µL of acetonitrile (ACN) was added and mixed for 1 min. Each protein extract was obtained by centrifugation (12,100 x g, 4 min). Matrix/sample method PM was optimized for quality spectra acquisition. For technical replication, each extract was spotted onto three individual wells, therefore yielding 9 spectra per strain.

3.2.2 Mass spectra acquisition

MALDI-TOF mass spectra were automatically acquired on an Ultraflex III TOF/TOF MS instrument (Bruker Daltonics®, Bremen, Germany), operating in positive ion detection at a SmartbeamTM laser at 200 Hz frequency, pulsed-ion extraction time of 120 ns, and the laser power adjusted between 45% to 80%. The voltage of the ion source was set as 25.00 kV (ion source 1), 23.55 kV (ion source 2) and 6.01 kV (lens). Samples were analyzed using the linear detector at high mass range m/z 2,000-20,000, and reflector detector at low mass range m/z 500-4,000. The final spectrum was an average accumulation of 800 single spectra (low mass range m/z 500-4,000) or 2,000 single spectra (high mass range m/z 2,000-20,000) gathered. Each single spectrum was recorded from 10 random raster spots.

The mass spectrometer was externally calibrated in every experiment at regular intervals, using the calibrant position in the middle of each tetrad of spots. For low mass range m/z 500-4,000, peptide II standard (Bruker Daltonics ®) (Bradykinin 1-7, [M + H]+ at m/z757.3992, Angiotensin II, [M + H]+ at m/z 1046.5418, Angiotensin I, [M + H]+ at m/z 1296.6848, Substance P, [M + H]+ at m/z 1347.7354, Bombesin, [M + H]+ at m/z 1619.8223, ACTH clip 1-17, [M + H]+ at m/z 2093.0862, ACTH clip 18-39, [M + H]+ at m/z 2465.1983 and Somatostatin 28, [M + H]+ at m/z 3147.4710) was used. For high mass range m/z 2,000-20,000, an in-house protein standard comprising Insulin, [M + H]+ at m/z 5734.52, Cytochrome C , [M + H]+ at 12360.99 and [M + H]2+ at 6180.99, Myoglobin, [M + H]+ at 16952.30 and [M + H]2+ at 8476.65), Aprotinin [M + H] + m/z 6511.51, and β-lactoglobulin [M + H] + m/z 18363.00 was used.

3.2.3 MALDI-TOF MS data analysis

Raw mass spectra were exported as .txt format using FlexAnalysis software (version 3.0. Bruker Daltonics®), and imported into software BioNumerics version 7.6 (Applied Maths). Spectra preprocessing was achieved at a default setting, but baseline subtraction with Rolling disc value was adjusted to 150. Kaiser Window value in smoothing and signal to noise ratio (S/N) in peak filtering were adjusted according to spectra quality.

A composite profile of each strain was obtained using 9 spectra derived from three technical replicates of each of three biological replicates. Cluster analysis was performed using the Pearson correlation coefficient and UPGMA (unweighted-pair group method with arithmetic mean) algorithm. The “goodness-of-fit” between calculated similarity values between all strains, and the clustering shown in the dendrogram, was calculated using the cophenetic correlation method, whereby a value of 1.0 indicates a perfect correlation of the dendrogram with the similarity matrix.

3.2.4 Machine learning-based analysis

MDS and PCA analyses are available in BioNumerics version 7.6. MDS was performed based on similarity matrix calculated using the metric algorithm Pearson Coefficient. Pearson coefficient is insensitive to global differences in background and intensity as it containing an average intensity correction, but sensitive to local differences in intensity, thus is recommended for typing purpose therefore adopted in our study (Vranckx et al. 2017). PCA and UMAP were executed on peak classes detected by "peak matching" using the default settings (high mass: constant tolerance 1.9, linear tolerance 550 ppm, peak detection rate 10; low mass: constant tolerance 0.5, linear tolerance 300 ppm, peak detection rate 50). PCA was calculated with quantitative values (not just absent/present) and options to Subtract average character value over the characters. UMAP is founded on the assumptions that the data is uniformly distributed on Riemannia manifold, the Riemannian metric is locally constant, and the manifold is locally connected, which was applied using the conda-forge packages for Python (https://umap-learn.readthedocs.io/en/latest/index.html).

3.3 Molecular Identification

3.3.1 DNA Extraction

26S rDNA sequencing was modified based on Baleiras Couto et al. (2005). A single colony from yeast isolates of interest was resuspended in 100 µL sterile deionised water and frozen at -80 °C overnight. DNA was obtained by disrupting yeast cells by subsequently heating at 95 °C for 5 min.

3.3.2 Partial sequencing of the 26S rDNA gene

Each PCR sequencing reaction was performed in a 20 µL system containing 10 x PCR Buffer (Qiagen), 2.5 mM MgCl2 (Qiagen), 62.5 µM of each dNTPs (Invitrogen), 0.75 µM of each primer (Invitrogen), 2 U taq polymerase (Qiagen) and 1 µL DNA suspension. Partial 26S large subunit ribosomal DNA fragments were amplified using forward NL-1 primer (5' - GCA TAT CAA TAA GCG GAG GAA AAG - 3') and reverse NL-4 primer (5' -GGT CCG TGT TTC AAG ACG G -3') (Invitrogen) in Multigene Gradient (Labnet International, Inc., USA) with an initial denaturation at 94 °C for 3 min, followed by 36 cycles with a temperature profile of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1.5 min, ended with an final extension period at 72 °C for 5 min and remained at 4 °C (Baleiras Couto et al. 2005). Afterwards, PCR products were purified with AxyPrep PCR Clean-up Kit (Biosciences) according to the protocol and sequenced in single direction with only prime NL-1 or NL-4 at the Bio-Protection sequencing facility (Lincoln University). The quality of sequences obtained were checked with Sequence Scanner software (version 1.0, Applied Biosystem) and

compared by BLASTn tool online (http://www.ncbi.nlm.nih.gov/BLAST/). For the sequences obtained using reverse primer NL-4, Chromas software (Version 2.6) was used to get the reverse sequences. Species identification was considered valid when the identity of a 26S DNA sequence and a reference sequence was ≥98%.

A phylogenetic tree of partial sequences and the corresponding type strain published in the Genbank database was constructed using software Mega 7 (Kumar et al. 2016) by Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale with branch lengths measured in the number of substitutions per site. Field isolates were identified based on the similarity value relative to strains of known identity.

3.3.3 PCR-RFLP analysis of the NTS2 region for *Saccharomyces* speciation

In the absence of a reference strain of *S. uvarum* in our collection, isolates requiring confirmation as either *S. uvarum* or *S. bayanus* were identified using the method described by Nguyen and Boekhout (2017). Twelve representative isolates from the MALDI-dendrogram were further validated and 4 type strains (*S. bayanus* NCYC 2578, *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, *S. pastorianus* NCYC 396) were adopted as the control. The non-transcribed spacer 2 (NTS2) of ribosomal DNA was amplified using primers NTSU-NTS2 (5' - AACGGTGCTTTCTGGTAG - 3') and ETSL-NTS2 (5'-TGTCTTCAACTGCTTT-3') (Nguyen and Boekhout 2017). The annealing temperature was set at 55 °C. Restriction digestion using *Alu*I (Thermo Scientific™) was carried out directly on 20 µL of PCR product at 37 °C for 3 hours according to the manufacturer's instruction.

Amplified DNA strands were checked by agarose gel electrophoresis at voltage of 100 V for 60 min (PowerPac™ Basic, BIO-RAD). 4 µL PCR products mixed thoroughly with 1 µL loading dye (0.2% w/v bromophenol blue, 0.25% Xylene cyanol, 60% w/v glycerol) and 6 µL DNA marker HyperLadder™ 25kb (Bioline) or HyperLadder™ 1kb (Bioline) were loaded in 2% Agarose gel in 1 X Tris-borate-EDTA (TBE) buffer staining with 2% ethidium bromide (EtBr). Restriction fragments were separated on a 2% Agarose gel in 0.5 X TBE buffer staining with 2% EtBr. DNA bands were visualized under UV light and digital images were acquired using Molecular Imager®Gel DocTM XR+ with Image LabTM software (BIO-RAD). Fragment patterns of our relevant isolates were compared with those of type and reference strains (Nguyen and Boekhout 2017) for speciation.

Chapter 4

An Improved Method for MALDI-TOF Analysis of Wine-associated Yeasts

4.1 Introduction

Wine grapes are frequently colonised by indigenous yeast species of diverse origin. Spontaneous fermentation carried out by naturally occurring yeast species present on grapes may be considered an integral part of *terroir* (Capozzi et al. 2015) and the extensive range of other metabolic by-products could confer more desirable complexity in wine. However, unwanted species such as *Brettanomyces* spp. and *Zygosaccharomyces* spp. may also increase the risk of spoilage or poor quality product, and thus significant economic loss (Knight et al. 2015, Kraková et al. 2017, Hart et al. 2019). Effective and timely fermentation monitoring is required to manage such factors but is hampered by the lack of rapid and cost-effective yeast identification methods. Furthermore, characterization and evaluation of yeast strains for specific applications (*e.g.* low alcohol wine production) also require reliable identification (Jolly et al. 2014, Quirós et al. 2014, Ciani et al. 2016).

Molecular techniques like Internal Transcribed Spacer (ITS) and 26S rDNA sequencing have been adopted for species identification, and Amplified Fragment Length Polymorphism (AFLP), PCR-Restriction Fragment Length Polymorphism (RFLP), and Comparative Genome Hybridization (CGH) may be used for strain typing purposes (Guillamón et al. 1998, Kurtzman 2006, Pope et al. 2007, Zhang et al. 2010, Hesham et al. 2014, Kurtzman 2015). However, despite their powerful discriminatory capacity, these methods are high cost, generally labour intensive and also involve complex processes (Ivey and Phister 2011). Alternatively, Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is an emerging technique, which has proven to be a rapid and reliable tool in wine yeast identification at the species/strain levels (Moothoo-Padayachie et al. 2013, Usbeck et al. 2014, Gutiérrez et al. 2017). As yet, this list is not exhaustive and to date, several species including *Saccharomyces*, *Kluyveromyces* and *Brettanomyces* spp. have not to our knowledge been examined. Although MALDI-TOF has only been recently applied to yeast species, its potential for species- and strain analysis seems well founded. Moothoo-Padayachie *et al.* (2013) demonstrated that MALDI-TOF MS was able to identify *S. cerevisiae* to the species level with 100% accuracy, and strain level with 90% accuracy. In addition, Kraková et al. (2017) determined that strains could be classified to genus level, even where individual species were not present in the database used for comparison. Furthermore, MALDI-TOF

MS analysis has shown potential in predicting the utility of individual yeast strains used in the production of different wine styles including Chardonnay, Beaujolais and Champagne (Usbeck et al. 2014).

For sound identification to species- and strain level, clearly a standardized and robust protocol is desirable, as the efficiency of mass spectra acquisition may be influenced by microbial cell culture conditions, steps in the sample preparation, MALDI instrument and even personnel (Wunschel et al. 2005, Oberle et al. 2016). A standardised method for yeast analysis has been described (Gutiérrez et al. 2017). This chapter describes our experience with the latter, and, as a result, an amended approach others may find useful. In addition, the value of mass ranges m/z 500-4,000 and m/z 2,000-20,000 in identification of yeast species of oenological significance were investigated, since to our knowledge, no information of the potential low mass range peaks has yet been reported for yeast characterisation. We believe this is the first study to describe MALDI-TOF MS for yeast characterization and identification in New Zealand.

4.2 Results

4.2.1 MALDI Sample preparation optimization

Each method produced distinct MALDI spectra patterns of the yeast strains examined at both high and low mass range, for which exemplars are provided in Figure 4.1. Profiles for each strain are presented in Figure A.2-A.4. For high mass spectra, the pre-mixing method (PM) produced more peaks covering a broader mass range from m/z 4,000 to 20,000, while using the dried-droplet method (DM), the main mass range was from m/z 4,000 to 12,000, with no evident peaks beyond this range. For most species, the overall absolute peak intensity was higher with DM than PM. In many such cases, the background was also higher.

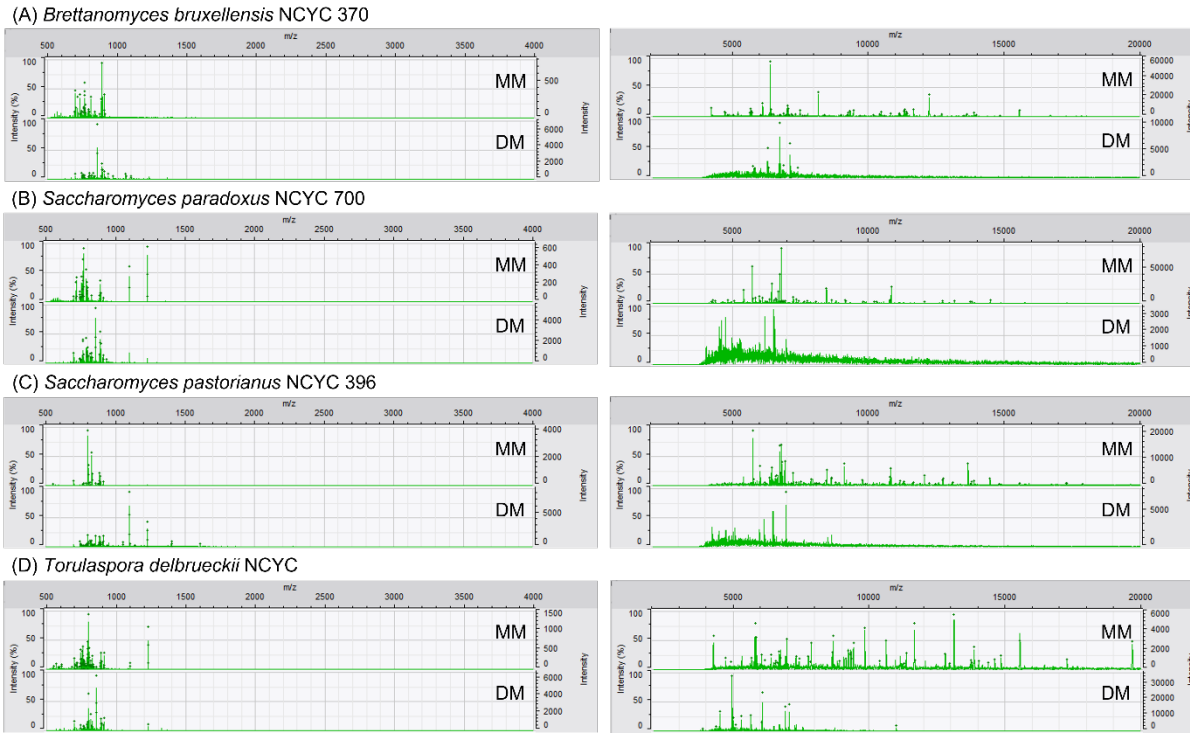


Figure 4.1 MALDI-TOF profiles of four representative wine-associated yeast strains at both low mass range (m/z 500-4,000) and high mass range (m/z 2,000-20,000) with Dried-droplet method (DM) and Pre-mixing method (PM).

Likewise, the low mass spectra obtained from two methods were different in peak number and intensity as well. The peaks were mainly found in m/z 500-1,000 using either method, but with a few species (*e.g. K. lactis* NCYC 416 and *K. wickerhamii* NCYC 546), DM elucidated peaks with a slightly broader mass range.

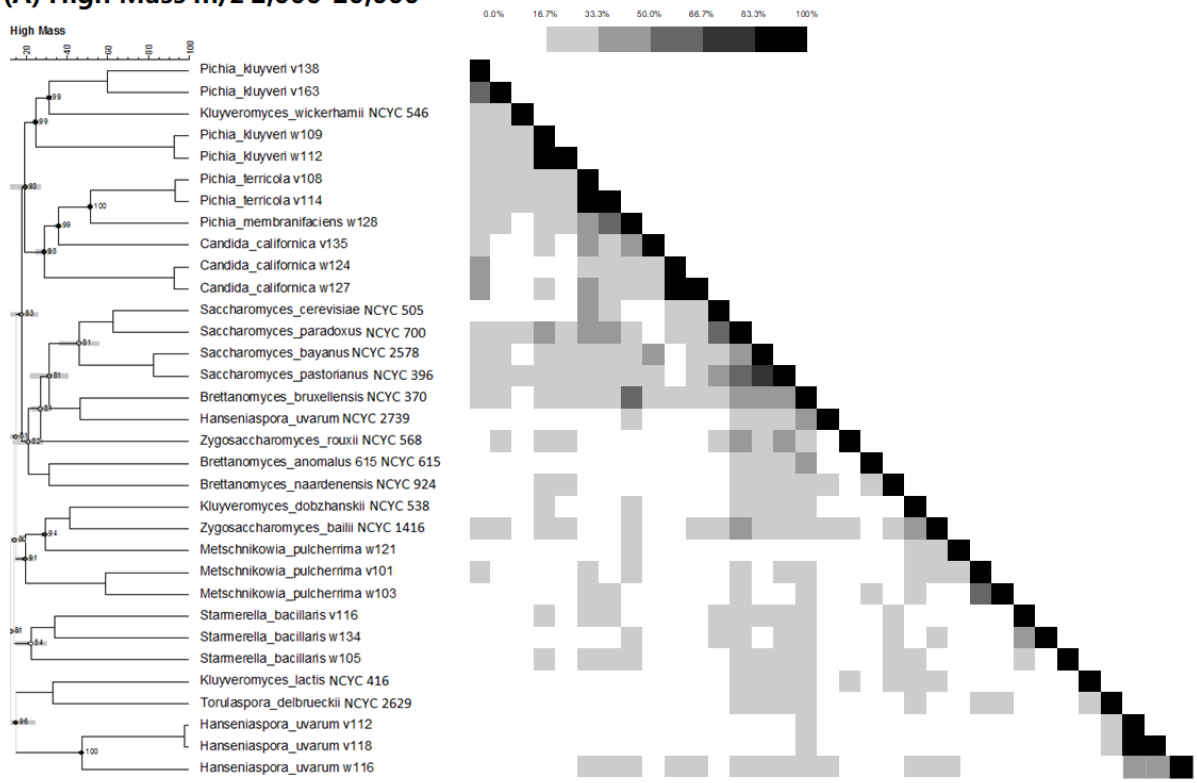
4.2.2 Cluster analyses of high, low and combined high-low mass spectral profiles

Dendrograms and similarity matrices derived from high, low, and combined spectral types for DM and PM sample preparation methods are shown in Figure 4.2 and 4.3 respectively. In the high mass spectral analyses, similarity values in PM-prepared extracts tended to be higher between strains of the same species compared to corresponding values from comparable DM-prepared extracts, allowing for accurate and effective species delineation at the 80% similarity level (Figure 4.3 (A)). Furthermore, the cluster analysis of the high-mass DM extracts divided *P. kluyveri, C. californica* and *M. pulcherrima* strains into two distinct groups, thus no single cut-off could accurately define all strains into distinct species (Figure 4.2 (A)).

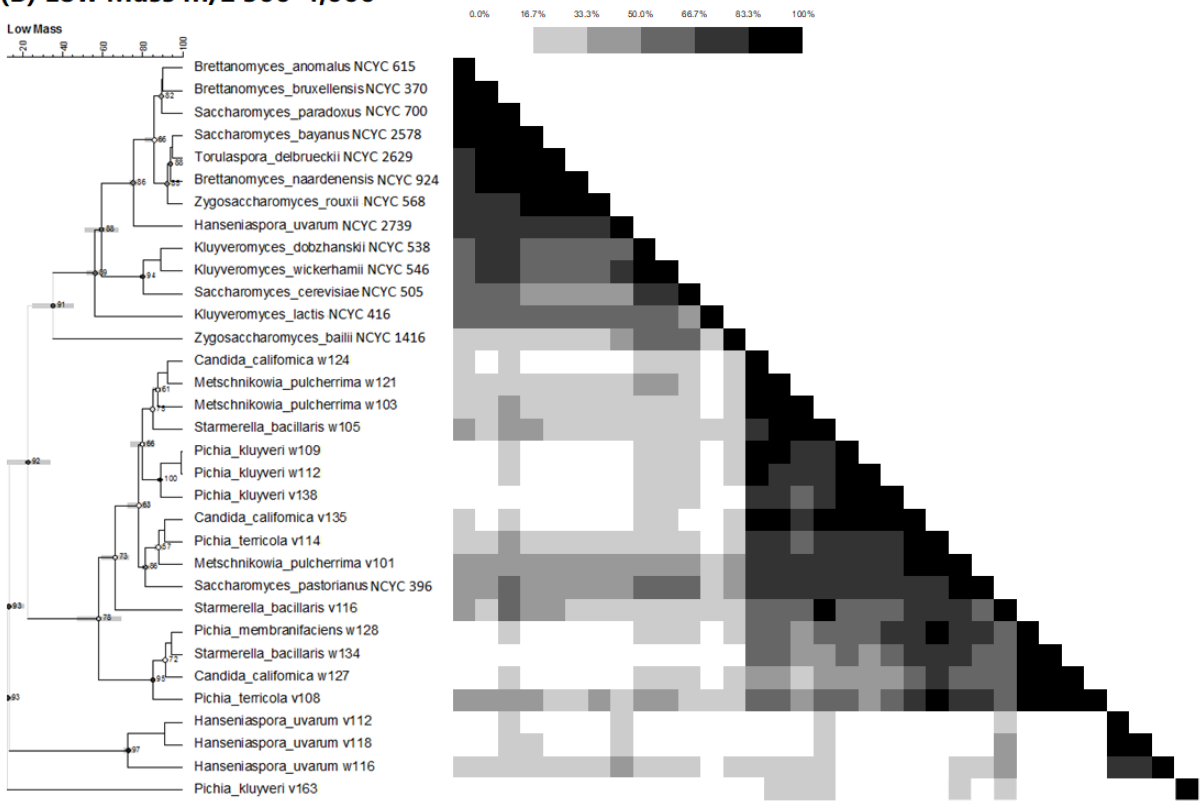
A similar trend was seen when dendrograms of low mass profiles from DM (Figure 4.2 (B)) and PM extraction protocols (Figure 4.3 (B)) were examined. In general, there were a greater number of higher scoring pairs of strains belonging to the same species with PM extracts compared to DM, however in each case a number of strains were found not to cluster with other species members. For

DM extracts, outlying strains from their member species group were seen in *H. uvarum*, *P. terricola, C. californica St. bacillaris* and *P. kluyveri.* For PM extracts, aberrant results were seen with *P. terricola, C. californica St. bacillaris* and *P. kluyveri.* Cluster analyses of dendrograms based on combined low and high mass data from DM and PM extraction profiles showed strains of *H. uvarum*, *St. bacillaris, C. californica* and *P. kluyveri* placed in outlying positions to other strains of these species in DM extracted profiles. However, all strains of these species clustered together with PM extracted profiles, albeit at lower similarity levels of 55% compared with high mass range data alone (Figure 4.3 (C)).

(A) High Mass m/z 2,000-20,000



(B) Low Mass m/z 500-4,000



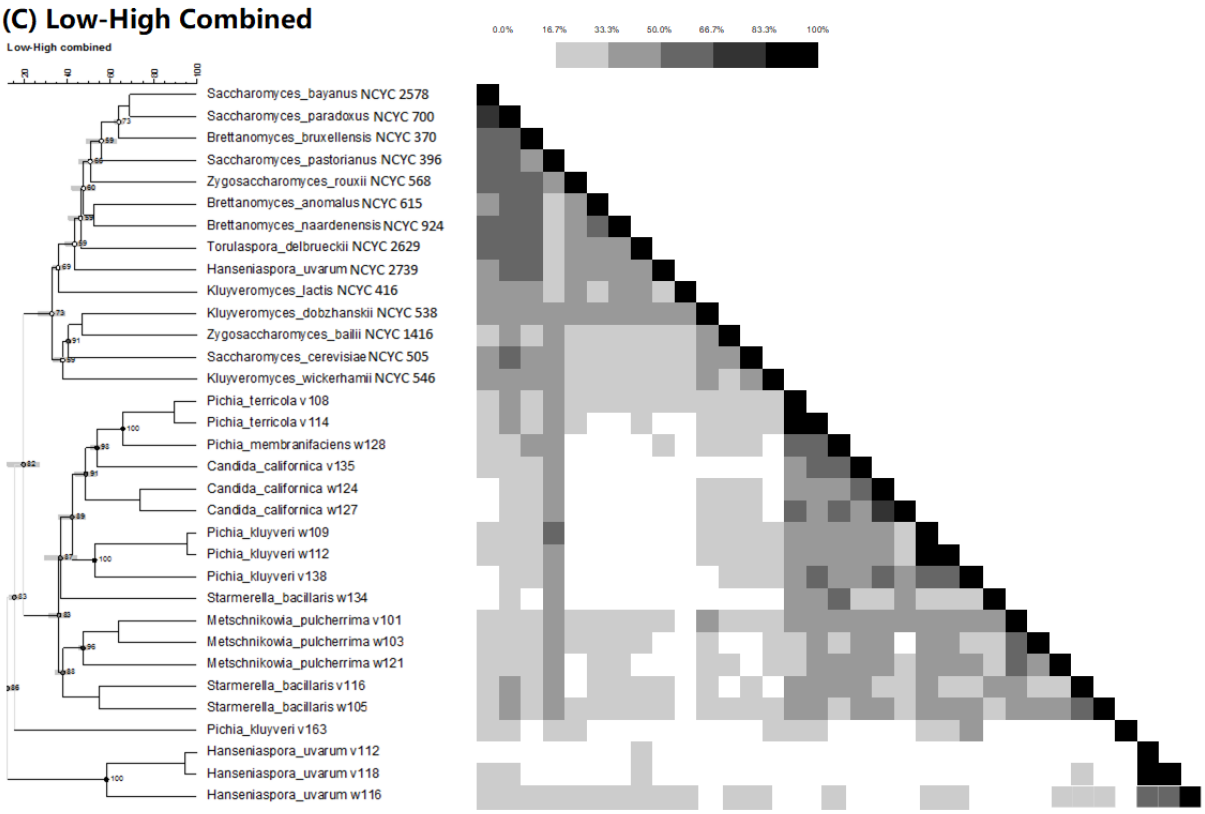
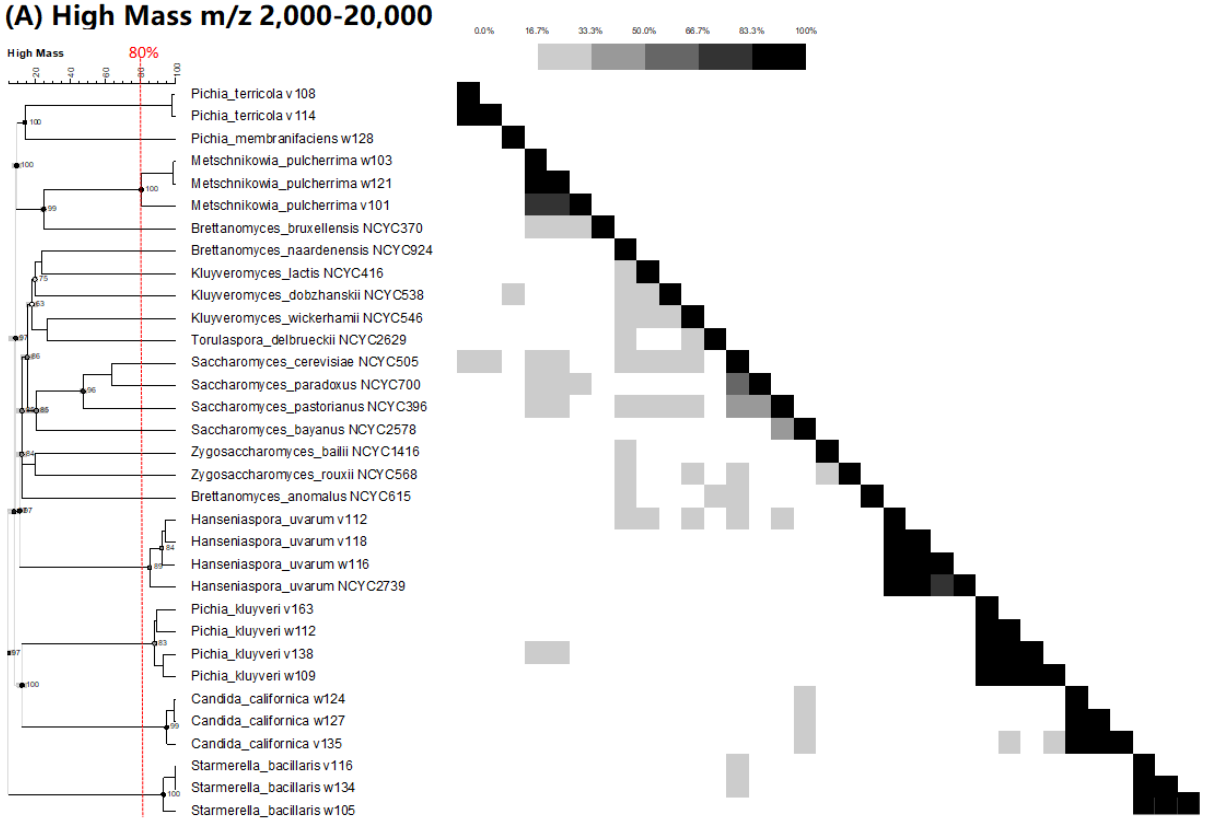


Figure 4.2 Dendrogram and similarity matrices derived from (A) High mass profiles, (B) Low mass profiles, and (C) Low-High Combined profiles using Dried-droplet method (DM)-prepared extracts.

(A) High Mass m/z 2,000-20,000



(B) Low Mass m/z 500-4,000

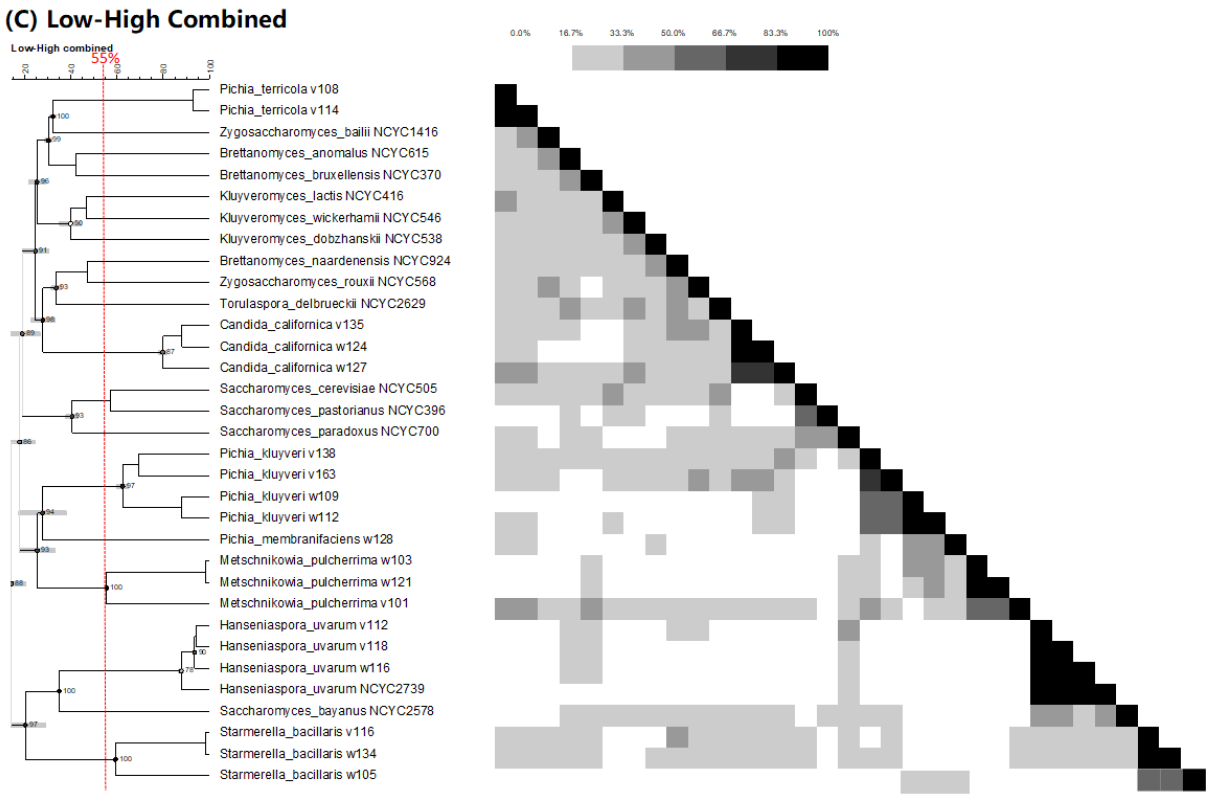


Figure 4.3 Dendrogram and similarity matrices derived from (A) High mass profiles, (B) Low mass profiles, and (C) Low-High Combined profiles using Pre-mixing method (PM)-prepared extracts.

4.3 Discussion

This study outlines the development of MALDI-TOF MS as a rapid and reliable tool in wine-related yeast identification and differentiation for use in New Zealand. Compared to molecular methods, MALDI-TOF MS has advantages of minimal and easier sample handling in a faster and cheaper way. For routine yeast identification analysis, more than 84.5% accuracy was achieved at species-level within 5.1 min at cost of $0.50 per sample (Dhiman et al. 2011). Prior to MALDI instrument analysis, the general sample preparation usually includes two steps of protein extraction or direct colony smear and matrix/sample reaction. In most cases, protein extraction is recommended as it enhances performance in microbial identification (Kim et al. 2017). In particular, unlike bacteria, yeast has a thick chitinous cell wall, for which a protein extraction step is usually adopted. Indeed for yeast analysis, results are either unsuccessful or variable depending on yeast species, using direct colony application (Gutiérrez et al. 2017). Our results support the claim made by Kim et al. (2017) since we obtained better results when a protein extraction step was included.

MALDI-TOF MS analysis potentially offers high sensitivity and mass accuracy better than 0.1%, but this technique is largely dependent on sample preparation (Claydon et al. 1996). The sample/matrix

method has the most significant influence on the performance of MALDI analysis, as the distribution of matrix/sample affects the matrix crystallization process, and therefore the laser beam energy absorption and efficiency for molecular desorption/ionization (Dai et al. 1999, Mello et al. 2017). The dried-droplet method (DM) is routinely used for bacterial identification, and a variation of this protocol was proposed as a standardised approach to characterise winemaking yeast using MALDI-TOF (Gutiérrez et al. 2017). The latter formed the basis of our initial studies, however although we determined it to yield peaks at high intensities, observations of high background noise and relatively low signals in the higher mass range encouraged us to explore the development of an alternative sample preparation method. We felt this prudent given that profiles were intended to be subjected to numerical analysis for identification purposes and more data with lower signal-to-noise ratio is an attractive prospect in this regard.

The levels of different proteins vary from fewer than 50 to more than 10^6 molecules per cell in yeast, however mass spectrometry seems strongly biased towards the detection of abundant proteins (Ghaemmaghami et al. 2003). Our results indicate the DM method is optimised for the detection of major cell components, since overall peak intensity was higher in DM extracts compared with PM data. However, the PM protocols clearly allows for the detection of more proteins, including those at low-abundance, with the added benefit of lower background noise. Since the distribution and homogeneity between matrix and sample is essential for the spectra acquisition with high quality and reproducibility (Dai et al. 1999, Williams et al. 2003, Rešetar et al. 2016, Gutiérrez et al. 2017), the PM mixing step may account for this improvement. Furthermore, a combination of more extensive protein species detection as well as improved profile clarity is likely to account for the improved species resolution of PM extracts compared with the DM method when data are then subjected to numerical analysis of high- and combined high- and low range profiles (Figure 4.2 and 4.3). Although, neither DM or PM protocols yielded low mass spectra that yielded unequivocal species-specific profiles, the incorporation of low mass proteins into future analyses may yield additional benefits when examining the potential utility of yeast species in, for example, the production of particular wine styles as has been intimated before (Usbeck et al. 2014). From this perspective it is noteworthy that the standardised DM method proposed by Gutiérrez et al. (2017) does not appear to display differences among *S. cerevisiae* strains: a result in contrast to those described by Usbeck et al. (2014).

4.4 Conclusion

In conclusion, our modified PM sample preparation improved the performance of MALDI-TOF analysis in wine-associated yeasts characterization. In our study, PM yeast preparations subjected to

MALDI-TOF MS seems suited to detect minor peak variations of *H. uvarum, C. californica*, *M. pulcherrima*, and *P. kluyveri,* while retaining the ability to accurately group strains at the species level. It has been previously reported that factors of isolation sources and geographic distance could influence the protein fingerprints and MALDI dendrogram (Kern et al. 2014, Zhang et al. 2015, Mello et al. 2017). Furthermore, the intraspecific variations in spectral patterns of yeast isolates may be reflected in differences in fermentative properties, as indicated previously (Usbeck et al. 2014). Overall, aside from its powerful, rapid and cost-effective identification capacity, MALDI-TOF MS may also have potential in selecting strains with special properties (*e.g.* low alcohol production, probiotic potential). For these reasons, we hope that our sample preparation method may be of interest to investigators wishing to reliably characterise yeast species using MALDI-TOF with an extended proteomic range, as we continue to pursue its potential to add value to the New Zealand winemaking sector.

Chapter 5

Application of MALDI-TOF Analysis to Reveal Diversity and Dynamics of Winemaking Yeast Species in Wild-fermented, Organically Produced, New Zealand Pinot Noir Wine

5.1 Introduction

The New Zealand export wine sector has enjoyed continued growth for more than a decade, and part of its success has been attributed to the diversity of styles and products available (NZ Winegrowers annual report 2019. https://www.nzwine.com/en/media/statistics/annual-report/). The range of varied geographic locations and climatic conditions contribute to wines possessing unique characteristics, and is often referred to as *terroir* (Parr et al. 2007). Environmental factors such as soil composition and climate play an important role in wine quality (Tonkin et al. 2015), and influence the chemical composition in terms of volatile and non-volatile compounds (Sagratini et al. 2012). Waipara Valley in South Island of New Zealand has a cool, dry, warm temperate climate with diverse soil types, which has been exploited to produce Pinot Noir wines with distinctive regional characteristics (Tomasino et al. 2013).

Yeast species/strains and population are also critical to the flavour and *terroir* of final wine products (Fleet 2008). Previous studies have indicated that yeast communities and populations associated with vines and wines were region-specific in NZ, and interpreted it as a microbial aspect to *terroir* (Gayevskiy and Goddard 2012, Taylor et al. 2014). The commercial use of “wild” or spontaneous fermentation with indigenous yeasts on grapes leverages this distinctiveness, but poses challenges for consistent production. Rapid identification of yeast strains facilitates timely technical interventions for commercial benefit, and indeed potential for using novel indigenous yeast species/strains for production of novel wine varieties.

Numerous identification techniques are available for winemaking-related yeasts (Ivey and Phister 2011, Hart et al. 2019). Although some molecular methods have been shown to be reliable for this purpose, they are overly expensive and time-consuming for the rapid identification of wine yeast species/strains during commercial fermentation (Hart et al. 2019). In contrast a proteomic approach based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry, (MALDI-TOF MS) has been shown to be a rapid, reliable and cost-effective tool in wine yeast identification at the species/strain levels (Moothoo-Padayachie et al. 2013, Usbeck et al. 2014, Gutiérrez et al. 2017). In

addition, the potential for predicting applications for specific yeast strains to produce individual wine- or beer styles or as probiotics, has been alluded to Usbeck et al. (2014) and Lauterbach et al. (2017).

Although yeast community diversity associated with wine and vine has been investigated previously in Gisborne, West Auckland, Waiheke Island, Hawkes Bay, Marlborough and Central Otago with molecular methods (Zhang et al. 2010, Taylor et al. 2014), no data of yeast community diversity in the Waipara region has been published. Furthermore, we are unaware of any studies investigating yeast diversity in organic wine production using solely indigenous yeast. We have previously described an optimised method for using MALDI-TOF spectra of winemaking-relevant yeasts for identification purposes (Zhang et al. 2020). This chapter examines the use of MALDI-TOF MS analysis to evaluate the dynamics of culturable yeast species during wine fermentation under each of two distinct forms of organic indigenous fermentation conditions in the Waipara region.

5.2 Results

5.2.1 Isolate identification

A total of 235 field isolates were isolated (Table 5.1) and subjected to MALDI-TOF MS analysis, and relationships determined in a dendrogram that also included 14 reference strains of known identity (Figure 5.1 (A)). Clusters containing distinct species were defined at the 40% similarity threshold (Figure 5.1 (A)). Results of the MALDI-based dendrogram were validated by partial 26S rDNA sequencing of 73 representative field strains (Figure 5.1 (B)), or PCR-RFLP for discrimination of *Saccharomyces* species (Nguyen and Boekhout 2017). These results confirmed the efficacy of MALDI-TOF for speciation.

A subgroup of four isolates closely related to, but distinct from our other *S. uvarum* isolates was observed in our MALDI-TOF analysis (Figure 5.1 (A)). These isolates exhibited a faint band ca. 350 bp in size in our PCR-RFLP analysis of these strains, in contrast with the *S. uvarum* type strain (Nguyen and Boekhout 2017), and from eight field isolates yielding a typical *S. uvarum* PCR-RFLP profile (Figure A.5). We consequently refer to these four isolates as *S. uvarum*-like, pending further study.

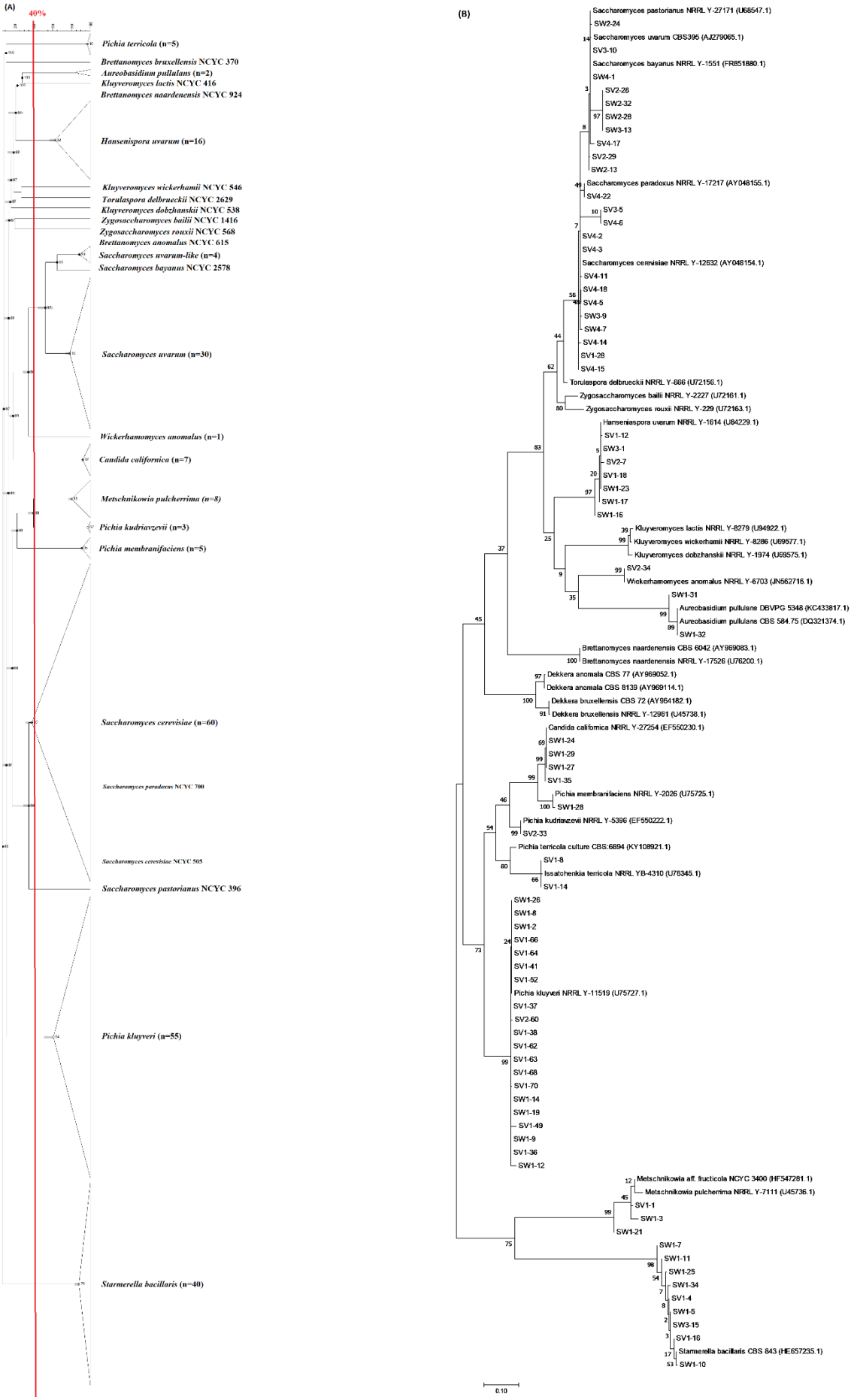

(A)
40%
Pichia terricola (n=5)
Brettanomyces bruxellensis NCYC 370
Aureobasidium pullulans (n=2)
Kluyveromyces lactis NCYC 416
Brettanomyces naardenensis NCYC 924
Hansenispora uvarum (n=16)
Kluyveromyces wickerhamii NCYC 546
Torulaspora delbrueckii NCYC 2629
Kluyveromyces dobzhanskii NCYC 538
Zygosaccharomyces bailii NCYC 1416
Zygosaccharomyces rouxii NCYC 568
Brettanomyces anomalus NCYC 615
Saccharomyces uvarum-like (n=4)
Saccharomyces bayanus NCYC 2578
Saccharomyces uvarum (n=30)
Wickerhamomyces anomalus (n=1)
Candida californica (n=7)
Metschnikowia pulcherrima (n=8)
Pichia kudriavzevii (n=3)
Pichia membranifaciens (n=5)
Saccharomyces cerevisiae (n=60)
Saccharomyces paradoxus NCYC 700
Saccharomyces cerevisiae NCYC 505
Saccharomyces pastorianus NCYC 396
Pichia kluyveri (n=55)
Starmerella bacillaris (n=40)
(B)
Saccharomyces pastorianus NRRL Y-27171 (U68547.1)
SW2-24
Saccharomyces uvarum CBS395 (AJ279065.1)
SV3-10
Saccharomyces bayanus NRRL Y-1551 (FR851880.1)
SW4-1
SV2-28
SW2-32
SW2-28
SW3-13
SV4-17
SV2-29
SW2-13
Saccharomyces paradoxus NRRL Y-17217 (AY048155.1)
SV4-22
SV3-5
SV4-6
SV4-2
SV4-3
Saccharomyces cerevisiae NRRL Y-12632 (AY048154.1)
SV4-11
SV4-18
SV4-5
SW3-9
SW4-7
SV4-14
SV1-28
SV4-15
Torulaspora delbrueckii NRRL Y-866 (U72156.1)
Zygosaccharomyces bailii NRRL Y-2227 (U72161.1)
Zygosaccharomyces rouxii NRRL Y-229 (U72163.1)
Hanseniaspora uvarum NRRL Y-1614 (U84229.1)
SV1-12
SW3-1
SV2-7
SV1-18
SW1-23
SW1-17
SW1-16
Kluyveromyces lactis NRRL Y-8279 (U94922.1)
Kluyveromyces wickerhamii NRRL Y-8286 (U69577.1)
Kluyveromyces dobzhanskii NRRL Y-1974 (U69575.1)
SV2-34
Wickerhamomyces anomalus NRRL Y-6703 (JN562716.1)
SW1-31
Aureobasidium pullulans DBVPG 5348 (KC433817.1)
Aureobasidium pullulans CBS 584.75 (DQ321374.1)
SW1-32
Brettanomyces naardenensis CBS 6042 (AY969083.1)
Brettanomyces naardenensis NRRL Y-17526 (U76200.1)
Dekkera anomala CBS 77 (AY969052.1)
Dekkera anomala CBS 8139 (AY969114.1)
Dekkera bruxellensis CBS 72 (AY964182.1)
Dekkera bruxellensis NRRL Y-12961 (U45738.1)
Candida californica NRRL Y-27254 (EF550230.1)
SW1-24
SW1-29
SW1-27
SV1-35
Pichia membranifaciens NRRL Y-2026 (U75725.1)
SW1-28
Pichia kudriavzevii NRRL Y-5396 (EF550222.1)
SV2-33
Pichia terricola culture CBS:6894 (KY109921.1)
SV1-8
Issatchenkia terricola NRRL YB-4310 (U76345.1)
SV1-14
SW1-26
SW1-8
SW1-2
SV1-66
SV1-64
SV1-41
SV1-52
Pichia kluyveri NRRL Y-11519 (U75727.1)
SV1-37
SV2-60
SV1-38
SV1-62
SV1-63
SV1-68
SV1-70
SW1-14
SW1-19
SV1-49
SW1-9
SV1-36
SW1-12
Metschnikowia aff. fructicola NCYC 3400 (HF547281.1)
Metschnikowia pulcherrima NRRL Y-7111 (U45736.1)
SV1-1
SW1-3
SW1-21
SW1-7
SW1-11
SW1-25
SW1-34
SV1-4
SW1-5
SW3-15
SV1-16
Starmerella bacillaris CBS 843 (HE657235.1)
SW1-10
0.10

Figure 5.1 (A) MALDI-dendrogram using Pearson correlation and UPGMA algorithm; (B) Molecular Phylogenetic analysis by Maximum Likelihood method based on the Tamura-Nei model.

Pichia membranifaciens and *Candida californica* pose similar identification challenges due to their close phylogenetic relationship (Wu et al. 2006). Nonetheless, our MALDI-TOF analysis clustered SW1-28 and SW1-29 together and differentiated these from *C. californica.* Isolates in white with reddish pigment leaching into the growth medium were typical of species of the genus *Metschnikowia*. Three isolates SV1-1, SW1-3 and SW1-21 were subjected to 26S rDNA sequencing, of which BLASTn results showed SV1-1 and SW1-21 had the highest similarity of 99% and 97% with *M. pulcherrima*, and SW1-3 showed 99% similarity with *M. fructicola*. These species are phylogenetically highly related (Kurtzman and Droby 2001). *Metschnikovia fructicola* has largely been explored as a biocontrol agent against soft rot in berries (Kurtzman and Droby 2001), and previous studies of yeast diversity in New Zealand grapes have not, to our knowledge, revealed its presence in vineyards examined in this country (Zhang et al. 2010, Gayevskiy and Goddard 2012). In contrast, *M. pulcherrima* is common in early stage ferments (Morata et al. 2019) and known to be present in New Zealand (Zhang et al. 2010), hence we consider these strains to be *M. pulcherrima*.

In summary, a total of 13 yeast species belonging to 8 genera were identified among our isolates.

5.2.2 Yeast species dynamics during fermentation

The distribution of the different yeast species identified in this study in wine fermentations undertaken in the winery and vineyard respectively during each of the four sampling periods is shown in Table 5.1. Enumerations of total yeast populations in each stage are also given. The initial yeast population of vineyard samples was 1.10 x 10^8 cfu/mL, almost 3 times higher than that of winery samples (3.40 x 10^7 cfu/mL). Nonetheless, the trend for yeast proliferation in each fermentation system was the same, with peak yeast numbers attained in the second stage and gradually declining after that. At the final stage, the winery sample yeast population (1.60 x 10^7 cfu/mL) was about 3 times higher than that of vineyard samples (5.40 x 10^6 cfu/ml).

Table 5.1 Yeast community dynamics during vineyard and winery fermentation.

		Vineyard fermentation				Winery fermentation			
	Non-*Saccharomycs*	**SV1**	**SV2**	**SV3**	**SV4**	**SW1**	**SW2**	**SW3**	**SW4**
1	*H. uvarum*	52%	7%	-	-	11%	+	+	-
2	*St. bacillaris*	39%	40%	30%	-	86%	12%	33%	-
3	*P. terricola*	8%	-	-	-				
4	*M. pulcherrima*	+	+	-	-	+	-	-	-
5	*P. kluyveri*	+	+	-	-	+	-	+ (1)	-
6	*C. californica*	+	-	-	-	+	-	-	-
7	*P. membranifaciens*	-	+ (3)	-	-	+ (2)	-	-	-
8	*A. pullulans*					+ (2)	-	-	-
9	*P. kudriavzevii*	-	+	-	-				
10	*W. anomalus*	-	+	-	-				
	Saccharomyces								
11	*S. cerevisiae*	+	48%	70%	100%	-	86%	67%	85%
12	*S. uvarum*	-			-	-			5%
13	*S. uvarum-like*					-		-	10%
Population (cfu/mL)		$1.10x10^8$	$1.27x10^{10}$	$5.60x10^8$	$5.40x10^6$	$3.40x10^7$	$1.08x10^{10}$	$3.30x10^8$	$1.60x10^7$

Note: "+" means detectable but at very low levels, the number in "()" means the number of isolates; "-" means undetectable.

In the earliest samples taken from the vineyard (SV1), the most prevalent species were *H. uvarum* (52%), *St. bacillaris* (39%), and *P. terricola* (8%), with other species identified as *M. pulcherrima*, *P. kluyveri*, and *C. californica;* only one isolate was identified as *S. cerevisiae*. In SV2, the prevalence of *Saccharomyces* species (*S. cerevisiae* and S*. uvarum*) increased to 48% of the detected yeast populations, and the proportion of *St. bacillaris* remained stable (40%), and *H. uvarum* decreased greatly (7%). Three previously undetected species were *P. membranifaciens*, *P. kudriavzevii*, *and Wickeraromycess anomalus*, whereas *C. californica* and *P. terricola* disappeared. In the following stages, *Saccharomyces* species (*S. cerevisiae* and *S. uvarum*) gradually took over the fermentation, with only *S. cerevisiae* detected in SV4. The yeast diversity and the frequency of their appearance varied between the vineyard samples and winery samples, although the grapes used in each ferment were from the same batch. In the first stage winery samples (SW1), *St. bacillaris* was the dominant species (86%), with *H. uvarum* in 11%. In the second stage (SW2), *Sacchromyces* species (*S. cerevisiae, S. uvarum* and *S. uvarum-like*) soared up to 86%, *St. bacillaris* and *H. uvarum* dropped sharply to 12% and 2%, respectively. Interestingly, our *S. uvarum-like* taxon was not isolated from SW3, but appeared in SW2 and SW4, while the proportion of *St. bacillaris* increased from 12% to 33%; sample size may help account for this finding. In the final stage, *Saccharomyces* species

completely obscured the non-*Saccharomyces* species. Similar to vineyard samples at the same stage, *S. cerevisiae* was dominant but a few *S. uvarum* and *S. uvarum-like* strains were detected.

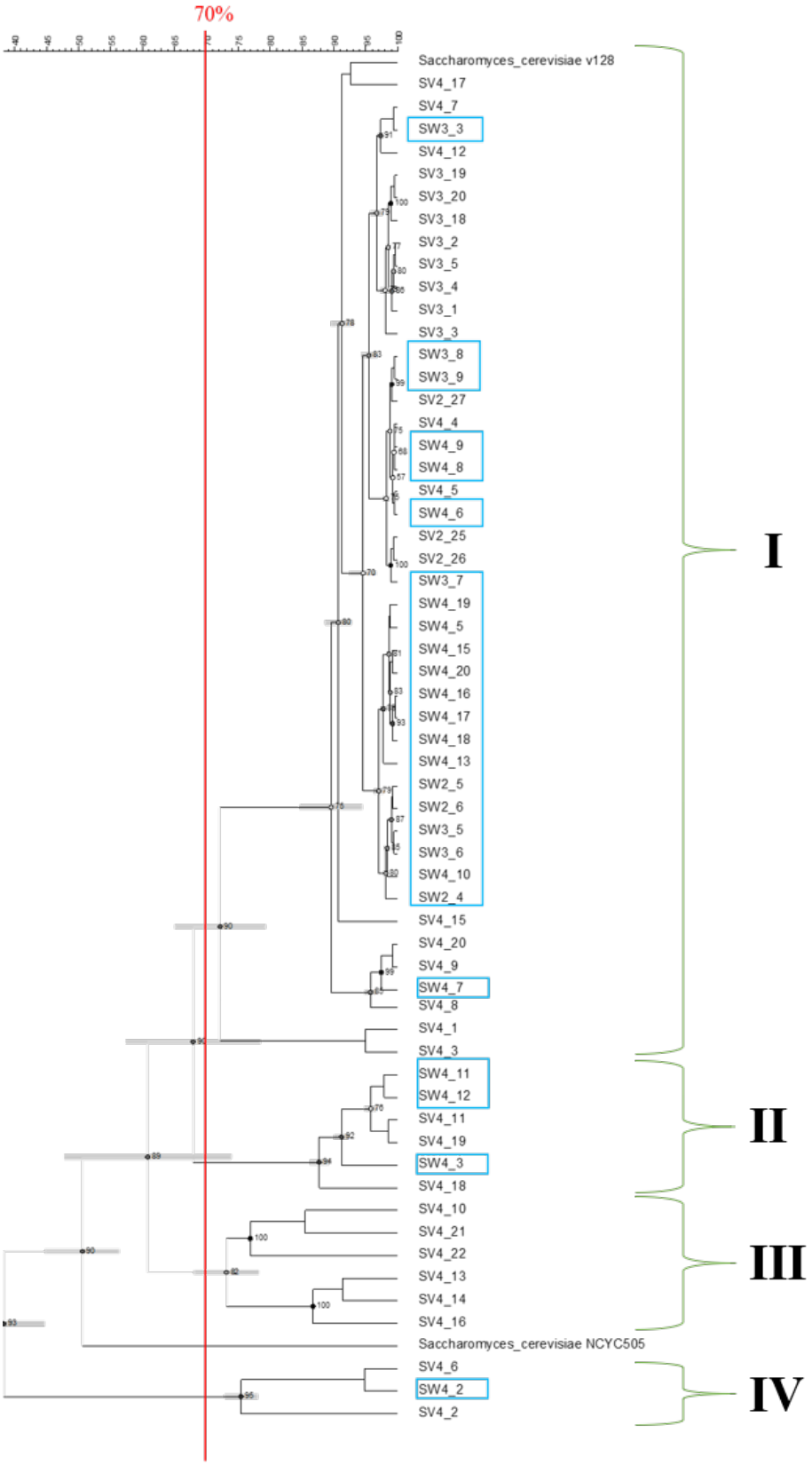
Overall, non-*Saccharomyces* yeast was most abundant at the start of the winemaking process, but were gradually replaced over time by *Saccharomyces* species, as is generally expected. *Saccharomyces cerevisiae* was in general the dominant species at the end of fermentation.

5.2.3 MALDI-based strain subtype analyses

5.4.3.1 *Saccharomyces* species

A total of sixty *S. cerevisiae* isolates obtained from winery fermentation (n=26) and vineyard fermentation (n=34) samples were identified in this study. Variance in MALDI-based profiles was evident both visually and in cluster analysis, where a 70% similarity value was selected as the threshold to define strain types (Figure 5.2). The *S. cerevisiae* isolates were distributed among 5 types, of which one is unique and represented by the type strain. Type I is predominant type (representing 23/34 vineyard and 22/26 winery strains) and was observed in every fermentation stage. The other three types were only detected in later ferment stages, with type III only found in vineyard samples (Figure 5.2 (A)). Figure 5.2 (B) presents the profile pattern of each *S. cerevisiae* type.

(A)


70%
Saccharomyces_cerevisiae v128
SV4_17
SV4_7
SW3_3
SV4_12
SV3_19
SV3_20
SV3_18
SV3_2
SV3_5
SV3_4
SV3_1
SV3_3
SW3_8
SW3_9
SV2_27
SV4_4
SW4_9
SW4_8
SV4_5
SW4_6
SV2_25
SV2_26
SW3_7
SW4_19
SW4_5
SW4_15
SW4_20
SW4_16
SW4_17
SW4_18
SW4_13
SW2_5
SW2_6
SW3_5
SW3_6
SW4_10
SW2_4
SV4_15
SV4_20
SV4_9
SW4_7
SV4_8
SV4_1
SV4_3
SW4_11
SW4_12
SV4_11
SV4_19
SW4_3
SV4_18
SV4_10
SV4_21
SV4_22
SV4_13
SV4_14
SV4_16
Saccharomyces_cerevisiae NCYC505
SV4_6
SW4_2
SV4_2
I
II
III
IV

(B)

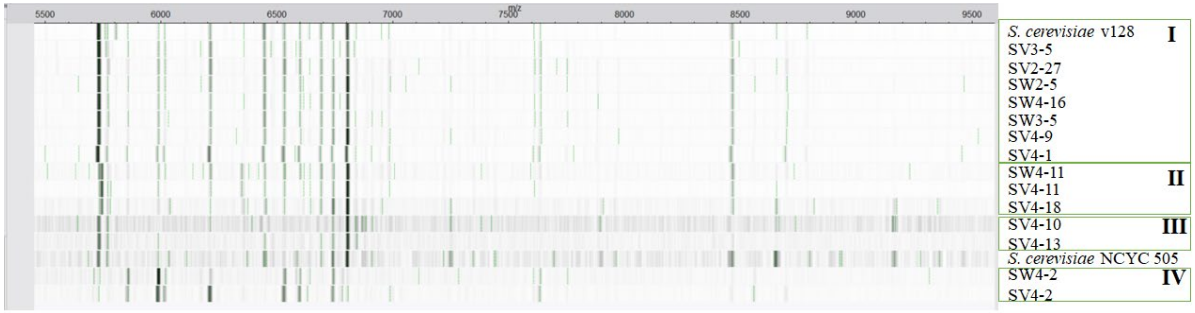
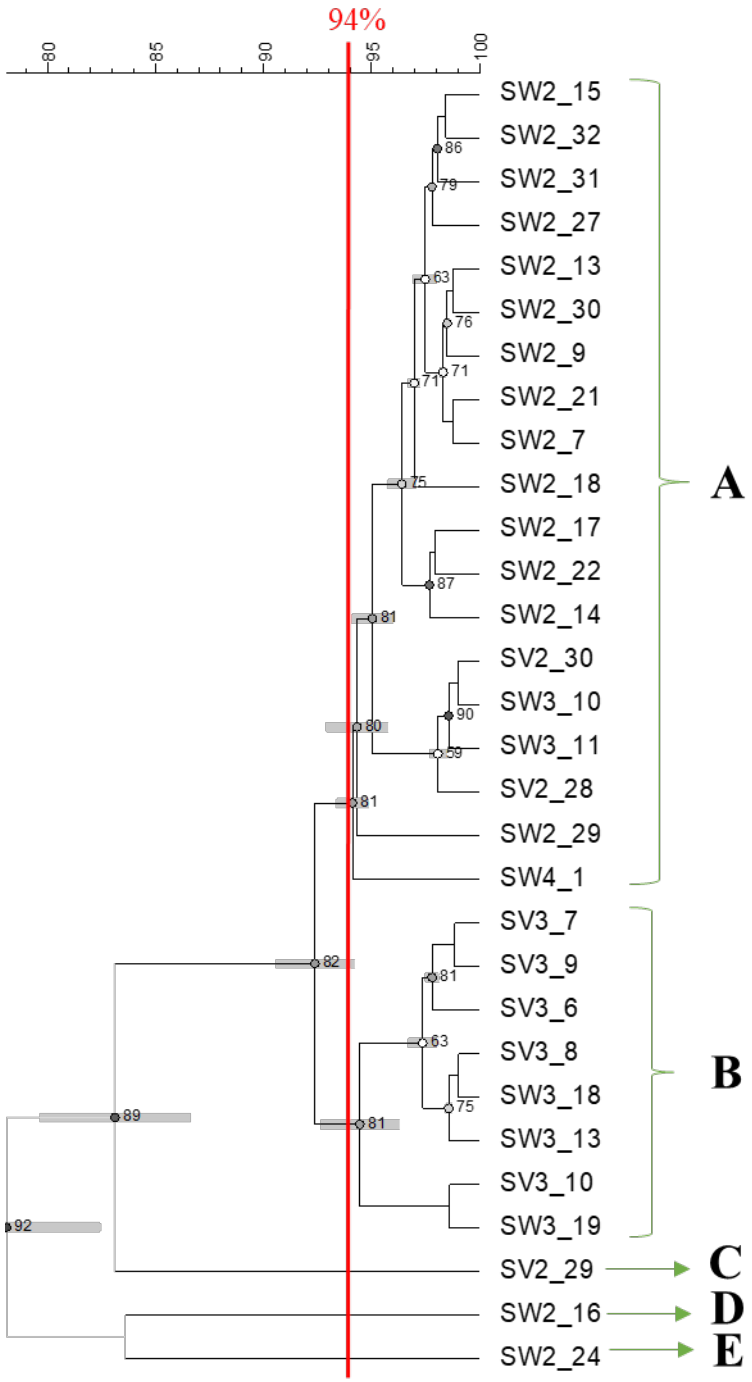


Figure 5.2 Cluster analysis of the (A) 60 *S. cerevisiae* isolates and one reference strain *S. cerevisiae* NCYC 505, all the winery isolates are marked in blue squares; 5 types were divided based on the 70% threshold; (B) band presentation (m/z 5,500-9,500) of representative strains from each *S. cerevisiae* types.

Thirty strains of *S. uvarum* were isolated in our study, of which eight were from vineyard samples and the remainder from winery ferments. Five types were defined (Figure 5.3), Figure 5.3 (B) presents the profile pattern of each type. Type A dominated the second stage ferments with type B most prolific in the third stage. Types A and B were closely related with profiles exhibiting relatively few differences. Their dominance at differing stages of the fermentative process may indicate a clonal relationship, with differences in phenotypic expression resulting from changes in the environment (*e.g.* pH, ˚Brix) during fermentation. Absence of *S. uvarum* in stage 1 ferment samples, and recovery of just one strain in the last stage, may indicate a specific sensitivity to environmental conditions.

(A)


94%
80
85
90
95
100
SW2_15
SW2_32
SW2_31
SW2_27
SW2_13
SW2_30
SW2_9
SW2_21
SW2_7
SW2_18
SW2_17
SW2_22
SW2_14
SV2_30
SW3_10
SW3_11
SV2_28
SW2_29
SW4_1
SV3_7
SV3_9
SV3_6
SV3_8
SW3_18
SW3_13
SV3_10
SW3_19
SV2_29
SW2_16
SW2_24
86
79
63
76
71
71
75
87
81
90
80
59
81
82
81
63
75
81
89
92
A
B
C
D
E

(B)

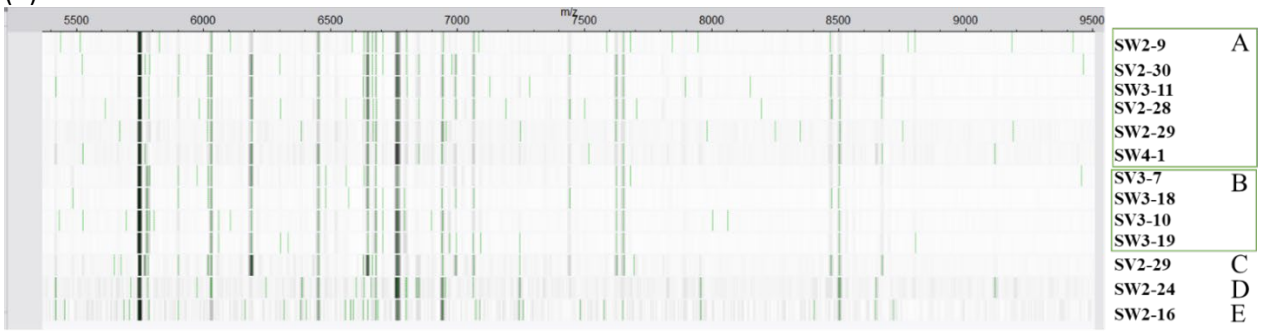


Figure 5.3 Cluster analysis of the (A) 30 *S. uvarum* isolates; 5 types were divided based on the threshold 94%; (B) band presentation (m/z 5,500-9,500) of representative strains from each *S. uvarum* types.

Regarding the four *S. uvarum-like* isolates, the absence of peak m/z 6,646 distinguished strain SW2-28 from the other isolates. The type strain of *S. bayanus* used as a reference was isolated from beer, and has been proposed to be a hybrid with genetic traits of *S. uvarum* (63%), *S. eubayanus* (37%) and minor *S. cerevisiae* constituents (<1%) (Nguyen and Boekhout 2017), which may explain its lower similarity (Figure 5.4) with our strains.

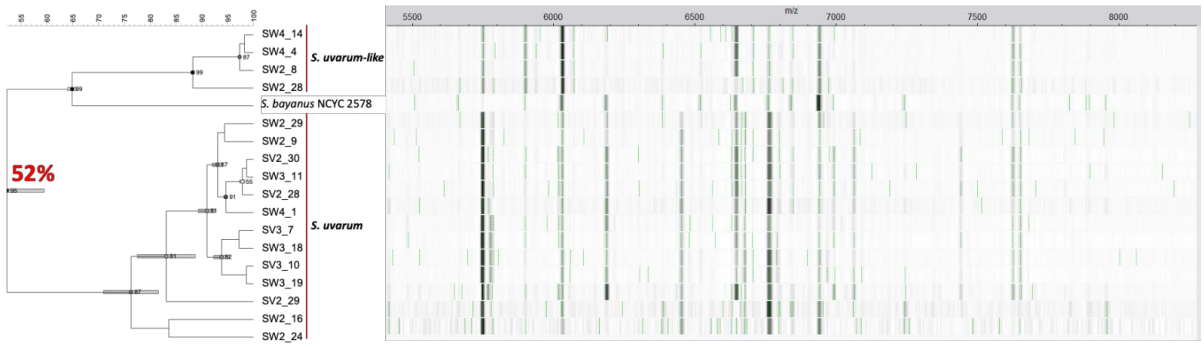


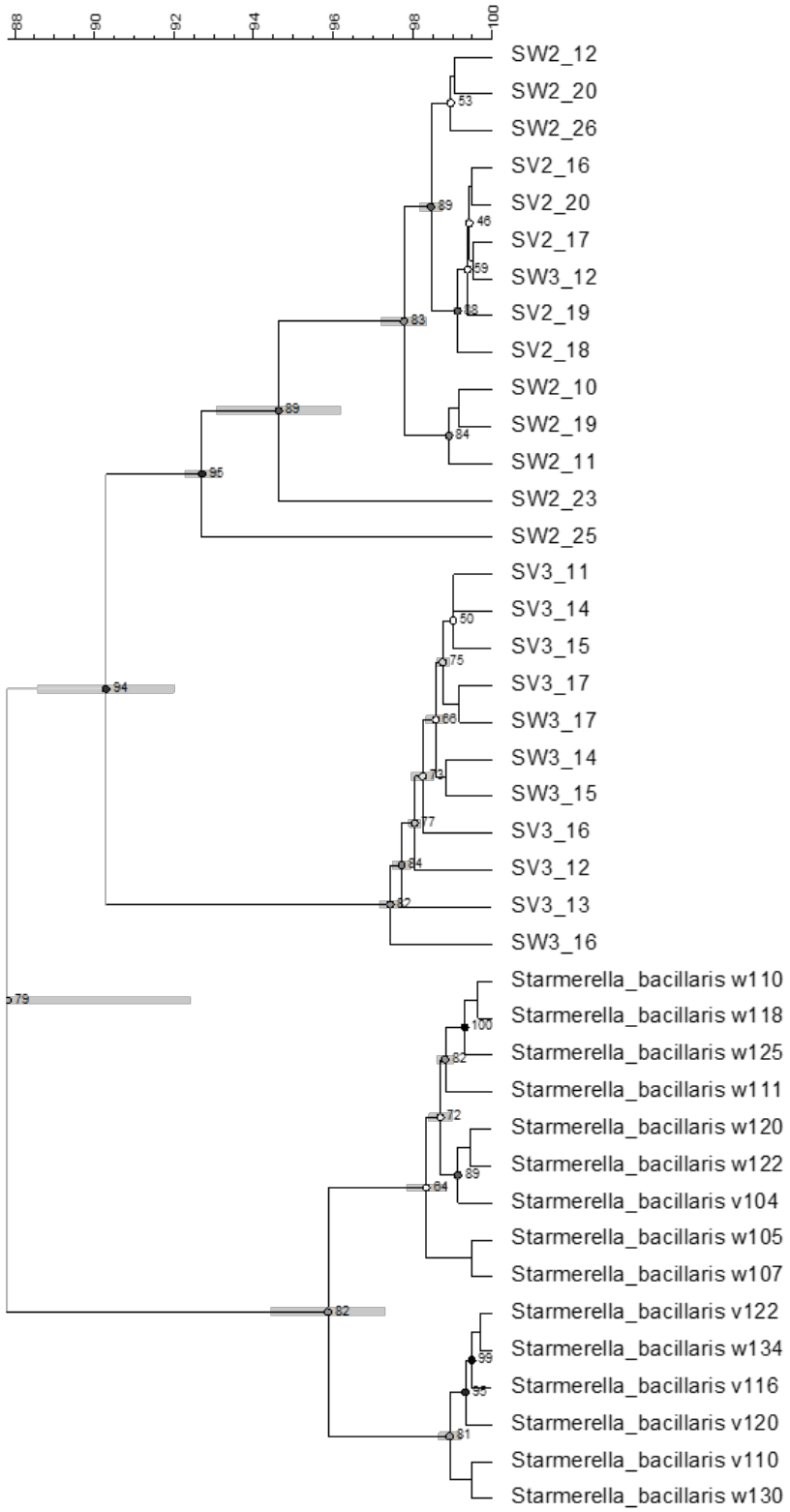
Figure 5.4 Cluster analysis of the *S. bayanus* NCYC 2578 and representative *S. uvarum* isolates combined with their band presentation of spectra patterns.

5.4.3.2 non-*Saccharomyces* species

Starmerella bacillaris and *H. uvarum* were the only two non-*Saccharomyces* yeasts detected over three successive ferment stages. Interestingly, *St. bacillaris* strains were divided into three clusters according to the ferment stage they were recovered from. These clusters were distinguished by differences in several prominent peaks, namely m/z 5,390; 5,405; 6,209; 10,779; 10,808 and 13,956 (Figure 5.5). Whole-genomic analyses have suggested that *St. bacillaris* may be capable of an intricatestress response (Lemos Junior et al. 2018), and the differences we observe may be the result of differential gene expression in relation to environmental changes as the wine ferments. Further studies are required to confirm this.

Three of the other non-*Saccharomyces* yeasts isolated in our study, *H. uvarum*, *C. californica*, *P. membranifaciens* showed similar trends to the *St. bacillaris* strain variation, with MALDI profile clustering generally aligned with the stage of ferment at which strains were recovered (Figure A.6-A.8). The variation observed among the *M. pulcherrima* strains distinguished three profile groups (Figure A.9) but these were not correlated with either source or ferment stage. *Pichia kluyveri* isolates demonstrated both considerable variation in their MALDI spectra and colony morphotypes (Figure A.10-11). *Candida californica* was only detected in the first ferment stage in both vineyard and winery ferments, and the variation in peak m/z 8,211 correlated with isolation source. Moreover, the isolates from two ferments showed different colony morphotypes (Figure A.11).

(A) ***St. bacillaris***



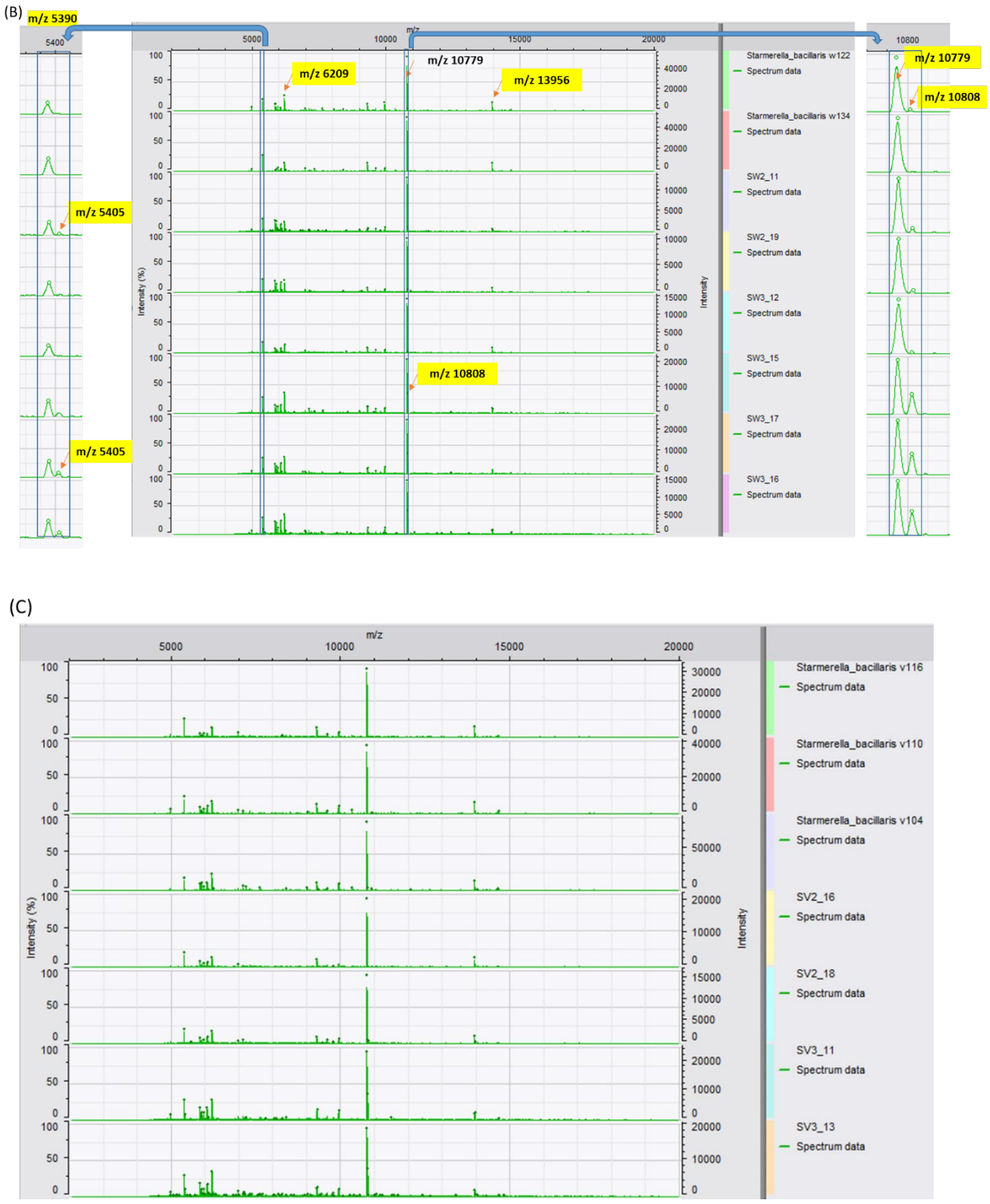


Figure 5.5 (A) Cluster analysis of the *St. bacillaris* isolates; (B) peak changes over fermentation in winery and (C) vineyard.

5.3 Discussion

It is well established that a variety of yeast species are present at the beginning of wine fermentation and that these may confer desirable or undesirable properties to the final products (Swiegers et al. 2005). Close monitoring of the wine's microbiological status during fermentation is desirable to support consistent quality, an aspect arguably more important in organic and biodynamic wine production, due to the limited interventions available.

The use of MALDI-TOF in clinical microbiology is now widespread due to its low cost-per-sample and rapid turnaround time (Dhiman et al. 2011), however for industrial applications the databases required for effective identification are more limited, often requiring bespoke approaches as described here and indeed elsewhere (Pavlovic et al. 2014, Gutiérrez et al. 2017). Critical to its further use in industrial applications is the development of suitable reference databases; however, in this study alone, we added a total of 249 spectra representing 25 species belonging to eight genera to our database in just 9 days (not considering primary isolation and confirmation). 26S rDNA gene sequence analysis was used to support the identification results inferred by cluster analysis of MALDI profiles where reference strains were not available. Our results clearly validate the efficacy of MALDI-TOF profiling as an identification tool, with phylogenetically distinct species clearly distinguished in the cluster analysis (Figure 5.1). Given the challenges described by others in differentiating oenologically-relevant yeast species (Kurtzman and Robnett 1998, Lopandic et al. 2008, Csoma et al. 2010, Kurtzman 2015, Gutiérrez et al. 2017), we consider MALDI-TOF analysis represents important progress in this field. In our study, 13 species belonging to eight genera were identified, comparable generally to the diversity seen in other studies of yeast in wine production (Romano et al. 2003, Fleet 2008).

In this study, the winemaker's fermentations were undertaken both indoors (winery) and outdoors (vineyard), allowing for comparisons between these two systems. Moreover, we saw several examples in both *Saccharomyces* and non-*Saccharomyces* yeasts in which the cluster analysis of MALDI profiles correlated with the isolation source and ferment stage. Proteomics is dynamic and depends on the environmental conditions (Silvestre et al. 2012), and it has been previously reported that isolation sources may influence the protein fingerprints and MALDI dendrogram (Kern et al. 2014, Zhang et al. 2015). Consequently, MALDI-TOF may offer particular advantages for characterizing winemaking yeast since it offers taxonomic accuracy together with resolution of strain differences expressed under differing environmental conditions.

In both systems, non-*Saccharomyces* yeasts *H. uvarum, St. bacillaris*, *C. californica*, *P. kluyveri*, *P. membranifaciens* and *M. pulcherrima* were isolated. The former two species were most abundant, as noted elsewhere (Jolly et al. 2014, Englezos et al. 2017). As far as we are aware, it is the first time *C. californica* has been isolated in a New Zealand vineyard. Farming practices or detection methodology may account for this, as *C. californica* appears absent or present at very low frequencies in conventional vineyards elsewhere (Agarbati et al. 2019). The marked difference in the proportions of these species between the two systems (Table 5.1) is noteworthy given that the grapes used had the same origins. Other species found only in vineyard ferments were *P. terricola, P. kudriavzevii* and *W. anomalus*. These findings were likely the combined results of microbial interactions (*e.g.*, killer phenotype of certain strains) (Clavijo et al. 2010) in the must, as well as the different environmental conditions to which they were exposed. Vineyard ferments suffered from erratic weather conditions whereas winery ferments were kept indoors in a more stable environment. Interestingly, although the mold species *A. pullulans* is frequently associated with the grape phyllosphere (Bozoudi and Tsaltas 2018), we recovered strains of this only from the winery ferments. Additional studies would be needed to determine if this species had, via its propensity to form biofilms (Bozoudi and Tsaltas 2018), become resident. Winery surfaces are considered as a potential reservoir for introduction to early wine fermentation communities, and play a role in shaping the microbiota of wine fermentation, in which the resident microbial consortia can be affected by the combination of facility design, age, ferment tank material and oxygen permeability, sanitation regimens, and processing decisions (Nguyen et al. 2010, Bokulich et al. 2013, del Alamo-Sanza et al. 2015). Nonetheless, since *A. pullulans* can influence wine flavor (Bozoudi and Tsaltas 2018), its presence is noteworthy.

Yeast cells can adapt their physiology to external stimuli in a rapid and robust way; for example, H_2O_2 treatment elicited the change of yeast proteomic response dramatically as early as 30 min from initiation of the oxidative stress (Breker et al. 2013). In our study, *Candida californica* in SV1 and SW1 were clearly differentiated by their MALDI protein profiles, in which the peak m/z 8,211 was present as a singlet in winery samples, while it displayed as a doublet m/z 8,211, 8,241 in vineyard samples, though they were sourced from the same batch of grape juice. Similar subtle changes in MALDI-TOF profiles of other microorganisms have been found to represent key phenotypic, including morphological, differences (Sousa et al. 2013, Flores-Trevino et al. 2019). Thus, such peak variation in our case may indicate the differential phenotypic evolution in *C. californica* during adaptations to environmental conditions, which could relate to their differing colony morphologies (Figure A.11). Indeed, it has been noted that yeast colony morphology can vary in response to environmental factors including medium composition, pH, cultivation time and temperature (de

Becze 1956, Vopalenska et al. 2005). *Pichia kluyveri* isolates also present multiple colony morphotypes, and it could be the reason that their MALDI proteomic fingerprints showed high intraspecific variation (Figure A.10-11), whereby MALDI-TOF MS was suggested as a potential colony morphotyping approach by Sousa et al. (2013). The importance of environmental factors resulting in heritable and detectable changes in yeast phenotype is well recognized, with differing potential underlying mechanisms identified (Halfmann et al. 2012, Holland et al. 2014). All yeast strains were subjected to several environmental stresses over fermentation, such as the nutrient starvation, oxygen shortage, high ethanol concentration, and low pH (Trabalzini et al. 2003). Differences between fermentations undertaken indoors and outdoors may account for the band- and peak differences observed in proteomic profiles of *St. bacillaris* in these conditions (Figure 5.5).

The occasional transformation between silence and reactivation- corresponding genes of certain phenotypes has been observed in *S. bayanus*, *S. cerevisae* and *S. uvarum*, therefore such instability may contribute to their high diversity in the fermenting yeast population (Csoma et al. 2010). Similarly, we observed significant variation in protein fingerprints of *S. cerevisiae* in particular (Figure 5.2), consistent with published genetic analysis (Csoma et al. 2010, Zhang et al. 2010, Zhang et al. 2015). Strain profiles were classified into four types, of which one appeared to be predominant, and observed across the whole fermentation process (Figure 5.2). The other three types were only detected in the last ferment stage. Two of these three profile types seemed sufficiently closely related to represent adaptation of the dominant strain to the changing environment, whereas Type IV appeared sufficiently distinct to potentially represent a novel strain that had been introduced, or recovered, during the later fermentation stages. Of *S. uvarum*, types A and B were close visually in profile patterns and cluster analysis, that may exhibit a transition of their proteomic responses from stage 2 (A) to stage 3 (B) (Figure 5.3).

The evolutionary divergence of industrial yeasts is considered to be shaped by both the industrial application and geographical origin, and they are genetically and phenotypically separated from wild stocks due to human selection and trafficking (Liti et al. 2009, Yarza et al. 2014, Gallone et al. 2016). Since type strains *S. cerevisiae* NCYC 505 and *S. bayanus* NCYC 2578 were originally isolated from beer, their divergence from our indigenous isolates in our MALDI dendrogram further supports MALDI-TOF MS as a powerful tool to discriminate and classify industrial yeast strains.

5.4 Conclusions

To our knowledge, this study is the first to describe the variation among and between yeast species in organic wine production facilities in the Waipara region of New Zealand over the course of

fermentation. In addition, the opportunity to examine the impact of differing (but co-located) production infrastructures on yeast growth dynamics is also, to our knowledge, novel. Our findings indicate that winery infrastructures do appear to have a significant effect in shaping yeast diversity and thus wine production dynamics. We are engaged with further studies to assess these dynamics over a more prolonged term.

MALDI-TOF MS analysis affords excellent taxonomic resolution for yeast identification, including closely related species, with the added advantage of low-cost, ease of operation and short-turnaround time. Development of reference databases of industrial interest is crucial to its further application in practice. Furthermore, as a proteomic tool, MALDI-TOF MS potentially portrays a more active insight into the dynamics of the wine fermentation process. We observed notable correlations of isolation source (*i.e.* between indoor and outdoor production conditions) and fermentation stage with MALDI-based clustering, indicating the importance of environmental conditions on yeast populations in winemaking, even where production systems are co-located. This observation has interesting implications on the microbial aspect of *terroir*, whereby even microconditions may exert subtle influences on product. Further studies are underway to examine the relationship between sensory characteristics, yeast dynamics and production systems in this regard. Nonetheless, the potential of employing MALDI-TOF MS in monitoring wine fermentation to actively support the consistency of high-quality wine products, and potentially for their development too, is advocated for in our study.

Chapter 6

The Influence of Growth Conditions on MALDI-TOF MS Spectra of Winemaking Yeast: Implications for Industry Applications

6.1 Introduction

Wine is the product of the metabolism of yeast species and strains on grape juice, resulting in a unique set, and concentration, of metabolites (Richter et al. 2013). In order to control the fermentation efficiently and ensure the homogeneity of wine products, the use of commercial wine yeast strains has become a common practice in winemaking (Valero et al. 2005, Donalies et al. 2008), usually belonging to *Saccharomyces sensu stricto* species. Wine commercial strains were predominantly isolated from vineyard environments and exploited for different purposes in winemaking due to their advantageous kinetic and metabolic characteristics (Camarasa et al. 2011). Furthermore, the diverse phenotypic variations among commercial strains allow their specific application according to the wine style and/or grape variety (Franco-Duarte et al. 2009, Richter et al. 2013). For example, Carew et al. (2013) demonstrated the use of yeast strain significantly affected both the concentration and composition of Pinot Noir wine tannins. Previous studies have shown that commercial wine yeasts are genetically and phenotypically separated from other industrial yeast strains (*i.e.* beer, bread, and sake), laboratory strains and "wild" yeasts, which also reflects a wide diversity in metabolic strategies to cope with the stressful environment (Palková 2004, Donalies et al. 2008, Schacherer et al. 2009, Camarasa et al. 2011, Gallone et al. 2016, Goncalves et al. 2016, Fay et al. 2019).

Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is an analytical method that can be used to separate, quantify and identify proteins, and has proven to be a rapid and reliable tool in wine yeast identification (Usbeck et al. 2014, Gutiérrez et al. 2017, Zhang et al. 2020). However, the highly dynamic nature of the proteome in living cells presents several interesting challenges and opportunities to the method, since differential protein expression levels of yeast under different growth conditions are regulated accordingly (Kolkman et al. 2005). During the winemaking process, the yeast often encounters high concentrations of sugars, and limited oxygen and nutrients (*e.g.* carbon and nitrogen). Enzymes involved in central carbon metabolism pathways showed a significant change in wild type *S. cerevisiae* under glucose- or ethanol-limited conditions (Kolkman et al. 2005); 51 proteins upregulated in response to glucose

limitation and 51 upregulated in response to ammonia limitation were identified in *S. cerevisiae*, illustrating the impact of growth conditions on the yeast proteome (Kolkman et al. 2006).

The influence of certain culture conditions including (*e.g.* oxygen availability, culture media, growth phase and cell concentration) on MALDI-TOF mass spectra of a few wine spoilage yeasts has been examined (Usbeck et al. 2013) but to our knowledge, such studies have not been undertaken on winemaking yeasts, where MALDI-TOF analyses have been proposed as a rapid and objective approach for assessing the best application of individual yeast strains for different wine styles (Usbeck et al. 2014). Furthermore, studies to date have not included the use of natural or artificial grape juices, that may provide a more nuanced and accurate evaluation of yeast strain application for industry.

We have previously described an optimised protocol for the MALDI-TOF profiling of winemaking yeast in chapter 4 (Zhang et al. 2020). In this chapter, we investigated a range of culture substrates including conventional laboratory media, and natural and artificial grape juices, to determine the best medium for the use of MALDI-TOF MS to predict wine yeast application. Eight *Saccharomyces* strains from international culture collections, commercial winemaking yeast providers, and a locally sourced isolate from a New Zealand winery, were used.

6.2 Results

6.2.1 Growth curve

The growth rate in liquid media of strains examined varied substantially (Figure 6.1). The corresponding cell number of strains *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, *S. pastorianus* NCYC 396, and *S. bayanus* NCYC 2578 determined by a calibration curve (Figure A.12) at $OD_{(600nm)}$ 0.5 was 4.15 x 10^6 cells/mL, 4.01 x 10^6 cells/mL, 6.07 x 10^6 cells/mL, 8.00 x 10^6 cells/mL, respectively, whereas the cell number was around 9.00x 10^6 cells/mL for the other four strains. Comparison of the growth rates enabled strains to be delineated into two groups; commercial wine strains (Lalvin RC 212, Lalvin QA 23 and Lalvin ICV D47) and wild isolate *S. cerevisiae* v128 that reproduced vigorously (Group I); and reference strains sourced from culture collections (Group II). More specifically, compared to group II, group I exhibited a shorter lag phase during which yeast cells become acclimatised to the new environment, and a higher growth rate during the log phase (Figure A.13).

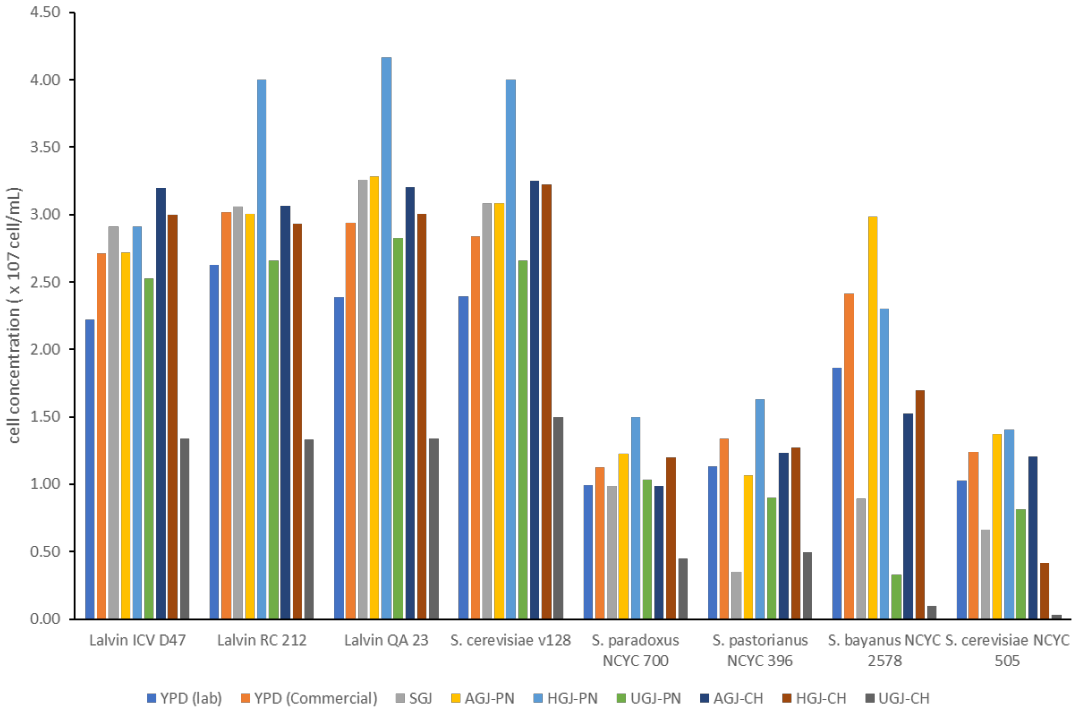


Figure 6.1 Biomass of each culture in different liquid culture media after 24 h incubation under 28°C. The cell concentration of each culture was calculated based on the growth curve and standard curve.

In most of the tested media, strains reached stationary phase at 24 h, however in YPD broth (Difco and lab) and UGJ (PN and CH), group I strains entered stationary phase earlier around 16-18 h. Therefore, 24 h was selected as the test time for MALDI sample preparation to maintain the consistency of the cell physiology.

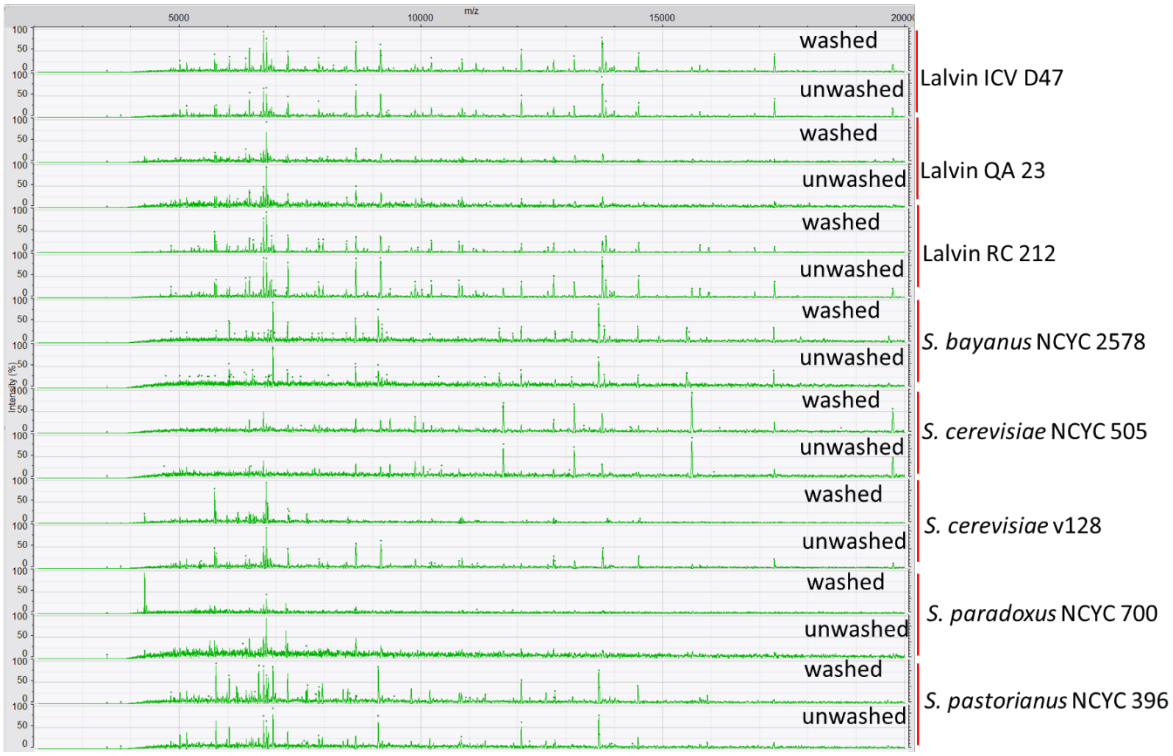
6.2.2 Effect of culture media on MALDI-TOF spectra

The quality of the MALDI-TOF profiles in terms of the background observed and number of clearly discernible informative peaks differed substantively between strains and depending on the medium used to culture them. The reference strains from international culture collections yielded spectra that possessed high background levels, or exhibited very few informative peaks, when cultured in natural or synthetic grape juices. In contrast, the results from strains obtained from a commercial supplier (Lalvin) with established use in winemaking, or an isolate from a New Zealand vineyard (v128) were generally more consistent, although strains cultured in Chardonnay juice did not generally yield suitable profiles, with the exception of *S. cerevisiae* strain Lalvin ICV D47, recommended by the supplier for the production of Chardonnay wine. The growth media that produced the spectra with the lowest background, and overall the most identifiable peaks, were commercially produced YPD broth (except *S. paradoxus,* discussed below), and commercially produced YPD agar. These media were also those that best supported yeast growth (Figure 6.1).

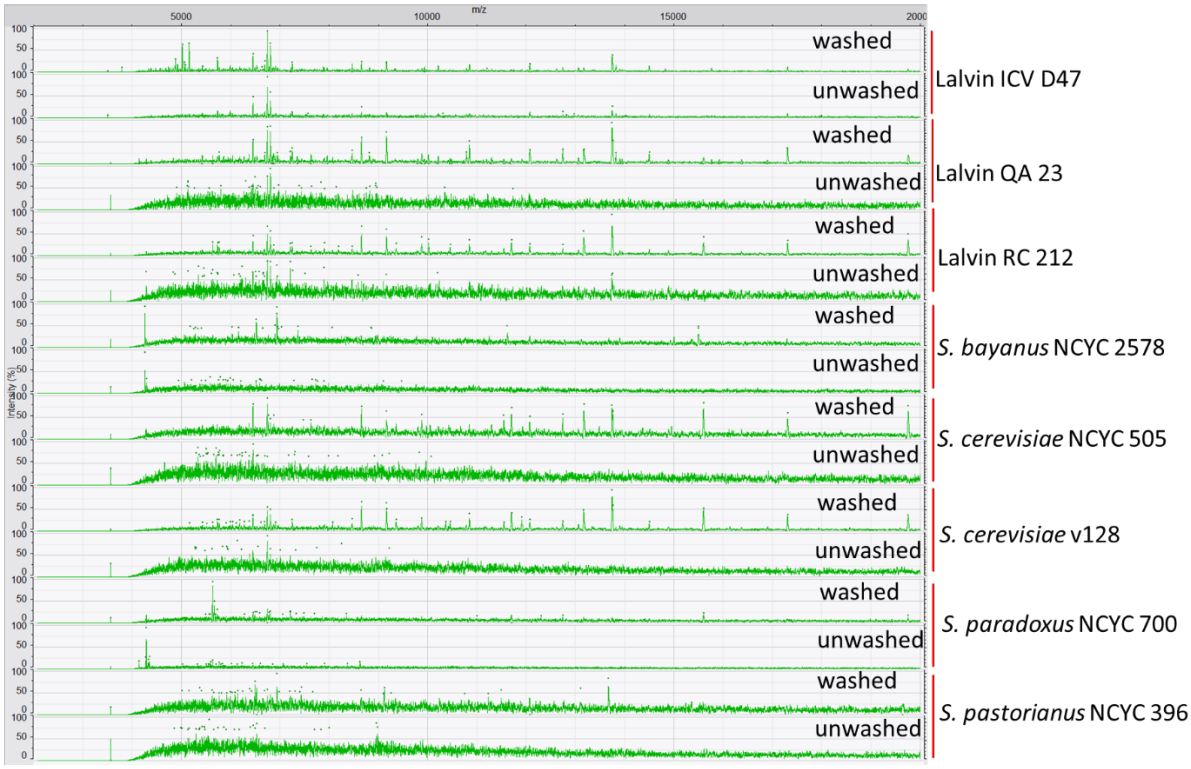
6.2.3 Effect of washing step on MALDI-TOF profile clarity

An additional washing step would increase the time- and labour- cost in practical operation, especially a large number of samples. In this study, inclusion of a wash step improved the spectra profiles cultured in grape juice significantly, but did not have a profound effect on YPD broth-derived profiles. Results from three media (YPD broth, Difco), PN-AGJ and CH-AGJ are presented here as examples (Figure 6.2). In order to detect the possible influence of the native grape proteins on MALDI spectra, the sediments of each treatment of both PN and CH were also subjected to the same MALDI procedures as the yeast strains (Figure A.14). Thus, we observed certain peaks appearing in unwashed samples that could be traced to the corresponding grape juice, *e.g.*, m/z 10,641 detected in unwashed *S. bayanus* NCYC 2578 cells (PN-HGJ and PN-UGJ).

(A) YPD broth (Difco)



(B) PN-AGJ



(C) CH-AGJ

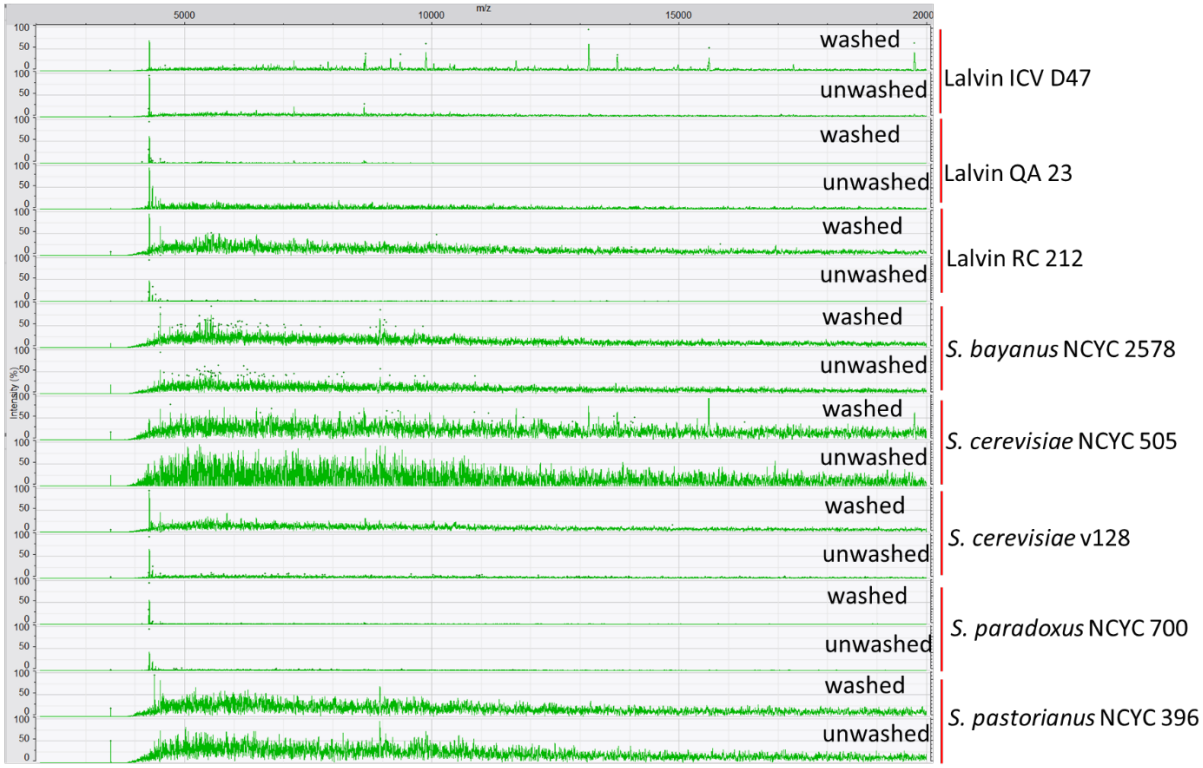


Figure 6.2 Comparison of the wash step impact on strains grown in (A) YPD broth (Difco), (B) PN-AGJ and (C) CH-AGJ.

6.2.4 Influence of culture conditions on cluster analysis

Results of cluster analyses (Figure 6.3) based on each of the growth conditions yielding clearly discernible peaks (*i.e.* YPD agar, YPD broth, and Lab-YPD broth) revealed subtly different results reflecting the differences observed between the corresponding MALDI-TOF spectra. YPD broth (Difco) gave the lowest similarity between strains reflecting the greater diversity of peaks among profiles, but the highest similarity (68.1%) between species *S. bayanus* NCYC 2578 and *S. pastorianus* NCYC 396, whereas the values were 40.8% and 52.4% in YPD agar- and YPD broth (lab)- dendrogram, respectively. *S. paradoxus* NCYC 700 was not separated from the *S. cerevisiae* group in the comparison using YPD agar-cultured strains, but was in comparable analyses from each of the two YPD broths used. The resultant similarity matrices (Figure 6.3) also exhibited a more straightforward visualization that profiles derived from cultures on YPD agar and YPD broth (lab) allowed for better species-level differentiation.

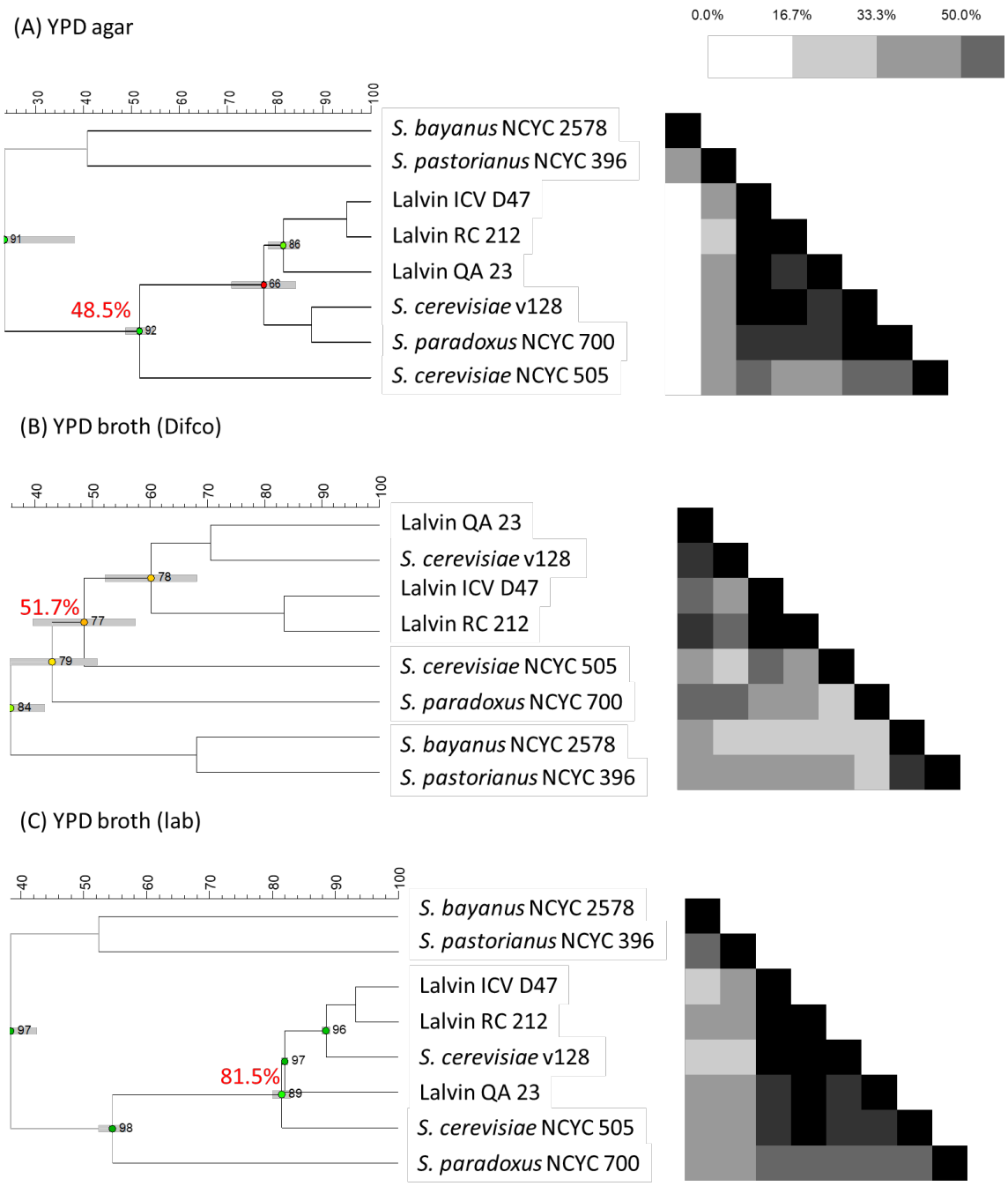


Figure 6.3 Cluster analysis and similarity matrices of eight strains derived from (A) YPD agar (Difco), (B) YPD broth (Difco), and (C) YPD broth (lab) using the Pearson correlation coefficient and UPGMA algorithm. Similarity of the *S. cerevisiae* branch was labelled in red, namely 48.5% in (A) YPD agar - Lalvin ICV D47, Lalvin RC 212, Lalvin QA 23, *S. cerevisiae* v128, *S. cerevisiae* NCYC 505 and *S. paradoxus* NCYC 700, 51.7% in (B) YPD broth (Difco) - Lalvin QA 23, *S. cerevisiae* v128, Lalvin ICV D47, Lalvin RC 212 and *S. cerevisiae* NCYC 505, 81.5% in (C) YPD broth (lab) - Lalvin ICV D47, Lalvin RC 212, *S. cerevisiae* v128, Lalvin QA 23, *S. cerevisiae* NCYC 505.

It is noteworthy that commercial strain Lalvin QA 23 is a hybrid (*S. cerevisiae* x *S. bayanus* var. *uvarum*) (Usbeck et al. 2014), however, its peak variation from the other *S. cerevisiae* strains is clearly displayed in profiles derived from both YPD media. As can be seen from Figure 6.4 (A), the major QA 23-specific peak pair m/z 5,733, 5,746 is differentiated from the single peak at m/z 5,733 in the other *S. cerevisiae* strains. The spectra differences among strains provided by YPD broth (Difco) (Figure 6.3 (B)) were greater than those of YPD agar (Figure 6.3 (A)), as indicated by the similarity of 48.5% and 51.7%, respectively; on the other hand, it is 81.5% where laboratory-synthesised YPD broth was used (Figure 6.3 (C)).

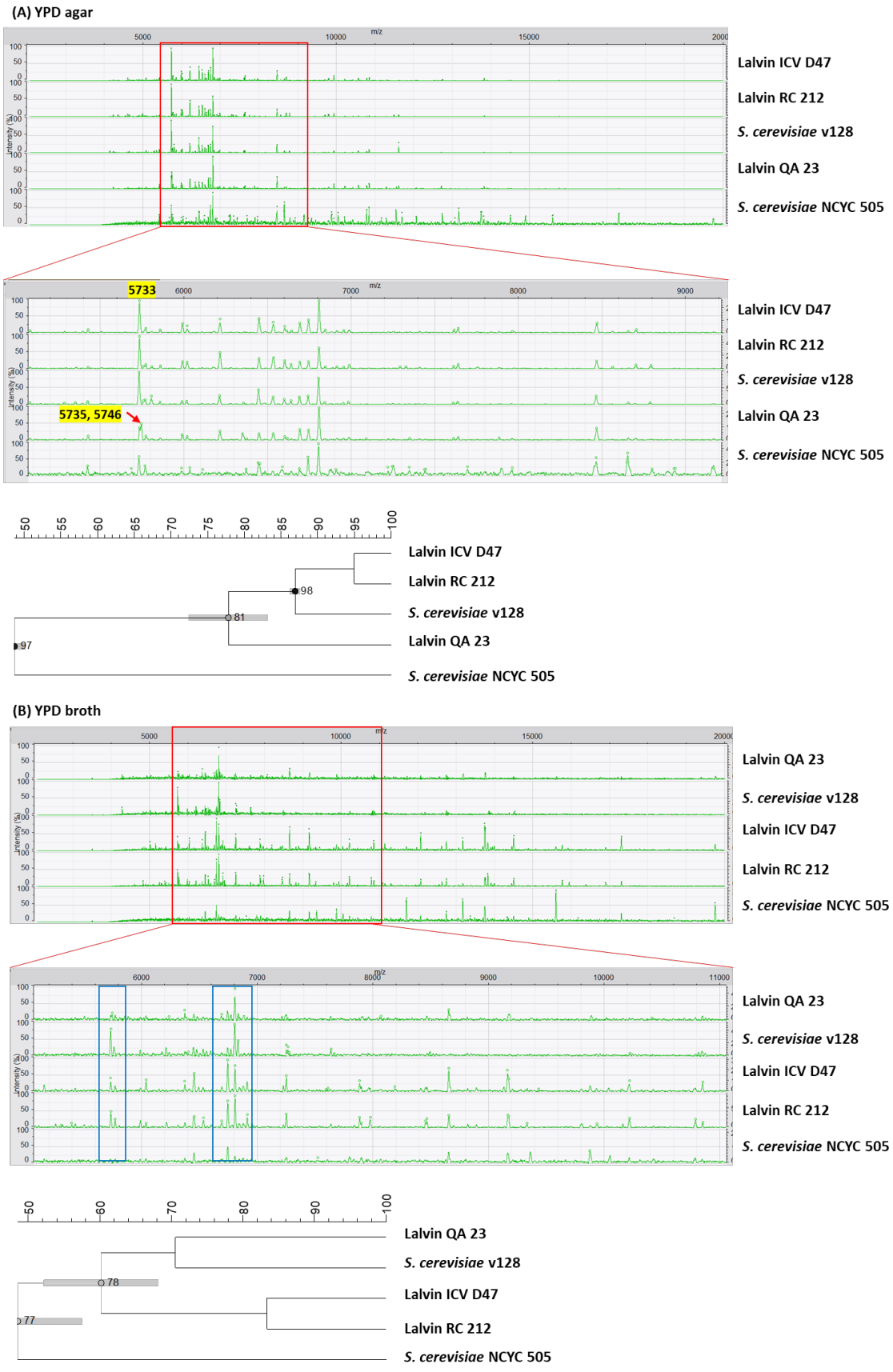


Figure 6.4 MALDI spectra and cluster analysis of five *S. cerevisiae* strains grown (A) on YPD agar and (B) in YPD broth. The number highlighted in yellow indicates the peak m/z 5733 in Lalvin ICV D47, and a doublet m/z 5,733, 5,746 in Lalvin QA 23.

Although most of the PN-derived spectra were similar among *S. cerevisiae* strains, Table 6.1 summarizes the discriminant peaks of each strain from closer visual inspection (Figure 6.5). For example, peak m/z 10,023 only appeared in RC 212, while m/z 10,368 was unique to *S. cerevisiae* v128.

Table 6.1 Discriminant peaks among five *S. cerevisiae* strains of diverse origins and application.

	Discriminant peaks (*m/z*)								**Application**
Lalvin ICV D47	-	-	-	-	-	-	-	-	Chardonnay
Lalvin QA 23	9,878	-	-	11,912	-	13,171	-	19,755	Sauvignon Blanc
Lalvin RC 212	9,878	10,023	-	11,912	11,703	13,171	15,601	19,755	Pinot Noir
S. cerevisiae NCYC 505	9,878	-	-	11,912	11,703	13,171	15,601	19,755	Reference (originally from Brewing)
S. cerevisiae v128	9,878	-	10,368	11,912	11,703	13,171	15,601	19,755	Pinot Noir vineyard isolate (Waipara, New Zealand)

Note: "-" means the absence of peak.

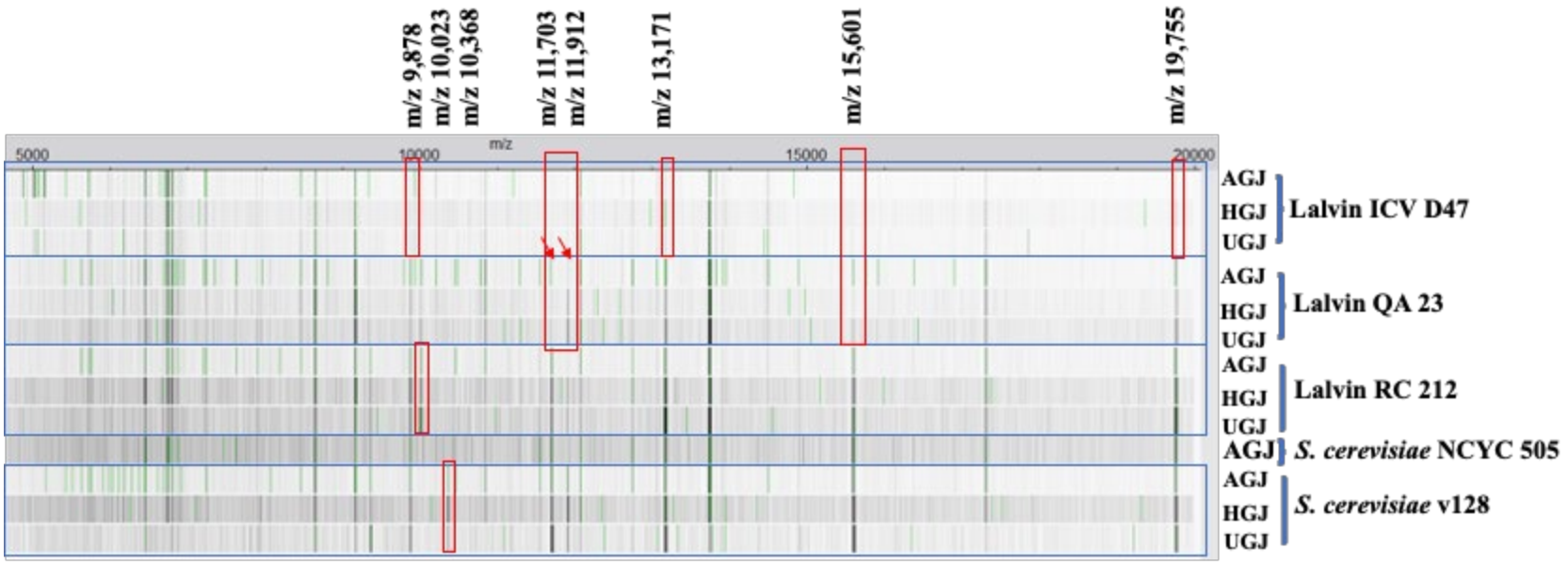


Figure 6.5 Band presentation of MALDI spectra from five *S. cerevisiae* strains grown in Pinot Noir grape juice.

6.3 Discussion

The extensive biological diversity evidenced among *Saccharomyces* species and indeed utilised to produce a wide range of alcoholic beverage styles was displayed in this study by the different growth rates seen among the media used, and also in the MALDI-TOF profiles derived from strains following cultivation.

Comparison of growth curves and the resulted spectra indicated that media that did not support vigorous growth of the strains tested yielded material that gave correspondingly poor quality MALDI-TOF spectra, this fact also has been reported by Wieme et al. (2014) and Luthje et al. (2017). However, despite the variation under different growth media, certain common peak classes remain stable, which might represent "Housekeeping genes" known to be constitutively expressed to maintain cellular function irrespective of the changing metabolic activities (Valentine et al. 2005). Peaks in the MALDI spectra have been reported to be mainly ribosomal- and mitochondrial-related proteins (Ryzhov and Fenselau 2001, Usbeck et al. 2013). It is well known that growth phase (or different physiological state) affected the yielded protein profiles (Vargha et al. 2006, Qian et al. 2008, Wieme et al. 2014), and Usbeck et al. (2013) suggested younger cultures showed better results, accordingly, 24 h was taken as the standard incubation time in this study given the consistent cell physiological state and ease of operation.

Most notably, the type strains used in this study as taxonomic references generally grew poorly in the natural and artificial grape juices used, yet strains recommended for use in winemaking, or isolated from a New Zealand vineyard, were clearly more suited for growth in the grape juice environment. Commercial strains are either natural isolates from vineyard or wineries shown superior properties for winemaking, or have been experimentally selected for specific purpose (Bradbury et al. 2006). Long-term domestication of type strains under optimized lab conditions might repress their some protective and adaptive mechanisms essential for survival in natural environments (Palková 2004). Certainly these strains showed weaker fermentative vigour during the growth in grape juice, and failed to obtain quality protein profiles. Compared to rich medium YPD, grape juice (low pH 2.9-3.8 and high osmolarity sugars of 200-300 g/L)) is highly unfavourable to most microorganisms, but is one to which wine yeasts are well adapted (Richter et al. 2013).

Walker et al. (2002) indicated the variations in spectra produced under different media reflected the differences in cell surface composition. Yeast cell wall is a complex and dynamic structure composed of β-1,3 and β-1,6-glucan, chitin and mannoproteins, the composition and structure vary depending on the growth conditions, *e.g.*, growth media, carbon source, nitrogen, pH, temperature and aeration (Kapteyn et al. 2001, Aguilar-Uscanga and Francois 2003, Orlean 2012). A low-pH environment was shown to induce stronger yeast cell resistance (Kapteyn et al. 2001, Aguilar-

Uscanga and Francois 2003), which may be partially responsible for the failure of quality spectra acquisition in CH (pH 3.0) and SGJ (pH 3.5), thereby impeding the efficiency of intracellular protein extraction. Such a situation caused by cell wall structure transformation may also apply to type strain *S. paradoxus* NCYC 700, whereby its tendency to form "clumps" or "flocs" (Figure A.15) may have contributed to its slow growth rate in liquid media and correspondingly poor quality MALDI-TOF spectra, as the "flocculins" (lectin-like proteins) that protrude from the cell wall lead to the phenomenon of flocculation (Verstrepen et al. 2003).

Preconditioning to the specific environmental conditions of grape juice appears to be significant for individual *Saccharomyces* strains to thrive as well as the yielded protein profiles. The physico-chemical properties of grape juice can be affected by the heat treatment in protein, pH, total acid, viscosity, ion-concentration, and brix (Ozcan et al. 2015), which reflected in the change of brix value and the liquid colour among three treatments. The autoclaved grape juice (AGJ) showed a darker colour especially in terms of the Chardonnay grape juice, which was due to the non-enzymic browning reaction at high temperature occurred between reducing sugars (*e.g.* glucose and fructose) and the amino acids (*e.g.* arginine, glutamine and proline) in grape juice (Bozkurt et al. 1999, Ozcan et al. 2015). Therefore, AGJ provided an absolutely sterilized situation for the yeast strains tested, whereas UGJ was similar to a spontaneous fermentation system where the tested strains would encounter the competitive stressor from the indigenous microbes. Short-term pasteurization would lead to proteins denaturation, except some proteins with a high degree of thermostability, such as the invertases and lipid-transfer proteins (Marangon et al. 2012). The denatured proteins caused by heat treatment may be more susceptible for utilization and thus favour the yeast metabolism, therefore an overall higher quality of protein profile was observed in AGJ and HGJ than in UGJ.

Additionally, the high concentration of background organic matter or ions in grape juice could impair the efficacy of spectra acquisition. A large number of low molecular weight interfering compounds such as salts or polyphenols can affect the signal intensity by increasing the chemical noise in the mass spectrum (Nunes-Miranda et al. 2013). Ion suppression is shown to affect mass spectrometry, during which the presence of ions in the media could block the ionization process, consequently, decreasing the efficiency of the analyte ionized (Annesley 2003, Johanson et al. 2007, Anderson et al. 2012). Alispahic et al. (2010) encountered similar problems with quality spectra acquisition when cultures on mCCD (modified charcoal cefoperazone deoxycholate) agar as the mCCD agar contaminants interfered with ionization process. However, a washing step often improved the clarity of spectra. The components in liquid media act as spectral contaminants that can interfere with quality spectrum acquisition. Grape proteins appear to have a major impact on spectra acquisition. Certain peaks (*e.g.* m/z 7,107, 9,050 and 10,641) observed in grape juice spectra were reported as 7.1 kDa, 9.1 kDa, 10.6 kD in Muscat of Alexandria wine, Chardonnay wine and Sauvignon blanc wine

(Weiss et al. 1998). Proteins with similar sizes can present in different lots of wines but may be in variable amounts (Weiss et al. 1998). Due to the high levels of insoluble tannins in red wines, it is believed that most proteins can be removed by precipitation in the form of tannin-protein complex (Nakanishi and Yokotsuka 1990), it may explain the higher quality spectra were obtained in PN than in CH, as the interference of PN-proteins may lower than that from CH.

Among the winemaking or vineyard-associated strains, differences were evident too. Despite these strains all showing an ideal growth rate in natural and synthetic grape juices, well-defined MALDI-TOF profiles were not obtained from all strains when cultured in these media. Only Lalvin ICV D47 consistently generated analyzable MALDI-TOF profiles from each growth medium, including Chardonnay grape juice, which otherwise yielded poor quality spectra from other strains (Figure A.16). Interestingly, Lalvin ICV D47 is recommended by the distributors for Chardonnay wine production. The MALDI-TOF profile of the strain is unique (Table 6.1); the absence of certain peaks observed in this, and Lalvin QA 23 (the only other strain examined recommended for the production of Sauvignon Blanc and other white wines) supports previous studies whereby the recommended application of winemaking yeast correlated with MALDI-TOF spectra (Usbeck et al. (2014). Furthermore, the use of YPD broth and YPD agar had a significant impact on taxonomic resolution at strain level. We have previously used MALDI-TOF analysis in conjunction with YPD agar cultures of over 20 different yeast species to demonstrate its efficacy as an identification tool (Zhang et al. 2020), even indicating its value to differentiate strains recovered from different fermentation environments (Zhang et al. 2021). Nonetheless, it is perhaps encouraging to note that YPD broth (Difco) generated a higher discriminatory power. Usbeck et al. (2014) claimed successful *S. cerevisiae* strain differentiation by using YPG broth; on the other hand, Gutiérrez et al. (2017) failed whereby the use of yeast malt agar (YMA) composed by 1% glucose, 0.5% proteose peptone n° 3, 0.3% yeast extract, 0.3% malt extract and 2% agar. Laboratory media may vary in the individual ingredients across different labs, thus rigorously consistent quality commercial YPD broth (Difco) is recommended for the consistent profile quality.

6.4 Conclusion

In conclusion, the selection of suitable culture media plays a key role in the discriminatory power of MALDI-TOF MS. Media that did not sustain optimal growth have a profound impact on the MALDI spectra patterns; both the synthetic and natural grape juice proved to be poor matrices for generating suitable MALDI-TOF profiles, although Pinot noir juice was more forgiving. The influence of culture media on final protein profiles may originate from the environment-induced cell physiological state change and the media components (*e.g.* grape proteins and ions in grape juice)

direct interference on the ionization process. Specific to the latter case, a simple centrifugation and wash steps can help improve the efficiency of MALDI-TOF MS.

It is encouraging that laboratory-defined media found to yield the best quality MALDI-TOF spectra in this study has been used previously to infer optimal strain utilization in winemaking and brewing (Usbeck et al. 2014, Lauterbach et al. 2017) making the potential wider use in strain prediction pragmatic. We conclude that a combination of commercially available YPD agar and YPD broth accompanied by a deionized water wash is recommended for in-house MALDI database construction and strain-level differentiation, respectively, as some microbial cells grown in liquid media may not be suited for MALDI analysis.

Chapter 7

Predictive Potential of MALDI-TOF Analyses for Wine and Brewing Yeast

7.1 Introduction

Wine is a complex product resulting from the interactions between yeasts and grape juice components, and each yeast strain within the same species has a specific impact on the final wine composition and sensory profile (Roullier-Gall et al. 2020). Diversity among the commercial strains was highlighted through the unique phenotypic patterns of each strain (Barbosa et al. 2014). The impact of the yeast on wine flavour is largely determined by the array of volatile substances (*e.g.* higher alcohols, acids, esters, carbonyls, and thiols) produced by the metabolism of grape juice components (Howell et al. 2006).

The adaptive divergence of genomics in response to different ecological niches allows the development of specific genetic groups of *S. cerevisiae* in different fermented food (*e.g.* wine, beer, dairy products, and bread) and the natural habitats (Legras et al. 2018). Along with the diverse fermentation environments, genotypes and phenotypes of *S. cerevisiae* are shaped via hybridization, polyploidization, pseudogenization, genome decay, gene duplication, and horizontal gene transfer to specifically adapt (Sicard and Legras 2011, Gibbons and Rinker 2015). Commercial wine yeast strains are closely related as demonstrated genetically by the microarray karyotyping analysis (Dunn et al. 2005), the differences in the fermentation and organoleptic properties of each strain may arise from a small number of genetic changes. Most quantitative trait alleles exert considerable phenotypic variations among *S. cerevisiae* strains and alter conserved amino acid positions within protein coding sequencing (Fay 2013).

As a novel proteomic approach, Matrix Assisted Laser Desorption/Ionization–Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has been proved to be a powerful tool in wine yeast identification at species (Usbeck et al. 2013, Gutiérrez et al. 2017, Zhang et al. 2020) and even strain level (Moothoo-Padayachie et al. 2013, Usbeck et al. 2014). Furthermore, Usbeck et al. (2014) demonstrated the role MALDI-TOF MS in revealing the relationship between wine yeast strains and their application potential, as well as comparable studies of brewing strains (Lauterbach et al. 2017). The underlying mechanism is the link between proteome and metabolism, Lafaye et al. (2005) showed that proteome and metabolic data could be correlated either positively or negatively depending on the growth conditions. Nonetheless, studies are few at this point and none to our knowledge have combined investigations on both wine and beermaking yeasts.

Machine learning is widely used to analyse complex data sets for prediction purposes (De Bruyne et al. 2011, Caglar et al. 2018). Principal component analysis (PCA), Multidimensional scaling (MDS) and Uniform Manifold Approximation and Projection (UMAP) are three dimensionality reduction techniques (DRTs) for data visualization of Machine Learning based methods (Mazher 2020). PCA is a parametric linear projection by capturing maximum variances in dataset but unable to capture the non-linear structure. MDS is the first non-parametric DRT that preserves topology and distances, it is able to capture non-linear structure but with limited capability (Mazher 2020). UMAP is a new non-parametric approach put forward by McInnes et al. (2018) builds on strong mathematical foundations, which is very efficient in handling very large datasets.

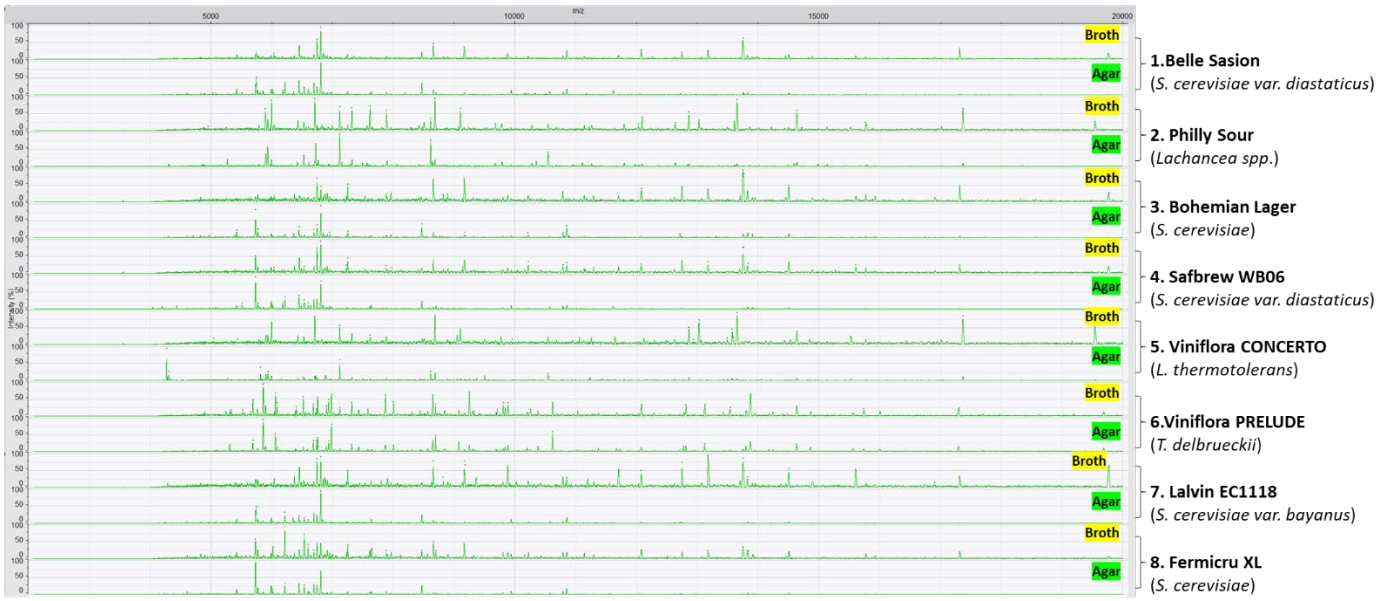
In this chapter, firstly, the culture medium (YPD agar and broth) and mass range (m/z 500-4,000 and m/z 2,000-20,000) were evaluated for the best fit based on our previous work (Zhang et al. 2020). Thereafter, the three algorithms listed above, in addition to a classical hierarchical clustering approach, were adopted to investigate the potential of MALDI profiles in industrial yeast strains differentiation (commercial wine and brewing strains) and the potential application prediction. Furthermore, the manufacturer's recommended application for each strain was incorporated to evaluate its potential in predicting strain utility for winemaking/beermaking.

7.2 Results

7.2.1 MALDI-TOF profiles of strains cultured on YPD Broth and YPD Agar

Good-quality MALDI profiles from each of the strains examined were obtained from cultures on each of the media used. Representative MALDI profiles of eight wine and brewing yeast strains are presented in Figure 7.1. Compared to strains grown on YPD agar, strains grown in YPD broth generated more peaks in a wider mass range, but the overall peak intensity was greatly decreased. Despite the visible differences of produced MALDI profiles, a set of common peaks with varying peak intensity (Low mass: m/z 712, 757, 767, 770, 891, 1100; High mass: m/z 5,735, 5,773, 6,535, 6,746, 6,809, 7,254, 7,887, 8,469, 8,658, 10,219, 10,792, 10,854, 12,750, 13,750, 13,829, 14,506) were observed in samples from both growth media.

(A) High Mass



(B) Low Mass

Figure 7.1 MALDI spectra of (A) high mass and (B) low mass of eight representative commercial strains cultured under YPD broth and YPD agar; 1-4: Brewing strains, 5-8: Wine strains.

7.2.2 Strain classification using cluster analysis and machine learning approaches

Although strain profiles produced from broth cultures contained more peaks, cluster (Figure A.17) and machine learning-based analyses (Figure A.18) tended to correlate poorly with extant information concerning the utility of individual strains. These results are not considered further.

Cluster analysis of all the *S. cerevisiae* strains (winemaking and brewing) exhibited different grouping based on their high-, low- and combined-mass spectra profiles (Figure A.19). With a thorough visual

examination on the spectra patterns, 95% and 85% were indicated as the threshold values in high mass and low mass dendrograms, respectively, resulting in 17 and 20 subclusters. Likewise, 18 subclusters were recognized in the high-low combined dendrogram when 85% was set as the threshold value. Compared to high mass clustering, the industrial strains differentiation was better illustrated by low mass profiles where all the brewing strains were clustered together (group 12-20). In either the high or low mass dendrogram, strains of Velluto Evolution, Fermi champ, Renaissance Vivace, Belgian Wit, Belle Saison, Verdant IPA, NWS Ale, LalBrew Köln and BRY97_American were affiliated. Three Lager strains of Californian Lager, Bohemian Lager and Saflager 23 clustered together in the low mass dendrogram analysis, while the former two strains were mixed with wine strains (Group 2) in the high mass dendrogram. Strains recommended for Champagne production (PDM) fell into three subclusters in both dendrograms, containing four different strains of *S. cerevisiae*, *S. cerevisiae* var. *cerevisiae*, *S. cerevisiae* x *S. cariocanus*, and *S. cerevisiae* var. *bayanus*.

Representation of inter-strain relationships among all strains examined using each of the multidimensional scaling techniques (MDS, PCA, and UMAP) was generally more nuanced. The PCA plot gave the poorest degree of association between strain utility and even species identity, with the most obvious outliers to be the major group represented by a local vineyard isolate of *S. cerevisiae*, and the type strain of *S. paradoxus* NCYC 700 (Figure 7.2 (D)). The UMAP analysis distributed most of the *S. cerevisiae* strains recommended for winemaking among five groups, although some of these contained strains recommended for beer and Champagne production (PDM) as well (Figure A.20 (A)). The MDS plot displayed a more consistent grouping of strains with better alignment of their recommended use and taxonomic relationship. Brewing-related strains (*S. cerevisiae* NCYC 505, *S. bayanus* NCYC 2578, and *S. pastorianus* NCYC 396) were aligned with the commercial brewing group (red dots), whereas *S. cerevisiae* v128 (indigenous yeast isolate) appeared close to, but distinct from, wine and PDM group strains, and quite close to the *S. paradoxus* type strain (Figure 7.2 (A)). Strains recommended for Champagne production (PDM) were somewhat at an interface between the wine- and beer producers.

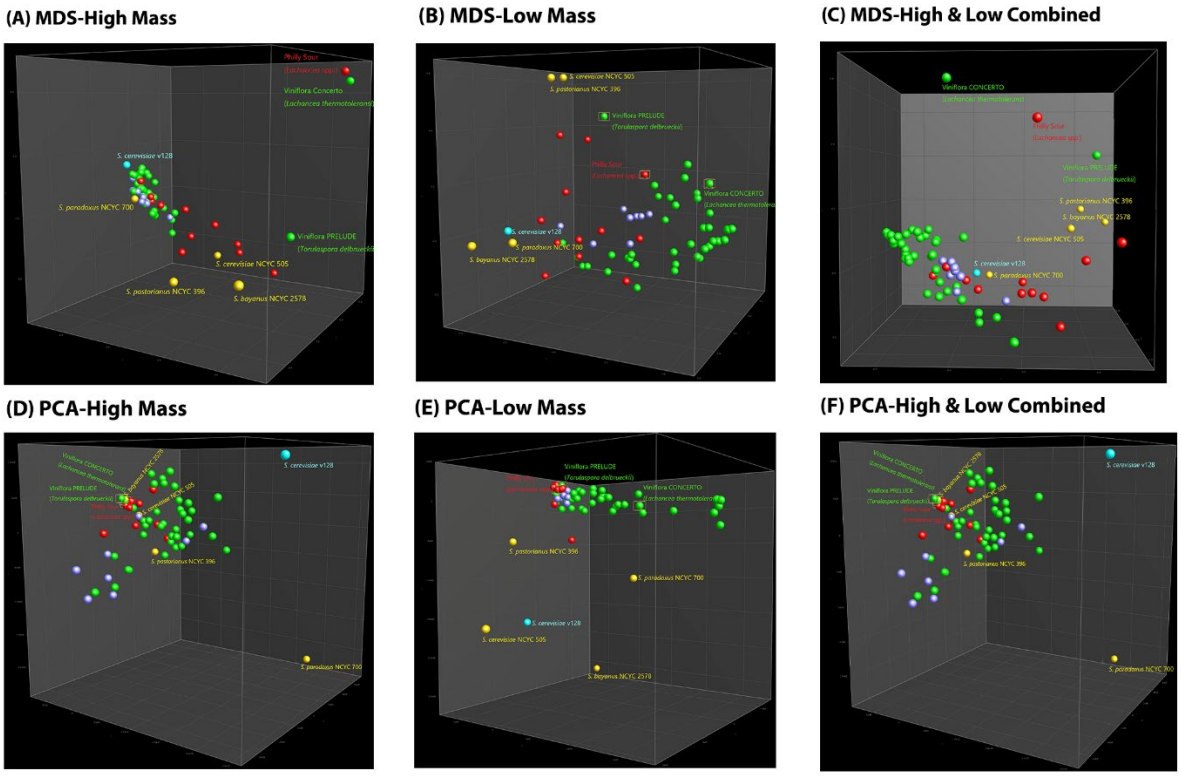


Figure 7.2 MDS analysis of (A) high mass, (B) low mass and (C) high & low combined data, PCA analysis of (D) high mass and (E) low mass and (F) high & low combined data of 62 yeast strains-45 wine strains (green/purple), 12 brewing strains (red), *S. cerevisiae* v128 (blue), *S. cerevisiae* NCYC 505 (yellow), *S. paradoxus* NCYC 700(yellow), *S. pastorianus* NCYC 396 (yellow), *S. bayanus* NCYC 2578 (yellow).

7.2.3 Separate analyses were undertaken on *S. cerevisiae* strains for which recommendations were extant for particular wine styles.

The 45 *Saccharomyces* wine yeast strains we selected cover a wide range of applications, which can be roughly divided as 9 categories, namely, for the production of white wine, red wine, red and white wine, white/rose/red wine, rose wine, white and rose wine, white/red/fruit/cider, white/rose/red/sparkling wine, and one fructophile yeast Fermicru Champ used for tackling stuck fermentation. MDS and PCA did not show appreciable groupings based on their purposes in winemaking for different wine styles (Figure A.21). However, UMAP distinguished five groups containing strains with some agreement where winemaking style recommendations were taken into account (Figure 7.3 and Figure A.22). Group 1 was dominated by strains recommended for red wine production. Group 2 contained the majority of strains used to produce PDM and was classified as *S. cerevisiae* var. *bayanus*. Compared to the other three groups of strains, these two groups seem to have a stronger tolerance to low fermentation temperature and high alcohol content according to the manufacturing information, and their overall peak intensity and peak numbers were relatively low (Figure A.23). Groups 3 and 4 are also well-populated with strains for red winemaking, and rosé too, in the case of Group 3. Group 5 contains mainly white wine yeast strains, mostly recommended for producing Sauvignon Blanc and Chardonnay wines.

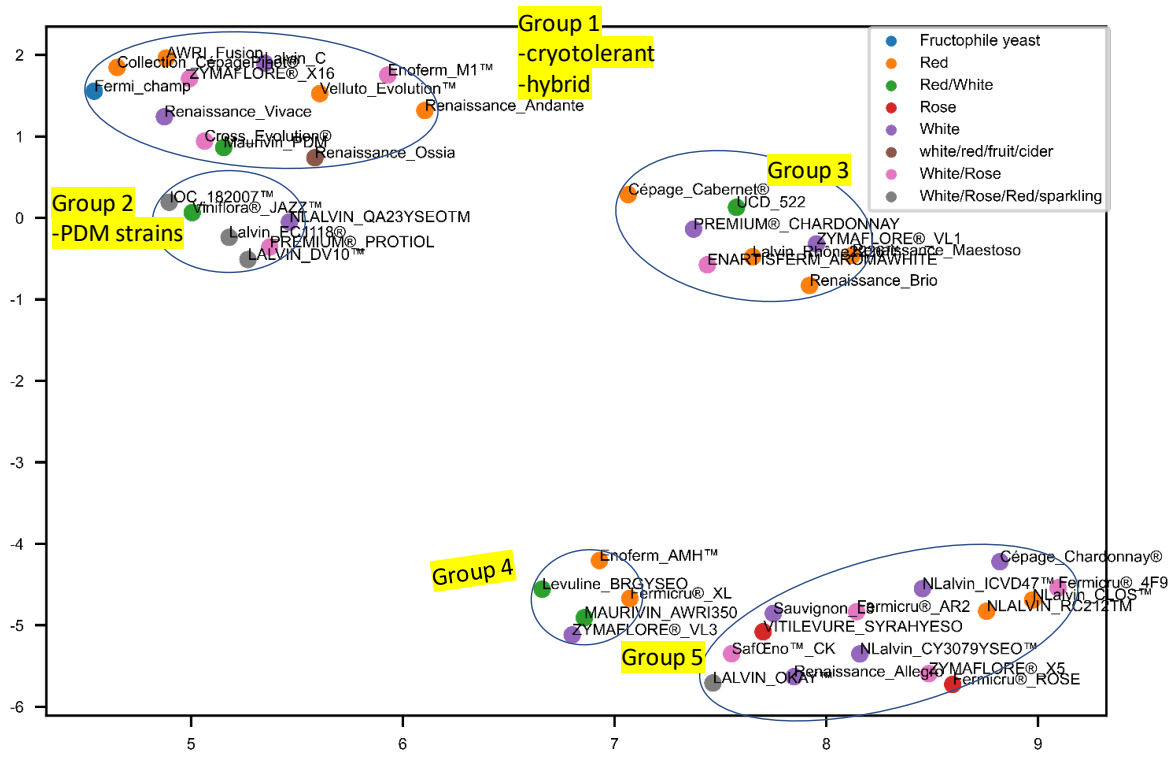


Figure 7.3 UMAP analysis of high mass profiles of 45 commercial wine *Saccharomyces* strains.

Although only 12 brewing strains were examined, strains belonging to wheat, lager and ale were grouped separately, in particular, when the high mass was analysed (Figure 7.4 and Figure A.24). The outlier ale yeast Belle Saison and wheat yeast Safbrew_WB06 were placed closer as their identity as *S. cerevisiae* var. *diastaticus*. The single strain representing the non-*Saccharomyces* species (*Lachanchea* spp.) on the left bottom is suggested to produce a sour beer.

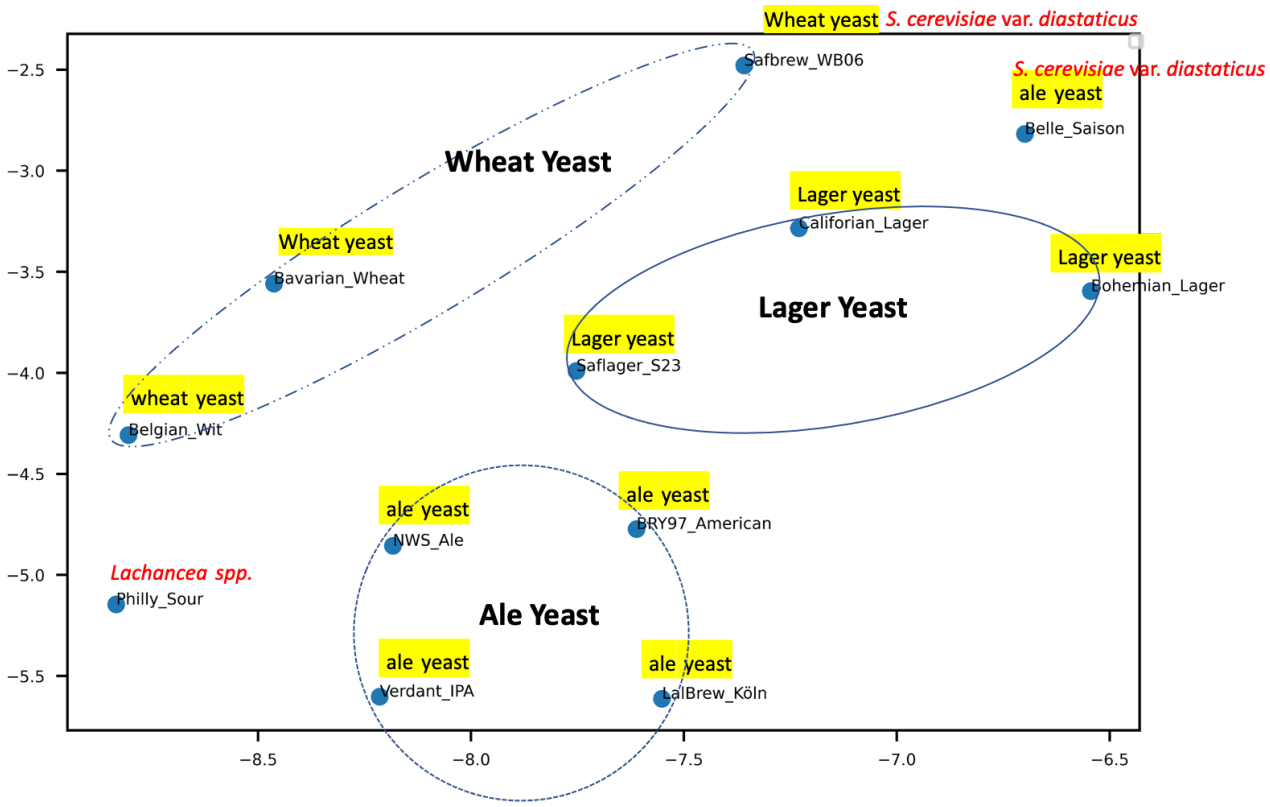


Figure 7.4 UMAP analysis of high mass profiles of 12 commercial brewing strains.

7.3 Discussion

The interaction between yeast strain and grape variety is integral to the flavour profile of the wine. During fermentation, the performance of each yeast strain is affected by the grape must composition, as well as the fermentation conditions. Therefore, the strain may not perform as expected if the growth condition (*e.g.* matrice and temperature) is not compatible with the expression of desired characters (Bisson 2017). Some strains can produce metabolites that enhance mouthfeel (*e.g.* Lalvin ICV D47 and Lalvin CLOS), modify varietal aroma through enzymatical and chemical cleavage of aroma precursors (*e.g.* Lalvin QA 23 with high β-glucosidase activity), and improve the wine stability by increasing yeast mannoproteins (Bisson 2017). Therefore, it is important to choose an appropriate yeast strain for making wine from a particular grape variety. We further examined the prospects of identifying strain utility for fermentation processes using proteome characterization by MALDI-TOF MS.

Based on optimized parameters described previously (Zhang et al. 2020, Zhang et al. 2021), YPD agar and YPD broth were selected as the culture media in this work. Although differences were observed among MALDI profiles, a set of core peaks remained constant, which was consistent with the reports from Reich et al. (2013), Usbeck et al. (2013) and Moothoo-Padayachie et al. (2013) who also stated

that the variations did not compromise the accurate identification on species/strain level. The common peaks are likely to be the ribosomal or housekeeping proteins, whose expression is vital to the basic cellular function irrespective of the growth conditions. Approximately half of the peaks in the MALDI spectra could be assigned to such highly abundant ribosomal proteins, with some peaks matched to post-translationally modified ribosomal proteins (Ryzhov and Fenselau 2001).

Wine yeast stains are genomically and phenotypically distinct from other industrial yeast strains (beer, bread, and sake), as well as laboratory strains, pathogenic strains, and 'wild' yeast strains (Richter et al. 2013). Dunn et al. (2012) pointed out that *NFT1*, *FLO1*, *AAD6*, and *AGP3* genes present in most wine yeast strains but absent in most non-wine yeast strains, are important marker genes to differentiate yeast strains based on their application. Likewise, MALDI profiles successfully differentiated the wine and brewing yeast strains tested in this work. The domestication of diverse industrial *S. cerevisiae* populations (*e.g.* wine, beer, and bread) has been achieved through long-term evolution under selective pressures of various sources, like ancient customs, human migration, and industrial practice, encouraging the development of customized genomes for better adaption in new ecological niches (Legras et al. 2007, Monerawela and Bond 2017, Legras et al. 2018). In addition, species *S. paradoxus*, *S. bayanus*, and *S. pastorianus* are also of industrial importance in food fermentation, as well as their interspecific/intraspecific hybrids (Krogerus et al. 2018). *S. paradoxus* is commonly found on the exudates and bark of deciduous trees (Fay and Benavides 2005). In wild environments, *S. paradoxus* rarely cross fertilizes with *S. cerevisiae*, but conditions in the intestine of some insects favour their hybridization, potentially creating an adaptive environment (Stefanini et al. 2016). Lager beer yeast *S. pastorianus*, especially amenable to cooler fermentation temperature, is a naturally occurring interspecies hybrid of *S. cerevisiae* and *S. eubayanus* (Monerawela and Bond 2017). Type strain *S. bayanus* NCYC 2578 is a hybrid between *S. eubayanus* and *S. uvarum* (Pérez-Través et al. 2014). Their genetic structure is reflected in our MALDI-TOF analysis, whereby *S. pastorianus* NCYC 396 and *S. cerevisiae* NCYC 505 are closer than NCYC 396 and NCYC 2578 in both high- (44.6% vs 40.8%) and low- (74.1% vs 9.8%) mass spectra. Moreover, their proximity to the brewing group of strains not only exhibited the capacity of this methodology as a powerful identification tool, but also showed the potential of MALDI-TOF MS as a predictive phenotypic tool.

Data interpretation is greatly affected by the algorithm used (Usbeck et al. 2013). Dimensionality reduction techniques (DRTs) can provide an in-depth insight into subgrouping with an intuitive data interpretation. In this study, MDS is calculated based on the similarity matrix based on the Pearson Coefficient, then each data point was assigned using a nonlinear least squares fit, minimizing the distances between the data points (De Bruyne et al. 2011). MDS appears to be a valuable alternative to the traditional clustering methods. In our study, PCA was the least informative of the DRT methods applied, yielding the poorest correlation of strain grouping with industry recommendation,

although it is one of the oldest and best-known DRTs. However, with the help of UMAP, 45 *Saccharomyces* commercial strains were classified into 5 groups using the high mass profiles, where MDS and PCA failed. It could be due to the fact that UMAP allows a more accurate representation of local trends, while PCA is better at the visualization of global data structure (Mazher 2020).

Low mass profiles allow for a rough classification of the industrial strains under MDS analysis (Figure 7.2 (B)), but its combination did not significantly enhance the differential capacity of high mass profiles (m/z 2,000-20,000). PCA and UMAP could not extract meaningful information from the limited peak classes (7 peak classes) as well. Interestingly, the data comparison between the UPGMA-based high- and low- dendrogram substantiated the potential of low mass data as a powerful biotyping tool. The grouping of certain strains in two dendrograms was observed to be consistent. Velluto Evolution, the only hybrid of *S. cerevisiae*/*uvarum*, was in a single branch in both dendrograms. A similar case applies to Fermi champ, a special strain for tackling stuck fermentation, which is claimed to be *S. cerevisiae* (ex *bayanus*) but separated from the other strains of *S. cerevisiae* (ex *bayanus*). It is reasonable to infer that the MALDI profile clustering is an interaction between the genetic and phenotypic traits of individual strains. Overall, low mass profiles allowed a more detailed strain classification but also affected by the phenotypes. In accordance with our previous inference (chapter 4), the low mass profiles did contribute to the added benefits of amplifying the intraspecific features (Zhang et al. 2020).

When looking at the UMAP subgroups, group 1 was dominated by the hybrid strains, which usually combines and exhibits superior phenotypic qualities over parent strains. Yeasts belonging to species of *S. bayanus* (*S. uvarum* x *S. eubayanus*)/*S. uvarum* are usually related to the ability to ferment at lower temperatures and greater production of aroma-active higher alcohols (Silhavy-Richter et al. 2020). For example, the natural intraspecific hybrid Cross Evolution (*S. cerevisiae* var. *cerevisiae*) is ideal for white and rosé wines with high aromatic intensity (including ester production) and low fermentation temperature, and the interspecific hybrid Velluto Evolution (*S. cerevisiae* x *S. uvarum*) is characterized by high production of glycerol, phenyl ethanol generation and good tolerance to low fermentation temperature (*e.g.* at 12 ˚C). Group 2 was represented by the PDM strains with two non-PDM strains of Premium Protiol and Viniflora Jazz. The collection of PDM strains is a special group from wine yeasts mainly described as *S. cerevisiae* var. *bayanus* (Eglinton et al. 2005), which is considered to be an intermediate group between non-wine and wine strains (Dunn et al. 2012). A related observation using the MDS and UMAP algorithms is that the PDM group is distributed at the interface between wine and brewing strains.

S. cerevisiae var. *bayanus* is a variety of *S. cerevisiae* that was reduced from its former species status (*S. bayanus*), as it could only be differentiated from *S. cerevisiae* by the fermentation of galactose

(Eglinton et al. 2005). The almost identical genotypes of the majority of the PDM group suggested that they may have arisen from a single progenitor strain, or a highly interrelated progenitor population (Borneman et al. 2016). Coi et al. (2017) inferred that the PDM group (Champagne related strains) may result from the cross between flor and wines gene pool, which benefits from the ability of flor strains under poor nutritional conditions and ethanol stress during the second fermentation of the "*Prise de mousse*" step that imposes a second anaerobic growth. In this sense, it explains its location as a neighbour beside the hybrid group 1, having an overall stronger tolerance to low fermentation temperature and high alcohol content, as well as the fructophile strain Fermicru Champ for tackling stuck fermentation. Zymaflore VL3 in group 4, Zymaflore X5, and Fermicru 4F9 in group 5 are representative "thiol-releasing" wine yeasts suitable for the full aroma potential development of Sauvignon Blanc wine (Hart et al. 2016). Similarly, Zymaflore X5 and Fermicru 4F9 were also shown to be a closer relationship in the study of Hart et al. (2016).

As stated by the manufacturer, Premium Protiol is a strain of *S. cerevisiae*, but Silhavy-Richter et al. (2020) inferred it could be an unidentified interspecific hybrid of *S. cerevisiae* and *S. bayanus*, and strain QA 23 could be a derivative of EC 1118. Microsatellite analysis is not affected by physiological parameters, whereby the two PDM strains QA 23 and EC118 as well as the non-PDM strain Premium Protiol were clustered together as our MALDI result indicated (Silhavy-Richter et al. 2020). In addition to the natural hybridization between *Saccharomyces* strains, gene transfer between Saccharomyces and non-Saccharomyces species was observed in strain EC1118, the major wine contaminant *Zygosaccharomyces bailii* was identified as one donor species (Novo et al. 2009). Additionally, aroma compounds produced were shown to be temperature dependent and vary between pure strain and hybrids; the best aroma producers at 28 ˚C were *S. cerevisiae* strains, whereas *S. uvarum* and some hybrids excelled at 12 ˚C (Gamero et al. 2013). It may corroborate our observation that an overall lower peak numbers and intensity (low protein expression) of Group 1 and 2 strains (most of the hybrids) under YPD agar (28 ˚C) was seen compared to the other three groups.

As discussed above, specific MALDI profiles obtained from yeasts grown on the YPD agar at 28 °C cannot reflect the real-time protein expression of yeast strains under winemaking conditions. Unlike the complex composition in grape must, YPD agar is a defined medium comprising four components (yeast extract, sugar, peptone, and agar). A previous study indicates different metabolites detected by MALDI-TOF analysis when winemaking yeast is cultured in these different conditions (Zhang et al. 2021). Nonetheless, the use of defined media for MALDI-TOF characterization of winemaking yeast is still recommended, based on the clarity of the spectra obtained and general support of yeast growth in comparable conditions (Zhang et al. 2021). The release of aroma compounds is strongly linked to the presence of aroma precursors in fermenting media (Gamero et al. 2011). The wine aromatic profiles can be modulated by employing different yeast species/strains and fermentation

temperature (Gamero et al. 2011, Gamero et al. 2013, Liang et al. 2013). For example, according to the manufacturer instructions, strain EnartisFerm Aroma White is recommended for the thiolic varieties such as Sauvignon Blanc and Pinot Blanc with more citrus and mineral notes produced at 14-16°C and more aromas of tropical white fruit produced at 17-20°C. Enoferm AMH is a colour-friendly yeast and particularly suited for Pinot Noir and Zinfandel partially due to its low-levels of enzymes production responsible for colour loss, and its long lag phase plus low-medium fermentation rate also allows the expression of indigenous microflora. In face of fluctuating environments, limitations in gene expression play a role in phenotypic diversity at the expense of growth rates (Kim et al. 2020). The early study of Batistote et al. (2006) suggested that the sugar types and concentration, the nitrogen source complexity, and the yeast genetic background collectively influenced the optimal industrial yeast fermentation performance. Moreover, the biotechnological application of yeast strains, sometimes, is often contradictory. According to the instruction, UCD522 (Group 3, red wine yeast group) is recommended for white and red wines, and more popular for red wines. However, Carrau et al. (2008) suggests it is more suitable for fermentation of neutral varieties. The data presented in this study corroborates the study of Usbeck et al. (2014) in indicating a role for rapid and cost-effective MALDI-TOF profiling to predict the potential of individual yeast strain for production of specific or distinct wine varietals. However, to better correlate the relationship between the MALDI data and the oenological traits of wine yeast strains, a more complete and objective analysis of metabolites produced is required.

7.4 Conclusion

In conclusion, MALDI profiles generated under YPD agar have a better performance for the purpose of industrial strains differentiation than YPD broth. Neither MDS nor PCA analysis could group wine strains according to their recommended application in winemaking. However, UMAP provided the predictive potential in clustering strains of similar functionality and/or organoleptic attribute. In summary, further studies and subsequent algorithm exploration and data mining are warranted to fully evaluate the relationship of the MALDI profile to practical application in wine production. MALDI-TOF MS is worth continuing investigation as a powerful tool for yeast strain application prediction, to simplify and expedite the selection of relevant indigenous wine yeasts for the development of new and interesting wine styles from an entirely natural base.

Chapter 8

Conclusion and Future Work

8.1 Conclusion

In this study, the potential of MALDI-TOF MS for application in the New Zealand wine industry is realised by, (1) developing an improved method for MALDI-TOF analysis; (2) application of the method to elucidate the extensive diversity of yeast species in wild ferments in an organic vineyard in the Waipara area of Canterbury; (3) optimising growth conditions for use of MALDI analysis to predict yeast strain functionality (*i.e.* which wine style may be best produced by a given strain); and (4) the proof of concept study investigation of MALDI-TOF MS in commercial yeast strain application prediction which is inspired by Usbeck et al. (2014).

To the best of our knowledge, this is the first study in New Zealand to utilize MALDI-TOF MS in the wine industry. Although MALDI-TOF mass spectrometric analysis has been applied to the characterisation of yeast species important in winemaking, relatively few taxa have so far been examined, and the value of low mass peaks for identification has not, to our knowledge, been previously determined. We described a modified (pre-mixing) procedure for extraction of low (m/z 500-4,000) - and high (m/z 2,000-20,000) mass range moieties detected by MALDI-TOF and compare it with a previously described, proposed standard method based on a dried-droplet approach (Gutiérrez et al. 2017). Thirty-three strains representing 21 yeast species were examined. Specific to the indigenous isolates and type strains, our modified protocol with premixing method (PM) is more suitable for the MALDI-TOF analysis and the identification purpose. We found our modified method consistently yielded more discriminatory peaks and a broader mass range detection (from m/z 4,000 to 20,000) than the proposed standard method (from m/z 4,000 to 12,000) for the species examined. Cluster analyses of MALDI-TOF profiles also indicated better separation between species when the pre-mixing method was used, especially where high mass features were used. For low mass features, due to the narrow mass range and low peak number produced, the profiles are of low inter-specific variation and intra-specific similarity, which failed to achieve species identification (Chapter 4), but appeared to be a superior tool for strain typing (Chapter 7).

Regarding strain-level resolution, Gutiérrez et al. (2017) concluded that *S. cerevisiae* strain differentiation by MALDI-TOF analysis was not sensitive, since these authors observed only three different mass spectra profiles among 109 different strains. With the improved PM methods described in this thesis, 17 (high mass) and 20 (low mass) individual mass spectra profiles were recognized from the 56 commercial *Saccharomyces* strains (Chapter 7). Data analysis was based on

the genetic information provided by the manufacturers, although some details have been doubted by some authors (Silhavy-Richter et al. 2020). For example, these authors inferred that Premium Protiol is an interspecific hybrid of *S. cerevisiae* and *S. bayanus* instead of *S. cerevisiae*, which is consistent with our low mass clustering where Premium Protiol was grouped with other *S. cerevisiae* var. *bayanus* or *S. cerevisiae* (ex *bayanus*). Also, an earlier research reported the widespread occurrence of mistakes or fraudulent practices by yeast producers (Fernández-Espinar et al. 2001), where only 30 individual strains have been identified among tested 45 commercial *Saccharomyces* wine yeast strains by different companies, as well as the case of re-identification of *S. uvarum* and *S. bayanus* to *S. cerevisiae*. From this point of view, it is necessary to investigate the genotypes of our tested commercial strains in follow-up research, providing an integrative genotypic and proteomic picture. Even with small to moderate variations in genome structures (*e.g.* gene copy number) between different wine yeast strains and within different isolates of a given strain, it is enough to result in the unique identification of strains, and possibly the fermentative and organoleptic properties (Dunn et al. 2005). In view of the potential biotyping ability of low mass spectra on commercial strains, it is encouraging to note the earlier work of Moothoo-Padayachie et al. (2013), who alluded the enormous potential of MALDI-TOF MS to be used as a biotyping tool for the *S. cerevisiae* strains discrimination. Usbeck et al. (2014) also came up with the possibility of MALDI-TOF MS to replace the time-consuming and laborious method for strain level differentiation.

Rapid yeast identification is of particular importance in monitoring wine fermentation and assessing strain application in winemaking. We examined the yeast diversity in wine organically produced in Waipara, New Zealand. A total of 235 yeast strains were isolated from 2018 wild Pinot Noir ferments, collected at four key fermentation stages, in two distinct fermentation systems (*i.e.* winery ferments and vineyard ferments, a typical fermentation in Greystone Wines). With the modified MALDI-TOF MS method, 13 indigenous species belonging to eight genera were identified from Pinot Noir ferments, with taxonomic diversity generally reducing as fermentation progressed, namely *H. uvarum, St. bacillaris, P. terricola, M. pulcherrima, P. kluyver, C. californica, P. membranifaciens, A. pullulans, P. kudriavzevii, W. anomalus, S. cerevisiae, S. uvarum* and *S. uvarum-like*, and the results were validated by 26S rDNA sequencing and PCR/RFLP. In comparison with well-established molecular methods, MALDI-TOF MS shows excellent performance on highly related species discrimination. In the MALDI-dendrogram, three pairs of species, *C. californica* and *P. membranifaciens*, *M. pulcherrima* and *M. fructicola*, *S. uvarum* and *S. uvarum-like* were separated properly in distinct branches. Within the 13 species, as far as we are aware, the isolation of *C. californica* from a New Zealand vineyard was reported for the first time.

The investigation of the yeast diversity and dynamics from winery and vineyard ferments provided further evidence for the microbial aspect of *terroir*. Although based on the same batch of grapes, the

yeast diversity and population, as well as the fermentation time, varied between vineyard ferments and winery ferments. At the proteome level, MALDI-TOF MS revealed more intriguing information. Within the same species, the observed proteomic differences of isolates were correlated to the isolation source (*i.e.* fermentation systems and the fermentation stages), which was especially evident in non-*Saccharomyces* yeasts. Some consistent proteomic differences between strains of *S. cerevisiae, Hanseniasporum uvarum*, *Candida californica*, *Pichia membranifaciens* and *Starmerella bacillaris* correlated with the different fermentation systems used. Furthermore, in the case of *C. californica* and *P. kluyveri*, we found the correlation between colony morphotypes and MALDI profiles. Isolates of *C. californica* from the two ferments presented two typical colony morphotypes along with a minor variation at peak m/z 8,211 in their MALDI profiles. Similarly, multiple colony morphotypes of *P. kluyveri* isolates were identified from more complex MALDI profiles. To sum up, the proteomic variations observed in their MALDI profiles were attributed to the physiology of individual yeast cell and the outer environments. The high speed, low cost, taxonomic resolution, and ability to characterise subtle changes in phenotype that may result from variations in environmental conditions makes MALDI-TOF analysis an attractive tool for further and wider applications in the wine industry. Such applications may include monitoring wine fermentation to actively support the consistency of high-quality wine products, and potentially for the development of such products too.

However, an issue that was not addressed in this study was whether the proteomic variations observed come from the strain identity or purely the environmental factors. Take *S. cerevisiae* as an example; among the 5 observed types at the Greystone winemakers, type I was dominant in both systems (68% in vineyard isolates and 85% in winery isolates) and detected across the whole fermentation process, in contrast, the remaining types were only observed in the final ferments. The unanswered question at present is, whether the emerging *S. cerevisiae* types are novel strains or the outcome of epigenetic traits. Epigenetic traits represent a stably heritable phenotype resulting from changes in a chromosome without alteration in the DNA sequence (Fuchs and Quasem 2014). The epigenetic programmes provide yeasts with phenotypic plasticity that allows them to respond to changing circumstances and thrive in niches. In our study, the yeast strains isolated from two fermentation systems were recovered on YPD agar in lab at the same time. Thus, the detected proteomic variations in MALDI profiles could be a heritable trait from the wine environments where the strains were isolated.

A typical example is *C. californica*, the isolates from vineyard and winery samples presents two distinct colony morphotypes, accompanied with nuanced MALDI profiles. This is very similar to the epigenetic switch occurring in pathogenic species *Candida albicans* between two cell types, referred to “white-opaque switching”, which was discovered by Slutsky et al. (1987). The cells switched heritably and reversibly between two phenotypes readily distinguishable by the colony size, shape,

and colour. The winery *C. californica* isolates formed a small and “white” hemispherical colony, whereas the vineyard isolates formed a larger, flatter and “opaque” colony, which is consistent to the description by Slutsky et al. (1987). Other than the colony morphotype, these two distinct cell types of *C. albicans* also differ in many aspects, such as metabolic states, mating behaviours, preferred niches in the host and most importantly, virulence, and the switching frequency can be significantly altered by the environmental cues (Lohse and Johnson 2009).

Thus, this is an important issue for future research, which will help us understand the potential of MALDI-TOF MS for the strain level differentiation and its ability to detect the epigenetic modification or regulation in yeast. To achieve this objective, whole-genome sequencing can be an ideal tool to compare strains on a genomic scale (Crauwels et al. 2014). The establishment and maintenance of epigenetic states (*e.g.* histone modifications) has been a hot topic to modify the yeast to present stable desired phenotypic traits (O'Kane and Hyland 2019). The dietary epigenetic compound has recently shown to impact wine chemical composition and its sensory profile (Suresh 2021). Given that, MALDI-TOF MS could be a rapid and economic approach to detect the stability of epigenetic inheritance and contribute to the study of epigenetics in fermented food innovation.

Whilst more work needs to be done to support its strain typing ability, it did substantiate the power of MALDI-TOF MS in identifying unknown indigenous yeasts at species level, as well as the potential in phenotypic screening due to its high sensitivity to external stimuli. On another note, such high sensitivity may also cast a doubt on its reproducibility to characterize wine yeasts under different growth conditions. However, it is also established that substrate composition influences protein expression, but the degree to which this may affect MALDI-TOF spectra (and analytical results thereof) had not been fully explored (Moothoo-Padayachie et al. 2013, Usbeck et al. 2013). To further inform assay optimisation, the influence on MALDI-TOF spectra was determined using eight *Saccharomyces* strains of diverse origins cultivated on grape juices from Pinot Noir and Chardonnay varieties, synthetic grape juice, and laboratory-grade artificial culture media (YPD broth and agar) to select the best medium to reflect the predictive potential of MALDI-TOF MS. Grape juice is the most familiar environment for wine yeasts where we expected more interesting findings. The result is somewhat frustrating, since most of the tested yeast strains grown in grape juice failed to obtain quality MALDI profiles.

To sum up, our results demonstrated significant influences of culture media on strain MALDI-TOF spectra and confirmed that unfavourable culture media is a significant factor affecting the quality profiles. Growth media composed of same ingredients, but different treatments or states (*e.g.* solid and liquid) produced distinct profile patterns, however, the main peaks remained constant. We are not aware of any other similar studies involving natural and artificial grape juices. The profiles

generated by cultures on YPD agar and YPD broth have similar species-level resolution, but the potential "floc" characteristic of certain strains in liquid media may impede the quality spectra acquisition, and the additional wash step would increase the time- and labour-cost in practical operation, thus yeast culture on YPD agar is recommended for taxonomic studies, with YPD broth culture of *S. cerevisiae* offering potential improved intra-subspecific differentiation.

Previous studies have shown MALDI-TOF MS to be a powerful tool in wine yeast identification and potential prediction of application (Usbeck et al. 2014). In chapter 7, the potential of MALDI-TOF profiling for predicting potential applications of yeast strains in the beverage sector was assessed. This work has been one of the first attempts to thoroughly examine the predictive potential of MALDI-TOF MS in wine/brewing yeast strains application. A panel of 59 commercial yeasts (47 wine and 12 brewing yeasts) were used to validate the concept whereby two culture media (YPD agar and YPD broth) as well as two mass ranges m/z 500-4,000 and m/z 2,000-20,000 were evaluated for the best fit. Three Machine Learning based algorithms PCA, MDS, and UMAP in addition to a hierarchical clustering method, were employed. We believe this is the first known application of UMAP to MALDI-TOF analysis. Profiles derived from broth cultures yielded more peaks, but these were less well defined compared with those from agar cultures.

The current study also highlights the importance of the algorithm selection. Hierarchical clustering more clearly resolved different species and gave a broad overview of potential strain utility, but more nuanced insights were provided by MDS and UMAP analyses. PCA-based displays were less informative. In our study, only UMAP analysis indicated that the clustering obtained can be correlated with functionality and/or organoleptic attributes. The strains clusters/groups with similar oenological properties are all very well in UMAP plotting but there is no objective data on the exact details of their performance/attributes. A more comprehensive study to objectively determine each strains performance in the production of critical wine organoleptic parameters such as higher alcohol, ester and terpene formation (reviewed by Swiegers et al. (2005) is required to better understand the relationships. Even so, simply according to the manufacturers' instructions for commercial yeast usage, there is a positive insight into the yeast strain grouping. Of the 5 wine yeast groups, group 1 is dominated by the hybrid strains of cryotolerant nature; group 2 can be considered as the champagne strains; group 5 is mainly composed by the strains for white wine production (*e.g.* Sauvignon Blanc and Chardonnay styles); group 3 and 4 are well populated with strains for red wine making and rosé. The 12 brewing strains can be delineated into three groups of wheat yeast, lager yeasts, and ale yeast.

In contrast, low mass application seems to be less useful in predicting strains application, but it does have a good indication of industrial application that wine strains can be differentiated from beer

strains and from champagne strains. A limitation of its use could be the relatively small sample size. Compared to the high mass detection (peak coverage of m/z 4,000-20,000), the low mass peaks are mainly distributed between m/z 500 and 1,500. Hence it is no wonder that low mass peaks carry less information. However, as discussed above, its potential to differentiate the strains within a given species is very promising. Thereby, more samples will be examined in further work and we believe more valuable information can be extracted with proper algorithms. Additionally, it is intriguing to figure out the proteins/peptides detected within low mass range (m/z 500-1,500). It is well known that m/z 2,000-20,000 represents the ribosomal and housekeeping proteins of taxonomic relevance, but to our knowledge, no work reported the low mass proteins of wine yeast MALDI profiles. According to current study, it could conceivably be hypothesised that these low mass proteins are strain-specific.

Thus far, the potential of MALDI-TOF proteomics in predicting the utility of yeast strains of commercial benefit is supported in our project, provided appropriate approaches are used for data generation and analysis. Look back upon the two totally contrasting ideologies where we start off the project, our results support Usbeck et al. (2014) over Gutiérrez et al. (2017) who claimed that there was no success in determining the existence of clusters not just for strain-level differentiation but also for ecotype-, winery-, or Designations of Origin (DO)-level differentiation using MALDI data.

In conclusion, this thesis contributes to the wine industry by establishing a sound MALDI-TOF MS system. Our work not only improved the capacity of MALDI-TOF analysis in wine-associated yeasts identification, but also proved its predictive potential for wine and brewing strains. With the integration of further work, the use of our MALDI-TOF MS systems will be landed on the indigenous yeasts commercialization and the technical support for the wine industry. With merely a single colony or small amount of wine samples, a wide range of information can be provided, such as the identification, application recommendation (*e.g.* grape varietal, aroma, and flavour) as well as the oenological properties of interests (*e.g.* alcohol, glycerol and SO_2). Likewise, the wine quality can be assured with such a rapid and accurate identification approach. The adaptive software package will be necessary to be developed as a comprehensive system, which cannot only provide the detection results but also the professional counselling and advice for winemaking, as well as the yeast recommendation for the desired wine styles.

Beyond the wine industry, this system can also be extended to brewing industry where the possibility we have attested, and other fermented beverage industries, like coffee or tea. In the same way, the lactic acid bacteria (LAB) should be also of value to explore, for instance, LAB (*e.g. Oenococcus oeni*) in malolactic fermentation (MLF).

8.2 Future Work

Therefore, a natural progression of this work is:

1. to objectively analyse the technological attributes (*e.g.* low-alcohol wine production, high glycerol production and enhanced activity of enzymes with oenological impact) of the commercial yeast stains in different UMAP clusters (in Chapter 7) so as to understand the relationship of the MALDI profiles to practical production;

2. to determine the high- and low mass-MALDI profiles of more isolates and explore its value in strain differentiation and potential prediction ability using multiple data analysis;

3. to analyse the wild isolates (in Chapter 5) using UMAP in addition to their fermentative attributes, which can contribute to the knowledge of non-*Saccharomyces* strains for their use in the wine industry;

4. to classify *S. cerevisiae* isolates (in Chapter 5) targeting their δ sequence region so as to examine the identification ability of MALDI-TOF MS on strain level, as well as the yeast diversity at strain level as an important factor for determining the microbial influence on the flavour properties of wine;

5. to examine all the strains of interests at a genomic scale by high-resolution whole genome sequencing, in particularly the isolates of *C. californica*, *P. kluyveri*, *S. cerevisiae* and *S. uvarum* sourced from different fermentation stage and fermentation systems;

6. to involve more New Zealand wine regions that will allow for a better understanding of the microbial *terroir* of the indigenous yeasts and the development of high throughput metabolic screenings;

7. to have further data mining may help correlate MALDI-TOF proteomics with the utility of wine yeast strains of commercial benefit accurately, and potentially yeasts used for other fermented products too.

The future possibilities of this research on yeast strains are as numerous as the products that can be made from them.

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Appendix A

Supplementary Data

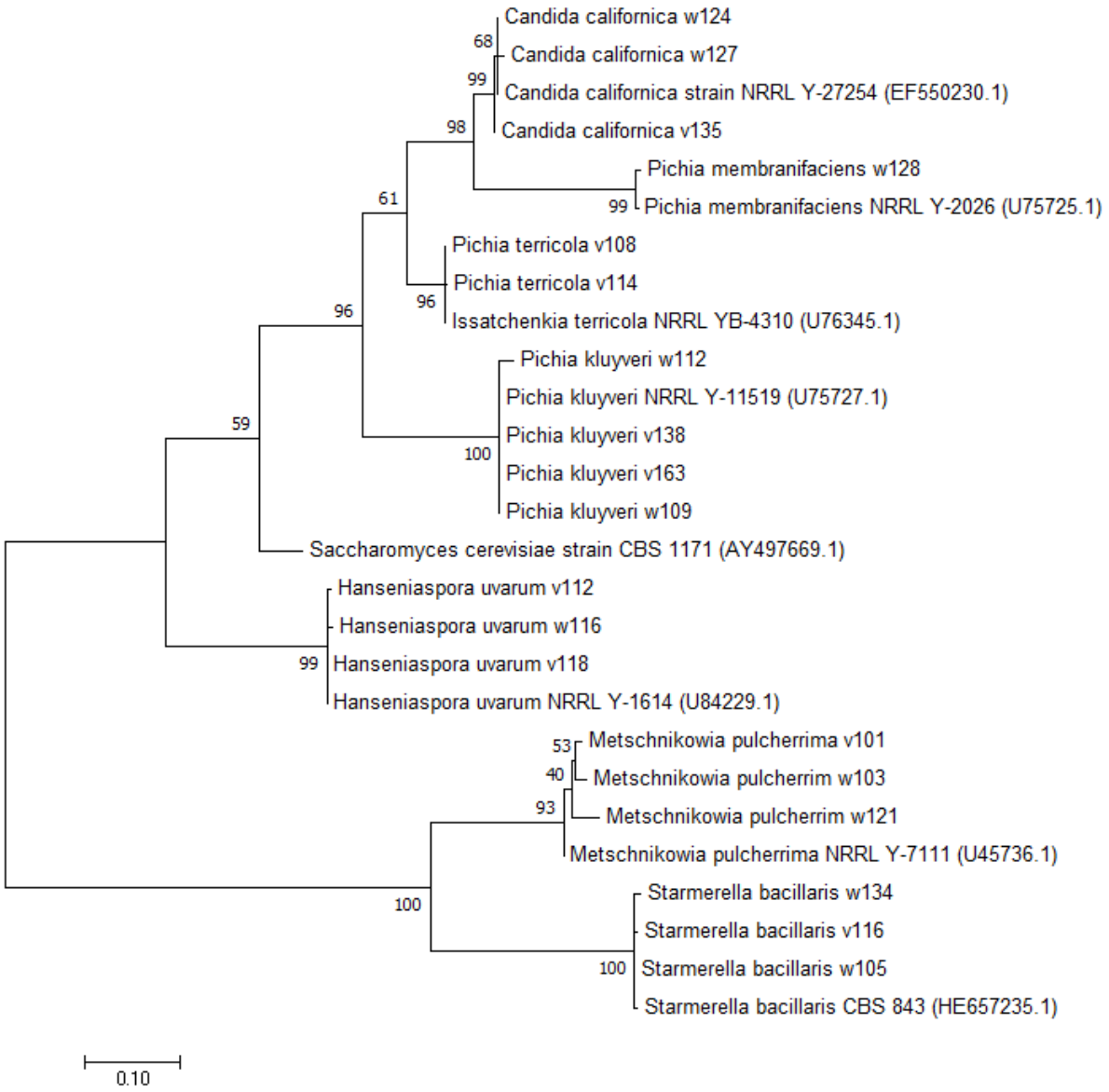


Figure A.1 Phylogenetic analysis of 19 yeast isolates from organic grape juice compared to authenticated type or reference strains using partial 26S rDNA sequences and Maximum Likelihood clustering.

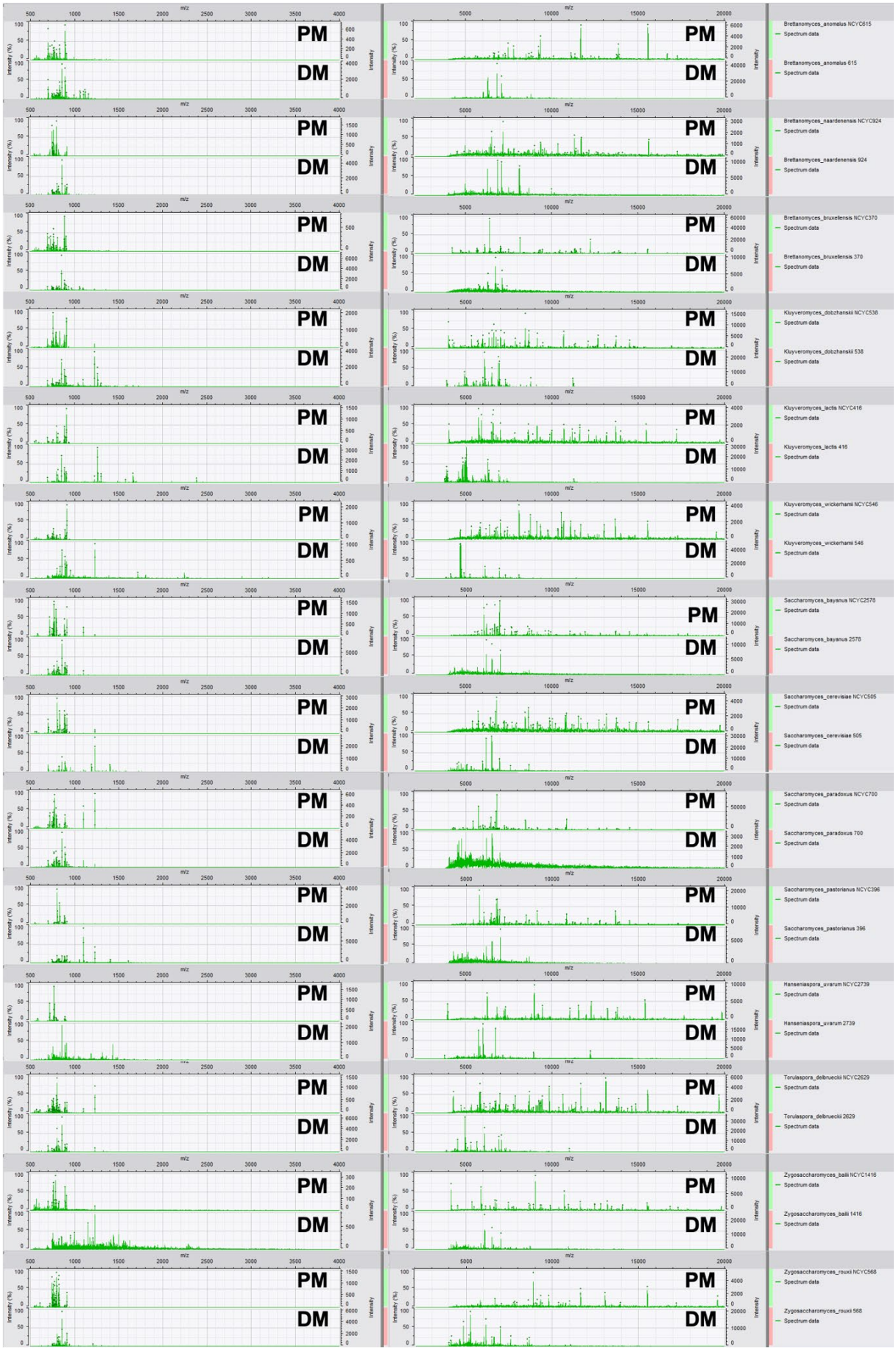
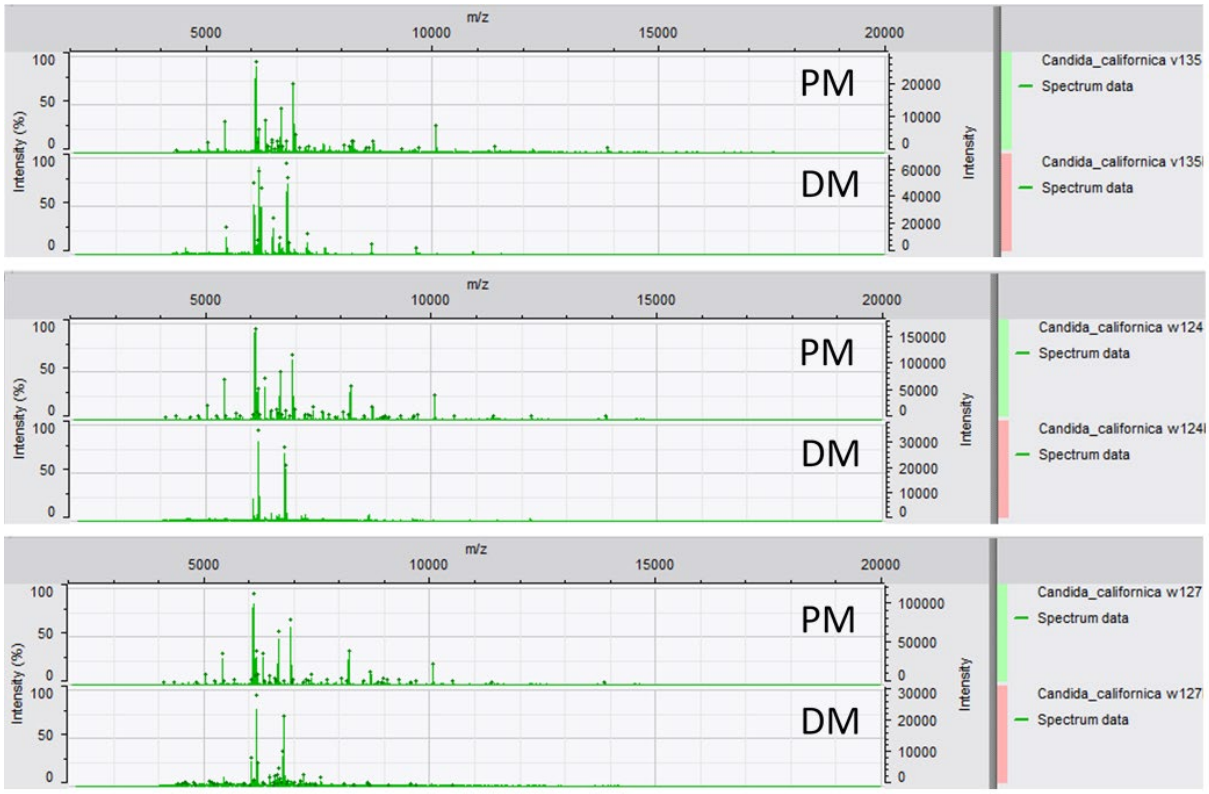
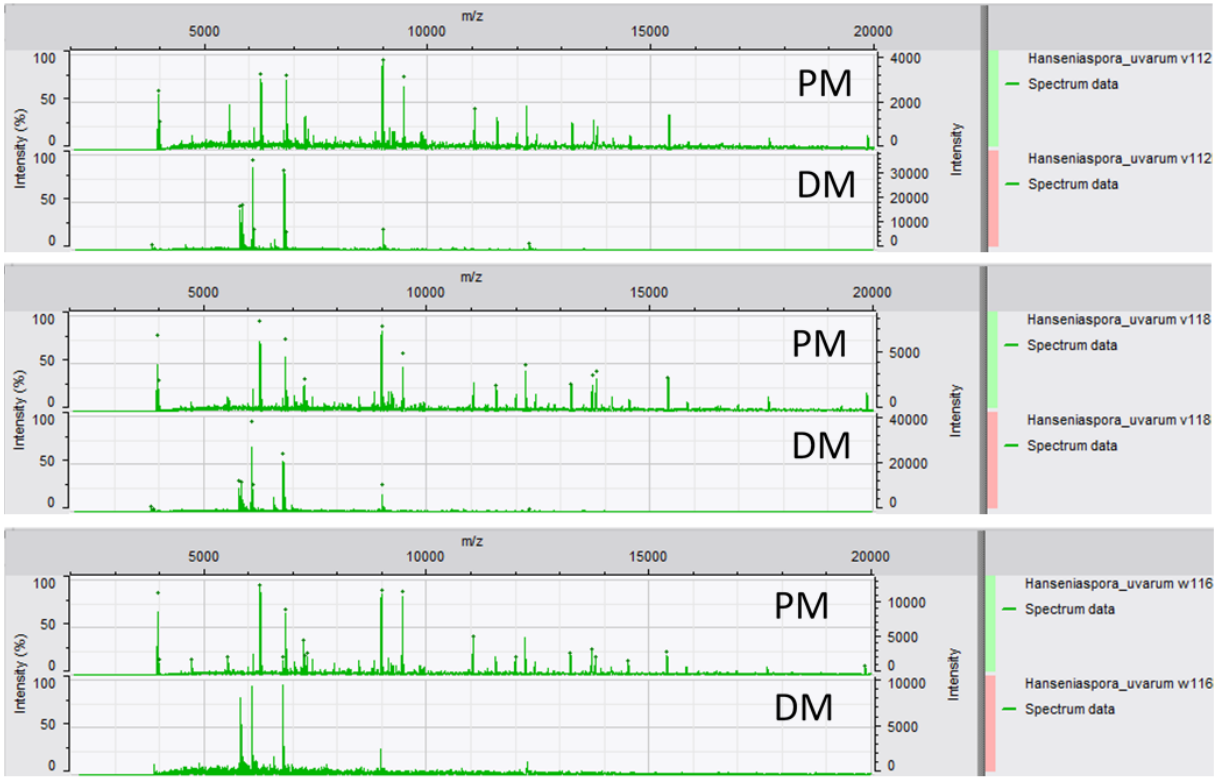

PM
DM
m/z
Intensity (%)
Intensity
Brettanomyces_anomalus NCYC615
Spectrum data
Brettanomyces_anomalus 615
Spectrum data
Brettanomyces_naardenensis NCYC924
Spectrum data
Brettanomyces_naardenensis 924
Spectrum data
Brettanomyces_bruxellensis NCYC370
Spectrum data
Brettanomyces_bruxellensis 370
Spectrum data
Kluyveromyces_dobzhanskii NCYC538
Spectrum data
Kluyveromyces_dobzhanskii 538
Spectrum data
Kluyveromyces_lactis NCYC416
Spectrum data
Kluyveromyces_lactis 416
Spectrum data
Kluyveromyces_wickerhamii NCYC546
Spectrum data
Kluyveromyces_wickerhamii 546
Spectrum data
Saccharomyces_bayanus NCYC2578
Spectrum data
Saccharomyces_bayanus 2578
Spectrum data
Saccharomyces_cerevisiae NCYC505
Spectrum data
Saccharomyces_cerevisiae 505
Spectrum data
Saccharomyces_paradoxus NCYC700
Spectrum data
Saccharomyces_paradoxus 700
Spectrum data
Saccharomyces_pastorianus NCYC396
Spectrum data
Saccharomyces_pastorianus 396
Spectrum data
Hanseniaspora_uvarum NCYC2739
Spectrum data
Hanseniaspora_uvarum 2739
Spectrum data
Torulaspora_delbrueckii NCYC2629
Spectrum data
Torulaspora_delbrueckii 2629
Spectrum data
Zygosaccharomyces_bailii NCYC1416
Spectrum data
Zygosaccharomyces_bailii 1416
Spectrum data
Zygosaccharomyces_rouxii NCYC568
Spectrum data
Zygosaccharomyces_rouxii 568
Spectrum data

Figure A.2 MALDI-TOF profiles of 14 reference strains at both low mass range (m/z 500-4,000) and high mass range (m/z 2,000-20,000) with Dried-droplet method (DM) and Pre-mixing method (PM).

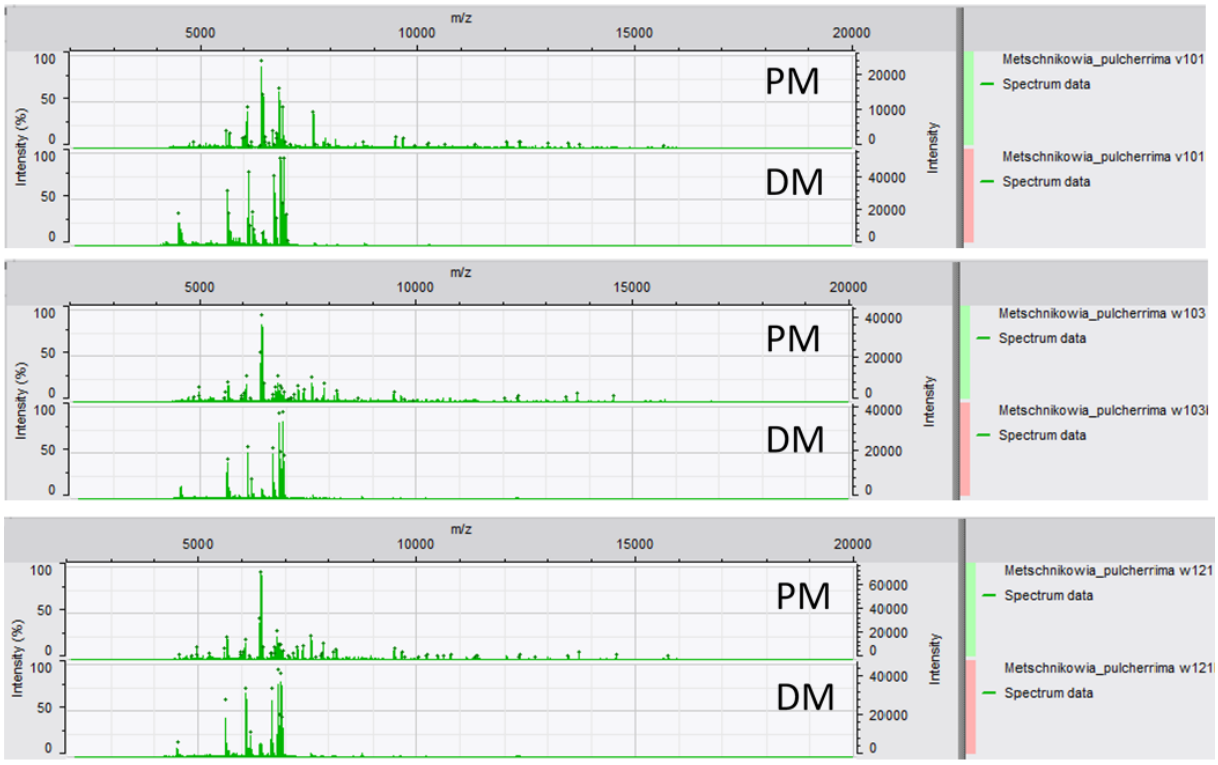
(A) *Candida californica*



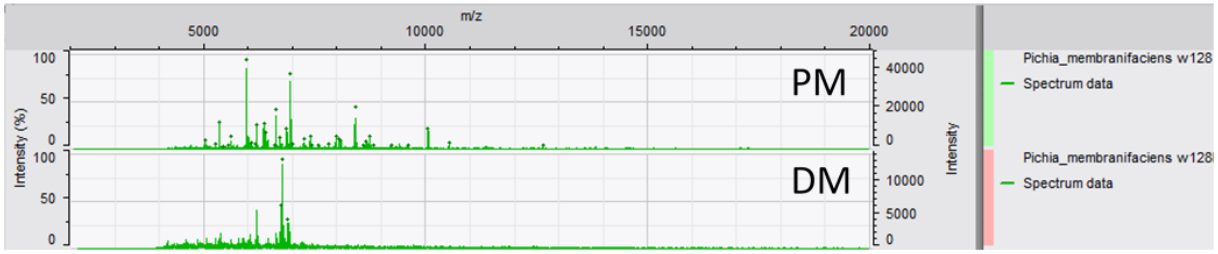
(B) *Hanseniaspora uvarum*



(C) *Metschnikowia pulcherrima*

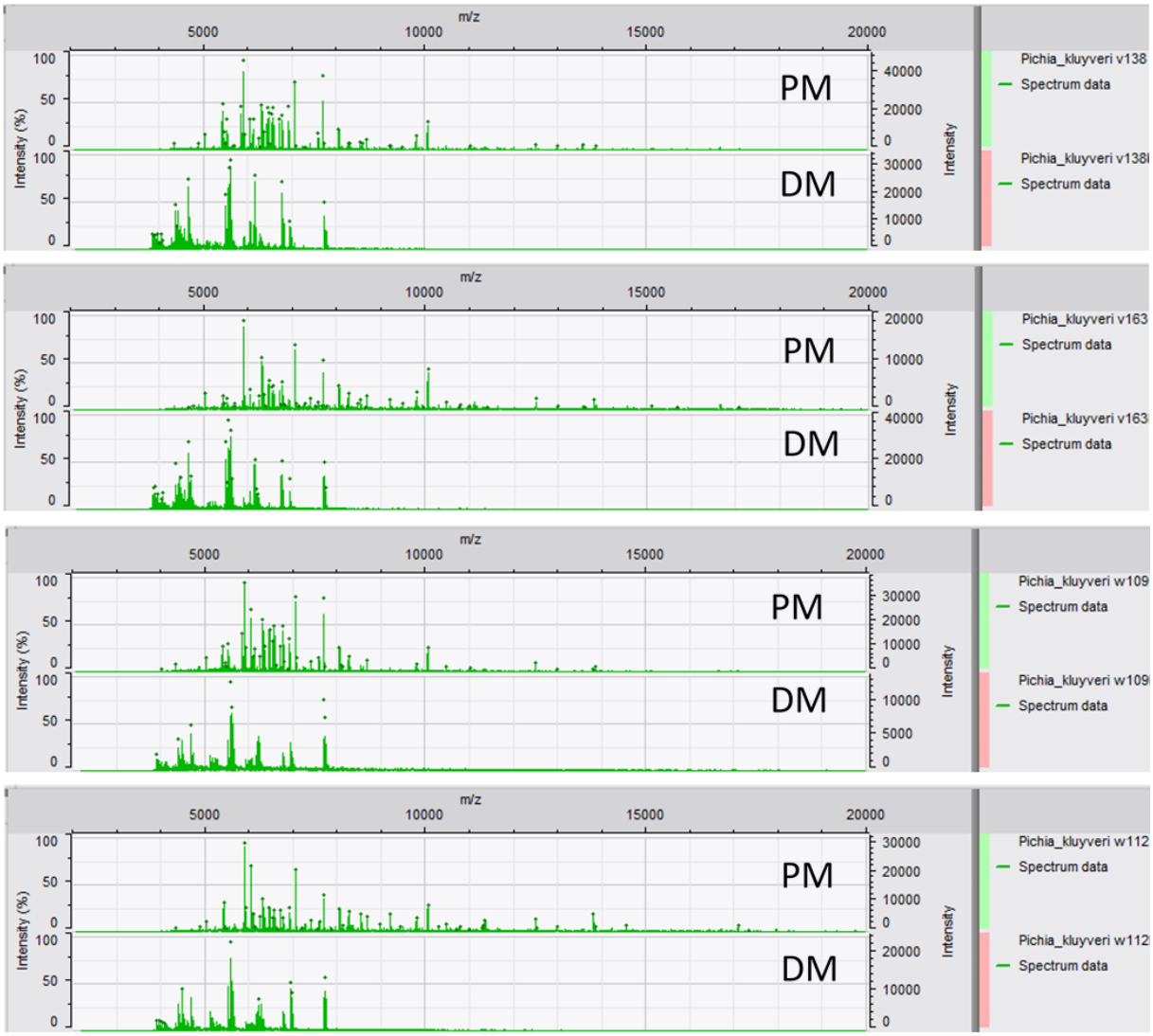


(D) *Pichia membranifaciens*



(E) *Pichia terricola*

(F) *Pichia kluyveri*



(G) *Starmerella bacillaris*

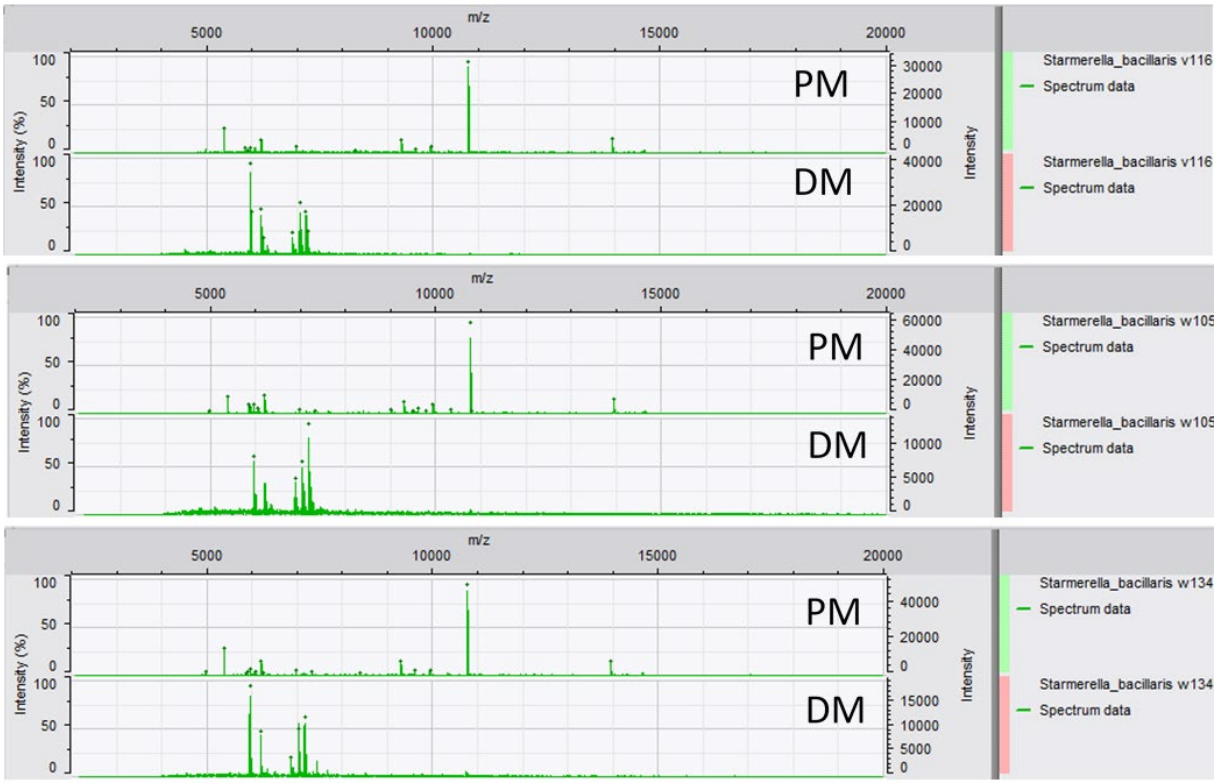
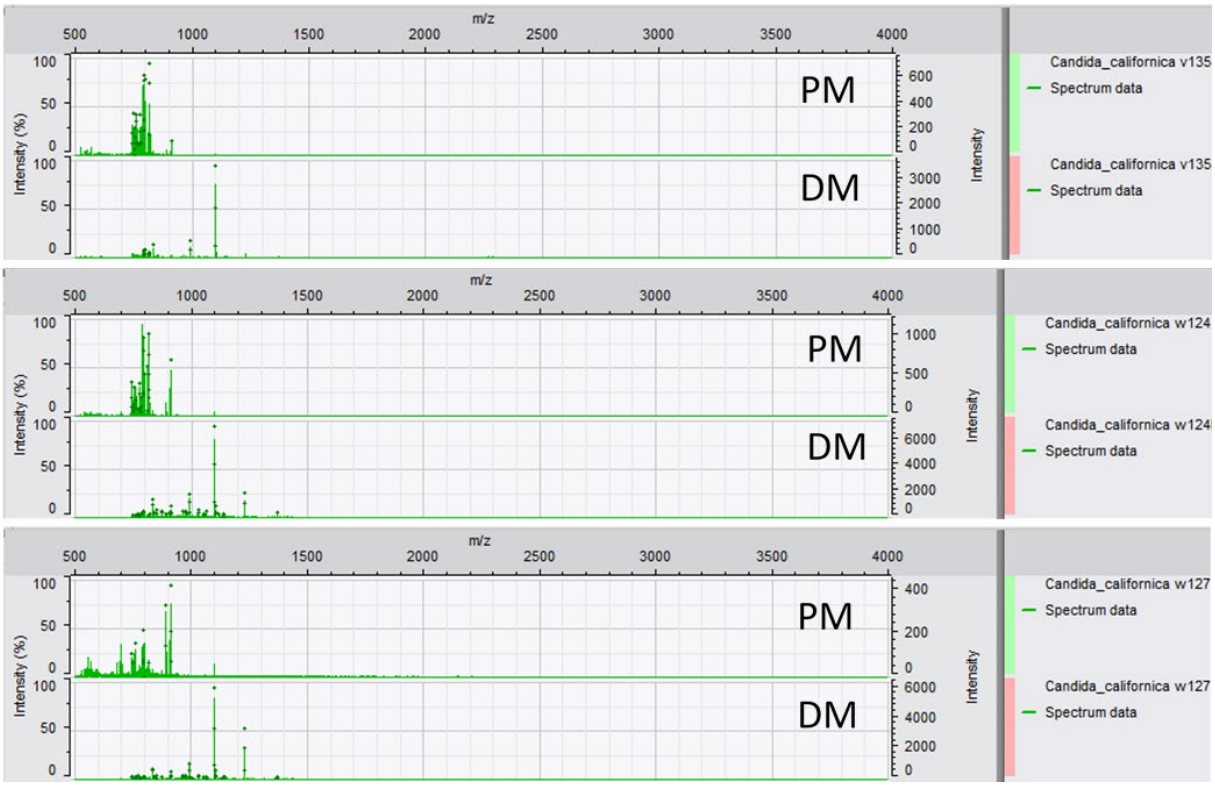
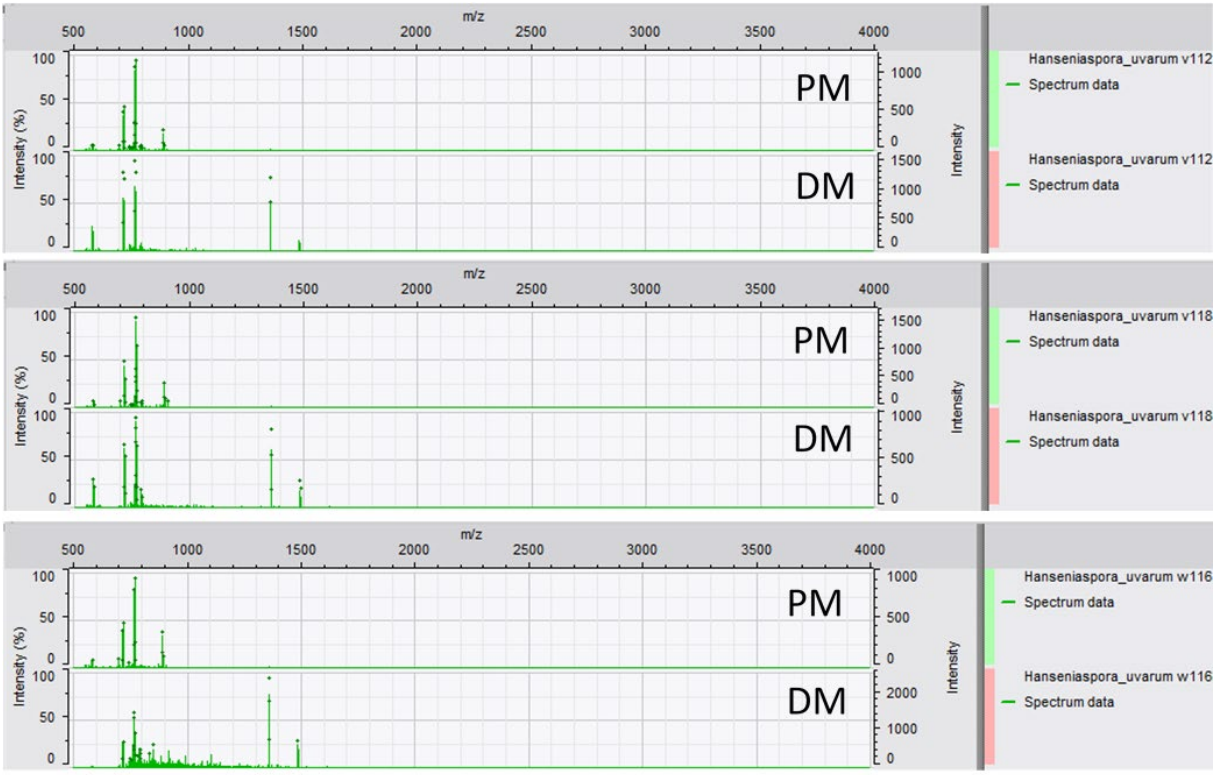


Figure A.3 MALDI-TOF profiles of 19 yeast isolates at high mass range (m/z 2,000-20,000) with Dried-droplet method (DM) and Pre-mixing method (PM).

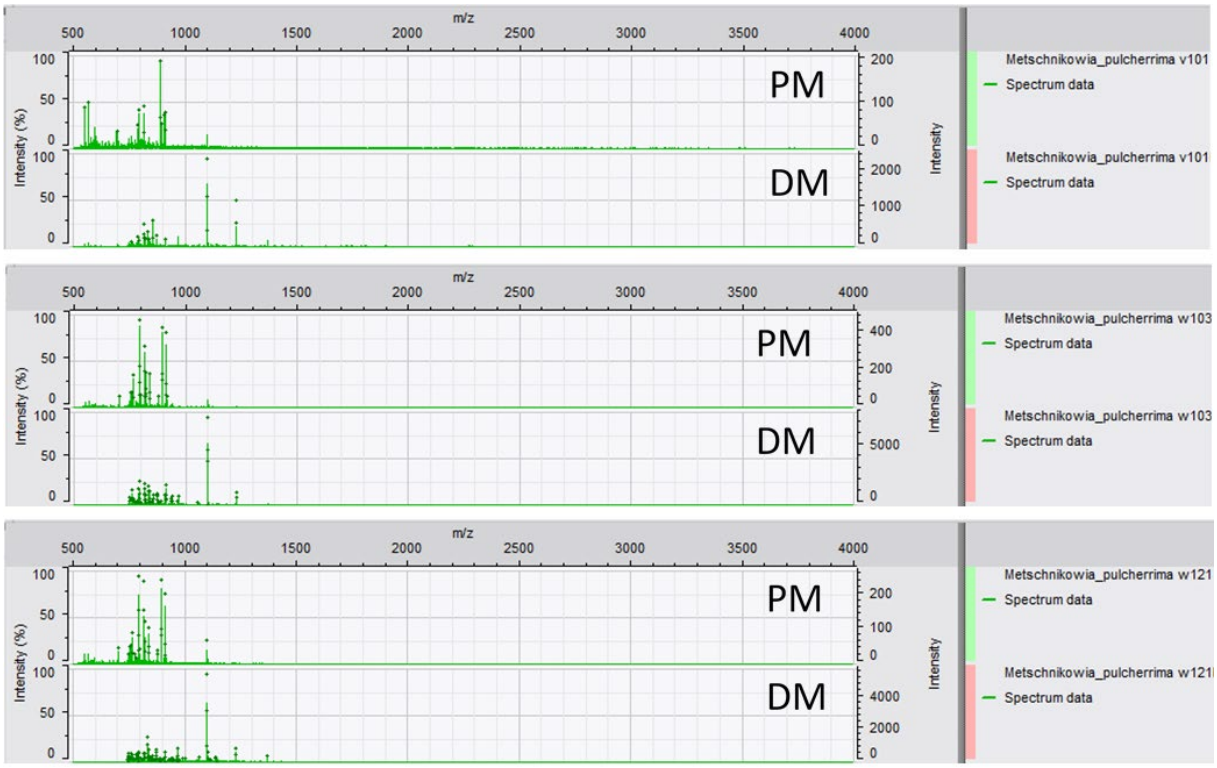
(A) *Candida californica*



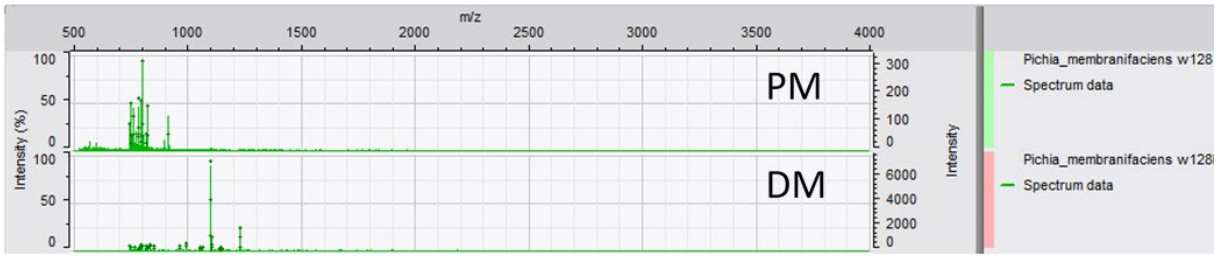
(B) *Hanseniaspora uvarum*



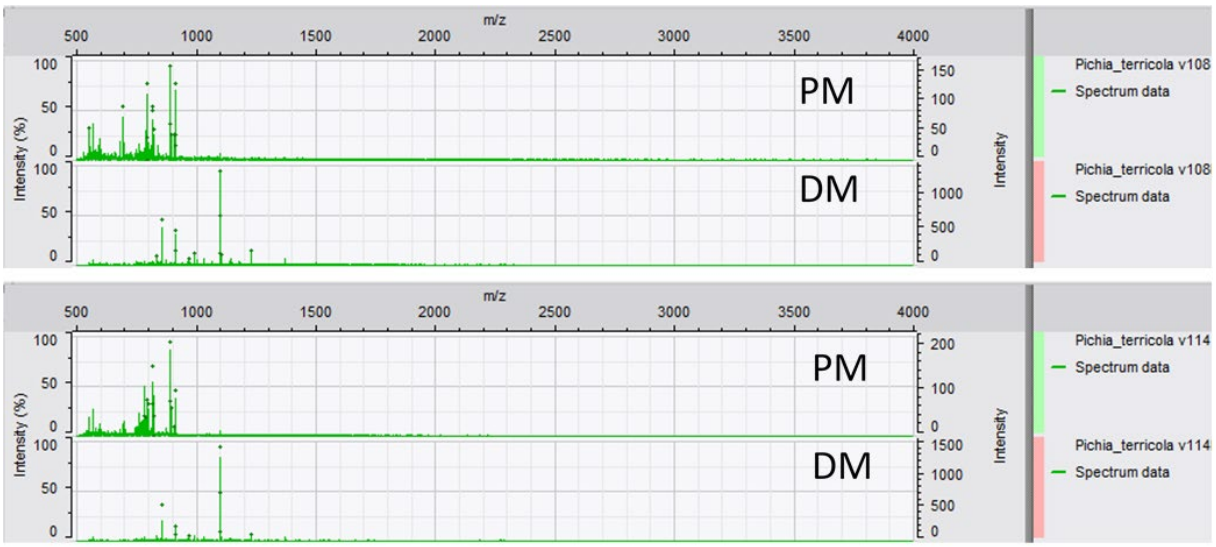
(C) *Metschnikowia pulcherrima*



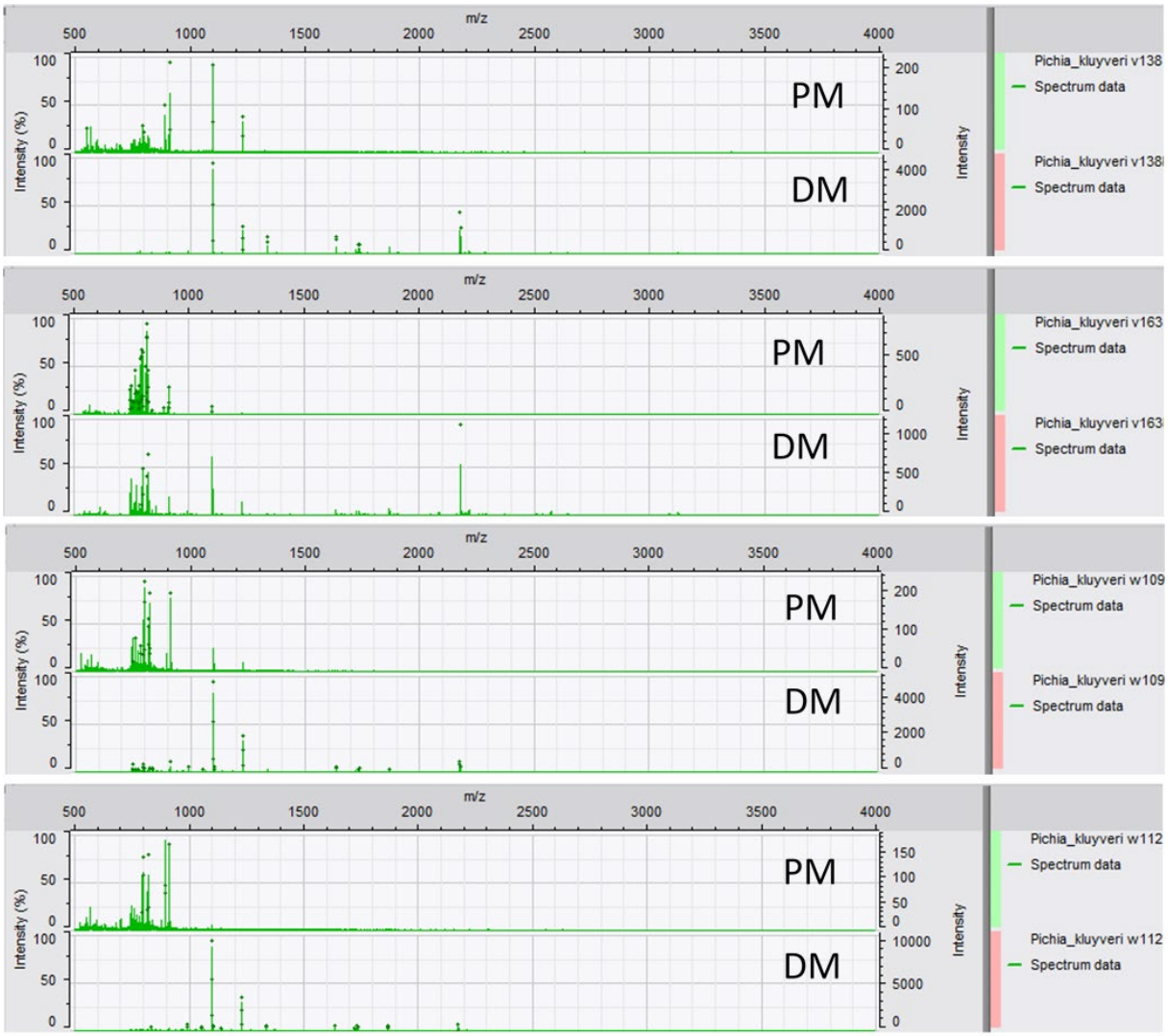
(D) *Pichia membranifaciens*



(E) *Pichia terricola*



(F) *Pichia kluyveri*



(G) *Starmerella bacillaris*

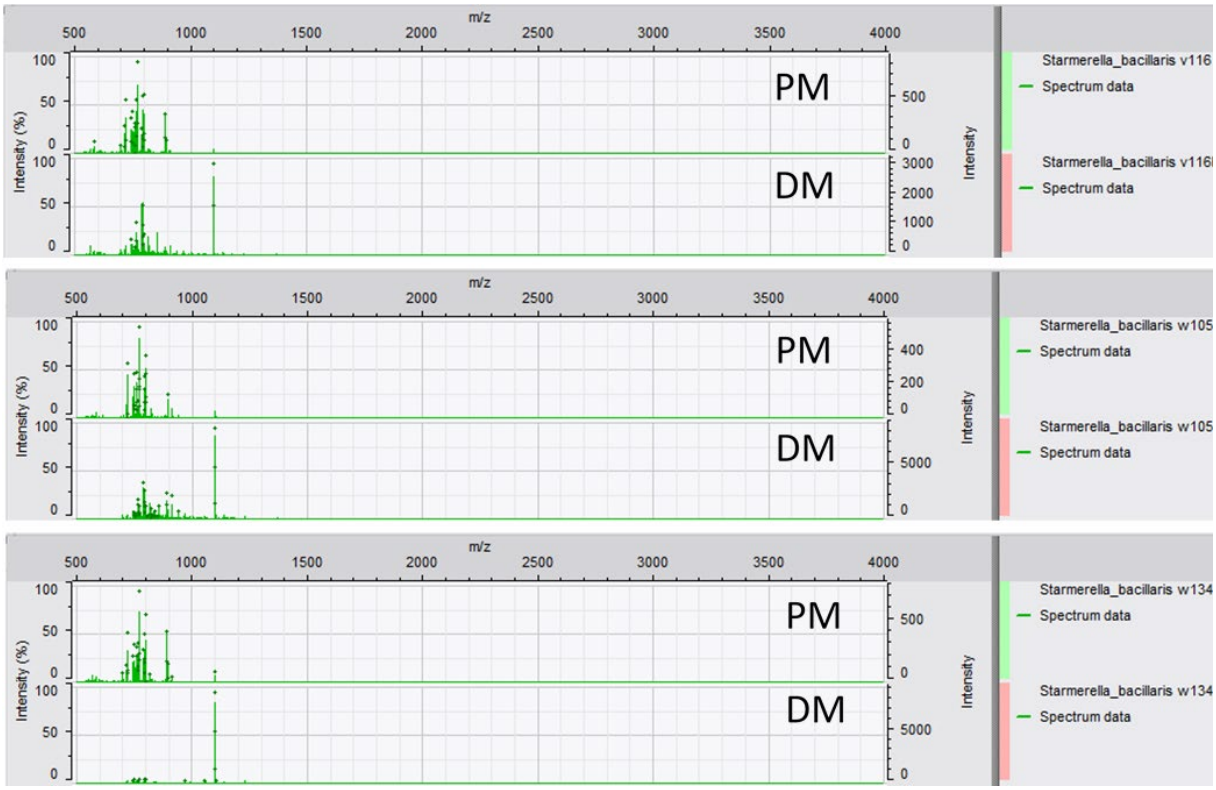
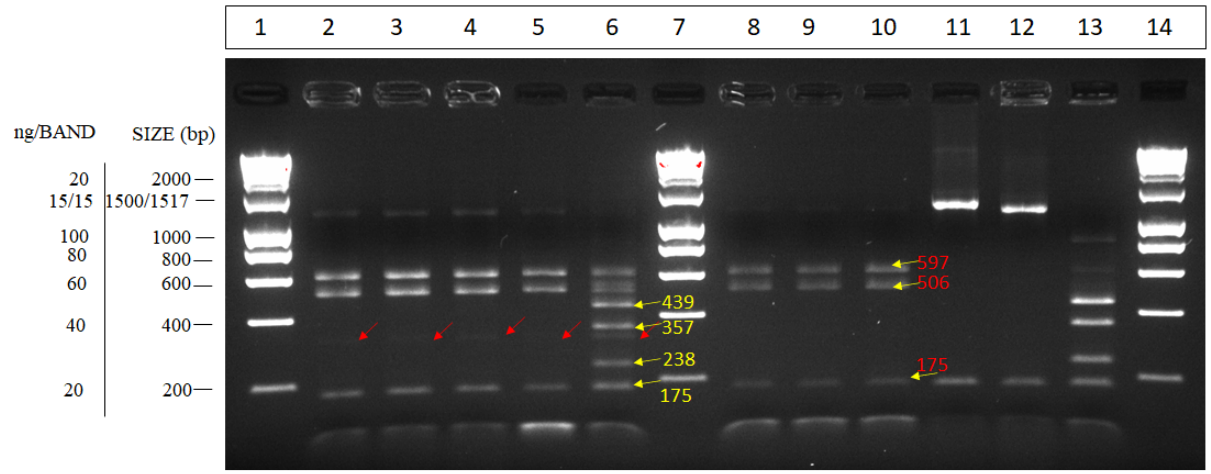
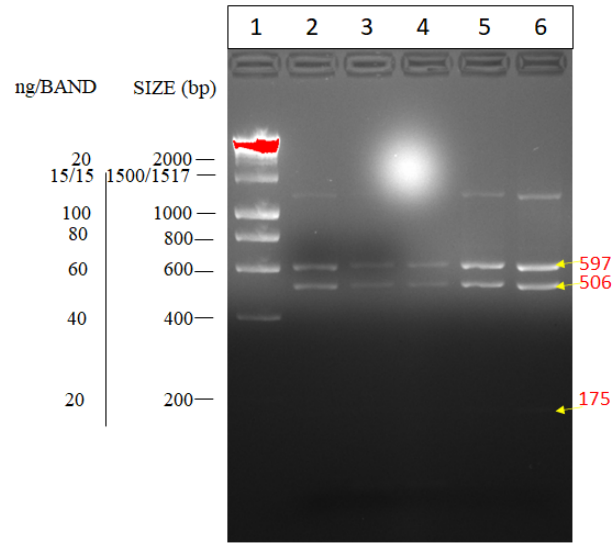


Figure A.4 MALDI-TOF profiles of 19 yeast isolates at low mass range (m/z 500-4,000) with Dried-droplet method (DM) and Pre-mixing method (PM).



1. HyperLadder™ 1kb – Bioline (Hyper Ladder I);
2. Strain SW4-14 (*S. uvarum-like*);
3. Strain SW4-4 (*S. uvarum-like*);
4. Strain SW2-8 (*S. uvarum-like*);
5. Strain SW2-28 (*S. uvarum-like*);
6. *S. bayanus* NCYC 2578;
7. HyperLadder™ 1kb – Bioline (Hyper Ladder I);
8. Strain SW2-32 (*S. uvarum*);
9. Strain SW2-13 (*S. uvarum*);
10. Strain SV2-28 (*S. uvarum*);
11. *S. cerevisiae* NCYC 505;
12. *S. paradoxus* NCYC 700;
13. *S. pastorianus* NCYC 396;
14. HyperLadder™ 1kb – Bioline (Hyper Ladder I);



1. HyperLadder™ 1kb – Bioline (Hyper Ladder I);
2. Strain SW4-1 (*S. uvarum*);
3. Strain SW3-13 (*S. uvarum*);
4. Strain SV3-10 (*S. uvarum*);
5. Strain SV2-29 (*S. uvarum*);
6. Strain SW2-24 (*S. uvarum*);

Figure A.5 NTS2 (rDNA) *Alu*I profiles of *S. uvarum* isolates, *S. bayanus* NCYC 2578, *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, and *S. pastorianus* NCYC 396.

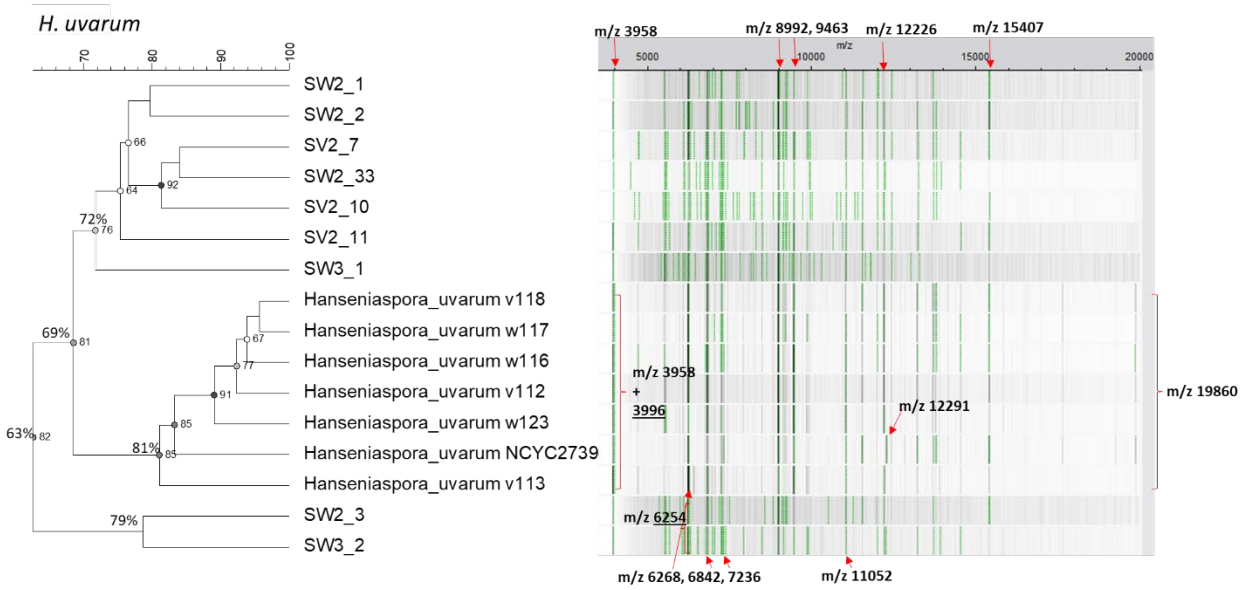


Figure A.6 Cluster analysis of the *H. uvarum* isolates and reference strain NCYC 2739 combined with their band presentation of spectra patterns. Red arrows marked their common peaks and two peak shifts m/z 6,254 and 12,291, the red brackets showed the unique peak.

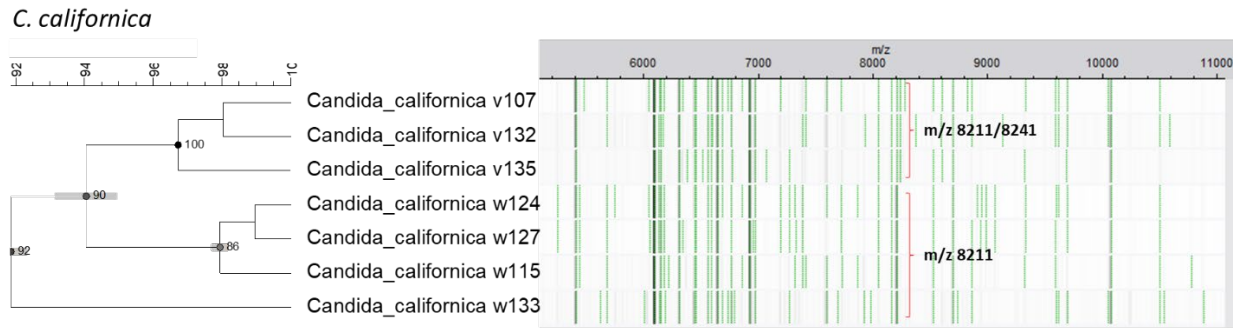


Figure A.7 Cluster analysis of the *C. californica* isolates combined with their band presentation of spectra patterns.

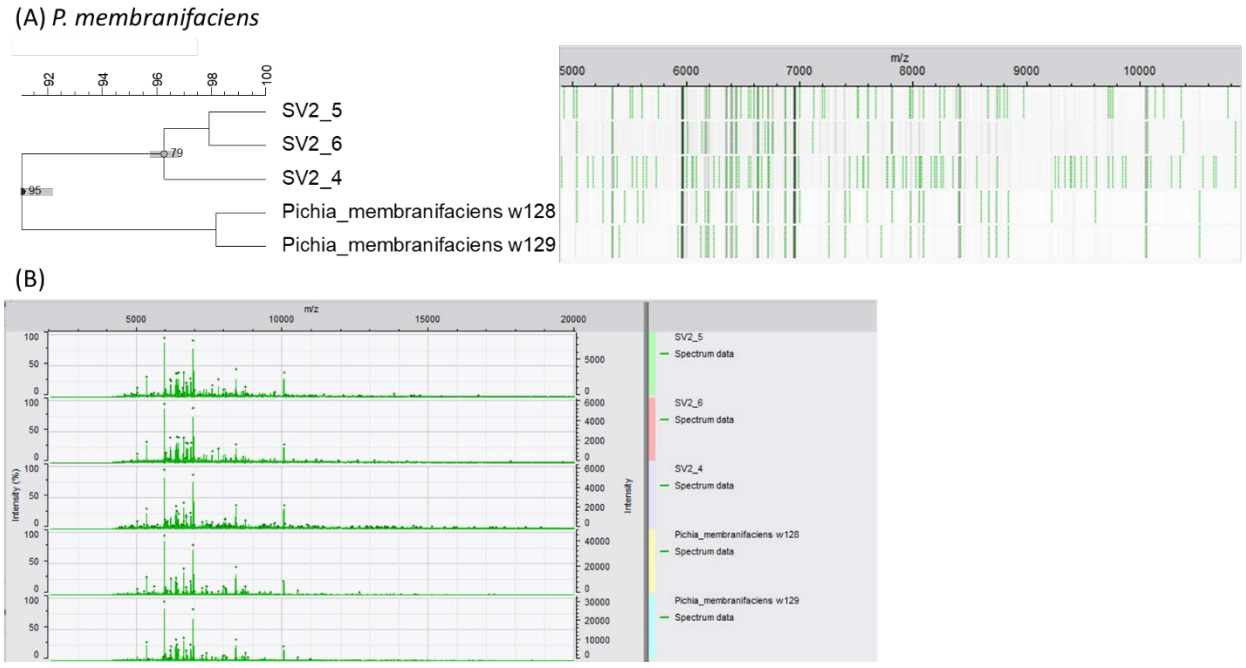


Figure A.8 (A) Cluster analysis of the *P. membranifaciens* isolates combined with their band presentation of spectra patterns and (B) spectra patterns.

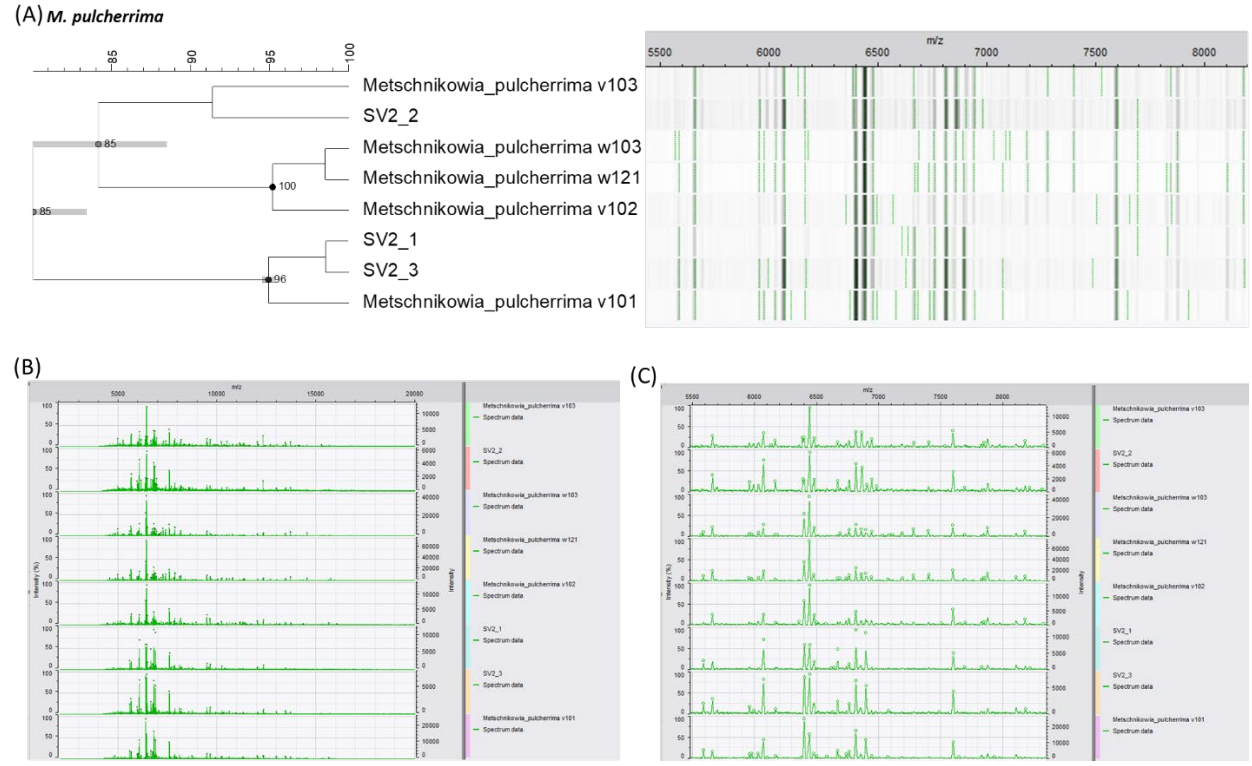


Figure A.9 (A) Cluster analysis of the *M. pulcherrima* isolates combined with their band presentation of spectra patterns; (B) comparison of eight spectra patterns and (C) the enlargement of m/z 5,500-8,300.

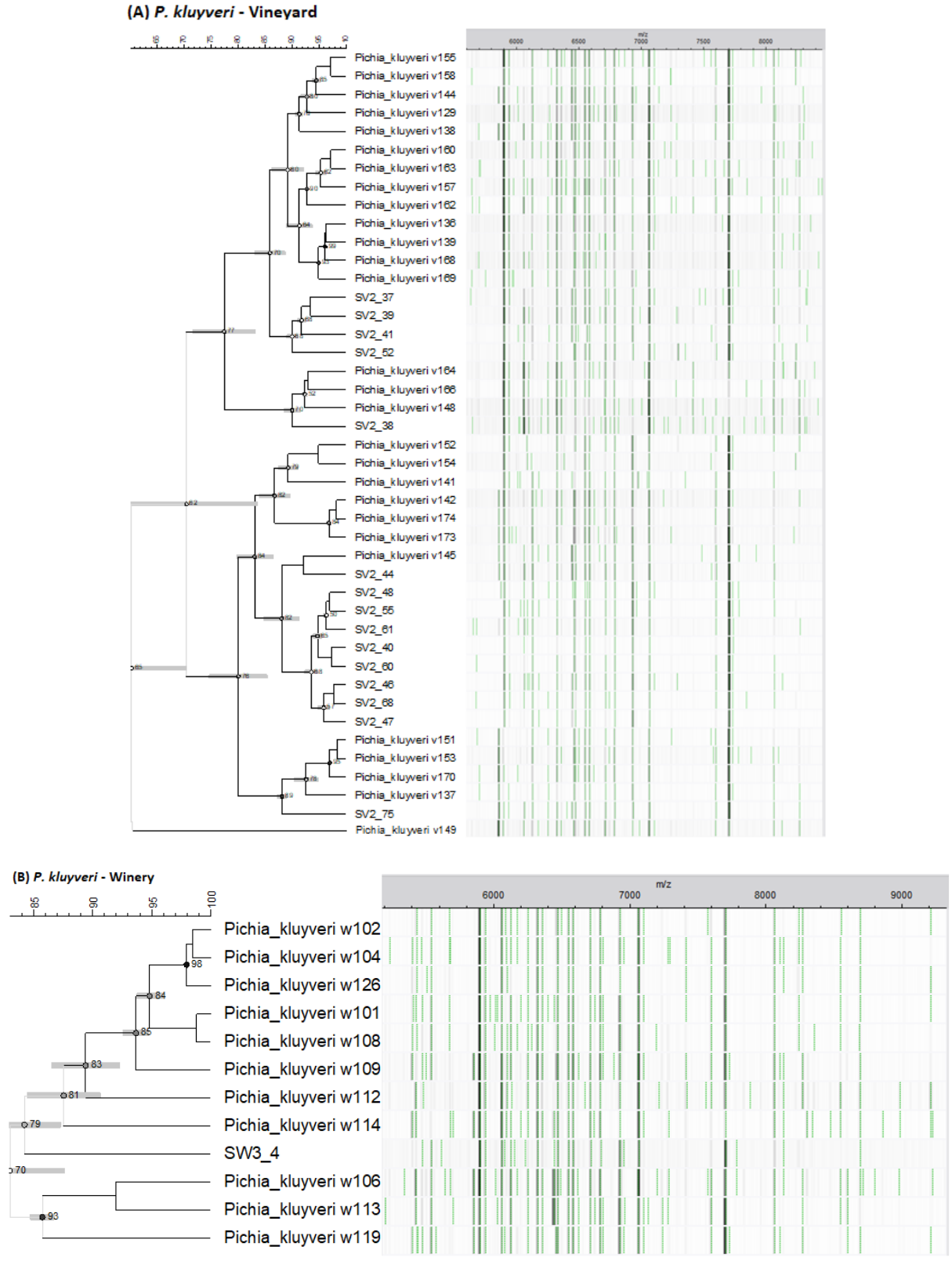


Figure A.10 Cluster analysis of the *P. kluyveri* isolates (A) from vineyard (B) winery, combined with their band presentation of spectra patterns.

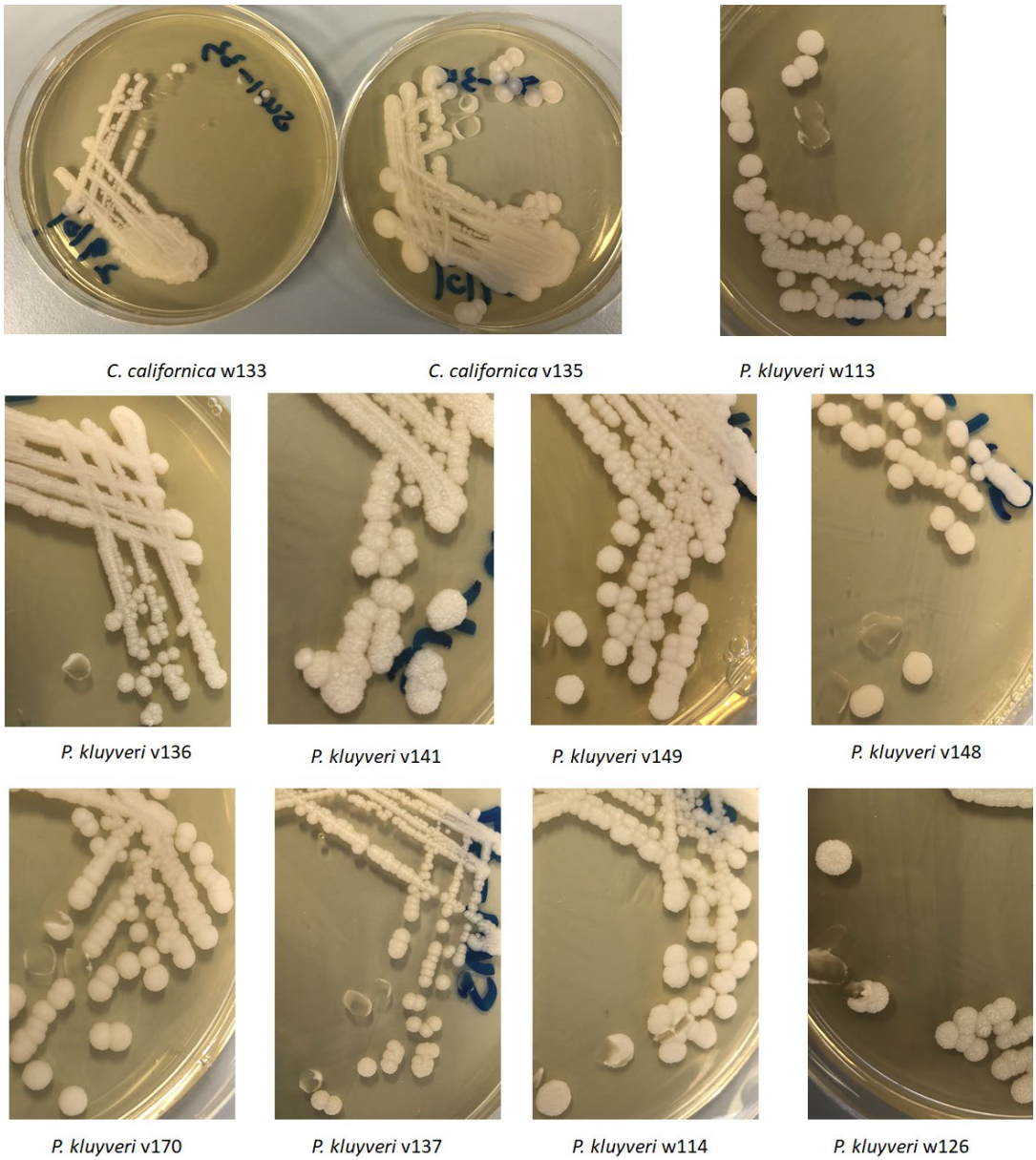


Figure A.11 Colony morphotypes of representative isolates from *C. californica* and *P. kluyveri*.

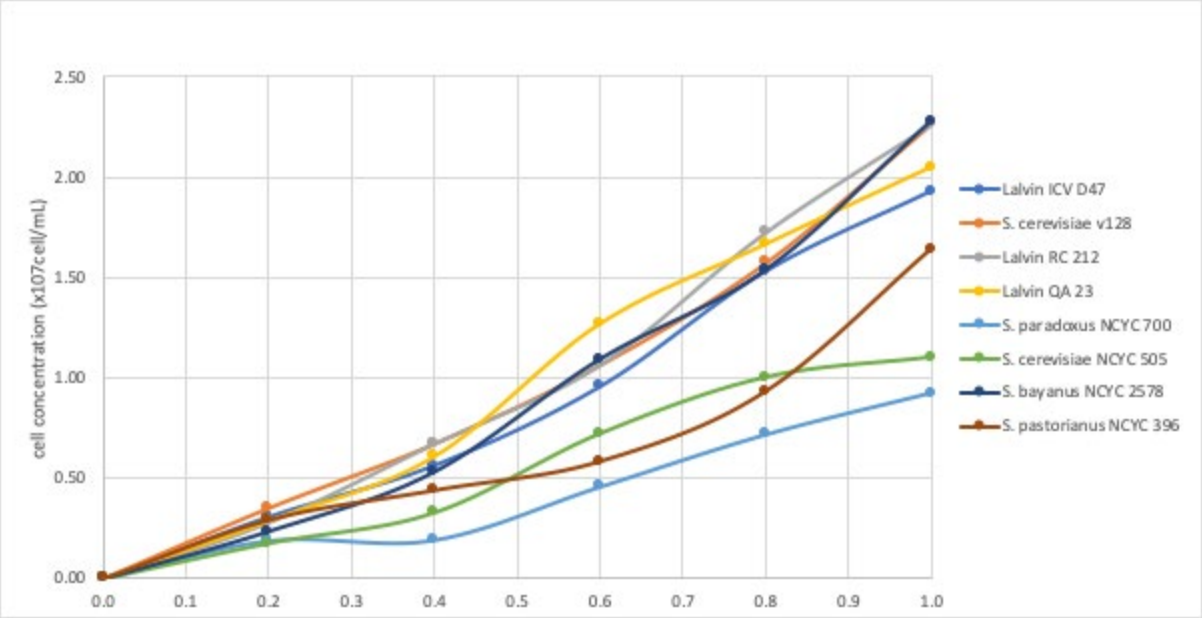


Figure A.12 Standard Curve of eight yeast strains, which was made by determine the relation between the OD $_{(600nm)}$ value and corresponding cell number using hemacytometer.

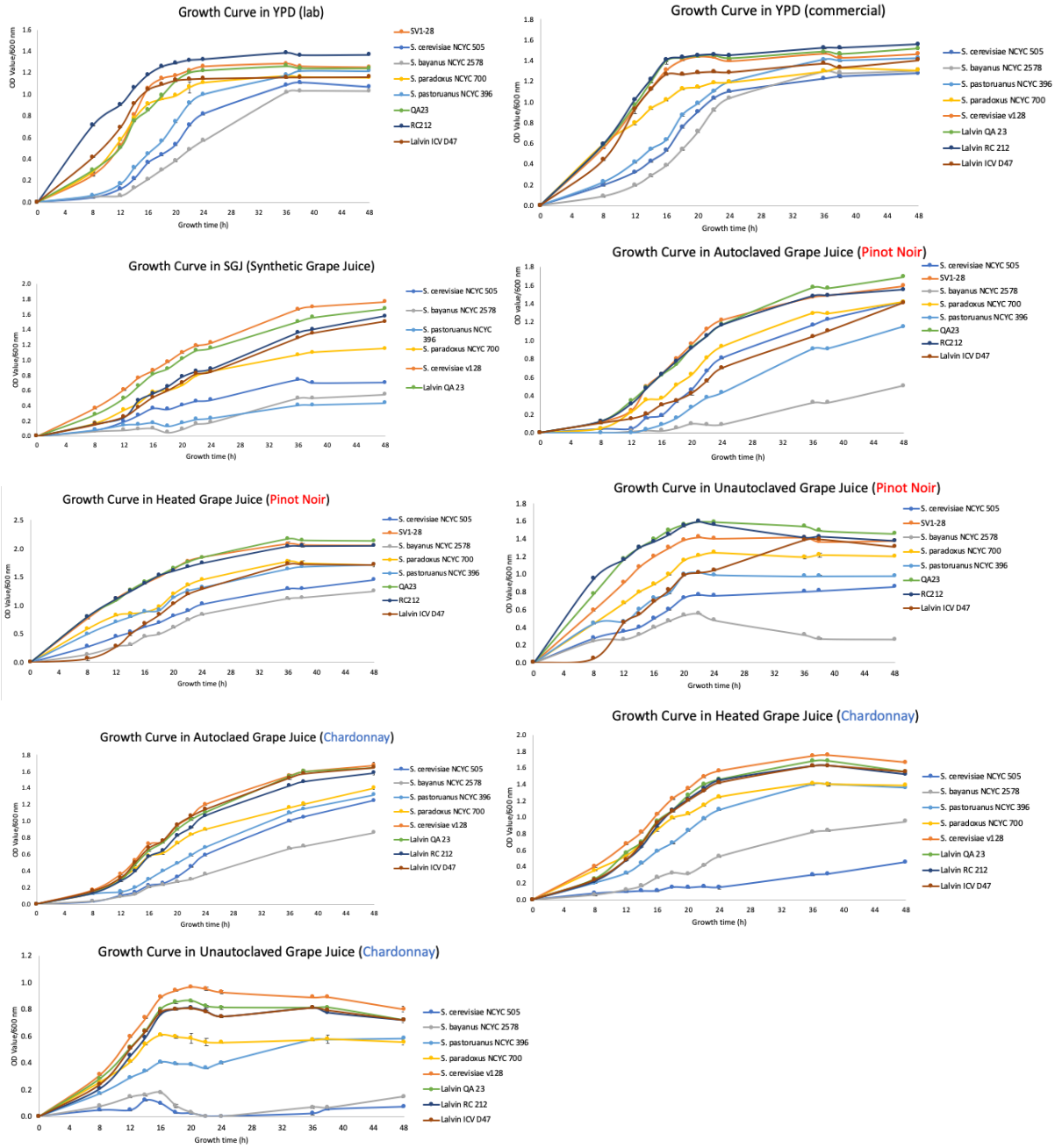


Figure A.13 Growth curves of eight yeast strains in nine culture media.

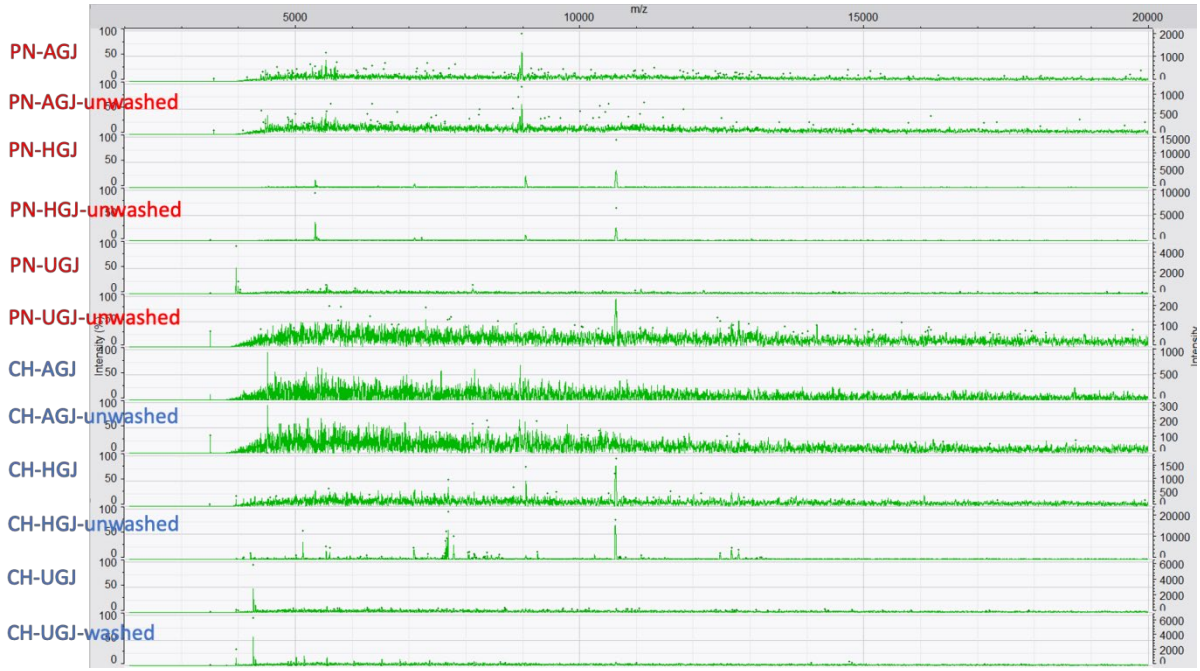


Figure A.14 MALDI spectra of Pinot Noir (PN) and Chardonnay (CH) in different treatments.

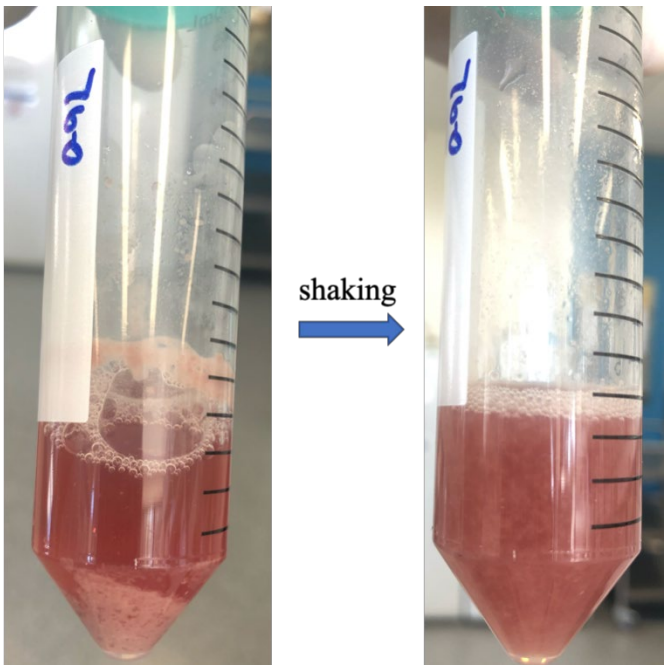
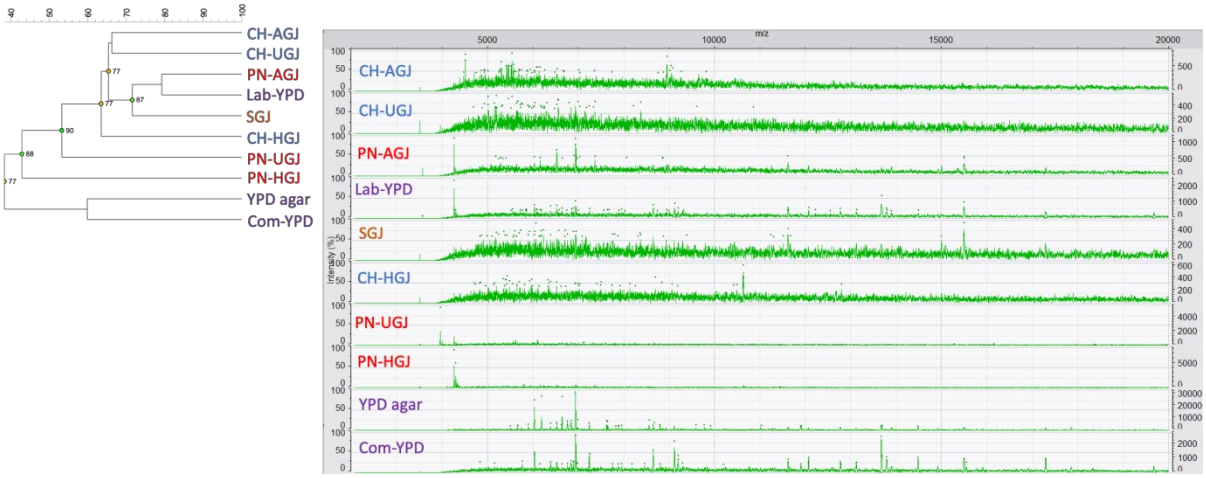
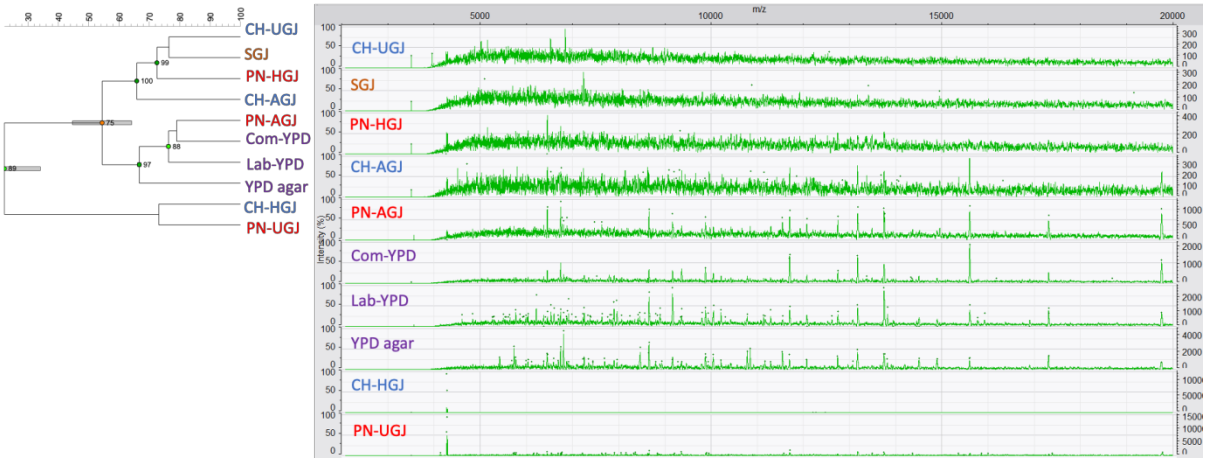


Figure A.15 *S. paradoxus* NCYC 700 grown in PN-HGJ after 24 h under 28 ˚C.

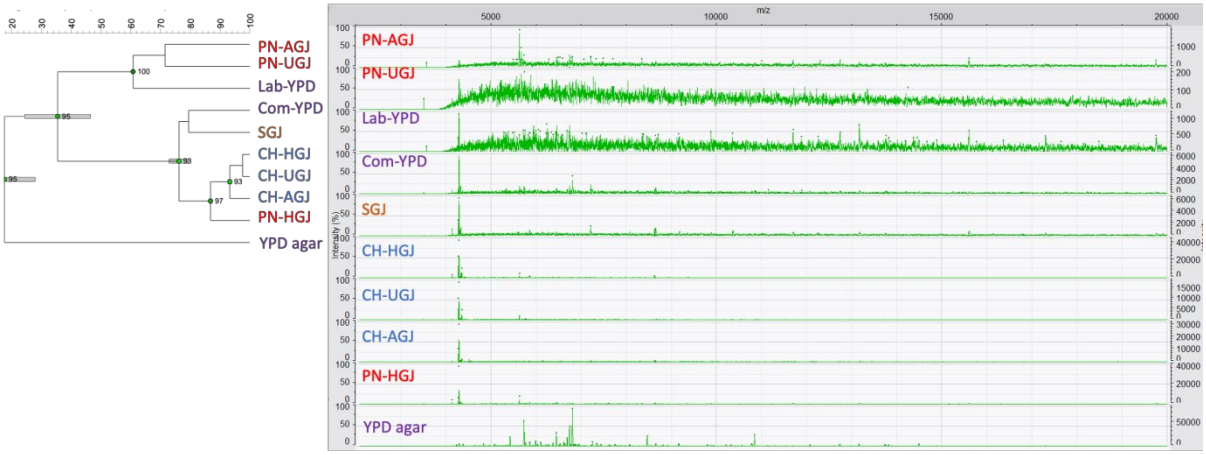
Reference strains-*S. bayanus* NCYC 2578



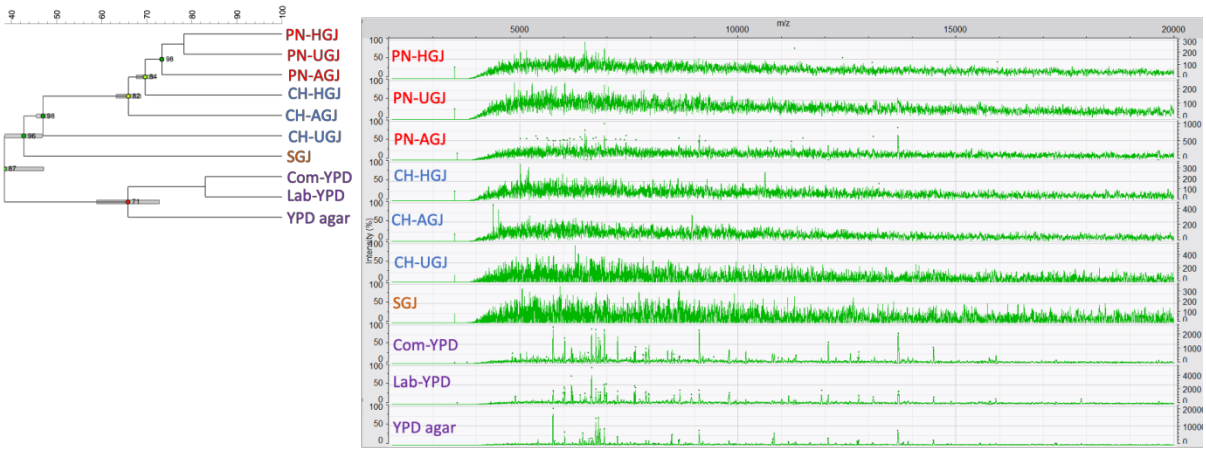
Reference strains-*S. cerevisiae* NCYC 505



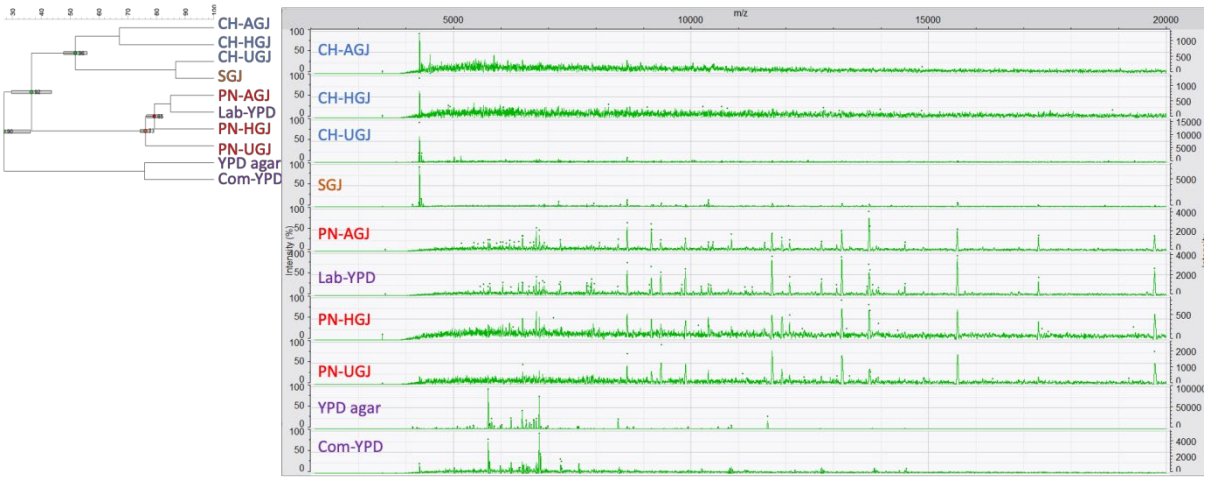
Reference strains-*S. paradoxus* NCYC 700



Reference strains-*S. pastorianus* NCYC 396



Wild isolate-*S. cerevisiae* v128



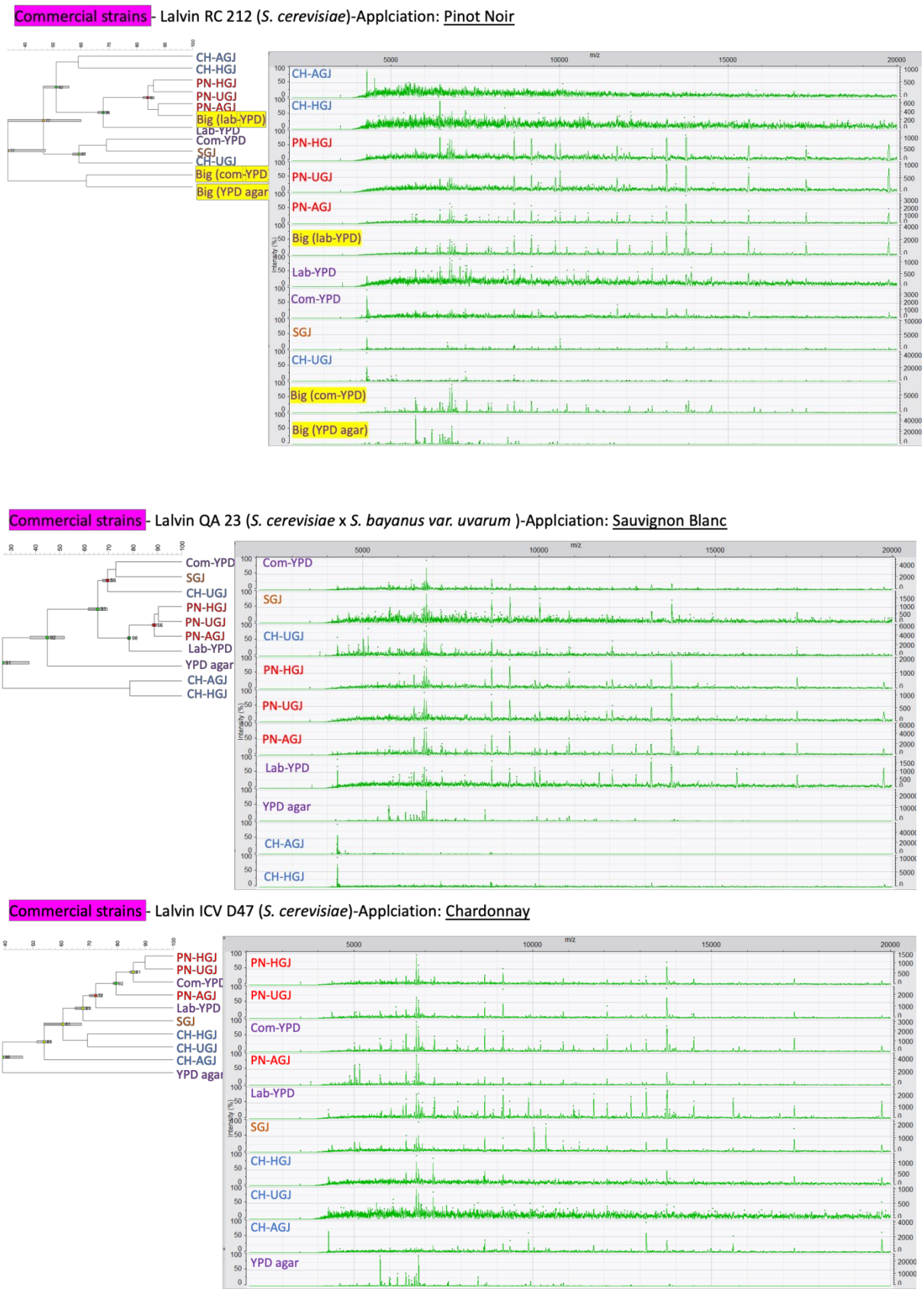
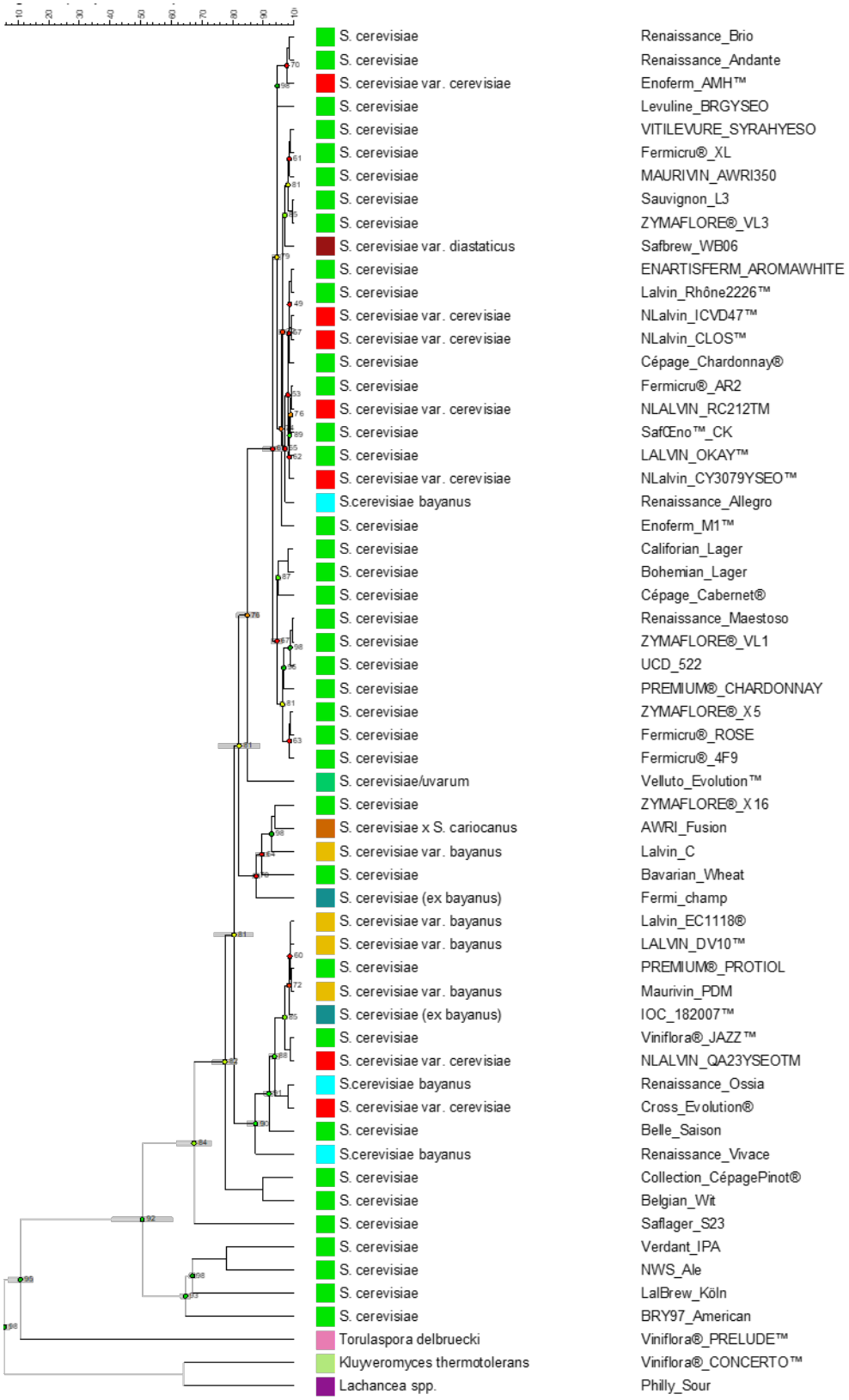


Figure A.16 Cluster analysis and corresponding spectra profiles of all tested strains grown in different media.

(A) YPD agar


S. cerevisiae Renaissance_Brio
S. cerevisiae Renaissance_Andante
S. cerevisiae var. cerevisiae Enoferm_AMH™
S. cerevisiae Levuline_BRGYSEO
S. cerevisiae VITILEVURE_SYRAHYESO
S. cerevisiae Fermicru®_XL
S. cerevisiae MAURIVIN_AWRI350
S. cerevisiae Sauvignon_L3
S. cerevisiae ZYMAFLORE®_VL3
S. cerevisiae var. diastaticus Safbrew_WB06
S. cerevisiae ENARTISFERM_AROMAWHITE
S. cerevisiae Lalvin_Rhône2226™
S. cerevisiae var. cerevisiae NLalvin_ICVD47™
S. cerevisiae var. cerevisiae NLalvin_CLOS™
S. cerevisiae Cépage_Chardonnay®
S. cerevisiae Fermicru®_AR2
S. cerevisiae var. cerevisiae NLALVIN_RC212TM
S. cerevisiae SafŒno™_CK
S. cerevisiae LALVIN_OKAY™
S. cerevisiae var. cerevisiae NLalvin_CY3079YSEO™
S.cerevisiae bayanus Renaissance_Allegro
S. cerevisiae Enoferm_M1™
S. cerevisiae Californian_Lager
S. cerevisiae Bohemian_Lager
S. cerevisiae Cépage_Cabernet®
S. cerevisiae Renaissance_Maestoso
S. cerevisiae ZYMAFLORE®_VL1
S. cerevisiae UCD_522
S. cerevisiae PREMIUM®_CHARDONNAY
S. cerevisiae ZYMAFLORE®_X5
S. cerevisiae Fermicru®_ROSE
S. cerevisiae Fermicru®_4F9
S. cerevisiae/uvarum Velluto_Evolution™
S. cerevisiae ZYMAFLORE®_X16
S. cerevisiae x S. cariocanus AWRI_Fusion
S. cerevisiae var. bayanus Lalvin_C
S. cerevisiae Bavarian_Wheat
S. cerevisiae (ex bayanus) Fermi_champ
S. cerevisiae var. bayanus Lalvin_EC1118®
S. cerevisiae var. bayanus LALVIN_DV10™
S. cerevisiae PREMIUM®_PROTIOL
S. cerevisiae var. bayanus Maurivin_PDM
S. cerevisiae (ex bayanus) IOC_182007™
S. cerevisiae Viniflora®_JAZZ™
S. cerevisiae var. cerevisiae NLALVIN_QA23YSEOTM
S.cerevisiae bayanus Renaissance_Ossia
S. cerevisiae var. cerevisiae Cross_Evolution®
S. cerevisiae Belle_Saison
S.cerevisiae bayanus Renaissance_Vivace
S. cerevisiae Collection_CépagePinot®
S. cerevisiae Belgian_Wit
S. cerevisiae Saflager_S23
S. cerevisiae Verdant_IPA
S. cerevisiae NWS_Ale
S. cerevisiae LalBrew_Köln
S. cerevisiae BRY97_American
Torulaspora delbrueckii Viniflora®_PRELUDE™
Kluyveromyces thermotolerans Viniflora®_CONCERTO™
Lachancea spp. Philly_Sour

(B) YPD broth

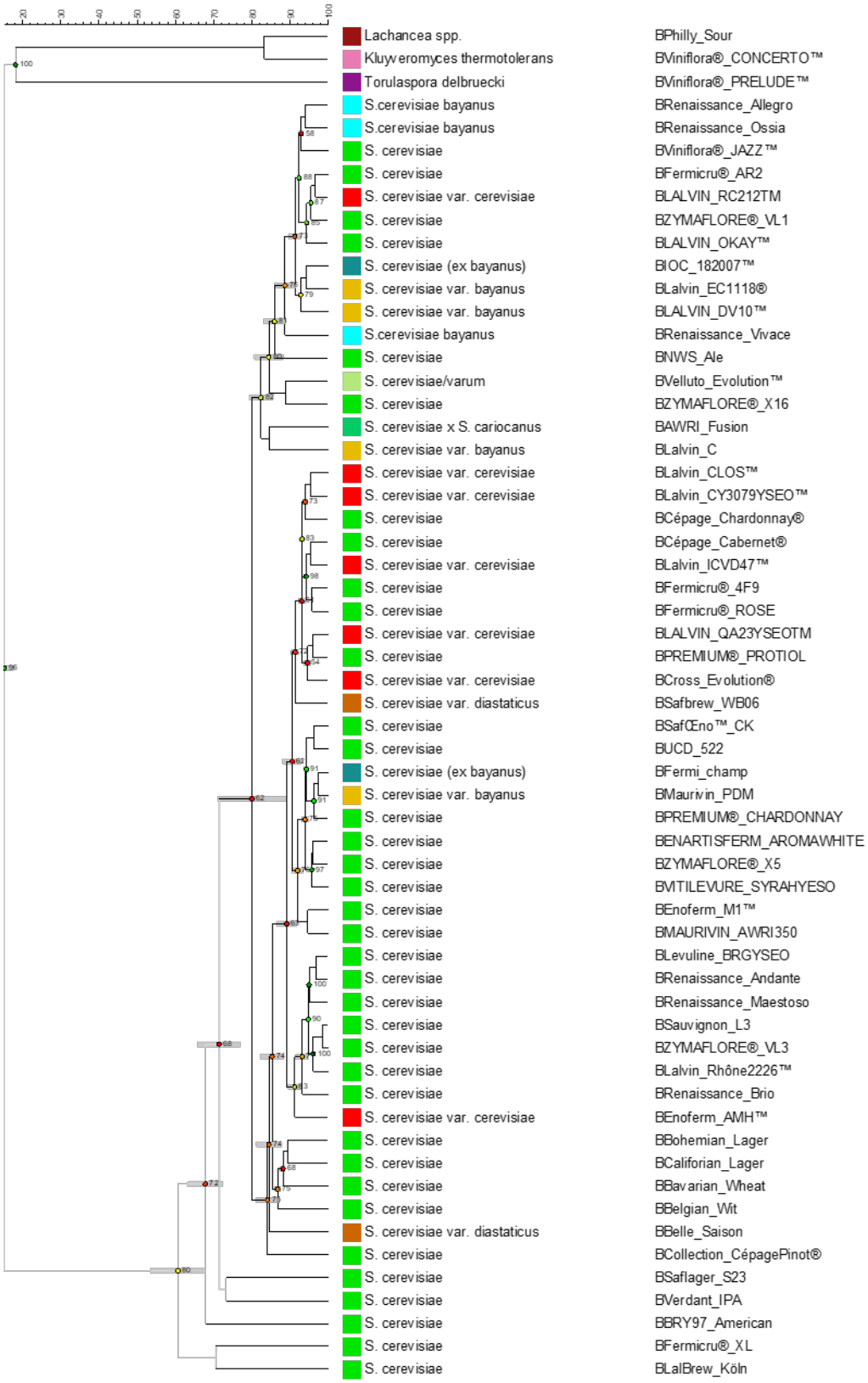


Figure A. 17 Cluster analysis of high mass profiles of 59 commercial strains (47 wine and 12 brewing strains) grown under (A) YPD agar and (B) YPD broth.

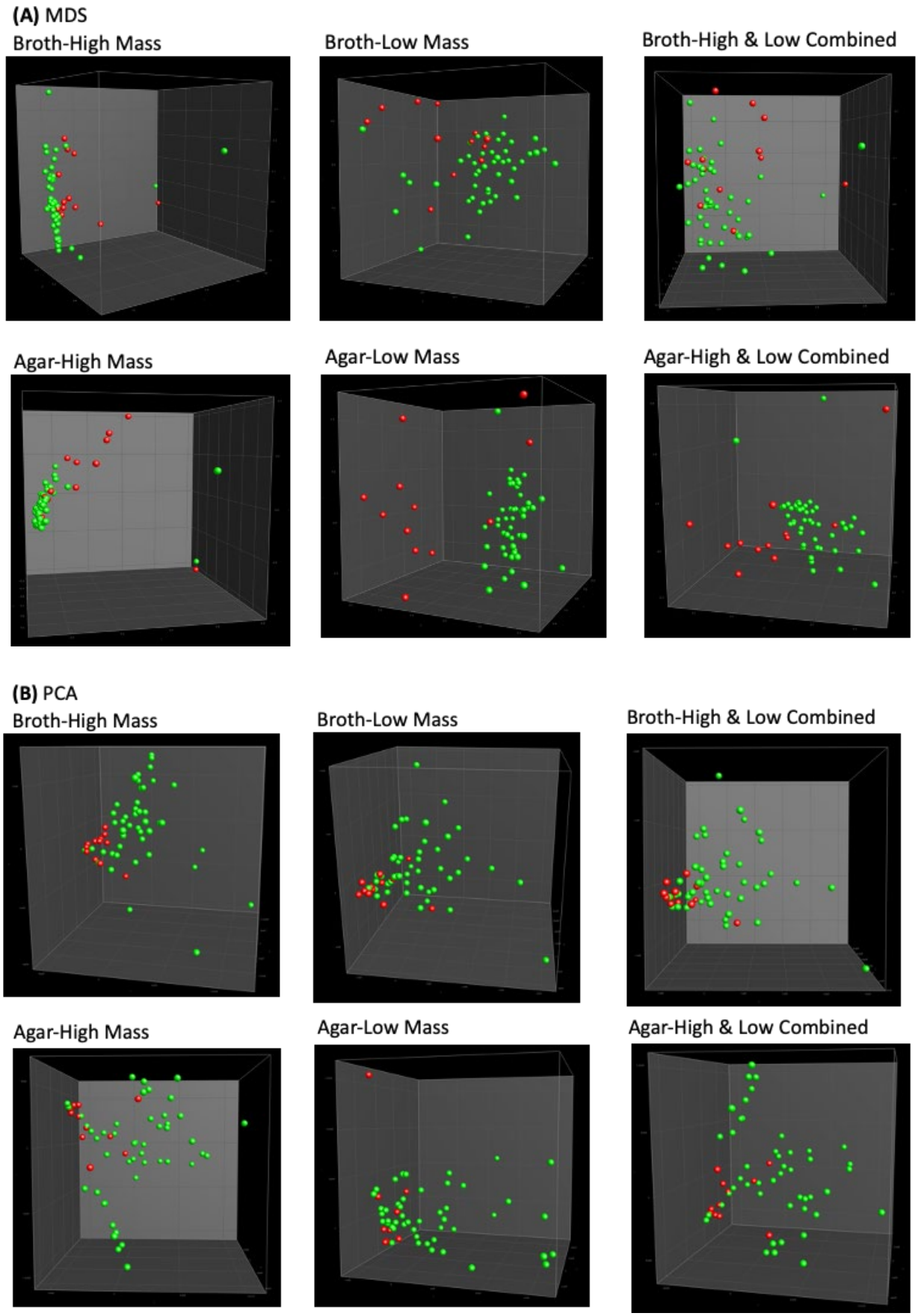
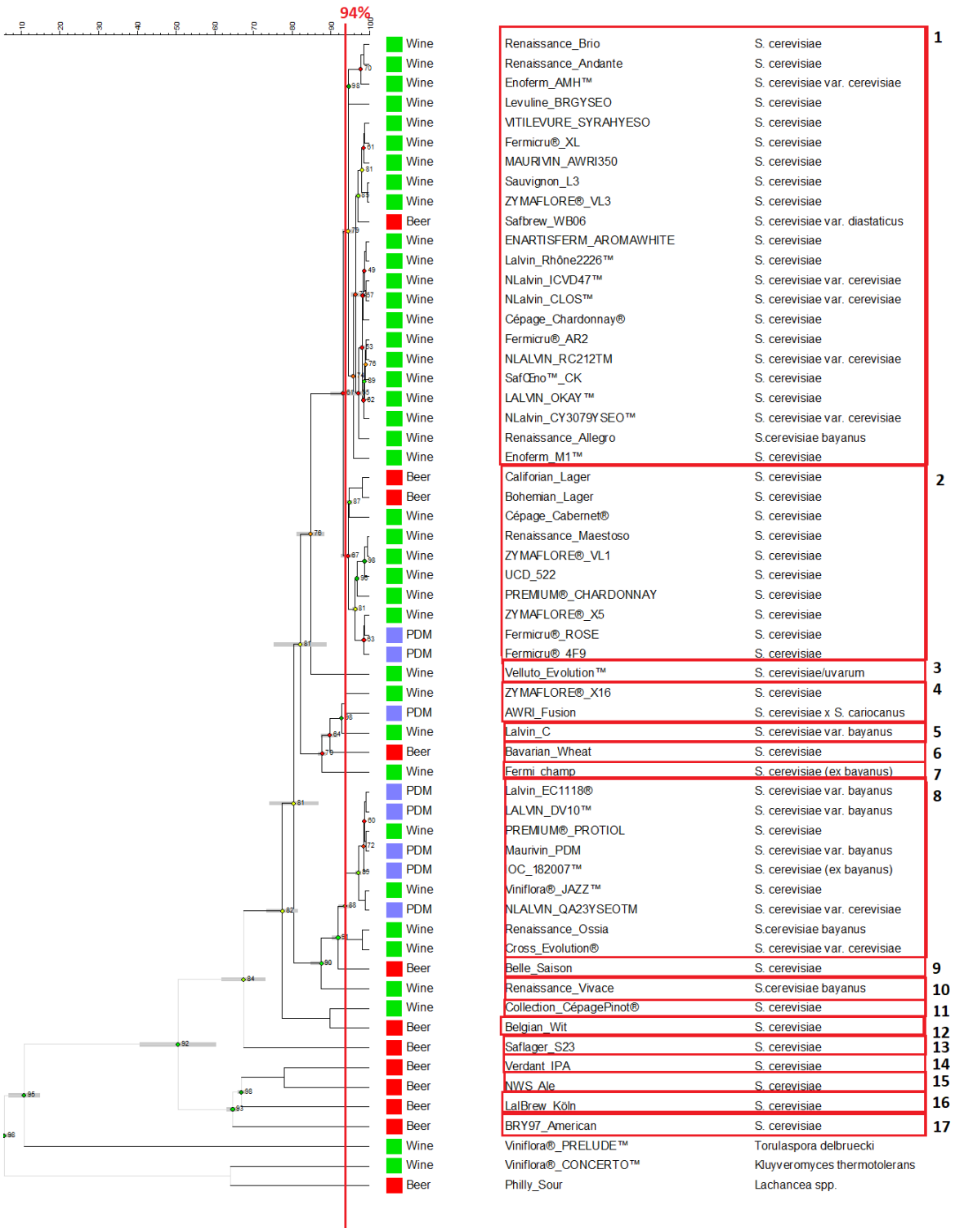


Figure A.18 (A) MDS analysis and (B) PCA analysis of high mass, low mass and high-low combined of 59 commercial yeast strains (47 wine and 12 brewing strains) under YPD broth and YPD agar.

(A) High Mass


94%
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Beer
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Wine
Beer
Beer
Wine
Wine
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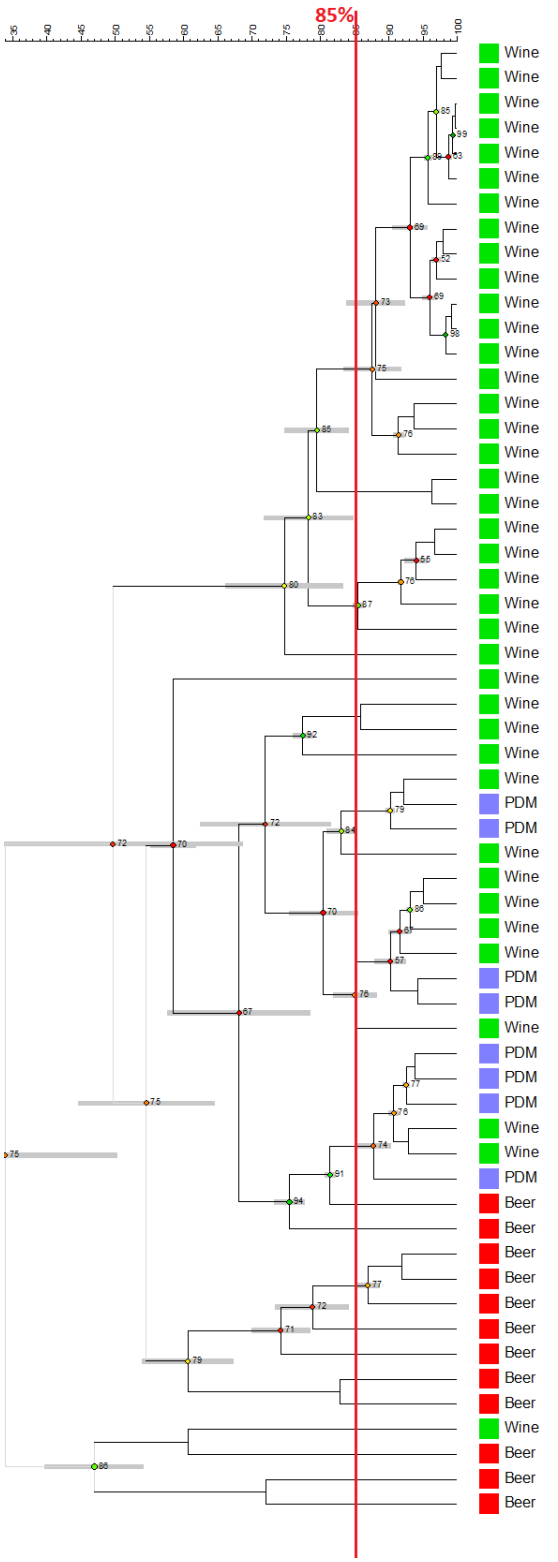
Beer
Renaissance_Brio S. cerevisiae 1
Renaissance_Andante S. cerevisiae
Enoferm_AMH™ S. cerevisiae var. cerevisiae
Levuline_BRGYSEO S. cerevisiae
VITILEVURE_SYRAHYESO S. cerevisiae
Fermicru®_XL S. cerevisiae
MAURIVIN_AWRI350 S. cerevisiae
Sauvignon_L3 S. cerevisiae
ZYMAFLORE®_VL3 S. cerevisiae
Safbrew_WB06 S. cerevisiae var. diastaticus
ENARTISFERM_AROMAWHITE S. cerevisiae
Lalvin_Rhône2226™ S. cerevisiae
NLalvin_ICVD47™ S. cerevisiae var. cerevisiae
NLalvin_CLOS™ S. cerevisiae var. cerevisiae
Cépage_Chardonnay® S. cerevisiae
Fermicru®_AR2 S. cerevisiae
NLALVIN_RC212TM S. cerevisiae var. cerevisiae
SafŒno™_CK S. cerevisiae
LALVIN_OKAY™ S. cerevisiae
NLalvin_CY3079YSEO™ S. cerevisiae var. cerevisiae
Renaissance_Allegro S.cerevisiae bayanus
Enoferm_M1™ S. cerevisiae
Californian_Lager S. cerevisiae 2
Bohemian_Lager S. cerevisiae
Cépage_Cabernet® S. cerevisiae
Renaissance_Maestoso S. cerevisiae
ZYMAFLORE®_VL1 S. cerevisiae
UCD_522 S. cerevisiae
PREMIUM®_CHARDONNAY S. cerevisiae
ZYMAFLORE®_X5 S. cerevisiae
Fermicru®_ROSE S. cerevisiae
Fermicru®_4F9 S. cerevisiae
Velluto_Evolution™ S. cerevisiae/uvarum 3
ZYMAFLORE®_X16 S. cerevisiae 4
AWRI_Fusion S. cerevisiae x S. cariocanus
Lalvin_C S. cerevisiae var. bayanus 5
Bavarian_Wheat S. cerevisiae 6
Fermi_champ S. cerevisiae (ex bayanus) 7
Lalvin_EC1118® S. cerevisiae var. bayanus 8
LALVIN_DV10™ S. cerevisiae var. bayanus
PREMIUM®_PROTIOL S. cerevisiae
Maurivin_PDM S. cerevisiae var. bayanus
OC_182007™ S. cerevisiae (ex bayanus)
Viniflora®_JAZZ™ S. cerevisiae
NLALVIN_QA23YSEOTM S. cerevisiae var. cerevisiae
Renaissance_Ossia S.cerevisiae bayanus
Cross_Evolution® S. cerevisiae var. cerevisiae
Belle_Saison S. cerevisiae 9
Renaissance_Vivace S.cerevisiae bayanus 10
Collection_CépagePinot® S. cerevisiae 11
Belgian_Wit S. cerevisiae 12
Saflager_S23 S. cerevisiae 13
Verdant_IPA S. cerevisiae 14
NWS_Ale S. cerevisiae 15
LalBrew_Köln S. cerevisiae 16
BRY97_American S. cerevisiae 17
Viniflora®_PRELUDE™ Torulaspora delbrueckii
Viniflora®_CONCERTO™ Kluyveromyces thermotolerans
Philly_Sour Lachancea spp.

(B) Low Mass



SafŒno™_CK	S. cerevisiae	1
ZYMAFLORE®_X5	S. cerevisiae	
ENARTISFERM_AROMAWHITE	S. cerevisiae	
VITILEVURE_SYRAHYESO	S. cerevisiae	
Lalvin_Rhône2226™	S. cerevisiae	
Sauvignon_L3	S. cerevisiae	
Enoferm_M1™	S. cerevisiae	
MAURIVIN_AWRI350	S. cerevisiae	
ZYMAFLORE®_VL3	S. cerevisiae	
Renaissance_Brio	S. cerevisiae	
Renaissance_Maestoso	S. cerevisiae	
Renaissance_Andante	S. cerevisiae	
UCD_522	S. cerevisiae	
Fermicru®_AR2	S. cerevisiae	
Levuline_BRGYSEO	S. cerevisiae	
ZYMAFLORE®_X16	S. cerevisiae	
NLalvin_ICVD47™	S. cerevisiae var. cerevisiae	
Viniflora®_CONCERTO™	Kluyveromyces thermotolerans	2
LALVIN_OKAY™	S. cerevisiae	
Fermicru®_XL	S. cerevisiae	3
NLalvin_CLOS™	S. cerevisiae var. cerevisiae	
Cépage_Chardonnay®	S. cerevisiae	
NLALVIN_RC212TM	S. cerevisiae var. cerevisiae	
Collection_CépagePinot®	S. cerevisiae	
Velluto_Evolution™	S. cerevisiae/uvarum	4
Viniflora®_JAZZ™	S. cerevisiae	5
Renaissance_Ossia	S.cerevisiae bayanus	6
Lalvin_C	S. cerevisiae var. bayanus	
Renaissance_Vivace	S.cerevisiae bayanus	7
Cross_Evolution®	S. cerevisiae var. cerevisiae	8
Fermicru®_ROSE	S. cerevisiae	
AWRI_Fusion	S. cerevisiae x S. cariocanus	
Fermi_champ	S. cerevisiae (ex bayanus)	9
ZYMAFLORE®_VL1	S. cerevisiae	10
NLalvin_CY3079YSEO™	S. cerevisiae var. cerevisiae	
Enoferm_AMH™	S. cerevisiae var. cerevisiae	
Cépage_Cabernet®	S. cerevisiae	
Fermicru®_4F9	S. cerevisiae	
NLALVIN_QA23YSEOTM	S. cerevisiae var. cerevisiae	
PREMIUM®_CHARDONNAY	S. cerevisiae	
Maurivin_PDM	S. cerevisiae var. bayanus	11
Lalvin_EC1118®	S. cerevisiae var. bayanus	
IOC_182007™	S. cerevisiae (ex bayanus)	
Renaissance_Allegro	S.cerevisiae bayanus	
PREMIUM®_PROTIOL	S. cerevisiae	
LALVIN_DV10™	S. cerevisiae var. bayanus	
Belle_Saison	S. cerevisiae	12
Philly_Sour	Lachancea spp.	
Californian_Lager	S. cerevisiae	13
Bohemian_Lager	S. cerevisiae	
Saflager_S23	S. cerevisiae	
Belgian_Wit	S. cerevisiae	14
Safbrew_WB06	S. cerevisiae var. diastaticus	15
NWS_Ale	S. cerevisiae	16
LalBrew_Köln	S. cerevisiae	17
Viniflora®_PRELUDE™	Torulaspora delbruecki	
BRY97_American	S. cerevisiae	18
Verdant_IPA	S. cerevisiae	19
Bavarian_Wheat	S. cerevisiae	20

(C) High & Low Combined

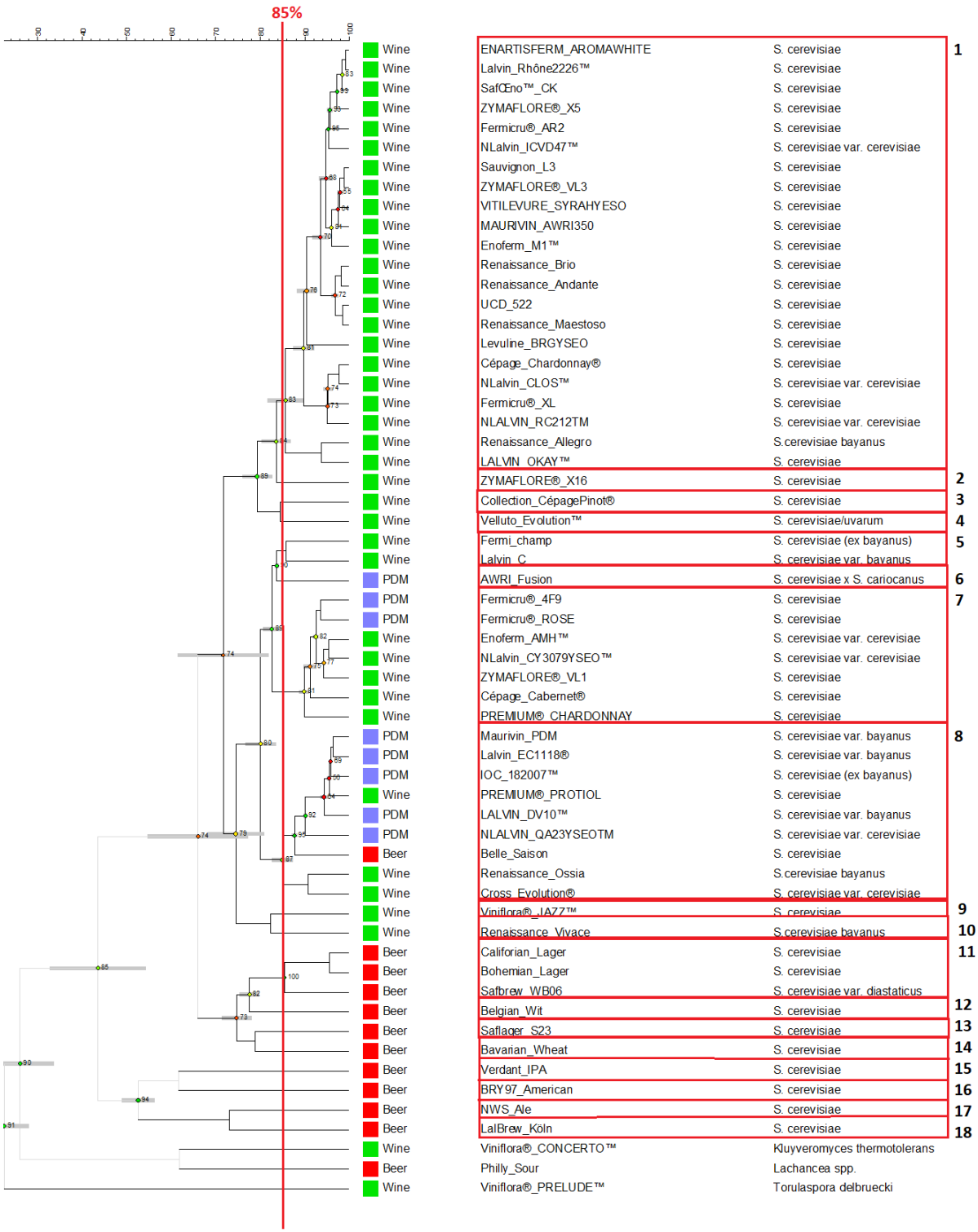
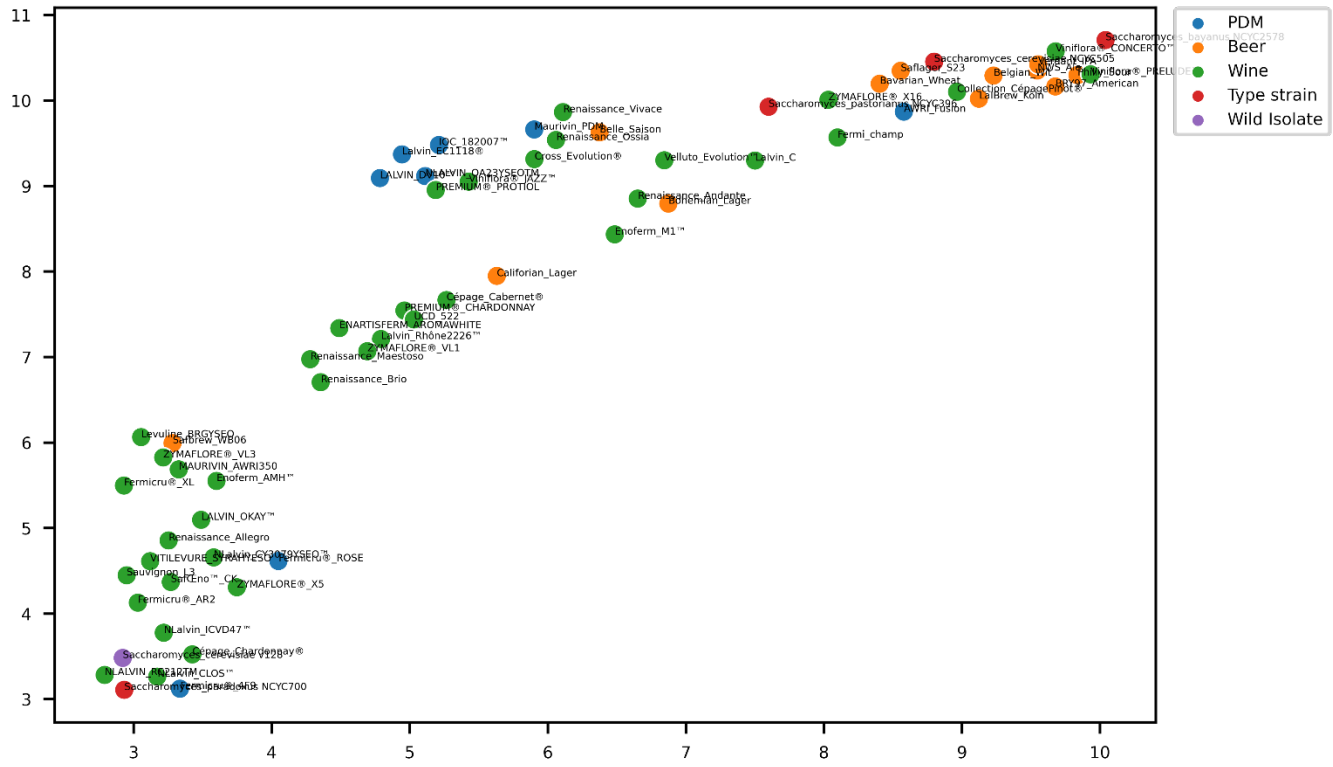
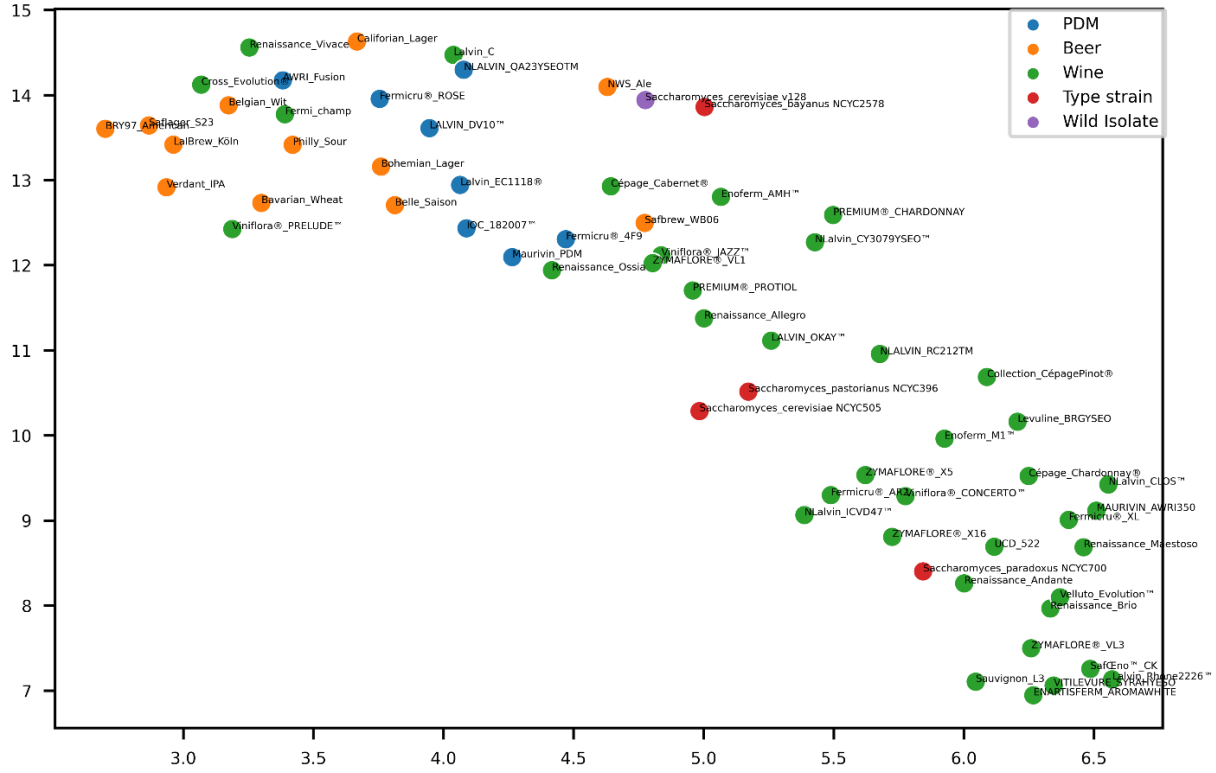


Figure A.19 Cluster analysis of high mass profiles of 59 commercial strains (47 wine and 12 brewing strains) grown on YPD agar (A) High Mass, (B) Low Mass and (C) High & Low Combined.

(A) High Mass



(B) Low Mass



(C) High & Low Combined

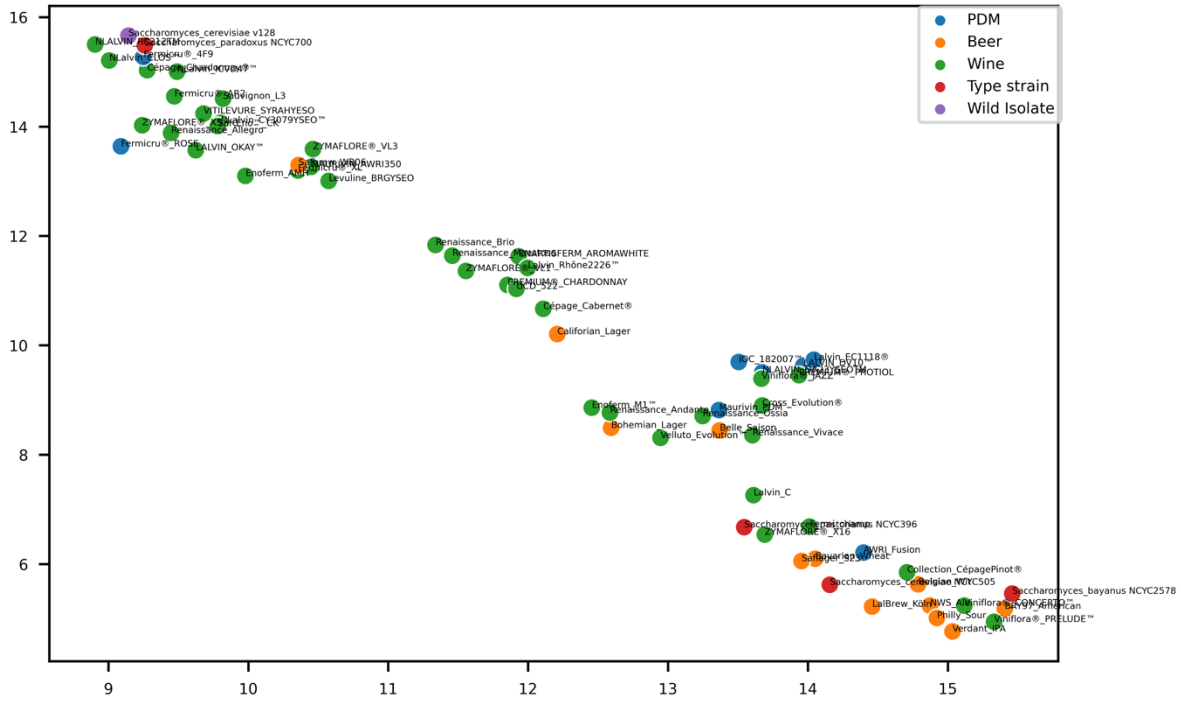


Figure A.20 UMAP analysis of (A) high mass, (B) low mass and (C) high & low combined data of 62 yeast strains-45 wine strains (green/purple), 12 brewing strains (red), *S. cerevisiae* v128 (blue), *S. cerevisiae* NYC 505 (yellow), *S. paradoxus* NCYC 700(yellow), *S. pastorianus* NCYC 396 (yellow), *S. bayanus* NCYC 2578 (yellow).

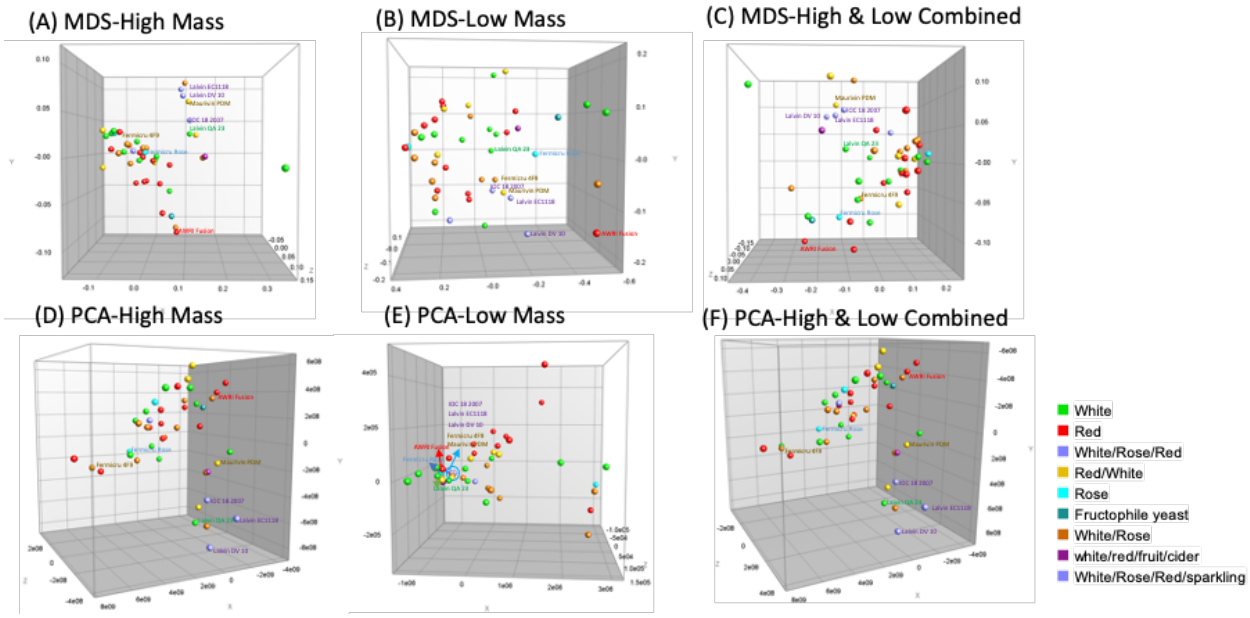
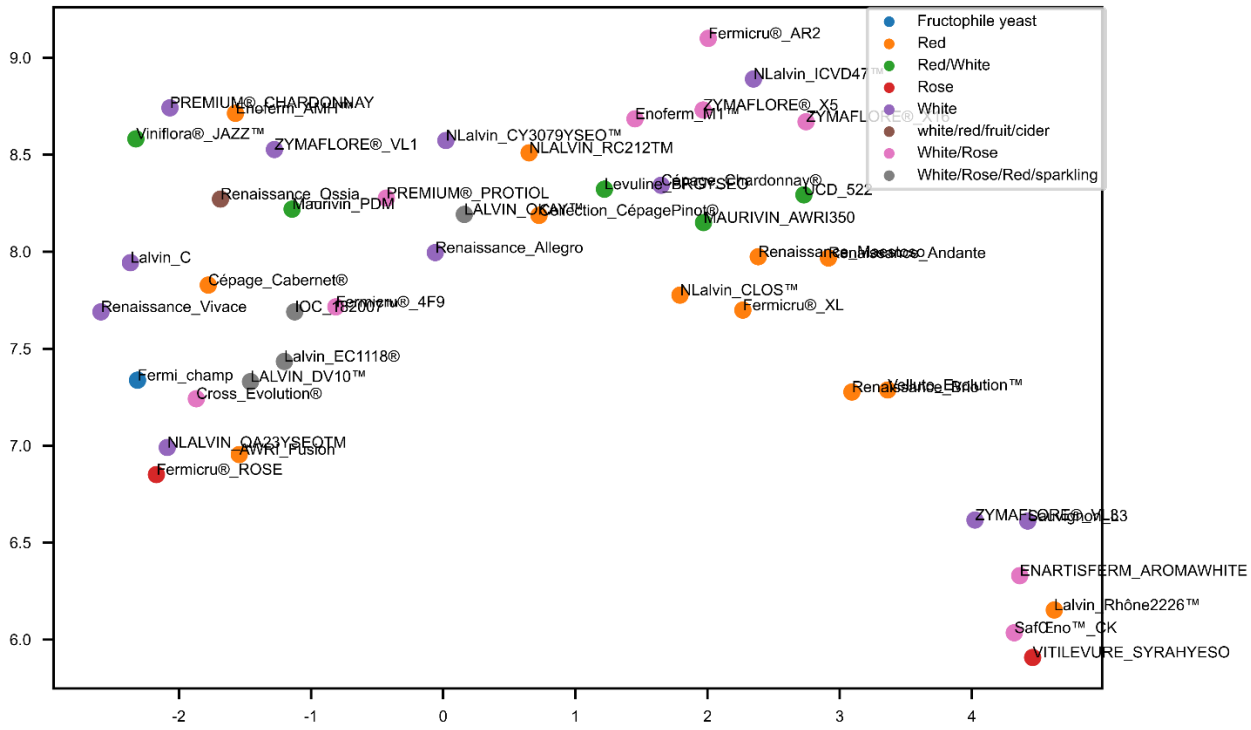


Figure A.21 MDS and PCA analysis of 45 commercial wine *Saccharomyces* strains.

(A) Low Mass



(B) High & Low Combined

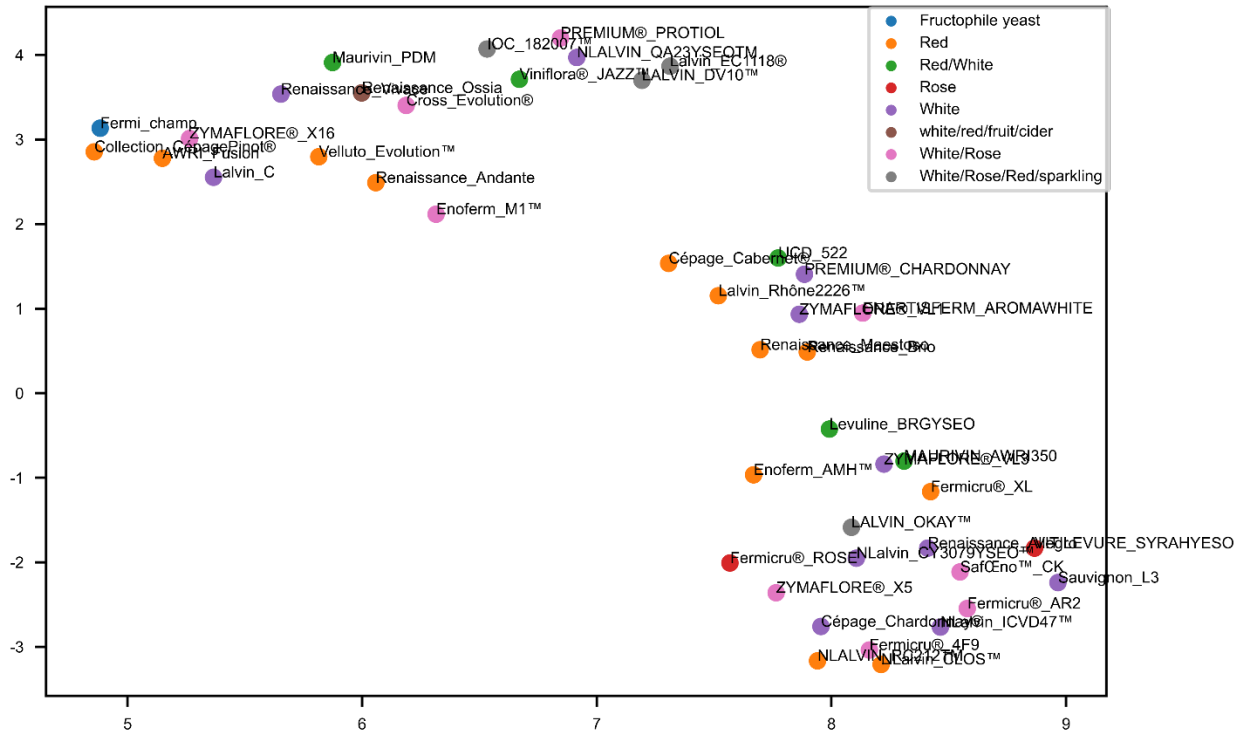


Figure A.22 UMAP analysis of (A) Low mass and (B) High & Low combined data of 45 commercial wine *Saccharomyces* strains.

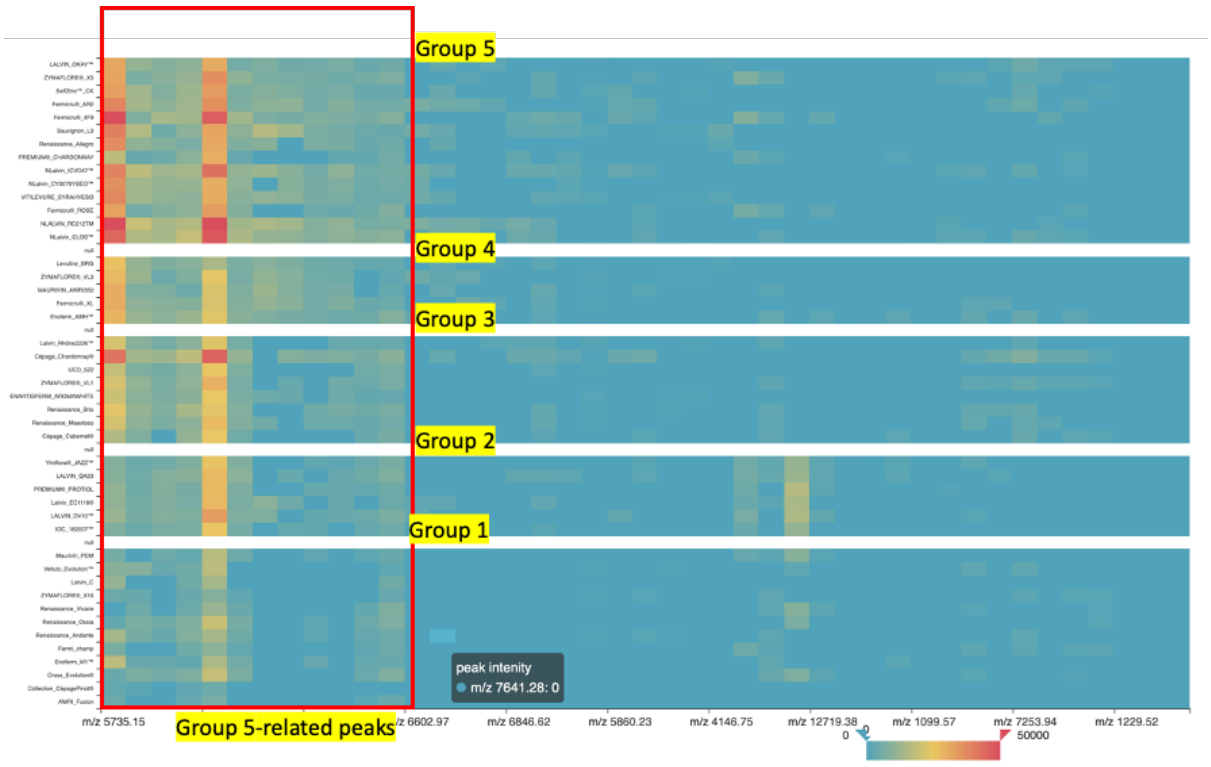
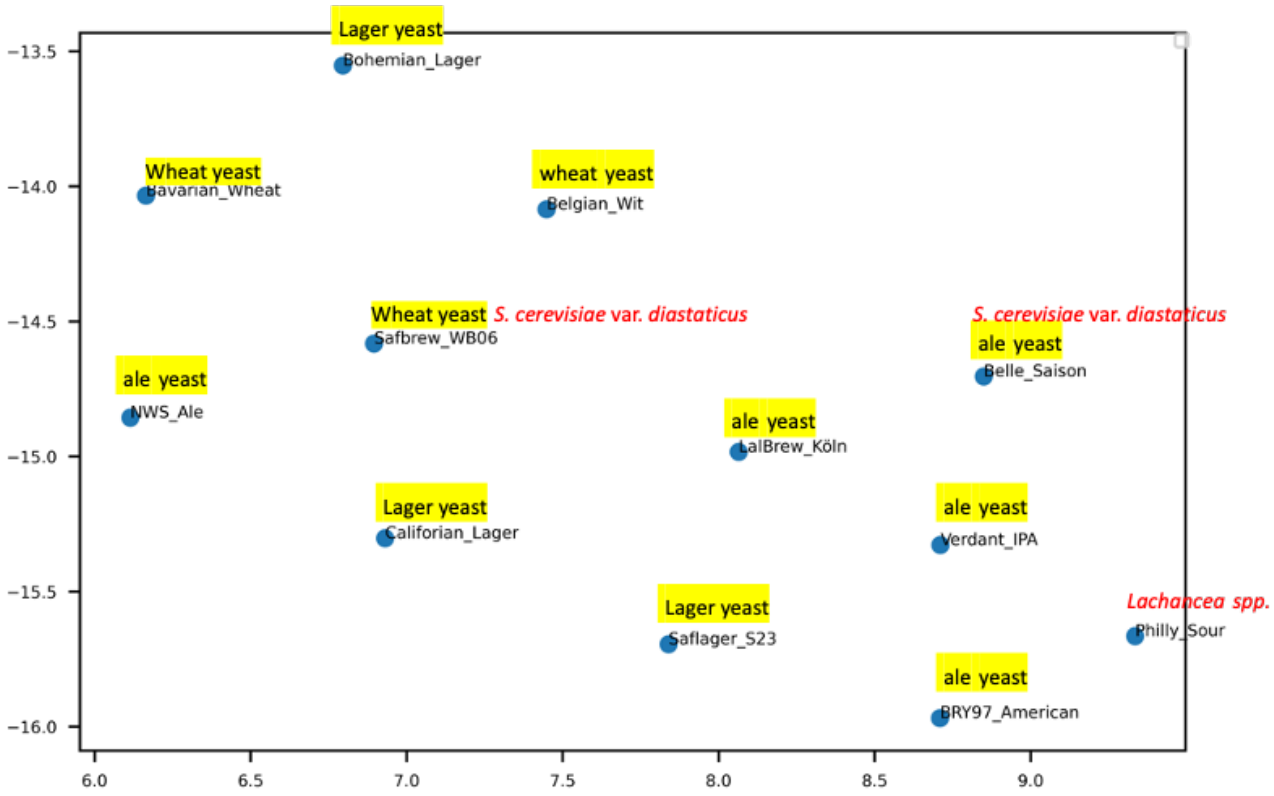


Figure A.23 Heatmap of peak classes detected from 45 commercial wine strains and grouped according to UMAP analysis. Red colour represents the highest peak intensity, whereas the blue colour represents the lowest peak intensity.

(A) Low Mass



(B) High & Low Combined

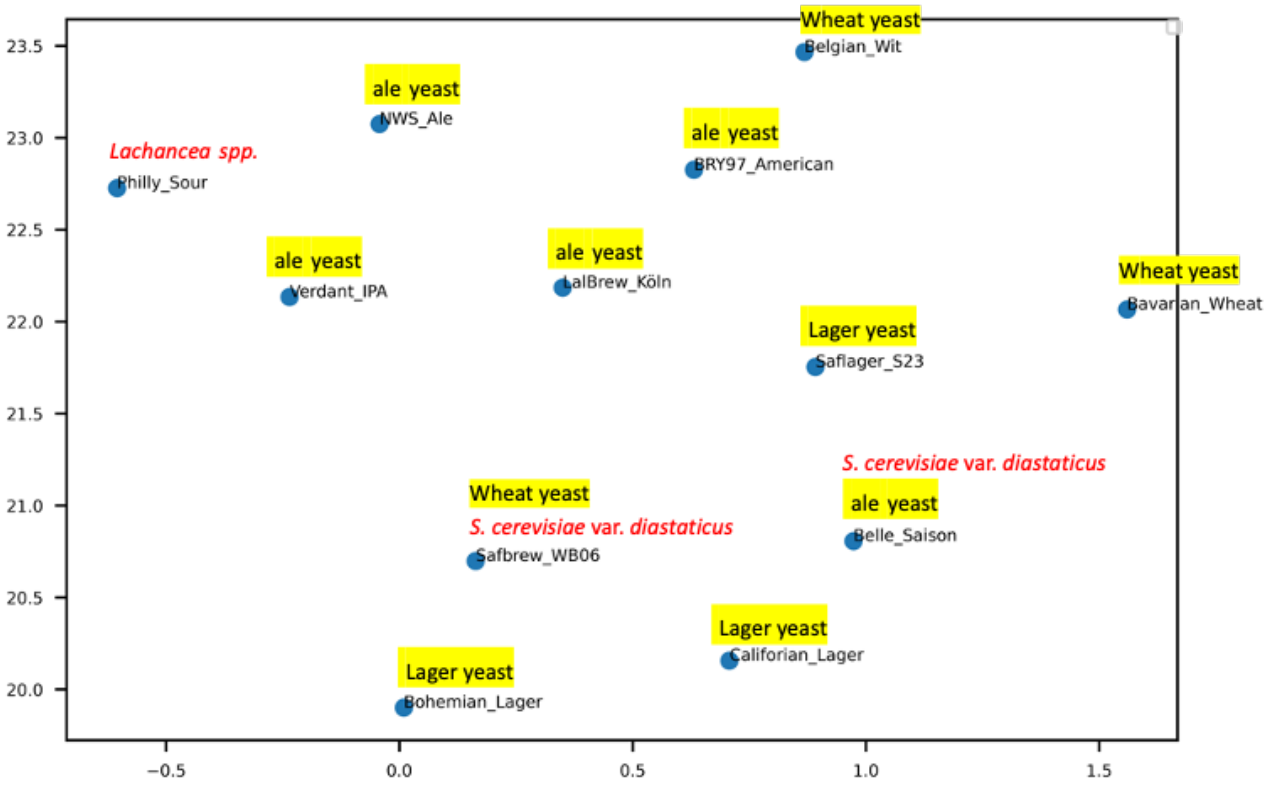


Figure A.24 UMAP analysis of (A) Low mass and (B) High & Low combined data of 12 commercial brewing strains.

Appendix B

26S rDNA Sequence Data

B.1 26S rDNA sequence BLAST results

Table B.1 26S rDNA sequencing results of 73 isolates

	Isolate	Species designation	Similarity	Fragment sequenced	Primer
1	SV1-1	*Metschnikowia Pulcherrima*	99%	439 bp	NL1
2	SV1-4	*Starmerella bacillaris*	99%	554 bp	NL1
3	SV1-8	*Pichia terricola*	100%	327 bp	NL1
4	SV1-12	*Hanseniaspora uvarum*	100%	435 bp	NL1
5	SV1-14	*Issatchenkia terricola*	100%	444 bp	NL1
6	SV1-16	*Starmerella bacillaris*	99%	360 bp	NL1
7	SV1-18	*Hanseniaspora uvarum*	100%	478 bp	NL1
8	SV1-28	*Saccharomyces cerevisiae*	100%	331 bp	NL1
9	SV1-35	*[Candida] californica*	100%	514 bp	NL4
10	SV1-36	*Pichia kluyveri*	100%	487 bp	NL1
11	SV1-37	*Pichia kluyveri*	100%	471 bp	NL1
12	SV1-38	*Pichia kluyveri*	100%	499 bp	NL1
13	SV1-41	*Pichia kluyveri*	100%	414 bp	NL1
14	SV1-49	*Pichia kluyveri*	98%	456 bp	NL1
15	SV1-52	*Pichia kluyveri*	100%	355 bp	NL1
16	SV1-62	*Pichia kluyveri*	100%	480 bp	NL1
17	SV1-63	*Pichia kluyveri*	100%	469 bp	NL1
18	SV1-64	*Pichia kluyveri*	100%	503 bp	NL1
19	SV1-66	*Pichia kluyveri*	100%	478 bp	NL1
20	SV1-68	*Pichia kluyveri*	100%	489 bp	NL1
21	SV1-70	*Pichia kluyveri*	100%	468 bp	NL1
22	SW1-2	*Pichia kluyveri*	100%	485 bp	NL1
23	SW1-3	*Metschnikowia aff. Fructicola*	99%	435 bp	NL1
24	SW1-5	*Starmerella bacillaris*	99%	404 bp	NL1
25	SW1-7	*Starmerella bacillaris*	96%	352 bp	NL1
26	SW1-8	*Pichia kluyveri*	100%	474 bp	NL1
27	SW1-9	*Pichia kluyveri*	100%	473 bp	NL1
28	SW1-10	*Starmerella bacillaris*	100%	415 bp	NL1
29	SW1-11	*Starmerella bacillaris*	99%	421 bp	NL1
30	SW1-12	*Pichia kluyveri*	98%	284 bp	NL1
31	SW1-14	*Pichia kluyveri*	100%	497 bp	NL1
32	SW1-16	*Hanseniasopora uvarum*	99%	317 bp	NL1
33	SW1-17	*Hanseniasopora uvarum*	100%	451 bp	NL1
34	SW1-19	*Pichia kluyveri*	100%	416 bp	NL1

35	SW1-21	*M. Pulcherrima*	97%	301 bp	NL1
36	SW1-23	*Hanseniasopora uvarum*	100%	511 bp	NL1
37	SW1-24	*[Candida] californica*	99%	513 bp	NL4
38	SW1-25	*Starmerella bacillaris*	98%	389 bp	NL1
39	SW1-26	*Pichia kluyveri*	100%	500 bp	NL1
40	SW1-27	*[Candida] californica*	100%	541 bp	NL4
41	SW1-28	*Pichia membranifaciens*	99%	550 bp	NL1
42	SW1-29	*C. californica*	100%	542 bp	NL1
		P. membranifaciens	98%		
43	SW1-31	*Aureobasidium pullulans*	99%	297 bp	NL1
44	SW1-32	*Aureobasidium pullulans*	100%	381 bp	NL1
45	SW1-34	*Starmerella bacillaris*	97%	342 bp	NL1
46	SV2-7	*Hanseniaspora uvarum*	100%	311 bp	NL1
47	SV2-28	*Saccharomyces uvarum*	100%	492	NL1
		Saccharomyces bayanus	100%		
48	SV2-29	*Saccharomyces uvarum*	99%	339	NL1
		Saccharomyces bayanus	99%		
49	SV2-33	*Pichia kudriavzevii*	100%	269 bp	NL1
50	SV2-34	*Wickerhamomyces anomalus*	100%	321 bp	NL1
51	SV2-60	*Pichia kluyveri*	100%	281 bp	NL1
52	SV3-5	*Saccharomyces cerevisiae*	97%	554 bp	NL1
53	SV3-10	*Saccharomyces uvarum*	100%	471 bp	NL1
		Saccharomyces bayanus	100%		
54	SV4-2	*Saccharomyces cerevisiae*	100%	437 bp	NL1
55	SV4-3	*Saccharomyces cerevisiae*	100%	339 bp	NL1
56	SV4-5	*Saccharomyces cerevisiae*	100%	399 bp	NL1
57	SV4-6	*Saccharomyces cerevisiae*	100%	297 bp	NL1
58	SV4-11	*Saccharomyces cerevisiae*	100%	236 bp	NL1
59	SV4-14	*Saccharomyces cerevisiae*	99%	334 bp	NL1
60	SV4-15	*Saccharomyces cerevisiae*	100%	340 bp	NL1
61	SV4-17	*Saccharomyces cerevisiae*	100%	252 bp	NL1
62	SV4-18	*Saccharomyces cerevisiae*	100%	206 bp	NL1
63	SV4-22	*Saccharomyces paradoxus*	100%	386 bp	NL1
64	SW2-13	*Saccharomyces uvarum*	100%	413 bp	NL1
		Saccharomyces bayanus	100%		
65	SW2-24	*Saccharomyces uvarum*	100%	271 bp	NL1
		Saccharomyces bayanus	100%		
66	SW2-28	*Saccharomyces uvarum*	100%	460 bp	NL1
		Saccharomyces bayanus	100%		
67	SW2-32	*Saccharomyces uvarum*	100%	448 bp	NL1
		Saccharomyces bayanus	100%		
68	SW3-1	*Hanseniaspora uvarum*	100%	326 bp	NL1
69	SW3-9	*Saccharomyces cerevisiae*	100%	399 bp	NL1
70	SW3-13	*Saccharomyces uvarum*	100%	414 bp	NL1

		Saccharomyces bayanus	100%		
71	SW3-15	*Starmerella bacillaris*	99%	285 bp	NL1
72	SW4-1	*Saccharomyces uvarum*	100%	287 bp	NL1
		Saccharomyces bayanus	100%		
73	SW4-7	*Saccharomyces cerevisiae*	99%	358 bp	NL1

B.2 26S rDNA sequences

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>SV1-4 NL1_2019-02-25_C08_2032

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>SV1-12 NL1_2019-02-25_F08_2032

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> SV1-16 NL1_2019-02-25_H08_2032

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> SV1-18-NL1_2019-02-26_A09_2033

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> SV1-37 NL1_2019-02-26_F09_2033

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> SV1-38 NL1_2019-02-26_G09_2033

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> SV1-41 NL1_2019-02-26_H09_2033

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> SV1-49 NL1_2019-02-26_A10_2033

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> SV1-52 NL1_2019-02-26_B10_2033

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> SV1-62 NL1_2019-02-26_C10_2033

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> SV1-63 NL1_2019-02-26_D10_2033

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TTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAC
AGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGC
GGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCG
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> SV1-64 NL1_2019-02-26_E10_2033

GGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACG
TGTGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTAGCGTGCATGTCGACACCTGTGA
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TTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAC
AGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGC
GGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCG
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>SV1-66 NL1_2019-02-26_F10_2033

GGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACG
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GGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATA
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AGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGC
GGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCG
GAGTGTTATAGCCTTTTGTAGATGCTGCGTATGGGGACCGAGG

> SV1-68 NL1_2019-02-26_G10_2033

GGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACG
TGTGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGA
GGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATA
TTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAC
AGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGC
GGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCG
GAGTGTTATAGCCTTTTGTAGATGCTGCGTATGGGGACCGAGGGCTGCGGCGGA

> SV1-70 NL1_2019-02-26_H10_2033

CAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTG
TGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGAGG
CCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATTG
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ACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGCGG
CGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCGGA
GTGTTATAGCCTTTTGTAGATGCTGCGTATGGT

> SW1-2 NL1_2019-02-25_A05_2031

CAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTG
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CCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATTG
GCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGC
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GTGTTATAGCCTTTTGTAGATGCTGCGTATGGGGACCGAGGGCTGCGGCG

> SW1-3 NL1_2019-02-25_B05_2031

CAAAAGCTCAAATTTGAAATCCCCCGGGAATTGTAATTTGAAGAGATTTGGGTCCGGCCGGCGGGGGTTAAGT
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AGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATACCGGCGAGAGACC

GATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATT
GTTGAAAGGGAAGGGCTTGCAAGCAGACACTTAACTGGGCCAGCATCGGGGCGGCGGGAAACAAAACCACC
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> SW1-5 NL1_2019-02-25_C05_2031

CAAGAGCTCAGATTTGAAAGGCACTTTTGTGCTGTTGGTATTCTGAAGTTAGGGTCCTGAGAACGATGCTTAA
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GTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAATATCTGCGAGAGACCGA
TAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTT
GAAATGGAAGGGTAGGCCGCTAACCATGTAGAGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCT
CCTCGGAGTATTATAGATGCAGTTCATATTCCCACCCGAGC

> SW1-8 NL1_2019-02-25_E05_2031

CGGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGAC
GTGTGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTAGCGTGCATGTCGACACCTGTG
AGGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAAT
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CAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGG
CGGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTC
GGAGTGTTATAGCCTTTTGTAGATGCTGCGTATGGGGAC

> SW1-9 NL1_2019-02-25_F05_2031

CGGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGAC
GTGTGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTG
AGGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAAT
ATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAA
CAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGG
CGGCGCTCCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTC
GGAGTGTTATAGCCTTTTGTAGATGCTGCGTATGGGG

> SW1-10 NL1_2019-02-25_G05_2031

CAAGAGCTCAGATTTGAAAGGCACTTTTGTGGGTTGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAAG
TCTTCTGGAAAGGAGCGCCATGGAGGGTGATAGCCCCGTCTAGCATTGACCTCATATAGGATCTTAACATGGA
GTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAATATCTGCGAGAGACCGA
TAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTT

GAAATGGAAGGGTAGGCCGCTAACCATGTAGAGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCT
CCTCGGAGTATTATAGATGCAGTTCATATTCCCACCCGAGCGCGAGGATCTC

>SW1-11 NL1_2019-02-25_H05_2031

CAGGCAAGAGCTCAGATTTGAAAGGCACTTTTGTGGGTTGGTATTCTGAAGTTAGGGTCCTGAGAAACGATGC
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ATGGAGTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAATATCTGCGAGAG
ACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAA
ATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAGAGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAAT
GTAGCTCCTCGGAGTATTATAGATGCAGTTCATATTCCCACCCCGAGCGCGAGGGATCT

> SW1-12 NL1_2019-02-25_A06_2031

GCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTGTGTGC
AAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGAGGCCCTTC
TGAAGAGGTGGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATTGGCGA
GAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCCCTTTGATAAGAGAGTGAAACA

> SW1-14 NL1_2019-02-25_B06_2031

CAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTG
TGTGCAAGTCCCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGAGG
CCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATTG
GCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGC
ACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGCGG
CGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCGGA
GTGTTATAGCCTTTTGTAGATGCTGCGTATGGGGACCGAGGGCTGCGGCGGACTCGTTTTCG

SW1-16 NL1_2019-02-25_D06_2031

CGGTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAG
GTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTCT
GTAAGACTTTTTTGAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATCTAAAGCT
AAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGTAAAGAGAGT
GAAAAAGTACGTGAAATTGTTGAAA

> SW1-17 NL1_2019-02-25_E06_2031

GAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCT
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CTTTTCTTTGTAAGACTTTTTCGAAGAGTCGAGTTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCAT
CTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAA
AGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTTTGCATGCA
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TAAGAATGTAGC

> SW1-19 NL1_2019-02-25_F06_2031

GCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGT
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GCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATT
GGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAG
CACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGCG
GCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAA

> SW1-21 NL1_2019-02-25_G06_2031

AAAAGCTCAAATTTGAAATCCCCCGGGAATTGTAATTTGAAGAGATTTGGGTCCGGCCGGCGGGGGTTAAGTC
CACTGGAAAGTGGCGCCACAGAGGGTGACAGCCCCGTGAACCCCTTTAAAGCCCTCATCCCAGGTCTCCAAGA
GTCGAGTTGGTTTGGGTATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATACCGGCGAGAGACC
GATAGCGAACAAGTACAGTGTTGCACAGATGAAAAGCACTTTGAAAAAAGAGTTAAAAAGTACGTGAAATTG
TTTTAAGGGA

> SW1-23 NL1_2019-02-25_H06_2031

GAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCT
TTGATTAGGTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATAC
CTTTTCTCTGTAAGACTTTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATC
TAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAA
GAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTTTGCATGCACT
CGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCACTGGGCCAACATCAATTCTGGCAGCAGGATAAATCATTA
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> SW1-24 NL4 2019-05-22

AGGAAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGT
GTTTCGGCACGAGTTGTAGAGTGTAGGTGGGAGTCTCTGCGGAGCACAGTGTCCAAGTCCCTTGGAACAGGG
CGCCTGAGAGGGTGAGAGCCCCGTGGGGTGCTGTGCGAAGCTTTGAGGCCCTGCTGACGAGTCGAGTTGTTT
GGGAATGCAGCTCTAAGCGGGTGGTAAATTCCATCTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAA
GTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAA
GGGTATTGGGCCCGACATGGGGAGTGCGCACCGCTGTCTCTTGTAGGCGGCGCTCTGGGCGCTCTCTGGGCC
AGCATCGGTTCCTGCTGCGAGAGAAAGGGTTCCGGAAAGTGGCTCTTCGGAGTGTTATAGCCGGGGCCAGAT
GTCGCGTGTGG

> SW1-25 NL1_2019-02-25_B07_2032

CAGGCAAGAGCTCAGATTTGAAAGGCACTTTTGTCGTGTTGTTATTCTGAAGTTAGGGTCCTGAGAACGATGC
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ATGGAGTCGAGTTTGTTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAATATCTGCGAG
AGACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTG
AAATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAGAGCCGTGTTTGGGGGGAAGATAAATGCTGTAGA
ATGTAGCTCCTCGGAGTATTATAGATG

> SW1-26 NL1_2019-02-25_C07_2032

CGGCAAGAGCTCAGATTTGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGAC
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AGGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAAT
ATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAA
CAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCTCGACATGGGATTTACGCATCGTTGCCTCTCGTGGG
CGGCGCTCTGGGTTTTTCCTGGGCCAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTC
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> SW1-27 NL4 2019-05-22

GCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAA
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GGGCGCCTGAGAGGGTGAGAGCCCCGTGGGGTGCTGTGCGAAGCTTTGAGGCCCTGCTGACGAGTCGAGTT
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ACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGG
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GCCAGCATCGGTTCCTGCTGCGAGAGAAAGGGTTCCGGAAAGTGGCTACTTCGGAGTGTTATAGCCGGGGCC
TAGATGTCGCGTGTGGGGACCGAGGACTGCGGCTTCTGTCT

> SW1-28

GCGGCGAGTGAGCGGCAAGAGCTTCAGATTTTGAAATCGTGCCAATTTTTTTTTGGCACGAGTTCGTAGAGTC
GTAGGCGGGAGTCTTTGTGGAGCACGGTGTCCAAGTCCCTTGGAACAGGGCGCCTGAGAGGGTGAGAGCCCC
GTGGGGTGTCGTGCGAAGCTTTGAGGCCCTGCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGTGGGT
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AATGGGTGCCGGAAAGTGGCTCTTCGGAGTGTTATAGCCGGCGCCAGATACCGCGTGCGGGGACCGAGGACT
GCGGCTTCTGTCTCGGATGCTGGCACAACGGCGCAATACCGCCCG

>SW1-29 NL1_2019-03-22

GCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGTTTCGGCACGAGTTGTAGAGTGTAGGTGGGA
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GTGCGAAGCTTTGAGGCCCTGCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGCGGGTGGTAAATTCCA
TCTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAA
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CTGTCTCTTGTAGGCGGCGCTCTGGGCGCTCTCTGGGCCAGCATCGGTTCCTGCTGCGAGAGAAAGGGTTCCG
GAAAGTGGCTCTTCGGAGTGTTATAGCCGGGGCCAGATGTCGCGTGTGGGGACCGAGGACTGCGGCTTCTGT
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> SW1-31 NL1_2019-02-25_G07_2032

AACAGCCTCAAATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTTGTAGAGGATGATTTGGGGAAGCCGCC
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AAATCTCCTTCCAACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAA
TATTGGCGAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAA
AAGCA

> SW1-32 NL1_2019-02-25_H07_2032

CTCAAATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTTGTAGAGGATGATTTGGGGAAGCCGCCTGTCTAA
GTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACCCTATGTAAATCTC
CTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGC
GAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAAAGCAC

GTGAAATTGTTGAAAGGGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTCGGCCGGTCTTCTGACCGGTTTAC
TCAGTTTGGACAGGCC

> SW1-34 NL1_2019-02-25_A08_2032

CAAGAGCTCAGATTTGAAAGGCACTTTTGTCCTGTTGGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAG
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AGTCGAGGTTGTTTTGGGATTGCAGCTCAAATGGGTGGGTATGCTCCATCTAAAGCTAAATATCTGCGAGAGA
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> SV2-7 NL1_2019-09-03

CTATGAAATCTGGTACTTTCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTT
CCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTTTGTAAGACTTTTTCGA
AGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAG
ACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAA
ATTGTTGAAAGGGAAGGGC

> SV2-28 NL1_2019-09-03

TCAAATTTGAAATCTGGTACCTTTGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGACCGTTCCTTGTCT
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CCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGG
CGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTA
CGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGCGCCCTCTGCTCCTTGTGGGTGGGG
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> SV2-29 NL1_2019-09-03

CAAAAGCTCAAATTTGAAATCTGGTACCTTTGGTGCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGACCGTT
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AAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAA
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> SV2-33 NL1_2019-09-03

GAGCTCAGATTTGAAATCGTGCTTTGCGGCACGAGTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCGGT
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CCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGGTAAATTCCATCTAAGGCTAAATACTG
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> SV2-34 NL1_2019-09-03

TCGAGTTGTAATTTGAAGATGGTAACCTTGGGTTTGGCTCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAG
GGTGAGAATCCCGTCTGATGAGATGCCCATTCCTATGTAAGGTGCTATCGAAGAGTCGAGTTGTTTGGGAATG
CAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTG
ATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTA
GATCAGACTTGGTGTTTTACGATTATCTTC

> SV2-60 NL1_2019-09-03

ATGAAATCTCACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTGTGTGCAAGTCCCT
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TCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAGGCTAAATATTGGCGAGAGACCGA
TAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGT

> SV3-5 NL1_2019-09-03

AAAAGCTCAAATTTGAAATCTGGTACCTTCGGTGCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTC
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AAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAA
TATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAA
AAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGG
GTCGTGGAATCTCGCATTTCACTGGGCCAGGGTCGGTCTTGGTGGCAGGTTAAATCCATAGGAATGTAGCTAG
CCTCGGTTAGTATTATAGCCTTGTGGGGAATCCTGCCAGTTGAAGAGGGAGCCCTGTGGTTTTTTTTCCTCCGC
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> SV3-10 NL1_2019-09-03

AAAGCTCAAATTTGAAATCTGGTACCTTTGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGACCGTTCC
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ATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAA
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> SV4-2 NL1_2019-09-03

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G

> SV4-3 NL1_2019-09-03

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> SV4-5 NL1_2019-09-03

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> SV4-6 NL1_2019-09-03

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> SV4-15 NL1_2019-09-03

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> SW2-24 NL1_2019-09-03

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> SW2-28 NL1_2019-09-02

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> SW3-1 NL1_2019-09-03

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> SW3-9 NL1_2019-09-02

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> SW3-15 NL1_2019-09-03

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GAGTCGAGTTTGTTTGGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAATATCTGCGAGAGA
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> SW4-1 NL1_2019-09-03

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> SW4-7 NL1_2019-09-03

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GTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATAT

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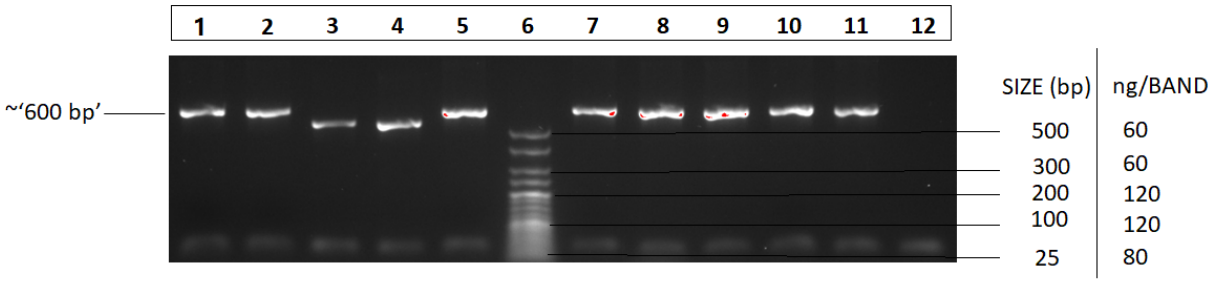
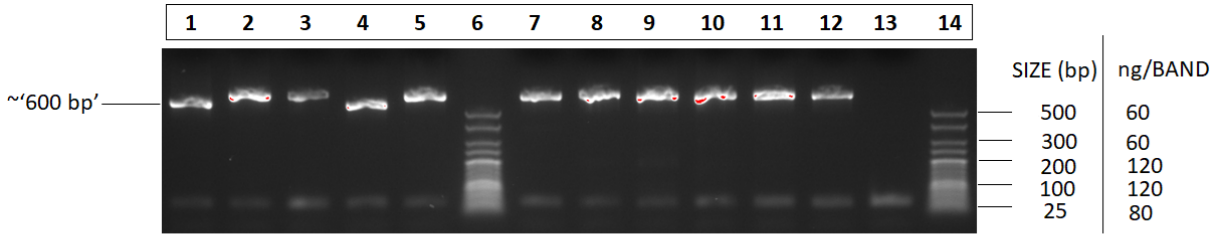
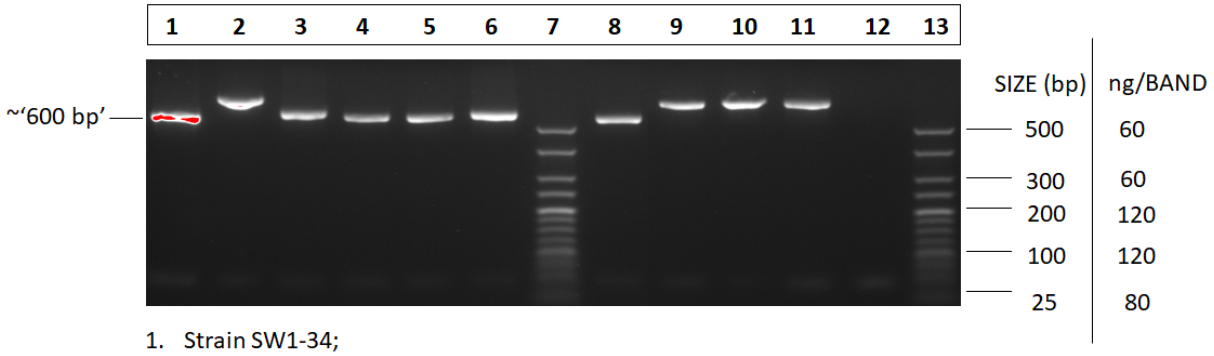
B.3 26S rDNA PCR products - gel electrophoresis



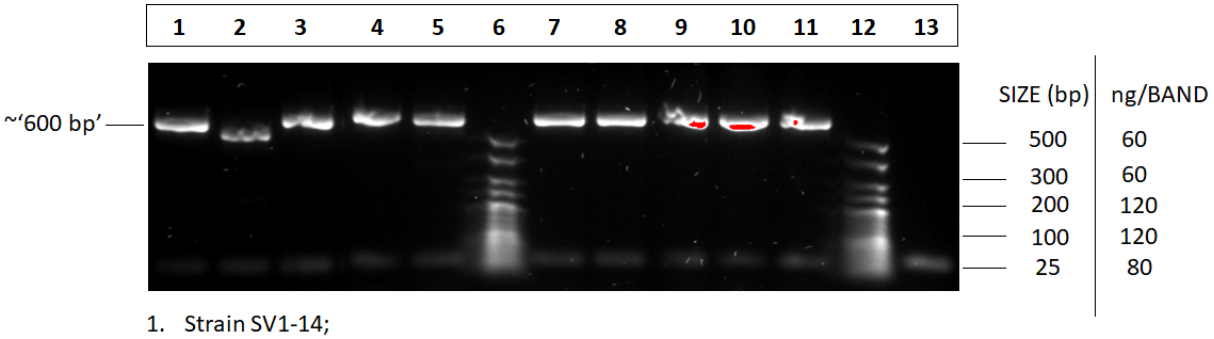
1. Strain SW1-8;
2. Strain SW1-9;
3. Strain SW1-10;
4. Strain SW1-11;
5. Strain SW1-12;
6. HyperLadder™ 25bp – Bioline;
7. Strain SW1-14;
8. Strain SW1-16;
9. Strain SW1-17;
10. Strain SW1-19;
11. PTC (Positive-template Control / SW1-2);
12. NTC(Non-template Control).



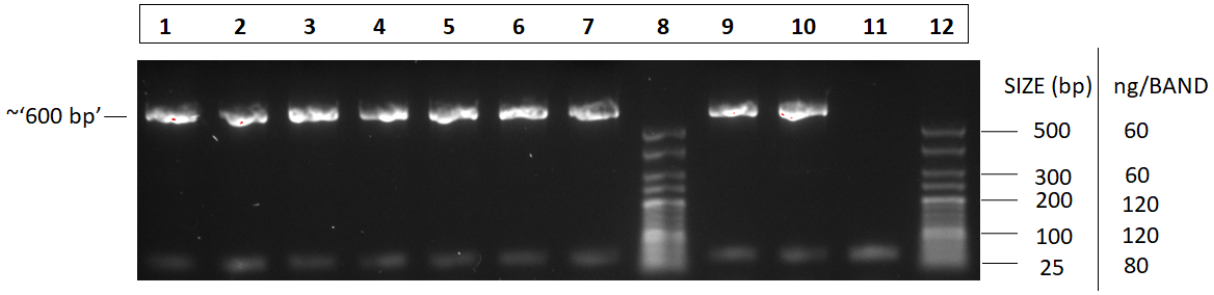
1. Strain SW1-21;
2. Strain SW1-23;
3. Strain SW1-24;
4. Strain SW1-25;
5. Strain SW1-26;
6. HyperLadder™ 25bp – Bioline;
7. Strain SW1-27;
8. Strain SW1-28;
9. Strain SW1-29;
10. Strain SW1-31;
11. Strain SW1-32;
12. PTC (Positive-template Control / SW1-2);
13. NTC(Non-template Control);
14. HyperLadder™ 25bp – Bioline.



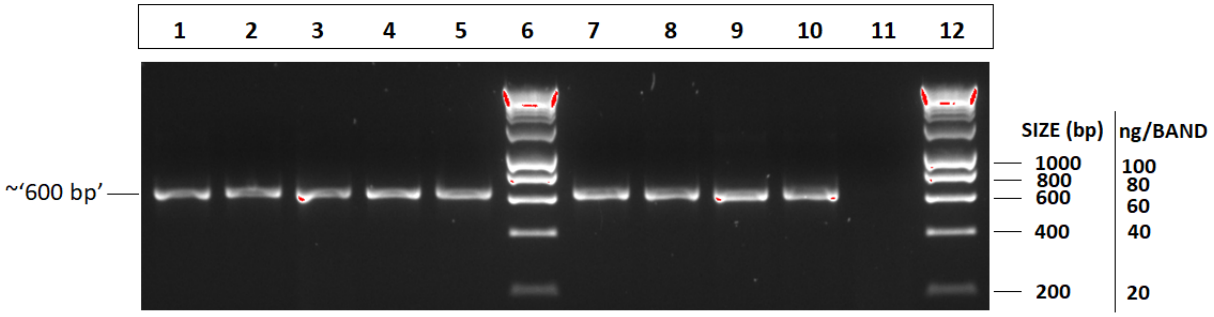
1. Strain SW1-34;
2. Strain SW1-2;
3. Strain SW1-3;
4. Strain SW1-5;
5. Strain SW1-7;
6. Strain SV1-1;
7. HyperLadder™ 25bp – Bioline;
8. Strain SV1-4;
9. Strain SV1-8;
10. Strain SV1-12;
11. PTC (Positive-template Control / SW1-2);
12. NTC(Non-template Control);
13. HyperLadder™ 25bp – Bioline.



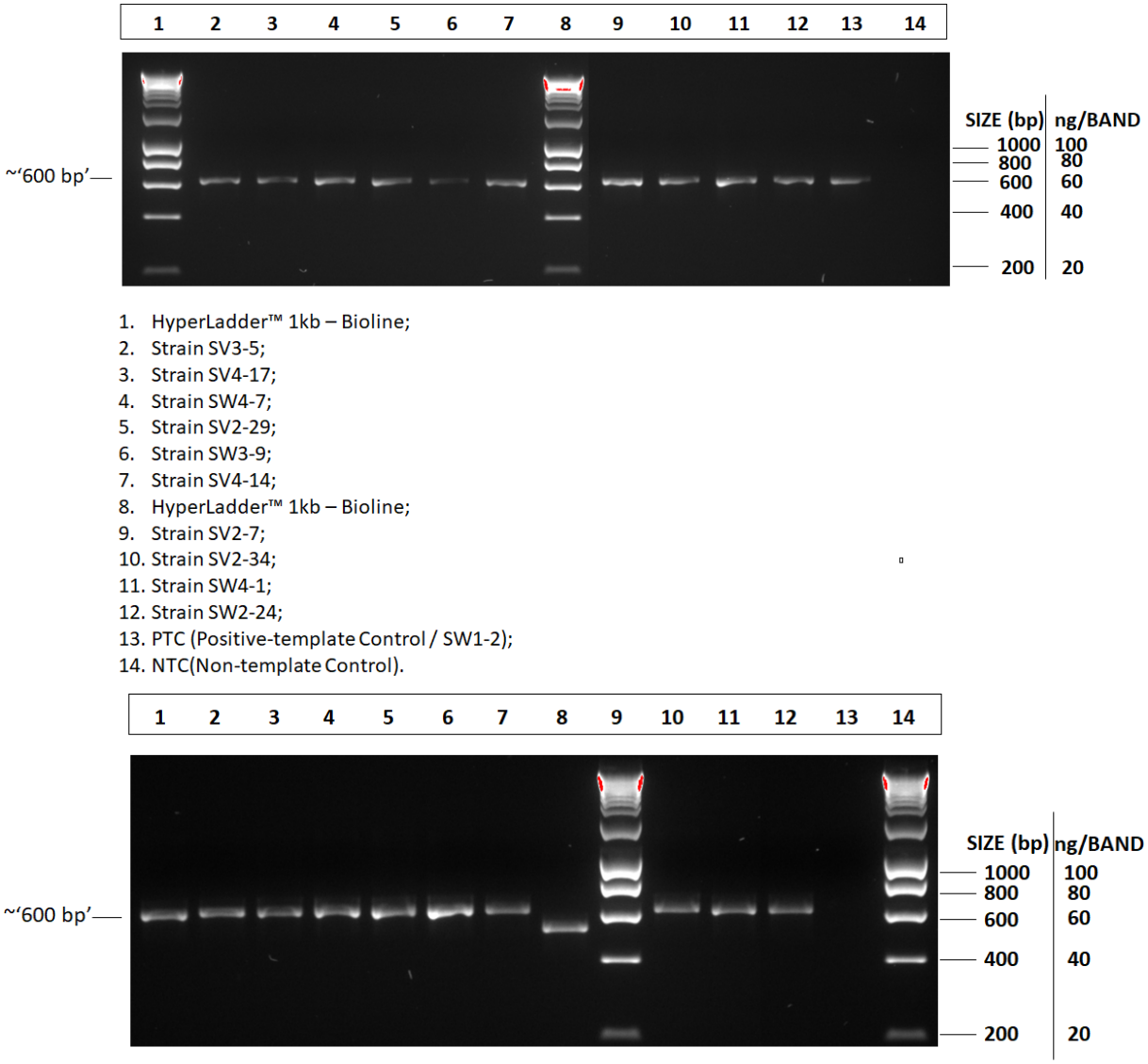
1. Strain SV1-14;
2. Strain SV1-16;
3. Strain SV1-18;
4. Strain SV1-28;
5. Strain SV1-35;
6. HyperLadder™ 25bp – Bioline;
7. Strain SV1-36;
8. Strain SV1-37;
9. Strain SV1-38;
10. Strain SV1-41;
11. PTC (Positive-template Control / SW1-2);
12. HyperLadder™ 25bp – Bioline;
13. NTC(Non-template Control).



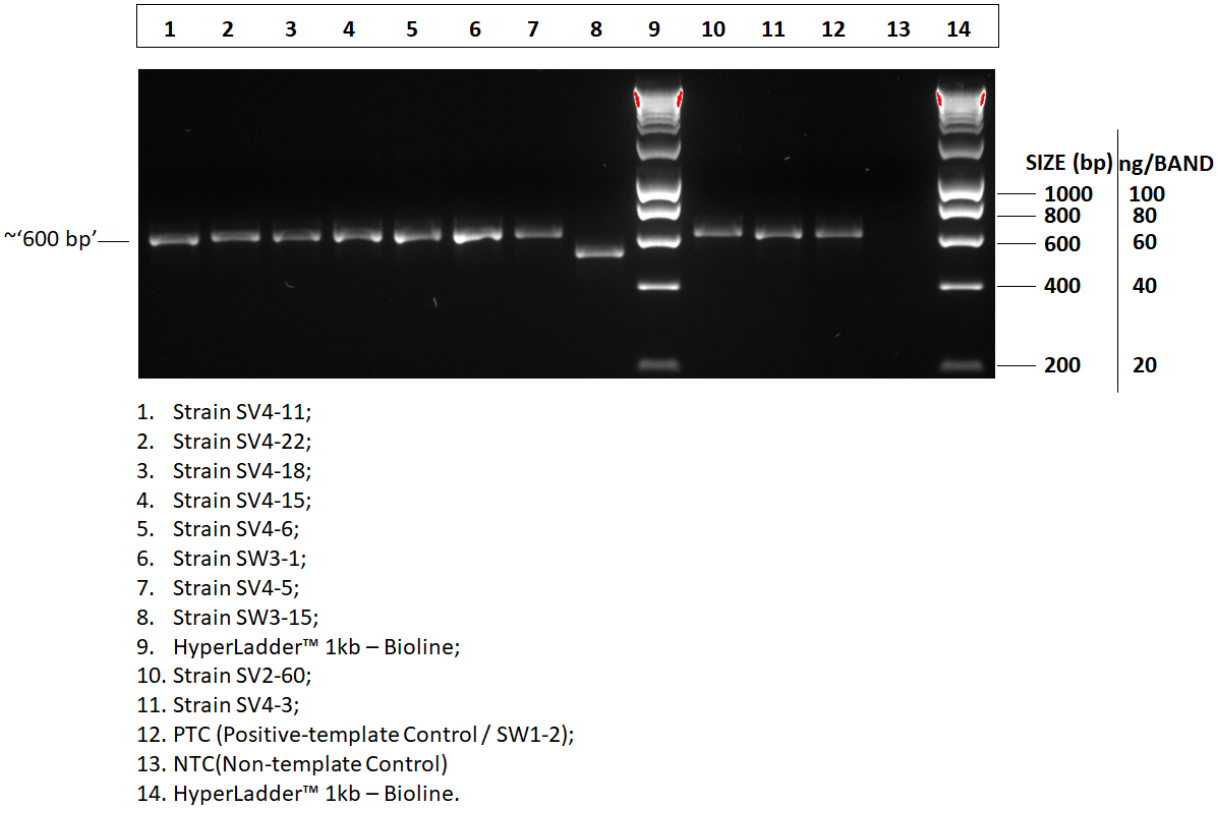
1. Strain SV1-49;
2. Strain SV1-52;
3. Strain SV1-62;
4. Strain SV1-63;
5. Strain SV1-64;
6. Strain SV1-66;
7. Strain SV1-68
8. HyperLadder™ 25bp – Bioline;
9. Strain SV1-70;
10. PTC (Positive-template Control / SW1-2);
11. NTC(Non-template Control);
12. HyperLadder™ 25bp – Bioline.



1. Strain SW2-32;
2. Strain SW2-13;
3. Strain SW2-28;
4. Strain SW3-13;
5. Strain SV4-2;
6. HyperLadder™ 1kb – Bioline;
7. Strain SV2-28;
8. Strain SV3-10;
9. Strain SV2-33;
10. PTC (Positive-template Control / SW1-2);
11. NTC(Non-template Control);
12. HyperLadder™ 1kb – Bioline.



1. HyperLadder™ 1kb – Bioline;
2. Strain SV3-5;
3. Strain SV4-17;
4. Strain SW4-7;
5. Strain SV2-29;
6. Strain SW3-9;
7. Strain SV4-14;
8. HyperLadder™ 1kb – Bioline;
9. Strain SV2-7;
10. Strain SV2-34;
11. Strain SW4-1;
12. Strain SW2-24;
13. PTC (Positive-template Control / SW1-2);
14. NTC(Non-template Control).



1. Strain SV4-11;
2. Strain SV4-22;
3. Strain SV4-18;
4. Strain SV4-15;
5. Strain SV4-6;
6. Strain SW3-1;
7. Strain SV4-5;
8. Strain SW3-15;
9. HyperLadder™ 1kb – Bioline;
10. Strain SV2-60;
11. Strain SV4-3;
12. PTC (Positive-template Control / SW1-2);
13. NTC(Non-template Control)
14. HyperLadder™ 1kb – Bioline.

Figure B.1 Gel electrophoresis of 26S rDNA PCR products.

Appendix C

Published Chapter

C.1 Chapter 4: Zhang, J., J. E. Plowman, B. Tian, S. Clerens and S. L. On (2020). "An improved method for MALDI-TOF analysis of wine-associated yeasts." Journal of microbiological methods 172: 105904.

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An improved method for MALDI-TOF analysis of wine-associated yeasts

Junwen Zhang[a,*], Jeffrey E. Plowman[b], Bin Tian[a], Stefan Clerens[b,c,d], Stephen L.W. On[a,*]

[a] Department of Wine, Food and Molecular Biosciences, Lincoln University, PO Box 85054, Lincoln, New Zealand
[b] Food and Bio-Based Products, AgResearch Ltd, Lincoln, New Zealand
[c] Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand
[d] Riddet Institute, Massey University, Palmerston North, New Zealand

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ABSTRACT

Although MALDI-TOF mass spectrometric analysis has been applied to the characterization of yeast species important in winemaking, relatively few taxa have so far been examined, and the value of low mass peaks for identification has not, to our knowledge, been previously determined. We describe a modified (pre-mixing) procedure for extraction of low (*m/z* 500–4000) - and high (m/z 2000–20,000) mass range moieties detected by MALDI-TOF and compare it with a previously described, proposed standard method based on a dried-droplet approach. Thirty-three strains representing 21 yeast species were examined. We found our modified method consistently yielded more discriminatory peaks and a broader mass range detection than the proposed standard method for the species examined. Cluster analyses of MALDI-TOF profiles also indicated better separation between species when the pre-mixing method was used, especially where high mass features were used. The use of low mass features may be useful for strain-level discrimination.

1. Introduction

Wine grapes are frequently colonised by indigenous yeast species of diverse origin. Spontaneous fermentation carried out by naturally occurring yeast species present on grapes may be considered an integral part of terroir (Capozzi et al., 2015) and the extensive range of other metabolic by-products could confer more desirable complexity in wine. However, unwanted species such as *Brettanomyces* spp. and *Zygosaccharomyces* spp. may also increase the risk of spoilage or poor quality product, and thus significant economic loss (Knight et al., 2015; Kraková et al., 2017; Hart et al., 2019). Effective and timely fermentation monitoring is required to manage such factors but is hampered by the lack of rapid and cost-effective yeast identification methods. Furthermore, characterization and evaluation of yeast strains for specific applications (e.g. low alcohol wine production) also require reliable identification (Jolly et al., 2014; Quirós et al., 2014; Ciani et al., 2016).

Molecular techniques like Internal Transcribed Spacer (ITS) and 26S rDNA sequencing have been adopted for species identification, and Amplified Fragment Length Polymorphism (AFLP), PCR- Restriction Fragment Length Polymorphism (RFLP), and Comparative Genome Hybridization (CGH) may be used for strain typing purposes (Guillamón et al., 1998; Kurtzman, 2006; Pope et al., 2007; Zhang et al., 2010; Hesham et al., 2014; Kurtzman, 2015). However, despite their powerful discriminatory capacity, these methods are high cost, generally labor intensive and also involve complex processes (Ivey and Phister, 2011). Alternatively, Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is an emerging technique, which has proven to be a rapid and reliable tool in wine yeast identification at the species/strain levels (Moothoo-Padayachie et al., 2013; Usbeck et al., 2014; Gutiérrez et al., 2017). As yet, this list is not exhaustive and to date, several species including *Saccharomyces*, *Kluyveromyces* and *Brettanomyces* spp. have not to our knowledge been examined. Although MALDI-TOF has only been recently applied to yeast species, its potential for species- and strain analysis seems well founded. Moothoo-Padayachie et al. (2013) demonstrated that MALDI-TOF MS was able to identify *S. cerevisiae* to the species level with 100% accuracy, and strain level with 90% accuracy. In addition, Kraková et al. (2017) determined that strains could be classified to genus level, even where individual species were not present in the database used for comparison. Furthermore, MALDI-TOF MS analysis has shown potential in predicting the utility of individual yeast strains used in the

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; ITS, internal transcribed spacer; AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; CGH, comparative genome hybridization; DM, dried-droplet method; PM, Pre-mixing method;; UPGMA, unweighted-pair group method with arithmetic mean

* Corresponding authors.
E-mail addresses: Cherie.Zhang@lincolnuni.ac.nz (J. Zhang), stephen.on@lincoln.ac.nz (S.L.W. On).

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0167-7012/

production of different wine varietals including chardonnay, beaujolais and champagne (Usbeck et al., 2014).

For sound identification to species- and strain level, clearly a standardised and robust protocol is desirable, as the efficiency of mass spectra acquisition may be influenced by microbial cell culture conditions, steps in the sample preparation, MALDI instrument and even personnel (Wunschel et al., 2005; Oberle et al., 2016). A standardised method for yeast analysis has been described (Gutiérrez et al., 2017). This paper describes our experience with the latter, and, as a result, an amended approach others may find useful. In addition, the value of mass ranges *m/z* 500–4000 and m/z 2000–20,000 in identification of yeast species of oenological significance were investigated, since to our knowledge, no information of the potential low mass range peaks has yet been reported for yeast characterization. We believe this is the first paper to describe MALDI-TOF MS for yeast characterization and identification in New Zealand.

2. Material and methods

2.1. Yeast strains

A panel of 33 yeast strains was used, including 14 type strains (*Brettanomyces anomalus* NCYC 615, *B. bruxellensis* NCYC 370, *B. naardenensis* NCYC 924, *Hanseniaspora uvarum* NCYC 2739, *Kluyveromyces dobzhanskii* NCYC 538, *K. lactis* NCYC 416, *K. wickerhamii* NCYC 546, *Saccharomyces bayanus* NCYC 2578, *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, *S. pastorianus* NCYC 396, *Torulaspora delbrueckii* NCYC 2629, *Zygosaccharomyces bailii* NCYC 1416 and *Z. rouxii* NCYC 568) purchased from NCYC (National Collection of Yeast Cultures); and 19 yeast isolates representing seven species (*Candida californica, Metschnikowia pulcherrima, Pichia membranifaciens, P. terricola, P. kluyveri, H. uvarum*, and *Starmerella bacillaris*) purified from Pinot Noir grape juice obtained from an organic winery, Greystone Wines, Waipara, New Zealand. These strains had been independently speciated by partial 26S rRNA sequence analysis using BLAST and subsequently phylogenetic analysis including comparable sequences of type strains (Supplementary Fig. 1).

2.2. MALDI-TOF MS

2.2.1. Sample preparation

Yeast strains were cultured on YPD agar (Difco) for 3 days at 28 °C on 3 different days to obtain 3 biological replicates. 1–3 colonies were picked using a sterile 200 µL pipette tip and emulsified into 300 µL deionized water. Afterwards, 900 µL absolute ethanol was added and vortexed for 1 min. After centrifugation (13, 400 rpm, 12,100 × *g*, 4 min) (Eppendorf AG, Minispin 5452), the pellet was kept and air-dried in laminar-flow hood. Prior to protein extraction, the samples were stored for up to two months at − 20 °C.

To extract proteins, 50 µL of 70% formic acid (*v*/v) was added to each yeast pellet and mixed thoroughly by vortexing for 1 min, then 50 µL of acetonitrile (ACN) was added, mixed for 1 min, centrifuged (12,100 × G, 4 min.) and the supernatant removed for analysis. To test the performance of two matrix/sample methods, strains were further analysed using each of two methods. The first represented a proposed standard "dried-droplet" method (hereafter DM) (Gutiérrez et al., 2017) with minor modifications, described below. Here, 1 µL of protein extract (compared with 2 µL used by Gutiérrez et al., 2017) was applied onto the MALDI ground steel target plate (MTP 384, Bruker Daltonics®) and allowed to dry. Thereafter, 1 µL α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (10 mg/mL in 75% ACN and 2.5% trifluoroacetic (TFA) was overlaid and dried at room temperature before analysis. The second method we describe as a pre-mixing method (PM), in which 8 µL of each protein extract and matrix solution were mixed well, before 1 µL of this mixture was deposited onto the target plate and allowed to dry. For technical replication, each extract was spotted onto three individual wells, therefore yielding 9 spectra per strain.

2.2.2. Mass spectra acquisition

MALDI-TOF mass spectra were automatically acquired on a Ultraflex III TOF/TOF MS instrument (Bruker Daltonics®, Bremen, Germany), operating in positive ion detection mode using a Smartbeam™ laser at 200 Hz; pulsed-ion extraction time of 120 ns; and laser power adjusted between 45% to 80%. The voltage of the ion source was set as 25.00 kV (ion source 1), 23.55 kV (ion source 2) and 6.01 kV (lens). Samples were analysed using the linear detector at high mass range *m/z* 2000-20,000, and reflector detector at low mass range *m/z* 500–4000. The final spectrum was an average accumulation of 800 single spectra (low mass range *m/z* 500–4000) or 2000 single spectra (high mass range *m/z* 2000–20,000) gathered. Each single spectrum was recorded from 10 random raster spots.

The mass spectrometer was externally calibrated in every experiment at regular intervals, using the calibrant position in the middle of each tetrad of spots. For low mass range *m/z* 500–4000, peptide II standard (Bruker Daltonics ®) (Bradykinin 1–7, [M + H] + at m/z757.3992, Angiotensin II, [M + H] + at m/z 1046.5418, Angiotensin I, [M + H] + at m/z 1296.6848, Substance P, [M + H] + at m/z 1347.7354, Bombesin, [M + H] + at m/z 1619.8223, ACTH clip 1–17, [M + H] + at m/z 2093.0862, ACTH clip 18–39, [M + H] + at m/z 2465.1983 and Somatostatin 28, [M + H] + at m/z 3147.4710) was used. For high mass range m/z 2000-20,000, an in-house protein standard comprising Insulin, [M + H] + at m/z 5734.52, Cytochrome C, [M + H] + at 12360.99 and [M + H]2+ at 6180.99, Myoglobin, [M + H] + at 16952.30 and [M + H]2+ at 8476.65), Aprotinin [M + H] + m/z 6511.51, and β-lactoglobulin [M + H] + m/z 18,363.00 was used.

2.2.3. MALDI-TOF MS data analysis

Raw mass spectra were exported as .txt format using FlexAnalysis software (version 3.0. Bruker Daltonics®), and imported into software BioNumerics version 7.6 (Applied Maths). Spectral pre-processing was achieved at a default setting, but baseline subtraction was performed with the Rolling disc method with a value adjusted to 150. Kaiser Window value in smoothing and signal to noise ratio (S/N) in peak filtering were adjusted manually according to spectral quality.

A composite profile of each strain was obtained using 9 spectra derived from three technical replicates of each of three biological replicates. Cluster analysis was performed using the Pearson correlation coefficient and UPGMA (unweighted-pair group method with arithmetic mean) algorithm. The "goodness-of-fit" between calculated similarity values between all strains, and the clustering shown in the dendrogram, was calculated using the cophenetic correlation method, whereby a value of 1.0 indicates a perfect correlation of the dendrogram with the similarity matrix. Data from high (*m/z* range 2000–20,000), low (500–4000), and combined high and low m/z spectra were analysed individually and in combination.

3. Results

3.1. MALDI sample preparation optimization

Each method produced distinct MALDI spectral patterns of the yeast strains examined at both high and low mass range, for which exemplars are provided in Fig. 1. Profiles for each strain are presented in Supplementary data (Supplementary Fig. 2–4). For high mass spectra, the pre-mixing method (PM) produced more peaks covering a broader mass range from *m/z* 4000 to 20,000, while using the dried-droplet method (DM), the main mass range was from m/z 4000 to 12,000, with no evident peaks beyond this range. For most species, the overall absolute peak intensity was higher with DM than PM. In many such cases, the background was also higher.

Likewise, the low mass spectra obtained from two methods were

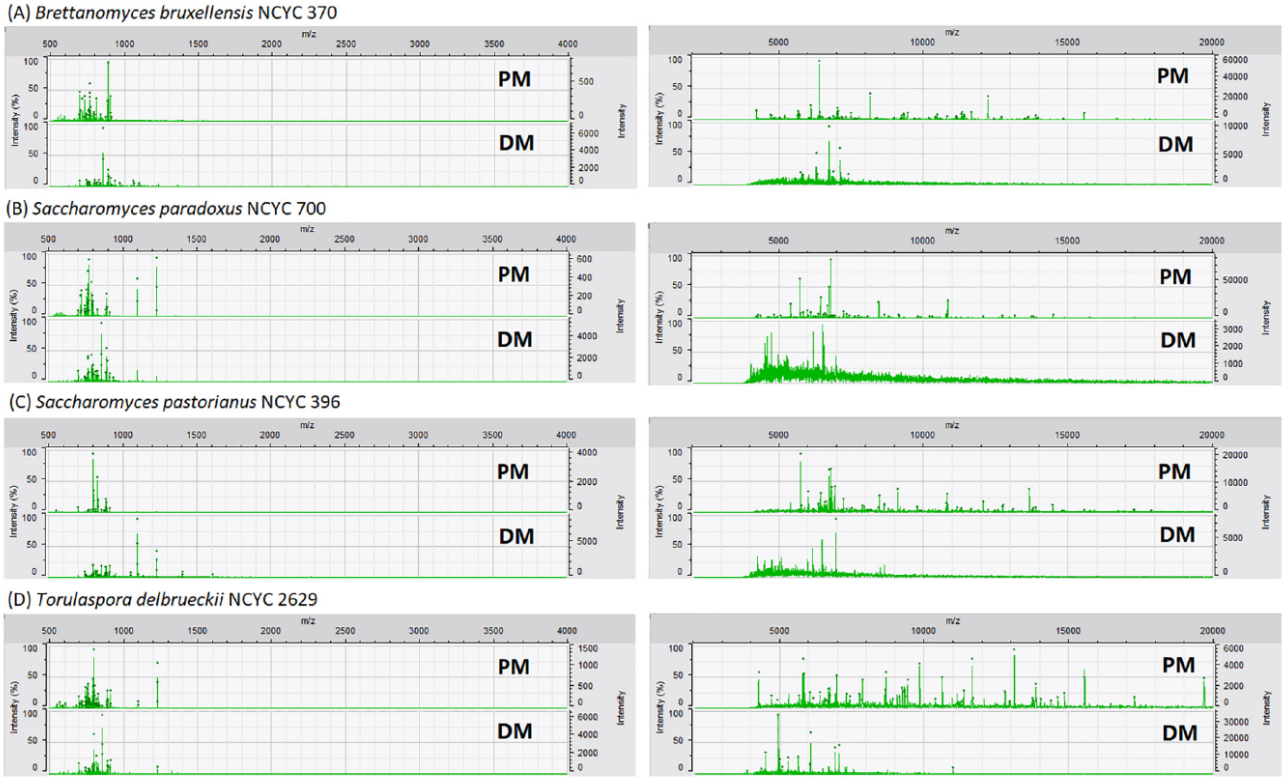

Fig. 1. MALDI-TOF profiles of four representative wine-associated yeast strains at both low mass range (*m/z* 500–4000) and high mass range (m/z 2000-20,000) with Dried-droplet method (DM) and Pre-mixing method (PM).

different in peak number and intensity as well. The peaks were mainly found in m/z 500–1000 using either method, but with a few species (e.g. *K. lactis,* NCYC 416 and *K. wickerhamii* NCYC 546), DM elucidated peaks with a slightly broader mass range.

3.2. Cluster analyses of high, low and combined high-low mass spectral profiles

Dendrograms and similarity matrices derived from high, low, and combined spectral types for DM and PM sample preparation methods are shown in Figs. 2 and 3 respectively. In the high mass spectral analyses, similarity values in PM-prepared extracts tended to be higher between strains of the same species compared to corresponding values from comparable DM-prepared extracts, allowing for accurate and effective species delineation at the 80% similarity level (Fig. 3A). Furthermore, the cluster analysis of the high-mass DM extracts divided *P. kluyveri, C. californica* and *M. pulcherrima* strains into two distinct groups, thus no single cut-off could accurately define all strains into distinct species (Fig. 2A).

A similar trend was seen when dendrograms of low mass profiles from DM (Fig. 2B) and PM extraction protocols (Fig. 3B) were examined. In general, there were a greater number of higher scoring pairs of strains belonging to the same species with PM extracts compared to DM, however in each case a number of strains were found not to cluster with other species members. For DM extracts, outlying strains from their member species group were seen in *H. uvarum*, *P. terricola, C. californica S. bacillaris* and *P. kluyveri*. For PM extracts, aberrant results were seen with *P. terricola, C. californica S. bacillaris* and *P. kluyveri*. Cluster analyses of dendrograms based on combined low and high mass data from DM and PM extraction profiles showed strains of *H. uvarum, S. bacillaris, C. californica* and *P. kluyveri* placed in outlying positions to other strains of these species in DM extracted profiles. However, all strains of these species clustered together with PM extracted profiles, albeit at lower similarity levels of 55% compared with high mass range data alone (Fig. 3C).

4. Discussion

This study outlines the development of MALDI-TOF MS as a rapid and reliable tool in wine-related yeast identification and differentiation for use in New Zealand. Compared to molecular methods, MALDI-TOF MS has advantages of minimal and easier sample handling in a faster and cheaper way. For routine yeast identification analysis, more than 84.5% accuracy was achieved at species-level within 5.1 min at cost of $0.50 per sample (Dhiman et al., 2011). Prior to MALDI instrument analysis, the general sample preparation usually includes two steps of protein extraction or direct colony smear and matrix/sample reaction. In most cases, protein extraction is recommended as it enhances performance in microbial identification (Kim et al., 2017). In particular, unlike bacteria, yeast has a thick chitinous cell wall, for which a protein extraction step is usually adopted. Indeed for yeast analysis, results are either unsuccessful or variable depending on yeast species, using direct colony application (Gutiérrez et al., 2017). Our results support the claim made by Kim et al. (2017) since we obtained better results when a protein extraction step was included.

MALDI-TOF MS analysis potentially offers high sensitivity and mass accuracy better than 0.1%, but this technique is largely dependent on sample preparation (Claydon et al., 1996). The sample/matrix method has the most significant influence on the performance of MALDI analysis, as the distribution of matrix/sample affects the matrix crystallization process, and therefore the laser beam energy absorption and efficiency for molecular desorption/ionisation (Dai et al., 1999; Mello et al., 2017). The dried-droplet method (DM) is routinely used for bacterial identification, and a variation of this protocol was proposed as

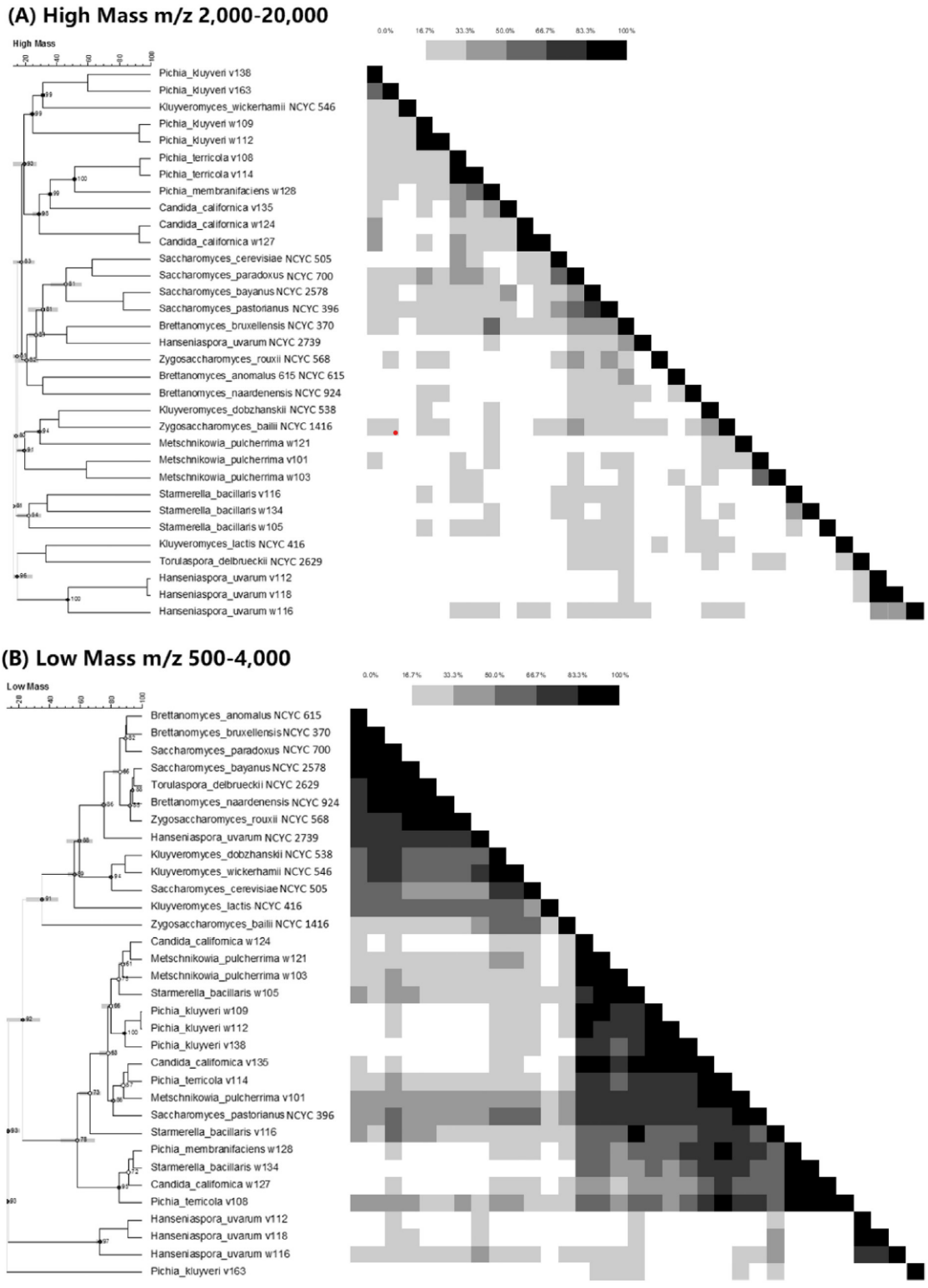

Fig. 2. Dendrogram and similarity matrices derived from (A) High mass profiles, (B) Low mass profiles, and (C) Low-High Combined profiles using Dried-droplet method (DM)-prepared extracts.

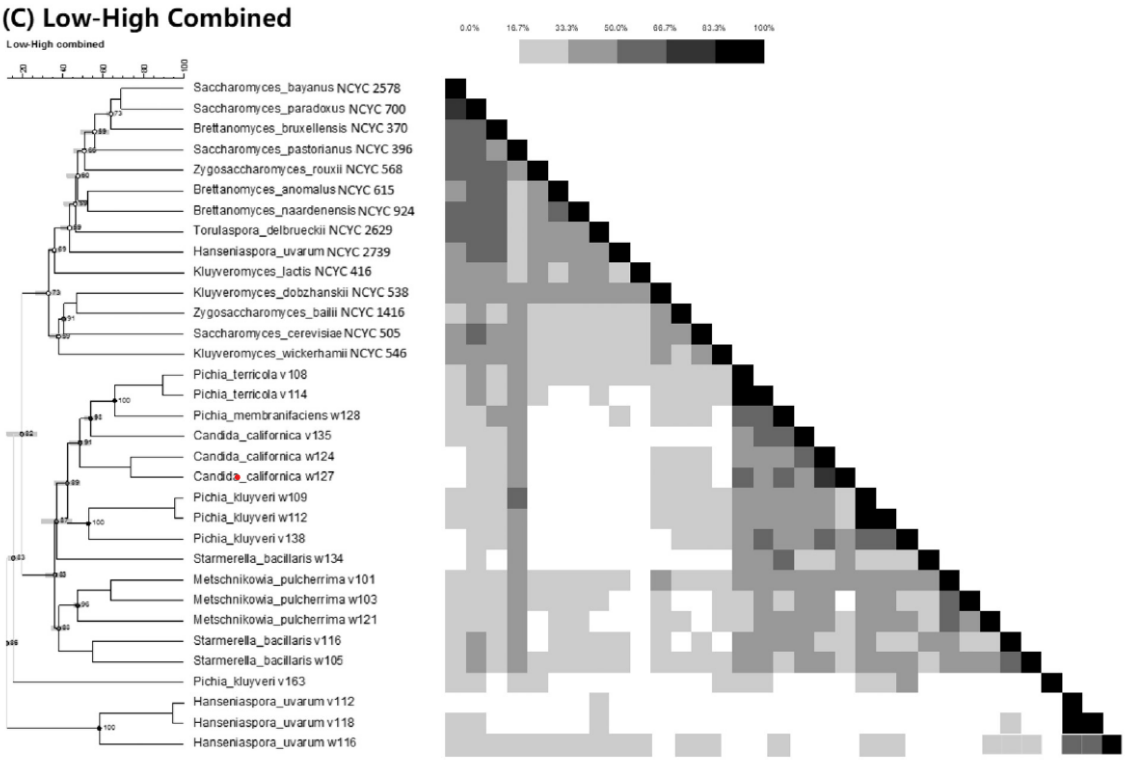

Fig. 2. (*continued*)

a standardised approach to characterise winemaking yeast using MALDI-TOF (Gutiérrez et al., 2017). The latter formed the basis of our initial studies, however although we determined it to yield peaks at high intensities, observations of high background noise and relatively low signals in the higher mass range encouraged us to explore the development of an alternative sample preparation method. We felt this prudent given that profiles were intended to be subjected to numerical analysis for identification purposes and more data with lower signal-to-noise ratio is an attractive prospect in this regard.

The levels of different proteins vary from fewer than 50 to more than 10^6 molecules per cell in yeast, however mass spectrometry seems strongly biased towards the detection of abundant proteins (Ghaemmaghami et al., 2003). Our results indicate the DM method is optimised for the detection of major cell components, since overall peak intensity was higher in DM extracts compared with PM data. However, the PM protocols clearly allows for the detection of more proteins, including those at low-abundance, with the added benefit of lower background noise. Since the distribution and homogenetiy between matrix and sample is essential for the spectra acquisition with high quality and reproducibilty (Dai et al., 1999; Williams et al., 2003; Rešetar et al., 2016; Gutiérrez et al., 2017), the PM mixing step may account for this improvement. Furthermore, a combination of more extensive protein species detection as well as improved profile clarity is likely to account for the improved species resolution of PM extracts compared with the DM method when data are then subjected to numerical analysis of high- and combined high- and low range profiles (Figs. 2 and 3). Although, neither DM or PM protocols yielded low mass spectra that yielded unequivocal species-specific profiles, the incorporation of low mass proteins into future analyses may yield additional benefits when examining the potential utility of yeast species in, for example, the production of particular wine styles as has been intimated before (Usbeck et al., 2014). From this perspective it is noteworthy that the standardised DM method proposed by Gutiérrez et al. (2017) does not appear to display differences among *S. cerevisiae* strains: a result in contrast to those described by Usbeck et al. (2014).

The evolutionary divergence of industrial yeasts is considered to be shaped by both the industrial application and geographical origin, and they are genetically and phenotypically separated from wild stocks due to human selection and trafficking (Gallone et al., 2016). In our study, PM yeast preparations subjected to MALDI-TOF MS seems suited to detect minor peak variations of *H. uvarum*, *C. californica*, *M. pulcherrima*, and *P. kluyveri*, while retaining the ability to accurately group strains at the species level. It has been previously reported that factors of isolation sources and geographic distance could influence the protein fingerprints and MALDI dendrogram (Kern et al., 2014; Zhang et al., 2015; Mello et al., 2017). Furthermore, the intraspecific variations in spectral patterns of yeast isolates may be reflected in differences in fermentative properties, as indicated previously (Usbeck et al., 2014). Overall, aside from its powerful, rapid and cost-effective identification capacity, MALDI-TOF MS may also have potential in selecting strains with special properties (e.g. low alcohol production, probiotic potential). For these reasons, we hope that our sample preparation method may be of interest to investigators wishing to reliably characterise yeast species using MALDI-TOF with an extended proteomic range, as we continue to pursue its potential to add value to the New Zealand winemaking sector.

Declaration of Competing Interest

None.

Acknowledgements

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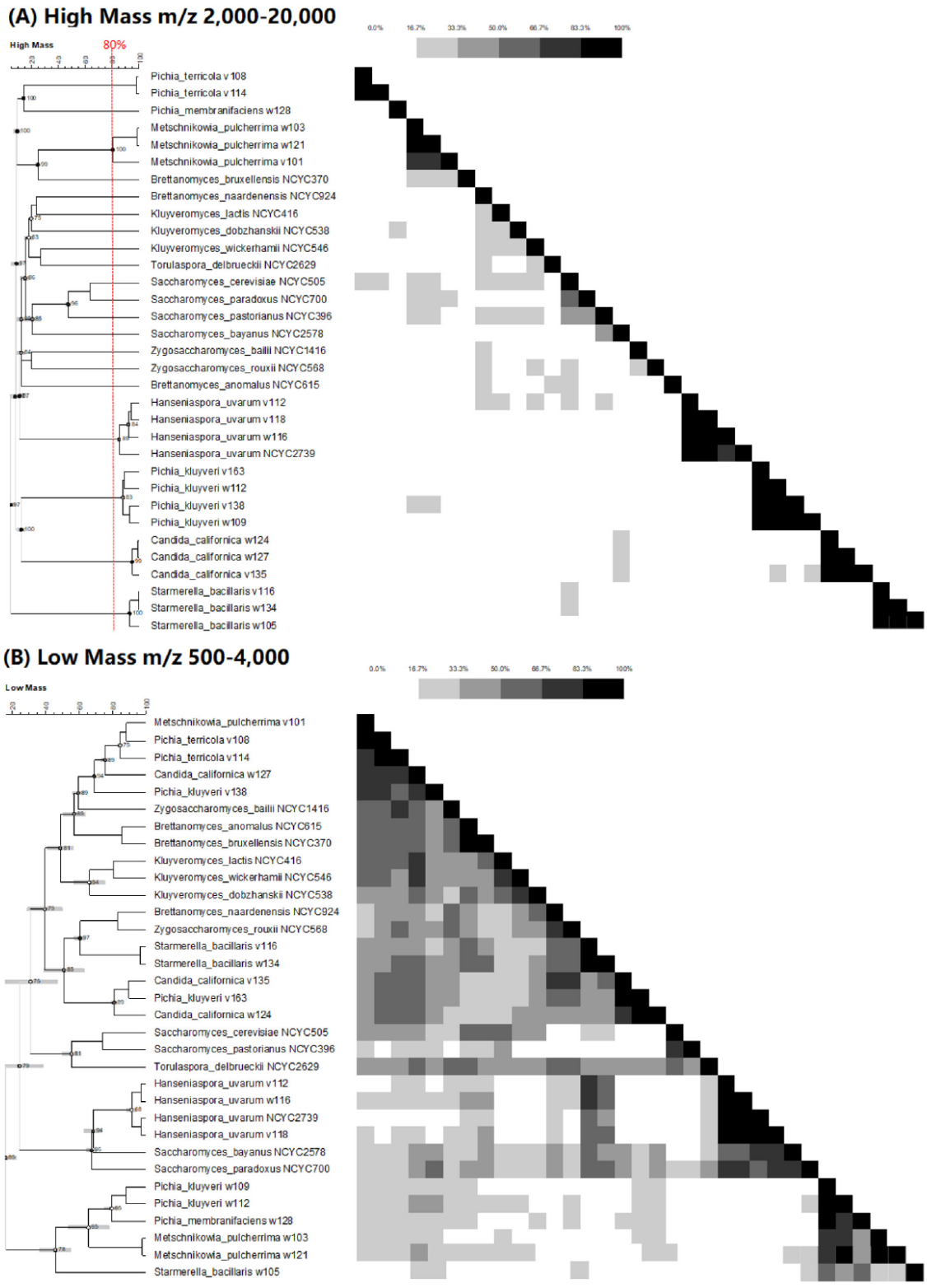

Fig. 3. Dendrogram and similarity matrices derived from (A) High mass profiles, (B) Low mass profiles, and (C) Low-High Combined profiles using Pre-mixing method (PM)-prepared extracts. The dotted lines in Fig. 3A and C indicate the most appropriate cut-off similarity (S-) level at which species were delineated. All species clustered at or above the 80% S-level in Fig. A, while for Fig. 3C, all species were distinguished at 55% apart from the *Saccharomyces* species that were separated at the 57.0% higher S-level.

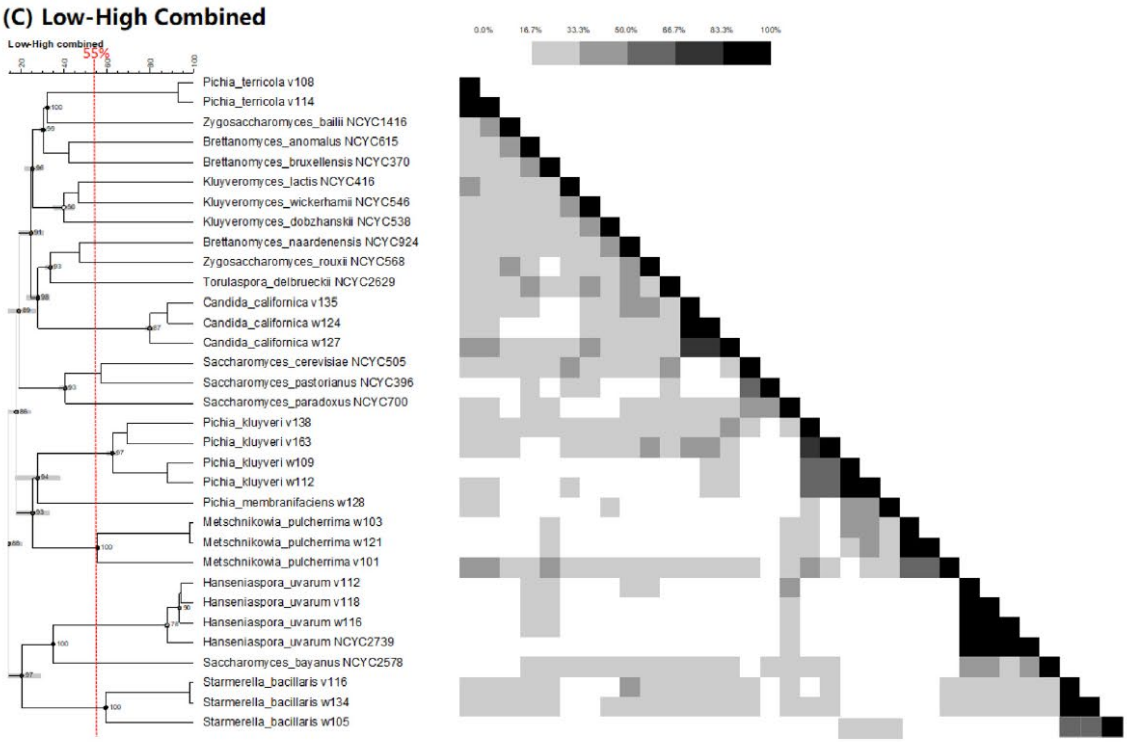

Fig. 3. (*continued*)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2020.105904.

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Application of MALDI-TOF analysis to reveal diversity and dynamics of winemaking yeast species in wild-fermented, organically produced, New Zealand Pinot Noir wine

Junwen Zhang [a], Jeffrey E. Plowman [b], Bin Tian [a], Stefan Clerens [b,c,d], Stephen L.W. On [a,*]

[a] Department of Wine, Food and Molecular Biosciences, Lincoln University, PO Box 85054, Lincoln, New Zealand
[b] AgResearch Ltd, Lincoln Research Centre, Lincoln, New Zealand
[c] Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand
[d] Riddet Institute, Massey University, Palmerston North, New Zealand

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ABSTRACT

Rapid yeast identification is of particular importance in monitoring wine fermentation and assessing strain application in winemaking. We used MALDI-TOF MS analysis supported by 26 S rRNA gene sequence analysis and *Saccharomyces*-specific PCR testing to differentiate reference and field strains recovered from organic wine production facilities in Waipara, New Zealand, in which Pinot Noir wine was produced by spontaneous fermentations in the vineyard and in the winery. Strains were isolated from each of four key stages of each ferment to evaluate changes in taxonomic diversity. MALDI-TOF MS analysis was confirmed as an excellent yeast identification method, with even closely related *Saccharomyces* species readily distinguished. A total of 13 indigenous species belonging to eight genera were identified from Pinot Noir ferments, with taxonomic diversity generally reducing as fermentation progressed. However, differences between the taxa recovered were observed between the vineyard and winery ferments, despite the grapes used being from the same batch. Furthermore, some consistent proteomic differences between strains of *S. cerevisiae, Hanseniasporum uvarum*, *Candida californica*, *Pichia membranifaciens* and *Starmerella bacillaris* correlated with the different fermentation systems used. The high speed, low cost, taxonomic resolution and ability to characterise subtle changes in phenotype that may result from variations in environmental conditions makes MALDI-TOF analysis an attractive tool for further and wider applications in the wine industry. Such applications may include monitoring wine fermentation to actively support the consistency of high-quality wine products, and potentially for the development of such products too.

1. Introduction

The New Zealand export wine sector has enjoyed continued growth for more than a decade, and part of its success has been attributed to the diversity of styles and products available (NZ Winegrowers annual report 2019. https://www.nzwine.com/en/media/statistics/annual-report/). The range of varied geographic locations and climatic conditions contribute to wines possessing unique characteristics, and is often referred to as "*terroir*" (Parr et al., 2007). Environmental factors such as soil composition and climate play an important role in wine quality (Tonkin et al., 2015), and influence the chemical composition in terms of volatile and non-volatile compounds (Sagratini et al., 2012). Waipara Valley in South Island of New Zealand has a cool, dry, warm temperate climate with diverse soil types, which has been exploited to produce Pinot Noir wines with distinctive regional characteristics (Tomasino et al., 2013).

Yeast species/strains and population are also critical to the flavor and "*terroir*" of final wine products (Fleet 2008). Previous studies have indicated that yeast communities and populations associated with vines and wines were region-specific in NZ, and interpreted it as a microbial aspect to *terroir* (Gayevskiy and Goddard 2012; Taylor et al., 2014). The commercial use of "wild" or spontaneous fermentation with indigenous yeasts on grapes leverages this distinctiveness, but poses challenges for consistent production. Rapid identification of yeast strains facilitates timely technical interventions for commercial benefit, and indeed potential for using novel indigenous yeast species/strains for production of novel wine varietals.

Numerous identification techniques are available for winemaking-

* Corresponding author.
E-mail address: Stephen.on@lincoln.ac.nz (S.L.W. On).

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related yeasts (Ivey and Phister 2011; Blattel et al., 2013; Hart et al., 2019). Although some molecular methods have been shown to be reliable for this purpose, they are overly expensive and time-consuming for the rapid identification of wine yeast species/strains during commercial fermentation (Hart et al., 2019). In contrast a proteomic approach based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry, (MALDI-TOF MS) has been shown to be a rapid, reliable and cost-effective tool in wine yeast identification at the species/strain levels (Moothoo-Padayachie et al., 2013; Usbeck et al., 2014; Gutiérrez et al., 2017). In addition, the potential for predicting applications for specific yeast strains to produce individual wine- or beer styles or as probiotics, has been alluded to Usbeck et al. (2014), Lauterbach et al. (2017).

Although yeast community diversity associated with wine and vine has been investigated previously in Gisborne, West Auckland, Waiheke Island, Hawkes Bay, Marlborough and Central Otago with molecular methods (Zhang et al., 2010; Taylor et al., 2014), no data of yeast community diversity in the Waipara region has been published. Furthermore, we are unaware of any studies investigating yeast diversity in organic wine production using solely indigenous yeast. We have previously described an optimized method for using MALDI-TOF spectra of winemaking-relevant yeasts for identification purposes (Zhang et al., 2020). This study examines the use of MALDI-TOF MS analysis to evaluate the dynamics of culturable yeast species during wine fermentation under each of two distinct forms of organic indigenous fermentation conditions in the Waipara region.

2. Materials and methods

2.1. Wine samples and yeast isolation

Pinot Noir grapes (clone: 115) sourced from the Greystone Block 5 vineyard were harvested on March 22, 2018. Spontaneous fermentation was carried out in 1.5 tons of tanks made of high density polyethylene (HDPE) which were placed (a) in the winery (an indoor environment) and (b) in the vineyard (an outdoor environment without temperature control) respectively. Pinot Noir grape juice ferment samples from each of the winery and vineyard environments were collected at four key different stages of fermentation, namely: (i) the first sign of fermentation; (ii) 6–8 °Brix dropped; (iii) half of °Brix dropped; and (iv) at the end of fermentation (Table 1).

Pinot noir grape juice ferment sample of 500 mL from each stage was collected in 750 mL wine bottles and quickly (within 90 min) transported on ice to the microbiology laboratory at Lincoln University, where 50 mL aliquots was centrifuged (3000×*g*, 10 min, 4 °C) (Heraeus Multifuge X3R, Thermo Scientific) and the pellet was then resuspended in YPD-30% (v/v) glycerol medium to facilitate storage of viable microorganisms at −80 °C. For yeast isolation, 1 mL of each defrosted Pinot Noir grape juice ferment sample from each fermentation system (i.e. winery and vineyard) was taken, and a ten-fold serial dilution series prepared (in 0.1% peptone water) from each. Then, 100 μL of each dilution was spread on YPD agar (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) with 0.1 g/L of chloramphenicol (Sigma) and 0.25 g/L of sodium propionate (Sigma) and incubated at 28 °C for 2–3 days. Experiments were performed in triplicate. Hence, each sample had been cultured on 30 agar media plates for microbial analysis and comparison. Total yeast colonies and the percentage of each species were counted using plates with 30–300 colonies, the results were given as cfu/mL (colony-forming units per milliliter). 3–5 colonies of each morphologically-classified isolate (i.e. colonies classified by their colour, shine, shape, edge and size) were selected and re-streaked on the same fresh media 3–4 times. Care was taken to ensure all morphologically distinct types were represented at each stage by careful examination of cultures from each dilution, including replicates. All isolates were subjected to MALDI-TOF MS analysis, and stored in 30% (v/v) glycerol stock at −80 °C.

Table 1
Pinot Noir ferments sampled from four key stages of fermentation in Winery and Vineyard wine production systems. Isolates were recovered from 500 mL aliquots of each sample as described in Materials and Methods. The number of isolates examined at each stage is given in square brackets. Isolate selection was based on careful and systematic screening (see materials and methods for details).

	S1 (beginning of fermentation)	S2 (6–8° Brix dropped)	S3 (half of ° Brix dropped)	S4 (end of fermentation)
Winery	SW1 [**34**] (morning, March 27, 2018)	SW2 [**33**] (afternoon, March 28, 2018)	SW3 [**19**] (morning, March 29, 2018)	SW4 [**20**] (April 12, 2018)
Vineyard	SV1 [**48**] (morning, March 26, 2018)	SV2 [**39**] (afternoon, March 26, 2018)	SV3 [**20**] (morning, March 27, 2018)	SV4 [**22**] (April 11, 2018)

2.1.1. Type strains

Fourteen type strains were also examined to provide comparators of known identity in the MALDI-TOF comparisons. These were: *Brettanomyces anomalus* NCYC 615, *B. bruxellensis* NCYC 370, *B. naardenensis* NCYC 924, *Hanseniaspora uvarum* NCYC 2739, *Kluyveromyces dobzhanskii* NCYC 538, *K. lactis* NCYC 416, *K. wickerhamii* NCYC 546, *Saccharomyces bayanus* NCYC 2578^T, *S. cerevisiae* NCYC 505^T, *S. paradoxus* NCYC 700^T, *S. pastorianus* NCYC 396^T, *Torulaspora delbrueckii* NCYC 2629, *Zygosaccharomyces bailii* NCYC 1416 and *Z. rouxii* NCYC 568. All strains were purchased from the NCYC (National Collection of Yeast Cultures, UK) and analysed by MALDI-TOF using the same methods described below as for our field isolates.

2.2. MALDI-TOF MS analysis

2.2.1. Sample preparation

An optimized sample preparation protocol has been established previously in our lab (Zhang et al., 2020). In brief, yeast strains were cultured on YPD agar (Difco) for 3 days at 28 °C on 3 different days to obtain 3 biological replicates. 1–3 colonies were picked using a sterile 200 μL pipette tip and emulsified into 300 μL deionized water. Afterwards, 900 μL absolute ethanol was added and vortexed for 1 min. After centrifugation (12,100×*g*, 4 min) (Eppendorf AG, Minispin 5452), the pellet was kept and air-dried in laminar-flow hood. Prior to protein extraction, the samples were stored for up to 2 months at −20 °C.

To extract proteins, 50 μL of 70% formic acid (v/v) was added to yeast pellet and mixed thoroughly by vortexing for 1 min, then 50 μL of acetonitrile (ACN) was mixed for the same time. Protein extract was obtained by centrifugation (12,100×*g*, 4 min). Equal volume of protein extract and α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (10 mg/mL in 75% ACN and 2.5% trifluoroacetic (TFA) were mixed well and 1 μL of this mixture was deposited onto the MALDI ground steel target plate (MTP 384, Bruker Daltonics®) till dry. For technical replication, each extract was spotted onto three individual wells, therefore yielding 9 spectra per strain.

2.2.2. Mass spectra acquisition

MALDI-TOF mass spectra were automatically acquired on an Ultraflex III TOF/TOF MS instrument (Bruker DaltonicBremen, Germany), operating in linear positive ion detection mode with a Smartbeam™ laser at 200 Hz frequency and covering the mass range m/z 2000–20,000. Pulsed-ion extraction time was 120 ns, and the laser power was adjusted between 45% and 80%. The voltage of the ion source was set as 25.00 kV (ion source 1), 23.55 kV (ion source 2) and 6.01 kV (lens). The final spectrum was an average accumulation of 2000 single spectra gathered, each single spectrum was recorded from 10 random raster spots.

The mass spectrometer was externally calibrated in every experiment

at regular intervals, using the calibrant position in the middle of each tetrad of spots, using an in-house protein standard comprising Insulin, [M + H]+ at m/z 5734.52, Cytochrome C, [M + H]+ at 12360.99 and [M + H]2+ at 6180.99, Myoglobin, [M + H]+ at 16952.30 and [M + H]2+ at 8476.65), Aprotinin [M + H] + m/z 6511.51, and β-lactoglobulin [M + H] + m/z 18363.00.

2.2.3. Data analysis

Raw mass spectra were exported as. txt format using FlexAnalysis software (version 3.0. Bruker Daltonics®), and imported into software BioNumerics version 7.6 (Applied Maths). Spectra pre-processing was achieved at a default setting but baseline subtraction with Rolling disc value was adjusted to 150. Kaiser Window value in smoothing and signal to noise ratio (S/N) in peak filtering were adjusted according to the quality of spectra.

A composite profile of each strain was obtained using 9 spectra derived from three technical replicates of each of three biological replicates. Cluster analysis was performed using the Pearson correlation coefficient and UPGMA (unweighted-pair group method with arithmetic mean) algorithm.

2.3. Molecular identification

2.3.1. DNA extraction

73 representative isolates from the MALDI-dendrogram were further validated using 26 S rDNA sequencing (Baleiras Couto et al., 2005) as before. A single colony from yeast isolates of interest was resuspended in 100 μL sterile deionized water and frozen at −80 °C overnight. DNA was obtained by disrupting yeast cells by subsequently heating at 95 °C for 5 min.

2.3.2. Partial sequencing of the 26 S rRNA gene

Each PCR sequencing reaction was performed in a 20 μL system containing 10 x PCR Buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 62.5 μM of each dNTPs (Invitrogen), 0.75 μM of each primer (Invitrogen), 2 U taq polymerase (Qiagen) and 1 μL DNA suspension. Partial 26 S large subunit ribosomal DNA fragments were amplified using forward NL-1 primer (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse NL-4 primer (5′-GGT CCG TGT TTC AAG ACG G-3′) (Invitrogen) in Multigene Gradient (Labnet International, Inc., USA) with an initial denaturation at 94 °C for 3 min, followed by 36 cycles with a temperature profile of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1.5 min, ended with an final extension period at 72 °C for 5 min and remained at 4 °C (Csoma et al., 2010). Afterwards, PCR products were purified with AxyPrep PCR Clean-up Kit (Biosciences) according to the protocol and sequenced in single direction with only prime NL-1 or NL-4 at the Bio-Protection sequencing facility (Lincoln University). The quality of sequences obtained were checked with Sequence Scanner software (version 1.0, Applied Biosystem) and compared by BLASTn tool online (http://www.ncbi.nlm.nih.gov/BLAST/). For the sequences obtained using reverse primer NL-4, Chromas software (Version 2.6) was used to get the reverse sequences. Species identification was considered valid when the identity of a 26 S DNA sequence and a reference sequence was ≥98%.

A phylogenetic tree of partial sequences and the corresponding type strain published in the Genbank database was constructed using software Mega 7 (Kumar et al., 2016) by Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale with branch lengths measured in the number of substitutions per site.

2.3.3. PCR-RFLP analysis of the NTS2 region for saccharomyces speciation

In the absence of a reference strain of *S. uvarum* in our collection, isolates requiring confirmation as either *S. uvarum* or *S. bayanus* were identified using the method described by (Nguyen and Boekhout 2017). Twelve representative isolates from the MALDI-dendrogram were further validated and 4 type strains (*S. bayanus* NCYC 2578, *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, *S. pastorianus* NCYC 396) were adopted as the control. The non-transcribed spacer 2 (NTS2) of ribosomal DNA was amplified using primers NTSU-NTS2 (5′-AACGGTGCTTTCTGGTAG-3′) and ETSL-NTS2 (5′-TGTCTTCAACTGCTTT-3′) (Nguyen and Boekhout 2017). The annealing temperature was set at 55 °C. Restriction digestion using *Alu*I (Thermo Scientific™) was carried out directly on 20 μL of PCR product at 37 °C for 3 h according to the manufacturer's instruction.

Amplified DNA strands were checked by agarose gel electrophoresis at voltage of 100 V for 60 min (PowerPac™ Basic, BIO-RAD). 4 μL PCR products mixed thoroughly with 1 μL loading dye (0.2% w/v bromophenol blue, 0.25% Xylene cyanol, 60% w/v glycerol) and 6 μL DNA marker HyperLadder™ 25 kb (Bioline) or HyperLadder™ 1 kb (Bioline) were loaded in 2% Agarose gel in 1 X Tris-borate-EDTA (TBE) buffer staining with 2% ethidium bromide (EtBr). Restriction fragments were separated on a 2% Agarose gel in 0.5 X TBE buffer staining with 2% EtBr. DNA bands were visualized under UV light and digital images were acquired using Molecular Imager®Gel Doc™ XR+ with Image Lab™ software (BIO-RAD). Fragment patterns of our relevant isolates were compared with those of type and reference strains (Nguyen and Boekhout 2017) for speciation.

3. Results

3.1. Isolate identification

A total of 235 field isolates were isolated (Table 1) and subjected to MALDI-TOF MS analysis, and relationships determined in a dendrogram that also included 14 reference strains of known identity (Fig. 1a). Clusters containing distinct species were defined at the 40% similarity threshold (Fig. 1a). Results of the MALDI-based dendrogram were validated by partial 26 S rDNA sequencing of 73 representative field strains (Fig. 1b), or PCR-RFLP for discrimination of *Saccharomyces* species (Nguyen and Boekhout 2017). These results confirmed the efficacy of MALDI-TOF for speciation.

A subgroup of four isolates closely related to, but distinct from our other *S. uvarum* isolates was observed in our MALDI-TOF analysis (Fig. 1a). These isolates exhibited a faint band ca. 350 bp in size in our PCR-RFLP analysis of these strains, in contrast with the *S. uvarum* type strain (Nguyen and Boekhout 2017), and from eight field isolates yielding a typical *S. uvarum* PCR-RFLP profile (Supplementary Fig. 1). We consequently refer to these four isolates as *S. uvarum*-like, pending further study.

Pichia membranifaciens and *Candida californica* pose similar identification challenges due to their close phylogenetic relationship (Wu et al., 2006). Nonetheless, our MALDI-TOF analysis clustered SW1-28 and SW1-29 together and differentiated these from *C. californica*. Isolates in white with reddish pigment leaching into the growth medium were typical of species of the genus *Metschnikowia*. Three isolates SV1-1, SW1-3 and SW1-21 were subjected to 26 S rDNA sequencing, of which BLASTn results showed SV1-1 and SW1-21 had the highest similarity of 99% and 97% with *M. pulcherrima*, and SW1-3 showed 99% similarity with *M. fructicola*. These species are phylogenetically highly related (Kurtzman and Droby 2001). *Metschnikovia fructicola* has largely been explored as a biocontrol agent against soft rot in berries (Kurtzman and Droby 2001), and previous studies of yeast diversity in New Zealand grapes have not, to our knowledge, revealed its presence in vineyards examined in this country (Zhang et al., 2010; Gayevskiy and Goddard 2012). In contrast, *M. pulcherrima* is common in early stage ferments (Morata et al., 2019) and known to be present in New Zealand (Zhang et al., 2010), hence we consider these strains to be *M. pulcherrima*.

Fig. 1. (a) MALDI-dendrogram using Pearson correlation and UPGMA algorithm (b) Molecular Phylogenetic analysis by Maximum Likelihood method based on the Tamura-Nei model.

In summary, a total of 13 yeast species belonging to 8 genera were identified among our isolates.

3.2. Yeast species dynamics during fermentation

The distribution of the different yeast species identified in this study in wine fermentations undertaken in the winery and vineyard respectively during each of the four key sampling periods is shown in Table 2. Enumerations of total yeast populations in each stage are also given. The initial yeast population of vineyard samples was 1.10×10^8 cfu/mL, almost 3 times higher than that of winery samples (3.4×10^7 cfu/mL). Nonetheless, the trend for yeast proliferation in each fermentation system was the same, with peak yeast numbers attained in the second stage and gradually declining after that. At the final stage, the winery sample yeast population (1.60×10^7 cfu/mL) was about 3 times higher than that of vineyard samples (5.40×10^6 cfu/mL).

In the earliest samples taken from the vineyard (SV1), the most prevalent species were *H. uvarum* (52%), *St. bacillaris* (39%), and *P. terricola* (8%), with other species identified as *M. pulcherrima*, *P. kluyveri*, and *C. californica;* only one isolate was identified as *S. cerevisiae*. In SV2, the prevalence of *Saccharomyces* species (*S. cerevisiae* and S. *uvarum*) increased to 48% of the detected yeast populations, and the proportion of *St. bacillaris* remained stable (40%), and *H. uvarum* decreased greatly (7%). Three previously undetected species were *P. membranifaciens*, *P. kludriavzevii*, *and Wickeraromycess anomalus*, whereas *C. californica* and *P. terricola* disappeared. In the following stages, *Saccharomyces* species (*S. cerevisiae* and *S. uvarum*) gradually took over the fermentation, with only *S. cerevisiae* detected in SV4. The yeast diversity and the frequency of their appearance varied between the vineyard samples and winery samples, although the grapes used in each ferment were from the same batch. In the first stage winery samples (SW1), *St. bacillaris* was the dominant species (86%), with *H. uvarum* in 11%. In the second stage (SW2), *Sacchromyces* species (*S. cerevisiae,* S. *uvarum* and S. *uvarum-like*) soared up to 86%, *St. bacillaris* and *H. uvarum* dropped sharply to 12% and 2%, respectively. Interestingly, our *S. uvarum-like* taxon was not isolated from SW3, but appeared in SW2 and SW4, while the proportion of *St. bacillaris* increased from 12% to 33%; sample size may help account for this finding. In the final stage, *Saccharomyces* species completely obscured the non-*Saccharomyces* species. Similar to vineyard samples at the same stage, *S. cerevisiae* was dominant but a few *S. uvarum* and *S. uvarum-like* strains were detected.

Overall, non-*Saccharomyces* yeast was most abundant at the start of the winemaking process, but were gradually replaced over time by *Saccharomyces* species, as is generally expected. *Saccharomyces cerevisiae* was in general the dominant species at the end of fermentation.

3.3. MALDI-based strain subtype analyses

3.3.1. Saccharomyces species

A total of 60 *S. cerevisiae* isolates obtained from winery fermentation (n = 26) and vineyard fermentation (n = 34) samples were identified in this study. Variance in MALDI-based profiles was evident both visually and in cluster analysis, where a 70% similarity value was selected as the threshold to define strain types (Fig. 3). The *S. cerevisiae* isolates were distributed among 5 types, of which one is unique and represented by the type strain. Type I was the predominant type (representing 23/34 vineyard and 22/26 winery strains) and was observed in every fermentation stage. The other three types were only detected in later ferment stages, with type III only found in vineyard samples (Fig. 3a). Fig. 3b presents the profile pattern of each *S. cerevisiae* type.

Thirty strains of *S. uvarum* were isolated in our study, of which eight were from vineyard samples and the remainder from winery ferments. Five types were defined (Fig. 4), Fig. 4b presents the profile pattern of each type. Type A dominated the second stage ferments with type B most prolific in the third stage. Types A and B were closely related with profiles exhibiting relatively few differences. Their dominance at differing stages of the fermentative process may indicate a clonal relationship, with differences in phenotypic expression resulting from changes in the environment (e.g. pH, °Brix) during fermentation. Absence of *S. uvarum* in stage 1 ferment samples, and recovery of just one strain in the last stage, may indicate a specific sensitivity to environmental conditions.

Regarding the four *S. uvarum-like* isolates, the absence of peak m/z 6646 distinguished strain SW2-28 from the other isolates. The type strain of *S. bayanus* used as a reference was isolated from beer, and has been proposed to be a hybrid with genetic traits of *S. uvarum* (63%), *S. eubayanus* (37%) and minor *S. cerevisiae* constituents (<1%) (Nguyen and Boekhout 2017), which may explain its lower similarity (Fig. 2) with our strains.

3.3.2. Non-saccharomyces species

Starmerella bacillaris and *H. uvarum* were the only two non-

Table 2
Yeast community dynamics during vineyard and winery fermentation.

		Vineyard fermentation				Winery fermentation			
	Non-*Saccharomycs*	SV1	SV2	SV3	SV4	SW1	SW2	SW3	SW4
1	*H. uvarum*	52%	7%	-	-	11%	+	+	-
2	*St. bacillaris*	39%	40%	30%	-	86%	12%	33%	-
3	*P. terricola*	8%	-	-	-				
4	*M. pulcherrima*	+	+	-	-	+	-	-	-
5	*P. kluyveri*	+	+	-	-	+	-	+ (1)	-
6	*C. californica*	+	-	-	-	+	-	-	-
7	*P. membranifaciens*	-	+ (3)	-	-	+ (2)	-	-	-
8	*A. pullulans*					+ (2)	-	-	-
9	*P. kudriavzevii*	-	+	-	-				
10	*W. anomalus*	-	+	-	-				
	Saccharomyces								
11	*S. cerevisiae*	+	48%	70%	100%	-	86%	67%	85%
12	*S. uvarum*	-			-	-			5%
13	*S. uvarum-like*					-		-	10%
Population (cfu/mL)		$1.10x10^8$	$1.27x10^{10}$	$5.60x10^8$	$5.40x10^6$	$3.4x10^7$	$1.08x10^{10}$	$3.30x10^8$	$1.60x10^7$

Note: "+" means detectable but at very low levels, the number in "()" means the number of isolates; "-" means undetectable.

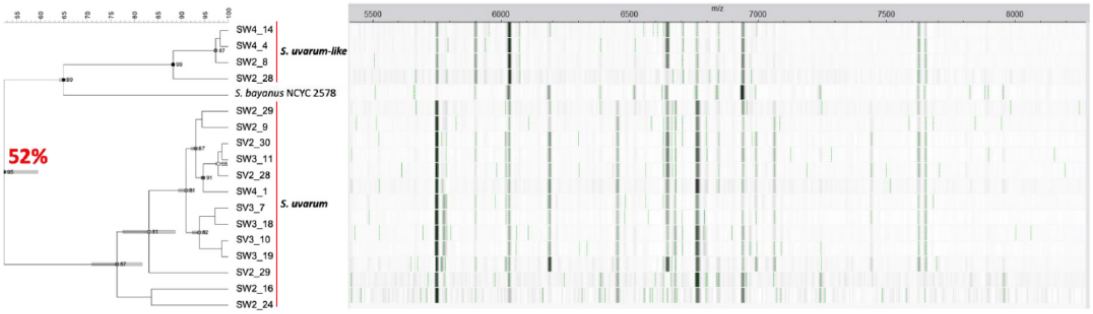

Fig. 2. Cluster analysis of the *S. bayanus* NCYC 2578 and representative *S. uvarum* isolates combined with their band presentation of spectra patterns.

Saccharomyces yeasts detected over three successive ferment stages. Interestingly, *St. bacillaris* strains were divided into three clusters according to the ferment stage they were recovered from. These clusters were distinguished by differences in several prominent peaks, namely m/z 5390; 5405; 6209; 10,779; 10,808 and 13,956 (Fig. 5). Whole-genomic analyses have suggested that *St. bacillaris* may be capable of an "elaborate" stress response (Lemos et al., 2018), and the differences we observe may be the result of differential gene expression in relation to environmental changes as the wine ferments. Further studies are required to confirm this.

Three of the other non-*Saccharomyces* yeasts isolated in our study, *H. uvarum*, *C. californica*, *P. membranifaciens* showed similar trends to the *St. bacillaris* strain variation, with MALDI profile clustering generally aligned with the stage of ferment at which strains were recovered (Supplementary Figs. 2–4). The variation observed among the *M. pulcherrima* strains distinguished three profile groups (Supplementary Fig. 5) but these were not correlated with either source or ferment stage. *Pichia kluyveri* isolates demonstrated both considerable variation in their MALDI spectra and colony morphotypes (Supplementary Figs. 6–7). *Candida californa* was only detected in the first ferment stage in both vineyard and winery ferments, and the variation in peak m/z 8211 correlated with isolation source. Moreover, the isolates from two ferments showed different colony morphotypes (Supplementary Fig. 7).

4. Discussion

It is well established that a variety of yeast species are present at the beginning of wine fermentation and that these may confer desirable or undesirable properties to the final products (Swiegers et al., 2005). Close monitoring of the wine's microbiological status during fermentation is desirable to support consistent quality, an aspect arguably more important in organic and biodynamic wine production, due to the limited interventions available.

The use of MALDI-TOF in clinical microbiology is now widespread due to its low cost-per-sample and rapid turnaround time (Dhiman et al., 2011), however for industrial applications the databases required for effective identification are more limited, often requiring bespoke approaches as described here and indeed elsewhere (Pavlovic et al., 2014; Gutiérrez et al., 2017). Critical to its further use in industrial applications is the development of suitable reference databases; however, in this study alone, we added a total of 249 spectra representing 25 species belonging to eight genera to our database in just 9 days (not considering primary isolation and confirmation). 26 S rDNA gene sequence analysis was used to support the identification results inferred by cluster analysis

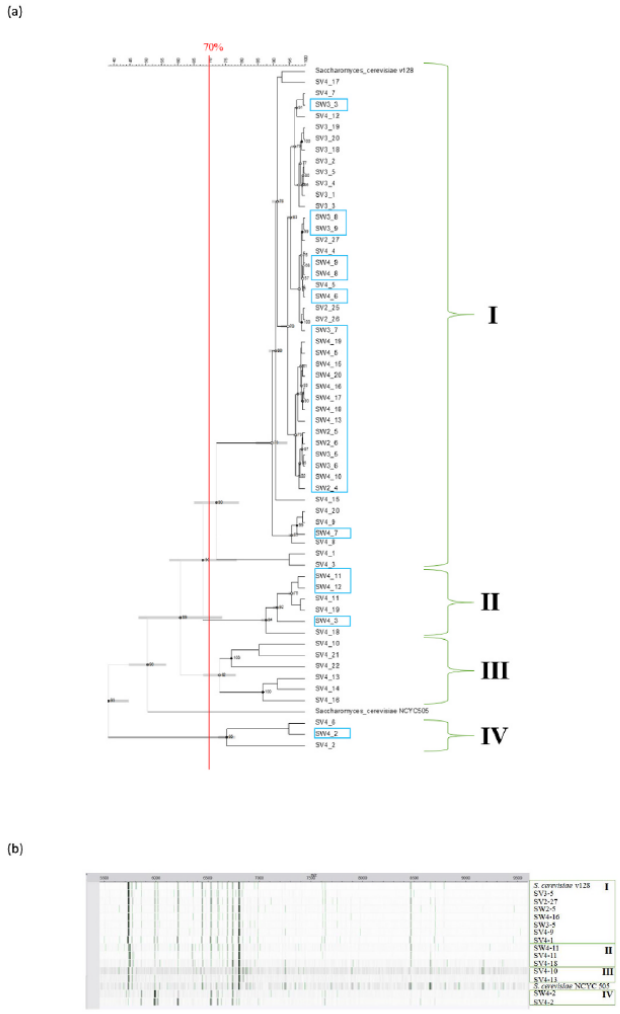

Fig. 3. Cluster analysis of the (a) 60 *S. cerevisiae* isolates and one reference strain *S. cerevisiae* NCYC 505, all the winery isolates are marked in blue squares; 5 types were divided based on the 70% threshold; (b) band presentation (m/z 5500–9500) of representative strains from each *S. cerevisiae* types. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of MALDI profiles where reference strains were not available. Our results clearly validate the efficacy of MALDI-TOF profiling as an identification tool, with phylogenetically distinct species clearly distinguished in the cluster analysis (Fig. 1). Given the challenges described by others in differentiating oenologically-relevant yeast species (Kurtzman and Robnett 1998; Lopandic et al., 2008; Csoma et al., 2010; Kurtzman 2015; Gutiérrez et al., 2017), we consider MALDI-TOF analysis represents important progress in this field. In our study, 13 species belonging to eight genera were identified, comparable generally to the diversity seen in other studies of yeast in wine production (Romano et al., 2003; Fleet 2008).

In this study, the winemaker's fermentations were undertaken both indoors (winery) and outdoors (vineyard), allowing for comparisons between these two systems. Moreover, we saw several examples in both *Saccharomyces* and non-*Saccharomyces* yeasts in which the cluster analysis of MALDI profiles correlated with the isolation source and ferment stage. Proteomics is dynamic and depends on the environmental conditions (Silvestre et al., 2012), and it has been previously reported that isolation sources may influence the protein fingerprints and MALDI dendrogram (Kern et al., 2014; Zhang et al., 2015). Consequently, MALDI-TOF may offer particular advantages for characterizing wine-making yeast since it offers taxonomic accuracy together with resolution of strain differences expressed under differing environmental conditions.

In both systems, non-*Saccharomyces* yeasts *H. uvarum, St. bacillaris*, *C. californica*, *P. kluyveri*, *P. membranifaciens* and *M. pulcherrima* were isolated. The former two species were most abundant, as noted elsewhere (Jolly et al., 2014; Englezos et al., 2017). As far as we are aware, it is the first time *C. californica* has been isolated in a New Zealand vineyard. Farming practices or detection methodology may account for this, as *C. californica* appears absent or present at very low frequencies in conventional vineyards elsewhere (Agarbati et al., 2019). The marked difference in the proportions of these species between the two systems (Table 2) is noteworthy given that the grapes used had the same origins. Other species found only in vineyard ferments were *P. terricola, P. kudriavzevii* and *W. anomalus*. These findings were likely the combined results of microbial interactions (e.g., killer phenotype of certain strains) (Clavijo et al., 2010) in the must, as well as the different environmental conditions to which they were exposed. Vineyard ferments suffered from erratic weather conditions whereas winery ferments were kept indoors in a more stable environment. Interestingly, although the mold species *A. pullulans* is frequently associated with the grape phyllosphere (Bozoudi and Tsaltas 2018), we recovered strains of this only from the winery ferments. Additional studies would be needed to determine if this species had, via its propensity to form biofilms (Bozoudi and Tsaltas 2018), become resident. Winery surfaces are considered as a potential reservoir for introduction of microorganisms to early wine fermentation communities, and play a role in shaping the microbiota of wine fermentation, in which the resident microbial consortia can be affected by the combination of facility design, age, ferment tank material and oxygen permeability, sanitation regimens, and processing decisions (Nguyen et al., 2010; Bokulich et al., 2013; del Alamo-Sanza, Laurie et al., 2015). Nonetheless, since *A. pullulans* can influence wine flavor (Bozoudi and Tsaltas 2018), its presence is noteworthy.

Yeast cells can adapt their physiology to external stimuli in a rapid and robust way; for example, H_2O_2 treatment elicited the change of yeast proteomic response dramatically as early as 30 min from initiation of the oxidative stress (Breker et al., 2013). In our study, *Candida californica* in SV1 and SW1 were clearly differentiated by their MALDI protein profiles, in which the peak m/z 8211 was present as a singlet in winery samples, while it displayed as a doublet m/z 8,211, 8241 in vineyard samples, though they were sourced from the same batch of grape juice. Similar subtle changes in MALDI-TOF profiles of other microorganisms have been found to represent key phenotypic, including morphological, differences (Flores-Trevino et al. (2019); Sousa et al. (2013)). Thus, such peak variation in our case may indicate the differential phenotypic evolution in *C. californica* during adaptations to environmental conditions, which could relate to their differing colony morphologies (Supplementary Fig. 7). Indeed, it has been noted that yeast colony morphology can vary in response to environmental factors including medium composition, pH, cultivation time and temperature (de Becze 1956; Vopalenska et al., 2005). *Pichia kluyveri* isolates also present multiple colony morphotypes, and it could be the reason that their MALDI proteomic fingerprints showed high intraspecific variation (Supplementary Figs. 6–7), whereby MALDI-TOF MS was suggested as a potential colony morphotyping approach by Sousa et al. (2013). The importance of environmental factors resulting in heritable and detectable changes in yeast phenotype is well recognized, with differing potential underlying mechanisms identified (Halfmann et al., 2012; Holland et al., 2014). All yeast strains were subjected to several environmental stresses over fermentation, such as the nutrient starvation, oxygen shortage, high ethanol concentration, and low pH (Trabalzini et al., 2003). Differences between fermentations undertaken indoors and outdoors may account for the band- and peak differences observed in proteomic profiles of *St. bacillaris* in these conditions (Fig. 5).

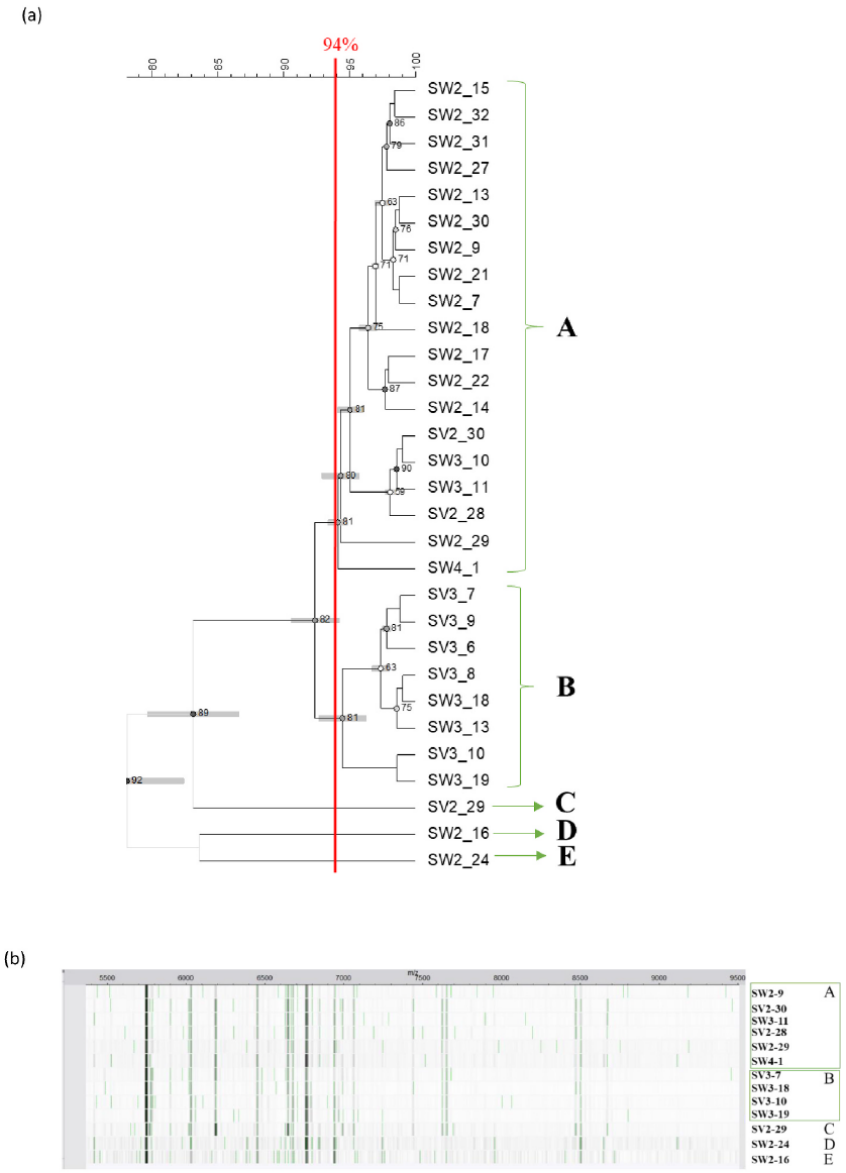

Fig. 4. Cluster analysis of the (a) 30 *S. uvarum* isolates; 5 types were divided based on the threshold 94%; (b) band presentation (m/z 5500–9500) of representative strains from each *S. uvarum* types.

The occasional transformation between silence and reactivation-corresponding genes of certain phenotypes has been observed in *S. bayanus*, *S. cerevisae* and *S. uvarum*, therefore such instability may contribute to their high diversity in the fermenting yeast population (Csoma et al., 2010). Similarly, we observed significant variation in protein fingerprints of *S. cerevisiae* in particular (Fig. 3), consistent with published genetic analysis (Csoma et al., 2010; Zhang et al., 2010; Zhang et al., 2015). Strain profiles were classified into four types, of which one appeared to be predominant, and observed across the whole fermentation process (Fig. 3). The other three types were only detected in the last ferment stage. Two of these three profile types seemed sufficiently closely related to represent adaptation of the dominant strain to the changing environment, whereas Type IV appeared sufficiently distinct to potentially represent a novel strain that had been introduced, or recovered, during the later fermentation stages. Of *S. uvarum*, types A and B were close visually in profile patterns and cluster analysis, that may represent a transition of their proteomic responses from stage 2 (A) to stage 3 (B) (Fig. 5).

The evolutionary divergence of industrial yeasts is considered to be shaped by both the industrial application and geographical origin, and they are genetically and phenotypically separated from wild stocks due to human selection and trafficking (Liti et al., 2009; Yarza et al., 2014; Gallone et al., 2016). Since type strains *S. cerevisiae* NCYC 505 and *S. bayanus* NCYC 2578 were originally isolated from beer, their divergence from our indigenous isolates in our MALDI dendrogram further supports MALDI-TOF MS as a powerful tool to discriminate and classify industrial yeast strains.

5. Conclusions

To our knowledge, this study is the first to describe the variation among and between yeast species in organic wine production facilities in

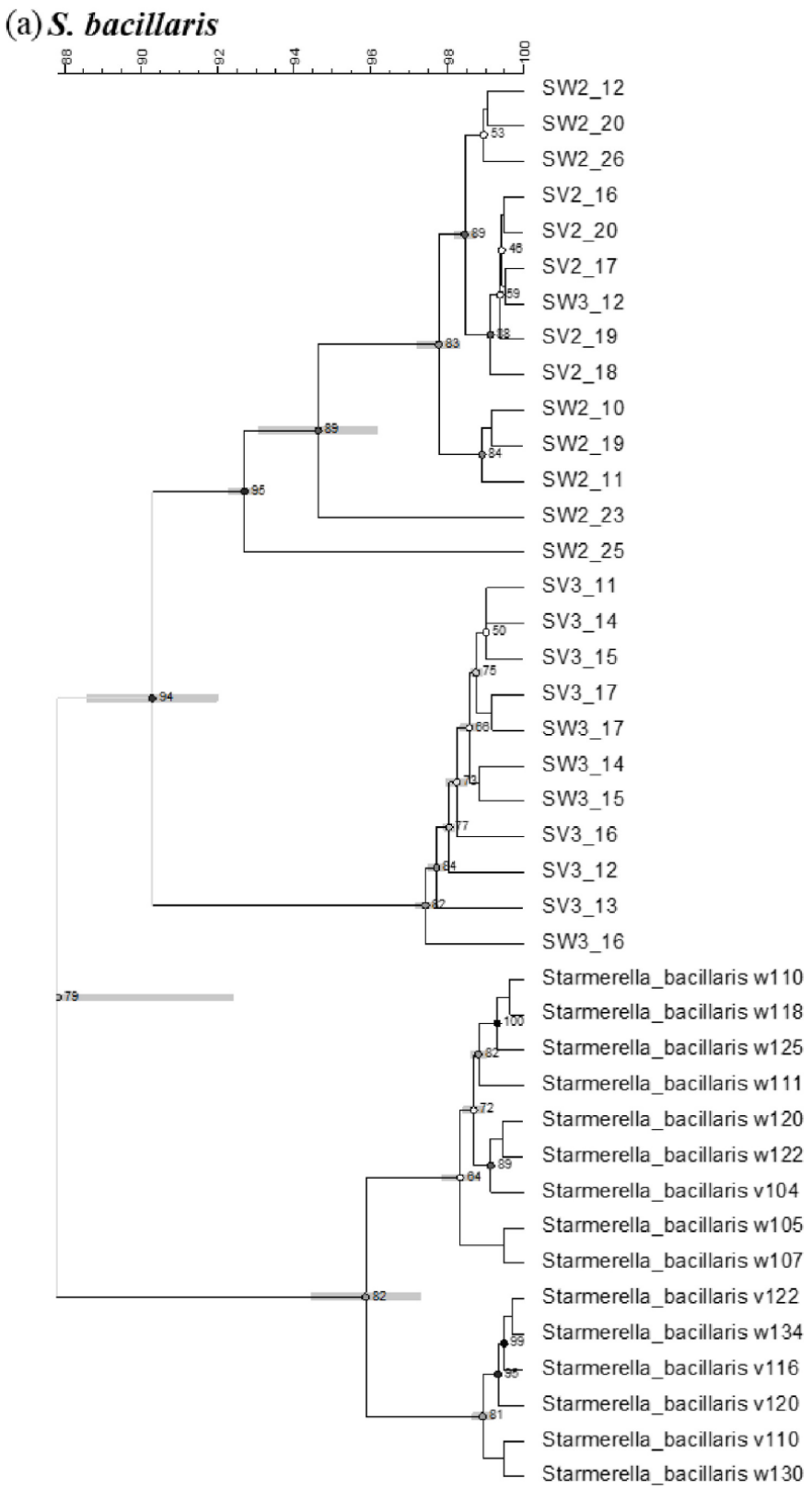

Fig. 5. (a) Cluster analysis of the *St. bacillaris* isolates; (b) peak changes over fermentation in winery and (c) vineyard.

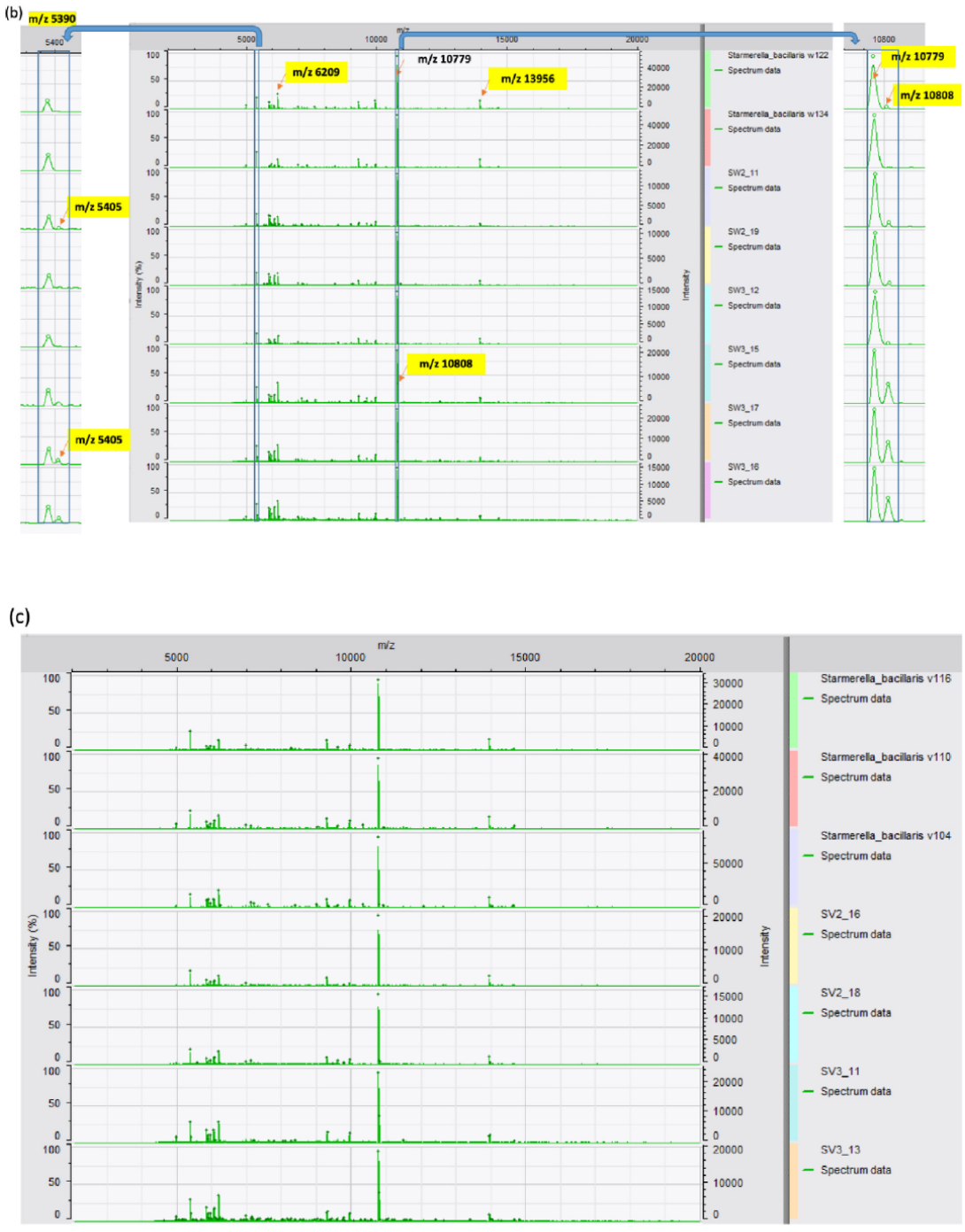

Fig. 5. (*continued*).

the Waipara region of New Zealand over the course of a typical fermentation. In addition, the opportunity to examine the impact of differing (but co-located) production infrastructures on yeast growth dynamics is also, to our knowledge, novel. Our findings indicate that winery infrastructures do appear to have a significant effect in shaping yeast diversity and thus wine production dynamics. We are engaged with further studies to assess these dynamics over a more prolonged term.

MALDI-TOF MS analysis affords excellent taxonomic resolution for yeast identification, including closely related species, with the added advantage of low-cost, ease of operation and short-turnaround time. Development of reference databases of industrial interest is crucial to its further application in practice. Furthermore, as a proteomic tool, MALDI-TOF MS potentially portrays a more active insight into the dynamics of the wine fermentation process. We observed notable correlations of isolation source (i.e. between indoor and outdoor production conditions) and fermentation stage with MALDI-based clustering, indicating the importance of environmental conditions on yeast populations

in winemaking, even where production systems are co-located. This observation has interesting implications on the microbial aspect of terroir, whereby even microconditions may exert subtle influences on product. Further studies are underway to examine the relationship between sensory characteristics, yeast dynamics and production systems in this regard. Nonetheless, the potential of employing MALDI-TOF MS in monitoring wine fermentation to actively support the consistency of high-quality wine products, and potentially for their development too, is advocated for in our study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103824.

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The influence of growth conditions on MALDI-TOF MS spectra of winemaking yeast: implications for industry applications

Junwen Zhang [a], Jeffrey E. Plowman [b], Bin Tian [a], Stefan Clerens [b,c,d], Stephen L.W. On [a,*]

[a] Department of Wine, Food and Molecular Biosciences, Lincoln University, PO Box 85054, Lincoln, New Zealand
[b] AgResearch Ltd, Lincoln Research Centre, Lincoln, New Zealand
[c] Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand
[d] Riddet Institute, Massey University, Palmerston North, New Zealand

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ABSTRACT

Previous studies have shown MALDI-TOF MS to be a powerful tool in wine yeast identification and potential prediction of application. However, it is also established that substrate composition influences protein expression, but the degree to which this may affect MALDI-TOF spectra (and analytical results thereof) has not been fully explored. To further inform assay optimisation, the influence on MALDI-TOF spectra was determined using eight *Saccharomyces* strains of diverse origins cultivated on grape juices from Pinot Noir and Chardonnay varieties, synthetic grape juice, and laboratory-grade artificial culture media (YPD broth and agar). Our results demonstrated significant influences of culture media on strain MALDI-TOF spectra. Yeast culture on YPD agar is recommended for taxonomic studies, with YPD broth culture of *S. cerevisiae* offering improved intra-subspecific differentiation Furthermore, our data supported a correlation between MALDI spectra and the potential industrial application of individual yeast strains.

1. Introduction

Wine is the product of the metabolism of yeast strains on grape juice, resulting in a unique set, and concentration, of metabolites (Richter et al., 2013). In order to control the fermentation efficiently and ensure the homogeneity of wine products, the use of commercial wine yeast strains has become a common practice in winemaking (Valero et al., 2005; Donalies et al., 2008), usually belonging to *Saccharomyces* sensu stricto species. Wine commercial strains were predominantly isolated from vineyard environments and exploited for different purposes in winemaking due to their advantageous kinetic and metabolic characteristics (Camarasa et al., 2011). Furthermore, the diverse phenotypic variations among commercial strains allow their specific application according to the wine style and/or grape variety (Franco-Duarte et al., 2009; Richter et al., 2013). For example, Carew et al. (2013) demonstrated the use of yeast strain significantly affected both the concentration and composition of Pinot Noir wine tannins. Previous studies have shown that commercial wine yeasts are genetically and phenotypically separated from other industrial yeast strains (i.e. beer, bread, and sake), laboratory strains and "wild" yeasts, which also reflects a wide diversity in metabolic strategies to cope with the stressful environment (Palková, 2004; Donalies et al., 2008; Camarasa et al., 2011; Gallone et al., 2016; Goncalves et al., 2016; Fay et al., 2019).

Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is an analytical method that can be used to separate, quantify and identify proteins, and has proven to be a rapid and reliable tool in wine yeast identification (Usbeck et al., 2014; Gutiérrez et al., 2017; Zhang et al., 2020). However, the highly dynamic nature of the proteome in living cells presents several interesting challenges and opportunities to the method, since differential protein expression levels of yeast under different growth conditions are regulated accordingly (Kolkman et al., 2005b). During the winemaking process, growth of yeast often encounters high concentrations of sugars, and limited oxygen and nutrients (e.g. carbon and nitrogen). Enzymes involved in central carbon metabolism pathways showed a significant change in wild type *S. cerevisiae* under glucose- or ethanol-limited conditions (Kolkman et al., 2005a); 51 proteins upregulated in response to glucose limitation and 51 upregulated in response to ammonia limitation were identified in *S. cerevisiae*, illustrating the impact of growth conditions on the yeast proteome (Kolkman et al., 2006).

The influence of certain culture conditions including (e.g. oxygen availability, culture media, growth phase and cell concentration) on

* Corresponding author.
E-mail address: stephen.on@lincoln.ac.nz (S.L.W. On).

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MALDI-TOF mass spectra of a few wine spoilage yeasts has been examined (Usbeck et al., 2013) but to our knowledge, such studies have not been undertaken on winemaking yeasts, where MALDI-TOF analyses have been proposed as a rapid and objective approach for assessing the best application of individual yeast strains for different wine styles (Usbeck et al., 2014). Furthermore, studies to date have not included the use of natural or artificial grape juices, that may provide a more nuanced and accurate evaluation of yeast strain application for industry.

We have previously described an optimized protocol for the MALDI-TOF profiling of winemaking yeast (Zhang et al., 2020). In this study, we investigate a range of culture substrates including conventional laboratory media, and natural and artificial grape juices, to determine the best medium for the use of MALDI-TOF MS to predict wine yeast application. Eight *Saccharomyces* strains from international culture collections, commercial winemaking yeast providers, and a locally sourced isolate from a New Zealand winery, were used.

2. Materials and methods

2.1. Yeast strains

Eight yeast strains were used including four *Saccharomyces* spp. reference strains (*S. bayanus* NCYC 2578 ^{T}, *S. cerevisiae* NCYC 505 ^{T}, *S. paradoxus* NCYC 700 ^{T}, *S. pastorianus* NCYC 396 ^{T}, purchased from NCYC [National Collection of Yeast Cultures]); three commercial strains (Lalvin RC 212, Lalvin QA23, Lalvin ICV D47) purchased as active dry yeast from C.A.L. Limited (New Zealand), and one wild isolate *S. cerevisiae* v128 purified from Pinot Noir grape juice obtained from an organic winery (Greystone Wines, Waipara, New Zealand).

2.2. Culture media

The culture media tested were YPD agar (Difco) (pH 6.5), YPD broth (Difco) (pH 6.5), Laboratory YPD broth (Yeast Extract (10 g/L)(Oxoid LP0021), Peptone (20 g/L)(Oxoid CM0509), D(+)-Glucose (20 g/L) (BDH AnalaR), Agar (15 g/L)(Oxoid LP0011), pH 6.5), synthetic grape juice (SGJ), Pinot Noir (PN) and Chardonnay (CH) grape juice.

Synthetic Grape Juice was made as described by Oro et al. (2014), and comprised solution A (110 g/L glucose, 110 g/L fructose, 10 mg/L ergosterol and1 ml/L Tween80), solution B (6 g/L tartaric acid, 3 g/L malic acid and 0.5 g/L citric acid), and solution C (1.7 g / L yeast nitrogen base with amino acids, 0.2 g $CaCl_2$, 2 g/L casamino acids, 0.8 g/L arginine-HCl, 1 g/L proline and 0.1 g/L tryptophan) were separately sterilized at 121 °C for 20 min and mixed in a laminar flow cabinet aseptically, in which solution B and C were adjusted to pH 3.5 using KOH and HCl, respectively. The final pH and °Brix was 3.5 and 19.

Pinot Noir (harvested in 2019) and Chardonnay (harvested in 2020) grapes were collected from the Lincoln University vineyard and stored at −20 °C. Pinot Noir grape juice was prepared according to Barbosa et al. (2018) with minor modifications. Briefly, grape juice was obtained by pressing Pinot Noir grapes in sterilized filter bags (Stomacher® lab system 400 classic, Seward). After homogenization, juice was transferred to 50 mL tubes and centrifuged at 4700 x*g* for 30 min (Heraeus™ Multifuge™ X3 Centrifuge, ThermoFisher Scientific), the supernatant was collected and split into three lots for further treatments. Chardonnay grape juice was processed and obtained directly from the Lincoln University winery and stored frozen. After thawing at room temperature, the juice was also split into three lots for the following treatments: i) Autoclaved Grape Juice (AGJ) - sterilized at 121 °C for 15 min, ii) Heated Grape Juice (HGJ) – heated in a water bath at 70 °C for 10 min to eliminate background yeast then immediately cooled and stored at −20 °C, iii) Unautoclaved Grape Juice (UGJ) without further treatment and stored at −20 °C.

Thereafter, the eight selected yeast strains were cultured on each of 10 culture media, i.e. YPD agar (Difco), YPD broth (Difco), Laboratory YPD broth, SGJ, PN-(AGJ, HGJ, UGJ) and CH-(AGJ, HGJ, UGJ), from which microbial growth was subjected to MALDI-TOF analysis.

Three treatments of PN juices had the same initial pH and °Brix at 3.8 and 23, but the three treatments of CH juices showed different °Brix with AGJ at 24, HGJ over 32, and UGJ at 21, respectively. The pH was measured at 3.0 for all three treatments of CH juices. The pH and °Brix were measured by an HI 9025 microcomputer pH meter (Hanna instruments) and a refractometer (Bellingham + Stanley).

2.3. Culture conditions

Reference strains and wild isolate *S. cerevisiae* v128 were maintained at −80 °C in YPD + 30% (*v*/v) glycerol stock and were recovered in YPD agar at 28 °C for 3 days. Commercial strains were activated by rehydrating directly in 15 mL YPD broth (Difco) followed by an anaerobic incubation overnight at 28 °C in a 50-mL tube covered tightly without agitation

MALDI sample preparation from liquid media was modified based on Usbeck et al. (2014). A single colony was picked to inoculate in 15 mL YPD broth (Difco) and subcultured twice prior to inoculation to the different liquid media used. Subsequently, 15 mL of nine fresh liquid growth media were inoculated with 4% (*v*/v) (approx. $OD_{(600nm)}$ 0.05) of the overnight culture and incubated as described above for 48 h to monitor the growth. Growth curves of the eight strains in each of the nine liquid media above were recorded using optical density $OD_{(600nm)}$ (SmartSpec™ 3000 Spectrophotometer, BIO-RAD) at various time points (8, 12, 14, 16, 18, 20, 22, 24, 36, 38, and 48 h), with corresponding media as the blank. Calibration curves for each strain were made to determine the relation between the $OD_{(600nm)}$ value and corresponding cell number by the hemacytometer. All experiments were conducted in triplicate.

2.4. MALDI-TOF MS

2.4.1. Sample preparation

The preparation of yeast strains harvested from YPD agar was as described previously (Zhang et al., 2020). Samples collected from the liquid media was according to Usbeck et al. (2013), in order to obtain sufficient quantities of yeast cells for MALDI analysis. Briefly, 900 µL culture of YPD broth (Difco, and in-lab produced) and 1.5 mL culture of the other seven media were transferred into a 1.5 mL tube (Safe-Lock, Eppendorf) and centrifuged at 12,100 x*g* for 4 min (Eppendorf AG, Minispin 5452). The supernatant was discarded, and the pellet was i) washed with 900 µL sterilized deionized water three times or ii) kept unwashed to verify the influence of the wash step on the final spectra. Subsequently, the pellet was resuspended into 300 µL deionized water, and vortexed for 1 min with 900 µL absolute ethanol. After centrifugation (12,100 x*g*, 4 min), the pellet was air-dried in a laminar-flow hood and stored at −20 °C prior to protein extraction.

To extract proteins, 50 µL of 70% formic acid (*v*/v) was added to yeast pellet and mixed thoroughly by vortexing for 1 min, then 50 µL of acetonitrile (ACN) was mixed for the same time. Protein extract was obtained by centrifugation (12,100 x*g*, 4 min). Equal volume of protein extract and α -cyano-4-hydroxycinnamic acid (HCCA) matrix solution (10 mg/mL in 75% ACN and 2.5% trifluoroacetic (TFA) were mixed well and 1 µL of this mixture was deposited onto the MALDI ground steel target plate (MTP 384, Bruker Daltonics®) till dry. For technical replication, each extract was spotted onto three individual wells, therefore yielding 9 spectra per strain.

2.4.2. Mass spectra acquisition

MALDI-TOF mass spectra were automatically acquired on a Ultraflex III TOF/TOF MS instrument (Bruker Daltonics®, Bremen, Germany), operating in positive ion detection mode using a SmartbeamTM laser at 200 Hz; pulsed-ion extraction time of 120 ns; and laser power 80%. The voltage of the ion source was set as 25.00 kV (ion source 1), 23.55 kV (ion source 2) and 6.01 kV (lens). Samples were analyzed using the

linear detector at high mass range *m/z* 2000-20,000. The final spectrum was an average accumulation of 2000 single spectra (high mass range m/z 2000-20,000) gathered. Each single spectrum was recorded from 10 random raster spots. The mass spectrometer was externally calibrated in every experiment at regular intervals, using the calibrant position in the middle of each tetrad of spots. an in-house protein standard comprising Insulin, [M + H] + at *m/z* 5734.52, Cytochrome C, [M + H] + at 12360.99 and [M + H]2+ at 6180.99, Myoglobin, [M + H] + at 16952.30 and [M + H]2+ at 8476.65), Aprotinin [M + H] + m/z 6511.51, and β-lactoglobulin [M + H] + m/z 18,363.00 was used.

2.4.3. Data analysis

Raw mass spectra were exported as .txt format using FlexAnalysis software (version 3.0. Bruker Daltonics®), and imported into software BioNumerics version 7.6 (Applied Maths). Spectra pre-processing was achieved at a default setting but baseline subtraction with Rolling disc value was adjusted to 150. Kaiser Window value in smoothing and signal to noise ratio (S/N) in peak filtering were adjusted according to the quality of spectra.

A composite profile of each strain was obtained using 9 spectra derived from three technical replicates of each of three biological replicates. Cluster analysis was performed using the Pearson correlation coefficient and UPGMA (unweighted-pair group method with arithmetic mean) algorithm. The "goodness-of-fit" between calculated similarity values between all strains, and the clustering shown in the dendrogram, was calculated using the cophenetic correlation method, whereby a value of 1.0 indicates a perfect correlation of the dendrogram with the similarity matrix.

3. Results

3.1. Growth curve

The growth rate in liquid media of strains examined varied substantially (Fig. 1). The corresponding cell number of strains *S. cerevisiae* NCYC 505, *S. paradoxus* NCYC 700, *S. pastorianus* NCYC 396, and *S. bayanus* NCYC 2578 determined by a calibration curve (Supplementary Fig. 1) at $OD_{(600nm)}$ 0.5 was 4×10^6 cells/mL, 4×10^6 cells /mL, 6×10^6 cells /mL, 8×10^6 cells /mL, respectively, whereas the cell number was 9×10^6 cells /mL for the other four strains. Comparison of the growth rates enabled strains to be delineated into two groups; commercial wine strains (Lalvin RC 212, Lalvin QA 23 and Lalvin ICV D47) and wild isolate *S. cerevisiae* v128 that reproduced vigorously (Group I); and reference strains sourced from culture collections (Group II). More specifically, compared to group II, group I exhibited a shorter lag phase during which yeast cells become acclimatised to the new environment, and a higher growth rate during the log phase (Supplementary Fig. 2).

In most of the tested media, strains reached stationary phase at 24 h, however in YPD broth (Difco and lab) and UGJ (PN and CH), group I strains entered stationary phase earlier around 16–18 h. Therefore, 24 h was selected as the test time for MALDI sample preparation to maintain the consistency of the cell physiology.

3.2. Effect of culture media on MALDI-TOF spectra

The quality of the MALDI-TOF profiles in terms of the background observed and number of clearly discernible informative peaks differed substantively between strains and depending on the medium used to culture them. The reference strains from international culture collections yielded spectra that possessed high background levels, or exhibited very few informative peaks, when cultured in natural or synthetic grape juices. In contrast, the results from strains obtained from a commercial supplier (Lalvin) with established use in winemaking, or an isolate from a New Zealand vineyard (v128) were generally more consistent, although strains cultured in Chardonnay juice did not generally yield suitable profiles, with the exception of *S. cerevisiae* strain Lalvin ICV D47, recommended by the supplier for the production of Chardonnay wine. The growth media that produced the spectra with the lowest background, and overall the most identifiable peaks, were commercially produced YPD broth (except *S. paradoxus,* discussed below), and commercially produced YPD agar. These media were also those that best

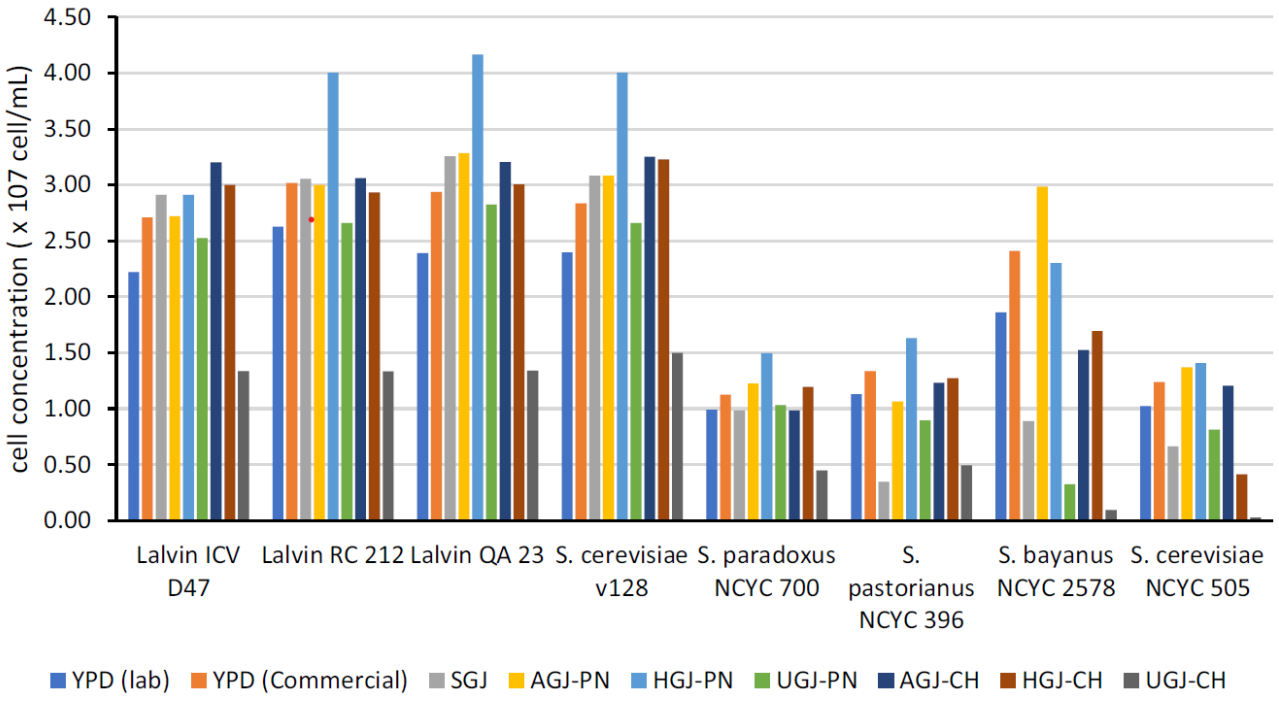

Fig. 1. Biomass of each culture in different liquid culture media after 24 h incubation under 28 °C.

supported yeast growth (Fig. 1).

3.3. Effect of washing step on MALDI-TOF profile clarity

An additional washing step would increase the time- and labour- cost in practical operation, especially a large number of samples. In this study, inclusion of a wash step improved the spectra profiles cultured in grape juice significantly, but did not have a profound effect on YPD broth-derived profiles. Results from three media (YPD broth, Difco), PN-AGJ and CH-AGJ are presented here as examples (Fig. 2). In order to detect the possible influence of the native grape proteins on MALDI spectra, the sediments of each treatment of both PN and CH were also subjected to the same MALDI procedures as the yeast strains (Supplementary Fig. 3). Thus, we observed certain peaks appearing in unwashed samples that could be traced to the corresponding grape juice, e.g., *m/z* 10,641 detected in unwashed *S. bayanus* NCYC 2578 cells (PN-HGJ and PN-UGJ).

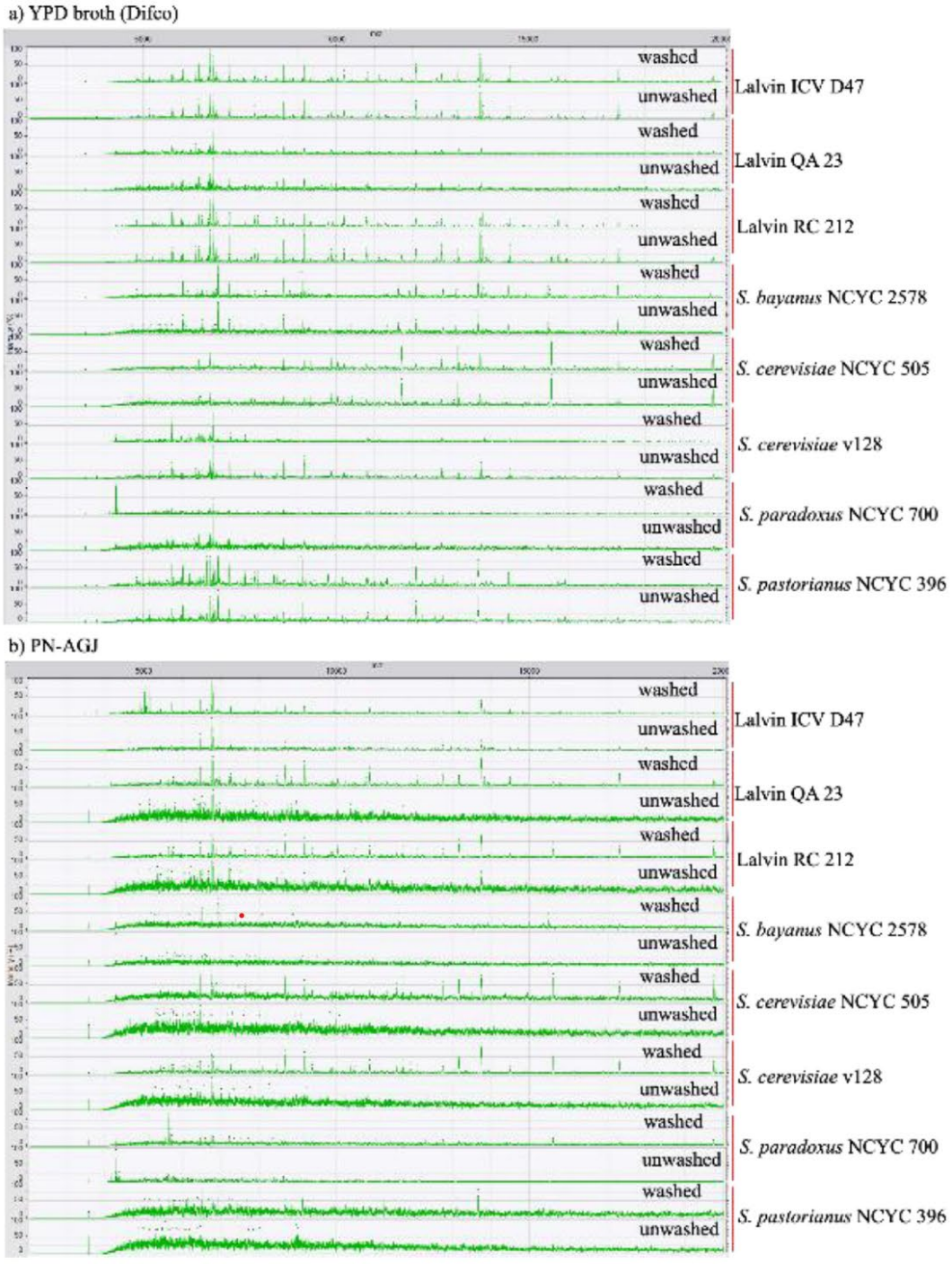

Fig. 2. Comparison of the wash step impact on strains grown in a) YPD broth (Difco), b) PN-AGJ and c) CH-AGJ.

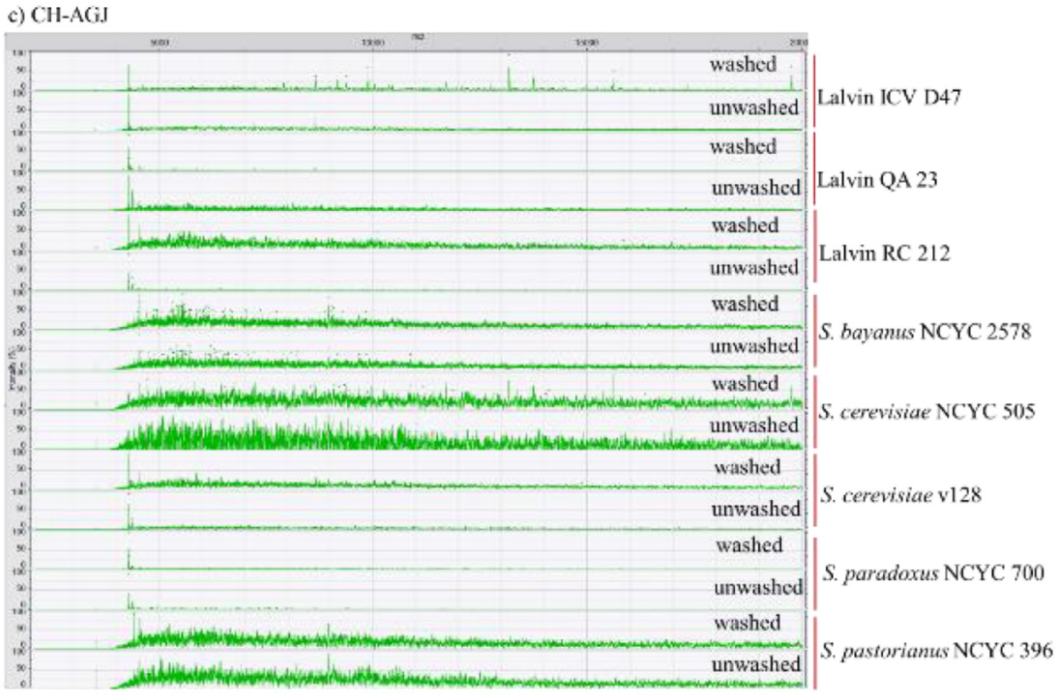

Fig. 2. (*continued*).

3.4. Influence of culture conditions on cluster analysis

Results of cluster analyses (Fig. 3) based on each of the growth conditions yielding clearly discernible peaks (i.e. YPD agar, YPD broth, and Lab-YPD broth) revealed subtly different results reflecting the differences observed between the corresponding MALDI-TOF spectra. YPD broth (Difco) gave the lowest similarity between strains reflecting the greater diversity of peaks among profiles, but the highest similarity (68.1%) between species *S. bayanus* NCYC 2578 and *S. pastorianus* NCYC 396, whereas the values were 40.8% and 52.4% in YPD agar- and YPD broth (lab)- dendrogram, respectively. *S. paradoxus* NCYC 700 was not separated from the *S. cerevisiae* group in the comparison using YPD agar-cultured strains, but was in comparable analyses from each of the two YPD broths used. The resultant similarity matrices (Fig. 3) also exhibited a more straightforward visualization that profiles derived from cultures on YPD agar and YPD broth (lab) allowed for better species-level differentiation.

It is noteworthy that commercial strain Lalvin QA 23 is a hybrid (*S. cerevisiae* x *S. bayanus* var. *uvarum*), however, its peak variation from the other *S. cerevisiae* strains is clearly displayed in profiles derived from both YPD media. As can be seen from Fig. 4 (a), the major QA 23-specific peak pair *m/z* 5733, 5746 is differentiated from the single peak at m/z 5733 in the other *S. cerevisiae* strains. The spectra differences among strains provided by YPD broth (Difco) (Fig. 3 (b)) were greater than those of YPD agar (Fig. 3 (a)), as indicated by the similarity of 48.5% and 51.7%, respectively; on the other hand, it is 81.5% where laboratory-synthesised YPD broth was used (Fig. 3 (c)).

Although most of the PN-derived spectra were similar among *S. cerevisiae* strains, Table 1 summarizes the discriminant peaks of each strain from closer visual inspection (Fig. 5). For example, peak *m/z* 10,023 only appeared in RC212, while m/z 10,368 was unique to *S. cerevisiae* v128.

4. Discussion

The extensive biological diversity evidenced among *Saccharomyces* species and indeed utilised to produce a wide range of alcoholic beverage styles was displayed in this study by the different growth rates seen among the media used, and also in the MALDI-TOF profiles derived from strains following cultivation.

Comparison of growth curve and the resulted spectra indicated that media that did not support vigorous growth of the strains tested yielded material that gave correspondingly poor quality MALDI-TOF spectra, this fact also has been reported by Wieme et al. (2014) and Luthje et al. (2017). However, despite the variation under different growth media, certain common peak classes remain stable, which might represent "Housekeeping genes" known to be constitutively expressed to maintain cellular function irrespective of the changing metabolic activities (Valentine et al., 2005). Peaks in the MALDI spectra have been reported to be mainly ribosomal- and mitochondrial-related proteins (Ryzhov and Fenselau, 2001; Usbeck et al., 2013). It is well known that growth phase (or different physiological state) affected the yielded protein profiles (Vargha et al., 2006; Qian et al., 2008; Wieme et al., 2014), and Usbeck et al. (2013) suggested younger cultures showed better results, accordingly, 24 h was taken as the standard incubation time in this study given the consistent cell physiological state and ease of operation.

Most notably, the type strains used in this study as taxonomic references generally grew poorly in the natural and artificial grape juices used, yet strains recommended for use in winemaking, or isolated from a New Zealand vineyard, were clearly more suited for growth in the grape juice environment. Commercial strains are either natural isolates from vineyard or wineries shown superior properties for winemaking, or have been experimentally selected for specific purpose (Bradbury et al., 2006). Long-term domestication of type strains under optimized lab conditions might repress their some protective and adaptive mechanisms essential for survival in natural environments (Palková, 2004). Certainly these strains showed weaker fermentative vigour during the growth in grape juice, and failed to obtain quality protein profiles. Compared to rich medium YPD, grape juice (low pH 2.9–3.8 and high osmolarity sugars of 200–300 g/L)) is highly unfavourable to most of microorganisms, but is one to which wine yeasts are well adapted (Richter et al., 2013).

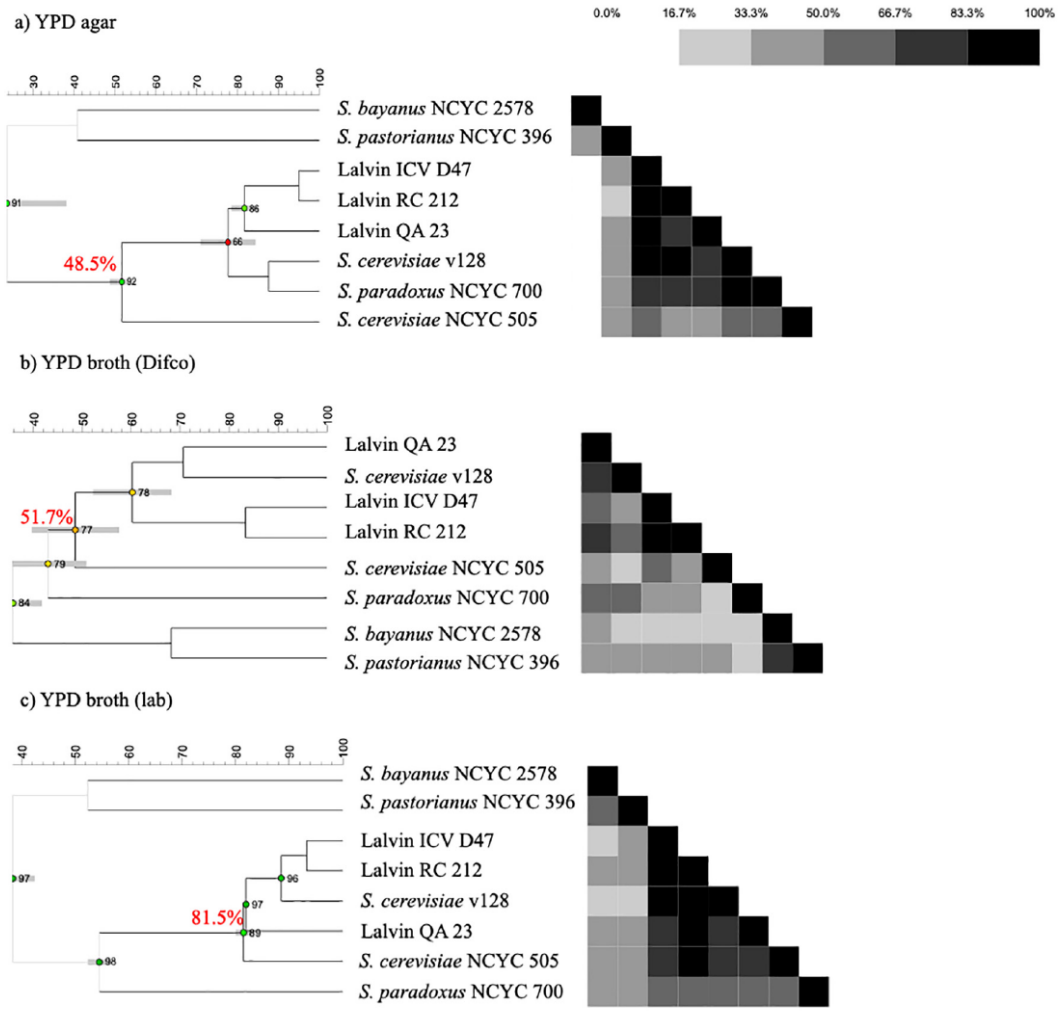

Fig. 3. Cluster analysis and similarity matrices of eight strains derived from a) YPD agar (Difco), b) YPD broth (Difco), and c) YPD broth (lab) using the Pearson correlation coefficient and UPGMA algorithm. Similarity of the *S. cerevisiae* branch was labelled in red, namely 48.5% in a) YPD agar - Lalvin ICV D47, Lalvin RC 212, Lalvin QA 23, *S. cerevisiae* v128, *S. cerevisiae* NCYC 505 and *S. paradoxus* NCYC 700, 51.7% in b) YPD broth (Difco) - Lalvin QA 23, *S. cerevisiae* v128, Lalvin ICV D47, Lalvin RC 212 and *S. cerevisiae* NCYC 505, 81.5% in c) YPD broth (lab) - Lalvin ICV D47, Lalvin RC 212, *S. cerevisiae* v128, Lalvin QA 23, *S. cerevisiae* NCYC 505. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Walker et al. (2002) indicated the variations in spectra produced under different media reflected the differences in cell surface composition. Yeast cell wall is a complex and dynamic structure composed of β-1,3 and β-1,6-glucan, chitin and mannoproteins, the composition and structure vary depending on the growth conditions, e.g., growth media, carbon source, nitrogen, pH, temperature and aeration (Kapteyn et al., 2001; Aguilar-Uscanga and Francois, 2003; Orlean, 2012). A low-pH environment was shown to induce stronger yeast cell resistance (Kapteyn et al., 2001; Aguilar-Uscanga and Francois, 2003), which may be partially responsible for the failure of quality spectra acquisition in CH (pH 3.0) and SGJ (pH 3.5), thereby impeding the efficiency of intracellular protein extraction. Such a situation caused by cell wall structure transformation may also apply to type strain *S. paradoxus* NCYC 700, whereby its tendency to form "clumps" or "flocs" (Supplementary Fig. 4) may have contributed to its slow growth rate in liquid media and correspondingly poor quality MALDI-TOF spectra, as the "flocculins" (lectin-like proteins) that protrude from the cell wall lead to the phenomenon of flocculation (Verstrepen et al., 2003).

Preconditioning to the specific environmental conditions of grape juice appears to be significant for individual *Saccharomyces* strains to thrive as well as the yielded protein profiles. The physico-chemical properties of grape juice can be affected by the heat treatment in protein, pH, total acid, viscosity, ionconcentration, and brix (Ozcan et al., 2015), which reflected in the change of brix value and the liquid colour among three treatments. The autoclaved grape juice (AGJ) showed a darker colour especially in terms of the Chardonnay grape juice, which was due to the non-enzymic browning reaction at high temperature occurred between reducing sugars (e.g. glucose and fructose) and the amino acids (e.g. arginine, glutamine and proline) in grape juice (Bozkurt et al., 1999; Ozcan et al., 2015). Therefore, AGJ provided an absolutely sterilized situation for the yeast strains tested, whereas UGJ was similar to a spontaneous fermentation system where the tested strains would encounter the competitive stressor from the indigenous microbes. Short-term pasteurization would lead to proteins denaturation, except some proteins with a high degree of thermostability, such as the invertases and lipid-transfer proteins (Marangon et al., 2012). The denatured proteins caused by heat treatment may be more susceptible for utilization and thus favour the yeast metabolism, therefore an overall higher quality of protein profile was observed in AGJ and HGJ than in UGJ.

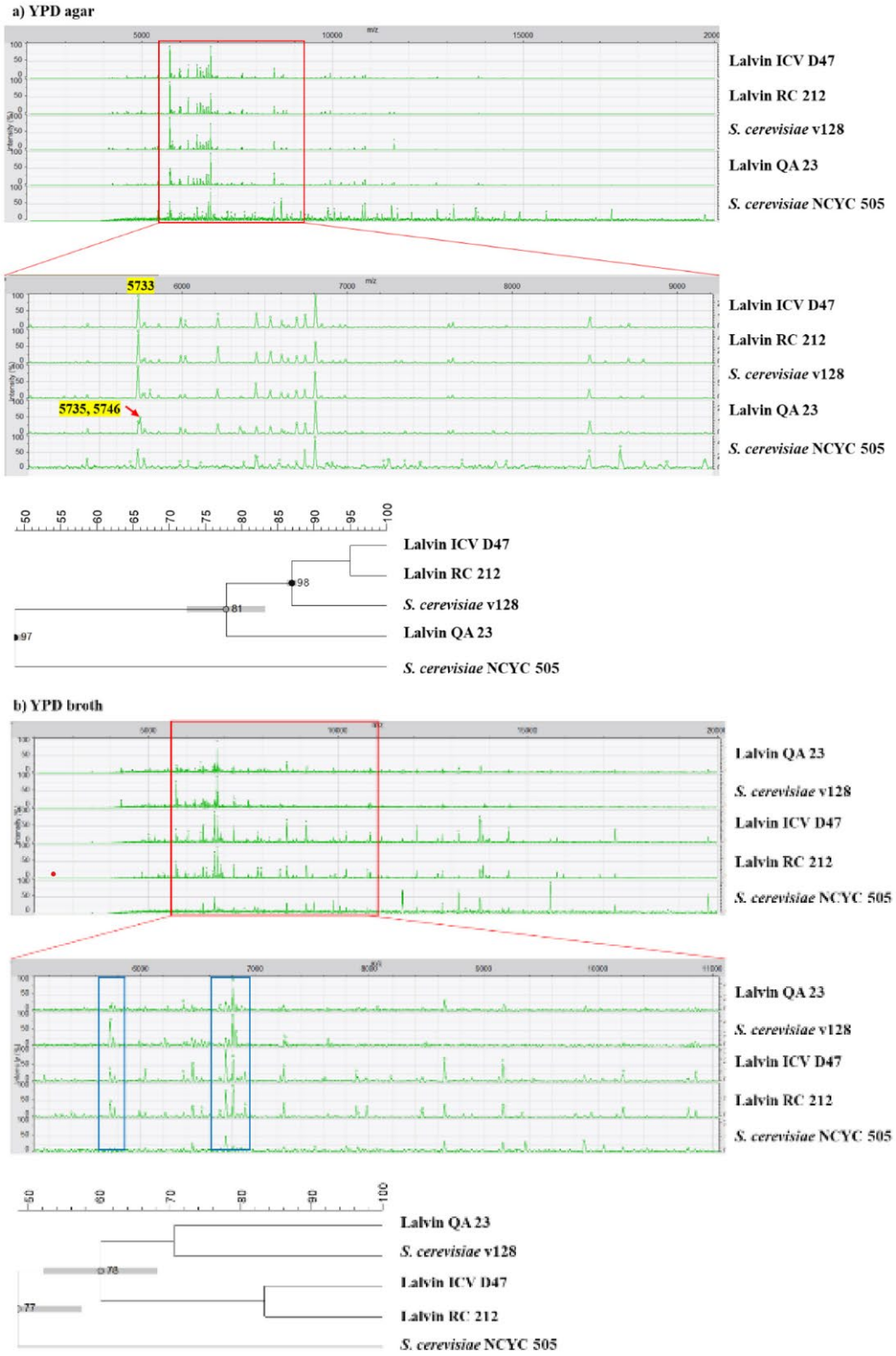

Fig. 4. MALDI spectra and cluster analysis of five *S. cerevisiae* strains grown a) on YPD agar and b) in YPD broth. The number highlighted in yellow indicates the peak m/z 5733 in Lalvin ICV D47, and a doublet *m/z* 5733, 5746 in Lalvin QA 23. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Discriminant peaks among five *S. cerevisiae* strains of diverse origins and application.

	Discriminant peaks (*m/z*)								Application
Lalvin ICV D47	–	–	–	–	–	–	–	–	Chardonnay
Lalvin QA 23	9878	–	–	11,912	–	13,171	–	19,755	Sauvignon Blanc
Lalvin RC 212	9878	10,023	–	11,912	11,703	13,171	15,601	19,755	Pinot Noir
S. cerevisiae NCYC 505	9878	–	–	11,912	11,703	13,171	15,601	19,755	References (originally from Brewing)
S. cerevisiae v128	9878	–	10,368	11,912	11,703	13,171	15,601	19,755	Pinot Noir vineyard isolate (Waipara, New Zealand)

Note: "–" means the absence of peak.

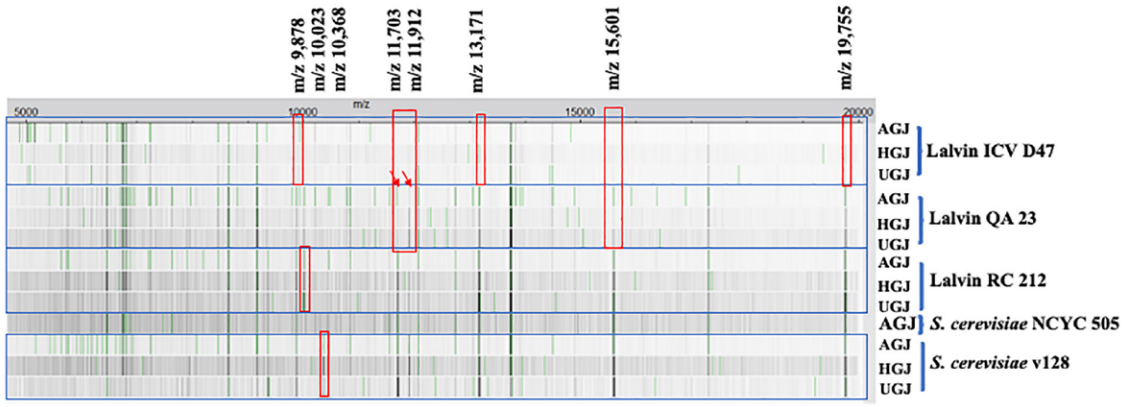

Fig. 5. Band presentation of MALDI spectra from five *S. cerevisiae* strains grown in Pinot Noir grape juice. The discriminant peaks among the five strains of diverse origins and application are labelled on top of the profiles.

Additionally, the high concentration of background organic matter or ions in grape juice could impair the efficacy of spectra acquisition. A large number of low molecular weight interfering compounds such as salts or polyphenols can affect the signal intensity by increasing the chemical noise in the mass spectrum (Nunes-Miranda et al., 2013). Ion suppression is shown to affect mass spectrometry, during which the presence of ions in the media could block the ionization process, consequently, decreasing the efficiency of the analyte ionized (Annesley, 2003; Johanson et al., 2007; Anderson et al., 2012). Alispahic et al. (2010) encountered similar problems with quality spectra acquisition when cultures on mCCD (modified charcoal cefoperazone deoxycholate) agar as the mCCD agar contaminants interfered with ionization process. However, a washing step often improved the clarity of spectra. The components in liquid media act as spectral contaminants that can interfere with quality spectrum acquisition. Grape proteins appear to have a major impact on spectra acquisition. Certain peaks (e.g. *m/z* 7107, 9050 and 10,641) observed in grape juice spectra were reported as 7.1 kDa, 9.1 kDa, 10.6 kD in Muscat of Alexandria wine, Chardonnay wine and Sauvignon blanc wine (Weiss et al., 1998). Proteins with similar sizes can present in different lots of wines but may be in variable amounts (Weiss et al., 1998). Due to the high levels of insoluble tannins in red wines, it is believed that most proteins can be removed by precipitation in the form of tannin-protein complex (Nakanishi and Yokotsuka, 1990), it may explain the higher quality spectra were obtained in PN than in CH, as the interference of PN-proteins may lower than that from CH.

Among the winemaking or vineyard-associated strains, differences were evident too. Despite these strains all showing an ideal growth rate in natural and synthetic grape juices, well-defined MALDI-TOF profiles were not obtained from all strains when cultured in these media. Only Lalvin ICV D47 consistently generated analyzable MALDI-TOF profiles from each growth medium, including Chardonnay grape juice, which otherwise yielded poor quality spectra from other strains (Supplementary Fig. 5). Interestingly, Lalvin ICV D47 is recommended by the distributors for Chardonnay wine production. The MALDI-TOF profile of the strain is unique (Table 1); the absence of certain peaks observed in this, and Lalvin QA 23 (the only other strain examined recommended for the production of Sauvignon Blanc and other white wines) supports previous studies whereby the recommended application of winemaking yeast correlated with MALDI-TOF spectra (Usbeck et al. (2014). Furthermore, the use of YPD broth and YPD agar had a significant impact on taxonomic resolution at strain level. We have previously used MALDI-TOF analysis in conjunction with YPD agar cultures of over 20 different yeast species to demonstrate its efficacy as an identification tool (Zhang et al., 2020), even indicating its value to differentiate strains recovered from different fermentation environments(Zhang et al., 2021). Nonetheless, it is perhaps encouraging to note that YPD broth (Difco) generated a higher discriminatory power. Usbeck et al. (2014) claimed successful *S. cerevisiae* strain differentiation by using YPG broth; on the other hand, Gutiérrez et al. (2017) failed whereby the use of yeast malt agar (YMA) composed by 1% glucose, 0.5% proteose peptone n° 3, 0.3% yeast extract, 0.3% malt extract and 2% agar. Laboratory media may vary in the individual ingredients across different labs, thus rigorously consistent quality commercial YPD broth (Difco) is recommended for the consistent profile quality.

5. Conclusion

In conclusion, the selection of suitable culture media plays a key role in the discriminatory power of MALDI-TOF MS. Media that did not sustain optimal growth have a profound impact on the MALDI spectra patterns; both the synthetic and natural grape juice proved to be poor matrices for generating suitable MALDI-TOF profiles, although Pinot noir juice was more forgiving. The influence of culture media on final protein profiles may originate from the environment-induced cell physiological state change and the media components (e.g., grape proteins and ions in grape juice) direct interference on the ionization process. Specific to the latter case, a simple centrifugation and wash steps

can help improve the efficiency of MALDI-TOF MS.

It is encouraging that laboratory-defined media found to yield the best quality MALDI-TOF spectra in this study has been used previously to infer optimal strain utilization in winemaking and brewing (Usbeck et al., 2014; Lauterbach et al., 2017) making the potential wider use in strain prediction pragmatic. We conclude that a combination of commercially available YPD agar and YPD broth accompanied by a deionized water wash is recommended for in-house MALDI database construction and strain-level differentiation, respectively, as some microbial cells grown in liquid media may not be suited for MALDI analysis.

Declaration of Competing Interest

We have no competing interests to declare. No funding was received for this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106280.

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Predictive Potential of MALDI-TOF Analyses for Wine and Brewing Yeast

Junwen Zhang [1], Jeffrey E. Plowman [2], Bin Tian [1], Stefan Clerens [2,3,4] and Stephen L. W. On [1,5,*]

[1] Department of Wine, Food and Molecular Biosciences, Lincoln University, P.O. Box 85054, Lincoln 7674, New Zealand; Cherie.Zhang@lincolnuni.ac.nz (J.Z.); Bin.Tian@lincoln.ac.nz (B.T.)
[2] AgResearch Ltd., Lincoln Research Centre, Lincoln 7674, New Zealand; Jeff.plowman@agresearch.co.nz (J.E.P.); Stefan.Clerens@agresearch.co.nz (S.C.)
[3] Biomolecular Interaction Centre, University of Canterbury, Christchurch 8041, New Zealand
[4] Riddet Institute, Massey University, Palmerston North 4472, New Zealand
[5] Centre for Foods for Future Consumers, Lincoln University, P.O. Box 85054, Lincoln 7674, New Zealand
* Correspondence: stephen.on@lincoln.ac.nz

Abstract: The potential of MALDI-TOF profiling for predicting potential applications of yeast strains in the beverage sector was assessed. A panel of 59 commercial yeasts (47 wine and 12 brewing yeasts) was used to validate the concept whereby 2 culture media (YPD agar and YPD broth), as well as two mass ranges m/z 500–4000 and m/z 2000–20,000, were evaluated for the best fit. Three machine learning-based algorithms, PCA, MDS, and UMAP, in addition to a hierarchical clustering method, were employed. Profiles derived from broth cultures yielded more peaks, but these were less well-defined compared with those from agar cultures. Hierarchical clustering more clearly resolved different species and gave a broad overview of potential strain utility, but more nuanced insights were provided by MDS and UMAP analyses. PCA-based displays were less informative. The potential of MALDI-TOF proteomics in predicting the utility of yeast strains of commercial benefit is supported in this study, provided appropriate approaches are used for data generation and analysis.

Keywords: MALDI-TOF analysis; commercial wine yeast; brewing yeast; winemaking; UMAP

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1. Introduction

Wine is a complex product resulting from the interactions between yeasts and grape juice components, and each yeast strain within the same species has a specific impact on the final wine composition and sensory profile [1]. Diversity among commercial strains was highlighted through the unique phenotypic patterns of each strain [2]. The impact of the yeast on wine flavour is largely determined by the array of volatile substances (e.g., higher alcohols, acids, esters, carbonyls, and thiols) produced by the metabolism of grape juice components [3].

The adaptive divergence of genomics in response to different ecological niches allows the development of specific genetic groups of *Saccharomyces cerevisiae* in different fermented food (e.g., wine, beer, dairy products, and bread) and their natural habitats [4]. Along with the diverse fermentation environments, genotypes and phenotypes of *S. cerevisiae* are shaped via hybridization, polyploidization, pseudogenization, genome decay, gene duplication, and horizontal gene transfer to specifically adapt [5,6]. Commercial wine yeast strains are closely related, as demonstrated genetically by the microarray karyotyping analysis [7]; differences in the fermentation and organoleptic properties of each strain may arise from a small number of genetic changes. Most quantitative trait alleles exert considerable phenotypic variations among *S. cerevisiae* strains and alter conserved amino acid positions within protein-coding sequencing [8].

Matrix Assisted Laser Desorption/Ionization–Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is a novel proteomic approach, which has been widely applied in the

identification and characterization of important microorganisms of food interest, such as pathogenic bacteria-*Listeria monocytogenes* [9], *Staphylococcus aureus* [10], *Thermophilic Campylobacter* [11], and non-typhoidal *Salmonella* [12]. In brief, MALDI-TOF MS is a technique based on "soft ionization", where microbial cells are embedded in a suitable matrix that extracts and crystallises the native proteins and assists in their ionisation when exposed to a laser beam. The ions are then accelerated through an electrostatic field, and separated according to their m/z ratio until they reach the detector [13]. The resulting complex profile represents a species-specific fingerprint, conveying the ion mass (m/z) (typically $z = 1$) on the x-axis, and the number of ions of a particular size that hit the detector (peak intensity) on the y-axis.

The first time that MALDI-TOF MS was applied to the identification of yeasts (*S. cerevisiae* isolates) from fermented beverages was, to our knowledge, conducted by Vallejo et al. (2013) [14]. Recently, MALDI-TOF MS has been proven to be a powerful tool in wine yeast identification at species [15–17] and even strain levels [18,19]. Furthermore, Usbeck and Wilde [18] demonstrated the role of MALDI-TOF MS in revealing the relationship between wine yeast strains and their application potential, as well as comparable studies of brewing strains [20]. The underlying mechanism is the link between proteome and metabolism. Lafaye and Junot [21] showed that proteome and metabolic data could be correlated either positively or negatively depending on the growth conditions. Nonetheless, studies are few at this point, and none to our knowledge have combined investigations on both wine and brewing yeasts.

Machine learning is widely used to analyse complex data sets for prediction purposes [22,23]. Principal component analysis (PCA), Multidimensional scaling (MDS), and Uniform Manifold Approximation and Projection (UMAP) are three dimensionality reduction techniques (DRTs) for data visualization of machine learning-based methods [24]. PCA is a parametric linear projection that captures maximum variances in the dataset but is unable to capture the non-linear structures. MDS is the first non-parametric DRT that preserves topology and distances; it is able to capture non-linear structures but with limited capability [24]. UMAP is a new non-parametric approach put forward by McInnes and Healy [25] that builds on strong mathematical foundations and efficiently handles very large datasets.

To assess the use of MALDI-TOF analyses to predict potential applications of yeast in wine and beer production, we investigated several factors. First, the culture medium (YPD agar and broth) and mass range (m/z 500–4000 and m/z 2000–20,000) were evaluated for the best fit based on our previous work [17,26]. Thereafter, the three algorithms listed above, in addition to a classical hierarchical clustering approach, were adopted to investigate the potential of MALDI profiles in industrial yeast strains differentiation (commercial wine and brewing strains) and the potential application prediction. Furthermore, the manufacturer's recommended application for each strain was incorporated to evaluate its potential in predicting strain utility for winemaking/beermaking.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

A collection of 47 commercial wine yeast strains and 12 brewing yeast strains were tested (Table 1). Four additional type strains *S. cerevisiae* NCYC 505 ^{T}, *S. paradoxus* NCYC 700 ^{T}, *S. pastorianus* NCYC 396 ^{T}, and *S. bayanus* NCYC 2578 ^{T} were purchased from NCYC (National Collection of Yeast Cultures, UK), and one isolate *S.cerevisiae* v128 was purified from Pinot Noir (PN) grape juice obtained from an organic winery, Greystone Wines, Waipara, New Zealand.

Table 1. A total of 47 commercial wine yeast strains and 12 commercial brewing yeasts strains used in this work, in which wine strains were kindly provided by Lincoln University Winery, and brewing strains were purchased from BREWSHOP.

Commercial Strains	Genetic Background
Wine strains	
AWRI Fusion *	*S. cerevisiae* × *S. cariocanus*
Cepage Cabernet	*S. cerevisiae*
Cepage Chardonnay	*S. cerevisiae*-Strain n° LW05
Collection Cepage Pinot	*S. cerevisiae*
Cross Evolution	*S. cerevisiae* var. *cerevisiae*
Enartisferm Aroma White	*S. cerevisiae*
Enoferm AMH™	*S. cerevisiae* var. *cerevisiae*
Enoferm M1	*S. cerevisiae*
Fermi champ	*S. cerevisiae* (ex *bayanus*)
Fermicru 4F9 *	*S. cerevisiae*-Strain n° 4F9
Fermicru AR2	*S. cerevisiae*-Strain n° L0122
Fermicru Rose *	*S. cerevisiae*-Strain n°LW10
Fermicru XL	*S. cerevisiae*-Strain n° CECTA 11947
IOC 18-2007 *	*S. cerevisiae* var. *bayanus*
Lalvin C	*S. cerevisiae* var. *bayanus*
Lalvin CLOS	*S. cerevisiae* var. *cerevisiae*
Lalvin CY 3079	*S. cerevisiae* var. *cerevisiae*
Lalvin DV10 *	*S. cerevisiae* var. *bayanus*
Lalvin EC1118 *	*S. cerevisiae* var. *bayanus*
Lalvin ICV D47	*S. cerevisiae* var. *cerevisiae*
Lalvin OKAY	*S. cerevisiae* var. *cerevisiae*
Lalvin RC212	*S. cerevisiae* var. *cerevisiae*
Lalvin Rhone 2226	*S. cerevisiae*
LalvinQA 23 *	*S. cerevisiae*
Levuline BRG	*S. cerevisiae*
Maurivin AWRI 350	*S. cerevisiae*
Maurivin PDM *	*S. cerevisiae* (var. *bayanus*)
Premium Chardonnay	*S. cerevisiae*
PREMIUM® PROTIOL	*S. cerevisiae*
Renaissance Allegro	*S. cerevisiae bayanus*
Renaissance Andante	*S. cerevisiae*
Renaissance Brio (Brioso)	*S. cerevisiae*
Renaissance Maestoso	*S. cerevisiae*
Renaissance Vivace	*S. cerevisiae bayanus*
Rennaissance Ossia	*S. cerevisiae*
Safoeno™ CK	*S. cerevisiae*
Sauvignon L3	*S. cerevisiae*
UCD522	*S. cerevisiae*
Velluto Evolution™	*S. cerevisiae*/*uvarum*
Viniflora Jazz	*S. cerevisiae*
Viniflora® PRELUDE™	*Torulaspora delbrueckii*
Viniflora® CONCERTO™	*Lachancea thermotolerans*
Vitilevure Syrah	*S. cerevisiae*
Zymaflore VL1	*S. cerevisiae*
Zymaflore VL3	*S. cerevisiae*
Zymaflore X5	*S. cerevisiae*
ZYMAFLORE® X16	*S. cerevisiae*
Brewing strains	
BRY-97 American West Coast Yeast	*S. cerevisiae*
LalBrew KÖln	*S. cerevisiae*
Belle Saison	*S. cerevisiae* var. *diastaticus*
Mangrove Jack's New World Strong Ale Yeast	*S. cerevisiae*
Philly Sour	*Lachancea* spp.

Table 1. *Cont.*

Commercial Strains	Genetic Background
LalBrew Verdant IPA	*S. cerevisiae*
Mangrove Jack's Californian Lager Yeast	*S. cerevisiae*
Mangrove Jack's Bohemian Lager Yeast	*S. cerevisiae*
Saflager S-23 Yeast	*S. cerevisiae*
Mangrove Jack's Bavarian Wheat Yeast	*S. cerevisiae*
Mangrove Jack's Belgian Wit Yeast	*S. cerevisiae*
Safbrew WB-06 Wheat Yeast	*S. cerevisiae* var. *diastaticus*

* Fermicru_ROSE, AWRI_Fusion, Lalvin DV10, Fermicru 4F9, Lalvin EC 1118, Lalvin QA 23, IOC 18-2007, and Maurivin PDM are associated with the *Prise de Mousse* (PDM) collection of Champagne production [27,28].

All commercial yeast strains were aseptically re-hydrated and inoculated into 15 mL YPD broth (Difco, c/o Thermo Fisher Scientific Ltd., Waltham, MA, USA) overnight at 28 °C. Afterward, the cultures were streaked onto YPD agar (Difco) and cultured under the same conditions for three days. Purified yeast strains were obtained and routinely stored at −80 °C in YPD glycerol stock (30%, v/v) after two-times subculture.

For MALDI-TOF MS analysis, yeast strains on YPD agar (Difco) were cultured for 72 h at 28 °C, whereas the strains in YPD broth (Difco) were cultured for 24 h at 28 °C.

2.2. MALDI-TOF MS

2.2.1. Sample Preparation

The preparation of yeast strains harvested from YPD agar (Difco) was as described previously [17]. Samples from the liquid media were collected according to Usbeck and Kern [16]. In order to obtain enough yeast cells for MALDI analysis, 900 µL culture of YPD broth (Difco) was transferred into a 1.5 mL tube (Safe-Lock, Eppendorf, Hamburg, Germany) and centrifugated at 12,100× g for 4 min (Eppendorf AG, Minispin 5452, Hamburg, Germany). The supernatant was discarded, and the pellet was washed using 900 µL sterilized deionized water (produced by an ultra-pure water system by Barnstead GenPure Pro, Thermo Scientific, Waltham, MA, USA) 3 times. Subsequently, the pellet was resuspended into 300 µL deionized water, and vortexed for 1 min with 900 µL absolute ethanol (Fisher Chemical, Chicago, IL, USA). After centrifugation (12,100× g, 4 min), the pellet was air-dried in laminar-flow hood and stored at −20 °C prior to protein extraction.

To extract proteins, 50 µL of 70% formic acid (v/v) (Fisher Chemical, Chicago, IL, USA) was added to the yeast pellet and mixed thoroughly by vortexing for 1 min, then 50 µL of acetonitrile (ACN) (Fisher Chemical, Chicago, IL, USA) was mixed for the same time. Protein extract was obtained by centrifugation (12,100× g, 4 min). An equal volume of protein extract and α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Bremen, Germany) matrix solution (10 mg/mL in 75% ACN and 2.5% trifluoroacetic (TFA)) were mixed well, and 1 µL of this mixture was deposited onto the MALDI ground steel target plate (MTP 384, Bruker Daltonics, Billerica, MA, USA) until dry. For technical replication, each extract was spotted onto 3 individual wells, therefore yielding 9 spectra per strain.

2.2.2. Mass Spectra Acquisition

MALDI-TOF mass spectra were automatically acquired on an Ultraflex III TOF/TOF MS instrument (Bruker Daltonics, Billerica, MA, USA), operating in positive ion detection mode using a SmartbeamTM laser at 200 Hz, pulsed-ion extraction time of 120 ns, and laser power 80%. The voltage of the ion source was set as 25.00 kV (ion source 1), 23.55 kV (ion source 2), and 6.01 kV (lens). Samples were analyzed using the linear detector at high mass range m/z 2000–20,000 and reflector detector at low mass range m/z 500–4000. The final spectrum was an average accumulation of 800 single spectra (low mass range m/z 500–4000) or 2000 single spectra (high mass range m/z 2000–20,000) gathered. Every single spectrum was recorded from 10 random raster spots.

The mass spectrometer was externally calibrated in every experiment at regular intervals, using the calibrant position in the middle of each tetrad of spots. For low mass range m/z 500–4000, peptide II standard (Bruker Daltonics, Billerica, MA, USA) (Bradykinin 1–7, $[M + H]^+$ at m/z 757.3992, Angiotensin II, $[M + H]^+$ at m/z 1046.5418, Angiotensin I, $[M + H]^+$ at m/z 1296.6848, Substance P, $[M + H]^+$ at m/z 1347.7354, Bombesin, $[M + H]^+$ at m/z 1619.8223, ACTH clip 1–17, $[M + H]^+$ at m/z 2093.0862, ACTH clip 18–39, $[M + H]^+$ at m/z 2465.1983 and Somatostatin 28, $[M + H]^+$ at m/z 3147.4710) was used. For high mass range m/z 2000–20,000, an in-house protein standard comprising Insulin, $[M + H]^+$ at m/z 5734.52, Cytochrome C, $[M + H]^+$ at 12,360.99 and $[M + H]^{2+}$ at 6180.99, Myoglobin, $[M + H]^+$ at 16,952.30 and $[M + H]^{2+}$ at 8476.65), Aprotinin $[M + H]^+$ m/z 6511.51, and β-lactoglobulin $[M + H]^+$ m/z 18,363.00 was used.

2.2.3. Data Analysis

Raw mass spectra were exported as .txt format using FlexAnalysis software (version 3.0. Bruker Daltonics, Billerica, MA, USA) and imported into software BioNumerics version 7.6 (Applied Maths, Kortrijk, Belgium). Spectra pre-processing was achieved at a default setting, but baseline subtraction with Rolling disc value was adjusted to 150. Kaiser Window value in smoothing and signal to noise ratio (S/N) in peak filtering were adjusted according to the quality of spectra.

A composite profile of each strain was obtained using 9 spectra derived from 3 technical replicates of each of 3 biological replicates. Cluster analysis was performed using the Pearson correlation coefficient and UPGMA (unweighted-pair group method with arithmetic mean) algorithm.

MDS and PCA analyses are available in BioNumerics version 7.6. MDS was performed based on a similarity matrix calculated using the metric algorithm Pearson coefficient. Pearson coefficient is insensitive to global differences in background and intensity as it contains an average intensity correction but is sensitive to local differences in intensity; thus, it is recommended for typing purposes and therefore adopted in our study [29]. PCA and UMAP were executed on peak classes detected by "peak matching" using the default settings (high mass: constant tolerance 1.9, linear tolerance 550 ppm, peak detection rate 10; low mass: constant tolerance 0.5, linear tolerance 300 ppm, peak detection rate 50). PCA was calculated with quantitative values (not just absent/present) and options to subtract the average character value over the characters. UMAP is founded on the assumptions that the data is uniformly distributed on the Riemannian manifold, the Riemannian metric is locally constant, and the manifold is locally connected, which was applied using the conda-forge packages for Python (Available online: https://umap-learn.readthedocs.io/en/latest/index.html (accessed on 19 January 2022)).

3. Results

3.1. MALDI-TOF Profiles of Strains Cultured on YPD Broth and YPD Agar

Good quality MALDI profiles from each of the strains examined were obtained from cultures on each of the media used. Representative MALDI profiles of eight wine and brewing yeast strains are presented in Figure 1. Compared to strains grown on YPD agar, strains grown in YPD broth generated more peaks in a wider mass range, but the overall peak intensity was greatly decreased. Despite the visible differences of produced MALDI profiles, a set of common peaks with varying peak intensity (Low mass: m/z 712, 757, 767, 770, 891, 1100; High mass: m/z 5735, 5773, 6535, 6746, 6809, 7254, 7887, 8469, 8658, 10,219, 10,792, 10,854, 12,750, 13,750, 13,829, 14,506) were observed in samples from both growth media.

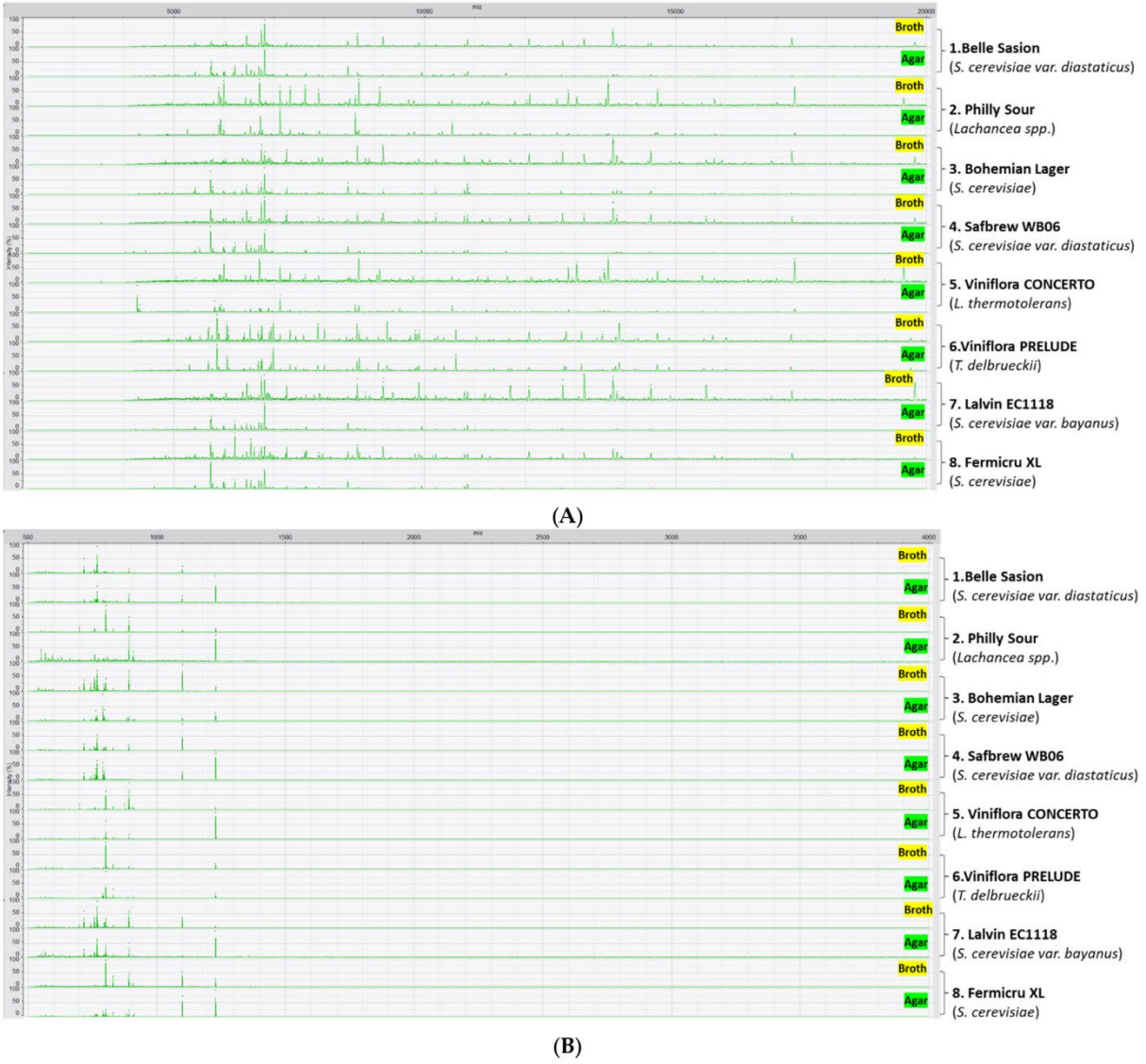

Figure 1. MALDI spectra of (**A**) high mass and (**B**) low mass of eight representative commercial strains cultured under YPD broth and YPD agar; 1–4: Brewing strains, 5–8: Wine strains.

3.2. Strain Classification Using Cluster Analysis and Machine Learning Approaches

Although strain profiles produced from broth cultures contained more peaks, cluster (Figure S1) and machine learning-based analyses (Figure S2) tended to correlate poorly with extant information concerning the utility of individual strains. These results are not considered further.

Cluster analysis of all the *S. cerevisiae* strains (winemaking and brewing) exhibited different grouping based on their high-, low- and combined-mass spectra profiles (Figure S3). With a thorough visual examination of the spectra patterns, 95% and 85% were indicated as the threshold values in high mass and low mass dendrograms, respectively, resulting in 17 and 20 subclusters. Likewise, 18 subclusters were recognized in the high-low combined dendrogram when 85% was set as the threshold value. Compared to high mass clustering, the industrial strains differentiation was better illustrated by low mass profiles

where all the brewing strains were clustered together (group 12–20). In either the high or low mass dendrogram, strains of Velluto Evolution, Fermi champ, Renaissance Vivace, Belgian Wit, Belle Saison, Verdant IPA, NWS Ale, LalBrew Köln, and BRY97_American were affiliated. Three Lager strains of Californian Lager, Bohemian Lager, and Saflager 23 clustered together in the low mass dendrogram analysis, while the former two strains were mixed with wine strains (Group 2) in the high mass dendrogram. Strains recommended for Champagne production (PDM) fell into three subclusters in both dendrograms, containing four different strains of *S. cerevisiae*, *S. cerevisiae* var. *cerevisiae*, *S. cerevisiae* × *S. cariocanus*, and *S. cerevisiae* var. *bayanus*.

Representation of inter-strain relationships among all strains examined using each of the multidimensional scaling techniques (MDS, PCA, and UMAP) was generally more nuanced. The PCA plot gave the poorest degree of association between strain utility and even species identity, with the most obvious outliers to be the major group represented by a local vineyard isolate of *S. cerevisiae*, and the type strain of *S. paradoxus* NCYC 700 (Figure 2D). The UMAP analysis distributed most of the *S. cerevisiae* strains recommended for winemaking among five groups, although some of these contained strains recommended for beer and Champagne production (PDM) as well (Figure S4A). The MDS plot displayed a more consistent grouping of strains with better alignment of their recommended use and taxonomic relationship. Brewing-related strains (*S. cerevisiae* NCYC 505, *S. bayanus* NCYC 2578, and *S. pastorianus* NCYC 396) were aligned with the commercial brewing group (red dots), whereas *S. cerevisiae* v128 (indigenous yeast isolate) appeared close to, but distinct from, wine and PDM group strains, and quite close to the *S. paradoxus* type strain (Figure 2A). Strains recommended for Champagne production (PDM) were somewhat at an interface between the wine and beer producers.

3.3. Separate Analyses Were Undertaken on S. cerevisiae Strains for Which Recommendations Were Extant for Particular Wine Styles

The 45 *Saccharomyces* wine yeast strains we selected to cover a wide range of applications, which can be roughly divided into 9 categories, namely, for the production of white wine, red wine, red and white wine, white/rose/red wine, rose wine, white and rose wine, white/red/fruit/cider, white/rose/red/sparkling wine, and one fructophile yeast Fermicru Champ used for tackling stuck fermentation. MDS and PCA did not show appreciable groupings based on their purposes in winemaking for different wine styles (Figure S5). However, UMAP distinguished five groups containing strains with some agreement where winemaking style recommendations were taken into account (Figure 3 and Figure S6). Group 1 was dominated by strains recommended for red wine production. Group 2 contained the majority of strains used to produce PDM and was classified as *S. cerevisiae* var. *bayanus*. Compared to the other three groups of strains, these two groups seem to have a stronger tolerance to low fermentation temperature and high alcohol content according to the manufacturing information, and their overall peak intensity and peak numbers were relatively low (Figure S7). Groups 3 and 4 are also well-populated with strains for red winemaking, and rosé too, in the case of Group 3. Group 5 contains mainly white wine yeast strains, mostly recommended for producing Sauvignon Blanc and Chardonnay wines.

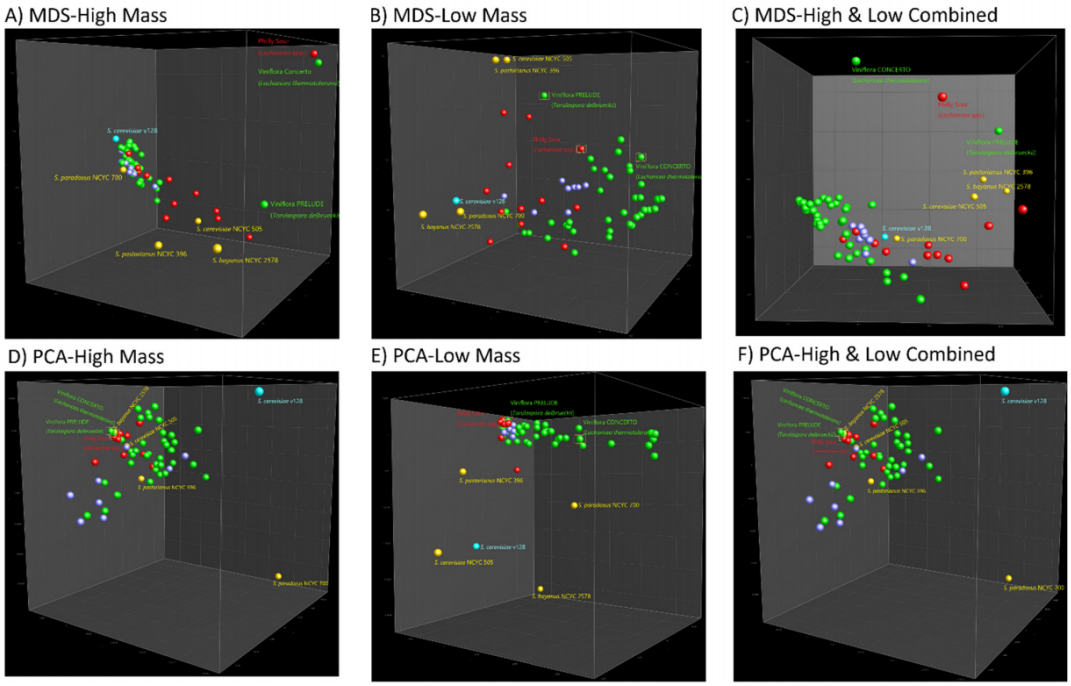

Figure 2. MDS analysis of (**A**) high mass; (**B**) low mass and (**C**) high and low combined data, PCA analysis of (**D**) high mass and (**E**) low mass, and (**F**) high and low combined data of 62 yeast strains-45 wine strains (green/purple), 12 brewing strains (red), *S. cerevisiae* v128 (blue), *S. cerevisiae* NCYC 505 (yellow), *S. paradoxus* NCYC 700(yellow), *S. pastorianus* NCYC 396 (yellow), *S. bayanus* NCYC 2578 (yellow).

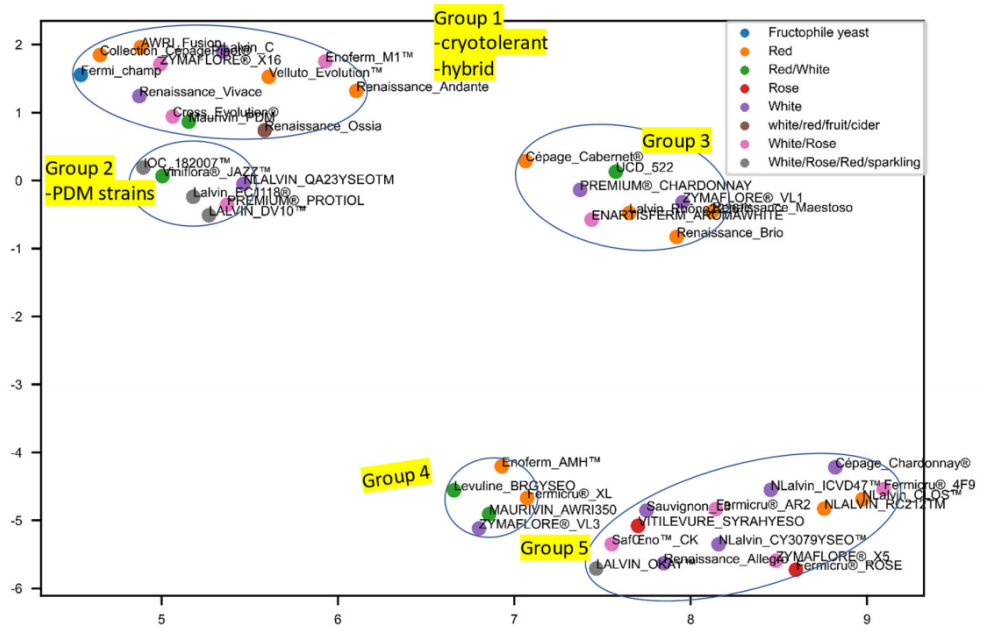

Figure 3. UMAP analysis of high mass profiles of 45 commercial wine *Saccharomyces* strains.

Although only 12 brewing strains were examined, strains belonging to wheat, lager, and ale were grouped separately, in particular, when the high mass was analysed (Figure 4 and Figure S8). The outlier ale yeast Belle Saison and wheat yeast Safbrew_WB06 were placed closer as their identity as *S. cerevisiae* var. *diastaticus*. The single strain representing the non-*Saccharomyces* species (*Lachanchea* spp.) on the left bottom is suggested to produce a sour beer.

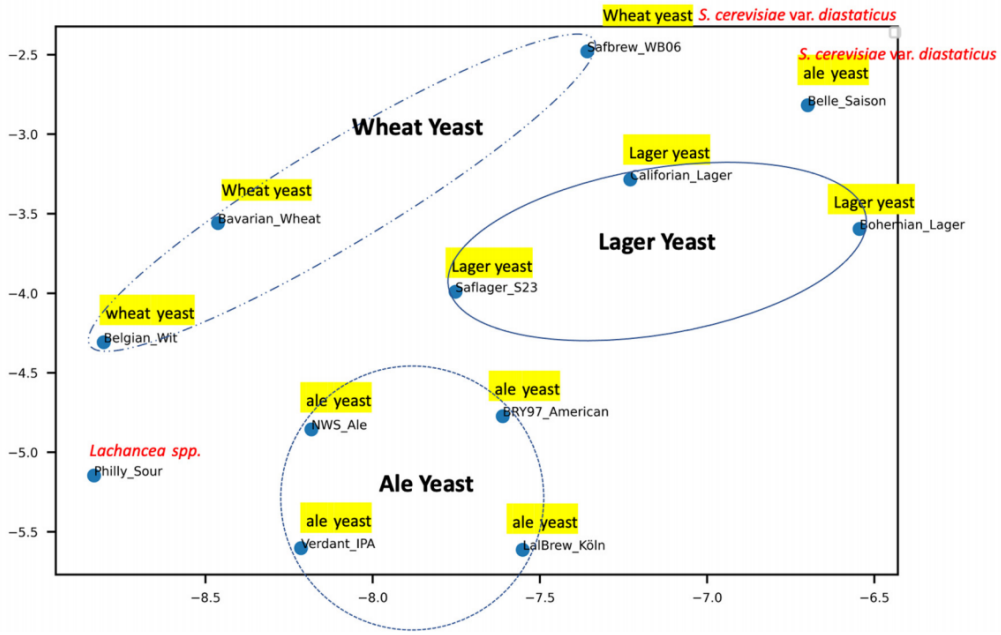

Figure 4. UMAP analysis of high mass profiles of 12 commercial brewing strains.

4. Discussion

The interaction between yeast strain and grape varietal is integral to the flavour profile of the wine. During fermentation, the performance of each yeast strain is affected by the grape must composition, as well as the fermentation conditions. Therefore, the strain may not perform as expected if the growth condition (e.g., matrice and temperature) is not compatible with the expression of desired characters [30]. Some strains can produce metabolites that enhance mouthfeel (e.g., Lalvin ICV D47 and Lalvin CLOS), modify varietal aroma through enzymatical and chemical cleavage of aroma precursors (e.g., Lalvin QA 23 with high β-glucosidase activity), and improve the wine stability by increasing yeast mannoproteins [30]. Therefore, it is important to choose an appropriate yeast strain for making wine from a particular grape variety. We further examined the prospects of identifying strain utility for fermentation processes using proteome characterization by MALDI-TOF MS.

Based on optimized parameters described previously [17,26], YPD agar and YPD broth were selected as the culture media in this work. Although differences were observed among MALDI profiles, a set of core peaks remained constant, which was consistent with the reports from Usbeck and Kern [16], Reich and Bosshard [31], and Moothoo-Padayachie and Kandappa [19], who also stated that the variations did not compromise the accurate identification on species/strain level. The common peaks are likely to be the ribosomal or housekeeping proteins, whose expression is vital to the basic cellular function irrespective of the growth conditions. Approximately half of the peaks in the MALDI spectra could be assigned to such highly abundant ribosomal proteins, with some peaks matched to post-translationally modified ribosomal proteins [32].

Wine yeast strains are genomically and phenotypically distinct from other industrial yeast strains (beer, bread, and sake), as well as laboratory strains, pathogenic strains, and 'wild' yeast strains [33]. Dunn and Richter [28] pointed out that *NFT*1, *FLO*1, *AAD*6, and *AGP*3 genes present in most wine yeast strains but absent in most non-wine yeast strains, are important marker genes to differentiate yeast strains based on their application. Likewise, MALDI profiles successfully differentiated the wine and brewing yeast strains tested in this work. The domestication of diverse industrial *S. cerevisiae* populations (e.g., wine, beer, and bread) has been achieved through long-term evolution under selective pressures of various sources, like ancient customs, human migration, and industrial practice, encouraging the development of customized genomes for better adaption in new ecological niches [4,34,35]. In addition, species *S. paradoxus*, *S. bayanus*, and *S. pastorianus* are also of industrial importance in food fermentation, as well as their interspecific/intraspecific hybrids [36]. *S. paradoxus* is commonly found on the exudates and bark of deciduous trees [37]. In wild environments, *S. paradoxus* rarely cross-fertilizes with *S. cerevisiae*, but conditions in the intestine of some insects favour their hybridization, potentially creating an adaptive environment [38]. Lager beer yeast *S. pastorianus*, especially amenable to cooler fermentation temperature, is a naturally occurring interspecies hybrid of *S. cerevisiae* and *S. eubayanus* [34]. Type strain *S. bayanus* NCYC 2578 is a hybrid between *S. eubayanus* and *S. uvarum* [39]. Their genetic structure is reflected in our MALDI-TOF analysis, whereby *S. pastorianus* NCYC 396 and *S. cerevisiae* NCYC 505 are closer than NCYC 396 and NCYC 2578 in both high-(44.6% vs. 40.8%) and low-(74.1% vs. 9.8%) mass spectra. Moreover, their proximity to the brewing group of strains not only exhibited the capacity of this methodology as a powerful identification tool, but also showed the potential of MALDI-TOF MS as a predictive phenotypic tool.

Data interpretation is greatly affected by the algorithm used [16]. Dimensionality reduction techniques (DRTs) can provide an in-depth insight into subgrouping with an intuitive data interpretation. In this study, MDS was calculated based on the similarity matrix based on the Pearson coefficient, and then each data point was assigned using a non-linear least-squares fit, minimizing the distances between the data points [23]. MDS appears to be a valuable alternative to the traditional clustering methods. In our study, PCA was the least informative of the DRT methods applied, yielding the poorest correlation of strain grouping with industry recommendation, although it is one of the oldest and best-known DRTs. However, with the help of UMAP, 45 *Saccharomyces* commercial strains were classified into 5 groups using the high mass profiles, where MDS and PCA failed. It could be due to the fact that UMAP allows a more accurate representation of local trends, while PCA is better at the visualization of global data structure [24].

Low mass profiles allow for a rough classification of the industrial strains under MDS analysis (Figure 2B), but its combination did not significantly enhance the differential capacity of high mass profiles (m/z 2000–20,000). PCA and UMAP could not extract meaningful information from the limited peak classes (7 peak classes) as well. Interestingly, the data comparison between the UPGMA-based high- and low- dendrogram substantiated the potential of low-mass data as a powerful biotyping tool. The grouping of certain strains in two dendrograms was observed to be consistent. Velluto Evolution, the only hybrid of *S. cerevisiae*/*uvarum*, was in a single branch in both dendrograms. A similar case applies to Fermi champ, a special strain for tackling stuck fermentation, which is claimed to be *S. cerevisiae* (ex *bayanus*) but separated from the other strains of *S. cerevisiae* (ex *bayanus*). It is reasonable to infer that the MALDI profile clustering is an interaction between the genetic and phenotypic traits of individual strains. Overall, low mass profiles allowed a more detailed strain classification but were also affected by the phenotypes. In accordance with our previous inference, the low mass profiles did contribute to the added benefits of amplifying the intraspecific features [17].

When looking at the UMAP subgroups, Group 1 was dominated by the hybrid strains, which usually combine and exhibit superior phenotypic qualities over parent strains. Yeasts belonging to species of *S. bayanus* (*S. uvarum* × *S. eubayanus*)/*S. uvarum* are usually related to

the ability to ferment at lower temperatures and greater production of aroma-active higher alcohols [40]. For example, the natural intraspecific hybrid Cross Evolution (*S. cerevisiae* var. *cerevisiae*) is ideal for white and rosé wines with high aromatic intensity (including ester production) and low fermentation temperature, and the interspecific hybrid Velluto Evolution (*S. cerevisiae* × *S. uvarum*) is characterized by high production of glycerol, phenyl ethanol generation, and good tolerance to low fermentation temperature (e.g., at 12 °C). Group 2 was represented by the PDM strains with two non-PDM strains of Premium Protiol and Viniflora Jazz. The collection of PDM strains is a special group from wine yeasts mainly described as *S. cerevisiae* var. *bayanus* [41], which is considered to be an intermediate group between non-wine and wine strains [28]. A related observation using the MDS and UMAP algorithms is that the PDM group is distributed at the interface between wine and brewing strains.

S. cerevisiae var. *bayanus* is a variety of *S. cerevisiae* that was reduced from its former species status (*S. bayanus*), as it could only be differentiated from *S. cerevisiae* by the fermentation of galactose [41]. The almost identical genotypes of the majority of the PDM group suggested that they may have arisen from a single progenitor strain or a highly interrelated progenitor population [27]. Coi and Bigey [42] inferred that the PDM group (Champagne related strains) might result from the cross between flor and wines gene pool, which benefits from the ability of flor strains under poor nutritional conditions and ethanol stress during the second fermentation of the "*Prise de mousse*" step that imposes a second anaerobic growth. In this sense, it explains its location as a neighbour beside the hybrid Group 1, having an overall stronger tolerance to low fermentation temperature and high alcohol content, as well as the fructophile strain Fermicru Champ for tackling stuck fermentation. Zymaflore VL3 in Group 4, Zymaflore X5, and Fermicru 4F9 in Group 5 are representative "thiol-releasing" wine yeasts suitable for the full aroma potential development of Sauvignon Blanc wine [43]. Similarly, Zymaflore X5 and Fermicru 4F9 were also shown to be a closer relationship in the study of Hart and Jolly [43].

As stated by the manufacturer, Premium Protiol is a strain of *S. cerevisiae*, but Silhavy-Richter, Hack [40] inferred it could be an unidentified interspecific hybrid of *S. cerevisiae* and *S. bayanus*, and strain QA 23 could be a derivative of EC 1118. Microsatellite analysis is not affected by physiological parameters, whereby the two PDM strains QA 23 and EC118, as well as the non-PDM strain Premium Protiol, were clustered together as our MALDI result indicated [40]. In addition to the natural hybridization between *Saccharomyces* strains, gene transfer between *Saccharomyces* and non-*Saccharomyces* species was observed in strain EC1118, the major wine contaminant *Zygosaccharomyces bailii* was identified as one donor species [44]. Additionally, aroma compounds produced were shown to be temperature dependent and vary between pure strain and hybrids; the best aroma producers at 28 °C were *S. cerevisiae* strains, whereas *S. uvarum* and some hybrids excelled at 12 °C [45]. It may corroborate our observation that an overall lower peak numbers and intensity (low protein expression) of Group 1 and 2 strains (most of the hybrids) under YPD agar (28 °C) was seen compared to the other 3 groups.

As discussed above, specific MALDI profiles obtained from yeasts grown on the YPD agar at 28 °C cannot reflect the real-time protein expression of yeast strains under winemaking conditions. Unlike the complex composition in grape must, YPD agar is a defined medium comprising four components (yeast extract, sugar, peptone, and agar). A previous study indicates different metabolites detected by MALDI-TOF analysis when winemaking yeast is cultured in these different conditions [26]. Nonetheless, the use of defined media for MALDI-TOF characterization of winemaking yeast is still recommended, based on the clarity of the spectra obtained and general support of yeast growth in comparable conditions [26]. The release of aroma compounds is strongly linked to the presence of aroma precursors in fermenting media [46]. The wine aromatic profiles can be modulated by employing different yeast species/strains and fermentation temperatures [45–47]. For example, according to the manufacturer instructions, strain EnartisFerm Aroma White is recommended for the thiolic varieties such as Sauvignon Blanc and Pinot Blanc with

more citrus and mineral notes produced at 14–16 °C and more aromas of tropical white fruit produced at 17–20 °C. Enoferm AMH is a colour-friendly yeast and particularly suited for Pinot Noir and Zinfandel, partially due to its low levels of enzyme production responsible for colour loss and its long lag phase plus low-medium fermentation rate also allows the expression of indigenous microflora. In the face of fluctuating environments, limitations in gene expression play a role in phenotypic diversity at the expense of growth rates [48]. The early study of Batistote and da Cruz [49] suggested that the sugar types and concentration, the nitrogen source complexity, and the yeast genetic background collectively influenced the optimal industrial yeast fermentation performance. Moreover, the biotechnological application of yeast strains is often contradictory. According to the instruction, UCD522 (Group 3, red wine yeast group) is recommended for white and red wines, and more popular for red wines. However, Carrau and Medina [50] suggest it is more suitable for fermentation of neutral varieties. The data presented in this study corroborates the study of Usbeck et al. (2014) [18] in indicating a role for rapid and cost-effective MALDI-TOF profiling to predict the potential of individual yeast strain for production of specific or distinct wine varietals. However, to better correlate the relationship between the MALDI data and the oenological traits of wine yeast strains, a more complete and objective analysis of metabolites produced is required.

5. Conclusions

In conclusion, MALDI profiles generated under YPD agar have a better performance for the purpose of industrial strains differentiation than YPD broth. Neither MDS nor PCA analysis could group wine strains according to their recommended application in winemaking. However, UMAP provided the predictive potential in clustering strains of similar functionality and/or organoleptic attribute. In summary, further studies and subsequent algorithm exploration and data mining are warranted to fully evaluate the relationship of the MALDI profile to practical application in wine production. MALDI-TOF MS is worth continuing investigation as a powerful tool for yeast strain application prediction to simplify and expedite the selection of relevant indigenous wine yeasts for the development of new and interesting wine styles from an entirely natural base.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10020265/s1, Figure S1: Cluster analysis of high mass profiles of 59 commercial strains (47 wine and 12 brewing strains) grown under (A) YPD agar and (B) YPD broth.; Figure S2: (A) MDS analysis and (B) PCA analysis of high mass, low mass and high-low combined of 59 commercial yeast strains (47 wine and 12 brewing strains) under YPD broth and YPD agar; Figure S3: Cluster analysis of high mass profiles of 59 commercial strains (47 wine and 12 brewing strains) grown on YPD agar (A) High Mass, (B) Low Mass and (C) High & Low Combined; Figure S4: UMAP analysis of (A) high mass, (B) low mass and (C) high & low combined data of 62 yeast strains-45 wine strains (green/purple), 12 brewing strains (red), *S. cerevisiae* v128 (blue), *S. cerevisiae* NYC 505 (yellow), *S. paradoxus* NCYC 700(yellow), *S. pastorianus* NCYC 396 (yellow), *S. bayanus* NCYC 2578 (yellow); Figure S5: MDS and PCA analysis of 45 commercial wine *Saccharomyces* strains; Figure S6: UMAP analysis of (A) Low mass and (B) High & Low combined data of 45 commercial wine *Saccharomyces* strains; Figure S7: Heatmap of peak classes detected from 45 commercial wine strains and grouped according to UMAP analysis. Red colour represents the highest peak intensity, whereas the blue colour represents the lowest peak intensity; Figure S8: UMAP analysis of (A) Low mass and (B) High & Low combined data of 12 commercial brewing strains.

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