UNIVERSIDAD DE SEVILLA
Facultad de Química
Departamento de Cristalografía, Mineralogía y Química Agrícola

ESTUDIO ECOLÓGICO DE *SACCHAROMYCES* EN LA SERRANÍA DE RONDA (MÁLAGA). COMPORTAMIENTO ENOLÓGICO DE CEPAS DE *SACCHAROMYCES* AUTÓCTONAS.

Memoria que presenta la
Licenciada ALMUDENA CLAVIJO RODRÍGUEZ para
optar al título de Doctor en Biología por la Universidad de Sevilla

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Doña MATILDE FORTEZA GONZÁLEZ, Profesora Titular de Universidad y Directora del Departamento de Cristalografía, Mineralogía y Química Agrícola de la Facultad de Química de la Universidad de Sevilla,

CERTIFICA: Que la Tesis Doctoral titulada "ESTUDIO ECOLÓGICO DE *SACCHAROMYCES* EN LA SERRANÍA DE RONDA (MÁLAGA). COMPORTAMIENTO ENOLÓGICO DE CEPAS DE *SACCHAROMYCES* AUTÓCTONAS.", que presenta la Lda. Almudena Clavijo Rodríguez para optar al grado de Doctor en Biología, ha sido realizado en el Área de Edafología y Química Agrícola de este Departamento bajo la dirección de las Dras. Patricia I. Paneque Macías y Mª Lourdes Morales Gómez, durante el tiempo requerido y reuniendo los requisitos exigidos en este tipo de trabajo.

Y para que así conste, firmo el presente certificado en Sevilla, Julio de 2010.

Fdo. Matilde Forteza González

PATRICIA I. PANEQUE MACÍAS, Profesora Colaboradora del Área de Edafología y Química Agrícola de la Universidad de Sevilla y Mª LOURDES MORALES GÓMEZ, Profesora Titular del Área de Nutrición y Bromatología de la Universidad de Sevilla,

CERTIFICAN: Que la Tesis Doctoral titulada "ESTUDIO ECOLÓGICO DE *SACCHAROMYCES* EN LA SERRANÍA DE RONDA (MÁLAGA). COMPORTAMIENTO ENOLÓGICO DE CEPAS DE *SACCHAROMYCES* AUTÓCTONAS.", ha sido realizada por la Lda. Dña. Almudena Clavijo Rodríguez, en el Departamento de Cristalografía, Mineralogía y Química Agrícola de la Universidad de Sevilla, bajo su dirección y que reune, a su juicio, las condiciones necesarias para optar al grado de Doctor en Biología por la Universidad de Sevilla.

Y para que así conste, firmamos en Sevilla, Julio de 2010.

Directora

Fdo. Patricia I. Paneque Macías

Directora

Fdo. Mª Lourdes Morales Gómez

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ÍNDICE

INTRODUCCIÓN

1. Microorganismos asociados a la elaboración del vino

La existencia de alimentos fermentados por levaduras como el pan, la cerveza y el vino es milenaria; es bien conocido que estos productos eran ya consumidos por los egipcios, y probablemente por pueblos vecinos y otros con los que comerciaban (Spencer & Spencer, 1997). Sin embargo, los agentes responsables de la fermentación no fueron observados por primera vez hasta finales del s. XVII (1680), cuando el comerciante danés Antonie van Leeuwenhoek haciendo uso de un rudimentario microscopio construido por él mismo, observó unos pequeños corpúsculos ovalados en la cerveza fermentada a los que llamó "animáculas". A pesar de que comunicó sus observaciones a la Real Sociedad de Londres, se desconoce hasta qué punto se especuló sobre la naturaleza de esos organismos o sobre su papel en la fermentación de la cerveza y del vino.

Cagniard-Latour en Fancia (1835) y Schawnn y Kutzing en Alemania (1837) observaron la presencia de organismos unicelulares en sedimentos procedentes de las vasijas de fermentación, y aunque no identificaron dichos organismos, demuestran que la causa de la fermentación está ligada a su desarrollo (Spencer & Spencer, 1997; Suárez Lepe & Iñigo Leal, 2004); en 1838, Meyen los clasifica como *Saccharomyces*, siendo más tarde conocidas como **levaduras** (de "levare" o levantar) (Hidalgo Togores, 2003).

Sin embargo, fue Louis Pasteur quien a través de sus trabajos *Etudes sur le Vin* (1866) y *Etudes sur la Bière* (1876) demostró de manera irrefutable la naturaleza biológica de las fermentaciones. Sin la presencia de levaduras no ocurre la fermentación y si otros organismos de morfología diferente están presentes, la fermentación no ocurre de forma deseada y el vino se estropea (Spencer & Spencer, 1997). Por primera vez obtiene cultivos de levaduras diferentes, establece el origen y ciclo de algunas de ellas en la naturaleza, precisando su inexistencia sobre la superficie de las uvas verdes y su asentamiento cuando están maduras, adheridas a través de la pruina, y también esclarece la naturaleza bacteriana de algunas enfermedades del vino (Hidalgo Togores, 2003; Suárez lepe & Iñigo Leal, 2004).

Desde Pasteur, la microbiología de las vinificaciones ha sido ampliamente estudiada revelando la gran complejidad de su ecología (Fleet, 1993). Aunque las levaduras juegan un papel central, las uvas, mostos y vinos pueden contener otros microorganismos, como hongos (otros diferentes a las levaduras), bacterias lácticas y acéticas y virus (Fleet, 1993; Pretorius, 2000; Hidalgo Togores, 2003). La intervención de estos microorganismos es limitada debido a la cantidad de interacciones que se producen entre ellos y a los efectos acumulados del etanol y el pH combinados con la escasez de nutrientes y a las medidas tomadas durante el proceso que evitan su desarrollo (Fugelsang, 1997). De esta manera, según Pardo et al. (1989), los hongos desaparecen rápidamente al inicio de la fermentación, la población de bacterias lácticas disminuye durante la fermentación alcohólica para luego incrementar y llevar a cabo la fermentación maloláctica y las levaduras, por el contrario, crecen rápidamente en el mosto y alcanzan su población máxima para el desarrollo de la fermentación alcohólica.

Las bacterias lácticas de interés enológico están representadas por los géneros *Lactobacillus, Pediococcus, Oenococcus* y *Leuconostoc* (Fugelsang, 1997). Su actividad influye positiva y negativamente en la producción del vino. Por un lado, su acción positiva está relacionada con la fermentación maloláctica (de la que es responsable principalmente *Oenococcus oeni*) a traves de la cual degradan el ácido málico a ácido láctico disminuyendo así la acidez de los vinos (esencial en los tintos) y además contribuye a la estabilidad microbiológica del producto final pues, por una parte, eliminan nutrientes residuales impidiendo que el vino pueda fermentar tras su embotellado comercial y, por otra producen bacteriocinas (Fleet, 2003; Muñoz et al., 2005). Por otro lado, pueden producir alteraciones que disminuyen la calidad del vino. Algunas son ya bien conocidas, como la degradación del glicerol o el denominado "picado láctico"; y otras se han descrito en la última década y todavía no se conocen con suficiente detalle; estas alteraciones afectan al plano sensorial ya que producen olores indeseables debidos a la producción de fenoles volátiles o a la formación de bases heterocíclicas aromáticas. También se ha estudiado en los últimos años, la implicación de las bacterias lácticas en la calidad higiénica de los vinos que derivan del metabolismo de los aminoácidos, como es la formación de carbamato de etilo y de aminas biógenas (Muñoz et al., 2005).

Otro factor de gran importancia económica en la industria, relacionado con las bacterias lácticas es la infección por **bacteriófagos** ya que provocan paradas en la

degradación de ácido málico durante la fermentación maloláctica, por pérdida de la viabilidad de las bacterias lácticas aunque aún no esta clara esta relación (Muñoz et al., 2005).

Las bacterias acéticas están representadas por 6 géneros: *Acetobacter, Gluconobacter, Gluconacetobacter, Acidomonas, Asaia* y *Kozakia*. Su crecimiento es indeseable ya que están asociadas a procesos de alteración del vino, fundamentalmente mediante la producción de ácido acético, acetaldehído y acetato de etilo (Fugelsang, 1997). Son responsables del picado acético en los vinos, pero también de la pudrición ácida en uvas y mostos, de la producción de polisacáridos extracelulares que dificultan el filtrado de los vinos, de la degradación del glicerol y de la producción de ácido glucónico y exofructosa como productos del metabolismo de los azúcares, los cuales poseen una elevada capacidad para unirse al SO_2, disminuyendo su capacidad antimicrobiana y antioxidante, entre otros efectos (Guillamón & Mas, 2005).

Los hongos filamentosos intervienen en diferentes etapas de la producción del vino aunque principalmente influyen durante el cultivo de la uva causando su deterioro (Fugelsang, 1997; Fleet, 2003). Los más importantes o habituales en la vid son *Oidium tuckeri* causante del oidio, *Plasmopara vitícola* causante del Mildiu, *Botrystis cinerea* que ocasiona la podredumbre gris o "botritis", *Phyllosticta ampelicida* responsable de la podredumbre negra, *Phomopsis vitícola* que provoca la excoriosis y *Eutipa lata*, causante de la eutipiosis (Cantoral & Collado, 2005). Por otro lado, el crecimiento de hongos como *Botrytis cinerea, Aspergillu*s spp. y *Penicillium* spp. puede dar lugar a la producción de diversos metabolitos y condiciones influyendo sobre la ecología y crecimiento de las levaduras durante la fermentación (Fleet, 2003). Sin embargo, se han encontrado algunas levaduras que poseen una fuerte actividad fungicida, como *Metschnikowia pulcherrima*, varias especies de *Candida*, *Pichia*, *Cryptococcus*, y algunas especies de *Saccharomyces* y *Zygosaccharomyces*; que ha propiciado la iniciativa de usarlas como agentes naturales en biocontrol (Fleet, 2003).

2. Las levaduras vínicas: su origen

Las levaduras son hongos unicelulares microscópicos, que se reproducen por gemación o escisión, constituyendo un grupo taxonómico complejo y heterogéneo, que incluyen Ascomicetos, Basidiomicetos y Hongos Imperfectos según su desarrollo

sexual (modo de formación de esporas), si bien la mayoría de levaduras vínicas pertenecen a los Ascomicetes (*Saccharomyces*, *Debaryomyces*, *Dekkera*, *Hansenula*, *Kluyveromyces, Pichia*, *Zygosaccharomyces*, *Torulospora*, *Hanseniaspora,* y *Schizosaccharomyces*) y a los Hongos Imperfectos (*Brettanomyces, Candida, Kloeckera, Cryptococcus, Rhodotorula* y *Metschnikowia*). Mientras que en los Ascomicetos las esporas o ascosporas haploides están contenidas en ascas (especie de sacos formados a partir de la célula vegetativa), en las levaduras asporógenas no se pudo evidenciar un modo de reproducción sexuada y están clasificadas entre los Hongos Imperfectos o Deuteromicetos (Ribéreau-Gayon et al., 2003 a; Hidalgo Togores, 2003).

El origen de las levaduras que participan en la fermentación ha sido tema muy discutido en estos últimos años. Según algunos estudios, se encuentra en los nidos o colmenas de los insectos, o incluso en el propio insecto formando parte de su microbiota intestinal (Mortimer, 2000). Las levaduras alcanzan el viñedo por los insectos o por efecto de diseminación del viento, que las transportan desde sus nidos o colmenas de forma que están presentes en las uvas desde el inicio de maduración (Lafon-Lafourcade, 1983; Mortimer & Porsinelli, 1999). La población de levaduras en el mosto fresco de uva sana y madura oscila entre 10^3-10^5 ufc/ml. Sin embargo, la población total de levaduras en el grano de uva, así como la proporción relativa de cada especie, depende de diversos factores como la temperatura, la pluviometría y otros factores climáticos, grado de madurez de la uva, uso de fungicidas o pesticidas, daño físico por hongos, insectos o pájaros, la altitud, la variedad de uva y la campaña de estudio (Fleet & Heard, 1993; Boulton et al., 1996; Sabate et al., 2002; Ribéreau-Gayon et al., 2003 a; Fleet, 2003). Los géneros *Kloeckera/Hanseniaspora* representan entre el 50-75% de la población total de levaduras en el grano de uva, aunque en menor proporción es posible encontrar otras especies de levaduras estrictamente oxidativas o débilmente fermentativas y poco tolerantes al etanol, pertenecientes a los géneros *Metschnikowia, Candida, Cryptococcus, Rhodotorula, Pichia, Kluyveromyces* y *Hansenula* (Fleet & Heard, 1993; Ribéreau-Gayon et al., 2003 a; Barata et al., 2008). Estas especies también son conocidas como no-*Saccharomyces*. Sin embargo, la presencia de *Saccharomyces* en las uvas es muy reducida o incluso nula (Fleet & Heard, 1993; Sabate et al., 2002; Ribéreau-Gayon et al., 2003 a; Ciani et al., 2004), siendo más abundantes en uvas rotas, dañadas y sobremaduras (De la Torre et al., 1999; Mortimer & Polsinelli, 1999; Sabate et al., 2002; Schuller et al., 2005; Barata et al., 2008). Por otro lado, Valero et al. (2007)

en su estudio en el viñedo encuentran una gran diversidad de *S. cerevisiae* y exponen que la biodiversidad de esta especie en el viñedo depende de la edad o el tamaño del mismo y de las condiciones climáticas. Resultados similares obtienen Schuller et al. (2005) que todas las colonias que aíslan en el viñedo pertenecen a *S. cerevisiae*. Sin embargo, aunque hay autores que defienden que las levaduras que participan en la fermentación provienen en parte del viñedo y en parte de la bodega (Le Jeune et al., 2006), Ciani et al. (2004) y Mercado et al. (2007) demuestran que la contribución en el proceso de fermentación de las levaduras *Saccharomyces cerevisiae* presentes en las uvas es poco significativa o nula.

Por otra parte, en las superficies y equipos de bodega, con la que el mosto puede entrar en contacto durante el proceso de vinificación, se desarrolla una microflora de levaduras característica conocida como residencial, constituida principalmente por *S. cerevisiae* aunque también han sido aisladas en este ambiente especies de los géneros *Kloeckera, Torulospora, Brettanomyces, Candida, Hansenula* y *Pichia* (Fleet & Heard, 1993). Según Martín & Martín (1990) ésta constituye la principal fuente de cepas indígenas de *S. cerevisiae* para el desarrollo de la fermentación. Por tanto, la población de levaduras que se desarrolle en la bodega dependerá de la naturaleza de la superficie y del grado de limpieza y estado sanitario en el que se encuentre (Fleet & Heard, 1993). Sin embargo, las condiciones en las que se realicen las fermentaciones determinan la naturaleza de las cepas *S. cerevisiae* que se encuentran en estas superficies. Así, el empleo de levaduras comerciales, la frecuencia de su uso y la edad de la bodega influyen en la población de levaduras autóctonas encontradas en las superficies y equipos de bodega y en las fermentaciones (Beltrán et al., 2002; Santamaría et al., 2005; Mercado et al., 2007).

3. Biología de las levaduras *Saccharomyces cerevisiae*

La levadura es el más simple de los organismos eucariotas. Son hongos unicelulares formados por dos envueltas celulares (pared y membrana plasmática), un citoplasma con orgánulos y un núcleo verdadero recubierto por una membrana encerrando los cromosomas. Pared y membrana plasmática son muy importantes para el desarrollo de la fermentación alcohólica y para la composición del vino ya que ceden algunos de sus componentes al medio (Ribéreau-Gayon et al., 2003 a).

Por su destacado papel en la vinificación, se describen a continuación algunos aspectos sobre la pared celular, las mitocondrias, la constitución genética, la reproducción y ciclos de vida, el metabolismo, el crecimiento y cinética de fermentación de *S. cerevisiae*.

3.1. La pared celular

La pared celular representa entre el 15 y el 25% del peso seco de la célula. Es de naturaleza esencialmente polisacarídica, formada por glucanos (60% del peso seco), manoproteínas (25-50%) y quitina (1-2%). Aunque es una envoltura rígida, está dotada de cierta flexibilidad.

Es una estructura dinámica, multifuncional, cuya composición y funciones evolucionan en el curso de la vida de la célula y según los factores de su medio ambiente. La pared celular le confiere a la levadura protección, su propia forma (por su organización macromolecular) y lugar de anclaje de moléculas que determinan ciertas interacciones celulares (como la unión sexual, la floculación y el factor *killer)* y de numerosas enzimas, generalmente hidrolasas, asociadas a la pared algunas y otras alojadas en el espacio periplasmático (entre la pared y la membrana plasmática) cuyos sustratos son sustancias nutritivas del medio en el que se desarrollan e incluso las macromoléculas de la pared, constantemente modificadas en el transcurso de la morfogénesis celular (Ribéreau-Gayon et al., 2003 a).

La liberación "artificial" de la pared mediante la acción hidrolítica de enzimas en una solución estabilizada osmóticamente origina un protoplasto o esferoplasto, lo que ha facilitado el estudio de la propia pared celular, el aislamiento de orgánulos y otros componentes celulares (como el ADN) y también estudios genéticos en los que se precisa la fusión y transformación de levaduras con material genético exógeno.

3.2. Las mitocondrias

Es fundamental el papel metabólico de las mitocondrias, los verdaderos orgánulos respiratorios de las levaduras. En aerobiosis la célula de *S. cerevisiae* contiene cerca de cincuenta. En anaerobiosis, estos orgánulos degeneran pero de forma reversible, de forma que si las células formadas en anaerobiosis son reubicadas en aerobiosis, las mitocondrias recuperan su forma normal (Ribéreau-Gayon et al., 2003 a).

Debido a su naturaleza fermentativa, pueden existir cepas de *Saccharomyces* sin mitocondrias (los denominados mutantes deficientes en respiración o "petite"). Durante la fermentación, debido a la elevada concentración de glucosa en el medio de cultivo, se produce una inhibición de la síntesis de enzimas implicados en el ciclo del ácido cítrico y de los citocromos de la cadena respiratoria, y la actividad oxidativa mitocondrial es baja. Este fenómeno se conoce como "represión catabólica de la glucosa". Sin embargo, durante algunas etapas de la vinificación, las levaduras realizan un metabolismo respiratorio, con una implicación muy importante de las mitocondrias (Hidalgo Togores, 2003; Aranda et al., 2005).

Las mitocondrias poseen un genoma cuyos genes codifican proteínas de este orgánulo implicadas en la cadena respiratoria (Barre et al., 1993; Ribéreau-Gayon et al., 2003 a).

3.3. El genoma

El genoma de *S. cerevisiae* y en general, el de las levaduras vínicas, es el más pequeño de los genoma eucariotas conocidos. Este material genético está formado por el ADN cromosómico, el mitocondrial y el plasmídico y según la cepa, dos moléculas de ARN ubicadas en el citoplasma que confieren el llamado factor *killer* (Barre et al., 1993).

3.3.1. ADN nuclear

El ADN nuclear está formado por el ADN cromosómico y el plasmídico.

El ADN cromosómico representa el 80-85% del ADN total en una cepa haploide y contiene alrededor de 14.000 kb, escaso comparado al de los eucariotas superiores. Está organizado en 16 cromosomas de tamaño comprendido entre 2.000 y 200 kpb que no pueden ser observados al microscopio óptico por su pequeño tamaño y su débil condensación. Sin embargo, las cepas vínicas son principalmente diploides, aneuploides y ocasionalmente poliploides, y altamente heterocigóticas mostrando un gran polimorfismo en la longitud cromosómica, de forma que el análisis de los cariotipos es uno de los principales criterios de identificación de las cepas (Barre et al., 1993; Pretorius, 2000; Pérez-Ortín et al., 2002; Ribéreau-Gayon et al., 2003 a).

Los cromosomas de las levaduras contienen pocos intrones y relativamente pocas secuencias repetidas; la mayoría de los genes sólo están presentes en un solo ejemplar

en un genoma haploide. Sin embargo, los genes de los ARN ribosómicos (5,8S, 18S y 26S) son abundantemente repetitivos, se agrupan en tándem formando unidades de transcripción que se repiten en el genoma entre 100 y 200 veces (Fernández-Espinar et al., 2005; Ribéreau-Gayon et al., 2003 a).

Además de los cromosomas, en el núcleo existe un plásmido de 2 μm. Se trata de una molécula circular de ADN que contiene 6 kpb, presentes en cantidades de 50 a 100 copias por célula, cuya función biológica es desconocida (Ribéreau-Gayon, 2003 a).

3.3.2. ADN mitocondrial

El ADN mitocondrial (ADNmt) consiste en ADN circular de 75 kpb cuyos genes confieren resistencia a ciertos antisépticos, sistemas de enzimas respiratorios y sistemas de componentes estructurales de la membrana mitocondrial (Suárez Lepe, 1997). La molécula posee dos propiedades notables: su alto grado de polimorfismo según la cepa y su estabilidad (baja tasa de mutación) durante la multiplicación vegetativa (Ribéreau-Gayon, 2003 a). Los distintos tamaños del ADNmt entre las distintas cepas de *S. cerevisiae* resultan de la presencia variable de algunos intrones y de variaciones de tamaño en las regiones intergénicas (Clark-Walter et al., 1981). Esta variabilidad ha sido utilizada para la caracterización de cepas unida a otra característica de esta molécula: su rica composición en regiones AT (75% de la molécula) además de unas 200 regiones ricas en GC (Barre et al., 1993; Fernández-Espinar et al., 2005).

3.3.3. ARN *killer*

La propiedad *killer*, que confiere a las cepas que la poseen la capacidad de matar a otras cepas de fenotipo *sensible*, consiste en genes nucleares y dos ARN citoplasmáticos de doble cadena: L y M. El ARN L está normalmente en las células, sólo el ARN M es específico de cepas de fenotipo *killer* o *neutras* ya que es el que codifica para la toxina *killer* y para el factor de inmunidad a toxinas. Se han descrito 5 tipos de toxinas *killer* en *S. cerevisiae*: K1, K2, K3 (Young & Yagiu, 1978), KT28 (Pfeiffer et al., 1982) y K3GR1 (Extremera et al., 1982) según la agresividad de éstas, siendo K1 la de mayor agresividad (Suárez Lepe, 1997). El tamaño del ARN M difiere según el tipo *killer* (2-1,5 kb) mientras que el del ARN L es prácticamente constante (4,7 kb). Las toxinas más ampliamente distribuidas entre las cepas vínicas son la K1 y la K2 aunque es la K2 la más importante en vinificación ya que es estable al pH del mosto. Los ARN L y M están en el citoplasma en el interior de una cápsida proteica similar a partículas de tipo

viral. El ARN L codifica para las proteínas de la cápsida y para una ARN polimerasa (Barre et al., 1993; Shimizu, 1993; Hidalgo Togores, 2003).

3.4. Reproducción y ciclo de vida

S. cerevisiae, como otras levaduras esporógenas que pertenecen a la clase de los Ascomicetos, puede reproducirse asexualmente por vía vegetativa o de forma sexual formando ascosporas.

3.4.1. Reproducción asexual

La reproducción vegetativa o asexual en *S. cerevisiae* se produce por gemación. El proceso se realiza en cuatro fases: la primera, en G1, es el periodo precedente a la síntesis de ADN; la segunda, S, donde se empieza a formar un brote o gema y el huso polar del núcleo se extiende fuera de éste hacia la citada protuberancia; la tercera, G2, en la que el núcleo empieza a estirarse para meterse en la gema, en la que también entran mitocondrias y pequeñas vacuolas; y por último, la cuarta fase corresponde a la mitosis (M) propiamente dicha, que está marcada por el estiramiento máximo del núcleo y la separación de la célula madre y de la célula hija, luego por la construcción de la pared de separación y finalmente, por el depósito de un anillo de quitina sobre la cicatriz de brotación en la célula madre (Hidalgo Togores, 2003; Ribéreau-Gayon et al., 2003 a).

En el mosto de uva, la duración de la gemación es de una a dos horas, de modo que, en la fase de crecimiento activo de las levaduras durante la fermentación, el número de células se duplica en dos horas (Ribéreau-Gayon et al., 2003 a).

3.4.2. Reproducción sexual

Cuando las células diploides de las levaduras esporógenas se encuentran en un medio que les es hostil, como por ejemplo carente de azúcares asimilables o pobre en nitrógeno o muy aireado, cesan de multiplicarse por gemación, transformándose las células en ascas con una gruesa pared y conteniendo cuatro ascosporas o esporas haploides procedentes de la división meiótica del núcleo (Hidalgo Togores, 2003).

En *S. cerevisiae*, las ascosporas son de dos tipos de signo sexual: a y α. En el asca existen dos de cada tipo. En condiciones favorables de esporulación, como la presencia de azúcares, la pared del asca se rompe liberando las ascosporas que se multiplican vegetativamente en el medio. Dos células de signo opuesto pueden entonces aparearse,

uniéndose sus núcleos y citoplasmas en un zigoto, formando una nueva levadura diploide (a/α), que entonces puede multiplicarse por gemación.

En ciertas especies de levaduras, mientras que las células haploides se multiplican, algunas de ellas tienen la capacidad de invertir su signo sexual gracias a un gen llamado HO, pudiendo entonces unirse entre sí para formar una nueva levadura diploide (a/a o α/α). Estas son conocidas como especies homotálicas y corresponden a la mayoría de las levaduras vínicas, a diferencia de las especies heterotálicas, que no presentan esta propiedad y pueden pasar varias generaciones manteniéndose en estado haploide y con signo sexual constante.

Las levaduras del género *Saccharomyces* son diploides y homotálicas y tienen un ciclo de reproducción diplofásico en el que la reducción o meiosis se produce antes de la formación de esporas de forma que siempre son diploides cuando se reproducen vegetativamente.

El jugo de uva y el vino no parecen ser un campo propicio para la esporulación de las levaduras, por lo que mayoritariamente se reproducirán asexualmente.

3.5. Metabolismo

Las levaduras en condiciones anaeróbicas realizan la *fermentación alcohólica*, en la que los azúcares presentes en el mosto de uva (glucosa y fructosa, principalmente), son transformados en alcohol etílico y anhídrido carbónico, además de otras sustancias, transformándose, entonces, en vino. Esta compleja reacción bioquímica se produce con desprendimiento de calor, pues en el catabolismo de los azúcares se produce un exceso de energía que no se utilizan en los procesos de anabolismo de construcción de las células o de su multiplicación.

Durante la fermentación alcohólica también se producen una serie de fenómenos bioquímicos colaterales de gran importancia en el desarrollo de la misma y en la composición del vino, como la fermentación gliceropirúvica, fermentación homoláctica, fermentación maloalcohólica, catabolismo y anabolismo de los aminoácidos y metabolismo de los compuestos azufrados.

Es necesario disponer de pequeñas cantidades de oxígeno durante la fermentación alcohólica con el objeto de que las levaduras se multipliquen y su desarrollo sea el adecuado. En este momento cobra gran importancia el metabolismo aerobio de las

levaduras, donde los azúcares u otros sustratos son transformados finalmente en agua y anhídrido carbónico mediante la *respiración*, produciéndose una energía veinte veces superior a la de la fermentación alcohólica que explica el mayor crecimiento de la población celular en estas condiciones (Hidalgo Togores, 2003).

Las levaduras deben sintetizar durante su crecimiento todas las proteínas que necesita. Para ello asimila del medio fermentativo los compuestos nitrogenados que contiene en forma de ion amonio o de aminoácidos, a partir de los cuales podrá sintetizar otros aminoácidos que formarán parte de las proteínas. Sin embargo, *Saccharomyces* es incapaz de asimilar las fuentes de nitrógeno inorgánico del medio (como nitratos y nitritos) así como las proteínas y polipéptidos, ya que carece de los sistemas de digestión extracelular para este tipo de compuestos, por lo que el contenido en nitrógeno de los mostos suele ser limitante para el crecimiento de *S. cerevisiae* desde el punto de vista nutricional y el principal causante de paradas de fermentación. En cuanto al catabolismo de los aminoácidos es importante destacar la formación de metabolitos como los alcoholes superiores y los ésteres derivados de éstos, ambos con una participación decisiva en la fracción aromática de los vinos (Aranda et al., 2005; Hidalgo Togores, 2003).

Respecto a los compuestos azufrados, las levaduras son capaces de incorporar y metabolizar sulfitos, sulfatos, aminoácidos azufrados, glutatión, tiamina, biotina, etc., pero también producir y segregar al medio otras sustancias azufradas, como sulfitos, sulfuro de hidrógeno, dimetilsulfuro, mercaptanos, tioésteres, sulfuros, etc. La mayor parte de ellos influyen en las cualidades organolépticas de los vinos (Hidalgo Togores, 2003).

3.6. Ciclo de crecimiento y cinética de fermentación

El desarrollo de la fermentación alcohólica está íntimamente ligado al ciclo de crecimiento de las levaduras, de forma que depende el uno del otro. En el ciclo de crecimiento se pueden apreciar seis fases de desarrollo (Aranda et al., 2005; Hidalgo Togores, 2003):

1. **Fase de latencia:** La población de levaduras no aumenta, ya que no se producen multiplicaciones celulares al estar adaptándose al medio.

2. **Fase de aceleración:** Las levaduras empiezan a multiplicarse, alcanzándose una población de unas 10^5 células/ml. Esta fase junto con la anterior transcurre en varios días, dependiendo de la temperatura, y termina cuando el mosto se satura de anhídrido carbónico. Si el proceso fermentativo se inicia mediante inóculos de aproximadamente 10^6 células/ml, esta fase puede acortarse.

3. **Fase de crecimiento exponencial:** La población de levaduras crece exponencialmente hasta alcanzar 10^7-10^8 células/ml, coincidiendo el número de células totales con las vivas y sucediéndose un máximo de 4 a 5 poblaciones de células. Esta fase dura entre 2 y 5 días y en ella la velocidad de fermentación es máxima y constante y suele consumirse entre 1/3 y 1/2 de la cantidad de azúcares iniciales.

4. **Fase de ralentización del crecimiento:** Corresponde a la última parte de la fase anterior, donde debido a los factores limitantes del medio fermentativo, la población de levaduras deja de crecer. Prácticamente la totalidad de las levaduras están vivas y activas.

5. **Fase estacionaria:** El crecimiento es nulo. Las levaduras no se multiplican, permaneciendo la población estacionaria durante unos 8 días. Sin embargo, las células siguen metabólicamente activas y la velocidad de fermentación continúa en su valor máximo.

6. **Fase de declive o muerte:** Transcurre durante varias semanas. La población de levaduras totales disminuye ligeramente pero son las levaduras vivas las que sufren una importante reducción hasta 10^5 células/ml, debiendo éstas de terminar de transformar los últimos azúcares del mosto en condiciones cada vez más adversas. La pérdida de viabilidad va acompañada de una disminución de la velocidad de fermentación debida no únicamente a la disminución del número de células viables sino también a una inhibición de la actividad metabólica de las células no proliferativas. La pérdida de capacidad fermentativa se ha relacionado con la depleción del ATP y la acumulación del etanol, que producen efectos negativos sobre la capacidad de transporte a través de las membranas. Las células mueren y por autolisis empiezan a excretar al medio las sustancias que contienen.

El mosto de uva es un medio fácilmente fermentable que contiene todos los nutrientes necesarios para permitir el crecimiento de las levaduras; por tanto, cuando cesa su multiplicación se debe a la aparición de uno o más "factores limitantes" que frenan su crecimiento. Estos factores pueden ser de carácter fisicoquímico o bien pueden estar relacionados con algunas necesidades nutritivas de las levaduras (Fleet & Heard, 1993), con la población inicial, diversidad e interacciones entre las diferentes especies y cepas presentes en el mosto así como con la incorporación de levaduras mediante inóculos (Fleet, 2003).

3.6.1. Factores fisicoquímicos

La temperatura de fermentación: Es el factor de mayor incidencia en la cinética fermentativa, duplicándose la intensidad de fermentación cada 10 ºC, hasta el máximo de 30 a 35 ºC (óptimo fermentativo). Se estima que por cada grado de temperatura aumentado, la levadura es capaz de fermentar un 10% más de azúcares. Las temperaturas comprendidas entre 5 a 40 ºC son posibles para el inicio y desarrollo de la fermentación alcohólica; por encima de 40 ºC se produce la muerte celular debido a la desnaturalización de sus proteínas. Este máximo de temperatura está ligado a la presencia de otros factores limitantes como la concentración de etanol, cuya toxicidad aumenta con la temperatura, ácidos grasos de cadena corta, e incluso también el ácido acético (Fleet, 2003; Hidalgo Togores, 2003). Las levaduras no-*Saccharomyces* son más sensibles a las altas temperaturas que las especies de *Saccharomyces* (Torija et al., 2003; Torrens et al., 2007). Las temperaturas demasiado elevadas en la fase de multiplicación de las levaduras, del orden de 25 a 30 ºC, aceleran la velocidad de fermentación, pero pueden ser causa de una parada posterior, generando casi siempre problemas de finalización de la misma, y además, provocan la evaporación del etanol y la pérdida de compuestos volátiles esenciales para las características organolépticas del vino. También tiende a aumentar la concentración de ácido acético y de glicerol y a disminuir las de acetaldehído y ácido succínico (Torija et al., 2003). Por otra parte, temperaturas bajas pueden inhibir la fermentación alcohólica, sin llegar a producir la muerte de las levaduras, debido a un funcionamiento deficiente de la ATPasa como "bomba de protones", produciéndose entonces una acidificación del citoplasma celular (Hidalgo Togores, 2003). *S. cerevisiae* muestra una temperatura óptima de crecimiento alrededor de los 30 ºC, aunque puede adaptarse a un amplio abanico de temperaturas, con un máximo de 40 ºC, a partir del cual se observa una caída de viabilidad (Watson,

1987). En general, la fermentación de los vinos blancos se tiende a hacer entre los 15 y 20 ºC, mientras que la temperatura para la elaboración de los tintos se sitúa en valores más elevados, entre los 20 y 30 ºC (Ough, 1992), para favorecer las extracción de las sustancias fenólicas típicas de los mismos.

El régimen de aireación: La presencia de oxígeno es necesaria para la multiplicación de las levaduras y para ciertas funciones de su metabolismo como la síntesis de esteroles y ácidos grasos insaturados que aumentan la permeabilidad de las membranas celulares y cuya ausencia es frecuentemente causa de paradas de fermentación (Hidalgo Togores, 2003). Sin embargo un exceso de oxígeno puede producir un aumento de acetaldehído y SH_2 y un descenso en la producción de ésteres aromáticos (Nykänen, 1986) y también inhibir la fermentación, lo que se conoce como *efecto Pasteur* (Aranda et al., 2005). Las levaduras no-*Saccharomyces* parecen ser menos tolerantes a la ausencia o baja disponibilidad de oxígeno que las *Saccharomyces* (Visser et al., 1990; Fleet, 2003).

El efecto del pH: El rango típico de pH en el mosto no representa una limitación para el crecimiento de *Saccharomyces*; sólo valores inferiores a pH 2,8 pueden plantear dificultades (Aranda et al., 2005). Los efectos nocivos del pH bajo se deben a que incrementan el efecto nocivo del etanol y del sulfito afectando al correcto funcionamiento de la ATPasa de protones de la membrana plasmática encargada de mantener el pH neutro en el interior celular (Hidalgo Togores, 2003).

La clarificación: La eliminación de partículas sólidas del mosto conlleva la eliminación conjunta de nutrientes asociados a ellas, sobre todo nitrogenados, que pueden dificultar el crecimiento de las levaduras (Ayestaran et al., 1995). Este factor es importante sobre todo en la producción del vino blanco en el que se realiza la clarificación previa a la fermentación.

El dióxido de azufre (SO_2): La adición del SO_2 en el mosto controla las reacciones de oxidación y restringe el crecimiento de la microbiota autóctona. Como antimicrobiano, el SO_2 presenta los siguientes efectos sobre la cinética de crecimiento de las levaduras durante la fermentación (Fleet & Heard, 1993): incrementa la fase de latencia y retrasa el inicio de la fermentación; disminuye la tasa de crecimiento e incrementa el tiempo para completar la fermentación aunque a dosis pequeñas es activador del crecimiento; acelera la fase de declive o muerte celular y ejerce una

selección sobre las especies o cepas que crecen en el mosto y desarrollan la fermentación. En general, restringe el crecimiento de las especies autóctonas de no-*Saccharomyces* y fomenta el desarrollo de *S. cerevisiae.*

3.6.2. Necesidades nutricionales o composición del mosto

La composición del mosto afecta a la tasa de fermentación y a que ésta se realice por completo así como a la concentración de compuestos aromáticos constituyentes del vino. Es importante tener en cuenta que tal composición varía con la variedad de uva, las condiciones del suelo, el uso de fertilizantes y la madurez de la uva al vendimiar (Fleet & Heard, 1993). Generalmente, el mosto contiene una cantidad suficiente de nutrientes para desarrollar la fermentación alcohólica; sin embargo, en mostos excesivamente desfangados en algunas ocasiones, se originan algunas carencias que pueden generar problemas en la finalización de la fermentación (Hidalgo Togores, 2003), destacando entre estas sustancias las nitrogenadas y las minerales y algunos factores de crecimiento y de supervivencia esenciales para el crecimiento de las levaduras (Ribéreau-Gayon et al., 2003 a).

Compuestos carbonatados: Los azúcares fermentables por las levaduras son la principal fuente de carbono, especialmente la glucosa y la fructosa. La concentración de azúcares que posee el mosto determina su velocidad de fermentación: es constante entre 20-200 g/l y decrece fuera de este rango (Hidalgo Togores, 2003; Fleet & Heard, 1993).

Compuestos nitrogenados: El mosto es bastante rico en compuestos nitrogenados de los que un 5-10% está constituido por la fracción mineral o amoniacal, un 20-30% por aminoácidos, un 30-40% por polipéptidos y un 5-10% por proteínas. Sin embargo, las levaduras, como se ha mencionado anteriormente, sólo utilizan las fuentes más fácilmente asimilables, como el ión amonio, los aminoácidos y algunos péptidos de bajo peso molecular. Generalmente el mosto contiene una cantidad suficiente de estas sustancias asimilables para el crecimiento de las levaduras y el desarrollo correcto de la fermentación, pero suelen ser factor limitante en mostos desfangados muy limpios o en determinadas vendimias empobrecidas en estas sustancias (Hidalgo Togores, 2003). Además, si existe carencia de nitrógeno durante la fase de crecimiento exponencial de las levaduras, éstas producen una gran cantidad de SH_2 (Jiranek et al., 1995), responsable del característico olor a huevos podridos (Pisarnitskii, 2001).

Otros compuesto que merecen especial atención son las **vitaminas**. Las levaduras vínicas no son tan dependientes de estos cofactores como otros organismos más complejos ya que son capaces de sintetizar sus propias vitaminas, excepto la biotina. No obstante, la presencia de estos compuestos en le mosto estimula el crecimiento y la actividad metabólica de las levaduras por lo que se les considera *factores de crecimiento* (Ribéreau-Gayon et al., 2003 a).

Compuestos minerales: Estos compuestos son importantes para asegurar las funciones vitales de las levaduras. La levadura seca contiene de 5 a 10% de compuestos minerales y son principalmente óxidos de fósforo, potasio, magnesio, calcio, azufre, sodio, hierro y sílice y el ión cloro (Ribéreau-Gayon, 2003).

En relación a los **lípidos**, las levaduras no pueden sintetizar ácidos grasos de cadena larga ni esteroles en las condiciones de anaerobiosis propia de la fermentación vínica. La carencia de este tipo de lípidos afecta a la estructura y función de la membrana plasmática, aumentando los efectos del etanol y dificultando la captación de glucosa. A estos compuestos se les denomina *factores de supervivencia* ya que su presencia es necesaria para la viabilidad celular aunque su adición no produce un aumento del crecimiento (Ribéreau-Gayon et al., 2003 a).

3.6.3. Interacciones entre levaduras. Efecto de la inoculación de levaduras seleccionadas y del factor *killer*

Aunque *S. cerevisiae* es la especie principal en la producción del vino, otras especies también juegan un papel importante y entre ellas se producen una gran diversidad de interacciones a lo largo de las diferentes etapas del proceso. Estas interacciones influyen tanto en la eficiencia de la producción como en la calidad del vino (Fleet, 2003). Las interacciones que ocurren entre las levaduras a causa de la inoculación de levaduras en la fermentación y el factor *killer* provocan la inhibición y muerte, respectivamente, de unas especies o cepas y determinan el predominio de otras que van a ser las responsables del desarrollo de la fermentación.

La inoculación de levaduras seleccionadas afectan al crecimiento de levaduras ya existentes en el mosto y, por tanto, al desarrollo de la fermentación, ya que se reduce la fase de latencia y el riesgo de contaminación del mosto por otros microorganismos indeseables y la fermentación se completa con mayor rapidez (Fleet & Heard, 1993). La ecología de las levaduras que participan en la fermentación se ve afectada por el inóculo

de cepas *S. cerevisiae* que suprime el crecimiento de las especies de no-*Saccharomyces* (Egli et al., 1998; Henick-Kling et al., 1998; Beltrán et al., 2002) y disminuye la diversidad de cepas *S. cerevisiae* autóctonas (Schütz & Gafner, 1993; Egli et al., 1998; Henick-Kling et al., 1998; Torija et al., 2001; Beltrán et al., 2002; Santamaría et al., 2005; Lopes et al., 2007), siendo este hecho más acentuado en bodegas de nueva construcción (Constantí et al., 1997; Beltrán et al., 2002; Santamaría et al., 2005).

El uso de inóculos con capacidad *killer* puede prevenir o reducir de forma importante la posibilidad de contaminaciones (Suárez Lepe, 1997). Aunque hay muchas variables en la vinificación que influyen en la expresión de los fenotipos *killer* o sensible, es evidente que las interacciones *killer* determinan la evolución de las especies y cepas de levaduras durante la fermentación (Fleet, 2003). Las cepas *S. cerevisiae* con fenotipo *killer* provocan la muerte de las cepas de fenotipo sensible disminuyendo la biodiversidad de las levaduras a lo largo de la fermentación; de esta forma garantizan su permanencia o incluso su dominio en la fermentación hasta el final (Vagnoli et al., 1993; Suárez Lepe, 1997, Sangorrín et al., 2001; Zargoc et al., 2001). Estos fenómenos de antagonismo entre levaduras *killer* y sensibles pueden provocar paradas o retrasos en la fermentación alcohólica, por lo que se hace necesario controlar estos procesos.

4. Fermentación alcohólica

La producción del vino implica la transformación del mosto por la actividad metabólica de las levaduras, principalmente *S. cerevisiae*, mediante el desarrollo de la *fermentación alcohólica*. Se trata de una ruta catabólica que tiene lugar en el citoplasma de las células y consiste en la transformación de las hexosas presentes en el mosto (glucosa y fructosa, principalmente) en etanol y CO_2 y puede expresarse mediante la siguiente ecuación global:

$$C_6H_{12}O_6 + 2\ ADP + 2\ HPO_4^- \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP + 2\ H_2O$$

4.1. Fermentación espontánea. Sucesión de especies de levaduras

La fermentación alcohólica natural o espontánea es un proceso microbiológico complejo en el que intervienen una gran biodiversidad de especies de levaduras que proceden del grano de uva o bien de la superficie de los equipos de bodega (Heard & Fleet, 1993) cuya composición ya se ha comentado en el apartado 2. En una

fermentación natural o espontánea (sin inóculo) dichas especies no actúan de forma simultánea, sino que crecen de forma secuencial a lo largo de la fermentación alcohólica (Fleet & Heard, 1993; Schütz & Gafner, 1993; Querol et al., 1994; Schütz & Gafner, 1994; Sabate et al., 1998; Torija et al., 2001; Santamaría et al., 2005; Maro et al., 2007; Mercado et al., 2007). La fermentación alcohólica es iniciada principalmente por especies de *Kloeckera, Hanseniaspora y Candida*, y en menor medida de *Hansenula* y *Pichia*, pero a medida que ésta avanza estos microorganismos van desapareciendo dando paso a otras especies de *Saccharomyces*, fuertemente fermentativas y tolerantes al etanol, capaces de finalizar la fermentación. La desaparición de las especies no-*Saccharomyces* parece estar relacionada con su baja tolerancia al etanol y a su incapacidad para sobrevivir en un medio con concentraciones crecientes de etanol producido por las especies de *Saccharomyces*. Sin embargo, Pérez-Nevado et al. (2006) apuntan que algunas no-*Saccharomyces* como *H. guilliermondii* y *H. uvarum* toleran grandes cantidades de etanol en cultivos puros pero mueren rápidamente cuando crecen en cultivos mixtos con *S. cerevisiae* por lo que postulan que la causa de muerte (y por tanto de la sucesión durante la fermentación alcohólica) podría ser distinta a la toxicidad por el etanol. Según Ribéreau-Gayon et al. (2003 a), además del etanol, la selección natural de *S. cerevisiae* se produce por otros tres factores: la anaerobiosis, el sulfitado de los mostos y la riqueza en azúcar, a los cuales las especies no-*Saccharomyces* son más sensibles que *S. cerevisiae*.

El empleo de técnicas de caracterización molecular ha puesto de manifiesto que durante la fermentación participan diversas cepas de *S. cerevisiae* encontrándose dos situaciones, fermentaciones en las que sólo un pequeño número de ellas son mayoritarias (1 a 3) representando el 70-80% de las colonias aisladas (Ribéreau-Gayón et al., 2003), o bien fermentaciones en las que ninguna cepa domina sobre las demás (Torija et al., 2001; Sabate et al., 1998). La aplicación de estas técnicas ha permitido además estudiar la distribución geográfica de cepas de *S. cerevisiae* salvajes o indígenas, demostrando la adaptación de algunas de ellas a microambientes específicos (Nadal et al., 1996; Schütz & Gafner, 1994).

Uno de los principales inconvenientes de las fermentaciones espontáneas, sin embargo, es la variabilidad en la población total de levaduras y en su composición de una campaña a otra, que por una parte puede no ser suficiente para asegurar el inicio de la fermentación alcohólica o que ésta sea muy lenta favoreciendo la proliferación de

levaduras contaminantes y, por otra, hace que las características del producto final de la fermentación varíe cada vendimia y que, por tanto, la fermentación espontánea resulte altamente impredecible (Pretorius, 2000).

4.2. Fermentaciones inoculadas

Frente al desarrollo de las fermentaciones espontáneas, a cargo de la microflora presente en los racimos de uva y la residencial en la bodega, las fermentaciones pueden ser dirigidas mediante la inoculación de levaduras seleccionadas, bien en forma de levaduras secas activas o bien mediante su adición a partir de una cepa conservada en laboratorio y debidamente propagada en escalonamientos sucesivos.

Sin embargo, antes de que se iniciaran los procesos de selección de cepas vínicas, para paliar los problemas expuestos anteriormente en relación a las fermentaciones espontáneas, se recurría al empleo de un *pie de cuba*, que no era más que un mosto en plena fermentación, conducida en las mejores condiciones para procurar el predominio de las mejores levaduras para el proceso enológico (Suárez Lepe, 1997). Se trata de una técnica antigua pero que hoy día se sigue utilizando en muchas bodegas para aquellas elaboraciones donde se desea que la población de levaduras proceda del propio ecosistema, sobre todo en vinos que presentan una fuerte expresión de su origen o "terroir".

El pie de cuba se prepara unos días antes de la vendimia a partir de racimos de uva sanos y maduros de los que se extrae el mosto que se deja fermentar de forma espontánea. Cuando está en fermentación tumultuosa y alcanzado una graduación alcohólica de entre 6-8 % vol, se añade a los depósitos de fermentación en proporción variable de 3-5%, que asegura la siembra de una población de 2 ó 3 x 10^6 levaduras/ml y la presencia de levaduras más favorables para el proceso de fermentación. Se ha de mezclar bien con el mosto a fermentar por remontado o mejor situándolo en el fondo del depósito antes de ser llenado, acto que le da nombre a la técnica *pie de cuba* (Suárez Lepe, 1997; Hidalgo Togores, 2003).

Por otra parte, el concepto de empleo de levaduras seleccionadas para conducir la fermentación tampoco es reciente. A principios del siglo pasado el instituto La Claire en Francia comenzó a aislar, mantener y producir a pequeña escala cepas puras de levadura en forma de cultivos líquidos que se utilizaban como pies de cubas en mostos, en un proceso que se iba escalonando hasta permitir el inóculo de los tanques industriales de

fermentación (Degre, 1993; Ramón., 2005). A nuestro país dicho concepto llegó de la mano de Tommaso Castelli en 1955, discípulo de Gino de Rossi. El objetivo era la obtención de una colección de microorganismos de distintas regiones vitivinícolas, entendiendo que las más abundantes en las distintas fases de fermentación (inicio, tumultuosa y final) serían potencialmente interesantes como futuras levaduras seleccionadas. Según Castelli, aunque la fermentación en pureza no era implacable, podría permitir una mayor regularidad, mayor producción de alcohol, mejor relación rendimiento alcohólico y menor acidez volátil. Tomasso Castelli y Baldomero Iñigo fueron los responsables de los primeros análisis microbiológicos de fermentaciones realizados en España a final de la década de los 50 del siglo XX (González et al., 2005).

No obstante, no fue hasta la década de los 60 cuando comienza la producción industrial de cepas enológicas de levaduras en forma de levaduras secas activas (LSA) en respuesta a las necesidades de una creciente producción de vinos en California, y aunque en principio el número de cepas disponibles era muy reducido (Montrachet y Pasteur champagne), en la actualidad rondan el centenar las cepas comerciales ofrecidas por diferentes empresas (Degre, 1993; González et al, 2005). El empleo de LSA, además de aumentar el grado de reproducibilidad del vino año tras año, asegura una rápida y completa fermentación del mosto y disminuye la fase de latencia de las levaduras (Fleet & Heard, 1993) reduciendo así el riesgo de contaminación y permitiendo un mayor control de la fermentación y calidad en los vinos (Henick-Kling et al., 1998). Otra de las ventajas de la LSA es su fácil manipulación en bodega, que no requiere personal especializado. Para su inoculación tan sólo es precisa una fase previa de rehidratación durante 15-20 minutos en agua caliente (30-40 ºC) a la que se le debe añadir mosto o sacarosa con objeto de evitar el choque osmótico.

Sin embargo, hay que tener en cuenta que el empleo de levaduras comerciales no asegura su total implantación durante la fermentación, que puede ser incluso baja o nula (Barrajón et al., 2009). El grado de implantación de las LSA depende principalmente de la dosis usada, de las condiciones de rehidratación y de la temperatura de inoculación, aunque también otros factores pueden afectar directamente a su viabilidad, vitalidad y actividad fisiológica y, por tanto, a su capacidad de competir con la microflora salvaje presente en el mosto, por ejemplo, el contenido intracelular en carbohidratos, contenidos de nitrógeno en el mosto, la presencia de factores de supervivencia y la temperatura de fermentación (revisado por Barrajón et al., 2009). En este sentido, las técnicas de

biología molecular son una herramienta importante que permite la monitorización de las fermentaciones y el control del grado de implantación de la levadura inoculada y, de este modo, tomar a tiempo las medidas pertinentes en caso de no observarse una adecuada implantación de la cepa inoculada.

A pesar de las ventajas del uso de levaduras comerciales, muchos autores defienden el empleo de levaduras autóctonas seleccionadas (Martini & Vaughan-Martini, 1990; Suárez Lepe & Iñigo Leal, 2004; Querol et al., 1992a; Regodón et al., 1997; Romano et al., 1998). Por un lado, estas últimas están mejor adaptadas a su microambiente y pueden imponerse más fácilmente garantizando la homogeneidad de la flora (Martini & Vaughan-Martini, 1990, Lema et al., 1996; Regodón et al., 1997; Pretorius, 2000; Esteve-Zarzoso et al., 2001; Romano et al., 2003; Lopes et al., 2007; Barrajón et al., 2009; Rodríguez et al., 2009). Por otro lado, esta práctica facilita el mantenimiento de la tipicidad del producto según la región de origen (Regodón et al., 1997; Nikolaou et al., 2006; Rementaria et al., 2006; Rodríguez et al., 2009). Por ello, el mantenimiento del patrimonio biológico es esencial para obtener inóculos capaces de desarrollar por completo los sabores y aromas típicos de los vinos originados en los diferentes viñedos (Pretorius, 2000).

5. Selección de levaduras autóctonas

Los beneficios de controlar el proceso fermentativo mediante la inoculación de levaduras frente al desarrollo de la fermentación de forma espontánea o natural han sido ya expuestos en el apartado anterior, así como las ventajas que supone el empleo de levaduras autóctonas seleccionadas frente al de levaduras comerciales en relación a la más fácil implantación de las primeras en el proceso fermentativo y en el mantenimiento de las características particulares de los vinos producidos en una región determinada.

El proceso de selección clonal de levaduras lleva consigo determinar las propiedades enológicas de cada cepa que la haga más adecuada para el proceso fermentativo, bien por su comportamiento tecnológico durante la fermentación, bien por las características que aporte al producto final. Si además la selección se lleva a cabo entre las levaduras autóctonas (o locales) de la propia región donde se vayan a emplear

en la vinificación se llegaría al concepto de *levadura local seleccionada* ya apuntado en 1964 por Iñigo Leal (Suárez Lepe & Iñigo Leal, 2004).

El primer paso, por tanto, en el proceso de selección de una levadura local o autóctona es el aislamiento e identificación de la microflora levaduriforme que participa en la fermentación espontánea de los mostos en una región determinada (Briones et al., 1995). Lo más habitual es realizar los aislamientos en el mosto en fermentación, normalmente en la última fase de la misma, aunque también pueden usarse otros sustratos como la propia uva, mosto fresco e incluso lías (González et al., 2005), para después proceder a la identificación y caracterización de las especies y cepas presentes.

A continuación las cepas identificadas son ensayadas de acuerdo a unos criterios de selección. Según Suárez Lepe & Iñigo Leal (2004) debe diferenciarse entre criterios primarios de selección, básicos o fundamentales, útiles para la selección de levaduras destinadas a fermentar cualquier mosto, y otros que una vez superados los primeros, se deben estudiar con carácter específico según el tipo de vino a elaborar. Consideran básicos un poder fermentativo elevado, baja producción de acidez volátil y una correcta cinética fermentativa. Otros criterios a tener en cuenta son la resistencia al SO_2, fenotipo *killer* y la ausencia de defectos olfativos (producción de SH_2). Posteriormente, la selección debe proseguir en función de su aplicación tecnológica, considerando la producción de vinos comunes, vinos tintos o blancos de calidad, o bien de vinos especiales como los de crianza biológica o los espumosos.

Degré (1993) resume algunas de las propiedades tecnológicas de las levaduras, clasificándolas en función de sus efectos en el proceso de vinificación y/o calidad del producto final como:

- **Favorables**: poder fermentativo, fermentación completa de azúcares, cinética fermentativa adecuada, alta tolerancia al etanol, capacidad de fermentar a diferentes temperaturas, resistencia al SO_2, factor *killer* o resistencia a toxinas *killer*, degradación del ácido málico, producción de β-glucosidasa y producción de glicerol.

- **Desfavorables**: producción acidez volátil, de SH_2, de acetaldehído, formación de precursores del carbamato de etilo, y formación de espuma.

5.1. Propiedades favorables

El *poder fermentativo* o poder alcoholígeno corresponde al máximo porcentaje de etanol que es capaz de producir una levadura al fermentar un mosto estéril con cantidad suficiente de azúcares; indica, por tanto, su capacidad para producir vinos de elevado grado alcohólico (Suárez Lepe & Iñigo Leal, 2004). Esta propiedad está asociada a alta velocidad de fermentación, fases de latencia cortas y *consumo casi total de los azúcares* presentes en el mosto (González et al., 2005), y constituye uno de los primeros criterios empleados en la selección de levaduras. Es muy variable entre las distintas especies de levaduras vínicas y, a menudo, entre diferentes cepas de una misma especie. Normalmente las cepas con mayor poder fermentativo son también las más *resistentes al etanol*. *S. cerevisiae* y otras levaduras vínicas pertenecientes a este género, son los microorganismos más tolerantes al etanol de entre los eucariotas, son capaces de crecer a concentraciones comprendidas entre 8-12% vol etanol y de producir una graduación alcohólica superior a 12% vol (Suárez Lepe, 1997).

Otra propiedad de interés en relación a la selección de una cepa de levadura es su *cinética fermentativa*, que se establece mediante la determinación de otros parámetros como la duración de la fase de latencia (relacionada con el tiempo que tarda en producirse el arranque de la fermentación), la regularidad fermentativa de la cepa, la duración total del proceso y la curva termodinámica de cada cepa, que deben definirse en las mismas condiciones experimentales (Suárez Lepe & Iñigo Leal, 2004).

En relación a la *temperatura de fermentación*, cada cepa tiene su óptimo de temperatura (Torija et al., 2003). Puede ser de gran utilidad contar con cepas seleccionadas capaces de fermentar a temperaturas elevadas en zonas vinícolas con altas temperaturas durante la vendimia y el período de fermentación y con bodegas que carecen de sistemas de refrigeración, situaciones que pudieran conducir a paradas de fermentación (Regodón et al., 1997, Nikolaou et al., 2006). Sin embargo, en otras ocasiones interesa fermentar a temperaturas bajas para favorecer la conservación de los aromas varietales y fermentativos en el vino, aunque disminuye la tasa de crecimiento de las levaduras y por tanto de la fermentación.

La *resistencia al sulfuroso* sigue siendo un carácter a tener en cuenta en la selección de cepas vínicas, especialmente cuando no se limita a especies de *S. cerevisiae*. El empleo de sulfito es una práctica habitual durante la vinificación,

justificada por su actividad antiséptica, entre otras, que ayuda a controlar el crecimiento de microorganismos indeseables en el mosto y en el vino. La resistencia a este compuesto es característica común de las cepas vínicas de *S. cerevisiae*, que poseen una copia del alelo SSU1-R del gen SSU1 que les confiere mayor resistencia a este compuesto que las cepas que poseen el alelo normal (González et al., 2005).

También parece interesante contar con cepas *killer* de *S. cerevisiae* como inóculos, capaces de liberar toxinas y de este modo matar cepas que no posean factor *killer* o cuyo factor *killer* es diferente (González et al., 2005) favoreciéndose la implantación de la cepa inoculada y evitando el desarrollo de levaduras sensibles que puedan contaminar y dañar el vino (Kunkee, 1984).

El ácido málico no sólo contribuye a la acidez en el vino provocando un desequilibrio organoléptico (Suárez Lepe, 1997), sino que también sirve de sustrato para contaminaciones por bacterias lácticas que pueden dañar el vino tras su embotellamiento. Por ello es esencial eliminar el exceso de ácido málico del vino para asegurar la estabilidad física, bioquímica y microbiológica y por tanto, la calidad del vino (Pretorious, 2000). La *capacidad de degradar ácido málico* del vino difiere con la especie y la cepa de levadura (Redzepovic et al., 2003).

El aroma varietal, o primario del vino puede verse afectado por la cepa de levadura que se emplee en la vinificación. La *producción de enzimas β-glucosidasa* por algunas cepas vínicas permite la hidrólisis de los precursores aromáticos procedentes de la uva, que suelen ser terpenos unidos por enlace β-glucosídico a un resto azucarado, liberando así el verdadero compuesto aromático, que es el terpeno libre (Fernández-González et al., 2003; Hernández et al., 2003). Por otra parte, la acción de otras enzimas hidrolíticas ayudan también a la extracción de componentes del aroma y color a partir de las paredes celulares de la uva, por lo que la producción de estas enzimas con estas actividades también se han propuesto como criterio de selección (Gonzaléz et al., 2005).

La *producción de glicerol* también se asocia con una mejora de las propiedades sensoriales del vino al proporcionarle dulzor, cuerpo y suavidad (Nikolaou et al., 2006). En algunos vinos puede ser, después del alcohol y el agua, el constituyente presente en mayor cantidad (Ribéreau-Gayon et al., 2003 b; Suárez Lepe & Iñigo Leal, 2004). Su formación transcurre en los primeros estadios de la fermentación alcohólica, como consecuencia de la reducción de la fosfohidroxiacetona, cuando todavía hay poco

acetaldehído formado. Teniendo en cuenta que cada vez existe una mayor demanda de vinos con baja graduación alcohólica, y que un aumento en la producción de glicerol implica un descenso en la de etanol, la selección de cepas productoras de glicerol tiene interés desde ambos puntos de vista.

5.2. Propiedades desfavorables

Desde un punto de vista negativo, un exceso de ácido acético y por tanto, una *producción de acidez volátil* elevada, es uno de los defectos más fácilmente detectables en el vino. A menudo un exceso de acético se asocia al desarrollo incontrolado de bacterias –normalmente acéticas aunque también lácticas- pero también puede haber una contribución apreciable de este compuesto como consecuencia del propio metabolismo de *S. cerevisiae* que dependerá de cada cepa (Suárez Lepe, 1997).

También un exceso en *sulfuro de hidrógeno* disminuye la calidad de los vinos acabados ya que produce un desagradable olor a huevos podridos (Pisarnitskii, 2001). Aunque todas las cepas de *Saccharomyces* pueden producir una pequeña cantidad de este compuesto (Gutiérrez et al., 1997), su producción depende de la cepa de levadura y de sus características fisiológicas (Rementaria et al., 2006). Existen cepas cuyas características genéticas les permite bloquear la síntesis de SH_2 cuando existe una traza de éste en el medio; estas cepas poseen lo que se conoce como "carácter SH_2 negativo" que consiste en un sistema de control específico para la reducción de sulfatos (Suárez Lepe & Iñigo Leal, 2004).

El *acetaldehído* es un compuesto muy reactivo que se une fácilmente al SO_2, impidiendo que ejerza sus funciones, y a otros constituyentes del vino, como los compuestos fenólicos provocando insolubilizaciones (Mareca Cortés, 1983). En exceso provoca la denominada maderización o gusto oxidado (Mesas & Alegre, 1999). Además, su oxidación produce ácido acético, aumentando así la acidez volátil de los vinos (Mareca Cortés, 1983).

El *carbamato de etilo* está relacionado con la calidad higiénica. Parece ser un agente cancerígeno cuyo principal precursor es probablemente la urea por reacción química entre ésta con el etanol. Durante la fermentación alcohólica, la urea es liberada bajo la acción de la arginasa en el transcurso de la metabolización de la arginina (Flanzy, 2003).

La *formación de espuma* durante la fermentación hace necesario dejar una cámara de aire en los depósitos de fermentación para evitar que rebosen y dificulta el transporte del mosto por las bombas (Kunkee, 1984), por ello es importante seleccionar las cepas que menos espuma produzcan.

5.3. Ensayos de selección

La determinación del comportamiento de las cepas de levaduras a ensayar en relación con los criterios seleccionados implica el empleo de medios sintéticos o bien de microfermentaciones en mosto natural en condiciones de laboratorio, así como la determinación de compuestos volátiles y análisis sensorial en los productos finales obtenidos en vinificaciones de laboratorio (Briones et al., 1995). Finalmente, el comportamiento de las cepas seleccionadas debe ensayarse también en vinificaciones en bodega (Regodón et al., 2000; Rementaria et al., 2006; Rodríguez et al., 2009).

En nuestro país se han llevado a cabo diversos trabajos de selección enfocados a la selección de alguna cepa de *S. cerevisiae* para su aplicación en una región vinícola dada: en la DO de Alicante (Querol et al., 1992a), en la DO de Valdepeñas (Briones et al., 1999), en Extremadura (Regodón et al., 1997), en La Mancha (Pérez-Coello et al., 1999), en El Penedés (Esteve-Zarzoso et al., 2000), en la DO Terra Alta (Torija, 2002) y, más recientemente, en la provincia de Cádiz (Rodríguez et al., 2009), entre otros. Trabajos similares han sido realizados recientemente en Grecia (Nikolaou et al., 2006) y en la Patagonia argentina (Lopes et al., 2007). En términos generales, los criterios de selección más frecuentemente empleados fueron la cinética fermentativa, tasa de consumo de azúcares, producción de acidez volátil, de SH_2 y de espuma, fenotipos *killer* y resistencia a diferentes concentraciones de SO_2, estudiados en la mayoría de ellos. Otras propiedades estudiadas, aunque en menor medida, fueron la tolerancia al etanol (Briones et al., 1995; Pérez-Coello et al., 1999), a diferentes temperaturas (Querol, 1992, Regodón et al., 1997, Nikolaou et al., 2006), la adaptación a concentraciones de azúcares elevadas (Querol, 1992), producción de fenoles volátiles (Briones et al., 1995; Pérez-Coello et al., 1999), de glicerol y aminas biógenas (Nikolaou et al., 2006). Por tanto, no existe un protocolo estándar para la selección de levaduras, hallándose diferencias en la totalidad de propiedades enológicas estudiadas así como en la prioridad que se le da a cada una de ellas en el proceso de selección. Sin embargo, todos los trabajos de selección coincidieron en la realización de microvinificaciones, con un

número reducido de cepas preseleccionadas, en las que se evalúan las propiedades sensoriales y, en algunos casos, se determinan el contenido en compuestos volátiles de los vinos obtenidos. Finalmente, en algunos de ellos se hacen vinificaciones en bodega para comprobar la implantación de la cepa seleccionada, su adaptación a las condiciones locales y las propiedades de los vinos elaborados (Briones et al., 1995, Esteve-Zarzoso et al., 2000, Rementeria et al., 2006).

6. Papel de las levaduras en el aroma del vino

El aroma de los vinos es de una gran complejidad que se debe en parte a su origen, pues es el resultado final de una larga secuencia biológica, bioquímica y tecnológica, y en parte al número elevado de constituyentes volátiles que contiene, cuyas concentraciones van desde algunos nanogramos por litro a algunas centenas de microgramos, o algunos miligramos. En el potencial aromático de los vinos hay que tener en cuenta otros compuestos ligados llamados también precursores del aroma (Bayonove et al., 2000).

Los constituyentes aromáticos del vino se clasifican según su origen (Bayonove et al., 2000; Romano et al., 2003), en:

- *Aromas varietales* que provienen de la uva y que dependen esencialmente de la variedad pero también de otros factores. En el caso de variedades poco aromáticas, estos constituyentes son esencialmente precursores: ácidos grasos, glucósidos, carotenoides y compuestos fenólicos.
- *Aroma prefermentativo* que se forma en las operaciones de extracción, maceración y acondicionamiento del mosto como consecuencia de la acción de enzimas sobre ciertos lípidos; son esencialmente compuestos de 6 átomos de carbono.
- *Aromas fermentativos*, formados por las levaduras durante la fermentación alcohólica y por las bacterias lácticas en aquellos vinos en los que tenga lugar la fermentación maloláctica. Cuantitativamente son los más abunadantes y son considerados como los responsables de la nota vinosa común a todos los vinos.
- Aromas postfermentativos, incluyen todos los compuestos volátiles que se forman durante el envejecimiento y la conservación del vino.

El aroma del vino es, por tanto, el resultado de los compuestos volátiles que lo forman de manera que el aroma que se percibe al oler, raramente puede corresponder a un compuesto específico. Sin embargo, no todos los compuestos contribuyen igual al aroma del vino. Los compuestos aromáticos del vino pueden ser clasificados atendiendo al papel aromático más relevante que pueden jugar en el vino. Cada compuesto se asignará a la clase correspondiente al papel aromático más importante que dicho componente puede jugar en toda la gama de vinos. De acuerdo con este criterio podemos establecer las siguientes categorías (Ferreira, 2007):

1. Compuestos impacto o altamente activos: Son los componentes que pueden transmitir de manera efectiva sus características específicas (caso de los impacto) o sus características primarias (altamente activos) sin la necesidad del soporte de más componentes químicos.

2. Familias de compuestos impacto: Se trata de familias de compuestos con similitud tanto en sus estructuras químicas como en sus características sensoriales. Varios de estos grupos están formados por series de compuestos homólogos, como es el caso de la lactonas.

3. Compuestos o familias de papel sutil o secundario: Se trata de aquellos componentes o grupos de componentes que no son capaces de transmitir sus notas aromáticas específicas al vino pero que contribuyen de manera neta a alguna nota aromática secundaria o genérica (como por ejemplo frutal, dulce). La nota aromática será el resultado de la interacción de estos componentes con otros compartiendo alguna similitud aromática. Los compuestos clasificados en las categorías 1 y 2, cuando no alcanzan suficiente concentración, o si incluso alcanzándola, concurren con muchos otros aromas potentes (tal y como ocurre en vinos complejos), pueden pertenecer a esta categoría.

4. Compuestos formando la base del aroma. Estos son los componentes genéricos de fermentación presentes en todos los vinos en concentraciones superiores al valor umbral e integrando el buffer aromático. El aroma de todos estos componentes está totalmente integrado, de manera que no es posible identificar las notas de los componentes individuales. Dentro de este grupo hay algunos compuestos que pueden actuar como potenciadores del aroma y otros que lo hacen como depreciadores.

5. “Off-flavours”. Son los componentes cuya presencia lleva asociada una disminución en la calidad general del vino.

Como se ha comentado, los compuestos responsables del aroma fermentativo tienen un gran peso en el aroma del vino y constituyen la base aromática común a todos los vinos. Se han identificado más de 1000 compuestos volátiles de los que más de 400 son producidos por las levaduras durante la fermentación (Nykänen, 1986).

Por tanto, la especie de levadura que participa en la fermentación es muy importante ya que es responsable de la naturaleza y la concentración de los productos finales del vino (Henick-Kling et al., 1998; Ribéreau-Gayon et al., 2003 a; Romano et al., 2003; Clemente-Jiménez et al., 2004), de forma que cada especie de levadura, e incluso cada cepa, proporciona peculiaridades distintas en el producto final (Lurton et al., 1995; Egli et al., 1998; Pérez-Coello et al, 1999; Romano et al., 2003; Estévez et al. 2004; Torrens et al., 2008). Existe una gran diversidad de cepas de *S. cerevisiae* que participan en una misma fermentación. Todas ellas contribuyen a la composición química y a las cualidades sensoriales del vino resultante (Lurton et al., 1995). Estas cualidades pueden ser deseables o indeseables al sabor y aroma de los vinos (Suárez Lepe, 1997), lo que ha conducido a la selección y utilización de aquellas cepas de levaduras que produzcan un aroma y un sabor más agradables. Además, el origen del mosto y su composición se ha de tener en cuenta al seleccionar una cepa de *S. cerevisiae*, pues la composición cualitativa y cuantitativa del mosto influye en las características de los vinos obtenidos (Romano et al., 2003) de modo que mostos de diferentes variedades fermentados con las mismas levaduras producen vinos distintos (Egli et al., 1998).

Las levaduras contribuyen en el aroma de los vinos a través de la fermentación alcohólica mediante diferentes mecanismos (Lambrechts & Pretorius, 2000; Fleet, 2003):

- utilizando constituyentes del mosto de uva,
- produciendo etanol y otros compuestos que ayudan a extraer los componentes aromáticos de las uvas,
- produciendo enzimas que transforman los compuestos neutros de la uva en compuestos aromáticos,
- produciendo centenas de aromas activos, metabolitos secundarios (por ejemplo, ácidos, alcoholes, ésteres, polioles, aldehídos, cetonas, compuestos azufrados volátiles)

- y a través de la autolisis de las levaduras muertas.

Los productos sintetizados mayoritariamente por las levaduras son el etanol, el glicerol y el dióxido de carbono (Henschke & Jiranek, 1993). Pero son los ácidos orgánicos, los alcoholes superiores y ésteres, y en menor medida los aldehídos, algunos fenoles volátiles los que constituyen el principal grupo de compuestos que forman el aroma fermentativo (Rapp & Versini, 1991). Cuando estos compuestos se encuentran en exceso pueden ser considerados indeseables (Romano et al., 2003). Al aroma también contribuyen sustancias derivadas de los ácidos grasos y de compuestos que contienen nitrógeno y azufre, entre otros, puesto que en las reacciones bioquímicas que éstos experimentan se producen como subproductos numerosas sustancias volátiles (Boulton et al., 1996).

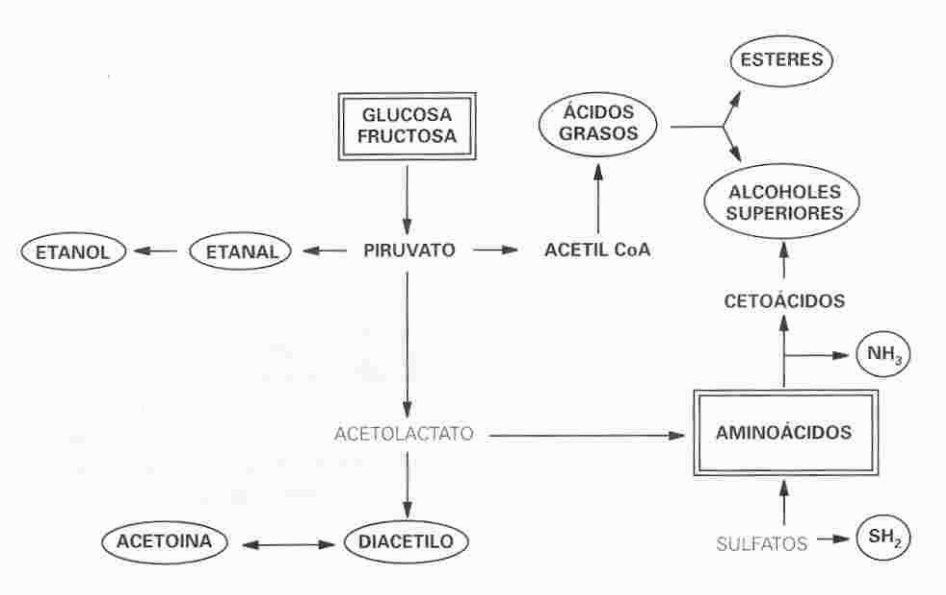

Figura 1. Implicaciones aromáticas del metabolismo microbiano de azúcares y aminoácidos (Suárez Lepe, 1997)

6.1. Ácidos grasos volátiles

El vino contiene una gran variedad de ácidos. En relación al aroma, el ácido acético interviene en la fracción aromática de los vinos: es el responsable principal de la acidez volátil produciendo el olor característico del vinagre, y por ello sus niveles están muy controlados y no deben superar los 600 mg/l, considerados valor umbral de aceptabilidad en un vino (Romano et al., 2003). La cantidad de ácido acético varía sensiblemente con las especies de levaduras que llevan a cabo la fermentación (Suárez

Lepe, 1997). *S. cerevisiae* lo produce en cantidades que varían entre 0.3-0.8 g/L según la cepa (Fleet & Heard, 1993).

Aunque el ácido acético es el más estudiado, dentro del grupo de los ácidos grasos se encuentran también los ácidos grasos de cadena corta (C6-C12) y los de cadena larga (C16 y C18), aunque estos últimos generalmente no aparecen en los vinos (Manzanares & Vallés, 2005). Los ácidos grasos de cadena corta de número par de átomos de carbono tales como el hexanoico, octanoico y decanoico, presentan aromas lácteos relacionados con notas negativas aromáticas (rancio, queso, aceitoso, agrio); sin embargo, son muy importantes en el equilibrio aromático de los vinos si se oponen a la hidrólisis de los ésteres etílicos correspondientes (Suárez Lepe, 1997; Torrens et al., 2008) y su presencia juega un papel importante en la complejidad aromática. Los ácidos grasos se generan en la levadura a partir del acetil-CoA procedente de la descarboxilación oxidativa del piruvato.

6.2. Alcoholes superiores

Son alcoholes con más de dos átomos de carbono cuyo peso molecular y punto de ebullición son superiores a los del etanol. Son los compuestos que cuantitativamente más influyen en el aroma del vino (Amerine et al., 1980). La capacidad de producir alcoholes superiores es una característica general de todas las levaduras pero la cantidad varía en función del género, la especie y la cepa, siendo además un carácter hereditario (Suárez Lepe, 1997). Se considera que a concentraciones inferiores a 300 mg/l contribuirían a la deseada complejidad del vino, mientras que valores superiores a 400 mg/l tendrían un efecto negativo en la calidad del mismo (Nykänen, 1986; Rapp & Versini, 1991; Lambrechts & Pretorius, 2000). Los alcoholes superiores producidos en mayor cantidad por las levaduras durante la fermentación alcohólica son: 1-propanol, 2-metil-1-propanol, 2-metill-1-butanol, 3-metill-1-butanol, 1-hexanol (herbáceo) y 2-feniletanol (rosa) (Henschke & Jiranek, 1993). Estos compuestos son también importantes como precursores en la formación de ésteres.

La formación de los alcoholes superiores se puede producir mediante dos vías (Aranda et al., 2005): la síntesis *de novo* a partir de azúcares, mediante la formación inicial de los correspondientes cetoácidos; o la conversión por transaminación y reducción, de aminoácidos de cadena lateral ramificada (valina, leucina, isoleucina y treonina); esta última vía es dependiente de la cantidad y tipo de fuente nitrogenada

presente en el mosto (Giudici et al., 1993). Su formación varía notablemente en función de las condiciones de aireación y temperatura de la fermentación (Suárez Lepe, 1997).

6.3. Ésteres

Es uno de los grupos de compuestos volátiles más importantes y numerosos que afectan al aroma y, a excepción del acetato de etilo, el acetato de metilo y el acetato de propilo, su contribución en el aroma es positiva ya que son responsables en gran medida del carácter afrutado y floral de diversos vinos. Los ésteres que contribuyen en mayor grado al aroma son los ésteres de alcoholes superiores y ácido acético (acetato de 2-feniletanol y acetato de isoamilo, entre otros) que proporcionan aromas a frutas tropicales, y los ésteres etílicos de ácidos grasos saturados de cadena media, como por ejemplo el hexanoato de etilo (manzana), octanoato de etilo (piña, pera), decanoato de etilo (floral); éstos dos últimos se consideran responsables directos de la calidad de los vino jóvenes (Suárez Lepe, 1997). Todos estos compuestos derivan principalmente del metabolismo de azúcares (Aranda et al., 2005). Los niveles finales de estos compuestos en el vino se ven afectados por dos factores: su hidrólisis durante las fases tempranas de la maduración del vino y la proporción en que son transferidos al medio, que disminuye con el aumento de la longitud de la cadena y está influida por la temperatura a la que se realiza la fermentación.

6.4. Compuestos carbonílicos

Los aldehídos volátiles de cadena corta contribuyen sobre todo a aromas tipo manzana, piña o limón (Aranda et al., 2005) aunque en exceso transmiten sabores lácteos y amargos indeseables (Mesas & Alegre, 1999). El más importantes es el acetaldehído (olor agrio, a piña verde) producido por descarboxilación del piruvato en la fermentación alcohólica de las levaduras o por oxidación del etanol.

Otro de los compuestos carbonílicos principales (en un 90% junto con el acetaldehído) es el diacetilo. Es una cetona que proporciona aroma a mantequilla y tiene su origen en la condensación y descarboxilación de dos moléculas de ácido pirúvico. (Aranda et al., 2005; Mesas & Alegre, 1999).

6.5. Compuestos azufrados

Su contribución al aroma del vino es muy importante, debido tanto a que son moléculas muy reactivas como a sus umbrales de detección extraordinariamente bajos, aunque en algunos casos son responsables de aromas indeseables (Aranda et al., 2005).

Existen muchos compuestos azufrados volátiles producidos por *Saccharomyces* durante la fermentación. Sulfuros, tioles (mercaptanos), tiolésteres y ésteres de ácido tioacético son los grupos funcionales más comunes. Estos metabolitos primarios reaccionan con otros compuestos formando una gran diversidad de volátiles azufrados (Bisson & Karpel, 2010; Flanzy, 2003).

El principal es el sulfuro de hidrógeno (SH_2). Su producción está relacionada con el metabolismo del nitrógeno y del azufre y ha sido estudiada en detalle en *S. cerevisiae* debido a que ocurre con frecuencia cuando se parte de mostos bajos en nitrógeno (Manzanares & Vallés, 2005).

Otros compuestos azufrados volátiles producidos por *S. cerevisiae* son el metionol y el dimetil sulfuro que provocan aromas a verdura cocida, o el metil mercaptano que produce olor fecal (Pisarnitskii, 2001).

6.6. Actividades enzimáticas que influyen en el aroma del vino

Durante la fermentación del vino, la actividad metabólica de las levaduras actúa en dos niveles diferentes: mediante la producción de compuestos aromáticos y por transformación de los precursores presentes en el mosto (Romano et al., 2003). En esta doble acción intervienen decisivamente enzimas producidas por las levaduras.

Glucosidasas

Los compuestos aromáticos varietales se pueden encontrar en *forma libre*, siendo percibidos por el olfato tal cual se encuentran en las uvas, o por el contrario en *forma combinada o ligada*, generalmente sin propiedades olfativas (Hidalgo Togores, 2003). El aroma varietal se debe mayoritariamente a la presencia de monoterpernos, principalmente linalol (aroma a rosa), geraniol (aroma a pelargonio), nerol (aroma a rosa), citronerol (aroma a cítrico) y α-terpineol (aroma a herbáceo) (Pisarnitskii, 2001), además de sesquiterpenos, pirazinas o norisoprenoides que juegan un papel dominante en la calidad del aroma (Hidalgo Togores, 2003). Según Pisarnitskii (2001) la mayoría de los terpenos de las uvas aparecen glucosilados, es decir, unidos a azúcares que

pueden ser glucosa, ramnosa, arabinosa, y apiosa, así como disacáridos. Las levaduras de fermentación poseen una cierta actividad glucosídica, siendo un importante factor de liberación de aromas varietales (Rosi et al., 1994; Fernández-González et al., 2003; Hernández et al., 2003). Cuando estos glucósidos son liberados por la acción de las enzimas glucosidasas, el contenido en terpenos en el mosto aumenta de dos a cinco veces.

La actividad glucosídica depende de la cepa de *S. cerevisiae* utilizada pero no es inhibida por la presencia de glucosa como es el caso de las glucosidasas de la uva y presenta un máximo de actividad al finalizar la fase de crecimiento de las levaduras, aunque luego decrece rápidamente (Hidalgo Togores, 2003). La actividad de esta enzima en la elaboración del vino son limitadas ya que las condiciones óptimas de actividad son pH 4 y a 40º C de temperatura (Rosi et al., 1994; Hernández et al., 2003).

La diana de acción de estas enzimas son las uniones entre un compuesto no glucídico (o aglicona) con otro glucídico formando heterósidos. Muchos precursores de aromas varietales se encuentran en forma de heterósidos, especialmente los que la aglicona es un terpenol, norisoprenoide o un fenol volátil. Las enzimas *β-D-glucosidasas* son capaces de hidrolizar estos compuestos, liberando así los aromas varietales.

6.6.2. Proteasas

Aunque las proteasas tienen un papel importante en el proceso de clarificación y estabilidad del vino al hidrolizar las proteínas (Canal-Llauberes, 1993) y en evitar la formación de espuma (Kunkee, 1984), también como consecuencia de esta actividad influyen indirectamente en el aroma del vino.

Las proteasas intervienen en la producción de compuestos aromáticos mediante la "fermentación alcohólica de los aminoácidos". La actividad de estas enzimas extracelulares producidas por algunas cepas de *S. cerevisiae*, hidrolizan las proteínas del mosto en los aminoácidos correspondientes (Bilinski et al., 1987; Úbeda Iranzo et al., 1998), estimulando su metabolismo y, al tomar los aminoácidos como fuente de nitrógeno para atender sus funciones vitales, excretan al medio los alcoholes superiores correspondientes como sustancias de desecho (Hidalgo Togores, 2003).

7. Técnicas de identificación y caracterización molecular de levaduras

El conocimiento de las levaduras presentes en la fermentación es fundamental, dada la influencia de éstas en la producción de compuestos que determinan el sabor y el perfil aromático de los vinos. Por ello, es preciso contar con técnicas de identificación y caracterización que permitan distinguir y determinar las especies de levaduras a nivel de cepa para llevar a cabo el estudio ecológico de levaduras responsables de la fermentación, los controles de producción, de comercialización, y de implantación de las levaduras seleccionadas utilizadas como inóculo, así como la constitución y el mantenimiento de las colecciones de levaduras autóctonas o seleccionadas.

Los primeros métodos de identificación y, por tanto, de clasificación de las levaduras, se basaron en el estudio de caracteres morfológicos, tales como la forma, dimensiones celulares, y formación de esporas. También se aplicaron criterios fisiológicos de asimilación de compuestos carbonados y nitrogenados, así como la necesidad de factores de crecimiento. Estos métodos eran muy laboriosos, su realización era lenta y costosa y su interpretación requería de un especialista. Además, estos factores fenotípicos no permitían la precisión suficiente para establecer una correcta clasificación y delimitación de las especies de levaduras (Hidalgo Togores, 2003, Fleet, 1993) ya que por otro lado, podían variar los resultados en función de las condiciones de cultivo y en ocasiones, las especies están delimitadas por una sola característica fisiológica en algunos casos determinada por un solo gen.

Sin embargo, a partir de la década de los ochenta del siglo pasado, como consecuencia del avance de la genética y de la tecnología molecular se han podido aplicar diversas técnicas de análisis del ADN que han permitido diferenciar entre especies e incluso entre cepas distintas. Entre ellas, se comentan a continuación aquellas que han sido utilizadas en el desarrollo de esta Tesis.

7.1. Métodos de identificación de especies

Los genes ribosomales 5,8S, 18S y 26S así como los espaciadores internos (ITS, del inglés *Internal Transcribed Spacer*) y los espaciadores intergénicos (NTS, del inglés *Non-Transcribed Spacer*) constituyen poderosas herramientas para el establecimiento de las relaciones filogenéticas y la identificación de especies (Kurztman & Robnett, 1998), debido a la existencia de secuencias conservadas en las mismas, así como por su evolución concertada, es decir, la similitud entre las unidades de transcripción repetidas

es mayor dentro de especies que entre unidades de distintas especies (Fernández Espinar et al., 2005). Esta técnica ha sido ampliamente usada en la identificación de levaduras vínicas (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Fernández-Espinar et al., 2000; Esteve-Zarzoso et al., 2001; Torija et al., 2001; Beltrán et al., 2002; Sabate et al., 2002; Clemente-Jiménez et al., 2004; Lopes et al., 2007; Maro et al., 2007; Valero et al., 2007; Barata et al., 2008; Jeyaram et al., 2008; Barrajón et al., 2009; Tofalo et al., 2009).

El análisis de estas regiones ribosomales se basa en la técnica de la Reacción en Cadena de la Polimerasa (PCR). Se trata de la amplificación de la región génica o secuencia diana deseada a la que se unen en cada cadena del ADN de la levadura, dos oligonucleótidos de secuencia conocida utilizados como cebadores para la enzima que lleva a cabo la reacción: la ADN polimerasa. Esta enzima, conocida como *Taq* polimerasa al haber sido aislada de la bacteria termófila *Thermophilus aquaticus*, es estable a las elevadas temperaturas a las que se desarrolla la PCR.

Esta técnica consiste en un número de ciclos (habitualmente entre 25 y 45) y cada uno de ellos consta de los siguientes pasos:

- *desnaturalización del ADN,* en la que se separan las dos cadenas de la molécula,

- *hibridación* o unión de los cebadores a cada cadena, y

- *extensión,* en la que la *Taq* polimerasa se une a los cebadores y a partir de ellos copia o replica la cadena de ADN molde.

El resultado es la amplificación del ADN, duplicando la cantidad de ADN diana en cada ciclo.

7.1.1. Análisis de restricción de regiones ribosomales

Se basa en la amplificación por PCR de la región 5,8S-ITS del ADN ribosómico (ADNr) a partir de los cebadores ITS1 e ITS4 descritos por White et al. (1999) y la posterior restricción de los amplificados. Es una técnica de fácil manipulación y gran reproducibilidad.

Aunque lo habitual es usar ADN directamente como molde en la reacción de amplificación (Guillamón et al., 1998; Fernández-Espinar et al., 2000; Torija et al., 2001; Beltrán et al., 2002; Sabate et al., 2002; Clemente-Jiménez et al., 2004), diversos

autores han utilizado como molde una pequeña cantidad de biomasa de una colonia aislada (Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Esteve-Zarzoso et al., 2001; Barata et al., 2008; Jeyaram et al., 2008; Tofalo et al., 2009). Esto supone un gran ahorro de tiempo y sólo precisa de un paso previo de 15 minutos a 95ºC en el protocolo de amplificación con objeto de liberar el ADN a la mezcla de reacción, para que pueda ser utilizado como molde.

Los perfiles de amplificación se visualizan en geles de agarosa al 1.4%. Los productos de amplificado de distinto tamaño corresponden a especies diferentes, sin embargo, cuando los amplificados son del mismo tamaño, no siempre corresponden a la misma especie. Siempre es necesario recurrir a la digestión de estos fragmentos para llegar a la definitiva identificación.

La digestión de los productos de PCR se lleva a cabo directamente sin necesidad de un paso previo de purificación. Para ello se emplean enzimas de restricción de ADN: son endonucleasas que reconocen una secuencia diana interna del ADN y generan un corte específico en dicha secuencia. Para la identificación de las especies de levaduras vínica las endonucleasas más empleadas son *Cfo*I (con la misma diana y corte en el ADN que la enzima *Hha*I), *Hae*III e *Hinf*I y en ocasiones *Dde*I (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Esteve-Zarzoso et al., 2001; Torija et al., 2001; Beltrán et al., 2002; Sabate et al., 2002; Barrajón et al., 2009).

Los fragmentos generados son separados por electroforesis en geles de agarosa al 3% y, una vez teñidos, revelan un perfil de bandas característico de especie.

7.1.2. Secuenciación de regiones ribosomales

La secuenciación es una técnica muy precisa y de aplicación relativamente rápida basada en la determinación y comparación de secuencias nucleotídicas de las regiones ribosomales. Las dos regiones más utilizadas son las correspondientes a los dominios D1 y D2 situados en el extremo 5' del gen 26S (Kurtzman & Robnett, 1998; Baleiras Couto et al., 2005; Maro et al., 2007; Tofalo et al., 2009) y el gen 18S (James et al., 1997), aunque la región 5,8S-ITS también ha sido empleada en algunos trabajos (Nisiotou & Gibson, 2005). La disponibilidad de las secuencias en bases de datos, sobre todo en el caso de la región D1/D2 del gen 26S, hacen que esta técnica sea muy útil para asignar una levadura desconocida a una especie concreta cuando el porcentaje de identidad de sus secuencias sea superior o igual a 99% (Kurtzman & Robnett, 1998).

La amplificación del dominio considerado se lleva a cabo por PCR a partir del ADN total. En este caso, los productos de PCR han de ser purificados mediante *kits* comerciales para eliminar los cebadores y el exceso de desoxinucleótidos que interferirían en la reacción de secuenciación.

7.2. Métodos de caracterización de cepas de la especie *S. cerevisiae*

Existe una gran diversidad de técnicas para la diferenciación entre cepas. Se han utilizado técnicas de hibridación (Querol et al., 1992b; Xufre et al., 2006), análisis del cariotipo electroforético (Schütz & Gafner, 1993; Schütz & Gafner, 1994; Briones et al., 1996; Izquierdo Canas et al., 1997; Pérez-Coello et al., 1999; Fernández-Espinar et al., 2001; Valero et al., 2007; Jeyaram et al., 2008), análisis de restricción de mtADN (Querol et al., 1992c; Querol et al., 1994; Constantí et al., 1997; Sabate et al., 1998; Esteve-Zarzoso et al., 2001; Torija et al., 2001; Beltrán et al., 2002; Sabate et al., 2002; Schuller et al., 2004; Santamaría et al., 2005; Lopes et al., 2007; Mercado et al., 2007; Garijo et al., 2008; Barrajón et al., 2009), métodos de amplificación por PCR ya sea *RAPD (Ramdon Amplified Polymorphic DNA)* (Baleiras Couto et al. 1996; Maro et al., 2007; Tofalo et al., 2009), *PCR de zonas repetitivas del genoma* (Masneuf-Pomarède et al., 2007) , *amplificación de secuencias δ* (Egli et al., 1998; Fernández-Espinar et al., 2001; Ciani et al., 2004; Le Jeune et al., 2006; Mercado et al., 2007; Domizio et al., 2009), y *AFLP* (*Amplified Fragment Length Polymorphism*) (Flores Berrios et al., 2005).

7.2.1. Análisis de restricción del ADNmt

El alto grado de polimorfismo que revela el ADNmt entre cepas vínicas de *S. cerevisiae* hace que el análisis de esta variabilidad sea uno de los más aplicados para la caracterización de este tipo de aislados (Querol et al., 1992c; Querol et al., 1994; Constantí et al., 1997; Sabate et al., 1998; Esteve-Zarzoso et al., 2001; Torija et al., 2001; Beltrán et al., 2002; Sabate et al., 2002; Schuller et al., 2004; Santamaría et al., 2005; Lopes et al., 2007; Mercado et al., 2007; Garijo et al., 2008; Barrajón et al., 2009). Además, no requiere equipos sofisticados ni una gran habilidad personal (Querol et al., 1992c).

Querol et al. (1992c) desarrollaron un método de análisis de ADNmt que evita la utilización de gradientes de cloruro magnésico y el uso de una ultracentrífuga, factor limitante para su uso en la industria. La simplificación de la técnica se basa en el hecho

de que el ADNmt de levaduras tiene un alto contenido en AT (75%) aunque contiene unas 200 regiones ricas en GC. Por tanto, digestiones del ADN total con enzimas del tipo GCAT no reconocen las secuencias ricas en GC ni las ricas en AT. Dado el bajo número de sitios de restricción en el ADNmt y el alto número de puntos de corte en el ADN nuclear, éste último se rompe en fragmentos de pequeño tamaño, lo que permite visualizar claramente las bandas correspondientes al ADNmt como bandas definidas, superpuestas a la sombra de degradación del ADN nuclear mediante una electroforesis convencional en gel de agarosa al 1%. No todos los enzimas revelan el mismo grado de polimorfismo y es muy dependiente de la especie. En el caso concreto de *S. cerevisiae*, el enzima más apropiado para la diferenciación a nivel de cepa es *Hinf*I (Querol et al., 1994).

8. Breve historia del vino en Málaga, sus Denominaciones de Origen (D.O.) y situación actual de la viticultura en Ronda

8.1. Breve historia del vino y D.O. en Málaga

Las primeras referencias del vino en Málaga se remontan a la llegada de los fenicios a sus costas en el s. VIII a.C., quienes no sólo introdujeron vinos procedentes del mediterráneo oriental sino que además cultivaron la vid en asociación con los grupos autóctonos elaborando vinos (http://www.vinomalaga.com).

Coincidiendo con la fundación de la mítica Mainake, hacia el año 600 a.C., los griegos se establecieron en Málaga y enseñaron a los indígenas a podar las viñas según Guillén Robles (1987).

En las ruinas romanas de Acinipo, en Ronda, se han encontrado numerosas monedas con la representación de un racimo de uvas, lo que da ha entender la importancia de la producción vitícola en ese territorio.

En las tierras malagueñas de Al andalus se siguió cultivando la viña para producir vino y pasas. El vino llamado *xarab al malaquí* se siguió consumiendo, fue muy famoso y cantado por los más destacados poetas. El cultivo de la pasa sigue realizándose en nuestros días siguiendo la más genuina tradición, protegida bajo la D.O. Pasas de Málaga.

Con el descubrimiento de América se produce un gran auge de la viticultura en toda Andalucía, enviándose las primeras partidas al Nuevo Mundo. Se empieza a exportar a Europa en el siglo XV, siendo en el siglo XVI cuando se establece oficialmente el mercado con Inglaterra y Dinamarca (http://www.serraniaderonda.cc). En este siglo, se constituye en Málaga la Hermandad de Viñeros, que subsiste en su faceta gremial en lo que hoy es el Consejo Regulador y en su faceta religiosa en la Muy Ilustre Antigua y Venerable Hermandad Sacramental de Ntro. Padre Jesús Nazareno de Viñeros, Ntra. Sra. del Traspaso y Soledad de Viñeros y S. Lorenzo Mártir (http://www.vinomalaga.com).

A finales del siglo XIX se produce de forma involuntaria, la renovación total del viñedo como consecuencia de la invasión por *Phylloxera vastatrix* (año 1876), insecto que ataca a la raíz de las cepas sensibles a esta plaga, responsable de la destrucción de prácticamente todos los viñedos establecidos en ese momento (http://www.serraniaderonda.cc).

En el año 1900 aparece el Reglamento de la Asociación Gremial de Criadores Exportadores de Vino de Málaga, para velar por los intereses generales del comercio de vinos expidiendo certificados, nombrando árbitros y peritos y sobre todo, garantizando por medio de su sello de origen, la legitimidad de los vinos que exportaban. En 1924 consiguen la inscripción de la marca "Málaga" (http://www.vinomalaga.com).

La Denominación de Origen Málaga es una de las más antiguas del país (1933). Ampara vinos cuyas uvas se recogen en cinco zonas de la provincia, alrededor de 1.030 hectáreas, repartidas por 66 municipios. Las zonas de producción son: *Norte, Axarquía, Montes, Costa Occidental* y *Serranía de Ronda*.

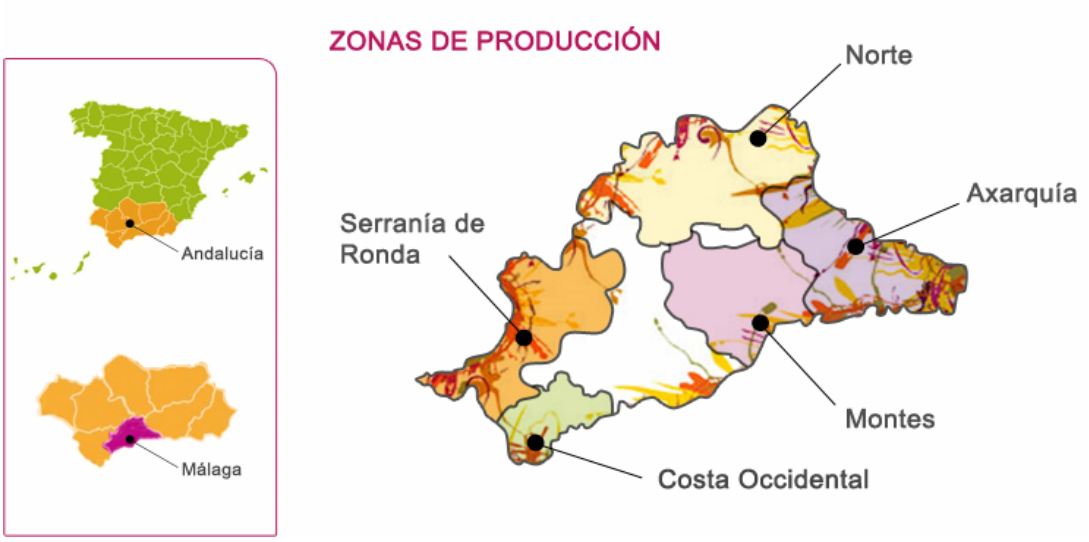

Figura 2. Zonas de producción de las DDO Málaga y Sierras de Málaga

En 2001 se crea la Denominación de Origen "Sierras de Málaga", subdenominación "Serranía de Ronda", compartiendo Consejo regulador con la D.O. "Vinos de Málaga".

Los vinos "Sierras de Málaga" se clasifican en blancos, tintos y rosados y se pueden elaborar con las variedades blancas: Pero Ximen (Pedro Ximénez), Moscatel de Alejandría, Moscatel Morisco (Moscatel de grano menudo), Chardonnay, Macabeo, Sauvignon Blanc, Lairén y Doradilla; y las tintas: Romé, Cabernet Sauvignon, Merlot, Syrah, Tempranillo, Garnacha, Cabernet Franc, Pinot Noir, Colombard y Petit Verdot. Ampara los vinos "tranquilos", aquellos que se obtienen mediante la fermentación natural de los mostos, con un contenido alcohólico de entre 10 y 15 %.

8.2. Situación actual de la viticultura en Ronda

La década de los sesenta trajo consigo una fuerte emigración que supuso el abandono del cultivo de la vid en muchas parcelas de la comarca de Ronda, aunque nunca se ha dejado totalmente de producir (http://www.serraniaderonda.cc).

Actualmente el cultivo de la vid está resurgiendo. Se están estableciendo plantaciones modernas, con densidades de plantación que van de 4.300 a 5.000 plantas por hectárea, formadas en espaldera, cultivo en la mayoría de los casos ecológico, utilizando variedades ya injertadas sobre pies seleccionados en función de un análisis realizado sobre el suelo donde se van a plantar. Estas variedades son mayoritariamente tintas, como Tempranillo, Merlot, Syrah, Cabernet Sauvignon, Pinot Noir, Garnacha

Tinta, Petit Verdot, y en menor medida con variedades blancas como la Chardonnay. La superficie existente en la actualidad ronda las 200 ha, comprendiendo éstas las antiguas (40 ha) y las de nueva plantación.

La corriente vitivinícola que se está desarrollando en la comarca es heredera de los cambios que se han producido en el sector a nivel español en la década de los ochenta y noventa, pasando de la producción en gran cantidad (destinada mayoritariamente a la destilación) a la producción de vinos de alta calidad y sobre todo vinos con identidad propia. También se ha optado por la fusión entre el viticultor y el elaborador de vinos en una misma persona, dejando de ser así el trabajo en bodega un proceso industrial y pasa a ser un proceso artesanal, donde cada vitivinicultor da al vino unas características propias que reflejan todo lo que el suelo donde se cultivan esas viñas tiene de particular y característico. Se compite así con un producto de altísima calidad y con una identidad diferente a lo que existe en el mercado.

9. Viticultura y vinos ecológicos

Se entiende por *agricultura ecológica, biológica, orgánica, biodinámica* o *biológico-dinámica* al sistema agrario cuyo objetivo fundamental es la obtención de alimentos de máxima calidad respetando el medio ambiente y conservando la fertilidad de la tierra, mediante la utilización óptima de los recursos y sin el empleo de los productos de síntesis. La *viticultura ecológica* es una consecuencia de la anterior, donde la uva como fruto de la vid puede ser objeto de una producción ecológica. Sin embargo, la elaboración de *vino ecológico* como tal es un término no amparado por la legislación comunitaria actual, ya que este tipo de vino no se encuentra recogido en la Organización Común del Mercado Vitivinícola de la Unión Europea, y por lo tanto, no se puede hablar de vino ecológico, si no más bien de "vino procedente de uvas de agricultura ecológica" (Hidalgo Togores, 2003).

Las parcelas destinadas a la agricultura ecológica deberán pasar por un periodo de conversión de al menos dos años antes de la siembra, o en el caso de cultivos vivaces distintos de las praderas de al menos tres años antes de la primera cosecha, aplicándose esta normativa en el caso del establecimiento de un viñedo ecológico.

La fertilidad y la actividad biológica del suelo deberán ser mantenidas o incrementadas mediante:

- El cultivo de leguminosas, abono verde o plantas de enraizamiento profundo, con arreglo a un programa de rotación plurianual adecuado.

- La incorporación de estiércol procedente de la producción ganadera ecológica, con un máximo de animales por hectárea equivalente a 170 kg de nitrógeno por hectárea y año.

- La incorporación de cualquier otro material orgánico procedente de explotaciones de producción ecológica.

- Podrán añadirse excepcionalmente y como complemento, fertilizantes establecidos, orgánicos o minerales, tales como estiércol, mantillo de excrementos sólidos o excrementos líquidos de animales, arcillas, serrín, sulfato de potasio, carbonato cálcico de origen natural, entre otras.

- Para la activación de compost podrán utilizarse preparados apropiados a base de vegetales o preparados a base de microorganismos, que no estén modificados genéticamente, pudiendo utilizarse los llamados preparados biodinámicos a base de polvo de roca, estiércol de granja o vegetales.

- Para mejorar el estado general del suelo o su disponibilidad de nutrientes, podrán utilizarse preparados apropiados a base de microorganismos, que no estén modificados genéticamente, cuando la necesidad de su empleo se reconozca expresamente por las autoridades.

La lucha contra parásitos, enfermedades y malas hierbas también está establecida mediante una serie de medidas determinadas a adoptar según el caso.

A pesar de no estar reglamentados los vinos ecológicos en la Unión Europea, en España, desde octubre de 2006, existen unas directrices para la elaboración vinos procedentes de uvas de agricultura ecológica (CAECV, 2010), donde destacan una serie de prácticas permitidas, entre otras:

- Transporte de la uva a la bodega en el mismo día y de forma que se evite su compactación y alteración.

- Sistemas mecánicos de prensado que no dañen o dislaceren los componentes sólidos del racimo.

- Fermentación con levaduras existentes de forma natural en el mosto, y con levaduras seleccionadas que no podrán ser genéticamente modificadas (OGMs) ni derivadas de OGMs.

- Sulfitado por combustión de azufre puro o de mezclas azufradas sobre soporte de celulosa o bien por adición de soluciones sulfurosas del 5-8 % de SO_2, pastillas de azufre que no permitan el goteo y gases líquidos a presión. La cifra de SO_2 total en el producto terminado y dispuesto para el consumo, deberá ser lo más bajo posible y no exceder de 100 o 120 mg/L en vinos tintos jóvenes o envasados con mas de un año, respectívamente, 120 o 160 mg/L para los blancos y rosados secos o dulces, semidulces y semisecos, respectivamente, y 120 mg/L para generosos y de licor y espumosos.

10. La bodega de estudio

El estudio realizado en esta Tesis se ha llevado a cabo la Bodega Joaquín Fernández, Finca los Frutales, en el municipio de Ronda. Situada en plena sierra a 723 m s.n.m., sobre suelo de textura franco arcillo arenosa, en una ladera orientada al sur por lo que recibe la luz del sol durante todo el día y, al estar en ladera, el aire frío se desplaza hacia las zonas bajas. La temperatura media en Ronda es de 15ºC y la precipitación anual de 648 L/m^2 (http://www.ronda.es). Los duros inviernos y el clima continental producen vinos intensos y con carácter.

La historia de esta bodega es bastante reciente (http://www.bodegajf.es). En el año 2001 se empezaron a preparar los terrenos para la plantación del viñedo en la Finca Los Frutales. A finales del invierno de 2002 se plantaron cerca de 2 ha de viña de las variedades Cabernet Sauvignon, Syrah, Merlot y Garnacha. En el año 2003 se amplió la superficie de viñedo con 2,2 hectáreas de Merlot, Syrah y Garnacha, alcanzando más de 4 hectáreas de viñedo. En 2003 se inscribió la finca en el Comité Andaluz de Agricultura Ecológica (CAAE)**,** organismo que controla y certifica la producción para que, de esta forma, el producto pueda ser vendido como "vino producido a partir de uva ecológica" tras el período de reconversión correspondiente. En nuestro país los vinos

ecológicos no gozan todavía del prestigio y aceptación que en otros países pioneros en estas labores, como Alemania o Estados Unidos, pero poco a poco se van aceptando como una nueva e innovadora manera de producir el vino de forma más natural, sostenible y respetuosa con el medio ambiente.

La bodega es también de reciente construcción, como otras de la Serranía de Ronda. La primera campaña de vinificación en esta bodega fue en el año 2003 con uvas procedentes de otra finca del mismo municipio, mediante fermentaciones inoculadas con levaduras secas comerciales con el fin de asegurar fermentaciones completas. Desde entonces, en cada campaña se utilizan varias levaduras comerciales distintas en sus vinificaciones. También fue práctica común en la bodega durante los años de inicio (2003-2005) esparcir las lías de las fermentaciones sobre el viñedo como abono ecológico.

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JUSTIFICACIÓN, OBJETIVOS Y PLAN DE TRABAJO

1. Justificación y Objetivos

La Tesis Doctoral que se presenta se ha desarrollado como consecuencia de mi participación como becaria en el proyecto "Mejora de la vinificación ecológica mediante el empleo de levaduras autóctonas seleccionadas en la Serranía de Ronda (Málaga)", contrato P-2005/1258, entre la Empresa Pública de Desarrollo Agrario y Pesquero (Junta de Andalucía) y la Universidad de Sevilla. Los investigadores que participan en él pertenecen al Área de Edafología y Química Agrícola, al de Nutrición y Bromatología y al Departamento de Genética de la Universidad de Sevilla.

Este proyecto tiene su origen en la convocatoria de concurso para la realización de proyectos de investigación y transferencia de tecnología en el ámbito de la agricultura y ganadería ecológica (Expdte.: 92162/1) de la Junta de Andalucía, siendo los procesos industriales agroalimentarios ecológicos una de las líneas de investigación y, en concreto, la mejora de los métodos de vinificación ecológica. Dentro de este contexto, el proyecto se presentó coordinado con una bodega recientemente establecida en la Serranía de Ronda con vocación de producir vinos, tintos particularmente, a partir de uva de cultivo ecológico.

A pesar de no existir una regulación europea sobre la elaboración de vinos ecológicos, a nivel nacional existen unas recomendaciones para la elaboración de dichos vinos; entre ellas la fermentación a partir de las levaduras presentes de forma natural en el mosto (fermentaciones espontáneas) o el empleo de levaduras seleccionadas.

Por tanto, el objetivo principal de nuestro trabajo era la mejora de la elaboración ecológica de los vinos en la bodega de estudio mediante el empleo de levaduras autóctonas de la Serranía de Ronda que proporcionen características únicas y propias de la zona de producción, y que contribuya a la conservación de la biodiversidad. Por otra parte, dada las características de la bodega y de su viñedo, ambos de reciente creación, ha sido práctica común el empleo de levaduras comerciales desde el inicio de la actividad de la bodega para asegurar el desarrollo de la fermentación.

El primer paso, por tanto, y antes de realizar los trabajos de selección, era conocer la microbiota levaduriforme propia (autóctona) de Ronda, nunca antes estudiada y crear una colección de cultivos puros de *Saccharomyces cerevisiae* autóctonas. El empleo de

las técnicas de identificación molecular nos permite identificar de forma fiable las cepas de levaduras a nivel de especie e incluso a nivel de cepa, permitiendo de este modo, distinguir entre cepas de *S. cerevisiae* autóctonas y aquellas comerciales usadas por la bodega.

De acuerdo con lo expuesto anteriormente, los objetivos principales de esta Tesis son:

1. Obtención, identificación y caracterización molecular de cultivos puros de *Saccharomyces* autóctonas.
2. Evaluación del grado de implantación en bodega de las levaduras comerciales empleadas y su posible diseminación en el viñedo.
3. Estudio del comportamiento tecnológico de las cepas autóctonas de *Saccharomyces* así como de las características fisicoquímicas y organolépticas (especialmente perfil aromático) de los productos obtenidos, en relación al de las levaduras comerciales empleadas en la bodega de estudio.
4. Evaluación de la idoneidad del uso de levaduras autóctonas seleccionadas frente al de levaduras comerciales en la bodega.

Esta Tesis se ha podido llevar a cabo gracias a la subvención de dos proyectos:

- P-2005/1258 "Mejora de la vinificación ecológica mediante el empleo de levaduras autóctonas seleccionadas en la Serranía de Ronda (Málaga)", entre la Empresa Pública de Desarrollo Agrario y Pesquero (Junta de Andalucía) y la Universidad de Sevilla .
- FEDER RM2007-00008-C02-00, "Identificación y caracterización de levaduras autóctonas del género *Saccharomyces* de la zona sur de España. Evaluación, conservación y ampliación de la colección de levaduras de la Universidad de Sevilla" dentro de la convocatoria de "Conservación de los recursos genéticos de interés agroalimentario"del INIA-Ministerio de Educación y Ciencia de España.

2. Plan de trabajo

El plan de trabajo diseñado para alcanzar los objetivos descritos consta básicamente de tres partes. La primera de carácter ecológico, con la finalidad de conocer la dinámica de cepas autóctonas y comerciales de *S. cerevisiae* durante el proceso de fermentación en la bodega de estudio y en el viñedo próximo, y de crear una colección de cultivos puros de cepas de *S. cerevisiae* autóctonas.

La segunda parte consiste en el estudio de algunas propiedades tecnológicas de las cepas de *S. cerevisiae* autóctonas aisladas y de las cepas comerciales más frecuentemente usadas por la bodega, con el fin de conocer el potencial enológico de las primeras y la idoneidad de su empleo frente a las comerciales. Para ello se determinan algunas propiedades en las cepas y se realizan diferentes ensayos de fermentación en el laboratorio que permitan seleccionar aquellas con mejor comportamiento. En la tercera parte, las levaduras seleccionadas se ensayan en fermentaciones a escala semi-piloto en la bodega de estudio.

Los resultados obtenidos se presentan en forma de Capítulos, cada uno correspondiente a un artículo científico; la metodología seguida se detalla al final de la Memoria en el Anexo I.

1. Estudio ecológico de levaduras vínicas en la Serranía de Ronda

1.1 . En bodega

Durante la campaña de 2005 el estudio ecológico se realizó durante el proceso de fermentación en dos depósitos de la bodega con mosto de la variedad Cabernet Sauvignon. Un depósito fue inoculado con una levadura comercial y el otro con un pie de cuba natural. En la campaña de 2007 se estudiaron 3 depósitos de la bodega, cada uno de una variedad de uva distinta, Merlot, Syrah y Cabernet Sauvignon. Los dos primeros depósitos se inocularon con un pie de cuba natural y el último con una levadura comercial. De cada depósito se aislaron cepas de levaduras en diferentes momentos de la fermentación. Además, a principios de 2007, una vez finalizada la campaña de 2006, se realizaron aislamientos a partir de diferentes superficies de la bodega (suelo, paredes y diversos equipos de la bodega).

Las cepas aisladas se identificaron a nivel de especie mediante PCR-RFLP de la región génica 5.8S-ITS del ADN ribosómico y mediante secuenciación de los dominios D1 y D2 del ADNr 26S. La caracterización de cepas de *S. cerevisiae* se realizó mediante análisis RFLP del ADN mitocondrial. Los resultados obtenidos se exponen y discuten en los **Capítulos 1 y 3**.

1.2 . En viñedo

El estudio de la población de *S. cerevisae* presente en el viñedo se realizó durante dos campañas consecutivas (2006 y 2007). Durante la campaña de 2006 se eligieron dos viñedos, uno junto a la bodega en la que se seleccionaron dos parcelas (una de la variedad Syrah y otra de Merlot) y otro viñedo de la variedad Syrah que no tenía ninguna bodega adyacente. Durante la campaña 2007, las muestras se tomaron sólo en tres parcelas del viñedo anexo a la bodega, cada una de una variedad de uva (Merlot, Syrah y Cabernet Sauvignon). Durante las dos campañas de estudio se establecieron diferentes fechas y puntos de muestreo. Los racimos se recolectaron de forma aséptica, y a partir de ellos se realizaron microfermentaciones en el laboratorio. Los aislamientos se efectuaron cuando en cada microfermentador se hubieron consumido 2/3 de los azúcares del mosto. La identificación y caracterización de las cepas aisladas se realizó siguiendo la misma metodología indicada en el apartado anterior. Los resultados obtenidos se exponen y discuten en el **Capítulo 2**.

2. Caracterización enológica y selección de levaduras

Para esta parte del estudio se seleccionaron aquellas cepas autóctonas que se aislaron con mayor frecuencia durante las campañas 2005 a 2007 y las comerciales más frecuentemente empleadas por la bodega. En primer lugar se estudiaron algunas propiedades de interés tecnológico para el proceso de vinificación como el factor *killer*, producción de SH_2, producción de espuma, resistencia a SO_2 y presencia de actividades enzimáticas β-glucosidasa y proteasa.

A continuación, se realizaron ensayos de fermentación con cada una de las cepas en mosto sintético con diferentes concentraciones de azúcar (225 y 275 g L^{-1}) y a dos temperaturas (20 y 30 ºC). Se estudió la cinética de fermentación, capacidad de agotar los azúcares del medio y la producción de etanol y de acidez volátil, entre otros.

Finalmente también se hicieron ensayos de fermentación con mosto natural (variedad Merlot) estéril con las cepas seleccionadas a partir de las pruebas anteriores. En las muestras de vino obtenidas se determinaron parámetros físico-químicos generales y se hicieron además análisis de compuestos volátiles y análisis sensorial. Los resultados obtenidos se exponen y discuten en el **Capítulo 4**.

3. Ensayos en bodega

Finalmente, se realizaron 5 vinificaciones en la bodega en depósitos de 100 litros de acero inoxidable con 5 cepas distintas. La implantación de las cepas inoculadas se valoró mediante análisis de restricción del ADN mitocondrial. En los productos finales se determinaron parámetros físico-químicos generales y se realizaron análisis sensorial y de compuestos volátiles. Los resultados obtenidos se exponen y discuten en el **Capítulo 5**.

Capítulo 1

Effect of the use of commercial *Saccharomyces* strains in a newly established winery in Ronda (Málaga, Spain)

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Enviado a *Antonie van Leeuwenhoek*.

Abstract

An ecological study of a spontaneous and an inoculated fermentation in red wine was carried out in the 2005 vintage in a winery located in the Denomination of Origin "Sierras de Málaga" (Malaga, southern of Spain). The winery operated by the first time with the 2003 vintage and since then has used commercial yeast inoculums to start alcoholic fermentation. Yeast isolates were identified by ribosomal DNA and mitochondrial DNA RFLP analysis. All the isolates were found to be strains of *Saccharomyces cerevisiae* whose mitochondrial restriction patterns matched to some of those of the commercial yeasts employed by the winery during the successive vintages. The same four restriction patterns were found in non inoculated and inoculated vats, although with different frequencies. No non-*Saccharomyces* yeasts or indigenous *Saccharomyces* yeasts were isolated during fermentation, and the former were only found in the fresh must before fermentation. The use of commercial yeast starter in a new established winery seems to have prevented the development of a resident indigenous *Saccharomyces* flora, and suppressed the growth of non-*Saccharomyces* yeasts during the early stages of the fermentation.

Keywords: *Saccharomyces cerevisiae*, alcoholic fermentation, inoculation, new winery

1. Introduction

The conversion of grape must to wine is a complex microbiological reaction involving the sequential development of various yeast and lactic acid bacteria species. Traditionally, wine has been produced by natural fermentation of grape juice by yeasts that originate from the grapes and winery equipment. As summarized by Beltran et al. (2002), the diversity, composition and evolution of yeast flora in grape musts depend on a variety of factors: geographic location, climatic conditions, age of the vineyard and grape variety. Yeast of the genera *Kloeckera*, *Hanseniaspora*, *Candida*, *Pichia* and, sometimes, *Hansenula* grow during the early stages of fermentation but eventually die off, leaving *Saccharomyces cerevisiae* as the dominant species to complete the fermentation (Heard and Fleet 1985).

On the other hand, using active dry yeast in fermentation is becoming one of the most common practices in winemaking because it ensures a reproducible product and reduces the lag phase and the risk of wine spoilage (Beltrán et al. 2002; Heard and Fleet 1985; Santamaría et al. 2005). The inoculation of a large population of *S. cerevisiae* strains could inhibit the growth of other *Saccharomyces* and also non-*Saccharomyces* species. Hence, inoculation limits the growth of wild yeasts (Santamaría et al. 2005). Thus, the winemaking community is still widely divided about this practice because of a widespread belief that native yeast strain gives a distinctive style and quality to wine (Beltrán et al. 2002).

This study shows the influence of the winery characteristics, in special, the age and the use of commercial yeasts, on the yeast ecology of the winery: the behaviour of the commercial strains used in different vintages in order to become a component of the winery yeast flora and participate in inoculated and non-inoculated fermentations.

2. Materials and Methods

2.1. The vineyard and the winery

The vineyard and the winery are located in the “Serranía de Ronda” (Malaga, South of Spain), both belonging to the Denomination of Origin “Sierras de Málaga”. The vineyard, established in 2002, has an extension of approximately four ha of cultivated grapevines (Merlot, Syrah, Cabernet Sauvignon and Garnacha) and it is managed under the routine practices of Integrated Production and Organic Farming.

The winery was built just prior to the 2003 vintage, and it is adjacent to the vineyard. Its first vinification took place during the 2003 and 2004 vintages but with grapes harvested in other vineyards from the same municipality. All the equipment in the winery was totally new and neither the winery, nor the equipment had been previously exposed to must, fruit juices, wines, or any beverage or food before the 2003 vintage. For this reason, the use of commercial starter has become a usual practice in this winery to ensure the alcoholic fermentation.

2.2. Wine fermentations

The conditions of fermentations were established by the management of the winery according to their common practises and commercial interests

Wines were produced from Cabernet Sauvignon grapes harvested during the 2005 vintage. The must was supplemented with sulfur dioxide (35 mg/liter) and was separated in two different vats of 700 liters each and kept at 10 ºC during 48 h. Next, Vat 1 was inoculated with the active dry yeast Excellence XR (Lamothe-Abiet, France), that was rehydrated prior to inoculation according to the instructions of the supplier (25 g/Hl); and Vat 2 was inoculated with a spontaneous fermenting must of the same variety prepared separately from the winery. This addition of natural preconditioned yeast cells is called the “pie de cuba” method of inoculation (*pied de cup*). The fermentations in both vats took place under the same conditions of temperature (25-30ºC) and kinetics of the fermentation was monitored by measuring the Baume degree.

2.3. Yeast isolation

Samples were taken periodically during the fermentation for the isolation and enumeration of yeasts. Density was monitored in each vat and three sampling points were considered: the beginning (14 Baume degree), the middle (6-7 Baume degree) and the end (less than 2 Baume degree) of the alcoholic fermentation. Additionally, a sample of the fresh must before filling the vats was also taken for microbiological analysis perfomance.

Aliquots (0.1 ml each) of serial dilutions were spread onto plates of YEPD agar (yeast extract 1% w/v, peptone 1 % w/v, glucose 2 % w/v, agar 2 % w/v). Plates were incubated at 28 ºC for 72 hours and, for each sample, 30 colonies were randomly chosen and submitted to further studies.

2.4. Yeast identification

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeast, every isolate was evaluated according to its ability to grow on L-lysine plates (Barnett et al. 1990). Isolates that were not able to grow using L-lysine as the sole nitrogen source were classified as *Saccharomyces*. This classification was confirmed by PCR of the ribosomal region encompassing the 5,8S ribosomal DNA (rDNA) gene and the two internal transcribed spacers. rDNA was amplified by PCR in a TC-312 Techne termocycler using primers ITS1 and ITS4, described by White et al. (1990). The species of the isolates were identified by PCR-RFLP (restriction fragment length polymorphism) digesting with the restriction endonucleases *Hinf*I, *Hha*I (same enzymatic activity than *Cfo*I) and *Hae*III (Amersham Biosciences) as previously described by Guillamón et al. (1998). PCR products and restriction fragments were separated on a horizontal 1.4% and 3 % (w/v) agarose gel, respectively, in TAE buffer and compared with standards (100-bp DNA ladder, Amersham Biosciences).

2.5. Strain differentiation

Mitochondrial DNA (mtDNA) restriction analysis was used to differentiate strains of *S. cerevisiae*. DNA extraction and restriction of mtDNA was performed according to Querol et al. (1992b). Electrophoresis was carried out as described before except that restriction products were separated on a 1 % (w/v) agarose gel and lambda DNA digested with PstI was used as standard. Results of restriction with *Hinf*I (Amersham Biosciences) were found to be the most informative (Querol et al. 1994).

2.6. Commercial yeasts

Samples of the active dry yeasts used in the winery since the first vinification (2003 vintage) were also analysed in order to distinguish between indigenous *Saccharomyces* strains and commercial ones. Table 1 shows the commercial yeasts used in the winery during the 2003 to 2005 vintages.

3. Results

Two vats from the winery specified in Materials and Methods were selected to study the evolution of *Saccharomyces* strains populations during the fermentation of Cabernet Sauvignon musts. One of the vats was inoculated with the commercial dry yeast strain coded as LSA7, and the other one was inoculated with a *pied de cup*

prepared with the same grape variety. Four sampling points were defined and a total of 206 yeast colonies were isolated.

3.1. Yeast identification

Wine yeasts isolated from the fresh must and during the fermentation of Cabernet Sauvignon must in both vats were initially tested for their capability to grow in L-lysine medium. According to this test, non-*Saccharomyces* yeasts were only found in the fresh must sample, but in a low proportion (five out of the 26 isolates), and the rest of the yeast strains were assigned to the genus *Saccharomyces*.

The PCR-amplified products from *Saccharomyces* ITS1-5,8S rDNA-ITS2 regions showed the same size (850 pb), indicating that those isolates belonged to the *Saccharomyces sensu-stricto* group (Fernández Espinar et al. 2000). The PCR products digested with *Hha*I, *Hae*III and *Hinf*I endonucleases were analysed comparing the sizes of the restriction products with those described previously (Esteve-Zarzoso et al. 1999). These results showed that all the *Saccharomyces* isolates correspond to the species *S. cerevisiae* (Figure 1).

3.2. Strain differentiation

The mtDNA restriction patterns of the *S. cerevisiae* isolates revealed scarce diversity at strain level, with only four different patterns characterized (A, B, C and D). The number and frequencies of the patterns of the isolates are presented in Table 2 and represented in Figure 2. In addition, when comparing these patterns with those obtained from the commercial yeasts used in the winery (Figure 3), we found that the patterns A, B, C and D were coincident with those of the commercial yeast coded as starter LSA2, LSA4, LSA3 and LSA7, respectively (Figure 4). More interestingly, no indigenous (wild) *Saccharomyces* strains were isolated during the fermentation and not even in the fresh must where, curiously enough, most of the isolates in the fresh must corresponded to commercial *Saccharomyces cerevisiae* strains but four non-*Saccharomyces* isolates (Table 2).

Yeast strains with each of the four patterns were found in both vats during the fermentation, but pattern C was scarcely represented. This mtDNA restriction pattern corresponds to commercial starter LSA3 that was used only during the 2004 vintage, prior to this study. Strains showing restriction patterns A, B and D were found in both

vats with some differences in their frequencies, particularly in Vat 1 (Figure 2). In Vat 2 the three main patterns (A, B and D) were found at slightly different frequencies, especially at the beginning and middle fermentation stages, thus, none of these yeast strains prevailed during the alcoholic fermentation.

Active dry yeast coded as starter LSA4 (pattern B) has been used every vintage since the start of the vinification activity in the winery (2003, 2004 and 2005 vintages). In both vats the number of isolates increased in the middle stage of the fermentation but decreased in the last stage.

Active dry yeast LSA2 (profile A) used in the winery in the 2005 vintage for the first time to inoculate other fermentation vats, was found in both vats at frequencies close to 30% (33.3 and 26.6 % in Vat 1 and 2, respectively).

Commercial starter LSA7 (profile D) has been used in the 2004 and 2005 vintage campaigns and for inoculating Vat 1 during this study. This fact explains its dominance in this vat and its prevalence until the end of the fermentation, representing 52.2 % of isolates.

Finally, no strain with a profile similar to commercial strain LSA1 (firstly used during the vintage campaign of this study) was isolated in any of the sampled vats.

4. Discussion

Different authors have studied the fermentation of grape juice into wine pointing out the role of yeasts, mainly *S. cerevisiae* but also other non-*Saccharomyces* species, as primarily responsible for the alcoholic fermentation (Heard and Fleet 1985; Querol et al. 1994; Torija et al. 2001). These studies demonstrate that *S. cerevisiae*, extremely rare on grape and vineyard (Le Jeune et al. 2006, Martini 1993, Pretorius 2000; Sabate et al. 2002) but mainly colonising the surface of winery equipment (Beltran et al. 2002; Ciani et al. 2004; Le Jeune et al. 2006; Rosini 1984; Sabate et al. 2002; Sangorrin et al. 2002; Vaughan-Martini and Martini 1995), dominates the fermentation process due to its efficient fermentative metabolism. The use of *S. cerevisiae* starters in the form of active dry yeast has become a usual practice to ensure the reproducibility of fermentations and the final product quality (Beltran et al. 2002; Henick-Kling et al. 1998; Querol et al. 1990, 1992b; Santamaria et al. 2005). Diverse molecular methods to clearly differentiate between the inoculated *S. cerevisiae* strain and the wild *S. cerevisiae* strains present in

musts are available (Le Jeune et al. 2006; Mercado et al. 2007; Querol et al. 1992a; Schuller et al. 2005; Schütz and Gafner 1993). Among these techniques, mitochondrial DNA restriction analysis has proven useful for monitoring inoculated wine fermentations due to the high mtDNA polymorphism among wine *Saccharomyces* strains (Constantí et al. 1997, Querol et al. 1992a).

In this work, we have stated how the use of commercial yeast in a modern winery since the first vintage influenced the composition of the winery yeast flora as to participate in inoculated (Vat 1) and non-inoculated (Vat 2) fermentations. According to our results, commercial *Saccharomyces* strains have been the most isolated both from the fresh must and during the alcoholic fermentation in the two vats sampled, we have not isolated any indigenous *Saccharomyces* strain from these sources and only a low population of non-*Saccharomyces* has been detected in the fresh must. The absence of indigenous *S. cerevisiae* strains results especially curious in the vat 2 inoculated with a natural *pied de cup* prepared separately of the winery. In this kind of inoculums cellular densities may achieve values of 10^6-10^7 cells/ml, thus it seems quite unlikely that indigenous or wild *S. cerevisiae* strains that occur in such a high concentration are completely suppressed by the winery's resident commercial *S. cerevisiae* strains, unless the natural flora is mainly composed of commercial *S. cerevisiae* strains. Therefore, it is important to bear in mind some considerations about the characteristics of our work. Both the vineyard and the winery were recently established, the winery equipment was also brand new and, thus, a resident *Saccharomyces* microbiota was not available to initiate and lead the fermentation, for this reason, the use of commercial yeasts has been a usual practice since the beginning of the winery activity in the 2003 campaign to ensure the alcoholic fermentation. In such circumstances, after several vintages and fermentations, the equipment, walls and the rest of the winery has become suitable for the development of a microbiota adapted to the winery environment (Martini et al. 1996), and it is likely that this winery resident microbiota is composed of commercial strains that have been selected for their good adaptation to the winemaking process (Boulton et al. 1996). On the other hand, it is of interest to note that during the two first vintages (2003 and 2004 vintages) the lees obtained from the fermentation vats, containing the commercial yeasts used in the winery, were spread along the closest vineyard with fertilizing purposes. Since the vineyards were recently established it seems that not only the winery but also the vineyard became "contaminated" with

commercial yeast strains. Valero et al. (2005) reported how commercial strains were isolated in the vineyard at very close proximity to the winery, especially after the harvest and once the winery activity have started, reflecting immediate dissemination. Thus, further studies about the evolution of these yeasts in the vineyard would be of great interest. Furthermore, our experiment took place when other grape varieties had already been harvested and fermented, thus the winery was already enriched with fermenting commercial *Saccharomyces* strains. It is well known than "cross-contamination" between adjacent vats by means of insects or winery equipment is frequent (Constantí et al. 1997, Ribereau.Gayon et al. 2000), even the air in itself could behave as a means for the dispersal of microorganisms within the winery and for the inoculation of tanks (Garijo et al. 2008), which may explain that commercial strains used during the vintage and contaminating the winery become dominant during fermentation.

All these reasons may explain the sole presence of commercial yeasts in both fermentation vats. However, the isolation of the different commercial yeasts used in the cellar does not occur to the same extent. The presence of starters LSA2, LSA4 and LSA7 was quite remarkable in both vats, while starter LSA3 (used in the 2004 vintage) and starter LSA1 (used in the 2005 vintage) were scarce or null, respectively. Santamaría et al. (2005) reported that as more vintages are studied in a winery, more previously detected strains appear, indicating that different *S. cerevisiae* strains remain in the winery ecosystem. According to these authors, the different conditions of each harvest could determine which specific strains develop during the fermentation.

Finally, a significant dominance of the inoculated yeast (starter LSA7) in Vat 1 could not been observed contrary to results described in other works when monitoring wine fermentations conducted by active dry yeast (Querol et al. 1992a). In our study, at the end of the fermentation, the frequency of isolation of the strain inoculated in Vat 1 was only 52.2 %, higher than in the non inoculated Vat 2 (33.3 %), but lower than that suggested by Delteil et al. (2001) to consider that the implantation is effective (80 %). So, it seems that in Vat 1, the inoculated starter strain LSA7 competed with strains LSA2, LSA3 already present in the fresh must and with LSA4 detected during fermentation, but did not completely suppress their growth (see Figure 2). This result agrees with that of Querol et al. (1992a), demonstrating the competence among *Saccharomyces* strains resident in the must before the inoculation, and the inoculated

one. Depending on the proportion of each yeast strain and their own nature, one or more strains will persist during the fermentation process (López et al. 2003, Schütz and Gafner 1993).

Our results confirm the influence of some winery characteristics, in special, the conjunction of the age of the winery (a newly built one) together with the practice of the use of commercial yeasts, on the yeast ecology of the winery. The use of active dry yeast can not only induce a decrease in the presence of non-*Saccharomyces* yeasts and indigenous *Saccharomyces*, as reported by other authors (Beltrán et al. 2002; Constanti et al. 1997; Henick-Kling et al. 1998; Santamaría et al. 2005), but hindering their development in the winery environment during the first vintages. Therefore, the practice of using wine lees as fertilizer facilitates the dissemination of commercial strains in the vineyard near to the winery, probably explaining their presence in the fresh must and in the fermentation vats when *pied de cup* starters are prepared, as it seems likely to have happened in our study. We also conclude that further studies on the yeast population on the vineyard, winery equipment and *pied de cup* would be of great interest in order to a better understanding of yeasts dynamics during the fermentation process. We consider these results to be taken into account in the case of newly established wineries and of particular relevance for winemakers that intend to implement Organic Farming and that consider avoiding the use of commercial strains and instead rely on indigenous (native) flora.

Acknowledgements

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Figures

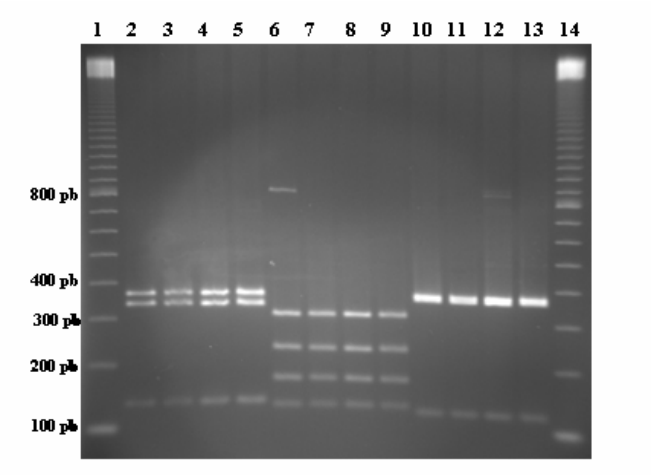

Figure 1 Electrophoretic patterns of rDNA PCR-RFLP of four *Saccharomyces* isolates (12/52, 12/50, 12/51 y 12/32) digested with endonucleases *Hha*I (lanes 2-5), *Hae*III (lanes 6-9) and *Hinf*I (lanes 10-13). Lanes 1 and 14: 100 bp size marker.

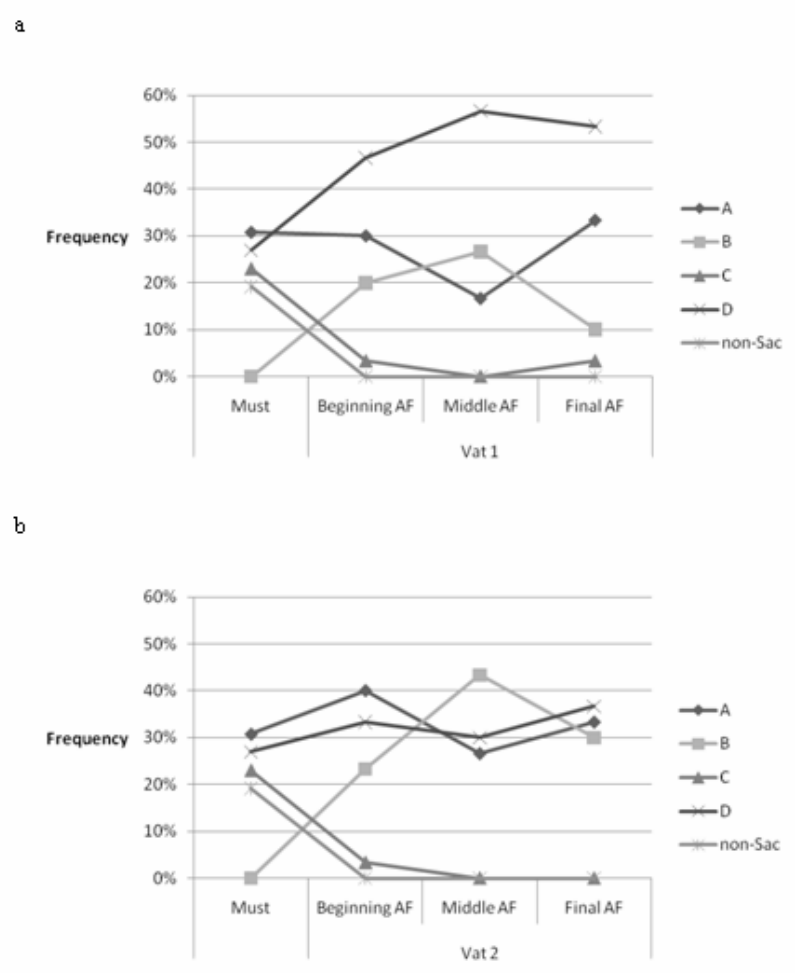

Figure 2 Frequencies of isolation of non-*Saccharomyces* yeasts (non-Sac) and *S. cerevisiae* strains with pattern A, B, C, D of mtDNA in each fermentation vat in must and during the alcoholic fermentation (AF). a) Vat 1 inoculated with commercial yeast LSA7 (pattern D); b) Vat 2 prepared with a "*pied de cup*"

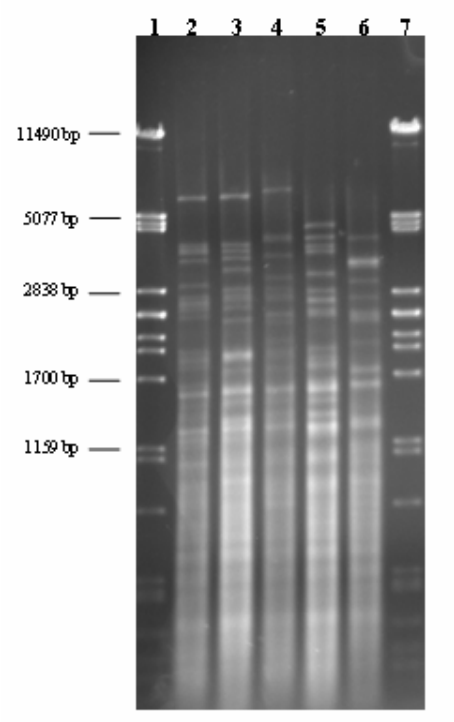

Figure 3 Electrophoretic patterns of mitochondrial DNA of commercial yeast strains used in the winery digested with *Hinf*I. Lanes 1 and 7: phage lambda digested with PstI (ladder); lane 2: pattern A (LSA2); lane 3: pattern B (LSA4); lane 4: pattern C (LSA3); lane 5: pattern D (LSA7); lane 6: pattern E (LSA1).

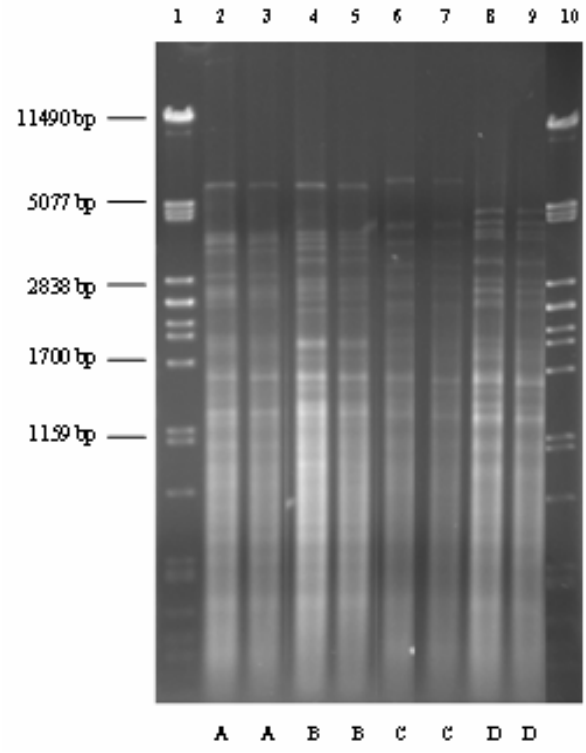

Figure 4 Comparison of patterns of mtDNA digested with *Hinf*I of commercial yeast strains used in the winery with some of the *Saccharomyces* strains isolated during the fermentation. Top: lanes 2, 4, 6 and 8 correspond to commercial starters (LSA2, LSA4, LSA3 and LSA7, respectively), lanes 3, 5, 7 and 9 correspond to the strain isolates (12/52, 12/50, 12/51 and 12/32, respectively) and lanes 1 and 10 correspond to phage lambda digested with PstI (ladder). Bottom: codes assigned to each mtDNA pattern.

Tables

Table 1. Commercial yeasts strains used in the winery during 2003-2005.

Commercial yeast	Assigned code	Vintage		
		2003	2004	2005
BDX, Lallemand	LSA4	X	X	X
Rhône 2056, Lallemand	LSA3	--	X	--
Excellence XR, Lamothe-Abiet	LSA7	--	X	X
Excellence SP, Lamothe-Abiet	LSA2	--	--	X
LA-BJL, Lamothe-Abiet	LSA1	--	--	X

X: used. --: Not used

Table 2. Number and frequencies (%) of *S. cerevisiae* strains presenting patterns A, B, C, D of mtDNA, isolated from each fermentation vat and yeast population in must and each stage of fermentation (BF: beginning; MF: middle; EF: end). Vat 1 inoculated with commercial yeast LSA7 (pattern D); Vat 2 prepared with a "*pied de cup*".

Strain/Pattern	Must	Vat 1			Vat 2		
		BF	MF	EF	BF	MF	EF
LSA2 / A	8 (31%)	9 (30%)	5 (16.7%)	10 (33.3%)	12 (40%)	8 (26.7%)	10 (33.3%)
LSA4 / B	0	6 (20%)	8 (26.7%)	3 (10%)	7 (23.4%)	13 (43.3%)	9 (30%)
LSA3 / C	6 (23%)	1 (3.3%)	0	1 (3.3%)	1 (3.3%)	0	0
LSA7 / D	7 (27%)	14 (46.7)	17 (56.6%)	16 (53.4%)	10 (33.3%)	9 (30%)	11 (36.7%)
***S. cerevisiae* isolates**	21	30	30	30	30	30	30

Capítulo 2

Influence of the winery environment in the yeast populations present in alcoholic fermentations inoculated with a natural "pied de cup" or a commercial yeast strain

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Abstract

The present study has been carried out in an organic winery established in 2003 in the Denomination of Origin "Sierras de Málaga" (Southern Spain) region, during the 2007 vintage. The aim of this work was to ascertain the yeast microflora present in the winery and the vinifications and to obtain by the first time a collection of autochthonous *S. cerevisiae* strains from this area. Yeast population from three vats containing fermenting musts from different grape varieties were analysed. Two of them were inoculated with a natural "pied de cup" while the third was sown with a rehydrated commercial yeast strain. A total of 382 yeasts were isolated and identified, initially by restriction analysis of ribosomal DNA and further by sequencing of this region. Non-*Saccharomyces* yeasts were found in all three musts but they practically disappeared as the fermentations progressed. Analysis of mitochondrial DNA RFLP revealed 13 different restriction patterns of *Saccharomyces cerevisiae* strains, five of them similar to those of commercial strains used in the winery. Commercial strains were found even in vats inoculated with "pied de cups" generated by spontaneous fermentation of a must sample. The analysis of samples recovered from different winery surfaces and equipments demonstrated that non-*Saccharomyces*, and both commercial and autochthonous *Saccharomyces* strains were part of the resident microflora in the winery. Biodiversity of autochthonous *S. cerevisiae* in fermentation vats was low but two of them were able to compete with the commercial ones and they were isolated even at the end of fermentations.

Keywords: *Saccharomyces cerevisiae*, commercial strains, alcoholic fermentation, inoculation, winery surfaces

1. Introduction

Wine is the result of the alcoholic fermentation of must by different yeast strains. *S. cerevisiae* is the main yeast species which carries out the fermentation (Fleet & Heart, 1993). Traditionally, wine has been produced by natural fermentation of grape juice by yeasts that originate from the grapes and winery equipment (Fleet & Heart, 1993; Le Jeune et al., 2006). However, Martini (1993) concluded that few, if any, cells of *S. cerevisiae* resident on the grapes provide an extremely limited contribution to spontaneous must fementation. These results have been confirmed by Ciani et al. (2004) that demonstrated that *S. cerevisiae* strains colonising the winery surfaces are responsible of the natural must fermentation. On the other hand, the use of active dry yeast in fermentation is becoming one of the most common practices in winemaking because it ensures a reproducible product and reduces the lag phase and the risk of wine spoilage (Beltrán et al., 2002; Heard & Fleet, 1985; Santamaría et al., 2005). The inoculation of a large population of *S. cerevisiae* strains could inhibit the growth of other *Saccharomyces* and also non-*Saccharomyces* species, thus limiting the growth of wild yeasts (Santamaría et al., 2005). Commercial yeast strains used in the wineries could become winery-resident strains and take over the spontaneous fermentation (Mercado et al., 2007; Santamaría et al., 2005). However, the use of starter cultures can also produce loss of complexity in the final wine (Mas et al., 2002) displacing or even eliminating from fermentation native yeast strains that confer a distinctive style and quality to the wine.

The "Serranía de Ronda" is a wine-producing area of Malaga (Southern Spain) characterized by recently new established small cellars, where the use of commercial strain in vinification is a common practice by winemakers, even in wineries producing organic wines as that in which this study was carried out. However, one of the recommendations is the use of autochthonous or native yeasts to carry out the fermentations (Hidalgo Togores, 2003). In this context, it is very important to isolate and identify yeast strains present in the media and to study their oenological characteristics in order to evaluate their potential in wine-making (Briones et al. 1995; Pérez-Coello et al., 1999). In a previous work, we isolated and identified the yeast microflora present in fermenting Cabernet Sauvignon musts, both in a vat that was inoculated with commercial yeast and in vat in which fermentation took place spontaneously. In that campaign (2005 vintage) only commercial *Saccharomyces* strains

used in that vintage and in previous ones were isolated, thus suggesting that they had become natural residents of the winery participating even in inoculated and non-inoculated fermentations. In the present study we have sampled fermenting vats during the 2007 campaign in order to follow up the evolution of the yeast microflora after several campaigns and to evaluate the implantation of the commercial strains in the vinification process. In addition, yeast population associated to the winery surfaces has also been investigated in order to asses their role in vinification. Finally, the obtainance of a collection of autochthonous *S. cerevisiae* strains of the "Serranía de Ronda", never accomplished before, will make it possible to evaluate their oenological properties in future studies.

2. Materials and Methods

2.1. The vineyard and the winery

The vineyard and the winery are located in the Serranía de Ronda (Malaga, South of Spain), both belonging to the Denomination of Origin "Sierras de Málaga". The vineyard, established in 2002, has an extension of approximately four ha of different cultivated grapevines (Merlot, Syrah, Cabernet Sauvignon and Garnacha) and is managed under the routine practices of Integrated Production and Organic Farming.

The winery that was built just prior to the 2003 vintage, is adjacent to the vineyard. Its first vinifications took place during the 2003 and 2004 vintages but with grapes harvested in other vineyards from the same area. All the equipment in the winery was totally new and neither the winery, nor the equipment had been previously exposed to must, fruit juices, wines, or any beverage or food before 2003 vintage. For this reason, the use of commercial starter became a usual practice in this winery in order to ensure the alcoholic fermentation. Table 1 shows the commercial strains used by the winery in the 2003 to 2007 vintages.

2.2. Wine fermentations

The conditions of fermentations were established by the management of the winery according to their common practises and commercial interests.

Wines were produced from Merlot, Syrah and Cabernet Sauvignon grapes harvested during the 2007 vintage. Musts were supplemented with 35 mg/liter of

sulphur dioxide. A "pied de cup" was prepared by allowing a sample of Merlot must to ferment spontaneously for approximately 9 days. In the Merlot fermentation, must was inoculated 9 days after cold maceration, with 20 L of the prepared "pied de cup". In the Syrah fermentation, the "pied de cup" was added to the vat only 24 hours after the extraction of the must because a strong spontaneous fermentation was detected. In the latter case, 10 L of fresh Syrah must was added to 10 L of the "pied de cup" prior to inoculation. Cabernet Sauvignon vat was inoculated five days after cold maceration, with the active dry yeast LSA7 (Excelence XR, Lamothe-Abiet, Bordeaux, France) that was rehydrated prior to inoculation according to the instructions of the supplier (25 g/Hl). All fermentation took place in vats containing 700 L of must kept at 25-30ºC.

2.3. Yeast isolation

Fermentation kinetics was monitored by density measurement. Five samples were considered for the isolation and enumeration of yeast: (1) grape juice before SO_2 addition; (2) must before inoculation; (3) inoculum; (4) at the middle (6.5º Baume) and (5) at the end (< 2º Baume) of alcoholic fermentation. However, in the case of Cabernet Sauvignon vat, since it was inoculated with a rehydrated commercial strain, the inoculum was not analysed; the sample corresponding to the middle of the fermentation was not taken due to accidental circumstances.

An additional study of the yeast microflora that was present in the winery surface previous to the 2007 campaign was also carried out. Once the 2006 campaign was over and all the winery equipment had been cleaned with a solution of NaOH, microbial analyses were conducted on winery surfaces and winery equipment. The isolation was performed as follows: sterile cotton plugs were streaked on surfaces of approximately 400 cm^2 each on the winery floor (two samples), on the winery wall (two samples), of the pneumatic press (two samples), of the destemmer (one sample), of the empty fermentation vats (internal and external, four samples), of the storage vat (two samples), of the barrels with wine (four samples) and of winery sink (one sample). Then, the cotton plugs were introduced in sterile tubes filled with 5 ml sterile Ringer solution and upon arrival to the laboratory where they were vigorously shaken for 1 hour at room temperature before plating.

Aliquots (0.1 ml each) of serial dilutions were spread onto plates of YEPD agar (yeast extract 1% w/v, peptone 1 % w/v, glucose 2 % w/v, agar 2 % w/v) and the plates

were incubated at 28 ºC for 72 hours. When possible, 30 colonies of each sample were randomly chosen and submitted to further studies.

2.4. Yeast identification

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeasts in samples from winery surfaces and equipment, every isolate was evaluated according to its ability to grow on L-lysine plates (Barnett et al., 1990). Isolates that were not able to grow using L-lysine as the sole nitrogen source were classified as *Saccharomyces*.

PCR of the ribosomal region encompassing the 5,8S rRNA gene and the two internal transcribed spacers was used to identify species of yeast isolates. rDNA was amplified by PCR in a TC-312 Techne termocycler using primers ITS1 and ITS4, described by White et al. (1990). The species of the isolates were identified by PCR-RFLP (Restriction Fragment Length Polymorphism), digesting the PCR products with the restriction endonucleases *Hha*I (same enzymatic activity as *Cfo*I), *Hae*III and *Hinf*I (Takara BIO INC, Otsu, Shiga, Japan) as previously described by Guillamón et al. (1998). PCR products and restriction fragments were separated on a horizontal 1.4% and 3% agarose gel, respectively, in TAE buffer and compared with standards (100-bp DNA ladder, Amersham Biosciences).

Species were confirmed by sequencing the D1/D2 variable domains of 26S rRNA gene, of at least two isolates of each species. Amplification of the fragments to be sequenced was carried out using the external primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). PCR reactions were performed in a 100 µl volume containing 0.25 µM of each primer, 80 µM of dNTP, 1 x buffer (MBL, Dominion), 1.5 mM $MgCl_2$, 5 U Taq polymerase (MBL Dominion, Cordoba, Spain), and DNA isolated according to Querol et al. (1992) and diluted to 1-50 µg/µl. PCR was performed in a TC-312 cycler (Techne) as follows: a first denaturation step at 95 ºC for 5 min, followed by 36 cycles with a temperature profile of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 1.5 min. An extension period of 5 min at 72 ºC was carried out after 36 cycles. The PCR products were purified using a Wizard® SV Gel and PCR Clean-Up system (Promega, Madrid, Spain) according to the manufacturer's instructions and then sequenced by the company Stab Vida (Portugal). A BLAST analysis

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed for the sequences obtained. Identification was considered to be valid when the identity was at least 98 %.

2.5. *S. cerevisiae* strain differentiation

Mitochondrial DNA (mtDNA) restriction analysis was used to differentiate strains of *S. cerevisiae*. DNA extraction and restriction of mtDNA was performed according to Querol et al. (1992). Electrophoresis was carried out as described before except that restriction products were separated on a 1 % agarose gel and lambda DNA digested with PstI was used as standard. Results of restriction with *Hinf*I (Takara BIO INC., Otsu, Shiga, Japan) were found to be the most informative (González et al., 2007; Querol et al., 1994).

Samples of the active dry yeasts used in the winery since the first vinification (2003 vintage) were also analysed in order to distinguish between indigenous *Saccharomyces* and commercial ones.

3. Results

3.1. Vinifications

A total of 390 colonies (30 colonies from 13 different samples) were isolated from the three tanks sampled. However, since a few isolates (eight) died off before being identified, only 146 colonies from Merlot, 146 from Syrah and 90 from Cabernet Sauvignon vats were analysed.

3.1.1. Yeast identification

Table 2 indicates the number of isolates of each species found during the fermentation of the musts. Analysis of RFLP of PCR 5.8-ITS rRNA region products revealed that 287 of 382 isolates were *S. cerevisiae* strains. The other species identified were *Hanseniaspora guilliermondii* (68 isolates), *H. uvarum* (18 isolates), *Issatchenkia orientalis* (2 isolates), *Pichia anomala* (6 isolates) and *Kluyveromyces thermotolerans* (only 1 isolate). Sequencing of the D1/D2 variable domains of 26S rDNA confirmed these results.

Non-*Saccharomyces* yeasts were isolated only in the grape juice (before SO_2 addition) in all the fermentations and, in the Cabernet Sauvignon vat, also in the must

before inoculation. Afterwards, *Saccharomyces* strains took over the fermentation in all cases (Table 2).

3.1.2. *Saccharomyces* strain differentiation

Mitochondrial DNA restriction analysis of the 287 *Saccharomyces* isolates revealed 13 different patterns (Figure 1). Five of them were coincident with those of the commercial yeast used in the winery, namely LSA2 (A), LSA3 (C), LSA7 (D), LSA8 (F) and LSA1/LSA9 (E) (LSA1 and LSA9 presented the same pattern) (Figure 2). The eight remaining patterns were different from those described of the 45 commercial strains described in Fernández-Espinar et al. (2006) and, thus, they were considered autochthonous or indigenous, although the possibility of them being commercial ones used in nearby wineries can not be discarded although we consider it to be unlikely. Patterns M and N had been also found during the 2006 campaign on the surface of the grapes from the surrounding vineyard, but the other 6 patterns coded V, W, X, Z, AG and AM were isolated by the first time in this zone.

However, as shown in Tables 3 to 5 not all the patterns were found in the three vats. *S. cerevisiae* strains corresponding to profiles A, C, D and F (commercial strains) and AG (autochthonous strain) were isolated in the three vats, although with different frequencies, but other patterns were only found in some of the vats. Merlot vat presented a higher *S. cerevisiae* diversity than Syrah and Cabernet Sauvignon vats. Six restriction profiles corresponding to one commercial (pattern E) and five autochthonous strains (M, N, V, W and Z) were only found in Merlot vat, while pattern X is exclusive from Syrah vat and pattern AM was common to Syrah and Cabernet Sauvignon vats.

Merlot grapes were vinificated in first place. In the grape juice, although non-*Saccharomyces* species were predominant, some *S. cerevisiae* yeasts were also present (4 colonies out of 27) (Table 3). Two of them corresponded to one of the commercial strains (LSA1/9, pattern E), and the other 2 isolates, to wild *S. cerevisiae* strains. After 9 days of cold maceration, in the must before inoculation, non-*Saccharomyces* yeasts had died off and only *S. cerevisiae* strains were found, mainly commercial ones; most of them corresponded to commercial strain LSA8. Autochthonous strains were also found but were different to those previously isolated in the grape juice: pattern AG (4 isolates) and V and W (one isolate each).

The inoculum, consisting in the same must that had fermented spontaneously during 9 days, contains almost 97% of commercial yeasts confirming the displacement of the non-*Saccharomyces*, by the *Saccharomyces* strains. However, the growth of autochthonous strains was not suppressed by commercial strains and in the middle of the fermentation, they constitute the 36.7% of the isolates and by the end of the alcoholic fermentation, commercial and autochthonous strains coexisted with similar frequency, but autochthonous strain with pattern AG was the most isolated.

Commercial strains were present throughout the fermentation but the different number of each fluctuated during the process, being LSA8 and LSA7 the most prevalent at the end of the fermentation.

The case of Syrah yeast population vats was somehow different (Table 4). Although grapes were harvested 9 days after Merlot grapes, vinification of both musts was carried out at the same time because it was inoculated with the "pied de cup" only 24 h after harvest because, according to the oenologist, the must began to ferment quickly. For the same reason, it was not possible to prepare a "pied de cup" with the Syrah must and that prepared for the Merlot vat was also used. During this brief fermentation a common feature with the Merlot must was found namely the disappearance of the non-*Saccharomyces* strains and the appearance of both commercial and autochthonous *Saccharomyces* strains. In this case, at the end of the fermentation the prevalence of commercial strains, mainly LSA7, LSA3 and LSA8, was evident but the autochthonous AMY could also be found.

In Cabernet Sauvignon vat, only non-*Saccharomyces* species were isolated in the fresh must and they were still present in the must after 5 days of cold maceration, although similarly to the other vats, *S. cerevisiae* strains took over the fermentation (Table 5). As expected, inoculation of this vat with LSA7 contributed significantly to the prevalence of this strain at the end of the fermentation. This inoculation implied a threefold increase of the LSA7 (from 7×10^6 to 2.5×10^7 cfu/ml), thus decreasing the proportion of cells corresponding to the other LSA strains.

Figure 3 reflects a whole view of the differences in the proportion of non-*Saccharomyces* and commercial and autochthonous *S. cerevisiae* yeasts throughout the fermentation of the three grape varieties. In Figure 4 we have represented the yeast

population of each type of strains calculated using total population and the proportion of each of them.

3.2. Analysis of winery surfaces and equipment microbiota

Yeast analysis was performed on winery surfaces and equipment in January 2007, upon completion of the 2006 campaign and after the winery equipment had been cleaned and disinfected. Total yeast count ranged from 10^1 to 10^4 cfu mL^{-1}. Of a total of 189 isolates, 93 of them were non-*Saccharomyces* yeasts and the rest (96) corresponded to *Saccharomyces* genus according to their differential growth in L-lysine media. Curiously enough, while non-*Saccharomyces* were found in all surfaces except the barrels, *Saccharomyces* strains were isolated almost exclusively or massively in the barrels, and in low proportion on the winery floor and the pneumatic press.

RFLP-PCR of 5,8S-ITS rRNA and mtDNA restriction analysis of the *Saccharomyces* isolates revealed four different *S. cerevisiae* strains (Table 6), one of which corresponded to LSA7 (pattern D), a commercial strains used in the previous campaigns and was only found in the pneumatic press accounting for the 3% of all *S. cerevisiae* isolates in the winery environment. The other three patterns (R, S and T) did not corresponded to any commercial strain; they had not been isolated before, neither there was found in the following campaign described in this paper.

4. Discussion

This study has been carried out in 2007 in a winery built in 2003 at the "Serranía de Ronda" (Southern Spain). In this wine growing region red wines are usually elaborated by induced fermentation with commercial yeast strains. The winery is surrounded by a vineyard with Merlot, Syrah, Cabernet Sauvignon and Garnacha grape varieties planted also in recent times. The circumstances of this winery are quite similar to other ones also recently established in the same region. In a previous work (submitted manuscript) carried out in the 2005 campaign, the third vinification campaign of this winery, we observed that the use of commercial yeasts since the first vinification campaign seemed to have propitiated that these strains become a component of the winery yeast flora participating in the fermentations. In that campaign, no wild *S. cerevisiae* yeasts were isolated even in fresh grape juice sample.

In the present study, we have isolated and identified the yeast microflora participating in the fermentation of different musts. Vats containing Merlot and Syrah musts were inoculated with a natural "pied de cup", e.g., a must in full fermentation to which no active dry yeasts had been added. A third vat containing Cabernet Sauvignon must was inoculated with rehydrated commercial yeast coded as LSA7.

Some differences have been found among the three vats. Although in all of them non-*Saccharomyces* species prevailed in the fresh musts, in the case of the Merlot and Syrah vats, they disappeared during the following days (9 and 1 day , respectively) during which they were kept in the cold. Moreover, they also disappeared from the "pied de cup", a sample of the Merlot must that had been spontaneously fermenting during 9 days. However, in the Cabernet Sauvignon vat non-*Saccharomyces* species coexisted with *S. cerevisiae* yeasts in a significant amount (43 %) of total isolates before inoculation. Commercial strains of *S. cerevisiae* predominated in Merlot and Cabernet Sauvignon musts respectively, whereas wild strains were mostly frequent in Syrah must. In the "pied de cup" employed for Merlot and Syrah vinifications, commercial strains (mainly LSA7 and LSA8, but also LSA2 and LSA3 strains) represented most of the isolations, and only one isolate in both musts corresponded to a wild strain with restriction pattern AG. In Syrah fermentation, commercial strains LSA3, LSA7 and LSA8 predominate in the fermentation and only the wild strain AMY (which accounted for the 81 % of the isolates in the must before adding the "pied de cup") was present until the end of the fermentation, although in a low proportion. However, in the case of Merlot vat, commercial strains (LSA3, LSA7 and LSA8) carried out the fermentation together with the wild strain AGY that represented the 50 % of the isolates at the end of the fermentation. These results might reflect the robustness of strain AGY since in Merlot must just before adding the "pied de cup" they only represented 14 % of the isolations and the commercial strains were predominant. However, the composition of the must seem to influence in this result since in Syrah must, strain AGY present in the "pied de cup" disappeared before the middle of the fermentation. In Cabernet Sauvignon vat both strains AGY and AMY were found at the end of the fermentation. These results suggest differences in the behaviour of both wild strains in different musts, both being well adapted to the vinification conditions since they are capable of surviving until the end of the fermentation together with commercial strains. However, according to Lopes et al. (2007) differences in the population ranges of the must before

inoculation and of the "pied de cup" could also explain the different behaviour of Merlot and Syrah fermentations. In the first case, the relationship between both populations (almost 10^7 and 10^9 cfu ml^{-1}, respectively) was lower than in Syrah fermentation where difference between the must and the "pied de cup" populations was higher (10^5 and 10^9 cfu ml^{-1}, respectively), and thus autochthonous strains AGY grew together with commercial strains in Merlot vat but the growth of strain AMY was highly inhibited by commercial strains in Syrah vat. It has to be pointed out that Syrah grapes were harvested and processed later than Merlot grapes. According to some authors (Beltrán et al., 2002) when other grape varieties had been previously harvested, there is enrichment in the cellar environment with already fermenting *Saccharomyces* strains. Moreover, Mercado et al. (2007) found that the yeast population on the winery surfaces also increases as the wine making goes on.

However, when analysing the yeast population present in the winery environment in January 2007, once completion of the 2006 campaign, the only commercial *S. cerevisiae* strain found was LSA7 (restriction profile D). LSA2, LSA3 and LSA8 were not isolated from the winery surfaces, but were present, although in low proportion, in the elaborated "pied de cup", in the must before inoculation and during the alcoholic fermentation in the three vats during 2007 campaign. Some authors have found that commercial strains used in the winery may remain in the equipment and participate actively in future spontaneous fermentations (Constantí et al., 1997; Mercado et al., 2007), even if their presence is scarce (Mercado et al., 2007). The presented results only partially agree with the cited literature since only one of them was found among the winery resident microflora and none of the other strains found, namely, wild strains, participated in the 2007 fermentations.

In our case, we do not find a correlation between the microflora isolated from the winery surfaces and equipment and that present in fermenting vats and "pied de cup". The former was composed mainly by non-*Saccharomyces* strains also present in the must probably derived from the grape surface. Among the *Saccharomyces* strains found in the equipment, only the commercial strain LSA7 was also found in different stages of fermentation in all three vats. It has to be pointed that this strain together with other commercial one, LSA4, were repeatedly used in the different vintages but the latter was not found in any of the samples analysed. Thus, other sources of yeast strains have to be postulated. On the other hand, the amount of wine yeasts present on the

winery equipment would depend on the standards of cleanliness of the winery and the nature of the surface. Irregular, unpolished surfaces and difficult to clean may support dense populations of winery yeasts (Pretorius, 2000), which could explain differences in the yeasts growth from different sources of samples from the winery surfaces. Alternatively, a scarce presence in the analysed equipment or grapes cannot be discarded. They would, thus, remain undetected by the sampling method used (Mercado et al., 2007). The conditions of the fermentations would select the most robust strains among others. Commercial strains such as LSA2, LSA3, LSA7 and LSA8 were isolated from fermentations inoculated with a spontaneous fermenting "pied de cup" (Merlot and Syrah vats) and even from Cabernet Sauvignon vat inoculated only with one of them. On the other hand, a low biodiversity of wild or autochthonous *S. cerevisiae* yeasts have been found, especially if we compare our results with those obtained from older wineries even if commercial strains were employed (Lopes et al., 2007; Santamaría et al., 2005; Torija et al., 2001). In our study only eight different restriction patterns corresponding to wild strains have been characterized. Among them, yeasts strains corresponding to pattern AG and AM seem to be competitive with commercial ones even at the end of the fermentation.

The present study constitutes the first one carried out in the recently established wineries in the "Serranía de Ronda" (Malaga). The presented results should be useful to other wineries with similar characteristics (recently established and use of commercial strains) present in the region. The isolation and preservation of natural genetics resources of technological importance is of great interest for further strain development, especially of autochthonous strains with very good performance during fermentation that could be used in a future as starters, thus avoiding the use of commercial strains. This practice could help in developing a more characteristic and singular wine.

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Figures

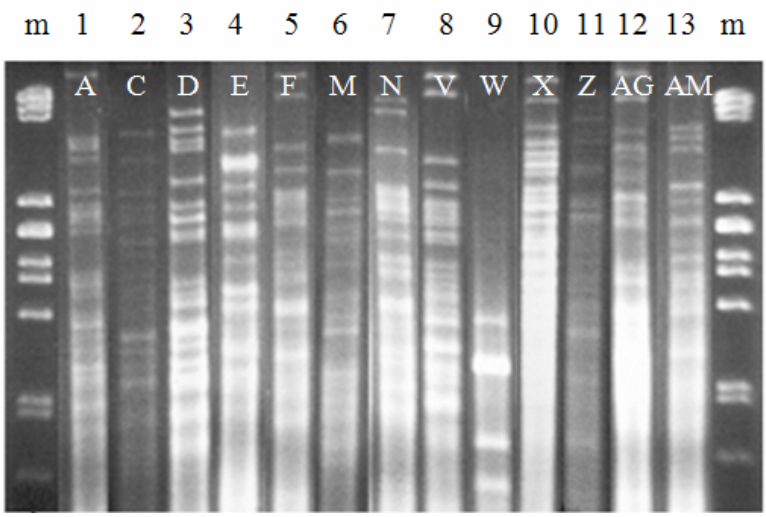

Figure 1. Electrophoretic patterns of mitochondrial DNA cut with *Hinf*I from *S. cerevisiae* isolated in the three vats under study. Lanes: 1, pattern A (LSA2); 2, pattern C (LSA3); 3, pattern D (LSA7); 4, pattern E (LSA1/9); 5, pattern F (LSA8); 6, pattern M; 7, pattern N; 8; pattern V; 9, pattern W; 10, pattern X; 11, pattern Z; 12, pattern AG; 13, pattern AM. *m*: size marker digested with PstI (λ-PstI).

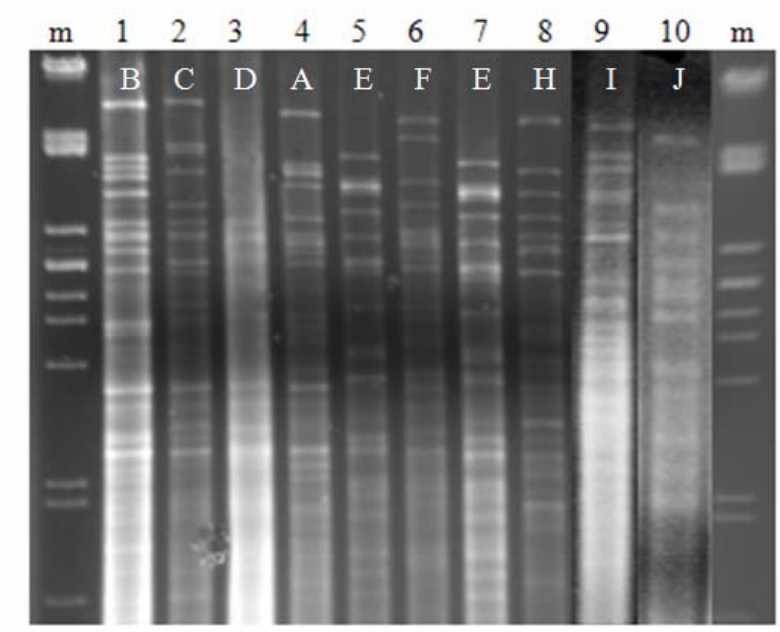

Figure 2. Electrophoretic patterns of mitochondrial DNA cut with *Hinf*I from of the commercial yeasts used in the winery during 2003 to 2007 vintages. Lanes: 1, LSA4; 2, LSA3; 3, LSA7; 4, LSA2; 5, LSA1; 6, LSA8; 7, LSA9; 8; LSA6; 9, LSA10; 10, LSA11 and *m* is the size marker digested with PstI (λ-PstI).

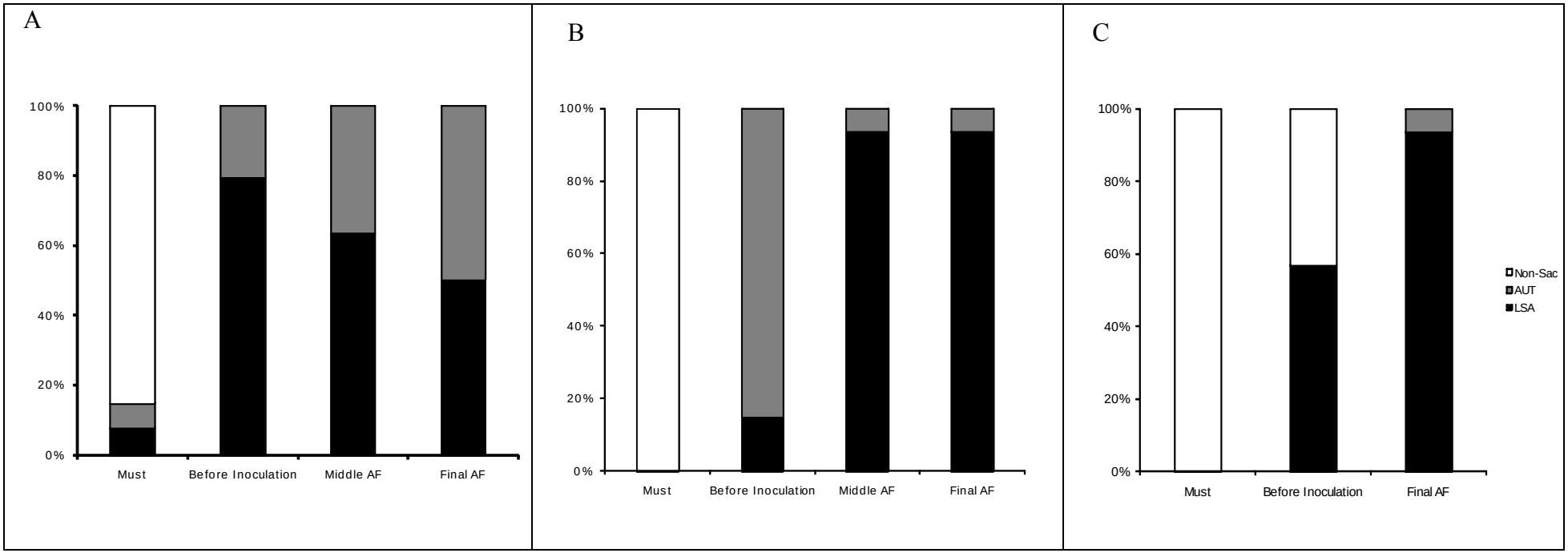

Figure 3. Relative proportion (%) of commercial and autochthonous *Saccharomyces* and non-*Saccharomyces* yeasts in samples from different stages of fermentations of the three grape varieties studied: A) Merlot vat; B) Syrah vat; C) Cabernet Sauvignon vat.

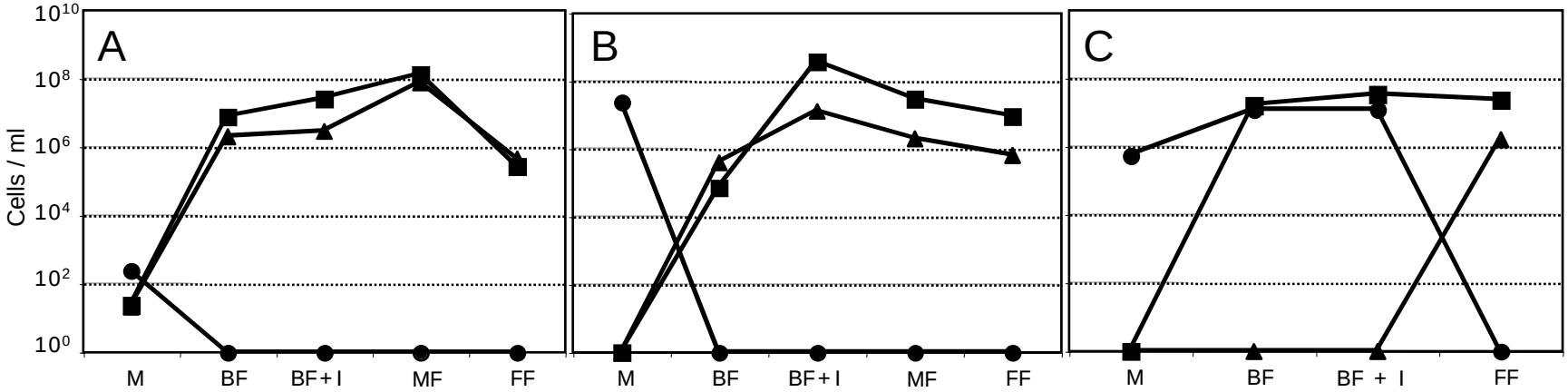

Figure 4. Yeast population (cells/mL) in samples from the three grape varieties studied: (A) Merlot vat; (B) Syrah vat; (C) Cabernet Sauvignon vat in different stages of fermentation: M, must; BF, before fermentation; BF+I, must before fermentation plus inoculum; MF, middle of fermentation; FF, end of fermentation. ▲ Autochthonous (AUT); ■ Commercial (LSA); ● non-*Saccharomyces* (Non-Sac) strains.

Tables

Table 1. Commercial yeasts strains used in the winery during 2003 to 2007 vintages.

Commercial yeast	Code	Vintage				
		2003	2004	2005	2006	2007
BDX, Lallemand	LSA 4	X	X	X	X	X
Rhône 2056, Lallemand	LSA 3	--	X	--	--	--
Excellence XR, Lamothe-Abiet	LSA 7	--	X	X	X	X
Excellence SP, Lamothe-Abiet	LSA 2	--	--	X	--	--
LA-BJL, Lamothe-Abiet	LSA 1	--	--	X	--	X
Fermol Premier Cru	LSA 8	--	--	--	X	--
Fermol Mediterranée	LSA 9	--	--	--	X	--
43, Lallemand	LSA 6	--	--	--	X	--
Chalenge Selection ES U42, Sepsa-Enartis	LSA 10	--	--	--	--	X
Star, Agrovin	LSA 11	--	--	--	--	X

X: used; --: not used

Table 2. Number of isolates of each species found in the samples from the three fermentations studied.

Date	Sampling point	Total Isolates	*S. cerevisiae*	*H. guilliermondii*	*H. uvarum*	*I. orientalis*	*P. anomala*	*K. thermotolerans*
11/09/2007	Grape juice	27	4	4	11	2	6	--
20/09/2007	Must before inoculation	29	29	--	--	--	--	--
20/09/2007	Inoculum (*"pied de cup"*)	30	30	--	--	--	--	--
26/09/2007	Middle of fermentation	30	30	--	--	--	--	--
04/10/2007	Final of fermentation	30	30	--	--	--	--	--
	Total	**146**	**123**	**4**	**11**	**2**	**6**	**0**
19/09/2007	Grape juice	29	--	22	7	--	--	--
20/09/2007	Must before inoculation	27	27	--	--	--	--	--
20/09/2007	Inoculum (*"pied de cup"*)	30	30	--	--	--	--	--
27/09/2007	Middle of fermentation	30	30	--	--	--	--	--
04/10/2007	Final of fermentation	30	30	--	--	--	--	--
	Total	**146**	**117**	**22**	**7**	**0**	**0**	**0**
19/10/2007	Grape juice	30	--	29	--	--	--	1
24/10/2007	Must before inoculation	30	17	13	--	--	--	--
24/10/2007	Inoculum (LSA7)	n/s[a]	--	--	--	--	--	--
	Middle of fermentation	n/s[a]	--	--	--	--	--	--
08/11/2007	Final of fermentation	30	30	--	--	--	--	--
	Total	**90**	**47**	**42**	**0**	**0**	**0**	**1**
	Total	**382**	**287**	**68**	**18**	**2**	**6**	**1**

[a]n/s: not samples

Table 3. Number of yeast strains showing different mtDNA restriction pattern isolated from different samples studied in the Merlot must containing vat.

Merlot			Must	Before Inoculation	Inoculum	Middle F[e]	Final F[f]
Species	Code[a]	Pattern					
S. cerevisiae	LSA2	A	--	--	4	2	--
"	LSA3	C	--	2	6	3	1
"	LSA7	D	--	4	10	7	4
"	LSA1/9	E	2	1	1	--	--
"	LSA8	F	--	16	8	7	10
"		M	1	--	--	--	--
"		AG	--	4	1	10	15
"		N	--	--	--	1	--
"		V	--	1	--	--	--
"		W	--	1	--	--	--
"		Z	1	--	--	--	--
H. guilliermondii			4	--	--	--	--
H. uvarum			11	--	--	--	--
I. orientalis			2	--	--	--	--
P. anomala			6	--	--	--	--
Total isolates			27	29	30	30	30
Population			2.9×10^2	9.7×10^6	8.9×10^8	2.1×10^8	7.3×10^5
Total LSA[b]			2	23	29	19	15
Total AUT[c]			2	6	1	11	15
Total non-Sac[d]			23	0	0	0	0

[a] Commercial yeast code; [b]LSA: Commercial yeasts; [c]AUT: Autochthonous yeasts; [d] non-Sac: Non-*Saccharomyces*; Middle F: [e] Middle of fermentation; [f]Final F: End of fermentation

Table 4. Number of yeast strains showing different mtDNA restriction pattern isolated from different samples studied in the Syrah must containing vat.

Syrah			Must	Before Inoculation	Inoculum	Middle F [e]	Final F [f]
Species	Code[a]	Pattern					
S. cerevisiae	LSA2	A	--	--	2	2	1
"	LSA3	C	--	1	10	10	6
"	LSA7	D	--	--	13	5	15
"	LSA8	F	--	3	4	11	6
"		AG	--	--	1	--	--
"		AM	--	22	--	2	2
"		X	--	1	--	--	--
H. guilliermondii			22	--	--	--	--
H. uvarum			7	--	--	--	--
Total			29	27	30	30	30
Population			2.4×10^7	4.6×10^5	2.0×10^9	3.1×10^7	9.6×10^6
Total LSA[b]			0	4	29	28	28
Total AUT [c]			0	23	1	2	2
Total non-Sac[d]			29	0	0	0	0

[a] Commercial yeast code; [b]LSA: Commercial yeasts; [c]AUT: Autochthonous yeasts; [d] non-Sac: Non-*Saccharomyces*; Middle F: [e] Middle of fermentation; [f]Final F: End of fermentation

Table 5. Number of yeast strains showing different mtDNA restriction pattern isolated from different samples studied in the Cabernet Sauvignon must containing vat.

Cabernet Sauvignon			Must	Before Inoculation	Final F[f]
Species	Code[a]	Pattern			
S. cerevisiae	LSA2	A	--	3	3
"	LSA3	C	--	1	2
"	LSA7	D	--	8	19
"	LSA8	F	--	5	4
"		AG	--	--	1
"		AM	--	--	1
H. uvarum			29	13	--
K. thermotolerans			1	--	--
Total			30	30	30
Population			5.4×10^5	2.8×10^7	2.4×10^7
Total LSA[b]			0	17	28
Total AUT[c]			0	0	2
Total non-Sac[d]			30	13	0

[a] Commercial yeast code; [b]LSA: Commercial yeasts; [c]AUT: Autochthonous yeasts; [d] non-Sac: Non-*Saccharomyces*; [f]Final F: End of fermentation

Table 6. Number of yeast colonies showing different mtDNA restriction pattern isolated from the winery surfaces and equipment.

Yeasts/Patterns	Surfaces		Equipment					
	WF[a]	WW[b]	PP[c]	D[d]	FV[e]	SV[f]	B[g]	WS[h]
S. cerevisiae	3	0	3	0	0	0	90	0
Pattern D[i]	--	--	3	--	--	--	--	--
Pattern R	--	--	--	--	--	--	29	--
Pattern S	--	--	--	--	--	--	18	--
Pattern T	3	--	--	--	--	--	43	--
Non-*Saccharomyces*	15	1	5	1	11	30	0	30
Total isolates	18	1	8	1	11	30	90	30

[a] WF: winery floor; [b] WW: winery wall; [c] PP: pneumatic press; [d] D destemmer; [e] FV: fermentation vats; [f] SV: storage vat; [g] B: barrels; [h] WS: winery sink; [i] corresponds to strain LSA7.

Capítulo 3

Diversity of *Saccharomyces* and non-*Saccharomyces* yeasts in three red grape varieties cultured in the *Serranía de Ronda* (Spain) vine-growing region

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Abstract

For the first time, an ecological survey of wine yeasts present in grapes growing in two vineyards located in the region of "Serranía de Ronda" (Malaga, southern Spain) has been carried out. During the 2006 and 2007 vintages, grapes from different varieties were aseptically collected and allowed to ferment spontaneously in the laboratory. From a total of 1586 colonies isolated from microvinifications, 1281 were identified according to ITS polymorphisms and their identity confirmed by sequencing of the D1/D2 region of 26S rDNA. Most of the isolates (84 %) corresponded to thirteen different non-*Saccharomyces* species with *Kluyveromyces thermotolerans*, *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum* and *Issatchenkia orientalis* accounting for 42.7 % of the total. Mitochondrial DNA restriction analysis from the *Saccharomyces cerevisiae* isolates revealed a low diversity since only eleven different profiles were found, nine of them corresponding to local strains and two to commercial ones that had been used in different campaigns and that very likely were disseminated from the winery to the adjacent vineyard. A different distribution of strains was found in the three grape varieties studied.

Keywords: biodiversity, vineyard, *Saccharomyces*, non-*Saccharomyces*, dissemination

1. Introduction

The transformation of grape juice into wine by natural alcoholic fermentation is the result of the combined action of several yeasts species that grow more or less sequentially throughout the fermentation process. Fermentations are initiated by various species of *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulospora* and *Zygosaccharomyces* in principle present on the grapes surface. Their growth is generally limited to the first two or three days of fermentation, after which they die off. Subsequently, the most strongly fermenting and more ethanol tolerant species of *Saccharomyces* take over the fermentation (Fleet & Heard, 1993). Different theories explain the early growth arrest of non-*Saccharomyces* strains: their low tolerance to ethanol or to other toxic compounds (Fleet, 2003, Pérez-Nevado et al., 2006) but also a cell-cell contact mechanism dependent on the presence of viable *S. cerevisiae* cells at high concentrations (Nissen et al., 2003).

Nowadays, fermentations inoculated with selected commercially available *S. cerevisiae* strains are prevalent in large-scale wine production due to the ease of control and homogeneity of fermentations. However, the use of local (autochthonous) strains of *S. cerevisiae* is preferable since they are better acclimatised to the environmental conditions (Degré, 1993, Martini & Vaughan-Martini, 1990, Regodón et al., 1997) thus assuring the maintenance of the typical sensory properties of the wines of a given region (Hernández et al., 2003).

In this context, the present work was performed to ascertain the biodiversity of *Saccharomyces cerevisiae* flora found in vineyards from the viticultural region "Serranía de Ronda" (Malaga, southern Spain) in order to define strategies for future wine yeasts selection programs. Besides, the establishment of a yeast strain collection will contribute to the preservation of genetic resources from a viticultural region never explored before.

In a previous study (submitted manuscript) yeasts from fermenting musts from a winery in the "Serranía de Ronda" (2005 vintage) were isolated and characterized. In that case, only commercial *S. cerevisiae* strains used in the winery were found in the fermenting vats. In this region, where most of the vineyards and wineries are recently established, the use of commercial selected yeasts in order to ensure the fermentation is

a usual practice. For the present study carried out during the 2006 and 2007 campaigns, grapes were aseptically collected from the vineyards and fermented in the laboratory to avoid contamination with the winery microbiota. Species diversity was evaluated by PCR of 5,8S-ITS rDNA region RFLP combined with the sequencing of D1/D2 region of 26S rDNA, and *S. cerevisia*e strains were characterized by mtDNA RFLP.

2. Materials and Methods

2.1. Sampling plan

During the 2006 harvest campaign, the sampling plan included a total of 6 sites in two plots of a vineyard located in the South of Spain ("Serranía de Ronda", Malaga) (summarized in Table 1). The grapevine varieties sampled were Syrah (plot BS) and Merlot (plot BM). In each plot, three sampling points were defined (BSI, BSII, BSIII and BMI, BMII, BMIII, respectively). The distance between the winery and the sampling sites varied between 20 and 200 m. Five sampling campaigns were performed in a time frame of about 5 weeks, in order to assess the diversity among fermentative yeast communities during the last stage of grape maturation, harvest and post-harvest stages. Samples were always collected from the same vine plant or, if necessary, from, plants at a distance of 1-2 m. Sampled vine plants were marked and not collected during regular harvest.

Additionally, during the 2006 harvest campaign another vineyard located far from the winery was also sampled (plot PS). In this case, the dissemination phenomenon of yeas strains from the winery to the vineyard was supposed to be lower. Three sampling sites were also defined (PSI, PSII, PSIII), all of them corresponding to the Syrah grapevine variety. Only two sampling campaigns were carried out: an early sampling stage before harvest, and a harvest sampling stage.

During the 2007 harvest, six points were sampled in the first vineyard. Four sampling sites corresponded to four of the sites of BS and BM plots sampled during the 2006 campaign (BSI, BSII, BMI, BMII), and two new points were sampled from Cabernet sauvignon grapevines (plot BCS, sampling points BCSI and BCSII). During this year, two sampling campaigns were carried out, in the harvest and post-harvest stages, with a gap of nine to ten days in between (Table 1).

2.2. Sample collection, microvinifications and yeast isolation

From each sampling point, approximately 1.5 to 2 kg of grapes were collected aseptically and directly placed into sterile plastic bags that were transported to the laboratory. Grapes were carefully crushed in the plastic bags, and 80 ml of juice was poured into 100 ml erlenmeyer flasks. Duplicates of microvinifications were carried out for all samples except for samples 15BH and 20AH from the 2006 campaign. The fermentors were placed at 25 ºC. Daily weight determination allowed the monitoring of the fermentation progress. When a consumption of about 2/3 of the sugar content was observed, diluted must samples were spread on YEPD plates (yeast extract 1% w/v, peptone 1% w/v, glucose 2% w/v, agar 2% w/v) and incubated for 48-72 hours at 28 ºC. Thirty colonies were selected at random from each spontaneous fermentation.

2.3. Yeast identification

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeasts, every isolate was evaluated according to its ability to grow on L-lysine plates (Barnett et al., 1990). Isolates that were not able to grow using L-lysine as the sole nitrogen source were classified as *Saccharomyces*.

The species of the yeast isolates was determined according to their rDNA polymorphism of the 5,8S-ITS gene region. This region was amplified by PCR using primers ITS1 and ITS4, described by White et al. (1990). The PCR products were digested with restriction endonucleases *Hinf*I, *Hae*III and *Hha*I (same activity than *Cfo*I) (Takara, Japan). PCR amplification products and restriction fragments were separated on a horizontal 1.4 and 3 % agarose gel, respectively, in TAE buffer and their size determined comparing with a standard (100-bp DNA ladder, Amersham Biosciences). An additional restriction endonuclease (*Dde*I) was used to differentiate *Hanseniaspora* species (Esteve-Zarzoso et al., 1999).

Strains of the *Lachancea* clade (*Zygosaccharomyces fermentati*, *Zygosaccharomyces cidri*, *Kluyveromyces thermotolerans* and *Kluyveromyces waltii*) with similar restriction profiles and yeast isolates with misidentified restriction profiles were identified by sequencing the D1/D2 variable domains of the large subunit rRNA gene. They were amplified using the external primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). PCR reactions were performed in a 100 µl volume

containing 0.25 μM of each primer, 80 μM of dNTP, 1 x buffer (MBL, Dominion), 1.5 mM $MgCl_2$, 5 U Taq polymerase (MBL, Dominion) and DNA isolated according to Querol *et al*. (1992) and diluted to 1-50 μg/μl. PCR was performed in a TC-312 cycler (Techne) as follows: a first denaturation step at 95 ºC for 5 min, followed by 36 cycles with a temperature profile of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 1.5 min. An extension period of 5 min at 72 ºC was carried out at the end of the 36 cycles. The PCR products were purified using a Wizard® SV Gel and PCR Clean-Up system (Promega) according to the manufacturer's instructions and then sequenced directly (StabVida, Portugal). A BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed for the sequences obtained. We considered identification to be valid when the identity was at least 98 %.

2.4. Characterization of *S. cerevisiae* isolates

The colonies identified as *S. cerevisiae* were also characterized at strain level by mtDNA restriction analysis as described by Querol et al (1992). Yeast DNA was digested with *Hinf*I, the fragments were separated in 1 % agarose gels and their size determined comparing with a standard (Lambda DNA digested with PstI).

2.5. Commercial yeast

Samples of the active dry yeasts used in the winery since the first vinification (2003 vintage) were also characterized in order to distinguish between indigenous *Saccharomyces* and commercial ones. Table 2 shows the commercial yeasts used in the winery during the 2003 to 2007 vintages.

3. Results

3.1. Yeast isolation and identification from microvinifications

Microfermentations of a total of 75 samples were attempted (54 and 21 during 2006 and 2007 harvest seasons, respectively), but only 58 of them were able to start fermentation, after more as 30 days of incubation. None of the samples of date 15BH (2 weeks before harvest) in the 2006 vintage fermented and few of the samples of date 7BH (one week before harvest) did. In general terms, fermentation took place for most of the samples only at harvest and post-harvest dates. For this reason, sampling dates 15BH and 7BH were discarded for the 2007 vintage campaign.

Yeast population ranged from 3 x 10^4 to 2.2 x 10^7 cfu/mL and, in general terms, it increased with the sampling date and campaign, with higher values from harvest sampling onwards, and in the 2007 campaign. In order to ascertain the natural yeast flora present in the vineyard, 1586 isolates from microvinifications carried out during the 2006 and 2007 vintages were selected for analysis, 1334 of them corresponded to non-*Saccharomyces* yeasts and the rest (252) to *Saccharomyce*s species as determined by their ability to grow on plates containing L-lysine as the sole nitrogen source (Barnett et al., 1990). However, 305 isolates of non-*Saccharomyces* yeasts (mainly belonging to sampling dates 7B and H from the 2006 vintage) died off before further analysis. The rest of the isolates were identified according to the ITS polymorphisms and the results confirmed by sequencing of the D1/D2 region of 26S rDNA of each PCR-RFLP pattern obtained and this technique was also applied to those yeast isolates with misidentified restriction profiles (Table 3). The PCR products sizes ranging from 400 to 850 bp were digested with *Hha*I, *Hae*III and *Hinf*I, whereby 12 different profiles were obtained. Nine of the 12 patterns were identified after comparing the molecular mass of the restriction products with those previously described (Esteve-Zarzoso et al., 1999, 2001, Granchi et al., 1999). These 9 groups were *Candida stellata*, *Candida parapsilosis, Issatchenkia orientalis, Metschnikowia pulcherrima*, *Pichia anomala, Pichia guilliermondii*, *Torulospora delbrueckii*, *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. An additional restriction analysis with endonuclease *Dde*I was necessary to differentiate between *H. guilliermondii, H. uvarum* and *H. vineae* (Esteve Zarzoso et al., 2001). Another group of similar restriction profiles (profile n. 11) included the species *Kluyveromyces thermotolerans*, *K. waltii*, *Zygosaccharomyces cidri* and *Z. fermentati*. Their 26S rRNA gene sequence results yielded 100 % similarity to *Kluyveromyces thermotolerans*. The last profile (n.12) was also identified by this technique as *Aureobasidium pullulans*.

As stated in Table 3, *S. cerevisiae, K. thermotolerans*, *H. uvarum* and *H. guilliermondii* were the yeast species most frequently isolated. Although non-*Saccharomyces* yeasts were mostly isolated, it is remarkable that the percentage of *S. cerevisiae* isolates (19.7 and 9.8 % in 2006 and 2007 vintages, respectively is higher than reported elsewhere (Fleet & Heard, 1993, Mortimer & Polsinelli, 1999). In most of the fermentations (45 out of 58) isolates corresponded only to non-*Saccharomyces* species. In 6 of the fermentations, only *S. cerevisiae* strains were isolated and both *S.*

cerevisiae and non-*Saccharomyces* were isolated in the rest of them (7 fermentations). Spontaneous fermentations were performed by one or more predominating species accompanied by a very heterogeneous yeast community with no prevalent species.

3.2. Yeast population diversity in spontaneous fermentation

Table 4 indicates the distribution of yeasts during spontaneous must fermentation according to sampling dates, grape variety and vintage. Although data of the 2006 vintage are incomplete, results seem to indicate that greater yeast diversity existed in sample 20AH (20 days after harvest) for Merlot and Syrah fermentations from BM and BS plots. Before harvest (sampling date 7BH), *Pichia anomala* was the predominant yeast in Merlot and Syrah musts (49.2 and 100 %, respectively) but it was hardly isolated after this date. In harvest and post-harvest samples (H, 10AH and 20AH sampling dates), *C. parapsilosis*, *H. guilliermondii, I. orientalis* and *S. cerevisiae* were frequently isolated but with some differences among Merlot and Syrah fermentations. .Whereas in Merlot samples *S. cerevisiae* yeasts were only found 10 days after harvest (date 10AH) and accounted for almost 100 % of the isolates, in Syrah samples they were isolated from harvest onwards (dates H, 10AH, 20AH), although in much lower frequencies (Table 4). During the 2006 campaign, another Syrah plot was sampled (PS), in this case there was no winery in the proximity of the vineyard. Only sampling date H experiments fermented. Again, *I. orientalis* and *H. guilliermondii* were mostly identified (20.0 and 18.9 %, respectively), together with *S. cerevisiae* (12.2 %),but in this case *Zygosaccharomyces bailii*, not isolated in BS or in BM plots, was also found (15.6 %).

When focusing in the 2007 results, we observed some differences in the species found in both the 2006 and 2007 campaigns (Table 3). *K. thermotolerans* and *H. uvarum* were the major species isolated in the 2007 (33.6 and 25.5 %, respectively), but they were hardly found in the 2006 campaign, at least considering the limitations of our work for that vintage. *H. guilliemondii* and *S. cerevisiae* yeast strains were also isolated, but in lower percentages than in 2006 (14.4 and 9.8 %, respectively). As in the 2006 harvest, differences were observed among the grape varieties studied, with Cabernet Sauvignon fermentations showing greater yeasts diversity than Merlot and Syrah assays (Table 4). Finally, *S. cerevisiae* strains were only isolated from after harvest samples (10AH) in Cabernet Sauvignon fermentations.

3.3. Characterization of *S. cerevisiae* strains

From the 1598 yeasts isolated, only 252 of them corresponded to *S. cerevisiae* (192 and 60 isolates during the 2006 and 2007 campaigns, respectively). They were characterized at strain level by RFLP of the mtDNA. Eleven different restriction profiles were identified (Figure 1), two of which corresponded to two of the commercial yeasts employed by the winery (Figure 2), namely profile C (LSA3) and D (LSA7).

Differences in the restriction profiles distribution were found according to grape varieties, sampling dates and sampling campaigns as reflected in Table 5. During the 2006 campaign, six restriction profiles were detected, four of them corresponded to local strains (patterns M, N, O and Q) and the rest (patterns C and D) to two commercial ones. In the Merlot fermentations, *S. cerevisiae* species were only isolated in post-harvest assays (sampling date 10AH) and autochthonous strains (restriction profiles N and O) and commercial strains (profiles C and D) were isolated with similar frequency. In Syrah assays, different *S. cerevisiae* strains were characterized depending on the sampling date. Autochthonous strains all from the same profile (M) were isolated exclusively at harvest date (H) in both BS and PS vineyards. However, only commercial strains were isolated in post-harvest sampling dates (profiles D and C in dates 10AH and 20AH, respectively) in BS plots. Thus, a succession of *S. cerevisiae* strains was observed during this vintage.

Autochthonous *S. cerevisiae* strains isolated in 2006 were different from those isolated in the 2007 campaign, with a higher diversity in the latter (Table 5). Five new different restriction profiles were characterized, but most of the isolates (85 %) corresponded to the pattern coded as AF. *S. cerevisiae* strains were only isolated from Cabernet Sauvignon fermentations in date 10AH. No commercial strains were found in this campaign.

S. cerevisiae strains were found in 13 microfermentations (Table 5). Interestingly, yeast with profile M were found only in Syrah grapes in both vineyards but in that located close to the winery (BS), it was substituted by the commercial strains LSA3 and LSA7 in samples taken after harvest (10AH and 20 AH). The cited commercial strains were found also in grapes from two sites of the BM vineyard, also located near the winery, but not in the BCS one. In this latter location, autochthonous strains with profile AF were predominant but other local strains were also found.

4. Discussion

One of the goals of this work was the isolation for yeast selection purposes of indigenous *S. cerevisiae* in the "Serranía de Ronda", a viticultural region where no ecological study on wine yeasts had been performed before. This task was not possible to fulfil in the 2005 vintage since only commercial strains were isolated from fermenting vats (publication submitted). Thus, microfermentations from grapes aseptically taken from the vineyard were carried out in the laboratory during the 2006 and 2007 campaigns. It is well known that the presence of *S. cerevisiae* in grapes is rather rare, according to Mortimer and Polsinelli (1999) only about one in a thousand berries is *S. cerevisiae* positive. However, in our case, during the 2006 and 2007 vintages, 15.6 % of the isolates corresponded to this species. As described by Valero et al. (2007) fermentations could perform as an enrichment media enhancing *S. cerevisiae* growth. Only six and five different profiles were characterized in each vintage, indicating a low biodiversity in contrast to the results found by other authors (Schuller et al., 2005, Torija et al., 2001, Valero et al., 2007). Moreover, two of the profiles matched commercial strains. As described by Martini et al. (1996), the youth of the vineyard (in this case, established between 2002 and 2003) influences the quantity and biodiversity of yeasts.

Several authors have reported studies about the biodiversity of yeasts associated with the vineyard in order to evaluate the dynamics of autochthonous populations of *Saccharomyces* and the impact of the use of commercial yeasts in wine regions of France and Portugal (Schuller et al., 2005, Valero et al., 2005, Valero et al., 2007). In our case, the presence of commercial strains has been detected in vineyards adjacent to the winery. This result indicates that dissemination of commercial yeasts from the winery to the nearby vineyard could be frequent, probably facilitated by vectors such as insects and wasps, but also by other common operations in the cellar (Mortimer & Polsinelli, 1999, Ribereau-Gayon et al., 2003). Valero et al. (2005) also indicate that the topography of the vineyard, for instance the fact that it is sloped could constitute a factor that facilitates the dissemination because of water run-off. This is the case of vineyards BS, BM and BCS. In our study, commercial yeasts were isolated at places located at distances less than to 200 m from the cellar, which, according to Valero et al. (2005), is, in general, the maximum radius of dissemination of yeasts. The practice of throwing out the fermentation lees from the winery to the neighbouring vineyard during

2003 to 2005 vintages could have facilitated the dissemination of commercial yeasts in the adjacent vineyards.

Different commercial strains have been used by the winery since 2003 (Table 2), but only Rhône 2056 (LSA3) and Excellence XR (LSA7) have been isolated in the present study. The latter has been used in the winery since 2004 in all vintages, while Rhône 2056 was only used in 2004. In contrast, the other widely used commercial strain (BDX, coded as LSA4) has not been isolated. Thus, there is no strict correlation between the utilisation level and the frequency of dissemination (Valero et al., 2005) and a permanent implantation of commercial strains in the vineyard did not occur, but instead these strains are subject to natural fluctuations of periodical appearance/disappearance like autochthonous strains do (Valero et al., 2005).

On the other hand, it should be borne in mind that some aspects of our methodology involving fermentations, plating on nutrient plates and isolation of a restricted number of colonies may limit our view about the microflora diversity. Killer phenotype may also play an important role in fermentation aptitude and competitive traits making possible that only certain strains participate or dominate in fermentation although their population was scarce at the beginning (Mercado et al., 2007, Van Vuuren & Jacobs, 1992). It is to be noted that the commercial strains Excellence XR (LSA7) and Rhône 2056 (LSA3) presented a killer-resistant phenotype, while strain BDX (LSA4) was sensitive to the killer toxin (data not shown).

Finally, 13 different non-*Saccharomyces* species have been identified. *H. guilliermondii*, *I. orientalis* and, to a lesser extent, *P. anomala* were the most frequent species isolated in the 2006 vintage, and *K. thermotolerans*, *H. uvarum* and *H. guilliermondii* in the 2007 campaign. According to the literature, the yeasts of *Kloeckera* and *Hanseniaspora* genera are the predominant species on the surface of the grapes, accounting for 50-75 % of the total yeast population (Fleet & Heard, 1993) and, to a lesser extent, it is also possible to detect the species of *Candida*, *Cryptococcus*, *Rhodotorula*, *Pichia*, *Metschnikowia* and *Kluyveromyces* (De la Torre et al., 1999, Fleet, 1993, Longo et al., 1991, Martini et al., 1996).

Although non-*Saccharomyces* wine yeasts have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage, the role of non-*Saccharomyces* yeasts in winemaking has been re-evaluated (Ciani et al., 2006,

Viana et al., 2008, Zott et al., 2008). Several studies have shown their capacities to contribute positively to wine flavour (Ciani and Maccarelli, 1998, Esteve-Zarzoso et al., 1998, Fleet, 2003, Romano et al., 2003), or considered the biotechnical interest of their enzymatic activities (Arévalo Villena et al., 2007, Fernández et al., 2000, Manzanares et al., 2000, Strauss et al., 2005, Úbeda Iranzo et al., 1998). For this reason, some authors have evaluated the possibility of using mixed starters to improve the quality of wines (Ciani et al., 2006, Clemente-Jiménez et al., 2005, Garde-Cerdán and Ancín-Azpilicueta, 2006, Moreira et al., 2008, Rojas et al., 2003, Viana et al., 2008, 2009). One of the species more frequently isolated in our study, *I. orientalis,* has been also isolated in Spanish fermentations by Clemente-Jimenez et al. (2004), who also experimented their capability to ferment Macabeo musts obtaining a very good profile of higher alcohols even if their did not consum all the initial glucose. This species has been proven to reduce the malic acid content in wines when co-fermenting with *S. cerevisiae* obtaining a higher score in sensory evaluations when compared to wines fermented with *S. cerevisiae* alone (Kim et al., 2008). On the other hand, Kapsopoulos et al (2007) used *K. thermotolerans* and *S. cerevisiae* in mixed culture fermentations of grape must and found that it provided an effective biological acidification during alcoholic fermentation by the production of L-lactic acid. The fermentation behaviour of *K. thermotolerans* has also been studied by Ciani et al (2006). Moreira et al. (2008) found that the growth of *H. guilliermondii* and *H. uvarum* during the first days of fermentation enhanced the production of desirable compounds, such as esters, and may not have a negative influence on the production of higher alcohols and heavy sulphur compounds. Studies on acetate ester formation in wine by mixed cultures of *S. cerevisiae* and *P anomala* and *H. guilliermondii* in laboratory fermentations have been carried out by Rojas et al. (2003), Viana et al. (2008) also accomplished a rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and oenological traits that involved different strains of the genera *Candida, Pichia*, *Hanseniaspora*, *Torulospora* and *Zygosaccharomyces*.

Accordingly, the current trend in the wine industry is to develop new technologies and promote the use of mixed and sequential cultures of non-*Saccharomyces* together with *S. cerevisiae* strains in order to increase the aromatic properties of wine while obtaining a desirable ethanol production. In this work, we have isolated and identified a

number of cultivable yeasts and also built a collection of yeast present in grapes which could participate in winemaking in the studied area.

Acknowledgements

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Figures

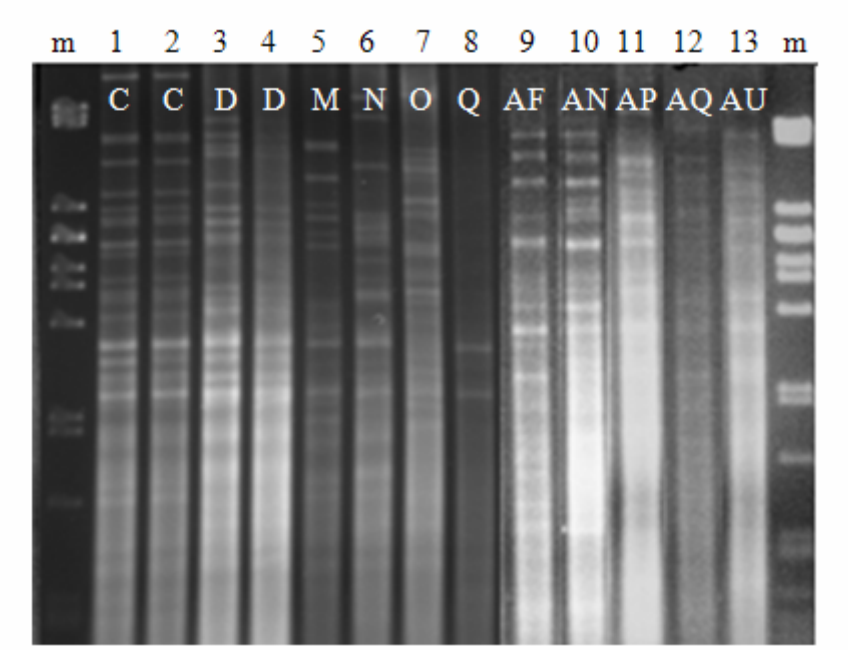

Figure 1. Mitochondrial DNA restriction patterns with *Hinf*I of *S. cerevisiae* isolates found during the 2006 and 2007 vintages. Lanes 1 and 2 correspond to commercial strain LSA3 and lanes 3 and 4, to commercial strain LSA7;*m* is the size marker (λ-PstI).

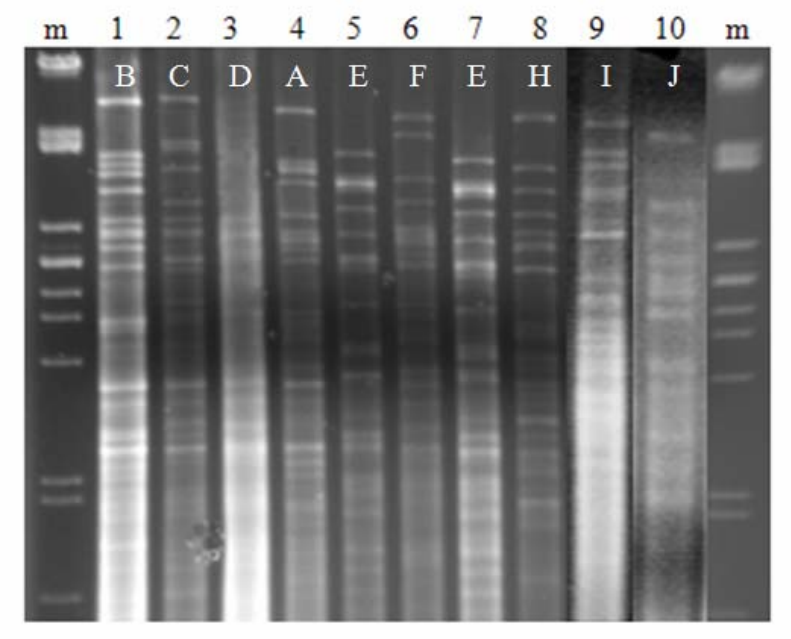

Figure 2. Mitochondrial DNA restriction patterns with *Hinf*I of the commercial yeasts used in the winery during 2003 to 2007 vintages. Lanes: 1, LSA4; 2, LSA3; 3, LSA7; 4, LSA2; 5, LSA1; 6, LSA8; 7, LSA9; 8; LSA6; 9, LSA10; 10, LSA11; *m*: size marker (λ-PstI).

Tables

Table 1. Vineyards, grapevine variety, sampling plots and sampling dates during the 2006 and 2007 campaigns.

Vineyard	Grapevine variety	Plot code	Sampling point	Sampling campaigns				
				15BH	7BH	H	10AH	20AH
2006 campaign								
B	Syrah		BSI	X	X	X	X	X
		BS	BSII	X	X	X	X	X
			BSIII	X	X	X	X	X
	Merlot		BMI	X	X	X	X	X
		BM	BMII	X	X	X	X	X
			BMIII	X	X	X	X	X
P	Syrah		PSI	X	--	X	--	--
		BP	PSII	X	--	X	--	--
			PSIII	X	--	X	--	--
2007 campaign								
B	Syrah	BS	BSI	--	--	X	X	--
		BS	BSII	--	--	X	X	--
B	Merlot	BM	BMI	--	--	X	X	--
		BM	BMII	--	--	X	X	--
B	Cabernet Sauvignon	BCS	BCSI	--	--	X	X	--
		BCS	BCSII	--	--	X	X	--

Vineyards: B (close to the winery); P (far from winery). Sampling campaigns: 15BH (15 days before harvest campaign); 7BH (7 days before harvest campaign); H (harvest campaign); 10AH (10 days after harvest campaign) and 20AH (20 days after harvest campaign)

Table 2. Commercial yeasts strains used in the winery during 2003-2007.

Commercial yeast	Code	Vintage				
		2003	2004	2005	2006	2007
BDX, Lallemand	LSA 4	X	X	X	X	X
Rhône 2056, Lallemand	LSA 3	--	X	--	--	--
Excellence XR, Lamothe-Abiet	LSA 7	--	X	X	X	X
Excellence SP, Lamothe-Abiet	LSA 2	--	--	X	--	--
LA-BJL, Lamothe-Abiet	LSA 1	--	--	X	--	X
Fermol Premier Cru	LSA 8	--	--	--	X	--
Fermol Mediterranée	LSA 9	--	--	--	X	--
43, Lallemand	LSA 6	--	--	--	X	--
Chalenge Selection ES U42, Sepsa-Enartis	LSA 10	--	--	--	--	X
Star, Agrovin	LSA 11	--	--	--	--	X

Table 3. Results of the comparison of the isolates with those present in GenBank from NCBI database and relative frequency of each species during the 2006 and 2007 campaigns.

Yeast species	Isolate designation	Sequencing product	Strain type	Gen Bank Data Base	% Identity	2006	2007
Saccharomyces cerevisiae	35/61	550	NRRL Y-12632	AY048154	100	19,7	9,8
Aureobasidium pullulans	BCSII/2	530*	T4B1c.7P	FJ490622	100	0,0	6,7
Candida parapsilosis	44/31	561	26.4	EU660860	100	3,7	0,0
Candida stellata	59/31	401*	A17-1-2	EF648014	98	2,9	0,3
Hanseniaspora guilliermondii	35/31	553	TY20	FJ972220	100	18,9	14,4
Hanseniaspora uvarum	$BCSI_2$/25	557	SYAKW	EU326135	99	3,2	25,5
Hanseniaspora vineae	BCSI/2	553	NRRL Y-27936	DQ655684	100	0,0	4,3
Issatchenkia orientalis	BCSII/2	551	NRRL Y-5396	EF550222	100	11,0	2,6
Kluyveromyces thermotolerans	BCSII/9	567	NRRL Y-8284	U69581	100	1,5	33,6
Metschnikowia pulcherrima	BCSI/15	483	MB510	DQ872871	100	0,0	0,8
Pichia anomala	25/31	566	Lv196	EU795420	100	7,0	0,0
Pichia guilliermondii	$BCSI_2$/32	573	NRRL Y-27949	DQ655689	100	0,1	0,7
Torulospora delbrueckii	46/66	565	CO4	EU879961	100	0,8	0,0
Zygosaccharomyces bailii	32/62	575	N2314	EU268642	100	2,9	0,0
Non identified Non-*Saccharomyces*	--	--	--	--	--	28,4	1,3

* Sequencing product from PCR 5,8S ITS

Table 4. Distribution of yeast (%) during spontaneous must fermentation according to sampling dates, grape variety (Merlot, Syrah, Cabernet Sauvignon) and sampling campaign (2006 and 2007)

	BM						BS						PS	BCS	
	2006				2007		2006				2007		2006	2007	
Yeast species	7BH	H	10AH	20AH	H	10AH	7BH	H	10AH	20AH	H	10AH	H	H	10AH
S. cerevisiae	--	--	99,2	--	--	--	--	19,6	7,8	15,6	--	--	12,2	--	25,0
A. pullulans	--	--	--	--	--	--	--	--	--	--	100	--	--	--	--
C. parapsilosis	--	--	--	--	--	--	--	--	33,3	6,7	--	--	--	--	--
C. stellata	--	--	--	31,1	--	--	--	--	--	--	--	--	--	--	0,8
H. guilliermondii	--	19,2	--	30,0	--	27,5	--	35,3	25,6	17,8	--	57,8	18,9	2,5	--
H. uvarum	--	--	--	34,4	--	22,5	--	--	--	--	--	42,2	--	44,2	15,8
H. vineae	--	--	--	--	--	--	--	--	--	--	--	--	--	21,7	--
I. orientalis	--	23,1	--	1,1	--	--	--	--	--	37,8	--	--	20,0	13,3	--
K. thermotolerans	3,1	--	0,8	3,3	--	50,0	--	--	--	10,0	--	--	--	7,5	56,7
M. pulcherrima	--	--	--	--	--	--	--	--	--	--	--	--	--	4,2	--
P. anomala	49,2	0,6	--	--	--	--	100	--	--	4,4	--	--	--	--	--
P. guilliermondii	--	--	--	--	--	--	--	--	--	1,1	--	--	--	0,8	1,3
T. delbrueckii	--	--	--	--	--	--	--	--	8,9	--	--	--	--	--	--
Z. bailii	--	--	--	--	--	--	--	--	--	--	--	--	15,6	--	--
Non identified	47,7	57,1	--	--	--	--	--	45,1	24,4	6,7	--	--	33,3	5,8	0,4

BM: Merlot vineyard; BS: Syrah vineyard; PS: Syrah vineyard far from winery; BCS: Cabernet Sauvignon vineyard. 7BH: one week before harvest; H: harvest; 10AH: ten days after harvest; 20AH: twenty days after harvest.

Table 5. Mitochondrial DNA RFLP patterns of *S. cerevisiae* yeast isolates from microvinifications of grapes samples collected in vineyard BM, BS, PS and BCS in harvest (sampling date H) and post harvest (sampling dates 10AH and 20AH) during the 2006 and 2007 campaigns.

Campaign	Vineyard	Sampling date	Isolates	Number of *S. cerevisiae*	Pattern
2006	BM	10AH	30	30	N
			30	30	O
			30	30	C
			30	28	D
				1	Q
	BS	H	30	30	M
		10AH	30	7	D
		20AH	30	14	C
	PS	H	30	9	M
			30	10	M
			30	2	M
			30	1	M
2007	BCS	10AH	30	29	AF
				1	AQ
			30	22	AF
				2	AN
				4	AP
				1	AQ
				1	AU

Capítulo 4

Selection of autocthonous *Saccharomyces* strains from Ronda (Malaga, Southern Spain) based on their oenological characteristics, for organic wine production

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Abstract

Eight autochthonous *Saccharomyces cerevisiae* strains isolated in a winery and its vineyard from "Serranía de Ronda" (Malaga, southern Spain) and five commercial yeasts commonly used by the same winery were evaluated according to their oenological properties. Most of them were found to present β-glucosidase activity and two of them protease activity as well. Some statistically significant differences were found among the strains when fermenting different glucose concentration media (225 and 275 g L^{-1}) at 30 ºC. However, none of the strains tested satisfied all the established criteria. For further studies, two commercial and one local strain were discarded because they were sensitive to killer yeast toxins; the rest, were used for micro-scale fermentations (330 ml of sterile Merlot must) carried out under controlled conditions. The results showed no differences among them in most of the analytical parameters of the fermented products. However, sensory analysis results and volatile composition of the produced wines showed that some autochthonous strains seem to produce wines with better quality. Finally, one commercial and four autochthonous strains were preselected for pilot scale fermentation in the winery.

Keywords: *Saccharomyces cerevisiae*, Yeast selection; Oenological characteristics; Volatile compounds; Sensory evaluation

1. Introduction

Wine fermentation is a complex microbial process involving the transformation of must into wine by the action of different species of yeasts and lactic acid bacteria originally present on the grapes and the winery equipment (Fleet & Heard, 1993). The main agents responsible of the alcoholic fermentation are strains of the species *Saccharomyces cerevisiae*. Commercially available dried yeast strains of *S. cerevisiae* can be inoculated into the grape juice in order to establish a high population and accomplish well-controlled must fermentations (Nikolau et al., 2006, Lopes et al., 2007). However, the use of local, autochthonous strains of *S. cerevisiae* is preferable since they are better acclimatised to the environmental conditions (Martini & Vaughan-Martini, 1990; Degré, 1993; Regodón et al., 1997) thus assuring the maintenance of the typical sensory properties of the wines of a given region (Hernández et al., 2003). In consequence, for local strains selection purposes, it is necessary the isolation and identification of yeast species present in the fermenting must, together with their evaluation in accordance with established oenological criteria (Briones et al., 1995; Pérez-Coello et al., 1999; Rodríguez et al., 2009). Among the most important oenological properties that wine strains should present are: tolerance to and high ethanol production, exhaustion of sugar potential and high fermentation activity, growth at low and high temperatures, growth at high sugar concentration, good glycerol production, resistance to and low sulphur dioxide production, low volatile acidity and hydrogen sulfide production, resistance to killer toxins but killer phenotype, good enzymatic profile (high β-glucosidase and proteolytic activities) and low acetaldehyde production (Ubeda Iranzo et al., 1998; Pérez-Coello et al., 1999; Esteve-Zarzoso et al., 2000, Rementeria et al., 2006).

The "Serranía de Ronda" (Malaga, southern Spain) is located at 5º 10' and 36º 44' northern latitude, and less than 750 m above sea level. The Denomination of Origin (DO) "Sierras de Málaga" accomplished red and white wines produced in the province of Malaga, but gives a special mention to those wines elaborated in the "Serranía de Ronda" thus recognizing the climatic and geographic peculiarities of this region. The use of commercial yeasts is rather common in the region since most of the wineries and vineyards are newly established and no study of the indigenous yeast population has been as far reported.

In a previous work, we isolated and identified the yeast population present in a newly built winery and in its neighbouring vineyard located in the "Serranía de Ronda" (DO Sierras de Málaga) during 2005 to 2007 campaigns (submitted publication). This population comprised non-*Saccharomyces* and *Saccharomyces* strains, both autochthonous and of commercial origin. This winery, as many other in the area, elaborates *organic wines* (wines produced with grapes from organic farming). Nowadays there is no an EU normative regulating the production of these wines. However, some recommendations for their elaboration and composition do exist in Spain (CAECV, 2010; Hidalgo Togores, 2003) and, among them, the use of selected autochthonous yeasts for the fermentation process.

In this work we have studied some relevant oenological characteristics of eight autochthonous *S. cerevisiae* strains isolated from the "Serranía de Ronda" area, and compared with that of five commercial strains commonly used by the winery. Some of the tested strains were finally used for microvinification assays carried out under controlled laboratory-scale conditions, and the produced wines were evaluated by sensory and instrumental analysis.

2. Materials and Methods

2.1 Yeast strains

For this study, eight autochthonous strains were selected on the basis of their frequency of isolation during 2005 to 2007 campaigns. They were coded MY, NY, OY, RY, SY, TY, AGY and AMY, defined for their particular molecular pattern of mtDNA-RFLP polymorphism analysis. Additionally, five of the most frequently employed commercial strains were also chosen for the same purpose, namely (in order to facilitate yeast designation, a code was assigned to each strain): Excellence SP (LSA2), Rhône 2056 (LSA3), BDX (LSA4), Excellence XR (LSA7) and Fermol Premier cru (LSA8).

To determine the killer production/sensitivity phenotype, the following *S. cerevisiae* strains were used: 1101 (K1 killer yeast) and 1384 (K2 killer yeast), provided by Dr. R. Esteban (University of Salamanca, Spain), and 47G (sensitive non-killer yeast) provided by Dr. T. Villa (University of Santiago de Compostela, Spain).

As a positive control for β-glucosidase activity, a strain of *S. cerevisiae* T73 (provided by Dr. A. Querol, IATA, Valencia, Spain) was used.

2.2. Oenological properties

To study hydrogen sulfide production, flasks containing 90 ml of YNB medium (Yeast Nitrogen Base, Difco 6.7 g L^{-1}) plus 20 % glucose, were inoculated with a suspension of each strain to achieve a cellular population of 10^6 cells mL^{-1}, and incubated at 25 ºC for 7 days without shaking. Paper impregnated with lead acetate was used to perform a qualitative control of SH_2 production (Ubeda Iranzo et al., 1998).

Foam production was examined according to Regodón et al. (1997). Yeasts were inoculated in test tubes containing 10 mL sterile must (cultivar Merlot) and incubated at 25 ºC. The must contained 305 g L^{-1} sugars, pH 3.6 and 5.3 g L^{-1} total acidity expressed as tartaric acid and 0.62 g L^{-1} malic acid. Foam height was measured everyday throughout the fermentation. Yeasts were classified into three categories based on the maximum foam height reached: F0 (foaming lower than 2 mm), F1 (foaming between 2 and 4 mm) and F2 (foaming greater than 4 mm).

For the test of resistance to sulfur dioxide, the strains were suspended in sterile must (cultivar Merlot) supplemented with 100 and 150 mg L^{-1} of total sulfur dioxide, and the time required by each strain to begin fermentation was determined. Higher concentrations of SO_2 were not tested because the winery produces organic wines and no more than 120 mg L^{-1} is allowed for red wines (CAECV, 2010).

Killer activity and sensitivity was determined as described by Sangorrín et al. (2001) with minor modifications: Each strain was tested for killer activity against the sensitive reference strain 47G whereas killer sensitivity was tested against the killer reference strains 1101 (K1) and 1384 (K2). Test plates were incubated for one week at 22ºC. A strain was designated as killer yeast (K) when its streak was surrounded by a clear zone of inhibition fringed with blue colour of the sensitive lawn. Yeasts that did not show immunity or resistance to the killer toxin produced by the killer reference strains were designated as sensitive (R^-), if they did as resistant (R^+) but if they, additionally, did not kill the sensitive strain, they were designated neutral (N). Yeast biotype characterization was performed against the two killer reference strains (K1 and K2 biotypes) as described by Sangorrín et al. (2007).

β-glucosidase activity was determined using the screening method described by Rosi et al. (1994), carried out on agar containing arbutin (Sigma-Aldrich, Steinheim,

Germany) as substrate. Strains with this activity hydrolyze the substrate and a dark brown colour develops in the agar. Strain T73 with known β-glucosidase activity was used as positive control.

Protease activity was determined by the method proposed by Bilinski et al. (1987). The strains were inoculated in radial streaks on skim milk agar (Oxoid Unipath Ltd., England) plates and the appearance of clear zones was observed. Protease from *Bacillus licheniformis* (EC.3.4.21.14, Sigma-Aldrich, Steinheim, Germany), was used as a positive control.

2.3. Fermentations

Synthetic media (Medium I and Medium II) containing two different glucose concentration (225 and 275 g L^{-1}, respectively) were used. Both media contained KH_2PO_4 (5 g L^{-1}), $(NH_4)_2SO_4$ (2 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.4 g L^{-1}), yeast extract (1 g L^{-1}) and malic acid (5 g L^{-1}); pH was adjusted to 3.8 and the media sterilized in the autoclave (115 ºC, 30 min). 250 mL of each medium were poured in Erlenmeyer flasks. Yeast strains were cultured in a liquid YEPD medium for 48 h, washed twice by centrifugation with sterile water and suspended in the 250 mL of Media I and II at a concentration of 10^6 cells mL^{-1}. Fermentations were conducted at two different temperatures (20 and 30 ºC) in duplicate. Fermentation kinetics was monitored by loss of weight in the glass flasks. The rate was expressed as loss of CO_2 (g L^{-1} h^{-1}), within periods of 24 and 72 h (Ubeda Iranzo et al., 1999).

2.4. Microvinifications

A number of the strains under study were preselected for microvinification experiments. Fresh Merlot must was treated by adding 1 mg L^{-1} of dimethyl di-carbonate (Fluka, Sigma-Aldrich, Stenheim, Germany) and stored at -20 ºC until use. A volume of 330 mL was fermented in 500 mL Erlenmeyer-flasks at 25 ºC in duplicate. Musts were inoculated with 10^6 cells mL^{-1} from 24 h cultures grown in liquid YEPD medium. Fermentation kinetics was monitored as described above, and fermenting musts were periodically sampled to enumerate yeast populations plating dilutions onto YEPD containing plates. At the end of the fermentation, settled solid were discarded, the samples were centrifuged and used for analytical and sensory assays.

2.5. Analytical methods

Some physicochemical parameters were analyzed in the products resulting from the fermentations and microvinifications. L-malic acid and glycerol were determined using enzymatic kits provided by Boehringer Mannheim (GmbH, Germany), pH by potentiometric method, total acidity (g L^{-1} tartaric acid) by indicator titration to pH 8.2 using standardized sodium hydroxide, volatile acidity (g L^{-1} acetic acid) by steam distillation and titration with standardized sodium hydroxide, reducing sugars by Rebelein method involving reaction of reducing sugars with copper (II) in alkaline solution, and ethanol by titrametric dichromate analysis.

2.6. Sensory analysis

Sensory analysis using orthonasal evaluation was performed by an expert panel composed of seven tasters (five females and two males). All members belonging to the laboratory staff had been trained according to international protocols (ISO, 1983 and ISO, 1985). 15 ml of wine sample was presented in dark glass covered with a plastic dish. Discriminant sensory evaluation was performed through triangular tests to assess significant differences between wines fermented by each yeast strain.

Quantitative descriptive analysis (QDA) was carried out using 13 sensory terms: green, floral, red fruit, ripe fruit, raisin, sweet, meat/sweat odour, nutty, citrus, balsamic, spicy, coffee and general impression. These terms to describe the samples were selected by the panel during preliminary sessions. The selected attributes were compiled in a tasting-card and panellists were asked to rank each descriptor on a 10-cm unstructured scale (from not noticeable to very strong).

2.7. Volatile compounds determination

The volatile compounds were determined using Headspace Sorptive Extraction and Gas Chomatography-Mass Spectrometry (HSSE-GC-MS).

The 48 standards of aroma compounds employed for the quantification were obtained from several commercial sources: Sigma-Aldrich (Stenheim, Germany); Merck (Darmstadt, Germany) and Fluka (Sigma-Aldrich, Stenheim, Germany). 4-methyl-2-pentanol (Merck, Darmstadt, Germany) was used as internal standard (IS).

The HSSE sampling conditions were as follows (Callejón et al., 2008): 5 mL of sample and 10 µL of 4-methyl-2-pentanol (IS) at 1045 mg L^{-1} was placed into a 20-mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with a 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put in an open glass insert and placed in the vial to carry out the extraction in the headspace. Then, the vial was tightly capped and heated for 60 minutes at 62 ºC in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water and dried with lint-free tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed in a glass tube 60 mm long, 6 mm o.d. and 4 mm i.d. This tube was then placed on the autosampler tray of the thermo desorption unit for GC-MS analysis.

Gas Chromatography analysis was carried out with a 6890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel). The thermal desorption was performed in splitless mode and with a flow rate of 90 mL min^{-1}. The desorption temperature program was the following: 35 ºC for 1 min, ramped at 60 ºC min^{-1} to 250 ºC held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner, was held at –35 ºC with liquid nitrogen for the total desorption time and then raised at 10 ºC s^{-1} to 290 ºC and held for 4 min. The solvent vent mode was used to transfer the sample to the analytical column. A CPWax-57CB column, 50 m x 0.25 mm, 0.20 µm film thickness (Varian, Middelburg, Netherlands) was used and the carrier gas was He at a flow rate of 1 mL min^{-1}. The oven temperature program was 35 ºC for 5 min, which was then raised to 220 ºC at 2.5 ºC min^{-1} (held for 5 min). The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 ºC, respectively.

Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35 to 350 amu.

All data were recorded using a MS ChemStation (Agilent Technologies, Santa Clara, California). The identity of peaks was assigned using the NIST 98 library and confirmed by standard retention indices when they were available. Quantification was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard (Callejón et al., 2008). The samples were

analyzed in triplicate and blank runs were made with empty glass tubes before and after each analysis. RIs were calculated from the retention times of n-alkanes by linear interpolation, in accordance with the literature (Silva Ferreira et al., 2003).

2.8. Statistical analysis

The Statistical version 7.0 software package (Statsoft, Tulsa, USA) was used to perform multiple range tests to compare sample means and correlation studies. One-way ANOVA analyses were carried out using Tukey's test to determine differences between means (statistical level of significance was set at $P \leq 0.05$).

3. Results and Discussion

3.1. Oenological properties and fermentation of synthetic media

In a first stage, 8 autochthonous and 5 commercial strains were evaluated in their technological and qualitative properties using both specific assays (SH_2 production, foam production, SO_2 tolerance, enzymatic activities, killer behaviour,) and small-scale fermentations carried out on synthetic media as described in Material and Methods.

As shown in Table 1, there were little differences in the behaviour of most of the isolates in relation to their oenological properties. Hydrogen sulfide causes an undesirable sulfurous off-flavour that is reminiscent of rotten eggs when concentrations exceeding its odour threshold in wine (50-80 μg L^{-1}). Despite the fact that chemical and biological factors affect the production of SH_2 in wines, most significantly, its production varies with the strain of *S. cerevisiae* (Rauhut, 1993). In our study, most of the strains screened were found to be moderate or high sulfide producers. Only indigenous RY strain was a non-SH_2 producer.

Another undesirable feature is the production of foam, which is usual during alcoholic fermentation and that is a problem of economic importance because fermentors can be commonly filled only to about two-thirds of their capacity to allow space for the foam. There seems to be a relationship between the amount of foam, the variety of grape, and the wine yeast carrying out the fermentation (Kunkee, 1984). In our case, all strains tested were medium (F1) or high (F2) foam producers. Finally, with respect to SO_2 tolerance, all strains were capable to initiate fermentation in less than 48 h when 100 mg L^{-1} of SO_2 was added to the must.

Enzymatic activities such as protease and β-glucosidase activities were also screened. Grape proteins may cause wine instability and haze formation due to aggregation of these molecules in the finished wine. The extracellular hydrolases produced by yeast may degrade wine proteins and other macromolecules thereby decreasing the formation of haze in wine (Esteve-Zarzoso et al., 1998, Dizy & Bisson, 2000, Strauss et al., 2001). Moreover, yeast proteases also play a major role during the autolysis process in wines kept on yeast lees during ageing (Esteve-Zarzoso et al., 1998, Dizy & Bisson, 2000). Additionally, yeast involved in winemaking could be important producers of pectinases, glycosidases and other enzymes. However, *S. cerevisiae*, the main yeast involved in wine making, is not recognised as a significant producer of extracellular hydrolases (Esteve-Zarzoso et al., 1998, Dizy & Bisson, 2000). Accordingly, in our study, only two autochthonous strains (MY and SY) produced clear zones on casein agar medium when screened for protease activity. On the other hand, β-glucosidase activity is desirable for wine strains since these enzymes would release conjugated terpenes enhancing the varietal characteristics (Arévalo Villena et al., 2006). Although this activity is frequent in non-*Saccharomyces* yeasts present in grapes and wine, we have found that 12 strains out of 14 were capable to produce a brown colour around the colony on the agar plates and, thus, hydrolysing the arbutin. Only the autochthonous strains SY and TY did not show β-glucosidase activity.

Killer wine yeast may offer some advantages over conventional ones when employed as fermentation starters to eliminate undesirable wild strains and dominate the wine fermentation. It is, thus, important to know the killer behaviour of yeast biota naturally present in the area where the selected strain is to be used, because different killer interactions between starter and wild strains could be established during fermentation affecting both the fermentative process, and the final quality of wine (Sangorrín et al., 2001).Accordingly, the yeast strains were tested for their capacity to kill a sensitive reference strain and their sensitivity to the most common killer toxins K1 and K2 produced by two killer reference strains. As shown in Table 1, all strains were sensitive to killer toxin K1 and strains LSA2, LSA4 and TY were also sensitive to K2 and did not present killer activity (K^-R^-), MY and SY were also sensitive to both toxins but presented killer activity (K^+R^-). Other strains with this activity were LSA3, LSA7, LSA8, NY, OY and RY (K^+R^-), these strains together with AGY and AMY were resistant to K2 toxin, although the latter were not killer phenotype (K^-R^-).

Fermentation in different glucose containing media (Medium I and Medium II) at different temperatures (20 and 30 ºC) were carried out. Table 2 presents the results obtained from the determination of physicochemical parameters (residual sugars, ethanol production and volatile acidity) in each experiment. At the early stages of the experiment, fermentation rates were, in all cases, higher than 0.2 g L^{-1} CO_2 which is consistent with the data presented by Pérez-Coello et al. (1999) and Nikolau et al. (2006). As for the rest of the parameters, statistically significant differences among the yeast strains tested were found, although not in all the assays. As reflected in Table 2, in the experiments carried out at 20ºC, no significant differences were found in either media, with the exception of the alcohol produced by RY which is significantly higher than the others in Medium II. Additionally, some differences were also found in the production of volatile acidity in this experiment. More differences were observed, however, in the experiments carried out at 30 ºC. In Medium I, differences were found specially in the production of volatile acidity, the highest value corresponding to strain RY In Medium II at 30ºC the strains showed major difference in their behaviour. None of them were capable to consume all the sugars from the medium but while for some strains (LSA3, LSA8 and NY) residual sugar was less than 25.7 g L^{-1}, for others (LSA2, LSA7, MY, RY and TY) was more than 60 g L^{-1}. They all produced more than 0.56 g L^{-1} of volatile acidity, but some strains even more than 0.90 g L^{-1} (LSA3, OY, RY, TY) expressed as acetic acid.

Differences were found when comparing the mean values of the four experiments (Table 3). In general, all strains were capable of fermenting sugars at a level < 5 g L^{-1} in both Medium I and Medium II at 20 ºC, but no strain did it when fermenting Medium II at 30 ºC (Table 2). On the other hand, and as expected, differences in initial sugar content in the media (225 or 275 g L^{-1} glucose) caused differences in the final ethanol produced, with greater values in Medium II at both temperatures (Table 3). Criterion for ethanol production was established depending on the glucose concentration of the media, being at least 8.5 % and 10.5 % vol for 225 and 275 g L^{-1} glucose media, respectively. Most of the strains reached ethanol values similar or higher than those established except for TY in Medium I at 30 ºC and LSA7, RY and TY in Medium II at 30 ºC (Table 2).

The production of volatile acidity, a highly undesirable trait with negative consequences in organoleptics properties of the wines (Ribéreau-Gayon et al., 2006),

was also measured. Significant differences between fermentations in Medium I and Medium II were found (Tables 2 and 3). At both temperatures, most strains (except for MY) produced values around or higher than 0.8 g L^{-1} acetic acid in Medium II, but less than this amount in Medium I, with the exception of strain RY.

Additionally, we have also compared the behaviour of commercial strains against autochthonous ones (Table 4). No significant differences between both types of yeasts were found in the experiments with Medium I at 20 ºC and in Medium II at 30 ºC for the considered analytical parameters. However, production of volatile acidity in Medium II at 20 ºC was higher for commercial strains than for autochthonous ones, and commercial strains produced higher alcohol degree in Medium I at 30 ºC, than the autochthonous ones.

Thus, it is difficult to select a strain having both good oenological and enzymatic properties together with a good behaviour when fermenting different sugars concentration media at different temperatures. For further studies, strains LSA2, LSA4 and TY strains were discarded since they are sensitive to both K1 and K2 killer toxins and therefore, did not present killer activity either, thus, if they were to be used as starters, this characteristic would make them less competitive against other fermenting yeasts. (Jacobs and Van Vuuren., 1991, Lopes et al., 2007). On the other hand, autochthonous strains AG and AM were highly isolated during campaign 2007 and it seemed interesting them to be included in this study. However, they were not submitted to synthetic media fermentations: results obtained in the rest of the strains (a total of eleven) were not clarifying for the selection of any particular strain among tested. Thus, once their oenological properties regarding SH_2 and foam production, SO_2 tolerance, killer phenotype and enzymatic activities were determined (Table 1), they were included for fresh must microvinifications assays.

3.2. Microvinifications

Using the eight preselected yeast strains indicated above, duplicate fermentations were carried out in 330 mL sterile grape must from Merlot cultivar. Fermentation pattern (fermentation rate and evolution of yeast population) was monitored and some physicochemical parameters, together with sensory and volatile compounds analysis, were performed. Figure 1 shows the growth of the yeast strains in Merlot must inoculated with pure cultures and the fermentation kinetics of each yeast

tested. All strains were able to grow in the must until the end of fermentation. The growth peaked at viable populations exceeding 10^7 cells x mL^{-1} and only commercial strains and two autochthonous strains (MY and AGY) reached 10^8 cells L^{-1}. At the end of the fermentation, viable populations of most of the strains fell down to 10^6 cells x mL^{-1} except for OY and AMY strains which reached 10^5 cells x mL^{-1}. Despite those differences in viable populations among the strains, all of them showed the same fermentation kinetics except MY fermentation that was slower. All fermentations began one day after inoculation and the population began to decline when most of the sugar had been exhausted.

Table 5 summarizes fermentation rate, fermentation days and general parameters of wines fermented with preselected yeasts. Again, some statistically significant differences were found among the yeast strains tested, mainly in the production of volatile acidity. In all cases, fermentations were complete (< 5 g L^{-1} sugars left), ethanol production was about 12.5 % vol except for SY and AGY strains (15 and 15.5. % vol, respectively) and volatile acidity was lower than 0.6 g L^{-1} for SY, AGY and AMY strains and equal or less than 0.2 g L^{-1} acetic acid for the rest of the strains.

Glycerol is quantitatively the most important fermentation product after ethanol and carbon dioxide (Ribéreau-Gayon et al., 2006). Although its importance in wine quality is questionable (Ribéreau-Gayon et al., 2006), some researchers postulate that it influences wine's smoothness and viscosity and, in general, it is considered to contribute positively to the sensory quality of wine (Wang et al., 2001). Glycerol levels in this study ranged between 6.7 and 10.4 g L^{-1}were considered comparable well with reported values for other wines (Amerine & Ough, 1976).

3.3. Volatile compounds

The profile analysis of the wines produced by microvinofication led to the identification of 38 compounds comprising mainly esters, alcohols, acids, aldehydes and acetals.

The higher amounts of volatiles were found in three wines produced by autochthonous yeasts OY, RY and SY. In general, the major volatile compound was acetaldehyde diethylacetal. As expected, alcohols were the most abundant group, followed by ethyl esters.

Although these wines present a wide variety of volatile compounds, their influence on the final aroma depends on their odor threshold that is related to the parameter known as "odor activity value" (OAV). This parameter is defined as the concentration/threshold ratio. Odorants with low OAVs, or low impact odorants (i.e. with typical values <1) are generally considered to be unimportant to the overall sensory perception (Ryan et al., 2008).

In our case, among all volatile compounds quantified, only 16 had an OAV greater than 1 (Table 6). Hence, these compounds would contribute mainly to wine aroma. One of the main sensorial qualities in young red wines is the fruity aroma which is closely related to esters. Our wines contented the following esters: ethyl octanoate, which showed OAVs between 28.9 and 98.2, followed by ethyl furoate with OAVs between 30.0 and 57.4 and ethyl hexanoate with the lowest values, 8.2-25.4, except to wine WLSA8 (Table 6). According to esters OAVs, the wines studied presented similar values, with the exception of the wine produced by the commercial yeast LSA8 due to the remarkable high content of ethyl hexanoate in this last sample. Other ester that had OAVs higher than 1 in all the samples, excepting WSY where it was not detected, was ethyl lactate. This compound is formed during malolactic fermentation and it does not contribute to the fruity aroma in wines.

Regarding alcohols, only 3-methyl-1-butanol, 2-methyl-1-butanol, 2-phenylethanol, furfuryl alcohol and isobutanol had an OAV greater than 1 (Table 6). This last compound together with 3-methyl-1-butanol and 2-methyl-1-butanol are related to solvent aroma. If we consider the sum of their OAVs, the commercial yeasts provided slightly higher values. On the other hand, 2-phenylethanol is the only alcohol described at a sensory level in pleasant terms (rose aroma) (Lorenzo et al., 2008). Although the range of values was quite similar in all the samples, the highest value was reached by the wine produced by the commercial yeast LSA3.

All the quantified acids had OAVs greater than 1, although this fact only was observed for decanoic acid in sample WSY. The acid that contributed mainly to the overall aroma of wine was isovaleric acid, showing OAVs ten times higher than the other acids. As it is well known, these compounds are not associated with the wine quality since they are related to rancid aroma. The highest amounts of acids were found in sample WSY.

Regarding ketones, acetoin was the only compound of this chemical group detected in the samples. This compound showed OAV higher than 1 in two samples, WRY and WSY. According to Enshani et al., (2009), acetoin at concentrations higher than its threshold level (around 150 mg L^{-1}) can confer an unpleasant buttery flavour on wines.

3.4. Sensory analysis

Wines obtained by microvinifications were submitted to quantitative descriptive analysis (QDA). In this sensory analysis, thirteen sensory attributes, mentioned in Materials and Methods, were used to define the aromatic profile of the samples. Among these attributes, ripe fruit, sweet, green and general impression reached the highest scores (Figure 2). The descriptor "general impression" is a hedonic descriptor that shows the general sensory quality of wines. When statistical correlations among the attributes were performed, we observed that general impression and sweet aroma are directly correlated. If we consider the chemical data, the total amount of alcohols and acids are inversely correlated with these two attributes. These compounds are generally related to "solvent" and "fatty aroma" respectively. Among the wines produced by autochthonous strains, the lowest general impression was reached in sample WSY and WAMY, and WLSA3 in wines produced by commercial yeasts.

On the other hand, the descriptor "meat/sweat odour" is a negative aromatic characteristic that means a defect in the wine. This attribute presented the scores 0.16, 0.21 and 0.46 in the samples WLSA8, WAMY and WLSA3, respectively. Therefore, two of the three commercial yeast strains tested seem to produce negative aroma in wines.

Taking into account the results obtained in sensory analysis, among autochthonous yeasts employed, we would discard SY and AMY, since they produce wines with lowest general impression values and high scores in green descriptor. For similar reasons, AMY would also be discarded because it produces wines with negative aromatic notes (meat/sweat odour) and LSA3 because it provides aromatic defects in the produced wines. The two remaining commercial yeasts produced wines with a similar sensory profile, since the panellists were not able to differentiate them in the triangular tests although, as indicated above, LSA8 seem to provide some meat/sweat odour to the wine.

The aim of this work was a preliminary selection of *S. cerevisiae* strains for the production of *organic wines* in the "Serranía de Ronda" (southern Spain). For this purpose we have studied the oenological properties of some autochthonous *S. cerevisiae* strains isolated from a winery and its surrounding vineyard in that region and compare them with those of commercial strains frequently employed in the same winery. As stated above, we have not found statistically significant differences in the behaviour of strains when fermenting different glucose concentration media at 20 ºC. However, differences were found when fermenting at 30 ºC, especially in medium containing 275 g L^{-1} glucose. Yeasts behaviour differed according to fermentation conditions, being the production of volatile acidity higher at 30 ºC than at 20 ºC and residual sugar higher in Medium II (275 g L^{-1} glucose) at 30 ºC. As expected, final alcohol degree was higher in experiments carried out with Medium II than in Medium I (275 and 225 g L^{-1} initial glucose, respectively).

Regarding fresh must microvinifications, differences were found in the production of volatile acidity, although mean values were lower than those obtained in synthetic media I and II. Sensory analysis indicated different preferences for the resulting wines from the fermentation of Merlot must. Autochthonous strains RY, SY and AMY and commercial strain LSA3 and LSA8 obtained the lowest general impression or showing some aromatic defects in the resulting wines. Accordingly, only commercial strain LSA7 and four autochthonous strains (MY, NY, OY and AG) were preselected for a pilot scale fermentation in the winery.

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Figures

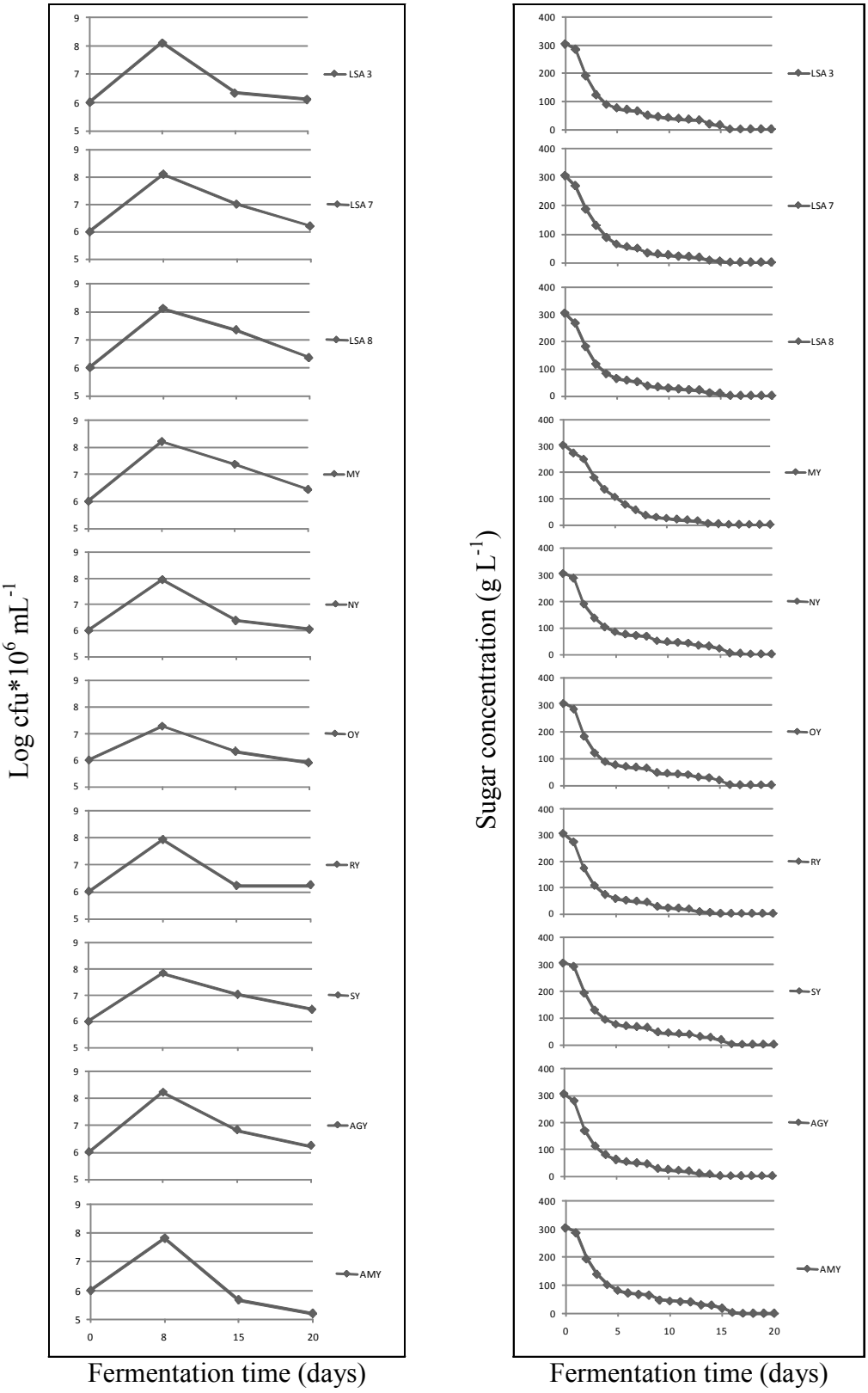

Figure 1. Yeast population evolution (left panel) and consumption of sugars (right panel) in must inoculated with different preselected yeasts

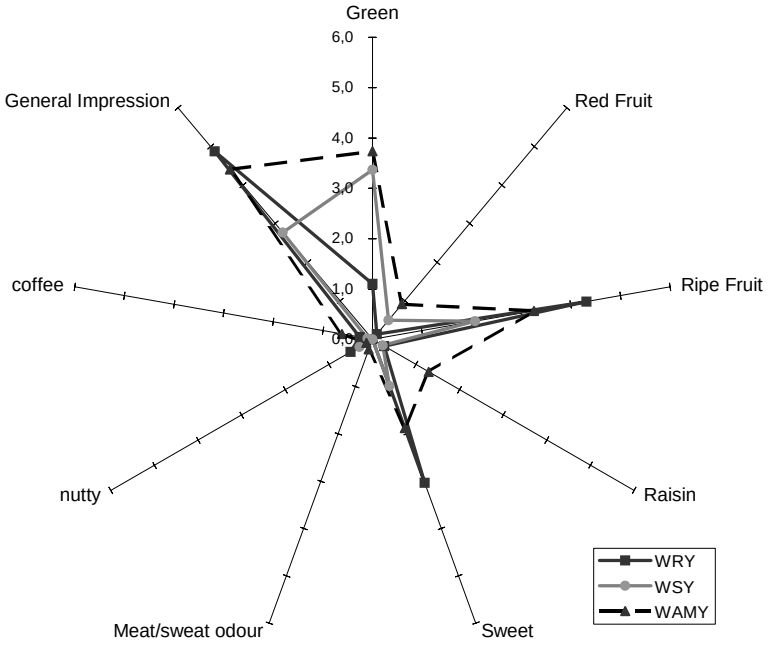

Figure 2A

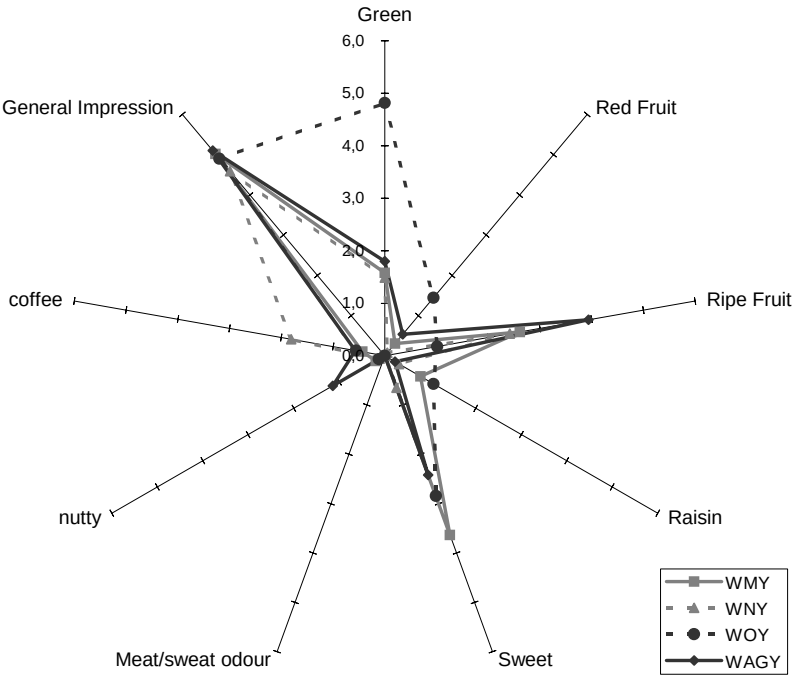

Figure 2B.

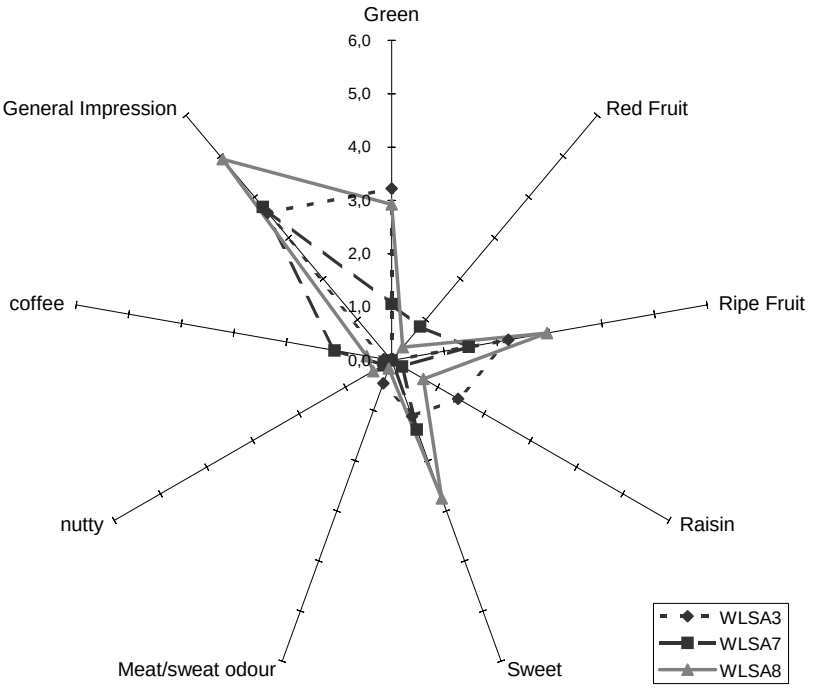

Figure 2C.

Figure 2. Sensory profiles of Merlot red wine samples: A and B, wines produced by autochthonous yeast strains; C, wines produced by commercial yeast strains

Tables

Table 1. Strain code, number of isolates, origin, identity and some technological and qualitative properties of commercial and autochthonous *S. cerevisiae* isolates

Strain code	Origin	SH_2	Foam	SO_2 tolerance (100 mg L^{-1})	Proteinase activity	β-glucosidase activity	Killer phenotype[a]	Killer biotype[b]
LSA2	Commercial	+	F2	+	-	+	K^-R^-	K1, K2
LSA3	Commercial	+	F2	+	-	+	K^+R^-	K1
LSA4	Commercial	+	F2	+	-	+	K^-R^-	K1, K2
LSA7	Commercial	+	F2	+	-	+	K^+R^-	K1
LSA8	Commercial	++	F2	+	-	+	K^+R^-	K1
MY	Grape	+	F2	+	+	+	K^+R^-	K1, K2
NY	Grape	+	F2	+	-	+	K^+R^-	K1
OY	Grape	+	F1	+	-	+	K^+R^-	K1
RY	Wine barrel surface	-	F2	+	-	+	K^+R^-	K1
SY	Wine barrel surface	++	F1	+	+	-	K^+R^-	K1, K2
TY	Wine barrel surface	++	F1	+	-	-	K^-R^-	K1, K2
AGY	Fermenting must	++	F2	+	-	+	K^-R^-	K1
AMY	Fermenting must	+	F2	+	-	+	K^-R^-	K1

[a] K^-R^-, sensitive; K^+R^-, killer/sensitive
[b] K1, sensitive to toxin K1; K2, sensitive to toxin K2.

Table 2. Fermentation rate and physicochemical parameters (RA: residual sugars, VA: volatile acidity and Eth: ethanol production) in fermentation duplicates from two media containing different glucose concentrations incubated at different temperatures (20 and 30 ºC).

	20 ºC			30 ºC		
	Medium I (225 g L^{-1} glucose)					
Strain	RS (g L^{-1})	VA (g L^{-1} AcH)	Eth (% vol)	RS (g L^{-1})	VA (g L^{-1} AcH)	Eth (% vol)
LSA2	22.7 ±25.6 a	0.43±0.22 a	9.6±0.1 a	1.5±0.2 a	0.49±0.06 cd	11.1±0.2 c
LSA3	0.9 ±0.5 a	0.78±0.10 a	8.4±0.8 a	1.4±0.2 a	0.56±0.01 de	11.0±0.2 c
LSA4	23.70±26.2 a	0.53±0.33 a	8.4±1.2 a	1.4±0.2 a	0.47±0.03 bcd	10.7±0.2 c
LSA7	19.5±22.1 a	0.35±0.16 a	9.3±1.0 a	27.6±2.6 c	0.36±0.01 ab	8.2±0.1 b
LSA8	0.8±0.9 a	0.63±0.03 a	10.2±0.2 a	1.3±0.2 a	0.53±0.01 de	10.8±0.2 c
MY	0.4±0.3 a	0.36±0.09 a	10.1±0.1 a	1.3±0.2 a	0.28±0.01 a	10.8±0.4 c
NY	0.5±0.3 a	0.46±0.04 a	10.2±0.1 a	1.3±0.3 a	0.38±0.01 abc	11.5±0.0 c
OY	0.3±0.4 a	0.40±0.04 a	9.7±0.1 a	1.4±0.2 a	0.54±0.04 de	8.8±0.1 b
RY	0.9±0.1 a	0.85±0.05 a	10.2±0.4 a	1.5±0.2 a	0.90±0.07 g	11.4±0.2 c
SY	1.0±0.2 a	0.68±0.03 a	9.9±0.5 a	6.4±0.4 b	0.70±0.01 f	11.5±0.4 c
TY	1.9±0.3 a	0.71±0.03 a	9.4±0.4 a	11.1±1.3 b	0.64±0.04 ef	4.8±0.4 a
	Medium II (275 g L^{-1} glucose)					
LSA2	1.6±0.1 a	0.79±0.06 ab	11.2±0.4 a	64.8±13.7 bc	0.75±0.04 ab	11.3±0.8 abc
LSA3	1.5±0.2 a	0.89±0.09 ab	10.5±0.4 a	7.4±1.6 a	0.92±0.02 bc	13.7±0.2 c
LSA4	1.4±0.2 a	0.91±0.06 ab	11.6±0.4 a	35.1±1.5 ab	0.83±0.00ab	11.8±0.7 abc
LSA7	1.6±0.2 a	0.90±0.01 ab	11.1±0.1 a	96.8±3.9 c	0.70±0.06ab	9.6±0.6 a
LSA8	1.4±0.2 a	0.88±0.03 ab	11.2±0.0 a	16.1±2.4 a	0.83±0.08 ab	13.2±0.1 c
MY	1.6±0.3 a	0.58±0.09 a	11.2±0.5 a	64.0±0.9 bc	0.56±0.00 a	10.5±0.8 ab
NY	1.2±0.1 a	0.72±0.10 ab	10.6±0.9 a	25.7±22.3 a	0.86±0.20 b	13.5±0.1 c
OY	1.4±0.1 a	0.62±0.23 a	10.7±0.0 a	39.9±5.3 ab	0.91±0.01 bc	13.6±01 c
RY	1.31±0.2a	0.92±0.00 ab	14.1±0.1 a	63.3±1.1 bc	1.16±0.01 c	10.2±0.6 ab
SY	1.3±0.2 a	1.06±0.10 b	11.1±0.4 a	37.9±2.3 ab	0.87±0.03 b	12.5±1.0 bc
TY	1.9±0.7 a	0.79±0.02 ab	11.2±0.1 a	75.6±3.1 c	0.95±0.02 bc	9.9±0.8 a

a, b, c : Data of a certain row with the same letter do not differ at a 95 % confidence level

Table 3. Mean values of reducing sugars (RS), volatile acidity (VA), ethanol produced (Eth) and days of fermentation for each fermentation experiment carried out in synthetic media I and II.

Medium	Temp (ºC)	RS (g L^{-1})	VA (g L^{-1} AcH)	Eth (% vol)	Days of fermentation
I	20	6.6±9.9 [a]	0.56±0.18 [a]	9.6±0.7 [a]	13.0±4.9 [a]
I	30	5.1±8.1[a]	0.53±0.17 [a]	10.0±2.0 [a]	11.5±1.9 [a]
II	20	1.5±0.2 [a]	0.82±0.14 [b]	11.3±1.0 [b]	18.3±5.5 [b]
II	30	47.9±27.2 [b]	0.85±0.15 [b]	11.8±1.6 [b]	12.3±1.6 [a]

a, b: Data with the same letter do not differ at 95 % confidence level

Table 4. Mean values of reducing sugars (RS), volatile acidity (VA) and ethanol produced (Eth) for commercial (C) and autochthonous (A) strains in each fermentation experiment carried out in synthetic media I and II.

	20 ºC			30 ºC		
	Medium I (225 g L^{-1} glucose)					
Strain	RS (g L^{-1})	VA (g L^{-1} AcH)	Eth (% vol)	RS (g L^{-1})	VA (g L^{-1} AcH)	Eth (% vol)
C	13.5 ± 20.6 a	0.54± 0.22 a	9.2± 0.9 a	6.7 ±11.1 a	0.48±0.57 a	10.3± 1.2 b
A	0.82 ± 0.58 a	0.58± 0.19 a	9.9± 0.4 a	3.8 ± 3.9 a	0.57±0.21 a	9.8±2.5 a
	Medium II (275 g L^{-1} glucose)					
C	1.5±0.1 a	0.87±0.06 b	11.1±0.5 a	44.0±34.8 a	0.81±0.09 a	11.9±1.6 a
A	1.4±0.4 a	0.78±0.20 a	11.5±1.3 a	51.1±20.4 a	0.89±0.19 a	11.7±1.7 a

a, b: Data with the same letter do not differ at 95 % confidence level

Table 5. Fermentation rate, days of fermentations and general parameters of Merlot wines fermented with commercial and authoctonous yeast strains

Strain	Fermentation rate ($g\ L^{-1}\ h^{-1}$)	Days of fermentation	Reducing Sugar ($g\ L^{-1}$)	Volatile acidity ($g\ L^{-1}$ acetic acid)	Ethanol (% vol)	pH	Total Acidity ($g\ L^{-1}$ tartaric acid)	Glycerol ($g\ L^{-1}$)	Malic Acid ($g\ L^{-1}$)
LSA3	1.67	12	1.2±0.1 a	0.2±0.0 abc	12.8±0.2 a	3.39±0.04 ab	6.0±0.1 a	8.8±0.2 a	0.6±0.1 bc
LSA7	1.46	12	1.1±0.1 a	0.1±0.0 ab	12.0±0.3 a	3.39±0.01 ab	6.2±0.1 a	6.7±0.2 a	0.5±0.0 b
LSA8	1.53	12	1.0±0.1 a	0.2±.0.1 abc	12.6±0.1 a	3.41±0.01 ab	5.6±0.1 a	8.0±0.4 a	0.4±0.0 a
MY	0.90	13	1.2±0.1 a	0.1±0.0 ab	12.4±0.1 a	3.37±0.02 a	5.6±0.3 a	8.4±0.6 a	0.5±0.0 b
NY	1.53	11	1.4±0.1 a	0.1±0.0 ab	12.6±0.1 a	3.39±0.01 ab	5.2±0.2 a	7.7±0.1 a	0.6±0.0 c
OY	1.69	11	1.3±0.3 a	0.1±0.0 abc	12.3±0.5 a	3.39±0.01 ab	5.3±0.1 a	7.6±0.1 a	0.6±0.0c
RY	1.77	11	1.6±0.0 a	0.2±0.0 abc	12.3±0.2 a	3.45±0.01 b	5.7±0.2 a	9.6±0.2 a	0.4±0.0 a
SY	1.77	12	2.1±1.1 a	0.6±0.3 c	15.8±2.9 a	3.40±0.03 ab	5.7±0.5 a	10.4±2.3 a	0.4±0.0 a
AGY	1.83	12	3.9±3.3 a	0.5±0.1 bc	15.5±2.0 a	3.41±0.03 ab	5.7±0.2 a	9.1±1.8 a	0.6±0.0 c
AMY	1.53	11	1.2±0.2 a	0.4±0.0 abc	12.6±0.1 a	3.42±0.03 ab	5.3±0.1 a	8.0±0.0 a	0.5±0.0 b

a, b, c: Data with the same letter do not differ at 95 % confidence level

Table 6. Odour activity value (OAV*) of volatile compounds in red wines (W) produced by commercial and autochthonous yeast strains.

Compounds	Wine samples									
	WLSA3	WLSA7	WLSA8	WAGY	WOY	WMY	WAMY	WNY	WRY	WSY
Acetaldehyde diethylacetal	3677	4572	4111	5086	9027	4524	2066	5371	9842	8880
Ethyl octanoate	62.2	80.2	72.08	66.6	95.0	28.9	94.5	98.2	55.2	66.2
Ethyl furoate	54.2	57.4	48.3	30.0	35.0	33.0	38.6	43.9	31.6	56.4
Ethyl lactate	26.4	28.0	21.9	26.0	28.9	60.3	21.9	24.0	17.2	0.00
Ethyl hexanoate	13.1	17.4	142.7	25.4	18.9	8.2	17.9	16.7	12.2	15.9
Isovaleric acid	20.2	32.8	23.0	20.3	21.7	32.8	14.7	16.8	33.7	32.5
3-Methyl-1-butanol	4.95	3.82	3.70	3.85	3.04	3.28	3.06	3.03	3.30	4.52
Hexanoic acid	3.64	4.91	3.04	2.10	3.64	1.31	2.78	3.19	3.90	4.87
2-Phenylethanol	5.74	4.35	2.65	2.06	4.76	3.01	3.25	4.89	2.44	4.68
Furfuryl alcohol	1.26	2.77	1.08	2.82	1.13	0.00	1.76	1.61	1.27	3.85
2-Methyl-1-butanol	1.39	1.18	0.00	1.11	2.08	0.00	1.57	1.74	0.00	0.00
Octanoic acid	4.63	3.94	1.47	0.00	2.50	0.00	2.02	4.22	2.11	6.87
Isobutanol	0.00	0.00	1.03	1.35	0.00	0.00	0.00	0.00	0.00	1.11
Acetoin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.0	1.0
Decanoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.05
Methyl acetate	0.00	0.00	0.00	1.37	0.00	0.00	0.00	0.00	0.00	0.00

*OAV calculated using the odour threshold showed in Ortin, 2006; Moyano et al., 2009

Capítulo 5

Volatile and sensory profile of organic red wines produced by different selected *Saccharomyces cerevisiae* strain. Autochthonous and commercial

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Abstract

Organic wines were produced at pilot scale to select the best autochthonous and commercial yeast strains to obtain wines with high organoleptic qualities. We tested the behaviour of five *S. cerevisiae* yeast strains and determined their volatile composition and organoleptic characteristics by sensory analysis. A total of 51 volatile compounds were quantified in the wines produced. The concentration of most of the volatile compounds was significantly influenced depending on which yeast strain was inoculated. The differences observed in the volatile composition of the wines appear to be quantitative rather than qualitative. In general, acetals were the most abundant group of volatile compounds in all the samples studied, followed by alcohols without ethanol. The highest contents of volatile compounds were found in two of the wines produced by autochthonous yeast strains. The results obtained in the sensory analysis suggest that autochthonous yeast produced wines of higher organoleptic quality because this sample gave the lowest value for the general impression attribute.

Keywords**:** organic red wine, yeast, volatile compounds, sensory analysis, HS-SBSE-TDU-GC-MS.

1. Introduction

Growing concern among consumers in developed countries regarding the health and the protection of the environment has increased the demand for organic food over the last decade (especially in Germany, the United Kingdom, Switzerland, New Zealand, Japan, and the United States). Among organic products, EU consumers have shown an increasing interest in organic wines. More and more new organically based wineries are being established and old traditional wineries are adding organic wine to their product lines (Azabagaoglus et al., 2007).

Wine quality is influenced, in part, by the composition of the grape juice and by the microbial communities present during the fermentation process. Aroma is one of the main characteristics that determine a wine's quality and value. The aroma of wine is a unique mixture of volatile compounds originating from grapes (varietal aromas), secondary products formed during the wine fermentation (fermentative aromas) and aging (post-fermentative aromas) (Swiegers et al., 2005). The volatile fraction of wine can be made up of more than 800 different compounds (Rapp, 1998) with a wide concentration range varying from hundreds of mg L^{-1} to ug L^{-1} or ng L^{-1} levels (Garde-Cerdán et al., 2008). This great variety of volatile compounds, with different polarities, volatilities and a wide range of concentrations, is responsible for the complexity of the wine's bouquet and ensures its specificity and character (Mauriello et al., 2009).

The particular importance of each compound to the final aroma is related to its odour perception threshold (Garde-Cerdán et al., 2008). General approaches to identifying "important" or high impact odorants are based on odour activity values (OAVs) and the concentration/threshold ratio. Odorants with low OAVs, or low impact odorants (i.e. with typical values <1) are generally considered to be unimportant to the overall sensory perception (Ryan et al., 2008).

However, Ryan et al. (2008) hypothesise that compounds with low OAVs could play a critical role in characterizing the overall odour of a sample. Furthermore, other authors think that OAVs provide only a rough evaluation of the real contribution of each compound to the overall aroma. In fact, the volatility and the perception of aroma compounds are significantly affected by the basic chemical composition of the wine, which can both mask the odour impact of certain compounds present in concentrations

above their detection thresholds and favour the detection of other molecules present in concentrations below theirs (Molina et al., 2009).

A vast number of volatile compounds are formed and modulated by yeast during alcoholic fermentation and significantly impact the flavour and overall quality of wines (King, et al., 2008). The volatile compounds synthesized by wine yeast include higher alcohols, medium- and long-chain volatile acids, acetate esters, ethyl esters and aldehydes among others (Delfini et al., 2001; Lambrechts & Pretorius, 2000). The capacity to form aroma depends not only on yeast species but also on the particular strain of the individual species (Torrens et al., 2008). Different strains of *S. cerevisiae* can produce significantly different flavour profiles when fermenting the same must. This is a consequence of both the differential ability of wine yeast strains to release varietal volatile compounds from grape precursors and the differential ability to synthesise de novo yeast-derived volatile compounds (Wondra & Boveric, 2001; Swiegers et al., 2006; Ugliano et al., 2006; Vilanova & Sieiro, 2006). Therefore, selecting the proper yeast strain can be critical for the development of the desired wine style (Molina et al., 2009). For this reason, modern wine makers prefer to employ selected yeast strains. For the production of young wine, the wineries select yeast strains that produce both the high levels of the esters and acetates needed for the desirable fruity taste and the low levels of higher alcohols that contribute negatively to aroma (Torrens et al., 2008). Moreover, employing selected starter yeast cultures provides technological advantages such as guaranteeing that the must ferments in the correct way. On the other hand, the use of autochthonous yeast strains, besides promoting biodiversity, is rather preferable since they are better acclimated to the environmental conditions and assure the maintenance of the typical sensory properties of the wines of any given region.

The aim of this work is to select the best yeast strains among autochthonous and commercial to obtain organic wines with high organoleptic qualities.

2. Experimental

2.1. Yeast strains and yeast implantation control

Five different strains of *S. cerevisiae* were tested; four autochthonous strains were compared with a commercial yeast strain frequently employed by the winery for the vinification of red wines. The commercial yeast was Excellence XR (XR) (Lamothe-Abiet, Bordeaux, France) and the autochthonous yeasts were coded as MY, NY, OY

and AGY. The commercial yeast was selected in accordance with the winery's preferences and the autochthonous strains were isolated from the same cellar and the neighbouring vineyard and selected according to their oenological traits in a previous selection study which evaluated ethanol production, sugar consumption, volatile acidity production, malic acid degradation, glycerol production, foam and SH_2 production, killer behaviour, volatile compounds production and the organoleptic characteristics of the experimental wines obtained (data not shown).

In order to assess the implantation of the inoculated yeast in each vinification vat, samples were taken at different stages of fermentation: 24 h after inoculation, in the middle (day 3) and at the end of the fermentation (day 6). In addition, a sample of fresh grape juice was taken to determine the yeast microflora in the must before the vats were filled and sown with the starter. Aliquots (0.1 mL each) of serial dilutions were spread onto plates of YEPD agar (yeast extract 1% w/v, peptone 1 % w/v, glucose 2 % w/v, agar 2 % w/v). Plates were incubated at 28 ºC for 72 hours and, for each sample, 20 colonies were randomly chosen and submitted to further studies.

Isolates from the fresh juice were identified by PCR of the ribosomal region encompassing the 5,8S rRNA gene and the two internal transcribed spacers. rDNA was amplified by PCR in a TC-312 Techne termocycler using primers ITS1 and ITS4, described by White et al. (1990). The species of the isolates were identified by PCR-RFLP (restriction fragment length polymorphism) digesting with the restriction endonucleases *Hinf*I, *Hha*I (same enzymatic activity as *Cfo*I) and *Hae*III (Takara Japan) as previously described by Guillamón et al. (1998). PCR products and restriction fragments were separated on a horizontal 1.4% and 3 % agarose gel, respectively, in TAE buffer and compared with standards (100-bp DNA ladder, Amersham Biosciences). Isolates from the five fermenting musts were directly characterized by restriction analysis of mitochondrial DNA in order to compare the resulting profiles with those of the *S. cerevisiae* strains inoculated in each vat. Thus, all these isolates were initially presumed to belong to *S. cerevisiae* species; PCR-RFLP would only be used to identify the rDNA if any of the profiles did not match any of the inoculated strains. DNA extraction and restriction analysis were performed following the method proposed by Querol et al. (1992). DNA (6 μL) was digested with *Hinf*I endonuclease (Takara Japan) and restriction fragments were separated by electrophoresis on 1% agarose gel with added ethidium bromide and visualized in a UV transilluminator. This

technique was also applied to each yeast strain used as starter. The genetic profiles of all the isolates were compared with the profiles of the inoculated yeasts and this enabled us to determine the implantation percentage of each strain tested.

2.2. Grape musts and pilot scale fermentation

Pilot scale fermentations took place in the winery in 100 L stainless vats with Merlot grape variety must. The physical and chemical must parameters were the following: pH 3.40, total acidity (g L^{-1} tartaric acid) 5.1, density 1.0945 and 30 mg L^{-1} of SO_2.

All the yeast inocula (including the commercial strain) came from pure cultures previously sown in a solid medium which were inoculated in a small volume of sterilized must to a final concentration of 10^6 cell mL^{-1} and incubated at 28 ºC for 24 h. The starters were progressively diluted with sterilized must in the laboratory and inoculated with a volume sufficient to obtain a cellular population of 10^7 cell mL^{-1} in the winery vat. In all the cases fermentation took place at room temperature and kinetics were monitored by measuring the Baume degree of the fermenting must.

At the end of the alcoholic fermentation, wines were run off and placed in a vat for malolactic fermentation to occur. For this purpose, wines were inoculated with commercial lactic acid bacteria *Oenococcus oeni* (Challenge EASY ML, Sepsa-Enartis, Spain) and malolatic fermentation took place at 20 ºC for 9 to 15 days depending on the vat. Subsequently, wines were racked twice and plate-and-frame filtered before bottling.

2.3. Physicochemical analysis

Some physicochemical parameters were analyzed in the resulting wines. L-malic acid and glycerol were determined using enzymatic kits from Boehringer Mannheim (GmbH, Germany), pH by using the potentiometric method, total acidity (g L^{-1} tartaric acid) by indicator titration to pH 8.2 using standardized sodium hydroxide, volatile acidity (g L^{-1} acetic acid) by steam distillation and titration with standardized sodium hydroxide, reducing sugars by Rebelein method which involves reacting reducing sugars with copper (II) in alkaline solution, and ethanol by titrametric dichromate analysis.

2.4. Sensory analysis

An expert panel composed of seven tasters (five females and two males) carried out sensory analysis using orthonasal evaluation. All members belonging to the laboratory staff were trained according to international protocols (ISO 1983; ISO 1985). A 15 ml wine sample was presented in dark glass covered with a plastic dish.

Quantitative descriptive analysis (QDA) was carried out using 13 sensory terms: green, floral, red fruit, ripe fruit, raisin, sweet, meat/sweat odour, nutty, citrus, balsamic, spicy, coffee and general impression. These terms were selected by the panel during preliminary sessions to describe the samples. The selected attributes were put on a tasting-card and panellists were asked to rank each descriptor on a 10-cm unstructured scale (from unnoticeable to very strong).

Discriminant sensory evaluation was performed through triangular tests to assess the significant differences caused by each yeast strain in each wine.

2.5. Volatile compound analysis

We used two methods of analysis because we expected to find volatile compounds with different volatilities and a wide range of concentrations. Therefore, those compounds present in high concentrations (major volatile compounds) were determined by direct injection using Gas Chromatography-Flame Ionization Detection (GC-FID). The minor compounds were extracted by Headspace Sorptive Extraction (HSSE) and then determined by Gas Chomatography-Mass Spectrometry (HSSE-GC-MS).

The 51 standards of aroma compounds used for quantification (see Table 2) were obtained from several commercial sources as follows: 2, 3, 14, 15, 19-21, 23-27, 29-32, 40-42, 45-51, (Sigma Aldrich, Madrid, Spain); 1, 4, 6-10, 13, 17, 18, 28, 34-39, 44, (Merck, Darmstadt, Germany); 5, 11, 12, 16, 22, 33, 43 (Fluka, Madrid, Spain). 4-methyl-2-pentanol (Merck) was used as the internal standard (IS).

2.5.1. GC-FID Analysis.

Ethyl acetate, acetaldehyde, methanol, propanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol were quantified by GC-FID using the method proposed by Morales et al. (2001). A 1 mL sample was filtered through 0.22 μm Millex-

GV13 filters and 10 µL of 4-methyl-2-pentanol at 102.14 mg L^{-1} was added as the internal standard (IS).

The samples were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID). 1 µL of filtered sample was injected in the split mode (1:60) into a CP-Wax 57 CB column, 50 m x 0.25 mm ID x 0.2 µm film thickness (Varian, Middelburg, Netherlands). The carrier gas was H_2 at 1 mL min^{-1}. The program temperature was: 35 ºC for 5 min, ramped at 4 ºC min^{-1} to 150 ºC and held for 17.5 min. The injector was set at 220 ºC and the detector at 250 ºC. The data acquisition software was the HPChemstation data processing system (Agilent Technologies, Santa Clara, California).

2.5.2. HSSE-GC-MS Analysis.

The HSSE sampling conditions were as follows (2008): 5 mL of sample and 10 µL of 4-methyl-2- pentanol (IS) at 1045 mg L^{-1} was placed into a 20-mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with a 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put in an open glass insert and placed in the vial to carry out the extraction in the headspace. Then, the vial was tightly capped and heated for 60 minutes at 62 ºC in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water and dried with lint-free tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed in a glass tube 60 mm long, 6 mm o.d. and 4 mm i.d. This tube was then placed on the autosampler tray of the thermo desorption unit for GC-MS analysis.

Gas Chromatography analysis was carried out with a 6890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel). The thermal desorption was performed in splitless mode and with a flow rate of 90 mL min^{-1}. The desorption temperature program was the following: 35 ºC for 1 min, ramped at 60 ºC min^{-1} to 250 ºC and held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner was held at –35 ºC with liquid nitrogen for the total desorption time and then raised at 10 ºC s^{-1} to 290 ºC and held for 4 min. The solvent vent mode was used to transfer the sample to the analytical column. A CPWax-57CB column, 50 m x 0.25 mm, 0.20 µm film thickness (Varian, Middelburg, Netherlands) was used and the carrier gas was He at a flow rate of 1 mL min^{-1}. The oven temperature program was 35 ºC for 5

min, which was then raised to 220 ºC at 2.5 ºC min^{-1} (held for 5 min). The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 ºC, respectively.

Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35 to 350 amu.

All data were recorded using a MS ChemStation (Agilent Technologies, Santa Clara, California). Peaks were identified using the NIST 98 library and confirmed by standard retention indices when they were available. Quantification was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard (Callejón et al., 2008). The samples were analyzed in triplicate and blank runs were made with empty glass tubes before and after each analysis. RIs were calculated from the retention times of n-alkanes by linear interpolation, in accordance with the literature (Silva Ferreira et al., 2003).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and principal component analysis (PCA) were performed using Statistica, version 7.0 software (Statsoft, Tulsa, USA).

3. Results and Discussion

3.1. Yeast analysis and fermentation kinetics

Forty yeast strains were isolated from the fresh juice before yeast inoculation. According to PCR-RFLP of the 5,8S rDNA gene, 38 isolates corresponded to non-*Saccharomyces* strains, and the remainder to the *Saccharomyces* genus. Further analysis characterised the last strains as the commercial yeast Excellence XR, frequently used by the winery.

To determine the implantation of the inoculated yeast in each vat, samples at different fermentation stages were analysed by mitochondrial DNA RFLP. Fig.1 shows the restriction profiles of the five strains tested, and Fig. 2 indicates the initial yeast population in the fresh must and during the fermentation progress (day 1, day 3 and day 6) in each vat. The restriction profiles of all the isolated colonies matched the profile of the inoculated yeast in their respective vats. Thus, in all the vinifications the inoculated yeast strains began to predominate immediately after inoculation (24 h, 100%

implantation) and the same results were obtained at the middle and the end of the fermentation. The inoculated yeast population in each vat was therefore sufficient to eliminate a significant quantity of wild no-*Saccharomyces* and *Saccharomyces* yeast throughout the course of fermentation.

Yeast enumeration in the fresh must before inoculation revealed an initial population of 3.1 x 10^4 cell mL^{-1}, mainly corresponding to non-*Saccharomyces* species as described above. On the other hand, starters of pure cultures were inoculated in sufficient volume to reach a final population of 10^7 cell mL^{-1} in the must. Thus, differences between the initial yeast population in the fresh must and the inoculated yeast population in each vat together with differences in the fermentative activity of the cells in each case justify the rapid implantation of the latter after 24 h.

The progress of the fermentations was monitored by measuring the Baume degree of the fermenting must. A comparison of fermentation kinetics in the five vats is shown in Fig. 3. According to this figure, the duration of all fermentations was about six days. In all the cases the rate of fermentation is very similar and the lag phase happened in only one day. Most of the sugar is consumed in the first three days for all the yeast strains tested, although the commercial strain (XR) showed a slower consumption rate.

3.2. Physicochemical analysis

Table 1 summarizes the chemical composition of the wines obtained. The residual sugar content showed that all yeast strains tested fermented the wines to dryness and the degree of alcohol ranged from 11.9 to 12.6 % vol, which agreed with the amount of sugars in the must. No L-malic acid was detected in any of the samples, total acidity ranged from 4.37 to 5.57 g L^{-1} expressed as tartaric acid, pH was close to 3.5 in all the samples and values for volatile acidity were no greater than 0.3 g L^{-1} acetic acid in any case. Finally, glycerol production varied from 6.2 to 8.6 g L^{-1}. Thus, although slight differences were observed in some parameters according to the inoculated yeast strain, no statistical differences among the physicochemical properties were found.

3.3. Volatile composition

A total of 80 volatile compounds were detected in the red wines analyzed. Among them 51 were quantified with their corresponding calibration curve. Remaining compounds (29) were tentatively identified by comparing their mass spectra with those

in a commercial library and with those reported in the literature. These compounds belong to several chemical classes including alcohols, esters of fatty acid (ethyl, methyl and isoamyl esters), acetic esters, acids, acetals, aldehydes, ketones, volatile phenols, lactones and terpenes (Table 2).

The differences observed in the volatile composition of the wines obtained from different yeast strains seem to be quantitative rather than qualitative, which agrees with previous studies (Mateo et al., 2001; Patel & Shibamoto, 2002; Romano et al., 2003). Hence, wines M and N had the highest total content of volatile compounds (> 2 g L^{-1}). The high total content of volatile compounds in N is caused by acetals (acetaldehyde diethylacetal), whilst M stands out for its high content in ethyl esters, alcohols, ketones and acids.

In general, acetals were the most abundant volatile compounds in all the samples studied, followed by alcohols without ethanol (Fig. 4). The principal compound of this alcoholic fraction is 3-methyl-1-butanol followed by methanol. As Table 2 shows, this last compound is present in higher concentrations in red wine and reached concentrations lower than expected in samples AG and XR (Ribéreau-Gayon, et al. 2006). Methanol results exclusively from enzymic hydrolysis of the methoxyl groups of the pectins during fermentation but it is not formed by alcoholic fermentation. However, the concentrations of higher alcohols in wine are important variables for differentiating between yeast strains because of their strict relation with yeast metabolism (Romano et al., 2003).These compounds have not been considered as factors of wine quality because they possess fusel-like odours (Mallouchos et al., 2002). Moderate concentrations of these, however, contribute to the wine's aromatic complexity. In this study, the value of higher alcohols varied significantly according to the yeast used (Table 2). Other alcohols that reached high concentrations in all the wines were 2-methyl-1-butanol, 2-phenylethanol, 1-propanol and isobutanol. However, only 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenylethanol (rose aroma) have an OAV greater than 1.0 in all the samples. According to Torrens et al. (2008), alcohols characterized by a "vegetal" and "herbaceous" aroma, such as 1-hexanol and *cis*-3-hexen-1-ol, seem to be linked to the yeast strains used. In fact, the highest producer of these compounds was the M strain while the lowest amounts were formed by the XR strain. Significantly different concentrations were found among the strains employed.

Esters in wine have two distinct origins: enzymic esterification during the fermentation process and chemical esterification during long-term aging. The same esters may be synthesized in either way (Ribéreau-Gayon et al., 2006). The most prevalent ester in wine is ethyl acetate. This compound adds complexity to the aroma of wines at low levels, but it can give an unpleasant odour (vinegary) to the wine at concentrations higher than 150 mg L^{-1} (Mallouchos et al., 2002). For all our wines, its content was always below the level considered negative (Table 2), the lowest amount of ethyl acetate being found in the wine made with the commercial yeast strain. However, this compound showed OAV > 1 in all the samples. The other acetic ester which was present in high concentrations was methyl acetate, as was expected in red wines (Ribéreau-Gayon et al., 2006). This compound makes a different contribution to wine aroma since it does not present the typical fruity aroma as the other acetic esters do. As Table 2 shows, concentrations of methyl acetate are higher in wines elaborated using autochthonous yeast strains than in those made using commercial strains.

Among acetates, isoamyl acetate has been considered as a quality factor by several authors especially in young wines (Ferreira et al., 1995; Van der Merwe & Van Wyk, 1981). This compound was the only acetate to reach an OAV greater than 1 and only in the XR wine. Moreover, its concentration was significantly higher compared with those provided by autochthonous yeast strains.

On the other hand, wine M had the highest amounts of ethyl esters due to its high content of ethyl lactate and diethyl succinate (malolactic esters).

All of the ethyl esters responsible for the fruity aroma in wine showed OAV >1. Ethyl octanoate, which has a sweet aroma, showed the highest concentrations followed by ethyl hexanoate (green apple aroma) and ethyl butyrate (acid fruit aroma), (Table 2). Ethyl furoate was the ethyl ester with the lowest concentrations, ranging between 54-46 μg L^{-1}. Ethyl isovalerate (strawberry aroma (Callejón et al., 2008)) was only found in the wine made with the commercial yeast strain.

AG and XR were the samples with the highest content of all "fruity" ethyl esters, including unquantified ones such as ethyl decanoate, ethyl *trans*-4-decenoate, ethyl dodecanoate, etc. Moreover, we detected some isoamylic esters (isoamyl hexanoate, octanoate and decanoate), the samples AG and XR particularly standing out for their high contents. Therefore, these samples should be the fruitiest wines.

According to Pérez-Coello et al. (1999), yeast strains differ in their ability to produce acetaldehyde depending on the enzymatic activity (alcoholic dehydrogenase). In our study, we found significant differences among the acetaldehyde produced by some yeast strains, independently of their origin (autochthonous or commercial) (Table 2).

Fatty acids have been described as giving rise to fruity, cheesy, fatty and rancid notes. Although, C_6-C_{10} fatty acids are usually related to the appearance of negative odours, they are very important for aromatic equilibrium in wines because they oppose the hydrolysis of the corresponding esters (Torrens et al., 2008), and their presence plays an important role in the complexity of the aroma (Shinohara, 1985). The production of medium chain fatty acid in wines depends on which yeast strain is inoculated. Hence, Torrens et al. (2008) observed that specific yeast strains stood out for their production level of characteristic fatty acids (Torrens et al., 2008). In our case, wine M presented the highest total amount of acids. Hexanoic acid was the most abundant acid followed by octanoic and isovaleric acid respectively, excepting wine N, whose main acid was octanoic. Accordingly, our results show that the difference among the yeast strains studied is due to the amount of acid and not the predominant acid. On the other hand, decanoic acid had the lowest concentrations in all samples. In fact, it was the only acid whose OAV did not exceed the unit, in similar way to that observed by Lorenzo et al. (2008).

We did not find notable differences between the wines regarding those compounds that were identified but not quantified, such as *trans*-3-hexanol, ethyl-3-methylthio-propanoate, dihydro-2-methyl-3-(2H)-thiophenone, and β-damascenone among others.

3.4. Sensory analysis

We carried out triangular tests to compare the wines obtained using the different yeast strains. In all cases, the wines were differentiated by the panel ($p<5$).

The aroma of the wines were assessed by seven tasters, using thirteen descriptors (green, floral, red fruit, ripe fruit, raisin, sweet, meat/sweat odour, nutty, citrus, balsamic, spicy, coffee and general impression) previously agreed upon as the best for describing sensory characteristics of wines.

Fig.5 shows a "spider-web" graph of the results obtained in quantitative descriptive analysis (QDA). The attributes that did not achieve scores higher than 1 in any sample were not represented in this graph. The sample that showed the highest sensory quality was N because it reached the highest score for attribute "general impression". In contrast, the wine obtained using commercial yeast (XR) had the lowest value for this attribute. In fact, this wine reached the highest value for the "meat/sweat odour" attribute, that is a negative aromatic characteristic.

If we consider the volatile composition of these wines, samples AG and XR should have the highest values for the attributes "ripe fruit" and "sweet aroma" due to their large quantities of fruity ethyl esters. Moreover, these were the only two wines that presented the unpleasant "meat/sweat odour" aroma. The presence of compounds responsible for the "meat/sweat odour" could hinder or distort the assessment of the descriptor "ripe fruit". On the other hand, we found some correlations between descriptors and volatile compounds such as "green" with ethyl acetate or "ripe fruit" with β-damascenone.

PCA of the data obtained in QDA was carried out. Fig. 6 shows the score plot of the first two PCs. The plan made of the first two PCs (Fig. 6) indicates that the samples are divided into three groups. One group is closely related to fresh and vegetable aroma (M, AG), another with fruit-sweet aroma (N, O), and the last group with the unpleasant aroma "meat/sweat odour" (XR). This last sample is also the most separated from the rest. These results are consistent with those obtained in the triangle tests. The samples differentiated by the panel with the highest percentages of success are those that appear more distant in Fig.6, such as: M-N, N-XR, M-O or AG-XR.

4. Conclusions

The concentrations of most of the volatile compounds were significantly influenced by the yeast strain. Furthermore, because the implantation percentage of the strain sown in each vat was 100 %, we can say that each strain tested is responsible for the volatile profile of the resulting wine. The differences observed in the volatile composition of wines obtained from different yeast strains appear to be quantitative rather than qualitative.

The results obtained in the sensory analysis suggest that autochthonous yeast produced wines of higher organoleptic quality.

Autochthonous yeast strains could be employed instead of commercial ones, thus enhancing the biodiversity.

Autochthonous yeast strains have been shown to be able to produce wines with different volatile profiles. Some of them have produced wines rich in alcohols and others have produce wines rich in fruity esters. All of them could be suitable for winemaking depending on the desired wine style. Moreover, several authors have demonstrated that coinoculation of wine yeast can be used in order to modulated the volatile composition and sensory profile of wines when a balanced yeast combination is used. Coinoculated fermentations are a promising tool for the wine industry, allowing winemakers to alter the aroma of wines according to market specifications (King et al., 2008). Future research will focus on coinoculated fermentations.

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Figures

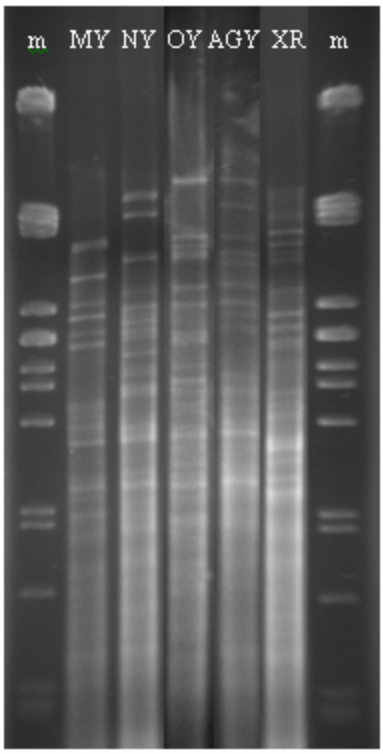


Figure 1. Electrophoretic profiles of mitochondrial DNA of yeast strains used in the study (MY, NY, OY, AGY and XR) digested with *Hinf*I. (m), marker: phage lambda digested with PstI.

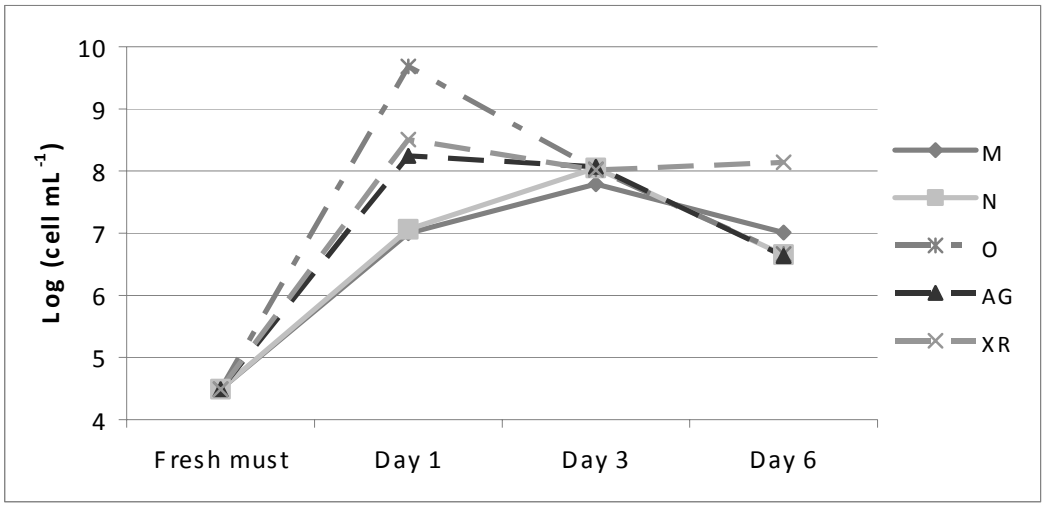


Figure 2. Yeast population in the fresh must and during the fermentation (day 1, day 2 and day 3) in each experiment.

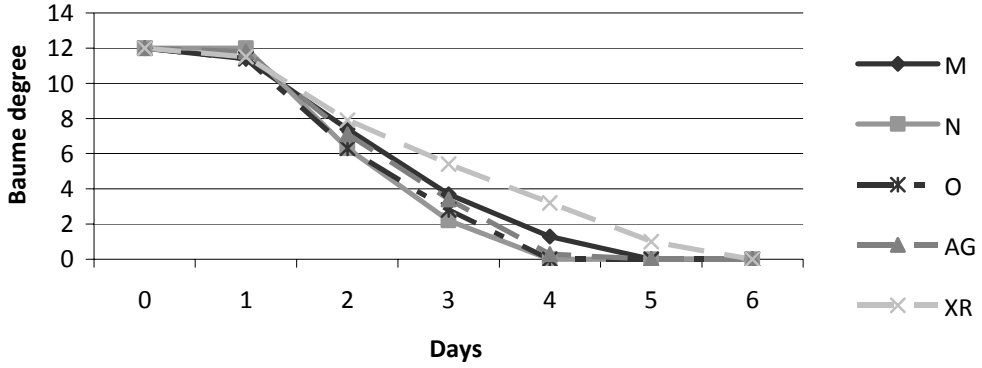


Figure 3. Fermentation kinetics of the five yeast strains monitored by Baume degree.

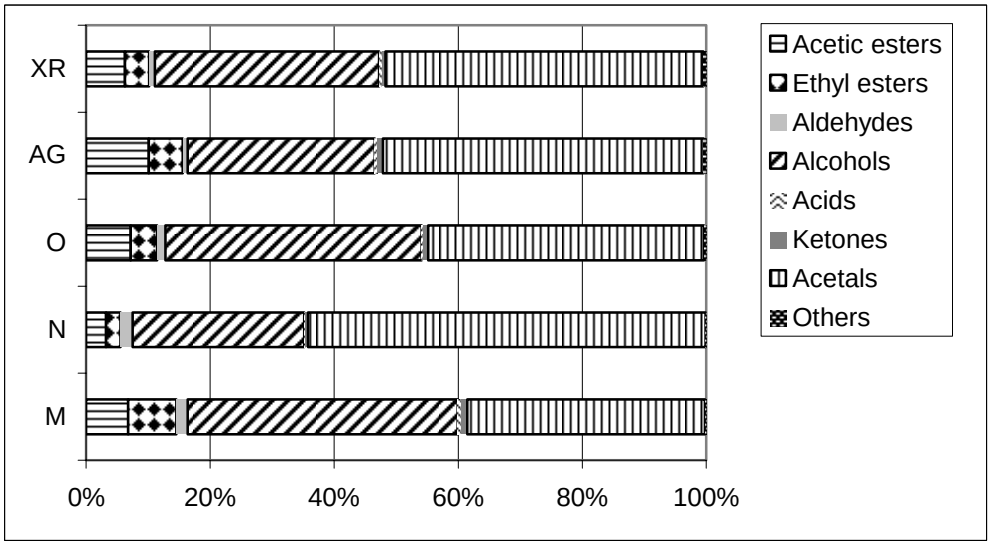


Figure 4. Contribution (%) of different groups of quantified volatile compounds to the volatile profile of wines.

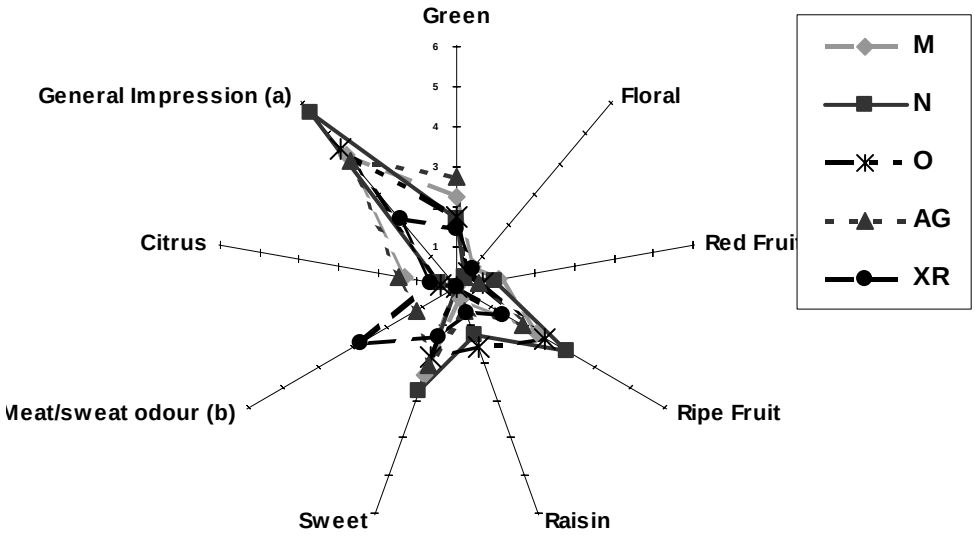


Figure 5. Sensory profiles of red wine samples (a: significant differences between samples produced by commercial and autochthonous yeast strains; b: significant differences between samples produced by commercial and O, N, and M yeast strains respectively.

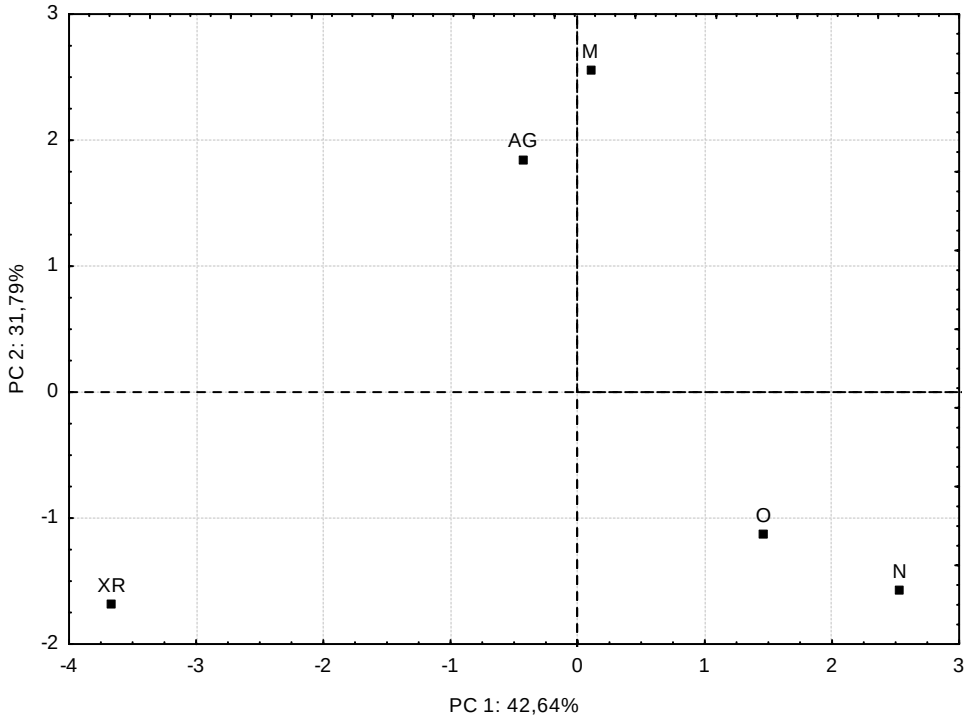


Figure 6.A

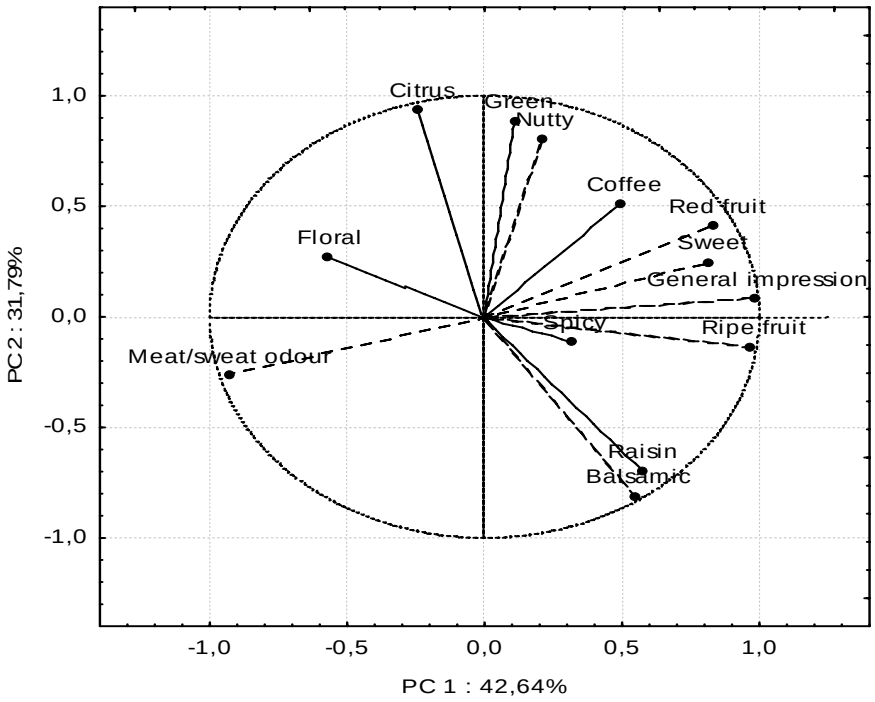


Figure 6.B

Figure 6. Score plot of sensory descriptive variables (A) and wine samples (B) in the plan made of the first two principal components (PC1 against PC2).

Tables

Table 1. Chemical composition of wines obtained.

Wines	% vol. ethanol	Residual sugars ($g\ L^{-1}$)	Glycerol ($g\ L^{-1}$)	L-malic acid ($g\ L^{-1}$)	Total acidity ($g\ L^{-1}$)	pH	Volatile acidity ($g\ L^{-1}$)	Free SO_2 ($mg\ L^{-1}$)	Total SO_2 ($mg\ L^{-1}$)
M	11.9±0.1	3.70±0.14	8.6±0.1	nd	5.57±0.06	3.4±0.0	0.26±0.01	20.9±2.3	193±23
N	12.4±0.0	1.55±0.07	6.7±0.0	nd	4.37±0.06	3.5±0.0	0.28±0.02	30.0±5.7	243±13
R	12.3±0.0	2.65±0.07	6.2±0.2	nd	4.73±0.34	3.5±0.0	0.27±0.01	29.5±3.3	178±21
AG	12.5±0.0	2.65±0.07	6.7±0.0	nd	4.93±0.06	3.5±0.0	0.27±0.01	27.9±2.5	141±19
XR	12.6±0.1	2.60±0.14	7.4±0.1	nd	5.29±0.11	3.4±0.0	0.26±0.03	35.9±1.9	111±4
mean	12.4±0.3	2.69±0.67	7.0±0.9	-	4.88±0.47	3.5±0.0	0.27±0.02	30.1±5.9	173±45

The data are mean values of duplicates; nd: no detected

Table 2. Volatile compounds of organic red wine.

Nº	Compound	Mean concentration (µg/L)				
		M	**N**	**O**	**AG**	**XR**
Aldehydes						
1	Acetaldehyde*	$36 \pm 4^{c,d,1}$	$53 \pm 6^{c,d,1}$	18.7 ± 0.4^{1}	$9.1 \pm 0.9^{a,e}$	$11 \pm 1^{a,e,1}$
2	Hexanal	$7.3 \pm 0.8^{b,d}$	$8.9 \pm 0.8^{b,c,d}$	$20.5 \pm 0.7^{a,c,d,e}$	$5.4 \pm 0.5^{a,b,d}$	$26.4 \pm 1.5^{a,b,c,e}$
3	2-Furfuraldehyde	$940 \pm 20^{a,b}$	$731 \pm 31^{b,e}$	$481 \pm 59^{a,c,d,e}$	779 ± 60^{b}	1081 ± 127^{b}
4	Benzaldehyde	$45 \pm 7^{a,b,c}$	n.q.	n.q.	n.q.	$39 \pm 3^{a,b,c}$
5	5-Methyl-2-furfuraldehyde	$114 \pm 13^{a,b,c}$	$55 \pm 7^{a,b}$	$30 \pm 4^{a,c,d,e}$	$64 \pm 3^{b,e}$	$57 \pm 8^{b.e}$
	Total aldehydes*	36.7	54.0	19.3	9.9	12.4
Acetal						
6	Acetaldehyde diethylacetal*	$761 \pm 82^{a,1}$	$1764 \pm 2^{b,c,d,e,1}$	$644 \pm 43^{a,1}$	$696 \pm 3^{a,1}$	$680 \pm 7^{a,1}$
Acetic esters						
7	Methyl acetate*	1.680 ± 0.003^{d}	1.450 ± 0.023^{d}	$2.05 \pm 0.05^{d,e}$	1.35 ± 0.09^{b}	$1.00 \pm 0.08^{a,b,e}$
8	Ethyl acetate*	$132.0 \pm 0.2^{a,b,d,1}$	$86 \pm 11^{c,e,1}$	$101 \pm 6^{c,e,1}$	$133 \pm 5^{a,b,d,1}$	$80 \pm 12^{c,e,1}$
9	Propyl acetate	$84 \pm 2^{a,b,c,d}$	$212 \pm 4^{b,c,d,e}$	$157 \pm 7^{a,c,d,e}$	$76.3 \pm 0.8^{a,b,d,e}$	$58 \pm 4^{a,b,c,e}$
10	Isobutyl acetate	n.q.	n.q.	$125 \pm 6^{a,d,e}$	$116.5 \pm 1.4^{a,b,d,e}$	n.q.
11	Butyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
12	Isoamyl acetate*	$0.99 \pm 0.03^{c,d}$	$1.03 \pm 0.02^{c,d}$	1.1 ± 0.1^{d}	$1.31 \pm 0.03^{a,d,e}$	$1.72 \pm 0.03^{a,b,c,e,1}$
13	Hexyl acetate	n.q.	n.q.	n.q.	n.q.	n.q.
14	Benzyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
15	2-Phenylethyl acetate	n.q.	n.q.	n.q.	n.q.	n.q.
	Total acetic esters*	134.7	88.7	104.0	135.5	83.2
Ketones						
16	Diacetyl*	n.q.	n.q.	n.q.	3.20 ± 0.22^{1}	n.q.
17	Acetoin*	20 ± 14	10.74 ± 0.03^{b}	11.45 ± 0.18^{a}	n.q.	n.q.
18	Acetophenone	n.q.	n.q.	n.q.	n.q.	n.q.
	Total ketones*	20.0	10.7	11.4	3.2	-

*: concentration in mg/L
n.d.: below detection limit
n.q.: below quantification limit
[1]: OAV >1
[a]: significant differences with sample N
[b]: significant differences with sample O
[c]: significant differences with sample AG
[d]: significant differences with sample XR
[e]: significant differences with sample M

Table 2. (Continued)

Nº	Compound	Mean concentration (µg/L)				
		M	N	O	AG	XR
Ethylic esters						
19	Ethyl propanoate	n.d.	n.d.	n.d.	n.d.	n.d.
20	Ethyl isobutyrate	n.q.	n.q.	n.q.	$130 \pm 2^{a,b,d,e,1}$	$103 \pm 2^{a,b,c,e,1}$
21	Ethyl butyrate	$426 \pm 15^{a,b,d,1}$	$457.4 \pm 1.5^{b,c,d,e,1}$	$386 \pm 9^{a,c,d,e,1}$	$669 \pm 14^{a,b,d,1}$	$456 \pm 30^{a,b,c,e,1}$
22	Ethyl 2-methylbutyrate	n.q.	n.q.	n.q.	n.q.	n.q.
23	Ethyl isovalerate	n.q.	n.q.	n.q.	n.q.	$63.7 \pm 1.7^{a,b,c,e,1}$
24	Ethyl valerate	n.q.	n.q.	n.q.	n.q.	n.q.
25	Ethyl hexanoate	$502 \pm 64^{c,d,1}$	$597 \pm 51^{c,d,1}$	$564 \pm 2^{c,d,1}$	$1138 \pm 63^{a,b,d,e,1}$	$833 \pm 58^{a,b,c,e,1}$
26	Ethyl heptanoate	n.q.	n.q.	n.q.	n.q.	n.q.
27	Ethyl lactate*	$148 \pm 6^{a,b,c,d,1}$	$59.9 \pm 0.9^{c,d,e,1}$	$59 \pm 7^{e,1}$	$69 \pm 2^{a,d,e,1}$	$45.5 \pm 0.7^{a,b,c,1}$
28	Ethyl octanoate	$1143 \pm 131^{c,d,1}$	$810 \pm 24^{c,d,1}$	$1030 \pm 117^{c,d,1}$	$1700 \pm 103^{a,b,e,1}$	$1723 \pm 96^{a,b,e,1}$
29	Ethyl furoate	$53.9 \pm 0.6^{a,1}$	$48 \pm 2^{e,1}$	51 ± 3^{1}	48.3 ± 1.8^{1}	46 ± 4^{1}
30	Ethyl benzoate	n.q.	n.q.	n.q.	n.q.	n.q.
31	Ethyl phenylacetate	n.q.	n.q.	n.q.	n.q.	n.q.
32	Diethyl succinate*	$3.5 \pm 0.3^{a,b,c}$	1.7 ± 0.3^{e}	$1.14 \pm 0.16^{d,e}$	$1.4 \pm 0.2^{d,e}$	$2.7 \pm 0.3^{b,c}$
	Total ethylic esters*	154.0	63.4	61.9	74.4	51.4
Alcohols						
33	Metanol*	$231 \pm 31^{a,c,d}$	$129 \pm 3^{c,d,e}$	$136.9 \pm 1.3^{c,d}$	$54 \pm 8^{a,b,e}$	$64 \pm 9^{a,b,e}$
34	1-Propanol*	$44 \pm 5^{a,b,c,d}$	$104 \pm 9^{b,c,d,e}$	$69.3 \pm 1.7^{a,c,d,e}$	$19 \pm 3^{a,b,e}$	$16.1 \pm 2.3^{a,b,e}$
35	Isobutanol*	39.4 ± 1.8^{b}	34.6 ± 1.3	27.0 ± 0.9^{e}	35.6 ± 1.4	33.5 ± 0.7
36	2-Methyl-1-butanol*	$87 \pm 3^{b,c,d,1}$	$82 \pm 5^{c,1}$	$75.8 \pm 0.5^{c,e,1}$	$48 \pm 7^{e,1}$	$57 \pm 7^{a,b,e,1}$
37	3-Methyl-1-butanol*	$369 \pm 10^{b,c,d,1}$	$330 \pm 23^{b,c,1}$	$234.5 \pm 1.4^{a,e,1}$	$208 \pm 25^{a,e,1}$	$246 \pm 36^{e,1}$
38	1-Hexanol	$2613 \pm 26^{a,b,c,d}$	$2351 \pm 21^{d,e}$	2212 ± 101^{e}	$2350 \pm 12^{d,e}$	$1902 \pm 36^{a,c,e}$
39	*Cis*-3-hexen-1-ol	$63.7 \pm 0.7^{a,b,c,d}$	48 ± 3^{e}	45 ± 26^{e}	45.0 ± 0.5^{e}	43.4 ± 2.1^{e}
40	Benzyl alcohol	$453.0 \pm 1.1^{b,c,d}$	437 ± 23^{d}	331 ± 3^{e}	$408 \pm 3^{b,d}$	$348 \pm 9^{a,c,e}$
41	Furfuryl alcohol	1083 ± 117^{c}	$1342 \pm 10^{b,c}$	$1228 \pm 24^{a,c}$	$2273 \pm 134^{a,b,d,e}$	1323 ± 52^{c}
42	2-Phenylethanol*	$92 \pm 4^{c,1}$	$77 \pm 4^{b,c,1}$	$51 \pm 4^{a,1}$	$37.94 \pm 0.01^{a,e,1}$	60 ± 8^{1}
	Total alcohols*	865.4	760.5	599.0	406.7	479.5

*: concentration in mg/L
n.d.: below detection limit
n.q.: below quantification limit
[1]: OAV >1
[a]: significant differences with sample N
[b]: significant differences with sample O
[c]: significant differences with sample AG
[d]: significant differences with sample XR
[e]: significant differences with sample M

Table 2. (Continued)

Nº	Compound	Mean concentration (µg/L)				
		M	**N**	**O**	**AG**	**XR**
Terpene						
43	α-Terpineol	n.d.	n.q.	n.d.	n.q.	n.d.
Acids						
44	Isovaleric acid	$2178 \pm 305^{a,b,c,1}$	$1104 \pm 28^{b,c,e,1}$	$715 \pm 34^{a,c,d,e,1}$	$1208 \pm 8^{a,b,e,1}$	$1343 \pm 99^{b,1}$
45	Hexanoic acid	$4571 \pm 593^{a,b,d,1}$	$1806 \pm 145^{c,e,1}$	$1915 \pm 256^{c,e,1}$	$3081 \pm 217^{a,b,d,1}$	$1965 \pm 221^{c,e,1}$
46	Heptanoic acid	n.q.	n.q.	n.q.	n.q.	n.q.
47	Octanoic acid	$3038 \pm 388^{b,c,1}$	$2358 \pm 322^{b,1}$	$836 \pm 124^{a,d,e,1}$	$1317 \pm 131^{e,1}$	$1885 \pm 278^{b,1}$
48	Nonanoic acid	n.q.	n.q.	n.q.	n.q.	n.q.
49	Decanoic acid	$802 \pm 122^{b,c,d}$	$960 \pm 127^{b,c,d}$	$172 \pm 26^{a,e}$	$172 \pm 3^{a,d,e}$	$278 \pm 32^{a,c,e}$
	Total acids*	10.6	6.2	3.6	5.8	5.5
Lactones						
50	γ-Butyrolactone	$4161 \pm 162^{b,d}$	$3426 \pm 342^{b,c,d}$	$5040 \pm 118^{a,e}$	5650 ± 552^{a}	$6167 \pm 490^{a,e}$
Phenols						
51	Guaiacol	$61.7 \pm 2.1^{a,b,c,d,1}$	$39 \pm 3^{b,c,e,1}$	$24 \pm 3^{a,e,1}$	$21.6 \pm 1.2^{a,e,1}$	$26 \pm 4^{e,1}$
	Total amounts*	2016	2763	1464	1355	1324

*: concentration in mg/L
n.d.: below detection limit
n.q.: below quantification limit
[1]: OAV >1
[a]: significant differences with sample N
[b]: significant differences with sample O
[c]: significant differences with sample AG
[d]: significant differences with sample XR
[e]: significant differences with sample M

CONCLUSIONES GENERALES

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se obtienen las siguientes conclusiones:

1. Durante la campaña de 2005 se realizaron análisis de la población de levaduras en dos depósitos de Cabernet Sauvignon. Todos los aislamientos realizados durante el proceso de fermentación correspondieron a cuatro cepas de *S. cerevisiae* comerciales empleadas en la bodega durante esa campañas y/o anteriores, con patrones de restricción A, B, C y D (levaduras comerciales LSA2, LSA4, LSA3 y LSA7, respectivamente). No se aislaron, por tanto, cepas de *S. cerevisiae* autóctonas o locales y sólo se aislaron cepas no-*Saccharomyces* a partir de la muestra del mosto fresco.
2. Durante la campaña de 2007 la participación de levaduras comerciales en el desarrollo de las fermentaciones en bodega siguió siendo evidente tanto en fermentaciones inoculadas con pie de cuba natural (depósitos de Merlot y Syrah) como en el depósito de Cabernet Sauvignon (inoculado con una levadura comercial), identificándose hasta 5 cepas diferentes de levaduras comerciales. Si bien, a diferencia de lo descrito para la campaña de 2005, se observó la presencia de cepas autóctonas de *S. cerevisiae* en el transcurso de las fermentaciones. Entre las 8 cepas autóctonas caracterizadas, dos de ellas, las de perfiles de restricción AG y AM destacaron en alguna de las fases de fermentación en los depósitos de Merlot y Syrah y fueron las únicas cepas autóctonas encontradas en el depósito de Cabernet Sauvignon al final de la fermentación, lo que podría indicar que se trata de cepas capaces de competir con las comerciales presentes en el mosto.

   Por otra parte, el análisis de diversas superficies de bodega reveló la presencia de diferentes cepas de *S. cerevisiae*, principalmente en las barricas muestreadas y, en menor medida, en la prensa. Se trataba mayoritariamente de tres cepas autóctonas de *S. cerevisiae*, y sólo en la prensa se aisló la cepa comercial LSA7; sin embargo, ninguna de las cepas autóctonas encontradas fue aislada después en las fermentaciones estudiadas durante la vendimia, sólo la comercial LSA7, usada para inocular el depósito de Cabernet Sauvignon y aislada en los tres depósitos.
3. El análisis de la microflora levaduriforme presente en el viñedo durante las campañas 2006 y 2007 a partir de uvas en diferentes estados de maduración, reveló

la presencia mayoritaria de especies no-*Saccharomyces* en todas las fechas muestreadas, principalmente *H. guilliermondii*, *Kluyveromyces thermotolerans, H. uvarum* e *Issatchenkia orientalis*; pero en muestras de vendimia y fechas posteriores a ella, también se aislaron diversas cepas de *S. cerevisiae*, llegando a representar cerca del 20 % y 10 % de los aislamientos en 2006 y 2007, respectivamente, por lo que su presencia parece estar ligada al grado de madurez de la uva. Entre las cepas de *S. cerevisiae* caracterizadas, solo dos corresponden a cepas comerciales (concretamente, LSA3 y LSA7) y fueron aisladas en la campaña de 2006, probablemente favorecida por la práctica de usar las lías de fermentación como fertilizantes en el viñedo en campañas anteriores, facilitando la diseminación de algunas de las cepas comerciales usadas en la bodega; el resto correspondió a diferentes cepas autóctonas de *S. cerevisiae* (4 en la campaña de 2006 y 5 en la de 2007) ninguna de las cuales se repitieron en cada campaña estudiada.

4. En total, durante las campañas de análisis microbiológico en bodega y viñedo durante 2005 a 2007, se han caracterizado 24 cepas de *S. cerevisiae*, 18 autóctonas y 6 comerciales. Estos resultados indican una baja biodiversidad de cepas *S. cerevisiae* tanto en las fermentaciones desarrolladas en bodega como en las de laboratorio a partir de racimos de uva, que puede deberse a las propias características de la bodega y del viñedo adyacente (construida en 2003 y de reciente plantación, respectivamente) y de las fermentaciones (realizadas en su mayoría con levaduras comerciales).

5. Algunas de las cepas, tanto autóctonas como comerciales, de *S. cerevisiae* fueron estudiadas en relación a sus propiedades enológicas (cepas MY, NY, OY, RY, SY, TY, AGY y AMY; y las comerciales LSA2, LSA3, LSA4, LSA7 y LSA8), no encontrándose diferencias significativas entre el comportamiento fermentativo de cada una de ellas en medio sintético con distinta concentración de glucosa a 20 ºC, pero sí a 30 ºC. Tampoco se encontraron diferencias estadísticamente significativas en los parámetros fisicoquímicos generales al fermentar mosto de Merlot estéril.

   De los 38 compuestos volátiles identificados en los vinos resultantes de dichos ensayos, principalmente esteres, alcoholes, ácidos, aldehídos y acetales, sólo 16 mostraron valores de OAV mayor que 1. El análisis sensorial indicó diferentes preferencias por los vinos obtenidos siendo las cepas autóctonas RY, SY y AMY y las comerciales LSA3 y LSA8 las que obtuvieron valores mas bajos en la impresión

general o mostraron algún defecto olfativo. Por todo ello, sólo las cepas autóctonas MY, NY, OY y AG, y la comercial LSA7 fueron seleccionadas para ensayos a escala piloto en la bodega.

6. El ensayo en bodega de las cinco cepas seleccionadas en mosto de la variedad Merlot indicó la total implantación de la cepa inoculada en cada depósito y un correcto desarrollo de la fermentación alcohólica, con consumo casi total de los azúcares, no encontrándose, además, diferencias significativas entre los vinos obtenidos en relación a los parámetros fisicoquímicos generales. Sin embargo, la cepa de levadura inoculada en cada ensayo influyó significativamente en la composición, cuantitativa mas que cualitativa, en compuestos volátiles. El análisis sensorial de los vinos obtenidos sugiere que las cepas autóctonas producen vinos con mejores cualidades sensoriales que las comerciales; mientras que algunas de ellas producen vinos más ricos en alcoholes, otras lo hacen en ésteres afrutados.

ANEXO I

1. Medio de crecimiento, selección, recuento y conservación de levadurasYEPD (Yeast Extract Peptone Dextrose) agar

Extracto de levadura (Cultimed)	10 g/L
Peptona (Cultimed)	10 g/L
Glucosa (Panreac)	20 g/L
Agar (Cultimed)	20 g/L

Se autoclava a 120 ºC, 20 min. y se distribuye en placas de Petri.

1.2. YEPD líquido

La misma composición del medio anterior pero sin agar.

1.3. Medio lisina (Barnet et al., 1990)

Este medio se emplea para diferenciar entre levaduras *Saccharomyces* y no-*Saccharomyces* (Barnet et al., 1990) ya que las primeras no pueden crecer en un medio con Lisina como única fuente de carbono.

YNB-aa-NH_4SO_4 (Yeast Nitrogen Base without aminoacids and ammonium sulfate) (Difco)	1,7 g/L
Lisina (Sigma)	2 g/L
Glucosa (Panreac)	10 g/L
Agar (Cultimed)	20 g/L

Se autoclava a 120 ºC, 20 min. y se distribuye en placas de Petri.

El medio control del experimento se prepara de la misma forma pero en vez de lisina, se añaden 5 g/L de sulfato amónico.

1.4. Mosto sintético (modificado de Querol, 1992)

Glucosa (Panreac)	225 ó 275 g/L
$K H_2 PO_4$ (Panreac)	5,0 g/L
$(NH_4)_2 SO_4$ (Panreac)	2,0 g/L
$MgSO_4 \cdot 7 H_2O$ (Panreac)	0,4 g/L
Acido málico (Merck)	5,0 g/L
Extracto de levadura (Cultimed)	1,0 g/L

Se autoclava a 115 ºC, 30 min.

1.5. Solución para recuento de Unidades Formadoras de Colonias (UFC)/mL (E.B.C., 1997)

Azul de metileno (Panreac)	0,01 g
Citrato sodio dihidratado (Panreac)	2,00 g
Agua destilada	10 mL

Se filtra (Ø 0,22 µm) y se lleva a un volumen de 100 mL con agua destilada estéril.

Se conserva en frasco topacio en oscuridad a 4 ºC.

1.6. Conservación células a -80 ºC

Se dejan crecer las cepas de levaduras en medio YEPD líquido a 28 ºC, 24 horas.

Se añade 800 µl del cultivo de células a 200 µl de glicerol (Panreac) estéril.

Se homogeniza con el vórtex y se congela.

2. Técnicas de Biología Molecular

2.1. Análisis de los perfiles de restricción (RFLP) de la región génica 5,8S-ITS del DNA ribosómico (ADNr)

PCR (Reacción en Cadena de la Polimerasa) (Guillamón et al., 1998)

La mezcla de la reacción de PCR (V_{final} = 100 µl) está formada por:

5,0 µl Primer ITS1[a] (10 µM, Invitrogen)
5,0 µl Primer ITS4[a] (10 µM, Invitrogen)
8,0 µl dNTPs (1 mM, Dominion)
10,0 µl Tampón Taq 10X, sin Mg (Dominion)
6,0 µl Cloruro Magnésico (25 mM, Dominion)
65,5 µl Agua Milli-Q

Con un palillo estéril se toma una muestra de cultivo fresco de la levadura a ensayar y se añade a la mezcla de reacción de PCR.

Se calienta a 95 ºC, 15 min. en un termociclador TC-312 (Techne) para romper las paredes y membranas celulares y añadir 0,5 µl *Taq* polimerasa (5 U/µl, Dominion)

Condiciones del programa de PCR:

- Desnaturalización inicial a 95 ºC durante 5 min.
- La PCR se realiza con un programa de 40 ciclos consistente en:

  Desnaturalización a 94 ºC durante 1 min.
  Hibridación a 55,5 ºC durante 2 min.
  Extensión a 72 ºC durante 2 min.

- Extensión final a 72 ºC durante10 min.

[a] Primers ITS1 e ITS4 descritos por White et al. (1990):
Primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3')
Primer ITS4 (5' TCCTCCGCTTATTGATATGC 3')

Gel para la visualización de los productos de PCR

Se prepara gel de agarosa al 1,4 % (D-1 low EEO, Pronadisa) en tampón TAE 1X[b].

La mezcla de carga (V_{final} = 10 µl) está formada por:

3 µl Agua Milli-Q
3 µl Tampón de carga[c]
4 µl ADN amplificado

El gel se tiñe con bromuro de etidio (0,001 µg/mL, Sigma)

Las bandas se visualizan mediante transiluminador de luz UV y se fotografian mediante un documentador de geles Uvitec. El tamaño de cada banda se estimó en comparación con un marcador estándar de longitudes de ADN (7 µl de la escalera de 100 pb, Amersham Biosciences).

[b] TAE 1X: preparado a partir de un stock de TAE 40X (trizma base (Sigma), 242 g/L; ácido acético glacial (Panreac), 57,1 mL; 0,5M EDTA (Panreac) (pH 8)).

[c] Tampón de carga: 50 % glicerol (Panreac), 0,1 M EDTA (Panreac), 5 µl azul de bromofenol (Sigma) al 0,25 %

Digestión del producto de PCR

La mezcla de digestión (V_{final} = 25 µl) consiste en:

15 µl ADN amplificado
3 µl Tampón según el enzima
1 µl Enzima de restricción*
6 µl Agua Milli-Q

Se incuba a 37 ºC durante toda la noche.

*Endonucleasas empleadas: *Hha*I, *Hae*III, *Hinf*I y *Dde*I y sus correspondientes tampones (Takara).

Gel para la visualización de la digestión de los productos de PCR

Se prepara gel de agarosa al 3 % (D-1 low EEO, Pronadisa) con tampón TAE 1X[b].

La mezcla de carga está formada por:

2,5 µl Tampón de carga[c]
25,0 µl ADN digerido

El gel se tiñe con bromuro de etidio (0,001 µg/mL, Sigma).

Las bandas se visualizan mediante transiluminador de luz UV y se fotografiaron mediante un documentador de geles Uvitec. El tamaño de cada banda se estimó en comparación con el marcador de 100 pb (7 µl, Amersham Biosciences).

2.2. Análisis de los perfiles de restricción (RFLP) del ADN mitocondrial (ADNmt)

Extracción del ADN genómico (modificado de Querol et al., 1992)

Se hacen crecer las células en 2 mL de YEPD líquido a 28 ºC durante una noche.

Se centrifuga 3 min. a 12000 rpm. Se elimina el sobrenadante y el precipitado se limpia con 1 mL de agua destilada estéril.

Se centrifuga 3 min. a 12000 rpm. Se elimina el sobrenadante y el precipitado se resuspende en 1 mL de la Solución 1[d].

Se añade 30 µl de zimoliasa (15 mg/mL, USBiological). La mezcla se incuba a 37 ºC durante 20 min.

Se centrifuga 3 min. a 12000 rpm. Se elimina el sobrenadante y el precipitado se resuspende en 500 µl de Solución 2[e].

Se adiciona 13 µl de SDS (Sigma) 10 % pH 7,2, se homogeniza en el vórtex y se incuba a 65 ºC durante 5 min.

Se añade 200 µl de acetato potásico[f] 5M/3M, se agita y se deja en hielo 10 min.

Se centrifuga 15 min. a 13000 rpm. Se transfiere el sobrenadante a un nuevo Eppendorf, se adicionan 700 µl de isopropanol (Sigma), se agita suavemente y se deja precipitar durante 10 min. a temperatura ambiente.

Se centrifuga 10 min. a 12000 rpm. Se elimina el sobrenadante y se lava con 500 µl de etanol (Panreac) 70 % (v/v).

Se centrifuga 5 min. a 12000 rpm. Se elimina el sobrenadante.

Se seca el ADN a temperatura ambiente o con una bomba de vacío.

Se resuspende el ADN en 18 µl de TE[g].

Se conserva a -20 ºC.

[d] Solución 1: 0,9 M sorbitol (Sigma); 0,1 M EDTA (preparado a partir de un stock de 0,5 M EDTA·$2H_2O$ (Panreac) pH 8).

[e] Solución 2: 50 mM Tris-HCl (preparado a partir de un stock de Tris-HCl 1M: 121g/L Trizma base (Sigma); 42 mL de ácido clorhídrico); 20 mM EDTA (preparado a partir de un stock de 0,5 M EDTA·$2H_2O$ (Panreac) pH 8).

[f] Acetato potásico 5M/3M: 294,7 g/L acetato potásico (Panreac); ácido acético glacial hasta pH 4,8.

[g] TE: 10 mM Tris-HCL (preparado a partir de un stock de Tris-HCl 1M: 121g/L Trizma base (Sigma); 42 mL de ácido clorhídrico); 1 mM EDTA (preparado a partir de un stock de 0,5 M EDTA·$2H_2O$ (Panreac) pH 8).

Digestión del ADN extraído

La mezcla de digestión (V_{final} = 19,5 µl) está compuesta de:

9,0 µl ADN extraído
2,0 µl RNasa (500 µg/mL, Sigma)
2,5 µl Tampón del enzima
1,0 µl Enzima de restricción *Hinf*I (Takara)
5,0 µl Agua Milli-Q

Se incuba a 37 ºC durante una noche.

Gel para la visualización de digestión del ADN extraído

Se prepara un gel de agarosa al 1 % (D-1 low EEO, Pronadisa) en tampón TAE 1X[b].

La mezcla de carga está formada por:

3 µl Tampón de carga BPB[h]
12 µl ADN digerido

El gel se tiñe con bromuro de etidio (0,001 µg/mL, Sigma).

Las bandas se visualizan mediante transiluminador de luz UV y se fotografiaron mediante un documentador de geles Uvitec. El tamaño de cada banda se estimó en comparación con el marcador λ-PstI (4 µl de la digestión del ADN del fago Lambda (Roche) con la endonucleasa PstI (Amersham Biosciences)).

[h] Tampón de carga BPB: 50 % glicerol; 20 % 0,5 M EDTA pH8; 0,25 % azul de bromofenol; 0,25 % xilen glicol

2.3. Secuenciación de la región D1/D2 del ADN ribosómico 26S

PCR (Reacción en Cadena de la Polimerasa) (Baleiras Couto et al., 2005)

La mezcla de reacción de PCR (V_{final} = 100 µl) está formada por:

2,5 µl Primer NL1[i] (10 µM, Invitrogen)
2,5 µl Primer NL4[i] (10 µM, Invitrogen)
8,0 µl dNTPs (1 mM, Dominion)
10,0 µl Tampón Taq 10X, sin Mg (Dominion)
6,0 µl Cloruro Magnésico (25 mM, Dominion)
66,5 µl Agua Mili-Q
0,5 µl Taq polimerasa (5 U/µl, Dominion)
4,0 µl ADN extraído

Condiciones del programa de PCR:

- Desnaturalización inicial a 94 ºC durante 3 min.
- La PCR se realiza con un programa de 36 ciclos consistente en:

  Desnaturalización a 94 ºC durante 1 min.
  Hibridación a 58 ºC durante 1 min.
  Extensión a 72 ºC durante 1,5 min.

- Extensión final a 72 ºC durante 5 min.

[i] Primer NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3')
Primer NL4 (5' GGTCCGTGTTTCAAGACGG 3')

Gel para la visualización de los productos de PCR

Se procede según lo explicado en el apartado 2.1

Purificación de los productos de PCR

Se realizó con el kit Wizard® SV Gel and PCR Clean-Up System (Promega) según las instrucciones del fabricante.

Cuantificación del ADN purificado

La cuantificación de ADN purificado se realizó usando el equipo Nanodrop 3.0.1. (Colema Tecnologies Inc.) midiendo la absorbancia en 1 µl de la muestra a 260 nm y 280 nm. El programa Nano-Drop 3.0.1. de NanoDrop Technologies calcula la concentración del ADN purificado a partir de la medida de 260 nm y mediante el cálculo de la relación A260/A280 proporciona una estimación de la pureza del ácido nucleico.

Secuenciación

La secuenciación del producto de PCR se encargó a una empresa externa (Stab Vida, Portugal).

Para cada cepa de levadura, se alinean las dos secuencias (*forward* y *reverse*) con el programa ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.htmL). Para ello se necesita la secuencia reversa complementaria de una de las dos secuencias a alinear. Ésta se consigue con el programa *Reverse Complement* (http://www.bioinformatics.org/sms/rev_comp.htmL).

La secuencia consenso obtenida se compara usando el servicio *Blast* de la base de datos *NCBI* (http://blast.ncbi.nlm.nih.gov/Blast.cgi) y del buscador *Fungal Genomes* (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl).

3. Determinación del Factor *killer* (Sangorrín et al., 2001)

Preparación del medio

Medio MBM.

El medio está formado por tres fracciones:

- Medio A: peptona, 20 g; extracto de levadura (Cultimed), 10 g; glucosa (Panreac), 20 g; agar (Cultimed), 20 g; agua destilada, 500 mL. Se esteriliza en autoclave a 121 ºC durante 15 min.

- Medio B: Tampón citrato-fosfato 1M pH 4,5 (500 mL). Se prepara mezclando 250 mL de citrato 1M con la cantidad necesaria de fosfato 1M (algo más de 450 mL), de aquí se toman los 500 mL necesarios. Se esteriliza por filtración (Ø 0,022 µm).

    - Citrato 1M: 10,5 g de ácido cítrico monohidratado (Panreac) en 50 mL de agua destilada.

    - Fosfato 1M: 21 g de fosfato dipotásico (Panreac) en 120 mL de agua destilada.

- Solución azul de metileno: azul de metileno (Panreac) al 0,003 % (Boone et al., 1990). Se esteriliza por filtración (Ø 0,022 µm).

Tras la esterilización por separado de las tres fracciones, éstas se mezclan asépticamente a 60 ºC y se dispensa el medio en placas de Petri.

Preparación de las cepas para el análisis

La preparación se hace para todas las cepas de levaduras a ensayar y para tres cepas control: 2 cepas con fenotipo *killer* (K1 y K2) y una con fenotipo sensible (S).

A. Siembra del césped:

Se crecen las cepas en 2 mL de YEPD líquido a 30 ºC durante una noche.
Se lava el cultivo con agua destilada estéril.
Se diluye 1/5 la solución celular.
Se siembran 100 µl de la dilución celular de cada cepa en el medio MBM.

B. Siembra de las cepas mediante réplica:

A partir de un cultivo fresco de levaduras a ensayar y de las tres cepas control se preparan tres "placas madres" en medio YEPD agar:

- "Placa cepas control": con las tres cepas control (K1, K2, S).

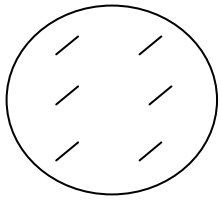
- "Placa autóctonas": con las 8 cepas autóctonas a ensayar y las 3 cepas control.
- "Placa comerciales": con las 5 cepas comerciales a ensayar y las 3 cepas control.

Las placas se incuban a 30 ºC durante una noche.

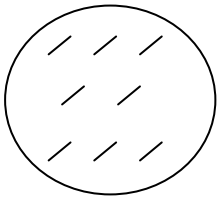
Las placas madres así preparadas se siembran mediante réplica sobre césped en medio MBM del siguiente modo:

- La "placa cepas control" se siembra sobre el césped de cada levadura autóctona y comercial a ensayar.
- Las placas "autóctonas" y "comerciales" se siembran sobre el césped de cada control (K1, K2 y S).

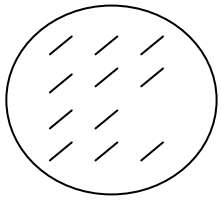
Cada ensayo se hace por duplicado y tras sembrar cada placa, se hace una réplica en una placa con medio YEPD como control de crecimiento para comprobar que se sembró una cantidad de biomasa celular suficiente.



Placa control
K1, K2 y S



Placa LSA
5 LSA y 3 controles
(K1, K2 y S)



Placa AUTÓCTONAS
8 autóctonas y 3 controles
(K1, K2 y S)

Análisis

Se distinguen cuatro fenotipos posibles:

Fenotipo killer/resistente ($K^{\pm}R^{\pm}$): cepas ensayo que producen halo en el césped sensible y los biotipos control K1 y/o K2 no lo producen cuando ellas son el césped.

Fenotipo killer/sensible ($K^{\pm}R^{-}$): cepas ensayo que producen halo en el césped sensible y los biotipos control K1 y K2 producen halo cuando ellas son el césped.

Fenotipo resistente ($K^{-}R^{\pm}$): cepas ensayo que no producen halo en el césped sensible y los biotipos control K1 y/o K2 tampoco lo producen cuando ellas son el césped.

Fenotipo sensible ($K^{-}R^{-}$): cepas ensayo que no producen halo en el césped sensible pero los biotipos control K1 y K2 sí lo producen cuando ellas son el césped.

4. Determinación de la producción de espuma (Regodón et al., 1997)

En un tubo de ensayo con 10 mL de mosto estéril (1g/L dimetil dicarbonato, Fluka) se inocula la cepa de levadura a ensayar (10^6 cel/mL) a partir de un cultivo fresco (24 h). Los ensayos se hacen por duplicado. A continuación los tubos se incuban a 25 ºC sin agitación. Los tubos se pesan a diario para el seguimiento de la fermentación alcohólica y se mide la altura de la espuma.

Análisis

Las levaduras se clasifican en tres categorías según la altura máxima alcanzada:

- **F0**: menos de 2 mm de espuma.
- **F1**: entre 2 – 4 mm de espuma.
- **F2**: más de 4 mm de espuma.

5. Determinación de la producción de SH_2 (Perez-Coello et al., 1999)

Preparación del medio YNBG

YNB (Difco)	6,7 g/L
Glucosa (Panreac)	20,0 g/L

El medio se esteriliza mediante filtración (Ø 0,22 μm).

Se dispensan aproximadamente 90 mL del medio en erlenmeyers de 100 mL, se taponan con algodón y se inserta una tira de papel de acetato de plomo (Panreac).

Se inoculan 10^6 ufc/mL de un cultivo fresco de la levadura a ensayar y se incuba a 25 ºC. Se lleva el control de la fermentación pesando a diario los matraces.

Análisis

Es una medida cualitativa de la producción de SH_2. Si existe producción de SH_2, se verá un precipitado de color negro en la tira de papel correspondiente al sulfuro de plomo (Pb S) formado como producto de la reacción del acetato de plomo (Pb (C_2 H_3 O_2)) con el SH_2 generado por la levadura.

$$Pb\ (C_2\ H_3\ O_2) \cdot 3\ H_20 + H_2S \rightarrow PbS + H_2O + C_2\ H_3\ O_2$$

6. Determinación de la resistencia a SO_2 (adaptación de Parish & Carroll, 1987)

Preparación del medio YNBG

YNB (Difco)	6,7 g/L
Glucosa (Panreac)	10,0 g/L

Se preparan 4 lotes en los que se añade 0, 50, 100 y 200 mg/L de metabisulfito sódico (Panreac).

Se ajusta el pH a 3,3 con HCl o NH_3.

Se esteriliza mediante filtración (Ø 0,45 μm). Se dispensan 10 mL de medio en tubos de ensayo (16 x 160 mm) con campana Durham y se taponan con algodón. Cada dosis se ensaya por duplicado.

Los tubos se inoculan por duplicado con 10^5 ufc/mL de un cultivo fresco de la levadura a ensayar y se incuban a 30 ºC.

Se controla a diario la fermentación por el desprendimiento de CO_2 observado en el desplazamiento de la campana Durham y el pesado de los tubos.

7. Determinación de actividades enzimáticas

7.1. β-Glucosidasa (Rosi et al., 1994)

Preparación del medio

Arbutina (Sigma)	5 g/L
Extracto de levadura (Cultimed)	1 g/L
Agar (Cultumed)	20 g/L

Se deposita 0,1 mL de una solución de cloruro de hierro (III) (Panreac) al 1 % en cada tubo de ensayo. Se dispensan a continuación 5 mL del medio fundido en cada tubo. Se autoclava a 1 atm, 15 min.

Análisis

Los tubos se siembran con un cultivo joven (48 horas en YEPD agar) de la cepa de levadura a ensayar. A continuación se incuban en estufa a 27 ºC durante 15 días. Como testigo se utiliza un tubo sin inocular, y como controles positivos, un tubo sembrado con la cepa T73 modificada genéticamente para ser β-Glucosidasa positiva y otro tubo en el que se dispensa la enzima β-Glucosidasa pura (EC 3.2.1.21, Sigma).

A los 15 días se compara la tonalidad del medio con la del tubo testigo. La sal férrica soluble reaccionará con la quinona libre procedente de la arbutina, dando lugar a la aparición de un color oscuro si actúa el enzima o queda del color original si no lo hace.

7.2. Proteasa (Bilinsky et al., 1987)

Preparación del medio

MYPG agar

Extracto de malta (Merck)	3 g/L
Extracto de levadura (Cultimed)	3 g/L
Peptona (Cultimed)	5 g/L
Glucosa (Panreac)	20 g/L
Agar (Cultimed)	20 g/L

Se dispensa en tubos de ensayo y se autoclava 20 min., a 120 ºC. Se deja enfriar con el agar inclinado.

Skim Milk agar

Este medio está formado por tres fracciones:

A) Se preparan 70 mL de:

Skim Milk (Difco)	100,00 g/L
Tampón fosfato pH 6,7	0,05 M
83,7 % Fosfato monopotásico (Panreac)	6,80 g/L

16,3 % Fosfato disódico (Panreac) 7,10 g/L

Se autoclava 10 min., a 100 ºC

B) Se preparan 60 mL de:

Fosfato monopotásico (Panreac)	24 g/L
Fosfato disódico (Panreac)	35 g/L

Se filtra (Ø 0,22 µm) y se mezcla con la fracción A.

C) Se preparan 480 mL de:

Glucosa (Panreac)	10 g/L
YNB sin aminoácidos ni sulfato amónico (Sigma)	2 g/L
Agar (Cultimed)	1 g/L

Se autoclava 20 min., a 120º C y se mezcla con la fracción restante.

Análisis

Las levaduras a ensayar se siembran en medio MYPG agar y se incuban a 30 ºC durante una noche. A continuación se siembran en el medio Skim Milk agar y se incuban a 27 ºC durante 48 h. La aparición de zonas claras alrededor de las colonias indica la actividad de la enzima. Como control positivo se utiliza Proteasa de *Bacillus licheniformis* (EC.3.4.21.14, Sigma)

8. Determinación de los compuestos volátiles del vino

Los compuestos volátiles se han determinado mediante dos métodos de análisis:

- Para los compuestos volátiles mayoritarios: *Cromatografía de gases con detección de ionización en llama (GC-FID)* por inyección directa.
- Para los compuestos minoritarios: *Extracción en espacio de cabeza por absorción con barras magnéticas agitadoras* y *Cromatografía de Gases-Espectrometría de Masas* (*HSSE-GC-MS*).

Los patrones de compuestos aromáticos empleados para la cuantificación se obtuvieron de varias fuentes comerciales: Sigma-Aldrich, Merck y Fluka (Sigma-Aldrich). Como patrón interno (PI) se utilizó 4-metil-2-pentanol (Merck).

8.1. Cromatografía de gases-detección de ionización de llama (GC-FID)

La cuantificación de acetato de etilo, acetaldehído, metanol, propanol, butanol, 2-metil-1-butanol, 3-metil-1-butanol y 2-feniletanol se realizó por *GC-FID* de acuerdo con el método propuesto por Morales et al. (2001).

Se filtra 1 mL de la muestra a través de filtros Millex-GV13 de 0,22 µm y se añaden 10 µL de 4-metil-2-pentanol (102,14 mg/L) como patrón interno (PI).

Las muestras se analizan utilizando un cromatógrafo de gases Hewlett-Packard 6890 equipado con detector de ionización de llama (FID). Se inyecta 1 µL de muestra filtrada

en el modo división de flujo (1:60) en una columna CP-WAX 57 CB de 50 m x 0,25 mm diámetro interno x 0,2 micras de espesor de película (Varian). El gas portador es H_2 a 1 mL/min.

La temperatura del programa es 35 ºC durante 5 min., se incrementa esta temperatura a razón de a 4 ºC/min. hasta 150 ºC y se mantiene durante 17,5 min.

La temperatura del inyector es 220 ºC y la del detector 250 ºC. El software de adquisición de datos es el sistema de procesamiento de datos HPChemstation (Agilent Technologies).

8.2. Extracción en espacio de cabeza por absorción con barras magnéticas agitadoras y Cromatografía de Gases-Espectrometría de Masas (HSSE-GC-MS)

La determinación de los compuestos minoritarios se llevó a cabo según el método establecido por Callejón et al. (2008). Las condiciones de muestreo *HSSE* fueron las siguientes:

1. En un vial de 20 mL se añaden 1,67 g de NaCl, 5 mL de muestra y 10 µL de 4-metil-2-pentanol (PI) a 1045 mg/L.

2. Se coloca un inserto de vidrio abierto en el vial donde se introduce la barra magnética de 10 mm recubierta de 0,5 mm de polidimetilsiloxano (PDMS) (Twister, Gerstel).

3. Se cierra el vial herméticamente y se calienta durante 60 min. a 62 ºC en un baño termostatizado.

4. Se extrae el twister con pinzas, se enjuaga con agua Milli-Q y se seca con papel.

5. Para la desorción térmica (TD), se introduce el twister en un tubo de vidrio de 60 mm de largo, 6 mm de diámetro exterior y 4 mm de diámetro interior. Este tubo se coloca en la bandeja del muestreador automático de la unidad de desorción térmica para el análisis por GC-MS.

El análisis de cromatografía de gases se lleva a cabo con un equipo Agilent 6890 acoplado a un detector de espectrometría de masas (MS) tipo cuadrupolo Agilent 5975 inert, equipado con un sistema de desorción térmica (TDS2) y un inyector con crioconcentrador CIS-4 PTV (Gerstel).

La desorción térmica se realizó en el modo sin división de flujo de 90 mL/min.

El programa de temperaturas de desorción fue el siguiente: 35 ºC durante 1 min., incrementando esta temperatura 60 ºC/min. hasta 250 ºC y se mantiene durante 5 min.

El inyector CIS-4 PTV se mantiene a -35 ºC con nitrógeno líquido durante el tiempo de desorción total; posteriormente se sube la temperatura a 10 ºC/s hasta 290 ºC y se mantiene durante 4 min.

Para transferir la muestra a la columna de análisis se utiliza el modo de inyección "venteo de solvente". Se usa una columna CPWax-57CB, de 50 m x 0,25 mm, 0,20 µm de espesor de película (Varian) y el gas portador es He, con un flujo de 1 mL/min.

El programa de temperatura del horno es de 35 ºC durante 5 min., que se eleva a razón de 2,5 ºC/min. hasta 220 ºC a y se mantiene durante 5 min.

El cuadrupolo, la fuente y la línea de transferencia se mantuvieron a 150, 230 y 280 ºC, respectivamente.

Se registran espectros de masas de ionización de electrones en el rango de 35 a 350 uma, a 70 eV de energía de electrones mediante un ChemStation MS (Agilent Technologies).

La identidad de los picos se asigna comparando con la biblioteca de espectros NIST 98 y se confirma por los tiempos de retención de los estándares.

La cuantificación se realiza utilizando el área relativa del ion cualificador de cada compuesto respecto a la del PI. Las muestras se analizan por triplicado y se realiza un blanco entre análisis.

9. Análisis sensorial del vino

El análisis sensorial se realiza mediante evaluación ortonasal por un panel de expertos integrado por siete catadores (cinco mujeres y dos hombres). Todos los miembros pertenecientes al personal de laboratorio han sido entrenados de acuerdo a los protocolos internacionales (ISO, 1983; ISO, 1985).

Se presentan 15 mL de muestra de vino en copas de vidrio negras cubiertas con placas de petri.

9.1 Análisis descriptivo cuantitativo

El análisis descriptivo cuantitativo se realiza utilizando 13 descriptores sensoriales: herbáceo, floral, frutos rojos, fruta madura, pasas, dulce, cárnico/sudor, frutos secos, cítricos, balsámico, especias, café e impresión general. Estos descriptores fueron seleccionados por el panel durante sesiones preliminares para describir las muestras. Los atributos seleccionados se recogen en una ficha de cata y a los expertos se les pide que puntúen cada descriptor en una escala de 10 cm no estructurada (que va desde no perceptible a muy fuerte).

9.2 Evaluación sensorial discriminante

La evaluación sensorial discriminante se realiza mediante pruebas triangulares para probar si existen diferencias significativas entre los vinos fermentados por cada cepa de levadura.

10. Análisis químico

10.1. Grado alcohólico (Ribéreau-Gayón et al., 1980)

Se fundamenta en la oxidación crómica de etanol (1) y posterior valoración del exceso de bicromato potasio mediante adición de una solución valorada de sulfato de hierro amoniacal (sal de Mohr) (2), según las reacciones:

(1)

$$2\ K_2CrO_7 + 8\ H_2SO_4 + 3\ CH_3CH_2OH \rightarrow CH_3COOH + 2\ Cr_2(SO_4)_3 + 2\ K_2SO_4 + 11\ H_2O$$

(2)

$$K_2CrO_7 + 8\ H_2SO_4 + 6\ Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O \rightarrow 3Fe_2(SO_4)_3 + Cr_2(SO_4)_3 + 2\ HKSO_4 + 6\ (NH_4)_2SO_4 + 13\ H_2O$$

Reactivos

Licor valorado de bicromato potásico:

Bicromato potásico (Panreac)		33,79 g
Agua destilada	c.s.p.	1 L (20 ºC)

Licor reductor de sal de Mohr:

Sal de Mohr (Panreac)		135,1 g
Ácido sulfúrico (Panreac)		20 mL
Agua destilada	c.s.p.	1 L (20 ºC)

Este valor es tal que 2 mL de este licor reducen 1 mL de licor de dicromato. Esta solución debe ser revalorada frecuentemente con respecto a la solución de bicromato.

Solución de permanganato de potasio:

Permanganato de potasio (Panreac)		1,083 g
Agua destilada	c.s.p.	1 L

Ácido sulfúrico diluido:

Ácido sulfúrico puro (Panreac)		500 mL (sobre agua)
Agua destilada	c.s.p.	1 L

Reactivo de ortofenantrolina ferrosa:

Sulfato ferroso (Panreac)		0,695 g
Agua destilada	c.s.p.	100 mL
Ortofenantrolina monohidrato (Panreac)		1,485 g

Análisis

A. Destilación

Se toma un volumen de vino V = 20 mL y se lleva a 200 mL con agua destilada. El vino así diluido se introduce en el balón de destilación del aparato de destilación y se alcaliniza añadiendo 10 mL de cal 4 N. El destilado se recoge sobre matraz aforado de 200 mL que se enrasa con agua destilada.

B. Oxidación

En un matraz Erlenmeyer de 250 ml con tapón esmerilado se introducen 20 mL de solución valorada de bicromato potásico, 20 mL de ácido sulfúrico al 50 % y se agita. Se agregan a continuación 10 mL de destilado. Se tapa el erlenmeyer, se agita y se esperan 30 min. como mínimo agitando de vez en cuando.

C. Valoración

El exceso de bicromato se valora mediante adición de solución de sal de Mohr con la ayuda de una bureta graduada. Cuando la coloración verde de la solución vira a verde-azulado, se agregan cuatro gotas de reactivo de ortofenantrolina. Se continúa la adición de sal de Mohr hasta que el medio pasa de verde-azulado a marrón.

Como a menudo se pasa un poco el viraje, hay que retornar al viraje preciso por medio de la solución de permanganato de potasio. La décima parte del volumen empleado de esta solución se resta del volumen de la solución de sal de Mohr: sea *n* esta diferencia.

La sal de Mohr debe valorarse, por otra parte, en relación con la solución de bicromato de potasio, como ya se indicó antes. Para ello se realiza la oxidación y valoración anteriormente indicadas de la misma manera con la salvedad de añadir en la oxidación, 10 mL de agua destilada en lugar del destilado. Sea *n'* el volumen de sal de Mohr que corresponde a 20 mL de solución de bicromato de potasio.

Cálculos

El grado alcohólico de ese líquido es:

$$\% \text{ vol } = 400 \cdot \frac{(n'-n)}{V \cdot n'}$$

10.2. Glicerol

El contenido en glicerol se determinó mediante kit enzimático específico (Boehringer Mannheim, Ref. 148270) siguiendo el protocolo del fabricante.

Principio del método

El glicerol es fosforilado por la gliceroquinasa, en presencia de adenosina-5-trifosfato dando lugar a glicerol-3-fosfato y adenosina-5-difosfato. Ésta es reconvertida en adenosina-5-trifosfato por el fosfoenolpiruvato mediante la piruvatoquinasa formándose piruvato. El piruvato es reducido a lactato por nicotinamida-adenina dinucleótido (NADH). La cantidad NADH oxidado a NAD^+ en este último paso es equivalente al glicerol presente en la reacción. Se mide en la región del espectro ultravioleta de 340 nm.

10.3. Azúcares reductores por el método Rebelein (García Barceló, 1990)

El método Rebelein está basado en la reducción de un reactivo cúprico-alcalino en presencia de los azúcares y valoración del cobre residual por yodometría.

Reactivos

<u>Solución de sulfato de cobre pentahidrato</u>:		
Sulfato cúprico	(Carlo Erba)	41,92 g
Ácido sulfúrico 0,5 M (Panreac)		10 mL
Agua destilada	c.s.p.	1 L
<u>Solución alcalina:</u>		
Tartrato sodio potasio (Panreac)		250 g en 400 mL de agua
Hidróxido sódico (Panreac)		80 g en 400 mL de agua

Se mezclan las dos soluciones y se completa a volumen de un litro cuando esté frio.

Solución ioduro potásico:		
Ioduro potásico (Panreac)		300g en 500 mL de agua
Hidróxido sódico 1 M (Panreac)		100 mL
Agua destilada	c.s.p.	1 L

Solución ácido sulfúrico al 16 %:		
Ácido sulfúrico puro (Panreac)		175 mL (sobre agua)
Agua destilada	c.s.p.	1 L

Solución almidón:	
Almidón (Panreac)	8 g en 500 mL de agua en ebullición (2 min.)
Ioduro potásico (Panreac)	20 g en 400 mL de agua
Hidróxido sódico 1 M (Panreac)	10 mL

Se mezcla y se completa con agua destilada al volumen de un litro.

Solución de tiosulfato:		
Tiosulfato sódico puro (Panreac)		13,777 g
Hidróxido sódico 1 M (Panreac)		50 mL
Agua destilada	c.s.p.	1 L

Análisis

A. Reducción

En un matraz Erlenmeyer de 250 mL se vierten 10 mL de solución cúprica, 5 mL de solución alcalina, 2 mL de la muestra de vino (de menos de 28 g de azúcar por litro) y un poco de piedra pómez.

Se hierve durante un min. y medio exactamente, enfriando rápidamente al chorro de agua fría.

Se añade 10 mL de solución de ioduro y 10 mL de ácido sulfúrico.

B. Valoración

Se valora con solución de tiosulfato sódico, hasta un color ligeramente amarillo.

En este momento se añaden 10 mL de almidón y se completa la valoración hasta una coloración crema claro.

Es necesario hacer un blanco tomando 2 mL de agua destilada en vez de vino.

Cálculos

El contenido en azúcares reductores es el siguiente:

$$\text{Azúcares reductores, g/L} = N' - N$$

Siendo:

N’ = mL de la solución de tiosulfato gastados en la valoración del blanco (con agua destilada)

N = mL de la solución de tiosulfato gastados en la valoración de la muestra de vino

Si el vino ha sido diluido, se deberá multiplicar por el factor de dilución.

10.4. Acidez total o titulable

Se considera acidez titulable de un vino (o acidez valorable) la suma de los ácidos titulables presentes en el mismo. Deben excluirse de ésta las acideces de adición como son el dióxido de carbono y el sulfuroso.

Para su determinación se ha seguido el procedimiento de la A.O.A.C., descrito por Amerine y Ough (1974).

Análisis

Se colocan 200 mL de agua hirviendo en un matraz Erlenmeyer de 500 mL.

Se añaden unas gotas de fenolftaleína al 1 % como indicador y se valora con hidróxido sódico 0,1 N hasta color rosa suave pero definido.

A continuación se añaden al mismo erlenmeyer 5 mL de mosto o vino y se valora con hidróxido sódico 0,1 N hasta el mismo color.

Cálculos

La acidez total o titulable se expresa en ácido tartárico mediante el siguiente cálculo:

$$\text{Acidez total, g/L ácido tartárico} = \frac{75 \cdot f \cdot V \cdot N}{v}$$

Siendo:
f = factor de disolución de la solución de NaOH 0,1 N
V = volumen de hidróxido sódico 0,1 N consumido en la valoración (mL)
N = normalidad de la solución de NaOH
v = volumen de la muestra de vino o mosto (mL)

10.5. pH

Este parámetro determina la concentración de hidrogeniones del medio, medida orientativa de los estados de combinación o ionización de los compuestos del vino.

La determinación se realiza directamente sobre la muestra, utilizando un pH-metro CRISSON (micropH 2000) de lectura directa y con sonda térmica para la corrección de la temperatura.

10.6. Acidez volátil (M.A.P.A., 1993)

La acidez volátil está constituida por parte de los ácidos grasos pertenecientes a la serie acética que se hallan en los vinos en estado libre o salificado.

El método empleado se basa en el descrito en los Métodos Oficiales de Análisis del M.A.P.A. (1993), cuyo fundamento es la valoración de los ácidos volátiles separados del vino por arrastre de vapor de agua y rectificación de los vapores. Para la destilación se ha empleado el aparato Mathieu.

Previamente debe eliminarse el dióxido de carbono; y la acidez del dióxido de azufre libre y combinado, destilados en dichas condiciones, debe restarse de la acidez del destilado.

Análisis

A. Destilación

Se introducen 20 mL de vino y un poco de piedra pómez en el matraz de destilación.
Se llena el embudo del aparato Mathieu de destilación con agua destilada.
Se inicia la destilación y, una vez recogidos 6 mL de destilado en la probeta, se añade del embudo otros 6 mL de agua; se recogen hasta 12 mL del destilado y se añade de nuevo agua para mantener el volumen en el matraz de destilación y así sucesivamente hasta recoger 24 mL de destilado.

B. Valoración

Se añaden dos gotas de fenolftaleína (1 %) como indicador y se valora el destilado con hidróxido sódico 0,1 N hasta aparición de color rosa suave pero definido.

C. Valoración del sulfuroso libre

Se añade una gota de ácido clorhídrico puro (para acidular nuevamente), un cristal de Ioduro de potasio y 2 mL de solución de almidón como indicador.
Se valora el SO_2 libre con la solución de iodo 0,01 N.

D. Valoración del ácido sulfuroso combinado con el acetaldehído

Se añade 20 mL de solución saturada de bórax; el líquido tomará un color rosa pálido.
Se valora nuevamente con solución de Iodo 0,01 N.

Cálculos

La acidez volátil, expresada como g/L de ácido acético, se determina mediante el siguiente cálculo:

$$\text{Acidez volátil aparente, g/L ácido acético} = \frac{0{,}6}{2} \cdot V$$

$$\text{Acidez volátil real, g/L ácido acético} = \frac{0{,}6}{2} \cdot \left(V - \frac{V'}{10} - \frac{V''}{20}\right)$$

Siendo:

V = volumen en mL de NaOH 0,1 N consumidos en la primera valoración

V'= volumen en mL de Iodo 0,01 N consumidos en la oxidación del anhídrido sulfuroso libre

V''= volumen en mL de Iodo 0,01 N consumidos en la oxidación del anhídrido sulfuroso combinado con el acetaldehído

10.7. Ácido málico

El contenido en ácido málico se determinó mediante kit enzimático específico (Boehringer Mannheim, Ref. 139068) siguiendo el protocolo del fabricante.

Principio del método

En presencia de L-malato dehidrogenasa, el L-málico o sus sales, es oxidado por el NAD a oxalacetato. La reacción, catalizada por la enzima glutamato-oxalacetato-transaminasa (GOT), el oxalacetato se convierte en L-aspartato, en presencia de l-

glutamato. La cantidad de NADH formada es estequiométrica con la concentración de L-malato. La NADH es la que provoca la absorbancia en la longitud de onda de 340 nm.

10.8. Dióxido de azufre libre y total por el método de Ripper doble (M.A.P.A., 1993)

Para esta determinación se ha seguido el método descrito en los Métodos Oficiales de Análisis del M.A.P.A. (1993). El dióxido de azufre libre se determina por valoración yodométrica directa; el dióxido de azufre combinado, tras hidrólisis alcalina, se determina también por valoración yodométrica. La suma del dióxido de azufre libre y combinado permite obtener el dióxido de azufre total.

En un matraz Erlenmeyer de 500 mL se introducen 50 mL de vino, 3 mL de ácido sulfúrico (Panreac) al 10%, 30 mg de EDTA (Panreac) y 5 mL de almidón (Panreac) como indicador. Se homogeneiza y se valora con solución de iodo (Panreac) 0.05 N hasta color azul persistente, siendo *V* el volumen de iodo empleado.

A continuación se añaden 8 mL de hidróxido sódico (Panreac) 4N, se agita una sola vez y se deja en reposo durante 5 minutos. Se añaden 10 mL de ácido sulfúrico (Panreac) (10%), se agitar enérgicamente y se valora con solución de iodo (Panreac) 0,05 N inmediatamente. El volumen *V'* será el del iodo gastado.

Por último se añaden 20 mL de hidróxido sódico (Panreac) 4N, se agita una vez y se deja en reposo durante minutos. Se diluye con 200 mL de agua destilada muy fría y se agita enérgicamente. Se añaden entonces 30 mL de ácido sulfúrico (Panreac) (10%) y se valora con la solución de iodo (Panreac) 0,05 N, siendo *V"* el volumen empleado.

Cálculos

El resultado se expresa en mg/L de SO_2:

$$SO_2 \text{ libre} = 32 \cdot f \cdot V$$

$$SO_2 \text{ combinado} = 32 \cdot f \cdot (V' + V'')$$

$$SO_2 \text{ total} = 32 \cdot f \cdot (V + V' + V'')$$

Siendo f el factor de titulación de la solución de iodo 0,05 N.

11. Elaboración del pie de cuba (Suárez Lepe, 1997)

Para el ensayo del comportamiento de las levaduras autóctonas y comerciales en bodega a escala semipiloto fue necesario preparar un pie de cuba de cada cepa con el que inocular el depósito de fermentación.

Para ello, dos semanas antes del inicio del ensayo, se extrajeron aproximadamente 50 L de mosto a partir de racimos maduros y sanos de uva de la variedad Merlot. El mosto se esterilizó en laboratorio mediante adición de 1 g/L de dimetil dicarbonato (se dejó actuar 6 horas a 20 ºC); a continuación se desfangó en frío y se repartió en tubos en

ensayos (10 mL), en matraces Erlenmeyer de 250 mL (150 mL) y en garrafas de 5 litros (3 litros), y se conservó a 4 ºC.

Para cada cepa a ensayar se inocularon 6 tubos con 10 mL de mosto estéril a partir de un cultivo fresco en YEPD agar y se incubaron a 25 ºC durante 24 horas.

A continuación se inocularon 3 matraces Erlenmeyer (150 mL) para cada cepa, cada uno con dos tubos de los sembrados el día anterior y se incubaron durante 48 h a 25 ºC.

Para cada cepa se inocula una garrafa con 3 L de mosto estéril con los 3 matraces Erlenmeyer sembrados 48 horas antes y se incubaron a 25 ºC durante 24-48 horas.

El pie de cuba se mantiene en fermentación tumultuosa añadiendo mosto fresco hasta el momento de inocular los depósitos en la bodega. Se comprueba mediante conteo de células viables que la población celular se mantenga en el orden de 10^8 ufc/mL.

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ANEXO II

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Volatile and sensory profile of organic red wines produced by different selected autochthonous and commercial *Saccharomyces cerevisiae* strains

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HS-SBSE-TDU-GC–MS

ABSTRACT

Organic wines were produced at pilot scale to select the best autochthonous and commercial yeast strains to obtain wines with high organoleptic qualities. We tested the behaviour of five *S. cerevisiae* yeast strains and determined their volatile composition and organoleptic characteristics by sensory analysis. A total of 51 volatile compounds were quantified in the wines produced. The concentration of most of the volatile compounds was significantly influenced depending on which yeast strain was inoculated. The differences observed in the volatile composition of the wines appear to be quantitative rather than qualitative. In general, acetals were the most abundant group of volatile compounds in all the samples studied, followed by alcohols without ethanol. The highest contents of volatile compounds were found in two of the wines produced by autochthonous yeast strains. The results obtained in the sensory analysis suggest that autochthonous yeast produced wines of higher organoleptic quality because this sample gave the highest value for the general impression attribute.

1. Introduction

Growing concern among consumers in developed countries regarding the health and the protection of the environment has increased the demand for organic food over the last decade (especially in Germany, the United Kingdom, Switzerland, New Zealand, Japan, and the United States). Among organic products, EU consumers have shown an increasing interest in organic wines. More and more new organically based wineries are being established and old traditional wineries are adding organic wine to their product lines [1].

Wine quality is influenced, in part, by the composition of the grape juice and by the microbial communities present during the fermentation process. Aroma is one of the main characteristics that determine a wine's quality and value. The aroma of wine is a unique mixture of volatile compounds originating from grapes (varietal aromas), secondary products formed during the wine fermentation (fermentative aromas) and aging (post-fermentative aromas) [2]. The volatile fraction of wine can be made up of more than 800 different compounds [3] with a wide concentration range varying from hundreds of mg L^{-1} to μg L^{-1} or ng L^{-1} levels [4]. This great variety of volatile compounds, with different polarities, volatilities and a wide range of concentrations, is responsible for the complexity of the wine's bouquet and ensures its specificity and character [5].

The particular importance of each compound to the final aroma is related to its odour perception threshold [4]. General approaches to identifying "important" or high impact odorants are based on odour activity values (OAVs) and the concentration/threshold ratio. Odorants with low OAVs, or low impact odorants (i.e. with typical values <1) are generally considered to be unimportant to the overall sensory perception [6].

However, Ryan et al. [6] hypothesise that compounds with low OAVs could play a critical role in characterizing the overall odour of a sample. Furthermore, other authors think that OAVs provide only a rough evaluation of the real contribution of each compound to the overall aroma. In fact, the volatility and the perception of aroma compounds are significantly affected by the basic chemical composition of the wine, which can both mask the odour impact of certain compounds present in concentrations above their detection thresholds and favour the detection of other molecules present in concentrations below theirs [7].

A vast number of volatile compounds are formed and modulated by yeast during alcoholic fermentation and significantly impact the flavour and overall quality of wines [8]. The volatile compounds synthesized by wine yeast include higher alcohols, medium- and long-chain volatile acids, acetate esters, ethyl esters and aldehydes among others [9,10]. The capacity to form aroma depends not only on yeast species but also on the particular strain of the individual species [11]. Different strains of *S. cerevisiae* can

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produce significantly different flavour profiles when fermenting the same must. This is a consequence of both the differential ability of wine yeast strains to release varietal volatile compounds from grape precursors and the differential ability to synthesise de novo yeast-derived volatile compounds [12–15]. Therefore, selecting the proper yeast strain can be critical for the development of the desired wine style [7]. For this reason, modern wine makers prefer to employ selected yeast strains. For the production of young wine, the wineries select yeast strains that produce both the high levels of the esters and acetates needed for the desirable fruity taste and the low levels of higher alcohols that contribute negatively to aroma [11]. Moreover, employing selected starter yeast cultures provides technological advantages such as guaranteeing that the must ferments in the correct way. On the other hand, the use of autochthonous yeast strains, besides promoting biodiversity, is rather preferable since they are better acclimated to the environmental conditions and assure the maintenance of the typical sensory properties of the wines of any given region.

The aim of this work is to select the best yeast strains among autochthonous and commercial to obtain organic wines with high organoleptic qualities.

2. Experimental

2.1. Yeast strains and yeast implantation control

Five different strains of *S. cerevisiae* were tested; four autochthonous strains were compared with a commercial yeast strain frequently employed by the winery for the vinification of red wines. The commercial yeast was Excellence XR (XR) (Lamothe-Abiet, Bordeaux, France) and the autochthonous yeasts were coded as MY, NY, OY and AGY. The commercial yeast was selected in accordance with the winery's preferences and the autochthonous strains were isolated from the same cellar and the neighbouring vineyard and selected according to their oenological traits in a previous selection study which evaluated ethanol production, sugar consumption, volatile acidity production, malic acid degradation, glycerol production, foam and SH_2 production, killer behaviour, volatile compounds production and the organoleptic characteristics of the experimental wines obtained (data not shown).

In order to assess the implantation of the inoculated yeast in each vinification vat, samples were taken at different stages of fermentation: 24 h after inoculation, in the middle (day 3) and at the end of the fermentation (day 6). In addition, a sample of fresh grape juice was taken to determine the yeast microflora in the must before the vats were filled and sown with the starter. Aliquots (0.1 mL each) of serial dilutions were spread onto plates of YEPD agar (yeast extract 1% w/v, peptone 1% w/v, glucose 2% w/v, agar 2% w/v). Plates were incubated at 28 °C for 72 h and, for each sample, 20 colonies were randomly chosen and submitted to further studies.

Isolates from the fresh juice were identified by PCR of the ribosomal region encompassing the 5.8S rRNA gene and the two internal transcribed spacers. rDNA was amplified by PCR in a TC-312 Techne termocycler using primers ITS1 and ITS4, described by White et al. [16]. The species of the isolates were identified by PCR-RFLP (restriction fragment length polymorphism) digesting with the restriction endonucleases HinfI, HhaI (same enzymatic activity as CfoI) and HaeIII (Takara Japan) as previously described by Guillamón et al. [17]. PCR products and restriction fragments were separated on a horizontal 1.4% and 3% agarose gel, respectively, in TAE buffer and compared with standards (100-bp DNA ladder, Amersham Biosciences). Isolates from the five fermenting musts were directly characterized by restriction analysis of mitochondrial DNA in order to compare the resulting profiles with those of the *S. cerevisiae* strains inoculated in each vat. Thus, all these isolates were initially presumed to belong to *S. cerevisiae* species; if any of the profiles did not match any of the inoculated strains, these isolates would be identified by PCR-RFLP of the rDNA as described above. DNA extraction and restriction analysis were performed following the method proposed by Querol et al. [18]. DNA (6 μL) was digested with HinfI endonuclease (Takara Japan) and restriction fragments were separated by electrophoresis on 1% agarose gel with added ethidium bromide and visualized in a UV transilluminator. This technique was also applied to each yeast strain used as starter. The genetic profiles of all the isolates were compared with the profiles of the inoculated yeasts and this enabled us to determine the implantation percentage of each strain tested.

2.2. Grape musts and pilot scale fermentation

Pilot scale fermentations took place in the winery in 100 L stainless vats with Merlot grape variety must. The physical and chemical must parameters were the following: pH 3.40, total acidity ($g\,L^{-1}$ tartaric acid) 5.1, density 1.0945 and 30 $mg\,L^{-1}$ of SO_2.

All the yeast inocula (including the commercial strain) came from pure cultures previously sown in a solid medium which were inoculated in a small volume of sterilized must to a final concentration of 10^6 cell mL^{-1} and incubated at 28 °C for 24 h. The starters were progressively diluted with sterilized must in the laboratory and inoculated with a volume sufficient to obtain a cellular population of 10^7 cell mL^{-1} in the winery vat. In all the cases fermentation took place at room temperature and kinetics were monitored by measuring the Baume degree of the fermenting must.

At the end of the alcoholic fermentation, wines were run off and placed in a vat for malolactic fermentation to occur. For this purpose, wines were inoculated with commercial lactic acid bacteria *Oenococcus oeni* (Challenge EASY ML, Sepsa-Enartis, Spain) and malolatic fermentation took place at 20 °C for 9–15 days depending on the vat. Subsequently, wines were racked twice and plate-and-frame filtered before bottling.

2.3. Physicochemical analysis

Some physicochemical parameters were analyzed in the resulting wines. L-Malic acid and glycerol were determined using enzymatic kits from Boehringer Mannheim (GmbH, Germany), pH by using the potentiometric method, total acidity ($g\,L^{-1}$ tartaric acid) by indicator titration to pH 8.2 using standardized sodium hydroxide, volatile acidity ($g\,L^{-1}$ acetic acid) by steam distillation and titration with standardized sodium hydroxide, reducing sugars by Rebelein method which involves reacting reducing sugars with copper (II) in alkaline solution, and ethanol by titrametric dichromate analysis.

2.4. Sensory analysis

An expert panel composed of seven tasters (five females and two males) carried out sensory analysis using orthonasal evaluation. All members belonging to the laboratory staff were trained according to international protocols [19,20]. A 15 mL wine sample was presented in dark glass covered with a plastic dish.

Quantitative descriptive analysis (QDA) was carried out using 13 sensory terms: green, floral, red fruit, ripe fruit, raisin, sweet, meat/sweat odour, nutty, citrus, balsamic, spicy, coffee and general impression. These terms were selected by the panel during preliminary sessions to describe the samples. The selected attributes were put on a tasting-card and panellists were asked to rank each descriptor on a 10-cm unstructured scale (from unnoticeable to very strong).

Discriminant sensory evaluation was performed through triangular tests to assess the significant differences caused by each yeast strain in each wine.

2.5. Volatile compound analysis

We used two methods of analysis because we expected to find volatile compounds with different volatilities and a wide range of concentrations. Therefore, those compounds present in high concentrations (major volatile compounds) were determined by direct injection using gas chromatography-flame ionization detection (GC-FID). The minor compounds were extracted by headspace sorptive extraction and then determined by gas chromatography–mass spectrometry (HSSE-GC–MS).

The 51 standards of aroma compounds used for quantification (see Table 2) were obtained from several commercial sources as follows: 2, 3, 14, 15, 19–21, 23–27, 29–32, 40–42, 45–51 (Sigma–Aldrich, Madrid, Spain); 1, 4, 6–10, 13, 17, 18, 28, 34–39, 44 (Merck, Darmstadt, Germany); 5, 11, 12, 16, 22, 33, 43 (Fluka, Madrid, Spain). 4-Methyl-2-pentanol (Merck) was used as the internal standard (IS).

2.5.1. GC-FID analysis

Ethyl acetate, acetaldehyde, methanol, propanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol were quantified by GC-FID using the method proposed by Morales et al. [21]. A 1 mL sample was filtered through 0.22 μm Millex-GV13 filters and 10 μL of 4-methyl-2-pentanol at 102.14 mg L^{-1} was added as the internal standard (IS).

The samples were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID). 1 μL of filtered sample was injected in the split mode (1:60) into a CP-Wax 57 CB column, 50 m × 0.25 mm i.d. × 0.2 μm film thickness (Varian, Middelburg, Netherlands). The carrier gas was H_2 at 1 mL min^{-1}. The program temperature was: 35 °C for 5 min, ramped at 4 °C min^{-1} to 150 °C and held for 17.5 min. The injector was set at 220 °C and the detector at 250 °C. The data acquisition software was the HPChemstation data processing system (Agilent Technologies, Santa Clara, California).

2.5.2. HSSE-GC–MS analysis

The HSSE sampling conditions were as follows [22]: 5 mL of sample and 10 μL of 4-methyl-2-pentanol (IS) at 1045 mg L^{-1} was placed into a 20-mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with a 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put in an open glass insert and placed in the vial to carry out the extraction in the headspace. Then, the vial was tightly capped and heated for 60 min at 62 °C in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water and dried with lint-free tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed in a glass tube 60 mm long, 6 mm o.d. and 4 mm i.d. This tube was then placed on the autosampler tray of the thermo desorption unit for GC–MS analysis.

Gas chromatography analysis was carried out with a 6890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel). The thermal desorption was performed in splitless mode and with a flow rate of 90 mL min^{-1}. The desorption temperature program was the following: 35 °C for 1 min, ramped at 60 °C min^{-1} to 250 °C and held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner was held at −35 °C with liquid nitrogen for the total desorption time and then raised at 10 °C s^{-1} to 290 °C and held for 4 min. The solvent vent mode was used to transfer the sample to the analytical column. A CPWax-57CB column, 50 m × 0.25 mm, 0.20 μm film thickness (Varian, Middelburg, Netherlands) was used and the carrier gas was He at a flow rate of 1 mL min^{-1}. The oven temperature program was 35 °C for 5 min, which was then raised to 220 °C at 2.5 °C min^{-1} (held for 5 min). The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively.

Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35–350 amu.

All data were recorded using a MS ChemStation (Agilent Technologies, Santa Clara, California). Peaks were identified using the NIST 98 library and confirmed by standard retention indices when they were available. Quantification was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard [22]. The samples were analyzed in triplicate and blank runs were made with empty glass tubes before and after each analysis. RIs were calculated from the retention times of n-alkanes by linear interpolation, in accordance with the literature [23].

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and principal component analysis (PCA) were performed using Statistica, version 7.0 software (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Yeast analysis and fermentation kinetics

Forty yeast strains were isolated from the fresh juice before yeast inoculation. According to PCR-RFLP of the 5.8S rDNA gene, 38 isolates corresponded to non-*Saccharomyces* strains, and the remainder to the *Saccharomyces* genus. Further analysis characterised the last strains as the commercial yeast Excellence XR, frequently used by the winery.

To determine the implantation of the inoculated yeast in each vat, samples at different fermentation stages were analyzed by mitochondrial DNA RFLP. Fig. 1 shows the restriction profiles of the five strains tested, and Fig. 2 indicates the initial yeast population in the fresh must and during the fermentation progress (day 1, day 3 and day 6) in each vat. The restriction profiles of all the isolated colonies matched the profile of the inoculated yeast in their respective vats. Thus, in all the vinifications the inoculated yeast strains began to predominate immediately after inoculation (24 h, 100% implantation) and the same results were obtained at the middle and the end of the fermentation. The inoculated yeast population in each vat was therefore sufficient to eliminate a significant quantity of wild no-*Saccharomyces* and *Saccharomyces* yeast throughout the course of fermentation.

Yeast enumeration in the fresh must before inoculation revealed an initial population of 3.1×10^4 cell mL^{-1}, mainly corresponding to non-*Saccharomyces* species as described above. On the other hand, starters of pure cultures were inoculated in sufficient volume to reach a final population of 10^7 cell mL^{-1} in the must. Thus, differences between the initial yeast population in the fresh must and the inoculated yeast population in each vat together with differences in the fermentative activity of the cells in each case justify the rapid implantation of the latter after 24 h.

The progress of the fermentations was monitored by measuring the Baume degree of the fermenting must. A comparison of fermentation kinetics in the five vats is shown in Fig. 3. According to this figure, the duration of all fermentations was about six days. In all the cases the rate of fermentation is very similar and the lag phase happened in only one day. Most of the sugar is consumed in the first three days for all the yeast strains tested, although the commercial strain (XR) showed a slower consumption rate.

3.2. Physicochemical analysis

Table 1 summarizes the chemical composition of the wines obtained. The residual sugar content showed that all yeast strains

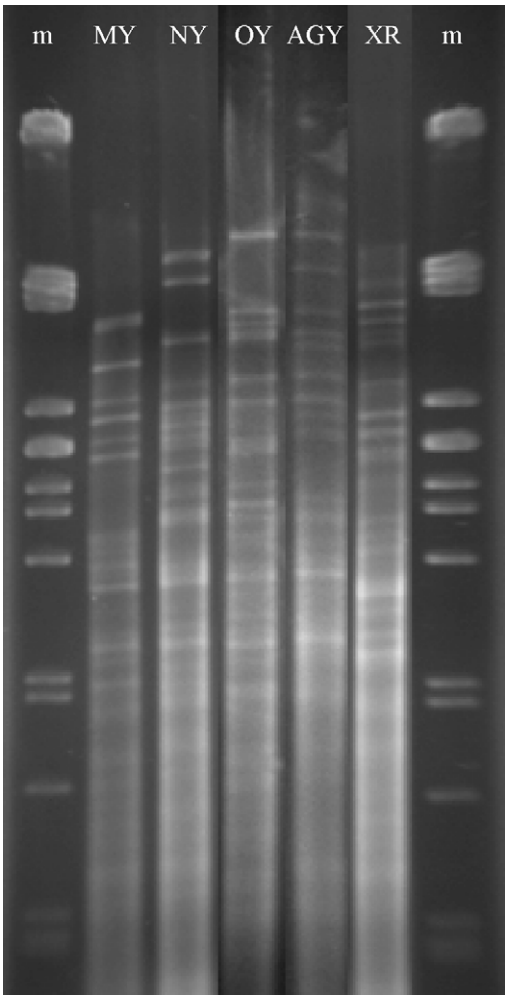

Fig. 1. Electrophoretic profiles of mitochondrial DNA of yeast strains used in the study (MY, NY, OY, AGY and XR) digested with HinfI. (m), marker: phage lambda digested with PstI.

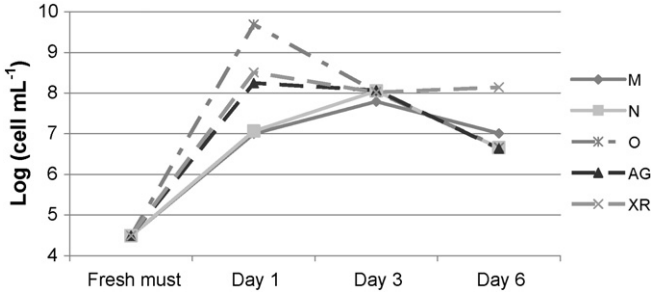

Fig. 2. Yeast population in the fresh must and during the fermentation (day 1, day 2 and day 3) in each experiment.

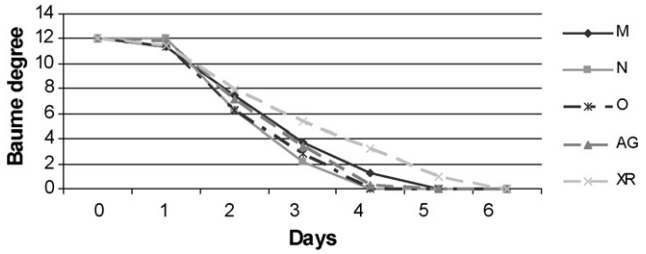

Fig. 3. Fermentation kinetics of the five yeast strains monitored by Baume degree.

tested fermented the wines to dryness and the degree of alcohol ranged from 11.9 to 12.6% vol, which agreed with the amount of sugars in the must. No L-malic acid was detected in any of the samples, total acidity ranged from 4.37 to 5.57 g L^{-1} expressed as tartaric acid, pH was close to 3.5 in all the samples and values for volatile acidity were no greater than 0.3 g L^{-1} acetic acid in any case. Finally, glycerol production varied from 6.2 to 8.6 g L^{-1}. Thus, although slight differences were observed in some parameters according to the inoculated yeast strain, no statistical differences among the physicochemical properties were found.

3.3. Volatile composition

A total of 80 volatile compounds were detected in the red wines analyzed. Among them 51 were quantified with their corresponding calibration curve. Remaining compounds (29) were tentatively identified by comparing their mass spectra with those in a commercial library and with those reported in the literature. These compounds belong to several chemical classes including alcohols, esters of fatty acid (ethyl, methyl and isoamyl esters), acetic esters, acids, acetals, aldehydes, ketones, volatile phenols, lactones and terpenes (Table 2).

The differences observed in the volatile composition of the wines obtained from different yeast strains seem to be quantitative rather than qualitative, which agrees with previous studies [24–26]. Hence, wines M and N had the highest total content of volatile compounds (>2 g L^{-1}). The high total content of volatile compounds in N is caused by acetals (acetaldehyde diethylacetal), whilst M stands out for its high content in ethyl esters, alcohols, ketones and acids.

In general, acetals were the most abundant volatile compounds in all the samples studied, followed by alcohols without ethanol (Fig. 4). The principal compound of this alcoholic fraction is 3-methyl-1-butanol followed by methanol. As Table 2 shows, this last compound is present in higher concentrations in red wine and reached concentrations lower than expected in samples AG and XR [27]. Methanol results exclusively from enzymic hydrolysis of the methoxyl groups of the pectins during fermentation but it is not formed by alcoholic fermentation. However, the concentrations of higher alcohols in wine are important variables for differentiating between yeast strains because of their strict relation with yeast metabolism [26]. These compounds have not been con-

Table 1
Chemical composition of wines obtained.

Wines	% vol. ethanol	Residual sugars (g L^{-1})	Glycerol (g L^{-1})	L-Malic acid (g L^{-1})	Total acidity (g L^{-1})	pH	Volatile acidity (g L^{-1})	Free SO_2 (mg L^{-1})	Total SO_2 (mg L^{-1})
M	11.9 ± 0.1	3.70 ± 0.14	8.6 ± 0.1	nd	5.57 ± 0.06	3.4 ± 0.0	0.26 ± 0.01	20.9 ± 2.3	193 ± 23
N	12.4 ± 0.0	1.55 ± 0.07	6.7 ± 0.0	nd	4.37 ± 0.06	3.5 ± 0.0	0.28 ± 0.02	30.0 ± 5.7	243 ± 13
R	12.3 ± 0.0	2.65 ± 0.07	6.2 ± 0.2	nd	4.73 ± 0.34	3.5 ± 0.0	0.27 ± 0.01	29.5 ± 3.3	178 ± 21
AG	12.5 ± 0.0	2.65 ± 0.07	6.7 ± 0.0	nd	4.93 ± 0.06	3.5 ± 0.0	0.27 ± 0.01	27.9 ± 2.5	141 ± 19
XR	12.6 ± 0.1	2.60 ± 0.14	7.4 ± 0.1	nd	5.29 ± 0.11	3.4 ± 0.0	0.26 ± 0.03	35.9 ± 1.9	111 ± 4
mean	12.4 ± 0.3	2.69 ± 0.67	7.0 ± 0.9	-	4.88 ± 0.47	3.5 ± 0.0	0.27 ± 0.02	30.1 ± 5.9	173 ± 45

The data are mean values of duplicates; nd: no detected.

Table 2
Volatile compounds of organic red wine.

No.	Compound	Mean concentration ($\mu g\,L^{-1}$)				
		M	N	O	AG	XR
Aldehydes						
1	Acetaldehyde[a]	$36 \pm 4^{b,c,d}$	$53 \pm 6^{b,c,d}$	18.7 ± 0.4^{d}	$9.1 \pm 0.9^{e,f}$	$11 \pm 1^{e,f,d}$
2	Hexanal	$7.3 \pm 0.8^{g,c}$	$8.9 \pm 0.8^{g,b,c}$	$20.5 \pm 0.7^{e,b,c,f}$	$5.4 \pm 0.5^{e,g,c}$	$26.4 \pm 1.5^{e,g,b,f}$
3	2-Furfuraldehyde	$940 \pm 20^{e,g}$	$731 \pm 31^{g,f}$	$481 \pm 59^{e,b,c,f}$	779 ± 60^{g}	1081 ± 127^{g}
4	Benzaldehyde	$45 \pm 7^{e,g,b}$	n.q.	n.q.	n.q.	$39 \pm 3^{e,g,b}$
5	5-Methyl-2-furfuraldehyde	$114 \pm 13^{e,g,b}$	$55 \pm 7^{e,g}$	$30 \pm 4^{e,b,c,f}$	$64 \pm 3^{g,f}$	$57 \pm 8^{g,f}$
	Total aldehydes[a]	36.7	54.0	19.3	9.9	12.4
Acetal						
6	Acetaldehyde diethylacetal[a]	$761 \pm 82^{e,d}$	$1764 \pm 2^{g,b,c,f,d}$	$644 \pm 43^{e,d}$	$696 \pm 3^{e,d}$	$680 \pm 7^{e,d}$
Acetic esters						
7	Methyl acetate[a]	1.680 ± 0.003^{c}	1.450 ± 0.023^{c}	$2.05 \pm 0.05^{c,f}$	1.35 ± 0.09^{g}	$1.00 \pm 0.08^{e,g,f}$
8	Ethyl acetate[a]	$132.0 \pm 0.2^{e,g,c,d}$	$86 \pm 11^{b,f,d}$	$101 \pm 6^{b,f,d}$	$133 \pm 5^{e,g,c,d}$	$80 \pm 12^{b,f,d}$
9	Propyl acetate	$84 \pm 2^{e,g,b,c}$	$212 \pm 4^{g,b,c,f}$	$157 \pm 7^{e,b,c,f}$	$76.3 \pm 0.8^{e,g,c,f}$	$58 \pm 4^{e,g,b,f}$
10	Isobutyl acetate	n.q.	n.q.	$125 \pm 6^{e,c,f}$	$116.5 \pm 1.4^{e,g,c,f}$	n.q.
11	Butyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
12	Isoamyl acetate[a]	$0.99 \pm 0.03^{b,c}$	$1.03 \pm 0.02^{b,c}$	1.1 ± 0.1^{c}	$1.31 \pm 0.03^{e,c,f}$	$1.72 \pm 0.03^{e,g,b,f,d}$
13	Hexyl acetate	n.q.	n.q.	n.q.	n.q.	n.q.
14	Benzyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
15	2-Phenylethyl acetate	n.q.	n.q.	n.q.	n.q.	n.q.
	Total acetic esters[a]	134.7	88.7	104.0	135.5	83.2
Ketones						
16	Diacetyl[a]	n.q.	n.q.	n.q.	3.20 ± 0.22^{d}	n.q.
17	Acetoin[a]	20 ± 14	10.74 ± 0.03^{g}	11.45 ± 0.18^{e}	n.q.	n.q.
18	Acetophenone	n.q.	n.q.	n.q.	n.q.	n.q.
	Total ketones[a]	20.0	10.7	11.4	3.2	-
Ethyl esters						
19	Ethyl propanoate	n.d.	n.d.	n.d.	n.d.	n.d.
20	Ethyl isobutyrate	n.q.	n.q.	n.q.	$130 \pm 2^{e,g,c,f,d}$	$103 \pm 2^{e,g,b,f,d}$
21	Ethyl butyrate	$426 \pm 15^{e,g,c,d}$	$457.4 \pm 1.5^{g,b,c,f,d}$	$386 \pm 9^{e,b,c,f,d}$	$669 \pm 14^{e,g,c,d}$	$456 \pm 30^{e,g,b,f,d}$
22	Ethyl 2-methylbutyrate	n.q.	n.q.	n.q.	n.q.	n.q.
23	Ethyl isovalerate	n.q.	n.q.	n.q.	n.q.	$63.7 \pm 1.7^{e,g,b,f,d}$
24	Ethyl valerate	n.q.	n.q.	n.q.	n.q.	n.q.
25	Ethyl hexanoate	$502 \pm 64^{b,c,d}$	$597 \pm 51^{b,c,d}$	$564 \pm 2^{b,c,d}$	$1138 \pm 63^{e,g,c,f,d}$	$833 \pm 58^{e,g,b,f,d}$
26	Ethyl heptanoate	n.q.	n.q.	n.q.	n.q.	n.q.
27	Ethyl lactate[a]	$148 \pm 6^{e,g,b,c,d}$	$59.9 \pm 0.9^{b,c,f,d}$	$59 \pm 7^{f,d}$	$69 \pm 2^{e,c,f,d}$	$45.5 \pm 0.7^{e,g,b,d}$
28	Ethyl octanoate	$1143 \pm 131^{b,c,d}$	$810 \pm 24^{b,c,d}$	$1030 \pm 117^{b,c,d}$	$1700 \pm 103^{e,g,f,d}$	$1723 \pm 96^{e,g,f,d}$
29	Ethyl furoate	$53.9 \pm 0.6^{e,d}$	$48 \pm 2^{f,d}$	51 ± 3^{d}	48.3 ± 1.8^{d}	46 ± 4^{d}
30	Ethyl benzoate	n.q.	n.q.	n.q.	n.q.	n.q.
31	Ethyl phenylacetate	n.q.	n.q.	n.q.	n.q.	n.q.
32	Diethyl succinate[a]	$3.5 \pm 0.3^{e,g,b}$	1.7 ± 0.3^{f}	$1.14 \pm 0.16^{c,f}$	$1.4 \pm 0.2^{c,f}$	$2.7 \pm 0.3^{g,b}$
	Total ethyl esters[a]	154.0	63.4	61.9	74.4	51.4
Alcohols						
33	Methanol[a]	$231 \pm 31^{e,b,c}$	$129 \pm 3^{b,c,f}$	$136.9 \pm 1.3^{b,c}$	$54 \pm 8^{e,g,f}$	$64 \pm 9^{e,g,f}$
34	1-Propanol[a]	$44 \pm 5^{e,g,b,c}$	$104 \pm 9^{g,b,c,f}$	$69.3 \pm 1.7^{e,b,c,f}$	$19 \pm 3^{e,b,f}$	$16.1 \pm 2.3^{e,g,f}$
35	Isobutanol[a]	39.4 ± 1.8^{g}	34.6 ± 1.3	27.0 ± 0.9^{f}	35.6 ± 1.4	33.5 ± 0.7
36	2-Methyl-1-butanol[a]	$87 \pm 3^{g,b,c,d}$	$82 \pm 5^{b,d}$	$75.8 \pm 0.5^{b,f,d}$	$48 \pm 7^{f,d}$	$57 \pm 7^{e,g,f,d}$
37	3-Methyl-1-butanol[a]	$369 \pm 10^{g,b,c,d}$	$330 \pm 23^{g,b,d}$	$234.5 \pm 1.4^{e,f,d}$	$208 \pm 25^{e,f,d}$	$246 \pm 36^{f,d}$
38	1-Hexanol	$2613 \pm 26^{e,g,b,c}$	$2351 \pm 21^{c,f}$	2212 ± 101^{f}	$2350 \pm 12^{c,f}$	$1902 \pm 36^{e,b,f}$
39	*Cis*-3-Hexen-1-ol	$63.7 \pm 0.7^{e,g,b,c}$	48 ± 3^{f}	45 ± 26^{f}	45.0 ± 0.5^{f}	43.4 ± 2.1^{f}
40	Benzyl alcohol	$453.0 \pm 1.1^{g,b,c}$	437 ± 23^{c}	331 ± 3^{f}	$408 \pm 3^{g,c}$	$348 \pm 9^{e,b,f}$
41	Furfuryl alcohol	1083 ± 117^{b}	$1342 \pm 10^{g,b}$	$1228 \pm 24^{e,b}$	$2273 \pm 134^{e,g,c,f}$	1323 ± 52^{b}
42	2-Phenylethanol[a]	$92 \pm 4^{b,d}$	$77 \pm 4^{g,b,d}$	$51 \pm 4^{e,d}$	$37.94 \pm 0.01^{e,f,d}$	60 ± 8^{d}
	Total alcohols[a]	865.4	760.5	599.0	406.7	479.5
Terpene						
43	α-Terpineol	n.d.	n.q.	n.d.	n.q.	n.d.
Acids						
44	Isovaleric acid	$2178 \pm 305^{e,g,b,d}$	$1104 \pm 28^{g,b,f,d}$	$715 \pm 34^{e,b,c,f,d}$	$1208 \pm 8^{e,g,f,d}$	$1343 \pm 99^{g,d}$
45	Hexanoic acid	$4571 \pm 593^{e,g,c,d}$	$1806 \pm 145^{b,f,d}$	$1915 \pm 256^{b,f,d}$	$3081 \pm 217^{e,g,c,d}$	$1965 \pm 221^{b,f,d}$
46	Heptanoic acid	n.q.	n.q.	n.q.	n.q.	n.q.
47	Octanoic acid	$3038 \pm 388^{g,b,d}$	$2358 \pm 322^{g,d}$	$836 \pm 124^{e,c,f,d}$	$1317 \pm 131^{f,d}$	$1885 \pm 278^{g,d}$
48	Nonanoic acid	n.q.	n.q.	n.q.	n.q.	n.q.
49	Decanoic acid	$802 \pm 122^{g,b,c}$	$960 \pm 127^{g,b,c}$	$172 \pm 26^{e,f}$	$172 \pm 3^{e,c,f}$	$278 \pm 32^{e,b,f}$
	Total acids[a]	10.6	6.2	3.6	5.8	5.5
Lactones						
50	γ-Butyrolactone	$4161 \pm 162^{g,c}$	$3426 \pm 342^{g,b,c}$	$5040 \pm 118^{e,f}$	5650 ± 552^{e}	$6167 \pm 490^{e,f}$
Phenols						
51	Guaiacol	$61.7 \pm 2.1^{e,g,b,c,d}$	$39 \pm 3^{g,b,f,d}$	$24 \pm 3^{e,f,d}$	$21.6 \pm 1.2^{e,f,d}$	$26 \pm 4^{f,d}$
	Total amounts[a]	2016	2763	1464	1355	1324

n.d.: below detection limit. n.q.: below quantification limit.
[a] Concentration in $mg\,L^{-1}$.
[b] Significant differences with sample AG.
[c] Significant differences with sample XR.
[d] OAV > 1.
[e] Significant differences with sample N.
[g] Significant differences with sample O.
[f] Significant differences with sample M.

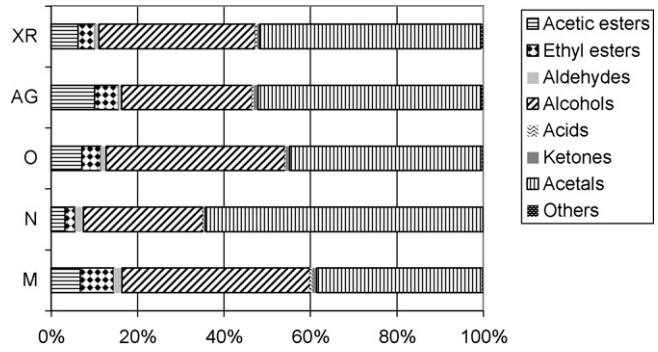

Fig. 4. Contribution (%) of different groups of quantified volatile compounds to the volatile profile of wines.

sidered as factors of wine quality because they possess fusel-like odours [28]. Moderate concentrations of these, however, contribute to the wine's aromatic complexity. In this study, the value of higher alcohols varied significantly according to the yeast used (Table 2). Other alcohols that reached high concentrations in all the wines were 2-methyl-1-butanol, 2-phenylethanol, 1-propanol and isobutanol. However, only 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenylethanol (rose aroma) have an OAV greater than 1.0 in all the samples. According to Torrens et al. [11], alcohols characterized by a "vegetal" and "herbaceous" aroma, such as 1-hexanol and *cis*-3-hexen-1-ol, seem to be linked to the yeast strains used. In fact, the highest producer of these compounds was the M strain while the lowest amounts were formed by the XR strain. Significantly different concentrations were found among the strains employed.

Esters in wine have two distinct origins: enzymic esterification during the fermentation process and chemical esterification during long-term aging. The same esters may be synthesized in either way [27]. The most prevalent ester in wine is ethyl acetate. This compound adds complexity to the aroma of wines at low levels, but it can give an unpleasant odour (vinegary) to the wine at concentrations higher than 150 mg L^{-1} [28]. For all our wines, its content was always below the level considered negative (Table 2), the lowest amount of ethyl acetate being found in the wine made with the commercial yeast strain. However, this compound showed OAV > 1 in all the samples. The other acetic ester which was present in high concentrations was methyl acetate, as was expected in red wines [27]. This compound makes a different contribution to wine aroma since it does not present the typical fruity aroma as the other acetic esters do. As Table 2 shows, concentrations of methyl acetate are higher in wines elaborated using autochthonous yeast strains than in those made using commercial strains.

Among acetates, isoamyl acetate has been considered as a quality factor by several authors especially in young wines [29,30]. This compound was the only acetate to reach an OAV greater than 1 and only in the XR wine. Moreover, its concentration was significantly higher compared with those provided by autochthonous yeast strains.

On the other hand, wine M had the highest amounts of ethyl esters due to its high content of ethyl lactate and diethyl succinate (malolactic esters).

All of the ethyl esters responsible for the fruity aroma in wine showed OAV > 1. Ethyl octanoate, which has a sweet aroma, showed the highest concentrations followed by ethyl hexanoate (green apple aroma) and ethyl butyrate (acid fruit aroma) (Table 2). Ethyl furoate was the ethyl ester with the lowest concentrations, ranging between 54 and 46 μg L^{-1}. Ethyl isovalerate (strawberry aroma [31]) was only found in the wine made with the commercial yeast strain.

AG and XR were the samples with the highest content of all "fruity" ethyl esters, including unquantified ones such as ethyl decanoate, ethyl *trans*-4-decenoate, ethyl dodecanoate, etc. Moreover, we detected some isoamylic esters (isoamyl hexanoate, octanoate and decanoate), the samples AG and XR particularly standing out for their high contents. Therefore, these samples should be the fruitiest wines.

According to Pérez-Coello et al. [32], yeast strains differ in their ability to produce acetaldehyde depending on the enzymatic activity (alcoholic dehydrogenase). In our study, we found significant differences among the acetaldehyde produced by some yeast strains, independently of their origin (autochthonous or commercial) (Table 2).

Fatty acids have been described as giving rise to fruity, cheesy, fatty and rancid notes. Although, C_6–C_{10} fatty acids are usually related to the appearance of negative odours, they are very important for aromatic equilibrium in wines because they oppose the hydrolysis of the corresponding esters [11], and their presence plays an important role in the complexity of the aroma [33]. The production of medium chain fatty acid in wines depends on which yeast strain is inoculated. Hence, Torrens et al. [11] observed that specific yeast strains stood out for their production level of characteristic fatty acids [11]. In our case, wine M presented the highest total amount of acids. Hexanoic acid was the most abundant acid followed by octanoic and isovaleric acid respectively, excepting wine N, whose main acid was octanoic. Accordingly, our results show that the difference among the yeast strains studied is due to the amount of acid and not the predominant acid. On the other hand, decanoic acid had the lowest concentrations in all samples. In fact, it was the only acid whose OAV did not exceed the unit, in similar way to that observed by Lorenzo et al. [34].

We did not find notable differences between the wines regarding those compounds that were identified but not quantified, such as *trans*-3-hexanol, ethyl-3-methylthio-propanoate, dihydro-2-methyl-3-(2H)-thiophenone, and β-damascenone among others.

3.4. Sensory analysis

We carried out triangular tests to compare the wines obtained using the different yeast strains. In all cases, the wines were differentiated by the panel ($p < 5$).

The aroma of the wines were assessed by seven tasters, using thirteen descriptors (green, floral, red fruit, ripe fruit, raisin, sweet, meat/sweat odour, nutty, citrus, balsamic, spicy, coffee and general impression) previously agreed upon as the best for describing sensory characteristics of wines.

Fig. 5 shows a "spider-web" graph of the results obtained in quantitative descriptive analysis (QDA). The attributes that did not achieve scores higher than 1 in any sample were not represented in this graph. The sample that showed the highest sensory quality was N because it reached the highest score for attribute "general impression". In contrast, the wine obtained using commercial yeast (XR) had the lowest value for this attribute. In fact, this wine reached the highest value for the "meat/sweat odour" attribute, that is a negative aromatic characteristic.

If we consider the volatile composition of these wines, samples AG and XR should have the highest values for the attributes "ripe fruit" and "sweet aroma" due to their large quantities of fruity ethyl esters. Moreover, these were the only two wines that presented the unpleasant "meat/sweat odour" aroma. The presence of compounds responsible for the "meat/sweat odour" could hinder or distort the assessment of the descriptor "ripe fruit". On the other hand, we found some correlations between descriptors and volatile compounds such as "green" with ethyl acetate or "ripe fruit" with β-damascenone.

PCA of the data obtained in QDA was carried out. Fig. 6 shows the score plot of the first two PCs. The plan made of the first two PCs (Fig. 6) indicates that the samples are divided into three groups.

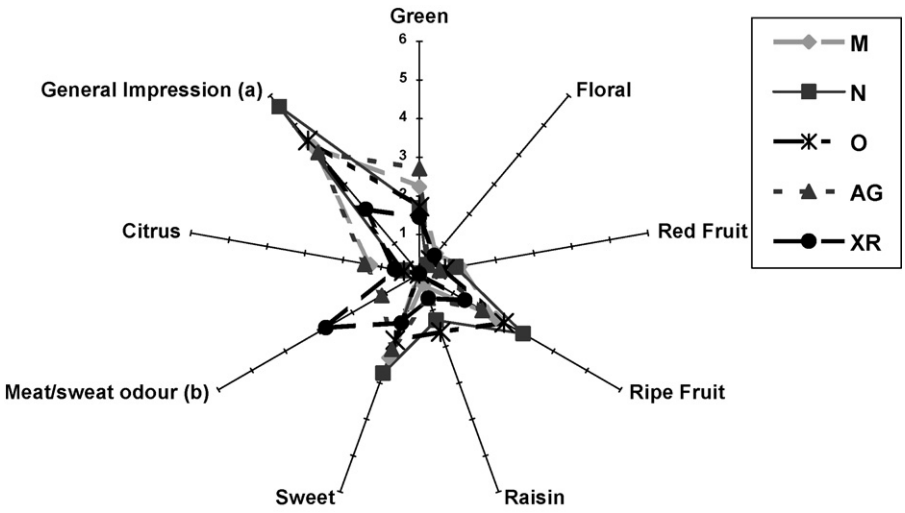

Fig. 5. Sensory profiles of red wine samples (a) significant differences between samples produced by commercial and autochthonous yeast strains; (b) significant differences between samples produced by commercial and O, N, and M yeast strains, respectively.

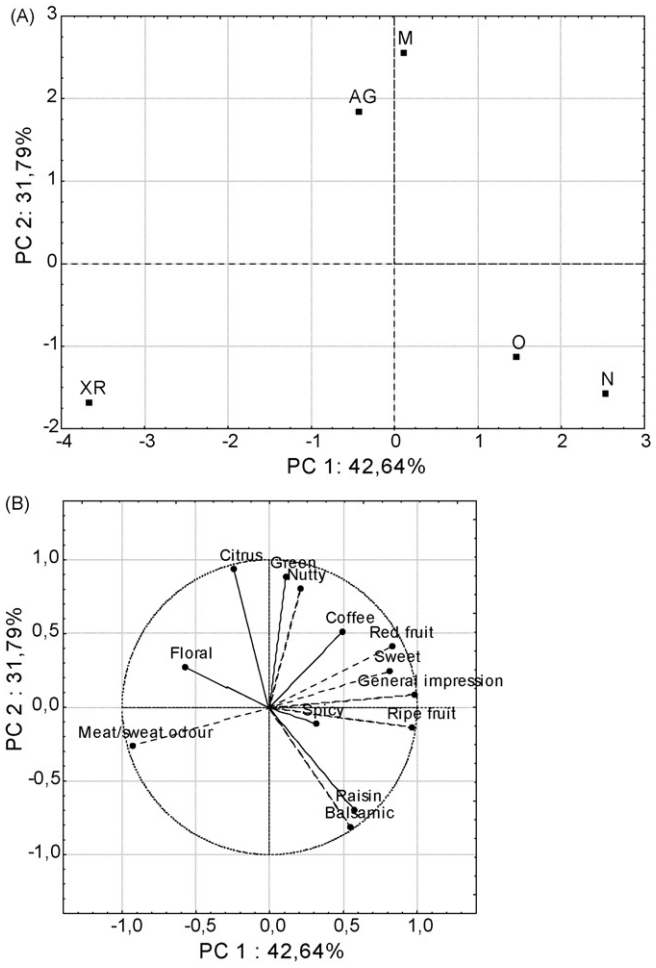

Fig. 6. Score plot of sensory descriptive variables (A) and wine samples (B) in the plan made of the first two principal components (PC1 against PC2).

One group is closely related to fresh and vegetable aroma (M, AG), another with fruit-sweet aroma (N, O), and the last group with the unpleasant aroma "meat/sweat odour" (XR). This last sample is also the most separated from the rest. These results are consistent with those obtained in the triangle tests. The samples differentiated by the panel with the highest percentages of success are those that appear more distant in Fig. 6, such as: M–N, N–XR, M–O or AG–XR.

4. Conclusions

The concentrations of most of the volatile compounds were significantly influenced by the yeast strain. Furthermore, because the implantation percentage of the strain sown in each vat was 100%, we can say that each strain tested is responsible for the volatile profile of the resulting wine. The differences observed in the volatile composition of wines obtained from different yeast strains appear to be quantitative rather than qualitative.

The results obtained in the sensory analysis suggest that autochthonous yeast produced wines of higher organoleptic quality.

Autochthonous yeast strains could be employed instead of commercial ones, thus enhancing the biodiversity.

Autochthonous yeast strains have been shown to be able to produce wines with different volatile profiles. Some of them have produced wines rich in alcohols and others have produce wines rich in fruity esters. All of them could be suitable for winemaking depending on the desired wine style. Moreover, several authors have demonstrated that coinoculation of wine yeast can be used in order to modulated the volatile composition and sensory profile of wines when a balanced yeast combination is used. Coinoculated fermentations are a promising tool for the wine industry, allowing winemakers to alter the aroma of wines according to market specifications [8]. Future research will focus on coinoculated fermentations.

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