UNIVERSIDAD COMPLUTENSE DE MADRID

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DEPARTAMENTO DE MICROBIOLOGÍA III

TESIS DOCTORAL

Estudio filo-funcional de levaduras de interés enológico para su aplicación industrial

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Ignacio Belda Aguilar

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Tesis Doctoral presentada por D. Ignacio Belda Aguilar para optar al grado de Doctor en Biología por la Universidad Complutense de Madrid

Madrid, 2016

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A mi familia

“In hard times, imagination is more important than knowledge”

Albert Einstein

Agradecimientos:

Esta Tesis es de mucha gente. Mucha gente que ha puesto medios económicos, materiales y, sobre todo, humanos para que pueda llevarse a cabo.

Obligatorio comenzar por agradecer a Antonio Santos la dedicación en cuerpo y alma en estos ya casi 5 años juntos. No ha habido hora ni día en que haya mostrado una negativa a poner sobre la mesa lo que fuera necesario por nuestro trabajo. Como decía al principio, esta Tesis es de mucha gente, pero desde luego tuya es. Tirando también del carro de la dirección, Eva Navascués, los pies en la tierra y la que nos enseño la ciencia que esconde una botella de vino. Apostó desde el principio por nosotros y, desde luego, este trabajo no sería una realidad sin su fe en nosotros y en nuestra manera de ver la Microbiología. Finalmente y, aunque no figura como Director de esta Tesis, suya es la culpa de que yo comenzara a trabajar en este grupo. De nuevo, una confianza ciega, que me otorgó sin apenas conocerme, o si… yo nunca lo sabré. Domingo, gracias por ese tiempo y esa puerta abierta que siempre has tenido para lo que aconteciera.

Ahora van mis compañeros de laboratorio, todos ellos responsables de mis ganas de venir a trabajar y pacientes de mis manías (que no detallaré). Alex, desde el comienzo, un buen compañero que se hizo amigo. Hemos compartido muchas horas y faenas para sacar esto adelante, enhorabuena por lo que te toca. Rocío, ya no está, se fue a la cumbre, pero sigue presente y ayudando en lo que puede. Fuiste clave en los inicios y sigues siendo muy importante en este final. Gracias por todo. Patri, a dos laboratorios de distancia y con distinta área de investigación, conoce al dedillo lo que hago, porque soy muy pesado y ella me escucha con un poleo delante. Por escuchar siempre, lo científico y lo no, gracias. Javi, llegó el último, pero arrimó el hombro como el primero. Con todas mis ganas le he trasmitido lo que sé y ahora ya es todo un científico, quédense con el Ruiz *et al*. Millones de gracias por tu dedicación. Albert, sonrisa siempre en la boca, dando vida a un laboratorio que inunda de anécdotas. Gracias por desenrranciarme siempre que está en tu mano. Y por último, Cris, nuestra pie en la química y en lo volátil. Gracias porque nunca haya horarios, por vivir con nosotros las prisas y presiones como si fueran tuyas. Al resto de compañeros que han pasado por este laboratorio o han paseado por el pasillo de esta planta, gracias por lo que hayáis podido aportar.

Echando la vista atrás, y unos pisos más abajo, ni más ni menos que responsables de que yo acabara con decencia esta preciosa carrera de Biología; Pati, Isa, Estela, Gerardo: gracias por todo lo vivido.

La vida científica sale fuera de las puertas de un laboratorio. Por su constante apoyo y momentos compartidos, quería agradecer a Víctor J. Cid sus consejos y su visión de la Ciencia y la vida científica. Un lujo haberme cruzado contigo. En el mismo pack, de los *home idols* con vocación cervecera, Ana Alastruey, mil gracias también por muchas horas de trabajo y consejos. Gobernarás el mundo, lo sé. De nuevo, un placer. A Mª Victoria Moreno-Arribas, porque la confianza no se paga con nada y tú lo has hecho en mí cada vez que lo he necesitado. Gracias por cada empujón y segundo dedicado. A Santiago Benito y Fernando Calderón, un pie en el laboratorio y otro en la bodega. Mucho de esta Tesis es suyo, no sólo resultados, si no un enorme respeto mío y de los míos por una dedicación admirable.

En el capítulo personal, gracias a mi familia, siempre. Una fe ciega y un cariño constante responsable de que sea quien soy. Papa, Mamá, Emi, Ani, Ahinoa, Nahia (preciosa) y Rocio. Gracias por todo. A esta última, Rocío, gracias especiales por todo lo que has puesto en esta tesis. Lo científico y lo no tanto, leyendo manuscrito tras manuscrito que componen esta tesis y aportando lo mejor de ti en ellos. Por mucho e impagable, Gracias.

Como decía al principio, esta Tesis es de mucha gente, y por supuesto debo reconocer el papel de los organismos y empresas que han confiado y financiado este trabajo a través de distintos proyectos. En primer lugar y, tanto en el plano institucional como al personal de Laboratorio, gracias a Agrovin S.A. (proyecto ENZIOXIVIN, IDI- 20130192) por su constante confianza y al Centro para el Desarrollo Técnico Industrial (CDTI, Ministerio de Economía y Competitividad) por su sustento. Este trabajo ha sido financiado también por Bodegas Pago de Carraovejas (proyecto IDI-20140448), Bodegas Emilio Moro, quienes con su valentía han sido personajes fundamentales en la transferencia a la industria del conocimiento generado en este trabajo.

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ABREVIATURAS

AC	Contenido en Antocianos / *Anthocyanin Content*
ADN/DNA	Ácido Desoxirribonucleico / Desoxiribonucleic Acid
ADNr/rDNA	Ácido Desoxirribonucleico ribosómico / Ribosomal Desoxiribonucleic Acid
Ap	*Aureobasidium pullulans*
ARN/RNA	Ácido Ribonucleico / Ribonucleic Acid
ARNr/rRNA	Ácido Ribonucleico ribosomal / ribosomal Ribonucleic Acid
BLAST	*Basic Local Alignment Search Tool*
CECT	Colección Española de Cultivos Tipo / *Spanish Type Culture Collection*
CFU	Unidades Formadoras de Colonias/ *Colony Forming Units*
CI	Intensidad de Color / *Color Intensity*
CO_2	Dióxido de Carbono / *Carbon dioxide*
C-S	Carbono-Azufre / *Carbon-Sulfur*
cv	Variedad Cultivada / *Cultivated Variety*
CYC	Colección Complutense de Levaduras */ Complutense Yeast Collection*
D.O.	Denominación de Origen / *Apellation of Origin*
DGGE	Electroforesis en Gel con Gradiente de Desnaturalización / *Denaturing Gradient Gel Electrophoresis*
EM	Bodegas Emilio Moro / *Emilio Moro winery*
G	Bodegas Gordonzello / *Gordonzello winery*
GC-MS	Cromatografía de Gases-Espectrometría de Masas / *Gass Cromatography-Mass Spectometry*
H_2S	Ácido sulfhídrico / *Hydrogen sulfide*
HCl	Ácido clorhídrico / *Hydrochloric acid*
HL	Homocigoto para el alelo íntegro */ Homozygous full-lenght allele*
HPLC	Cromatografía líquida de alta eficacia / *High-Performance Liquid Chromatography*
HS	Homocigoto para el alelo delecionado / *Homozygous Short allele*
HTS	Cribado de alto rendimiento / *High-Throughput Screening*
HT	Alelo heterocigoto / *Heterozygous allele*
L.	Linneo / *Linneaus*
Lt	*Lachancea thermotolerans*
DMDS	Dimetil disulfuro / *Dimethyl disulfide*
Mp	*Metschnikowia pulcherrima*
MTL	Metanotiol / *Methanethiol*
NaCl	Cloruro Sódico / Sodium chloride
NCBI	*National Center for Biotechnology Information*
NCR	Represión Catabólica por Nitrógeno / *Nitrogen Catabolite Repression*
NTU	Unidades Nefelométricas de Turbidez / *Nephelometric Turbidity Units*
O	Bodegas Ossian / *Ossian winery*

OD	Densidad Óptica / *Optical Density*
OIV	Organización Internacional para la Vid y el Vino / *International Organisation of Vine and Wine*
PCs	Componentes principales / *Principal Components*
PCA	Análisis de Componentes principales / *Principal Component Analysis*
PDC	Bodegas Pago de Carraovejas / *Pago de Carraovejas winery*
PYCC	Colección Portuguesa de Levaduras / *Portuguese Yeast Culture Colection*
Sc	*Saccharomyces cerevisiae*
SM	*Coinoculación simultánea* / *Simultaneous co-inoculation*
SMC	S-metil-L-cisteína / *S-methyl-L- cysteine*
SO_2	Dióxido de azufre / Sulfur dioxide
sp.	Especie / *Species*
SPME	*Microextracción en Fase Sólida* / *Solid Phase Microextraction*
SQ	Inoculación secuencial / *Sequential inoculation*
Td	*Torulaspora delbrueckii*
TIC	Cromatograma de Iones Totales / *Total Ion Chromatogram*
TPI	Índice de Polifenoles Totales / *Total Polyphenol Index*
UPGMA	*Unweighted Pair Group Method with Arithmetic Mean*
YCB	Base de Carbono para Levaduras / *Yeast Carbon Base*
YNB	Base de Nitrógeno para Levaduras / *Yeast Nitrogen Base*
YMA	Agar extracto de malta-extracto de levadura / *Yeast Malt Agar*
YMA-MB	Agar extracto de malta-azul de metileno / *Yeast Malt Agar-Methylene Blue*
YPD	Extracto de levadura-Peptona-Dextrosa / *Yeast Peptone Dextrose*
YPGE	Extracto de levadura-Peptona-Glicerol-Etanol / *Yeast Peptone Glycerol Ethanol*
vs.	*Versus*
v/v	Volumen/Volumen / *Volume/Volume*
wt/vol	Peso/Volumen / *Weight/Volume*
3-MH	3-Mercaptohexanol / *3-Mercaptohexanol*
3-MHA	Acetato de 3-mercaptohexilo / *3- mercaptohexyl acetate*
4-MMP	4-Mercapto-4metilpentan-2-ona / *4-mercapto-4-methylpentan-2-one*

RESUMEN & SUMMARY

RESUMEN

La microbiología asociada al proceso de fermentación espontánea de un mosto de uva consiste en una compleja sucesión de especies de levaduras que establecen una dinámica poblacional determinada, fundamentalmente, por la presión selectiva que ejerce la creciente concentración de etanol en el medio. En este contexto, es conocido cómo *Saccharomyces cerevisiae* logra dominar el proceso, aún estando en concentraciones relativamente bajas en la población inicial del mosto, a través de la estrategia de producción-acumulación-consumo de etanol. Esta estrategia ha permitido a *S. cerevisiae* hacer, de un ambiente fundamentalmente antrópico como son las fermentaciones, uno de sus principales nichos ecológicos. No obstante, el proceso de fermentación vínica no es un proceso axénico y, dado el papel crucial de *S. cerevisiae* en el proceso, la bibliografía tradicional ha denominado al resto de especies de levadura involucradas en la fermentación vínica como levaduras "no-*Saccharomyces*".

Así, las levaduras no-*Saccharomyces* constituyen un grupo heterogéneo de especies fundamentalmente involucradas en las primeras etapas del proceso de fermentación. Puede establecerse una clasificación de las mismas en función de su poder fermentativo y, por tanto, del mayor o menor tiempo que, *a priori,* podrán mantenerse activas en la fermentación. Existen levaduras eminentemente oxidativas (*Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Cryptococcus* spp.), levaduras poco fermentativas (*Hanseniaspora spp.*) y levaduras con cierta o notable capacidad fermentativa (*Metschnikowia* spp*., Kluyveromyces* spp., *Lachancea* spp*., Torulaspora* spp. y *Zygosaccharomyces* spp.). Su fisiología y su contribución a la composición y calidad del vino están todavía poco estudiadas, aunque en la actualidad, tanto científicos como enólogos, son conscientes de su potencialidad en la mejora de la calidad de los vinos, tanto en aspectos sensoriales como tecnológicos y de seguridad alimentaria.

El presente trabajo abordó, en una primera etapa, el establecimiento de una amplia colección de levaduras no-*Saccharomyces*, aisladas de mostos sin fermentar de tres Denominaciones de Origen diferentes y a lo largo de tres vendimias. En esta colección, compuesta por un total de 770 aislamientos, fueron identificadas 15 especies distintas, entre las cuales dominaron notablemente las pertenecientes a los géneros *Hanseniaspora*, *Lachancea* y *Metschnikowia*. En el estudio de la producción de enzimas de interés enológico por parte de las levaduras de la colección, se observó como ciertas actividades como β-

glucosidasa o proteasa estaban ampliamente distribuidas en el total de aislamientos y, por el contrario, actividades como α-L-arabinofuranosidasa, pectinasa y celulasa estaban restringidas a un bajo número de especies y cepas con, además, baja representación relativa en la población total. En una posición intermedia se encontraron las actividades β-xilosidasa, sulfito reductasa y β-liasa, destacando esta última por encontrarse en niveles de intensidad moderados en la mayoría de especies que la mostraban. Los resultados de este estudio no sólo permitieron establecer características generales de cada especie, si no observar diferencias intraespecíficas que, además, respondían a patrones dependientes de origen, por lo que fue posible establecer *clusters* de cepas dependiendo del lugar y el año en que fueron aisladas considerando sus patrones de producción de actividades enzimáticas en el correspondiente análisis de componentes principales.

Dada la dificultad del estudio de las 8 actividades enzimáticas mencionadas en la colección de 770 aislamientos con los métodos descritos hasta el momento, este trabajo requirió de la adaptación de los mismos a un formato de *screening* de alto rendimiento o del desarrollo de métodos nuevos para actividades, como la β-liasa, para los que hasta el momento no existía un método de evaluación directo mas allá de la valoración de los compuestos volátiles derivados de su acción sobre sus precursores. Así en el presente trabajo se describe un medio de cultivo diferencial para levaduras con alta actividad β-liasa basado en el uso de la S-Metil-L-Cisteína como sustrato de dicha actividad análogo a los sustratos cisteinilados presentes en el mosto y con el amonio derivado de su hidrólisis como única fuente de nitrógeno. Conocido el gen *IRC7* de *S. cerevisiae* como principal responsable de la liberación de tioles voláties a partir de sus precursores cisteinilados en el mosto y conocida también la existencia de dos isoformas del mimo, una activa y una inactiva, el medio de cultivo descrito permitió la diferenciación, por su crecimiento, de aquellas cepas que presentaban en homocigosis o heterocigosis la copia de funcional del gen *IRC7*. De igual manera, el medio de cultivo permitió identificar algunas cepas de especies como *Torulaspora delbrueckii* o *Kluyveromyces marxianus* como cepas con elevada actividad β-liasa, comprobándose en fermentaciones en mosto blanco de la variedad verdejo, la gran capacidad de la cepa *T. delbrueckii* Viniferm NS-TD para la liberación de los dos principales compuestos volátiles tiólicos (3-MH y 4-MMP).

Tras el estudio filo-funcional, en el que pudieron establecerse los perfiles de producción de enzimas de las 15 especies identificadas en la colección, se procedió al estudio de la repercusión de levaduras con actividad poligalacturonasa sobre el proceso de extracción de color y mejora del proceso de clarificación de vinos tintos. En este estudio pudo comprenderse como la especie *Metschnikowia pulcherrima,* considerada como moderadamente fermentativa logra incidir más notablemente sobre los citados parámetros que la otra especie pectinolítica, *Aureobasidium pullulans*, fundamentalmente oxidativa. Además, de estos resultados se deriva la importancia de la baja temperatura en los procesos de maceración prefermentativa, que no sólo mejora la extracción de ciertos pigmentos por motivos químicos de solubilidad de la matriz acuosa que se mantiene por retraso de aparición del etanol, si no por favorecer el desarrollo de ciertas especies de levaduras no-*Saccharomyces* mas criófilas que *S. cerevisiae* y por el retraso en el desarrollo de ésta.

Con el objetivo de mejorar la calidad sensorial de los vinos tintos, se llevó a cabo el estudio de la incidencia de la cepa *Torulaspora delbrueckii* Viniferm NS-TD mediante inoculación simultánea y secuencial con *S. cerevisiae*. Como primera conclusión se deriva que, a pesar del notable carácter fermentativo de *T. delbrueckii*, la coinoculación simultánea de ésta con *S. cerevisiae* en concentraciones iguales limita notablemente su desarrollo. En el caso de los vinos producidos con inoculación secuencial de *T. delbrueckii* Viniferm NS-TD seguido de *S. cerevisiae* tras un descenso de 15 g/L en la densidad del mosto, se pudo apreciar un notable incremento en la calidad de estos en comparación con lo exclusivamente fermentados con *S. cerevisiae*. Esta mejora pudo asociarse con un notable incremento en el contenido en manoproteínas de estos vinos, así como un ligero descenso en la acidez y en la concentración de alcoholes superiores de los mismos.

Finalmente este trabajo abordó la aplicación de cepas de levadura no convencionales en crianza sobre lías para la mejora de las propiedades sensoriales de los vinos tintos, analizando, como factor principal, la liberación de manoproteínas durante el proceso. Los resultados mostraron que, nuevamente, la cepa *T. delbrueckii* Viniferm NS-TD logra liberar concentraciones muy elevadas de manoproteínas también durante la crianza sobre lías, alcanzando niveles ligeramente superiores a los mostrados por la cepa, superproductora de manoproteinas, *S. cerevisiae* Viniferm 3D. Notablemente por debajo, aunque superando las concentraciones de manoproteínas liberadas por la cepa control *S. cerevisiae* Viniferm CT007

y por la cepa *Lachacea thermotolerans* NS-G-32, la cepa *M. pulcherrima* NS-EM-34, mostró una producción dos veces mayor a la de dicha cepa control. Destacó la mayor liberación de aminoácidos mostrada por esta cepa de *M. pulcherrima*, aunque debe mencionarse que esto no supuso en ningún caso un incremento en la concentración de aminas biógenas en los vinos tras los 4 meses de crianza.

En resumen, el presento trabajo aborda la línea de investigación en levaduras no-*Saccharomyces* desde el punto de vista de sus propiedades enzimáticas de interés en enología. Los resultados obtenidos no se limitan al establecimiento de los patrones generales de producción de enzimas en las especies estudiadas, si no que demuestran su incidencia sobre parámetros determinados de la calidad de los vinos, tanto en ensayos a escala de laboratorio como a escala industrial. El conocimiento generado sobre el metabolismo y la fisiología en fermentación de las cepas de la colección establecida en este trabajo, abren una amplia línea de investigación futura en el estudio de las bases genéticas y los factores transcripcionales que determinan un correcto desarrollo de estas cepas de levadura no-*Saccharomyces* facilitando su su uso exitoso como inóculos en la industria enológica.

SUMMARY

Microbiology associated to the spontaneous fermentation process of grape must comprises a complex series of yeast species that stablish a certain population dynamics, basically due to the selection pressure exerted by the increasing ethanol concentration present in the environment. In this context, it is known how *Saccharomyces cerevisiae* gets to dominate the process, even when present at relatively low concentrations in the initial must population, by an ethanol production-accumulation-consumption strategy. This strategy has allowed *S. cerevisiae* to turn an essentially antropic environment such as fermentations into one of its main ecologic niches. Nevertheless, wine fermentation is not a single-species process and, given the crucial role of *S. cerevisiae*, traditional bibliography refers to all the other yeast species involved in wine fermentation as 'non-*Saccharomyces* yeasts'.

Thus, non-*Saccharomyces* yeasts comprise a heterogeneous group of species largely/mainly involved in the first stages of the fermentation process. These can be classified according to their fermentative capacity and, therefore, to the amount of time they could potentially stay active during the fermentation. There are essentially oxidative yeasts (*Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Cryptococcus* spp.), low fermentative yeasts (*Hanseniaspora spp*) and notably fermentative yeasts (*Metschnikowia* spp., *Kluyveromyces* spp., *Lachancea* spp., *Torulaspora* spp. y *Zygosaccharomyces* spp). Their physiology and contribution to wine composition and quality are still poorly studied, although nowadays both scientifics and enologists are aware of their potential use in the improvement of wine quality, both in sensorial and technologic and food-safety aspects.

The current work addressed, in a first phase, the establishment of a wide non-*Saccharomyces* yeast collection isolated from unfermented musts from 3 different Certificates of Origin during 3 harvests. In this collection, formed by a total of 770 isolates, 15 different species were identified, among which the ones belonging to the genera *Hanseniaspora*, *Lachancea* and *Metschnikowia* largely predominated. In the study of the production of enzymes with enologic interest by yeasts from the collection, certain activities such as β-glucosidase or protease activities were largely distributed in all the isolates and, on the contrary, activities such as α-L-arabinofuranosidase, pectinase y celulase were restricted to a low number of species and strains with also low relative representation in the total population.

β-xilosidase, sulfite reductase and β-lyase activities were in an intermediate situation, standing out the latter for being in moderate intensity levels in most of the species that showed this activity. The results of this study allowed us not only to establish general characteristics of each species, but also to observe intraspecific differences that, in addition, varied depending on the origin; thus, we were able to establish clusters of strains depending on the location and year when they were isolated taking into account their enzymatic activity patterns in the corresponding analysis of principal components.

Given the difficulty of studying the 8 above-mentioned enzymatic activities in the 770 isolates collection with the described methods to date, this work needed to either adapt them to a high throughput screening format or develop new methods for activities such as β-liase, which, to date, lack a direct evaluation method other than the valoration of volatile compounds derived from their action on their precursors. Thus, in the present work we describe a differentiating culture medium for yeasts with high β-liase activity based on the use of S-Methil-L-Cysteine as the substrate for this activity, analogue to the cysteinylated substrates present in the must, and the use of the ammonium resulting from its hydrolysis as the only nitrogen source. Knowing that the *IRC7* gene of *S. cerevisiae* is the main responsible for the release of volatile thiols from cysteinylated precursors in must, and the existence of two isoforms of it, one active and the other one inactive, the described culture medium allowed for the differentiation by their growing of those strains that were homozygotic or heterozygotic for the functional copy of the *IRC7* gene. In a similar way, the culture medium allowed us to identify some strains of species such as *Torulaspora delbrueckii* or *Kluyveromyces marxianus* as strains with high β-liase activity, testing in fermentations of white musts from 'verdejo' variety the great ability of the strain *T. delbrueckii* NS-TD to release the two main volatile compounds (3-MH and 4-MMP).

After the phylo-funcional study in which we could establish the enzymatic production profile of the 15 species identified in the collection, we went on to study the incidence of yeasts with polygalacturonase activity on the color extraction process and on the improvement of the red wine clarification process. In this study we could elucidate how *Metschnikowia pulcherrima,* considered as moderately fermentative, could affect more notably the mentioned parameters than the other pectinolytic species, *Aureobasidium pullulans,* mainly oxidative. In addition, from these results we could highlight the importance of low temperatures during

pre-fermentative maceration processes, which not only improve the extraction of certain pigments due to chemical reasons of solubility of the aqueous matrix that is maintained due to the delay in the ethanol appearance, but also favor the development of certain species of non-*Saccharomyces* yeasts that are more cryophilic than *S. cerevisiae* and the delay in the development of the latter.

With the aim of improving the sensorial quality of red wines, we performed a study about the incidence of the strain *Torulaspora delbrueckii* NS-TD by simultaneous and sequential inoculation with *S. cerevisiae*. As a first conclusion, we found that despite the important fermentative role of *T. delbrueckii,* simultaneous co-inoculation with *S. cerevisiae* at the same concentrations notably limits its development. In the case of wines produced by sequential inoculation of *T. delbrueckii* NS-TD followed by *S. cerevisiae,* after a 15 g/L decrease in must density, we noted a high increase in must quality as compared to the ones only fermented by *S. cerevisiae.* This improvement was related to a notable increase in the mannoprotein content of these wines, as well as to a slight decrease in their acidity and higher alcohols concentration.

Finally, this work addressed the application of non-conventional yeast strains in wine ageing over-lees for the improvement of sensorial properties of red wines, analyzing the release of mannoproteins as the main factor during the process. The results showed that, again, the strain *T. delbrueckii* NS-TD was able to release very high concentrations of mannoproteins also during the ageing over-lees, reaching levels slightly higher than the mannoprotein-overproducer strain *S. cerevisiae* Viniferm NS-TD. Considerably below but also over the concentration of mannoproteins released by the control strain *S. cerevisiae* Viniferm CT007 and by the strain *Lachancea thermotolerans* NS-G-32, the strain *M. pulcherrima* NS-EM-34 showed a two-fold production compared to the control strain. The higher amino acids release showed by this *M. pulcherrima* strain must be highlighted, although we it should be mentioned that this did not correlate with an increase in the concentration of biogenic amines in wine after 4 months ageing.

In summary, this work address the non-*Saccharomyces* topic in enology industry from an original enzymatic point of view. These results not only establish the enzymatic properties of some yeast species of enological interest, but also proving their incidence on wine quality in both laboratory and industrial scale. This metabolic and physiological knowledge allow us to foresee a large number of future works on the study of the genetic basis and the transcriptional factors that determine the usefulness and the commercial success of non-*Saccharomyces* yeasts in wine industry.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Microbiología, enología e industria enológica

La enología, como ciencia pluridisciplinar, emplea conocimientos derivados de la química, la microbiología y la tecnología. Los avances en investigación permiten hoy en día, mediante su interacción con la industria, anticiparse a las demandas del consumidor desarrollando herramientas tecnológicas, bioquímicas y biológicas que marcarán el futuro de la enología (Belda *et al*., 2015a).

Desde sus inicios, la microbiología enológica ha suscitado el interés de los enólogos sobre el proceso fermentativo y los determinantes microbiológicos que condicionan de la calidad final de los vinos que producen. En este campo, los trabajos iniciados a principios del siglo XIX por el químico y enólogo Müller-Thurgau comenzaron a desarrollar las herramientas microbiológicas básicas para el control tecnológico y sensorial del proceso de fermentación mediante el uso de fermentaciones dirigidas inoculadas con cepas seleccionadas de *Saccharomyces cerevisiae* (Pretorius, 2000). Sin embargo, la naturaleza inherente al proceso de fermentación vínica no se restringe únicamente a la presencia de dicha especie, si no que surge de la participación secuencial de una gran número de especies de levaduras y bacterias, y de su interacción con las propiedades varietales de la uva (Fleet, 1990). Este consorcio microbiano, como fruto de sus propiedades metabólicas, será responsable de gran parte de la calidad sensorial de los vinos (Liu *et al*., 2015), que es en ultima instancia el objeto de esta ciencia hedonista. La difícil elección entre la complejidad sensorial asociada a las fermentaciones espontáneas y la seguridad industrial de las fermentaciones dirigidas ha llevado en las últimas décadas al desarrollo de una intensa línea de investigación para el completo entendimiento de la microbiología de ambos procesos para aunar las virtudes de ambas tendencias, incrementando de forma paralela la calidad organoléptica y la seguridad higiénica e industrial de los vinos.

Si bien las buenas prácticas vitícolas son determinantes para la llegada a la bodega de una materia prima de calidad, es labor del enólogo la elección de las herramientas adecuadas para el desarrollo de un proceso de fermentación que permita la revelación de las propiedades varietales de cada uva. Para ello es fundamental la comprensión de la fisiología en fermentación de las distintas cepas de levaduras disponibles hoy en el mercado, así como el desarrollo de nuevas cepas que amplíen el espectro de aplicaciones de las levaduras a lo largo del proceso de elaboración de vino (Pretorius, 2000; Cordente *et al*., 2012; Pretorius *et al*., 2012; Belda *et al*., 2016a).

1.2. Las levaduras en enología

Las uvas constituyen uno de los principales reservorios naturales de levaduras, entre las que se establecen comunidades microbianas muy heterogéneas (Liu *et al*., 2015). Hasta 93 especies de levadura de 30 géneros distintos han sido descritos en la literatura, considerando los resultados de estudios que copan 49 variedades de uva distintas procedentes de 22 países (Barata *et al*., 2008; 2012; Bisson y Joseph, 2009). Renouf *et al*. (2007) pudo identificar, en un solo estudio y mediante técnicas moleculares clásicas de PCR-DGGE, un total de 47 especies de levadura pertenecientes a 22 géneros a partir de la superficie de uvas de 6 variedades diferentes *Aureobasidium, Auriculibuller, Brettanomyces, Bulleromyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenkia, Kluyveromyces, Lipomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Sporidiobolus, Sporobolomyces, Torulaspora, Yarrowia, Zygoascus* y *Zygosaccharomyces*. Al margen de esta gran diversidad microbiana, la densidad poblacional en la superficie de las uvas es baja en las primeras etapas de maduración de la uva (10^1-10^3 UFC/g), incrementándose en varios órdenes de magnitud (10^3-10^6 UFC/g) hasta el momento de la vendimia (Jolly *et al*., 2003; Prakitchaiwattana *et al*., 2004; Renouf *et al*., 2005; Setati *et al*., 2012). La disponibilidad de nutrientes en el hollejo, las condiciones climáticas, la variedad de uva y su estado fitosanitario van a determinar la distribución y el balance en la población de levaduras asociadas, aunque la influencia de cada uno de estos factores sobre dicha población no está carente de controversia (Liu *et al*., 2015).

La población de levaduras en la superficie de la uva comienza a establecerse durante la etapa de maduración de las uvas en la que su superficie aumenta conforme aumenta también la disponibilidad de nutrientes y decrece la acidez (Combina *et al*., 2005; Cadez *et al*., 2010). Las características fisiológicas y bioquímicas de cada variedad de uva pueden contribuir a determinar la población de levaduras que se establezca en su superficie (Guerzoni y Marchetti, 1987; De La Torre *et al*., 1999; Sabate *et al*., 2002; Renouf *et al*., 2005; Nisiotou *et al*., 2007). También las condiciones climáticas, como el grado de humedad generado por la pluviometría de cada año, muestra en la mayoría de estudios una relación directa con el incremento de la población de hongos y levaduras sobre las uvas (Longo *et al*., 1991; De la Torre *et al*., 1999; Combina *et al*., 2005; Cadez *et al*., 2010). Sin embargo, a pesar de esta mayor proliferación fúngica, no se ha observado relación entre las condiciones climáticas y la diversidad de levaduras en un viñedo (Barata *et al*., 2012). Los tratamientos vitícolas, según sean orgánicos, convencionales respetuosos o altamente agresivos, van a determinar también la heterogeneidad de levaduras en un viñedo (Comitini y Ciani, 2008; Cadez *et al*., 2010; Cordero-Bueso *et al*., 2011; Schmid *et al*., 2011; Tofalo *et al*., 2011; Tello *et al*., 2012; Milanovic *et al*., 2013; Martins *et al*., 2014; Belda *et al*., 2016a). A este respecto, cabe destacar la influencia del estado fitosanitario de las uvas en la población de levaduras en su superficie. Por ejemplo, la presencia de *Botrytis cinerea* en las uvas afecta la composición nutricional de las uvas y su superficie, por lo que se verá afectada la población de levaduras en ella (Nisiotou y Nychas, 2007; Barata *et al*., 2008). A su vez, la capacidad de antibiosis de ciertas especies del género *Metschnikowia* por la liberación de ácido pulquerrimínico genera un efecto inhibitorio del desarrollo de otras levaduras y hongos que hace que estén particularmente presentes en uvas afectadas por ese tipo de infecciones fúngicas (Sipikzci, 2006). En este sentido, cabe mencionar la producción de toxinas *killer* por parte de ciertas levaduras sobre la propia ecología microbiana asociada al proceso fermentativo, así como su uso en biocontrol (Marquina *et al*., 2002; Alonso *et al*., 2015; Velazquez *et al*., 2015).

Los estudios metagenómicos llevados a cabo por Bokulich *et al.* (2013, 2014) permiten concluir que la microbiota asociada a un viñedo no es aleatoria, si no que responde a unos patrones marcados por la situación geográfica, la variedad de uva y los factores climáticos.

Si bien la población de levaduras que se establezca en la superficie de las uvas formará parte de la "materia prima" para el proceso de fermentación, las condiciones y prácticas enológicas en bodega, antes, durante y después del proceso de fermentación, determinarán la evolución de la microbiota asociada a ella. El proceso de fermentación espontánea de un mosto de uva está definido por la sustitución secuencial de una serie de especies de levaduras en función de la presión selectiva que ejerce la creciente concentración de etanol en el medio (Figura 1). Así, en su mayor parte, y en especial en sus últimas etapas, el proceso está dominado por cepas de la especie *Saccharomyces cerevisiae,* muy tolerantes al etanol. La estrategia de "producción-acumulación-consumo" de etanol, característica de las especies del género *Saccharomyces* (y compartida con el género *Dekkera*), confiere la ventaja competitiva responsable de su dominancia en el proceso (Marsit y Dequin, 2015). Esto es debido al rápido consumo de los azúcares del mosto, su conversión en etanol que actúa como inhibidor del crecimiento del resto de especies y el posterior consumo del cierta cantidad del etanol acumulado tras la dominancia del nicho ecológico (Thomson *et al.*, 2005; Piškur *et al.*, 2006; Rozpędowska *et al.*, 2011; Dashko *et al.*, 2014). Así, la presencia y prevalencia de *S. cerevisiae* en el proceso es un hecho esperado y deseado para la correcta llegada a término del proceso de fermentación (Jolly *et al.*, 2014).

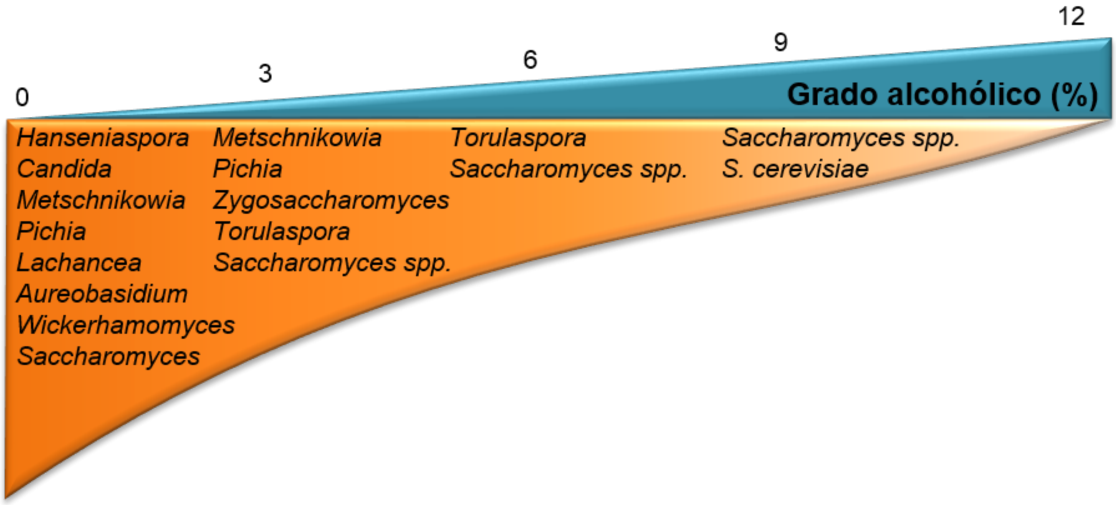

Figura 1. Esquema representativo de la evolución de la población de levaduras a lo largo del proceso de fermentación espontánea de un mosto de uva. Se aprecia la dominancia de *Saccharomyces cerevisiae* en las etapas finales del proceso, conforme aumenta la concentración de etanol en el medio.

Sin embargo, la coexistencia de *S. cerevisiae* con el resto de especies presentes en el mosto de uva y en las primeras etapas de la fermentación parece ser relevante para la complejidad y calidad final en los vinos. Dado el papel crucial de *S. cerevisiae* en el proceso, la bibliografía tradicional ha denominado al resto de especies de levadura involucradas en el proceso como levaduras "no-*Saccharomyces*". La presencia de ciertas especies de levaduras no-*Saccharomyces* en las etapas iniciales e intermedias de la fermentación vínica contribuye, a través de su fisiología y su interacción con *S. cerevisiae,* a modular las propiedades sensoriales de los vinos, dotándolos de una mayor complejidad (Zironi *et al*., 1993; Gil *et al*., 1996; Lema *et al*., 1996; Toro y Vázquez, 2002; Ciani *et al*., 2006; Viana *et al*., 2008). Al margen de la carga y diversidad microbiana asociada a la uva desde el viñedo, existen diferentes prácticas que favorecen el desarrollo de las levaduras no-*Saccharomyces* durante las primeras etapas fermentativas, retardando en cierta manera el desarrollo de *S. cerevisiae* y el consiguiente inicio de la fermentación tumultuosa que acabará con la mayor parte de las levaduras no-*Saccharomyces*. Una adición moderada de SO_2, muy efectivo frente a la mayoría de especies no-*Saccharomyces*, o el desarrollo de etapas de maceración prefermentativa pueden favorecer el desarrollo de las poblaciones de levaduras no-*Saccharomyces* en el mosto, obteniendo una mayor incidencia de sus beneficios. Sin embargo, el desconocimiento *a priori* de la microbiota asociada a esas uvas puede provocar la aparición de desviaciones sensoriales inesperadas. Fleet (2008) comenzó a sentar las bases de lo que hoy se conoce como fermentaciones combinadas, secuenciales o *multistarter* controladas y su aplicación en la industria mediante la producción de inóculos a gran escala de levaduras no-*Saccharomyces*. Las primeras aplicaciones de las levaduras no-*Saccharomyces* para la mejora de las características organolépticas de los vinos se produjeron a mediados del siglo XX, cuando Cantarelli (1955) y Castelli (1955) realizaron trabajos de reducción del contenido en ácido acético de los vinos haciendo uso de cepas seleccionadas de *Torulaspora delbrueckii*. Sin embargo, la industria siguió relacionando este grupo heterogéneo de levaduras no-*Saccharomyces* con ciertas desviaciones sensoriales, paradas de fermentación, incremento de la acidez volátil de los vinos o la aparición de especies perjudiciales para el vino como *B. bruxellensis*. Los progresivos trabajos de caracterización fisiológica de las distintas levaduras implicadas en el proceso, ha permitido vislumbrar aquellas que parecen susceptibles de aplicación en la industria para la mejora de la calidad de los vinos en aspectos de mejora sensorial (Egli *et al*. 1998; Esteve-Zarzoso *et al*. 1998; Fleet y Heard 1993; Gil *et al*. 1996; Henick-Kling *et al*. 1998; Lambrechts yPretorius 2000; Fleet 2003, 2008; Romano *et al*. 2003; Viana *et al*. 2008; Belda *et al*., 2015b; Benito *et al*., 2015), tecnológica (Belda *et al*., 2016b) e incluso de la seguridad nutricional de los mostos y vinos (Oro *et al*., 2014; Alonso *et al*., 2015).

1.2.1. *Saccharomyces cerevisiae*

Como levadura responsable de la mayor parte del proceso de fermentación vínica *S. cerevisiae* ha copado el interés de microbiólogos y enólogos en el estudio de sus propiedades metabólicas que determinan gran parte de las características finales de los vinos. *S. cerevisiae* ha acompañado a la humanidad a lo largo de gran parte de su desarrollo, sufriendo, a través de su uso en fermentaciones, un proceso de "domesticación" (Liti *et al.*, 2009). Se han establecido dos eventos de domesticación, uno asociado a la elaboración del Sake y el otro asociado al proceso de vinificación (Fay y Benavides, 2005). Desde el descubrimiento de las levaduras como responsables del proceso de fermentación (Pasteur, 1860) una gran cantidad de cepas de *S. cerevisiae* han sido aisladas, la mayoría de ellas asociadas a ambientes fermentativos, y estudiadas a nivel genómico y metabólico. Sin embargo, a pesar de lo antiguo de este proceso de domesticación, determinado por las prácticas fermentativas, no fue hasta finales del siglo XIX cuando dos circunstancias determinaron el futuro del uso industrial de *S. cerevisiae*. A principios de la década de 1880, Emile Christian Hansen, en el *Carlsberg laboratory* (Dinamarca), desarrolló el primer inóculo axénico que fue usado en fermentaciones experimentales pocos años después. En 1890, Müller-Thurgau realizó la primera inoculación de un mosto de uva con un cultivo puro de levadura, aunque este proceso no fue adecuadamente desarrollado en la industria hasta la década de los 70 del siglo XX. Desde ese momento se incrementaron los procesos de selección de cepas de levaduras con propiedades enológicas óptimas para el desarrollo de procesos de fermentación controlados (Marsit y Dequin, 2015). La disponibilidad en un mercado global de un número limitado de cepas de *S. cerevisiae* provoca que los grandes esfuerzos de las bodegas en su viticultura por obtener una vendimia de calidad e identificativa de su *terroir*, queden atenuados o mermados durante la posterior fermentación por el uso de cepas comerciales de levaduras aisladas de orígenes distintos al propio entorno de la bodega y comunes para bodegas diferentes. A este respecto surgen los procesos de selección de levaduras autóctonas adaptadas al entorno vitivinícola del cual han sido aisladas y donde posteriormente serán utilizadas como inóculos (Lopes *et al.*, 2002; Capece *et al.*, 2010), incrementando la tipicidad.

El complejo metabolismo de las cepas industriales de *S. cerevisiae*, debido a su complejo genoma, deriva en la existencia de una gran diversidad intraespecífica, que permite dotar al enólogo de un extenso catálogo de cepas de *S. cerevisiae* comerciales para la elaboración de sus vinos. Las propiedades óptimas y deseables de una cepa de *S. cerevisiae* para su uso como inóculo en enología son bien conocidas (Figura 2). Estas propiedades pueden ser diferenciadas y agrupadas en: propiedades fermentativas, propiedades sensoriales y propiedades tecnológicas.

Figura 2. Resumen de propiedades tecnológicas y sensoriales deseables en cepas industriales de levaduras *Saccharomyces cerevisiae*.

Como se ha comentado anteriormente, las propiedades fermentativas óptimas parecen ser exclusivas de ciertas especies del género *Saccharomyces*. Sin embargo, parece claro que la diversidad de cepas de *S. cerevisiae* no cubre todas las demandas exigidas por los enólogos y no permite la revelación completa del potencial sensorial de las distintas variedades de uva. Por el contrario, las posibilidades que brinda la enorme diversidad de especies de levaduras asociadas a las primeras etapas del proceso fermentativo permitirán suplir las carencias que muestran la inmensa mayoría de cepas de *S. cerevisiae* (Jolly *et al*., 2014). Estas levaduras, conocidas en su conjunto como levaduras no-*Saccharomyces*, constituyen en la actualidad uno de los focos de interés en I+D para el desarrollo y comercialización de nuevas cepas de levaduras con propiedades innovadoras (Tabla 1).

1.2.2. Levaduras no-*Saccharomyces*

La taxonomía actual reconoce un total de 149 géneros de levaduras que comprenden unas 1500 especies (Kurtzman *et al*., 2011), de las cuales más de 90 han podido ser aisladas de mostos de uva (Liu *et al*., 2015).

La prevalencia de las diferentes especies no-*Saccharomyces* durante el proceso de fermentación vínica depende de su vigor fermentativo y de su resistencia al etanol, más que de la concentración celular inicial que presenten en la uva. Así, al igual que ocurre con *S. cerevisiae,* levaduras que se encuentran generalmente en bajas concentraciones en la uva pero que muestran un cierto poder fermentativo, como es el caso de *T. delbrueckii*, y algunas especies de los géneros *Kluyveromyces* y *Lachancea*, perduran en la fermentación hasta las etapas más tardías. En función de su poder fermentativo, las levaduras no-*Saccharomyces* pueden dividirse en 3 grupos: levaduras estrictamente aerobias (*Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Cryptococcus* spp.), levaduras apiculadas con bajo poder fermentativo (*Hanseniaspora uvarum* (y su anamorfo *Kloeckera apiculata*) y *Hanseniaspora guilliermondii*) y levaduras con metabolismo fermentativo (*Metschnikowia pulcherrima, Kluyveromyces marxianus, Torulaspora delbrueckii* y *Zygosaccharomyces bailii*) (Jolly *et al*., 2014). En base a esto, las levaduras de este último grupo parecen ser las de mayor potencial para su uso en cultivos combinados con *S. cerevisiae,* puesto que serán las únicas capaces de desarrollar de forma suficiente su metabolismo para que pueda tener una incidencia en la calidad del vino (Ciani *et al*., 2010).

1.2.2.1. Aplicaciones actuales

- Grado alcohólico. Uno de los objetivos de mayor interés en la enología actual, y que está mostrando resultados muy prometedores, es la disminución del contenido en etanol de los vinos (Contreras *et al*., 2014; Quirós *et al*., 2014). Este objetivo se aborda mediante el aprovechamiento del menor rendimiento alcohólico de ciertas especies no-*Saccharomyces.* Dichas levaduras presentan un metabolismo, cuya regulación incluso en condiciones de alta presión osmótica, les permite consumir parte de los azúcares de forma oxidativa generando una cantidad de etanol menor. El uso de *M. pulcherrima* en inoculación secuencial *con S. cerevisiae* ha permitido lograr, en condiciones controladas y en ausencia de desviaciones sensoriales asociadas al metabolismo respiratorio (excesiva liberación de ác. acético) la disminución de hasta 4% (v/v) de etanol en los vinos (Morales *et al*., 2015). Unido al distinto uso de los azúcares en fermentación, el incremento de la producción de glicerol en los vinos es una de las virtudes mas destacadas de las fermentaciones espontáneas y corroboradas en el desarrollo de fermentaciones secuenciales con levaduras no-*Saccharomyces*. Diversas especies levaduras no-*Saccharomyces* en fermentación contribuyen a un incremento significativo en el contenido en glicerol en los vinos, sin embargo los resultados mas prometedores a este respecto se han encontrado en el uso de cepas de *L. thermotolerans* y *Candida zemplinina* (Ciani y Ferraro, 1998; Soden *et al*., 2000; Comitini *et al*., 2011).

- Acidez de mostos y vinos. En el estudio del metabolismo de las levaduras no-*Saccharomyces*, su incidencia sobre la acidez de los vinos ofrece herramientas útiles para su control. Ha sido ampliamente demostrada la incidencia de *T. delbrueckii* sobre la acidez volátil de los vinos logrando una reducción significativa (Moreno *et al*. 1991; Bely *et al*. 2008; Renault *et al*. 2009; Belda *et al*., 2015b). También, las desviaciones en la acidez total inicial de los mostos pueden ser corregidas mediante la disminución de la acidez málica o el incremento de la liberación de ácido láctico durante la fermentación. Benito *et al*. (2013) demostró la capacidad de cepas seleccionadas de *SchizoSaccharomyces pombe* para la degradación completa del ácido málico del vino. *Zygosaccharomyces bailii* y *T. delbrueckii* presentan también cierta capacidad de degradación del ácido málico en fermentación (Romano y Suzzi, 1993; Belda *et al*., 2015b). En cuanto al incremento de la acidez de los vinos, el uso de *L. thermotolerans* ha permitido una mejora muy significativa del perfil sensorial de los vinos mediante la liberación de ácido láctico (Comitini *et al*., 2011; Gobbi *et al*., 2013).

-Perfil aromático. El principal objetivo del uso de levaduras no-*Saccharomyces* es el incremento del carácter varietal y la intensidad aromática de ciertas variedades de uva debido a su potencial enzimático. Si bien las propiedades enzimáticas de distintas especies no-*Saccharomyces* han sido estudiadas desde hace mucho tiempo, en especial las glicosidasas por su efecto sobre el perfil varietal de vinos blancos (Rosi *et al*., 1994; Manzanares *et al*., 1999; McMahon *et al*., 1999; Mendes-Ferreira *et al*., 2001; Mateo *et al*., 2011), la confirmación de su repercusión sobre las propiedades del vino es una tarea difícil y que requiere mayor estudio (Belda *et al*., 2015c). A este respecto, la búsqueda de levaduras con un determinado perfil enzimático ha permitido identificar nuevas aplicaciones de las levaduras no-*Saccharomyces* para la mejora de otras propiedades aromáticas, como el incremento del carácter tiólico de ciertos vinos blancos por actividades β-liasas alternativas (Belda *et al.*, 2016c),

Tabla 1. Resumen de las propiedades enológicas a destacar de levaduras no-*Saccharomyces*. Contribuciones positivas y negativas a la composición y calidad de los vinos.

Especie	**Propiedades enológicas positivas**	**Efectos indeseables**	**Referencias**
Torulaspora delbrueckii	Disminución del contenido en ácido acético. Incremento en la liberación de manoproteínas. Incremento del carácter varietal (liberación de aromas tiólicos y terpénicos). Disminución de astringencia (consumo de ácido málico).	Cinética fermentativa más lenta. Liberación de H_2S.	Castelli, 1955; King & Dickson, 2000; Renault *et al.*, 2009; Azzolini *et al.*, 2012, 2015; Taillander *et al.*, 2014; Belda *et al.*, 2015b, 2016d; Velazquez *et al.*, 2016)
Metschinkowia pulcherrima	Intensa liberación de ésteres aromáticos (fruta blanca). Incremento del carácter varietal (liberación de aromas tiólicos y terpénicos). Incremento en la liberación de manoproteínas. Elevada actividad pectinolítica (extracción de color y proceso de clarificación). Reducción del grado alcohólico de los vinos.	Retrasos o paradas de fermentación por acción antimicrobiana.	Bisson & Kunkee, 1991; Pallman *et al.*, 2001; Clemente-Jimenez *et al.*, 2004b; Rodriguez *et al.*, 2010; Sadoudi *et al.*, 2012; Oro *et al.*, 2014; Contreras *et al.*, 2015; Belda *et al.*, 2016b
Lachancea thermotolerans	Liberación de ácido láctico. Producción elevada de glicerol. Liberación de 2-feniletanol.	Obtención de vinos con atributos "ácidos" o "picantes".	Kapsopoulou *et al.*, 2007; Comitini *et al.*, 2011; Gobbi *et al.*, 2013; Benito *et al.*, 2016)
Candida zemplinina	Alta producción de glicerol. Consumo preferencial de fructosa. ↑ liberación de terpenos. ↓ producción de alcoholes superiores y aldehidos.	Efecto poco predecible (cepa-dependiente) en el perfil aromático	Ciani & Maccarelli, 1998; Soden *et al.*, 2000; Andorra *et al.*, 2010; Di Maio *et al.*, 2012; Sadoudi *et al.*, 2012
Hanseniaspora vineae	Liberación de 2-feniletil acetato. Liberación de terpenos (alta actividad β-glucosidasa).	Elevada acidez volatil Producción de H_2S Formación de aminas biógenas Retraso en el inicio de la fermentación	Caruso *et al.*, 2002; Rojas *et al.*, 2003; Viana *et al.*, 2009; De Benedictis *et al.*, 2011; Belda *et al.*, 2016b; Martin *et al.*, 2016; Lleixá *et al.*, 2016
Zygosaccharomyces bailii	↓ producción de H_2S Y SO_2. Degradación de ácido málico. Liberación de polisacáridos. Consumo preferencial de fructosa. Elevado vigor fermentativo (reactivación de paradas de fermentación).	Elevada producción de ácido acético	Romano & Suzzi, 1993; Domizio *et al.*, 2011, Loureiro & Malfeito-Ferreira., 2003; Sütterlin, 2010
Schizosaccharomyces pombe	Degradación de ácido málico y ácido glucónico	Alta producción de acetaldehido, propanol y 2,3-butanediol	Peinado *et al.*, 2004; Benito *et al.*, 2013, 2015

Finalmente, de la coinoculación de levaduras no-*Saccharomyces* junto con *S. cerevisiae* ha mostrado una repercusión muy significativo sobre el perfil de aromas fermentativos en los vinos, mediante, por ejemplo, una reducción del contenido en alcoholes superiores que en ocasiones tienen a monopolizar el aroma de los vinos en detrimento de su complejidad sensorial (Belda *et al.*, 2015b; Benito *et al.*, 2016). Estudios recientes revelan que el uso de levaduras no-*Saccharomyces* en fermentaciones combinadas con *S. cerevisiae* no sólo modula el perfil aromático de los vinos por el propio metabolismo de las primeras, si no que su mera coexistencia provoca variaciones muy significativas en el patrón de expresión génica, y por tanto en el metabolismo, de la cepa de *S. cerevisiae* que lleva a cabo la mayoría del proceso fermentativo (Barbosa *et al.*, 2015). Así, los verdaderos mecanismos que determinan la variación en el perfil aromático de los vinos en fermentaciones mixtas han de ser estudiado en profundidad en un futuro próximo.

-Liberación de manoproteínas. Recientemente la liberación de manoproteínas por ciertas especies no-*Saccharomyces* ha sido identificada como una característica con gran repercusión en la calidad de los vinos. Ello es debido a que ciertas levaduras presentan en su pared una mayor cantidad de manoproteínas y a que dichas levaduras sufren un proceso de lisado previo a la finalización de la fermentación vínica (Domizio *et al.*, 2014). Este hecho es especialmente acusado en cepas seleccionadas de *T. delbrueckii*, posiblemente por el gran desarrollo y prevalencia que esta levadura alcanza en fermentación (Belda *et al.*, 2015b) aunque, recientemente, se han demostrado los beneficios de su aplicación durante la crianza sobre lías para modificar el perfil sensorial de los vinos (Belda *et al.*, 2016d).

-Control biológico y seguridad alimentaria. Por último, el control biológico es una de las aplicaciones mas prometedoras de las levaduras no-*Saccharomyces*, aunque todavía en fases muy preliminares. La producción de toxinas *killer* en especies como *Pichia membranifaciens, Ustilago maydis* y ciertas especies del género *Kluyveromyces* y de otras moléculas que generan antibiosis, como el ácido pulquerrimímico de *M. pulcherrima,* han sido utilizados en el control biológico en pre- y post-cosecha de especies deteriorantes como *B. bruxellensis* (Comitini *et al.*, 2004; Santos *et al.*, 2009, 2011; Mehlomakulu *et al.*, 2014; Oro *et al.*, 2014) o en el control de la contaminación de mostos concentrados por parte de especies osmófilas (Alonso *et al.*, 2015) u hongos fitopatógenos como *Botrytis cinerea* (Santos *et al.*, 2004). Asimismo, en seguridad alimentaria el uso de inóculos de levaduras activas o inactivas para la adsorción de ocratoxina A está siendo estudiada para paliar los problemas de salud asociados a su consumo (Petruzzi *et al*., 2014).

1.2.2.2. Perspectivas de investigación y aplicaciones futuras

El desarrollo de las tecnologías ómicas, en especial de la metagenómica han puesto de manifiesto la verdadera diversidad microbiana asociada a cada una de las etapas del proceso de vinificación, desde la viña hasta la crianza de los vinos (Bokulich *et al*., 2014, Liu *et al*., 2015). Complementariamente, la metabolómica ha permitido identificar los determinantes químicos los vinos de distintas variedades de uva y provenientes de regiones vitivinícolas diferentes, así como la influencia con que las distintas levaduras implicadas en el proceso de vinificación contribuyen a la composición final del vino (Hong 2011; Alañón *et al*., 2015). De esta forma, comienzan a establecerse las bases analíticas de la repercusión que las distintas técnicas agronómicas y enológicas muestran sobre el perfil sensorial de los vinos y, que desde hace décadas se vienen observando de forma empírica. Siguiendo con la tendencia de tecnologías de alto rendimiento, uno de los objetivos abordados en esta tesis doctoral ha sido el desarrollo y adaptación de los métodos y procesos de selección de levaduras no-*Saccharomyces* en base a sus propiedades enzimáticas (Belda *et al*., 2016a, 2016c). Los datos genómicos disponibles hasta el momento para la mayoría de especies de levadura no-*Saccharomyces* dificulta el establecimiento de las bases genéticas su metabolismo, así como su respuesta transcripcional en distintas condiciones enológicas. Los numerosísimos estudios de regulación génica llevados a cabo en *S. cerevisiae* durante las últimas décadas de *S. cerevisiae* hasta la actualidad han permitido un profundo conocimiento sobre los parámetros importantes en la fermentación para el control y la optimización al máximo de las distintas posibilidades que *S. cerevisiae* ofrece a nivel metabólico. De esta forma la tarea a realizar en un futuro próximo consistirá en estudiar la estructura y función génicas en las especies no-*Saccharomyces* para las que existen ya inóculos comerciales (*T. delbrueckii, L. thermotolerans, M. pulcherrima* y *P. kluyvery*, fundamentalmente) así como sus mecanismos de regulación para controlar y optimizar su uso en bodega. Este será el trabajo que garantizará el establecimiento real de los no-*Saccharomyces* en la industria, ofreciendo al enólogo una opción cada vez mas real en su uso como herramientas para el control y modulación del perfil sensorial de los vinos.

1.3. Microbiología y elaboración de vino

El enólogo tras el delicado proceso de vendimiado de las uvas y su transporte hasta la bodega hace uso de su conocimiento y del conjunto de técnicas físicas, químicas y biológicas para obtener el mejor resultado, en forma de vino, tras el proceso de fermentación. Centrándonos en el proceso microbiológico de fermentación, se puede dividir el conjunto de las operaciones de vinificación en 3 grandes etapas. Así, flanqueando al proceso fermentativo, existirá una etapa prefermentativa y una postfermentativa (Figura 3). Ambas presentan una duración variable y el conjunto de técnicas que se pueden aplicar en ambas etapas es también muy amplio y determinará junto con el proceso fermentativo, las propiedades finales de los vinos.

1.3.1. Etapa prefermentativa.

Comprende desde la llegada de la uva a la bodega hasta el inicio de la fermentación alcohólica. Durante esta etapa se corrigen posibles defectos que traiga la uva consigo o para la mejora de las propiedades del mosto de partida para la vinificación.

En zonas cálidas es necesaria la acidificación de los mostos para el mantenimiento de ciertas características de frescura y longevidad deseadas por los consumidores, especialmente en vinos blancos. El método más habitual es la adición de ácido tartárico. Por el contrario, en climas fríos, cuando las uvas no alcanzan una madurez completa su acidez puede ser elevada y se lleva a cabo una desacidificación, bien química o biológica. La adición de agentes antimicrobianos como el SO_2, que ayuden a estabilizar la microbiota indígena que trae consigo la uva, es necesario para el disminuir el riesgo de desviaciones microbianas a lo largo del proceso de elaboración y crianza.

En la elaboración de vinos tintos, se procede al estrujado y despalillado de la uva previa a su introducción en los depósitos donde se llevará a cabo la fermentación. Una operación prefermentativa opcional es la técnica de maceración preferemntativa en frío (MPF) que consiste en el encubado de la uva entera a baja tempertura (10-12ºC) durante un intervalo de tiempo variable (4-7 días) a fin de incrementar el potencial aromático y colorante de los vinos. En vinos blancos las operciones prefermentativas son el despalillado, prensado de la uva, para la extracción de mosto y desfangado o clarificación de los mismos, realizándose la fermentación en ausencia de hollejos y otras partes sólidas de la uva. El desfangado consiste en las eliminación de sustancias pécticas del mosto, localizadas en las paredes de las células del hollejo. El mosto de uva contiene enzimas pectinolíticas, (pectín metil esterasa y poligalacturonasa) a la degradación de las pectinas que logrando un descenso en la viscosidad de los mostos.

Al margen de estas actividades endógenas, es frecuente el uso de preparados enzimáticos comerciales en enología para abordar distintos procesos de mejora tecnológica y sensorial de los vinos. En primer lugar, la adición de enzimas pectinolíticas (poligalacturonasa, pectín metil esterasa y pectín liasa) acelera el proceso de clarificación de mostos evitando los riesgos de iniciar la fermentación sobre mosto no clarificado. En el caso de los vinos tintos, estos preparados enzimáticos se emplean durante el proceso de maceración para la mejora de la extracción de compuestos fenólicos al mosto que contribuyan al color de los vinos así como a su estructura.

1.3.2. Fermentación alcohólica

La fermentación alcohólica del vino consiste en la transformación de los azúcares presentes en el mosto en alcohol etílico y CO_2. Sin embargo, el proceso de fermentación de un vino es un complejo proceso bioquímico en el que diversas rutas metabólicas de los microorganismos implicados en ella y diversos procesos físico-químicos determinan las propiedades finales del vino. En el caso de los vinos blancos, la fermentación se lleva a cabo sobre un mosto clarificado en ausencia de hollejos y otras partes sólidas de la uva. En elaboración de vino tinto, la fermentación transcurre a la vez que la maceración, es decir se fermenta en contacto con los hollejos, y pepitas.

Aunque sigue habiendo vinificaciones particulares mediadas por la microbiota espontanea de la uva, lo habitual es la fermentación controlada mediante la adición de un inóculo seleccionado de levadura *S. cerevisiae* (Pretorius, 2000). Actualmente el elaborador dispone de cientos de cepas para elegir en función de las características del vino buscado. En los últimos cinco años, se están trasladando a la industria el empelo de inóculos secuenciales de una levadura no-*Saccharomyces* seleccionada seguida de *S. cerevisiae* para la finalización del proceso fermentativo (Fleet, 2008; Jolly *et al*., 2014).

Como ya se dijo anteriormente, en el proceso de fermentación espontánea de un mosto de uva se suceden una serie de especies de levaduras siendo *S. cerevisiae* la encargada de llevar a término el proceso. Sin embargo, al contrario que en las fermentaciones en las que se adiciona un cultivo seleccionado de *S. cerevisiae*, su desarrollo y dominancia en el proceso espontáneo es más lento. Esta es una de las explicaciones de las virtudes de las fermentaciones espontáneas, que gozan de una etapa prefermentativa más extensa que aquellas inoculadas con *S. cerevisiae* por lo que la flora de levaduras no-*Saccharomyces* salvajes presentes en el mosto puede desarrollarse en mayor medida (Constantini *et al*., 1998; Egli *et al*., 1998).

Centrándonos en el desarrollo de fermentaciones controladas, la inoculación de *S. cerevisiae* en concentraciones en torno a los 10^6 células/mL garantiza en la mayoría de casos la implantación de esta cepa sobre la flora autóctona de la uva, iniciándose rápidamente el proceso de fermentación de los azúcares del mosto y produciéndose un vino de características relativamente predichas en función del mosto de partida y la cepa de levadura de elección (Pretorius, 2000). Por el contrario, el uso de procesos complejos de fermentación secuencial cuentan con dos momentos de inóculo cuyo objetivo es sacar el mayor fruto de una primera etapa de menor rendimiento fermentativo llevada a cabo por una cepa no-*Saccharomyces* y una segunda etapa de fermentación alcohólica clásica llevada a cabo por *S. cerevisiae*. Así, de alguna forma se pueden obtener los beneficios de las fermentaciones espontáneas sin asumir el riesgo que ellas implican (Fleet, 2008). El intenso conocimiento sobre la fisiología y la regulación génica de *S. cerevisiae* ha llevado en la actualidad a la existencia de un enorme catálogo de cepas comerciales que el enólogo puede emplear en función de sus intenciones. En inicio, el uso de las levaduras no-*Saccharomyces* fue meramente empírico (Cantarelli, 1955), sin embargo, los intensos trabajos de investigación llevados a cabo hoy en día permiten un uso dirigido de las distintas especies con fines de mejora de la calidad de los vinos ofreciendo productos innovadores al consumidor (Belda *et al*., 2015a). La fermentación alcohólica se considera finalizada cuando se han agotado los azúcares existentes en el medio, de forma que progresivamente las levaduras quedan depositadas en el fondo, deteniéndose su metabolismo. En el caso de los vinos tintos, tras la fermentación alcohólica es frecuente el desarrollo de una fermentación maloláctica. Este proceso, llevado a cabo por bacterias de la especie *Oenococcus oeni* tiene como objetivo la conversión del ácido málico del vino en ácido láctico, disminuyendo la sensación de acidez y ayudando a la estabilidad microbiología del vino, pues el ácido láctico no es degradado.

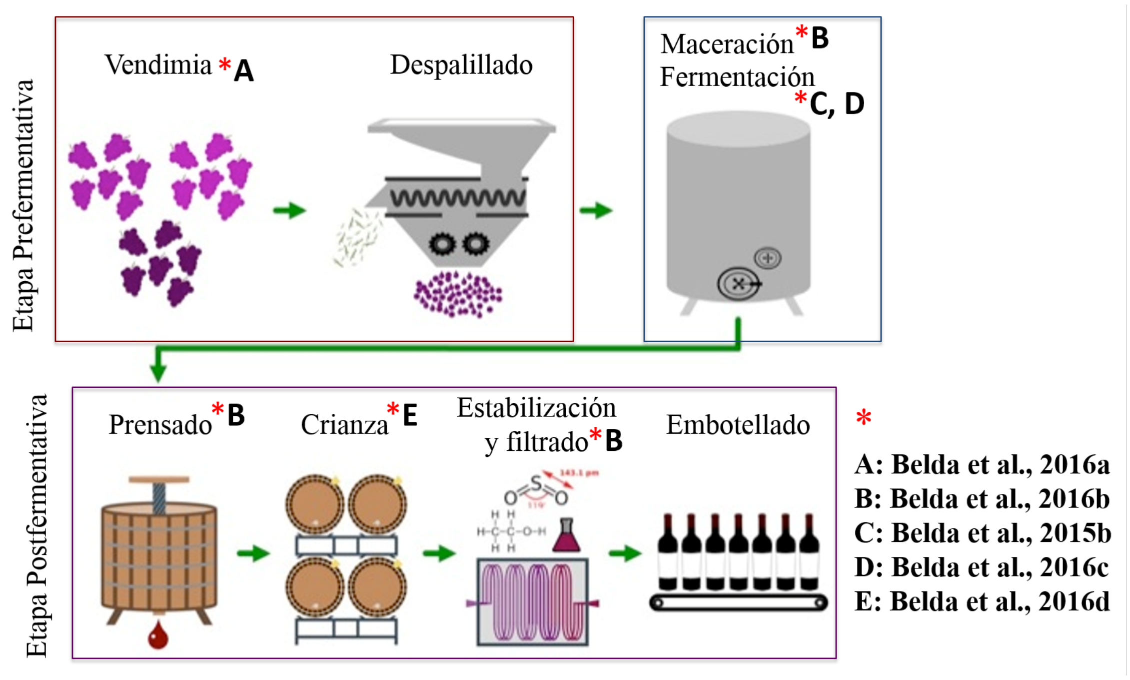

Figura 3. Esquema de un proceso clásico de elaboración de vino tinto. Las letras destacadas (*) hacen referencia a los trabajos presentados en esta Tesis Doctoral con origen o incidencia en los procesos enológicos sobre los que se sitúan.

1.3.3. Etapa post fermentativa

Finalizada la fermentación alcohólica, y maloláctica en su caso, el vino estaría listo para su consumo en forma de vino joven tras las operaciones de estabilización, clarificación y filtración. En el caso de los vinos tintos, tras la fermentación alcohólica, se lleva a cabo el prensado de los hollejos.

Además de estas operaciones básicas postfermentativas, existen una serie de opciones que comprenden el proceso de crianza de los vinos que contribuyen a incrementar su complejidad y tipicidad. Tradicionalmente la crianza de los vinos se ha llevado a cabo en barricas, generalmente de roble, durante un tiempo variable de tiempo desde los pocos meses hasta varios años, que conllevaba un efecto positivo sobre las características sensoriales de los vinos (Pérez-Prieto *et al*., 2003). La composición del vino cambia de forma continua durante su crianza incidiendo significativamente sobre su composición en compuestos fenólicos y aromáticos. El elevado coste de este método tradicional de crianza por su duración, control y almacenaje, ha llevado a la aparición de una serie de tecnologías que permiten acelerar dicho proceso obteniendo resultados beneficiosos similares sin los riesgos y el coste asociados a una crianza prolongada (Tao *et al*., 2014). Estas tecnologías pueden resumirse en el uso de fragmentos o *chips* de madera que aceleran el efecto de la madera sobre el vino por aumentar la superficie de contacto con él.

Durante esta fase, tanto en barrica como en depósito, el vino puede someterse al proceso de crianza sobre lías que permite la extracción de ciertos metabolitos de interés, en especial de manoproteínas, tras la lisis de las levaduras muertas tras la fermentación.

1.4. Incidencia de las levaduras en la composición del vino

1.4.1. Consumo de azúcares y liberación de etanol

El alcohol etílico es el producto fundamental derivado del metabolismo fermentativo de *S. cerevisiae*, con la consecuente producción de CO_2 (Figura 4) (Piskur *et al*., 2006). El nivel de etanol en los vinos varía en concentraciones entre 8 y 16 % dependiendo de la variedad de uva, su estado de maduración y el metabolismo fermentativo de las levaduras empleadas en su fermentación. El etanol juega un papel relevante en el aroma del vino debido fundamentalmente a su interacción con otros compuestos. Tiene un efecto en la volatilidad de compuestos aromáticos, cuyo incremento o detrimento puede influir en el aroma global del vino (Voilley y Lubbers, 1998; Chambers y Koppel, 2013). El etanol provoca también la modificación en la conformación de las proteínas, reduciéndose la adsorción de compuestos aromáticos a las mismas. En consecuencia, estos compuestos son liberados al medio (Havkin-Frenkel y Belanger, 2008).

Hoy en día existe una tendencia generalizada a la reducción del contenido alcohólico (etanol) en los vinos. Esto es debido a dos factores fundamentales: en primer lugar, por la creciente concienciación de la población en temas de salud, además del incremento en la severidad de las leyes de estatales en cuestiones de consumo de alcohol (Nevoigt, 2008). En segundo lugar, y más relacionado con la preferencia de los consumidores, porque ha sido demostrado el impacto de niveles elevados de etanol en los vinos con el enmascaramiento de la complejidad aromática de los mismos, siendo posible la percepción de un mayor número de determinantes sensoriales a concentraciones menores de etanol (Frost *et al.*, 2015). La reducción del etanol en vinos puede llevarse a cabo mediante diferentes métodos físicos como procesos de ósmosis inversa, adsorción, evaporación, filtración a través de membranas o procesos de fermentación parcial. Sin embargo, estos métodos traen consigo altos costes de maquinaria y procesamiento, así como un impacto considerable en el aroma y el sabor final del vino. Una alternativa a estos métodos es el uso de cepas de levadura que produzcan cantidades menores de etanol durante la fermentación completa de los azúcares del mosto. La estricta legislación en lo que respecta al uso de organismos modificados genéticamente en productos alimentarios obliga a la búsqueda y selección de cepas con un rendimiento alcohólico bajo que, de forma natural o mediante técnicas de evolución dirigida, presenten estos bajos niveles de producción de etanol en las fermentaciones (Tilloy *et al.*, 2014). Sin embargo, la inmensa mayoría de cepas de *S. cerevisiae* comerciales disponibles en la actualidad generan vinos con diferencias que apenas superan el 0,5 % v/v de etanol finalizado el proceso (Palacios *et al.*, 2007; Varela *et al.*, 2008) en función de su rendimiento alcohólico. Así, surge en los últimos años el estudio del potencial de las levaduras no-*Saccharomyces* en la reducción de alcohol en los vinos mediante el control de su metabolismo, menos fermentativo y mas respiratorio que el de *S. cerevisiae* (Contreras *et al.*, 2014; Gobbi *et al.*, 2014; Quirós *et al.*, 2014). Los resultados más prometedores a este respecto están siendo obtenidos en el empleo de cepas seleccionadas de *M. pulcherrima* en condiciones de oxigenación controladas (Morales *et al.*, 2015), si bien cabe destacar la reducción en los niveles de etanol obtenidos mediante inoculación secuencial de *T. delbrueckii* (Bely *et al.*, 2008; Belda *et al.*, 2015b). Por ello, y como se comentara ya con anterioridad, el estudio de las levaduras no-*Saccharomyces* como herramienta para la reducción del contenido en etanol en los vinos constituye uno de los pilares que sustenta su interés tanto para investigadores como para la industria.

1.4.2. Glicerol

El glicerol es, cuantitativamente, el producto de fermentación más importante tras el alcohol etílico. Es un polialcohol incoloro e inodoro con una elevada viscosidad. Su presencia en vinos tiene un efecto positivo, aportando suavidad, dulzor y densidad en boca (Nieuwoudt *et al.*, 2002).

El glicerol deriva de la degradación glicolítica de los azúcares en las etapas iniciales de la fermentación (Figura 4). Aproximadamente un 8% de los azúcares del mosto van a ser degradados por fermentación gliceropirúvica, generando glicerol y ácido pirúvico. El contenido final de glicerol en los vinos depende de diversos factores, que fundamentalmente son el contenido inicial en azúcares del mosto, la temperatura de fermentación y la cepa de levadura utilizada como inóculo.

La concentración de glicerol suele ser mayor en vinos tintos que en rosados o blancos, oscilando entre los 4 y 15 g/L (Nurgel y Pickering, 2005). La obtención de concentraciones que superiores a 10 g/L presenta un efecto muy significativo en la apreciación sensorial de las propiedades de redondez en boca antes comentadas (Gawel *et al.* 2007; Jones *et al.* 2008). Si bien existen diferencias significativas en la producción de glicerol por distintas cepas de *S. cerevisiae*, la obtención de concentraciones mayores en el desarrollo de fermentaciones espontáneas hizo vislumbrar el uso de especies no-*Saccharomyces* para el incremento del contenido en glicerol en los vinos (Ciani y Ferraro, 1996). A este respecto, estudios sucesivos han demostrado que ciertas especies como *L. thermotolerans* y *C. zemplinina* son capaces de producir grandes concentraciones de glicerol durante la fermentación (Ciani y Ferraro, 1998; Soden *et al.*, 2000; Comitini *et al.*, 2011; Englezos *et al.*, 2015).

1.4.3. Ácidos

En el vino se pueden encontrar distintos tipos de compuestos ácidos con propiedades diferentes. Estos compuestos han sido divididos en dos grandes grupos; son los ácidos volátiles y no volátiles que se describen a continuación.

1.4.3.1. Ácidos volátiles

La acidez volátil de un vino está compuesta por un conjunto de ácidos orgánicos de cadena corta. El ácido acético constituye aproximadamente el 90% de los estos ácidos volátiles jugando, por tanto, un papel fundamental en la calidad del vino (Eglinton y Henschke, 1999). A altas concentraciones, otorga sabores avinagrados que comienzan a ser muy patentes a concentraciones superiores a 0,7 g/L (Fowles, 1992). Las diferentes cepas de *S. cerevisiae* pueden producir cantidades muy variables de ácido acético (de 0,03 g/L a 1 g/L) dependiendo de su metabolismo y las condiciones de fermentación por lo que la selección de cepas adecuadas para su uso industrial constituye una de las mayores herramientas para el control de la acidez volátil durante la fermentación alcohólica (Reynolds *et al*., 2001; Paraggio y Fiore, 2004). Se ha descrito que el uso en fermentación de especies alternativas del género *Saccharomyces*, *S. bayanus* y *S. uvarum,* suelen producir niveles inferiores de ácido acético que la inmensa mayoría de cepas de *S. cerevisiae* (Giuduci *et al*., 1995; Tosi *et al*., 2009). Asimismo, el uso de ciertas especies no-*Saccharomyces,* como *T. delbrueckii,* como inóculo en fermentaciones secuenciales ha demostrado tener una influencia significativa en el descenso de la acidez volátil y el contenido en ácido acético de los vinos fermentados exclusivamente con *S. cerevisiae* (Bely *et al*., 2008; Azzolini *et al*., 2012; Belda *et al*., 2015b).

1.4.3.2. Ácidos no volátiles

La acidez de un mosto o de un vino tiene una influencia directa en sus características sensoriales, así como en su estabilidad bioquímica y microbiológica (Fowles, 1992). Los ácidos no volátiles más abundantes en los mostos de uva son el ácido tartárico y el ácido málico, que constituyen el 90% de la acidez detectable en los mostos. El ácido tartárico es resistente a la actividad microbiana, permaneciendo casi inalterable durante la fermentación y siendo, por tanto, independiente de las variables microbiológicas. Los ácidos láctico y cítrico, aunque son menos abundantes, también contribuyen a la acidez del mosto (Havkin-Frenkel y Belanger, 2008). Cabe destacar también el ácido pirúvico por como precursor clave de ciertas rutas anabólicas como la de síntesis de determinados pigmentos estables (Benito *et al*., 2011).

1.4.3.2.1. Ácido málico

El ácido málico constituye cerca de la mitad de la acidez total de los vinos. Su concentración en la uva disminuye conforme avanza la maduración, especialmente en periodos cálidos en las fases finales de la maduración. El ácido málico en los vinos aporta un carácter acídulo que en vinos tintos de crianza es deseable eliminar.-Aunque la reducción de la concentración de ácido málico en los vinos generalmente se lleva a cabo mediante el proceso bacteriano de fermentación maloláctica, el uso de levaduras capaces de degradarlo constituye una alternativa, aún por valorar a nivel industrial, cuando quiera prescindirse de la incidencia subyacente de dicho proceso sobre las características sensoriales de los vinos (Suárez-Lepe *et al.* 2012; Su *et al.* 2014). Siendo la mayoría de cepas de *S. cerevisiae* muy poco eficaces para la degradación de ácido málico (Su *et al.*, 2014), los resultados mas exitosos han sido obtenidos mediante el uso de cepas seleccionadas de *Schizosaccharomyces pombe* (Benito *et al.* 2013, 2014, 2015) y *Pichia kudriavzevii* (Del Mónaco *et al.*, 2014), aunque cabe también mencionar la acción, menos patente aunque significativa, de *T. delbrueckii* en la reducción del contenido en ácido málico en los vinos (Belda *et al.*, 2015b).

1.4.3.2.2. Ácido láctico

La presencia de ácido láctico en el mosto es nula y tampoco se aprecia incremento de este ácido en fermentación alcohólica. Este ácido es un indicativo de actividad bacteriana en vinos o mostos, generándose a partir de azucares (picado láctico) o a partir de ácido málico. Como bacteria responsable del proceso enológico de fermentación maloláctica, *O. oeni* es capaz de descarboxilar el ácido málico transformándolo en ácido láctico. El principal beneficio de este proceso es la conversión del sabor áspero de los vinos, derivado de la elevada presencia de ácido málico (dicarboxílico), en sabores más suaves propios de ácidos monocarboxílicos como el ácido láctico.

A pesar de la incapacidad de *S. cerevisiae* en para la producción de ácido láctico, *L. thermotolerans* ha demostrado ser útil como levadura productora de elevadas concentraciones de ácido láctico (superiores a 3 g/L) en fermentaciones combinadas con *S. cerevisiae*, lo que la convierte en una herramienta útil para la corrección de la acidez en mostos poco ácidos (Su *et al.*, 2014; Kapsopoulou *et al.*, 2007; Gobbi *et al.*, 2013). Recientemente ha sido propuesto el uso combinado de *S. pombe* como levadura degradadora de ácido málico y *L. thermotolerans* como productora de ácido láctico como alternativa al desarrollo de la fermentación maloláctica bacteriana en condiciones que dificulten su desarrollo como en vinos con elevado pH o elevado contenido en etanol (Benito *et al.*, 2015).

1.4.4. Compuestos nitrogenados

Las uvas contienen una gran variedad de compuestos nitrogenados entre los que podemos destacar los α-aminoácidos, el amonio y pequeños péptidos. Sin embargo, la concentración de estos compuestos es relativamente pequeña por lo que la tasa de consumo de éstos por parte de las levaduras puede ser crítica en ciertos casos (Kunkee, 1991). Bajas concentraciones de Nitrógeno Fácilmente Asimilable (NFA) a lo largo del proceso de fermentación pueden dar como resultado fermentaciones lentas e incluso detenidas (Beltrán *et al*., 2005). Por ello, las bodegas incorporan a las fermentaciones suplementos nutricionales en forma de nitrógeno orgánico (hidrolizados de levaduras) o inorgánico (sales de amonio). Por ello, es importante el conocimiento del metabolismo del nitrógeno en las especies y cepas de levadura presentes en las fermentaciones. El nitrógeno en forma de amonio se consume en primer lugar agotándose, en condiciones normales, en un periodo aproximado de 48 horas. A partir de ese momento la fuente de nitrógeno principal son los aminoácidos. Todos los aminoácidos presentes en el mosto son metabolizados por las levaduras en condiciones de anaerobiosis a excepción de la prolina (Zamora, 2009) que sólo puede ser asimilada por *S. cerevisiae* en condiciones aerobias, y que precisamente es el aminoácido más abundante en el mosto.

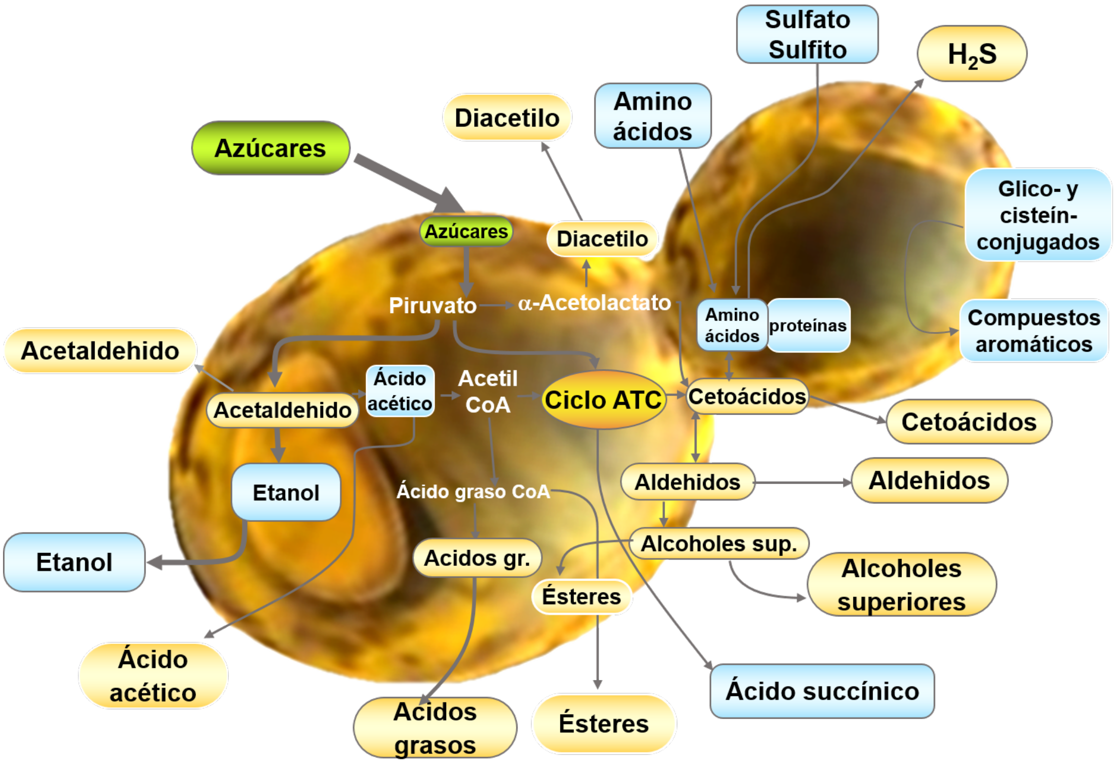

Figura 4. Esquema representativo del metabolismo básico de las levaduras en fermentación haciendo referencia a algunos de los metabolitos de mayor interés enológico.

La importancia del control del nitrógeno en fermentación radica tanto en la consecución correcta del proceso por parte de *S. cerevisiae* por motivos nutricionales (Navascués, 2015) como en la influencia de ciertas formas de nitrógeno sobre la represión de rutas de síntesis de aromas mediante Represión Catabólica por Nitrógeno (NCR) siendo determinante en el perfil sensorial de los vinos (Cooper y Sumrada, 1983; Thibon *et al.*, 2008). Esta importancia ha provocado que, a día de hoy la nutrición nitrogenada en bodega sea uno de las intervenciones enológicas más consideradas y controladas en el desarrollo de fermentaciones inoculadas con *S. cerevisiae* (Ugliano *et al.*, 2007), aunque el metabolismo y los requerimientos nutricionales de las distintas especies de levadura no-*Saccharomyces*, incluso de aquellas ya disponibles como inóculos, apenas han sido estudiados (Kemsawasd *et al.*, 2015). Un desarrollo excesivo de las poblaciones de levaduras no-*Saccharomyces* durante las etapas iniciales de la fermentación puede desencadenar un elevado consumo de nutrientes que genere a su vez problemas en el posterior desarrollo e implantación de *S. cerevisiae*. Sin embargo, el estudio de los mecanismos de NCR en especies no-*Saccharomyces*, que en *S. cerevisiae* provocan una liberación muy limitada de compuestos aromáticos como los tioles, puede dar lugar al desarrollo de protocolos de fermentación secuencial que mejoren la intensidad aromática de los vinos.

1.4.5. Compuestos aromáticos

1.4.5.1. Ésteres

Existen dos clases de ésteres en la composición aromática de los vinos, los ésteres etílicos y los ésteres de acetato. En éstos últimos el grupo acilo deriva del acetato (en forma de acetil-coA) y el grupo alcohol es el etanol o un alcohol complejo derivado del metabolismo de los aminoácidos. Los ésteres de acetato más abundantes son: el acetato de etilo (aroma desagradable a disolvente, especialmente en concentraciones elevadas, aunque aporta cierto aroma frutal en concentraciones muy bajas), el acetato de isoamilo (olor a plátano, que tiende a monopolizar el aroma de los vinos en concentraciones elevadas) y el 2-fenil acetato (aromas florales). Por otro lado, los ésteres etílicos se componen de etanol como grupo alcohólico y un grupo acilo que deriva de un ácido graso de cadena mediana (Saerens *et al.*, 2010). Éstos ésteres, como por ejemplo el hexanoato y octanoato de etilo aportan aromas a fruta, descriptores de manzana y otras frutas blancas (Cordente *et al.*, 2012). La síntesis de esteres se produce a lo largo del proceso de fermentación y su producción dependerá tanto de la presencia de los citados sustratos requeridos para la síntesis de ambos grupos de ésteres, como de la actividad de las enzimas responsables de su síntesis e hidrólisis (acil transferasas y esterasas). *S. cerevisiae* es responsable de gran parte de los ésteres presentes en un vino puesto que su metabolismo es el responsable de la mayor parte del proceso fermentativo. De los 5 genes que codifican las enzimas descritas como responsables de su síntesis en *S. cerevisiae* (*ATF1, ATF2, EHT1, EEB1* e *IAH1*) es la alcohol acetiltransferasa Atf1p la que ha presentado la mayor actividad (Sumby *et al.* 2010). Si bien las técnicas de modificación genética, como pueda ser la estrategia de sobreexpresión de *ATF1* en *S. cerevisiae,* han demostrado un incremento en la síntesis de ésteres de acetato en general (Lilly *et al.* 2000, 2006; Verstrepen *et al.* 2003), la liberación excesiva de acetato de etilo contribuye de forma muy negativa a las propiedades sensoriales de esos vinos, por lo que, al margen de la imposibilidad en el uso de organismos modificados genéticamente, el complejo metabolismo de estos compuestos dificulta la optimización de esta estrategia (Cordente *et al*., 2012). En este contexto, el uso de *M. pulcherrima* como inóculo combinado con *S. cerevisiae* presenta como una de sus principales contribuciones aromáticas la liberación de altas concentraciones de ésteres (Bisson y Kunkee, 1991; Rodríguez *et al*., 2010; Sadoudi *et al*., 2012) especialmente de ésteres característicos de frutas blancas como el octanoato de etilo (Lambrechts y Pretorius, 2000; Clemente-Jimenez *et al*., 2004). La concentración final de ésteres en un vino dependerá del balance entre la actividad de enzimas de síntesis de ésteres y enzimas esterasas. Si bien es conocida la existencia de esterasas extracelulares en *S. cerevisiae* que contribuyen a la disminución de la concentración final de ésteres en los vinos (Ubeda-Iranzo *et al*., 1998), su existencia en especies no-*Saccharomyces* como *M. pulcherrima* debe ser investigada, pero una menor actividad de dichas esterasas podría contribuir a explicar el aumento en la concentración de estos compuestos (Jolly *et al*., 2014).

1.4.5.2. Alcoholes superiores y ácidos grasos volátiles

Además de la formación de esteres, la fermentación alcohólica va acompañada de la síntesis de alcoholes alifáticos y aromáticos conocidos como alcoholes superiores o alcoholes de fusel. Altas concentraciones de estos compuestos contribuyen negativamente al aroma de los vinos, por el contrario concentraciones moderadas de estos alcoholes y sus ésteres son parte fundamental de la complejidad aromática de los mismos (Lambrechts y Pretorius 2000; Nykanen *et al*., 1977). Concretamente, el 2-feniletanol es considerado como uno de los alcoholes aromáticos más importantes en el aroma de los vinos. Nuevamente, la producción de alcoholes superiores durante la fermentación es un proceso complejo, que se produce a partir de α-cetoácidos mediante la degradación de aminoácidos por la vía de Erlich en levaduras (Hazelwood *et al.*, 2008) cuyo funcionamiento en *S. cerevisiae* se ha relacionado con 20 genes distintos; 4 que regulan la fase inicial de transaminación que resulta en la síntesis del α-cetoácido (*BAT1, BAT2, ARO8* y *ARO9*), 5 genes que codifican enzimas que regulan su posterior descarboxilación hasta formar un aldehído de fusel (*PDC1, PDC5, PDC6, ARO10* y *THI3*) y finalmente otros 11 genes que codifican las oxidoreductasas responsables de la formación final del alcohol de fusel (*ADH1-ADH7, SFA1, GRE2, YPR1* y *AAD6*) (Bisson y Karpel, 2010). Nuevamente, la complejidad de estas rutas y su conexión con otras determinantes del metabolismo de las levaduras hace compleja la optimización de protocolos de modificación genética para la optimización de la liberación, por incremento o disminución, de alcoholes superiores en el vino. Como ya se ha indicado, en general, concentraciones elevadas de alcoholes superiores en el vino no son deseables por monopolizar el aroma de los mismos disminuyendo así su complejidad. En este sentido, la formación de alcoholes superiores por la mayoría de especies no-*Saccharomyces* es, generalmente, menor que en *S. cerevisiae* (Romano *et al.*, 1992, 1993; Romano y Suzzi, 1993; Zironi *et al.*, 1993) quedando probado que parte del efecto de *T. delbrueckii* sobre el perfil sensorial de los vinos es debido a la disminución de la concentración de estos alcoholes en los mismos (Belda *et al.*, 2015b).

1.4.5.3. Terpenos

Los terpenos forman parte del aroma de los vinos siendo los compuestos determinantes del carácter varietal de ciertas variedades de uva blanca definidas por notas herbáceas y frescas (Albariño, Moscatel, Riesling). Los compuestos terpénicos mas abundantes en el vino son el linalol, nerol, geraniol, citronelol, y α-terpineol. Parte de los terpenos se encuentran en el mosto en forma libre, aunque en mayor medida lo están, conjugados a azúcares. Estos compuestos glicosilados no son volátiles y, por tanto, carecen de carácter odorante por lo que la liberación de los terpenos bloqueados será necesaria para el incremento de la concentración de aromas terpénicos en los vinos. La hidrólisis de estos conjugados por parte de enzimas glicosidasas de las levaduras es la responsable de esta liberación. Este proceso se produce en dos fases requeridas para la hidrólisis del diglucósido que retiene el terpeno aromático (Belda *et al.*, 2015a). En primer lugar, una de las siguientes enzimas: α-L-ramnosidasa, α-L-arabinofuranosidasa, β-D-xilosidasa o β-D-apiosidasa actúa sobre la molécula y posteriormente una enzima β-D-glucosidasa libera el terpeno bloqueado (Figura 5) (Flipphi *et al.*, 1993; LeClinche *et al.*, 1997; Zietsman *et al.*, 2011). Aunque ciertas actividades como la β-D-xilosidasa se encuentran presentes en *S. cerevisiae*, la ausencia en la mayoría de cepas de actividad β-D-glucosidasa en condiciones enológicas hace que esta especie sea incapaz de completar el proceso de liberación de terpenos de forma natural. En este caso la ingeniería genética si ha permitido el diseño de cepas de *S. cerevisiae* capaces de incrementar la liberación de terpenos en fermentación (Pretorius y Bauer 2002; Manzanares *et al.*, 2003; Schuller y Casal 2005), aunque nuevamente su aplicación industrial carece de perspectiva.

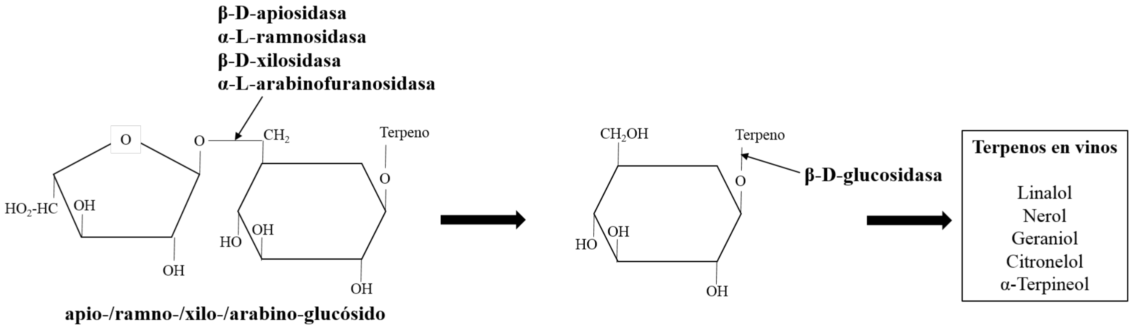

Figura 5. Esquema representativo del mecanismo de liberación de terpenos volátiles a partir de sus precursores glicosídicos no odorantes. Adaptado de Belda *et al.* (2016a).

El incremento del carácter varietal de los vinos por liberación de compuestos aromáticos como los terpenos es uno de los principales objetivos que persigue la investigación en levaduras no-*Saccharomyces* (Belda *et al.*, 2015c). La inmensa mayoría de especies no-*Saccharomyces* presentan actividad β-D-glucosidasa por lo que, a priori, parecen una herramienta útil para este fin (Belda *et al.*, 2016a). Algunas cepas de *C. zemplinina*, *T. delbrueckii* y *M. pulcherrima* han sido relacionadas con el incremento de la concentración de terpenos en el vino (Jolly *et al.*, 2014), sin embargo, su actividad glicolítica en fermentación y su represión catabólica por glucosa deben ser estudiados en profundidad para confirmar su aplicación industrial.

1.4.5.4. Compuestos azufrados

El compuesto azufrado más estudiado en enología, por su implicación negativa sobre el aroma de los vinos es el ácido sulfhídrico (H_2S), descriptor del olor a huevos podridos. Su metabolismo en *S. cerevisiae* ha sido profundamente estudiado identificándose los determinantes genéticos y nutricionales de su producción (Swiegers y Pretorius, 2007). Las carencias en nitrógeno son la condición fermentativa que más contribuye a la liberación de este compuesto por parte de las levaduras (Figura 4). Sin embargo, existe un grupo reducido de compuestos tiólicos cuya presencia en el vino confiere a este aromas característicos a frutas tropicales, pomelo y boj. Estos compuestos son la 4-mercapto-4-metil-pentan-2-ona (4-MMP), el 3-mercaptohexan-1-ol (3-MH) y su derivado acetilado el acetato de 3-mercaptohexilo (3-MHA). Su presencia en el mosto, de igual forma que los terpenos, se encuentra en bajas concentraciones en forma libre aromática y en mayor medida bloqueados mediane su unión a cisteína o glutatión (Tominaga *et al*., 1998; Peyrot Des Gachons *et al*., 2002; Fedrizzi *et al*., 2009; Roly*et al*., 2010). La actividad responsable de la hidrólisis de este complejo es la actividad β-liasa de las levaduras que, por ser intracelular, su actividad dependerá de la internalización de los precursores a través de transportadores específicos o generales de aminoácidos (Figura 6) (Darriet *et al*., 1995). A pesar de que el umbral de percepción de estos compuestos está en el orden de los 3-60 ng/L (Dubourdieu *et al*., 2006), los recursos microbiológicos actuales mediante el uso de las cepas de *S. cerevisiae* disponibles comercialmente apenas permiten la liberación de una pequeña fracción de estos precursores. Esto es debido tanto a la ineficacia de los transportadores y las enzimas con actividad β-liasa responsables de la internalización e hidrólisis del compuesto conjugado, así como a la represión que distintas fuentes de nitrógeno (orgánicas e inorgánicas) ejercen sobre ellas mediante NCR. Los genes *CYS3, STR3, BNA5* e *IRC7* codifican enzimas responsables de la hidrólisis de precursores cisteinilados naturales o sintéticos de 3-MH y 4-MMP.

IRC7 ha podido ser confirmado como responsable de la liberación de la práctica totalidad de 4-MMP (Thibon *et al*., 2008; Roncoroni *et al*., 2011) aunque contribuye también a la de 3-MH, mientras que *STR3* parece mostrar mayor repercusión sobre la liberación de 3-MH (Holt *et al*., 2011). Los determinantes genéticos de la liberación de 3-MH y 4-MMP están caracterizados en *S. cerevisiae* y su sobreexpresión por técnicas de ingeniería genética permite el incremento significativo de la liberación de tioles en fermentación (Howell *et al*., 2005;

Subileau *et al*., 2008; Thibon *et al*., 2008; Holt *et al*., 2011; Roncoroni *et al*., 2011; Belda *et al*., 2016c). Debido a la limitación de las cepas naturales disponibles actualmente en la liberación de tioles, recientemente ha sido investigado el potencial uso de levaduras no-*Saccharomyces* como herramienta para la mejora de este proceso, concluyendo que el uso de cepas seleccionadas de *T. delbrueckii* incrementa notablemente la liberación de tioles en fermentación (Belda *et al*., 2016c; Renault *et al*., 2016).

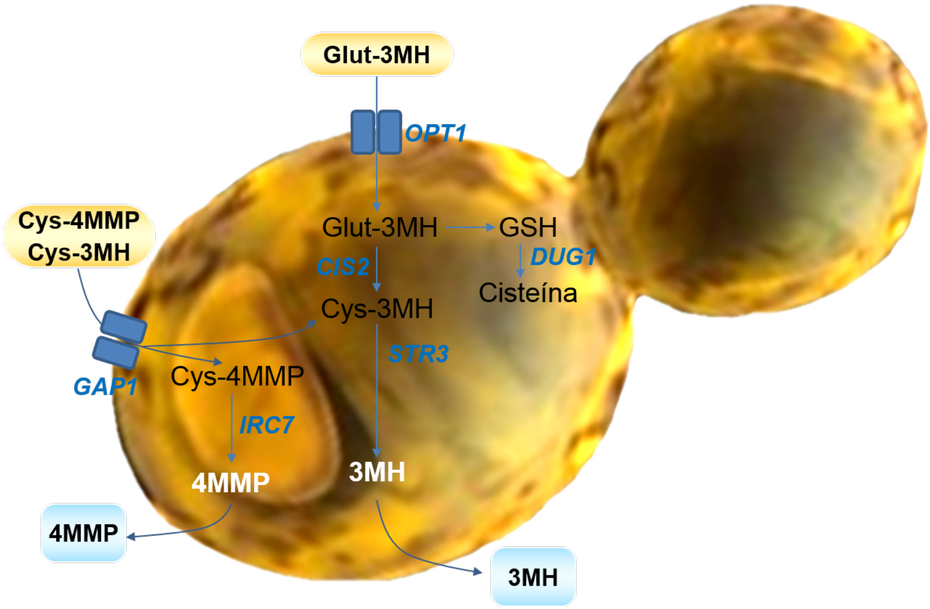

Figura 6. Esquema representativo de los genes y metabolitos implicados en la internalización e hidrólisis de los precursores cisteinilados y glutationilados de tioles volátiles en *Saccharomyces cerevisiae*.

2. OBJETIVOS

2. OBJETIVOS

El objetivo general de la tesis consistió en sentar las bases de la variabilidad fenotífica, en lo que a producción de enzimas de interés enológico se refiere, de una amplia colección de levaduras no-*Saccharomyces* para su futura aplicación en la mejora de parámetros de calidad tecnológica y sensorial de los vinos. Así mismo, se planteó el estudio de la contribución de ciertas especies no-*Saccharomyces* a la composicón en manoproteinas de los vinos.

1. Establecimiento de una colección de levaduras fermentativas y no fermentativas asociadas a distintas regiones vitivinícolas.

2. Desarrollo de métodos rápidos para la evaluación de las propiedades enzimáticas de las levaduras con interés en enología. Estudio inter- e intraespecífico de la producción de enzimas de interés enológico.

3. Selección y aplicación de levaduras no-*Saccharomyces* pectinolíticas en maceración prefermentativa para la mejora tecnológica de vinos tintos.

4. Selección y aplicación de *Saccharomyces cerevisiae* y levaduras no-*Saccharomyces* para la mejora de las propiedades sensoriales de los vinos: manoproteinas y tioles varietales.

5. Estudio de la incidencia en la calidad de vinos tintos de la crianza sobre lías de levaduras no convencionales.

3. CAPÍTULO 1

3.1. Estudio de la diversidad de especies de levaduras asociadas a distintas regiones vitivinícolas y estudio inter- e intraespecífico de la producción de enzimas de interés enológico.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 January 2016
doi: 10.3389/fmicb.2016.00012

Unraveling the Enzymatic Basis of Wine "Flavorome": A Phylo-Functional Study of Wine Related Yeast Species

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Non-*Saccharomyces* yeasts are a heterogeneous microbial group involved in the early stages of wine fermentation. The high enzymatic potential of these yeasts makes them a useful tool for increasing the final organoleptic characteristics of wines in spite of their low fermentative power. Their physiology and contribution to wine quality are still poorly understood, with most current knowledge being acquired empirically and in most cases based in single species and strains. This work analyzed the metabolic potential of 770 yeast isolates from different enological origins and representing 15 different species, by studying their production of enzymes of enological interest and linking phylogenetic and enzymatic data. The isolates were screened for glycosidase enzymes related to terpene aroma release, the β-lyase activity responsible for the release of volatile thiols, and sulfite reductase. Apart from these aroma-related activities, protease, polygalacturonase and cellulase activities were also studied in the entire yeast collection, being related to the improvement of different technological and sensorial features of wines. In this context, and in terms of abundance, two different groups were established, with α-L-arabinofuranosidase, polygalacturonase and cellulase being the less abundant activities. By contrast, β-glucosidase and protease activities were widespread in the yeast collection studied. A classical phylogenetic study involving the partial sequencing of 26S rDNA was conducted in conjunction with the enzymatic profiles of the 770 yeast isolates for further typing, complementing the phylogenetic relationships established by using 26S rDNA. This has rendered it possible to foresee the contribution different yeast species make to wine quality and their potential applicability as pure inocula, establishing species-specific behavior. These consistent results allowed us to design future targeted studies on the impact different non-*Saccharomyces* yeast species have on wine quality, understanding intra and interspecific enzymatic odds and, therefore, aiming to predict the most suitable application for the current non-*Saccharomyces* strains, as well as the potential future applications of new strains. This work therefore contributes to a better understanding of the concept of wine microbiome and its potential consequences for wine quality, as well as to the knowledge of non-*Saccharomyces* yeasts for their use in the wine industry.

Keywords: microbial terroir, enological enzymes, non-*Saccharomyces*, phylo-functional study, targeted yeast selection

OPEN ACCESS

Edited by:
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Reviewed by:
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Stellenbosch University, South Africa
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 November 2015*
Accepted: *08 January 2016*
Published: *20 January 2016*

Citation:
Belda I, Ruiz J, Alastruey-Izquierdo A, Navascués E, Marquina D and Santos A (2016) Unraveling the Enzymatic Basis of Wine "Flavorome": A Phylo-Functional Study of Wine Related Yeast Species. Front. Microbiol. 7:12. doi: 10.3389/fmicb.2016.00012

INTRODUCTION

Microorganisms coexist and interact in many environments and processes, and this fact is of practical relevance for both the environmental and industrial fields (Ivey et al., 2013). Grape musts naturally contain a mixture of yeast species, and wine fermentation is not a "single-species" process (Fleet, 1990). Despite the dominance of *Saccharomyces cerevisiae* in fermentation, which is expected and welcomed to avoid stuck and sluggish fermentations, the indigenous non-*Saccharomyces* yeasts, already present in the musts, play a critical role during the early stages of fermentation. While these yeast species are not the ones mainly responsible for alcoholic fermentation, they can release a wide variety of hydrolytic enzymes depending on their diversity (Jolly et al., 2014). Non-*Saccharomyces* yeasts were originally held responsible for microbe-related problems in wine production due to their isolation from spoiled wines. However, in recent years both empiric and scientific knowledge has emerged concluding that, in some cases, higher microbial diversity improves wine complexity.

The concept of vineyard and wine microbiome has been addressed in recent years, obtaining extensive and meaningful results on the microbial complexity of the fermentation process (Liu et al., 2015). These population studies, carried out by both classical molecular methods and metagenomics, are currently ongoing to better understand and establish the concept of "microbial terroir" (Bokulich et al., 2013, 2014; Gilbert et al., 2014). Considering that a wide variety of yeast species have been identified in different scientific studies (Bisson and Joseph, 2009; Barata et al., 2012), the role of all these yeast species and their intraspecific variations need to be known. There is an intense debate over the pertinence of the concept of microbial terroir in vineyards and wine fermentation. Several factors have been described as determinants of microbial diversity in enological environments. Robust results reported by Bokulich et al. (2014) and Wang et al. (2015) have concluded that grape-associated microbial biogeography is non-randomly associated with regional, varietal and climatic factors across multi-scale viticultural areas. However, this concept should be studied in depth, encompassing a strain-typing level and its final influence on wine quality.

A non-*Saccharomyces* strain was first used intentionally in wine fermentation in the 1960s, when Cantarelli (1955) significantly reduced the volatile acidity of wines by using selected *Torulaspora delbrueckii* strains. Nowadays, there is a wide variety of current and expected applications of non-*Saccharomyces* yeasts whose metabolic heterogeneity not only allows overcoming certain shortcomings detected in most *S. cerevisiae,* but also enables the development of innovative fermentation processes to obtain wines with new properties in sensorial, technological and safety aspects.

Apart from reducing volatile acidity in wines (Moreno et al., 1991; Renault et al., 2009), other specific applications have been attributed to certain wine yeast species, such as alcohol reduction (Contreras et al., 2014), modulation of acidity (Gobbi et al., 2013; Benito et al., 2015), increased glycerol content (Ciani and Ferraro, 1998; Soden et al., 2000), mannoprotein release (Belda et al., 2015), and the modulation of wine aroma profiles and other microbial products (reviewed by Jolly et al., 2014). In addition to fermentative aromas, mainly dependent on *S. cerevisiae* metabolism, non-*Saccharomyces* yeasts have long been described as a useful tool for revealing the varietal profile of certain grape varieties, whose aroma-determinant components are usually found as odorless conjugated precursors (Gunata et al., 1990; Tominaga et al., 1998). Trace amounts of terpenes and thiols could be present in grapes in a free form, although during fermentation yeasts may also release them from their corresponding odorless precursors. The cleavage of terpenic glycosides is dependent on the hydrolytic activity of glycosidases (Mateo and Di Stefano, 1997) and β-lyases for cysteine-conjugated thiols (Swiegers et al., 2009).

However, the improvement of the aromatic properties of wine is not the only aspect dependent on the enzymatic properties of yeasts, as other sensorial and technological features can be enhanced by other hydrolytic activities. Pectinolytic enzymes (mainly polygalacturonase) are widely used in enology to help degrade the plant cell wall polysaccharides of the grape skin and pulp. They can also help to improve clarification and filterability processes, releasing more color and flavor compounds entrapped in the grape skin, and facilitating the release of phenolic compounds (Lang and Dornenburg, 2000; Van Rensburg and Pretorius, 2000). Finally, the use of proteases in winemaking is not a widely extended practice at the present time, with bentonite being used more frequently to solve protein haze problems. The use of bentonite usually impairs the sensorial properties of wines, so the use of proteases for this purpose may be a potential solution (Marangon et al., 2012).

On the other hand, the presence of sulfite reductase in wine yeast strains is responsible for the production of hydrogen sulfide in wine fermentations, with the consequent appearance of the characteristic rotten egg off-flavor (Swiegers and Pretorius, 2007).

This paper explores the knowledge established between the concepts of wine microbiome and microbial terroir, linking the phylogenetic data provided with the enzymatic characteristics determined in a wide yeast collection. These results have allowed us to establish a general enzymatic phenotypical characterization of several wine-related yeast species and their intraspecific variability, predicting the impact of yeast microbiome on wine flavor. Thus, since the wine microbial terroir has been defined as the distinctive autochthonous microbiome of a wine region and it has been experimentally demonstrated as a determining feature of wine qualities (Bokulich et al., 2014), this work provides a compelling basis to understand the influence of these microbial differences on the wine flavor identity, developing the new concept of wine yeast flavorome and also providing some of its enzymatic basis.

MATERIALS AND METHODS

Grape Samples and Yeast Isolation

Grape samples were collected from three different Spanish wine appellations: Tierra de León (vineyard named in this study as G), Ribera del Duero (vineyards named as PDC and EM) and

Rueda (vineyard named as O). G is a young (20–40 years old) vineyard with vines of the Prieto Picudo variety; the PDC and EM vineyards are between 25 and 91 years old, with vines of the Tempranillo variety; and O is an ancient vineyard with pre-Phylloxera vines between 100 and 200 years old of the Verdejo variety, and also involves biodynamic agricultural practices. Representative samples were taken by analyzing a variety of different sample points depending on the particular agronomical heterogeneity of each vineyard. Three samples points were selected in vineyard G, 10 in vineyard PDC, 5 in vineyard EM and 9 in vineyard O.

Seventy-three yeasts were isolated from vineyard G during the 2012 harvest; 450 yeasts were isolated from vineyards PDC and EM during the 2013 and 2014 harvests; and finally, 247 yeasts were isolated from vineyard O during the 2013 and 2014 harvests (Table S1).

For the isolation of non-*Saccharomyces* yeasts, grape samples weighing about 0.5 kg were taken from healthy grape bunches. After pressing, to reduce the number of ubiquitous *A. pullulans* and basidiomycetous species of no interest to the enological objectives of this work, grape musts were incubated overnight at 20°C. A suitable diluted aliquot of grape must was then spread onto a lysine agar medium (Oxoid) plates at 28°C for 48 h. As stated above, 770 discrete colonies were isolated, and then restreaked on the same medium to obtain pure cultures that were cryopreserved and included in a yeast collection.

These yeast isolates were identified by partial sequencing of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al., 1992), and the DNA for sequencing was amplified by using an Eppendorf Mastercycler, with 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′) (Kurtzman and Robnett, 1997). The sequences obtained to identify yeasts were analyzed and compared by BLAST-search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers listed in Table S1.

Phylogenetic Tree Analysis

Phylogenetic analyses were conducted with InfoQuest FP Software (version 4.5 Bio-Rad Laboratories, Madrid, Spain). The clustering was performed following the Neighbor joining (NJ) method, with Kimura two-parameter correction.

Culture Media and Enzymatic Screening Procedures

Glycosidase Activities

β-Glucosidase activity was evaluated as reported by Villena et al. (2005) on a medium containing 0.5% cellobiose (4- O-β-D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base (Difco) and 2% agar. This medium was adjusted to pH 3.5 as follows. The components of the medium were sterilized separately to avoid agar hydrolysis. Agar and cellobiose were autoclaved, and the yeast nitrogen base was adjusted to pH 3.5 with HCl and then filtered (0.22 μm). Both fractions were subsequently mixed when the agar solution was around 60°C. A loop full of each yeast strain was spread onto the medium surface and incubated at 28°C for 3 days. Any significant growth of the colonies indicated the presence of β-glucosidase activity. A positive control (*Rhodotorula glutinis* CECT 10143) and a negative one (*Torulaspora delbrueckii* CECT 10676) were used as reference for growth determinations.

Additionally, β-D-xylosidase and α-L-arabinofuranosidase activities were evaluated using the corresponding methylumbelliferyl-conjugated substrates (methylumbelliferyl-β-D-xylopyranoside (MUX) and methylumbelliferyl-α-L-arabinofuranosidase (MUA), respectively; Sigma-Aldrich), according to the method described by Manzanares et al. (1999), with slight modifications for their development in 96-well microplates. Methylumbelliferone release was measured by detecting fluorescence using a Varioskan Flash Mutimode Reader (Thermo Scientific) with an excitation wavelength at 355 nm and emission at 460 nm. Once again, *R. glutinis* CECT 10143 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

β-Lyase Activity

β-Lyase activity was evaluated on a medium containing the following: 0.1% S-methyl-L-cysteine (Sigma-Aldrich), 0.01% pyridoxal-5′-phosphate (Sigma-Aldrich), 1.2% Yeast Carbon Base (Difco, Detroit, MI, USA) and 2% agar. This medium was adjusted to pH 3.5 and sterilized as described above to avoid agar hydrolysis. The agar solution was autoclaved, and all the other components were adjusted to pH 3.5 with HCl and filtered (0.22 μm), then both fractions were mixed when the agar solution was around 60°C. Any significant growth of the colonies after 48–72 h indicated the presence of β-lyase activity (Patent pending). *T. delbrueckii* CECT 10676 and *R. glutinis* CECT 10143 were used as positive and negative controls, respectively.

Pectinase Activities

Yeast isolates were screened for polygalacturonase activity in a polygalacturonate agar medium containing 1.25% polygalacturonic acid (Sigma), 0.67% yeast nitrogen base (YNB, Difco), 1% glucose and 2% agar, adjusted to a final pH 3.5, as previously described (Strauss et al., 2001), with slight modifications. Agar was sterilized separately by autoclaving, and all the other components were adjusted to pH 3.5 and boiled. Both solutions were mixed when agar reached a temperature of around 60°C. *Metschnikowia pulcherrima* CECT 11202 and *Lachancea thermotolerans* CECT 1951 were used as positive and negative controls, respectively.

Protease Activities

Protease activity was evaluated on YPD plates (containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar) with 2% skim milk powder (Sigma-Aldrich). The plates were incubated for 5 days at 30°C. A clear zone around the colony identified protease activity (Strauss et al., 2001). *Wickerhamomyces anomalus* PYCC 2495 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Cellulase Activities

Cellulase production was determined on YPGE plates (containing 1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol) with 0.4% carboxymethylcellulose, as previously described (Teather and Wood, 1982). *Aureobasidium pullulans* CECT 2660 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Sulfite Reductase Activity

Hydrogen sulfide production was evaluated by using a modification of the lead acetate method (Linderholm et al., 2008) described by Belda et al. (2015) for its use in 96-well microplates. Briefly, this method detects volatile H_2S in the headspace of a culture medium containing 1.17% yeast carbon base (Difco), 4% glucose anhydrous, and 0.5% ammonium sulfate. Yeasts were grown at 28°C for 3 days in 96-well microplates containing 200 µl of medium with orbital agitation (200 rpm). Hydrogen sulfide formation was initially detected by using paper strips (Whatman filter paper) that had been previously embedded with a 0.1 M lead acetate solution and allowed to dry at 65°C for 10 min and deposited over microplate wells. Hydrogen sulfide formation was qualitatively measured based on the degree of blackening of the lead acetate strip, and quantitatively estimated by densitometric measurement of the color intensity (Software "My Image Analysis v1.1" Thermo Scientific). *R. glutinis* CECT 10143 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Statistical Analysis of Enzymatic Data

Enzymatic activity was coded on a scale from 1 (no activity) to 5 (highest activity) and loaded into InfoQuest FP Software (version 4.5 Bio-Rad Laboratories, Madrid, Spain) as a character type. A similarity matrix was calculated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Groups were assigned according to the identification of the strains by 26S analysis. Group separation was calculated with the Jackknife method. Principal Components Analysis (PCA) was performed with InfoQuest FP Software.

The species distribution per sample site was introduced into R program (R Core Team, 2013). The function vegdist from the package vegan version 2.2-1 (Oksanen et al., 2015) was used to calculate a dissimilarity matrix between samples.

RESULTS

Description of Yeast Populations

In this work, 770 yeast isolates from grape musts of different origins were identified by partial sequencing of the 26S rRNA gene (Table S1). Fifteen different species were found among the yeast collection studied here (**Figure 1**), which consisted of a wide range of yeast species usually found in vineyards, and mostly having been reported to be of enological interest (Fleet, 2008; Jolly et al., 2014). *Hanseniaspora uvarum* was the most abundant species, making up more than half of the total isolates, followed by *Metschnikowia* sp. (comprising *M. pulcherrima* and *M. fructicola*) and *Lachancea thermotolerans,* with the other 12 yeast species only present at levels of less than 4% (**Figure 1**). In spite of this small diversity of species, the high sample size (770 isolates) allowed us to conduct a functional analysis of the yeast collection in question. Considering the complete yeast collection used here, a phylogenetic analysis of the 770 isolates, belonging to 15 yeast species identified on the basis of rDNA 26S sequences, was carried out in order to confirm the success of the molecular identification process (Figure S1). It should be noted that *M. fructicola* and *M. pulcherrima* could not be properly differentiated by 26S sequence analysis (Guzmán et al., 2013), and are henceforth referred to here as *Metschnikowia* sp.

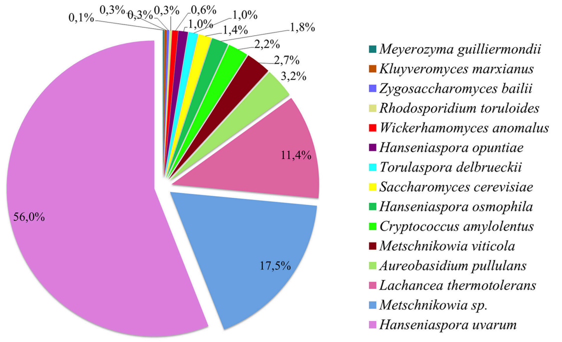

FIGURE 1 | Population distribution across the 770 yeast isolates.

Notable differences between the diversity and richness of yeast species in the different vineyards sampled were observed (**Figure 2**, Table S3). Furthermore, some differences could be perceived between yeast populations of different vintages from the same vineyard. Particular note should be taken of the low diversity of yeast species in the EM vineyard, which had only three yeast species, all of which were identified in both the 2013 and 2014 vintages, with *H. uvarum* accounting for more than three quarters of the total of 196 isolates, followed by *L. thermotolerans* and *Metschnikowia* sp. (**Figure 2A**).

In the case of the PDC vineyard (**Figure 2B**), a total of 254 yeast isolates, comprising eight species, were obtained. *H. uvarum, Metschnikowia* sp. and *L. thermotolerans* were once again the most dominant species (39, 24.8, and 19.7% of the total population, respectively). However, in this case, significant differences could be observed between vintages. There was a significant decrease in *L. thermotolerans* isolates in the 2014 vintage, and there was a higher diversity. The other species identified were *Aureobasidium pullulans*, *Cryptococcus amylolentus*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus,* and *Torulaspora delbrueckii,* jointly accounting for less than 16.6% of the PDC population and 5.4% of the total population.

Similar diversity was observed in the O vineyard, with six yeast species being identified among the 247 isolates (**Figure 2C**). *H. uvarum* was again the most abundant, accounting for 64.4% of the total, with the key observation being the low abundance of *L. thermotolerans* (one of 247 isolates). It should be noted that in this vineyard *M. viticola* was identified as an additional

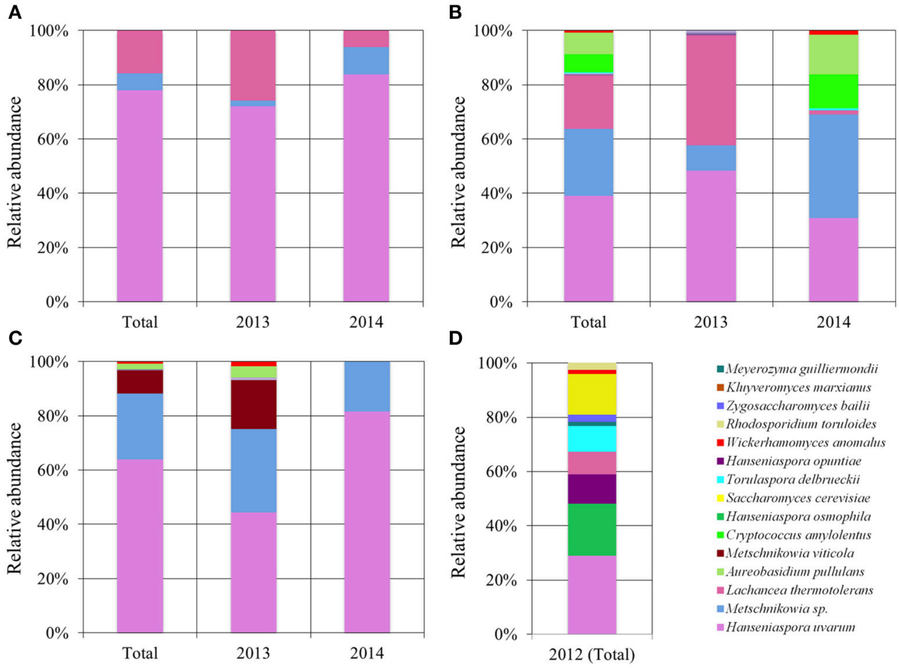

FIGURE 2 | Total and vintage-specific population distribution from the four sampled vineyards. (A) Population distribution of EM vineyard. **(B)** Population distribution of PDC vineyard. **(C)** Population distribution of O vineyard. **(D)** Population distribution of G vineyard.

Metschnikowia species. Contrary to what was observed in the PDC vineyard, a higher diversity was found in the 2013 vintage, when compared to 2014, when only *H. uvarum* and *Metschnikowia* sp. were isolated.

The G vineyard comprised 10 yeast species (nine non-*Saccharomyces* species along with some *Saccharomyces cerevisiae* isolates). *Hanseniaspora* genus was distributed among isolates of three species: *H. uvarum* (28.8%), *H. osmophila* (19.2%), and *H. opuntiae* (11%) (**Figure 2D**). Apart from *Hanseniaspora* species and *L. thermotolerans,* in the other vineyards the other five non-*Saccharomyces* species were either not isolated (*Meyerozyma guilliermondii, Zygosaccharomyces bailii,* and *Rhodosporidium toruloides*) or rarely isolated (*W. anomalus* and *T. delbrueckii*). In this case, the absence of isolates from different vintages made it impossible to establish any population trends. Finally, contrary to what was expected due to the use of a lysine medium, 11 yeast isolates were identified as *S. cerevisiae*; nevertheless, they were not removed from the collection, but instead used as a comparative control for the enzymatic study.

Phylo-Functional Study

To address a targeted use of non-*Saccharomyces* species in the wine industry, it is required a better understanding of their specific metabolic properties and their strain-dependent features. Different yeast species have been reported to modulate wine flavor and aroma, in part because of their enzymatic properties (Hernández-Orte et al., 2008; Maturano et al., 2015). The main aim of this work was to robustly establish the wine-related enzymatic profile of a large collection of wine yeasts.

A combined analysis of phylogenetic and enzymatic data (β-glucosidase, α-L-arabinofuranosidase, β-D-xylosidase, β-lyase, protease, polygalacturonase (pectinase), cellulase, and sulfite reductase) was performed to observe whether there were any overall differences in enzyme abundances and their presence among different phylogenetic groups, inferring species-specific behaviors (**Figure 3**, Figure S1). In this context, two different groups of highly and less abundant enzymes could be established, highlighting α-L-arabinofuranosidase, polygalacturonase and cellulase as the least abundant activities and, on the other hand, β-glucosidase and protease as the most widespread activities throughout the yeast collection studied.

Figure 3 shows the overall abundance and activity level of the different enzymes studied in the 770 yeast isolates, and their distribution among the 15 species identified. β-Glucosidase was widespread among wine yeast species. All the strains of *Z. bailii* and *L. thermotolerans* were observed to be β-glucosidase negative, whereas most of the strains belonging to *A. pullulans, T. delbrueckii* and *S. cerevisiae* were also found to be β-glucosidase negative, without any species-specific behavior. On the other hand, note should be taken of the activity of *H. osmophila, H. opuntiae, M. guilliermondii,* and *R. toruloides* (**Figure 3**, Figure S1). Regarding the other two glycosidases, the abundance of β-D-xylosidase and α-L-arabinofuranosidase was found to be of medium and low, respectively. Special mention should be

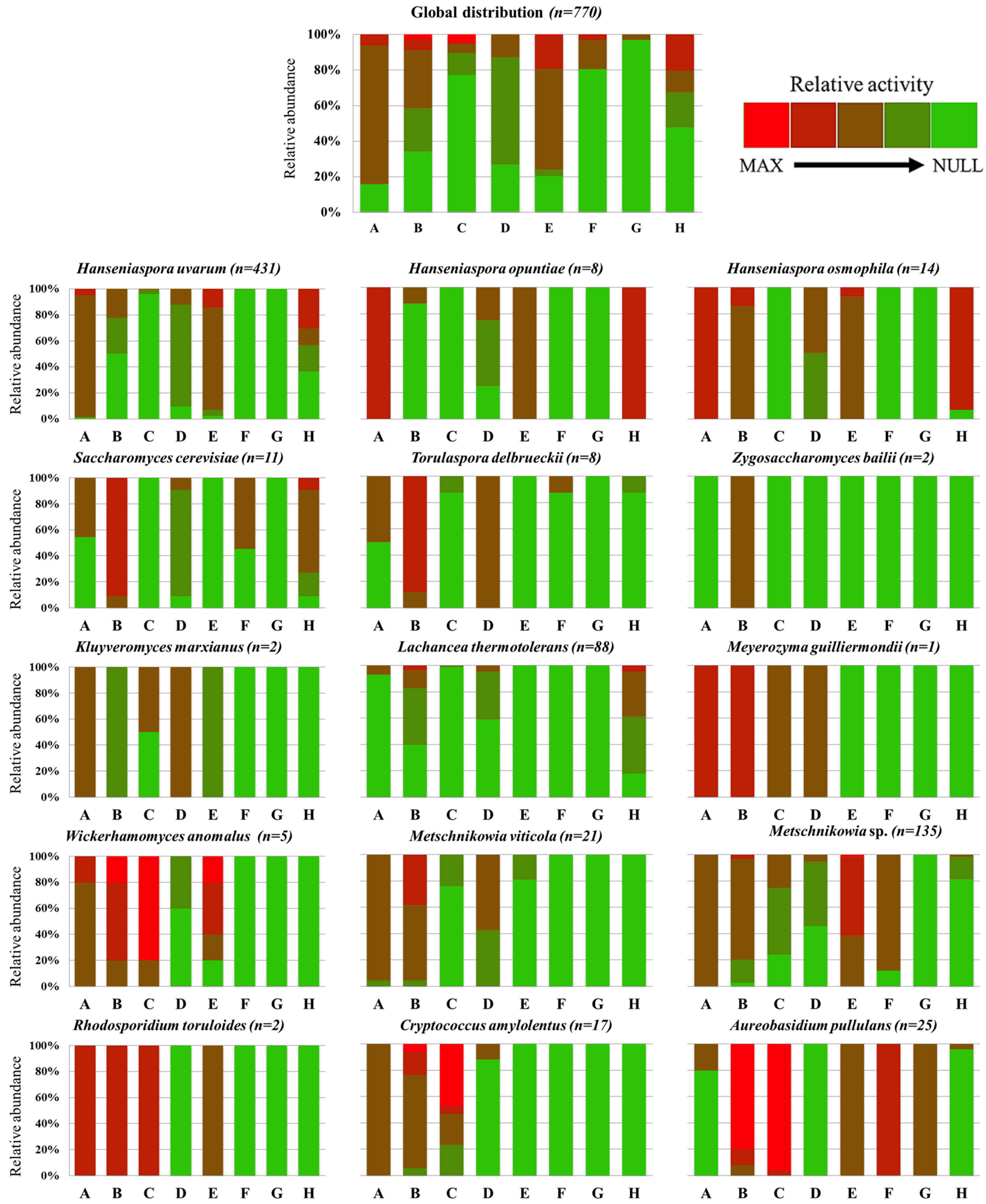

FIGURE 3 | Abundance and distribution of enzymatic activities among the total yeast collection, individually considering the 15 yeast species identified. The eight enzymatic activities evaluated were: (A) β-glucosidase; (B) β-D-xylosidase; (C) α-L-arabinofuranosidase; (D) β-lyase; (E) Protease; (F) Polygalacturonase; (G) Cellulase; (H) Hydrogen sulfide production. Enzymatic activity was determined on a scale from 1 (no activity) to 5 (highest activity) corresponding to a progressive color code from green to red.

made of the production of β-D-xylosidase in *S. cerevisiae, T. delbrueckii, M. guilliermondii, W. anomalus, R. toruloides,* and *A. pullulans,* with the production of α-L-arabinofuranosidase being only noteworthy in the three latter species, as well as in *C. amylolentus*. Overall, a glycosidase-active cluster could be observed in the basidiomycetous group (*C. amylolentus* and *R. toruloides*), together with the yeast-like fungus *A. pullulans*, all of them located at the bottom of the phylogenetic tree (Figure S1).

β-Lyase activity was widespread, albeit in most cases with moderate activity throughout the isolates. Only *T. delbrueckii*, *M. guilliermondii,* and *K. marxianus* had a wholly positive specific behavior.

Protease activity was, together with β-glucosidase, the most abundant activity in the yeast population studied. However, 40% of the yeast species (six out of 15) had no protease activity. This apparent contradiction can be explained by the small representation these species have in the total number of yeast isolates. It should be mentioned that protease activity was fully absent in the phylogenetically related species *S. cerevisiae*, *Z. bailii,* and *T. delbrueckii,* as well as in *L. thermotolerans*, *M. guilliermondii,* and *C. amylolentus* (**Figure 3**).

On the other hand, pectinase and cellulase activities had a restricted distribution, with pectinase having only a significant presence in *Metschnikowia* sp. and *A. pullulans,* and cellulase only in *A. pullulans*. Apart from that, almost half of *S. cerevisiae* and a few *T. delbrueckii* isolates had pectinase activity. It should be mentioned that protease and pectinase activities are the main phenotypic differences between *M. viticola* and the other two *Metschnikowia* species isolates.

Finally, hydrogen sulfide production due to the activity of sulfite reductase was remarkably high in some *H. uvarum* and in most *H. osmophila* and *H. opuntiae* isolates, confirming a genus-related behavior. Regarding the other yeast species, only *S. cerevisiae* and *T. delbrueckii* had certain H_2S-producer strains.

Thus, Figure S1 shows an active cluster at the lower region of the phylogenetic tree composed by basidiomycetous species (*C. amylolentus* and *R. toruloides*) and by *Metschnikowia* sp. and *A. pullulans* isolates. A highly inactive cluster in enzymatic terms could also be observed in the lower-middle zone.

Origin-Dependent Intraspecific Study

In order to study the concept of microbial terroir in depth, an intraspecific analysis was conducted on the enzymatic properties associated to every strain. **Figure 4** shows the intraspecific clustering of the isolates of different species (five species isolated from more than one origin) by carrying out a PCA analysis using enzymatic data.

Considering the three less abundant species analyzed (*T. delbrueckii*, *A. pullulans,* and *W. anomalus*), it was possible to clearly establish origin-dependent strain clusters composed of homogeneous populations that could be distinguished by their enzymatic profiles. *T. delbrueckii* was isolated from the G (seven isolates) and PDC (one isolate) vineyards in the 2012 and 2014 vintages, respectively. Two different groups could be

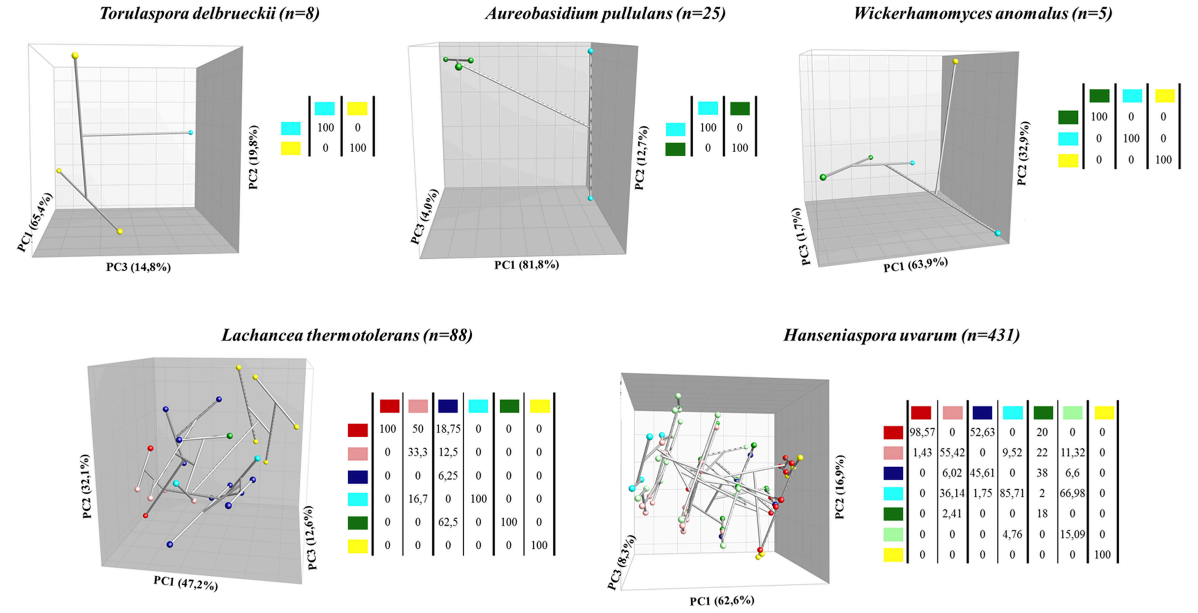

FIGURE 4 | Intraspecific distribution of isolates from the four origins and their corresponding vintages sampled. Tridimensional plots correspond to the PCA analysis of specific populations considering their enzymatic activities, and group separation was calculated with the Jackknife method. Color legends: red (EM 2013), pink (EM 2014), blue (PDC 2013), cyan (PDC 2014), dark green (O 2013), pale green (O 2014), and yellow (G 2012). Tridimensional visualization was captured in order to optimize group distinction.

statistically identified (with two Principal Components (PCs) explaining 85.2% of the differences, and three PCs explaining 100%), showing a clear origin-dependent differentiation with β-glucosidase and pectinase mostly affecting this clustering (**Figure 4**, Table S2a). *A. pullulans* was also isolated from two vineyards: PDC (2014) and O (2013), with 20 and 5 isolates, respectively. In this case, two different groups were established depending on the isolation origin, composing 100% homogeneous population groups (**Figure 4**). The PCA analysis allowed us to statistically support this clustering, with the first two PCs explaining 94.55% of these differences, and three PCs explaining 98.51%. In this case, β-glucosidase and β-D-xylosidase were the factors mostly responsible for affecting this clustering, by greatly contributing to the first PC, which alone explains 81.84% of the established differences (Table S2b). *W. anomalus* was isolated from three different vineyards: G (2012), PDC (2014), and O (2013), with 1, 2 and 2 isolates, respectively, and these five isolates again described a phenotypic cluster according to their origin, composing three different phylo-functional groups (**Figure 4**). This clustering was again statistically significant in the PCA analysis, explaining 96.8% of the differences with the first two PCs, and 97.5% with three PCs. Protease activity was the most responsible factor, explaining the origin-dependent cluster separation, and contributing significantly to the first PC, which could explain 63.88% of the differences detected (Table S2c).

Due to their large sample size, the other two species evaluated (*L. thermotolerans* and *H. uvarum*) generate more complex clustering but, in most cases, some statistically homogeneous groups could be composed depending on the origin-dependent strain phenotype. Regarding *L. thermotolerans*, a total of 88 isolates were analyzed from G (2012), PDC (2013, 2014), EM (2013, 2014), and O (2013), with 6, 50, 31 and 1 isolates, respectively.

Clusters were established for the isolates from the four different vineyards, although a less precise separation could be established between the isolates of different years from the same vineyard. **Figure 4** shows that *L. thermotolerans* isolates from EM (2013), PDC (2014), O (2013), and G (2012) established statistically homogeneous groups, defining their own enzymatic profile. Isolates from EM (2014) did not form a homogeneous group, but 50% of these isolates could be assigned to the EM (2013) enzymatic profile. Regarding PDC (2013) isolates, it was not possible to establish a uniform profile, with most of its isolates being similar to the enzymatic profiles from other origins. Apart from that, the PCA of the enzymatic properties of the total *L. thermotolerans* population could explain 79.28% of the differences between origins, considering the first two PCs, and 91.87% considering the first three PCs. These differences could be attributed mostly to β-D-xylosidase activity, H_2S production, and β-glucosidase activity (Table S2d). Finally, regarding the largest species population in this study, the analysis of *H. uvarum* enzymatic profile generated the most complex clustering, although in some cases an origin-dependent enzymatic profile could be defined. *H. uvarum* was isolated from all the vineyards, reaching a total of 431 isolates from all sampled origins. Three origins established consistent groups: EM (2013), PDC (2014), and G (2012). On the other hand, *H. uvarum* isolates from O (2013 and 2014) did not establish a consistent enzymatic profile of their own, with most of the isolates being statistically attributed to other origin profiles. Finally, in an intermediate situation, EM (2014) and PDC (2013) originated not-fully consistent groups, with their enzymatic profile overlapping with the profile described by other vineyards from the same appellation (EM 2014 with PDC 2014; PDC 2013 with EM 2013) (**Figure 4**), describing a wider origin-specific profile. The PCA analysis of these data gives us statistical evidence of the significance of these clustering results. Sulfite reductase and β-D-xylosidase activities contributed notably to these differences, significantly affecting PC1, which could alone explain 62.62% of the differences between groups, and also PC2, which accumulates an explanation of 79.48% of the differences (**Figure 4**, Table S2e).

DISCUSSION

Diversity and Richness of Yeast Species

The main aim of this work was to establish a large collection of non-*Saccharomyces* yeasts isolated from different Spanish wine appellations in order to perform a joint phylo-functional analysis, linking phylogenetic and phenotypic data on the enzymatic properties of the yeast species identified. Furthermore, an attempt has been made to relate certain enzymatic activities, which are usually associated with certain yeasts, to the potential role they could play in enology.

The experimental approach developed for this study was based on culture-dependent techniques in order to obtain a yeast collection of enological origin that may have a use in winemaking. From a general point of view, our population data (**Figure 1**) were in line with other studies reporting that, apart from the *Aureobasidium* and *Rhodotorula* species that were intentionally avoided in this study as described in the yeast isolation procedure, *Hanseniaspora* spp., *Metschnikowia* spp., and *L. thermotolerans* dominate yeast communities in fresh musts (Prakitchaiwattana et al., 2004; Pinto et al., 2015), with *H. uvarum* accounting for more than half of the total yeast population isolated (Beltran et al., 2002; Wang et al., 2015).

There has recently been confirmation of the major differences in population richness values between culture-dependent and independent approaches in enological environments (Wang et al., 2015). Our overall results of yeast diversity using a culture-dependent approach are wider than those obtained in other similar studies. Wang et al. (2015) have managed to identify a total of three species (*H. uvarum, Issatchenkia terricola,* and *Starmerella bacillaris*) from a collection of 179 yeasts isolated from nine different origins by using a lysine medium, and five species (the three previously mentioned, together with *S. cerevisiae* and *Hanseniaspora valbyensis*) in 183 isolates from the same nine samples using YPD plates. The higher diversity obtained in our work (15 vs. 5 species) could be explained by both the larger sample size (770 vs. 362 isolates) and the greater heterogeneity in sampling areas (**Figure 1**). According to data reported by Beltran et al. (2002), several differences in yeast diversity were observed between years, as shown in **Figure 2**.

Differences in the microbial composition among vintages, grape varieties, climate and location have been widely reported by Bokulich et al. (2014), and could account for the differences observed for yeast diversity found in the G vineyard compared to the diversity found in the other three vineyards studied (Figure S2, Table S3). The different microclimatic conditions, vineyard location and vine variety of this vineyard, with only the 2012 vintage sampled, could account for such a difference. The 2012 vintage in most Spanish wine appellations was characterized by low rainfall (Figure S2), which could restrict the filamentous fungi overgrowth that could displace some of the yeast species present in the grape microbial consortia (Liu et al., 2015). Additionally, as we show in this work, not only were the diversity and richness of yeast species affected by location, but also the phenotypic profile of the same yeast species differed across vineyards, and even in consecutive vintages (**Figure 4**).

Although most of the current population studies using culture-independent molecular methods report higher diversity values for fresh must than those reported here (Bokulich and Mills, 2013; David et al., 2014; Pinto et al., 2015), a wide variety of yeast species of enological interest (Jolly et al., 2014) were represented in the yeast collection established for their enzymatic characterization.

Enzyme Abundance and Species-Specific Distribution

Regarding enzymatic screening, eight enzymatic activities were evaluated to establish an enzymatic profile of enological interest for the 15 yeast species studied (**Figure 3**). A group of three glycosidases (β-glucosidase, β-D-xylosidase, and α-L-arabinofuranosidase) were determined, recording different performances in terms of activity, distribution and abundance. According to other works (Fia et al., 2005), β-glucosidase was a widespread activity among wine yeasts. Our results have highlighted the β-glucosidase production of *Hanseniaspora* species, as well as of *M. guilliermondii* and *W. anomalus*. These results are also consistent with other enzymatic screenings that additionally reported the ability of some *H. uvarum* strains to produce versatile β-glucosidase enzymes with no repression by glucose and with no significant activity decrease in a wide range of pH values (López et al., 2015). Delcroix et al. (1994) and Hernández et al. (2002) evidenced a loss of stability of β-glucosidase in *S. cerevisiae,* with a strong reduction in its enzymatic activity (about 80%) when changing from pH 5 to pH 3, while other authors have reported a notable decrease in most non-*Saccharomyces* species at pH values below 4 (Rosi et al., 1994). However, Mateo et al. (2011) have reported that *W. anomalus* reached its maximum β-glucosidase activity at pH 3.2, also recording lower rates of catabolic repression by glucose. Thus, with β-glucosidase being the final activity responsible for the release of wine terpenes from their glycosylated precursors, both *Hanseniaspora* species and *W. anomalus* seem to be a useful tool to increase wine terpenics, as suggested by Mendes-Ferreira et al. (2001) and Mateo et al. (2011), respectively.

Regarding the other two glycosidases analyzed (β-D-xylosidase and α-L-arabinofuranosidase), different abundances were observed among the yeast population studied. Contrary to what was observed in β-glucosidase activity, *Hanseniaspora* spp. had neither β-D-xylosidase (with the exception of *H. osmophila* and a few *H. uvarum* strains) nor α-L-arabinofuranosidase activities, which was in complete agreement with previous observations reported by Manzanares et al. (1999). However, they also highlighted a remarkable β-D-xylosidase activity for some *W. anomalus* and *H. uvarum* strains at the usual enological pH values of 3–3.8, with their use also being recommended for terpene release in wine fermentation. Furthermore, lower repression levels by glucose and ethanol have been reported for *W. anomalus* glycosidase activities (Mateo et al., 2011). Regarding the other yeast isolates, a β-D-xylosidase-active cluster was observed in the phylogenetically related species *T. delbrueckii, Z. bailii,* and *S. cerevisiae*. However, a high glucose-dependent repression has been observed in these species (Gueguen et al., 1995; Mateo and Di Stefano, 1997; Mateo et al., 2011), restricting their use in terpene release in wine fermentation.

Finally, α-L-arabinofuranosidase, as the least distributed glycosidase, was observed in *M. guilliermondii*, *W. anomalus*, *A. pullulans*, *R. toruloides,* and *C. amylolentus*. McMahon et al. (1999) have reported the major ability *A. pullulans* glycosidases have to release wine terpene glycosides. According to Mateo et al. (2011), α-L-arabinofuranosidase, together with α-L-rhamnosidase, is the least glucose-repressed glycosidase in wine yeasts, so both are of enological interest. Regarding *Metschnikowia* spp., most of them had remarkable β-glucosidase and β-D-xylosidase activities, although a considerable number of *Metschnikowia* sp. (not considering *M. viticola* isolates) had also α-L-arabinofuranosidase activity. Along these lines, it has been reported that a commercial strain of *M. pulcherrima* could increase volatile terpenes in wine due to its α-L-arabinofuranosidase activity (Lallemand, 2013).

Overall, our results are in agreement with other works reporting that *Pichia, Wickerhamomyces,* and *Hanseniaspora* genera are major producers of glycosidase enzymes (Manzanares et al., 1999) and, furthermore, we report the remarkable glycosidase activity of wine-related basidiomycetes, such as *R. toruloides* and *C. amylolentus*.

β-Lyase activity, which is also directly related to varietal aroma enhancement, recorded a moderate distribution in the overall yeast collection studied. **Figure 3** shows moderate β-lyase activity in the majority of yeast species, with its production being remarkable in *T. delbrueckii*, *K. marxianus,* and *M. guilliermondii*. Although this activity has been studied in depth in *S. cerevisiae* wine strains (Howell et al., 2005; Thibon et al., 2008; Roncoroni et al., 2011), actual information on the ability of non-*Saccharomyces* to release volatile thiols in wine is very scarce. Zott et al. (2011) have reported that β-lyase activity is a strain-dependent characteristic in non-*Saccharomyces* yeasts, as described in *S. cerevisiae* (Roncoroni et al., 2011; Santiago and Gardner, 2015). Accordingly, **Figure 3** shows that β-lyase activity has great intraspecific variability. Zott et al. (2011) have reported that, apart from *T. delbrueckii*, some *M. pulcherrima* and *L. thermotolerans* strains have the ability to release volatile thiols in Sauvignon Blanc wines, but only a few strains of these species have recorded β-lyase activity in our *in vitro* assays. Regarding the *Hanseniaspora* genus, and as occurred with β-D-xylosidase, *H. osmophila* recorded higher β-lyase activity compared to *H. opuntiae* and *H. uvarum* species. These phenotypical differences were consistent with the observations made in the phylogenetic tree (Figure S1), in which *H. osmophila* was distant from the *Hanseniaspora* genus cluster. Due to the high nitrogen catabolic repression affecting β-lyase activity in *S. cerevisiae,* which restricts its contribution to thiol release in wine fermentation (Thibon et al., 2008), these alternative yeasts should be studied in depth as a way to improve volatile thiol release in enological conditions.

H_2S production, as a result of sulfite reductase activity, is a rare feature among the majority of non-*Saccharomyces* species. Furthermore, as occurred with β-lyase (the other sulfur-related activity), major intraspecific variability could be observed in species such as *H. uvarum* and *L. thermotolerans,* as well as in *S. cerevisiae,* as previously reported by Linderholm et al. (2008). Given that the nitrogen composition of musts has been described to affect H_2S production by yeasts (Linderholm et al., 2008), and since non-*Saccharomyces* yeasts have high nutritional demands (Jolly et al., 2014), the lack of sulfite reductase activity in most of them is a positive characteristic for their application without the risk of wine reduction.

Protease, pectinase and cellulase have been studied for their involvement in several technological processes in winemaking. **Figure 3** shows that protease is a widespread activity when the total population of yeasts is considered, in agreement with previous works (Lagace and Bisson, 1990; Chomsri, 2008). This is caused by the protease activity of the most abundant species (*Hanseniaspora* species and *Metschnikowia* sp.), although other species of enological interest with a lower relative abundance recorded no activity (*S. cerevisiae, T. delbrueckii,* and *L. thermotolerans*, among others). In addition, protease and pectinase seem to be the main differential activities between *M. viticola* and the other *Metschnikowia* species isolated. The use of proteases in winemaking is not a widely extended practice at the moment, with bentonite being used more often to solve protein haze problems. The use of bentonite usually impairs the sensorial properties of wines, so the use of proteases for this purpose seems to be a potential future application (Marangon et al., 2012). Special note should be taken of the high protease activity of *W. anomalus*, especially in the NS-PDC-167 strain (**Figure 3**, Figure S1), which should be studied in depth for its application in protein haze prevention. In fact, an aspartate-protease from *M. pulcherrima* has been characterized and expressed in *S. cerevisiae* by Reid et al. (2012) for its potential wine application, but the role of proteases from yeasts in winemaking is still poorly understood.

Regarding pectinolytic activity, different studies have confirmed that most yeast species are unable to produce pectic enzymes. It should be mentioned that polygalacturonase activity has been reported in a few wine yeast isolates without establishing a species-specific behavior (Strauss et al., 2001; Merín et al., 2011). In this context, our results suggest that *M. pulcherrima*, *M. fructicola* (jointly identified here as *Metschnikowia* sp.), and *A. pullulans* are leading candidates for their use as a source of pectinase in winemaking. Following the confirmed usefulness of pectinases from *A. pullulans* in winemaking conditions (Merín and Morata de Ambrosini, 2015), the impact of *M. pulcherrima,* improving phenolic extraction and clarification processes in some pectinase-dependent wine properties, has recently been confirmed (Belda et al., unpublished). Furthermore, in light of the behavior of *A. pullulans*, this was the only cellulase-active species in the collection studied, in contrast with data reported by Strauss et al. (2001) and Merín et al. (2015) which describe the presence of cellulase activity in some ascomycetous yeasts (*Candida stellata*, *M. pulcherrima,* and *H. uvarum*) and in the basidiomycetous yeast *Rhodotorula dairenensis*, respectively.

It has been reported that at least 75% of the *S. cerevisiae* enological strains have limited pectinolytic activity (Blanco et al., 1994). However, Merín et al. (2011) and Merín and Morata de Ambrosini (2015) have confirmed the existence of a constitutive pectinase activity not repressed by glucose in non-*Saccharomyces* species, in contrast with what occurred in *S. cerevisiae* (Radoi et al., 2005). In this context, our results confirm that the vast majority of *Metschnikowia* sp. and *A. pullulans* strains are of interest for their use as pectinase sources in enology, opening a new research line for their industrial application.

Origin-Dependent Intraspecific Phenotypic Profiles

Metagenomic approaches have allowed researchers to definitively establish the concept of microbial terroir, relating location and climatic factors to specific microbial populations in vineyards (Bokulich et al., 2014). This finding has been put forward as a determinant in the differential flavor and aroma profiles of wines from different origins (Gilbert et al., 2014). Additionally, our results confirm that significant phenotypical differences could be observed between strains of the same species from different origins, delving further into the concept of microbial terroir, for the first time at strain level.

The results shown in **Figure 4** allow us to confirm the possibility of separating single species populations based on their enzymatic properties establishing origin-dependent clusters. It has been suggested that high-throughput screening (HTS) assays are crucial for discovering interesting enzymes and new sources (Goddard and Reymond, 2004). Here, we also report the potential these techniques have to develop phylo-functional analyses of yeast communities to perform innovative ecological studies. A similar approach has recently been adopted by Zhang et al. (2015) to establish phylo-functional differences among the gut microbiota of different human populations.

The connecting lines shown in **Figure 4** have allowed us to decipher the phylogenetic relationships among groups of isolates according to their phenotypical similarities. The tridimensional plot for *T. delbrueckii*, *A. pullulans,* and *W. anomalus* shows highly defined origin-dependent clusters with significant percentages of statistical differences among groups, bearing in mind that they were scarcely isolated. The population distribution of *L. thermotolerans* and *H. uvarum* isolates shown in the tridimensional plot could be better interpreted considering numerical data for group homogeneity (**Figure 4**) because of the high number of isolates considered. The results for both species isolated from Ribera del Duero vineyards (EM and PDC) suggest that the EM population isolated in 2014 was more heterogeneous when compared with data for 2013. In contrast, yeast populations from the PDC vineyard followed the opposite trend, with the populations being more homogeneous in 2014 for both species, as compared to 2013. These differences, together with the different behavior of EM and PDC populations shown in **Figure 2**, could be related to microclimatic determinants and to viticulture practices conditioning the health status of grapes that could determine microbial populations in them (Sipiczki, 2006; Barata et al., 2008). In the case of *H. uvarum* isolates from the O vineyard (Rueda wine appellation), the populations obtained in both the 2013 and 2014 vintages were very heterogeneous. As they were the only species analyzed for consecutive vintages in this vineyard, it is not possible to draw a wider conclusion about the intraspecific consistency in the O vineyard. It may be the case that the biodynamic practices applied in this vineyard contribute to a great microbial diversity, as suggested by Setati et al. (2012). The wide gap between the G population and the other population groups could be explained by geographic and climatic reasons, as it has been isolated in a wine appellation (Tierra de León) with several climatic and orographic differences with respect to its Ribera del Duero and Rueda counterparts, as well as in a different vintage (2012) with certain weather peculiarities (remarkably low rainfall).

In summary, the phenotypical characterization of our yeast population goes deep into the concept of microbial terroir, considering the yeast diversity at strain level as an important factor for determining the microbial influence on the flavor properties of wines. This intraspecific phenotypical clustering could not have been explored with current metagenomic approaches. However, the exponential growth of genomic data for non-*Saccharomyces* species and the versatility of high throughput genomic techniques, together with data on the species-specific enzymatic profiles reported in this work, open new possibilities for future comparative genomic works that will allow for the targeted development of high throughput metabolic screenings.

AUTHOR CONTRIBUTIONS

AS, EN, and DM conceived the project; IB, AS, EN, and JR designed and performed the experiments; IB, AA, and AS analyzed the data, and IB and AS wrote and edited the manuscript.

ACKNOWLEDGMENTS

The funding for the research described in this paper was provided by Agrovin S.A, within the framework of the project IDI-20130192 (Centre for Industrial Technological Development-CDTI, Spain) and by Pago de Carraovejas, within the framework of the project IDI-20140448 (Centre for Industrial Technological Development-CDTI, Spain). We thank Dr. Cristina Gutiérrez for her technical support, and Rocío Ramírez for reading the paper and contributing to its final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00012

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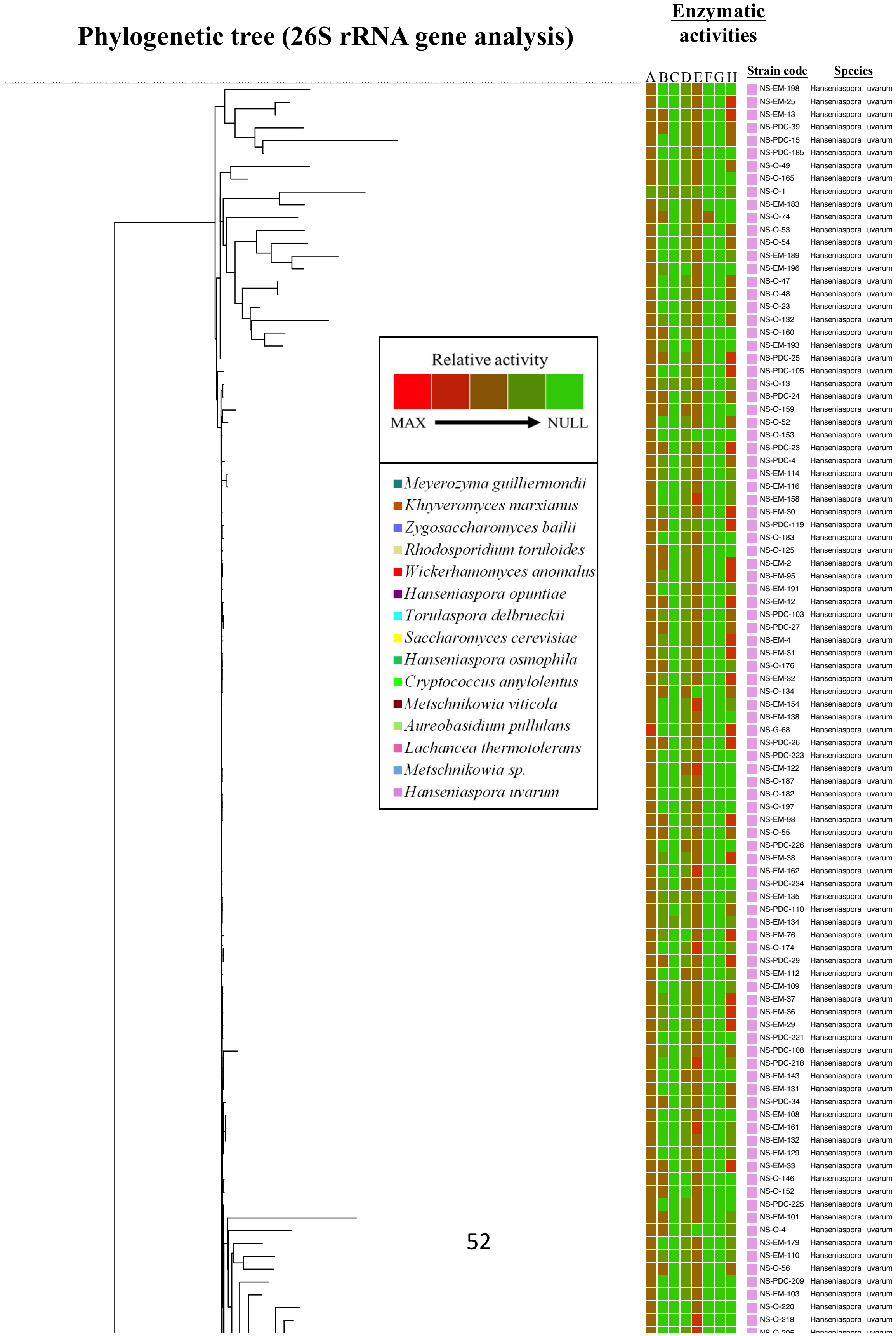
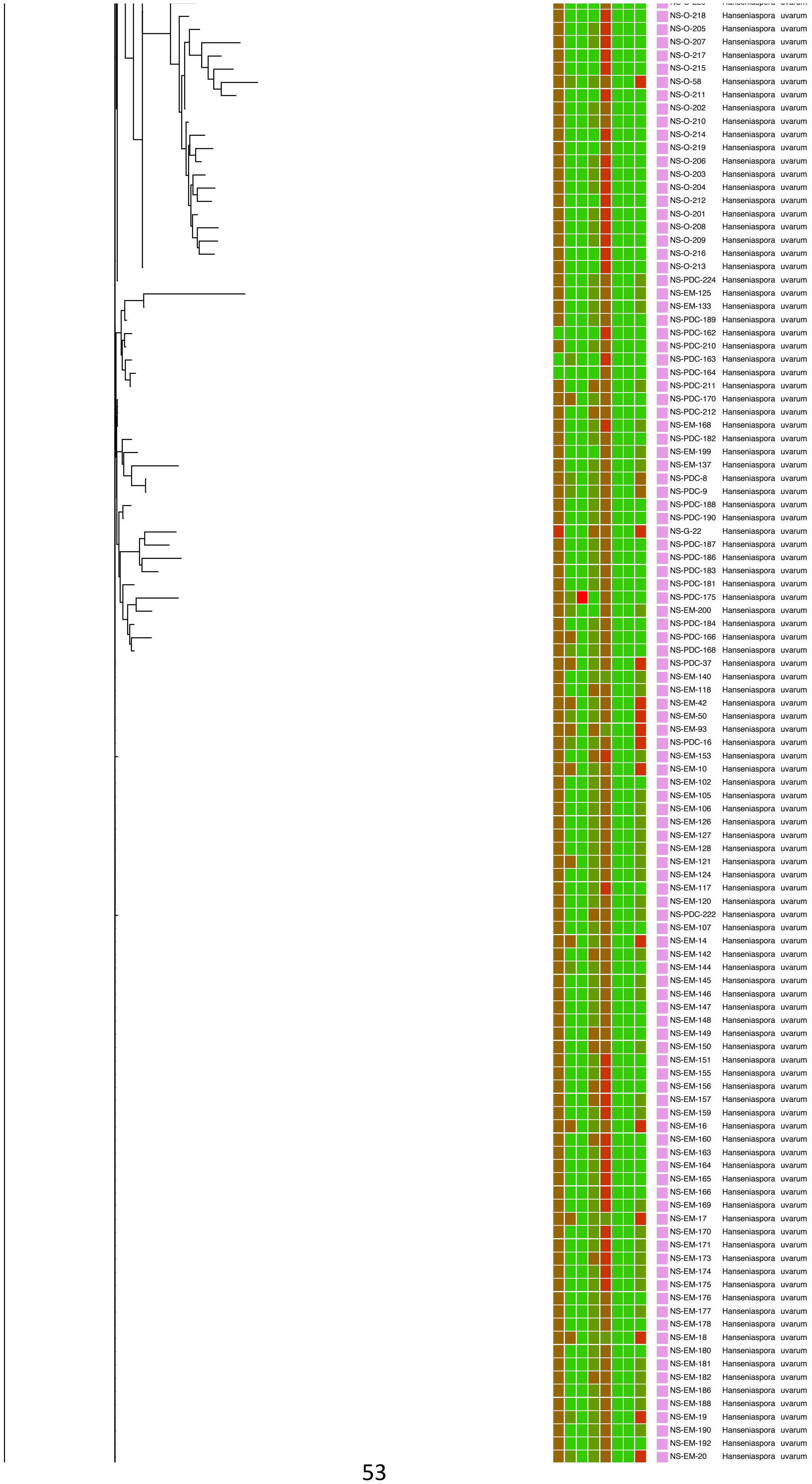
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material. Belda et al.

Unraveling the enzymatic basis of wine “flavorome”: a phylo-functional study of wine related yeast species.

Figure S1. Phylogenetic tree for the sequences obtained of the 26S rRNA gene for the 770 isolates described in this work. In addition, the activity of these isolates for seven enzymatic activities with enological relevance was also included. The 8 enzymatic activities evaluated were: A) β-glucosidase; B) β-D-xylosidase; C) α-L-arabinofuranosidase; D) β-lyase; E) Protease; F) Polygalacturonase; G) Cellulase; H) Hydrogen sulfide production.

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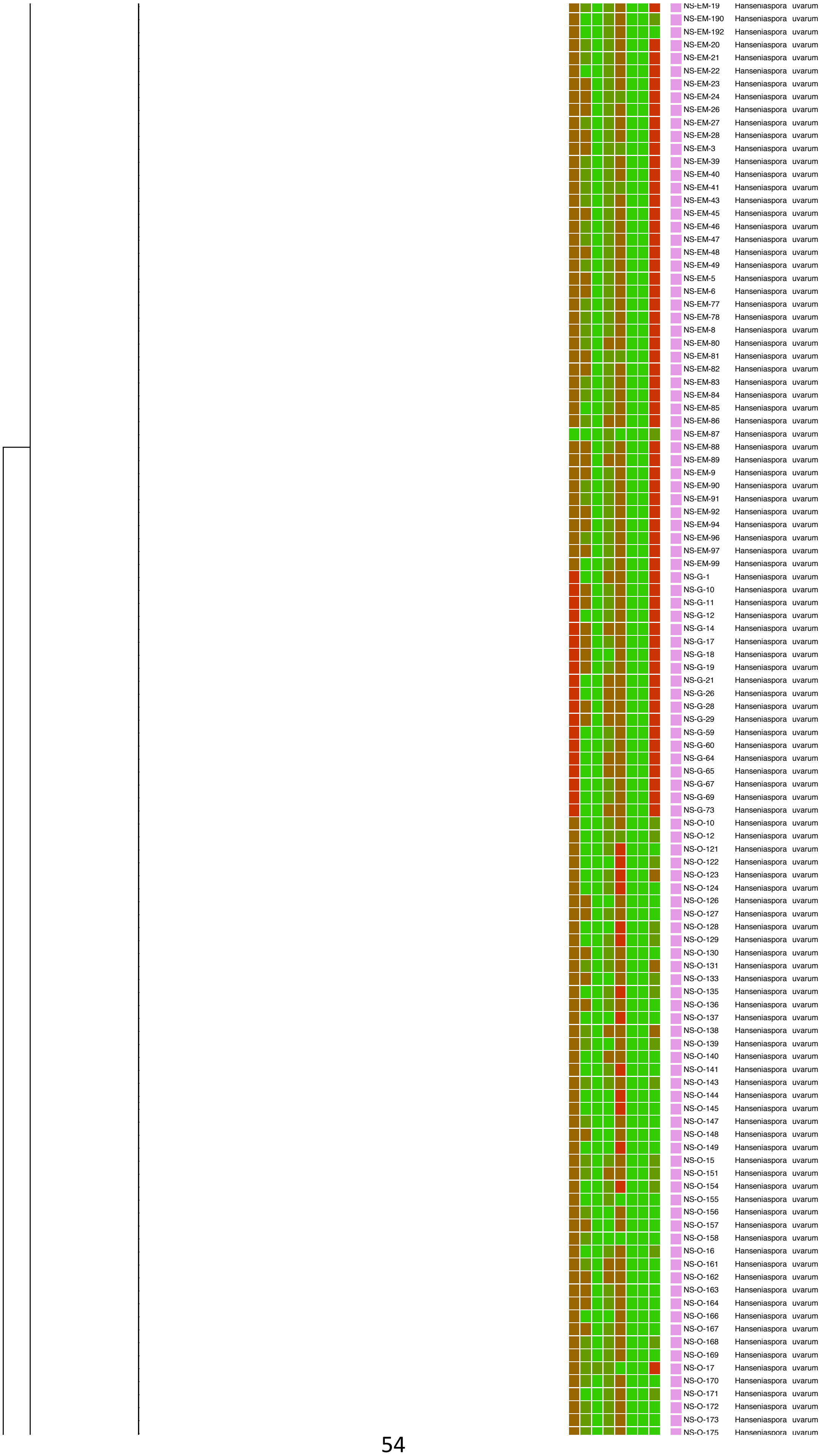
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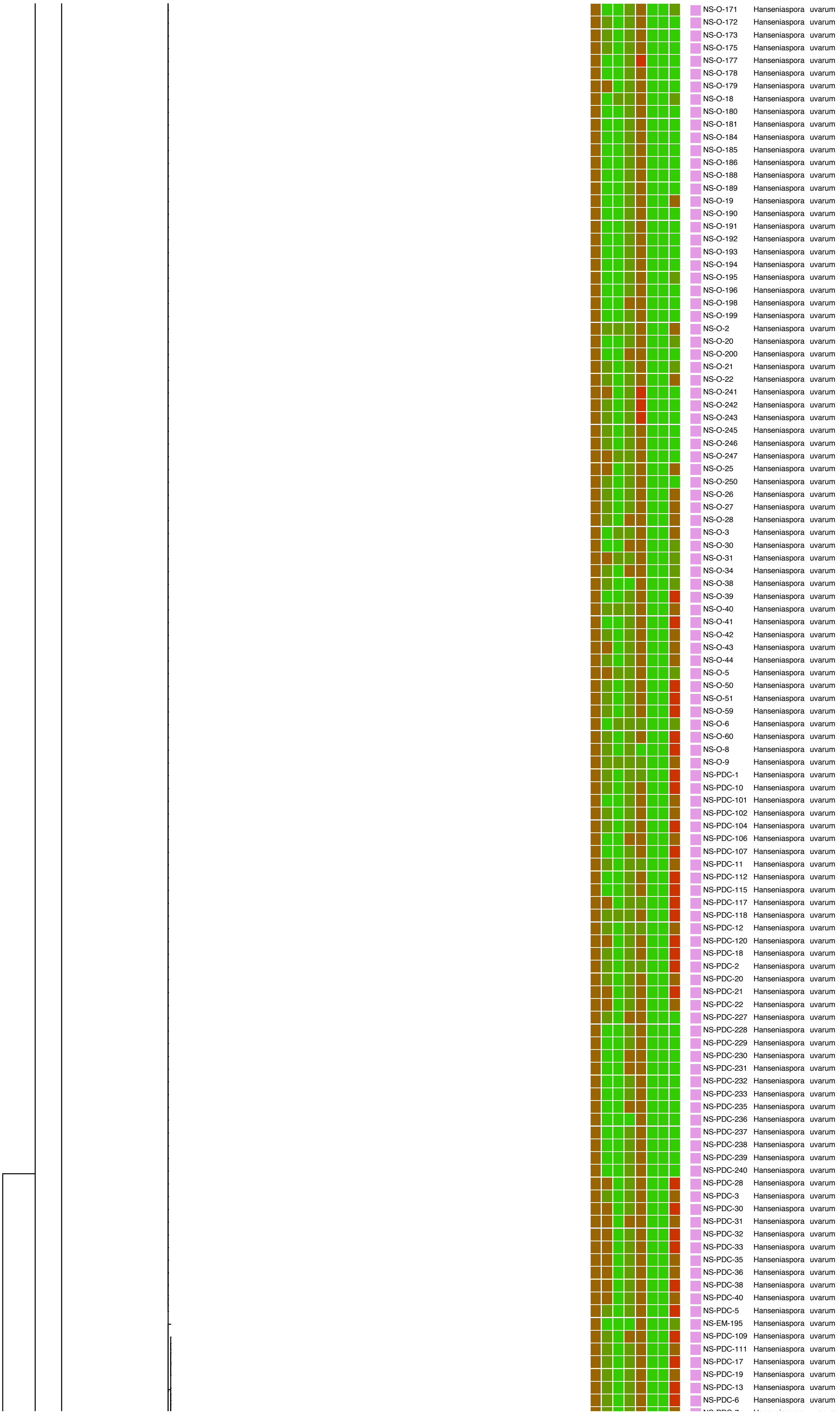
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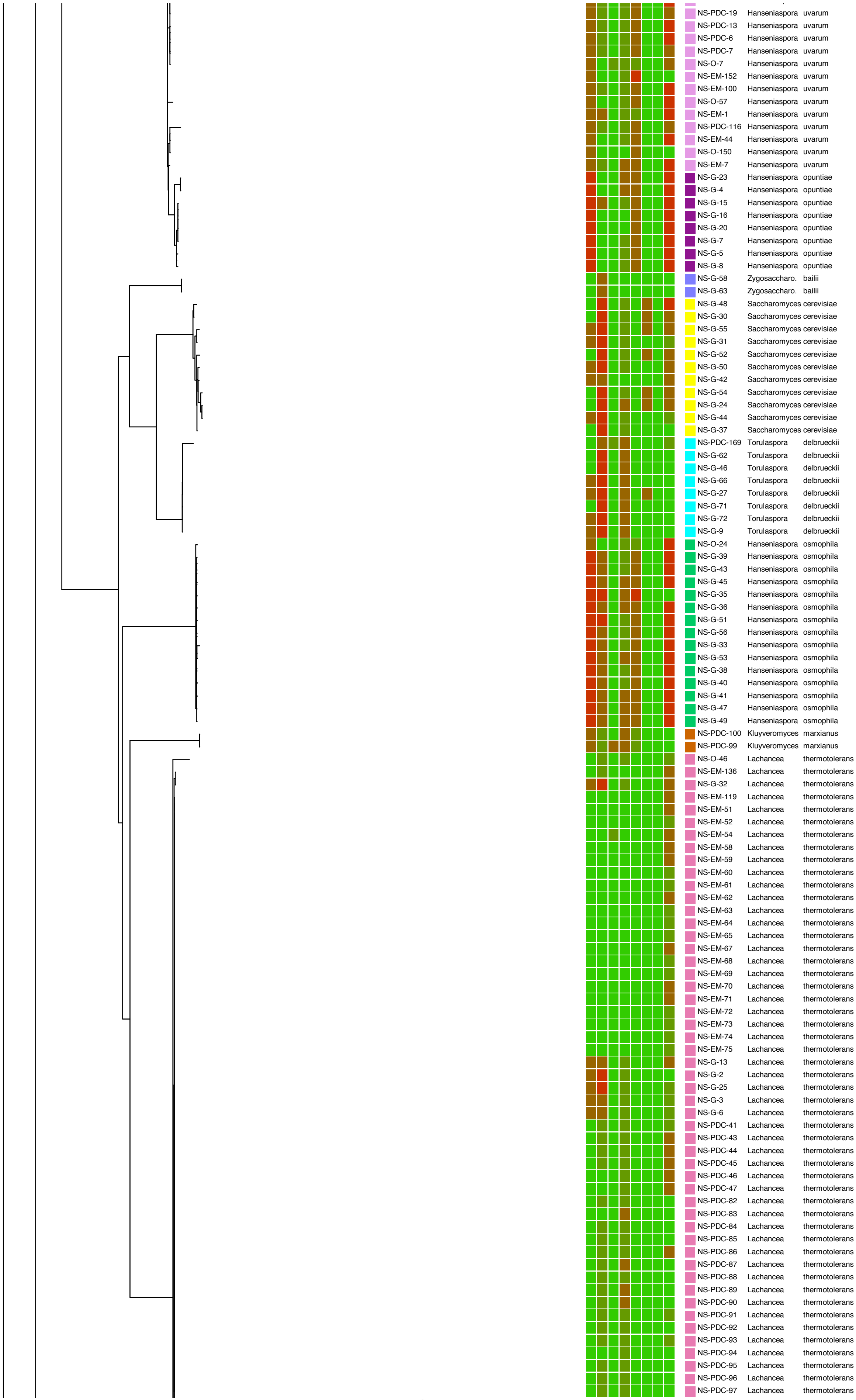
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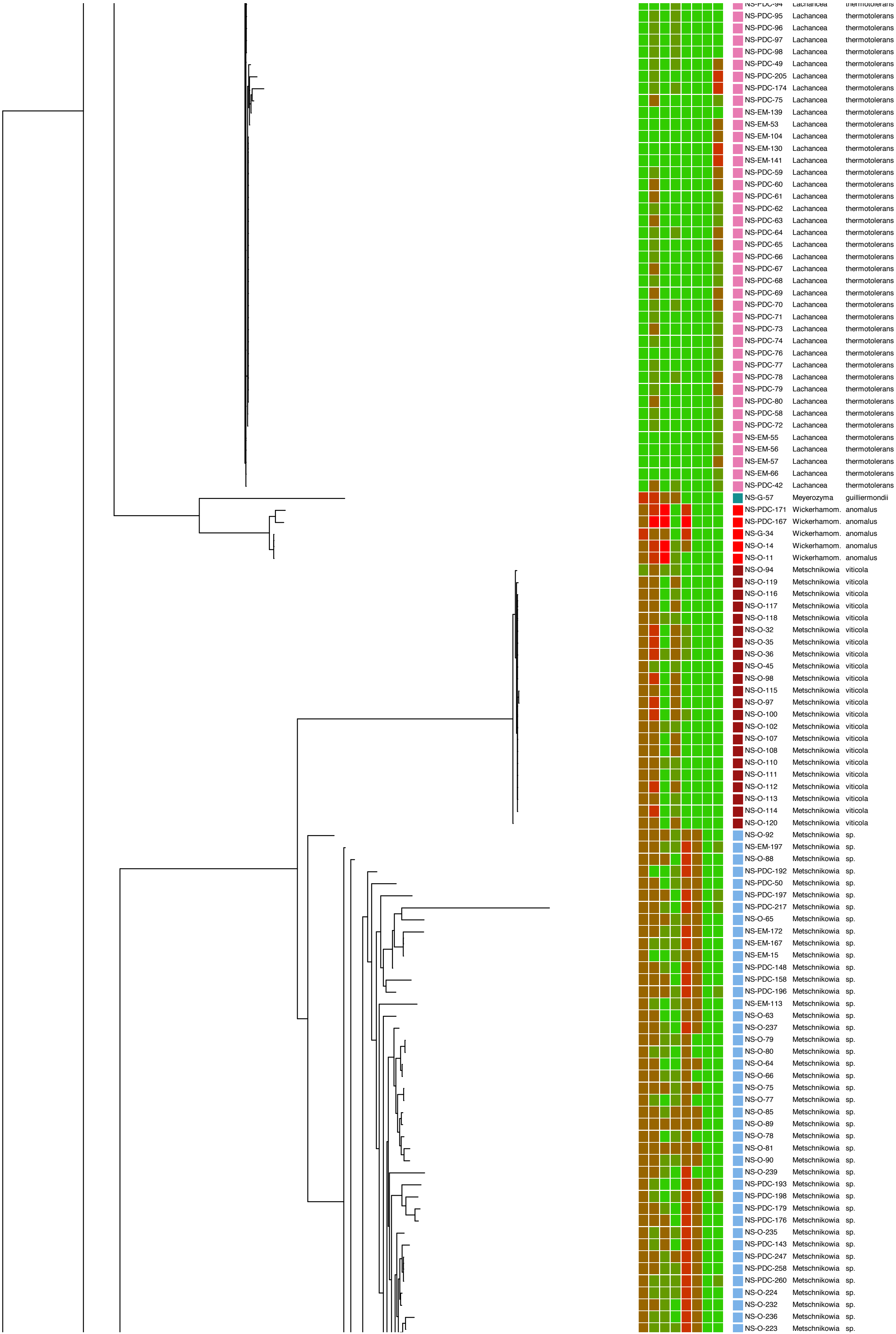
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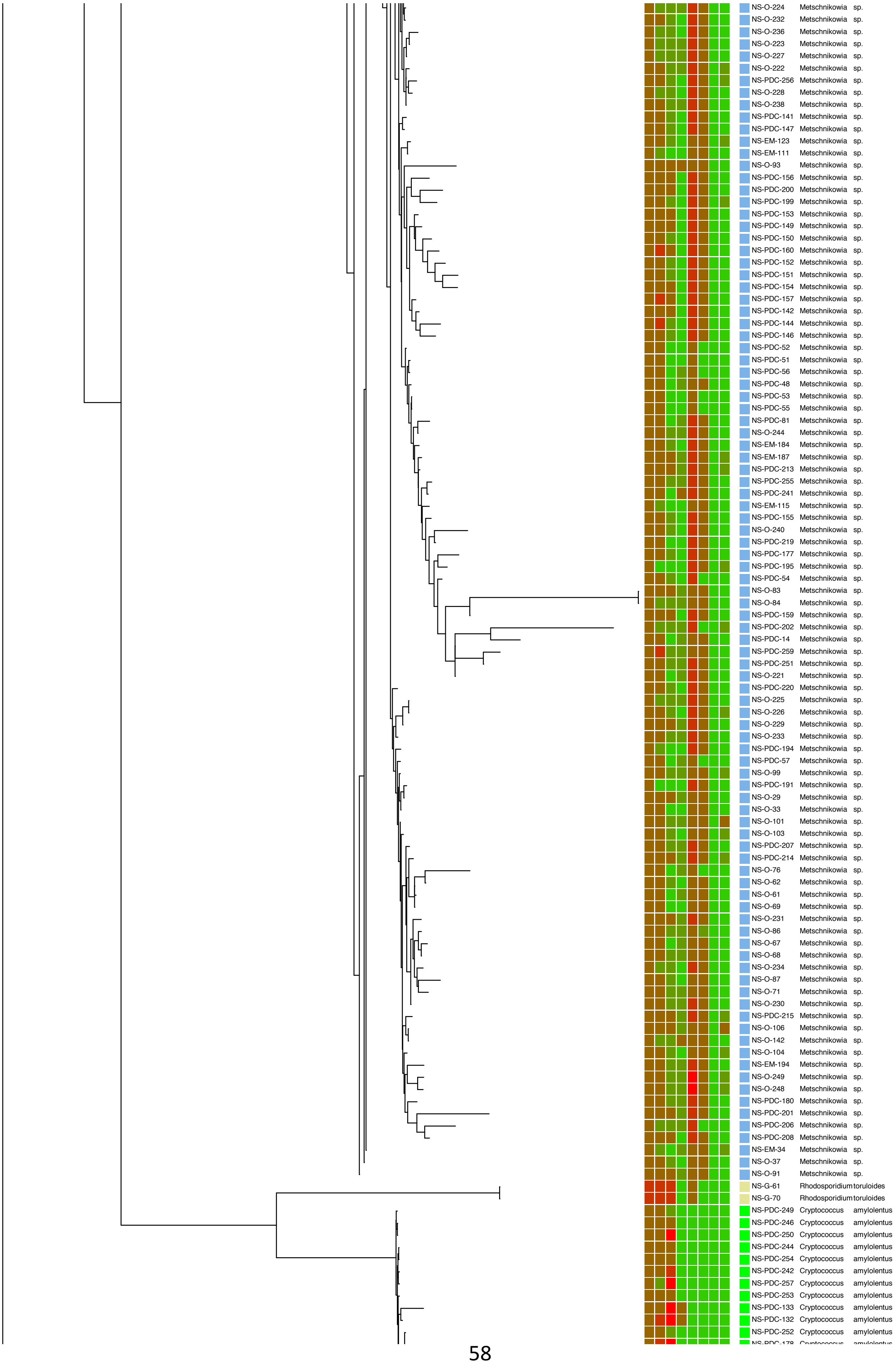
NS-PDC-77 Lachancea thermotolerans
NS-PDC-78 Lachancea thermotolerans
NS-PDC-79 Lachancea thermotolerans
NS-PDC-80 Lachancea thermotolerans
NS-PDC-58 Lachancea thermotolerans
NS-PDC-72 Lachancea thermotolerans
NS-EM-55 Lachancea thermotolerans
NS-EM-56 Lachancea thermotolerans
NS-EM-57 Lachancea thermotolerans
NS-EM-66 Lachancea thermotolerans
NS-PDC-42 Lachancea thermotolerans
NS-G-57 Meyerozyma guilliermondii
NS-PDC-171 Wickerhamom. anomalus
NS-PDC-167 Wickerhamom. anomalus
NS-G-34 Wickerhamom. anomalus
NS-O-14 Wickerhamom. anomalus
NS-O-11 Wickerhamom. anomalus
NS-O-94 Metschnikowia viticola
NS-O-119 Metschnikowia viticola
NS-O-116 Metschnikowia viticola
NS-O-117 Metschnikowia viticola
NS-O-118 Metschnikowia viticola
NS-O-32 Metschnikowia viticola
NS-O-35 Metschnikowia viticola
NS-O-36 Metschnikowia viticola
NS-O-45 Metschnikowia viticola
NS-O-98 Metschnikowia viticola
NS-O-115 Metschnikowia viticola
NS-O-97 Metschnikowia viticola
NS-O-100 Metschnikowia viticola
NS-O-102 Metschnikowia viticola
NS-O-107 Metschnikowia viticola
NS-O-108 Metschnikowia viticola
NS-O-110 Metschnikowia viticola
NS-O-111 Metschnikowia viticola
NS-O-112 Metschnikowia viticola
NS-O-113 Metschnikowia viticola
NS-O-114 Metschnikowia viticola
NS-O-120 Metschnikowia viticola
NS-O-92 Metschnikowia sp.
NS-EM-197 Metschnikowia sp.
NS-O-88 Metschnikowia sp.
NS-PDC-192 Metschnikowia sp.
NS-PDC-50 Metschnikowia sp.
NS-PDC-197 Metschnikowia sp.
NS-PDC-217 Metschnikowia sp.
NS-O-65 Metschnikowia sp.
NS-EM-172 Metschnikowia sp.
NS-EM-167 Metschnikowia sp.
NS-EM-15 Metschnikowia sp.
NS-PDC-148 Metschnikowia sp.
NS-PDC-158 Metschnikowia sp.
NS-PDC-196 Metschnikowia sp.
NS-EM-113 Metschnikowia sp.
NS-O-63 Metschnikowia sp.
NS-O-237 Metschnikowia sp.
NS-O-79 Metschnikowia sp.
NS-O-80 Metschnikowia sp.
NS-O-64 Metschnikowia sp.
NS-O-66 Metschnikowia sp.
NS-O-75 Metschnikowia sp.
NS-O-77 Metschnikowia sp.
NS-O-85 Metschnikowia sp.
NS-O-89 Metschnikowia sp.
NS-O-78 Metschnikowia sp.
NS-O-81 Metschnikowia sp.
NS-O-90 Metschnikowia sp.
NS-O-239 Metschnikowia sp.
NS-PDC-193 Metschnikowia sp.
NS-PDC-198 Metschnikowia sp.
NS-PDC-179 Metschnikowia sp.
NS-PDC-176 Metschnikowia sp.
NS-O-235 Metschnikowia sp.
NS-PDC-143 Metschnikowia sp.
NS-PDC-247 Metschnikowia sp.
NS-PDC-258 Metschnikowia sp.
NS-PDC-260 Metschnikowia sp.
NS-O-224 Metschnikowia sp.
NS-O-232 Metschnikowia sp.
NS-O-236 Metschnikowia sp.
NS-O-223 Metschnikowia sp.


NS-O-224 Metschnikowia sp.
NS-O-232 Metschnikowia sp.
NS-O-236 Metschnikowia sp.
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NS-O-227 Metschnikowia sp.
NS-O-222 Metschnikowia sp.
NS-PDC-256 Metschnikowia sp.
NS-O-228 Metschnikowia sp.
NS-O-238 Metschnikowia sp.
NS-PDC-141 Metschnikowia sp.
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NS-O-93 Metschnikowia sp.
NS-PDC-156 Metschnikowia sp.
NS-PDC-200 Metschnikowia sp.
NS-PDC-199 Metschnikowia sp.
NS-PDC-153 Metschnikowia sp.
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NS-PDC-52 Metschnikowia sp.
NS-PDC-51 Metschnikowia sp.
NS-PDC-56 Metschnikowia sp.
NS-PDC-48 Metschnikowia sp.
NS-PDC-53 Metschnikowia sp.
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NS-PDC-81 Metschnikowia sp.
NS-O-244 Metschnikowia sp.
NS-EM-184 Metschnikowia sp.
NS-EM-187 Metschnikowia sp.
NS-PDC-213 Metschnikowia sp.
NS-PDC-255 Metschnikowia sp.
NS-PDC-241 Metschnikowia sp.
NS-EM-115 Metschnikowia sp.
NS-PDC-155 Metschnikowia sp.
NS-O-240 Metschnikowia sp.
NS-PDC-219 Metschnikowia sp.
NS-PDC-177 Metschnikowia sp.
NS-PDC-195 Metschnikowia sp.
NS-PDC-54 Metschnikowia sp.
NS-O-83 Metschnikowia sp.
NS-O-84 Metschnikowia sp.
NS-PDC-159 Metschnikowia sp.
NS-PDC-202 Metschnikowia sp.
NS-PDC-14 Metschnikowia sp.
NS-PDC-259 Metschnikowia sp.
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NS-O-221 Metschnikowia sp.
NS-PDC-220 Metschnikowia sp.
NS-O-225 Metschnikowia sp.
NS-O-226 Metschnikowia sp.
NS-O-229 Metschnikowia sp.
NS-O-233 Metschnikowia sp.
NS-PDC-194 Metschnikowia sp.
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NS-O-99 Metschnikowia sp.
NS-PDC-191 Metschnikowia sp.
NS-O-29 Metschnikowia sp.
NS-O-33 Metschnikowia sp.
NS-O-101 Metschnikowia sp.
NS-O-103 Metschnikowia sp.
NS-PDC-207 Metschnikowia sp.
NS-PDC-214 Metschnikowia sp.
NS-O-76 Metschnikowia sp.
NS-O-62 Metschnikowia sp.
NS-O-61 Metschnikowia sp.
NS-O-69 Metschnikowia sp.
NS-O-231 Metschnikowia sp.
NS-O-86 Metschnikowia sp.
NS-O-67 Metschnikowia sp.
NS-O-68 Metschnikowia sp.
NS-O-234 Metschnikowia sp.
NS-O-87 Metschnikowia sp.
NS-O-71 Metschnikowia sp.
NS-O-230 Metschnikowia sp.
NS-PDC-215 Metschnikowia sp.
NS-O-106 Metschnikowia sp.
NS-O-142 Metschnikowia sp.
NS-O-104 Metschnikowia sp.
NS-EM-194 Metschnikowia sp.
NS-O-249 Metschnikowia sp.
NS-O-248 Metschnikowia sp.
NS-PDC-180 Metschnikowia sp.
NS-PDC-201 Metschnikowia sp.
NS-PDC-206 Metschnikowia sp.
NS-PDC-208 Metschnikowia sp.
NS-EM-34 Metschnikowia sp.
NS-O-37 Metschnikowia sp.
NS-O-91 Metschnikowia sp.
NS-G-61 Rhodosporidium toruloides
NS-G-70 Rhodosporidium toruloides
NS-PDC-249 Cryptococcus amylolentus
NS-PDC-246 Cryptococcus amylolentus
NS-PDC-250 Cryptococcus amylolentus
NS-PDC-244 Cryptococcus amylolentus
NS-PDC-254 Cryptococcus amylolentus
NS-PDC-242 Cryptococcus amylolentus
NS-PDC-257 Cryptococcus amylolentus
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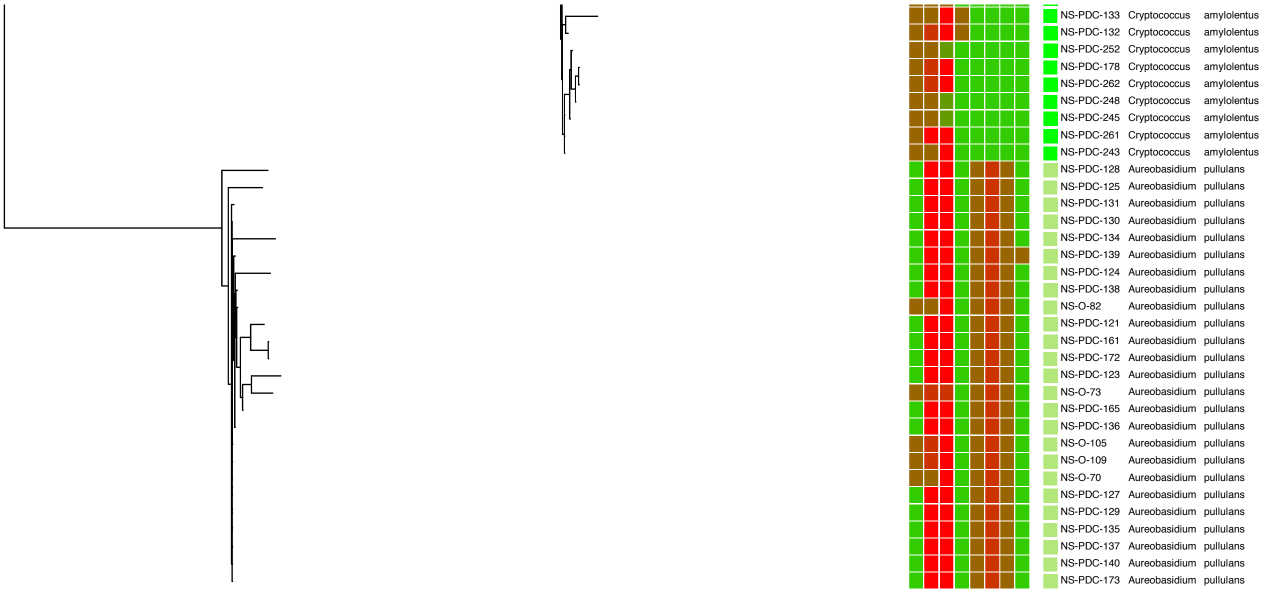
NS-PDC-133 Cryptococcus amylolentus
NS-PDC-132 Cryptococcus amylolentus
NS-PDC-252 Cryptococcus amylolentus


NS-PDC-133 Cryptococcus amylolentus
NS-PDC-132 Cryptococcus amylolentus
NS-PDC-252 Cryptococcus amylolentus
NS-PDC-178 Cryptococcus amylolentus
NS-PDC-262 Cryptococcus amylolentus
NS-PDC-248 Cryptococcus amylolentus
NS-PDC-245 Cryptococcus amylolentus
NS-PDC-261 Cryptococcus amylolentus
NS-PDC-243 Cryptococcus amylolentus
NS-PDC-128 Aureobasidium pullulans
NS-PDC-125 Aureobasidium pullulans
NS-PDC-131 Aureobasidium pullulans
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NS-PDC-138 Aureobasidium pullulans
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NS-PDC-121 Aureobasidium pullulans
NS-PDC-161 Aureobasidium pullulans
NS-PDC-172 Aureobasidium pullulans
NS-PDC-123 Aureobasidium pullulans
NS-O-73 Aureobasidium pullulans
NS-PDC-165 Aureobasidium pullulans
NS-PDC-136 Aureobasidium pullulans
NS-O-105 Aureobasidium pullulans
NS-O-109 Aureobasidium pullulans
NS-O-70 Aureobasidium pullulans
NS-PDC-127 Aureobasidium pullulans
NS-PDC-129 Aureobasidium pullulans
NS-PDC-135 Aureobasidium pullulans
NS-PDC-137 Aureobasidium pullulans
NS-PDC-140 Aureobasidium pullulans
NS-PDC-173 Aureobasidium pullulans

Supplementary material. Belda et al.

Unraveling the enzymatic basis of wine "flavorome": a phylo-functional study of wine related yeast species

Figure S2. Wine appellations sampled in the study indicating geographical and climatic data.

G vineyard
Wine apellation: Tierra de León
Grape variety: Prieto Picudo
Altitude: 747 m
Rainfall (total annual data): 224,9 mm (2012)
Mean maximum temperature: 18,5 ºC (2012)
Mean minimum temperature: 5,2 ºC (2012)

EM & PDC vineyards
Wine apellation: Ribera del Duero
Grape variety: Tempranillo
Altitude: 746 m (EM) & 754 m (PDC)
Rainfall (total annual data): 258,2 mm (2012) 444,8 mm (2013); 378,2 mm (2014)
Mean maximum temperature: 18,7 ºC (2012); 18 ºC (2013); 19,2 ºC (2014)
Mean minimum temperature: 5,4 ºC (2012); 5,7 ºC (2013); 7,1 ºC (2014)

O vineyards
Wine apellation: Rueda
Grape variety: Verdejo
Altitude: 842 m
Rainfall (total annual data): 280 mm (2012) 416,98 mm (2013); 350,7 mm (2014)
Mean maximum temperature: 19 ºC (2012); 18,6 ºC (2013); 19,1 ºC (2014)
Mean minimum temperature: 5,6 ºC (2012); 5,6 ºC (2013); 6,6 ºC (2014)

Supplementary material. Belda et al.
Unraveling the enzymatic basis of wine “flavorome”: a phylo-functional study of wine related yeast species

Table S1: Yeast collection with genbank accession numbers

ISOLATES IDENTIFICATION

Origin	Strain code	Genbank accession number	Identification (species)
🟩	NS-O-1	KT922724	*Hanseniaspora uvarum*
	NS-O-2	KT922725	*Hanseniaspora uvarum*
	NS-O-3	KT922726	*Hanseniaspora uvarum*
	NS-O-4	KT922727	*Hanseniaspora uvarum*
	NS-O-5	KT922728	*Hanseniaspora uvarum*
	NS-O-6	KT922729	*Hanseniaspora uvarum*
	NS-O-7	KT922730	*Hanseniaspora uvarum*
	NS-O-8	KT922731	*Hanseniaspora uvarum*
	NS-O-9	KT922732	*Hanseniaspora uvarum*
	NS-O-10	KT922733	*Hanseniaspora uvarum*
	NS-O-11	KT922734	*Wickerhamomyces anomalus*
	NS-O-12	KT922735	*Hanseniaspora uvarum*
	NS-O-13	KT922736	*Hanseniaspora uvarum*
	NS-O-14	KT922737	*Wickerhamomyces anomalus*
	NS-O-15	KT922738	*Hanseniaspora uvarum*
	NS-O-16	KT922739	*Hanseniaspora uvarum*
	NS-O-17	KT922740	*Hanseniaspora uvarum*
	NS-O-18	KT922741	*Hanseniaspora uvarum*
	NS-O-19	KT922742	*Hanseniaspora uvarum*
	NS-O-20	KT922743	*Hanseniaspora uvarum*
	NS-O-21	KT922744	*Hanseniaspora uvarum*
	NS-O-22	KT922745	*Hanseniaspora uvarum*
	NS-O-23	KT922746	*Hanseniaspora uvarum*
	NS-O-24	KT922747	*Hanseniaspora osmophila*
	NS-O-25	KT922748	*Hanseniaspora uvarum*
	NS-O-26	KT922749	*Hanseniaspora uvarum*
	NS-O-27	KT922750	*Hanseniaspora uvarum*
	NS-O-28	KT922751	*Hanseniaspora uvarum*
	NS-O-29	KT922752	*Metschnikowia sp.*
	NS-O-30	KT922753	*Hanseniaspora uvarum*
	NS-O-31	KT922754	*Hanseniaspora uvarum*
	NS-O-32	KT922755	*Metschnikowia viticola*
	NS-O-33	KT922756	*Metschnikowia sp.*
	NS-O-34	KT922757	*Hanseniaspora uvarum*
	NS-O-35	KT922758	*Metschnikowia viticola*
	NS-O-36	KT922759	*Metschnikowia viticola*
	NS-O-37	KT922760	*Metschnikowia sp.*
	NS-O-38	KT922761	*Hanseniaspora uvarum*
	NS-O-39	KT922762	*Hanseniaspora uvarum*

O (2013)	NS-O-40	KT922763	*Hanseniaspora uvarum*
	NS-O-41	KT922764	*Hanseniaspora uvarum*
	NS-O-42	KT922765	*Hanseniaspora uvarum*
	NS-O-43	KT922766	*Hanseniaspora uvarum*
	NS-O-44	KT922767	*Hanseniaspora uvarum*
	NS-O-45	KT922768	*Metschnikowia viticola*
	NS-O-46	KT922769	*Lachancea thermotolerans*
	NS-O-47	KT922770	*Hanseniaspora uvarum*
	NS-O-48	KT922771	*Hanseniaspora uvarum*
	NS-O-49	KT922772	*Hanseniaspora uvarum*
	NS-O-50	KT922773	*Hanseniaspora uvarum*
	NS-O-51	KT922774	*Hanseniaspora uvarum*
	NS-O-52	KT922775	*Hanseniaspora uvarum*
	NS-O-53	KT922776	*Hanseniaspora uvarum*
	NS-O-54	KT922777	*Hanseniaspora uvarum*
	NS-O-55	KT922778	*Hanseniaspora uvarum*
	NS-O-56	KT922779	*Hanseniaspora uvarum*
	NS-O-57	KT922780	*Hanseniaspora uvarum*
	NS-O-58	KT922781	*Hanseniaspora uvarum*
	NS-O-59	KT922782	*Hanseniaspora uvarum*
	NS-O-60	KT922783	*Hanseniaspora uvarum*
	NS-O-61	KT922784	*Metschnikowia sp.*
	NS-O-62	KT922785	*Metschnikowia sp.*
	NS-O-63	KT922786	*Metschnikowia sp.*
	NS-O-64	KT922787	*Metschnikowia sp.*
	NS-O-65	KT922788	*Metschnikowia sp.*
	NS-O-66	KT922789	*Metschnikowia sp.*
	NS-O-67	KT922790	*Metschnikowia sp.*
	NS-O-68	KT922791	*Metschnikowia sp.*
	NS-O-69	KT922792	*Metschnikowia sp.*
	NS-O-70	KT922793	*Aureobasidium pullulans*
	NS-O-71	KT922794	*Metschnikowia sp.*
	NS-O-73	KT922795	*Aureobasidium pullulans*
	NS-O-74	KT922796	*Hanseniaspora uvarum*
	NS-O-75	KT922797	*Metschnikowia sp.*
	NS-O-76	KT922798	*Metschnikowia sp.*
	NS-O-77	KT922799	*Metschnikowia sp.*
	NS-O-78	KT922800	*Metschnikowia sp.*
	NS-O-79	KT922801	*Metschnikowia sp.*
	NS-O-80	KT922802	*Metschnikowia sp.*
	NS-O-81	KT922803	*Metschnikowia sp.*
	NS-O-82	KT222663	*Aureobasidium pullulans*
	NS-O-83	KT922804	*Metschnikowia sp.*
	NS-O-84	KT922805	*Metschnikowia sp.*
	NS-O-85	KT922806	*Metschnikowia sp.*
	NS-O-86	KT922807	*Metschnikowia sp.*

🟩	NS-O-87	KT922808	*Metschnikowia sp.*
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	NS-O-91	KT922812	*Metschnikowia sp.*
	NS-O-92	KT922813	*Metschnikowia sp.*
	NS-O-93	KT922814	*Metschnikowia sp.*
	NS-O-94	KT922815	*Metschnikowia viticola*
	NS-O-97	KT922816	*Metschnikowia viticola*
	NS-O-98	KT922817	*Metschnikowia viticola*
	NS-O-99	KT922818	*Metschnikowia sp.*
	NS-O-100	KT922819	*Metschnikowia viticola*
	NS-O-101	KT922820	*Metschnikowia sp.*
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	NS-O-106	KT922825	*Metschnikowia sp.*
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	NS-O-118	KT922837	*Metschnikowia viticola*
	NS-O-119	KT922838	*Metschnikowia viticola*
	NS-O-120	KT922839	*Hanseniaspora uvarum*
🟩	NS-O-121	KT922840	*Hanseniaspora uvarum*
	NS-O-122	KT922841	*Hanseniaspora uvarum*
	NS-O-123	KT922842	*Hanseniaspora uvarum*
	NS-O-124	KT922843	*Hanseniaspora uvarum*
	NS-O-125	KT922844	*Hanseniaspora uvarum*
	NS-O-126	KT922845	*Hanseniaspora uvarum*
	NS-O-127	KT922846	*Hanseniaspora uvarum*
	NS-O-128	KT922847	*Hanseniaspora uvarum*
	NS-O-129	KT922848	*Hanseniaspora uvarum*
	NS-O-130	KT922849	*Hanseniaspora uvarum*
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	NS-O-134	KT922853	*Hanseniaspora uvarum*

	NS-O-135	KT922854	*Hanseniaspora uvarum*
	NS-O-136	KT922855	*Hanseniaspora uvarum*
	NS-O-137	KT922856	*Hanseniaspora uvarum*
	NS-O-138	KT922857	*Hanseniaspora uvarum*
	NS-O-139	KT922858	*Hanseniaspora uvarum*
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	NS-O-141	KT922860	*Hanseniaspora uvarum*
	NS-O-142	KT922861	*Metschnikowia sp.*
	NS-O-143	KT922862	*Hanseniaspora uvarum*
	NS-O-144	KT922863	*Hanseniaspora uvarum*
	NS-O-145	KT922864	*Hanseniaspora uvarum*
	NS-O-146	KT922865	*Hanseniaspora uvarum*
	NS-O-147	KT922866	*Hanseniaspora uvarum*
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	NS-O-149	KT922868	*Hanseniaspora uvarum*
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	NS-O-153	KT922872	*Hanseniaspora uvarum*
	NS-O-154	KT922873	*Hanseniaspora uvarum*
	NS-O-155	KT922874	*Hanseniaspora uvarum*
	NS-O-156	KT922875	*Hanseniaspora uvarum*
	NS-O-157	KT922876	*Hanseniaspora uvarum*
	NS-O-158	KT922877	*Hanseniaspora uvarum*
	NS-O-159	KT922878	*Hanseniaspora uvarum*
	NS-O-160	KT922879	*Hanseniaspora uvarum*
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	NS-O-162	KT922881	*Hanseniaspora uvarum*
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	NS-O-173	KT922892	*Hanseniaspora uvarum*
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	NS-O-177	KT922896	*Hanseniaspora uvarum*
	NS-O-178	KT922897	*Hanseniaspora uvarum*
	NS-O-179	KT922898	*Hanseniaspora uvarum*
	NS-O-180	KT922899	*Hanseniaspora uvarum*

O (2014)	NS-O-181	KT922900	*Hanseniaspora uvarum*
	NS-O-182	KT922901	*Hanseniaspora uvarum*
	NS-O-183	KT922902	*Hanseniaspora uvarum*
	NS-O-184	KT922903	*Hanseniaspora uvarum*
	NS-O-185	KT922904	*Hanseniaspora uvarum*
	NS-O-186	KT922905	*Hanseniaspora uvarum*
	NS-O-187	KT922906	*Hanseniaspora uvarum*
	NS-O-188	KT922907	*Hanseniaspora uvarum*
	NS-O-189	KT922908	*Hanseniaspora uvarum*
	NS-O-190	KT922909	*Hanseniaspora uvarum*
	NS-O-191	KT922910	*Hanseniaspora uvarum*
	NS-O-192	KT922911	*Hanseniaspora uvarum*
	NS-O-193	KT922912	*Hanseniaspora uvarum*
	NS-O-194	KT922913	*Hanseniaspora uvarum*
	NS-O-195	KT922914	*Hanseniaspora uvarum*
	NS-O-196	KT922915	*Hanseniaspora uvarum*
	NS-O-197	KT922916	*Hanseniaspora uvarum*
	NS-O-198	KT922917	*Hanseniaspora uvarum*
	NS-O-199	KT922918	*Hanseniaspora uvarum*
	NS-O-200	KT922919	*Hanseniaspora uvarum*
	NS-O-201	KT922920	*Hanseniaspora uvarum*
	NS-O-202	KT922921	*Hanseniaspora uvarum*
	NS-O-203	KT922922	*Hanseniaspora uvarum*
	NS-O-204	KT922923	*Hanseniaspora uvarum*
	NS-O-205	KT922924	*Hanseniaspora uvarum*
	NS-O-206	KT922925	*Hanseniaspora uvarum*
	NS-O-207	KT922926	*Hanseniaspora uvarum*
	NS-O-208	KT922927	*Hanseniaspora uvarum*
	NS-O-209	KT922928	*Hanseniaspora uvarum*
	NS-O-210	KT922929	*Hanseniaspora uvarum*
	NS-O-211	KT922930	*Hanseniaspora uvarum*
	NS-O-212	KT922931	*Hanseniaspora uvarum*
	NS-O-213	KT922932	*Hanseniaspora uvarum*
	NS-O-214	KT922933	*Hanseniaspora uvarum*
	NS-O-215	KT922934	*Hanseniaspora uvarum*
	NS-O-216	KT922935	*Hanseniaspora uvarum*
	NS-O-217	KT922936	*Hanseniaspora uvarum*
	NS-O-218	KT922937	*Hanseniaspora uvarum*
	NS-O-219	KT922938	*Hanseniaspora uvarum*
	NS-O-220	KT922939	*Hanseniaspora uvarum*
	NS-O-221	KT922940	*Metschnikowia sp.*
	NS-O-222	KT922941	*Metschnikowia sp.*
	NS-O-223	KT922942	*Metschnikowia sp.*
	NS-O-224	KT922943	*Metschnikowia sp.*
	NS-O-225	KT922944	*Metschnikowia sp.*
	NS-O-226	KT922945	*Metschnikowia sp.*

🟩	NS-O-227	KT922946	*Metschnikowia sp.*
	NS-O-228	KT922947	*Metschnikowia sp.*
	NS-O-229	KT922948	*Metschnikowia sp.*
	NS-O-230	KT922949	*Metschnikowia sp.*
	NS-O-231	KT922950	*Metschnikowia sp.*
	NS-O-232	KT922951	*Metschnikowia sp.*
	NS-O-233	KT922952	*Metschnikowia sp.*
	NS-O-234	KT922953	*Metschnikowia sp.*
	NS-O-235	KT922954	*Metschnikowia sp.*
	NS-O-236	KT922955	*Metschnikowia sp.*
	NS-O-237	KT922956	*Metschnikowia sp.*
	NS-O-238	KT922957	*Metschnikowia sp.*
	NS-O-239	KT922958	*Metschnikowia sp.*
	NS-O-240	KT922959	*Metschnikowia sp.*
	NS-O-241	KT922960	*Hanseniaspora uvarum*
	NS-O-242	KT922961	*Hanseniaspora uvarum*
	NS-O-243	KT922962	*Hanseniaspora uvarum*
	NS-O-244	KT922963	*Metschnikowia sp.*
	NS-O-245	KT922964	*Hanseniaspora uvarum*
	NS-O-246	KT922965	*Hanseniaspora uvarum*
	NS-O-247	KT922966	*Hanseniaspora uvarum*
	NS-O-248	KT922967	*Metschnikowia sp.*
	NS-O-249	KT922968	*Metschnikowia sp.*
	NS-O-250	KT922969	*Hanseniaspora uvarum*
🟦	NS-PDC-1	KT922471	*Hanseniaspora uvarum*
	NS-PDC-2	KT922472	*Hanseniaspora uvarum*
	NS-PDC-3	KT922473	*Hanseniaspora uvarum*
	NS-PDC-4	KT922474	*Hanseniaspora uvarum*
	NS-PDC-5	KT922475	*Hanseniaspora uvarum*
	NS-PDC-6	KT922476	*Hanseniaspora uvarum*
	NS-PDC-7	KT922477	*Hanseniaspora uvarum*
	NS-PDC-8	KT922478	*Hanseniaspora uvarum*
	NS-PDC-9	KT922479	*Hanseniaspora uvarum*
	NS-PDC-10	KT922480	*Hanseniaspora uvarum*
	NS-PDC-11	KT922481	*Hanseniaspora uvarum*
	NS-PDC-12	KT922482	*Hanseniaspora uvarum*
	NS-PDC-13	KT922483	*Hanseniaspora uvarum*
	NS-PDC-14	KT922484	*Metschnikowia sp.*
	NS-PDC-15	KT922485	*Hanseniaspora uvarum*
	NS-PDC-16	KT922486	*Hanseniaspora uvarum*
	NS-PDC-17	KT922487	*Hanseniaspora uvarum*
	NS-PDC-18	KT922488	*Hanseniaspora uvarum*
	NS-PDC-19	KT922489	*Hanseniaspora uvarum*
	NS-PDC-20	KT922490	*Hanseniaspora uvarum*
	NS-PDC-21	KT922491	*Hanseniaspora uvarum*
	NS-PDC-22	KT922492	*Hanseniaspora uvarum*

PDC (2013)	NS-PDC-23	KT922493	*Hanseniaspora uvarum*
	NS-PDC-24	KT922494	*Hanseniaspora uvarum*
	NS-PDC-25	KT922495	*Hanseniaspora uvarum*
	NS-PDC-26	KT922496	*Hanseniaspora uvarum*
	NS-PDC-27	KT922497	*Hanseniaspora uvarum*
	NS-PDC-28	KT922498	*Hanseniaspora uvarum*
	NS-PDC-29	KT922499	*Hanseniaspora uvarum*
	NS-PDC-30	KT922500	*Hanseniaspora uvarum*
	NS-PDC-31	KT922501	*Hanseniaspora uvarum*
	NS-PDC-32	KT922502	*Hanseniaspora uvarum*
	NS-PDC-33	KT922503	*Hanseniaspora uvarum*
	NS-PDC-34	KT922504	*Hanseniaspora uvarum*
	NS-PDC-35	KT922505	*Hanseniaspora uvarum*
	NS-PDC-36	KT922506	*Hanseniaspora uvarum*
	NS-PDC-37	KT922507	*Hanseniaspora uvarum*
	NS-PDC-38	KT922508	*Hanseniaspora uvarum*
	NS-PDC-39	KT886435	*Hanseniaspora uvarum*
	NS-PDC-40	KT922509	*Hanseniaspora uvarum*
	NS-PDC-41	KT922510	*Lachancea thermotolerans*
	NS-PDC-42	KT922511	*Lachancea thermotolerans*
	NS-PDC-43	KT922512	*Lachancea thermotolerans*
	NS-PDC-44	KT922513	*Lachancea thermotolerans*
	NS-PDC-45	KT922514	*Lachancea thermotolerans*
	NS-PDC-46	KT922515	*Lachancea thermotolerans*
	NS-PDC-47	KT922516	*Lachancea thermotolerans*
	NS-PDC-48	KT922517	*Metschnikowia sp.*
	NS-PDC-49	KT922518	*Lachancea thermotolerans*
	NS-PDC-50	KT922519	*Metschnikowia sp.*
	NS-PDC-51	KT922520	*Metschnikowia sp.*
	NS-PDC-52	KT922521	*Metschnikowia sp.*
	NS-PDC-53	KT922522	*Metschnikowia sp.*
	NS-PDC-54	KT922523	*Metschnikowia sp.*
	NS-PDC-55	KT922524	*Metschnikowia sp.*
	NS-PDC-56	KT922525	*Metschnikowia sp.*
	NS-PDC-57	KT922526	*Metschnikowia sp.*
	NS-PDC-58	KT922527	*Lachancea thermotolerans*
	NS-PDC-59	KT922528	*Lachancea thermotolerans*
	NS-PDC-60	KT922529	*Lachancea thermotolerans*
	NS-PDC-61	KT922530	*Lachancea thermotolerans*
	NS-PDC-62	KT922531	*Lachancea thermotolerans*
	NS-PDC-63	KT922532	*Lachancea thermotolerans*
	NS-PDC-64	KT922533	*Lachancea thermotolerans*
	NS-PDC-65	KT922534	*Lachancea thermotolerans*
	NS-PDC-66	KT922535	*Lachancea thermotolerans*
	NS-PDC-67	KT922536	*Lachancea thermotolerans*
	NS-PDC-68	KT922537	*Lachancea thermotolerans*

NS-PDC-69	KT922538	*Lachancea thermotolerans*
NS-PDC-70	KT922539	*Lachancea thermotolerans*
NS-PDC-71	KT922540	*Lachancea thermotolerans*
NS-PDC-72	KT922541	*Lachancea thermotolerans*
NS-PDC-73	KT922542	*Lachancea thermotolerans*
NS-PDC-74	KT922543	*Lachancea thermotolerans*
NS-PDC-75	KT922544	*Lachancea thermotolerans*
NS-PDC-76	KT922545	*Lachancea thermotolerans*
NS-PDC-77	KT922546	*Lachancea thermotolerans*
NS-PDC-78	KT922547	*Lachancea thermotolerans*
NS-PDC-79	KT922548	*Lachancea thermotolerans*
NS-PDC-80	KT922549	*Lachancea thermotolerans*
NS-PDC-81	KT922550	*Metschnikowia sp.*
NS-PDC-82	KT922551	*Lachancea thermotolerans*
NS-PDC-83	KT922552	*Lachancea thermotolerans*
NS-PDC-84	KT922553	*Lachancea thermotolerans*
NS-PDC-85	KT922554	*Lachancea thermotolerans*
NS-PDC-86	KT922555	*Lachancea thermotolerans*
NS-PDC-87	KT922556	*Lachancea thermotolerans*
NS-PDC-88	KT922557	*Lachancea thermotolerans*
NS-PDC-89	KT922558	*Lachancea thermotolerans*
NS-PDC-90	KT922559	*Lachancea thermotolerans*
NS-PDC-91	KT922560	*Lachancea thermotolerans*
NS-PDC-92	KT922561	*Lachancea thermotolerans*
NS-PDC-93	KT922562	*Lachancea thermotolerans*
NS-PDC-94	KT922563	*Lachancea thermotolerans*
NS-PDC-95	KT922564	*Lachancea thermotolerans*
NS-PDC-96	KT922565	*Lachancea thermotolerans*
NS-PDC-97	KT922566	*Lachancea thermotolerans*
NS-PDC-98	KT922567	*Lachancea thermotolerans*
NS-PDC-99	KT922568	*Kluyveromyces marxianus*
NS-PDC-100	KT922569	*Kluyveromyces marxianus*
NS-PDC-101	KT922570	*Hanseniaspora uvarum*
NS-PDC-102	KT922571	*Hanseniaspora uvarum*
NS-PDC-103	KT922572	*Hanseniaspora uvarum*
NS-PDC-104	KT922573	*Hanseniaspora uvarum*
NS-PDC-105	KT922574	*Hanseniaspora uvarum*
NS-PDC-106	KT922575	*Hanseniaspora uvarum*
NS-PDC-107	KT922576	*Hanseniaspora uvarum*
NS-PDC-108	KT922577	*Hanseniaspora uvarum*
NS-PDC-109	KT922578	*Hanseniaspora uvarum*
NS-PDC-110	KT922579	*Hanseniaspora uvarum*
NS-PDC-111	KT922580	*Hanseniaspora uvarum*
NS-PDC-112	KT922581	*Hanseniaspora uvarum*
NS-PDC-115	KT922582	*Hanseniaspora uvarum*
NS-PDC-116	KT922583	*Hanseniaspora uvarum*

■	NS-PDC-117	KT922584	*Hanseniaspora uvarum*
	NS-PDC-118	KT922585	*Hanseniaspora uvarum*
	NS-PDC-119	KT922586	*Hanseniaspora uvarum*
	NS-PDC-120	KT922587	*Hanseniaspora uvarum*
□	NS-PDC-121	KT922588	*Aureobasidium pullulans*
	NS-PDC-123	KT922589	*Aureobasidium pullulans*
	NS-PDC-124	KT922590	*Aureobasidium pullulans*
	NS-PDC-125	KT922591	*Aureobasidium pullulans*
	NS-PDC-127	KT922592	*Aureobasidium pullulans*
	NS-PDC-128	KT922593	*Aureobasidium pullulans*
	NS-PDC-129	KT922594	*Aureobasidium pullulans*
	NS-PDC-130	KT922595	*Aureobasidium pullulans*
	NS-PDC-131	KT922596	*Aureobasidium pullulans*
	NS-PDC-132	KT922597	*Cryptococcus amylolentus*
	NS-PDC-133	KT922598	*Cryptococcus amylolentus*
	NS-PDC-134	KT922599	*Aureobasidium pullulans*
	NS-PDC-135	KT922600	*Aureobasidium pullulans*
	NS-PDC-136	KT922601	*Aureobasidium pullulans*
	NS-PDC-137	KT922602	*Aureobasidium pullulans*
	NS-PDC-138	KT922603	*Aureobasidium pullulans*
	NS-PDC-139	KT922604	*Aureobasidium pullulans*
	NS-PDC-140	KT922605	*Aureobasidium pullulans*
	NS-PDC-141	KT922606	*Metschnikowia sp.*
	NS-PDC-142	KT922607	*Metschnikowia sp.*
	NS-PDC-143	KT922608	*Metschnikowia sp.*
	NS-PDC-144	KT922609	*Metschnikowia sp.*
	NS-PDC-146	KT922610	*Metschnikowia sp.*
	NS-PDC-147	KT922611	*Metschnikowia sp.*
	NS-PDC-148	KT922612	*Metschnikowia sp.*
	NS-PDC-149	KT922613	*Metschnikowia sp.*
	NS-PDC-150	KT922614	*Metschnikowia sp.*
	NS-PDC-151	KT922615	*Metschnikowia sp.*
	NS-PDC-152	KT922616	*Metschnikowia sp.*
	NS-PDC-153	KT922617	*Metschnikowia sp.*
	NS-PDC-154	KT922618	*Metschnikowia sp.*
	NS-PDC-155	KT922619	*Metschnikowia sp.*
	NS-PDC-156	KT922620	*Metschnikowia sp.*
	NS-PDC-157	KT922621	*Metschnikowia sp.*
	NS-PDC-158	KT922622	*Metschnikowia sp.*
	NS-PDC-159	KT922623	*Metschnikowia sp.*
	NS-PDC-160	KT922624	*Metschnikowia sp.*
	NS-PDC-161	KT922625	*Aureobasidium pullulans*
	NS-PDC-162	KT922626	*Hanseniaspora uvarum*
	NS-PDC-163	KT922627	*Hanseniaspora uvarum*
	NS-PDC-164	KT922628	*Hanseniaspora uvarum*
	NS-PDC-165	KT922629	*Aureobasidium pullulans*

PDC (2014)	NS-PDC-166	KT922630	*Hanseniaspora uvarum*
	NS-PDC-167	KT922631	*Wickerhamomyces anomalus*
	NS-PDC-168	KT922632	*Hanseniaspora uvarum*
	NS-PDC-169	KT922633	*Torulaspora delbrueckii*
	NS-PDC-170	KT922634	*Hanseniaspora uvarum*
	NS-PDC-171	KT922635	*Wickerhamomyces anomalus*
	NS-PDC-172	KT922636	*Aureobasidium pullulans*
	NS-PDC-173	KT922637	*Aureobasidium pullulans*
	NS-PDC-174	KT922638	*Lachancea thermotolerans*
	NS-PDC-175	KT922639	*Hanseniaspora uvarum*
	NS-PDC-176	KT922640	*Metschnikowia sp.*
	NS-PDC-177	KT922641	*Metschnikowia sp.*
	NS-PDC-178	KT922642	*Cryptococcus amylolentus*
	NS-PDC-179	KT922643	*Metschnikowia sp.*
	NS-PDC-180	KT922644	*Metschnikowia sp.*
	NS-PDC-181	KT922645	*Hanseniaspora uvarum*
	NS-PDC-182	KT922646	*Hanseniaspora uvarum*
	NS-PDC-183	KT922647	*Hanseniaspora uvarum*
	NS-PDC-184	KT922648	*Hanseniaspora uvarum*
	NS-PDC-185	KT922649	*Hanseniaspora uvarum*
	NS-PDC-186	KT922650	*Hanseniaspora uvarum*
	NS-PDC-187	KT922651	*Hanseniaspora uvarum*
	NS-PDC-188	KT922652	*Hanseniaspora uvarum*
	NS-PDC-189	KT922653	*Hanseniaspora uvarum*
	NS-PDC-190	KT922654	*Hanseniaspora uvarum*
	NS-PDC-191	KT922655	*Metschnikowia sp.*
	NS-PDC-192	KT922656	*Metschnikowia sp.*
	NS-PDC-193	KT922657	*Metschnikowia sp.*
	NS-PDC-194	KT922658	*Metschnikowia sp.*
	NS-PDC-195	KT922659	*Metschnikowia sp.*
	NS-PDC-196	KT922660	*Metschnikowia sp.*
	NS-PDC-197	KT922661	*Metschnikowia sp.*
	NS-PDC-198	KT922662	*Metschnikowia sp.*
	NS-PDC-199	KT922663	*Metschnikowia sp.*
	NS-PDC-200	KT922664	*Metschnikowia sp.*
	NS-PDC-201	KT922665	*Metschnikowia sp.*
	NS-PDC-202	KT922666	*Metschnikowia sp.*
	NS-PDC-205	KT922667	*Lachancea thermotolerans*
	NS-PDC-206	KT922668	*Metschnikowia sp.*
	NS-PDC-207	KT922669	*Metschnikowia sp.*
	NS-PDC-208	KT922670	*Metschnikowia sp.*
	NS-PDC-209	KT922671	*Hanseniaspora uvarum*
	NS-PDC-210	KT922672	*Hanseniaspora uvarum*
	NS-PDC-211	KT922673	*Hanseniaspora uvarum*
	NS-PDC-212	KT922674	*Hanseniaspora uvarum*
	NS-PDC-213	KT922675	*Metschnikowia sp.*

	NS-PDC-214	KT922676	*Metschnikowia sp.*
	NS-PDC-215	KT922677	*Metschnikowia sp.*
	NS-PDC-217	KT922678	*Metschnikowia sp.*
	NS-PDC-218	KT922679	*Hanseniaspora uvarum*
	NS-PDC-219	KT922680	*Metschnikowia sp.*
	NS-PDC-220	KT922681	*Metschnikowia sp.*
	NS-PDC-221	KT922682	*Hanseniaspora uvarum*
	NS-PDC-222	KT922683	*Hanseniaspora uvarum*
	NS-PDC-223	KT922684	*Hanseniaspora uvarum*
	NS-PDC-224	KT922685	*Hanseniaspora uvarum*
	NS-PDC-225	KT922686	*Hanseniaspora uvarum*
	NS-PDC-226	KT922687	*Hanseniaspora uvarum*
	NS-PDC-227	KT922688	*Hanseniaspora uvarum*
	NS-PDC-228	KT922689	*Hanseniaspora uvarum*
	NS-PDC-229	KT922690	*Hanseniaspora uvarum*
	NS-PDC-230	KT922691	*Hanseniaspora uvarum*
	NS-PDC-231	KT922692	*Hanseniaspora uvarum*
	NS-PDC-232	KT922693	*Hanseniaspora uvarum*
	NS-PDC-233	KT922694	*Hanseniaspora uvarum*
	NS-PDC-234	KT922695	*Hanseniaspora uvarum*
	NS-PDC-235	KT922696	*Hanseniaspora uvarum*
	NS-PDC-236	KT922697	*Hanseniaspora uvarum*
	NS-PDC-237	KT922698	*Hanseniaspora uvarum*
	NS-PDC-238	KT922699	*Hanseniaspora uvarum*
	NS-PDC-239	KT922700	*Hanseniaspora uvarum*
	NS-PDC-240	KT922701	*Hanseniaspora uvarum*
	NS-PDC-241	KT922702	*Metschnikowia sp.*
	NS-PDC-242	KT922703	*Cryptococcus amylolentus*
	NS-PDC-243	KT922704	*Cryptococcus amylolentus*
	NS-PDC-244	KT922705	*Cryptococcus amylolentus*
	NS-PDC-245	KT922706	*Cryptococcus amylolentus*
	NS-PDC-246	KT922707	*Cryptococcus amylolentus*
	NS-PDC-247	KT922708	*Metschnikowia sp.*
	NS-PDC-248	KT922709	*Cryptococcus amylolentus*
	NS-PDC-249	KT922710	*Cryptococcus amylolentus*
	NS-PDC-250	KT922711	*Cryptococcus amylolentus*
	NS-PDC-251	KT922712	*Metschnikowia sp.*
	NS-PDC-252	KT922713	*Cryptococcus amylolentus*
	NS-PDC-253	KT922714	*Cryptococcus amylolentus*
	NS-PDC-254	KT922715	*Cryptococcus amylolentus*
	NS-PDC-255	KT922716	*Metschnikowia sp.*
	NS-PDC-256	KT922717	*Metschnikowia sp.*
	NS-PDC-257	KT922718	*Cryptococcus amylolentus*
	NS-PDC-258	KT922719	*Metschnikowia sp.*
	NS-PDC-259	KT922720	*Metschnikowia sp.*
	NS-PDC-260	KT922721	*Metschnikowia sp.*

🟦	NS-PDC-261	KT922722	*Cryptococcus amylolentus*
	NS-PDC-262	KT922723	*Cryptococcus amylolentus*
🟥	NS-EM-1	KT922276	*Hanseniaspora uvarum*
	NS-EM-2	KT922277	*Hanseniaspora uvarum*
	NS-EM-3	KT922278	*Hanseniaspora uvarum*
	NS-EM-4	KT922279	*Hanseniaspora uvarum*
	NS-EM-5	KT922280	*Hanseniaspora uvarum*
	NS-EM-6	KT922281	*Hanseniaspora uvarum*
	NS-EM-7	KT922282	*Hanseniaspora uvarum*
	NS-EM-8	KT922283	*Hanseniaspora uvarum*
	NS-EM-9	KT922284	*Hanseniaspora uvarum*
	NS-EM-10	KT922285	*Hanseniaspora uvarum*
	NS-EM-12	KT922286	*Hanseniaspora uvarum*
	NS-EM-13	KT922287	*Hanseniaspora uvarum*
	NS-EM-14	KT922288	*Hanseniaspora uvarum*
	NS-EM-15	KT922289	*Metschnikowia sp.*
	NS-EM-16	KT922290	*Hanseniaspora uvarum*
	NS-EM-17	KT922291	*Hanseniaspora uvarum*
	NS-EM-18	KT922292	*Hanseniaspora uvarum*
	NS-EM-19	KT922293	*Hanseniaspora uvarum*
	NS-EM-20	KT922294	*Hanseniaspora uvarum*
	NS-EM-21	KT922295	*Hanseniaspora uvarum*
	NS-EM-22	KT922296	*Hanseniaspora uvarum*
	NS-EM-23	KT922297	*Hanseniaspora uvarum*
	NS-EM-24	KT922298	*Hanseniaspora uvarum*
	NS-EM-25	KT922299	*Hanseniaspora uvarum*
	NS-EM-26	KT922300	*Hanseniaspora uvarum*
	NS-EM-27	KT922301	*Hanseniaspora uvarum*
	NS-EM-28	KT922302	*Hanseniaspora uvarum*
	NS-EM-29	KT922303	*Hanseniaspora uvarum*
	NS-EM-30	KT922304	*Hanseniaspora uvarum*
	NS-EM-31	KT922305	*Hanseniaspora uvarum*
	NS-EM-32	KT922306	*Hanseniaspora uvarum*
	NS-EM-33	KT922307	*Hanseniaspora uvarum*
	NS-EM-34	KT222665	*Metschnikowia sp.*
	NS-EM-36	KT922308	*Hanseniaspora uvarum*
	NS-EM-37	KT922309	*Hanseniaspora uvarum*
	NS-EM-38	KT922310	*Hanseniaspora uvarum*
	NS-EM-39	KT922311	*Hanseniaspora uvarum*
	NS-EM-40	KT922312	*Hanseniaspora uvarum*
	NS-EM-41	KT922313	*Hanseniaspora uvarum*
	NS-EM-42	KT922314	*Hanseniaspora uvarum*
	NS-EM-43	KT922315	*Hanseniaspora uvarum*
	NS-EM-44	KT922316	*Hanseniaspora uvarum*
	NS-EM-45	KT922317	*Hanseniaspora uvarum*
	NS-EM-46	KT922318	*Hanseniaspora uvarum*

EM (2013)	NS-EM-47	KT922319	*Hanseniaspora uvarum*
	NS-EM-48	KT922320	*Hanseniaspora uvarum*
	NS-EM-49	KT922321	*Hanseniaspora uvarum*
	NS-EM-50	KT922322	*Hanseniaspora uvarum*
	NS-EM-51	KT922323	*Lachancea thermotolerans*
	NS-EM-52	KT922324	*Lachancea thermotolerans*
	NS-EM-53	KT922325	*Lachancea thermotolerans*
	NS-EM-54	KT922326	*Lachancea thermotolerans*
	NS-EM-55	KT922327	*Lachancea thermotolerans*
	NS-EM-56	KT922328	*Lachancea thermotolerans*
	NS-EM-57	KT922329	*Lachancea thermotolerans*
	NS-EM-58	KT922330	*Lachancea thermotolerans*
	NS-EM-59	KT922331	*Lachancea thermotolerans*
	NS-EM-60	KT922332	*Lachancea thermotolerans*
	NS-EM-61	KT922333	*Lachancea thermotolerans*
	NS-EM-62	KT922334	*Lachancea thermotolerans*
	NS-EM-63	KT922335	*Lachancea thermotolerans*
	NS-EM-64	KT922336	*Lachancea thermotolerans*
	NS-EM-65	KT922337	*Lachancea thermotolerans*
	NS-EM-66	KT922338	*Lachancea thermotolerans*
	NS-EM-67	KT922339	*Lachancea thermotolerans*
	NS-EM-68	KT922340	*Lachancea thermotolerans*
	NS-EM-69	KT922341	*Lachancea thermotolerans*
	NS-EM-70	KT922342	*Lachancea thermotolerans*
	NS-EM-71	KT922343	*Lachancea thermotolerans*
	NS-EM-72	KT922344	*Lachancea thermotolerans*
	NS-EM-73	KT922345	*Lachancea thermotolerans*
	NS-EM-74	KT922346	*Lachancea thermotolerans*
	NS-EM-75	KT922347	*Lachancea thermotolerans*
	NS-EM-76	KT922348	*Hanseniaspora uvarum*
	NS-EM-77	KT922349	*Hanseniaspora uvarum*
	NS-EM-78	KT922350	*Hanseniaspora uvarum*
	NS-EM-80	KT922351	*Hanseniaspora uvarum*
	NS-EM-81	KT922352	*Hanseniaspora uvarum*
	NS-EM-82	KT922353	*Hanseniaspora uvarum*
	NS-EM-83	KT922354	*Hanseniaspora uvarum*
	NS-EM-84	KT922355	*Hanseniaspora uvarum*
	NS-EM-85	KT922356	*Hanseniaspora uvarum*
	NS-EM-86	KT922357	*Hanseniaspora uvarum*
	NS-EM-87	KT922358	*Hanseniaspora uvarum*
	NS-EM-88	KT922359	*Hanseniaspora uvarum*
	NS-EM-89	KT922360	*Hanseniaspora uvarum*
	NS-EM-90	KT922361	*Hanseniaspora uvarum*
	NS-EM-91	KT922362	*Hanseniaspora uvarum*
	NS-EM-92	KT922363	*Hanseniaspora uvarum*
	NS-EM-93	KT922364	*Hanseniaspora uvarum*

🟥	NS-EM-94	KT922365	*Hanseniaspora uvarum*
	NS-EM-95	KT922366	*Hanseniaspora uvarum*
	NS-EM-96	KT922367	*Hanseniaspora uvarum*
	NS-EM-97	KT922368	*Hanseniaspora uvarum*
	NS-EM-98	KT922369	*Hanseniaspora uvarum*
	NS-EM-99	KT922370	*Hanseniaspora uvarum*
	NS-EM-100	KT922371	*Hanseniaspora uvarum*
🟥	NS-EM-101	KT922372	*Hanseniaspora uvarum*
	NS-EM-102	KT922373	*Hanseniaspora uvarum*
	NS-EM-103	KT922374	*Hanseniaspora uvarum*
	NS-EM-104	KT922375	*Lachancea thermotolerans*
	NS-EM-105	KT922376	*Hanseniaspora uvarum*
	NS-EM-106	KT922377	*Hanseniaspora uvarum*
	NS-EM-107	KT922378	*Hanseniaspora uvarum*
	NS-EM-108	KT922379	*Hanseniaspora uvarum*
	NS-EM-109	KT922380	*Hanseniaspora uvarum*
	NS-EM-110	KT922381	*Hanseniaspora uvarum*
	NS-EM-111	KT922382	*Metschnikowia sp.*
	NS-EM-112	KT922383	*Hanseniaspora uvarum*
	NS-EM-113	KT922384	*Metschnikowia sp.*
	NS-EM-114	KT922385	*Hanseniaspora uvarum*
	NS-EM-115	KT922386	*Metschnikowia sp.*
	NS-EM-116	KT922387	*Hanseniaspora uvarum*
	NS-EM-117	KT922388	*Hanseniaspora uvarum*
	NS-EM-118	KT922389	*Hanseniaspora uvarum*
	NS-EM-119	KT922390	*Lachancea thermotolerans*
	NS-EM-120	KT922391	*Hanseniaspora uvarum*
	NS-EM-121	KT922392	*Hanseniaspora uvarum*
	NS-EM-122	KT922393	*Hanseniaspora uvarum*
	NS-EM-123	KT922394	*Metschnikowia sp.*
	NS-EM-124	KT922395	*Hanseniaspora uvarum*
	NS-EM-125	KT922396	*Hanseniaspora uvarum*
	NS-EM-126	KT922397	*Hanseniaspora uvarum*
	NS-EM-127	KT922398	*Hanseniaspora uvarum*
	NS-EM-128	KT922399	*Hanseniaspora uvarum*
	NS-EM-129	KT922400	*Hanseniaspora uvarum*
	NS-EM-130	KT922401	*Lachancea thermotolerans*
	NS-EM-131	KT922402	*Hanseniaspora uvarum*
	NS-EM-132	KT922403	*Hanseniaspora uvarum*
	NS-EM-133	KT922404	*Hanseniaspora uvarum*
	NS-EM-134	KT922405	*Hanseniaspora uvarum*
	NS-EM-135	KT922406	*Hanseniaspora uvarum*
	NS-EM-136	KT922407	*Lachancea thermotolerans*
	NS-EM-137	KT922408	*Hanseniaspora uvarum*
	NS-EM-138	KT922409	*Hanseniaspora uvarum*
	NS-EM-139	KT922410	*Lachancea thermotolerans*

EM (2014)	NS-EM-140	KT922411	*Hanseniaspora uvarum*
	NS-EM-141	KT922412	*Lachancea thermotolerans*
	NS-EM-142	KT922413	*Hanseniaspora uvarum*
	NS-EM-143	KT922414	*Hanseniaspora uvarum*
	NS-EM-144	KT922415	*Hanseniaspora uvarum*
	NS-EM-145	KT922416	*Hanseniaspora uvarum*
	NS-EM-146	KT922417	*Hanseniaspora uvarum*
	NS-EM-147	KT922418	*Hanseniaspora uvarum*
	NS-EM-148	KT922419	*Hanseniaspora uvarum*
	NS-EM-149	KT922420	*Hanseniaspora uvarum*
	NS-EM-150	KT922421	*Hanseniaspora uvarum*
	NS-EM-151	KT922422	*Hanseniaspora uvarum*
	NS-EM-152	KT922423	*Hanseniaspora uvarum*
	NS-EM-153	KT922424	*Hanseniaspora uvarum*
	NS-EM-154	KT922425	*Hanseniaspora uvarum*
	NS-EM-155	KT922426	*Hanseniaspora uvarum*
	NS-EM-156	KT922427	*Hanseniaspora uvarum*
	NS-EM-157	KT922428	*Hanseniaspora uvarum*
	NS-EM-158	KT922429	*Hanseniaspora uvarum*
	NS-EM-159	KT922430	*Hanseniaspora uvarum*
	NS-EM-160	KT922431	*Hanseniaspora uvarum*
	NS-EM-161	KT922432	*Hanseniaspora uvarum*
	NS-EM-162	KT922433	*Hanseniaspora uvarum*
	NS-EM-163	KT922434	*Hanseniaspora uvarum*
	NS-EM-164	KT922435	*Hanseniaspora uvarum*
	NS-EM-165	KT922436	*Hanseniaspora uvarum*
	NS-EM-166	KT922437	*Hanseniaspora uvarum*
	NS-EM-167	KT922438	*Metschnikowia sp.*
	NS-EM-168	KT922439	*Hanseniaspora uvarum*
	NS-EM-169	KT922440	*Hanseniaspora uvarum*
	NS-EM-170	KT922441	*Hanseniaspora uvarum*
	NS-EM-171	KT922442	*Hanseniaspora uvarum*
	NS-EM-172	KT922443	*Metschnikowia sp.*
	NS-EM-173	KT922444	*Hanseniaspora uvarum*
	NS-EM-174	KT922445	*Hanseniaspora uvarum*
	NS-EM-175	KT922446	*Hanseniaspora uvarum*
	NS-EM-176	KT922447	*Hanseniaspora uvarum*
	NS-EM-177	KT922448	*Hanseniaspora uvarum*
	NS-EM-178	KT922449	*Hanseniaspora uvarum*
	NS-EM-179	KT922450	*Hanseniaspora uvarum*
	NS-EM-180	KT922451	*Hanseniaspora uvarum*
	NS-EM-181	KT922452	*Hanseniaspora uvarum*
	NS-EM-182	KT922453	*Hanseniaspora uvarum*
	NS-EM-183	KT922454	*Hanseniaspora uvarum*
	NS-EM-184	KT922455	*Metschnikowia sp.*
	NS-EM-186	KT922456	*Hanseniaspora uvarum*

🟥	NS-EM-187	KT922457	*Metschnikowia sp.*
	NS-EM-188	KT922458	*Hanseniaspora uvarum*
	NS-EM-189	KT922459	*Hanseniaspora uvarum*
	NS-EM-190	KT922460	*Hanseniaspora uvarum*
	NS-EM-191	KT922461	*Hanseniaspora uvarum*
	NS-EM-192	KT922462	*Hanseniaspora uvarum*
	NS-EM-193	KT922463	*Hanseniaspora uvarum*
	NS-EM-194	KT922464	*Metschnikowia sp.*
	NS-EM-195	KT922465	*Hanseniaspora uvarum*
	NS-EM-196	KT922466	*Hanseniaspora uvarum*
	NS-EM-197	KT922467	*Metschnikowia sp.*
	NS-EM-198	KT922468	*Hanseniaspora uvarum*
	NS-EM-199	KT922469	*Hanseniaspora uvarum*
	NS-EM-200	KT922470	*Hanseniaspora uvarum*
🟨	NS-G-1	KT922970	*Hanseniaspora uvarum*
	NS-G-2	KT922971	*Lachancea thermotolerans*
	NS-G-3	KT922972	*Lachancea thermotolerans*
	NS-G-4	KT922973	*Hanseniaspora opuntiae*
	NS-G-5	KT922974	*Hanseniaspora opuntiae*
	NS-G-6	KT922975	*Lachancea thermotolerans*
	NS-G-7	KT922976	*Hanseniaspora opuntiae*
	NS-G-8	KT922977	*Hanseniaspora opuntiae*
	NS-G-9	KT922978	*Torulaspora delbrueckii*
	NS-G-10	KT922979	*Hanseniaspora uvarum*
	NS-G-11	KT922980	*Hanseniaspora uvarum*
	NS-G-12	KT922981	*Hanseniaspora uvarum*
	NS-G-13	KT922982	*Lachancea thermotolerans*
	NS-G-14	KT922983	*Hanseniaspora uvarum*
	NS-G-15	KT922984	*Hanseniaspora opuntiae*
	NS-G-16	KT922985	*Hanseniaspora opuntiae*
	NS-G-17	KT922986	*Hanseniaspora uvarum*
	NS-G-18	KT922987	*Hanseniaspora uvarum*
	NS-G-19	KT922988	*Hanseniaspora uvarum*
	NS-G-20	KT922989	*Hanseniaspora opuntiae*
	NS-G-21	KT922990	*Hanseniaspora uvarum*
	NS-G-22	KT922991	*Hanseniaspora uvarum*
	NS-G-23	KT922992	*Hanseniaspora opuntiae*
	NS-G-24	KT922993	*Saccharomyces cerevisiae*
	NS-G-25	KT922994	*Lachancea thermotolerans*
	NS-G-26	KT922995	*Hanseniaspora uvarum*
	NS-G-27	KT922996	*Torulaspora delbrueckii*
	NS-G-28	KT922997	*Hanseniaspora uvarum*
	NS-G-29	KT922998	*Hanseniaspora uvarum*
	NS-G-30	KT922999	*Saccharomyces cerevisiae*
	NS-G-31	KT923000	*Saccharomyces cerevisiae*
	NS-G-32	KT222664	*Lachancea thermotolerans*

G (2012)	NS-G-33	KT923001	*Hanseniaspora osmophila*
	NS-G-34	KT923002	*Wickerhamomyces anomalus*
	NS-G-35	KT923003	*Hanseniaspora osmophila*
	NS-G-36	KT923004	*Hanseniaspora osmophila*
	NS-G-37	KT923005	*Saccharomyces cerevisiae*
	NS-G-38	KT923006	*Hanseniaspora osmophila*
	NS-G-39	KT923007	*Hanseniaspora osmophila*
	NS-G-40	KT923008	*Hanseniaspora osmophila*
	NS-G-41	KT923009	*Hanseniaspora osmophila*
	NS-G-42	KT923010	*Saccharomyces cerevisiae*
	NS-G-43	KT923011	*Hanseniaspora osmophila*
	NS-G-44	KT923012	*Saccharomyces cerevisiae*
	NS-G-45	KT923013	*Hanseniaspora osmophila*
	NS-G-46	KT923014	*Torulaspora delbrueckii*
	NS-G-47	KT923015	*Hanseniaspora osmophila*
	NS-G-48	KT923016	*Saccharomyces cerevisiae*
	NS-G-49	KT923017	*Hanseniaspora osmophila*
	NS-G-50	KT923018	*Saccharomyces cerevisiae*
	NS-G-51	KT923019	*Hanseniaspora osmophila*
	NS-G-52	KT923020	*Saccharomyces cerevisiae*
	NS-G-53	KT923021	*Hanseniaspora osmophila*
	NS-G-54	KT923022	*Saccharomyces cerevisiae*
	NS-G-55	KT923023	*Saccharomyces cerevisiae*
	NS-G-56	KT923024	*Hanseniaspora osmophila*
	NS-G-57	KT923025	*Meyerozyma guilliermondii*
	NS-G-58	KT923026	*Zygosaccharomyces baillii*
	NS-G-59	KT923027	*Hanseniaspora uvarum*
	NS-G-60	KT923028	*Hanseniaspora uvarum*
	NS-G-61	KT923029	*Rhodosporidium toruloides*
	NS-G-62	KT923030	*Torulaspora delbrueckii*
	NS-G-63	KT923031	*Zygosaccharomyces baillii*
	NS-G-64	KT923032	*Hanseniaspora uvarum*
	NS-G-65	KT923033	*Hanseniaspora uvarum*
	NS-G-66	KT923034	*Torulaspora delbrueckii*
	NS-G-67	KT923035	*Hanseniaspora uvarum*
	NS-G-68	KT923036	*Hanseniaspora uvarum*
	NS-G-69	KT923037	*Hanseniaspora uvarum*
	NS-G-70	KT923038	*Rhodosporidium toruloides*
	NS-G-71	KT923039	*Torulaspora delbrueckii*
	NS-G-72	KT923040	*Torulaspora delbrueckii*
	NS-G-73	KT923041	*Hanseniaspora uvarum*

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Table S2a. Component scores of the PCA analysis of *Torulaspora delbrueckii* isolates.

	PC1	**PC2**	**PC3**	**PC4**
PCA	65,39%	19,84%	14,77%	0,00%
β-glucosidase	-2,7525972	-0,4350818	0,4836448	0
β-D-xylosidase	-0,454887	-0,2758188	-0,7694166	0
α-L-arabinofuranosidase	0,454887	0,2758188	0,7694166	0
β-lyase	0	0	0	0
Protease	0	0	0	0
Pectinase	-1,0191866	1,5443728	-0,2759914	0
Cellulase	0	0	0	0
Hydrogen sulfide	0,454887	0,2758188	0,7694166	0

Table S2b. Component scores of the PCA analysis of *Aureobasidium pullulans* isolates.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PCA	81,84%	12,71%	3,96%	1,49%	0,00%
β-glucosidase	-3,9674513	-0,0657378	0,3954627	-0,3140354	0
β-D-xylosidase	2,9145225	0,0674117	0,6352186	-0,3708255	0
α-L-arabinofuranosidase	0,3608266	-0,0006769	-0,7884007	-0,45632	0
β-lyase	0	0	0	0	0
Protease	0	0	0	0	0
Pectinase	0	0	0	0	0
Cellulase	0	0	0	0	0
Hydrogen sulfide	0,2349292	-1,9454368	0,0089224	-0,0020793	0

Table S2c. Component scores of the PCA analysis of *Wickerhamomyces anomalus* isolates.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PCA	63,88%	32,87%	1,67%	1,57%	0,00%
β-glucosidase	0,3119884	0,8279487	0,1125434	0,0670679	0
β-D-xylosidase	0,1701489	-1,3376974	0,4051581	0,1321433	0
α-L-arabinofuranosidase	-0,6239768	-1,6558975	-0,2250869	-0,1341357	0
β-lyase	-0,9734537	-0,1301428	-0,1692056	0,4547748	0
Protease	3,0021804	-0,3965892	-0,136305	0,1051224	0
Pectinase	0	0	0	0	0
Cellulase	0	0	0	0	0
Hydrogen sulfide	0	0	0	0	0

Table S2d. Component scores of the PCA analysis of *Lachancea thermotolerans* isolates.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
PCA	47,17%	32,11%	12,59%	7,54%	0,58%	0,00%
β-glucosidase	-2,7019002	-2,2142606	0,1526593	3,1838648	0,0110519	0
β-D-xylosidase	-6,0620138	-3,9149433	-1,269482	-1,4245284	-0,0158298	0
α-L-arabinofuranosidase	0,1343755	-0,0228551	0,0113386	0,0581786	-0,9831297	0
β-lyase	-3,4338728	0,8871775	4,1287642	-0,4391873	-0,0059975	0
Protease	0	0	0	0	0	0
Pectinase	0	0	0	0	0	0
Cellulase	0	0	0	0	0	0
Hydrogen sulfide	4,7074035	-5,6646144	1,464285	-0,3290471	0,0096475	0

Table S2e. Component scores of the PCA analysis of *Hanseniaspora uvarum* isolates.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**
PCA	62,62%	16,86%	8,30%	6,90%	2,58%	2,43%	0,32%	0,00%
β-glucosidase	1,6052499	-1,2517011	-0,2888223	1,890322	-5,3872489	0,9172722	-0,0133567	0
β-D-xylosidase	10,202646	12,8537732	1,0154342	2,1261501	-0,0610447	0,044465	-0,0316283	0
α-L-arabinofuranosidase	-0,0271649	0,6111734	-0,4786401	-1,0740953	-1,1139738	-5,2980603	0,0130678	0
β-lyase	1,6205529	-2,3458289	-5,5246202	7,2533963	1,0370313	-0,6165931	-0,0059899	0
Protease	-4,0082486	-2,3418536	8,2548803	4,7039366	0,3929968	-0,6703662	0,0010808	0
Pectinase	-0,0563053	0,2368151	-0,0156737	0,0796928	-0,0304144	0,0417455	1,9804444	0
Cellulase	0	0	0	0	0	0	0	0
Hydrogen sulfide	25,3287538	-5,317611	1,2685261	-0,6968938	0,3605937	-0,1482685	0,0185575	0

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Table S3. Dissimilarity matrix for the similarity found during vintages and wine appellations.

	EM 2013	EM 2014	PDC 2013	PDC 2014	O 2013	O 2014	G 2012
EM 2013	0.00						
EM 2014	0.20	0.00					
PDC 2013	0.24	0.36	0.00				
PDC 2014	0.66	0.58	0.58	0.00			
O 2013	0.53	0.45	0.45	0.34	0.00		
O 2014	0.27	0.11	0.45	0.51	0.37	0.00	
G 2012	0.63	0.65	0.63	0.68	0.69	0.71	0.00

Values in the matrix range from 0 to 1, with 0 representing no differentiation between samples and 1 meaning no similarity.

4. CAPÍTULO 2

4.1. Aplicación de levaduras pectinolíticas en maceración prefermentativa para la mejora tecnológica de vinos tintos

International Journal of Food Microbiology 223 (2016) 1–8

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking

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A R T I C L E   I N F O

Article history:
Received 26 October 2015
Received in revised form 15 January 2016
Accepted 3 February 2016
Available online 4 February 2016

Keywords:
Metschnikowia pulcherrima
Non-*Saccharomyces*
Pectinase
Wine clarification
Phenolic extraction

A B S T R A C T

Pectinase enzymes have shown a considerable influence in both, sensitive and technological properties of wines. They can help to improve clarification process, releasing more color and flavor compounds entrapped in grape skin, facilitating the liberation of phenolic compounds. This work aims to find yeasts that, because of their native pectinases, can be applied on combined fermentations with *Saccharomyces cerevisiae* obtaining significant benefits over single-inoculated traditional fermentations. 462 yeast strains isolated from wineries were identified and tested for several enzymatic activities of recognized interest for enology industry. Considering the 7 identified species, only *Aureobasidium pullulans*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola* showed polygalacturonase activity. Because of its interest in winemaking, due to its reported incidence in wine flavor, the impact of *M. pulcherrima* as a source of pectinolytic enzymes was analyzed by measuring its influence in filterability, turbidity and the increase on color, anthocyanin and polyphenol content of wines fermented in combination with *S. cerevisiae*. Among the strains screened, *M. pulcherrima* NS-EM-34 was selected, due to its polygalacturonase activity, for further characterization in both, laboratory and semi-industrial scale assays. The kinetics concerning several metabolites of enological concern were followed during the entire fermentation process at microvinification scale. Improved results were obtained in the expected parameters when *M. pulcherrima* NS-EM-34 was used, in comparison to wines fermented with *S. cerevisiae* alone and combined with other pectinolytic and non-pectinolytic yeasts (*A. pullulans* and *Lachancea thermotolerans*, respectively), even working better than commercial enzymes preparations in most parameters. Additionally, *M. pulcherrima* NS-EM-34 was used at a semi-industrial scale combined with three different *S. cerevisiae* strains, confirming its potential application for red wine improvement on the mentioned sensorial and technological properties.

1. Introduction

Current research in the wine industry pursues different objectives in agronomic, biochemical and microbiological aspects. The hallmarks of enological microbiology are informed by three different targets: the sensory, technological and fermentative properties of microbial strains. The enzymatic properties of the different microorganisms involved in the winemaking process have been studied for a long time (Van Rensburg and Pretorius, 2000; Belda et al., 2016).

Pectinase enzymes have a considerable influence on both the sensory and technological properties of wines (Merín and Morata de Ambrosini, 2015). They can help to improve the clarification and filtration process, releasing more of the color and flavor compounds contained in the grape skin, and facilitating the liberation of phenolic compounds (Van Rensburg and Pretorius, 2000). The addition of commercial enzyme preparations, with filamentous fungi as the main source, can be costly for industry. Within this context, researchers have focused their attention on the native pectinases of yeasts (Alimardani-Theuil et al., 2011; Merín et al., 2011, 2015; Pretorius, 2000). It has been reported that at least 50% of the *Saccharomyces cerevisiae* enological strains tested had limited pectinolytic activity (Fernández-González et al., 2004) There has recently been increasing interest in the application of non-*Saccharomyces* wine yeasts, but the ability these yeasts have to secrete efficient pectinases needs to be studied in depth.

Traditionally, the commercial pectinases used in winemaking comprise the mixtures of polygalacturonase, pectate lyase and pectin methylesterase enzymes (Lang and Dornenburg, 2000). Of these, two types of polygalacturonases, endo- and exo-polygalacturonase, are mainly responsible for pectinolytic activity, and hence are enzymes of particular importance to industry. Furthermore, cold-active pectinolytic enzymes have a number of potential advantages such as their functionality during the prefermentative cold soak process that contributes to the color and flavor stability of wines (Merín and Morata de Ambrosini, 2015).

Combined fermentations using non-*Saccharomyces* and *S. cerevisiae* strains, as sequential inocula in wine fermentations, have a significant impact on the sensorial properties of wines (Ciani et al., 2010; Fleet,

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.02.003
0168-1605/

2008; Lambrechts and Pretorius, 2000). Most studies have been developed at laboratory scale but scarcely validated on an industrial or semi-industrial scale, questioning their applicability at cellar (Jolly et al., 2014). Some enzymatic activities related to aroma enhancement (glycosidases and β-lyase for terpene and thiol release, respectively) and the release of some interesting products such as glycerol and mannoproteins, among others, are the highlights that justify the increasing interest in these mixed fermentations (Ciani et al., 2010; Rojas et al., 2001). In this context, combined fermentations are a very useful tool to improve wine fermentations in which aromatic complexity of spontaneous fermentations and the safety of industrial targeted fermentations are joined (Ciani et al., 2010; Romano et al., 2003). The wine industry is currently demanding new yeast strains in order to innovate and improve wine quality. Within this context, positive results in industrial assays with selected yeast strains have an added value, and may contribute to the deployment of non-*Saccharomyces* strains in the enology industry.

Since the incidence of *M. pulcherrima* on overall wine quality in combined fermentations has been described (Parapouli et al., 2010), modifying wine aroma by releasing high amounts of esters (Sadoudi et al., 2012) or decreasing ethanol content of wines (Contreras et al., 2015; Quirós et al., 2014) and also the potential use of its antimicrobial activity (Oro et al., 2014), the study of its pectinolytic activity to improve clarification and phenolic extraction has not been carried out yet.

This work aims to validate the industrial use of a selected *M. pulcherrima* strain that improves different aspects of wine quality, such as polyphenol and anthocyanin content, color intensity, turbidity or filterability.

2. Materials and methods

2.1. Isolation and molecular identification of yeast strains

Grape samples were collected from different districts in the Spanish Designation of Origin (DO) Ribera del Duero. Samples were taken from *Vitis vinifera* L.cv. Tempranillo grapes during the 2013 and 2014 harvests, at appropriate ripeness and in good sanitary conditions. After pressing, a suitably diluted aliquot of grape must was spread onto lysine agar medium (Oxoid) plates at 28 °C for 48 h. Four hundred and sixty-two yeast colonies were taken and restreaked on the same medium to obtain pure cultures. All the isolates were conserved at −80 °C and deposited in the Complutense Yeast Collection. These isolates were identified by partial sequencing of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al., 1992), and the DNA for sequencing was amplified by using an Eppendorf Mastercycler apparatus, with 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′) (Kurtzman and Robnett, 1997). The sequences obtained were analyzed and compared by BLAST-search for yeast identification (BLAST; www.ncbi.nlm.nih.gov).

Six yeast strains, three *S. cerevisiae* and three non-*Saccharomyces*, were selected for the conducted trials of this study at a microvinification scale and an industrial scale. The *S. cerevisiae* strains were: *S. cerevisiae* CVA (Genbank accession number KT222660) and VRI (Genbank accession number KT222662) from CYC (Complutense Yeast Collection, Madrid, Spain) and Viniferm RVA (Genbank accession number KT222661) from Agrovin S.A., (Alcázar de San Juan, Spain). The non-*Saccharomyces* strains were: *Metschnikowia pulcherrima* NS-EM-34 (Genbank accession number KT222665), *Aureobasidium pullulans* NS-O-82 (Genbank accession number KT222663) and *Lachancea thermotolerans* NS-G-32 (Genbank accession number KT222664) from CYC.

2.2. Enzymatic characterization of yeast strains

The 462 yeast strains were screened for polygalacturonase, protease, cellulase and β-glucosidase activities. Polygalacturonase activity was determined in polygalacturonate agar medium containing 1.25% polygalacturonic acid (Sigma), 0.67% yeast nitrogen base (YNB, Difco), 1% glucose and 2% agar, adjusted to a final pH 3.5, as previously described (Strauss et al., 2001).

Protease activity was evaluated on YPD plates containing 2% skim milk powder (Sigma-Aldrich). The plates were incubated for five days at 30 °C. A clear zone around the colony identified protease activity (Strauss et al., 2001).

Cellulase production was determined on YPGE plates (containing 1% yeast extract, 2% peptone, 3% glycerol and 2% ethanol) with 0.4% carboxymethylcellulose, as previously described (Teather and Wood, 1982).

β-glucosidase activity was evaluated as reported by Villena et al. (2005), on a medium containing 0.5% cellobiose (4-O-β-D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base (Difco) and 2% agar.

2.3. Pectinolytic activity on microvinifications

A microvinification assay was conducted to confirm the pectinolytic activity of *M. pulcherrima*, in sequential fermentations combined with the commercial *S. cerevisiae* Viniferm RVA strain. *M. pulcherrima* NS-EM-34 and *A. pullulans* NS-O-82 strains were used as polygalacturonase active strains, and *L. thermotolerans* NS-G-32 as a negative control. These non-*Saccharomyces* strains were selected among the complete yeast collection analyzed due to their pectinolytic properties and reported enological usage (Jolly et al., 2014). Initial cellular concentrations in must were of about 10^6 cells/ml for every strain in sequential fermentations with an inocula ratio of 1:1. Forty-eight hours after the inoculation of non-*Saccharomyces* strains, *S. cerevisiae* Viniferm RVA was used to develop sequential fermentations.

Additionally, in order to compare with usual industrial practices, two commercial enzyme preparations, Enozym Clar and Enozym Lux (Agrovin S.A.) with high and medium polygalacturonase activity, respectively, were used as positive controls. The time of action of both enzymes was four hours prior to inoculation, according to the manufacturer instructions. After this time, *S. cerevisiae* RVA was inoculated. All assays were compared with a control assay inoculated solely with *S. cerevisiae* RVA.

Furthermore, two temperature conditions were evaluated in the assays; first, applying a controlled prefermentative cold soak (12 °C during the first 48 h, and 25 °C during the remainder of the fermentation) and, second, an assay at a constant temperature of 25 °C from the start without prefermentative cold soak.

The assays were conducted, in triplicate, by using 50 ml Falcon® tubes containing 40 g of Tempranillo crushed and destemmed grapes in their own juice. The cap was immersed daily during vinification to simulate winemaking procedures.

The Color Intensity (CI), Total Polyphenol Index (TPI) and Anthocyanin Content (AC) of the wines were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with a 1 cm path-length quartz cuvette. The CI value was calculated as the sum of the absorbances at 420, 520, and 620 nm (Glories, 1984). TPI was measured spectrophotometrically at 280 nm using wine 1/100 (*v*/*v*) diluted with distilled water (Ribéreau-Gayon et al., 2006). AC was analyzed by determining the absorbance at 520 nm of wine 1/100 (*v*/*v*) diluted with 1% (*v*/*v*) of HCl (Ruiz-Hernández, 2004).

Wine filterability was measured by filtration through a 0.22 μm filter (25 mm diameter) applying a vacuum force of 0.1 bars, as described by Haight and Gump (1994) with slight modifications, and expressed as the seconds needed to filtrate 1 ml of wine. Additionally, the turbidity of wines produced in microvinifications was evaluated by measuring the nephelometric turbidity units on a nephelometer (2100N Turbidimeter, Hach, Loveland, USA). All the experiments were conducted in triplicate.

2.4. Semi-industrial assays

2.4.1. Semi-industrial fermentations

All semi-industrial fermentations were undertaken using *V. vinifera* L. cv. Tempranillo must. 700 kg of freshly pressed grapes were placed in 1000 L PVC fermentation tanks, and sulfur dioxide (40 mg/kg) was added. The initial must density was 1104 g/L, the yeast assimilable nitrogen was 250 mg/L and pH 3.42. All the fermentations were carried out at a cellar temperature of approximately 20 °C. During the first 48 h, temperature was set at 17 °C and then fermented at cellar temperature until the end of the fermentation.

In order to determine the oenological properties of *M. pulcherrima* NS-EM-34 with independence of the *S. cerevisiae* strain used to complete the wine fermentation process, seven assays, 700 kg each, were conducted. First, three fermentations inoculated with three different *S. cerevisiae* strains: CVA, VRI and Viniferm RVA as sole inocula (Sc fermentations). Second, three fermentations performed by inoculation of *M. pulcherrima* NS-EM-34 on combined fermentations with the previously mentioned three different *S. cerevisiae* strains (Mp + Sc fermentations). Finally, one fermentation inoculated solely with *M. pulcherrima* NS-EM-34 followed by spontaneous fermentation (Mp + Spt fermentation).

Cultures were adjusted in order to reach an initial cellular concentration in must of about 10^6 cells/ml for every strain, developing mixed cultures with an inocula ratio of 1:1. During co-fermentations, aliquots were taken periodically, and further tenfold dilutions were made serially. Growth kinetics were followed by plating 50 μL of the appropriate dilution on Sabouraud glucose agar with chloramphenicol (total yeast counts) and lysine media (non-*Saccharomyces* counts). Colonies were counted after growth at 30 °C for 48–72 h. *M. pulcherrima* colonies were differentiated because of the reddish-brown halo developed surrounding them in lysine agar.

2.4.2. Analytical determinations of wines

Glucose, fructose, malic acid, lactic acid, acetic acid, glycerol, ammonium, primary amino nitrogen (PAN), yeast assimilable nitrogen (YAN), SO_2, TPI and CI were all determined using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain). These analyses were performed using the appropriate kits supplied by the manufacturer (BioSystems, Barcelona, Spain).

Total acidity, pH, ethanol, turbidity and density of wines were determined following the methods described in the Compendium of International Methods of Analysis of Musts and Wines (OIV, 2014).

2.5. Statistical analysis

All the statistical analyses were performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p < 0.05$ for the ANOVA matrix F value. Furthermore, the multiple-range test was used to compare the means. Hypothesis contrast was used to compare means on the industrial scale assays, setting the significance to $p < 0.05$ and remarked significance values to $p < 0.01$. A principal component analysis (PCA) of the analytic features determined in wines was also performed.

3. Results

3.1. Yeast population and screening of enzymatic properties

A total of 462 yeast isolates, pertaining to 9 different species, were tested for different enzymatic activities of enological interest. β-glucosidase, pectinase (polygalacturonase), protease and cellulase activities were analyzed because of their influence on certain technological properties, such as turbidity and filterability (Table S1).

β-glucosidase and protease activities were widely distributed among the yeast collection. Three species, *L. thermotolerans*, *A. pullulans* and *Torulaspora delbrueckii* showed full negative β-glucosidase activity and also four *Hanseniaspora uvarum* isolates (from a total of 260 isolates) were negative for this activity. All *L. thermotolerans*, *Cryptococcus amylolentus* and *T. delbrueckii* isolates were negative for protease activity and also *Kluyveromyces marxianus* showed a moderate activity. It should be mentioned that *H. uvarum* NS-EM-87, one of the β-glucosidase negative isolates, also showed no protease activity. It should be also outstanding the remarkably high protease activity of the *H. uvarum* isolates from EM-A (2014) vineyard that showed a distinctive behavior when compared with the most of the other *H. uvarum* isolates from other origins. In the same line, it is noteworthy the distinctive protease activity of some *M. pulcherrima* isolates from EM-B (2013), EM-B (2014), PDC-C (2013) and PDC-D (2013) vineyards that showed lower, but positive, protease activities compared with most of the other *M. pulcherrima* isolates. Polygalacturonase activity was present only in *M. pulcherrima* (88.5% positive isolates), *M. fructicola* (88.9% positive isolates) and *A. pullulans* strains (100% positive isolates), with the highest activity in this latter species. Cellulase activity was found only in *A. pullulans* (Table S1).

Due to its pectinolytic activity and common usage in winemaking, an additional characterization was conducted to analyze the influence of *M. pulcherrima* on red wine fermentations. In order to analyze the influence of the polygalacturonase activity of *M. pulcherrima* in some sensorial and technological characteristics of red wines, in both microvinifications and semi-industrial fermentations, we decide to use *M. pulcherrima* NS-EM-34 as the strain with the lowest acetic acid production and the highest sugar consumption and ethanol production rates (data not shown) among the studied *M. pulcherrima* strains, being the most suitable for winemaking. The results were compared with those obtained by using *A. pullulans* as a pectinolytic, but not recommended for winemaking, and *L. thermotolerans* as non-pectinolytic but of enological interest. *A. pullulans* NS-O-82 and *L. thermotolerans* NS-G-32 were selected in representation of the isolates of their own species.

3.2. Microvinifications

Fermentations were carried out at laboratory scale to evaluate the influence of different microbial or enzymatic pectinase sources on different enological parameters of enological interest (filterability, turbidity, TPI, AC and CI). The evolution of TPI (Fig. S1), AC (Fig. S2) and CI (Fig. S3) during the entire fermentative process was evaluated.

As indicated in Table 1, only the prefermentative cold soak conducted at 12 °C during 48 h with *M. pulcherrima* NS-EM-34 generated statistically significant differences on CI and TPI compared with *S. cerevisiae* RVA alone. *A. pullulans* has a similar effect on the same features, but no significant differences can be established. The final AC data of wines showed no significant differences between microvinifications, but a noticeable increment in AC was seen when non-*Saccharomyces* were used. However, the AC extraction rate at the start of the fermentation process is related to the efficiency of the cold soak process and also critical in the final CI of wines, being precursors of stable color pigments (Panprivech et al., 2015). In this sense, it should be highlighted the maximum AC value obtained during the fermentation in the different assays (Fig. S2). When the prefermentative cold soak was applied, both pectinolytic yeasts, *A. pullulans* and *M. pulcherrima*, reached mean values of 46.6 ± 3.93 and 45.1 ± 5.17 at the fifth day of fermentation, respectively, whereas wines inoculated with *L. thermotolerans* reached maximum AC values of 42.1 ± 5.03 (day 13 of fermentation), wines fermented solely with *S. cerevisiae* Viniferm RVA reached values of 38.4 ± 1.84 (day 9 of fermentation) and finally, those treated with Enovin Clar enzymes reached 41.4 ± 2.42 (day 5 of fermentation) or Enozym Lux enzymes reached AC values of 41.3 ± 4.58 (day 2 of fermentation) (Fig. S2a). These results indicated that the prefermentative cold soak conducted by certain non-*Saccharomyces* are of interest in winemaking for color increment purposes, being their effect

Table 1
Analytical results for the studied microvinifications applying prefermentative cold soak at 12 °C or at a constant temperature of 25 °C.

Assays	CI		TPI		AC		Filterability (s/mL)		Turbidity (NTU)	
	12 °C	25 °C	12 °C	25 °C	12 °C	25 °C	12 °C	25 °C	12 °C	25 °C
Sc	9.97 ± 0.42^{b}	11.57 ± 0.40^{a}	70.13 ± 2.76^{b}	77.73 ± 2.25^{a}	38.40 ± 1.84^{a}	40.73 ± 1.01^{bcd}	125.92 ± 8.56^{a}	158.79 ± 28.57^{a}	42.97 ± 5.33^{a}	27.17 ± 10.20^{b}
Ap	10.57 ± 0.40^{ab}	10.60 ± 0.92^{ab}	72.57 ± 4.50^{ab}	74.77 ± 3.46^{ab}	41.20 ± 6.34^{a}	48.47 ± 6.91^{a}	65.53 ± 2.79^{c}	104.24 ± 17.08^{bc}	24.00 ± 2.69^{b}	2.60 ± 1.68^{c}
Mp	11.23 ± 0.71^{a}	10.70 ± 0.70^{ab}	75.53 ± 0.59^{a}	74.90 ± 3.08^{ab}	41.57 ± 2.22^{a}	42.17 ± 2.11^{bc}	79.74 ± 11.61^{bc}	119.00 ± 12.43^{bc}	11.28 ± 7.76^{c}	8.44 ± 1.85^{c}
Lt	10.43 ± 0.38^{b}	11.50 ± 0.36^{a}	72.83 ± 3.43^{ab}	74.07 ± 0.51^{ab}	42.13 ± 5.03^{a}	44.80 ± 1.01^{ab}	89.35 ± 18.43^{b}	130.97 ± 11.93^{b}	2.83 ± 0.70^{d}	45.45 ± 1.34^{a}
Clar	10.33 ± 0.35^{b}	9.97 ± 0.40^{b}	73.97 ± 3.63^{ab}	68.77 ± 3.10^{c}	39.17 ± 2.99^{a}	35.50 ± 2.00^{d}	88.64 ± 19.00^{b}	96.88 ± 5.04^{c}	3.79 ± 2.8^{cd}	1.55 ± 0.25^{c}
Lux	9.97 ± 0.25^{b}	10.13 ± 0.93^{b}	69.23 ± 0.50^{b}	70.40 ± 3.81^{bc}	37.90 ± 1.40^{a}	37.30 ± 1.45^{c}	26.99 ± 0.88^{d}	31.51 ± 0.86^{d}	2.23 ± 0.81^{cd}	3.98 ± 2.39^{c}

Results represent the mean SD for three replicates. Means in the same row with the same letter are not significantly different ($s < 0.05$). Ap: *A. pullulans* followed by *S. cerevisiae* Viniferm RVA; Mp: *M. pulcherrima* followed by *S. cerevisiae* Viniferm RVA; Lt: *L. thermotolerans* followed by *S. cerevisiae* Viniferm RVA; Sc: *S. cerevisiae* Viniferm RVA alone; Clar: *S. cerevisiae* Viniferm RVA previously treated with Enovin Clar; Lux: *S. cerevisiae* Viniferm RVA previously treated with Enozym Lux. CI, Color Intensity; TPI, Total Polyphenol Index; AC, Anthocyanin Content.

quantitatively similar to the use of commercial enzyme preparations of fungal origin.

Enzymatic treatment with industrial preparations do not increase CI, TPI and AC values of wines, but both enzymatic treatments have a very significant effect on the filterability and turbidity of wines, highlighting the effect of Enozym Lux on wine filterability, accordingly to its high polygalacturonase activity. The influence of non-*Saccharomyces* on wine filterability was also directly related to their pectinase activity, showing that the highest polygalacturonase activity observed (in plate assays) corresponded to the lowest filtration time reached in wines. Regarding turbidity data, all treatments had a statistically significant effect compared with fermentations with *S. cerevisiae* Viniferm RVA as sole inoculum, standing out the unexpected effect of *L. thermotolerans* NS-G-32 on this parameter applying cold soak (Table 1).

The maintenance of a constant temperature of 25 °C (no prefermentative cold soak) during the entire fermentation process notably reduced the differences between treatments (Table 1). No significant differences were observed between *S. cerevisiae* Viniferm RVA and non-*Saccharomyces* treatment for CI and TPI, and only *A. pullulans* was able to significantly increase the final value of AC. The initial extraction of anthocyanins, as occurred in the other studied parameters when prefermentative soak was developed at 25 °C, the pectinolytic effect of both, non-*Saccharomyces* and enzyme preparations, was not clear being less dependent of those pectinase sources. Only the delay in the start of the fermentation process contributed to the differences observed between the presence or absence of non-*Saccharomyces* yeasts at the early stages. In this case *A. pullulans*, *M. pulcherrima* and *L. thermotolerans* reached similar maximum mean AC values of 51.3 ± 3.90, 51.4 ± 3.18 and 50.9 ± 0.70 at the fifth day of fermentation, respectively. Nevertheless, wines fermented with *S. cerevisiae* Viniferm RVA and treated with pectinolytic enzymes reached AC values of 44.0 ± 4.03, 39.4 ± 1.64 and 42.5 ± 1.92, respectively, at the fifth day of fermentation (Fig. S2b).

Furthermore, the effect observed at 25 °C on filterability was also slighter in both *S. cerevisiae* Viniferm RVA and non-*Saccharomyces* strains. According to the results observed at 12 °C, the effect on filterability of the enzyme preparation Enozym Lux was higher than the effect of Enovin Clar; however, in both cases their effect was higher than any other microbial treatment. The turbidity data were similar to those observed at a low temperature, but in this case, *L. thermotolerans* NS-G-32 recorded the highest turbidity value, contrary to that observed with the same strain at a low temperature (Table 1).

3.3. Semi-industrial fermentations

In order to confirm the incidence of *M. pulcherrima* NS-EM-34 on wine properties, due to its remarkable pectinase activity, seven industrial trials were performed. Three fermentations were inoculated solely with one of the following three *S. cerevisiae* strains: RVA, CVA and VRI (Sc fermentations). Three sequential fermentations (Mp + Sc) of *M. pulcherrima* NS-EM-34 were carried out in combination with every one of the mentioned three *S. cerevisiae* strains (Mp + RVA, Mp + CVA and Mp + VRI). Finally, another fermentation was inoculated solely with *M. pulcherrima* NS-EM-34 followed by spontaneous fermentation (Mp + Spt).

3.3.1. Fermentation kinetics

Fermentations were carried out at a cellar temperature (20 °C approximately) requiring between six (Sc fermentations) and eight days (Mp + Spt fermentations) to finalize (Fig. 1). Mp + Spt fermentations recorded the slowest fermentation kinetics due to the absence of a *S. cerevisiae* inoculum, with *S. cerevisiae* wild yeasts being responsible for completing the fermentation process. The other three combined fermentations (Mp + RVA, Mp + CVA and Mp + VRI) showed no noticeable differences in the fermentation kinetics compared with their respective Sc fermentations, completing the process after seven days.

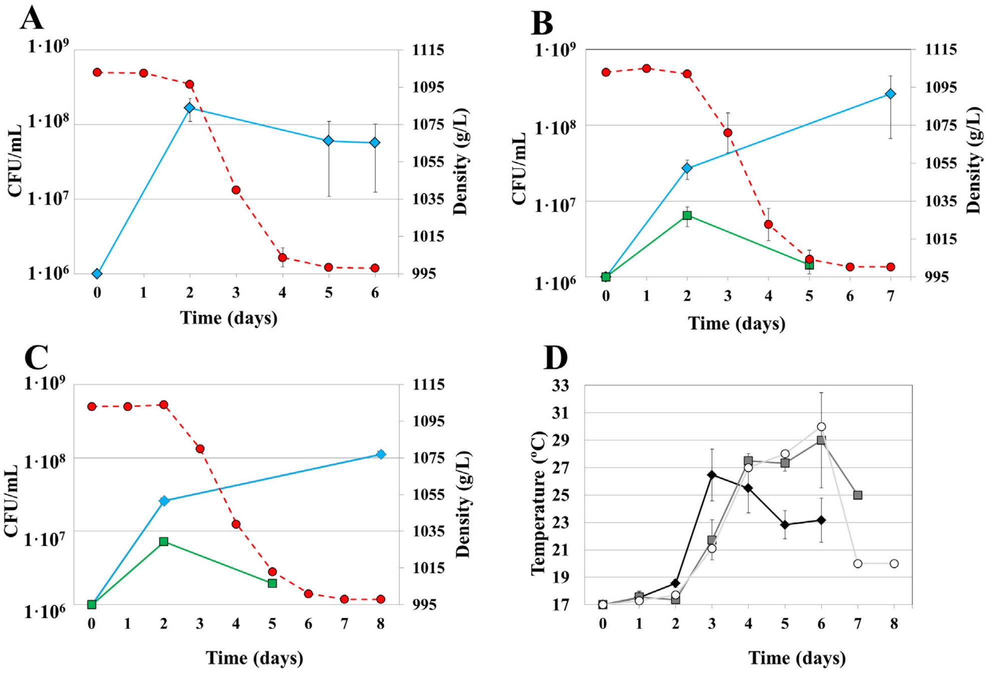

Fig. 1. Fermentation kinetics of semi-industrial trials. A) Sc fermentations. Total yeast cell counts (blue) and must density evolution (red) of Sc fermentations. B) Mp + Sc fermentations. Total yeast cell counts (blue), *M. pulcherrima* NS-EM-34 cell counts (green) and must density (red) of Mp + Sc fermentations. C) Mp + Spt fermentations. Total yeast cell count (blue), *M. pulcherrima* NS-EM-34 cell count (green) and must density decrease (red) of Mp + Spt fermentations. D) Temperature evolution in Sc (black), Sc + Mp (gray) and Mp + Spt (white) fermentations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

S. cerevisiae maintained high cell viability until the end of fermentation in both, as the sole inoculum (Fig. 1a) or sequentially co-inoculated with *M. pulcherrima* (Fig. 1b). The total cell counts from Sc fermentations recorded a higher growth rate at the early stage of fermentation. However, the final yeast population was higher in combined fermentations (Mp + Sc). Besides the slower fermentation kinetics, Mp + Spt recorded lower biomass counts compared with, Sc and Mp + Sc (Fig. 1c).

3.3.2. Wine composition

In order to detect and highlight differences between wines fermented with single or mixed inocula, principal component analysis (PCA) was applied to all the analytical data obtained for the final composition of wines (Fig. 2). Wines positioned in the right quadrants of Fig. 2a correspond to combined fermentations, forming a homogeneous group. The fermentations inoculated solely with *S. cerevisiae* formed a heterogeneous group, and are positioned in the left quadrants of Fig. 2a. Fig. 2b represents the two-dimensional projection of the data according to the parameters used, explaining 77.8% of the variability in the first two dimensions. PC1 accounted for 52.9%, and PC2 accounted for an additional 24.8% of the total variability. PC1, which accounts for almost a half of the total variability, was positively loaded by pectinase-dependent parameters such as CI and TPI, detected in higher values in fermentations where *M. pulcherrima* were involved. It should be mentioned that PC1 is also loaded in the other direction by turbidity data, detected in lower values in Mp + Spt and Mp + Sc fermentations. Thus, PCA analysis showed that the global characteristics of the wines can be used to separate them into two defined groups depending on the presence of *M. pulcherrima* as inoculum, and notably influenced by pectinase-dependent parameters.

Table 2 shows the final chemical composition of wines that, according to PCA results, only showed significant differences in a few parameters. Apart from CI, TPI and turbidity, only malic and lactic acid recorded

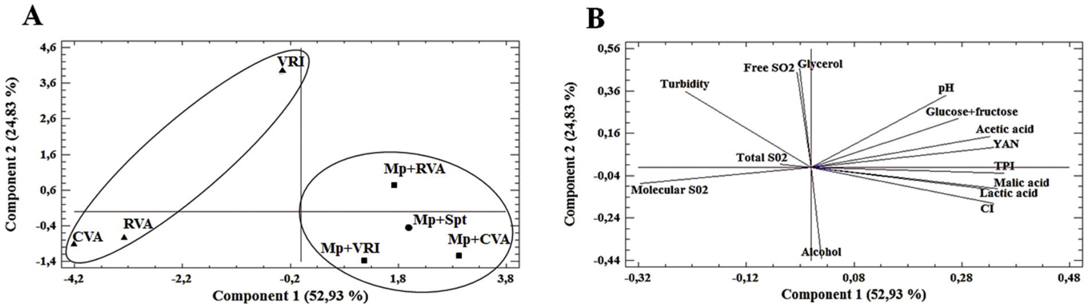

Fig. 2. Principal Component Analysis (PCA) of the analytical composition of wines. A) Scores for the seven wine samples for the two first principal components. Wines were fermented by using *S. cerevisiae* CVA, RVA and VRI strains and their combinations in sequential fermentations with *M. pulcherrima* NS-EM-34 (Mp + CVA, Mp + RVA, Mp + VRI). Finally, a fermentation with *M. pulcherrima* NS-EM-34 followed by a spontaneous fermentation (Mp + Spt) was conducted. B) Loadings of the variables on the two first principal components. The compounds considered, listed by PC1 loading value, were: TPI, lactic acid, CI, YAN, malic acid, acetic acid, pH, sugars, alcoholic grade, free SO_2, glycerol, total SO_2, turbidity, and molecular SO_2.

Table 2
Analytical results of the semi-industrial assays developed with *S. cerevisiae* as sole inoculum (Sc) or combined with *M. pulcherrima* NSEM 34 (Mp + Sc).

Parameter	*S. cerevisiae* (Sc)			Combined fermentations (Mp + Sc)				Mean values	
	RVA	CVA	VRI	Mp + RVA	Mp + CVA	Mp + VRI	Mp + Spt	$\overline{X}$ Sc	$\overline{X}$ Mp + Sc
Glucose + Fructose (g/L)	0.21	0.25	0.3	0.33	0.3	0.26	0.29	0.25 ± 0.05	0.30 ± 0.03
Acetic acid (g/L)	0.21	0.18	0.27	0.26	0.3	0.24	0.27	0.22 ± 0.05	0.27 ± 0.03
Malic acid (g/L)	3.13	3.18	3.22	3.5	3.42	3.44	3.45	3.18 ± 0.05	3.45 ± 0.03**
Lactic acid (g/L)	0.21	0.2	0.24	0.28	0.31	0.3	0.27	0.22 ± 0.02	0.29 ± 0.02**
Glycerol (g/L)	7.7	7.8	8.4	7.7	7.9	7.5	7.7	7.97 ± 0.38	7.70 ± 0.16
Ammonium (mg/L)	34	31	39	41	39	37	38	34.7 ± 4.0	38.8 ± 1.7
PAN (mg/L)	59	54	81	93	91.2	88	106	64.7 ± 14.4	94.6 ± 7.9*
YAN (mg/L)	86	78	111	125	121.6	107. 0	136	91.7 ± 17.2	122.4 ± 12.0*
Alcohol (% *v*/*v*)	14.09	14.05	13.98	14.03	14.12	14.08	14.02	14.04 ± 0.06	14.06 ± 0.05
pH	3.66	3.58	3.8	3.73	3.73	3.72	3.71	3.68 ± 0.11	3.72 ± 0.01
Free SO_2 (mg/L)	99	99	121	111	93	104	94	106.3 ± 12.7	100.5 ± 8.6
Molecular SO_2 (ppm)	1.38	1.65	1.23	1.32	1.1	1.26	1.17	1.42 ± 0.21	1.21 ± 0.10
Total SO_2 (mg/L)	168	193	164	199	162	152	187	175 ± 15.7	175 ± 21.7
CI	7.16	7.05	7.54	11.81	13.62	10.74	12.6	7.25 ± 0.26	12.20 ± 1.22**
TPI	65.8	63.6	73.8	85.8	84.6	83.1	83.5	67.7 ± 5.4	84.3 ± 1.2**
Turbidity (NTU)	587	441	614	451	235	255	324^{a}	547 ± 93	316 ± 98*

Results in the seven left columns show the values for the seven individual assays. The two right columns represent the mean SD for the three single (Sc) fermentations (Viniferm RVA, CVA and VRI) and the four combined (Mp + Sc) fermentations (Mp + RVA, Mp + CVA, Mp + VRI and Mp + Spt). Means in the same row with single asterisk (*) indicate significantly different ($p < 0.05$) and with double asterisk (**) indicate significantly different ($p < 0.01$). RVA: *S. cerevisiae* Viniferm RVA alone; CVA: *S. cerevisiae* CVA alone; VRI: *S. cerevisiae* VRI alone; Mp + RVA: *M. pulcherrima* NSEM-34 followed by *S. cerevisiae* Viniferm RVA; Mp + CVA: *M. pulcherrima* followed by *S. cerevisiae* CVA; Mp + VRI: *M. pulcherrima* NSEM-34 followed by *S. cerevisiae* VRI; Mp + Spt: *M. pulcherrima* NSEM-34 followed by spontaneous fermentation.

significant differences between wines fermented with or without *M. pulcherrima*.

4. Discussion

The present work has afforded the study of the enzymatic properties of a wide collection of yeasts isolated from the winemaking environment that comprised eight species of enological interest. The studied enzymatic properties were those related with the implementation of clarification and color extraction processes in winemaking and the study was reinforced by the application of these strains in enological conditions.

4.1. Population and enzymatic distribution

Two different groups of high and low distributed enzymatic activities, among the yeast collection studied, were established. According to several studies, the presence of β-glucosidase activity is widespread in most wine-related yeast species (Fia et al., 2005), although it is scarce in *S. cerevisiae* strains. Moreover, the proteolytic activity is also abundant among yeasts (Chomsri, 2008), being the most extended activity across the 462 yeast isolates studied in this work. On the other hand, pectinase and cellulase activities are the most restrictively distributed activities. Contrary to other authors that reported the presence of cellulase activity in some yeast species (*Candida stellata*, *M. pulcherrima* and *Kloeckera apiculata*) (Strauss et al., 2001), in this work cellulase activity was detected only in *A. pullulans* strains (Table S1).

It should be mentioned that polygalacturonase activity has been reported in a few wine yeast isolates without establishing a species-specific behavior (Merín et al., 2011; Strauss et al., 2001). Within this context, the selection of pectinolytic yeast strains for their use as inoculum in industrial fermentations seems to be a useful tool to produce higher quality wines without the addition of expensive commercial enzyme preparations.

4.2. Incidence of different polygalacturonase sources on wine composition

The results obtained in this work not only contributes to the knowledge about the usefulness of *M. pulcherrima*, but also open a new research line on the influence of different variables, such as temperature, on its metabolic efficiency. Data shown in Table 1 reveals the effect of low temperature on non-*Saccharomyces* metabolism and, therefore, on the final composition of the wine. Differences between assays were observed depending on the pectinolytic activity of the strains when prefermentative cold soak was applied. Nevertheless, these differences decreased significantly when a constant temperature (25 °C) was applied from the start of the process, obtaining more homogeneous results.

Most winemakers usually apply a prefermentative cold soak to improve certain aspects of wine quality, especially those related with color intensity and stability. Apart from the fact that the extraction of some color compounds increases when a cold soak is applied, due to the increased permeabilization of the grape's cellular membranes as a result of longer contact time, our results suggest that the presence of certain yeast species contributes significantly to the increase in phenolic and color extraction. Those results were strengthened by the fact that *L. thermotolerans* inoculation (with no pectinolytic activity) did not increase color extraction rates, if compared with *S. cerevisiae* fermentations, in spite of the delay caused by the prefermentative cold soak (Table 1). We may therefore posit that a prefermentative cold soak not only contributes to wine composition by chemical means, but also that microbiological aspects are involved through the intervention of certain non-*Saccharomyces* yeasts. It has been recently reported that a longer prefermentative cold soak leads to higher color intensity values at the end of this process, but such differences usually disappear at the end of alcoholic fermentation (Panprivech et al., 2015). It should be mentioned that, in this study, the higher values reached for CI, TPI and AC in wines fermented without a prefermentative cold soak (Table 1; 25 °C vs. 12 °C) could be explained by the faster extraction of phenolic compounds due to the effect of temperature and of ethanol as solvent (Sacchi et al., 2005), so only an internal comparison of the effect of yeast strains in assays with or without a cold soak can be made with any certainty. Thus, the results shown in Table 1 prove that there are significant increases in CI and TPI when *M. pulcherrima* was inoculated during the prefermentative cold soak. These increases were not very remarkable, although statistically significant. It could be explained because of the experimental conditions at a laboratory scale where fermentations were performed in 50 mL Falcon® tubes that increase the grape-juice contact surface in comparison to semi-industrial assays. Confirming these facts in real winemaking conditions, Table 2 shows that the mean increments observed in TPI and CI values for semi-industrial assays when *M. pulcherrima* was inoculated were 19.7% and 40.6%, respectively, compared with wines only inoculated with *S. cerevisiae* (Table 2). These increases were remarkably higher than those obtained by applying a conventional cold soak (Panprivech et al., 2015) and also higher compared with other studies using genetic engineering approaches (Radoi et al., 2005; Fernández-González et al., 2005) and other innovative treatments for wine phenolic extraction, such as the pulsed electric fields technology (Puértolas et al., 2009).

Additionally, as reported by Panprivech et al. (2015), and in agreement with our results with pectinolytic yeasts (Fig. S2a), greater rates of anthocyanin extraction during the early stages of fermentation are observed when a cold soak was used. Furthermore, increased maximum values of anthocyanin along the process have been reached (Fig. S2a) that could contribute to the final CI values because of the formation of stable pigments by copigmentation (Casassa et al., 2013). However, our results show that, during the prefermentative cold soak, the increase in the anthocyanin extraction rate only occurs when a source of pectinases is applied, contrary to observed with a prefermentative soak at 25 °C, where the lack of action of non-*Saccharomyces* yeasts eliminates the differences between treatments with pectinolytic (*A. pullulans* and *M. pulcherrima*) and non-pectinolytic (*L. thermotolerans*) yeasts. It may be concluded that a prefermentative cold soak contributes positively to the extraction rates of color compounds when a source pectinase (microbial or enzymatic) was added. Due to the difficulty of analytically-monitoring semi-industrial fermentations, AC extraction rates were not quantified, but the notable increase in CI for Mp + Sc compared with Sc wines (Table 2) could be related with both, the higher TPI values observed and, possibly, with greater AC extraction rates.

Filterability values were observed to be directly related with the pectinolytic activity of yeast strains, diminishing when the polygalacturonase activity from yeasts or enzyme preparations increased (Table 1). The reduction of filtration time obtained with pectinolytic non-*Saccharomyces* yeasts (*A. pullulans* and *M. pulcherrima*) was slightly higher than the obtained with the less active pectinolytic enzyme preparation (Enovin Clar) when cold soak was applied. Furthermore, the reduction time was slightly lower when cold soak was not applied; and in all cases it was notably lower than that obtained with the high efficient pectinolytic enzyme preparation (Enozyn Lux) (Table 1).

Some industrial methods have been developed to reduce turbidity in winemaking; their effects have been recently evaluated by Fernandes et al. (2015) showing great turbidity reductions, especially when yeast protein extracts were applied as fining agents. All of these techniques were applied in postfermentative stages, with the time and economic costs that involved. Our results show that there was a clear improvement in this parameter with both sources of pectinases, from enzymatic or microbial origin, as well as with or without cold soak. *A. pullulans* showed a remarkable effect at higher prefermentative temperatures reducing turbidity values in a 90.4% but only a 44.2% when cold soak was applied. This fact are partially in agreement with Merín et al. (2011) that reported pectinolytic activity in *A. pullulans* at 12 °C, however our results show that this activity are lower than the activity found at 25 °C. On the contrary, *M. pulcherrima* showed a noticeable effect at both prefermentative temperatures, reducing 73.8% and 68.9% by applying cold and conventional soak, respectively (Table 1). These turbidity reduction rates are not really far from those obtained by Fernandes et al. (2015) (turbidity reduction of 81.3%) using their best fining agent (yeast protein extracts) as an additional post-fermentative treatment and that in all cases are less efficient than the commercial enzyme preparations evaluated in this study (Table 1). Additionally, as occurred with CI and TPI values, the effect of *M. pulcherrima* in wine turbidity at semi-industrial scale confirmed its usefulness for this objective with a mean value of turbidity reduction of 42.23% with its greatest effect (58.47%) combined with *S. cerevisiae* VRI (Table 2).

Special note should be taken on the effect of *L. thermotolerans* on wine turbidity when it was inoculated during the prefermentative cold soak. There was a sharp decrease in wine turbidity, recording the highest microbial yield for this parameter. As previously reported, *L. thermotolerans* is positively promoted at lower temperatures (20 °C vs. 30 °C) (Gobbi et al., 2013); however, the cryophilic nature of *L. thermotolerans* at temperatures close to 12 °C has not yet been reported, as far as we know. An increased metabolic rate of *L. thermotolerans* at low temperatures could explain the decrease in wine turbidity through the release of higher amounts of organic compounds, such as proteins, which contribute to the precipitation of suspended particles (Deckwart et al., 2014) as observed by Fernandes et al. (2015) by applying yeast protein extracts as fining agent, but this fact should be studied in depth.

The industrial use of *M. pulcherrima* NS-EM-34 on a semi-industrial scale combined with three different *S. cerevisiae* strains (two autochthonous ones and a commercial one) allows us to robustly confirm its global application, independently of the *S. cerevisiae* strain used for alcoholic fermentation. The fermentation kinetics and population dynamics (Fig. 1) recorded similar results to those reported in studies of sequential fermentations with *M. pulcherrima* (Sadoudi et al., 2012), where the presence of *M. pulcherrima* is limited to the first half of the fermentation process. This moderate implantation allowed *S. cerevisiae* to easily govern the alcoholic fermentation, achieving the completion of the process without significant delays.

According to other studies (Sun et al., 2014), *M. pulcherrima* does not modify enological analytical parameters such as ethanol or glycerol concentrations (Table 2). However, it should be mentioned the differences observed in the malic acid concentrations between assays, that was higher using *M. pulcherrima* (Table 2). It has been reported the capability of other non-*Saccharomyces* species such as *Schizosaccharomyces pombe* to consume malic acid during wine fermentation modifying the final sensorial properties of wines that has been described to be less bitter (Benito et al., 2015). It could be also mentioned the repercussion of *M. pulcherrima* NS-EM-34 in acetic acid concentrations that are slightly higher compared with fermentations inoculated only with *S. cerevisiae*, however this increase cause no significant differences. This fact is of importance to winemaking industry, since an excessive increase of acetic acid in wines has been traditionally associated with the presence of non-*Saccharomyces* yeasts during the fermentation process (Jolly et al., 2014). On the same line, other non-*Saccharomyces* species such as *T. delbrueckii* has been described to keep or slightly reduce the acetic acid content of wines (Bely et al., 2008; Belda et al., 2015). This work has reported a novel usage of *M. pulcherrima* through the exploitation of its polygalacturonase activity. Tables 1 and 2 indicates that CI, TPI and turbidity data show significant differences that could be related to pectinolytic activity, Furthermore, PCA analysis of semi-industrial wines confirm that wines inoculated with *M. pulcherrima* NS-EM-34 can be clearly distinguished from wines fermented solely with *S. cerevisiae*, with these differences mostly affecting CI, TPI and turbidity (Fig. 2).

This study broadly contributes to the knowledge on the enzymatic properties of non-*Saccharomyces* yeasts and their applicability in winemaking, and specifically to the understanding of the behavior of *M. pulcherrima* in wine fermentations. In addition to the previously reported impact on sensorial aspects of wines such as their aromatic complexity and alcoholic content (Morales et al., 2015), this work confirms the usefulness of *M. pulcherrima* NS-EM-34 to improve some aforementioned technological aspects of wines like clarification and phenolic extraction processes. In this sense, the increase in the knowledge about the physiological properties and the metabolic determinants of non-*Saccharomyces* yeasts will be the only way to achieve their deployment in the enology industry.

Acknowledgments

The funding for the research in this paper was provided by Agrovin S.A, under the framework of the project IDI-20130192-ENZIOXIVIN (Centre for Industrial Technological Development—CDTI, Spain). We thank Dra. María de los Ángeles Gómez-Flechoso for helpful discussion on statistical analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.02.003.

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Supplementary material – International Journal of Food Microbiology

Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking

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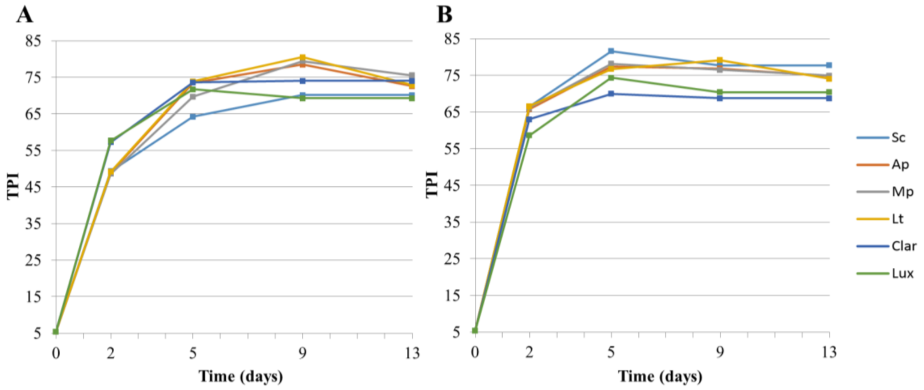

Figure S1. Evolution of Total Polyphenol Index (TPI) during microvinification assays. A) Fermentations carried out applying prefermentative cold soak; B) Fermentations carried out at a constant temperature. Ap (orange line): *A. pullulans* followed by *S. cerevisiae* RVA**; Mp (grey line):** *M. pulcherrima* followed by *S. cerevisiae* RVA; **Lt (yellow line):** *L. thermotolerans* followed by *S. cerevisiae* RVA; **Sc (light blue line):** *S. cerevisiae* RVA alone; **Clar (dark blue line):** *S. cerevisiae* RVA previously treated with Enovin Clar; **Lux (green line):** *S. cerevisiae* RVA previously treated with Enozym Lux.

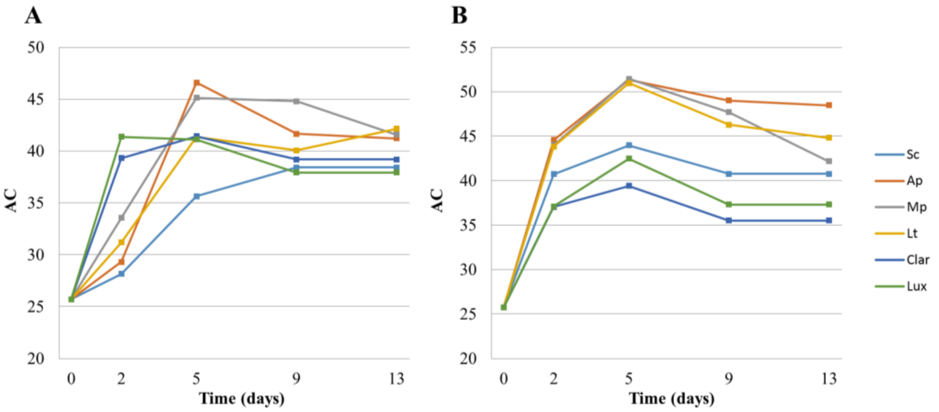

Figure S2. Evolution of Anthocyanin Content (AC) during microvinification assays. A) Fermentations carried out applying prefermentative cold soak; B) Fermentations carried out at a constant temperature. Ap (orange line): *A. pullulans* followed by *S. cerevisiae* RVA**; Mp (grey line):** *M. pulcherrima* followed by *S. cerevisiae* RVA; **Lt (yellow line):** *L. thermotolerans* followed by *S. cerevisiae* RVA; **Sc (light blue line):** *S. cerevisiae* RVA alone; **Clar (dark blue line):** *S. cerevisiae* RVA previously treated with Enovin Clar; **Lux (green line):** *S. cerevisiae* RVA previously treated with Enozym Lux.

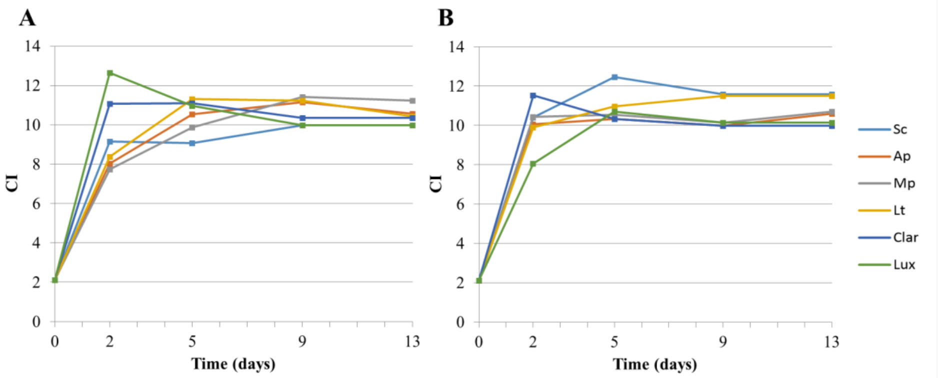

Figure S3. Evolution of Color Intenstity (CI) during microvinification assays. A) Fermentations carried out applying prefermentative cold soak; B) Fermentations carried out at a constant temperature. Ap (orange line): *A. pullulans* followed by *S. cerevisiae* RVA**; Mp (grey line):** *M. pulcherrima* followed by *S. cerevisiae* RVA; **Lt (yellow line):** *L. thermotolerans* followed by *S. cerevisiae* RVA; **Sc (light blue line):** *S. cerevisiae* RVA alone; **Clar (dark blue line):** *S. cerevisiae* RVA previously treated with Enovin Clar; **Lux (green line):** *S. cerevisiae* RVA previously treated with Enozym Lux.

Table S1. Identification of the yeast collection analyzed (species and isolation source) and enzymatic characterization.

Collection number	Identification	Isolation source (vineyard code and year)	β-Glucosidase	Protease	Polygalacturonase	Cellulase
	Aureobasidium pullulans **(n=22)**					
NS-PDC-121	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-122	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-123	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-124	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-125	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-126	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-127	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-128	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-129	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-130	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-131	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-134	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-135	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-136	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-137	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-138	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-139	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-140	*A. pullulans*	PDC-H (2014)	-	+	++	+
NS-PDC-161	*A. pullulans*	PDC-J (2014)	-	+	++	+
NS-PDC-165	*A. pullulans*	PDC-J (2014)	-	+	++	+
NS-PDC-172	*A. pullulans*	PDC-J (2014)	-	+	++	+
NS-PDC-173	*A. pullulans*	PDC-J (2014)	-	+	++	+
	Cryptococcus amylolentus **(n=17)**					
NS-PDC-132	*C. amylolentus*	PDC-H (2014)	+	-	-	-
NS-PDC-133	*C. amylolentus*	PDC-H (2014)	+	-	-	-

NS-PDC-178	*C. amylolentus*	PDC-K (2014)	+	-	-	-
NS-PDC-242	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-243	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-244	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-245	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-246	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-248	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-249	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-250	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-252	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-253	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-254	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-257	*C. amylolentus*	PDC-O (2014)	+	-	-	-
NS-PDC-261	*C. amylolentus*	PDC-P (2014)	+	-	-	-
NS-PDC-262	*C. amylolentus*	PDC-P (2014)	+	-	-	-
	Hanseniaspora uvarum **(n=260)**					
NS-EM-1	*Hanseniaspora uvarum*	EM-A (2013)	+	+-	-	-
NS-EM-2	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-3	*Hanseniaspora uvarum*	EM-A (2013)	+	+-	-	-
NS-EM-4	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-5	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-6	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-7	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-8	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-9	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-10	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-11	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-12	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-13	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-14	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-

NS-EM-16	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-17	*Hanseniaspora uvarum*	EM-A (2013)	+	+-	-	-
NS-EM-18	*Hanseniaspora uvarum*	EM-A (2013)	+	+-	-	-
NS-EM-19	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-20	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-21	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-22	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-23	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-24	*Hanseniaspora uvarum*	EM-A (2013)	+	+-	-	-
NS-EM-25	*Hanseniaspora uvarum*	EM-A (2013)	+	+	-	-
NS-EM-26	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-27	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-28	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-29	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-30	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-31	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-32	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-33	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-35	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-36	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-37	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-38	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-39	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-40	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-41	*Hanseniaspora uvarum*	EM-B (2013)	+	+-	-	-
NS-EM-42	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-43	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-44	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-45	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-46	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-

NS-EM-47	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-48	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-49	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-50	*Hanseniaspora uvarum*	EM-B (2013)	+	+	-	-
NS-EM-76	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-77	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-78	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-79	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-80	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-81	*Hanseniaspora uvarum*	EM-D (2013)	+	+-	-	-
NS-EM-82	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-83	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-84	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-85	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-86	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-87	*Hanseniaspora uvarum*	EM-D (2013)	-	-	-	-
NS-EM-88	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-89	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-90	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-91	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-92	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-93	*Hanseniaspora uvarum*	EM-D (2013)	+	+-	-	-
NS-EM-94	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-95	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-96	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-97	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-98	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-99	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-100	*Hanseniaspora uvarum*	EM-D (2013)	+	+	-	-
NS-EM-101	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-

NS-EM-102	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-103	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-105	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-106	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-107	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-108	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-109	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-110	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-112	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-114	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-116	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-117	*Hanseniaspora uvarum*	EM-B (2014)	+	++	-	-
NS-EM-118	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-120	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-121	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-122	*Hanseniaspora uvarum*	EM-B (2014)	+	++	-	-
NS-EM-124	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-125	*Hanseniaspora uvarum*	EM-B (2014)	+	+	-	-
NS-EM-126	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-127	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-128	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-129	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-131	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-132	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-133	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-134	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-135	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-137	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-138	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-140	*Hanseniaspora uvarum*	EM-D (2014)	+	+-	-	-

NS-EM-142	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-143	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-144	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-145	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-146	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-147	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-148	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-149	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-150	*Hanseniaspora uvarum*	EM-D (2014)	+	+	-	-
NS-EM-151	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-152	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-153	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-154	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-155	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-156	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-157	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-158	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-159	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-160	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-161	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-162	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-163	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-164	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-165	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-166	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-168	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-169	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-170	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-171	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-173	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-

NS-EM-174	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-175	*Hanseniaspora uvarum*	EM-A (2014)	+	++	-	-
NS-EM-176	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-177	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-178	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-179	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-180	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-181	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-182	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-183	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-185	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-186	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-188	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-189	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-190	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-191	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-192	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-193	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-195	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-196	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-198	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-199	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-EM-200	*Hanseniaspora uvarum*	EM-E (2014)	+	+	-	-
NS-PDC-1	*Hanseniaspora uvarum*	PDC-A (2013)	+	+-	-	-
NS-PDC-2	*Hanseniaspora uvarum*	PDC-A (2013)	+	+-	-	-
NS-PDC-3	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-4	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-5	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-6	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-7	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-

NS-PDC-8	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-9	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-10	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-11	*Hanseniaspora uvarum*	PDC-A (2013)	+	+-	-	-
NS-PDC-12	*Hanseniaspora uvarum*	PDC-A (2013)	+	+-	-	-
NS-PDC-13	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-15	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-16	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-17	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-18	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-19	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-20	*Hanseniaspora uvarum*	PDC-A (2013)	+	+	-	-
NS-PDC-21	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-22	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-23	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-24	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-25	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-26	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-27	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-28	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-29	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-30	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-31	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-32	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-33	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-34	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-35	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-36	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-37	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-38	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-

NS-PDC-39	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-40	*Hanseniaspora uvarum*	PDC-B (2013)	+	+	-	-
NS-PDC-101	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-102	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-103	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-104	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-105	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-106	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-107	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-108	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-109	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-110	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-111	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-112	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-113	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-114	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-115	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-116	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-117	*Hanseniaspora uvarum*	PDC-G (2013)	+	+-	-	-
NS-PDC-118	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-119	*Hanseniaspora uvarum*	PDC-G (2013)	+	+-	-	-
NS-PDC-120	*Hanseniaspora uvarum*	PDC-G (2013)	+	+	-	-
NS-PDC-162	*Hanseniaspora uvarum*	PDC-J (2014)	-	++	-	-
NS-PDC-163	*Hanseniaspora uvarum*	PDC-J (2014)	-	++	-	-
NS-PDC-164	*Hanseniaspora uvarum*	PDC-J (2014)	-	+	-	-
NS-PDC-166	*Hanseniaspora uvarum*	PDC-J (2014)	+	+	-	-
NS-PDC-168	*Hanseniaspora uvarum*	PDC-J (2014)	+	+	-	-
NS-PDC-170	*Hanseniaspora uvarum*	PDC-J (2014)	+	+	-	-
NS-PDC-175	*Hanseniaspora uvarum*	PDC-J (2014)	+	+	-	-
NS-PDC-181	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-

NS-PDC-182	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-183	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-184	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-185	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-186	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-187	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-188	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-189	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-190	*Hanseniaspora uvarum*	PDC-C (2014)	+	+	-	-
NS-PDC-203	*Hanseniaspora uvarum*	PDC-M (2014)	+	++	-	-
NS-PDC-209	*Hanseniaspora uvarum*	PDC-M (2014)	+	+	-	-
NS-PDC-210	*Hanseniaspora uvarum*	PDC-M (2014)	+	+	-	-
NS-PDC-211	*Hanseniaspora uvarum*	PDC-M (2014)	+	+	-	-
NS-PDC-212	*Hanseniaspora uvarum*	PDC-M (2014)	+	+	-	-
NS-PDC-218	*Hanseniaspora uvarum*	PDC-M (2014)	+	++	-	-
NS-PDC-221	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-222	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-223	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-224	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-225	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-226	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-227	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-228	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-229	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-230	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-231	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-232	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-233	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-234	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-235	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-

NS-PDC-236	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-237	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-238	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-239	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
NS-PDC-240	*Hanseniaspora uvarum*	PDC-N (2014)	+	+	-	-
	Kluyveromyces marxianus **(n=2)**					
NS-PDC-99	*Kluyveromyces marxianus*	PDC-F (2013)	+	+-	-	-
NS-PDC-100	*Kluyveromyces marxianus*	PDC-F (2013)	+	+-	-	-
	Lachancea thermotolerans **(n=81)**					
NS-EM-51	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-52	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-53	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-54	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-55	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-56	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-57	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-58	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-59	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-60	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-61	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-62	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-63	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-64	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-65	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-66	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-67	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-68	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-69	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-70	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-71	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-

NS-EM-72	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-73	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-74	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-75	*Lachancea thermotolerans*	EM-C (2013)	-	-	-	-
NS-EM-104	*Lachancea thermotolerans*	EM-B (2014)	-	-	-	-
NS-EM-119	*Lachancea thermotolerans*	EM-B (2014)	-	-	-	-
NS-EM-130	*Lachancea thermotolerans*	EM-D (2014)	-	-	-	-
NS-EM-136	*Lachancea thermotolerans*	EM-D (2014)	-	-	-	-
NS-EM-139	*Lachancea thermotolerans*	EM-D (2014)	-	-	-	-
NS-EM-141	*Lachancea thermotolerans*	EM-D (2014)	-	-	-	-
NS-PDC-41	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-42	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-43	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-44	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-45	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-46	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-47	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-49	*Lachancea thermotolerans*	PDC-C (2013)	-	-	-	-
NS-PDC-58	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-59	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-60	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-61	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-62	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-63	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-64	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-65	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-66	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-67	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-68	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-69	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-

NS-PDC-70	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-71	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-72	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-73	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-74	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-75	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-76	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-77	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-78	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-79	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-80	*Lachancea thermotolerans*	PDC-E (2013)	-	-	-	-
NS-PDC-82	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-83	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-84	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-85	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-86	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-87	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-88	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-89	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-90	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-91	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-92	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-93	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-94	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-95	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-96	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-97	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-98	*Lachancea thermotolerans*	PDC-F (2013)	-	-	-	-
NS-PDC-174	*Lachancea thermotolerans*	PDC-J (2014)	-	-	-	-
NS-PDC-205	*Lachancea thermotolerans*	PDC-M (2014)	-	-	-	-

	***Metschnikowia fructicola* (n=18)**					
NS-EM-15	*Metschnikowia fructicola*	EM-A (2013)	+	+	+	-
NS-EM-113	*Metschnikowia fructicola*	EM-B (2014)	+	+	+	-
NS-EM-172	*Metschnikowia fructicola*	EM-A (2014)	+	++	+	-
NS-EM-194	*Metschnikowia fructicola*	EM-E (2014)	+	++	+	-
NS-PDC-14	*Metschnikowia fructicola*	PDC-A (2013)	+	+	+	-
NS-PDC-54	*Metschnikowia fructicola*	PDC-D (2013)	+	++	-	-
NS-PDC-57	*Metschnikowia fructicola*	PDC-D (2013)	+	+	-	-
NS-PDC-177	*Metschnikowia fructicola*	PDC-K (2014)	+	++	+	-
NS-PDC-191	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-192	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-193	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-194	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-195	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-196	*Metschnikowia fructicola*	PDC-L (2014)	+	++	+	-
NS-PDC-207	*Metschnikowia fructicola*	PDC-M (2014)	+	++	+	-
NS-PDC-208	*Metschnikowia fructicola*	PDC-M (2014)	+	++	+	-
NS-PDC-214	*Metschnikowia fructicola*	PDC-M (2014)	+	++	+	-
NS-PDC-220	*Metschnikowia fructicola*	PDC-M (2014)	+	++	+	-
	***Metschnikowia pulcherrima* (n=61)**					
NS-EM-34	*Metschnikowia pulcherrima*	EM-B (2013)	+	+	+	-
NS-EM-111	*Metschnikowia pulcherrima*	EM-B (2014)	+	+	+	-
NS-EM-115	*Metschnikowia pulcherrima*	EM-B (2014)	+	+	+	-
NS-EM-123	*Metschnikowia pulcherrima*	EM-B (2014)	+	+	+	-
NS-EM-167	*Metschnikowia pulcherrima*	EM-A (2014)	+	++	+	-
NS-EM-184	*Metschnikowia pulcherrima*	EM-E (2014)	+	++	+	-
NS-EM-187	*Metschnikowia pulcherrima*	EM-E (2014)	+	++	+	-
NS-EM-197	*Metschnikowia pulcherrima*	EM-E (2014)	+	++	+	-
NS-PDC-48	*Metschnikowia pulcherrima*	PDC-C (2013)	+	+	+	-
NS-PDC-50	*Metschnikowia pulcherrima*	PDC-C (2013)	+	+	+	-

NS-PDC-51	*Metschnikowia pulcherrima*	PDC-D (2013)	+	+	-	-
NS-PDC-52	*Metschnikowia pulcherrima*	PDC-D (2013)	+	+	-	-
NS-PDC-53	*Metschnikowia pulcherrima*	PDC-D (2013)	+	+	-	-
NS-PDC-55	*Metschnikowia pulcherrima*	PDC-D (2013)	+	+	-	-
NS-PDC-56	*Metschnikowia pulcherrima*	PDC-D (2013)	+	+	-	-
NS-PDC-81	*Metschnikowia pulcherrima*	PDC-F (2013)	+	++	+	-
NS-PDC-141	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-142	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-143	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-144	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-145	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-146	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-147	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-148	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-149	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-150	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-151	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-152	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-153	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-154	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-155	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-156	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-157	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-158	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-159	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-160	*Metschnikowia pulcherrima*	PDC-I (2014)	+	++	+	-
NS-PDC-176	*Metschnikowia pulcherrima*	PDC-K (2014)	+	++	+	-
NS-PDC-179	*Metschnikowia pulcherrima*	PDC-K (2014)	+	++	+	-
NS-PDC-180	*Metschnikowia pulcherrima*	PDC-K (2014)	+	++	+	-
NS-PDC-197	*Metschnikowia pulcherrima*	PDC-L (2014)	+	++	+	-

NS-PDC-198	*Metschnikowia pulcherrima*	PDC-L (2014)	+	++	+	-
NS-PDC-199	*Metschnikowia pulcherrima*	PDC-L (2014)	+	++	+	-
NS-PDC-200	*Metschnikowia pulcherrima*	PDC-L (2014)	+	++	+	-
NS-PDC-201	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-202	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	-	-
NS-PDC-204	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-206	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	-	-
NS-PDC-213	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-215	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-216	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-217	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-219	*Metschnikowia pulcherrima*	PDC-M (2014)	+	++	+	-
NS-PDC-241	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-247	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-251	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-255	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-256	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-258	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
NS-PDC-259	*Metschnikowia pulcherrima*	PDC-O (2014)	+	+	+	-
NS-PDC-260	*Metschnikowia pulcherrima*	PDC-O (2014)	+	++	+	-
	Torulaspora delbrueckii **(n=1)**					
NS-PDC-169	*Torulaspora delbrueckii*	PDC-J (2014)	-	-	-	-
	Wickerhamomyces anomalus **(n=2)**					
NS-PDC-167	*Wickerhamomyces anomalus*	PDC-J (2014)	+	+++	-	-
NS-PDC-171	*Wickerhamomyces anomalus*	PDC-J (2014)	+	++	-	-

5. CAPÍTULO 3

5.1. Desarrollo de un métodos rápido para la selección de levaduras con elevada actividad β-liasa

International Journal of Food Microbiology 225 (2016) 1–8

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Improvement of aromatic thiol release through the selection of yeasts with increased β-lyase activity

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ARTICLE INFO

Article history:
Received 1 August 2015
Received in revised form 22 January 2016
Accepted 1 March 2016
Available online 3 March 2016

Keywords:
Wine fermentation
Selective medium
β-lyase activity
Aromatic thiols

ABSTRACT

The development of a selective medium for the rapid differentiation of yeast species with increased aromatic thiol release activity has been achieved. The selective medium was based on the addition of S-methyl-L-cysteine (SMC) as β-lyase substrate. In this study, a panel of 245 strains of *Saccharomyces cerevisiae* strains was tested for their ability to grow on YCB-SMC medium. Yeast strains with an increased β-lyase activity grew rapidly because of their ability to release ammonium from SMC in comparison to others, and allowed for the easy isolation and differentiation of yeasts with promising properties in oenology, or another field, for aromatic thiol release. The selective medium was also helpful for the discrimination between those *S. cerevisiae* strains, which present a common 38-bp deletion in the *IRC7* sequence (present in around 88% of the wild strains tested and are likely to be less functional for 4-mercapto-4-methylpentan-2-one (4MMP) production), and those *S. cerevisiae* strains homozygous for the full-length *IRC7* allele. The medium was also helpful for the selection of non-*Saccharomyces* yeasts with increased β-lyase activity. Based on the same medium, a highly sensitive, reproducible and non-expensive GC–MS method for the evaluation of the potential volatile thiol release by different yeast isolates was developed.

1. Introduction

Several cysteine-S-conjugates found in foods and beverages (garlic, onion, grape must, etc.) are β-lyase substrates. Cysteine-S-conjugated compounds are precursors of potent aromatic thiols that contribute to aroma descriptors such as grapefruit, passion fruit, citrus and boxwood of many white wines with a sensory perception threshold range in the parts per trillion (Bailly et al., 2006; Bouchilloux et al., 1998; Darriet et al., 1995). These aromatic thiols are practically absent in grape juice and develop only during the alcoholic fermentation. This explains the commonly held notion that the wine yeast *Saccharomyces cerevisiae* is responsible for the formation of volatile thiols during fermentation. Darriet et al. (1995) found that volatile thiols occur in the grape in the form of aroma-free, non-volatile, and cysteine-bound compounds and that yeast, is only involved in releasing the aromatic thiols from the aroma-free grape precursor compounds. For example, the aromatic thiols 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP) are released from odorless cysteine-S-conjugated precursors of the grape must during fermentation (Holt et al., 2012; Swiegers et al., 2009).

Some genes (*BNA3*, *CYS3*, *GLO1*, *IRC7*, *STR3*) have been suggested to be involved in volatile thiol release (Howell et al., 2005; Thibon et al., 2008). Recently, *IRC7* and *STR3* genes have been confirmed to be responsible for 4MMP and 3MH production due to their encoded carbon-sulfur β-lyase activity (EC 4.4.1.8) (Holt et al., 2011; Roncoroni et al., 2011). However, only *irc7*Δ mutant had shown significant reductions in the release of both, 4MMP and 3MH, independently of the initial cysteine-S-conjugated precursor concentration indicating its central role in volatile thiol release. The presence of carbon–sulfur β-lyase activity has been determined to be the main responsible for cleavage of cysteine-S-conjugated forms of 3MH and 4MMP into free thiols (Harsch and Gardner, 2013; Howell et al., 2005; Swiegers and Pretorius, 2007; Tominaga et al., 1998). The release of aromatic thiols by other microorganisms has been related to the activity of cystathionine γ- and β-lyases (Irmler et al., 2008; Martínez-Cuesta et al., 2006; Troccaz et al., 2008; Wu and Morris, 1973). Furthermore, apart from their role in aromatic thiol release, cystathionine β-lyases catalyze the conversion of cystathionine into homocysteine in an α, β-elimination reaction, which generates methionine and the by-products pyruvate and ammonia employing pyridoxal-5′-phosphate as cofactor (Thomas and Surdin-Kerjan, 1997) (Fig. S1).

It is generally accepted that grape harvesting practices and processing can have an important influence on thiol yield (Allen et al., 2011; Capone and Jeffery, 2011). However, conversion of the cysteinylated precursors into their corresponding thiols is accepted to be very limited, typically less than 5%, taking into account an efficient *S. cerevisiae* strain (Coetzee and du Toit, 2012; Murat et al., 2001; Peña-Gallego et al., 2012;

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.03.001
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Roland et al., 2011; Winter et al., 2011). For instance, Str3p showed a modest side activity, being able to release an amount of free 3MH and 4MMP corresponding to approximately 0.1% and 0.6%, respectively, of the specific activity against L-cystathionine (Holt et al., 2012). β-lyases are enzymes involved in amino acid metabolism that do not normally catalyze a β-lyase reaction, but catalyze a non-physiological related β-lyase side reaction that depends on the electron-withdrawing characteristics of the cysteine S-conjugates (Cooper et al., 2010). Due to the very low sensory perception threshold range, even a modest increase in yeast β-lyase activity, due to this side reaction, could alter the composition of volatile thiols in wine and improve flavor (Murat et al., 2001). Many types of yeast, including *Saccharomyces* sp., possess limited capabilities in terms of enzymatic hydrolysis of precursors and formation of volatile products. Cleavage of volatile thiols during fermentation appears to be strain dependent and a particular strain ability to release one thiol does not seem to be linked to the formation of a different thiol (Holt et al., 2012; Roncoroni et al., 2011). Research suggests that by using different strains, differences in the release of these volatile thiols can be achieved.

Due to the demanding nature of modern winemaking practices and an increasingly consumer quality demand, there is a growing need for wine strains possessing a wide range of improved, optimized or novel enological features. One challenge today is the development of screening methods to identify strains that improve wine quality from the great, unexplored diversity of natural grape yeasts. Based on several criteria, winemakers often use selected strains to improve flavor, palate structure and alcohol and phenolic content, among others (Belda et al., 2016; Pretorius, 2000). Commercial wine *S. cerevisiae* has been selected on the basis of enhanced tropical fruity characters produced during fermentation and similarly several non-*Saccharomyces* yeasts have been shown to release significant concentrations of volatile thiols. Indeed, *Pichia kluyveri* was recently commercialized (Frootzen, Chr. Hansen, Denmark) for winemaking with the aim of enhancing fruity flavors (Anfang et al., 2009; Zott et al., 2011). Therefore, selection programs of wine yeast starters able to produce more volatile thiols constitute an important goal for the wine industry, which has never conducted rationally.

Selective media are formulated to support the growth of one group of organisms, but inhibit the growth of another. The aim of this study was to develop a selective medium for the differentiation of yeast species according to their β-lyase activity and to discuss their potential application in oenology. Additionally, based on the same medium, a highly sensitive, reproducible and non-expensive method for the evaluation of the potential volatile thiol release by different yeast isolates has been developed.

2. Material and methods

2.1. Strains and general media

223 *S. cerevisiae* strains, originally isolated from wineries from *Designation of Origin* (D.O.) Ribera del Duero, D.O. Rueda and D.O. Tierra de León (deposited in CYC, Complutense Yeast Collection, Complutense University of Madrid, Spain) and 22 industrial strains (Agrovin S.A., Alcázar de San Juan, Spain).

Sabouraud-Chloramphenicol (Oxoid, Hampshire, UK) was routinely used for *S. cerevisiae* isolation and Lysine Agar (Oxoid) was used for the isolation of non-*Saccharomyces* strains.

In order to determine if the methods described in this work were also helpful with non-*Saccharomyces* yeasts, a selection of 13 non-*Saccharomyces* strains isolated from wineries was used for the determination of their β-lyase activity. These non-*Saccharomyces* strains were identified by partial sequencing of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al., 1992), and the DNA for sequencing was amplified by using an Eppendorf Mastercycler apparatus, with 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′) (Kurtzman and Robnett, 1997). Isolates were compared for aromatic thiol release with *Torulaspora delbrueckii* Viniferm NSTD, a strain of industrial usage for thiol release as described below (Fig. 4).

2.2. Interdelta analysis for S. cerevisiae strain fingerprinting

Eight hundred and eighty *S. cerevisiae* strains isolated from wineries in this study were checked for fingerprinting on interdelta polymorphisms by PCR amplification using delta12 (5′-TCAACAATGGAATCCC AAC-3′) and delta21 (5′- CATCTTAACACCGTATATGA-3′) primers (Legras and Karst, 2003). Agarose gels were stained with GelRed® and analyzed under an UV transilluminator. The selection of the *S. cerevisiae* strains was achieved according to the different patterns observed using SigmaGel software.

2.3. The selective YCB-SMC medium

The selective medium described in this work was based on the reaction catalyzed by the β-lyase activity over cysteinylated thiol precursors. The YCB-SMC medium was: 0.1% (wt/vol) S-methyl-L-cysteine (Sigma-Aldrich, Madrid, Spain), 0.01% (wt/vol) pyridoxal-5′-phosphate (Sigma-Aldrich) and 1.2% (wt/vol) Yeast Carbon Base (Difco, Detroit, MI, USA). For solid medium 2% agar was added. This medium was adjusted to pH 3.5 with HCl. All components, except agar, were sterilized by using 0.22 μm filters. The medium was kept under refrigeration at 4 °C before use. The strains were seeded on YCB-SMC medium following the procedure for nitrogen assimilation tests used for classical methods on yeast taxonomy. Yeasts grown on a rich medium may carry a reserve of nitrogen in the form of protein. Possible errors due to this reserve are eliminated by making two serial transfers in the YCB-SMC medium. When the first transfer was three days old, one loopful was transferred to a second plate of the YCB-SMC medium containing the same source of nitrogen. Colony growth was checked after 48–72 h at 20 °C.

Based on the YCB-SMC medium and with the objective of validating it, a Cys-4MMP based medium, instead of SMC, was developed as follows: 0.1% (wt/vol) S-4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP), 0.01% (wt/vol) pyridoxal-5′-phosphate (Sigma-Aldrich), and 1.2% (wt/vol) Yeast Carbon Base (Difco). Cys-4MMP was synthetized according to the procedure of Howell et al. (2004). Purity was determined by ^{1}H-NMR (Bruker DPX 300 MHz) and ESI-MS (HPLC) with Bruker EsquireLC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany) (Fig. S7).

2.4. PCR analysis of IRC7 genotypes

With the initial aim being to gather new information on the comparison of the *IRC7* sequences of the industrial and wild *S. cerevisiae* strains, a PCR protocol was conducted with the primers PF6, 5′-AGCTGGTCTGGA GAAAATGG-3′ and PR7, 5′-TCTTCTGCGAGACGTTCAAA-3′ (Roncoroni et al., 2011). The DreamTaq Green PCR Master Mix (Life Technologies Ltd., Paisley, UK) was used. The PCR reaction conditions were an initial denaturing step of 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s and 72 °C for 1 min and then a final extension at 72 °C for 5 min. The PCR products were run on 2% agarose gels with an appropriate molecular weight marker and the size of the amplified products was checked (Fig. S3 and Fig. S4). Six strains were selected in representation of the three *IRC7* genotypes (Fig. 1). The selected *S. cerevisiae* strains were: (homozygous for the short *IRC7* allele: 1- SEM-73, 2- SEM-25, 3- SEM-107, 4- SEM-251, 5- SEM-271 and 6- SO-320); (heterozygous strains for *IRC7* allele: 7-SEM-10, 8- SO-166, 9- SO-353, 10- SO-10, 11- SEM-113, 12- SEM-294) and (homozygous for the full-length *IRC7* allele: 13- SO-213, 14- SO-335, 15- SO-331, 16- SEM-115, 17- SEM-129 and 18- SO-203).

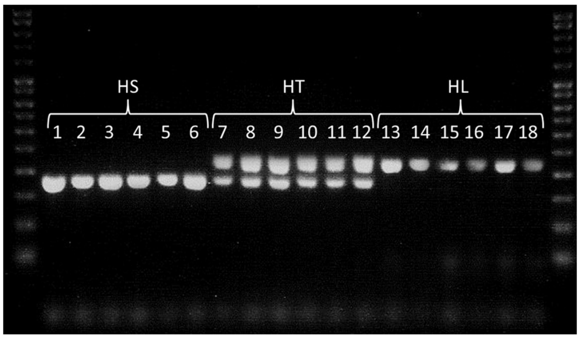

Fig. 1. PCR products of the main three *IRC7* genotypes present in *S. cerevisiae* amplified with PF6 and PR7 primers. *S. cerevisiae* strains used as PCR templates were as follows: HS (1- SEM-73, 2- SEM-25, 3- SEM-107, 4- SEM-251, 5- SEM-271, 6- SO-320), HT (7-SEM-10, 8- SO-166, 9- SO-353, 10- SO-10, 11- SEM-113, 12- SEM-294, 13- SO-213) and HL (14-SO-335, 15- SO-331, 16- SEM-115, 17- SEM-129 and 18- SO-203).

2.5. *IRC7* genotype differentiation based on the spot growth assay on YCB-SMC medium

In order to determine whether there is a correlation between the growth observed in the YCB-SMC medium and the different *IRC7* alleles, the six wild strains belonging to each of the three *IRC7* genotypes referred in this work were grown on solid YCB-SMC and YCB-DAP media. Every strain was seeded onto YCB-SMC medium and incubated at 20 °C for 48–72 h and restreaked on the same medium to avoid nutrient carryover. Then the strains were suspended in saline buffer to reach an optical density at $\lambda_{600\ \mathrm{nm}}$ of 0.5. The indicated strains were tested for growth by applying 5 μl of serial dilutions from 10^{-1} to 10^{-6} onto YCB-SMC and YCB-DAP media. YCB-DAP medium (containing the same components and concentrations of YCB-SMC medium but containing 0.1% diammonium phosphate instead of SMC) was used as positive control for growth (Fig. 2).

2.6. Blind test for validation of YCB-SMC medium

Two different *S. cerevisiae* strains, S-EM-129 and S-EM-251; in representation of two *IRC7* genotypes (the homozygous for the full-length *IRC7* allele and the homozygous for the short *IRC7* allele, respectively) were grown in YCB-SMC agar medium during 48 h to develop a cellular suspension of 10^6 cells/ml in NaCl 0.9%. Both strains were mixed together (ratio 1:1) and then serially diluted and spread in YCB-SMC agar medium. Plates were incubated at 20 °C during 72 h and colony development was followed. Plates were photographed with a Nikon Eclipse 50i microscope at 40× magnification using a ProgRes® CT3 camera with a ProgRes® CapturePro 2.6 software (Jenoptik Laser, Optik, Systeme GmbH). Colony area was calculated by using the colony diameter expressed in number of pixels. Based on their size, colonies were selected and analyzed by PCR for *IRC7* genotypes as described before (Fig. S5).

2.7. Quantification of thiol release by gas chromatography–mass spectrometry in YCB-SMC medium

The liquid YCB-SMC medium was used to prepare inocula in 20 ml headspace vials with magnetic screw caps (Supelco Inc., Bellefonte, Pennsylvania, USA) at 10 ml per tube. Inocula of the different yeast strains were standardized at an $OD_{600\ \mathrm{nm}}$ of 0.1. The strains used are listed in Fig. 3 (*S. cerevisiae* strains) and Fig. 4 (non-*Saccharomyces* strains). These liquid cultures were incubated with shaking during 24 h at 28 °C. Then, the production of methanethiol (MTL), and its dimer (dimethyl disulfide, SMDS), were determined in the headspace by GC–MS. A Varian gas chromatograph CP-3800 coupled to a Saturn 2200 ion trap mass spectrometer was used to analyze 200 μl of the sample headspace. The properties and basic settings of the GC–MS were as follows. The GC column used in this instrument was a capillary column Phenomenex ZB-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The volatilized compound was carried in a helium flow at 1 ml/min. The spectrometer operated in full-scan mode in a mass interval between 30 and 400 amu. The injection-port temperature was set at 260 °C. The temperature program was initially set at 30 °C for 2 min

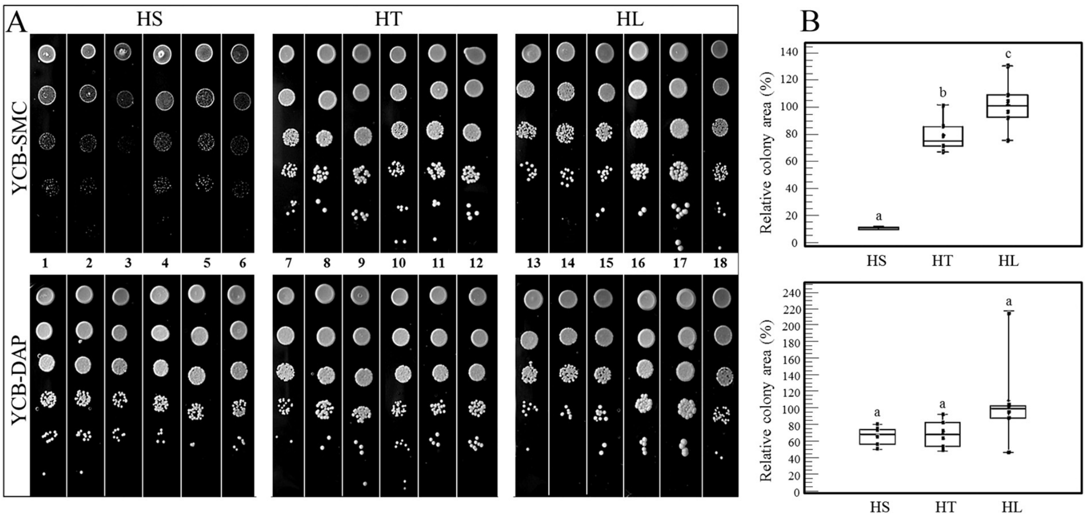

Fig. 2. Plot test study developed in order to determine the growth ability in YCB-SMC and YCB-DAP media of the six strains selected belonging to the three *IRC7* allele groups present in *S. cerevisiae* (A). The first group (HS) was formed by the strains homozygous for the short *IRC7* allele (1- SEM-73, 2- SEM-25, 3- SEM-107, 4- SEM-251, 5- SEM-271 and 6- SO-320), that showed a slow growth in YCB-SMC. The second group (HT) was composed by the heterozygous strains for *IRC7* allele (7-SEM-10, 8- SO-166, 9- SO-353, 10- SO-10, 11- SEM-113, 12- SEM-294). (HL) Group of strains homozygous for the full-length *IRC7* allele (13- SO-213, 14-SO-335, 15- SO-331, 16- SEM-115, 17- SEM-129 and 18- SO-203) that showed the highest growth in YCB-SMC, but very similar to the heterozygous strains for *IRC7*. The same medium supplemented with diammonium phosphate (DAP), instead of SMC, was used as control. Box plots (B) represent data for the six strains analyzed of the three different genotypes (HS, HT, HL). Different letters among boxes represent different statistical groups ($p < 0.05$).

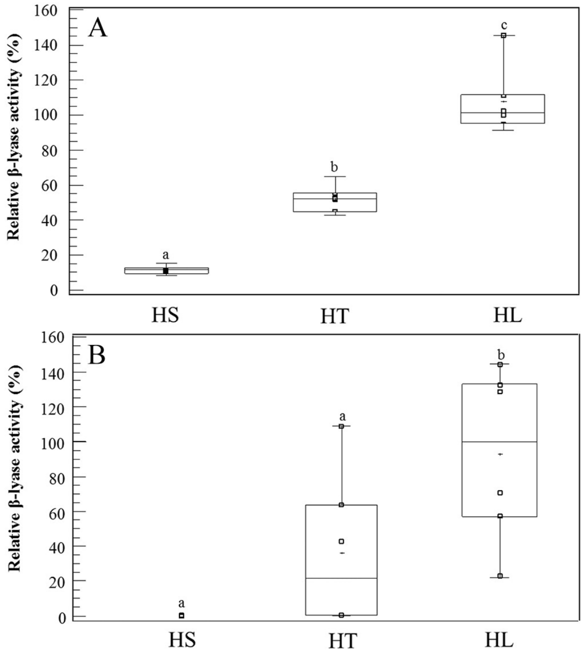

Fig. 3. β-lyase activity of 18 *S. cerevisiae* wild strains determined by GC–MS using YCB-SMC (A) and YCB-Cys-4MMP (B) as substrates for β-lyase activity. The strains homozygous for the short *IRC7* allele (SO-320, SEM-271, SEM-251, SEM-107, SEM-25 and SEM-73) had a residual activity (HS). The heterozygous strains for *IRC7* allele (SO-353, SO-166, SO-10, SEM-294, SEM-113 and SEM-10) reached intermediate activity values (HT). The highest β-lyase activity (HL) was obtained for the homozygous *S. cerevisiae* strains for the full-length *IRC7* allele (SO-335, SO-331, SO-213, SO-203, SEM-129 and SEM-115). The same pattern of β-lyase activity was observed for both substrates (SMC or Cys-4MMP). Box plots represent data for the six strains analyzed of the three different genotypes (HS, HT, HL). Different letters among boxes represent different statistical groups ($p < 0.05$).

and ramped to 40 °C at 2 °C/min, then to 100 °C at 15 °C/min and finally ramped to 200 °C at 25 °C/min and stated at this temperature during 5 min. The total program was 20 min. A TIC (Total Ion Chromatogram) was created by summing up intensities of all mass spectral peaks belonging to the same scan.

In the conditions tested, S-methyl-L-cysteine was transformed to MTL, pyruvate and ammonium due to the β-lyase activity of yeasts. In these conditions, MTL dimerized in part through reoxidation to DMDS, and so, both compounds were detected simultaneously by GC–MS (Fig. S6). Sodium methanethiolate and dimethyl disulfide (Sigma) were used as standards for quantification. Each determination was done by triplicate in three independent assays. The results obtained were expressed as relative β-lyase activity.

2.8. Quantification of thiol release by gas chromatography–mass spectrometry in YCB-Cys-4MMP medium

With the aim of validating the results obtained with the new described methodology and also to compare them with the results obtained with naturally occurring substrates, the β-lyase activity of the same *S. cerevisiae* strains was analyzed (Fig. 3b) in a medium supplemented with Cys-4MMP instead of SMC. The method for the determination of 4MMP production was based in the method described by Howell et al., 2004, but detecting 4MMP directly from the vial headspace without the use of SPME fiber. The detailed method was exactly the same described above for SMC products (MTL/DMDS) but with the injection of 1000 μl of the sample headspace instead of 200 μl.

2.9. Statistical analysis

All the statistical analyses were performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p < 0.05$ for the ANOVA matrix F value. Furthermore, the multiple-range test was used to compare the means.

3. Results

3.1. Interdelta analysis for S. cerevisiae strain fingerprinting

With the initial aim of checking a significant number of yeast isolates with oenological interest, 880 isolates of *S. cerevisiae*, from wineries of three different D.O., were checked for interdelta polymorphisms. The analysis of the polymorphisms in agarose gels obtained for the entire collection of *S. cerevisiae* isolates revealed the existence

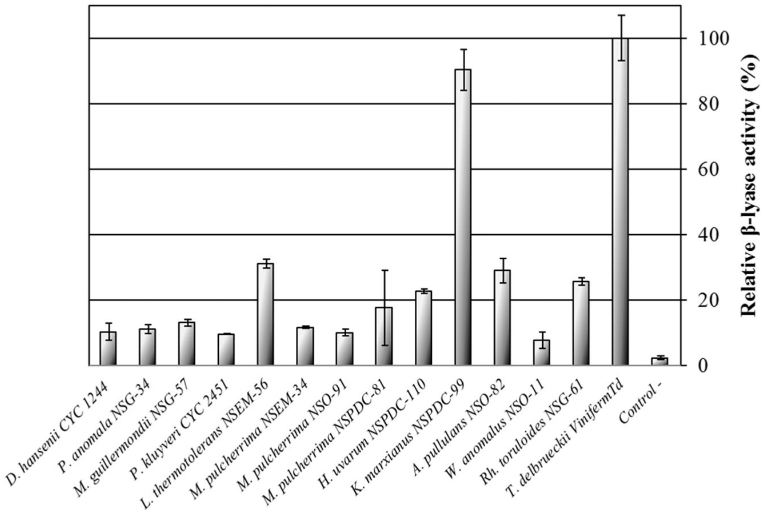

Fig. 4. β-lyase activity of 14 non-*Saccharomyces* strains determined by GC–MS by using the selective medium developed in this work. Non-*Saccharomyces* strains had a low β-lyase activity with the exception of *T. delbrueckii* Viniferm NSTD and *K. marxianus* NSPDC-99.

of 223 different polymorphisms (Fig. S2), named in this study as "wild strains".

3.2. Growth industrial and wild S. cerevisiae strains on YCB-SMC medium

Two growth phenotypes on YCB-SMC medium were observed among the 245 industrial and wild strain collections. Approximately, 15% of the strains were able to grow more efficiently on the selective SMC medium, generating bigger colonies. The percentage of this phenotype varied between the industrial (10 of 22; 45.5%) and the wild strains (27 of 223; 12%) isolated in this study, showing the influence of the selection processes of industrial strains.

3.3. Determination of IRC7 genotypes in wild and industrial strains

With the aim of determining the presence of different genotypes in *IRC7*, according to the findings by Roncoroni et al. (2011), 223 wild isolates were checked for the determination of the presence of a 38-bp deletion commonly found between yeasts isolates (Liti et al., 2009). As expected, two sizes of PCR products were detected, 219 bp for the full-length copy and 181 bp for the 38-bp deletion of *IRC7*. The percentage of wild yeast strains with the reported 38-bp deletion in *IRC7* was 88% (196 strains of 223). Additionally, there were 2.7% (6 strains of 223) of yeast strains homozygous for the full-length *IRC7* allele and 9.4% (21 strains of 223) that were found to be heterozygous for the full-length allele (Fig. 1; Fig. S3).

In addition, the industrial collection (Agrovin, S.A.) of *S. cerevisiae* strains was tested in the same way (Fig. S4). In this collection, 23% (5 strains of 22) of the strains were found to be homozygous for the full-length *IRC7* allele and an additional 23% was found to be heterozygous for the full-length *IRC7* allele.

3.4. Relationship between ICR7 genotypes and phenotypes detected in YCB-SMC medium

Comparison of the *IRC7* genotypes of *S. cerevisiae* and the growth ability in YCB-SMC medium indicated that there is a clear relationship between those features. The strains homozygous and heterozygous for the full-length *IRC7* allele were able to grow faster in YCB-SMC medium than those strains homozygous for the short-length *IRC7* allele (Fig. 2). It was also noticeable that, according to colony growth, *S. cerevisiae* strains described to be homozygous and heterozygous for the full-length *IRC7* allele were difficult to differentiate in YCB-SMC medium. The homozygous and the heterozygous strains for the full-length *IRC7* allele were able to grow faster in YCB-SMC medium, giving a phenotype clearly different than the observed for the strains that were homozygous for the short-length *IRC7* allele (Fig. 2). The average colony area comprised by the group of strains homozygous for the full-length allele was 17.3 times higher than the homozygous for the short-length allele and 1.26 times higher than the heterozygous strains for *IRC7* allele, indicating that the YCB-SMC medium generated a selective advantage over the group of strains that were homozygous for the short *IRC7* allele (Fig. S5a). These observations gave the opportunity to easily differentiate one of the main *IRC7* genotypes.

Two strains (S-EM-129 and S-EM-251; in representation of the homozygous full- and the homozygous short-length *IRC7* alleles, respectively) were mixed (ratio 1:1) and used as inocula to develop a blind test to differentiate *IRC7* genotypes based on the phenotypes showed in YCB-SMC medium. As expected, yeast colonies, isolated from YCB-SMC medium, showed different sizes and were ascribed to two colony size groups. Ten colonies of each group were subjected to PCR for *IRC7* genotype differentiation (Fig. S5b). The group of smaller colonies was observed to have the reported 38-bp deletion in *IRC7* whereas the group of bigger colonies had the homozygous full-length allele. 100% of the strains were correctly assigned to their respective genotype.

3.5. Determination of the β-lyase activity on YCB-SMC medium by GC–MS

With the aim of determining if the YCB-SMC medium was useful for quantification purposes of the β-lyase activity, the YCB-SMC medium was inoculated with the aforementioned *S. cerevisiae* strains and the β-lyase reaction products (MTL and DMDS) determined by GC–MS. According to the results, it was possible to establish a relationship between the *IRC7* genotypes of the *S. cerevisiae* strains and the release of thiols (Fig. 3). The homozygous strains for the full-length *IRC7* allele (SEM-115, SEM-129, SO-203, SO-213, SO-331 and SO-335) had, approximately, a 50% more β-lyase activity than the heterozygous strains for *IRC7* allele (SEM-10, SEM-113, SEM-294, SO-10, SO-166 and SO-353). It was also noticeable that the activity showed by the strain SEM-129, being 30–40% higher than the rest of the strains belonging to the same group. The β-lyase activity showed for the strains SEM-25, SEM-73, SEM-107, SEM-251, SEM-271 and SO-320 bearing a deletion in the *IRC7* sequence, was comprised between 5 and 10% in comparison to the average value of β-lyase activity obtained for the homozygous strains for the full-length *IRC7* allele (Fig. 3a).

Among the selection of non-*Saccharomyces* strains used in the present study, only *Kluyveromyces marxianus* NSPDC-99 was observed to cleave an important amount of SMC, in comparison to *T. delbrueckii* Viniferm NSTD, a commercial strain indicated for volatile thiol release (Fig. 4).

3.6. β-lyase activity on YCB-Cys-4MMP medium

Six strains belonging to each of the three *IRC7* genotypes described were inoculated in a liquid medium in which SMC was substituted by Cys-4MMP as the natural thiol-cysteine precursor present in grapes. The products were detected by GC–MS. The strains bearing a full-length copy of *IRC7* (SEM-115, SEM-129, SO-203, SO-213, SO-331 and SO-335) had the best production of 4-MMP when growing in YCB-Cys-4MMP medium, followed by the heterozygous strains for *IRC7* allele (SEM-10, SEM-113, SEM-294, SO-10, SO-166 and SO-353). The strains found to be homozygous for the deleted *IRC7* allele (SEM-25, SEM-73, SEM-107, SEM-251, SEM-271 and SO-320) showed no production of 4-MMP or a production under the detection limits.

4. Discussion

4.1. The selective YCB-SMC medium

The final aromatic profile of wine is one of the most important factors when determining its quality and intrinsic value. Winemakers generally focus on maximizing aroma intensity and complexity while minimizing aromas that may dominate or produce a negative perception for the production of wines with varietal distinction. The aroma of a wine is one of the most important determinants of its quality (Selli et al., 2004). Current strategies intended for the increment of wine aroma comprise the choice of grape variety to optimize production of thiol precursors, the choice of yeast strain to optimize release of volatile thiols in the winery and the coinoculation with yeast blends. Heretofore, the yeast selection programs do not have a tool for the selection of yeasts with an increased capacity of varietal thiol release. In this work a selective medium for the isolation of yeasts with an increased capacity for volatile thiol release has been developed. Cysteine S-conjugate β-lyases are pyridoxal 5′-phosphate-dependent enzymes that catalyze β-elimination reactions with cysteine S-conjugates that possess an electron-withdrawing group attached at the sulfur. The end-products of the β-lyase reaction are pyruvate, ammonium and a sulfur-containing fragment. The selective medium was designed by using S-methyl-L-cysteine (SMC) as the only nitrogen source to provide an easy and rapid method for isolation of yeast strains for their ability to cleave thiol precursors to release varietal thiol aroma. SMC was chosen because it structurally resembled naturally occurring cysteinilated precursors in grape must, being commercially available, water-soluble and non-expensive, avoiding the process of synthesis or extraction and purification of naturally occurring cysteinilated precursors in grapes. Additionally, SMC was found to be less toxic than other similar cysteinylated compounds, such as S-ethyl-L-cysteine, and it is metabolized to less-toxic end-products (Maw, 1961).

4.2. The selective YCB-SMC medium and IRC7 in S. cerevisiae

As described by Roncoroni et al. (2011) in *S. cerevisiae* strains the main β-lyase activity, responsible for the release of the varietal thiol 4MMP, is coded by the *IRC7* gene (Howell et al., 2005; Roncoroni et al., 2011). Two alleles have been described for this gene; the most common allele has a 38-bp deletion, missing a conserved region found in other β-lyase genes, which generates a C-terminally truncated protein of about 340 amino acids instead of the 400 amino acids protein present in other yeasts and bacteria (Hall et al., 2005). This deletion implies the production of an enzyme, which is characterized by its low β-lyase activity, and therefore a lower 4MMP production (Roncoroni et al., 2011). The observation, also reported by Roncoroni et al. (2011), that the short allele of *IRC7* is present in a higher proportion in commercial wine yeasts helps to explain why certain commercial strains are known as high 4MMP releasers (Lee et al., 2008; Masneuf et al., 2002, 2006). On the contrary, 88% of the autochthonous *S. cerevisiae* strains isolated in this study bear the truncated *IRC7* allele (Fig. S3), and being therefore low 4MMP producers. The reasons about why this short allele of *IRC7* have been selected by nature remains to be elucidated. The high diversity observed among natural strains reinforces the usefulness of the screening proposed to be added to the current yeast selection criteria used. However, it must be considered that the findings described in this work could be affected by the existence of additional considerations such as the intrinsic characteristics of the strains which could affect metabolism behavior or the variable toxicity of the precursors that are substrates of β-lyase (Santiago and Gardner, 2015a). In our laboratory, in addition to SMC, other potential substrates of β-lyase have been considered (S-benzyl-L-cysteine, S-(2-aminoethyl)-L-cysteine and S-(4-tolyl)-L-cysteine) but without valuable results due to growth inhibition in the conditions tested (not shown).

In addition, the described method for yeast isolation was also useful when performed to quantitatively detect β-lyase activity by determining the release of MTL/DMDS (Fig. S6). This method uses SMC as substrate for β-lyase avoiding the use of natural precursors such as Cys-4MMP. SMC is commercially available, water soluble and non-toxic for yeast cells in the conditions tested. In order to phenotypically differentiate between the three different *IRC7* genotypes present in *S. cerevisiae* strains, six *S. cerevisiae* strains belonging to each genotype were selected and inoculated in the YCB-SMC medium and then subjected to analysis for MTL/DMDS production. According to the results presented in the Fig. 3a, the strains with the full-length copy of *IRC7* were the higher MTL/DMDS producers indicating an increased β-lyase activity. On the contrary, those strains with the 38-bp deletion produced small amounts of MTL/DMDS indicating a less efficient β-lyase activity. Furthermore, this is in agreement with recent findings that indicate that a full-length copy of *IRC7* allele is required for the cleavage of two thiol precursors (cysteine-4MMP and glutathione-3MH) in a panel of *S. cerevisiae* strains (Santiago and Gardner, 2015b).

In order to validate the proposed methods for, qualitatively and quantitatively, determine the β-lyase activity in yeast isolates, the use of SMC was compared with the natural precursor Cys-4MMP (Fig. 3). The results indicate that although the reported data are not exactly the same, the conclusions for these findings were similar; confirming that yeast strains possessing a long size copy of *IRC7* are more efficient for thiol aroma release and YCB-SMC medium is a suitable simplified method for the isolation of yeasts with good properties for thiol aroma production. These results indicate that the same β-lyase activity could be the responsible for the cleavage of both substrates, Cys-4MMP and SMC. The GC–MS method described in this work was developed in the hope that it could be helpful for the determination of the β-lyase activity avoiding the use of natural substrates such as Cys-4MMP, more difficult to detect by GC–MS and not widely available as commercial products. The detection of MTL/DMDS by GC–MS was observed to be simplest, because the method only considered injection of a small headspace volume without the utilization of a SPME fiber to concentrate the products of the reaction, as it was described to be required for the detection of 4MMP (Howell et al., 2004).

4.3. Contribution of non-Saccharomyces to thiol release

In addition to the presence of a highly functional *IRC7*-coded β-lyase, it is known that Nitrogen Catabolite Repression (NCR) affects concentrations of varietal thiols in wine through the repression of *IRC7*, specially by inorganic nitrogen forms such as ammonium, usually added as diammonium phosphate during the middle and final fermentation stages (Subileau et al., 2008; Thibon et al., 2008), and also low fermentation temperatures diminish 4MMP production (Howell et al., 2004; Masneuf et al., 2006). That resulted in a small conversion of the cysteinylated thiol precursors into their corresponding free thiols, usually about 5%, or even less, (Murat et al., 2001; Roland et al., 2011; Winter et al., 2011; Coetzee and du Toit, 2012; Peña-Gallego et al., 2012). Although some genetic engineering techniques have been developed to enhance the cleavage of cysteinylated precursors into free thiols (Swiegers et al., 2007), these approaches are not acceptable for consumers and winemakers because of the use of genetically modified organisms. Exploiting the natural genetic variations offered by different *S. cerevisiae* (Liti et al., 2009) and non-*Saccharomyces* strains is a powerful tool to improve wine yeast characters (Zott et al., 2011).

Non-*Saccharomyces* species are limited to the early stages of fermentation, while *Saccharomyces* dominated towards the end of the alcoholic fermentation. However, the presence of non-*Saccharomyces* (autochthonous or commercial strains) generates significant differences in wine quality (Belda et al., 2015; Benito et al., 2015). For that reason YCB-SMC medium was intended for the selection of both, *S. cerevisiae* and non-*Saccharomyces* yeasts. Due to the cleavage of SMC, yeasts with an increased β-lyase activity were able to grow rapidly probably because of the ammonium released in the β-lyase reaction and, so, generating big colonies (Fig. 2). Furthermore, non-*Saccharomyces* yeast strains, such as *T. delbrueckii* Viniferm NSTD, were able to grow faster on YCB-SMC medium, being also able to release higher amounts of MTL/DMDS as detected by GC–MS (Fig. 4b), indicating that the medium was useful for the selection of a wide variety of yeast species.

In conclusion, there is a good margin to further multiply thiol production by non-*Saccharomyces* yeasts by breeding. In that sense, non-*Saccharomyces* yeasts are dominant during the first stages of wine fermentation, such as pre-fermentative cold soak (Zott et al., 2008). In accordance with that, their impact on wine quality is mainly circumscribed to the early stages in winemaking, when the addition of inorganic nitrogen sources (as the major catabolic repressor) could be avoided or substituted by the addition of organic nitrogen complexes that has been shown to be less repressive. YCB-SMC medium was useful for the determination of the potential of varietal thiol release by both, *S. cerevisiae* and non-*Saccharomyces* strains, making possible the efficient selection of yeasts with increased volatile thiol release. This possibility allows us to contemplate the possibility of using non-*Saccharomyces* yeasts as tools to increase the volatile thiol release in a time in which multi-starter fermentations are increasingly being used for organoleptic and quality improvement (Belda et al., 2015; Ciani et al., 2010). According to Zott et al. (2011), *T. delbrueckii* and *K. marxianus* had a high capacity to release MTL/DMDS indicating their high β-lyase activity. However, the strains of *Metschnikowia pulcherrima* tested in our study were found to have a moderate β-lyase activity (Fig. 4). Other yeasts species usually found in wineries released low amounts of SMC-related thiols, indicating their poor contribution to thiol production. Taking into account that NCR has not been studied in depth in several non-*Saccharomyces* species, and that the relationship between NCR and thiol release has been studied only in *S. cerevisiae*, our ongoing research is to investigate whether the positive effect of our promising non-*Saccharomyces* yeasts on thiol release is less affected by NCR in wine, giving the opportunity to develop a promising application for exploiting the potential thiol content of grape musts.

Acknowledgement

Funding for the research in this paper was provided by the Agrovin S.A, under the framework of the project IDI20130192-ENZIOXIVIN (Centre for Industrial Technological Development-CDTI, Spain). We are grateful to Dra. Cristina Gutiérrez from the Mass Spectrometry Centre of the Complutense University of Madrid for technical assistant and helpful discussions. We also thank Dr. Luis Sánchez and Julia Buendía from Analytical Chemistry Department of the Complutense University of Madrid for their help with 4MMP and Cys-4MMP synthesis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.03.001.

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Supplementary material

Figure S1. Cystathionine β-lyases catalyze the conversion of cystathionine into homocysteine and the by-products pyruvate and ammonia, employing pyridoxal-5’-phosphate as cofactor. They can also release the aromatic thiols 3MH and 4MMP from their respective cysteine-S-conjugated precursors present in grape must. The selective medium described in this work is composed by S-methyl-L-cysteine (SMC). Yeasts with an increased β-lyase activity are able to grow efficiently due to the assimilation of the released ammonia from SMC.

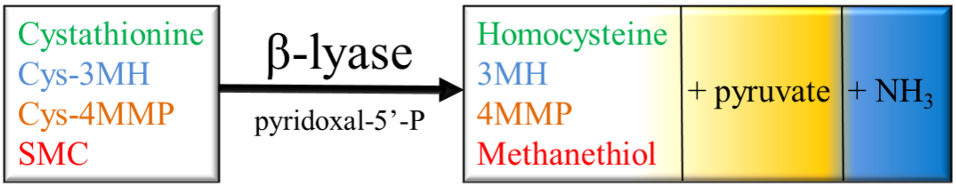
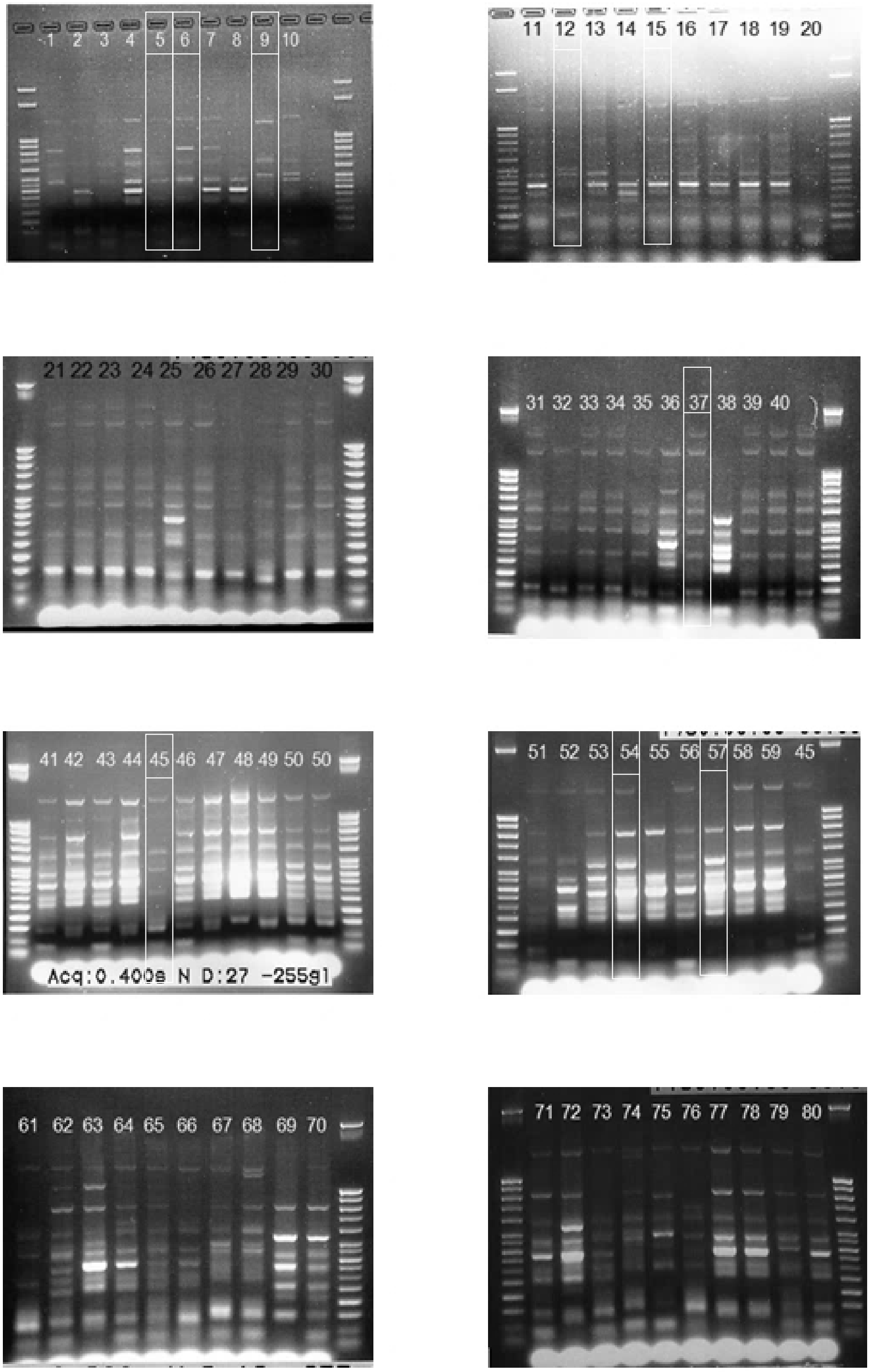
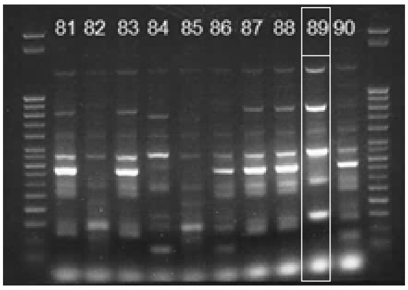


Figure S2a. PCR amplification of inter-delta regions. Electrophoretical patterns obtained with delta1-2 and delta 2-1 primers for the 120 *S. cerevisiae* isolates obtained from cellars of the D.O. *Tierra de Leon*. Highlighted lanes represents every one of the 223 different polymorphisms selected in the whole study.

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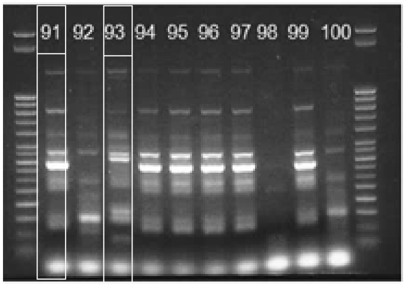
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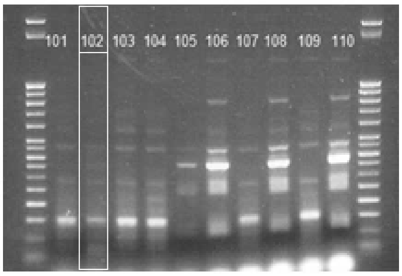
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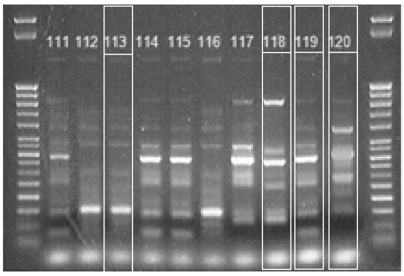
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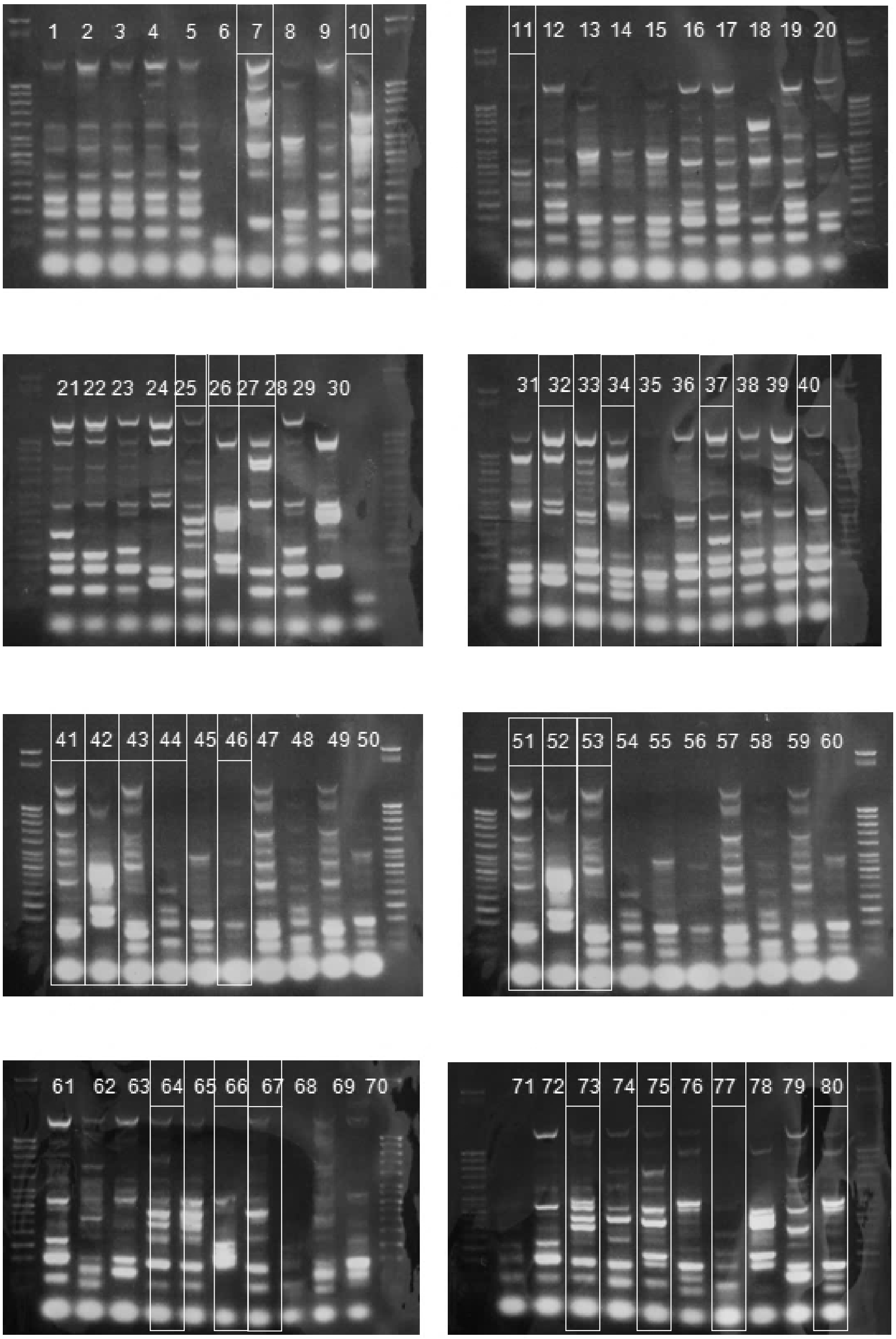
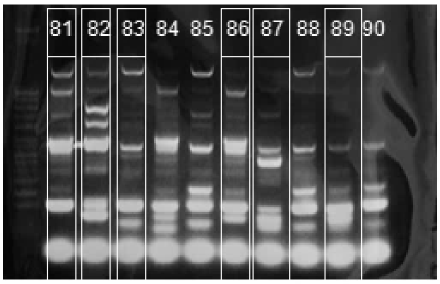
Figure S2b. PCR amplification of inter-delta regions. Electrophoretical patterns obtained with delta1-2 and delta 2-1 primers for the 380 *S. cerevisiae* isolates obtained from cellars of the D.O. *Ribera de Duero*. Highlighted lanes represents every one of the 223 different polymorphisms selected in the whole study.

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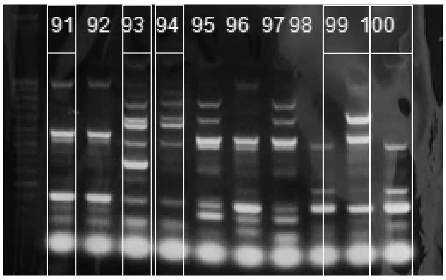
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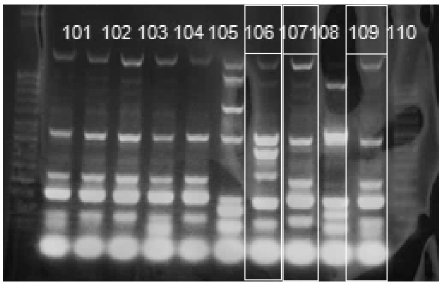
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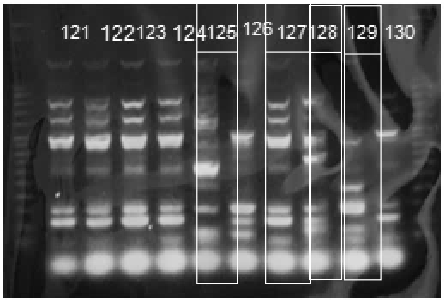
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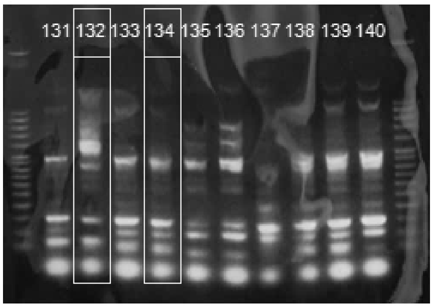
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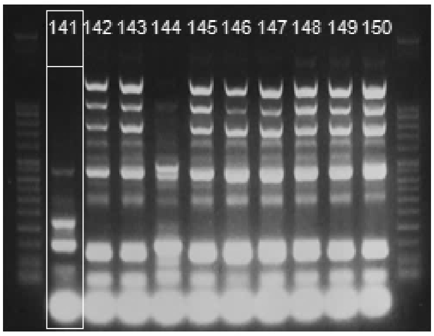
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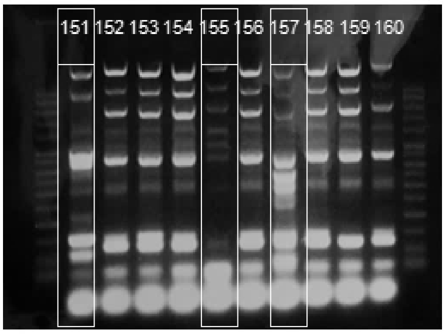
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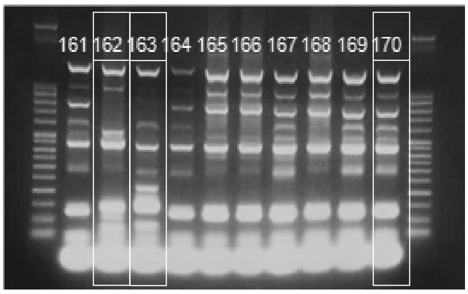
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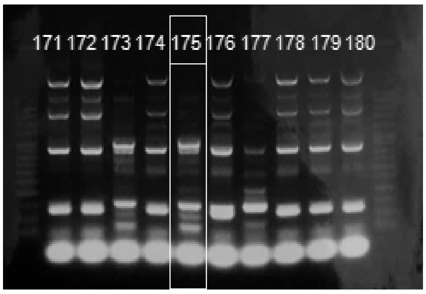
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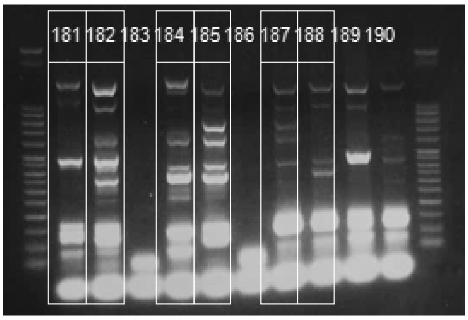
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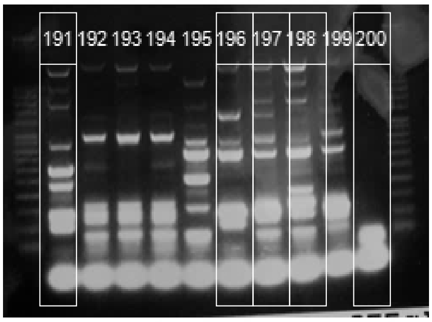
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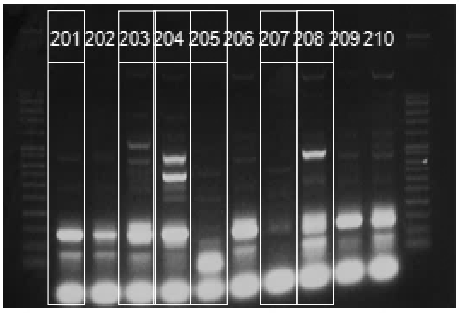
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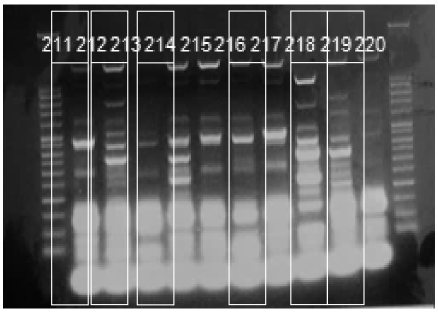
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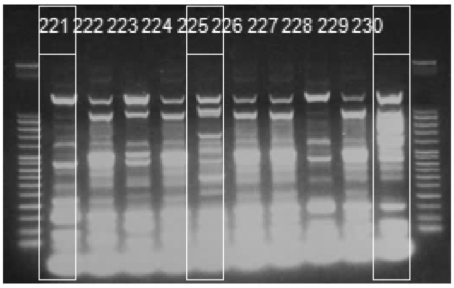
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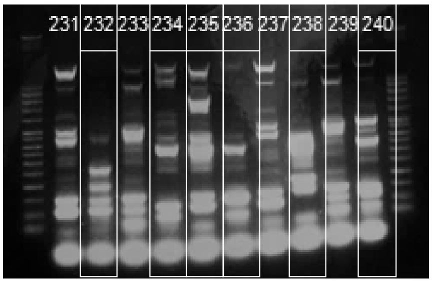
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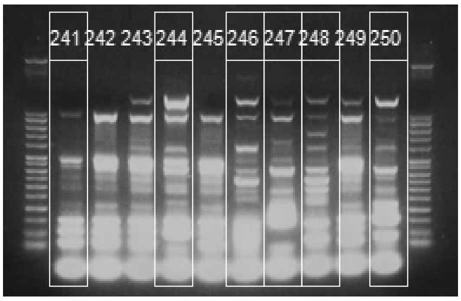
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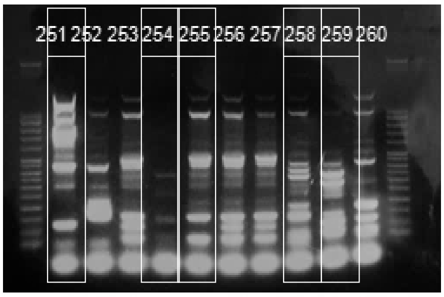
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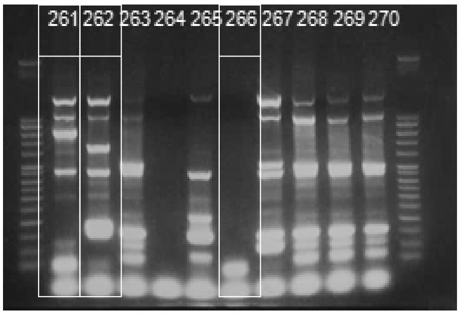
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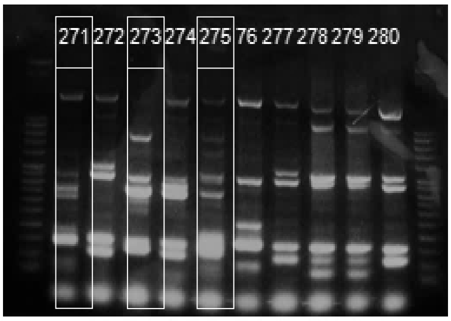
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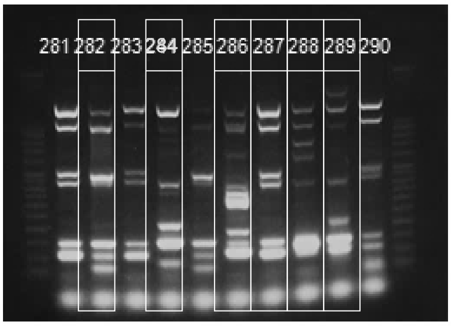
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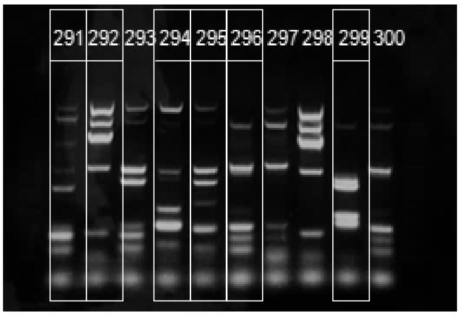
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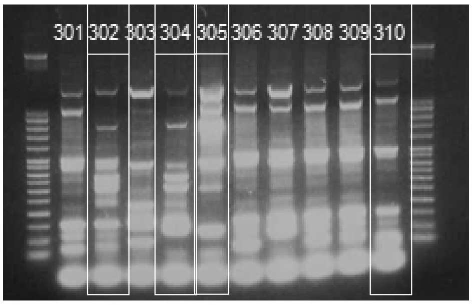
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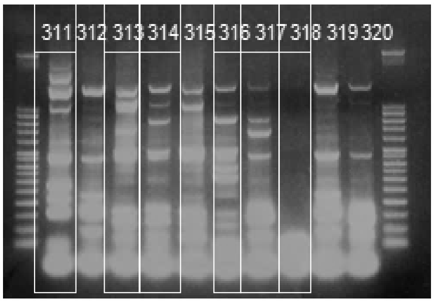
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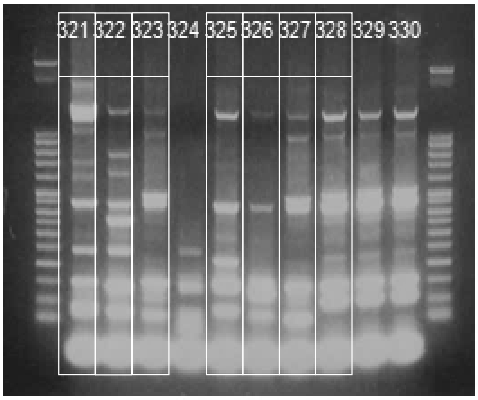
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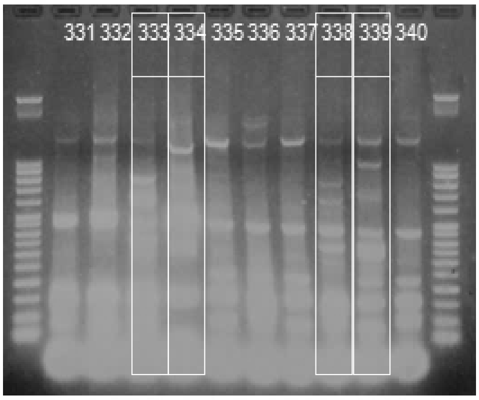
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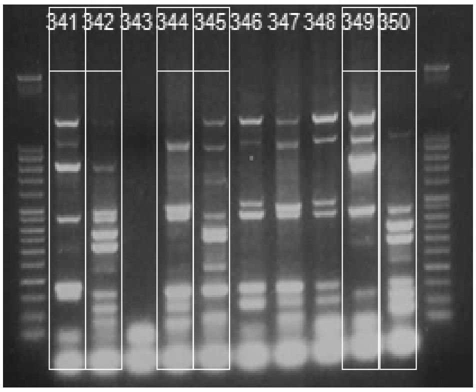
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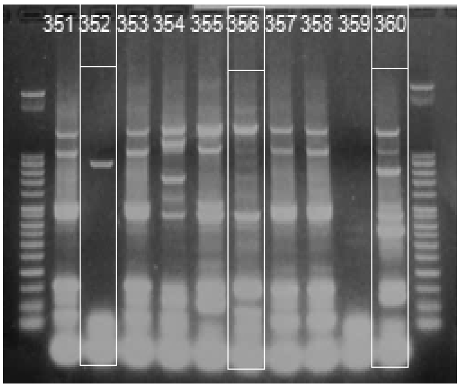
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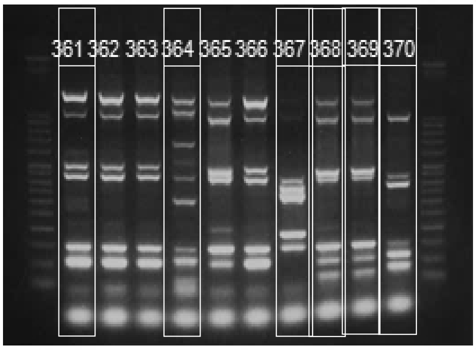
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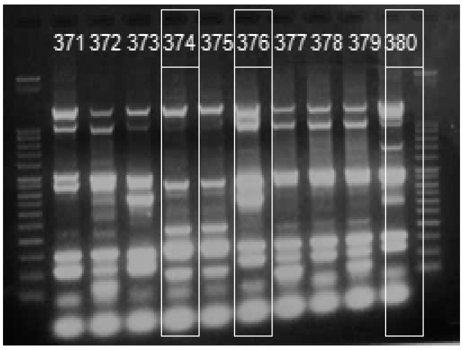
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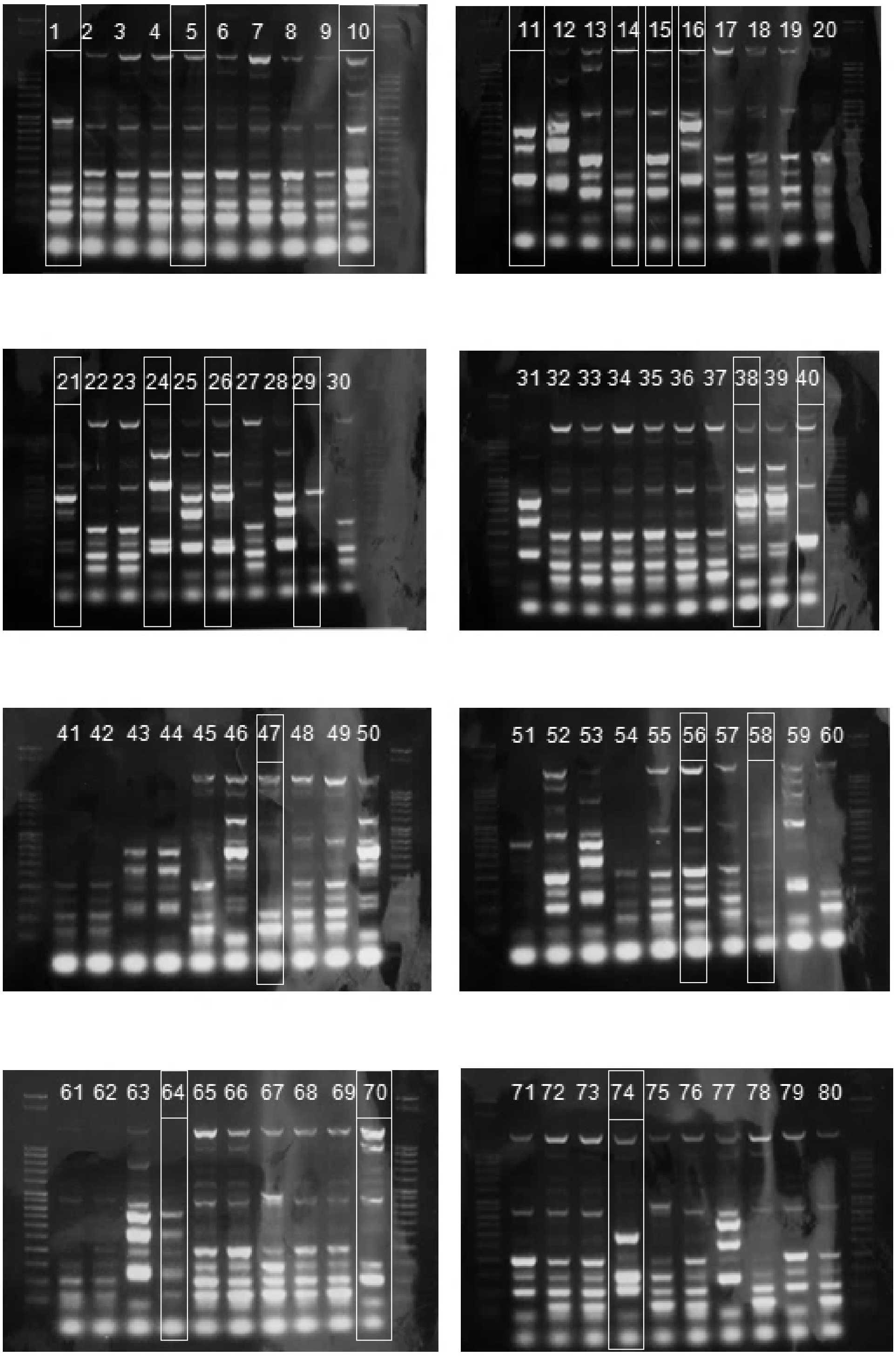
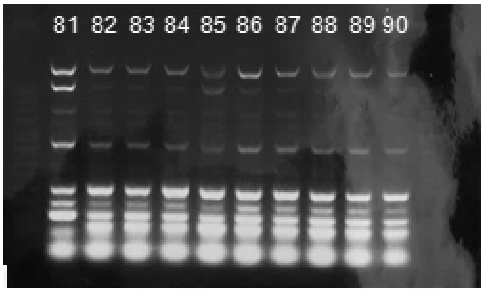
Figure S2c. PCR amplification of inter-delta regions. Electrophoretical patterns obtained with delta1-2 and delta 2-1 primers for the 380 *S. cerevisiae* isolates obtained from cellars of the D.O. *Rueda*. Highlighted lanes represents every one of the 223 different polymorphisms selected in the whole study.

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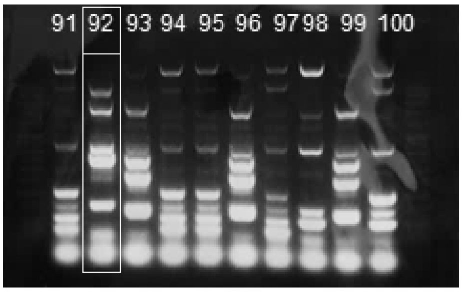
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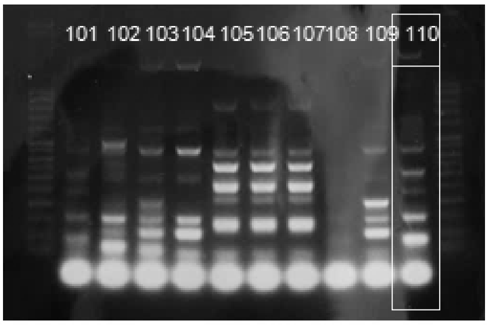
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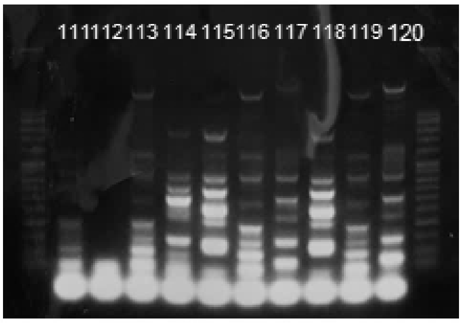
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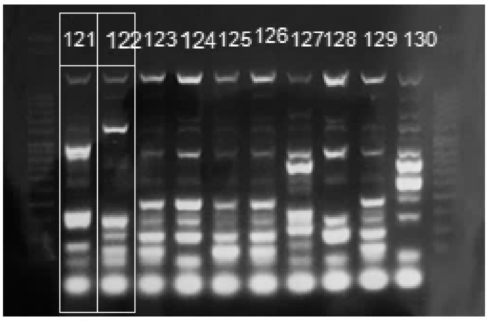
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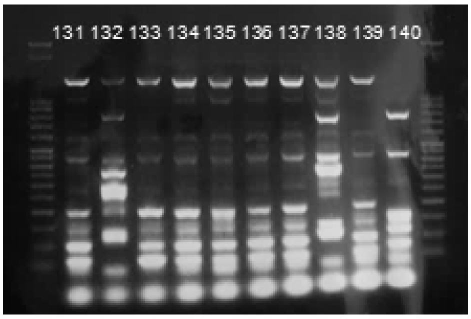
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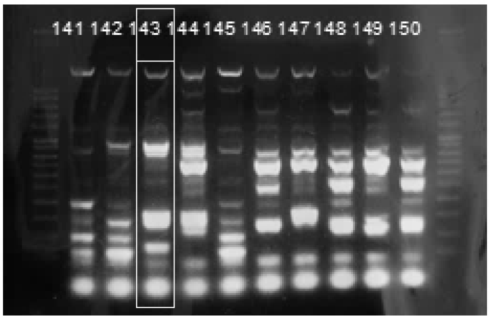
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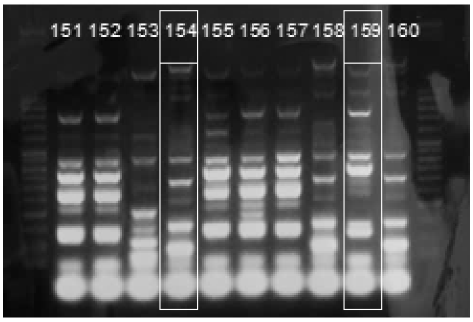
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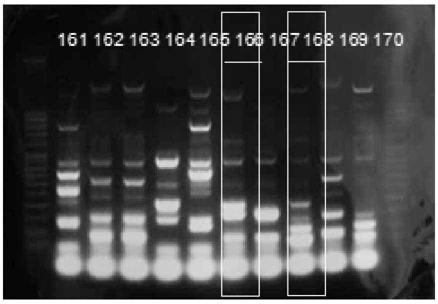
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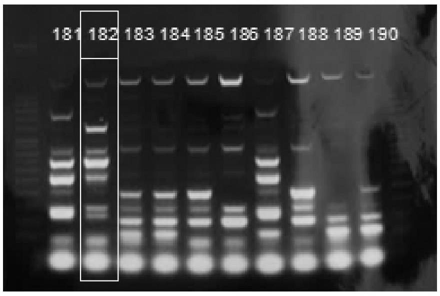
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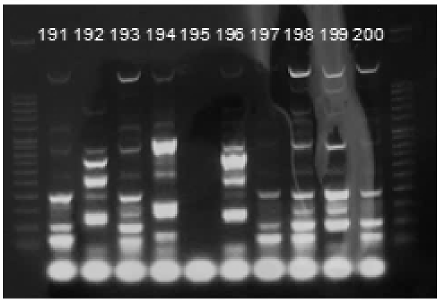
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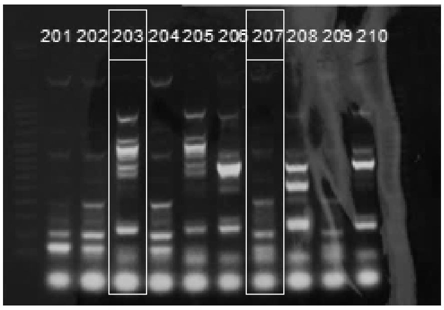
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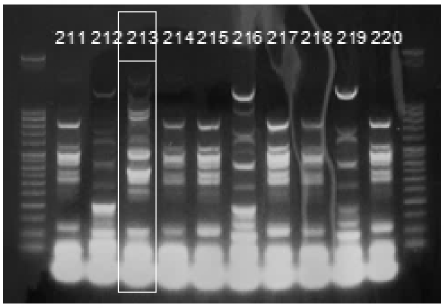
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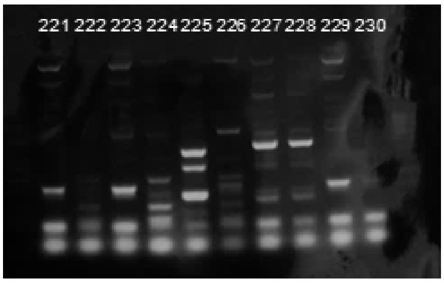
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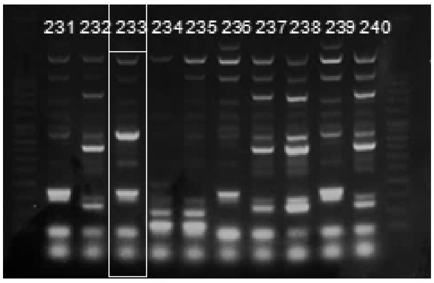
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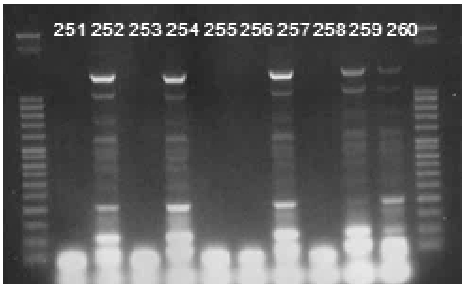
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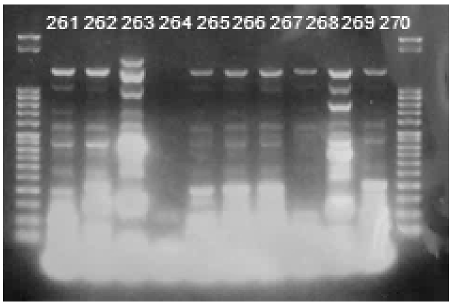
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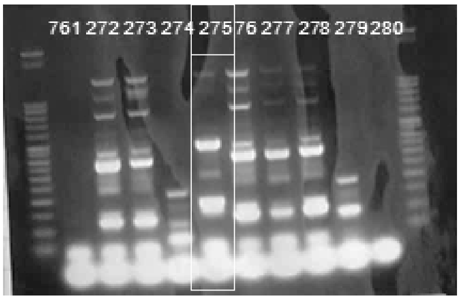
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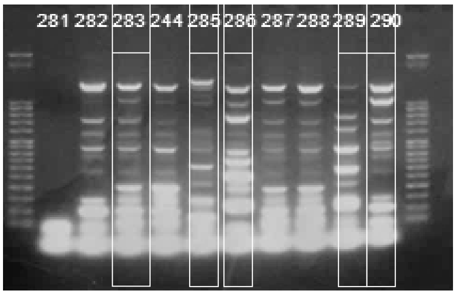
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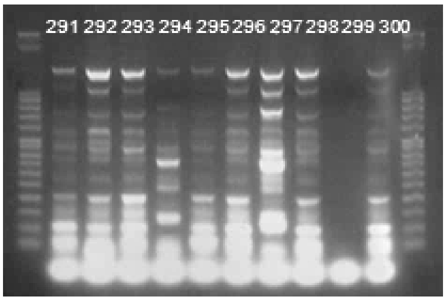
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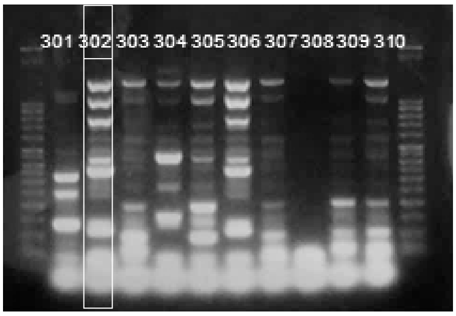
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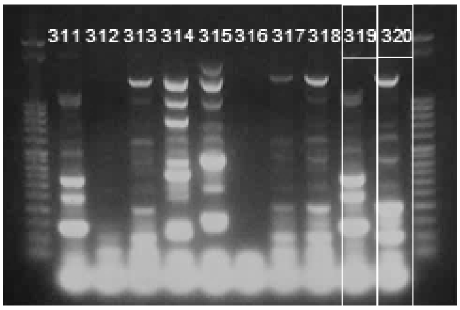
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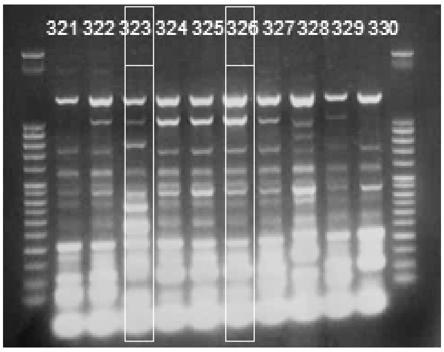
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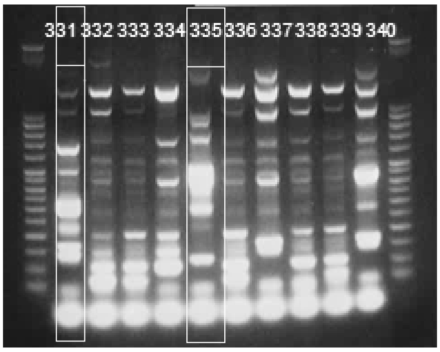
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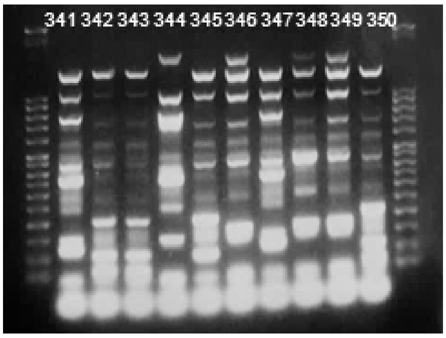
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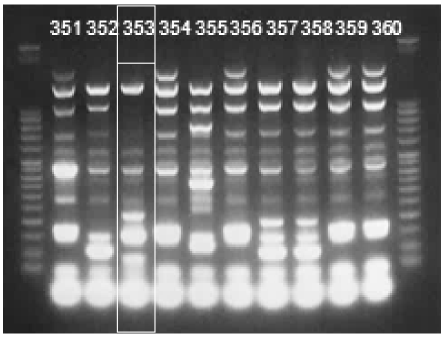
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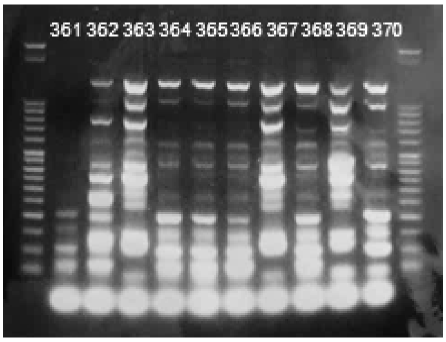
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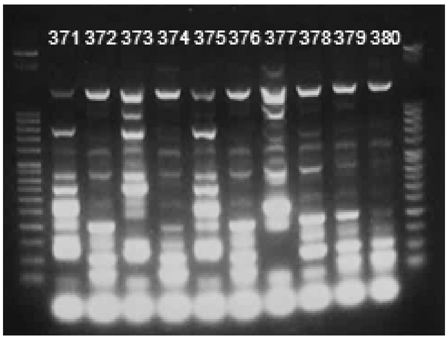
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Figure S3. The main three *IRC7* genotypes present in 223 wild strains of *S. cerevisiae*. PCR products of yeast genomic DNA amplified with PF6 and PR7 primers. Yeast strains used as PCR templates were named as indicated in the figure.

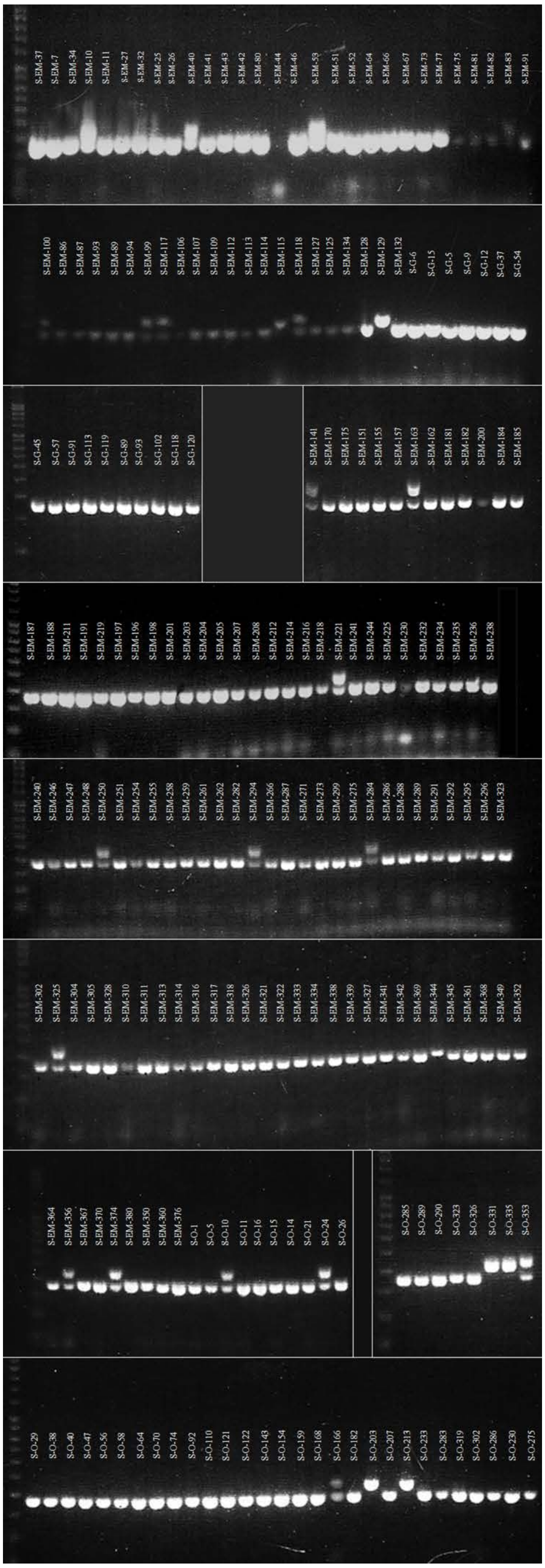


Figure S4. PCR amplification of *IRC7* for the 22 industrial *S. cerevisiae* strains obtained from Agrovin, S.A.

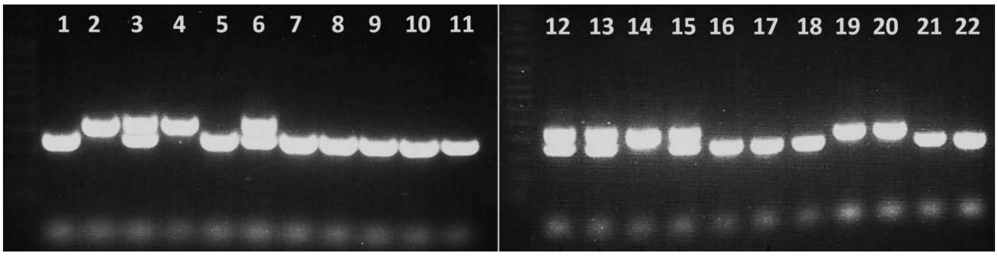


Figure S5. A micrograph taken with a phase-contrast microscopy (X40) after 24 h of cultivation of the colonies developed in SMC medium (A) in a blind test developed by mixing two strains of *S. cerevisiae* (S-EM-129 and S-EM-251; in representation of the homozygous full- and the homozygous short-length *IRC7* alleles, respectively, as detected by PCR (B).

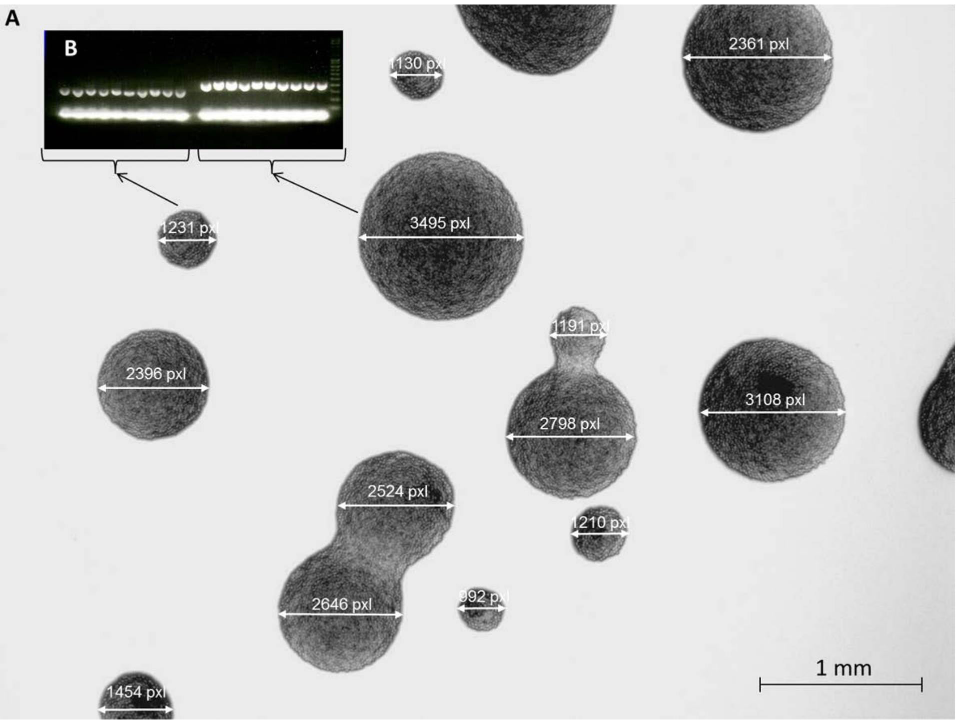


Figure S6. Detection by GC-MS of the end-products (MTL/DMDS) of the SMC metabolism for the determination of the β-lyase activity. Yeast strains with an increased β-lyase activity released high amounts of MTL in comparison with those strains with low β-lyase activity. MTL dimerized, in part, to dimethyl disulfide (DMDS) and both were simultaneously detected by GC-MS.

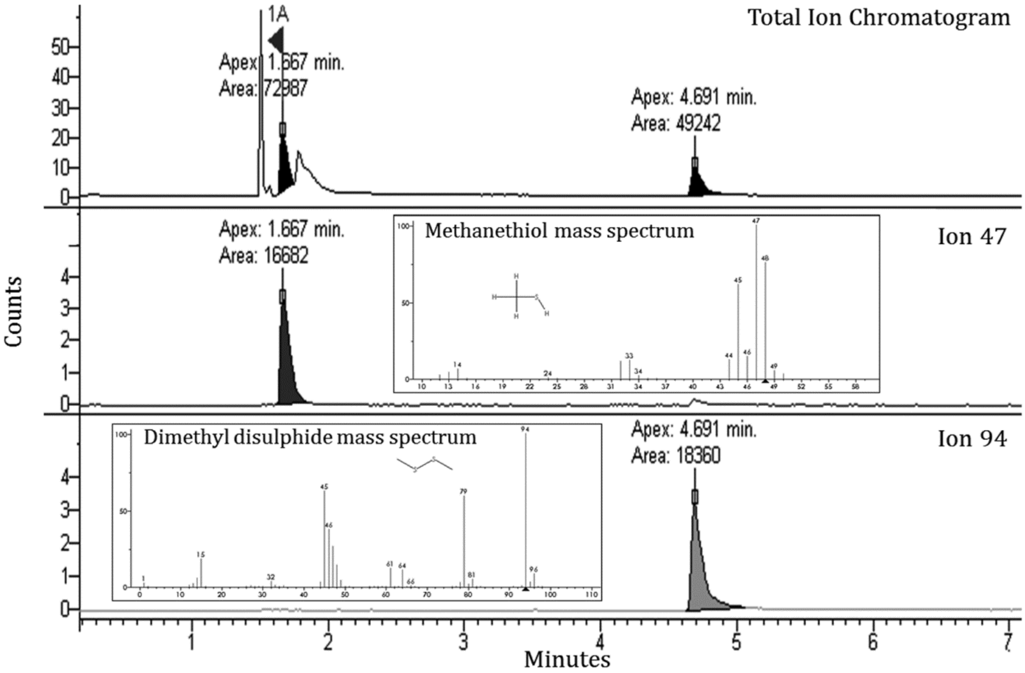
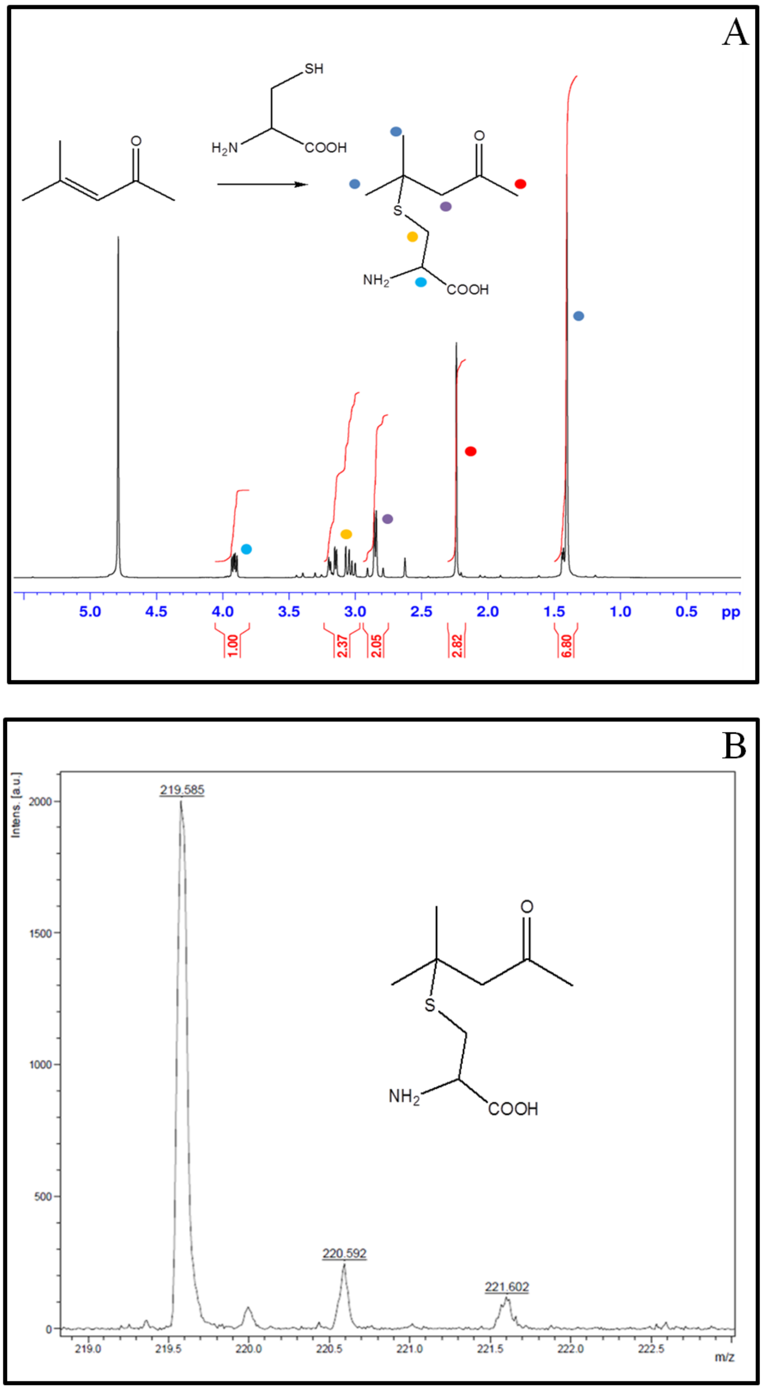


Figure S7. Cys-4MMP was synthetized according to the procedure of Howell et al. (2004). Cys-4MMP purity was determined by (A) ^{1}H-NMR (Bruker DPX 300MHz) and (B) ESI-MS (HPLC) with Bruker EsquireLC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany).



5.2. Caracterización de la fisiología en fermentación de *Torulaspora delbrueckii* y su contribución a la complejidad de vinos tintos.

Appl Microbiol Biotechnol (2015) 99:1911–1922
DOI 10.1007/s00253-014-6197-2

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality

Ignacio Belda · Eva Navascués · Domingo Marquina · Antonio Santos · Fernando Calderon · Santiago Benito

Received: 12 August 2014 / Revised: 26 October 2014 / Accepted: 27 October 2014 /

Abstract This work examines the physiology of a new commercial strain of *Torulaspora delbrueckii* in the production of red wine following different combined fermentation strategies. For a detailed comparison, several yeast metabolites and the strains implantation were measured over the entire fermentation period. In all fermentations in which *T. delbrueckii* was involved, the ethanol concentration was reduced; some malic acid was consumed; more pyruvic acid was released, and fewer amounts of higher alcohols were produced. The sensorial properties of final wines varied widely, emphasising the structure of wine in sequential fermentations with *T. delbrueckii*. These wines presented the maximum overall impression and were preferred by tasters. Semi-industrial assays were carried out confirming these differences at a higher scale. No important differences were observed in volatile aroma composition between fermentations. However, differences in mouthfeel properties were observed in semi-industrial fermentations, which were correlated with an increase in the mannoprotein content of red wines fermented sequentially with *T. delbrueckii*.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6197-2) contains supplementary material, which is available to authorized users.

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Keywords *Torulaspora delbrueckii* · Manno proteins · Glyceropyruvic pathway · Malic acid · Pyruvic acid · Combined fermentation

Introduction

Many research groups are currently studying non-*Saccharomyces* yeasts (Comitini et al. 2011; Contreras et al. 2014; Garde-Cerdán and Ancín-Azpilicueta 2006; Jolly et al. 2006) due to their unique physiological metabolic properties, which may be advantageous in winemaking. The presence of non-*Saccharomyces* wild yeasts in fermentations has been associated, traditionally, with high levels of acetic acid and other off-flavours. Nevertheless, nowadays, researchers and winemakers are aware of the influence of non-*Saccharomyces* in wine aroma complexity (Egli et al. 1998; Esteve-Zarzoso et al. 1998; Fleet 2003, 2008; Fleet and Heard 1993; Gil et al. 1996; Henick-Kling et al. 1998; Lambrechts and Pretorius 2000; Romano et al. 2003; Viana et al. 2008). The difficulty with which non-*Saccharomyces* wine yeast finishes the alcoholic fermentation requires the development of combined fermentation with *Saccharomyces cerevisiae* as a binding partner. Some enzymatic activities related to aroma enhancement (glycosidases and β-lyase for terpene and thiol release, respectively) and the release of some interesting products such as glycerol and mannoproteins, among others, are the highlights that justify the interest in these mixed fermentations (Ciani et al. 2010; Rojas et al. 2001). In this context, combined fermentations are a very useful tool to improve wine fermentations in which aromatic complexity of spontaneous fermentations and the safety of industrial targeted fermentations are joined (Ciani et al. 2010; Romano et al. 2003).

Some studies have analysed the use and influence of different non-*Saccharomyces* species in wine fermentations, such as *Kloeckera apiculata* (Herraiz et al. 1990), other apiculated yeasts like *Hanseniaspora uvarum* (Zironi et al. 1993), *Torulaspora delbrueckii*, *Kluyveromyces thermotolerans*, *Hansenula anomala*, and *Metschnikowia pulcherrima* (Ciani et al. 2006; Izquierdo-Cañas et al. 2011, 2014; Oro et al. 2014).

Despite that studies of industrial or semi-industrial use of *T. delbrueckii* and its repercussion on wine quality are scarce, most scientific studies report its relationship with wines with low acetic acid content and great mouthfeel properties (Bely et al. 2008). Furthermore, the fermentative capacity of *T. delbrueckii* (Quirós et al. 2014) allows its implantation at the beginning of fermentation process, contrary to other strictly oxidising non-*Saccharomyces* yeasts. At the same time that several authors are studying the potential use of non-*Saccharomyces* yeasts in wine fermentations (De Benedictis et al. 2010; Domizio et al. 2011; Viana et al. 2008), the enology industry has been able to accept this trend, and most wine yeast distribution companies already have non-*Saccharomyces* strains for its use in winery.

The possibility to modulate the flavour and style of wine by different fermentation strategies forced the study on all possible combinations of non-*Saccharomyces* and *Saccharomyces* yeast strains (Azzolini et al. 2012). In this sense, most of studies analyse fermentations carried out with non-*Saccharomyces* strains by itself, with mixed fermentations by simultaneous and sequential inoculation, comparing all of them with the alcoholic fermentation with *S. cerevisiae* by itself.

This study aims to validate the industrial use of a new commercial strain of *T. delbrueckii* from Agrovin S.A., studying their physiology throughout fermentation in order to explain the chemical composition, aromatic profile and sensorial properties of the red Tempranillo wines produced by different mixed cultures of the strain *T. delbrueckii* NSA-1 with *S. cerevisiae.*

Most of studies reported to analysing the properties and that advantages of some non-*Saccharomyces* yeast are developed following a microvinification trend, but results are rarely validated in an industrial or semi-industrial scale, questioning its potential applicability (Jolly et al. 2014) due to the influence of scale on yeast gene expression (Rossouw et al. 2012). In order to validate microvinification results in this study, semi-industrial fermentation was carried out in 100-L stainless tanks.

Materials and methods

Microorganisms

Yeast strains and molecular identification

S. cerevisiae CT007 and *T. delbrueckii* NSA-1 Viniferm NS-TD were obtained from the Agrovin S.A. (Alcázar de San Juan, Spain) collection and identified by using molecular methods as follows. Yeast isolates were identified by sequence analysis of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al. 1992), and DNA for sequencing was amplified using an Eppendorf Mastercycler apparatus as described by Kurtzman and Robnett (1997) with 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′). Sequences obtained to identify yeasts were analysed and compared by BLAST-search (GenBank;www.ncbi.nlm.nih.gov). The 26S rRNA nucleotide sequences has been submitted to Genbank-NCBI under accession numbers KM434246 (*S. cerevisiae* CT007) and KM434245 (*T. delbrueckii* NSA-1).

Additionally, *S. cerevisiae* CT007 identification was confirmed by the polymerase chain reaction amplification of the interdelta region of *S. cerevisiae* (Legras and Karst 2003) using delta12 (5′-TCAACAATGGAATCCCAAC-3′) and delta21 (5′- CATCTTAACACCGTATATGA-3′) primers.

Characterisation of yeast strains

β-Glucosidase activity was evaluated as reported by Rosi et al. (1994), on a medium containing 0.5 % cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), 0.67 % yeast nitrogen base (Difco) and 2 % agar. Yeast strains were inoculated as above and incubated at 28 °C for 3 days. A significant growth of the colonies indicated the presence of β-glucosidase activity. Additionally, β-D-xylosidase and α-L-arabinofuranosidase activities were evaluated using the correspondent methylumbelliferyl-conjugated substrates (methylumbelliferyl-β-D-xylopyranoside and methylumbelliferyl-α-L-arabinofuranosidase, respectively), according to the method described by Manzanares et al. (1999). Strains of *T. delbrueckii* CECT 10676 from the Spanish Type Culture Collection (CECT Valencia, Spain) and *Rhodotorula mucilaginosa* NSG-61 from the Complutense Yeast Collection (CYC Madrid, Spain) were used as were used as negative and positive controls, respectively.

Production of hydrogen sulfide was evaluated by using a modification of the lead acetate method (Linderholm et al. 2008). This method detects volatile H_2S in the headspace of the fermentation in a culture medium containing 1.17 % yeast carbon base (Difco), 4 % glucose anhydrous, and 0.5 % ammonium sulfate. Yeasts were grown at 28 °C for 3 days in 96-well microplates containing 200 μl of medium with orbital agitation (200 rpm). Hydrogen sulfide formation was initially detected by using paper strips (Whatman filter paper) that had been previously embedded with a 0.1 M lead acetate solution and allowed to dry at 65 °C for 10 min and deposited over microplate wells. Hydrogen sulfide formation was qualitatively measured based on the degree of blackening of the lead acetate strip and quantitatively estimated by densitometric measure of the intensity (Software "My Image Analysis v1.1" Thermo Scientific).

Killer activity was measured by the method described by Santos et al. (2009). Yeast to be tested for killer activity were inoculated in ~1-cm diameter concentrated zones onto YMA-MB plates (1 % glucose, 0.3 % yeast extract, 0.3 % malt extract and 0.5 % proteose peptone no. 3, supplemented with 30 mg/L of methylene blue, 3 % NaCl and 2 % agar) previously seeded with a lawn (5.0×10^5 cells/ml) of the sensitive yeast (*S. cerevisiae* Hansen BY4741). The sensitive strain was grown on YMA medium (YMA-MB without NaCl and methylene blue) and suspended in sterile water just before inoculation. The plates were incubated for a week at 20 °C. Killer yeasts were identified by a clear zone of inhibition surrounding them (Llorente et al. 1997).

Biomass production

S. cerevisiae CT007 was obtained as active dry yeast and rehydrated following the manufacturer's instructions. *T. delbrueckii* cultures were obtained by using an enriched must medium (12.5 % concentrated must (final concentration, 50 g/L glucose+fructose), 1 % yeast extract, 0.5 % proteose peptone no.3, pH 3.5) at 25 °C. Upon reaching the necessary cell concentration, *T. delbrueckii* was concentrated by decantation and then used as inoculum for vinifications.

Microvinifications and growth kinetics

All fermentations were prepared using the must from *Vitis vinifera* L.cv. Tempranillo grapes from El Socorro (Experimental Vineyard, Madrid, Spain) and processed accordingly to the methods described previously with slight modifications (Benito et al. 2012; Sampaio et al. 2007). Fresh must was bleeding from crushed-grapes (3.5 L) and placed in 4.9-L glass fermentation vessels, leaving enough space for carbon dioxide emission. Sulphur dioxide (40 mg/L) (Panreac, Barcelona, Spain) was added to each vessel. The sugar content was 247 g/L, yeast assimilable nitrogen 188 mg/L, pH 3.42.

By triplicate, four assays were performed: (1) inoculation with *T. delbrueckii* (Td), (2) sequential inoculation (SQ) with *T. delbrueckii* followed by *S. cerevisiae* CT007 after 15 g/L sugar consume was detected, (3) simultaneous co-inoculation (SM) with *T. delbrueckii* and *S. cerevisiae* CT007 and (4) inoculation with *S. cerevisiae* CT007 (Sc).

Cultures were adjusted in order to reach an initial cellular concentration in must of about 10^6 cells/ml for every strain, developing mixed cultures with an inocula ratio of 1:1. During co-fermentations, aliquots were taken periodically, and further tenfold dilutions were made serially. Growth kinetics were followed by plating 50 μL of the appropriate dilution on Sabouraud glucose agar with chloramphenicol (total yeast counts) and lysine media (non-*Saccharomyces* counts). Colonies were counted after growth at 30 °C for 48–72 h.

All fermentation processes were carried out at 20 °C. Once fermentation of sugars was completed (deemed to be represented by a remaining glucose+fructose concentration lower than 3 g/L), 50 mg/L of sulphur dioxide was added in potassium metabisulfite form to the wines, and they were racked and stabilised during 7 days at 4 °C, and the final product was bottled. Bottles were placed horizontally in a climate chamber TR2V120 (La Sommelie`re, Saint-Saturnin, France) at 18 °C and 70 % relative humidity. These conditions were maintained until the sensory evaluation took place.

Semi-industrial fermentations

All semi-industrial fermentations were undertaken using *V. vinifera* L. cv. Tempranillo must. Eighty kilograms of fresh crushed grapes were placed in 100 L stainless steel fermentation tanks, leaving enough space for the emission of carbon dioxide. Sulphur dioxide (40 mg/kg) was added to each. The sugar content was 247 g/L, yeast assimilable nitrogen 188 mg/L, pH 3.42.

Four assays were performed as described above for microvinifications. All fermentation processes were carried out at winery temperature of 20 °C. Once fermentation of sugars was complete (deemed to be represented by a remaining glucose fructose concentration lower than 3 g/L), the wines fermented with *T. delbrueckii* were racked and stabilized during 15 days at 4 °C, and the final product was bottled. Fifty milligrams per liter of sulphur dioxide were added in potassium metabisulfite form. Corked bottles were placed as described above. These conditions were maintained for 7 weeks until the sensory evaluation took place.

Analytical determinations of non-volatile compounds

Glucose fructose, malic acid, lactic acid, acetic acid, glycerol, pyruvic acid and colour intensity were all determined using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain). These analyses were performed using the appropriate kits supplied by the manufacturer (www.biosystems.pt).

Total acidity, pH, ethanol and density were determined following the methods in the Compendium of International Methods of Analysis of Musts and Wines (OIV 2014).

Analytical determinations of volatile compounds

Volatile compounds from microvinifications The concentration of volatile compounds (Tables 2 and S2), all of which influence wine quality, were measured at the end of alcoholic fermentations by gas chromatography using an Agilent Technologies 6850 gas chromatograph with a flame ionisation detector (Hewlett Packard, Palo Alto, CA, USA) (Ortega et al. 2001). The apparatus was calibrated with a 4-methyl-2-pentanol internal standard. Gas chromatography quality compounds (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland) were used to provide standard patterns. Higher alcohols were separated as described in the Compendium of International Methods of Analysis of Musts and Wines (OIV 2014). The detection limit was 0.1 mg/L. Minor compounds were quantified by gas chromatography–mass spectrometry as described by Lopez et al. (2002) with the modifications introduced by Loscos et al. (2007).

Analysis of mannoprotein content of wines Total soluble wine polysaccharides were evaluated in duplicate by using a HPLC apparatus (Surveyor Plus chromatograph, Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector (Surveyor RI Plus Detector) as reported (Quirós et al. 2012). The column employed was a 300 × 7.7 mm PL Hi-Plex Pb 8 lm (Varian, Inc., Shropshire, UK). MilliQ water was used as the mobile phase at a flux of 0.6 mL/min and a column temperature of 70 °C. The retention time valued was between 0 to 30 min.

Sensorial analysis The final wines were assessed (blind) by a panel of ten experienced wine tasters, all members of the staff of the Food Technology Department of the Polytechnic University of Madrid. Assessments took place in standard sensory analysis chambers with separate booths. Following the generation of a consistent terminology by consensus, two visual descriptors, five aromas and four taste attributes were chosen to describe the wines. Formal assessment consisted of two sessions held on different days where wine tasters tasted all fermented triplicates. The panelists used a 10 cm unstructured scale, from 0 (no character) to 10 (very strong character), to rate the intensity of ten attributes.

Statistical analysis All statistical analyses were performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p<0.05$ for the ANOVA matrix F value. The multiple-range test was used to compare the means.

Results

Fermentation kinetics

Population dynamics

S. cerevisiae population showed the typical growth kinetic where, in all cases, it maintained high cell viability until the end of fermentations, both as only inoculum or as coinoculated with *T. delbrueckii* (Fig. 1). Figure 1a shows microbial kinetics of a fermentation carried out with *T. delbrueckii* by itself (Td), so total viable cells counted in Sabouraud medium are relating to the wild yeasts in the must. The sequential inoculation (SQ), in which *S. cerevisiae* was inoculated at day 4, showed a similar fermentation kinetic compared with Td fermentation, but with greater homogeneity in yeast populations between replicates (Fig. 1b). In these fermentations, non-*Saccharomyces* can be isolated until advanced stages of the process (day 17) contrary to what could be observed in the simultaneous inoculation of *T. delbrueckii* and *S. cerevisiae* (SM), where non-*Saccharomyces* can be only observed until the day 7 (Fig. 1c). Figure 1d shows the total cell count corresponding to the fermentation inoculated only with *S. cerevisiae* (Sc).

Sugar consumption and ethanol production

Figure 2 shows the different fermentation kinetics of microvinifications and semi-industrial fermentations by sugar consumption. In the case of the laboratory-scale assays, fermentations which were started with *T. delbrueckii* by itself (Td) and sequentially (SQ) with *S. cerevisiae* required 24 and 21 days, respectively, to complete fermentation, despite fermentations with *S. cerevisiae* by itself (Sc), and its simultaneous (SM) inoculation with *T. delbrueckii* only required 14 days to finish (Fig. 2a). Regarding semi-industrial fermentations, all of them required only 12 days to complete fermentation, despite fermentations started only with *T. delbrueckii* (Td and SQ) followed slower kinetics at the beginning compared with fermentations started with *S. cerevisiae* (Sc and SM) (Fig. 2b). The final alcohol content of the wines obtained in fermentations involving *T. delbrueckii* NSA-1 was lower than those only fermented by *S. cerevisiae* CT007 (Table 1). The semi-industrial fermentations confirmed this reduction, so final alcohol degree produced in different fermentations was gradually lower, depending on the higher *T. delbrueckii* presence (Supplementary material, Table S1).

Acetic acid and malic acid production

Slight differences in acetic acid production were observed between assays (Fig. 3). Figure 3a shows the acetic acid release kinetics in microvinifications, where SQ and SM fermentations produced final acetic acid concentrations ranging from 0.29 to 0.32 g/L, similar to Sc fermentations (0.31 g/L). Similar data were obtained from semi-industrial fermentations in which SQ fermentation shows the minimum acetic acid release (0.29 g/L) (Fig. 3b). SM and Sc fermentations show again similar acetic acid content (0.35 and 0.33 g/L, respectively) (Fig. 3b).

In addition, lower levels in total acidity and higher pH values in *Torulaspora* related fermentations were detected

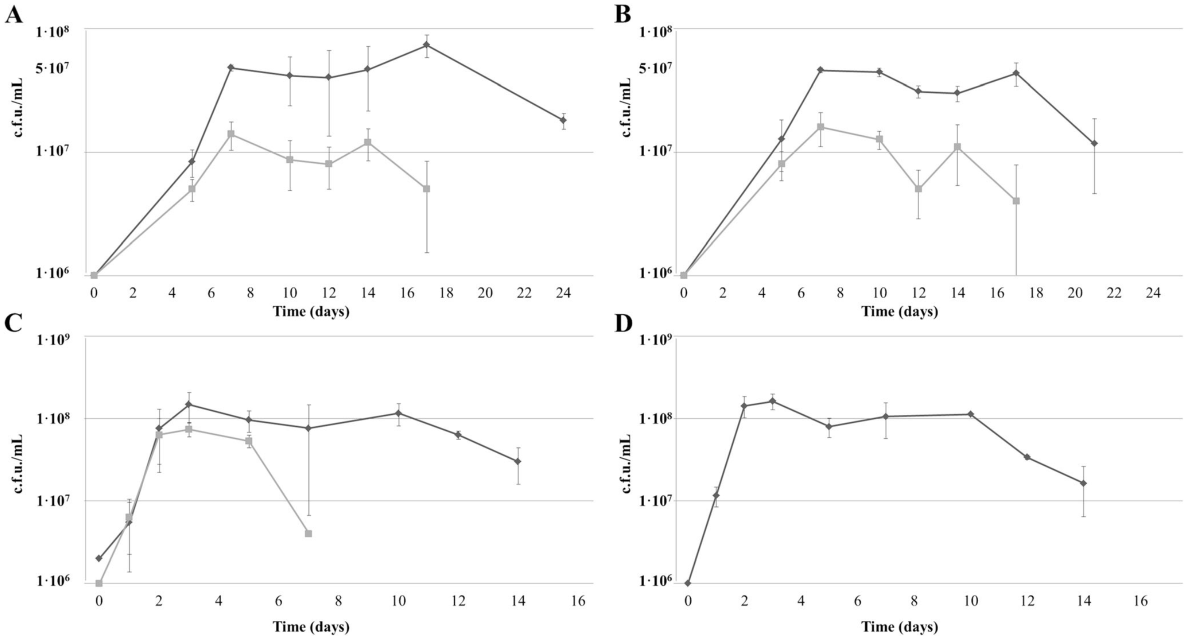

Fig. 1 Total yeast cell count (*black triangle*) and *T. delbrueckii* NSA-1 cell count (*black circle*) during fermentation. **a** Fermentation inoculated only with *T. delbrueckii* NSA-1. **b** Sequential inoculation of *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007. **c** Simultaneous inoculation of *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007. **d** Fermentation inoculated only with *S. cerevisiae* CT007

(Table 1). It also could be related to the higher malic acid consumption by *T. delbrueckii*.

Fermentations involving *T. delbrueckii* NSA-1 consumed part of the malic acid present in the must, which showed an initial malic acid content of 1.77 g/L. The final values in fermentations involving *T. delbrueckii* NSA-1 were lower than Sc fermentation ones (Table 1), detecting a maximum malic acid reduction rate of 13.56 % in Td fermentation and a 4.52 % of malic acid reduction in Sc fermentation. Table 1

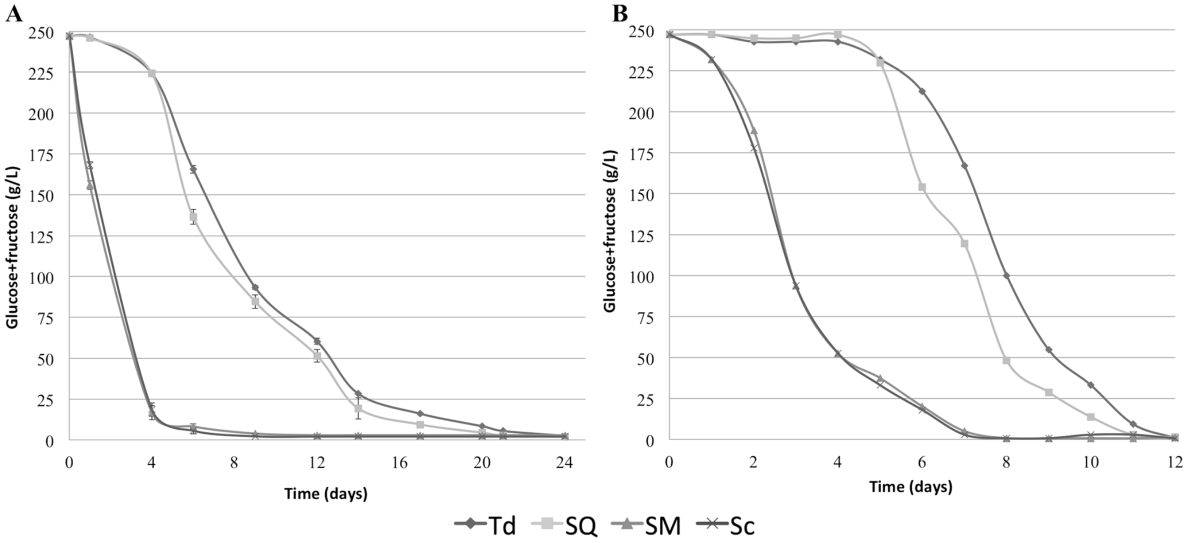

Fig. 2 Change in glucose fructose concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1 *S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial-scale assays

Table 1 Analytical results for the wines produced by the different fermentation systems

Assays	Glucose fructose (g/l)	Acetic acid (g/l)	Malic acid (g/l)	Lactic acid (g/l)	Glycerol (g/l)	Free SO2 (mg/L)	Total SO2 (mg/l)	Total acidity(g/l)	Alcohol (%v/v)	pH	Colour intensity
Td	2.49±0.47a	0.37±0.02a	1.53±0.03a	0.11±0.02a	6.70±0.03a	25.13±3.21a	82.13±3.21a	6.62±0.08a	14.38±0.07a	3.56±0.01a	3.16±0.05a
SQ	1.99±0.37a	0.29±0.02b	1.57±0.04b	0.10±0.02a	6.71±0.02a	23.42±1.21a	79.31±2.33ab	6.67±0.06a	14.39±0.03a	3.54±0.02c	3.09±0.03a
SM	.88±0.13b	0.32±0.02b	1.61±0.02b	0.09±0.01a	6.63±0.02b	26.38±2.46a	77.13±2.21ab	6.75±0.02b	14.46±0.02b	.53±0.02c	2.96±0.02b
sss	2.03±0.22a	0.31±0.01b	1.69±0.02c	0.09±0.01a	6.63±0.01b	24.13±1.76a	4.13±2.02b	6.77±0.01b	14.53±0.02b	.51±0.04c	2.77±0.02c

Results represent the mean SD for three replicates. Means in the same row with the same letter are not significantly different (*s*<0.05)

Td T. delbrueckii NSA-1 alone, *SQ* sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007, *SM* simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* CT007, *Sc* fermentation with *S. cerevisiae* CT007 alone

shows final concentrations of lactic acid; the absence of malolactic fermentation confirmed that no contamination by lactic acid bacteria occurred.

Pyruvic acid and glycerol production

S. cerevisiae CT007 by itself (Sc) and SM fermentation showed maximum pyruvic acid production at fourth day, reaching 111 and 141 mg/L, respectively, in microvinifications (Fig. 4a). Td and SQ fermentations showed higher values with maximum figures of 156 and 143 mg/L, respectively, at day 6. Similar values and kinetics can be observed in the semi-industrial fermentations where *T. delbrueckii* contributed to the pyruvic acid production obtaining its maximum values times depending on the different fermentation kinetics (Fig. 4b).

The glycerol content in Td and SQ fermentations was also slightly higher than the one observed in Sc and SM fermentations in microvinifications (Table 1) and semi-industrial trials (Supplementary material, Table S1).

Volatile compounds

Table 2 shows that fermentations involving *T. delbrueckii* produced lower concentrations of higher alcohols; nevertheless, all fermentations produced these compounds in moderate quantities. A similar effect was observed in the case of esters and fatty acids. Some compounds such acetaldehyde, diacetyl and ethyl acetate were detected in higher values when *Torulaspora* was used alone. These results were confirmed in semi-industrial-scale vinifications (Supplementary material, Table S2). No differences of terpenic compounds between assays (Supplementary material, Table S2) were found in this trial.

Sensorial analysis

Figure 5 shows a “spider web” diagram for the average scores of some olfactory and taste attributes. Light differences in the perception of acidity were recorded. Colour intensity perception was higher in those fermentations in which *T. delbrueckii* NSA-1 took place. Fermentation with *S. cerevisiae* CT007 alone produced slightly stronger sensations of oxidation. None of the wines that involved fermentation with *T. delbrueckii* had any perceptible organoleptic problems; indeed, sequential and mixed fermentations received the best scores from all tasters. The greatest virtue attributed to SQ fermentation was the complexity and structure of its mouthfeel properties.

Mannoproteins content in semi-industrial fermentations

Final content of mannoproteins in semi-industrial scale fermentation in tanks containing 80 kg of crushed grapes were

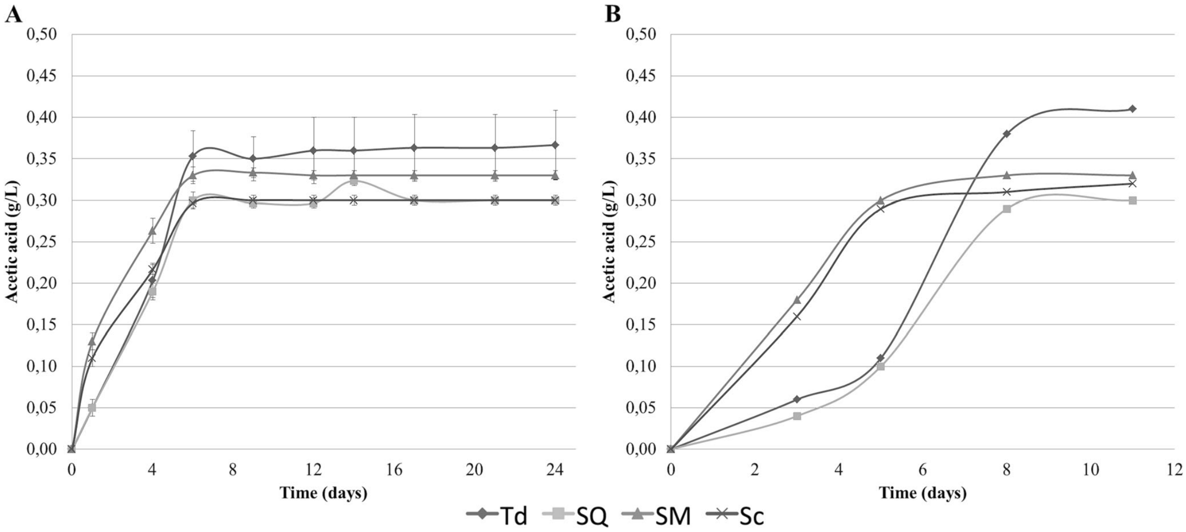

Fig. 3 Change in acetic acid concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial scale assays

analysed. In Fig. 6, the increase of mannoproteins can be seen in the fermentations in which *T. delbrueckii* acts for longer, especially in controlled sequential fermentation.

Discussion

Similar results in fermentation kinetics and population dynamics (Fig. 1) can be seen in recent analogous studies using non-*Saccharomyces* yeasts, where simultaneous inoculation of both, *S. cerevisiae* and non-*Saccharomyces* strains, limited the presence of non-*Saccharomyces* to the early stages of fermentation (Azzolini et al. 2012; Oro et al. 2014). In this work, the studied yeast strains were observed to present killer phenotype and were active against the sensitive strain used as control (*S. cerevisiae* BY4741). However, there was no cross-activity between them, so killer activity was not considered as a relevant feature in the growth kinetics of both strains.

The sugar consumption results showed in this work (Fig. 2) agree with the lower fermentative power of *Torulaspora* spp.

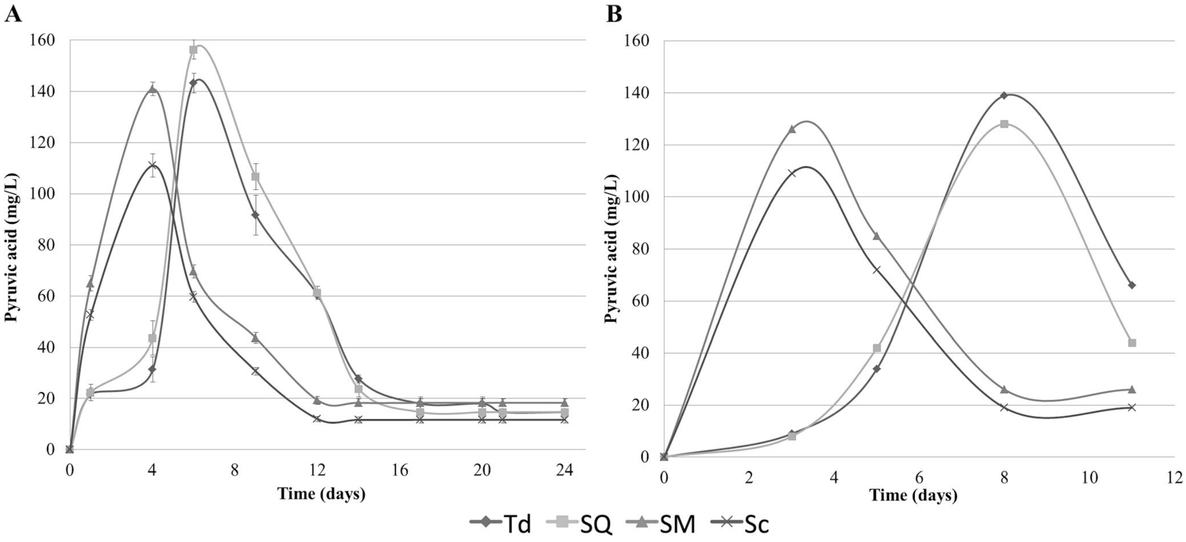

Fig. 4 Change in pyruvic acid concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial scale assays

Table 2 Volatile compounds (micrograms per liter) detected in the different fermentations

Compounds	Sc	SM	SQ	Td
1-Hexanol	1,835.33±80.03b	1,983.00±45.90a	1,557.00±72.63c	1,859.00±77.32ab
3-Hexanol	684.67±7.02a	680.67±21.94a	457.67±17.95c	564.33±23.59b
Isoamylalcohol	391,316.33±13,882.09a	403,590.33±4,815.73a	334,420.00±9,634.85b	386,564.67±9,624.89a
Isobutanol	55,502.00±1,213.69b	60,904.33±1,608.61a	54,783.00±1,371.60b	61,803.33±1,533.15a
Alcohols	449,337.99±12,961.9b	467,158.33±4,523.64a	391,217.67±8,922.84c	450,791.33±8,930.99b
Acetaldehyde	332.27±631.43a	7,332.27±631.43a	7,254.34±672.34a	11,342.13±792.23b
Diacetyl	1,243.45±65.13a	1,425.32±88.43b	1,223±77.34a	1,634.43±104.47c
Carbonyl compounds	8,575±584.41b	9,846±687.29b	8,477.34±622.67c	1,2976±741.61a
Ethyl acetate	21,365.23±1,365.45b	23,456.34±1,567.76ab	21,897.65±1,645.54b	5,764.26±1,876.54a
Ethyl butyrate	157.33±4.73b	182.00±5.29a	144.67±7.02c	181.67±6.51a
Ethyl decanoate	86.33±4.52a	95.00±6.00a	74.33±4.06b	80.00±8.73ab
Ethyl hexanoate	613.67±17.16a	652.33±16.56a	339.67±20.60c	462.33±23.29b
Ethyl lactate	3,700.33±121.38a	3,711.33±104.31a	2,832.67±120.02c	2,832.67±120.02c
Ethyl octanoate	372.00±33.15a	425.67±46.74a	297.00±12.53b	377.67±36.74a
Isoamyl acetate	1,381.67±43.25a	1,073.33±47.48b	974.00±12.53c	1,136.00±79.54d
Esters	2,7676.23±1,200.65bc	29,596±1,396.32b	26,559.99±1,494.77c	31,156.93±1,706.91a
hexanoic acid	3,614.33±140.47a	3,521.33±151.64a	3,154.00±157.29b	3,383.33±161.98ab
Isobutyric acid	3,614.33±140.47a	3,521.33±151.64a	3,154.00±157.29b	3,383.33±161.98ab
isovaleric acid	385.67±7.51a	368.00±4.58b	331.33±13.61c	363.67±6.66b
octanoic acid	9,919.33±74.59a	10,125.67±145.29a	6,703.00±220.96c	8,544.00±224.01b
valeric acid	598.67±12.22a	584.33±20.40a	531.33±25.20b	567.67±11.72ab
2-Phenylethanol	54,308.67±1,151.98a	53,194.00±2,022.95ab	52,531.67±1,170.21ab	51,485.33±719.18b
2-phenylethyl acetate	8.00±1.00a	7.67±2.31a	7.67±1.53a	7.00±1.00a
Phenols	8.00±1.00a	53,201.67±2,022.82ab	52,539.34±1,170.13ab	51,492.33±719.13b

Results represent the mean SD for three replicates. Means in the same row with the same letter are not significantly different ($s<0.05$)

T. delbrueckii NSA-1 alone (Td); Sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (SQ); Simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* CT007 (SM); fermentation with *S. cerevisiae* CT007 alone (Sc)

compared with *S. cerevisiae* reported by other authors (Bisson and Kunkee 1991; Jolly et al. 2006), due to the fact that, in the last stages, only *Saccharomyces* was detected (Azzolini et al. 2012). The slower kinetics of *T. delbrueckii* fermentations (Fig. 2a) was attributed to a high nutrient demand by these species that limited the later *S. cerevisiae* fermentation activity (Romano et al. 2003). Data obtained for fermentation kinetics in semi-industrial fermentations confirm this trend. The fact

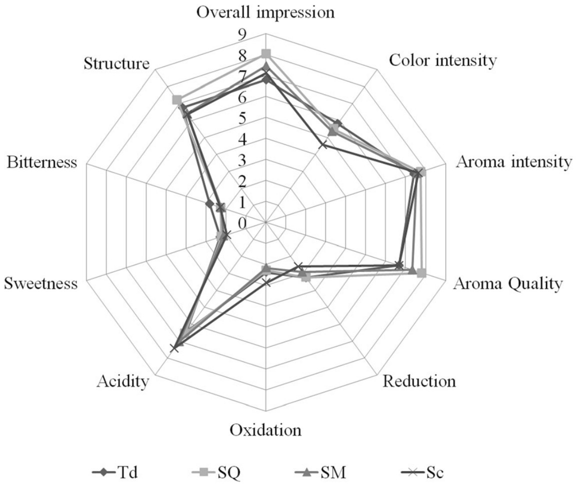

Fig. 5 Taste and olfactory attribute scores for the final wines

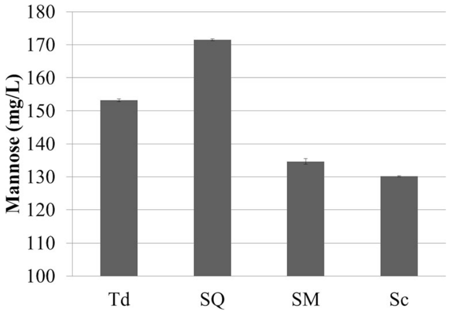

Fig. 6 Mannoprotein content (milligrams per liter of mannose) of wines fermented at semi-industrial scale with: *S. cerevisiae* CT007 (*Sc*); *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007 by using simultaneous inoculation (*SM*); sequential inoculation (*SQ*); and *T. delbrueckii* NSA-1 alone (*Td*)

that the semi-industrial fermentation kinetics was faster than microvinifications (Fig. 2) can be explained because of the different composition of the must. In microvinifications, the fermentative media contained only fresh must and must with crushed grapes in semi-industrial fermentations, which contribute to nutrient enrichment of musts.

Several authors argue the usefulness of non-*Saccharomyces* yeast in the production of lower concentrations of alcohol in wines (Contreras et al. 2014; Kutyna et al. 2010), reporting reductions higher than 1 % in final alcohol content. These previous results agree with the lower final alcohol content of the wines produced in fermentations involving *T. delbrueckii* NSA-1 in this work (Table 1); however, in this assay, the ethanol reduction was lower than 0.2 %. Sugar consumption could also be used to produce alternative compounds to ethanol, such as glycerol or pyruvic acid, or to increase the yeast biomass by *T. delbrueckii* due to its reported lower Crabtree effect (Bely et al. 2008; Merico et al. 2007).

Higher acetic acid values from Td fermentations (Table 1) than the others, both in microvinifications and in semi-industrial fermentations, can be attributed to the wild *Saccharomyces* yeasts that finish the fermentation, observing the increase in the release of acetic acid at the beginning of tumultuous fermentation (Fig. 3) and confirming the usefulness of selected strains to reduce the acetic acid content in wines. One of the questions raised by winemakers is the excessive increase of acetic acid in wines with high presence of non-*Saccharomyces* yeasts (Jolly et al. 2014). Our results show that using *T. delbrueckii* in mixed fermentations does not cause an increase of acetic acid (Table 1), according to the results reported in similar studies (Azzolini et al. 2012). Other authors also described *T. delbrueckii* as a low acetic acid producer compared with most non-*Saccharomyces* yeasts (Bely et al. 2008; Moreno et al. 1991; Renault et al. 2009).

The higher decrease in malic acid content observed in the trials involving *T. delbrueckii* (Table 1) agrees with the reported by other authors who confirm that malic acid can be metabolised by several yeast species (Benito et al. 2013, 2014a, 2014b; Suárez-Lepe et al. 2012; Su et al. 2014) in levels lower than 20 %, unless *Schizosaccharomyces* species are used.

Previous pyruvic acid-based selection studies on *S. cerevisiae* strains returned maximum values of 60–132 mg/L after 4 days of fermentation (Morata 2004), values below those obtained in the present study with *T. delbrueckii* fermentations (Fig. 4a). A strong correlation has been reported between the amount of pyruvic acid released into the medium and the formation of vitisin A (Morata et al. 2003, 2012) which is also related to colour stability. Other authors have reported before a high production of other acid in yeast metabolism by *T. delbrueckii* such as succinic acid (Ciani and Maccarelli 1998). Different non-*Saccharomyces* yeasts have been found to have influence in intensity and stability of wine colour (Benito et al. 2011, 2014c; Morata et al. 2012). Final OD values in colour intensity of 3.16, 3.09, 2.96 and 2.77 were returned for Td, SM, SQ and Sc fermentations, respectively (Table 1). The formation of highly stable pigments such as vitisin A, due to the higher pyruvic acid formation, could explain these chromatic differences between wines. Also, colour material absorption could be different between species and strains (Morata et al. 2005). Furthermore, higher total sulphur dioxide levels (Table 1) in fermentations involving *T. delbrueckii* could also be explained by higher combinations of anthocyanins with pyruvic acid during fermentation (Morata et al. 2003).

The increase of glycerol content in wines is one of the most recognised contributions of non-*Saccharomyces* species to the quality of wines (Jolly et al. 2006). However, some authors reported that an increase in glycerol production is usually linked with a rise in acetic acid production (Prior et al. 2000), which can be detrimental to wine quality. This fact was observed in *Candida stellata* strains that can produce elevated concentrations of glycerol (10 to 14 g/L) compared with *S. cerevisiae* (4 to 10 g/L); on the contrary, our results confirm that these facts seem to be irrelevant in the case of *T. delbrueckii*.

The values observed in pyruvic acid and glycerol production could indicate that *T. delbrueckii* possesses a highly active glyceropyruvic pathway (Ciani and Maccarelli 1998; Renault et al. 2009). Besides, some authors have been reported that there is a big difference in glycerol production depending on strain level (Loira et al. 2012).

The detected lower production of higher alcohols by *T. delbrueckii* could have increased the varietal Tempranillo aroma perception. Different non-*Saccharomyces* yeasts produce different levels of higher alcohols (Lambrechts and Pretorius 2000; Romano et al. 1992). This can be important because a large concentration of higher alcohols can generally not be desired, whereas lower values can contribute to wine complexity (Romano and Suzzi 1993). Non-*Saccharomyces* yeasts often form lower levels of these alcohols than *S. cerevisiae*, but there is great strain variability (Romano et al. 1992; Zironi et al. 1993). The higher values detected in acetaldehyde, diacetyl and ethyl ethanol when *Torulaspora* was used alone could be attributed to wild high fermentative non-selected yeasts involved in a spontaneous process. Other authors have reported a higher production of terpenic compounds by *T. delbrueckii* in Muscat variety (King and Dickson 2000). In contrast, our results show no differences between assays in this kind of compounds (Supplementary material, Table S2). However, this ability is attributed to specific strains and *T. delbrueckii* NSA-1 does not possess the terpenic-related enzymatic properties that were analysed (β-glucosidase, β-D-xylosidase and α-L-arabinofuranosidase).

The recorded differences in acidity perception could be related to the small malic acid consumption detected in fermentations in which *T. delbrueckii* NSA-1 was involved and to the lower total acidity levels obtained in these wines (Table 1). Differences in colour intensity perception could be partially explained because of the higher pyruvic acid content detected and its influence in high stable colour forms (Benito et al. 2011). Other authors described that wines fermented by coinoculation with *T. delbrueckii* and *S. cerevisiae* are better than the regular *S. cerevisiae* control for the varieties Sauvignon Blanc, Chenin Blanc and Amarone (Azzolini et al. 2012; Jolly et al. 2003). In this work, a similar effect was found for the Tempranillo variety. The tasters perceived higher aroma quality in the specific cases of SQ and SM fermentations, but no strong difference in aroma compounds was observed. This could be explained due to a lower higher-alcohol content which generally overlays other minor compounds that contribute to the wine aroma complexity. Mannoproteins are one of the main microbial metabolites related with the complexity of wine mouthfeel properties. The better mouthfeel structure of SQ fermentations that contributed to the higher overall score could be related to this fact (Fig. 6). In addition, a higher perception in sweetness was detected, probably due to the malic acidity consumed by *T. delbrueckii*, but also influenced by higher levels of mannoproteins in wines.

One of the main contributions of non-*Saccharomyces* yeasts during wine fermentation are their repercussion on the mouthfeel properties (Suárez-Lepe and Morata 2012). Macromolecules derived from the yeast cell wall, particularly mannoproteins, have capital importance in the mouthfeel properties (Gonzalez-Ramos et al. 2008), and enological empirical experience carried out to date with *T. delbrueckii* talks about a remarkable complexity and roundness in mouthfeel (Guadalupe et al. 2007). Recently, *T. delbrueckii* has been described as a wine yeast with a higher content of wall polysaccharides (Domizio et al. 2014).

This study contributes to confirm the role of non-*Saccharomyces* in wine fermentation by analysing metabolic and physiological properties of a new industrial strain of *T. delbrueckii*. A significant effect in some major aroma compounds (higher alcohols and esters), as in pyruvic, malic and acetic acids and in alcohol content were found in microvinifications carried out with *T. delbrueckii* NSA-1 industrial strain using different combined fermentation strategies, concluding that sequential fermentation is the most appropriate. Scaling assays for validating the industrial use of yeasts are a key factor and the bottleneck of the yeast selection process. In this study, we validate the use of the new *T. delbrueckii* NSA-1 strain in a semi-industrial assay, and similar results can be found for all parameters analyzed. Furthermore, sensorial analysis of these semi-industrial fermentations emphasized the improvement of mouthfeel properties in fermentations in which *T. delbrueckii* was involved. This fact could be explained, aside from the chemical and aromatic properties already mentioned, because of the increase in mannoprotein content of these wines. The use of non-*Saccharomyces* yeast in winemaking implies its adaptation to a cellar environment, so semi-industrial and industrial assays should be considered as important and ought to be included in scientific reports.

Acknowledgements Funding for the research in this paper was provided by Agrovin S.A, under the framework of the project IDI20130192-ENZIOXIVIN (Centre for Industrial Technological Development-CDTI, Spain).

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Supplementary material - Applied Microbiology and Biotechnology

Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality

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Table S1. Analytical results for the wines produced by the different fermentation systems on a semiindustrial scale

Assays	Glucose + Fructose (g/l)	Acetic Acid (g/l)	Malic Acid (g/l)	Lactic Acid (g/l)	Glycerol (g/l)	Total Acidity (g/l)	Alcohol (% v/v)	pH
Td	0.83	0.43	1.71	0.11	5.78	6.55	14.34	3.51
SQ	1.39	0.29	1.93	0.13	5.77	7.27	14.38	3.48
SM	1.33	0.35	1.73	0.09	5.59	6.67	14.44	3.54
Sc	0.57	0.33	1.80	0.09	5.44	6.75	14.48	3.53

Results represent the value for a single semiindustrial sample

Table S2. Volatile compounds (µg/l) detected in the different fermentations on a semiindustrial scale

Compounds	**Sc**	**SM**	**SQ**	**Td**
1-Hexanol	1750	2020	1690	1980
3-Hexanol	330	340	250	290
Isoamylalcohol	322470	363570	323620	386560
Isobutanol	55280	60040	59050	69020
Alcohols	379830	425970	384610	457850
Acetaldehyde	11940	16460	10080	18850
Diacetyl	1080	2970	910	4310
Carbonyl compounds	13020	19430	10990	23160
Ethyl acetate	32900	33070	32190	36030
Ethyl butyrate	140	170	150	170
Ethyl decanoate	70	110	70	100
Ethyl hexanoate	190	620	260	430
Ethyl lactate	3650	2740	3220	2790
Ethyl octanoate	310	410	290	350
Isoamyl acetate	1160	1170	1090	1200
Esters	38420	38290	37270	41070
hexanoic acid	990	2480	1170	2150
Isobutyric acid	1860	1580	1880	2260
isovaleric acid	1740	1760	1620	2430
octanoic acid	950	2200	1210	1790
valeric acid				
Fatty acids	5540	8020	5880	8630
2-Phenylethanol	85740	67460	79710	57100
2-phenylethyl acetate	N.D	N.D.	10	N.D.
Phenols	85740	67460	79720	57100
Linalool (p.p.b)	19.68	18.27	17.89	18.81
linalool acetate (p.p.b)	1.81	1.74	2.06	2.28
α-Terpineol (p.p.b)	1.45	1.26	1.24	1.28

β-Citronellol (p.p.b)	10.09	12.46	10.34	10.01
Geraniol (p.p.b)	5.86	4.17	4.71	4.6
Terpenes (p.p.b)	38.89	37.9	36.24	36.98

Results represent the value for a single semiindustrial sample

ANEXO

AGROVIN

Levaduras

Ficha técnica

viniferm NS TD

Levadura No *Saccharomyces* para fermentación secuencial.
Incremento aromático y estructura en boca

CARACTERÍSTICAS

Viniferm NS TD es una cepa de *Torulaspora delbrueckii* seleccionada por su aptitud a la mejora sensorial de los vinos.

En nariz: Incrementa la complejidad y espectro aromático de los vinos. Intensifica los registros florales por producción de β-fenil etanol (rosa, flor blanca) y el carácter varietal por su potente actividad B-liasa (pomelo, boj).

En boca: Confiere redondez y volumen a los vinos por su importante liberación de manoproteínas.

ORIGEN

Torulaspora delbrueckii. Colección Agrovin.

Procedencia de la cepa: D.O. Ribera del Duero

Equipo investigador: Departamento Microbiología III. Universidad Complutense de Madrid.

Referencias:

» *Dynamic analysis of physiological properties of Torulaspora delbrueckii in wine fermentations and its incidence on wine quality.* (Belda *et al.* 2015 Applied Microbiology and Biotechnology)

» *Actividades enzimáticas de levaduras no Saccharomyces para su aplicación enológica* (Belda *et al.* 2015 ACE Enologia)

» *Directed metabolomic approaches for the characterization and development of new yeast strains.* (Belda *et al.* 2015 BIO Web of Conferences OIV2015)

CUALIDADES ORGANOLÉPTICAS

Incremento de complejidad en los registros aromáticos del vino. Intensifica las cualidades varietales. Fuerte impacto gustativo, acentúa las sensaciones de volumen y untuosidad en boca, limando sensaciones de astringencia y prolongando el postgusto.

APLICACIÓN

» Vinos blancos y tintos de corte varietal en los que interese acentuar las sensaciones en boca.

» Fermentaciones controladas de variedades neutras en los que se desee aumentar la complejidad aromática.

» Elaboración de vinos rosados de corte floral y con aumento de volumen en boca.

Caracterización del perfil aromático y analisis sensorial.

Producción de manoproteinas

1/2

AGROVIN

Levaduras

Ficha técnica

PROPIEDADES ENOLÓGICAS

» Poder alcoholígeno 9,5 %vol. Requiere inoculación posterior de cepa de *Saccharomyces cerevisiae.*

» Temperatura de trabajo 17 °C - 28 °C

» Necesidades de NFA medias.

» Producción de sulfhídrico ausente.

» Producción de acidez volátil, muy baja.

» Producción de compuestos carbonílicos (acetaldehído, acetoína), muy baja.

» Cinética fermentativa, media.

» Resistencia al sulfuroso, baja.

» El empleo de fermentaciones secuenciales No *Saccharomyces* + *Saccharomyces cerevisiae*, permite la obtención de vinos con menor graduación alcohólica.

Viniferm NSTD ha sido galardonada con el Premio a la Innovación Enomaq 2015.

DOSIS

Vinificación 20-30 g/HL

MODO DE EMPLEO

Para obtener los mejores resultados es indispensable asegurar la buena implantación de la cepa en el medio, por lo tanto es importante:

» Mantener una buena higiene en la bodega.
» Añadir la levadura lo antes posible.
» Respetar la dosis prescrita.
» Rehidratar bien la levadura.

Rehidratación:

1.- Añadir las levaduras secas en 10 veces su peso en agua a 35 °C - 40 °C (10 litros de agua por 1 kg de levadura).

2.- Esperar 10 minutos.

3.- Agitar la mezcla.

4.- Esperar 10 minutos e incorporar al mosto, procurando que no haya una diferencia de más de 10 °C entre el medio rehidratado y el mosto.

Precauciones de trabajo:

- En cualquier caso, la levadura no deberá estar rehidratándose más de 30 minutos en ausencia de azúcares.

- El respeto del tiempo, temperatura y modo de empleo descrito garantizan la máxima viabilidad de la levadura hidratada.

- La siembra secuencial de levadura Saccharomyces debe realizarse cuando se evidencie una bajada de la densidad (48-72 horas en función de la temperatura). No se recomienda la siembra simultanea de ambas cepas.

- Protocolo de trabajo: ver ficha adjunta.

PROPIEDADES MICROBIOLÓGICAS Y FISICOQUÍMICAS

Recuento de levaduras (***Torulaspora delbrueckii***.) [UFC/g]	$> 10^{10}$
Otras levaduras [UFC/g]	$< 10^{5}$
Mohos [UFC/g]	$< 10^{3}$
Bacterias lácticas [UFC/g]	$< 10^{5}$
Bacterias acéticas [UFC/g]	$< 10^{4}$
Salmonella [UFC/25 g]	Ausencia
E. coli [UFC/g]	Ausencia
Staphylococcus aureus [UFC/g]	Ausencia
Coliformes totales [UFC/g]	$< 10^{2}$
Humedad [%]	< 8
Pb [mg/kg]	< 2
Hg [mg/kg]	< 1
As [mg/kg]	< 3
Cd [mg/kg]	< 1

ASPECTO FÍSICO

Gránulos de color tostado, desprovistos de polvo.

PRESENTACIÓN

Paquetes de 500 g envasados al vacío en envuelta multilaminar de aluminio en cajas de 10 kg.

CONSERVACIÓN

El producto conforme a los estándares cualitativos se conserva en su envase sellado al vacío durante un periodo de cuatro años en cámara refrigerada entre 4 °C y 10 °C.

Eventuales exposiciones prolongadas a temperaturas superiores a 35 °C y/o con humedad reducen su eficacia.

Registro:
R.G.S.A: 31.00391/CR
Producto conforme con el Codex Enológico
Internacional y el Reglamento CE 606/2009.
VINIFERM NSTD EP 871 / Rev.: 1 / Fecha: 14/09/15

6. CAPÍTULO 4

6.1. Estudio de la incidencia en la calidad de vinos tintos de la crianza sobre lías de levaduras no convencionales

Outlining the influence of non-conventional yeasts in wine ageing over-lees

Artículo aceptado para su publicación en la revista “Yeast”

(doi: 10.1002/yea.3165)

Outlining the influence of non-conventional yeasts in wine ageing over-lees

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Abstract

During the last decade, the use of innovative yeast cultures of both *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts as alternative tools to manage the winemaking process have turned the oenology industry. Although the contribution of different yeast species to wine quality during fermentation is increasingly understood, the information about their role in

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/yea.3165

wine ageing over-lees is really scarce. This work aims to analyse the incidence of 3 non-*Saccharomyces* yeast species of oenological interest (*Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Metschnikowia pulcherrima*) and of a commercial mannoprotein-overproducer *S. cerevisiae* strain compared with a conventional industrial yeast strain during wine ageing over-lees. To evaluate their incidence in mouthfeel properties of wine after 4 months of ageing, mannoprotein content of wines was evaluated, together with other wine analytic parameters such as colour and aroma, biogenic amines and amino acids profile. Some differences among the studied parameters were observed during the study, especially regarding the mannoprotein concentration of wines. Our results suggest that the use of *T. delbrueckii* lees in wine ageing is a useful tool for the improvement of overall wine quality by notably increasing mannoproteins, reaching values higher than obtained using a *S. cerevisiae* overproducer strain.

Keywords: *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, ageing over-lees, mannoprotein, amino acids.

Introduction

The incidence of yeasts in winemaking not only works during the alcoholic fermentation process, but also in both pre- and postfermentative stages. In an industrial context that demands products with increasingly high quality and safety, the development of new techniques to improve wine attributes and to avoid a global-market wine homogeneity is advisable (Moreno-Arribas and Polo, 2005).

The technique of ageing over-lees is gaining importance in the production of red wine because of its impact on wine mouthfeel properties. Mannoproteins have been recognized to have many positive sensorial attributes such as improving wine mouthfeel and roundness (Vidal *et al.,* 2004), increasing aromatic persistence (Chalier *et al.,* 2007), and decreasing astringency (Saucier *et al.,* 2002). Additionally, some technological advantages have been described for mannoproteins in wines, by reducing protein and tartrate instability (González-Ramos *et al*., 2008) and also by removing (by absorption) ochratoxin A from wine (Ringot *et al*., 2005). On the other hand, the use of wine ageing over-lees can also involve certain risks such as the formation of biogenic amines or the release of their amino acid precursors (Martínez-Rodríguez and Polo, 2000).

Keeping in contact the wine with the resting dead yeast cells after the alcoholic fermentation may cause the release of the polysaccharide fraction from the yeast cell wall due to the autolysis process and to the action of the yeast-derived enzymes β-glucanase and cell wall mannosidase (Charpentier and Freyssinet, 1989). Nowadays, the influence of different *Saccharomyces cerevisiae* strains in wine ageing over-lees is known (Loira *et al*., 2013). It also has been reported a strain-dependent behaviour regarding to the mannoprotein release rate during alcoholic fermentation, nevertheless this performance is not directly correlated with mannoprotein release during ageing over-lees (del Barrio-Galán *et al*., 2015).

The yeast cell-wall composition is variable between species and the polysaccharide composition of some relevant wine yeast species have already been described (Domizio *et al*., 2014). There is an increasing interest in non-*Saccharomyces* yeasts to improve wine quality (Jolly *et al*., 2006, 2014) and it should be mentioned that certain yeast species, such as *Torulaspora delbrueckii,* have been reported as adequate to increase mannoprotein content of wines during wine fermentation (Belda *et al*., 2015; Domizio *et al*., 2014). However, the information about their influence during wine ageing over-lees is really scarce. The influence of certain non-*Saccharomyces* yeasts such as *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Wickerhamomyces anomalus* and *Pichia mambranifaciens* has been evaluated for over-lees ageing (Palomero *et al*., 2009). Other species such as *T. delbrueckii* (Azzolini *et al*., 2015; Belda *et al*., 2015; Renault *et al*., 2015), *Lachancea thermotolerans* (Benito *et al*., 2015a; Benito *et al*., 2016; Comitini *et al*., 2011; Gobbi *et al*., 2013) and *Metschnikowia pulcherrima* (Belda *et al*., 2016; Benito *et al*., 2015b; Contreras *et al*., 2014; Jolly *et al*., 2003) have been proved to improve wine quality during alcoholic fermentation. However, they have not been studied in ageing over-lees yet.

This work aims to study the incidence of some of the currently most used non-*Saccharomyces* yeasts in wine industry (*T. delbrueckii, L. thermotolerans* and *M. pulcherrima*) and also of a mannoprotein-overproducer *S. cerevisiae* commercial strain in wine composition during red wine ageing over-lees.

Materials and Methods

Yeasts used in experimental over-lees ageing

Five different yeast strains, pertaining to 4 different species, were used: *Saccharomyces cerevisiae* CT007 (Agrovin S.A., Ciudad Real, Spain) which was used as a control; *S. cerevisiae* 3D (Agrovin S.A.), as a mannoprotein overproducing industrial strain. *Torulaspora delbrueckii* NS-TD (Agrovin S.A.; also referred in past literature as *T. delbrueckii* NSA-1 from Complutense Yeast Collection-CYC, Madrid, Spain) (Genbank accession number KM434245), *Lachancea thermotolerans* NS-G-32 (CYC) (Genbank accession number KT222664), and *Metschnikowia pulcherrima* NS-EM-34 (CYC) (Genbank accession number KT222665) were used as non-*Saccharomyces* yeasts.

The yeast biomass used in the over-lees ageing assay was obtained according to Palomero *et al*. (2009) with some modifications. Briefly, yeasts were grown using an enriched must medium (12.5 % concentrated must [final concentration, 50 g/L glucose+fructose], 1 % yeast extract, 0.5 % proteose peptone no.3, pH 3.5) at 25 °C with orbital agitation (100 rpm) (Orbital shaker Infors AG, Bottminger CH-4103, Switzerland) for 48 hours. After that, the yeast biomass was washed with 10:1 volumes of sterile distilled water, centrifuged at 3000 rpm for 2 min, and the supernatant discarded. This procedure was repeated twice to provide yeast biomass with no remains of nutrients. Finally, these yeasts were lyophilised using a Cryodos apparatus (Telstar, Spain) and added, under sterile conditions (laminar flow cabinet Telstar PV-100, Spain), to wines at a concentration of 0.8 g/L.

Wine ageing over-lees assays were performed, by triplicate, in 1L crystal bottles (Fisherbrand FB-800-1000, UK) with its proper hermetic seal (Fisherbrand GL45, UK) filling up the entire bottle to avoid any oxidation problems during ageing. Wine ageing was carried out for 4 months, at a constant temperature of 16ºC, using a young commercial wine (var. Tempranillo; Bodegas Urbina S.L., Spain) from La Rioja wine appellation.

Analytical determinations of non-volatile compounds

Color Intensity (CI), Total Polyphenol Index (TPI), Anthocyanin Content (AC), urea, acetaldehyde, acetic acid, lactic acid, malic acid, glucose/fructose citric acid and glycerol were measured using a Y15 enzymatic autoanalyzer (Biosystems S.A, Barcelona, Spain) and their corresponding kits (http://www.biosystems.es/products/). Ethanol and pH were determined following the methods in the Compendium of International Methods of Analysis of Musts and Wines (http://www.oiv.int/oiv/info/enmethodesinternationalesvin).

Analysis of mannoprotein content of wines

Mannoprotein concentration of wines were evaluated in duplicate by using a HPLC apparatus (Surveyor Plus chromatograph, Thermo Fisher Scientific, Waltham, MA) equipped with a refractive index detector (Surveyor RI Plus Detector) as reported (Quirós *et al.,* 2012). The column employed was a 300 x 7.7 mm PL Hi-Plex Pb 8 µm (Varian, Inc., Shropshire, UK). MilliQ water was used as the mobile phase at a flux of 0.6 ml/min and a column temperature of 70ºC. The retention time valued was between 0 and 30 minutes.

Analytical determinations of amino acids

Amino acids were analyzed using a Jasco (Tokyo, Japan) UHPLC chromatograph series X-LCTM, equipped with a fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile, 50:50, v/v) and B (sodium acetate /tetrahydrofuran, 99:1, v/v) were used in a C18 (HALO, USA) column (100 mm × 2.1 mm; particle size 2.7 µm) as follows: 90 % B (0.25 mL/min) from 0 to 6 min, 90–78 % B linear (0.2 mL/min) from 6 to 7.5 min, 78 % B from 7.5 to 8 min, 78–74 % B linear (0.2 mL/min) from 8 to 8.5 min, 74 % B (0.2 mL/min) from 8.5 to 11 min,74–50 % B linear (0.2 mL/min) from 11 to 15 min, 50 % B (0.2 mL/min) from 15 to 17 min, 50–20 % B linear (0.2 mL/min) from 17 to 21 min, 20–90 % B linear (0.2 mL/min) from 21 to 25 min and re-equilibration of the column from 25 to 26 min. Detection was performed by scanning in the 340–455 nm range. Quantification was performed by comparison against external standards of the studied amino acids. The different amino acids were identified by their retention times.

Analytical determinations of biogenic amines

Biogenic amines were analyzed using a Jasco (Tokyo, Japan) UHPLC chromatograph series X-LCTM, equipped with a fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile, 50:50, v/v) and B (sodium acetate /tetrahydrofuran, 99:1, v/v) were used in a C18 (HALO, USA) column (100 mm × 2.1 mm; particle size 2.7 µm) as follows: 60% B (0.25 ml/min) from 0 to 5 min, 60–50% B linear (0.25 ml/min) from 5 to 8 min, 50% B from 8 to 9 min, 50-20% B linear (0.2 ml/min) from 9 to 12 min, 20% B (0.2 ml/min) from 12 to 13 min, 20–60% B linear (0.2 ml/min) from 13 to 14.5 min, and re-equilibration of the column from 14.5 to 17 min. Detection was performed by scanning in the 340–420 nm range. Quantification was performed by comparison against external standards of the studied amines. The different amines were identified by their retention times.

Analytical determination of volatile compounds

Volatile compounds were quantified by headspace gas chromatography–mass spectrometry (HS-GC-MS). Analyses were carried out using a Perkin-Elmer Clarus 500 gas chromatograph with a flame ionization detector, coupled to a mass spectrometer single quadrupole Clarus 560 S, all coupled to an automatic headspace sampler Turbomatrix 110 Trap (Perkin-Elmer, Massachusetts, USA). The headspace sampler conditions were: temperature of thermostating: 80ºC; time of thermostating: 45 min; type of trap: Tenax TA; cycles of purge and trap: 4; temperature of trap capture: 45ºC; desorption temperature of the trap: 290ºC; time of dry trap purge: 10 min; desorption time of trap: 2 min; trap cleaning time: 5 min; needle temperature: 110ºC; temperature of HS-GC transfer line: 150ºC; vial pressure: 30 psi; and constant pressure column: 28 psi. A Free Fatty Acid Phase (FFAP) capillary column (60 m × 0.25 mm DI x 0.25 µm film thickness) was used. Helium (Air Liquide, Spain) was used as carrier gas. Gradient analysis was run using the following temperature program: 40ºC (3 min); 40–80ºC (2ºC/min); 80-180ºC (3ºC/min); and 210ºC (5 min). Identification of individual compounds was based on a comparison of the obtained mass spectra of the individual chromatographic peaks with those valid for the standards and available from the National Institute of Standards and Technology (Gaithersburg, MD) software library. We also compared the retention times valid for individual peaks from the wine samples with those of the known volatile components used as standard patterns. To this effect, we used Gas chromatography quality compounds as the sets of the volatile standards (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland).

Sensory analysis

The final wines were assessed (blind test) by a panel of 15 experienced wine tasters; all staff members of the Chemistry and Food Technology Department of Polytechnic University of Madrid and the Department of Microbiology of the Biology Faculty of the Complutense University of Madrid. Following the generation of a consistent terminology by consensus, two aromas and nine taste attributes were chosen to describe the wines. The panellists used an 8-cm unstructured scale, from 0 (no character) to 8 (very strong character), to rate the intensity of the 11 attributes.

Statistical analysis

All statistical analyses were performed using PC Statgraphics v. 5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p < 0.05$ for the ANOVA matrix F value. The multiple range test was used to compare the means.

Results and Discussion

General chemical analyses

No statistical differences were observed among most of the different studied basic chemical parameters (Table 1). These results could be related with no deviations during the studied ageing over-lees period. Nevertheless, differences in other parameters are explained below as consequence of the ageing over-lees process.

Mannoproteins

One of the main reasons to use yeast lees for wine ageing is to improve its mouthfeel properties by releasing most of the mannoproteins contained in the yeast cell wall. Figure 1 shows that the use of different *S. cerevisiae* strains lees could increase, in a significant way, the mannoprotein content of wines after a short ageing period. However, several differences in mannoprotein content of about 240 mg/L were observed between CT007 and 3D assays, as expected taking into account that *S. cerevisiae* 3D is a commercial mannoprotein overproducer strain. Our results show that the use of *T. delbrueckii* lees was able to greatly increase the mannoprotein concentration after four months of wine ageing, reaching significant higher values of about three times when compared with the conventional *S. cerevisiae* CT007 strain and being also slightly higher (7,9%) than *S. cerevisiae* 3D strain, but without establishing significant statistical differences in this last case. In the same line, *M. pulcherrima* showed a significant increase in the final mannoprotein content of wines, being remarkably higher than CT007 and Lt levels but lower than the concentrations obtained using *T. delbrueckii* lees. These results are in agreement with the results of Domizio *et al.* (2014) reporting the higher mannoprotein release of both *M. pulcherrima* and *T. delbrueckii,* during alcoholic fermentation, when compared with *S. cerevisiae* or other non-*Saccharomyces* such as *L. thermotolerans*. Here we confirm, for the first time, the usefulness of these yeast species, not only during the alcoholic fermentation, but also during wine ageing over-lees by releasing their mannoproteins. The ability of *T. delbrueckii* NS-TD to release significant amounts of mannoproteins during the alcoholic fermentations has been described (Belda *et al.,* 2015) and, on the other hand, the contrary has been observed using *M. pulcherrima* NS-EM-34 (Belda *et al.,* unpublished). However, other authors have reported different results of high mannoprotein release levels using other *M. pulcherrima* strain (Domizio *et al.,* 2014). In this sense, del Barrio-Galán *et al.* (2015), showed a strain-dependent behaviour in mannoprotein release for their *S. cerevisiae* strains used during both alcoholic fermentation and wine ageing over-lees period. Commercial information of *S. cerevisiae* 3D strain (http://www.agrovin.com/agrv/pdf/enologia/levaduras/en/Viniferm_3D_en.pdf) shows that using this strain during the alcoholic fermentation causes an increase of about 30% compared with a conventional *S. cerevisiae* strain. Our results show that the use of their lees could increase this difference, making it more interesting for winemakers to improve wine mouthfeel properties. In summary, the use of *T. delbrueckii* seems to be a useful tool to increase mannoprotein concentration in wines in both fermentation process and, as we show here, during wine ageing over-lees.

Colour characteristics

Colour Intensity (CI), Total Polyphenol Index (TPI) and Anthocyanin Content (AC) of wines were evaluated, since they have been described as influenced by ageing process over-lees (Loira *et al.,* 2013; Palomero *et al.,* 2009). Table 2 shows different final values of CI, with certain statistical differences depending on the assay. Wine ageing has been related with both colour stabilization and colour loss depending on the physicochemical environment of the ageing process and also the characteristics of yeast cells, such as their adsorption capacity depending on their porosity, in the case of over-lees ageing processes (Gómez-Cordovés and Gonzalez-San José, 1995; Morata *et al.,* 2003). Our results showed, in all cases, a CI loss with slight differences among treatments after 4 months of ageing. Trials Td and Lt showed higher values in colour measurements at 520 nm than CT007 trial, up to 0.4 absorbance units, keeping better their red colour. The role of mannoproteins in the stabilization of colour properties of wines has been previously reported (Feuillat *et al.,* 2001; Fuster and Escot, 2002; Saucier *et al.,* 2002) by preventing the precipitation of anthocyanins and tannins (Escot *et al.,* 2001; Francois *et al.,* 2007) and reducing the oxidation process on polyphenols (Salmon, 2005), but other authors reported no improvements in wine colour properties when ageing over-lees was developed (del Barrio-Galán *et al.,* 2015; Loira *et al.,* 2013; Rodrigues *et al.,* 2012). Our results are partially in agreement with both data, since Td showed both the lowest CI and TPI value decreases and also the highest mannoprotein release values and Mp showed lower TPI decreases and also higher mannoprotein values when compared with the other ageing over-lees assays. However, other factors, such us cell wall adsorption, could be also contributing to the final colour properties of wines, since Lt showed the lowest enhancement in mannoprotein but final CI values similar to Td assay.

Degradation of pigments present in wine or their development into other compounds, which, in both cases, can lead to lower colour indices, have been described before (Palomero *et al.,* 2009). Furthermore, other authors have reported before adsorption of anthocyanins phenomena related to yeast structure (Morata *et al.,* 2003). In this case, a higher decrease in AC using *M. pulcherrima* lees has been observed when compared with the other assays that performed ageing over-lees, but no significant differences could be established among the other assays.

Amino acids

Higher levels in amino acids were reported for the treatments that performed ageing over-lees. Although this increase was observed in all the trials, due to the cell lysis process, the differences obtained in their amino acid profile could be related with the different amino acidic content of the different yeast strain used (Vaughan-Martini *et al.,* 1979). Table 3 shows statistical differences among the different amino acids concentration at levels of some units in mg/l, except for the case of alanine and asparagine, where differences up to tens in mg/l were observed. Mp assay reached higher significant levels than all the other treatments in histidine, aspartic acid, alanine, arginine, phenylalanine, isoleucine, leucine, serine and tyrosine. Td repetitions showed higher levels in histidine and tryptophan while Lt showed higher levels in lysine and threonine. Lt reported higher final levels in alanine, lysine and serine (Table 3). CT007 and 3D produced the highest concentrations in leucine and threonine and 3D also scored the highest value in ornithine.

The statistical differences reported in histidine, phenylalanine, ornithine and tyrosine show that ageing over-lees processes could increase the content of biogenic amine precursors that could be metabolized to biogenic amines by the action of microorganisms with decarboxylase enzymes activities (Alcaide-Hidalgo *et al.,* 2007; Lehtonen, 1996; Ribéreau-Gayon *et al.,* 2006; Smit *et al.,* 2008).

Biogenic amines

Histamine is the most studied biogenic amine, although it is not the most abundant in wine, because of their likely occurring health risks such as headaches, low blood pressure, heart palpitations, oedema, vomiting (Moreno-Arribas and Polo*,* 2008a). Other biogenic amines such as Tyramine or putrescine may also influence human health (Jansen *et al.,* 2003; Kanny *et al.,* 2001; Maynard *et al.,* 1996; Moreno-Arribas and Polo*,* 2008a; Romano *et al.,* 2007;). The final levels of histamine were always lower than 1 mg/l (Table 4). A histamine value of 2 mg/L is considered the most restricted level in some countries due to food safety legislation (Lehtonen, 1996; Martuscelli *et al.,* 2013). It has been described that most biogenic amines are produced during malolactic fermentation and wine ageing (Alcaide-Hidalgo *et al.,* 2007; Benito *et al.,* 2015; Lonvaud-Funel, 1999). Nevertheless, our results prove that a controlled ageing over-lees process, without any deviation performed by lactic acid bacteria, does not produce higher levels of biogenic amines than a regular control, even in the cases where the amino acids precursors were increased. Thus, the previous increases reported in the literature could be related to preservation conditions and bacteria presence, as several factors can influence the presence of biogenic amines (Del Petre *et al.,* 2009; Marqués *et al.,* 2008; Moreno-Arribas and Polo, 2008b). Reductions in biogenic amines were reported (Table 4) depending on the biogenic amine and yeast strain. Treatments Mp and 3D were more effective in removing biogenic amines. Other authors have reported other non-*Saccharomyces* species such as *Hanseniaspora vineae* as highly effective reducing histamine contents (Medina *et al.,* 2013).

Analytical determination of volatile compounds

Slight differences were found among the studied volatile compounds (Table 5), especially for 3D treatment, that show statistical differences in 1-propanol and 2-methyl-butanol with the other assays. Nevertheless, since the final higher alcohol content was below 300 mg/l in all cases, this fact doesn't influence negatively wine quality (Rapp and Mandery, 1986). Other authors have also reported differences in higher alcohols (Loira *et al.,* 2013) after an ageing over-lees process. It has been also reported increases in ethyl lactate (Loira *et al.,* 2013) and in 2-Phenyl-ethanol (Liu *et al.,* 2015) after an ageing over-lees process. This phenomenon was not observed in this work, so it could depend on specific strains.

Sensory analysis

The most significant differences were found in mouth volume, persistence and structure (Fig. 2). These factors could depend on mannoprotein content (Belda *et al.,* 2015) in the case of *T. delbrueckii* treatment that could have influenced other parameters such as preference or overall impression. Also, *M. pulcherrima* together with *S. cerevisiae* 3D as high producers of mannoproteins scored relatively higher values in mouth volume, persistence and structure. However, Mp assay showed the lowest values in preference and overall impression. Thus, it indicates that mannoprotein release during wine ageing over-lees is an important factor but there are many others that also pose an important influence in wine perception.

Conclusions

Overall, we can conclude that the aged over-lees processes and the different strains used influenced, in a significant way, some of the studied wine quality parameters. Since not too many differences have been detected in the analytical aroma profile among assays, several differences were observed in their sensorial analysis. *T. delbrueckii* released higher amounts of mannoproteins that not only improved the wine mouthfeel properties but also seems to contribute to its colour stabilization. All the trials increased the amino acids content of wines. Furthermore, biogenic amines are generated from their amino acids precursors, mainly by microbial decarboxylation. The origin, detection, and quantification of biogenic amines in wine are extremely important for oenology, because of their health risks. In spite of the fact that some strains increased biogenic amines precursors, according to our results, we cannot conclude that an ageing process is directly related to biogenic amines increases. However, an exhaustive microbiological control should be performed during these processes to avoid the presence of biogenic amines related bacteria. Finally, different sensory profiles of wines were observed depending on the strain used for ageing over-lees, and they were mainly related with mannoproteins content. In conclusion, the use of certain non-*Saccharomyces* and non-conventional *S. cerevisiae* strains lees during ageing of wines could be a successful postfermentative alternative to improve the sensorial characteristics of young wines and to produce more distinctive wines.

Acknowledgements

Funding for the research in this paper was provided by Agrovin S.A, under the framework of the project IDI20130192-ENZIOXIVIN (Centre for Industrial Technological Development-CDTI, Spain). The authors are very grateful to the accredited laboratory Estación Enológica de Haro directors, Montserrat Iñiguez and Elena Meléndez for performing the amino acids and biogenic amines analyses.

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Figure legends

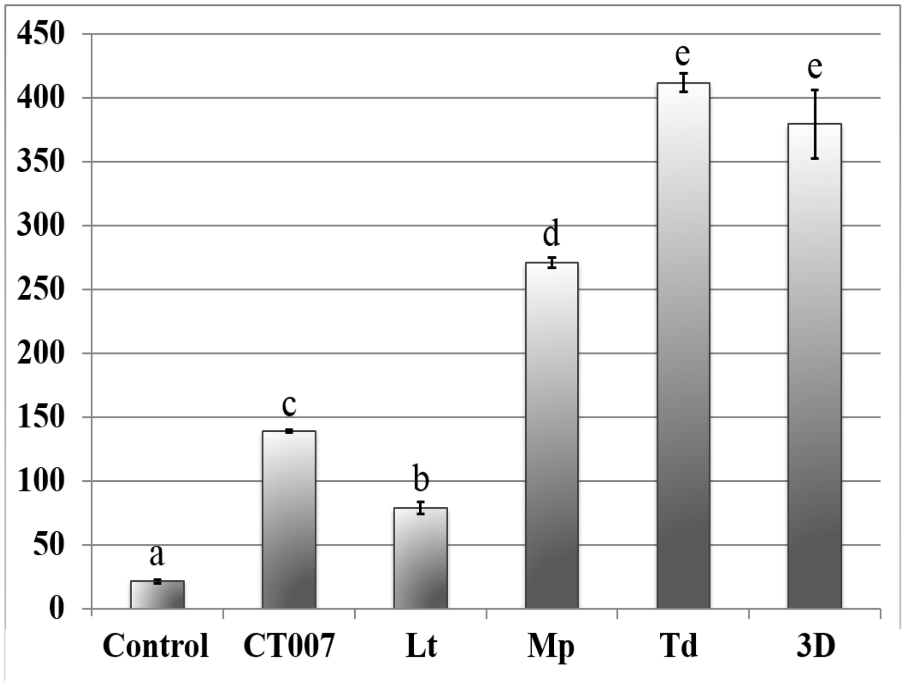

Figure 1. Mannoprotein content (milligrams per liter of mannose) of the initial control wine (Control) and of the wines aged over-lees with *S. cerevisiae* CT007 (CT007), *S. cerevisiae* 3D (3D), *L. thermotolerans* NS-G-32 (Lt), *M. pulcherrima* NS-EM-34 (Mp) and *T. delbrueckii* NS-TD (Td). Results represent the mean±SD for three replicates. Bars marked with the same letter showed no significant differences ($p<0.05$).

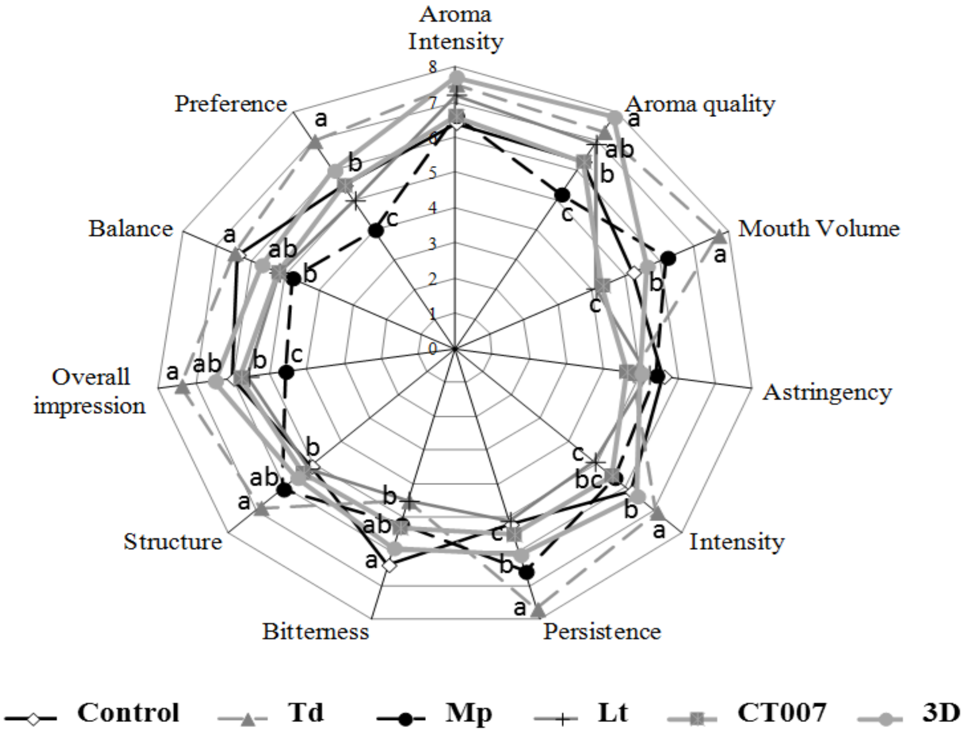

Figure 2. Taste and olfactory attribute scores for the initial control wine (Control) and for the final wines aged over-lees with *S. cerevisiae* CT007 (CT007), *S. cerevisiae* 3D (3D), *L. thermotolerans* NS-G-32 (Lt), *M. pulcherrima* NS-EM-34 (Mp) and *T. delbrueckii* NS-TD (Td). Means marked with the same letter showed no significant differences (p<0.05).

Table 1. Final analysis after ageing over-lees with *S. cerevisiae* CT007 (CT007), *L. thermotolerans* (Lt), *S. Cerevisiae* 3D (3D), *M. pulcherrima* (Mp) and *T. delbrueckii* (Td).

Compounds	Control	CT007	Lt	3D	Mp	Td
L-Lactic Acid (g/L)	1.62 ± 0.13	1.68 ± 0.15	1.65 ± 0.16	1.69 ± 0.15	1.64 ± 0.14	1.62 ± 0.17
L-Malic Acid (g/L)	0.24 ± 0.07	0.29 ± 0.09	0.25 ± 0.11	0.24 ± 0.07	0.27 ± 0.08	0.24 ± 0.07
Acetic Acid (g/L)	0.38 ± 0.09	0.4 ± 0.12	0.38 ± 0.07	0.41 ± 0.08	0.39 ± 0.09	0.36 ± 0.13
Glucose+Fructose (g/L)	0.32 ± 0.11	0.41 ± 0.09	0.36 ± 0.08	0.50 ± 0.16	0.32 ± 0.06	0.38 ± 0.09
Glycerol (g/L)	8.34 ± 0.18	8.12 ± 0.13	8.22 ± 0.20	8.36 ± 0.14	8.31 ± 0.13	8.29 ± 0.16
pH	3.51 ± 0.03	3.53 ± 0.06	3.51 ± 0.05	3.54 ± 0.06	3.52 ± 0.04	3.53 ± 0.05
Urea (mg/L)	14.51 ± 1.18	15.32 ± 2.14	14.31 ± 1.99	14.76 ± 1.87	15.13 ± 1.66	14.28 ± 2.06
Citric Acid (g/L)	0.41 ± 0.07	0.43 ± 0.09	0.39 ± 0.10	0.38 ± 0.09	0.42 ± 0.11	0.45 ± 0.08
Ethanol (% *v/v*)	14.51 ± 0.14	14.46 ± 0.13	14.58 ± 0.17	14.55 ± 0.14	14.53 ± 0.11	14.52 ± 0.16

Results represent the mean±SD for three replicates. No significant differences were observed among assays in the evaluated parameters ($p<0.05$).

Table 2. Final colour analysis after ageing over-lees with *S. cerevisiae* CT007 (CT007), *L. thermotolerans* (Lt), *S. Cerevisiae* 3D (3D), *M. pulcherrima* (Mp) and *T. delbrueckii* (Td).

Colour Measurements (Absorbance Units)	Control	CT007	Lt	3D	Mp	Td
420nm	5.07±0.09b	4.73±0.16a	4.75±0.18a	4.86±0.12ab	4.75±0.15a	4.81±0.14a
520nm	6.37±0.08c	5.75±0.14a	5.96±0.16ab	6.10±0.16b	5.89±0.11ab	6.17±0.10b
620nm	0.84±0.01a	0.83±0.02a	0.84±0.02a	0.86±0.02a	0.83±0.02a	0.84±0.02a
CI	12.28±0.07c	11.31±0.13a	11.55±0.15ab	11.77±0.13b	11.47±0.12a	11.88±0.11b
TPI	43,3±0,00d	32,33±0,12a	33,87±0,95c	32,87±0,49ab	33,6±0,36bc	33,93±0,71c
AC	158±0,00c	135,33±5,03b	127,67±4,04a	133,67±3,51b	130±0,00ab	132±2,65ab

Results represent the mean±SD for three replicates. Means in the same row with the same letter are not significantly different ($p<0.05$).

Table 3. Final amino acids analysis after ageing over-lees with *S. cerevisiae* CT007 (CT007), *L. thermotolerans* (Lt), *S. Cerevisiae* 3D (3D), *M. pulcherrima* (Mp) and *T. delbrueckii* (Td).

Compounds	Control	CT007	Lt	3D	Mp	Td
Histidine (mg/l)	2.54±0.00a	3.63±0.35b	3.92±0.47b	3.78±0.42b	5.92±0.55c	6.94±0.72c
Aspartic acid (mg/l)	4.82±0.00a	6.29±0.56c	6.53±0.61c	6.48±0.54c	8.79±0.93d	5.14±0.18b
Alanine (mg/l)	24.09±0.00a	28.44±1.28c	30.18±1.52c	29.11±1.33c	38.28±1.99d	25.42±0.24b
Arginine (mg/l)	26.53±0.00a	30.68±2.43bc	34.53±2.12c	31.18±2.31bc	38.26±2.45d	28.45±0.92b
Asparagine (mg/l)	16.86±0.00a	20.18±1.56b	18.24±1.12b	20.26±1.27b	28.52±2.24c	20.54±1.62b
Phenylalanine (mg/l)	2.48±0.00a	4.77±0.23c	4.58±0.19c	4.89±0.56c	6.77±0.51d	3.43±0.14b
Glycine (mg/l)	2.89±0.00a	5.16±0.59b	4.67±0.51b	5.26±0.61b	5.78±0.72b	4.95±0.63b
Tryptophan (mg/l)	1.78±0.00a	3.72±0.23b	3.56±0.21b	3.92±0.38b	3.82±0.36b	5.16±0.56c
Isoleucine (mg/l)	1.56±0.00a	3.15±0.24c	3.64±0.32c	3.21±0.23c	4.87±0.46d	2.43±0.18b
Lysine (mg/l)	14.12±0.00a	18.12±1.78b	25.36±2.56c	19.26±1.96b	23.21±2.16c	18.14±1.12b
Leucine (mg/l)	2.76±0.00a	5.62±1.08c	3.98±0.42b	5.98±0.92c	6.12±1.26c	3.72±0.35b
Ornithine (mg/l)	2.46±0.00a	5.14±0.42c	4.82±0.38c	5.55±0.48c	5.26±0.32c	3.22±0.21b
Serine (mg/l)	2.15±0.00a	3.98±0.31b	4.89±0.64bc	4.11±0.36b	5.86±0.58c	4.02±0.35b
Tyrosine (mg/l)	2.74±0.00a	5.13±0.42b	5.55±0.51b	5.38±0.43b	6.82±0.62c	5.16±0.53b
Threonine (mg/l)	14.24±0.00a	19.44±1.18c	18.92±1.11c	19.86±1.22c	16.11±1.02b	16.03±0.98b

Results represent the mean±SD for three replicates. Means in the same row with the same letter are not significantly different ($p<0.05$).

Table 4. Final biogenic amines analysis after ageing over-lees with *S. cerevisiae* CT007 (CT007), *L. thermotolerans* (Lt), *S. Cerevisiae* 3D (3D), *M. pulcherrima* (Mp) and *T. delbrueckii* (Td).

Compounds	Control	CT007	Lt	3D	Mp	Td
Histamine (mg/l)	0.98 ± 0.00b	0.86 ± 0.03 a	0.89± 0.05 a	0.83 ± 0.03a	0.81± 0.03a	0.88 ± 0.04a
Tyramine (mg/l)	1.34 ± 0.00 c	1.20 ± 0.04 ab	1.27 ± 0.03 b	1.16 ± 0.05 a	1.14 ± 0.04 a	1.26 ± 0.03 b
Phenylethylamine (mg/l)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Putrescine (mg/l)	3.12± 0.00 c	2.75± 0.06 ab	2.81 ± 0.05 ab	2.71 ± 0.04 a	2.66 ± 0.05 a	2.82 ± 0.06b
Cadaverine (mg/l)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Results represent the mean±SD for three replicates. Means in the same row with the same letter are not significantly different ($p<0.05$), n.d: not detected.

Table 5. Final analysis of volatile compounds (mg/L) after ageing over-lees with *S. cerevisiae* CT007 (CT007), *L. thermotolerans* (Lt), *S. Cerevisiae* 3D (3D), *M. pulcherrima* (Mp) and *T. delbrueckii* (Td).

Compounds	**Control**	**CT007**	**Lt**	**3D**	**Mp**	**Td**
Acetaldehyde	14.22±1.67a	15.42±2.02a	13.86±1.48a	12.98±1.78a	15.38±2.13a	14.37±1.55a
Ethyl lactate	42.26±3.48a	45.52±3.52a	41.34±2.77a	40.11±3.02a	47.22±3.75a	42.98±3.16a
Ethyl acetate	29.45±1.85a	30.06±1.54a	28.76±1.91a	28.14±1.74a	31.26±2.12a	29.89±1.98a
Diacetyl	3.78±0.44a	3.69±0.48a	3.82±0.56a	3.22±0.38a	3.64±0.68a	3.66±0.52a
Isoamyl acetate	2.37±0.32a	2.34±0.38a	2.46±0.35a	2.46±0.48a	2.23±0.39a	2.41±0.51a
1-Propanol	37.82±3.52a	35.46±3.61ab	37.06±3.33a	30.82±2.25b	38.13±3.68a	34.35±3.68ab
Isobutanol	41.32±3.82a	37.62±3.33a	41.24 ±3.16a	35.62±3.76a	42.87±3.58a	38.14±2.88a
3-Methyl-butanol	34.33±2.13a	33.78±1.98a	34.46±2.44a	33.12±1.18a	34.69±2.06a	33.03±1.24a
2-Methyl-butanol	86.38±4.36a	82.24±4.55ab	85.79±3.98a	75.16±3.88b	87.54±4.58a	83.34±4.26ab
Isobutyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl butyrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Phenyl-ethanol	24.58±2.96a	22.93±3.08a	25.33±3.12a	25.78±3.46a	21.72±3.21a	26.22±3.88a
2- Phenyl ethyl acetate	3.26±0.39a	3.22±0.44a	3.02±0.41a	3.48±0.52a	3.06±0.47a	3.56±0.48a
Hexanol	3.48±0.26a	3.28±0.32a	3.52±0.34a	3.16±0.29a	3.58±0.42a	3.21±0.37a

Results represent the mean±SD for three replicates. Means in the same row with the same letter are not significantly different ($p<0.05$), n.d: not detected.

7. DISCUSIÓN

7. DISCUSIÓN GENERAL

El proceso de elaboración de vino, a pesar su origen antrópico, constituye uno de los principales hábitats y reservorios naturales de las levaduras, tanto en el viñedo como en el proceso de fermentación (Pretorius, 2000). La diversidad de especies de levaduras asociadas al proceso de vinificación lo convierte en un interesante foco de estudios de ecología microbiana, así como de la influencia que sobre la misma tienen tanto factores climáticos y oro-geográficos, como las distintas prácticas vitivinícolas (Lachance y Stramer, 1998; Liu *et al*., 2015). Esta diversidad microbiana, unida a la complejidad nutricional de un sustrato como el mosto de uva, cuya metabolización determinará en parte la composición final del vino, hacen de la enología una interesante ciencia para el estudio y aplicación de la diversidad metabólica asociada a especies y cepas de levaduras (Belda *et al*., 2016a).

7.1. Diversidad microbiana y metabólica asociada al proceso de fermentación: estudio filo-funcional de levaduras de interés enológico

El microbioma asociado a un vino o viñedo está en la base del concepto de *terroir* microbiano, que pretende dar explicación a la incidencia de los microorganismos en el perfil sensorial de los vinos de una determinada región vitivinícola (Bokulich *et al*., 2014; Gilbert *et al*., 2014). Los estudios poblacionales llevados a cabo para el establecimiento de estos conceptos han arrojado resultados notables respecto a la complejidad microbiana del proceso de fermentación vínica (Liu *et al*., 2015). Un total de 93 especies de levadura pertenecientes a 30 géneros distintos han sido identificadas en ambientes enológicos (Barata *et al*., 2008; 2012; Bisson y Joseph, 2009), de las cuales aproximadamente 25 han sido directamente relacionadas con el proceso de vinificación (Jolly *et al*., 2014) aunque su papel en el proceso y, por tanto, su potencial aplicación han sido escasamente estudiados.

En este contexto, el objetivo inicial de este trabajo, recogido en su primer capítulo, consistió en el estudio de la diversidad de especies de levaduras asociadas a distintas zonas vitivinícolas de España (D.O. Ribera del Duero, D.O. Rueda y D.O. Tierra de León) y la posterior caracterización de sus propiedades metabólicas en lo referente a la producción de enzimas de interés enológico (β-glucosidasa, β-D-xilosidasa, α-L-arabinofuranosidasa, β-liasa, proteasa, pectinasa, celulasa y sulfito reductasa). El establecimiento de una colección amplia de 770 levaduras pertenecientes a 15 especies distintas permitió el estudio de la variabilidad metabólica inter- e intraespecífica para las ocho actividades enzimáticas citadas previamente (Belda *et al*., 2016a).

El diseño del muestreo y la metodología de aislamiento de levaduras fueron adaptados a los objetivos generales del trabajo consistentes en la obtención de una diversidad razonable de especies de levaduras no-*Saccharomyces* de interés enológico para su caracterización enzimática y su posterior aplicación en fermentaciones combinadas con *Saccharomyces cerevisiae.* Por ello, se procuró evitar el aislamiento tanto de esta última como de otras especies de levaduras basidiomicetes y del hongo levaduriforme *Aureobasidium pullulans*, de metabolismo eminentemente oxidativo y con una abundancia relativa elevada en la comunidad microbiana presente en las uvas. Considerados estos elementos que pudieran condicionar los datos de diversidad microbiana de la colección establecida, se pudo comprobar la existencia de unos datos poblacionales globales (considerando los 4 orígenes muestreados) similares a lo descrito en otros trabajos, destacando la dominancia de especies de los géneros *Hanseniaspora*, *Metschnikowia* y *Lachancea* (Cocolin *et al*., 2000; Pinto *et al*., 2015). Éstas suponen más del 85% de la población de levaduras aisladas en este trabajo, pertenecientes a 12 géneros distintos, siendo la especie *H. uvarum* responsable de más del 50% de dicha población, de acuerdo a lo obtenido en trabajos de enfoque similar (Beltrán *et al*., 2002; Wang *et al*., 2015).

A excepción del comportamiento de las especies dominantes, *H. uvarum*, *Metschnikowia* sp. (agrupando *M. pulcherrima* y *M. fructicola*) y *Lachancea thermotolerans*, la población de levaduras aisladas en las bodegas muestreadas en la D.O. Ribera del Duero (EM y PDC) presentó una composición y evolución distinta en los años 2013 y 2014, manteniéndose relativamente estable en la primera de ellas (EM) y variando significativamente la otra (PDC), en la que pudo aislarse una diversidad mayor en la vendimia 2014. La gran cercanía geográfica de ambas bodegas (zonas de viñedo muestreadas) y, por tanto, la similitud de condiciones climáticas y orográficas hace pensar que las prácticas agrícolas o la casuística microclimática pudieron determinar este comportamiento diferencial.

En los muestreos realizados en la D.O. Rueda, se observó el fenómeno contario y la diversidad de levaduras aislada en la vendimia de 2013 fue considerablemente mayor que la observada en 2014.

En el caso de la bodega muestreada en la D.O. Tierra de León, se registra gran diversidad microbiana obtenida en un solo año de muestreo y con un número moderado de aislamientos (73 aislamientos), pudiendo identificarse un total de 10 especies distintas. En este caso, y aunque nuevamente no existen evidencias concluyentes de su relación con la diversidad poblacional, destacan las características microclimáticas del año 2012 (un año extraordinariamente seco) en el que fue muestreada la bodega de la D.O. Tierra de León con respecto a los años 2013 y 2014 en los que se muestrearon las otras bodegas. Una menor humedad durante el desarrollo de la uva y particularmente durante su vendimia conlleva un menor desarrollo de poblaciones invasivas de hongos filamentosos lo que facilita el desarrollo y asilamiento de las de una mayor diversidad de especies de levaduras.

La caracterización de las propiedades enzimáticas del conjunto de la colección establecida permitió observar, ahora sí, patrones fenotípicos intraespecíficos característicos de las distintas regiones y bodegas muestreadas. Considerando las 8 actividades enzimáticas evaluadas, el uso adecuado de herramientas estadísticas de *clustering* nos permitió la diferenciación a nivel funcional, de subgrupos de levaduras de la misma especie aislados en distintos orígenes. Esta diferenciación fenotípica de las cepas de aisladas en distintos orígenes sí está en consonancia con el concepto de *terroir* microbiano comentado anteriormente. La relación potencial de estos patrones fenotípicos con las propiedades sensoriales de los vinos llevó al desarrollo del concepto *wine flavorome*, como una evidencia más de la contribución de la microbiota característica de cada zona vitivinícola a las propiedades sensoriales diferenciadoras de los vinos de una región.

En cuanto a la distribución de las actividades enzimáticas a nivel interespecífico, se pudieron establecer 3 grupos de actividades en función de su abundancia global, dependiendo esta tanto del número de especies que muestran dicha actividad como de su abundancia relativa en la población total. Así, destacan la actividad β-glucosidasa y proteasa como actividades altamente distribuidas y contrariamente se sitúan las actividades α-L-arabinofuranosidasa, pectinasa y celulasa restringidas a un bajo número de especies y, en ocasiones, con baja representación en la comunidad de levaduras asociadas a mostos de uva.

La importancia de las enzimas glicosidasas como responsables de la liberación de terpenos en vinos ha sido ampliamente estudiada, siendo estos los descriptores aromáticos principales de ciertas variedades de uva como la moscatel, albariño o riesling (Marais, 1983). Se ha descrito la producción de ciertas enzimas glicosidasas por parte de cepas de *S. cerevisiae*, sin embargo la mayoría de ellas no presentan o presentan una actividad β-D-glucosidasa muy limitada (Úbeda-Iranzo *et al.*, 1998; Van Rensburg *et al.*, 2005), necesaria para la etapa final en la liberación de aromas terpénicos a partir de sus conjugados glicosilados. Nuestros resultados mostraron que la mayoría de especies de levadura no-*Saccharomyces* poseen en mayor medida esta actividad, de acuerdo con lo descrito en otros trabajos (Fia *et al.*, 2005), además de, en muchos casos, actividad β-D-xilosidasa e incluso α-L-arabinofuranosidasa, estando esta última restringida a unas pocas especies. En este contexto, la comunidad de levaduras no-*Saccharomyces* parecen ser un foco interesante de estudio para su utilización como herramientas para incrementar la revelación del perfil varietal de variedades de uva terpénicas o el revelado de aromas en variedades neutras.

En el presente estudio, destacó la producción de actividad β-D-glucosidasa de las especies evaluadas del género *Hanseniaspora*, así como de *Meyerozyma guilliermondii* y *Wickerhamomyces anomalus*. El uso de cepas de *H. uvarum* y *W. anomalus* para la liberación de compuestos terpénicos en vinos ha sido previamente demostrada (Mendes-Ferreira *et al.*, 2001; Fernández-González *et al.*, 2003; Mateo *et al.*, 2011) destacándose su actividad en un amplio rango de pH y con bajos índices de represión por glucosa (Mateo *et al.*, 2011; López *et al.*, 2015). En este mismo sentido, la actividad β-D-xilosidasa mostrada por el grupo de especies relacionadas *S. cerevisiae, Torulaspora delbrueckii y Zygosaccharomyces bailii,* presenta, en la mayoría de cepas, un alto grado de represión por glucosa, limitando, de nuevo, su uso para la liberación de terpenos en fermentaciones vínicas (Delcroix *et al.*, 1994; Rosi *et al.*, 1994; Gueguen *et al.*, 1995; Mateo y Di Stefano, 1997; Hernández *et al.*, 2002; Mateo *et al.*, 2011).

Tanto la literatura científica como la actividad industrial indican también la capacidad de ciertas cepas de *M. pulcherrima* para la liberación de terpenos glicosilados mediante la producción de actividad α-L-arabinofuranosidasa (Fernández-González *et al.*, 2003). Nuestros resultados muestran que, si bien es cierto que la actividad β-D-glucosidasa se encuentra muy ampliamente distribuida en el conjunto de cepas analizadas de *Metschnikowia* sp., la producción de α-L-arabinofuranosidasa está restringida a unas pocas cepas.

En resumen, en lo referente a las actividades glicosidasas estudiadas y, de acuerdo con lo previamente descrito por Manzanares *et al.* (1999), *Pichia*, *Wickerhamomyces,* y *Hanseniaspora* constituyen géneros con altos grados de actividad. Adicionalmente, nuestro trabajo destaca la producción de enzimas glicosidasas por parte de ciertas especies de basidiomicetes ampliamente distribuidas en ambientes enológicos como son *Rhodosporidium toruloides* y *Cryptococcus amylolentus*.

La actividad β-liasa, relacionada con la liberación de compuestos tiólicos mostró niveles de abundancia moderados en la colección de levaduras estudiada. Aunque no puede considerarse una actividad exclusiva de pocas especies, la mayoría de ellas muestran niveles de actividad muy moderados, destacando la actividad de *T. delbrueckii*, *K. marxianus* y *M. guilliermondii*. Aunque esta actividad ha sido ampliamente estudiada en *S. cerevisiae,* siendo muy variable entre cepas (Howell *et al*., 2005; Thibon *et al*., 2008; Roncoroni *et al*., 2011; Santiago y Gardner, 2015; Belda *et al*., 2016c;), su presencia en levaduras no-*Saccharomyces* ha sido escasamente evaluada (Zott *et al*., 2011; Belda *et al*., 2016c). De forma análoga a lo estudiado en el caso de las actividades glicosidasas y su represión por glucosa, y puesto que la actividad β-liasa de *S. cerevisiae* presenta niveles elevados de represión catabólica por nitrógeno, la caracterización de dicha actividad en levaduras no-*Saccharomyces* en diferentes condiciones enológicas debe ser realizada para su aplicación como herramientas útiles en el incremento del perfil tiólico de vinos blancos.

Las actividades proteasa, pectinasa y celulasa fueron evaluadas por su implicación en la mejora de ciertas características tecnológicas de los vinos, fundamentalmente relacionadas con el proceso de clarificación, extracción de compuestos fenólicos y prevención de la quiebra proteica (Marangon *et al*., 2012; Belda *et al*., 2016b). La actividad proteasa mostró gran abundancia en la colección de levaduras evaluada, sin embargo, un gran número especies de notable interés enológico mostraron valores bajos o nulos de dicha actividad, como *S. cerevisiae, T. delbrueckii, Z. bailii* o *L. thermotolerans*, entre otros. Por el contrario, el conjunto de cepas evaluadas del género *Metschnikowia*, a excepción de las pertenecientes a la especie *M. viticola*, mostraron niveles elevados de esta actividad, por lo que al margen reconocida contribución a la mejora del perfil sensorial de los vinos, su uso como herramienta de prevención de la quiebra proteica de vinos debe ser estudiada en futuros ensayos. En la misma línea, y mostrando valores muy elevados de esta actividad, cabe destacar el comportamiento de ciertas cepas de la especie *W. anomalus*, cuya contribución a la mejora del perfil sensorial de los vinos también ha sido descrita con anterioridad (Domizio *et al.*, 2011; Izquierdo-Cañas *et al.*, 2011).

En el extremo opuesto en cuanto a su abundancia se encuentran las actividades pectinasa y celulasa en la colección de levaduras evaluada. La primera de ellas, si bien pudo detectarse en aproximadamente el 50% de las cepas evaluadas de *S. cerevisiae,* su funcionalidad en condiciones enológicas se encuentra de nuevo condicionada por la presencia de glucosa (Radoi *et al.*, 2005), contrariamente a lo observado en ciertas especies no-*Saccharomyces* (Merín *et al.*, 2011; Merín y Morata de Ambrosini, 2015). Nuestros resultados muestran que, al margen de unas pocas cepas de *T. delbrueckii*, la presencia de actividad pectinolítica parece estar reservada a las especies del género *Metschnikowia* y a *A. pullulans* (única especie productora de actividad celulasa)*,* habiéndose demostrado en este trabajo su incidencia en el proceso de clarificación y extracción fenólica en vinos tintos (Belda *et al.*, 2016b).

Finalmente destaca la ausencia de actividad sulfito reductasa en la práctica totalidad de especies no-*Saccharomyces* analizadas, a excepción de los elevados niveles de producción mostrados por las especies del género *Hanseniaspora*.

7.2. Aplicación de levaduras pectinolíticas en maceración de vinos tintos

El uso de enzimas pectinolíticas en las fases de maceración, previas al comienzo de la fermentación tumultuosa propiamente dicha, es una práctica habitual en enología para la elaboración de vinos tintos. Su aplicación persigue el incremento de color, a través de la extracción de polifenoles y antocianos, lográndose además, una mejor clarificación del vino resultante (Van Rensburg y Pretorius, 2000; Merín y Morata de Ambrosini, 2015). La adición de pectinasas se realiza en forma de preparados enzimáticos de origen fúngico Estos preparados comerciales suelen consistir en una mezcla de enzimas con distintas actividades (poligalacturonasa, pectín-liasa y pectín-metilesterasa), entre las que las poligalacturonasas son las principales responsables de la actividad pectinolítica en vinos (Lang y Dornenburg, 2000). En este contexto, existe un interés científico e industrial en la búsqueda de levaduras como fuente de enzimas pectinolíticas y su uso como herramientas biológicas en la mejora del proceso de maceración. Por ello, en el presente trabajo se abordó la búsqueda y selección de levaduras con actividad poligalacturonasa para su posterior aplicación como inóculos durante la maceración prefermentativa para la mejora de los parámetros antes comentados. Además, dado el interés en el desarrollo de los procesos de maceración prefermentativa en condiciones de baja temperatura controlada para la mejora de la extracción y estabilización del color (Merín y Morata de Ambrosini, 2015), se valoró la actividad de dichas actividades enzimáticas aplicando procesos de maceración prefermentativa fría (12ºC) y convencional (25ºC).

Los resultados mostrados en el primer capítulo de este trabajo confirman que la presencia de actividad poligalacturonasa en levaduras de interés enológico está reservado a unas pocas especies, fundamentalmente *M. pulcherrima* y *A. pullulans* (Belda *et al.*, 2016a). Los resultados de los trabajos mostrados en el segundo capítulo de este trabajo (Belda *et al.*, 2016b) demuestran la funcionalidad de ambas especies para la mejora de los parámetros de calidad derivados de su actividad poligalacturonasa, fundamentalmente en condiciones de maceración prefermentativa en frio (MPF). Esto parece indicar que el control de temperaturas bajas en las etapas previas a la fermentación no sólo contribuye a la extracción de polifenoles y antocianos por motivos químicos de solubilidad de la matriz acuosa como se venía creyendo hasta el momento (Delteil, 2004, Hernández-Jiménez *et al.*, 2012), sino que quizá un mayor desarrollo de especies no-*Saccharomyces*, favorecido por las bajas temperaturas y el consecuente retraso en el inicio de la fermentación alcohólica propiamente dicha (Mendoza *et al.*, 2009; Andorrá *et al.*, 2010), contribuya también a este hecho mediante la actuación de las actividades pectinolíticas provenientes de éstas, tanto en fermentaciones espontáneas como mediante la inoculación de cepas no-*Saccharomyces* seleccionadas.

Los resultados mostrados en el mencionado trabajo (Belda *et al.*, 2016b), prueban que la incidencia de las levaduras pectinolíticas sobre los parámetros estudiados dependientes de las mismas es más acentuada cuando se aplican en MPF., destacando los resultados mostrados por *M. pulcherrima*. Ésta, en ensayos de fermentación secuencial junto con *S. cerevisiae* a escala de laboratorio, logró incrementar el contenido en polifenoles y la extracción de antocianos, así como los valores finales de intensidad de color en un 10%, 21% y 15%, respectivamente, en comparación con el control exclusivamente inoculado con *S. cerevisiae*. Asimismo, mostró una influencia positiva sobre la turbidez y el tiempo de filtración de los vinos con reducciones en dichos valores del 57% y el 34%, respectivamente, aunque en este caso, los valores similares mostrados por *L. thermotolerans* (levadura control sin actividad poligalacturonasa), parecen indicar que existen otros factores, al margen de la actividad pectinolítica de las levaduras, que contribuyen a este hecho.

Finalmente, se demostró la potencial aplicación de la cepa *M. pulcherrima* NS-EM-34 en la mejora de los parámetros analizados a escala semi-industrial obteniéndose incrementos de la intensidad de color de los vinos superiores al 40% y del contenido en polifenoles cercano al 20%, así como una reducción de la turbidez de los vinos del 42%. Estos resultados mejoran significativamente lo mostrado en otros trabajos aplicando técnicas de MPF (Panprivech *et al*., 2015) e incluso en el uso de *S. cerevisiae* cepas modificadas genéticamente para la sobreexpresión del gen *PGU1* codificante para una enzima poligalacturonasa en dicha especie (Fernández-González *et al*., 2005; Radoi *et al*., 2005).

Si bien la incidencia de *M. pulcherrima* sobre la composición aromática y el contenido en etanol de los vinos ha sido descrita con anterioridad (Parapouli *et al*., 2010; Rodríguez *et al*., 2010; Sadoudi *et al*., 2012; Quirós *et al*., 2014; Contreras *et al*., 2015), este trabajo describe por primera vez la incidencia de *M. pulcherrima* NS-EM-34 durante el proceso de maceración prefermentativa en la mejora de los procesos de clarificación y extracción de color. Así, fue posible confirmar mediante análisis estadístico de componentes principales (PCA) que, en el estudio de un elevado número de parámetros de composición química del vino, aquellos relacionados con dichos procesos son responsables de la diferenciación analítica de los vinos fermentados exclusivamente con *S. cerevisiae* o en fermentación secuencial con *M. pulcherrima* (Belda *et al*., 2016b).

7.3. Selección y aplicación de levaduras en fermentación para la mejora de las propiedades sensoriales de los vinos

Como se muestra en los capítulos segundo y cuarto de este trabajo, las levaduras no sólo tienen incidencia en la composición del vino durante de fermentación alcohólica. Durante años, se han sucedido los trabajos de selección de levaduras *S. cerevisiae* para dotar a la industria de inóculos con los que desarrollar las fermentaciones en bodega con garantías de calidad y seguridad fermentativa (Pretorius, 2000). Estos inóculos eran seleccionados con dos objetivos generales; finalizar eficientemente el proceso de fermentación del mosto y producir vinos de alta calidad. A este respecto, la literatura clásica estableció dos grandes grupos de propiedades a evaluar en los procesos de selección de cepas de *S. cerevisiae* como inóculo (Zambonelli, 1998): propiedades tecnológicas (tolerancia al etanol, poder fermentativo, resistencia al SO_2, capacidad de crecimiento en medio líquido, crecimiento en amplio rango de temperaturas, presencia de factor *killer*, etc.) y propiedades sensoriales (generación de subproductos de la fermentación: ácido acético, glicerol, acetaldehído y alcoholes superiores; producción de compuestos azufrados: H_2S y SO_2; y producción de enzimas hidrolíticas: β-glucosidasa, esterasa, enzimas proteolíticas). Dado el elevado número de requisitos, la presencia de cepas salvajes con una combinación óptima de propiedades tecnológicas y sensoriales es muy baja (Rainieri y pretorius, 2000), por ello, la optimización en los procesos de selección, mediante el desarrollo de métodos de *screening* metabólico de alto rendimiento, parece ser la forma ideal de afrontar este reto (Figura 7).

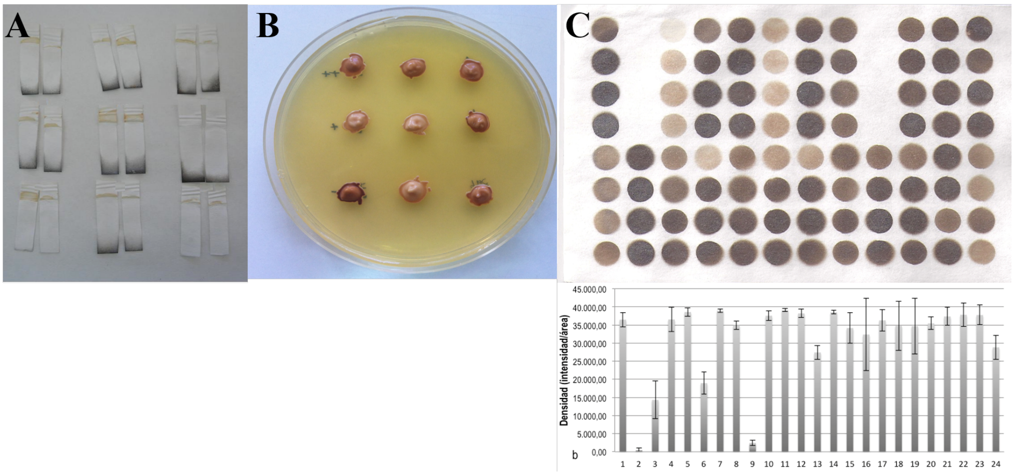

Figura 7. Adaptación de métodos de detección de liberación de H_2S a formato de alto rendimiento, con elevada reproducibilidad y posibilidad de cuantificación por densitometrado. Adaptado de Belda *et al*. (2013). A) método tradicional de detección por acetato de plomo; B) Detección en medio Biggy; C) Adaptación del método de detección por acetato de plomo a formato de alto rendimiento que permite su cuantificación por densitometrado.

En este contexto, la primera parte del tercer capítulo de este trabajo, muestra el desarrollo de un método de selección de levaduras con elevada actividad β-liasa, responsable de la liberación de aromas tiólicos en vinos (Patente presentada a la Oficina Española de Patentes y Marcas bajo el número de registro P-201500195). Esta enzima es responsable de la liberación de los compuestos volátiles responsables del aroma tiólico de los vinos blancos, 3-mercaptohexanol (3-MH) y 4-mercapto-4-metilpentan-2-ona (4-MMP), mediante la ruptura de sus precursores no volátiles (cisteinilados) (Swiegers *et al.*, 2009; Holt *et al.*, 2012). Si bien la liberación del primer compuesto no es responsabilidad exclusiva de un único gen, aunque el gen *STR3* de *S. cerevisiae* ha sido descrito como responsable mayoritario de tal hecho (Holt *et al.*, 2011, 2012), la liberación de 4-MMP desde su precursor cisteinilado ha podido ser atribuida en su práctica totalidad a la acción del gen *IRC7* en *S. cerevisiae,* cuya inactivación ocasiona también una reducción considerable en la liberación de 3-MH (Roncoroni *et al.*, 2011). De las dos isoformas de *IRC7* que han sido descritas, una de ellas conteniendo una deleción que determina una disminución de actividad en la enzima, la isoforma íntegra y, por tanto, más eficaz de la enzima, se encuentra muy poco presente entre las cepas salvajes de *S. cerevisiae,* en niveles inferiores al 3% (Belda *et al.*, 2016c). Por primera vez, en este trabajo se describe la existencia de cepas con un genotipo heterocigoto para el gen *IRC7*, cuyo fenotipo, aunque de menor actividad β-liasa, tiende a asemejarse al del genotipo homocigoto para el gen íntegro, y su abundancia relativa en la población de cepas salvajes evaluada fue del 9,4%. El análisis del genotipo *IRC7* en una colección de 22 cepas de levadura industriales (Agrovin S.A.) dio como resultado una distribución de 23% de cepas homocigotas para el gen íntegro, 23% para el genotipo heterocigoto y 54% de cepas homocigotas para el gen truncado. Este incremento en la proporción de cepas de genotipo *IRC7* íntegro, con respecto a las cepas salvajes evaluadas, es fácilmente explicable por el filtro de calidad sensorial al que se someten las cepas de levadura para su selección previa a su comercialización en las que los parámetros de calidad aromática juegan un papel decisivo (Masneuf-Pomarède *et al.*, 2002, 2006; Lee *et al.*, 2008).

El umbral de percepción, extremadamente bajo, de estos compuestos tiólicos (3 ng/L para 4-MMP y 60 ng/L para 3-MH) hace que, pequeños incrementos en la liberación de estos compuestos puedan modular significativamente el perfil de aromas tiólicos de un vino, y por tanto la selección de levaduras con alta actividad β-liasa constituye un reto de interés (Murat *et al.*, 2001). El medio de cultivo descrito en el primer apartado del capítulo tercero de este trabajo presenta en su composición un sustrato químico comercial, la S-metil-cisteína (M-6626, Sigma-Aldrich), de estructura y enlace C-S análogos a los de los precursores cisteinilados naturales presentes en la uva para su uso como agente selectivo de levaduras en función de su actividad β-liasa. Su presencia en el medio como única fuente de nitrógeno permite seleccionar aquellas cepas capaces de usar el amonio derivado de su hidrólisis como única fuente de nitrógeno. De esta forma, se pueden realizar procesos de aislamiento y selección de levaduras con elevada actividad β-liasa en función de su capacidad de crecimiento en el medio sólido descrito, incrementando las probabilidades de éxito en un contexto de baja abundancia en la naturaleza. Además de la utilidad para la diferenciación intraespecífica de *S. cerevisiae* en función de la integridad y funcionalidad de su gen *IRC7*, este medio de cultivo permite la selección de levaduras no-*Saccharomyces* con elevada actividad β-liasa, siempre con la precaución necesaria a la hora de establecer comparaciones a nivel interespecífico dadas las diferencias basales de *fitness* entre especies. A este respecto, los resultados mostrados en el trabajo de Belda *et al.* (2016c), destacan la elevada actividad β-liasa mostrada por ciertas cepas de *T. delbrueckii* y *K. marxianus*, muy destacadas entre un amplio rango de especies no-*Saccharomyces* de origen enológico evaluadas.

La incidencia de *T. delbrueckii* para el incremento del perfil tiólico de vinos blancos ha sido recientemente descrita por Renault *et al.* (2016) quienes muestran un efecto sinérgico en la liberación de 3-MH mediante la coinoculación de *T. delbrueckii* y *S. cerevisiae* en mostos de la variedad Sauvignon blanc. Si bien sus resultados descartan la liberación de 4-MMP por parte de *T. delbrueckii*, y han de ser interpretados con cautela por no detallarse el genotipo del gen *IRC7* de la cepa de *S. cerevisiae* utilizada (Zymaflore X5), este comportamiento parece ser dependiente de cepa, ya que resultados recientes derivados de esta Tesis Doctoral confirman la utilidad de la cepa *T. delbrueckii* Viniferm NS-TD para el incremento del perfil tiólico de vinos blancos (variedad verdejo) mediante la liberación de 3-MH y, especialmente, de 4-MMP (Figura 8).

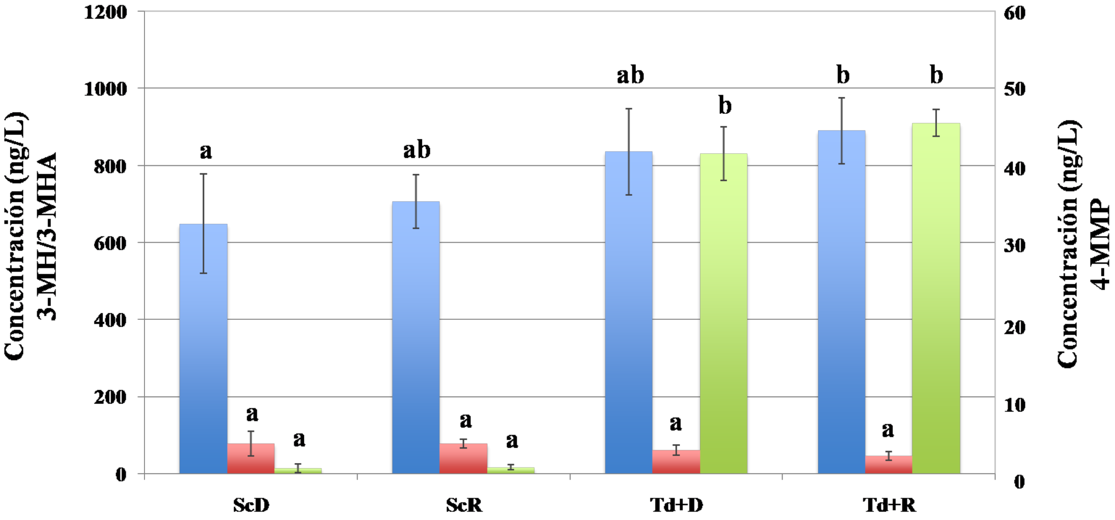

Figura 8. Liberación de tioles varietales (ng/L) en fermentación de mosto de la variedad verdejo con cepas *S. cerevisiae* con genotipo *IRC7* funcional (*S. cerevisiae* Viniferm Revelacion (ScR)) y no funcional (*S. cerevisiae* Viniferm Diana (ScD)) y su uso en inoculación secuencial con *T. delbrueckii* Viniferm NS-TD (Td+R y Td+D, respectivamente). 3-MH (azul): 3-mercaptohexanol; 3-MHA (rojo): acetato de 3-mercaptohexilo; 4-MMP (verde): 4-mercapto-4-metilpentan-2-ona. Datos procedentes de análisis ejecutados por la compañía Nyseos (Montpellier, Francia). Letras diferentes sobre los valores del mismo parámetro indican diferencias significativas entre ensayos en el correpondiente análisis ANOVA ($p<0,05$).

Probada la incidencia de *T. delbrueckii* Viniferm NS-TD en el perfil sensorial de la variedad Verdejo, como una de las variedades blancas de mayor incidencia en la viticultura española, se procedió a la caracterización de dicho impacto en la composición y propiedades sensoriales en fermentaciones de vinos tintos de la variedad Tempranillo (Belda *et al.*, 2015b).

En este caso, se realizaron fermentaciones a escala de laboratorio (5 L) y a escala semi-industrial (100 L) para el estudio de la evolución de parámetros analíticos básicos del vino así como para la caracterización del perfil final de compuestos aromáticos volátiles. Así mismo se llevó a cabo un seguimiento de la cinética de fermentación y la dinámica poblacional en los ensayos, tanto exclusivamente inoculados con *S. cerevisiae* o *T. delbrueckii* por separado, o en su inoculación conjunta de forma simultánea o secuencial. La inoculación simultánea no mostró diferencias notables ni en cinética fermentativa ni en composición analítica de los vinos comparada con la fermentación control inoculada exclusivamente con *S. cerevisiae*, lo que puede explicarse por la rápida imposición de la población de ésta última especie de acuerdo con lo observado en estudios similares con esta y otras especies no-*Saccharomyces* (Azzolini *et al.*, 2012; Oro *et al.*, 2014). *T. delbrueckii*, a pesar de ser una de las levaduras no-

Saccharomyces con mayor poder fermentativo (Jolly *et al*., 2014), éste sigue siendo inferior al mostrado por *S. cerevisiae* (Bisson y Kunkee 1991; Jolly *et al*., 2006), lo que junto a una mayor demanda nutricional atribuida a las especies no-*Saccharomyces* (en términos de consumo de recursos nitrogenados y vitaminas) y que puede dificultar la posterior actividad por parte de *S. cerevisiae* en su inoculación secuencial (Romano *et al*., 2003) explicaría la cinética fermentativa mas lenta mostrada en dicho ensayo. En cuanto a los parámetros analíticos evaluados, destacó una ligera reducción en la acidez volátil de los vinos fermentados con *T. delbrueckii* en inoculación secuencial, de acuerdo a lo previamente descrito en la bibliografía (Moreno *et al*., 1991; Bely *et al*., 2008; Renault *et al*., 2009; Azzolini *et al*., 2012). En cuanto al metabolismo de ácidos orgánicos, pudo observarse de nuevo una ligera reducción en el contenido en ácido málico en los vinos fermentados en presencia de *T. delbrueckii* Viniferm NS-TD, aunque esta parece ser una característica dependiente de cepa, discrepando con los resultados de ligero incremento mostrados por la cepa de *T. delbrueckii* evaluada por Sun *et al*. (2014). Más significativo es el incremento en el contenido en ácido pirúvico observado en los vinos fermentados en presencia de *T. delbrueckii* Viniferm NS-TD, observándose picos máximos de liberación muy superiores a los observados en las fermentaciones inoculadas exclusivamente con *S. cerevisiae*. En el metabolismo del ácido pirúvico no debe considerarse su valor final en el vino, si no el valor máximo de producción obtenido, normalmente durante la fermentación tumultuosa, ya que mas tarde este subproducto metabólico es consumido como fuente de carbono. Además, la cantidad de ácido pirúvico liberado en fermentación ha sido relacionada con la formación de pigmentos como la vitisina A que aportan estabilidad al color de los vinos (Morata *et al*., 2003, 2012). Ensayos previos de selección de cepas de *S. cerevisiae* en base a su liberación de ácido pirúvico, lograban el aislamiento de cepas productoras de valores máximos entre 60 y 132 mg/L que, en cualquier caso son inferiores a los valores máximos obtenidos en el uso secuencial de la cepa *T. delbrueckii* Viniferm NS-TD en nuestro ensayo que alcanza valores medios cercanos a 160 mg/L en las fermentaciones a escala de laboratorio. Así, en estos ensayos puede observarse una relación directa entre la cantidad de ácido pirúvico liberada y la intensidad de color final de los vinos siendo ésta significativamente superior en los vinos inoculados con *T. delbrueckii* exclusivamente y en fermentación secuencial con *S. cerevisiae*. En paralelo a este incremento en la liberación de ácido pirúvico, estas dos fermentaciones mostraron niveles superiores de glicerol lo que contribuye a confirmar la mayor actividad de la ruta gliceropirúvica en *T. delbrueckii* con respecto a la mayoría de cepas de *S. cerevisiae*

(Ciani y Maccarelli, 1998; Renault *et al*., 2009). Así mismo, esta mayor liberación de glicerol lleva asociada una ligera reducción en el contenido en etanol de los vinos así como de otros alcoholes superiores volátiles. Este hecho puede relacionarse parcialmente con la mejor valoración general atribuida por el panel de cata en la calidad general y aromática de los vinos fermentados mediante inoculación secuencial con *T. delbrueckii*, ya que ha sido probado el incremento en la calidad y complejidad aromática de los vinos con ligeras reducciones en su contenido en etanol y otros alcoholes por tender estos a la monopolización del aroma general de los vinos (Frost *et al*., 2015). No obstante, las pequeñas diferencias que se obtuvieron en estos parámetros analíticos no justificaban del todo la diferencia en las puntaciones que el panel de cata otorgó a los vinos en los que destacaban tanto a nivel general como, en especial, en el volumen en boca, los vinos fermentados con *T. delbrueckii* en inoculación secuencial con *S. cerevisiae*. Esto llevó a la valoración del contenido en manoproteínas de los vinos fermentados a escala semiindustrial, siendo éstos los que mostraron las diferencias mas patentes a nivel organoléptico. Como puede observarse en el trabajo de Belda *et al*. (2015b) contenido en la segunda parte del tercer capítulo de esta Tesis Doctoral, el contenido en manoproteínas fue el parámetro que mostró unas mayores diferencias entre los distintos ensayos, incrementándose su concentración en las fermentaciones que contaron con más tiempo de desarrollo y actuación de la cepa *T. delbrueckii* Viniferm NS-TD. Así, en paralelo a lo mostrado en el apartado anterior en referencia al impacto de esta cepa sobre el perfil aromático de vinos blancos mediante su potente actividad β-liasa, se pudo concluir que su mayor aportación a la calidad de los vinos tintos estaba determinada por su liberación de manoproteínas al vino, corroborando lo sugerido previamente por Domizio *et al*. (2014).

En base a estos resultados y dada la falta de información en referencia al uso de levaduras no-*Saccharomyces* en crianza sobre lías, el presente trabajo abordó el estudio de la influencia de algunas de las especies no-*Saccharomyces* de mayor implantación en la industria enológica actual (*T. delbrueckii*, *L. thermotolerans* y *M. pulcherrima*) sobre el perfil sensorial de vinos tintos durante la crianza sobre lías.

7.4. Aplicación de levaduras no convencionales en fases postfermentativas de crianza sobre lías

El último objetivo del presente trabajo consistió en la evaluación de la influencia del uso de lías de levaduras no-*Saccharomyces* y cepas industriales seleccionadas de *S. cerevisiae* (*S. cerevisiae* Viniferm CT007 como cepa control de uso habitual en bodega y *S. cerevisiae* Viniferm 3D, como cepa superproductora de manoproteínas, ambas de la colección de Agrovin S.A.) sobre la composición analítica y el perfil sensorial de vinos tintos. En el trabajo de Belda *et al.* (2016d) se evaluó la evolución de parámetros analíticos básicos, parámetros de color, perfil aromático, composición de aminoácidos y contenido en manoproteínas de vinos tintos tras un periodo corto de 4 meses en crianza sobre lías, siendo éste el mayor foco de interés en el estudio. En primer lugar, cabe destacar que no se observaron diferencias ni desviaciones en los parámetros enológicos básicos evaluados, lo que prueba el correcto desarrollo del proceso de crianza sin intervención alguna de bacterias o levaduras contaminantes ajenas al estudio. En referencia al contenido en manoproteínas de los vinos, y como era de esperar, se observaron diferencias muy significativas entre los ensayos en crianza sobre lías de las dos cepas de *S. cerevisiae* en estudio, siendo los valores mostrados por la cepa *S. cerevisiae* Viniferm 3D, 2,7 veces superiores que los mostrados por la cepa *S. cerevisiae* Viniferm CT007. En la misma línea y, en consonancia con lo obtenido en la comparación de las mismas cepas en su uso en fermentación alcohólica, los valores mostrados en el uso de *T. delbrueckii* Viniferm NS-TD fueron de aproximadamente el triple con respecto a la cepa *S. cerevisiae* Viniferm CT007. *M . pulcherrima* NS-EM-34 logró un incremento del doble en el contenido en manoproteínas con respecto a la cepa *S. cerevisiae* Viniferm CT007, lejos de los valores mostrados por *S. cerevisiae* Viniferm 3D y *T. delbrueckii* Viniferm NS-TD. Estos resultados están en consonancia con el estudio de liberación de manoproteínas llevado a cabo por Domizio *et al.* (2014) que mostraba que ambas especies presentaban ratios de liberación de manoproteínas por peso seco de pared celular muy superiores a la cepa de referencia *S. cerevisiae* EC1118. A pesar de los resultados mostrados para la cepa *M . pulcherrima* NS-EM-34 en crianza sobre lías, cabe destacar que, en estudios industriales llevados a cabo en nuestro grupo de investigación, en el contexto del trabajo de Belda *et al.* (2016b), esta cepa no mostró un incremento significativo del contenido en manoproteínas en su uso como inóculo mixto en fermentación. En este sentido, del Barrio-Galán *et al.* (2015), describen un comportamiento cepa-dependiente en los resultados de liberación de manoproteínas durante la fase de fermentación alcohólica y durante la crianza sobre lías. Sus resultados sugieren que cepas altamente liberadoras de manoproteínas durante la fermentación, no necesariamente lo son durante la crianza sobre lías y vice-versa. Esto es comprensible atendiendo a los mecanismos que determinan ambos procesos y que no tienen porque estar igualmente regulados en las distintas cepas o especies. En el caso de la liberación de manoproteínas en fermentación, ésta está relacionada con la propia división celular o con procesos de respuesta a estrés (Charpentier *et al.*, 1986; Fleet, 1991), mientras que durante el proceso de crianza sobre lías su liberación al medio está determinada por la degradación de biopolímeros por acción de endo-hidrolasas inducidas durante el proceso de lisis celular (Feuillat *et al.*, 1989; Fornairon-Bonnefond *et al.*, 2002). Así, mientras que en el caso de la cepa *T. delbrueckii* Viniferm NS-TD ambos procesos parecen determinar la liberación de manoproteínas al vino, en el caso de *M. pulcherrima* NS-EM-34, su contribución al contenido en manoproteínas de los vinos parece estar limitado a la fase postfermentativa de crianza sobre lías. En cuanto a la cepa *L. thermotolerans* NS-G-32, ésta mostro niveles ligeramente inferiores a los de la cepa *S. cerevisiae* Viniferm CT007, en relación con lo mostrado también por Domizio *et al.* (2014) durante la fermentación alcohólica. Si bien estos resultados, meramente observacionales, dan idea sobre la potencial aplicación de estas cepas en procesos industriales de crianza sobre lías, los mecanismos moleculares que determinan la liberación de estos compuestos en las distintas especies de interés deben ser evaluados en profundidad. El contenido en manoproteínas de los vinos parece ser el factor que determinó la preferencia en calidad de los vinos determinada por el panel de cata en su análisis sensorial, si bien, pudo comprobarse una clara preferencia en parámetros como estructura, volumen en boca o impresión general en aquellos ensayos con mayores concentraciones de manoproteínas y dado que no se observaron diferencias notables en el perfil analítico de composición aromática de los diferentes ensayos.

El incremento del contenido en manoproteínas de los vinos es uno de los principales objetivos en el desarrollo de procesos de crianza sobre lías, no obstante, este proceso presenta una influencia notable sobre la estabilidad e intensidad de color y la composición en polifenoles de los vinos (Palomero *et al.*, 2009; Loira *et al.*, 2013). Aunque se ha descrito una relación entre la presencia de manoproteínas y la estabilización del color en los vinos (Feuillat *et al.*, 2001; Fuster y Escot, 2002; Saucier *et al.*, 2002) por prevención de la precipitación de antocianos y taninos (Escot *et al.*, 2001; Francois *et al.*, 2007) y del proceso de oxidación de los polifenoles (Salmon, 2005) nuestros resultados no pueden ser del todo explicados por este hecho. Todos los ensayos de crianza sobre lías mostraron un descenso en los parámetros de color evaluados tras los 4 meses de crianza, aunque este descenso fue menos acusado en los ensayos que generaron un incremento en el contenido en manoproteínas (*M. pulcherrima* NS-EM-34 y *T. delbrueckii* Viniferm NS-TD). No obstante, los datos de intensidad de color obtenidos en el ensayo usando *L. thermotolerans* NS-G-32 (cepa poco productora de manoproteínas durante la crianza sobre lías) fueron similares a los obtenidos en el ensayo con *T. delbrueckii* Viniferm NS-TD, por lo que otros factores adicionales al contenido en manoproteínas, como por ejemplo la adsorción de pigmentos a la pared celular de dichas cepas, determinada por la porosidad, juegan un papel clave en el proceso (Gómez-Cordovés y Gonzalez-San José, 1995; Morata *et al*., 2003).

Finalmente, se evaluó el contenido en aminas biógenas y aminoácidos, como potenciales precursores de estas, en los vinos tras la crianza sobre lías. Las aminas biógenas y, en particular la histamina por su mayor prevalencia, son estudiadas con interés en el vino dada su implicación negativa en la salud humana (Moreno-Arribas y Polo, 2008).

El contenido en aminoácidos de una célula es propio de la cepa (Vaughan-Martini *et al*., 1979) y, por tanto, contribuirá de distinta forma al perfil de aminoácidos del vino tras la lisis de las lías durante la crianza. Así, el contenido en aminoácidos en los distintos ensayos evaluados en este trabajo mostró diferencias notables, destacando los elevados valores mostrados en el uso de las lías de *M. pulcherrima* NS-EM-34. Un mayor contenido en histidina, fenilalanina y tirosina podría implicar un mayor riesgo de aparición de las aminas biógenas de las que éstos son precursores por acción de la actividad descarboxilasa de ciertas bacterias (Lehtonen, 1996; Ribéreau-Gayon *et al*., 2006; Alcaide-Hidalgo *et al*., 2007; Smit *et al*., 2008). Sin embargo, el desarrollo de estos ensayos en condiciones higiénicas y de asepsia adecuados permitió obtener valores, en los distintos ensayos de crianza sobre lías, inferiores a los mostrados por el vino control de partida en consonancia con lo mostrado en otras especies no-*Saccharomyces* como *Hanseniaspora vineae* (Medina *et al*., 2013) y sin superar en ningún caso los límites de máximos establecidos en seguridad alimentaria (Martuscelli *et al*., 2013).

En resumen, los resultados obtenidos permiten demostrar la utilidad de la cepa *T. delbrueckii* Viniferm NS-TD para la mejora de la calidad de vinos tintos en procesos cortos de crianza sobre lías, obteniendo niveles de manoproteínas ligeramente superiores a los mostrados por la cepa comercial superproductora de manoproteínas *S. cerevisiae* Viniferm 3D, sin detrimento de la estabilidad del color de dichos vinos y sin poner en riesgo la seguridad alimentaria del vino al no contribuir a la producción de aminas biógenas o sus precursores aminoacídicos.

7.5. Perspectivas futuras

La información disponible sobre el metabolismo y la fisiología en fermentación de las levaduras no-*Saccharomyces* aumenta a un ritmo considerable, existiendo una intensa investigación al respecto. Sin embargo, este conocimiento dista todavía del disponible para *S. cerevisiae* y, quizá, del necesario para poder convertirse en una realidad industrial. En este contexto, los resultados de la presente Tesis Doctoral contribuyen al conocimiento general sobre la fisiología de estas levaduras, así como a la información básica acerca de su diversidad fenotípica en cuanto a la producción de enzimas de interés enológico. Estos resultados abren las puertas a una línea de investigación futura acerca de las bases genéticas y transcripcionales de la fisiología de estas levaduras, que permita una mejor comprensión y aprovechamiento industrial de sus especiales características metabólicas.

Comprendida la base genética, en *S. cerevisiae*, de muchos de los procesos metabólicos de interés en el proceso de fermentación vínica, el incremento exponencial de la información genómica disponible sobre las distintas especies de levadura permite la búsqueda de genes, ortólogos a los identificados en *S. cerevisiae,* para el estudio de su funcionalidad y regulación. De esta forma se podrán estudiar en detalle los mecanismos de adaptación y respuesta de las distintas especies de levadura de interés a las condiciones de fermentación vínica (temperatura, nutrición nitrogenada, coinoculación *S. cerevisiae/*no-*Saccharomyces*) permitiendo un uso racional y optimizado de las mismas y dotándolas, por tanto, de una mayor presencia en la industria.

8. CONCLUSIONES

8. CONCLUSIONES

1. El estudio poblacional realizado, considerando un total de 770 aislamientos de levaduras no-*Saccharomyces* procedentes de tres Denominaciones de Origen durante tres vendimias consecutivas, no demuestra la existencia de una distribución de especies característica de dichos orígenes y sostenida en el tiempo, mostrándose como determinantes las prácticas agrícolas y la casuística microclimática de la zona. La agrupación en *clusters* intraespecíficos de las levaduras aisladas considerando su origen, y en base a características fenotípicas con incidencia en la calidad del vino, está en consonancia con el concepto de *terroir* microbiano como comunidad de microorganismos asociada a un territorio y determinante de las propiedades sensoriales de sus vinos.

2. La diversidad de especies de levaduras no-*Saccharomyces* asociadas al vino constituye un espacio fenotípico a explorar para la aplicación de caracteres de su fisiología que contribuyan a la calidad y complejidad de los vinos fermentados exclusivamente con *Saccharomyces cerevisiae*. La ausencia en la mayoría de cepas de *S. cerevisiae* de ciertas actividades enzimáticas (β-glucosidasa, β-liasa, pectinasa) o su baja actividad en condiciones de fermentación vínica, hace interesante el estudio de dichas actividades en especies no-*Saccharomyces* que puedan presentar distintas regulaciones metabólicas y mecanismos de adaptación al entorno fermentativo.

3. El uso de la cepa *Metschnikowia pulcherrima* NS-EM-34, seleccionada en base a su actividad pectinolítica, permitió la mejora de ciertos parámetros de calidad de los vinos tintos, incrementando sus índices de color y mejorando el rendimiento en el proceso de clarificación. La aplicación de dicha cepa en maceración prefermentativa en frío multiplicó sus efectos positivos sobre la calidad de los vinos tintos elaborados.

4. El medio de cultivo desarrollado basado en el uso de S-metil-L-Cisteína (SMC) como única fuente de nitrógeno y de estructura análoga a los precursores cisteinilados naturales de los aromas tiólicos presentes en la uva, resultó útil para

la selección de levaduras con alta actividad β-liasa y, por tanto, para discernir el potencial de liberación de aromas tiólicos en cepas de levaduras *S. cerevisiae* y no-*Saccharomyces*.

En *S. cerevisiae*, conocida la existencia del gen *IRC7* como principal responsable de la liberación de tioles a partir de sus precursores cisteinilados, el medio de cultivo descrito permitió diferenciar, en base a su crecimiento, las cepas que en homocigosis presentan el gen *IRC7* delecionado y, por tanto, con poca capacidad de liberación de aromas tiólicos, de las cepas que en homocigosis o heterocigosis presentan el gen *IRC7* intacto y, en consecuencia, con mayor actividad β-liasa. Estas últimas están poco representadas en en poblaciones autóctonas de *S. cerevisiae*, por lo que el desarrollo de este medio de cultivo permite dirigir los procesos de selección de levaduras en base a su actividad β-liasa.

5. El uso de SMC como análogo del sustrato natural en el mosto de uva (Cisteína-4-MMP) de las enzimas con actividad β-liasa, también permitió desarrollar un método simplificado para cuantificar la actividad β-liasa de las levaduras seleccionadas. Los productos de la actividad β-liasa sobre dicho sustrato (metanotiol, y su dímero dimetildisulfuro) fueron detectados por cromatografía de gases acoplada a espectrometría de masas con resultados análogos a los obtenidos en la detección del compuesto volátil natural (4-MMP), pero simplificando la metodología requerida para su valoración.

6. La cepa *T. delbrueckii* Viniferm NS-TD mostró los mayores niveles de actividad β-liasa entre una amplia colección de levaduras no-*Saccharomyces* evaluada, seguida por la cepa *Kluyveromyces marxianus* NS-PDC-99. La aplicación de *T. delbrueckii* Viniferm NS-TD en fermentación secuencial con *S. cerevisiae* en mosto fresco de la variedad Verdejo mostró un incremento muy significativo en la liberación tanto de 3-MH como de 4-MMP con independencia del genotipo *IRC7* presentado por la cepa de *S. cerevisiae* con la que se coinocule.

7. El uso de la cepa *T. delbrueckii* Viniferm NS-TD, en fermentación secuencial con *S. cerevisiae* en vinos tintos, mostró una mejora significativa en su calidad

sensorial. Esta mejora se atribuyó al notable incremento en el contenido en manoproteínas. Así mismo, cabe destacar el mantenimiento de la acidez volátil de estos vinos en comparación con el control exclusivamente inoculado con *S. cerevisiae,* así como una ligera reducción de su contenido en ácido málico y de la liberación de alcoholes superiores.

8. Se confirmó el interés de determinadas cepas de levaduras no-*Saccharomyces* en la liberación de manoproteínas en fases postfermentativas de crianza sobre lías. En ellas, destacó la cepa *T. delbrueckii* Viniferm NS-TD, superando ligeramente los valores mostrados por la cepa superproductora de manoproteínas *S. cerevisiae* Viniferm 3D, así como la cepa *M. pulcherrima* NS-EM-34 que, aunque en valores notablemente menores, logró duplicar el contenido en manoproteinas de los vinos tratados con la cepa control *S. cerevisiae* Viniferm CT007.

9. BIBLIOGRAFÍA

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