Characterisation of Non-*Saccharomyces* Yeasts Using Different Methodologies and Evaluation of their Compatibility with Malolactic Fermentation

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Although *Saccharomyces cerevisiae* is the yeast species predominantly used for alcoholic fermentation, non-*Saccharomyces* yeast species are also important because they produce secondary metabolites that can contribute to the final flavour and taste of wines. In this study, 37 strains representing seven non-*Saccharomyces* species were characterised and evaluated for potential use in wine production, as well as for their effects on malolactic fermentation (MLF). Contour-clamped homogeneous electric field (CHEF) gel electrophoresis and matrix-assisted laser desorption ionisation using a time-of-flight mass spectrometer (MALDI-TOF MS) were used to verify species identity and to determine intra-species variation. Extracellular enzyme production, malic acid degradation and the fermentation kinetics of the yeasts were also investigated. CHEF karyotyping and MALDI-TOF MS were useful for identifying and typing *Hanseniaspora uvarum, Lachancea thermotolerans, Candida zemplinina* (synonym: *Starmerella bacillaris*) and *Torulaspora delbrueckii* strains. Only *H. uvarum* and *Metschnikowia pulcherrima* strains were found to have β-glucosidase activity. *M. pulcherrima* strains also had protease activity. Most of the strains showed limited malic acid degradation, and only *Schizosaccharomyces pombe* and the *C. zemplinina* strains showed mentionable degradation. In synthetic wine fermentations, *C. stellata*, *C. zemplinina, H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were shown to be slow to medium fermenters, whereas *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters. The effect of the yeasts on MLF varied, but inhibition was strain dependent.

INTRODUCTION

Yeasts play a key role in wine production. They are present on the grapes and winery equipment, or are added as starter cultures, and are responsible for alcoholic fermentation by which the grape must is transformed into wine. These yeasts can arbitrarily be divided into two categories: *Saccharomyces* and non-*Saccharomyces* (wild yeasts). *Saccharomyces cerevisiae* may be present at very low numbers on the grape skins, but are normally found in greater numbers on the winery equipment (Fleet *et al*., 2002; Ribéreau-Gayon *et al*., 2006). Non-*Saccharomyces* yeast genera frequently found on grapes and in must include *Hanseniaspora* (*Kloeckera*), *Candida*, *Metschnikowia*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula, Zygosaccharomyces, Cryptococcus* and the black pigmented yeast-like fungi, *Aureobasidium pullulans* (Fleet *et al*., 2002; Jolly *et al*., 2003a; Ribéreau-Gayon *et al*., 2006; Romano *et al*., 2006; Jolly *et al*., 2014; Alessandria *et al*., 2015; Capozzi *et al*., 2015). In the initial phase of spontaneous fermentations, strains from the genera *Kloeckera* and *Candida* usually dominate (Ribéreau-Gayon *et al*., 2006; Romano *et al*., 2006). As the ethanol levels increase, the more ethanol-tolerant *Saccharomyces* yeast strains dominate.

Malolactic fermentation (MLF) is a secondary but important fermentation process conducted by lactic acid

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bacteria (LAB), usually *Oenococcus oeni* (Bauer & Dicks, 2004; Lerm *et al*., 2010). Malolactic fermentation is not a true "fermentation", but rather an enzymatic reaction in which malic acid is decarboxylated to lactic acid and CO_2. This process is often desired in the production of red wines, and in certain white and sparkling wine styles (Wibowo *et al*., 1985; Bartowsky *et al*., 2015), because it increases wine microbiological stability and enhances aroma and flavour (Davis *et al*., 1985; Bartowsky *et al*., 2002, Lerm *et al*., 2010; Sumby *et al*., 2014).

In the last decades, research has focused on the role that non-*Saccharomyces* yeasts play in wine production. The use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae*. These mixed fermentations lead to the production of wines with more desirable characteristics, and starter cultures containing non-*Saccharomyces* yeasts, namely *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri* and *Metschnikowia pulcherrima*, are available commercially (Jolly *et al*., 2014). Specific compounds produced by non-*Saccharomyces* yeasts that can affect wine aroma and flavour include acetaldehyde, acetic acid, esters, glycerol, higher alcohols, terpenoids and other by-products (Romano *et al*., 1997; 2003; Jolly *et al*., 2006; Comitini *et al*., 2011; Jolly *et al*., 2014). Non-*Saccharomyces* yeasts also possess various degrees of β-glucosidase activity, which plays a role in releasing volatile compounds from non-volatile precursors (Rosi *et al*., 1994; Hernández-Orte *et al*., 2008). Extracellular proteolytic and pectinolytic enzymes of non-*Saccharomyces* yeasts might also be beneficial by improving wine processing through the facilitation of juice extraction and clarification, wine filtration and colour extraction (Van Rensburg & Pretorius, 2000; Strauss, 2003; Reid, 2012). Strains of *Candida stellata*, *C. zemplinina* (synonym: *Starmerella bacillaris*), *Hanseniaspora uvarum*, *M. pulcherrima* and *P. anomala* have been found to produce a variety of extracellular enzymes (Charoenchai *et al*., 1997; Strauss, 2003; Mostert, 2013).

Considering the great diversity and potential applications of different non-*Saccharomyces* yeast strains within the same species, it is important to devise simple and reliable molecular typing techniques to discriminate at the subspecies level. The application of karyotyping electrophoresis techniques, such as contour-clamped homogeneous electric field (CHEF) gel electrophoresis, has been useful to differentiate non-*Saccharomyces* yeasts at species and strain level (Esteve-Zarzoso *et al*., 2001; Sipiczki, 2004; Alcoba-Flórez *et al*., 2007; Van Breda *et al*., 2013). Its high discriminatory power and repeatability also justify why this technique is often considered favourably in comparison with other typing methods. Matrix-assisted laser desorption ionisation, using a time-of-flight mass spectrometer (MALDI-TOF MS), is a 'soft' or non-destructive method that can be used for the identification of yeasts and bacteria at the genus and species level (Van Veen *et al*., 2010). Studies using MALDI-TOF MS to identify yeasts have focused more on clinical *Candida* strains (Marklein *et al*., 2009) than on wine-associated yeasts (Moothoo-Padayachie *et al*., 2013; Kántor & Kačániová, 2015).

The interactions between different non-*Saccharomyces* yeasts (naturally present and inoculated) and LAB, as well as their impact on MLF, have received little attention. The resulting impact on wine aroma/flavour is also uncertain. With the increasing number of non-*Saccharomyces* yeasts available commercially, the need for a better understanding of the interactions between the wine yeast, *S. cerevisiae*, the non-*Saccharomyces* yeasts and LAB is critical. Therefore, the aims of this study were to characterise strains from seven non-*Saccharomyces* species by means of CHEF karyotyping, MALDI-TOF bio-typing, enzyme activity and malic acid degradation in order to investigate their use in wine production and to evaluate their compatibility with MLF.

MATERIALS AND METHODS

Characterisation

Isolation and cultivation of micro-organisms

The yeast strains used in this study are listed in Table 1 and included one *C. stellata*, seven *C. zemplinina* (synonym: *St. bacillaris*), 11 *H. uvarum* (anamorph: *Kloeckera apiculata*), two *L. thermotolerans* (previously *Kluyveromyces thermotolerans*), seven *M. pulcherrima* (anamorph: *Candida pulcherrima*), one *Schizosaccharomyces pombe*, eight *Torulaspora delbrueckii* (anamorph: *Candida colliculosa*) and six *S. cerevisiae* strains. Strain *L. thermotolerans* Viniflora® Rhythm™ (Chr. Hansen, Denmark) and *T. delbrueckii* strains Viniflora® Harmony™ (Chr. Hansen), (Level^2 TD™ (Lallemand Inc., France) and Zymaflore® Alpha $^{TD\ n.\ Sacc.}$ (Laffort Oenologie, France), were isolated from commercial active dried yeast blends (Van Breda *et al*., 2013 and this study) and included as reference strains. All the yeasts were stored under cryo-preservation at -80°C. When required, the yeasts were grown on yeast peptone dextrose agar (YPDA, Merck, South Africa) at 28°C for 48 hours, or until sufficient growth was observed. Single colonies were then selected and transferred to 10 mL YPD broth and grown for 24 hours at 28°C before inoculation. *Oenococcus oeni* (Viniflora® oenos, Chr. Hansen) was used to induce MLF according to the supplier's instructions.

Electrophoretic karyotyping

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to investigate the strain diversity of the non-*Saccharomyces* yeasts, and the intact chromosomal DNA was prepared using the embedded agarose technique described by Hoff (2012). A CHEF DRIII electrophoretic apparatus (Bio-Rad Laboratories, Inc., Richmond, USA) and the method described by Hoff (2012) were used with the following changes to the running conditions: 34-hour programme, initial pulse was 30 s and final pulse was 215 s at an angle of 120 degrees at a constant 6 volt; 72-hour programme, initial and final pulse of 1 800 s at an angle of 106 degrees at a constant 2.5 volt. *Saccharomyces cerevisiae* reference strain CBS 432 was used as the standard reference strain for all CHEF gels and was loaded on the outer lanes of each gel. Agarose gels at a concentration of 1.2% and 0.8% were used to separate yeasts run on the 34 and 72 hour programmes respectively.

TABLE 1
Yeasts used in this study

Species name	Strain code	Strain, origin and source information	References*
Saccharomyces cerevisiae	S1	N 96, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012
	S2	VIN 13, commercial yeast from Anchor Wine Yeast, South Africa	Jolly *et al*., 2003b; 2003c; Hoff, 2012; Van Breda *et al*., 2013; Minnaar *et al*., 2015
	S3	NT 112, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012
	S4	NT 202, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012; Scholtz, 2013
	S5	VIN 7, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012
	S6	CBS 432, from Centraalbureau voor Schimmelcultures (CBS), Netherlands	
Candida stellata	Cs	CBS 157^{T}, from CBS, Netherlands	Sipiczki, 2004; Csoma & Sipiczki, 2008
Candida zemplinina (synonym: *Starmerella bacillaris*)	C1	CBS 9494, type strain from CBS, Netherlands	Sipiczki, 2004; Csoma & Sipiczki, 2008, Magyar *et al*., 2014
	C2	VEN 2097, from the University of California, Davis	Bokulich *et al*., 2012
	C3	770**, from the ARC Infruitec-Nietvoorbij, South Africa	Jolly *et al*., 2003b**
	C4	788, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	C5	841, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	C6	971, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	C7	C2-19, from the ARC Infruitec-Nietvoorbij, South Africa	This study
Hanseniaspora uvarum (anamorph*:* *Kloeckera apiculata*)	H1	752, from the ARC Infruitec-Nietvoorbij, South Africa	Jolly *et al*., 2003b
	H2	791, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H3	802, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H4	897, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H5	899, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H6	913, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H7	918, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H8	932, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H9	934, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H10	961, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	H11	980, from the ARC Infruitec-Nietvoorbij, South Africa	This study
*Lachancea thermotolerans (*previously *Kluyveromyces thermotolerans)*	L1	Viniflora® Rhythm™, commercial yeast from Chr. Hansen, Denmark	This study
	L2	548, from the ARC Infruitec-Nietvoorbij, South Africa	This study
Metschnikowia pulcherrima (anamorph: *Candida pulcherrima)*	M1	825, from the ARC Infruitec-Nietvoorbij, South Africa	Jolly *et al*., 2003b; 2003c
	M2	C1/15, from the ARC Infruitec-Nietvoorbij, South Africa	Jolly *et al*., 2003c
	M3	780, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	M4	870, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	M5	950, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	M6	O2/16, from the ARC Infruitec-Nietvoorbij, South Africa	This study
	M7	O2/17, from the ARC Infruitec-Nietvoorbij, South Africa	This study
Schizosaccharomyces pombe	Sp	CBS 5557, CBS, Netherlands	This study

TABLE 1 (CONTINUED)

Species name	Strain code	Strain, origin and source information	References*
Torulaspora delbrueckii (anamorph: *Candida colliculosa)*	T1	CBS 1146^{T}, CBS, Netherlands	Van Breda *et al*., 2013
	T2	CBS 4663, CBS, Netherlands	Van Breda *et al*., 2013
	T3	Level^{2} TD™, commercial strain from Lallemand Inc, France	This study
	T4	Zymaflore® Alpha $^{TD\ n.\ Sacc.}$, commercial strain from Laffort, France	This study
	T5	Viniflora® Harmony™, commercial yeast from Chr. Hansen, Denmark	Van Breda *et al*., 2013
	T6	M2/1, from the ARC Infruitec-Nietvoorbij, South Africa	Jolly *et al*., 2003b; Van Breda *et al*., 2013
	T7	654, from the ARC Infruitec-Nietvoorbij, South Africa	Van Breda *et al*., 2013; Minnaar *et al*., 2015
	T8	301, from the ARC Infruitec-Nietvoorbij, South Africa	Van Breda *et al*., 2013

*Publications in which strains have been investigated.
**Strain 770 was classified as *Candida stellata* in this paper.

Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide (Bio-Rad Laboratories, Inc.). Normalisation of gels and comparison of banding patterns were done using FPQuest™ software (Bio-Rad Laboratories, Inc.) and the normalised electrophoretic patterns were grouped. Similarities (s) were obtained using the Dice coefficient, before cluster analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA).

MALDI-TOF bio-typing

Single colonies of each yeast strain were selected for identification and bio-typing by MALDI-TOF MS. One micro-litre of wine yeast protein extract was spotted onto a MTP 384 polished steel target plate as described by Moothoo-Padayachie *et al*. (2013) and Deak *et al.* (2015). Thereafter, the spotted target plate was inserted into a Bruker UltrafleXtreme MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) apparatus. Generation of yeast protein mass spectra using MALDI-TOF/TOF MS was conducted according to the standard National Agricultural Proteomics Research & Services Unit method (obtainable from the National Agricultural Proteomics Research & Services Unit (NAPRSU), University of the Western Cape, South Africa). Mass spectra for all strains were acquired in triplicate. The spectrum acquired for each sample was compared to the Bruker reference database, which contains 4 110 microorganisms (NAPRSU, May 2015).

Enzyme screening and malic acid degradation

Polygalacturonase/pectinase activity was determined as described by McKay (1988), β-glucosidase activity was determined through the screening method of Strauss *et al*. (2001) and acid protease activity was determined following the method of Charoenchai *et al*. (1997). The ability of yeasts to degrade malic acid was determined using the plate assay method described by Mocke (2005). The medium used for malic acid degradation was also modified slightly by excluding the agar and bromocresol green to determine malic acid degradation in a liquid medium. Aliquots of 10 mL of medium were dispensed into 42 test tubes and autoclaved. After this, single colonies of the yeast strains were inoculated into the test tubes containing the MLF broth and kept at an ambient temperature of 22°C for up to 40 days. Malic acid concentration was measured by enzymatic analysis (Arena 20XT enzyme robot, Institute for Wine Biotechnology, Stellenbosch University).

Evaluation of yeasts

Fermentation trial

Laboratory-scale alcoholic fermentation trials were conducted in a chemically defined grape juice as described by Costello *et al*. (2003). Yeasts were grown in 10 mL of YPD broth at 30°C prior to inoculation. Pure cultures of the different yeast strains were inoculated into sterilised 375 mL glass bottles containing 250 mL of filter-sterilised synthetic grape juice and fermented to dryness. Each yeast strain had three biological repeats. After the alcoholic fermentation (AF), the resultant synthetic wine of each yeast treatment was pooled, aseptically filtered (0.22 µm) and used for the MLF trial. Fifty millilitres of the synthetic wine were aliquoted into sterilised 250 mL bottles before inoculating with LAB. Two treatments were applied, *viz.* (1) addition of *O. oeni* only and (2) addition of nutrients as described by Costello *et al*. (2003) prior to the addition of *O. oeni* (Viniflora® oenos). Alcoholic and malolactic fermentations were conducted at ±22°C.

Chemical analyses

The Ripper method as described by Iland *et al*. (2000) was used to determine free and total SO_2. The sugar concentration, pH, malic acid, total acidity (TA), alcohol and volatile acidity (VA) of the synthetic wines were determined using an OenoFoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark).

RESULTS AND DISCUSSION

The role of non-*Saccharomyces* yeasts in wine production is not as well researched as the role of *S. cerevisiae* (Jolly

et al., 2014). Although *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* are receiving much more attention due to the availability of commercial products, a variety of other non-*Saccharomyces* yeast species have been investigated (Jolly *et al*., 2003b; Comitini *et al*., 2011; Jolly *et al*., 2014; Padilla *et al*., 2016). In this investigation, 37 non-*Saccharomyces* strains representing seven different non-*Saccharomyces* species, *viz. H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *Sc. pombe*, *C. zemplinina*, *C. stellata* and *T. delbrueckii*, were characterised by CHEF karyotyping, MALDI-TOF bio-typing, enzyme assays and malic acid degradation. The aforementioned non-*Saccharomyces* yeasts were compared to five commercial *S. cerevisiae* strains (N 96, NT 112, NT 202, VIN 7 and VIN 13), and their interactions with one *O. oeni* strain were investigated in synthetic grape juice. As the species-level identities of the yeasts used in this study were already known, CHEF karyotyping and MALDI-TOF bio-typing were used to study strain diversity within the different species (Figs 1, 2 and 3).

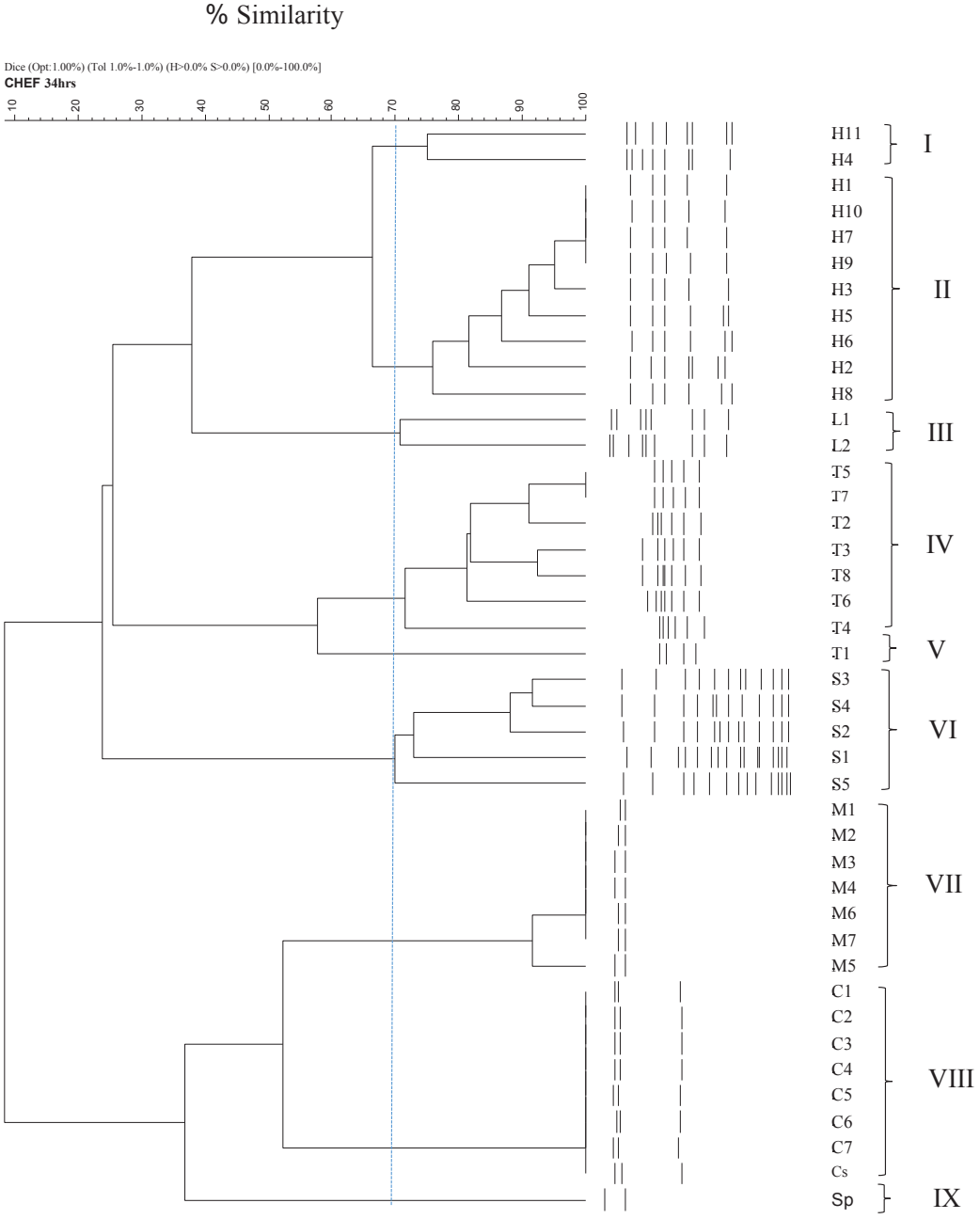

FIGURE 1

Dendrogram showing the clustering of yeast strains obtained by numerical analysis of CHEF karyotypes using a 34-hour programme. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cluster I and II: *Hanseniaspora uvarum* strains; III: *Lachancea thermotolerans* strains; IV and V: *Torulaspora delbrueckii* strains; VI: *Saccharomyces cerevisiae* strains; VII: *Metschnikowia pulcherrima*; VIII: *Candida zemplinina* (*Starmerella bacillaris*) and *Candida stellata* strains; and IX: *Schizosaccharomyces pombe*.

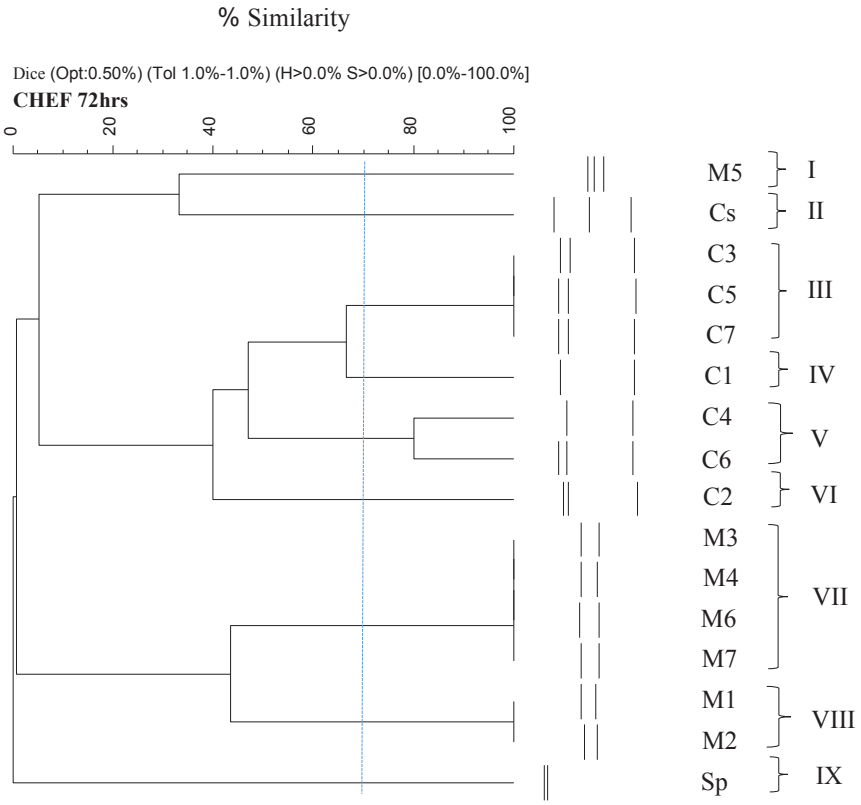

FIGURE 2

Dendrogram showing the clustering of yeast strains obtained by numerical analysis of CHEF karyotypes using the 72-hour programme. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cluster I: *Metschnikowia pulcherrima*; Cluster II: *Candida stellata*; Clusters III, IV, V and VI: *C. zemplinina*; Clusters VII and VIII: *M. pulcherrima*; and Cluster IX: *Schizosaccharomyces pombe*.

Electrophoretic karyotyping

The results of the CHEF karyotyping of the 34- and 72-hour programmes are shown in Figs 1 and 2 respectively. The Dice coefficient was used to group the yeasts based on the similarities of the electrophoretic banding patterns obtained. The 34-hour programme enabled the various yeasts to be separated to species and, in some cases, also to strain level (Fig. 1). The species could be separated into nine distinct clusters at a similarity (*s*) limit of 70%.

Cluster I was delineated at *s* = 75% and comprised two *H. uvarum* strains, H4 and H11, which were different from the other nine *H. uvarum* strains. Cluster II was delineated at *s* = 76% and included the remaining *H. uvarum* strains, *viz.* H1, H2, H3, H5, H6, H7, H8, H9 and H10. Within this cluster, strains H1, H7, H9 and H10 had an almost identical karyotype and were delineated at *s* = 100%. Strains H9 and H10 were isolated from grapes from the same location and may well be the same strain, but strains H1 and H7 were isolated from different areas within the Western Cape. This indicates that *H. uvarum* strains might not be as heterogeneous as *S. cerevisiae* strains. Cluster III comprised the two *L. thermotolerans* strains, L1 (Vinflora® Rhythm™) and L2, delineated at *s* = 70%. There were clear differences between the karyotypes of these two strains. Seven *T. delbrueckii* strains, *viz.* T2 (CBS 4663), T3 (Level 2^{Td}), T4 (Zymaflore® Alpha $^{TD\ n.\ Sacc.}$), T5 (Viniflora® Harmony™), T6, T7 and T8, formed cluster IV at *s* = 70%. *T. delbrueckii*-type strain, T1 (CBS 1146), clustered alone in cluster V at *s* = 58%.

Cluster VI comprised the five *S. cerevisiae* strains at *s* = 70% and these strains showed a high level of heterogeneity. These results confirmed reports by Hoff (2012) and Moothoo-Padaychie *et al*. (2013) on the heterogeneity of *S. cerevisiae* wine yeast strains. The *M. pulcherrima* strains formed cluster VII at *s* = 92%. All the strains had a similarity of 100%, except strain M5. The only difference for the *M. pulcherrima* karyotypes was the spacing between bands within the banding patterns. Cluster VIII was delineated at *s* = 100%, comprised all the *C. zemplinina* strains, including the type strain (CBS 9494), and also contained the *C. stellata*-type strain, Cs (CBS 157). These two species are closely related and were only reclassified as two different species when Sipiczki (2003; 2004) revealed the differences between them. More recently, Duarte *et al*. (2012) recommended the reinstatement of *Starmerella bacillaris* comb. nov. with the name *C. zemplinina* as obligate synonym, which has not been widely accepted (Magyar *et al*., 2014). As in the case of the *M. pulcherrima* cluster, the patterns of the *C. zemplinina* strains are very similar, with small spacing differences. *Sc. pombe* grouped on its own to form cluster IX at *s* = 38%, but showed some similarity with the *M. pulcherrima* strains, which also had only two bands.

The 34-hour CHEF programme was very useful for

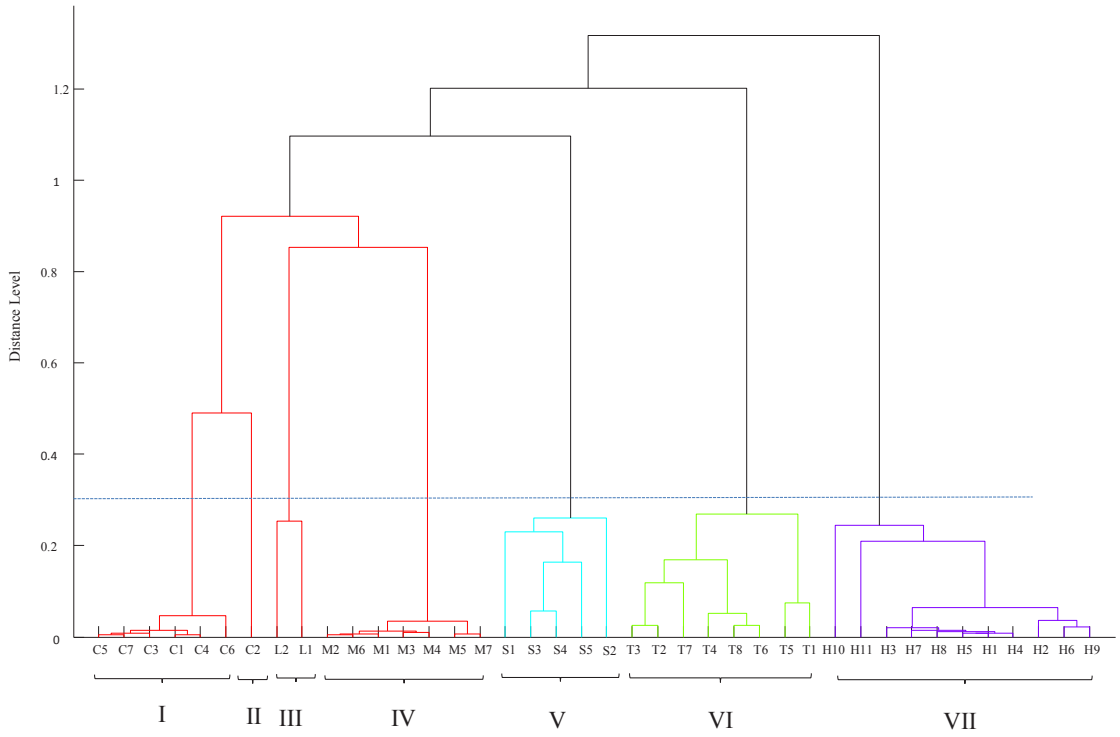

FIGURE 3

Dendrogram created from the mass spectral profiles of yeast strains using MALDI Biotyper software. Cluster I and II: *Candida zemplinina* (*Starmerella bacillaris*) strains; III: *Lachancea thermotolerans* strains; IV: *Metschnikowia pulcherrima*; V: *Saccharomyces cerevisiae* strains; VI: *Torulaspora delbrueckii* strains; and VII: *Hanseniaspora uvarum* strains.

the typing of the *S. cerevisiae* strains and strains within the *H. uvarum*, *L. thermotolerans* and *T. delbrueckii* clusters. However, it was not nearly as effective for typing *M. pulcherrima* and *C. zemplinina* strains. This confirms reports by Van Breda (2012) about the usefulness of CHEF for the typing of *T. delbrueckii* strains. However, the 34-hour programme could not be used to distinguish between *M. pulcherrima* and *C. zemplinina* at a strain level, therefore an extended 72-hour CHEF programme was investigated.

The clustering analysis of the 72-hour programme is shown in Fig. 2. Nine clusters could be discerned at $s = 70\%$. Cluster I was delineated at $s = 33\%$ and comprised only *M. pulcherrima* strain M5. The banding pattern of this strain was different to the other *M. pulcherrima* strains, and this was also evident in the grouping of the strains using the 34-hour programme (Fig. 1). Cluster II comprised the *C. stellata*-type strain (Fig. 2). Cluster III contained three *C. zemplinina* strains, C3, C5 and C7, at $s = 100\%$. These *C. zemplinina* strains had identical karyotypes, indicating that these isolates are possibly the same strain. Strains C3 and C7 were isolated from grapes on the same farm and may well be the same strain. Despite being isolated from a different area, it is possible that strain C5 might be the same strain as C3 and C7. Cluster IV was delineated at $s = 66\%$ and comprised only strain C1 (CBS 9494). Cluster V was delineated at $s = 80\%$ and comprised strains C4 and C6. Cluster VI was delineated at $s = 40\%$ and comprised one strain, C2. More differences were observed among the *C. zemplinina* strains with the 72-hour programme than with the 34-hour programme. The *M. pulcherrima* strains formed clusters VII (M3, M4, M6 and M7) and VIII (M1 and M2) at $s = 44\%$. Strains M4, M6 and M7 were isolated from the same location and could possibly be the same strain. This would explain the similarity between these strains. However, strain M3 was isolated from a different area within the Western Cape (South Africa). As was observed with the 34-hour programme, the karyotypes of the different strains were very similar. This indicates a high level of conserved genetic material within the small group of strains investigated. Cluster IX contained the one *Sc. pombe* strain, which had a completely different banding pattern from the other species, and this was also confirmed by a low similarity value.

More differences were observed between strains from the *C. zemplinina* and *M. pulcherrima* clusters with the 72-hour programme than the 34-hour programme. *Candida zemplinina* strains showed a higher level of heterogeneity than the *M. pulcherrima* strains with the 72-hour programme. This indicates that the CHEF programmes used in this study were not adequate for the typing of *M. ulcherrima* strains and that more optimisation is required. Differences were observed between the karyotypes of *C. emplinina* and *C. stellata* strains using the 72-hour programme, which is in agreement with the findings of Sipiczki (2004) and Csoma and Sipiczki (2008), who performed electrophoretic karyotyping over 99 and 96 hrs respectively. Similar results were obtained in this study, but using a shorter running time (72 h). This study confirmed that CHEF is a reliable technique for the identification of non-*Saccharomyces* yeast to the species and strain level. However, more optimisation and refinement are required for the typing of *M. pulcherrima* strains.

MALDI-TOF bio-typing

The results of the MALDI-TOF MS analyses (Fig. 3) show that the non-*Saccharomyces* and *S. cerevisiae* yeasts formed distinct groups. The identity of *H. uvarum*, *M. pulcherrima*, *S. cerevisiae*, *Sc. pombe* and *T. delbrueckii* could all be verified to species level using the MALDI Biotyper database. As *L. hermotolerans*, *C. zemplinina* and *C. stellata* were not in the MALDI Biotyper database, it could not be used to identify these strains. However, the MALDI-TOF MS profiles could be used to differentiate between strains within a species. The six non-*Saccharomyces* species could be grouped into seven clusters following cluster analysis of the mass spectra obtained at a phylogenetic distance level of 0.3, indicated by the dotted line in Fig. 3. Cluster I and II comprised the *C. zemplinina* strains, with strain C2 positioning on its own. The strains in cluster I showed a high level of similarity and grouped closely together. The composition of the *C. zemplinina* groupings differed from the groupings obtained using the 72-hour CHEF programme. Cluster III consisted of the two *L. thermotolerans* strains, which clearly differed from each other. Cluster IV consisted of all the *M. pulcherrima* strains, which also showed a high level of similarity and grouped closely together. Cluster V comprised the *S. cerevisiae* strains and appear to be a heterogeneous cluster. The *T. delbrueckii* strains grouped together in cluster VI and three sub-groups can be differentiated within this cluster. These strains show a high degree of variation. Cluster VII comprised the *H. uvarum* strains, which showed a high level of similarity, although four sub-groups could be differentiated. The *H. uvarum* strains H10 and H11 differed from the other strains and formed separate sub-groups. Strains H2, H6 and H9 also formed a separate sub-group. Strains H1, H3, H4, H5, H7 and H8 all grouped together and had a level of similarity. The sub-groups differed from the groupings obtained using CHEF karyotyping, indicating that isolates that were considered to be identical might be different strains.

The MALDI-TOF MS results were easier and faster to obtain than the CHEF karyotyping results. In both cases, software was needed for normalisation and clustering analyses. Both CHEF and MALDI-TOF MS were useful for species identification and could clearly type strains from *S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii* and *H. uvarum*, with the MALDI-TOF MS profiles showing slightly more variation. Neither technique was effective for the typing of *C. zemplinina* and *M. pulcherrima* strains, with MALDI-TOF MS revealing slightly more differences among the *M. pulcherrima* strains, and the 72-hour CHEF programme being more effective for the typing of *C. zemplinina* strains. For the typing of species with high genetic similarity, *i.e. M. pulcherrima* strains, alternative methods, such as amplified fragment length polymorphism (Spadaro *et al*., 2008) or tandem repeat-tRNA PCR (Barquet *et al*., 2012), could be considered. This study showed that MALDI-TOF MS can be used for the identification and typing of non-*Saccharomyces* yeasts and confirms the findings of Kántor and Kačániová (2015) about the usefulness of MALDI-TOF MS to differentiate between wine yeast species. However, MALDI-TOF MS was not as effective for typing *C. zemplinina* and *M. pulcherrima* strains.

Enzyme production

The ability of the eight non-*Saccharomyces* yeast species to produce acid protease, polygalacturonase/pectinase and β-glucosidase enzymes and to degrade malic acid is shown in Table 2. The *S. erevisiae* strains used in this study did not produce any extracellular enzymes. Charoenchai *et al*. (1997) reported some β-glucosidase activity in some *S. cerevisia*e strains, but Mostert (2013) found that the *S. cerevisiae* strain they tested did not have β-glucosidase or acid protease activity, but produced pectinase enzymes. The *C. stellata* strain was only positive for protease production and this is in agreement with the findings of Strauss (2003), who also showed that some *C. stellata* strains showed pectinolytic activity. Protease activity could be beneficial during fermentation by liberating assimilable nutrient sources, such as amino acids and peptides (Englezos *et al*., 2015). All the *C. zemplinina* strains tested negative for all three enzyme activities. Di Maio *et al*. (2012) and Englezos *et al*. (2015) reported medium to low β-glucosidase activity for *C. zemplinina* strains. Englezos *et al*. (2015) reported protease activity in 48 of 63 *C. zemplinina* strains studied, but none of the strains had pectinase activity.

The *H. uvarum* strains tested positive for β-glucosidase and negative for the other two enzyme activities. This confirmed the findings of Rodríguez *et al*. (2004) and Hernández-Orte *et al*. (2008), namely that *H. uvarum* strains have β-glucosidase activity. Strauss (2003) and Mostert (2013) also reported on *H. uvarum* strains that had protease and pectinase activity.

The two *L. thermotolerans* strains tested negative for all three enzyme activities. This is in contrast to Comitini *et al*. (2011) and Mostert (2013), who reported β-glucosidase activity in two *L. thermotolerans* strains. As in the case with the other species, enzyme activity appears to be strain dependent. All the *M. pulcherrima* strains were positive for protease and β-glucosidase activity, which is in agreement with the literature (Strauss, 2003; Mostert, 2013). The one *Sc. pombe* strain showed protease activity. Visintin *et al*. (2016) also reported on a *Sc. pombe* strain that had protease activity and a different *Sc. pombe* strain that produced pectinase. The results of this study confirmed the conclusion of Ganga and Martínez (2004) that enzyme production is not characteristic of a particular genus or species, but depends on the yeast strain analysed.

Malic acid degradation

The *S. cerevisiae* strains showed no malic acid degradation on the plate assay, but showed low activity in the broth, with S5 (VIN 7) utilising about 24% of the malic acid (Table 2). The low malic acid utilisation by *S. cerevisiae* is well documented (Gao & Fleet, 1995; Volschenk *et al*., 2003; Ribéreau-Gayon *et al*., 2006). The ability of the non-*Saccharomyces* strains to degrade malic acid varied greatly and there also were clear differences between the results of the plate and broth assays. The results indicate that the plate assay for malic acid utilisation is not very reliable, as it gave a lot of negative results as well as false positives. The *C. stellata* strain produced a positive reaction for malic acid utilisation on the plate assay, but could only utilise 9% of the malic acid in the broth assay. All the *C. zemplinina* strains

TABLE 2
Screening of *Saccharomyces* and non-*Saccharomyces* yeasts for production of extracellular enzymes and the ability to degrade malic acid.

Species name	Strain code	Enzyme activities			Malic acid degradation		
		Protease	Pectinase	β-Glucosidase	Plate assay	Broth	% Utilised
Saccharomyces cerevisiae	S1	-	-	-	-	-	13
	S2	-	-	-	-	-	11
	S3	-	-	-	-	-	11
	S4	-	-	-	-	-	12
	S5	-	-	-	-	-	24
Candida stellata	Cs	+	-	-	+	-	9
Candida zemplinina	C1	-	-	-	+	+	54
	C2	-	-	-	+	+	34
	C3	-	-	-	+	+	37
	C4	-	-	-	+	+	33
	C5	-	-	-	+	+	34
	C6	-	-	-	+	+	51
	C7	-	-	-	+	+	47
Hanseniaspora uvarum	H1	-	-	+	+	+	10
	H2	-	-	+	+	+	30
	H3	-	-	+	+	+	9
	H4	-	-	+	+	+	11
	H5	-	-	+	+	+	12
	H6	-	-	+	+	+	14
	H7	-	-	+	+	-	8
	H8	-	-	+	+	-	7
	H9	-	-	+	+	-	9
	H10	-	-	+	+	-	10
	H11	-	-	+	+	-	7
Lachancea thermotolerans	L1	-	-	-	+	+	20
	L2	-	-	-	+	-	10
Metschnikowia pulcherrima	M1	+	-	+	-	-	15
	M2	+	-	+	-	+	23
	M3	+	-	+	+	+	22
	M4	+	-	+	-	+	24
	M5	+	-	+	-	+	28
	M6	+	-	+	-	+	26
	M7	+	-	+	-	+	20
Schizosaccharomyces pombe	Sp	+	-	-	+	+	78
Torulaspora delbrueckii	T1	-	-	-	-	-	14
	T2	-	-	-	-	-	11
	T3	-	-	-	-	+	19
	T4	-	-	-	-	+	31
	T5	-	-	-	-	+	18
	T6	-	-	-	-	-	8
	T7	-	-	-	-	+	18
	T8	-	-	-	-	-	11

gave positive results for malic acid utilisation on the plate assay and in the broth, with malic acid utilisation ranging from 33 to 54%.

All the *H. uvarum* strains also gave positive reactions for malic acid utilisation on the plate assay, but only strain H2 showed real malic acid utilisation (30%) in the broth. The other *H. uvarum* strains utilised only between 7% and 14% of the malic acid in the broth. *T. delbrueckii* strains gave negative results for malic acid utilisation on the plate assay, but showed variable malic acid utilisation (11% to 31%) in the broth, with strain T4 (Zymaflore® Alpha $^{TD\ n.\ Sacc.}$) showing the most activity (31%). The above results are in agreement with reports of low malic acid utilisation for *C. stellata*, *T. delbrueckii* and *H. uvarum* (Gao & Fleet, 1995; Saayman & Viljoen-Bloom, 2006). The *L. thermotolerans* strains were also able to degrade malic acid on the plate assay, but were not as efficient in the broth, with strain L1 (Vinflora® Rhythm™) managing to utilise 20% of the malic acid. Only strain M3 gave a positive reaction on the plate assay, but all the *M. pulcherrima* strains showed some malic acid utilisation (15% to 28%).

As expected, the *Sc. pombe* strain gave a positive reaction on the plate assay and utilised 78% of the malic acid in the broth. Strains of *Sc. pombe* can degrade high concentrations of L-malate, but only if glucose or another assimilable carbon source is present (Baranowski & Radler, 1984; Rodriquez & Thornton, 1989, Benito *et al*., 2013; 2014).

Evaluation of yeasts

Fermentation trial

The ability of the non-*Saccharomyces* yeast to ferment synthetic juice and the progress of alcoholic fermentation are shown in Figs 4 to 8. The fermentations were monitored regularly for 40 days, but the final wine chemical analyses were carried out after 180 days, when the wines produced with the slow-fermenting yeasts were found to be dry (glucose/fructose < 4 g/L). *Candida zemplinina* strains showed variable fermentation abilities, with strains C1 (CBS 9494) and C2 (VEN 2097) standing out as the strongest fermenters, although still not comparable to the *S. cerevisiae* strains (Fig. 4). According to Csoma and Sipiczki (2008), *C. zemplinina* strains can be found throughout white and red wine fermentations and usually have sustained presence until the end of alcoholic fermentation. This study showed that some of the *C. zemplinina* strains have enough fermentation potential to be used in mixed culture fermentations.

The *H. uvarum* strains were slow to moderate fermenters, with strain H11 being the strongest fermenter (Fig. 5). The low fermentation activity of *H. uvarum* is in agreement with Ciani and Maccarelli (1998). The *M. pulcherrima* strains were also slow fermenters and most were still fermenting after 40 days, with the exception being strain M6 (Fig. 6). This concurs with reports from other studies (Jolly *et al*., 2003c; Mostert & Divol, 2014). The *T. delbrueckii* strains were strong fermenters and had fermentation rates that were comparable to the *S. cerevisiae* reference strains (Fig. 7). This concurs with the reports of Van Breda *et al*. (2013) and Renault e*t al*. (2015). The two *L. thermotolerans* strains were also strong fermenters and comparable to the *S. cerevisiae* strains (Fig. 8). These results confirmed the findings of Comitini *et al*. (2011) and Mostert and Divol (2014). The fact that both *T. delbrueckii* and *L. thermotolerans* are such strong fermenters is probably one of the reasons why strains from these species were selected for use as commercial starters in mixed culture fermentations with *S. cerevisiae* (Jolly *et al*., 2014). The *Sc. pombe* strain is a moderate

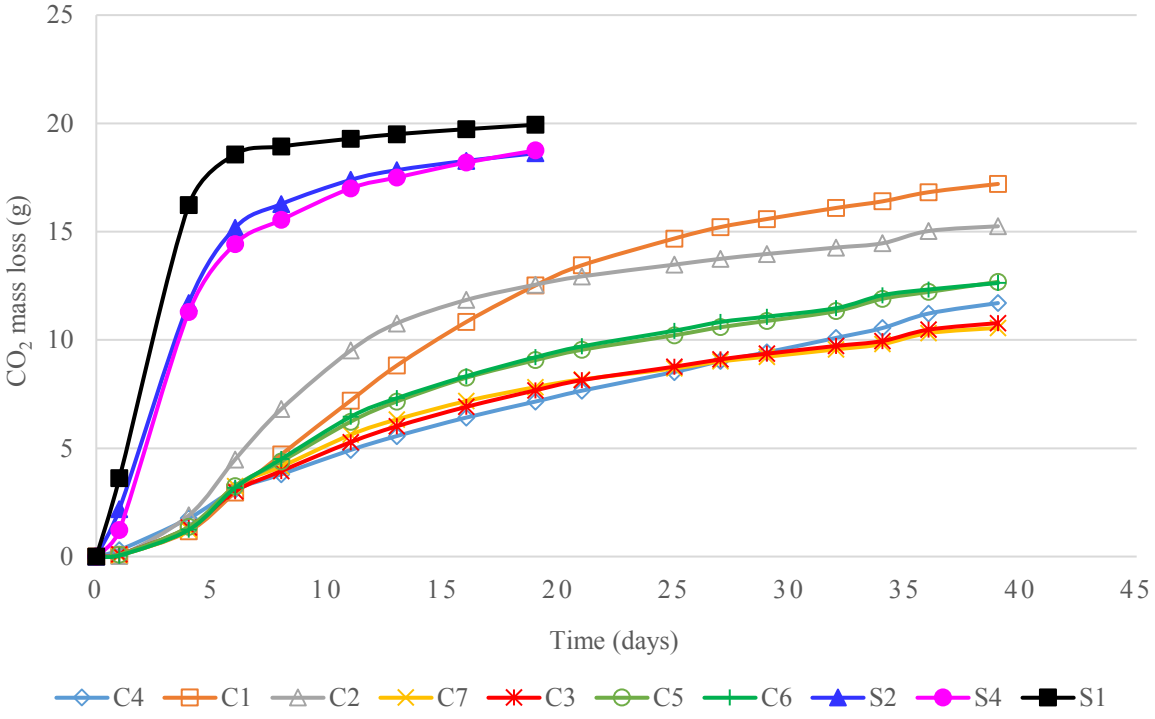

FIGURE 4
Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Candida zemplinina* strains in synthetic grape juice.

fermenter and fermentation activity may vary between strains (Benito *et al*., 2012; 2013). The *C. stellata* strain was a slow fermenter.

Chemical analyses

The results of the chemical analyses of synthetic wines produced with the different yeast species are listed in Table 3. The fermentations conducted by the slow-fermenting yeasts

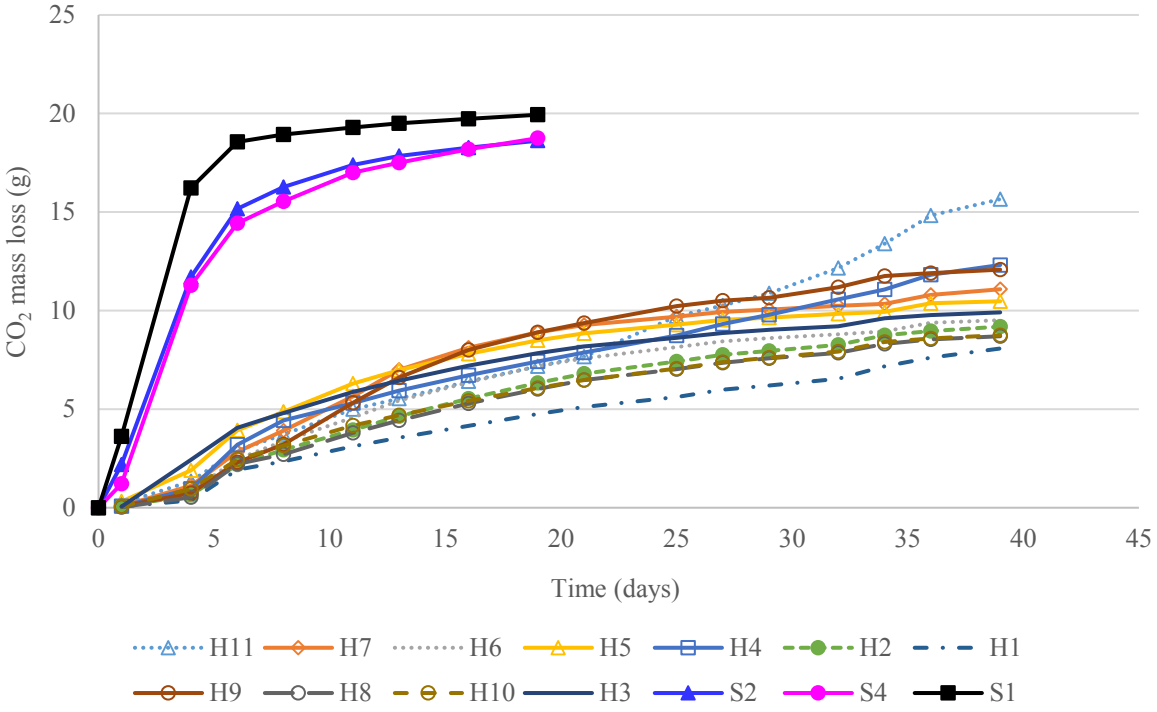

FIGURE 5

Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* strains in synthetic grape juice.

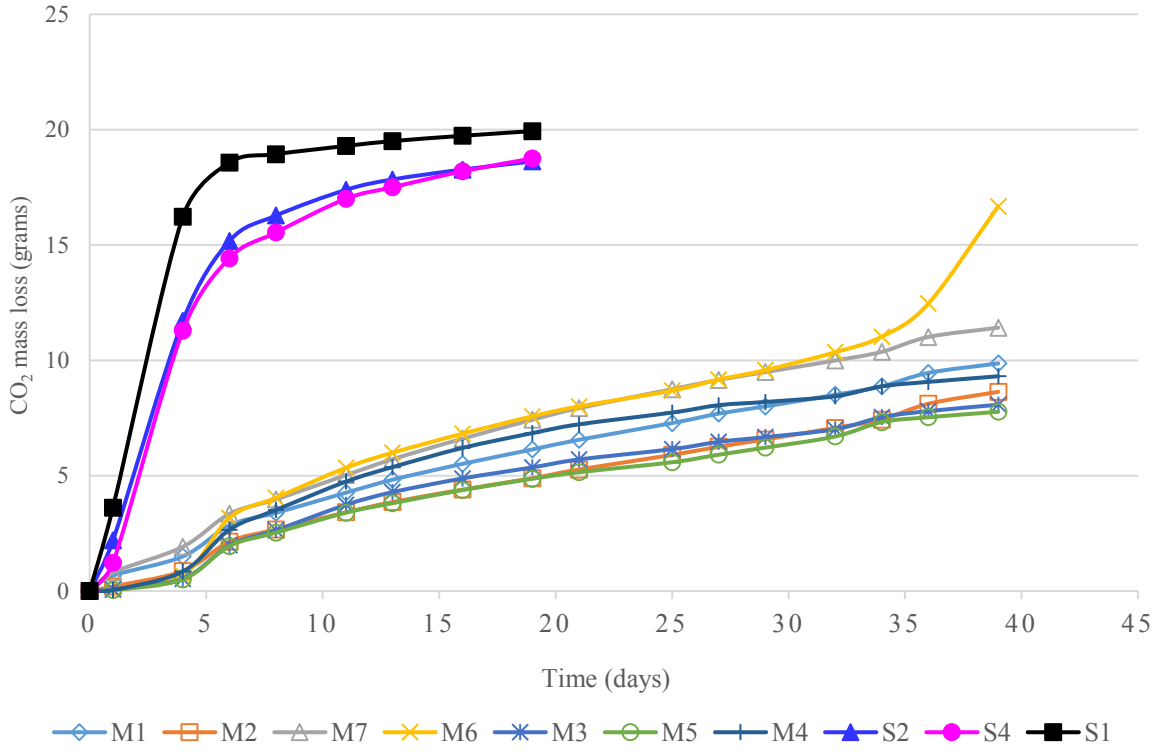

FIGURE 6

Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Metschnikowia pulcherrima* strains in synthetic grape juice.

TABLE 3
Chemical analyses and duration of alcoholic fermentation (AF) in synthetic wine produced with different yeast strains.

Species name	Strain code	Residual sugar (g/L)	Total acidity (g/L)	pH	Ethanol (% v/v)	Malic acid (g/L)	Volatile acidity (g/L)	Duration of AF (days)
Saccharomyces cerevisiae	S1	1.90 ± 0.25	3.54 ± 0.02	3.48 ± 0.01	9.93 ± 0.03	2.35 ± 0.04	0.40 ± 0.01	20
	S2	1.76 ± 0.19	3.43 ± 0.03	3.56 ± 0.03	9.88 ± 0.14	2.56 ± 022	0.34 ± 0.04	19
	S3	1.56 ± 0.13	3.52 ± 0.04	3.53 ± 0.02	10.02 ± 0.25	2.29 ± 0.12	0.29 ± 0.05	20
	S4	1.66 ± 0.55	3.54 ± 0.04	3.54 ± 0.04	10.07 ± 0.19	2.36 ± 0.07	0.30 ± 0.10	20
	S5	1.27 ± 0.13	3.37 ± 0.03	3.59 ± 0.03	9.37 ± 0.23	1.96 ± 0.22	0.57 ± 0.08	22
Candida stellata	Cs	1.01 ± 0.31	3.49 ± 0.15	3.67 ± 0.05	9.34 ± 0.14	2.20 ± 0.13	0.39 ± 0.06	180
Candida zemplinina	C1	3.36 ± 0.16	3.28 ± 0.02	3.70 ± 0.02	9.82 ± 0.04	2.07 ± 0.08	0.27 ± 0.01	42
	C2	2.41 ± 0.36	3.20 ± 0.23	3.70 ± 0.06	8.6 ± 0.92	1.67 ± 0.23	0.30 ± 0.14	45
	C3	1.60 ± 0.24	3.11 ± 0.06	3.78 ± 0.02	9.52 ± 0.21	1.81 ± 0.1	0.32 ± 0.02	40
	C4	1.21 ± 0.17	3.34 ± 0.04	3.74 ± 0.04	9.80 ± 0.09	1.83 ± 0.49	0.50 ± 0.05	180
	C5	1.05 ± 0.12	3.22 ± 0.04	3.73 ± 0.02	9.87 ± 0.04	1.57 ± 0.07	0.37 ± 0.02	180
	C6	1.47 ± 0.39	2.99 ± 0.06	3.72 ± 0.02	9.86 ± 0.31	1.43 ± 0.07	0.36 ± 0.04	45
	C7	1.94 ± 0.15	3.17 ± 0.15	3.74 ± 0.05	9.91 ± 0.12	1.63 ± 0.37	0.33 ± 0.08	68
Hanseniaspora uvarum	H1	1.65 ± 0.16	3.77 ± 0.40	3.66 ± 0.05	9.84 ± 0.11	1.89 ± 0.14	0.47 ± 0.34	180
	H2	1.57 ± 0.43	3.73 ± 0.50	3.75 ± 0.02	9.73 ± 0.26	1.81 ± 0.38	0.72 ± 0.50	180
	H3	1.19 ± 0.08	3.78 ± 0.05	3.74 ± 0.02	10.08 ± 0.08	1.77 ± 0.22	0.84 ± 0.08	180
	H4	1.68 ± 0.01	3.62 ± 0.16	3.67 ± 0.04	10.08 ± 0.26	2.13 ± 0.08	0.56 ± 0.06	42
	H5	3.20 ± 0.47	3.33 ± 0.02	3.65 ± 0.05	9.55 ± 0.07	2.17 ± 0.16	0.20 ± 0.05	180
	H6	1.95 ± 0.45	3.37 ± 0.30	3.69 ± 0.06	9.73 ± 0.38	1.91 ± 0.22	0.35 ± 0.25	180
	H7	1.77 ± 0.52	3.52 ± 0.25	3.72 ± 0.16	9.2 ± .21	1.99 ± 0.25	0.43 ± 0.06	180
	H8	1.93 ± 0.24	3.49 ± 0.46	3.70 ± 0.01	9.43 ± 0.25	1.87 ± 0.27	0.22 ± 0.02	180
	H9	1.88 ± 0.09	3.31 ± 0.07	3.70 ± 0.01	8.80 ± 0.12	1.79 ± 0.13	0.16 ± 0.05	180
	H10	1.97 ± 0.75	3.74 ± 0.09	3.74 ± 0.02	9.91 ± 0.14	1.74 ± 0.09	0.88 ± 0.04	180
	H11	1.86 ± 0.44	4.18 ± 0.63	3.71 ± 0.07	9.64 ± 0.76	2.34 ± 0.40	0.51 ± 0.23	45
Lachancea thermotolerans	L1	1.12 ± 0.10	2.84 ± 0.02	3.59 ± 0.01	9.31 ± 0.02	1.81 ± 0.22	0.18 ± 0.04	22
	L2	2.29 ± 1.20	3.10 ± 0.08	3.51 ± 0.02	10.35 ± 0.12	2.12 ± 0.18	0.10 ± 0.07	25
Metschnikowia pulcherrima	M1	0.96 ± 0.02	3.79 ± 0.31	3.51 ± 0.02	9.12 ± 0.20	1.91 ± 0.26	0.35 ± 0.15	180
	M2	0.86 ± 0.63	3.88 ± 0.04	3.59 ± 0.01	9.11 ± 0.01	1.83 ± 0.11	0.26 ± 0.11	180
	M3	0.56 ± 0.34	3.89 ± 0.27	3.63 ± 0.04	8.93 ± 0.65	1.61 ± 0.20	0.37 ± 0.05	180
	M4	0	3.94 ± 0.04	3.70 ± 0.03	9.51 ± 0.36	1.71 ± 0.12	0.25 ± 0.14	180
	M5	0	3.87 ± 0.18	3.65 ± 0.03	9.58 ± 0.16	1.50 ± 0.12	0.52 ± 0.03	180
	M6	2.63 ± 2.15	3.63 ± 0.23	3.65 ± 0.22	8.10 ± 1.27	1.78 ± 0.27	0.21 ± 0.11	46
	M7	0.65 ± 0.62	3.84 ± 0.25	3.53 ± 0.11	8.97 ± 1.15	1.62 ± 0.17	0.33 ± 0.20	180
Schizosaccharomyces pombe	Sp	1.87 ± 0.24	1.82 ± 0.03	3.69 ± 0.04	10.13 ± 0.03	0.56 ± 0.01	0.07 ± 0.04	39

TABLE 3 (CONTINED)

Species name	Strain code	Residual sugar (g/L)	Total acidity (g/L)	pH	Ethanol (% v/v)	Malic acid (g/L)	Volatile acidity (g/L)	Duration of AF (days)
Torulaspora delbrueckii	T1	1.60 ± 0.55	3.18 ± 0.05	3.69 ± 0.13	10.00 ± 0.14	1.85 ± 0.07	0.12 ± 0.02	180
	T2	1.80 ± 0.64	3.63 ± 0.13	3.58 ± 0.04	9.36 ± 0.92	2.48 ± 0.23	0.19 ± 0.01	31
	T3	1.83 ± 0.12	2.86 ± 0.16	3.60 ± 0.07	9.84 ± 0.27	2.31 ± 0.02	0.07 ± 0.01	24
	T4	3.16 ± 2.32	3.28 ± 0.25	3.78 ± 0.26	9.28 ± 0.21	2.16 ± 0.37	0.19 ± 0.05	39
	T5	3.70 ± 0.31	3.01 ± 0.03	3.59 ± 0.01	9.51 ± 0.22	2.39 ± 0.01	0.06 ± 0.01	39
	T6	3.00 ± 1.31	3.16 ± 0.11	3.59 ± 0.03	9.75 ± 0.51	2.52 ± 0.18	0.07 ± 0.02	20
	T7	1.46 ± 0.09	3.11 ± 0.06	3.58 ± 0.01	10.12 ± 0.10	2.32 ± 0.12	0.05 ± 0.05	20
	T8	2.93 ± 1.21	3.17 ± 0.06	3.61 ± 0.02	9.82 ± 0.27	2.46 ± 0.10	0.05 ± 0.04	20

were considered to be dry (residual sugar < 4 g/L) after 180 days. A great degree of variation was observed among the ethanol, malic acid and volatile acidity (VA) levels of the different non-*Saccharomyces* yeast species and strains. *Candida zemplinina* strains produced low VA and were similar to the *S. cerevisiae* strains, although *C. zemplinina* strains can be low or high VA producers (Magyar & Toth, 2011; Magyar *et al*., 2014; Englezos *et al*., 2015). Synthetic wines produced with *H. uvarum* contained high VA levels, especially wines produced with strains H2, H3 and H10. In contrast, synthetic wines produced with strains H5, H6, H7, H8 and H9 had low VA levels, which indicate strain variation within this species. Wines produced by other non-*Saccharomyces* yeasts contained lower VA levels than *H. uvarum*, which is in agreement with findings by other researchers (Ciani & Picciotti, 1995; Rojas *et al*., 2003). Wines produced with the *Sc. pombe* strain and *T. delbrueckii* strains contained the lowest VA levels. This is in agreement with Moreno *et al*. (1991) and Renault *et al*. (2009), who showed that pure cultures of *T. delbrueckii* produced lower VA levels than *S. cerevisiae*. Benito *et al*. (2012; 2013; 2014) showed that *Sc. pombe* can be moderate to high VA producers, depending on the strain. Most of the *M. pulcherrima* strains produced low VA levels, except for strain M5, which produced slightly higher VA levels (0.52 g/L). *M. pulcherrima* is not normally associated with VA production, but rather with relatively high concentrations of esters (Bisson & Kunkee, 1991).

The malic acid levels were lower in all synthetic wines, indicating loss due to precipitation, but also some degradation (Table 3). In most cases, synthetic wines fermented with non-*Saccharomyces* yeasts had lower malic acid levels than synthetic wines fermented with *S. cerevisiae* strains. Wines fermented with *Sc. pombe* had a malic acid reduction of > 77%, while the reduction by the other non-*Saccharomyces* yeast varied. These results are in agreement with those obtained for the malic acid utilisation in the malic acid broth.

Malolactic fermentation

The effect of various yeast strains on *O. oeni* growth and its ability to complete MLF prior to inoculation, with or without nutrient supplementation, is presented in Table 4. There were clear differences between the MLF treatments that were applied. In most cases, MLF proceeded quickly and without delays. However, in some cases where delays occurred, nutrient supplementation improved the progress of MLF or completely eliminated the delays. None of the yeasts produced high enough levels of SO_2 to inhibit LAB, but there were some variations between the species and among strains from the same species. Despite producing low levels of SO_2, there were differences among the *S. cerevisiae* strains. Strains S1 and S5 had the least inhibitory effect on MLF, which was completed after seven days (Table 4). Strain S3 had an inhibitory effect on MLF, and this was evident in both treatments. In this case inhibition could be due to SO_2, but the production of other inhibitory compounds is more likely. Yeasts can inhibit LAB, and therefore MLF, by depleting nutrients or by producing toxic metabolites such as ethanol, SO_2, medium-chain fatty acids and proteins or peptides

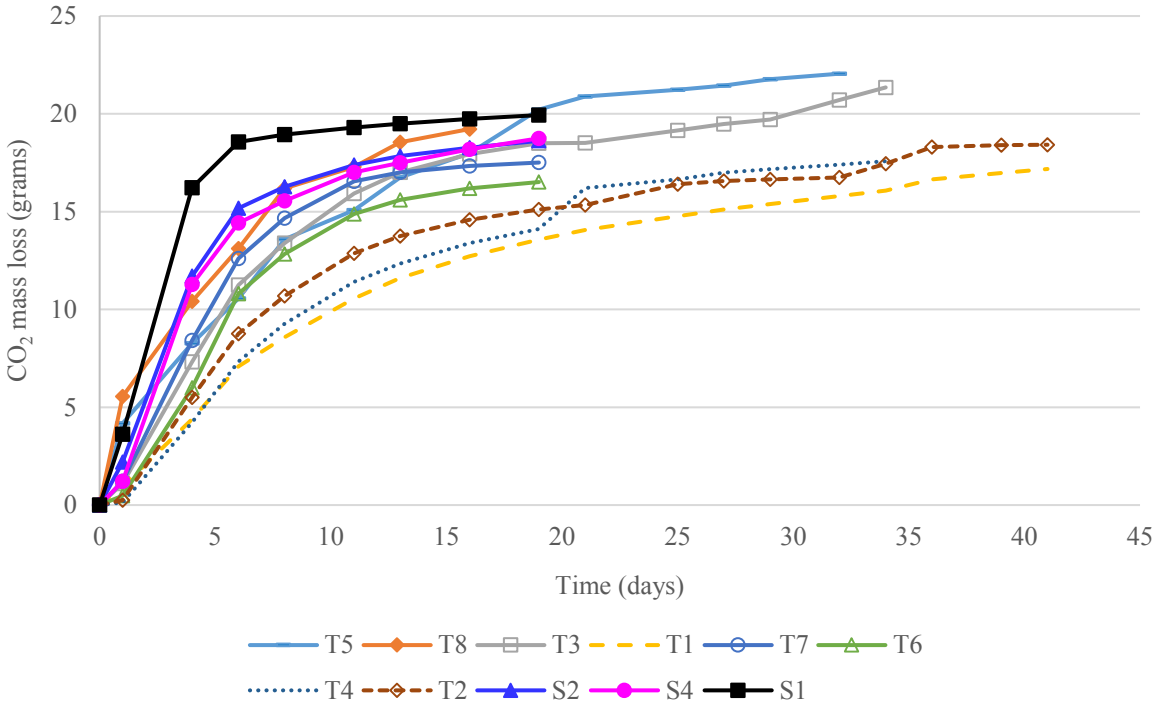

FIGURE 7
Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* strains in synthetic grape juice.

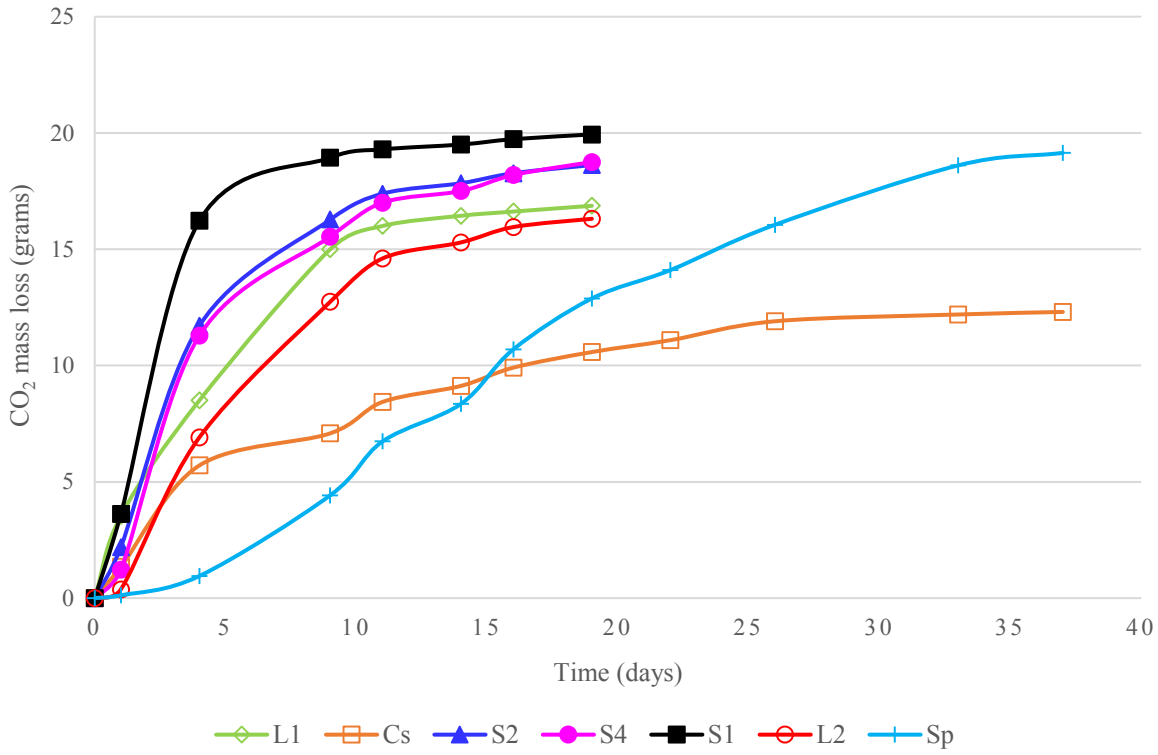

FIGURE 8
Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae*, *Lachancea thermotolerans* and *Schizosaccharomyces pombe* strains in synthetic grape juice.

TABLE 4
Free and total SO_2 levels and duration of malolactic fermentation (MLF) in synthetic wines fermented with different yeasts.

Species name	Strain code	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Duration of MLF (days)	
				Treatment 1*	Treatment 2**
Saccharomyces cerevisiae	S1	3	8	7	7
	S2	4	9	13	7
	S3	5	9	14	13
	S4	5	9	13	7
	S5	2	5	7	6
Candida stellata	Cs	2	9	26	21
Candida zemplinina	C1	2	4	7	7
	C2	2	5	7	7
	C3	2	5	7	7
	C4	2	6	7	7
	C5	2	5	7	7
	C6	2	5	7	7
	C7	2	6	20	7
Hanseniaspora uvarum	H1	2	10	7	7
	H2	1	8	7	7
	H3	2	8	10	7
	H4	2	9	7	7
	H5	2	8	13	13
	H6	1	8	7	7
	H7	2	6	14	14
	H8	2	6	13	7
	H9	1	8	13	7
	H10	2	9	7	7
	H11	2	8	7	7
Lachancea thermotolerans	L1	2	5	7	7
	L2	2	5	7	7
Metschnikowia pulcherrima	M1	3	9	7	7
	M2	3	10	7	7
	M3	3	9	7	7
	M4	2	9	7	7
	M5	2	10	7	7
	M6	3	10	7	7
	M7	3	10	7	7
Torulaspora delbrueckii	T1	2	10	7	7
	T2	2	8	14	14
	T3	2	4	13	7
	T4	2	5	7	7
	T5	2	5	7	7
	T6	3	5	7	7
	T7	3	5	7	10
	T8	2	5	7	7

*Treatment 1: Sequential inoculation with commercial *Oenococcus oeni* strain.
**Treatment 2: Nutrient supplementation (Costello *et al*., 2003) prior to sequential inoculation with *O. oeni* strain.

(Alexandre *et al*., 2004, Comitini *et al*., 2005; Nehme *et al*., 2008). Strains S2 and S4 also had an inhibitory effect on MLF (treatment 1), but the inhibition could be overcome by nutrient supplementation (treatment 2). The antagonistic effect of some *S. cerevisiae* on MLF has been reported, and yeast and LAB compatibility is an important factor to consider for successful MLF (Henick-Kling & Park 1994; Costello *et al*., 2003).

The *C. stellata* strain (Cs) had an inhibitory effect on MLF (26 days) and resulted in MLF taking longer to complete (Table 4). However, delayed MLF could be partially alleviated by nutrient supplementation (treatment 2), but MLF still took 21 days. Inhibition by *C. stellata* could be partially due to nutrient depletion, but other inhibitory compounds are a more likely explanation. In general, the *C. zemplinina* strains did not have an inhibitory effect on MLF, except for strain C7, which took 20 days to complete MLF. The inhibitory effect of C7 was completely eliminated by nutrient supplementation.

Hanseniaspora uvarum strains H5 and H7 had a slight inhibitory effect on all MLF treatments. SO_2 levels were not excessively high in these wines, indicating that some other inhibitory compound(s) was probably produced. Strains H3, H8 and H9 also had an inhibitory effect on MLF, but the inhibitory effect could be eliminated by nutrient supplementation. The *L. thermotolerans* and *M. pulcherrima* strains completed MLF quickly and were finished within seven days. No variations with regard to MLF were observed for strains within these species. The *M. pulcherrima* strains had the highest total SO_2 levels of all the non-*Saccharomyces* yeast, but these did not affect the progression of MLF.

The results indicate that some of the yeast strains had a higher nutrient demand or uptake, which resulted in slower progression of MLF. The duration of MLF varied between the yeasts used, but none of the yeasts completely inhibited MLF. In the case of delayed MLF it appears to be strain dependent. SO_2 was ruled out as a reason for the delays, but other toxic metabolites were not investigated. The metabolites produced by these inhibitory strains need further investigation. The results obtained in synthetic wine should be confirmed in real grape juice and wine fermentations because the interaction between the non-*Saccharomyces* yeast and LAB might be different in a real wine matrix.

CONCLUSIONS

Both CHEF karyotyping and MALDI-TOF MS were effective techniques for identifying wine non-*Saccharomyces* yeast species and could also be used for the typing of *C. zemplinina*, *H. uvarum*, *L. thermotolerans* and *T. delbrueckii* strains. Both techniques were unable to adequately type *M. pulcherrima* strains, but CHEF karyotyping showed more potential for the typing of these strains. Yeast enzyme activity appears to be strain dependent, and most of the species investigated did not have extracellular β-glucosidase, pectinase and protease activity. In the synthetic wine fermentations, the *C. stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were shown to be slow to medium fermenters. The *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters and comparable to *S. cerevisiae*. Further investigations are needed to evaluate the *L. thermotolerans* and *T. delbrueckii* strains as potential single inoculations or co-inoculations with *S. cerevisiae* in grape must, while the *H. uvarum* and *M. pulcherrima* strains need to be evaluated in co- or sequential inoculations with *S. cerevisiae*. The effect of non-*Saccharomyces* yeast species on MLF varied and inhibition was found to be strain dependent. All *M. pulcherrima* and *L. thermotolerans* strains used in this study were compatible with the *O. oeni* strain and conducive to MLF. In most cases, delays in MLF could be alleviated by nutrient supplementation. Many of the non-*Saccharomyces* yeast strains evaluated showed potential for use in wine production and warrant further investigation.

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