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NOVEL LIQUID STARTER CULTURES FOR MALOLACTIC FERMENTATION IN WINE

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Valencia, Febrero de 2014

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The purpose of this thesis:

The malolactic fermentation is essential for the quality of wines, especially in red wines. This fermentation can be a powerful tool in the winemaking process, not only to reduce its acidity, but also because it influences on the aroma and on the sensory profile of the wine. This fermentation is carried out by lactic acid bacteria, but it is not always accomplished at the right time and under optimal conditions, since many factors affect the lactic acid bacteria growth and metabolism.

One strategy used to control the fermentation consists on its induction by the inoculation of selected lactic acid bacteria, mainly with *Oenococcus oeni* strains. Nowadays, many commercial starter cultures of lactic acid bacteria have difficulties to perform the malolactic fermentation and to be implanted in some wines, due to the different processing conditions and to the intrinsic characteristics of these wines. Furthermore, the conditions are getting worse for the growth and metabolism of these bacteria because of the climate change: the increase of the alcoholic content and the reduction of the nutrient content of the wines make life quite difficult for these microorganisms. For this reason, wineries are claiming for new malolactic fermentation starters with an easier use and more effectively tailored to potentiate the differential characteristics of their own wine, so more information about malolactic fermentation and *O. oeni* and how to improve starter cultures are necessary.

This work is the result of an agreement between the University of Valencia and Agrovin S.A. which was formalized by a V Segles Universitat-Empresa fellowship program. In this project, the viability of liquid cultures of *O. oeni* inoculated directly to wine has been evaluated and we have improved the understanding of malolactic fermentation with the practical techniques of Agrovin S.A. and the University research experience. Moreover, the wineries' needs have been translated to the University, where the design of liquid starter cultures and useful methods for the selection and characterization of *O. oeni* strains have been developed.

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Abbreviations

ACE	Acetic acid bacterium medium
AF	Alcoholic Fermentation
ANOVA	Analysis Of Variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATP	Adenosine Tri-Phosphate
AU	Arbitrary Units
BA	Biogenic Amine
bp	Base pair
CECT	Spanish Type Culture Collection
cfu/mL	Colony-forming units per millilitre
CO_2	Carbon dioxide
DCM	Delignified Cellulosic Material
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
DSM	German Collection of Microorganisms and Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden–Meyerhof–Parnas
GPYA	Glucose Peptone Yeast Extract Agar
HDC	Histidine Decarboxylase
HIM	Histamine
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
LAB	Lactic Acetic Bacteria
Lb.	*Lactobacillus*
Lc.	*Leuconostoc*
MDB	Medium for Detection of Biogenic amines
MLE	Malolactic Enzyme
MLF	Malolactic Fermentation
MLO	Medium for Leuconostoc Oenos
MRS	de Man, Ragosa and Sharpe Agar
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
O.	*Oenococcus*
O_2	Oxygen
OD	Optical Density
OMP	*Oenococcus* Medium Production
P.	*Pediococcus*
PCR	Polymerase Chain Reaction
PVA	Polyvinyl Alcohol
RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomic Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomic Ribonucleic Acid
S.	*Saccharomyces*
SO_2	Sulfur dioxide
TAE	Tris-acetate-EDTA
Tris	Tris hidroximetil aminomethane
UPGMA	Unweighted Pair Group Method with Arithmetic mean
v	Volts
v:v	Relation volume-volume
W.	*Weissella*
w:v	Relation weight-volume

Resumen

Introducción

La fermentación maloláctica es fundamental para la calidad del vino, especialmente en el vino tinto. Se trata de la transformación del ácido L-málico procedente de las uvas, de un gusto fuerte y áspero, en ácido L-láctico, de gusto más agradable, liberándose además CO_2. Esta fermentación puede ser una poderosa herramienta en la elaboración del vino, no solo por reducir su acidez, sino también por influir positivamente en el aroma y perfil sensorial del vino. Esta fermentación es llevada a cabo por bacterias lácticas, pero no siempre se realiza en el momento oportuno ni en las condiciones óptimas, ya que son muchos los factores que influyen en su crecimiento y desarrollo. Una de las estrategias utilizadas para controlar la fermentación de forma adecuada, consiste en la inducción de ésta con la inoculación de bacterias lácticas seleccionadas, principalmente con cepas de *Oenococcus oeni*.

En la actualidad, muchos de los cultivos iniciadores malolácticos de bacterias lácticas disponibles en el mercado presentan problemas de implantación debido a las diferentes condiciones de elaboración y características intrínsecas de los vinos. Las bodegas reclaman nuevos cultivos iniciadores más fáciles de usar y con una mayor eficacia. Y además, con mayor frecuencia, cultivos obtenidos a partir de cepas propias y diferenciales de la bodega. La necesidad de obtener más información sobre la fermentación maloláctica, sobre *O. oeni* y sobre cómo mejorar los cultivos iniciadores, llevó a desarrollar esta investigación, resultado de un acuerdo entre la Universidad de Valencia y Agrovin S.A. En esta tesis doctoral se ha evaluado la posibilidad de emplear cultivos iniciadores líquidos de *O. oeni* inoculados directamente en vino y se han ampliado los conocimientos sobre la fermentación maloláctica y *O. oeni*.

Objetivos

El objetivo general de esta tesis doctoral ha sido seleccionar cepas de *O. oeni* y evaluar la efectividad del empleo de cultivos iniciadores líquidos en la inducción de la fermentación maloláctica. Para llevar a cabo este objetivo general, se han planteado los siguientes objetivos específicos:

Seleccionar una cepa de *O. oeni* de la colección Enolab, con el fin de elegir aquella capaz de resistir y crecer en un amplio rango de valores de pH, en elevados niveles de etanol, e incapaz de formar aminas biógenas.

Diseñar un medio de cultivo líquido favorable para el crecimiento de *O. oeni* que permita la producción de altos niveles de biomasa, y al mismo tiempo una adaptación a las condiciones del vino.

Llevar a cabo el escalado del proceso de producción para la obtención de niveles industriales de biomasa de *O. oeni* utilizando el medio de cultivo diseñado.

Reducir el contenido de histamina presente en los vinos de una bodega mediante la selección e inoculación de una cepa autóctona de *O. oeni* cultivada en el medio de cultivo líquido diseñado previamente.

Optimizar los procesos de conservación e inoculación de cultivos iniciadores, con el fin de mantener una adecuada actividad maloláctica y viabilidad celular en vino.

Desarrollar métodos alternativos de inoculación de cultivos iniciadores malolácticos en vino con el fin de mejorar la adaptación a las condiciones del vino, coinmovilizando *O. oeni* con *Saccharomyces cerevisiae*.

Metodología y resultados

Selección de una cepa de *O. oeni* como cultivo iniciador maloláctico

Se realizó un proceso de selección entre 40 cepas de *O. oeni* pertenecientes a la colección Enolab. Este proceso se llevó a cabo siguiendo diversos criterios para elegir las mejores desde el punto de vista de actividad maloláctica, como son, capacidad para crecer y desarrollar la fermentación maloláctica en un amplio rango de pHs, alto grado alcohólico y ausencia de capacidad de sintetizar aminas biógenas. Las cepas de *O. oeni* se cultivaron en MLO con 3 valores de pHs diferentes (3,2, 3,5 y 3,8). Las 12 cepas de *O. oeni* con mayor crecimiento en los 3

pHs fueron cultivadas en MLO con concentraciones de 9 % y 11 % de etanol (v:v). Los resultados obtenidos mostraron que todas las cepas analizadas fueron capaces de desarrollarse con 9 % de etanol (v:v). Sin embargo, la adición de una concentración mayor de etanol (11 % v:v), mostró diferencias en el comportamiento entre las distintas cepas de *O. oeni*. Las cepas que presentaron un mayor crecimiento poblacional con un nivel de etanol del 11 % (v:v) fueron E4061, E5003 y E3874. Se analizó la actividad maloláctica de estas 3 cepas a diferentes niveles de pH, mediante un sistema de microplaca y azul de bromofenol. La cepa E5003 mostró los mejores resultados, consumiendo el ácido málico en los niveles de pH más bajos.

Con el objetivo de estudiar la formación de aminas biógenas por parte de las 3 cepas seleccionadas, se llevó a cabo el análisis mediante cromatografía líquida de alta resolución. Para ello, se inoculó el medio de cultivo MDB-mod con $2x10^7$ ufc/mL de estas cepas por separado. Tras 15 días, se analizó el contenido en histamina, tiramina, cadaverina y putrescina. Ninguna de las cepas seleccionadas fue capaz de sintetizar bajo estas condiciones las aminas analizadas.

Tras inocular una concentración final de $1x10^6$ ufc/mL de las 3 cepas seleccionadas en vino tinto por separado, se observó que la cepa E5003 mantenía la mejor viabilidad celular y consumía el ácido málico más rápidamente, en 15 días, por lo que fue elegida como cultivo iniciador maloláctico.

Diseño de un medio de cultivo líquido para la producción del cultivo iniciador maloláctico

Tras la selección de la cepa de *O. oeni*, se diseñó un medio de cultivo adecuado para el cultivo de las bacterias y producción de biomasa. Los medios de laboratorio suelen producir unas cantidades elevadas de biomasa, pero con una baja o nula capacidad de adaptación de estas células al vino. Con el fin de mejorar estas limitaciones, se diseñó un medio de cultivo que preadaptase las bacterias a un medio hostil como es el vino, pero teniendo en cuenta las condiciones óptimas de crecimiento de la bacteria para conseguir los máximos niveles de biomasa y con una óptima actividad metabólica y viabilidad tras su inoculación en vino. Por ello, se analizaron 27 medios de cultivo, con diferentes pHs, niveles de etanol y concentración de

azúcares. El medio de cultivo optimizado (OMP) era el que contenía mosto blanco comercial diluido 1/6, 4 % (v:v) de etanol y pH 3,8.

Una vez seleccionada la cepa de *O. oeni* y diseñado un medio de cultivo apropiado, se realizó el escalado del proceso para la obtención de biomasa suficiente para probar su viabilidad como cultivo iniciador maloláctico en grandes volúmenes de vino. Se efectuó un escalado partiendo de un cultivo de 50 mL con una concentración celular de $1x10^9$ ufc/mL. Este cultivo se inoculó en 500 mL de medio OMP y se incubó hasta llegar a una población final de $1x10^9$ ufc/mL. Una vez conseguida esta biomasa, se inoculó en 8 L de dicho medio de cultivo, obteniéndose el cultivo iniciador líquido para ser inoculado en vino. Durante todo el proceso de escalado, se llevaron a cabo controles de contaminación de los medios de cultivo y cultivos obtenidos. Los resultados revelaron una correcta esterilidad del medio de cultivo y un buen desarrollo de la población de las bacterias producidas. La biomasa conseguida tras el proceso de producción fue elevada, obteniéndose una población de la cepa seleccionada de *O. oeni* de $1,3x10^9$ ufc/mL en 6 días en el fermentador de 8 L.

Con el fin de estudiar la actividad maloláctica del cultivo iniciador, se procedió a la inoculación de vino tinto. Se inoculó el vino con el cultivo procedente del fermentador de 8 L en proporciones de 1/10 y 1/100 y 1/1000 y se midieron las concentraciones de ácido málico y láctico a lo largo del tiempo mediante cromatografía líquida. En todos los casos, se consumió todo el ácido málico presente en el vino tras 6 días de incubación. Se realizó el último paso de escalado del proceso a nivel industrial en Agrovin S.A. Allí, se elaboraron 80 L de biomasa bacteriana para su utilización como cultivo iniciador maloláctico líquido, obteniéndose $1x10^9$ ufc/mL en 6 días en este último paso. Tras el éxito de estos ensayos, la cepa E5003 cultivada en OMP se comercializa como cultivo iniciador maloláctico líquido con excelentes resultados en bodega. La aplicación de este cultivo iniciador maloláctico es mucho más fácil que la de los habituales cultivos iniciadores liofilizados ya que no necesita rehidratación, ni aclimatación y las células están activas, realizando la fermentación maloláctica rápidamente.

Diseño de un cultivo iniciador maloláctico para una bodega

Frente a la problemática planteada por una bodega de la Denominación de Origen Ribera del Duero, con elevados niveles de histamina en sus vinos, se planteó diseñar una estrategia para disminuir dicha concentración y obtener productos más saludables y con menos barreras para su comercialización y venta. Se buscaron los microorganismos responsables de la síntesis de histamina en los vinos y se seleccionó una cepa autóctona de *O. oeni* no productora de histamina para su utilización como cultivo iniciador maloláctico propio. Para el aislamiento de la microbiota autóctona, se tomaron muestras de 13 depósitos, antes y después de la fermentación maloláctica, y se sembraron en medio MLO y MRS sólido. Se identificaron 8 perfiles de bandas tras la tipificación mediante la técnica molecular de RAPD-PCR, que correspondieron a 8 cepas diferentes de *O. oeni*. De las 8 cepas, 3 de ellas fueron capaces de producir histamina en medio MDB-mod. De las cepas no productoras, se seleccionó la cepa de *O. oeni* V6B1 para constituir cultivo iniciador maloláctico propio para la bodega, ya que además de no producir histamina en MDB-mod estaba presente en los depósitos con el mínimo contenido de esta amina.

En la siguiente vendimia, se realizó la producción de biomasa de la cepa seleccionada (V6B1) en el medio OMP siguiendo el escalado descrito anteriormente. Se produjeron 24 L de cultivo iniciador maloláctico para la inoculación un depósito de 200 HL en la bodega. La cinética de crecimiento fue similar a la previamente descrita para la cepa E5003, alcanzándose una concentración de $1x10^9$ ufc/mL.

Tras la inoculación del depósito, se analizó la viabilidad del cultivo iniciador, la concentración de histamina y la degradación de ácido málico durante el proceso de vinificación, comparándose con un depósito no inoculado en la misma bodega. En ambos depósitos se llevó a cabo la fermentación maloláctica, pero en el depósito inoculado acabó antes. En ambos casos, durante la fermentación maloláctica, los niveles poblacionales de *O. oeni* llegaron a $1x10^7$ ufc/mL. Tras la fermentación descendieron, pero aun así las poblaciones superaban $1x10^5$ ufc/mL al transferirse el vino a las barricas. En cuanto a la concentración de histamina, antes de la fermentación maloláctica no se detectó en ninguno de los 2 depósitos. Tras la fermentación, se detectó 5 veces menos histamina en el depósito inoculado. Tras un año de envejecimiento en barrica los niveles de histamina aumentaron en ambos depósitos pero encontramos 3 veces

menos histamina en el depósito inoculado que en el no inoculado. Estos resultados mostraron el éxito de la utilización de cultivos autóctonos malolácticos líquidos para la disminución del contenido de histamina. Esta metodología innovadora permite la disminución de aminas biógenas en el vino final, manteniendo la microbiota propia y por tanto conservando la personalidad del vino.

Conservación de *O. oeni*

Conservación del cultivo iniciador maloláctico líquido

Tras la selección de la cepa de *O. oeni* E5003 y el diseño del medio de cultivo OMP para el cultivo iniciador maloláctico, se estudiaron diferentes métodos para su conservación. Se comparó la conservación a temperatura de 4 ºC y a -20 ºC, con la liofilización. Tras la aplicación de estas metodologías se analizó la viabilidad de los cultivos a lo largo del tiempo. Mediante la liofilización se observó una gran pérdida de viabilidad inmediata debido al propio proceso de liofilización, pero posteriormente, la viabilidad se estabilizó y se mantuvo en el tiempo. Este método de conservación por tanto, sería el adecuado para la conservación del cultivo durante un largo periodo de tiempo.

La congelación a -20 ºC fue la metodología más adecuada para el mantenimiento del cultivo líquido durante 30 días, ya que durante este tiempo presentó los resultados más altos de viabilidad celular, manteniéndose en $7{,}19 \times 10^8$ ufc/mL. La conservación mediante refrigeración a 4 ºC resultó la mejor opción para tiempos de conservación de entre 2 y 4 meses ya que presentó la mejor viabilidad, manteniéndose en $2{,}56 \times 10^7$ ufc/mL tras 124 días.

Conservación de *O. oeni* en lías

Con la intención de evaluar su idoneidad como alternativa a los cultivos líquidos refrigerados, se estudió la conservación de cultivos de *O. oeni* en lías procedentes de vino tinto. Este sistema permitiría adaptar las bacterias a las condiciones del vino a la vez que conservarlas durante un largo periodo de tiempo. Con estos objetivos se inoculó la cepa E5003 de *O. oeni* en tres lías diferentes procedentes de la vinificación de Tempranillo. Se analizó la viabilidad de las

bacterias mediante este método de conservación y la actividad maloláctica de las bacterias tras su inoculación en vino. Los resultados mostraron que la viabilidad de *O. oeni* a lo largo del tiempo dependía del tipo de lías, obteniéndose una mejor respuesta en las lías con alto contenido en polisacáridos.

Coinmovilización de *S. cerevisiae* y *O. oeni* en material celulósico y almidón

La inmovilización de cultivos iniciadores proporciona numerosas ventajas en comparación con la utilización de células libres, ya que el soporte de inmovilización actúa como un agente de protección contra los efectos fisicoquímicos de vino. La inoculación de una elevada concentración de bacterias que permite estos sistemas, proporciona tiempos de fermentación más cortos, la eliminación de la fase de crecimiento y procesos de fermentación en continuo. Del mismo modo, permite una fácil recuperación, regeneración y reutilización del producto. Para este estudio, se utilizaron las cepas de *S.cerevisiae* AXAZ-1 aislada de uva en Grecia y la cepa de *O. oeni* E5003. Los resultados obtenidos mediante microscopía electrónica manifestaron el logro de la coinmovilización. Con el fin de comprobar su efectividad en vino, se inocularon 0,3 g y 0,03 g de coinmovilizado en 50 mL de vino y se analizó mediante cromatografía líquida el contenido en azucares, ácido málico y ácido láctico a lo largo del tiempo. Estas fermentaciones se compararon con la inoculación simultanea de células libres de *S. cerevisiae* AXAZ-1 y *O. oeni* E5003 en concentraciones de 10^6 ufc/mL y 10^7 ufc/mL. Las fermentaciones alcohólica y maloláctica se desarrollaron de forma paralela, consumiéndose el ácido málico en 5 días y los azúcares en 5-6 días, al inocular 0,3 g de coinmovilizado o 10^7 ufc/ml de células libres, reduciendo de este modo considerablemente el tiempo habitual del proceso de vinificación y permitiendo una rápida estabilización del vino. Al inocular 0,03 g de coinmovilizado en 50 mL de mosto tinto la fermentación alcohólica y maloláctica también se desarrollaron paralelamente, consumiéndose el ácido málico y los azucares en 8 días. Sin embargo al inocular simultáneamente células libres de *S. cerevisiae* y *O. oeni* en una concentración de 10^6 ufc/mL, la fermentación alcohólica tuvo lugar en 8 días pero el ácido málico no fue consumido, de modo que a concentraciones de 10^6 ufc/mL la coinmovilización es más eficaz que la inoculación simultanea de ambos microorganismos.

Conclusiones

1. La cepa de *O. oeni* E5003 ha sido elegida como cultivo iniciador maloláctico, ya que es capaz de crecer en un amplio rango de pH, en medios con niveles elevados de etanol, no produce aminas biógenas, y presenta la mejor actividad maloláctica y viabilidad celular tras su inoculación en vino.
2. El método de cuantificación de aminas biógenas mediante cromatografía líquida empleado hasta el momento ha sido optimizado utilizando una nueva tecnología de columnas de cromatografía de núcleo duro más rápida y barata.
3. El ensayo basado en microplacas usando azul de bromofenol, como indicador de pH, ha permitido la selección de cepas malolácticas en 24 horas, en términos de degradación de ácido málico.
4. Un nuevo medio de cultivo (OMP) para la obtención de elevadas cantidades de biomasa de *O. oeni* E5003 ha sido diseñado. La composición del medio de cultivo proporciona niveles poblacionales de $1x10^9$ ufc/mL y permite la adaptación de las bacterias a las condiciones del vino.
5. Se ha escalado el proceso de producción de biomasa de *O. oeni* utilizando 80 L del medio OMP. El tiempo total de producción son 22 días y la población bacteriana alcanza niveles de $1x10^9$ ufc/mL.
6. Una cepa autóctona de *O. oeni* (V6B1) de una bodega ha sido seleccionada para ser empleada como cultivo iniciador maloláctico propio, por su alta efectividad maloláctica y su incapacidad para producir histamina. El uso del cultivo iniciador maloláctico líquido permite reducir 3 veces el contenido en histamina en un vino final comparado con un vino no inoculado en la bodega.
7. El método de conservación más adecuado del cultivo iniciador maloláctico de *O. oeni* E5003 depende del tiempo de almacenamiento del mismo. La liofilización permite la conservación del cultivo largos periodos de tiempo, mientras que la congelación a -20 ºC es óptima para la conservación durante 1 mes. La refrigeración a 4 ºC es adecuada para periodos de almacenamiento de hasta 4 meses.

8. El cultivo iniciador maloláctico liofilizado presenta mejores resultados de actividad maloláctica y viabilidad en vino al ser rehidratado con una solución de fructosa, arginina y acido málico, que al ser rehidratado con NaCl.
9. Un método de conservación alternativo de cultivos iniciadores malolácticos consiste en la conservación en lías de vino tinto. La mejor viabilidad celular se obtiene con lías que poseen un mayor contenido en polisacáridos.
10. El cultivo iniciador generado mediante la coinmovilización de *S. cerevisiae* y *O. oeni* en material celulósico y almidón permite una fermentación alcohólica y maloláctica en vino simultáneas, reduciéndose el tiempo de vinificación considerablemente.

1. Introduction

1.1 Wine and vinification process

1.1.1 History

Wine has always been an essential witness of any event and around it many significant agreements and historical happenings have been signed.

In Roman Empire, it was thought that wine had curative and destructive powers: it could cure depression, memory lost, gout, breath, snake bit and dizziness, but the excess of wine produced soul fury. Wine has always been surrounded by mystery and magic, and if something characterizes wine is that its presence in the civilizations was previous to the vinification process knowledge. Wine production came from the Neolithic, and the archaeological remains were found in the region that today occupies Georgia, Armenia and Iran. Later on, wine consumption was extended to Egypt and from there to all Eurasia. The ancient Greece and Rome played an important role in wine history as the technology knowledge and the production wine process were extended widely around the Empire.

Viticulture had its major development because of the Christianity propagation, where wine was necessary for the mass celebration. Monasteries were the precursors of viticulture and winemaking with its own elaboration and production of wines.

The role of yeasts in alcoholic fermentation (AF), particularly in the transformation of grape must into wine, was only clearly established in the middle of the 19th century. The ancients explained the 'boiling' during fermentation as a reaction between substances that came into contact with each other during crushing. In 1680, Antonie van Leeuwenhoek, was the first person that observed yeasts in beer using a microscope but he did not establish a relationship between these 'corpuscles' and AF. It was not until the end of the 18th century when Lavoisier began the chemical study of AF. Gay-Lussac continued Lavoisier's research into the next century (Ribéreau- Gayon *et al.* 2000).

Louis Pasteur gave definitive credibility to the vitalist viewpoint of AF (Köning and Fröhlich 2009). He demonstrated that the responsible yeasts in spontaneous fermentation of

grape must or crushed grapes came from the surface of the grape; he isolated several species. He even conceived the notion that the nature of the yeast carrying out the AF could influence the gustatory features of wine. He also pointed out the effect of O_2 on the assimilation of sugar by yeasts. Louis Pasteur proved that the yeast produced secondary products such as glycerol in addition to alcohol and CO_2. Since Pasteur, yeasts and AF have incited a considerable amount of research, making use of progress in microbiology, biochemistry and now genetics and molecular biology (Ribéreau- Gayon *et al.* 2000).

1.1.2 Winemaking process

The winemaking process is the set of operations and practices carried out to transform grapes into wine, respecting the intrinsic qualities of grapes to obtain the best result (Suárez 1990). Winemaking process formally begins when the grapes, or juice, reach the winery. The basic steps in the production of wines are outlined in **Figure 1**. This process includes 2 main phases carried out by microorganisms: AF developed by yeast and malolactic fermentation (MLF) developed by lactic acid bacteria (LAB) (Fleet *et al.* 1984).

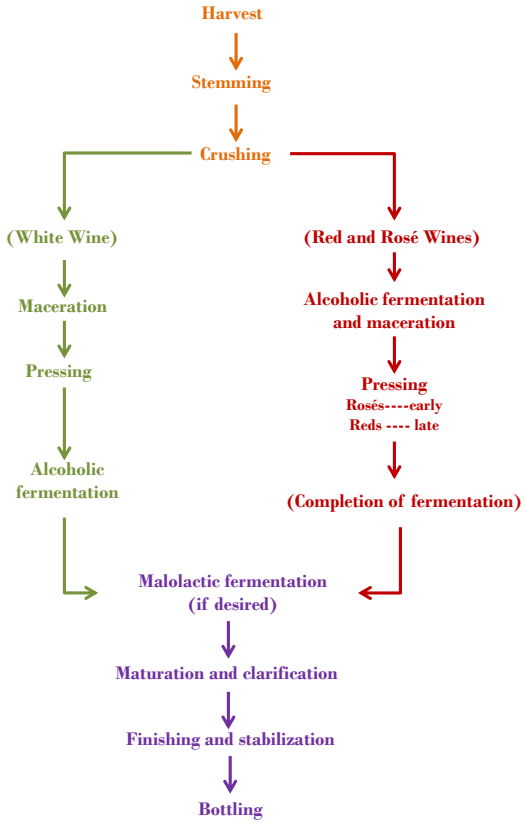


Figure 1. Operations and practices carried out during the winemaking process

Alcoholic fermentation

Alcoholic fermentation is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and CO_2. This process is carried out by yeast. However, AF is a much more complex process. At the same time as this reaction occurs, a lot of other biochemical, chemical and physicochemical processes take place, making it possible to turn the grape juice into wine. Besides ethanol, several other compounds are produced throughout AF such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin and 2,3-butanediol. Simultaneously, some compounds of grape juice are also transformed by yeasts. Without the production of these other substances, wine would have little organoleptic interest (Fleet 1993; Moreno-Arribas and Polo 2005).

At the start of the winemaking process, several species of yeast are present in the grape juice. This biodiversity depends on several factors such as grape variety, the ripening stage at harvest, the antifungal treatments, the climatic conditions, the development of grey rot or other fungal plagues, and the viticultural practices. All contact of grapes and must during harvest, transport and, in particular winery operations, significantly influence the final distribution of yeasts at the beginning of AF (Constantí *et al.* 1997; Mortimer and Polsinelli 1999). Different yeast species participate in spontaneous AF even when SO_2 is present (Constantí *et al.* 1997). *Kloeckera*, *Hanseniaspora* and *Candida* usually predominate in the early stages of AF. Later, *Pichia* and *Metschnikowia* prevail in the middle stages. Finally, during the latter stages of fermentation, *Saccharomyces cerevisiae* is the predominant yeast because of its greater resistance to high ethanol concentration (Fleet 1993). Some other yeast species, such as *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces*, *Zygosaccharomyces* and *Brettanomyces* may also be present during AF and even in the wine itself, which may cause some organoleptic alterations (Ribéreau- Gayon *et al.* 2000). The different yeast species succession throughout AF influences the final composition of wine (Chatonnet *et al.* 1995). Nowadays, most wineries inoculate selected dry yeast in grape must in order to guarantee AF without any deviation. However, other wineries, especially traditional wine cellars, continue to use spontaneous AF because they give wines a greater complexity (Moreno-Arribas and Polo 2005).

Malolactic fermentation

For a long time spontaneous acid reduction observed in wine was related only to precipitation of tartaric acid, but in 1891, Müller-Thurgau postulated that acid reduction could be due to bacterial activity. In 1913 Müller-Thurgau and Osterwalder, with their investigation into LAB in wine, explained bacterial degradation of malic acid to lactic acid and CO_2 according to the formula (**Figure 2**) (Davis *et al.* 1985):

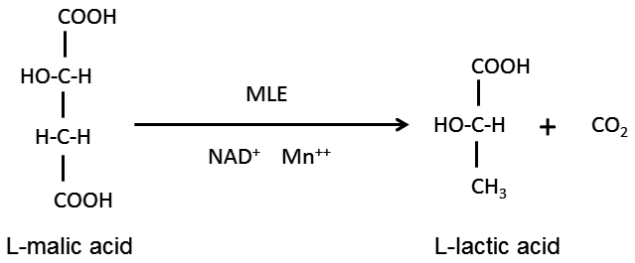


Figure 2. MLF: Conversion of L-malic acid into L-lactic acid and CO_2 by the malolactic enzyme (MLE)

This phenomenon was called malolactic fermentation. However this term is not correct since the transformation is not a fermentative pathway, but a decarboxylation. Radler findings (1967) showed that LAB of grape must and wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and more recently to *Oenococccus* (Dicks *et al.* 1995). Different LAB enter into grape juice and wine from grape berry surfaces, stems, leaves, soil and winery equipment. However, due to the high selective environment of different juices and wines, only few species of LAB are able to grow in wine (Wibowo *et al.* 1985; Krieger 2009). Malolactic fermentation is considered extensively in **Section 1.3**.

1.2 Lactic acid bacteria in wine

1.2.1 General description

In 1873, ten years after L. Pasteur studied lactic acid fermentation (between 1857 and 1863), the first pure culture of LAB '*Bacteriumlactis*' was obtained by J. Lister. Starter cultures for cheese and sour milk production were introduced in 1890, while fermented food has been

used by man for more than 5,000 years (Schlegel 1999; Stiles and Holzapfel 1997). The first monograph about LAB by S. Orla-Jensen appeared in 1919 (Köning and Fröhlich 2009).

Lactic acid bacteria appear in habitats with a rich nutrition supply. They exist in decomposing plant material, fruits, dairy products, fermented fish and meat, beets, potatoes, beverages, plants, water, fruit juices, sewage and cavities of human and animals. The typical LAB features are: gram-positive, non-sporing, catalase negative, aerotolerant, organotrophic and strictly fermentative rod or coccus producing lactic acid as a major final product. These bacteria are usually non-motile and they have requirements for the complex growth factors such as vitamins and amino acids (Köning and Fröhlich 2009).

1.2.2 Taxonomy

Nowadays, 5 genera of LAB have been isolated from must and wine, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Weissella* (Axelsson 2004; Köning and Fröhlich 2009). These 5 genera have tolerance against ethanol and acidic conditions. Generally they are inhibited at ethanol concentrations above 8 % (v:v) but *O. oeni* tolerates 14 % (v:v). *Lactobacillus brevis*, *Lactobacillus fructivorans* and *Lactobacillus hilgardii* can be found even in fortified wines up to an ethanol concentration of 20 %. LAB isolated from wine grow between 15 °C and 45 °C in the laboratory with an optimal growth range between 20 °C and 37 °C. The best growth in wine during MLF is obtained around 20 °C (Köning and Fröhlich 2009).

Genus *Lactobacillus*

Lactobacilli are straight gram-positive, non-sporulating, non-motile or rarely motile rods, with sometimes a coccobacilli form with a 0.5-1.2 µm diameter and a 1-10 µm length. They can form chains; the tendency towards chain formation varies between species and even strains. It depends on the growth phase and the medium pH. The length and curvature of the rods depend on the composition of the medium and the oxygen pressure. They are aciduric or acidophilic. The highest pH allowing growth is about 7.2. The G + C content of the DNA ranges from 32 to 53 mol%. They can tolerate O_2 or live anaerobically. They have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, nucleic acid derivates,

vitamins and minerals (Köning and Fröhlich 2009). Out of about 174 described species/subspecies, 16 have been found in must and wine. The type species is *Lactobacillus delbrueckii* CECT 286^T (Fugelsang 2007; Köning and Fröhlich 2009).

Genus *Leucosnostoc*

Leuconostocs are straight gram-positive with a spherical or lenticular form, pairs or chains with a 0.5-0.7 μm diameter and a 0.7-1.2 μm length. Leuconostocs are heterofermentative cocci producing only D-lactic acid from glucose and are unable to produce ammonia from arginine. Sugars are fermented by the 6-P-gluconate/phosphoketolase pathway with D-lactic acid, ethanol/acetate and CO_2 as end products. They are are nonproteolytic and they do not reduced nitrate. Leuconostocs share many features with the heterofermentative lactobacilli (Dellaglio *et al.* 1994). Dextrans, which have industrial importance, are produced by leuconoctocs, especially *Leuconostoc mesenteroides*, from sucrose. The G + C content of the DNA ranges between 37 and 41 mol%. Only *Lc. mesenteroides*, *Leuconostoc citreum* and *Leuconostoc fructosum* have been isolated from must and wine. The type species is *Lc. mesenteroides* ssp. *mesenteroides* CECT 219^T (Dicks and Endo 2009; Köning and Fröhlich 2009; Mesas *et al.* 2011).

Genus *Pediococcus*

Pediococcus are gram-positive, non-sporulating, spherical and never elongated as it is the case of leuconostocs and oenococci. The cell size is 0.36–1.43 μm. They form short chains by pairs of cells or tetrads (Garvie 1986b). Glucose is fermented by the Embden–Meyerhof–Parnas pathway to D- or L-lactate. A wide range of carbohydrates is used such as hexoses, pentoses, disaccharides, trisaccharides and polymers such as starch. All wine-related species grow only in presence of carbohydrates. They are nonproteolytic and nitrate is not reduced. Pediococci are catalase negative and non-motile (Simpson *et al.* 2002). The G + C content of the DNA ranges between 34 and 44 mol%. The species are differentiated by their range of sugar fermentation, hydrolysis of arginine, growth at different pH levels (4.5, 7.0), the configuration of lactic acid produced (Axelsson 2004) and ribotyping (Satokari *et al.* 2000). Four species have been found in must or wine (*Pediococcus damnosus*, *Pediococcus inopinatus*, *Pediococcus parvulus* and

Pediococcus pentosaceus). The type species is *P. damnosus* CECT 793^T (Köning and Fröhlich 2009).

Genus *Weissella*

Weissellas are gram-positive, spherical, lenticular or irregular rods. They are heterofermentative species, which produce D or L-lactic acid, while *Weisella paramesenteroides* forms D-lactic acid from glucose. *W. paramesenteroides* is the only species of this genus isolated from must and wine. The type species is *Weissella viridescens* CECT 283^T (Köning and Fröhlich 2009; Mesas *et al.* 2011).

Genus *Oenococcus*

Special attention must be given to this genus because from all the LAB, *O. oeni* is the predominant species conducting MLF in wine (Davis *et al.* 1985; Maicas *et al.* 1999a) and it is recognized as the bacterium most tolerant to the wine conditions, such as low pH, high SO_2, and alcohol content (Versari *et al.* 1999; Liu 2002).

Oenococci have been isolated from must and wine (Garvie 1986a). They are gram-positive, non-motile, non-sporulating, form spherical or lenticular cells, pairs or chains with a diameter of 0.5-0.7 μm and a length of 0.7-1.2 μm. The DNA G + C content ranges from 41 to 43 mol%. *O. oeni* can grow below pH 3.0 and above 10 % of ethanol. The optimum growth temperature is 20-30 ºC. Heat shock proteins and special membrane lipids are produced under these environmental conditions (Coucheney *et al.* 2005b). *O. oeni* can use the glucose and fructose hexoses, while not all strains use trehalose (Garvie 1986a). L-arginine can be degraded to CO_2, ammonia and ornithine. Oenococci can be distinguished from less acid tolerant *Leuconostoc* species by using saccharose, lactose and maltose as substrate (Garvie 1986a). The type species is *O. oeni* CECT 217^T.

O. oeni (before classified as *Leuconostoc oenos*) was separated from genus *Leuconostoc* in 1995 (Dicks *et al.* 1995; Schleifer *et al.* 1995) (**Figure 3**). *L. oenos* was distinguished from other *Leuconostoc spp.* because they grow in acidic media, by their requirement for a growth factor in tomato juice, and by a number of carbohydrate fermentation characteristics. In addition, the

results of a total soluble cell protein analysis performed with other *Leuconostoc* spp. clearly indicated that *L. oenos* was distinguished from the other *Leuconostoc* species (Dicks and van Vuuren 1990). Further evidence showing *L. oenos* was a distinct taxon came from genotypic investigations. Chromosomal DNA-DNA pairing was used extensively to determine the genetic relationships of *Leuconostoc* members (Farrow *et al.* 1989; Schillinger *et al.* 1989; Dicks and van Vuuren 1990). *L. oenos* was determined as distinct species by this approach, more significant were the very low levels of DNA identity detected between this species and other members of the *Leuconostoc* genus (Dicks *et al.* 1995). DNA-rRNA hybridization analyses (Schillinger *et al.* 1989) and rRNA sequence analyses (Yang and Woese 1989; Martínez-Murcia and Collins 1990) also revealed the distinctness of *L. oenos* (Dicks *et al.* 1995).

O. oeni was considered "*tachytelic*" by Yang and Woese (1989), and this is explained because Oenococci exhibit a high mutability due to the lack of the mismatch repair genes *mutS* and *mutL* (Marcobal *et al.* 2008), which may facilitate the speciation process. Several specific methods for the rapid detection or differentiation of *O. oeni* strains in must and wine samples have been developed (Kelly *et al.* 1993; Zavaleta *et al.* 1997; Reguant and Bordons 2003; Larisika *et al.* 2008; Araque *et al.* 2009; Claisse and Lonvaud-Funel 2012). Among all the methods, the randomly amplified polymorphic DNA (RAPD) technique, in conjunction with the PCR, appeared to be a suitable method for typing and monitoring *O. oeni* strains in winemaking. It is a method that incorporates a single arbitrarily designed oligonucleotide primer in the amplification reaction to generate DNA fragment polymorphisms (Welsh and McClelland 1990; Williams *et al.* 1990). This technique is specific, because the entire genome of an organism is used as the basis for generating a DNA profile. The technique of RAPD-PCR is very sensitive, rapid, simple and highly discriminating. It has been used successfully with a number of LAB (Cocconcelli *et al.* 1995; ML Johansson 1995; Rebecchi *et al.* 1998; Sohier *et al.* 1999), and with *O. oeni* isolated from wine (Zapparoli *et al.* 2000; Capozzi *et al.* 2010; Ruiz *et al.* 2010; Solieri *et al.* 2010; Bordas *et al.* 2013).

Recently, another species has been described as *Oenococcus*, so two species belong to this genus, *O. oeni* and *Oenococcus kitaharae* (isolated from a composting distilled sochu residue) (Endo and Okada 2006). Nevertheless, only *O. oeni* is present in wine and able to perform the MLF.

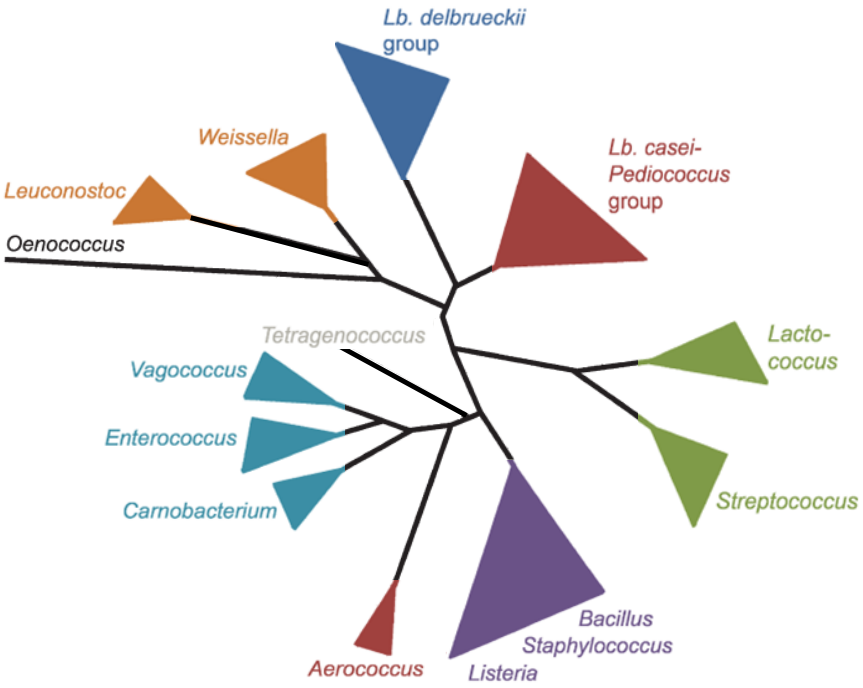


Figure 3. Schematic unrooted phylogenetic tree of LAB and related genera (adapted from Alexon 2004)

1.2.3 Nutrients

Metabolism of sugars

Lactic acid bacteria obtain their energy mainly from sugars' fermentation. They use both main hexoses of wine, glucose and fructose, as energy and carbon sources. These sugars are in the same quantity in grape must, and the total concentration varies between 150 to 250 g/L. Pentoses as L-arabinose, D-xylose, D-ribose and L-rammnose are present in grape must in low concentrations. Lactic acid bacteria metabolize sugars by 3 different pathways: homofermentative, heterofermentative or facultative homofermentative pathways (**Table 1**). Species from genus *Pediococcus* metabolize hexoses by homofermentative pathway, genera *Oenococcus* and *Leuconostoc* by the heterofermentative pathway, and *Lactobacillus* by heterofermentative or homofermentative pathway depending on the species (Moreno-Arribas and Polo 2005; Köning and Fröhlich 2009).

Table 1. Features of wine-related lactic acid genera (Köning and Fröhlich 2009)

Genus	Morphology	Carbohydrate fermentation	Lactic acid isomer
Lactobacillus	Rods coccobacilli cells single or chains	Homo- or heterofermentative, facultatively hetrofermentative	D, L, DL
Leuconostoc	Spherical lenticular cells pairs or chains	heterofermentative	D
Oenococcus	Spherical lenticular cells pairs or chains	heterofermentative	D
Pediococcus	Spherical cells pairs tetrads	Homofermentative, facultatively heterofermentative	DL, L
Weissella	Spherical lenticular irregular cells	heterofermentative	D, DL

Homofermentative LAB ferment hexoses to 2 moles each of lactate and ATP per mole of hexose used, through the EMP pathway (**Figure 4**). They are not able to metabolize pentoses. Only *Lactobacillus vini*, a wine homofermentative LAB ferment pentoses exclusively yielding lactic acid as end product (Rodas *et al.* 2006).

Hexoses and pentoses are fermented by heterofermentative LAB via the phosphoketolase pathway to 1 mole each of lactate, ethanol, CO_2 and ATP per mole of sugar consumed (**Figure 4**). If fructose acts as an electron acceptor, is reduced to mannitol. Consequently, acetyl phosphate formed during hexose fermentation is converted to acetate instead of being reduced to ethanol, generating an additional ATP (Pilone *et al.* 1991; Salou *et al.* 1994). Facultative homofermentative LAB metabolize hexoses by homofermentative pathway and pentoses via the pentose phosphate pathway with the formation of 1 mole each of lactate and ethanol/acetate and 2 moles ATP per mole of pentose used (**Figure 4**) (Ribéreau- Gayon *et al.* 2000).

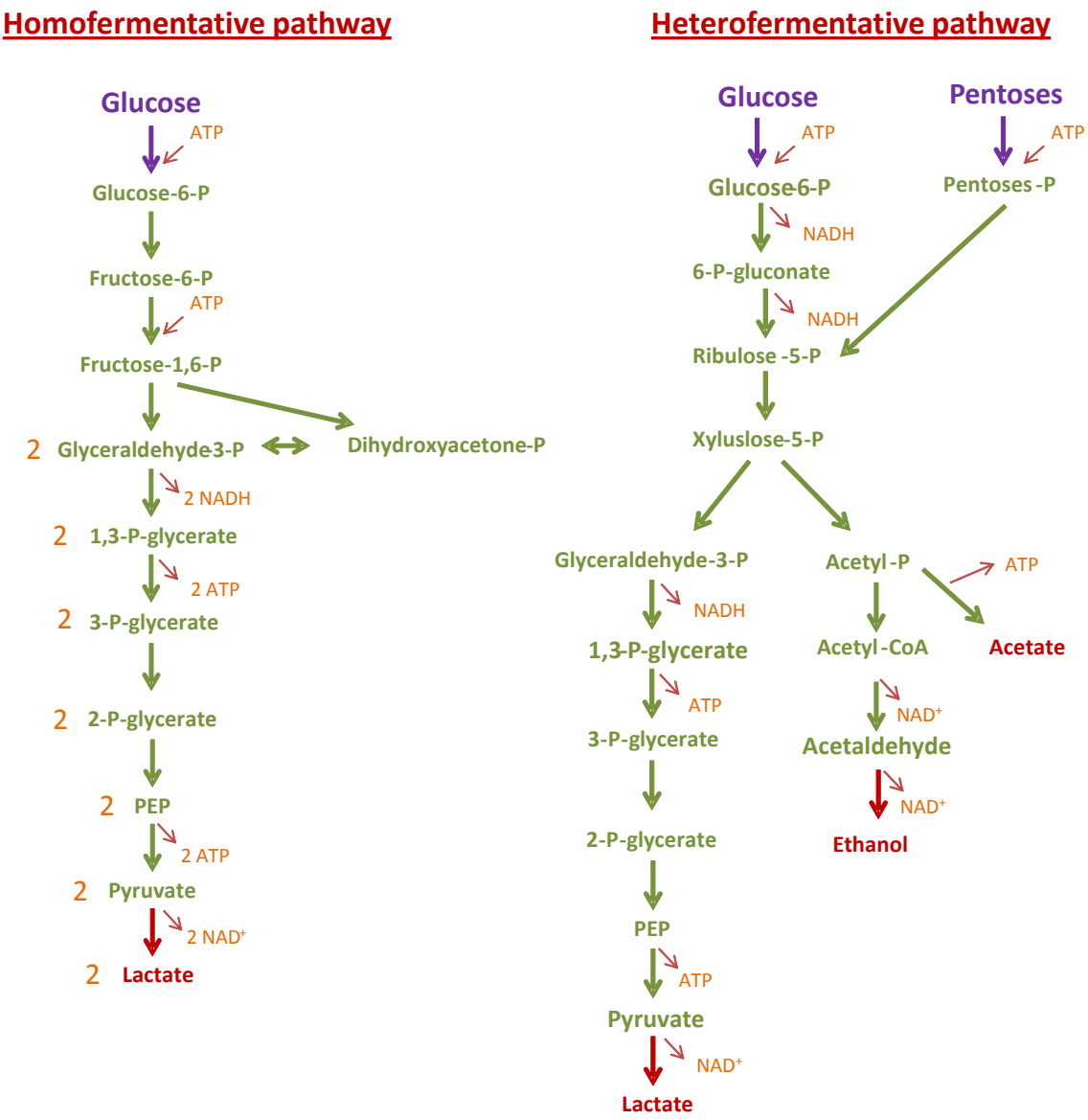


Figure 4. Homofermentative and heterofermentative pathways in LAB

Metabolism of organic acids

Lactic acid bacteria can also metabolize three main acids of grape must to obtain energy: tartrate, citrate and malate. Tartrate can be converted to lactate, acetate and CO_2 by the homofermentative LAB and to acetate and CO_2 or fumaric acid by heterofermentative LAB although only a few strains can do it. If tartrate is metabolized, volatile acidity increases and it is detrimental to wine. Citrate is transformed to lactate, acetate, diacetyl, acetoin and 2,3-butanediol. The most important oenological significance associated to citrate fermentation is the production of diacetyl, with a buttery flavor note. In general, wines that have undergone

MLF have higher concentrations of diacetyl (Martineau and Acree 1995). The final level of diacetyl in wine is affected by different factors, such as bacterial strain, wine type, SO_2 and O_2 (Martineau *et al.* 1995; Nielsen and Richelieu 1999). Many LAB, including oenococci, contain diacetyl reductase that converts the flavourful diacetyl to the much less flavourful acetoin and 2,3-butanediol (Ramos *et al.* 1995) (**Figure 5**). Metabolism of malic acid is considered extensively in the **Section 1.3** (Malolactic fermentation).

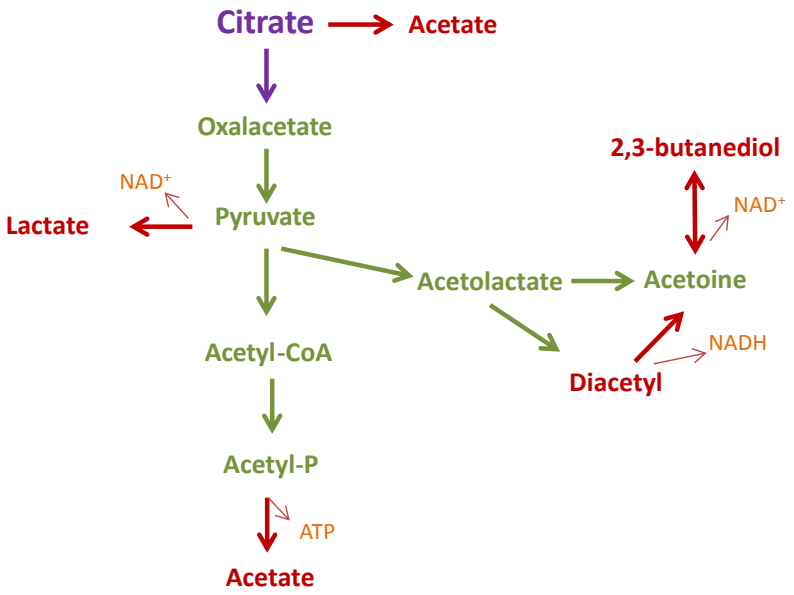


Figure 5. Metabolic pathway for citric degradation by LAB

Metabolism of amino acids

Lactic acid bacteria can use amino acids both for their own anabolism, and for catabolism. As an example, the amino acid decarboxylation to biogenic amines (BAs) may play an important role in microbial physiology that might cause adaptive advantages. Indeed, detailed studies have demonstrated that BAs production is enhanced when growth conditions become less favourable owing to the absence of fermentable substrates and the presence of ethanol. These findings have suggested that amino acid decarboxylases might function as an additional mechanism for energy generation (Lonvaud-Funel 2001).

For the histidine decarboxylation, the energetic advantage for the cell might be due to an electrogenic exchange between histidine and histamine, as it was found in *Lactobacillus buchneri*:

the amino acid enters the cell in the neutral form while histamine is excreted with one positive charge, generating a proton motive force of sufficient value to drive ATP synthesis (Le Jeune *et al.* 1995; Lonvaud-Funel 2001). This proton consuming decarboxylation also generates a trans-membrane pH gradient that enables the cells to protect themselves against the adverse effects of the acidic environment. Tyrosine and other amino acid decarboxylations, have been envisaged an energetic advantage through a mechanism similar to that described above, but studies on this matter are still incomplete and further investigations are required to establish the physiological importance of these reactions (Köning and Fröhlich 2009).

Another health concern in wine is the presence of ethyl carbamate (Izquierdo Cañas *et al.* 2008). This compound attracts the attention of many researchers because of its carcinogen potential and its possible origin from precursors produced by microbiological catabolism of amino acids. Ethyl carbamate could be originated in wine as a consequence of a spontaneous, non-enzymatic reaction between ethanol and a compound containing a carbamyl group, such as urea (produced from arginine breakdown by yeasts), citrulline or carbamylphosphate (produced from arginine breakdown by LAB) (Mira de Orduña *et al.* 2000). This alcoholysis of carbamyl compounds has been shown to be directly dependent on both concentration of reactants and temperature (Ough *et al.* 1988), the reaction occurs faster with carbamyl phosphate and slowler in the cases of urea and citrulline at normal wine storage temperatures. Consequently, ethyl carbamate levels are usually low or non-detectable in young wines and variable in aged or stored wines, depending on cellar or storage temperature (Köning and Fröhlich 2009).

1.2.4 Ecology of lactic acid bacteria in wine

On grape berries the bacterial population is low, or at least only a few bacteria are able to grow when they are sampled on nutritional medium such as De Man-Rogosa-Shape (MRS) (De Man *et al.* 1960) under laboratory conditions. The population varies from about 10^2 cfu/mL to 10^4 cfu/mL, depending on the climatic conditions during the final days of grape maturation. It is mainly correlated to pH; the higher pH, the higher total LAB population. (Lonvaud-Funel 1999).

At the end of AF, LAB population generally decreases to around 10^2 cfu/mL. Most important, not only total bacterial counts diminish but the original diversity of the species does also. In most cases *O. oeni* predominates at the end of AF. The natural selection of *O. oeni* during fermentation is mainly due to the progressive increment in ethanol and other products of yeast metabolism. MLF starts when the LAB population reaches 10^6 cfu/mL after a fast growth rate which may begin sooner or later after the end of AF. The lag phase between the two fermentations mainly depends on temperature, pH and ethanol contents, the main factors determining bacterial growth in wine (Lafon-Lafourcade *et al.* 1983b).

Some other factors affect the LAB population dynamics in wines. As examples, fatty acids such as decanoic and dodecanoic acid are powerful inhibitors of LAB growth (Edwards *et al.* 1990). Like ethanol they alter the bacterial membrane (Lonvaud-Funel *et al.* 1988). Some yeast strains may also produce large amounts of SO_2 from their sulfur compound metabolism (King and Beelman 1986). In addition to the increasing toxicity of the medium, some nitrogen lack can occur, at least during the exponential phase of yeast growth. However, this relative starvation is transitory since yeasts release amino acids, especially at the end of fermentation. From all LAB species, *O. oeni* is probably the best adapted to overcome these obstacles. However, some *Pediococcus* and *Lactobacillus* strains can survive, and they are potent spoilage agents during winemaking (Lonvaud-Funel *et al.* 1993a).

When all malic acid is degraded, wines are stabilized by sulphating. Most bacteria and possibly the remaining yeasts are sensitive to SO_2. In high pH wines, the ineffectiveness of SO_2 and the lesser medium stringency mean that bacteria survive easier. In such wines, high levels of viable cells may be found reaching sometimes 10^5 or 10^6 cfu/mL, even during the ageing. In these conditions *Lactobacillus* and *Pediococcus* are frequently predominant and can induce spoilage (Lafon-Lafourcade *et al.* 1983b; Köning and Fröhlich 2009).

1.3 Malolactic fermentation

1.3.1 General description

MLF is considered a simple phenomenon but with a practical relevance in wine. The main value of the MLF in vinification is the biological de-acidification, which results from the transformation of L-malic acid into L-lactic acid by LAB. This induces a pH increase, and a change in wine taste. Decarboxylation of malic acid into L-lactic acid is catalyzed by the malolactic enzyme (MLE) which is different from the malic enzyme leading to pyruvate (Davis *et al.* 1985; Battermann and Radler 1991). The decarboxylation of L-malic acid involves the active transport of malic acid into the cell, decarboxylation, and the transport of lactic acid out of the cell into the surrounding medium (Poolman *et al.* 1991; M Salema 1994; Zaunmüller *et al.* 2006). The three genes involved in the malolactic reaction, *mle*RAP, have been cloned and their respective proteins characterised; *mle*A encodes the malolactic enzyme, *mle*P encodes the malate permease, and *mle*R is a LysR-type regulatory protein (Bartowsky 2005) (**Figure 6**). The expression of *mle* genes is influenced by pH and ethanol; the expression increases at low pH values and decreased in the presence of ethanol (Miller *et al.* 2011).

The malolactic activity is strictly dependent on the integrity of the bacterial membrane. The optimum pH of the MLE is around 5.8, it needs cofactors (Mn^{2+}, NAD^{+}), and its activity is affected by many wine components as ethanol, carboxylic acids and polyphenols (Bartowsky 2005; Miller *et al.* 2011). Malolactic fermentation does not produce energy-rich phosphate bond intermediates directly, however the electrochemical energy can be generated via an indirect electrical potential ($\Delta\Psi$). A proton is consumed in the decarboxylation reaction increasing the internal pH. Cytoplasm alkalinization results in a chemical potential of protons across the membrane (ΔpH), and together with the $\Delta\Psi$, it forms the proton motive force across the cytoplasmic membrane (chemiosmotic mechanism) (Poolman *et al.* 1991; Salema *et al.* 1996; Versari *et al.* 1999; Zaunmüller *et al.* 2006). The MLF rate in wine depends on the LAB species, strain, cell density, specific malolactic activity and physiological state of cells (Nault *et al.* 1995).

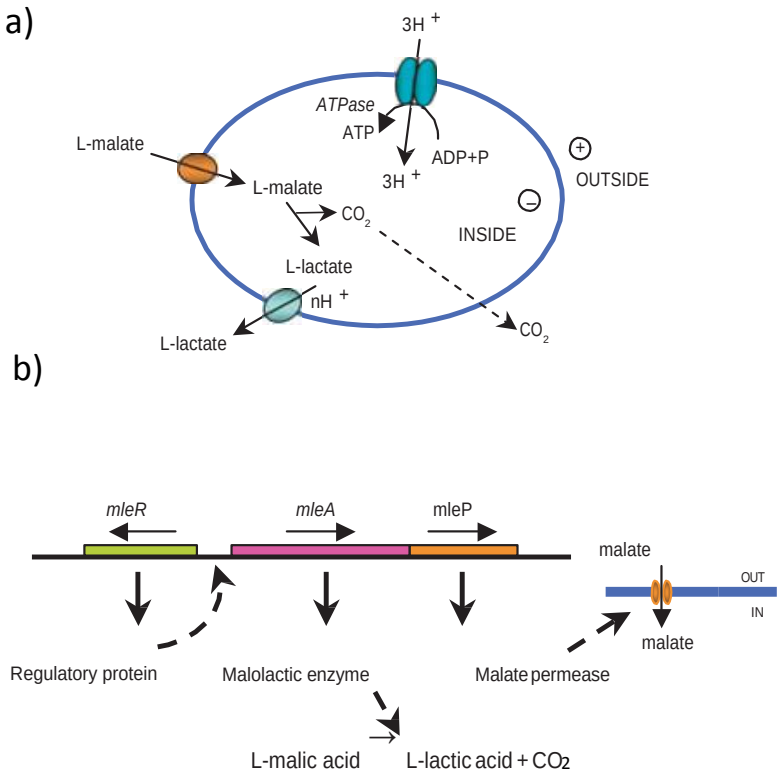


Figure 6. Malolactic fermentation. **a)** The decarboxylation of L-malic acid to L-lactic acid involves the active transport of malic acid into the cell, decarboxylation, and the transport of lactic acid out of the cell into the surrounding medium; **b)** The three genes involved in the malolactic reaction in *O. oeni* are *mle*A, *mle*P and *mle*R (Bartowsky 2005)

1.3.2 Factors affecting malolactic fermentation

Recent findings into MLF have provided better understanding of biological wine de-acidification, and the limiting factors in wine impacting on the growth of LAB, responsible for the biotransformation.

pH

The pH is one of the essential factors for biological stability and is a selective factor over the species that participate in the winemaking process and also influences the bacteria growth kinetics and malolactic activity (Wibowo *et al.* 1985). Wines usually have a pH value between 3 and 4; wines with a pH over 3.3 do not have problems to perform the MLF, while wines with a pH under 3.3 present difficulties to carry out the MLF (Kunkee 1967). From all LAB, *O. oeni* is the most tolerant bacterium to low pH (Lonvaud-Funel 1999; Versari *et al.* 1999; Guzzo *et al.*

2000). Nowadays, climate change is increasing potassium levels and lower total acidities thus a combined effect on increased pH levels. Low pH values are a cornerstone of microbiological stability. Accordingly, the trend towards higher pH values, if not corrected, more risk of increased microbial contamination in wine (Mira de Orduña 2010).

SO_2

The SO_2 is widely used in winemaking as an antioxidant, antimicrobial and antienzymatic (Carreté *et al.* 2002).Winemakers usually add 160 mg/L as maximum values of total SO_2 in red wine and 210 mg/L in white wine. Despite its value and potency, a majority of consumers view sulfite additions to wine as unnatural an unhealthy. Nowadays, winemakers are challenged tu restrict or even eliminate sulfites during winemaking process, while maintaining high product quality (Jackowetz and Mira de Orduña 2012). The chemistry of SO_2 in wine is complex. Upon addition to wine, total SO_2 enters a pH-dependent equilibrium consisting of a bound SO_2 or free SO_2 (molecular, bisulfite and sulfite ions) (**Figure 7**). The molecular fraction is the form that enters in the bacteria cell by diffusion and is transformed to HSO_3^-, which reacts with enzymes causing the cell death (Bauer and Dicks 2004). Fang and Dalmasso (1993) found that SO_2 was more effective against LAB at pH 3.4 than at pH 3.8. Concentrations of molecular SO_2 of 0.5 mg/L are high enough to inhibit LAB growth in red wine, but tolerance varies between species and strains. Strains from *O. oeni* are more sensitive to SO_2 than *Pediococcus* and *Lactobacillus* (Davis *et al.* 1985).

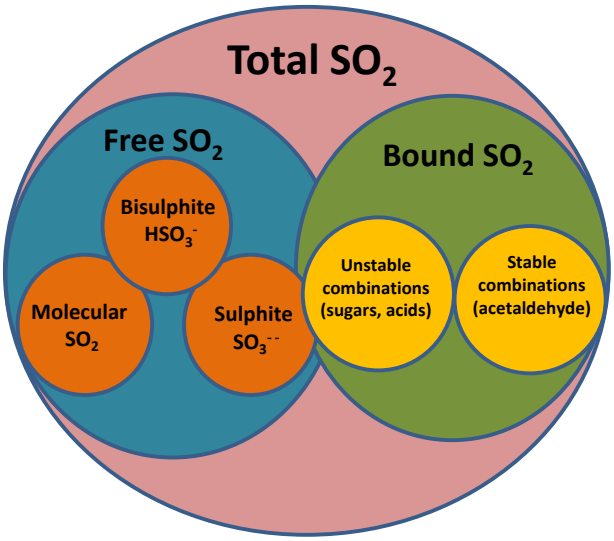


Figure 7. Forms of SO_2 that can be present in wine

Ethanol

The ethanol inhibits bacterial growth interacting with the membrane lipids, being toxic for the majority of microorganisms. Wine usually contains between 8.5 and 15.5 % (v:v) of ethanol. The ability of LAB to survive and grow in wine decreases as the alcohol concentration increases, even though some LAB strains are able to grow in wines with a concentration above 20 % (v:v) (Davis *et al.* 1985; Wibowo *et al.* 1985). One of the effects of the climatic change is the increase of the sugar content and the concomitant increase in the alcoholic content of the wine (Mira de Orduña 2010). The inhibitory effect of ethanol can be so important that some authors have focused their studies on this aspect on trying different strategies to attain a good MLF. The ethanol tolerance is species and strain dependent, and *O. oeni* is recognized as the most tolerant bacterium (Versari *et al.* 1999). Many authors point to the bacteria acclimation as a crucial step for a successful MLF (Zapparoli *et al.* 2009).

Temperature

The temperature affects the MLF because it influences the bacterial growth, the lag phase and the toxic effect of SO_2 and ethanol. The MLF is carried out between ranges of temperatures from 10 ºC to 32 ºC, depending on the LAB strain. The MLF is very slow under 15 ºC and above 30 ºC (Henick-Kling 1993).

It is important to point out that pH, SO_2, ethanol and temperature are interrelated in the MLF, so it is necessary to consider all these parameters together in practical terms.

Phenolic compounds

Phenolic compounds affect the MLF trough the influence on growth and bacterial viability (Rodríguez *et al.* 2009). Some polyphenols as the vanillic acid and tannins have a bactericide effect that can produce a delay of the MLF in barrels. Nevertheless, some compounds as gallic acid and anthocyans can produce a stimulant effect because some LAB are able to use them to obtain energy (Vivas *et al.* 1997; Figueiredo *et al.* 2008). Malolactic fermentation by *O. oeni* is

stimulated in presence of phenolic compounds as catechin and quercetin, but MLF is delayed with p-coumaric acid (Reguant *et al*. 2000).

Fatty acids

Medium-chain fatty acids, present in many alcoholic beverages, are toxic for LAB. Edwards *et al*. (1990) suggested that these compounds may result in bacterial antagonism during AF. Decanoic acid, reported to be present in some wines depending on must composition and winemaking conditions, was found to suppress the growth of *O. oeni* at 5-10 mg/L. The inhibitory effect of fatty acids on malolactic activity and cell growth is concentration and pH-dependent. Free fatty acids are present in wine solution (pH > 3.2) as undissociated molecules (C_{10} has a p*K*a > 4.9). Once inside the bacterial cell, they dissociate with a consequent accumulation of intracellular hydrogen ion and dispersion of the trans-membrane proton gradient, inhibiting intracellular enzymes and ΔpH-dependent transport systems (Capucho and Sanromao 1994). Also, alcohol, temperature and pH can modify the fatty acid composition of the cell membrane in wine LAB, and in particular the saturated/unsaturated fatty acids ratio affects the viability of these bacteria (Henick-Kling 1995). Ethanol (10 % v:v) decreases the unsaturated/saturated fatty acid ratio in the microbial membrane. The fatty acid composition of *O. oeni* varies not only according to environmental conditions, it is also strain dependent (Drici-Cachon *et al*. 1996).

Interactions between yeast and bacteria

Numerous reports suggest that the strain of *S. cerevisiae* responsible for the AF can affect the subsequent growth of LAB and the conduction of MLF (Fornachon 1968; Mayer 1978; Beelman 1982). Some strains of *S. cerevisiae* may stimulate the growth of these bacteria eliminating inhibitors (polyphenols, fatty acids) and supplying nutrients and mannoproteins from its autolysis, others may inhibit the growth by the production of inhibitors as SO_2, CO_2, ethanol, medium-chain fatty acids, fumaric acid, proteins or antibacterial compounds, etc. (Osborne and Edwards 2007; Mendoza *et al*. 2010).

Interactions between lactic acid bacteria

The production of antimicrobial peptides as bacteriocins constitutes a potent adaptation advantage for those strains that dominate in a medium such as wine, and play an important role in the ecology of wine microorganisms. Bacteriocins are secreted by some bacteria; which are thus adapted for competition against other microorganisms growing in the same medium (Navarro *et al.* 2000). A few bacteriocinegenic LAB strains from wines have been so far described and they are potential tools for wine microbiological control (Lonvaud-Funel *et al.* 1993b; Strasser de Saad and Manca de Nadra 1993; Díez *et al.* 2012). Strasser de Saad and Manca de Nadra (1993) reported one *P. pentosaceus* strain with inhibitory activity against other LAB strains which included: *Pediococcus* spp., *O. oeni* and *Lactobacillus* spp. Lonvaud-Funel *et al.* (1993b) found inhibitory activities by one *P. pentosaceus* and one *Lb. plantarum* strains against *O. oeni* and *Lc. mesenteroides*. Díez *et al.* (2012) determined that *O. oeni* resulted more sensitive to pediocin produced by *P. acidilactici* J347-29 than other LAB.

1.3.3 Influence of lactic acid bacteria and malolactic fermentation on wine composition

Lactic acid bacteria transform substrates, and consequently organoleptic characters are modified during MLF. Some metabolic activities are favorable, some are without consequence, and others are totally detrimental to wine quality (Lonvaud-Funel 1999). Many of these changes in the chemical composition of wine have been described in reviews especially, for *O. oeni* (**Figure 8**) (Versari *et al.* 1999; Liu 2002; Bartowsky 2005).

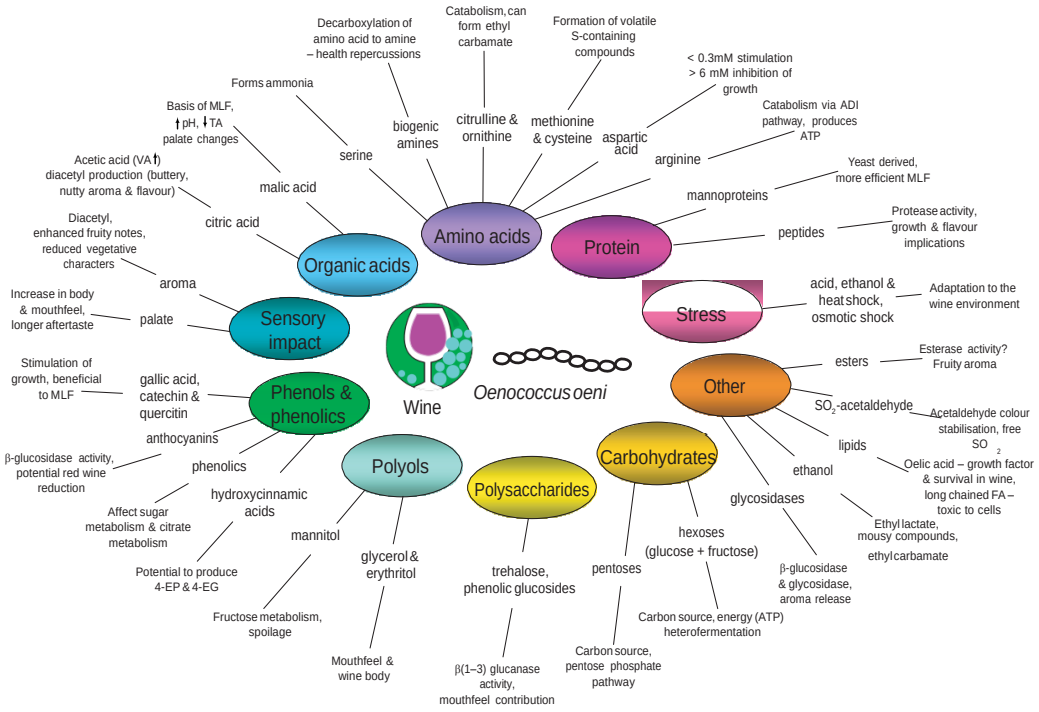


Figure 8. Summary of the characterized biochemical changes which occur during MLF and *O. oeni* metabolism (Bartowsky 2005)

Malolactic fermentation can be produced by 2 strategies: by spontaneous MLF or by inoculation with starter cultures. Traditionally, a natural process of MLF is carried out by indigenous bacteria such as *O. oeni* or other LAB. These bacteria develop spontaneously and grow in wine. This MLF method can take weeks or months (Zhang and Lovitt 2006). The starter cultures use inducing MLF can, in some cases, ensure faster fermentation, reduce potential spoilage by other LAB and, furthermore, allow the control and selection of the strain responsible for the MLF and its contribution to the wine quality (Mira de Orduña *et al.* 2001; Pozo-Bayón *et al.* 2005). Despite the advantages of inducing MLF through the inoculation of commercial strains of *O. oeni*, it is not effective sometimes, since wine's environment is very harsh for bacterial growth (Coucheney *et al.* 2005a). Thus, the use of an autochthonous starter culture well-adapted to the conditions of a specific wine-producing area has been recommended. The use of selected strains from indigenous wine microbiota of each region takes advantage of the natural adaptation of the strains to the wine characteristics, and may simultaneously preserve the qualities of regional wines (Henick-Kling *et al.* 1989; Nielsen *et al.* 1996; Izquierdo *et al.* 2004; Capozzi *et al.* 2010; Cañas *et al.* 2012). As we will discuss later on, many researchers

have focused their studies on the selection of LAB strains to carry out effective MLF (Costello *et al.* 2003; Coucheney *et al.* 2005a), and several parameters affecting the bacterial activity have been considered for starters' selection (Liu and Gallander 1983; Teixeira *et al.* 2002; Rosi *et al.* 2003; Reguant *et al.* 2005a). Furthermore, because of the current climate change, the sugar content of grapes is increasing, and so the alcoholic content of the wine is increasing concomitantly (Mira de Orduña 2010). One of the most significant problems, due to the increase of the alcoholic content, is the growth limitation for LAB. This inhibitory effect of ethanol and other substances as polyphenols or SO_2 can cause an unreliable and inconsistent MLF. For this reason, several strategies are being developed in order to enhance the wine MLF, most of them focussing on the bacteria themselves (Maicas *et al.* 2001).

Beneficial effects of lactic acid bacteria in wine

- Acidity reduction

Most wines contain 0.35 % to 0.55 % tritable acid, and MLF is a major method to reduce such acidity. The biological deacidification results from the transformation of L-malic acid (dicarboxylic acid) to L-lactic acid (monocarboxylic acid). Reduction in wine acidity by MLF can vary from 0.1 % to 0.3 %, and pH may rise by 0.1 to 0.5 units. This is beneficial for wines with low pH (Lonvaud-Funel 1999).

- Flavor and aroma modification

During the MLF, the development of LAB brings changes in the sensory properties of wine. Apart from producing lactic acid as the major end-product, LAB are well known for their ability to produce flavor compounds, such as acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2, 3-butanediol (Martineau and Henick-Kling 1995; Ramos *et al.* 1995; Nielsen and Richelieu 1999).

One of the most significant descriptors for sensory panelists is 'buttery'. This is directly linked to the increased concentration of a diacetyl which is recognized as the major contributor to aroma change during MLF (Bertrand *et al.* 1984; Davis *et al.* 1985). Acetoinic compounds

which comprise diacetyl, acetoin and 2,3-butanediol result from citric acid metabolism. In general, wines that have undergone MLF have higher concentrations of diacetyl (Martineau and Acree 1995). The final level of diacetyl in wine is affected by different factors, such as bacterial strain, wine type, SO_2 and O_2 (Martineau and Henick-Kling 1995; Nielsen and Richelieu 1999). It should be pointed out that diacetyl is formed chemically from the oxidative decarboxylation of α-acetolactate, an unstable intermediary compound produced during citrate metabolism. Many LAB, including *O. oeni*, contain diacetyl reductase that converts the diacetyl to acetoin and 2,3-butanediol (Ramos *et al.* 1995). The formation of diacetyl by LAB enhances the buttery aroma of wine, whereas the reduction of diacetyl decreases it (Martineau and Acree 1995).

- Microbiological stability

One of the main reasons supporting MLF is that wines which have undergone MLF are, in a microbiological sense, more stable than those that have not sustained MLF, because the growth of malolactic bacteria means that there are not available substrates for spoilage bacteria, decreasing the risk of MLF occurring in the bottle (Davis *et al.* 1985).

Lactic acid bacteria as spoilage agents of wine

- Increase of wine acidity

Lactic acid bacteria can spoil wine during winemaking, during maturation or in bottle ageing. In the first case, bacteria which are going to perform the MLF grow too early. Therefore, bacteria ferment carbohydrates, particularly hexoses, which have not been totally fermented by yeasts. At this stage, most of the bacterial population is composed of heterofermentative strains, mainly *O. oeni*. Besides ethanol and CO_2, which are also formed by yeasts, the major products of fermentation are acetic acid and D-lactic or L-lactic acid, depending on the lactate dehydrogenase isoenzymes. In consequence, the volatile and total acidities of wine increase. This accident named '*piqûre lactique*' occurs when the end of AF is too slow or when it stops. High sugar concentration, low pH and nitrogen deficiency, together with the excretion of toxic yeast metabolites, make the yeast activity very difficult. In these

conditions the bacteria growth can start. They may ferment several sugars, producing acids (Lonvaud-Funel 1999).

- Ropiness of wine

This deterioration is characterized by an abnormal increase in viscosity, to such an extent that wine runs thicker than oil. The ropy wines contain polysaccharides produced from residual sugars by strains of species such as *P. damnosus*. This species is normally present in grape and must and disappears almost completely during the winemaking process. Sometimes *P. damnosus* also plays a role in MLF. Most of the *P. damnosus* strains are not spoilage agents, only some of them can synthetize exocellular polysaccharides (Llaubères *et al.* 1990).

- Production of off-flavors

During and after winemaking, off-flavors sometimes appear. These defects are not exactly characterized, either by the substances concerned or by the reason for their formation. Special attention has been given in recent years to the 'animal' phenolic odors due to excessive amounts of volatile phenols in red wines, 4-ethylphenol and 4-ethyl guaiacol. Most often the volatile phenols formation occurs during barrel aging and it is attributed to the activity of *Brettanomyces* sp. strains (Valentão *et al.* 2007; Oelofse *et al.* 2009; Curtin *et al.* 2013) . However, authors as Calvin *et al.* (1993) and Silva *et al.* (2011) showed that some LAB (*Lb. plantarum, Lb. brevis* and *Pediococcus*) could metabolize phenol carboxylic acids, ferulic and p-coumaric acids to form these volatile phenols.

'Mousy' off-flavors are also produced by LAB in wine and three heterocyclic volatile bases are responsible: 2-acetyltetrahydropyrine, 2-ethyltetradropyridine and 2-acetyl1-pyrroline. Costello *et al.* (1996) studied the ability for wine LAB to produce such compounds in grape juice and wine-based medium. Some heterofermentative species, such lactobacilli and *O. oeni* were the responsible for these compounds (Lonvaud-Funel 1999).

Sorbic acid (2,4-hexadienoic acid) can be used as a chemical preservative in sweetened wines at bottling to prevent yeast fermentation after packaging. However, several LAB species,

including *O. oeni* strains, are able to metabolize sorbic acid resulting in the formation of 2-ethoxyhexa-3,5-diene, which has an odor reminiscent of crushed geranium leaves (Bartowsky 2009).

Metabolism of several sugars and polyols by bacteria can result in wine spoilage. Bitterness in wine can develop from metabolism of glycerol, mainly by *Lactobacillus* sp. The bitter taste is thought to result from the reaction of red wine phenolics with acrolein (Bartowsky 2009).

Fructose metabolism by heterofermentative LAB including *O. oeni* can result in the formation of mannitol, a six-carbon sugar alcohol. Mannitol is usually also accompanied by high acetic acid, D-lactic acid, n-propanol and 2-butanol. Such spoiled wine can also be perceived as having a slimy texture with a vinegar-estery aroma and slightly sweet taste (Richter *et al.* 2003).

1.4 Biogenic amines

1.4.1 Biogenic amines in wine: characteristics

One of the main problems that can cause the presence of LAB in wine is the production of BAs. They are nitrogenous low molecular weight organic bases that can have an aliphatic, aromatic or a heterocyclic structure and are widely present in foods, especially in fermented foods, mostly as a consequence of the decarboxylation of their respective free precursor amino acids, through the action of substrate-specific microbial decarboxylases (Silla Santos 1996; Martuscelli *et al.* 2013). Hence, the amines histamine, tyramine, putrescine, cadaverine, 2-phenylethylamine, agmatine and tryptamine originate from the precursor amino acids histidine, tyrosine, ornithine and arginine, lysine, phenylalanine, arginine and tryptophan, respectively. Other amines, possibly present in wines, include the aliphatic volatile amines (methylamine, ethylamine and isoamylamine), that can be originated by the amination of non-nitrogen compounds, such as aldehydes and ketones (Bauza *et al.* 1995) and the polyamines, spermine and spermidine, that can be produced from putrescine (1,4-diaminobutane), through methylation reactions involving *S*-adenosyl-methionine. The most frequent amines in wine are histamine, tyramine putrescine and cadaverine (**Figure 9**) (Landete *et al.* 2005b).

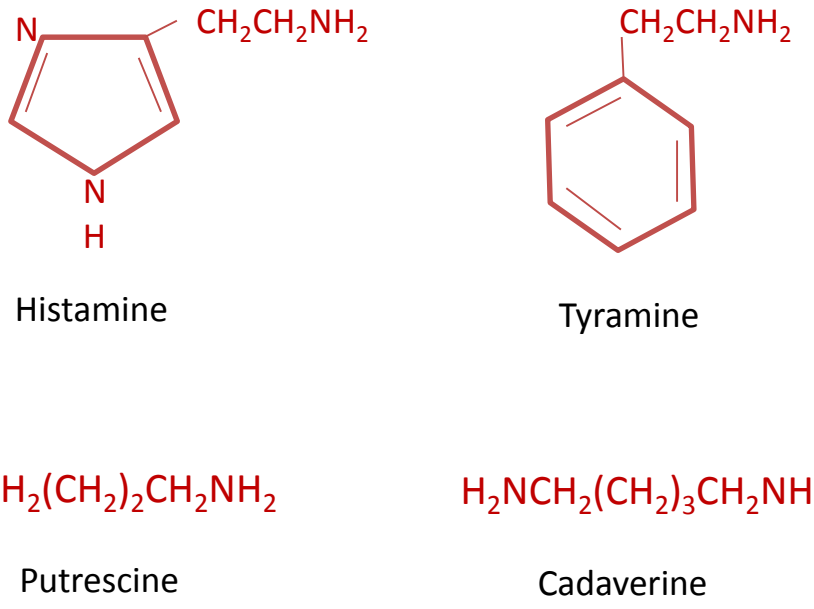


Figure 9. Examples of the most frequent BAs present in wine

All these amines have the potential to cause physiological distress in the human organism if ingested in relatively high concentration by sensitive people (Shalaby 1996). More specifically, histamine, the most toxic and studied BA, may induce headaches, hypotension, heart palpitation, cutaneous and gastrointestinal disorders (Arrieta and Prats-Moya 2012). The aromatic amines, tyramine and 2-phenylethylamine are known to cause migraines and hypertensive crises. The polyamines (putrescine, agmatine, cadaverine, spermine and spermidine), although non-toxic themselves, potentiate the effects of the toxic amines, being also able to inhibit enzymes such as the amino-oxidases, that catalyse the oxidative deamination of amines and constitute the main detoxifying system of BAs in humans (ten Brink *et al.* 1990). The volatile monoamines, in spite of their poor physiological significance, are active as irritants and thus may negatively affect the sensorial profile of foods (Lehtonen 1996).

Fresh musts usually contain low levels of BAs, almost entirely represented by putrescine produced from the vine. Wines are usually characterized by a significant higher content of BAs than their respective fresh musts, and red wines are generally characterized by BAs content significantly higher than white wines. Biogenic amines presence in wine has been roughly considered as a consequence of MLF (Smit *et al.* 2013). Biogenic amines formation in wine

requires the presence of both precursor amino acids and microorganisms with amino acid decarboxylase activity, besides environmental conditions allowing microbial growth and enzyme activity (ten Brink *et al.* 1990; Martuscelli *et al.* 2013).

Biogenic amines accumulation in wine was long considered as index of poor hygiene in the winery and/or the result of wine spoilage from strains of LAB belonging to the genera *Pediococcus, Lactobacillus, Leuconostoc* or *Oenococcus*. Indeed, several strains of different species of these four genera, isolated from wines containing BAs, proved to be able to produce BAs in the presence of their precursor amino acids (Moreno-Arribas *et al.* 2003; Landete *et al.* 2007).

1.4.2 Histamine production

Synthesis

Histamine is produced by enzymatic decarboxylation of the amino acid histidine (**Figure 10**). It is assumed that most histamine in wine is produced by histidine decarboxylases from LAB during MLF (Davis *et al.* 1985; Gerbaux *et al.* 1997; Lonvaud-Funel 2001; López-Rituerto *et al.* 2013). As *O. oeni* is the dominant species during the MLF in wines (Davis *et al.* 1986; van Vuuren and Dicks 1993), many authors have considered *O. oeni* the main organism that produces histamine in wine (Coton *et al.* 1998; Landete *et al.* 2005a; Lucas *et al.* 2008; López *et al.* 2009). However, this conclusion may not be the only explanation for histamine production in wine. Other LAB species occur during wine production and have been found in the grape must, during and after MLF. These are generally species of *Lactobacillus* and *Pediococcus* which are also histamine producers (Landete *et al.* 2005a; Costantini *et al.* 2006).

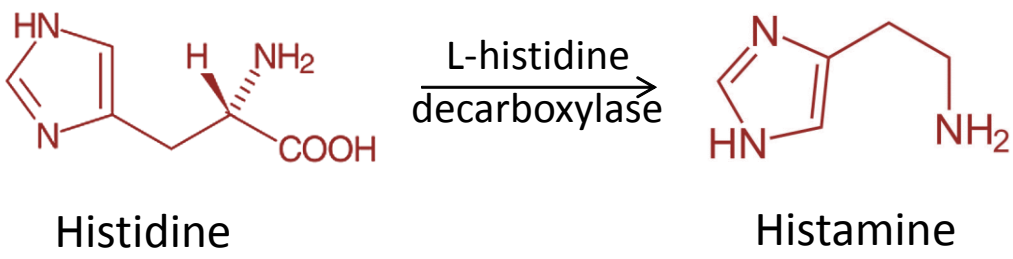


Figure 10. Enzymatic decarboxylation of the amino acid histidine to form histamine

Nowadays there is a controversy regarding whether or not *O. oeni* is the principal bacterial species implicated in histamine production in wine (García-Moruno and Muñoz 2012). García-Moruno and Muñoz (2012) conclude that the array of methods used to determine histamine production by *O. oeni* has given some inconsistent data, and that evidence supporting the hypothesis that *O. oeni* is responsible for histamine production in wines, needs to be questioned and re-examined. Furthermore, an international collaborative study to develop reliable, standard methodology is also needed to be applied in laboratories to verify this hypothesis, and to evaluate the histamine producing potential in the global diversity of *O. oeni* strains.

Methods for the histamine detection and quantification

During the last two decades, many methods for the detection of BA-producing bacteria have been developed. Several detection methods are based on differential growth media pointed the pH increase upon BAs formation (Maijala 1993; Bover-Cid and Holzapfel 1999). Others are enzymatic methods, especially for the histamine quantification (Landete *et al.* 2004), thin-layer chromatography and plate determination, particularly for tyramine and histamine determination (Roig-Sagués *et al.* 1997; García-Moruno *et al.* 2005; Landete *et al.* 2007) and ELISA (Marcobal *et al.* 2005). Nevertheless, high-performance liquid chromatography (HPLC) is the most often technique used to determine these amines due to its sensibility and selectivity, (Ferreira 1993; Hernández-Orte *et al.* 2003; Hernández-Orte *et al.* 2006; Peña-Gallego *et al.* 2009). Molecular methods, mainly based on the PCR, have been reported for detection of histamine-producing bacteria by amplifying the *hdc* genes that encode histidine decarboxylase enzymes (Marcobal *et al.* 2006; Landete *et al.* 2007). Also, new tools as ^{13}C nuclear magnetic resonance had been use to study the metabolism or transformation of histidine into histamine or histaminol during the AF and MLF (López-Rituerto *et al.* 2013).

1.4.3 Strategies for biogenic amines' diminution

Biogenic amines' elimination

Some studies are done to reduce the amines' content in wine. One strategy is the use of lysozyme, an enzyme isolated from hen egg albumen. Lysozyme lytic activity against most of

LAB associated with wine has recently found to be useful in controlling bacterial activity in wines (Gao *et al.* 2002). Lysozyme has several applications, including delaying MLF, allowing longer macerations and micro-oxigenation to improve color stabilization and polyphenolic extraction. It can prevent the increase of volatile acidity during stuck or sluggish AF and can maintain low histamine levels in wine (López *et al.* 2009). Nevertheless, other studies as the reported by Cejudo-Bastante *et al.* (2010) indicate that the pre-fermentative lysozyme, tannin or sulphites addition to grape must had no specific influence on the final amount of BA. The studies of Callejón *et al.* (2013) and García-Ruiz *et al.* (2011) proposed the use of LAB to degrade BAs through the production of amine oxidase enzymes. García-Ruiz *et al.* (2011) examined the ability of 85 LAB strains isolated from wines and other oenological sources to degrade amines. The 25 % of LAB were able to degrade histamine but no more of 16 % of this amine was degraded in wine. Callejón *et al.* (2013) searched for enzymatic activities responsible BAs degradation in LAB strains isolated from wine, their identification, and the evaluation of their applicability for reducing BAs in wine. Fifty-three percent of the 76 LAB cell extracts showed activity against a mixture of histamine, tyramine, and putrescine when analyzed in-gel. The quantification of the degrading ability for each individual amine was tested in a synthetic medium and wine, and most of the bacteria analyzed were able to degrade the three amines in both conditions.

Prevention of biogenic amines formation

Other strategy that can be performed is the prevention of the BAs formation by the use of malolactic starters (Moreno-Arribas *et al.* 2003; Manfroi *et al.* 2009; Beneduce *et al.* 2010; Pramateftaki *et al.* 2012). Moreno-Arribas *et al.* (2003) demonstrated that the application of commercial malolactic starters in wines was useful to reduce the BAs amounts; in inoculated wines, BAs concentrations were significantly lower when compared to those not inoculated. In non-inoculated wines the amounts of BAs were higher, probably because MLF was conducted by indigenous malolactic bacteria producers of BAs. These results suggest that the use of selected malolactic starters can minimise the production of BAs. Also Manfroi *et al.* (2009) showed that the addition of starter culture during MLF compared to spontaneous fermentation, provided better quality wine with reduced accumulation of putrescine (*L. plantarum* DSM 4361) and cadaverine (*L. plantarum* DSM 4361 or *O. oeni* DSM 12923).

1.5 Starter cultures

1.5.1 Malolactic starter cultures: Description

One method to control that the MLF is carried out in winemaking process, and not detrimental compounds are formed, is inoculating high concentration of LAB using bacterial starter cultures. Starter cultures are defined as preparations which contain living microorganisms which are applied with the intention of making use of their microbial metabolism (Hammes 1990). Generally, the preparations used as starters can be classified within 3 categories: undefined cultures, single-strain cultures and multiple-strain cultures. Undefined cultures are based on fermenting substrate use, taken from a selected process that resulted in good-quality end products. These starters are propagated and distributed commercially. The other categories are represented by single-strain cultures, and multiple-strain cultures, which contain one or more selected strains, respectively. General requirements for starter cultures regarding safety, technological effectiveness, and economics are summarized in **Table 2**.

Table 2. General criteria for starter cultures (adapted from Buckenhüskes 1993)

1. Safety
1.1 Starter organisms are not in possession of any pathogen or toxic activity
1.2 The preparations are free from hygienic infections or substances
2. Technological effectiveness
2.1 Starter microorganisms dominate over the spontaneous microbiota
2.2 Starter organisms perform to required metabolic activity
3. Economic aspects
3.1 The propagation must be feasible from economical point of view
3.2 The starter culture can be preserved by refrigeration, freezing or freeze-drying with little practical loss of activity
3.3 The important properties are stable under defined storage conditions for several months
3.4 The handling of the starter culture must be as easy as possible

Liquid malolactic cultures have been available and used for decades until early 1980's when frozen and freeze-dried malolactic bacteria starter cultures were developed. The 1990's saw the development of direct inoculation freeze-dried malolactic starter cultures. Most of commercial starter cultures available for winemaking are storage under refrigerate or frozen conditions in their original and unopened package. The container should not be opened until just before use. In addition, these freeze-dried bacteria should avoid contact with oxygen, excess moisture and high temperature, because these conditions are detrimental to bacteria survival (Krieger 2009).

Table 3. A selection of malolactic starter cultures available in the industry (adapted from Torriani *et al.* 2011)

Company	Culture	Presentation
AEB group	Biolact Acclimatée; Biolact Acclimatée BM; Biolact Acclimatée PB1025 ; Biolact Acclimatée 4R;	Freeze-dried
Anchor	NT202 Co-inoculant	Freeze-dried
CHR Hansen	Viniflora Oenos; Viniflora CH11; VinifloraCH16; Viniflora CH35 Viniflora plantarum Viniflora Freasy CH16	Freeze-dried Frozen
Enartis Vinquiry	ML One; ML Silver; MCW-Freeze-Dried MCW-Liquid; ML-34	Freeze-dried Liquid
EverIntec	Extremo X 03	Freeze-dried
Laffort	Lactoenos 350 Preac; Lactoenos 450 Preac ; Lactoenos SB3 Instant; Lactoenos B16 Standard; Lactoenos B28 Preac	Freeze-dried
LAMOTHE-ABIET	Oeno 1; Oeno 2	Freeze-dried
Lallemand	Lalvin 31; Lalvin VP41; Lavin PN4; Lavin ICC Elios; Uvapherm Alpha; Uvapherm Beta; Duo Merlot; Duo Riesling; Duo Red-Y-Fruits; V22	Freeze-dried
Oenofrance	FML Expertise C; FML Expertise S; FML Expertise Extrême	Freeze-dried
SGBIOTECH	SRW	Frozen
Tebaldi.it	ExperTi oeni; ExperTi oeni Alcol; ExperTi oeni pH	Freeze-dried
WYEAST	ER1A; EY2D; 4007 Blend	Liquid

Nowadays many different malolactic starter cultures exist in industry, liquid, frozen or freeze-dried that are adapted to the wine specific necessities (**Table 3**).

In some cases an acclimation phase is required to activate the culture before its inoculation in wine. Usually, the bacterial culture is mixed with a pH>3.5 wine, at a temperature between 20 and 25 ºC during 18-24 hours. This step must be generally done, especially when the wine presents a high alcohol content and low pH. Evermore, sometimes is necessary to add nutritional supplements for a successful induction of the MLF. Also some freeze-dried cultures need a previous rehydration phase, dissolving the content in water during 20 minutes. Nevertheless, most of the modern malolactic starter cultures have a direct inoculation in wine what reduces the time for the starter preparation. Most commonly *O. oeni* starter cultures are used, but there are also some preparations with lactobacilli reported to give good results (Prahl *et al.* 1988; Krieger 2009). Also, new strategies have been developed to carry out MLF in wine, like the use of immobilized LAB as starter cultures. This technology provides advantages compared to free cells because the support acts as a protection agent against the effects of pH, temperature, ethanol, etc. (Berbegal *et al.* 2012). The high bacterial concentration permits a faster MLF, fermentation at low temperatures, easier recovery and reuse of the starter culture (Kourkoutas *et al.* 2004).

From the 80s, many studies have been done to immobilize LAB in different materials (Prévost *et al.* 1994; Maicas 2001; Kourkoutas *et al.* 2004). An example of these works is the study of Rossi and Clementi (1984). They studied the catabolism of malic acid in *O. oeni* immobilized in polyacrylamide gel. Other materials investigated for immobilization were k-carragean (McCord and Ryu 1985; Crapisi *et al.* 1987) and alginate gel (Spettoli *et al.* 1982). Later on, polyacrylamide, silica gel, chitosan and wood chips were used to immobilize *Lactobacillus* and *O. oeni* with satisfactory results (Naouri *et al.* 1991), where they were able to induce decarboxylation of a red wine at low pH (3.0) and low temperature (<20 ºC). Maicas *et al.* (2001) studied the immobilization of *O. oeni* in a positively-charged cellulose sponge for carry out the MLF in wine. This system enabled L-malic acid conversion rates higher than 50 % in repeated periods of 24 hours without loss of activity. Agouridis *et al.* (2008) immobilized *O. oeni* in cellulosic material, evaluated its stability in repeated MLF and the production of volatile compounds. The results showed that the cellulosic material was a good support for *O. oeni*

immobilization. Latest experiments have been done with new materials as Lentikats, a polyvinyl alcohol (PVA) matrix (Rodríguez-Nogales *et al.* 2013). The entrapment of *O. oeni* in this PVA was successfully reused for six cycles, retaining an efficacy of 75 % for the conversion of malic acid into lactic acid. The immobilized cells gave better performance than free cells due to the increase of the alcohol tolerance. Moreover, the immobilization of *O. oeni* cells in natural materials for carry out the MLF in white wine has been studied (Genisheva *et al.* 2013). *O. oeni* cells were immobilized on corn cobs, grape skins and grape stems and the immobilized bacteria were more resistant against the inhibitory effect of high concentrations of ethanol, SO_2 and elevated temperatures. They also were capable to perform repetitive MLF for long periods of time, at least 5 months.

Recently, a combination of materials has been studied for the immobilization of microorganisms. Callone *et al.* (2008) investigated the immobilization of yeast and bacteria in alginate microbeads coated with silica. The results showed that the subsequent coating with a silica gel layer improved the mechanical stability and reduced the leakage. In a posterior work, Guzzon *et al.* (2012) immobilized *O. oeni* cells in alginate microbeads covered with a methyltrietoxysilane membrane using a specially designed pilot scale apparatus for Ca-alginate bead production. The results showed that the use of immobilized bacteria allowed simultaneous AF and MLF in grape must and the achievement of MLF in wine with lysozyme added to suppress wild LAB. The porosity of the siliceous membrane constituted an effective barrier for lysozyme, enabling the MLF by immobilized LAB and preventing the activity of wine spoilage bacteria.

1.5.2 Selection criteria of lactic acid bacteria for winemaking starter culture preparation

As explained previously, relying on indigenous bacteria to complete spontaneously and timely a desirable MLF can be unpredictable. Even when desirable LAB are established in a winery, the onset of the MLF may take several months and may occur in some barrels and vats but not in others. For this reason, induction of the MLF by the use of selected bacterial starter cultures is becoming the preferred option. Selecting strains of *O. oeni*, which are the best in terms of performance and interesting in flavor production, is a challenging task. The physiology

and the genetic profiles of new and interesting strains are determined in the laboratory and in pilot vinifications. Different selection criteria have been established to find the best MLF starters. Some of the most important criteria to study are the resistance to low pH, ethanol and low temperatures, kinetics of survival and kinetics of malate degradation. The selected strains must also be suitable for industrial preparation; this requires that the bacteria are easily cultivated on large scale and that they are highly stable during the storage. An example of selection criteria is pointed in **Table 4**.

Table 4. LAB selection criteria for induction of MLF in wine (adapted from Buckenhüskes 1993)

1st order criteria
Resistant to low pH
Resistant to ethanol
Tolerant to low temperatures
Limited metabolism of hexoses and pentoses
2nd order criteria
Count of living organisms after propagation
Time of propagation
Yield by propagation
Kinetics of survival
Kinetics of malate degradation
3rd order criteria
Limited interactions with yeast of the AF
Limited interactions with other LAB
Phage resistance
Resistant to pesticides
No formation of BAs
Citrate metabolism under aerobe conditions
Potential to form dyacetil and acetoine
Limited potential to form volatile acids from hexoses and pentoses
Probably no formation of acetic acid
Limited metabolism of organic acids of wine
No glycerine degradation
No sensorial alterations of wine

At the present time, many approaches have been done in order to select *O. oeni* strains to produce malolactic starters. An example of these studies is the work from Sico *et al.* (2008), where the malolactic activity, SO_2 resistance, acetaldehyde metabolism, and other technological characteristics were tested to characterize and select *O. oeni* strains isolated from Aglianico wines. The results showed that none strain was able to grow in presence of 14 % of ethanol. About 80 % of *O. oeni* strains degraded more than 80 % of acetaldehyde and, 4 isolates from 9, were sensitive to 60 mg/L of SO_2. Considering their technological characteristics, 5 *O. oeni* strains were selected as starter cultures. The specificity of indigenous strains could be interesting for local producers as a way to induce MLF with native strains. Bordas *et al.* (2013) isolated 21 different strains of *O. oeni* from south of Catalonia (wines easily reach an ethanol content of 14 %), with a high ethanol tolerance, to serve as promising malolactic starters in wines with similar qualities. Other authors have studied the characterization of *O. oeni* strains with molecular and physiological tools in order to select malolactic starters (Coucheney *et al.* 2005b; Renouf *et al.* 2009; Capozzi *et al.* 2010; Ruiz *et al.* 2010). The molecular techniques can be carried out more quickly and more reliably than physiological ones for the selection and production of malolactic strains for direct inoculation into wine. The idea is to correlate molecular with physiological techniques, and using a molecular analysis, select strains with specific physiological characteristics. As example, Coucheney *et al.* (2005b) measured malolactic and ATPase activities and evaluated the expression of a small heat shock protein Lo18 in 3 commercial malolactic starters of *O. oeni*. The results showed on intact cells at pH 3 differences for the global malolactic activity between strains, and the level of induction of the small heat shock protein Lo18. The strain that presented the highest malolactic activity and the highest level of Lo18 induction showed a high growth rate and a high kinetics of malate consumption. Also, Renouf *et al.* (2009) by targeting the *rpo*B gene (RNA polymerase *beta* subunit) during spontaneous winemaking, found 2 types of *O. oeni* strains resulting from a single mutation in the *rpo*B region, generating 2 different electrophoretic profiles, L and H. The results confirmed the relevance of *rpo*B mutation for characterizing the 2 *O. oeni* subgroups. The L strains reacted favorably to the grape must environment, and H strains were most resistant to post-fermentation stress, specially the addition of SO_2. The relationship between the physiological response to stress and the *rpo*B allele raise a technological interest to select an *O. oeni* strain to become malolactic starter.

1.5.3 *O. oeni* starter culture biomass preparation

Successful inoculation of the bacterial starter culture into wine depends not only on the suitable strains of *O. oeni*, but also on the preparation and use of the starter culture. For the production of a suitably large biomass for industrial use, selected strains of *O. oeni* are grown under conditions which permit rapid growth and result in a high cell yields with high malolactic activity and high survival rate in wine. The conditions for bacterial growth in wine are very different: the optimum temperature for growth is near 30 ºC, the temperature of wine is commonly between 15 ºC and 25 ºC; synthetic media for the production of bacterial biomass do not contain inhibitory substances such ethanol, SO_2 or phenolic compounds, yet wine does. If a bacterial culture grown under much different conditions, is inoculated directly into wine, it will lose much viability (Henick-Kling 1988). So, it is necessary to grow the bacteria in an appropriate medium to obtain biomass but also the medium must prepare cells to the harsh wine conditions. This entails to find the optimal growth conditions to achieve the maximum cell population with the subsequent high cell viability in wine. Therefore, investigations into better understanding of the growth behaviour are needed in order to develop chemically defined media under optimal conditions.

Some studies have been focused on the perfect medium to produce *O. oeni* biomass. *O. oeni* is usually grown in complex culture media such as MRS (De Man *et al.* 1960), FT80 (Cavin *et al.* 1988) and AGB (Dicks *et al.* 1990). Most of them frequently contain grape juice (Davis *et al.* 1985) or apple juice (Champagne *et al.* 1989b). They are generally supplemented with other nutrients like yeast extract, peptone, and Tween 80, increasing the biomass production (Pilone and Kunkee 1972; Kunkee 1974; Champagne *et al.* 1989a; Krieger *et al.* 1990; Guerrini *et al.* 2002). The difficulty in obtaining and storing fresh juice has been a major factor in the high cost of producing malolactic cultures. Also, the juice quality varies with supplier and growing season. Differences in juice quality can significantly change the rate of growth and cell yield (Zhang and Lovitt 2006). The growth of *O. oeni* has been also investigated over single sugars and their mixtures. The best growth was obtained with sugar mixtures (glucose-fructose) instead of growth on a single sugar (Maicas *et al.* 1999b; Zhang and Lovitt 2005). Other components as manganese or yeast mannoproteins and nutrient requirements have been studied. Results revealed that the essential bacterial nutrients were strain specific and *O. oeni*

strains showed a large number of auxotrophies (Theobald *et al.* 2005; Terrade and Mira de Orduña 2009; Diez *et al.* 2010).

During the preparation of malolactic starter cultures, two groups of factors have to be considered: those affecting cell growth and viability, and those affecting the activity of the malolactic system. Desired conditions must be chosen to give a large yield of biomass that retains high cell viability and malolactic activity. The use of a basal medium with known composition and supplemented with specific nutrients at specific stages of growth is required in order to optimize cell viability and malolactic activity. Hayman and Monk (1982) evaluated the effect of adding wine to a medium for the production of *O. oeni* and found that a content of 40-80 % wine in the medium improved survival and malolactic activity of the culture in the wine. The best time for harvesting *O. oeni* was 18–24 hours after the culture had reached the stationary phase; the cells had the highest survival and malolactic activity after inoculation into wine (Krieger *et al.* 1990). Also, the effects of temperature and pH have to be considered. Optimum pH for growth is from 4.5 to 4.8, while optimum temperature is between 28 ºC and 32 ºC. In both cases this is strain dependent (Champagne *et al.* 1989a; Maicas *et al.* 1999b) and thus more studies have to be done in each case.

1.5.4 Starter culture storage and inoculation in wine

Failures in MLF also occur because of the adaptation lack of cultures to wine or because of cellular damage of the malolactic starter during the storage. In the study of Maicas *et al.* (2000), the culture medium used (MP medium) reduced the preparation time of the *O. oeni* starter culture, prepared cells to survive after the storage, and allowed the growth in wine in comparison to MLO culture medium. The storage at -20 ºC was an effective procedure. Similar results were obtained freeze-drying the *O. oeni* starter culture.

During the freeze-drying, bacterial cell survival depends on many factors, including previous growth conditions, protective medium and initial cell concentration, freezing temperature, and rehydration conditions. Protective agents play an important role in preservation and cell viability (Zhao and Zhang 2009b).

Rehydration is also a critical step in the recovery of freeze-dried bacteria. Direct rehydration in wine can result in a mortality of 95 % of the freeze-dried culture (Davis *et al.* 1985). If a rehydration medium is used, its molarity and the rehydration conditions can significantly affect the rate of recovery. Storage conditions can affect also the stability of the freeze-dried culture. LAB cultures are usually stored at a temperature below 5 ºC. Many compounds offer protection to freeze-drying itself but do not prevent cell viability losses during storage. Different studies have evaluated the influences of protectants, rehydration media and storage of LAB after freeze-drying (Champagne *et al.* 1996; Zhao and Zhang 2009a; Zhao and Zhang 2009b).

Inoculation time of starter cultures is an important factor influencing the success of induced fermentations, and various studies have been carried out to determine the effect of bacterial inoculation time on vinification kinetics, chemical composition and sensory and sanitary attributes of wines (Rosi *et al.* 2003; Alexandre *et al.* 2004; Abrahamse and Bartowsky 2011; Mendoza *et al.* 2011). Results from some of these studies have shown that simultaneous yeast and bacteria inoculation poses important risks, such as the development of undesirable and antagonistic interactions between the two microorganisms, stuck AF, interruption of AF before sugar depletion, wines with increased concentrations of acetic acid that render them unacceptable for consumption, or the production of possible off-odors. On the contrary, other authors recommend simultaneous inoculation of yeast and bacteria into the grape must (Zhang and Lovitt 2006; Cañas *et al.* 2012; Pan *et al.* 2011). They think that the bacteria can in this way get use and grow better in the presence of ethanol and with a big amount of nutrients at their disposal, and that these conditions will not necessarily lead to an excessive acetic acid production (Beelman and Kunkee 1985). In this case, the interactions between the yeasts and bacteria must be taken into account (King and Beelman 1986; Costello *et al.* 2003; Larsen *et al.* 2003; Comitini and Ciani 2007). The microbiological stability of the wine seems to be another advantage of the yeasts and bacteria co-inoculation, earlier MLF avoids the maintaining of the wine under dangerous conditions without SO_2 for long lasting periods, and avoid exposition to the development of different contaminant microorganisms, like the undesirable acetic acid bacteria (Ribéreau-Gayon *et al.* 2003). However, co-inoculation is not yet a very common practice and new and more exhaustive studies are needed to gain more knowledge of the influence of early bacterial inoculation on the winemaking process and on wine quality (Cañas *et al.* 2012).

2. Objectives

The main objective of this work is to select *O. oeni* strains well adapted to red wines and to evaluate the success of *O. oeni* liquid starter cultures to lead the MLF.

To carry out this goal the following specific objectives are set out:

- To select an *O. oeni* strain to carry out the MLF, choosing the best one, able to resist and grow at wide range of pH levels, at the highest level of ethanol, and that was not able to form BA.

- To design a liquid culture medium for growing *O. oeni* that would permit high levels of biomass production, but also an adequate adaptation to wine conditions.

- To carry out the scale up of the process to obtain industrial levels of *O. oeni* biomass, using the culture medium designed, what would permit the realization of starter cultures with the desired *O. oeni* strain, depending on the winery necessities and also to study the viability and malolactic activity of the MLF starter culture in large volumes of wine.

- To reduce the histamine content of a red wine by selecting and inoculating an autochthonous strain of *O. oeni* in a cellar, previously grown in the designed culture medium.

- To optimize the preservation of the MLF starter, finding the best way to inoculate the *O. oeni* culture in wine with the maximum malolactic activity and cell viability.

- To develop alternative methods of malolactic starter inoculation in wine thought the immobilization of the bacteria, improving the adaptation of *O. oeni* to wine conditions and increasing cell viability. The coimmobilization of *O. oeni* with *S. cerevisiae* is postulated.

3. Materials and methods

3.1 Microorganisms

3.1.1 *Oenococcus oeni* strains

Forty *O. oeni* strains from the Enolab collection (**Table 5**) isolated previously from red wines with spontaneous MLF and preserved in our laboratory, were included in the selection process to carry out the first objective of the study. The aim was to select those ones that were able to survive and grow at the highest levels of ethanol (9, 11, 13 % (v:v)) lowest pH levels (3.2, 3.5, 3.8) and that were not able to produce BAs.

Table 5. *O. oeni* strains from Enolab collection used in the selection process.

***O. oeni* strains**	
E3841	E4065
E3874	E4066
E3995	E4067
E3996	E4069
E4046	E4070
E4047	E4071
E4048	E4072
E4052	E4134
E4053	E4135
E4054	E4136
E4055	E4154
E4056	E4155
E4057	E4157
E4058	E4158
E4059	E4159
E4060	E4161
E4061	E4701
E4062	E4709
E4063	E4959
E4064	E5003

3.1.2 *Saccharomyces cerevisiae* strains

Two different *S. cerevisiae* strains were used to carry out the AF in red must in the experiments throughout the project (**Table 6**).

Table 6. *S. cerevisiae* strains used to conduct the AF in red must

S. cerevisiae strain	Origin
Viniferm B4	Agrovin S.A.
AXAZ-1	Greek grapes

3.2 Culture media, must and wine

3.2.1 Culture media

ACE (medium for acetic bacteria)

Glucose	25 g/L
Yeast extract	10 g/L
Agar (only for solid media)	20 g/L

GPYA (medium for yeast and moulds)

Glucose	40 g/L
Mycological peptone	5 g/L
Yeast extract	5 g/L
Agar (only for solid media)	20 g/L

MRS (General medium for LAB) (De Man *et al.* 1960)

MRS BROTH Oxoid CM 359	52 g/L
L- cysteine	0.5 g/L
Agar (only for solid media)	20 g/L

pH 6.5 (except if growth with different pHs is studied)

MLO (medium for *O. oeni*) (Zúñiga *et al.* 1993)

Tryptone	10 g/L
Yeast extract	5 g/L
Glucose	10 g/L
Fructose	5 g/L
$MgS0_4.7H_2O$	0.2 g/L
$MnSO_4.H_2O$	0.05 g/L
Diamonic citrate	3.5 g/L
L-cysteine	0.5 g/L
Tween 80	1 mL
Tomato juice*	100 mL
Natamycine*	0.075 g/L
Agar (only for solid media)	20 g/L

pH 4.8 (except if growth with different pH is studied)

The pH is adjusted and autoclaved at 115 ºC, 30 minutes. The agar is sterilized in the autoclave separately with the water.

***Tomato juice**

Tomato juice	1 L
Distilled water	2 L

Macerate at 4 ºC overnight

Centrifuge for 20 minutes at 14000 rpm

Filter thought filter paper

Preserve at -20 ºC to its use

***Natamycine**

Natamycine is only added when yeast growth must be inhibited. The amount of 0.15 g/L of Actistab (corresponding to 0.075 g/L of the antibiotic Natamycine) were dissolved in 10 mL of DMSO (dimethyl sulfoxide) and were added to the MLO culture medium after the autoclave sterilization to prevent the yeast growth.

MDB-mod (medium for BAs detection) (Landete *et al.* 2005a)

Meat extract	8 g/L
Triptone	5 g/L
Yeast extract	4 g/L
Glucose	1.5 g/L
Fructose	1 g/L
Tween 80	0.5 g/L
$S0_4Mg$.$7H_2O$	0.2 g/L
$MnSO_4$ .H_2O	0.05 g/L
$FeSO_4$	0.04 g/L
$CaCO_4$	0.1 g/L
Pyridoxal hydrochloride	0.25 g/L
Bromocresol purple	0.03 g/L
Histidine	2 g/L

pH 5.2

OMP (medium designed in this thesis for *O. oeni* growth)

Yeast extract	5 g/L
Tomato juice*	23 mL/L
Tween 80	0.5 mL/L
L-malic acid	3 g/L
White concentrate must diluted 1/6	576.5 mL/L
White concentrate wine reconstituted	400 mL
Ethanol	4% (v:v)

pH 3.8

The pH is adjusted and autoclaved at 115 ºC, 30 or 45 minutes. The ethanol content (4 %) is added after the sterilization.

PCA (General medium for bacteria)

Triptone	5 g/L
Yeast extract	2.5 g/L
Glucose	1 g/L
Agar	20 g/L

3.2.2 Must and wine

Grape must and wine were used for the media production and for the MLF experiments.

Reconstituted concentrate commercial white wine (Agrovin S.A.)

Glucose	0.13 g/L
Fructose	0.14 g/L
Malic acid	0.42 g/L
Lactic acid	0.59 g/L
Tartaric acid	3.90 g/L
Acetic acid	0.07 g/L

pH 3.54

Reconstituted (1/6) concentrate commercial white must (Agrovin S.A.)

Glucose	58 g/L
Fructose 5	9 g/L
Malic acid	1.48 g/L
Lactic acid	0.03 g/L
Tartaric acid	0.91 g/L
Acetic acid	0 g/L

pH 3.54

Red wine

All red wines used in the project (except wine from Ribera del Duero region) were winemade at the laboratory using a red commercial grape must (Don Simón), inoculated with *S. cerevisiae* Viniferm B4 (Agrovin S.A.) in a final concentration of $2x10^6$ ufc/mL, and were incubated at 28 ºC, 10 days, until the AF was finished. When the sugar concentration was less than 1 g/L, the ethanol was adjusted to 12 %, 3 g/L of L-malic acid were added, and the pH was adjusted to 3.5. Then, it was sterilized by filtering through 0.22 µm pore filter and stored at 15 ºC until its use.

Red commercial grape must (Don Simón)

Glucose	83 g/L
Fructose	83 g/L
Malic acid	0.30 g/L
Lactic acid	0.04 g/L
Tartaric acid	4.7 g/L
Acetic acid	0.001 g/L

3.2.3 Wine lees

Three different Tempranillo wine lees from Utiel-Requena region were used to preserve *O. oeni* E5003 (**Table 7**).

Table 7. Chemical analysis of the three red wine lees (A, B and C)

	A Lees	B Lees	C Lees
Malic acid (g/L)	0.01	0.02	0
Lactic acid (g/L)	1.52	1.32	1.79
Glucose (g/L)	0.92	0.06	0.52
Fructose (g/L)	0.35	006	0.16
pH	3.96	3.92	3.97
Polyphenols (IPT)	27.9	13.5	34.3
Polysaccarids (mg/L)	1200	1350	2835

3.3 Microorganisms' observation and cell counting methods

Different counting methods have been used to enumerate the *O. oeni* strains in culture media and red wine.

3.3.1 Absorbance spectroscopy

Bacterial growth kinetics was measured by absorbance at 600 nm in a CE 373 spectrophotometer (CECIL).

Data from growth kinetics measured as DO (600 nm), was transformed in a value of area under the curve (AU) with the GraphPad Prism 5 software. The area under the curve is an integrated amount of a cumulative measurement of growth. This area is computed using the trapezoid rule. It simply connects a straight line between every set of adjacent points defining the curve, and sums up the areas beneath these areas. A curve is simply a series of connected XY points, with equally spaced X values. **Figure 11** shows two of these points and the baseline as a dotted line. The area under that portion of the curve, a trapezoid, is shaded. The area, therefore, is ΔX*(Y1+Y2)/2. This formula is repeatedly for each adjacent pair of points defining the curve (Gagnon and Peterson 1998).

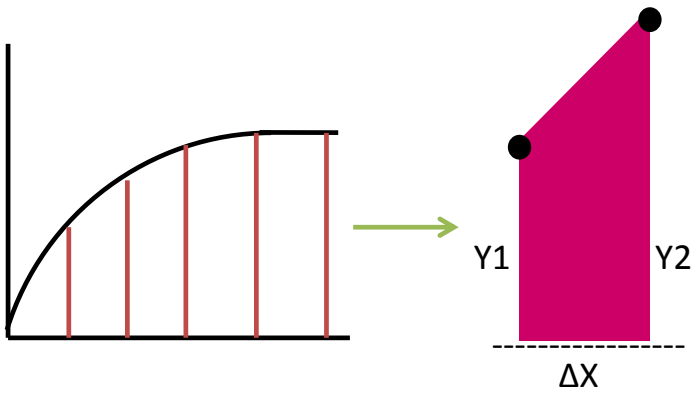


Figure 11. Value estimation of the area under the curve using the trapezoid rule

3.3.2 Total cell count

Total bacterial count was realized using a Thoma counting chamber (0.100 mm x 0.0025 mm^2) using the 40X objective from a Leica DM LB microscope. The cell population was estimated from the average of chains counted in the 20 squares of the diagonal of the counting chamber.

3.3.3 Viable cell count

Cell viability in culture media and in wine was studied by plate counting. The volume of 0.1 mL of decimal serial dilutions in sterile saline solution were spreaded by duplicate on MLO or MRS agar plates and were incubated at 28 ºC for 7 days and then the colonies were counted.

3.3.4 Fluorescence-based assays

Bacterial viability was determined by LIVE/DEAD BacLight bacterial Viability Kit (Invitrogen). One mL of culture or wine sample was centrifuged at 8000 rpm 5 minutes. The pellet was resuspended in 100 µL of distillated water and 0.3 µL of the mix (1:1) (solution A: solution B) from the kit was added. After 20 minutes of incubation in the dark, the samples were observed at 100X with immersion oil with a fluorescence microscope Leica. Dead bacteria were observed in red and live bacteria in green.

3.3.5 Scanning electron microscopy

O. oeni and *S. cerevisiae* on cellulosic material with starch gel were monitored by scanning electron microscopy. The biocatalyst sample was coated with gold blazers SCD 004 sputter coater for 2 minutes and examined under JSM-6300 scanning electron microscope.

3.4 Microorganisms' preservation

A range of microorganism preservation methods were employed to maintain the microorganisms for short and long term, and to investigate also how they affect the bacterial culture viability.

3.4.1 Crioprotection

The crioprotection was carried out growing the bacteria in culture medium until the end of exponential phase, and then bacterial cultures and sterile glycerol at 30 % (v:v) in water were mixed 50 % each. The mixture was frozen at -20 ºC in tubes of 2 mL.

Also freezing was achieved growing *O. oeni* until the exponential phase in *Oenococcus* Medium Production (OMP) medium. Bacterial cultures were preserved by freezing at -20 ºC in aliquots of 8 mL for 8 months.

3.4.2 Freeze-drying

Freeze-drying was realized after growing the bacteria until the end of the exponential phase in 50 mL of culture medium (MLO or OMP). The cells were recovered though centrifugation at 6000 rpm 15 minutes in a Heraeus Multifuge 1 S-R centrifuge. Then, the cells were washed twice with glutamic acid 5 %, recovered with the same above centrifugation conditions, and resuspended in 2 mL of 15 % glutamic acid. The bacteria solution was distributed in 400 µL per tube. Tubes were frozen at – 20 ºC, 1 hour. The freeze-dying was performed at -60 ºC for 18 hours under vacuum (Virtis Sentry). The tubes were vacuum sealed and stored at 4 ºC under dark.

3.4.3 Refrigeration

Refrigeration was realized growing *O. oeni* until the exponential phase in OMP medium and keeping the cultures at 4 ºC in aliquots of 8 mL for 8 months.

3.4.4 Preservation in wine lees

Wine lees from Tempranillo wine from Utiel-Requena region were sterilized by autoclaving at 115 ºC, 30 minutes. 10 mL of wine lees were inoculated with $3x10^7$ cfu/mL of *O. oeni*. The inoculated wine lees were stored for 4 months at room temperature.

3.5 Bacterial isolation

Bacterial isolation from wine was carried out by plating 0.1 mL (from the different serial dilutions of each wine sample in NaCl 9 % solution), on MRS and MLO supplemented with 0.15 mg/L of Actistab (0.075 mg/L natamycin) (Gist-Brocades) to prevent the development of yeast and molds. All plates were incubated at 28 ºC for 7 days. After that time, typical colonies of LAB with different morphologies were selected and purified by streaking in fresh media. Then the isolates were crioprotected.

3.6 Typing by random amplified polymorphic DNA PCR

Wine isolates identified as *O. oeni* were strain characterized using the RAPD-PCR technique, with the M13 primer Eurofins mwg/operon (5' GAG GGT GGC GGT TCT 3') (Zapparoli *et al.* 1998). DNA amplification was carried out in 50 µL PCR mixture containing 200 mM dNTPs, 1 mM M13 primer, 10 mM Tris/HCl (pH 8.8), 50 mM KCl, 0.1 % Triton X-100, 2 mM MgCl, 1 U of DyNazyme II DNA polymerase (Finnzymes) and 1 µL of the cell suspension (1 colony suspended in 10 µL sterile milli-Q water). PCR was performed in a PTC-100™ Thermal cycler (MJResearch, Watertown, USA) following the conditions described by Rodas *et al.* (2005). PCR products were resolved by electrophoresis (80 v, 3 hours) in 1.2 % (w/v) SeaKem LE agarose (FMC, USA) gels in 0.5xTBE (45 mM Tris-HCl, 45 mM boric acid and 1mM EDTA pH 8.0), and stained with ethidium bromide (0.5 µg/mL). Gels with amplification fragments were visualized and photographed under UV light with the GelPrinter Plus (TDI, Madrid, Spain), and 1 Kb Plus DNA Ladder (Invitrogen) was used as molecular marker to know the size of the fragments.

BioNumerics software (version 6.5, Applied Maths, Kortrijk, Belgium) was used to register RAPD-PCR patterns, normalize densitometries traces, calculate the Pearsons' correlation coefficient, and perform cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA). To determine the minimum percentage of similarity (*r*) necessary for strain discrimination, a reproducibility study was carried out (Ruiz *et al*. 2008).

3.7 Sugar consumption and malolactic activity

Sugars and malic acid consumption and the lactic acid formation in grape must, medium or wine were determined by HPLC equipment (Agilent series 1200) with an isocratic pump (Agilent G1310A) following the procedure described by Frayne (1986) with minor modifications. The mobile phase consisted of a solution of 0.75 mL of 85 % H_3PO_4 per liter of deionized water with a flow rate of 0.7 mL/min. An Agilent G1322A degasser was employed. Samples were injected automatically (Agilent G1367B). The separation of the components was carried out using an Aminex HPX-87H precolumn (Bio-Rad) coupled with two ion exclusion columns of 300 mm by 7.8 mm Aminex HPX-87H (Bio-Rad) thermostatically controlled at 65 °C (Agilent G1316A). Compounds were detected by a G1314B variable-wavelength detector (Agilent) set to 210 nm and a refractive index detector (Agilent G1362A) in series. The elution time was 45 minutes. External calibration was performed. All samples were centrifuged and filtered through a membrane filter with a mean pore size of 0.22 μm before used. Quantification was performed by measuring peak height compared to external standards.

3.8 Determination of biogenic amines formation

The BAs determination method was improved using the new core-shell HPLC column technology to reduce time and cost analysis.

3.8.1 Standard solutions and bacteria inoculation

Stock solutions of histamine, tyramine, cadaverine and putrescine were made as 2000 ppm in methanol. The working solutions were prepared by dilutions with milli-Q water. The

standards were stored at -20 ºC. Ten ppm of histamine, tyramine, putrescine and cadaverine were added to a red wine (pH 3.5, 12 % ethanol (v:v)) as a control for the study.

Wine samples preparation and the internal standard (IS) addition were realized following the recommended protocol proposed by Hernández-Orte *et al.* (2006) with some modifications. One mL sample of wine was centrifuged at 8000 rpm for 10 minutes and the filtered through a membrane filter with a mean pore size of 0.22 µm. A solution was prepared with 760 µL of filtered wine and 40 µL of 2-amino heptanoic acid (IS, 8 ppm).

The *O. oeni* strains were firstly grown in MLO (pH 4.5), washed with distilled water and inoculated in MDB-mod media at $2x10^7$ cfu/mL. MDB cultures were incubated at 28 ºC during 15 days and analyzed by HPLC.

3.8.2 Extraction and pre-column fluorescence derivatizacion

The isolation of the BAs was performed using a method based on solid-phase extraction with mixed-mode resins (oasis MCX, reverse–phase and ion exchange) developed by Peña-Gallego *et al.* (2009). Resin was conditioned by passing 2 mL of methanol followed by 2 mL of milli-Q water. 0.6 mL of the sample with the IS were percolated. Three consecutive washings were made: (a) 2 mL of 10 mM H_3PO_4:MeOH (90:10) solution; (b) 2 mL of 10 mM NaOH:MeOH (70:30) solution; (c) 2 mL of 10 mM $CaCl_2$:MeOH (70:30) solution. The elute was collected in a vial containing 100 µL of 1.2 M HCl. Between each loading, washing and eluting stage, the column was washed with 1 mL of milli-Q water. The derivatization of the standards and samples was carried out following the method described by Cohen and De Antonis (1994) with the modifications proposed by Hernández-Orte *et al.* (2006). 20 µL of sample were buffered with 50 µL of a 0.2 M solution of sodium borate at pH 8.8, which contained 5 mM of disodium EDTA. The derivatization reaction was made by adding 30 µL of the AccQ-Fluor™ Reagent Kit.

3.8.3 High performance liquid chromatography analysis

The HPLC analysis was performed using an Agilent 1200SL HPLC system equipped with an in-line degasser (Agilent G1322A), autosampler (Agilent G1367B), column heater (Agilent G1316A) and fluorescence detector (Agilent G1321A). The chromatographic separation was performed using two different HPLC columns: a 250 mm x 4.6 mm column (Luna C18 silica, Phenomenex) with a 20 mm x 4.6 mm precolumn, or a 100 mm x 4.6 mm column (Kinetex 2.6 µm PFP, Phenomenex) with a guard column (Krudkatcher Ultra, Phenomenex).

A solution of 140 mM sodium acetate trihydrate and 17 mM TEA adjusted to pH 5.052 was used as mobile phase A, and methanol was used as mobile phase B. The gradient elution program used in Luna C18 silica column was described by Peña-Gallego *et al.* (2006). The flow rate was 1 mL/min. The total elution time was 50 minutes. Two different elution programs were used for the Kinetex PFP column. Program I was from the beginning with a gradient elution as can be seen in **Table 8**. The flow rate was 2 mL/min. The total elution time was 10 minutes. In the other elution program used, program II, the chromatographic separation started with an isocratic elution to separate the histamine peak from others that were close to it. The flow rate was 0.8 mL/min. The total elution time was 25 minutes (**Table 9**).

The excitation and emission wavelengths of the fluorescence detector were set at 250 and 395 mm respectively in all cases. A volume of 5 µL was injected for each analysis. The HPLC column temperature was kept at 65 ºC. Quantification was performed by measuring peak areas compared to external standards.

Table 8. Gradient for the separation of BAs with the Kinetex PFP using elution program I

t (minutes)	% A	% B
0	75	25
2.30	60	40
6.42	35	65
7.19	20	80
7.34	0	100
10.00	0	100

Table 9. Gradient for the separation of BAs with Kinetex PFP using the elution program II

t (minutes)	% A	% B
0	75	25
5.76	75	25
7.30	70	30
9.30	60	40
13.30	40	60
17.30	20	80
21.30	0	100
25.00	0	100

3.8.4 Calibration Curves

Five calibration points were determined at the following concentrations: 0, 5, 10, 25 and 50 ppm of standard solutions of histamine, tyramine, putrescine and cadaverine. The analyses were carried out in triplicate.

3.9 Statistical analysis

The SPSS Statistics 19 program was used to calculate means and standard deviations of the results. Data were normalized and the analysis of variance (ANOVA) test and the Tukey *post-hoc* test were used to determine significant differences between the experiment results.

3.10 *O. oeni* selection

The selection program began with a phenotypic characterization, choosing those *O. oeni* strains that were able to grow at wide range of pH and in the highest levels of ethanol. Then, the BAs formation and the malolactic activity and cell viability in red wine of these strains were studied.

3.10.1 Bacterial growth at pH levels of 3.2, 3.5 and 3.8

For the experiment, tubes with 10 mL of MLO at different pHs were used (3.2, 3.5, and 3.8). The pH level was adjusted with NaOH and HCl. The *O. oeni* strains were firstly grown in MLO (pH 4.5), washed with distilled water and inoculated in the different media at final concentration of 10^6 cfu/mL. The cultures were incubated at 28 ºC.

The bacterial growth was measured at 600 nm every 24 hours during 10 days. The kinetics of each *O. oeni* strain at the three pH levels was studied. Experiments were carried out in duplicate. Quantification was performed by measuring the area under the curve (Gagnon and Peterson 1998).

3.10.2 Bacterial growth at ethanol levels of 9, 11 and 13 % (v:v)

Those *O. oeni* strains with the highest growth in the media with the 3 pH levels were grown in MLO (pH 4.5), washed with distilled water and inoculated in the different media (MLO (pH 4.5) with 9, 11, 13 % of ethanol (v:v)) at 10^6 cfu/mL. The cultures were incubated at 28 ºC.

The bacterial growth was measured at 600 nm every 24 hours during 20 days. The kinetics of each *O. oeni* strain at the three pH levels was studied. Experiments were carried out in duplicate. Quantification was performed by measuring the area under the curve (Gagnon and Peterson 1998).

3.10.4 Malolactic activity measurement in microplate

Two pH indicators were tested to find the most suitable to be used in the miniaturized system; bromophenol blue (Probus) that changes the colour between pH levels of 3 (yellow) and 4.6 (purple), and Congo red (Panreac) that changes colour between pH levels of 3 (violet-blue) and 5.2 (orange-red). The idea was to find which of these pH indicators had the best chromatic result without interfering with the cell viability and with the metabolic properties. The absorbance was measured at different wavelengths (405, 485, 570 and 600 nm), and different pH indicator concentrations were used (0.01, 0.04, 0.06 g/L).

After the selection of the best pH indicator, its concentration and detection wavelength, the *O. oeni* strains that were selected after the selection program (E5003, E4061 and E3874), were grown in liquid MLO to a final concentration of $2x10^9$ cfu/mL, washed twice with distilled water, and inoculated at $1x10^8$ cfu/mL in a final volume of 300 µL of a solution that contained monosodium phosphate (200 mM), L-malic acid (3 g/L) and bromophenol blue (0.04 g/L) in duplicate, without any nutrient, so the bacterial growth and other metabolic activities were inhibited. The pH was adjusted with NaOH and H_2PO_4 (pH 3.08, 3.18, 3.22, 3.36, 3.46, 3.75). Once the cells were inoculated into the microwells, the microplate was incubated at 28 ºC, 24 hours. The absorbance measures were done with a microplate reader (FLUOstar optima, BMG Labtech) set at 600 nm, each two hours during one day. Then, the pH was measured in all microwells with a pH electrode (slimtrode, Hamilton) to relate the absorbance changes to the pH variation, and the malic acid consumed was measured by HPLC to relate it with the pH variation.

3.10.5 Biogenic amines' formation

To study if the *O. oeni* strains selected had the ability to form BAs, the improved HPLC method was employed. The bacteria were grown in liquid MLO to a final concentration of $2x10^9$ cfu/mL. Then, MDB-mod liquid media with the BAs precursors was inoculated to a final concentration of $2x10^7$ cfu/mL and was incubated for 15 days at 28 ºC and was analized by HPLC.

3.10.6 Malolactic activity and cell viability in red wine

The *O. oeni* strains that were able to grow in culture medium with 11 % of ethanol (v:v) and were not able to form amines, were grown in liquid MLO to a final concentration of $2x10^9$ cfu/mL, and then were inoculated in red wine fermented in the laboratory in a final concentration of $2x10^6$ cfu/mL. The cultures were incubated for 15 days at 28 ºC in triplicate to analyze which one had the best malolactic activity and viability. Samples were taken at 1, 3, 7 and 15 days and the malic acid consumption and lactic acid formation were analyzed by HPLC. The viability in wine was studied by viable cell counting on MLO plates.

3.11 Starter culture medium design

To achieve the production of bacterial biomass from the selected strain, a semisynthetic medium was designed, based on the previous knowledge in our laboratory. It was called *Oenococcus* Medium Production (OMP). The culture medium should contain mainly must and wine for adapting cells to the harsh conditions of wine. White wine and must were used because the sterilization of red must and wine caused precipitates, and also the turbidity did not allow following the bacterial growth. Concentrate commercial wine and must were used in order to standardize the culture medium. Even though, this permitted to adjust the ethanol concentration to the desired amount, because the sterilization by the autoclave of commercial wine caused the partial evaporation of ethanol. Nutritional supplements were included in the production medium to benefit the bacterial growth as yeast extract, tween 80, and tomato juice. Also, L-malic acid was incorporated to the medium to activate the MLE during the growth before the inoculation in wine.

The base medium of OMP composition was:

- White reconstituted concentrate must (576.5 mL/L)
- White reconstituted concentrate wine (400 mL/L)
- Tomato juice (23 mL/L)
- Tween 80 (0.5 mL/L)
- L-malic acid (3 g/L)

From this base medium, the influence of the sugar concentration, pH and ethanol concentration in growth and wine adaptation were studied. Concentrate white must was diluted 4 times (175 g/L of sugars), 6 times (117 g/L of sugars) and 8 times (87 g/L of sugars). Also, different levels of pH were studied: the media were adjusted to 3.8, 4 and 4.5. The ethanol influence was measured adding it to the medium, 4 %, 6 % and 8 % (v:v). The final concentration of sugars and malic and lactic acids were measured by HPLC. Thus, a combination of 27 media was obtained (**Table 10**). The *O. oeni* E5003 strain was inoculated in these 27 different media in a final concentration of $2x10^6$ cfu/mL and growth was studied. The cultures were incubated at 28 ºC, 7 days. The growth was measured with the optical density at

600 nm and quantification was performed by measuring area under the curve for the 27 media (Gagnon and Peterson 1998).

The cultures that reached a value of the area under the curve was above 2 AU after 7 days, were inoculated into red wine in a final concentration of $2x10^6$ cfu/mL. The malolactic activity was measured as the malic acid consumption followed by HPLC. From the culture media studied, the cultures that consumed all the malic firstly were chosen.

Table 10. Combination of diluted must (1/4, 1/6 or 1/8), ethanol content (4, 6 or 8 %) and pH level (3.8, 4 or 4.5) resulted in 27 different media for the biomass production

Medium	Must	Ethanol	pH
1	1/4	4	3.8
2	1/4	4	4
3	1/4	4	4.5
4	1/4	6	3.8
5	1/4	6	4
6	1/4	6	4.5
7	1/4	8	3.8
8	1/4	8	4
9	1/4	8	4.5
10	1/6	4	3.8
11	1/6	4	4
12	1/6	4	4.5
13	1/6	6	3.8
14	1/6	6	4
15	1/6	6	4.5
16	1/6	8	3.8
17	1/6	8	4
18	1/6	8	4.5
19	1/8	4	3.8
20	1/8	4	4
21	1/8	4	4.5
22	1/8	6	3.8
23	1/8	6	4
24	1/8	6	4.5
25	1/8	8	3.8
26	1/8	8	4
27	1/8	8	4.5

3.12 Biomass production

3.12.1 Fermenters

The 0.5 L fermenter was built using a Pyrex bottle with a screw cap GL 45 with three ports GL 14 thread (Duran). One of these ports was closed with a screw cap GL 14, the second one was connected to a set for pressure compensation (Duran) to permit fermentation gases to vent out, and the third one was connected with a screw Cap GL 14 for hose connection (Duran) with a joint silicone diameter 0.6 mm and a silicone tube of 3x6 mm. This silicone tube was ended with a 3.2 mm luer lock male connector to join one of the ways of the PVDF luer lock 3 way valve (Cole Palmer) of the 10 L fermenter.

The 10 L fermenter was built using a Pyrex bottle with a screw cap GL 45 with three ports GL 14 thread (Duran). One of these ports was connected to a set for pressure compensation (Duran) due to permit fermentation gases vent out, and the other two were connected with a screw cap GL 14 for hose connection (Duran) with a joint silicone diameter 0.6 mm and silicone tubes of 3x6 mm. One of these tubes was used to inoculate the *O. oeni* E5003 liquid culture starter from the 0.5 L fermenter, and the other one was used to collect samples during the biomass production. At the end of both silicone tubes, a PVDF luer lock 3 way valve (Cole Palmer) was joined with a 3.2 mm luer lock male connector. In both valves, a 0.2 µm PTFE Syringe-driven filter unit (Millipore) was added to permit the inoculation from the 0.5 L fermenter. In the other valve connection, 50 mL luer lock syringes were connected to the silicone collection tube to collect the samples (**Figure 12**).

Figure 12. Assembly of the 10 L fermenter

A 100 L fermenter (Bioprocess technology Bio-pro 100 L) was used in Agrovin S.A. to carry out the industrial biomass production of the selected *O. oeni* strain with the designed medium OMP (**Figure 13**).

Figure 13. 100 L fermenter used in Agrovin S.A.

3.12.2 Scale up of the biomass production process

The scale up started with a culture of 10^6 cfu/mL of *O. oeni* E5003 in 50 mL of liquid OMP medium. The culture was incubated statically at 28 ºC. When the bacterial population arrived to $2x10^9$ cfu/mL, the 50 mL were inoculated into the 0.5 L fermenter that contained 0.5 L of OMP sterile medium (**Figure 14**). The inoculated fermenter was incubated at 28 ºC until the bacteria population reached $1x10^9$ cfu/mL. Then the *O. oeni* culture was inoculated into the 10 L fermenter with 8 L of OMP sterile medium and was incubated stirred at room temperature to a final concentration of $1x10^9$ cfu/mL.

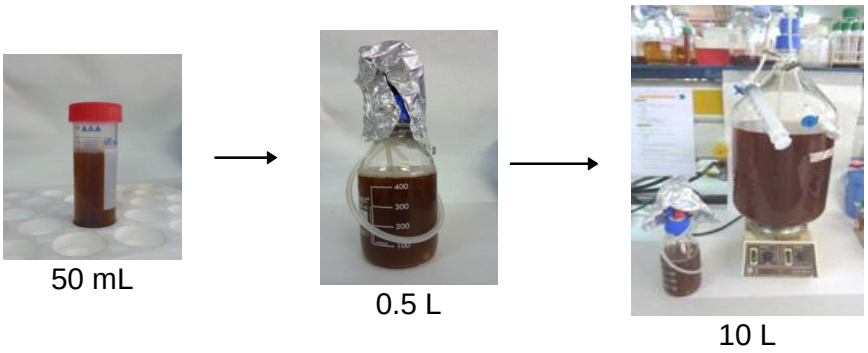


Figure 14. Scale up of the biomass production process at laboratory

The industrial biomass production process was carried out inoculating the 10 L of the *O. oeni* culture into a fermenter in Agrovin S.A. with 80 L of OMP sterile medium. The inoculated culture was incubated at 28 ºC until the bacteria population reached 1x10^9 cfu/mL.

3.12.3 Biomass production controls

A microbiological analysis of the three scale-up steps carried out at the laboratory was made to certify the correct sterilization of the culture medium. After the sterilization of 50 mL and 0.5 L fermenter of OMP medium by autoclave, 115 ºC 30 minutes, and the fermenters of 10 L and 100 L, 115 ºC 45 minutes, 0.1 mL sample of each fermenter were plated on ACE, MRS, MLO, PCA and GPYA media. The plates were incubated at 28 ºC for seven days.

Moreover, the *O. oeni* cultures were also analyzed microbiologically to confirm their identity, plating 0.1 mL of all the samples collected during the biomass production after dilution, on ACE, MRS, MLO, PCA and GPYA media. The plates were incubated at 28 ºC for 7 days. The identity of the *O. oeni* strain was verified by the molecular technique of RAPD-PCR. The isolates RAPD-PCR patterns from the different cultures were compared to the one from the original *O. oeni* strain kept in glycerol 15 % at -20 ºC, employing the software Bionumerics 6.5.

The viability of the culture from the 10 L fermenter was determined by LIVE/DEAD BacLight bacterial Viability Kit (Invitrogen).

The sugars' consumption and the lactic acid formation by the *O. oeni* strain were followed during the biomass production. Samples were taken and analyzed by HPLC at 1, 3 and 6 days after the bacterial inoculation.

3.12.4 Malolactic activity and cell viability in red wine

To study the malolactic activity and viability of the liquid MLF starter, red wine made in the laboratory was inoculated to a final concentration of 10^8 cfu/mL, 10^7 cfu/mL and 10^6 cfu/mL. The malic acid and lactic acid were measured by HPLC at 1 and 5 days and the cell viability was studied by colony counting, plating 0.1 mL of decimal serial dilution of the taken samples on MLO culture medium.

3.13 Cellar malolactic fermentation starter design

The identification of the microorganism responsible for histamine formation in a cellar located in Ribera del Duero region (Spain), as the selection of an autochthonous *O. oeni* for MLF liquid starter culture were carried out as it is shown in the **Figure 15**.

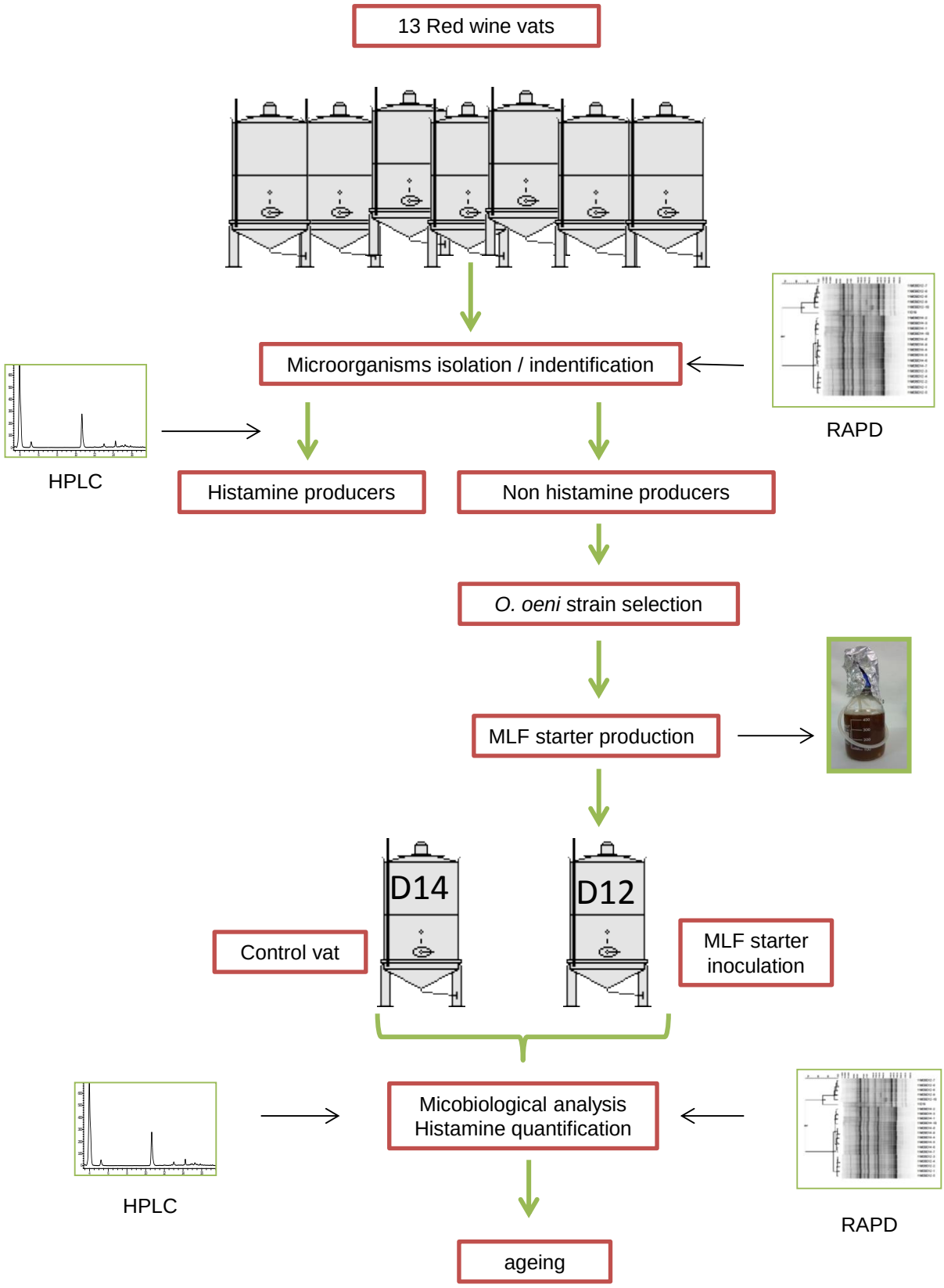


Figure 15. Diagram of the cellar MLF starter design

3.13.1 Wine samples and histamine quantification

Wine samples were taken before and after the MLF from 13 vats with 200 HL of red wine from the cellar in 2010, and histamine quantification was carried out by HPLC.

3.13.2 Isolation and identification of microorganisms

Total cell and viable counts were made from all the samples before and after the MLF with the Thoma chamber counting and plating decimal serial dilutions on MLO plates.

From each sample, before and after the MLF, 10 colonies were randomly isolated from MLO plates and inoculated on new culture medium. After growth, the molecular technique of RAPD-PCR was performed to differentiate *O. oeni* strains.

3.13.3 Histaminogenic activity

The different *O. oeni* strains isolated from all samples on MLO plates were grown in liquid MLO until a final population of $2x10^9$ cfu/mL. Then, $2x10^7$ cfu/mL were inoculated in MDB-mod and incubated 15 days at 28 ºC. The cultures were analyzed by HPLC to determine if histamine had been produced.

3.13.4 Malolactic fermentation starter production

The following year, in 2011, for the biomass production of the selected strain, a scale-up was made at laboratory from 150 mL culture medium to a 30 L culture medium, passing through a 1.5 L step, as in the previous biomass production. A vat of 200 HL of red wine was inoculated with this 24 L starter culture at the cellar. A microbiological analysis of the three scale-up steps was made to certify the correct sterilization of the culture medium. After the sterilization, the culture medium was plated on ACE, MRS, MLO, PCA and GPYA media. The plates were incubated at 28 ºC for 7 days.

3.13.5 Cellar malolactic fermentation starter inoculation

In the cellar, after the AF, two separate vats (D12 and D14) of 200 HL of red wine were mixed and divided again in the two vats to obtain the same physicochemical conditions. The vat D12 was inoculated with the MLF starter previously prepared in a 1/1000 dilution to achieve a final concentration of $1x10^6$ cfu/mL of bacteria. The D14 vat was kept as non-inoculated vat control.

Just before the inoculation, samples from the two vats were taken and physicochemical and bacteriological analyses were made (D12BML and D14BML).

Wine samples were taken from vats D12 and D14, also during the winemaking process, one week after the inoculation (1W), after the MLF (AML), after the SO_2 addition (SO_2), when wine was transferred to barrels (BL), and after one year in barrels (BL1Y), to analyze the malic and lactic acid, histamine contents and bacterial viability of both vats.

To identify the *O. oeni* strains that were in the different samples and to study the starter implantation, 10 colonies were randomly taken from each stage and vat and they were isolated on new MLO culture medium. The molecular technique of RAPD-PCR was applied to all the isolates from all the samples, and the RAPD-PCR patterns obtained were compared to the band profile from the *O. oeni* strain inoculated, employing the software BioNumerics.

3.14 Starter culture preservation and alternative preservation techniques

3.14.1 Starter culture preservation with different methods

The three methodologies were studied, freeze-drying, refrigeration at 4 ºC, and freezing at -20 ºC.

Firstly, E5003 *O. oeni* strain was grown in 500 mL of OMP media to a final concentration of $1x10^9$ cfu/mL. Then, 300 mL were divided in aliquots of 8 mL. Half of the aliquots were

maintained at 4 ºC and the other half were kept at -20 ºC. The remaining 200 mL culture was used to freeze-dry the *O. oeni*.

Once all the preservation methodologies were done, the viability analyses were carried out taking samples, after 24 hours, 15 days, 1 month, 3 months and 8 months. Decimal dilutions were done and plated on MLO culture media.

3.14.2 Cell preservation in red wine lees

Three different Tempranillo wine lees from Utiel-Requena region (**Table 7**) were used to preserve *O. oeni* E5003. The *O. oeni* strain was inoculated in the 3 wine lees in a final concentration of $3x10^7$ cfu/mL. After 2 weeks of preservation of the *O. oeni* strain in the wine lees, 10 colonies from each sample were randomly taken from the MLO plates and the molecular technique of RAPD-PCR was performed to certify the implantation of the *O. oeni* strain in the wine lees. Cell viability of the inoculated cultures in wine lees was studied through 16 weeks by cell plate counting on MLO medium.

A proportion of 1/10 and 1/100 of each of the wine lees, incubated for 15 days with the *O. oeni* strain, were inoculated in red wine made at the laboratory, to see how the preservation in wine lees affected the cell viability in wine. Wine fermentations were carried out in triplicate. Wines were incubated at 28 ºC. Samples were collected for cell viability analysis during 14 days and, malic acid consumption was measured by HPLC.

3.15 Effect of different rehydration solutions on the survival of freeze-dried cultures

One of the strategies to make a starter to achieve the MLF is to freeze-drying the *O. oeni* culture. The *O. oeni* E5003 strain was grown in OMP to a final concentration of $1x10^9$ cfu/mL and freeze-dried. The malolactic activity in wine was compared using NaCl (9 g/L) or a mix of 5 g/L fructose, 5 g/L arginine and 3 g/L L-malic acid as rehydration solutions for freeze-drying MLF starters.

3.15.1 Rehydration with NaCl

The quantity of 0.1 g of the freeze-dried culture ($1x10^{10}$ cfu/g) was rehydrated with 10 mL of NaCl 0.9 % for 1 hour. Then the culture was inoculated in red wine in final concentrations of $1x10^{6}$ cfu/mL and $1x10^{7}$ cfu/ mL, and incubated at 28 ºC. During the MLF, samples were collected for analysis by HPLC.

3.15.2 Rehydration with arginine, fructose and malic acid

The quantity of 0.1 g of the freeze-dried culture ($1x10^{10}$ cfu/g) was rehydrated with 10 mL of a mix solution that contained 5 g/L arginine, 5 g/L fructose and 3 g/l acid L-malic for 1 hour. Then the culture was inoculated in red wine in a final concentration of $1x10^{6}$ cfu/mL and $1x10^{7}$ cfu/ mL and incubated at 28 ºC. During the MLF samples were collected for analysis by HPLC.

3.16 Coimmobilization of *S. cervisiae* and *O. oeni*

3.16.1 Coimmobilization of yeast and bacteria

The yeast *S. cerevisiae* and the bacterium *O. oeni* were coimmobilized in delignified cellulosic material (DCM) and wheat starch gel. For the immobilization, both microorganisms were grown in liquid culture media (MLO for bacteria and GPYA for yeast) to their maximum absorbance and then centrifuged to remove the liquid. Then *O. oeni* E5003 was firstly immobilized on DCM. This material was produced from wood sawdust after treatment with 1 % NaOH solution and heating for 3 hours at the boiling point for removal of lignin. For the immobilization, 40 g of DCM, 200 mL of fresh MLO liquid medium and 4 g of wet *O. oeni* cells were placed in a flask and allowed to ferment overnight at room temperature. The mix was then filtered to remove the liquid and was dried at 35 ºC for 48 hours. The immobilization of the bacteria was certified by scanning electron microscope. When *O. oeni* was immobilized, *S. cerevisiae* was entrapped in wheat starch gel as follows: 4 g of wheat starch were mixed with 50 mL of deionized water heated to 90 ºC and then left to cool at room temperature. Then 5 mL of GPYA were mixed with 5 g of wet weigh of *S. cerevisiae* AXAZ-1 and with the prepared gel.

Both microorganisms were collected by dry impregnation; DCM with the already immobilized *O. oeni* was added to the starch gel with *S. cerevisiae* until its maximum absorption capacity. The mix was then incubated at 30 ºC for 24 hours and then a thermal dry at 35 ºC for 48 hours was carried out. The immobilization of both microorganisms was observed by scanning electron microscope.

3.16.2 Alcoholic and malolactic fermentations in wine

The biocatalyst was used for AF and MLF experiments. An amount of 0.03 g and 0.3 g of the immobilized biocatalyst were introduced into 50 mL of red grape must at 28 ºC without any agitation. Inoculating this amount, the estimated final bacterial population in wine were around 10^6 cfu/mL and 10^7 cfu/mL respectively. The biocatalyst was compared to a simultaneous inoculation of free cells of *S. cerevisae* AXAZ-1 and *O. oeni* E5003. *S. cerevisiae* was grown in GPYA and *O. oeni* in OMP medium. Both microorganisms were inoculated simultaneously in a final concentration of 10^6 cfu/mL or 10^7 cfu/mL in red grape must. During the fermentations, samples were collected for analysis by HPLC. The fermentations were carried out in triplicate.

4. Results and discussion

4.1 Biogenic amines' determination

The first step for the starter culture selection was to improve the BAs determination method, using the new core-shell HPLC column technology (Kinetex PFP) in order to reduce time and cost analysis for the following experiments performed in this project.

4.1.1 Comparison between HPLC columns and elution programs

Two HPLC columns were used, the Luna C18, with the elution program already described (Peña-Gallego *et al*. 2009), and Kinetex PFP HPLC column. For the Kinetex PFP column, two elution programs were designed to study the differences in the histamine elution. The retention time for each BA, employing the two different HPLC columns with the different elution programs described in materials and methods, were established (**Table 11**). The HPLC data showed a reduction in the elution time of each BA studied, using the Kinetex PFP HPLC column, with a 5-fold decrease in run time with program I and with a 2-fold decrease with program II compared to the Luna C18 column.

Table 11. Retention time of each BA using Luna C18 and Kinetex PFP columns

Amine	Retention time (minutes)		
	Luna C18	Kinetex PFP (Program I)	Kinetex PFP (Program II)
Histamine	8.59	1.81	5.2
Tyramine	23.84	4.05	13.39
Putrescine	29.12	4.97	15.22
Cadaverine	32.37	5.38	15.60
Total run time	50	10	25

Results from the standards' analysis evidenced that no interference peaks at the respective retention times for each BA occurred using the Luna C18 and the Kinetex PFP HPLC columns (**Figure 16**). Both columns were able to distinguish the analytes from the interference components. However, Kinetex PFP was better HPLC column in the chromatographic separation for the BAs determination because all the components were nearly baseline resolved in few minutes with good resolution.

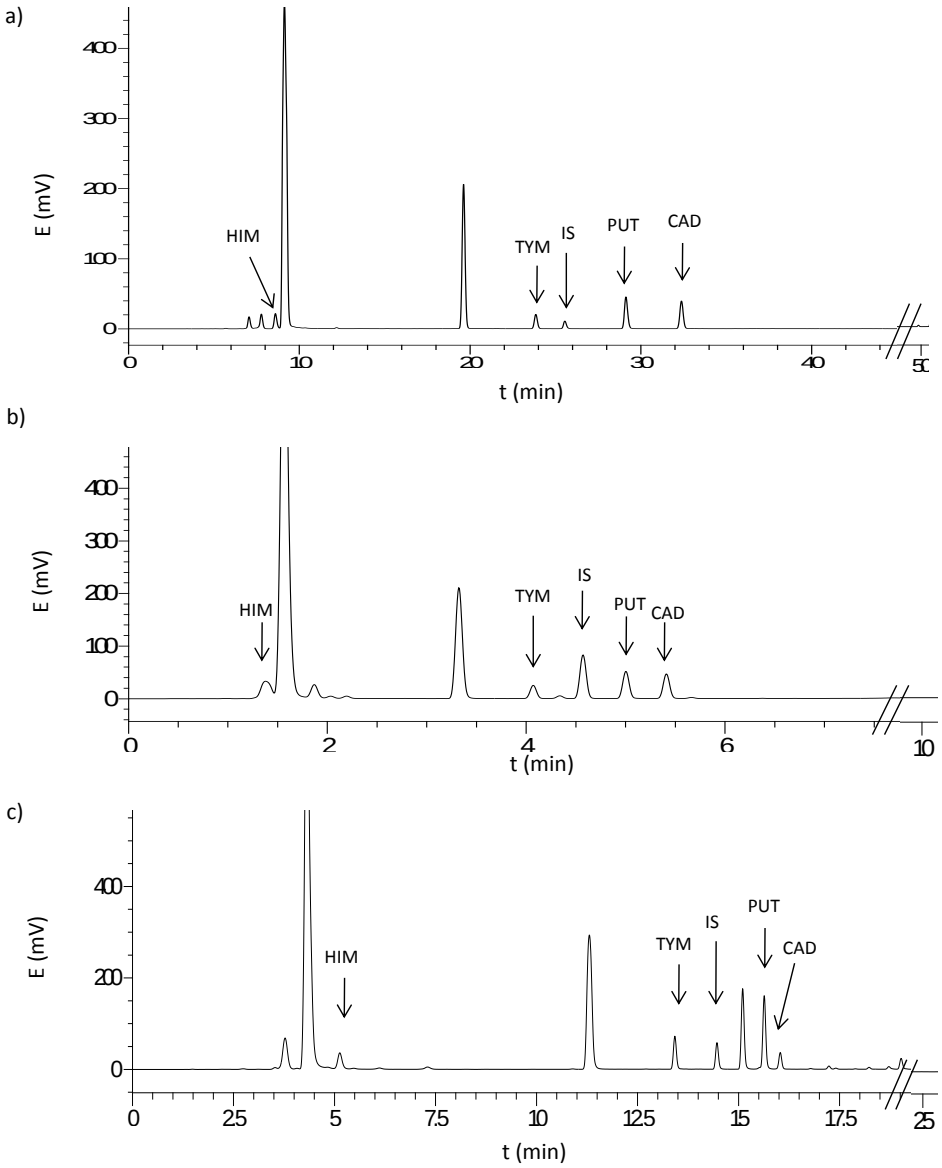


Figure 16. Chromatograms obtained from a standard solution of 50 ppm of the four BAs, histamine (HIM), tyramine (TYM), putrescine (PUT), cadaverine (CAD) and the internal standard (IS) with: **a)** Luna C18 HPLC column , **b)** Kinetex PFP HPLC column with elution program I and **c)** Kinetex PFP HPLC column with elution program II

The two elution programs used with the Kinetex PFP HPLC column showed differences related manly to the histamine peak (HIM) (**Figures 16b and 16c**). The chromatograms showed that when a temporary isocratic elution was added in the gradient (program II), the histamine peak was eluted later; causing its isolation and therefore its determination and quantification of this BA was easier.

4.1.2 Calibration curves

The calibration curves were calculated from the representation of the ratio between peak area of BAs versus the amine concentration forcing to zero. Data obtained from calibration curves were submitted to linear regression analysis and showed an excellent linear relationship in all cases. As example, with the PFP HPLC column (elution program II), the correlation coefficients (R^2) varied between 0.991 and 0.997 (**Figure 17**).

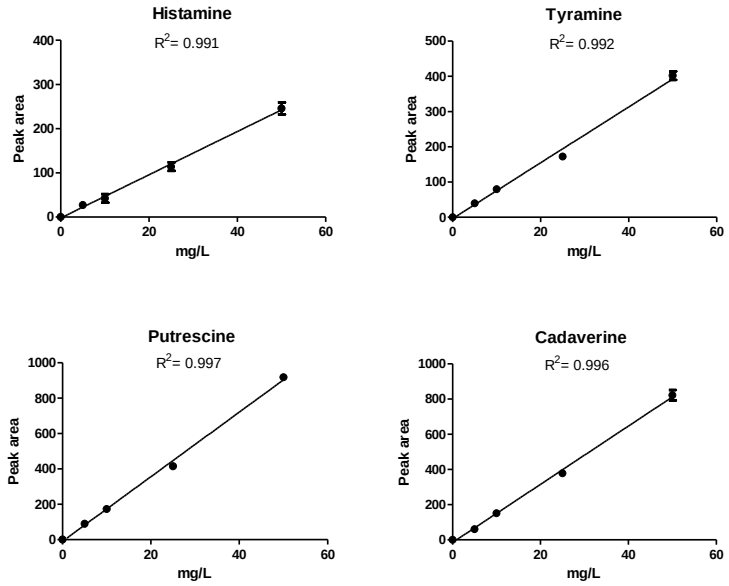


Figure 17. Calibration curves determined following the concentration of 0, 5, 10, 25 and 50 ppm for histamine, tyramine, putrescine and cadaverine amines using the PFP HPLC column with the elution program II

4.1.3 Sample analysis

To confirm that the method was suitable for wine analysis, a red wine was spiked with 10 ppm of the four amines. The results showed a good resolution with both Luna C18 and Kinetex PFP HPLC columns using both elution programs, allowing the amines identification and quantification (**Figure 18**). Like previous analysis of the standard solutions, the histamine peak was better resolved using the elution program II with the Kinetex PFP HPLC column. The use of this new core-shell technology permitted less mobile phase use, spending 47 mL per sample with the Luna HPLC column and 20 mL using the Kinetex PFP HPLC column. Time reduction represented also an important UV lamp use saving.

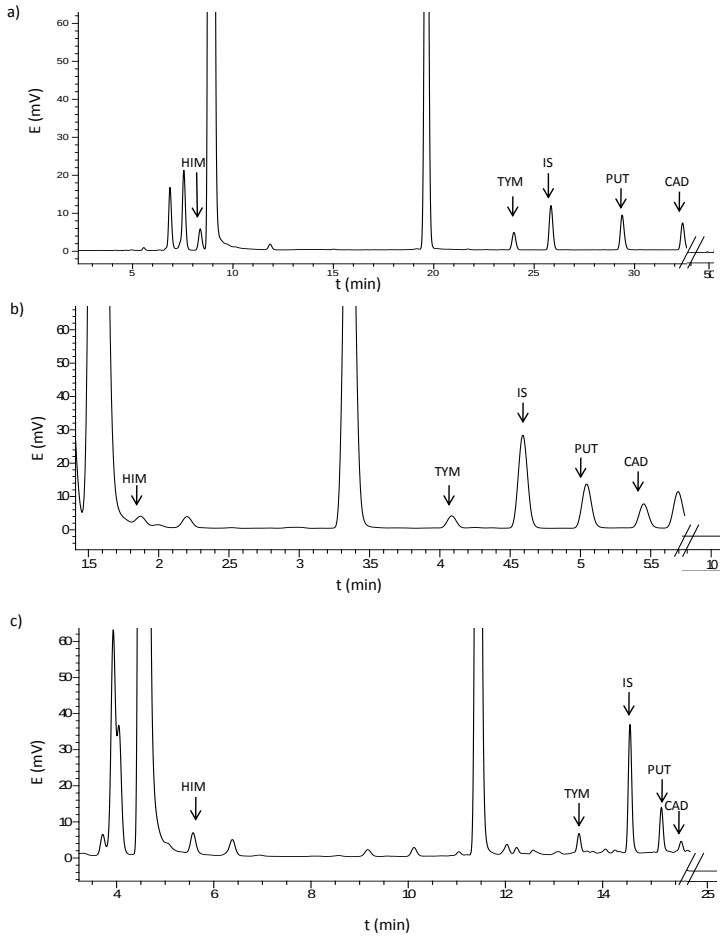


Figure 18. Chromatograms obtained from a wine with the four BAs (10 ppm), histamine (HIM), tyramine (TYM), putrescine (PUT), cadaverine (CAD) and the internal standard (IS) with: **a)** Luna C18 HPLC column, **b)** Kinetex PFP HPLC column with elution program I and **c)** Kinetex PFP HPLC column with elution program II

Compared to already described Luna C18 HPLC column (Peña-Gallego *et al.* 2009), the main advantage of the presented technique is the reduction of run times and solvent volumes. Results showed a reduction in the chromatographic separation time for each BA with the Kinetex 2.6 μm PFP HPLC column. Moreover, because it was a little time-consuming, it reduces considerably the spend volume of organic solvents and even, the Kinetex PFP column is commercially cheaper than the Luna C18 HPLC column. Furthermore, depending on which amine we are interested in, the gradient elution program I or the isocratic and gradient program II can be used. Histamine was the main BA studied in this project, therefore the PFP HPLC column with elution program II was employed in the following experiments.

4.2 *O. oeni* strain selection

To design an *O. oeni* MLF starter, the first objective was to select an *O. oeni* strain able to grow at wide range of pH, at high ethanol levels and not able to produce BAs. Forty *O. oeni* strains from the Enolab collection were firstly cultured from freeze-dried cultures in MLO medium to certify the viability and the culture purity (**Table 5**). Only E4158 was not able to grow, therefore this *O. oeni* strain was not included in the subsequent selection program.

4.2.1 Bacterial growth at pH levels between 3.2 and 3.8

All the *O. oeni* strains were grown in MLO liquid medium with 3 different levels of pH (3.2, 3.5 and 3.8) in duplicate. Wine pH varies usually between these pH levels, hence, the objective was to select the *O. oeni* strains with the highest growth in the 3 pH levels. The bacterial growth was measured by OD at 600 nm, and the area under the curve (AU) was calculated for each *O. oeni* strain at the 3 different conditions (**Figure 19**).

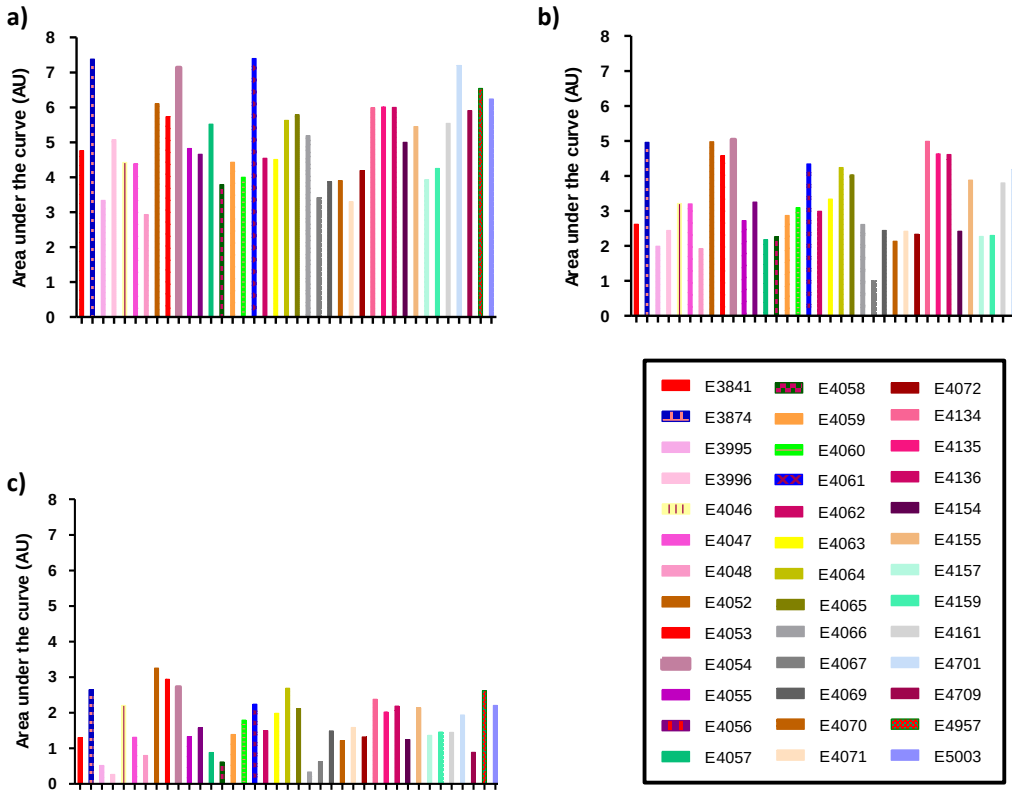


Figure 19. Values of the area under the curve (AU) of the *O. oeni* strains under different pH conditions in MLO; **a)** pH 3.8, **b)** pH 3.5 and **c)** pH 3.2

All *O. oeni* strains grew in the 3 pH levels. Bacterial growth in culture media with a pH of 3.8 was noteworthy, going above a value of 2.5 AU of area under the curve in all cases. The average was 5.08 AU and reaching to 7 AU sometimes (**Figure 18a**). Growing the *O. oeni* strains at pH level of 3.5 showed that in all cases, the values of the area under the curve decreased, observing between 1 and 5 AU with an average of 3.26 AU. Data of growing the *O. oeni* strains at pH level of 3.2 showed values lower in all cases and an average of 1.65 AU of area under the curve (**Table 12**).

Table 12. Descriptive statistics of area under the curve for the *O. oeni* strains under different pH conditions: pH 3.8, pH 3.5, and pH 3.2

pH	Average	SD
3.8	5.07	1.18
3.5	3.26	1.05
3.2	1.65	0.74

Results were analyzed using an ANOVA test. This analysis revealed a significant effect for pH level ($p< 0.05$). These observations are in agreement with those presented by Champagne *et al.* (1989a) and Ribéreau- Gayon *et al.* (2000) as they establish that the pH influence in growth rate and yield. There was also a significant effect for the *O. oeni* strain ($p<0.05$). The Tukey *post-hoc* test grouped the 39 strains in 23 groups considering the area under the curve of the 3 pH levels. The 12 strains from the 7 groups with the major value of area under the curve (E3874, E4052, E4053, E4054, E4061, E4064, E4134, E4135, E4136, E4701, E4957 and E5003) were selected to undergo to the next selection criteria, high ethanol tolerance. These strains were the ones that better grew in the 3.2 pH level (getting over a value of 2 UA of area under the curve), hence these strains were able to grow also with the lowest pH level (**Figure 19c**).

4.2.2 Bacterial growth at ethanol levels of 9, 11 and 13 % (v:v)

The 12 selected strains (E3874, E4052, E4053, E4054, E4061, E4064, E4134, E4135, E4136, E4701, E4957, E5003) were grown in MLO with 9, 11, 13 % of ethanol (v:v). The objective was to select the *O. oeni* strains with the maximum growth in the highest ethanol content.

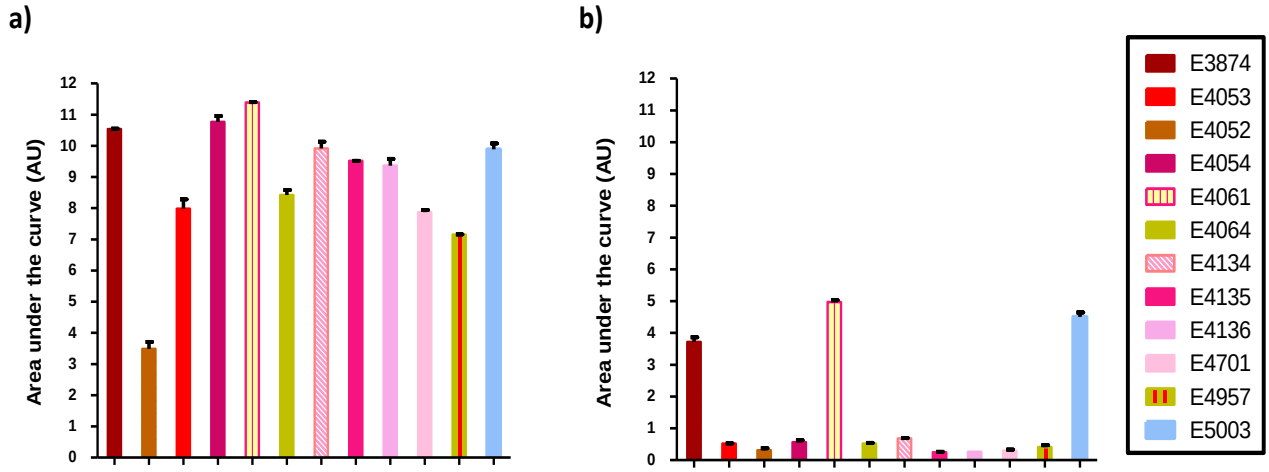


Figure 20. Values of the area under the curve of the *O. oeni* strains under different ethanol level in MLO culture media: **a)** at 9 % (v:v) and **b)** at 11 % (v:v)

At 13 % (v:v) ethanol level, none of the studied strains were able to grow (data not shown). A significant growth of all bacteria was obtained at 9 % (v:v) ethanol level, excepting for E4052 strain (**Figure 20a**). The area under the curve average at this ethanol level was 8.82 AU. Data of bacteria growth in culture medium with an ethanol level of 11 % (v:v) showed an average of 1.4 AU (**Table 13**). Nevertheless, there were big differences between 3 strains (E3874, E4061, E5003) and the rest of them at this ethanol level (**Figure 20b**).

Table 23. Descriptive statistics of area under the curve for the *O. oeni* strains under different pH conditions: pH 3.8, pH 3.5, and pH 3.2

Ethanol	Average	SD
9 %	8.82	2.05
11 %	1.40	3.18

Results of area under the curve of growth at 9 % and 11 % (v:v) ethanol level were analyzed using an ANOVA test. This analysis revealed a significant effect for ethanol level ($p<$ 0.05). As the pH level, the ethanol plays an important role in the bacterial growth. These observations are in accordance with those presented by Vaillant *et al.* (1995) and Ribéreau-Gayon *et al.* (2000). The ANOVA analysis revealed also a significant effect for *O. oeni* strain ($p<0.05$). The Tukey *post-hoc* test grouped the 12 strains in 6 groups considering the area under the curve of the 2 ethanol levels. The 3 strains from the 2 groups with the major value of area under the curve (E3874, E4061 and E5003) were selected to undergo to the next selection criteria that was the malolactic activity. These 3 strains were the only ones able to get over a value of 2 UA of area under the curve with a level of 11 % (v:v) (**Figure 20b**), therefore these *O. oeni* were able to grow with the highest ethanol content.

4.2.3 Malolactic activity measurement in microplate

Microplate readers have been useful assistant of researchers for several decades. This experiment was focused on the application of a simple absorbance microplate reader (96-well format with temperature control and shaking option) in malolactic activity research. The idea to use the microplate reader in combination with colour indicator for monitoring pH changes have been employed by different authors to study the yeast physiology (Marešová and Sychrová 2007) or the bacterial growth in spoilage foods (Kuda *et al.* 2004).

Congo red and bromophenol blue were tested as pH indicators in this experiment. Congo red can be used as a pH indicator, due to a color change from blue to red at pH 3.0-5.2, but the absorbance variation between two values using this pH indicator were lower than with the bromophenol blue, making more difficult the observation of pH changes, so this pH indicator was rejected for the malolactic activity mesaurement in microplate.

Bromophenol blue was used as pH indicator to detect an increase of pH when the L-malic acid is transformed to lactic acid because its color variation is related to the absorbance at 600 nm. So by measuring the absorbance variation, a color change was detected, and therefore an increase of pH. The amount of 0.04 g/L of the bromophenol blue as pH indicator was the optimum concentration to see the pH variation and the dye did not interfere with the viability

metabolic activity of the cells. The curve presented a third order polynomial regression with a R^2 of 0.999 (**Figure 21**). A higher or lower concentration of pH indicator (0.01 and 0.06 g/L) did not provide the expected results, making more difficult the observation of the color changes.

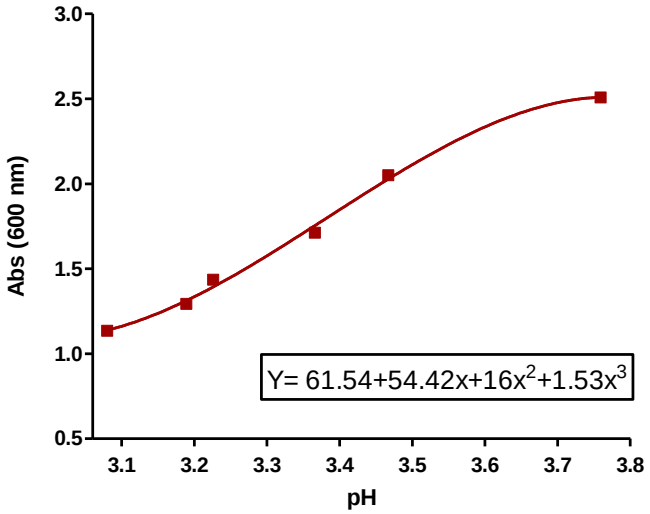


Figure 21. Calibration curve (A600 nm) of the pH indicator bromophenol blue with a concentration 0.04 g/L. The results presented a third order polynomial regression with a R^2 of 0.999

The use of pH as indicator in the bacterial incubation with the microplate reader allowed us to observe little differences of L-malic degradation between the *O. oeni* strains at pH levels of 3.08, 3.18, 3.22, 3.36, 3.46 and 3.75. As can be seen in the **Figure 22**, at higher pH, greater absorbance variation was observed. At pH levels of 3.18 and 3.08 the absorbance increment was very low, compared to higher pH levels, due to the difficulties of carry out the MLF at these pH conditions.

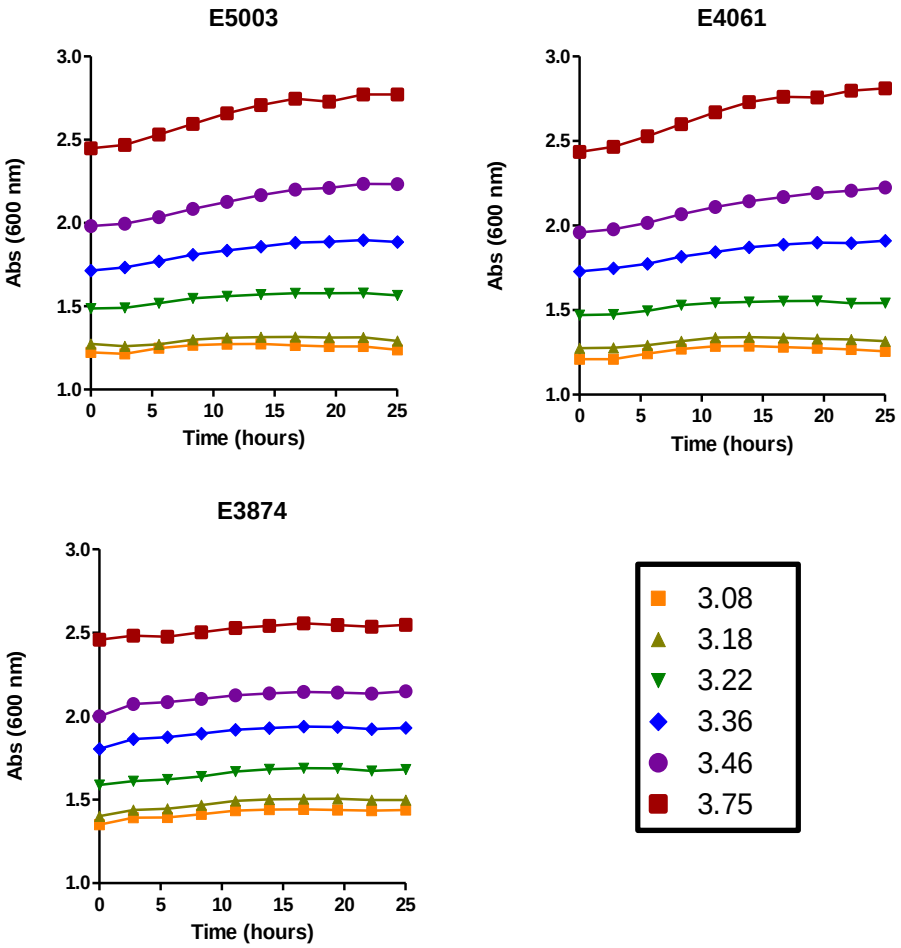


Figure 22. Kinetics of the absorbance variation (A600 nm) of the *O. oeni* strains E5003, E4061 and E3874 in the malic acid and bromophenol blue solutions with pH levels from 3.08 to 3.75, obtained with the microplate reader during 24 hours of incubation (control)

The data revealed that the *O. oeni* strain E5003 was the best one in terms of malic acid degradation at low pHs. As can be seen in **Figure 23**, the color variation after 24 hours of incubation at 28 ºC was notable in E5003 microwells, showing color differences with the control microwells until a pH level of 3.08. These color variation were verified with the pH increment measurement in all microwells after the 24 hours incubation with cells, and with the malic acid quantification by HPLC.

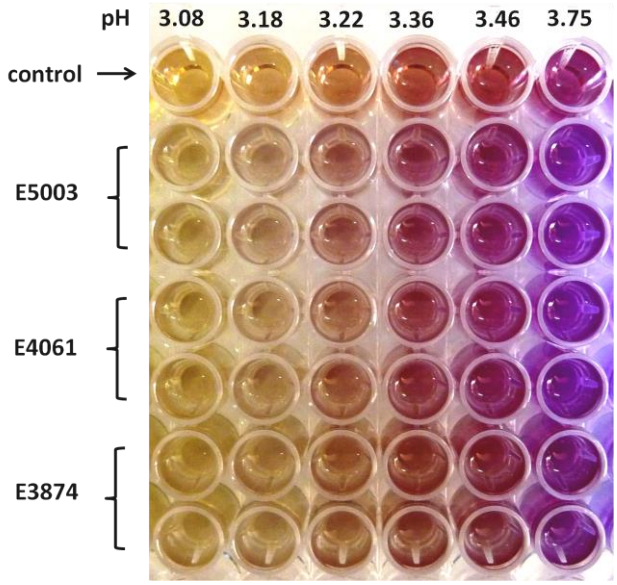


Figure 23. Aspect of the microplate after the 24 hours incubation of 1x10^8 cfu/mL from the *O. oeni* strains E5003, E4061 and E3874 by duplicate. The malic acid degradation was detected by a color change from levels of pH of 3.08 to 3.75 compared to the microwells without inoculation

The pH measurement showed that in all cases the E5003 presented an increase of the original pH, from 0.02 units at pH level of 3.08 to 0.332 units at pH level of 3.75. *O. oeni* E3874 showed the worst results, with a very low increase of the pH and only at pH level of 3.75 presented a significant increase of 0.127 units (**Figure 24**).

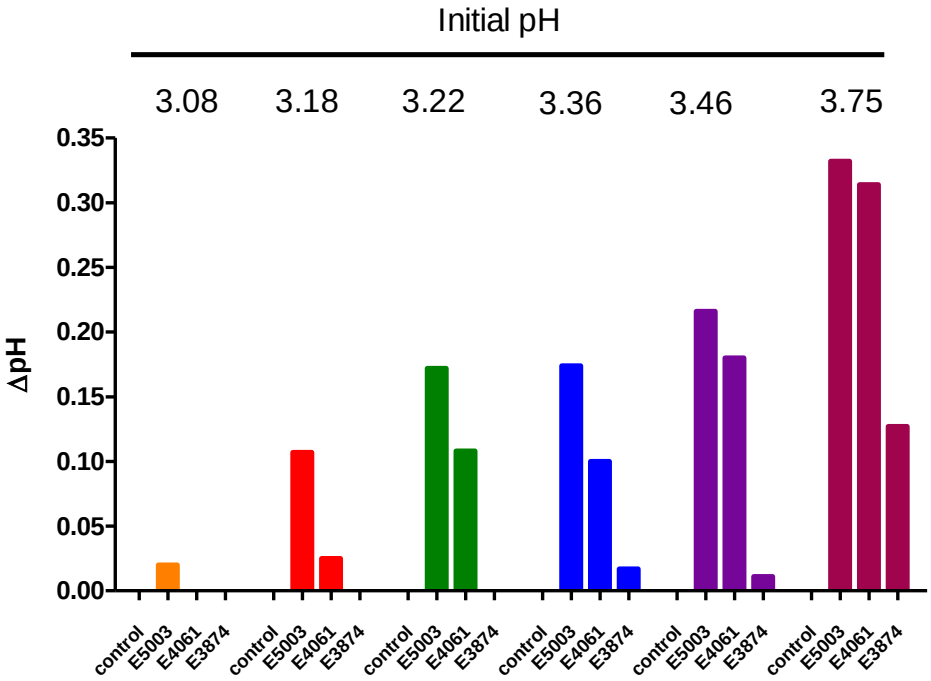


Figure 24. Increment of the pH level of the *O. oeni* strains E5003, E4061 and E3874 incubated during 24 hours, compared to the original solutions without inoculation

These results confirmed that the best *O. oeni* strain to be used as malolactic starter was E5003. To verify that the malic acid degradation was carried out at these conditions, the cultures were analysed by HPLC after the 24 hours of incubation, and the L-malic acid was quantified. The data showed that at pH level of 3.75, the *O. oeni* strain E5003 consumed the 3 g/L of L-malic acid (100%), the pH level variation was 0.33 units and the absorbance increased 0.322 units. On the other hand, when the pH level was 3.08, 2.14 g/L of malic acid (71.33%) were consumed, the pH increased only 0.02 units therefore the absorbance increased 0.015 units (**Table 14**). These data suggest that at least 2 g/L of malic acid must be consumed to visualize a clear pH variation and a color change.

Table 14. Data obtained from the malic acid degradation of *O. oeni* strain E5003, the increase of the absorbance at 600 nm and the increase of the pH level at the different pH levels studied in the miniaturized system

pH_i	Malic acid degradation (%)	$\Delta Abs_{600\ nm}$	ΔpH
3.08	71.33	0.015	0.02
3.18	93.67	0.018	0.11
3.26	96.00	0.078	0.17
3.36	97.67	0.171	0.18
3.46	98.33	0.252	0.22
3.75	100.00	0.322	0.33

The use of the microplates was shown to have several advantages. The volume of media was reduced to 300 µL, which minimized the expenses required for the testing procedures, and also this method allowed the automated study of many cultures at the same time without manipulating them to obtain the absorbance measurements. This method provides a tool for rapid selection of malolactic strains in 24 hours. The use of a microplate reader and a pH indicator permitted the selection of the best *O. oeni* strain in terms of malolactic acid degradation in a short time and spending the minimum culture media.

4.2.4 Biogenic amines' formation

To study if the *O. oeni* selected strains, E3874, E4061 and E5003 were able to form BAs, the developed HPLC method was employed with the elution program II. The three bacteria were grown in MDB-mod (Landete *et al.* 2005a) liquid media with the amino acids precursors and incubated 15 days at 28 ºC. The chromatograms revealed that none of the three *O. oeni* strains studied were able to produce BAs in the culture media with the new developed HPLC method. Therefore E3874, E4061, E5003 were submitted to the next selection criteria to study their ability to grow and to carry out the MLF in wine.

Nowadays, there are contradictory data as to whether or not *O. oeni* strains are histamine producers (García-Moruno and Muñoz 2012). In this experiment, none of these strains were able to produce histamine in culture medium, but in experiments that we will see later on this thesis, we found other *O. oeni* strains able to produce it so we support the fact that some strains of this species are able to produce this BA.

4.2.5 Malolactic activity and cell viability in wine

The *O. oeni* strains able to grow in culture medium with 11 % of ethanol (v:v) and were not able to form amines, E4061, E3874 and E5003, were previously grown in MLO and then inoculated by triplicate in red wine, made at the laboratory, in a final concentration of $2x10^6$ cfu/mL, and were incubated for 15 days at 28 ºC to analyze which one had the best malolactic activity and viability. Samples were taken at 1, 3, 7 and 15 days and the malic acid consumption and lactic acid formation were analyzed by HPLC (**Figure 25**). The results showed that the E5003 strain consumed all the malic acid in 15 days, producing 3.20 g/L of lactic acid. *O. oeni* E4061 and E3874 were not able to consume all the malic acid, remaining 2.87 g/L 3.71 g/L respectively.

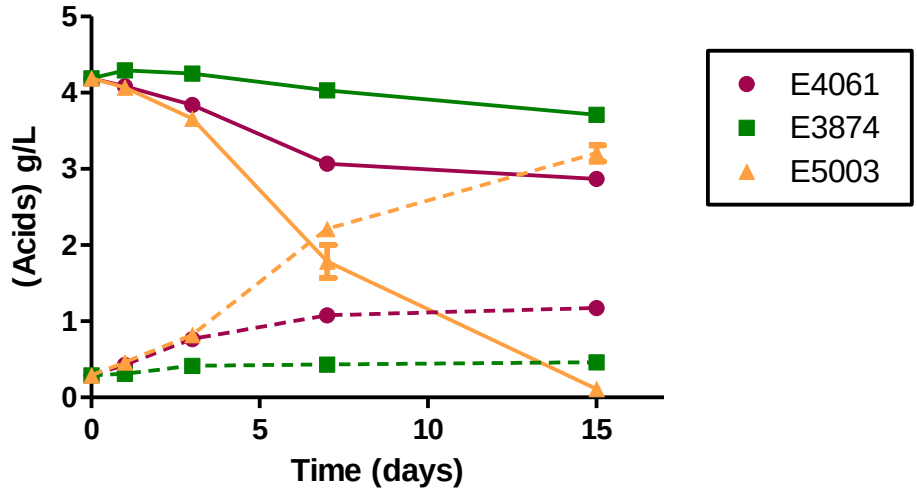


Figure 25. Malic acid consumption (—) and lactic acid formation (---) of E4061, E3874 and E5003 *O. oeni* strains in wine during 15 days at 28º C

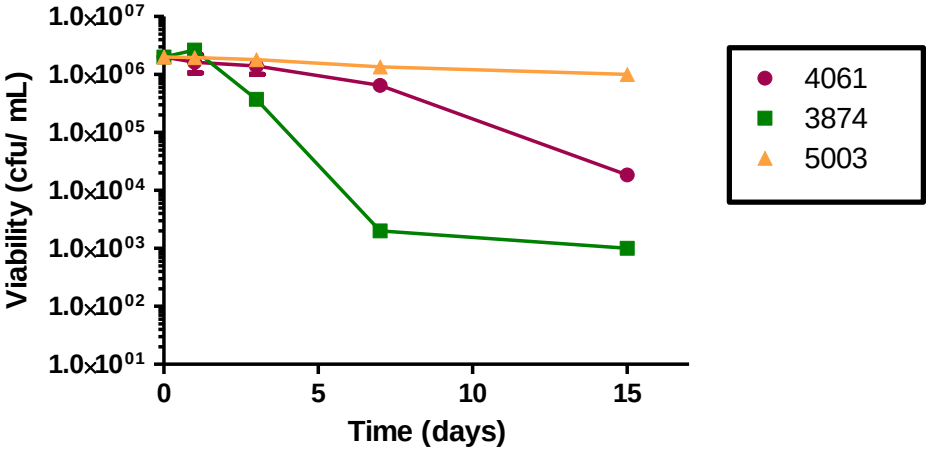


Figure 26. Cell viability of E4061, E3874 and E5003 *O. oeni* strains in wine during 15 days at 28 ºC

Results matched up with the cell viability data (**Figure 26**), seeing that the viability of E4061 reminded over 10^6 cfu/mL during the first 7 days, that was the period in which this strain consumed the malic acid. After that, the cell viability decreased and the lactic acid formation stopped. E3874 population went down in the first 3 days to a concentration of less than 10^6 cfu/mL, explaining the low consumption of malic acid of this *O. oeni* strain. However, the *O. oeni* strain E5003 was over 10^6 cfu/mL during the 15 days, showing the best results for cell viability and so malic acid consumption in wine. These observations are in accordance with those presented by many authors as Wibowo *et al.* (1988), Kunkee (1991)

and Versari *et al*. (1999); MLF occur in wine when bacterial population is about 10^6 cfu/mL. In this experiment only the *O. oeni* strain E5003 was over 10^6 cfu/mL, and so was the only strain that consumed all the malic acid. Also, this strain had the best results in terms of malic acid degradation at low pHs in the microplate experiment and it had not the ability to form BAs in MDB-mod. For this reason this strain was selected to be used in the majority of the forthcoming experiments as MLF starter.

4.3 Design of a culture medium for the biomass production of *O. oeni* malolactic starter

4.3.1 Bacterial growth in the different culture media

If propagation of *O. oeni* is carried out under favorable conditions before inoculating wine, higher cell yields and productivities are possible. The induction of MLF with a high concentration of cells enables a better control and increases the success of the process (Gao and Fleet 1994; Formisyn *et al.* 1997; Maicas *et al.* 1999c). Therefore, the optimization of growth and production of batch cultures is very important to obtain a high productivity of biomass that can be utilized as a starter culture (Zhang and Lovitt 2005). The previously selected strain, *O. oeni* E5003, was grown in 27 media with different sugar, ethanol contents and pH levels (**Table 10** from materials and methods) to design the best production medium (OMP) to reach the highest *O. oeni* E5003 biomass. Bacterial biomass was measured as OD (600 nm) at the 27 media. The value of area under the curve was calculated for each medium after 7 days of incubation at 28 ºC.

As can be seen in the **Figure 27**, when the white concentrate must was diluted 4 times (media 1-9), the growth was lower than when the must was diluted 6 (media 10-18) or 8 (media 19-27) times. Between the must diluted 6 or 8 times the differences were lower, although there was a slightly better growth with the must diluted 6 times.

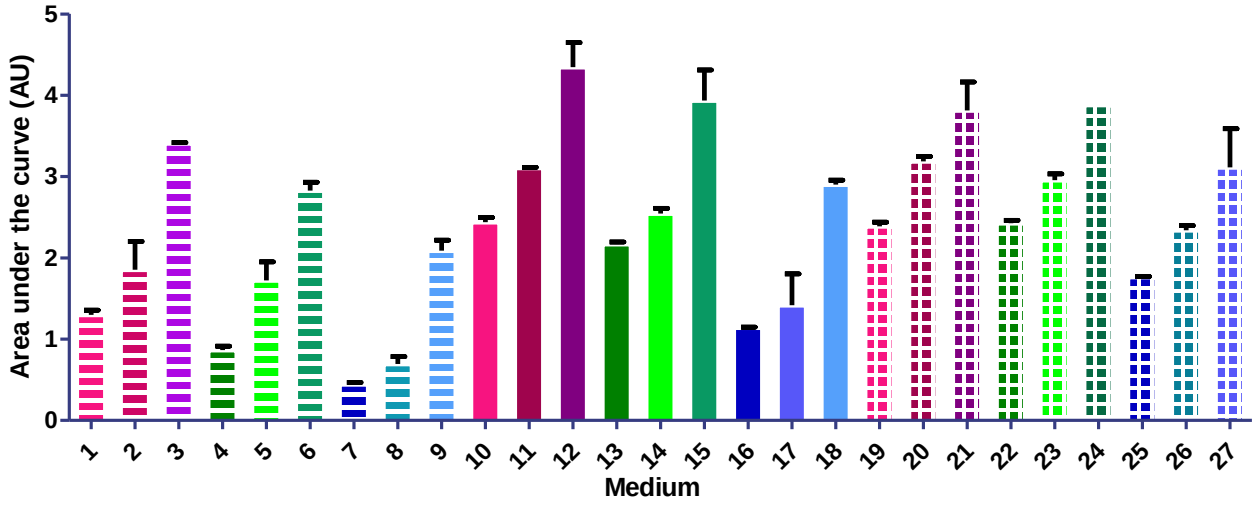


Figure 27. Values of *O. oeni* E5003 area under the curve (AU) in the 27 different culture media

The best growth was obtained with ethanol levels of 4 % (v:v) (1-3, 10-12, 19-21), and the worst results were found with ethanol levels of 8 % (v:v) (7-9, 16-18, 25-27). In all cases, growth with a pH of 4.5 (media 3, 6, 9, 12, 15, 18, 21, 24, 27) resulted in a major value of area under the curve followed by growth with a pH of 4 (media 2, 5, 8, 11, 14, 17, 20, 23, 26) and the minor value was obtained with the pH of 3.8 (media 1, 4, 7, 10, 13, 16, 19, 22, 25). It was observed that at lower pH, more time was needed to reach to the same O.D (600 nm). The best results were found, as could be expected, when the must was diluted 6 or 8 times with the lower ethanol content (4 %) and with the highest pH (4.5) (media 12 and 21) and the worst growth was observed with the must diluted 4 times, the highest ethanol content (8 %) and 3.8 pH (medium 7) (**Figure 27**).

Results were analysed using an ANOVA test. This analysis revealed a significant effect ($p< 0.05$) for must, ethanol, pH and for the interactions must-ethanol and must-pH. Through the Tukey *post hoc* test, the media with the lowest area under the curve were rejected (1, 2, 4, 5, 7, 8, 16, 17, and 25). All these media obtained an area under the curve below 2 AU, and their pH levels were 3.8 or 4. The cultures from the 18 remaining media were selected and its malolactic activity in wine was studied.

The composition of OMP medium was based on previous studies of growth, metabolism and biomass production of *O. oeni*. Champagne *et al.* (1989b) studied the production of *O. oeni* biomass in apple juice media and grape media. Their results showed that there were variations between strains in their ability to grow in grape or apple juice media and the optimum pH was also strain dependent, varying from 4.5 to 4.8. The supplementation by yeast extract was most beneficial for growth. In our study, 5 g/L of yeast extract was added to the media and a range of pH was studied but always below 4.8 because this value was too high, compared to the wine pH. Also malic acid was added because it stimulates LAB growth (Terrade *et al.* 2009) The studies of Zhang and Lovitt (2005) and Maicas *et al.* (1999b) showed that better growth of *O. oeni* was obtained in presence of sugar mixtures compared to sole sugar, when glucose-fructose (1:1) mixture was used in culture medium. The biomass formation showed little variation over the range from pH 3.5 to 5, while at pH 6 biomass was about half that obtained at pH 4.5. The optimal growth of *O. oeni* was at pH 4.5, with the maximum biomass yield and specific growth rate. In our experiment, the best biomass also was obtained when the bacterium was growth at

pH level of 4.5 and the medium contained a sugar mixture of glucose:fructose (1:1). When the must was diluted 8 times it contained around 45 g/L of glucose and 45 g/L of fructose, when was diluted 6 times it contained around 72 g/L of glucose, and 72 g/L of fructose and when was diluted 4 times, the must contained 90 g/L of glucose and 90 g/L of fructose. In this case, the bacterium grew less than when the must was diluted 1/6 or 1/8 probably because of the osmotic pressure.

4.3.2 Malolactic activity in wine

Henick-Kling (1988) said that if a bacterial culture, grown under much different conditions, is inoculated directly to the wine, it will lose much of its viability; that is because a large number of the bacteria will be killed or injured upon inoculation. To survive and grow in the new environment, the bacteria must make physical adjustments to maintain the cell's viability in the new environment. To induce properly a MLF with a bacterial starter culture, the loss of cell viability upon transfer must be minimized and bacterial cells must rapidly degrade malate. The procedures generally involve the successive transfer of the culture from synthetic nutrient media with high pH to media like wine containing grape juice or other fruit juices with pH values between 3.5 and 4 (Hayman and Monk 1982; Lafon-Lafourcade *et al.* 1983a; Beelman and Kunkee 1985; Wibowo *et al.* 1985).

In this study, after growing the selected bacteria E5003 in the different culture media, the cultures that had a value of area under the curve above 2 (3, 6, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 26, 27) (**Figure 27**) were inoculated in red wine made at laboratory. All cultures were inoculated in a final concentration of $2x10^6$ cfu/mL and the malolactic activity was analyzed (as consumption of malic acid (%) in wine). Some differences were found (**Figure 28**); these differences in malolactic activity in wine depended mainly on the pH of the medium where the bacteria were grown before. The cultures that were grown in a medium with a pH of 4.5, resulted in a slower consumption of malic acid in red wine. The best results in wine were found when *O. oeni* was grown in a medium with a pH of 3.8. This can be correlated obviously to the fact that this pH was more similar to the wine pH (3.5), so the cells were more adapted to it.

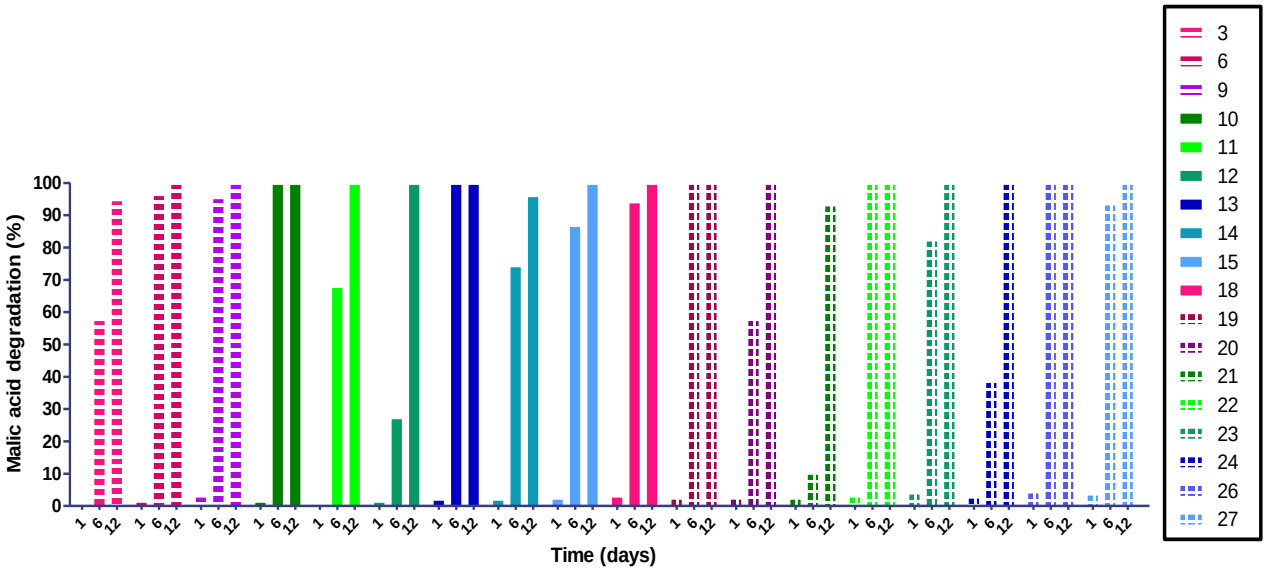


Figure 28. Consumption of malic acid (%) in red wine of the *O. oeni* E5003 strain previously grown in the different culture media (OMP) with different conditions (3, 6, 9, 10, 11, 12, 13, 14, 15, 18,19, 20, 21, 22, 23, 24, 26, 27 (**Table 9**))

From these results, the 5 cultures that consumed all the malic acid from red wine in 6 days (10, 13, 19, 22, and 26) were chosen as no differences in malolactic activity were found between them. From these 5 media, 4 of them (10, 13, 19 and 22) had been grown in a pH of 3.8, proving the importance of the pH as growth factor in the culture media to carry out the MLF in wine later on.

Because these 5 cultures had identical results of malolactic activity in wine, the culture with the highest biomass in the culture medium was chosen. The medium 10 was the one with the highest value of area under the curve (2.44 AU) obtained in the growth experiment (**Figure 29**), so this was chosen to be the biomass production medium, OMP. Consequently, we could obtain the maximum biomass with the same malolactic activity, so more liters of red wine could be inoculated with this liquid malolactic culture.

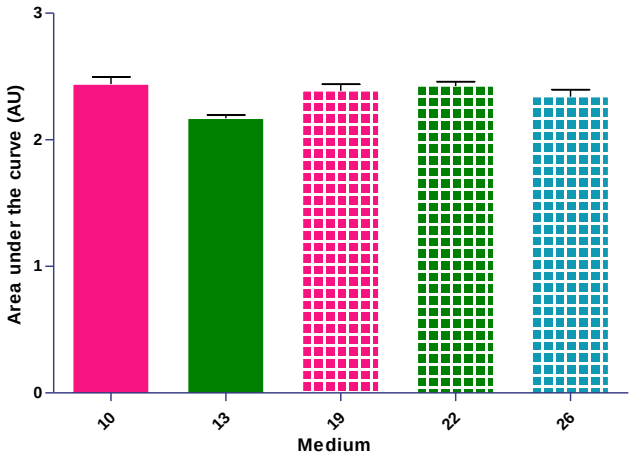


Figure 29. Values of the area under the curve of the *O. oeni* E5003 strain under different culture media (10, 13, 19, 22 and 26). Data taken out from Figure 27

A compromise between biomass production and malolactic activity in wine was found with the best production medium composition. That was the medium with the must diluted 6 times, with an ethanol content of 4 % and with a pH of 3.8. The HPLC analysis of this medium before inoculation, showed a concentration of around 40 g/L of glucose, 40 g/L of fructose, between 3.5-4 g/L of malic acid and 1 g/L of lactic acid.

4.3.3 Scale up of the biomass production process at laboratory

Sterilization controls

To reach a higher biomass production of the selected strain of *O. oeni,* E5003 in the OMP medium, a scale up was made from a 50 mL culture medium to 0.5 L and 10 L culture media. A microbiological analysis of the three scaled up steps was made to certify the correct sterilization of the OMP culture medium. The results showed that the sterilization of all media was adequate, not appearing any growth in any control media ($<1x10^2$ cfu/mL in all plate media).

O. oeni biomass production

The scale up started with a culture of 10^6 cfu/ mL *O. oeni* E5003 in 50 mL of OMP medium. When the bacterial population reached $1x10^9$ cfu/mL after 6 days incubation, the 50 mL were

inoculated into the 0.5 L fermenter that contained 0.5 L of OMP sterile medium. The inoculated fermenter was incubated at 28 ºC until the bacteria population reached $1x10^9$ cfu/mL after 4 days of incubation. Then the *O. oeni* culture was inoculated into the 10 L fermenter with 8 L of OMP sterile medium and was incubated at room temperature with agitation to a final concentration of $1x10^9$ cfu/mL after 6 days of incubation. To certify the bacterial viability, an analysis was done out using LIVE/DEAD BacLight bacterial Viability Kit (Invitrogen). The kit showed a 90 % viability of the culture, seeing as green (alive) the majority of the bacterial cells (**Figure 30**).

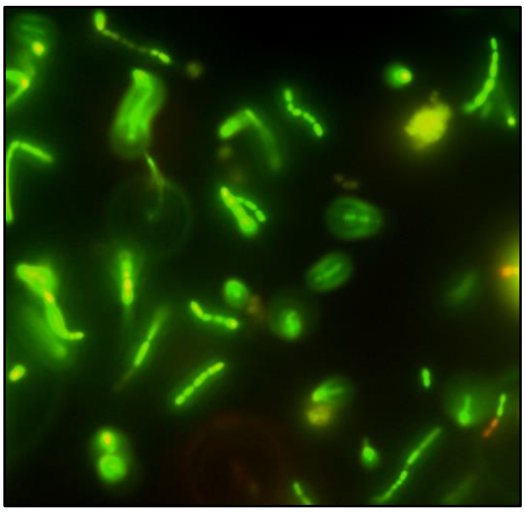


Figure 30. Cell viability assay of E5003 *O. oeni* strain from the 10 L fermenter with the LIVE/DEAD BacLight bacterial Viability Kit. In green live cells and in red dead cells

All the samples collected under sterile conditions during the biomass production in the 10 L fermenter were also analyzed microbiologically, counting the bacterial cells by Thoma counting chamber and plating 0.1 mL of serial dilutions on MLO medium to study the culture growth dynamics. The *O. oeni* cultures were also plated on ACE, MRS, PCA and GPYA media to check the purity and to reject any putative contamination. The plates were incubated at 28 ºC for 7 days. The data showed that no contamination was observed during the biomass production, the final bacteria concentration was $1.3x10^9$ cfu/mL after 6 days of incubation at room temperature (**Table 15**). The total time to obtain 8 L of a *O. oeni* liquid starter culture at laboratory with a final population of $1.3x10^9$ cfu/mL were 16 days.

Table 15. Viability cells analysis of the 10 L fermenter in a typical batch through the time in different culture media

	7 days / 28 ºC					
Time (days)	**Total count (chains/mL)**	**MLO (cfu/mL)**	**PCA (cfu/mL)**	**ACE (cfu/mL)**	**MRS (cfu/mL)**	**GPYA (cfu/mL)**
0	$8x10^7$	$8x10^7$	$<1x10^2$	$<1x10^2$	$<1x10^2$	$<1x10^2$
1	$1.6x10^8$	$1.1x10^8$	$<1x10^2$	$<1x10^2$	$<1x10^2$	$<1x10^2$
2	$3.2x10^8$	$2.2x10^8$	$<1x10^2$	$<1x10^2$	$<1x10^2$	$<1x10^2$
3	$6.4x10^8$	$5.7x10^8$	$<1x10^2$	$<1x10^2$	$<1x10^2$	$<1x10^2$
6	$2x10^9$	$1.3x10^9$	$<1x10^2$	$<1x10^2$	$<1x10^2$	$<1x10^2$

Strain identity

The identity of the *O. oeni* strain was verified by the molecular technique of RAPD-PCR. This technique is very sensitive, rapid, simple and high discriminating, and it has been used successfully with *O. oeni* (Reguant *et al.* 2005b; Ruiz *et al.* 2010; Solieri *et al.* 2010). The RAPD-PCR patterns of the isolates from the 0.5 L and 10 L cultures were compared to the pattern from the original *O. oeni* strain kept under glycerol at 15 %. The RAPD-PCR patterns were identical, confirming that the *O. oeni* E5003 was the same strain during all the production process (**Figure 31).**

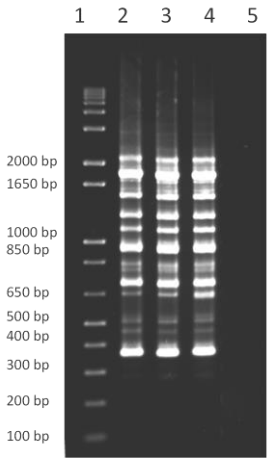


Figure 31. Electrophoresis of theRAPD-PCR of the two isolates of E5003 *O. oeni* strain from the 0.5 L and 10 L fermenters. Line 1: 1 KB plus ladder (Invitrogen). Line 2: band profile from the original *O. oeni* strain. Line 3: *O. oeni* strain isolated from the 0.5 L fermenter, Line 4: *O. oeni* strain isolated from the 10 L fermenter. Line 5: negative control

Sugars and malic acid consumption

The sugars consumed and the lactic acid formation by the *O. oeni* strain were followed during the biomass production of 10 L. Samples were taken and analyzed by HPLC at 1, 3 and 6 days after the bacterial inoculation. The results showed that the *O. oeni* strain consumed most of the malic acid during the first day. After that, the bacteria consumed the sugars, increasing the final amount of lactic acid (**Table 16**).

Table 16. HPLC analysis of the 10 L culture media (OMP) of a typical batch at 0, 1, 3 and 6 days after the bacteria inoculation

Time (Days)	Glucose (g/L)	Fructose (g/L)	Malic Ac. (g/L)	Lactic Ac. (g/L)
0	39.86	44.02	3.84	1.10
1	39.54	43.09	0.30	5.92
3	37.53	38.47	0.29	7.87
6	33.82	30.54	0.25	10.59

Malolactic activity in wine

As in the previous experiment, to study the malolactic activity and the cell viability of the starter, red wine made at the laboratory was inoculated with 10^8, 10^7 and 10^6 cfu/mL. The malic acid and lactic acid were measured by HPLC at 1 and, 6 days and 0.1 mL of serial dilution of the taken samples were plated on MLO. In all cases most of the malic acid was consumed in 6 days (**Table 17**).

Table 17. Malic and lactic acid HPLC analysis of red wine inoculated with $1x10^8$, $1x10^7$ and $1x10^6$ cfu/mL of the starter culture E5003 grown in OMP

Sample	Day 0		Day 1		Day 6	
	Malic acid (g/L)	Lactic acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)
Uninoculated wine	3.1	0.39	3.1	0.39	3.1	0.40
$1x10^8$ cfu/mL	3.1	0.39	1.33	2.87	0.08	4.09
$1x10^7$ cfu/mL	3.1	0.39	2.80	0.75	0	4.1
$1x10^6$ cfu/mL	3.1	0.39	2.96	0.42	0.39	3.40

The data showed that the viability of the cultures with less concentration (1x10^6 cfu/mL) increased; maintaining the bacterial population in the three cases over 1x10^6 cfu/mL, permitting the consumption of most of the malic acid in 6 days (**Figure 32**).

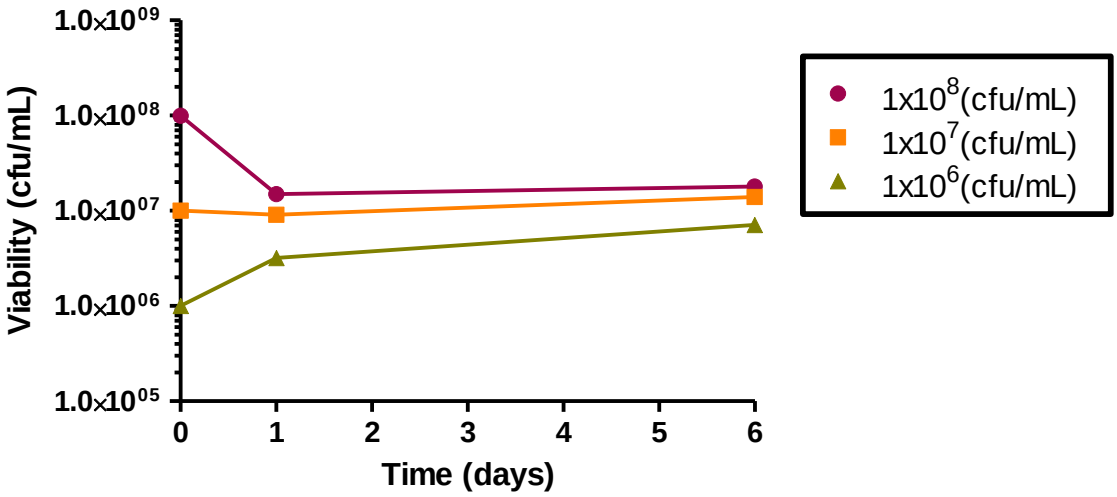


Figure 32. Viability of E5003 *O. oeni* strain in red wine when 1x10^8, 1x10^7 and 1x10^6 cfu/mL were inoculated, during 6 days at 28 ºC

4.3.4 Industrial biomass production

The technology and the know-how of the process were transferred to Agrovin S.A. and the last step of the biomass production was carried out in the industry with a production of 80 L in a 100 L fermenter. The 8 L of *O. oeni* culture was inoculated into the 100 L fermenter of OMP medium and was incubated at 28 ºC with agitation to a final concentration of 1x10^9 cfu/mL. The culture reached this cell concentration in 6 days (**Figure 33**). The total time of the production process, to obtain 80 L of a *O. oeni* liquid starter culture, from the stock culture at the laboratory, with a final population of 1x10^9 cfu/mL were 22 days.

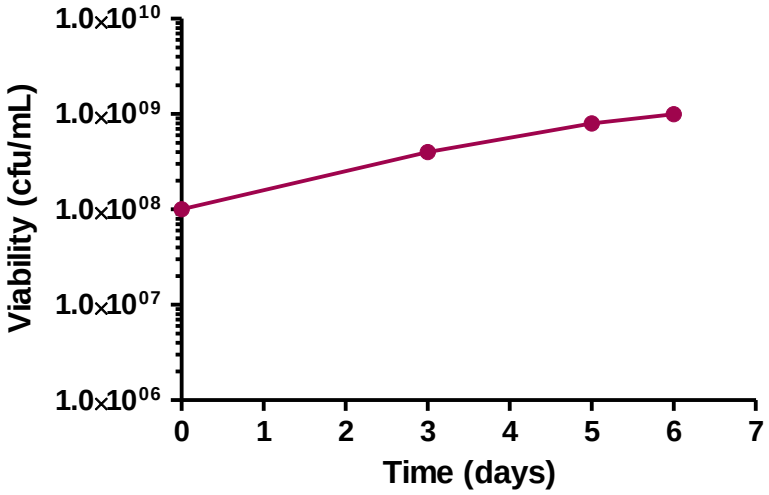


Figure 33. Kinetics of viable *O. oeni* E5003 cells (cfu/mL) in the 100 L fermenter during 6 days

Agrovin S.A. industry produces 800 L of the malolactic starter per year and all biomass is commercialized in containers of 1 L, 500 mL and 30 mL depending on the wine volume that has to be inoculated in the cellars (**Figure 34**).

Figure 34. Liquid malolactic starters of *O. oeni* E5003 commercialized by Agrovin S.A.

In Agrovin S.A. industry, the MLF starter culture was preserved at 4 ºC during 170 days (**Figure 35**). The starter culture showed a 60 % of cell viability during 120 days. After that time cell viability decreased to 50 % after 150 days.

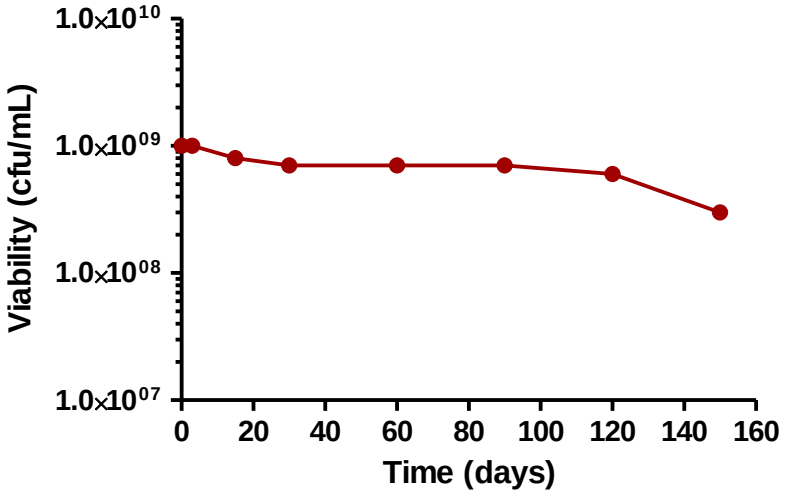


Figure 35. Cell viability of MLF starter culture *O. oeni* E5003 during storage at 4 ºC

Nowadays, the product is commercialized in Spain and France, and the experience shows that the *O. oeni* culture is implanted in most of the inoculated wines although they are produced in different regions and with different characteristics, carrying out a successful MLF in short time and with pleasant organoleptic properties in the final wine. This liquid malolactic starter does not need rehydration and acclimation, and has an easy application in wine, compared to the usual freeze-dried starters.

This result showed a commercial success with a stable and viable product. It was confirmed that a scale-up of the biomass production process could be done and the growth rate and yield and the malolactic activity were not affected. As we have seen before, many authors have studied the medium composition to reach the maximum biomass production and to improve the malolactic activity of *O. oeni* in wine (Henick-Kling 1988; Champagne *et al.* 1989a; Champagne *et al.* 1989b; Krieger *et al.* 1992; Zhang and Lovitt 2005) but in our case, we have demonstrated that the production of a liquid active malolactic starter is possible in a short time, and scalable.

4.4 Cellar malolactic fermentation starter culture design

This part objective was to find the responsible microorganism of histamine formation in a cellar located in Ribera del Duero region, with a high histamine concentration in its red wines, and to design a *O. oeni* MLF starter from the same cellar employing autochthonous strains grown in OMP medium to reduce as much as possible the histamine content of the final wines.

At present, there are no regulations regarding to the level of BAs in wine. However, it may happen that wines will not be accepted in some markets because the high levels of BAs. Moreover, it is usual that winemakers feel concerned with this problem and take it into account in order to produce high quality wines (Lonvaud-Funel 2001). To meet consumer demand, nowadays wines produced are less acid than in the past. Grape maturity is prolonged as far as possible to increase the extractability of phenolic compounds and the concentration of aroma precursors. Also, because of the climate change, total acidity is lower and pH higher. In the studied cellar, wine pH was typically between 3.7 and 3.8. At this high pH, SO_2 added at the end of malolactic fermentation is rather inefficient. Relatively high lactic acid bacteria populations are often encountered several months after vinification. Even if they are not growing, such latent populations survive and are still metabolically active. For example, they still produce histamine. Moreover, several decarboxylation pathways have been shown to provide energy to LAB, so amino acid-decarboxylating strains might survive longer than those that do not decarboxylate (Leuschner *et al.* 1998).

4.4.1 Characterization of the microbiota and selection of an autochthonous *O. oeni* strain

Histamine determination in wine samples

Samples of 13 wine vats were used for the analysis, before (samples A) and after (samples B) the MLF. The histamine quantification was made using the HPLC method previously developed.

The results showed that there was not histamine in wine samples from vats before the MLF was carried out (A), so yeasts did not produce histamine during the AF (**Table 18**). Otherwise, histamine was found in vat samples after MLF (B), confirming that LAB were the microorganisms responsible of the histamine formation. The HPLC data showed histamine contents from 2 mg/L to 37 mg/L (**Table 18**). These results were in accordance with those obtained by Aerny (1985) and Vázquez-Lasa *et al.* (1998) where BAs, and specially histamine, seemed to be more abundant in wines where MLF had occurred. This fact and the relatively high amount of precursors can explain why the highest concentrations of histamine are found in red wines. Landete *et al.* (2005b) studied the evolution of BAs before and after the MLF, observing an increase of histamine during MLF and after 6 months in bottle storage.

Table 18. Histamine content of the 13 wine vats sampled before MLF (A) and after (B) MLF

Vat	Histamine (mg/L)	
	A	B
V1	0	25
V2	0	21
V3	0	5
V4	0	5
V5	0	37
V6	0	2
V7	0	16
V8	0	23
V9	0	28
V10	0	2
V11	0	5
V12	0	17
V13	0	15

Bacteria isolation, identification and typing

Total cell and viable count were made from all samples before (A) and after (B) the MLF. In all samples growth was observed on MLO culture media and no growth was observed on MRS plates. In all cases, typical *O. oeni* morphology was observed under the microscope and on the plates (**Figure 36**).

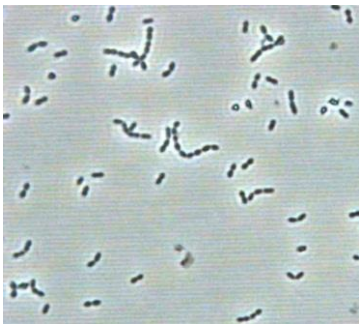


Figure 36. Microscopic morphology of *O. oeni* found in all wine vat samples

The cell count revealed an initial population of less than 10^4 cfu/mL of *O. oeni* in all samples, and bacteria population between 10^6 cfu/mL and $8x10^6$ cfu/mL after MLF (**Table 19**). These results suggest that *O. oeni* was the responsible of the MLF and the putative bacteria responsible of the histamine formation during the MLF. Usually *O. oeni* is the dominant species during the MLF, so many authors have considered it the main organism producing histamine in wine (Landete *et al.* 2005a; Lucas *et al.* 2005; Costantini *et al.* 2006).

Table 19. Total and viable count of *O. oeni* cells from the 13 wine vats studied before (A) and after (B) the MLF

Sample	A (cfu/mL)		B (cfu/mL)	
	Total chains (Thoma C.)	Viable cells (MLO)	Total chains (Thoma C.)	Viable cells (MLO)
V1	$<10^5$	9.0x10	$1.6x10^7$	$5.6x10^6$
V2	$<10^5$	$9.0x10^2$	$8.0x10^6$	$7.5x10^6$
V3	$<10^5$	$6.5x10^2$	$2.0x10^7$	$6.4x10^6$
V4	$<10^5$	$5.0x10^2$	$1.2x10^7$	$9.0x10^6$
V5	$<10^5$	$1.3x10^3$	$1.6x10^7$	$8.0x10^6$
V6	$<10^5$	$1.0x10^2$	$8.0x10^6$	$4.0x10^6$
V7	$<10^5$	$4.0x10^2$	$8.0x10^6$	$1.2x10^6$
V8	$<10^5$	9.0x10	$2.0x10^7$	$6.8x10^6$
V9	$<10^5$	7.0x10	$1.6x10^7$	$4.7x10^6$
V10	$<10^5$	2.0x10	$1.2x10^7$	$3.3x10^6$
V11	$<10^5$	9.0x10	$2.0x10^7$	$4.9x10^6$
V12	$<10^5$	2.0x10	$1.2x10^7$	$1.0x10^6$
V13	$<10^5$	$1.0x10^2$	$1.2x10^7$	$9.0x10^6$

The ability of different *O. oeni* to grow and undergo MLF in wine was tested by studying their population dynamics using RAPD-PCR with M13 primer. This method has already proven its utility in studying the population dynamics of unknown set of autochthonous bacterial strains (Ruiz *et al.* 2010; Solieri *et al.* 2010). From each sample, before and after the MLF, 10 colonies were randomly isolated from MLO plates and inoculated on new culture medium. The molecular technique of RAPD-PCR was realized for these 260 isolates. Dendograms were constructed from the cluster analysis of the isolates RAPD-PCR patterns. The value for the similarity coefficient (r=94%) derived from the reproducibility study was applied to calculate similarities between *O. oeni* strains. The results illustrated that the 260 isolates were grouped in eight different clusters. The 8 different RAPD-PCR patterns can be seen at **Figure 37**.

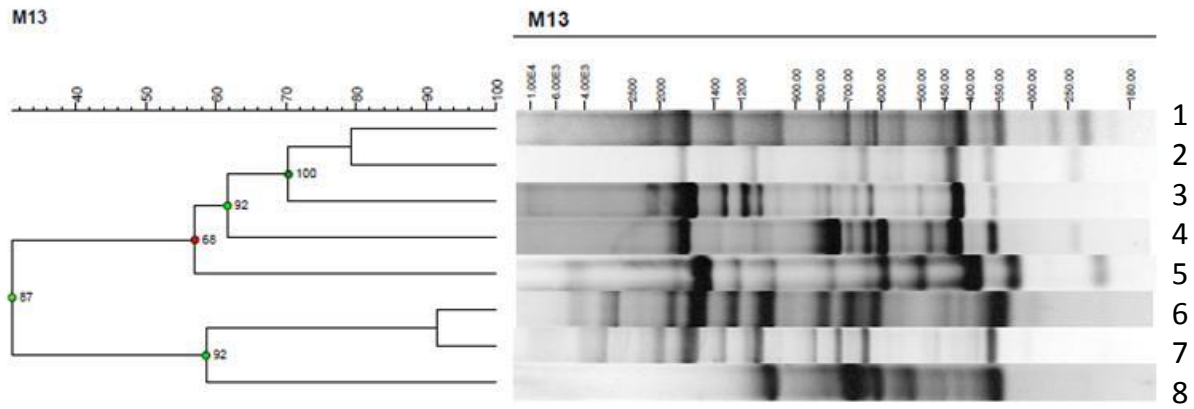


Figure 37. Dendrogram of the 8 different RAPD-PCR patterns of the *O. oeni* isolates from the 13 vats before and after the MLF

The findings showed in all cases a typical RAPD-PCR pattern from the bacterium *O. oeni,* and in most of the samples more of one pattern was obtained (**Table 20**). The results indicated that the RAPD-PCR pattern 6 was present in almost all vats before and after the MLF, but was not present in vats V6 and V10. In one cluster, all the isolates of V6B and V10B were grouped with the same RAPD-DNA pattern (cluster 1); showing that was the same *O. oeni* strain (**Table 20**). In both vats the histamine content was the lowest, with 2 mg/L. Subsequently, this strain was considered a good option to be the MLF starter.

Table 20. Different patterns (by RAPD-PCR with M13 primer), from *O. oeni* isolates, found in the wine vats before (A) and after (B) the MLF and histamine content in vats after the MLF (B)

Vat	RAPD-PCR patterns		Histamine (mg/L)
	A	B	B
V1	4-6	6	25
V2	4-6	3-6	21
V3	5-6-8	6	5
V4	6	6	5
V5	6-7	6-7	37
V6	1	1	2
V7	3-4-5-6	6	16
V8	6	6	23
V9	2-4-6	4-6	28
V10	1-2	1	2
V11	2-4	6	5
V12	6	4-5-6	17
V13	4-6	6	15

Histaminogenic activity

The different *O. oeni* strains isolated from all samples on MLO media, one of each cluster, were grown in MDB-mod for 15 days. After that time, the cultures were analyzed by HPLC to determinate if the isolates were able to produce histamine (**Table 21**).

Table 21. Histamine content produced from the *O. oeni* isolates (one per each cluster) grown in MDB-mod for 15 days at 28 ºC

RAPD-PCR patterns	Isolate	Histamine (mg/L)
1	V6B1	0
2	V9A1	0
3	V7A1	0.41
4	V13A1	0
5	V12B7	0
6	V5B2	17.37
7	V510	0
8	V3A7	6.86

The HPLC data showed that there were both *O. oeni* strains histamine producers and non-producers in these wines. The three isolates from the clusters 3, 6 and 8, were able to produce histamine in MDB-mod. From these 3 strains, V5B2 from cluster 6 produced the highest histamine content in MDB-mod. As can be seen in **Table 20**, the RAPD-PCR pattern 6 appeared in almost all vats before and after the MLF, explaining why all vats where it was present had the highest histamine levels.

Nevertheless, the isolate V6B1 (cluster 1) was not able to produce histamine in MDB-mod. This strain was present in vats V6 and V10 after the MLF, the vats with the lowest histamine level, therefore this would confirm that this *O. oeni* strain was the best option to become the autochthonous MLF starter, and this strain was selected to perform the following experiments. The strain was preserved by crioprotection to be used the following year in the cellar as MLF starter.

4.4.2 Malolactic fermentation starter production

Biomass production

To produce biomass of the V6B1 isolate, a scale-up was made from three cultures of 50 mL to three 10 L culture medium passing through a 0.5 L step as in the previous biomass production experiment, to inoculate a vat of 200 HL of red wine in the cellar. A microbiological analysis of the three scale-up steps was made to certify the correct sterilization of the culture medium. After the sterilization of 50 mL, 0.5 L and 10 L fermenters, 0.1 mL were sampled on ACE, MRS, MLO, PCA and GPYA media. The plates were incubated at 28 ºC for 7 days. The results showed that the sterilization of all media was adequate, not appearing any growth on control plates ($<10^2$ cfu/mL).

The scale-up started with a culture of 10^6 cfu/mL *O. oeni* V6B1 in 3 cultures of 50 mL of OMP medium. The culture was incubated at 28 ºC. When the bacterial population reached $1x10^9$ cfu/mL in 6 days, the 50 mL tubes were inoculated into three 0.5 L fermenters that contained 0.5 L of OMP sterile medium. The inoculated fermenters were incubated at 28 ºC until the bacteria population reached $1x10^9$ cfu/mL in 4 days. Then, the *O. oeni* cultures were

inoculated into the three 10 L fermenters with 8 L of OMP sterile medium and were incubated at room temperature with agitation to a final concentration of $1x10^9$ cfu/mL.

All the samples collected under sterile conditions during the biomass production in the 10 L fermenters were also analyzed microbiologically, counting the bacterial cells by Thoma counting chamber and plating 0.1 mL of serial dilutions on MLO medium to study the culture growing dynamics. The plates were incubated at 28 ºC for 7 days. The final bacteria concentration in the three fermenters was $1x10^9$ cfu/mL after 6 days of incubation at room temperature (**Figure 38**). Growth kinetics was the same than in the previous experiment, therefore this medium can be applied successfully to different *O. oeni* strains biomass production.

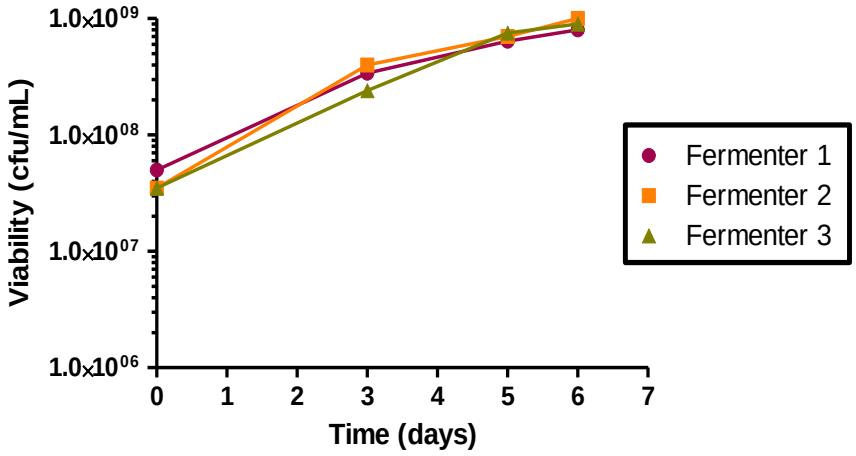


Figure 38. Kinetics of viable V6B1 *O. oeni* cells (cfu/mL) from the three 10 L fermenters during 6 days

HPLC analysis

The sugars' consumption and the lactic acid formation by the *O. oeni* strain were followed during the biomass production. Samples were taken and analyzed by HPLC at 2 and 5 days after the bacterial inoculation. The outcome showed that the *O. oeni* strain consumed most of the malic acid in less than two days. After that, the bacteria consumed the sugars, increasing the final amount of lactic acid as happened in the previous experiment with the E5003 *O. oeni* strain (**Table 22**).

Table 22. HPLC analysis of sterile culture medium and the cultures from the 1st, 2nd and 3rd 10L fermenters at 2 and 5 days after the bacteria inoculation

Time (days)	Fermenter (10 L each)	Glucose (g/L)	Fructose (g/L)	Malic Ac. (g/L)	Lactic Ac. (g/L)
0	Control	30.71	47.36	4.37	0.9
2	1st	28.43	42.74	0.2	4.98
	2nd	28.82	41.69	0.13	5.06
	3rd	27.27	37.8	0.15	5.48
5	1st	22.55	37.38	0.14	6.15
	2nd	23.99	34.88	0.14	6.75
	3rd	23.33	30.47	0.08	7.17

4.4.3 Malolactic fermentation cellar starter inoculation

In the cellar, after the AF, two vats of 200 HL of red wine were mixed and divided again in two vats (D12 and D14) to have the same physicochemical conditions. The D12 vat was inoculated with the MLF starter previously prepared (combining the three 10 L fermenters content) in a 1/1000 dilution to obtain a final concentration of $1x10^6$ cfu/mL bacteria. The other vat, D14, was kept as non-inoculated vat control.

Just before the inoculation, samples from the two vats were taken and physicochemical (**Table 23**) and bacteriological analyses were made from D12 and D14 vats. The HPLC results showed that wine had a content of 2 g/L of malic acid, a high content of ethanol (16.6 %) (v:v) and a high level of pH (3.73), confirming the favorable conditions for the production of BA by LAB. Landete *et al.* (2005b) studied the influence of enological factors on the histidine decarboxylase genes (*hdc*) expression and on the histidine decarboxylase enzyme (HDC) activity in *L. hilgardii*, *P. parvulus* and *O. oeni*. They concluded that histamine synthesis was negatively correlated to malic acid, citric and glucose content, and positively correlated to ethanol content

(122 % activity for 10 % ethanol) and for the pH, and optimal value of 5 for whole cells was found however, *O. oeni* cells keep more than 75 % of HDC activity at a pH of 3.

Table 23. HPLC analysis of D12 and D14 vats before the inoculation

Sample	Glucose (g/L)	Fructose (g/L)	Malic Ac. (g/L)	Lactic Ac. (g/L)	Ethanol % (v:v)	pH
D12	0	0.48	2.08	0.39	16.58	3.73
D14	0	0.47	2.12	0.34	16.58	3.73

4.4.4 Evolution of *O. oeni* populations and malolactic fermentation in wine

Wine samples were taken from both vats (D12 and D14) also during the winemaking process, just after the inoculation (BML), one week after the inoculation (1W) after the MLF (AML), after the SO_2 addition (SO_2), and when where transfer to barrels (BL), to analyze the malic and lactic acid, histamine contents, and bacterial viability.

Malic acid consumption

The HPLC data revealed that in both vats the MLF was performed, but in the inoculated vat (D12) the fermentation started before and probably ended before than the non-inoculated vat (D14). From the day that the D12 was inoculated until the MLF was finished in both vats went on 24 days (**Figure 39**).

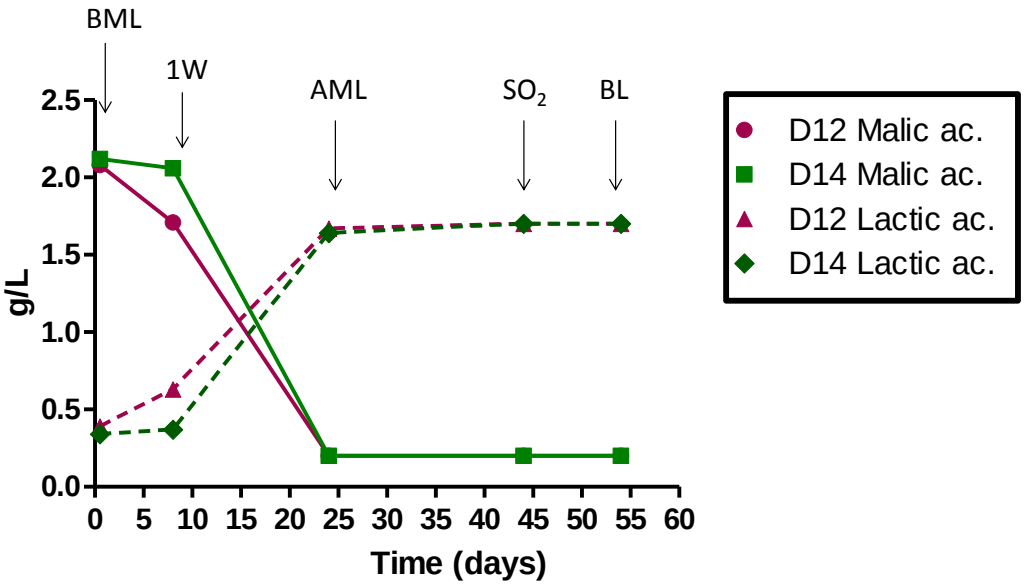


Figure 39. Malic acid consumption and lactic acid formation from the different samples taken from the inoculated vat (D12) and the non-inoculated vat (D14), before MLF (BML), after 1 week from the inoculation (1W), after the MLF (AML), after the SO_2 addition (SO_2), and when wine was transferred to barrels (BL)

Cell viability assay of *O. oeni* populations

Samples from both vats were analyzed microbiologically to study the kinetics of the bacterial populations. Samples that were taken before MLF (BML) shown that in the D12 vat, the viability of the MLF starter was the adequate with $1.3x10^6$ cfu/mL, while in the non-inoculate vat the endogenous bacteria population was in a concentration of $6x10^3$ cfu/mL. In both vats, the bacteria population increased until the MLF was ended (AML), to a population that went beyond 10^7 cfu/mL. After the SO_2 addition, *O. oeni* decreased until $1.92x10^6$ cfu/mL in the D12 vat and $3.06x10^6$ cfu/mL in the D14 vat, but in both cases the populations were still high and were maintained when were transferred to barrels (BL) (**Figure 40**).

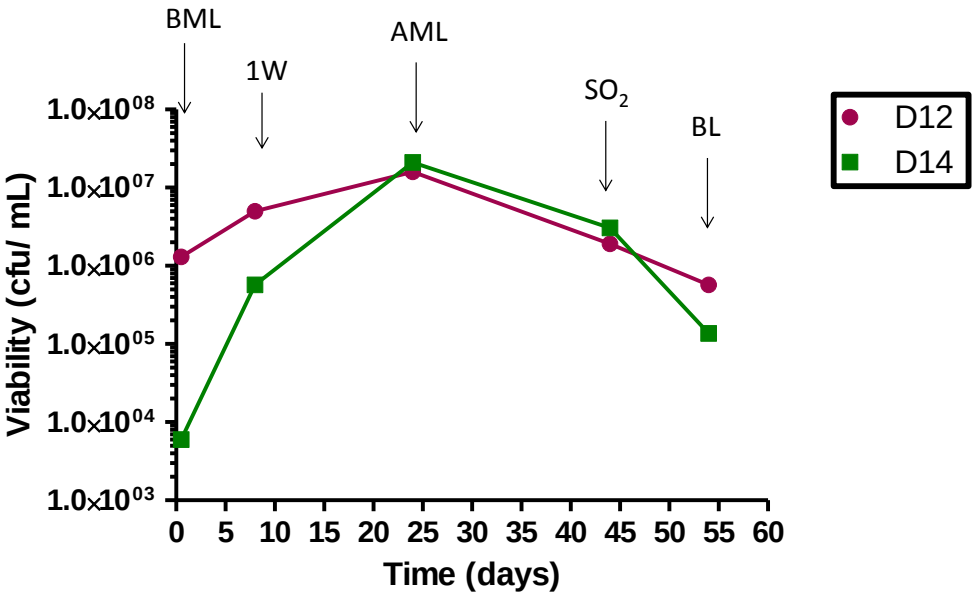


Figure 40. Cell viability of the *O.oeni* population (cfu/mL) from the different samples taken from the inoculated vat (D12) and the non-inoculated vat (D14), before MLF (BML), after 1 week from the inoculation (1W), after the MLF (AML), after the SO_2 addition (SO_2), and when wine was transferred to barrels (BL)

Histamine quantification

The histamine content from the samples BML, AML and BL was quantified by the HPLC method designed previously. Also, the histamine content was analyzed in wine samples after aging one year in barrels (BL1Y). The chromatograms showed that histamine was not found before the MLF in any vat (BML), but when the MLF was finished (AML) 0.98 mg/L and 5.1 mg/L were quantified in the D12 and D14 respectively. At this moment, there was 5 times less histamine content in the inoculated vat than in the non-inoculated one. This amount increased in both vats and when wines were moved to barrels, vat D12 maintained 2 mg/L of histamine; however in the vat D14 the histamine content increased to 12 mg/L. After one year of aging in barrels (BL1Y), the amounts of histamine resulted in 6 mg/L in vat D12 and 18 mg/L in vat D14, proving that the inoculation of the MLF starter caused significant differences between the vats (**Figure 41**). This dramatic reduction is significant at the commercial wines, as some importers demand histamine levels under 10 mg/L and their content should be as low as possible because their presence may pose a toxicological risk for consumers.

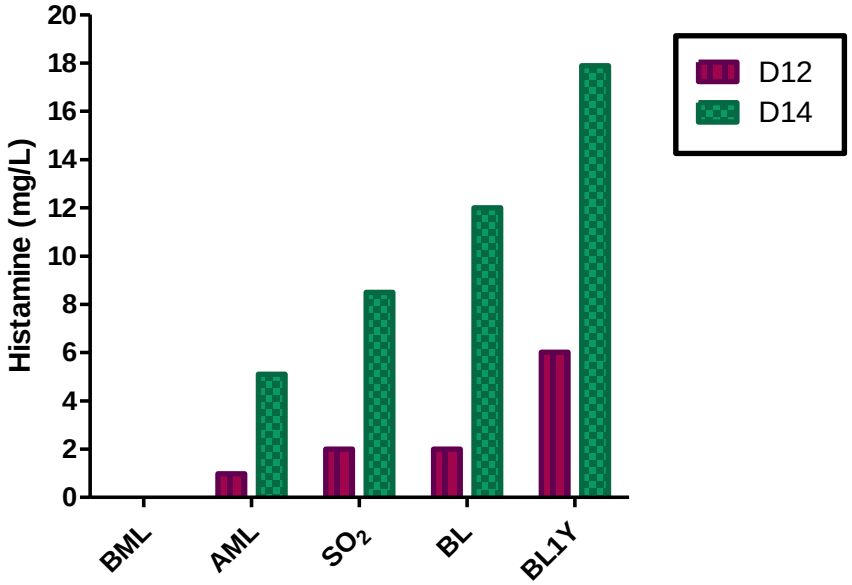


Figure 41. Evolution of the histamine content (mg/L) from the different samples taken from the inoculated vat (D12) and the non-inoculated vat (D14), before MLF (BML), after 1 week from the inoculation (1W), after the MLF (AML), after the SO_2 addition (SO_2), when wine was transferred to barrels (BL) and after one year of aging in barrels (BL1Y)

Evolution of *O. oeni* populations

To identify the *O. oeni* strains present in the different samples, 10 colonies were randomly taken from each stage and isolated on MLO plates, excepting from samples BL1Y because no viable bacteria were found at this point. The molecular technique of RAPD-PCR with the M13 primer was applied to all the isolates from all the samples, and the patterns obtained were compared to the pattern from the *O. oeni* strain inoculated (V6B1) employing the BioNumerics software. The value for the similarity coefficient (r=73%) derived from the reproducibility study was applied to compare similarities between *O. oeni* strains.

- Vat D12

The dendrogram of all the isolates from the inoculated vat (D12) samples was divided in two clusters with a cutoff of 73%. One cluster contained all the isolates from the sample taken from the barrel (D12BL) except the isolate D12BL-3. This isolate was in the other cluster with all the rest isolates and the RAPD-PCR pattern from the V6B1 strain. So, two

different *O. oeni* strains were in the vat D12, one that predominate at the first stages and the other one that appeared in the barrel stage (**Figure 42**). These results would explain why the content of histamine increased when wine was transferred to barrels (D12BL); possibly the inoculated strain was displayed by a histamine producer *O. oeni* strain.

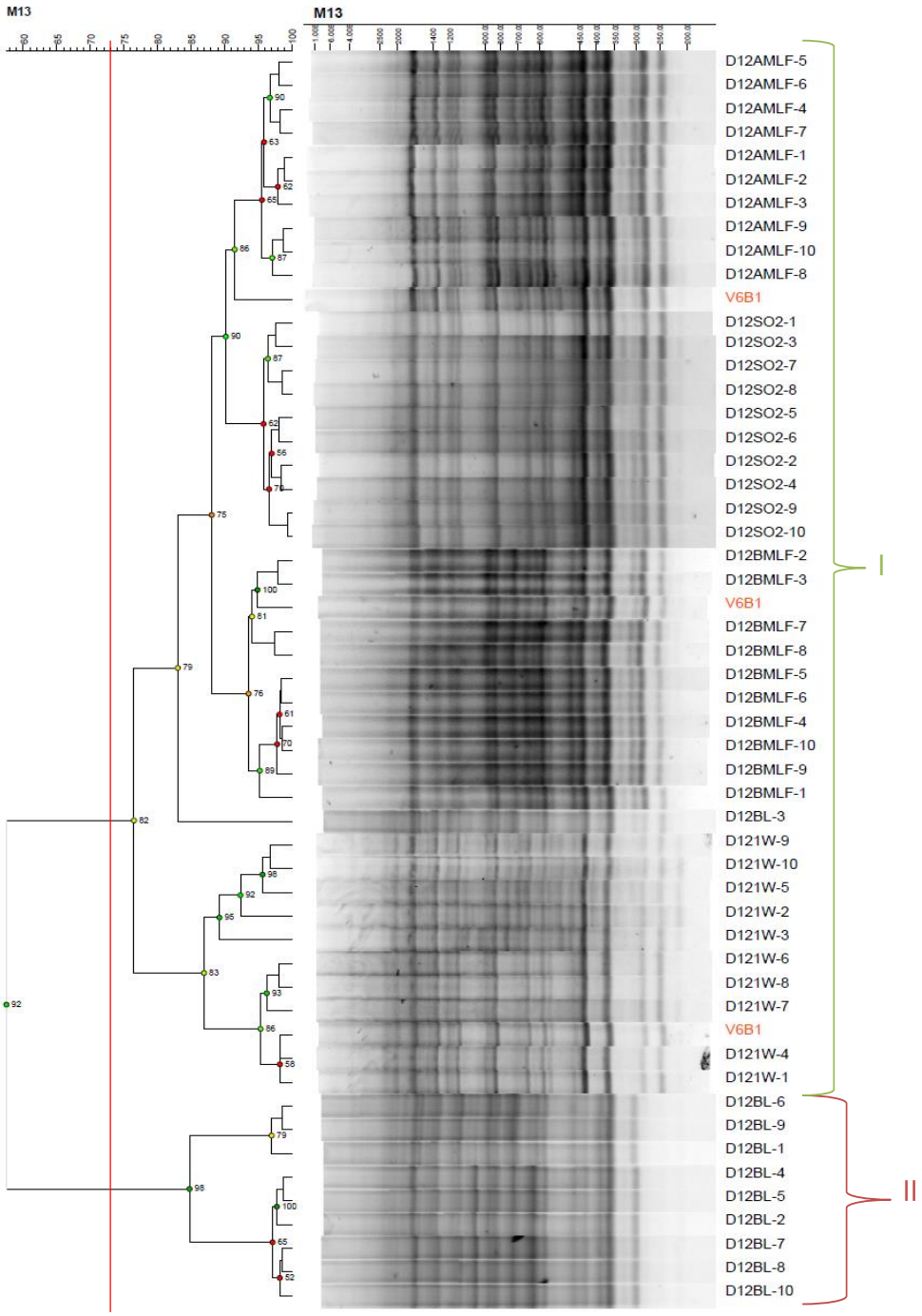


Figure 42. Dendrogram obtained from the all RAPD-PCR patterns from the D12 vat isolates from the different samples taken before MLF (BMLF 1-10), after 1 week from the inoculation (1W 1-10), after the MLF (AMLF 1-10), after the SO_2 addition (SO_2 1-10) and when wine was transferred to barrels (BL 1-10) and the control strain V6B1

- Vat D14

When the cluster analysis for the D14 vat was obtained, the dendrogram was divided in two clusters by a cutoff of 73 %. One cluster contained only one pattern from an isolate (D14AMLF-4). The remaining RAPD-patterns where included in one cluster with the V6B1 strain (**Figure 43**). So the inoculated strain predominated also in the non-inoculate vat.

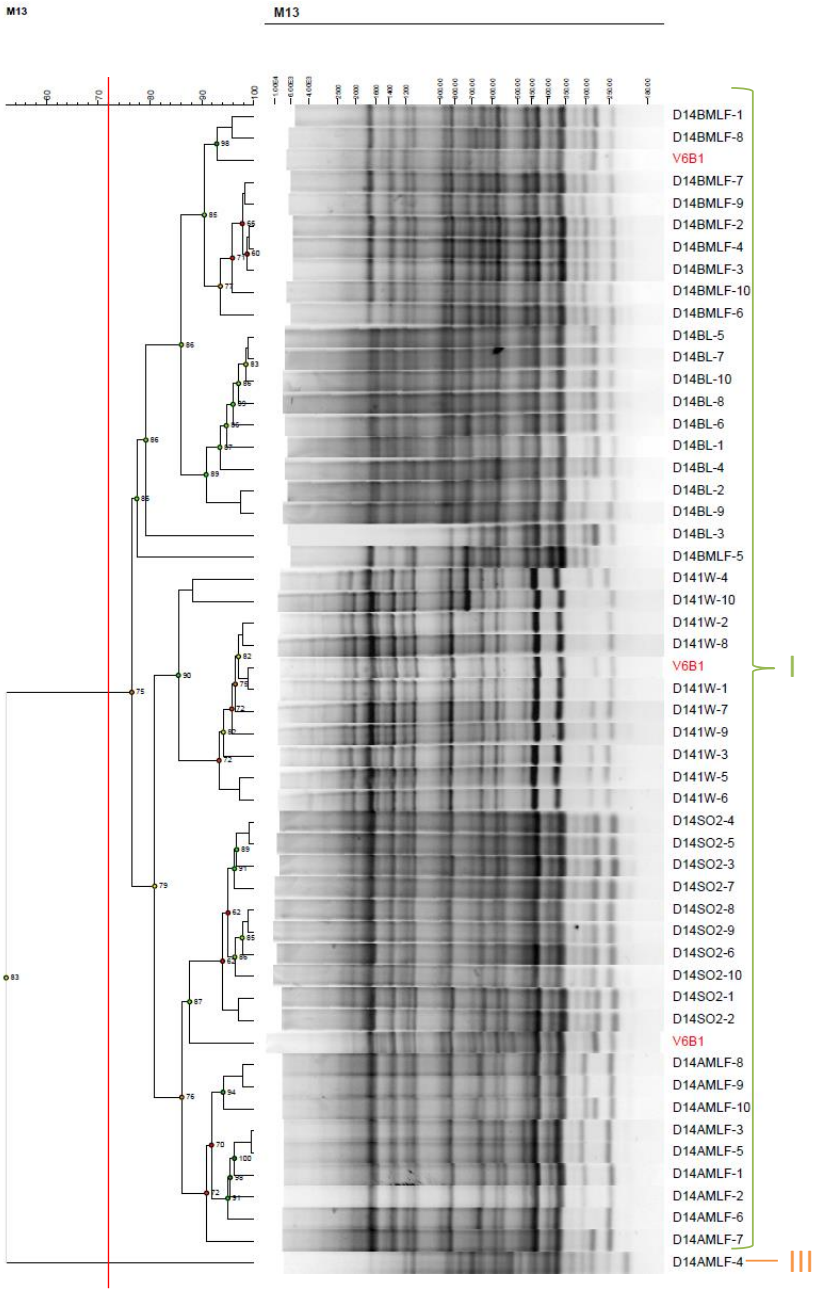


Figure 43. Dendrogram obtained from the all patterns from the D14 vat isolates from the different samples taken before MLF (BMLF 1-10), after 1 week from the inoculation (1W 1-10), after the MLF (AMLF1-10), after the SO_2 addition (SO_2 1-10) and when wine was transferred to barrels (BL 1-10) and the control strain V6B1

These apparently controversial results can be explained by diverse reasons. Possibly the molecular technique of RAPD-PCR using the M13 primer cannot distinguish between different strains because they have very similar patterns and what we considered one cluster was formed by more than one strain. Or maybe, a very low number of isolates had been taken, 10 colonies per each sample, and although there are other populations in less concentration, only the predominant strains had been detected. And, because the inoculated strain is an autochthonous strain from the cellar, was not strange that appeared in the non-inoculated vat.

4.4.5 Rapid detection of the histaminogenic activity of the autochthonous microbiota

In order to study if in wine samples from the non-inoculate vat, there were *O. oeni* strains in low populations able to produce histamine different from the inoculated strain, a rapid method of enrichment and histamine detection was carried out. Samples after the MLF from the 2 vats were taken (D12AML and D14AML), since it was the sampling time in which appeared the major difference in the histamine concentration between the two samples. The pellet obtained from the centrifugation of 8 mL of wine from the 2 AML samples were inoculated in 2 mL of MDB-mod and incubated for 3 days at 28 ºC. After that time, an HPLC analysis was made to quantify the histamine contents. The differences of histamine content between the two vats were again remarkable with 163.2 mg/L in the sample MDBD12 and 254.88 mg/L in the sample MDBD14 (**Figure 44**). As the results obtained in wine, there is more histamine content in the samples from vat D14 than in the samples from vat D12.

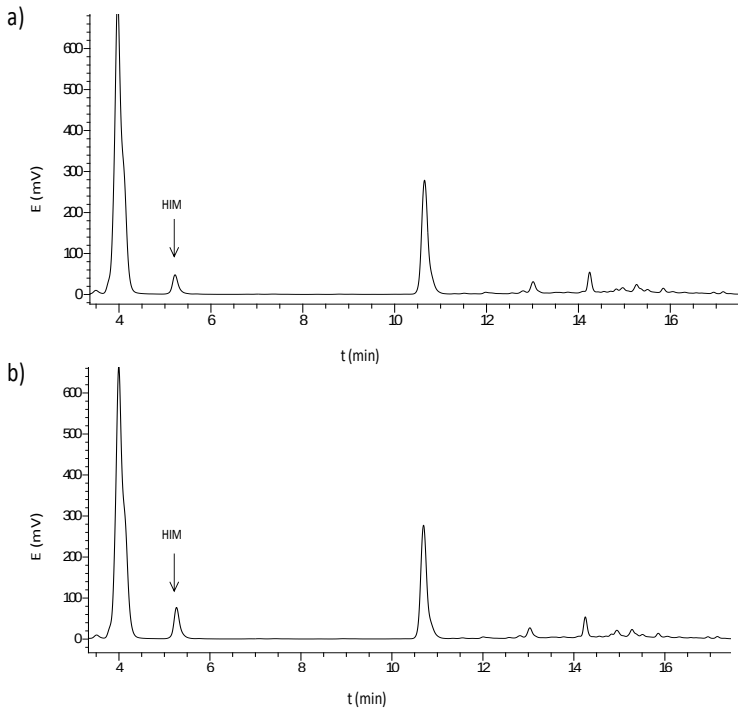


Figure 44. Chromatograms obtained from the HPLC analysis. Histamine (HIM) detection in: **a)** MDBD12 culture medium and **b)** MDBD14 culture medium

This method permitted to study the ability of the populations of forming histamine in only 3 days of incubation. The medium has a high concentration of histidine precursor, and low sugar concentration, so the histamine production is quick and the quantities are high, so this method can be used to predict and confirm the AB formation in wine before they appear.

Serial dilutions were made from the two MDB-mod cultures (MDBD12 and MDBD14) and were plated on MLO. After the incubation at 28 ºC for 7 days, 10 colonies were randomly taken from each sample and the molecular technique of RAPD-PCR with the M13 primer was performed. The patterns of both samples were compared to the V6B1 pattern with a cutoff of 73 %. Two different strains of *O. oeni* appeared in the dendrogram. In one cluster (I) five isolates from MDB12 were grouped with the V6B1 band profile, and in the other (IV) all the isolates from the sample MDBD14 with half of the MDBD12 isolates were grouped. This RAPD-PCR pattern (IV) did not appear directly in wine samples taken before from vats D12 and D14 (**Figure 45**).

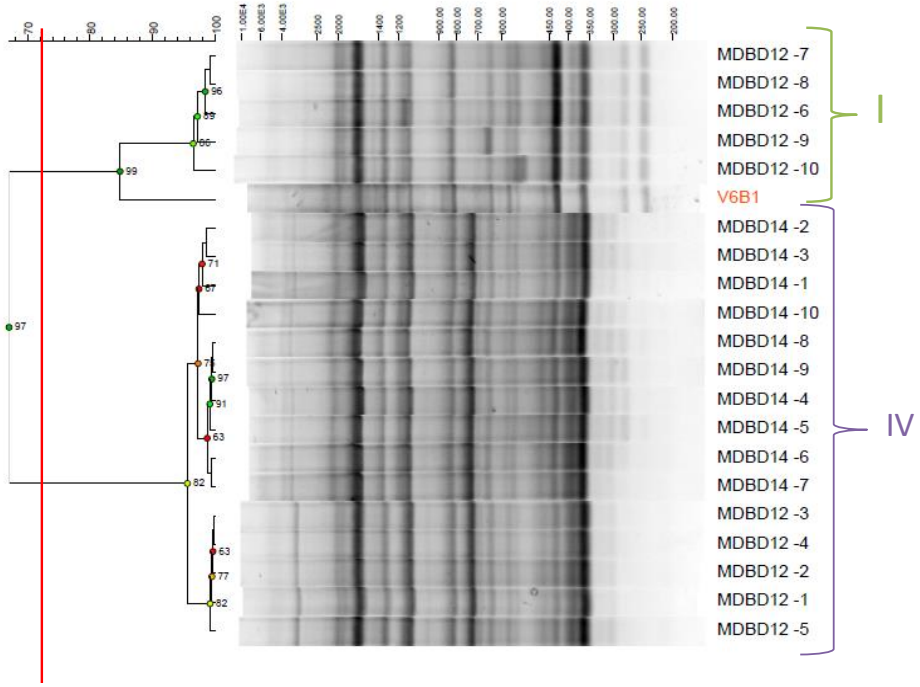


Figure 45. Dendrogram of isolates obtained from the MDBD12 and MDBD14 samples and V6B1 control strain

On one hand, these results could explain that the *O. oeni* strain (cluster IV) present in the sample MDBD14 was in a lower concentration than 10 % in the original wine at the sampling moment, since this RAPD-PCR pattern did not appear in the AMLF sample under the detection limits of this experiment. But when the bacteria were inoculated in MDB-mod medium with histidine, this strain had advantage over the V6B1 strain, displacing it, growing and producing high histamine content. On the other hand, in the D12 vat, also this histamine producer strain was found explaining why this sample had histamine. In the MDBD12 also the V6B1 strain was found, what could explain that the histamine content was lower than in the MDBD14 sample.

To verify this hypothesis, the isolates MDBD14-1 (cluster IV), MDBD12-1 (cluster IV) and MDBD12-6 (cluster I) were grown in liquid media MLO at 28 ºC. When the population arrived to $2x10^9$ cfu/mL, the pellet obtained from the centrifugation of 8 mL of the culture was inoculated in 2 mL of MDB-mod for 3 days at 28 ºC. After that time an HPLC analysis was made to quantify the histamine produced (**Table 24**).

Table 24. Histamine content produced by the different isolates (MDBD14-1, MDBD12-1 and MDB12-6) in MDB-mod medium incubated 3 days at 28 ºC

Sample	Histamine (mg/L)	Cluster
MDBD14-1	8.30	IV
MDBD12-1	9.74	IV
MDBD12-6	0	I

The chromatograms showed that the isolates from the cluster IV, MDBD14-1 and MDBD12-1, were able to produce histamine, this confirmed that this strain was the responsible of the histamine formation is samples MDBD12 and MDBD14 and probably was producing histamine in the wine samples but the bacterial concentration was lower than the 10 % so the strain and the RAPD-PCR pattern was not detected. The isolate MDBD12-6 from cluster I, with the same pattern than the V6B1 strain did not produce histamine, confirming that the malolactic starter was not able to produce histamine (**Table 24**).

4.4.6 Implications of wine inoculation with an autochthonous starter culture

When a dendogram with of all the RAPD-PCR patterns from isolates obtained from the vats D12 and D14 was performed, including the isolates from the MDB-mod samples and the strain V6B1, four clusters were obtained (**Figure 46**). The largest group (I) had the RAPD-PCR pattern from the strain V6B1 and most of the patterns excepting 9 isolates from the barrel sample from the D12 vat (D12BL) (cluster II). This results showed a displacement of the V6B1 *O. oeni* strain by the pattern of group II when the wine is transferred to the barrel, remaining only a 10 % of the pattern from V6B1. And, although the pattern from group IV did not appeare in the samples after the MLF (AML), this strain must be initially in a proportion lower than a 10 %. When this sample is inoculated in MDB, the population level of the pattern group IV increased. This strain can be producing histamine, and this would explain the 2 mg/L of histamine that were synthesized the D12 vat.

On the other hand, in the D14 vat, the predominant strain in all samples was the same strain that was inoculated in D12. Even so, in the sample D14BML, before the MLF occurred, the band profile D14BMLF-5 was found (cluster IV). This RAPD-PCR pattern was grouped

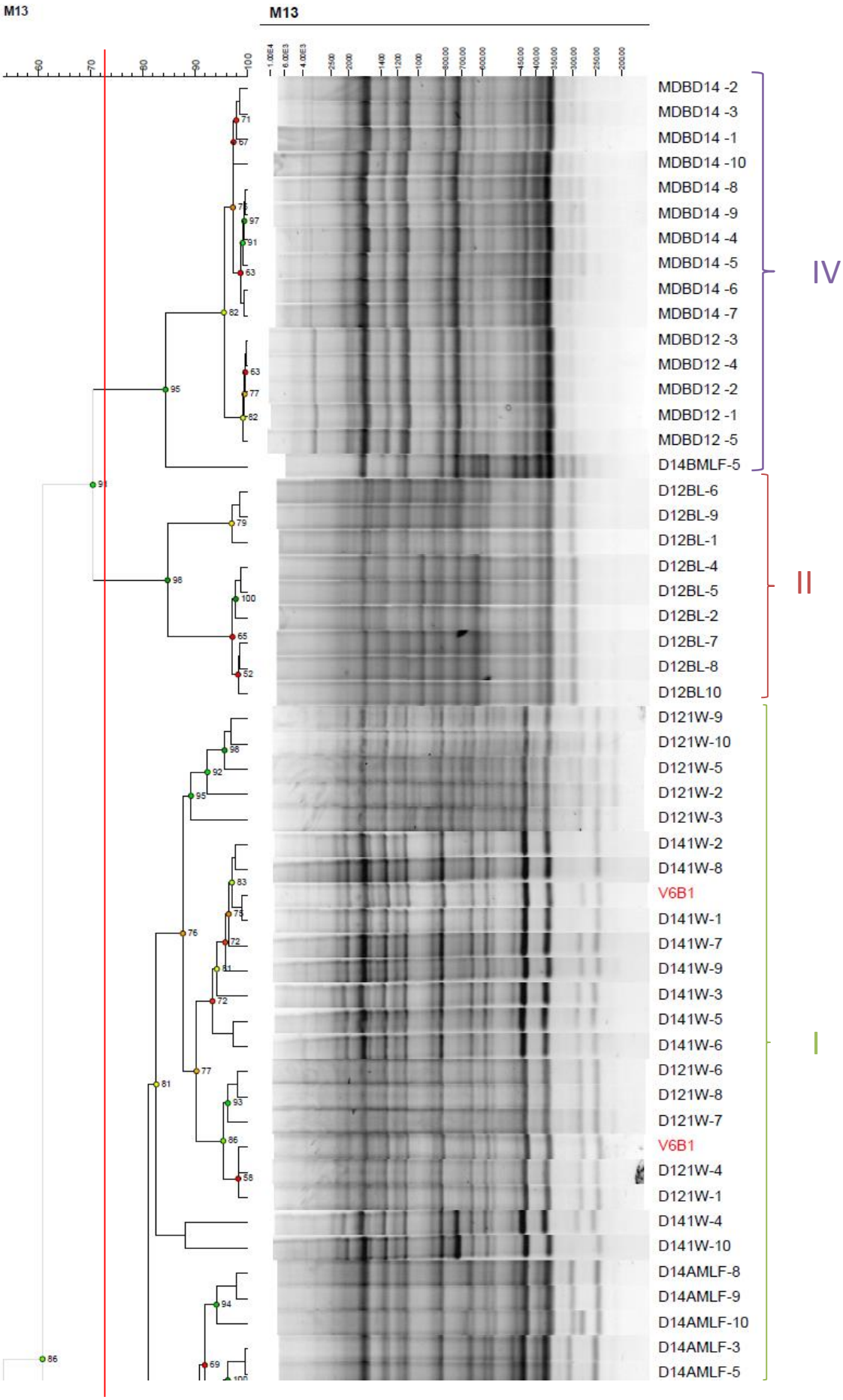
with all the isolates from the MDBD14 sample in the cluster IV (**Figure 46**); therefore, although this strain was in a lower proportion, it may produce histamine from the beginning of the MLF and producing more histamine content than in the vat D12.

After all, the inoculated wine with the selected strain (vat D12) showed 5 times less histamine content than the non-inoculated wine after the MLF, and 3 times less histamine content after one year of ageing in barrels. This experiment shows the success of a selection program of an autochthonous *O. oeni* strain in a cellar to reduce dramatically the levels of histamine. This can be an important way of prevention of BA production based on the use of selected non-histamine producing starters to carry out the MLF in wine. So this strategy can be used in any cellar to reduce the level of histamine content using autochthonous strains adapted to its characteristics and preserving the wine character.

The inoculation of the pellet wine sample (AML) in MDB-mod permitted the detection of histaminogenic activity in a short time (3 days) and permitted also to isolate *O. oeni* strains histamine producers that were not found in the wine original sample. This strategy can be used to detect histamine producer bacteria in wine and to predict the histamine formation in wine during the winemaking process.

Studies from Moreno-Arribas *et al.* (2003) and Manfroi *et al.* (2009) demonstrated that the application of commercial malolactic starters in wines were useful to reduce the BAs amounts, since in the inoculated wines, BAs concentrations were significantly lower when compared to those not inoculated. Otherwise, the use of an autochthonous starter culture well-adapted to the conditions of a specific wine-producing area has been recommended (Henick-Kling *et al.* 1989; Nielsen *et al.* 1996; Izquierdo *et al.* 2004; Capozzi *et al.* 2010; Cañas *et al.* 2012). Nevertheless, there are no references about reducing the histamine content with an autochthonous malolactic starter, so this study initiates a new research field to prevent the formation of AB in wine.

This strategy can be used also to select an autochthonous *O. oeni* strain adapted to specific or hard wine conditions as high ethanol content, low pH, etc. The selection of a specific autochthonous LAB and the biomass production using the OMP medium permits to produce MLF starter cultures '*à la carte*' for specific necessities of the cellars.


M13
M13
MDBD14 -2
MDBD14 -3
MDBD14 -1
MDBD14 -10
MDBD14 -8
MDBD14 -9
MDBD14 -4
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MDBD14 -6
MDBD14 -7
MDBD12 -3
MDBD12 -4
MDBD12 -2
MDBD12 -1
MDBD12 -5
D14BMLF-5
IV
D12BL-6
D12BL-9
D12BL-1
D12BL-4
D12BL-5
D12BL-2
D12BL-7
D12BL-8
D12BL10
II
D121W-9
D121W-10
D121W-5
D121W-2
D121W-3
D141W-2
D141W-8
V6B1
D141W-1
D141W-7
D141W-9
D141W-3
D141W-5
D141W-6
D121W-6
D121W-8
D121W-7
V6B1
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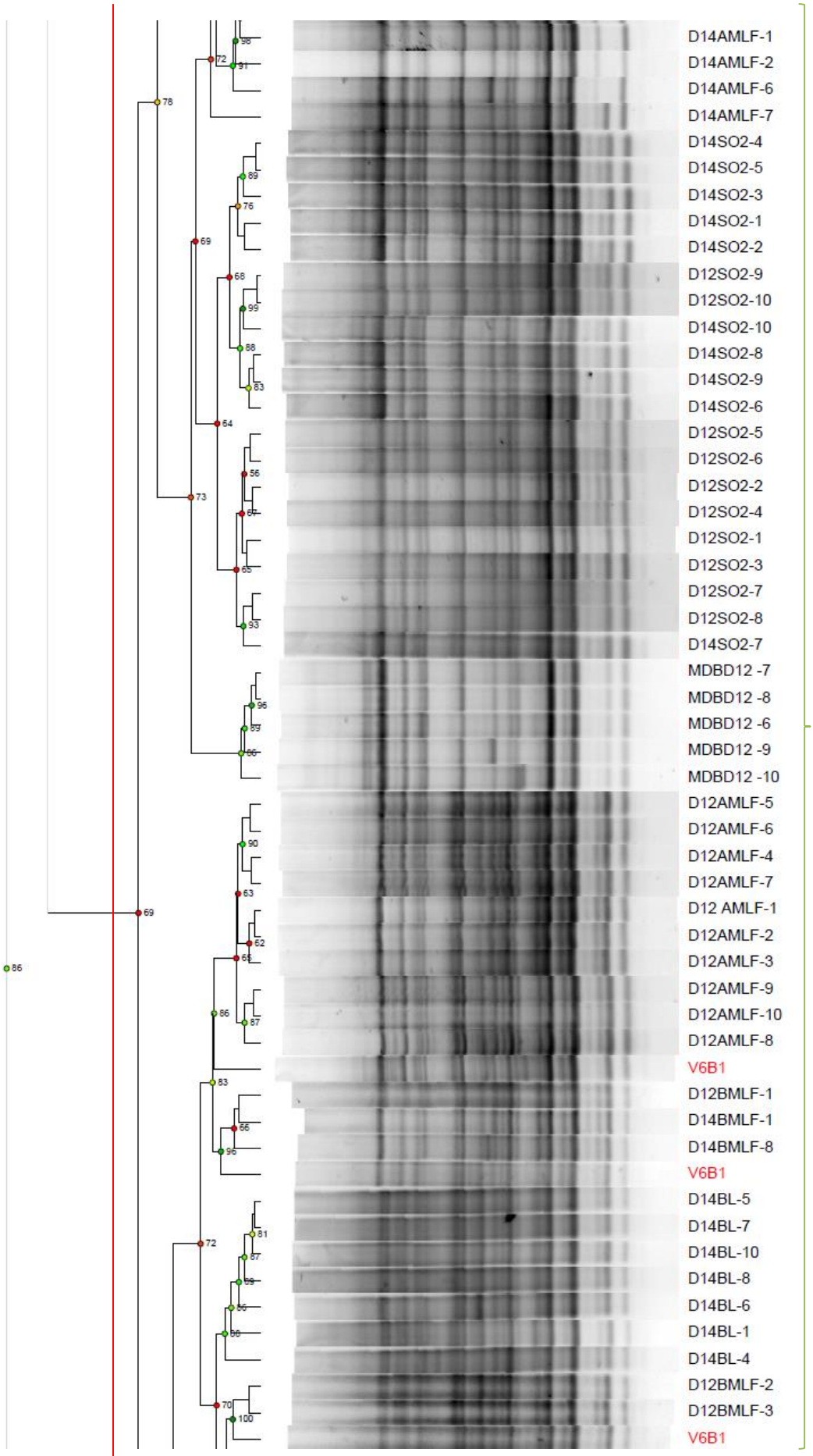
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D14SO2-6
D12SO2-5
D12SO2-6
D12SO2-2
D12SO2-4
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D12SO2-8
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MDBD12 -9
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D12AMLF-7
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D12AMLF-8
V6B1
D12BMLF-1
D14BMLF-1
D14BMLF-8
V6B1
D14BL-5
D14BL-7
D14BL-10
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D14BL-6
D14BL-1
D14BL-4
D12BMLF-2
D12BMLF-3
V6B1

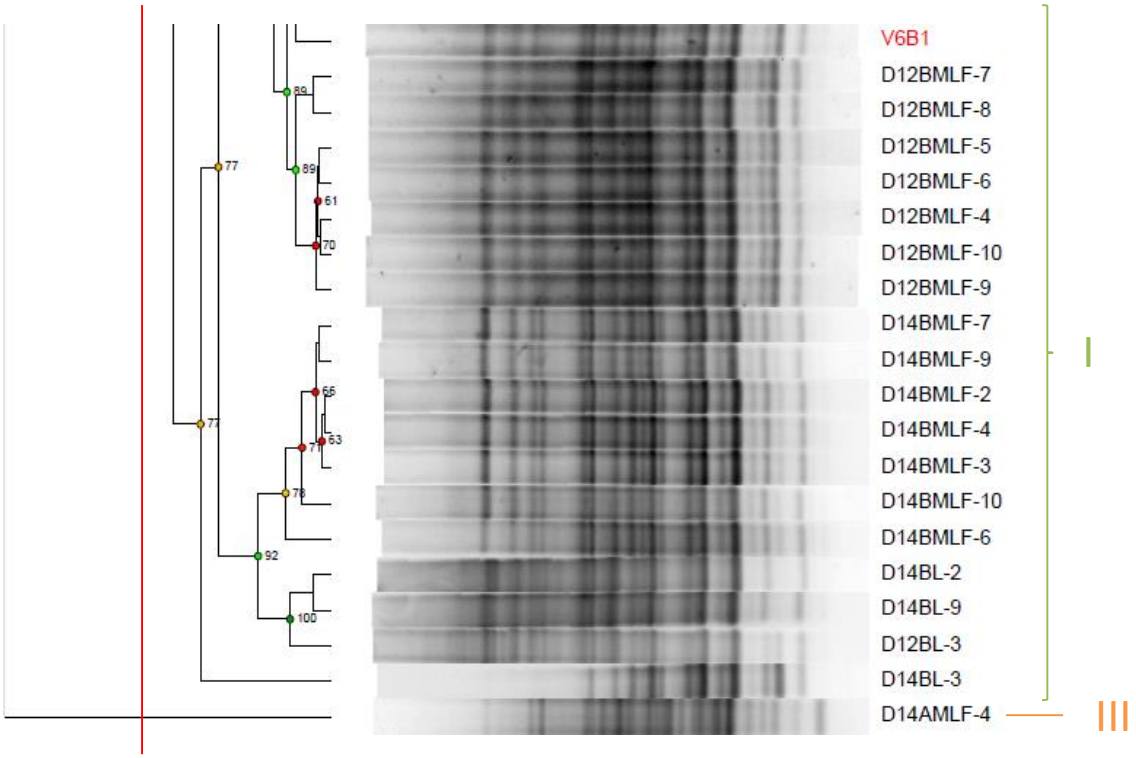

Figure 46. Dendrogram containing all the RAPD-PCR patterns from the isolates from vat D12 and vat D14, before the MLF (BMLF 1-10), one week after the starter culture inoculation (1W 1-10), after the MLF (AMLF 1-10), after the SO_2 addition (SO_2 1-10) and after the transfer to barrels (BL 1-10). All patterns were compared to band profiles from the V6B1 strain

4.5 Techniques of starter culture preservation

The most habitual techniques of starter culture preservation are freeze-drying and low temperatures. In this study, the maintenance of the liquid starter culture produced in OMP medium has been studied using freezing at -20 ºC and refrigeration at 4 ºC. These methods have been compared to the freeze-drying as it is the most common technique for the MLF starter culture preservation.

An alternative to the habitual preservation methods could be the use of wine lees. Traditionally, wine lees have been used empirically as a storing up system of microorganism in cellars, using them as starter culture year after year. These wine lees contain nutrients and a favorable environment for the bacterial survival along the time. The use of these wine lees as a preservation system would allow preserving selected microorganism and adapting them to the wine conditions. This system has also been studied in this project.

4.5.1 Malolactic starter culture preservation by low temperatures and wine inoculation

Malolactic starter culture preservation

The aim of this study was to compare different preservation techniques by low temperatures to establish the methodology that least affects the starter culture viability through the time, keeping the technological properties intact. The three methodologies that were studied were freeze-drying, refrigeration at 4 ºC, and freezing at -20 ºC, after growing the *O. oeni* E5003 strain in OMP medium.

After the 3 treatments, the cell viability and the activity of the culture with the 3 preservation techniques was analyzed. Firstly, the effect of the preservation methodology was studied. Results from the freeze-drying technique showed a large loss of cell viability, falling from $1.52x10^9$ to $3x10^8$ cfu/mL, so the viability after the process was 20 %. At time zero of

refrigeration and freezing, the viability of the cultures were 29.34 % and 43.34 % respectively. So the process of freeze-drying was the one that more affected cell viability.

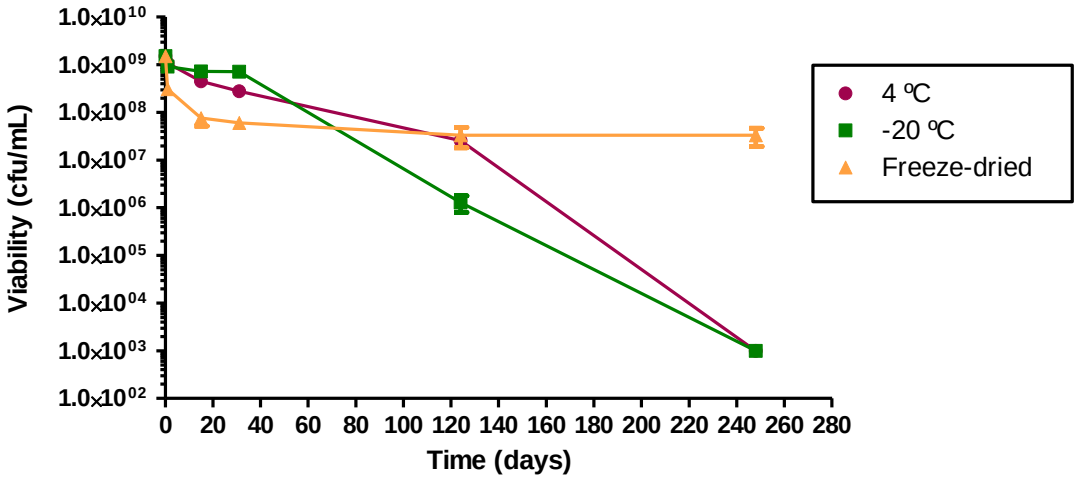

Figure 47. Cell viability of the E5003 *O. oeni* starter culture preserved by refrigeration (4 ºC), freezing (-20 ºC) and freeze-drying during 250 days

The culture preservation after the process of freeze-drying, showed that the cell viability was stabilized and maintained during 8 months. At this point this method presented the best viability results, so this preservation method would be the most adequate for long time storage (**Figure 47**).

On the other hand, freezing at -20 ºC presented a very low fall of cell viability thought the first month. This technique would be the most adequate for the culture preservation during 30 days, as during this time the freezing method presents the best results, preserving the bacterial population over $7x10^8$ cfu/mL (51.31 %). After that period the mortality increases drastically (**Figure 47**).

The preservation by refrigeration at 4 ºC was better than freeze-drying for preserving the bacteria culture through short period of time, until 50 days, but it was worse than freezing at -20 ºC. Therefore, for preservation from 2 to 4 months, the refrigeration was the best option because the cell viability was over $2.5x10^7$ cfu/mL after 124 days. After 4 months the cell viability dropped fast (**Figure 47**).

Depending on the preservation time that was required, one technique was better than the others. For a long storage period freeze-drying, would be the best option. Nevertheless, until 4 months, the storage at 4 ºC was the best choice, and for 1 month stores period the freezing at -20 ºC would be the preferred option (**Figure 47**). Many authors had studied the effect of different preservation methods of *O. oeni* and its influence on MLF, mainly freeze-drying conditions of survival of *O. oeni* for MLF in wine (Maicas *et al.* 2000; G-Alegria *et al.* 2004; Zhao and Zhang 2005; Zhao and Zhang 2009a). Maicas *et al.* (2000) studied the effects of freezing at -20 ºC in glycerol (40 % v:v) and freeze-drying in 0.067M of *O. oeni* upon induction of MLF in red wine and the results obtained in freeze-drying experiments were similar to those described for storage at -20 ºC, but the ability to induce the MLF in wine depended on the medium where the cells were previously grown before were stored freeze or freeze-dried.

As a conclusion, the refrigeration of the liquid starter is a good proposal for starter cultures. It provides a good viability for periods up to 3 or 4 months, and it is cheaper and easier to store and distribute than freeze-dried or frozen cultures. Besides as less manipulations are needed, less contamination risks occur.

Evaluation of the rehydration solution of freeze-dried cultures and inoculation in wine

The freeze-drying is a good method for long terms preservation as was confirmed in the previous experiment and it is the most common method, but the direct inoculation in wine can be an aggressive technique that kills most of the bacterial population, decreasing its viability and therefore, its malolactic activity. The usual way to inoculate a freeze-dried culture in wine at the laboratory is with a previous rehydration with NaCl 9 g/L. In this study the malolactic activity in wine was compared using NaCl or a mix of 5 g/L fructose, 5 g/L arginine and 3 g/L malic acid as rehydration solutions. Arginine and fructose were used because the cultivation of *O. oeni* in a fructose and arginine supplemented medium prior to wine exposure protects bacteria against subsequent wine shock. Arginine in combination with fructose triggers the expression of subset genes which are stress-responsive (Bourdineaud 2006). Malic acid was added to de rehydration mix to see if the MLF was carried out in wine faster. Krieger *et al.* (1992) support that cells should be grown in media with malate in order to prepare cells with high malolactic activity.

The *O. oeni* E5003 strain was grown in OMP and freeze-dried with glutamic acid 5 % as crioprotector. The amount of 0.1 g of the freeze-dried culture was rehydrated with 10 mL of both solutions and was incubated 1 hour at 28 ºC. After that time, cell count and viability was estimated. In both cases, the total count was $1x10^{11}$ cells/g and the viability of the culture was $8x10^{10}$ cfu/g, showing an 80 % of survival after the freeze-drying process. After the rehydration, the culture was inoculated from both rehydration solutions in red wine at $1x10^{7}$ cfu/mL and $1x10^{6}$ cfu/mL concentrations and samples were analyzed by HPLC. The malic acid consumption of the freeze-dried culture was compared to a culture grown in OMP and preserved at 4 ºC for 15 days and inoculated in wine in a final concentration of $1x10^{6}$ cfu/mL.

The HPLC data showed better results if $1x10^{7}$ cfu/mL were inoculated from the freeze-dried culture than if $1x10^{6}$ cfu/mL were inoculated. Comparing both rehydration solutions, the malolactic activity with the mix solution of fructose, arginine and malic acid yielded the best results. Furthermore, after three weeks, when $1x10^{6}$ cfu/mL were inoculated from the mix rehydration, the bacteria consumed all the malic acid. The worst result was observed when $1x10^{6}$ cfu/mL after a NaCl rehydration of the freeze-dried culture were inoculated in wine, there remained 2.73 g/L of malic acid after 21 days (**Figure 48**).

After all, comparing those results to a direct inoculation in red wine, from the bacteria grown in OMP and conserved at 4 ºC, it was observed that the freeze-dried cultures performed the MLF in a longer term. The liquid starter needed 6 days to consume all the malic acid (3.1 g/L) whereas the freeze-dried culture needed at least 2 weeks to carry out the MLF. So inoculating the same bacterial concentration in wine, the liquid starter culture preserved at 4 ºC showed a better adaptation to wine than the freeze-dried cultures.

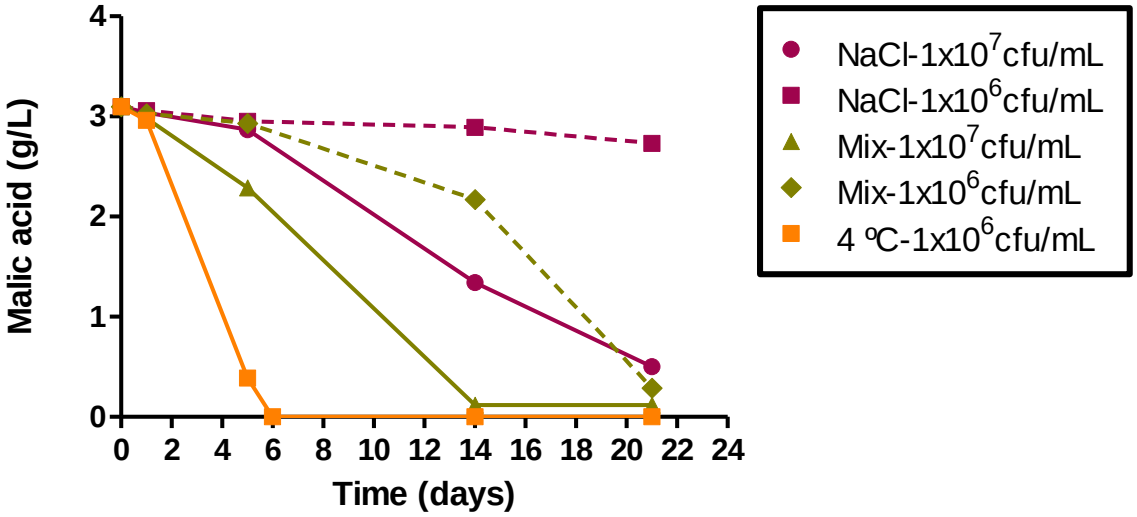

Figure 48. Malic acid consumption when the freeze-dried starter culture was rehydrated with NaCl or with rehydration mix (5 g/L fructose, 5 g/L arginine and 3 g/L malic acid) and inoculated in red wine in a final concentration of $1x10^6$ or $1x10^7$ cfu/mL compared to the starter culture refrigerate at 4 ºC inoculated in wine wine in a final concentration of $1x10^6$ cfu/mL

4.5.2 Preservation of bacteria in wine lees

The aim of this study was to employ wine lees from red wine as a system to preserve *O. oeni* cells. Three different Tempranillo wine lees (A, B and C) (**Table 7** from materials and methods) were used to preserve E5003 *O. oeni* to study the cell viability and malolactic activity in red wine after the preservation in these wine lees.

After the sterilization in the autoclave at 115 ºC, 30 min, samples of each wine lees were taken and 0.1 mL were plated on MLO, MRS, GPYA and ACE media. In all cases no growth was observed; therefore the sterilization was carried out adequately.

All the wine lees were inoculated with $3x10^7$ cfu/mL of E5003. After 2 weeks at room temperature, samples were taken and serial dilutions were made and plated on MLO. The molecular technique of RAPD-PCR was performed. The RAPD-PCR pattern of the 10 random colonies from each wine lees were identical than the pattern from E5003 strain, confirming *O. oeni* strain implantation.

The cell viability of the *O. oeni* strain in the 3 wine lees was followed by taking samples and plating serial dilutions on MLO during 16 weeks. Results showed that in A and B wine lees, the cell viability decreased under $1x10^6$ cfu/mL before the first 2 weeks in case A, and before 6 weeks in case B. However, in C wine lees, the cell viability was kept over $1x10^6$ cfu/mL for 12 weeks, and even the bacterial population increased in the first 2 weeks to $8x10^7$ cfu/mL (**Figure 49**). Therefore the C wine lees exhibited the best results and were the best lees to preserve *O. oeni* cells.

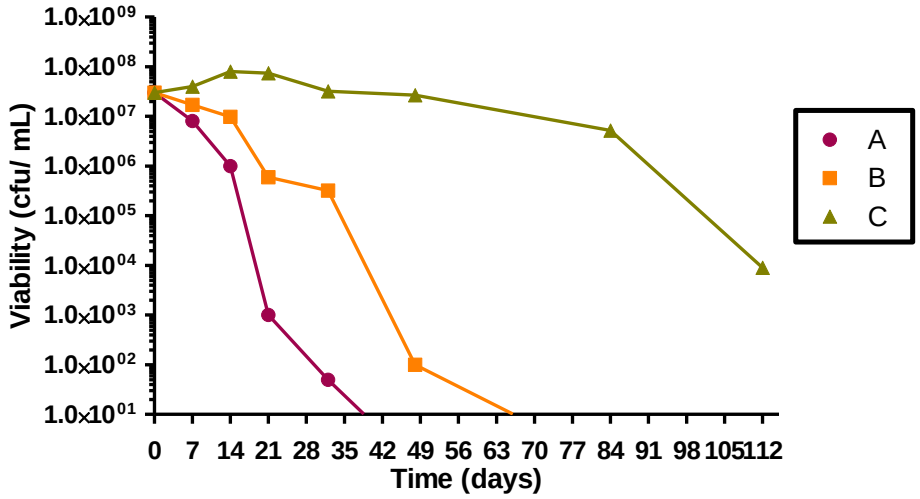

Figure 49. Viability of *O. oeni* E5003 in the three wine lees (A, B and C) during 16 weeks

The analysis of the 3 Tempranillo wine lees reveal that C wine lees had the highest content in polysaccharides (**Section 3.14.2**). Wine polysaccharides are grouped in 2 families according to their origin: those originating from grape primary cell walls, and those released by microorganisms. Polysaccharides from grape berries have pectin as one main constituent and neutral pectin substances (Vidal *et al.* 2003). The second most abundant family of polysaccharides in red wine is that of mannoproteins (Aymerich *et al.* 2006; Guadalupe and Ayestarán 2007). The origin of these macromolecules is yeast cell walls (Juega *et al.* 2014). Results from Diez *et al.* (2010) revealed that *O. oeni* growth was not inhibited by polysaccharide extract from Tempranillo wine lees, whose composition was mainly yeast mannoproteins (71.5 %) and that *O. oeni* growth was activated by 200 mg/L or lower concentrations of commercial mannoproteins. Hence, in this experiment, the high polysaccharides content of C could promote the high viability of *O. oeni* in those wine lees.

After 2 weeks of *O. oeni* preservation in wine lees, a red wine made at laboratory was inoculated in proportions of 1/10 and 1/100 with each of the 3 wine lees to study the cell viability and malolactic activity in wine. Samples were taken at 1, 6 and 14 days after the inoculation, and serial dilutions were plated on MLO to examine the cell viability (**Figure 50a**).

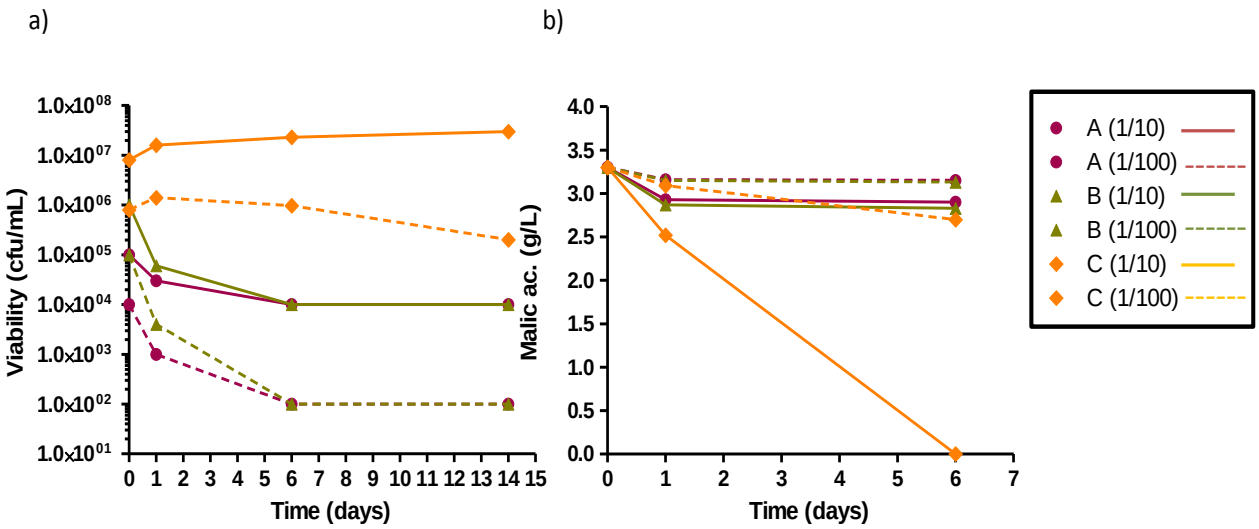

Figure 50. a) Cell viability assay and **b)** malic acid consumption of *O. oeni* E5003 in red wine, inoculating a portion of 1/10 and 1/100 of the 3 different wine lees (A, B and C) where the *O. oeni* strain had been previously preserved for 2 weeks

The data reveal that the best results appeared when C wine lees with the *O. oeni* E5003 cells were inoculated in red wine in a 1/10 proportion. In this case the initial bacterial population was $8x10^6$ cfu/mL permitting all the malic acid be consumed in a short time as can be seen in **Figure 50b**. In A and B wine lees, the initial cell concentration was too low because of the viability fell in the preservation period from $3x10^7$ cfu/mL to $1x10^7$ cfu/mL and $1x10^8$ cfu/mL respectively (**Figure 49**). In wine, the bacterial population was $1x10^6$ cfu/mL when A lees were inoculated in a proportion of 1/10 and $1x10^5$ cfu/mL in a proportion of of 1/100. When B lees were inoculated in a proportion of 1/10 in wine, the bacterial population was $1x10^5$ cfu/mL and $1x10^4$ cfu/mL and $1x10^4$ cfu/mL in a 1/100 proportion. In all cases less than $1x10^6$ cfu/mL were inoculated, for that reason the MLF was not developed and the malic acid was not consumed (**Figure 50**).

This experiment showed that C Tempranillo wine lees were an adequate system to preserve *O. oeni* cells probably because its high polysaccharide content. Also, the red wine inoculation with these lees showed that the bacteria possessed malolactic activity and satisfactory cell viability that permitted growth in wine and the consumption of all the malic acid in 6 days.

Although the loss of cell viability is higher that when the *O. oeni* culture is frozen, freeze-dried or refrigerated, the use of wine lees can be an alternative method to preserve the cells. To our knowledge no references have been reported before about this technique, so more studies should be done to find the best characteristics and conditions of the preservation in wine lees.

4.6 Coimmobilization of *S. cerevisiae* and *O. oeni* in delignified cellulosic material and starch gel

4.6.1 Coimmobilization of yeast and bacteria

Delignified cellulosic material have been used as immobilization supports of yeasts (Bardi 1994) and bacteria (Agouridis *et al.* 2005; Agouridis *et al.* 2008) for winemaking, demonstrating their promotional effect on AF and MLF and reduction of the activation energy (Ea) even at very low temperatures. DCM is a material of food-grade purity, cheap and abundant in nature, and the immobilization method is simple and easy. Furthermore, it was recently demonstrated that DCM contains nano- and micro-tubes (**Figure 51**) facilitating extremely low temperature fermentation and other bioprocesses. Moreover, other biomaterials, such as starch gel have been interesting for yeast entrapment for use in wine fermentation (Kandylis *et al.* 2008).

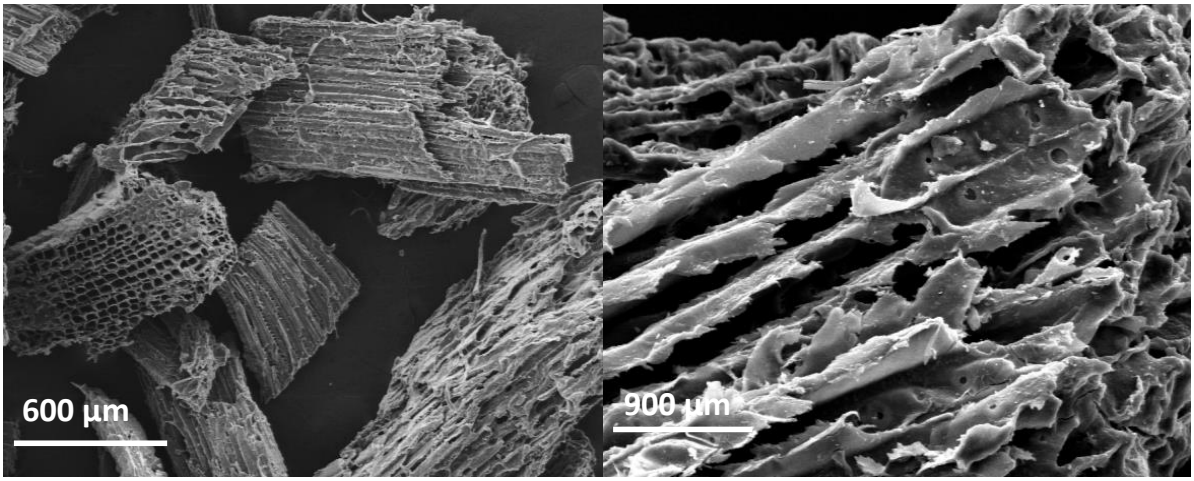

Figure 51. Electron micrographs of microtubes of DCM

O. oeni E5003 was firstly immobilized in DCM and then coimmobilized with *S. cerevisiae* AZAX-1 using wheat starch gel. The immobilized biocatalyst was successfully thermally dried in 48 hours. Cell immobilization was confirmed by electron microscopy (**Figure 52**), showing *O. oeni* immobilized in DCM.

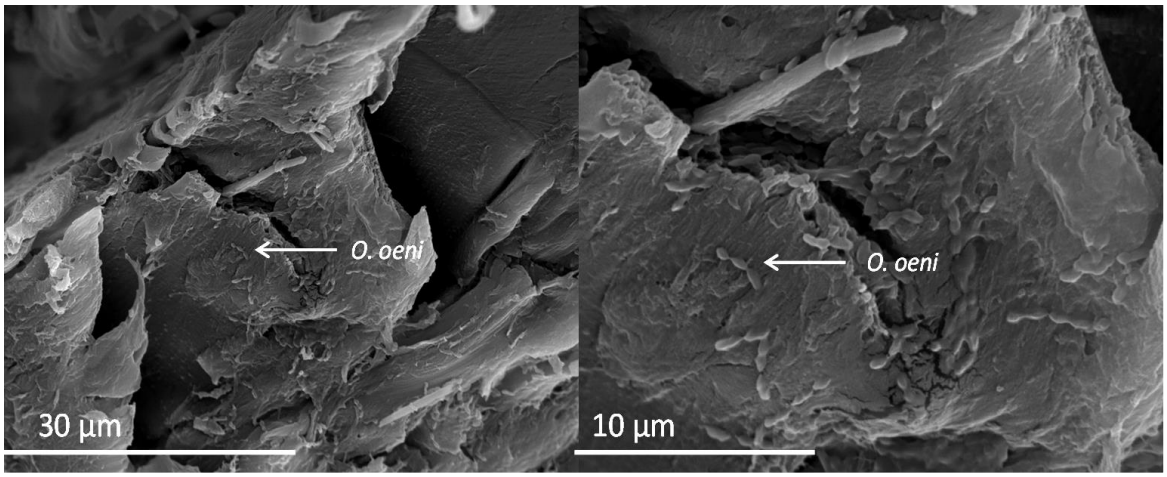

Figure 52. Electron micrographs of DCM and starch gel with the immobilized cells of *O. oeni* E5003

At **Figure 53**, bacteria cells on the surface of DCM and covered by the starch gel and yeast entrapped inside the gel can be observed.

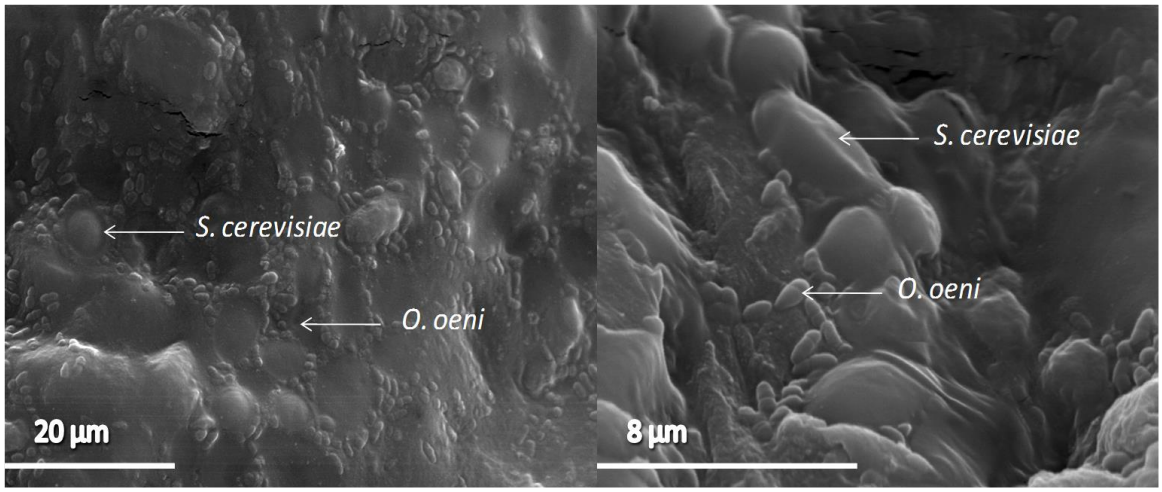

Figure 53. Electron micrographs of DCM and starch gel with the coimmobilized cells of *S. cerevisiae* AXAZ-1 and *O. oeni* E5003

4.6.2 Alcoholic and malolactic fermentations in wine

The evaluation of the coimmobilization of yeasts and bacteria for AF and MLF in wine was made in terms of malic degradation and sugars' consumption in red grape must. An amount of 0.3 g or 0.03 g of the immobilized biocatalyst was introduced into 50 mL of red grape must. Inoculating this amount, the estimated final bacterial population in wine were

around 10^6 cfu/mL and 10^7 cfu/mL respectively. The coimmobilization was compared to a simultaneous inoculation of 10^6 cfu/mL or 10^7 cfu/mL of free cells of *S. cerevisiae* and *O. oeni*. For the analysis, samples were taken through 8 days. The HPLC results showed the consumption of the 3 g/L of malic acid within 5 days, inoculating 0.3 g of the biocatalyst or 10^7 cfu/mL of free cells, and sugars were consumed in 4-5 days (**Figure 54b and 54d**). Therefore there were no initial differences between inoculating 0.3 of biocatalyst g or 10^7 cfu/mL of cells. In both cases, the AF and the MLF were carried out simultaneously, so the *O. oeni* strain inoculated in high concentrations with the yeast permits a rapid MLF what reduces considerably the winemaking process time.

When 0.03 g of the biocatalyst were inoculated in 50 mL of red grape must, sugars were consumed in 7 or 8 days and malic acid was consumed in 8 days (**Figure 54a**). Compared to the simultaneous inoculation of 10^6 cfu/mL of free yeast and bacteria, the MLF was faster. When free *S. cerevisiae* and *O. oeni* cells were inoculated simultaneously in a final concentration of 10^6 cfu/mL in grape must, the AF was carried out in 7-8 days but the MLF was not finished within this time, more than 2 g/L of malic acid were left (**Figure 54c**). Hence, for a simultaneous AF and MLF with 10^6 cfu/mL *S. cerevisiae* and *O. oeni*, the coimmovilization in DCM and starch gel presented an advantage compared to the co-inoculation of yeast and bacteria, completing both fermentations in 8 days.

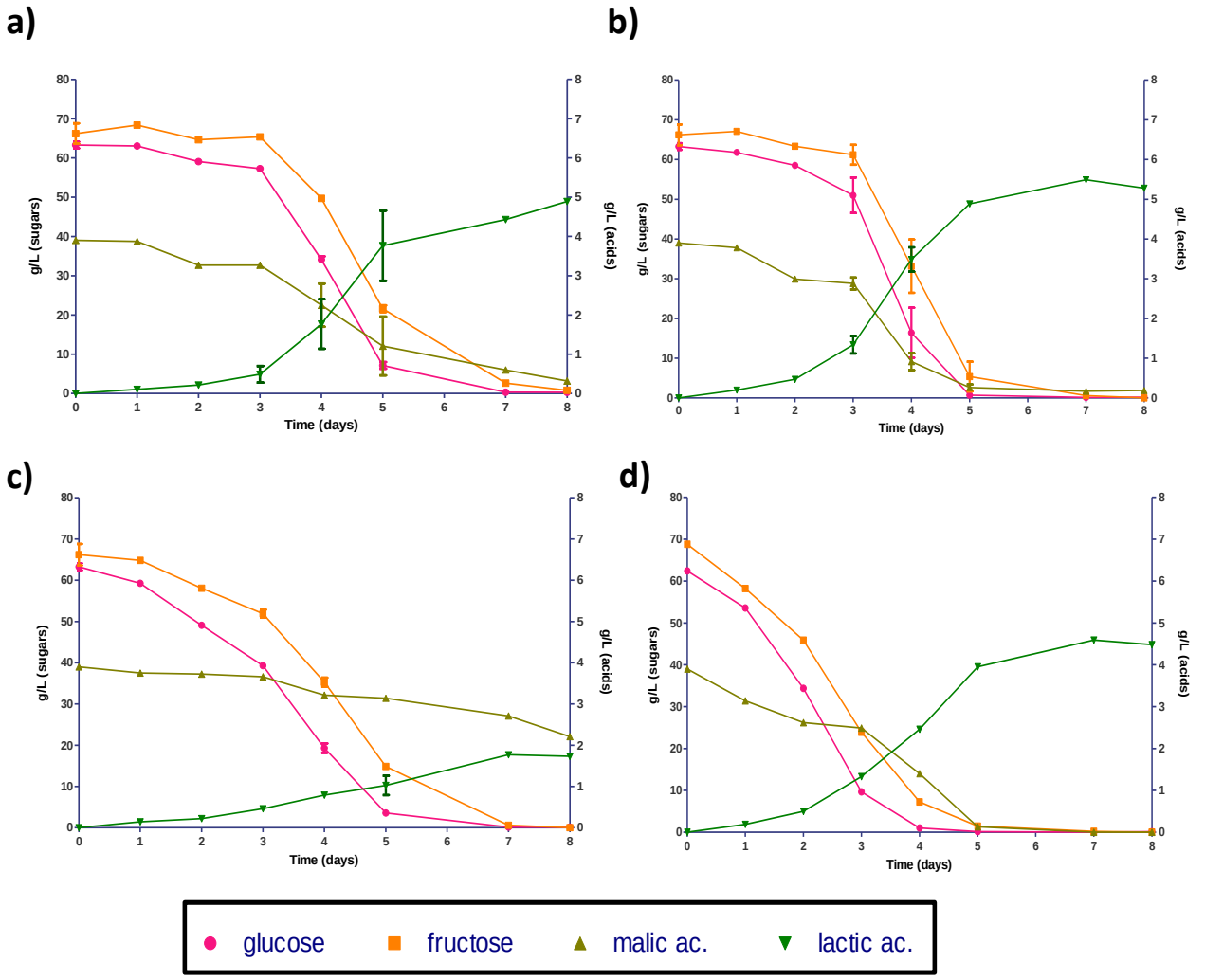

Figure 54. Glucose, fructose and malic acid consumption and lactic acid formation during 8 days by: **a)** 0.03 g of the coimmobilized biocatalyst, **b)** 0.3 g of the coimmobilized biocatalyst, **c)** simultaneous 10^6 cfu/mL yeast and bacteria inoculation, **d)** simultaneous 10^7 cfu/mL yeast and bacteria inoculation

The biocatalyst (**Figure 55**) could be packed in a container of thin paper or cloth holding (like a tea bag) with a measured amount to carry out the MLF in wine, avoiding the loss of cells, and permitting its reuse in consecutive fermentations. Moreover, this material could be used to immobilize yeast and bacteria '*à la carte*', depending on the needs of the industry or the cellar, or could be used also as a preservation system of microorganisms. Nevertheless, more experiments must be done to study the number or consecutive fermentations that the biocatalyst can carry out, the behaviour in grape must with different pH, sugars concentrations, temperatures, etc. Also more experiments must been done to find suitable yeast-bacterial combinations.

Figure 55. Aspect of the DCM with the coimmobilized *S. cerevisiae* AXAZ-1 and *O. oeni* E5003

From the above results, we can conclude that *O. oeni* and *S. cerevisiae* were successfully co-immobilized in DCM and starch gel, as clearly observed under scanning electron micrographs. Such DCM have been previously proved as very good support increasing the fermentation rates compared to those obtained by free cells, reducing the winemaking costs and time, and the fermentation for wine production even at very low temperatures (Koutinas 1994; Agouridis *et al.* 2008). As well the starch gel increases the fermentation rate compared to free cells (Kandylis *et al.* 2008). In addition, the coimmobilization of both microorganisms provides a novel biocatalyst that facilitates the process of winemaking because of the inoculation of only one starter for both fermentations, and the MLF finishes before than if the fermentation occurs after the AF. The use of immobilized *O. oeni* in DCM leds to improvements in developing MLF in winemaking (Agouridis *et al.* 2008).

5. Conclusions

- The *O. oeni* E5003 strain has been selected as MLF starter because is able to grow in media at wide range of pH levels, at the highest levels of ethanol, is not able to form BAs, and has the best malolactic activity and cell viability in wine.

- An improvement of the BAs determination and quantification method has been done employing the new core-shell HPLC column technology. This method allows the reduction of run times and solvent volumes.

- The use of a microplate reader and a pH indicator (bromophenol blue) permits the selection of malolactic strains in terms of malic acid degradation in 24 hours, spending a minimum of culture media.

- A liquid culture medium has been designed (OMP) for growing *O. oeni* and obtain high levels of biomass production, but also an adequate adaptation to the wine conditions. The composition of the biomass production medium permits to reach a population of *O. oeni* of $1x10^9$ cfu/mL in 6 days.

- A scale-up of the process to obtain industrial levels of E5003 *O. oeni* biomass has been done using 80 L of the culture medium designed (OMP). The bacterial population reaches $1x10^9$ cfu/mL in 22 days from the laboratory stock.

- The use of an autochthonous *O. oeni* liquid starter culture in a cellar has reduced the presence of histamine 3 times in the final wine compared to a non-inoculated control vat.

- For a long storage period, of the E5003 *O. oeni* starter culture, freeze-drying is the best option; nevertheless, until 3 months of preservation, the storage at 4 ºC is the best choice, and for 1 month store period, the freezing at -20 ºC is the best option.

- The rehydration of the freeze-dried malolactic starter culture with a mix of fructose, arginine and malic acid shows better results of malolactic activity in wine than if it is rehydrated with NaCl.

- The preservation strategies of *O. oeni* in wine lees can be useful. The best preservation is obtained when a high content in polysaccharides is found in the wine lees.

- The coimmobilization of *O. oeni* E5003 and *S. cerevisiae* AZAX-1 in DCM with starch gel permitted a simultaneous AF and MLF, reducing the winemaking process time.

6. Conclusiones

- La cepa de *O. oeni* E5003 ha sido elegida como cultivo iniciador maloláctico, ya que es capaz de crecer en un amplio rango de pH, en medios con niveles elevados de etanol, no produce aminas biógenas, y presenta la mejor actividad maloláctica y viabilidad celular tras su inoculación en vino.

- El método de cuantificación de aminas biógenas mediante cromatografía líquida empleado hasta el momento ha sido optimizado utilizando una nueva tecnología de columnas de cromatografía de núcleo duro más rápida y barata.

- El ensayo basado en microplacas usando azul de bromofenol, como indicador de pH, ha permitido la selección de cepas malolácticas en términos de degradación de ácido málico en 24 horas.

- Un nuevo medio de cultivo (OMP) para la obtención de elevadas cantidades de biomasa de *O. oeni* E5003 ha sido diseñado. La composición del medio de cultivo proporciona niveles poblacionales de $1x10^9$ ufc/mL y permite la adaptación de las bacterias a las condiciones del vino.

- Se ha escalado el proceso de producción de biomasa de *O. oeni* utilizando 80 L del medio OMP. El tiempo total de producción son 22 días desde el cultivo de laboratorio y la población bacteriana alcanza niveles de $1x10^9$ ufc/mL.

- Una cepa autóctona de *O. oeni* de una bodega ha sido seleccionada para ser empleada como cultivo iniciador maloláctico propio, por su alta efectividad maloláctica y su incapacidad para producir histamina. El uso del cultivo iniciador maloláctico permite reducir 3 veces el contenido en histamina en un vino final comparado con un vino no inoculado en la bodega.

- El método de conservación más adecuado del cultivo iniciador maloláctico de *O. oeni* E5003 depende del tiempo de almacenamiento del mismo. La liofilización permite la conservación del cultivo largos periodos de tiempo, mientras que la congelación a -20 ºC es óptima para la conservación durante 1 mes. La refrigeración a 4 ºC es adecuada para periodos de almacenamiento de hasta 4 meses.

- El cultivo iniciador maloláctico liofilizado presenta mejores resultados de actividad maloláctica y viabilidad en vino al ser rehidratado con una solución de fructosa, arginina y acido málico, que al ser rehidratado con NaCl.

- Un método de conservación alternativo de cultivos iniciadores malolácticos consiste en la conservación en lías de vino tinto. La mejor viabilidad celular se obtiene con lías que poseen un mayor contenido en polisacáridos.

- El cultivo iniciador generado mediante la coinmovilización de *S. cerevisiae* AZAX-1 y O. *oeni* E5003 en material celulósico y almidón permite una fermentación alcohólica y maloláctica en vino simultáneas, reduciéndose el tiempo de vinificación considerablemente.

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