María Arévalo Villena

ESTUDIO DE LA ACTIVIDAD β-GLUCOSIDÁSICA EN LEVADURAS VÍNICAS Y SU APLICACIÓN EN ENOLOGÍA

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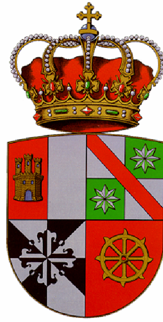

UNIVERSIDAD DE CASTILLA-LA MANCHA
Facultad de Ciencias Químicas
Departamento de Química Analítica y Tecnología de Alimentos

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DEPARTAMENTO DE
QUIMICA ANALÍTICA Y TECNOLOGÍA DE ALIMENTOS

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María Arévalo Villena

Visado en Ciudad Real, 16 de Junio de 2005

Fdo.: Ana Isabel Briones Pérez
Profesora Titular del Departamento de Química Analítica y Tecnología de Alimentos
Universidad de Castilla-La Mancha

Fdo.: Juan Francisco Úbeda Iranzo
Catedrático de Escuela Universitaria del Departamento de Química Analítica y Tecnología de Alimentos
Universidad de Castilla-La Mancha
Fdo.: María Arévalo Villena
Licenciada en Ciencia y Tecnología de Alimentos.

DEPARTAMENTO DE
QUIMICA ANALÍTICA Y TECNOLOGÍA DE ALIMENTOS

Facultad de Ciencias Químicas

Dª ANTONIA GARCÍA RUIZ, Profesora Titular de Escuela Universitaria y Secretaria del Departamento de Química Analítica y Tecnología de Alimentos de la Universidad de Castilla-La Mancha

CERTIFICA:

Que el presente trabajo de investigación titulado "**Estudio de la actividad β-glucosidásica en levaduras vínicas y su aplicación en Enología**" constituye la Tesis Doctoral que presenta **Dª MARÍA ARÉVALO VILLENA** para aspirar al Grado de Doctor en Ciencia y Tecnología de Alimentos, y ha sido realizado en los laboratorios de este Departamento bajo la dirección de la Dra. Dª. Ana Isabel Briones Pérez y la Dr. D. Juan Francisco Úbeda Iranzo.

Y para que así conste, expido y firmo el presente certificado en Ciudad Real a diez de marzo de dos mil cinco.

Vº Bº

Fdo. Maria Dolores Cabezudo Ibáñez	Fdo. Antonia García Ruiz
Directora del Departamento	***Secretaria del Departamento***

Sonríele a la vida…, conseguirás contagiarla

A mis padres

A mi prima

Espero no extenderme demasiado con los agradecimientos, aunque me sobran los motivos.

Con mucho cariño y admiración les doy las gracias a mis directores de tesis, la Doctora Ana Briones y el Doctor Juan Úbeda, Ana y Juan, por enseñarme a aprender, por su profesionalidad, y por dirigir tan brillantemente esta ilusión llamada Tesis, que no hubiera sido tan satisfactoria sin la calidad humana que han mostrado en todo momento.

Al Institute for Wine Biotechnology, por la oportunidad de trabajar allí. Al Doctor Ricardo Cordero, por "estrujarme" tanto en tan poco tiempo, aprovechando al máximo mis estancias en Sudáfrica. A sus "chicos", y con especial cariño a Johnny, Nivi, Vasu y Sarath, por abrirme la puerta de su mundito, algo tan importante cuando estás tan lejos.

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A mis hermanos, no sé si gracias o enhorabuena, porque llevar a la niña a veces es todo un reto.

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A mi prima, ¿cómo expresarlo? Apoyo incondicional sobre todo en este último año. Si no fuera por esas risas…

Y sobre todo a mis padres, olvidados injustamente en ocasiones por el papel que les toca. Porque cada día me demuestran su amor, pase lo que pase. Gracias por TODO.

JUSTIFICACIÓN DEL TRABAJO

En la transformación del mosto de uva en vino, el proceso bioquímico más importante es la fermentación alcohólica, llevada a cabo por levaduras del género *Saccharomyces.* Pero también es cierto que en la biota espontánea están presentes levaduras no *Saccharomyces*, que son capaces de aportar ciertas características sensoriales a determinados tipos de vinos.

Estas levaduras, juegan un papel relevante en las primeras etapas de la fermentación, y aunque posteriormente son desplazadas por las condiciones del proceso, que conllevan la imposición de *Saccharomyces*, merecen ser consideradas por sus efectos beneficiosos, debiéndose potenciar más su presencia para conseguir vinos más complejos organolépticamente.

Por otra parte, en Castilla la Mancha, durante la última década se han efectuado considerables inversiones, apoyadas por los fondos estructurales de la UE, destinadas a la renovación de equipos y maquinaria y a la reconversión del viñedo. Estos hechos obligan a unos cambios que pasan por la mejora de la calidad de los vinos y el estudio del mercado al que van dirigidos, entre otros. Dado que el aroma es uno de los atributos sensoriales más valorado en la actualidad, su potenciación, en especial en aquellos vinos de variedades menos aromáticas, es uno de los retos biotecnológicos que tienen actualmente planteados los centros de investigación aplicada relacionados con el sector vitivinícola.

El mercado del vino está sujeto continuamente a cambios impuestos por las modas que determinan los consumidores. Actualmente se tiende a vinos blancos, jóvenes, afrutados, cuyo signo de identidad son sensaciones frescas y ligera

vienen dadas por una mayor una liberación de aromas que la conseguida hasta hace un par de décadas.

Para ello en Enología se emplean preparados enzimáticos que ayudan a valorizar los vinos, situándolos en una posición competitiva respecto a los países con biotecnología de vanguardia. El estudio de estas actividades, sentaría las bases para el inicio de una línea de investigación sobre biotecnología aplicada en el campo de enzimas “ad hoc” obtenidas de levaduras de ecosistemas vínicos naturales, y cuyo uso respondería a las tendencias actuales dirigidas hacia productos naturales y genuinos. Esto supondría una alternativa al uso de los preparados enzimáticos comerciales de origen fúngico que se usan hoy día en las bodegas.

Todo ello ha provocado la aparición de numerosos estudios sobre aroma en vinos jóvenes, ligados a la búsqueda y caracterización de actividades enzimáticas, más concretamente β-glucosidasas, procedentes de levaduras vínicas. La ingente bibliografía, plantea cierta confusión e incluso a veces contradicción a la hora de trabajar, tanto en el sector de la investigación como a pie de bodega (sectores que deben estar estrechamente unidos), por lo que se creyó oportuno orientar la presente Tesis Doctoral hacia la puesta a punto de un método rápido que permitiera cuantificar la actividad β-glucosidásica en levaduras vínicas, caracterizar las enzimas halladas y comprobar su efecto en vinos elaborados con variedades de uvas blancas con diferente potencial aromático procedentes de cultivares de Castilla la Mancha.

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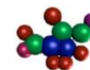
1. Introducción general

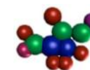


El término enzima deriva del latín donde significa "en levaduras", ambas palabras son la clave principal del proceso de vinificación, donde la fermentación alcohólica es sin duda la transformación bioquímica más importante. En el desarrollo de la fermentación intervienen levaduras con alta capacidad para producir etanol a partir del azúcar del mosto, cuya composición depende principalmente de la variedad de vinífera, del suelo y de la climatología.

La composición del mosto, junto con los microorganismos que intervienen en el proceso y la tecnología de la que se dispone, son las variables que determinan el éxito de la fermentación alcohólica.

En la elaboración tradicional del vino, la fermentación del mosto se produce de forma espontánea y natural con levaduras propias de la materia prima, sin embargo, la aplicación de nuevas tecnologías, así como el deseo de conseguir vinos de calidad uniforme y adecuada, han hecho que en la actualidad el proceso se lleve a cabo con levaduras seleccionadas, que en algunos casos son además autóctonas de la región y/o bodega elaboradora.

Las cepas responsables de conducir la fermentación alcohólica pertenecen al género *Saccharomyces*, pero si limitáramos el proceso de elaboración del vino sólo a los cambios bioquímicos llevados a cabo por estas especies, no aprovecharíamos todo el potencial de la materia prima. Por el contrario, al considerar otros géneros y tener en cuenta la composición del mosto, se podría llegar a vinos más diversos, ya que las levaduras no *Saccharomyces* aportan características peculiares al producto final al poseer actividades enzimáticas que resultan de interés en Enología. En principio, esta flora autóctona, se podría usar tanto desde el comienzo del proceso con el uso de cultivos mixtos iniciadores, como de forma más controlada mediante el aislamiento, selección, caracterización y posterior adición al producto acabado de la levadura o de su extracto enzimático.



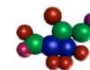
1.1 BIODIVERSIDAD DE LEVADURAS VÍNICAS

El proceso bioquímico tan simple, definido por Louis Pasteur, por el que el mosto de uva se convierte en vino mediante la fermentación espontánea de los azúcares, dando etanol, dióxido de carbono y otros metabolitos, es hoy en día infinitamente más complejo y sofisticado, aunque indudablemente sigue siendo la base de la elaboración del vino (Pretorius 2000).

En la actualidad se sabe que dicho proceso incluye la interacción de mohos, levaduras, bacterias ácido lácticas y bacterias acéticas, pero de todos ellos, son las levaduras el foco principal de la interacción bioquímica producida en los mostos de las variedades *Vitis vinifera*. (Fleet 1993, Fleet 1998).

Las levaduras son hongos unicelulares que pertenecen en su mayoría a la clase de los *Ascomycetes, Basidiomycetes* y *Deuteromycetes.* Para su crecimiento necesitan una fuente de carbono, hidrógeno, nitrógeno, fósforo y sales minerales. Son microorganismos quimioorganotróficos que utilizan compuestos orgánicos como fuente de carbono y de energía para sintetizar sus propias estructuras. De los 100 géneros de levadura representados por 700 especies descritas en la última edición de "The Yeast, A taxonomic Study" (Kurtzman and Fell 1998), 15 están asociados con el proceso de vinificación: *Brettanomyces* y su forma sexual *Dekkera*, *Candida*, *Cryptococcus, Debaryomyces, Hanseniaspora* y su forma asexual *Kloeckera*, *Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomyces, Saccharomycodes, Schizosaccharomyces* y *Zigosaccharomyces.* (Pretorius 2000).

Diversos estudios han proporcionado datos cuali y cuantitativos sobre la ecología de mostos y vinos (Barnett et al., 1972, Davenport 1974, Goto y Yokotsuka 1977, Bureau et al., 1982, Rosini et al., 1982, Parish y Carrol 1985, Izquierdo et al., 1995, Izquierdo et al., 1996 a, Sapis-Domercq 1997, Torija et al., 2001, Beltrán et al., 2002, Mas et al., 2003). Las levaduras responsables de la fermentación alcohólica



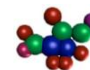
tienen su origen en el hollejo de la uva, en la maquinaria de la bodega y en la adición de cultivos iniciadores.

En las uvas encontramos una microflora constituida por mohos, bacterias y levaduras con características morfológicas y fisiológicas diversas, que en algunos casos son poco importantes en el proceso de fermentación. Esta flora la determina una serie de factores tales como la variedad de uva, temperatura, pluviosidad, tipo de suelo, fertilización, regadío, prácticas de cultivo, podredumbres o tipo de recolección, entre otros. (Arévalo Villena 2003).

De hecho se sabe que la fermentación se inicia por el crecimiento de algunas de estas especies (*Kloeckera, Hanseniaspora, Brettanomyces, Debaryomyces, Candida, Metschnikowia, Pichia y Zygosaccharomyces)* (Manzanares et al., 2000, Mendes-Ferreira et al., 2001), y que las especies fermentativas de *Saccharomyces* se encuentran en un principio en concentraciones realmente bajas, sobre todo en uvas sanas (Martín 1993).

Kloeckera y *Hanseniaspora* (*apiculata* y *uvarum* respectivamente) son las especies predominantes. Numéricamente por debajo de estas levaduras apiculadas encontramos especies de *Candida* como *C. stellata* o *C. pulcherrima*, *Brettanomyces* (*B. intermedius, B. lambicus* y *B. custeri*), *Cryptococcus, Kluyveromyces, Metschnikowia, Pichia* o algunas especies de *Rhodotorula* como *R. minuta* (Fleet, 1993; Fleet, 1998).

Por otra parte, el mosto de uva es un medio rico en nutrientes, pero teniendo en cuenta su bajo pH, que oscila entre 3 y 4 y su alto contenido en azúcar, se impone una fuerte selección de especies microbianas, de tal forma que aunque en un principio la flora espontánea esté así constituida, sólo unas especies de levaduras y otros microorganismos pueden proliferar. Del mismo modo, el ambiente anaerobio que se genera durante la vinificación y la adición de SO_2, usado por su poder antioxidante y

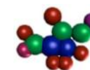


antimicrobiano, inhiben el crecimiento de microorganismos aerobios como las bacterias acéticas, los mohos y las levaduras oxidativas. (Henschke 1997).

Por tanto, las levaduras no *Saccharomyces*, con poca capacidad fermentativa debido a su baja tolerancia al etanol, son sustituidas por las dos especies de *Saccharomyces* que participan en el proceso (*Saccharomyces cerevisiae* y *Saccharomyces bayanus*) (Boulton et al., 1998, Fleet 1998, Charoncheai et al., 1997).

Sin embargo, a pesar de la selección natural que sucede de forma espontánea y que garantiza la imposición de *Saccharomyces*, la diversidad de especies al inicio del proceso, ha originado que en los últimos años se haya impuesto el uso regular de cultivos iniciadores. No obstante, otra vertiente del sector enológico está considerando la utilización de cultivos mixtos combinando distintos géneros de levaduras, lo que reflejaría la biodiversidad levaduriforme de cada región (Fernández 1999, Heard 1999, Pretorius 2000, Holm-Hansen 2001, García A. et al., 2002). Con ello se enriquecería el producto final desde el punto de vista del aumento del flavour de los vinos, ya que parece que las levaduras no *Saccharomyces* incrementan la concentración de compuestos volátiles responsables del aroma afrutado (Herráiz et al., 1990, Lema et al., 1996, Ciani y Ferraro 1995, Fernández et al., 2000, Strauss et al., 2001, Romano et al., 2003). Además, el uso de cultivos mixtos aislados en cada bodega y usados como iniciadores, reflejaría la biodiversidad levaduriforme de cada región.

En resumen, en la fermentación espontánea de los mostos de uva se suceden distintas etapas biológicamente diferenciadas, en las que intervienen especies de levaduras con marcadas diferencias fisiológicas entre sus cepas. Pero sólo las mejor adaptadas resisten hasta el final del proceso siguiendo el principio de sustitución poblacional, aunque una inadaptación a las condiciones extremas o una baja tolerancia al stress fermentativo, no significa que las cepas participantes en los primeros estadíos no aporten peculiaridades específicas a los caldos. En definitiva la



elaboración del vino es un proceso microbiológico dinámico en el que hay una sucesión de géneros, especies y de cepas de levaduras.

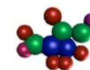
1.2 COMPUESTOS TERPÉNICOS

Los compuestos terpénicos contribuyen al carácter varietal que tipifica el vino, pudiéndose clasificar en tres categorías que están interrelacionadas entre sí. En primer lugar, el grupo más complejo es el que forman los compuestos del aroma que se encuentran en forma libre, como el linalol, geraniol, nerol y los piranos y furanos de los óxidos del linalol. Sin embargo, dependiendo de algunos factores como el clima o el tratamiento del mosto, se pueden encontrar en este grupo, algunos monoterpenos adicionales tales como el citronelol, α-terpineol, ho-trienol óxidos del nerol, mircenol, ocimenoles o algunos aldehídos o hidrocarburos.

Un segundo grupo lo constituyen los polialcoholes o formas polihidroxiladas de estos monoterpenos, que no contribuyen directamente en el aroma del vino, aunque algunos pueden hidrolizarse pasando a ser compuestos volátiles, como es el caso del diendiol, cuyo fraccionamiento origina ho-trienol y un óxido del nerol. (Williams et al., 1981).

Por último están las formas glicoconjugadas de los monoterpenos que tampoco son volátiles debido a su estructura. Estos glicósidos son, en la mayoría de los casos, las formas más abundantes de los tres tipos descritos y se conocen como precursores del aroma. (Mateo y Jiménez 2000).

En la **Figura 1** se muestra la evolución de estos precursores y de los terpenos libres durante las distintas fases de la maduración del fruto. En ella se observa cómo la concentración de los precursores es mucho más elevada en cualquiera de las etapas de maduración de la uva y que además los terpenos libres no sólo tienen una concentración baja a lo largo de todo el proceso, sino que a partir del final de la cuarta



etapa disminuyen, por lo que en el momento de la vendimia, esta diferencia entre los precursores y los terpenos libres es aún más acusada. (Arévalo Villena 2003).

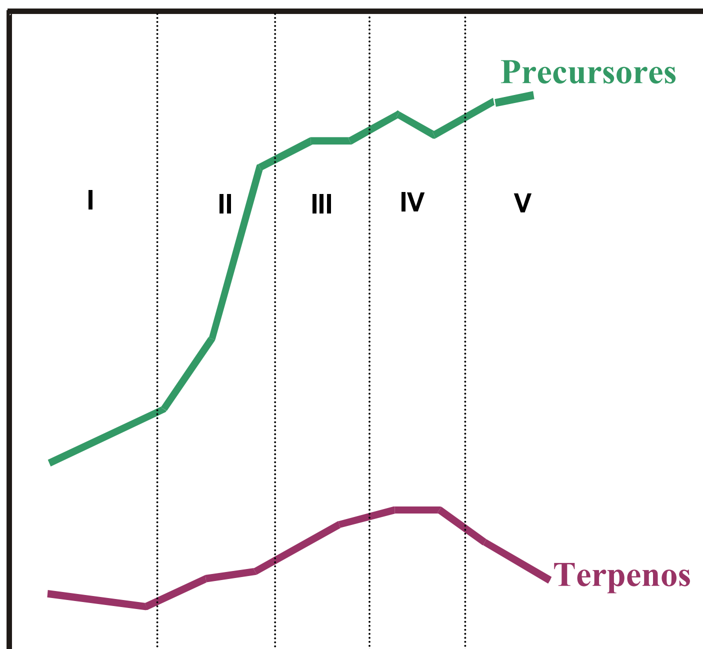
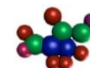


Figura 1. Evolución de monoterpenos de los grupos 1 y 3 en función de los días de maduración de la uva.

1.2.1 Estructura de los glicósidos.

Los compuestos glicosídicos identificados en frutas y plantas son muy complejos y presentan gran diversidad, sobre todo con respecto a la aglicona. La fracción glucídica, consiste en un β-D-glucopiranósido y diferentes diglicósidos: 6-O-α-L-arabinofuranosil-β-D-glucopiranósido, 6-O-α-L-arabinopiranosil-β-D-glucopiranósido (vicianósidos), 6-O-α-L-ramnopiranosil-β-D-glucopiranósido (rutinósidos) o 6-O-β-apiofuranosil-β-D-glucopiranósido, 6-O-β-glucopiranosil-β-D-glucopiranósido y 6-O-β-xilipiranosil-β-D-glucopiranósido (primeverósidos) (Gunata et al., 1985, Vasserot et al., 1991). En casos excepcionales también se han aislado trisacáridos glicoconjugados (Winterhalter y Skouroumounis 1997). La parte aglicona normalmente está formada



por terpenoles, aunque también se pueden encontrar óxidos del linalol o dioles y trioles terpénicos.

En la **Figura 2** se recogen algunos glicósidos encontrados en uvas de la variedad Moscatel.

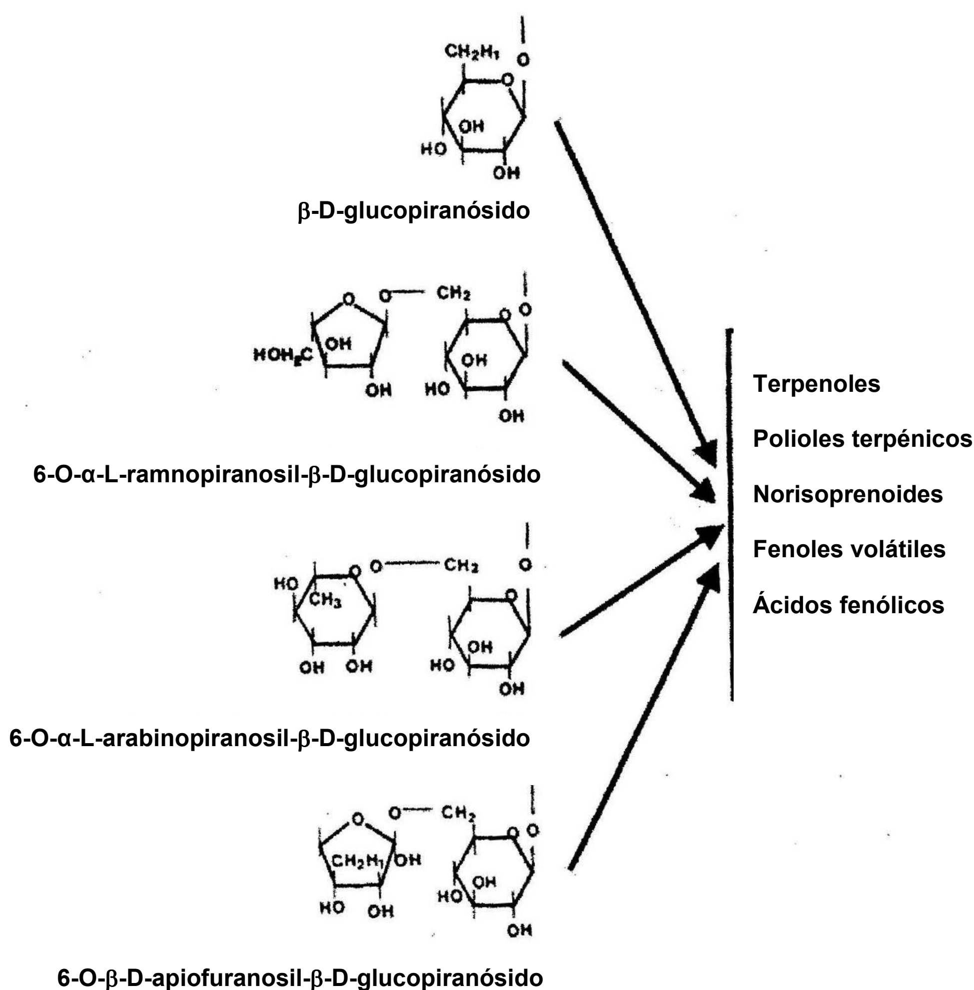
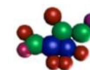


Figura 2. Glicósidos de la variedad Moscatel.

Además, existe otro tipo de compuestos, también considerados como precursores del aroma, cuyas agliconas pueden ser alcoholes lineales o cíclicos tales como el hexanol, feniletanol, bencil alcohol, norisoprenoides en C_{13}, ácidos fenólicos y fenoles volátiles como la vainillina.

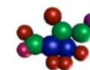


En la **Figura 3** se muestran los monoterpenos más frecuentes en el mosto y en el vino (Allen et al., 1989, Schwab y Schreier 1990; Sarry y Gunata 2002). Se pueden identificar alrededor de 70 terpenos, pero sólo los que se encuentran en concentraciones superiores a su umbral de percepción son los que realmente aportan el perfume característico a los vinos.

Consideran los glicósidos y las agliconas más frecuentes, se puede generalizar diciendo que los precursores más abundantes son los apiosilglicósidos (por encima del 50 % dependiendo de la variedad), seguidos de los rutinósidos (del 6 al 13%) y finalmente los glucosilglicósidos (de 4 al 9 %). Diversos estudios muestran que no todos los glicósidos están presentes en todas las variedades y que sus proporciones son también diferentes dependiendo del tipo de uva (Bayonove et al., 1993), oscilando entre un rango de 500 hasta 1700 μg / L de mosto (Gunata et al., 1985).

Los estudios que se recogen en la bibliografía consideran como variedades de *Vitis vinifera* aromáticas las uvas de Gewüerztraminer, Riesling, Moscatel, Cabernet sauvignon, Merlot, Monastrell, Ruby cabernet, Syrah, Cencibel, Macabeo, Xarel.lo, Parellada, Chardonnay, Nosiola, Garganega, Malvasía o Brachetto, entre otras (Nicolini et al., 1993, Razungles et al., 1993, Di Stefano et al., 1995, Arrhenius et al., 1996, Mccloskey et al., 1996, Reynolds y Wardle 1997, Lao et al., 1997, Zoecklein et al., 1997, Reyero et al., 2000, García-Moruno et al., 2000).

Figura 3. Monoterpenos del mosto y del vino: 1, 2, 8, 9 óxidos del linalol, 3 linalol, 4 ho-trienol, 5 neral, 6 α-terpineol, 7 geranial, 10 citronelol, 11 nerol, 12 geraniol, 13, 15 dioles, 14, endiol, 16 hidroxicitronelol, 17 8-hidroxi-dihidro-linalol, 18 hidroxinerol, 19 8-hidroxilinalol, 20 hidroxigeraniol, 21 8-hidroxilinalol, 22 ácido geránico, 23 triol.

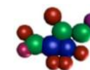


Ya que estos compuestos representan una reserva inmovilizada del aroma, romper el enlace entre el terpeno y el azúcar podría contribuir a aumentar su intensidad y por lo tanto el flavour de los vinos, siempre y cuando se trate de una variedad de uva rica en precursores. Además, hay que tener en cuenta que ninguno de estos compuestos por separado aporta las características varietales al vino, pero sí lo hace la mezcla de todos ellos; cuyo umbral olfativo es más bajo que cada uno de sus componentes individuales (Ribereau-Gayon et al., 1998).

1.2.2 Hidrólisis de los terpenos glicosilados

Desde hace unos años se ha estudiado la hidrólisis de los glicósidos, pero los trabajos presentan a menudo contradicciones. Algunos de ellos se basan en la hidrólisis ácida por calentamiento, pero las agliconas resultantes aparecen dañadas y por consiguiente no contribuyen a la fracción olorosa del producto (Usseglio Tomaste y Di Stefano 1980). Sin embargo algunos autores (Williams et al., 1982, Di Stefano 1989, Mateo et al., 1997, Sarry y Gunata 2004) han puesto de manifiesto que esta forma de liberar los terpenos simula las reacciones que tienen lugar durante el envejecimiento de los vinos y que los alcoholes terpénicos aparecen en concentraciones similares en ambas situaciones.

Otros autores (Katrina et al., 1998) estudiaron la hidrólisis de los precursores en boca llevada a cabo por la microflora presente en la saliva, pero esta hipótesis necesita ser confirmada usando un sustrato natural como el vino y no uno sintético (hexil β-D-glucósido). Por último, otra vía de estudio, probablemente la más utilizada, es la hidrólisis enzimática, donde entran en juego las β-glucosidasas.



1.3 ENZIMAS DE INTERÉS EN ENOLOGÍA

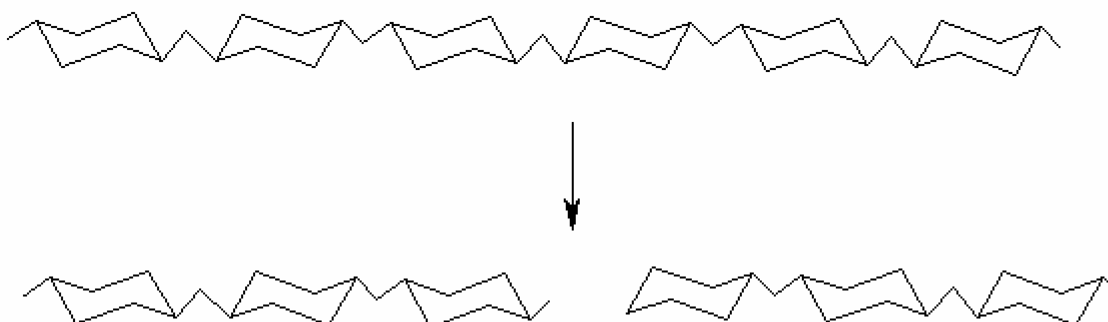
Las enzimas son proteínas especializadas en la catálisis de las reacciones biológicas. Se encuentran entre las más notables de las biomoléculas conocidas debido a su extraordinaria especificidad y a su elevado poder catalítico.

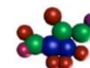
Su importancia en el proceso de vinificación es conocida, de hecho, en un principio no se usó el nombre de enzima, sino el de "fermentos" debido a que se sabía la existencia de catalizadores biológicos que intervenían en la fermentación del azúcar para dar etanol. En Enología interesan principalmente las enzimas oxidorreductasas, pectinasas, proteasas y glicosidasas (Fernández 1999).

1.3.1 Glucosidasas. Clasificación y modo de acción

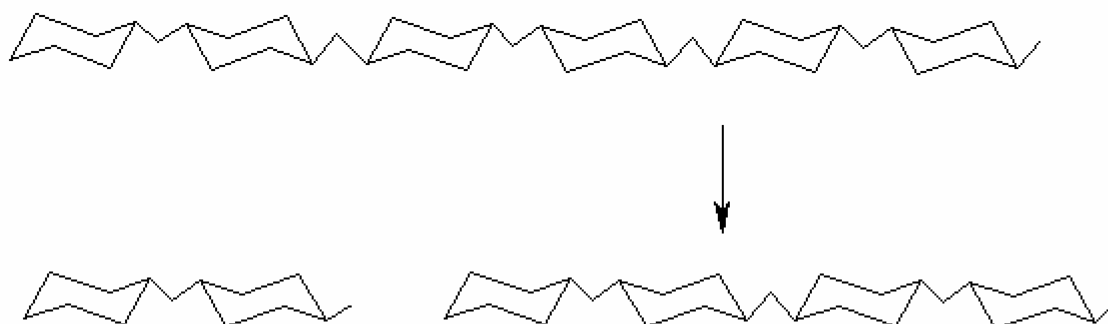
Las glucohidrolasas β-D-glucósido (E.C. 3.2.1.21), más conocidas como β-glucosidasas, pertenecen a la familia de las β-glucanasas, y son responsables del catabolismo de una amplia gama de hidratos de carbono; pueden ser endo o exoglucanasas, cuya diferencia es el punto por el que inician la hidrólisis del polisacárido (Schomburg y Schomburg 2003)

Así por ejemplo las endoglucanasas hidrolizan al azar cualquier punto del interior de la molécula liberando polisacáridos de menor tamaño.





Por el contrario, las exoglucanasas actúan de forma ordenada a partir de los extremos no reductores de la macromolécula, dejando libres residuos de celobiosa.



Y son estos residuos no reductores de celobiosa el sustrato de las β-glucosidasas, que atacan el disacárido liberando dos moléculas de glucosa.

En la actualidad se conoce perfectamente la estructura de los precursores del aroma y el mecanismo de hidrólisis de las β-glucosidasas, siendo un proceso secuencial formado por dos hidrólisis consecutivas (Gunata et al., 1988). En un primer paso se produce la liberación del monoterpenil β-D-glucósido más el correspondiente azúcar residual. Esta reacción es llevada a cabo por una α-L-arabinosidasa, una α-L-ramnosidasa, o una β-apiosidasa con la liberación de una arabinosa, una ramnosa o una apiosa respectivamente, más el correspondiente glucósido unido a la aglicona por un enlace β-D-glucosídico cuyo enzima específico de hidrólisis es la β-glucosidasa. Una vez liberado el terpeno al medio, éste pasa a ser volátil formando parte de la fracción aromática del vino ***(Figura 4)***.

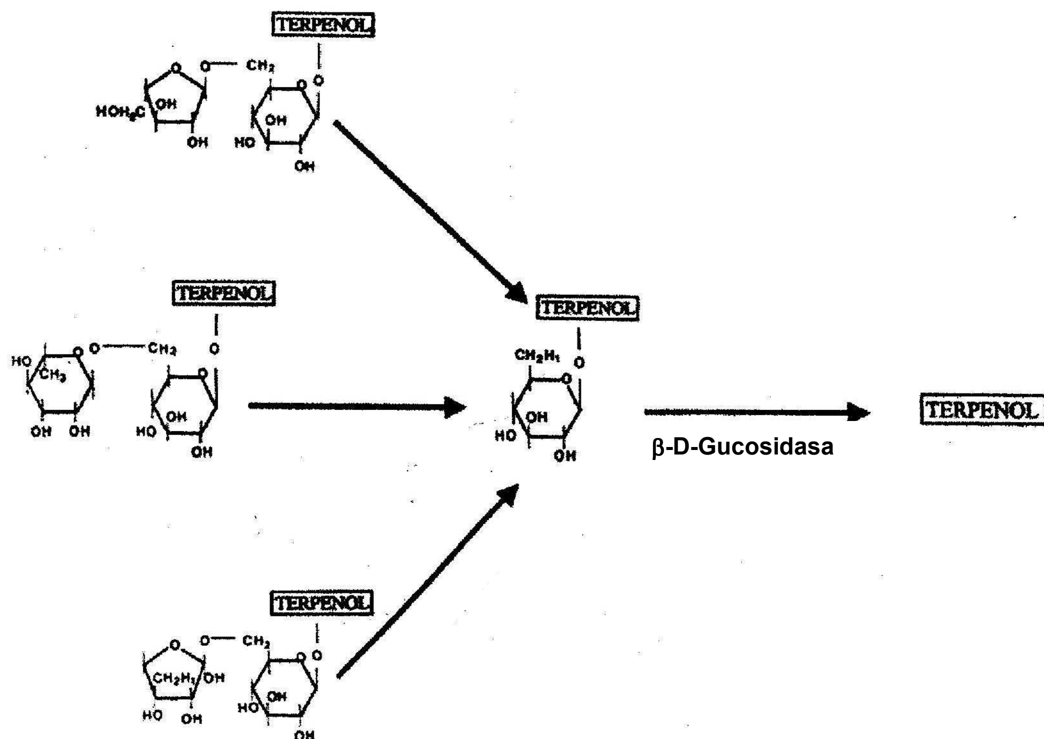
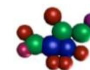


Figura 4. Hidrólisis enzimática secuencial de los glucósidos del mosto.

1.3.2 Procedencia de las β-glucosidasas

Las β-glucosidasas se han aislado de distintas fuentes. En humanos, se han identificado hasta tres β-glucosidasas nativas (Hays et al., 1996). Dos de ellas formando parte de las membranas biológicas y con alta especificidad de sustrato, mientras que la tercera se localiza en el citosol de las células hepáticas y en el intestino de los mamíferos (Mellor y Layne 1971, Daniels et al., 1981).

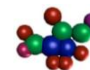
En insectos las β-glucosidasas se asocian a la hidrólisis de di y oligosocáridos derivados de las hemicelulosas y celulosa, estando implicadas en la interacción insecto-planta (Terra y Ferreira, 1994).



En algunas plantas, están implicadas en distintas rutas metabólicas relacionadas con la respuesta al crecimiento, defensa de patógenos y herbicidas o hidrólisis de fitohormonas conjugadas. Existen numerosos estudios acerca de su especificidad (Gunata et al., 1990 c, Cicek et al., 2000), modo de acción Bathia et al., 2002) o capacidad para ser clonadas (Geerling et al., 2000). Entre estas β-glucosidasas se encuentran las procedentes de la uva, con un pH óptimo de 5, dando bajas actividades a pH comprendidos entre 3 y 4 (Lecas et al., 1991) siendo fuertemente inhibidas por altas concentraciones de glucosa y etanol (Bayonove et al., 1984, Aryan et al., 1987). Son incapaces de hidrolizar azúcares conjugados con alcoholes terciarios como el linalol o el α-terpineol, y su actividad se ve afectada por ciertas operaciones como la clarificación y centrifugación. Por todo esto resultan poco útiles para su aplicación en Enología.

Los microorganismos son una fuente muy importante de enzimas y aunque las características de una enzima microbiana pueden ser muy diferentes en función de la fisiología, las β-glucosidasas de microorganismos comparten muchas funciones, entre las que se encuentran la conversión de la celulosa hasta etanol, la liberación de compuestos del aroma en frutas y vinos y la degradación de la materia orgánica en el suelo y en ecosistemas marinos (Potgieter 2004).

En cuanto a su localización, las β-glucosidasas microbianas pueden ser extracelulares, cuando son excretadas al medio de crecimiento, o intracelulares, cuando permanecen en el interior de las estructuras. Respecto a su síntesis son constitutivas, cuando el microorganismo las sintetiza independientemente de las condiciones de crecimiento; o inducidas, cuando lo hace sólo bajo determinadas condiciones, como podría ser la presencia en el medio de crecimiento de una fuente de carbono con un enlace glucosídico β-1,4. (Arévalo Villena, 2003).



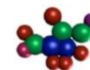
1.3.3 Las β-glucosidasas y el vino

Actualmente en la elaboración del vino, para incrementar la liberación de aromas se usan preparados enzimáticos comerciales de origen fúngico, principalmente procedentes de *Aspergillus spp*.

La composición de estos preparados no está en ocasiones, bien definida, y en realidad son una mezcla de glucanasas; así, por ejemplo, uno de los preparados más utilizados en bodega posee actividades exo β-glucanásica, endo-β-1,3-glucanásica, exo-β-1,6-glucanásica y actividad β-glucosidásica (Van Rensburg y Pretorius 2000).

El inconveniente de su uso radica en que son inespecíficos, por lo que pueden desencadenar otras reacciones de hidrólisis colaterales (Riou et al 1998). Las actividades enzimáticas presentes en estos cócteles enzimáticos se inhiben por la presencia de glucosa y pH ácido, no obstante son eficaces porque consiguen liberar los compuestos de interés y mejorar el aroma. Un estudio comparativo llevado a cabo por Tingle y Halvorson (1971) dio como resultado que la actividad β-glucanásica, sólo era responsable del 3 % de la liberación de los precursores del aroma mientras que una enzima β-glucosidasa específica hidrolizó la totalidad.

Una alternativa al empleo de los preparados enzimáticos de origen fúngico, sería el uso de enzimas específicas de levaduras de ecosistemas vínicos que se encuentran adaptadas a su medio ecológico. Dichas enzimas liberarían los terpenos conjugados sin afectar al resto de los componentes y se conseguiría aumentar la tipicidad de los vinos, tendencia actual cada vez más valorada por los consumidores.



1.4 VISIÓN GENERAL DE LAS GLICOSIDASAS MICROBIANAS. ESTUDIOS PRELIMINARES

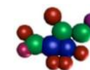
Los microorganismos que poseen glucosidasas con implicaciones enológicas se han dividido en tres grupos taxonómicas distintos: bacterias, levaduras y mohos (Gunata et al., 1990 b, Boio et al., 2002).

1.4.1 Glicosidasas de bacterias lácticas

Aunque no existe mucha información acerca de las β-glucosidasas procedentes de bacterias ácido lácticas implicadas en Enología, *Oenococcus oeni* es la especie que posee este tipo de enzimas (Henick.-Kling 1995, Maicas et al., 1999).

Diversos estudios demostraron la efectividad de las β-glucosidasas de la bacteria sobre el malvidin-3-glucósido y sobre los terpenil glicósidos del vino (Grimaldi et al., 2000, Boio et al., 2002, Barbagallo et al., 2004, D'Incecco et al., 2004). Sin embargo McMahon et al. (1999), observó mínima actividad sobre extractos glicosídico y Mansfield et al. (2002), aun detectando la existencia de enzimas en algunas cepas de *O. oeni*, no consiguieron hidrolizar con ellas los glicósidos aislados directamente de uvas.

Ugliano et al. (2003), estudiaron durante la fermentación maloláctica la hidrólisis de los precursores enzimáticos llevada a cabo por diversas cepas de usadas como cultivos iniciadores de *O. oeni,* observando que la actividad β-glucosidásica era cepa dependiente y que algunas de ellas contribuían notablemente a la liberación de volátiles, mejorando las características sensoriales de los vinos.



1.4.2 Glicosidasas de levaduras

Las β-glucosidasas de las levaduras han sido probablemente las enzimas microbianas más estudiadas.

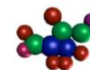
A. Actividad β-glucosidásica en cepas Saccharomyces.

Existe controversia sobre la capacidad β-glucosidásica de la levadura vínica. Hay autores que han puesto de manifiesto esta actividad, y en cambio otros defienden la idea de que la síntesis de esta enzima es nula en este género.

Como defensores de su capacidad enzimática, Darriet et al., (1988), observaron que las oxidasas localizadas en el espacio periplásmico de *Saccharomyces* tenían capacidad de hidrolizar los monoterpenos glicosilados de las uvas Moscatel, encontrando que esta actividad era glucosa independiente.

Más tarde Rosi et al., (1994) realizaron un estudio con 370 levaduras de origen vínico de las que 153 eran *Saccharomyces*, y de éstas, sólo una presentaba actividad β-glucosidásica. En el "screening" previo se usó arbutina como fuente de carbono, y la actividad se cuantificó con el sustrato sintético pNPG (paranitrofenil-β-D-glucósido). La síntesis de la enzima se inducía cuando el microorganismo crecía en aerobiosis y se inhibía por la presencia de glucosa; por otra parte también se observó, que la actividad de la enzima, se inhibía ante la presencia de glucosa o etanol.

Mateo y Di Stefano (1997) encontraron actividad β-glucosidásica intracelular (asociada a la pared) en *Saccharomyces,* basándose en la hidrólisis del pNPG y glucósidos aislados del vino de la variedad Moscatel. Esta actividad se inducía por la presencia de salicina como única fuente de carbono durante el crecimiento. El problema era que la enzima se veía fuertemente inhibida con tan solo un 5 % de etanol, por lo que su uso sólo sería viable en las primeras etapas de la fermentación. Además demostraron que el pNPG no era el sustrato idóneo para la búsqueda de la

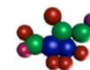


actividad, ya que los valores de su hidrólisis eran mayores que los resultados hallados sobre los glicósidos del vino.

Hernández et al (2002) por su parte, encontraron actividad β-glucosidásica en *Saccharomyces cerevisiae.* Probaron cualitativamente distintos medios de cultivos tales como YPD, SC y mosto de uva a los que se le adicionaban las siguientes fuentes de carbono: esculina, arbutina, pNPG y 4-metilumberifenil β-D-glucopiranósido (MUG). Posteriormente cuantificaron la actividad usando como sustrato pNPG y pNPX (paranitrofenil-β-D-xilopiranósido), y estudiando el pH y la temperatura óptimos para la síntesis enzimática. Observaron que una de las cepas de *Saccharomyces* estudiadas era capaz de hidrolizar los precursores del aroma aislados de mostos en las condiciones de vinificación, aunque esta hidrólisis era menor que la observada en la condiciones óptimas de la enzima. Por otra parte mostraron que la proteína tenía distinta afinidad por los diversos sustratos estudiados.

Finalmente, Fernández et al., (2003) hallaron que alguna cepa vínica de *Saccharomyces* era capaz de hidrolizar algunos glicósidos aislados de mosto de la variedad Moscatel.

Sin embargo, aunque estos autores detectaban actividad en *Saccharomyces*, no aseguraban su aplicación en Enología. Por otra parte en la bibliografía existen otros estudios que avalan que las levaduras de dicho género no poseen actividad β-glucosidásica capaz de hidrolizar los precursores del aroma en los procesos de vinificación. Así, Gunata et al., (1986) midieron la concentración de terpenos libres y ligados en uvas de la variedad Moscatel antes y después de la fermentación. El resultado fue que existían pequeñas variaciones en las concentraciones de estos compuestos, pero no eran lo suficientemente significativas como para clasificar a *Saccharomyces* como productora de enzimas β-glucosidasas.



Dada la controversia respecto al género *Saccharomyces* y a lo poco eficaz que resulta su actividad en Enología, se recurrió a la Biología Molecular para obtener cepas recombinantes que expresaran esta actividad. Así, Adam y Polaina (1991), transformaron *Saccharomyces cerevisiae* con el gen codificante de la actividad β-glucosidásica procedente de *Bacillus polimixa* consiguiendo que la nueva cepa creciera en celobiosa como única fuente de carbono.

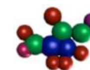
Posteriormente, se clonó el gen procedente de *Cellulomonas biazotea* en *Escherichia coli* para luego transformar *Saccharomyces cerevisiae*, dando resultados positivos al crecer las cepas transformadas en placas de esculina (Rajoka et al., 1998).

Estudios más recientes clonaron el gen BGL II responsable de la actividad β-glucosidásica de *Pichia etchellsii* en *E. coli.* La proteína fue caracterizada y purificada y mostró hasta 2.5 veces más actividad que la proteína natural sobre sustratos como salicina y amigdalina. (Bathia et al., 2005).

De forma similar se han realizado otros muchos trabajos relacionados con actividad β-glucosidásica y cepas de levadura recombinantes (Pérez-González et al., 1993, Adam et al., 1995, Janbon et al., 1995, Skory y Freer, 1995, Van Rensburg et al., 1998, Muller et al., 1998, Bathia et al., 2002, Liu et al., 2005, Van Rensburg et al., 2005), pero la Legislación no permite el uso de microorganismos recombinantes transgénicos en la Industria Alimentaria por lo que no han tenido incidencia en los procesos de vinificación.

B. Actividad β-glucosidásica en cepas no Saccharomyces.

Aunque no se debe descartar del todo la actividad β-glucosidásica en *Saccharomyces*, las levaduras más estudiadas en los últimos años han sido las no



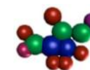
Saccharomyces, generalmente vínicas, aunque también se pueden encontrar en la bibliografía ensayos realizados con levaduras de origen no vínico como *Candida molischiana y Candida wickeramii* (Gunata et al., 1990 b, Sánchez-Torres et al., 1998), con actividades β-glucosidásicas menos sensibles a la glucosa y con una gran especificidad por la aglicona.

La β-glucosidasa de *C. molischiana* fue inmovilizada mediante una resina de Duolite A-569 mostrando propiedades físicoquímicas similares a las que presentó la enzima libre. La proteína inmovilizada resultó estable en las condiciones de vinificación y se podía usar varias veces para hidrolizar los precursores del aroma (Gueguen et al., 1996).

Uno de los campos más estudiados ha sido la puesta a punto de métodos que permitieran realizar una búsqueda de la actividad sobre un amplio número de levaduras y determinar de forma fiable su capacidad β-glucosidásica.

Los sustratos más usados han sido los glucósidos del paranitrofenol, tales como pNPG, p-nitrofenil-α-L-ramnopiranósido y p-nitrofenil-α-L-arabinofiranosido (Gunata et al 1990 a, Delcroix et al., 1994, Rosi et al., 1994, Charoncheai et al., 1997, Manzanares et al., 2000, Mendes-Ferreira et al., 2001, Hernández et al., 2002). Esta determinación no es específica, ya que la hidrólisis del sustrato puede venir dada por la acción de una actividad glucanásica residual, más inespecífica, y que por lo tanto, aún hidrolizando los glucósidos del paranitrofenol, no sea capaz de producir cambio alguno en los precursores del aroma, para lo que se necesita una especificidad mayor.

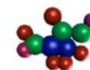
Por esto, para la determinación de la actividad se empezó a trabajar bien con glicósidos más específicos de la enzima, usando aquellos extraídos previamente del mosto o bien por cuantificación directa mediante cromatografía de gases de los monoterpenos libres en el vino (Rosi et al., 1994, Yannai y Sato, 1999, McMahon et al., 1999, Fernández et al., 2003).



En cuanto a la extracción de los glicósidos existen numerosos trabajos que utilizan distintos métodos para conseguirlo. Williams et al (1982) utilizaban cartuchos C_{18} en fase inversa para la retención de los precursores y que eluían con solventes apolares capaces de arrastrar los glicósidos. Di Stefano et al., (1991) mejoraron este protocolo usando otro tipo de solventes en la extracción. Otro de los adsorbentes empleados fue el Amberlite XAD-2 (Gunata et al., 1985) a su vez mejorado por Di Stefano (1989). Ninguno de los dos fue totalmente efectivo debido a que las resinas retenían glucosa libre que no se podía eliminar y que interferiría en la cuantificación. Los cartuchos de sílica gel en fase inversa parecían finalmente los más adecuados, aislando los glicósidos en un solo paso (Williams et al., 1995), aunque el método más reciente encontrado en la bibliografía extraía los precursores mediante microondas (Bureau et al., 1996), pero no se llegó a resultados concluyentes.

Otros trabajos se han dirigido al estudio de la síntesis de la enzima. Algunos autores observaron que la actividad era constitutiva (Vasserot et al., 1989), mientras que otros la inducían usando compuestos con enlaces β-glucosídicos, empleados en algunos casos como única fuente de carbono para el crecimiento de la levadura. Este criterio se usaba para la selección de las levaduras con actividad β-glucosidásica, de modo que las que no crecían con estas fuentes de carbono se rechazaban por no mostrar actividad en el ensayo cualitativo. De este modo se han usado en el medio de crecimiento el 4-metilumbeliferil-β-D-glucósido (Manzanares et al., 2000, Hernández et al., 2002), la arbutina (Rosi et al., 1994, Mendes-Ferreira et al., 2001, Hernández et al., 2002), la celobiosa (Yanai y Sato 1999), la esculina (Hernández et al., 2002), o la salicina (Miklósy y Polos 1995, Mateo et al., 1997).

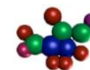
En cuanto a la localización celular de la enzima hay autores que detectaron la actividad en el sobrenadante de los cultivos (Rosi et al., 1994); algunos en célula entera (McMahon et al., 1999, Manzanares et al., 2000, Mendes-Ferreira et al., 2001)



y otros muchos la asociaron al interior celular (Rosi et al., 1994, Miklosy y Polos 1995, McMahon et al., 1999, Strauss et al., 2001). Algunos trabajos diferenciaron las fracciones obtenidas con la permeabilización de la célula, pudiendo detectarse entonces actividad a nivel de pared celular, espacio periplásmico, membrana o interior celular (Miklósy y Polos, 1995, Rosi et al., 1994, Mcmahon et al., 1999, Yanai y Sato 1999, Mendes-Ferreira et al., 2001). Incluso para este fraccionamiento se aplicaban diversos protocolos que en muchos casos eran contradictorios.

La influencia de la aireación en la síntesis enzimática ha sido otro factor estudiado creciendo las levaduras bajo distintos grados de aerobiosis. (Mendes-Ferreira et al., 2001, Hansen et al., 2001, Hernández et al., 2002).

Finalmente se estudió el efecto de la inmovilización de la enzima por unión física o química a soportes sólidos, o por su entrampamiento en polímeros que retuvieran sus propiedades catalíticas. Esto sería una forma de solucionar ciertos inconvenientes que derivan del uso industrial de enzimas, ya que éstas se pierden al final del tratamiento y no son, por lo tanto reutilizadas, lo que supone costes adicionales en el proceso. La inmovilización permitiría la reutilización en operaciones continuas o discontinuas con un control más preciso del proceso, haciéndolo además más rentable. La literatura existente en el campo de la inmovilización es muy amplia y variada por lo que se refiere a la enzima inmovilizada, el tipo de enlace entre la enzima y el soporte, la temperatura y pH del proceso, los agentes de entrecruzamiento y su activación y los soportes y sustratos utilizados, entre otros. Riccio et al., (1999), inmovilizaron la enzima mediante un método en un paso sobre hidroxiapatita observando una fuerte caída de la actividad aun cuando la proteína era mucho más estable que en su forma libre. Otro estudio más reciente (Dincer et al., 2005) utilizó quitosano como matriz polimérica, encontrando que la estabilidad de la enzima aumentó sin que disminuyera notablemente en esta ocasión la capacidad hidrolítica. En cualquier caso las variables que definen la eficacia de todos estos procesos de



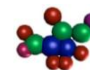
inmovilización son la constante de Michaelis-Menten (Km) y la velocidad inicial máxima (Vmáx).

1.4.3 Glicosidasas de mohos

El uso de este tipo de enzimas está muy extendido en la industria enológica para la liberación de los terpenos en los vinos. Su eficacia se basa en que no son selectivas y toleran relativamente bien altas concentraciones de etanol, aunque su pH óptimo oscile entre 5 y 6.

Normalmente se trata de preparaciones enzimáticas de *Aspergillus niger* con actividad β-glucosidásica residual. Gunata et al (1990 b) observaron cómo dos preparados comerciales de este tipo, que en principio debían actuar de forma similar sobre los precursores del aroma, tenían comportamientos diferentes en los vinos, lo que demostró su inespecificidad.

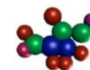
Por otra parte, el estudio de purificación de enzimas y el uso de sustratos sintéticos y métodos analíticos para determinar el grado de liberación, son necesarios en el desarrollo de la hidrólisis enzimática de los terpenil glicósidos (Wiiliams et al., 1982, Bitteur et al., 1989, Voirin et al., 1990, 1992; Skouroumounis et al., 1995). Se han aislado y purificado enzimas de preparaciones de origen fúngico, extractos vegetales o medios de cultivo sintéticos inoculados con mohos mediante diferentes técnicas cromatográficas (gel filtración, cromatografía de intercambio iónico, cromatografía de afinidad) (Guanta et al., 1988, Dupin et al., 1992, Spagna et al., 1998). También se han realizado ensayos de inmovilización de extractos enzimáticos de *A. niger* mediante perlas de alginato cálcico (Shoseyov et al., 1988), acrilato (Shoseyov et al., 1990) o γ-alúmina activada con dodecametilendiamina y glutaraldehído, comprobando por cromatografía de gases que las enzimas inmovilizadas hidrolizaban los precursores del aroma.



Aspergillus oryzae excretó al medio de crecimiento dos β-glucosidasas cuya síntesis era inducida por la quercitina, y aunque la enzima mayoritaria estaba fuertemente inhibida por la glucosa, la excretada en menor grado hidrolizó glicosil terpenos en los vinos. (Riou et al., 1998). No obstante se ha demostrado que estas enzimas están presentes en concentraciones bajas en los preparados enzimáticos comerciales, donde predominan otro tipo de actividades (Dupin et al., 1992).

Por otra parte, si los preparados enzimáticos se adicionaban al principio del proceso, la actividad se inhibía notablemente por la alta concentración de glucosa. (Gunata et al., 1990 b). Además, los terpenos que se pudieran liberar en estas primeras etapas de la fermentación no contribuirían organolépticamente al vino, ya que gran parte de ellos se volatilizarían y serían arrastrados por el CO_2 producido, por lo que durante el intercambio de metabolitos entre el medio de fermentación y el ambiente se perderían. En cualquier caso esta pérdida no sería preocupante, ya que en algunas variedades todavía quedaría gran cantidad de precursores susceptibles de ser hidrolizados mediante tratamientos enzimáticos.

En cualquier caso, diversos estudios han puesto de manifiesto que el empleo de estos preparados, provocan a veces aromas anómalos indeseables, incluso cuando se produce liberación de terpenos. Se han detectado altas concentraciones de vinil fenoles (4-vinil fenol y 4-vinil guayacol) llegando a concentraciones superiores a 1 mg / l. Algunos autores piensan se debe a la acción de dos sistemas enzimáticos. En primer lugar se produce la hidrólisis de los ésteres cinámicos mediante esterasas que se encuentran en los cócteles, por lo que el medio se enriquece de ácidos ferúlico y p-cumárico, que a su vez son descarboxilados por acción de las levaduras produciendo los vinil fenoles; aunque resulta difícil de explicar la formación de estos compuestos cuando los preparados enzimáticos se usaban sobre el vino acabado, donde ya no



entraría en juego el metabolismo de las levaduras, (Gunata 1993, Sefton y Williams 1993).

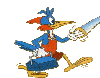
Por otra parte, la actividad hidrolítica de los preparados puede degradar los antocianos (Huang 1955, Blom 1983). El efecto de decolaración producido por este tipo de mezclas protéicas ha sido observado en frambuesas, fresas (Rwahahizi y Wrolstad 1988), moras (Yang y Steele 1958) y antocianos de las uvas (Fu-mian et al., 1994). Por lo tanto, su uso estaría desaconsejado en vinos con bajo contenido en antocianos.

Finalmente, las preparaciones enzimáticas procedentes de mohos generan diversos productos de oxidación durante la hidrólisis de los glicósidos (Sefton y Williams 1994).

Todo ello hace que en Enología la elección de las enzimas sea un factor a considerar, obligando a ampliar la búsqueda de enzimas β-glucosidasas para la mejora del aroma de los vinos, ya que las compañías transnacionales del sector vitivinícola no se han preocupado en profundizar en este tipo de investigaciones debido posiblemente a un mercado todavía poco exigente, por lo que existe una gran carencia de estudios relacionados con la síntesis de enzimas con potencial uso en alimentación, por parte de levaduras, mohos filamentosos y bacterias.

Estrategias mixtas como el uso combinado de levaduras seleccionadas de la microbiota autóctona y enzimas provenientes de los mismos ecosistemas, inmovilizados o no, constituyen en la actualidad una interesante y práctica línea de trabajo. Por ello, se planteó que la presente Tesis Doctoral fuese dirigida a la obtención de enzimas con actividad glucosidásica procedentes de levaduras GRAS aisladas del nicho ecológico, que fueran capaces de liberar aromas relacionados con el perfil floral y afrutado en los vinos blancos de distintas variedades de Castilla la Mancha.

2. Objetivos y planificación del trabajo

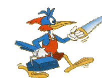


El objetivo principal de la Tesis Doctoral fue detectar actividad β-glucosidásica en levaduras vínicas, y que además dicha actividad cumpliera ciertos requisitos como ser extracelular, estable en las condiciones de vinificación y no ser inhibida por altas concentraciones de glucosa, etanol o ácido acético. Por otro lado la enzima debeía ser capaz de hidrolizar los precursores del aroma de tal forma que dicha liberación fuera percibida por los catadores. Esto, ofrecería la posibilidad de su uso en bodegas bien en forma de extracto, inmovilizada y/o clonada, como una alternativa al uso de los preparados enzimáticos comerciales de origen fúngico.

La planificación del trabajo experimental con el fin de lograr el objetivo propuesto, se fue modificando en virtud de los resultados obtenidos a lo largo del desarrollo del trabajo, contemplando siempre otras alternativas. Por otro lado las estancias en el laboratorio del IWBT han reportado una indudable ventaja no solo en la experiencia científica sino también en el terreno personal.

El trabajo realizado se divide en distintos bloques:

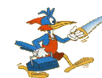
- Búsqueda de la actividad β-glucosidásica en levaduras vínicas: levaduras aisladas de distintas bodegas se chequearon cualitativamente en YNB celobiosa para detectar la actividad enzimática deseada. Las cepas se identificaron mediante la técnica de PCR-RFLP.



- Cuantificación de la actividad: puesta a punto de un método para cuantificar la actividad β-glucosidásica en las cepas que habían mostrado hidrólisis cualitativa, basado en la ruptura de un sustrato con enlace β-glucosídico específico como la celobiosa. Se optimizó por una parte, la síntesis de la proteína estudiándose la influencia del medio de cultivo y condiciones de crecimiento, y por otra, las variables del ensayo enzimático (concentración de sustrato, tiempo y temperatura de contacto). Por último las células se fraccionaron para conocer la localización de la enzima.
- Microvinificaciones con cultivos mixtos (*Debaryomyces pseudopolymorphus* y *Saccharomyces cerevisiae)* para comprobar si la actividad β-glucosidásica se expresaba durante la fermentación.

Los resultados obtenidos en este bloque del trabajo, dieron lugar a las dos primeras publicaciones: “Characterization of the β-glucosidase activity produced by enological strain of non *Saccharomyces* yeasts”, cuya parte experimental se realizó durante la primera estancia en Sudáfrica con cepas autóctonas; y “Optimization of a rapid method for studying the cellular location of β-glucosidase activity in wine yeasts”.

- Optimización de las condiciones de síntesis de la enzima en *Debaryomyces pseudopolymorphus* en cuanto a los requerimientos de la levadura: fuente de carbono, grado de aireación y tiempo de cosechado del cutivo. Se purificó parcialmente la enzima a partir del sobrenadante y se caracterizó en finción de



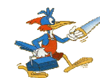
sus parámetros bioquímicos (pH y temperatura óptimos, estabilidad térmica, parámetros cinéticos, especificidad de sustrato y efecto sobre su actividad de iones metálicos y otros compuestos).

Estos resultados generaron la publicación titulada "Characterization of an exocellular β-glucosidase from *Debaryomyces pseudopolymorphus*", cuyo trabajo de laboratorio de llevó a cabo durante la segunda estancia en el IWBT.

- Estudio del potencial aromático de distintas variedades de uva blanca. Era primordial disponer de precursores del aroma para ensayar la actividad β-glucosidasa encontrada en *Db pseudopolymorphus*. Para ello se puso a punto un método rápido de extracción y cuantificación de los precursores del aroma basado en la retención, elución e hidrólisis ácida de los terpenil- glicosídos, valorando mediante un kit enzimático la glucosa liberada tras la hidrólisis.

El método está patentado y pendiente de publicación bajo el título "A rapid method for quantifying aroma precursors". Application to grape extract, musts and wines made from several varieties.

- Aplicación de la enzima en glicósidos naturales, mostos y vinos para conocer si era susceptible de hidrolizar los precursores del aroma. Se trabajó con distintas presentaciones de la proteína, liofilizando e inmovilizando los extractos purificados del sobrenadante, que se probaron sobre glicósidos aislados de



mostos de distintas variedades de uva. Se estudiaron la dosis y tiempo de contacto más adecuadas y su efecto se comparó con diversos preparados enzimáticos comerciales de origen fúngico empleados en Enología. Por último se llevaron a cabo microvinificaciones con mosto Moscatel y se emplearon los extractos enzimáticos más adecuados estudiando si existía hidrólisis mediante dos métodos analíticos: protocolo de aislamiento y cuantificación de los precursores del aroma y cromatografía de gases / espectometría de masas.

Estos datos se recogieron en el trabajo llamado "Relationship between *Debaryomyces pseudopolymorphus* enzymatic extracts and release of terpenes in wine".

- Por último se vinificaron tres variedades de uva de la zona con distinto potencial aromático (Airén, Riesling y Moscatel), y los vinos resultantes se trataron con el extracto β-glucosidásico de la levadura. El grado de hidrólisis se cuantificó con los mismos métodos del apartado anterior. Las muestras se evaluaron organolépticamente, siendo ésta la prueba definitiva del desarrollo experimental. El estudio se completó determinando la capacidad de formación de fenoles volátiles indeseados (4-vinil-fenol y 4-vinil-guaiacol).

Esta información originó el último artículo que cierra la presente Tesis Doctoral: "Aroma enhancement in wine using wine yeast β-glucosidases".

3. Publicaciones derivadas del trabajo de investigación

1. Cordero Otero R.R., Ubeda Iranzo J.F., Briones-Prerez A.I., Potgieter N., Arévalo Villena M., Pretorius I.S., Van Rensburg P., (2003). Characterisation of β-glucosidase activities in non-*Saccharomyces* wine-related yeasts. Journal of Food Science 68, 2564-2569
2. Arévalo Villena M., Úbeda Iranzo J.F., Cordero Otero R.R., Briones Pérez A.I. (2005a). Optimisation of a rapid method for studying the cellular location of β-glucosidase activity in wine yeasts. Journal of Applied Microbiology. (En prensa).
3. Arévalo Villena M., Ubeda Iranzo J.F., Gundllapalli S., Briones Pérez A., Cordero Otero R., (2005b). Characterization of a novel highly glucose-tolerant β-glucosidase from *Debaryomyces pseudopolymorphus.* Enzyme and Microbial Technology. (Enviado).
4. Arévalo Villena M., Díez Pérez J., Ubeda Iranzo J., Navascués E., Briones Pérez A., (2005c). A rapid method for quantifying aroma precursors. Application to grape extract, musts and wines made from several varieties. Food Chemistry. (Aceptado).
5. Arévalo Villena M., Ubeda Iranzo J., Briones Pérez A., (2005d). Relationship between *Debaryomyces pseudopolymorphus* enzymatic extracts and release of terpenes in wine. International Journal of Food Microbiology. (Enviado).
6. Arévalo Villena M., Ubeda Iranzo J., Briones Pérez A., (2005e). Aroma enhancement in wines using wine yeast β-glucosidase. Australian Journal of Grape and Wine Research. (Enviado).

JFS: Food Microbiology and Safety

Characterization of the β-Glucosidase Activity Produced by Enological Strains of Non-*Saccharomyces* Yeasts

R.R. CORDERO OTERO, J.F. UBEDA IRANZO, A.I. BRIONES-PEREZ, N. POTGIETER, M.A. VILLENA, I.S. PRETORIUS, AND P. VAN RENSBURG

ABSTRACT: The β-glucosidase activities of 20 wine-related non-*Saccharomyces* yeasts were quantified, characterized, and assessed for their efficiency in releasing aroma-enhancing compounds during the winemaking process. Of these enzymatic activities, the β-glucosidase activity of *Debaryomyces pseudopolymorphus* revealed the most suitable combination of properties in terms of functionality at wine pH, resistance to wine-associated inhibitory compounds (glucose, ethanol, and sulfur dioxide), high substrate affinity, and large aglycone-substrate recognition. Its potential as a wine aroma–enhancing enzyme was confirmed by the significantly increasing concentrations of free volatiles (citronellol, nerol, and geraniol) during the fermentation of Chardonnay juice inoculated with both *D. pseudopolymorphus* and a widely used commercial starter culture strain of *Saccharomyces cerevisiae*, VIN13.

Keywords: *Debaryomyces*, *Saccharomyces*, β-glucosidase, wine, yeast

Introduction

Terpenes are a class of compounds that contribute to the varietal aroma of a number of fruits and their fermented products. Previous studies on wine have revealed that in addition to a free fraction of volatile terpenols, there are nonodorous and nonvolatile precursors that represent an important source of fragrant compounds (Cordonnier and others 1986; Williams and others 1995). These nonodorous precursors consist of terpenols that are linked to sugars such as 6-*O*-α-L-rhamnopyranosyl-, 6-*O*-α-L-arabinofuranosyl-, and 6-*O*-β-D-apiofuranosyl-β-D-glucosides. The aglycone part mainly contains monoterpenols and aromatic alcohols, such as benzyl and 2-phenylethyl alcohol (Gunata and others 1985). In general, bound glycoside forms are more abundant than the free ones (Dimitriadis and Williams 1984; Gunata and others 1985) and, in grapes, the ratio between the potential (bound) and free monoterpenes varies between 5:1 in Muscat of Alexandria and 1:1 in some nonmuscat varieties such as Chardonnay (Williams and others 1982). Acid hydrolysis of grape glycosides has been studied as a method for the release of bound monoterpenes with a view to enhancing the aroma of grape juice through the formation of free volatiles. This method, which is promoted by heating, causes a rearrangement of the monoterpene aglycones (Williams and others 1982). As an alternative, enzymatic hydrolysis has attracted much interest with regard to the enhancement of wine flavor. Unlike acidic hydrolysis, enzymatic hydrolysis is highly efficient and does not alter the aglycone (Gunata and others 1990). β-Glucosidase (1,4-β-D-glucosidase; EC 3.2.1.21) is a key enzyme in the enzymatic release of these bound monoterpenols from their glycosidic precursors (Shoseyov and others 1990; Guegen and others 1996) and functions through the sequential hydrolysis of the glycosidic bonds. Depending on the precursors, the glycosidic linkages are first cleaved by an α-L-arabinofuranosidase, β-L-rhamnopyranosidase, or a β-D-apiosidase. The 2nd step involves the liberation of the monoterpenols by β-glucosidase (Gunata and others 1988; Guegen and others 1996). Limited hydrolysis of glycosides occurs during berry maturation by endogenous fruit β-glucosidases (Cordonnier and others 1986). These plant-derived β-glucosidases are characterized by a restricted specificity with respect to aglycone and are inhibited by a glucose concentration higher than 1% (Bayonove and others 1984; Aryan and others 1987). Because these enzymes cannot liberate all of the aromatic potential in grape must, hydrolytic experiments were performed with exogenous β-glucosidases (Shoseyov and others 1990; Rosi and others 1994; Saha and Bothast 1996). Trials conducted with β-glucosidases of fungal origin have indicated that these enzymes can indeed enhance the varietal aroma of certain wines. These enzymes can only be introduced into the wine after the yeasts cells have depleted the glucose, as they are strongly inhibited by glucose (Aryan and others 1987; Gunata and others 1993). According to some reports, certain strains of *Saccharomyces cerevisiae* also possess β-glucosidase activity (Delcroix and others 1994; Hernández and others 2002

This activity appears to be very limited and therefore recent studies have rather focused on non-*Saccharomyces* yeasts, such as *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, and *Pichia* (Vasserot and others 1989; Rosi and others 1994; McMahon and others 1999; Fernández and others 2000; García and others 2002). Results obtained from studies on yeast glycosidases suggest that specific yeast strains can affect the varietal aroma of wines (Rosi and others 1994). For glycosidases to be exploited in the enhancement of wine aroma, they must satisfy a few prerequisites. These include a high affinity for grape-derived terpenoid aglycones, optimal activity at

MS 20030278 Submitted 5/20/03, Revised 7/17/03, Accepted 9/4/03. *Authors Cordero Otero, Potgieter, Pretorius, and van Rensburg are with the Inst. for Wine Biotechnology, Dept. of Viticulture & Oenology, Stellenbosch Univ., Stellenbosch ZA 7600, South Africa. Author Ubeda Iranzo, Briones-Perez, and Arevalo Villena are with the Dept. de Química Analítica, Tecnología de Alimentos, Universidad de Castilla-La Mancha, Ciudad Real, Spain. Author Pretorius is also with The Australian Wine Research Inst., Waite Rd., Urrbrae, Adelaide, Australia. Direct inquiries to author van Rensburg (E-mail: pvr@sun.ac.za).*

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wine pH, resistance to glucose inhibition, and high tolerance to ethanol. The need for more efficient aroma-liberating enzymes has led us to search for novel β-glucosidases that fit the above mentioned criteria. With this in mind, the purpose of this investigation was to screen wine-related non-*Saccharomyces* yeasts (species belonging to *Brettanomyces, Candida, Debaryomyces, Kloeckera*, and *Zygosaccharomyces*) for the appropriateness of their β-glucosidase by quantifying, characterizing, and comparing these activities. *Debaryomyces pseudopolymorphus* was identified as the strain with the highest β-glucosidase activity and with a high tolerance of both glucose and ethanol. The effect of cofermentation with *D. pseudopolymorphus* and *S. cerevisiae* (VIN13) on the terpenic glycosides in wine was also investigated.

Materials and Methods

Yeast strains

The names and the origins of the 20 yeast strains that were used in this study are summarized in Table 1. Four of the strains are from the yeast collection of the Dept. of Microbiology, Univ. of the Free State (UFS), South Africa. The remaining 16 strains were obtained from the Wine and Fermentation Technology Div., ARC Infruitech-Nietvoorbij, Stellenbosch, South Africa. These indigenous strains were isolated from different wine production regions in the Western Cape, South Africa, namely Constantia (represented by C), Robertson (represented by M), Slanghoek (represented by O), and Stellenbosch (represented by R) (Strauss and others 2001). The strain collection used in this study was compiled from vineyard and winery isolates. Yeast strains were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30 °C for 48 h.

Table 1—Yeast strains used in this study

Species	Isolates	Institution	Origin
Candida colliculosa	C2/5	Nietvoorbij[a]	Cellar
C. guilliermondii	M1/30	Nietvoorbij	Vineyard
C. hellenica	O1/16	Nietvoorbij	Vineyard
C. lambica	M2/4	Nietvoorbij	Cellar
C. oleophila	O1/29	Nietvoorbij	Vineyard
C. pelliculosa	R1/17	Nietvoorbij	Vineyard
C. sorbosa	R2/29	Nietvoorbij	Cellar
C. stellata	C2/20	Nietvoorbij	Cellar
C. valida	O1/22	Nietvoorbij	Vineyard
Debaryomyces hansenii	R2/15	Nietvoorbij	Cellar
D. maramus	Y-0896	UFS[b]	N.A.
D. polymorphus var. *africanus*	Y-0064	UFS	N.A.
D. pseudopolymorphus	Y-0541	UFS	N.A.
Kloeckera apiculata	R2/21	Nietvoorbij	Cellar
Kloeckera spp.	R2/30	Nietvoorbij	Cellar
Pichia carsonii	Y-0895	UFS	N.A.
P. farinose	R1/15	Nietvoorbij	Vineyard
P. kluyveri	R2/11	Nietvoorbij	Cellar
Zygosaccharomyces bailii	O1/25	Nietvoorbij	Vineyard
Brettanomyces spp.	O1/33	Nietvoorbij	Cellar
Saccharomyces cerevisiae	VIN13	IWBT[c]	IWBT[c]

[a]ARC Infruitech-Nietvoorbij
[b]University of the Free State (UFS)
[c]Institute for Wine Biotechnology (IWBT)

Isolation of cell-associated protein

YPD broth (50 mL) was inoculated at an optical density at 600 nm (od_{600}) of 0.1 from an overnight culture and grown at 30 °C for 48 h. Cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 5 mL of 50 mM Tris (pH 7.5, containing 10 mM $NaCl_2$) buffer. Glass beads (amount, 0.1 g; dia, 0.2 mm) were added and the cells were vortexed vigorously for 3 min. After centrifugation at 6000 rpm for 2 min, the supernatant, containing the crude cell protein extract, was carefully removed and used for the enzyme assays.

Protein determination and enzyme assays

Protein concentration was determined by using Pierce protein reagent with bovine serum albumin as the standard (Bradford 1976). β-Glucosidase activity was assayed using a modified protocol from Lloyd and Whelan (1969). Because the β-glucosidase of most tested yeasts was cell-associated, all enzymatic tests were conducted on total crude cell extracts. Crude cell extract (40 µL) was mixed with 40 µL of a 0.4% (wt/vol) cellobiose solution in 0.1 M citrate-phosphate buffer (pH 3.4). Water was added to the reaction mixture to obtain a total volume of 100 µL. The mixture was incubated at 25 °C for 30 min, and the amount of glucose released from the hydrolyzed cellobiose was determined using the Glucose Trinder Kit (Sigma), according to the specifications of the supplier. Enzyme and reagent blanks were included and subtracted from the absorbance obtained for the enzyme-substrate reaction mixture (A_{505} – reaction blank/standard – reaction blank × 100 = [glucose] in mg/100 mL). The specific amount of glucose release was expressed as mg/100 mL/mg of protein/30 min. The effect of ethanol, SO_2, and glucose on β-glucosidase activity was assessed through the addition of different concentrations of these substances to the reaction mixtures prior to incubation at 25 °C. Cultures were grown in duplicate and assays were conducted in quadruplicate. The standard deviations ranged from 3% to 5%. One unit of β-glucosidase activity was defined as the amount of crude enzyme required to produce 1 µmol of glucose under the assay conditions employed. The calculation of the specific activity (U/mg) was based on the level of enzymatic activity divided by the protein concentration of the crude enzyme preparations.

Microvinification

Microvinifications were carried out with single-strain samples of either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13), as well as with a mixed-culture sample (*D. pseudopolymorphus* plus *S. cerevisiae* VIN13). Chardonnay must (700 mL for each sample) with an SO_2 concentration of 35 mg/L was inoculated with either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13) to cell concentrations of 1×10^7 cells/mL and 1×10^6 cells/mL, respectively. The same concentration of inocula was used on the 1st day of fermentation for the mixed-culture samples. The musts were fermented in 1 L glass bottles for 10 d at 25 °C ± 2 °C, and the enzymatic assays were carried out in quadruplicate.

Standard wine analysis

Fourier transform infrared spectroscopy, using the GrapeScan 2000 instrument (FOSS Electric, Denmark), was used to determine the chemical characteristics of the fermented wines. The commercial calibrations for the analysis of the wine were provided by FOSS, and all calibrations were validated.

Quantitative determination of monoterpene concentrations

The determination of monoterpene concentrations was performed using a modified protocol from Ferreira and others (1993). A total of 10 mL of wine was mixed with a solution of 2 µL of 2,6-dimethylheptenol (400 mg/L in ethanol as internal standard) before liquid-liquid extraction with 200 µL of Freon 113. The Freon extracts of the terpenes were analyzed by gas chromatography on a Supelco SPB5 column using HE as carrier gas. Three standard solutions

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were prepared for all the terpenes. The pure chromatographic standards of analytical quality were obtained from Fluka and Sigma-Aldrich (____Germany). 1,1,2-Trichloro-1,2,2-trifluoroethane (Freon 113) of high-performance liquid chromatography quality was obtained from Aldrich. The compounds were identified by a flame ionization detector and compared with the retention times of the pure chemical standards. The amount of each terpene was quantified by plotting the heights of peaks on the chromatograms compared with the terpene concentration in wine. Five calibration points were used for each of the terpenes compound.

Results and Discussion

The role of microbial β-glucosidases in the liberation of monoterpenols from glycoside precursors has been reported in numerous studies (Gueguen and others 1996; Iwashita and others 1999; Yanai and Sato 1999). Therefore, the potential of β-glucosidases to release flavor compounds from glycosidically bound nonvolatile precursors is of great significance to the wine industry. Previous work has shown that, among yeasts, different strains of *Candida* (Gunata and others 1990), *Debaryomyces* (Rosi and others 1994), *Metschnikowia* (Fernández and others 2000), and *Brettanomyces* (McMahon and others 1999) exhibit activity toward various β-glucosides.

In the present study, a total of 20 yeast strains isolated from vineyards and wine cellars (Table 1) were tested for β-glucosidase activity. A preliminary evaluation of β-glucosidase activity on different protein fractions (extracellular, intracellular cytosol, and membranes) for all the strains showed that the activity was primarily cell-associated, and extracellular activity was detected only for *D. pseudopolymorphus* and *C. oleophila* (data not shown). Figure 1 shows that *Candida lambica*, *Candida valida*, *Debaryomyces hansenii*, and *Kloeckera apiculata* have very low β-glucosidase activity with none of the strains releasing more than 0.03 mg/100 mL of glucose from cellobiose. The only *Brettanomyces* strain (a yeast usually associated with wine spoilage) used in this study showed the highest activity, viz. 4.84 mg/100 mL of glucose released from cellobiose.

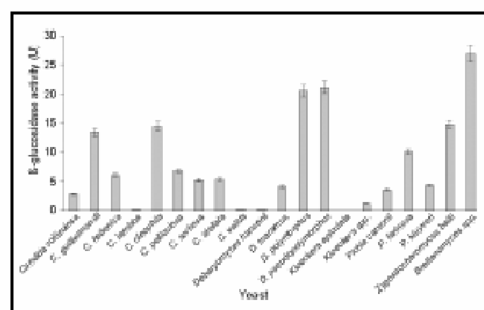

Figure 1—Cellobiose hydrolysis by yeast β-glucosidases. Cellobiose degradation by yeast was analyzed using the glucose oxidase/peroxidase method to determine the formation of glucose from the substrate. Glucose was assessed after incubation of cell crude extract for 30 min at 25 °C in the presence of 8 mg of cellobiose in 100 mM citrate-phosphate buffer, pH 3.4. Cultures were grown in duplicate and the assays repeated 4 times. The specific activity of the enzymes was expressed as 1 μmol of glucose produced per mg of protein, equivalent to U/mg. The standard deviations were between 3% and 5%.

The other species with high β-glucosidase activity were *D. polymorphus* (3.69 mg/100 mL of glucose), *D. pseudopolymorphus* (3.79 mg/100 mL of glucose) and, to a lesser extent, the 2 strains *C. oleophila* (2.58 mg/100 mL of glucose) and *Zygosaccharomyces bailii* (2.66 mg/100 mL of glucose). The 4 yeasts with the highest activity were selected to investigate the effect of ethanol, glucose, and SO_2 on their β-glucosidase activities.

Previously, it has been found that alcohol stimulates β-glucosidase activity in many fungal and yeasts species (Delcroix and others 1994; Yan and Lin 1997). Pemberton and others (1980) proposed that alcohol activation of some β-glucosidases might be due to their glycosyl transferase activity. However, some other yeast β-glucosidases (Mateo and Di Stefano 1997), as well as grape β-glucosidases (Aryan and others 1987) have been reported to be inhibited by ethanol. As shown in Figure 2a, we also observed that ethanol inhibited β-glucosidase activity at all concentrations tested for the 4 different crude extracts evaluated. The most prominent inhibition was observed for the β-glucosidase activity of *C. oleophila*, which exhibited a 21% reduction (compared with the total activity of the untreated samples) in enzyme activity at 14% ethanol. The β-glucosidases of *D. pseudopolymorphus* and the *Brettanomyces* sp. showed the most resistance to ethanol inhibition and maintained 56% and 66% of their activity, respectively, at 14% ethanol. Spagna and others (2002) have hypothesized that ethanol can cause an alteration of the enzyme conformation, and consequently of its active site, by changing the polarity of the medium and thus reducing its activity. Inhibition by glucose, which, although there are some exceptions (Rosi and others 1994; Saha and Bothast 1996; Riou and others 1998), is a common characteristic of β-glucosidases (Guegen and others 1995; Spagna and others 2002), is an important constraint for the industrial use of these enzymes in winemaking. Our results on the effect of different glucose concentrations (4%, 8%, and 15%) on β-glucosidase activity is presented in Figure 2b. The results depicted in Figure 2b show that the β-glucosidase of the *Brettanomyces* sp. was strongly inhibited by the presence of glucose, whereas the β-glucosidase activities derived from the other yeasts were, to varying degrees, more resistant to glucose inhibition. At a glucose concentration of 15%, the *Brettanomyces* β-glucosidase activity was only 0.12% (ratio of treated reaction mixture/untreated reaction mixture) of the maximum activity. The *D. polymorphus* β-glucosidase activity appeared to be insensitive to glucose, whereas the activity of both *C. oleophila* and *D. pseudopolymorphus* were markedly increased throughout the whole glucose concentration spectrum that was tested. The *D. polymorphus* β-glucosidase showed an initial 30% increase in activity, which was maintained throughout the glucose series tested. At a glucose concentration of 15%, the *D. polymorphus* β-glucosidase displayed a 5-fold increase in activity respectively. *C. oleophila*, showing a 3-fold increase in β-glucosidase activity at a concentration of 15%, exhibited a gradual increase in activity, which correlates to an increase in glucose concentration. The most outstanding increase in β-glucosidase activity was observed in *D. pseudopolymorphus*, which exhibited a marked increase of 4-fold at 8% glucose and 5-fold at 15% glucose.

Little is known about the influence of sulfur dioxide on β-glucosidase activity. Delcroix and others (1994) found that an SO_2 concentration of 50 mg/L had no effect on enzyme activity. As shown in Figure 2c, our data are in agreement with this observation. None of the SO_2 concentrations tested had a marked effect on the β-glucosidase activity of the 4 crude protein extracts tested. The only exception was in the case of *D. polymorphus*, which showed a significant increase in activity throughout the entire SO_2 series tested. The *D. polymorphus* β-glucosidase exhibited an overall increase in activity throughout the series tested, reaching a 3-fold induction at

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Table 2—Analysis of wines at 10 d of fermentation[a]

Inoculated strain	pH	Volatile acid[b]	Total acid[b]	Reducing sugar[b]	Malic acid[b]	Glucose + Fructose[b]	Ethanol[c]	Density
D. pseudopoly-morphus	2.91 ± 0.25	0.22 ± 0.04	5.58 ± 0.4	200.19 ± 16.04	3.65 ± 0.34	187.39 ± 15.33	0.42 ± 0.03	1.08 ± 0.01
S. cerevisiae	2.92 ± 0.01	0.52 ± 0.01	6.24 ± 0.02	50.88 ± 0.35	4.02 ± 0.02	52.07 ± 0.19	9.82 ± 0.01	1.02 ± 0.01
D. pseudopoly-morphus and *S. cerevisiae*	2.85 ± 0.01	0.56 ± 0.01	6.47 ± 0.01	43.46 ± 0.19	4.01 ± 0.03	44.33 ± 0.11	10.22 ± 0.01	1.01 ± 0.01

[a]Fermentation assays were carried out in quadruplicate using 700 mL of Chardonnay must for each sample. The juice was sulfited at 35 mg/L. *Debaryomyces pseudopolymorphus* and *Saccharomyces cerevisiae* VIN13 strain samples were inoculated at a level of 1 × 10^7 cells/mL and 1 × 10^6 cells/mL, respectively. The same value of the inoculums applied for the single-culture samples was used on the 1st day of fermentation for the mixed-culture samples. The juices were fermented in 1 L glass bottles at 25 ± 2 °C for 10 d.
[b]g /L
[c]% vol/vol

60 ppm. At 60 ppm, the *C. oleophila* and *Brettanomyces* β-glucosidases maintained 87.4% and 86.9% of their maximum activity, respectively. *D. pseudopolymorphus* showed a 20% increase in β-glucosidase activity at 30 ppm SO_2 and still maintained its maximum activity at 60 ppm. No explanation for this increase can be offered at this point in time, and this aspect needs further investigation.

Microorganisms of enological origin do not always produce enzymes that can be used in wine conditions (Mateo and Di Stefano 1997; Spagna and others 2002). However, the enzymatic properties exhibited by *D. pseudopolymorphus*, that is, high activity at higher glucose and sulfur dioxide concentrations and relative resistance to high ethanol values, suggest that this yeast has an appropriate kinetic profile to be used in the wine industry. Therefore, microvinifications were performed with single-strain samples consisting of either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13; a commercial wine starter culture strain), as well as with a mixed-culture sample consisting of both these yeasts. No obvious differences in the pH and density of the wines were observed between the single-strain and mixed-culture inocula after 10 d of fermentation (Table 2). *D. pseudopolymorphus*, as a single-strain inoculum, is not an effective fermenting wine yeast under the fermentation conditions tested. After 10 d of fermentation, the wine still contained a reducing sugar content of 200.19 g/L, as well as 187.39 g/L of glucose and fructose, and an ethanol content of 0.42% (vol/vol). The volatile acid and total acid content of the single-culture fermentations were slightly lower than that of the mixed culture fermentation (Table 2). Furthermore, it appears that the mixed-culture inoculum was more effective in driving these particular fermentations than the single-strain *S. cerevisiae* VIN13 inoculum because the wine had a reducing sugar content of 43.46 g/L, 44.33 g/L of glucose and fructose, and an ethanol content of 10.22% (vol/vol) after 10 d of fermentation.

Whether *D. pseudopolymorphus* produces any off-flavors in wine was not determined in this study because a comprehensive analysis of the alcohol, acid, and phenolic compounds was not done. Rather, the influence of the cofermentation of *D. pseudopolymorphus* and *S. cerevisiae* on the concentration of free volatiles in Chardonnay wine was studied (Table 3). In general, the concentration of the primary alcohols obtained with the single-strain *S. cerevisiae* fermentation was slightly higher than that obtained with the single-strain *D. pseudopolymorphus* fermentation, except for the concentration of geraniol, which was 7-fold higher in the latter. No significant differences were found in the linalool and α-terpineol concentrations produced with either the single-strain or mixed-culture fermentation. The results clearly indicate that fermentation with the mixed-culture inoculum resulted in a significant increase in the concentration of citronellol (37.2 μg/L) and geraniol (21.2 μg/L) after 10 d. In the case of nerol, fermentation with the mixed-culture inoculum resulted in only a 2- to 3-μg/L increase in concentra-

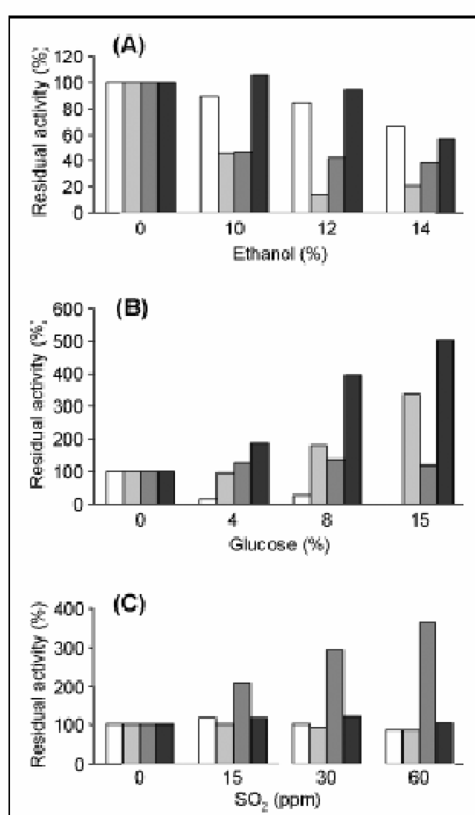

Figure 2—Effects of ethanol (A), glucose (B), and SO_2 (C) on β-glucosidase activity of *Brettanomyces* spp. (white bars), *Candida oleophila* (gray bars), *Debaryomyces polymorphus* (dark gray bars), and *D. pseudopolymorphus* (black bars). Residual activity was assayed after incubation of cell crude extract for 30 min at 25 °C in 100 mM citrate-phosphate buffer, pH 3.4, complemented with different values of ethanol, glucose, and SO_2. The scale of residual activity indicates the percentage of the experimental value for various enzyme reactions relative to the maximum value (100%) of the non-spiked reaction mixture of each enzyme. The values are means of triplicate experiments, and the relative error was less than 5%.

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Table 3—Influence of cofermentation with *Debaryomyces pseudopolymorphus* and *Saccharomyces cerevisiae* VIN13 strain on the concentration of free volatiles in Chardonnay wine

	Inoculated yeast strain		
Volatile compound	*D. pseudo-polymorphus*[a]	*S. cerevisiae* VIN13[a]	*D. pseudopoly-morphus* and *S. cerevisiae* VIN13[a]
Linalool	34.7 ± 1.2	34.9 ± 1.4	33.6 ± 1.5
Terpineol	36.4 ± 1.1	35.7 ± 1.3	33.3 ± 1.1
Citronellol	3.1 ± 0.2	7.2 ± 0.3	37.2 ± 1.1
Nerol	4.6 ± 0.3	5.6 ± 0.2	7.9 ± 0.2
Geraniol	7.4 ± 0.2	1.3 ± 0.1	21.2 ± 1.0

[a]µg/L. The numbers represent mean values and deviations from the mean of quadruplicate fermentation experiments.

tion in comparison to the fermentation that was performed with the 2 single-strain inocula. This could be explained by the fact that certain β-glucosidases display selectivity to specific aglycones (Williams and others 1995; Mansfield and others 2002). Grape β-glucosidases, for example, act on precursors that have a primary alcohol as aglycone, such as geraniol and citronellol, but do not cleave glycosides bonding a tertiary alcohol, such as linalool or α-terpineol (Gunata and others 1985). Citronellol, in particular, was produced in high concentrations by the cofermentation. Di Stefano and others (1992) suggested that this compound could originate from the reduction of geraniol and nerol by yeast during fermentation. Chardonnay is one of the varieties known to contain low levels of terpenes or their glycosides (Dimitriadis and Williams 1984; Sefton and others 1993) and is not dependent on monoterpenes for flavor (Strauss and others 1986). The limited presence of flavor precursors, such as neryl β-D-gluopyranoside, can explain the low concentration of nerol produced during wine fermentation.

Conclusions

This study makes an important contribution to an extensive survey aimed at the exploitation of the hidden oenological potential of the untapped non-*Saccharomyces* yeasts and their enzymes (Pretorius and others 1999; Fernández and others 2000; Strauss and others 2001; Fernández-Gonzélez and others 2003; Jolly and others 2003a, 2003b, 2003c). The data presented here suggest that the β-glucosidase activity of *D. pseudopolymorphus* possesses suitable properties in terms of optimum pH, resistance to wine-associated inhibitory compounds (glucose, ethanol, and sulfur dioxide), high substrate affinity, and large aglycone-substrate recognition. The potential of this newly discovered β-glucosidase to increase the aromatic varietal character of wines through the hydrolysis of flavor glucosidic precursors is underpinned by its capability to increase the concentrations of citronellol, nerol, and geraniol during the fermentation of Chardonnay juice inoculated with both *D. pseudopolymorphus* and a commercial wine yeast, *S. cerevisiae* VIN13. However, additional work is required to determine the influence of *D. pseudopolymorphus* on wine aroma, as well as the effectiveness of the β-glucosidase enzyme in large-scale winemaking trials. This study also lays the foundation for the cloning and expression of the *D. pseudopolymorphus* β-glucosidase gene in a commercial wine yeast. Such recombinant wine yeast would release grape-derived varietal aroma compounds from the nonvolatile, nonodorous precursors during single culture fermentations, thereby increasing the sensorial quality of wine—the single most important aspect in winemaking.

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Optimization of a rapid method for studying the cellular location of β-glucosidase activity in wine yeasts

M. Arévalo Villena[1], J.F. Úbeda Iranzo[1], R.R. Cordero Otero[2] and A.I. Briones Pérez[1]

[1]Tecnología de Alimentos, Universidad de Castilla La Mancha, Ciudad Real, Spain, and [2]Institute for Wine Biotechnology, University of Stellenbosch, Stellenbosch, South Africa

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ABSTRACT

M. ARÉVALO VILLENA, J.F. ÚBEDA IRANZO, R.R. CORDERO OTERO AND A.I. BRIONES PÉREZ. 2005.

Aims: To improve a method for determining β-glucosidase activity and to apply it in yeasts isolated from wine ecosystems from 'La Mancha' region and to know its cellular location.

Methods and Results: A total of 82 wine yeasts were identified (PCR/RFLP) and evaluated for their β-glucosidase activity. First, they were qualitatively evaluated by growth on YNB cellobiose, the activity was quantified using different culture media, under aerobic and anaerobic conditions and cells after 24–72 h of growth. To study the location activity, five fractions were obtained (supernatant, whole cell, cell wall, cytosol and cell membrane). The enzymatic assays were optimized, being: growth in YP cellobiose for 72 h in aeration conditions and, after cell removing, enzyme analysis with 128 g l^{-1} of cellobiose as substrate, for 30 min at 30°C. The genus that displayed the greatest activity were *Pichia*, *Hanseniaspora* and *Rhodotorula*, and the activity was intracellular.

Conclusions: The study showed that β-glucosidase activity was induced by the carbon source and was aerobic dependent. The non-*Saccharomyces* species displayed the greatest activity, which was intracellular and strain-dependent.

Significance and Impact of the Study: This study developed a reliable method for screening β-glucosidase activity in yeasts isolated from wine ecosystems. This activity is very important in the release of monoterpenols from glycoside precursors for the enhancement of wine aromas.

Keywords: β-glucosidase activity, cellular location, non-*Saccharomyces*, quantification, wine yeasts.

INTRODUCTION

Spontaneous grape-must fermentation begins with the growth of species belonging to such genera as *Kloeckera*, *Hanseniaspora*, *Brettanomyces*, *Debaryomyces*, *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* (Manzanares *et al.* 2000; Mendes-Ferreira *et al.* 2001). These yeasts, which display low fermentative capacity and low ethanol tolerance (Charoenchai *et al.* 1997; Fleet and Heard 1997; Boulton *et al.* 1998) could enhance wine flavour by increasing concentrations of the volatile compounds responsible for the fruity aroma, through hydrolysis of aromatic precursors prompted by β-glucosidase enzyme activity (Herráiz 1990; Ciani and Picciotti 1995; Fernández *et al.* 2000; Strauss *et al.* 2001).

To date, β-glucosidases have been isolated from plants, from the grape itself or from micro-organims. The first two sources are of little oenological value, as in winemaking conditions β-glucosidases are strongly inhibited by the low pH, high initial sugar concentrations and high final ethanol concentrations (Williams *et al.* 1982; Cordonnier *et al.* 1989; Lecas *et al.* 1991). Hence, our research is directed to β-glucosidase from micro-organism.

At present, aroma release is enhanced using commercial enzyme preparations of fungal origin, mainly *Aspergillus* spp.

Correspondence to: R.R. Cordero Otero, Institute for Wine Biotechnology, University of Stellenbosch, Victoria St., 7600 Stellenbosch, South Africa (e-mail: ana.briones@uclm.es).

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The composition of these preparations varies and they are actually a mixture of nonspecific glucanases. Tingle and Halvorson (1971) found that these cocktails hydrolysed only 3% of precursors, but that this was sufficient to ensure an analytically and organoleptically detectable increase in free terpenes. At the same time, however, as glucanases are nonspecific, they may trigger collateral reactions detrimental to the product such as hydrolysis of anthocyans, which are also stabilized by binding to sugar molecules (Riou *et al.* 1998). For these reasons, the best option would be to use specific enzymes, which are not excessively active; ideally, they should also be contained in yeasts forming part of the wine ecosystem, and thus adapted to their ecological environment.

Some authors have reported β-glucosidase activity in *Saccharomyces cerevisiae* (Rosi *et al.* 1994; Hernández *et al.* 2002); others argue that while the enzyme can be synthesized, it is rendered inactive by processing conditions (Gunata *et al.* 1986; Delcroix *et al.* 1994; Mateo and Di Stefano 1997), and that the required activity can only be ensured using non-*Saccharomyces* yeasts (Cordero Otero *et al.* 2003). Published reports differ with regard to the active strains used, constitutive (Vasserot *et al.* 1989) *vs* induced synthesis (Rosi *et al.* 1994; Miklósy and Polos 1995; Mateo and Di Stefano 1997; Yanai and Sato 1999; Mendes-Ferreira *et al.* 2001; Hernández *et al.* 2002) and location on microbial cultures (Rosi *et al.* 1994; Miklósy and Polos 1995; McMahon *et al.* 1999; Yanai and Sato 1999; Manzanares *et al.* 2000; Mendes-Ferreira *et al.* 2001; Strauss *et al.* 2001). Most of these studies additionally use nonspecific substrates, so the activity cannot be quantified as readily as with natural glycosides isolated from must or wine or quantified directly in wine by gas chromatography (Gunata *et al.* 1990a).

Cordero Otero *et al.* (2003) quantified the β-glucosidase activity in non-*Saccharomyces* wine yeasts using cellobiose as substrate. The activity was only determined in crude extract.

The aim of this work was to improve upon a method used by these authors, so we optimized the cellobiose concentration and assayed different culture conditions, harvested cell time and incubation temperature. On the contrary, we applied the new method proposed for screening this activity in 82 wine yeasts isolated from 'La Mancha' region and studied its cellular location.

MATERIALS AND METHODS

Yeasts: recovery and identification

This study used a total of 82 *Saccharomyces* and non-*Saccharomyces* strains isolated from nine wine cellars belonging to the 'La Mancha' Denomination of Origin, all of which use traditional methods for making red and white wines (Table 1).

Strains were recovered from pure cultures stored at −70°C by preculture and growth in YPD medium and they were identified by direct 5·8 S-ITS rDNA region amplification and later RFLP analysis using the enzymes *Hinf*I, *Hae*III, *Cfo*I and *Hpa*II (Esteve-Zarzoso *et al.* 1999; Fernández *et al.* 2000).

Improvement of a method for determining β-glucosidase activity

The improvement of the method was carried out with a positive control, *Candida molischiana* known for its strong β-glucosidase activity (Gunata *et al.* 1990b) and a negative control (*S. cerevisiae*, 321 UCLM) isolated from the winemaking ecosystems from la Mancha region (Briones *et al.* 1995). The assays were performed in triplicate.

Culture conditions. In order to determine whether the activity was constitutive or induced, various carbon sources (glucose, cellobiose and arbutin) were added to YP or YNB medium at a range of concentrations.

Yeasts were grown for 48 h at 30°C in 10 ml of each of the following media:

- i YPD (2% yeast extract, 1% peptone, 1% glucose).
- ii YPC (2% yeast extract, 1% peptone, 1% cellobiose).
- iii YPD + C (2% yeast extract, 1% peptone, 0·2% glucose, 0·8% cellobiose).
- iv YNB-A (0·67% YNB, 0·5% arbutin).
- v YNB-C (0·67% YNB, 0·5% cellobiose).
- vi YNB-G (0·67% YNB, 0·5% glucose).
- vii YNB-A + G (0·67% YNB, 0·4% arbutin, 0·1% glucose).
- viii YNB-C + G (0·67% YNB, 0·4% cellobiose, 0·1% glucose).

Table 1 Origin of the yeasts. All of them were isolated from different wine cellar of the Mancha Region

Origin	Yeasts
Cencibel (red must)	1M, 18M, 2Mt, 4Mt, 6Mt, 9Mt, 24Mt, 30Mt, 31Mt, 1C, 2C, 3C, 4C, 5C, 6C, 7C, 8C, 9C, 11C, 12C, 13C, 14C, 15C
Airen (white must)	84M, 85M, 97M, 98M, 132M, 167M, 1B, 3B, 6B, 7B, 8B, 9B, 14B, 15B, 16B, 18B, 20B, 24B, 25B, 26B, 28B, 29B, 30B, 32B, 34B, 35B, 37B, 33B, 38B, 17B
Macabeo (white must)	47Mt, 48Mt, 52Mt, 17C, 18C, 20C, 21C, 22C, 23C, 24C, 25C, 26C, 27C, 28C, 29C, 30C, 31C, 32C, 33C, 34C, 41C, 44C, 45C, 244C, 247C, 248C, 250C, 256C, 341C

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To determine the effect of oxygen, yeasts were grown in both strictly anaerobic and aerobic conditions and were shaken at 150 rev min^{-1}. To ascertain the timing of peak enzyme synthesis, cells were harvested after 24, 48 and 72 h of growth.

Enzyme assay. Enzymatic activity was evaluated by determining the amount of glucose released from the cellobiose substrate. Quantification was optimized by calculating the most suitable cellobiose concentration for hydrolysis (32, 64, 80, 96, 128 or 160 g l^{-1}), the required enzyme-substrate contact time (30 and 60 min) and the incubation temperature for the reaction (30 and 45°C).

A 40 μl of substrate was added to 50 μl of cell extract in 10 μl 100 mmol l^{-1} citrate phosphate buffer (pH 3·5). Released glucose was measured using the Trinder kit (Sigma Aldrich) with the following modifications: 5 μl of reaction mixture were added to 1 ml of Trinder reagent; after 18-min incubation at room temperature, absorbance was measured at 505 nm. Blanks were prepared both for the reagent and for the different cell fractions obtained.

Search for yeasts displaying β-glucosidase activity

The 82 strains (Table 1) were grown for 6 h at 30°C in carbon-free YNB broth and maximum shaking to consume residual sugars and then 5 μl of this medium were inoculated on yeast nitrogen base cellobiose agar (YNB-cel); 0·67% YNB (Difco), 0·5% cellobiose, 2% agar) for 24–48 h. at 30°C.

Quantification of the activity and its cellular location

Activity was quantified in all the cases using the best conditions found in the previous charter; assays were performed in triplicate using the yeasts displaying the greatest growth in YNB-cel agar, two randomly chosen low-growth strains (6Mt and 32C) and a commercial *S. cerevisiae* strain (UCLMS 325). *Candida molischiana* was used as the positive control.

Enzyme location in cells. Enzymatic activity was evaluated in whole cells, supernatant, cell wall, cytosol and membrane. For this purpose, 6 ml of culture medium were centrifuged at 3000 *g* for 3 min. Two fractions were obtained: the supernatant, of which 1 ml was kept refrigerated until analysis, and the pellet, which was washed twice in sterile saline solution and split into two batches. One batch was used for the whole-cell evaluation and the other was resuspended in 500 μl of lysis solution (500 μl CLB, 10 mmol l^{-1} containing NaCl, 10 mmol l^{-1}; KH_2PO_4, 1 mmol l^{-1}; $NaHCO_3$, 5 mmol l^{-1}; $CaCl_2$, 1 mmol l^{-1} and $MgCl_2$, 0·5 mmol l^{-1}, 25 μl EDTA, 0·1 mol l^{-1}; 10 μl IP and 1 μl PMSF 100 mmol l^{-1}); after 5 min in an ice bath, the pellet was broken up with a Dounce homogenizer. The resulting fluid was centrifuged at 700 *g* for 5 min, yielding a cell-wall and a supernatant, which was in turn centrifuged at 23 000 *g* for 30 min, to obtain cytosol and cell membrane. (Delcroix 4 *et al.* 1994; Mateo and Di Stefano 1997; McMahon *et al.* 1999; Yanai and Sato 1999; Mendes-Ferreira *et al.* 2001).

All fractions were stored at 4°C prior to analysis of β-glucosidase activity using the proposed enzyme assay method.

Glucose released from cellobiose was quantified by plotting absorbance values on a calibration curve. Results were expressed as μg glucose ml^{-1} supernatant or ng glucose mg^{-1} cell dry weight, which was obtained by filtering 1 ml of culture on four micro filters that were dried at 70°C until the weight was constant.

Statistical treatment

A one-factor analysis of variance (statistical software package SPSS Version 11·0) was used to test for significant differences between cell fractions for each yeast.

RESULTS

Of the 12 profiles differentiated by PCR/RFLP analysis, 30 strains were *S. cerevisiae*, 2 *Debaryomyces hansenii*, 2 *Mestchnikowia pulcherrima*, 2 *Rhodotorula mucilaginosa*, 7 *Kluyveromyces thermotolerans*, 1 *Pichia anomala*, 2 *Pichia membranaefaciens*, 1 *Pichia kluyveri*, 2 *Candida stellata*, 4 *Hanseniaspora uvarum*, 4 *Hanseniaspora osmophila* and 25 *Torulaspora delbrueckii* (Esteve-Zarzoso *et al.* 1999). The Fig. 1 is an example of some of these identifications. Table 2 set out the length (bp) of PCR amplification products and the number and length (bp) of the restriction fragments.

Improvement of a method for determining β-glucosidase activity

The improvement of the method was carried out with a positive control (*C. molischiana*) and a negative control (*S. cerevisiae*) for which the data are not given because they were always close to 0.

Culture conditions. Examination of the growth medium indicated whether the activity was induced or constitutive. Table 3a shows the β-glucosidase activity for each medium after 48 h of growth, maximum values in both supernatant and whole cells were obtained when cellobiose was used as the only carbon source, although values were not greatly different from those obtained using glucose and cellobiose in the YP medium. These findings largely coincide with those

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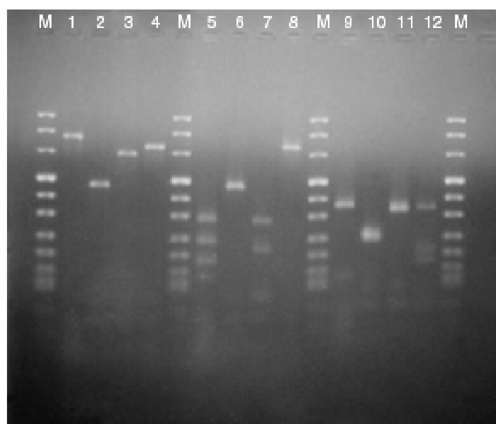

Fig. 1 Agarose gel with the PCR products from amplification of the 5·8S-ITS region of rDNA from four different species (lanes 1–4). Digestion products of the amplificates obtained using the restriction enzymes *Hae*III (lanes 5–8) and *Hin*fI (lanes 9–12). Lanes M: marker VIII (1114, 900, 692, 501–489, 404, 320, 242, 190, 147, 124, 110, 67, 37/34/34/26/19 bp) (Boehringer, Mannheim GmbH, Germany). Lanes 1, 5 and 9: *Saccharomyces cerevisiae* (18C); lanes 2, 6 and 10: *Candida stellata* (17B); lanes 3, 7 and 11: *Kluyveromyces thermotolerans* (30Mt); lanes 4, 8 and 12: *Hanseniaspora uvarum* (38B)

of other authors: McMahon *et al.* (1999) used arbutin for yeast growth, while Gunata *et al.* (1988) used cellobiose; Yanai and Sato (1999) induced activity with 2% cellobiose, whereas Mateo and Di Stefano (1997) used salicin. Although Vasserot *et al.* (1989) argue that the enzyme is constitutive, the present study found that activity was more intense when a glucoside-bond-containing compound was added to the medium.

With regard to the degree of oxygen exposure, the activity was found in aerobic conditions. There was no difference with Erlenmeyer flasks *vs* test-tubes, so yeasts were grown in 50-ml Erlenmeyer flasks containing 10-ml culture medium. In anaerobic conditions, even the positive control displayed some inhibition of growth. These results are consistent with the findings reported by other authors (Rosi *et al.* 1994; Mendes-Ferreira *et al.* 2001; Hernández *et al.* 2002).

The β-glucosidase activity at different incubation time using YPC medium, peak activity in both supernatant and whole cells was recorded after 72-h growth is given in Table 3b.

Enzyme assay To optimize the enzyme analysis, the range of cellobiose concentrations indicated in the Materials and methods section was tested: the best concentration proved to be 128 g l^{-1} (Fig. 2). Higher concentrations were rejected because of cellobiose precipitation. An enzyme-substrate contact incubation time of 30 min was selected, although there was a slight increase in hydrolysis by 60 min, the difference was not sufficient to warrant prolonging overall assay time. Finally, no significant difference was observed between incubation temperatures of 30 and 45°C, so 30°C was selected.

Search for yeasts displaying β-glucosidase activity

For plate-screening of β-glucosidase activity, yeast growth on YNB-cel agar was classed on quality grounds (i.e. in terms of the biomass produced after 24–48 h growth) as: no growth (−), weak (+), moderate (++) and intense (+++). Almost 75% of the yeasts displayed no growth or weak growth, whilst the remaining 25% showed good development. Behaviour of *Saccharomyces* and non-*Saccharomyces* on YNB-cel was: growth of non- *Saccharomyces* strains ranged from nonexistent to intense (*D. hansenii*, *M. pulcherrima*, *H. uvarum*, *H. osmophila* and *R. mucilaginosa*), whilst all except one *Saccharomyces* strain displayed weak growth, possibly because of residual glucanase activity in these strains.

Quantification of the activity and its cellular location

Having optimized the method and the conditions for enzyme synthesis, assays were performed to locate the activity in the cell. The yeasts displaying the most intense growth on cellobiose (1M, 97M, 98M, 132M, 2Mt, 4Mt, 9Mt, 24Mt, 30Mt, 31Mt, 52Mt, 38B, 7C, 9C, 33C, 34C, 41C, 44C, 45C and 341C), together with two randomly selected weak-growth strains (*P. membranaefaciens*, strain 6Mt and *K. thermotolerans*, strain 32C), the commercial strain *S. cerevisiae* (UCLMS 325) and *C. molischiana* (positive control) were used.

All strains displaying activity were fractionated as indicated under the Materials and methods section, and five extracts were obtained (supernatant, whole cell, cell wall, cytosol and membrane). Table 4 shows mean values and significant differences.

Comparison of the results for the same fraction in different yeasts with respect to the positive control *C. molischiana*, showed that in the case of supernatant none of the yeasts exceeded the positive control value; the only striking result was for *H. uvarum* (38B) with a value of 205·1 μg glucose ml^{-1} supernatant (Table 4). This suggests that extracellular activity was very weak, in contrast to the findings reported by other authors (Rosi *et al.* 1994).

Whole-cell results were more favourable: the strain 98M (*P. membranaefaciens*) recorded a value of 4286·3 ng glu-

Table 2 Summary of molecular profiles (PCR-RFLP) and identification of the yeasts

	Restriction fragments				Yeasts	
PCR	*Hin*fI	*Hae*III	*Cfo*I	*Hpa*II	Isolates	Identification
850	375 + 365 + 110	325 + 230 + 170 + 125	375 + 325 + 150	725 + 125	1B, 3B, 6B, 7B, 9B, 14B, 15B, 16B, 18B, 20B, 24B, 25B, 26B, 28B, 29B, 30B, 32B, 34B, 35B, 37B, 1C, 2C, 3C, 4C, 5C, 17C, 18C, 41C, 256 C, 18M	*S. cerevisiae*
650	325 + 325	420 + 150 + 90	300 + 300 + 50	–	1M, 48Mt	*D. hansenii*
700	355 + 345	310 + 215 + 90 + 90	315 + 285 + 95	–	85M, 30Mt, 47Mt, 33B, 27C, 29C, 32C	*K. thermotolerans*
475	235 + 235	475	215 + 110 + 80 + 60	–	167M, 17B	*C. stellata*
400	200 + 190	280 + 100	205 + 100 + 95	–	132M, 9Mt	*M. pulcherrima*
650	310 + 310	600 + 50	575	–	97M	*P. anomala*
450	250 + 200	370 + 80	175 + 115 + 80 + 80	–	84M	*P. kluyveri*
500	275 + 200	330 + 90 + 50	175 + 110 + 90 + 75	–	98M 6Mt	*P. membranaefaciens*
800	410 + 380	800	330 + 220 + 150 + 100	–	4Mt, 8C, 11C, 12C, 13C, 14C, 15C, 17C, 20C, 21C, 22C, 23C, 24C, 25C, 26C, 28C, 30C, 31C, 33C, 34C, 244C, 247C, 248C, 249C, 250C	*T. delbrueckii*
640	340 + 225 + 75	425 + 215	320 + 240 + 80	–	2Mt, 341C	*R. mucilaginosa*
750	350 + 200 + 180	750	320 + 310 + 105	–	24Mt, 31Mt, 52Mt, 38B	*H. uvarum*
750	390 + 360	460 + 12090 + 80	275 + 150 + 135 + 95 + 75	–	7C, 9C, 44C, 45C	*H. osmophila*

Table 3 β-glucosidase activity (absorbance values at 505 nm) of *Candida molischiana* for different culture conditions: (a) growth medium and (b) growth time. Triplicate assays were carried out

Medium	Supernatant	Pellet
(a) Growth medium assay performed at 48 h of growth		
YPD	0·04	1·53
YPD + C	0·19	1·71
YPC	0·26	2·03
Time (h)	**Supernatant**	**Pellet**
(b) Growth time assay performed with YPC as carbon source		
24	0·12	0·88
48	0·23	1·80
72	0·78	3·31
96	0·56	2·34

cose mg^{-1} dry weight, i.e. 80% of the positive control value; the strain 6Mt (*P. membranaefaciens*) recorded 2932·0 ng glucose mg^{-1} dry weight, a relative activity of 55%, while strain 97M (*P. anomala*) displayed a relative activity of 46% (2457·6 ng glucose mg^{-1}).

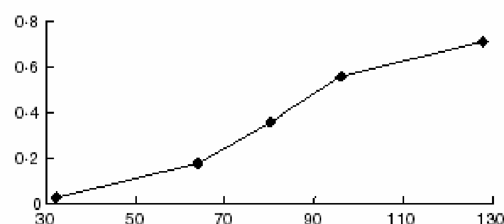

Fig. 2 β-Glucosidase activity (absorbance values at 505 nm, 'y' values) of *Candida molischiana* for different cellobiose concentration (g l^{-1}), 'x' values. Triplicate assays carried out

These three yeasts also displayed cell-wall activity greater than that of the positive control. It may thus be concluded that the *Pichia* strains studied had the activity linked to the cell wall.

Only two yeasts (*P. membranaefaciens*, 98M and *H. osmophila*, 44C) displayed cytosol activity values lower than those of the positive control; *M. pulcherrima* (132M) displayed a relative activity of 825% with respect to

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Table 4 β-Glucosidase activity in different cell fractions. Significant differences ($P \le 0{\cdot}05$) in enzyme activity between different yeast fractions. (Triplicate assays carried out)

	SPNT	WC	CW	CT	MBRN
D. hansenii (1M)	90·8	35·9^{a}	66·3^{a}	1211·9^{b}	367·9^{c}
K. thermotolerans (30Mt)	20·7	13·3^{a}	53·6^{a}	2660·8^{b}	587·9^{c}
K. thermotolerans (32C)	20·4	106·0^{a}	50·7^{a}	612·9^{b}	2356·4^{c}
M. pulcherrima (132M)	69·9	4·7^{a}	124·7^{a}	4066·6^{b}	440·2^{a}
M. pulcherrima (9Mt)	0·0	13·7^{a}	74·2^{a}	2890·9^{b}	381·3^{c}
P. anomala (97M)	82·1	2457·6^{a}	5131·0^{b}	3403·6^{c}	429·3^{d}
P.membranaefaciens (98M)	2·4	4286·3^{a}	4713·5^{a}	405·8	3043·4^{b}
P. membranaefaciens (6Mt)	86·7	2932·0^{a}	4453·2^{b}	0·0^{c}	2712·5^{a}
H. uvarum (24Mt)	40·2	377·9^{a}	235·9^{a}	6946·8^{b}	882·7^{c}
H. uvarum (31Mt)	6·4	0·0^{a}	199·7^{a,c}	2470·2^{b}	652·2^{c}
H. uvarum (52Mt)	7·1	194·1^{a}	110·4^{a}	2052·3^{b}	754·7^{c}
H. uvarum (38B)	205·1	30·7^{a}	110·5^{a}	1602·3^{b}	527·6^{c}
H. osmophila (7C)	55·4	0·0^{a}	0·0^{a}	19710·0^{b}	2617·6^{c}
H. osmophila (9C)	1·8	0·0^{a}	0·0^{a}	98025·3^{b}	19197·6^{c}
H. osmophila (44C)	0·0	25·9^{a}	62·2^{a}	1604·3^{b}	93·4^{a}
H. osmophila (45C)	0·0	0·0^{a}	0·0^{a}	2815·4^{b}	206·7^{c}
R. mucilaginosa (2Mt)	4·4	0·0^{a}	52·5^{a}	0·0^{a}	1092·3^{b}
R. mucilaginosa (341C)	30·9	380·2^{a}	573·9^{a}	757·3^{b}	1081·6^{b}
T. delbrueckii (34C)	20·1	996·5^{a}	444·1^{a,b}	0·0^{b}	2478·8^{c}
T. delbrueckii (4Mt)	0·0	92·3^{a}	105·7^{a}	2267·7^{b}	439·1^{c}
T. delbrueckii (33C)	38·2	1563·9^{a}	618·3^{b}	2277·1^{c}	497·1^{b}
S. cerevisiae (41C)	0·0	139·6^{a}	106·6^{a}	1601·0^{b}	678·2^{c}
S. cerevisiae (UCLMS325)	77·1	888·9^{a}	661·0^{a}	1164·7^{b}	1382·7^{b}
C. molischiana	939·5	5370·3^{a}	1519·3^{b}	492·9^{c}	952·7^{b,c}

SPNT: activity in supernatant expressed as μg de glucose ml^{-1}.
WC, CW, CT and MBRN: activity in whole cell, cell wall, cytosol and membrane, respectively, expressed as ng de glucose mg^{-1} dry weight. Different letters show significant differences.

C. molischiana (4066·6 ng glucose mg^{-1} dry weight), while three *Hanseniaspora* strains displayed a relative activity of over 1000%, one *H. uvarum* (24Mt) at 6946·8 ng glucose mg^{-1} dry weight and two *H. osmophila* strains (7C and 9C), one of which recorded an activity of 20 000% (98025·3 ng glucose mg^{-1} dry weight).

Finally, 35% of the yeasts displayed membrane activity greater than that of the positive control, although values in all the cases were lower than those recorded for cytosol.

A one-factor ANOVA was used to test for significant differences in enzyme activity between the fractions of a single yeast; results are given in Table 4, where significant differences ($P \le 0{\cdot}05$) are marked by different letters. Data for supernatant were not tested, comparison being pointless as values were expressed in different units. No significant differences in enzyme activity were found between the whole cell and the cell wall of any given yeast, with the exception of *D. hansenii* (97M), *P. membranaefaciens* (6Mt) and *T. delbrueckii* (34C). Significant differences were found between these two fractions and both cytosol and membrane, as well as between cytosol and membrane, where differences were statistically significant for all except *R. mucilaginosa* strain, 341C.

DISCUSSION

The method used by Cordero Otero *et al.* (2003) was improved in this study. We found that the great activity shown by the cells was obtained when they grew on YP cellobiose instead of YPD and for 72 h (not 48). The substrate concentration in the enzymatic assay was 128 g l^{-1} and the incubation temperature 30°C.

Although it is not easy to draw general conclusions regarding the portion of the cell involved in β-glucosidase activity, the present results suggest that extracellular activity is weak and intracellular activity much more marked; in most strains, and particularly in *Hanseniaspora*, activity would appear to be chiefly located in the cytosol.

Given the cell-wall activity observed in some strains, future research might usefully focus on obtaining a crude cell extract to enable swift identification of the individual yeasts displaying the most intense β-glucosidase activity.

Non-*Saccharomyces* species displayed greater activity, although *Saccharomyces* strains also displayed weak hydrolysis of β-glucoside bonds, possibly because of residual glucanase activity.

Activity was strain-dependent, as shown by the four *H. osmophila* strains tested, two of which showed intense

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cytosol activity while the other two exhibited virtually no activity at all.

In principle, these results are of little value for industrial application because the activity was intracellular; however, attempts should still be made to identify individual strains from vineyard-winecellar ecosystems with more intense extracellular activity.

ACKNOWLEDGEMENTS

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Characterisation of an exocellular β-glucosidase from *Debaryomyces pseudopolymorphus*

María Arévalo Villena [1*], Juan F. Úbeda Iranzo [1], Sarath B. Gundllapalli [2], Ricardo R. Cordero Otero [2] and Ana I. Briones Pérez [1]
[1] *Departamento de Química Analítica, Tecnología de Alimentos, Universidad de Castilla-La Mancha, Ciudad Real 13071, Spain.*
[2] *Institute for Wine Biotechnology, Department of Viticulture & Oenology, Stellenbosch University, Stellenbosch ZA 7600, South Africa.*

Abstract

When grown in complex media containing 20 g of cellobiose per litre, *Debaryomyces pseudopolymorphus* secreted a β-glucosidase. The synthesis of this enzyme was repressed by glucose. Most of the enzyme was concentrated in the supernatant, with only 10% of the total activity being cell associated. This β-glucosidase (designated Dp-BgI) was purified and shown to be a monomer with a native molecular mass of approximately 100.000 Da. It demonstrated optimal activity at a pH of 4 and, in the short term (no more than 2 h), at a temperature of 40°C. Temperature-stability analysis revealed that the enzyme was labile at 50°C and above. It had a strong affinity for cellobiose and maltose, and degraded laminarin. It was inhibited by Ca^{++}, Zn^{++}, Mg^{++} and acetic acid, but apparently not by glucose and ethanol.

Keywords*:* β-glucosidase, *Debaryomyces pseudopolymorphus*, exocellular, glucose resistance, wine aroma

INTRODUTION

Monoterpenols play an invaluable role in the flavour and aroma of grapes and wine and are present as free, volatile and odorous molecules, as also as flavourless, non-volatile glycosidic complexes (Williams *et al*. 1995). Depending on the precursors, the glycosidic linkages of these complexes are first cleaved by α-L-arabinofuranosidase, an α-L-rhamnopyranosidase or a β-D-apiosidase, followed by a second step that involves the liberation of the monoterpenols (Gueguen *et al*. 1996). Fungal, bacterial and some yeast β-

glucosidases (1,4-β-D-glucosidase; EC 3.2.1.21) may be effective aroma liberators (Gueguen *et al*. 1996), but these enzymes are not always suitable for use under the harsh conditions that prevail during winemaking (i.e. low pH, low temperatures, and high ethanol and glucose concentrations). The limited enzyme activities of the above mentioned microorganisms have necessitated a search among non-*Saccharomyces* yeasts for β-glucosidases that can withstand these conditions (Gueguen *et al*. 1994, Cordero Otero *et al*. 2003, Arévalo Villena *et al*. 2005).

Previously, it was studied the β-glucosidase activities of 20 wine-associated non-*Saccharomyces* yeasts, which were quantified, characterised and assessed to determine the efficiency with which they could liberate monoterpenols from their terpenyl-glycosides (Cordero Otero *et al*. 2003). *Debaryomyces pseudopolymorphus* β-glucosidase (Dp-Bgl) exhibited the most suitable combination of properties in terms of functionality at wine pH, resistance to wine-associated inhibitory compounds (glucose, ethanol and sulphur dioxide), high substrate affinity, and large aglycone-substrate recognition. In conjunction with *S. cerevisiae* VIN13, this yeast strain was also used for small-scale fermentation, which suggested that the β-glucosidase of *D. pseudopolymorphus* has definite potential as a wine aroma-enhancing enzyme, for the reduction of citrus bitterness in juices, and as a main enzymatic component of the synergistic hydrolysis of cellulosic biomass for bio-ethanol production.

In the present study, it was isolated and characterised an exocellular β-glucosidase from cellobiose cultures of *D. pseudopolymorphus*. The effect of different carbon sources on enzymatic biosynthesis in the supernatant fluid of *D. pseudopolymorphus* was evaluated due to previous studies demonstrated that the β-glucosidase synthesis was depending strain (Arévalo Villena *et al,* 2005). It was also studied the influence of pH, temperature, thermal stability, kinetic properties, substrate specificity and effect of metal ions and compounds, confirming that this β-glucosidase is potentially useful for the enhancement of wine aroma.

MATERIAL AND METHODS

Growth and β-glucosidase production

The yeast strain used was *Debaryomyces pseudopolymorphus.* It was studied the time of growth in which the synthesis was maximum. Erlenmeyer flasks filled to 20% of their volume with YPD (10 $g{\cdot}l^{-1}$ yeast extract,

20 g·l^{-1} peptone, 20 g·l^{-1} glucose) or YPC medium, and inoculated with 24 hour-old culture to an initial OD_{600} of 0.5, were used for aerobic cultures. The cultures were incubated for 72 h at 30°C on a gyratory shaker at 200 rpm. At intervals, the cells' dry biomass and enzyme activity were evaluated. The enzyme activity was determined by the method proposed for Arévalo Villena *et al* (2005), where the glucose released by cellobiose hydrolysis was quantified using the Glucose Go Kit (Sigma) according to the supplier's specifications, and the amount of glucose produced was calculated from a standard curve for glucose. The activity was expressed in nmol glucose ml^{-1} mg^{-1} dry cells.

Enzyme purification

- Enzyme isolated:

The cells from a 40 hour-old culture were harvested by centrifugation at 5000 rpm for 5 min at 4°C and 50 ml of supernatant was brought to 80% saturation by the addition of pre-chilled saturated acetone solution and left overnight at 4°C. Precipitates were collected by centrifugation and dissolved in 50 mM citrate buffer at pH 6.0. The solution was subsequently diafiltered on a 50 kDa cut-off and 100 kDa Amicon membrane (Amicon) with 50 mM citrate buffer at pH 6.0. All the fractions obtained were evaluated quantifying the β-glucosidase activity.

The proteins were then separated on a Bio-Rad Automated Econo system by anion exchange chromatography equipped with a DEAE sepharose column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl and the monitored fractions showing activity were pooled. The purified enzyme was dialysed against 50 mM citrate buffer at pH 4.8 and concentrated by ultra-filtration (100 kDa cut-off membrane, Amicon). The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard.

- SDS-PAGE:

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed with a 10% gel according to the method of Laemmli (Laemmli *et al*. 1970), using a mini-protean system (Bio-Rad). The gel was stained with 0.1% Coomassie brilliant blue in a solution containing 50% methanol and 15% acetic acid. The fraction that showed the greatest activity together with an aliquot of supernatant and another one from acetone precipitation was confirmed by used of this method.

(SigmaMarker™ high molecular weight range used as marker).

- Antibody preparation

Antibodies to almond β-glucosidase (Sigma) were obtained as described before (Bellstedt *et al*. 1987), except for the use of purified β-glucosidase for the primary immunisation of the rabbit (1 ml of 1 $mg \cdot ml^{-1}$). Antigen boosts were performed at three weeks and five weeks after primary immunisation, and the antiserum was collected on the 28th and 42nd days.

- Immunochemical determination of Dp-Bgl.

Samples were electrophoresised in SDS-PAGE gels and were transferred to polyvinylidene fluoride (PVDF) microporous membrane (Immobilon™-P Transfer Membrane, Millipore) using standard procedures (Towbin *et al*. 1979). Western-blot protein analysis was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech). Blotted proteins were identified immunochemically by sequential addition of anti-β-glucosidase serum, followed by goat anti-(rabbit IgG) Ig conjugated with alkaline phosphatase (Bio-Rad). The secondary antibody was detected at A_{405} with a microtiter plate reader (Bio-Tek Instruments Inc.), and converted to quantities of Dp-βgl in culture supernatants, which were calculated using standard curves obtained with linearly increasing concentrations of purified almond β-glucosidase (Sigma).

Effect of temperature and pH on β-glucosidase activity

The temperature profile for Dp-βgl was measured between 20 and 70°C in 50 mM citrate buffer at pH 4.0. The effect of pH on the activity of the isolated enzyme was investigated in the pH range of 3-7 at 40°C, using citrate phosphate buffer for the pH range 3.0-5.0 and Tris-HCl buffer for the pH range 6.0-7.0.

Enzyme thermal stability

The temperature stability of the isolated Dp-βgl was investigated by incubating the enzyme preparations in 50 mM citrate buffer at pH 4.0 in airtight tubes at 40°C. At different times, 100 μl samples were withdrawn and stored on ice until the residual activity was determined.

Kinetic properties

The kinetic parameters, V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$), and Michaelis-Menten constant, K_m (mM), were determined from Michaelis-Menten plots of specific activities at six to 10 concentrations of substrate, and the rates were measured in duplicate, ranging from 0.2 to five times the value of K_m. The values of

V_{max} and K_m were determined by nonlinear regression, analysis using the graph pad prism program.

Substrate specificity

To study if the enzyme could hydrolyse β-Glucosidic links of specific substrates, the β-Glucosidase activity was assayed by measuring the amount of glucose released from cellobiose (Sigma) substrate, using the Temperature and pH optimum. Substrate specificity of the β-glucosidase enzyme was determined using two different concentrations (1 and 10 mM) of different polymers, containing either β-1, 4, β-1, 3, α-1, 4 linkages, or β-aryl-glycosides such as lactose, maltose, laminarin, paranitrophenyl β-D-glucoside (pNPG), arbutine, N-octil-β-D-glucoside or mandenonitrile glucoside.

Effect of metal ions and compounds

The effect of different cations and reagents on β-glucosidase activity was assessed by adding 1 or 10 mM of various divalent and monovalent cations (Ca $^{+2}$, Co $^{+2}$, Mg $^{+2}$, Zn, K and Na), glycerol, EDTA, acetic acid, SDS, triton x 100, glucose and ethanol to the reaction mixtures prior to incubation at 40 °C.

In all cases cultures were grown in duplicate, and assays were conducted in quadruplicate. The value ascribed to the maximum β-glucosidase activity obtained in the non-spiked reaction mix is 100% (always done with cellobiose as sustrate).

RESULTS

Growth and β-glucosidase production

The effect of different carbon sources on enzymatic biosynthesis in the supernatant fluid of *D. pseudopolymorphus* is shown in Figure 1. The yeast grew well on both substrates, although cell mass was about 1.2 times higher in glucose-containing media than in cellobiose-containing media. The differences in the growth yield were not, however, correlated with the differences in total enzyme activity. A preliminary investigation using cellobiose media showed that intracellular activity remained at about 5% of the maximum activity in the supernatant fluid. The greatest production of β-glucosidase was observed during the aerobic growth of *D. pseudopolymorphus* in a growth medium with cellobiose as the sole carbon source, being therefore a synthesis induced as showed previous studied (Arévalo Villena *et al,* 2005). Maximum enzymatic synthesis occurs in the supernatant fluid after 40 h of growth of *D. pseudopolymorphus*, but

the β-glucosidase activity in the medium decreased after 48 h. Enzyme production during aerobic growth in the medium with 2% glucose as sole carbon source was less than 1% of the maximum value in YPC. Cellobiose is thus a good carbon source for exocellular β-glucosidase production from *D. pseudopolymorphus*. In contrast, glucose has a repressive effect, possibly through the catabolic repression of β-glucosidase synthesis, which was reported for an intracellular β-glucosidase from *Debaryomyces hansenii* (Riccio *et al*. 1999). However, the constitutive extracellular β-glucosidase isoforms for this yeast are not repressed by glucose, in contrast to our findings. The inhibition of exocellular Dp-βgl reported by us may therefore result from a different mechanism.

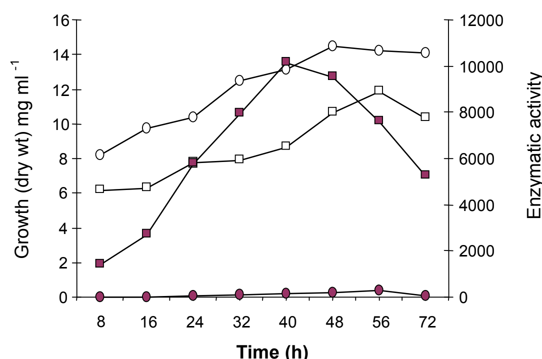

Fig. 1. Kinetics of growth and β-glucosidase biosynthesis in *D. pseudopolymorphus*. The enzymatic activity is expressed as nmol glucose produced per ml of supernatant fluid h^{-1}. Growth in YPD (○) and in YPC (□); enzymatic activity in supernatant YPD (●) and YPC (■). The standard deviations were between 5% and 10%.

Purification of the enzyme

The purification of enzyme from 50 ml of supernatant was performed as described earlier in materials and methods. The purity of the β-glucosidase preparation was confirmed by SDS-PAGE electrophoresis and compared with different extracts obtained during the purification process (Fig. 2). β-glucosidase activity assays and the gel showed that the enzyme of interest was a monomer with a native molecular mass of approximately 100.000 Da and that the purification process with that criterion was correct. On the other hand, the Dp-βgl determination was confirmed by Western-blot protein analysis.

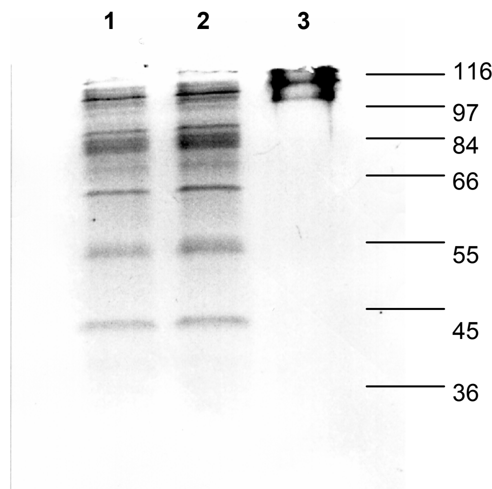

Fig. 2. SDS-PAGE electrophoresis of the various steps of β-glucosidase isolation. Lane 1, supernatant fraction; lane 2, after acetone precipitation; lane 3, after ultrafiltration, fraction with greatest activity (≥ 100 kDa). The numbers to the right of the figure indicate the position and molecular weight in KDa of the marker (SigmaMarker™ high molecular weight range).

Effect of temperature and pH on β-glucosidase activity

A temperature profile was constructed to investigate the temperature optimum for the β-glucosidase from *D. pseudopolymorphus*. Enzyme activity as a percentage of maximum activity was plotted for a range of temperatures (Fig. 3A). The exocellular β-glucosidase from *D. pseudopolymorphus* has a temperature optimum around 40°C. The temperature activity profile was extended into lower temperatures, showing that specific activities at 20°C and 30°C were 60% and 80% of the maximum respectively. The enzyme showed a very high deactivation rate at incubation temperatures higher than 40°C, with only 20% and 10% of maximum activity remaining at 50°C and 70°C respectively.

The pH dependence of β-glucosidase activity was measured using reaction mixtures between pH 3 and 7 (Fig. 3B). The enzyme had a pH optimum of 4, and the specific activities at pH 3.6 and 7 were 80%, 94% and 82% of the maximum respectively. *D. pseudopolymorphus* β-glucosidase activity thus remained quite high in acidic reaction mixtures.

Enzyme thermal stability

To determine the thermal stability of the β-glucosidase, residual activity was measured after heat treatment at 40°C for various periods (Fig. 4). The residual activity after 3 h of incubation decreased to approximately 30% of the maximum activity.

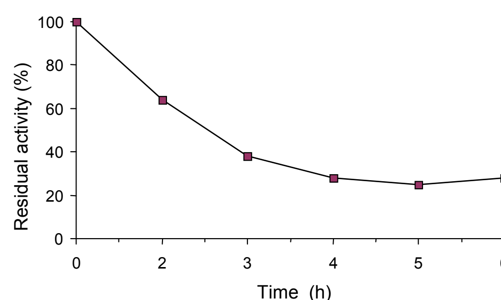

Fig. 4. Thermostability of the β-glucosidase. The scale of residual activity (%) indicates the percentage of experimental value to the maximum. The stability over time is of purified β-glucosidase incubated at 40°C and pH 4. The values shown here are means from quadruplicated assays ± 5% standard deviation.

Kinetic properties

The kinetics of cellobiose hydrolysis were determined using purified enzyme preparation at 40°C and pH 4. The enzyme had an apparent K_m value of 11.9 mM, and a V_{max} value of 70.2 $\mu mol.min^{-1}.mg\ protein^{-1}$ for the hydrolysis of cellobiose.

Substrate specificity

Substrate specificity of the β-glucosidase enzyme was determined using a number of different polymers, containing either β-1,4, β-1,3, α-1-4 linkages, or β-aryl-glycosides.

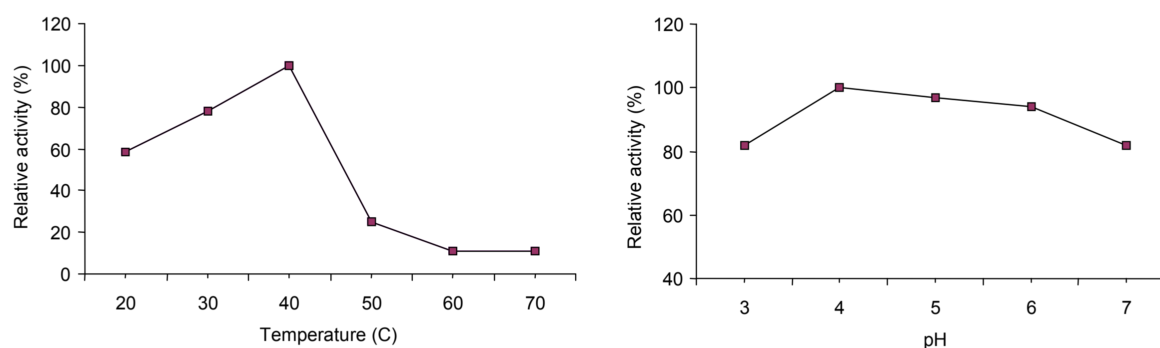

Fig. 3. The relative enzyme activity at different temperatures (A) and pH (B) values for the purified β-glucosidase. The scale of relative activity (%) indicates the percentage of the experimental value at various temperatures and pH's relative to the maximum. The values shown here are means from quadruplicated assays ± 5% standard deviation.

The enzyme showed high reactivity towards cellobiose and p-nitrophenyl-β-D-glucoside, but lower quantities of reducing sugars were liberated from maltose, laminarin, N-octil-β-D-glucoside and mandelonitrile-D-glucoside (Table 1). The soluble polysaccharide laminarin, usually a good substrate for β-glucosidase enzymes, was hydrolysed at 60% of the maximum hydrolysis rate, confirming the endo activity of the purified peptide. The enzyme hydrolysed pNPG, N-octil-β-D-glucoside and mandelonitrile-D-glucoside at 100%, 43% and 52% of the maximum relative hydrolysis rate respectively. With regard to the arbutin, it was not possible to determinate the activity due to there was some interference with the colour development (the problem sample did not even come to the reading of the blank). On the other hand the enzyme showed no activity on lactose, what was waited due to the link is not β-glucosidic, in spite of the this substrate is hydrolysed by other β-glucosidases. The β-glucosidase Dp-βgl that we describe thus has broad specificity for different kind of substrates, and very good activity for mono-β-D-glycosides.

Effect of metal ions and compounds

The effect of various cations and compounds, at concentrations of 1 mM and 10 mM, was tested on the activity of β-glucosidase (Table 2). The enzyme was moderately inhibited by 10 mM Ca^{2+}, Mg^{2+} and Zn^{2+}, showing 69%, 81% and 53% of maximum activity respectively. At the same concentration of the Co^{2+}, K^{+} and Na^{+} cations, the enzyme showed no inhibition. The β-glucosidase was activated to a lesser degree by Co^{2+}, Mg^{2+}, K^{+} and Na^{+} at 1 mM. It was also investigated the action of some enzyme compound effectors at the same concentrations and found that

Table 1. Effect of the Dp-βgl of *D. pseudopolymorphus* on different substrates.

Substrate	Configuration of glycoside linkage	Relative initial rate of hydrolysis (%)
Cellobiose	(1→4)–βGlc	100
pNPG	(1→4)–βGlc	100
Laminarin	(1→3)–βGlc	60
Maltose	(1→4)–αGlc	52
Mandelonitrile glucoside	(1→4)–βGlc	52
N-octil β-D-glucoside	(1→4)–βGlc	43
Arbutin	(1→4)–βGlc	ND
Lactose	(1→4)–βGal	ND

ND, not detected. Standard deviations ≤ 10 % in all cases.

Table 2. Effect of different cations and compounds on Dp-βgl from *D. pseudopolymorphus.*

	Residual activity (%)			Residual activity (%)	
Cation	**1mM**	**10 mM**	**Compounds**	**1mM**	**10 mM**
Ca^{2+}	93	69	**Glycerol**	119	103
Co^{2+}	124	113	**EDTA**	132	94
Mg^{2+}	115	81	**Acetic ac.**	119	47
K^{+}	123	115	**SDS**	30	54
Na^{+}	128	108	**Triton X100**	75	71
Zn^{2+}	111	53	**Glucose**	70	120
Control	100	100	**Ethanol**	154	63

Standard deviations ≤ 10 % in all cases.

the enzyme was moderately activated by 1 mM acetic acid and ethanol, but inhibited by the same compounds at 10 mM, showing 47% and 63% of maximum activity respectively. The β-glucosidase showed 70% of maximum residual activity with 1 mM glucose, and was moderately activated by this hexose at 10 mM. Finally, it showed slight activation in 1 mM glycerol (119% of maximum), but was not affected by 10 mM glycerol.

DISCUSSION

Previously, we characterised the β-glucosidase activity of wine-related non-*Saccharomyces* yeasts (Cordero Otero *et al*. 2003, Arévalo Villena *et al*. 2005). The β-glucosidase activity of *Debaryomyces pseudopolymorphus* showed a promising, suitable combination of good functionality at wine pH, temperature, resistance to wine-associated inhibitory compounds, high substrate affinity and wide aglycone-substrate recognition. In this study we isolated and characterised an exocellular, active form of β-glucosidase from the same yeast. The lower value of β-glucosidase activity observed in the medium was found to be dependent on the presence of glucose, anaerobic conditions for growth, and the physiological state of the cells. Rosi *et al*. (1994) obtained similar results for *D. hansenii* strain 4025, but not with respect to glucose regulation. On the other hand, the study showed that to quantify β-glucosidase activity in wine yeasts, is necessary to study the optimum time of growth (between 36 and 72 hours) for each one of the strains, since the enzyme synthesis is strain depending (Arévalo Villena *et al.* 2005).

The purified enzyme from *D. pseudopolymorphus* clearly shows two different glycosylated forms, rather than a diffuse heterogeneity (Fig. 2), with an apparent MW of 100 ± 5kDa, which is similar to that reported for *Candida molischiana* and *D. hansenii* (Vasserot *et al*. 1991, Riccio *et al*. 1999).

The optimal activity conditions for this enzyme are similar to those reported for the exocellular yeast β-glucosidases from *Dekkera intermedia* and *Hanseniaspora vineae* (Vasserot *et al*. 1989), namely pH 4-5 at 40°C. The trend for higher specific activity at pH values lower than the optimum of 4 reported for most of the β-glucosidases might be due to an acid-base catalytic mechanism, such as that shown for β-glucosidases (Legler 1990). Moreover, the decrease in stability at pH 3 might be correlated with the isoeletric point of the *D. pseudopolymorphus* enzyme, with the increasing number of intra- and intermolecular interactions protecting its three-dimensional structure from a lower unfolding rate (thermal denaturation) (Mozhaev & Martinek 1990). Dp-βgl displayed maximal activity at 40°C, and retained 60% of its activity at the temperature of wine fermentation (approximately 20°C), a temperature at which most of the characterised β-glucosidases retain only 10% to 40% of their maximum activity (Belancic *et al*. 2003). In addition, both the pH and temperature

optima for Dp-βgl activity (pH 4-5, 40°C) correspond to those reported for a *D. hansenii* purified extracellular enzyme (Riccio *et al*. 1999), and to those for purified enzyme from *D. vanrijiae* (Belancic *et al*. 2003). The kinetic parameters calculated for the Dp-βgl on cellobiose (K_m 11.9 mM and V_{max} 70.2 μmol.min^{-1}.mg^{-1}) are not in agreement with those for a β-glucosidase purified from *D. vanrijiae* (K_m 57.9 and V_{max} 84.3, Belancic *et al*. 2003).

β-Glucosidases may be divided into three groups on the basis of their substrate specificity: aryl-β-glucosidases, cellobiases, and β-glucosidases with broad substrate specificity (Gueguen *et al*. 1994). In this study it demonstrates that Dp-βgl may be included in the first group, with many others isolated from both fungi (Gueguen *et al*. 1994, Gueguen *et al*. 1995) and bacteria (Kempton & Withers 1992). Furthermore, Dp-βgl shares the capacity to hydrolyse both α- and β-glucosides with only two other β-glucosidases, namely those isolated from *Botrytis cinerea* (Gueguen *et al*. 1995) and *Aspergillus oryzae* (Riou *et al*. 1998). The low inhibition action of the chelating agent EDTA (Table 2) allowed us to conclude that the active site of this enzyme is not dependent on divalent cations for enzyme activation. The partial inhibition of Dp-βgl by both sodium dodecyl sulphate (SDS) and triton x 100 indicates that the integrity of its three-dimensional structure is critical for its catalytic activity. The enhancement of its activity by ethanol is shared with other yeast β-glucosidases (Vasserot *et al*. 1989, 1991, Gueguen *et al*. 1994, Rosi *et al*. 1994, Cordero Otero *et al*. 2003), and may involve glucosyltransferase activity. However, the inhibition of Dp-βgl activity by higher ethanol concentrations was probably attributable to protein denaturation, which may also explain the inhibition of the activity of this enzyme in the presence of acetic acid (Table 2).

In conclusion, the properties shown by the β-glucosidase of *D. pseudopolymorphus* (higher activity at acidic pH, at relatively high glucose, acetic acid and ethanol concentrations), together with its capacity to hydrolyse terpenic glycosides (Cordero Otero *et al*. 2003) make this enzyme a good candidate for applications in the development of the varietal characters of wines and fruit juices. However, additional work is required to determine the influence of *D. pseudopolymorphus* on wine aroma, and the effectiveness of the immobilised β-glucosidase enzyme in large-scale winemaking trials. The present study also lays the foundation for the cloning and expression of the

D. pseudopolymorphus β-glucosidase gene in commercial wine yeast. Such recombinant wine yeast would release grape-derived varietal aroma compounds from the non-volatile, non-odorous precursors during single-culture fermentations, thereby increasing the sensorial quality of wine – the single most important aspect of winemaking.

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Analytical, Nutritional and Clinical Methods

A rapid method for quantifying aroma precursors: Application to grape extract, musts and wines made from several varieties

M. Arévalo Villena [a], J. Díez Pérez [b], J.F. Úbeda [a], E. Navascués [b], A.I. Briones [a,*]

[a] *Tecnología de Alimentos, Universidad de Castilla La Mancha, Avda. Camilo José Cela s/n, 13071 Ciudad Real, Spain*
[b] *Agrovin S.A. Polígono Industrial Los Alces s/n, Alcázar de San Juan, Ciudad Real, Spain*

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Abstract

This paper reports on a quick and simple method for reliably quantifying aroma precursors in grape extract, musts and wines of different grape varieties. The method, which is intended mainly for use by wineries and analytical laboratories, is based on isolation of terpenyl-β-D-glycosides through selective retention on a C_{18} reversed-phase column, followed by hydrolysis to liberate terpenes, yielding an equimolar proportion of free aglycons and glucose. Sugar was measured using an enzyme analysis kit. Aroma precursors were quantified using this method, in a range of crushed grapes, musts and wines produced in the Castilla La Mancha region. The method was also applied on a larger scale in a wine analytical laboratory, to chart the behavior of terpenyl glycosides during the final stages of ripening of grapes from different viticulture Spanish regions.

The results obtained provided a reliable indication of the aroma potential of the varieties studied. The method is simple, practical and readily applicable in wineries.

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Keywords: Terpenil β-D-glycosides; Aroma precursors quantification; Grape extract; Must; Wine

1. Introduction

The aroma potential of a grape must derives from aromatic free volatiles and from non-volatile, odorless precursors, which may be hydrolyzed during the wine-making process. Over recent years, increasing attention has been paid to these glycosidically bound precursors, since – in certain varieties of must – some monoterpenes, norisoprenoids and shikimic acid derivatives are bound to sugars, and glycosidase-catalyzed hydrolysis releases the volatiles associated with the fruity aroma so highly appreciated in white wines.

The major precursors include structures such as β-D-glucopyranoside, α-L-arabinofuranosyl-β-D-glucopyranoside, α-L-ramno-pyranosyl-β-D-glucopyranoside and β-apiosyl-β-D-glucopyranoside (Gunata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1988; Voirin, Baumes, Bitteur, Gunata, & Bayonove, 1990; Williams, Strauss, Wilson, & Massy-Westropp, 1982).

Quantification of these molecules is of particular interest to winemakers, since they are seen as a quality parameter in white grape varieties used for young fruity wines. It is also of interest when using treatments involving commercial enzyme preparations, in order to establish the right dose of each product to be added, and to predict the outcome of treatment.

Reports indicate that aroma precursor glycosides are not present in all grape varieties, and where present are not always to be found at the same concentrations (Arrhenius, McCloskey, & Sylvan, 1996; Di Stefano, Borsa, Maggiorotto, & Corino, 1995; Garcia-Moruno, Ribaldone, & Stefano, 2000; Gunata, Bayonove, Baumes, & Cordonnier, 1985; Lao, López Tamanes, Lamuela Raventos, Buxaderas, & Torre Boronat,

* Corresponding author. Tel.: +34 926 295300x3424; fax: +34 926 295318.
E-mail address: Ana.Briones@uclm.es (A.I. Briones).

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56 1997; McCloskey, Sylvan, & Arrhenius, 1996; Nicolini, 57 Versini, & Adalla, 1993; Razungles, Gunata, Pinatel, 58 Baumes, & Rayonove, 1993; Reyero et al., 2000; Rey-59 nolds & Wardle, 1997; Zoecklein, Marcy, Williams, & 60 Jasinski, 1997). Two-thirds of glycosides are found in 61 the must, and the other third in the grape skin; thus they 62 can be almost completely recovered by simple pressing. 63 By contrast, free terpenes and anthocyanins are almost 64 exclusively found in skin, and maceration is thus re-65 quired for their extraction (Boulton, Singleton, Bisson, 66 & Kunkee, 1998).

67 Methods used for glycoside analysis generally involve 68 complex procedures combining extraction and detection 69 by gas chromatography (Voirin, Baumes, Gunata, Bit-70 teur, & Bayanove, 1992; Voirin, Baumes, Sapis, & Baya-71 nove, 1992), although Williams et al. (1995), have 72 proposed a very simple method based on quantification 73 of the glucose released through hydrolysis of precursors.

74 This paper reports on a modification of the method 75 developed by Williams et al. (1995). Since hydrolysis 76 of glycosides yields an equimolar proportion of aglycons 77 and D-glucose, determination of the glucose liberated by 78 hydrolysis will permit an estimation of the total concen-79 tration of glycosylated secondary metabolites present in 80 the substrate. This modified method was applied to 81 grape extract, musts and wines (mainly white). This 82 method will provide wineries with a simple, readily ap-83 plied test to determine the aroma potential of musts 84 and wines and to know the effectiveness of the enzyme 85 treatments used.

86 2. Materials and methods

87 The method proposed by Williams et al. (1995) was 88 modified at each stage (glycoside retention on a C_{18} re-89 versed-phase column, washing to remove polar sub-90 stances, recovery of precursors by elution, acid 91 hydrolysis and measurement of the glucose released). 92 Standard solutions of the synthetic glycoside *N*-octyl 93 β-D-glucoside (Sigma) were used; glucose and ethanol 94 concentrations varied depending on the parameter 95 studied.

96 The measurement method developed as described be-97 low was then applied to real samples of grapes, musts 98 and wines, first in our own laboratory and later in a 99 commercial wine analytical laboratory.

100 In order to identify the appropriate modifications to 101 the original method, the following parameters were 102 examined.

103 *2.1. Column recovery capacity*

104 This was tested using 20 ml of a range of *N*-octyl β-D-105 glucoside solutions of known concentration (0.00, 0.10, 106 0.25, 0.50, 1.00 and 1.25 μmol *N*-octyl ml^{-1}).

2.2. Flow rate 107

A 0.5 μmol ml^{-1} solution of *N*-octyl β-D-glucoside 108 was passed through the column at three different flow 109 rates: low (2 ml min^{-1}), medium (3 ml min^{-1}) and high 110 (4 ml min^{-1}). 111

2.3. Number of uses per column 112

Using an *N*-octyl solution (0.2 μmol ml^{-1}), liberated 113 glucose was measured after using the same column 1–4 114 times. 115

2.4. Interference of other compounds 116

The potential interference of flavonols in the determi- 117 nation of aroma precursors was studied, using both syn- 118 thetic and natural flavonols. 119

- *Synthetic flavonols.* Various concentrations of the 120 synthetic flavonol quercetin 3-β-D-glucopyranoside 121 (Fluka) (0, 0.03, 0.06 and 0.12 μmol ml^{-1}) were 122 added to a 0.3 μmol ml^{-1} *N*-octyl β-D-glucopyrano- 123 side solution, and the amount of glucose released 124 was measured for each assay. 125
- *Natural flavonols.* A Muscat was used, both at natural 126 pH and at pH 10 adjusted with concentrated NaOH 127 (Iland, Cynkar, Francis, Williams, & Coombe, 128 1996). Both were eluted through the column and sub- 129 jected to acid hydrolysis. The pH 10 sample was 130 divided into two fractions: one was used to analyze 131 glucose released after acid hydrolysis, and the other 132 was eluted again; the first milliliter of eluate – con- 133 taining the flavonols which might interfere in mea- 134 surements – was discarded. 135

2.5. Resulting protocol 136

Modification of the various steps yielded the follow- 137 ing quantification protocol: 138

Step 1, sample preparation. 139

(a) Musts and wines:. They were centrifuged at 4500 rpm for 5 min.

(b) Grape extract: Grape samples were homogenized using a homogenizer with a dispersing head; 10 g of homogenate were added to 10 ml of a 50% ethanol solution and were agitated at 200 rpm for 2 h at room temperature. Homogenate was then centrifuged at 4500 rpm for 5 min; 5 ml of the clean product were dilute with water to 25 ml in order to obtain the appropriate alcohol concentration (always less than 15% v/v).

152

Step 2, isolation of glycosides. C_{18} cartridges were pre- 153 treated with 10 ml methanol (HPLC quality) followed 154

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by 10 ml Milli-Q water (Williams et al., 1995). An appropriate volume of each sample (15 ml for must and grape extract, and 20 ml for wine) was loaded onto the cartridge. These volumes, which differ from those indicated in the original protocol, were considered more suitable for column retention capacity, because the precursor content in musts is greater than in wine due to collateral hydrolysis of terpenyl glycosides during fermentation. The cartridge was then washed with water (3×15 ml for grape extract and wine, 3×20 ml for must). Glycosides were eluted with 1.5 ml 100% HPLC-quality ethanol followed by 3 ml water, and adjusted to a final volume of 5 ml with water. In all cases, the flow rate was approximately 3 ml min^{-1}.

Step 3, acid hydrolysis. No modification was made to the original method: to aliquots (0.5 ml) of the above glycoside eluate was added H_2SO_4 (1.0 ml, 2.25 M) to give solutions for hydrolysis containing 1.5 M H_2SO_4 and 10% v/v ethanol. A control solution was similarly prepared for each eluate, with water (1.0 ml) added in place of the H_2SO_4 solution, for determination of the free (i.e., non-glycosidic) glucose concentration of the eluate. A reagent blank was made with 30% ethanol in place of the glycoside eluate. The samples and the reagent blank were brought to boiling for 1 h, while controls were held at room temperature.

Step 4, analysis of D-*glucose*. The D-glucose released in each of the assays was measured using a glucose oxidase assay kit (Sigma). Aliquots of the solutions after hydrolysis (262 μl) were transferred to plate wells to which 3 M NaOH solution (260 μl) was added. For the control solutions, water was substituted for the NaOH solution. It was found that with 144 μl of a 95 μg ml^{-1} glucose solution all test samples lay within the detection range of the enzyme kit. To 200 μl of sample, 400 μl of the kit solution were added, and the mixture was then held at 37 °C for 30 min; the reaction was stopped by addition of 400 μl 6 M H_2SO_4, following supplier's recommendations. Absorbance was then read at 540 nm, a wavelength absorbing the pinkish color formed by glucose oxidation to glyconic acid and the subsequent reaction with *o*-dianisidine. Resulting values were interpolated into a glucose calibration curve.

2.6. Application to real samples

In all cases, musts and grapes were collected from various different wine cellars and vineyards around the Castilla la Mancha region. The following test samples were used.

- *Grape extract*. Grapes of the following varieties were processed as indicated earlier: Airén, Chardonnay, Chenín, Garnacha, Muscat, Rosanne, Sauvignon blanc and Verdejo.
- *Must and wine*. The following varieties were used: Airén, Chardonnay, Gewürztraminer, Macabeo, Muscat, Sauvignon blanc and Riesling. Musts were fermented in the laboratory in 3-l flasks using the commercial *Saccharomyces cerevisiae* strain UCLM 325 (Biospringer). Fermentation was monitored for weight loss and considered to have finished when the sugar concentration fell below 5 g l^{-1}.
- *Must, wine and enzyme-treated wine*. Three varieties were used: Airén, Macabeo and Muscat. A fraction of the wines were made from each variety were treated with the recommended dose of a commercial enzyme preparation designed specifically to enhance aroma release in white varieties; this preparation was kept in contact with the wine for 15 days at room temperature.

2.7. Application in an analytical laboratory (Agrovin SA, Spain)

The aim was to ascertain whether this method was routinely applicable at a larger scale in an analytical laboratory receiving samples from different Spanish regions (Galicia, Andalucía, Castilla la Mancha,and Castailla León). Aroma precursors were measured in the following varieties: Muscat, Albariño, Macabeo (irrigated), Airén (irrigated and rainfed), Gewürztraminer and Chardonnay. Precursor levels were also charted over the last 15 days of ripening prior to harvesting.

All assays were performed in duplicate.

2.8. Statistical analysis

A one-way analysis of variance was performed, using the SPSS statistical software package, to detect significant differences between samples both for the assays carried out when developing the method and those performed when applying the method to real samples.

3. Results and discussion

3.1. Column recovery capacity

The retention capacity of the column was tested using *N*-octyl β-D-glucoside solutions of known concentration, covering the range for the various varieties assayed. Total glucose liberated from known concentrations was calculated, and the result was compared to that obtained applying the precursor recovery and quantification protocol. As Fig. 1 shows, the percentage recovery was excellent; the linear correlation coefficient (r^2) of 0.996, recorded even with very high glycoside concentrations, suggests that the method effectively addressed this problem and was applicable to any grape variety.

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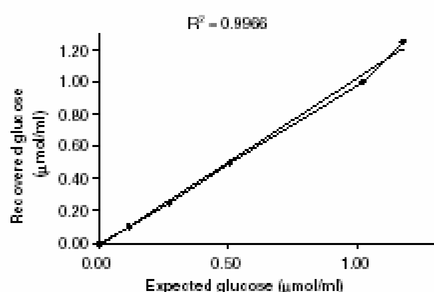

Fig. 1. Recovery of *N*-octyl-β-D-glycoside solutions of known concentration (0.00, 0.10, 0.25, 0.50, 1.00, 1.25 µmol *N*-octyl ml^{-1}).

3.2. Flow rate

A medium concentration of *N*-octyl β-D-glucoside (0.5 µmol ml^{-1}) was used to measure glucose released at the three different flow rates indicated under Section 2 (2–4 ml min^{-1}). Measured glucose ranged from 0.49 to 0.51 µmol min^{-1}. One-way ANOVA indicated no significant differences (95% confidence) between the values obtained at different flow rates and the expected real value. This is of methodological importance, since it allows the analyst a certain margin in this parameter, which is difficult to control.

3.3. Number of uses per column

The *N*-octyl β-D-glucoside concentration used was that most commonly recorded for precursor concentrations (0.2 µmol ml^{-1}). Liberated glucose was measured from four equal samples run on the same column. Table 1 shows mean glucose values obtained for each use of the column. Recovery rates were good until the third use, falling off considerably on the fourth use. ANOVA showed significant differences (95% confidence) between the amount of glucose recovered on the fourth use of the column and the real expected value, while no significant difference was found between the real value and the values recorded for the first, second and third runs on the same column. This suggests that each column may be used up to three times with 95% reliability, which represents an economic advantage for wineries.

Table 1
Glucose released from a 0.2 µM *N*-octyl β-D-glucoside solution after repeated use of the same column

Number of uses	1	2	3	4
Glucose (µmol ml^{-1})	0.20	0.20	0.22	0.10*

* Significant differences at 95% confidence.

3.4. Interference of other compounds

- *Synthetic flavonols*. Rising concentrations of quercetin 3-β-D-glucopyranoside (0, 0.03, 0.06 and 0.12 µmol ml^{-1}) were added to a 0.3 µmol ml^{-1} *N*-octyl solution, and the amount of glucose released by acid hydrolysis following isolation on C18 columns was measured for each assay. Table 2 shows mean values obtained for glucose released by the various samples. ANOVA showed that quantification of the fourth solution (0.12 µM) was significantly lower (95% confidence), suggesting interference at that concentration with the enzyme reaction color development. This however, poses no problem since that flavonol concentration is never reached in real samples (Boulton et al., 1998).
- *Natural flavonols*. Muscat must samples were treated as indicated under Section 2. Table 3 shows mean precursor enzyme values expressed as amount of *N*-octyl ml^{-1} of must. ANOVA showed no significant differences between the various samples assayed, confirming – as in the previous test – that flavonols did not interfere with precursor quantification in real samples.

False positives, i.e., the recovery and quantification of flavonols as though they were aroma precursors, was a potential source of interference due to the structural similarity between the two types of molecules. No false positives were recorded.

3.5. Application to real samples

- *Grape extract*. Grapes of the following varieties were processed as indicated under Section 2: Airén, Chardonnay, Chenín, Garnacha, Muscat,

Table 2
Glucose released from 0.3 µmol ml^{-1} *N*-octyl solutions containing different concentrations of quercetin 3-β-D-glucopyranoside

Sample	A	B	C	D
Glucose (µmol ml^{-1})	0.28	0.29	0.28	0.25*

A, 0.00 µmol ml^{-1}; B, 0.03 µmol ml^{-1}; C, 0.06 µmol ml^{-1}; D, 0.12 µmol ml^{-1}.
* Significant differences at 95% confidence.

Table 3
Concentration of aroma precursors expressed as nmol glucose ml^{-1} must released from terpenyl glycosides in Muscat must treated in different conditions to remove flavonols

Sample	A	B	C
Glucose (nmol ml^{-1})	252.18	260.58	260.58

A, glycosides from must at natural pH; B, glycosides from must at pH 10; C, glycosides from must at pH 10 discarding the first milliliter on a second run.

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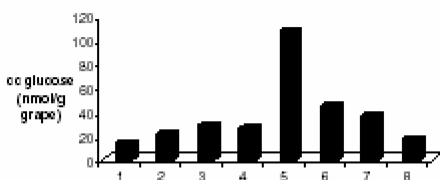

Fig. 2. Aroma precursor concentrations (expressed as nmol glucose g^{-1} grape) in grapes of different varieties: Airén (1); Chardonnay (2); Chenin (3); Garnacha (4); Muscat (5); Rosanne (6); Sauvignon blanc (7); Verdejo (8).

Rosanne, Sauvignon blanc and Verdejo. Fig. 2 shows the glucose measurements obtained following hydrolysis of the glycosides isolated. One-way ANOVA showed significant differences (95% confidence) between all varieties, with the exception of Chenín and Garnacha, which – though similar to each other – differed significantly from the other varieties. As the figure shows, Muscat was the most aromatic variety, and Airén the least aromatic; this confirms results reported in the literature (Boulton et al., 1998; Gunata, Bayonove, Baumes, & Cordonnier, 1986; Iland et al., 1996; Tingle & Halvorson, 1971; Williams et al., 1995).

- *Must and wine*. The following varieties were used: Airén, Chardonnay, Gewürztraminer, Macabeo, Muscat, Sauvignon Blanc and Riesling. Precursors were measured in musts and corresponding wines produced in the laboratory after fermentation with *S. cerevisiae* UCLM 325 strain. Fig. 3 shows, again, the considerable variation in precursor concentrations from one variety to another; the ANOVA showed significant differences (95% confidence) in glycosilated terpene levels between all must varieties. Similar results were recorded for wines, except that no significant difference was found between Airén and Sauvignon Blanc. The highest value was found for Gewürztraminer, followed closely by Muscat. Riesling also displayed high aroma precursor concentrations, whereas the lowest values were again found for Airén. The decline in precursor levels following fermentation of some varieties may have been due to the residual glucanase activity of certain *S. cerevisiae* strain used (Arévalo Villena, 2003; Boulton et al., 1998; Cordero Otero et al., 2003; Hernández, Espinosa, Fernández-González, & Briones, 2002). It is perhaps surprising that so many studies address the use of enzyme treatments to enhance aroma release (Charoenchai, Fleet, Henschke, & Todd, 1997; Delcroix, Günata, Sapis, Salmon, & Bayonove, 1994; Fernández, Úbeda, & Briones, 2000; Gunata, Bayonove, Tapiero, & Cordonnier, 1990; Manzanares, Rojas, Genovés, & Vallés, 2000; Mendes-Ferreira, Clímaco, & Mendes Faia, 2001; Strauss, Jolly, Lambrechts, & van Rensburg, 2001), given the large amount released during fermentation, particularly considering the low detection threshold of this type of compound, which should permit their complete detection in the final product. However, it would appear that despite the release observed through precursor measurement, there is little organoleptic difference; this may be because during fermentation most of these terpenes are volatilized, drawn off by the CO_2 produced during the process, and lost during the metabolite exchange taking place between the fermentation medium and the atmosphere. In any case, the loss is not a source of concern, since – as the figure shows – in some varieties a large amount of substrate remains, and can be released by enzyme treatment.
- *Must, wine and enzyme-treated wine*. In view of the results obtained in the previous section a new set of musts were analyzed, as were the wines produced from them, some of which were then subjected to a commercial enzyme treatment designed specifically to enhance aroma release. Three varieties were used for this purpose: Airén, Macabeo and Muscat. As Fig. 4 shows, the highest

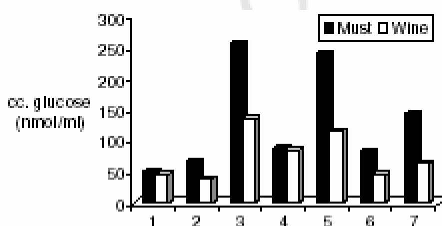

Fig. 3. Aroma precursor concentrations (expressed as nmol glucose ml^{-1}) in musts and wines of different varieties: Airén (1); Chardonnay (2); Gewürztraminer (3); Macabeo (4); Muscat (5); Sauvignon blanc (6); Riesling (7).

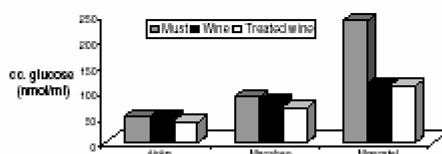

Fig. 4. Aroma precursor concentrations (expressed as nmol glucose ml^{-1}) in musts, untreated wines and enzyme-treated wines of different varieties.

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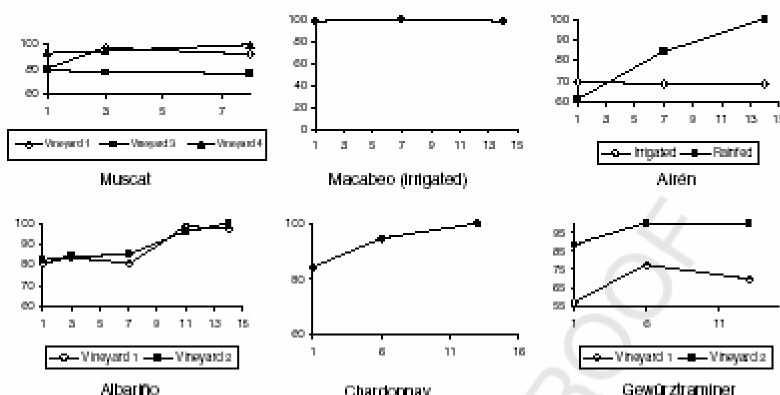

Fig. 5. Behavior of aroma precursors during the days prior to harvesting, expressed as % glucose released as a function of time (days).

values were again displayed by Muscat, followed by Macabeo and Airén. One-way ANOVA was applied separately to each variety to detect significant differences (95% confidence) between must, untreated wine and treated wine. The three products differed significantly for Airén, although since this is a neutral variety, this does not necessarily ensure a difference in sensory perception. For Macabeo products, aroma precursor concentrations were significantly higher in enzyme-treated than in untreated wine, suggesting an appropriate use of enzyme treatment; no difference was found between must and untreated wine, presumably because yeast had no significant effect on terpenyl glycosides for this variety. In Muscat, by contrast, significant differences were detected between must and wines (treated and untreated), but not between treated and untreated wines, suggesting that enzyme treatment is unnecessary with this variety. However, these results need to be confirmed by organoleptic testing, since each terpene has a a different detection threshold, and it is the interaction between terpenes that characterizes each variety (Ribereau-Gayon, Glories, Maujena, & Dubourdieu, 2000).

3.6. Application in an analytical laboratory

In order to ascertain whether this method was routinely applicable on a larger scale, it was tested in a wine analytical laboratory, using the following varieties: Muscat, Macabeo (irrigated), Airén (irrigated and rainfed), Albariño, Chardonnay and Gewürztraminer. Grapes were from vineyards in Pontevedra, Málaga, Cuenca, Huesca and Burgos, sampled over the 15 days immediately prior to harvesting. Fig. 5 shows the behavior of aroma precursors of each variety. Precursor concentrations were expressed as percentage glucose released as a function of time; the last day in each case corresponded to harvesting.

Fig. 5 shows precursor concentrations in different samples of Muscat, Macabeo and Airén. For Muscat, vineyard 1 displayed a slight increase in concentrations towards the end of ripening, while precursor levels in the other two vineyards remained stable. According to the laboratory, the only difference between vineyards 3 and 4, is that vines in vineyard 4 were trained up tall frames; this method of training may thus promote aroma precursor formation when other conditions are constant. Macabeo precursor concentrations remained constant throughout the period studied. The only difference between the two Airén samples was that one was from an irrigated, and the other from a rainfed, vineyard. In the irrigated vineyard, precursors had already formed by the start of the study, whereas samples from the rainfed vineyard displayed a marked increase in precursor levels during ripening, and higher final concentrations. The same figure shows the results for Albarino, Chardonnay and Gewürztraminer, for which the laboratory provided no vineyard information. All three varieties displayed a slight increase in precursor concentrations in the final stages of ripening. A difference was noted in precursor levels between the two Gewürztraminer vineyards.

4. Conclusion

The results obtained here provide a reliable indication of the aroma potential of the varieties studied. The method is simple, practical and readily applicable

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in wineries, allowing wine-makers to determine aroma precursor levels in grapes, musts and/or wines, to chart their behavior over the days immediately prior to harvesting and to test the effectiveness of any enzyme treatments used. The method will thus be valuable when taking decisions on the timing of harvesting and/or the use of enzyme treatments (dose, duration) applied to enhance aroma release.

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Relationship between *Debaryomyces pseudopolymorphus* enzymatic extracts and release of terpenes in wine

María Arévalo Villena , Juan F. Úbeda Iranzo and Ana I. Briones Pérez
Departamento de Química Analítica, Tecnología de Alimentos, Universidad de Castilla-La Mancha, Ciudad Real 13071, Spain.

Abstract

Due to the interest that exits on the liberation of aromas in young wines, it were obtained some different enzymatic extracts (purified extract, P, lyophilised purified extract LP, immobilised purified extract, IP and immobilised lyophilised purified extract ILP) with β-glucosidase activity form *Debaryomyces pseudopolymorphus* that excreted the enzyme at growth medium. The extracts were added to natural glycosides isolated from different grape varieties. The results were compared with the effect of 7 commercial enzyme preparations (CEP), obtained from moulds used on wine making. It was showed that some yeast extracts had similar effect than the CEP, for what the next step was to use them on wine samples elaborated at laboratory. It was studied the effect at 9 and 16 days of contact quantifying both the precursors that were stayed and the liberated terpenes. The results were compared with a control wine (without any extract) and with treated wine with a commercial enzyme preparation specially indicated to liberation of aromas. It was observed that the enzymatic extracts from *Debaryomyces pseudopolymorphus* hydrolysed the precursors in wine and that they could compete with the commercial preparations since that liberation it was produced even in less time.

Keywords: Terpenes, aroma precursors, β-glucosidase activity, wine yeasts, enzymatic extracts

1. Introduction

The essence of wine lies primarily in its colour, aroma and flavour. The seemingly endless and fascinating diversity of these attributes is defined by a set of compounds largely derived from naturally-occurring constituents of the grape berry (including sugars,

acids, nutrients and glycosylated aglycons), oak wood when used, and microorganisms during fermentation (Bartowsky et al. 2004).

Winemakers are continually seeking techniques to unleash the latent pool of volatile aglycons masked in the flavourless grape-derived glycoconjugates, in order to enhance varietal character and to modulate and enhance the sensorially-diverse qualities of wine. These aglycons include norisoprenoids (e.g. damascenone), volatile phenols and other benzene derivatives (e.g. raspberry ketone), aliphatic alcohols (e.g. hexanol), sexquiterpenes, resveratrol and monoterpenes (e.g. citronellol, geraniol, hotrienol, linalool, nerol and α-terpineol).

Monoterpenes and monoterpene alcohols play a key role in the aroma and flavour of Muscat grape varieties and wine. However, these compounds also occur in other cultivars, such as the non-floral variety, Chardonnay, in which 200 different aglycons have been identified (Sefton et al. 1993). In the latter, these compounds exist as subtle supporting aromas and flavours, although they are less pronounced, and other odorous flavour compounds might play a larger role in the varietal aroma and flavour of the wine. The fragrances and flavours they impart are, therefore, not limited to the perfume-like aroma of muscat, but also include other aromas, such as spicy, peppery, smoky and grassy odours. The major fractions of the monoterpenes and monoterpene alcohols occur in the grape as glycosidically-bound forms (Williams et al. 1982, Gunata et al. 1985, Voirin et al. 1992), which render them non-volatile and flavourless. The flavourless glycoconjugates are either glucosides or disaccharide or trisaccharide glycosides (Bartowsky et al. 2004).

The glycosidic bonds present in glycoconjugates can be released by two methods: the first, acid hydrolysis, has the disadvantage of possibly changing the intrinsic varietal aroma of the wine. The second method is by enzyme hydrolysis, which has attracted attention as a means of improving the varietal aroma and flavour of wine. The mechanism by which this hydrolysis works is now well established, and entails specific glycosidases active in two successive steps (Gunata et al, 1985; 1988). In the first step, the action of an α-arabinofuranosidase, α-rhamnosidase, β-xylosidase or β-apiosidase is necessary to cleave the intersugar-linkages; this is followed by a second step in which β-glucosidases release aglycons (Gunata et al. 1988). Where the disaccharide moiety consists of two glucose units, only the action of a β-glucosidase is required to facilitate the complete reaction (Haisman et al.

1967). The release of these molecules can also help to improve wine aroma and flavour, as well as having other beneficial properties, especially with regard to the release of the antioxidant resveratrol (Vrhovsek et al. 1997*;* Becker et al. 2003).

Glycosidases occur in nearly all organisms, and perform a variety of functions. However, not all of these enzymes are suitable for use in winemaking; for example, those from plants exhibit high pH optima (pH 5 for *Vitis vinifera* grapevine), and are virtually inactive at pH 3 – 4, the pH of wines and grape juices (Aryan *et al,* 1987). Glycosidases from fungi are notoriously inhibited by glucose concentrations as low as 1 to 1.5%. These enzymes are also more active at high pH values (Woodward and Wiseman, 1982). Several yeast β-glucosidases have been studied with a view to future application (Raynal and Guerineau, 1984; Kuranda and Robbins, 1987; Machida et al, 1988; Delcroix et al. 1994, Gunata et al, 1990a; Gueguen et al, 1994; Rosi et al, 1994; Gueguen et al, 1995; Saha and Bothast, 1996; Skory et al, 1996; Mateo and Di Stefano, 1997; Yan and Lin, 1997; Riou et al, 1998, Fernández et al, 2000; Manzanares et al, 2000; Mendes Ferreira et al, 2001; Strauss et al, 2001; Hernández et al, 2002; Cordero et al, 2003, Arévalo et al, 2005a)

Bacterial glycosidases generally have the disadvantage of being active at high temperatures (50ºC and higher) (Woodward and Wiseman, 1982). Commercial strains of the yeast (*Saccharomyces cerevisiae*) and bacterium (*Oenococcus oeni*) most commonly used to initiate alcoholic fermentation and malolactic fermentation, respectively, have only limited aroma-enhancing enzyme activities; this leaves a substantial pool of grape-derived glycosides untapped (D'Incecco *et al,* 2004).

At present, aroma release is enhanced using commercial enzyme preparations of fungal origin, mainly *Aspergillus spp*. The composition of these preparations varies, and they are actually a mixture of non-specific glucanases. Tingle and Halvorson (1971) found that these cocktails hydrolyzed only 3% of precursors, but that this was sufficient to ensure an analytically- and organoleptically-detectable increase in free terpenes. At the same time, however, since glucanases are non-specific, they may trigger collateral reactions detrimental to the product, such as hydrolysis of anthocyans which are also stabilised by binding to sugar molecules (Riou et al. 1998). For these reasons, the best option would be to use specific enzymes which are not excessively active; ideally, they should also be contained in yeasts forming part

of the wine ecosystem, and thus adapted to their ecological environment.

An alternative to this would be to use mixed yeast cultures to initiate wine fermentations. Recently, it has been reported that co-fermentation of a Chardonnay grape must using a *Debaryomyces pseudopolymorphus* strain with commercial wine yeast, *S. cerevisiae* VIN13, increased the liberation of citronellol, geraniol and nerol (Cordero Otero et al. 2003). Thus, the presence of non-*Saccharomyces* species at the onset of alcoholic fermentation might have a greater potential to contribute to the liberation of some aglycons from the flavourless precursor glycoside during fermentation. At the same time, however, their presence during fermentation might also cause off-flavours.

Debaryomyces pseudopolymorphus has an extracellular β-glucosidase displaying good activity on synthetic substrates, and biochemical characterisation has shown that it is stable and active over the pH range used in wine fermentation, as well as in the presence of glucose, ethanol and other metabolites that may be present in wine. (Arévalo Villena et al, 2005).

The purpose of this study was to examine the effect of various forms of yeast-derived enzyme extract on natural terpenyl glycosides isolated from several grape varieties. The effect of these extracts on aroma precursors when added directly to wine was also studied. Finally, dose and contact time were optimised, and the results were compared to the effects of a commercial enzyme preparation commonly used by winemakers.

2. Material and Methods

2.1.Debaryomyces seudopolymorphus enzymatic extracts

The yeast was grown under aerobic conditions in 250-ml flasks containing 50 ml YP cellobiose, and incubated at 30 ºC for 42 h. The culture was centrifuged, and the following enzyme extracts were obtained from the supernatant:

Purified extract (P): supernatant was purified by size exclusion, using filter cartridges designed for the concentration and purification of small peptides, oligonucleotides, nucleic acids, enzymes, antibodies and other molecules (Pall). The molecule size selected was 100 KDa, considered the most suitable for excreted protein (Arévalo Villena et al, 2005 b). Samples were run for 30 min., 1 h and 1.5 h at 4500 rpm, and the β-glucosidase activity of the different fractions was checked by determining the amount of glucose released (Sigma GO glucose enzyme kit) from cellobiose

hydrolysis (Sigma) (Arévalo Villena et al. 2005a).

Lyophilised purified extract (LP): The extract obtained above was subjected to several laboratory-scale lyophilisation tests at a temperature of -51.8 ºC and a pressure of 2.6 e^{-2} mB pressure for 24 h, in a lyophilizer (Pelstar). Skimmed milk was used for cryoprotection, at a range of concentrations (0, 10 and 20 % v/v). β-glucosidase activity of lyophils was determined by measuring cellobiose hydrolysis capacity.

Immobilised purified extract (IP): Purified extract was immobilised in 5 % sodium alginate (Sigma) using 0.05 M calcium chloride as gelling agent. Entrapped activity in the 3–mm beads thus obtained was checked using the method indicated earlier.

Immobilised lyophilised purified extract (ILP): The lyophil obtained was also immobilised using the above method.

2.2. Isolation of natural glycosides

Natural glycosides were isolated from musts of the varieties: Airén, Muscat, Macabeo, Gerwürztraminer, Sauvignon Blanc and Riesling, all of them from cultivars used in Castilla La Mancha (Spain). Terpenyl glycosides were isolated and recovered following a protocol developed by Arévalo Villena et al. (2005c) using reversed-phase C_{18} columns (Waters).

This method provides a rapid overall quantification of aroma precursors in musts, wine or enzyme-treated wine, but does not differentiate between compounds.

2.3. Hydrolysis of natural glycosides obtained from grape varieties, by Debaryomyces pseudopolymorphus enzymatic extracts

In order to ascertain the extent to which enzyme extracts were able to hydrolyse the aroma precursors isolated as shown above, the following assays were performed in triplicate:

- *Purified extract (P):* Purified extract activity was examined at three different concentrations. For each variety under study, 2 ml of each glycoside solution form the different varieties was added: a) to the amount of extract displaying the same activity on cellobiose as the recommended dose of the commercial enzyme preparation used by winemakers; b) to three times that amount; and c) half that amount. Samples were kept for 15 days at 30ºC, checking precursor hydrolysis by quantification of released glucose.
- *Purified (P), immobilised (IP), lyophilised (LP), immobilised lyophilised (ILP) extracts.* Each extract was placed in contact with 2 ml of a solution of Muscat natural precursors. The doses used were the recommended dose and half the recommended dose; contact time was 20 days at ambient temperature.

2.4. Hydrolysis of natural Muscat natural glycosides by commercial enzyme preparations.

A study was made of the β-glucosidase activity of several commercial enzyme preparations (CEP), obtained from moulds, widely used by winemakers to enhance wine aroma. Seven enzyme cocktails were added to 2 ml of the glycosides isolated from Muscat, at the concentrations recommended by the manufacturers. Contact time was 20 days at ambient temperature, and the degree of hydrolysis for each cocktail was determined by measuring released glucose. The enzyme preparations used are shown in Table 1.

2.5. Microvinification

The last part of the study sought to determine the ability of enzyme extracts to hydrolyse aroma precursors in real wine samples. For this purpose, Muscat grapes were crushed and pressed in a pilot plant. Reducing sugars (degree Brix), pH, total acidity and precursor content were measured in the must thus obtained (Arévalo Villena et al, 2005c).

Microvinification was performed in a multiple-tube laboratory fermenter: 5 L of must were inoculated with 10^6 cells/ml of a commercial *Saccharomyces cerevisiae* strain (UCLM 325), and 50 ppm of SO_2 was added. The fermentation was carried out at 18 ºC until the sugars were consumed. Thereafter, wines were decanted and fractionated for the next assay.

2.6. Hydrolysis of aroma precursors in wine by Debaryomyces pseudopolymorphus extracts

Enzyme extracts P, IP and LP were added to 200 ml of wine , at manufacturers' recommended doses. Both ILP and the use of other doses were excluded in the light of the results of previous assays. Wine with no added enzyme extract was used as negative control, and a commercial enzyme preparation widely used in the La Mancha region of Spain (preparation E) was used as positive control. Samples were kept at ambient temperature for between 9 and 16 days, after which bentonite (20 g/hl) was added to remove enzyme extract. Samples were then decanted and stored at 4 ºC until analysed.

Hydrolysis of terpenyl glycosides in wine samples was quantified using two methods:

- *Quantification of non-hydrolysed aroma precursors:* This was performed following the protocol described above under “Isolation of natural glycosides” (Arévalo Villena et al, 2005c) .
- *Quantification of released terpenes by gas chromatography*: Wine volatiles were analysed following the method reported by Salinas and Alonso (1997), based on a dynamic headspace

Table 1. Commercial mould-derived enzyme preparations widely used by winemakers.

Enzyme preparation	*Description*	*Indications*
A	Pectolytic enzyme preparation with residual β-glucosidase activity	To enhance expression of tone and structure, acting collaterally on aromatic substances
B	Pectolytic enzyme preparation with secondary hemicellulase and cellulase activity	Extraction of colour, tannins and aroma precursors in high-potential grapes
C	Pectolytic enzyme preparation with secondary hemicellulase and cellulase activity	Extraction of colour, tannins
D	Pectinase especially intended for complementary glycosidase activity and low extraction activities (cellulases and hemicellulases)	To release of aroma in white-grape varieties rich in aroma precursors
E	Pectolytic enzyme preparation with considerable β-glucosidase activity	To enhance aroma
F	Mixture of pectinases and β-glucosidases	Release aromas in young white and rosé wines
G	Mixture of pectinases and maltodextrins	For use in winemaking

using thermal desorption, gas chromatography and mass spectrometry. Methyl caprilate was used as an internal standard (IS), adding 3 uL (1% in absolute ethanol) to 50 mL of wine. Isolation was carried out in triplicate using a Dynamic Thermal Stripper 1000 (Dynatherm Analytical Instruments Inc.). The purge phase used 50 mL of wine; helium was bubbled through the sample for 20 minutes at a flow rate of 84.3 mL/min and at 30 ºC; the dry phase lasted 5 minutes under the same conditions. Volatiles were adsorbed on 0.17 g of Tenax-TA (60 mesh; Alltech) contained in a metal tube at 25 ºC introduced into the desorption thermal equipment (Perkin-Elmer Desorption ATD 400) under the following conditions: oven at 300 ºC; desorption time, 4 min; cold trap, -30ºC with 0.02 g Tenax adsorbent. The inlet, outlet and desorption flows were 45, 9 and 53 mL/min, respectively. The compounds passed through a transfer line at 200 ºC into an HP 6890 gas chromatograph coupled to an LC 3D mass detector with a fused silica capillary column (BP21 stationary phase 50 m length, 0.22 mm i.d., and 0.25 µm film thickness). The chromatography program was: 50ºC (2.5 ºC/min), 180 ºC (2 min) and up to 200 ºC (1ºC/min). In the mass spectrometry analysis, electron impact mode (EI) at 70 eV was used. The mass range varied from 35

to 500 u and the detector temperature was 150 ºC. (Salinas et al, 2003).

Compound identification was carried out using the NIST library and with data from authentic compounds. Chemical standards were supplied by Aldrich (Gillingham, UK), Sigma and Chemservice (West Chester, PA). Quantification was performed in SIM mode; peak heights were checked to ascertain whether they were three times the baseline.

3. Results and discussion

3.1.Debaryomyces pseudopolymorphus enzymatic extracts

Of the three times tested for partial enzyme purification (30 min., 1 h and 1.5 h), 1 h was selected, since although purification and concentration was a bit better at 1.5 hours, the added 0.5 h was not justified by the results obtained.

For lyophilisation, 10 % skimmed milk was selected as cryoprotective agent, since differences in activity on cellobiose were negligible with respect to other concentrations, and the texture of the lyophil with 10% cryoprotector was found more suitable.

3.2. Hydrolysis of natural glycosides by Debaryomyces pseudopolymorphus enzymatic extracts

- *Purified extract (P)*. Precursors from the grape varieties tested were kept in contact with the three test concentrations of this extract, at 30 ºC for 15 days; released glucose was quantified at 1, 3, 7 and 15 days. Precursor hydrolysis over time, for the different varieties and the three extract concentrations used, is shown in Figure 1. All varieties displayed similar behaviour, and 100% of precursors were hydrolysed by day 15, at all three test concentrations. However, in Airén, Sauvignon Blanc and Macabeo, complete hydrolysis took place within the first few days of contact, since these varieties are more neutral and thus have a lower precursor content. These results confirm that the β-glucosidase activity of this yeast strain is sufficient to hydrolyse natural glycosides and thus contribute to wine aroma.

With regard to the concentrations used, hydrolysis with the triple dose was slower in Macabeo, Riesling and Gewürztraminer, possibly because excess enzyme interfered with the substrate, preventing hydrolysis (saturation-inhibition effect). There were no marked differences between the other two concentrations; thus, similar results may be obtained using less enzyme. This is of particular interest to winemakers, in that it represents a considerable financial saving.

Having confirmed the ability of the yeast enzyme extract to hydrolyse terpenyl glycosides in all the varieties

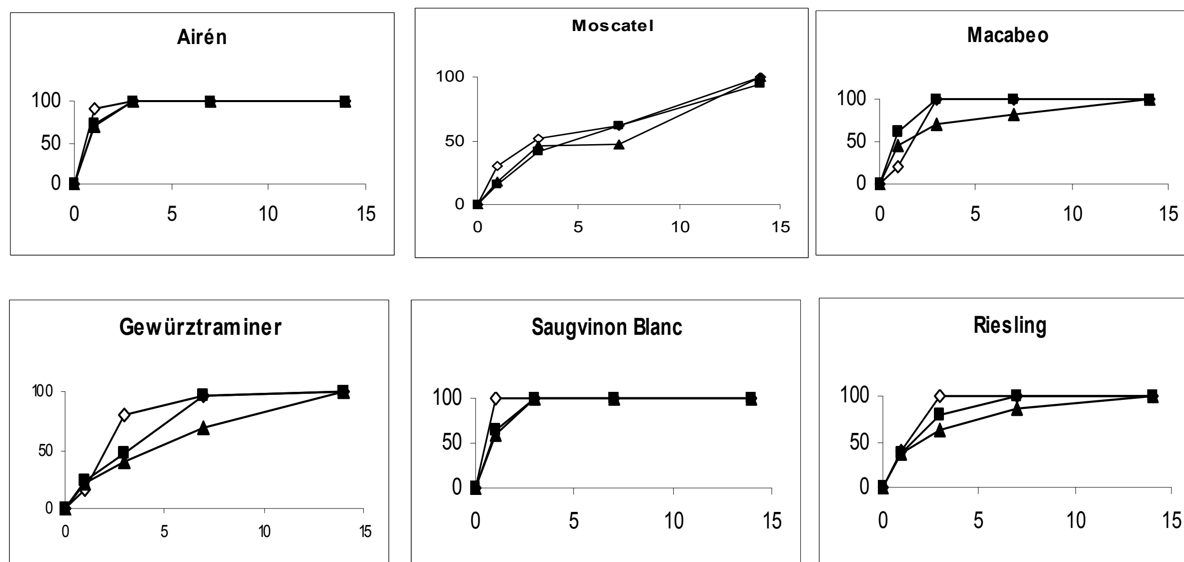

Figure 1. β-glucosidase activity of *Db. pseudopolymorphus* microbial enzyme on aroma precursors isolated from different grape varieties, expressed as percentage hydrolysed glycosides vs. time (days). Assay performed at 30 ºC using three enzyme concentrations: ◊ half-dose, ■ recommended dose and ▲ triple dose.

tested, subsequent assays were performed only with Muscat, which has one of the highest precursor contents.

- *Purified (P), immobilised (IP), lyophilised (LP) and immobilise lyophilised (ILP) extracts.* Glycosides extracted from Muscat must were placed in contact with P, IP, LP and ILP extracts. In view of the results described above, only two concentrations were used: the recommended dose and half that dose. Contact time was 20 days at ambient temperature, thus reproducing real winemaking conditions. Hydrolysis was measured by quantifying released glucose. As Figure 2 shows, the change in temperature affected enzyme activity, since neither of the concentrations used achieved 100% hydrolysis. Although better results were obtained with the recommended dose, this poses no problem for industrial applications; given the low detection threshold of these compounds, sensory perception of release is reported with as little as 3% hydrolysis (Tingle *et al,* 1971).

Hydrolysis of aroma precursors was not identical for all forms of extract tested. Lyophilised extract displayed the greatest activity, at both concentrations, followed by purified extract. However, purified extract may be the more suitable form for industrial use; it guarantees a similar rate of precursor hydrolysis, and is less expensive and time-consuming than lyophilised extract. Immobilised extract displayed a lower rate of β-glucosidase activity.

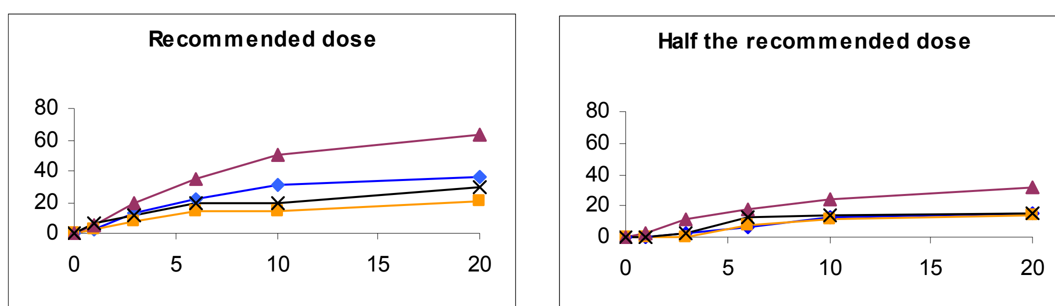

Figure 2. β-glucosidase activity of *Db. pseudopolymorphus* enzyme extracts on aroma precursors isolated from Muscat, expressed as percentage hydrolysed glycosides vs. time (days). Assay performed at ambient temperature with various forms of extract: ▲ lyophil, χ immobilised lyophil, ■ immobilised purified extract, and ◊ purified extract.

3.3. Hydrolysis of natural Muscat glycosides by commercial enzyme preparations.

Glycosides extracted from Muscat must were placed in contact with commercial preparations (recommended doses) for 20 days at ambient temperature. The results obtained are shown in Figure 3. All preparations hydrolysed aroma precursors to a greater or lesser degree, even though some – according to the commercial literature – lacked specific β-glucosidase activity. Terpene release was greatest with preparation G, and lowest with preparation D; curiously, the former is not especially indicated for aroma release in precursor-rich varieties, whereas the latter is. This would suggest that these cocktails are not too pure, and may have side-effects that could prove detrimental to the wine. Some preparations displayed behaviour similar to that of the yeast extract tested; these include preparation E, whose references for aroma-release applications were highly favourable.

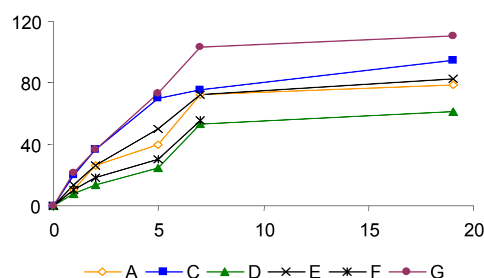

Figure 3: β-glucosidase activity of commercial enzyme preparations on aroma precursors isolated from Muscat musts, expressed as percentage hydrolysed glycosides vs. time(days). Assay performed at ambient temperature.

3.4. Hydrolysis of aroma precursors in wine by Debaryomyces pseudopolymorphus extracts

The Muscat must used for wine-making had the following properties: 237 g/l reducing sugars; pH 3.35; total acidity 7.3 g/l and terpenyl glycoside content 218 nmoles glucose/ml must. Wines made in the laboratory were placed in contact with enzyme extracts under the conditions described in "Material and Methods".

- *Quantification of non-hydrolysed aroma precursors:* these were quantified at 9 and 16 days in wines treated with the various yeast enzyme

extracts (P, IP, LP) and with the commercial enzyme preparation. Precursor content was also measured in the initial must and in non-enzyme-treated control wines. Results, expressed as nmoles glucose / ml must or wine, are shown in Table 2. The precursor content of control wines was lower than that of musts; this finding, reported in previous studies (Arévalo Villena, et al, 2005c), may be due to the residual hydrolytic activity of the *Saccharomyces* strain used in fermentation. Nevertheless, although precursors are hydrolysed during fermentation, enzyme treatment is advisable for releasing aromas, since part of the released terpenes may be volatilised and removed by the CO_2 produced during the fermentation process.

Hydrolysis of wine precursors was greater using the purified yeast extract, suggesting that this form displays greater β-glucosidase activity.

Days	***Control***	Treated wines			
		P	**PI**	**PL**	**CEP**
9	110 b	81 *, c	96 a	92 a	106 *, b, a
16	110 b	64 *, a	88 b, c	81 a, c	77 *, a, c

Table 2. Aroma precursors (nmoles glucose / ml wine) at 9 and 16 days in control and treated with various enzyme extracts wines.

P: Purified extract; PI: immobilised purified extract; PL: lyophilised purified extract; and CEP: commercial enzyme preparation.
* Significant differences ($p \leq 0.05$) between treatment times for the same enzyme preparation. Different letters indicate significant differences (95% confidence) between wines treated with different extracts for 9 and 16 days.

Data were subjected to one-way analysis of variance to study for significant differences between contact times and/or enzyme extracts.

Results for contact times showed significant differences ($P \leq 0.05$) (Table 2, marked *) only between wines treated with the purified extract and those treated with the commercial enzyme preparation.

At 9 days, wines treated with the purified extract differed significantly from those treated with other forms of extract; those treated with the lyophilised (LP) and the immobilised purified extract (IP) displayed no significant differences with respect to the commercial enzyme preparation (CEP), although they differed significantly from control wines. Precursor hydrolysis was lowest using the CEP, results being similar to those obtained in controls.

At 16 days, the purified yeast extract also displayed greater Muscat precursor hydrolysis than the other treatments. At this stage, wines treated with P, PL and CEP all showed significant differences with respect to control; by contrast, no significant difference was noted for the immobilised extract, indicating that immobilisation was a less effective means of precursor release.

- *Quantification of released terpenes by gas chromatography:*

Table 3 shows the content of volatile terpenes, together with that of certain esters and phenyl ethanol in treated and control wines. To facilitate the interpretation of results, a value of 100% is assigned to control wines for each compound measured.

As expected, treated wines in all cases displayed higher content of volatile terpenes than untreated control. After only 9 days' contact, wines treated with yeast extracts exhibited higher concentrations than those treated with the fungal preparation. Similar results were observed for 2 phenyl ethanol, isoamyl acetate, ethyl hexanoate, hexyl acetate and 2 phenyl ethyl.

Results of the one-way ANOVA performed to check for differences in wine terpene content after the two treatment times are shown in Table 2. Significant differences between both contact times were only observed in wines treated with the commercial enzyme preparation, suggesting that the *Debaryomyces pseudopolymorphus* extract requires less contact time to achieve the desired effect.

In the absence of significant differences in the performance of yeast enzyme extracts as a function of treatment times, the results obtained at 9 days were subjected to one-way ANOVA to determine differences in extract performance (Table 3).

Greater amounts of 1-hexanol were released by purified extract (P), followed by immobilised purified extract (IP). There were no significant differences between the four treatments, and only P and LP differed significantly from control. Geraniol content was greater with P and LP, with no significant difference between the two; however, these differed significantly both from the other preparations and from control. There were no differences between the enzyme extracts with respect to alpha-terpineol content, only were significantly differences between purified extract (P) and commercial preparation (CEP). There were no significant differences between treatments with respect to nerol content, though all treatments differed from control.

With regard to 2-phenyl ethanol content, only wines treated with P and LP differed significantly from control. Higher isoamyl acetate values were recorded for P and LP, which differed significantly from other treatments. Ethyl hexanoate and hexyl acetate contents were similar for all treated wines, which differed significantly from control. Wines treated with P, LP and CEP displayed similar values for ethyl octanoate content; differences between these and both PI-treated and control wines were significant. Finally, treated wines showed no significant differences from control with respect to 2-phenyl

Table 3. Content of volatiles in control and treated with various enzyme extracts wines at 9 and 16 days, expressed as % with respect to controls.

	Control wines	***Treated wines***							
		P		**PI**		**PL**		**CEP**	
Days		**9**	**16**	**9**	**16**	**9**	**16**	**9**	**16**
1- hexanol	100 [a]	135 [b]	131	124 [b]	138	123 [a,b]	130	121 [a, b]	124
3-hexen-1-ol	100	124	112	108	122	102	102	112	102
Geraniol	100 [a]	168 [b]	166	133 [a]	110	149 [b,c]	161	133 [c]	171
Linalol	100	113	116	116	123	103	120	104	109
Hotrienol	100	110	113	92	93	91	107	96	99
α-terpineol	100 [a, b]	104 [b]	113	98 [a, b]	102	87 [a, b]	107	81 [a]*	108*
Citronelol	100	108	129	109	115	115	111	94	123
Nerol	100 [a]	128 [b]	136	116 [a, b]	107	117 [a,b]	124	131 [b]	144
2-phenyl ethanol	100 [a]	122 [b]*	136*	112 [a, b]	112	118 [b]	133	113 [a, b]*	160*
Ethyl acetate	100	108	118	110	129	100	98	108	114
Ethyl butyrate	100 [a]	143 [b]	133	129 [a]	145	128 [a]	132	138 [a]	120
Isoamyl acetate	100 [a]	201 [b]	186	133 [c]	167	210 [b]	208	147 [c]*	210*
Ethyl hexanoate	100 [a]	190 [b]	199	191 [b]	197	183 [b]	191	164 [b]	193
Hexyl acetate	100 [a]	152 [b]	141	139 [b]	145	143 [b]	141	135 [b]	134
Ethyl octanoate	100 [a]	118 [b]	115	106 [a]	109	117 [b]	112	123 [b]	122
2-phenyl ethyl acetate	100 [a, b, c]	111 [b, c]	114	104 [a, b, c]	117	109 [c]	114	93 [a]*	104*

P: Purified extract; PI: immobilised purified extract; PL: lyophilised purified extract; and CEP: commercial enzyme preparation.
* Significant differences ($p \leq 0.5$) between treatment times for the same enzyme preparation. Different letters indicate significant differences (95% confidence) between wines treated with different extracts for 9 and 16 days.

ethyl acetate content, although concentrations were higher in wines treated with P and LP.

These results suggest that yeast enzyme extracts display comparable and adequate β-glucosidase activity, which is in many cases greater than that of commercial mould preparations. Of the yeast forms tested, purified

extract proved to be the most suitable, treated wines exhibiting the highest volatile terpene content; this was followed by lyophilised purified extract. The immobilised form released the lowest amount of terpenes; these findings matched those recorded for wine precursors. With regard to treatment times, at 9 days wines treated with purified yeast extract differed significantly from control; this difference was not observed for the commercial preparation. Thus, one advantage of the yeast extract is that it allows winemakers to obtain the same aroma release using shorter enzyme treatment times.

Although the results obtained here need to be confirmed by sensory analysis, optimisation would appear wholly feasible, since the volatile extraction method used for GC requires no sample handling; the sample is handled in exactly the same way as the sample presented to the taster in an organoleptic study.

4. Conclusions

Some non-*Saccharomyces* wine yeasts posses β-glucosidase activity, evident not only on substrates with β-glycosidic bonds such as cellobiose, but also on natural glycosides from various grape varieties.

Purified extract of *Debaryomyces pseudopolymorphus* hydrolyses aroma precursors in Muscat wine, and requires less time than commercial enzyme preparations. Purified extract may also be lyophilised with similar results. Use of this extract instead of commercial enzyme preparations is currently being studied in wines from various grape varieties.

The results obtained here highlight the agreement between GC quantification of free terpenes and quantification of total aroma precursors.

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Aroma enhancement in wines using wine yeast β-glucosidase

M. Arévalo Villena , J.F. Úbeda Iranzo and A.I. Briones Pérez
Departamento de Química Analítica, Tecnología de Alimentos, Universidad de Castilla-La Mancha, Ciudad Real 13071, Spain.

Abstract

Due to the interest that exits on the liberation of aromas in young wines, and to the results obtained in previous studies, it was decided to study the effect of an enzymatic extract purified with β-glucosidase activity from *Debaryomyces pseudopolymorphus* on different wine varieties from La Mancha region (Muscat, Riesling and Airén). It was studied the effect at 6 and 12 days of contact quantifying both the precursors that were stayed and the liberated terpenes (GC). The results were compared with a control wine (without any extract) and with treated wine with a commercial enzyme preparation specially indicated to liberation of aromas. It was observed that the enzymatic extract from *Db. pseudopolymorphus* hydrolysed the precursors in wine and that it could compete with the commercial preparations since that liberation it was produced even in less time. It was observed that the terpenes found to each variety were different and so what not all the treatments to liberate the aroma should be similar between each other. On the other hand, It was done a sensory analysis of the different wines and were studied some collateral reactions (POF character). With regard to the POF character, it was found that none of the β-glucosidases decarboxylated p-cumaric and ferulic acids.

Keywords: Terpenes, aroma precursors, β-glucosidase activity, wine yeasts, enzymatic extracts

INTRODUCTION

Over the last thirty years, aroma precursors in grapes have been the object of considerable research (Di Stefano, 1982, Williams et al. 1982, Aryan et al. 1987, Gunata et al. 1988, Cordonnier et al. 1989, Sefton et al. 1993, Cordero et al. 2003, Hernández et al. 2002, Fernández et al. 2003, Arévalo et al. 2005 a, b, c, d). These odourless compounds can be modified

by various biological, biotechnological and physical-chemical factors to release odorous compounds.

Heteroside aroma precursors occur in grapes mainly in the form of β-D-glucopyranosides/glucosides, 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides (arabinosyl glucosides), 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides) and 6-O-β-L-apiofuranosyl-β-D-glucopyranosides (apiosyl glucosides), (Williams et al. 1982, Gunata et al. 1988, Voirin et al. 1992).

The aglycone moiety often consists of terpenols, primarily linalool, nerol and geraniol; linalool oxides, terpene diols and triol, along with other compounds such as cyclic or straight-chain alcohols (hexanol, hesenols), aromatic alcohols (phenylethanol, benzyl alcohol), C_{13} norisoprenoids and volatile phenols such as vanillin (Williams et al. 1981, Di Stefano 1982, Gunata et al. 1985). These compounds are bound to aglycone by a β-glucoside bond which is hydrolyzed by a β-glucosidase.

At present, aroma release is enhanced using commercial enzyme preparations of fungal origin, mainly *Aspergillus spp*. The composition of these preparations varies, and they are actually a mixture of non-specific glucanases. Tingle and Halvorson (1971) found that these cocktails hydrolyzed only 3% of precursors, but that this was sufficient to ensure an analytically- and organoleptically-detectable increase in free terpenes. At the same time, however, since glucanases are non-specific, they may trigger collateral reactions detrimental to the product, such as hydrolysis of anthocyans which are also stabilised by binding to sugar molecules (Riou et al. 1998).

For some years, therefore, research has focused on seeking β-glucoside enzymes from yeasts present in wine ecosystems which might enhance typical wine aromas (Pretorius 2000). Several β-glucosidases obtained from *Saccharomyces* (Darriet et al. 1988; Rosi et al. 1994; Mateo y Di Stefano, 1997;Hernández et al. 2002), and non-*Saccharomyces* yeasts have been studied with a view to future application in winemaking. There are some studies about genetic engineer (Adam y Polaina, 1991; Pérez Gonzalez et al. 1993; Adam et al. 1995; Skori and Freer, 1995; Rajoka et al. 1998; Muller et al. 1998; Van Rensburg et al. 1998; Van Rensburg et al. 2005). Other authors have studied some yeasts wich origin is not from wine (Gunata et al. 1990a; Sanchez Torres et al. 1998; Gueguen et al. 1994; Gueguen et al. 1995; Saha and Bothast, 1996) or non-*Saccharomyces* yeasts isolated from wine (Delcroix et al. 1994, Gunata et al.

1990b; Rosi et al. 1994; Mateo and Di Stefano, 1997; Yan and Lin, 1997; Riou et al. 1998, Fernández et al. 2000; Manzanares et al. 2000; Mendes Ferreira et al. 2001; Strauss et al. 2001; Hernández et al. 2002; Cordero et al. 2003, Arévalo et al. 2005a).

Other sources of these enzymes include certain bacteria such as *Oenococcus oeni* (Bartowsky et al. 2004; D'Incecco et al. 2004), and genetically-modified microorganisms (Becker et al. 2003).

Debaryomyces pseudopolymorphus has an extracellular β-glucosidase displaying good activity on synthetic substrates, and its biochemical characterisation has shown that it is stable and active over the pH range used in wine fermentation, as well as in the presence of glucose, ethanol and other metabolites that may be present in wine (Arévalo Villena et al. 2005 b).

Arevalo-Villena et al (2005c) studied the effects of various forms of yeast-derived enzyme extracts on natural terpenyl glycosides isolated from several grape varieties. The effect of these extracts on aroma precursors when added directly to Muscat wine was also assayed, together with the influence of dose and contact time. The results showed that volatile terpene concentrations were greatest in wines treated with *Debaryomyces pseudopolymorphus* enzyme extract.

In studies of this kind, however, results obtained by instrumental analysis need to be backed up by sensory analysis, in order to ascertain whether the terpene release detected by GC is also apparent to a panel of tasters. This paper reports on the effects of purified enzyme extract obtained from *Debaryomyces pseudopolymorphus* on three wines made at laboratory scale (Muscat, Riesling and Airén). The results were compared to the effects of a commercial enzyme preparation commonly used by winemakers. Terpene release was studied using gas chromatography, quantification of residual aroma precursors and sensory analysis.

Possible undesirable collateral reactions attributable to enzyme extracts, such as POF character, were checked for using the same times and dose-rates as for hydrolysis.

MATERIAL AND METHODS

Debaryomyces pseudopolymorphus enzyme extract

Purified enzyme extract, which had yielded the best results in previous studies (Arévalo Villena et al. 2005 d), was used for the present purpose. Yeast was grown in optimal conditions (Arévalo Villena et al. 2005 b);

supernatant was harvested after 42 hours' growth and partially purified by size exclusion (100 KDa) (Arévalo Villena et al. 2005 b). Extract, kept at 4 ºC until use, was added to wine samples in order to test their capacity to hydrolyze aroma precursors.

Microvinification

Three white grape varieties with different aroma-precursor content, harvested from Castilla-La Mancha cultivars, were used: Airén (the most widely-grown local variety), Riesling and Muscat, which has been successfully grown in this area for several years. They were harvested at optimum ripeness, met all health and hygiene requirements and also complied with European regulations on fungal treatments. The grapes were crushed and pressed in a pilot plant.

Microvinifications, by duplicated, were performed in a multiple-tube laboratory fermenter: 5 L of must with 50ppm of SO_2 were inoculated with 10^6 cells/ml of a commercial *Saccharomyces cerevisiae* strain (UCLM 325). Fermentation was carried out at 18 ºC until sugars were consumed. Thereafter, wines were decanted and fractionated for the next assay.

Hydrolysis of aroma precursors in wine by Debaryomyces pseudopolymorphus extract

Fractions of 2.5 L of each variety were added with:

- Yeast enzyme extract (from *Db. pseudopolymorphus*) at recommended dose (P)
- Commercial enzyme preparation widely employed in the La Mancha region of Spain (Lallenzyme beta, as positive control) (CEP).

As negative control were used samples with the same volume of each variety without any enzyme added (C).

In order to reproduce real wine-making conditions in terms of volume, airing and light exposure, the decanted wines were kept in the fermenter at room temperature for between 6 and 12 days (less time than in previous studies), after which bentonite (20 g/hl) was added to remove enzyme extract. Samples were then decanted and stored at 4 ºC until analysed (assays done for triplicate in all cases)

Quantification of non-hydrolysed aroma precursors

Terpenyl glycosides (in treated wines, and in positive and negative controls) were isolated and recovered following a protocol developed by Arévalo Villena et al. (2005 c) using reversed-phase C_{18} columns (Waters).

This method provides a rapid overall quantification of aroma precursors in musts, untreated wine or enzyme-treated wine, but does not differentiate between compounds, measuring only the content of aroma precursors susceptible to hydrolysis by β-glucosidases.

Quantification of released volatiles by gas chromatography

Released volatiles were quantified following the method reported by Salinas and Alonso (1997), based on a dynamic headspace using thermal desorption, gas chromatography and mass spectrometry (Arévalo Villena et al. 2005 d). The advantage of this method is that the volatile extraction method used for GC requires no sample handling; the sample is handled in exactly the same way as the sample presented to the taster in an organoleptic study.

Sensory analysis

To ascertain the correlation between analytical methods and sensory analysis, wines of all three varieties were evaluated in a standard tasting room [Spanish Standard 87004:1979 (AENOR, 1997)], using regulation glasses [Spanish Standard UNE 87022:1992 – ISO Standard 3591:1977 (AENOR, 1997)].

The panel consisted of 10 tasters familiar with the product, who had been provided with sufficient information on the wines to be tasted and on the treatments applied.

A triangular test was designed to detect differences between wines treated (after 12 days) and untreated wines. Tasting took place over three consecutive days (Muscat wines on day 1, Riesling on day 2 and Airén on day 3). Evaluations were carried out at daily sessions between 10:00 a.m. and 12:00 noon to prevent taster fatigue. Two daily sessions were held: untreated wine vs. yeast-extract treated wine in the first session, and yeast extract vs. commercial preparation in the second (Kobler, 1996)

POF character

The capacity of yeast extract to decarboxylate phenol compounds was determined using solutions of coumaric acid and ferulic acid (0.8 and 0.58 % respectively) in ethanol. 1 ml of each solution was added to a final volume of 50 ml containing the amount of yeast extract used in wines. Parallel tests were run with the commercial enzyme preparation and a negative control (solutions without extract).

Mixtures were kept at room temperature for 6 and 12 days. Decarboxylation of ferulic acid to 4 vinyl phenol was quantified by sensorial analysis, whilst decarboxylation of coumaric acid to 4 vinyl guayacol was measured spectrophotometrically. Coumaric acid absorbs at 290 nm, whilst its decarboxylation product peaks at 260 nm. (Briones et al. 1995)

Stadistical treatment

A one factor analysis of variance (statistical software package SPSS Version 11.0) was used to test for significant differences between:

- Treatment times of each enzyme preparation (CEP and P, marked with *).
- Treatment kind for each time (6 and 12 days marked with normal letters and letters with ‘ respectively)

It was applied in both quantification methods: non hydrolyzed aroma precursors and liberation of volatiles by chromatographic gas. Differences were deemed significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Quantification of non-hydrolysed aroma precursors

These were quantified at 6 and 12 days in wines treated with the yeast enzyme extracts (P) and with the commercial enzyme preparation (CEP). Precursor content was also measured in the initial must and in negative control wines of each variety.

Results, expressed as nmol glucose / ml must or wine, are shown in Table 1. The precursor content of control wines was lower than that of musts; this finding, reported in previous studies (Arévalo Villena, et al. 2005c), may be due to the residual hydrolytic activity of the *Saccharomyces* strain used in fermentation. Nevertheless, although precursors are hydrolysed during fermentation, enzyme treatment is advisable for releasing aromas, since part of the released terpenes may be volatilised and removed by the CO_2 produced during the fermentation process.

As shown in Table 1, the various musts used here displayed different precursor contents and thus different aroma potentials. Muscat was the most aromatic variety with 214.3 nmol glucose/ml, while Airén – a more neutral variety – had the lowest precursor content at 88.3 nmol glucose/ml (data reported in Arévalo Villena et al. 2005 c).

In treated Airén wines, the commercial preparation released the same amount of terpenes at 6 days as at 12 days (marked with * in Table 1)); this was not

Table 1. Aroma precursors (nmoles glucose / ml wine) at 6 and 12 days in must, control wine and wines treated with the enzyme extracts.

VARIETY	MUST	CONTROL	TREATED WINE			
			CEP		*P*	
			6	**12**	**6**	**12**
AIREN	88.3	72.5$^{a'}$	48.9	48.9$^{b'}$	59.9 *	26.8$^{*, c'}$
RIESLING	163.9	124.5$^{a, a'}$	115.1$^{*, a}$	97.7$^{*, b'}$	66.2$^{*, b}$	47.3$^{*, c'}$
MOSCATEL	214.3	146.6$^{a, a'}$	129.2$^{*, b}$	110.3$^{*, b'}$	78.8$^{*, c}$	60.0$^{*, c'}$

PYE: Purified yeast extract; CEP: commercial enzyme preparation.
(*)Significant differences ($p \leq 0.05$) between treatment times for the same enzyme preparation in each variety. Different letters indicate significant differences (95% confidence) between wines treated with different extracts for 6 (normal letters) and 12 days (letters with superscript) for each variety

the case for the purified yeast extract. Comparison of controls with treated wines by ANOVA revealed no significant differences at 6 days (marked with letters), while significant differences were recorded at 12 days between all samples; purified yeast extract hydrolyzed the largest number of precursors.

For Riesling samples, both enzyme treatments released more terpenes at 12 than at 6 days. At 6 days, there was no significant difference between controls and wines treated with the commercial enzyme preparation, although significant differences were found between the latter and wines treated with purified yeast extract. At 12 days, there were significant differences between all three samples, thus confirming that the commercial fungal preparation requires longer to achieve the desired release, a finding reported in previous studies (Arévalo Villena et al. 2005 d).

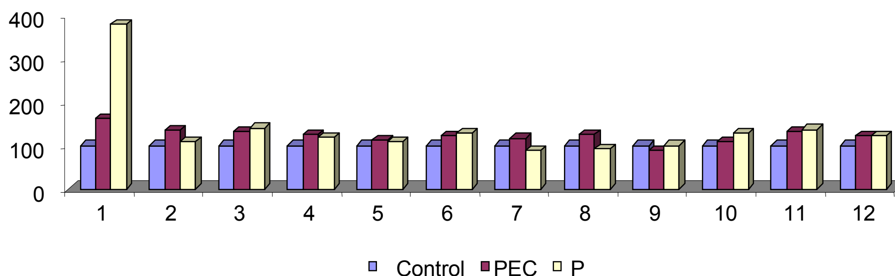
For Muscat, significant differences were observed between all samples as a function of both treatment and time.

Quantification of released volatiles by gas chromatography

These were quantified at 6 and 12 days in wines of each variety treated with yeast enzyme extract and with the commercial enzyme preparation. To facilitate the interpretation of results, a value of 100% is assigned to control wines for each compound measured.

For Airén wines (Figure 1) significant differences as a function of contact time were apparent only between wines treated with the commercial preparation and those treated with the purified yeast extract, suggesting that *Debaryomyces pseudopolimorphus* extract requires less time to achieve the desired effect. With regard to the different enzyme extracts, small fluctuations were noted at 6 days for

A.



B.

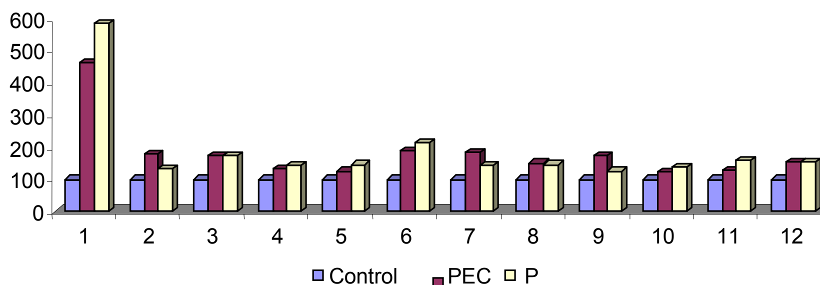
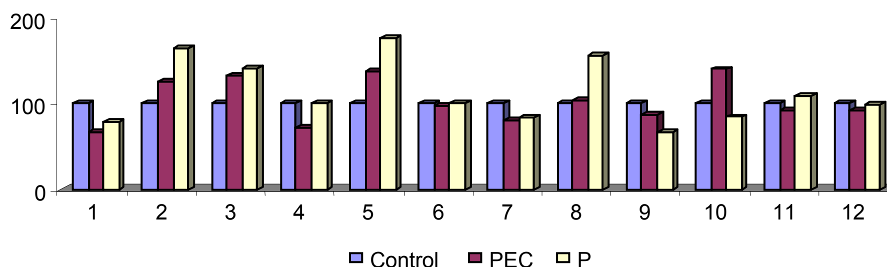


Figure 1. Release of volatile compounds (% hydrolysis with respect to controls) in Airén control wines (C) and in wines treated with yeast extract (P) and a commercial enzyme preparation (CEP),after 6 and 12 days' treatment (A and B, respectively)
1: limonene, 2: 1-hexanol, 3: 3-hexen-1-ol, 4: citronelol, 5: 2-phenylethanol, 6: ethyl acetate, 7: ethyl butyrate, 8: isoamyl acetate, 9: ethyl hexanoate, 10: hexyl acetate, 11: ethyl octanoate, 12: 2-phenylethyl acetate.

some compounds, although in most cases there were no significant differences, and wines were similar to controls. At 12 days, treated wines displayed greater volatile content. Both extracts (yeast and commercial) showed similar behaviour, although the yeast extract released more limonene, citronelol, 2-phenylethanol, ethyl acetate, hexyl acetate and ethyl octanoate (Figure 1B).

For Riesling samples (Figure 2) significant differences were found between treated wines as a function of contact time. At 6 days, the two enzyme treatments yielded similar volatile contents, although wines treated with yeast extract displayed higher values for 3-hexen-1-ol, linalol, citronelol, 2 phenyl ethanol and isoamyl acetate (Figure 2A). By contrast, at 12 days (Figure 2B), treatments displayed similar efficacy, although wines treated with the commercial enzyme preparation displayed higher values for citronelol, 2-phenyl ethanol, ethyl

A.



B.

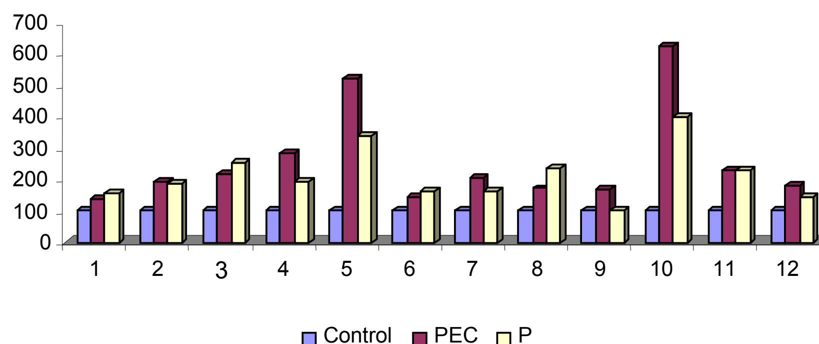
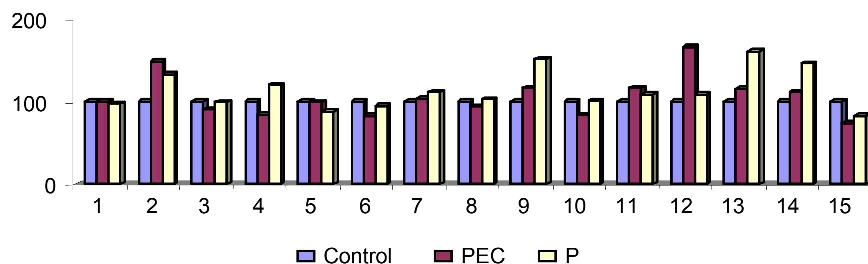


Figure 2. Release of volatile compounds (% hydrolysis with respect to controls) in Riesling control wines (C) and in wines treated with yeast extract (P) and a commercial enzyme preparation (CEP),after 6 and 12 days' treatment (A and B, respectively). 1: 1-hexanol, 2: 3-hexen-1-ol, 3: linalol, 4: citronelol, 5: 2-phenylethanol, 6: ethyl acetate, 7: ethyl butyrate, 8: isoamyl acetate, 9: ethyl hexanoate, 10: hexyl acetate, 11: ethyl octanoate, 12: 2-phenylethyl acetate.

butyrate, ethyl hexanoate, hexyl acetate and 2-phenylethyl acetate. This highlights again that the fungal preparation requires greater contact time and also that its effect is less controlled; there maybe excessive release, which was not seen in wines treated with the *Debaryomyces* extract.

For Muscat wines (Figure 3) differences were found for both enzyme treatments as a function of contact time (i.e. 6 vs. 12 days); these differences were more marked in wines treated with the commercial preparation. At 6 days (Figure 3A), volatile-compound content was higher in the two treated wines than in untreated controls, and the yeast extract released larger amonts of geraniol, linalol, alpha terpineol, and citronelol. At 12 days (Figure 3B), treated wines displayed higher ester content. By contrast, wines treated with

A.



B.

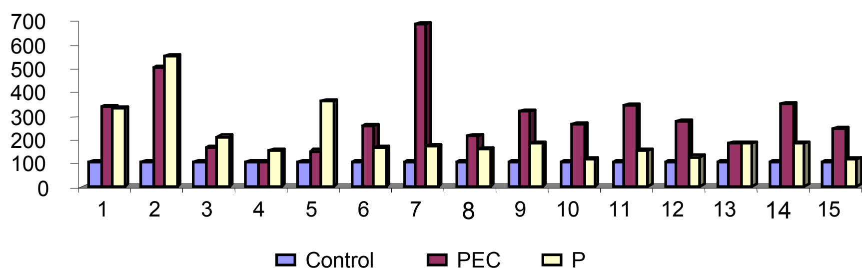


Figure 3. Release of volatile compounds (% hydrolysis with respect to controls) in Riesling control wines (C) and in wines treated with yeast extract (P) and a commercial enzyme preparation (CEP),after 6 and 12 days' treatment (A and B, respectively). 1: 1-hexanol, 2: 3-hexen-1-ol, 3: geraniol, 4: linalol, 5: ho-trienol, 6: alpha terpineol, 7: citronelol, 8:2-phenylethanol, 9: ethyl acetate, 10: ethyl butyrate, 11: isoamyl acetate, 12: ethyl hexanoate, 13: hexyl acetate, 14: ethyl octanoate, 15: 2-phenylethyl acetate.

the yeast extract exhibited greater volatile terpene content (geraniol, linalol, ho-trienol).

As expected, volatile-compound content was higher in the two treated wines than in untreated controls, even after only 6 days' contact in the case of yeast extract. Volatile compound concentrations increased over time, the increase being more marked in wines treated with the commercial preparation, particularly in the more aromatic varieties. This again highlights the indiscriminate release triggered by commercial preparations, which may prove detrimental to the product (Tingle and Halvorson, 1971); for example, wines of all varieties treated with the commercial product displayed higher ethyl butyrate values at 12 days.

These results suggest that the β-glucosidase activity of yeast enzyme extract is comparable to, and in many cases more controlled than, that of commercial fungal preparations. A further advantage of the yeast extract in

wine-making is that it shortens the enzyme treatment time required to obtain the same aroma release.

Different terpenes were obtained for each grape variety, terpene content being an intrinsic characteristic of the variety (Bayonove et al., 1993): limonene was found only in Airén samples, which displayed low content for other terpenes. By contrast, Riesling yielded some linalool, being Muscat the greatest variety of terpenes (linalol, geraniol, ho-trienol and alpha terpineol).

Comparison of the two methods used to evaluate the effectiveness of enzyme treatments (non-hydrolyzed precursors and release of volatile compounds) showed that both yielded similar results; although the data in the Figures and in Table 1 appear contradictory, it should be remembered that the methods quantify different kind of compounds: the precursors method only quantifies terpenil glycosides meanwhile that GC showed the terpenes and other volatile compounds (esters).

Sensory analysis

Sensory analysis was performed to confirm analytical results. Triangular tests were carried out as described earlier. For Muscat, tasters found significant (99.9%) differences between untreated controls and wines treated with yeast extract, and 95% differences between those treated with yeast extract and those treated with the commercial preparation.

For Riesling samples, 99% differences were reported for the wines in the two triangular tests (control vs. yeast extract and yeast extract vs. commercial enzyme preparation).

Tasters found it more difficult to distinguish between treatments in Airén samples, since they contain fewer aroma precursors. Nevertheless, a 90% difference was reported between untreated controls and wines treated with purified enzyme extract, while no significant difference was reported between the two enzyme treatments.

These results agree with those obtained by chemical analysis; the greater degree of terpene release observed in treated Muscat and Riesling wines was also detected by tasters.

POF character

Finally, we tested the ability of the yeast enzyme extract and the commercial preparation to decarboxylate phenolic compounds to form volatile phenols which might give rise to off-flavours.

It was found that none of the β-glucosidases decarboxylated p-coumaric and ferulic acid, and thus

neither 4 vinyl- guayacol nor 4 vinyl-phenol were formed.

CONCLUSIONS

Grape varieties have different aroma precursor content, giving rise to wines with different profiles. Nevertheless, precursors can be hydrolyzed via enzyme treatment to enhance wine aroma.

The good results obtained here confirm those obtained in previous studies, and show that *Debaryomyces pseudopolymorphus* yeast extract is suitable for the release of volatile terpenes in white grape varieties, and required less time than the commercial fungal preparation tested here, to produce a liberation more controlled, which not only is reflected on analytical methods since also in sensory analysis.

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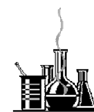
4. Resumen de resultados



En este capítulo se intenta dar una visión particular de cada uno de los artículos publicados que engloban todo el trabajo de investigación realizado.

_____ ARTÍCULO 1: **Caracterización de la actividad β-glucosidásica en levaduras vínicas no *Saccharomyces*.**

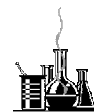
El objetivo del trabajo fue el estudio de la capacidad β-glucosidásica de levaduras aisladas en ambientes vínicos y su aplicación en la vinificación de un mosto de la variedad Chardonnay mediante el uso de cultivos mixtos.

La actividad enzimática fue cuantificada en el sobrenadante (actividad extracelular), en la célula completa (enzimas ubicadas en la pared celular y orientadas hacia el exterior) y en el extracto crudo (intracelulares y asociadas a la pared celular pero orientadas hacia el citosol). El análisis se basó en la liberación de glucosa a partir de la celobiosa. Las levaduras se crecieron por duplicado en medio YPD durante 48 horas a 30 ºC sin consideerarse la influencia del medio de cultivo ni las condiciones de crecimiento.

Sólo se detectó actividad extracelular en dos cepas, *Debaryomyces pseudopolymorphus* y *Candida oleophila*, aunque hubo más levaduras capaces de hidrolizar la celobiosa mediante enzimas asociados a la pared celular (extracto crudo), entre las que destacaron las dos anteriores junto con una cepa de *Debaryomyces polymorphus* y de *Brettanomyces spp*.

Se estudió, por triplicado, la influencia de algunos compuestos presentes en el vino (etanol, glucosa $SO_{2)}$ sobre la actividad β-glucosidásica de los extractos enzimáticos anteriores.

El etanol redujo la actividad de la enzima, siendo las levaduras menos sensibles *Brettanomyces spp*. y *D. pseudopolymorphus*. Este efecto posiblemente se debió al cambio conformacional de las estructuras de la proteína que se traduciría en



una variación de la polaridad del medio, afectando directamente al centro activo de la enzima.

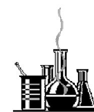
Las elevadas concentraciones de glucosa inhibieron notablemente la capacidad de *Brettanomyces spp*, sin embargo activaron las enzimas producidas por *D. pseudopolymorphus* y *C. oleophila*, no influyendo en aquellas procedentes de *D. polymorphus.*

Por último, se observó que el SO_2 no afectó a la actividad β-glucosidásica, excepto para el extracto de *D. polymorphus* que hidrolizó la celobiosa en mayor medida.

La elección de la cepa más adecuada para su uso como cultivo iniciador mixto junto con una cepa comercial de *Saccharomyces,* se hizo en función de la localización de la enzima y de los resultados anteriores. *D. pseudopymorphus* fue la mejor candidata, al excretar las enzimas al medio de crecimiento, no ser fuertemente inhibida por el etanol, potenciada por la glucosa e insensible al sulfuroso.

Como era de esperar, los mostos inoculados sólo con *Debaryomyces* levadura no fermentaron adecuadamente. Los vinos elaborados con los cultivos mixtos (*D. pseudopolymorphus* y *S. cerevisiae*) no presentaron diferencias significativas con respecto a los fermentados solo con *Saccharomyces* en cuanto al contenido en ácido acético, acidez total, pH o densidad. Los azúcares residuales aparecieron en concentraciones elevadas en ambos casos, lo que indicó que la cepa comercial de *Saccharomyces* escogida no pudo acabar la fermentación, aunque los vinos inoculados con los cultivos mixtos lograron un grado alcohólico ligeramente más alto.

Respecto al contenido en terpenos volátiles, se apreciaron diferencias en los vinos elaborados con los cultivos mixtos, que presentaron mayor concentración en citronelol, geraniol y nerol. Tanto el análisis químico de los vinos como la determinación de los terpenos volátiles se llevó a cabo por cuadriplicado.



Esta investigación se desarrolló durante la primera estancia predoctoral en el Institute for Wine Biotecnology.

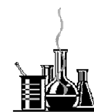
_____ **ARTÍCULO 2**: **Optimización de un método rápido para el estudio de la localización celular de la actividad β-glucosidásica en levaduras vínicas.**

Este estudio intentó dar respuesta a ciertos interrogantes surgidos en el trabajo anterior referentes al efecto del medio de cultivo y condiciones de crecimiento sobre la síntesis enzimática, la optimización de variables en la cuantificación de la actividad β-glucosidásica (hidrólisis de celobiosa) o la localización exacta de las enzimas en la célula.

Los objetivos planteados fueron:

- Influencia del medio de crecimiento en la síntesis enzimática (enzima inducida o constitutiva), tiempo de cosechado de las células y grado de aireación.
- Optimización del ensayo enzimático para cuantificar la actividad sobre celobiosa, estudiando la concentración del sustrato y el tiempo y temperatura de incubación.
- Localización de la enzima en la célula. Puesta a punto de un protocolo de fraccionamiento que permitiera el chequeo de la actividad enzimática en las distintas fracciones de la célula.
- Aplicación, del método optimizado, en la búsqueda de levaduras con activiadad β-glucosidásica aisladas en bodegas de Castilla la Mancha.

Las condiciones óptimas de la síntesis enzimática se evaluaron mediante ensayos por triplicado usando un control positivo y otro negativo (*Candida molischiana* y *Saccharomyces cerevisiae, 321* respectivamente). La producción de la β-glucosidasa resultó estar inducida por la fuente de carbono del medio de crecimiento (máxima síntesis cuando se usó únicamente celobiosa), fue mayor en condiciones de



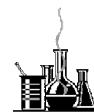
aerobiosis y tras un tiempo de crecimiento de 72 horas, a partir del cual comenzaba a disminuir la actividad.

En cuanto al ensayo enzimático de cuantificación sobre la celobiosa, se observó que la actividad aumentaba con la concentración de sustrato (inducción por saturación), no viéndose significativamente afectada por la temperatura ni el tiempo de incubación, por lo que estas variables quedaron fijadas a 30 ºC durante 30 minutos.

Puesto a punto el método, se aplicó para estudiar la actividad β-glucosidásica de 82 levaduras vínicas de Castilla la Mancha, previamente identificadas por técnicas moleculares (PCR-RFLP). Un primer screening donde se crecían las cepas sobre YNB celobiosa tras agotamiento previo de fuentes de carbono, permitió detectar cualitativamente aquellas que poseían la actividad buscada.

Todas a excepción de una, eran no *Saccharomyces* perteneciendo a las siguientes especies: *Debaryomyces hansenii* (1)*, Kluyveromyces thermotolerans* (2), *Mestchnikowia pulcherrima* (2), *Pichia anomala* (1), *Pichia membranaefaciens* (2), *Hanseniaspora uvarum* (4), *Hanseniaspora osmophila* (4), *Rodotorula mucilaginosa* (2) y *Torulaspora delbrueckii* (3). Para los siguientes estudios, además de éstas, se emplearon dos levaduras, elegidas al azar, con crecimiento débil en YNB-celobiosa, la cepa comercial de *Saccharomyces* (325) y el control positivo *C. molischiana*, llevando a cabo cada ensayo por triplicado.

Resultó de interés conocer la localización exacta de las enzimas, para lo que se puso a punto un protocolo de lisis celular que diferenciaba entre sobrenadante, célula completa, pared celular, citosol y membrana. La única cepa que mostró actividad extracelular de forma significativa fue *H. uvarum*. En célula completa los resultados eran más favorables, destacando sobre todo las dos *P. membranaefaciens* y *P. anomala* que también mostraron hidrólisis más elevadas que el control positivo en la pared celular, lo que indicaba que el género *Pichia* poseía actividad asociada a la



pared. En cuanto al citosol, todas excepto dos, presentaron valores superiores a *C. molischiana*, destacando principalmente *M. pulcherrima* y tres cepas de *Hanseniaspora*, dos *uvarum* y una *osmophila*. En la membrana se detectó actividad en la mayoría de las levaduras aunque en ningún caso los valores fueron tan elevados como en las fracciones anteriores.

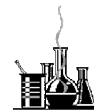
Aunque resultó difícil generalizar en qué parte de la célula se encontraba asociada la actividad β-glucosidásica, a la vista de estos resultados se dedujo que era difícil que las levaduras excretaran las enzimas al medio siendo por tanto, una actividad marcadamente intracelular.

Estos resultados en principio no eran favorables con vistas a su aplicación industrial. No obstante, en este punto del trabajo y con previsión a lo que debía durar la tesis doctoral se decidió trabajar con las levaduras no *Saccharomyces* que habían presentado las mejores aptitudes (*D. pseudopolymorphus* y *H. uvarum*), sin descartar futuras búsquedas de levaduras en el ecosistema viñedo-bodega castellano manchego con el propósito de encontrar alguna que presentara mayor actividad extracelular, estudio que se está desarrollando en la actualidad y en el que ya se han detectado posibles candidatas.

_____ ARTÍCULO 3: Caracterización de una β-glucosidasa exocelular de *Debaryomyces pseudopolymorphus*.

Una vez elegida la cepa más adecuada los objetivos de este trabajo, fueron:

- Optimización de las condiciones de crecimiento apropiadas para la cepa con el fin de conseguir la máxima síntesis (fuente de carbono y tiempo de cosechado).
- Purificación parcial de la enzima.
- Caracterización de la enzima: efecto de la temperatua y pH sobre la actividad β-glucosidásica, estabilidad térmica, propiedades cinéticas (velocidad máxima y



constante de Michaelis Menten), especificidad a nivel de sustrato y efecto de diferentes compuestos e iones metálicos sobre la actividad.

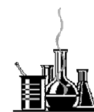
La cepa elegida fue *D. pseudopolymorphus*, ya que de las dos candidatas (*D. pseudopolymorphus* y *H. uvarum*), fue la que mayor actividad β-glucosidásica presentó. La optimización de las condiciones de crecimiento fue necesaria al haber observado que la actividad era cepa dependiente. Así, se confirmó que la síntesis era inducida, excretándose en mayor medida cuando se usaba celobiosa como única fuente de carbono, hecho que no afectó al crecimiento de la levadura. El tiempo de cosechado más favorable fue aproximadamente a las 40 horas de crecimento, una temperatura de 30 ºC y una agitación de 200 rpm.

En todos los ensayos los cultivos ses crecieron por duplicado y la actividad enzimática se cuantificó por cuadriplicado.

La purificación parcial de la enzima y su confirmación por Western-blot mostró que se trataba de un monómero cuyo peso molecular estaba en torno a 100 KDa.

El efecto de la temperatura y del pH se estudió empleando alícuotas parcialmente purificadas y concentradas (extractos obtenidos tras precipitación con acetona y ultrafiltración con membranas de 100 KDa). La actividad se cuantificó a 20, 30, 40, 50, 60 y 70 ºC manteniendo el pH a 4. La hidrólisis máxima de la celobiosa se obervó a los 40 ºC, cayendo la actividad hasta el 20 % a 50 ºC. El perfil de la actividad a temperaturas menores fue más favorable, conservándose un 60 y 80 % a 20 y 30 ºC respectivamente. En cuanto al pH, se estudió su influencia incubando a 40 ºC a pH de 3, 4, 5, 6 y 7. Esta variable influyó menos en la actividad, y aunque la máxima se obtuvo a 4, para el resto de valores de pH, la actividad no disminuyó más de un 20 %.

La estabilidad térmica, entendida como actividad enzimática a lo largo del tiempo bajo las condiciones óptimas de hidrólisis, se midió manteniendo los extractos β-glucosídicos a 40 ºC y pH 4 durante 6 horas, y tomando alícuotas para observar la



evolución de la hidrólisis a tiempo 0, 2, 3, 4, 5, y 6 horas. La curva de estabilidad mostró que la enzima era inestable a partir de las tres horas, ya que después de este tiempo la actividad disminuyó hasta un 30 %. Este hecho posiblemente justifique la caída observada después de 40 horas de crecimiento del cultivo.

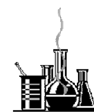
Las propiedades cinéticas de la enzima se calcularon usando celobiosa como sustrato de la hidrólisis que se realizó a 40 ºC, pH 4 y 30 minutos de incubación en todos los casos. La K_m obtenida fue de 11.9 mM siendo V_{max} 70.2 μmol.min^{-1}.mg protein^{-1}.

En cuanto a la especificidad a nivel de sustrato, se cuantificó la hidrólisis sobre celobiosa (control positivo al que se asignó un 100 % de hidrólisis), p-nitrofenil-β-D-glucósido, laminarina, maltosa, mandelonitrilo-β-D-glucósido, N-octil-β-D-glucósido, arbutina y lactosa. Se observó que la enzima tenía una amplia especificidad por diferentes tipos de sustratos presentando buena actividad sobre los mono-β-D-glucósidos.

Con respecto a la influencia de los cationes o compuestos de interés, aunque las evaluaciones fueron útiles para la caracterización enzimática, en ningún caso destacaron valores que pudieran incidir, por activación o inhibición, en la aplicación industrial de esta β-glucosidasa en procesos de vinificación.

_____ **ARTÍCULO 4**: **Método rápido para la cuantificación de precursores del aroma. Su aplicación en extractos de uva, mostos y vinos de diversas variedades.**

Una vez aislada la enzima, parcialmente purificada y caracterizada, era necesario conocer su comportamiento sobre el sustrato natural en el que se quería aplicar, es decir, los precursores del aroma de las distintas variedades de uva. Se



pensó en el empleo de un método rápido, reproducible y de fácil aplicación en el laboratorio y en bodega que permitiera conocer el potencial aromático de uvas, mostos o vinos.

Los objetivos del presente trabajo fueron:

- Mejora de un método para cuantificar los precursores del aroma (terpenos glicosilados) en distintas variedades de uva.
- Su aplicación, a escala de laboratorio, a muestras de uva, mostos y vinos de Castilla la Mancha tratados y sin tratar enzimáticamente.
- Su aplicación, a mayor escala, en un laboratorio de análisis sobre muestras reales de uvas vinos de otras regiones vitivinícolas.

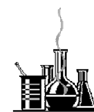
Se partió del método propuesto por Williams et al. (1995), basado en la proporción equimolar entre la aglicona y la glucosa que se da en los precursores del aroma. El proceso consistía en la retención de los glicósidos sobre cartuchos C_{18} en fase inversa, lavado con agua para eliminar las sustancias polares, recuperación de los precursores, hidrólisis ácida y cuantificación de la glucosa liberada. Para modificar aquellas etapas en las que se detectó algún tipo de interferencia, se utilizaron soluciones ideales de N-octil-β-D-glucósido adicionadas o no de otro tipo de compuestos dependiendo del parámetro a ensayar.

Las variables estudiadas fueron el grado de recuperación de las columnas, flujo de elución por cartuchos, número de usos de los cartuchos e interferencia con otros compuestos, realizando los ensayos por triplicado en todos los casos. El protocolo resultante fue:

Paso 1, preparación de la muestra.

a) Mostos y vinos: Se centrifugaron a 4500 rpm durante 5 minutos.

b) Vendimia estrujada: Las uvas se procesaron en un homogeneizador de paleta y 10 g de esta digestión se mantuvieron en contacto con 10 ml de etanol al 50 % durante 2 horas a temperatura ambiente con una

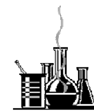


agitación de 200 rpm. El homogeneizado se centrifugó a 4500 rpm durante 5 minutos y 5 ml del producto limpio se aforaron a 25 ml para conseguir el grado alcohólico adecuado (siempre menor del 15 % v/v).

Paso 2, aislamiento de los glucósidos. Los cartuchos se activaron con 10 ml de metanol (calidad HPLC) seguidos de 10 ml de agua Milli-Q y se cargó un volumen apropiado de cada muestra: 15 ml de mosto y de vendimia estrujada y 20 ml de vino. Estos volúmenes difieren del protocolo original, ya que se consideraron más apropiados para la capacidad de retención de la columna, al existir más precursores en el mosto que en el vino debido a la hidrólisis colateral de los terpenil glucósidos durante la fermentación. El cartucho se lavó con agua, 3 x 15 ml en el caso de vendimia estrujada y vino y 3 x 20 ml en el de mosto. Los glicósidos se eluyeron con 1.5 ml de etanol (calidad HPLC) al 100 % seguidos de 3 ml de agua, enrasando a un volumen final de 5 ml. En todos los casos el flujo de paso por la columna fue de 2-3 ml/min aproximadamente.

Paso 3, hidrólisis ácida. Se realizó según el método propuesto sin existir en este caso ninguna modificación: a 0.5 ml de los eluídos anteriores se le añadieron 1 ml de H_2SO_4 2.25 M resultando las mezclas de reacción 1.5 M H_2SO_4 y 10 % v/v de etanol. De forma paralela se llevaron a cabo controles en los que el H_2SO_4 se sustituyó por 1 ml de agua con objeto de determinar la glucosa libre que no procedía de la hidrólisis de los precursores. En el blanco del reactivo el eluído se sustituyó por etanol al 30 %. Las muestras y el blanco del reactivo se hirvieron durante 1 hora permaneciendo los controles a temperatura ambiente.

Paso 4, análisis de D-Glucosa. La D-glucosa liberada en cada uno de los ensayos se cuantificó por el kit enzimático glucosa go (Sigma). A 262 µl de cada prueba se le adicionaron 260 µl de NaOH 3 M en el caso de las hidrólisis ácidas y de agua en los controles, 144 µl de una solución de glucosa 95 µg/ml permitió que todas



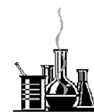
las muestras analizadas se encontraran en el rango de detección del kit enzimático. 200 µl de esta mezcla se añadieron a 400 µl del kit manteniéndose la mezcla a 37 ºC durante 30 minutos y parando la reacción con 400 µl de H_2SO_4 6 M según indicaciones del suministrador. A continuación se leyó la absorbancia a 540 nm, longitud de onda a la que absorbe la coloración rosada formada por la oxidación de la glucosa a ácido glucónico y posterior reacción con la o-dianisidina. Los valores se interpolaron en una curva de calibrado de glucosa.

A su vez se ajustaron los parámetros que se consideraban más importantes para la viabilidad y reproducibilidad del método: capacidad de recuperación de la columna, velocidad de flujo y número de usos de cada cartucho, así como la posible interferencia de otros compuestos con estructuras similares a los glicosil glicósidos.

El protocolo se aplicó para cuantificar los precursores del aroma en muestras de uva de las variedades Airén, Chardonnay, Chenín, Garnacha Tintorera, Moscatel, Rosanne, Saugvinon Blanc y Verdejo. Se observó que Moscatel era la variedad más aromática, mientras que Airén presentó el valor mínimo.

El método permitió analizar también los precursores de distintos mostos (Airén, Chardonnay, Gerwürztraminer, Macabeo, Moscatel, Saugvinon Blanc y Riesling) y de sus correspondientes vinos, obtenidos en el laboratorio por microvinificaciones con la cepa comercial UCLM 325. De nuevo se observó la gran variabilidad en la cantidad de precursores en función de la variedad de uva, y su disminución tras la fermentación. Esto, posiblemente se debía a la actividad glucanásica residual que poseen algunas cepas de *Saccharomyces cerevisiae*. No obstante, los vinos contenían todavía precursores susceptibles de hidrolizarse mediante la adición de enzimas exógenas para exaltar el aroma.

Tras estos resultados se decidió analizar nuevos mostos, y sus vinos no tratados y tratados con un preparado enzimático comercial, especialmente indicado



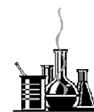
para la liberación de aromas. Para ello se utilizaron tres variedades: Airén, Macabeo y Moscatel. Los mostos y vinos de la variedad Airén fueron significativamente diferentes, aunque esto no aseguró una percepción sensorial al tratarse de una variedad neutra. En el caso de Macabeo, la hidrólisis de precursores del aroma fue significativamente mayor en el vino tratado enzimáticamente que en el no tratado, lo que indicó que el uso de enzimas era adecuado. Por último, en Moscatel, se detectaron diferencias significativas entre el mosto y los vinos.

En la última parte del estudio el método se aplicó en un laboratorio de análisis homologado, con el fin de comprobar si se podía usar de forma rutinaria y a mayor escala. Se usaron uvas de las variedades Moscatel, Macabeo (regadío), Airén (regadío y secano), Albariño, Chardonnay y Gerwürztraminer, procedentes de distintas fincas de Pontevedra, Málaga, Cuenca, Huesca y Burgos muestreadas durante los últimos 15 días previos a la vendimia.

El estudio permitió conocer la evolución de los precursores del aroma, parámetro de interés en el muestreo de uvas para determinar el momento óptimo de la cosecha. Se observaron diferencias en el contenido de los precursores en función del tipo de formación del cultivo: así, en la variedad Moscatel, se formaban más cuando se trataba de un cultivo en espaldera. Por otra parte, el desarrollo de los precursores fue distinto en plantíos de secano o de regadío: en la variedad Airén los terpenil glicósidos aparecieron antes en viñas regadas, pero en el momento de la vendimia, la concentración era más elevada en los cultivos de secano.

La cuantificación de los precursores para cada tipo de muestra se llevó a cabo por triplicado en todos los casos.

El estudio confirmó la diferencia de concentración de los terpenil glicósidos en las distintas variedades de uva, indicando que no todos los vinos requerían el mismo tratamiento enzimático para exaltar los aromas. A su vez sugirió que la adición de la



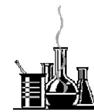
enzima β-glucosidasa debía hacerse sobre vinos una vez acabada la fermentación, momento en el que la concentración de terpenos glicosilados era aún elevada en las variedades ricas en este tipo de compuestos y ya no existía riesgo de pérdida por arrastre de CO_2.

_____ ARTÍCULO 5: Relación entre el extracto enzimático de *Debaryomyces pseudopolymorphus* y la liberación de terpenos en vino.

Los objetivos planteados fueron:

- Estudiar el comportamiento de las distintas formas de presentación del extracto enzimático de *Debaryomyces pseudopolymorphus* (enzima parcialmente purificado, P; inmovilizado, PI; liofilizado, PL y liofilizado inmovilizado, PLI) sobre terpenil glicósidos aislados de distintas variedades de uva mediante el protocolo anterior.
- Observar el efecto de estos extractos cuando se adicionaban directamente al vino y comparar los resultados con algunas preparaciones enzimáticas comerciales de origen fúngico empleadas en Enología.
- Calcular la dosis de extracto y el tiempo de contacto más adecuado para el tratamiento de los vinos.

Para obtener los distintos extractos enzimáticos, la levadura se creció en las condiciones óptimas de síntesis y se recuperó el sobrenadante del cultivo que fue parcialmente purificado y concentrado en función del tamaño de la enzima. Se usaron cartuchos de exclusión molecular con un tamaño de poro de 100 KDa durante 1 hora a 4500 rpm y 4 ºC, obteniendo el extracto purificado. Para conseguir el extracto purificado liofilizado se tomó una alícuota de la fracción anterior y se le adicionó un 10 % de leche descremada. La mezcla se liofilizó a -51.8 ºC con una presión de 2.6 e^{-2} mB en un liofilizador. Las fracciones inmovilizadas del extracto purificado y liofilizado

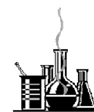


se obtuvieron usando alginato sódico al 5 % y cloruro cálcico 50 mM como agente gelificante.

Para comprobar si la actividad β-glucosidásica de los distintos extractos enzimáticos hidrolizaba los precursores del aroma, se extrajeron los terpenil glicósidos de mostos de las variedades Airén, Moscatel, Macabeo, Gerwürztraminer, Sauvignon blanc y Riesling, todos ellos procedente de viñedos de Castilla la Mancha.

Tres dosis del extracto purificado (mitad de dosis, dosis recomendad y dosis triple) se pusieron en contacto con los precursores del aroma, a 30 ºC durante 15 días, cuantificándose la glucosa liberada. Todas las variedades tuvieron un comportamiento similar, y a los 15 días los precursores se hidrolizaron al 100 %. Cuando se triplicó la cantidad de enzima la hidrólisis fue más lenta en alguna de las variedades más aromáticas; posiblemente debido a un efecto de inhibición por saturación. Esto es interesante desde el punto de vista tecnológico, ya que supone un ahorro económico al poder usar menos cantidad de enzima.

Una vez comprobado que el extracto enzimático de la levadura era capaz de hidrolizar los terpenil glicósidos en todas las variedades estudiadas, se decidió continuar los ensayos con la variedad Moscatel por ser una de las de mayor contenido en precursores. Se probaron todas las presentaciones enzimáticas obtenidas (P, PI, PL y PLI). Para intentar reproducir las condiciones de vinificación, las muestras se trataron a temperatura ambiente durante 20 días y se emplearon sólo dos dosis de enzima (la más elevada se eliminó debido a los resultados del estudio anterior). Se observó que el cambio de temperatura afectaba a la actividad enzimática, ya que en ningún caso se alcanzó el 100 % de hidrólisis, y al emplear la dosis recomendada se obtuvieron los mejores resultados. No obstante, esto no supone un problema para la aplicación a nivel industrial, ya que está demostrado que con sólo un 3 % de hidrólisis

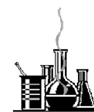


ya existen mejoras sensoriales por la liberación producida, debido al bajo umbral de precepción de este tipo de compuestos.

Por otra parte no todas las presentaciones del extracto enzimático hidrolizaron por igual los precursores del aroma, siendo el liófilo el que más actividad presentó, seguido del extracto purificado, posiblemente el más adecuado para el uso en la industria por su relación coste-eficacia. El extracto inmovilizado fue el que menos actividad β-glucosidásica mostró.

El siguiente paso fue comparar la eficacia de la enzima de la levadura con la de 8 preparados comerciales de origen fúngico. Los glicósidos extraídos de la variedad Moscatel se pusieron en contacto durante 20 días a temperatura ambiente con las dosis recomendadas en cada caso de los preparados comerciales. Todos hidrolizaron los precursores del aroma en mayor o menor medida, a pesar de que algunos no poseían actividad β-glucosidásica específica o al menos así constaba en la información comercial. Este hecho indicó que se trataba de mezclas no demasiado puras con algún efecto colateral que en ocasiones podían ir en detrimento del vino. Otros, cuyas referencias para su aplicación en la liberación de aromas eran muy favorables, tuvieron un comportamiento similar al extracto de levadura, y ello determinó el tratamiento directo de vinos con los extractos enzimáticos de *Debaryomyces pseudopolymorphus*.

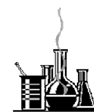
Para ello, las uvas de la variedad Moscatel se procesaron a escala piloto y las microvinificaciones del mosto se llevaron a cabo en el laboratorio en un fermentador de tubos múltiples. 5 L de mosto adicionados de 50 ppm de SO_2 se inocularon con una población de 10^6 células/ml de una cepa de *Saccharomyces cerevisiae* comercial (UCLM 325). Las fermentaciones, por duplicado, se llevaron a cabo a 18 ºC hasta agotamiento de azúcares.



Terminado el proceso, los vinos se decantaron y fraccionaron. A 200 ml de vino se le añadieron las dosis recomendadas por las casas comerciales de los extractos enzimáticos P, PI y PL, descartándose el PLI y el uso de otras dosis por los resultados obtenidos en los ensayos anteriores. Como control negativo se usó el vino sin adición de extracto enzimático y el control positivo fue el vino adicionado de un preparado enzimático comercial muy usado en la región de la Mancha (preparado E). Las muestras se mantuvieron a temperatura ambiente durante 9 y 16 días. Una vez finalizado el tiempo estimado a los vinos se les adicionó bentonita (20 g/ Hl) para eliminar los extractos enzimáticos, se decantaron y se mantuvieron a 4 ºC hasta su análisis.

El grado de hidrólisis se cuantificó empleando dos métodos: cantidad de precursores del aroma no hidrolizados, mediante el método de retención y recuperación de glicósidos en columnas $C_{18,}$ y cuantificación de volátiles liberados por cromatografía de gases y espectometría de masas, realizando en todos los casos los ensayos por triplicado.

De los resultados se desprende que los extractos enzimáticos de levadura poseen una adecuada actividad β-glucosidásica comparable, y en muchos casos mayor a la de los preparados fúngicos comerciales. De ellos el más adecuado fue el purificado ya que sus vinos fueron los que presentaron mayor contenido en terpenos volátiles (geraniol, linalol, hotrienol, α-terpineol, citronelol y nerol), en 2 fenil etanol y en algunos ésteres como el acetato de isoamilo, hexanoato de etilo, acetato de hexilo y de 2 fenil etilo. A éste le siguió el extracto liofilizado, siendo de nuevo la forma inmovilizada la que menos terpenos liberó, resultados acorde con los obtenidos en el apartado anterior sobre los precursores hidrolizados en el mosto. Respecto a los tiempos de tratamiento, a los 9 días de contacto los vinos tratados con el extracto purificado de levadura ya eran significativamente distintos al vino control, hecho



observado en los vinos que habían estado en contacto con el preparado comercial. Este resultado presentó una indudable ventaja porque permitiría acortar en bodega los tiempos de tratamiento enzimático obteniendo la misma liberación de aromas.

Aunque los resultados obtenidos debían ser corroborados por un análisis sensorial, se presumía una óptima concordancia con los métodos analíticos, ya que la extracción de volátiles realizada para la CG ofrece un resultado idéntico al que percibe un catador.

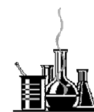
_____ ARTÍCULO 6: Mejora del aroma del vino mediante β-glucosidasas de levaduras vínicas.

Los objetivos del último trabajo fueron:

- Estudiar el efecto del extracto enzimático purificado de *Debaryomyces pseudopolymorphus* sobre vino de tres variedades de uva blanca (Airén, Riesling y Moscatel) y comparar los resultados con los obtenidos por un preparado enzimático comercial.
- Análisis sensorial de los vinos tratados y sin tratar.
- Determinación de reacciones colaterales no deseadas: formación de 4.vinil fenol y 4 vinil guayacol.

En este caso el extracto que se empleó fue el purificado de *D. pseudopolymorphus*, por ser el más adecuado para su empleo en la industria y por los buenos resultados mostrados. También se empleó el preparado enzimático comercial E.

Las vinificaciones se llevaron a cabo igual que en el trabajo anterior, fermentando por duplicado tres variedades distintas: Airén, Riesling y Moscatel. Para reproducir las condiciones reales que se dan en bodega en cuanto a volumen, aireación y exposición a la luz, las muestras de vino permanecieron en el fermentador



a temperatura ambiente. Los extractos enzimáticos de la levadura y del moho se añadieron al vino, siendo una fracción sin tratar el control negativo.

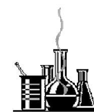
El estudio de la liberación de volátiles se hizo usando los mismos métodos que en el trabajo anterior: cuantificación de los glicósidos no liberados y de los volátiles presentes en las muestras, a los 6 y 12 días de tratamiento (tiempos menores debido a los resultados previos obtenidos).

Como era de esperar, los vinos tratados presentaron mayor contenido en volátiles que el vino sin tratar, incluso con solo 6 días de contacto con el extracto de levadura. Su concentración aumentó con el tiempo, siendo más acusado el incremento para los vinos tratados con el preparado enzimático comercial, a los 12 días, sobre todo en las variedades más aromáticas. Esto volvió a reflejar la liberación, a veces indiscriminada, que provocan este tipo de preparados comerciales que en muchos casos va en detrimento del producto, así por ejemplo los vinos de todas las variedades tratados con el preparado comercial presentaron a los 12 días valores más elevados de butirato de etilo.

De los resultados se desprende que los extractos enzimáticos de levadura poseían una adecuada actividad β-glucosidásica comparable, y en muchos casos más adecuada que los preparados fúngicos comerciales, aportando además una indudable ventaja: acortar en bodega los tiempos de tratamiento enzimático.

Se observó cómo los terpenos encontrados eran distintos para cada variedad de uva. Así, el limoneno solo apareció en la variedad Airén que resultó pobre en el resto de terpenos. Por el contrario, en Riesling apareció el linalol, siendo la variedad Moscatel la que ofreció mayor diversidad de terpenos (linalol, geraniol, ho-trienol y alfa terpineol).

Resulta interesante puntualizar que al comparar los dos métodos usados en el estudio para evaluar la eficacia de los tratamientos enzimáticos (precursores no



hidrolizados y liberación de volátiles), a priori parecían contradictorios, pero hay que tener en cuenta que no cuantificaban lo mismo, y que sólo se podían comparar cuando nos referíamos a terpenos volátiles correlacionándolos con los precursores del aroma.

Con el fin de corroborar los resultados analíticos se realizó un análisis sensorial, prueba determinante en este tipo de estudios. Se llevaron a cabo pruebas triangulares, en las que los catadores encontraron o no diferencias significativas dependiendo de la muestra problema. Los resultados obtenidos fueron acordes con los hallados en el análisis químico, reflejando una mayor liberación de terpenos en los vinos tratados de Moscatel y Riesling (intervalos de confianza mayores), que en la variedad Airén, donde no hubo diferencias entre ambos tratamientos enzimáticos pero sí, aunque solo al 90 % de confianza, entre el control y los vinos tratados con el extracto de *Debaryomyces*.

Por último y, considerando la capacidad de formación de compuestos conocidos como off-flavours que poseen algunos preparados enzimáticos usados para la liberación de aromas, se estudió la aparición de 4-vinil fenol y 4-vinil guaiacol en muestras tratadas con el extracto purificado de levadura y con el preparado fúngico empleado en el estudio. Se observó que ninguna de las β-glucosidasas descarboxilaron los ácidos p-cumárico y ferúlico y por tanto no se detectaron los correspondientes fenoles volátiles. Esto coincide con la teoría de que para la formación de este tipo de compuestos se requiere la acción combinada de las enzimas de los cócteles enzimáticos exógenos y de las levaduras fermentativas que en este ensayo no estaban presentes.

5. Conclusiones generales

1. Las levaduras aisladas de fermentaciones vínicas espontáneas de mosto blanco, identificadas mediante PCR-RFLP, pertenecen a los géneros *Saccharomyces, Debaryomyces, Kluyveromyces, Candida, Metchnikowia, Pichia, Toluraspora, Rhodotorula* y *Hanseniaspora*.
2. Sólo las levaduras no *Saccharomyces* poseen actividad β-glucosidásica específica sobre celobiosa, siendo su síntesis enzimática característica de cada cepa. Este hecho no permite generalizar en cuanto al género o especie, localización en la célula, fuente de carbono o condiciones óptimas de crecimiento para la síntesis enzimática.
3. No obstante la actividad es marcadamente intracelular, aunque *Hanseniaspora uvarum* y *Debaryomyces pseudopolymorphus* son capaces de excretar la enzima al medio de crecimiento, siendo la última cepa la más apta para su uso en vinificación.
4. *Debaryomyces pseudopolymorphus* usada junto con *Saccharomyces cerevisiae* como cultivo iniciador mixto en la fermentación de un mosto Chardonnay, no modifica significativamente los parámetros convencionales del vino. En cuanto a la liberación de terpenos, no existe aumento significativo en la concentración de linalol o α-terpineol, pero sí en la de nerol, citronelol y geraniol.
5. Las condiciones óptimas de crecimiento para que la cepa de *Debaryomyces pseudopolymorphus* sintetice la enzima y exprese la máxima actividad β-glucosidásica son YP- celobiosa como medio de crecimiento, incubando a 30 ºC durante 40-45 horas con una agitación de 200 rpm.
6. Las enzimas del sobrenadante parcialmente purificadas y caracterizadas bioquímicamente, son proteínas monoméricas con un tamaño en torno a 100

KDa, pH y temperaturas óptimas de 4 y 40 ºC respectivamente, sensibles al calor, pero que conservan el 60 y el 80 % de la actividad a los 20 y 30 ºC respectivamente. Sus parámetros cinéticos para la celobiosa son V_{max} 70.2 μmol.min^{-1}.mg proteína^{-1} y Km 11.9 mM. En cuanto a la especificidad a nivel de sustrato, la enzima tiene una buena actividad sobre los mono-β-D-glucósidos. Los cationes o compuestos químicos presentes en el vino no potencian ni inhiben su actividad significativamente.

7. El protocolo de extracción y cuantificación de los precursores del aroma en mostos y vinos, ha sido útil para chequear de forma rápida el contenido en glicosil-glicósidos de los distintos sustratos, factor a considerar en la elección del tratamiento enzimático requerido. Por otra parte resulta útil para conocer la actividad β-glucosídásica de los distintos preparados enzimáticos.
8. Las enzimas β-glucosidasas de *Debaryomyces pseudopolymorphus* son capaces de hidrolizar los precursores del aroma de distintas variedades de uva por la adición directa de los extractos parcialmente purificados, liofilizados o inmovilizados. No obstante, aunque el tratamiento más eficaz es el de la enzima parcialmente purificada, la liofilización podría ser una forma alternativa de su uso.
9. El extracto purificado de *Debaryomyces pseudopolymorphus* contribuye a la liberación de terpenos en la variedad Moscatel, Riesling y en menor medida en Airén, incluso en un menor tiempo que los preparados enzimáticos comerciales. Este resultado presenta una indudable ventaja ya que permitiría acortar en bodega los tiempos de tratamiento enzimático obteniendo la misma liberación de aromas.

10. El Análisis Sensorial pone de manifiesto que los vinos tratados con enzimas son distintos al control y los catadores son capaces de diferenciar entre los distintos tratamientos.

11. Por tanto, esta levadura propia del ecosistema vínico posee una adecuada actividad β-glucosidásica, comparable a la de los preparados enzimáticos comerciales de origen fúngico y que resulta menos agresiva. Ello hace que las compañías transnacionales del sector vitivinícola puedan emplear el extracto enzimático de *Debaryomyces pseudopolymorphus* como alternativa a los cócteles actuales, cubriendo las necesidades impuestas por las nuevas tendencias dirigidas hacia vinos genuinos.

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