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Departamento de Biotecnología de los Alimentos

LEVADURAS NO-*SACCHAROMYCES* PARA MODULAR EL AROMA SECUNDARIO DE LOS VINOS: INCREMENTO DEL ACETATO DE 2-FENILETILO MEDIANTE CULTIVOS INICIADORES MIXTOS

Memoria presentada por:

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CERTIFICAN: Que D. Fernando Viana Garrido, Licenciado en Enología por la Universidad Politécnica de Valencia, ha realizado bajo su dirección el trabajo titulado: "Levaduras no-*Saccharomyces* para modular el aroma secundario de los vinos: incremento del acetato de 2-feniletilo mediante cultivos iniciadores mixtos", que presenta para optar al grado de Doctor.

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 1 de julio de 2011.

Dra. Paloma Mª Manzanares Mir Dr. Salvador Vallés Alventosa

RESUMEN

Actualmente los enólogos se enfrentan a la necesidad de mejorar el proceso fermentativo y las cualidades sensoriales del vino en un sector cada vez más competitivo y global. Entre estos retos, destaca la necesidad de producir vinos más atractivos y complejos desde el punto de vista aromático. En este contexto, las levaduras vínicas no-*Saccharomyces* podrían representar una herramienta adecuada dado su potencial para formar compuestos volátiles. En este trabajo se ha evaluado la capacidad de estas levaduras para producir ésteres de acetato, y su posible inclusión en cultivos iniciadores mixtos, capaces no sólo de llevar a cabo la fermentación alcohólica sino de introducir características aromáticas diferenciales en los vinos así obtenidos. Como resultado del escrutinio inicial se seleccionó la cepa *Hanseniaspora vineae* 1471 por su destacada producción de acetato de 2-feniletilo así como por sus buenas características enológicas. Posteriormente se diseñaron cultivos iniciadores mixtos con *Saccharomyces cerevisiae*, confirmándose las propiedades de *H. vineae* 1471, y la posibilidad de modular la concentración de acetato de 2-feniletilo variando las proporciones iniciales de estas levaduras en el cultivo mixto. Así mismo, empleando mostos naturales no estériles, se confirmó el crecimiento de *H. vineae* 1471, inoculada de forma secuencial, a pesar de la presencia de una microbiota nativa elevada, y la obtención de vinos con concentraciones incrementadas de acetato de 2-feniletilo. Por último la inmovilización de *H. vineae* 1471 en geles de alginato cálcico resultó una técnica adecuada para controlar su permanencia y producción de acetato de 2-feniletilo durante la fermentación.

ABSTRACT

Due to the shifting consumer preferences and the globalized wine markets the winemaking industry needs permanent technological innovations to improve the vinification process and the sensory qualities of wine. Among these challenges, the need to enhance the aromatic profile to obtain more attractive and flavour-unique wines stands out. In this context, non-*Saccharomyces* wine yeasts may represent a suitable tool due to their potential to produce volatile compounds. The suitability of these yeasts to produce acetate esters and their effect as part of mixed cultures able to carry out the alcoholic fermentation and produce wines with a wide range of flavour composition has been evaluated. As a result of the initial screening *Hanseniaspora vineae* strain 1471 was found to be a strong producer of 2-phenylethyl acetate while keeping good enological traits. Subsequently we confirmed the potential of using *H. vineae* 1471 in mixed starters with *Saccharomyces cerevisiae* to increase the levels of 2-phenylethyl acetate in wine. Moreover, it was found that the ratio of both yeast strains in the mixed culture modulates ester concentration leading to wines with a wide range of ester levels. Also we showed that *H. vineae* as a part of a sequential mixed starter is able to compete with native yeasts present in a non-sterilised natural must and produce the desired effect in the final wine. Finally, *H. vineae* 1471 immobilization on calcium alginate beads was a suitable technique to control yeast permanence during must fermentation and thus modulate 2-phenylethyl acetate production.

RESUM

Actualment els enòlegs s'enfronten a la necessitat de millorar el procés fermentatiu i les qualitats sensorials del vi en un sector cada vegada més competitiu i global. Entre estos reptes, destaca la necessitat de produir vins més atractius i complexos des del punt de vista aromàtic. En este context, els llevats vínics no-*Saccharomyces* podrien representar una ferramenta adequada donat el seu potencial per a formar compostos volàtils. En este treball s'ha avaluat la capacitat d'estos llevats per a produir esters d'acetat, i la seua possible inclusió en cultius iniciadors mixtos, capaços no sols de dur a terme la fermentació alcohòlica sinó d'introduir característiques aromàtiques diferencials en els vins així obtinguts. Com resultat de l'escrutini inicial es va seleccionar la soca *Hanseniaspora vineae* 1471 per la seua destacada producció d'acetat de 2-feniletil així com per les seues bones característiques enològiques. Posteriorment es van dissenyar cultius iniciadors mixtos amb *Saccharomyces cerevisiae*, confirmant-se les propietats de *H. vineae* 1471, i la possibilitat de modular la concentració d'acetat de 2-feniletil variant les proporcions inicials d'estos llevats en el cultiu mixt. També, emprant mostos naturals no estèrils, es va confirmar el creixement de *H. vineae* 1471, inoculada de forma seqüencial, a pesar de la presència d'una microbiota nativa elevada, i l'obtenció de vins amb concentracions incrementades d'acetat de 2-feniletil. Finalment la immobilització de *H. vineae* 1471 en gels d'alginato càlcic va resultar una tècnica adequada per a controlar la seua permanència i producció d'acetat de 2-feniletil durant la fermentació.

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“El futuro pertenece a quienes creen

en la belleza de sus sueños”

Eleanor Roosevelt, (1884-1962)

ÍNDICE

ABREVIATURAS

AATFasa	Alcohol acetil transferasa
CECT	Colección Española de Cultivos Tipo
CoA	Coenzima A
DNA	Ácido desoxiribonucleico
EDTA	Ácido etilen diamino tetraacético
ITS	*Internal transcribed spacer* (Espaciador interno transcrito)
LSA	Levadura seca activa
mtDNA	DNA mitocondrial
PCR	Reacción en cadena de la polimerasa
RNA	Ácido ribonucleico
RAPD	*Random amplified polymorphic* DNA
rDNA	DNA ribosómico
RFLP	*Restriction Fragment Length Polimorphism* (Polimorfismo de la longitud de los fragmentos de restricción)
ufc	Unidades formadoras de colonias

1. Introducción

La actual coyuntura vitivinícola hace que el sector atraviese momentos difíciles que se manifiestan, principalmente, en un importante descenso en el consumo mundial de vino, con una disminución en 2009 de 6,8 millones de hectolitros en relación al año anterior. Esta situación se ha visto agravada año tras año por una producción constante de vino a nivel mundial, lo que desequilibra al sector. Esta realidad hace que la industria enológica, sometida a una gran competitividad, se enfrente a desafíos continuos en el actual mercado global siendo claves la tecnología y el conocimiento para un nuevo y mejor posicionamiento en el mercado. Para ello, es fundamental que el sector adapte sus productos e identifique las tendencias y gustos del consumidor actual.

En este contexto, la nueva biotecnología enológica busca la obtención de vinos más atractivos y complejos desde el punto de vista organoléptico con el objetivo de satisfacer estas necesidades, poniendo a disposición del mercado “vinos a la carta”. Encontrar nuevas formas de mejorar la fermentación, y las cualidades sensoriales del vino son objetivos prioritarios para los profesionales de la enología.

En la actualidad existe una demanda continua de nuevas y mejores cepas de levaduras adaptadas a diferentes tipos y estilos de vinos. Este trabajo pretende satisfacer esta demanda a través de una selección de levaduras vínicas no-*Saccharomyces* y su inclusión en cultivos iniciadores mixtos, capaces no sólo de llevar a cabo la fermentación alcohólica sino de introducir características aromáticas diferenciales en los vinos obtenidos.

1.1 Vino y aroma: importancia del aroma fermentativo

Aroma y sabor son dos de las características organolépticas más importantes que definen la calidad de un vino. El aroma viene determinado por compuestos de naturaleza volátil, como son, alcoholes, ésteres, aldehídos, cetonas e hidrocarburos. Por el contrario, en el sabor influyen compuestos no volátiles tales como azúcares, ácidos orgánicos, derivados fenólicos y sustancias minerales. En general, la contribución de estos últimos compuestos al sabor del vino sólo se manifiesta cuando se hallan presentes en concentraciones iguales o superiores a 10 g/L, mientras que los compuestos volátiles pueden ser percibidos a concentraciones mucho más bajas, ya que sus umbrales de percepción varían entre 10^{-4} y 10^{-12} g/L (Guadagni *et al.,* 1963).

Al igual que en otros muchos alimentos, el aroma de un vino está determinado por varios cientos de compuestos volátiles de diversa naturaleza química. Hasta la fecha, se han identificado más de 680 compuestos volátiles, lo que indica su complejidad (Schreier, 1979; Maarse y Vissher, 1994; Rapp, 1998; Guth y Sies, 2002). La concentración de estos compuestos en el producto final depende de factores asociados al cultivo de la uva, tales como el clima, el suelo, el riego y el momento de la vendimia, así como de las numerosas variables del proceso de fermentación (pH, temperatura, nutrientes y microflora) y de las operaciones que integran la elaboración del vino, como los procesos de filtración o clarificación entre otros. El aroma final derivará del balance y de la interacción de todos estos compuestos, ya que pequeñas variaciones en su concentración pueden marcar la diferencia entre vinos de alta gama y vinos de mesa.

En términos enológicos y atendiendo al origen de los compuestos que lo constituyen, el perfil aromático de un vino se clasifica en tres categorías: aroma varietal o primario, aroma fermentativo o secundario y bouquet o aroma terciario (Schreier, 1979; Boulton *et al.,* 1995; Rapp, 1998).

El aroma varietal se compone de aquellas sustancias que proceden directamente de la variedad de uva utilizada, tales como los ésteres del ácido acético y los monoterpenos, estos últimos característicos de la variedad de uva Moscatel (Rapp y Mandery, 1986). Dentro de este grupo también se incluyen compuestos que se generan en el transcurso de la manipulación, preparación, extracción y acondicionamiento del mosto en la bodega y entre ellos cabe destacar aldehídos, cetonas y diferentes tipos de alcoholes (Stevens *et al.,* 1967; Ramshaw y Hardy, 1969; Schreier, 1979).

El aroma fermentativo es el que se atribuye a los compuestos generados, durante la fermentación alcohólica, por el metabolismo de las levaduras, fundamentalmente de la especie *Saccharomyces cerevisiae*, aunque no se debe ignorar la contribución de las levaduras oxidativas y apiculadas, denominadas de forma genérica no-*Saccharomyces,* presentes durante los primeros días de la fermentación, y de las distintas especies de bacterias ácido lácticas durante el desarrollo de la fermentación maloláctica.

En cuanto al "bouquet" de maduración, éste aparece como consecuencia de una serie de reacciones enzimáticas y/o físico-químicas que tienen lugar en el proceso de envejecimiento y crianza del vino. Según el tipo de envejecimiento se distinguen dos tipos de "bouquet", el de oxidación, originado por la crianza en madera y el de reducción, generado durante el envejecimiento en botella (Rapp y Mandery, 1986).

Partiendo del hecho de que los vinos producidos con una variedad específica presentan las características aromáticas propias de la uva, hay que tener en cuenta además, que muchos de estos compuestos se liberan y/o modifican por la acción de las levaduras y bacterias y que además el propio metabolismo microbiano genera una serie de volátiles con gran incidencia en el aroma del vino. Esta es la razón por la cuál el perfil aromático del vino es mucho más complejo que el del mosto de uva del que procede, demostrando la importancia del aroma fermentativo (Figura 1).

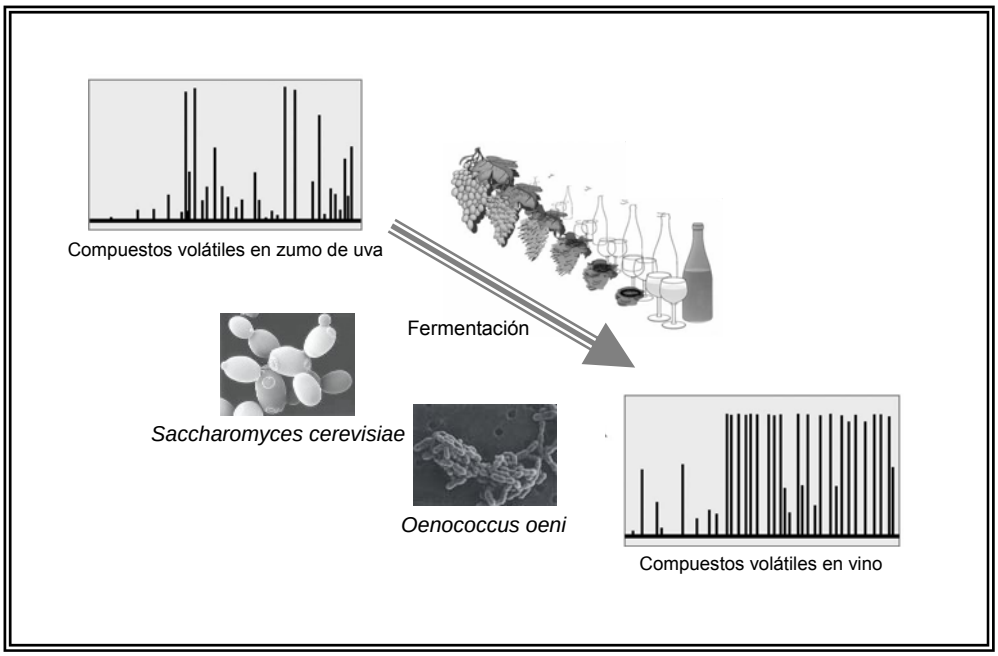


Figura 1. Diagrama representativo de la modulación microbiana del perfil de compuestos volátiles en vino (tomado de Swiegers *et al*., 2005).

En este contexto, durante la fermentación alcohólica las levaduras no sólo convierten los azúcares en etanol y dióxido de carbono, sino que también producen una serie de metabolitos volátiles detallados en la Figura 2 que, aunque minoritarios, determinan de manera fundamental el carácter aromático propio del vino (Schreier, 1979; Etiévant, 1991; Guth, 1998; Rapp, 1998; Lambrechts y Pretorius, 2000; Romano *et al*., 2003). El perfil aromático de un producto tan complejo como el vino no es atribuible a un solo compuesto de impacto, sino que es el resultado de la combinación e interacción entre los distintos compuestos aromáticos. A pesar de ello, su aroma genérico de fondo se atribuye mayoritariamente a alcoholes y ésteres, que le otorgan su calidad e intensidad aromática. (Noble, 1994; Cole y Noble, 1995; Lambrechts y Pretorius, 2000).

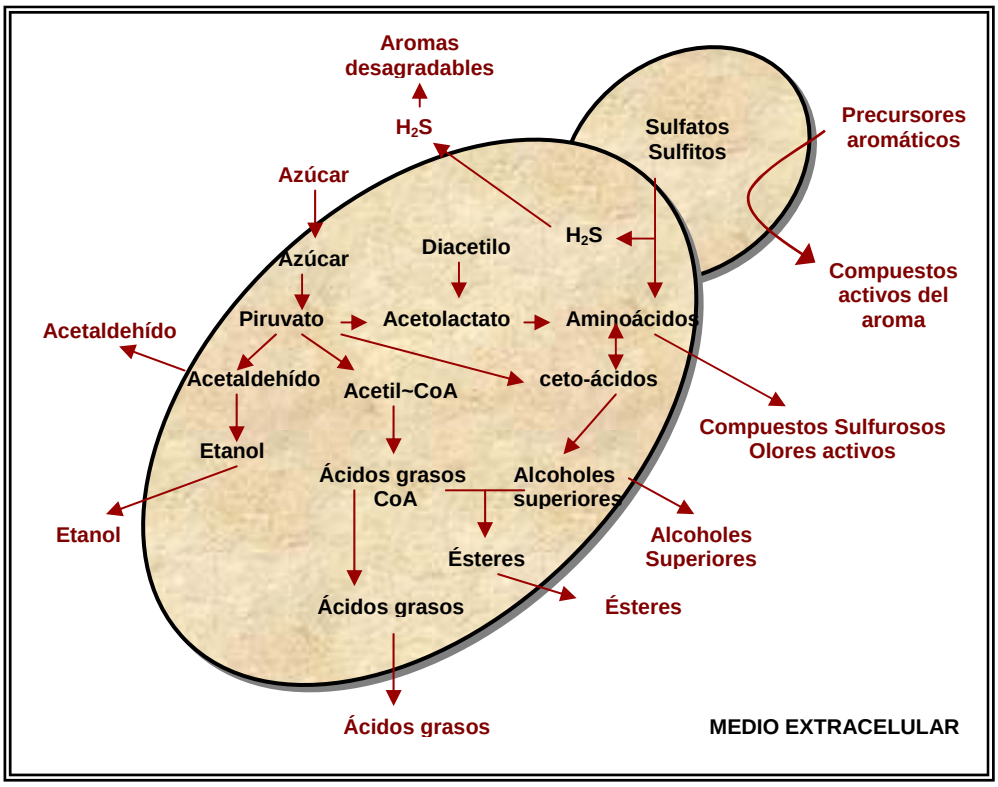


Figura 2. Formación de compuestos aromáticos durante la fermentación alcohólica (tomado de Rojas, 2002).

Por lo que respecta a los alcoholes superiores, término que engloba a aquellos alcoholes que poseen más de dos átomos de carbono y un peso molecular y punto de ebullición superior al del etanol, su principal característica es su olor penetrante. Generalmente se producen en cantidades elevadas, del orden de 100 a 400 mg/L, y cuando sus concentraciones exceden este intervalo originan un efecto negativo en la percepción sensorial del producto (Rapp y Mandery, 1986). Los alcoholes superiores, reflejados en la Tabla 1, se clasifican en alcoholes alifáticos y aromáticos. Entre los primeros se incluyen el propanol, isobutanol, hexanol y alcohol isoamílico, siendo éste último el más significativo cuantitativa y

cualitativamente, mientras que los alcoholes aromáticos incluyen el tirosol y el 2-feniletanol. Independientemente de la influencia que puedan ejercer por sí mismos sobre las propiedades organolépticas del vino, la importancia enológica de los alcoholes superiores también radica en ser los compuestos precursores de los ésteres de acetato (Soles *et al.,* 1982), tal y como se esquematiza en la Figura 3.

Tabla 1. Concentración en vino, umbrales de detección y aroma característico de algunos alcoholes superiores producidos por *S. cerevisiae* durante la fermentación alcohólica.

Alcohol superior	Concentración en vino (mg/L)	Umbral de detección (mg/L)	Aroma
Propanol	9-68	500* 800**	Disolvente
Isobutanol	9-28	500* 200**	Alcohol
Hexanol	0'3-12	4*	Verde, hierba
Alcohol isoamílico	45-490	300* 70*	Mazapán
Tirosol			Miel
2-Feniletanol	10-180	125**	Floral, rosas

Tomado de Lambrechts y Pretorius, 2000. (*) En vino; (**) En cerveza.

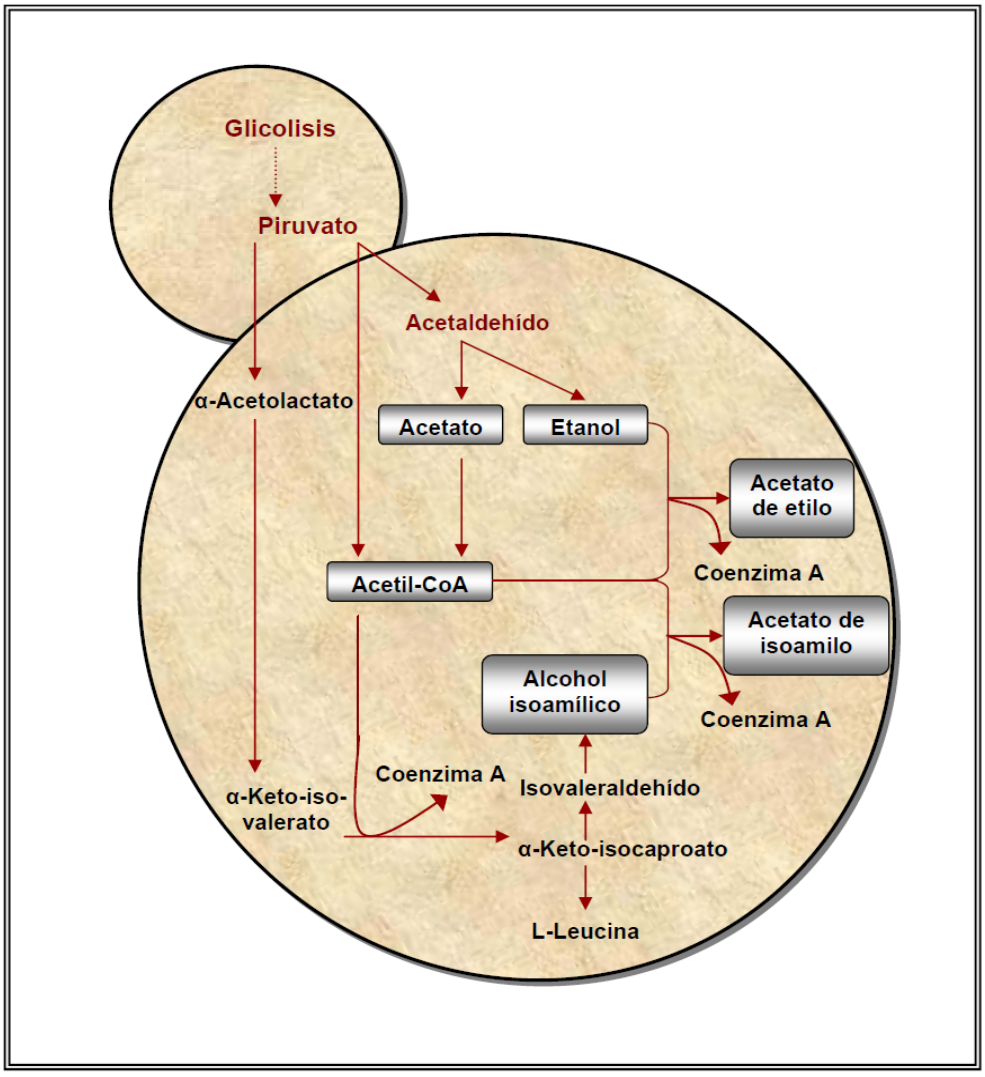


Figura 3. Representación esquemática de la formación de acetato de etilo y acetato de isoamilo en levaduras vínicas (adaptado de Swiegers *et al*., 2005).

En cuanto a los ésteres, estos compuestos son, dentro de todos los grupos funcionales encontrados en el vino, los más importantes numéricamente, habiéndose identificado aproximadamente unos 160. Los ésteres, compuestos volátiles con fragancias aromáticas agradables, son generados en pequeñas cantidades pero a una concentración superior a su umbral de percepción. Los más importantes son los ésteres polares (2-etilhidroxipropionato, succinato de dietilo, etil-4-hidroxibutanoato, malato de dietilo o isopentil-2-hidropropionato), usualmente responsables de dar al vino cuerpo y consistencia, y los ésteres apolares donde se incluyen los ésteres de acetato de alcoholes superiores (acetato de etilo, acetato de isoamilo, acetato de isobutilo, acetato de hexilo y acetato de 2-feniletilo) y los ésteres de etilo de ácidos grasos saturados (butanoato de etilo, hexanoato o caproato de etilo, octanoato o caprilato de etilo y decanoato o caprato de etilo) (Baumes *et al.,* 1986). En general, los ésteres apolares se asocian al aroma frutal, floral y fresco de los vinos jóvenes. Así por ejemplo, en vinos blancos, la presencia de una mezcla de estos compuestos contribuye a proporcionar una percepción afrutada, mientras que en vinos tintos contribuyen a modular su calidad aromática (Ferreira *et al.,* 1995).

En la Tabla 2 se muestran los ésteres más importantes, junto a su concentración, su umbral de percepción y su aroma característico. Cabe destacar que la contribución de los ésteres al aroma tiene un efecto sinérgico, y que raramente una propiedad aromática particular se asocia con un éster en concreto (Van Rooyen *et al.,* 1982).

Tabla 2. Concentración en vino, umbrales de detección y aroma característico de ésteres principales producidos por *S. cerevisiae* durante la fermentación alcohólica.

Éster	Concentración en vino (mg/l)	Umbral de detección (mg/l)	Aroma
Acetato de etilo	10–100	15–20	Barniz, afrutado
Acetato de 2-feniletilo	0'01–4'5	0'01–8'0	Rosa, miel, afrutado, floral
Acetato de isoamilo	0'03–8'1	0'26	Plátano, pera
Acetato de isobutilo	0'01–0'8	1'6 (cerveza)	Plátano
Acetato de hexilo	0–4'8	0'67–2'4	Manzana madura
Butanoato de etilo	0'01–3	0'4 (cerveza)	Floral, afrutado
Caprato de etilo	Trazas–2'1	0'5	Floral
Caprilato de etilo	0'05–3'8	0'258–0'58	Piña, pera
Caproato de etilo	Trazas–3'4	0'08	Manzana, plátano, violetas
Isovaleriato de etilo	n.d.–0'7		Manzana, afrutado
2- metil butanoato de etilo	n.d.–0'9		Fresa, piña

n.d.: no detectado. Tomado de Salo, 1970a y b; Peddie, 1990; Riesen, 1992; Boulton *et al*., 1995 y Lambrechts y Petrorius, 2000.

El acetato de etilo es, cuantitativamente, el éster mayoritario, con unas concentraciones que oscilan entre 50 y 100 mg/L. Niveles iguales o inferiores a 50 mg/L contribuyen positivamente al aroma en su conjunto, mientra que por encima de 150 mg/L el efecto es negativo, proporcionando notas a barniz y vinagre (Jackson, 1994).

La presencia de acetato de isoamilo y de acetato de 2-feniletilo en el vino se considera una cualidad positiva, ya que confieren fragancias afrutadas, y por consiguiente, realzan su calidad. Se ha descrito que la

combinación de ambos ésteres en un mismo vino conduce a una mejor percepción sensorial de las notas florales (Cacho, 2006).

En cuanto a los ésteres de etilo de ácidos grasos, la cantidad transferida desde la levadura al vino disminuye a medida que aumenta la cadena de los ácidos grasos: pasa al medio el 100% del caproato de etilo, el 54-68% del caprilato de etilo y el 8-17% del caprato de etilo (Nykänen *et al.,* 1977). El caproato de etilo se asocia a aroma de manzanas y violetas, el caprilato de etilo a aromas de pera y piña, mientras que las notas florales caracterizan al caprato de etilo (Boulton *et al*., 1995).

Aunque son muchos los factores que pueden afectar a la producción de ésteres y alcoholes aromáticos durante la fermentación, el grado de madurez y el contenido en azúcar de la uva (Houtman *et al.,* 1980a y 1980b), la temperatura del proceso fermentativo (Piendl y Geiger, 1980), el método de vinificación empleado (Herraiz y Ough, 1993; Gómez *et al.,* 1994), el proceso de envejecimiento y la temperatura o el tipo de almacenamiento (Marais y Pool, 1980; Ramey y Ough, 1980), hay que destacar entre todos ellos la cepa de levadura utilizada (Lambrechts y Pretorius, 2000).

Dado que la propia levadura es la que proporciona mayores posibilidades para modular el aroma fermentativo, el control de la ecología de la fermentación así como la selección/inoculación de levaduras con características específicas son herramientas a disposición del enólogo para obtener vinos con perfiles aromáticos determinados.

1.2 Fermentaciones espontáneas frente a inoculadas: potencial de las levaduras no-*Saccharomyces* en vinificación

Desde un punto de vista microbiológico, la obtención de vino a partir de mosto de uva es un proceso complejo que implica la participación de diferentes tipos de microorganismos siendo *S. cerevisiae*, junto con otros géneros y especies de levaduras, la principal responsable de la fermentación alcohólica.

Para fermentar el mosto, existen dos posibilidades, bien llevar a cabo una fermentación natural o espontánea donde se deja evolucionar la propia microbiota del mosto, o bien lo que se conoce como fermentación inoculada, donde se potencia la imposición de una determinada cepa de *S. cerevisiae* mediante su inoculación en forma de levadura seca activa (LSA).

Tradicionalmente la fermentación del mosto se lleva a cabo mediante la fermentación natural o espontánea, donde según distintos estudios existe un crecimiento secuencial de distintas especies de levadura. El proceso lo inician las levaduras apiculadas, poco tolerantes al etanol y pertenecientes al género *Hanseniaspora/Kloeckera*, que son reemplazadas por *S. cerevisiae* la cual continúa y finaliza la fermentación (Martini, 1993). Durante las diferentes etapas de la misma es posible aislar levaduras pertenecientes a los géneros *Candida*, *Torulaspora*, *Kluyveromyces* y *Metschnikowia* (Fleet *et al*., 1984; Pardo *et al*., 1989), capaces de sobrevivir a niveles significativos (hasta 10^6-10^7 ufc/mL) durante la fermentación y por períodos más largos que los previamente descritos.

La desaparición o permanencia de las levaduras no-*Saccharomyces* a lo largo de la fermentación está influenciada por varios factores fisicoquímicos y microbiológicos. Entre los primeros destacan la temperatura de fermentación y la concentración de oxígeno. Se ha descrito que a menor temperatura hay mayor resistencia a etanol y por tanto mayor permanencia (Gao y Fleet, 1988; Erten, 2002), mientras que a menor

cantidad de oxígeno disponible, la supervivencia es menor (Hansen *et al*., 2001). Por lo que se refiere a los factores microbiológicos, la supervivencia de las levaduras no-*Saccharomyces* depende del número de células viables de *S. cerevisiae* debido a posibles interacciones célula-célula que provocan la inhibición del crecimiento (Nissen y Arneborg, 2003; Nissen *et al.*, 2003) o a la producción de compuestos tóxicos (Pérez-Nevado *et al*., 2006), aunque los autores no lograron identificar la naturaleza de estos compuestos.

En la actualidad, se tiende a inocular el mosto con cepas de *S. cerevisiae* en forma de LSA, con el fin de controlar mejor las fermentaciones, evitar incidentes organolépticos y conseguir una calidad homogénea añada tras añada. La disponibilidad comercial de estos cultivos de *S. cerevisiae* ha contribuido a que la inoculación del mosto de uva se haya popularizado, siendo una práctica atractiva y cómoda para las bodegas (Kraus *et al*., 1983; Barre y Vezinhet, 1984). Por estos motivos, el uso de cultivos seleccionados es una práctica habitual tanto en los nuevos países productores de vino (Estados Unidos, Sudáfrica, Australia, Chile y Argentina) como en países más tradicionales (Francia, España e Italia) (Reed y Nagodawithana, 1988; Fleet y Heard, 1993).

A pesar de las numerosas ventajas inherentes a las fermentaciones inoculadas, se ha constatado durante estos últimos años que el control del proceso de fermentación va siempre acompañado de una pérdida de tipicidad aromática de los vinos, lo que no sucede en las fermentaciones espontáneas. Por tanto, existe una controversia dentro del ámbito de la enología relativo al empleo de fermentaciones espontáneas o inoculadas, particularmente con respecto a la calidad organoléptica de los vinos obtenidos. Por este motivo e independientemente de que las fermentaciones se inoculen con *S. cerevisiae*, los enólogos tienen la posibilidad de decidir si se potencia o no la microbiota no-*Saccharomyces* con el fin de aprovechar sus características enológicas.

En este contexto, durante los últimos años se está reevaluando la influencia de las levaduras no-*Saccharomyces* sobre la calidad del vino. Estos estudios se han abordado considerando tanto la producción de enzimas como la de metabolitos secundarios, relacionados ambos con el aroma del vino.

Por lo que respecta a las actividades enzimáticas de las levaduras vínicas no-*Saccharomyces* que pueden influir en la calidad del vino, se deben considerar las denominadas de maceración que mejoran ciertas etapas del proceso de vinificación, como la clarificación y filtración, y las glicosidasas, responsables de la liberación de terpenos. Desde el punto de vista aromático, las enzimas más relevantes son éstas últimas, entre las que destaca la actividad β-D-glucosidasa. Esta enzima, parece ser característica de las levaduras no-*Saccharomyces*, ya que su presencia se ha demostrado en diversas especies pertenecientes a los géneros *Candida*, *Debaryomyces*, *Hanseniaspora/Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces* y *Zygosaccharomyces* (Strauss *et al*., 2001; Manzanares *et al*., 2000; McMahon *et al*., 1999; Charoenchai *et al*., 1997; Rosi *et al*., 1994). Por lo que se refiere a otras glicosidasas, como β-D-xilosidasa, α-L-arabinofuranosidasa y α-L-ramnosidasa, si bien también participan en los procesos de liberación de terpenos, son pocas las especies de levaduras no-*Saccharomyces* que las poseen. Un resumen de las enzimas producidas por diferentes especies de levaduras no-*Saccharomyces* se detalla en las Tablas 3 y 4, y una revisión sobre este tema puede consultarse en Manzanares *et al.,* (2005).

Tabla 3. Enzimas de maceración producidas por levaduras no-*Saccharomyces* (tomado de Manzanares *et al*., 2005).

	Enzimas de maceración						
Levaduras	PG	PME	CEL	GLU	XYL	PR	Referencia
Candida albicans			X				(7)
Candida flavus						X	(1)
Candida hellenica			X	X			(1)
Candida krusei		X					(3)
Candida lambica				X			(1)
Candida lipolytica						X	(4)
Candida norvegensis	X						
Candida olea						X	(1)(4)
Candida oleophila	X				X		(1)
Candida pelliculosa					X		(1)
Candida pulcherrima	X		X	X	X	X	(1)(4)
Candida silvae	X						
Candida sorbosa				X			(1)
Candida stellata	X		X	X	X	X	(1)(6)
Candida tropicalis	X						(2)
Candida valida	X						(1)
Candida wickerhamii			X				(8)
Cryptococcus sp.			X				(11)
Cryptococcus albidus	X						(12)
Debaryomyces hansenii						X	(1)
Debaryomyces membranaefaciens		X					(3)
Hanseniaspora guilliermondii						X	(5)
Kloeckera apiculata						X	(1)(4)(5)
Kloeckera thermotolerans	X						(6)
Kluyveromyces marxianus	X						(10)
Metschnikowia pulcherrima						X	(1)(6)
Pichia anomala	X						(6)
Pichia guilliermondii	X						(6)
Pichia kluyveri	X	X					(9)
Pichia membranaefaciens	X						(6)

PG = poligalacturonasa; PME = pectin metilesterasa; CEL = celulasa; GLU = ß-glucanasa; XYL = xilanasa; PR = proteasa.

(1) Strauss *et al*., 2001; (2) Luh y Phaff, 1951; (3) Bell y Etchells, 1956; (4) Lagace y Bisson, 1990; (5) Dizy y Bisson, 2000; (6) Fernández *et al*., 2000; (7) Chambers *et al*., 1993; (8) LeClerc, *et al*., 1984; (9) Masoud y Jespersen, 2006; (10) Serrat *et al*., 2004; (11) Thongekkaew *et al*., 2008; (12) Servili *et al*. 1990.

Tabla 4. Glicosidasas producidas por levaduras no-*Saccharomyces* (tomado de Manzanares *et al*., 2005).

	Glicosidasas				
Levaduras	BGL	XYL	RAM	ARA	Referencia
Brettanomyces bruxellensis	X				(1)
Candida stellata	X				(2),(3)
Candida pulcherrima	X				(3),(4)
Candida cacaoi	X				(20)
Candida cantarelli	X				(5)
Candida colliculosa	X				(3)
Candida dattila	X				(5)
Candida domerquiae	X				(5)
Candida famata	X				(3)
Candida guilliermondii	X		X		(4),(6)
Candida hellenica	X				(2)
Candida krusei	X				(3)
Candida molischiana	X				(7),(21)
Candida parapsilosis	X				(6)
Candida peltata	X				(8)
Candida utilis		X			(9)
Candida vinaria	X				(5)
Candida vini	X				(5)
Candida wickerhamii	X				(21)
Cryptococcus albidus		X			(19)
Debaryomyces hansenii	X				(10),(11)
Debaryomyces vanrijiae	X				(12),(13)
Hanseniaspora guilliermondii	X				(5)
Hanseniaspora osmophila	X	X			(5),(14)
Hanseniaspora uvarum	X	X			(5),(14),(15)
Kloeckera apiculata	X				(2),(3),(4),(6)
Metschnikowia pulcherrima	X				(5),(6)
Pichia anomala	X	X		X	(3),(5),(14),(16)
Pichia capsulata				X	(17)
Pichia membranaefaciens	X				(5)
Pichia stipitis		X			(18)
Zygosaccharomyces bailii	X				(5)
Zygosaccharomyces mellis	X				(5)
Zygosaccharomyces rouxii	X				(5)

BGL = ß-glucosidasa; XYL = ß-xilosidasa; RAM = α-ramnosidasa; ARA = α-arabinofuranosidasa.

(1) Mansfield *et al*., 2002; (2) Strauss *et al*., 2001; (3) Charoenchai *et al*., 1997; (4) Rodríguez *et al*., 2004; (5) Manzanares *et al*., 2000; (6) McMahon *et al*., 1999; (7) Genovés *et al*., 2003; (8) Saha y Bothast, 1996; (9) Yanai y Sato, 2001; (10) Yanai y Sato, 1999; (11) Riccio *et al*., 1999; (12) Belancic *et al*., 2003; (13) García *et al*., 2002; (14) Manzanares *et al*., 1999; (15) Fernández-González *et al*., 2003; (16) Spagna *et al*., 2002; (17) Yanai y Sato, 2000; (18) Lee *et al*., 1986; (19) Peciarová y Biely, 1982; (20) Drider *et al*. 1993. (21) Gunata *et al*. 1990.

Por lo que respecta a los metabolitos secundarios, tradicionalmente las levaduras no-*Saccharomyces* se han considerado productoras de metabolitos con incidencia negativa sobre la calidad del vino, por lo que siempre se las ha catalogado como levaduras alterantes. Entre estos metabolitos destacan el ácido acético, el acetaldehído, la acetoína y el acetato de etilo, junto con los vinil y etil fenoles, éstos últimos asociados principalmente al desarrollo de *Brettanomyces/Dekkera* spp. (Chatonnet *et al.*, 1995). Sin embargo, la producción de todos estos compuestos es dependiente de cepa (Lambretchs y Pretorius, 2000), lo que permitiría con un buen programa de selección, identificar aquellas levaduras más apropiadas.

Contrariamente, otros estudios han puesto de manifiesto el papel positivo de estas levaduras en las propiedades químicas y sensoriales del vino (Fleet, 2008; Pretorius, 2000; Romano *et al*., 2003a; Swiegers *et al*., 2005). De especial interés es la capacidad de las levaduras no-*Saccharomyces* para sintetizar ésteres de acetato que, como ya se ha expuesto anteriormente, juegan un papel clave en la calidad aromática de los vinos jóvenes. Además de la ya comentada capacidad de producción de acetato de etilo, superior a la de las cepas vínicas de *S. cerevisiae* (Nykänen, 1986; Ough *et al.,* 1968), el género *Rhodotorula* y *Pichia* han sido señalados como productores de acetato de isoamilo (Suomalainen y Lehtonen, 1979; Rojas *et al.,* 2001), mientras que diferentes especies del género *Hanseniaspora* son descritas como buenas productoras tanto de acetato de 2-feniletilo como de acetato de isoamilo (Moreira *et al.,* 2005, 2008; Plata *et al.,* 2003; Rojas *et al.,* 2001, 2003). Estos estudios demuestran que las levaduras no-*Saccharomyces* pueden ser seleccionadas en base a su capacidad para producir metabolitos secundarios favorables que contribuyan a mejorar la calidad del vino, minimizando sus efectos negativos. Esta posibilidad ha llevado a diferentes autores a proponer su utilización en cultivos iniciadores mixtos junto a *S. cerevisae*. Con esta propuesta se aprovecharían las características ventajosas de las levaduras no-*Saccharomyces* a la vez que se

normalizarían los procesos de vinificación, representando una alternativa frente a las fermentaciones espontáneas, a veces impredecibles y potencialmente problemáticas. Además, se introduciría también diversidad y complejidad aromática en las fermentaciones llevadas a cabo con cepas comerciales de *S. cerevisiae*.

1.3 Cultivos iniciadores mixtos en vinificación

Aunque la propuesta de los cultivos iniciadores mixtos que permitan utilizar las características positivas de las levaduras no-*Saccharomyces* es una de las tendencias actuales dentro de la biotecnología enológica, ya a mediados del siglo pasado se propuso esta estrategia para controlar la acidez de los vinos (Castelli, 1955, 1969; Peinaud y Sudrad, 1962; Rankine, 1966). En la actualidad se ha confirmado la viabilidad de esta estrategia para reducir la acidez volátil mediante el empleo de cultivos mixtos de *T. delbrueckii* y *S. cerevisiae*, tanto con inoculación conjunta como secuencial. En concreto se demostró que este cultivo mixto en una proporción de inóculo 20:1 redujo un 53% la acidez volátil y un 60% el acetaldehído, con respecto a cultivos puros de *S. cerevisiae* (Bely *et al*., 2008). También en este contexto, la desacidificación biológica de mostos y/o vinos mediante la reducción del contenido en ácido málico puede alcanzarse mediante la inoculación secuencial de *Schizosaccharomyces pombe y S. cerevisiae* (Snow y Gallender, 1979; Satyanarayana *et al*., 1988). Con este mismo objetivo, recientemente Kim *et al*., (2008) estudiaron el empleo de un cultivo mixto de *Issatchenkia orientalis* y *S. cerevisiae* consiguiéndose una reducción del 70% en el contenido de ácido málico.

Otro aspecto de gran interés en enología es la corrección de la baja acidez de algunos mostos de uva. Este defecto pudo corregirse utilizando un cultivo mixto de *K. thermotolerans* y *S. cerevisiae* capaz de aumentar en un 70% la acidez total mediante la producción de ácido láctico lo que originó una reducción de 0,3 unidades de pH (Kapsopoulou *et al*., 2007).

Una de las levaduras más evaluadas para su utilización como cultivo iniciador en combinación con *S. cerevisiae* es *Candida stellata*, debido a su capacidad para producir glicerol. Esta especie en concreto presenta una alta tolerancia a concentraciones elevadas de etanol (hasta el 12%) y preferencia por el consumo de fructosa, lo que complementa el carácter glucofílico de *S. cerevisiae*. Además del aumento en glicerol, los vinos así obtenidos presentan una mayor complejidad aromática, ya que se sintetizan más cantidad de compuestos volátiles (Ciani y Ferraro, 1998; Romano *et al.,* 2003; Ciani y Comitini, 2006). Resultados similares también fueron descritos por Toro y Vázquez (2002) empleando *C. cantarellii* en cultivos mixtos.

Otros estudios han puesto de manifiesto el interés de llevar a cabo fermentaciones mixtas para aumentar la fracción aromática de los vinos. En concreto, García *et al.* (2002) propusieron el empleo de un cultivo mixto de *Debaryomyces vanriji* y *S. cerevisiae* para incrementar terpenos, especialmente el geraniol, en vino elaborado con la variedad de uva Moscatel. Más recientemente, fueron propuestas en co-fermentación *S. cerevisiae* y *Pichia kluyveri* para incrementar las concentraciones de tioles varietales en Sauvignon Blanc (Anfang *et al.,* 2009).

El incremento de las concentraciones de ésteres de acetato también ha sido objetivo en fermentaciones mixtas. Estos estudios se han llevado a cabo principalmente con levaduras apiculadas, bien conocidas por su capacidad de producción de dichos ésteres. En concreto, la especie *H. guilliermondii* destaca por su producción de acetato de 2-feniletilo tanto en medio microbiológico (Rojas *et al*., 2001; Moreira *et al*., 2005) como en vino (Rojas *et al*., 2003; Moreira *et al*., 2008). En vinificación, la producción de acetato de 2-feniletilo tiene lugar cuando se utilizan cultivos puros de dicha especie y también en vinos obtenidos con fermentaciones mixtas de *H. guilliermondii* y *S. cerevisiae*. Sin embargo, aunque las principales características enológicas de los vinos obtenidos mediante cultivos mixtos fueron similares a las de aquellos vinos obtenidos con cultivos puros de *S. cerevisae*, todos ellos presentaron el inconveniente de una formación

excesiva de acetato de etilo. Algunas de estas cepas de *H. guilliermondii* también son buenas productoras de acetato de isoamilo, característica compartida con la especie *H. uvarum* (Moreira *et al*., 2008). Algunos de los ejemplos correspondientes al empleo de cultivos mixtos se resumen en la Tabla 5.

Tabla 5. Cultivos mixtos de levaduras no-*Saccharomyces* y *S. cerevisiae* propuestos para la elaboración del vino (tomado de Ciani *et al.,* 2010).

Especies empleadas	Objetivo	Proceso	Referencias
S. cerevisiae *T. delbrueckii*	Reducción en la producción de ácido acético	Cultivos secuenciales	Castelli (1969); Herraiz *et al*., (1990); Ciani *et al*., (2006); Salmon *et al*., (2007); Bely *et al*., (2008)
S. cerevisiae *S. pombe*	Degradación del ácido málico	Cultivos secuenciales Células inmovilizadas (proceso en batch) Células inmovilizadas (proceso en continuo)	Snow y Gallender (1979); Magyar y Panyik (1989); Yokotsuka *et al*., (1993), Ciani (1995)
S. cerevisiae *C. stellata*	Aumento del contenido de glicerol	Células inmovilizadas (pretratamiento o cultivos secuenciales)	Ciani y Ferraro (1996); Ciani y Ferraro (1998); Ferraro *et al.,* (2000)
S. cerevisiae *C. cantarelli*	Aumento del contenido de glicerol	Cultivos mixtos o secuenciales	Toro y Vázquez (2002)
S. cerevisiae *C. stellata*	Mejora del perfil aromático del vino	Cultivos mixtos o secuenciales	Soden *et al.,* (2000)
S. cerevisiae *H. uvarum (K. apiculata)*	Simulación de la fermentación natural (mejora de la complejidad aromática)	Cultivos mixtos o secuenciales	Herraiz *et al.,* (1990); Zironi *et al.,* (1993); Moreira (2005); Ciani *et al.,* (2006); Moreira *et al.,* (2008); Mendoza *et al.,* (2007)

Tabla 5 (continuación)

S. cerevisiae *K. thermotolerans*	Reducción de la producción de ácido acético Aumento de la acidez total	Cultivos secuenciales	Mora *et al.,* (1990); Ciani *et al.,* (2006); Kapsopoulou *et al.,* (2007)
S. cerevisiae *Issatchenkia orientalis*	Reducción del contenido de ácido málico	Fermentación mixta	Kim *et al*., (2008)
S. cerevisiae *Pichia fermentans*	Mayor complejidad aromática	Cultivos secuenciales	Clemente-Jiménez *et al.,* (2005)
S. cerevisiae *Pichia kluyveri*	Incremento de tioles varietales	Fermentación mixta	Anfang *et al.,* (2009)
S. cerevisiae *Candida pulcherrima*	Mejora del perfil aromático del vino	Fermentación mixta	Zohre y Erten (2002); Jolly *et al.,* (2003)
S. cerevisiae *Debaryomyces vanriji*	Incremento de la concentración de geraniol	Fermentación mixta	García *et al.,* (2002)
S. cerevisiae *Schizosaccharomyces* spp. *Saccharomyces* spp. *Pichia* spp.	Influencia en las propiedades sensoriales y físico-químicas del vino	Etapas de envejecimiento durante la maduración del vino	Palomero *et al.,* (2009)

1.3.1 Interacciones entre las levaduras integrantes del cultivo iniciador

La bibliografía existente pone de manifiesto que el comportamiento enológico de las levaduras no-*Saccharomyces* no es igual en cultivo puro que en presencia de *S. cerevisiae*, lo cual se debe a las interacciones entre las levaduras integrantes del cultivo iniciador. De hecho, se ha demostrado que cuando las levaduras se desarrollan juntas en condiciones de fermentación, no coexisten de forma pasiva, sino que interactúan y producen compuestos impredecibles y/o diferentes cantidades de

productos de fermentación, los cuales pueden afectar a la composición química y aromática de los vinos (Howell *et al.,* 2006; Anfang *et al.,* 2009).

Estas interacciones, tanto fisiológicas como metabólicas, se concretan, entre otras, en el propio crecimiento de las levaduras, variaciones en el grado de floculación y formación de compuestos volátiles. Por lo que se refiere al primero de ellos, se ha demostrado que si bien se reduce la densidad celular máxima alcanzada por ambos tipos de levadura en la fermentación conjunta con respecto a sus cultivos puros, aumenta la viabilidad celular y permanencia de las no-*Saccharomyces* en las fermentaciones donde se emplearon los cultivos mixtos (Ciani *et al.,* 2006; Mendoza *et al.,* 2007). Respecto a la floculación, en cultivos mixtos de una cepa floculante de *K. apiculata* con una cepa no floculante de *S. cerevisiae*, la primera induce la co-floculación de ambas (Sosa *et al.,* 2008). Por último, en cuanto a la formación de compuestos volátiles, se observó un aumento en las concentraciones de ésteres en comparación con las fermentaciones puras (Garde-Cerdán y Ancín-Azpilicueta, 2006) lo que también ha sido descrito en fermentaciones mixtas de levaduras apiculadas y *S. cerevisiae* (Moreira *et al*., 2008). Estos últimos autores confirmaron que en el caso del acetato de etilo, éster que a concentraciones elevadas tiene un impacto negativo sobre la calidad aromática del vino, su concentración se reducía en aquellos vinos fermentados en cultivo mixto, dato que ya había sido descrito por Rojas *et al*., (2003). Además, resulta de interés resaltar que la presencia de las levaduras no-*Saccharomyces* no afectó la formación de ésteres etílicos por parte de *S. cerevisiae* (Rojas *et al*., 2003).

1.3.2 Implantación de los cultivos iniciadores mixtos

Además de tener en cuenta las posibles interacciones entre los componentes del cultivo iniciador mixto, resulta imprescindible determinar la influencia de la microbiota autóctona en la implantación de dicho cultivo. De hecho, la implantación de un cultivo iniciador, aunque se trate de una

cepa comercial de *S. cerevisiae* inoculada en forma de LSA, no está en absoluto garantizada. Diversos estudios han demostrado que varias cepas de esta levadura, inoculadas como cultivos iniciadores, no son capaces de competir con éxito frente a las cepas indígenas, no siendo por tanto las responsables de la fermentación alcohólica (Querol *et al*., 1992; Schutz & Gafner, 1994; Constanti *et al*., 1998; Egli *et al*., 1998; Gutiérrez *et al*., 1999; Ganga y Martínez, 2004; Santamaría *et al*., 2005; Capece *et al*., 2010). Esta situación tiene consecuencias prácticas muy importantes, ya que el coste económico que supone la inoculación de LSA no va acompañado de un control real de la fermentación.

A pesar de los numerosos estudios llevados a cabo sobre la ecología de las fermentaciones tanto inoculadas como espontáneas, no es fácil entender las causas por las que una determinada cepa de levadura no es capaz de competir con la microbiota presente en el mosto y la propia de la bodega. No hay que olvidar la complejidad microbiológica de las fermentaciones de mosto de uva, donde por ejemplo, en el caso *de S. cerevisiae* se han llegado a describir la sucesión de más de 10 cepas en una única fermentación (Sabaté *et al*., 1998; Pramateftaki *et al*., 2000; Cocolin *et al*., 2004; Ganga y Martínez, 2004; Sipiczki *et al*., 2004; Santamaría *et al*., 2005). En el caso de levaduras no-*Saccharomyces* también ha sido descrita esta evolución de cepas a lo largo de la fermentación (Schutz y Gafner, 1994; Povhe-Jemec *et al*., 2001).

Todos estos estudios se han podido realizar gracias al desarrollo de las técnicas moleculares, que permiten la diferenciación tanto de especies como de cepas de levadura a lo largo del proceso fermentativo. Las técnicas moleculares que más se han utilizado para diferenciar las distintas especies de levaduras vínicas son la electroforesis de cromosomas (Nadal *et al*., 1996; Schütz y Gafner, 1993), análisis de restricción de la región 5.8 S-ITS (Rodríguez *et al*., 2004; Granchi *et al*., 1999; Pramateftaki *et al*., 2000; Torija *et al*., 2001), análisis de restricción de otras regiones ribosomales (Van Keulen *et al*., 2003) o la combinación de más de una técnica, como RAPD y mtDNA (Torriani *et al*., 1999). De entre ellos

destaca por su sencillez la amplificación por PCR de las regiones antes nombradas del DNA ribosomal y posterior restricción de los amplificados. En la Figura 4 se esquematiza esta técnica que se caracteriza por su fácil manipulación y su reproducibilidad.

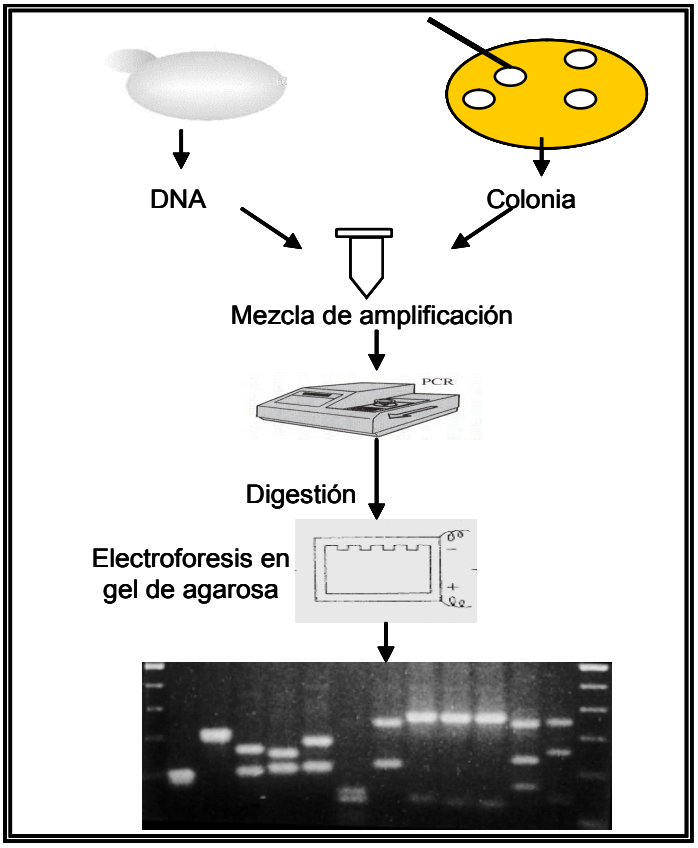


Figura 4. Método basado en el análisis de regiones ribosomales mediante amplificación por PCR y posterior restricción (tomado de Fernández-Espinar *et al*., 2005).

Para la diferenciación a nivel de cepa se pueden utilizar diversas técnicas entre las que se incluyen la electroforesis de cariotipos (Blondin y Vezinhet, 1998; Yamamoto *et al*., 1991), análisis de restricción del mtDNA (Querol *et al*., 1992; Schuller *et al*., 2004), amplificación de elementos δ

(Legras y Karst, 2003) y microsatélites (González Techera *et al*., 2001). En general es necesaria la combinación de varias de estas técnicas moleculares para la caracterización definitiva de aislados (Fernández-Espinar *et al*., 2001).

Estas herramientas han permitido asociar determinadas cepas autóctonas con características sensoriales de los vinos, así como establecer su sensibilidad al SO_2 (Egli *et al*., 1998). También se han aplicado para analizar la influencia que determinadas cepas de *S. cerevisiae* inoculadas en distintos porcentajes tienen sobre la supervivencia de las levaduras no-*Saccharomyces,* y la relación con la concentración de determinados metabolitos (Erten *et al*., 2006).

De todo lo expuesto hasta ahora queda demostrado el potencial de los cultivos iniciadores mixtos basados en levaduras no-*Saccharomyces* seleccionadas por sus características positivas. Queda también establecida la necesidad de considerar las interacciones entre las especies del iniciador así como su implantación en mostos naturales. Sin embargo a pesar de todos estos estudios científicos el enólogo es todavía reacio a trabajar con levaduras no-*Saccharomyces,* ya que las sigue asociando a alteraciones organolépticas del vino. Este hecho puede ser una de las causas de que en la actualidad solamente dos empresas comercialicen cultivos iniciadores mixtos comerciales en formato LSA, por un lado uno que contiene *S. cerevisiae, K. thermotolerans* y *T. delbrueckii* (Vinflora ® Harmony.nsac; Christian Hansen) y otro comercializado por Lallemand con la mezcla de esta última levadura y *S. cerevisiae* (Level2^{TM} TD; www.lallemandwine.com). Desde el punto de vista de la aplicabilidad en bodega, resultaría más atractivo un cultivo iniciador con los porcentajes mínimos de levadura no-*Saccharomyces* que permitieran obtener el efecto deseado y a la vez modular la supervivencia de la levadura. Otra posibilidad sería la utilización de levaduras no-*Saccharomyces* inmovilizadas que pudieran ser retiradas del tanque de fermentación una vez conseguido el efecto buscado.

1.3.3 Cultivos iniciadores inmovilizados

Los microorganismos inmovilizados presentan ciertas ventajas entre las que cabe destacar su posible reutilización, el control preciso del tiempo de permanencia en el depósito de fermentación y la reducción del riesgo de contaminación microbiana debido a la alta densidad celular y actividad fermentativa (Kourkoutas *et al*., 2004).

En enología, una de las técnicas más empleadas para la inmovilización de las levaduras es la inclusión. Esta técnica consiste en entrampar los microorganismos en un gel o cápsula que impida la salida de la célula fuera de éstos, aunque permitiendo la entrada y salida del medio a través del soporte, lo que conduce al desarrollo de la transformación biológica deseada. En concreto, los geles de alginato cálcico son considerados los más apropiados para su uso en la fermentación alcohólica (Colagrande *et al.,* 1994), aunque las sales de sodio, calcio y bario de alginatos también han sido utilizadas para el entrampamiento celular. En la Tabla 6 se recogen algunas de las aplicaciones de microorganismos inmovilizados en la elaboración del vino.

Tabla 6. Técnicas y soportes de inmovilización celular que han sido propuestos en la elaboración del vino (tomado de Kourkoutas *et al.,* 2004).

Microorganismo	Soporte de inmovilización	Empleo	Producto	Referencias
S. cerevisiae + *S. cerevisiae f.r. bayanus*	Esferas de alginato	Fermentación alcohólica	Vino espumoso	Fumi *et al*., (1987)
S. pombe	Esferas de alginato de doble capa	Fermentación maloláctica	Mosto de uva desacidificado	Taillandier *et al*., (1994)
S. cerevisiae	Membranas de microfiltración	Fermentación alcohólica secundaria	Vino espumoso	Lemonnier y Duteurtre, (1989); Ramón-Portugal *et al*., (2003)

Tabla 6 (continuación)

S. cerevisiae	Material celulósico delignificado	Fermentación alcohólica	etanol; vino	Bardi y Koutinas, (1994)
S. cerevisiae	Hollejos de uva	Fermentación alcohólica	Vino	Mallouchos *et al*., (2002)
S. cerevisiae	Trozos de manzana	Fermentación alcohólica	Vino	Kourkoutas *et al*., (2001; 2002a)
S. cerevisiae	Microfiltros a contraflujo	Crecimiento celular; Fermentación alcohólica	Biomasa; vino	Takaya *et al*., (2002)
S. cerevisiae	Pellets de gluten	Fermentación alcohólica a baja temperatura	Vino	Iconomopoulou *et al*., (2002)
S. cerevisiae	Alginato cálcico	Prevenir paradas de fermentación	Vino	Silva *et al*., (2002a)
S. cerevisiae	Alginato cálcico	Fermentación alcohólica secundaria	Vino espumoso	Silva *et al*., (2002b; 2003)
S. cerevisiae	Trozos de membrillo	Fermentación alcohólica a baja temperatura	Vino	Kourkoutas *et al*., (2003b)
C. stellata	Esferas de alginato	Fermentación alcohólica	Vino	Ferraro *et al*., (2000)
L. casei	Pectato de calcio; kitosán modificado	Fermentación maloláctica	Vino	Kosseva *et al*., (1998)

Como se observa en la Tabla, la inmovilización celular de levaduras no-*Saccharomyces* para su uso en vinificación está poco explotada. En concreto, esta técnica se ha aplicado a *C. stellata*, para aumentar la concentración de glicerol en vino (Ciani y Ferraro, 1996), y a *Schizo.*

pombe (Silva *et al.,* 2003) y a *Issatchenkia orientalis* (Hong *et al*., 2010) para degradar ácido málico en mosto y vino.

En el caso de *C. stellata*, esta levadura se ha aplicado inmovilizada tanto en cultivo puro como en combinación con *S. cerevisiae*. Estos estudios mostraron que las células de *C. stellata* inmovilizadas en geles de alginato cálcico fueron capaces de incrementar aproximadamente 30 veces su velocidad de fermentación y la concentración de etanol, mientras que redujeron la producción de acetaldehído y acetoína comparadas con las células libres (Ciani y Ferraro ,1996). En estudios posteriores (Ferraro *et al.*, 2000) se llevaron a cabo fermentaciones secuenciales a escala piloto y en condiciones no estériles de vinificación, para lo cual se emplearon células inmovilizadas de *C. stellata* y libres de *S. cerevisiae,* estas últimas inoculadas al tercer día de fermentación. Se evaluó la dinámica poblacional de las levaduras y su influencia en el perfil analítico del vino, observándose que aunque la actividad de las levaduras indígenas no fue completamente suprimida, se incrementó en un 70% el contenido en glicerol de los vinos.

Por lo que se refiere a la degradación de ácido málico, Silva *et al.,* (2003) emplearon una cepa inmovilizada de *Schizo. pombe* al inicio de la fermentación. Una vez obtenido el efecto deseado, se retiraron las esferas de alginato y se inoculó *S. cerevisiae* para completar la fermentación alcohólica. La evaluación sensorial mostró que los vinos obtenidos empleando *Schizo. pombe* tuvieron una mejor calidad organoléptica que los vinos sin desacidificar. Los análisis de algunos compuestos aromáticos tales como SH_2, acetaldehído, metanol, isopropanol y alcoholes amílicos e isoamílicos al final de la fermentación alcohólica no mostraron diferencias significativas en comparación con la fermentación control. Además, los tests de calidad de las esferas de alginato mostraron que las células inmovilizadas podrían ser recicladas hasta cinco veces sin la liberación de células de levadura al medio. Recientemente se ha descrito la inmovilización de una cepa de *Issatchenkia orientalis* capaz de reducir en un 90% la concentración de ácido málico presente en vino (Hong *et al*., 2010).

En la actualidad, la empresa portuguesa Proenol ofrece cuatro productos basados en levaduras inmovilizadas. Tres de ellos denominados ProElif, ProDessert y ProRestart corresponden a cepas de *S. cerevisiae*, mientras que el cuarto, denominado ProMalic corresponde a una cepa de *Schizo. pombe.* Estas levaduras son suministradas a las bodegas para su inclusión en unos sacos permeables que permiten una fácil extracción y uso de los mismos durante o una vez finalizado el proceso fermentativo, bien de los depósitos o de las barricas en caso de fermentaciones en madera, tal y como se aprecia en la Figura 5.

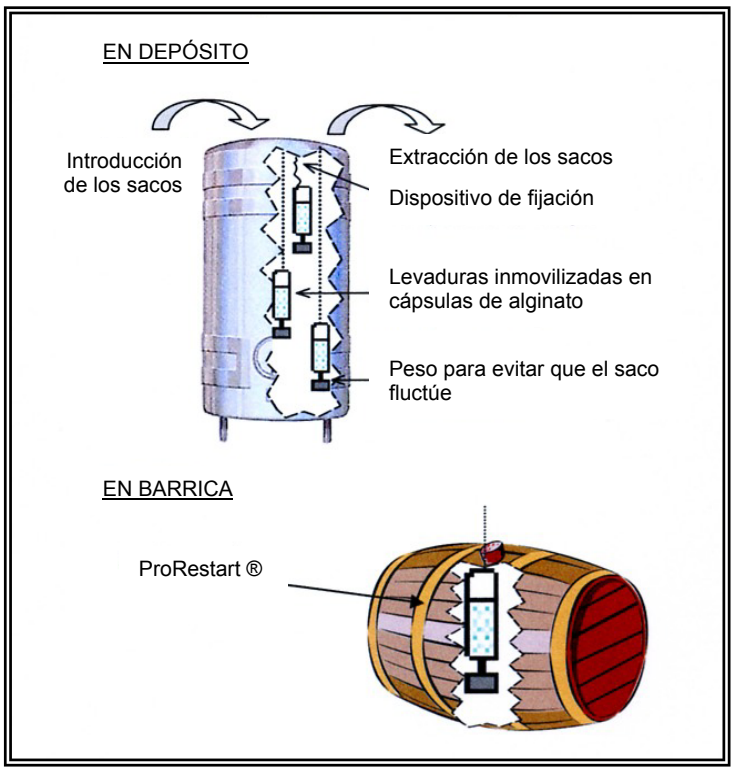


Figura 5. Ejemplos de aplicación de levaduras inmovilizadas en geles de alginato y su empleo en depósitos de fermentación y barricas de roble introducidas en sacos permeables (tomada de Proenol, Industria Biotecnológica Lda., Portugal).

2. Objetivos

En la actualidad, la biotecnología enológica busca la obtención de vinos más atractivos y complejos desde el punto de vista organoléptico con el objetivo de satisfacer las demandas del mercado. En este contexto, el presente trabajo pretende evaluar el potencial de las levaduras no-*Saccharomyces* como productoras de ésteres de acetato y su inclusión en cultivos iniciadores mixtos, capaces no sólo de llevar a cabo la fermentación alcohólica sino de introducir características aromáticas diferenciales en los vinos obtenidos. Los objetivos parciales se detallan a continuación:

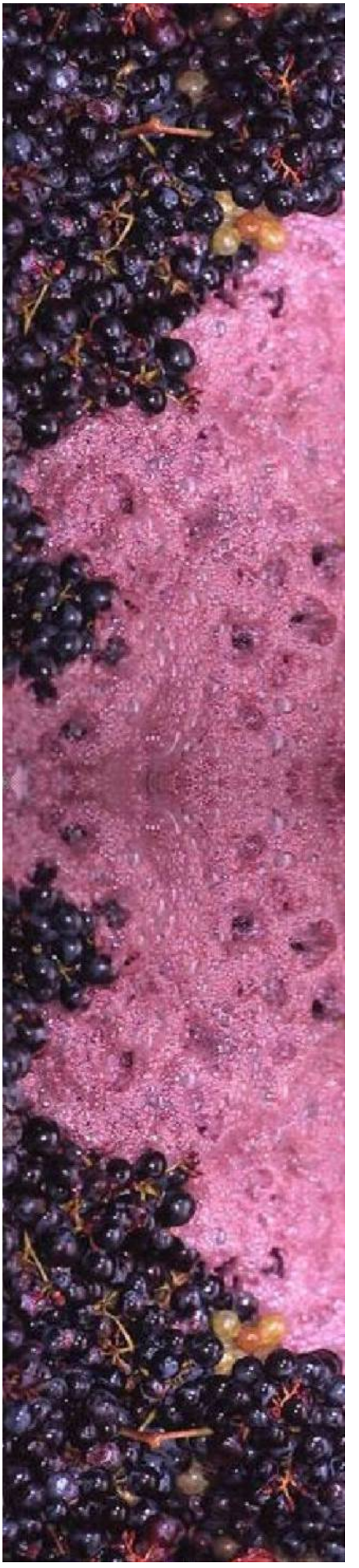
Objetivo 1: selección de levaduras no-*Saccharomyces*, para ser posteriormente incluidas en cultivos iniciadores mixtos, basada en el perfil de ésteres de acetato producidos tanto en medio microbiológico como en mosto de uva, y en la caracterización de sus propiedades enológicas.

Objetivo 2: diseño de cultivos iniciadores mixtos empleando levaduras seleccionadas en el objetivo anterior junto con *S. cerevisiae*, estudiando su comportamiento en ensayos de microvinificación. Se evaluará principalmente la producción de acetato de 2-feniletilo, así como su modulación variando en el cultivo mixto los porcentajes de las levaduras integrantes.

Objetivo 3: para simular condiciones habituales de vinificación, se estudiará la implantación de los cultivos iniciadores mixtos en mostos naturales no estériles, comparando dos tipos de inoculación, secuencial y conjunta.

Objetivo 4: aplicación de la técnica de inmovilización de la levadura no-*Saccharomyces* seleccionada, con el fin de controlar su permanencia y, consecuentemente, modular la concentración final de acetato de 2-feniletilo en vino.

3. Resultados y discusión



Esta tesis es una compilación de una serie de publicaciones que se indican a continuación. El presente apartado de *resultados y discusión* está formado por tales publicaciones introducidas como tal en su orden correspondiente. Además, al final del apartado se incluye una discusión general que engloba y enlaza todos los temas tratados en cada uno de los artículos:

I. Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. **Fernando Viana**, José V. Gil, Salvador Genovés, Salvador Vallés and Paloma Manzanares. *Food Microbiology* 25: 778-785, 2008.

II. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. **Fernando Viana**, José V. Gil, Salvador Vallés and Paloma Manzanares. *International Journal of Food Microbiology* 135: 68-74, 2009.

III. Monitoring a mixed starter of *Hanseniaspora vineae*-*Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. **Fernando Viana**, Carmela Belloch, Salvador Vallés and Paloma Manzanares. Artículo aceptado en *International Journal of Food Microbiology* (DOI: 10.1016/j.ijfoodmicro.2011.09.005).

IV. 2-Phenylethyl acetate formation by immobilized cells of *Hanseniaspora vineae* in sequential mixed fermentations. **Fernando Viana**, Patricia Taillandier, Salvador Vallés, Pierre Strehaiano and Paloma Manzanares. *American Journal of Enology and Viticulture* 62: 122-126, 2011.

Artículo I

Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits

ABSTRACT

Thirty-eight yeast strains belonging to the genera *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* were screened for ester formation on synthetic microbiological medium. The genera *Hanseniaspora* and *Pichia* stood out as the best acetate ester producers. Based on ester profile *Hanseniaspora guilliermondii* 11027 and 11102, *Hanseniaspora osmophila* 1471 and *Pichia membranifaciens* 10113 and 10550 were selected for further characterization of enological traits. When growing on must *H. osmophila* 1471, which displayed a glucophilic nature and was able to consume more than 90 % of initial must sugars, produced levels of acetic acid, medium chain fatty acids and ethyl acetate within the ranges described for wine. On the other hand it was found to be a strong producer of 2-phenylethyl acetate. Our data suggest *H. osmophila* 1471 is a good candidate for mixed starters, although the possible interactions with *S. cerevisiae* deserve further research.

Keywords: non-*Saccharomyces*, yeast selection, *Hanseniaspora*, 2-phenylethyl acetate, mixed starters.

1. Introduction

Nowadays a number of viticultural and winemaking practices are being investigated to improve wine quality. In this context there is a growing demand for new and improved wine yeast strains adapted to different types and styles of wines. Industrial wine fermentations are currently conducted by starters of selected wine yeast strains of *Saccharomyces cerevisiae* in contrast to traditional spontaneous fermentations conducted by the flora present on the grapes and in the winery. Despite the advantages of using pure cultures of *S. cerevisiae* with regard to the easy of control and homogeneity of fermentations, wine produced with pure yeast monocultures lacks the complexity of flavour, stylistic distinction and vintage variability caused by indigenous yeasts (Lambrechts and Pretorius, 2000; Romano et al., 2003). In recent years the inclusion of non-*Saccharomyces* wine yeast species as part of mixed starters together with *S. cerevisiae* to improve wine quality has been suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Jolly et al., 2003; Romano et al., 2003; Rojas et al., 2003; Ciani et al., 2006).

Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage (Sponholz, 1993; Ciani and Picciotti, 1995) the potential positive role they play in the organoleptic characteristics of wine has been emphasized in numerous studies (reviewed in Fleet, 2003). Metabolic interactions between non-*Saccharomyces* wine yeasts and *S. cerevisiae* during fermentation could positively or negatively interfere with the growth and fermentation behaviour of yeast species, particularly *S. cerevisiae*. In this context, positive interactions between fructophilic non-*Saccharomyces* yeasts and glucophilic *S. cerevisiae* strains have been described (Ciani and Fatichenti, 1999). By contrast, negative interactions have been reported, caused by the production of compounds that inhibit to *S. cerevisiae* such as medium-chain fatty acids or killer factors (Bisson, 1999). Given the strain biodiversity of non-*Saccharomyces* yeasts in regard

to their production levels of enzymatic activities (Manzanares et al., 1999, 2000; Mendes-Ferreira et al., 2001; Strauss et al., 2001) and fermentation metabolites (Romano et al., 1992, 2003; Capece et al., 2005) of enological importance, suitable strains should be selected in order to be able to design mixed starters capable of provide beneficial contributions to wine quality.

Among fermentation metabolites, it is generally described that esters make the greatest contribution to the characteristic fruity odours of wine fermentation bouquet (Rapp and Mandery, 1986). Acetate esters such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate, recognised as important flavour compounds in wine and other grape-derived alcoholic beverages, can be formed in relatively high concentrations by non-*Saccharomyces* wine yeasts (Rojas et al., 2001, 2003). In the present study we characterize the ester profile of non-*Saccharomyces* wine yeast strains when grown in synthetic medium and verify their fermentation behaviour in must with the final aim of selecting those strains of biotechnological interest to be included in wine mixed starters.

2. Materials and methods

2.1 Yeast strains and culture media

A total of 38 yeast strains mainly isolated from grapes and wines belonging to the genera *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* (Table 1) were obtained from the Spanish Type Culture Collection (CECT). Nine commercial *Saccharomyces cerevisiae* wine yeast strains, Fermol Primeurs, Fermol Rouge, Fermol Bouquet and Fermol Clarifiant (AEB Group, Brescia, Italy), Lalvin T73 (Lallemand Inc, Rexdale, Ontario), UCLM S377 (Springer Oenologie, Bio Springer, Maisons-Alfort, France), Uvaferm CEG (Danstar Ferment AG, Zug, Switzerland), Fermiblanc Arom and Fermicru Primeur (DSM Oenology, Delft, The Netherlands) were also included in the present study. Commercial strains were rehydrated following the supplier's protocol. Yeasts strains were maintained on GPY plates (5 g yeast extract, 5 g peptone, 40 g glucose, 20 g agar per litre, pH 5.5). For ester formation in microbiological medium yeast strains were grown in 40 mL of GPYM medium (containing 5 g yeast extract, 5 g peptone, 40 g glucose, 5 g malt extract, 1 g $MgSO_4 \cdot 7H_2O$ per litre, pH 6) in 100 mL flasks at 30°C and 200 rpm. Cultures were inoculated with 10^6 cells per mL from 24-48 h pre-cultures grown in the same medium.

Table 1. Yeast species used in this study.

Species	CECT number	Work Code
Candida cantarelli	11150	1
Candida cantarelli	11170	2
Candida dattila	10387	3
Candida dattila	10559	4
Candida dattila	1962	5
Candida stellata	11046	6
Candida stellata	11109	7
Candida stellata	11110	8
Hanseniaspora guilliermondii	11027	9
Hanseniaspora guilliermondii	11102	10
Hanseniaspora osmophila	1471	11
Hanseniaspora uvarum	10389	12
Hanseniaspora uvarum	11105	13
Hanseniaspora uvarum	11106	14
Hanseniaspora uvarum	11107	15
Pichia anomala	10410	16
Pichia anomala	10571	17
Pichia anomala	10572	18
Pichia anomala	10590	19
Pichia anomala	10591	20
Pichia anomala	10593	21
Pichia anomala	10594	22
Pichia fermentans	10064	23
Pichia membranifaciens	10037	24
Pichia membranifaciens	10113	25
Pichia membranifaciens	10550	26
Pichia membranifaciens	10565	27
Pichia membranifaciens	10568	28
Pichia membranifaciens	10569	29
Pichia membranifaciens	10570	30
Torulaspora delbrueckii	1879	31
Torulaspora delbrueckii	1880	32
Torulaspora delbrueckii	10558	33
Torulaspora delbrueckii	10589	34
Torulaspora delbrueckii	10676	35
Zygosaccharomyces bailii	11042	36
Zygosaccharomyces rouxii	11136	37
Zygosaccharomyces rouxii	11189	38

2.2 Analytical determinations

To measure ester, fatty acid and higher alcohol formation by yeast strains culture aliquots were taken (at 24 h of growing in GPYM medium or at the end of wine fermentation) and analysed by headspace solid-phase microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-Aldrich, Barcelona, Spain) and gas chromatography (GC) as described by Ortiz-Serrano and Gil (2007) with some modifications. Aliquots of 3 mL of the samples and 0.6 g of NaCl were placed in 7 mL vials and 2 µg of 2-heptanone (Sigma Chemical Co., St. Louis, MO) as internal standard were added. The vials were closed with screwed caps and 1.5-mm thick teflon septa. Solutions were stirred for 1 h at 25°C to get the required headspace-liquid equilibrium. PDMS fibres were injected to the vial septum and exposed to the headspace for 30 min at 25°C and then desorbed during 4 min in an HP 5890 series II gas chromatograph equipped with a HP-VOC column (Agilent, Englewood, CO, USA) (length, 30 m; inside diameter 0.20 mm; film thickness, 0.10 µm). The injection block and detector temperatures were kept constant at 220 and 300°C, respectively. The oven temperature was programmed as follows: 40°C (10 min) to 150°C at 1.5°C per min, to 170°C at 20°C per min and to 250°C at 20°C per min and then kept at 250°C for 2 min. Ester, fatty acids and higher alcohol concentrations were calculated using standard solutions (Fluka, Buchs, Switzerland) and are given as the mean of three independent cultures or two independent vinifications.

2.3 Enzymatic activities

2.3.1 Sulphite reductase activity

The H_2S-production potential of the yeasts was determined by plating the yeasts onto a solid juice indicator agar (Strauss et al., 2001). After 24-48 h of incubation at 30°C, a low H_2S-producing colony was

identified by its white colour whereas a high H_2S-producing colony had a black colour.

2.3.2 Hydroxycinnamic acid decarboxylase activity

Decarboxylation of ferulic and ρ-coumaric acids by yeasts was determined following the protocol described by Prim et al. (2002) with some modifications. Detection of activity was performed using YPD plates containing 0.01% (w/v) bromocresol purple (Sigma Aldrich) supplemented with 0.145% (w/v) ferulic or ρ-coumaric acids (Fluka). Aliquots (10 µL) of cell extracts prepared in 10 mM phosphate buffer pH 7 from 24h must cultures were laid on the surface of the plates and incubated for 1-2 h at 37°C. Hydroxycinnamic acid decarboxylase activity can be detected by a colour shift from yellowish to purple as a result of a pH increase due to the decarboxylation of the hydroxycinnamic acid, which leads to an alkalization of the sample environment.

2.4 Vinifications

Duplicate vinifications were carried out in grape must from Muscat cultivar (Godelleta, Valencia, Spain) with an initial sugar content of 200 g/L and supplemented with 1 g/L of a complex yeast nutrient (Fermaid K, Lallemand). Fresh must was treated by adding 1 mg/L of dimethyl di-carbonate (Fluka) and stored at -20°C until use. Aliquots of 90 mL of must were fermented in 100 mL bottles at 20°C. Musts were inoculated with 10^6 cells per mL from 24 h pre-cultures grown in the same must. Fermenting musts were sampled to enumerate yeast populations by plating on GPY medium (containing 5 g yeast extract, 5 g peptone, 40 g glucose and 20 g agar per litre, pH 5.5) and incubated at 30°C for 72 h. Ester, fatty acid and higher alcohol concentrations were determined as specified above and given as the mean of two independent vinifications.

2.5 Enological parameters

Glucose and fructose consumption throughout the fermentation process as well as the concentration of glycerol, acetaldehyde and acetic acid in wines were measured enzymatically in an Echo-Enosys analyzer (Tecnova, San Sebastián de los Reyes, Spain) following the supplier's instructions. Ethanol concentration in wines was determined using the R-Biopharm enzymatic assay (R-Biopharm AG, Darmstadt, Germany).

2.6 Statistical analysis

Fisher's least significant difference procedure (LSD) was used for mean separation (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

3. Results and discussion

3.1 Ester production in liquid microbiological medium

The commonly held opinion is that ester production during wine fermentation contributes significantly to the desirable fermentation bouquet of wine and that it is closely related to the particular yeast species involved. Non-*Saccharomyces* wine yeasts, known as good producers of esters, have traditionally been associated with the negative effects of high ethyl acetate formation and few studies have focused on the so-called fruity acetate esters, such as isoamyl acetate (banana-like aroma) and 2-phenylethyl acetate (fruity and flowery flavour). Previous studies showed different *Hanseniaspora guilliermondii* strains to be strong producers of 2-phenylethyl acetate and ethyl acetate in both synthetic microbiological medium and must (Rojas et al., 2001, 2003; Moreira et al., 2005). With the aim of further selecting the non-*Saccharomyces* wine yeasts able to contribute positively to wine aroma we have screened 38 yeast strains for ester production in liquid microbiological medium.

Different studies have shown the production of certain metabolites depends on the yeast species. Here we have grouped ester production by yeast genera and carried out a statistical analysis of the main esters formed as shown in Table 2. For better comparison, nine commercial *S. cerevisiae* strains were included in the study. Interestingly there are some significant differences among yeast genera. Results show the genus *Hanseniaspora* to be the best acetate ester producer, which stands out given the production of 2-phenylethyl acetate. The genus *Pichia* showed similar ethyl acetate levels to *Hanseniaspora* and was the second best producer of isobutyl acetate and isoamyl acetate. There were no significant differences among the genera *Candida*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* for acetate ester production.

Table 2. Mean values and standard deviations of ester concentrations (mg/L) produced by yeast genera*.

Genus (number of strains)	Ethyl acetate	Isobutyl acetate	Isoamyl acetate	2-Phenylethyl acetate	Ethyl caproate	Ethyl caprilate
Candida (8)	4.91 ± 9.80[a]	0.009 ± 0.004[a]	0.035 ± 0.032[a]	0.011 ± 0.010[a]	0.002 ± 0.001[a]	0.0014 ± 0.0006[a]
Hanseniaspora (7)	223 ± 132[b]	0.171 ± 0.097[c]	1.639 ± 1.805[c]	12.68 ± 20.39[b]	0.003 ± 0.001[a]	0.0021 ± 0.0010a
Pichia (15)	204 ± 129[b]	0.067 ± 0.048[b]	0.630 ± 0.570[b]	0.563 ± 0.611[a]	0.007 ± 0.005[a]	0.0012 ± 0.0007[a]
Saccharomyces (9)	1.42 ± 0.72[a]	0.023 ± 0.008[a]	0.239 ± 0.081[ab]	0.143 ± 0.061[a]	0.026 ± 0.018[b]	0.0012 ± 0.0006[a]
Torulaspora (5)	3.87 ± 1.84[a]	0.012 ± 0.010[a]	0.023 ± 0.011[a]	0.013 ± 0.005[a]	0.009 ± 0.014[a]	0.0052 ± 0.0092[b]
Zygosaccharomyces (3)	0.91 ± 0.56[a]	0.012 ± 0.001[ab]	0.033 ± 0.024[ab]	0.412 ± 0.256[a]	n.d.	0.0006 ± 0.0003[a]

*Mean values for three independent experiments. N.d.: not detected. Data with the same letter do not differ at 95% confidence level (LSD procedure).

With respect to ethyl esters the genus *Saccharomyces* was the best producer of ethyl caproate, whereas the genus *Torulaspora* stood out for ethyl caprilate formation. There were no significant differences among the genera *Candida*, *Hanseniaspora*, *Pichia* and *Zygosaccharomyces* for ethyl ester production.

With the aim of selecting those non-*Saccharomyces* yeast strains that produce the highest levels of 2-phenylethyl acetate and isoamyl acetate, but that also avoid an excessive formation of ethyl acetate, yeast strains were arranged from high to low levels of ethyl acetate production as shown in Figure 1. The best producers of 2-phenylethyl acetate corresponded to *H. osmophila* 1471 (work code 11, see Table 1; 37 mg/L) and *H. guilliermondii* strains 11102 (code 10; 13 mg/L) and 11027 (code 9; 3.5 mg/L). The four strains tested of *H. uvarum* (code 12-15) did not produced 2-phenylethyl acetate. Interestingly strains producing the highest levels of 2-phenylethyl acetate were also the best producers of isoamyl acetate together with *P. membranifaciens* 10550 (code 26). Based on these results *H. osmophila* 1471 and both *H. guilliermondii* strains 11102 and 11027 were selected for further studies. Moreover these strains produced different levels of ethyl acetate ranging from approximately 250 mg/L (*H. osmophila* 1471) to 100 mg/L (*H. guilliermondii* 11102). Also *P. membranifaciens* 10550 (code 26) and 10113 (code 25) were selected for their isoamyl acetate production (1.9 and 1.2 mg/L, respectively) although both reached levels of approximately 300 mg/L of ethyl acetate.

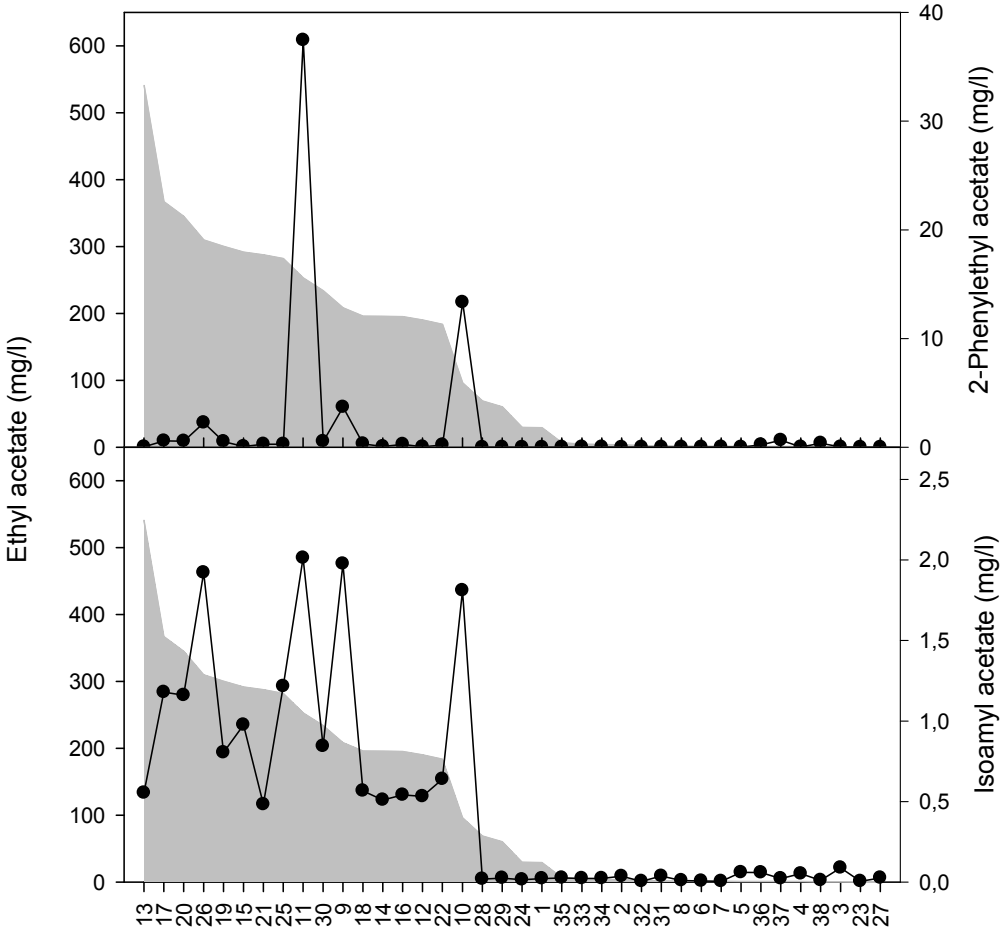


Fig. 1. Production of acetate esters by yeast strains. Panel A: 2-phenylethyl acetate (black circles). Panel B: isoamyl acetate (black squares). Ethyl acetate is represented in both panels as an area plot. For strain codes see Table 1.

3.2 Sulphite reductase and hydroxycinnamic acid decarboxylase activities

The production of wine off-flavours such as hydrogen sulphide and volatile phenolic compounds by *Saccharomyces* and non-*Saccharomyces* yeast strains has been previously reported (Shinohara et al., 2000; Mendes-Ferreira et al., 2002). Solid media containing grape juice and bismuth citrate are effective for visually screening the potential production of H_2S by wine related yeasts. The variation in colony colour intensity suggests significant differences in sulfite reductase activity. In our study from the five yeasts selected for their ability to produce esters, the two strains of *P. membranifaciens* did not produce H_2S (white colonies), whereas *H. guilliermondii* and *H. osmophila* strains proved higher producers (black colonies). The eight commercial *S. cerevisiae* wine yeast strains were also tested and all of them showed intermediate production of H_2S (brown colonies). Although bismuth containing indicator medium is an indication of the maximum genetically determined sulfite reductase activity for a given strain, the activity does not necessarily predispose a strain to excessive H_2S production in complete media (Jiranek et al., 1995) pointing out the importance of evaluating H_2S production using appropriate natural musts. Several studies have reported strain-dependent production of H_2S for the species *Candida stellata* and *Kloeckera apiculata* (Strauss et al., 2001) and also for commercial strains of *S. cerevisiae* (Mendes-Ferreira et al., 2002).

Production of vinyl- and ethylphenols can impart a phenolic off-odour in wine. Traditionally ethylphenol producers have been ascribed to the genus *Brettanomyces*/*Dekkera* whereas the production of vinylphenols varied among non-*Saccharomyces* and *Saccharomyces* wine yeasts (Chatonnet et al., 1992). We have screened potential decarboxylation of ferulic and ρ-coumaric acids into vinylphenols through hydroxycinnamic acid decarboxylase activity but have found that none of the yeast strains tested was able to decarboxylate either ferulic or ρ-coumaric acids under the conditions tested.

3.3 Wine fermentations

To characterize the fermentation pattern of selected yeasts, must inoculations were carried out and the evolution of yeast population and consumption of glucose and fructose monitored. *P. membranifaciens* 10550 was unable to grow in must 10 days after inoculation under the conditions tested and was thus discarded. Figure 2 (left panel) shows the growth of *H. guilliermondii* strains 11027 and 11102, *H. osmophila* 1471 and *P. membranifaciens* 10113 in must inoculated with pure cultures. The growth peaked at viable populations exceeding 10^7 cfu/mL but only *H. guilliermondii* 11102 viable population reached 10^8 cfu/mL. *H. osmophila* population dropped quickly and no viable cells were detected on day 10, whereas at the end of fermentation *H. guilliermondii* strains 11102 and 11027 and *P. membranifaciens* 10113 viable populations reached 10^6, 10^4 and 10^5 cfu/mL, respectively. As a general trend the population of *S. cerevisiae* strains reached 10^8-10^9 cfu/mL and kept practically constant around 10^7-10^8 cfu/mL till the end of the fermentation. A typical example of *S. cerevisiae* growth is shown in Figure 2 (panel E, right) for Lalvin T73.

The differences in glucose and fructose consumption among non-*Saccharomyces* wine yeasts selected for mixed starters could have a positive effect on the fermentation behaviour of *S. cerevisiae*. The latter usually displays a glucophilic nature and consequently residual sugar in fermented musts usually contains more fructose than glucose (Berthels et al., 2004). Apart of causing undesirable sweetness in dry wines, residual fructose may be responsible for sluggish fermentations (Gafner and Schütz, 1996). Figure 2 (right panel) shows the fermentation profiles measured by glucose and fructose consumption. The residual glucose plus fructose content varied from 100 g/L, in wines fermented by *P. membranifaciens* 10113 to a value of 12 g/L in *H. osmophila* 1471 wines. Even though the fermentation process started with approximately equal amounts of glucose and fructose, in wines produced by *H. osmophila* 1471 fructose was used up more slowly over the course of fermentation, leading to wines with a fructose (11 g/L) concentration significantly higher than glucose (1 g/L).

This discrepancy between glucose and fructose levels was not found in any other wine. These results suggest that under the conditions tested *H. osmophila* 1471 displays a glucophilic nature whereas the remaining strains consume glucose and fructose equally. Several wine strains of *H. osmophila* have also been described as glucophilic (Granchi et al., 2002) whereas *H. uvarum* and *H. guilliermondii* strains have been reported as fructophilic (Ciani and Fatichenti, 1999). In our experimental conditions all commercial yeast strains tested utilised glucose faster than fructose confirming its glucophilic nature (see Fig 2 panel E left for strain Lalvin T73). Although the consumption of fructose was slower along fermentation, final wines contained less than 1 g/L of sugar.

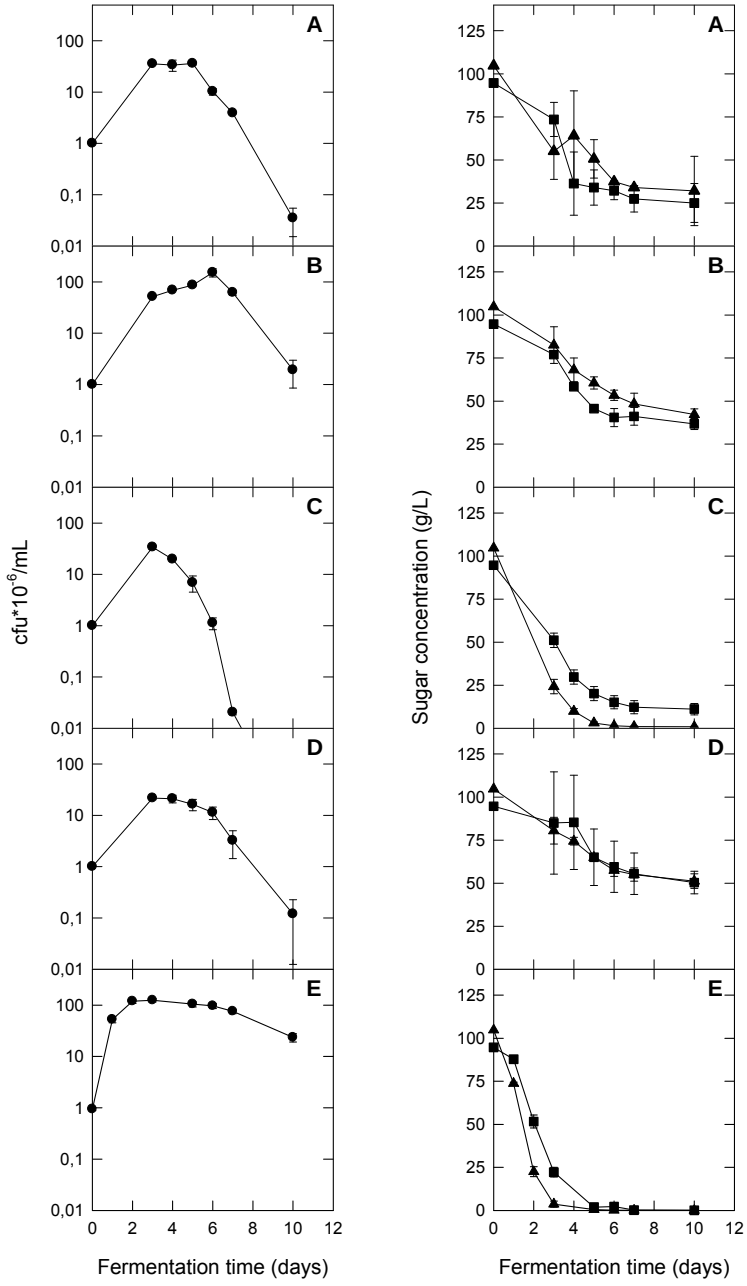


Fig. 2. Yeast population evolution (left panel) and consumption of glucose and fructose (right panel) in must inoculated with selected yeasts. A: *H. guilliermondii* 11027 (work code 9); B: *H. guilliermondii* 11102 (work code 10); C: *H. osmophila* 1471 (work code 11); D: *P. membranifaciens* 10113 (work code 25); E: *S. cerevisiae* Lalvin T73. Glucose: black triangles; fructose: black squares.

Another selection criterion concerned the production of certain fermentation metabolites that can be potentially detrimental to wine quality. Table 3 shows the effect of non-*Saccharomyces* yeasts on the concentrations of such metabolites. For better comparison the range of metabolite concentrations produced by the nine commercial *S. cerevisiae* strains has been included. As expected from sugar consumption there was a higher ethanol concentration in wines obtained by *H. osmophila* fermentation, whereas *P. membranifaciens* wines gave the lowest levels. There were no significant differences in the acetaldehyde concentration produced by the four yeast strains tested, similar to that found for the commercial *S. cerevisiae* strains and in the range of 6-190 mg/L found in wines (Then and Radler, 1971). Romano et al. (1997) found a range of acetaldehyde production for non-*Saccharomyces* wine strains of 2.5-81.5 mg/L in synthetic medium. Acetic acid becomes unpleasant at concentrations near its flavour threshold of 0.7-1.1 g/L and usually values between 0.2 and 0.7 g/L are considered optimal (Lambretchs and Pretorius, 2000). The highest quantity of acetic acid, approximately 1 g/L, was produced by *H. guilliermondii* 11102, whereas the other yeast strains yielded amounts around 0.6-0.7 g/L. The possible inhibitory effect of acetic acid against *S. cerevisiae* has been described, although the minimum inhibitory concentrations observed (4.5-7.5 g/L) were higher than the concentrations produced by the selected yeast strains (Edwards et al., 1999). Glycerol production ranged from 3.85 to 4.91 g/L, the best producers being *H. guilliermondii* 11027 and *H. osmophila* 1471. High levels of medium-chain fatty acids produced during alcoholic fermentation can inhibit yeast growth and cause stuck fermentations. No significant differences were found for hexanoic and decanoic acid levels among non-*Saccharomyces* yeast strains tested, and ranged from 0.51 to 0.76 and from 0.45 to 0.62 mg/L, respectively. *H. osmophila* 1471 produced the lowest levels of octanoic acid (0.15 mg/l) whereas *P. membranifaciens* 10113 produced 0.53 mg/L. These levels are similar to those found by Herraiz et al. (1990) in wines fermented with non-*Saccharomyces* yeasts and they are much lower than the concentrations of 5 mg/L hexanoic acid,

9 mg/L octanoic acid and 5 mg/L decanoic acid found in stuck fermentations (Edwards et al., 1990).

With respect to higher alcohols significant differences among strains were found only for 2-phenylethyl alcohol and isoamyl alcohol. *H. osmophila* 1471 yielded a concentration of the latter approximately 3-4-fold higher than those formed by *H. guilliermondii* strains and *P. membranifaciens*. The total higher alcohol concentrations varied from 86 to 167 mg/L and were lower than the amount produced by commercial *S. cerevisiae* strains. Concentrations below 300 mg/L are considered to contribute positively to wine flavour complexity (Rapp and Mandery, 1986).

3.4 Ester production in wine

Further studies to characterise the selected yeast strains included the analysis of ester profiles at the end of must fermentation (Table 4). Also the range of ester concentrations determined in wines fermented by the commercial *S. cerevisiae* strains has been included. According to ester formation on synthetic medium, ethyl acetate was the ester found in the highest quantities (39.9-292.8 mg/L). Ethyl acetate, the main ester in wine, can impart spoilage character at levels of 150-200 mg/L. In this context the levels of ethyl acetate produced by *H. osmophila* 1471 are similar to those found in wines fermented by our commercial *S. cerevisiae* strains and those reported in the literature (Fleet and Heard, 1993). The next highest ester concentrations found corresponded to 2-phenylethyl acetate (0.18-12.9 mg/L) which contributes with fruity and flowery notes to wine, and diethylsuccinate (0.84-2.54) contributing more to the body of a wine (Lambrechts and Pretorius, 2000). The highest concentration of these compounds was observed in wines fermented by *H. osmophila* 1471. Interestingly the levels of 2-phenylethyl acetate produced by *H. osmophila* were approximately 10-fold greater than those produced by commercial *S. cerevisiae* strains. As a general trend for the three selected *Hanseniaspora* strains, lower levels of 2-phenylethyl acetate were detected in wine than in

synthetic medium. No significant differences in isoamyl acetate were found in musts fermented by the non-*Saccharomyces* yeast strains tested which produced less than 0.5 mg/L, whereas in synthetic medium they produced between 1 and 2 mg/L. Isobutyl acetate which also contributes to the desirable qualities of wine bouquet was produced by all yeasts tested with the exception of *H. osmophila* 1471. With respect to ethyl esters no differences were found among yeasts for ethyl caprilate, whereas *H. osmophila* 1471 wines showed the highest quantity of ethyl caproate. The levels of ethyl esters produced by non-*Saccharomyces* yeasts were much lower than those detected in *S. cerevisiae* wines accordingly to our previous work (Rojas et al., 2003).

This study has revealed the potential of non-*Saccharomyces* wine yeasts to produce acetate esters, and specifically the genera *Hanseniaspora* and *Pichia*. Moreover the production of 2-phenylethyl acetate seems to be restricted to the genus *Hanseniaspora*. Based on technological traits *H. osmophila* 1471 seems to be a good candidate for designing mixed starters although further studies are necessary on its potential to produce H_2S as well as the possible interactions with *S. cerevisiae*.

Table 3. Effects of yeast strains on wine pH and fermentation product concentrations[a].

	Yeast strain				
	S. cerevisiae[b]	*H. guilliermondii* 11027	*H. guilliermondii* 11102	*H. osmophila* 1471	*P. membranifaciens* 10113
pH	3.21-3.42	3.33 ± 0.01^{B}	3.37 ± 0.01^{B}	3.23 ± 0.06^{A}	3.34 ± 0.01^{B}
Ethanol (%, v/v)	10.9-11.7	8.6 ± 0.1^{B}	7.9 ± 0.6^{B}	11.6 ± 0.5^{C}	6.3 ± 0.3^{A}
Acetaldehyde (mg/L)	14.9-20.3	11.5 ± 1.4^{A}	12.7 ± 1.4^{A}	10.6 ± 1.2^{A}	15.3 ± 1.4^{A}
Acetic acid (g/L)	0.13-0.16	0.68 ± 0.05^{A}	1.09 ± 0.07^{B}	0.57 ± 0.01^{A}	0.62 ± 0.01^{A}
Glycerol (g/L)	5.72-5.94	4.91 ± 0.01^{B}	3.85 ± 0.06^{A}	4.73 ± 0.13^{B}	3.95 ± 0.01^{A}
Hexanoic acid (mg/L)	2.3-4.1	0.58 ± 0.07^{A}	0.51 ± 0.06^{A}	0.76 ± 0.09^{A}	0.52 ± 0.05^{A}
Octanoic acid (mg/L)	3.0-6.4	0.42 ± 0.05^{BC}	0.26 ± 0.09^{AB}	0.15 ± 0.05^{A}	0.53 ± 0.09^{C}
Decanoic acid (mg/L)	0.51-2.3	0.54 ± 0.01^{A}	0.62 ± 0.02^{A}	0.55 ± 0.27^{A}	0.45 ± 0.08^{A}
2-Phenylethyl alcohol (mg/L)	30.7-112	27.5 ± 2.7^{C}	11.5 ± 2.8^{A}	23.8 ± 3.5^{BC}	15.7 ± 3.6^{AB}
Isoamyl alcohol (mg/L)	190-240	30.6 ± 0.1^{A}	21.2 ± 1.5^{A}	89.6 ± 11.3^{B}	22.4 ± 0.6^{A}
Propanol (mg/L)	4.3-10.9	7.2 ± 1.7^{A}	5.9 ± 0.3^{A}	10.8 ± 2.1^{A}	5.9 ± 1.2^{A}
Isobutanol (mg/L)	45.2-60.5	43.4 ± 4.0^{A}	47.2 ± 4.2^{A}	43.1 ± 2.5^{A}	48.9 ± 4.9^{A}
Total higher alcohols (mg/L)	270-423	108.8	85.8	167.4	92.9

[a]Mean values for two independent experiments and standard deviations. Data with the same letter do not differ at 95% confidence level (LSD procedure).
[b]Ranges obtained for the nine *S. cerevisiae* commercial strains.

Table 4. Effect of yeast strains on wine ester concentrations[a].

	Yeast strain				
	S. cerevisiae[b]	*H. guilliermondii* 11027	*H. guilliermondii* 11102	*H. osmophila* 1471	*P. membranifaciens* 10113
Ethyl acetate (mg/L)	31.3-55.7	114.8 ± 6.2^{AB}	228.2 ± 18.9^{BC}	39.9 ± 16.8^{A}	292.8 ± 82.3^{C}
Isoamyl acetate (mg/L)	1.5-4.4	0.33 ± 0.05^{A}	0.39 ± 0.04^{A}	0.37 ± 0.03^{A}	0.28 ± 0.06^{A}
2-Phenylethyl acetate (mg/L)	0.05-1.51	1.55 ± 0.32^{A}	2.98 ± 0.07^{A}	12.9 ± 3.5^{B}	0.18 ± 0.01^{A}
Isobutyl acetate (µg/L)	30.8-78.7	12.1 ± 1.1^{A}	26.9 ± 2.5^{B}	n.d.	23.0 ± 5.2^{B}
Hexyl acetate (µg/L)	15.3-24.8	10.0 ± 2.3^{A}	19.2 ± 3.2^{A}	8.6 ± 0.1^{A}	15.8 ± 4.7^{A}
Ethyl caproate (µg/L)	81.3-295	9.6 ± 1.0^{B}	6.8 ± 1.1^{AB}	22.2 ± 2.0^{C}	4.9 ± 0.5^{A}
Ethyl caprylate (µg/L)	210-775	3.2 ± 0.5^{A}	3.1 ± 1.0^{A}	3.5 ± 1.0^{A}	3.5 ± 0.5^{A}
Diethyl succinate (mg/L)	0.51-3.2	1.64 ± 0.14^{B}	0.93 ± 0.07^{A}	2.54 ± 0.05^{C}	0.84 ± 0.09^{A}

[a]Mean values for two independent experiments and standard deviations. Data with the same letter do not differ at 95% confidence level (LSD procedure).
[b]Ranges obtained for the nine *S. cerevisiae* commercial strains.

4. Acknowledgements

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Artículo II

Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*

ABSTRACT

The impact of mixed cultures of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae* with different initial yeast ratios on wine composition has been examined. The mixed culture significantly affected sugar consumption, the main enological parameters and ester concentrations, with the exception of glycerol, isoamyl acetate and diethyl succinate levels. Remarkably, in wines obtained with mixed cultures the concentration of 2-phenylethyl acetate was approximately 3- to 9-fold greater than that produced by *S. cerevisiae* pure culture. Moreover sensory evaluation revealed a stronger fruity character in wines fermented with mixed cultures than in control wines. Independently of the mixed culture used, all wines showed concentrations of acetic acid and ethyl acetate within the ranges described for wines. Our data suggest that a mixed culture of *H. osmophila* and *S. cerevisiae* can be used as a tool to increase 2-phenylethyl acetate in wine and that its concentration can be controlled by modulating the initial yeast ratio in the culture.

Keywords: 2-phenylethyl acetate, mixed culture, *Hanseniapora osmophila*, yeast ratio, wine, fruity character.

1. Introduction

Wine fermentation is a complex microbiological process in which yeasts play a fundamental role. Spontaneous wine fermentation gives rise to a succession of yeasts: non-*Saccharomyces* yeast species grow during the early stages of the process whereas *Saccharomyces cerevisiae* strains dominate at the later stages of fermentation due to their ethanol resistance (Fleet and Heard, 1993). Nowadays, fermentations inoculated with selected *S. cerevisiae* strains are prevalent in large-scale wine production due to the ease of control and homogeneity of fermentations. However, spontaneous fermentations reinforce wine flavour complexity, stylistic distinction and vintage variability (Lambrechts and Pretorius, 2000; Romano et al., 2003). An alternative to both fermentation practices is the use of mixed starters of selected yeasts combined with a commercial strain of *S. cerevisiae* to avoid stuck fermentations, which takes advantage of the potential positive role that non-*Saccharomyces* wine yeast species play in the organoleptic characteristics of wine (reviewed in Fleet, 2003).

Several studies have evaluated the feasibility of mixed starters to improve wine quality. In this context, studies have proposed *Candida stellata* to enhance glycerol content (Soden et al., 2000) and *Torulaspora delbrueckii,* in combination with *S. cerevisiae,* to improve the analytical profile of sweet wines (Bely et al., 2008). Similarly, geraniol concentration was increased in Muscat wines by using a mixed culture with *Debaryomyces vanriji* (Garcia et al., 2002); whereas *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* grown as mixed cultures in grape must increased the 2-phenylethyl acetate and isoamyl acetate content of wines, respectively (Rojas et al., 2003; Moreira et al., 2008). Considering the technological potential of mixed cultures, studies have been made into the influence of fermentation parameters such as oxygen and temperature on the fermentation behaviour of mixed starters (Holm Hansen et al., 2001; Ciani and Comitini, 2006) as well as the effect of several inoculation protocols (Soden et al., 2000). Moreover, some studies have characterized the nature and kinetics of the death of non-*Saccharomyces* yeasts in mixed

cultures that seems to be mediated by a cell-cell contact-mediated mechanism rather than to nutrient depletion or the presence of toxic compounds (Nissen and Arneborg, 2003; Nissen et al., 2003).

With the aim of selecting yeast strains able to modulate the aromatic profile of wines, we have screened non-*Saccharomyces* wine yeasts for their potential to produce acetate esters and studied their effect as part of mixed cultures on acetate ester concentrations in wine (Rojas et al., 2001; 2003). However, due to their excessive production of ethyl acetate, their applicability to winemaking was limited. Recently, we characterized the ester profile of non-*Saccharomyces* wine yeast strains when grown in synthetic medium and verified their fermentation behaviour in must. This study allowed us to select a *Hanseniaspora osmophila* strain yielding high levels of 2-phenylethyl acetate, while producing levels of acetic acid and ethyl acetate within the ranges described for wine (Viana et al., 2008). In the present work we report the impact of mixed cultures of *H. osmophila* and *S. cerevisiae* on wine fermentation and ester formation. The influence of the yeast ratios in the mixed starter on the final wine is also evaluated.

2. Materials and methods

2.1 Yeast strains and culture media

Hanseniaspora osmophila 1471 from the CECT Collection (Spanish Type Culture Collection) and *Saccharomyces cerevisiae* Lalvin T73 (Lallemand Inc, Rexdale, Ontario) were used. Yeast strains were maintained on GPY plates (5 g/L yeast extract, 5 g/L peptone, 40 g/L glucose, 20 g/L agar, pH 5.5).

2.2 Fermentation conditions

Triplicate fermentations were carried out in red grape must from a Bobal cultivar (Utiel-Requena, Valencia, Spain) with an initial sugar content of 185 g/L, pH 3.2 and supplemented with 1 g/L of a complex yeast nutrient (Fermaid K, Lallemand). Fresh must was sterilised by adding 1 mg/L of dimethyl di-carbonate (Fluka) and stored at -20°C until use. Aliquots of 90 mL of must were fermented in 100 mL bottles at 25°C. Musts were inoculated with 10^6 cells/mL from 24 h pre-cultures grown in the same must. Mixed culture fermentations with *H. osmophila*:*S. cerevisiae* at ratios of 90:10, 75:25, 50:50, 25:75, 10:90 and 5:95 were tested. Pure fermentations with *S. cerevisiae* (0:100) were also carried out.

2.3 Cell population counts

Fermenting musts were sampled to enumerate yeast populations. *H. osmophila* cells were counted by plating on lysine agar (66 g/L lysine medium (Oxoid Ltd, Basingstoke, UK), 10mL/L 50% potassium lactate, 1mL/L 10% lactic acid, pH 4.8) and total yeast cells were counted using GPY plates. Plates were incubated at 30°C for 72 h.

2.4 Wine analysis

Glucose and fructose consumption throughout the fermentation process as well as the concentration of glycerol, acetaldehyde and acetic acid in wines were measured enzymatically in an Echo-Enosys analyzer (Tecnova, San Sebastián de los Reyes, Spain) following the supplier's instructions. Ethanol concentration in wines was determined using the R-Biopharm enzymatic assay (R-Biopharm AG, Darmstadt, Germany). Higher alcohols, esters and fatty acids in wines were analysed by headspace solid-phase microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-Aldrich, Barcelona, Spain) and gas chromatography (GC) as described previously (Viana et al., 2008). Ester, fatty acids and higher alcohol concentrations were calculated using standard solutions (Fluka, Buchs, Switzerland) and are given as the mean of three independent vinifications.

Specific rates for sugar consumption were calculated adjusting experimental data to a mathematical model using the XLfit curve fitting software (IDBS, Guildford, UK) for Microsoft® Excel. V10, V50 and V80 were defined as consumption rates of the 10, 50 and 80% must sugar concentration, respectively. Rates corresponded to a glucose consumption of 9.7 g/L, 48.5 g/L and 77.6 g/L, respectively and a fructose consumption of 8.8 g/L, 44 g/L and 70.4 g/L, respectively.

2.5 Sensory evaluation

Fermentation trials for sensory evaluation were carried out in fresh red grape must from a Bobal cultivar with an initial sugar content of 200 g/L, pH 3.5, supplemented with Fermaid K and sterilised with dimethyl di-carbonate as specified above. Nine liters of must were fermented in 10 L bottles at 25°C. Musts were inoculated with 10^6 cells/mL from 24 h pre-cultures grown in the same must. Mixed culture fermentations with *H. osmophila*:*S. cerevisiae* at a 90:10 ratio and pure fermentations with *S.*

cerevisiae were carried out. Fermentations were done in duplicate until dry and wines were cold stabilized, filtered and bottled. Cell population counts and wine analyses were carried out as specified above.

A panel of 9 experienced wine judges was assembled for a sensory evaluation of the wines. They used a standard tasting chamber and standard glasses. Intensity, quality of aroma (as a parameter of overall acceptability) and fruity aroma were the properties graded. A points system of positive numbers was used.

2.6 Statistical analysis

Fisher's least significant difference procedure (LSD) was used for mean comparison (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

3. Results and discussion

3.1 Yeast growth during fermentation

The use of new fermentation technologies for optimizing wine quality and producing wines with particular flavour profiles is one of the worldwide trends in enology. In this context there is a growing demand for new and improved wine yeast strains adapted to different types and styles of wines. This demand could be met by non-*Saccharomyces* wine yeasts, which are described as producers of high concentrations of fermentation metabolites of enological importance (Romano et al., 2003). Of these metabolites, it is generally described that esters make the greatest contribution to the characteristic fruity odours of wine fermentation bouquet (Rapp and Mandery, 1986).

H. osmophila 1471 was previously selected based on ester profile and enological traits. Specifically, when grown in must *H. osmophila* 1471 produced around 10-13 mg/L of 2-phenylethyl acetate and less than 50 mg/L of ethyl acetate (Viana et al., 2008). 2-phenylethyl acetate is recognised as an important flavour compound in wine and contributes to the fruity notes of wine aroma. With the aim of evaluating its possible applicability to obtain wines with increased levels of 2-phenylethyl acetate, we designed mixed cultures of *H. osmophila* and *S. cerevisiae* with different initial yeast percentages. To imitate the proportion of yeast species at the beginning of spontaneous fermentation we designed the first mixed culture of *H. osmophila* and *S. cerevisiae* with a ratio of 90:10. Previous studies with mixed cultures have shown that this ratio is appropriate for obtaining the desired effect on wine composition (Rojas et al., 2003), but there is a lack of data on the minimum percentage of non-*Saccharomyces* yeasts in mixed starters that can influence the analytical profile of wines. Also, considering the possible biotechnological application of the starter, it is more likely that a mixed culture with a reduced percentage of non-*Saccharomyces* yeast would be more acceptable to the winemaking industry. Thus the studies reported here were set up to characterize the impact of different yeast ratios, from 90:10 to 5:95, on wine fermentations.

Yeast growth and sugar depletion were monitored during fermentation. Figure 1 shows population evolution of *H. osmophila* and *S. cerevisia*e yeasts in must inoculated with mixed cultures. In all cultures tested, *S. cerevisiae* reached a maximum population of approximately 10^8 cfu/mL and then slowly declined as fermentation progressed to completion after 8 days. In panels A and B, in which *S. cerevisiae* comprises only a minor proportion of the culture, *H. osmophila* population exceeded that of *S. cerevisiae* cells during the first two days of fermentation and remained practically at the same level as *S. cerevisiae* until day 5, when it started to decline. As the *H. osmophila* percentage in the initial starter decreased (panels C to F), its population decreased more rapidly and, at day 5, it varied from 10^6 cfu/mL (panel C) to 10^2 cfu/mL (panel F). The growth and survival of *H. osmophila* in mixed cultures did not differ markedly to those previously obtained in single cultures (Viana et al., 2008). Similar results have also been described for *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* that showed similar growth parameters in both pure and mixed cultures (Moreira et al., 2008). Traditionally, the disappearance of non-*Saccharomyces* species during must fermentation has been associated with their lower tolerance to ethanol or to other toxic compounds (Fleet, 2003). However recent studies have shown that the early growth arrest of some non-*Saccharomyces* yeast species in mixed cultures cannot be explained by nutrient depletion or the presence of toxic compounds and, instead, seems to be due to a cell-cell contact mechanism (Nissen et al., 2003).

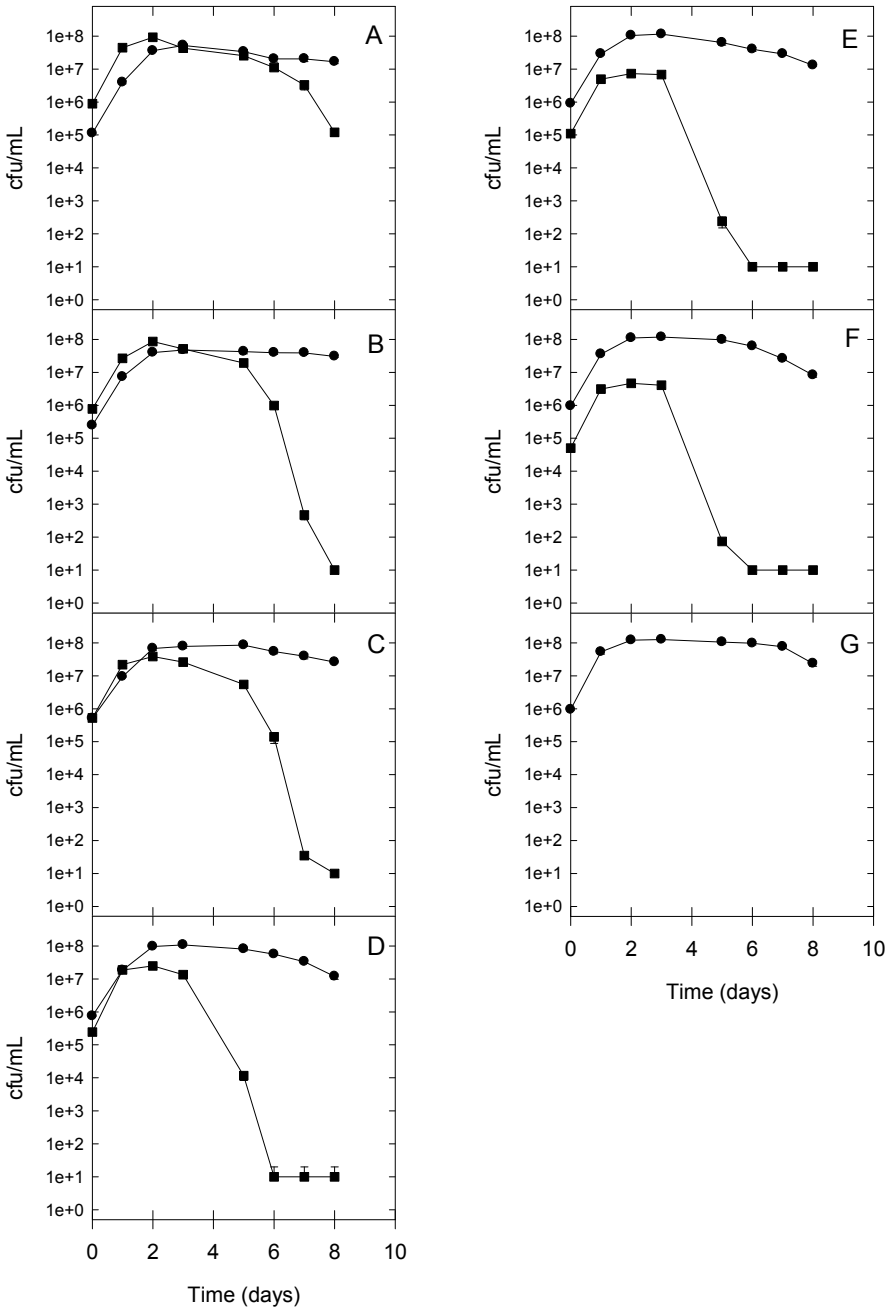

Fig. 1. Evolution of yeast populations in musts inoculated with mixed starters. (●): *S. cerevisiae*; (■): *H. osmophila*. Letters A-G refer to *H. osmophila:S. cerevisiae* culture ratios. A: 90:10; B: 75:25; C: 50:50; D: 25:75; E: 10:90; F: 5:95; G: 0:100.

Fermentation profiles were measured by glucose and fructose consumption. For better comparison among fermentation trials, consumption rates of the 10%, 50% and 80% of must sugar concentrations were calculated and summarized in Table 1. As a general trend, a delay in sugar consumption was observed when the ratio of *H. osmophila* increased in the mixed culture. Nevertheless, in all the trials alcoholic fermentation was completed and, at day 8, all wines contained less than 1 g/L of sugar. In all fermentations, glucose was consumed faster than fructose, confirming the observed glucophilic nature of both *S. cerevisiae* T73 and *H. osmophila* in single cultures (Viana et al., 2008). There were practically no differences (less than 10%) among rates calculated for *S. cerevisiae* single cultures and mixed cultures with a proportion of *H. osmophila* up to 25 %. When *H. osmophila* was the main species in the initial mixture, lower rates for both glucose and fructose consumption were observed. In comparison to *S. cerevisiae* in monoculture, glucose consumption rates were about 10-20% lower for the culture ratio 75:25, whereas reductions of approximately 20-30% were found for the culture ratio 90:10. Higher reductions in fructose consumption rates were observed (50%), mainly for V10 corresponding to the first stages of fermentation. When fermentation progressed, reductions of around 10-20% and 30% were found for mixed culture ratios 75:25 and 90:10, respectively (see Table 1).

Table 1. Sugar consumption rates by the mixed cultures and, in brackets, their percentages relative to the rate determined for *S. cerevisiae* monoculture.

Rate (g/L x day)		Mixed culture (% *H.osmophila*:% *S. cerevisiae*)						
		0:100	5:95	10:90	25:75	50:50	75:25	90:10
Glucose	V10	15.58	15.14 (97.2)	16.24 (104.2)	15.14 (97.2)	14.80 (95.0)	15.89 (102.0)	12.10 (77.7)
	V50	33.36	32.80 (98.4)	33.15 (99.4)	32.02 (96.0)	30.44 (91.2)	29.54 (88.5)	27.62 (82.8)
	V80	37.33	35.80 (95.9)	35.78 (95.8)	34.97 (93.7)	31.94 (85.6)	29.73 (79.6)	27.30 (73.1)
Fructose	V10	13.21	13.96 (105.7)	13.69 (103.6)	14.03 (106.2)	12.94 (98.0)	6.98 (52.8)	6.68 (50.6)
	V50	19.79	19.75 (99.8)	19.60 (99.0)	19.76 (99.8)	18.94 (95.7)	17.49 (88.4)	14.21 (71.8)
	V80	21.47	20.97 (97.7)	20.72 (96.5)	21.19 (98.7)	18.77 (87.4)	17.66 (82.3)	15.13 (70.5)

3.2 Analytical profile of wines

The main enological characteristics of the wines produced are presented in Table 2. All of the parameters, with the exception of glycerol, were affected by the presence of *H. osmophila* in the starter. Glycerol, which confers fullness and softness to wines, varied from a concentration of 6.6 g/l to 6.9 g/l; within the range of levels usually found in wines (Ribéreau-Gayon et al., 2000). Some of the parameters such as pH (ranging from 2.82-3.25), ethanol (11.5% - 12.3%) and acetaldehyde (30.8 - 45.9 mg/l) showed statistically significant differences among trials, but these differences were not correlated with the percentage of *H. osmophila* in the mixed starter. In contrast to our results, ethanol and glycerol levels obtained in mixed cultures of apiculate yeasts (*H. uvarum* and *H. guilliermondii*) with *S. cerevisiae* were found to be lower than those produced by a pure culture of *S. cerevisiae* (Moreira et al., 2008). Remarkably, our selected strain of *H. osmophila* in pure culture was able to produce similar ethanol (11.6%) and glycerol (4.7 mg/L) levels to those produced by *S. cerevisiae* (Viana et al., 2008). There are also recent reports of some strains of *Hanseniaspora* species that may have ethanol tolerances similar to *S. cerevisiae* (Rojas et al., 2003; Pina et al., 2004).

The high production of acetic acid is recognized as a common pattern in apiculate yeasts and thus they have been considered as spoilage yeasts (du Toit and Pretorius, 2000). However, great variability in acetic acid production, from about 0.6 g/L to more than 3.4 g/L has been observed (Romano et al., 2003; Viana et al., 2008). In our experiments, mixed fermentation with *H. osmophila* produced a substantial increase in acetic acid concentration, reaching levels between 0.39 and 0.42 g/L in the mixed culture ratios 75:25 and 90:10, respectively. Although acetic acid levels were approximately 3-fold higher than those produced by *S. cerevisiae* (0.13 g/L), they were within the optimal concentration range of 0.2 - 0.7 g/L described for wines (Lambrechts and Pretorius, 2000).

Table 2. Effects of mixed cultures on wine pH and fermentation product concentrations[a].

	Mixed culture (% *H.osmophila*:% *S. cerevisiae*)						
	0:100	5 :95	10 :90	25 :75	50 :50	75 :25	90:10
pH	2.82 ± 0.02^{D}	3.25 ± 0.02^{A}	3.27 ± 0.04^{A}	3.24 ± 0.02^{AB}	3.25 ± 0.03^{A}	3.17 ± 0.09^{B}	2.93 ± 0.01^{C}
Ethanol (%, v/v)	11.5 ± 0.2^{C}	12.3 ± 0.3^{A}	12.3 ± 0.5^{AB}	11.9 ± 0.1^{ABC}	11.9 ± 0.3^{ABC}	11.8 ± 0.1^{BC}	11.9 ± 0.1^{ABC}
Acetaldehyde (mg/L)	43.1 ± 2.9^{AB}	42.9 ± 3.9^{AB}	45.9 ± 2.9^{A}	43.7 ± 4.5^{AB}	38.9 ± 4.5^{B}	30.8 ± 1.2^{C}	45.9 ± 2.0^{A}
Acetic acid (g/L)	0.13 ± 0.01^{D}	0.16 ± 0.02^{CD}	0.19 ± 0.01^{C}	0.19 ± 0.02^{C}	0.26 ± 0.05^{B}	0.39 ± 0.02^{A}	0.42 ± 0.02^{A}
Glycerol (g/L)	6.9 ± 0.1^{A}	6.8 ± 0.3^{A}	6.6 ± 0.2^{A}	6.8 ± 0.1^{A}	6.9 ± 0.3^{A}	6.6 ± 0.1^{A}	6.6 ± 0.3^{A}
Hexanoic acid (mg/L)	4.9 ± 0.5^{A}	4.6 ± 0.7^{AB}	3.8 ± 0.3^{BC}	3.4 ± 0.2^{C}	3.5 ± 0.2^{C}	3.8 ± 0.5^{BC}	3.4 ± 0.2^{C}
Octanoic acid (mg/L)	6.9 ± 0.9^{A}	7.4 ± 0.2^{A}	5.8 ± 0.6^{B}	4.9 ± 0.1^{BC}	4.3 ± 0.1^{C}	4.1 ± 0.2^{C}	4.4 ± 0.5^{C}
Decanoic acid (mg/L)	3.7 ± 0.6^{A}	3.4 ± 0.5^{A}	2.8 ± 0.1^{AB}	2.8 ± 0.1^{AB}	2.9 ± 0.3^{AB}	2.2 ± 0.3^{B}	2.2 ± 0.7^{B}
2-Phenylethyl alcohol (mg/L)	114.9 ± 10.4^{A}	95.2 ± 21.8^{AB}	80.5 ± 7.9^{BC}	83.5 ± 6.5^{BC}	63.0 ± 16.4^{CD}	46.6 ± 6.7^{DE}	25.5 ± 2.5^{E}
Isoamyl alcohol (mg/L)	291.5 ± 45.6^{A}	256.5 ± 6.3^{AB}	227.4 ± 1.5^{BC}	202.8 ± 9.2^{C}	192.8 ± 5.7^{C}	202.0 ± 10.7^{C}	217.4 ± 3.9^{C}
Propanol (mg/L)	5.8 ± 0.7^{A}	4.8 ± 1.5^{AB}	3.9 ± 0.2^{BC}	4.5 ± 0.1^{ABC}	3.8 ± 0.3^{BC}	3.7 ± 0.7^{BC}	3.0 ± 0.1^{C}
Isobutanol (mg/L)	40.3 ± 1.4^{E}	44.0 ± 1.0^{DE}	47.9 ± 6.1^{CD}	52.1 ± 0.4^{BC}	56.9 ± 1.6^{AB}	58.4 ± 1.5^{A}	60.3 ± 3.5^{A}
Total higher alcohols (mg/L)	452.5	400.5	359.7	342.9	316.5	310.7	306.2

Data with the same letter do not differ at 95% confidence level (LSD procedure).
[a]Mean values for three independent experiments and standard deviations.

Medium-chain fatty acid concentrations decreased in wines obtained from mixed cultures. Reductions of about 30-40% in the levels of hexanoic, octanoic and decanoic acids were observed in the 90:10 trials, in comparison to *S. cerevisiae* monoculture fermentations. Similar results were observed by Herraiz et al. (1990) when comparing *S. cerevisiae* monocultures with *K. apiculata* and *S. cerevisiae* mixed starters; although in those trials the levels were also affected by the inoculation protocol, *i.e*. either mixed or sequential.

Higher alcohols, recognizable by their strong and pungent smell and taste, can have a significant influence on the taste and character of wine (Lambrechts and Pretorius, 2000). Higher alcohols are also important precursors for ester formation (Soles et al., 1982). Different studies have shown that apiculate yeasts in pure and mixed starters produced lower amounts of higher alcohols when compared to *S. cerevisiae* (Romano et al., 2003; Rojas et al., 2003; Moreira et al. 2008). In our assays, the total amount of higher alcohols varied from approximately 300 to 450 mg/L within the broad range typically found in wines (Nÿkanen, 1986). Nevertheless, higher alcohols decreased up to 30% in wines fermented in the presence of *H. osmophila*, although the concentration of each alcohol was affected differently. Only the isobutanol concentration increased (by approximately 50%). The two main alcohols formed by yeasts, 2-phenylethyl alcohol and isoamyl alcohol showed a significant decrease. The highest reduction (about 80%) corresponded to the level of 2-phenylethyl alcohol that ranged from about 115 mg/L in the *S. cerevisiae* pure culture trials to 25 mg/L in the fermentations obtained with the highest percentage of *H. osmophila*. The levels of isoamyl alcohol also decreased in wines fermented in the presence of *H. osmophila* (approximately 30%), although the concentration remained constant in trials involving percentages of *H. osmophila* higher than 25%. The concentration of propanol decreased from approximately 6 mg/L (pure culture) to 3 mg/L (mixed culture 90:10).

A linear model can be used to describe the relationship between enological parameters and *H. osmophila* proportion in the mixed culture. With the exceptions of hexanoic acid and isoamyl alcohol, a significant correlation was found between enological parameters and the composition of the starter. Two compounds, acetic acid and isobutanol showed a positive relationship with the percentage of *H. osmophila;* while an inverse correlation was found for the concentration of medium-chain fatty acids, 2-phenylethyl alcohol and propanol. The fitted model for acetic acid and 2-phenylethyl alcohol showed the lowest P-values (0.0001 and 0.0005, respectively).

3.3 Ester production

Further studies to characterize wines produced by mixed cultures included the analysis of ester concentrations at the end of must fermentation. Figure 2 shows the concentration of the main acetate and ethyl esters that contribute, with the exception of ethyl acetate, to fruit and flower notes to wine aroma. Diethyl succinate, which contributes more to the body of a wine, was also included in the study.

All the acetate esters determined were significantly affected by the inclusion of *H. osmophila* in the starter, with the exception of isoamyl acetate levels that ranged from 4.30 to 6.84 mg/L, suggesting that *S. cerevisiae* metabolism is mainly responsible for isoamyl acetate formation under the conditions tested. It is worth noting that *H. osmophila* in monoculture produced around 0.40 mg/L of isoamyl acetate (Viana et al., 2008). We obtained similar results in wines fermented with mixed cultures of *H. guilliermondii* and *Pichia anomala* with *S. cerevisiae* (Rojas et al., 2003). However higher levels of isoamyl acetate were found in wines produced with *H. uvarum,* whether in pure or mixed cultures, in comparison to those produced by *S. cerevisiae* monoculture (Moreira et al., 2008).

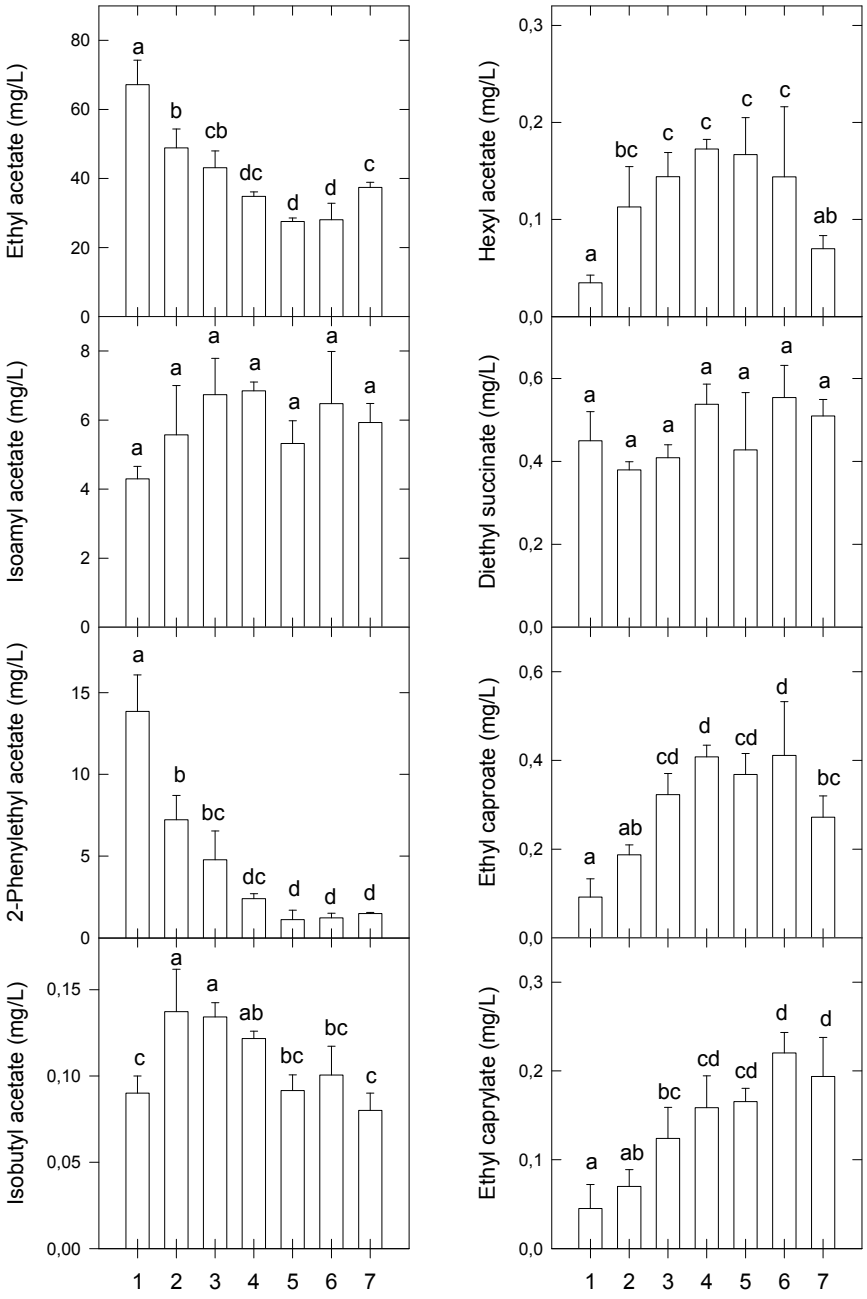

Fig. 2. Concentration of esters in wine obtained with different *H. osmophila:S. cerevisiae* culture ratios. 1: 90:10; 2: 75:25; 3: 50:50; 4: 25:75; 5: 10:90; 6: 5:95; 7: 0:100. Bars indicate standard deviations for three independent fermentations. Data with the same letter do not differ at 95% confidence level (LSD procedure).

Ethyl acetate and 2-phenylethyl acetate concentrations in wine were higher when the proportion of *H. osmophila* in the culture increased. The quantity of 2-phenylethyl acetate was significantly different from that formed by *S. cerevisiae* monoculture if the percentage of *H. osmophila* in the culture was at least 50%. In wines fermented with the culture ratio 90:10, the concentration of 2-phenylethyl acetate was approximately 9-fold greater than that produced by *S. cerevisiae* pure culture, and was similar to that found in *H. osmophila* monoculture (Viana et al., 2008). Significant increases of 2-phenylethyl acetate in comparison to *S. cerevisiae* monoculture were also found in wines fermented with the culture ratios 50:50 (3-fold greater) and 75:25 (5-fold greater). These results are also in agreement with the lower levels of 2-phenylethanol, the precursor, together with acetyl-CoA, for 2-phenylethylacetate synthesis by the action of alcohol acetyltransferase (Yoshioka and Hashimoto, 1981), typical in such wines (see Table 2). The genus *Hanseniaspora* has been described as a good producer of acetate esters, particularly for the production of 2-phenylethyl acetate (Viana et al., 2008). Previous studies have shown different strains of *H. guilliermondii* to be strong producers of such esters, either in pure or mixed fermentations (Rojas et al., 2003; Moreira et al., 2008).

Ethyl acetate, the main ester in wine, can impart spoilage character at levels of 150-200 mg/L (Jackson, 1994). These levels have been traditionally associated with the growth of apiculate yeasts and, in some wines obtained with mixed cultures, such high levels have been found (Rojas et al., 2003; Moreira et al., 2008). However, the concentration of ethyl acetate in our experiments was only approximately 2-fold higher in wines fermented with the culture ratio 90:10 (67 $\pm$ 7 mg/L) in comparison to monoculture wines (37 $\pm$ 1 mg/L). Moreover, the levels produced by all the mixed cultures tested are similar to those found in wines fermented with *S. cerevisiae* strains (Fleet and Heard, 1993; Viana et al., 2008).

Minor amounts of isobutyl acetate (banana aroma) and hexyl acetate (sweet aroma) were found in wines ranging from 0.09 to 0.14 mg/L and from 0.03 to 0.17 mg/L, respectively. Although there were statistically

significant differences in both acetate ester concentrations among wines fermented with the different starters, the variations did not correlate with the proportion of non-*Saccharomyces* yeast in the starter. This was also observed for some other enological parameters such as acetaldehyde concentration and may be due to the complex ecological relationships established between the two yeast populations in the mixed starter and how certain yeast proportions may influence the development of the fermentation.

The ethyl esters of fatty acids contribute pleasant fruity and floral odours to wine aroma. Their production has been shown to be significantly higher in wines produced by a pure culture of *S. cerevisiae* whereas the inoculation of apiculate yeasts resulted in a decreased of these esters (Herraiz et al., 1990). Our results showed lower levels of ethyl caproate and ethyl caprylate when wines were obtained using mixed cultures with a minimum proportion of *H. osmophila* of 50%, in comparison to those levels found in wines obtained with *S. cerevisiae* monoculture. Recently, Moreira et al. (2008) found that *S. cerevisiae* produced wines with levels of ethyl caproate that were not affected by the presence of apiculate yeasts in the starter, confirming results found by Rojas et al. (2003). With respect to diethyl succinate, its concentration (0.38 - 0.55 mg/L) was not affected by the composition of the initial culture.

The concentrations of ethyl acetate, 2-phenylethyl acetate, ethyl caproate and ethyl caprylate showed a statistically significant relationship with *H. osmophila* proportion in the mixed culture when fitted to a linear model. Both acetate esters showed a positive relationship with the percentage of *H. osmophila* in the mixed culture whereas a negative relationship was found for the two ethyl esters. Ethyl caprylate and 2-phenylethyl acetate showed the lowest P-values (0.0002 and 0.002, respectively).

3.4 Sensory analysis

The mixed culture ratio 90:10 was selected to perform sensory evaluations in comparison with wines produced by *S. cerevisiae* monoculture. Vinifications (9 L) were carried out and wine analysis results confirmed higher concentrations of 2-phenylethyl acetate and ethyl acetate in wines fermented by the mixed culture (1.53 mg/L and 41.1 mg/L, respectively) with respect to those obtained with *S. cerevisiae* (0.39 mg/L and 29.5 mg/L, respectively). Although approximately a 4-fold increased in 2-phenylethyl acetate concentration was found, ester levels produced by the mixed culture did not reach those recorded in the microvinifications (see Figure 2), probably due to differing vinification conditions. No significant differences between the mixed and the monoculture wines were found for the remaining esters. Accordingly to the microvinifications experiments lower levels of higher alcohols and medium chain fatty acids and higher levels of acetic acid were found in wines obtained with the mixed culture with respect to those obtained with *S. cerevisiae*. Among enological parameters similar pH values and ethanol, glycerol and acetaldehyde levels were found in both wines.

With respect to the sensorial attributes considered, only fruity aroma differed significantly between wines obtained with the mixed culture and those obtained with *S. cerevisiae* alone. The fruity character was stronger in the wines fermented with both *H. osmophila* and *S. cerevisiae* compared to the control wines ($p < 0.05$) whereas no differences were found for the overall parameters of intensity and quality of aroma. The sensory impact of wines with increased levels of 2-phenylethyl acetate deserves further research.

The role of non-*Saccharomyces* yeasts in wine-making is far from negligible and their impact on the analytical composition and sensory characteristics of wine has been reported in literature (Fleet, 2003; Romano et al., 2003). While the elevated production of ethyl acetate and acetic acid by apiculate yeasts has been widely reported, also the effect of strain

variation has been pointed out (Plata et al., 2003; Romano et al., 2003; Ciani et al., 2006). Here we show that careful selection of non-*Saccharomyces* yeast strains can facilitate the design of wine yeast starters with optimised ester-producing capabilities without compromising wine quality. Results obtained in this work suggest the potential of using *H. osmophila* 1471 in mixed cultures with *S. cerevisiae* to increase the levels of 2-phenylethyl acetate in wines. Moreover, the ratio of both yeast strains in the mixed culture modulates ester concentrations leading to wines with a wide range of flavour compositions. Further studies including the suitability of the mixed culture for semi-pilot vinifications and its effect on different musts are in progress.

4. Acknowledgements

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Artículo III

Monitoring a mixed starter of *Hanseniaspora vineae-Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production

ABSTRACT

The effect of simultaneous or sequential inoculation of *Hanseniaspora vineae* CECT 1471 and *Saccharomyces cerevisiae* T73 in non-sterile must on 2-phenylethyl acetate production has been examined. In both treatments tested, no significant differences in *Saccharomyces* yeasts growth were found, whereas non-*Saccharomyces* yeasts growth was significantly different during all days of fermentation. Independently of the type of inoculation, *S. cerevisiae* was the predominant species from day 3 till the end of the fermentation. The dynamics of indigenous and inoculated yeast populations showed *H. vineae* to be the predominant non-*Saccharomyces* species at the beginning of fermentation in sequentially inoculated wines, whereas the simultaneous inoculation of *S. cerevisiae* did not permit any non-*Saccharomyces* species to become predominant. Differences found in non-*Saccharomyces* yeast growth in both fermentations influenced the analytical profiles of final wines and specifically 2-phenylethyl acetate concentration which was two-fold increased in sequentially inoculated wines in comparison to those co-inoculated. In conclusion we have shown that *H. vineae* inoculated as part of a sequential mixed starter is able to compete with native yeasts present in a non-sterile must and modify the wine aroma profile.

Keywords: wine mixed starter, *Hanseniaspora vineae*, 2-phenylethyl acetate, non-sterile must, simultaneous inoculation, sequential inoculation.

1. Introduction

Many studies have been carried out on the ecology of wine yeasts and established the complexity of alcoholic fermentation, whether spontaneous or inoculated. It is now accepted that wine fermentation involve the growth of non-*Saccharomyces* and *Saccharomyces* species, and that the former play a relevant role in the organoleptic characteristics of wine (Fleet, 2008).

Mixed fermentations using controlled inoculation of *S. cerevisiae* and selected non-*Saccharomyces* yeasts represent a feasible tool to obtain different types and styles of wines (Ciani et al., 2010). Mixed fermentations were initially proposed as a way of simulating spontaneous fermentations to confer greater complexity to final wine (Herraiz et al., 1990; Zironi et al., 1993). The combined use of *S. cerevisiae* and non-*Saccharomyces* yeasts has also been proposed to increase or decrease a specific wine compound such as glycerol (Ciani and Ferraro, 1996; Toro and Vazquez, 2002) or acetic acid (Bely et al., 2008). The increase of specific volatile compounds to improve wine aroma such as geraniol (García et al., 2002), varietal thiols (Anfang et al., 2009) and acetate esters (Rojas et al., 2003; Moreira et al., 2008) can be also achieved through the use of selected mixed cultures.

When using particular yeast cultures to obtain a special character or style in the final product a dominant growth of the inoculated strain(s) is required. There are many factors that might affect the kinetic of yeast growth during wine fermentation. In this context the influence of oxygen and temperature (Holm Hansen et al., 2001; Ciani and Comitini, 2006) and inoculation protocol (Soden et al., 2000) on the fermentation behaviour of mixed starters have been studied. Although the death of non-*Saccharomyces* yeasts during the early stages of fermentation has been related to ethanol sensitivity, other mechanisms such as glucose uptake ability (Nissen et al., 2004), killer factors (Yap et al., 2000) or cell-cell interactions (Nissen and Arneborg, 2003; Nissen et al., 2003) have been described.

With the aim of designing mixed starters to obtain wines with increased concentrations of acetate esters we selected a *Hanseniaspora vineae* (formerly *H. osmophila*) strain CECT 1471 able to yield high levels of 2-phenylethyl acetate, while producing levels of acetic acid and ethyl acetate within the ranges described for wine (Viana et al., 2008). Recently we proposed mixed starters *H. vineae-S. cerevisiae* with different yeast ratios as a tool to modulate the concentration of 2-phenylethyl acetate in wine (Viana et al., 2009). In the present work, to further study the potential of the *H. vineae-S. cerevisiae* starter, we have monitored by means of molecular techniques the evolution of yeasts in natural non-sterile must fermentation conducted by the inoculated mixed culture. The influence of simultaneous or sequential inoculation on yeast population dynamics and aroma profile of final wines is also evaluated.

2. Materials and methods

2.1 Yeast strains and fermentation conditions

The strains used in this study were *Hanseniaspora vineae* (formerly *H. osmophila*) CECT 1471 (Spanish Type Culture Collection) and *Saccharomyces cerevisiae* Lalvin T73 (Lallemand Inc, Rexdale, Ontario). Yeast strains were maintained on GPYA medium (2% glucose, 0.5% yeast extract, 0.5% peptone and 2% g/L agar, pH 5.5). Exponentially growing yeast cultures of *H. vineae* and *S. cerevisiae* were prepared from 24 h growing yeasts at 28ºC on GPY liquid medium for inoculation of grape must.

Triplicate fermentations were carried out in red grape must from a Tempranillo cultivar (Requena, Valencia, Spain) with an initial sugar content of 257 g/L, pH 3.6, treated with with SO_2 (30 mg/L) and supplemented with 1 g/L of complex yeast nutrient (Fermaid K, Lallemand). Fermentations were carried out using 250 mL flasks containing 225 mL of Tempranillo must. Flasks were inoculated with 10^6cells/mL and incubated at 25ºC. Two inoculation strategies were used to achieve the desired mixed fermentation i) simultaneous inoculation of both yeast, *H. vineae* and *S. cerevisiae*, at 90:10 ratio respectively and, ii) sequential inoculation of both yeasts, *H. vineae* inoculated at the beginning of fermentation and *S. cerevisiae* inoculated at the fourth day of fermentation, at the same inoculation ratios as above.

2.2 Cell counts and isolation of yeasts

Fermenting must samples were taken from each flask at days 0, 1, 3, 5 and 7 (final day) of fermentation. Each fermentation sample was diluted in saline solution and plated on lysine agar medium (Oxoid Ltd, Basingstoke, UK) and GPYA medium plates and incubated at 28ºC for 48h. Lysine medium provided non-*Saccharomyces* yeast cell counts, whereas general purpose GPYA medium yielded total yeast counts. Statistically

representative dilution plates were counted and around 30 colonies from every fermentation sample (10 colonies from each triplicate) were randomly selected and purified on GPYA plates for further yeast identification and characterization.

2.3 Identification of yeast isolates and mtDNA RFLPs of S. cerevisiae

Isolated yeasts were inoculated into 1 mL of GPY liquid medium and incubated for 24 h at 28ºC. Total DNA was extracted following the procedure of Querol et al. (1992a). DNA was used for PCR amplification of the ITS1-5.8S-ITS2 rDNA region. Yeast identification was performed by RFLPs of PCR amplified ITS1-5.8S-ITS2 rDNA region as described by Esteve-Zarzoso et al. (1999). The RFLP patterns of the yeast isolates were compared with those included in the Yeast-id database (http://www.yeast-id.com) and assigned to a known yeast species.

2.4 Mitochondrial DNA restriction analysis

DNA extraction and determination of mtDNA restriction patterns of *S. cerevisiae* strains was carried out as described elsewhere (Querol et al., 1992a and b). DNA was digested with the restriction endonuclease *Hin*fI (Roche, Spain), according to the supplier's instructions. Restriction fragments were separated on horizontal agarose gels in 0.5x TBE buffer (44.5 mM Tris-borate, 1 mM EDTA, pH 8), stained with ethidium bromide (0.5 pg/mL) and visualised under UV light.

2.5 Wine analysis

Reducing sugars consumption throughout the fermentation process as well as the concentration of glycerol, acetaldehyde and acetic acid in wines were measured enzymatically in an Echo-Enosys analyzer (Tecnova, San Sebastián de los Reyes, Spain) following the supplier’s instructions.

Fermentation process was considered complete when reducing sugars concentration was lower than 2 g/L. Ethanol concentration in the final wines was determined using the R-Biopharm enzymatic assay (R-Biopharm AG, Darmstadt, Germany). Higher alcohols and esters in wines were analysed by headspace solid-phase microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-Aldrich, Barcelona, Spain) and gas chromatography (GC) as described previously (Viana et al., 2008).

2.6 Statistical analysis

Student's t-test was used for mean comparison at 95% confidence level (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

3. Results

3.1 Yeast growth during fermentation

Yeast growth and sugar depletion were monitored during fermentation. Cells counts revealed a high initial population in Tempranillo must (4.7 x 10^6 cfu/mL) of both non-*Saccharomyces* (91.5% of total count) and *Saccharomyces sp.* (8.5% of total count) yeasts. Figure 1 shows population evolution and sugar consumption of *Saccharomyces sp.* and non-*Saccharomyces* yeasts in must inoculated simultaneously (panel A) or sequentially (panel B) with the mixed culture. Independently of the type of inoculation strategy used, there were not significant differences in *Saccharomyces* yeasts growth during fermentation. In both treatments tested, *Saccharomyces* cells reached a maximum population of approximately 10^8 cfu/mL after 2 days and then decreased slightly till 8 x 10^7 cfu/mL at day 7. With respect to non-*Saccharomyces* yeasts, cell growth was significantly different during all days of fermentation in both treatments tested. In simultaneous fermentations non-Saccharomyces reached a maximum population at day 1 (9.3 x 10^7 cells/mL) and then it started to decline (2.9 x 10^7 cells/mL at day 2). In sequential fermentations maximum population was also reached at day 1 but it was kept constant at day 2 (7.9 x 10^7 and 8 x 10^7 cells/mL, respectively). It is worthwhile to note that at day 2 in sequential fermentations there were not significant differences between non-*Saccharomyces* and *Saccharomyces* cell numbers (8 x 10^7 and 1 x 10^8 cells/mL, respectively; p value= 0.264; Student's t-test) whereas in simultaneous fermentations non-*Saccharomyces* population represented only 30% of the total yeast count. Fermentation profiles were obtained by measuring consumption of reducing sugars (Figure 1). Significant differences in sugar consumption were found from day 2 till the end of fermentations. Fermentation dynamics was slightly faster in the sequential fermentation and for both fermentation types glucose was consumed faster than fructose (data not shown). In both fermentations at day 4, inoculation day of S. cerevisiae T73 in the sequential fermentation, remaining reducing sugars were lower than 5 g/L.

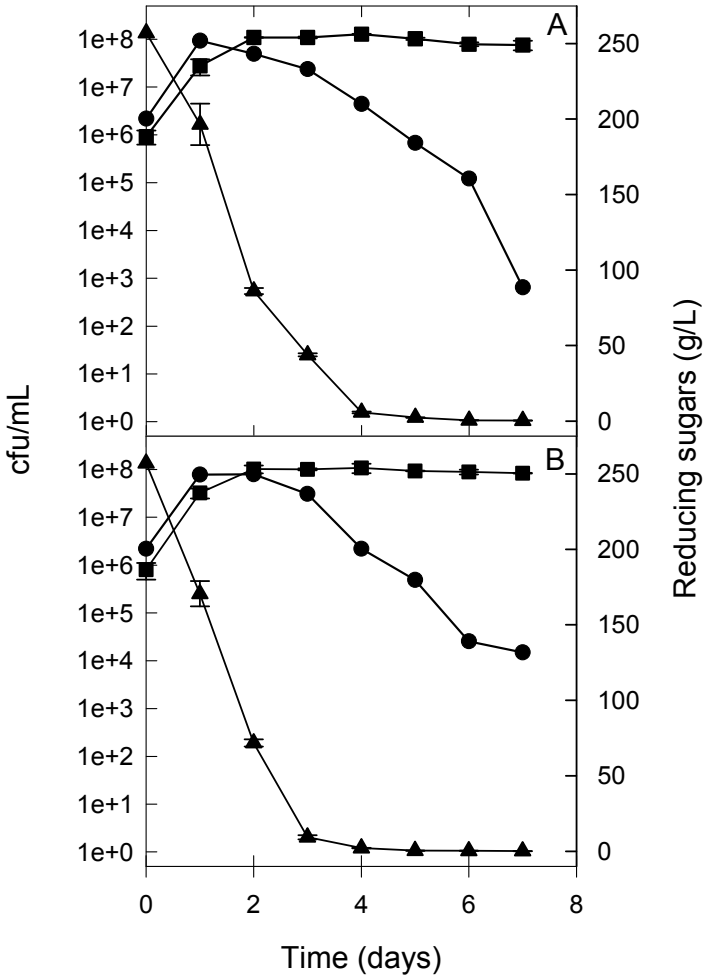

Figure 1. Evolution of yeast population and sugar consumption in musts simultaneously (A) or sequentially (B) inoculated with *H. vineae* CECT 1471 and *S. cerevisiae* T73. (●) non-*Saccharomyces* yeasts; (■) *Saccharomyces sp.* yeasts; (▲) reducing sugars.

3.2 Yeast isolation and identification

A total of 234 yeasts colonies were isolated from GPYA plate counts. Identification of the isolated yeasts was accomplished by comparison of the RFLPs of the ITS1-5.8S-ITS2 rDNA region and comparison with the Yeast-id database. All yeast isolates could be classified by comparison of the patterns obtained with digestion with three enzymes, *Cfo*I, *Hae*III and *Hin*fI, except for isolates pertaining to the species *H. guilliermondii* and *H. uvarum*, which were differentiated by restriction with an additional enzyme *Dde*I. The 234 yeasts isolated were classified into eight yeast species frequently found in fermenting grape musts (Table 1).

Percentage and distribution of the yeast strains identified from both fermentation types are represented in Figure 2. The RFLP ITS1-5.8S-ITS2 pattern corresponding to *H. vineae* did not appear in the Tempranillo must before inoculation of *H. vineae* 1471, therefore all isolates found in the fermentations showing that pattern correspond to the inoculated strain. On the other hand, isolates of *S. cerevisiae* were present from day 0 of fermentation and the inoculated *S. cerevisiae* T73 was indistinguishable from other native *S. cerevisiae* present in the Tempranillo must. At the first day of fermentation, remarkable differences in the percentages of the different yeast species between both fermentation types surfaced. In the sequentially inoculated fermentation, the percentage of *S. cerevisiae* yeasts was similar to the one found in the initial must; however, in the simultaneously inoculated fermentation the percentage of *S. cerevisiae* yeasts was, as expected, much higher. In case of *H. vineae* 1471, the percentage of this yeast in the sequentially inoculated fermentation was more than two-fold of the percentage of the same yeast in the simultaneously inoculated fermentation.

Table 1. Yeast species isolated from Tempranillo fermentation inoculated simultaneously or sequentially with *H. vineae* 1471 and *S. cerevisiae* T73.

Species	PCR product (bp)	Restriction fragments (bp)			
		*Hae*III	*Hinf*I	*Cfo*I	*Dde*I
Hanseniaspora guilliermondii	775	775	360+200+160	340+320+105	360+160+120+90
Hanseniaspora osmophila	775	460+120+100	390+370	270+180+150+80	
Hanseniaspora uvarum	775	775	370+200+160	340+320+100	300+170+100+80
Hanseniaspora vineae[a]	775	660+110	390+370	270+180+150+90	
Metschnikowia pulcherrima	400	280+100	200+190	205+100+95	
Saccharomyces cerevisiae	850	320+230+180+150	365+155	385+365	
Torulaspora delbrueckii	800	800	410+380	330+220+150+100	
Zygosaccharomyces cidri	700	300+210+95+95	340+340	310+280+90	

[a] The RFLP pattern corresponds solely to the strain *H. vineae* CECT 1471.

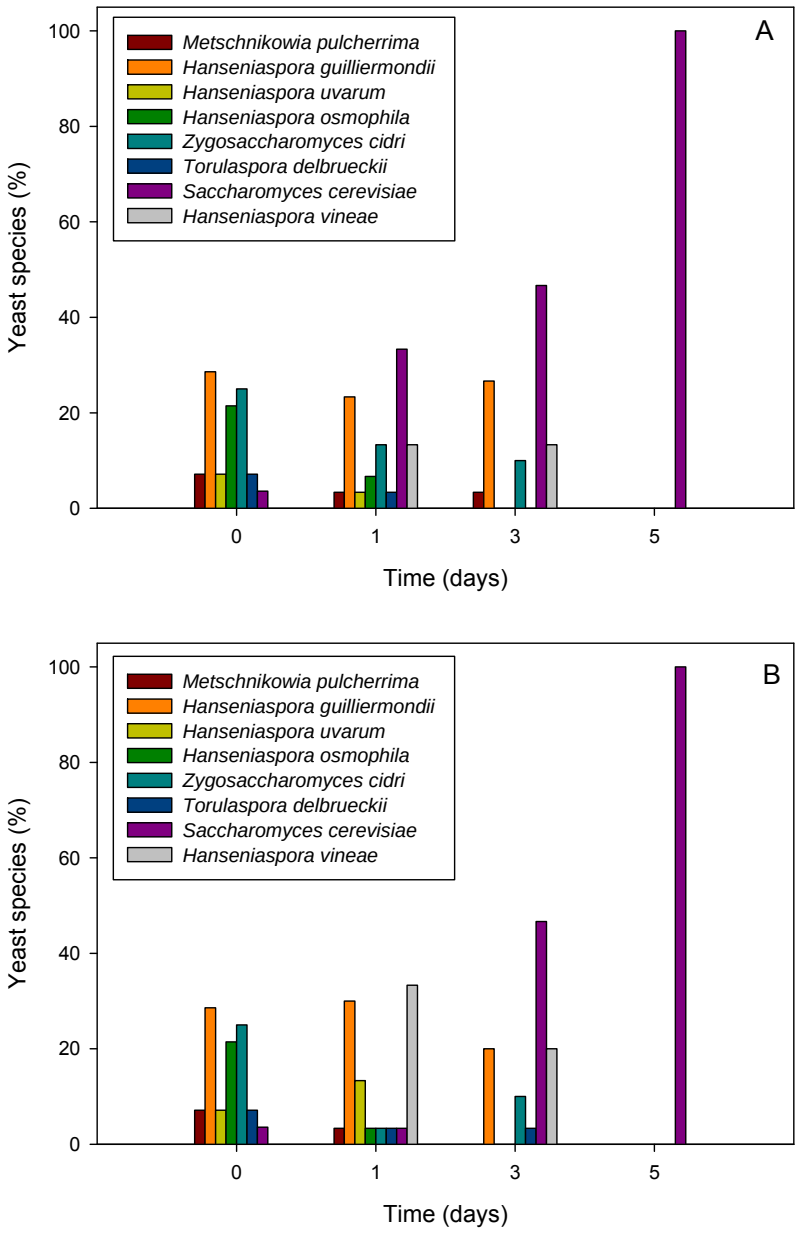

Figure 2. Yeast species dynamics in musts simultaneously (A) or sequentially (B) inoculated with *H. vineae* CECT 1471 and *S. cerevisiae* T73.

Comparison between days 0 and 1 in the simultaneously inoculated fermentation revealed a decrease of cell numbers in all non-*Saccharomyces* after inoculation of *S. cerevisiae* and *H. vineae*. In the sequentially inoculated fermentation, non-*Saccharomyces* yeasts showed also a decrease in cell numbers except for *H. guilliermondii* and *H. uvarum* whose cell numbers increased to some extent.

At the third fermentation day, only slight differences in the percentage of yeast species could be observed between both fermentation types. The dominant yeast species was *S. cerevisiae*, although *H. guilliermondii*, *H. vineae* and *Z. cidri* were also present in substantial numbers. Comparison of both fermentation types showed the same percentage of *S. cerevisiae*, although *H. vineae* cell counts were still higher in the sequentially inoculated fermentation than in the simultaneously inoculated one.

At the fifth fermentation day both fermentations were dominated by the presence of *S. cerevisiae.* No non-*Saccharomyces* yeast species could be isolated on GPYA from the Tempranillo fermenting must, although the counts on lysine medium indicated the presence of non-*Saccharomyces* in both fermentations (Figure 1).

Differentiation of *S. cerevisiae* strains by RFLPs of mtDNA demonstrated the presence of different native strains in the non-inoculated must, however none of them shared the pattern displayed by *S. cerevisiae* T73. At the end of fermentation, the RFLPs of mtDNA revealed that none of the *S. cerevisiae* strains present in the must was predominant. Up to 8 patterns in the sequential and 9 in the simultaneous fermentation were isolated; in the first case only 10% of the isolates corresponded to the inoculated T73 strain, and in the second case the percentage increased slightly to 20%.

3.3 Analytical profile of wines

The main enological characteristics of the wines produced are presented in Table 2. Ethanol and glycerol levels and wine pH were not affected by the type of inoculation, whereas acetaldehyde and acetic acid levels were significantly higher in sequentially inoculated fermentations. All of the higher alcohols levels were affected by the inoculation type, although the trend was different depending on the individual alcohol. 2-phenylethyl alcohol and isobutanol levels were higher in wines obtained by sequentially inoculated fermentation whereas isoamyl alcohol and propanol concentrations were lower in those wines.

Among acetate esters, ethyl acetate and 2-phenylethyl acetate were higher in wines obtained by sequentially inoculated fermentation. On the contrary isoamyl acetate concentration was lower in such fermentations. Both ethyl esters were higher in mixed inoculated wines.

The main differences between both fermentations were reflected in the two-fold increase in acetaldehyde, 2-phenylethyl acetate and ethyl acetate concentrations in wines obtained by sequential fermentations. Moreover, these wines showed two-fold decrease in propanol and ethyl caprylate levels in comparison to those obtained by simultaneous fermentations.

Table 2. General composition and major volatile compounds[a] of wines produced by simultaneous and sequential fermentations of *H. vineae-S. cerevisiae*.

	Simultaneous fermentation	Sequential fermentation
pH	3.97 ± 0.06	3.81 ± 0.07
Ethanol (%, v/v)	12.7 ± 0.4	12.6 ± 0.3
Acetaldehyde (mg/L)*	30.6 ± 2.1	65.5 ± 10.6
Acetic acid (g/L)*	0.45 ± 0.02	0.64 ± 0.07
Glycerol (g/L)	7.0 ± 0.1	6.9 ± 0.1
2-Phenylethyl alcohol (mg/L)*	16.0 ± 0.2	18.2 ± 0.9
Isoamyl alcohol (mg/L)*	166.9 ± 3.6	156.1 ± 2.8
Propanol (mg/L)*	1.15 ± 0.10	0.60 ± 0.10
Isobutanol (mg/L)*	38.0 ± 0.8	48.0 ± 0.4
Ethyl acetate (mg/L)*	81.8 ± 5.3	138.2 ± 5.6
Isoamyl acetate (mg/L)*	5.83 ± 0.23	4.09 ± 0.19
2-Phenylethyl acetate (mg/L)*	0.81 ± 0.06	1.70 ± 0.32
Hexyl acetate (mg/L)	0.27 ± 0.04	0.20 ± 0.01
Ethyl caproate (mg/L)*	0.37 ± 0.02	0.31 ± 0.01
Ethyl caprylate (mg/L)*	0.40 ± 0.01	0.23 ± 0.01

[a]Mean values for three independent experiments and standard deviations.

*denotes significant differences (P<0.05; Student's t-test)

4. Discussion

In a previous work the growth and the production of 2-phenylethyl acetate of a mixed culture of *H. vineae* and *S. cerevisiae* on a sterilized must was studied. Moreover we showed that 2-phenylethyl acetate concentration showed a positive relationship with the percentage of *H. vineae* in the mixed culture, when fitted to a linear model (Viana et al., 2009). The studies reported here were conducted to evaluate the growth of *H. vineae* as a part of a mixed culture with *S. cerevisiae* in presence of native yeasts and its effect on wine volatiles, particularly 2-phenylethyl acetate. Inoculation of non-sterilized Tempranillo must was done using two approaches, simultaneous or sequential inoculation of both yeasts. In the first approach, both yeasts were inoculated at day 0 of fermentation and, in the second one *H. vineae* was inoculated at day 0 of fermentation and *S. cerevisiae* at day 4.

Total yeast cell counts found in Tempranillo must (4.7×10^6 cfu/mL) were in the range reported for sulphited musts of different grape varieties such as Garnatxa (10^6-10^7 cfu/mL) and Xarel.lo (10^5-10^7 cfu/mL) (Beltrán et al., 2002). Slightly lower cell counts were described for sulphited Riesling (10^5-10^6 cfu/mL; Egli et al., 1998) and Malbec musts (10^4-10^6 cfu/mL; Combina et al., 2005). As previously reported non-*Saccharomyces* yeasts were the most usual microorganisms in grape must, probably because they are present in grape skins and vineyards. Density of non-*Saccharomyces* yeasts in freshly extracted grape musts may range from about 10^3-10^6 cfu/mL (Granchi et al., 1998). Six different species belonging to 4 genera were identified in Tempranillo must. In general, the yeast species identified have been previously described in spontaneous fermentations. There was no one clearly predominant yeast species in Tempranillo must, although the most frequent species (approximately 60%) belonged to the genus *Hanseniaspora*. Usually *H. uvarum* (*Kloeckera apiculata*) is one of the most frequently described species in fresh musts from different places, although in our study only represented a 7 % of total count, which is lower than the percentages of *H. guilliermondii* (28.6%) and *H. osmophila* (21.4%). *Z. cidri*

was the second most frequent species (25%). Although yeasts of the *Zygosaccharomyces* genus are very rare in sound grapes and are considered spoilage yeasts, the presence of different species has been described in fresh must (Ganga and Martínez, 2004; Ocón et al., 2010). *M. pulcherrima* and *T. delbrueckii*, present in Tempranillo must, are also among the common species isolated from spontaneous fermentations.

Previous observations about the infrequent presence of *S. cerevisiae* in grape musts have been stated by different authors (Gutiérrez et al., 1999; Martini et al., 1996; Povhe Jemec et al., 2001; Schütz and Gafner, 1993;). However, several studies, as the one reported here, have detected the presence of this species in must samples and at the beginning of the fermentation (Beltrán et al., 2002; Combina et al., 2005; Ocón et al., 2010; Querol et al., 1992b). In Tempranillo must *S. cerevisiae* represented less than 10% of total yeast count. As reported by several authors, the presence of *S. cerevisiae* at the beginning of the fermentation may explain the relatively low population diversity found of non-*Saccharomyces* species (Beltrán et al., 2002; Ocón et al., 2010).

As a general trend, both inoculation strategies appeared not to have any influence on must fermentation dynamics, as fermentation processes ended on the same day and all enological parameters were within the range of levels usually found in wines. Independently of the type of inoculation, *S. cerevisiae,* as expected*,* was the predominant species from day 3 till the end of fermentation and at day 4 practically none fermentable sugars remained. Therefore, inoculation of *S. cerevisiae* T73 at the fourth day in the sequential fermentation had neither effect on the fermentation kinetics nor on the *S. cerevisiae* population at the end of fermentation. With respect to non-*Saccharomyces* yeasts, sequential inoculation allowed *H. vineae* to be, together with *H. osmophila*, the predominat species at day 1 of fermentation, and at day 3 to be only overcome by native *S. cerevisiae*. On the contrary the simultaneous inoculation of *S. cerevisiae* did not allow any non-*Saccharomyces* species to be the predominant species. It is known that inoculation of *S. cerevisiae* strains can inhibit the growth of non-

Saccharomyces yeasts and influence the development of wild *Saccharomyces* strains (Heard and Fleet, 1985). Also it has been showed in mixed fermentations trials that non-*Saccharomyces* yeasts show marked effects on *S. cerevisiae* growth (Comitini et al., 2010). Our results suggest that *H. vineae* is able to compete better with native non-*Saccharomyces* yeasts in the absence of inoculated *S. cerevisiae* (see day 1). Apparently, the presence of *S. cerevisiae* in high numbers in the simultaneously inoculated fermentation inhibited the growth of inoculated *H. vineae*. In this sense, high cell densities of *S. cerevisiae* seem to mediate the early death of *Kluyveromyces thermotolerans* and *T. delbrueckii* by a cell-to-cell contact mechanism (Nissen and Arneborg, 2003). However this effect was not observed in *H. vineae*-*S. cerevisiae* mixed cultures in sterile musts (Viana et al., 2009).

Interestingly, differences in non-*Saccharomyces* growth found between simultaneous and sequential fermentations influenced the analytical profile of final wines. Of note, 2-phenylethyl acetate concentration was approximately two-fold (1.70 mg/L) in sequentially inoculated wines in comparison to those co-inoculated (0.81 mg/L). In sterile musts we had previously reported increases in 2-phenylethyl acetate concentrations 3- to 9-fold greater in wines obtained by mixed *H. vineae*-*S. cerevisiae* cultures than those produced by *S. cerevisiae* pure cultures. However, the quantity of 2-phenylethyl acetate was only significantly different from that formed by *S. cerevisiae* monoculture if the percentage of *H. vineae* in the culture was at least 50%. In those conditions *H. vineae* population exceeded that of *S. cerevisiae* cells during the first day of fermentation, remained constant until day 3 and then it started to decline (Viana et al., 2009). In this study the initial percentage of *H. vineae* in the culture was 90%, but represented 30% of non-*Saccharomyces* growth at day 1 of sequentially inoculated fermentation in comparison to 5% of *S. cerevisae*. On the contrary, in the co-inoculated wines *H. vineae* reached a slightly lower population at day 1, representing 15% of the total count, whereas, *S. cerevisiae* represented more than 30%.

2-Phenylethyl acetate levels in wine vary from traces to 0.96 mg/L whereas its aroma threshold is around 0.25 mg/L (Francis and Newton, 2005). The inoculated strain of *S. cerevisiae* T73 in pure culture fermentations carried out in sterilized must with an initial sugar content of 200 g/L was able to produce 2-phenylethyl acetate levels in the range 0.4-1.5 mg/L (Viana et al., 2009). Similarly, several strains of *H. guilliermondii*, the predominat yeast species in Tempranillo must (28.6 %), have been described as good producers of 2-phenylethyl acetate, but also of ethyl acetate (Rojas et al., 2001, 2003; Viana et al., 2008; Moreira et al., 2008). Therefore the contribution of native yeast, and particularly *H. guilliermondii* and *S. cerevisiae* metabolism to the final ester concentration in both fermentations could not be discarded. In a recent work, Moreira et al (2011) described the contribution of an inoculated *H. guilliermondii* strain in a natural must in comparison to a spontaneous fermentation. They found that must inoculated with *H. guilliermondii* led to the production of wine with higher concentration of 2-phenylethyl acetate than wine resulting from the spontaneous fermentation (0.733 vs 0.356 mg/L).

Ethyl acetate and acetic acid concentrations, usually associated with apiculated yeast growth, were also increased in sequentially inoculated wines. In our experiments acetic acid concentration reached levels of 0.64 g/L, within the optimal concentration range of 0.2-0.7 g/L described for wines (Lambrechts and Pretorius, 2000). Ethyl acetate, the main ester in wine, can impart spoilage character at 150-200 mg/L (Jackson, 1994), which is slightly higher than the levels found in our fermentation trials. *H. vineae* either in pure or mixed fermentations in sterilised musts produced levels of ethyl acetate in the range 40-67 mg/L (Viana et al., 2008, 2009). In this study ethyl acetate concentrations reached approximately 140 mg/L, probably due to the growth of apiculated yeasts already present in the initial must. This ethyl acetate level is much lower than that found in wines obtained by inoculation of *H. guilliermondii* (791 mg/L; Moreira et al., 2011).

The use of starter cultures in wine production is currently a common practice that promotes the optimization and standardization of the

fermentation process. Nevertheless inoculated strains must dominate the fermentation process and play a fundamental role in the quality of the final product. Here we show that *H. vineae* inoculated as part of a sequential mixed starter is able to compete with native yeasts present in a non-sterilised must and produce the desired effect in the final wine. Taking into account that initial yeast population in natural musts has an impact on population evolution, the feasibility of *H. vineae* CECT 1471 as a commercial starter deserves further research.

5. Acknowledgements

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Artículo IV

2-Phenylethyl acetate formation by immobilized cells of *Hanseniaspora vineae* in sequential mixed fermentations

ABSTRACT

Hanseniaspora vineae 1471, characterized as a strong producer of 2-phenylethyl acetate in wine, has been immobilized on calcium alginate beads. Cell immobilization did not affect the levels of 2-phenylethyl acetate. Removal of *H. vineae* immobilized cells from the fermenting must at different time points followed by inoculation of free cells of a commercial strain of *Saccharomyces cerevisiae* can be used as a tool to modulate the concentration 2-phenylethyl acetate in wine. Analysis of enological characteristics and volatile compounds showed the feasibility of the use of the mixed sequential cultures to produce wines with different chemical characteristics.

Keywords: immobilization, *H. vineae*, 2-phenylethyl acetate, alginate, wine.

1. Introduction

During the last few decades, yeast impact on wine fermentation, wine chemistry and wine sensory properties has been elucidated (for reviews see Lambrechts and Pretorius 2000, Romano et al. 2003). Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage (Sponholz 1993, Ciani and Picciotti 1995) the potential positive role they play in the organoleptic characteristics of wine has been emphasized in numerous studies (reviewed in Fleet 2003). This knowledge has provided new challenges for innovation in wine fermentation technology and tailoring wines for new consumer demands. In this context, mixed fermentations using controlled inoculation of *S. cerevisiae* and non-*Saccharomyces* yeasts have been proposed as a tool to take advantage of the wine flavour complexity obtained from spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (reviewed in Fleet 2008, Ciani et al. 2010). Moreover blends of active dried yeasts of *S. cerevisiae*/*Kluyveromyces thermotolerans*/*Torulaspora delbrueckii* are commercially available (Vinflora® Harmony.nsac, Christian Hansen, Denmark).

Yeast immobilization for winemaking is also an expanding research area with the purpose of improving alcohol productivity and overall product quality (review in Kourkoutas et al. 2004). Fermentation tests with immobilized non-*Saccharomyces* yeasts have been reported. *Candida stellata* immobilized on alginate in combination with an inoculum of *S. cerevisiae* has been proposed to enhance glycerol formation in wine (Ciani and Ferraro 1996; Ferraro et al. 2000). Immobilized cells of *Schizosaccharomyces pombe* (Silva et al. 2003) and *Issatchenkia orientalis* (Hong et al. 2010) showed potential for reduction of malic acid contents in wine, and the former is now commercially available in an immobilized form (Promalic®; Proenol, Portugal).

In a previous work we selected a *Hanseniaspora vineae* strain (Spanish Type Culture Collection, CECT 1471, previously identified as *Hanseniaspora osmophila*) yielding high levels of 2-phenylethyl acetate, while producing levels of acetic acid and ethyl acetate within the ranges described for wine (Viana et al. 2008). In addition we examined the impact of mixed cultures of *H. vineae* and *S. cerevisiae* on wine composition and our results showed that the mixed culture can be used as a tool to increase 2-phenylethyl acetate in wine and that ester concentration can be controlled by modulating the initial yeast ratio in the culture (Viana et al. 2009). In the present study we report *H. vineae* immobilization on calcium-alginate beads and its behaviour in sequential fermentation tests in combination with free cells of *S. cerevisiae*. The appropriate time for *H. vineae* removal and *S. cerevisiae* inoculation to obtain wines with different levels of 2-phenylethylacetate is also evaluated.

2. Materials and methods

2.1 Yeast strains and culture media

Hanseniaspora vineae 1471 from the CECT Collection and *Saccharomyces cerevisiae* Lalvin T73 (Lallemand Inc, Rexdale, Ontario) were used. Yeast strains were maintained on GPY plates (5 g/L yeast extract, 5 g/L peptone, 40 g/L glucose, 20 g/L agar, pH 5.5). To prepare *H. vineae* cells for immobilization, cells were grown in GPYM medium (5 g/L yeast extract, 5 g/L peptone, 40 g/L glucose, 5 g/L malt extract, 1 g/L $MgSO_4$ $7H_2O$, pH 6).

2.2 Immobilization of yeast cells

H. vineae biomass was grown at 30ºC in GPYM medium in an orbital shaker (200 rpm) for 24 h, harvested by centrifugation and washed twice with sterile 0.9% NaCl solution. To prepare the beads, sterile 4% (w/v) Na-alginate solution was added to cell suspensions. The mixture was then dripped into a 170 g/L $CaCl_2$ solution to induce gelation. After 30 min, the beads were washed several times with sterile distilled water and used immediately.

2.3 Fermentation conditions

Triplicate immobilized *H. vineae* fermentations were carried out in commercial pasteurized Muscat must with an initial sugar content of 256 g/L, pH 3.6 and supplemented with 1 g/L of a complex yeast nutrient (Fermaid K, Lallemand). Aliquots of 225 mL were fermented in 250 mL bottles at 20ºC. Musts were inoculated with 10 g/L of beads (corresponding approximately to 10^7 cells/mL). As a control triplicate free *H. vineae* fermentations were carried out in the same conditions, inoculating the musts with 10^7 cells/mL from 24 h pre-cultures grown in the same must.

Duplicate mixed culture fermentations with immobilized *H. vineae* and free *S. cerevisiae* cells were carried out in red grape must from a Bobal cultivar (Utiel-Requena, Valencia, Spain) with an initial sugar content of 185 g/L, pH 3.2 and supplemented with 1 g/L of Fermaid K. Fresh must was sterilised by adding 1 mg/L of dimethyl di-carbonate (Fluka) and stored at –20 ºC until use. Aliquots of 225 mL of must were fermented in 250 mL bottles at 25ºC. Sequential fermentations were carried out with an inoculum of 10 g/L of *H. vineae* beads. After 4, 5 and 6 days, the beads were removed from the fermenting must and 10^6 cells/mL of *S. cerevisiae* from 24 h pre-cultures grown in the same must, were added. Single culture fermentations with immobilized *H. vineae* cells (10 g/L) were also carried out.

2.4 Analyses

Fermenting musts were sampled to enumerate yeast populations. *S. cerevisiae* free cells and *H. vineae* immobilized cells were counted by plating in GPY plates (5 g/L yeast extract, 5 g/L peptone, 40 g/L glucose, 20 g/L agar, pH 5.5). For the evaluation of *H. vineae* cell viability, beads were maintained under agitation in 10% (w/v) sodium acetate solution to release the cells. Plates were incubated at 30 ºC for 48 h.

Glucose and fructose consumption throughout the fermentation process as well as the concentration of glycerol, acetaldehyde and acetic acid in wines were measured enzymatically in an Echo-Enosys analyzer (Tecnova, San Sebastián de los Reyes, Spain) following the supplier's instructions. Ethanol concentration in wines was determined using the R-Biopharm enzymatic assay (R-Biopharm AG, Darmstadt, Germany). Higher alcohols, esters and fatty acids in wines were analysed by headspace solid-phase microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-Aldrich, Barcelona, Spain) and gas chromatography (GC) as described previously (Viana et al. 2008).

3. Results and discussion

Cell immobilization for winemaking offers several advantages such as enhanced fermentation productivity and feasibility of continuous processing (Kourkoutas et al. 2004). Another advantage of immobilized yeasts is that they can be easily removed from fermenting must or wine and thus they have been proposed to facilitate sparkling wine production (Fumi et al. 1988; Yokotsuka et al. 1997). Also immobilized cells of *Schiz. pombe* were used at the beginning of fermentation until partial or total consumption of L-malic acid and then removed to prevent off-flavor production (Silva et al. 2003).

We have shown the suitability of *H. vineae* 1471 in mixed cultures with *S. cerevisiae* to increase the levels of 2-phenylethylacetate (Viana et al. 2009), ester that contributes to the fruity notes of wine aroma. Considering the potential application to winemaking, it is more likely that a fermentation with *H. vineae* immobilized cells and their removal from must after 2-phenylethylacetate formation followed by *S. cerevisiae* inoculation, would be more acceptable to the industry. Thus the studies reported here were set up to evaluate the effect of *H. vineae* immobilization in calcium alginate beads on fermentation behaviour and mainly on 2-phenylethylacetate production with the final aim of designing sequential mixed fermentations.

3.1 Effect of immobilization on must fermentation

Various supports and immobilization techniques have been proposed for application in winemaking (Kourkoutas et al. 2004). In this study we have used calcium alginate gels, a simple and versatile methodology considered suitable for alcoholic fermentation, as immobilization support for *H. vineae*.

Fermentation trials of free and immobilized *H. vineae* cells were carried out with the aim of evaluating the effect of immobilization on

fermentation behaviour. Figure 1A shows population evolution of *H. vineae* cells in must. In both fermentations, biomass remained close to the concentration of initial inoculum until day 3 when it started to decline. Only at the end of fermentation (days 10 and 11) differences between free and immobilized populations were observed. Leakage of cells from beads into the must at day 3 was found to be lower than 1000 ufc/ml. With respect to sugar consumption along fermentation, a delay was observed in trials carried out with immobilized cells, although final wines contained less than 2 g/L of sugar (Figure 1B).

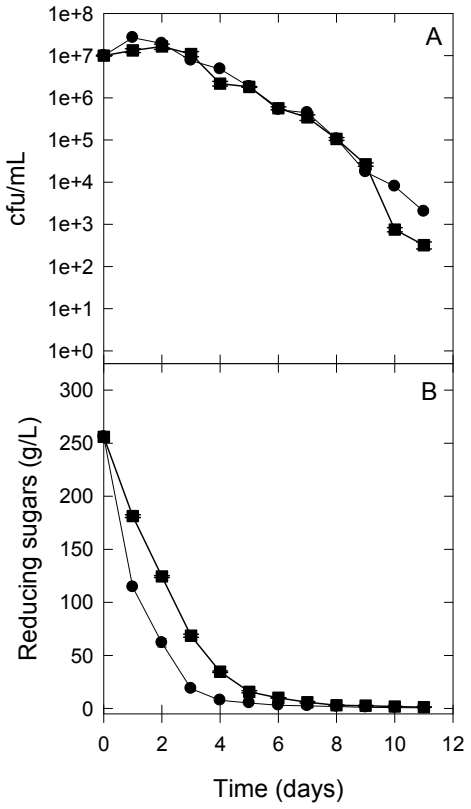

Figure 1. Yeast population evolution (A) and consumption of reducing sugars (B) in must inoculated with free (●) and immobilized (■) *H. vineae* 1471 cells.

The final major compounds of alcoholic fermentation as well as some volatile compounds are summarized in Table 1. Remarkably the levels of acetic acid and ethyl acetate were not affected by immobilization since their concentration did not differ significantly between the wines obtained using free and immobilized yeasts. The immobilization process did not alter the ability of *H. vineae* to produce 2-phenylethyl acetate, which concentration, in agreement with previous results, reached levels of approximately 10 mg/L (Viana et al. 2008). No significant differences were detected in main higher alcohol production whereas ethanol and acetaldehyde levels were higher in immobilized trials as well as the concentration of diethyl succinate. On the contrary, lower levels of glycerol and medium-chain fatty acids were found in wines fermented with immobilized *H. vineae* cells in comparison with free cell fermentation trials. As suggested by different studies, the differences between the concentrations of volatile compounds in wines made using immobilized yeasts and free yeasts may partly be ascribed to differences in the metabolic features of the yeasts in these two states (Yajima and Yokotsuka 2001).

Table 1. General composition and major volatile compounds of wines produced using free and immobilized *H. vineae* 1471 cells.

	Free cells	Immobilized cells
pH	3.38 ± 0.03	3.40 ± 0.03
Ethanol (%, v/v)*	9.1 ± 0.2	10.3 ± 0.6
Acetaldehyde (mg/L)*	11.2 ± 1.9	17.8 ± 1.6
Acetic acid (g/L)	0.44 ± 0.04	0.45 ± 0.01
Glycerol (g/L)*	6.2 ± 0.2	5.5 ± 0.04
Hexanoic acid (mg/L)*	1.2 ± 0.04	1.0 ± 0.1
Octanoic acid (mg/L)*	0.3 ± 0.02	0.2 ± 0.02
Decanoic acid (mg/L)*	0.6 ± 0.02	0.5 ± 0.04
2-Phenylethyl alcohol (mg/L)	7.4 ± 0.5	7.1 ± 1.5
Isoamyl alcohol (mg/L)	114.8 ± 6.6	119.6 ± 20.5
Propanol (mg/L)	0.22 ± 0.03	0.23 ± 0.04
Ethyl acetate (mg/L)	36.4 ± 1.1	37.3 ± 6.2
Isoamyl acetate (mg/L)	0.45 ± 0.03	0.41 ± 0.02
2-Phenylethyl acetate (mg/L)	9.96 ± 0.79	10.39 ± 1.61
Diethyl succinate (mg/L)	1.54 ± 0.10	2.19 ± 0.11

*denotes significant differences (P<0.05; Student's t-test)

3.2 Design of sequential mixed fermentations

To obtain wines with different levels of 2-phenylethyl acetate, mixed immobilized *H. vineae* and free *S. cerevisiae* fermentations were designed. Previously and with the aim of selecting the time points for *H. vineae* beads removal and *S. cerevisiae* cell inoculation, the kinetics of 2-phenylethyl acetate formation by immobilized *H. vineae* cells was studied. Figure 2 shows the evolution of the acetate ester as well as yeast population. 2-phenylethyl acetate was detected from day 3 till the end of fermentation, reaching a concentration of approximately 10.5 mg/L. Day 4, 5 and 6 corresponding to 2-phenylethyl acetate levels of around 2, 5 and 8 mg/L, respectively were selected for sequential inoculation. Yeast population in the beads did not vary significantly from the initial concentration and started to decline at day 6. Free cells in the must, at day 3, were found to be lower than 1000 cfu/mL.

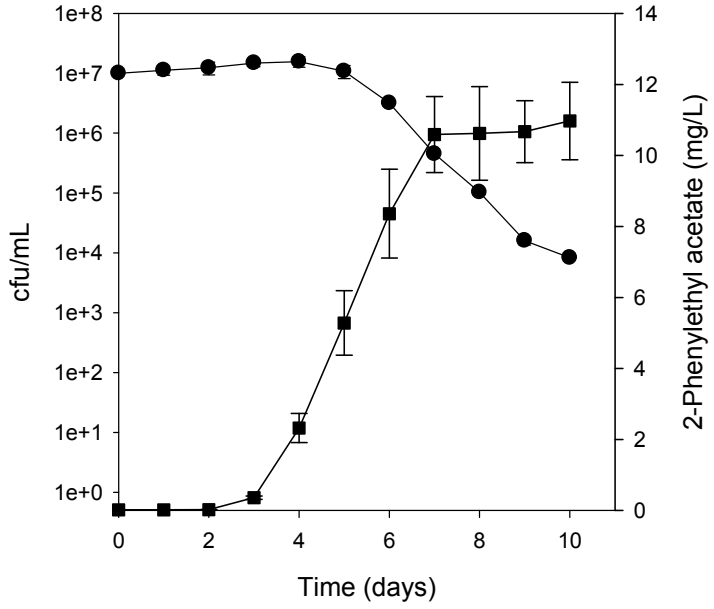

Figure 2. Yeast population evolution (●) and 2-phenylethyl acetate production (■) in must inoculated with immobilized *H. vineae* 1471 cells.

In sequential mixed fermentations *H. vineae* population evolution did not significantly differ from that showed in Figure 2. *S. cerevisiae* population slightly increased during 2 days after the inoculation time and reached a maximum population of approximately 5, 4 and 3 $\times$ 10^6 cells/mL in wines inoculated at day 4, 5 and 6, respectively. No differences in sugar consumption among the three sequential fermentations were found (data not shown).

The main enological characteristics of the wines produced are presented in Table 2. As a general trend and independently of the sequential mixed culture used, all of the parameters were within the range of levels usually found in wines suggesting the feasibility of the sequential fermentations. All of them, with the exception of glycerol and ethanol, were affected by the inoculation time of *S. cerevisiae*. Some of the parameters such as acetaldehyde, 2-phenylethyl alcohol and ethyl acetate showed statistically significant differences among fermentations, but these differences were not correlated with the time of removal of *H. vineae* beads. The concentration of most of the compounds such as medium-chain fatty acids, higher alcohols, isoamyl acetate and diethyl succinate, was slightly lower in wines inoculated with *S. cerevisiae* at day 6 whereas acetic acid and pH slightly increased in those wines. Remarkably *S. cerevisiae* growth did not have any effect on the concentrations of 2-phenylethyl acetate produced by *H. vineae* since the concentrations obtained in the sequential fermentations were similar to those obtained in *H. vineae* single fermentations (Figure 2). Although we have shown that wines obtained with mixed cultures of *H. vineae* and *S. cerevisae* have a stronger fruity character than those fermented with *S. cerevisiae* pure culture (Viana et al. 2009), the sensory impact of wines with increased levels of 2-phenylethyl acetate and obtain with immobilized cells deserves further research.

Results obtained in this work suggest the potential of using immobilized *H. vineae* 1471 cells in sequential mixed cultures with free cells of *S. cerevisiae* to modulate 2-phenylethyl acetate concentration in wine and thus obtain wines with a wide range of flavour compositions.

Further studies including the suitability of different immobilization supports in winery conditions are in progress.

Table 2. Effect of sequential mixed fermentations with different time points for *H. vineae* 1471 bead removal on the general composition and major volatile compounds of wines[a].

	Bead removal at day 4	Bead removal at day 5	Bead removal at day 6
pH	2.93 ± 0.05^{A}	2.90 ± 0.01^{A}	3.16 ± 0.03^{B}
Ethanol (%, v/v)	11.4 ± 0.1^{A}	11.2 ± 0.04^{A}	11.4 ± 0.2^{A}
Acetaldehyde (mg/L)	32.1 ± 3.0^{B}	23.5 ± 1.6^{A}	27.3 ± 2.9^{AB}
Acetic acid (g/L)	0.20 ± 0.01^{A}	0.21 ± 0.01^{AB}	0.23 ± 0.01^{B}
Glycerol (g/L)	5.45 ± 0.01^{A}	5.27 ± 0.11^{A}	5.35 ± 0.14^{A}
Hexanoic acid (mg/L)	4.8 ± 0.3^{B}	4.3 ± 0.1^{AB}	4.2 ± 0.0^{A}
Octanoic acid (mg/L)	6.27 ± 0.29^{B}	5.82 ± 0.03^{B}	5.17 ± 0.07^{A}
Decanoic acid (mg/L)	3.20 ± 0.13^{C}	2.85 ± 0.02^{B}	2.54 ± 0.07^{A}
2-Phenylethyl alcohol (mg/L)	21.5 ± 0.1^{A}	32.8 ± 0.5^{C}	25.2 ± 0.6^{B}
Isoamyl alcohol (mg/L)	183.1 ± 7.7^{B}	137.3 ± 6.1^{A}	153.6 ± 3.7^{A}
Propanol (mg/L)	2.40 ± 0.01^{C}	2.13 ± 0.05^{B}	1.91 ± 0.01^{A}
Ethyl acetate (mg/L)	26.2 ± 0.9^{B}	32.8 ± 0.6^{C}	21.2 ± 1.1^{A}
Isoamyl acetate (mg/L)	0.56 ± 0.01^{C}	0.46 ± 0.01^{B}	0.41 ± 0.01^{A}
2-Phenylethyl acetate (mg/L)	2.69 ± 0.03^{A}	4.75 ± 0.01^{B}	6.36 ± 0.11^{C}
Diethyl succinate (mg/L)	0.38 ± 0.01^{C}	0.35 ± 0.01^{B}	0.30 ± 0.01^{A}

Data with the same letter do not differ at 95% confidence level (LSD procedure).
[a]Mean values for two independent fermentations and standard deviations.

4. Conclusion

The current study contributes to the assessment of the use of controlled mixed fermentations using non-*Saccharomyces* and *Saccharomyces* wine yeast species that can affect both the chemical and the aromatic composition of wines. Moreover *H. vineae* immobilization in combination with free *S. cerevisiae* cells provides a tool for the implementation of new fermentation technologies and an easy approach to ensure the elimination of non-*Saccharomyces* yeasts once the positive effect has been reached.

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4. Discusión general

El estudio de diferentes estrategias para conseguir mayor complejidad aromática a los vinos, es una inquietud constante dentro del campo de la enología. En este sentido, el presente trabajo pretende poner a disposición de los bodegueros cepas de levadura capaces de incidir positivamente sobre la calidad aromática de los vinos. En concreto, la estrategia empleada ha sido la selección de levaduras no-*Saccharomyces* que, formando parte de cultivos mixtos con *S. cerevisiae*, sean capaces de incrementar la concentración de acetato de 2-feniletilo, ya que este compuesto se asocia con un aroma afrutado, floral y con notas de miel y tabaco (Thurston *et al*., 1981). En vinos jóvenes y rosados la concentración de este compuesto varía desde trazas hasta 0.96 mg/L, siendo su umbral de detección 0.25 mg/L (Francis y Newton, 2005).

En la síntesis de los ésteres de acetato está implicada la alcohol acetil-transferasa (AATFasa), que utiliza como sustratos alcohol y acetil~CoA (Ac~CoA) (Peddie, 1990), siendo los factores que gobiernan el metabolismo de este último, fundamentales para la producción de ésteres. Diversos autores han puesto de manifiesto que durante la segunda parte de la fermentación, cuando ya ha cesado la síntesis de lípidos, se produce un aumento en la producción de ésteres, que es proporcional a la concentración de Ac~CoA disponible (Thurston *et al*., 1981; 1982).

La aplicación de los conocimientos a nivel molecular de los genes de *S. cerevisae* que controlan la formación de ésteres de acetato durante la fermentación alcohólica, denominados *ATF1* y *ATF2* (Fujii *et al*., 1994; Nagasawa *et al*., 1998), permitió la construcción de levaduras vínicas transgénicas mediante la sobreexpresión de dichos genes. Lilly *et al*. (2000) clonaron el gen *ATF1* de una levadura vínica comercial y lo integraron en el genoma de tres cepas vínicas comerciales. Los vinos elaborados con las levaduras transgénicas presentaron concentraciones de acetato de 2-feniletilo y acetato de isoamilo de 5.4 y 65.1 mg/L, respectivamente, suponiendo un aumento entre 10 y 12 veces las cantidades producidas por las cepas no transformadas. Así mismo, se observó un incremento considerable de acetato de etilo (600 mg/L), que

distorsionaba, desde el punto de vista organoléptico, el carácter afrutado aportado por los ésteres antes citados. En esta misma línea de investigación, la sobrexpresión/deleción de diferentes genes implicados en el metabolismo de los esteres de acetato ha demostrado su eficacia para modular las concentraciones de los mismos (Verstrepen *et al*., 2003). Sin embargo, los problemas de aceptación social relativos a la utilización de organismos modificados genéticamente en agroalimentación en general, y en enología en particular, sobre todo en la Unión Europea, hacen que esta posibilidad tenga que ser considerada más como alternativa de futuro que de presente.

Tal y como se ha expuesto en la Introducción, en los últimos años, diversos autores han re-evaluado el papel de las levaduras no-*Saccharomyces* en el proceso de vinificación, resaltando su contribución positiva al aroma y presentándolas como una alternativa eficaz a la estrategia antes citada. De hecho, en estos momentos se dispone de cultivos iniciadores mixtos comercializados por Chr. Hansen (Viniflora®; www.chr-hansen.com) y Lallemand (Level2™ TD; www.lallemandwine.com). En el primer caso es posible elegir una mezcla de 2 (*K. thermotolerans y S. cerevisiae*) o 3 levaduras (*T. delbrueckii, K. thermotolerans y S. cerevisiae*) mientras que Lallemand comercializa un mezcla de *T*. *delbrueckii* y *S. cerevisiae*.

Las levaduras no-*Saccharomyces* se han asociado tradicionalmente a la formación de ésteres de acetato, aunque la biosíntesis de los mismos ha sido mucho menos estudiada que en el caso de *S. cerevisiae*. En esta línea de investigación, nuestro grupo evaluó la capacidad para formar diferentes ésteres de acetato, lo que llevó a la selección de una cepa de origen vínico, *H. guilliermondii* 11104, que destacó como potente productora de acetato de 2-feniletilo (Rojas *et al*., 2001). Sin embargo, el inconveniente de la cepa seleccionada fue la producción excesiva de acetato de etilo en mosto en fermentación, de forma similar a cuando se sobreexpresó en levadura vínica el gen *ATF1* (Lilly *et al*., 2000). Cuando la cepa seleccionada se empleó, junto con *S. cerevisiae* en un cultivo mixto,

se observó en el vino un descenso de la concentración de acetato de 2-feniletilo (4.5 mg/L) y de acetato de etilo (200 mg/L), con respecto a los vinos producidos en cultivo puro (8.8 y 400 mg/L, respectivamente) (Rojas *et al*., 2003). Esto sugiere que la participación de *S. cerevisiae* en el iniciador modula la producción de ambos ésteres, lo que podría ser una ventaja en el caso del acetato de etilo. Este hecho fue confirmado con posterioridad por Moreira *et al*. (2008) empleando un cultivo mixto similar al descrito con una cepa diferente de *H. guilliermondii.* Sin embargo, estos vinos, seguían presentando una concentración de acetato de etilo (170 mg/L) superior a la máxima recomendada (150-200 mg/L; Lambrechts y Pretorius, 2000).

A la vista de estos antecedentes resulta evidente la capacidad de la especie *H. guilliermondii* para producir acetato de 2-feniletilo en vino, aunque con el inconveniente de la producción excesiva de acetato de etilo. Para soslayar este problema, en este estudio se planteó como primer objetivo, un escrutinio empleando como criterio de selección la capacidad de producción tanto de acetato de 2-feniletilo como de acetato de etilo. También se incluyó como cualidad positiva la producción de acetato de isoamilo, que confiere fragancias afrutadas que realzan la calidad del vino. El escrutinio se realizó empleando 38 cepas de levaduras no-*Saccharomyces* de origen vínico, pertenecientes a la Colección Española de Cultivos Tipo (CECT). Se incluyeron especies del género *Candida* (*C. cantarelli*, *C. dattila* y *C. stellata*), *Hanseniaspora* (*H. guilliermondii*, *H. uvarum y H. vineae*), *Pichia* (*P. anomala*, *P. fermentans* y *P. membranifaciens*), *Torulaspora* (*T. delbrueckii*) y *Zygosaccharomyces* (*Z. bailii y Z. rouxii*). A efectos comparativos, se incluyeron también en el estudio 9 cepas comerciales de *S. cerevisiae*. Muchas de las especies de levaduras no-*Saccharomyces* estudiadas, habían sido descritas previamente como posibles integrantes de cultivos iniciadores mixtos (revisado en Ciani *et al*., 2010). Con respecto a la formación de ésteres de acetato, además de la ya comentada capacidad de formación de acetato de 2-feniletilo de la especie *H. guilliermondii*, otras especies del mismo

género y también del género *Pichia* habían sido descritas como buenas productoras de acetato de isoamilo (Rojas *et* al., 2001; Plata *et al*., 2003; Moreira *et al*., 2008).

Como resultado de este escrutinio, se seleccionaron 5 cepas de levadura pertenecientes a los géneros *Hanseniaspora* y *Pichia* (*H. guilliermondii* 11027 y 11102, *H. vineae* 1471, *P. membranifaciens* 10113 y 10550). Debido a que la producción de SH_2 y la formación de vinil- y etilfenoles por parte de las levaduras vínicas impacta negativamente en el aroma del vino, se evaluó, en estas levaduras seleccionadas, su actividad sulfito reductasa y la actividad cinamato descarboxilasa. Por lo que respecta a la actividad sulfito reductasa, no se detectó en la especie *Pichia membranifaciens*, pero sí en las especies de *Hanseniaspora*, aunque a niveles similares a los detectados en cepas comerciales de *S. cerevisiae*. Por el contrario, la actividad cinamato descarboxilasa no se detectó en ninguna de las cepas seleccionadas.

Con el objetivo de seleccionar, de entre estas cinco cepas, aquella mas apropiada para formar parte un cultivo iniciador mixto con *S. cerevisiae*, se determinó su perfil enológico. Este perfil incluyó consumo de glucosa y fructosa, por su posible efecto sobre el comportamiento fermentativo de *S. cerevisiae*, debido a su naturaleza glucofílica. Así mismo también se consideró la formación de etanol, acidez volátil, acetaldehído, glicerol, ácidos grasos de cadena corta, ésteres y alcoholes superiores. En concreto, el ácido acético, además de suponer un problema organoléptico cuando su concentración se aproxima a su umbral de detección (0.7-1.1 mg/L), también puede inhibir el crecimiento de *S. cerevisiae*, aunque para ello son necesarias concentraciones notablemente superiores (4.5-7.5 g/L) (Edwards *et al*., 1999). Este efecto de inhibición también se asocia a concentraciones excesivas de ácidos grasos de cadena corta, capaces de provocar paradas de fermentación (Edwards *et al*., 1990).

De las levaduras evaluadas, *P. membranifaciens* 10550 fue incapaz de crecer en mosto, por lo que fue descartada para su caracterización

enológica. Las 4 cepas restantes, en mayor o menor medida, fermentaron el mosto, alcanzándose una concentración entre 12 y 100 g/L de azúcares residuales y entre 6 y 11.6 % (v/v) de etanol. En general, el análisis de los compuestos de fermentación indicó que las 4 cepas produjeron niveles de acetaldehído y glicerol ligeramente inferiores a los niveles producidos por *S. cerevisiae*. La producción de ácido acético se situó en el intervalo 0.57-1.09 g/L, correspondiendo el valor más alto a *H. guilliermondii* 11102, por lo que fue descartada. Destacar así mismo que, en general, las cepas evaluadas produjeron niveles de ácido hexanoico y octanoico notablemente inferiores a *S. cerevisiae*, mientras que la concentración de ácido decanoico se acercó más a los niveles inferiores producidos por esta levadura. En el caso de los alcoholes superiores, su concentración, notablemente inferior a la producida por *S. cerevisiae*, se encontraba en el rango descrito como apropiado para vino (< 300 mg/L; Lambrechts y Pretorius, 2000). Estos resultados por tanto no se consideraron determinantes como criterio de exclusión.

El parámetro al que se dio mayor relevancia en el escrutinio fue la formación de acetato de etilo y de 2-feniletilo, lo que nos permitió seleccionar *H. vineae* 1471 como la cepa más adecuada (acetato de etilo: 39.9 mg/L; acetato de 2-feniletilo: 12.9 mg/L). En cuanto a la formación de acetato de isoamilo, ninguna de las cepas reprodujo en mosto los resultados obtenidos en medio microbiológico. Curiosamente estos resultados concuerdan con estudios previos de nuestro grupo, ya que la cepa *P. anomala* 10590, seleccionada por su elevada producción de acetato de isoamilo en medio microbiológico, produjo cantidades mínimas del éster en vino (Rojas *et al*., 2003). Por el contrario, Moreira *et al.* (2008) describieron una cepa de *H. uvarum* capaz de producir 5.1 mg/L de acetato de isoamilo durante la fermentación del mosto de uva.

Seleccionada *H. vineae* 1471, como segundo objetivo de este trabajo y empleando diferentes porcentajes de la misma, se abordó el diseño de cultivos iniciadores mixtos. Para ello, se evaluaron distintos iniciadores que incluyeron como base la proporción no-*Saccharomyces* y

S. cerevisiae al inicio de las fermentaciones espontáneas (90:10, % *H. vineae*: % *S. cerevisiae;* Schütz *et al*. (1993)), así como cultivos en los que se fue reduciendo de forma progresiva el porcentaje de *H. vineae* (75:25, 50:50, 25:75, 10:90, 5:95 y 0:100). La finalidad de este objetivo fue el diseño de iniciadores más acordes con el punto de vista del bodeguero, que tiende a valorar mejor aquellos cultivos que contienen porcentajes elevados de *S. cerevisiae*. En este contexto, señalar que algunos de los cultivos iniciadores mixtos comerciales antes citados pueden adquirirse en dos formatos que varían en el porcentaje inicial de las levaduras no-*Saccharomyces*, 20% (Viniflora® HARMONY.nsac y Viniflora® SYMPHONY.nsac) o 40% (Viniflora® MELODY.nsac y Viniflora® RHYTHM.nsac), según el efecto, más suave o más acusado, que se desee obtener (www.chr-hansen.com).

A la vista de los resultados obtenidos, fue posible reducir el porcentaje de *H. vineae* hasta un 50%, sin comprometer el efecto beneficioso buscado, ya que la cantidad de acetato de 2-feniletilo obtenida fue significativamente superior a la producida por *S. cerevisiae*. Los ensayos indicaron que, en general, todos los parámetros enológicos, a excepción del glicerol, se vieron afectados por la presencia de *H. vineae* en el iniciador. Se observó una relación lineal positiva con el porcentaje de levadura para las concentraciones de acético, isobutanol, acetato de etilo y acetato de 2-feniletilo, mientras que fue negativa para las de ácidos grasos de cadena corta, 2-feniletanol, propanol y los ésteres etílicos.

Otra conclusión extraída de estos experimentos fue que, independientemente de su porcentaje inicial, *H. vineae* fue capaz de crecer en presencia de *S. cerevisiae* de forma similar a su crecimiento en cultivo puro. Moreira *et al*. (2008) también describieron parámetros de crecimientos similares para *H. uvarum* y *H. guilliermondii* en cultivos puros y mixtos. Sin embargo, otras especies, como *K. thermotolerans* y *T. delbrueckii*, no presentan este comportamiento, ya que su crecimiento se ve seriamente afectado por la presencia de *S. cerevisiae* en el iniciador (Nissen *et al*., 2003). Se ha sugerido que la muerte de estas dos especies

estaría relacionada con un mecanismo de contacto célula a célula dependiente de la presencia de un alto número de células viables de *S. cerevisiae* (Nissen y Arneborg, 2003). Recientemente, también se ha descrito que determinadas cepas de *S. cerevisiae* secretan péptidos que inhiben el crecimiento de algunas levaduras no-*Saccharomyces* (Albergaria *et al*., 2010).

En nuestras condiciones de fermentación, la población de *H. vineae* decreció tras alcanzar el máximo después de 2 días de fermentación, hasta su práctica desaparición a los 8 días, con la excepción de los vinos inoculados con el mayor porcentaje. Tradicionalmente, se ha asociado la desaparición de las levaduras no-*Saccharomyces,* particularmente las especies del género *Hanseniaspora*, después de los primeros 3-4 días de fermentación, a su poca tolerancia al etanol (Fleet, 2003). Sin embargo, en concordancia con nuestros resultados, un estudio reciente señala que las levaduras apiculadas pueden sobrevivir a lo largo de la fermentación alcohólica durante periodos de tiempo mucho más largos que los inicialmente pensados. En concreto, se han descrito diferentes especies de *Hanseniaspora* con una tolerancia al etanol similar a la de *S. cerevisiae* (Rojas *et al*., 2003; Pina *et al*., 2004), que sobreviven en fermentaciones espontáneas en presencia de una concentración de etanol del 12.5% (Moreira *et al*., 2011).

Teniendo en cuenta que las fermentaciones realizadas en bodegas, no se realizan con mostos estériles, sino con mostos que, a pesar de ser tratados con sulfuroso, presentan una microbiota propia, como tercer objetivo de este trabajo se estudió la implantación de algunos de los cultivos iniciadores diseñados en mostos naturales no estériles. Para ello se empleó mosto de la variedad Tempranillo, con un recuento inicial de levaduras elevado (>10^6 cfu/ml). El 90% de estas levaduras fueron no-*Saccharomyces* y aunque no hubo ninguna especie predominante, *H. guilliermondii*, *H. osmophila* y *Zygosaccharomyces cidri*, representaron, cada una de ellas, entre un 20 y un 30% de la microbiota. Es de destacar

que en el mosto no se detectó *H. vineae*, lo que facilitó el seguimiento de la cepa inoculada.

Dado que algunas firmas comerciales recomiendan la aplicación de los cultivos mixtos de forma secuencial para conseguir una mejor implantación, se incluyó este tipo de inoculación comparándola con la inoculación conjunta. El tipo de inoculación no influyó de manera notable sobre la cinética de fermentación, aunque sí sobre las especies predominantes. En la inoculación secuencial, *H. vineae* fue la especie de levadura no-*Saccharomyces* predominante al inicio de la fermentación (días 1 y 3), lo que no se observó en el caso de la inoculación conjunta. Estos resultados parecerían sugerir que la elevada densidad celular de *S. cerevisiae* en la fermentación conjunta provocada por la inoculación podría estar inhibiendo, al menos en parte, el crecimiento de *H. vineae*. Este hecho podría estar relacionado con el posible mecanismo célula a célula, ya comentado, causante de la desaparición de *K. thermotolerans* y *T. delbrueckii* en cultivos mixtos cuando el número de células de *S. cerevisiae* es elevado (Nissen y Arneborg, 2003). Sin embargo, este efecto no se observó en los cultivos mixtos *H. vineae*-*S. cerevisiae* en mostos estériles.

En concordancia con el mayor crecimiento de la cepa de *H. vineae* en las fermentaciones secuenciales, la concentración de acetato de 2-feniletilo (1.7 mg/L) fue superior, con significancia estadística, a la obtenida en los vinos inoculados conjuntamente (0.8 mg/L). Para poner en contexto estas cantidades de acetato de 2-feniletilo con las obtenidas en mostos estériles, hay que recordar que el porcentaje mínimo de *H. vineae* en el iniciador para que se alcanzara una concentración significativa del éster fue del 50%. Con este porcentaje se alcanzó una concentración celular de *H. vineae* superior a la de *S. cerevisiae* durante los tres primeros días de fermentación, lo que no fue posible en el caso del mosto no estéril a pesar de utilizar un inóculo 90:10. Es obvio, que la presencia de la microbiota nativa en este último caso influyó en el crecimiento de *H. vineae* y por tanto no se alcanzaron concentraciones tan elevadas de acetato de 2-feniletilo como las obtenidas en mostos estériles. Por último, señalar, como ya se ha

comentado anteriormente, que no se puede descartar la contribución de la microbiota nativa a la concentración final de acetato de 2-feniletilo, sobre todo teniendo en cuenta la presencia de *H. guilliermondii* en el mosto y durante los primeros días de fermentación, ya que se trata de una especie caracterizada en esta memoria y en trabajos anteriores por su capacidad de formación de dicho éster (Rojas *et al*., 2001, 2003; Moreira *et al*., 2008).

A pesar del interés despertado por las levaduras no-*Saccharomyces* y la posibilidad de emplear cultivos iniciadores mixtos en vinificación existen pocos trabajos en los que se aborde la implantación de un iniciador de este tipo en mostos naturales. En uno de ellos, Moreira *et al*. (2011) observaron el crecimiento de una cepa inoculada de *H. guilliermondii,* seleccionada por su capacidad de formación de acetato de 2-feniletilo (Moreira *et al*., 2008), junto con una cepa nativa de *H. uvarum*. Durante la fermentación, ambas mantuvieron su viabilidad, mientras que las levaduras nativas no-*Saccharomyces* no-apiculadas desaparecieron a partir del quinto o sexto día de fermentación cuando las cepas nativas de *S. cerevisiae* fueron predominantes. Al igual que en nuestro estudio, la concentración de acetato de 2-feniletilo en los vinos obtenidos en presencia de *H. guilliermondii* fue mucho menor que la cantidad obtenida en fermentaciones llevadas a cabo en mostos estériles con cultivos iniciadores mixtos *H. guilliermondii*: *S. cerevisae* (0.733 mg/L frente a 17.3 mg/L). Cabe destacar que la concentración de acetato de etilo en los vinos obtenidos a partir de mostos no estériles (791 mg/L) fue muy superior a la detectada en nuestras fermentaciones (82 y 140 mg/L en las fermentaciones secuenciales y simultáneas, respectivamente).

Por último, con el fin de controlar el periodo de permanencia de *H. vineae* en el mosto en fermentación, como cuarto objetivo de esta tesis se planteó el estudio de la inmovilización celular de la levadura apiculada. De esta forma, se podría asegurar la práctica desaparición de la levadura, además de modular la cantidad de acetato de 2-feniletilo en función del momento de la retirada. Esta estrategia, aunque más explorada en el caso de *S. cerevisiae*, ha sido aplicada con éxito en el caso de *Schizo. pombe* al

inicio de la fermentación alcohólica para lograr el consumo parcial o total de ácido L-málico y evitar una vez conseguido el efecto, el desarrollo de aromas extraños característicos de esta levadura (Silva *et al*., 2003).

Como periodos de permanencia de *H. vineae* durante la fermentación, se seleccionaron 3 tiempos a los cuales retirar las células inmovilizadas e inocular *S. cerevisiae* en forma libre. Considerando que el periodo de permanencia se relaciona con la producción de acetato de 2-feniletilo se seleccionaron los días 4, 5 y 6 de fermentación, correspondientes a 2, 5 y 8 mg/L de dicho éster. Con la inoculación de *S. cerevisiae* a estos mostos (en los que se había consumido un 25, 50 y 60% de los azúcares iniciales), se completó la fermentación sin que prácticamente se viese afectada la concentración de acetato de 2-feniletilo en los vinos obtenidos. En estos vinos, no puede descartarse la contribución a la formación del éster por parte de *S. cerevisiae*, ya que esta cepa en cultivo puro fue capaz de producir aproximadamente 1.5 mg/L de acetato de 2-feniletilo, a partir de una concentración de azucares de 185 g/L. Dado que las concentraciones de azúcares en estos experimentos fueron inferiores, 130, 90 y 70 g/L, cuando se inoculó *S. cerevisiae* es muy probable que su producción de acetato de 2-feniletilo sea menor que en cultivo puro.

Con estos ensayos se ha demostrado el potencial de *H. vineae* inmovilizada como integrante de un cultivo mixto secuencial junto con células libres de S*. cerevisiae*, para modular la concentración de acetato de 2-feniletilo en vino, y por tanto ofrecer la posibilidad de obtener vinos, con parámetros enológicos apropiados, y composiciones aromáticas diferentes.

Una vez demostrado, desde el punto de vista analítico, la posibilidad de obtener vinos con distintas concentraciones de acetato de 2-feniletilo, bien mediante cultivos mixtos con células libres o combinado células inmovilizadas y libres, se planteó la posibilidad de confirmar estos resultados a nivel sensorial. Para ello se realizaron fermentaciones a mayor escala empleando el cultivo mixto que había producido la mayor

concentración de acetato de 2-feniletilo en microfermentaciones con células libres (90:10). En estas nuevas condiciones de vinificación se alcanzó una concentración de acetato de 2-feniletilo (1.5 mg/L) cuatro veces superior a la obtenida en vinificaciones con *S. cerevisiae* en cultivo puro (0.39 g/L), aunque estos niveles fueron inferiores a los obtenidos en las microvinificaciones. Con respecto a los atributos sensoriales considerados, intensidad, calidad del aroma y aroma frutal, sólo este último fue más intenso, con significancia estadística, en los vinos fermentados con el cultivo mixto, mientras que la intensidad y calidad del aroma fueron similares en ambos vinos. Estos resultados, aunque pueden ser considerados como preliminares, sí que demostrarían a nivel sensorial el impacto de *H. vineae* en las características aromáticas del vino.

Considerando todo lo expuesto en esta memoria, es evidente que la utilización de cultivos iniciadores mixtos con levaduras no-*Saccharomyces* seleccionadas con criterios enológicos razonables, es una alternativa prometedora que merece la pena continuar siendo explorada, tal y como nuestro grupo ha contribuido a poner de manifiesto.

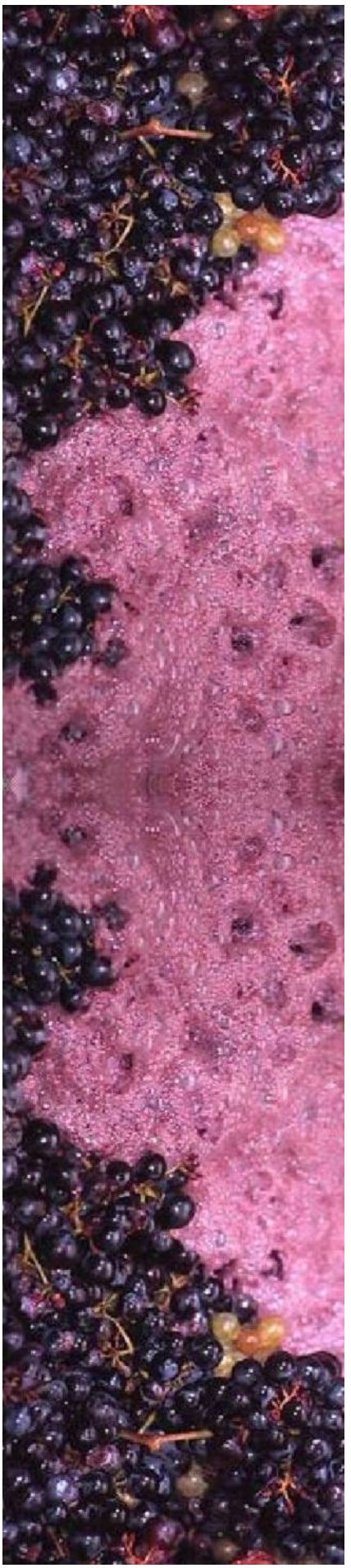
En este sentido se ha demostrado que *H. vineae* 1471, como integrante de cultivos iniciadores, produce vinos cuyos parámetros enológicos se encuentran en el intervalo descrito como apropiado, y es capaz de incrementar los niveles de acetato de 2-feniletilo con respecto a los producidos por *S. cerevisiae*. Sin embargo, dada la complejidad aromática del vino, el incremento de un único compuesto, podría no repercutir de forma significativa en el aroma global. En este sentido, existen estudios que señalan las interacciones, tanto de sinergia como de supresión, entre diferentes compuestos aromáticos (Francis y Newton, 2005). En nuestro caso, los análisis organolépticos indicaron un carácter afrutado más intenso en los vinos obtenidos empleando cultivos iniciadores mixtos, lo que sugiere que el incremento de la concentración del acetato de 2-feniletilo puede tener repercusión sobre el aroma global. Teniendo en cuenta que estos resultados corresponden a un vino en concreto, sería conveniente llevar a cabo estudios de escalado que permitieran abordar

distintos métodos de vinificación que podrían optimizar la producción de acetato de 2-feniletilo.

Por lo que respecta a la inmovilización, sería interesante desarrollar estudios más exhaustivos que tuvieran en cuenta la reutilización de las células inmovilizadas u otros soportes que minimizaran la liberación de las células entrampadas. En este sentido, se han descrito esferas de alginato de doble capa que minimizan la liberación de células durante la fermentación (Taillandier *et al*., 1994) así como la posibilidad de deshidratar las células inmovilizadas en los geles de alginato para aumentar su periodo de almacenamiento (Silva *et al*., 2003). La inmovilización, poco explotada en el caso de levaduras no-*Saccharomyces*, podría aportar tecnologías de vinificación novedosas y atractivas, para lo que resultaría imprescindible profundizar en su estudio.

Por último, un aspecto importante que no se ha abordado en este trabajo, y que facilitaría la aplicación de *H. vineae* a nivel industrial sería su producción en forma de levadura seca activa como los iniciadores de *S. cerevisiae.* Es de esperar que dada la experiencia de las empresas productoras de levaduras, la producción de no-*Saccharomyces* en forma de LSA no presente grandes dificultades, y que en los próximos años se incremente la oferta comercial de este tipo de iniciadores.

5. Conclusiones



1. Este estudio ha confirmado el potencial de las levaduras no-*Saccharomyces* pertenecientes a los géneros *Pichia* y *Hanseniaspora* para producir ésteres de acetato.
2. La selección de levaduras no-*Saccharomyces* basada en criterios enológicos bien definidos facilita el diseño de cultivos iniciadores con una capacidad óptima de producción de ésteres de acetato, sin comprometer la calidad del vino.
3. Como resultado del escrutinio llevado a cabo con el fin de aumentar la concentración de acetato de 2-feniletilo en vino mediante cultivos iniciadores mixtos con *Saccharomyces cerevisiae*, se ha seleccionado la levadura *Hanseniaspora vineae* CECT 1471 como la cepa más adecuada.
4. La inclusión de *H. vineae* 1471 en el cultivo iniciador mixto permite obtener vinos con niveles de acetato de 2-feniletilo superiores a los obtenidos con *S. cerevisiae* en monocultivo.
5. La proporción inicial de *H. vineae* 1471 y *S. cerevisiae* en el iniciador modula la concentración de ésteres de acetato, lo que permite obtener vinos con diferente perfil aromático. En nuestras condiciones de vinificación, una proporción de *H. vineae* del 50% en el iniciador, es suficiente para producir vinos con perfiles aromáticos distintos a los obtenidos con *S. cerevisiae*.
6. La presencia de *S. cerevisiae* en el iniciador no afectó el crecimiento de *H. vineae* 1471, en comparación con su crecimiento en cultivo puro, independientemente de los porcentajes iniciales de ambas levaduras.
7. *H. vineae* 1471 es capaz de crecer en mostos no estériles con una microbiota nativa elevada. Sin embargo, para conseguir las concentraciones de acetato de 2-feniletilo apropiadas, debe inocularse de forma secuencial con *S. cerevisiae*.

8. La inmovilización en geles de alginato es adecuada para el entrampamiento de *H. vineae* 1471, puesto que ni su viabilidad ni su capacidad de producción de acetato de 2-feniletilo se ven afectadas.
9. La utilización de *H. vineae* 1471 en forma inmovilizada permite controlar su permanencia y, consecuentemente, su efecto durante la fermentación, consiguiendose así modular la concentración final de acetato de 2-feniletilo en vino.
10. *H. vineae* 1471, como integrante de un cultivo mixto, es capaz de producir vinos con perfiles aromáticos cuantitativamente diferentes a los obtenidos con *S. cerevisiae,* que además presentan a nivel sensorial un carácter más afrutado.

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Anexos

LISTA DE PUBLICACIONES

Esta tesis es una compilación de las siguientes publicaciones:

I. **Fernando Viana**, José V. Gil, Salvador Genovés, Salvador Vallés and Paloma Manzanares (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology* 25: 778-785.

II. **Fernando Viana**, José V. Gil, Salvador Vallés and Paloma Manzanares (2009). Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 135: 68-74.

III. **Fernando Viana**, Carmela Belloch, Salvador Vallés and Paloma Manzanares. Monitoring a mixed starter of *Hanseniaspora vineae-Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. Artículo aceptado en *International Journal of Food Microbiology* (DOI: 10.1016/j.ijfoodmicro.2011.09.005).

IV. **Fernando Viana**, Patricia Taillandier, Salvador Vallés, Pierre Strehaiano and Paloma Manzanares (2011). 2-Phenylethyl acetate formation by immobilized cells of *Hanseniaspora vineae* in sequential mixed fermentations. *American Journal of Enology and Viticulture* 62: 122-126.

Anexo 1

Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits

Fernando Viana, José V. Gil, Salvador Genovés, Salvador Vallés and Paloma Manzanares

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Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits

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ABSTRACT

Thirty-eight yeast strains belonging to the genera *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* were screened for ester formation on synthetic microbiological medium. The genera *Hanseniaspora* and *Pichia* stood out as the best acetate ester producers. Based on the ester profile *Hanseniaspora guilliermondii* 11027 and 11102, *Hanseniaspora osmophila* 1471 and *Pichia membranifaciens* 10113 and 10550 were selected for further characterization of enological traits. When growing on must *H. osmophila* 1471, which displayed a glucophilic nature and was able to consume more than 90% of initial must sugars, produced levels of acetic acid, medium chain fatty acids and ethyl acetate, within the ranges described for wine. On the other hand, it was found to be a strong producer of 2-phenylethyl acetate. Our data suggest that *H. osmophila* 1471 is a good candidate for mixed starters, although the possible interactions with *S. cerevisiae* deserve further research.

1. Introduction

Nowadays a number of viticultural and winemaking practices are being investigated to improve wine quality. In this context, there is a growing demand for new and improved wine yeast strains adapted to different types and styles of wines. Industrial wine fermentations are currently conducted by starters of selected wine yeast strains of *Saccharomyces cerevisiae* in contrast to traditional spontaneous fermentations conducted by the flora present on the grapes and in the winery. Despite the advantages of using pure cultures of *S. cerevisiae* with regard to the easy control and homogeneity of fermentations, wine produced with pure yeast monocultures lacks the complexity of flavour, stylistic distinction and vintage variability caused by indigenous yeasts (Lambrechts and Pretorius, 2000; Romano et al., 2003). In recent years the inclusion of non-*Saccharomyces* wine yeast species as part of mixed starters together with *S. cerevisiae* to improve wine quality has been suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Jolly et al., 2003; Romano et al., 2003; Rojas et al., 2003; Ciani et al., 2006).

Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage (Sponholz, 1993; Ciani and Picciotti, 1995), the potential positive role they play in the organoleptic characteristics of wine has been emphasized in numerous studies (reviewed in Fleet, 2003). Metabolic interactions between non-*Saccharomyces* wine yeasts and *S. cerevisiae* during fermentation could positively or negatively interfere with the growth and fermentation behaviour of yeast species, particularly *S. cerevisiae*. In this context, positive interactions between fructophilic non-*Saccharomyces* yeasts and glucophilic *S. cerevisiae* strains have been described (Ciani and Fatichenti, 1999). By contrast, negative interactions have been reported, caused by the production of compounds that inhibit to *S. cerevisiae* such as medium-chain fatty acids or killer factors (Bisson, 1999). Given the strain biodiversity of non-*Saccharomyces* yeasts in regard to their production levels of enzymatic activities (Manzanares et al., 1999, 2000; Mendes-Ferreira et al., 2001; Strauss et al., 2001) and fermentation metabolites (Romano et al., 1992, 2003; Capece et al., 2005) of enological importance, suitable strains should be selected in order to be able to design mixed starters capable of providing beneficial contributions to wine quality.

Among fermentation metabolites, it is generally described that esters make the greatest contribution to the characteristic fruity odours of wine fermentation bouquet (Rapp and Mandery, 1986). Acetate esters such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate, recognised as important flavour

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Anexo 2

Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*

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Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*

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ABSTRACT

The impact of mixed cultures of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae* with different initial yeast ratios on wine composition has been examined. The mixed culture significantly affected sugar consumption, the main enological parameters and ester concentrations, with the exception of glycerol, isoamyl acetate and diethyl succinate levels. Remarkably, in wines obtained with mixed cultures the concentration of 2-phenylethyl acetate was approximately 3- to 9-fold greater than that produced by *S. cerevisiae* pure culture. Moreover sensory evaluation revealed a stronger fruity character in wines fermented with mixed cultures than in control wines. Independently of the mixed culture used, all wines showed concentrations of acetic acid and ethyl acetate within the ranges described for wines. Our data suggest that a mixed culture of *H. osmophila* and *S. cerevisiae* can be used as a tool to increase 2-phenylethyl acetate in wine and that its concentration can be controlled by modulating the initial yeast ratio in the culture.

1. Introduction

Wine fermentation is a complex microbiological process in which yeasts play a fundamental role. Spontaneous wine fermentation gives rise to a succession of yeasts: non-*Saccharomyces* yeast species grow during the early stages of the process whereas *Saccharomyces cerevisiae* strains dominate at the later stages of fermentation due to their ethanol resistance (Fleet and Heard, 1993). Nowadays, fermentations inoculated with selected *S. cerevisiae* strains are prevalent in large-scale wine production due to the ease of control and homogeneity of fermentations. However, spontaneous fermentations reinforce wine flavour complexity, stylistic distinction and vintage variability (Lambrechts and Pretorius, 2000; Romano et al., 2003). An alternative to both fermentation practices is the use of mixed starters of selected yeasts combined with a commercial strain of *S. cerevisiae* to avoid stuck fermentations, which takes advantage of the potential positive role that non-*Saccharomyces* wine yeast species play in the organoleptic characteristics of wine (reviewed in Fleet, 2003).

Several studies have evaluated the feasibility of mixed starters to improve wine quality. In this context, studies have proposed *Candida stellata* to enhance glycerol content (Soden et al., 2000) and *Torulaspora delbrueckii*, in combination with *S. cerevisiae*, to improve the analytical profile of sweet wines (Bely et al., 2008). Similarly, geraniol concentration was increased in Muscat wines by using a mixed culture with *Debaryomyces vanriji* (Garcia et al., 2002); whereas *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* grown as mixed cultures in grape must increased the 2-phenylethyl acetate and isoamyl acetate content of wines, respectively (Rojas et al., 2003; Moreira et al., 2008). Considering the technological potential of mixed cultures, studies have been made into the influence of fermentation parameters such as oxygen and temperature on the fermentation behaviour of mixed starters (Holm Hansen et al., 2001; Ciani and Comitini, 2006) as well as the effect of several inoculation protocols (Soden et al., 2000). Moreover, some studies have characterized the nature and kinetics of the death of non-*Saccharomyces* yeasts in mixed cultures that seems to be mediated by a cell–cell contact-mediated mechanism rather than to nutrient depletion or the presence of toxic compounds (Nissen and Arneborg, 2003; Nissen et al., 2003).

With the aim of selecting yeast strains able to modulate the aromatic profile of wines, we have screened non-*Saccharomyces* wine yeasts for their potential to produce acetate esters and studied their effect as part of mixed cultures on acetate ester concentrations in wine (Rojas et al., 2001, 2003). However, due to their excessive production of ethyl acetate, their applicability to winemaking was limited. Recently, we characterized the ester profile of non-*Saccharomyces* wine yeast strains when grown in synthetic medium and verified their fermentation behaviour in must. This study allowed us to select a *Hanseniaspora osmophila* strain yielding high levels of 2-

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Anexo 3

2-Phenylethyl Acetate Formation by Immobilized Cells of *Hanseniaspora vineae* in Sequential Mixed Fermentations

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Research Note

2-Phenylethyl Acetate Formation by Immobilized Cells of *Hanseniaspora vineae* in Sequential Mixed Fermentations

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Abstract: *Hanseniaspora vineae* 1471, characterized as a strong producer of 2-phenylethyl acetate in wine, has been immobilized on calcium alginate beads. Fermentation trials of free and immobilized cells showed that immobilization did not affect the levels of 2-phenylethyl acetate. The removal of *H. vineae* immobilized cells from the fermenting must at different time points followed by the inoculation of free cells of a commercial strain of *Saccharomyces cerevisiae* can be used as a tool to modulate the concentration of 2-phenylethyl acetate in wine. Analysis of enological characteristics and volatile compounds showed the feasibility of the use of the mixed sequential cultures to produce wines with different chemical characteristics.

Key words: immobilization, *Hanseniaspora vineae*, 2-phenylethyl acetate, alginate, wine

During the last few decades, yeast impact on wine fermentation, wine chemistry, and wine sensory properties has been elucidated (for reviews see Lambrechts and Pretorius 2000, Romano et al. 2003). Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavors, and wine spoilage (Sponholz 1993, Ciani and Picciotti 1995), the potential positive role they play in the sensory characteristics of wine has been emphasized in numerous studies (reviewed in Fleet 2003). This knowledge has provided new challenges for innovation in wine fermentation technology and tailoring wines for new consumer demands. Mixed fermentations using controlled inoculation of *S. cerevisiae* and non-*Saccharomyces* yeasts with optimized ratios have been proposed as a tool to take advantage of the wine flavor complexity obtained from spontaneous fermentations without running the risk of stuck fermentation or wine spoilage (reviewed in Fleet 2008, Ciani et al. 2010). Moreover, blends of active dried yeasts of *S. cerevisiae*, *Kluyveromyces thermotolerans*, and *Torulaspora delbrueckii* are commercially available (Viniflora Harmony. nsac, Chr. Hansen, Hoersholm, Denmark).

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Yeast immobilization for winemaking is also an expanding research area with the purpose of improving alcohol productivity and overall product quality (review in Kourkoutas et al. 2004). Immobilized yeasts can be easily removed from fermenting must or wine, and they have been proposed to facilitate sparkling wine production (Fumi et al. 1988, Yokotsuka et al. 1997). Immobilized cells of *Schizosaccharomyces pombe* were used at the beginning of fermentation until partial or total consumption of L-malic acid and then removed to prevent off-flavor production (Silva et al. 2003). This strain is now commercially available in an immobilized form (Promalic; Proenol, Portugal). Fermentation tests with immobilized *Candida stellata* (Ciani and Ferraro 1996, Ferraro et al. 2000) and *Issatchenkia orientalis* cells (Hong et al. 2010) have also been proposed to enhance glycerol formation and to reduce L-malic acid content in wine, respectively.

In a previous work, we selected a *Hanseniaspora vineae* strain (Spanish Type Culture Collection, CECT 1471, previously identified as *Hanseniaspora osmophila*) yielding high levels of 2-phenylethyl acetate, while producing levels of acetic acid and ethyl acetate within the ranges described for wine (Viana et al. 2008). In addition, we examined the impact of mixed cultures of *H. vineae* and *S. cerevisiae* on wine composition. Results showed that the mixed culture can be used to increase 2-phenylethyl acetate in wine and that ester concentration can be controlled by modulating the initial yeast ratio in the culture (Viana et al. 2009). Considering the potential application to winemaking, it is more likely that a fermentation with *H. vineae* immobilized cells and their removal from must after 2-phenylethyl acetate formation followed by *S. cerevisiae* inoculation would be more acceptable to the industry. Thus, the studies reported here were set up to evaluate the effect of *H. vineae* immobilization in calcium alginate beads on fermentation behavior and mainly on 2-phenylethyl acetate production, with the final aim of designing sequential mixed fermentations. The appropriate time for *H. vineae* removal