PROTEOMIC AND METABOLOMIC STUDY OF WINE YEASTS IN FREE AND IMMOBILIZED FORMATS, SUBJECTED TO DIFFERENT STRESS CONDITIONS

Jaime Moreno García

Vitenol Research Group

Department of Agricultural Chemistry

Department of Microbiology

Dissertation to achieve the Doctoral degree by the University of Córdoba, International Mention.

Córdoba, 2017

TITULO: *Proteomic and metabolomic study of wine yeasts in free and immobilized formats, subjected to different stress conditions*

AUTOR: *Jaime Moreno García*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS: Estudio proteómico y metabolómico de levaduras vínicas en formato libre e inmovilizado, sometidas a diferentes condiciones de estrés.

THESIS TITLE: Proteomic and metabolomic study of wine yeasts in free and immobilized formats, subjected to different stress conditions

DOCTORANDO/A: Jaime Moreno García

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El trabajo presentado por D. Jaime Moreno García, Licenciado en Ciencias Biológicas por la Universidad de Córdoba se encuadra en la línea de investigación "Proteómica y metabolómica de levaduras" de reciente implantación en el grupo de investigación "Vitenol" (http://www.uco.es/grupos/vitenol/) que está formado por profesores del Departamento de Química Agrícola y Edafología y del Departamento de Microbiología de la UCO. Esta línea de investigación tiene como objetivo principal relacionar la influencia que la composición química de medios de interés enológico y las condiciones físicas en las que se desarrollan las levaduras, ejercen sobre las proteínas intracelulares expresadas y los metabolitos excretados al medio externo, particularmente aquellos relacionados con la calidad organoléptica de las bebidas fermentadas.

El doctorando Jaime Moreno García, ha desarrollado desde su incorporación al programa de doctorado *Biociencias y Ciencias Agroalimentarias* en 2012, una intensa actividad investigadora, cuya novedad e interés para la comunidad científica internacional está avalada por la publicación de siete artículos de investigación como primer autor y dos como segundo autor,

que han sido publicados en revistas indexadas en el JCR en las áreas temáticas de Microbiology, Food depart and Technology, Multidisciplinary Chemistry y Chemical Engineering. Además de estos artículos ya publicados, incorpora a su tesis tres manuscritos en las que será primer autor, preparados para enviar a revistas indexadas en el primer cuartil.

La revisión bibliográfica efectuada le ha proporcionado una base sólida sobre su tema de investigación y le ha permitido elaborar una introducción que consta de una parte dedicada a proteómica de levaduras vínicas y otra parte a los metabolitos responsables del aroma de los vinos que proceden de la variedad de uva, de las levaduras de fermentación y de las levaduras de crianza biológica.

La investigación experimental realizada ha puesto de manifiesto la importancia del análisis de proteínas para determinar la respuesta de las células de levadura a condiciones de estrés y abre nuevas fronteras para establecer criterios de selección de levaduras en función de las condiciones fisicoquímicas en las que van a ser utilizadas. Este hecho posee aplicaciones de interés en las industrias de elaboración de bebidas fermentadas y en las de obtención de bioetanol, dada la diversidad de composición de medios a fermentar y las condiciones en que las levaduras deben desarrollar su actividad metabólica.

El número de artículos publicados y de manuscritos preparados para enviar a publicación, que constituyen el cuerpo de la Tesis son 12 en total, que se han agrupado en capítulos relacionados con las dos hipótesis de partida y con los objetivos planteados al comienzo de la tesis.

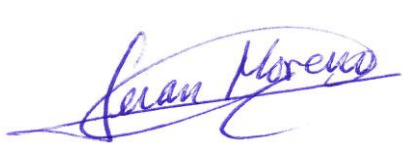
La realización de una estancia de tres meses de duración en la Universidad de Sassari Italia), financiada por el IDEP de la Universidad de Córdoba, que ha sido tutorizada por la Dra. Marilena Budroni, donde ha mejorado sus conocimientos de genética de levaduras, le permiten acceder a la mención de Doctorado Internacional. Además cuenta con una estancia de un año de duración en la Universidad de California, Davis (Estados Unidos), financiada por la Fundación Fulbright y tutorizada por la Dra. Bisson, que le ha permitido desarrollar un proyecto de investigación predoctoral sobre los factores que influyen en la formación de biofilm por las levaduras. Dicha estancia fue autorizada por el Ministerio de Educación Cultura y Deporte como complemento de formación en centro de

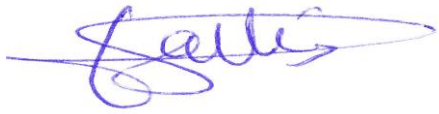
investigación extranjero, durante un periodo de 7 meses, que coincidió con el disfrute de su beca de formación de profesorado universitario.

Por todo lo expuesto consideramos que la investigación desarrollada y recogida en la presente Memoria reúne los requisitos de originalidad y calidad exigidos para una Tesis Doctoral por la Universidad de Córdoba, con mención de Doctorado Internacional e informamos favorablemente la presentación de la Tesis Doctoral de D. Jaime Moreno García.

Córdoba, 15 de junio de 2017

Firma de los directores





Fdo. Juan José Moreno Vigara Fdo. Mª Teresa García Martínez

Firma del tutor

Fdo. Juan Carlos García Mauricio

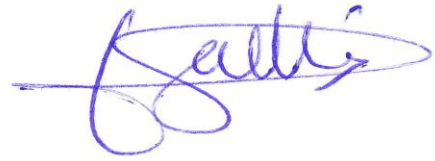
PhD. Juan José Moreno Vigara, Full Professor at the Department of Agricultural Chemistry, PhD. María Teresa García Martínez, Assistant Professor in the Department of Microbiology as directors, and PhD. Juan Carlos García Mauricio, Full Professor in the Department of Microbiology as tutor, all of them belonging to the "Vitenol" research group of the University of Córdoba,

AUTHORIZE to:

Jaime Moreno García to present this dissertation, entitled "Proteomic and metabolomic study of wine yeasts in free and immobilized formats, subjected to different stress conditions" to obtain the Doctoral degree by compendium of articles and with International Mention.

This work is the results of the investigation performed in our laboratories of Agricultural Chemistry and Microbiology, at the University of Córdoba, under our supervision and completed with a three months stage in the laboratory of the Department of Agriculture, Università degli Studi di Sassari (Italy), under the supervision of PhD. Marilena Budroni and a twelve months stage in the laboratory of the Department of Viticulture and Enology at the University of California (Davis, USA) under the supervision of PhD. Linda F. Bisson.

Signed in Córdoba, 6 June 2017



Dr. Juan José Moreno Vigara **Dra. Mª Teresa García Martínez**

Dr. Juan Carlos García Mauricio

Mención internacional del Doctorado

Mediante la exposición y defensa de esta memoria se pretende optar a la obtención de la mención de “Doctorado Internacional”, habida cuenta que el doctorando reúne los requisitos exigidos para tal mención:

1. Se cuenta con los informes favorables de doctores pertenecientes a instituciones de Enseñanza Superior, de países distintos al nuestro.

2. En el Tribunal constituido para evaluar la Tesis, existe un miembro de un centro de Enseñanza Superior de un país europeo distinto al nuestro.

3. Parte de la redacción y defensa de la Memoria se realizará en la lengua oficial de un país europeo distinto al nuestro.

4. El doctorando ha realizado una parte del trabajo experimental de investigación en las instalaciones del Departamento de Agricultura y Medio Ambiente de la Universidad de Sassari (Cerdeña, Italia), tutorizado por la Dra. Budroni.

5. El doctorando ha realizado una estancia en el Laboratorio de la Dra. Bisson, del Departamento de Viticultura y Enología de la Universidad de California (Davis, EEUU), mediante la fianaciación externa de la Fundación Fulbright. Dicha estancia fue autorizada por el Ministerio de Educación Cultura y Deporte como complemento de formación en centro de investigación extranjero, durante un periodo de siete meses, que coincidió con el disfrute de su contrato de Formación de Profesorado Universitario.

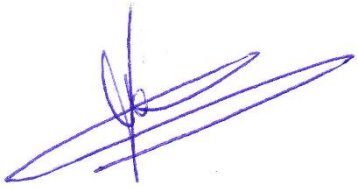
Informe de los artículos publicados según el Journal Citation Report

Los artículos que se exponen en la presente memoria han sido publicados en revistas cuyos factores de impacto se encuentran en el Journal Citation Report del ISI web of Knowledge y están recogidos en la siguiente tabla:

Journal	***JCR***	***Category***	***Rank***	***Quartile***	***Nº***
Food Research International	3.050	Food Science and Technology	14/123	**Q1**	1
International Journal of Food Microbiology	3.082	Food Science and Technology	12/123	**Q1**	1
Food Microbiology	3.682	Food Science and Technology	12/125	**Q1**	2
Process Biochemistry	2.529	Chemical Engineering	35/135	**Q2**	1
Data in Brief	0.187 (SJR)	Multidisciplinary		**Q3**	1
International Journal of Molecular Sciences	3.257	Multidisciplinary Chemistry	52/163	**Q2**	1
Food Chemistry	4.052	Food Science and Technology	7/125	**Q1**	1
Frontiers in Microbiology	4.165	Microbiology	23/123	**Q1**	1
Total publications in Quartile 1					**6**
Total publications in Quartile 2					**2**
Total publications in Quartile 3					**1**

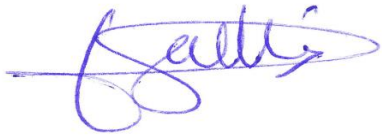
“ESTUDIO PROTEÓMICO Y METABOLÓMICO DE LEVADURAS VÍNICAS EN FORMATO LIBRE E INMOVILIZADO, SOMETIDAS A DIFERENTES CONDICIONES DE ESTRÉS”

Trabajo presentado para aspirar al grado de Doctor por:



Jaime Moreno García

Directores:



Juan J. Moreno Vigara **Mª Teresa García Martínez**

Tutor:

Juan Carlos García Mauricio

AGRADECIMIENTOS

A todas las personas e instituciones que han contribuido a la realización de las tareas, que han culminado en la elaboración del presente documento, cuyo objetivo es obtener el Grado de Doctor por la Universidad de Córdoba, con mención internacional.

En primer lugar, considero de justicia dedicar un especial agradecimiento a los directores de este trabajo, los doctores D. Juan José Moreno Vigara, Dª María Teresa García Martínez y D. Juan Carlos García Mauricio, por la oportunidad que me han brindado de avanzar en mi formación mediante la realización de la presente tesis. Durante estos años de convivencia y aprendizaje han sido un ejemplo de vocación, esfuerzo, motivación y dedicación a las múltiples tareas que desarrolla un profesor de Universidad, haciéndome comprender la responsabilidad que conlleva ser miembro de una institución como es la Universidad de Córdoba.

Al programa de doctorado "Biociencias y Ciencias Agroalimentatias", al grupo de investigación "Vitenol" y a los miembros del Departamento de Química Agrícola y Edafología y del Departamento de Microbiología de la Universidad de Córdoba, por aceptarme como estudiante de posgrado y por facilitarme la realización de mi labor investigadora.

A las diferentes instituciones que han financiado este trabajo: Ministerio de Educación, Cultura y Deporte la beca de Formación de Profesorado Universitario FPU14/02661. Al Instituto de Estudios de Postgrado (IDEP), por la ayuda a la estancia de tres meses en un centro de investigación europeo, al XXI Programa Propio de Fomento de la Investigación y a los Servicios Centrales de Apoyo a la Investigación, de la Universidad de Córdoba.

A la Dra. Marilena Budroni, del Departmento de Ciencias Agrícolas de la Universidad de Sassari, (Sassari, Cerdeña, Italia), por su magnífica acogida y a los Dres. Anna Lisa Coi, Giacomo Zara, Severino Zara e Ilaria Mannazzu, por su colaboración en la realización e interpretación de los experimentos con mutantes específicos de su colección de levaduras y por

acogerme durante mi estancia en Sassari, ofreciéndome apoyo constante y un cálido ambiente de trabajo.

Una mención especial de agradecimiento debo dedicar a la Fundación FULBRIGHT España, por la financiacíon de una estancia predoctoral de un año de duración en la primera universidad del mundo en temas agrícolas, como es la Universidad de California, Davis (USA).

A la Dra. Linda F. Bisson, directora del Laboratorio Bisson de Microbiología Enológica del Departamento de Viticultura y Enología en la Universidad de California, Davis (CA, USA) y tutora del proyecto Fulbright realizado, por su apoyo constante, sus sabios consejos, inolvidables momentos compartidos y por hacerme sentir como en casa. Un agradecimiento especial a la Dra. Vidhya Ramakrishnan por su gran amabilidad y disponibilidad para resolver problemas de laboratorio y por supuesto, a mis compañeros, Gordon Walker, Peter luong y Minami Ogawa que compartieron conmigo su experiencia y me ofrecieron su amistad.

A mis compañeros del laboratorio de Química Agrícola y Edafología y del Departamento de Microbiología de la Universidad de Córdoba por su disponibilidad a la hora de ayudar y su inmensa simpatía.

A todos ¡MUCHAS GRACIAS!

ACKNOWLEDGEMENTS

To all the people and institutions that have contributed to the accomplishment of obtaining my Ph.D. Degree by the University of Cordoba with International Mention.

First of all, I consider to give special thanks to all the supervisors of this work, Professor Juan José Moreno Vigara, Ph.D. María Teresa García Martínez and Professor Juan Carlos García Mauricio, for the opportunity they gave me to train me through the realization of this thesis. These years of learning have been an example of vocation, effort, motivation and dedication to multiple tasks that a university professor develops, providing me with understanding the responsibility that entails the fact of being a member of an institution such as the University of Cordoba.

I would like to thank the doctoral program "Biociencias y Ciencias Agroalimentarias", to the research group "Vitenol" and the members of the Department of Agricultural Chemistry and Soil Science and the members of the Department of Microbiology at the University of Cordoba, for accepting me as a PhD candidate.

Thanks to the different institutions that have funded this work: Ministerio de Educación, Cultura y Deporte from Spain, as sponsor of the "Formación de Profesorado Universitario" or "FPU" scholarship. To the IDEP for granting me an international stay of three months in a European research center, to the "XXI Programa Propio de Fomento de la Investigación" and the Central Support Services for Research, at the University of Cordoba.

To Ph.D. Marilena Budroni, from the Department of Agricultural Sciences of the University of Sassari (Sassari, Sardinia, Italy), for her magnificent reception, and Ph.D.'s Anna Lisa Coi, Giacomo Zara, Severino Zara and Ilaria Mannazzu for their collaboration in the experiments and hosting me during my stay in Sassari as well as offering constant support and a warm working environment.

A special mention of gratitude devoted to the FULBRIGHT Spanish Program, for funding a one-year pre-doctoral stay at the first University in the world on agricultural topics, the University of California, Davis (USA).

Professor Linda F. Bisson, Ph.D., director of the Bisson Laboratory from the Department of Enology and Viticulture, University of California, Davis; and supervisor of the Fulbright project, for her constant support, wise advice and unforgettable moments that made me feel like at home. A special thank you to Ph.D. Vidhya Ramakrishnan for her great kindness and willingness to solve laboratory problems and of course my colleagues, Gordon Walker, Peter Luong and Minami Ogawa who shared with me their experience at work and offered me their friendship.

To all my colleagues in the Laboratory of Agricultural Chemistry and Soil Science and the Department of Microbiology at University of Córdoba for their willingness to help and their immense sympathy.

To all of you, THANK YOU!

Dedicatoria/dedication:

Me gustaría dedicar el trabajo realizado durante estos cinco últimos años a las personas que realmente me importan contando los que ya no están. Gracias a mi madre, a mi padre y mi hermano por vuestro amor y apoyo en los momentos más duros. Gracias a mis amigos y a mi novia por vuestra constante confianza. ¡GRACIAS!

I would like to devote the work I have done over the past five years to the people who really matter to me including those who are no longer here. Thank you to my mother, my father and my brother for your love and support in the hardest moments. Thanks to my friends and my girlfriend for your constant confidence. THANK YOU!

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

ACRONYMS AND ABBREVIATIONS

2DE	*2 Dimension Electrophoresis*
3MH	*3-Mercaptohexan-1-ol*
3MHA	*3-Mercaptohexyl Acetate*
4MMP	*4-Mercapto-4-methylpentan-2-one*
AF	*Alcoholic Fermentation*
ANOVA	*Analysis of Variance*
ATTC	*American Type Culture Collection*
BFC	*Biofilm Formation Condition*
BLAST	*Basic Local Alignment Search Tool*
CA	*Cluster Analysis*
CAS	*Chemical Abstrac Services*
CHAPS	*3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate*
CID	*Collision-Induced Dissociation*
CIS	*Cooled Injection System*
CoA	*Coenzyme-A*
DRS	*Deconvolution-Reporting Software*
DTT	*Dithiothreitol*
EDTA	*Ethylenediaminetetraacetic Acid*
EI	*Electron Ionisation*
emPAI	*Exponentially Modified Protein Abundance Index*
FAS	*Fatty Acid Synthase*
FID	*Flame Ionization Detector*
FWHM	*Full-Width at Half-Maximum*
GC	*Gas Chromatography*
GC-MS	*Gas Chromatography–Mass Spectrometry*
Gly	*Glycerol*
GO	*Gene Ontology*
GPI	*Glycosylphosphatidylinositol*
GRAS	*Generally Recognized As Safe*
HCD	*Higher-Energy Collisional Dissociation*
HG	*Homogeneous Groups*
HPLC	*High Performance Liquid Chromatography*
IEF	*Isoelectric Focussing*
Kv	*Constant Reaction Rate*
LC	*Liquid Chromatography*
LDA	*Linear Discriminant Analysis*
LPO	*Lipoxygenase*
LRI	*Linear Retention Index*
LTQ	*Linear Trap Quadropole*

MALDI	*Matrix-Assisted Laser Desorption/Ionization*
MCFA	*Medium Chain Fatty Acid*
MLF	*Malolactic fermentations*
Mr	*Molar Mass*
MRLA	*Multi Regression Lineal Analysis*
MS	*Mass Spectrometry*
MSC	*Multiple-Sample Comparison*
mtDNA	*Mitochondrial DNA*
MVA	*Multiple Variable Analysis*
OAV	*Odor Activity Value*
OIV	*The International Organisation of Vine and Wine*
OPT	*Odor Perception Threshold*
ORF	*Open Reading Frame*
OS	*Odorant Serie*
PAI	*Protein Abundance Index*
PCA	*Principal Component Analysis*
PCR	*Polymerase Chain Reaction*
PDMS	*Polydimethylsiloxane*
PMSF	*Phenylmethane Sulfonyl Fluoride*
ROS	*Reactive Oxygen Species*
SBSE	*Stir Bar Sorptive Extraction*
SGD	*Saccharomyces Genome Database*
TCA	*Tricarboxylic Acid Cycle*
TDU	*Termal Desorption Unit*
TOF	*Time-Of-Flight*
TPI	*Total Polyphenols Index*
UCD	*University of California, Davis*
UCO	*University Of Cordoba*
UNISS	*Università degli Studi di Sassari*
YNB	*Yeast Nitrogen Base*
YPD	*Yeast extract Peptone Dextrose*

ABSTRACT

RESÚMEN

Background

The aroma of wine is the first sensory perception that affects its quality. The knowledge acquired to date ensures that the unique aroma of a specific wine is the result of the sensation that in our sense of smell cause the molecules of more than 800 volatile compounds present in the hydroalcoholic solution that is the wine.These compounds come from the grapes, the prefermentative treatments, the alcoholic fermentation and the aging or preservation of the wine. It is a proven fact that fermentative yeasts exert an important influence on the aroma of wine, because they excrete odorant molecules to the medium which are products of their metabolism.

Winemaking is a highly-skilled biotechnological process where a large number of commercial strains of *Saccharomyces* and non-*Saccharomyces* yeasts have been selected for their ethanol and/or flavour compounds production and comerciallized. During winemaking, yeast cells undergo different stress conditions, including high osmotic pressure induced by sugar content, increased concentration of ethanol, reactive oxygen species (ROS) derived from oxygen metabolism and high temperature. Yeasts cope with these conditions by adapting their response of their biological information system (genome-transcriptome-proteome-metabolome) in order to prevent induced cell dysfunctions and to acquire stress tolerances. On the other hand, it has been verified that the immobilized yeasts present better behavior than the free yeasts against certain stress conditions. An example is the formation of flor velum biofilm by some strains of *Saccharomyces cerevisiae* to tolerate elevated contents in ethanol.

The response of yeast *S. cerevisiae* to stress conditions has been studied fundamentally from the genomics and transcriptomics point of view. Studies on the production of ethanol and volatile compounds related to aroma and sensorial quality of wine are also numerous. However, the proteome of

yeasts under different stress conditions and their relationship with volatile metabolites excreted in the medium is a line of research little explored until the present moment.

The wine industry needs yeasts that are better adapted to grow under different stress conditions because of its interest in the elaboration of new types of wine according to the consumer preferences. Elucidating the proteomic response of yeasts to different stress conditions and knowing how this response affects the aroma-related metabolites are highly important for the selection and genetic improvement of yeast strains used in the alcoholic fermentation industries in general and wine in particular.

Hypotheses

Based on this background, the following starting hypotheses were established, on which the investigation reflected in this Doctoral Thesis has been developed:

1. The intracellular proteins expressed and the metabolites excreted are related with the yeast strain used and the physical and chemical conditions of the medium in which they grow. Therefore, it is possible to know the response of different yeasts to different stress conditions using analytical methods for the determination of key exometabolites and proteins.
2. Moreover, it is hypothized that certain properties related to the flor velum formation influence the yeast biocapsule formation and their characteristics.

Results

Among the yeast strains treated in this Doctoral Thesis, two were selected to study their metabolomic comportment due to their importance in the actual trends in wine microbiology and fermentation processes. A non-*Saccharomyces* yeast, as is *Torulaspora delbrueckii*, and a *S. cerevisiae* showed different behaviours towards the same

fermentative condition in terms of growth and metabolism, thus indicating that they have a different strategy to adapt to the osmotic stress, produced by high sugar contents.

The metabolomic and proteomic profiling of the flor yeast *S. cerevisiae* G1, highlights the different response of this strain to different conditions: a fermentative and an oxidative when the yeast form a biofilm, so-called velum. These results also shedded new light on several features of these special yeast and in particular, they have revealed the extent of proteome remodeling imposed by the biofilm life-style.

Finally, the utilization of the flor yeast abilities to consume ethanol in wines with high alcoholic degree resulted in a change on their metabolomic and aroma profiles, but still accepted after a sensory test. On the other hand, the flor yeast ability to form biofilm positively affects the rates of yeast immobilization among other parameters, when co-adhering with the GRAS (Generally Recognized As Safe) fungus *Penicillium chrysogenum* in the biocapsules immobilization system.

Antecedentes

El aroma del vino es la primera percepción sensorial que afecta a su calidad. Los conocimientos adquiridos hasta la fecha permiten asegurar que el aroma único de un vino es el resultado de la sensación que provocan en nuestro sentido del olfato las moléculas de más de 800 compuestos volátiles presentes en la disolución hidroalcohólica que es el vino. Estos compuestos proceden de la uva, de los tratamientos prefermentativos, de la fermentación alcohólica y de la crianza o conservación del vino. Es un hecho contrastado que las levaduras fermentativas ejercen una importante influencia sobre el aroma del vino, debido a que excretan al medio moléculas odorantes procedentes de su metabolismo.

La elaboración de vino es un proceso biotecnológico altamente tecnificado para el que actualmente existe en el mercado un amplio número de cepas comerciales de levaduras *Saccharomyces* y no-*Saccharomyces*, que han sido seleccionadas por su producción de etanol y/o aromas. Durante la elaboración del vino, las células de levadura se someten a diferentes condiciones de estrés, incluyendo la alta presión osmótica inducida por el contenido de azúcar, el aumento de la concentración de etanol, las especies reactivas de oxígeno (ROS) derivadas del metabolismo del oxígeno y la elevada temperatura. Las levaduras hacen frente a estas condiciones adaptando la respuesta de su sistema de información biológica (genoma-transcriptoma-proteoma-metaboloma) con objeto prevenir las disfunciones celulares inducidas y para adquirir tolerancias de estrés. Por otro lado se ha comprobado que las levaduras inmovilizadas presentan un mejor comportamiento que las levaduras libres frente a determinadas situaciones de estrés, siendo la formacion de velo de flor por algunas cepas de *Saccharomyces cerevisiae* un ejemplo de tolerancia a elevados contenidos en etanol.

La respuesta de las levaduras *S. cerevisiae* a condiciones de estrés se ha estudiado fundamentalmente desde el punto de vista de la genómica y de la transcriptómica. También son numerosos los estudios sobre la producción de

etanol y compuestos volátiles relacionados con el aroma y la calidad sensorial del vino. Sin embargo el proteoma de las levaduras en diferentes condiciones de estrés y su relación con los metabolitos volátiles excretados al medio es una línea de investigación poco explorada hasta el momento actual.

La industria vitivinícola necesita levaduras mejor adaptadas para crecer en diferentes condiciones de estrés por el interés en la elaboración de nuevos tipos de vino según la demanda real de los consumidores. Elucidar la respuesta proteómica de las levaduras a diferentes condiciones de estrés y como incide esta respuesta sobre los metabolitos volatiles relacionados con el aroma es de suma importancia para la selección y para la mejora genética de las cepas levaduras utilizadas en las industrias de las fermentaciones alcohólicas en general y del vino en particular.

Hipótesis

En base a estos antecedentes se establecieron las siguientes hipótesis de partida, sobre las que se han desarrollado las investigaciones reflejadas en la presente Tesis Doctoral:

1. Las proteínas endocelulares expresadas y los metabolitos excretados están relacionados con la cepa de levadura utilizada y las condiciones físicas y químicas del medio en el que crecen. Por tanto, conociendo esta relación es posible establecer modelos de predicción de comportamiento y criterios más consistentes para la mejora de levaduras.
2. Ciertas propiedades relacionadas con la formación de velo de flor por las levaduras *S. cerevisiae* influyen en la formación de la biocápsulas de levaduras y en sus características.

Resultados

Entre las cepas de levadura tratadas en esta Tesis Doctoral, se seleccionaron dos para estudiar su comportamiento metabolómico por su actual relevancia en la microbiología del vino y en los procesos de fermentación. Una cepa no-*Saccharomyces*, como es *Torulaspora delbrueckii* y otra cepa de *S. cerevisiae*, que mostraron diferentes pautas de crecimiento y metabolismo frente a la misma condición de fermentación, lo que indica que poseen una estrategia diferente de adaptación al estrés osmótico, producido por altos contenidos de azúcar.

La caracterización metabolómica y proteómica de la levadura de flor *S. cerevisiae* G1, pone de manifiesto una respuesta diferente de esta cepa a diferentes condiciones: una fermentativa y otra oxidativa, cuando la levadura forma el biofilm denominado velo de flor. Estos resultados también aportan información sobre varias características de estas levaduras especiales, y en particular, han revelado la remodelación del proteoma impuesta por las condiciones del medio de formación de velo.

Por último, los nuevos conocimientos sobre la utilización de las capacidades de la levadura de flor para consumir etanol, en vinos de elevado contenido de este alcohol, dieron como resultado un cambio en los perfiles metabólómicos y del aroma de los vinos, que fueron bien evaluados en un análisis organoléptico. Por otro lado, la capacidad de la levadura de flor para formar biofilm afecta positivamente a su tasa de inmovilización de levaduras, entre otros parámetros, cuando se adhiere al hongo GRAS (Generally Recognized As Safe) *Penicillium chrysogenum* en la formación de biocápsulas.

1. INTRODUCTION

1. INTRODUCTION

1.1. Wine yeasts

In general terms, the elaboration process of wine is comprised of grape harvesting, alcoholic (AF) and malolactic (MLF) fermentations, wine aging and bottling (Romano et al., 2003) (Figure 1).

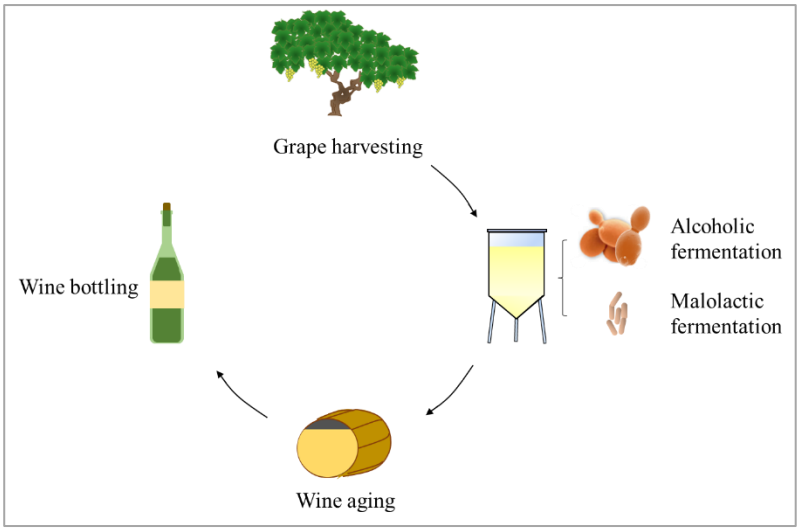


Figure 1. Schematic outline of the main steps in wine-making.

Wine microbiota is heterogeneous and is mainly composed by yeast, bacteria and filamentous fungi; with considerable potential interaction among them (Fleet, 2007; Fugelsang and Edwards, 2007). Yeasts, among the wine microbiota, detain a predominant role in the contribution to the wine chemistry through the AF (Fleet and Heard, 1993; Fugelsang, 1997) (Figure 2). On the other hand, MLF is carried by lactic acid bacteria, mainly *Oenococcus oeni* and *Lactobacillus plantarum* species (Lerm et al., 2010) (Figure 2).

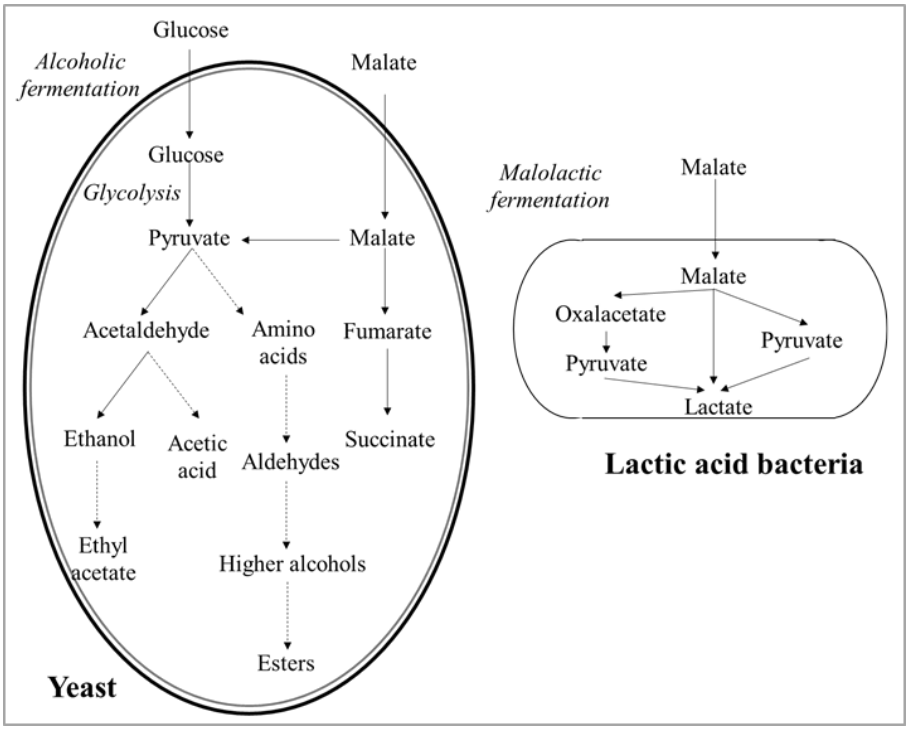


Figure 2. Pathways involved in formation of the major flavor compounds during alcoholic fermentation by yeasts and malolactic fermentation by lactic acid bacteria.

Traditional AF is considered as a natural spontaneous process performed sequentially by the different yeast species and/or strains present on the grapes or in the winery. *Pichia*, *Candida*, *Hanseniaspora* and *Metschnikowia* species start the AF when sugar and sulfur dioxide concentrations are highly abundant in the must. Then, within a few days, when nitrogen nutrients are depleted and the *Saccharomyces* spp. metabolism increases ethanol concentration and temperature, the non-*Saccharomyces* species are rapidly substituted by *S. cerevisiae* that will remain until the end of the AF being sometimes the only species detectable at this last stage (Fleet, 2003; Fleet and Heard, 1993). Consequently, in the spontaneous fermentations, the different yeast adaptations to wine environment and thus, the proportion to relative abundance, will affect on how each species and strain influence the aromatic properties of the final wine product (Romano et al., 2003).

Due to the yeast microbiota complexity of AF, wine yeasts has been conventionally clustered in two groups based on the fundamental role that the species of the genus *Saccharomyces*, play on the alcoholic fermentation (Ribéreau-Gayon et al., 2006a): *Saccharomyces* and non-*Saccharomyces*, the later more important at the initial stages of the AF. From now on, this chapter has been divided according to the prevalence yeasts during the spontaneous fermentations, into non-*Saccharomyces* and *Saccharomyces* sections. Further, special enphasis is placed on the non-*Saccharomyces* *Torulaspora delbrueckii* and the *Saccharomyces* flor yeasts as they are the main topics of the PhD thesis.

1.1.1. Non-*Saccharomyces* yeasts: *Torulaspora delbrueckii*

Non-*Saccharomyces* yeasts, as the name suggests, refers to all yeast species found in wine production with the exception of *S. cerevisiae*. Non-*Saccharomyces* yeasts along with other microorganisms that are naturally present on grape berries, govern the microbiota of early phases of spontaneous fermentations and although they are not able to entirely metabolize the sugars and produce a high quantity of ethanol, they contribute to the organoleptic properties of the wine in a fundamental way (Table 1).

Non-*Saccharomyces* yeasts found in grape must and during fermentation can be divided into three groups (Bisson and Kunkee, 1991; Combina et al., 2005; Fleet et al., 1984; Longo et al., 1991; Lonvaud-Funel, 1996; Lorenzini, 1999; Querol et al., 1990; Torija et al., 2001):

i) Aerobic yeasts.
ii) Apiculate yeasts with low fermentative activity.
iii) Fermentative yeasts.

Table 1. Principal oenological characteristic of non-*Saccharomyces* yeast of wines. Table modified from Capozzi et al. (2015).

Yeast genera	Oenological properties	Negative effect	References
Candida	High glycerol producer (up to 14 g/L), low acetic acid concentration; Increased concentrations of terpenol; decreased concentrations of aldehydes and acetate esters.	Production of sulphur compounds; slower kinetics rate (low ethanol concentration).	Ciani and Maccarelli, 1998; Sadoudi et al., 2012; Jolly et al., 2014
Hanseniaspora	Increased amounts of 2-phenyl-ethyl acetate, higher alcohols, acetate, ethyl esters and medium-chain fatty acids; reduced level ocratoxine A.	Negative compounds (volatile acidity, sulphur compounds, etc.); biogenic amine production; production of acetoin; sluggish or stuck fermentation.	Angioni et al., 2007; Caruso et al., 2002; De Benedictis et al., 2011; Jolly et al., 2014; Rojas et al., 2003; Viana et al., 2009
Issatchekia	Increase the amount of free monoterpenes and non-isoprenoids; reduction of malic acid content.	Production of biogenic amines.	González-Pombo et al., 2011; Kim et al., 2008; Tristezza et al., 2013
Kluyveromyces	Enhancement of aroma and flavor; increased concentrations of lactic acid, glycerol and 2-phenylethanol.	Higher "spicy" and "acidity" attributes.	Comitini and Ciani, 2011; Gobbi et al., 2013; Kapsopoulou et al., 2007
Metschnikowia	High concentration of esters; increase wine flavor and aroma; antimicrobial activity (pulcherrimin).	Delays in fermentation due to antimicrobial activity.	Rodriguez et al., 2010; Sadoudi et al., 2012; Oro et al., 2014

Table 1. Continued.

Yeast genera	Oenological properties	Negative effect	References
Pichia	Increased concentrations of volatile compounds (acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl octanoate, 2,3-butanediol and glycerol); increased concentration of polysaccharides.	Antimicrobial activity against *S. cerevisiae*.	Clemente-Jimenez et al., 2004; Domizio et al., 2011
Schizosaccharomyces	Degradation of malic and gluconic acid.	Increased concentration of acetaldehyde, propanol and 2,3-butandiol; low concentration of esters.	Benito et al., 2013
Torulaspora	Low concentration of acetic acid.	Slower fermentation rate; production of sulphur compounds.	Bely et al., 2008; Azzolini et al., 2015
Zygosaccharomyces	Low concentration of acetic acid, H_2S, SO_2, malic acid degradation, high fermentative power; increased concentration of polysaccharides.	High amount of acetic acid.	Domizio et al., 2011; Loureiro and Malfeito-Ferreira 2003

All species listed in table 1, sequentially succeed among each other during the spontaneous fermentation. Nevertheless, despite the constant presence of some non-*Saccharomyces* yeasts, the majority do disappear during the early stages of a vigorous fermentation (Fleet et al., 1984; Henick-Kling et al., 1998). The reason might be the effects of a combination of stresses (high concentrations of SO_2 and ethanol, low pH, oxygen deficiency and low fermentative capacity) that slow the yeast growth (Combina et al., 2005; Heard and Fleet, 1988). Further, nutrient limitation and dominance of *S. cerevisiae* can also have a suppressive effect (Granchi et al., 1998).

Importance of these strains resides on their effect on the wine aroma and flavor profile, which may be either negative or positive (Table 1). When only using non-*Saccharomyces* yeast pure cultures, wine shows several problems because of the yeasts metabolite compounds production or fermentative behavior. On the other hand, non-*Saccharomyces* enhance the biodiversity level of inoculated AF and contribute to aroma complexity and improve wine quality such as mouth-feel (texture), complexity and integration of flavors (Ciani et al., 2010; Comitini et al., 2011; Viana et al., 2008). Thus, research is required with the aim to prevent their negative effect on wine production while exploiting their beneficial contributions to wine quality (Ciani and Picciotti, 1995; Jolly et al., 2003b).

Metabolism of non-*Saccharomyces* yeast can be also advantageous for other purposes besides affecting wine flavor in a direct manner. Due to the depletion of essential nutrients in the media and formation of toxic metabolites, these yeasts can inhibit the growth of lactic acid bacteria, essential for the secondary malolactic fermentation in wine (Costello et al., 2003; Fornachon, 1968; Ribereau-Gayon et al., 2006). On the other hand, non-*Saccharomyces* by-products can have a stimulating effect on lactic acid bacteria. Non-*Saccharomyces* yeasts have as well been used to lower ethanol yields as it is a current trend of consumption of wines containing lower ethanol (Ciani and Ferraro, 1996; Ciani et al., 2006; Comitini et al., 2011; Di Maio et al., 2012; Ferraro et al., 2000; Kutyna

et al., 2010; Magyar and Toth, 2011; Sadoudi et al., 2012; Soden et al., 2000).

These facts together highlight the importance of obtaining a broad and deep knowledge about both non-*Saccharomyces* and *Saccharomyces*. Moreover, the wine industry has been directed towards the use of controlled mixed fermentations due to the positive influence of non-*Saccharomyces* yeasts (Benito et al., 2015; Ciani et al., 2006; Jolly et al., 2006; Rojas et al., 2003; Romano et al., 2003). Nonetheless, the wideness of yeast biodiversity leaves room for undiscovered hidden potentials that could be utilized for exploitation in wine production (Fleet, 2008; Pretorius et al., 1999; Pretorius, 2000). Investigation of the biodiversity of non-*Saccharomyces* yeasts and the potential risks on safety and quality, are very important before their application in order to select starter strains denoted by different oenological characteristics. Thus, proper mixed starter management during fermentation will allow winemakers to tailor wines to the changing demands of consumers.

Torulaspora delbrueckii.

T. delbrueckii is one of the few non-*Saccharomyces* species that are commercially available for use in wine and beer production in which it has been used for years. First, it was released as a component of a yeast blend along with *S. cerevisiae* and *K. thermotolerans* (Anonymous, 2004) and CHR Hansen (2013a, b); and subsequently *T. delbrueckii* was released on its own by CHR Hansen (2013a, b). The genera *Torulaspora* is present in most of the relevant wine regions in the world (reviewed by Capozzi et al., 2015). This yeast was formerly classified as *Saccharomyces rosei* and was previously suggested for winemaking of grape juices with low sugar and acid contents in commercial production of red and rose wines in Italy (Castelli, 1955). *T. delbrueckii* is a yeast characterized by its low resistance to the lack of oxygen and besides other yeasts that are affected and die because of the ethanol toxicity, this yeast might die due to low oxygen levels (Holm Hansen et al., 2001; Lachance and Kurtzman, 2011).

Also Nissen et al. (2003) reported a high sensitivity to cell–cell contact with *S. cerevisiae*.

Several studies confirm the role of *T. delbrueckii* on wine aroma and its potential use in winemaking. Moreno et al. (1991) and Renault et al. (2009) have shown that pure cultures of *T. delbrueckii* produce lower levels of volatile acidity than *S. cerevisiae* strains. For this reason, Bely et al. (2008) proposed *T. delbrueckii* as a good yeast candidate for the production of wines derived from botrytized grapes and highlighted its great potential in high-sugar fermentation, considering its lower production of volatile acidity, and other undesirable volatile compounds, like hydrogen sulphide and volatile phenols, than *S. cerevisiae* (Bely et al., 2008; Renault et al., 2009). Other compounds produced include succinic acid (Ciani and Maccarelli, 1998) and linalool (for specific strains), a monoterpene alcohol responsible of the varietal aroma of Muscat type wines (King and Dickson, 2000). In terms of wine flavor and aroma, *T. delbrueckii* co-inoculated with *S. cerevisiae* produced wines (Sauvignon Blanc and Chenin Blanc) that resulted in a better evaluation than *S. cerevisiae* reference wines 5 and 18 months after production (Jolly et al., 2003b).

Sadoudi et al. (2012) also confirmed previous reports stating that *T. delbrueckii* produced the least amount to acetic acid and volatile acidity. They showed that its chemical profile is related with the yeast *L. thermotolerans* but showed relatively higher concentrations of the sulfur containing compounds 3-(methylthio)-1-propanol, 3-[(2-hydroxyethyl)thio]-1-propanol, thietane (trimethylene sulfide), 3-(methylthio)-propanoic acid ethyl ester, and 1,3-oxathiane. This might be due to two likely causes: *T. delbrueckii* itself assimilates and catabolizes methionine more readily than *S. cerevisia*e or that *T. delbrueckii* creates a poor level of amino acid environment and facilitates the formation of these compounds by *S. cerevisiae*. These compounds have generally very low sensory thresholds. 3-(methylthio)-1-propanol smells like raw potato, sulfurous, onion, soup and

vegetable while 3-(methylthio) propanoic acid ethyl ester has been described as sulfurous, metallic, pineapple, fruity, and ripe pulpy tomato. One or the other at high a concentration would undoubtedly contribute to a wine fault. Whitener et al. (2016) found that while *T. delbrueckii* may reduce acetic acid in the final fermentation, it does little to positively enhance the overall aroma profile. Higher levels of off-odor thiol compounds were produced compared to other fermentations carried out by other yeasts. Their study showed that after a sensorial panel, results were similar between *T. delbrueckii* and other non-*Saccharomyces* yeasts like *L. thermotolerans*, *P. kluyveri* and *M. pulcherrima*.

In a recent study carried by Whitener et al. (2015), 10 out of 69 significant differences were positive fold changes in the *T. delbrueckii* case and 14 out of 62 for *S. cerevisiae* when fermenting Sauvignon blanc and Syrah. *T. delbrueckii* fermentations significantly lacked a number of esters, demonstrating a negative fold change, only with the exception of isobornyl acetate, isoeugenyl phenylacetate, and phenethyl propionate. A great difference was found in the metabolism of ethyl 2-hydroxy-4-methyl pentanoate among varieties used for the fermentation: a positive fold change in the Syrah and a negative fold change in the Sauvignon blanc. Phenethyl propionate and 5-methylfurfural were found over 50 and 60 times higher in *T. delbrueckii* fermentations, respectively, although only in the Sauvignon blanc while not significantly different in the Syrah fermentations in the case of 5-methylfurfural. Finally, the sulfur compound 3-methylthio-1-propanol was 5 fold higher in the Syrah must but not significantly different in the Sauvignon blanc.

In general, *Torulaspora* genera provides positive oenological properties such as low concentration of acetic acid and negative effects such as slower fermentation rate and production of sulphur compounds (Azzolini et al., 2015; Bely et al., 2008; reviewed by Capozzi et al., 2015).

1.1.2. *Saccharomyces cerevisiae*: flor yeasts

As ethanol concentrations rise in wine fermentation the species diversity of the ecosystem is diminished and the environment becomes more selective for *Saccharomyces cerevisiae* (Heard and Fleet 1985). Intentional inoculation of selected *S. cerevisiae* has been carried out for many years in order to avoid negative effects of spontaneous fermentation, i.e. unpredictable products from vintage to vintage, microbial spoilage, stuck fermentation and arrests, production of undesired metabolites (Capozzi et al., 2011; Spano et al., 2010). Its use is well diffused among production of table wine and modern wineries to steer fermentations.

Saccharomyces yeasts, specifically *S. cerevisiae* and some closely related species, have a remarkable characteristic called the Crabtree effect. This is defined as the ability to produce and accumulate ethanol even under aerobic conditions and it is distinguished among two types: the short-term and the long-term Crabtree effects. The first one is the immediate occurrence of alcoholic fermentation after the addition of sugar to sugar-limited and respiratory cultures due to an overflow in sugar metabolism (Pronk et al., 1996; Vemuri et al., 2007). The second is explained as a limited respiratory capacity reflecting the repression of respiratory genes (Alexander and Jeffries 1990; Postma et al. 1989) but it has recently been suggested that overflow metabolism is also the fundamental mechanism behind the Crabtree effect (Hagman and Piškur, 2015).

Overflow metabolism allows yeast to produce energy more rapidly and enable rapid consumption of glucose which may cause hyperosmotic stress. The glucose-mediated repression of respiration may have developed as a later step to increase overflow and ethanol production thus, inhibiting the growth of other microbes. Further, *Saccharomyces* are also able to efficiently catabolize ethanol, this is called the ‘make-accumulate-consume’ strategy. Such strategy consists of rapid consumption of sugars, transforming them into ethanol that inhibits other species growth and establishing their dominance within the ecological niche, and subsequently consume

ethanol (Dashko et al. 2014; Piškur et al. 2006; Rozpedowska et al. 2011; Thomson et al. 2005).

S. cerevisiae was the first eukaryotic genome to be completely sequenced (Goffeau et al. 1996) and has been widely studied because it is used as a model for understanding eukaryotic cellular processes. However, this yeast has only recently been considered as a model for ecological studies and evolutionary genetics (Landry et al. 2006). *S. cerevisiae* wine strains have attracted considerable interest in recent years.

Most pioneering research in the physiology, evolutionary biology, genetics, and genomics of yeasts used in winemaking has been developed in *S. cerevisiae* and to a lesser extent on other *Saccharomyces* species.

Flor yeasts.

Several strains of *S. cerevisiae* have the ability to form biofilm that is a type of spontaneous yeast immobilization, on the surface of wine after fermentation and develop oxidative metabolism (biological aging of wine). This process occurs in an environment where there is a low level of fermentable sugars, low oxygen concentration, low pH, the presence of sulphites (approx. 30 mg/L total SO_2) and a high concentration of ethanol (14-16% (v/v)) (Alexandre, 2013). The biofilm is known as flor velum and the yeasts "flor yeast" (recently been reviewed in Benitez et al., 2011; Pozo-Bayón and Moreno-Arribas, 2011). Biological aging is a method used in areas such as Spain, France, Italy, South Africa, Armenia, California and southern Australia. The most well-known wine in which elaboration the flor yeasts are used for the flor velum is Sherry wine (Benitez et al., 2011; Pozo-Bayón and Moreno-Arribas, 2011). After fermentation of the grape must, the wine is fortified with wine alcohol to 15% (v/v) (with some exceptions, e.g. Vin Jaune from France, Vernaccia di Oristano from Italy, Montilla-Moriles Fino wine from Spain) and transferred into oak casks for storage after being clarified by sedimentation. The wine can be stored for a minimum of two years and can last more than 10 years when

the flor yeast velum develops naturally on the surface of the wine. Fino Sherry in the Jerez and Montilla-Moriles in Spain regions are characterized by the undergoing of Sherry wine aging system, so-called "criaderas" and "solera" (Charpentier et al., 2002), more detailed in chapter 3.

Flor velum is mainly formed by *S. cerevisiae* strains, but other yeasts and bacteria might be present (Pozo-Bayón and Moreno-Arribas, 2011). Yeast species such as *Pichia*, *Candida* and *Hansenula* have also been found in velum (Suarez-Lepez and Iñigo-Leal, 2004). Martinez et al. (1995) reported *T. delbrueckii* (formerly named *Saccharomyces montuliensis*), together with other *S. cerevisiae* in the velum.

Flor yeasts are not found during alcoholic fermentation, probably because their cell concentration is too low and it is only after wine is fortified (the addition of alcohol) when flor yeasts were detected in the medium (Esteve-Zarzoso et al., 2001). Nevertheless, our group found flor yeast strains during fermentation in the elaboration of Fino Sherry wines from Montilla-Moriles. It should be considered as well that the aging process is different among Sherry wines, thus also differences among biological aging processes. Ibeas et al. (1997) found that in different cellars, different flor yeast strains dominate and persist during aging, also confirmed by Esteve-Zarzoso et al. (2001).

Flor yeast strains have been thought to be physiologically and genetically different to other wine yeasts, e.g. low chromosomal polymorphism (Ibeas and Jimenez, 1996; Martinez et al., 1995). These differences may be associated with an adaptation to a medium depleted in fermentable sugars, high levels of ethanol and acetaldehyde (Castrejon et al., 2002; Ibeas et al., 1997; Infante et al., 2003; Martinez et al., 1998; Mesa et al., 1999, 2000; Ristow et al., 1995). Biofilm-forming and oxidative metabolism are regarded as an adaptive mechanisms that allow cells to access oxygen and grow on non-fermentable carbon sources such as ethanol (Zara et al., 2005). It should be mentioned with this in

respect that *S. cerevisiae* biofilm could be formed on other reduced non-fermentable carbon sources like ethyl acetate or glycerol (Zara et al., 2010). Biofilm formation property was primarily attributed to a high hydrophobicity of the cell surface due to specific cell wall composition (Iimura et al., 1980; Martinez et al., 1997a,b). Cell wall glucanes and mannoproteins contribute to the surface hydrophobicity (Alexandre et al., 1998; Alexandre et al., 2000; Martinez et al., 1997c). Flor yeast lipid content and composition (greater chain lengths and unsaturation levels) also influence the velum ability (Farris et al., 1993; Zara et al., 2009; 2012).

Further, genes are involved in this phenotype, being *FLO11* (*MUC1*), which encodes a hydrophobic cell wall glycoprotein, the most currently studied gene (Fidalgo et al., 2006; Ishigami et al., 2004; Purevdorj-Gage et al., 2007; Reynolds and Fink, 2001; Zara et al., 2005). It should be mentioned that not all *S. cerevisiae* strains that have the gene are able to form a velum because the promoter and/or ORF (Open Reading Frame) sequence are different. A flor forming yeast *FLO11* gene has a promoter 0.1 kb shorter, a coding sequence or ORF 1 kb larger, with deletions and rearrangements mutations in both the promoter and the ORF (Fidalgo et al., 2006). Hence, the floating and biofilm formation ability may be explained since a deregulation of the *FLO11* promoter leading to a higher Flo11p synthesis and, consequently, greater hydrophobicity. Indeed, the longer coding sequence would be explained by an increased number of the repeated sequences in the central domain correlated to the yeast flor floatability (Fidalgo et al., 2006). These authors also show that the sequence also varies depending on the flor yeast strain.

The ratio and/or distribution of repetitive units and the transcription level of *FLO11* are correlated to the floatability and biofilm formation in wild flor yeasts (Fidalgo et al., 2008; Zara et al., 2009). Regarding to its transcription level, it depends on presence or absence of the 111 bp sequence in the *FLO11* promoter, the presence of two short repeats sequences

and an epigenetic regulation (Fidalgo et al., 2006; Zara et al., 2009). Verstrepen and Klis (2006) suggested that *FLO11* is repressed when glucose is present which supports the hypothesis that flor formation requires utilization of non-fermentable carbon sources, i.e. ethanol, instead of fermentable sources, i.e. glucose (Iimura et al., 1980). On the other hand, *FLO11* was found to be highly expressed when using either ethanol or glucose as the sole carbon source (Ishigami et al., 2006) and even the development of a yeast biofilm on the surface despite the presence of sugar (40 g/L) was observed by Kovacs et al. (2008). This fact might be related to the repression of the regulatory mechanisms of *FLO11*, as among the mutations in the gene, one is a deletion in the promoter, which relieves the gene from repression (Fidalgo et al., 2006). However, the influence of glucose on *FLO11* expression still needs to be clarified. Glycosylation of Flo11p is also required for biofilm formation (Meem and Cullen, 2012).

Other genes such as *HSP12*, *NRG1* and *BTN2* are involved in film formation as well (Espinazo-Romeu et al., 2008; Ishigami et al., 2004; Zara et al., 2002). Further, cell wall proteins Ccw7p, Pir2p and Hsp150p differ between laboratory and film-forming yeast strains (Kovacs et al., 2008). Recently, Zara et al. (2009) reported the existence of an extracellular polysaccharide matrix which deserves further investigation in order to clarify its role together with its composition.

In a winemaking context, flor yeasts owe their importance to the wine protection from oxidation and to their metabolism during biological aging both contributing to the wine sensorial and aromatic properties. Flor yeasts metabolism has been treated in more detail in the chapter 3.

Research on flor yeasts is promising because the control of biofilm formation is important not only for practical applications in winemaking industrial settings, but also in the biomedical and environmental fields. Innovative research on

flor yeasts related with the origin of these yeasts, their ecology, their restricted distribution and phylogenetic relationships. Also, questions like i) whether flor yeasts could be found on grapes or are only present in the cellar and ii) whether flor yeast only appear after alcoholic fermentation or are present both during alcoholic fermentation and aging; need to be addressed. Factors favoring velum formation, persistence and also the sinking of the velum need to be more deeply be studied as they are relevant for the production of quality wine (Charpentier et al., 2004; Villamiel et al., 2008). Indeed, the complexity and specificity of the flor yeasts genome are two reasons that make flor yeasts an interesting model for studies into adaptive evolution based on mutations in the *FLO11* gene and into speciation of *S. cerevisiae* (Fidalgo et al., 2006).

Recently, the flor yeasts widened their spectrum of applications with the use of bio-immobilization systems which will open new perspectives for fermentation processes, with substantial technical and economic advantages compared to traditional fermentation methods using free yeast cells (García-Martínez et al., 2011; 2012). Nedovic et al., 2015 proposed future insights into the function of *FLO11* in flor yeasts to further improve cell-immobilization technologies. Indeed, flor yeasts are also unique models for mixed biofilm and interspecies interaction studies, as bacteria and other yeasts can also be present in the velum.

1.2. Yeast immobilization

Immobilization systems are techniques that aims to enclose the biocatalyst into a certain region of space in a manner that they retain their activities and can be reutilized several times for long periods of time. Biocatalysts include enzymes, cellular organelles, microbial, animal and plant cells. Karel et al. (1985) defined specifically the cell immobilization as the physical confinement/localization of intact cells to a defined space with preservation of some desired catalytic activity. Nowadays, yeast cells immobilization is applied to several areas which include

environment, health and biotechnology. The last area includes applications as industrial fermentation for bioethanol production and alcoholic beverages production such as winemaking, malolactic fermentation products, brewing, cider-making and ethanol and distillates production (Kourkoutas et al., 2004).

The use of yeast cell immobilization in industrial fermentations provides advantages like increased and prolonged productivity, better control and stability of the yeast strain, cell protection against shear forces, the possibility of performing continuous fermentation and the reducing cost of processes due to the ease for cell recovery and reutilization (Baptista et al., 2006; Groboillot et al., 1994; Kourkoutas et al., 2004; Sakurai et al., 2000; Strehaiano et al., 2006;Williams and Munnecke, 1981).

For a wine point of view, it will provide the opportunity to increase productivities of aroma and other metabolites by increasing volumetric cell densities by packing the cells into a small defined volume. As general, for the production alcoholic beverages purposes (i.e. bacteria or yeasts), the cell carrier has to comply with certain requirements (Freeman, 1984; Martin, 1991):

i) Big surface, with functional groups for cells to adhere.
ii) Easy to handle and regenerate.
iii) High and retained cell viability and operational stability.
iv) Catalytic activity not affected.
v) Uniform and controllable porosity to allow free exchange of substrates, products, cofactors and gases.
vi) Good mechanical, chemical, thermal and biological stability.
vii) Easy, cost effective and amenable to scale-up immobilization technique.
viii) Not affect product quality.

Cell growth, physiology and metabolic activity alterations may be induced by cell immobilization although they are difficult to predict (Melzoch et al., 1994; Norton and D'Amore, 1994; Walsh and Malone, 1995).

Yeasts can be naturally immobilized. *S. cerevisiae* can perform various multicellular manners of existence (i.e. flocculation, adhesion, filament formation and biofilm formation) in which the cells cooperate to fully utilize available resources and to maximize their chances of survival through improved resistance to stress (Honigberg, 2011). Some of these behaviours are industrially beneficial, like flocculation in the elaboration of sparkling wine and brewing or biofilm for biological aging in the elaboration of Sherry as it was previously treated. An analogous stress avoidance mechanism is encapsulation of cells for immobilized yeast fermentation (Sun et al., 2007a, 2007b).

In terms of wine sensory quality, results vary from different studies (Genisheva et al., 2012; Gonzalez-Pombo et al. 2014; Mallios et al., 2004; Tsakiris et al. 2004a; 2004b;). Tsakiris et al. (2004a, 2004b) asked consumers to evaluate the pleasantness of red wine samples produced by immobilized cells and they found scores were slightly higher for immobilization but the difference was not statistically different. Knowledge about the aroma and flavor profile of wine yeasts combined with the employment of a mixed non-*Saccharomyces*/*Saccharomyces* starter and the application of yeast cell immobilization systems for sequential inoculations allow winemakers to use them in a scientifically controlled manner to craft wine styles that match consumer expectations in a diverse range of market segments.

In winemaking, cell immobilization is a rapidly expanding investigation area but their applications in an industrial scale are still limited. Purposes cover the improvement of alcohol productivity and overall product aroma, taste and quality, as well as the re-use of biocatalysts in continuous operation bioreactor systems, physical and

chemical protection of the cells, avoidance of microbial contamination, and ability to perform low-temperature fermentation. A specific wine application was the biological deacidification of wines to improve the organoleptic characteristics of products (Genisheva et al., 2012; 2013, 2014; Vilela et al., 2013). Further, Servetas et al. (2013) co-immobilized *S. cerevisiae* and *O. oeni* to simultaneously perform alcoholic and malolactic wine fermentations. For industrialization issues, wheat grain-supported biocatalyst efficiency for winemaking was studied in a scaled-up system of 80 L resulting in not negatively affected fermentative ability and improved aromatic profile compared to free cells (Kandylis et al., 2010a). For commercial issues, application of yeast immobilization technologies for wine production was successful (Kopsahelis et al., 2012).

Finally, the potential of storage for long periods of thermally dried immobilized yeast on different materials (delignified brewer's spent grains, gluten pellets, delignified cellulosic material and freeze dried wheat) without loss of cell viability/fermentation activity and producing wines with similar organoleptic characteristics to those of fresh cultures; accentuates the commercial potential for industrial application (Kandylis et al., 2010b; Kourkoutas et al., 2010; Tsaousi et al., 2010, 2011;). Due to these reasons, immobilization of microbial cells can improve cell metabolism, even under stress fermentation conditions (i.e. low and high temperatures, high sugar content) and can be used for biological deacidification or controlled liberation of flavor-active compounds, all of these improving the efficiency of the whole process and the quality of the end products. Indeed, the long-term storage of immobilized cells and their utilization in higher scales, will promote the industrialization of immobilized technology in winemaking.

Until the date, different methods for yeast immobilization have been developed depending on by the mechanism of cell localization and the nature of support material (Figure 3):

i) Immobilization on a support surface. The adsorption of yeast cells to some support material by covalent bonding between the cell membrane and the carrier, or by electrostatic forces.

ii) Flocculation of yeast cells. It consists in the non-sexual aggregation of single-celled organisms in suspension to form a larger unit or aggregates of many cells known as flocs (Jin and Speers, 1998).

iii) Mechanical containment behind a barrier. The most common one are the use of microporous or ultraporous membrane filters and entrapment of cells in a microcapsule.

iv) Entrapment in a porous matrix. Cells are incorporated in a rigid network preventing them from diffusing into the surrounding medium, while still allowing mass transfer of substrates and metabolism products.

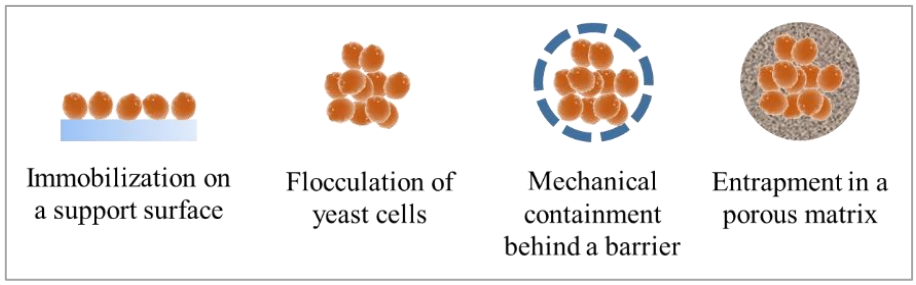


Figure 3. Basic methods of cell immobilization.

Based on the carrier chemical composition, according to Kourkoutas et al. (2010) Nedović et al. (2010), carrier materials can be categorized into:

i) Natural supports. Mainly of food grade purity and are used with minimum or no pre-treatment like wood, sawdust, pieces of fruit, gluten pellets, brewer's spent grains, delignified cellulosic material, etc.

ii) Organic supports. Synthetically made (like plastic) or extracted from natural sources by more complex

processes (like polymeric hydrogels) regardless of their food grade purity.

iii) Inorganic. A number of inorganic materials like porous ceramics, porous glass, polyurethane foam, etc., have been proposed as yeast cell carrier materials for many fermentation processes.

iv) Membrane systems.

v) Cells attached through various types of interaction (i.e. Van der Waals' forces, ionic bonds, hydrogen bridges).

vi) Multi-functional agents. Several functionalities are integrated into a single miniaturized device, i.e. glutaraldehyde-based system.

A high number of immobilization supports have been proposed by various researchers for alcoholic beverages production. The advantages related with the production of potable alcohol using cell immobilization are clear but, attention should be also focused on the improvement of products quality. Certain production systems seem to result in overall improvement of the sensory characteristics of the final products, e.g. beer, wine and cider, by promoting aroma production during the fermentation process. Thus, efforts should be made on abundant, cheap, able to be stored long-term, non-destructive and food-grade purity immobilization supports, which will improve quality and give distinctive aroma profiles to the final product. All of these features are needed for the promotion of industrial application of immobilized cell systems.

1.2.1. Biocapsules

During the last decades, a novel concept of yeast immobilization has arised. It consists of the attachment of yeasts to the mycelium that are generally recognized as safe (GRAS) filamentous fungus (*Rhizopus* sp. and *Penicillium* sp.) (Nyman et al., 2013; Peinado et al. 2004) which can be sorted among an ‘‘entrapment in a porous matrix’’ and a ‘‘natural’’ immobilization systems; and complies with several

required feature for the promotion of industrial application: abundant, cheap, able to be stored of long-term, non-destructive and food-grade, etc. Co-immobilizing *Penicillium chrysogenum* and yeast cells, results in the formation of hollow, spherical bodies that constitute a natural matrix system. This system has been known as "yeast biocapsules" (Figure 4).

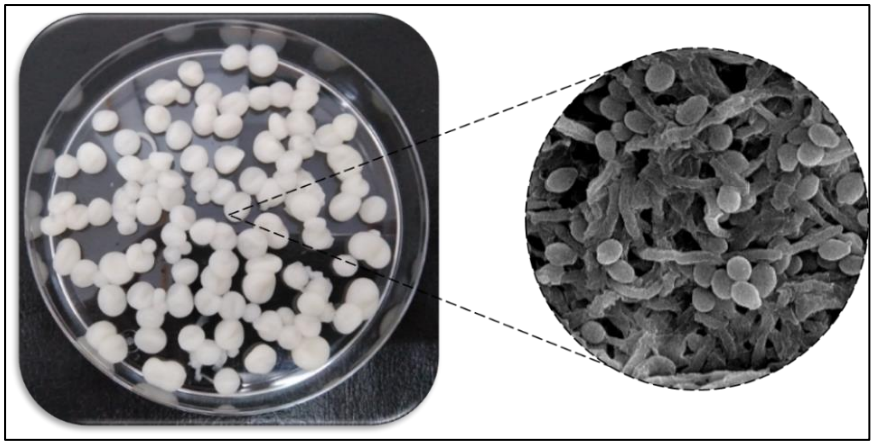


Figure 4. Yeast biocapsules from *Saccharomyces cerevisiae* and *Penicillium chrysogenum.*

If compared to other immobilization systems, yeast biocapsules eliminate the cost of inert supports and since it takes advantage of natural adhesion properties of yeast and filamentous fungus cell walls, it minimizes changes to the yeast metabolism and/or yeast viability. Further, biocapsules permit diffusion of nutrients and products among the external medium and the yeast due to the porous structure of the filamentous fungus (Peinado et al. 2004; García-Martínez et al. 2011). Once biocapsules are used for fermentation, the fungus die and the yeast cells colonize and invade all hyphae just remaining as a mere support for the yeast cells and not affecting products excreted to the medium (Peinado et al. 2006). At this respect, García-Martínez et al. (2011) by dialysis experiments, found that the death of the filamentous fungus was due to the yeast via a cell-hypha contact-mediated mechanism. Biocapsules can then be easily recovered from fermentations retaining the yeast viability and fermentative

capacity and thus, enabling multiple rounds of reuse (Peinado et al. 2004; García-Martínez et al., 2011). García-Martínez et al. (2012) reported that the successive reuse of the immobilized flor yeast resulted in a gradual adaptation to the fermentation conditions and an increasingly uniform behavior, in terms of the fermentation kinetics and production of metabolites. For all of these reasons, biocapsules are considered as a low-cost, natural, suitable alternative and hence as a promising technique for industrial-scale fermentation purposes.

During the last few years, yeast biocapsules were applied to entrap osmotolerant *S. cerevisiae* strains and produce sweet wine from raisin must through partial fermentations to overcome the growth lowering of yeasts under osmotic stress (López de Lerma et al., 2012; García-Martínez et al., 2013). Compounds related to the osmoregulatory were reported in higher concentrations when using biocapsules, i.e. glycerol, acetaldehyde, acetoin and butanediol as well as aromatic volatiles like ethyl octanoate, 4-butyrolactone, ethyl hexanoate, acetaldehyde, isoamyl alcohols, 2-phenylethanol, ethyl acetate, and 2,3-butanediol resulting in an increased complexity of wine aroma (García-Martínez et al., 2013).

Other applications that have been attempted are: production of white and sparkling wines and bioethanol production from starch and molasses (García-Martínez et al. 2012; García-Martínez et al. 2015; Peinado et al. 2005; Peinado et al. 2006; Puig-Pujol et al. 2013). For sparkling wine elaboration, biocapsule immobilization was compared to calcium alginate beads, producing the first a wine with improved enological characteristics and lower calcium ion content (Puig-Pujol et al., 2013). Wines produced did not show significant differences after a triangular sensory test. These facts together with other limitations of calcium alginate beads, like mechanical instability in high-capacity bioreactors (Kourkoutas et al., 2010), make yeast biocapsules a good alternative in the industrial production of sparkling wines.

Must from white grapes was fermented using yeast biocapsules and free yeasts resulting in higher contents of acetaldehyde produced by the biocapsules (84 vs. 63 mg/L, respectively), isobutanol (217 vs. 194 mg/l), L-proline (7.7 vs. 6.5 mM) and aspartic acid (0.42 vs. 0 mM) in final wine. All of these analyzed compounds range between the limits of concentration described in the literature and no off flavors were detected.

Yeast biocapsules have been originally used for the immobilization of flor yeast trying to exploit the adhesion and biofilm formation abilities of these strains (Peinado et al., 2006). A higher immobilization efficiency of flor yeast versus non-flor yeast on the filamentous fungi has been shown. The use of bio-immobilization systems will widen the spectrum of potential applications of flor yeast strains, which will open future perspectives for fermentation processes, with substantial economic and technical advantages over free yeast-based traditional fermentation methods (García-Martínez et al., 2012). Future research into the role of the *FLO11* gene as well as other genes involved in biofilm formation, in flor yeast will help to improve cell-immobilization technologies by avoiding release of yeast cells to the external medium (Nedovic et al., 2015).

1.3. Wine–omics

The term –omics refers to a field of study in biology ending in –omics, such as genomics, transcriptomics, proteomics or metabolomics and aims to the characterization/quantification of pools of biological molecules of an organism: genes (genome), transcripts (transcriptome), proteins (proteome) and metabolites (metabolome).

–Omics technologies have been largely used for the study of *S. cerevisiae* as a model organism, and these approaches have been extended to industrial strains.

Nowadays –omic sciences are applied to a wide variety of fields of knowledge and among them, the food industry. Particularly in enology, there exists a trend to establish relationships among proteomic responses in the vine and the microorganisms which are the responsible of the production of flavor and aroma compounds during the wine elaboration process. According to Giacometti et al., (2013) improvements in current techniques and developments of new –omics strategies and techniques, will allow new studies to be carried out in order to build databases that include exo-metabolites and their relationships with proteins implied in their formation. These estudies will lead to a greater understanding of the interactions between the biological information system genome-transcriptome-proteome-metabolome and the wine-making industry, will be the major beneficiaries of the generated knowledge (Bornemam et al., 2007). Indeed, a more generic perspective is needed to know how *S. cerevisiae* regulates its biological system under different conditions and to establish the exo-metabolomic profiles, especially those of small volatile molecules (Ding et al., 2010). Further, the identification of proteins and molecules produced and excreted in the medium by a yeast in a medium of a known composition, constitutes an important advance for the detection of problems related to the quality, safety and improvement of the food chain. According to Saghatelian and Cravatt (2005), functional interactions between proteome and exo-metabolome of yeasts will lead in the very near future to design strategies to improve the brewing of fermented beverages and to establish new applications of yeasts.

1.3.1. Proteomics

The proteome is defined as the set of protein species present in an organism, in a specific developmental state and under certain environmental conditions. Proteomic studies have experienced a boom in the last 20 years due to the development of analytical techniques and bioinformatic programs that allow the analysis of the data generated by a single experiment. According to Jorrín-Novo et al. (2009), the analytical methodology of proteomics consists of several

stages: experimental design, sampling, sample preparation, protein extraction, modification, separation, MS (Mass Spectrometry) analysis, identification, statistical analysis and validation. More specifically, the methodology of the proteomic studies is based on the previous separation of the protein mixture using two-dimensional polyacrylamide gels (2DE) techniques or more recently with off-gel electrophoretic techniques followed by HPLC-MS (High Performance Liquid Chromatography- Mass Spectrometry) analysis.

Proteomic studies about the biotechnology of fermentations, highlight that the response of yeasts changes during the fermentation and and a direct relationship between mRNA and protein transcripts is not observed (Rossignol et al., 2009). According to Rodríguez et al., (2012), the information provided by proteomic anlysis is useful to control fermentation and to guarantee the quality of the finished wine, and also offers excellent prospects for the improvement of fermentation processes in the near future.

Proteomic analyses on wine yeasts have been mostly focused on the fermentation process. Trabalzini et al. (2003) and Zuzuarregui et al. (2006) tracked changes on the proteomic profile along the fermentation finding an induction of stress response proteins and enzymes that participate in the carbon metabolism before the yeasts enter in the stationary growth phase. Ctt1p, Tdh1p, Rps0bp, Eno1p and Gpp2p involved in the hyperosmolarity response; and Cct1p, Sod1p, Hsp26p, Pst2p, Sba1p, Eno1p, Tfs1p, Ubi4p, Rps0bp, Tsa1p and Zwf1p in the oxidative response, both groups of proteins directed against the effects of ethanol, were found in high contents along the fermentation. Zuzuarregui et al. (2006) found proteins involved in the stress responses, glycolysis and fermentation over-repressented in a wine yeast strain when compared with another one with worse fermentative behavior that did not complete the vinification process. Glycolysis and ethanol production transcripts and proteins are one of the most

expressed categories at both transcript and protein levels (Rossignol et al., 2009).

Aditionally, protein folding, synthesis and degradation categories were over-represented during fermentation (Trabalzini et al., 2003). Bmh1p, Bmh2p, Eno1p, Gpm1p, Ubi4p, Tfs1p, Sod1p, Pup3p and Vma4p, up regulated through the fermentation, contribute to the protein degradation or autoproteolysis process which occur following several type of stress and aims to enrich the cytoplasmic amino acid pool or to contribute to the turnover by providing protein building blocks. Autoproteolysis mainly targeted towards particular isoforms of glycolytic enzymes (Trabalzini et al., 2003). On the other hand, these authors observed a repression of proteins involved in protein synthesis, cell growth and RNA metabolism.

Besides these processes, Rossignol et al. (2009) reported a higher representation of amino acid and sulfur metabolism functional categories through transcriptome and proteome analyses. Amino acid and fermentative metabolic enzymes, and the corresponding genes are strongly correlated over time and between different yeast strains, suggesting a strong transcriptional control of such enzymes (Rossouw et al., 2010). These authors also obtained differences in the ratios of several transcripts and proteins involved in the synthesis of aromatic amino acids (namely, Aro1p, Aro3p, Aro4p, and Aro8p) and differences in the concentrations of the metabolites of these pathways. Amino acid metabolism is interesting for winemaking because these molecules are precursors of some important volatile aroma compounds.

Most of research on yeast protection has been done by using 2D electrophoresis on gels, right before mass spectrometry by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization- Time Of Flight) (Usbeck et al., 2014). More recently, third-generation proteomics has been based on gel-free (OFF-GEL) and label-free (also MudPIT quantitative, shot-gun) techniques. The OFF-GEL is an

alternative to first-dimensional separation to fractionate peptides or proteins according to their pI values whereby the separated components are recovered in liquid phase (Horth et al., 2006) allowing interface with up- or downstream sample processing steps like protein digestion and liquid chromatography for multi-dimensional separations of complex samples. Overall, this technology provides fractions with greater protein richness than 2D techniques and allows to identify a higher number of proteins (Essader et al., 2005).

1.3.2. Metabolomics

Metabolomics has been defined as the characterization of the entire composition in small metabolites below 1.5 kDa, of a particular system or organism (Dunn y Ellis, 2005). Metabolites are the final products of the biological information system: genome-transcriptome-proteome-metabolome. Therefore, metabolomic platforms find a great field of study in the food sciences (Cevallos-Cevallos et al., 2009; Ebeler et al., 2006, 2009; Herrero et al., 2012 and Castro-Puyana et al., 2013). Indeed, the term foodomics, has emerged as a result of the discipline that studies the food and nutrition domains through the application of –omics technologies and some applications are also used in food safety, food quality and food traceability in the last few years. The methods of the study of cellular metabolism were developed very rapidly in the last decades and constitute a fundamental support for the innovation and the development of fermentation processes.

Until now, most of these studies have focused on the inside of the cell (endo-metabolome) but the transformations that occur in the extracellular environment (exo-metabolome) are also important. Daviss (2005) defines endometabolomics as the study of the fingerprinting in the inside of the cell while the exometabolomics, also known as metabolic footprinting, studies the transformations that the cells produce in the small molecules of the medium where they grow. This approach provides important information for establishing the cellular phenotype, avoiding the problems of determination of

intracellular metabolites. In this regard, Allen et al. (2003) indicate that the methods of sample preparation used in exo-metabolomics are more robust and have greater discriminant power between different cell growth states and among mutants than those used in endo-metabolomics. Moreover, the analysis of the chemical composition of a biological sample in a given condition allows for comparative studies between different conditions, so that the representative substances of each of them can be established.

The *"winomic"* word was used for first time in a publication by Skogerson from the University of California (Davis) in 2008. Arbulu, (2014) pushed forward the growing need to identify and measure the molecules present in foods and beverages in the production of microorganisms, such as wine. In a recent review, Alañón et al., (2015) relate the advances in wine chemistry during the last years with the development of metabolomic approaches. In this regard, is well known that wine is a complex hidro-alcoholic matrix composed of molecules of organic and inorganic nature with diverse structures (carbohydrates, alcohols, acids, esters, N-compounds, S-compounds, phenolics, proteins, etc.) in a wide range of concentrations (Moreno and Peinado, 2012). In addition, the aroma of a wine is the result of the contribution of about 800 volatile compounds of various chemical nature, ranging from several mg/L to a few ng/L, or even less (Ebeler, 2001; Etiévant, 1991; Nykänen, 1986; Schreier, 1979). Figure 5 show their classification according to its origin.

There exists an extense literature about metabolomics in wine. From now on, each type of compound potentially found in wines has been described to contextualize the yeast metabolites and distihguish them among other compounds that come from different sources that are not in the scope of this thesis.

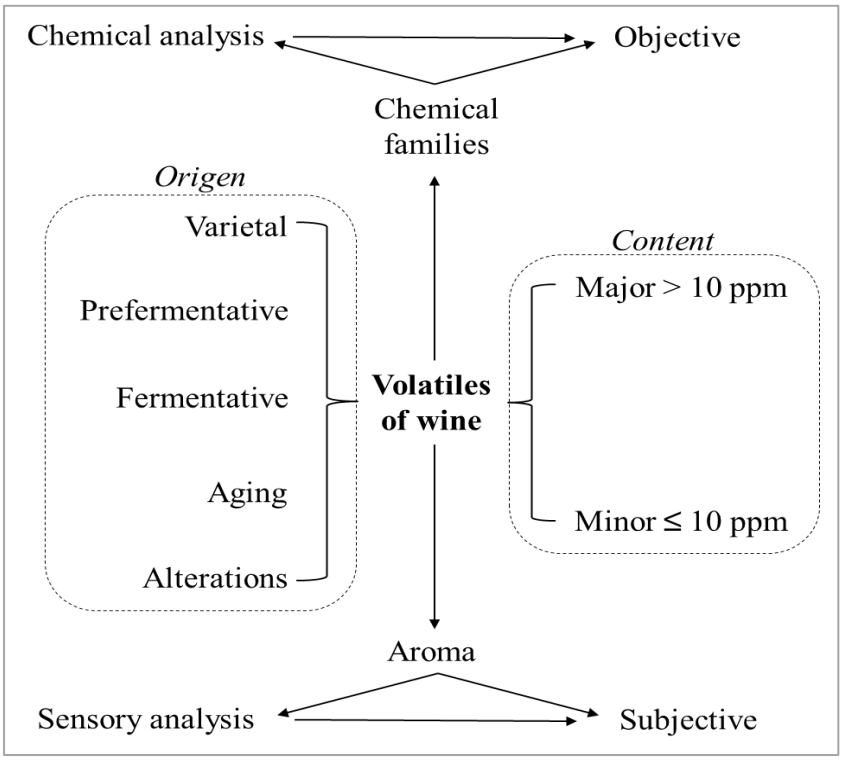


Figure 5. Analysis and classification of volatile metabolites.

i) Varietal aromas

The varietal or primary aroma comes from the metabolism of the grapevine and the grapes and plays a determining role in the quality and the uniqueness of a wine. It is therefore, the relationship between concentrations of certain molecules that distinguishes the typical aroma of each variety of grape. Certain "key" compounds identify grapes and wines as is the case of mono-terpenols in the Moscatel varieties and the methoxy-pyrazines in the Sauvignon varieties.

Although the varietal aromas depend basically on the grape variety (Günata et al., 1985; López-Tamames et al., 1997), its content is also influenced by soil-climatic factors of the vine growing zone (Sabon et al. 2002), vine management (Fragasso et al., 2012), diseases and the application of phytosanitary treatments (González-Rodríguez et al., 2011; Reboredo-Rodriguez et al., 2014), among others.

The grape constituents conditioning the aromatic potential and constituting the varietal aroma of wine, are classified into two groups: free aroma (volatile molecules) and combined non-volatile aromas which are odorless or precursors (glycosidic combinations, phenolic acids, fatty acids, among others). These last aromas originate odorant compounds during the winemaking process and constitute the so-called hidden aroma of the grape.

Free varietal aromas

The varietal aromas are constituted by small volatile molecules released spontaneously from the musts, immediately after crushing the grape. They have a specific odor and are classified from a chemical point of view into terpene compounds, norisoprenoids of 13 carbon atoms, methoxypyrazines and volatile thiols.

Terpene compounds

About 70 terpenic compounds have been identified in grapes and wine, most of which are monoterpenes and some sesquiterpenes with different functional groups. The monoterpenes are found as simple hydrocarbons (limonene, myrcene, etc.), aldehydes (linalal, geranial, etc.), alcohols (linalool, geraniol, etc.), acids (linalic acid, geranic acid, etc.) and even esters (linalyl acetate, etc.) with monoterpene alcohols being the most potent odorants (Linalool, α-terpineol, nerol, geraniol, citronellol, and hotrienol). They are responsible for fruit, citrus and floral aromas although some have resin or balsamic odours (α-terpinene, p-cymene, myrcene and farnesol). These compounds are metabolites synthesized by the plant and accumulated in the grape, being important to the varietal aroma and particularly abundant in the Moscatel varieties (Mateo and Jiménez, 2000).

Terpene biosynthesis follows a similar pathway in plants, microorganisms and animals, from glucose through the mevalonic acid pathway, from which the isopentenyl pyrophosphate (active isoprene) is formed. The first terpenoid molecule formed is the geranylpyrophosphate (IV), which is the basic skeleton for the synthesis of mono-terpenes (Figure 6) and more condensed terpenes are synthesized.

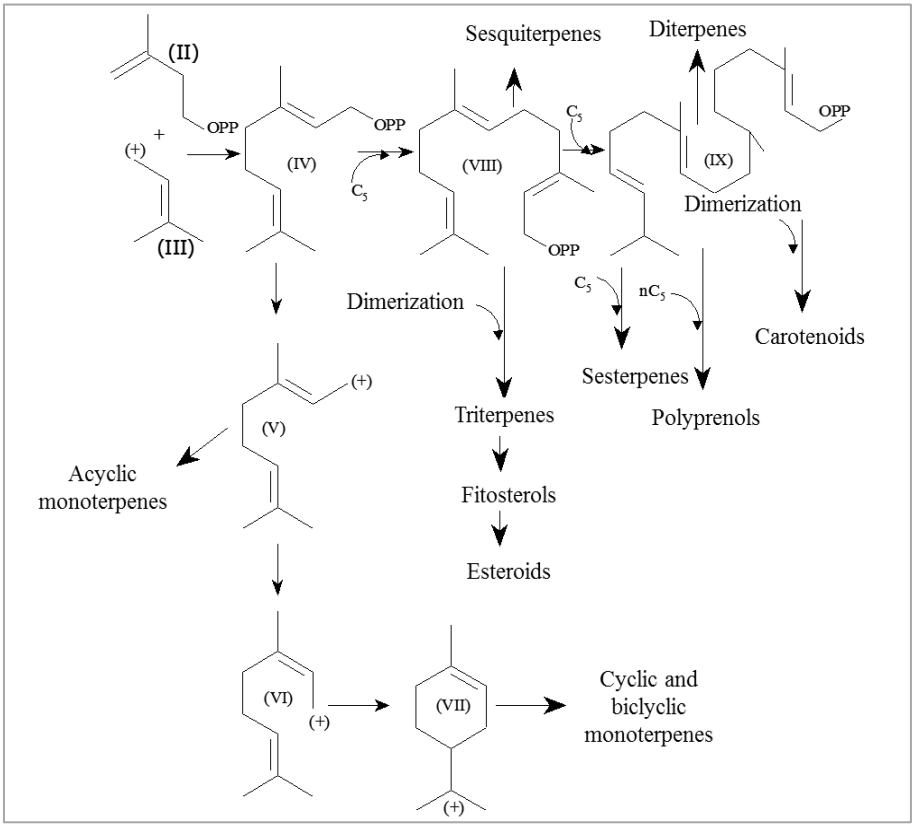


Figure 6. Biosynthesis of terpenes from mevalonic acid.

The structures of the most common monoterpenols and some derived oxides are shown in Figure 7.

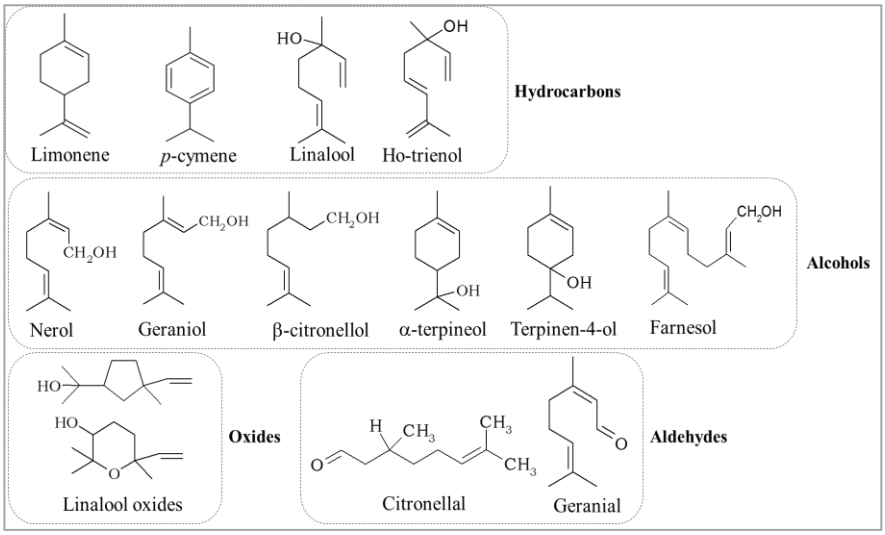


Figure 7. Monoterpenes of grapes and wines.

More than 50% of the content of the grapes in the free terpenes are found in the solids of the berry or grape (pulp and peel), where geraniol and nerol are more abundant in the skin (90% of the total of the grape) and linalool making up 50% of its content in the pulp and grape must.

C13 norisoprenoids

C13 nor-isoprenoids are formed by oxidation reactions of carotenoids, tetraterpene compounds that have 40 carbon atoms. The carotenoids are found in the pulp of the grape and in greater proportion in the skins. The oxidases enzymes degrade carotenoids giving rise to norisoprenoid compounds with 9, 10, 11 or 13 carbon atoms that are more volatile and odorous than their precursors (Figure 8).

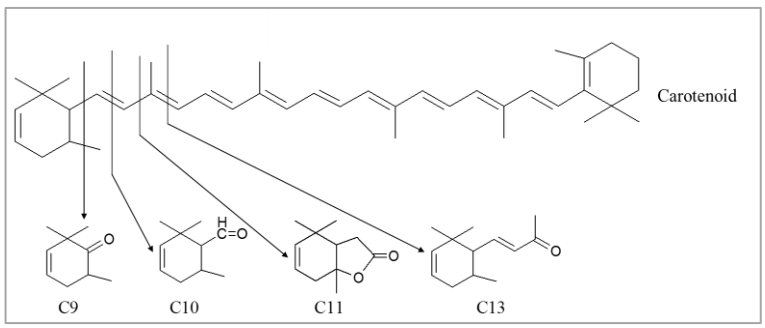


Figure 8. Formation of nor-isoprenoids from carotenoids.

The C13 derivatives are important because of their flowers and fruit odors at small concentrations (Baumes, 2009).

The nor-isoprenoids are two major molecular structures: the megastigman forms and the non-megastigmans (Figure 9). Mega-stigman forms include β-damascenone, with aromatic descriptors ranging from blackberry to prunes, and β-ionone, with violet notes.

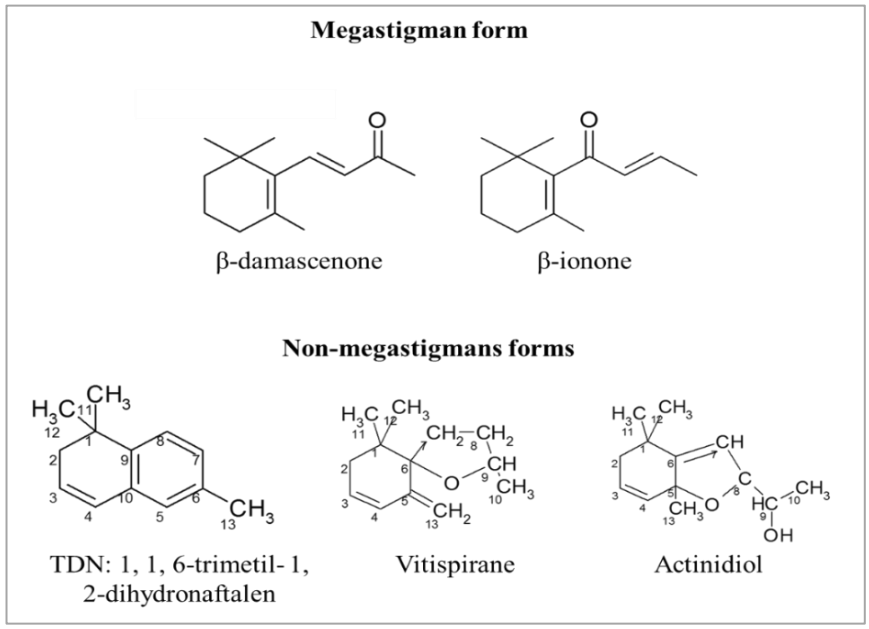


Figure 9. Molecular structures of nor-isoprenoids.

Methoxypyrazines

Heterocyclic compounds of nitrogen, which come from the metabolism of amino acids (Figure 10) are located mainly in grape skins. The most abundant methoxypyrazines are 2-methoxy-3-isobutyl-pyrazine, 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-sec-butylpyrazine, which are particularly abundant in grapes and wines from Sauvignon Blanc, Cabernet Sauvignon, contributing to the green pepper and asparagus scents.

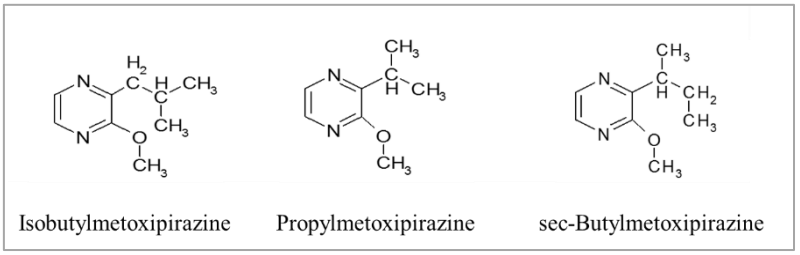


Figure 10. Structure of the principal metoxipirazines of Sauvignon wines.

Thiols

Thiols, as are the 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 3-mercapto-2-methylpropanol, have been identified as key compounds of the aroma of the grape varieties Sauvignon Blanc, Macabeo, Gewürztraminer, Riesling, Moscatel, Semillon, Verdejo, Grenache or Merlot (Murat et al., 2001; Tominaga et al., 2000). They have characteristic scents reminiscent of tropical fruits casis, passion fruit and grapefruit (Dubourdieu et al., 2006). 4MMP, 3MH and 3MHA are released during fermentation from odorless precursor molecules found in grapes of these varieties.

Bound varietal aromas or non-volatile precursors

They constitute the called hidden aroma of the grape and they are specific secondary metabolites of certain grape varieties. The aroma precursors of the most important wines are fatty acids, phenolic acids, some monoterpene alcohols, carotenoids and the glycosidic combinations of certain volatile and odorant molecules.

Fatty acids

The fatty acid content in the grapes is about 500 mg/kg and linoleic and linolenic acids account for more than half of the berry's fatty acids. There have been hardly any changes in the fatty acid content in berries of different varieties, so the few differences found are probably due to edaphic and non-genetic factors (Flanzy, 2003). Unsaturated fatty acids are

precursors of aldehydes and alcohols of 6 carbon atoms whith herbaceous, green aromas.

Phenolic acids

Hydroxycinnamic acids (caffeic, p-coumaric and ferulic) are esterified with tartaric acid in the pulp and grape skins and may undergo fermentation reactions of conversion, isomerization, oxidation, reduction and hydrolysis that transform them into volatile phenols as (4-vinyl) and (4-ethyl) -phenols and ferulic acid to (4-vinyl) and (4-ethyl) -guaiacols. Some of the esters of phenolic acids are presented as glycosidic combinations (Voirin et al., 1990). In fact, volatile phenols such as vanillin, propiovanillone, homovanilic alcohol, methyl vanillate can all be hypothesized to be derived from ferulic acid and have been identified in hydrolyzed grape glycoside extracts of various varieties (Voirin et al., 1990). All these compounds have a vanilla smell and therefore influence the aroma of the wine (Bayonove et al., 2000).

Bound terpenic alcohols

Monoterpenic compounds are generally present in grape skins as glycosidic combinations in greater proportion than free forms. The monoterpenols are sensitive to the hydration and oxidation reactions that occur during winemaking and cause the transformation of one into another, as in the case of linalool that isomerizes to nerol, geraniol, α-terpineol (Ribéreau-Gayon et al., 2006). There is an extensive bibliography about the terpene glycosides, which are recently compilated in an excellent review by González-Barreiro et al. (2015).

Carotenoids

Lutein and β-carotene, followed by neoxanthin, flavoxanthin and violaxanthin (most abundant) are located in the cells of the grape pulp and skins (Franke et al., 1994). The rupture of carotenoids by light and oxidases results in smaller molecules of 9, 10, 11 and 13 carbon atoms, more volatile and odorous.

Glycosidic bound precursors

The generic name of glycosides are attributed to the combinations of sugars with other molecules by oxygen bridges. Combinations of odorous molecules such as mono-terpenes and nor-isoprenoids with various sugars that have no odor have been identified but are a potential source of aroma, since their odorous part can be released during the winemaking process.

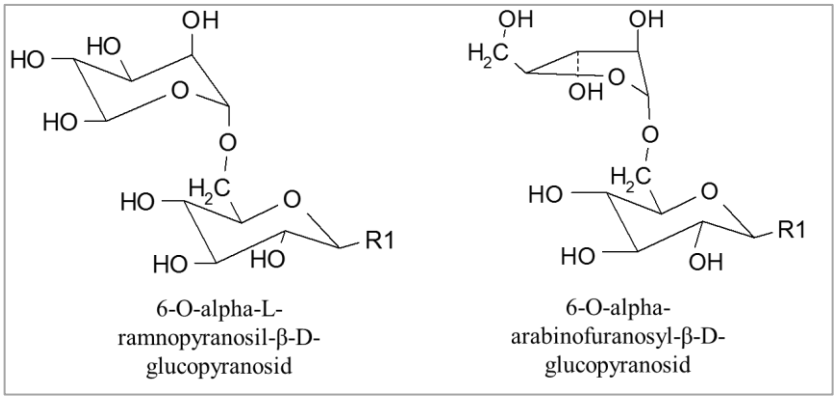


Figure 11. Glycosidic combinations of monoterpenes or nor-isoprenoids (R1).

The non-sugar (or aglycone) part is responsible for the odor and is generally constituted by monoterpenols, C13 nor-isoprenoids, volatile phenols, C6 alcohols, benzyl alcohol, 2-phenylethanol and some other volatile compounds (Cox et al., 2005; Winterhalter and Skouroumounis, 1997).

The hydrolysis of the glicosidic combination is produced by enzymes beta-glycosidases or chemically and recently, Tress et al., (2006) have isolated some yeast that are capable of hydrolyzing up to 40% of the beta-glycosidic combinations of Moscatel grapes.

ii) Pre-fermentative aromas

The cellular structure of the grapes is broken during the operations prior to the alcoholic fermentation of the must, mainly during the harvest and transport to the winery, destemming, crushing, pressing and maceration in the presence of solid parts. Consequently, the cellular enzymes

are released and brought into contact with their substrates, causing reactions giving rise to specific aroma compounds. The best known is the formation of C6 aldehydes and alcohols, all them with herbaceous odors and formed from polyunsaturated fatty acids with 18 carbon atoms by the action of lipoxygenase (LPO) enzymes and oxygen (Figure 12).

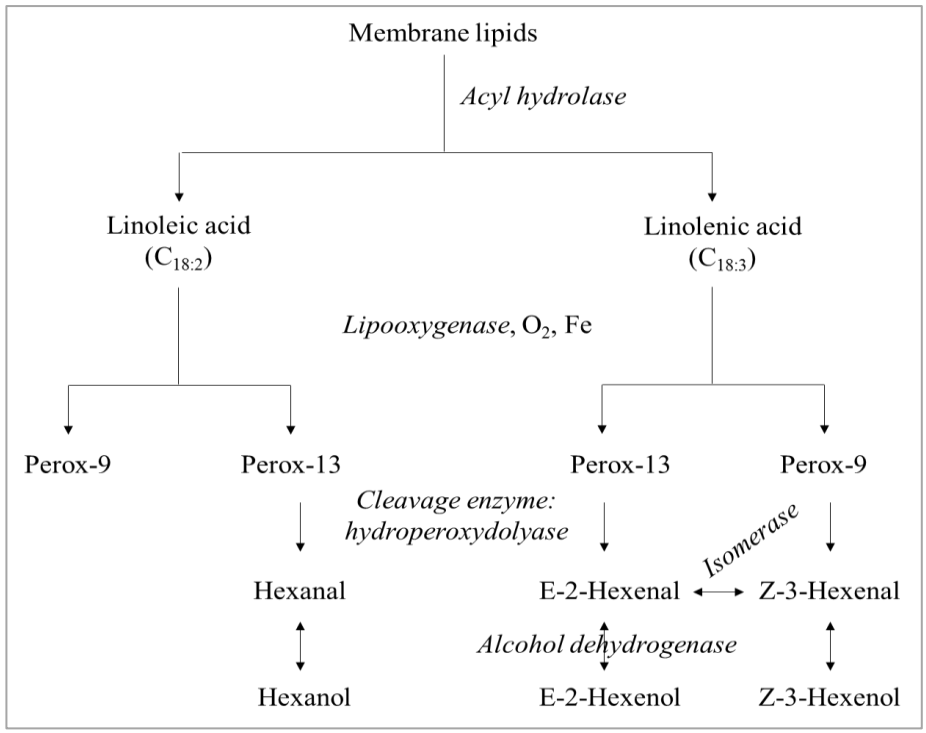


Figure 12. Enzymatic formation of aldehydes and C6 alcohols.

iii) Fermentative aromas

The aromas of fermentation are formed by the yeasts during the transformation of the grape must into wine and some other microorganism as are the lactic acid bacteria that grow later contribute to the wine aroma (Velázquez, 2015). The alcoholic fermentation results mainly in the production of ethanol, carbon dioxide and glycerin and other secondary compounds influencing the aroma of wine (Figure 13). These compounds are grouped into different chemical families: alcohols, acids, esters, carbonyl compounds, sulfur compounds and volatile phenols, among others.

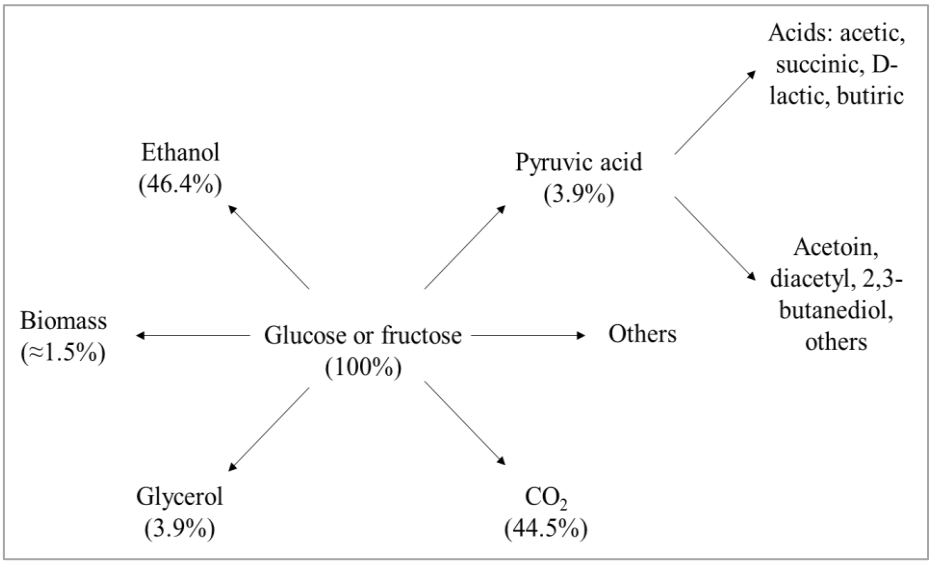


Figure 13. Alcoholic fermentation and yeast flavoring.

Higher alcohols

These alcohols are quantitavely the most abundant volatile compounds produced during yeast fermentation and contribute to essential aroma and flavors in fermented beverages and food (Hazelwood et al. 2008). This chemical family includes two types: the major higher alcohols, formed by those with concentrations higher than 10 mg/L and the minor higher alcohols, which have contents below this value.

Propanol, isobutanol (2-methyl-1-propanol), iso-amylic alcohols (2 and 3-methyl-1-butanol) and 2-phenylethanol, are the major higher alcohols in wines and 1-butanol, 3- and 4-metil-1-pentanol, furfuryl alcohol, benzyl alcohol and tyrosol are some of the minor higher alcohols. Higher alcohols are formed through the Ehrlich pathway or via central carbon metabolism from the corresponding α-keto acid and aldehyde, which is reduced by an alcohol dehydrogenase NADH-dependent to the alcohol (Hazelwood et al. 2008).

Fusel alcohols can have positive or negative sensory impacts, depending on their concentration. Fusel alcohol concentrations are yeast dependent (Moreno et al., 1991; Swiegers and Pretorius 2005). Propanol, butanol, and isobutanol have an alcoholic odor, while the isoamyl alcohols have a marzipan-like or banana aroma (Lambrechts and Pretorius 2000). Tyrosol and 2-phenylethanol impart honey-like and rose floral aromas, respectively (Lambrechts and Pretorius, 2000).

Acetates and ethyl esters

Volatile esters are the most important set of yeast-derived aroma-active compounds. Acetates and ethyl esters are responsible of highly desired fruity, candy and perfume-like aroma character of beer, wine and sake (Saerens et al., 2010). They are formed by enzymatic or chemical reactions, which occurr between the alcohols and acids. Their formation requires an alcohol molecule, acetyl-CoA and ATP. Both the production and degradation of esters are tightly regulated. The two main ester types produced during fermentation are the esters of acetic acid with the higher alcohols and the esters of ethanol with other organic acids.

The acetate esters include ethyl acetate, 2-methylbutyl and 3-methylbutyl acetates and phenylethyl acetate, having pineapple, banana, and rose-like odor respectively. Ethyl acetate is the most abundant acetate ester in alcoholic beverages. Levels higher than 170 mg/L in white wines and 160 mg/L in red wines, are considered unacceptables because they provide nail polish, varnish and chemical solvent odorous notes.

The synthesis of acetate esters is controlled by two alcohol acetyl transferases (AATase), Atf1p and Atf2p (Yoshimoto et al., 1998; Verstrepen et al., 2003) and is also substrate dependent, relying on the availability of fusel alcohols (Lilly et al., 2006).

The esterification reaction of ethanol with the medium chain fatty acids (MCFA) leads to the formation of the ethyl esters, which have sensorial attributes remembering the fruity descriptors such as apple, strawberry and pear (Saerens et al., 2010). Some polar ethyl esters of short chains fatty acids are responsibles for giving the wine body and consistency.

The fatty acid intermediates are released from the cytoplasmic fatty acid synthase (FAS) complex, influencing the ester synthesis (Plata et al., 1998; 2003; Taylor and Kirsop, 1977). The MCFAs are activated by coenzyme A and esterified with the ethanol by intermediation of esterases and ATP. There are three pathways responsible for the release of MCFAs and subsequent production of ethyl esters, which were described by Saerens et al., 2006 and Dufour et al., 2008 and according with Hirst and Richter (2016), several genes are involved in the esters formation and hydrolysis.

Carbonyl compounds

The main source of the carbonyl compounds (aldehydes and ketones) is the metabolism of sugars and α-keto acids in the synthesis of amino acids and higher alcohols by yeasts (Lambrechts and Pretorius, 2000), being reduced to the corresponding alcohols by action of dehydrogenases and reductases (Perestrelo et al., 2006; Ugliano and Henschke, 2009). Only a few carbonyl compounds (acetaldehyde, diacetyl, benzaldehyde, 2-furfural and 5-hydroxymethyl-2-furfural) contribute to the aroma of wines because of their low sensorial perception threshold.

Acetaldehyde is the precursor of ethanol and acetate and its contents in wine depend on the yeast strain that predominates during alcoholic fermentation (Romano et al., 1994). Their perception threshold is 10 mg/L and in young wines, it accounts for 90% of the total aldehyde concentration (approximately 200 mg/L). At low concentrations, it gives pleasant fresh fruit aromas to green apple and at high concentrations turn into herbaceous nuances, rotten apple and chemical solvent, which are very penetrating (Styger et al.,

2011). Medium chain saturated aldehydes (C3-C9) provide herbaceous, green, fruity and spicy aromas (Ebeler and Sapulding, 1998).

Diacetyl is a well-known odorant in food industries, it has a toasty, butterscotch, and nutty aroma descriptor, an odor threshold of 8 mg/L and smells like rancid butter at high concentrations (Swiegers and Pretorius 2005). Yeasts synthesize diacetyl during fermentation, but in red wines, diacetyl is predominantly produced by lactic acid bacteria (Laurent et al., 1994). According Suomalainen and Ronkainen (1968), diacetyl is formed by extracellular decarboxylation of α-acetolactate and converted to acetoin and 2,3-butanediol, which have a higher odor threshold (Swiegers and Pretorius, 2005).

Acetoin is a ketone whith a hydroxyl group in its molecular structure (3-hydroxybutanone or acetylmethyl-carbinol). It is a key compound in the biosynthesis of 2,3-butanediol and diacetyl. Generally, the wine contents do not exceed their threshold (150 mg/L, according Ferreira, 2005), giving rise to debate on its contribution to the dairy aroma of wine. Yeasts synthesize acetoin from pyruvate, by condensation of acetaldehyde with acetyl-CoA to form diacetyl and reduction to acetoin and finally, by condensation of two acetaldehyde molecules. Yeasts of the genera *Kloeckera* and *Hanseniaspora*, are the major producers. In contrast, *S. cerevisiae* produces low levels of acetoin and metabolize those formed by other yeasts during wine fermentations (Romano and Suzzi, 1996).

Benzaldehyde has a characteristic odor of bitter almonds and is important because of its negative impact on white wine odor, where it has a perception threshold of 3-3.5 mg/L (Delfini, 1987). This odor disappears when the benzaldehyde is oxidized to benzoic acid or reduced to benzyl alcohol by yeast. Finally, the aldehydes derived from furfural come from the dehydration and cyclization of carbohydrates

and usually come from the roasted staves inside the barrels, along the wine aging.

Other compounds formed by condensation reactions of acetaldehyde or other aldehydes with the major alcohols of the wine, are hemiacetals and acetals. Acetals derived from acetaldehyde are notable for their contribution to aroma in Sherry wines, 1,1-diethoxyethane (or diethyl acetal) reaching concentrations close to 150 mg/L and 1,1-di (3-methylbutoxy) ethane, which has contents close to 6 mg/L (Etiévant, 1991).

Lactones

These compounds are cyclic esters of the 4- or 5-hydroxycarboxylic acids. The intramolecular esterification takes place spontaneously when the ring that is formed is five- or six-membered. In this way, the 4-hydroxyacids give rise to γ-lactones, which are more abundant (Etievant, 1991) and the 5-hydroxyacids give the δ-lactones. Two groups of lactones have been established: a group consisting of γ-butyrolactones substituted or not substituted in 4-position and a second group consisting of the alkyl-γ-lactones (γ-C5 to C10 and C12). The γ-butyrolactones of the first group have as precursors 2-oxoglutaric and 4-oxobutanoic acids (Fagan et al., 1981). Pantolactone and sotolones are two important lactones of the second group. Pantolactone is a byproduct of pantothenic acid metabolism formed by the enzymatic reduction of 2-ketopantoyl lactone (Hata et al., 1987) and sotolone has a curry odor and a low perception threshold and increase their contents during wine aging (Sponholz, 1993). As for 4-alkyl-γ-lactones and 5-alkyl-γ-lactones, they are secondary products of glucose metabolism by yeast, via levulinic acid for γ-pentalactone (Tressl et al., 1978), or from the fatty acid metabolism (Dufossé et al., 1994). Quantitatively, the three most abundant lactones in the wine are γ-butyrolactone (caramel odor) 4-carbethoxy-γ-butyrolactone and 4-ethoxy-γ-butyrolactone, whose concentrations range in the order of mg/L, while the others lactones show concentration values of concentrations in the order of μg/L.

Other lactones contribute significantly to the flavor of fruits and fermented beverages and are used as flavorants. Some examples are γ-decalactone, (peach flavor); δ-decalactone and γ-dodecalactone, which have a creamy coconut/peach flavor, a description which also fits γ-octalactone (4-octanolide), although it also contains herbaceous character and lastly γ-nonalactone, which has an intense coconut flavor (Berger, 2007).

Sulfur compounds

According to Rauhut, (1993), there are five categories of sulfur-containing compounds described in wines: sulfides, polysulfides, thiols, thioesters, and heterocyclic compounds. These compounds have a very low odorant threshold and are described as having a cabbage, onion or rotten eggs aroma. Some of them originate from sulfur-containing fungicides or from the degradation of sulfur-containing amino acids during fermentation; however, the pathways responsible for these processes have not been fully elucidated.

Hydrogen sulfide (H_2S) contributes to one of the primary sulfur off-notes; it is produced during fermentation by the sulfate-reduction sequence pathway (Spiropoulos and Bisson 2000). Sulfide combines with O-acetyl-serine or O-acetyl-homoserine to form homocysteine (Swiegers and Pretorius 2005). Under methionine and cysteine limited conditions, O-acetylserine and O-acetylhomoserine are also limited, resulting in excess sulfide that is converted to H_2S (Thomas and Surdin-Kerjan 1997). Hydrogen sulfide is very reactive and is being responsible of additional off-notes in wine as those derived from ethanethiol, which is formed when H_2S reacts with ethanol (Spiropoulos and Bisson 2000).

The most odorant thiols are 4MMP, 3MH, and 3MHA.The pre-fermentative aromas (E)-2-hexenal and its corresponding alcohol, (E)-2-hexen-1-ol, are precursors to the formation of 3MH, enhancing the 3MH and 3MHA in the presence of a sulfur donor (Harsch et al., 2013).

The most odorant thiols are 4MMP, 3MH, and 3MHA.The pre-fermentative aromas (E)-2-hexenal and its corresponding alcohol, (E)-2-hexen-1-ol, are precursors to the formation of 3MH, enhancing the 3MH and 3MHA in the presence of a sulfur donor (Harsch et al., 2013).

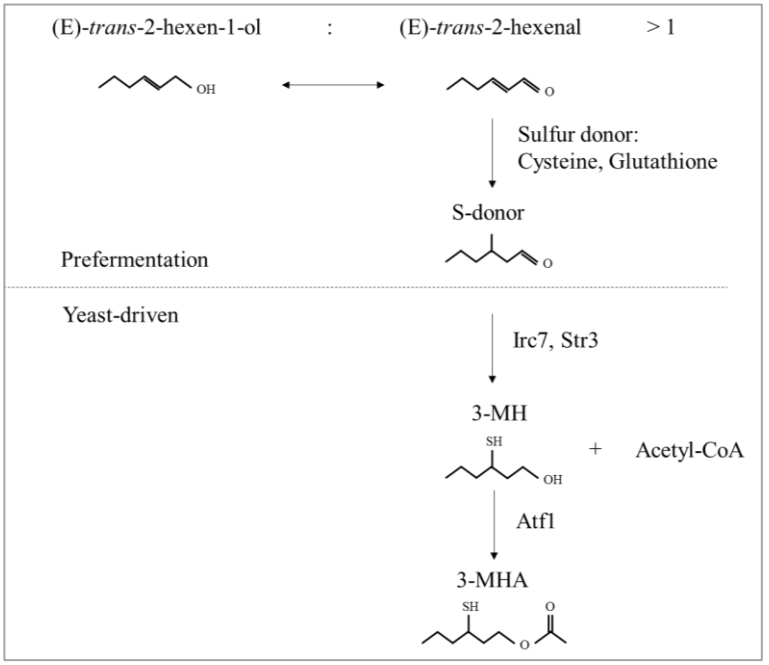


Figure 14. Thiols released in wines.

In contrast to other sulfur compounds, these volatile thiols have aromas described as boxwood, passion fruit, black currant, or grapefruit. These compounds are S-cysteine- or glutathione-bound in grapes (Figure 14) (Tominaga et al. 1998).

Volatile phenols

Volatile phenols are important for the taste, color and odor of wines. The most important in white wines are the vinylphenols and in red wines the ethylphenols (Chatonnet et al., 1997). Their presence in wines is due to the ability of some *S. cerevisiae* yeasts to produce 4-vinylphenols by enzymatic decarboxylation of the corresponding cinnamic acids (Figure

15). These compounds have odors and tastes very unpleasant, remembering to horse stable and sweat and higher contents have its origin in the action of yeasts *Brettanomyces bruxellensis* or *Dekkera bruxellensis* (Du Toit et al. 2005; Smith and Divol, 2016; Schifferdecker et al., 2014).

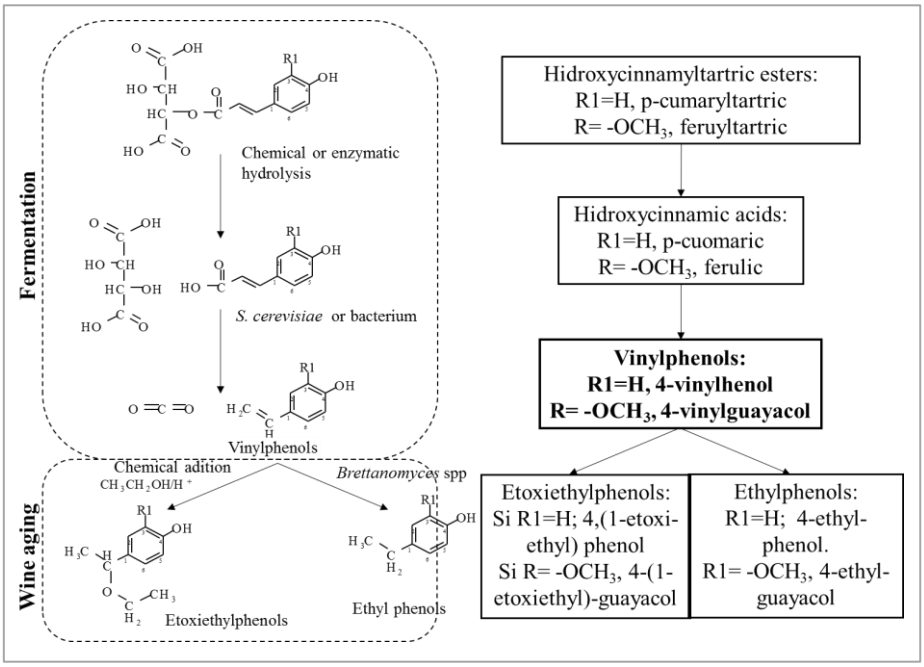


Figure 15. Formation of ethyl and vinylphenols by yeasts.

iv) Post-fermentative aromas (aged wine aroma)

Once grape-must has been fermented, some wines are subjected to special aging processes, aimed to generate specific changes in their composition in order to obtain other sensorial properties over time. In some cases, the wines are deposited in inert tank (glass or stainless steel), which do not contribute to change the composition of the wine and do not participate in the chemical reactions that occur in the wine matrix and in other cases, wines are deposited in wooden vessels, that are active from this point of view. This way, it can be distinguish among two aging models, according to the used vessels.

In the case of wood barrel aging, the physical and chemical reactions which have occurred in the wine matrix are influenced by components derived from the active container and the presence of the oxygen. On the other hand, the bottle aging can be defined as a process in which the natural physical and chemical properties of wine change over time in inert containers and in absence of the oxygen from the atmosphere.

Oxidation phenomena occur in porous containers such as wood, which permit the interchanges with the atmosphere, whereas reduction occurs in containers made of glass or stainless steel. These two types of aging are known as chemical aging, since they only involve chemical reactions and are not dependent on microorganisms. Additionally, another type of wine aging exist, which is named as biological aging and involve the use of oak casks and specific *Saccharomyces* yeast strains, called flor velum yeasts. The following revision is focussed to this special winemaking process.

Biological aging

After the alcoholic fermentation of the musts from some white grape varieties, the wines are racked off their lees and stored in inert vats made of cement or stainless steel until it is ready to be transferred to a dinamic or static system for biological aging. During this time, the wine undergoes malolactic fermentation and often develops a thin biofilm of flor yeasts on its surface. This wine is known by the term “sobretablas”.

The traditional system of biological aging consist in a dinamic system called as "criaderas" and "solera" that starts when the sobretablas wine is transferred to used American oak casks. These casks are filled to approximately four-fifths or five-sixths of their capacity to allow room for the flor yeast to grow on the surface of the wine. The barrels are then stacked on top of each other in three or four numbered rows known as criaderas. Each row contain wine of the same type and aging

stage. The bottommost row is called the solera (derived from the word suelo, in Spanish or floor in English language) and contains the oldest wine. No more than 40% of the content of each barrel on this row can be bottled per year.This wine is normally taken out three or four times a year and is replaced by an identical volume of wine from the row above, known as the first criadera. The barrels in the first criadera, in turn, are replenished with wine from the second criadera, and so on up to the topmost criadera, which contains the youngest wine. Finally, this criadera is refilled with the sobretablas wine.

The system described above ensures that the solera wine is a complex, uniform blend of wines from different years and means that the bottled products all have similar organoleptic properties, regardless of the harvest. The procedure also brings younger wine into contact with older wine, with the former providing the latter with the nutrients it needs to keep its flor active.

The changes that affect the composition of biologically aged wines are linked to the metabolism of flor yeasts, although they also undergoes changes common to all the wines long time stored. Several publications carried out along the last decades, about the flor yeast action on the composition of wine, have been summarized by Jackson (2008), Peinado and Mauricio (2009) and Moreno and Peinado (2012).

The main carbon sources used by flor yeasts are ethanol, glycerol and acetic acid. As nitrogen source, the amino acid L-proline is used. The ethanol, glycerol, and acetaldehyde contents are the compounds most changed by the metabolism of flor yeasts, but other compounds such as organic acids, nitrogen compounds, higher alcohols, esters, lactones, polyphenols, acetoin and diacetyl are also affected (Blandino et al., 1997; Mauricio and Ortega, 1993; Martınez et al., 1998; Charpentier et al., 2002). Yeast autolysis occurred during the biological aging time, results in the release of amino acids, peptides, nucleotides, mannoproteins, esters, alcohols, aldehydes, acids, and lactones (Villamiel et al.,

2008). These compounds make an important contribution to the organoleptic properties of the biologically aged wines.

Ethanol will be diminished in content because its evaporation and conversion to compounds such as acetaldehyde, acetic acid, butanediol, diacetyl, and acetoin (Figure 16). The remainder is metabolized by the tricarboxylic acids pathway and incorporated into cell material in the form of carbohydrates, fatty acids and proteins (Suárez-Lepe and Iñigo-Leal, 2004). The result is a decrease in ethanol content that is dependent on the dominant yeast strain in the velum, the yeast growth phase and the time that wine is subjected to the criaderas and soleras system.

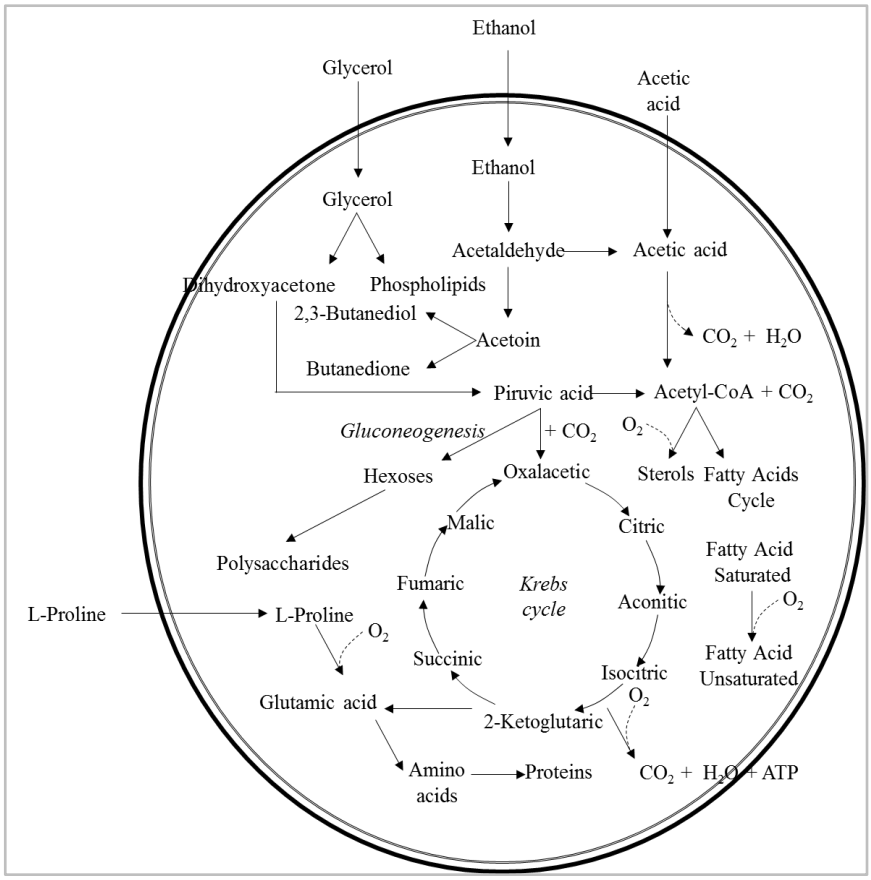


Figure 16. Metabolism of carbon and nitrogen sources by flor yeast.

More ethanol is consumed in the flor-formation phase than in the maintenance phase. By the end of biological aging,

up to 1% (v/v) of ethanol may be lost. At this respect, Cortés (2002), showed an ethanol consumption near to 4% (v/v) in laboratory experiments, carried out with *S. cerevisiae* yeasts in glass vessels.

Glycerol is the third component in abundance of the wines. The flor yeast grow in stress conditions imposed by high ethanol content, low pH values and nutrient depletion and furthermore, use glycerol as a carbon source. This compound is considered as critical stress protectant for yeasts (Shima and Takagi, 2009). The glycerol contents in wines decrease with the biological aging time, showing amount rarely exceeding 1 g/L in wines aged for 4 or 6 years.

Cellular macromolecules, including proteins, nucleic acids and membranes, are seriously damaged under the stress conditions, leading to the inhibition of cell growth and cell viability (Hallsworth, 1998). To avoid lethal damages, yeast cells acquire a variety of stress-tolerant mechanisms and according Matallana and Aranda (2016), all the metabolic pathways involved in the yeasts response to environmental changes work in a coordinated fashion. Stress response genes are usually regulated by more than one of the transcription factors, which causes the called cross-protection, i.e., a previous exposure to one type of stress protects against another type of environmental condition (Gasch et al., 2000; Causton et al., 2001).

Acetaldehyde is the more important compound produced by yeast during the biological aging, reach levels between 350 and 450 mg/L and occasionally about 1000 mg/L at the end of the process. It has a major impact on organoleptic properties and is responsible for the intense aroma that characterizes the special wines aged using this method. Acetaldehyde is mostly formed by enzymatic oxidation of ethanol throught alcohol dehydrogenase II (*ADH2*) with the formation of NADH. This isoenzyme is repressed by glucose and it plays an active role in the flor yeasts.

The most marked changes in acetaldehyde contents occurs when the yeast metabolism is more intense (i.e., in the barrels containing the youngest wines (Peinado and Mauricio, 2009).

The acetaldehyde participate in numerous chemical reactions (Figure 17), highlighting its reaction with the sulphurous acid to form the bounded sulphur dioxide and their reaction with ethanol to form the diethyl acetal (1,1-diethoxyethane). The levels of this acetal are related to ethanol and acetaldehyde contents and can exceed 100 mg/L in wines aged for over 5 years (Muñoz et al., 2005). It is the only acetal that contributes to the wine aroma with nutty, licorice aromas (Etiévant, 1991).

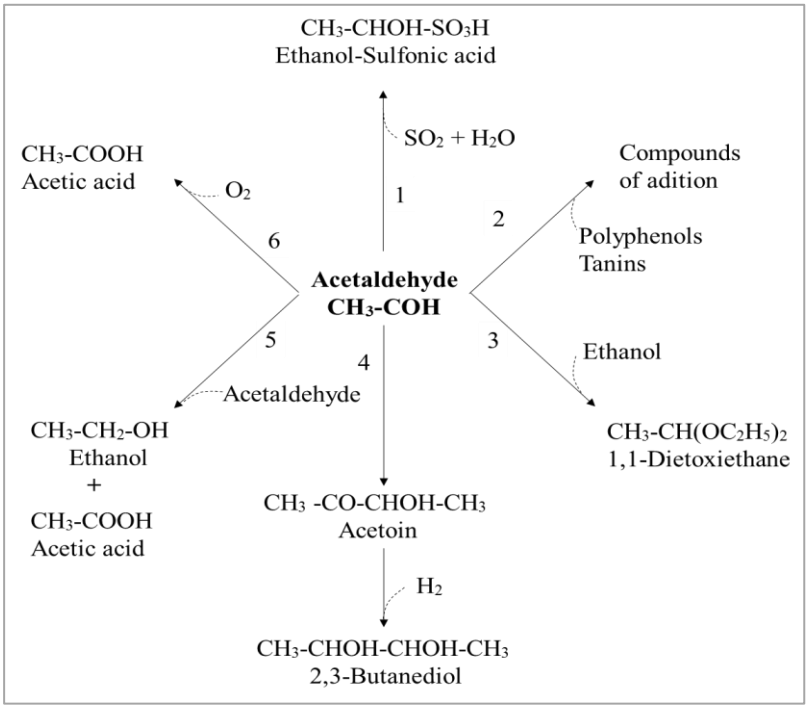


Figure 17. Reactions of acetaldehyde during the biological aging.

Acetaldehyde is involved in the formation of acetoine and 2,3-butanediol, throught the yeast metabolism (Romano and Suzzi, 1996), and takes part in the synthesis of sotolon

(Guichard et al., 1997; Pham et al., 1995). This molecule confers to sherry wines a strong spicy-curry-nut notes (Dubois et al., 1976; Martin et al., 1992). In other reactions with some polyphenols such as tannins and procyanidins, it acts as a "bridging molecule" (Haslam and Lilley, 1988). Lastly, acetaldehyde can be oxided to acetic acid by the oxygen action or also two molecules of acetaldehyde can undergo a self oxidation-reduction reaction to form acetic acid and ethanol.

Amino acids, ammonium ion and urea are the main sources of nitrogen for yeasts and their contents in the wine are strongly affected during biological aging. In this respect flor yeasts use L-proline as a nitrogen source and synthesize all other amino acids required for its growth (Peinado and Mauricio, 2009; Martinez et al., 1995). Other aminoacids as threonine, methionine, cysteine, tryptophan, and proline can be released to the wine to restore the intracellular redox balance by means of the oxidation of NADH in excess (Berlanga et al., 2006; Mauricio et al., 2001; Valero et al., 2003).

Regarding the effect of biological aging on the content of the acids, the malolactic fermentation is usually accomplished in the young wines, and as a result the wine built-in to the aging system, do not contain malic acid. The decrease in tartaric acid is a result of the precipitation of their salts potassium bitrartrate and calcium tartrate during the aging time. Other acids as the gluconic acid proceed from the rotten grapes and the contents below 1 g/L are allowed for biological aging. It is demonstrated by Peinado et al. (2003; 2006) that flor yeasts metabolize this acid during the biological aging without altering the quality of the final wine.

Acetic acid concentration in white wines rarely exceeding 0.7 g/L after the fermentation process and it is recommended contents below 0.5 g/L for the wines subjected to the biological aging. Flor yeasts metabolize this acid via acetyl-CoA and incorporation into the Krebs cycle and fatty acid synthesis during the biological aging process. Short chain

organic acids as butanoic, isobutanoic and 2- and 3-methylbutanoic are, result from the metabolism of flor yeasts and increase their contents with the aging time. Medium-chain fatty acids (hexanoic, octanoic and decanoic) exhibit the opposite behaviour and can even disappear from the medium after a prolonged aging time (Cortés, 2002).

The higher alcohols show different changes in their contents during the biological aging. In this way, isobutanol, 2-phenylethanol and isoamyl alcohols (2-methyl-1-butanol and 3-methyl-butanol) show few changes with time, nevertheless, the propanol content can be doubled during this process. Flor yeasts show a important activity in view these compounds, preferentially along the fourth, third and second criaderas (Peinado and Mauricio, 2009). The yeast autolysis has also a strong effect on the contents in higher alcohols, as shown by the presence of propanol, isobutanol and isoamyl alcohols in yeast extracts from wines under biological aging (Muñoz et al., 2001).

Ester concentrations change by effect of the hydrolysis and esterification reactions that occurs along the aging time at the acid pH value of the wine and also as a result of the enzymatic activity of the flor yeasts. This last effect is related with the specific yeast strain growing in the medium and with their physiological status (Mauricio et al., 1993; Plata et al., 1998). In general, the concentration of acetates of higher alcohols in wines subjected to biological aging, decreases along the first few months and the ethyl esters of organic acids (particularly lactic and succinic) increase with time (Peinado and Mauricio, 2009).

The lactones of wines α-butyrolactone and pantolactone come from the fermentative metabolism of yeasts. They are described also as typical compounds in the wines biological aged and are associated with specific flor yeast strains (Zea et al., 1995) and aging time (Cortes et al., 1998). Other lactones, as solerone (4-acetyl-γ-butyrolactone) and sotolon (3-hydroxy-4,5-dimethylfuranone) contribute to the aroma of the

oldest wines subjected to the biological aging. Solerone is an important contributor to their aroma, however, studies of Martin and Etiévant, (1991), show that this lactone has no impact on wine aroma. Sotolon is also detected in Jerez, Jura and some botrytized wines (Dubois et al., 1976). It possesses a strong, typical nutty and curry odour and a very low perception threshold of 10 μg/L (Da Silva Ferreira et al., 2002). The flor yeasts form this lactone via the aldolization reaction of α-ketobutyric acid, with acetaldehyde (Guichard et al., 1997; Pham et al., 1995). Its content depends on the aging time and reach levels about 200 μg/L in very old aged wines (Moreno et al., 2004). The criaderas and solera system supporting the biological aging process use very old oak casks, that are rarely emptied and washed. As a result, only the oldest wines contain low amounts of lactones, which are extracted from the wood, such as is the β-methyl-γ-octalactone (Chatonnet et al., 1990).

Collin et al. (2011) identified other new compounds in Jura flor Sherry wine, abhexon that confers sweet-caramel note of coffee beverages and might come from the reaction between α-ketobutyric acid and propanal. Dihydrodehydro-β-ionone and 4-hydroxy-7,8-dihydro-β-ionone are thought to be generated from theaspirane oxidation.

Lastly, flor yeast consumes the oxygen solved in the wine and the biofilm formed on the wine surface protects it from of the contact with the air of the atmosphere. As a result, the browning reactions due to the oxidation of polyphenol compounds are very limited and the wines remain with a pale yellow colour, when biological aging is carried out according to good cellar practices (Barón et al., 1997; Merida et al., 2005).

Applications based on Gas Chromatography–Mass Spectrometry of metabolomics have been used in different fields of research, such as medical sciences, plant physiology (Humston et al., 2010; Schmarr et al., 2010) and microbiology (Tian et al., 2008). Identification of peaks through database of

mass spectra is simple due to the reproducible and extensive fragmentation patterns obtained in full-scan mode (Koek et al., 2011). Nevertheless, it is possible to elute peaks that give more complex electron ionisation (EI) mass spectra by the coelution of compounds, requering the use of specialised GC–MS deconvolution-reporting software (DRS). Deconvolution is a mathematical method that "separates" overlapping mass spectra into "cleaned" spectra of the individual peaks (Meng et al., 2011). GC–MS coupled with deconvolution software has been applied to identify a wide range of chemical compounds in wine (Villas-Boas et al., 2005), including amino acids, organic acids, nucleic acids, sugars (Ruiz-Matute et al., 2011), amines and alcohols (Fiehn et al., 2000; Halket et al., 2005; Matsumoto et al., 1996; Skogerson et al., 2009).

Wishart et al. (2009) describes some of the newly emerging computational ways in metabolomics that are being used to identify metabolites with MS technology from different matrices. The most successful compound-identification strategies typically involve deconvoluting and matching the spectral features of the unknown compounds with reference compounds from a homemade database (Fiehn et al., 2011). This technique is known as the identification of "known unknowns" (Bowen et al., 2010; Hiller et al., 2009).

Several techniques have been employed to ensure the full characterisation of the volatile profiles of grapes and wines: dynamic headspace sampling (Rosillo et al., 1999), liquid extraction with organic solvents (Perestrelo et al., 2006; Recamales et al., 2011; Cadahia et al., 2009; Cortes and Diaz, 2011), solid-phase microextraction (Dominguez et al., 2010; Sun et al., 2012; Welke et al., 2012), solvent-assisted flavour evaporation (Mo et al., 2010; Tomiyama et al., 2011) or solid-phase extraction (Lopez et al., 2011; Weldegergis et al., 2011). Recently, Stir Bar Sorptive Extraction (SBSE) has been applied to aqueous matrices (Sampedro et al., 2009) and wine analysis (Hayasaka et al., 2003; Castro et al., 2008).The SBSE technique used for analyte extraction by direct

inmersion has been used by several authors for the analysis of higher alcohols (Sanchez Rojas, 2009), off-flavours (Franc et al., 2009), volatile constituents (Caven-Quantrill et al., 2011; Pedroza et al., 2010; Coelho et al., 2009), and esters. Other studies identified the presence of volatile compounds in wines (Weldegergis et al., 2007) and wine vinegars (Callejon et al., 2008) using the headspace technique with thermal desorption in GC–MS (Zalacain, et al., 2007).

The combination of powerful and robust analytical techniques provides a multi-dimensional data matrix, which require advanced chemometric tools. In this sense, metabolomics studies applied to wine sciences offer the analysis of as many metabolites as possible to carry out discrimination and/or classification studies according to the factors influencing their composition.

2. HYPOTHESES AND AIMS

2. HYPOTHESES AND AIMS

Currently, the –omic sciences are applied to a wide variety of areas of knowledge being Food Sciences one of them. Particularly, in the Oenology field, there is a tendency to establish relationships between the composition in metabolites of wines and the proteomic/transcriptomic /genetic information of life organisms involved in the wine-making process, like vines, yeasts and bacteria. Special interest in wine science and technology, are the establishment of the relationship between the response of the cellular proteome to an external condition with those exo-metabolites responsible of the organoleptics propierties, as an useful method to ensure the authenticity, traceability, origin and in general, for the quality assurance of this product. For these reasons, the application of –omic technologies in the study of the non-*Saccharomyces* and the *Saccharomyces cerevisiae* flor yeasts, can be interesting for the differents sectors involved in the wine industries.

With regards to the yeast-filamentous fungus co-immobilization technology, it was observed that flor yeasts among other strains, form biocapsules with high consistency and mechanical resistance. These yeasts have a high capacity to form biofilm aggregates (i.e. flor velum) at liquid-air interfaces under certain conditions and form an extracellular matrix among cells also revealed when forming biocapsules. Thus, it could be interesting to study which factors of flor yeast can positively affect the co-adhesion with the filamentous fungus to form the yeast biocapsules.

2.1. Starting hypotheses

The intracellular proteins expressed and the metabolites excreted are related with the yeast strain used and the physical and chemical conditions of the medium where they grow. Therefore, it is possible to know the response of different yeasts to different stress conditions using analytical methods for the determination of key exometabolites and proteins.

Moreover, it is hypothized that certain properties related to the flor velum formation influence the biocapsule formation and their characteristics.

2.2. Generic and specific aims:

1. To identify the metabolic and proteomic responses of non-*Saccharomyces* and *Saccharomyces* yeasts, to several stress conditions of enological interest.

1.1. To establish the profile of exo-metabolites of a *Torulaspora delbrueckii*, a non-*Saccharomyces* yeast, growing in a synthetic medium. Treated in the article to be published, shown in appendix 1.

1.2. To identify the influence of media with high sugar contents and media with high ethanol and glycerol contents on the proteins expressed and the small metabolites excreted by a *S. cerevisiare* flor velum-forming yeast strain. Treated in published articles shown in appendices 2 to 8 and in non-published article in appendix 9.

2. To study flor yeast applications: improvement of the sensorial quality of wines and the alcoholic fermentation processes to obtain new products.

2.1. To use the flor yeast velum for modulating colour, ethanol and major aroma compound contents in red wine. Treated in published article, shown in appendix 10.

2.2. To study the factors affecting to the co-adhesion phenomena between yeasts and a filamentous fungus in the formation of biocapsules immobilization system. Treated in appendix 11, article to be published.

The following publications should be included after each aim, as follows:

Objetivo 1.1 / Aim 1.1: This aim is addressed in:

Moreno-García et al. (2017). Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts. To be published in Q1 journal in Food Science and Technology. (Appendix 1)

Objetivo 1.2 / Aim 1.2: Addressed in:

Moreno-García, et al. (2013). *Food Res. Int.* 54, 285–292. DOI:10.1016/j.foodres.2013.07.031. (Appendix 2)
Moreno-García, et al. (2014). *Int. J. Food Microbiol.* 172, 21-29. DOI: 10.1016/j.ijfoodmicro.2013.11.030. (Appendix 3)
Moreno-García, et al. (2015). *Food Microbiol.* 46, 25-33. DOI: 10.1016/j.fm.2014.07.001. (Appendix 4)

Moreno-Garcia, et al. (2015). *Food Microbiol.* 51, 1-9. DOI: 10.1016/j.fm.2015.04.005. (Appendix 5)
Moreno-García, J., et al. (2016). *Process Biochem.* 51, 578–588. DOI: 10.1016/j.procbio.2016.02.011. (Appendix 6)
Moreno-García, J., et al. (2016). *Data Brief.* 7, 1021–1023. DOI: 10.1016/j.dib.2016.03.072. (Appendix 7)
Moreno-García, et al. (2017). *Int. J. Mol. Sci.* 720, 1-18. DOI: 10.3390/ijms18040720. (Appendix 8)
Moreno-García et al. (2017). Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain. To be published in Q1 journal in Food Science and Technology. (Appendix 9)

Objetivo 2.1 / **Aim 2.1**: Addressed in:

Moreno, J. et al. (2016). *Food Chem.* 213, 90-97. DOI: 10.1016/j.foodchem.2016.06.062. (Appendix 10)

Objetivo 2.2 / **Aim 2.2**: Addressed in:

Moreno-García et al. (2017). Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system. To be published in Q1 journal in Food Science and Technology. (Appendix 11)

3. MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Microorganisms and experimental conditions

Yeast and fungus strains from the Department of Microbiology, University of Cordoba (UCO), the Department of Agriculture of Università degli Studi di Sassari (UNISS) and the Department of Viticulture and Enology, University of California, Davis (UCD) collection, were used in this work (Table 2).

Table 2. Yeast and fungus strains used in the experiments. Number in the "experiment" column corresponds to the different aims of the thesis.

Strain	Strain details	Collection	Experiment
ATTC-MYA-426	*Torulaspora delbrueckii*	UCO	1.1.
G1 (ATTC-MYA-2451)	*Saccharomyces cerevisiae* (Sherry wine (Spain))	UCO	1.1., 1.2., 2.1., 2.2.
P3-D5 MAT α	*Saccharomyces cerevisiae* (Jura, France)	UNISS	1.2.
932	*Saccharomyces cerevisiae*	UCD	2.2.
77	*Saccharomyces cerevisiae* (wine, champagne)	UCD	2.2.
519	*Saccharomyces cerevisiae* (Sherry wine)	UCD	2.2.
580	*Saccharomyces cerevisiae* (Sherry wine)	UCD	2.2.
595	*Saccharomyces cerevisiae* (commercial dry wine yeast)	UCD	2.2.
661	*Saccharomyces cerevisiae* race *bayanus* (wine, champagne)	UCD	2.2.

Table 2. Continued.

Strain	Strain details	Collection	Experiment
662	*Saccharomyces cerevisiae* race *bayanus* (wine, champagne)	UCD	2.2.
726	*Saccharomyces cerevisiae* (wine)	UCD	2.2.
775	*Saccharomyces cerevisiae* (wine, Champagne)	UCD	2.2.
777	*Saccharomyces cerevisiae* race *bayanus* (commercial wine yeast)	UCD	2.2.
804	*Saccharomyces cerevisiae* (commercial wine yeast, champagne)	UCD	2.2.
814	*Saccharomyces cerevisiae* race *bayanus* (commercial wine yeast)	UCD	2.2.
854	*Saccharomyces cerevisiae* (Ale, England)	UCD	2.2.
1109	*Saccharomyces cerevisiae* (must)	UCD	2.2.
1162	*Saccharomyces cerevisiae* (unknown)	UCD	2.2.
2034	*Saccharomyces cerevisiae* (commercial yeast)	UCD	2.2.
2547	*Saccharomyces cerevisiae* (wine Spain)	UCD	2.2.
2865 B11 or BA11	*Saccharomyces cerevisiae*	UCD	2.2.
Original 594 (prise de mousse)	*Saccharomyces cerevisiae*	UCD	2.2.
H3	*Penicillium chrysogenum*	UCO	2.2.

All experiments were performed in triplicate and all material was sterilized by autoclave at 120 ºC for 20 min taking a special care with ethanol in media containing it to avoid its evaporation. Ethanol was added by filtration once the medium was autoclaved and cooled. The experiments were designed and performed using synthetic media or in wines

with a controlled composition, in order to have all the parameters monitored and standardized (Table 3).

For yeast INFORS AG (Switzerland) incubation shaker was utilized. The fermentations were monitored by measuring the amount of CO_2 released via the weight loss of the medium. Yeast population sizes were determined in a Beckman Coulter particle counter Z2 (U.S.A.) previously performing the appropriate dilution and then mixing with a ratio 0.1:39.9 of Isoton dilution solution according to the recommendations of the Beckman Coulter manufacturer. Also, cell, yeasts and fungus spores, counting was performed by using Beckman Coulter DU 640 spectrophotometer (U.S.A.) and by direct counting using a Haemocytometer grid under the microscope at 40x objective. Cell viability was determined by spreading 100 µL volumes of diluted suspension onto YPD (Yeast extract Peptone Dextrose) agar plates and counting colonies after 48 h at 28 ºC. If necessary, in order to track oxidative metabolism, the dissolved oxygen content was measured through the Oxymeter device CRISON OXI 92 (Spain).

Yeast cells were separated from the medium by centrifugation at 4600 rpm (Hettich centrifuge, U.S.A., 1717, 5000 rpm, 10 x 60g) for 10 minutes at 4 °C in a Rotina 38R Hettich centrifuge (U.S.A.). In the case of biofilm forming cells, they were collected by suction from the surface of each Erlenmeyer flask once the velum was fully formed.

A population of 1×10^6 cells/mL was inoculated in the fermentative (both *T. delbrueckii* vs. flor yeast *S. cerevisiae*) and velum formation media while 4×10^6 and 6×10^7 cell/mL in the flocculation medium and flor medium (Govender et al., 2010), respectively. For the formation of biocapsules, 4×10^6 yeast cells/mL and 4×10^6 *P. chrysogenum* spores were co-inoculated in the same medium.

Table 3. Media used in the experiment. Number in the "experiment" column corresponds to the Thesis aims.

Medium		Growth condition	Experiment
Name	**Composition**		
Fermentative (250 g/L glucose)	0.67% YNB w/o amino acids, 250 g/L glucose	175 rpm, 28 ºC, 10 days	1.1.
YPD or YPD-agar	1% yeast extract, 2% peptone, 2% dextrose, 2% agar (if required)	175 rpm, 28 ºC, 24 hours	1.1., 1.2., 2.2.
Fermentative	0.67% YNB w/o amino acids, 17% glucose, 10 mM glutamic acid	Slight agitation, 21 ºC, 12 hours	1.2.
Biofilm	0.67% YNB w/o amino acids, 1% (w/v) glycerol, 10 mM glutamic acid, 10% (v/v) ethanol	21 ºC, 29 days	1.2.
YNB 4% ETOH	0.67% YNB w/o amino acids, 4% (v/v) ethanol	Static condition, 30 ºC, 5 days	1.2.
Red wine 4% ETOH	Diluted standard red wine until reaching 4% (v/v) ethanol	Static condition, 30 ºC, 5 days	1.2.
FSM 4% ETOH	See Coi et al. (2016)	Static condition, 30 ºC, 5 days	1.2.
Fino wine elaborated in Montilla-Moriles	Characterized in Appendix 2	Ambient moisture > 85%, 15–20 °C, 72 months (1st type of wine sample) or 60–66 months (2nd type of wine sample)	2.1.
YP + 3% glycerol	1% yeast extract, 2% peptone, 3% glycerol (w/v)	175 rpm, 28 ºC, 3 days	2.2.

Table 3. Continued.

Medium		Growth condition	Experiment
Name	**Composition**		
Sporulation medium	1.7% corn meal agar, 0.1% yeast extract, 0.2% glucose and 2% agar	28 ºC, 7 days	2.2.
Flor medium (Govender et al., 2010)	0.67% YNB w/o amino acids, 3% ethanol, pH 3.5	21 ºC, 5 days	2.2.
Flocculation medium	See Giudici et al. (1994) and Spiropoulos et al. (2000)	Slight agitation, 25 ºC, 5 days	2.2.
Biocapsule formation medium (Peinado et al., 2004)	0.67% YNB w/o amino acids, 0.36% KH_2PO_4, 1.08% $Na_2HPO_47H_2O$, 0.556% D-gluconic acid, 2% (of the gluconic acid mass) acetic acid, pH 7	175 rpm, 28 ºC, 6 days	2.2.

3.2. Proteome analysis

Yeast cells were collected at the middle of the growth exponential phase, when the number of detectable proteins is large (Ghaemmaghami et al., 2003; Salvadó et al., 2008). Later, cells were resuspended in 1 mL of extraction buffer (100 mM Tris–HCl at pH 8. 0.1 mM Ethylenediaminetetra-acetic acid (EDTA), 2 mM Dithiothreitol (DTT) and 1 mM Phenylmethane Sulfonyl Fluoride (PMSF)) supplemented with Protease Inhibitor Cocktail tablets (Roche, Switzerland). Cell walls were broken by vortexing in a Vibrogen Cell Mill V6 (Edmund Bühler, Germany), using a volume of glass beads equivalent to that of the extraction buffer with the pellet (viz., 15 pulses of 1 min followed by 30 s on ice). Glass beads and cell debris were discarded by centrifugation at 500 g for 5 min. Protein precipitation was carried out by incubation overnight at 20ºC after the addition of 4 volumes of icecold

acetone and 10% (w/v) trichloroacetic acid to the supernatant. Samples were centrifuged at 16,000 g for 30 min, and the resulting protein pellets vacuum dried and resuspended in solubilization buffer (8 M urea, 2 M thiourea, 4% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 1% DTT). The Bradford (1976) assay was used to estimate the protein concentrations retrieved. Prior to the analysis, samples were stored at -80ºC.

The OFFGEL High Resolution kit pH 3-10 (Agilent Technologies, Palo Alto, CA, U.S.A.) was used for protein preparative isoelectric focussing (IEF) in solution. Protein samples (ca. 450 mg) were solubilized in Protein OFFGEL fractionation buffer containing thiourea, urea, DTT, glycerol and buffer with ampholytes (Agilent Technologies, Part number 5188-6444). Aliquots were evenly distributed in a 12-well 3100 OFFGEL Fractionator tray, also from Agilent Technologies, according to the supplier's instructions. Preset program OG12PR00 separation limits were used, namely: 4500 V, 200 mW and 50 mA; starting voltage 200-1500 V; ending voltage 5000-8000 V; and a constant voltage for protein separation zones after application of 20 kVh.

Peptides from each well obtained from a trypsin digestion, were fragmented on an LTQ (Linear Trap Quadropole) Orbitrap XL MS (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nano LC Ultimate 3000 system (Dionex, Germany). The electrospray voltage was set to 1300 V and the capillary voltage to 50 V at 190 ºC. The LTQ Orbitrap was operated in the parallel mode, which enabled accurate measurement of the precursor survey scan (400-1500 m/z) in the Orbitrap selection and afforded a 60,000 full-width at half-maximum (FWHM) resolution at m/z 400. This was concurrent with the acquisition of three collision-induced dissociation (CID) Data-Dependent MS/MS scans for peptide sequence, followed by three Data-Dependent higher-energy collisional dissociation (HCD) MS/MS scans (100-2000 m/z) with 7500 FWHM resolution at m/z 400 for peptide sequencing and quantitation. The collision energy used was 40% for HCD

and 35% for CID. Maximum injection times for MS and MS/MS were set to 50 and 500 ms, respectively. The precursor isolation width was 3 Da and the exclusion mass width 5 ppm. Monoisotopic precursor selection was allowed and singly charged species were excluded. The minimum intensity threshold for MS/MS was set to 8000 counts for the Orbitrap and 500 counts for the linear ion trap.

A database search with Uniprot (http://www.uniprot.org/) including fixed modification (viz., carbamidomethylation in Cys) against Proteome Discoverer v. 1.0 (Thermo Fisher Scientific Software, San Jose, CA, USA) was performed and the proteome results were statistically analysed with the same software.

After identification, a protein selection was carried out depending on the aim of the work (e.g. proteins located in mitochondria, proteins involved in the carbon metabolism, stress response proteome, etc.) in accordance with Gene Ontology (GO) terminology by using the Gene Ontology section on the *Saccharomyces* genome database or SGD (http://www.yeastgenome.org) or Uniprot. The SGD manuallycurated method was used to identify the GO annotations best reflecting the database as assigned by its curators, suggested in the literature for each gene or reported elsewhere. Further, the tool "GO Term finder" from SGD was used to determine the p-value for each annotation of proteins with GO terms, which is the probability or chance of seeing at least "x" number of ORFs out of the total "n" ORFs inthe list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO Term.

The amounts of proteins measured under both types of conditions were compared via the exponentially modified protein abundance index (emPAI; Ishihama et al., 2005), which is calculated as follows:

$$emPAI = 10^{\mathrm{PAI}} - 1$$

The PAI (Protein Abundance Index) value for each specific protein was obtained by dividing the number of peptides observed, with provision for charge state and missed cleavages, by the number observable peptides, which was estimated by using the software MS Digest from the web page: http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest

Fragmentation spectra matching the same peptide sequence but with a different charge or modification state, or containing a maximum of 3 missed cleavage sites, were counted separately. Protein relative contents under each condition were calculated from.

$$\text{Protein content (\% molar)} = \frac{emPAI}{\sum(emPAI)} \times 100$$

or

$$\text{Protein content (\% weight)} = \frac{emPAI \times Mr}{\sum(emPAI \times Mr)} \times 100$$

where Mr is the protein molar mass.

3.3. Genetic analysis

In order to elucidate the function of proteins highlighted in the proteomic analysis, null mutants of the genes that code for these proteins were constructed to later analyse their phenotypes. For the construction of deletion cassettes (Ble), phleomycin markers flanked by sequences homologous to genes removed (*CCW14* and *YGP1*), were amplified using oligos (Table 4) through a 50 µl PCR (Polymerase Chain Reaction) reaction mix: 5 µl 10X PCR Buffer-$MgCl_2$ Invitrogen, 2 mM dNTP Mix, 50 mM $MgCl_2$, 10 mM of each construction primer (table 1), 25 ng pUG66 template DNA, 2.5 U Taq DNA Polymerase Invitrogen. PCR conditions were: 94°C for 3 min, 94°C for 45 sec, 55°C for 30 sec, 72°C for 1 min 30 sec (30 cycles), 72°C for 10 min. The PCR product was purified (Qiagen, Inc.) and used to transform the

strains P3-D5 MAT α (Güldener et al., 1996). To select the transformants we used YPD plates containing 150 µg/mL phleomycin.

Further, to confirm the deletion of the allele, a PCR was ran using primers (Table 4) and consisting in a 12.5 µL PCR reaction mix: 1.25 µl 10X PCR Buffer-MgCl2 Invitrogen, 2 mM dNTP Mix, 50 mM MgCl2, 10 mM of each verification primer and 10 µM for Ble1 and Ble 2 (Table 4) (VerFw-VerRv for the first PCR verification, VerFw-Ble1 for the second and VerRv-Ble2 for the third), 15 µg template DNA, 0.625 U Taq DNA Polymerase Invitrogen.

Table 4. DNA primers.

Primer	Sequence	Purpose
CCW14 PhleFw	5' CAGCACTACTAGACTCGTTCAACACTCGTTATATA TTATCGTACGCTGCAGGTCGACAAC 3'	Construction of the *CCW14*::*PHLEO* cassette from pUG66
CCW14 PhleRv	5' GATAGATACCTTAACCCATTAGAAATAAAGTGATAGATAAACTATAGGGAGACCGGCAGA 3'	Construction of the *CCW14*::*PHLEO* cassette from pUG66
CCW14 VerFw	5' CCAGAATACGACGAGGACGG 3'	Verification of insertion of *CCW14*::*PHLEO* cassette
CCW14 VerRv	5' CCCAGATATGTACCGCCACC 3'	Verification of insertion of *CCW14*::*PHLEO* cassette
YGP1 PhleFw	5' TCTACTGGATTAATCGTCAGTTAAGTAATACAGTAAT AGAAAGTACGCTGCAGGTCGACAAC 3'	Construction of the *YGP1*::*PHLEO* cassette from pUG66
YGP1 PhleRv	5' AAAGAATCTCTATGCTTCGCTAGATTTAATATCTATCAGTACTATAGGGAGACCGGCAGA 3'	Construction of the *YGP1*::*PHLEO* cassette from pUG66
YGP1 VerFw	5' CGGCTTCTCGATGCTACAGT 3'	Verification of insertion of YGP1::PHLEO cassette
YGP1 VerRv	5' AGAAGGGGGTGAGATCCCTT 3'	Verification of insertion of *YGP1*::*PHLEO* cassette
Ble1	5' GTGGGCGAAGAACTCCAG 3'	Verification of insertion of *PHLEO* cassette
Ble2	5' GTTCTACCGGCAGTGCAAAT 3'	Verification of insertion of *PHLEO* cassette

The PCR conditions used were identical to those of cassette construction. After PCR amplification and

purification (Qiagen PCR purification kit), the insertion of deletion cassettes was verified also by Sanger sequencing (GATC-Biotech sequencing service) using their respective verification primers. DNA homology searches were performed using the BLAST (Basic Local Alignment Search Tool) algorithm through the National Center for Biotechnology Information and *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

3.4. Exo-metabolome analysis

General wine analysis

The common enological variables as the reducing sugars, titratable acidity, volatile acidity, pH and ethanol, were quantified by using the methods compiled by the The International Organisation of Vine and Wine (OIV, 2008). The parameters related to the color of the wines, when applicable, were measured as absorbances at 280, 420, 520 and 620 nm, and performed on a Perkin-Elmer (MA, U.S.A.) lambda 25 spectrophotometer. Color intensity and tonality were calculated according to Glories (1984) and Zamora (2013).

Aroma compounds analysis

There are several hundred of volatile compounds identified that contribute to the unique aroma sensation of each specific wine. These compounds are grouped into major volatile compounds, if their concentration is greater than or equal to 10 mg/L and in minor volatile compounds, if their content is lower than this value.

Major aroma compounds and polyols

These metabolites, released to the wine by the fermenting yeasts, were quantified in triplicate following the method of Peinado et al. (2004), by direct injection of 1µL of 10 mL wine samples previously added with 1 mL internal standard solution of 4-methyl-2-pentanol at 1 g/L. An Agilent 6890 Gas Chromatograph (GC) (Palo Alto, CA, U.S.A.) provided with a CP-WAX 57 CB capillary column (60 m, 0.25 mm i.d., 0.4 µm film thickness) from Varian (Palo Alto, CA, U.S.A.), and a Flame Ionization Detector (FID) was used. Each

compound was identified and quantified by a calibration table, build with standard solutions and analysed in the same conditions as the wine samples.

Minor aroma compounds

Volatile compounds contributing to the sensory properties of wine despite their low concentrations —less than 10 mg/L— were determined by using the analytical platform SBSE-TD-GC–MS (Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry). This platform includes a GC-7890A chromatograph, an MS 5975 detector from Agilent Technologies (U.S.A.) and a Multi-Purpose-Sampler from Gerstel (Mülheim an der Rühr, Germany), all governed via Chemstation (Agilent Technologies) and Maestro (Gerstel) software. Minor aroma compounds were analysed in triplicate according to the method of Vararu et al. (2016), taking 1 mL of wine sample and 0.1 mL of internal standard solution (0.446 mg/L ethyl nonanoate in ethanol), which were transferred to a 10 mL vial. The volume was completed to 10 mL with a buffered hydroalcoholic solution (12% v/v ethanol, pH 3.5 with 2.6 g/L tartaric acid and 2.2 g/L potassium bitartrate). Then, samples were stirred with a polydimethylsiloxane (PDMS) coated stir bar (Twister) with 0.5 mm film thickness and 10 mm length, from Gerstel (GmbH, Mülheim an der Rühr, Germany) at 1200 rpm for 100 min at 20 °C on a Variomag Multipoint 15 magnetic stirrer from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Then, the Twister was removed from the vial, rinsed with distilled water, dried with a cellulose tissue and finally, transferred into a thermal desorption unity (TDU), from Gerstel. Compounds were thermally desorbed at an initial temperature of 35 ºC for 0.1 s, using a 120 ºC/min ramp to 280 ºC for 10 min and a helium stream at 16 mL/min in the splitless mode into a Cooled Injection System (CIS-4) from Agilent Technologies furnished with an inlet liner packed with Tenax (3 × 2 mm). The CIS-4 was programmed as follows: 25 ºC (hold 0.05 s), 12 ºC/s ramp to 280 ºC (7 min); helium inlet flow, 16 mL/min. The 7890A GC instrument was equipped with an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness)

from Agilent Technologies (Wilmington, DE, USA). The oven initial temperature was set at 50 ºC for 2 min and then increased with 4 ºC/min to a final temperature of 190 ºC that was maintained for 10 min. The MS was used at 70 eV in the electron impact mode, using the mass range from 35 to 550 Da at 150 ºC. Each sample was measured in triplicate. Peak identification of the aroma components (all indicated along with its Chemical Abstrac Services number or CAS) was achieved by comparison of mass spectra with the mass spectral data collection from Wiley7N and NIST08 libraries and confirmed by comparing the calculated GC linear retention indices (LRI) using Van der Dool and Kratz (1963) equation with those of standards compiled in the NIST web book of Chemistry (Table 5). Some chromatographic peaks were also effectively identified by comparing its mass spectrum from those of pure compounds from our lab. Peak total ion areas relative to the IS (ethyl nonanoate) were used for comparison and statistical analysis since certain reference standards were unavailable.

Contribution of aroma compounds to the wine aroma

Each volatile compound is associated to a specific aroma according to their odour descriptor and several odorant series (OS) are stablished by grouping those compounds with the same or similar descriptors (Table 5). From a quantitative point of view, the contribution of each compound either to an specific OS, or to the overall aroma sensation can be estimated by their odorant activity values (OAV), which is calculated by dividing the content by the odor perception threshold (OPT) for each compound. This procedure relates the information provided by the chemical analysis, which are objective, with the sensory attributes of wines that are most subjective. Some authors as Zea et al. (2001; 2007), Moyano et al. (2002), Muñoz et al. (2007) and Noguerol-Pato et al. (2012a), Vararu et al. (2016) use these concepts to simplify the study of the wine aromas and Noguerol-Pato et al. (2012b) to study the aroma of grapes.

It is accepted that compounds showing OAV≥1, are the most important contributors to the wine aroma (Zea et al., 2001; López de Lerma et al., 2011, 2012). However, studies by Gómez-Míguez et al. (2007) and Rocha et al. (2004), have shown that some volatile compounds contribute to aroma when their OAV is higher than 0.2. As a result, the minor aroma compounds, which are present at trace levels can highly contribute to the wine aroma, while others major aroma compounds, more abundant can do so in a smallest extent.

The OPT from an aroma compound is dependent on its volatility and how it interact with the matrix where it is dissolved. Apolar volatile compounds have a high vapour pressure and show a low solublity in the polar solvents as the aqueous matrices and can to reach our nose easily. In contrast, the compounds showing a high polarity are more soluble in aqueous solutions and in minor extension in hydroalcoholic solutions as is the wine. The interaction of an odorous compound with other compounds is another problem occuring during the sensorial analysis of complex matrices, fundamentally due to the synergystic or antagonic effects among specific compounds. In this respect, a synergistic effect between the propyl acetate and ethyl propionate is described because the perceived odour is more intense when they are together in the same dissolution that when only one is present.

On the other hand, the ethyl acetate show an antagonic effect from a certain level of concentration, suppressing the propyl acetate and butyl acetate odours. The OAV show as a shortcoming where it assumes a linear relationship between the concentration of each compound and the intensity of perception, which is contradictory with one fundamental Psychophysics law's that establishes a exponential relationship. Althought the OAV is an approximate parameter, it does provide a quick and simple idea of the sensory importance of a particular compound and can be considered as an interesting approach to the quantification of the aroma intensity of the wines (Sánchez-Palomo et al., 2010).

Standars and chemical reagents

All reagents and standards used were analytical grade and supplied by Sigma-Aldrich, Riedel de Haën, Fluka or Merck. Ethanol absolute from Merck and tartaric acid and potassium bitartrate from Panreac (Barcelone, Spain). Pure water was obtained from Milli-Q purification system (Millipore). A C7-C40 hydrocarbon mixture in hexane (Sigma-Aldrich, Cat. Number U-49451) was used for the determination of LRI in the HP-5MS, GC capillary column.

3.5. Statistical analysis

The Software Package Statgraphics® Centurion XVI from Stat Points Technologies, Inc. (Warrenton, Virginia, USA) was used for statistical analysis. The following method were used in this work: ANOVA (Analysis Of Variance) and Fisher test for the establishment of Homogeneous Groups (HG) at a significance level $p \leq 0.05$, Multiple-Sample Comparison (MSC), Multiple Variable Analysis (MVA), Cluster Analysis (CA), Linear Discriminant Analysis (LDA), Multi Regression Lineal Analysis (MRLA), Principal Component Analysis (PCA) and calculation of Pearson coefficient for correlations among parameters.

Table 5. Major and minor aroma compounds identified in the wines.

No.	Compound name	CAS	LRI[a]	LRI[b]	Odor descriptor	OPT(mg/L)	OS
			Major compounds				
Alcohols							
1	Methanol	67-56-1	924.49	879	Chemical, medicinal, pungent fruity	668[VII]	1,2
2	1-Propanol	71-23-8	1067.60	1060	Fusel alcohol, ripe fruit	830[VII]	1,2
3	Isobutanol	78-83-1	1125.88	1108	Like wine, nail polish	40[VII]	1
4	Isoamyl alcohols	123-51-3	1242.61	1230	Like wine, nail polish, whisky, ripe fruit	30[VII]	1,2
5	Phenylethyl Alcohol	60-12-8	2011.18	1892	Rose talc, honey	10[VII]	A 5
Aldehides and ketones							
6	Acetaldehyde	75-07-0	687.84	800	Pungent, stewed apple	10[VII]	1,2
7	Acetoin	513-86-0	1336.76	1309	A Sour yogurt, sour milk	30[VII]	4
Esters							
8	Ethyl acetate	141-78-6	906.00	885	Pineapple, varnish, balsamic	7.5[VII]	1,2
9	Diethyl succinate	123-25-1	1729.46	1702	Overripe melon, lavender	100[VII]	2,5
Polyols							
10	2,3-Butanediol (levo)	513-85-9	1607.49	1545	Buttery, creamy	A 668[VII]	3,4
11	2,3-Butanediol (meso)	5341-95-7	1646.78	1585	Buttery, creamy	A 668[VII]	3,4
12	Glycerol	56-81-5	2475.54	n.f.	-	-	-

Table 5. Continued. Minor aroma compounds.

No.	Compound name	CAS	LRI[a]	LRI[b]	Odor descriptor	OPT(mg/L)	OS
			Minor aroma compounds.				
Alcohols							
13	2-furanmethanol	98-00-0	850.77	851	Alcoholic, chemical, caramel, bread, coffee	15[IV]	1,8
14	1-hexanol	111-27-3	866.94	867	Grass just cut	2.5[IV]	6
15	2-ethyl, 1-hexanol	104-76-7	1027.86	1027	Citrus, fresh floral	8[IX]	5,7
Aldehydes and ketones							
16	2-furancarboaldehyde	98-01-1	830.67	830	Fusel alcohol, toasted bread	0.77[IV]	1,8
17	5-methyl furfural	620-02-0	962.28	961.2	Toasted	1.1[IV]	87
18	Nonanal	124-19-6	1103.65	1103	Citrus, fatty, green, slightly pungent	0.01[I]	1,7
Carboxylic acids							
20	Octanoic acid	124-07-02	1177.39	1178	Fatty, waxy, rancid, oily	0.5[IV]	3
21	Decanoic acid	334-48-5	1369.11	1368.2	Unpleasant, rancid, sour	1[IV]	3
22	Tetradecanoic acid (Myristic acid)	544-63-8	1755.06	1758.2	Waxy, fatty, soapy	10[VIII]	3

Table 5. Continued. Minor aroma compounds.

No.	Compound name	CAS	LRI[a]	LRI[b]	Odor descriptor	OPT(mg/L)	OS
Esters							
23	Acetic acid, methyl ester	79-20-9	693.41	n.f.	Sweet, ether	0.7[X]	2
24	Propanoic acid, ethyl ester	105-37-3	707.80	705	Sweet fruity, grape, pineapple	5.5[VIII]	2
25	Ethyl isobutanoate	97-62-1	753.40	755	Fruity, apple, strawberry	0.015[VII]	2
26	Isobutyl acetate	110-19-0	771.13	779	Fruity, strawberry. banana	1.6[IX]	2
27	Ethyl butyrate	105-54-4	801.77	802	Fruity, sweet, tutti frutti. apple	0.02[II]	2
28	Butyric acid, 2-methyl, ethyl ester	7452-79-1	846.30	846	Sharp, sweet, green apple, fruity	0.02[II]	2,6
29	Butyric acid, 3- methyl, ethyl ester	108-64-5	849.62	847	Fruity, Lemon, apple	0.003[II]	2
30	Isoamyl acetate	123-92-2	874.77	876	Sweet, fruity, banana, solvent	0.03[VII]	2
31	Acetic acid, hexyl ester	142-92-7	1013.47	1015	Green apple	1.5[IX]	2
32	Heptanoic acid, ethyl ester	106-30-9	1098.38	1095	Fruity, pineapple, sweet, banana	0.002[VIII]	2
33	Octanoic acid, ethyl ester	106-32-1	1198.00	1196	Pineapple, pear, soapy	0.005[IV]	2
34	Octanoic acid, 2-methyl, ethyl ester	30982-02-6	1227.23	n.f.	n.f.	n.f.	-
35	2-Phenylethyl acetate	103-45-7	1256.11	1256	Fruity, rose, sweet, honey	0.25[II, III, IV]	2,5
36	Decanoic acid, ethyl ester	110-38-3	1395.38	1397	Sweet, fruity, nuts and dried fruit	0.2 [II, IX]	2

Table 5. Continued. Minor aroma compounds.

No.	Compound name	CAS	LRI[a]	LRI[b]	Odor descriptor	OPT(mg/L)	OS
Esters							
37	Dodecanoic acid, ethyl ester	106-33-2	1593.85	1593	Sweet	1.5[IX]	4,5
38	Tetradecanoic acid, ethyl ester	124-06-1	1792.97	1793	Sweet fruit, butter, fatty odor	2[IX]	4
39	Hexadecanoic acid, ethyl ester	628-97-7	1991.65	1993	Fatty, rancid, fruity, sweet	1.5[IX]	4
Lactones							
40	2 (5H)-Furanone (γ-crotonolactone)	497-23-4	913.40	915.6	Toasty, caramel	1[X]	8
41	Decalactone	706-14-9	1470.43	1470	A Peach, milky	0.01[IV]	2,4
Volatile phenols							
42	2-methoxy-4-vinylphenol	7786-61-0	1312.57	1313.6	Spices, clove, peanut, woody	0.04[VI]	9,10
43	Phenol, 2,6-dimethoxy-	91-10-1	1357.81	1359	Medicine, phenol, smoky	0.57[VI]	1,8

LRI: Linear retention index using definition of Van den Dool and Kratz (1963) in a *CPWAX57-CB capillary column (60 m/0.25 mm/0.40 μm. He) and **HP-5MS capillary column (30 m/0.25 mm/0.25 μm. He). .n.f. – not found in faced conditions. a Calculated values b Data collected from the NIST Webbook of Chemistry. http://webbook.nist.gov/chemistry (only orientative data for major volatile compounds). OPT: Odor Perception Threshold. OS: Odorant Series. 1. Chemistry. 2. Fruity. 3. Fatty. 4. Buttery. 5. Floral. 6. Fresh grass. 7. Citric fruit. 8. Toasty/smoky. 9. Spicy. 10. Dry grass/woody. I. – Culleré. (2011). II.- Gómez-Minguez. (2007). III.- Guth. (1997). IV.- López de Lerma. (2012a). V.- López de Lerma. (2012b). VI. López. (2002). VII.- Moreno. (2005). VIII..- Welke. (2014a). IX.- Yong-Sheng. (2009). X.- http://www.leffingwell.com/odorthre.htm

4. RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Objective 1. To identify the metabolic and proteomic responses of non-*Saccharomyces* and *Saccharomyces* yeasts, to several stress conditions of enological interest.

> ***4.1.1.*** To establish the profile of exo-metabolites of a *Torulaspora delbrueckii*, a non-*Saccharomyces* yeast, growing in a synthetic medium.

Non-*Saccharomyces* yeasts predominate among the microbiota present in the early stages of the spontaneous grape must fermentation and contribute to the organoleptic properties of the wine in a fundamental way. This fact facilitated great interest among the researchers and winemakers because it presents and increases the possibilities of diversification, contribution to the elaboration of new types of wines (Pretorius and Høj, 2005).

Among the non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* is highlighted due to several facts:

i) It is one of the few non-*Saccharomyces* species commercially available for its use in winemaking and beer production.

ii) It is present in most of the relevant wine regions around the world (Capozzi et al., 2015).

iii) It has high resistance to ethanol toxicity, sensitivity to lack of oxygen and to cell–cell contact with *S. cerevisiae* (Holm Hansen et al., 2001; Lachance and Kurtzman, 2011; Mauricio et al., 1998; Nissen et al., 2003).

iv) It shows low production of acetic acid, low fermentation rates and production of sulphur compounds (Bely et al., 2008; Azzolini et al., 2015).

In order to establish a footprint based on the metabolites secreted by *T. delbrueckii*, (ATTC-MYA 426) and avoid other factors that influence its contents, inoculation of yeast pure

cultures in synthetic media of known composition were performed. 1 x 10^6 total cells/mL population of pure cultures of *T. delbrueckii* and *S. cerevisiae* G1 (ATTC-MYA 2451), the last used as a reference strain, were inoculated separately in a fermentable medium consisiting in 0.67% YNB without amino acids and 250 g/L glucose. Alcoholic fermentations were carried out in 250 mL flasks, closed with hydrofobic cotton, under 175 rpm agitation and monitored by loss in weight. Experiments were terminated at the stationary yeast growth phase, when no changes in weigth were obtained for two consecutive days. Samples were taken 10 days after the inoculation, and the metabolite analyses were performed according to the methods explained in the material and methods section.

The obtained results for this experiment gave rise to the Manuscript attached in the Appendix 1, which will be sent as soon as posible to a journal indexed in Q1 of Food Science and Technology area.

Appendix 1. Article under preparation. Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts.

This manuscript reports that *T. delbrueckii* has a longer lag phase than *S. cerevisiae*, catabolizes less glucose, produces higher levels of succinic acid and lower concentrations of ethanol, acetaldehyde, acetoin, 2,3-butanediol, glycerol and acetic acid than the yeast used as the reference (Figure 18). As it is shown in this figure, *T. delbrueckii* directs 9.5% of the glucose to the formation of glycerol and 20.2% to secondary products, while *S. cerevisiae* yeast metabolizes around 1/4 of the total glucose by means of glycero-piruvic pathway and shows a ratio of 1:1 glycerol/secondary products.

A higher sensitivity to low oxygen contents by *T. delbrueckii* explains the slow growth rates (Ciani and Picciotti, 1995; Mauricio et al., 1991). Another reason could

be due to sensitivity of the high osmotic pressures to solutions with high sugar concentrations, as in the grape-must. The higher fermentation rates of *S. cerevisiae* make this yeasts consume sugars more rapidly and produce glycerol, an osmolyte that yeast cells accumulate when subjected to hyperosmotic conditions (Albertyn et al., 1994; Ansell et al., 1997;). Glycerol production provides an advantage to *S. cerevisiae* over *T. delbrueckii*, to better overcome this stress condition and to grow faster in the hyperosmotic media.

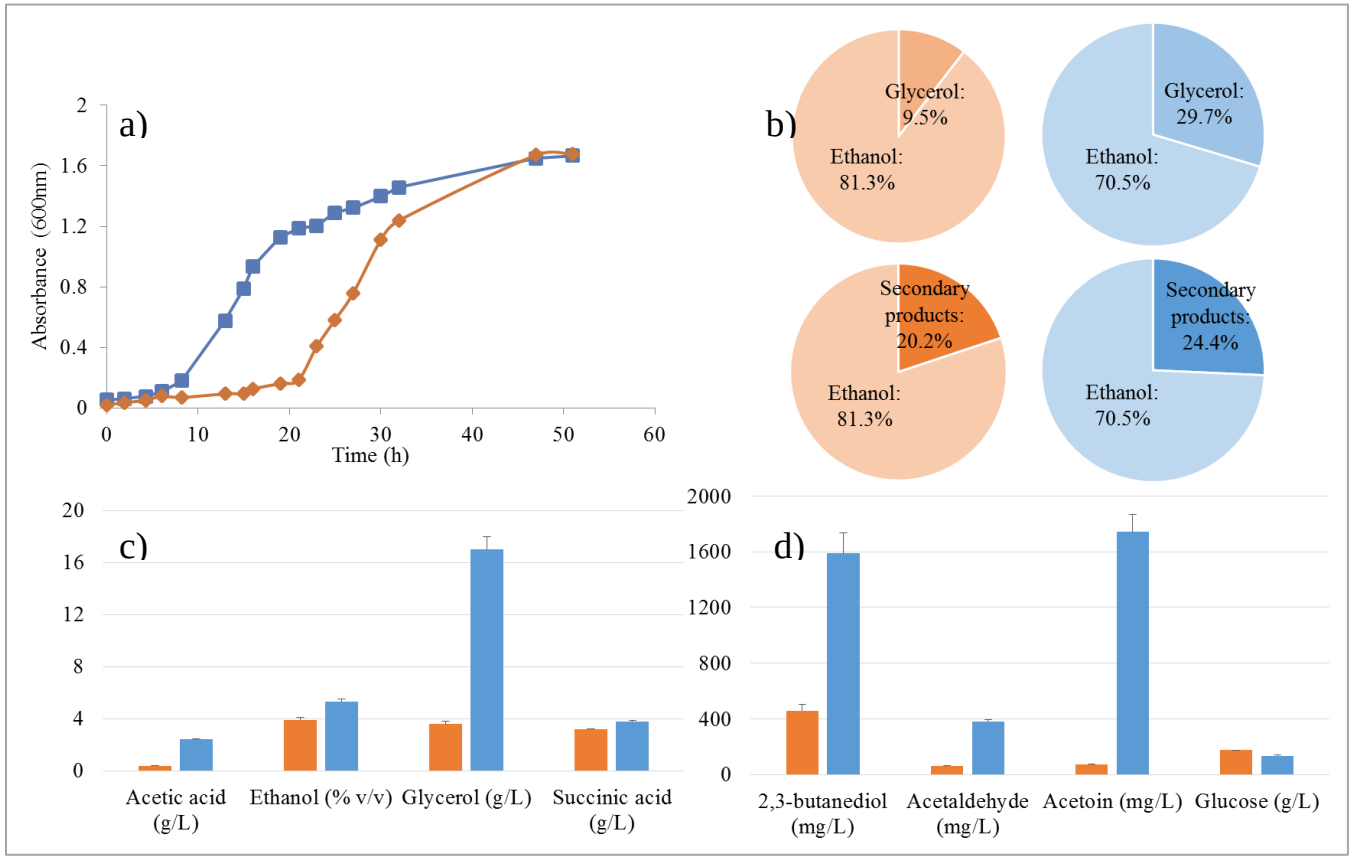


Figure 18. Growth and metabolism aspects of *Torulaspora delbrueckii* (orange) and *Saccharomyces cerevisiae* (blue) at YNB w/o amino acids and 250 g/L glucose: a) yeast cell population expressed in absorbance measured at 600 nm; b) percentages of glucose transformed to ethanol, glycerol and secondary products during fermentation; c and d) concentration of main metabolites that distict between yeasts.

4.1.2. To identify the influence of media with high sugar contents and media with high ethanol and glycerol contents on the proteins expressed and the small metabolites excreted by a *S. cerevisiae* flor velum-forming yeast strain.

In contrast with *T. delbrueckii*, flor yeasts perform their role in the last stages in the wine-making process, once fermentation is over. As previously mentioned, flor yeasts are responsible of the process known as biological aging. They are mainly *S. cerevisiae* strains that have the ability to form biofilm on the surface of wine after fermentation and develop of oxidative metabolism when low levels of fermentable sugar and high concentrations of ethanol and glycerol are reached (Alexandre, 2013). The ethanol, glycerol, and acetaldehyde are the compounds most modified in content by effect of flor yeast, but other compounds such as organic acids, nitrogen compounds, higher alcohols, esters, lactones, polyphenols, acetoin, and diacetyl are also affected (Blandino et al., 1997; Mauricio and Ortega, 1993; Martinez et al., 1998; Charpentier et al., 2002).

The effect of flor yeast metabolism on wine composition was investigated through a biological aging in a "criaderas" and "solera" system and also under controlled laboratory conditions. Additionally, biological processes and organelles that are essential for the flor yeasts to survive when forming biofilm and when fermenting were studied. From this, seven articles (Appendixes 2-8) have been published in the international scientific journals "Food Research International", "International Journal of Food Microbiology", "Food Microbiology", "Process Biochemistry", and "International Journal of Molecular Science", all in Q1 or Q2:

Appendix 2. Moreno-García, J., Raposo, R. M., Moreno, J. (2013). Biological aging status characterization of Sherry type wines using statistical and oenological criteria. *Food Research International, 54,* 285–292.

Appendix 3. Moreno-García, J., García-Martínez, T., Moreno, J., Millán, C., Mauricio, J. (2014). A proteomic and metabolomic approach for understanding the role of the flor yeast mitochondria in the velum formation. *International Journal of Food Microbiology*, *172*, 21-29.

Appendix 4. Moreno-García, J., García-Martínez, T., Moreno, J., Mauricio, J. (2015). Proteins involved in flor yeast carbon metabolism under biofilm formation conditions. *Food Microbiology*, *46*, 25-33.

Appendix 5. Moreno-Garcia, J., García-Martínez, T., Millán, M. C., Mauricio, J. C., Moreno, J. (2015). Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions. *Food Microbiology*, *51*, 1-9.

Appendix 6. Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T. (2016). Stress responsive proteins of a flor yeast strain during the early stages of biofilm formation. *Process Biochemistry*, *51*, 578–588.

Appendix 7. Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T. (2016). Functional analysis of stress protein data in a flor yeast subjected to a biofilm forming condition. *Data in brief*, *7*, 1021–1023.

Appendix 8. Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T. (2017). Differential proteome analysis of a flor yeast strain under biofilm formation. *International Journal of Molecular Science*, *720*, 1-18.

Appendix 9. Article under preparation. Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain.

Metabolomic analysis of a wine subjected to biological aging in a "criaderas" and "solera" *system.*

In Andalusia, Spain, biological aging takes place in a dynamic system known as "criaderas" and "solera". It starts after alcoholic and malolactic fermentations and when spontaneous stabilization of wine is concluded. The wine produced is placed into casks (≈ 600 L capacity) that are set into different scales, or rows called criaderas, containing wine with the same aging time and numbered from the floor to the top. The first is the "solera" lying on the floor and contains the most aged wine, while the scale above is the "first criadera" and subsequently the second, third, etc. The uppermost row is the "sobretabla" and it is where the year's harvest wine is loaded. The wineries often have 4-5 criaderas, including the solera. In this system, youngest wines are periodically added, then wine from each cask is blended with wine from other casks in the same criadera and subsequently wines are sequentially transferred through the criaderas thus, blending with a more aged wine. The aim of this technique is to obtain a mixture of wines obtained from different harvests with similar sensorial properties.

To address the second objective of this doctoral thesis, the biological aging of a Sherry fino wine made from Pedro Ximénez grapes and elaborated in the Protected Denomination of Origin Montilla–Moriles in Spain, was tracked through the "criaderas" and "solera" system, from the youngest wines in the first criadera til the oldest in the solera. Chemical and statistical analyses of the samples were made as indicated in the "Material and methods" section.

In 6 different biological aging statuses in the "criaderas" system (Figure 19), twenty variables, volatile aroma compounds and polyols were quantified. It is observed that during the criaderas system, the ethanol content remains constant (Martinez et al., 1998) while the glycerol rapidly declines as it is consumed (Bravo, 1995). Further, a significant increase in content of compounds like diethyl

succinate or acetaldehyde were reported, where the last is a precursor molecule of aroma compounds such as 1,1-diethoxyethane, diacetyl, acetoin, 2,3-butanediol and the four carbon atom organic acids (Casas, 1985; Cortés et al., 1998; Zea et al., 1995). The accumulation of acetaldehyde is responsible of conferring Sherry wines for their special aroma.

In addition, by using statistical tools it was possible to select 6 aroma compounds that differ among wines of different aging status: 1,1-diethoxyethane, 1-propanol, isoamylic alcohols, ethyl lactate, 2-phenylethanol and glycerol. Indeed, it was possible to establish a kinetic equation based on the glycerol concentration to calculate the aging time required by a young wine to achieve a certain characteristics of a solera wine and also the glycerol concentration after a known aging time.

Acording to the results, the differential and integrated equation of rate for glycerol in a standard winery, in the conditions of temperature (15-20ºC), moisture (more than 85%) and *Saccharomyces cerevisiae* flor yeast grown in a criadera and solera system, as those recommended by the PDO council from Montilla- Moriles, is expressed as the following:

$$-\frac{d|\,\mathrm{Gly}\,|}{dt} = Kv \times |\,\mathrm{Gly}\,|^{1}; \qquad -\int_{c_0}^{c_t} \frac{d|\,\mathrm{Gly}\,|}{|\,\mathrm{Gly}\,|} = \int_0^t Kv \times dt;$$

$$Ln\,|\,\mathrm{Gly}\,|_t - Ln|\,\mathrm{Gly}\,|_0 = -Kv \times t\ ;$$

$$Ln\,|\,\mathrm{Gly}\,|_t = Ln|\,\mathrm{Gly}\,|_0 - Kv \times t\,;$$

$$Kv = 0{,}032\,(\mathrm{months})^{-1}\,;$$

$$t = \frac{Ln\,|\,\mathrm{Gly}\,|_0 - Ln|\,\mathrm{Gly}\,|_t}{Kv}$$

Gly is the abbreviation of glycerol, C_0 are the initial concentration, and C_t the reached concentration in a time *t*,

which is the time in months and *Kv* is the specific constant reaction rate.

In the traditional biological aging system used in Montilla-Moriles cellars, this equation provides advantages over a traditional model based on acetaldehyde accumulation: i) glycerol is less volatile; ii) its content is hardly modificable as it is being consumed by the yeast and can not be added to the wine as the acetaldehyde; and iii) glycerol is reactive by lees. Moreover, other equations proposed by Baker et al. (1952) and Jackson (2008), are strictly mathematical considering variables like: number of criaderas, frequency and proportion of wine transferred through the criaderas system; and their application in the dynamic aging of oxidative type are used for other white and sweet wines and not for Sherry wines subjected to biological aging (Moreno and García-Mauricio, 2013; Moreno and Peinado, 2012).

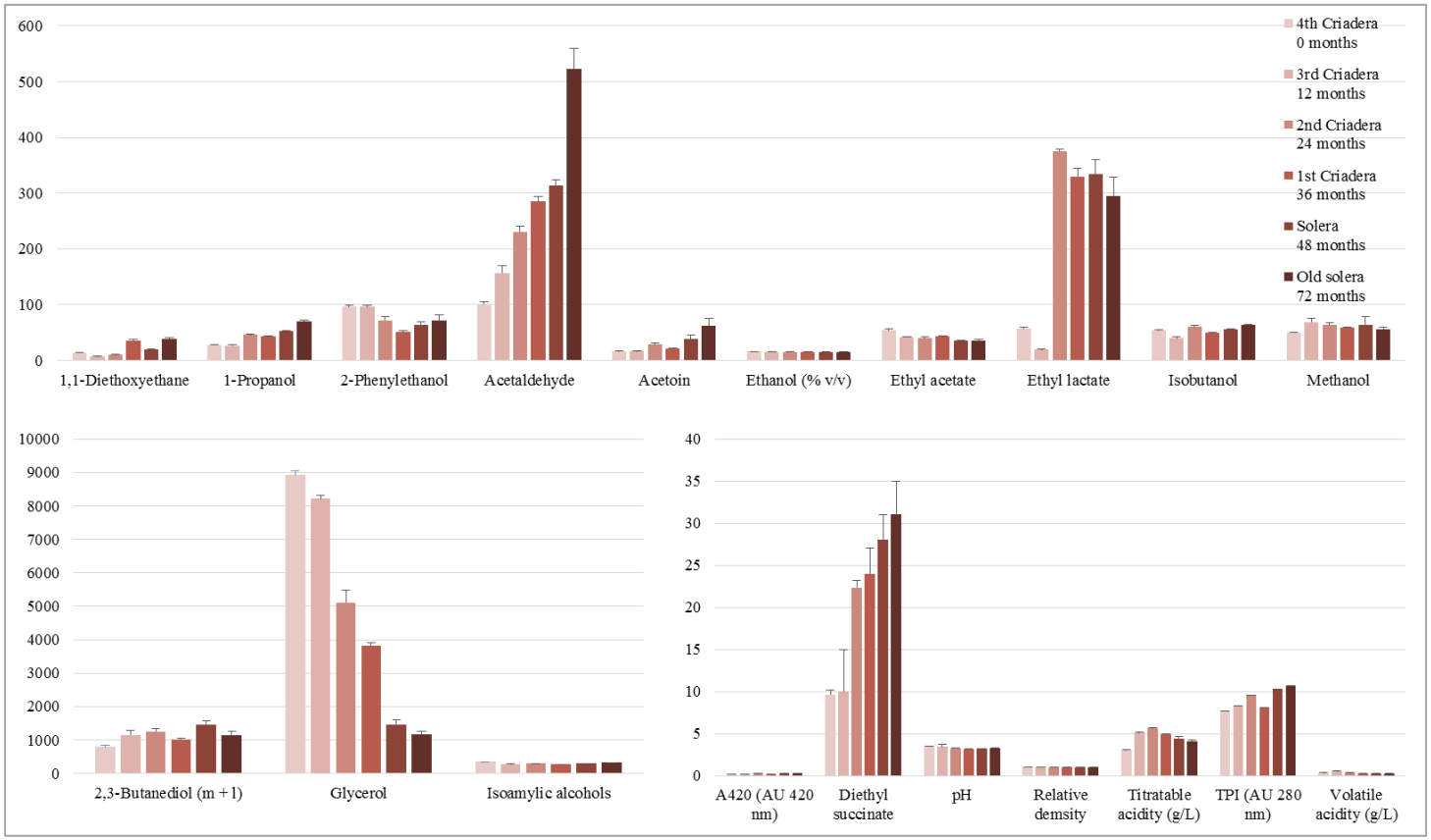


Figure 19. Averages and standard deviations for enological variables, aroma compounds and polyols in wines subjected to biological aging by the criaderas system in a winery. Concentration in volatile compounds and polyols are expressed as mg/L.

Metabolomic and proteomic responses of a flor yeast in fermentative and biological aging media.

Although changes in flavor-related molecules and their associated sensory properties been examined in depth (Alexandre, 2013; Cortes et al., 1998; Mauricio et al., 2001; Mesa et al., 2000; Muñoz et al., 2005, 2007; Peinado and Mauricio, 2009; Pham et al., 1995; Villamiel et al., 2008; Zea et al., 2001, 2007), the proteome of flor yeasts and its relation with the changes on the concentration of aroma compounds remain poorly known.

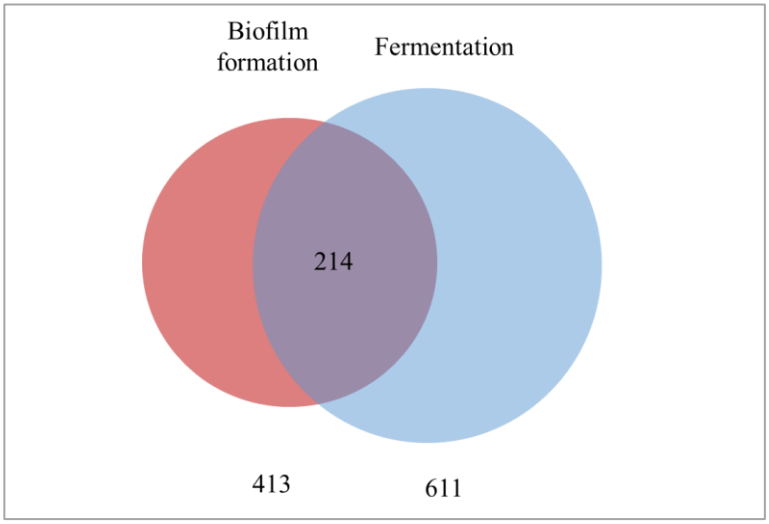


Figure 20. Venn diagram showing proteins detected when forming biofilm, when fermenting and detected under both conditions.

To explore the relationship between changes in the flor yeast proteome and exometabolome, and its influence on the organoleptic properties of wine, we inoculated pure cultures of flor yeasts in known-composition synthetic media (biofilm formation condition such as biological aging and a fermentative condition used for reference) and conducted proteomic and exometabolomic studies at the exponential growth phase. Proteomic and exometabolomic analyses were performed by using modern techniques such as OFFGEL fractionator and LTQ-Orbitrap; and SBSE-TD-GC-MS,

respectively (Appendix 5). For more detailed information about media chemical composition and techniques, see material and methods section.

We reported the presence of 413 flor yeast proteins when forming biofilm and 611 while fermenting (Figure 20). Among them, 214 was common in both conditions in which 67 and 42 proteins were more abundant in biofilm flor yeasts and when fermenting, respectively; being the rest of the common proteins (105) quantified in similar amounts.

Figure 21 shows concentration changes of aroma compounds with an OAV higher than 1 (b) where proteins related to their catabolism/anabolism (c) and the OAV (a). The dashed line to the right are represented by those aromas and proteins related to acetates quantified over their odour threshold; from the dashed line to the left those aromas and proteins related to compounds quantified over their odour threshold metabolized via downstream glycero-pyruvic and/or ethanol degradation pathways.

Flor yeast ethanol consumption and acetaldehyde accumulation were reported when forming biofilm and through the biological aging (Figure 20 and 22). Nevertheless, glycerol content shows minor changes in contrast with the significant decrease found in the "criaderas" and "solera" system (Appendix 5, Table 1 and Figure 20). Muñoz et al. (2005), where slight changes in glycerol concentration were observed during the early stages of biofilm formation and an increased consumption at the flor velum-maintanance phase. While flor biofilm is forming, the yeasts consume ethanol instead of glycerol (García-Maiquez, 1988, 1995; Gutiérrez et al., 2010; Martínez et al., 1998; Rodríguez et al., 2013). Thus, these observations support the results obtained through the metabolomic analysis (Figure 21).

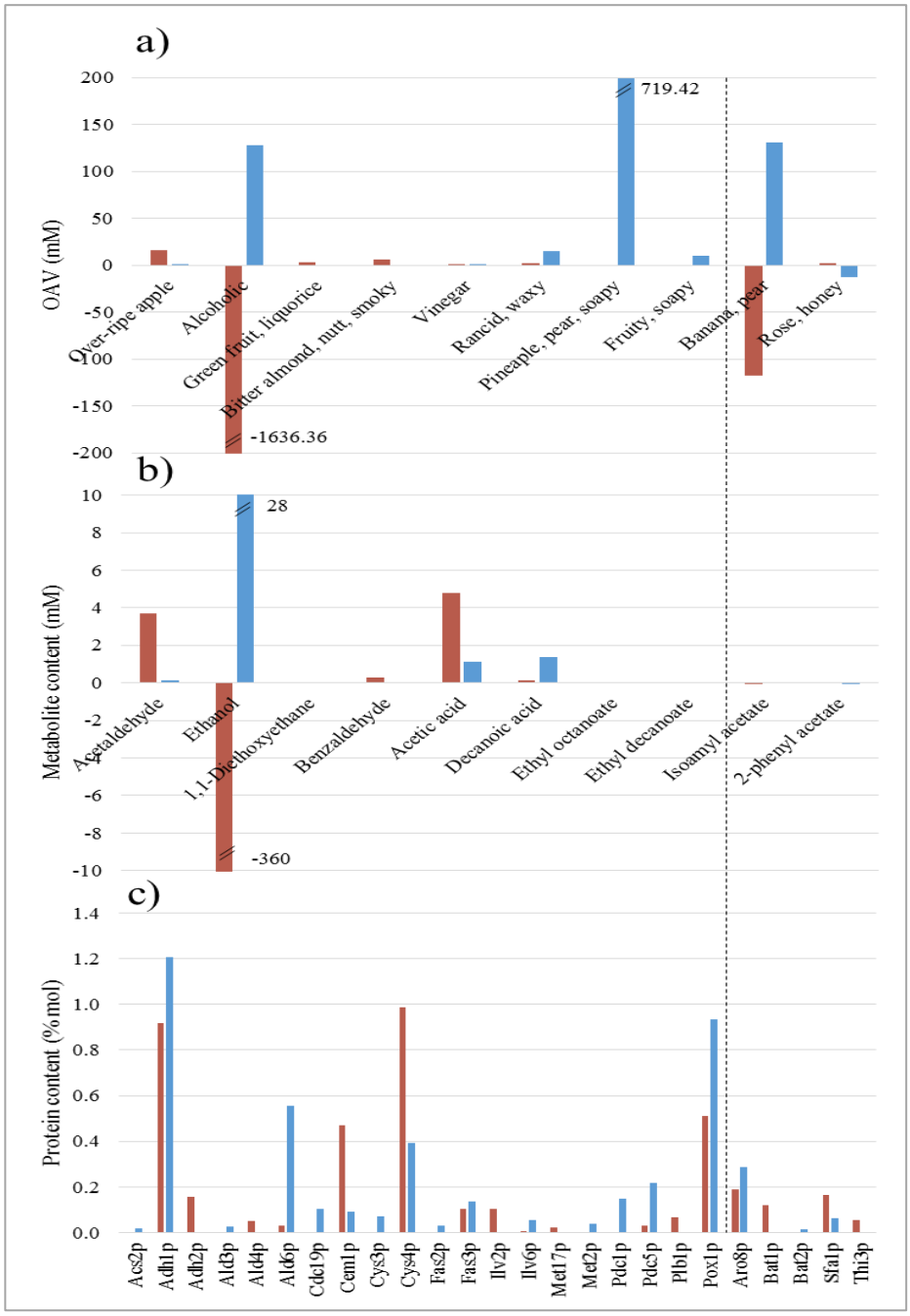


Figure 21. Odour activity value (OAV) (a), metabolomic (b) and proteomic profile (c) of a flor yeast under biofilm formation (red) and fermentative (blue) conditions.

The presence of enzymes as Adh2p, that catalyzes the oxidation of ethanol to acetealdehyde and Ald4p, converts acetaldehyde to acetic acid and explains the decrease in ethanol and increase in acetaldehyde and acetic acid, respectively, when flor yeasts form biofilm. Acetic acid is then converted to acetyl-CoA and this molecule is corporated into the glyoxylate cycle or Krebs cycle to obtain energy and intermediate metabolites, or into lipid and amino acid synthesis (Alexandre, 2013). Aranda and del Olmo (2003), previously found aldehyde dehydrogenase activity in flor yeasts that exceed fermentative yeasts. As expected, acetaldehyde was the compound with highest OAV when flor yeasts were foming velum (Figure 22). Proteins involved in the production of acetate by metabolization of the amino acids (Cys3p, Cys4p, Met17p and Met2p) were also detected. Cem1p was quantified 3-fold the content when fermenting. This is the mitochondrial beta-keto-acyl synthase with a potential role in fatty acid synthesis, which is derived from the ethanol consumption.

When comparing with the fermentative reference condition, proteins associated to processes such as non-fermentable carbon uptake, glyoxylate and tricarboxylic acid cycle (TCA), cellular respiration and inositol metabolism were detected at higher concentrations (Appendix 4). Further we did not report yeast consumption of glycerol (Appendix 5, Table 1). Enzymes participating in its degradation were identified in the flor yeasts forming velum: Gut2p and Dak1p. Cat8p and Cit2p, both involved in the glyoxylate cycle, which were only found when forming biofilm. This pathway is essential for yeast growth on two-carbon compounds such as ethanol or acetate and plays an anaplerotic role in the provision of precursors for biosynthesis. Efficient use of two-carbon compounds by yeasts also requires the TCA cycle and gluconeogenesis, which are coordinately regulated with the glyoxylate cycle. All TCA cycle proteins detected were much more abundant when oxidatively metabolized than when fermenting. The TCA cycle is known to play a central role in producing reducing power for cellular respiration, supplying

molecules for gluconeogenesis and producing amino acids and fatty acids. Cor1p, Mam33p, Mdh1p, Mic17p, Pet9p, Cox4p, Cox6p and Qcr6p are proteins that act in the cellular respiration founded in higher contents when forming biofilm. Inositol-3-phosphate synthase or Ino1p was found to be 5 times more abundant when flor yeasts were forming biofilm. Inositol is used to anchor the protein Flo11p which is essential for velum formation to the yeast cell surface (Ishigami et al., 2004; Fidalgo et al., 2006; Purevdorj-Gage et al., 2007; Reynolds et al., 2001; Zara et al., 2005).

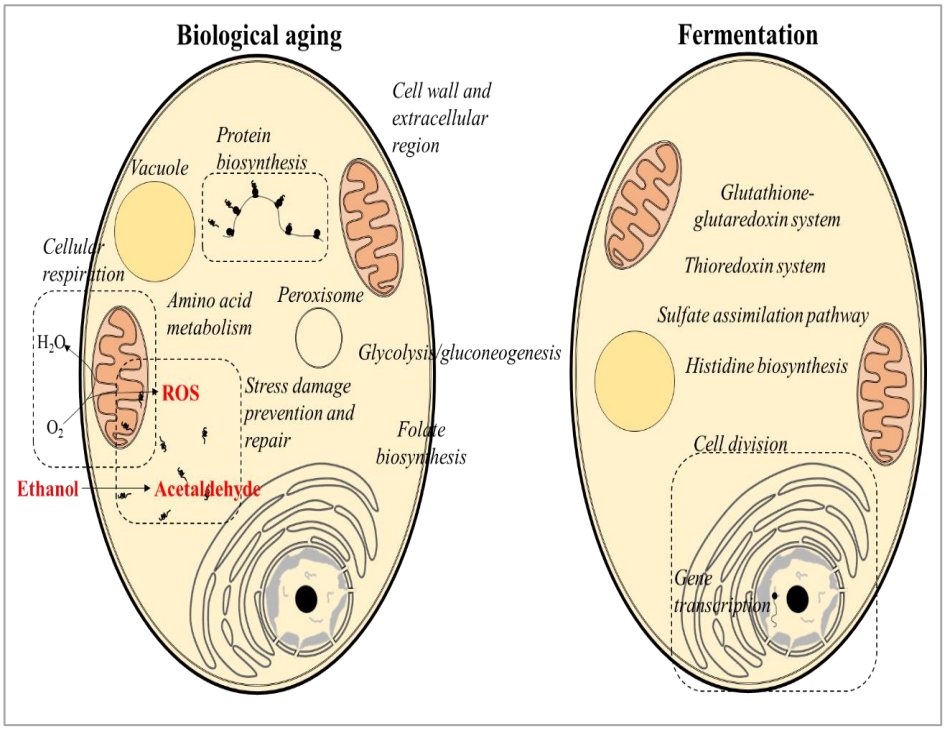


Figure 22. Yeast biological processes and organelles highlighted by the abundance of proteins detected under biological aging and fermentation.

Many of the proteins identified and related to the aroma compounds are located in the mitochondrion. This together with other organelles play important roles in the flor yeast oxidative metabolism. In order to detect organelles, biological processes and pathways contribute to the oxidative metabolism but also to the flor yeasts' survival to biological

aging conditions (Appendix 3). We used the GO Term finder tool of the database SGD and a GO Term or pathway ratio and compared values obtained from the reference fermentative condition to highlight "biological processes", "cellular components" and "pathways" (Appendix 8). Figure 22 sumarizes all processes, organelles and pathways highlighted under the two studied conditions.

In this work (Appendix 8), oxidative metabolism of the flor yeasts while forming velum was tracked through oxygen consumption (Figure 4, Appendix 8). Mitochondrial proteins and those involved in oxidative metabolism were found to be highly synthetized in the biofilm yeasts. Some authors have considered this metabolism as an adaptive mechanism allowing cells to survive under biological aging conditions in wine (Alexandre, 2013). Among the mitochondrial proteins, the mitochondrial malate dehydrogenase or Mdh1p was reported in very high content. It catalyzes the interconversion of malate and oxaloacetate in the TCA cycle and it is also a component of the NADH cytoplasm/mitochondria shuttle.

Mitochondrion plays a relevant role in flor yeast when forming biofilm because the metabolism is purely oxidative (Mauricio et al., 1997) and for its role in the elaboration of the stress responses. For instance, proteins like those forming part of the TCA cycle or in the respiratory electron chain (Reinders et al., 2006) and manganese superoxide dismutase (Sod1 and 2p) that confer oxidative stress protection (van Loon et al., 1986), are essential for flor yeasts when forming biofilm. Mitochondrial proteins related to the ethanol resistance, cell respiration, mitochondrial genome maintenance, and apoptosis were detected when flor yeast was forming flor velum (Appendix 3). It was found that, almost all of the ethanol resistance proteins contribute to the ethanol consumption. Respiration proteins detected included TCA cycle proteins like Aco1p, Cit1p, Idh1p, Idh2p, Kgd1p, kgd2p, Lsc1p, Mdh1p and Sdh1p; electron transport chain proteins like Cor1p, Cox4p, Cox6p and Qcr6p, among others. A high frequency of proteins involved in the mitochondrial

genome maintenance has been explained since despite adaptive responses to ROS presence, where cells exhibit an oxidative damage basal level to their macromolecules, especially those from mitochondria (Cabiscol et al., 2000). Thus, mtDNA can be affected by these species. Finally, two apoptosis-related proteins were detected in biofilm yeasts Cpr3 and Por1p. Yeasts under oxidative conditions can undergo apoptosis (Madeo et al., 2004). According to Newmeyer and Ferguson-Miller, (2003) mitochondria plays a central role in the process. Fröhlich and Madeo (2000) suggested that apoptosis originated in yeast as an altruistic response to severe oxidative damage.

Another organelle important for the flor yeast adaptation, the cell wall, showed high values of GO Term ratio and low GO Term p-values in biofilm formation conditions, revealing its importance (Figure 23 and Figure 1, Appendix 8). Unexpectedly, no evidence was reported about the glycosylphosphatidylinositol or GPI-anchored cell surface glycoprotein Flo11p, possibly due to its major expression during the stationary phase (Zara et al., 2009). Nonetheless, proteins that mediate its transcription and anchored membrane components were copious under the biofilm condition. Other cell wall proteins like glucan synthetases and mannoproteins also involved in the flor formation, were also found abundant; particularly Bgl2p, that participate in cell wall maintenance and incorporation of newly synthesized mannoprotein molecules (Gasch et al., 2000; Snowdon et al., 2009), and Ygp1p, an extensively N-glycosylated cell wall-related secretory protein. Ygp1p synthesis positively affects the biofilm formation (Vandenbosch et al., 2013) and until recently, has only been localized out of the cell (Curwin et al., 2009). These facts may evidence a proteomic composition of the extra-cellular matrix among flor yeast forming the velum revealed by Zara et al. (2009) and remains undiscovered until now.

Another cell wall glycoprotein related to biofilm formation (Andersen et al., 2014), Ccw14p, was quantified in

very high values when the yeast was forming velum (Appendix 6). Up-regulation of these glycoproteins and their function in biofilm may be indicative of an important role during flor yeast velum formation, such as Flo11p. With the collaboration of members of the Department of Agriculture, Università degli Studi di Sassari, we clarified this hypothesis by deleteding the *CCW14* and *YGP1* genes of a haploid flor yeast strain separately and tested the velum formed in terms of appearance, cell adherence and dry weight (Appendix 9). Material and methods for genetic analysis and biofilm formation and adherence assays are described in Appendix 9. This study revealed that yeast strain deficient in Ygp1p synthesis produced less velum biomass than the wildtype strain, thus showing its positive effect on velum formation while there were no significant differences for Ccw14p, a protein that can be associated to other process rather than the velum formation.

Proteins that participate in the prevention and protection of the damages caused by ROS stress that derived from the cellular metabolism, are highlighted: NADPH regenerators, proteins that maintain the structure and integrity of the mitochondrial genome (Aco1p or Hsp78p), damaged protein refolding, among others (Figure 23 and Figure 2, Appendix 8). High contents of proteins involved in translation were reported too, especially the Hyp2p, where some are strongly induced by stress (Appendix 7). These findings may indicate that flor yeasts refold or replace the proteins damaged by the stresses. Zara et al. (2010) observed through transcription analyses a 4.3-fold higher frequency of translation genes over-expressed in biofilm cells compared to non-biofilm cells, thus, supporting this hypothesis. Furthermore, peroxisome and vacuolar proteins involved in the biofilm formation and response to stresses were over-represented.

Proteins that have a role in the response to other typical stresses in biological aging besides oxidative stress, such as very low sugar and high ethanol content and high

acetaldehyde levels; were reported when forming biofilm but also when fermenting (Appendix 6 and 7). Eighty-five stress response proteins were found more abundantly in the flor than in the pelagic yeasts. Among them, a majority were involved in the response to the oxidative stress (46), to the ethanol stress (39), lack of fermentable carbon source (18) and to the acetaldehyde toxicity (3). Relative values show a higher importance of proteins involved mostly in the response to the oxidative stress and lack of fermentable carbon source (Figure 23). Moradas-Ferreira et al. (1996) stated that flor velum endurance depends on yeast tolerance to hostile conditions, above all, oxidative stress.

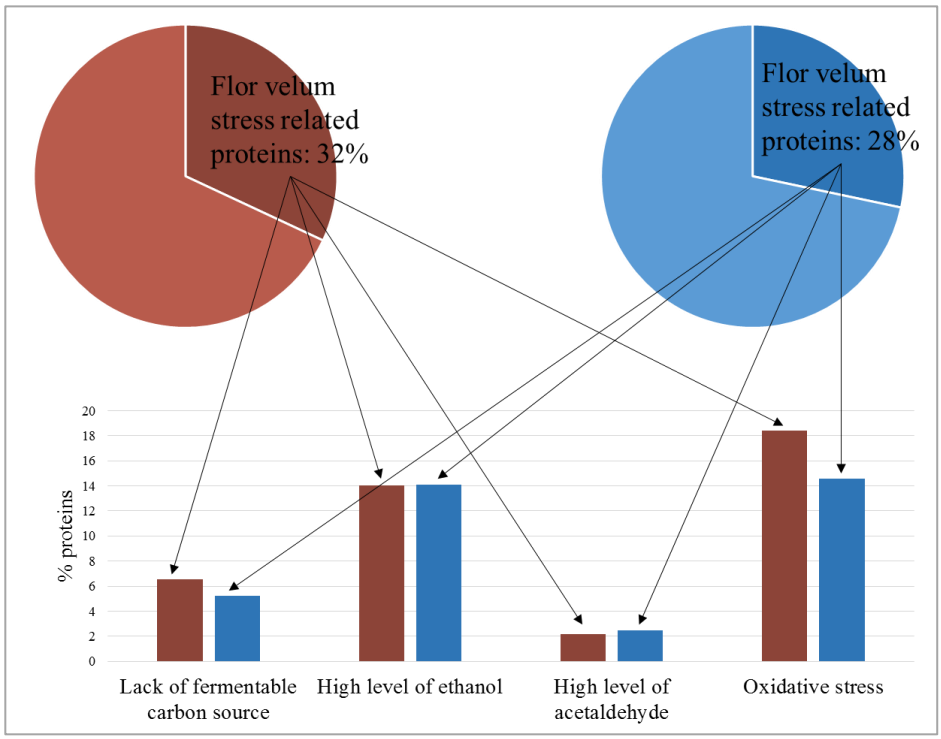


Figure 23. Total proteins identified in flor yeast when forming velum (red) and when fermenting (blue), and percentages of proteins involved in the typical flor velum formation stresses (darker colours).

Among proteins related to the oxidative stress response, high proportions of proteins were involved in processes like: cell wall organization or biogenesis (Ecm33p, Act1p, Mtl1p

and Gas1p), ribosome assembly (Nsr1p, Rpl25p and Rpl5p) or DNA-templated transcriptional elongation (Tfg1p, Asf1p and Npl6p). All proteins related to the response of lack of a fermentable carbon source are involved in the biofilm formation with the exception of Mtl1p, known to be required for general stress response activation under conditions of glucose starvation and also in the resistance to the oxidative stress (Ivanova et al., 2010; Vilella et al., 2005). Eighteen proteins found to be related with more than one biological aging stress plus only eight additional transcription-related proteins were detected or more abundant under BFC or Biofilm Formation Condition. All of these mentioned proteins would be potential targets for over-expression aiming to improve a strain that faces better the adverse biological aging conditions.

Furthermore, abundant proteins if compared with the reference fermentative condition are implied in the bioconversion of the carbon source (ethanol) to other compounds, such as polysaccharides, amino acids (glycine, isoleucine and arginine) or folate (Appendix 8). Gcv3p, found in high amounts, is involved in the glycine catabolism and connects the metabolism of one-, two- and three-carbon compounds (Sinclair et al., 1995; 1996; Ogur et al., 1977).

When flor yeasts were fermenting at the time of the exponential growth, proteins associated with cell division and gene transcription were more abundant than when forming biofilm.

4.2. Objective 2. To study flor yeast applications: improvement of the sensorial quality of wines and the alcoholic fermentation processes to obtain new products.

Once the ability of flor yeasts to tolerate the stresses and consume ethanol under natural and synthetic biological aging conditions were confirmed and studied, we utilized their skills as an alternative to improve the organoleptic properties of red wines and to enhance the alcoholic fermentations.

> ***4.2.1.*** To use the flor yeast velum for modulating colour, ethanol and major aroma compound contents in red wine.

During the last decades, a lag between industrial and phenolic grape ripeness because of global warming has been observed. To obtain an acceptable colour is only possible when the ethanol content of wine is high. At the same time, an actual trend of marketing low ethanol content wines has arised. To overcome such situations, the consumption of ethanol by flor yeasts can be seen as a suitable alternative as it is compatible with completing must fermentations until a certain ethanol content and later be reduced until 12–13% which is general for table wines (Fleet, 2008; Swiegers et al., 2005; Ciani et al., 2016).

We aimed to optimize the conditions for the maximum ethanol consumption and the minimum acetaldehyde production, in a short time period. Results obtained has been published in the international scientific journal "Food Chemistry" as indicated (Appendix 10):

Appendix 10. Moreno, J., Moreno-García, J., López-Muñoz, B., Mauricio, J. C., Martínez-García, T. (2016). Use of a flor velum yeast for modulating color, ethanol and major aroma compound contents in red wine. *Food Chemistry*. *213*, 90-97.

For such purpose, a young red wine from the 2014's vintage with a high ethanol content (14.5% v/v) and total

polyphenols index (TPI) of 74.12 was inoculated with 5×10^6 cells/mL of the flor yeast *S. cerevisiae* G1 (ATCC: MYA-2451). Experimental methods tried to simulate the habitual practice of winemakers during the traditional biological aging process. Further, sampling was made when ethanol content reached a content of 12.5 ± 0.5% (v/v), which occurred 40 days after the wine surface was completely covered with the flor velum. Enological parameters, quantification of major aroma compounds and statistical analysis were made as indicated in the "Material and methods" section. Finally, OAVs, OS and sensory analyses were performed for the biologically aged wines.

In this work, it was shown that flor yeast which grow in a short time under velum aging conditions (Figure 24), decreases the ethanol and volatile acidity contents, has a favourable effect on the colour and astringency and significantly changes the wine content of metabolites like 1-propanol, isobutanol, acetaldehyde, 1,1-diethoxiethane and ethyl lactate. From the sensory analysis, chemical, fat and floral aromas were less perceived and were well accepted by young consumers, thus suggesting that flor yeasts can be used as fining agent and supports new perspectives for the elaboration of a new wine type. The favourable effect on the colour is due to the high content of acetaldehyde which mediates the combination among anthocyanins and flavan-3-ols that result in a new and more stable pigment (vitisins) responsible of the red color of wines. Furthermore, the same reason could explain a decrease in perceived astringency through the increase of the polymeric pigments and encouragement of tannin modification.

Another factor that can benefits the colour of red wine is the solved oxygen consumption by flor yeasts and the retention of brown compounds in the cell walls that prevents or slow wine browning. In general, the aroma compounds quantified show a similar evolution than those obtained in studies carried out in our laboratories with white wines subjected to biological aging with same yeast and

experimental conditions (Cortés, et al., 1998; Moreno and Peinado, 2012; Muñoz et al., 2005, 2006; Peinado and Mauricio, 2009). Due to the high volatility, low threshold and high chemical reactivity, acetaldehyde has a strong influence on the organoleptic properties of wines subjected to the flor yeast.

Figure 24. Flor yeasts forming velum in a red wine-air interphase.

4.2.2. To study the factors affecting to the co-adhesion phenomena between yeasts and a filamentous fungus in the formation of biocapsules immobilization system.

Yeast immobilization allows higher cell densities than traditional fermentation methods, improves alcoholic fermentation processes, allows the reutilization of the biocatalyst, open new possibilities to improve industrial processes, etc. Biocapsules are defined as a yeast immobilization system that consists of hollow and spheric shape bodies wherein the hyphae of a filamentous fungus (i.e. *Penicillium chrysogenum*) serves as an inert support facilitating the entrapment, attachment and adherence of yeast cells (Peinado et al. 2004). A recent review has been published

by Gallo et al. (2016) about this immobilization system and its applications.

From previous studies (Peinado et al., 2004; García-Martínez et al., 2011; 2012), it was observed that flor yeast strains resulted to be the best at forming the most consistent biocapsules when compared to non-flor yeast strains. The capacity of these yeasts to form biofilm or the mere fact to attach to other surrounding yeast cells, like flocculent yeasts, may explain the higher consistency of the biocapsules formed.

To clarify which of the factors impact the co-adhesion with the fungus hyphae, biofilm formation or flocculation, a screening of strains able to form biofilm and/or flocculate was carried out and biocapsules were made with those strains showing different biofilm/flocculation patterns. For this purpose, we collaborated with the Department of Viticulture and Enology of the University of California, Davis (institution hosting one of the largest collection of wine yeast strains in the world) that provided the yeasts to be tested. Biocapsule parameters like percentage of immobilized yeasts, number of biocapsules, total volume, diameter, consistency and dry weight, were evaluated.

Currently, we are working on a manuscript to be submitted to a scientific international journal and which will be entitled as follows: "Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system" (Appendix 11).

The first results obtained from this research, allow to conclude that the ability of forming biofilm is positively correlated to the consistency of yeast-fungus biocapsules and that flocculant yeasts make more inconsistent, smaller biocapsules but in more abundance (Figure 26). It is hypothesized that flor yeasts form biofilm within the matrix of the fungus hyphae of biocapsule walls as the hyphae frame work environment is similar than the Sherry wine-air interphase were velum is formed. Hyphae framework may

retain oxygen from the agitation during biocapsule formation which is required to oxidatively metabolize the gluconic acid that is a non-fermentable carbon source such as ethanol in Sherry wines. This hypothesis is supported with the observation of an extracellular matrix in the velum biofilm formed by some strains (Zara et al. 2009) that is similar to that observed in the biocapsule walls among yeasts and the filamentous fungus (Peinado et al., 2006).

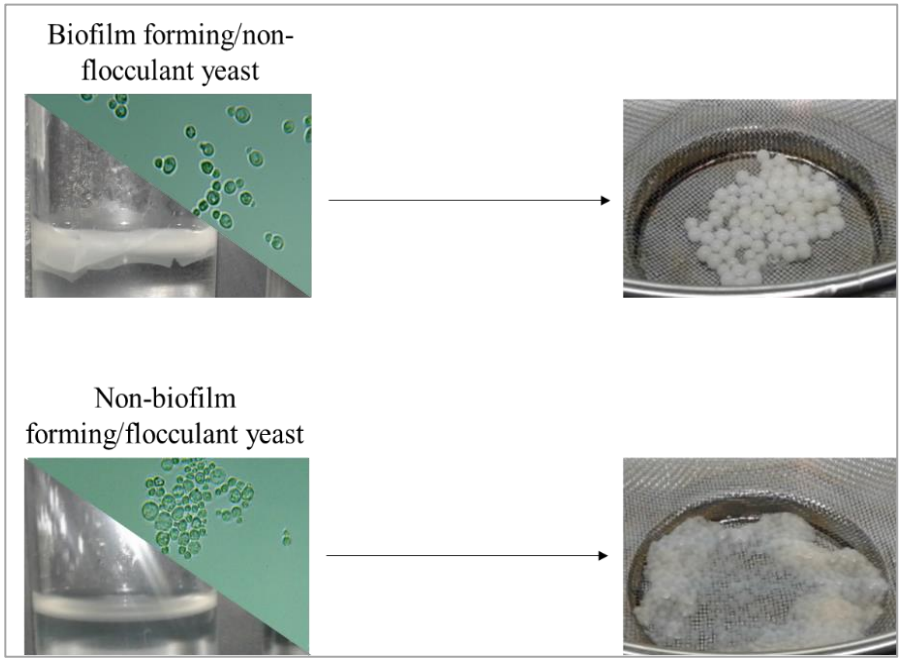


Figure 25. Biocapsules formed (right) with a biofilm forming/non-flocculant yeast (left up) and a non-biofilm forming/flocculant yeast (left down).

The yeast's competence to form biofilm, allowing it to strongly attach to the fungus hyphae and produce extracellular polymers that facilitate matrix formation, may explain the strong resistance conferred by the biocapsules to withstand the forces applied without breaking. On the other hand, flocculation is a homotypic process involving only one type of cell in the interactions (Stewart, 2009; Stratford, 1992), thus, not including *P. chrysogenum*. Flocculent yeasts inoculated in medium such as the yeast immobilization medium, allow for strong yeast to yeast cell attachments but

relatively weak cell to fungus hyphae attachments. In this study, these yeast cells were found to adhere to the filamentous fungus hyphae rather than to each other. This can be speculated to be because the fungal hyphae acts as platform to where yeast can initiate flocculation.

Moreover, it should be noted that specific lectin-like proteins are only present on flocculent cells. These proteins recognize and interact with carbohydrate residues of α-mannans (receptors) of neighboring cells (Miki et al. 1982). While flocculation lectins are only present in flocculent cells, the α-mannans are present in flocculent and non-flocculent cells, such as *P. chrysogenum*. This phenomenon explains the stronger connections among flocculent yeasts interacting with lectins and receptors from both sides while, on the other hand, explains the weaker connection among the yeasts and fungus hyphae that are only interacting with lectins and receptors from the one of the sides. Yeasts may also start to make flocs, impeding the filamentous fungus to grow in size and thus resulting in smaller, but more numerous biocapsules.

Yeasts that flocculate in agitation congregate with each other and form flocs that can potentially interfere with the fungus and make inconsistent biocapsules.

5. CONCLUSIONS

Identification of the metabolic and proteomic responses of non-*Saccharomyces* and *Saccharomyces* yeasts, to several stress conditions of enological interest.

The conclusions obtained to the aim 1.1, by analysis of the excreted metabolites in pure cultures of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*, grown in synthetic media with high sugar contents, were extracted from:
Moreno-García et al. (2017). Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts. To be published in Q1 journal in Food Science and Technology. (Appendix 1)

- *T. delbrueckii* has a longer lag phase than *S. cerevisiae*, and produces higher levels of succinic acid and lower contents of ethanol, acetaldehyde, acetoin, 2,3-butanediol, glycerol and acetic acid.
- *T. delbrueckii* and *S. cerevisiae* show different strategies to overcome the osmotic stress derived from the high sugar content in the medium and directs different glucose levels to the shynthesis of glycerol and by-products.
- *T. delbrueckii* can be regarded as a good candidate to elaborate sweet wines with low acidity and low alcoholic content.

The conclusions for the aim 1.2, about the identification of the influence of media with high ethanol and glycerol contents were obtained from:
Moreno-García, et al. (2013). *Food Res. Int.* 54, 285–292. DOI: 10.1016/j.foodres.2013.07.031. (Appendix 2).

- A kinetic model, based in the evolution of glycerol content, allows calculating the required time to achieve a specific glycerol concentration and also its content after a known aging time. The model is useful to control the biological aging process of sherry wines in the cellars.

On the other hand, the conclusions obtained from the study about the identification of the influence of media with high sugar contents and media with high ethanol and glycerol contents, on the proteins expressed and the small metabolites excreted by a *S. cerevisiae* flor velum-forming yeast strain, were extracted from the appendix 3-9 and compiled in Appendix 12, DOI: 10.3389/fmicb.2016.00503.

Moreno-García, et al. (2014). *Int. J. Food Microbiol.* 172, 21-29. DOI: 10.1016/j.ijfoodmicro.2013.11.030. (Appendix 3)

Moreno-García, et al. (2015). *Food Microbiol.* 46, 25-33. . DOI: 10.1016/j.fm.2014.07.001. (Appendix 4)

Moreno-Garcia, et al. (2015). *Food Microbiol*. 51, 1-9. DOI: 10.1016/j.fm.2015.04.005. (Appendix 5)

Moreno-García, J., et al. (2016). *Process Biochem*. 51, 578–588. DOI: 10.1016/j.procbio.2016.02.011. (Appendix 6)

Moreno-García, J., et al. (2016). *Data Brief.* 7, 1021–1023. DOI: 10.1016/j.dib.2016.03.072. (Appendix 7)

Moreno-García, et al. (2017). *Int. J. Mol. Sci.* 720, 1-18. DOI: 10.3390/ijms18040720. (Appendix 8)

Moreno-García et al. (2017). Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain. To be published in Q1 journal in Food Science and Technology. (Appendix 9)

- The proteome analysis provides knowledge about some specific features of the flor yeast, and reveals that proteome remodeling under biofilm-forming conditions is also related to the production of aroma compounds. Appendix 5, DOI: 10.1016/j.fm.2015.04.005.
- Qualitative and quantitative differences in the proteins involved in the metabolism of glycerol, ethanol, higher alcohols, acetaldehyde and its derivatives, organic acids and ethyl esters, has been revealed for the flor yeast growing in two studied media. Appendix 5, DOI: 10.1016/j.fm.2015.04.005.
- Under velum-formation conditions, associated proteins to non-fermentable carbon uptake, glyoxylate and

tricarboxylic acid cycles (TCA), cellular respiration and inositol metabolism, were detected at higher concentrations than under fermentative conditions. Appendix 4, DOI: 10.1016/j.fm.2014.07.001.

- By compiling the proteomic data, it can be considered that *S. cerevisiae* flor yeast, at the early stages of velum formation, shows an oxidative metabolism and builds a protein machinery that prevents and protects the yeast against the reactive oxygen species coming from the respiration. On the other hand, gene transcription and cell division proteins prevailed under fermentative condition, indicating that yeast reproduction is highly active at this point. Appendix 8, DOI: 10.3390/ijms18040720.
- Flor yeast when forming biofilm, synthetize mitochondrial proteins that participate mainly in processes like cell respiration, mitochondrial genome maintenance and ethanol resistance. Appendix 3, DOI: 10.1016/j.ijfoodmicro.2013.11.030
- Cell wall glycoproteins Ccw14p and Ygp1p, were over-expressed in the flor yeast velum. *YGP1* null mutant differed significantly with the wild type in terms of biofilm formation, thus, concluding that this protein is associated with velum biofilm formation while no significant differences were reported in regards to the *CCW14* null mutant and the wild type. Appendix 9, to be published.
- Proteins related to the oxidative stress response were the most numerous among other stress proteins under biofilm-formation condition. In addition, more proteins were found to be involved in the ethanol stress response than those involved in response to acetaldehyde. Treated in appendix 6, DOI: 10.1016/j.procbio.2016.02.011, and 7, DOI: 10.1016/j.dib.2016.03.072

Application of flor yeasts: improvement of the sensorial quality of wines and the alcoholic fermentation processes to obtain new products.

The conclusions obtained from the objective 2.1, about the use of flor yeast velum for modulating colour, ethanol and major aroma compound contents in red wine were extracted from:
Moreno, J. et al. (2016). Use of a flor velum yeast for modulating color, ethanol and major aroma compound contents in red wine. *Food Chem.* 213, 90-97. DOI: 10.1016/j.foodchem.2016.06.062. (Appendix 10)

- Red wines subjected to a short time of flor velum growing showed better color and tonality values than the initial wine. There were decreases in ethanol, glycerin and acetic acid and increases in acetaldehyde and 1,1-diethoxyethane contents, leading to a change in the aroma profile. These changes were well evaluated by the panel judges by means of a sensorial test.
- Flor velum yeasts can be used as fining agents, increasing the red colour stability and decreasing astringency and ethanol content of red wines, thus, supporting new perspectives for the elaboration of new wine types.

The following conclusions were obtained from the study of the factors affecting the co-adhesion phenomena between yeasts and a filamentous fungus, in the formation of biocapsules immobilization system:
Moreno-García et al. (2017). Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system. To be published in Q1 journal in Food Science and Technology. (Appendix 11)

- The co-immobilization between *S. cerevisiae* yeast strains and the filamentous fungus *P. chrysogenum* to form biocapsules, is dependent on the yeast ability to flocculate and aggregate to form biofilms.

- Biofilm forming yeast strains show higher rates of immobilization and form spheric biocapsules with a bigger diameter and more consistent, while strains able to flocculate form more abundant biocapsules but with smaller diameter and less consistent.

Final considerations

As a general conclusion, it is established that endocellular proteins and metabolites excreted to the medium are related to the specie and yeast strain and the physicochemical characteristics of the growing environment. The study of the expression of certain proteins as adaptation markers to the different media opens new perspectives for the selection and improvement of yeast´s species of interest in the elaboration of new fermented beverages and/or in the production of bioethanol.

Moreover, with this thesis, we shed light on the flor yeast abilities to consume ethanol and to form biofilm and we provided information for further apply these *S. cerevisiae* strains to modulate ethanol content in wines and to improve the yeast immobilization technologies, thus, going forward on basic and applied sciences.

Identificación de las respuestas metabólicas y proteómicas de levaduras no-*Saccharomyces* y *Saccharomyces* a condiciones de estrés de interés enológico.

Los resultados obtenidos mediante el análisis de metabolitos excretados por *Torulaspora delbrueckii* y *Saccharomyces cerevisiae*, crecidas en cultivos puros en un medio sintético de alto contenido en azúcar, han permitido establecer las siguientes conclusiones, extraidas del apéndice 1.
Moreno-García et al. (2017). Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts. To be published in Q1 journal in Food Science and Technology. (Apéndice 1)

- *T. delbrueckii* presenta una fase de latencia más prolongada y produce mayores contenidos de ácido succínico y menores de etanol, acetaldehído, acetoína, 2,3-butanodiol, glicerol y ácido acético que *S. cerevisiae*.
- *T. delbrueckii* y *S. cerevisiae* muestran diferentes estrategias para superar el estrés osmótico derivado del alto contenido de azúcar en el medio y destinan diferentes cantidades de glucosa al anabolismo de glicerol y productos secundarios.
- El uso de *T. delbrueckii* constituye una buena opción para elaborar vinos dulces con baja acidez volátil y contenido alcohólico aunque su uso como único inóculo puede provocar paradas en la fermentación.

Las conclusiones para el estudio del objetivo 1.2, sobre la influencia del alto contenido en etanol y glycerol sobre los metabolitos del vino durante la crianza biológica en bodega, mediante el sistema tradicional de criaderas y solera, se extrajeron del artículo:
Moreno-García, et al. (2013). *Food Res. Int.* 54, 285–292. DOI: 10.1016/j.foodres.2013.07.031. (Apéndice 2).

- Mediante un modelo cinético, basado en la evolución del contenido de glicerol, es posible calcular el tiempo de

crianza necesario para alcanzar una concentración determinada de glicerol y también su contenido después de un tiempo conocido. El modelo es útil para controlar el proceso de crianza biológica de los vinos tipo sherry.

Por otro lado, las conclusiones obtenidas del estudio sobre la influencia de medios con alto contenido de azúcares y medios con altos contenidos de etanol y glicerol sobre las proteínas endocelulares y los metabolitos del aroma excretados por la levadura formadora de velo de flor de *S. cerevisiae*, se extrajeron de los apéndices 3-9 y se recopilaron en el Apéndice 12, DOI: 10.3389/fmicb.2016.00503.

Moreno-García, et al. (2014). *Int. J. Food Microbiol*. 172, 21-29. DOI: 10.1016/j.ijfoodmicro.2013.11.030. (Apéndice 3)

Moreno-García, et al. (2015). *Food Microbiol*. 46, 25-33. . DOI: 10.1016/j.fm.2014.07.001. (Apéndice 4)

Moreno-Garcia, et al. (2015). *Food Microbiol*. 51, 1-9. DOI: 10.1016/j.fm.2015.04.005. (Apéndice 5)

Moreno-García, J., et al. (2016). *Process Biochem*. 51, 578–588. DOI: 10.1016/j.procbio.2016.02.011. (Apéndice 6)

Moreno-García, J., et al. (2016). *Data Brief*. 7, 1021–1023. DOI: 10.1016/j.dib.2016.03.072. (Apéndice 7)

.Moreno-García, et al. (2017). *Int. J. Mol. Sci*. 720, 1-18. DOI: 10.3390/ijms18040720. (Apéndice 8)

Moreno-García et al. (2017). Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain. To be published in Q1 journal in Food Science and Technology. (Apéndice 9)

- El análisis del proteoma revela características específicas de la levadura de flor cuando crece formando velo y una remodelación del proteoma que está relacionada con la producción de compuestos del aroma. Apéndice 5, DOI: 10.1016/j.fm.2015.04.005.
- Se obtienen diferencias cuali y cuantitativas en las proteínas implicadas en el metabolismo de glicerol, etanol, alcoholes

superiores, acetaldehído y sus derivados, ácidos orgánicos y ésteres etílicos dependiendo del medio inoculado. Apéndice 5, DOI: 10.1016/j.fm.2015.04.005.

- Las levaduras desarrolladas en condiciones de formación de velo presentan mayores contenidos que en condiciones fermentativas, de las proteínas asociadas al consumo de fuentes de carbono no fermentables, al ciclo del ácido glioxilico y de los ácidos tricarboxílicos (TCA), a la respiración celular y al metabolismo de inositol. Apéndice 4, DOI: 10.1016/j.fm.2014.07.001.
- La compilación de los datos proteómicos permite deducir que la levadura de flor *S. cerevisiae* muestra un metabolismo oxidativo en las primeras etapas de la formación del velo y construye una estructura proteíca que previene y protege a la levadura de las especies reactivas de oxígeno. Por otro lado, las proteínas de transcripción genética y de división celular predominan en la fermentación, lo que indica que la reproducción de la levadura es más activa. Apéndice 8, DOI: 10.3390/ijms18040720.
- Cuando la levadura de flor forma velo sintetiza proteínas mitocondriales que participan en procesos como la respiración celular, el mantenimiento del genoma mitocondrial y la resistencia al etanol. Apéndice 3, DOI: 10.1016/j.ijfoodmicro.2013.11.030
- Las glucoproteínas de la pared celular Ccw14p e Ygp1p, se encontraron sobre-expresadas en la levadura de flor cuando forma velo. El mutante nulo de *YGP1* difirió significativamente del tipo silvestre respecto a la formación de velo, lo que permite concluir que esta proteína está asociada a su formación, mientras que no se obtuvieron diferencias significativas con respecto al mutante nulo de *CCW14*. Apéndice 9, pendiente de publicación.
- En condiciones de formación de velo se observan más proteínas relacionadas con la respuesta al estrés oxidativo que a otros tipos de estrés. También se detectan más

proteínas de respuesta al contenido en etanol que de respuesta a acetaldehído. Extraídas de los apéndices 6, DOI: 10.1016/j.procbio.2016.02.011, y 7, DOI: 10.1016/j.dib.2016.03.072

Aplicaciones de las levaduras de flor: mejora de la calidad sensorial de los vinos y de los procesos de fermentación alcohólica para la obtención de nuevos productos.

Las conclusiones obtenidas para el estudio del objetivo 2.1, sobre el uso de la levadura de velo de flor para modular el contenido en etanol, el color y los aromas mayoritariso de los vinos tintos fueron extraidas del artículo:

Moreno, J. et al. (2016). Use of a flor velum yeast for modulating color, ethanol and major aroma compound contents in red wine. *Food Chem.* 213, 90-97. DOI: 10.1016/j.foodchem.2016.06.062. (Apéndice 10).

- Los vinos tintos sometidos a un corto período de crecimiento de levadura bajo velo de flor, mostraron un mejor color rojo y tonalidad que el vino inicial. Se obtuvo un descenso del contenido en etanol, glicerina y ácido acético y un aumento de los contenidos en acetaldehído y 1,1-dietoxietano, que producen un cambio en el perfil del aroma. Estos cambios fueron positivamente evaluados por catadores en pruebas sensoriales.
- Las levaduras de velo de flor pueden utilizarse como agentes de acabado para aumentar la estabilidad del color rojo y disminuir la astringencia y el contenido de etanol de los vinos tintos. Este hecho abre nuevas perspectivas para aumentar la diversificación de vinos.

Las siguientes conclusiones, sobre los factores que afectan los fenómenos de co-adhesión entre las levaduras y el hongo filamentoso (GRAS) *Penicillium chrysogenum*, en la formación del sistema de inmovilización de las biocápsulas, fueron extraidas de:

Moreno-García et al. (2017). Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system. To be published in Q1 journal in Food Science and Technology. (Apéndice 11).

- La co-inmovilización entre las cepas de levadura *S. cerevisiae* y el hongo filamentoso *P. chrysogenum* para formar biocápsulas, depende de la capacidad de la levadura para flocular y para formar biofilm.
- Las cepas de levadura que forman biofilm poseen una mayor tasa de inmovilización y forman biocápsulas esféricas más consistentes y de mayor diámetro, mientras que las cepas capaces de flocular forman biocápsulas más abundantes pero con menor consistencia y diamétro.

Consideraciones finales

Como conclusión general se establece que las proteínas endocelulares y los metabolitos excretados por las levaduras están relacionados con la especie y cepa de levadura y las características físico-químicas del medio donde se desarrollan. El estudio de la expresión de ciertas proteínas como indicadores de la adaptación a los diferentes medios abre nuevas perspectivas para la selección y mejora de especies de levaduras de interés en la elaboración de nuevas bebidas fermentadas y/o en la obtención de bioetanol.

Con esta tesis, hemos pretendido avanzar en el conocimiento de la capacidad de las levaduras de flor para consumir etanol y formar biofilm. La información obtenida se ha aplicado a la modulación del contenido en etanol de los vinos y a la mejora de la tecnología de inmovilización de levaduras, estableciendo de esta manera conexiones entre las ciencias básicas y aplicadas.

6. REFERENCES

6. REFERENCES

Alañón, M.E., Pérez-Coello, M.S. and Marina, M.L. (2015). Wine Science in the metabolomics era. Trends Anal. Chem., 74: 1-20.

Albertyn, J., Hohmann, S., Thevelein, J.M. and Prior, B.A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase is essential for growth under osmotic stress in *Saccharomyces cerevisiae* and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol., 14: 4135-4144.

Alexander, M.A. and Jeffries, T.W. (1990). Respiratory efficiency and metabolite partitioning as regulatory phenomena in yeasts. Enzyme Microb. Tech., 12: 2-19.

Alexandre, H. (2013). Flor yeasts of *Saccharomyces cerevisiae*-their ecology, genetics and metabolism. Int. J. Food. Microbiol., 167: 269-75.

Allen J., Davey , H.M., Broadhurst D., Heald J.K., Rowland J.J., Oliver S.G. and Kell, D.B. (2003). High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. Nat. Biotechnol., 21(6):692-6.

Andersen, K.S., Bojsen, R., Sørensen, L.G. R., Nielsen, M.W., Lisby, M., Folkesson, A. and Regenberg, B. (2014). Genetic basis for *Saccharomyces cerevisiae* biofilm in liquid medium. G3 (Bethesda), 4: 1671-1680.

Angioni, A., Caboni, P., Garau, A., Farris, A., Orro, D., Budroni, M. and Cabras, P. (2007). In vitro interaction between Ochratoxin A and different strains of *Saccharomyces cerevisiae* and *Kloeckera apiculata*. J. Agric. Food Chem. 55 (5), 2043-2048.

Anonymous (2004a). Viniflora HARMONY.nsac. Product information CHR Hansen A/S, 10-12 Bøge All e, DK-2970 Hørsholm (Denmark).

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M. and Adler, L. (1997). The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J 16, 2179-2187.

Aranda, A. and del Olmo, M.L. (2003). Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several *ALD* genes and is mediated by the general stress response pathway. Yeast, 20: 747-759.

Arbulu, M. (2014). Development of analytical methodologies applied to metabolomic studies in Graciano wine variety. Doctoral Thesis. University of País Vasco, (Spain).

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F. and Zapparoli, G. (2015). Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol., 31: 277-293.

Baker, G.A., Amerine, M.A. and Roessler, E.B. (1952). Theory and application of fractional-blending systems. Hilgardia, 21, 383-409.

Baptista, C., Cóias, J.M.A., Oliveira, A.C.M., Oliveira, N.M.C., Roche, J.M.S. and Dempsey, M.J. (2006). Natural immobilization of microorganisms for continuous ethanol production. Enzyme Microbiol. Technol., 40: 127-131.

Bardi, E., Koutinas, A.A. and Kanellaki, M. (1997). Room and low temperature brewing with yeast immobilized on gluten pellets. Process Biochem., 32: 691-696.

Baumes, R. (2009). Wine aroma precursors. In: Wine chemistry and biochemistry. Springer, New York, NY (U.S.A.). pp: 251-274.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I. and Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii*-*Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol., 122: 312-320.

Benitez, T., Rincon, A.M. and Codon, A.C. (2011). Yeasts used in biologically aged wines. In: Carrascosa Santiago, A.V., Munoz, R. and Gonzalez-García, R. (Eds.). Molecular Wine Microbiology. Academic Press. pp: 51-84.

Benito, A., Calderón, F., Palomero, F.E. and Benito, S. (2015). Combineuse of selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. Molecules, 20: 9510-9523.

Benito, S., Palomero, F., Calderón, F., Palmero, D. and Suárez-Lepe, J.A. (2013). Selection of appropriate *Schizosaccharomyces* strains for winemaking. Food Microbiol., 42: 218-224.

Berger, R.G. (Eds.) (2007). Flavours and fragrances chemistry, bioprocessing and sustainability. Springer, Berlin (Germany).

Berlanga, T., Millán, C., Mauricio, J.C. and Ortega, J.M. (2006). Influence of nitrogen on biological aging of sherry wine. J. Agric. Food Chem., 86: 2113-2118.

Bisson, L.F. and Kunkee, R.E. (1991). Zeikus, J.G. and Johnson, E.A., (Eds.). Microbial interactions during wine production. Mixed cultures in biotechnology. McGraw-Hill, Inc., New York, NY (U.S.A.). pp: 39-68.

Blandino, A., Caro, I. and Cantero, D. (1997). Comparative study of alcohol dehydrogenase activity in flor yeast extracts. Biotechnol. Lett., 19: 651-654.

Blein-Nicolas, M., Albertin, W., Valot, B., Marullo, P., Sicard, D., Giraud, C., Huet, S., Bourgais, A., Dillmann, C., de Vienne, D. and Zivy, M. (2013). Yeast proteome variations reveal different adaptive responses to grape must fermentation. Mol. Biol. Evol., 30:, 1368-1383.

Borneman, A.R., Chambers, P.J. and Pretorius, I.S. (2007). Yeast systems biology: modelling the winemaker's art. Trends Biotechnol., 25: 349-355.

Bowen, B.P. and Northen, T.R.J. (2010). Dealing with the unknown: metabolomics and metabolite atlases. Am. Soc. Mass Spectrom., 21: 1471-1476.

Braconi, D., Bernardini, G., Possenti, S., Laschi, M., Arena, S., Scaloni, A., Geminiani, M., Sotgiu, M. and Santucci, A. (2008). Proteomics and redox-proteomics of the effects of herbicides on a wild-type wine *Saccharomyces cerevisiae* strain. J. Proteome Res., 8: 256-267.

Bravo, F. (1995). Del vino y otros temas. Madrid, Spain: EYPASA.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E. and Ros, J. (2000). Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. J. Biol. Chem., 275: 27393-27398.

Cadahia, E., Fernandez de Simon, B., Sanz, M., Poveda, and Colio, P. (2009). Chemical and chromatic characteristics of Tempranillo, Cabernet Sauvignon and Merlot wines from DO Navarra aged in Spanish and French oak barrels. J. Food Chem., 115: 639-649.

Callejon, R.M., Gonzalez, A.G., Troncoso, A.M. and Morales, M.L. (2008). Optimization and validation of headspace sorptive extraction for the analysis of volatile compounds in wine vinegars. J. Chromatogr. A, 1204: 93-103.

Capozzi, V., Garofalo, C., Chiriatti, M.A., Grieco, F. and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. Microbiol. Res., 181: 75-83.

Capozzi, V., Ladero, V., Beneduce, L., Fernández, M., Alvarez, M.A., Benoit, B., Laurent, B., Grieco, F. and Spano, G. (2011). Isolation and characterization of tyramine-producing *Enterococcus faecium* strains from red wine. Food Microbiol., 28: 434-439.

Caruso, M., Capece, A., Salzano, G. and Romano, P. (2002). Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine. Lett. Appl. Microbiol., 34: 323-328.

Casas, J.F. (1985). Descripción resumida de la técnica enológica de los vinos de Jerez. III Jornadas Universitarias sobre el Jerez. Press 84-600-4052-6. University of Cádiz, Cádiz (Spain). pp: 333-361.

Castelli, T. (1955). Yeasts of wine fermentations from various regions of Italy. Am. J. Enol. Vitic., 6: 18-20.

Castro, R., Natera, R., Duran, E. and Garcia-Barroso, C. (2008). Application of solid phase extraction techniques to analyse volatile compounds in wines and other enological products. Eur. Food Res. Technol., 228: 1-18.

Castro-Puyana, M. and Herrero, M. (2013). Review: metabolomics approaches based on mass spectrometry for food safety, quality and traceability. Trends Anal. Chem., 52: 74-87.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell., 12: 323-337.

Caven-Quantrill, D.J. and Buglass, A. (2011). Comparison of volatile constituents extracted from model grape juice and model wine by stir bar sorptive extraction–gas chromatography–mass spectrometry. J. Chromatogr. A, 1218: 875-881.

Cevallos-Cevallos, J.M. Reyes de Corcuera, J.I., Etxeberria, E., Danyluk, M.D. and Rodrick, G.E. (2009). Metabolomic analysis in food science: a review. Trends Food Sci.Technol., 20: 557-566.

Charpentier, C., Dos Santos, A.M. and Feuillat, M. (2002). Contribution à l'étude du métabolisme des levures à voile dans l'élaborationdes vins jaunes du Jura. Rev. Fr. d'oenologie 195, 33-36.

Charpentier, C., Dos Santos, A.M. and Feuillat, M. (2004). Release ofmacromolecules by *Saccharomyces cerevisiae* during ageing of French flor sherry wine "Vin Jaune". Int. J. Food Microbiol., 96: 253-262.

Chatonnet, P., Boidron, J.N. and Pons, M. (1990). Élevage des vins rouges es fût de chene: évolution de certains composés volatils et de leur impact aromatique. Science Aliments, 10: 565-578.

Chatonnet, P., Viala, C. and Dubourdieu, D. (1997). Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. Am. J. Enol. Vitic., 48: 443-448.

CHR Hansen (2013a). VINIFLORA Yeast specialities. www.chr-hansen.com/products/product-areas/wine-ingredients (accessed 17 May 2013).

CHR Hansen (2013b). 'FrootZenTM' - the first Pichia kluyverii yeast for winemakers. www.chr-hansen.com/products/product-areas/wine-ingredients/specialty-yeast-pichia-kluyveri-frootzen.html (accessed 20 May 2013).

Ciani, M. and Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. World J. Microbiol. Biotechnol., 14: 199-203.

Ciani, M. and Picciotti, G. (1995). The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. Biotechnol. Lett., 17: 1247-1250.

Ciani, M., Beco, L. and Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multi starter wine yeast fermentations. Int. J. Food Microbiol., 108: 239-245.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G. and Romano, P. (2016). Yeast interactions in inoculated wine fermentation. Front. Microbiol., 7: 1-32.

Ciani, M., Comitini, F., Mannazzu, I. and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. FEMS Yeast Res., 10: 123-133.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J. and Rodríguez-Vico, F. (2004). Influence of sequential yeast mixtures on wine fermentation. Int. J. Food Microbiol., 98: 301-308.

Coelho, E., Coimbra, M.A., Nogueira, J.M.F. and Rocha, S.M. (2009). Quantification approach for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties by stir bar sorptive extraction with liquid desorption. Anal. Chim. Acta, 635: 214-221.

Coi, A.L., Legras, J.L., Zara, G., Dequin, S. and Budroni, M. (2016). A set of haploid strains available for genetic studies of *Saccharomyces cerevisiae* flor yeasts. FEMS Yeast Res., 16: fow066

Collin, S., Nizet, S., Claeys Bouuaert, T. and Despatures, P. M. (2011). Main odorants in Jura flor-sherry wines. Relative contributions of sotolon, abhexon, and theaspirane-derived compounds. J. Agric. Food Chem., 60(1), 380-387.

Comitini, F. and Ciani, M. (2011). *Kluyveromyces wickerhamii* killer toxin: purification and activity towards *Brettanomyces*/*Dekkera* yeasts in grape must. FEMS Microbiol. Lett. 316: 77-82.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I. and Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol., 28: 873-882.

Cortés, M.B. (2002). Aplicación de cultivos puros de *Saccharomyces cerevisiae* a la crianza biológica de vinos finos de la D.O. Montilla-Moriles. Doctoral Thesis. University of Córdoba (Spain).

Cortés, M.B., Moreno, J., Zea, L., Moyano, L. and Medina, M. (1998). Changes in aroma compounds of sherry wines during their biological aging carried out by *Saccharomyces cerevisiae* races *bayanus* and *capensis*. J. Agric. Food Chem., 46: 2389-2394.

Cortes, S. and Diaz, E. (2011). Characterization of autochthonous monovarietal wines from red Brancellao L. cultivars. J. Food Compos. Anal., 24: 154-159.

Costello, P.J., Henschke, P.A. and Markides, A.J. (2003). Standardised methodology for testing malolactic bacteria and wine yeast compatibility. Aust. J. Grape Wine Res., 9: 127-137.

Cox, A., Skouroumounis, G.K., Elsey, G.M., Perkins, M.V. and Sefton, M.A. (2005). Generation of (E)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene from C13-norisoprenoid precursors. J. Agric. Food Chem., 53: 6777-6783.

Curwin, A.J. Fairn, G.D. and McMaster, C.R. (2009). Phospholipid transfer protein sec14 is required for trafficking from endosomes and regulates distinct trans-golgi export pathways. J. Biol. Chem. 284 7364-7375.

Da Silva-Ferreira, A.C., Barbe, J.C. and Bertrand, A. (2002). Heterocyclic acetals from glycerol and acetaldehyde in port wine: Evolution with aging. J. Agric. Food Chem., 50, 2560-2564.

Dashko, S., Zhou, N., Compagno, C. and Piškur J. (2014). Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Res., 14: 826-832.

Daviss, B. (2005). Growing pains for metabolomics: the newest 'omic science is producing results and more data than researchers know what to do with. Scientist, 19: 25-28.

De Benedictis, M., Bleve, G., Grieco, F., Tristezza, M., Tufariello, M. and Grieco, F. (2011). An optimized procedure for the enological selection of non-*Saccharomyces* starter cultures. Anton. Leeuw., 99: 189-200.

Delfini, C. (1987). Observations expérimentals sur l'origine et la dispa-rition de l'alcool benzylique et de l'aldéhyde bezöique dans les moûts et les vins. Bull. O.I.V., 60: 463-473.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M. and Oliva, D. (2012). Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. S. Afr. J. Enol. Vitic., 33: 80-87.

Ding, M.Z., Zhou, X. and Yuan, Y.J. (2010). Metabolome profiling reveals adaptive evolution of *Saccharomyces cerevisiae* during repeated vacuum fermentations. Metabolomics, 6: 42-55.

Dominguez, A.M. and Agosin, E. (2010). Gas chromatography coupled with mass spectrometry detection for the volatile profiling of *Vitis vinifera* cv. Carménère wines. J. Chil. Chem. Soc., 55: 385-391.

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I. and Ciani, M. (2011a). Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association *with Saccharomyces cerevisiae* for grape juice fermentation. Int. J. Food Microbiol., 147: 170-180.

Du Toit, W., Pretorius, I. and Lonvaud-Funel, A. (2005). The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. J. Appl. Microbiol., 98: 862-871.

Dubois, P., Rigaud, J. and Dekimpe, J. (1976). Identification de la diméthyl-4,5-terahydrofuranedione-2,3-dans le vin jaune du Jura. Lebensm.Wissen Technol. Food Sc. Technol., 9: 366-368.

Dubourdieu, D., Tominaga, T., Masneuf, I., Peyrot des Gachons, C. and Murat, M. (2006). The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc. Am. J. Enol. Vitic., 57: 81-88.

Dufossé, L., Latrasse, A. and Spinnler, H.E. (1994). Importance des lactones dans les arômes alimentaires: structure, distribution, propiétés sensorielles et biosynthese. Sci. Aliments, 14: 17-50.

Dufour, J.P., Malcorps, P.H. and Silcock, P. (2008). Control of ester synthesis during brewery fermentation. In: 2nd edn. Smart, K. (Eds.). Brewing yeast fermentation performance. Blackwell Science, Oxford (UK). Pp: 213-233.

Dunn, W.B. and Ellis, D.I. (2005). Metabolomics: current analytical platforms and methodologies. Trends Anal. Chem., 24: 285-294.

Ebeler, S.E. (2001). Analytical chemistry: Unlocking the secrets of wine flavor. Food Rev. Int., 17: 45-64.

Ebeler, S.E. and Sapaulding, R.S. (1998). Characterization and measurement of aldehydes in wine. In: Waterhouse, A.L. and Ebeler, S.E. (Eds). Chemistry of wine flavour. ACS Symposium Series. American Chemical Society, Washington DC (U.S.A.). Pp: 186-179.

Ebeler, S.E. and Thorngate, J.H. (2009). Wine chemistry and flavor: Looking into the crystal glass. J. Agric. Food Chem., 57: 8098-8108.

Ebeler, S.E., Takeoka, G.R. and Winterhalter, P. (2006). Authentication of Food and Wines. ACS Symposium Series 952, (Eds), American Chemical Society, Washington DC (U.S.A.).

Essader, A.S., Cargile, B.J., Bundy, J.L. and Stephenson, J.L., Jr. (2005). A comparison of immobilized pH gradient isoelectric focusing and strong-cation-exchange chromatography as a first dimension in shotgun proteomics. Proteomics, 5: 24-34.

Esteve-Zarzoso, B., Fernandez-Espinar, M.T. and Querol, A. (2004). Authentication and identification of *Saccharomyces cerevisiae* "flor" yeast races involved in sherry ageing. Anton Leeuw. 85, 151-158.

Esteve-Zarzoso, B., Peris-Toran, M.J., Garcia-Maiquez, E., Uruburu, F., Querol, A. (2001). Yeasts population dynamics during the fermentation and biological aging of sherry wines. Appl. Environ. Microbiol., 67: 2056-2061.

Etiévant, P.X. (1991). Wine. In: Maarse, H. (Eds.). Volatile compounds in foods and beverages. Marcel Decker, New York, NY (U.S.A.).

Fagan, G.L., Kepner, R.E. and Webb, A.D. (1981). Biosynthesis of certain γ-butyrolactones present in film sherries. Am. J. Enol. Vit-ic., 32: 163-167.

Farris, G.A., Sinigaglia, M., Budroni, M. and Elisabetta, M. (1993). Cellular fatty acid composition in film-forming strains of two physicological races of *Saccharomyces cerevisiae*. Lett. Appl. Microbiol., 17: 215-219.

Ferraro, L., Fatichenti, F, and Ciani, M. (2000). Pilot scale vinification process using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. Process Biochem., 35: 1125-1129.

Ferreira, V. (2005). Aroma, aromas y olores del vino. In:. 2nd edn. Agrícola Española and MAPA (Eds.), Casal del Rey, J. (Coordinator). Análisis sensorial y cata de los vinos de España. Madrid, (Spain). pp:: 206-247.

Fidalgo, M., Barrales, R.R. and Jimenez, J. (2008). Coding repeat instability in the *FLO11* gene of *Saccharomyces* yeasts. Yeast, 25: 879-889.

Fidalgo, M., Barrales, R.R., Ibeas, J. and Jimenez, J. (2006). Adaptive evolution by mutations in the *FLO11* gene. Proc. Natl. Acad. Sci., 103: 11228-11233.

Fiehn, O., Kind, T. and Barupal, D.K. (2011). Biology of plant metabolomics. Annu. Plant Rev., 43: 367-406.

Fiehn, O., Kopka, J., Dormann, P., Altmann, T. and Trethewey, R.N. and Willmitzer, L. (2000). Metabolite profiling for plant functional genomics. Nat. Biotechnol., 18: 1157-1161.

Flanzy, C. (2003). AMV-Mundi-Prensa (Eds.). Enología: Fundamentos Científicos y Tecnológicos. Madrid (Spain).

Fleet, G. (2003). Yeast interactions and wine flavour. Int. J. Food Microbiol., 86: 11-22.

Fleet, G.H. (2008). Wine yeasts for the future. FEMS Yeast Res., 8: 979-995.

Fleet, G.H. and Heard, G.M. (1993). Yeasts growth during fermentation. In: Fleet, G.H. (Eds.). Wine Microbiology and Biotechnology. Harwood Academic, Chur (Switzerland). pp: 27-54.

Fleet, G.H., Lafon-Lafourcade, S. and Ribéreau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux Wines. Appl. Environ. Microbiol., 48: 1034-1038.

Fornachon, J.C.M. (1968). Influence of different yeasts on the growth of lactic acid bacteria in wine. J. Sci. Food Agric., 19: 374-378.

Fragasso, M., Antonacci, D., Pati, S., Tufariello, M., Baiano, A., Forleo, L. R., Caputo, A.R. and La Notte, E. (2012). Influence of training system on volatile and sensory profiles of Primitivo grapes and wines. Am. J. Enol. Vitic., ajev-2012.

Franc, C., David, F. and de Revel, G. (2009). Multi-residue off-flavour profiling in wine using stir bar sorptive extraction–

thermal desorption–gas chromatography–mass spectrometry. J. Chromatogr. A, 1216: 3318-3327.

Franke, A.A., Custer, L.J., Cerna, C.M. and Narala, K.K. (1994). Quantitation of phytoestrogens in legumes by HPL. J. Agric. Food Chem., 42: 1905-1913.

Freeman, A. (1984). Gel entrapment of whole cells and enzymes in cross-linked, prepolymerized polyacrylamide hydrazide. Ann. N. Y. Acad. Sci., 434: 418-426.

Fröhlich, K.U. and Madeo, F. (2000). Apoptosis in yeast a monocellular organism exhibits altruistic behaviour. FEBS Lett., 473: 6-9.

Fugelsang, K.C. (1997). Wine Microbiology. Chapman and Hall Pub. Co, New York, NY (U.S.A.).

Fugelsang, K.C. and Edwards, C.G. (2007). Wine Microbiology: Practical Applicationsand Procedures. Springer, NY (USA.).

Gallo, M., Speranza, B., Corbo, M.R., Sinigaglia, M. and Bevilacqua, A. (2016). Novel microbial immobilization techniques. In: Novel Food Fermentation Technologies. Springer, Berlin (Germany). pp: 35-55.

García-Maiquez, E. (1988). Les levures de voile dans l' elaboration des vins de Xeres. In: Proceedings of Aapplication a l'oenologie des progres recents en microbiologie et en fermentation. OIV, Paris (France). pp: 341-351.

García-Maiquez, E. (1995). Microorganismos de los vinos de Jerez. Microbiol. Soc. Esp. Microbiol., 11: 51-58.

García-Martínez, T., López de Lerma, N., Moreno, J., Peinado, R.A., Millán, M.C. and Mauricio, J. C. (2013). Sweet wine production by two osmotolerant *Saccharomyces cerevisiae* strains. J. Food Sci,. 78: 74-879.

García-Martínez, T., Moreno, J., Mauricio, J.C., and Peinado, R. (2015). Natural sweet wine production by repeated use of yeast cells immobilized on *Penicillium chrysogenum*. LWT-Food Sci. Technol., 61: 503-509.

García-Martínez, T., Peinado, R.A., Moreno, J., García-García, I. and Mauricio, J.C. (2011). Co-culture of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* leading to the immobilization of yeast. J. Chem.Technol.Biotechnol., 86: 812-817.

García-Martínez, T., Puig-Pujol, A., Peinado, R.A., Moreno, J. and Mauricio, J.C. (2012). Potential use of wine yeasts immobilized on *Penicillium chrysogenum* for ethanol production. J. Chem.Technol. Biotechnol., 87: 351-359.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell., 11: 4241-4257.

Genisheva, Z., Macedo, S., Mussatto, S.I., Teixeira, J.A. and Oliveira, J.M. (2012). Production of white wine by *Saccharomyces cerevisiae* immobilized on grape pomace. J. Inst. Brew., 118: 163-173.

Genisheva, Z., Mussatto, S.I., Oliveira, J.M. and Teixeira, J.A. (2013). Malolactic fermentation of wines with immobilised lactic acid bacteria- influence of concentration, type of support material and storage conditions. Food Chem., 138: 1510-1514.

Genisheva, Z., Teixeira, J.A. and Oliveira, J.M. (2014). Immobilized cell systems for batch and continuous winemaking. Trends Food Sci. Tech., 40: 33-47.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K. and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature. 425: 737-741.

Giacometti, J., Tomljanović, A.B. and Josić, D. (2013). Application of proteomics and metabolomics for investigation of food toxins. Food Res. Int., 54: 1042-1051.

Glories, Y. (1984). La coleur des vins rouges. II partie: mesure, origine et interpretation. Connaiss Vigne Vin, 4: 253-271.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I. and Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33: 271-281.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S.G. (1996). Life with 6000 Genes. Science, 274: 546-567.

Gómez-Míguez, M.J., Cacho, J.F., Ferreira, V., Vicario, I.M. and Heredia, F.J. (2007). Volatile components of Zalema white wines. Food Chem., 100: 1464-1473.

González-Barreiro, C., Rial-Otero, R., Cancho-Grande, B. and Simal-Gándara J. (2015). Wine aroma compounds in grapes: a critical review. Crit. Rev. Food Sci. Nutr., 55:202-18.

González-Pombo, P., Fariña, L., Carrau, F., Batista-Viera, F. and Brena, B.M. (2011). A novel extracellular -glucosidase from *Issatchenkia terricola*: isolation, immobilization and application for aroma enhancement of white Muscat wine. Process. Biochem., 46: 385-389.

González-Pombo, P., Fariña, L., Carrau, F., Batista-Viera, F. and Brena, B.M. (2014). Aroma enhancement in wines using co-immobilized *Aspergillus niger* glycosidases. Food Chem., 143: 185-191.

González-Rodríguez, R.M., Noguerol-Pato, R., González-Barreiro, C., Cancho-Grande, B. and Simal-Gándara, J. (2011).

Application of new fungicides under good agricultural practices and their effects on the volatile profile of white wines. Food Res. Int., 44: 397-403.

Govender, P., Bester, M. and Bauer, F.F. (2010). *FLO* gene-dependent phenotypes in industrial wine yeast strains. Appl. Microbiol. Biotechnol., 86: 931-945.

Granchi, L., Ganucci, D., Messini, A., Rosellini, D. and Vicenzini, M. (1998). Dynamics of yeast populations during the early stages of natural fermentations for the production of Brunello de Montalcino wines. Food Technol. Biotechnol., 36: 313-318.

Groboillot, A., Boadi, D.K., Poncelet, D. and Neufeld, R.J. (1994). Immobilization of cells for application in the food industry. Crit. Rev. Biotechnol., 14: 75-107.

Guichard, E., Pham, T.T. and Charpentier, C. (1997). Le sotolon, marqueur de la typicité de l'arome des vins du Jura. Rev. Oenologues, 82: 32-34.

Güldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic acids res. 24, 2519-2524.

Gutiérrez, P., Roldán, A., Caro, I. and Pérez, L. (2010). Kinetic study of the velum formation by *Saccharomyces cerevisiae* (beticus sp.) during the biological aging of wines. Process Biochem., 45: 493-499.

Hagman, A. and Piškur, J. (2015). A study on the fundamental mechanism and the evolutionary driving forces behind aerobic fermentation in yeast. PLoS One, 2015, 10: 468-477.

Halket, J.M., Waterman, D., Przyborowska, A.M., Patel, R.K.P., Fraser, P.D. and Bramley, P.M. (2005). Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. J. Exp. Bot., 56: 219-243.

Hallsworth, J.E. (1998). Ethanol-induced water stress in yeast. J. Ferm. Bioeng., 85: 125-137.

Harsch, M.J., Benkwitz F., Frost, A., Colonna-Ceccaldi, B., Gardner R.C. and Salmon J.M. (2013). New precursor of 3-mercaptohexan-1-ol in grape juice: Thiol-forming potential and kinetics during early stages of must fer mentation. J. Agric. Food Chem., 61: 3703-3713.

Haslam, E. and Lilley, T.H. (1988). Natural astringency in foodstuffs. A molecular interpretation. Crit. Rev. Sci. Nutr., 2: 1-40.

Hata, H., Shimizu, S. and Yamada, H. (1987). Enzymatic production of D-(-)-pantonyl lactona from ketopantonyl lactone. Agric. Biol. Chem., 51: 3011-3016.

Hayasaka, Y., MacNamara, K., Baldock, G.A., Taylor, R.L. and Pollnitz, A.P. (2003). Application of stir bar sorptive extraction for wine analysis. Anal. Bioanal. Chem., 948-955.

Hazelwood, L.A., Daran, J.M., van Maris, A.J., Pronk, J.T. and Dickinson, J.R. (2008). The Ehrlich pathway for fusel alcohol production: A century of research on Saccharomyces cerevisiae metabolism. Appl. Environ. Microbiol., 74:2259-2266.

Heard, G.M. and Fleet, G.H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microb., 50:7 27-728.

Heard, G.M. and Fleet, G.H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. J. Appl. Bacteriol., 65: 23-28.

Henick-Kling, T., Edinger, W., Daniel, P. and Monk, P. (1998). Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J. Appl. Microbiol., 84: 865-876.

Herrero, M., Simo, C., Garcia-Cañas, V., Ibañéz, E. and Cifuentes, A. (2012). Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrom. Rev., 31: 49-69.

Hiller, K., Hangebrauk, J., Jager, C., Spura, J., Schreiber, K. and Schomburg, D. (2009). MetaboliteDetector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis. Anal. Chem., 81: 3429-3439.

Hirst, M.B. and Richter, C.L. (2016). Review of aroma formation through metabolic pathways os *Saccharomyces cerevisiae* in beverage fermentations. Am. J. Enol. Vitic., 67: 361-370.

Holm Hansen, E., Nissen, P., Sommer, P., Nielsen, J.C. and Arneborg, N. (2001). The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with Saccharomyces cerevisiae. J Appl. Microbiol., 91: 541-547.

Honigberg, S.M. (2011). Cell signals, cell contacts, and the organizatio of yeast communities. Eukaryot. Cell, 10: 466-473.

Horth, P., Miller, C.A., Preckel, T. and Wenz, C. (2006). Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. Mol. Cell. Proteomics, 5: 1968-1974.

Humston, E.M., Knowles, J.D., McShea, A. and Synovec, R.E. (2010). Quantitative assessment of moisture damage for cacao bean quality using two-dimensional gas chromatography combined with time-of-flight mass spectrometry and chemometrics. J. Chromatogr. A, 1217: 1963-1970.

Iimura, Y., Hara, S. and Otsuka, K. (1980). Cell surface hydrophobicity as a pellicle formation factor in film strain of *Saccharomyces*. Agric. Biol. Chem., 44: 1215-1222.

Infante, J.J., Dombek, K.M., Rebordinos, L., Cantoral, J.M. and Young, E.T. (2003). Genome-wide amplifications caused by chromosomal rearrangements play amajor role in the adaptive evolution of natural yeast. Genetics, 165: 1745-1759.

Ishigami, M., Nakagawa, Y., Hayakawa, M. and Iimura, Y. (2004). *FLO11* is essential for flor formation caused by the C-terminal deletion of *NRG1* in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett., 237: 425-430.

Ishigami, M., Nakagawa, Y., Hayakawa, M. and Iimura, Y. (2006). *FLO11* is the primary factor in flor formation caused by cell surface hydrophobicity in wild-type flor yeast. Biosci. Biotechnol. Biochem., 70: 660-666.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., Mann, M. (2005). Exponentially modified protein abundance Index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol. Cell. Proteomics. 4: 1265-1272.

Ivanova Petkova, M. Pujol-Carrion, N. Arroyo, J. Garcia-Cantalejo, J. and de la Torre-Ruiz, M.A. (2010). Mtl1 is required to activate general stress response through tor1 and ras2 inhibition under conditions of glucose starvation and oxidativestress, J. Biol. Chem., 285: 19521-19531.

Jackson, R.S. (2008). Specific and distinctive wine styles. In: 3rd edn. Jackson, R.S. (Eds.). Wine science: Principles and applications. Academic Press-Elsevier, S. Diego, CA (U.S.A.). pp: 520-576.

Jiménez-Martí, E., Zuzuarregui, A., Gomar-Alba, M., Gutiérrez, D., Gil, C., and Del Olmo, M. (2011). Molecular response of *Saccharomyces cerevisiae* wine and laboratory strains to high sugar stress conditions. Int. J. Food Microbiol., 145: 211-220.

Jin, Y.L. and Speers, R.A. (1998). Flocculation of *Saccharomyces cerevisiae*. Food Res. Int., 31: 421-440.

Jolly, N.P., Augustyn, O.P.H. and Pretorius, I.S. (2003b). The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic., 24: 55-62.

Jolly, N.P., Varela, C. and Pretorius, I.S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res., 14: 215-237.

Jorrín-Novo, J.V., Maldonado, A.M., Echevarría-Zomeño, S., Valledor, L., Castillejo, M.A., Curto, M., Valero, J., Sghaier, B., Donoso, G. and Redondo, I. (2009). Plant proteomics update (2007-2008): second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfill MIAPE standards, increase plant proteome coverage and expand biological knowledge. J. Proteomics, 72: 285-314.

Kana, K., Kanellaki, M. and Koutinas, A.A. (1992). Volatile by-products formed in batch alcoholic fermentations: effect of γ-alumina and kissiris supported biocatalysts. Food Biotechnol., 6: 65-74.

Kandylis, P., Dimitrellou, D. and Koutinas, A.A. (2012a). Winemaking by barley-supported yeast cells. Food Chem., 130: 425-431.

Kandylis, P., Drouza, C., Bekatorou, A. and Koutinas, A.A. (2010a). Scale-up of extremely low temperature fermentations of grape must by wheat-supported yeast cells. Biores. Technol., 101: 7484-7491.

Kandylis, P., Manousi, M.E., Bekatorou, A. and Koutinas, A.A. (2010b). Freeze-dried wheat supported biocatalyst for low temperature wine making. LWT- Food Sci. Technol., 43: 1485-1493.

Kandylis, P., Mantzari, A., Koutinas, A.A. and Kookos, I.K. (2012b). Modelling of low temperature wine-making, using immobilized cells. Food Chem., 133: 1341-1348.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M. and Nerantzis, E. (2007). Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol., 23: 735-739.

Karel, S.F., Libicki, S.B. and Robertson, C.R. (1985). The immobilization of whole cells-engineering principles. Chem. Eng. Sci., 40: 1321-1354.

Kim, D.H., Hong, Y.A. and Park, H.D. (2008). Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine. Biotechnol. Lett. 30: 1633-1638.

King, A. and Dickson, J.R. (2000). Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromuces lactis*. Yeast, 16: 499-506.

Koek, M.M., Jellema, R.H., Greef, J., Tas, A.C. and Hankemeier, T. (2011). Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. Metabolomics, 7: 307-328.

Kopsahelis, N., Bosnea, L., Kanellaki, M. and Koutinas, A.A. (2012). Volatiles formation from grape must fermentation using a cryophilic and thermotolerant yeast. Appl. Biochem. Biotechnol., 167: 1183-1198.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R. and Koutinas, A.A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol., 21: 377-397.

Kourkoutas, Y., Manojlović, V. and Nedović, V.A. (2010). Immobilization of microbial cells for alcoholic and malolactic fermentation of wine and cider. In: Nedović, V.A. and Zuidam, N.J. (Eds). Encapsulation technologies for Active food

ingredients and food processing. Springer, London (U.K.). pp: 327-343.

Kovacs, M., Stuparevic, I., Mrsa, V. and Maraz, A. (2008). Characterization of Ccw7p cell wall proteins and the encoding genes of *Saccharomyces cerevisiae* wine yeast strains: relevance for flor formation. FEMS Yeast Res., 8: 1115-1126.

Kutyna, D.R., Varela, C., Henschke, P.A., Chambers, P.J. and Stanley, G.A. (2010). Microbiological approaches to lowering ethanol concentration in wine. Trends Food Sci. Technol., 21: 293-302.

Lachance, M.A. and Kurtzman, C.P. (2011). Kurtzman, C.P., Fell, J.W. and Boekhout, T., (Eds.). *Lachancea Kurtzman* (2003). Vol. 2, 5th Edn. The Yeasts, a Taxonomic Study. Elsevier Science Publishers, Amsterdam (The Netherlands). pp: 511-519.

Lambrechts, M. and Pretorius, I. (2000). Yeast and its importance to wine aroma - A review. S. Afr. J. Enol. Vitic., 21:97-129.

Landry, C.R., Townsend, J.P., Hartl, D.L. and Cavalieri, D. (2006). Ecological and evolutionary genomics of *Saccharomyces cerevisiae*. Mol. Ecol., 15: 575-591.

Laurent, M.H., Henickling, T. and Acree, T. (1994). Changes in the aroma and odor of Chardonnay wine due to malolactic fermentation. Wein-Wiss., 49: 3-10.

Lerm, E., Engelbrecht, L. and du Toit, M. (2010). Malolactic fermentation: the ABC's of MLF. S. Afr. J. Enol. Vitic., 31: 186-212.

Lilly, M., Bauer, F.F., Styger, G., Lambrechts, M.G. and Pretorius, I.S. (2006). The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS Yeast Res., 6:726-743.

Longo, E., Cansado, J., Agrelo, D. and Villa, T.G. (1991). Effect of climatic conditions on yeast diversity in grape musts from Northwest Spain. Am. J. Enol. Vitic., 42: 141-144.

Lonvaud-Funel, A. (1996). Microorganisms of winemaking. Cerevisia, 21: 55-58.

López de Lerma, N. and Peinado, R.A. (2011). Use of two osmoethanol tolerant yeast strain to ferment must from Tempranillo dried grapes. Effect on wine composition. International J. Food Microbiol., 145:342-348.

López de Lerma, N., García-Martínez, T., Moreno, J., Mauricio, J.C. and Peinado, R.A. (2012). Sweet wines with great aromatic complexity obtained by partial fermentation of must from Tempranillo dried grapes. Eur. Food Res. Technol., 234: 695-701.

Lopez, R., Gracia-Moreno, E., Cacho, J. and Ferrreira, V. J. (2011). Development of a mixed-mode solid phase extraction method and further gas chromatography mass spectrometry for the

analysis of 3-alkyl-2-methoxypyrazines in wine. Chromatogr. A, 1218: 842-848.

Lorenzini, F. (1999). Spontaneous fermentation on red vintage: example with Pinot noir. Rev. Sui. Vitic., Arboricult. Hort., 31: 191-195.

Loureiro, V. and Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry (review). Int. J. Food Microbiol., 86: 23-50.

Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T. and Fröhlich, K.U. (2004). Apoptosis in yeast. Curr. Opin. Microbiol., 7: 655-660.

Magyar, I. and Toth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. Food Microbiol., 28: 94-100.

Mallios, P., Kourkoutas, Y., Iconomopoulou, M., Koutinas, A.A., Psarianos, C., Marchant, R. and Bana, I.M. (2004). Low temperature wine-making using yeast immobilized on pear pieces. J. Sci. Food Agric., 84: 1615-1623.

Martin, B. and Etievant, P.X. (1991). Quantitative determination of solerone and sotolona in flor sherries by two dimensional-capillary GC. J. High Resol. Chromat. Chromat. Communications, 14: 133-135.

Martin, B., Etievant, P.X., Le Quere, J.L. and Schlich, P. (1992). More clues about sensory impact of sotolon in some flor sherry wines. J. Agric. Food Chem., 40: 475-478.

Martínez, P., Codon, A.C., Pérez, L. and Benitez, T. (1995). Physiological and molecular characterisation of flor yeasts: polymorphism of flor yeast populations. Yeast, 11: 1399-1411.

Martínez, P., Pérez Rodríguez, L. and Benítez, T. (1997a). Factors which affect velum formation by flor yeasts isolated from sherry wine. Syst. Appl. Microbiol., 20: 154-157.

Martínez, P., Pérez Rodríguez, L. and Benítez, T. (1997b). Evolution of flor yeast population during biological aging of fino sherry wine. Am. J. Enol. Vitic., 48: 160-168.

Martínez, P., Valcarcel, M.J., Perez, L. and Benıtez, T. (1998). Metabolism of *Saccharomyces cerevisiae* flor yeasts during fermentation and biological aging of fino sherry: byproducts and aroma compounds. Am. J. Enol. Vitic., 49: 240-250.

Matallana, E. and Aranda, A. (2016). Biotechnological impact of stress response on wine yeast. Lett. Appl. Microbiol., 64: 103-110.

Mateo, J.J. and Jiménez, M. (2000). Monoterpenes in grape juice and wines. J. Chromatogr. A, 881: 557-567.

Matsumoto, I. and Kuhara, T. (1996). A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry—rapid, practical, and simultaneous urinary metabolites analysis. Mass Spectrom. Rev., 15: 43-57.

Mauricio, J.C. and Ortega, J.M. (1993). Influence of fermentation conditions on specific activity of the enzymes alcohol and aldehyde dehydrogenase from yeasts. Microbios, 75: 95-106.

Mauricio, J.C., Guijo, S. and Ortega, J.M. (1991). Relationship between phospholipid and sterol contents in *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and their fermentation activity in grape musts. Am. J. Enol. Vitic., 42: 301-308.

Mauricio, J.C., Millán, M.C. and Ortega, J.M. (1998). Influence of oxygen on the biosynthesis of cellular fatty acids, sterols and phospholipids during alcoholic fermentation by *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. World J. Microbiol. Biotechnol., 14: 405-410.

Mauricio, J.C., Moreno, J. and Ortega, J.M. (1997). In vitro specific activities of alcohol and aldehyde dehydrogenases from two flor yeasts during controlled wine aging. J. Agric. Food Chem., 45: 1967-1971.

Mauricio, J.C., Moreno, J.J., Valero, E.M., Zea, L., Medina, M. and Ortega, J.M. (1993). Ester formation and specific activities of in vitro alcohol acetyltransferase and esterase by *Saccharomyces cerevisiae* during grape must fermentation. J. Agric. Food Chem., 41: 2086-2091.

Mauricio, J.C., Valero, E., Millán, C. and Ortega, J.M. (2001). Changes in nitrogen compounds in must and wine during fermentation and biological aging by flor yeasts. J. Agric. Food Chem., 49: 3310-3315.

Meem, M.H., Cullen, P.J. (2012). The impact of protein glycosylation on Flo11-dependent adherence in *Saccharomyces cerevisiae*. FEMS Yeast Res., 12: 809-818.

Melzoch, K., Rychtera, M. and Habova, V. (1994). Effect of immobilization up on the properties and behavior of *Saccharomyces cerevisiae* cells. J. Biotechnol., 32: 59-65.

Meng, C.K., Szelewski, M., Zweigenbaum, J., Furst, P. and Blanke, E. (2011). In: M. Stoytcheva (Eds.). Pesticides in the Modern World - Trends in Pesticides Analysis, InTech. pp: 391-412.

Merida, J., Lopez-Toledano, A., Marquez, T., Millan, C., Ortega, J.M. and Medina, M. (2005). Retention of browning compounds by yeasts involved in the winemaking of sherry type wines. Biotechnol. Lett., 27: 1565-1570.

Mesa, J.J., Infante, J.J., Rebordinos, L. and Cantoral, J.M. (1999). Characterization of yeasts involved in the biological ageing of sherry wines. Food Sci. Technol. Res. 32, 114-120.

Mesa, J.J., Infante, J.J., Rebordinos, L., Sanchez, J.A. and Cantoral, J.M. (2000). Influence of the yeast genotypes on enological characteristics of sherry wines. Am. J. Enol. Vitic., 51: 15-21.

Miki, B.L., Poon, N.H., James A.P. and Seligy, V.L. (1982). Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. J. Bacteriol., 150: 878-89.

Mo, X., Fan, W. and Xu, Y. (2010). Characterization of aroma compounds in Chinese rice wine Qu by solvent-assisted flavor evaporation and headspace solid-phase microextraction. J. Agric. Food Chem., 58: 2462-2469.

Moradas-Ferreira, P. Costa, V. Piper, P. and Mager, W. (1996). The molecular defences against reactive oxygen species in yeast. Mol. Microbiol., 19: 651-658.

Moreira, N., Mendes, F., Hogg, T. and Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixes cultures of apiculate wine yeasts. Int. J. Food. Microbiol., 103: 285-294.

Moreno J. and Peinado R.A. (2012). Enological Chemistry. Academic Press-Elsevier, San Diego CA (U.S.A.).

Moreno, J., Millán, C., Ortega, J.M. and Medina, M. (1991). Analytical differentiation of wine fermentations using pure and mixed yeast cultures. J. Ind. Microbiol., 7: 181-189.

Moreno, J.A., Zea, L., Moyano, L. and Medina, M. (2004). Aroma compounds as markers of the changes in sherry wine subjected to biological ageing. Food Control, 16: 333-338.

Moreno, J.J. and García-Mauricio, J.C. (2013). Pedro Ximénez and Malaga. In: Mencarelli, F. and Tonutti P. (Eds.). Sweet, reinforced and fortified wines. Wiley-Blackwell, Hoboken, NJ (U.S.A.). pp: 251-267.

Moyano, L., Zea, L., Moreno, J. and Medina, M. (2002). Analytical study of aromatic series in sherry wines subjected to biological aging. J. Agric. Food Chem., 50: 7356-7361.

Muñoz, D., Peinado, R.A. and Moreno, J. (2007). Biological aging of sherry wines under periodic and controlled microaerations with *Saccharomyces cerevisiae* var. capensis: Effect on odorant series. Food Chem., 100: 1188-1195.

Muñoz, D., Peinado, R.A., Medina, M. and Moreno, J. (2005). Biological aging of sherry wines using pure cultures of two flor yeast strain under controlled microaeration. J. Agric. Food Chem., 53: 5258-5264.

Muñoz, D., Peinado, R.A., Medina, M. and Moreno, J. (2006). Higher alcohols concentration and its relation with the biological aging evolution. Eur. Food Res. Technol. Chem., 5-6; 629-635.

Muñoz, D., Valero, E., Moreno, J., Moyano, L., Zea, L. and Medina, M. (2001). Estudio del aprovechamiento de la biomasa procedente de la crianza biológica de vinos finos. In: XXII Jornadas de Viticultura y Enología Tierra de Barros (Eds.). Centro Cultural Santa Ana, Almendalejo, Badajo (Spain). pp: 243-252.

Murat, M., Tominaga, T. and Dubourdieu, D. (2001). Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce Rose wine by assaying the cysteinylated

precursor of 3-mercaptohexan-1-ol. J. Agric. Food Chem., 49: 5412-5417.

Nedovic, V., Gibson, B., Mantzouridou, T.F., Bugarski, B., Djordjević, V., Kalušević, A., Paraskevopoulou, A., Sandell, M., Šmogrovičová, D. and Yilmaztekin, M. (2015). Aroma formation by immobilized yeast cells in fermentation processes. Yeast, 32: 173-216.

Nedović, V.A., Manojlovic, V., Bugarski, B. and Willaert, R. (2010). State of the art in immobilized/encapsulated cell technology in fermentation processes. In: Nedović, V.A. and Zuidam, N.J. (Eds.). Encapsulation technologies for active food ingredients and food processing. Springer: London (U.K.). pp: 119-146.

Newmeyer, D.D. and Ferguson-Miller, S. (2003). Mitochondria: releasing power for life and unleashing the machineries of death. Cell, 112: 481-490.

Nissen, P., Nielsin, D. and Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. Yeast, 20: 331-341.

NIST. National Institute of Standards and Technology. Web book of Chemistry. Available online: http://webbook.nist.gov/chemistry/ (last access on 2 September 2016).

Noguerol-Pato, R., González-Álvarez, M., González-Barreiro, C., Cancho-Grande, B. and Simal-Gándara, J. (2012a). Aroma profile of Garnacha Tintorera-based sweet wines by chromatographic and sensorial analyses. Food Chem., 134: 2313-2325.

Noguerol-Pato, R., González-Barreiro, C., Simal-Gándara, J., Martinez, M.C., Santiago, J.L. and Cancho-Grande, B. (2012b). Active odorants in Mouraton grapes from shoulders and tips into the bunch. Food Chem., 133: 1362-1372.

Norton, S. and D'Amore, T. (1994). Physiological effects of yeast cell immobilization: applications for brewing. Enzyme Microb. Technol., 16: 365-375.

Nykänen, L. (1986). Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. Am. J. Enol. Vitic., 37: 84-96.

Nyman, J., Lacintra, M.G., Westman, J.O., Berglin, M., Lundin, M., Lennartsson, P.R. and Taherzadeh, M.J. (2013). Pellet formation of zygomycetes and immobilization of yeast. New Biotechnol., 30: 516-522.

Ogur, M., Liu, T.N., Cheung, I., Paulavicius, I., Wales, W., Mehnert, D. and Blaise, D. (1977). "Active" one-carbon generation in *Saccharomyces cerevisiae*. J. Bacteriol., 129: 926-933.

OIV. (2008). Recueil des méthodes Internationales des vins et des mouts. Organisation Internationale de la Vigne et du vin. Paris, France.

Oro, L., Ciani, M. and Comitini, F. (2014). Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. J. Appl. Microbiol., 116: 1209-1217.

Pedroza, M.A., Zalacain, A., Lara, J.F. and Salinas, M. (2010). Global grape aroma potential and its individual analysis by SBSE–GC–MS. R. Food Res. Int., 43: 1003-1008.

Peinado, R.A. and Mauricio, J.C. (2009). Biologically Aged Wines. In: Moreno-Arribas, M.V., and Polo, M.C. (Eds.). Wine Chemistry and Biochemistry. Springer, New York, NY (U.S.A.). pp: 81-101.

Peinado, R.A., Mauricio, J.C. and Moreno, J. (2006). Aromatic series in sherry wines with gluconic acid subjected to different biological aging conditions by *Saccharomyces cerevisiae* var. *capensis*. Food Chem., 94: 232-239.

Peinado, R.A., Mauricio, J.C., Moreno, J., Ortega, J.M., Medina, M., Mérida, J., Millán, M.C., Mayén, M., Zea, L., Moyano, M.L., Valero, E., Muñoz, D., A. López and O. Maestre. (2004). Method of obtaining yeast biocapsules, biocapsules thus obtained and applications of same. World International Property Organization. Publication nr: WO2004029240 A1.

Peinado, R.A., Moreno, J.A., Muñoz, D., Medina, M. and Moreno, J. (2004). Gas-chromatographic quantification of major volatile compounds and polyols in wine by direct injection. J. Agric. Food Chem., 52: 6389-6393.

Peinado, R.A., Moreno, J.J., Maestre, O. and Mauricio, J.C. (2005). Use of a novel immobilization yeast system for winemaking. Biotechnology letters, 27(18), 1421-1424.

Peinado, R.A., Moreno, J.J., Ortega, J.M. and Mauricio, J.C. (2003). Effect of gluconic acid consumption during simulation of biological aging of sherry wines by a flor yeast strain on the final volatile compounds. J. Agric. Food Chem., 51: 6198-6203.

Peinado, R.A., Moreno, J.J., Villalba, J.M., González-Reyes, J.A., Ortega, J.M. and Mauricio, J.C. (2006).Yeast biocapsules: a new immobilization method and their applications. Enzyme Microb.Technol., 40: 79-84.

Perestrelo, R., Fernandes, A., Albuquerque, F.F., Marques, J.C. and Camara, J.S. (2006). Analytical characterization of the aroma of Tinta Negra Mole red wine: Identification of the main odorants compounds. Anal. Chim. Acta, 563: 154-164.

Pham, T.T., Guichard, E., Schlich, P. and Charpentier, C. (1995). Optimal conditions for the formation of sotolon from α-ketobutyric acid in the french Vin Jaune. J. Agric. Food Chem., 43, 2616-2619.

Piskur, J., Rozpedowska, E., Polakova, S., Merico, A. and Compagno, C. (2006). How did *Saccharomyces* evolve to become a good brewer? Trends Genet., 22: 183-186.

Plata, M.C., Mauricio, J.C., Millán, C., and Ortega, J.M. (1998). In vitro specific activity of alcohol acetyl-transferase and esterase in two flor yeast strains during biological aging of Sherry wines. J. Ferment. Bioeng., 85: 369-374.

Plata, M.C., Millán, M.C., Mauricio, J.C. and Ortega, J.M. (2003). Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol., 20: 217-224.

Postma, E., Verduyn, C., Scheffers, W. A. and Van Dijken, J.P. (1989). Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microb., 55: 468-77.

Pozo-Bayón, A. and Moreno-Arribas, V. (2011). Sherry wines. Adv. Food Nutr. Res., 63: 17-40.

Pretorius, I.S. and Høj, P. B. (2005). Grape and wine biotechnology: challenges, opportunities and potential benefits. Aust. J. Grape Wine Res. 11: 83-108.

Pretorius, I.S.,Van Der Westhuizen, T.J. and Augustyn, O.P.H. (1999). Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry-A review. S. Afr.J.Enol.Vitic., 20: 61-75.

Pronk, J.T., Steensma, H.Y. and Van Dijken, J.P. (1996). Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast, 12: 1607-33.

Puig-Pujol, A., Bertran, E., García-Martínez, T., Capdevila, F., Mínguez, S. and Mauricio, J.C. (2013). Application of a new organic yeast immobilization method for sparkling wine production. Am. J. Enol. Vitic., 63: 386-394.

Purevdorj-Gage, B., Orr, M.E., Stoodley, P., Sheehan, K.B., Hyman, L.E. (2007). The role of *FLO11* in *Saccharomyces cerevisiae* biofilm development in a laboratory based flow cell system. FEMS Yeast Res., 7: 372-379.

Querol, A., Jiménez, M. and Huerta, T. (1990). Microbiological and enological parameters during fermentation of musts from poor and normal grape harvests in the region of Alicante (Spain). J. Food. Sci., 55: 1603-1606.

Rauhut, D. (1993). Yeasts - production of sulfur compounds. In: Fleet, G.H. (Eds.).Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland. pp: 183-223.

Reboredo-Rodriguez, P., Gonzalez-Barreiro, C., Rial-Otero, R., Cancho-Grande, B. and Simal-Gandara, J. (2014). Wine aroma compounds in grapes: A critical review. Crit.Rev. Food Sci Nutr., 55: 202-218.

Recamales, A.F., Gallo, V., Hernanz, D., Gonzalez-Miret, M.L. and Heredia, F.J. (2011). Effect of time and storage conditions on

major volatile compounds of Zalema white wine. J. Food Qual., 34: 100-110.

Reinders, J., Zahedi, R.P., Pfanner, N., Meisinger, C. and Sickmann, A. (2006). Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. J. Proteome Res., 7: 1543-1554.

Renault, P., Miot-Sertier, C., Marullo, P., Hernández-Orte Lagarrigue, L., Lonvaud-Funel, A. and Bely, M. (2009). Genetic characterisation and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. Int. J. Food. Microbiol., 134: 201-210.

Reynolds, T.B. and Fink, G.R. (2001). Bakers'yeast, a model for fungal biofilm formation. Science, 291: 878-881.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. and Lonvaud-Funel, A. (2006). Vol. 1, 2nd edn. Handbook of enology: the microbiology of wine and vinifications. Wiley and Sons, Chichester (U.K.).

Ribéreau-Gayon, P., Glories, Y., Maujean, A. and Dubourdieu, D. (2006). Handbook of Enology (Vol. 2). The chemistry of wine and stabilization and treatments. John Wiley and Sons, Ltd, New York, NY (U.S.A.).

Ristow, H., Seyfarth, A. and Lochmann, E.R. (1995). Chromosomal damages by ethanol and acetaldehyde in *Saccharomyces cerevisiae* as studied by pulsed field gel electrophoresis. Mutat. Res., 326: 165-170.

Rocha, S.M., Rodrigues, F., Coutinho, P., Delgadillo, I. and Coimbra, M.A. (2004). Volatile composition of Baga Red Wine Assessment of the identification of the would-be impact Odourants. Anal. Chim. Acta, 513: 257-262.

Rodríguez, M.E., Infante, J.J., Mesa, J.J., Rebordinos, L. and Cantoral, J.M. (2013). Enological behaviour of biofilms formed by genetically-characterized strains of sherry flor yeast. Open Biotechnol. J., 7: 23-29.

Rodriguez, M.E., Lopes, C.A., Barbagelata, R.J., Barda, N.B. and Caballero, A.C. (2010). Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. Int. J. Food Microbiol., 138: 19-25.

Rodríguez, M.E., Rebordinos, L., Muñoz-Bernal, E., Fernández-Acero, F.J. and Cantoral, J.M. (2012). Application of gel electrophoresis techniques to the study of wine yeast and to improve winemaking. Gel Electrophoresis-Advanced Techniques, 1.

Rojas, V., Gil, J.V., Pinaga, F. and Mazanares, P. (2003). Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol., 86: 181-188.

Romano, P. and Suzzi, G. (1996). A review: origin and production of acetoin during wine yeast fermentation. Appl. Environ. Microbiol., 62: 309-315.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. and Capece, A. (2003). Function of yeast species and strains in wine flavour. Int. J. Food. Microbiol., 86: 169-180.

Romano, P., Suzzi, G., Turbanti, L. and Polsinelli, M. (1994). Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiol Lett., 118: 213-218.

Rosillo, L., Salinas, M.R., Garijo, J. and Alonso, G.L. (1999). Study of volatiles in grapes by dynamic headspace analysis: Application to the differentiation of some *Vitis vinifera* varieties. J. Chromatogr. A, 847: 155-159.

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L. and Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. J. Appl. Microbiol., 107: 47-55.

Rozpedowska, E., Hellborg, L., Ishchuk, O.P. Orhan, F., Galafassi, S., Merico, A., Woolfit, M., Compagno, C. and Piskur J. (2011). Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. Nat. Commun., 2: 1-7.

Ruiz-Matute, A.I., Hernandez-Hernandez, O., Rodriguez-Sanchez, S., Sanz, M.L. and Martinez-Castro, I. (2011). Derivatization of carbohydrates for GC and GC–MS analyses. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci., 879: 1226-1240.

Sabon, I., de Revel, G., Kotseridis, Y. and Bertrand, A. (2002). Determination of volatile compounds in Grenache wines in relation with different terroirs in the Rhone Valley. J. Agric. Food Chem., 50: 6341-6345.

Saccharomyces Genome Database. Available online: http://www.yeastgenome.org (accessed on 2 September 2016).

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.J. and Ballester, J. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol., 32: 243-253.

Saerens, S.M., Verstrepen, K.J, Van Laere, S.D, Voet, A.R, Van Dijck, P., Delvaux, F.R. and Thevelein, J.M. (2006). The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J. Biol. Chem., 281:4446 - 4456.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J. and Thevelein, J.M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microbial Biotechnol., 3: 165-177.

Saghatelian, A. and Cravatt, B.F. (2005). Global strategies to integrate the proteome and metabolome. Curr. Opin. Chem. Biol., 9: 62-68.

Sakurai. A., Nishida, Y., Saito, H. and Sakakibara, M. (2000). Ethanol production by repeated batch culture using yeast cells immobilized within porous cellulose carriers. J. Biosci. and Bioeng., 90: 526-529.

Sampedro, M.C., Goicolea, M.A., Unceta, N., Sanchez-Ortega, A. and Barrio, R.J. (2009). Sequential stir bar extraction, thermal desorption and retention time locked GC–MS for determination of pesticides in water. J. Sep. Sci., 32: 3449-3456.

Sánchez-Palomo, E., García-Carpintero, E., Alonso-Villegas, R., and González-Viñas, M.A. (2010). Characterization of the aroma compounds of Verdejo white wines from the La Mancha region by odour activity values. Flavour Frag. J., 25: 456-462.

Sanchez-Rojas, F. (2009). A review of stir bar sorptive extraction. Chromatographia, 69: S79-S94.

Schifferdecker, A.J., Dashko, S., Ishchuk, O.P. and Piškur, J. (2014). The wine and beer yeast *Dekkera bruxellensis*. Yeast. 31: 323-332.

Schmarr, H.-G. and Bernhardt, J. (2010). Profiling analysis of volatile compounds from fruits using comprehensive two-dimensional gas chromatography and image processing techniques. J. Chromatogr. A, 1217: 565-574.

Schreier, P. (1979). Flavor compounds of wines: a review. Crit.Rev. Food Sci Nutr., 12: 59-111.

Servetas, I., Berbegal, C., Camacho, N., Bekatoroua, A., Ferrer, S., Nigam, P., Drouza, C. and Koutinas, A.A. (2013). *Saccharomyces cerevisiae* and *Oenococcus oeni* immobilized in different layers of a cellulose/starch gel composite for simultaneous alcoholic and malolactic wine fermentations. Process Biochem., 48: 1279-1284.

Shima, J. and Takagi, H. (2009). Stress-tolerance of baker's-yeast (Saccharomyces cerevisiae) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol. Appl. Biochem. 53: 155-164.

Sinclair, D.A. and Dawes, I.W. (1995). Genetics of the synthesis of serine from glycine and the utilization of glycine as sole nitrogen source by *Saccharomyces cerevisiae*. Genetics, 40: 1213-1222.

Sinclair, D.A., Hong, S.P. and Dawes, I.W. (1996). Specific induction by glycine of the gene for the P-subunit of glycine decarboxylase from *Saccharomyces cerevisiae*. Mol. Microbiol., 19: 611-623.

Skogerson, K., Runnebaum, R., Heymann, H. and Fiehn, O. (2008). Chemical basis for wine body: Exploratory study of GC-MS and NMR metabolite identification in white wines. Int. Am. J. Enol. Vitic., 59: 336-336.

Skogerson, K., Runnebaum, R., Wohlgemuth, G., de Ropp, J., Heymann, H. and Fiehn, O. (2009). Comparison of gas chromatography-coupled time-of-flight mass spectrometry and 1H nuclear magnetic resonance spectroscopy metabolite identification in white wines from a sensory study investigating wine body. J. Agric. Food Chem., 57: 6899-6907.

Smith, B.D. and Divol, B. (2016). *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Food Microbiol., 59: 161-175.

Snowdon, C., Schierholtz, R., Poliszczuk, P., Hughes, S. and van der Merwe, G. (2009). *ETP1*/*YHL010c* is a novel gene needed for the adaptation of *Saccharomy cescerevisiae* to ethanol. FEMS Yeast Res., 9: 372-380.

Soden, A., Francis, I.L., Oakey, H. and Henschke, P.A. (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape. Wine Res., 6: 21-30.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero,V., Alvarez, M., Fernández, M., Lopez, de Palencia, P.F., Corbi, A., Trip, H., and Lolkema, J S .(2010). Biogenic amines in fermented foods. Eur. J. Clin. Nutr., 64: S95-100.

Spiropoulos, A. and Bisson. L.F. (2000). MET17 and hydrogen sulfide formation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 66:4421- 4426.

Sponholz, W.R. and Hünhn, T. (1993). 4,5-dimethyl-3-hidroxy-2-(5H)-furanon (sotolona), einBotrytisindicator? Vitic. Enol. Sci., 49: 37-39.

Stewart, G.G. (2009). The Horace Brown Medal lecture: forty years of brewing research. J. Inst. Brew., 115: 3-29.

Stratford, M. (1992). Yeast flocculation: a new perspective. Adv. Microb. Physiol., 33: 2-71.

Strehaiano, P., Portugal, F.R. and Tailandier, P. (2006). Querol, A. and Fleet, G.H. (Eds.).Yeast as biocatalysts, in yeasts in food and beverage. Springer-Verlag, Berlin (Germany). pp: 243-285.

Styger, G., Prior, B. and Bauer, F.F. (2011). Wine flavor and aroma. J. Ind. Microbiol. Biotechnol., 38: 1145-1159.

Suárez-Lepe, J.A. and Íñigo-Leal, B. (2004). Microbiología Enológica. In: Mundi-Prensa (Eds.). Fundamentos de vinificación. Madrid (Spain).

Sun, S.Y., Jiang, W.G. and Zhao, Y.P. (2012). Comparison of aromatic and phenolic compounds in cherry wines with different cherry cultivars by HS-SPME-GC-MS and HPLC. Int. J. Food Sci. Technol., 47: 100-106.

Sun, Z.J., Lv, G.J., Li SY, Xie, Y.B., Yu, W.T., Wang, W. and Ma, X.J. (2007a). Probing the role of microenvironment for

microencapsulated Saccharomyces cerevisiae under osmotic stress. J. Biotechnol., 128: 150-161.

Sun, Z.J., Lv, G.J., Li, S.Y., Yu, W.T., Wang, W., Xie, Y.B. and Ma, X. (2007b). Differential role of microenvironment in microencapsulation for improved cell tolerance to stress. Appl. Microbiol. Biotechnol., 75: 1419-1427.

Suomalainen, H. and Ronkainen P. (1968). Mechanism of diacetyl formation in yeast fermentation. Nature, 220:792-793.

Swiegers, J.H. and Pretorius, I.S. (2005). Yeast modulation of wine flavor. Adv Appl. Microbiol., 57:131-175.

Taylor, G.T. and Kirsop, B.H. (1977). The origin of the medium chain length fatty acids present in beer. J. Inst. Brewing., 83:241-243.

Thomas, D. and Surdin-Kerjan Y. (1997). Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev., 61:503-532.

Thomson, J.M., Gaucher, E.A., Burgan, M.F., De Kee, D.W., Li, T., Aris, J.P. and Benner, S.A. (2005). Resurrecting ancestral alcohol dehydrogenases from yeast. Nat. Genet., 37: 630-635.

Tian, J., Shi, C., Gao, P., Yuan, K., Yang, D., Lu, X. and Xu, G. (2008). Phenotype differentiation of three E. colistrains by GC-FID and GC-MS based metabolomics. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci., 871: 220-226.

Tominaga, T., Baltenweck-Guyot, R., Gachons, C. and Dubourdieu, D. (2000). Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. Am. J. Enol. Vitic., 51: 178-181.

Tominaga, T., Peyrot des Gachons, C. and Dubourdieu, D. (1998). A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon blanc: S-cysteine conjugates. J. Agric. Food Chem., 46: 5215-5219.

Tomiyama, K., Aoki, H., Oikawa, T., Sakurai, K., Kasahara, Y. and Kawakami, Y. (2011). Characteristic volatile components of Kabosu (*Citrus sphaerocarpa* Hort. ex Tanaka). Nat. Prod. Commun., 6: 403-408.

Torija, M.J., Rozes, N., Poblet, M., Guillamon, J.M. and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between different wine-producing areas over a period of three years. Anton. Leeuw., 79: 345-352.

Trabalzini, L., Paffetti, A., Ferro, E., Scaloni, A., Talamo, F., Millucci, L., Martelli, P. and Santucci, A. (2003). Proteomic characterization of a wild-type wine strain of *Saccharomyces cerevisiae*. Ital. J. Biochem., 52: 145-153.

Tressl, R., Apetz, M., Arrieta, R. and Grünewald, K.G. (1978). Formation of lactones and terpenoids by microorganism. In: Charambous G. and Inglett G.E. (Eds.). Flavor of Foods and

Beverages. Academic Press, New York, NY (U.S.A.). pp: 145-168.

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., Mita, G. and Grieco, F. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. Food Microbiol. 36: 335-342.

Tsakiris, A., Bekatorou, A., Psarianos, C., Marchant, R. and Banat, I.M. (2004a). Immobilization of yeast on dried raisin berries for use in dry white wine-making. Food Chem., 87: 11-15.

Tsakiris, A., Sipsas, V., Bekatorou, A., Mallouchos, A. and Koutinas, A.A. (2004b). Red wine making by immobilized cells and influence on volatile composition. J. Agric. Food Chem., 52: 1357-1363.

Tsaousi, K., Koutinas, A.A., Bekatorou, A. and Loukatos, P. (2010). Fermentation efficiency of cells immobilized on delignified brewers' spent grains after low and high-temperature thin layer thermal drying. Appl. Biochem. Biotechnol., 162: 594-606.

Tsaousi, K., Velli, A., Akarepis, F., Bosnea, L., Drouza, C., Koutinas, A.A. and Bekatorou, A. (2011). Low-temperature winemaking by thermally dried immobilized yeast on delignified brewer's spent grains. Food Technol. Biotechnol., 49: 379-384.

Ugliano, M. and Henschke, P.A. (2009). Yeasts and wine flavour. In: Moreno-Arribas, M.V. and Polo M.C. (Eds.). Wine Chemistry and Biochemistry. Springer, Madrid (Spain).

Uniprot. Available online: http://www.uniprot.org/ (last access on 2 September 2016).

Usbeck, J.C., Wilde, C., Bertrand, D., Behr, J. and Vogel, R.F. (2014). Wine yeast typing by MALDI-TOF MS. Appl. Microbiol. Biotechnol., 98: 3737-3752.

Valero, E., Millán, C., Ortega, J.M. and Mauricio, J.C. (2003). Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. J. Sci. Food Agric., 83: 830-835.

Van Der Dool, H. and Kratz, P.D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J. Chromatogr. A, 1: 463-471.

Van Loon, A.P., Pesold-Hurt, B. and Schatz, G. (1986). A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. Proc. Natl. Acad. Sci. U. S. A., 83: 3820-3824.

Vandenbosch, D., De Canck, E., Dhondt, I., Rigole, P., Nelis, H.J. and Coenye, T. (2013). Genomewide screening for genes involved in biofilm formation and miconazole susceptibility in *Saccharomyces cerevisiae*. FEMS Yeast Res., 13: 720-730.

Van Zyl, J.A., De Vries, M.J. and Zeeman, A.S. (1963). The microbiology of South African winemaking III. The effect of different yeasts on the composition of fermented musts. S. Afr. J. Agric. Sci., 6: 165-179.

Vararu, F., Moreno-Garcia, J., Zamfir, C.I., Cotea, V.V. and Moreno, J. (2016). Selection of aroma compounds for the differentiation of wines obtained by fermenting musts with starter cultures of commercial yeast strains. Food Chem., 197: 373-381.

Velázquez, R. (2015). Estudio de nuevas levaduras killer *Saccharomyces cerevisiae* y *Torulaspora delbrueckii* para elaborar vinos tranquilos y espumosos. Doctoral Thesis. University of Extremadura (Spain).

Vemuri, G.N., Eiteman, M.A., McEwen, J.E., Olsson, L. and Nielsen, J. (2007). Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. P. Natl. Acad. Sci. U.S.A., 104: 2402-7.

Verstrepen, K.J. and Klis, F.M. (2006). Flocculation, adhesion and biofilm formation in yeasts. Mol. Microbiol., 60: 5-15.

Verstrepen, K.J., Van Laere, S.D., Vanderhaegen, B.M., Derdelinckx, G., Dufour, J.P., Pretorius, I.S., Winderickx, J., Thevelein, J.M. and Delvaux, F.R. (2003). Expression levels of the yeast alcohol acetyltransferase genes *ATF1*, Lg-*ATF1*, and *ATF2* control the formation of a broad range of volatile esters. Appl. Environ. Microbiol., 69:5228-5237.

Viana, F., Gil, J.V., Genove´s, S., Valle´s, S. and Manzanares, P. (2008). Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol., 25: 778-785.

Viana, F., Gil, J.V., Vallés, S. and Manzanares, P. (2009). Increasing the levels of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. Int. J. Food Microbiol., 135: 68-74.

Vilela, A., Schuller, D., Mendes-Faia, A. and Côrte-Real, M. (2013). Reduction of volatile acidity of acidic wines by immobilized *Saccharomyces cerevisiae* cells. Appl. Microbiol. Biotechnol., 97: 4991-5000.

Vilella, F. Herrero, E. Torres, J. de la Torre-Ruiz, M.A. (2005). Pkc1 and the upstreamelements of the cell integrity pathway in *Saccharomyces cerevisiae*, Rom2 and Mtl1, are required for cellular responses to oxidative stress. J. Biol. Chem., 280: 9149-9159.

Villamiel, M., Carmen-Polo, M. and Moreno-Arribas, M.V. (2008). Nitrogen compounds and polysaccharides changes during the biological ageing of Sherry wines. Lebensm. Wiss. Technol., 41: 1842-1846.

Villas-Boas, S.G., Mas, S., Aakesson, M., Smedsgaard, J. and Nielsen, J. (2005). Mass spectrometry in metabolome analysis. Mass Spectrom. Rev. 24: 613-646.

Voirin, S., Baumes, R., Bitteur, S., Gunata, Y. and Bayonove, C. (1990). Novel monoterperne disaccharide glycosides of *Vitis vinifera* grapes. J. Agric. Food Chem., 38: 1373-1378.

Walsh, P.K. and Malone, D.M. (1995). Cell growth in immobilization matrices. Biotechnol. Adv., 13: 13-43.

Weldegergis, B.T., Crouch, A.M., Gorecki, T. and de Villiers, A. (2011). Solid phase extraction in combination with comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry for the detailed investigation of volatiles in South African red wines. Anal. Chim. Acta, 701: 98-111.

Weldegergis, B.T., Tredoux, A.G.J. and Crouch, A.M. (2007). Application of a headspace sorptive extraction method for the analysis of volatile components in South African wines. J. Agric. Food Chem., 55: 8696-8702.

Welke, J.E., Manfroi, V., Zanus, M., Lazarotto, M. and Alcaraz Zini, C. (2012). Characterization of the volatile profile of Brazilian Merlot wines through comprehensive two dimensional gas chromatography time-of-flight mass spectrometric detection. J. Chromatogr. A, 1226: 124-139.

Whitener, M.E.B., Carlin, S., Jacobson, D., Weighillb, D., Divolb, B., Conternoa, L., Du Toit, M. and Vrhovsek U. (2015). Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: A targeted approach. LWT-Food Sci. Technol., 64: 412-422.

Whitener, M.E.B., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M. and Vrhovsek, U. (2016). Untangling the wine metabolome by combining untargeted SPME-GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. Metabolomics, 12(3), 1-25.

Winterhalter, P. and Skouroumounis, G.K. (1997). Glycoconjugated aroma compounds: occurence, role and biotechnological transformation. Adv. Biochem. Eng. Biotechnol., 55: 73-105.

Wishart, D.S. (2009). Computational strategies for metabolite identification in metabolomics. Bioanalysis, 1: 1579-1596.

Yoshimoto, H., Fujiwara, D., Momma, T., Ito, C., Sone, H., Kaneko, Y. and Tamai, Y. (1998). Characterization of the *ATF1* and Lg-*ATF1* genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. J. Ferment. Bioeng., 86:15-20.

Zalacain, A., Marín, J., Alonso, G.L. and Salinas, M.R. (2007). Analysis of wine primary aroma compounds by stir bar sorptive extraction. Talanta, 71: 1610-1615.

Zamora, F. (2013). La química del color del vino. ACE. Dossier 12-18-2013.

Zara, G., Zara, S., Pinna, C., Marceddu, S. and Budroni, M. (2009). *FLO11* gene length and transcriptional level affect biofilm-

forming ability of wild flor strains of *Saccharomyces cerevisiae*. Microbiology, 155: 3838-3846.

Zara, S., Antonio Farris, G., Budroni, M. and Bakalinsky, A.T. (2002). *HSP12* is essential for biofilm formation by a Sardinian sherry strain of *S. cerevisiae*. Yeast, 19: 269-276.

Zara, S., Bakalinsky, A.T., Zara, G., Pirino, G., Demontis, M.A., Budroni, M. (2005). *FLO11*-based model for air-liquid interfacial biofilm formation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 71: 2934-2939.

Zara, S., Gross, M.K., Zara, G., Budroni, M. and Bakalinsky, A.T. (2010). Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 76: 4089-4091.

Zea, L., Moreno, J., Ortega, J.M., Mauricio, J.C. and Medina, M. (1995). Comparative study of the γ-butyrolactone and pantolactone contents in cells and musts during vinification by three *Saccharomyces cerevisiae* races. Biotechnol. Letters, 17: 1351-1356.

Zea, L., Moyano, L., Moreno, J., Cortes, B. and Medina, M. (2001). Discrimination of the aroma fraction of Sherry wines obtained by oxidative and biological ageing. Food Chem., 75: 79-84.

Zea, L., Moyano, L., Moreno, J., Cortes, B. and Medina, M. (2007). Aroma series as fingerprints for biological ageing in fino sherry-type wines. J. Sci. Food Agric., 87:2319-2326.

Zuzuarregui, A., Monteoliva, L., Gil. C, and del Olmo, M. (2006). Transcriptomic and proteomic approach for understanding the molecular basis of adaptation of *Saccharomyces cerevisiae* to wine fermentation. Appl. Environ. Microbiol., 72: 836-847.

Appendix 3.

A proteomic and metabolomic approach for understanding the role of the flor yeast mitochondria in the velum formation.

Moreno-García, J., García-Martínez, T., Moreno, J., Millán, M.C., Mauricio, J.C.
International Journal of Food Microbiology. Volume 172, 17 February 2014, Pages 21-29.
https://doi.org/10.1016/j.ijfoodmicro.2013.11.030

Máster en Biotecnología Molecular, Celular y Genética.

CeIA$_3$.*Universidad de Córdoba*.

Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts

Jaime Moreno García

Departamento de Microbiología, Facultad de Ciencias.

Tutor del trabajo:

Dr. Juan Carlos García Mauricio

Directores:

Dra. María Teresa García Martínez Dr. Juan José Moreno Vigara

Juan Carlos García Mauricio, tutor del Trabajo Fin de Máster "Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts", realizado por Jaime Moreno García propone como revisores de dicho trabajo a los profesores:

1. José Peinado Peinado
2. Isidoro García García

Tutor del trabajo:

Dr. Juan Carlos García Mauricio

Directores:

Dra. María Teresa García Martínez Dr. Juan José Moreno Vigara

Index

Metabolomic comparative study of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, two wine yeasts

Abstract

Fermentations with pure cultures of *Saccharomyces cerevisiae* G1 (ATTC: MYA 425) and *Torulaspora delbrueckii* (ATTC: MYA 426) were carried out separately in YNB medium without amino acids and with 250 g of glucose/L. Fermentation rate, cellular growth, and the formation of major volatile compounds were analyzed. The obtained results showed that *S. cerevisiae* has a minor lag phase and it produces higher contents of ethanol, glycerol, volatile acidity, acetaldehyde, acetoin and 2,3-butanediol and lower contents of succinic acid than *T. delbrueckii*. *S. cerevisiae* metabolizes around 25% of the total glucose by means of glycero-piruvic pathway, showing a ratio of 1:1 glycerol/secondary products. In contrast, *T. delbrueckii* metabolizes 9.5% of the glucose to the formation of glycerol and 20.2% to secondary products. This study demonstrated that *T. delbrueckii* may have a different strategy to adapt to the osmotic stress than *S. cerevisiae*. The last activates the glycero-piruvic fermentation, which involves acetic acid high production for maintenance of redox potential, but not in *T. delbrueckii*.

Keywords: *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, synthetic medium, metabolome, fermentation.

Objectives

The aim of the present study is to compare the results obtained in major products of the fermentative metabolism of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* developed in pure culture in a synthetic medium and also to complement with the proteome results that are currently in process. Then, it will be possible to relate the yeast metabolome to the proteome in order to determine their enological potential and to provide winemakers knowledge and useful tools that allow them to develop better control of yeast fermentation and innovations in the wine sector. This information might thus lead to the production of wines with more predictable and desirable characteristics.

Introduction

The traditional role of wine yeasts, i.e., that of transforming grape sugars into ethanol, has been significantly widened by the advent of modern oenological microbiology. Spontaneous fermentation of grape must is a complex process involving sequentially different yeast genera and species whose ecology, biochemistry, physiology and molecular biology and its influence on wine composition and sensory properties has been widely studied in recent years (Pretorius, 2000; Fleet, 2003, 2008; Swiegers *et al.,* 2005). *Saccharomyces cerevisiae* is the main yeast responsible for spontaneous fermentation of musts, although in early stages non-*Saccharomyces* yeasts predominate. However, these are less resistant to ethanol, have a lower fermentation capacity and die when the ethanol content exceeds a threshold,

specific for each species (Heard and Fleet, 1985, 1986). On the other hand, many traditional studies (Ciani and Maccarelli, 1998; Fleet *et al*., 1984; Heard and Fleet, 1985; Pardo *et al*., 1989) and culture-independent techniques (Andorrra *et al.*, 2008; Hierro *et al*., 2006; Zott *et al.,* 2010) on winemaking ecology have shown that non-Saccharomyces genera (*Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*) can survive during fermentation at significant levels for longer periods than previously thought (Zott *et al*., 2010). Indeed, several authors (Fleet *et al*., 1984, Heard and Fleet, 1985, Cabrera *et al*., 1988, Pardo *et al.,* 1989, Herraiz *et al*., 1990; Ciani and Picciotti, 1995; Ciani and Ferraro, 1998, Lema *et al*., 1996, Egli *et al*., 1998; Fleet, 2003, Romano *et al.,* 2003 and Pina *et al*., 2004) have shown that these certain non-*Saccharomyces* yeasts produce moderate amounts of ethanol and have a great influence on the sensory quality of wine. With this regard, new trends in winemaking are aimed at the selection of suitable yeast for the production of quality wines according to each type of wine (Suarez-Lepe *et al*., 2012). Even more, the fact of knowing the specific proteins from the specific yeast able to synthesize important compounds will provide very relevant information applicable in a near future.

It is known from *Saccharomyces* proteome that these yeasts and also non-*Saccharomyces* release enzymes that are able to transform neutral compounds of grape berries into active odorant compounds, a process that enhances the sensory attributes of wines. For instance, enzymes like β-glucosidases are hydrolases capable of breaking down the links in terpenyl-β-glucosides, which means they release volatile and active odorant compounds

from their non-volatile combinations (Blasco *et al*., 2006; Charoenchai *et al*., 1997; Esteve-Zarzoso *et al*., 1998; Fernández *et al*., 2000). Carbohydrolases such as pectinases, cellulases, hemicellulases and amylases that can degrade structural grape polysaccharides together with turbidity-active proteins by proteases allowing the clarification and filtration process to improve during winemaking (Colagrande *et al*., 1994; Esteve-Zarzoso *et al*., 1998; Haight and Gump, 1994; Jayani *et al*., 2005; Louw *et al*., 2006; van Rensburg and Pretorius, 2000). Nowadays, there are multiple purposes where enzymatic treatments of grapes, musts and wines are carried out, since they positively influence wine filtration and clarification, juice yield, color and aroma extraction as well as wine stability (Rogerson *et al*., 2000). Furthermore, enzymes used in the winemaking process could be extracted from wine yeast for commercial production purposes (Charoenchai *et al*., 1997; Fernández *et a*l., 2000; Strauss *et al*., 2001).

An important non-*Saccharomyces* yeast, *Torulaspora delbrueckii* known as *Saccharomyces rosei* and has been described by Cabrera *et al*. (1988), Herraiz *et al*. (1990), Mauricio *et al*. (1991), Moreno *et al*. (1991); Ciani and Picciotti (1995), Ciani and Maccarelli (1998), as a large producer of compounds responsible for aroma and taste of alcoholic beverages, like acetoin, acetaldehyde, acetate and ethyl acetate (Cabrera et al., 1988; Herraiz *et al*., 1990; Martínez *et al*., 1990, Ciani and Ferraro, 1998). This yeast under standard conditions, in mixed or sequential culture with *S. cerevisiae*, has been proposed as a way of reducing acetic acid content in wine due to its high fermentation purity (Herraiz *et al*., 1990; Ciani and Picciotti, 1995; Ciani *et al*., 2006). Acetic acid is responsible of the volatile acidity that plays a significant

role in wine aroma and excessive concentrations of this alcoholic fermentation by-product are highly detrimental to wine quality. *T. delbrueckii* is less tolerant than *S. cerevisiae* to low available oxygen conditions and ethanol (Mauricio *et al*., 1998, Holm Hansen *et al*., 2001), but there is still not much knowledge about its potential in high sugar fermentations. *Torulaspora* and *Saccharomyces* genera produce a broad range of enzymes that are implicated in the alcoholic synthesis routes (alcohol deshydrogenase, pyruvate descarboxylase or pyruvate kinase) (Hansen *et al*., 1996), and it has also been reported as good producers of exogenous enzymes like β-glucosidases, pectinases, proteases and those involved in xylan degradation, which have enological interest. These can contribute to hydrolysis of natural precursors and consequently affect the aromatic characteristics and quality of the wine (Charoenchai *et al*., 1997; Ganga and Martínez, 2004; Manzanares *et al*., 1999; Masoud and Jespersen, 2006; Maturano *et al*., 2012, Pérez *et al*., 2011; Romo-Sánchez *et al*., 2010). β-glucosidases can also be found from the yeast (Blasco *et al*., 2006; Charoenchai *et al*., 1997; Esteve-Zarzoso *et al*., 1998; Fernández *et al*., 2000). Nevertheless, the secretion of each enzyme depends on the yeast strain analyzed and not in a particular genus or specie (Ganga and Martínez, 2004). The enzymatic activity in yeast from enological environments is influenced by different parameters like pH and temperature, as well as the presence of inhibitors such as sugars and ethanol (Barbagallo *et al*., 2004; Cordero Otero *et al*., 2003; Hernández *et al*., 2002; Jurado et al., 2004; Manzanares *et al*., 1999; McMahon *et al*., 1999; Rosi *et al*., 1994) but studies of these enzymatic activities in winemaking and fermentations are still scarce (Blasco *et al*., 2006; Fia *et al*., 2005; Zamuz *et al*., 2004). However, Maturano *et al*. (2012)

demonstrated that high concentrations of sugars did not affect enzymatic activity. By contrast, β-glucosidase and pectinase activities diminished with increasing fermentation time due to the increase in ethanol.

During fermentation, the yeast has to physiologically adapt to a whole range of stresses. Among them, high osmotic pressure, carbon source depletion, increase in the ethanol concentration are the most important ones. The yeast responds to these stresses changing its proteome throughout protein repression, induction and also intracellular proteolysis. Some of these induced proteins are those implicated in the heat shock and in the trehalose metabolism, needed to stabilize membranes and proteins and to avoid the protein aggregation. Another enzymes identified during fermentation were those related with the phosphate pentoses pathway, gluconeogenesis and glycerol synthesis (Cheng *et al*., 2008). Salvadó *et al*. (2008) reported that during the first stages of fermentation changes in concentration of enzymes related to the carbon and nitrogen metabolism and also cellular stress were happened.

Recently, metabolome studies carried out with *T. delbrueckii* fermentations on synthetic media in YPD and high sugars by Bely *et al*. (2008), in YEPD media by Renault *et al*. (2009), and by Maturano *et al*. (2012) have shown that this yeast produces less than 14% v/v of ethanol, has low cell growth and the fermentation is slow, but stands out as a low producer of residual reducing sugars, volatile acidity and glycerol and behaves differently to *S. cerevisiae* under osmotic stress conditions. According to Moreno *et al*. (1991) is possible to differentiate the wines obtained by fermentation with *S. cerevisiae*, *T. delbrueckii* and other indigenous yeasts in pure culture and mixed with the

contents in eleven fermentative metabolites, most notably through its concentration isoamilic alcohols, 2-phenyl ethanol, isobutanol, ethyl lactate and some minority esters. Comparisons between *S. cerevisiae* and *T. delbrueckii* made by Ciani and Picciotti (1995) and Ciani and Macarelli (1998) in fermentations of grape musts, showed that the first specie is capable of producing large amounts of ethanol while low amounts of secondary products, presenting a direct relationship between the production of acetic acid and ethanol formation, while other products such as acetoin and 2,3-butanediol have few changes in their production. *S. cerevisiae* produces more amount of ethanol, both in synthetic media, as in grape must and increases production of volatile acidity and glycerol in response to hyperosmotic media (Singh and Kunkee, 1976; Silver *et al*., 2003; Bely *et al*., 2008; Renault *et al*., 2009; García-Martínez *et al*., 2011). Finally, the two yeast species act on the primary aromas of the wine that come from grapes, transforming some monoterpenes into others (King, 2000).

This study compares the results obtained about the fermentation rate, cellular-yeasts growth and the major products of fermentative metabolism of *T. delbrueckii* and *S. cerevisiae* developed in pure culture in a medium with high glucose concentration without amino acids, and it has to be complemented with the proteome results, using OFFGEL prefractionation, that are currently in process. Its main objective is to relate the yeast metabolome to the proteome in order to provide winemakers knowledge and useful tools that allow them to better control of yeast fermentation and develop innovations in the wine sector.

Materials and methods

Microorganisms and fermentation conditions.

The yeast strains used in this study were *Saccharomyces cerevisiae* G1 (ATCC: MYA-425) and *Torulaspora delbrueckii* (ATCC: MYA-426), from the collection of the Department of Microbiology, University of Cordoba. Each strain was grown separately in YPD medium (1% yeast extract, 2% peptone and 5% glucose) for 24 hours, and then yeasts were used to inoculate the synthetic fermentation medium composed of 0.67% YNB without amino acids and 250 g/L glucose, with 1 x 10^6 total cells/mL.

The fermentations were performed in triplicate in 500 mL flasks at 28 ℃ and shaking at 175 rpm (INFORS AG, Switzerland). The fermentations were monitored by measuring the amount of CO_2 released via the weight loss of the medium. Samples were taken for the study of the metabolome at 10 days after inoculation. The number of yeast cells was determined in a Beckman Coulter particle counter Z2 previously performing the appropriate dilution. Yeast cells were separated from medium by centrifugation at 4600 rpm for 10 minutes at 4 ℃ in a Rotina 38R Hettich centrifuge.

Metabolome analysis.

Fermentable sugars, titratable acidity, volatile acidity, fixed acidity, pH and ethanol content were analyzed using the methods recommended by the European Union (EEC, 1990).

The major volatile compounds and polyols, which are by-products of fermentation, were quantified by gas chromatography and direct injection of the

sample according to the method of Peinado *et al*. (2004). The gas chromatograph Agilent 6890 (Palo Alto, California, USA), was equipped with a capillary column CP-WAX 57 CB, 60 m length and 0.25 mm inner diameter and 0.4 microns thick coating, a flame ionization detector and an injector in split mode with a 30:1 flow split. It was injected 0.5 µL of a mixture consisting of 10 mL of sample and 1 mL of a solution containing 1 g/L of 4-methyl-2-pentanol as internal standard. Quantification was performed using the response factors of each compound previously calculated by subjecting standard solutions of known concentration to the same treatment as the samples. The temperature program started at 45 ℃ (15 min) with a ramp of 4 ℃/min and a final temperature of 190 ℃ (35 min). The temperatures of injector and detector were 270 ℃ and 300 ℃, respectively, and the carrier ga s (helium) was originally scheduled to 0.7 mL/min for 16 min, followed by a ramp of 0.2 mL/min to a value of 1.1 mL/min, which was maintained for 52 min.

Proteome analysis.

Protein extracts preparations. Yeast cells were collected from the media by using centrifugation 4500 x *g* during 10 minutes (Rotina-38). After that, yeast cells were broken and extracted using a buffer solution and glass beds in a Vibrogen Zellmühle (Edmund Bühler). Then, the proteins were precipitated with TCA-acetone. Bradford method (1976) was used in order to quantify proteins.

OFFGEL. The OFFGEL High Resolution kit pH 3−10 (Agilent Technologies) was used for pI-based protein preparative isoelectric focusing (IEF) in solution. Protein samples (500 µg aprox. of total yeast protein) were solubilized in Protein OFFGEL fractionation buffer supplied by the manufacturer

(containing urea, thiourea, DTT, glycerol, and buffer with ampholytes), and aliquots were evenly distributed in a 12-well 3100 OFFGEL Fractionator (Agilent Technologies) tray according to supplier instructions: preset program OG12PR00 (separation limits: 4500 V, 200 mW, and 50 µA; starting voltage, 200-1500 V as recommended by the manufacturer; ending voltage, 5000-8000 V; after the application of 20 kVh, the protein separation zones were maintained at constant voltage).

HPLC. The liquid fractions were recovered and ultrafiltered in order to concentrate them. The buffer was changed to ammonium bicarbonate. Reduction and alquilation were carried out previously to the digestion with trypsin. Samples were cleaned and solid phase was extracted, then concentrated and eluted. At last and before injecting to the HPLC, nitrile acetate was dried off and changed into mobile phase.

Statistical analysis.

We used Statgraphics Centurion XVI (Manugistics, Inc., Rockville, MD) for analysis and comparison of means homogeneous groups. Proteome results will be statistically analyzed with the Proteome Discovered program.

Results and discussion

Development of fermentation.

Figure 1 shows the absorbance values at 600 nm during the first 50 hours of fermentation. This measure is related to the number of total cells and

shows that *T. delbrueckii* required an adaptation period greater than *S. cerevisiae*. Figure 2 shows the kinetics of fermentation as CO_2 release rate *versus* time and it is observed that the two yeasts show a high speed in the first 24 hours of fermentation. Nevertheless, *T. delbrueckii* has a higher speed, and it was stopped sharply before that *S. cerevisiae*, which was continued softer until the experiment was finished. In the assayed conditions not sufficient ethanol was reached for the inhibition of *T. delbrueckii* growth, and as consequence reached cell population was similar in both yeasts (Table 1). On the other hand, Mauricio *et al*. (1991), and Ciani and Picciotti (1995), showed in grape must that the slower growth of *T. delbrueckii* is not due to a deficit of assimilable nitrogen in the fermentation medium, rather to the increase of the concentration of ethanol and to the lack of oxygen.

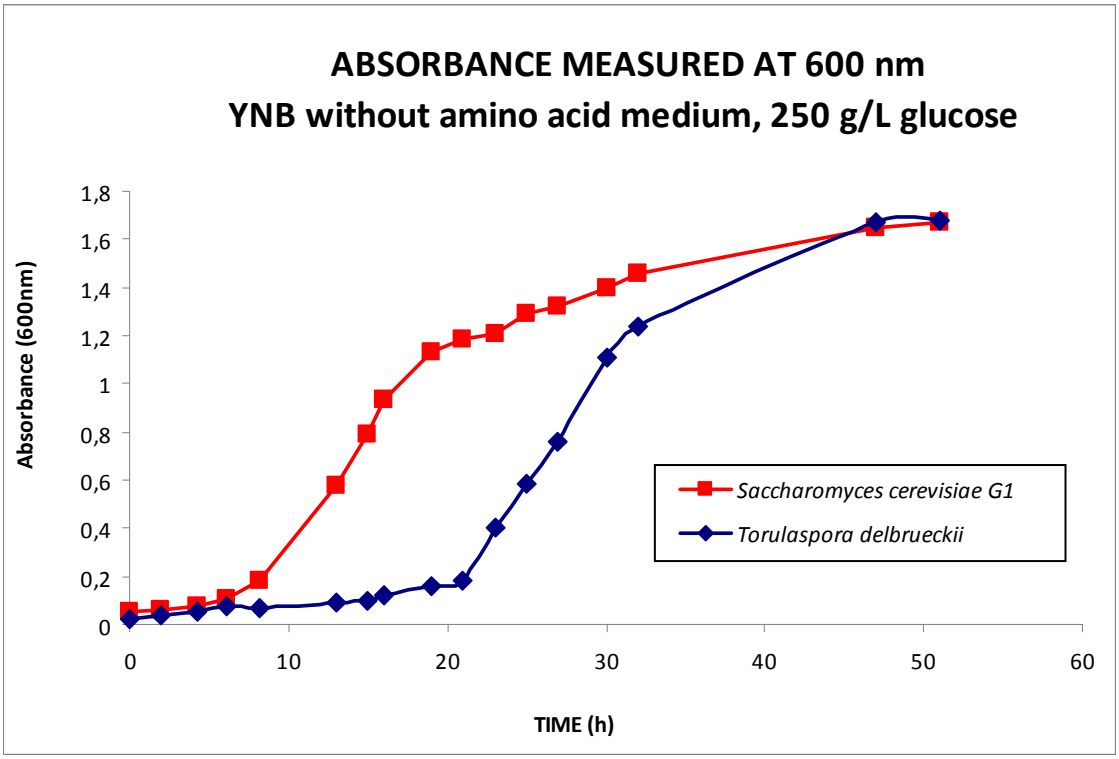


Figure 1. Absorbance measured at 600 nm of two yeasts during alcoholic fermentation in a synthetic medium.

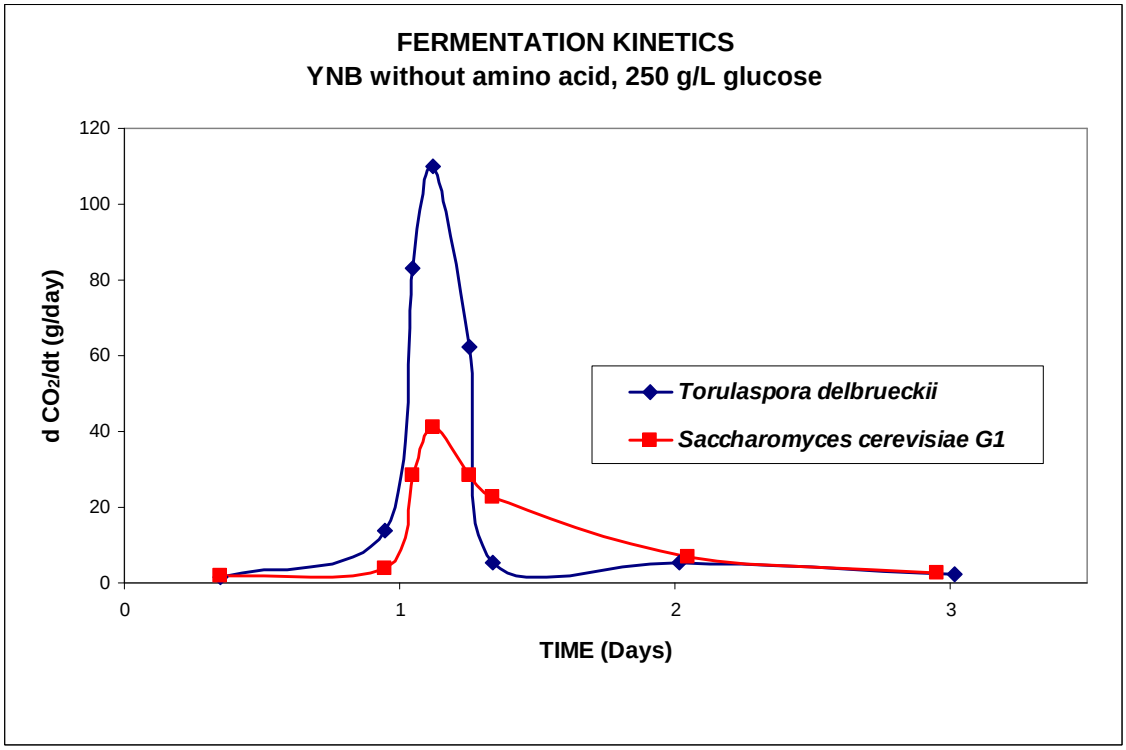


Figure 2. Mean values of CO_2 release per day of two yeasts during alcoholic fermentation in a synthetic medium.

Characteristics of fermented media.

Table 1 shows the major metabolites of enological interest contained in the analyzed mediums. Highlighting *S. cerevisiae* by increased production of ethanol, glycerol and volatile acidity, while *T. delbrueckii* notable for its low production of volatile acidity and increased production of fixed acidity. In the fermentation conditions tested *T. delbrueckii* presents an efficiency in the production of ethanol 0.42 g ethanol/g glucose (equivalent to 19 g glucose /% v/v ethanol) versus 0.36 g ethanol/g glucose (equivalent to 21.7 g glucose /% v/v ethanol) *S. cerevisiae*. Also *T. delbruecckii* presents a higher value of fixed acidity (mainly succinic acid) and a lower ratio volatile acid/ethanol, confirming the differences between the two species observed by Cabrera *et al*. (1988);

Herraiz *et al*. (1990); Martínez *et al*. (1990); Ciani and Picciotti (1995); Ciani and Ferraro (1998) and Bely *et al*. (2008). In regard to the pH of the medium, both yeast caused a decrease due to the formation of weak acids from neutral substrates and the medium was not buffered, according to Moreno and Peinado (2010).

Fraction or compound	***Initial medium***		***Saccharomyces cerevisiae***			***Torulaspora delbrueckii***		
	Mean	**SD**	**Mean**	**SD**	**HG**	**Mean**	**SD**	**HG**
Total cells (x 10^6/mL)	1.0	0.1	77	7	a	81	10	a
Ethanol (% v/v)	ND		5.3	0.2	a	3.9	0.2	b
Glucose (g/L)	250.0	0.1	135	5	a	176	1	b
Volatil acidity (g/L)	ND		2.40	0.05	a	0.40	0.01	b
Titulable acidity (g/L)	0.99	0.03	3.8	0.04	a	3.2	0.02	a
Fixed acidity (equiv/L)	0.013	0.001	0.011	0.001	b	0.035	0.001	a
pH	3.10	0.02	2.30	0.02	a	2.30	0.02	a
g/L Glucose / % Ethanol (v/v)			21.7	2.5	a	19.0	1.5	a
g Ethanol/g Glucose			0.36	0.04	a	0.42	0.03	a
g/L Acetic acid / % Ethanol (v/v)			0.45	0.01	a	0.10	0.01	b

Table 1. Main metabolites of oenological interest produced by the two yeasts after alcoholic fermentation in a synthetic medium.
(ND = not detected) SD: Standard deviation. HG: Homogeneous groups among the two yeasts. Different letters show significant differences with a confidence level of 95 % according to the Bonferroni test. The alphabetical order shows the content order.

By-products and balances.

Higher alcohols, esters and carbonyl compounds are secondary compounds from the yeast metabolism that contribute to the aroma of fermented beverages by their volatility. Their contents are found in Table 2.

Fraction or compound	***Initial medium***		***Saccharomyces cerevisiae***			***Torulaspora delbrueckii***		
	Mean	**SD**	**Mean**	**SD**	**HG**	**Mean**	**SD**	**HG**
Methanol (mg/L)	4.8	0.5	8.0	0.8	b	15.6	1.0	a
1-Propanol (mg/L)	ND		31.8	0.9	a	7.7	0.2	b
Isobutanol (mg/L)	ND		22.6	0.2	a	7.3	0.1	b
Isoamyl alcohols (mg/L)	ND		22.0	0.2	a	13.9	0.1	b
2-Phenylethanol (mg/L)	2.1	0.2	5.7	1.5	a	4.2	0.4	a
∑ **Alcohols (mg/L)**	7.1	0.5	90.0	0.9	a	49	2	b
Glycerol (g/L)	ND		17	1	a	3.6	0.2	b
Acetaldehyde (mg/L)	6.7	0.1	382	15	a	61	3	b
Acetoin (mg/L)	ND		1742	124	a	71	4	b
2,3-Butanediol *l* (mg/L)	ND		1177	108	a	276	28	b
2,3-Butanediol *m* (mg/L)	ND		413	38	a	182	18	b
∑ **Glyceropiruvic products (mg/L)**	6.7	0.1	3714	321	a	591	65	b
Ethyl acetate (mg/L)	ND		36	1	a	6.8	0.3	b
Ethyl lactate (mg/L)	ND		35	4	a	16	1	b
Diethyil succinate (mg/L)	ND		3.4	0.4	a	2	2	a
∑ **Esters (mg/L)**	ND		75	6	a	25	2	b

Table 2. Secondary compounds: major volatile compounds and polyols produced by the yeasts in the fermentation of the synthetic medium. (ND = not detected) SD: Standard deviation. HG: Homogeneous groups among the two yeasts. Different letters show significant differences with a confidence level of 95 % according to the Bonferroni test. The alphabetical order shows the content order.

It is noted in Table 2 that *S. cerevisiae* produced significantly greater amounts of 1-propanol, isobutanol, and isoamyl alcohols (2 and 3-metilisobutanol) than *T. delbrueckii*, while the last produced the greatest amount

of methanol. Also ethyl esters of acetic and lactic acids had significantly higher content in the fermentation carried out with *S. cerevisiae* and diethyl succinate content showed no significant difference between the two yeasts. Finally, *S. cerevisiae* produced the highest concentrations of the products of the glycero-piruvic fermentation as glycerol, acetaldehyde, acetoin and 2,3-butanediol (levo and meso forms). These results agree with the obtained ones for other authors (Ciani and Macarelli,1998; Ciani and Picciotti, 1995; Renault *et al.,* 2009).

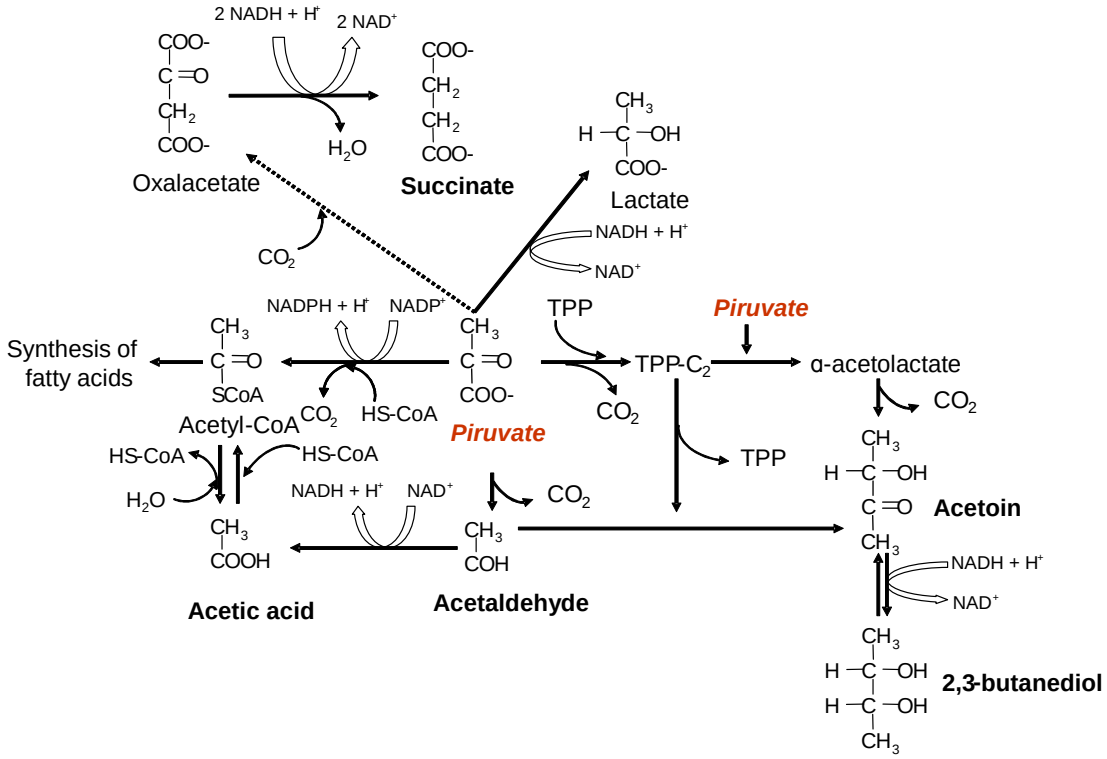


Figure 3. Relation of piruvate with glycerol-piruvic by-products. Broken arrows indicate a via not very active.

Yeasts transform throughout alcoholic fermentation one glucose or fructose molecule into two molecules of ethanol and two of CO_2. However, while the yeast accumulates sufficient acetaldehyde and reducing power to transform into ethanol, glycero-pyruvic fermentation occurs, with formation of a glycerol molecule and a molecule of pyruvic acid per molecule of glucose. Pyruvic acid is decarboxylated and forms acetaldehyde, which if not reduced to ethanol, forms various secondary products, among them, acetoin, 2,3-butanediol, succinic acid and acetic acid. It is therefore possible to take a balance of the

amounts of glucose that each yeast uses for the production of each of the metabolites quantified in this study (Figure 3).

Table 3 and Figure 4 show the concentrations expressed in mmol/L calculated from the mean levels obtained.

Compounds or fraction and ratio	***Saccharomyces cerevisiae***	***Torulaspora delbrueckii***
Residual Glucose	750	977
Consumed Glucose	639	411
Ethanol	901	669
Glycerol (G)	190	39
Secondary Products (PS)	156	83
Ratio ∑ PS/G	0.82	2.12
% Consumed Glucose	46.0	29.6
% Glucose to Ethanol	70.5	81.3
% Glucose to Glycerol	29.7	9.5
% Glucose to Secondary Products	24.4	20.2
% Glucose to Ethanol and Glycerol	100.2	90.9
% Glucose to Ethanol and Sec. Products	94.9	101.5

Table 3. Balance of alcoholic and glycero-pyruvic fermentations for the assayed yeasts. Concentrations have been expressed in mmol/L. The sum of the by-products from the glycero-pyruvic fermentation has been calculated according to the expression: ∑= 2a+5s+2m+2b+h. Being a= acetic acid; s= succinic acid; m= acetoin; b= 2,3-butanediol; h= acetaldehide.

It is remarkable that *S. cerevisiae* destines 70.5% of glucose consumed in the formation of ethanol and 29.7% glycerol formation, while *T. delbrueckii* makes it in an extension of 81.3 and 9.5% respectively. Thus under the conditions tested, this balance explain virtually 100% of glucose consumed by *S. cerevisae* while for *T. delbrueckii* consumption only accounts for 90.9% of the

initial glucose in the medium. As mentioned above, for every molecule of glycerol produced is obtained another molecule of pyruvic acid, so that if one considers the sum of concentrations of the products formed from pyruvic acid it should be obtained a ratio of 1 or close to unity. The formation reactions expressed by Ribéreau-Gayon *et al*. (2000) lead to the following expression, given by Moreno and Peinado (2010):

Initial concentration of pyruvic acid = 2 a + 5 s + 2 m + 2 b + h

Expressing all concentrations in mmol/L and with a = acetic acid, s = succinic acid, m = acetoin, b = 2,3-butanediol and h = free acetaldehyde.

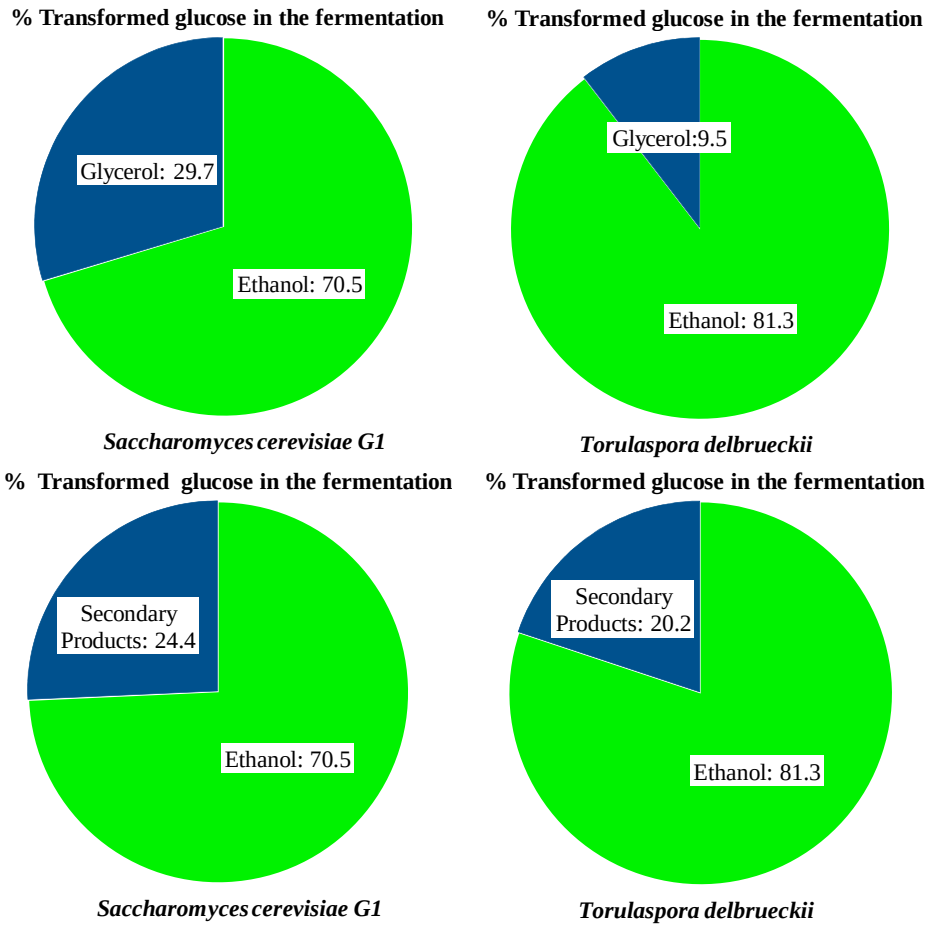


Figure 4. Metabolite balances in the *S.cerevisiae* and *T. delbruechkii*.

According to the results of Table 3, *S. cerevisiae* destines 24.4% of glucose consumed to secondary products, almost the same rate as glycerol, obtaining a relationship between both of 0.82, close to 1. In contrast, *T. delbrueckii* allocates 20.2% to secondary products and has a ratio of 2.1 compared to the glycerol formed. This percentage, added to the spent by the yeast in the formation of ethanol, complete virtually 100% of glucose metabolized and confirms that *T. delbrueckii* exhibits greater activity of the metabolic pathways involved in the formation of secondary compounds from pyruvic acid against the formation of glycerol. These results might be better explained when the results of the proteoma should be obtained.

Conclusions

Saccharomyces cerevisae produces higher amounts of glycerol, volatile acid, acetaldehyde, acetoin and 2,3-butanediol than *Torulaspora delbrueckii* in YNB medium without amino acids and 250 of glucose. *S. cerevisiae* metabolized about 25% of the total glucose by glycero-pyruvic fermentation and has a ratio glycerol/secondary products close to unity. In contrast, *T. delbrueckii* allocates 9.5% of glucose and glycerol formation by 20.2% to secondary products.

The results indicate that *S. cerevisiae* has the glicero-piruvic pathway more enhanced that *T. delbrueckii* in a high sugar medium, which might indicate different strategies to approach the osmotic stress.

Acknowledgements

This research was part of a project co-funded by Ministry of Economy and Competitiveness of Spain (INIA-CCAA) and FEDER (RTA2011-00020-C02-02).

References

Andorra, I., Landi, S., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B. "Effect of oenological practices on microbial populations using culture independent techniques" *Food Microbiology*, 25, 2008, 849–856.

Barbagallo, R.N., Spagna, G., Palmeri, R., Restuccia, C., Giudici, P. "Selection, characterization and comparison of β-glucosidase from mould and yeasts employable for enological applications" *Enzyme and Microbial Technology*, 35, 2004, 58–66.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu D. "Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation" *International Journal of Food Microbiology*, 122, 2008, 312–320.

Blasco, L., Veiga-Crespo, P., Poza, M., Villa, T.G. "Hydrolases as markers of wine aging" *World Journal of Microbiology and Biotechnology*, 22, 2006, 1229–1233.

Cabrera, M.J., Moreno, J., Ortega, J.M., Medina, M. "Formation of ethanol, higher alcohols, esters and terpenes by five yeast strains in must from Pedro Ximenez grapes in various degrees of ripeness" *American Journal of Enology and Viticulture*, 39, 1988, 283–287.

CEE. *Diario Oficial de las Comunidades Europeas.* L-272. Madrid (España). Ed. Mundi–Prensa. 1990.

Charoenchai, C., Fleet, G.H., Henschke, P.A., Todd, B.E.N. "Screening of non-Saccharomyces wine yeast for the presence of extracellular hydrolytic enzymes" *Australian Journal of Grape and Wine Research* 3, 1997, 2–8.

Cheng, J., Qiao, B. and Yuan, Y. "Comparative proteome analysis of robust *Saccharomyces cerevisiae* insights into industrial continuous and batch fermentation" *Applied Microbiology and Biotechnology* 81, 2008, 327–338.

Ciani, M., Beco, L., Comitini, F., "Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations" *International Journal of Food Microbiology* 108, 2006, 239–245.

Ciani, M., Ferraro, L. "Combined used of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines" *Journal of Applied Microbiology,* 85, 1998, 247–254.

Ciani, M., Maccarelli, F. "Enological properties of non-*Saccharomyces* yeasts associated with wine-making" *Biotechnology Letters*, 17, 1998, 1247-1250.

Ciani, M., Picciotti, G. "The growth Kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making" *World Journal Microbiology & Biotechnology*, 14, 1995, 199-203.

Cordero Otero, R.R., Ubeda Iranzo, J.F., Briones-Perez, A.I., Potgieter, N., Villena, M.A., Pretorius, I.S., van Rensburg, P. "Characterization of the β-glucosidase activity produced by enological strains of non-Saccharomyces yeasts" *Food Microbiology and Safety,* 68, 2003, 2564–2569.

Egli, C.M., Edinger, W.D., Mitrakul, C.M., Henick-Kling, T. "Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines" *Journal of Applied Microbiology* 85, 1998, 779–789.

Esteve-Zarzoso, B., Manzanares, P., Ramon, D., Querol, A. "The role of non-*Saccharomyces* yeasts in industrial wine-making" *International Microbiolog,* 1, 1998, 143–148.

Fernández, M., Ubeda, J.F., Briones, A.L. "Typing of non-Saccharomyces yeasts with enzymatic activities of interest in wine-making" *International Journal of Food Microbiology*, 59, 2000, 29–36.

Fia, G., Giovani, G., Rosi, I. "Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity" *Journal of Applied Microbiology*, 99, 2005, 509–517.

Fleet, G. H. "Wine yeasts for the future" *FEMS Yeast Research*, 8, 2008, 979–995.

Fleet, G.H. "Yeast interactions and wine flavor" *International Journal of Food Microbiology*, 86, 2003, 11–22.

Fleet, G.H., Lafon-Lafourcade, S., Ribéreau-Gayon, P. "Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines" *Applied and Environmental Microbiology*, 48, 1984, 1034–1038.

García-Martínez, T., Bellincontro, A., Lopéz de Lerma, M.N., Peinado, R.A., Mauricio, J.C., Mencarelli, F., Moreno, J. "Discrimination of sweet wines partially fermented by two osmo-ethanol tolerant yeasts by gas chromatographic analysis and electronic nose" *Food Chemistry*. 127, 2011, 1391–1396.

Ganga, M.A., Martínez, C. "Effect of wine yeast monoculture practice on the biodiversity of non-Saccharomyces yeasts" *Journal of Applied Microbiology*, 96, 2004 76–83.

Hansen, J., Kielland-Brandt, M. C. "Modification of biochemical pathways in industrial yeasts" *Journal of Biotechnology* 49, 1996, 1- 12.

Heard, G.M., Fleet, G.H. "Occurrence and growth of yeast species during the fermentation of some Australian wines" *Food Technology in Australia* 38, 1986, 22–25.

Heard, G.M., Fleet, G.H. "Growth of natural yeast flora during the fermentation of inoculated wines" *Applied and Environmental Microbiology*, 50, 1985, 727–728.

Hernández, L.F., Espinosa, J.C., Fernández-González, M., Briones, A. "β-Glucosidase activity in a Saccharomyces cerevisiae wine strain" *International Journal of Food Microbiology*, 80, 2002, 171–176.

Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P.J., Cabezudo, M. "The influence of the yeast and type of culture on the volatile composition of wine fermented without sulphur dioxide" *American Journal of Enology and Viticulture*, 41, 1990, 313–318.

Hierro, N., González, A., Mas, A., Guillamón, J.M. "Diversity and evolution of non-Saccharomyces yeast populations during wine fermentation: effect of grape ripeness and cold maceration" *FEMS Yeast*, 6, 2006, 102–111.

Holm Hansen, E., Nissen, P., Sommer, P., Nielsen, J.C., Arneborg, N. "The effect of oxygen on the survival of non-Saccharomyces yeasts during mixed culture fermentations grape juice with Saccharomyces cerevisiae" *Journal of Applied Microbiology,* 91, 2001, 541–547.

Jurado, E., Camacho, F., Luzón, G., Vicaria, J.M. "Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature" *Enzyme and Microbial Technology*, 34, 2004, 33–40.

King, A., Dickinson, J. R. "Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*" *Yeast*, 16, 2000, 499-506.

Lema, C., Garcia-Jares, C., Orriols, I., Angulo, L. "Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma" *American Journal of Enology and Viticulture*, 47, 1996, 206–216.

Manzanares, P., Ramon, D., Querol, A. "Screening of non-*Saccharomyces* wine yeasts for the production of β-D-xylosidase activity" *International Journal of Food Microbiology,* 46, 1999, 105–112.

Martínez, J., Toledano, F., Millán, C., Ortega J.M. "Development of alcoholic fermentation in non-sterile musts from Pedro Ximénez grapes inoculated with pure cultures of selected yeasts" *Food Microbiology*, 7, 1990, 217-225.

Masoud, W., Jespersen, L. "Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa" *International Journal of Food Microbiology,* 110, 2006, 291–296.

Maturano, Y. P., Rodríguez-Assaf, L. A., Toro, M. E., Nally, M. C., Vallejo, M., Castellanos de Figueroa, L. I., Combina, M., Vazquez, F., "Multi-enzyme production by pure and mixed cultures of Saccharomyces and non-Saccharomyces yeasts during wine fermentation" *International Journal of Food Microbiology* 155, 2012, 43–50.

Mauricio, J.C., Guijo, S., Ortega, J.M. "Relationship between phospholipids and sterol contents in *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and their fermentation activity in grape musts *American Journal of Enology and Viticulture*, 42, 1991, 301–308.

Mauricio, J.C., Millan, C., Ortega, J.M., "Influence of oxygen on the biosynthesis of cellular fatty acids, sterols and phospholipids during alcoholic fermentation by *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*" *World Journal of Microbiology & Biotechnology*, 14, 1998, 405-410.

McMahon, H., Zoecklein, B.W., Fugelsang, K., Jasinski, Y. "Quantification of glycosidase activities in selected yeasts and lactic acid bacteria" *Journal of Industrial Microbiology & Biotechnology*, 23, 1999, 198–203.

Moreno, J., Peinado, R.A. *Química Enológica*. Madrid (España). AMV Ediciones. 2010.

Moreno, J.J., Millan, C., Ortega, J.M., Medina, M. "Analytical differentiation of wine fermentations using pure and mixed yeast cultures" *Journal of Food Microbiology*, 7, 1991, 181–190.

Pardo, I., Garcia, M.J., Zuniga, M., Uruburu, F. "Dynamics of microbial populations during fermentation of wines from Utiel-Requena region of Spain" *Applied and Environmental Microbiology*, 55, 1989, 539–541.

Peinado, R.A., Moreno J.A., Muñoz, D., Medina, M., Moreno J. "Gas chromatographic quantification of major volatile compounds and polyols in wine by direct injection" *Journal Agricultural Food Chemistry*, 52, 2004, 6389–6393.

Pérez, G., Fariña, L., Barquet, M., Boido, E., Gaggero, C., Dellacassa, E., Carrau, F. "A quick screening method to identify β-glucosidase activity in native wine yeast strains: application of esculin glycerol agar (EGA) medium" *World Journal of Microbiology and Biotechnology,* 27, 2011, 47–55.

Pina, C., Santos, C., Couto, J.A., Hogg, T. "Ethanol tolerance of five non-*Saccharomyces* wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*—influence of different culture conditions" *Food Microbiology*, 21, 2004, 439–447.

Pretorius IS. "Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking" *Yeast*, 16, 2000, 675–729.

Renault, P., Miot-Sertier, C., Marullo, P., Hernández-Orte, P., Lagarrigue, L., Lonvaud-Funel, A., Bely, M. "Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry" *International Journal of Food Microbiology,* 134, 2009, 201–210.

Ribéreau-Gayon, P., Dubordieu, D., Donèche, B., Lonvaud, A. *Handbook of Enology*. *Vol I. The microbiology of wine and vinifications*. Ed. John Wiley & Sons Ltd. Chichester. England. 2000.

Rogerson, F.S.S., Vale, E., Grande, H.J., Silva, M.C.M. "Alternative processing of port-wine using pectolytic enzymes" *Ciencia y Tecnologia Alimentaria*, 2, 2000, 222–227.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A. "Function of yeast species and strains in wine flavor" *International Journal of Food Microbiology*, 86, 2003, 169–180.

Romo-Sánchez, S., Alves-Baffi, M., Arévalo-Villena, M., Úbeda-Iranzo, J., Briones-Pérez, A. "Yeast biodiversity from oleic ecosystems: study of their biotechnological properties" *Food Microbiology*, 27, 2010, 487–492.

Rosi, I., Vinella, M., Domizio, P. "Characterization of β-glucosidase activity in yeasts of enological origin" *The Journal of Applied Bacteriology*, 77, 1994, 519–527.

Salvadó, Z., Chiva, R., Rodríguez-Vargas, S., Rández-Gil, F., Mas, A. and Guillamón, M. J. "Proteomic evolution of a wine yeast during first hours of Fermentation" *FEMS Yeast Res* 8, 2008, 1137–1146.

Singh, R., Kunkee, R. "Alcohol dehydrogenase activities of wine yeasts in relation to higher alcohol formation" *Applied Environmental Microbiology*, 32, 1976, 666–670.

Strauss, M., Jolly, N., Lambrechts, M., van Rensburg, P. "Screening for the production of extracellular hydrolytic enzymes by non-Saccharomyces wine yeasts" *Journal of Applied Microbiology,* 91, 2001, 182–190.

Suarez-Lepe, JA., Morata, A. "New trends in yeast selection for winemaking" *Trends in Food Science & Technology*, 23, 2012, 39-50.

Swiegers , JH., Bartowsky, EJ., Henschke, PA., Pretorius IS. "Yeast and bacterial modulation of wine aroma and flavor" *Australian Journal Grape Wine Research*, 11, 2005, 139–173.

Zamuz, S., Sestelo, A.B.F., Poza, M., Villa, T.G. "Enzymes as potential markers of wine aging" *Journal of the Institute of Brewing*, 110, 2004, 220–226.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., Masneuf-Pomarede, I. "Characterization of the yeast ecosystem in grape must and wine using real-time PCR" *Food Microbiology,* 27, 2010, 559–567.

Appendix 2.

Biological aging status characterization of Sherry type wines using statistical and oenological criteria.

Moreno-García, J., Raposo-Ortega, R.M., Moreno, J.
Food Research International. Volume 54 (1), 1 November 2013, Pages 285-292.
https://doi.org/10.1016/j.foodres.2013.07.031

Food Research International 54 (2013) 285–292

Contents lists available at SciVerse ScienceDirect

Food Research International

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

Biological aging status characterization of Sherry type wines using statistical and oenological criteria

Jaime Moreno-García, Rafaela M. Raposo, Juan Moreno *

Departamento de Química Agrícola y Edafología, University of Córdoba, Spain

ARTICLE INFO

Article history:
Received 24 February 2013
Accepted 7 July 2013
Available online 13 July 2013

Keywords:
Wine
Biological aging
Volatile compounds
Chemometric classification
Kinetic model

ABSTRACT

20 chemical compounds were quantified in wines from 6 different biological aging statuses in an industrial "criadera" system. A Conglomerated Analysis (CA) realized with 7 selected compounds allowed the differentiation of wines according to their aging. By a Linear Discriminant Analysis (LDA) the 6 most discriminant compounds were identified and a correct classification of 100% of wine samples was achieved. The wine aging time can be calculated by a 3 compound equation obtained by Multiple Regression Analysis (MRA). The last two models were applied to wines with unknown aging time and their results related obtaining a $R = 0.958$. A kinetic model, based in the glycerol content evolution, allows the calculation of the required time to achieve the specific concentration of a status and the glycerol concentration after a known aging time. These models can help to protect the quality of wines elaborated by the traditional biological aging system.

1. Introduction

The process for the elaboration of biologically aged white wines through the so-called "criaderas" system can be considered as the most important contribution of the South of Spain to the Enology field worldwide. The obtained wines are known as "Fino" wines and are characterized by a light, subtle flavor; a very pale yellow color; a dry, fragrant, slightly bitter taste and a sugar content below 1 g/L. The sensorial properties of Fino wines are also known as "flor" or velum bouquet (López-Alejandre, 2005). The development and conservation of this velum, a yeast biofilm in the wine–air interface, is the principal aim of the industrial biological aging process.

The Fino-type wines are elaborated traditionally in the Andalucia community, fundamentally in the Protected Denomination of Origin (PDO) Jerez–Xerez–Sherry (JXS), Montilla–Moriles (MM) and Condado de Huelva (CH). According with Hidalgo (1999), these grape-growing areas belong to zone V of Winkler's classification, with an effective temperature, considered as the sum of degree-days below 10 °C, exceeding 2597 °C on the complete vegetative cycle grapevines. Palomino-Fino (PF), Pedro Ximenez (PX) and Zalema (ZA) white grapes are the predominant varieties cultivated respectively in the three above mentioned PDOs.

The criaderas system is a dynamic wine-aging technique applied to the elaboration of some special wine types. The process for the elaboration of Fino wine under the action of flor yeasts starts after alcoholic and malolactic fermentations and when the spontaneous stabilization of wine is concluded. Essentially it involves the aging of wines in used American oak casks with 625 L volume that are filled to 5/6 of their capacity in order to facilitate the development of the flor yeasts. Traditionally, the casks have been previously conditioned through grape-musts fermentations or whisky maturation. In this way the oak-flavor extraction, that might otherwise mask the bouquet of Fino wine, is minimized. A typical velum developed by *Saccharomyces cerevisiae* var. *capensis* on the wines containing around 15–15.5% (v/v) ethanol shows a white-ivory color, a thick film of several millimeters and a rough surface (Cortés, Moreno, Zea, Moyano, & Medina, 1998, 1999; Moreno & Peinado, 2012)

Basically, the set of casks in a criadera system is grouped into different scales, so called criaderas, each of which contains wine with the same aging time. The system consists of several rows that are numbered from the floor to the top. The first row, lying directly on the floor of the aging cellar, is called the "solera" and contains wine with the greatest aging time; the row above is the "first criadera" and subsequently the second, third, etc. The year's vintage wine is loaded in the "sobretabla" (the uppermost row) thereby containing the youngest wine. As a general rule, the wineries have from 4 to 5 criaderas, including the solera. The criadera system is a method of fractional blending, where the youngest wines are periodically added and sequentially transferred through the criaderas containing a more aged wine. Before transfer to the next stage, the wine drawn from each oak cask (about 1/4 of its volume) is blended with wine from other casks in the same criadera. The transfer frequency depends on development of the wine,

Abbreviations: AC, Analysis of Conglomerates; CA, Chemical Analysis; CH, Condado de Huelva; DF, Discriminant Function; GC, Gas Chromatograph; GCA, Gas-Chromatography Analysis; JXS, Jerez, Xeres, Sherry; LDA, Linear Discriminant Analysis; MCA, Multiple Comparison Analysis; MM, Montilla–Moriles; MRA, Multi Regression Analysis; MSE, Mean Square Error; PDO, Protected Denomination of Origin; PF, Palomino Fino; PX, Pedro Ximénez; R^2, variability percentage explained; ZA, Zalema.

* Corresponding author. Tel.: +34 957 218636; fax: +34 957 212146.
E-mail address: qe1movij@uco.es (J. Moreno).

0963-9969/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.foodres.2013.07.031

Appendix 3.

A proteomic and metabolomic approach for understanding the role of the flor yeast mitochondria in the velum formation.

Moreno-García, J., García-Martínez, T., Moreno, J., Millán, M.C., Mauricio, J.C.
International Journal of Food Microbiology. Volume 172, 17 February 2014, Pages 21-29.
https://doi.org/10.1016/j.ijfoodmicro.2013.11.030

International Journal of Food Microbiology 172 (2014) 21–29

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

A proteomic and metabolomic approach for understanding the role of the flor yeast mitochondria in the velum formation

Jaime Moreno-García [a,1], Teresa García-Martínez [a,1], Juan Moreno [b], M. Carmen Millán [a,1], Juan Carlos Mauricio [a,*]

[a] Department of Microbiology, Severo Ochoa (C6) building, Agrifood Campus of International Excellence CeiA3, University of Cordoba, Ctra. N-IV-A Km 396, 14014 Cordoba, Spain
[b] Department of Agricultural Chemistry, Marie Curie (C3) building, Agrifood Campus of International Excellence CeiA3, University of Cordoba, Ctra. N-IV-A, Km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 7 August 2013
Received in revised form 14 November 2013
Accepted 25 November 2013
Available online 4 December 2013

Keywords:
Saccharomyces cerevisiae
Flor yeast
Velum
Mitochondria
Proteome
Metabolome

ABSTRACT

Saccharomyces cerevisiae "flor" yeast shows a strong tolerance to high ethanol concentrations and develops a velum (biofilm) on the wine surface after the alcoholic fermentation of grape must. This velum remains along several years during the so called "biological aging" process in the elaboration of some special wines carried out in specific regions around the world and it contributes to the typical organoleptic characteristics of these wines. In order to grow in this condition, flor yeast has to elaborate a response where the mitochondrial function is essential. The objective of this study is to elucidate the role of the mitochondria in the response of a flor yeast, *S. cerevisiae* G1, growing in a controlled velum formation condition. For this purpose, proteome and metabolome were characterized by comparing data with those from an initial fermentative condition used as reference. The obtained proteomic profiles show more mitochondrial proteins related with the ethanol resistance (13), cell respiration (18), mitochondrial genome maintenance (13), and apoptosis (2) detected under the velum formation condition. Also, the finger-printing obtained by means of the exo-metabolites directly related with the quality of fermented beverages and quantified in the velum condition shows important differences from those obtained in the reference condition.

1. Introduction

Some special Sherry type wines are elaborated by a characteristic process called biological aging. This process is performed by specific *Saccharomyces cerevisiae* strains that are capable of growing on the wine surface where they form a biofilm, known as "flor" or "velum". It has been reported by some authors that these yeasts are responsible of a wide range of organoleptic properties for these special wines (Martínez et al., 1998: Mesa et al., 2000; Moyano et al., 2002; Muñoz et al., 2005, 2007; Peinado and Mauricio, 2009).

Biological aging takes place in the wines after the alcoholic fermentation when the glucose content is practically depleted and ethanol together with glycerol reach concentrations about 15% (v/v) and 8 g/L, respectively (Moreno and Peinado, 2012; Moreno-García et al., 2013). To metabolize these remaining molecules, *S. cerevisiae* flor yeast requires oxygen and at the time being consumed from the medium, cells aggregate each other close to the wine-air interphase. This zone is characterized by high dissolved oxygen content and constitutes the place where the velum is developed. In these conditions, flor yeast metabolism is purely oxidative (Mauricio et al., 1997); ethanol is oxidized to acetaldehyde and acetate, and glycerol is steadily consumed (Martínez et al., 1998). For these purposes and for the elaboration of the stress response to the environmental conditions established by this medium, yeast cells need to synthesize a range of essential proteins. Many of the required proteins are mitochondrial-located proteins since they are necessary to perform an oxidative metabolism (cellular respiration) of the non-fermentable carbon sources and they are also responsible for the elaboration of the response to stresses.

Some authors reported that a mitochondrial-located protein, manganese superoxide dismutase (Sod2p) confers protection against ethanol, acetaldehyde and reactive oxygen species (ROS) (Costa et al., 1993, 1997; van Loon et al., 1986; Piper, 1999; Ma and Liu, 2010; Fierro-Risco et al., 2013). Also the copper–zinc superoxide dismutase (Sod1p) located in the cytoplasm or in the mitochondrial intermembrane space provides resistance against oxidative stress. As it is known, other mitochondrial proteins are involved in the tricarboxylic acid cycle (TCA cycle) or in the respiratory electron chain (Reinders et al., 2006).

A proteome and metabolome data complementation will provide a fairly complete information about the yeast response under a typical velum formation condition, which will contribute to a better knowledge about flor yeast and also about its response to high ethanol content media. On one hand, the proteomic analysis will provide a list of proteins that the yeast synthesizes as a response to the environmental conditions and, on the other hand, the level of some metabolites could be regarded as the ultimate response of the biological systems to

* Corresponding author. Tel.: +34 957218640.
E-mail address: mi1gamaj@uco.es (J.C. Mauricio).
[1] Tel.: +34 957218640.

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.030

Appendix 4.

Proteins involved in flor yeast carbon metabolism under biofilm formation conditions.

Moreno-García, J., García-Martínez, T., Moreno, J., Mauricio, J.C.
Food Microbiology. Volume 46, April 2015, Pages 25-33.
https://doi.org/10.1016/j.fm.2014.07.001

Food Microbiology 46 (2015) 25–33

Contents lists available at ScienceDirect

Food Microbiology

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

Proteins involved in flor yeast carbon metabolism under biofilm formation conditions

Jaime Moreno-García [a], Teresa García-Martínez [a], Juan Moreno [b], Juan Carlos Mauricio [a, *]

[a] Department of Microbiology, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Severo Ochoa Building, Ctra. N-IV-A km 396, 14014 Cordoba, Spain
[b] Department of Agricultural Chemistry, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Marie Curie Building, Ctra. N-IV-A km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form
30 June 2014
Accepted 2 July 2014
Available online 18 July 2014

Keywords:
Flor yeast
Proteins
OFFGEL
Biofilm
Carbon metabolism

ABSTRACT

A lack of sugars during the production of biologically aged wines after fermentation of grape must causes flor yeasts to metabolize other carbon molecules formed during fermentation (ethanol and glycerol, mainly). In this work, a proteome analysis involving OFFGEL fractionation prior to LC/MS detection was used to elucidate the carbon metabolism of a flor yeast strain under biofilm formation conditions (BFC). The results were compared with those obtained under non-biofilm formation conditions (NBFC). Proteins associated to processes such as non-fermentable carbon uptake, the glyoxylate and TCA cycles, cellular respiration and inositol metabolism were detected at higher concentrations under BFC than under the reference conditions (NBFC). This study constitutes the first attempt at identifying the flor yeast proteins responsible for the peculiar sensory profile of biologically aged wines. A better metabolic knowledge of flor yeasts might facilitate the development of effective strategies for improved production of these special wines.

1. Introduction

Biologically aged wines require special production methods. Fino wine from grapes grown in the Sherry or Montilla–Moriles region, the production process for which is described in detail elsewhere (Peinado and Mauricio, 2009), is probably the best known type of wine obtained by biological ageing. Following fermentation of the starting must, biologically aged wine spontaneously develops a natural biofilm known as “flor velum”. The biofilm is formed by aggregated yeasts called “flor yeasts”, available knowledge about which was recently reviewed by Alexandre (2013).

Although biologically aged wines undergo changes similar to those in all aged wines including chemical reactions between compounds, extraction of specific compounds from the container wood and salt precipitation, most of the changes affecting their composition and sensory properties are associated to the metabolism of flor yeasts (Martínez et al., 1998; Mesa et al., 2000; Moyano et al., 2002; Muñoz et al., 2005). Flor yeasts in biologically ageing wines use previously fermented must—which contains high concentrations of fermentative products such as ethanol and glycerol but no fermentable sugars—as substrate. Composition changes in the medium cause flor yeasts to metabolize other molecules via an oxygen-requiring process; this requires cells to migrate to the wine surface once dissolved oxygen in the medium is depleted. Therefore, formation of a yeast biofilm can be regarded as an adaptive mechanism ensuring access to oxygen and allowing yeasts to cope with harsh environmental conditions (Alexandre, 2013). In this situation, yeasts adopt an oxidative metabolism involving consumption of non-fermentable carbon sources (Mauricio et al., 1997).

Controlled biological ageing experiments with pure cultures of *Saccharomyces cerevisiae* grown on natural wines showed ethanol to be metabolized preferentially at the velum formation stage rather than the velum maintenance stage (Rodríguez et al., 2013). Ethanol, which constitutes the most abundant source of carbon and energy for flor yeasts, is partly converted into other metabolites such as acetaldehyde and acetic acid. Acetaldehyde, which is the main metabolite excreted to the medium by flor yeasts, has a strong impact on the sensory properties of the resulting wine; in fact, it is the primary source of the strong aroma typical of biologically aged wines. This compound results mainly from enzymatic oxidation of

* Corresponding author. Tel.: +34 957218640; fax: +34 957218650.
E-mail address: mi1gamaj@uco.es (J.C. Mauricio).

http://dx.doi.org/10.1016/j.fm.2014.07.001
0740-0020/

Appendix 5.

Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions.

Moreno-García, J., García-Martínez, T., Moreno, J., Millán, M.C., Mauricio, J.C., Moreno, J.
Food Microbiology. Volume 51, October 2015, Pages 1-9.
https://doi.org/10.1016/j.fm.2015.04.005

Food Microbiology 51 (2015) 1–9

Contents lists available at ScienceDirect

Food Microbiology

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

Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions

Jaime Moreno-García [a], Teresa García-Martínez [a], M.Carmen Millán [a], Juan Carlos Mauricio [a], Juan Moreno [b, *]

[a] Department of Microbiology, Severo Ochoa (C6) building, Agrifood Campus of International Excellence CeiA3, University of Cordoba, Ctra. N-IV-A mm 396, 14014 Córdoba, Spain

[b] Department of Agricultural Chemistry, Marie Curie (C3) building, Agrifood Campus of International Excellence CeiA3, University of Córdoba, Ctra. N-IV-A, km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form
19 March 2015
Accepted 13 April 2015
Available online 24 April 2015

Chemical compounds studied in this article:
Acetaldehyde
(PubChem CID: 177)
Acetic acid
(PubChem CID: 176)
Benzaldehyde
(PubChem CID: 240)
Decanoic acid
(PubChem CID: 2969)
1,1-Diethoxyethane
(PubChem CID: 7765)
Ethanol
(PubChem CID: 702)
Ethyl decanoate
(PubChem CID: 8048)
Ethyl octanoate
(PubChem CID: 7799)
Glycerol
(PubChem CID: 753)
Isoamyl acetate
(PubChem CID: 31276)
2-Phenethyl acetate
(PubChem CID: 7654)

Keywords:
Saccharomyces cerevisiae
Flor yeast
Biofilm
Proteome
Exometabolome

ABSTRACT

A proteomic and exometabolomic study was conducted on *Saccharomyces cerevisiae* flor yeast strain growing under biofilm formation condition (BFC) with ethanol and glycerol as carbon sources and results were compared with those obtained under no biofilm formation condition (NBFC) containing glucose as carbon source. By using modern techniques, OFFGEL fractionator and LTQ-Orbitrap for proteome and SBSE-TD-GC-MS for metabolite analysis, we quantified 84 proteins including 33 directly involved in the metabolism of glycerol, ethanol and 17 aroma compounds. Contents in acetaldehyde, acetic acid, decanoic acid, 1,1-diethoxyethane, benzaldehyde and 2-phenethyl acetate, changed above their odor thresholds under BFC, and those of decanoic acid, ethyl octanoate, ethyl decanoate and isoamyl acetate under NBFC.

Of the twenty proteins involved in the metabolism of ethanol, acetaldehyde, acetoin, 2,3-butanediol, 1,1-diethoxyethane, benzaldehyde, organic acids and ethyl esters, only Adh2p, Ald4p, Cys4p, Fas3p, Met2p and Plb1p were detected under BFC and as many Acs2p, Ald3p, Cem1p, Ilv2p, Ilv6p and Pox1p, only under NBFC. Of the eight proteins involved in glycerol metabolism, Gut2p was detected only under BFC while Pgs1p and Rhr2p were under NBFC. Finally, of the five proteins involved in the metabolism of higher alcohols, Thi3p was present under BFC, and Aro8p and Bat2p were under NBFC.

* Corresponding author.
E-mail address: qe1movij@uco.es (J. Moreno).

http://dx.doi.org/10.1016/j.fm.2015.04.005
0740-0020/

Appendix 6.

Stress responsive proteins of a flor yeast strain during the early stages of biofilm formation.

Moreno-García, J., Mauricio, J.C., Moreno, J., García-Martínez, T. Process Biocehmistry. Volume 51 (5), May 2016, Pages 578-588. https://dx.doi.org/10.1016/j.procbio.2016.02.011

Process Biochemistry 51 (2016) 578–588

Contents lists available at ScienceDirect

Process Biochemistry

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

Stress responsive proteins of a flor yeast strain during the early stages of biofilm formation

Jaime Moreno-García[a], Juan Carlos Mauricio[a,*], Juan Moreno[b], Teresa García-Martínez[a]

[a] Department of Microbiology, Severo Ochoa (C6) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra, N-IV-A, Km 396, 14014 Cordoba, Spain

[b] Department of Agricultural Chemistry, Marie Curie (C3) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra, N-IV-A, Km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form 26 January 2016
Accepted 15 February 2016
Available online 20 February 2016

Keywords:
OFFGEL
LC–MS
Flor velum
Stress proteins

ABSTRACT

Flor yeasts metabolism configures organoleptic properties in Sherry type wines during biological aging. Along this process, yeasts form a biofilm known as *flor* velum, being the presence of certain stress proteins essential for survival and making them excellent organisms for stress response investigations. In this study, an OFFGEL fractionator coupled to LTQ Orbitrap XL MS equipment was used to identify in a flor yeast strain, the maximum possible number of stress proteins under biofilm formation conditions (BFC) and under non-biofilm formation conditions (NBFC), used as reference. A total of 85 stress response proteins have been detected in a higher content than in the reference condition of which 18 are involved in the response to the lack of fermentable carbon source, 39 in the response to the ethanol stress, 3 in the acetaldehyde resistance and 46 in the oxidative stress response. Three proteins of these 85 were found to respond to three different stresses (Por1p and Tps2p in lack of fermentable carbon source, resistance to ethanol and resistance to oxidative stress; and Tkl1p in the resistance to ethanol, resistance to acetaldehyde and resistance to oxidative stress). Results of this study might lead to the genetic improvement of flor yeast strains.

1. Introduction

In some wine-producing regions around the world: California (USA), Sardinia (Italy), Jura (France), Jerez and Montilla-Moriles (Spain), among others, biological aging method is used for the elaboration of some special white types of wine known as Sherry wines. During this process, the final organoleptic properties of the oenological product are modified principally due to the metabolism performed by peculiar yeast strains, so-called "flor yeasts" [1,2].

Flor velum formation takes place after fermentation when flor yeasts become predominant [3]. In this moment, the medium is characterized by very low sugar and high ethanol content that result from a previous fermentative metabolism and an oxidative stress coming from a non-fermentable carbon respiratory metabolism developed by the yeasts when fermentation is finished. In addition, high acetaldehyde levels are achieved during biological aging, being well known to be more toxic than ethanol for yeasts [4]. In order to survive in the mentioned harsh environmental conditions, flor yeasts have the capacity to develop biological systems where proteome plays an important role [1,5,6]. For instance, formation of the biofilm appears to be an adaptive mechanism because it ensures access to oxygen and therefore permits continued growth on non-fermentable carbon sources [7]. Proteins like Flo11p, by increasing surface hydrophobicity [8], Hsp12p found to be essential to the biofilm formation [9] and Ccw7p which exerts molecular reorganization of the cell wall during stress adaptation [10]; all contribute to the biofilm development. On the other hand, high ethanol and acetaldehyde content together with the oxidative stress also provokes a proteomic stress response. Alcohol dehydrogenase I and II isoenzymes have a role in the redox balance maintenance during hostile biofilm formation conditions [11] and Hsp12p both respond to ethanol and acetaldehyde high concentration [12] meanwhile superoxide dismutases (Sod1p and Sod2p) are known to be implicated in the response to ethanol and oxidative stresses [13].

Nowadays, genes that codify stress response proteins have been used as targets for the genetic improvement of wine yeast strains to enhance the fermentation performance [14]. Also, in the biological aging process, a higher quality in velum and a higher cell viability have been attained by the overexpression of *FLO11*, *SOD1*, *SOD2* and *HSP12* genes [15–17]. Certainly, another proteins involved in

* Corresponding author. Fax: +34 957218650.
E-mail address: mi1gamaj@uco.es (J.C. Mauricio).

http://dx.doi.org/10.1016/j.procbio.2016.02.011
1359-5113/

Appendix 7.

Functional analysis of stress protein data in a flor yeast subjected to a biofilm forming condition.

Moreno-García, J., Mauricio, J.C., Moreno, J., García-Martínez, T.
Data in Brief. Volume 7, June 2016, Pages 1021-1023.
https://dx.doi.org/10.1016/j.dib.2016.03.072

Data in Brief 7 (2016) 1021–1023

Contents lists available at ScienceDirect

Data in Brief

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

Data Article

Functional analysis of stress protein data in a flor yeast subjected to a biofilm forming condition

Jaime Moreno-García [a], Juan Carlos Mauricio [a,*], Juan Moreno [b], Teresa García-Martínez [a]

[a] Department of Microbiology, Severo Ochoa (C6) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra. N-IV-A, Km 396, 14014 Cordoba, Spain
[b] Department of Agricultural Chemistry, Marie Curie (C3) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra. N-IV-A, Km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 23 February 2016
Received in revised form
16 March 2016
Accepted 21 March 2016
Available online 28 March 2016

ABSTRACT

In this data article, an OFFGEL fractionator coupled to LTQ Orbitrap XL MS equipment and a SGD filtering were used to detect in a biofilm-forming flor yeast strain, the maximum possible number of stress proteins under the first stage of a biofilm formation conditions (BFC) and under an initial stage of fermentation used as reference, so-called non-biofilm formation condition (NBFC). Protein functional analysis – based on cellular components and biological process GO terms – was performed for these proteins through the SGD Gene Ontology Slim Mapper tool. A detailed analysis and interpretation of the data can be found in "Stress responsive proteins of a flor yeast strain during the early stages of biofilm formation" [1].

1. Specifications Table

Subject area	*Biology, Microbiology and Biochemistry* *Proteomics, Bioinformatics*

DOI of original article: http://dx.doi.org/10.1016/j.procbio.2016.02.011
* Corresponding author.
E-mail address: mi1gamaj@uco.es (J.C. Mauricio).

http://dx.doi.org/10.1016/j.dib.2016.03.072

Appendix 8.

Differential Proteome Analysis of a Flor Yeast Strain under Biofilm Formation.

Moreno-García, J., Mauricio, J.C., Moreno, J., García-Martínez, T.
International Journal of Molecular Sciences. Volume 18 (4), Article 720, 28 March 2017.
https://www.mdpi.com/1422-0067/18/4/720

International Journal of
Molecular Sciences

MDPI

Article

Differential Proteome Analysis of a Flor Yeast Strain under Biofilm Formation

Jaime Moreno-García [1], Juan Carlos Mauricio [1], Juan Moreno [2] and Teresa García-Martínez [1,*]

[1] Department of Microbiology, Agrifood Campus of International Excellence ceiA3, University of Cordoba, 14014-Cordoba, Spain; b62mogaj@uco.es (J.M.-G.); mi1gamaj@uco.es (J.C.M.)
[2] Department of Agricultural Chemistry, Agrifood Campus of International Excellence ceiA3, University of Cordoba, 14014-Cordoba, Spain; qe1movij@uco.es
* Correspondence: mi2gamam@uco.es; Tel./Fax: +34-957218640

Academic Editor: Martin Welch
Received: 10 February 2017; Accepted: 21 March 2017; Published: 28 March 2017

Abstract: Several *Saccharomyces cerevisiae* strains (flor yeasts) form a biofilm (flor velum) on the surface of Sherry wines after fermentation, when glucose is depleted. This flor velum is fundamental to biological aging of these particular wines. In this study, we identify abundant proteins in the formation of the biofilm of an industrial flor yeast strain. A database search to enrich flor yeast "biological process" and "cellular component" according to Gene Ontology Terminology (GO Terms) and, "pathways" was carried out. The most abundant proteins detected were largely involved in respiration, translation, stress damage prevention and repair, amino acid metabolism (glycine, isoleucine, leucine and arginine), glycolysis/gluconeogenesis and biosynthesis of vitamin B9 (folate). These proteins were located in cellular components as in the peroxisome, mitochondria, vacuole, cell wall and extracellular region; being these two last directly related with the flor formation. Proteins like Bgl2p, Gcv3p, Hyp2p, Mdh1p, Suc2p and Ygp1p were quantified in very high levels. This study reveals some expected processes and provides new and important information for the design of conditions and genetic constructions of flor yeasts for improving the cellular survival and, thus, to optimize biological aging of Sherry wine production.

Keywords: *Saccharomyces cerevisiae*; flor velum; GO Terms; OFFGEL electrophoresis; LTQ Orbitrap XL MS

1. Introduction

Saccharomyces cerevisiae flor yeast strains are very interesting for winemaking purposes due to their influence on the sensory properties of Sherry type wines [1,2]. These wines are produced from a process named "biological aging", a process conducted in many regions around the world, including Spain, France, Italy, South Africa, Hungary, Armenia, USA (California) and Southern Australia. In Southern Spain, wine is biologically aged in a so-called "criaderas system", which is regarded as the greatest contribution of this region to winemaking worldwide.

During biological aging, flor yeasts develop an oxidative metabolism (respiration) under harsh survival conditions, including high ethanol and acetaldehyde concentrations, oxidative stress and low pH. Buoyancy and spontaneous, natural immobilization by formation of a biofilm (flor velum) on the surface of wine provide an effective mechanism that protects the cells and facilitates survival and proliferation under the adverse conditions during the biological aging. In fact, the formation of a biofilm at the wine-air interface brings yeasts into contact with an oxygen-rich zone where they can efficiently metabolize ethanol and glycerol. Yeast proliferation under the prevailing oxidative conditions of biological aging is also facilitated by the antioxidant defense system protecting cells from

Appendix 9.

Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain.

Moreno-García, J. et al.
Food Microbiology. In preparation to sent.

Study of the role of two cell surface glycoproteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain

*Co-authors

*Affiliations

Abstract

In the Sherry wine elaboration process carried out in specific regions around the world, *Saccharomyces cerevisiae* "flor" yeast develops a velum (biofilm) on the wine surface. This velum remains along several years during the so-called "biological aging" process and it contributes to the typical wine organoleptic properties. In this study, null mutants have been constructed using a haploid flor yeast strain (P3-D5) and phenotypes study have been carried to study the function within the biofilm development of two potentially related velum formation proteins, Ccw14p and Ygp1p. Results show that *YGP1* might be related to the velum formation as significant differences considering the dry weight of yeast forming velum, were detected among Δ*ygp1* and wild type. This study together with other complementary, also applied to other flor yeast strains, could confirm the implication of this protein in the velum formation of flor yeast and further lead to the genetic improvement of strains aimed to enhance their survival during the biological aging process and hence the whole wine elaboration process.

Introduction

Flor yeasts are *Saccharomyces cerevisiae* strains whose interest within the enological field lies in their influence on the sensorial properties of a special type of wine, so-called "Sherry wines". This organoleptic change takes place during a process known as "biological aging" which is carried in many different areas around the world (Spain, France, Italy, South Africa, Armenia, Hungary, California and Southern Australia). After

the must fermentation, flor yeast start to consume ethanol and glycerol while other chemicals like acetaldehyde or acetic acid are produced, these affecting the wine color, texture, flavor and taste properties (Peinado and Mauricio, 2009). The resulting wines are characterized by sensorial properties known as “flor” or velum bouquet (López-Alejandre, 2005).

Along this process, flor yeasts have to face several harsh conditions for survival (low oxygen concentration, high ethanol concentration, low pH, etc.) (Alexandre, 2013). One of the principal is the lack of a fermentable carbon source like glucose or fructose already consumed during the previous fermentation process. Zara et al., (2010) considered the formation of the biofilm (also called “flor” or “velum”) as an adaptation system of the flor yeast to a medium which lacks a fermentable carbon source. Biofilm formation in the wine-air area allows the yeast to reach an oxygen-rich zone where it is possible to catalyze ethanol or glycerol, both produced in fermentation. This property was first attributed to a high cell surface hydrophobicity (Martínez et al., 1997b) which would be due to a specific cell wall composition. It was suggested by some authors that the presence of molecules, as β-glucans or mannoproteins increase the cell surface hydrophobicity (Iimura et al., 1980; Martínez et al., 1997a,b; Alexandre et al., 1998; Alexandre et al., 2000). Also specific cell wall proteins such as the glycosylphospatidylinositol (GPI) anchored cell surface glycoprotein Flo11p has an essential role in the biofilm formation (Reynolds and Fink, 2001; Zara et al., 2005; Ishigami et al., 2004; Fidalgo et al., 2006; Purevdorj-Gage et al., 2007). Moreover, Hsp12p and Nrg1p are also involved (Ishigami et al., 2004; Zara et al., 2002). Btn2p, encoding a v-snare interacting protein involved in intracellular protein trafficking, may indirectly affect velum flor formation as deletion of this gene in flor yeast affects biofilm formation (Espinazo-Romeu et al., 2008).

Here, attending to results obtained from proteome analysis of a flor yeast, *Saccharomyces cerevisiae* G1, in a biofilm formation condition (Moreno-García et al., 2014) a protein selection was performed with the aim to discover new proteins involved in the velum formation process. In this study, we analysed the role of two proteins, Ccw14p and Ygp1p, within the velum formation process in a flor yeast strain. These proteins are known to participate in biofilm formation (Andersen et al., 2014; Vandenbosch et al., 2013) and were observed by Moreno-García et al. (data not yet published) to be highly expressed in a biofilm forming condition if compared to a reference non-biofilm formation condition. For this purpose null mutants have been constructed for each of the protein gene and analyses of velum formation have been performed.

Material and methods

Yeast strains and media.

Saccharomyces cerevisiae G1 (ATCC: MYA-2451), a wild type industrial flor strain from the Department of Microbiology (University of Córdoba, Spain) collection has been used for the previously made proteome analysis (Moreno-García et al., 2014) and for spore viability studies. As the ploidy of this strain is not yet known, an already characterized and genetically-easy to manipulate haploid flor yeast strain from the Department of Agricultural Sciences - Microbiology Institute (University of Sassari, Italy) collection, *S. cerevisiae* P3-D5 MAT α (Jura, France), was used for the genotype studies.

Different parameters (appearance, cell adherence and dry weight) of velum formation were studied in three different media: 0.67% (w/v) YNB without amino acids (Difco, Detroit, Mich.) and 4% (v/v) ethanol (YNB 4% ETOH); red wine, a diluted

standard red wine until reaching 4% (v/v) ethanol (red wine 4% ETOH) and a flor synthetic medium (Coi et al., in publication) with 4% (v/v) ethanol (FSM 4% ETOH).

Screening of proteins.

Proteins extracted from the G1 flor yeast strain under a biofilm formation condition and under a reference non-biofilm formation condition (Moreno-García et al., 2014) were subjected to a filter by using the YeastMine tool of SGD database (http://yeastmine.yeastgenome.org/) in order to obtain those proteins related to the biofilm formation phenotype. From them, another filter was applied resulting in those that were quantified with a value 2-fold higher than that in the reference condition. At last, parameters as type of biofilm, induction transcription of the protein genes, viability of null mutant and the relation with the biofilm formation process; were taken into account for the final selection of proteins in which genotype studies were performed.

Genetic studies.

Phleomycin markers flanked by sequences homologous to *CCW14* and *YGP1* were amplified using oligos indicated in Table 1, for the construction of deletion cassettes (Fig. 1) called Ble. A 50 µl PCR reaction mix contained: 5 µl 10X PCR Buffer-$MgCl_2$ Invitrogen, 2 mM dNTP Mix, 50 mM $MgCl_2$, 10 mM of each construction primer (table 1), 25 ng pUG66 template DNA, 2.5 U *Taq* DNA Polymerase Invitrogen. PCR conditions were: 94°C for 3 min, 94°C for 45 sec, 55°C for 30 sec, 72°C for 1 min 30 sec (30 cycles), 72°C for 10 min. The PCR product was purified (Qiagen, Inc.) and used to transform the strains P3-D5 MAT α using the protocol described by Güldener et al. (1996). Selection of transformants was performed on YPD (1% yeast extract, 2% peptone, 2% glucose and 2% agar) plates containing 150 µg/mL phleomycin. Confirmation of allele deletion was made by PCR using primers indicated in the Table 1 and consisting in a 12.5 µL PCR

reaction mix: 1.25 µl 10X PCR Buffer-$MgCl_2$ Invitrogen, 2 mM dNTP Mix, 50 mM $MgCl_2$, 10 mM of each verification primer and 10 µM for Ble1 and Ble 2 (Table 1) (VerFw-VerRv for the first PCR verification, VerFw-Ble1 for the second and VerRv-Ble2 for the third), 15 µg template DNA, 0.625 U *Taq* DNA Polymerase Invitrogen. PCR conditions were the same as in the cassette construction. The proper insertion of deletion cassettes in place of *CCW14* or *YGP1* was verified also by Sanger sequencing (GATC-Biotech sequencing service) using their respective verification primers, after PCR amplification and purification (Qiagen PCR purification kit) of the corresponding regions. DNA homology searches were performed using the BLAST algorithm through the National Center for Biotechnology Information and *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

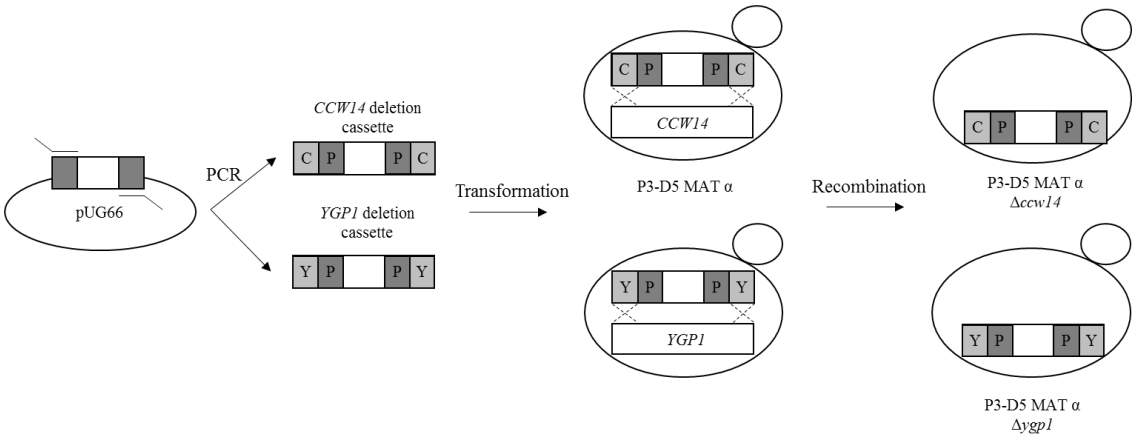


Fig. 1. Strategy to delete the *CCW14* and *YGP1* genes in a haploid flor yeast using the marker Phleo. P indicates the sequence section of Phleo in the cassette amplified in the PCR while C and P are the regions with homology to the oligos used to amplify the deletion cassette for *CCW14* and *YGP1* genes, respectively.

Table 1. DNA primers.

Primer	Sequence	Purpose
CCW14 PhleFw	5' CAGCACTACTAGACTCGTTCAACACTCGTTATATA TTATCGTACGCTGCAGGTCGACAAC 3'	Construction of the *CCW14*::*PHLEO* cassette from pUG66
CCW14 PhleRv	5' GATAGATACCTTAACCCATTAGAAATAAAGTGATAGATAAAC TATAGGGAGACCGGCAGA 3'	Construction of the *CCW14*::*PHLEO* cassette from pUG66
CCW14 VerFw	5' CCAGAATACGACGAGGACGG 3'	Verification of insertion of CCW14::PHLEO cassette
CCW14 VerRv	5' CCCAGATATGTACCGCCACC 3'	Verification of insertion of *CCW14*::*PHLEO* cassette
YGP1 PhleFw	5' TCTACTGGATTAATCGTCAGTTAAGTAATACAGTAAT AGAAAGTACGCTGCAGGTCGACAAC 3'	Construction of the *YGP1*::*PHLEO* cassette from pUG66
YGP1 PhleRv	5' AAAGAATCTCTATGCTTCGCTAGATTTAATATCTATCAGTACT ATAGGGAGACCGGCAGA 3'	Construction of the *YGP1*::*PHLEO* cassette from pUG66
YGP1 VerFw	5' CGGCTTCTCGATGCTACAGT 3'	Verification of insertion of *YGP1*::*PHLEO* cassette
YGP1 VerRv	5' AGAAGGGGGTGAGATCCCTT 3'	Verification of insertion of *YGP1*::*PHLEO* cassette
Ble1	5' GTGGGCGAAGAACTCCAG 3'	Verification of insertion of *PHLEO* cassette
Ble2	5' GTTCTACCGGCAGTGCAAAT 3'	Verification of insertion of *PHLEO* cassette

G1 flor yeast characterization

Spore viability analyses have been carried out. G1 yeast was grown during 6 days in agar sporulation medium containing potassium acetate (1%), bacto-yeast extract (0.1%), 0.5 g glucose (0.05%). Asci were digested with a solution 0.2% of the enzyme Lyticase (from Arthrobacter luteus, crude. Sigma) for 5 min and spores were separated using a micromanipulator (Singer Instrument, MSM technology). They were grown in YPD-agar for 5 days at 37 ºC. Grown spores were then inoculated again in an agar sporulation medium. Counting of spores were performed after the first and second spore growing. Further ploidy studies are required to adjust strategies for genetic studies of this flor yeast strain.

Biofilm formation analyses.

Air-liquid interfacial biofilm formation assay.

Formation of an air-liquid interfacial biofilm was performed as follows: P3-D5 MAT α wild-type and mutant strains were grown in 10 ml of YPD overnight at 30°C in an incubator-shaker, recovered by centrifugation, washed twice in sterile distilled water, and resuspended in three different media (YNB 4% ETOH, red wine 4% ETOH and FSM 4% ETOH). Three replicates of 2 ml (10^7 cells/ml) were aliquoted in 24-wells polystyrene microplates. Samples were incubated at 30°C for 3 to 5 days under static conditions and finally photographed. Technical and biological replicates were made for each sample.

Yeast forming biofilm adherence to polystyrene.

Strains were pre-cultivated overnight on SC (Yeast Nitrogen Base wo amino acid 0,67%) + 2% glucose, washed once and resuspended in SC + 0.1% glucose. 10 repetitions of 100 µl of each strain (OD_{600} nm=1) were aliquotated in 96-wells microplates (flat bottom, 353072, Becton-Dickinson Labware) and incubated 3 h in static conditions at 25°C. Cells that adhered to plastic were colored with 100 µl crystal violet (1% w/v). After 30 min wells were washed with sterile water and 100 µl 10 % SDS were added to each well. After 30 min 100 µl of sterile water were added, plates were photographed and adherence was quantified by measuring absorbance at 570nm.

Biofilm weight.

Strains were pre-cultivated overnight on 10 mL of YPD, and then cultivated in static conditions in 50 mL of YNB 4% ETOH medium for 4 days at 25°C. Biofilm was collected by aspiration with a vacuum pump and filtered with 0.45 µm nitrocellulose filters. Filters were dried and finally weighted. Biofilm dry weight was expressed as difference between filter + dried biofilm and the original filter before filtration of the velum.

Results and discussion

A total of 52 proteins were detected in the flor yeast proteome analysis by Moreno-García et al., 2014 (data not published) to be involved in the biofilm formation process. Among them, 37 were identified under the biofilm condition and 40 under the reference non-biofilm condition. Further, 17 in higher contents under the biofilm condition were found, being proteins Ccw14p and Ygp1p, both cell surface glycoproteins, those identified with highest differences among studied conditions: 0.53% (of the total protein weight) in the biofilm vs. 0.20% in the non-biofilm condition in the Ccw14p case and 1.12% compared to none in the non-biofilm condition in the Ygp1p case.

Based on the observation that *CCW14* and *YGP1* are somehow implicated in the formation of the yeast biofilm on a hydrophobic solid surface (Andersen et al., 2014; Vandenbosch et al., 2013), we reasoned that they would also be required for biofilm formation at the air-liquid interface, as gas-liquid interfaces are excellent models for hydrophobic surface-liquid interfaces (Hunter et al., 1990). Null mutants Δ*ccw14* and Δ*ygp1* were constructed replacing the ORF of *CCW14* and *YGP1*, respectively, with a cassette of Phleomycin in the haploid flor strain P3-D5 MAT α and their phenotypes related to the biofilm formation process were analyzed. Both mutants showed differences comparing with the wild type when inoculated in the YNB 4% ETOH medium in the air-liquid interfacial biofilm formation assay (Fig. 2). Contrasts under the other two studied conditions were not such marked probably because of a lesser identity with the biofilm formation condition used for the proteome analyses by Moreno-García et al. (2014) (data not published). Although these results seemed promising, one of the biological replicates exhibited similar biofilms within different strains so no relation among these proteins and velum formation can be established considering all replicates. Regarding to the adherence to polystyrene tests non-differences were reported (Fig. 3).

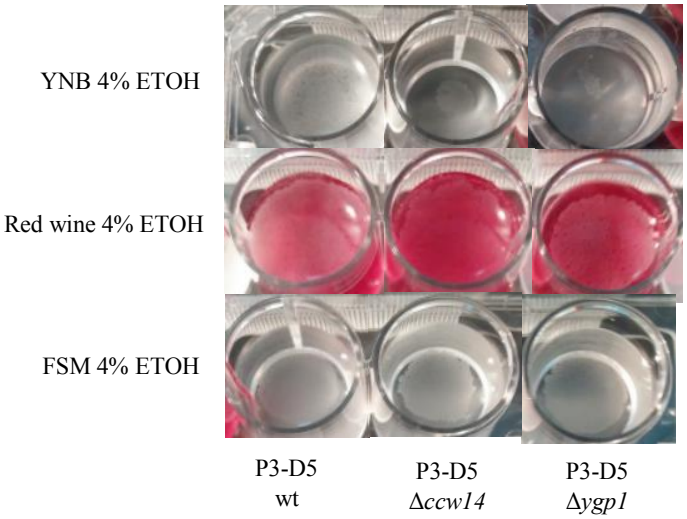


Figure 2. Test for velum formation. Cells were resuspended in 2 ml of medium. The wells were photographed after 3 days of static incubation at 30ºC.

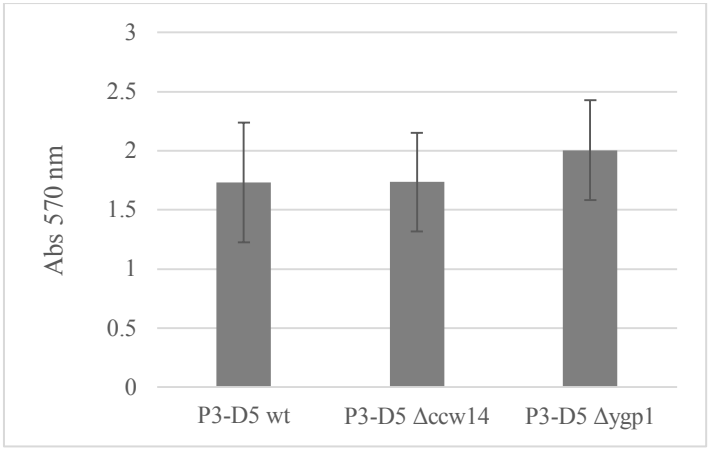


Fig. 3. Abs 570 nm obtained from the adherence to polystyrene tests.

On the other hand, significant differences were found with respect to the dry weight measurements of biofilm if considered the mutant Δ*ygp1* and the wild type (3.3 mg vs. 1.1 mg) as it is shown in the Fig. 4.

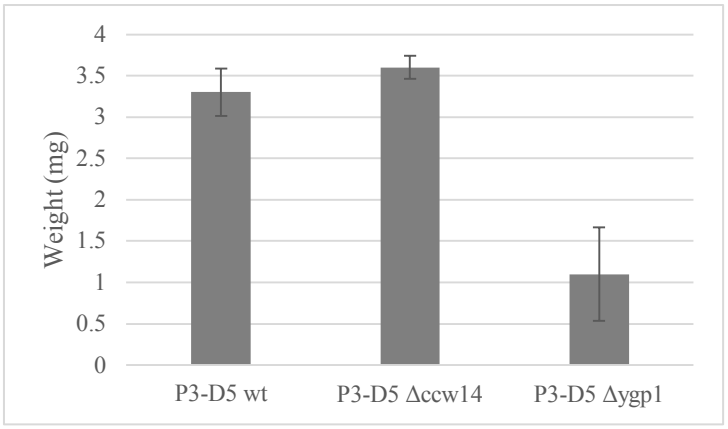


Fig. 4. Weight in mg obtained from the different strains velum forming yeasts in YNB 4% ETOH medium.

Sporulation tests have been performed for the flor yeast strain *Saccharomyces cerevisiae* G1 and from 67 spores inoculated, 18 were viable corresponding to a percentage of 26,87%. Further, from these 18, 12 were viable (66,7%). These results are within the range of spore viability values obtained from tests carried by Budroni et al. (2005) in flor yeasts. Moreover, the fact of finding spores in the second counting indicates that this strain is homothallic.

As no differences were perceived among velum formed by Δ*ccw14* null mutant and the wild type, it can be concluded that this protein is not involved in velum formation and hence, a higher content reported in the biofilm formation condition (Moreno-García et al., data not published) might indicates that Ccw14p participates in other biological processes also important in a biological aging condition. Ccw14p shares several characters with the velum formation-related protein Flo11p that makes it a potential protein involved in velum formation: GPI-anchoring, extensively O-glycosylated mannoprotein, cell surface location (Mrsa et al., 1999; Moukadiri et al., 1997) and SER-rich amino acid sequence; however no relation with velum making in flor yeast was perceived. Beside biofilm formation, Ccw14p is also involved in biological processes such as resistance to starvation (Davey et al., 2012) and fungal-type cell wall organization

(Moukadiri et al., 1997) being relevant for the resistance to stresses such as oxidative, high ethanol and acetaldehyde contents. To definitively confirm this hypothesis, similar genetic studies have to be expanded to the G1 flor yeast strain from which the basal proteomic data were extracted as well as analyses of different phenotypes in different flor yeast strains will be required.

Meanwhile Ygp1p, is a cell wall-related secretory extensively N-glycosylated glycoprotein; induced by nutrient deprivation-associated growth arrest, upon entry into stationary phase and repressed by high glucose concentrations (Destruelle et al., 1994; Byrne and Wolfe, 2005; Chang et al., 2001; Pardo et al., 1999; Riou et al., 1997). This protein is involved in the cell wall assembly and cellular amino acid metabolic process (Pardo et al., 1999). In this study, some influences have been observed considering this protein, mainly in the dry weight comparison. The presence of this protein did not show influence on the adherence of the cells, this indicating that might be contributing to the biofilm formation through another parameter such as hydrophobicity. For instance, Flo11p does contribute to the biofilm formation through increasing the adherence of the cells but also the hydrophobicity of the cell surface (Reynolds and Fink, 2001). As it has only been localized in the extracellular region, Ygp1p might form a portion of the composition of an extracellular matrix which until now has not been described although presence has been revealed (Zara et al., 2009).

It could be concluded that Ccw14p is associated to other process rather than the velum formation, like stress resistance also relevant for the flor yeast survival.

On the other hand, some evidences have been released relating flor yeast velum formation and the presence of the Ygp1p. It has been suggested that this protein may form a proportion of the biofilm matrix which composition has not been elucidated until now. Similar genetic analyses, including ploidy studies and genome sequencing, are required

for the flor yeast strain G1 to elucidate the role of Ccw14p and Ygp1p, as this strain was that used for the proteome analysis used as starting point.

References

Alexandre, H. 2013. Flor yeasts of *Saccharomyces cerevisiae*-Their ecology, genetics and metabolism. Int. J. Food Microbiol. 167, 269-275.

Alexandre, H., Bertrand, F., Charpentier, C. 1998. Ethanol induced yeast film formation with cell surface hydrophobicity as a major determinant. Food Technol. Biotechnol. 36, 27-30.

Alexandre, H., Blanchet, S., Charpentier, C., 2000. Identification of a 49 kDa hydrophobic cell wall mannoprotein present in velum yeast which may be implicated in velum formation. FEMS Microbiol. Lett. 185, 147-150.

Andersen, K. S., Bojsen, R. K., Sorensen, L. G., Nielsen, M. W., Lisby, M., Folkesson, A., Regenberg, B. 2014. Genetic basis for *Saccharomyces cerevisiae* biofilm in liquid medium. G3 (Bethesda) 4, 1671-1680.

Budroni M., Zara, S., Zara, G., Pirino, G., Mannazzu, I. 2005. Peculiarities of flor strains adapted to Sardinian sherry-like wine ageing conditions. FEMS Yeast Res. 951-958.

Byrne, K. P., Wolfe, K. H. 2005. The yeast gene order browser: combining curated homology and syntenic context reveals gene fate in polyploid species. Genome Res. 15, 1456-1461.

Chang, Y. W., Howard, S. C., Budovskaya, Y. V., Rine, J., Herman, P. K. 2001. The rye mutants identify a role for Ssn/Srb proteins of the RNA polymerase II holoenzyme during stationary phase entry in *Saccharomyces cerevisiae*. Genetics 157, 17-26.

Davey, H. M., Cross, E. J., Davey, C. L., Gkagkas, K., Delneri, D., Hoyle, D. C., Oliver, S. G., Kell, D. B., Griffith, G. W. 2012. Genome-wide analysis of longevity in nutrient-deprived *Saccharomyces cerevisiae* reveals importance of recycling in maintaining cell viability. Environ. Microbiol. 14, 1249-1260.

Destruelle, M., Holzer, H., Klionsky, D. J. 1994. Identification and characterization of a novel yeast gene: the YGP1 gene product is a highly glycosylated secreted protein that is synthesized in response to nutrient limitation. Mol. Cell. Biol. 14, 2740-2754.

Espinazo-Romeu, M., Cantoral, J.M., Matallana, E., Aranda, A., 2008. Btn2p is involved in ethanol tolerance and biofilm formation in flor yeast. FEMS Yeast Res. 8, 1127–1136.

Fidalgo, M., Barrales, R.R., Ibeas, J., Jimenez, J., 2006. Adaptive evolution by mutations in the FLO11 gene. Proc. Natl. Acad. Sci. 103, 11228-11233.

Güldener, U., Heck, S., Fielder, T., Beinhauer, J., Hegemann, J. H. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic acids res. 24, 2519-2524.

Hunter, J. R., Kilpatrick, P. K., Carbonell, R. G. 1990. Lysozyme adsorption at the air/water interface. J. Colloid Interface Sci. 137, 462-482.

Iimura, Y., Hara, S., Otsuka, K., 1980. Cell Surface hidrophobicity as a pellicle formation factor in film strain of Saccharomyces. Agric. Biol. Chem. 44, 71-74.

Ishigami, M., Nakagawa, Y., Hayakawa, M., Iimura, Y., 2004. FLO11 is essential for flor formation caused by the C-terminal deletion of NRG1 in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 237, 425-430.

López-Alejandre, M. M. Los vinos de Montilla–Moriles (2nd ed. Sevilla, Spain: Junta de Andalucía, Consejería de Agricultura y Pesca, 2005).

Martínez, P., Pérez-Rodríguez, L., Benítez, T., 1997a. Factors which affect velum formation by flor yeasts isolated from sherry wine. Syst. Appl. Microbiol. 20, 154-157.

Martínez, P., Perez-Rodriguez, L., Benitez, T., 1997b. Velum formation by flor yeasts isolated from sherry wines. Am. J. Enol. Vitic. 48, 55-62.

Moreno-García, J., García-Martínez, T., Moreno, J., Millán, M. C., Mauricio, J. C. 2014. A proteomic and metabolomic approach for understanding the role of the flor yeast mitochondria in the velum formation. Int. J. Food Microbiol. 172, 21-29.

Moukadiri, I., Armero, J., Abad, A., Sentandreu, R., Zueco, J. 1997. Identification of a mannoprotein present in the inner layer of the cell wall of *Saccharomyces cerevisiae*. J. Bacteriol .179, 2154-2162.

Mrsa, V., Ecker, M., Strahl-Bolsinger, S., Nimtz, M., Lehle, L., Tanner, W. 1999. Deletion of new covalently linked cell wall glycoproteins alters the electrophoretic mobility of phosphorylated wall components of *Saccharomyces cerevisiae*. J. Bacteriol. 181, 3076-3086.

Pardo, M., Monteoliva, L., Pla, J., Sánchez, M., Gil, C., Nombela, C. 1999. Two-dimensional analysis of proteins secreted by *Saccharomyces cerevisiae* regenerating protoplasts: a novel approach to study the cell wall. Yeast 15, 459-472.

Peinado, R. A., Mauricio, J. C. 2009. Biologically aged wines, p. 81–103. In: Moreno-Arribas, M.V., Polo, M.C. (Eds.), Wine Chemistry and Biochemistry. Springer.

Purevdorj-Gage, B., Orr, M.E., Stoodley, P., Sheehan, K.B., Hyman, L.E., 2007. The role of FLO11 in *Saccharomyces cerevisiae* biofilm development in a laboratory based flow-cell system. FEMS Yeast Res. 7, 372-379.

Reynolds, T. B., Fink, G. R., 2001. Bakers' yeast, a model for fungal biofilm formation. Science 291, 878-881.

Riou, C., Nicaud, J. M., Barre, P., Gaillardin, C. 1997. Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13, 903-915.

Vandenbosch, D., De Canck, E., Dhondt, I., Rigole, P., Nelis, H. J., Coenye, T. 2013. Genomewide screening for genes involved in biofilm formation and miconazole susceptibility in *Saccharomyces cerevisiae*. FEMS Yeast Res. 13, 720-730.

Zara, G., Zara, S., Pinna, C., Marceddu, S., Budroni, M. 2009. FLO11 gene length and transcriptional level affect biofilm-forming ability of wild flor strains of *Saccharomyces cerevisiae*. Microbiology 155, 3838-3846.

Zara, S., Bakalinsky, A. T., Zara, G., Pirino, G., Demontis, M. A., Budroni, M. 2005. FLO11-based model for air–liquid interfacial biofilm formation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 71, 2934-2939.

Zara, S., Farris, A. G., Budroni, M., Bakalinsky, A.T., 2002. HSP12 is essential for biofilm formation by a Sardinian sherry strain of *S. cerevisiae*. Yeast 19, 269-276.

Zara, S., Gross, M. K., Zara, G., Budroni, M. and Bakalinsky, A. T. 2010. Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. Appl. Environ. Microb. 76, 4089-4091.

Appendix 10.

Use of a flor velum yeast for modulating colour, ethanol and major aroma compound contents in red wine.

Moreno, J., Moreno-García, J., López-Muñoz, B., Mauricio, J.C., García-Martínez, T.
Food Chemistry. Volume 213, 15 December 2016, Pages 90-97
https://doi.org/10.1016/j.foodchem.2016.06.062

Food Chemistry 213 (2016) 90–97

Contents lists available at ScienceDirect

Food Chemistry

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

Use of a flor velum yeast for modulating colour, ethanol and major aroma compound contents in red wine

Juan Moreno [a,*], Jaime Moreno-García [b], Beatriz López-Muñoz [a], Juan Carlos Mauricio [c], Teresa García-Martínez [c]

[a] Department of Agricultural Chemistry, Marie Curie (C3) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra. N-IV-A, Km 396, 14014 Cordoba, Spain
[b] Department of Viticulture and Enology, University of California, Davis, 595 Hilgard Lane, Davis, CA 95616, USA
[c] Department of Microbiology, Severo Ochoa (C6) Building, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Ctra. N-IV-A Km 396, 14014 Cordoba, Spain

ARTICLE INFO

Article history:
Received 20 March 2016
Received in revised form 19 June 2016
Accepted 20 June 2016
Available online 21 June 2016

Chemical compounds studied in this article:
Ethanol (PubChem CID: 702)
Acetic acid (PubChem CID: 176)
Methanol (PubChem CID: 887)
1-Propanol (PubChem CID: 1031)
Isobutanol (PubChem CID: 6560)
2-Methyl-1-butanol (PubChem CID: 31260)
3-Methyl-1-butanol (PubChem CID: 8723)
Phenethyl alcohol (PubChem CID: 6054)
Acetaldehyde (PubChem CID: 177)
Acetal (1,1-diethoxyethane): (PubChem CID: 7765)
Acetoin (PubChem CID: 179)
Ethyl acetate (PubChem CID: 8857)
Ethyl lactate (PubChem CID: 7344)
Diethyl succinate (PubChem CID: 31249)
2,3-Butanediol (PubChem CID: 262)
Glycerol (PubChem CID: 753)

Keywords:
Flor yeast
Saccharomyces cerevisiae
Red wine
Colour
Ethanol
Aroma compounds

ABSTRACT

The most important and negative effect of the global warming for winemakers in warm and sunny regions is the observed lag between industrial and phenolic grape ripeness, so only it is possible to obtain an acceptable colour when the ethanol content of wine is high. By contrast, the actual market trends are to low ethanol content wines. Flor yeast growing a short time under velum conditions, decreases the ethanol and volatile acidity contents, has a favorable effect on the colour and astringency and significantly changes the wine content in 1-propanol, isobutanol, acetaldehyde, 1,1-diethoxiethane and ethyl lactate. The Principal Component Analysis of six enological parameters or five aroma compounds allows to classify the wines subjected to different velum formation conditions. The obtained results in two tasting sessions suggest that the flor yeast helps to modulate the ethanol, astringency and colour and supports a new biotechnological perspective for red winemakers.

1. Introduction

In an extensive study about the assessment of historical trends in wine grape maturity dates from vineyards located in geographically diverse growing areas in Australia, Webb, Wheton, and Barlow (2011) show an average advance in the vine phenology of 1.7 days per year along the 1993–2009 period. In addition, the review made by Mozell and Thach (2014), shows how an upward shift in seasonal temperature will dramatically change the growing season thereby modifying the normal pattern of grape development, especially in warm, sunny regions. According to Mira de Orduña (2010), climate change is exerting an increasingly profound influence on vine phenology, grape composition, wine

* Corresponding author.
E-mail address: qe1movij@uco.es (J. Moreno).

http://dx.doi.org/10.1016/j.foodchem.2016.06.062
0308-8146/

Appendix 11.

Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system.

Moreno-García, J., García-Martínez, T., Moreno, J., Mauricio, J.C., Ogawa, M., Luong, P., Bisson, L.F.
American Journal Of Enology and Viticulture. 2017. Sent to publish.

Impact of yeast flocculation and biofilm formation on yeast-fungus co-adhesion in a novel immobilization system

*Co-authors

*Affiliations

Abstract

A novel method of yeast immobilization, called biocapsules, has been developed in which yeasts are attached to the hyphae body of the fungus, *Penicillium chrysogenum*. Yeast immobilization can be considered beneficial because it facilitates higher cell densities than traditional fermentation methods, improves yield, allows the reutilization of the biocatalyst, etc. After a yeast screening for fungus co-immobilization, flor yeast strains resulted to be best at forming the most consistent biocapsules. Flor yeasts are commonly known to naturally form biofilms on the liquid surface of Sherry wines. Biofilms form in interphases (solid or liquid) via the attachment of cell-cell and/or cell-surface. Flocculation occurs in liquid media and refers to the attachment of cells to each other when grown dispersed. To elucidate the influence of biofilm formation versus flocculation on the yeast-fungus co-immobilization, a screening of selected strains from the Viticulture and Enology Department collection at University of California, Davis was carried out and their ability to flocculate and form biofilm was quantified. Eighteen yeast strains capable of biofilm formation and flocculations were concluded from this screening. Strains displaying differential capabilities in flocculation or biofilm formation plus two control strains were further evaluated for their ability to specifically immobilize with *P. chrysogenum.* Seven strains were found to show different patterns of flocculation and biofilm formation. Yeast strains that are able to form biofilm correlates with higher rates of immobilization with *P. chrysogenum* to form more consistent biocapsules. On the other hand, strains able to flocculate develop smaller, inconsistent biocapsules but in abundance. These results shed light on parameters that influence yeast-fungus co-immobilization, may lead to an improvement of biocapsules' consistency and further the field of application for this new immobilization system.

Introduction

The use of microbial immobilized systems for alcoholic products offers many advantages over conventional free cell fermentations. These advantages include: high yeast cell densities, product yield improvement, lowered risk of microbial contamination and reuse of the biocatalyst (Kourkoutas et al. 2004). The various supports used for cell immobilization are classified as inorganic, organic, or natural in addition to membrane systems. Natural supports derive from materials that are generally food grade purity with minimal or no pre-treatment intervention such as components of fruit, wood, sawdust, etc. (Kourkoutas et al. 2004). Immobilization supports and techniques have been applied to alcoholic beverage as well as potable or fuel ethanol production.

A novel yeast immobilization system, termed "biocapsules", offers a natural support consisting of a filamentous fungus (e.g. *Penicillium chrysogenum*), wherein the hyphae of the fungus serves as a platform for yeast cell attachment and adherence (Peinado et al. 2004). Biocapsules are hollow, spherical bodies that constitute a natural matrix system which eliminates the cost of inert supports and since it takes advantage of natural adhesion properties of yeast and filamentous fungus cell walls, it minimizes changes to the yeast metabolism and/or yeast viability. Additionally, biocapsules enable diffusion of nutrients and products to the yeast due to the porous structure of the filamentous fungus (Peinado et al. 2004; García-Martínez et al. 2011). When these biocapsules are placed in a fermentation medium, the yeast cells colonize and invade all hyphae, thereby causing the fungus to die and remain as a mere support for the yeast cells (Peinado et al. 2006). The yeast physically attach to the exterior of the mold hyphae in the form of a rounded or ball-type structure depending upon the conditions of formation that are large enough to readily settle under static conditions. Biocapsules can therefore be easily recovered from fermentations and the yeast retain viability and fermentative capacity enabling multiple rounds of reuse (Peinado et al. 2004). Because of these features, biocapsules have been considered a promising technique for industrial-scale fermentation purposes and have already been utilized in alcoholic beverage production like white wine, sparkling wine and natural sweet wine as well as for bioethanol

production from starch and molasses (Peinado et al. 2005; Puig-Pujol et al. 2013; García-Martínez et al. 2015; Peinado et al. 2006; García-Martínez et al. 2012).

Peinado et al. (2004) observed that when co-inoculated with the fungus, flor yeast strains form biocapsules with relatively high consistency and mechanical resistance; defined by the biocapsule's ability to withstand shear forces without breaking. Flor yeasts differ from other yeast in their capacity to auto-immobilize forming biofilm aggregates at a liquid-air interfaces under certain conditions (Esteve-Zarzoso et al. 2001; Aranda et al. 2002; Alexandre, 2013). Flor yeasts are used for the fermentation of Sherry wines due to their ability to survive in a post fermentation environment where fermentable carbon sources are nearly exhausted and only ethanol and glycerol remain (Esteve-Zarzoso et al. 2001). Oxygen is required in order to metabolize these carbon resources. The biofilm formation process allows the flor yeasts to access regions where oxygen is rich, i.e. the wine-air interface. Zara el al. (2009) revealed an existence of an extracellular matrix among flor yeast forming the velum. The composition of the velum is unknown.

García-Martínez et al. (2011) investigated the interactions of flor yeast with the fungal hyphal matrix within a biocapsule using transmission electron microscopy. Yeast were observed to be directly attached to the cell surface of the fungus and the attachment persisted after loss of fungal viability. The integrity of the biocapsules was retained following fermentation (Peinado et al., 2006) enabling reuse of the capsules. García-Martínez et al. (2011) established that biocapsules formed naturally, stabilized yeast fermentative activity and viability, and retained integrity after loss of viability of the fungus due to cell-hypha contact. The yeast were able to adapt to high ethanol conditions and complete fermentations. However, previous works were largely conducted with only one strain of flor yeast which may produce a spectrum of aroma compounds not normally found in table wine yeast (Peinado et al., 2005; 2006; García-Martínez, 2015).

The main focus of this work was to study the factors of yeast strains that affect co-adhesion with *P. chrysogenum* when forming biocapsules. Although immobilization effectiveness

of biocapsules made with non-flor yeast vs flor yeast was compared (García-Martínez et al., 2012), the effect of yeast properties such as flocculation and biofilm forming abilities on the formation of co-adhesion had not been systematically investigated. In this work we defined methodologies for assessment of flocculation and biofilm forming ability and assessed the impact of these properties on biocapsule formation. Known flocculent and biofilm forming yeast strains were initially screened to observe and quantify flocculation and biofilm formation phenotypes and strains displaying differences in these phenotypes were selected for subsequent analysis. The ability of these yeasts to form biocapsules was assessed and biocapsule parameters like percentage of immobilized yeasts, number of biocapsules, total volume, diameter, consistency and dry weight, were evaluated.

Materials and methods

Microorganisms and growth media

Saccharomyces cerevisiae yeast strains from the Department of Microbiology (University of Cordoba, Spain (UCO)) and the Department of Viticulture and Enology (University of California, Davis (UCD)) collection were used in this work (Table 1). *S. cerevisiae* G1 and 932 were utilized as positive and negative controls for flocculation/biofilm formation, respectively. Eighteen strains were chosen from the UCD collection due to their reported ability to form flocs and/or biofilms. This strain set included *S. cerevisiae* yeasts isolated from sherry, sparkling, dry and standard wine, beer and must, as well as commercial strains (Table 1). After analyzing and quantifying strain flocculation and biofilm formation phenotypes, seven strains showing different flocculation/biofilm formation patterns were selected together with G1 and 932 control strains, for co-immobilization experiments with the filamentous fungus and biocapsule formation. Cell population size for strains forming flocs in liquid media are difficult to quantify under the microscope or spectrophotometrically, producing false values. Therefore yeasts were pre-grown on solid media, YPD-agar (1% yeast extract: 2% peptone, 2% glucose and 2% agar), and the colonies transferred to an Eppendorf tube containing the liquid growth medium and agitated for 5 minutes to avoid flocs and generate the inocula.

Yeasts were co-immobilized with the filamentous fungus strain *P. chrysogenum* H3 from UCO collection. The fungus was pre-grown in a sporulation medium (SM) containing 1.7% corn meal agar, 0.1% yeast extract, 0.2% glucose and 2% agar for seven days at 28 ºC.

Flocculation assessment

To assess flocculation ability, 4 x 10^6 yeasts cell/ mL were inoculated into 5 mL of synthetic grape juice medium "minimal must media" (MMM), prepared at 123 mg/L yeast nitrogen equivalents (Giudici et al. 1994, Spiropoulos et al. 2000). The cells were then incubated at 25ºC under agitation conditions, on a rotary drum for 5 days. For the semi-quantitative analysis, pictures were taken at day five after the inoculum both macroscopically and microscopically, the later by using the Celestron microscope (Figure 1). To quantify non-flocculated verses flocculated yeast population sizes, cultures were filtered though a Hydrophilic Nylon membrane filter 30 µm ⌀ pore size (NY3004700 | Nylon mesh filter, hydrophilic, 30 µm, 47 mm) that allowed passage of free or suspended cells but retained flocs of 6 or more cells on the filter matrix. The dry weight of the suspended yeasts and the flocculent yeasts were then measured following drying to a constant weight. The filters were dried in an oven at 80 ºC constant temperature overnight.

Strain	Strain details	Flocculation	Biofilm formation	Biocapsule formation
G1 (positive control)	*S. cerevisiae* (Sherry wine (Spain))	+	++	
UCD 932 (negative control)	*S. cerevisiae*	-	-	
UCD 77	*S. cerevisiae* (wine, champagne)	+	+	
UCD 519	*S. cerevisiae* (Sherry wine)	-	+++	
UCD 580	*S. cerevisiae* (Sherry wine)	-	+++	
UCD 595	*S.cerevisiae* (commercial dry wine yeast)	-	+	
UCD 661	*S. cerevisiae* race bayanus (wine, champagne)	-	+	
UCD 662	*S. cerevisiae* race bayanus (wine, champagne)	-	+	
UCD 726	*S. cerevisiae* (wine)	-	-	
UCD 775	*S. cerevisiae* (wine, Champagne)	-	+	
UCD 777	*S. cerevisiae race bayanus* (commercial wine yeast)	+	-	
UCD 804	*S. cerevisiae* (commercial wine yeast, champagne)	-	-	

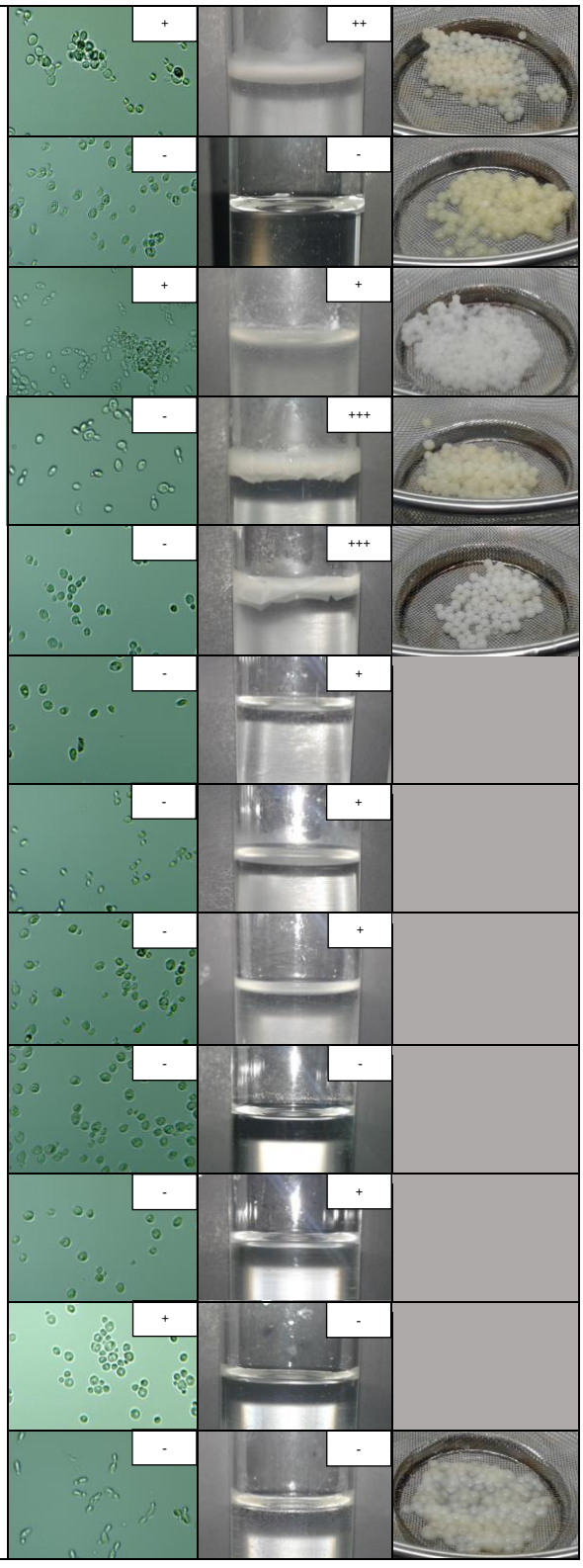


Figure 1. Results from the flocculation, biofilm and biocapsule formation semi-quantitative assays of the yeast strains. Symbols -, +, ++ and +++; indicate the phenotype qualification. Biocapsules were only made with those yeast strains showing different flocculation/biofilm formation patterns.

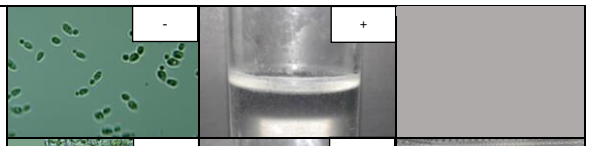
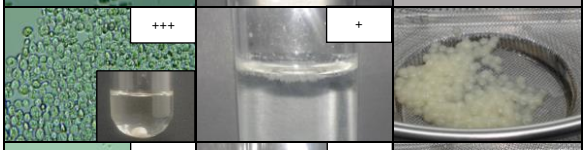
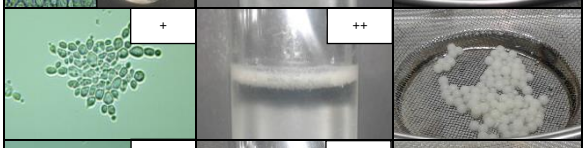
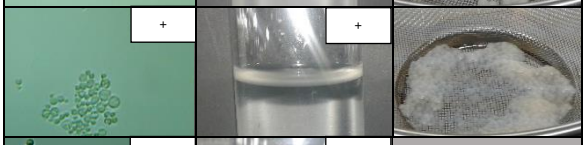
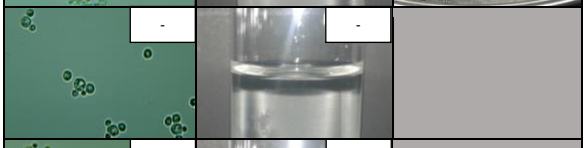
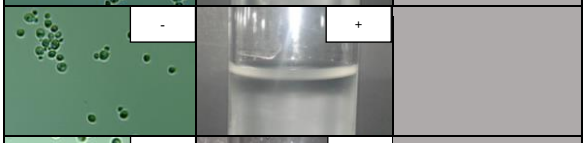
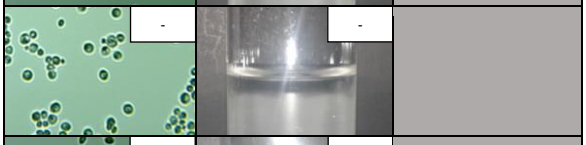
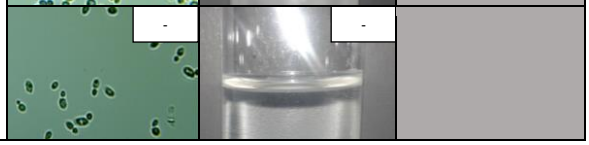
Strain	Strain details	Dynamic flocculation	Biofilm formation
UCD 814	*S. cerevisiae* race *bayanus* (commercial wine yeast)		
UCD 854	*S. cerevisiae* (Ale, England)		
UCD 1109	*S. cerevisiae* (must)		
UCD 1162	*S. cervisiae* (possible flor yeast)		
UCD 2034	*S. cerevisiae* (Commercial yeast)		
UCD 2547	*S. cerevisiae* (Wine Spain)		
UCD 2865 B11 or BA11	*S. cerevisiae*		
Original 594 (prise di mousse)	*S. cerevisiae*		

Figure 1 (continued)

Biofilm formation and assessment

Approximately $6 \cdot 10^7$ yeasts cells/ mL were inoculated into 10 mL of flor medium in a glass test tube containing 0.67% YNB without amino acids and 3% ethanol adjusted to pH 3.5; and incubated at 21 ºC for 5 days (Govender et al. 2010; Ishigami et al. 2004). Biofilms were defined as forming a coating on the top of the tube or across the air/liquid interface. Pictures were taken macroscopically at day 5 from inoculation. For quantification, biofilm forming yeast and non-biofilm forming yeasts (precipitated and suspended) dry weight were measured. Biofilms

were carefully extracted with a 5 mL pipette and a spatula when they were completely covering the medium surface at day 5 (Govender et al. 2010) while non-biofilm forming yeasts were collected by centrifugation (4500 rpm for 15 minutes).

Biocapsule formation and assessment of biocapsule properties

S. cerevisiae strains were pre-grown for 3 days in YP + 3% glycerol medium (175 rpm, 28 ºC). Yeast nitrogen base medium without amino acids (Difco); containing 5 g/L gluconic acid as a carbon source and buffered to pH 7 with sodium and potassium phosphate, was used as a biocapsule formation medium (BFM). The medium used for the co-immobilization is suitable for yeast to express its flocculation and biofilm formation phenotypes. Conditions that are most beneficial for yeasts to flocculate include pH range of 2.5 to 9, moderate agitation and aeration, absence of sugars, and incubation length overpassing logarithmic phase of growth (Stratford 1992; Miki et al. 1982a; Dengis et al. 1995; Stratford 1996; Soares and Seynaeve 2000; Kida et al. 1989; Soares et al. 1991; Miki et al. 1982b; Straver et al. 1993; Soares, 2011; Verstrepen and Klis 2006). Similarly, biofilm formation phenotype is expressed after depletion of fermentable carbon sources. Flor yeasts, when placed in an environment lacking nitrogen and sugar, as is the case at the end of alcoholic fermentation of Sherry type wines, form suspended multicellular aggregates followed by an air–liquid biofilm forming on the wine surface (Esteve-Zarzoso et al. 2001; Aranda et al. 2002; Alexandre, 2013). Additionally, the presence of other non-fermentable carbon sources can induce biofilm formation (Zara et al. 2010).

Three flasks per yeast strain, each containing sterile, autoclaved 150 mL BFM, were inoculated with $4 \bullet 10^6$ yeast cells mL^{-1} and $4 \bullet 10^6$ *P. chrysogenum* spores. The flasks were then shaken at 175 rpm and at 28 ºC for 6 days. Under these conditions, spontaneous immobilization occurred and yeast biocapsules were produced. The immobilization procedure used is the same as that patented by Peinado et al. (2004). The capacity of cells to be immobilized in the co-adhesion assay was determined by quantifying the following parameters: number of non-immobilized yeasts, immobilized yeasts, % yeast immobilized, number of biocapsules, diameter, total volume, consistency and dry weight. Yeast biocapsules were separated from the medium and

washed with distilled water prior to their analysis. Biocapsules were counted in each of the flasks, then the diameter size and the volume occupied by all biocapsules were measured. Biocapsule consistency was quantified by a TA.XT2 texture analyzer. For the immobilized yeasts counting, ten biocapsules were broken, fungus separated from yeast cells, cells counted and then referred to the total number of biocapsules in each replica. Biocapsules were broken by placing them into a NaCl 100 mM solution, crushed by a mortar for 2 minutes and then vortexed for 20 seconds. As a result, a mixture of yeast cells and *P. chrysogenum* was obtained. To get rid of *P. chrysogenum* hyphae, successive differential filtrations were carried out: i) by using a collander; ii) a 180 µm ⌀ filter (NY8H04700 | Nylon mesh filter, hydrophobic, 180 µm, 47mm) and iii) a 30 µm ⌀ filter (NY3004700 | Nylon mesh filter, hydrophilic, 30 µm, 47 mm). Yeast population sizes (non-immobilized and immobilized yeasts) were determined by direct counting using a Haemocytometer grid under the microscope at 40x objective. The remaining biocapsules were used for the measurement of the dry weight (80 ºC constant temperature overnight) and finally referred to the total number of biocapsules.

Statistical analyses

Data obtained from the quantified yeast strain and biocapsule parameters were subjected to statistical analyses through the software package Statgraphics Centurion XVI (Manugistics, Inc., Rockville, MD, USA). ANOVA and the Kruskal-Wallis tests were used to detect parameters which quantified values depending on the yeast strain analyzed. Kruskal-Wallis test is a nonparametric method to test whether a set of data comes from the same population. Intuitively, it is identical to ANOVA but replacing data by category. It is an extension of the U test of Mann-Whitney for 3 or more groups.

Further, in order to detect correlations among yeast strain and biocapsule parameters a multivariate analysis was performed and a Pearson coefficient and p-value were provided for each couple of variables (Table 2). The range of the Pearson coefficient goes from -1 to +1, and it measures the lineal relation among variables: 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation. The p-value tests the statistical significance

of the estimated correlations. P-values below 0.05 indicate correlations significantly different from zero, with a confidence level of 95.0%.

Results

Screen of flocculation and biofilm formation phenotype: Eighteen yeast strains were selected from the UCD collection to screen for flocculation and/or biofilm formation potential (Table 1). Yeast were categorized visually for the formation of cell aggregates with constant mixing (dynamic conditions) (Figure 1). Biofilm formation capacity was evaluated by assessing the formation of a film on the surface of the tube or growing on the side of the tube. Seven out of the 18 yeast strains tested (77, 519, 580, 804, 854, 1109 and 1162 plus the controls G1 and 932) showed different flocculation/biofilm patterns (see phenotypic qualification and physical aspect in Figure 1). 77 and 1109 displayed biofilm formation and flocculation. 519 and 580 formed a strong biofilm but were not flocculent. 804 neither flocculated nor formed a biofilm. 854 showed high levels of flocculation but did not form a significant biofilm. 1162 did show flocculation under dynamic growth and formed a biofilm although the size of the biofilm was less thick than that of 77 and 1109. Because of these differences in ability to flocculate and form surface biofilms, these strains were chosen for further analysis in *P. chrysogenum* co-adhesion assays. G1, the original strain evaluated by Peinado et al. (2004) for biocapsule formation, and 932, a non-flocculating strain that was not able to form a biofilm, were also included in subsequent assays as controls.

Quantification of flocculation and biofilm formation: The set of 9 strains were then evaluated in quantitative assays for assessment of flocculation ability and biofilm formation capacity (Figure 2). Strains showing flocculation levels over that of the negative control strain 932 (73.0 ±4.7%) were considered flocculent (Figure 2a). Highest flocculation capacity was performed by the yeast strain *S. cerevisiae* 854 isolated from British Ale beer (Figure 1 and 2 a), flocculating 39.83 out of 40.96 mg, or 97.5%. 854 flocs could even attain sizes at macroscopical dimensions. Figure 1 shows that strain 580 does not form flocs, but Figure 2a shows a high biomass that was attached to the filter. Although not flocculating, 580 seems to multiply, forming

a net of cells which might have collapsed the filter used, thus explaining the high values in Figure 2a. In addition, it should be taken into account that this was a biofilm forming yeast strain. Such strains synthetize proteins essential for the biofilm formation, like Flo11p, which also contribute to attachment of inert supports such as nylon, the material of the membrane filter used (Fidalgo et al., 2008).

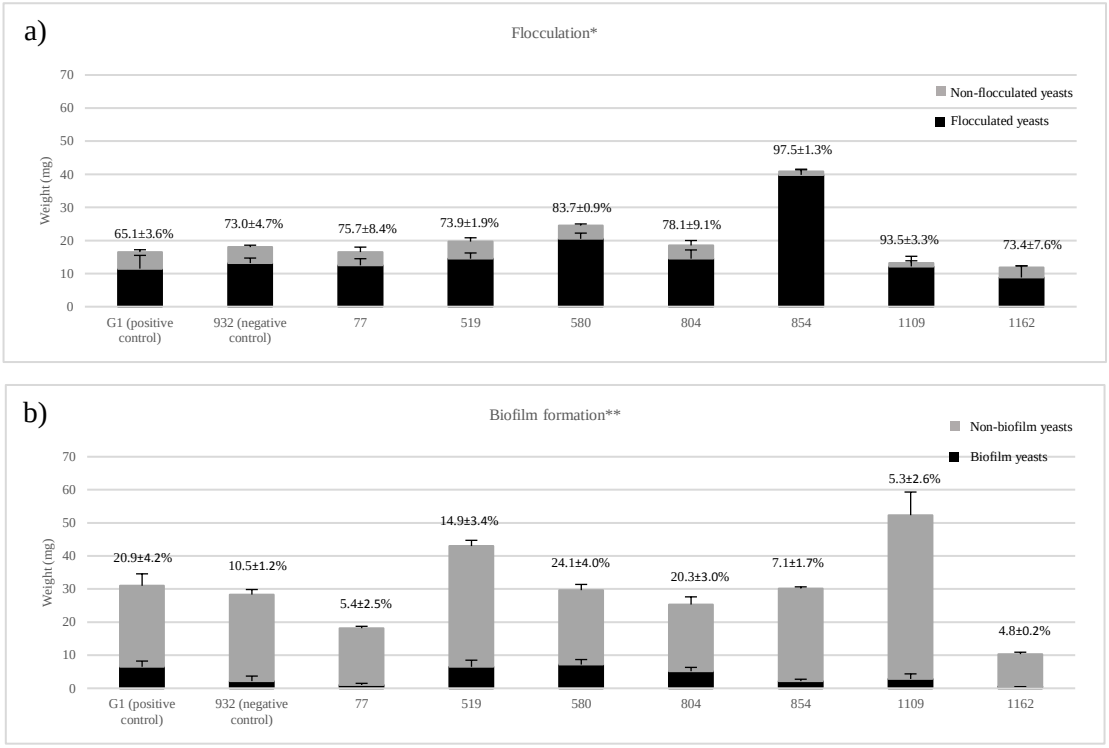


Figure 2. Yeast strains flocculation (a) and biofilm biofilm formation (b). In a, black bars represent yeasts flocculated, in b, biofilm yeasts. Grey bars represent yeasts non-flocculated and non-biofilm yeasts in a and b, respectively. Significance level as per the Kruskal-Wallis test: **p < 0.01; *p < 0.05; N.S p > 0.05; was provided.

580 displayed the highest biofilm formation capacity among the screening yeast strains (Figure 2b), both in terms of biofilm total dry weight and biofilm dry weight percentage, reaching up to 7.17 mg of biofilm out of 29.67 mg of total cells or 24.05%. This strain was closely followed by G1 with a 6.5 mg biofilm dry weight out of a total of 31 mg (20.94%) and 804 with 5.17 out of 25.33 mg (20.33%). The first two strains, 580 and G1, are flor yeasts used for Sherry wine elaboration while 804 is a commercial wine yeasts commonly used for champagne production. 1109 isolated from must and the Sherry wine yeast 519 grew better in the flor medium than the rest of the strains reaching total dry weights of 52.33 and 42 mg, respectively. 1162 and 77

displayed the lowest biofilm percentage values: 0.5 out of 10.33 mg (4.85%) and 1 out of 18.17 mg (5.41%), respectively. Figure 1 G1 picture demonstrates the formation of flocs when grown in MMM and agitating while Figure 2a shows low biomass values. It might be that G1 flocs are less consistent than others and yeast cells were disaggregated during the filtration process thus remaining less yeasts in the filters and obtaining low dry weight values.

Biocapsule formation: The Figure 3 shows values of all biocapsules parameters measured (a-f). Through the Kruskal-Wallis test all parameters excluding "biocapsule total volume", were reported to be dependent on the type of strain of yeast with a p-value < 0.01 (biofilm formation, yeast immobilization, biocapsule number and consistency and dry weight) and < 0.05 (flocculation and biocapsule diameter).

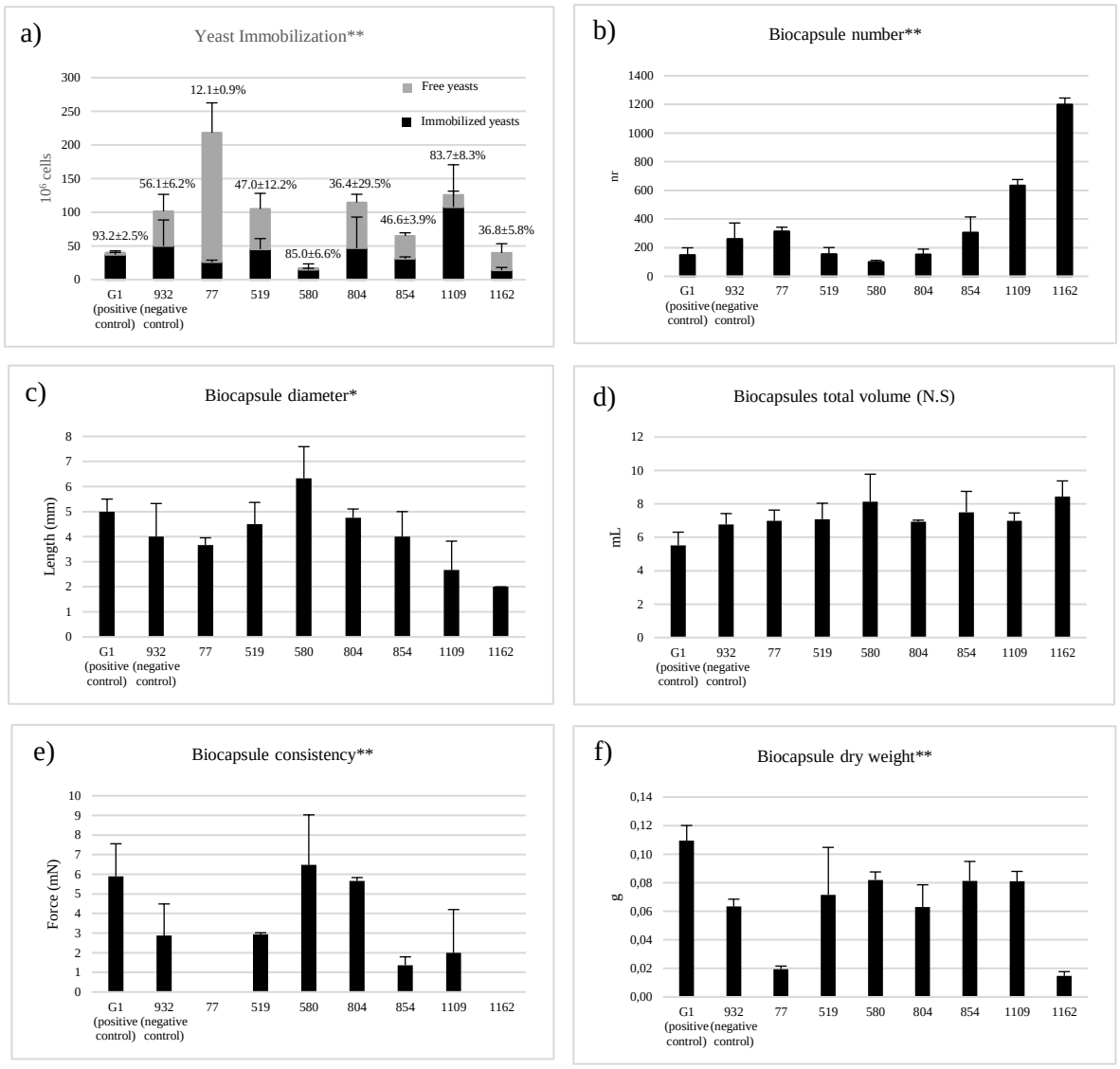


Figure 3. Yeast strains biocapsule related parameters. In a, black bars represent yeasts immobilized in biocapsules. Grey bars represent yeasts non-immobilized or free yeasts. Significance level as per the Kruskal-Wallis test: **p < 0.01; *p < 0.05; N.S p > 0.05; was provided.

A p-value lower than 0.01 indicates that the percentage of yeast immobilized with the biocapsule system strongly depends on the yeast strain (Figure 3). The highest immobilization capacity was observed for the positive control case *S. cerevisiae* G1 flor yeast with 93.2% of the yeasts cells adhering to the filamentous fungus hyphae. Another strain of flor yeast, 580, showed the second highest adherence percentage with 85.0% of the cells attached to the *Penicillium* hyphae. Both of these strains showed high biofilm forming capacity. G1 total yeast cells in the biocapsule formation medium (BFM) were more abundant than 580, 40.31 vs. 17.01 10^6 cells,

respectively. The highest number of immobilized yeast cells were found with strain 1109 reaching 107.99 10^6 cells, however, the percentage of immobilization was lower, 83.7%. It should be noted that this strain reached the highest cell population under biofilm formation, though possessed relatively low biofilm formation percentages altogether, and presented low total cell mass under the flocculation condition. 77, a yeast strain isolated from sparkling wine production, showed the lowest immobilization efficiency (12.08%) but the highest total population (218.05 x 10^6 cells) in BFM. Abundant yeasts found dispersed in the medium and with few cells attached to the fungus may indicate that the strain does not need the attachment to the fungus to grow in a medium like BFM. It should be noted that 77 is a strain with a very low ability to form biofilm, only 5.41% of the total biomass and flocculation close to the strain average value (79.28 ± 4.53%).

When considering number of biocapsules formed, highest values were attained with the co-immobilization of 1162 (1198 biocapsules). This value was significantly above those of other strains. 1162 is characterized mainly by its scarce ability to form biofilm (4.85%) and values of flocculation below average. Higher than the average (363 biocapsules), immobilization of 1109 strain resulted in 632 biocapsules. 1162, 1109, 854 and 77 formed more biocapsules and exhibited less ability to form biofilm while biofilm-forming yeast strains G1, 519, 580 and 804 immobilization resulted in the lowest number of biocapsules. This fact may indicate that the yeast biofilm formation feature is inversely related with the number of biocapsules formed. Furthermore, yeast strains with lowest biocapsule diameters coincide with those with highest number of biocapsules (Figure 3), thus indicating that there may be a positive correlation among “biocapsule diameter” and “biofilm formation” (Figure 4).

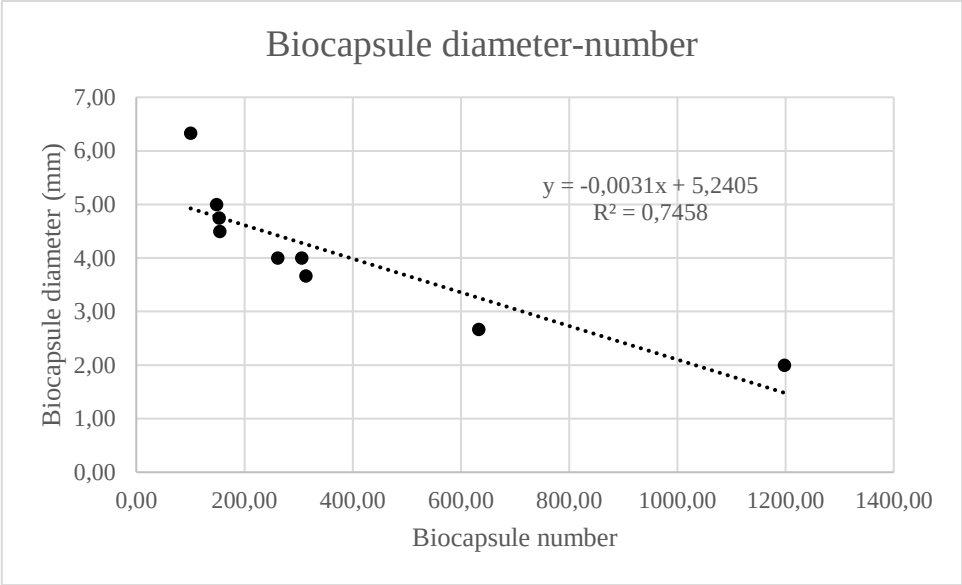


Figure 4. Linear regression among the "biocapsule diameter" and "biocapsule number" parameters.

Biocapsule total volume was not significantly different among the yeast strains immobilized. This study showed that those strains with higher capacity to form biofilm produce bigger biocapsules but in a lower amount than those made with yeast strains with lower capacity to form biofilm. Ultimately, both type of yeast strains will sum up to similar biocapsule total volumes.

On the other hand, more resistant biocapsules were those with the highest biofilm forming ability strains G1, 580 and 804 and less consistent biocapsules were obtained by immobilizing the strains with lower biofilm forming capacity 77, 854, 1109 and 1162. Similarly, heaviest biocapsules were obtained with the flor yeast G1 yeast strain while lightest with the non-biofilm forming 77 and 1162. The rest did not differ significantly in dry weight with an average of 0.07 g.

After applying the Pearson test to detect correlations among yeast strain and biocapsule parameters, lowest p-values were obtained among the following parameter couples: "biocapsule diameter"-"yeast forming biofilm (%)","biocapsule consistency"- "biofilm yeast weight" and "biocapsule consistency"- "yeasts forming biofilm (%)" (Table 2). For all these couples of parameters, the Pearson coefficients were over 0.7, indicating that a positive correlation exists for

each, reaching 0.93 in the case of “biocapsule consistency” and “yeasts forming biofilm (%)”. Bigger and more consistent biocapsules are obtained by immobilizing biofilm forming yeast strains. This result is consistent with the observation made by Peinado et al. (2004) who proposed flor yeasts as better candidates to make biocapsules (in terms of consistency) than those without the ability to form biofilm. On the other hand, flocculation under dynamic conditions appears to be inversely correlated with the biocapsule consistency: “non-floc yeast weight”-“biocapsule consistency” showed a Pearson value of 0.4274 and a p-value of 0.0262.

However, Table 2 also shows a negative correlation among the yeast’s ability to form biofilm and the number of yeast immobilized in biocapsules: positive correlation (Pearson coefficient = 0.6849; p-value = 0.0001) among the number of yeasts immobilized and the amount of yeasts that did not make biofilm in the flor medium.

Biocapsule dry weight is both positively correlated with the biofilm yeast weight plus the weight of non biofilm forming yeasts. This indicates that biocapsule weight is positively correlated with the total amount of yeast growing under this biofilm forming condition. However, it has to be considered that: i) the p-value for “biofilm yeast weight” was lower than that for “non biofilm yeast weight”, and ii) the “biocapsule dry weight” is also positively correlated with a p-value < 0.005 to the percentage of yeast forming biofilm, meaning that biofilm formation positively affects the weight of the biocapsules. The same case was repeated with the percentage of yeasts immobilized within biocapsules.

Lastly, it is shown that the number of biocapsules obtained at day 6 of the fungus-yeast co-inoculation is positively correlated to the yeast capacity to flocculate under agitation but negatively correlated in a significant level to biofilm formation.

Discussion

Here, we used yeast strains that significantly differ in flocculation and biofilm formation phenotypes (Figure 1 and 2). It was shown that these yeasts immobilize differently with the

filamentous fungus *P. chrysogenum* (Figure 3). Values from all parameters evaluated changed with a p-value < 0.05 with the exception of "total volume of biocapsules".

Biofilm formation is defined as a biological process in which planktonically growing microorganisms grow at a liquid-air interface, which is the case for flor or velum; or on a solid substrate under the flow of a liquid, like the filamentous fungus hyphae surface. The same proteins in *Saccharomyces* participate in the formation of biofilm on different areas (Kuchin et al. 2002; Ishigami et al. 2006; Fidalgo et al. 2008). This discovery means that flor yeast strains capable of biofilm formation, can further extend a film on a solid substrate. In the case of our subject, flor yeast that are able to form a velum on the Sherry wine-air interphase are able to form a biofilm on the hyphae surface of filamentous fungus. It must also be considered that the matrix formed by the filamentous fungus may trap air during agitation. Accordingly, flor yeast may encounter an environment in the hyphae matrix similar to that during biological aging where oxygen is necessary to metabolize the carbon source present in the medium. Both conditions promote the onset of biofilm formation. Once the biofilm forming yeasts are attached to the hyphae, they can form an extracellular matrix as previously revealed in the velum formed by some strains (Zara et al. 2009). This extracellular matrix was later detected in the biocapsule walls by Peinado et al. (2006) who observed this connection of yeasts to hyphae. When forming biofilm, yeasts produce extracellular polymers that facilitate matrix formation, resulting in a change in the organisms' growth rate and gene transcription. The ability of flor yeasts to form this fibrillary material, which until now was of unknown composition, may be engrossing the biocapsules formed and therefore explain the larger size of the biocapsules (Figure 2 b and Table 2). Biocapsule dry weight and percentage of yeasts immobilized were both directly correlated with the biofilm yeast weight and the weight of the non-biofilm forming yeasts. Nonetheless, they were also positively correlated with a p-value < 0.005 to the percentage of yeast forming biofilm, thus the formation of the biofilm positively affected the weight of the biocapsules. As shown in Figure 3 and 4, the number and diameter of the biocapsules appear inversely correlated: the more abundant the number of biocapsules, the smaller the biocapsule diameter; and the less number of biocapsules formed

larger diameter of each biocapsule. Consequently, the total volume remains consistent. It was also observed that the smaller biocapsules in larger quantities were formed with the non-biofilm forming strains (1162, 1109, 854 and 77) while biofilm forming yeasts (G1, 519, 580 and 804) immobilized to develop biocapsules that are larger in diameter but smaller in quantity. This fact is further confirmed by a negative significant Paerson coefficient for "biocapsule number"-"biofilm yeast weight" and "biocapsule number"-"yeasts forming biofilm (%)" and by a positive significant Paerson coefficient for "biocapsule diameter"-"biofilm yeast weight" and "biocapsule diameter"-" yeasts forming biofilm (%)" (Table 2). These facts can be explained with the same reasons exposed to explain the biofilm positive affect on biocapsule diameter. The biomass addressed to the biocapsule size is not addressed to the biocapsule number and thus, explains the negative correlation among "biocapsule number"-"biofilm yeast weight" and "biocapsule number"-"yeasts forming biofilm (%)".

Unlike biofilm formation, the yeast's ability to flocculate under agitation allows for a greater number of biocapsules formed but smaller average diameter of each biocapsule (Table 2). Flocculation and biofilm are two different types of cell aggregation. Flocculation is defined as the reversible, non-sexual aggregation of single-celled organisms in suspension to form aggregates of many cells known as flocs. Because flocculation is a homotypic process, involving only one type of cell in the interactions (Stratford 1992; Stewart 2009), it does not include *P. chrysogenum*. Flocculent yeasts inoculated in medium such as BFM, allow for strong cell to cell attachments but relatively average cell to fungus attachments. In this study, yeast cells were found to adhere to the filamentous fungus hyphae rather than to each other. This can be speculated to be because the fungus hyphae acts as platform where yeast can initiate flocculation. Moreover, it should be noted that specific lectin-like proteins are only present on flocculent cells. These proteins recognize and interact with carbohydrate residues of α-mannans (receptors) of neighboring cells (Miki et al. 1982a). While flocculation lectins are only present in flocculent cells, the receptors are present in flocculent and non-flocculent cells, due to the presence of mannans on the outer layer of *S. cerevisiae* and other fungi cell walls, such as *P. chrysogenum*. This phenonmenon

explains the stronger connections among flocculent yeasts interacting with lectins and receptors from both sides while, on the other hand, explains the weaker connection among the yeasts and fungus hyphae that are only interacting with lectins and receptors from one side. Yeasts may also start to make flocs, impeding the filamentous fungus to grow in size and thus resulting in smaller, but more numerous biocapsules.

Biocapsule consistency was shown to be yeast strain-dependent (Figure 3e). This parameter strongly relates with the yeast ability to form biofilm, reaching Pearson coefficients of over 0.7 for the correlation of biofilm yeast weight and percentage of yeasts forming biofilm at the time that is inversely correlated by the capacity of flocculates (Table 2). The yeast's competence to form biofilm, allowing it to strongly attach to the fungus hyphae and produce extracellular polymers that facilitate matrix formation, may explain the strong resistance conferred by the biocapsules to withstand the forces applied without breaking. Contrary, yeasts that flocculate congregate with each other and form flocs that can potentially interfere with the fungus and make inconsistent biocapsules.

A Pearson coefficient of 0.6849 (p-value = 0.0001) among the number of yeasts immobilized and the amount of yeasts that did not make biofilm in the flor medium, indicates that flor yeasts under the immobilization condition may attach to the fungal hyphae though not at a very high frequency. However, they maintain consistence by the polymer-nature matrix production within the fungal hyphae in the biocapsule. Another reason could be that flor yeasts were too strongly attached to fungal hyphae and there was not enough time for hyphae-yeast separation for immobilized yeast to be released from the biocapsule in order to be counted.

Yeast purpose to co-adhere with the filamentous fungus *P. chrysogenum* may come from several reasons. Consider the biocapsule matrix as a place that entraps and allows air to be stored in their matrix gaps. This matrix is beneficial for yeast and makes it a suitable environment to oxidatively metabolize the non-fermentable carbon source, gluconic acid, when attached to the hyphae. Further, as the main purpose of flocculation, yeast-hyphae association can act as a communitarian mechanism of survival: the external cells from the floc structure can directly

protect the inside cells against harmful environments by physically shielding them. Flocculent yeasts when exposed to several negative conditions such as nutrients starvation, ethanol toxicity, cold-shock, and osmotic stress induce the onset of flocculation (Gibson et al. 2007). Furthermore, another intention of flocculation that could be attributed to the biocapsule formation, is that when the environment becomes exhausted of nutrients, cells can cooperate within flocs. Autolysis or even apoptosis (programmed cell death) of some cells in the center of the floc will contribute compounds (proteins, carbohydrates and vitamins), which can support the survival of the other cells of the floc, most likely younger and healthier cells that have a higher chance of viability (Herker et al. 2004). Thus, yeast flocculation and biocapsule formation make it possible for long-term survival of a cellular community of yeast cells in an unfavorable environment such as nutrient starvation. Another hypothetical reason is that yeast and fungus establish a symbiotic relationship in which the yeast obtain gluconic acid sub-products that are easier to metabolize. Peinado et al. (2006) observed through transmission electron microscopy, within zones of contact with hyphae, vesicular structures are located near the contact zone in the cytoplasm of yeast cells, thus providing a polarized aspect to these cells.

S. cerevisiae and *P. chrysogenum* can utilize gluconic acid as a carbon source and incorporate it to the carbohydrate acid metabolism, both through its phosphorylation and entry to pentose phosphate pathway. In the case of flor yeasts, it has been observed that gluconic acid in contents lower than 5 g $L^{-}1$ is assimilated during the aerobic biological aging process of sherry wines (Cortés et al. 1999) but nevertheless its consumption initiated after 18 days (Peinado et al. 2003). This delayed time may occur because the protein that catalyzes the phosphorylation, the cytoplasmic putative gluconokinase *YDR248C,* is up-regulated in amino acid starvation (Gasch et al. 2000). Aversely, Schmitz et al. (2013) reported that *P. chrysogenum* has higher uptake rates for gluconate than for glucose. Hence, during the biocapsule formation, the first 6 days after the yeast-fungus co-inocculation, *YDR248C* may not yet be present, and yeasts are forced to establish a symbiotic relationship with the fungus to obtain an alternate carbon source from the fungus

gluconic acid degradation. Expression info is required for the *P. chrysogenum* probable gluconokinase Pc22g05190p to test this hypothesis.

Conclusion

In this study, we found that yeast *S. cerevisiae* immobilization in the filamentous fungus *Penicillium chrysogenum* and the consequent formation of biocapsules depends on the yeast ability to flocculate and aggregate in biofilms. Biofilm forming yeast strains have higher rates of immobilization and form bigger and more consistent biocapsules, while strains able to flocculate form more abundant biocapsules but smaller and less consistent. 1109 was the strain with the highest absolute values of cells immobilized above the positive control used G1 making this strain a good candidate to immobilize.

Understanding the yeast molecular mechanisms of flocculation and biofilm formation and their relationship with the yeast immobilization in *P. chrysogenum* is not only important in terms of potential industrial application, but also for our understanding of possible evolutionary mechanisms linked to physical interactions between different organisms in shared ecological niches. The way in which mixed species communities control their cell-cell interactions in complex habitats may provide novel insights into ecosystem evolution. Such physical associations can increase the probability of metabolic exchange between cells of different species, and support symbiotic associations as conditioned by the selective pressures of various fermentation environments.

Further research on elucidating the molecular mechanisms in *P. chrysogenum* of flocculation and biofilm formation and their relation with the yeast immobilization will complete this study and will have fundamental importance for understanding ecological processes and the role of direct cell-cell interactions between different species in shared environments. Sequencing and expression profiling of co-flocculation genes in *S. cerevisiae* and *P. chrysogenum* would also provide an important part of the interaction picture. In addition, the relevance of yeast and fungus

metabolism in the biocapsule systems needs further investigation to conclude if there exist a symbiosis among the organisms.

References

Alexandre, H. 2013. Flor yeasts of *Saccharomyces cerevisiae*-Their ecology, genetics and metabolism. Int. J. Food Microbiol. 167:269-275.

Aranda, A., A. Querol, and M.I. del Olmo. 2002. Correlation between acetaldehyde and ethanol resistance and expression of *HSP* genes in yeast strains isolated during the biological aging of sherry wines. Arch. Microbiol. 177:304-312.

Cortés, M.B., Moreno, J., Zea, L., L. Moyano and Medina, M. 1999. Biological aging of sherry wines with high content in gluconic acid. In Proceedings, V National Congress of Enologists. pp. 141-144. Extremadura Enologists Association, Mérida, Spain.

Dengis, P.B., L.R. Nelissen, and P.G. Rouxhet. 1995. Mechanisms of yeast flocculation: comparison of top-and bottom-fermenting strains. Appl. Environ. Microbiol. 61:718-728.

Esteve-Zarzoso, B., Peris-Torán, M.J., García-Maiquez, E., F. Uruburu and A. Querol. 2001. Yeast population dynamics during the fermentation and biological aging of sherry wines. Appl. Environ. Microbiol. 67:2056-2061.

Fidalgo, M., R.R. Barrales and J. Jimenez. 2008. Coding repeat instability in the *FLO11* gene of *Saccharomyces* yeasts. Yeast. 12:879-889.

García-Martínez, T., Moreno, J., J.C. Mauricio and R. Peinado. 2015. Natural sweet wine production by repeated use of yeast cells immobilized on *Penicillium chrysogenum*. LWT Food Sci. Technol. 61:503-509.

García-Martínez, T., Peinado, R.A., Moreno, J., I. García-García and J.C. Mauricio. 2011. Co-culture of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* leading to the immobilization of yeast. J. Chem. Technol. Biotech. 86:812–817.

García-Martínez, T., Puig-Pujol, A., Peinado, R.A., J. Moreno and J.C. Mauricio. 2012. Potential use of wine yeasts immobilized on *Penicillium chrysogenum* for ethanol production. J. Chem. Technol. Biotechnol. 87:351-359.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., D. Botstein and P.O. Brown. 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell. 11:4241-57.

Gibson, B.R., Lawrence, S.J., Leclaire, J.P., C.D. Powell and K.A. Smart. 2007. Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31:535-569.

Giudici, P. and R.E. Kunkee. 1994. The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. Am. J. Enol. Vitic. 45:107-112.

Govender, P., M. Bester and F.F. Bauer. 2010. *FLO* gene-dependent phenotypes in industrial wine yeast strains. Appl. Microbiol. Biotechnol. 86:931-45.

Herker, E., Jungwirth, H., Lehmann, K.A., Maldener, C., Fröhlich, K.U., Wissing, S., Büttner, S., Fehr, M., S. Sigrist and F. Madeo. 2004. Chronological aging leads to apoptosis in yeast. J. Cell Biol. 16:501-507.

Ishigami, M., Nakagawa, Y., M. Hayakawa and Y. Iimura. 2004. *FLO11* is essential for flor formation caused by the C-terminal deletion of *NRG1* in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 237:425-30.

Ishigami, M., Nakagawa, Y., M. Hayakawa and Y. Iimura. 2006. *FLO11* is the primary factor in flor formation caused by cell surface hydrophobicity in wild-type flor yeast. Biosci. Biotechnol. Biochem. 70:660-666.

Kida, K., Yamadaki, M., Asano, S.I., T. Nakata and Y. Sonoda. 1989. The effect of aeration on stability of continuous ethanol fermentation by a flocculating yeast. J. Biosci. Bioeng. 68:107-111.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., M. Roger and A.A. Koutinas. 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol. 21:377-397.

Kuchin, S., V.K. Vyas and M. Carlson. 2002. Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate *FLO11*, haploid invasive growth, and diploid pseudohyphal differentiation. Mol. Cell Biol. 22:3994-4000.

Miki, B.L., N.H. Poon and V.L. Seligy. 1982b. Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. J. Bacteriol. 150:890-899.

Miki, B.L., Poon, N.H., A.P. James and V.L. Seligy. 1982a. Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. J. Bacteriol. 150:878-89.

Peinado, R.A., Mauricio, J.C., Moreno, J., Ortega, J.M., Medina, M., Mérida, J., Millán, M.C., Mayén, M., Zea, L., Moyano, M.L., Valero, E., Muñoz, D., A. López and O. Maestre. 2004. Method of obtaining yeast biocapsules, biocapsules thus obtained and applications of same. World International Property Organization. Publication nr: WO2004029240 A1.

Peinado, R.A., Moreno, J.J., J.M. Ortega and J.C. Mauricio. 2003. Effect of gluconic acid consumption during simulation of biological aging of sherry wines by a flor yeast strain on the final volatile compounds. J. Agric. Food Chem. 51:6198-203.

Peinado, R.A., Moreno, J.J., O. Maestre and J.C. Mauricio. 2005. Use of a novel immobilization yeast system for winemaking. Biotechnol. Lett. 27:1421-1424.

Peinado, R.A., Moreno, J.J., Villalba, J.M., González-Reyes, J.A., J.M. Ortega and J.C. Mauricio. 2006. Yeast biocapsules: a new immobilization method and their applications. Enz. Microb. Technol. 40:79-84.

Puig-Pujol, A., Bertran, E., García-Martínez, T., Capdevila, F., S. Mínguez and J.C. Mauricio. 2013. Application of a new organic yeast immobilization method for sparkling wine production. Am. J. Enol. Vitic. 64:386-394.

Schmitz, K., Peter, V., Meinert, S., Kornfeld, G., Hardiman, T., W. Wiechert and S. Noack. 2013. Simultaneous utilization of glucose and gluconate in *Penicillium chrysogenum* during overflow metabolism. Biotechnol Bioeng. 110:3235-3243.

Soares, E.V. 2011. Flocculation in *Saccharomyces cerevisiae*: a review. J. Appl. Microbiol. 110:1-18.

Soares, E.V. and J. Seynaeve. 2000. Induction of flocculation of brewer's yeast strains of *Saccharomyces cerevisiae* by changing the calcium concentration and pH of culture medium. Biotechnol. Lett. 22:1827-1832.

Soares, E.V., J.A. Teixeira and M. Mota. 1991. Influence of aeration and glucose concentration in the flocculation of *Saccharomyces cerevisiae*. Biotechnol. Lett. 13:207-212.

Spiropoulos, A. and L.F. Bisson. 2000. *MET17* and hydrogen sulfide formation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 66:4421-4426.

Stewart, G.G. 2009. The Horace Brown Medal lecture: forty years of brewing research. J. Inst. Brew. 115:3-29.

Stratford, M. 1992. Yeast flocculation: a new perspective. Adv. Microb. Physiol. 33:2-71.

Stratford, M. 1996. Induction of flocculation in brewing yeasts by change in pH value. FEMS Microbiol. Lett. 136:13-18.

Straver, M.H., Aar, P., G. Smit and J.W. Kijne. 1993. Determinants of flocculence of brewer's yeast during fermentation in wort. Yeast. 9:527-532.

Verstrepen, K.J. and F.M. Klis. 2006. Flocculation, adhesion and biofilm formation in yeasts. Mol. Microbiol. 60:5-15

Zara, G., Zara, S., Pinna, C., S. Marceddu and M. Budroni. 2009. *FLO11* gene length and transcriptional level affect biofilm-forming ability of wild flor strains of *Saccharomyces cerevisiae*. Microbiology. 155:3838-3846.

Zara, S., Gross, M.K., Zara, G., M. Budroni and A.T. Bakalinsky. 2010. Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. Appl Environ. Microbiol. 76:4089-4091.

Appendix 12.

Flor Yeast: New Perspectives Beyond Wine Aging.

Legras, J.L, Moreno-Garcia, J., Zara, S., Zara, G., Garcia-Martinez, T., Mauricio, J.C., Mannazzu, I., Coi, A.L., Bou Zeidan, M., Dequin, S., Moreno, J., Budroni, M.

Frontiers of Microbiology. Volume 7, 14 April 2016, Article 503
https://doi.org/10.3389/fmicb.2016.00503

frontiers in Microbiology

REVIEW
published: 14 April 2016
doi: 10.3389/fmicb.2016.00503

Flor Yeast: New Perspectives Beyond Wine Aging

*Jean-Luc Legras[1], Jaime Moreno-Garcia[2], Severino Zara[3], Giacomo Zara[3], Teresa Garcia-Martinez[2], Juan C. Mauricio[2], Ilaria Mannazzu[3], Anna L. Coi[3], Marc Bou Zeidan[4], Sylvie Dequin[1], Juan Moreno[5] and Marilena Budroni[3]**

[1] SPO, Institut National de la Recherche Agronomique – SupAgro, Université de Montpellier, Montpellier, France, [2] Department of Microbiology, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Cordoba, Spain, [3] Department of Agricultural Sciences, University of Sassari, Sassari, Italy, [4] Department of Agri-Food Sciences, Holy Spirit University of Kaslik, Jounieh, Lebanon, [5] Department of Agricultural Chemistry, Agrifood Campus of International Excellence ceiA3, University of Cordoba, Cordoba, Spain

The most important dogma in white-wine production is the preservation of the wine aroma and the limitation of the oxidative action of oxygen. In contrast, the aging of Sherry and Sherry-like wines is an aerobic process that depends on the oxidative activity of flor strains of *Saccharomyces cerevisiae*. Under depletion of nitrogen and fermentable carbon sources, these yeast produce aggregates of floating cells and form an air–liquid biofilm on the wine surface, which is also known as velum or flor. This behavior is due to genetic and metabolic peculiarities that differentiate flor yeast from other wine yeast. This review will focus first on the most updated data obtained through the analysis of flor yeast with *-omic* tools. Comparative genomics, proteomics, and metabolomics of flor and wine yeast strains are shedding new light on several features of these special yeast, and in particular, they have revealed the extent of proteome remodeling imposed by the biofilm life-style. Finally, new insights in terms of promotion and inhibition of biofilm formation through small molecules, amino acids, and di/tri-peptides, and novel possibilities for the exploitation of biofilm immobilization within a fungal hyphae framework, will be discussed.

Keywords: flor yeast, wine, biofilm, -omic tools, immobilization, biofilm management, biocapsules

OPEN ACCESS

Edited by:
Giuseppe Spano, University of Foggia, Italy

Reviewed by:
Maurizio Ciani, Università Politecnica delle Marche, Italy
Francesco Grieco, National Research Council, Italy
Isabel Pardo, University of Valencia, Spain

****Correspondence:***
Marilena Budroni
mbudroni@uniss.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *12 February 2016*
Accepted: *29 March 2016*
Published: *14 April 2016*

Citation:
Legras J-L, Moreno-Garcia J, Zara S, Zara G, Garcia-Martinez T, Mauricio JC, Mannazzu I, Coi AL, Bou Zeidan M, Dequin S, Moreno J and Budroni M (2016) Flor Yeast: New Perspectives Beyond Wine Aging. Front. Microbiol. 7:503. doi: 10.3389/fmicb.2016.00503

INTRODUCTION

Saccharomyces cerevisiae flor yeast are responsible for the biological aging of Sherry and Sherry-like wines. The main feature of these yeast is that at the end of alcoholic fermentation, when they are under nitrogen and sugar depletion, they shift from fermentative to oxidative metabolism (i.e., the diauxic shift) and rise to the wine surface to form multicellular aggregates. This aggregation leads to the build-up of a biofilm, or velum or flor (Esteve-Zarzoso et al., 2001; Aranda et al., 2002; Alexander, 2013).

Biofilm formation is strongly dependent on the nutritional status of the wine. It is well known that biofilm starts when the concentration of any fermentable carbon source is imperceptible or null (Martínez et al., 1997a). In addition, the presence of other carbon sources, such as glycerol and ethyl acetate, can induce biofilm formation (Zara et al., 2010). Thus, biofilm formation is not limited to aerobic growth on ethanol, but occurs also on other reduced non-fermentable carbon sources that provide sufficient energy input. Moreover, biofilm formation is affected by the availability of nitrogen. It has been shown that in wine lacking nitrogen sources, the flor yeast do not form a