Characterization and improvement of non-conventional *Saccharomyces* yeasts to solve new challenges in the wine industry: Application of omics technologies

María Lairón Peris
PhD Thesis

Supervised by
Dr. Amparo Querol Simón
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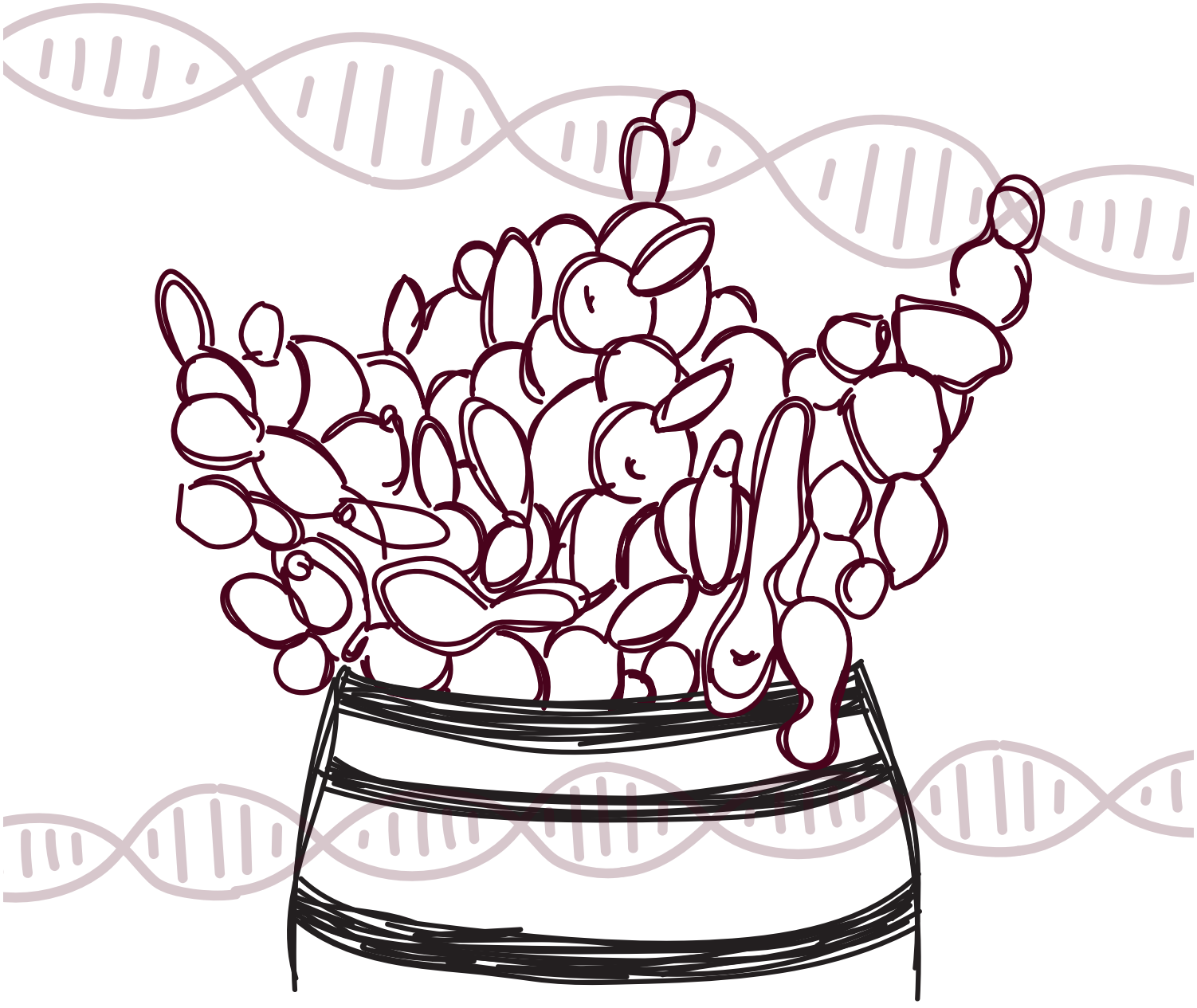
Valencia, 2021



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La Dra. Amparo Querol Simón, Profesora de investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA) y el Dr. Eladio Barrio Esparducer, Catedrático del Departamento de Genética de la Universitat de València.

CERTIFICAN

Que Dña. María Lairón Peris, graduada en Biotecnología por la Universitat de València, ha realizado bajo su dirección el trabajo titulado: ”Characterization and improvement of non-conventional *Saccharomyces* yeasts to solve new challenges in the wine industry: Application of omics technologies”, que presenta para optar al grado de Doctor en el programa de Biomedicina y Biotecnología por la Universitat de València. Asimismo, certifica haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que conste a los efectos oportunos, en cumplimiento de la legislación vigente, firman el presente certificado en

Valencia, a 29 de marzo de 2021

Los directores:

Fdo. Dra. Amparo Querol Simón

Fdo. Dr. Eladio Barrio Esparducer

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Wine is alive.
From the moment you pick the grapes.
till the moment you drink it,
it's alive.

Thibault Morey

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Glossary

5-FAA 5-fluoroanthranilic acid. 10, 136, 143

5-FOA Fluoroorotic acid. 62, 136

ALE Adaptive Laboratory Evolution. 64, 65, 205, 207, 209, 212, 222–224

ATP Adenosine triphosphate. 22, 23, 49, 75

CCNV Chromosome copy number variation. 37, 38, 210, 213, 215, 223

CDS Coding sequence. 185

CerP Ceramide 1-phosphates. 6, 94

CF Carboxyfluorescein. 85, 103

CL Cardiolipin. 6, 7, 26, 94, 95, 99

CNV Copy number variation. 37, 38, 172, 210

cr coverage ratio. 180

DE Differential expression. 13, 118, 153, 185, 193

DG Diacylglycerol. 6, 7, 95, 96, 99, 107, 219

DNA Deoxyribonucleic Acid. 35, 49, 75, 173

EEP Early Exponential Phase. 113, 122, 125

ER Endoplasmic Reticulum. 49

FDR False discovery rate. 142, 153, 174

GABA gamma-aminobutyric acid. 180

GAP General Aminoacids Permease. 49

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase. 180

GARMe Genome autoreduction in meiosis . 162

GMO Genetically Modified Organism. 3, 4, 9, 60, 62, 205, 225

GO Gene Ontology. 142, 153, 159, 166, 174, 190, 230, 231

GP Generalized Polarization. 84, 105, 176, 196, 210

GPA Glycerophosphatidic acid. 6, 7, 94, 95, 99, 219, 224

GPCho Glycerophosphocholine. 191, 193

GPDH Glycerol-3-phosphate dehydrogenase. 58, 59

GPEth Glycerophosphatidylethanolamine. 6, 7, 95, 99, 107, 193, 219, 224

GPGro Glycerophosphoglycerol. 99, 107, 195

GPIns Glycerophosphoinositols. 219

GPP Glycerol-3-phosphatase. 59

GPSer Glycerophosphatidylserine. 6, 7, 95, 99, 191, 195, 219, 224

GPY Glucose Peptone Yeast extract. 77, 80, 83, 86, 93, 175, 195, 207, 208, 211, 219

HOG High-osmolarity glycerol. 47

HSPs Heat shock proteins. 49

InDel Insertion–deletion. 40, 66

IPCC Intergovernmental Panel on Climate Change. 53

ITS Internal transcribed spacer. 29

LAB Lactic Acid Bacteria. 21

LC Liquid Chromatography. 83, 211

LCMS Liquid Chromatography Mass Spectrometry. 6, 13, 83

LEP Late Exponential Phase. 113, 122, 125

LOH Loss of Heterozygosity. 13, 35, 37, 39, 228

M-SM Modified Synthetic must. 206

MAPK Mitogen-activated protein kinase. 47

MAT(a/α) mating type locus (a/α). 39, 41, 42

MCFA Medium chain fatty acids. 27

MG Monoacylglicerol. 6, 7, 95, 96, 99

MIC Minimum inhibitory concentration. 5, 10, 55, 81, 89, 92, 105, 114, 135, 147

MLF Malolactic fermentation. 21

MM Minimal Medium. 62

MM Tandem mass spectrometry. 83

MS Mass Spectrometry. 83, 191, 211

mtDNA Mitocondrial DNA. 64, 208

NAD Nicotinamide adenine dinucleotide. 22

NCR Nitrogen Catabolite Repression. 52

NGS Next Generation Sequencing. 4, 68, 69, 111, 225

NIC Non-inhibitory concentration. 5, 10, 55, 81, 89, 92, 105, 114, 135, 147

OD_{600} Optical density at 600 nm wavelength. 44, 80, 86, 113, 116, 135, 140, 171, 173, 208, 209, 212

ORF Open Reading Frame. 34, 183, 185

PA Phosphatidic acid. 26

PC Phosphatidylcholine. 7, 26, 108, 195, 201

PCA Principal Component Analysis. 85, 105, 142, 152, 153, 157, 158, 183

PCR Polymerase Chain Reaction. 10, 64

PE Phosphatidylethanolamine. 7, 13, 26, 100, 105, 108, 109, 121, 195, 201, 219, 224, 226, 230, 231

PG diphosphatidylglycerol. 26

PI Phosphatidylinositol. 7, 26

PL Phospholipids. 26

POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. 83, 175

POPE 1-palmitoyl-2-oleoyl-sn-glycero-3-phos-phoethanolamine. 83, 84, 175

POPG 1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoglycerol. 83, 175

POPS 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine. 84, 175

PS Phosphatidylserine. 26, 100, 109, 195

QTL Quantitative Trait Locus. 205

RAPD Random Amplified Polymorphic DNA. 64

RFLP Restriction Fragment Length Polymorphism. 10, 64

RNA Ribonucleic Acid. 174

RNA-seq RNA sequencing. 13, 111

ROS Reactive Oxygen Species. 49, 75

rpm revolutions per minute. 83

SGD Saccharomyces Genome Database. 174, 213

SM Synthetic must. 12, 135, 145, 207

SNC *Saccharomyces* non *cerevisiae*. 46

SNP Single Nucleotide Polymorphism. 13, 15, 37, 40, 66, 140, 141, 151, 172, 178, 198, 210, 215, 217, 223

SP Stationary Phase. 113, 122, 125

SV Structural Variant. 37, 38

TA Tartaric acid. 171

TAE Tris-acetic acid-EDTA. 208

TG Triacylglycerol. 96, 99, 107, 191, 195

TLC Thin Layer Chromatography. 6, 7, 13, 84, 100, 175, 191, 201

UPR Unfolded Protein Response. 49, 107, 124

WGS Whole Genome Sequencing. 205

YAN Yeast assimilable nitrogen. 51

YEPD Yeast extract peptone dextrose. 171

YNB Yeast Nitrogen Base. 10, 80, 86, 89

α-AA α-aminoadipic. 10, 62, 136, 143

Resumen amplio en castellano

Las levaduras son microorganismos eucariotas que llevan a cabo multitud de procesos fermentativos con gran importancia biotecnológica. Entre ellas, el género *Saccharomyces* es uno de los más estudiados, ya que participa en distintos procesos fermentativos de valor en la industria alimentaria. En la actualidad, ocho especies se engloban en el género: *S. arboricola*, *S. cerevisiae*, *S. eubayanus*, *S. jurei*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, y *S. uvarum*, siendo la segunda y la última las únicas especies que han colonizado los ambientes fermentativos. Además, también es posible encontrar híbridos entre dos o más especies de *Saccharomyces* principalmente en fermentaciones, pero también en ambientes naturales.

La industria vínica utiliza levaduras seleccionadas para llevar a cabo la fermentación del vino de una manera controlada y que produzca un vino homogéneo, con la misma calidad año tras año. A consecuencia del cambio climático, existe un desequilibrio en las características de las uvas a partir de las cuales se obtienen los vinos. En el momento de la recolección, la uva presenta un desfase entre su madurez industrial y fenólica, lo que provoca que el vino final tenga un grado alcohólico mayor, un pH mayor y una acidez total menor a la esperada, características no deseables en el vino.

Entre las demandas del sector enológico está la utilización de cepas *Saccharomyces*

con las que se obtengan vinos con un menor contenido en etanol, con pH más bajos y con cierta acidez. Además, es necesario que el vino tenga un buen aroma y que su contenido en glicerol sea alto, para darle cuerpo al vino, ya que todo esto agrada al consumidor final.

En los últimos años, se han estudiado las características de cepas de levadura de distintas especies del género *Saccharomyces* con potencial para ser utilizadas en la industria vínica. La finalidad de estos trabajos es tener caracterizadas y así poder seleccionar aquellas que resulten de interés para un proceso fermentativo concreto.

Hay que tener en cuenta que las diferencias entre levaduras del género *Saccharomyces* se encuentran tanto a nivel de especie como de cepa, por lo que es necesario estudiar el comportamiento de cada cepa de levadura de manera individual. A grandes rasgos, las levaduras de la especie *S. cerevisiae* han sido las más utilizadas en la industria vínica, ya que poseen una elevada tolerancia al etanol, que es el principal estrés al que se enfrentan las levaduras durante el proceso de fermentación. Sin embargo, en los últimos años se ha intentado utilizar en la producción de vino cepas de otras especies como *S. uvarum* y *S. kudriavzevii*, así como sus híbridos con *S. cerevisiae*, ya que producen un mayor contenido en glicerol, una mayor diversidad de aromas, un menor contenido en etanol y son más tolerantes a las bajas temperaturas.

Una posible estrategia para conservar los aromas del vino es llevar a cabo las fermentaciones a temperaturas bajas, ya que facilitan la retención de los aromas. No obstante, estas condiciones no son las más idóneas para el crecimiento de las levaduras, y podría dar lugar a fermentaciones largas o, incluso, a paradas de fermentación. Una parada de fermentación representa un gran problema para la industria, ya que se pretende que toda la uva se transforme en vino en el menor tiempo posible y sin percances que generen grandes pérdidas económicas. En este sentido, la tolerancia de *S. kudriavzevii* y *S. uvarum* a las bajas temperaturas supondría una ventaja a la hora de llevar a cabo

estos procesos a esas temperaturas.

Otra posible causa de una parada de fermentación es la cantidad creciente de etanol que las levaduras van produciendo a lo largo de la fermentación, especialmente a partir de concentraciones altas de azúcares. El etanol resulta tóxico para el crecimiento de la propia levadura y lamentablemente, las levaduras de las especies *S. kudriavzevii* y *S. uvarum* son menos tolerantes que las de *S. cerevisiae*, por lo que su utilización se vería favorecida si se pudiese mejorar su tolerancia.

En términos generales, la presencia de etanol durante la fermentación es el mayor estrés al que se ven sometidas las levaduras durante los procesos industriales. La primera barrera con el medio exterior que presentan las células es la membrana, y el etanol -también la temperatura- altera la organización de los lípidos presentes en la misma, modificando su fluidez y provocando cambios en su fisiología.

Una de las estrategias para seleccionar una levadura que lleve a cabo un proceso fermentativo concreto, es su estudio en condiciones controladas para evaluar su comportamiento durante las situaciones de estrés a las que se puede enfrentar como son las fermentaciones en las que se añade una cantidad controlada de alcohol, fermentaciones que son realizadas a bajas temperaturas, etc. También, es posible llevar a cabo fermentaciones con una cepa de levadura concreta y evaluar la composición de los distintos metabolitos presentes en el vino final. Esto nos permite clasificar a las levaduras, según sean mejores o peores, para ser utilizadas en un contexto fermentativo determinado.

En otras ocasiones no se busca seleccionar una cepa de levadura ya existente y presente en la naturaleza, sino mejorar el comportamiento de una cepa de levadura de interés. En Europa, la mejora genética mediante la obtención de Organismos Modificados Genéticamente (OMGs, en inglés GMOs), está muy limitada por la legislación y la

percepción social; pero existen estrategias de mejora genética cuya levadura resultante no es considerada un OMG. Entre ellas destacan la evolución adaptativa en laboratorio y la obtención de híbridos entre dos cepas de levadura.

La obtención de híbridos permite que se forme una nueva cepa de levadura que puede presentar propiedades fisiológicas de ambos parentales. Por ejemplo, es posible obtener un híbrido que reúna la capacidad de fermentar de manera rápida los azúcares y la alta tolerancia al alcohol de *S. cerevisiae*, y la tolerancia a las bajas temperaturas y la producción de un buen perfil aromático y de composición final del vino de *S. kudriavzevii* y *S. uvarum*.

Otra posible estrategia para la mejora de una cepa de levadura es llevar a cabo su evolución adaptativa en el laboratorio. Esta técnica, considerada no GMO, permite obtener cepas adaptadas a ciertas condiciones mediante el cultivo de una población grande y heterogénea de la levadura de interés durante un periodo largo de tiempo en unas condiciones selectivas crecientes, los individuos que mejor resistan a dichas condiciones, se reproduzcan y seleccionen en detrimento del resto de individuos peor adaptados.

Si se utiliza alguna de estas estrategias de mejora, además de comprobar que la nueva cepa de levadura tiene ventajas con respecto a las cepas originales, también es importante comprobar que la nueva cepa obtenida sea estable, es decir que su genoma no se altera después de su obtención, y que se siga comportando igual con el paso del tiempo en las distintas situaciones, no solo en condiciones de laboratorio, sino también a nivel industrial.

Desde hace unos años, el auge de las tecnologías ómicas nos permite obtener datos complejos de organismos de interés, como en el caso de las levaduras. Con las tecnologías de secuenciación de nueva generación (NGS, del inglés), es posible estudiar

el genoma y el transcriptoma de los individuos seleccionados u obtenidos tras llevar a cabo una estrategia de mejora. Esto nos permite relacionar cambios fenotípicos en las cepas de levadura con cambios a nivel genético en las mismas. En el caso de que una levadura haya sido seleccionada por su capacidad de resistencia a un factor con influencia en la membrana plasmática, como la temperatura o el etanol, resulta muy útil el estudio de la composición de membrana plasmática de la misma. Esto se puede realizar con estudios lipidómicos que utilizan la espectrometría de masas para identificar los lípidos presentes en un organismo concreto. Así, podemos intentar relacionar composiciones concretas de membrana plasmática con una mayor resistencia a factores de estrés.

Teniendo en cuenta todo lo expuesto, en esta tesis nos propusimos la caracterización y la mejora de distintas cepas de levadura del género *Saccharomyces* con el fin de perfeccionar su comportamiento durante el proceso de fermentación vínica y de obtener un mejor producto: el vino final. Nos hemos centrado en la mejora de la tolerancia a etanol, ya que como hemos mencionado, la presencia de una elevada concentración de alcohol durante los procesos fermentativos supone un factor de estrés de alto impacto para las levaduras. Además, hemos hecho hincapié en tratar de relacionar el distinto comportamiento de las levaduras ante el etanol con su composición de membrana.

Con el conocimiento previo de que las cepas de *S. cerevisiae* son las más tolerantes al etanol, en el primer capítulo (Capítulo 1) de la presente tesis se seleccionaron un total de 61 cepas de esta especie, aisladas de distintos ambientes fermentativos y con distintos orígenes, para llevar a cabo su caracterización respecto a su tolerancia al etanol. Para ello, se analizó el crecimiento de estas cepas en medios sólido y líquido con distintas concentraciones de etanol. El crecimiento en medio líquido con concentraciones crecientes de un tóxico (el etanol) puede ser modelizado hasta obtener dos parámetros: el NIC (Concentración no inhibitoria) y el MIC (Concentración mínima inhibitoria) que informan de la susceptibilidad y resistencia al etanol de las distintas cepas. Cuanto

más altos son estos parámetros, menos susceptible y más resistente es una cepa a etanol, respectivamente. El crecimiento en medio sólido consiste en observar si un microorganismo es capaz de crecer o no en una determinada concentración del tóxico.

Después de analizar el crecimiento de las cepas, se procedió a seleccionar 5 de ellas que mostraron comportamientos distintos: AJ4 (cepa comercial de la empresa Lallemand), que resultó la cepa más tolerante de todas ante altas concentraciones de etanol; MY26 (cepa aislada de agave) que mostró una baja tolerancia al etanol, MY29 (cepa usada en la fermentación de los vinos de flor) con una tolerancia intermedia y MY3 y MY14, dos cepas vínicas que crecieron bien en medio sólido, pero presentaron problemas de crecimiento en medio líquido.

Estas 5 cepas fueron coinoculadas en fermentaciones con un 0%, 6% y 10% de etanol. AJ4 resultó ser la cepa dominante en los medios de 0% y 10% de etanol, mientras que MY29 fue la cepa dominante en el medio con un 6% de etanol. Se investigó la composición de la membrana de estas 5 cepas en presencia y ausencia de etanol. La composición lipídica de cada cepa se estudió por espectrometría de masas acoplada a la cromatografía líquida (LCMS) y por cromatografía de capa fina (TLC).

Los estudios lipidómicos mediante LCMS demostraron que la cepa que mostraba una composición lipídica más distinta a la del resto de cepas cuando fue crecida en un medio sin etanol era MY29. En concreto, esta cepa mostró una menor cantidad de ceramida-1-fosfatasa (CerP), diacilglicerol (DG), ácido glicerofosfatídico, (GPA), glicerofosfatidilserina (GPSer) y monoacilgilcerol (MG) y mayor cantidad de cardiolipina (CL) y glicerofosfatidiletanolamina (GPEth). Sin embargo, cuando se crecieron las cepas en un medio con un 6% de etanol, MY29 sufre una gran variación en su composición de membrana, que se hizo más similar a la del resto de cepas.

Se estudiaron también los cambios en la saturación y longitud de las cadenas y

aunque no hubo cambios entre cepas en la longitud de las mismas, sí que se encontraron diferencias en cuanto a la saturación. MY29 presentó una menor cantidad de cadenas saturadas en especies DG y en GPSer monoinsaturadas; en cambio, presentó una mayor cantidad en GPA, GPEth y GPSer saturadas; en CL monoinsaturadas, y en MG poliinsaturadas. MY29 volvió a ser la cepa con más diferencias en cuanto a especies saturadas en un 0% de etanol y cuando hubo presente un 6% de etanol en el medio, las membranas de las cepas tuvieron una composición más parecida entre sí.

En los estudios mediante TLC, la cepa menos tolerante de todas, MY26, mostró una mayor concentración de fosfatidiletanolamina (PE) en 0% y 6% de etanol, lo que podría indicar que este lípido está relacionado con la sensibilidad a etanol. En estudios previos se había relacionado una mayor cantidad de PC y de PI con una mayor tolerancia a etanol, y nosotros aquí asociamos una mayor cantidad de PE con una menor tolerancia a etanol.

También se midió la fluidez de la membrana de cada cepa en distintos puntos de fermentaciones con y sin etanol haciendo uso de una sonda fluorescente, el Laurdan, que es sensible a la polaridad del ambiente. La fluidez de las membranas de levadura disminuyó con el tiempo de cultivo y AJ4, la cepa más tolerante a etanol fue la cepa cuya membrana se hizo más fluida en presencia de etanol. MY26, cepa poco tolerante, resultó ser la cepa que menos fue capaz de modular su fluidez de membrana.

Una vez caracterizadas las membranas lipídicas de 5 de las cepas de *S. cerevisiae*, tanto en ausencia como en presencia de etanol, nos interesamos en dilucidar cuáles son los mecanismos moleculares que hacen que distintas cepas presenten tanto tolerancias a etanol, como composiciones de membrana distintas. Por ello, en el Capítulo 2 de esta tesis, decidimos llevar a cabo un crecimiento por triplicado de 3 de las cepas (AJ4, MY3 y MY26) en medio de cultivo GPY con etanol (un 6% y un 10%) y sin etanol para poder tomar muestras en distintos puntos y así llevar a cabo un estudio más completo a nivel transcriptómico de las tres.

El primer punto de muestreo o tiempo cero, fue tomado durante la primera hora de crecimiento en GPY sin etanol, y fue utilizado como la condición con la que comparar el resto de puntos. Tras añadir etanol en las concentraciones ya indicadas, se tomaron para cada cepa muestras en fase exponencial temprana (t1 o ESP); en fase exponencial tardía (t2 o LEP) y en fase estacionaria (t3 o SP) y se comparó con el t0.

Tras la extracción del RNA mensajero presente en estas muestras, su retrotranscripción a cDNA, y secuenciación en un equipo Illumina HiSeq, se llevó a cabo un análisis transcriptómico, que consistió en alinear las lecturas obtenidas contra un pangenoma de referencia (*mapping*), el conteo de la expresión de cada gen haciendo uso de htseq-count y el análisis de expresión diferencial de cada gen en cada muestra con respecto al t0 para lo que se utilizaron los paquetes de R, DESeq2 y limma.

Comparando las listas de genes que cambian su expresión significativamente entre cepas, nos dimos cuenta de que su número era muy grande, y por ello nos centramos en llevar a cabo un análisis de enriquecimiento de términos GO con los genes sobreexpresados e infraexpresados, lo que nos permite agruparlos en categorías funcionales. Es interesante destacar que la categoría de biosíntesis de ergosterol, un compuesto lipídico de membrana que le proporciona fluidez a la misma, está infrarrepresentada en las cepas MY26 y MY3 cuando hay etanol (6% y 10%) mientras que en AJ4 esto no sucede. Sin embargo, la expresión de estos genes en las tres cepas cuando no hay etanol presente en el medio es muy similar. En cambio, AJ4 tiene sobreexpresados distintos genes de la ruta de biosíntesis de ergosterol en presencia de etanol entre los que cabe destacar *ERG20* y a *ERG1*.

Además, la cepa AJ4 presenta una expresión significativamente más alta en presencia de etanol de *HMN1* y de *EKI1* que son genes que codifican enzimas presentes en la ruta de síntesis de fosfolípidos de membrana. Es interesante destacar que todos estos genes están regulados por el factor de transcripción Ino2p. Su secuencia presenta dos

mutaciones en la cepa AJ4, que son compartidas con cepas altamente tolerantes a etanol (datos provenientes de un artículo científico en el que se analizan 1000 cepas de *S. cerevisiae*). Lo mismo sucede con el activador transcripcional Gnc4p, que presenta cambios en su secuencia en la cepa AJ4 y está sobreexpresado en distintas condiciones durante el crecimiento en etanol de AJ4.

Todos estos datos generados nos han provisto de conocimiento a nivel molecular de qué genes pueden estar implicados en la distinta tolerancia al etanol. Estos genes son susceptibles de ser utilizados como dianas específicas para su edición genómica y comprobar su importancia en la tolerancia a etanol de las cepas. Sin embargo, la modificación de un solo locus mediante esta técnica, además de laborioso, puede tener un efecto ligero o moderado en la mejora de la tolerancia al etanol, ya que se trata de un carácter poligénico que depende de múltiples loci.

Por ello, en el Capítulo 3 de la presente tesis nos planteamos mejorar una cepa *S. uvarum* de interés haciendo uso de otra técnica: la obtención de un híbrido de esta cepa con una cepa *S. cerevisiae* altamente tolerante a etanol. Como ya se ha mencionado, las cepas de levadura de *S. uvarum* son criotolerantes y presentan características muy interesantes para ser utilizadas en la industria vínica, ya que generalmente producen vinos con alto contenido en glicerol y aromas y con poco ácido acético. Sin embargo, su tolerancia a etanol es menor que la de cepas de *S. cerevisiae*. Por este motivo, se propuso obtener mediante una técnica conocida como 'rare mating' un híbrido interespecífico *S. cerevisiae* x *S. uvarum* con el fin de aunar las ventajas de ambas especies en un solo híbrido. Esta técnica se ha utilizado con anterioridad en numerosos trabajos de nuestro grupo y ha permitido obtener híbridos con características muy interesantes y aptos para ser usados en la industria al no ser considerados GMO.

La cepa de *S. uvarum* que se quiso mejorar fue la Velluto BMV58^{TM} (BMV58), seleccionada en nuestro grupo para su uso en la industria por su bajo rendimiento en

etanol y alto rendimiento en glicerol. En primer lugar, se analizó el crecimiento en etanol de distintas cepas *S. cerevisiae* proporcionadas por Lallemand para seleccionar aquella con mayor tolerancia a etanol, que resultó ser AJ4, la misma que se seleccionó en el Capítulo 1 como una cepa muy tolerante a etanol. Estas dos cepas, se crecieron por separado en placas de agar α-AA y 5-FAA con el objetivo de obtener espontáneamente mutantes auxotrófos lys^- y trp^-, respectivamente. De esta manera se seleccionaron un auxóstrofo lys^- de AJ4 y uno trp^- de BMV58, que fueron usados para obtener híbridos mediante el procedimiento de 'rare mating', los cuales fueron recuperados en medios de selección y comprobados mediante amplificación por PCR de distintos genes y el subsiguiente análisis de restricción (RFLPs). Algunos de los híbridos obtenidos fueron capaces de esporular y de estos derivados monospóricos se comprobó también su estabilidad. Todos aquellos que resultaron estables fueron evaluados y clasificados según su crecimiento en mosto sintético con un 6,5% de etanol.

Dado que su crecimiento fue el mejor en condiciones de estrés por etanol, se seleccionó el derivado monospórico H14A7 y se analizó, junto al de sus parentales AJ4 y BMV58, su tolerancia al etanol mediante la estima de sus valores de NIC y MIC a 15ºC y 25ºC en medio YNB. El valor de NIC de H14A7 a 15ºC fue el más alto de las 3 cepas, y su valor de MIC fue intermedio al de AJ4 y BMV58 en ambas temperaturas. A continuación, se evaluaron las propiedades enológicas de H14A7, AJ4 y BMV58 llevando a cabo fermentaciones en mosto de vino Verdejo a 15 y a 25ºC. Se concluyó que la rápida actuación durante la fermentación y la producción de ácidos orgánicos de H14A7 es similar a la del parental *S. cerevisiae* y su alta síntesis de glicerol a la del parental *S. uvarum*.

Para determinar la constitución genómica del híbrido obtenido, se llevó a cabo la secuenciación de los genomas de H14A7 y de AJ4 mediante el sistema Illumina Miseq. El genoma anotado de BMV58 ya estaba disponible de un trabajo previo en nuestro

laboratorio y el genoma de AJ4 fue ensamblado y anotado para la presente tesis. Las lecturas del híbrido se mapearon contra los genomas de AJ4 y de BMV58. El análisis de composición genómica se complementó con un análisis de citometría de flujo. Aunando los resultados se determinó que H14A7 es un alotriploide y no un alodiploide como se esperaría tras la esporulación de un alotretraploide. Este alotriploide tendría un subgenoma *S. cerevisiae* diploide, con dos copias heterozigotas de cada cromosoma, y un subgenoma *S. uvarum* haploide. La única excepción es el cromosoma III, en el que ambos subgenomas presentan solo una copia. Esta constitución sugiere que el híbrido original a partir del que se obtiene el derivado monospórico H14A7 era el resultado de un evento de 'rare mating' entre una célula competente *S. cerevisiae* diploide y una célula *S. uvarum* haploide o diploide con distinto locus *MAT*.

Durante las fermentaciones en mosto de vino Verdejo se tomaron muestras de RNA que fueron secuenciadas para llevar a cabo un estudio transcriptómico que permitiera comparar la expresión génica de H14A7 a lo largo del proceso. La expresión de H14A7 fue comparada estudiando las diferencias de expresión entre los subgenomas de H14A7 así como con la de sus parentales AJ4 y BMV58. En el análisis de expresión comparativa entre los subgenomas del híbrido, las diferencias más significativas se dieron en la fase de latencia. A 15ºC, el subgenoma *S. cerevisiae* sobreexpresa genes relacionados con actividad catalítica y toma de nutrientes (iones, unión de proteínas, cofactores, etc.) mientras que el subgenoma *S. uvarum* tiene una alta expresión de genes de biogénesis de ribosomas, involucrados en la maquinaria de traducción necesaria para el crecimiento y división, y en el metabolismo de ergosterol.

En resumen, en este apartado del trabajo conseguimos obtener y tener caracterizado un derivado monospórico estable, H14A7, que presenta ventajas con respecto a las cepas parentales AJ4 y BMV58 para poder ser utilizado para llevar a cabo la fermentación vínica. Como resultado de este trabajo, H14A7 está siendo comercializado en la actualidad por

Lallemand Inc. con el nombre comercial de Velluto Evolution™.

En el Capítulo 4 de la tesis quisimos estudiar qué sucedía si sometíamos a este híbrido estable a unas condiciones de alto estrés que simularan el mosto presente en etapas tardías de una fermentación vínica y así estudiar a nivel genómico y fenotípico este híbrido entre *S. uvarum* y *S. cerevisiae*. Para ello, llevamos a cabo distintas rondas de fermentación en un mosto sintético (SM) modificado con alto contenido en metabisulfito, y concentraciones crecientes de etanol y decrecientes de azúcares. El metabisulfito, $K_2S_2O_5$, en el mosto se convierte en sulfito, un conservante que se utiliza en la industria vínica para prevenir la oxidación y la contaminación del vino, y que al igual que el etanol, puede resultar tóxico para la levadura.

La caracterización fenotípica del híbrido adaptado, al que llamaremos H14A7-etoh, mostró que esta cepa era ligeramente más resistente a etanol. Aunque se mejoró la tolerancia a etanol de H14A7, su impacto fue muy leve si lo comparamos con la mejora que se produjo en la cepa BMV58 tras su hibridación con AJ4, ya que el híbrido H14A7 ya es mucho más tolerante a etanol que BMV58 (Capítulo 2). Sin embargo, sí que mejoramos de manera clara la tolerancia de H14A7 al sulfito.

Se testó el comportamiento de H14A7-etoh en fermentaciones en mosto Verdejo a 15ºC y a 25ºC y se observó que este híbrido evolucionado había empeorado su capacidad de fermentar los azúcares presente en el vino. Este hecho demuestra que cuando se busca evolucionar una cepa para una característica concreta, esta cepa puede también cambiar alguna de sus propiedades a una no deseable.

El genoma de H14A7-etoh fue secuenciado y se detectaron diferentes señales de adaptación, siendo la más relevante de todas la fijación de aneuploidías durante el proceso selectivo, observándose la duplicación del cromosoma III de *S. cerevisiae* y del cromosoma VII-XVI de *S. uvarum*. Además, se observó en H14A7-etoh una pérdida del

cromosoma I de *S. uvarum* y una pérdida de heterozigosidad (LOH) en el cromosoma I de *S. cerevisiae*. El genoma de H14A7-etoh también presenta pequeñas deleciones y duplicaciones, así como la fijación de algunos SNPs. La duplicación del cromosoma III de *S. cerevisiae* podría ser el resultado de la restauración de su diploidia para igualarse a la de los restantes cromosomas *S. cerevisiae* de H14A7 o bien el resultado de una duplicación por adaptación a etanol, ya que un incremento del número de copias del cromosoma III se ha demostrado que está relacionado con un incremento de la tolerancia a etanol. En cuanto a la duplicación del cromosoma VII-XVI de *S. uvarum*, este presenta una translocación afecta al promotor del gen *SSU1*, que codifica un trasportador de sulfito, y da lugar a un incremento de su expresión, lo que confiere una mayor resistencia al sulfito. Por tanto, la duplicación de este cromosoma translocado de *S. uvarum* es una señal clara de adaptación a un estrés mediante el aumento del número de copias.

Tras llevar a cabo un estudio de RNA-seq se evaluó la expresión diferencial (DE) entre los genes presentes en H14A7-etoh y en H14A7 durante las fermentaciones vínicas, prestando especial atención a los genes presentes en el cromosoma III de S. *cerevisiae* y en el cromosoma VII-XVI de *S. uvarum*. En términos generales, H14A7-etoh sobreexpresa estos genes, debido al incremento de la dosis génica y en particular se expresan más en H14A7-etoh a 25ºC los genes *SSU1* y *FZF1*, que codifica un factor de transcripción de *SSU1*, y que también se localiza en el cromosoma VII-XVI duplicado.

Se estudió también la composición de membrana de estas dos cepas, mediante LCMS y TLC. La diferencia más significativa que se encontró fue que H14A7-etoh presenta menor cantidad de PE; lo que podría ser una respuesta adaptativa a estrés por etanol. La PE regula la fluidez de la membrana, ya que a mayor cantidad, menor es la fluidez de la membrana. Se llevaron a cabo experimentos de fluidez de membrana con Laurdan, que confirmaron que la membrana de H14A7-etoh es más fluida que la de H14A7, tal como indicaban los resultados de TLC.

Como la adaptación de H14A7 en unas condiciones estresantes con alto etanol y con sulfito consiguieron mejorar ligeramente la tolerancia al etanol de esta cepa, nos planteamos utilizar una estrategia similar para evolucionar adaptativamente otras cepas del género *Saccharomyces* en el laboratorio y ver si se conseguía mejorar su resistencia al etanol. Así pues, en el quinto capítulo de la tesis doctoral, llevamos a cabo una estrategia de evolución adaptativa en el laboratorio de dos cepas *S. uvarum* (CECT 12600 y BMV58) y de dos cepas *S. kudriavzevii* (CR85 y CA111). Todas estas cepas son menos tolerantes al etanol que H14A7 y nunca se ha llevado a cabo una evolución adaptativa de ellas en presencia de este compuesto, por lo que nos interesaba estudiar qué pasaba en sus genomas si se las sometía a este estrés.

En un primer momento se procedió a crecer las cepas en un mosto sintético, simulando una parada de fermentación, hasta alcanzar una concentración de un 8% de etanol en el caso de BMV58 y de CR85 y una concentración de un 9% en el caso de CA111 y de 12600. Las cepas así adaptadas fueron denominadas BMV58-EVO8, 12600-EVO9, CA111-EVO9, CR85-EVO8. En una segunda etapa se continuó la evolución siguiendo una estrategia de cuellos de botella en los que se sometía a las levaduras a un choque con una concentración aún más elevada de etanol (16% en placas) seguidos por periodos de estabilización en un mosto sintético con una composición normal de azúcares. Así, obtuvimos cepas evolucionadas hasta un 11% de etanol: BMV58-EVO11, 12600-EVO11, CA111-EVO11 y CR85-EVO11. La tolerancia al etanol de las cepas finalmente obtenidas fue caracterizada, revelando así que todas las cepas mejoraron su tolerancia al etanol, con la una única excepción de BMV58.

A su vez, se secuenciaron y analizaron los genomas de estas cepas evolucionadas observándose también distintas señales adaptativas en cada cepa de levadura. Las cepas que presentan unos cambios más drásticos a nivel de genoma fueron las cepas evolucionadas de *S. kudriavzevii*: CA111 y CR85, ya que se producen duplicaciones de

cromosomas completos durante su evolución. CA111-EVO9 adquirió una copia extra de su cromosoma VIII (pasa de 2 a 3 copias) para después volver a perder una de las copias en un paso de evolución posterior, es decir, CA111-EVO11 vuelve a presentar dos copias de este cromosoma. Por su parte, CR85-EVO8 adquirió una copia extra de sus cromosomas II, IX y XVI y estas copias extra se mantuvieron también en algunos individuos de la población secuenciada CR85-EVO11, pero en otros solo se mantiene la copia extra del cromosoma IX y se pierde la del II y la del XVI.

Se estudiaron también mutaciones puntuales y cambios en los SNPs de todas estas cepas evolucionadas en comparación a los de las cepas originales, resultando ser CECT 12600 la cepa que más cambios no sinónimos presentaron.

Los ensayos de fluidez de membrana mostraron que las cepas evolucionadas de BMV58 y CR85 presentaban membranas más fluidas ante la presencia de etanol con respecto a las cepas originales. La cepa CA111 evolucionada en el primer punto de la evolución, CA111-EVO9, adquiere fluidez de membrana, mientras que CA111-EVO11 presenta la misma rigidez que la cepa original. Este cambio podría estar relacionado con la ganancia y posterior pérdida del cromosoma VIII durante la evolución. El resto de cepas no mostraron cambios significativos en cuanto a fluidez de membrana. En cuanto a la composición lipídica de las cepas, CA111-EVO9 y 12600−EVO11 fueron las que presentaron una composición lipídica más distinta en cuanto al número de especies identificadas para las clases lipídicas principales.

Las conclusiones obtenidas durante esta tesis doctoral son varias. Por un lado, se comprobó que la tolerancia a etanol es variable entre distintas cepas de *S. cerevisiae* y que se puede correlacionar con la composición de membrana y con la respuesta transcriptómica en presencia de etanol de cada una de ellas. Por otro lado, se determinó que es posible obtener mediante hibridación una nueva cepa de levadura que mejore a dos parentales con características de interés distintas entre sí, en nuestro caso, la alta

tolerancia a etanol y la buena producción de aromas, de glicerol y tolerancia a las bajas temperaturas. A su vez, un híbrido así obtenido puede ser adaptado en presencia de una concentración alta de sulfito y de concentraciones crecientes de etanol, lo que provoca que se seleccionen distintas características genómicas que al final le proporcionan una mayor tolerancia a estos factores de estrés. En último lugar, se determinó que la evolución adaptativa de distintas especies del género *Saccharomyces* en un medio con etanol provoca cambios distintos en sus genomas y en la fluidez de membrana de las mismas, revelando así la presencia de una gran variedad de mecanismos evolutivos que pueden actuar en presencia de etanol.

Introduction

1. The origins of alcoholic beverages

Nowadays, alcohol is present in all human cultures, but it also played a central role in the beginnings of civilizations. Our ancestors have produced fermented food and beverages from sugar sources available in their local habitats (McGovern et al., 2004), as they early realized that the product of the fermentation, ethanol, seemed to provide beverages and food health benefits, as it preserves fermented foods from undesired microbes.

Food fermenting practices seem to have emerged independently in ancient civilizations worldwide (Hornsey, 2003). The early hunter-gatherer societies were spurred by these nutritious and mind-altering alcoholic beverages, that bring people together. Indeed, there is evidence of intentional production of fermented beverages since the Neolithic (McGovern et al., 1997), when Neolithic people settlements made possible the domestication of plants and animals (Zeder, 2006) and the storage and processing of food (Tamang et al., 2020). Archaeologists have found different pieces of evidence of fermented beverages in form of pottery vessels, paintings, and bronze sculptures. The first documented evidence of a fermented beverage was found in China at ca. 7000

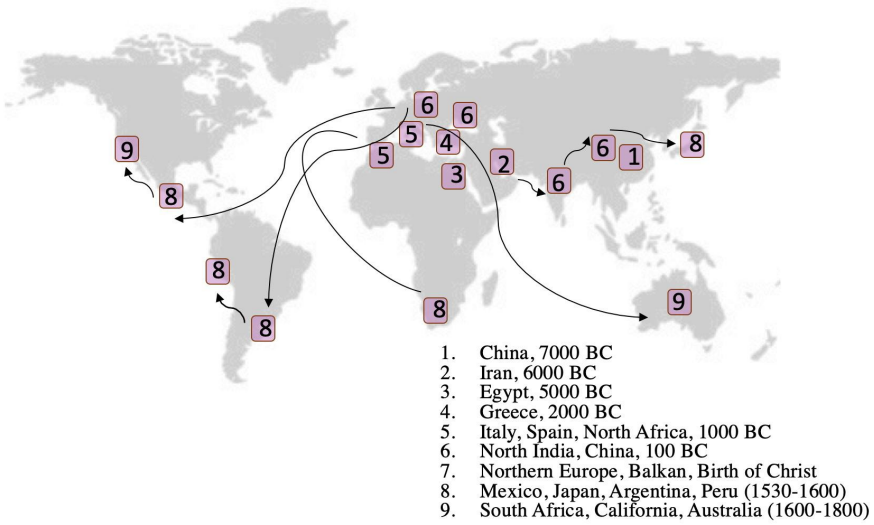


FIGURE 1 The early spreading and world distribution of fermented beverages (Figure adapted from Pretorius (2000)

BC (McGovern et al., 2004) and consisted of a mixture of rice, honey, and fruit. Other archaeological evidence of the forebears of modern grape wines and barley beers are documented in Iran at ca. 6000 BC (McGovern et al., 1997).

The conversion from Nomadic farming to agriculture development changed the bases of society (Underhill, 2002), leading to economic and social progress and allowing the development of the first great civilizations (Katz and Voigt, 1986). It is believed that from Mesopotamia, beverage production spread across the Mediterranean Sea throughout the World (Legras et al., 2007). This was an intense process, especially led by Romans colonization. By 500 BC wine was spread out through the Mediterranean, being produced in Italy, Sicily, France, Spain, Portugal, and North Africa (Pretorius, 2000).

European conquistadors took vines into the New World in the 16th century. In 1530 the Spanish explorers planted *Vitis vinifera*, the common grape vine, in Mexico, Argentina, Peru, and Chile, and in the 17th century, Dutch also planted vineyards in South Africa and shortly after, in California and Australia (Jagtap et al., 2017; Pretorius, 2000; Pretorius et al., 2015) (Figure 1).

Fermented beverages were very popular and present in all civilizations. The reason for this is that our ancestors find on them different beneficial characteristics, compared with no processed foods. Apart from an enhanced nutritional and sensorial value of food and beverages, people perceived pharmacological, analgesic, disinfectant, and mind-altering or psychopharmacological effects in them. Thus, religious and social traditions have been tightly associated with the control of these foods (McGovern, 2003; Earle, 2002; Katz and Voigt, 1986).

At the time, our ancestors used covered containers or recipients in which they introduced fruits and grains. After leaving these foods for a long time, they were converted into beverages as the first wines and beers, but it was not fully understood how this process occurred (Alba-Lois and Segal-Kischinevzky, 2010). In Europe, this process was named fermentation, about the word ”fervere”, which means ”to boil” in Latin, because when substances react during crushing, they produce bubbles, as though they were boiling (Alba-Lois and Segal-Kischinevzky, 2010). Through observation, producers learned that two key factors led to a successful fermentation process: temperature and air exposure. Moreover, production time influenced the process too: if the mixture was not left enough time, alcohol was not produced.

It was not until the 17th century, when Antonie van Leeuwenhoek, a Dutch cloth merchant, was able to observe yeasts, the actual responsible for converting the fruits into alcoholic beverages, in beer worst by using high-quality lenses. However, he did not establish a relation between yeasts and alcoholic fermentation (Ribereau-Gayon et al., 2006). In the following years, several chemists, as Lavoisier and Gay-Lussac, began the study of alcoholic fermentation. Finally, the chemist Louis Pasteur experimentally demonstrated that sugars, as glucose, were converted into alcohol in the absence of oxygen because microorganisms, such as yeasts, were capable of carrying out fermentation (Barnett, 2000).

Fermentations were then considered spontaneous processes, in which yeasts present in fermentation vessels were transferred fermentation after fermentation. In the later 19th century, new discoveries changed this paradigm. During these years, yeast metabolism was studied, leading to fundamental discoveries in biochemical and cell biology fields, and *Saccharomyces cerevisiae* was described as the main yeast responsible for carrying out alcoholic fermentation (Lachance, 2003), as being able to grow in a media containing increasing amounts of ethanol (Boulton et al., 1999). The first yeast pure culture was obtained in 1888 by Emil Christian Hansen in Carlsberg foundation, Copenhaguen; and the first wine yeast pure culture in 1890 by Müller-Thurgau (Dequin, 2001).

The industrial production of wine yeasts as we know it today began in the 1960s in the form of a product with the pressed baker's yeast with 70% moisture. However, this product was difficult to maintain, and in 1964 active dried yeasts were developed (González et al., 2011).

2. Winemaking process and alcoholic fermentation

The production of wine, winemaking, or vinification is a process that starts with the selection of the grapes, continues with their fermentation into alcohol, and finishes with the bottling of the wine liquid. Wine production is a highly conserved process, as industrial wineries usually follow a production process that has not been essentially modified through the years. It consists of 5 steps: harvesting of the grape, crushing and pressing, fermentation, clarification, and aging and bottling (Figure 2).

The quality of the the final wine depends on multiple factors at different stages of the process. The first one is the cultivar of the grape. Most grapes come from *Vitis vinifera* cultivars. This grapevine is native to the Mediterranean and Central Asia regions. The quality and quantity of the fruit depends on multiple factors that are conditioned by

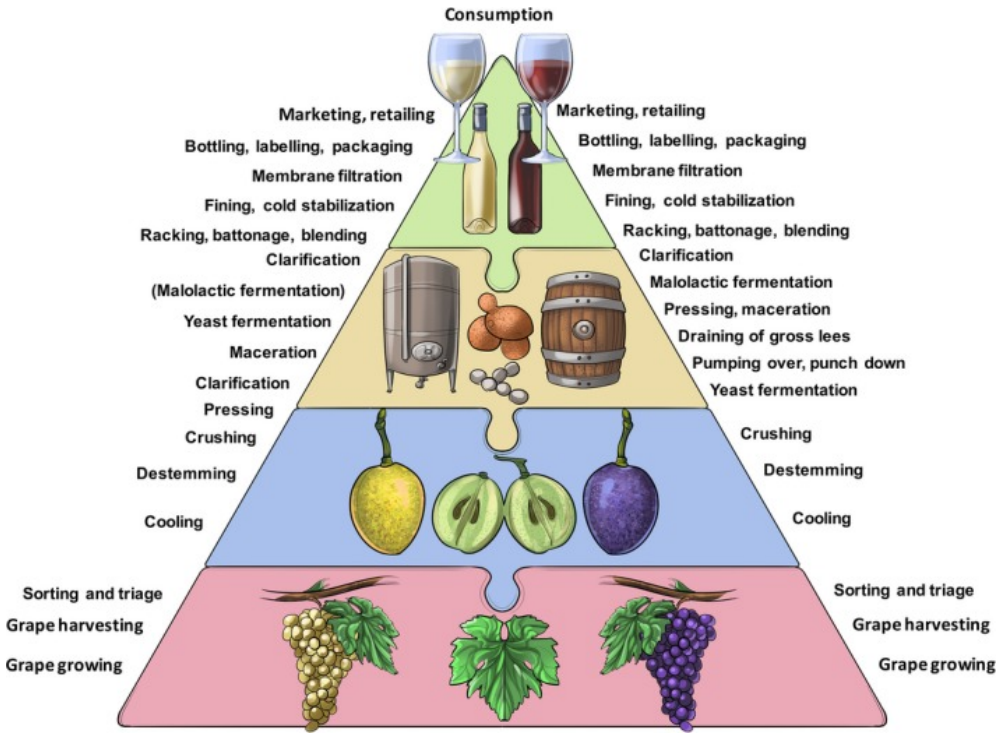


FIGURE 2 A schematic outline of wine production process (Goold et al., 2017).

the plant's environment. Climate and weather are the main factors because the plant needs heat and sunlight in combination with CO_2 and water to grow. Then, when the grapes reach a great balance between sugar levels and maturity, fruits are selected and harvested. After that, grapes are subjected to destemming, crushing, macerating, and pressing. In the case of red wines, alcoholic fermentation occurs at the same time as maceration, that is, fermentation is carried out in contact with the grape skins and seeds. In the case of Rosé wine, the macerating time is reduced in comparison with red wines. However, white wines are usually fermented directly after crushing without maceration (Querol et al., 2018). Alcoholic fermentation is the main stage of the process, and it is conducted mainly by yeasts of the *Saccharomyces* genus. Yeast converts most of the sugars into ethanol and carbon dioxide. After alcoholic fermentation, a second type of fermentation, called malolactic fermentation (MLF), takes place. This is carried out by lactic acid bacteria (LAB), which consume malolactic acid from wines and produce lactic acid and other metabolites. This step softens the acidic taste of the wines and changes

their aroma profile (Bartowsky, 2005; Cappello et al., 2017). The maturation of wine takes from months to years. Red wine is sometimes stored in oak barrels for maturation. Before bottling, a clarification step is needed to remove suspended material in wines.

Alcoholic fermentation is usually conducted by *Saccharomyces* yeasts which are naturally present in the grape or fruit (Pretorius, 2000) or are inoculated in the fermenter to control the fermentation process in industrial conditions (Querol et al., 1992). Fermentation consists of the degradation of six-carbon molecules, usually glucose and fructose, to the two-carbon compound ethanol, as well as CO_2 (Barnett, 2000).

Under aerobic conditions, yeast can degrade sugars using two metabolic pathways: alcoholic fermentation and respiration. During wine conditions, high glucose concentrations are present in the must, and *Saccharomyces* yeasts prefer to metabolize sugars by the fermentative pathway. The first step of fermentation is the conversion of sugar to pyruvate and is a common step in both alcoholic fermentation and respiration. This metabolic process is called glycolysis and is important because it generates ATP, and thus energy. During glycolysis, the redox cofactor NAD+ is reduced to NADH. This reduced NADH needs to be reoxidized. In the case of alcoholic fermentation, NAD+ is regenerated by converting pyruvate to ethanol and CO_2. This process is necessarily carried out by *S. cerevisiae* under anaerobic conditions, when oxygen is not available. However, *S. cerevisiae* also outperforms alcoholic fermentation even if oxygen is present in the media, according to this final stoichiometry:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

During alcoholic fermentation, around 90-95% of sugars are transformed into ethanol and carbon dioxide to produce ATP, and only 1-2% of the carbon source is used for cell growth. It is important to note that the 4-9% is transformed into secondary metabolites, such as glycerol, acetic acid, high alcohols, and esters (Boulton et al., 1999). Yeast

metabolism determines the proportion of sugars that are converted into each compound.

The ability to conduct alcoholic fermentation under high sugar concentration conditions and even in the presence of oxygen was called the Crabtree effect (De Deken, 1966). This phenomenon is produced in grape must, at any level of aeration, when yeasts are only capable of fermenting because of the high glucose and fructose concentrations (Ribereau-Gayon et al., 2006). In the first stages, glucose represses respiration, but after sugar is depleted from the medium, yeast metabolism switches to aerobic consumption of ethanol. Crabtree effect represented a paradox at first, as respiration generates much more ATP via the citric acid cycle and electron transport chain, but *S. cerevisiae*, the predominant yeast in fermentations, only uses respiration during sugar-limited cultivation and in the presence of oxygen.

More recently, it was discussed that the outstanding capability of *S. cerevisiae* is an advantage that guaranteed its implementation success in grape juice (Hagman et al., 2013). *S. cerevisiae* and other *Saccharomyces*' yeasts superiority during wine fermentation can be explained by the "make-accumulate-consume" strategy. These yeasts rapidly consume the sugars present in the must, transforming them into ethanol, which inhibits the growth of other competing microorganisms. Then, when all fermentable sugars are depleted and *Saccharomyces*' yeasts are the only microorganisms present in the media, the ethanol is consumed (Dashko et al., 2014; Piskur et al., 2006; Thomson et al., 2005). Ethanol is a toxic compound that affects most of the microorganisms, and *Saccharomyces* is imposed on their competitors killing them by producing ethanol, and lately, consuming the ethanol when needed (Piskur et al., 2006).

At present, it is well known that the fermentation of grape must and the production of quality wines is a complex ecological and biochemical process (Pretorius, 2000). Fermentation can be spontaneously carried out by the microorganisms present in the wine must. Microorganism composition on grape surfaces varies according to

climatic conditions, stage of grape ripening, physical damages of the grapes, viticultural practices, and the presence of fungicides in vineyards (Pretorius, 2000). The grape microbiota includes fungi, yeasts, lactic acid bacteria, acetic acid bacteria, as well as the mycoviruses and bacteriophages (Fleet and Heard, 1993; Fleet, 1998; Pretorius, 2000). The spontaneous alcoholic fermentation of grape must is initiated by fermentative yeasts, most of them weakly and oxidative yeasts (Baker et al., 2015; Ghosh et al., 2015; Jolly et al., 2003). Traditionally, these yeasts are indigenous and present on the grapes or are resident in the cellar. Physicochemical conditions of the fermentation influence the metabolic activity of yeasts, and hence, their prevalence (Mendoza et al., 2009; Sainz et al., 2003).

3. Yeasts

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi with a vegetative growth based on budding or fission mitotic divisions, and which do not form their sexual states within or upon a fruiting body (Kurtzman et al., 1998a). Yeasts are saprophyte organisms that can grow in an enormous variety of niches, especially in sugar-rich ones, and can also be plant or animal parasites.

Yeasts have been used for millennia because they are responsible for a lot of beneficial activities for human beings and they are the major producer of biotechnology products worldwide. This way, yeasts produce a high variety of fermented food and beverages, antibiotics, vitamins, and enzymes, whose annual biomass production exceeds the millions of tons, being the microorganism with higher economic revenue in industrial processes. Nevertheless, yeasts are also responsible for harmful activities, like food spoilage, and can cause infectious diseases to both animals and humans.

If we follow the classification of ”The Yeasts, A Taxonomic Study” (Kurtzman

et al., 2011) there are 15 genera associated with winemaking: *Brettanomyces* and its sexual ('perfect') equivalent *Dekkera*; *Candida*; *Cryptococcus*; *Debaryomyces*; *Hanseniaspora* and its asexual counterpart *Kloeckera*; *Kluyveromyces*; *Metschnikowia*; *Pichia*; *Rhodotorula*; *Saccharomyces*; *Saccharomycodes*; *Schizosaccharomyces*; and *Zygosaccharomyces* (Pretorius et al., 2017; Pretorius, 2000).

Commercially and genetically, *Saccharomyces* is the most studied yeast genus and the yeast species with the highest biotechnological interest is *Saccharomyces cerevisiae* (Moyad, 2007).

3.1 Yeasts cytology

Yeasts are the most simple of the eukaryotic microorganisms, but unicellular yeast cells contain all subcellular structures typical of eukaryotes. Yeast cellular architecture consists of different parts, which are, from outside to inside: a cell wall, the cell membrane, a cytoplasm with the organelles, and a nucleus surrounded by a membrane, which encloses the chromosomes (Ribereau-Gayon et al., 2006). The cytoplasm and the membrane conform the protoplast or spheroplast, cells whose cell wall have artificially been removed. The two cellular envelopes (the cell wall and the cell membrane) play an essential role during wine fermentation as they release constituents that are added to the resulting wine's composition (Ribereau-Gayon et al., 2006).

3.1.1 Cell wall

It is the first yeast barrier, and its primary function is to protect yeast cells. Without the cell wall, cells are lysed because of the internal osmotic pressure. Cell wall composition consists of β-glucans (about 60% of the dry weight of the *S. cerevisiae* cell wall) and mannoproteins (25–50% of the cell wall of *S. cerevisiae*) and a small proportion of chitin (Ribereau-Gayon et al., 2006).

3.1.2 Membrane

The yeast membrane is a selective barrier that controls the exchanges between the cell and the environment. Its composition in *S. cerevisiae* consists of 40% lipids and 50% protein, with a small proportion in glucans and mannans. The lipids of the membrane are essentially phospholipids (PL) and sterols, but also sphingolipids and glycerophospholipids are present in membranes (Figure 3) (Daum et al., 1998; Ribéreau-Gayon et al., 2006). They are amphipathic molecules (a polar head composed of phosphorylated alcohol and a hydrophobic part composed of fatty acid chains) that spontaneously form bimolecular films or lipid bilayers in an aqueous medium.

The simplest phospholipid is a phosphatidic acid (PA), and it acts as a biosynthetic precursor for the formation (directly or indirectly) of all the lipids in the cell. Various molecules such as choline, ethanolamine, serine, myoinositol, and glycerol can be linked to the phosphoryl group of the PA to form the phospholipids (López-Malo, 2013).

The three principal phospholipids in yeast membranes are phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI) which represent 70–85% of the total (Ribereau-Gayon et al., 2006). Phosphatidylserine (PS) and diphosphatidylglycerol or cardiolipin (PG, CL) are less prevalent (López-Malo, 2013; Tronchoni, 2011). PI is a phospholipid that is essential for yeast (Nikawa and Yamashita, 1997), whereas PS is a minor component of total cell phospholipids, but an important intermediate in *de novo* synthesis of PE and PC. The *de novo* pathway is the major route for PE synthesis in yeast, and it takes place through decarboxylation of PS. It is also possible to generate PE and PC through the Kennedy pathway, which converts ethanolamine to these compounds. Phospholipids present in yeast membranes play a key role in ethanol tolerance and in low temperature adaptation (Chi et al., 1999b; Redón et al., 2012). For years, PC has been considered the main phospholipid that has a role in ethanol tolerance (Mishra and Kaur, 1991). However, the positive influence of PI on

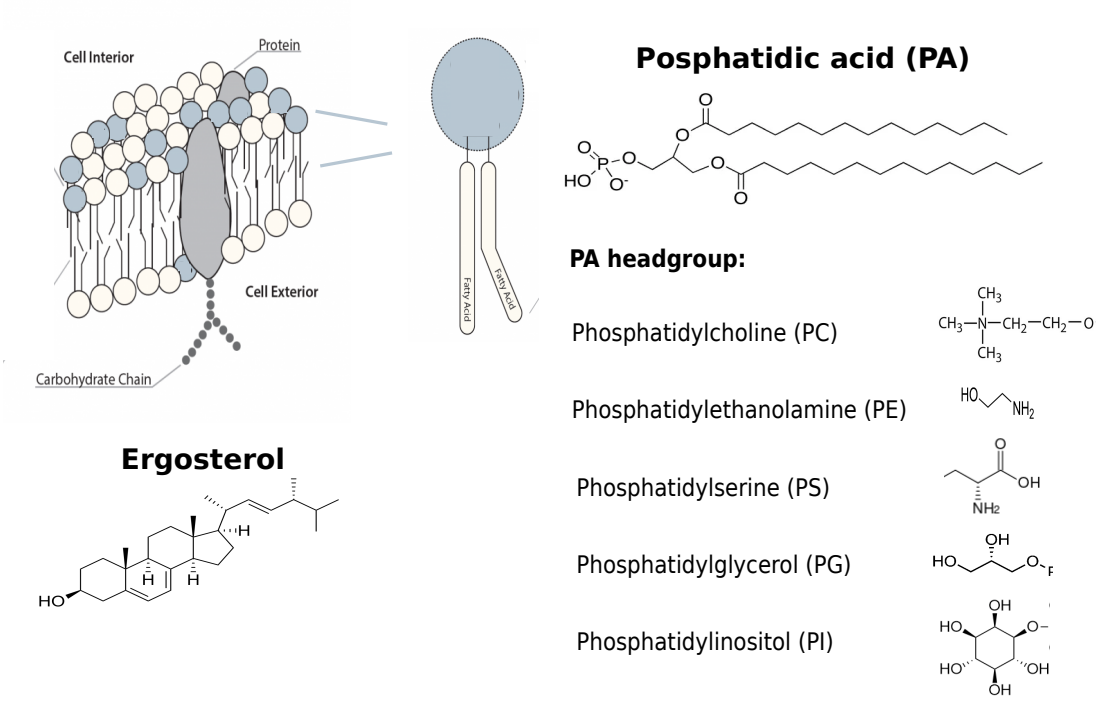


FIGURE 3 **Yeast membrane composition** adapted from Van Der Rest et al. (1995); Lisa (2016)

ethanol tolerance has been demonstrated (Chi et al., 1999b).

The fatty acids of the membrane phospholipids contain an even number (14 to 24) of carbon atoms (Ribereau-Gayon et al., 2006). The most abundant are C16 and C18 acids as oleic acid (18:1) and palmitoleic acid (16:1), linoleic acid (18:2), linolenic acid (18:3), palmitic acid (16:0), and stearic acid (18:0) (Daum et al., 1998).

Acyl chains of phospholipids and glycolipids determine membrane fluidity. In general terms, short-chain fatty acids or with cis-unsaturations decrease transition temperature, favoring the transition from a gel state (solid) to a liquid crystal state (more fluid). The medium-chain fatty acids (MCFA) (from C6 to C14) are present in a lower proportion in the membranes but their concentration increases during fermentations (Redón et al., 2009).

Apart from phospholipids, sterols are present in a high proportion in the membrane, being ergosterol the main sterol in fungi (Daum et al., 1998; López-Malo, 2013).

Membrane composition is important to preserve both the function and the activity of membrane-associated proteins and transporters (López-Malo, 2013). This way, yeasts have a membrane composition that can adapt its fluidity and properties depending on the ambient and which has been correlated with tolerance to stresses (Alexandre et al., 1994; Bisson, 1999; Navarro-Tapia et al., 2018).

3.2 *Saccharomyces* genus

The *Saccharomyces* genus (previously called *Saccharomyces sensu stricto*) belongs to the kingdom Fungi, the phylum Ascomycota (as the sexual reproduction is based on the formation of ascospores), the subphylum Saccharomycotina, the class Saccharomycetes, the order Saccharomycetales and the family Saccharomycetaceae.

The species included in this genus have been revised several times during the 20th century. All over the years, researchers have added and removed many taxa based on morphological or physiological properties, like nitrogen and carbon assimilation, which are not found in other genera. Nevertheless, phylogenetic analyses which started being done in the final years of the 20th century delimited *Saccharomyces* genus classification (Kurtzman, 2003; Naumov, 1996; Vaughan-Martini and Martini, 1995).

As mentioned before, a singularity in the *Saccharomyces* genus is their ability to carry out fermentation, either in the presence or in absence of oxygen, to transform sugars into ethanol. *Saccharomyces* yeasts are involved in a myriad of biotechnological applications, from wine fermentation to bioethanol production (Sicard and Legras, 2011; Walker and Walker, 2018). Currently, and based on increasing number of sequenced strains of the *Saccharomyces* genus, eight species are considered when we refer to this genus: *S. cerevisiae*, *S. kudriavzevii*, *S. uvarum*, *S. paradoxus*, *S. jurei*, *S. mikatae*, *S. arboricola*, and *S. eubayanus* (Borneman and Pretorius, 2015; Boynton and Greig, 2014; Dujon and Louis, 2017; Naseeb et al., 2017) (Figure 4); although other species within this genus may

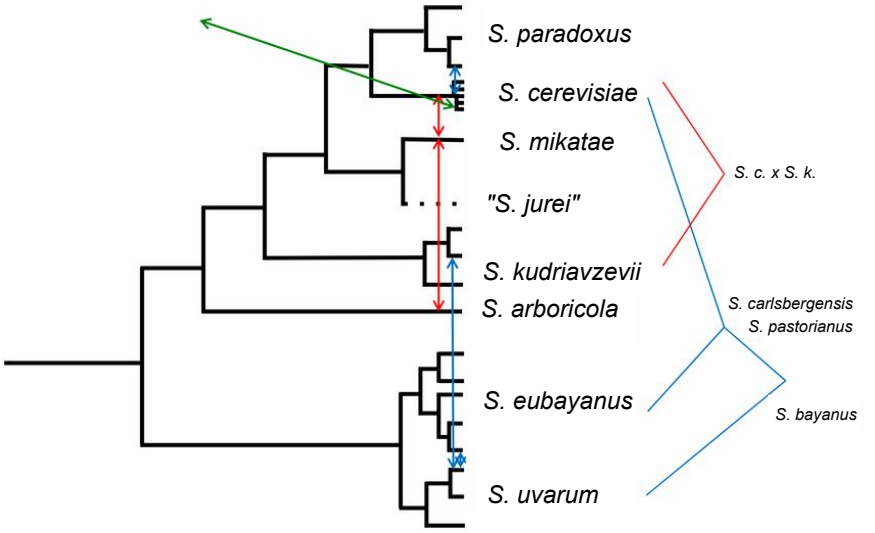


FIGURE 4 Phylogeny of the *Saccharomyces* (formerly *S. sensu stricto*) group (Dujon and Louis, 2017)

remain to be isolated (Legras et al., 2018; Peter et al., 2018). They can be differentiated based on the sequences of their internal transcribed spacer (ITS) and 26S rRNA D1/D2 regions (Naseeb et al., 2017; Kurtzman and Robnett, 1998b, 2003).

Moreover, numerous natural hybrid strains between two *Saccharomyces* species, have been found in industrial processes (Almeida et al., 2016; Borneman and Pretorius, 2015; Boynton and Greig, 2014; Hittinger, 2013; Legras et al., 2018; Naseeb et al., 2017; Peter et al., 2018), many of them associated with human biotechnological processes. Two former species were later classified as species hybrids: *S. bayanus* (*S. eubayanus* x *S. uvarum*) and *S. pastorianus* (*S. cerevisiae* x *S. eubayanus*). Other natural hybrids such as *S. cerevisiae* x *S. kudriavzevii*, *S. cerevisiae* x *S. uvarum*, and triple hybrids *S. cerevisiae* x *S. kudriavzevii* x *S. uvarum* (González et al., 2006; Lopes et al., 2010; Pérez-Torrado et al., 2018; Pérez-Través et al., 2014b; Peris et al., 2012a,b, 2018) have been also reported. The most important species due to their relevance in the wine industry are *S. cerevisiae*, *S. uvarum*, and *S. kudriavzevii* as well as their natural hybrids.

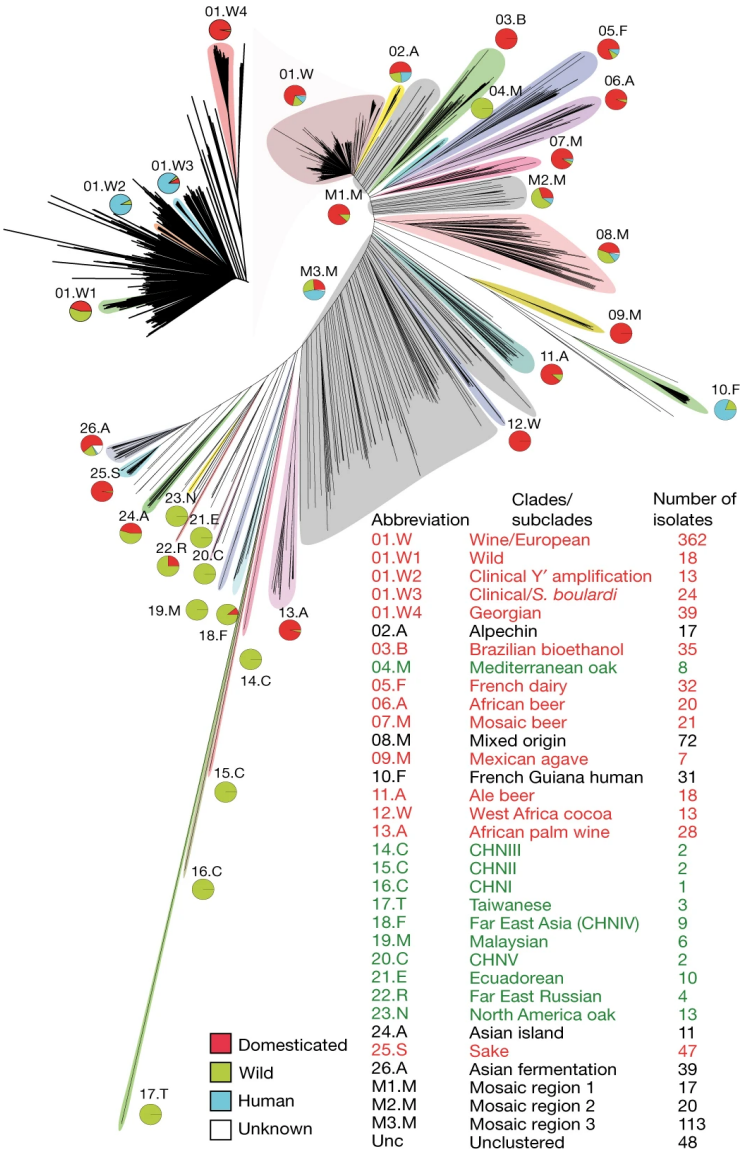
3.2.1 *S. cerevisiae*

Saccharomyces cerevisiae is the most studied eukaryotic organism besides the human

being. The study of *Saccharomyces cerevisiae* as a model organism has contributed to the development of different scientific areas as cell biology, biochemistry and genomics (Goffeau et al., 1996). This species has been found in different environments, including soil, plant exudates, animal tissues, and vineyards in different geographical areas (Fay and Benavides, 2005; Landry et al., 2006).

Different authors have addressed the study of *S. cerevisiae* origin. This task is challenging, as this species is present in a different number of niches, but with the widespread use of whole-genome data, we now have a better understanding. Goddard and Greig (2015) proposed that *S. cerevisiae* is a natural yeast with no niche, that changes its location using insects as vectors (Buser et al., 2014). Some independent domestication events may have taken place to give rise to different geographically separated domesticated lineages. Liti et al. (2009) classified them into five major clades: Wine/European, Malaysian, West African, North America, and Sake groups, and a series of mosaic strains with genetic admixtures of these groups (Figure 6). Last studies suggest that *S. cerevisiae* originated in Far-East Asia ('out-of-China' origin) and that various independent events eventually led to the domestication of this species into the aforementioned clades (Peter et al., 2018; Wang et al., 2012) (Figure 5).

S. cerevisiae is the predominant species in the production of wine, beer, sake, and other traditional fermented beverages. This *Saccharomyces* species exhibits the highest ethanol tolerance (Arroyo-López et al., 2010b) and is better suited to survive at high temperatures, with an optimal temperature of 32.3ºC and a maximum growth temperature of 45.4ºC (Salvadó et al., 2011b). As a high ethanol content is one of the selective pressures faced by yeasts during fermentation, *S. cerevisiae* is the most widely used species in the wine industry. It is also used for bioethanol production, as it also allows the achievement of high ethanol yields (Greetham et al., 2014; Wimalasena et al., 2014).



Abbreviation	Clades/ subclades	Number of isolates
01.W	Wine/European	362
01.W1	Wild	18
01.W2	Clinical Y′ amplification	13
01.W3	Clinical/*S. boulardi*	24
01.W4	Georgian	39
02.A	Alpechin	17
03.B	Brazilian bioethanol	35
04.M	Mediterranean oak	8
05.F	French dairy	32
06.A	African beer	20
07.M	Mosaic beer	21
08.M	Mixed origin	72
09.M	Mexican agave	7
10.F	French Guiana human	31
11.A	Ale beer	18
12.W	West Africa cocoa	13
13.A	African palm wine	28
14.C	CHNIII	2
15.C	CHNII	2
16.C	CHNI	1
17.T	Taiwanese	3
18.F	Far East Asia (CHNIV)	9
19.M	Malaysian	6
20.C	CHNV	2
21.E	Ecuadorean	10
22.R	Far East Russian	4
23.N	North America oak	13
24.A	Asian island	11
25.S	Sake	47
26.A	Asian fermentation	39
M1.M	Mosaic region 1	17
M2.M	Mosaic region 2	20
M3.M	Mosaic region 3	113
Unc	Unclustered	48

FIGURE 5 Neighbor-joining tree of 1011 *S. cerevisiae* strains (Peter et al., 2018)

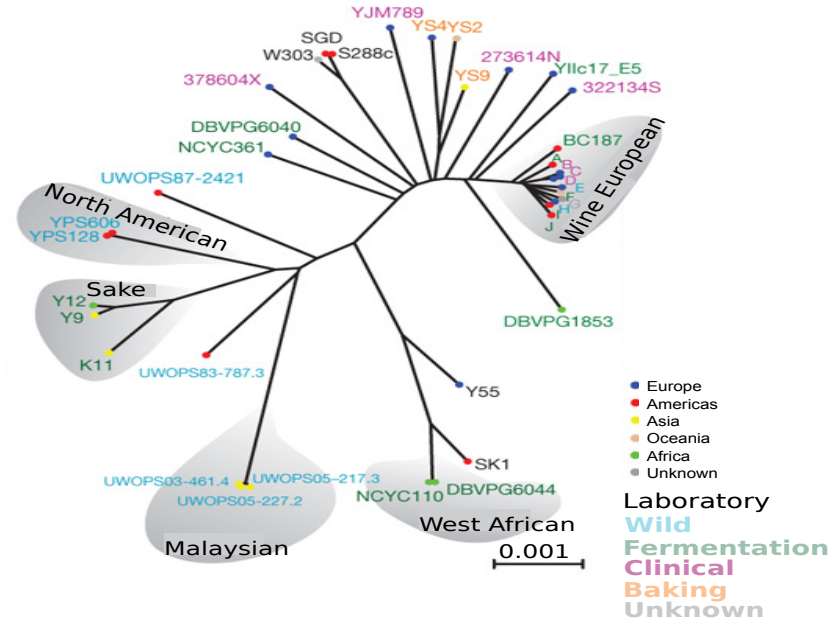


FIGURE 6 Neighbor-joining tree of *S. cerevisiae* strains (Figure adapted from Liti et al. (2009).

3.2.2 *S. uvarum*

S. uvarum is a cryophilic species in the *Saccharomyces* genus, whose fermentation profile in grape must differs from *S. cerevisiae*. It has been mainly found in human-related niches, such as wine and cider fermentations performed at low temperatures in regions of oceanic and continental climates (Demuyter et al., 2004; González Flores, 2019; Naumov et al., 2000b, 2001; Rodríguez et al., 2014), although it has also been isolated from insects, tree fluxes and mushrooms (Naumov et al., 2003; Stribny, 2016).

When used in wine fermentations, it produces lower levels of amyl alcohols and ethanol, but more glycerol, succinic acid, malic acid, isobutyl alcohol, isoamyl alcohol (Bertolini et al., 1996; Giudici et al., 1995; Sipiczki, 2008). It also generates numerous secondary compounds during alcoholic fermentation, such as phenylethanol and phenylacetate (Masneuf-Pomarède et al., 2010). These compounds are volatile, and wines produced with *S. uvarum* yeasts are perceived as more aromatic than those produced by *S. cerevisiae* (Coloretti et al., 2006; Gamero et al., 2013). These

characteristics make commercial *S. uvarum* strains a very interesting starter to produce several types of wines and ciders, usually at low temperatures.

3.2.3 *S. kudriavzevii*

S. kudriavzevii is also a cryophilic species within the *Saccharomyces* genus, and it has been reported as the best adapted to cold temperatures among all the *Saccharomyces* species (Salvadó et al., 2011a). It was first isolated from decayed leaves in Japan (Naumov et al., 2000a), but later also from oak trees in France, Portugal and Spain (Erny et al., 2012; Lopes et al., 2010; Sampaio and Gonçalves, 2008), as well as in Taiwan (Naumov et al., 2013). Although *S. kudriavzevii* isolates are distributed in different geographical areas, they show low divergence in their genomes (Hittinger et al., 2010).

S. kudriavzevii is a potential starter to be used in the wine industry; besides its capability to conduct fermentation at low temperature (Tronchoni et al., 2012), it gives interesting oenological properties to the final wine. Wines produced by *S. kudriavzevii* contain more glycerol and less ethanol (González et al., 2007; Pérez-Torrado et al., 2018; Peris et al., 2016), with no increase in the acetic acid levels (Alonso-del Real et al., 2017a; Henriques et al., 2018). This species also generates higher content in aromatic higher alcohols and 2-phenylethanol (rose aroma) at low temperatures (Coloretti et al., 2006; Stribny et al., 2015). However, it is the species within the *Saccharomyces* with the lower ethanol tolerance (Arroyo-López et al., 2010b), a trait that is necessary to conduct wine fermentations.

3.2.4 Natural hybrid strains

Saccharomyces interspecific hybrids are frequent, and they have also been isolated in nature. Prezygotic reproductive barriers are absent or very limited between *Saccharomyces* species (Gorter de Vries et al., 2019; Morales and Dujon, 2012). This fact has facilitated hybridization, although spore viabilities of the resulting hybrids are very

low (Kurtzman et al., 2011; Naumov et al., 2000a; Sampaio and Gonçalves, 2008).This sterility is likely due to the inability of diverged homeologous chromosomes to recombine during meiosis (Greig et al., 2003; Greig, 2009).

Despite this, several hybrids have been found associated with human-related environments, and they usually combine beneficial traits from their parental species, resulting in especial interest to conduct fermentative processes. The most known *Saccharomyces* hybrid is *S. pastorianus*, responsible for lager brewing. Their strains are hybrids between *S. cerevisiae* and *S. eubayanus*, and it has been proposed that *S. eubayanus* parental complements the fermentation capability of *S. cerevisiae* parental with its cryotolerance (Bing et al., 2014; Libkind et al., 2011; Peris et al., 2014). *S. bayanus* strains are hybrids between *S. uvarum* and *S. eubayanus* and are found in cider fermentation processes (Naumov et al., 2001).

Double hybrids between *S. cerevisiae* and *S. kudriavzevii* or *S. uvarum*, as well as triple hybrids among these three species, have been found in wine and cider fermentations (Belloch et al., 2008; González et al., 2008; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998; Querol and Bond, 2009). Hybrids often show a dynamic genome and their phenotype can change with the genomic content in very few generations (Morard et al., 2020b; Van den Broek et al., 2015).

3.3 *Saccharomyces* genome characteristics related to the domestication history

S. cerevisiae was the first eukaryotic genome to be sequenced in 1996 (Dolinski and Botstein, 2005; Goffeau et al., 1996). The characterization of the laboratory strain S288c revealed that *S. cerevisiae* genome was relatively small, with 5885 open reading frames (ORFs) which defined the same number of potential protein-encoding genes. The first studies revealed that about 60% of *S. cerevisiae* genes have orthologs in the human genome, and that important metabolic and cell signaling pathways are also present.

With the advent of new whole-genome sequencing technologies, a huge number of strains belonging to the *Saccharomyces* genus have been sequenced so on (Almeida et al., 2016; Baker et al., 2015; Barbosa et al., 2016; Liti et al., 2009; Nespolo et al., 2020; Peter et al., 2018; Scannell et al., 2011; Strope et al., 2015; Walther et al., 2014; Zhang et al., 2015). The different species of the *Saccharomyces* genus have a small highly packed 12 Mb genome, that is composed of sixteen chromosomes and the 2-µm plasmid in the nucleus, and the mitochondrial DNA. Chromosome synteny is generally conserved, except for some translocation events (Borneman and Pretorius, 2015). For example, *S. cerevisiae* and *S. uvarum* genomes are largely syntenic, except for 3 large reciprocal translocations in chromosomes (Kellis et al., 2003) and in their telomeres (Brown et al., 2010; Kellis et al., 2004). Some *S. cerevisiae* strains also have different transposable elements on its genomes, like Ty retrotransposons.

Saccharomyces strains can have different ploidies. The ploidy is the number of complete sets of chromosomes in a cell. *Saccharomyces* strains exist as stable haploid, diploid, or polyploid (e.g. triploid and tetraploid) cells (Todd et al., 2017). Aneuploidy is an abnormal chromosome number, due to the gain or loss of chromosomes.

S. cerevisiae industrial strains show a wide range of ploidies and different levels of aneuploidy (Strope et al., 2015). Moreover, polyploid strains have different heterozygosity levels in their genomes, which are related to differences in strain life cycles (Magwene et al., 2011). High heterozygosity levels are present in strains present in industrial environments rather than in natural strains, as these strains reproduce asexually (Gallone et al., 2016; Morard et al., 2019; Peter et al., 2018). Several events of loss of heterozygosity (LOH) are also present in heterozygous strains, especially in industrial strains.

Some of the *S. cerevisiae* genomes particularities are signs of domestication, which could have recently been characterized. The genomes of *Saccharomyces* species

related to food and beverage fermentations have been shaped by the selective pressures introduced by man practices throughout the history (Steensels et al., 2019). This process started unconsciously in the Neolithic, at the same time as the domestication of plants and animals. However, the effect of microbial domestication is less evident and was a less-controlled process. During spontaneous fermentation, there was a practice called backslopping, which consisted of transferring material (and so microbes) from the last fermentation product to the new batch. Artisans unconsciously promoted the adaptation of microbes to the human-manipulated fermentation environment in that way. As a result of this passive domestication, nowadays, industrial strains are genetically distinct from wild strains and, with a few exceptions, they cluster together according to their related product (wine, beer, bread, fermented milk, sake, etc.) and source of isolation rather than to their geographic origins, which is an evidence of their domestication and selection history (Gallone et al., 2016; Legras et al., 2007; Peter et al., 2018; Steensels et al., 2019).

Yeasts are used in a wide range of fermentation processes, and that has led to the selection of strains with specific phenotypes, that eventually are tailored to specific industrial applications (Dequin and Casaregola, 2011; Marsit et al., 2017).

For example, wine yeasts are more tolerant to sulfites and copper (sterilization agents used in both the winery and the vineyard) and beer yeasts can metabolize maltotriose, a sugar present in barley (Marsit et al., 2015, 2017; Pérez-Ortín et al., 2002; Underhill, 2002; Warringer et al., 2011).

In the *Saccharomyces* genus, the emergence of interspecific hybrids is an adaptation to man-made fermentation environments. As we previously mentioned, these hybrids often combine the vigorous fermentation capacity of *S. cerevisiae* with the tolerance to cold temperatures of cryotolerant species, such as *S. eubayanus*, *S. kudriavzevii* or *S. uvarum* (Baker et al., 2015; Libkind et al., 2011; Peris et al., 2012b). There are some genetic changes related to domestication in *S. cerevisiae* strain genomes. Within the

possible adaptation mechanisms described for yeast, it is worth mentioning SNPs, CNVs, SVs, CCNVs, chromosomal rearrangements, and LOH.

3.3.1 SNPs

The presence of Single Nucleotide Polymorphisms (SNPs) in *Saccharomyces* from different lineages is one genome trait related to domestication in *Saccharomyces* strains. Some studies report that SNP variation has changed sugar metabolism and reduced undesired flavors in yeasts (Bergström et al., 2014; Gallone et al., 2016; Gonçalves et al., 2016). Although SNPs only represent a small fraction of genome variation, they are easy to detect using short-read sequencing technologies.

3.3.2 Gene copy number variations

Another hallmark of adaptation to fermentation practices is the presence of Copy Number Variations (CNV). CNVs are small genetic loci, such as genes or clusters of few genes, which due to deletions and duplications vary in their absolute number across individuals from a population (Bergström et al., 2014). One example is the copy number variation of the gene *CUP1*, which encodes a copper-binding protein. This gene is present in a higher copy number in wine strains because it protects against a fungicide used in vineyards (Strope et al., 2015). In beer yeasts, *MAL* genes (encoding maltose transporters) improve consumption of maltose and maltotriose, the main sugars available during the fermentation of beer wort, and they are also in a higher number in these strains (Gallone et al., 2019).

3.3.3 Chromosomal copy number variation

Another domestication strategy is chromosomal copy number variation (CCNV) or karyotype variation (chromosome loss or gain), which leads to the previously mentioned aneuploidy state. Karyotype variations are well-tolerated by yeasts and they are often observed in yeast when they adapt to new, stressful environments (Dunham et al., 2002;

Gresham et al., 2008; Voordeckers and Verstrepen, 2015b; Yona et al., 2012), and CCNV are frequently involved in industrially relevant traits acquired during evolutionary engineering (Gorter de Vries et al., 2017). One example is the increase in the copy number of chromosome VIII, which harbors the *CUP1* gene, and improves copper tolerance in yeast (Zimmer et al., 2014). Another example is the gain of chromosome III involved in high-temperature and ethanol tolerances, or chromosome V, involved in high pH tolerance (Yona et al., 2012)), and the increase of chromosome III numbers related to ethanol tolerance (Morard et al., 2019; Voordeckers et al., 2015a).

3.3.4 Large-scale structural variants

Large-scale structural variants (SV) are other structural variations that are larger than CNV. This is the case of large deletions and duplications, inversions, reciprocal translocations, transpositions and novel insertions (Marsit et al., 2017). SVs are difficult to trace with traditional short-read sequencing but with the advent of new sequencing platforms, such as those of Pacific Biosciences (PacBio) and Oxford Nanopore, we now have long-read sequences and continuous assemblies of each chromosome of a strain, with the especially complex genomic regions, as repetitive or telomeres regions, resolved (Chin et al., 2013; Gordon et al., 2016; Yue et al., 2017).

In *Saccharomyces* spp., long-read sequencing revealed that *S. cerevisiae* more rapidly accumulates unbalanced rearrangements (deletions and duplications, insertions) in its chromosomal core compared with its non-domesticated sister-species *S. paradoxus* which faster accumulates balanced rearrangements (inversions, reciprocal translocations and transpositions) (Marsit et al., 2017). Besides, *S. cerevisiae* shows a higher degree of interchromosomal reshuffling in its subtelomeric regions.

3.3.5 Chromosome rearrangements

Chromosome rearrangements or interchromosomal reshuffling are other examples

of genome adaptive strategies on *Saccharomyces* chromosomes. A well-characterized example is the modification of the upstream region of the *SSU1* gene, which encodes a sulfite pump that confers sulfite resistance. At least two mechanisms have been documented in *Saccharomyces* yeasts to adapt to the high levels of sulfite present in wine. The first-documented gross chromosomal rearrangement was amongst VIII-t-XVI chromosomes (produced by a cross-over between 5' upstream regions of the *SSU1* and *ECM34* genes) (Pérez-Ortín et al., 2002). The second documented translocation involved in sulfite tolerance is amongst chromosomes XV-t-XVI, and it involves the promoter region of *ADH1* and the gene *SSU1* (Zimmer et al., 2014). These two translocations are present in different domesticated yeast strains, and it is proposed that they were selected by human activity. More recently, an inversion in chromosome XVI (inv-XVI) that increases the sulfite resistance capacity of a wine yeast strain was observed (García-Ríos et al., 2019d).

3.3.6 Loss of Heterozygosity

The term Loss of Heterozygosity (LOH) refers to genomic regions that have become homozygous for the polymorphisms present in them. They are produced during mitotic cell divisions when recombination events take place in the chromosomes. There are two types of LOH: interstitial events (conversions) resulting in a short LOH and terminal events (mostly cross-overs) in which the LOH tract extends to the end of the chromosome (Sui et al., 2020).

3.4 The life cycle of *Saccharomyces*

Yeast cells have both diploid and haploid modes of existence and can multiply either asexually or sexually. *Saccharomyces* yeasts present a sexual locus *MAT* with two possible alleles: *MAT*a and *MAT*α (*MAT(a/α)*) that determine the mating type and thus display simple sexual differentiation. There are three different cell types: haploids of two mating types, a and α, and a/α diploids (Herskowitz, 1988; Madhani, 2007). Most of the

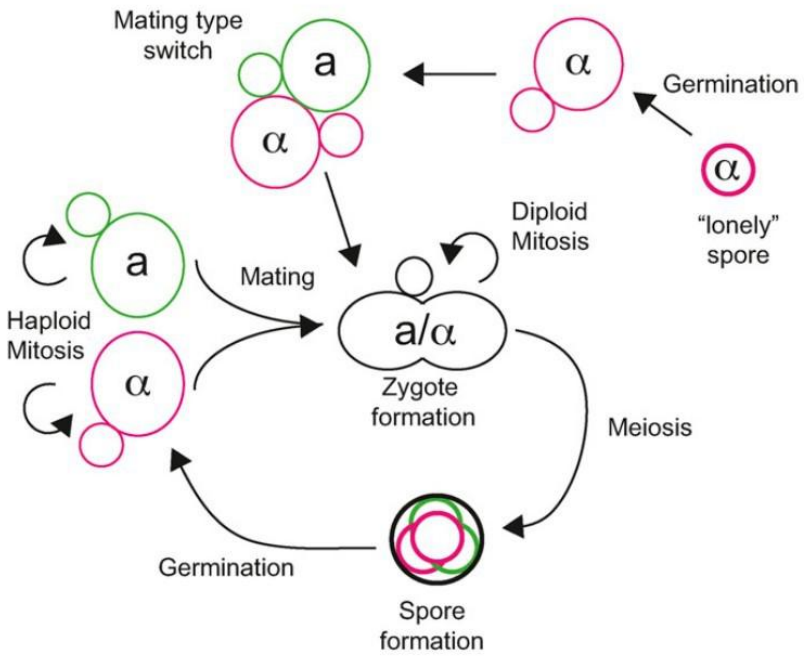


FIGURE 7 Schematic life cycle of *S. cerevisiae* from Hanson and Wolfe (2017).

yeast cells are diploid, which is the ideal state, and are heterozygous for the *MAT* locus (they possess *MAT*a and *MAT*α alleles). *Saccharomyces* haploids can turn into diploids by three different strategies: by the mating of unrelated haploids (amphimixis), by the mating between spores from the same tetrad (automixis or intratetrad mating) and by the mating between a mother and daughter cells after the type switching of one of the cells involved (haplo-selfing) (Hanson and Wolfe, 2017; Knop, 2006) (Figure 7).

Both haploid and diploid yeasts, can multiply either asexually by vegetative growth (mitosis), or sexually by sporulation and crossing (meiosis). The vegetative multiplication process is the predominant way of reproduction under optimal nutritional conditions (on average only one meiotic cycle per 1000 mitotic divisions) (Ruderfer et al., 2006; Steensels et al., 2014a; Tsai et al., 2008; Zörgö et al., 2012).

During these asexual reproductive cycles, spontaneous mutations, such as point mutations (SNPs), InDels and recombination events, can arise on yeast genomes (Steensels et al., 2014b). Vegetative multiplication can be divided into four phases: M,

G1, S and G2. M corresponds with mitosis, G1 is the period preceding S, which is the synthesis of DNA and G2 is the period before cell division. Once mitosis is concluded, the nascent nucleus and organelles migrate into the bud, cytokinesis starts and the septum is formed in the isthmus between mother and daughter cells, completing cell division.

If conditions in the culture media are nutrient-poor, diploids can undergo sporulation (meiosis followed by spore formation). This results in the conversion of a diploid cell into four haploid spores, two with mating type a and two with mating type α (Steensels et al., 2014a).

Moreover, most *Saccharomyces* strains are homothallic, meaning that the two types of haploid can mate if an *a* and an *α* cells meet when growing vegetatively, resulting in a diploid cell *MAT(a/α)* (Martin et al., 2013). *MATα* cells secrete α factor pheromone, a 13 residue peptide, and respond to a-Factor. *MATa* cells secrete a-Factor, a 12 residue peptide that is covalently attached to a lipid (farnesyl) group, and respond to α-Factor. If a yeast cell secreted its pheromone (a-Factor or α-Factor) and a nearby yeast cell with the receptor for this factor is stimulated by it, its receptor Ste3 and Ste2 (for a and α factor, respectively), activates a signaling response which leads to ultimately fuse the membranes and nuclei of the mating partners. The entire process takes about 4h (Bardwell, 2004; Martin et al., 2013).

In homothallic strains, the haploid derivatives can also switch their mating type, that is, a haploid *a* cell can become a haploid *α* cell, by changing its genotype at the mating-type (*MAT*) locus from *MATa* to *MATα*, or vice versa. This process is mediated by an endonuclease, encoded by the *HO* gene, that cleaves DNA specifically at the *MAT* locus (Steensels et al., 2014b). The *MAT* locus is located on chromosome III flanked by Hidden *MAT* Left and Right (*HML* and *HMR*, respectively), carrying a silenced copy of *MATa* and *MATα*, respectively. After the breakdown of the *MAT* locus by exonucleases, a gene conversion event occurs, where *HML* or *HMR* is used as a template to repair

the DNA strand. The mating-type switch occurs frequently because cells often prefer to change their former mating type, that is, a *MATa* cell will rather use *HMR* as a template to copy *MAT*α and viceversa (Herskowitz, 1988; Steensels et al., 2014b).

If a mating type-switched cell crosses with a near sister cell of the opposite mating type, the result is a homozygous diploid MAT(a/α) homozygous for all of the genes except the *MAT* locus (Steensels et al., 2014b). In heterothallic strains, the *HO* gene is typically inactive and therefore haploid derivatives cannot switch their mating types (Steensels et al., 2014b). Laboratory strains are usually heterothallic and this increases their stability.

4. Growth kinetics in fermentation

4.1. Yeast population dynamics during wine fermentation

In a typical fermentation, yeasts follow growth kinetics very similar to a standard microbial growth curve. It comprises a predictable succession of events divided into four main stages: latency or lag phase, exponential or log phase, diauxic phase, and stationary phase (Figure 8).

The lag phase is the first stage, in which yeasts adapt to the new environment and its duration depends on the appropriateness of the media conditions and of the initial population size. In this phase, oxygen is important as it is needed for lipids' biosynthesis and to end a successful fermentation and long-term health of the culture. During the lag phase, yeasts acclimatize to the must and prepare to consume massive amounts of sugars, amino acids, peptides, other proteins and nutrients, and finally, they start synthesizing the ribosomes and enzymes that are needed to reach a higher growth rate.

The second growth stage, the exponential phase, or log phase, starts once yeast cells start metabolizing actively, DNA replication starts and cells divide. While the cells are

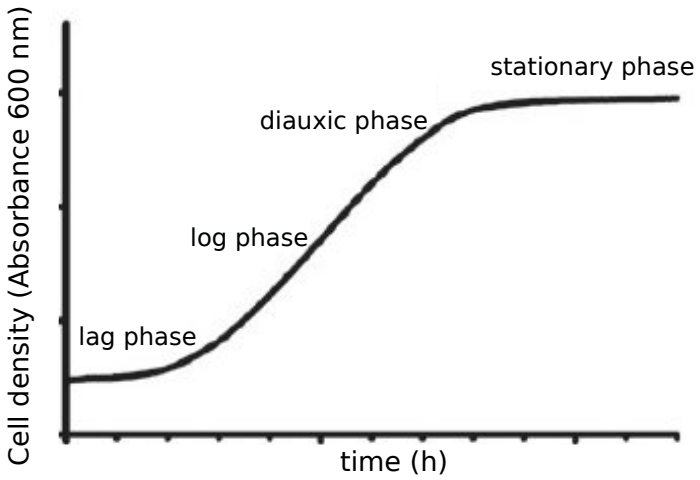


FIGURE 8 A schematic outline of yeast growth phases.

reproducing, nutrients are consumed. During this period yeasts rapidly multiply, reaching the specific maximal growth rate (μmax). Under optimal nutritional conditions, yeasts reproduce asexually by budding; and this generates a daughter cell that is genetically identical to the parental strain. Generation time is the time needed for a population to double its size, it is usually 90-120 min. During this exponential phase, many aromatic compounds which are by-products of cell growth are synthesized.

The third stage is the diauxic phase, a slow growth period. Due to a lack of fermentable carbon sources in the media, yeasts change their fermentative metabolism to a respiratory one in which they metabolize ethanol. The stationary phase is the last stage, which happens when the remaining sugars or nutrients are depleted or when there are growth inhibitors in the media, that prevent yeast cells to continue growing. In this phase, the yeast population reaches maximum density and the yeasts begin to prepare for a possible period of starvation. Yeasts can survive during long periods thanks to modifications in their cell wall and their storage of carbon, but after prolonged periods in the stationary phase, cells may die and autolysate.

One strategy to assess microbial growth data in the media, and be aware of the growing stage of the yeast, consists of taking absorbance measurements of the yeast in the media. Yeast growth can be conducted by taking optical density (OD) measurements at a wavelength of 600 nm: OD_{600}.

This permits the obtainment of kinetic parameters, which are further transformed into variables such as lag time; maximum growth rate, which is the slope of the tangent of exponential phase; and maximum population density, the asymptotic level of OD (Miranda Castilleja et al., 2017).

4.2 Stresses suffered by yeasts during wine fermentation

Fermentative yeasts' main purpose is simply to convert simple sugars into ethanol (Pretorius, 2000). Several environmental factors affect the yeast ability to multiply and ferment in the media. Some of these environmental stresses are the temperature fluctuations, the high osmotic pressure and high sugar initial concentrations, low pH, high ethanol presence, low O_2 in the media, sulfite presence and nutrient starvation, especially nitrogen (Bauer and Pretorius, 2000; García-Ríos, 2016; García-Ríos and Guillamón, 2019b; Marks et al., 2008; Su, 2020). Although they are stressing factors for all microorganisms, *S. cerevisiae* yeasts possess different physiological features to overcome these stresses that made this species very suitable for alcoholic fermentation and explain its competitive advantage over other yeast species. These stress factors, however, can cause ”stuck” and ”sluggish” fermentations. Incomplete or ”stuck” fermentations are defined as those fermentations having a higher levels of residual sugars in the final alcoholic product. The ideal sugar content should be lower than 2-4 g L^{-1}. Slow or ”sluggish” fermentations are those which need a big period of time, or that are delayed, to consume all the sugars present in the initial must (Bisson, 1999; García-Ríos, 2016).

We refer as a stress response to both the physiological and molecular response of

an organism to changes in the environment; while the ability to withstand unfavorable or unstable external conditions is called stress ”resistance” or stress ”tolerance” (Bauer and Pretorius, 2000). It has been studied in several organisms, *S. cerevisiae* included, that their exposure to mild stress results in improved resistance to subsequent exposures to more severe forms of the same stress or other related stresses. This phenomena has been defined as ”cross-protection” or ”acquired stress resistance” and have its basis in the fact that the molecular response to a stress actives pathways that are common to different stresses (Bauer and Pretorius, 2000; Ruis and Schüller, 1995; Siderius and Mager, 1997).

4.2.1 Temperature

Today, most wine fermentations are conducted under temperature-controlled conditions; red wine fermentation is performed at 18-25°C and white and rosé fermentations at 10°C-15°C. This temperature of fermentation directly affects the microorganisms present in the fermentation process, their ability to grow and their metabolism (Fleet, 2003; García-Ríos, 2016). Every living microorganism has an optimal growth temperature. In the case of yeasts, apart from that temperature range, which varies between species and even strains, during fermentations, cells release a significant amount of energy in the form of heat, and every temperature change is perceived as a stress by the cell (Bauer and Pretorius, 2000; Piper, 1997).

Temperature affects yeast biochemical reactions, and as a result, the formation of secondary metabolites such as glycerol, acetic acid, succinic acid, higher alcohols, acetate esters, and ethyl esters, etc (Lafon-Lafourcade, 1983; Torija et al., 2003). These aromatic compounds are essential for the organoleptic profile of wines (Saerens et al., 2010).

Low-temperature fermentation improves the production and retention of these volatile compounds (Ough and Killian, 1979). This leads to the current tendency of conducting fermentations at low temperatures, so that the resulting wines present richer and more

complex aroma profiles acquired during the process. However, *S. cerevisiae* has a higher optimal growth temperature, and at low temperatures the duration of fermentation processes, especially the lag phase, and the risk to stuck increases (Bisson, 1999; Salvadó et al., 2011b).

In particular, low temperatures seriously compromise *S. cerevisiae* wine yeasts, as the composition of the growth substrate, the must, is not optimal, and the high levels of ethanol produced during their growth, mutually affect and amplify cellular sensitivity to both stresses (Bauer and Pretorius, 2000; Deed et al., 2015; Piper, 1995). *S. kudriavzevii* and *S. uvarum* strains are better adapted to grow at low temperatures as a result of enhanced translation, glycolysis and amino acid metabolism (García-Ríos et al., 2016a).

Besides, if we modify the temperature, we have the risk of non-*Saccharomyces* yeast prevalence (Fleet, 2003). From a biotechnological point of view, the application of cryotolerant *Saccharomyces* species, different from *S. cerevisiae* (SNC), as starters for wine fermentation at low temperatures could avoid the colonization by undesirable microorganisms (Alonso-del Real et al., 2017a; Ciani and Comitini, 2006). Previous studies carried out in our research group, have shown that unconventional SNC yeast species, such as *S. kudriavzevii* and *S. uvarum*,are good candidates to use at low temperatures fermentations. They resist lower temperatures, and also produce wines with interesting traits, such as aromatic profiles, high content of glycerol and low content of ethanol (Alonso-del Real et al., 2017b; Arroyo-López et al., 2010a; González et al., 2008; Lopandic et al., 2007; Salvadó et al., 2011a).

During fermentation at low temperatures, cell viability is increased (Beltran et al., 2006; Du et al., 2012). This may be due to the presence of stress-protective compounds that are induced during these conditions, such as heat shock proteins, trehalose, and to changes in the fatty acid and sterol composition of the cell membrane (Beltran et al., 2008; Beney et al., 2001; Deed et al., 2015; Gasch and Werner-Washburne, 2002).

Besides, low temperature rearranges lipid membrane composition, whose fluidity decreases with temperature, and affects the transport of metabolites (Tronchoni et al., 2012, 2009). Recently, genes *AHP1*, *MUP1*, and *URM1* related to low-temperature resistance have been identified (García-Ríos et al., 2016b). Moreover, four genomic regions involved in the adaptation at low temperature were recently described in García-Ríos et al. (2017). Three of these regions are located in subtelomeric regions of chromosomes XIII, XV, and XVI.

4.2.2 High osmotic pressure

At the beginning of wine fermentations, fermentable sugars (glucose and fructose) are present in the must in high concentrations. These musts usually contain 16-26% (w/v) but it may be as high as 50% (w/v) for the production of noble late-harvest or ice wines (Fleet and Heard, 1993; Margalit, 1997). That causes osmotic stress on yeast cells, because they lose intracellular water and turgor (Hohmann, 1997). Some authors have related yeast growth with sugar concentration (Carrasco et al., 2001; Zuzuarregui and Del Olmo, 2004) and in the case of *S. cerevisiae*, if the initial concentration of sugar is above 200 g/L its growth rate and completeness of the fermentation will decay (Lafon-Lafourcade, 1983; Monk and Osmond, 1984). The response of yeast to osmotic stress is regulated by the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway (Chen and Thorner, 2007; Gustin et al., 1998).

4.2.3 Low pH

Grape must acidity is due to its low pH. Natural must have a different composition, that influences its pH, with ranges from 2.75 to 4.20 (Arroyo-López et al., 2009; Belloch et al., 2008). White wines are usually in ranges from 3 to 3.3 and red wines in the range of 3.3-3.6, but there are remarked exceptions depending on the grape variety, climate, region, or viticultural and enological practices. Most *S. cerevisiae* strains grow in a wide

pH range between 2.50 and 8.50, but they grow better under acidic conditions with optimal pH ranges from 4.00 to 6.25 (Carmelo et al., 1996; Liu et al., 2015; Narendranath and Power, 2005).

The ability to grow at low pH depends on temperature, the presence of the oxygen and the strain; but it could be considered common to all species in the *Saccharomyces* group, and consequently, grape must or beer with low pH should not be considered a stress factor for yeasts in alcoholic fermentation (Belloch et al., 2008; Liu et al., 2015; Serra et al., 2005), but it affects other microorganisms growth and prevents contamination. However, a lot of research about low pH or weak acid stress on *S. cerevisiae* has been made (Liu et al., 2015). The cell wall structure can be affected by weak acids, such as acetic acid (Zhao et al., 2014), lactic acid (Abbott et al., 2009), citric acid (Nielsen and Arneborg, 2007), benzoic acid (Hazan et al., 1999) and sorbic acid (Papadimitriou et al., 2007). These acids affect both the conformation of proteins and the lipid organization and function of membranes (Liu et al., 2015; Torija et al., 2003).

4.2.4 Ethanol

Among all the environmental stresses that yeast cells undergo during alcoholic fermentation, ethanol is considered the main one. Ethanol is a toxic compound, that from a physiological point of view, inhibits yeast growth and viability, affects different transport systems such as the general amino acid permease system and glucose uptake, and inhibits the activity of key glycolytic enzymes (Alexandre and Charpentier, 1998; Alexandre et al., 2001; Bisson, 1999).

The main target of ethanol is the plasma membrane, the fluidity of which is altered during ethanol stress even for small concentrations in the order of 1% (Jones and Greenfield, 1987; Lloyd et al., 1993; Marza et al., 2002; Navarro-Tapia et al., 2018). As ethanol has a small size and a hydroxyl group, it is soluble in both aqueous and lipidic

media, being able to cross the plasmatic membrane and increase its fluidity.

This alteration results in changes in permeability to ionic species, especially protons (Cartwright et al., 2009). Moreover, ethanol damages mitochondria, reduces respiratory flux and ATP levels, and leads to the formation of ROS and acetaldehyde, generating DNA damage, lipid peroxidation, and oxidative stress (Alexandre et al., 2001; Du and Takagi, 2007; Costa and Moradas-Ferreira, 2001). Another direct effect caused by ethanol is the inhibition of nutrient transport across the membrane. In this way, glucose, maltose, and ammonia transport system are affected, as well as the general amino acid permease (GAP), due to the alcohol and hydrophobic membrane regions interaction, that finally destabilize all proteins embedded (Leão and Van Uden, 1984).

Yeast cells have developed a panel of stress responses and adaptation mechanisms to cope with the deleterious effects of ethanol. This way, the synthesis of trehalose and heat shock proteins (HSPs) has been reported to occur during ethanol stress (Alexandre et al., 2001; Singer and Lindquist, 1998). Trehalose is considered a stress protectant, and HSPs have been reported to stabilize membranes and proteins and suppress protein aggregation (Singer and Lindquist, 1998). The role of these proteins remains to be fully understood, and it has to be determined whether they play a similar role that those exhibit during heat shock, where they prevent aggregation and assist the posterior refolding of proteins.

Recently, ethanol stress has been directly described as an activator of the unfolded protein response UPR, a conserved intracellular signaling pathway that regulates the transcription of ER homeostasis-related genes (Navarro-Tapia et al., 2016, 2017). These authors observed up-regulation of key genes, including *INO1*, involved in lipid metabolism and also significant changes in lipid composition, which correlate with major alterations of membrane fluidity by this amphipathic molecule (Navarro-Tapia et al., 2018).

4.2.5 Low oxygen levels

Oxygen is a structural component in numerous organic molecules (Visser et al., 1990). During alcoholic fermentation, *Saccharomyces* yeasts do not strictly need oxygen for their energy production, but it is an essential compound for its efficient growth, mainly in the early fermentation hours. The addition of oxygen at the beginning of fermentation prevents stuck or sluggish fermentations, as yeasts grow better with a small quantity of oxygen that generates survival factors. Moreover, aeration during specific fermentation phases has beneficial effects on fermentation kinetics (Fleet and Heard, 1993; Ribereau-Gayon et al., 2000). The presence of oxygen is also relevant to increase yeast ethanol resistance, as oxygen is needed to generate the unsaturated fatty acids and ergosterol present in yeast membranes, which better resist the high levels of ethanol (Alexandre et al., 1994; Bauer and Pretorius, 2000).

4.2.6 Sulfite concentration

Sulfite (SO_3^{2-}) is a normal but potentially toxic intermediate metabolite of microorganisms. It is widely used as a preservative in wine-making because yeasts can still grow normally (Divol et al., 2012) under high sulfite concentrations. Sulfite is produced by the dissolution of sulfur dioxide (SO_2) in water, and it is usually added in wine fermentation in the form of metabisulfite ($K_2S_2O_5$). Yeast can cope with sulfite toxicity through different strategies- for a review see (García-Ríos and Guillamón, 2019a). Among them, we can cite the increase of acetaldehyde production, up-regulation of sulfate uptake and assimilation pathway, and sulfite efflux from the cell by the membrane pump Ssu1p (Casalone et al., 1992; Nadai et al., 2016; Park and Bakalinsky, 2000). The most common mechanism to cope with sulfites in *S. cerevisiae* is the latter one, via promoting the sulfite efflux through the plasma membrane pump encoded by the *SSU1* gene (Avram and Bakalinsky, 1997; Avram et al., 1999).

The presence of sulfite in must wines is also interesting, as it forms complexes with aldehydes and ketones generating hydrogen sulfide and mercaptan, aromatic ingredients which improve the stability of flavor and the quality of wine products (Liu et al., 2017).

4.2.7 Nutrient starvation

Another stressing factor for yeasts is the limitation or lack of certain nutrients, especially in wine fermentations, where the phosphate limitation (Boulton et al., 1999), zinc starvation (Lyons et al., 2002), copper starvation (Gross et al., 2000) and nitrogen starvation (Sui et al., 2020) has provoked fermentation problems. Of all of these nutrients, nitrogen is the main limiting nutrient during wine fermentation. Although it is naturally present in the wine must, changes in the availability of specific nitrogen-containing compounds often represent a stress for yeasts.

4.2.8 Nitrogen composition

Nitrogen is an essential nutrient in alcoholic fermentation (Agenbach, 1977; Cramer et al., 2002). Grape musts contain different nitrogen sources such as ammonium ions, amino acids, and peptides, but not all of them can be metabolized under fermentation conditions, and yeasts do not prefer all of these nitrogen sources equally (Tesnière et al., 2015). The utilization of nitrogen-containing compounds by *S. cerevisiae* follows a complex, relatively well-established pattern during wine fermentation, and although it depends on the yeast strain, in general, a minimum of 140 mg/L of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998).

Although wine yeasts can metabolize more than 20 substances as unique sources of nitrogen (Tesnière et al., 2015), *S. cerevisiae* starts metabolizing preferentially aspartate, glutamate, glutamine, and ammonium, while their presence in the media represses the uptake of other, less efficient nitrogen sources, an effect known like nitrogen catabolite

repression (NCR) (Tesnière et al., 2015). Once these sources have been depleted, other nitrogen compounds like proline and other amino acids will be utilized (Cooper, 1982*a*,*b*). These compounds are mainly derived from the nitrogen of the amino acid alpha amines (Henschke and Jiranek, 1993) and ammonium. When yeast cells have to switch from a preferred nitrogen source to another compound because of the availability, they experience mild stress, that in laboratory conditions, results in a transient reduction in growth rate. On the other hand, in later phases of fermentation, nitrogen starvation could become a real problem, as it compromises both the fermentation kinetics and the formation of yeast metabolites. In most cases, nitrogen starvation is a consequence of the presence of ethanol, which inhibits the uptake (Boulton et al., 1999).

Recently, Su et al. (2019) demonstrated the existence of a differential behavior in nitrogen requirements among strains of the cryotolerant species *S. uvarum*, *S. eubayanus*, and *S. kudriavzevii*. Another recent review has focused on the consequences of nitrogen addition and its effect on volatile compound composition (Gobert et al., 2019).

5. Current wine market challenges and the use of tailored yeast starters

5.1 Wine industry problems, demands, and trends

The wine industry is facing different challenges related to both the market demand and the production process. The number of wine drinkers has declined due to a variety of health and lifestyle reasons. Nowadays, there are diverse and interesting offers in spirits, beer and cider, and consumers are more exigent with wine characteristics and quality. For instance, sweeter wines and fruity aromas fit better with young people's preferences. There is also an increasing consciousness about the effects of alcohol uptake on health as

well as road safety. Therefore, the wine industry must respond to these trends producing aromatic wines with lower ethanol content.

Moreover, the composition and properties of the grape have varied due to climate change (Borneman et al., 2013). The vast majority of the scientific community agrees on the reality of climate change caused by human activities (IPCC, 2014).

Among human activities, agriculture -and in particular viticulture- is highly dependent upon climatic conditions. Global warming and climate change make it more difficult to identify the point of enological maturity in vineyards. This enological maturity corresponds to the optimum harvesting moment of the grapevine which permits the production of the best wine in a given year and under specific conditions. This depends on three factors: industrial maturity, aromatic ripeness, and phenolic ripeness (Querol et al., 2018).

Industrial maturity depends on the sugar content and acidity of the starting must, which determines the final ethanol concentration in the wine. Phenolic ripeness depends on polyphenolic compound concentration, which gives color and astringency to the wine and aromatic aromas.

Higher temperatures accelerate the sugar maturity of grapes, lower the grape acidity- particularly the malic acid content- and disrupt phenolic maturity, thus, provoking an unbalance between these two factors (Jones et al., 2005; Mozell and Thachn, 2014; van Leeuwen and Darriet, 2016). In addition, high temperatures negatively affect relevant secondary metabolites involved in red wine color, like anthocyanins (Spayd et al., 2002). Thus, an imbalance between sugar content and phenolic maturity is present in grapevines, and a correct enological maturity point cannot be reached (Jones et al., 2005; Querol et al., 2018).

If wineries wait until the phenolic maturity is achieved in grapes before harvesting, the higher amount of sugars in the fruit results in the overproduction of ethanol in the final

wine. Thus, winemakers are forced to produce more alcoholic wines, with a decrease in the color intensity and stability and a different aroma profile. However, if the grapes are harvested earlier, when sugar content is optimum to prevent the overproduction of ethanol, the grape tannins and phenols have not reached their optimal maturation state, which results in astringent wines (Querol et al., 2018).

Consumers tend to dislike these wines -both the ones with high ethanol content and the ones with unripe tannins- (Querol et al., 2018). To avoid a lack of competitiveness in the wine sector, the industry is adopting different solutions. One of them is the use of yeasts whose metabolism produces lower ethanol and higher glycerol yields, as this combination of compounds balances wine astringency (Querol et al., 2018; White et al., 2006).

5.2 Wine strain selection

At present, most wine-producing companies add a pure *Saccharomyces* yeast strain to the must, also known as a starter cultures, to have a reproducible fermentation process and to maintain a high final product quality (González et al., 2011; Querol et al., 2018). To provide suitable yeast strains for specific industrial processes, as wine production, many strategies have been carried out, being the selection of strains the most used as it is the simplest one.

S. cerevisiae is the preferred yeast strain to initiate the fermentation process (Jolly et al., 2014), due to its high fermentation performance and ethanol tolerance. “Ethanol tolerance” is a term that is frequently used in the literature referring to the ability of yeasts to grow and survive in the presence of ethanol and ethanol resistance is a term that is commonly used as a synonym of ethanol tolerance (Morard et al., 2019; Snoek et al., 2016).

Different *S. cerevisiae* strains are known, and it is possible to characterize on a laboratory scale the differences among them for the desired trait to then use the selected

strain as a starter for the industrial process. Yeasts are a group of organisms with high diversity, and besides the differences among species, even strains of the same species show a high level of genetic divergence and different industrial behaviors (Steensels et al., 2014b).

For example, related to ethanol resistance, it is possible to quantitatively estimate the ethanol tolerance of a set of *S. cerevisiae* strains by growing the yeast under controlled amounts of this stressor, and then model the growing curves to obtain two parameters: MIC and NIC. MIC is considered the lowest concentration at which no growth is observed, while NIC is the lowest concentration at which any inhibitory effect is observed (Miranda Castilleja et al., 2017). This strategy can be followed to select strains with better performance under other stressors, as can be drugs, SO_2, etc. (Medina et al., 2012; Miranda-Castilleja et al., 2015; Sánchez-Rubio et al., 2017; Türkkan and Erper, 2014).

However, in recent years, stress tolerance is not the only factor that should be taken into account to choose a yeast strain as a starter for carrying out a fermentative process. This way, curbing wine ethanol content and enhancing aromas in wines is highly desirable, and this is possible with the usage of yeasts with a different metabolism that permits the generation of the compounds of interest. A lot of research in the use of alternative starters for winemaking has been made, as fungal diversity is high and the current industrial strains are only representing a small fraction of the natural biodiversity available (Steensels et al., 2014b; Tilloy et al., 2015). Moreover, it is possible to use different species to *S. cerevisiae*. These yeasts can be either *Saccharomyces* or non-*Saccharomyces* species with oenological properties, which can be selected to conduct the wine fermentation.

5.2.1 The selection of non-*Saccharomyces* yeasts

Under spontaneously fermenting wine, a succession of non-*Saccharomyces* yeasts of *Candida*, *Cryptococcus*, *Hanseniaspora (Kloeckera)*, *Metschnikowia*, *Pichia* and

Rhodotorula genera, are present in the fermentation (Jolly et al., 2003, 2014). However, as natural *S. cerevisiae* strains have a higher tolerance to the fermentation stresses, they dominate the middle and end of the fermentation (Bagheri et al., 2015; Ghosh et al., 2015; Jolly et al., 2003; Portillo and Mas, 2016). *Saccharomyces* strains quickly outcompete non-*Saccharomyces* species and so non-*Saccharomyces* contribution to final wine flavor is low (Bellon et al., 2011).

For this reason, industrial wineries are interested in the co-inoculation or sequential inoculation of these non-*Saccharomyces* species with one or more wine strains of *S. cerevisiae*. non-*Saccharomyces* species have a different respire-fermentative metabolism and Crabtree effect distribution, which allows them to reduce the final content in ethanol (González et al., 2013). They also contribute to wine flavors, secreting metabolites with impact in the primary and secondary aroma of wines, glycerol production, release of mannoproteins, low volatile acidity, or contributions to wine color stability (Bely et al., 2008; Canonico et al., 2016; Goold et al., 2017; Varela, 2016). Moreover, several interactions between yeast species result in different yeast population dynamics during fermentation (Rossouw et al., 2015). Finally, it is known that the sensory profile of a fermentation product varies if a combination of *S. cerevisiae* and non-*Saccharomyces* yeasts is used (Canonico et al., 2015; Varela, 2016).

Oliveira and Ferreira (2019) proved that the sequential inoculation of non-*Saccharomyces* yeasts (*Pichia kluyveri*, *Torulaspora delbrueckii* and *Lachancea thermotolerans*) followed by *S. cerevisiae* produced wines with aromatic changes, such as lower levels of isoamyl alcohol, etc. In González-Royo et al. (2015), the sequential inoculation of *T. delbrueckii* and *Saccharomyces cerevisiae* increased glycerol concentration and reduced volatile acidity among other interesting properties.

Non-*Saccharomyces* yeast species usage needs to be more investigated because although they reduce the ethanol yield in favor of biomass production and by-product

formation, because their compatibility with *S. cerevisiae* needs to be determined (Contreras et al., 2014; Esteve-Zarzoso et al., 1998; Gobbi et al., 2014).

5.2.2 *Saccharomyces* non-*cerevisiae* yeast selection based on growth at low temperature

One of the trends in enology is to conduct low-temperature fermentation, as wines produced at low temperatures more efficiently keep volatile aroma compounds and final wines have better sensory attributes. If *S. cerevisiae* strains are used in low-temperature fermentation, their growth rate is reduced and the risk of stuck or sluggish fermentations is high (López-Malo et al., 2013).

Different studies have been performed to understand *S. kudriavzevii* and *S. uvarum* behavior during fermentation. Tronchoni et al. (2012) determined that 3 *S. kudriavzevii* strains (CR85, CA111 and IFO1802) required less time than the T73 *S. cerevisiae* strain to consume sugars at 12ºC wine fermentations. These authors also studied the membrane composition of this yeast species, as it can confer a better adaptation to low temperature. Moreover, *S. kudriavzevii* produces less alcohol than *S. cerevisiae* (Torija et al., 2003).

S. uvarum behavior has also been analyzed at low-temperature fermentations. In another study, *S. uvarum* strains showed a shorter lag phase and the ability to complete alcoholic fermentation at 13ºC when compared with *S. cerevisiae* (Masneuf-Pomarède et al., 2010).

5.2.3 *Saccharomyces* non-*cerevisiae* yeast selection based on high glycerol production and low ethanol yield

Other winemaking trend that can be achieved by the use of *Saccharomyces* non-*cerevisiae* yeasts is the production of wines with higher glycerol yields and lower ethanol yields. Glycerol is a compound that contributes to wine quality because it provides sweetness, smoothness and fullness to wine, while reducing wine astringency (Goold

et al., 2017; Remize et al., 2000). It is the third by-product of alcoholic fermentation, after ethanol and carbon dioxide, in quantitative terms. The quantity of glycerol produced by yeasts is highly dependent on the environment, and its production can be optimized by using specific cultivation conditions. (Arroyo-López et al., 2010a).

Glycerol seems to play also an important role in low-temperature tolerance as a cryoprotectant agent in yeasts (Izawa et al., 2004), as it gives resistance to osmotic and cold stress. It has been reported that cryotolerant wine strains produce more glycerol than non- cryotolerant yeasts (Bertolini et al., 1996; Castellari et al., 1994). Under stressful conditions such as low temperature, low pH, and high sugar concentration, carbon flux is directed towards glycerol instead of ethanol (Arroyo-López et al., 2010a) and this effect is more remarkable in *S. uvarum* and *S. kudriavzevii*.

Arroyo-López et al. (2010a) observed that the *S. kudriavzevii* type strain (IFO 1802) produces higher glycerol concentrations under fermentation at low temperature (14ºC) when compared with a wine *S. cerevisiae* strain (T73). Pérez-Torrado et al. (2016) also proposed that the reason for higher glycerol content in wines produce by *S. kudriavzevii* is the differentiated import/efflux capacity under hyperosmotic stress.

In the presence of sulfite, the fermentation of glucose by yeasts produces equivalent quantities of glycerol, carbon dioxide, and acetaldehyde in its bisulfite form. This is called glyceropyruvic fermentation (Ribereau-Gayon et al., 2006). In this kind of fermentation, glycerol has an important role in keeping redox balance in the cell oxidizing NADH to NAD^+ (Hohmann, 1997). Since the acetaldehyde combined with sulfite cannot be reduced into ethanol, dihydroxyacetone phosphate (DHAP) becomes the terminal electron acceptor instead (Figure 9).

Thus, glycerol is synthesized from DHAP in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (GPDH), which reduces DHAP to

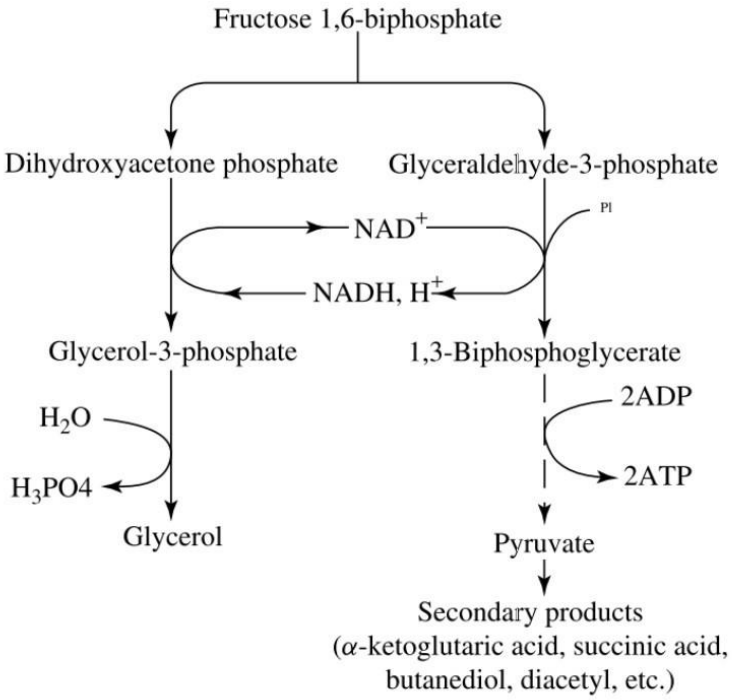


FIGURE 9 Glyceropyruvic fermentation (Ribereau-Gayon et al., 2006)

glycerol-3-phosphate and this to glycerol by the glycerol-3-phosphatase (GPP). The rate-controlling step of glycerol production is GPDH (Hohmann, 1997). This enzyme exists as two isoenzymes, Gpd1p, which is osmotically induced, and Gpd2p, which is constitutive and plays a key role in maintaining the NADH/ NAD+ ratio (Ansell et al., 1997; Remize et al., 2001).

A low ethanol yield in the final wine can be achieved using different strategies. In previous studies, it has been demonstrated that the combination of sulfite with acetaldehyde prevents acetaldehyde from being reduced to ethanol (Albertyn et al., 2015; Ansell et al., 1997; Remize et al., 2001). This reduces glycolytic flux due to a shortage of NAD^+ that would have been produced during ethanol fermentation, which can be restored by redirecting carbon to dihydroxyacetone-1-phosphate (DHAP), which becomes electron acceptor of NADH produced during glycolysis, regenerating NAD^+ (Goold et al., 2017; Petrovska et al., 1999; Tilloy et al., 2015) and producing glycerol instead of ethanol. All this knowledge is necessary to further produce tailored yeasts with these physiological

differences.

5.3 Strategies to improve yeast strains

In the previous sections, we have commented on the use of some alternative yeasts to conduct wine fermentations. However, industrial fermentations sometimes require strains with phenotypic traits that might not be encountered in nature. Therefore, to fulfill the selective and specific conditions of each industrial process, several techniques have been developed to improve yeast's behavior (Steensels et al., 2014b). This way, there is the possibility to generate artificial diversity in yeasts, using different methods. Current legislation in different countries limit the use of Genetically Modified Organisms (GMOs) in food (Álvarez-Pérez et al., 2014; Jolly et al., 2014; Lambert and Pearson, 2000). According to the definition of the Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001, GMO means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

Apart from the general and strict legislation on GMOs, consumers have also concerned in their use in the wine industry (Cebollero et al., 2007). For that reason, we will first focus on non-GMO techniques to create artificial diversity in yeasts.

5.3.1 Artificial hybridization

Hybridization among closely related species of *Saccharomyces* yeasts has been proposed as a good method for obtaining new *Saccharomyces* strains that are suitable for its use under enological conditions (Pérez-Través, 2015). This practice is similar to 'selective breeding' or 'artificial selection' that has been used in agriculture and animal breeding for thousands of years for the crossbreed of superior plants and domestic animals (Chambers et al., 2009; Steensels et al., 2012, 2014a). Similarly, human intervention may have given rise to new chimeric *Saccharomyces* strains in industrial environments where

two *Saccharomyces* species with interesting attributes, were put non-intentionally together and eventually crossed. Although these processes occurred naturally, recent knowledge on yeast physiology and technological advances have led to the targeted breeding of yeast strain by hybridization (Steensels et al., 2014b). Since then, many works report the successful hybridization of yeast strains, some of them are summarized in Table 1.

TABLE 1 List of artificial hybrids obtained among *Saccharomyces* species

Species combination	Hybrid phenotype	Reference
S. cerevisiae x *S. uvarum*	Ability to perform low-temperature fermentations; higher production of flavor compounds in wine	Kishimoto (1994)
S. cerevisiae x *S. bayanus*	Greater fermentative vigor; wider temperature range; intermediate compound production	Zambonelli et al. (1997)
S. cerevisiae × *S. uvarum*	Low-temperature fermentation capacity	García-Ríos et al. (2019c)
S. cerevisiae x *S. uvarum*; *S. cerevisiae* x *S. kudriavzevii*	Increased ethanol and glycerol production and better sugar consumption than their parental strains	Lopandic et al. (2016)
S. cerevisiae x *S. uvarum*	Low ethanol production, high glycerol synthesis, growth at low temperature; malic acid production with a particular aroma profile	Origone et al. (2018)
S. cerevisiae × *S. kudriavzevii*		Pérez-Través et al. (2012)
S. cerevisiae × *S. kudriavzevii*		Ortiz-Tovar (2018)
S. cerevisiae x *S. paradoxus*; *S. cerevisiae* x *S. kudriavzevii*	Different volatile fermentation product profiles	Bellon et al. (2011)
S. cerevisiae x *S. eubayanus*	Improved low-temperature fermentation and fruitier cider production	Magalhães et al. (2017)
S. cerevisiae × *S. uvarum*	Different secondary metabolite production profiles	Da Silva et al. (2015)
S. cerevisiae x *S. arboricola* *S. cerevisiae* x *S. eubayanus* *S. cerevisiae* x *S. mikatae* *S. cerevisiae* x *S. uvarum*	Improved low-temperature fermentation in lager brewing conditions	Nikulin et al. (2018)
S. cerevisiae × *S. bayanus*	Improved low-temperature fermentation	Sato et al. (2002)

Hybrids often show heterosis or hybrid vigor, which refers to the common superiority of hybrids over their parents for quantitative traits (Petrizzelli et al., 2019). The mainly used methodologies to obtain these artificial hybrids are rare mating and spore to spore mating. Spore to spore mating has been widely used, but it is a time-consuming method; whereas rare-mating is an easier methodology to generate these hybrids (Cebollero et al., 2007; Pretorius and Hoj, 2005; Schilter and Constable, 2002). Although there are other methods as protoplasts fusion to generate hybrids, they are considered GMO strategies and so the resulting hybrids cannot be transferred to the industry.

5.3.2 Rare mating

The rare mating technique is based on the natural rare event of mating-type switching in industrial yeasts, which are normally diploids. This results in the occurrence of mating cells at a low frequency, that can then conjugate with a known laboratory mating strain of either *a*, *aa*, *α* or *αα* mating type (Pérez-Través et al., 2012).

The first step to conduct a rare mating strategy is to select two strains (the ones with the properties of interest that we want to merge in the hybrid) that carry different auxotrophic markers. Auxotrophy is defined as the inability of an organism to synthesize a particular organic compound required for its growth. The selection of natural auxotrophic parental strains can be done by seeding onto plates with a selective agent that only allows the growth of a strain if they have a mutant genotype. For example, α-aminoadipic (α-AA) plates are used to select lys− auxotrophs and fluoroorotic acid (5-FOA) agar plate for ura3− auxotrophs (Spencer and Spencer, 1996).

Then, auxotrophs are placed together to let switching mating type and subsequent hybridization occur. After this incubation, the culture is spread on Minimal Media (MM) plates. If prototrophic colonies grow in this minimal media, they are isolated and purified to check their hybrid nature. If hybrids are obtained, their phenotypes are assessed to see

if the obtained hybrid has improved the characteristics of their parental strains.

Over the years, many natural interspecific *Saccharomyces* hybrids have been isolated from wine-related habitats (Boeke et al., 1987; Zaret and Sherman, 1985), and these hybrids show intermediate characteristics from both their parental strains that make them suitable for wineries demands. When hybrids are obtained by rare mating they often contain the complete genome of both parents (González et al., 2007; Lopandic et al., 2007; Pérez-Través et al., 2015; Peris et al., 2016).

However, the newly formed hybrids sometimes experiment a genome reduction and rearrangements in their genomes. If hybrids are going to be used in industrial processes, their genomes need to be stable, to ensure that the same strain is always used and the produced wines are consistent in successive vintages (Gunge and Nakatomi, 1972; Krogerus et al., 2016).

Some studies have addressed how to generate stable hybrids through a stabilization process (Pretorius, 2000). A major aspect of hybrids obtainment is the careful selection of stabilization conditions. The stabilization process consists on inoculate the obtained hybrids in media and at the end of the fermentation in that media characterize different obtained colonies.

It has also been proved that the use of selective pressure, mimicking the unfavorable conditions found in industrial environments, can be imposed during the stabilization. Sporulation of hybrids has also been applied as a stabilization method that accelerates the genome reduction process (Belloch et al., 1997, 1998; Fernandez-Espinar et al., 2003; Querol et al., 1992).

To check that the hybrid genomes are stable after the stabilization process, different characterization methods can be used to compare the profile present in the hybrid before and after the stabilization. Some of them are inter-δ sequences, random amplified

polymorphic DNA–PCR (RAPD–PCR) analyses and mtDNA-restriction fragment length polymorphism (mtDNA-RFLP) patterns (Antunovics et al., 2005; Bellon et al., 2013; Pérez-Través et al., 2012, 2014b). After the genetic stabilization of the hybrids, they are assessed for their industrial applicability on a laboratory scale.

5.3.3 Adaptive laboratory evolution

Another scientific approach towards the improvement of yeast strains is the use of adaptive laboratory evolution (also known as directed evolution or ALE). Darwin's theory of evolution describes how species change over time through variation and selection. Classical evolutionary theory says that genetic variation is the major source of heritable variation and natural selection acts on this basis (Bódi et al., 2017). This way, if a heterogeneous big population is under fluctuating or stressful conditions, only the individuals with the better traits will reproduce.

Adaptive evolution is based on a long-term adaptation of yeast under environmental or metabolic constraints, that finally lead to evolution. This strategy is useful for microorganisms as they can rapidly adapt to different environmental conditions. During microbial ALE, a microorganism is cultivated for a prolonged period which allows the selection of the improved phenotypes, under clearly defined conditions (Dragosits and Mattanovich, 2013).

ALE has been used to improve yeast strains for biotechnological applications, including wine making (Çakar et al., 2005; McBryde et al., 2006; Stanley et al., 2010c; Wisselink et al., 2009). Some examples of evolved strains to improved different traits can be seen in Table 2. Among them, and due to their potential application in the wine industry, we can highlight the obtainment of a *S. cerevisiae* yeast that enhances their parental glycerol production by 41% (Kutyna et al., 2012), and the evolution of a *S. cerevisiae* yeast that improved the growth of the parental strain at low temperatures (López-Malo et al., 2015).

TABLE 2 List of strains evolved in the laboratory belonging to *Saccharomyces* species

Evolved species	Evolved trait	Reference
S. cerevisiae	Oxidative stress, ethanol stress, heat stress, freezing/thawing stress	Çakar et al. (2005)
S. cerevisiae	Winelike fermentation stresses	McBryde et al. (2006)
S. cerevisiae	Ethanol stress	Stanley et al. (2010c)
S. cerevisiae	Glycerol production	Kutyna et al. (2012)
S. cerevisiae	Consumption of xylose and arabinose	Wisselink et al. (2009)
S. cerevisiae	Consumption of gluconate	Cadière et al. (2011)
S. cerevisiae	Fermentation at low temperatures	López-Malo et al. (2015)
S. cerevisiae	Ethanol	Voordeckers et al. (2015a)
S. cerevisiae	Heat, high pH	Yona et al. (2012)
S. cerevisiae x *S. eubayanus*	Lager-brewing conditions	Gorter de Vries et al. (2019)
S. cerevisiae, *S. paradoxus*, *S. mikatae*, *S. uvarum*, and *S. uvarum x S. cerevisiae*	Growth on media with sulfate limitation	Sanchez et al. (2017)
S. cerevisiae	Methanol assimilation	Espinosa et al. (2020)
S. cerevisiae	Growth on high temperature	García-Ríos et al. (2021)

One common method to perform ALE is the use of batch cultivation in shake flasks that propagate microbial cells (Dragosits and Mattanovich, 2013) in controlled environmental conditions and factors like temperature and spatial culture homogeneity. At regular intervals, an aliquot of the culture is transferred to a new flask with a fresh medium. This strategy allows massive parallel cultures on a cheap equipment. Shake flasks can be replaced with systems with well plates with smaller culture volumes, thus allowing the growth of hundreds of microbial cultures in parallel (Chambers et al., 2009; González et al., 2013).

During the ALE process, a new population mainly composed of cells with beneficial

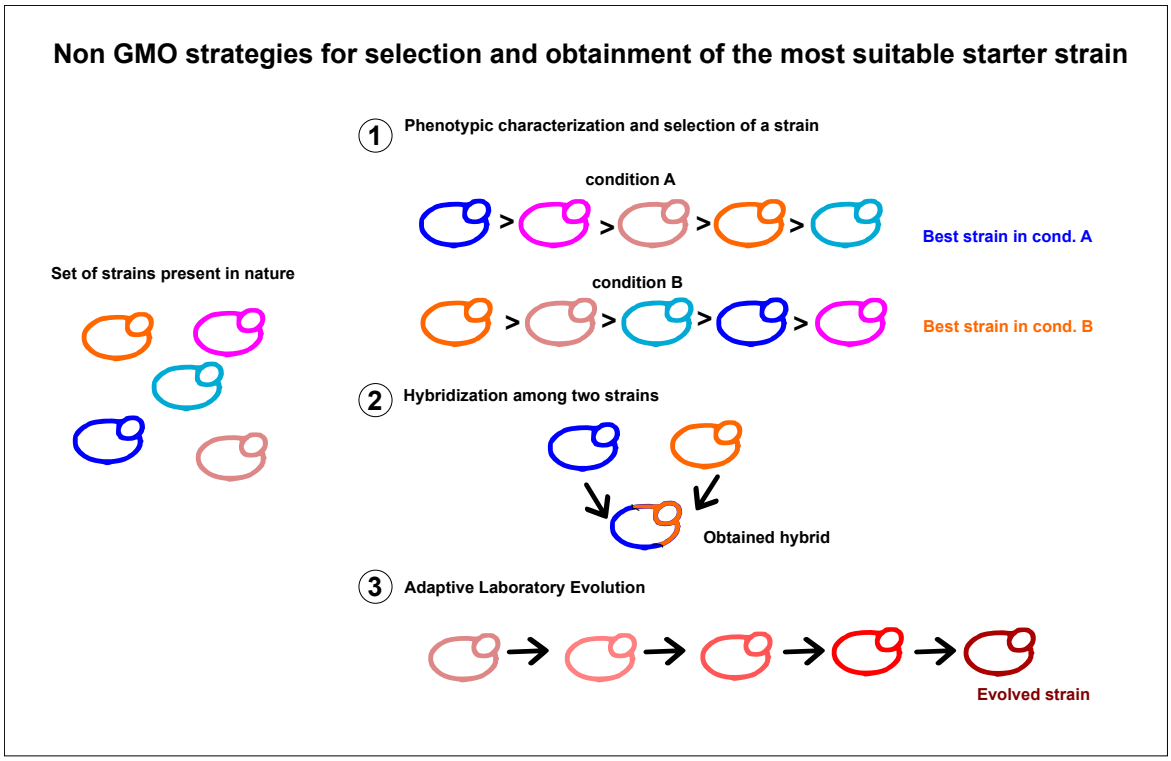


FIGURE 10 Strategies to select and obtain strains for industrial applications.

mutations can be obtained. Multiple genetic characteristics that naturally occur in yeast strains are selected and, as a consequence, mutations in the evolved cells can be observed in the form of SNP, InDels, large deletions and duplications, translocations and changes in ploidy.

A summary of the three strategies which are most frequently used for strain selection and obtainment can be seen in Figure 10.

6. Omics technologies

Currently, new techniques are available to study in-depth the genome, transcriptome, proteome and metabolome of a strain of particular interest in defined conditions. These methods aim to globally characterize a biological sample at a certain level (genes in genomics, mRNA in transcriptomics, proteins in proteomics and metabolites in

metabolomics). Metabolomics, at the same time, encompasses more specific techniques such as lipidomics, which studies the lipids present in a biological sample. All of all these techniques, and the integration of them from a systems biology perspective are known as 'Omic' technologies (Delneri et al., 2001; García-Ríos, 2016; Horgan and Kenny, 2011; Kitano, 2002; Oliver, 2002; Oliver et al., 2002; Paget et al., 2014; Petranovic and Nielsen, 2008).

The functional genomics field comprises different techniques that allow the biology study at different levels. It is important to differentiate between studies at the genome level, as genomic DNA of a particular strain is condition independent; from gene expression studies at the level of mRNA, or proteomics and metabolomics studies at protein or metabolite levels, as they are strongly dependent on environmental conditions and growth phase (Horgan and Kenny, 2011). Thus, comparative analyses of strains at these levels have to be carried out under carefully defined conditions (Saerens et al., 2010).

For that reason, using genomics to identify the different genes present in an organism with its genome sequenced, would be the first approach to have an initial idea of the genome composition and evolutionary story of that strain. Then, to elucidate the roles that play those genes, it is necessary to apply a functional study that involves transcriptomics, proteomics, or metabolomics. These three classes of functional genomic analysis are distinct. Messenger RNA molecules, the subject of transcriptome analysis, are not functional entities within the cell, but simply transmitters of the instructions for synthesizing proteins, and so transcriptome analyses only indirectly approach functionality, while both proteins and metabolites represent true functional entities within cells (Delneri et al., 2001). However, global gene expression analysis at the level of proteins (proteomics) is more laborious, less sensitive, and less reproducible than transcriptomics, as the sequencing technologies available for both genome and transcriptome understanding are well implemented and are easy to follow.

6.1 Sequencing technologies

Since two decades from now, we are experiencing a “genomic revolution” that has provided new knowledge in science. The advent of genome and transcriptome sequencing has enabled the studies of a wide variety of yeast strains and species. They are widely used as functional genomics tools that provide information about genes, their function and mechanisms of regulation, and which role play them on biology at different levels.

The first available sequencing method was the Sanger technology (Sanger et al., 1977), which sequences a single DNA fragment at a time. It uses dideoxynucleotides to terminate the chain amplification.

The advent of next-generation sequencing technologies (NGS) marked the start of a genetic and genomic revolution (Giordano et al., 2017). NGS significantly lowered the cost of sequencing using massively parallel sequencing methods (Goodwin et al., 2016; Liu et al., 2014). Two major paradigms are present in next-generation sequencing (NGS) technology: short-read and long-read sequencing (Goodwin et al., 2016). Short-read sequencing approaches provide accurate data at a low cost. The typical length of the generated fragments is between 50 and 400 bases long (Goodwin et al., 2016).

The first commercially successful next-generation system was Roche 454, which used pyrosequencing technology, that uses the detection of pyrophosphate released during nucleotide incorporation. Another next-generation sequencing system is AB SOLiD (Sequencing by Oligo Ligation Detection) (Mardis, 2008). It uses 8 base-probe ligations that complement the template strand and emit a fluorescent signal.

The last next-generation sequencing system, which is nowadays the most commonly used, is the Illumina GA/HiSeq System. Illumina uses a sequencing by synthesis (SBS) technology which consists of the addition of labeled nucleotides as the DNA chain is copied in a massively parallel approach that results in less time-consuming. Depending on the

study, Illumina reads can be either used in *de novo* assembly projects, for the assembly of an organism without using a reference or in resequencing projects for the detection of variants by mapping the Illumina reads from a strain onto a reference genome from the same species (Wolfe et al., 2019).

The problem in NGS technologies that generate short reads is that these reads are not able to solve complex genome features like highly repetitive regions longer than sequenced reads or copy number variations (Giordano et al., 2017). To solve this, long-read sequencing technologies have been developed. Pacific Biosciences and Oxford Nanopore MinION technologies produce long sequencing reads with average fragment lengths of over 10 000 base-pairs. These long fragments, which can reach the 100 000 base-pair, allow the obtainment of complete genomes with contig continuity even in problematic and repetitive regions. Their major drawbacks are the higher rate of sequencing errors (5–20%), their lower high-throughput, and their higher price (Giordano et al., 2017), but these issues are being solved, especially the error rate.

These sequencing technologies can be used with a large number of organisms. Yeast genomes are relatively small and easy to characterize by using these sequencing technologies. After the obtainment of this data, the different bioinformatic analyses need to be performed on them.

Objectives

In recent decades, the wine-producing sector in Spain has experienced important growth, becoming the world's leading exporter. Unfortunately, this has not been translated into a higher economic benefit, mainly due to the low average price of exported wine and a reduction in domestic consumption. To maintain competitiveness and consolidate its international market, the sector must take steps to adapt to both new market demands and the challenges imposed by climate change. On one hand, consumers demand new products with lower alcohol content and with more fruity aromas. On the other, climate change entails changes in the characteristics of the grape must (acidity, sugar or tannin content, etc.) that affect the quality of the final product.

Previous projects have shown that non-conventional *Saccharomyces* species, such as *S. uvarum* and *S. kudriavzevii*, seem to be good candidates to achieve such objectives: they exhibit good fermentation properties at low temperatures and produce wines with lower alcohol and higher glycerol content than *S. cerevisiae* and result in a good aromatic profile. Despite their potential, these species cannot compete on an industrial level with *S. cerevisiae*, which has greater resistance to ethanol and the ability to ferment at higher temperatures.

The main demand within the oenological sector is to select and use yeast strains which

perform well and resist to high-stress conditions that yeasts have to face during industrial processes. Wine fermentations are carried out under high ethanol concentrations, which are toxic to yeasts. The first target of this stressful situation is the plasma membrane of the cell because it acts as a barrier between the external environment and the inside of the cell.

Accordingly, in the present thesis, I focus on characterizing and improving different *Saccharomyces* yeast strains that have interesting physiological characteristics, suitable for their use in the wine industry. The global objective is to provide the market with yeast strains that both fulfill the sector demands; especially high ethanol tolerant yeasts for the industrial processes and consumers' demands: yeasts that produce final wines with a lower ethanol and higher glycerol contents and good aroma profiles.

This global aim has been subdivided into five partial objectives:

1) Physiological characterization of *S. cerevisiae* strains from different origins. Study of their ethanol resistance and membrane composition.
2) Transcriptome analysis of three selected *S. cerevisiae* strains with different ethanol tolerances
3) Improving the ethanol tolerance of a *S. uvarum* strain by obtaining a *Saccharomyces cerevisiae* x *S. uvarum* hybrid. Characterization, genomic and transcriptional analysis of this artificial hybrid and their parental strains.
4) Study of the *Saccharomyces cerevisiae* x *S. uvarum* artificial hybrid adaptation to a must media similar to that present in wine fermentation at advanced stages: high ethanol, high sulfites, and low sugar concentrations. Analysis of the genome, membrane composition, and transcriptome of the adapted hybrid.
5) Improvement of ethanol tolerance through adaptive evolution in the laboratory of different strains of *S. uvarum* and *S. kudriavzevii*. Study of its genome and

composition of the membrane.

This thesis is organized into 5 chapters:

1) Analysis of lipid composition reveals mechanisms of ethanol tolerance in the model yeast *Saccharomyces cerevisiae*.
2) Transcriptome analysis in *S. cerevisiae* strains under ethanol stress reveals different specific responses related to the synthesis of membrane lipids.
3) Differential contribution of the parental genomes to a *S. cerevisiae* × *S. uvarum* hybrid, inferred by phenomic, genomic, and transcriptomic analyses, at different industrial stress conditions.
4) Adaptive response to wine selective pressures shapes the genome of a *Saccharomyces* interspecies hybrid.
5) Adaptive evolution of *S. kudriavzevii* and *S. uvarum* strains under ethanol stress.

CHAPTER 1

Analysis of lipid composition reveals mechanisms of ethanol tolerance in the model yeast *Saccharomyces cerevisiae*

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1.1 Introduction

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism that has been employed as a model organism to study diverse relevant phenomena in biology at molecular level (Smith and Snyder, 2006). Due to its high fermentative capability, it is also widely used in the biotechnology field for the performance of industrial fermentations of products such as wine, beer or bread (Legras et al., 2007) or traditional Latin American beverages like pulque, masato, chicha, tequila or cachaça (Arias García, 2008; Badotti et al., 2014; Stringini et al., 2009; Suárez Valles et al., 2005). *S. cerevisiae* also has a relevant role in bioethanol production (van Zyl et al., 2007).

S. cerevisiae has been isolated from different sources and environments all over the world, including fruits, soils, cactus, insects, oak, and cork tree barks (Eberlein et al., 2015; Liti et al., 2009). The physiological and genetic diversity among the *Saccharomyces* genus is high, due to their colonization of different environments; the most studied species are those associated with industrial processes of economic importance as wine production (Alba-Lois and Segal-Kischinevzky, 2010; Camarasa et al., 2011; Franco-Duarte et al., 2014; Querol et al., 2003, 1994; Schuller et al., 2012), cider (Pando Bedriñana et al., 2010) and beer (Alba-Lois and Segal-Kischinevzky, 2010).

Saccharomyces yeasts that have been selected to carry out these fermentations in a controlled manner, show particular characteristics, as selective pressures imposed by the fermentative environment, such as low pH and the high ethanol levels in the media, favor yeasts with the most efficient fermentative catabolism, particularly *S. cerevisiae* strains, but there are species in the *Saccharomyces* genus which are also found spontaneously in these fermentation products including *S. uvarum* (Pretorius and Lambrechts, 2000). Depending on the fermentation process, other factors apart from alcohol concentration, as temperature, can be considered stress factors (Ganucci et al., 2018; Salvadó et al.,

2011a,b).

Ethanol (CH_3CH_2OH) is a small molecule containing a methyl group and a hydroxyl group and consequently it is soluble in both aqueous and lipidic phases. Because of these properties, it can penetrate inside cells, which generates important stresses; incorporation into the cell membrane can increase fluidity, which is a fundamental driver of membrane properties (Jones and Greenfield, 1987; Lloyd et al., 1993).

This fluidity change induces a loss of membrane integrity, becoming more permeable (Marza et al., 2002). Ethanol causes other detrimental effects to the cells, including alterations on mitochondrial structure, reducing ATP levels and respiratory frequency and favoring acetaldehyde and reactive oxygen species (ROS) generation, which can cause lipid peroxidation, DNA damage and oxidative stress (Alexandre et al., 2001; Yang et al., 2012). As a consequence, a notable reduction in cellular viability occurs.

Cell membranes are composed of lipids (mainly phospholipids and sterols, but also sphingolipids and glycolipids) and proteins. Membrane lipids are amphipathic, possessing hydrophobic (apolar) and hydrophilic (polar) regions. Embedded membrane proteins are strongly associated with the apolar core of the bilayer and peripheral proteins are more loosely associated with the membrane via several mechanisms. A key factor contributing to membrane fluidity is the fatty acids and sterol composition of the membrane (Zinser et al., 1991).

The molecular structure of ethanol allows passive diffusion across the membrane and likely incorporation into the bilayer structure (Peña and Arango, 2009). When this happens, van der Waals attractive forces decrease, increasing membrane fluidity (Ingram and Buttke, 1985). Using fluorescence anisotropy studies, a direct relationship between plasma membrane fluidity and ethanol concentration has been reported (Sánchez-Gallego, 2009; Simonin et al., 2008). This increase in fluidity, together with

the loss of structural integrity previously mentioned, result in loss of various intracellular components components including amino acids and ions (Marza et al., 2002), producing alterations in a cellular homeostasis.

The alterations in membrane properties are fundamental in the mechanism of ethanol toxicity, but the physical changes that the membrane structure undergoes as a result of ethanol presence in the media have not been completely described. It is widely accepted that ethanol is intercalated in lipidic heads of the membrane, with the OH group of the ethanol associated with the phosphate group of the lipidic heads and the hydrophobic tails aligned with the hydrophobic core of the membrane. When this interaction takes place, ethanol molecules substitute interfacial water molecules, generating lateral spaces between polar heads, and, as a consequence, spaces in the hydrophobic core (Chiou et al., 1992). These gaps result in unfavorable energy, so the system tries to minimize it by creating an interdigitated phase. This modification in the membrane causes a decrease in its thickness of at least a 25% (Kranenburg et al., 2004; Vanegas et al., 2010) and as a consequence of this thinning, alterations in membrane protein structure and function can occur, leading to cellular inactivation during the fermentation process (Lee, 2004).

It has been demonstrated that membrane thickness affects membrane protein functionality, in which maximum activity takes place with a defined thickness (Montecucco et al., 1982; Yuan et al., 2004). If this thickness changes, exposure of hydrophobic amino acid residues in integral membrane proteins can take place, resulting in a phenomenon known as hydrophobic maladjustment (Lee, 2004), that can lead to aggregation of membrane proteins to minimize the exposition of their hydrophobic parts in the aqueous media (Leão and Van Uden, 1984). Studies that use membrane models formed by phosphatidylcholine and ergosterol that are exposed to different ethanol concentrations have demonstrated that lipid composition protects the membrane because interdigitated phase formation is delayed (Tierney et al., 2005).

In Arroyo-López et al. (2010b), different *Saccharomyces* species were characterized for their ethanol tolerance, identifying *S. cerevisiae* as the most ethanol tolerant one. In the present work, we have selected 61 *S. cerevisiae* strains, from different origins and isolation sources. The purpose of this study was to establish differences in the behavior of strains that represent the different *S. cerevisiae* groups, to determine the most resistant ones, so they are better to perform industrial fermentations. With this aim, we both monitored the growth in a liquid medium with different ethanol concentrations, using absorbance measurements, and in a solid media, carrying out drop test analysis on ethanol plates. Growth data were statistically analyzed for each of the *S. cerevisiae* strains and strains showing a different behavior under ethanol stress were selected to conduct membrane studies that allow correlations of lipid composition in yeast populations with responses to environmental stress such as ethanol.

1.2 Materials and Methods

1.2.1 Strains and media conditions

The *Saccharomyces cerevisiae* yeast strains used in this study are listed in Table 1.1. A total number of 61 strains from different isolation sources were selected. These strains were maintained in GPY-agar medium (%w/v: yeast extract 0.5, peptone, 0.5, glucose 2, agar 2). Yeast identity was confirmed by sequencing the D1/D2 domain of the 26S rRNA gene (Kurtzman and Robnett, 1998b).

Table 1.1: **List of the 61 *Saccharomyces cerevisiae* strains used in this work.**

Strain name	Strain repository / Collection	Isolation source and origin	Strain properties / Description
Wine comercial fermentation strains			
MY1	Lallemand	Wine	White and rosé wines
MY2	Lallemand	Wine	White wines
MY3	Lallemand	Wine	Rosé and red wines
MY4	Lallemand	Wine	White and rosé wines
MY6	Lallemand	Wine	White, rosé and red wines
MY7	Lallemand	Wine	Red wines
MY8	Lallemand	Wine	Red wines
MY11	Lallemand	Wine	White wines
MY12	Lallemand	Wine	Red wines
MY13	Lallemand	Wine	White, red and rosé wines
MY14	Lallemand	Wine	Sparkling wines, fruit wines and ciders
MY15	Lallemand	Wine	White wines
MY16	Lallemand	Wine	White, red and rosé wines
MY17	Lallemand	Wine	White wines
MY18	Lallemand	Wine	Stuck fermentations
MY19	Lallemand	Wine	Red wines
MY20	Lallemand	Wine	Red wines
MY21	Lallemand	Wine	Red wines
MY51	Lallemand / AQ29	Wine	Red wines
MY62	Lallemand	Wine	White wines[a]
MY63	Lallemand	Wine	White and rosé wines
Wine non comercial fermentation strains			
MY52	AQ1336	Wine, South Africa	-
MY53	AQ923	Wine, Spain	-
MY54	AQ924	Wine, Spain	-
MY55	AQ2371	Bili wine, West Africa	-
MY56	AQ2375	Bili wine, West Africa	-
MY61	AQ[b]	Wine, Hungary	High Temperature
MY28	AQ2492	Flor wine, Spain	-
MY29	AQ2356	Flor wine, Spain	-
MY30	AQ94	Flor wine, Spain	-
MY31	AQ636	Flor wine, Spain	-
Other commercial fermentation strains			
AJ4	Lallemand	Fermentations	-
MY50	Lallemand	Fermenting cacao	-
MY60	Fermentis	Bioethanol	Ethanol Red

Table 1.1 continued from previous page			
Strain name	**Strain repository / Collection**	**Isolation source and origin**	**Strain properties / Description**
Other non commercial fermentation strains			
MY25	AQ2579	*Agave salmiana, Peru*	-
MY26	AQ2493	*Agave salmiana*, México	-
MY27	AQ2591	Chicha de jora, Perú	-
MY32	AQ594	Sake, Japan	-
MY33	AQ1312	Sakeye, Japan	-
MY34	AQ1314	Sakeye, Japan	-
MY35	AQ2332	Chicha de jora, Perú	-
MY36	AQ2469	Chicha de jora, Perú	-
MY37	AQ2363	Masato, Perú	-
MY38	AQ2473	Masato, Perú	-
MY43	AQ1180	Cider, Ireland	-
MY44	AQ1182	Cider, Ireland	-
MY45	AQ1184	Cider, Ireland	-
MY46	AQ2851	Sugar cane, Brazil	-
MY47	AQ2543	Sugar cane, Brazil	-
MY48	AQ2506	Sugar cane, Brazil	-
MY57	AQ843	Beer, Belgium	-
MY58	AQ1323	Sorghum beer, Burkina Faso	-
MY49	AQ1085	Fermenting cacao, Indonesia	-
MY59	UFLA	Bioethanol, Brazil	-
Natural Environmental strains			
MY22	AQ2458	*Agelaia vicina*, Peru	-
MY23	AQ2163	*Quercus faginea*, Spain	-
MY24	AQ997	*Prunus armeniaca*, Hungary	-
Clinical strains			
MY39	AQ2587	Dietetic product, Spain	-
MY40	AQ2654	Faeces, Spain	-
MY41	AQ435	Vagina, Spain	-
MY42	AQ2717	Lung, Spain	-

[a] *S. cerevisiae* strain containing a limited amount of *S. kudriavzevii* genome (Erny et al., 2012)

[b] Kindly provided by M. Sipiczki

AQ = Amparo Querol Collection

UFLA = Universidade Federal de Lavras

1.2.2 Drop test experiments. Assay in ethanol plates

To assess yeast strains' ethanol tolerance, drop test experiments were carried out. Rectangular GPY plates supplemented with different ethanol percentages (0, 6, 10, 14, 16 and 18%) were prepared. Yeast cells were grown overnight at 28ºC on GPY media and diluted to an OD_{600}= 0.1 in sterile water. Then, serial dilutions of cells (10^{-1} to 10^{-3}) were transferred on the plates with replicates and incubated at 28ºC for ten days with the plates wrapped in parafilm to avoid ethanol evaporation. Each strain was inoculated twice on the same plate but at different positions, and an exact replicate of the plate was made. With this method, four biological replicates of each strain were performed. Growth values were assigned to each of the replicates: 0 no growth, 1 weak growth, 2 intermediate growth and 3 remarked growth. Median growth values were assigned for each ethanol concentration. Hierarchical clustering used in heatmap plot was elaborated using www.heatmapper.ca tool, (Babicki et al., 2016) with Euclidean distance measurement method and group clustering was based on growth in different ethanol media averages (average linkage).

1.2.3 Growth in liquid media. Optical density measurements.

GPY precultures of each strain were prepared and incubated at 28ºC overnight. These cultures were washed with sterile water and adjusted to an OD_{600} = 0.1 in each one of the culture media (YNB liquid media supplemented with different ethanol percentages (0, 1, 6, 8, 10, 13, 16 and 18 %)). YNB is composed of 6.7 g/L of aminoacids and ammonium sulfate (YNB, Difco) and is supplemented with 20 g/L of D-glucose as carbon source. Growth was monitored in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 28ºC. NuncTM MicroWellTM 96 well plates (ThermoFisher Scientific) wrapped in parafilm and with water in each of its 4 repositories were employed. Measurements were taken at 600 nm every 30min, with 10 seconds of preshaking before each measurement until 64 hours of growth monitoring. All the experiments were carried out in triplicate.

1.2.4 Estimation of the NIC and MIC parameters

The basis of the technique, used as in Arroyo-López et al. (2010b) is the comparison of the area under the OD–time curve of positive control (absence of ethanol, optimal conditions) with the areas of the tested condition (presence of ethanol, increasing inhibitory conditions). As the amount of inhibitor in the well increases, the effect on the growth of the organism also increases. This effect on the growth is manifested by a reduction in the area under the OD–time curve relative to the positive control at any specified time.

Briefly, the areas under the OD–time curves were calculated by integration using GCAT software (http://gcat-pub.glbrc.org/). Then, for each ethanol condition and strain replicate, the fractional area (fa) was obtained by dividing the tested area between the positive control area (fa = (test area) / (positive control area). The plot of the fa vs log10 ethanol concentration produced a sigmoid-shape curve that could be well fitted with the modified Gompertz function for decay (Lambert and Pearson, 2000):

$$f\text{a} = \text{A} + \text{C} \times \exp^{-\exp^{\text{B}(x-\text{M})}}$$

After this modelling, the NIC (non-inhibitory concentration) and MIC (Minimum Inhibitory Concentration) parameters could be estimated as in Lambert and Pearson (2000):

$$\text{NIC} = 10^{[\text{M}-(1.718/\text{B})]} \qquad \text{MIC} = 10^{[\text{M}+(1/\text{B})]}$$

To check for significant differences among yeast species for NIC and MIC parameters, an analysis of variance was performed using the one-way ANOVA module of Statistica 7.0 software. Tukey test was employed for mean comparison. ggplot2 package (Wickham, 2009) implemented in R software, version 3.2.2 (R Core Team, 2013) was employed for graphic representation of these NIC and MIC values.

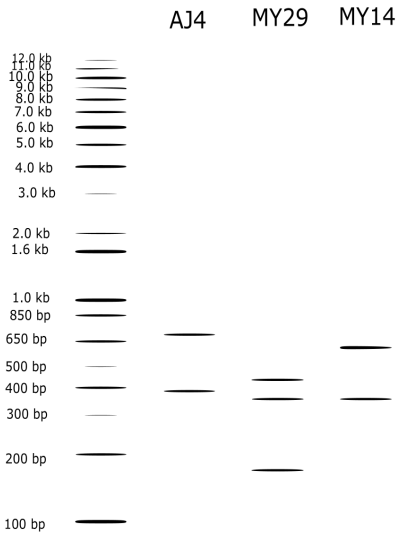


FIGURE 1.1 Theoretical restriction profile of *MMS1* region digested with enzyme *RsaI*. Lane 1 corresponds to AJ4; lane 2 to MY29; and lane 3 to MY14. Calculations were made based on strain specific haplotypes. MY14 shares an haplotype with MY29 that is indicated in blue for lane 3, and has differencial haplotype which is represented by black lines in lane 3.

1.2.5 Strains selection and competition fermentation

5 strains were selected based on their different growth in liquid media and in solid media with ethanol: AJ4, MY3, MY14, MY26 and MY29. Competition fermentations were carried out in 30 mL GPY, GPY+6% ethanol and GPY+10% ethanol in triplicate. 0.1 OD of each of the 5 strains were inoculated in every initial culture. Every 3-5 days 1 mL of the culture was transferred into the corresponding fresh media. After 5 and 10 rounds, culture plates of samples from every tube were obtained. 20 colonies from every plate were randomly picked for their identification. This was carried out by means of mitochondrial digestion profile identification (Querol et al., 1992), which allowed differentiation of all of the strains except for MY14 and MY29, which shared the same exact profile. As an alternative, as we had available the genome sequences of MY14 and MY29 (Morard et al., 2019), we identified a divergent region among these two strains which encomprises gene *MMS1*. We amplified a region of gene *MMS1* with primers f1 (AACGGATCCTTTTTCCCAAC) and r1 (CGGTCGCAAAAATTAACG) and used *RsaI* digestion to differentiate specially these two strains. Theoretical results for digestion bands sizes in a agarose were calculated based on Sanger sequencing of the amplicon for the strains of interest (Figure 1.1).

1.2.6 Lipid extraction and quantification by ammonium ferrothiocyanate assay

Yeast precultures of each one of the five selected strains (AJ4, MY3, MY14, MY26 and MY29) were first propagated in 25 mL of GPY media at 200 rpm and 28°C. The cultures were harvested after 24 h and total lipids were extracted using a modified Bligh and Dyer protocol (Spickett et al., 2011). To quantify the lipids, 10 µL sample was taken from the above 100 µL reconstituted lipids in chloroform and added to 2 mL chloroform with 1 mL of assay reagent (0.1M $FeCl_3.6H_2O$, 0.4 M ammonium thiocyanate) in a 15 mL glass tube. Samples were vortexed for 1 min and centrifuged at 14,500 g for 5 mins. The lower layer was collected into quartz cuvettes. The absorbance was measured at 488 nm, and the concentration of lipid was determined by comparison with a standard curve of a mixture of phospholipid standards (POPC, POPE and POPG) (Sigma).

1.2.7 Mass spectrometry of lipids present in the strains

The lipids from each of the five yeast strains extracted as previously described were reconstituted in 100 µL chloroform to contain 5 µg/µL lipid as determined by ammonium ferrothiocyanate assay, and then diluted 1 in 50 in solvent A (50:50 acetonitrile:H_2O, 5 mM ammonium formate and 0.1% v/v formic acid). Analysis of 10 µL samples was performed by LCMS. LC was performed on a U3000 UPLC system (Thermo scientific, Hemel Hempstead) using a Kinetex C18 reversed phase column (Phenomenex, 2.6 µm particle size, 2.1 mm x 150 mm), at a flow rate of 200 µL/min with a gradient from 10% solvent B to 100% solvent B (85:10:5 isopropanol: acetonitrile: H_2O, 5 mM ammonium formate and 0.1% v/v formic acid) with the following profile: t=0 10% A, t=20 86%A, t=22 96%A, t=26 95%A. MS analysis was carried out in positive and negative ionization mode on a Sciex 5600 Triple TOF. Source parameters were optimized on infused standards. Survey scans were collected in the mass range 250-1250 Da for 250 ms. MM data was collected using top 5 information dependent acquisition and dynamic exclusion for 5 s, using a fixed collision energy of 35V and a collision energy spread of 10V for 200 ms per scan. ProgenesisQI® was used for quantification and LipidBlast (https://fiehnlab.ucdavis.edu/projects/LipidBlast) for

identification. All data were manually verified and curated. Data were analyzed by two-way ANOVA and Tukey's multiple comparisons test, where n = 5. Data sets were uploaded to: https://doi.org/10.17036/researchdata.aston.ac.uk.00000495

1.2.8 TLC analysis

Yeast lipids extracted as above after 24 h growth were analyzed by TLC. Briefly, 20 µg of lipid sample and 10 µg phospholipid lipid standards (POPE and POPS) (Sigma) were loaded onto silica gel TLC plates (Sigma) and separated using chloroform/methanol/acetic acid/water 25:15:4:2. The plates were air dried and sprayed with ninhydrin reagent (0.2% ninhydrin in ethanol) (Sigma) and charred at 100ºC for 5 mins. Images of plates were captured with a digital camera and spot intensity was determined using ImageJ software.

1.2.9 Laurdan membrane fluidity assay

Yeast cultures were set up in GPY and incubated at 200 rpm and 28ºC overnight. Then, 25 mL of GPY media containing 0% ethanol, 6% ethanol or 10% ethanol was inoculated to an OD_{595} of 0.5. Samples were taken at different time points during the fermentation, and live yeast were diluted to an OD_{595} of 0.4 in GPY and incubated with 5 µM Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) for 1 h. Fluorescence emission of these cells stained with Laurdan was taken using a microplate reader (Mithras, Berthold) with the following filters; λex=460 λem=535. Generalized Polarization (GP), derived from fluorescence intensities at critical wavelengths, can be considered as an index of membrane fluidity and is calculated as $GP = (I_{460} - I_{535})/(I_{460} + I_{535})$ Data were analyzed by one-way ANOVA and Tukey's multiple comparisons test, where n = 3.

1.2.10 Carboxyfluorescein dye leakage assay

Lipids for each of the five selected yeast strains extracted as described previously were used to generate 400 nm liposomes loaded with 100 mM Carboxyfluorescein (CF) in protein buffer (50 mM tris, 50 mM NaCl, pH 7.4). Dye leakage assays were performed with at 0.125 mg/mL liposomes and increasing concentrations of ethanol in protein buffer at room temperature, and the fluorescence emission measured (λex= 492 nm, λem=512 nm). Liposomes were treated with 5% Triton X-100 to fully disrupt them, and fluoresecence measurements were normalized to the maximum reading for each liposome composition. Data were analyzed by one-way ANOVA and Tukey's multiple comparisons test, where n = 3.

1.2.11 PCA analysis

To visualize the relationships among different ethanol tolerance parameters and lipid composition of the selected *S. cerevisiae* strains, a principal component analysis (PCA) was performed using the prcomp function and ggbiplot (0.55 version) and ggplot (3.2.1 version) implemented in R.

1.3 Results

1.3.1 Ethanol tolerance of the strains in solid media

A total of 61 yeast strains belonging to *S. cerevisiae* were selected to asses the ethanol tolerance. The sequencing of the D1/D2 26S rRNA gene of these strains were deposited in GenBank with the accession numbers MW559910-MW559970. 21 are industrial strains and were selected for their use in winemaking and 40 of them belong to the IATA-CSIC collection. The sources from which these 40 strains were retrieved are diverse: agave, beer, bioethanol, chicha, cider, cocoa, honey water, masato, sake, sugar cane, wine, natural wild strains, etc. *S.*

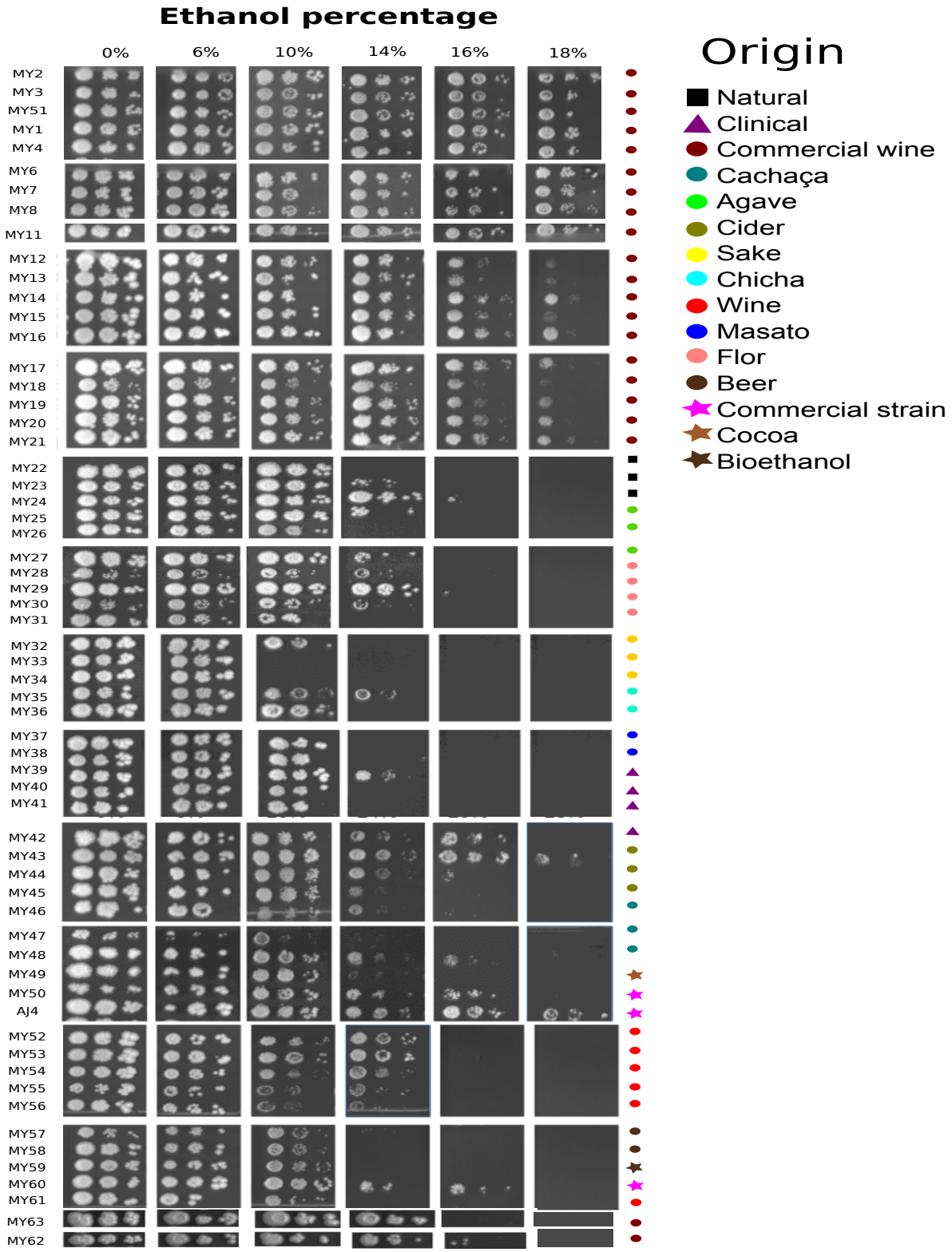
cerevisiae yeast strains' ethanol tolerance was first assessed in plates with GPY + different ethanol percentages. To observe the influence of ethanol on these strains we performed four biological replicates of each strain growth in 6 different media. One biological replicate for each of the strains and media can be seen in Supplementary Figure 1.1.

With the growth data of each of the strains and taking into account, the 4 replicates values of growth for each strain, a heatmap with the growth data in ethanol was constructed (Figure 1.2). This heatmap is hierarchically clustered into two big clusters with different subclusters. The first cluster is made up of the strains which are more tolerant of ethanol (a total number of 22 of the 61 strains) and another one with the rest of the strains which show intermediate and low growth with this compound (39 strains). Among the first cluster, with the most tolerant strains, it is interesting that 19 of the 22 strains belong to commercial wine strains. The other 3 strains which are included in this heatmap are AJ4, a Lallemand commercial strain, which is also one of the most tolerant strains of all the screened ones; MY48, a cachaça strain and MY43, a cider yeast strain.

The other cluster, with the 39 intermediate-low tolerant strains, appears to be divided into two subclusters too. One of the subclusters is composed of MY33 and MY34, which are the less ethanol tolerant strains, and that belong to the sake group. It is interesting to note that in the other subcluster, there are strains with different behaviors. As an example, strains MY46 (cachaça) and MY44 (cider) growth in ethanol media are affected by low ethanol concentrations (ethanol percentage of 6%), but they can grow (at a low rate) until 16% of ethanol is present in solid media. On the other hand, there are other strains, such as MY37 (Masato) and MY22 (natural), whose growth is not affected until 10% of ethanol is present in GPY solid media but in the next ethanol step (14%) they do not grow at all.

1.3.2 Ethanol tolerance of the strains in liquid media

Ethanol tolerance of the set of *S. cerevisiae* strains was evaluated in minimal YNB liquid media at 28ºC. Yeast growth was evaluated by OD_{600} determination in microtiter plates containing this media with different ethanol concentrations and for each strain, the area under the curve



SUPPLEMENTARY FIGURE 1.1 Images with one of the replicates per plate of the ethanol drop tests. For each one of the 61 strains, 4 replicates in GPY plates containing 6 different ethanol percentages (0%, 6%, 10%, 14%, 16% and 18%) were performed, using 3 serial dilutions of cells (10^{-1} to 10^{-3} OD).

FIGURE 1.2 Heatmap representation of growth values (from 0 to 3) of the analyzed strains at plates with increasing ethanol concentrations. Each line corresponds to a strain (AJ4, MY1-MY63) and each column to a particular ethanol concentration (0%, 6%, 10%, 14%, 16% and 18%). The color key bar at the top indicates growth values, from yellow (low growth value) to pink (high growth value). Hierarchical clustering is showed on the left. Color dots on the right of the Figure indicate the source/origin of each one of the strains. In Supplementary Figure 1 can be seen one of the four replicates from which these heatmap was constructed.

during these growths was calculated. With the area under the curve reduction due to the addition of ethanol, NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) parameters were calculated for 57 of the 61 strains. Not all of the 61 strains could be evaluated following this method: the data obtained with flor strains MY28 and MY31 could not be used because these strains flocculate and the data obtained with them are not reproducible. The data obtained with the strains MY55 and MY56 was not used as they have problems growing in minimal media YNB. The complete list with the NIC and MIC values for each one of the selected strains can be found in Table 1.2. Figure 1.3 depicts a graph representing these values for each one of the strains.

1.3.3 Strain selection

After performing the phenotypic characterization in ethanol of our collection of 61 strains, to further characterize some representatives of the different behaviors we decided to select 5 of them as they showed a range of tolerances: AJ4, MY3, MY14, MY26, and MY29. Figure 1.4A shows the results of the drop test in GPY+ethanol media of these 5 strains and Figure 1.4B the NIC and MIC parameters of growth in YNB liquid media+ethanol.

AJ4 shows high NIC and MIC values during YNB growth in liquid media, and in solid media in GPY + ethanol it clusters amongst the most tolerant *S. cerevisiae* strains too. This strain, is a Lallemand commercial strain that has been reported as a highly tolerant ethanol strain (Lairón-Peris et al., 2020). It has a high NIC value 11.62% ± 0.33%, which means that a high concentration of ethanol is needed to affect its growth.

MY29, which is a flor strain isolated from sherry wine, is classified within the second cluster with the strains that show an intermediate growth in GPY+ethanol in solid media. It grows well until 14% ethanol; however, viability is reduced in 16% ethanol, and it is unable to grow at 18% ethanol. Regarding the liquid assay in YNB+ethanol, its MIC value is amongst the highest MIC values of all of the strains (15.41% ± 2.93%), but its NIC value (7.5% ± 1.48%) can be classified as a medium-low value. This result shows that MY29 is a *S. cerevisiae* strain whose behavior can be

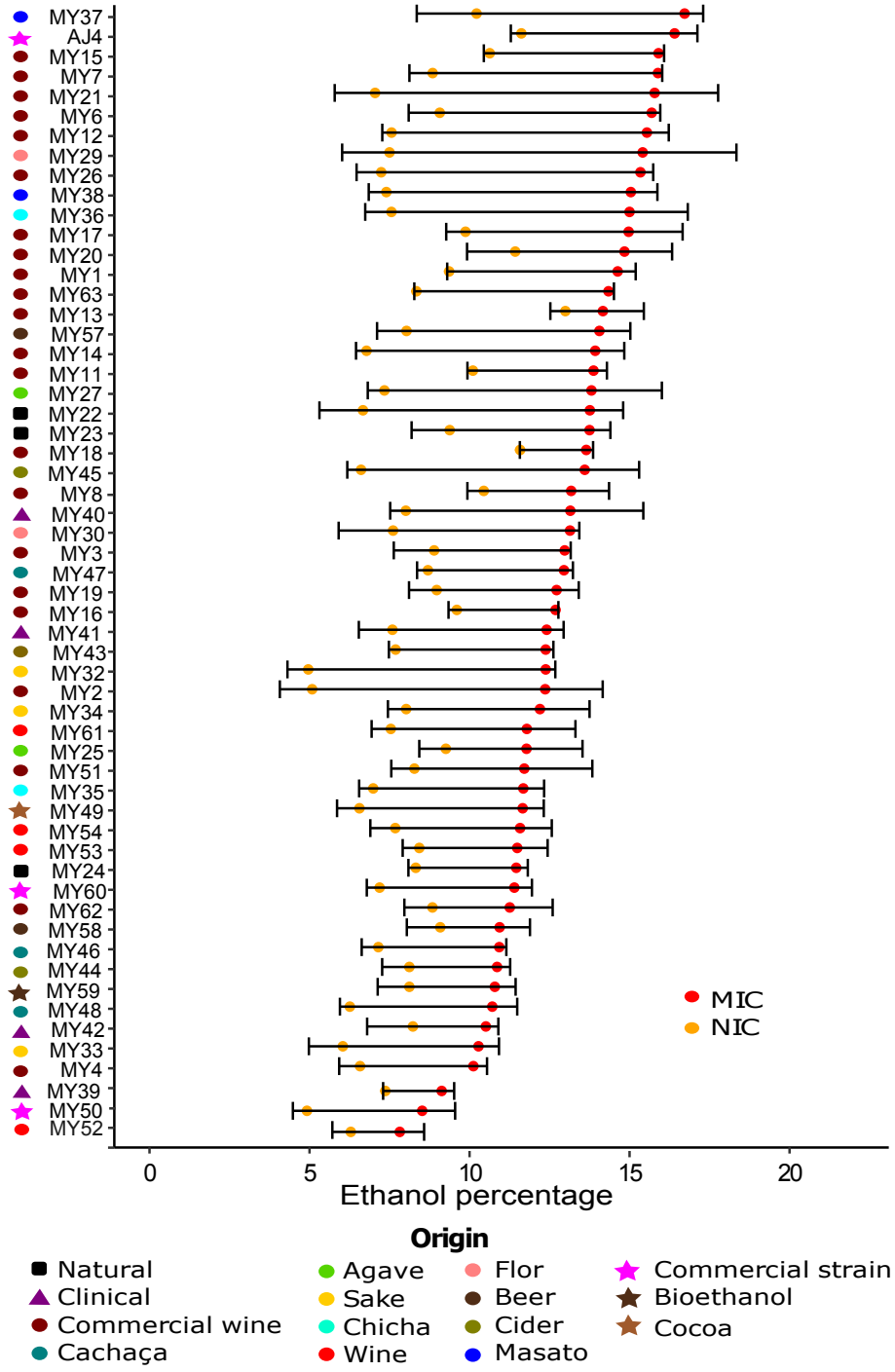


FIGURE 1.3 Representation of each strain NIC (yellow) and MIC (red) parameters in relation with its ethanol tolerance (%). Values are averages from triplicate experiments and standard deviation is represented too. Color dots on the right of the Figure indicate the source/origin of each one of the strains. Strains are ordered by MIC value.

TABLE 1.2 **NIC and MIC values of the *Saccharomyces cerevisiae* strains evaluated**

Strain	NIC (%)	MIC (%)	Strain	NIC (%)	MIC (%)
MY2	5.08 ± 1.01	12.36 ± 1.80	MY35	6.99 ± 0.44	11.68 ± 0.65
MY8	10.44 ± 0.51	13.18 ± 1.18	MY36	7.56 ± 0.82	15.00 ± 1.82
MY6	9.07 ± 0.97	15.69 ± 0.27	MY37	10.22 ± 1.87	16.72 ± 0.58
MY16	9.60 ± 0.26	12.69 ± 0.09	MY38	7.40 ± 0.55	15.04 ± 0.83
MY17	9.87 ± 0.60	14.97 ± 1.69	MY39	7.37 ± 0.07	9.13 ± 0.39
MY4	6.58 ± 0.65	10.12 ± 0.43	MY40	8.01 ± 0.49	13.15 ± 2.28
MY3	8.89 ± 1.26	12.97 ± 0.19	MY41	7.59 ± 1.05	12.41 ± 0.53
MY11	10.10 ± 0.17	13.87 ± 0.42	MY42	8.23 ± 1.43	10.51 ± 0.39
MY19	8.97 ± 0.87	12.72 ± 0.69	MY43	7.69 ± 0.21	12.38 ± 0.24
MY20	11.43 ± 1.51	14.84 ± 1.49	MY44	8.12 ± 0.85	10.86 ± 0.41
AJ4	11.62 ± 0.33	16.41 ± 0.71	MY45	6.61 ± 0.43	13.60 ± 1.70
MY10	4.97 ± 0.60	9.51 ± 0.00	MY46	7.15 ± 0.52	10.93 ± 0.22
MY5	5.54 ± 0.59	8.30 ± 0.25	MY47	8.70 ± 0.34	12.95 ± 0.28
MY51	8.28 ± 0.72	11.71 ± 2.13	MY48	6.26 ± 0.31	10.71 ± 0.78
MY1	9.36 ± 0.06	14.63 ± 0.56	MY49	6.56 ± 0.70	11.66 ± 0.66
MY21	7.05 ± 1.26	15.78 ± 1.98	MY50	4.92 ± 0.44	8.52 ± 1.03
MY7	8.85 ± 0.73	15.89 ± 0.13	MY52	6.29 ± 0.59	7.82 ± 0.76
MY9	7.28 ± 1.00	13.48 ± 1.12	MY53	8.43 ± 0.52	11.49 ± 0.95
MY15	10.63 ± 0.18	15.91 ± 0.17	MY54	7.68 ± 0.78	11.58 ± 0.99
MY22	6.67 ± 1.36	13.76 ± 1.04	MY59	8.12 ± 0.99	10.79 ± 0.65
MY23	9.38 ± 1.19	13.75 ± 0.65	MY60	7.19 ± 0.40	11.40 ± 0.55
MY24	8.32 ± 0.23	11.46 ± 0.36	MY61	7.54 ± 0.60	11.79 ± 1.52
MY25	9.26 ± 0.83	11.78 ± 1.75	MY14	6.78 ± 0.33	13.93 ± 0.91
MY26	7.24 ± 0.77	15.34 ± 0.40	MY18	11.58 ± 0.01	13.65 ± 0.21
MY27	7.34 ± 0.52	13.81 ± 2.20	MY13	12.99 ± 0.47	14.17 ± 1.28
MY29	7.50 ± 1.48	15.41 ± 2.93	MY12	7.56 ± 0.29	15.54 ± 0.68
MY30	7.61 ± 1.70	13.14 ± 0.29	MY57	8.03 ± 0.92	14.06 ± 0.97
MY32	4.96 ± 0.65	12.38 ± 0.30	MY58	9.09 ± 1.05	10.94 ± 0.95
MY33	6.04 ± 1.06	10.28 ± 0.64	MY62	8.84 ± 0.88	11.26 ± 1.34
MY34	8.02 ± 0.57	12.20 ± 1.55	MY63	8.34 ± 0.06	14.34 ± 0.17

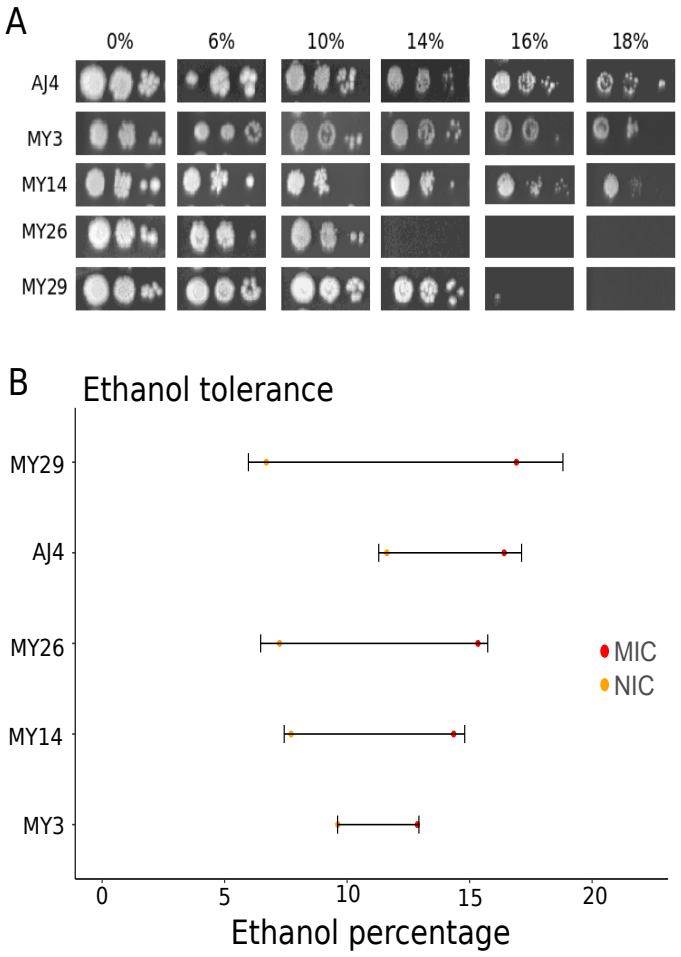


FIGURE 1.4 Photograph of the drop tests in ethanol plates (A) and the NIC and MIC parameters (B) for each one of the 5 selected strains.

classified as intermediate in ethanol conditions. Moreover, MY29 is the most tolerant sherry wine strain of the five strains analyzed.

MY26, which is an agave strain, is among the least tolerant strains in solid media and is also the strain which shows the lowest growth among the three agave strains that we selected for our study. In liquid media, its NIC value is also low, being affected by an ethanol concentration of 7.24% ± 0.77% but its MIC value is high (15.34% ± 0.4%). This strain shows similar behavior in liquid media as MY29, but in solid media, it proved to be less tolerant as it was not able to grow in 14% ethanol plates, and MY26 could grow in this condition too.

MY3 and MY14 are commercial wine strains, which are classified in the cluster of the most

tolerant strains regarding their growth on ethanol plates. Nevertheless, MY14 appears to be affected by the ethanol at low concentrations (NIC value of 6.78% ± 0.33% and MIC value of 13.93% ± 0.91%) and MY3 seems to start being affected by ethanol at higher concentrations but has a low range, as it has a low MIC value (NIC 8.89% ± 1.26% and MIC 12.97% ± 0.13%).

1.3.4 Competition fermentations

These five strains, AJ4, MY3, MY14, MY26, and MY29 were selected for their different behavior regarding ethanol susceptibility. They were inoculated into mixed culture fermentations to assess the correlation between ethanol tolerance and competition capacity under different ethanol concentrations (0%, 6%, and 10%). As one GPY fermentation would be insufficient for observing domination of the culture by one single strain, we followed a method in which we inoculated a sample of the culture after sugar depletion into new fresh media with the corresponding ethanol concentration.

After the tenth pass, AJ4 completely dominated the 0% and the 10% fermentations. However, in 6% fermentations, MY29 strain completely dominated one of the three replicate fermentations and clearly dominated the other two. The other 2 strains which are present in this 6% fermentation when sugar is depleted are AJ4 and MY14, although in low proportion. Neither MY3 nor MY26 colonies were found in any of the fermentation (Figure 1.5).

AJ4 dominating high ethanol concentration cultures was quite an expected result regarding its ethanol tolerance determined in the present work. However, it does not seem clear why MY29 dominates 6% ethanol cultures, given it moderate tolerance compared to other strains such as AJ4, MY3 or even MY14. Here, probably, complex interaction among strains play an important role in domination, which has been studied previously for another set of strains (Rossouw et al., 2015), and demonstrated to be of importance together with growth capacity under the studied media conditions (Alonso-del Real et al., 2017b).

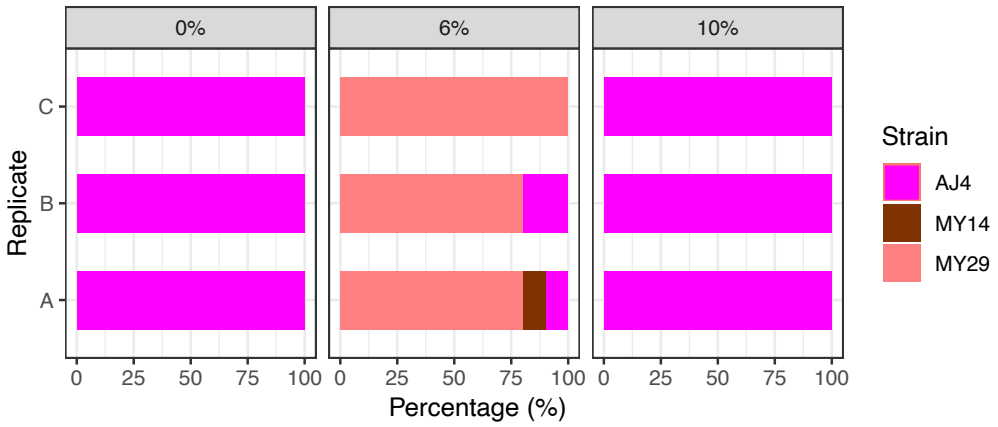


FIGURE 1.5 Percentage of strains present in GPY+ethanol media determined by molecular identification after 10 rounds of fermentations. Every biological replicate is indicated by letters A, B and C and the ethanol concentration present in the media in the X axis.

1.3.5 Lipid composition and membrane properties

Several studies have demonstrated that yeast are able to adapt their membrane composition in response to ethanol stress (Alexandre et al., 1994; Beaven et al., 1982; Chi and Arneborg, 1999a). To better understand the effects of ethanol upon the yeast strains, we investigated the properties of the membranes in the presence and absence of ethanol. We determined the total lipid composition of each of the strains by mass spectrometry. The number of species identified for major lipid classes for strains grown in media containing 0% or 6% ethanol is shown in Figure 1.6.

For the strains grown in the absence of ethanol, for ceramide 1-phosphates (CerP), there were significantly fewer species observed in MY29 (109.6 ± 6.61) compared to AJ4 and MY3 (128.2 ± 1.49 and 130 ± 0.55), where $P < 0.01$ (two-way anova and Tukey's multiple comparisons test) and MY14 (126.6 ± 1.86) where $P < 0.05$. For cardiolipin species (CL), there were significantly fewer observed in AJ4 and MY3 (3.0 ± 0.45 and 3.0 ± 0.31); ($P < 0.01$), and MY14 and MY26 (4.2 ± 1.3 and 4.0 ± 0.55); ($P< 0.05$) when compared to MY29 (9.67 ± 1.8). There were fewer diacylglycerols observed in MY29 compared to MY3 (180.2 ± 1.93 and 193.0 ± 1.41); ($P < 0.05$). For glycerophosphatidic acid (GPA) species, there were significantly fewer species identified for MY29 (126.4 ± 15.17) compared to AJ4 (178.0 ± 2.28; $P < 0.0001$), MY3 (175.0 ± 1.05; $P < 0.001$), MY14 (170.4 ± 5.30; $P < 0.001$), and MY26 (167.8 ± 6.67; $P < 0.01$). There were also fewer

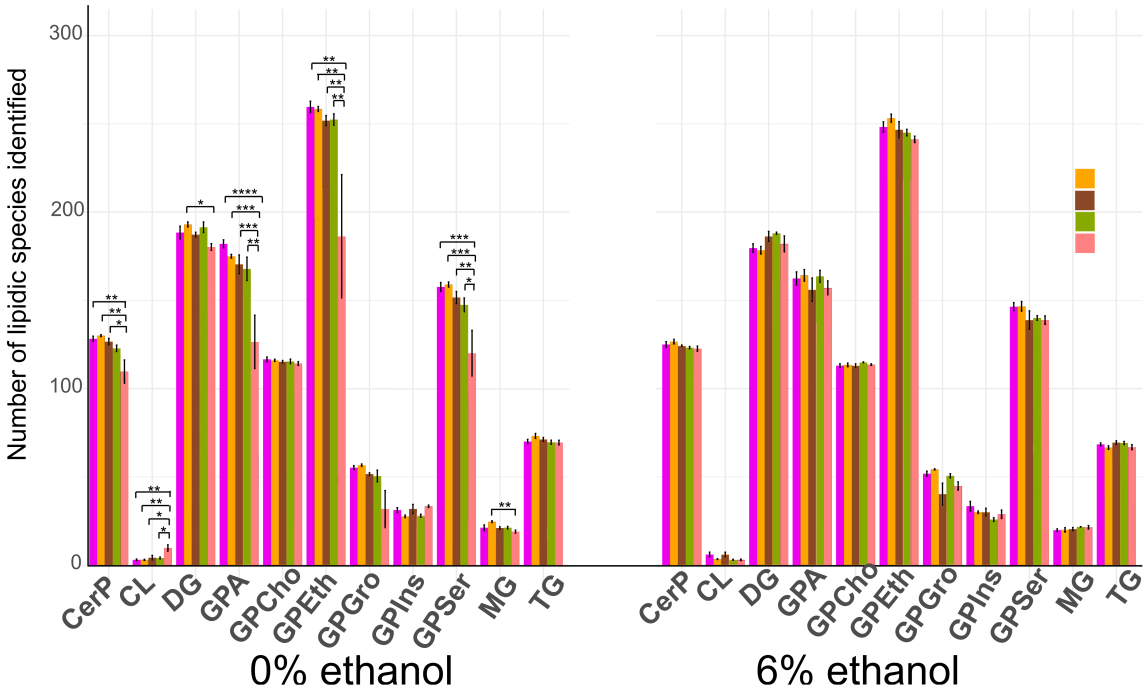
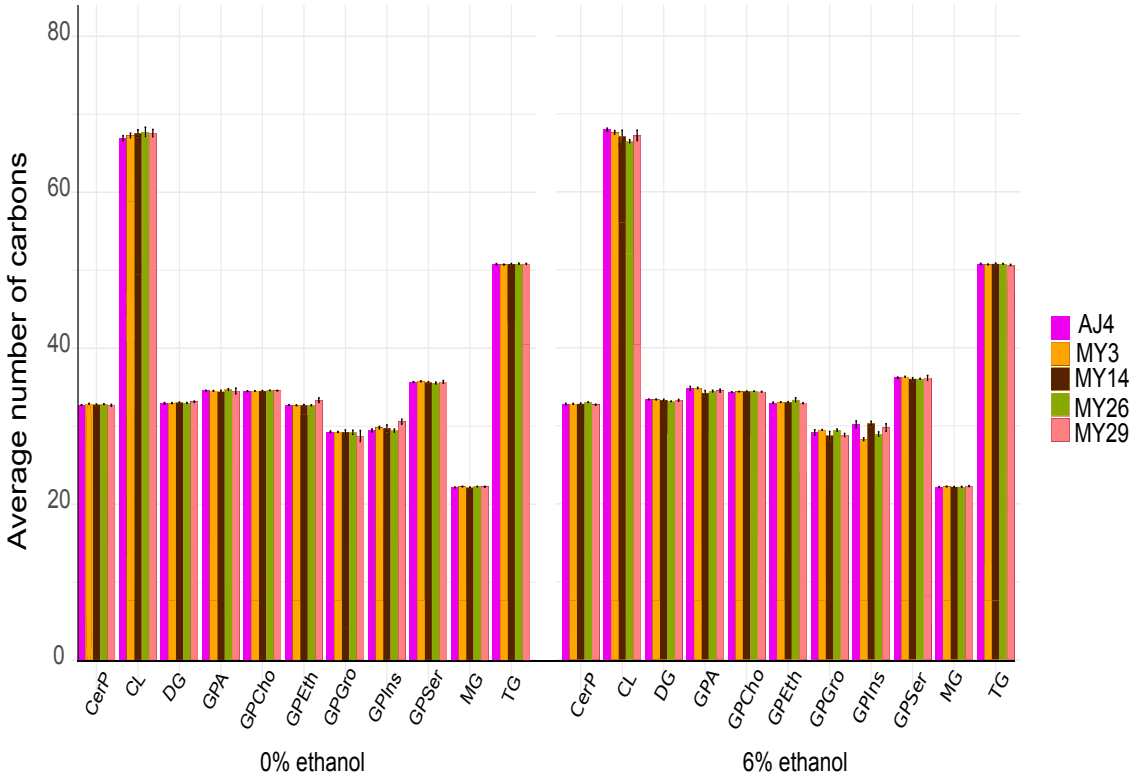


FIGURE 1.6 Number of species identified by lipid class for AJ4, MY3, MY14, MY26 and MY29 strains. Lipids were extracted and analyzed by LC-MS in positive and negative ion mode. n = 5.

glycerophosphatidylethanolamine (GPEth) species identified for MY29 compared to each of the strains ($P < 0.01$ in each case) (259.6 ± 3.2 AJ4; 258.4 ± 1.36 MY3; 254.8 ± 2.85 MY14; 252.4 ± 3.26 MY26 and 186.2 ± 35.034 for MY29). For glycerophosphoserine species (GPSer), there were fewer species in MY29 (120.0 ± 12.99) compared to AJ4 and MY3 (157.6 ± 2.50 and 159 ± 1.41; $P < 0.001$), MY14 (151.6 ± 3.41; $P < 0.01$) and MY26 (147.4 ± 3.94; $P < 0.05$). Lastly, there were less monoacylglycerols (MG) species observed in MY29 (19.0 ± 0.84) than for MY3 (24.6 ± 0.51; $P < 0.01$).

There were no significant differences observed between the species grown in the presence of 6% ethanol; however, significant changes were seen between the 0% and 6% ethanol samples. For CL, there were significantly fewer species observed for MY29 grown in 6% compared to 0% ethanol (3.0 ± 0.44 and 9.66 ± 1.80; $P < 0.01$). For DG, there were more species in 0% MY3 than 6% (193.0 ± 1.41 and 178.4 ± 2.13; $P < 0.05$), for GPA there were significantly fewer species in MY29 at 0% compared to 6% (126.4 ± 15.17 and 157.0 ± 4.03; $P < 0.05$), and for GPEth there were also significantly fewer species in MY29 at 0% compared to 6% ethanol (186.2 ± 35.04 and



SUPPLEMENTARY FIGURE 1.2 Average carbon length of the acyl chains for AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 0% ethanol and 6% ethanol. Lipids were extracted and analysed by LC-MS in positive and negative ion mode (n = 5).

241.2 ± 1.82; $P < 0.05$). There were significantly more MG species in MY3 at 0% (24.6 ± 0.51 and 20 ± 1.22; $P < 0.05$) and more TG species in MY3 at 0% compared to 6% ethanol (73.2 ± 1.39 and 66.6 ± 1.03; $P < 0.01$). Strikingly, MY29 seems to have the most different total lipid composition at 0% ethanol and to remodel this most dramatically, in terms of species diversity, at 6%. However, at 6% ethanol, species diversity in MY29 is similar to the other strains, perhaps indicating an optimal membrane composition for ethanol tolerance.

Acyl chain length and saturation have been shown to be important factors in regulating membrane fluidity and ethanol tolerance in yeast (Alexandre et al., 1994; Beaven et al., 1982; Chi and Arneborg, 1999a). We therefore investigated this for AJ4, MY3, MY14, MY26 and MY29 strains in both 0% and 6% ethanol. While there were no significant to changes in average carbon length of the acyl chains for each of the strains grown in 0% compared to 6% ethanol (Supplementary Figure 1.2), there were significant differences in saturation (Figure 1.7).

For the strains grown in 0% ethanol (Figure 1.7A), DG species contained a significantly lower

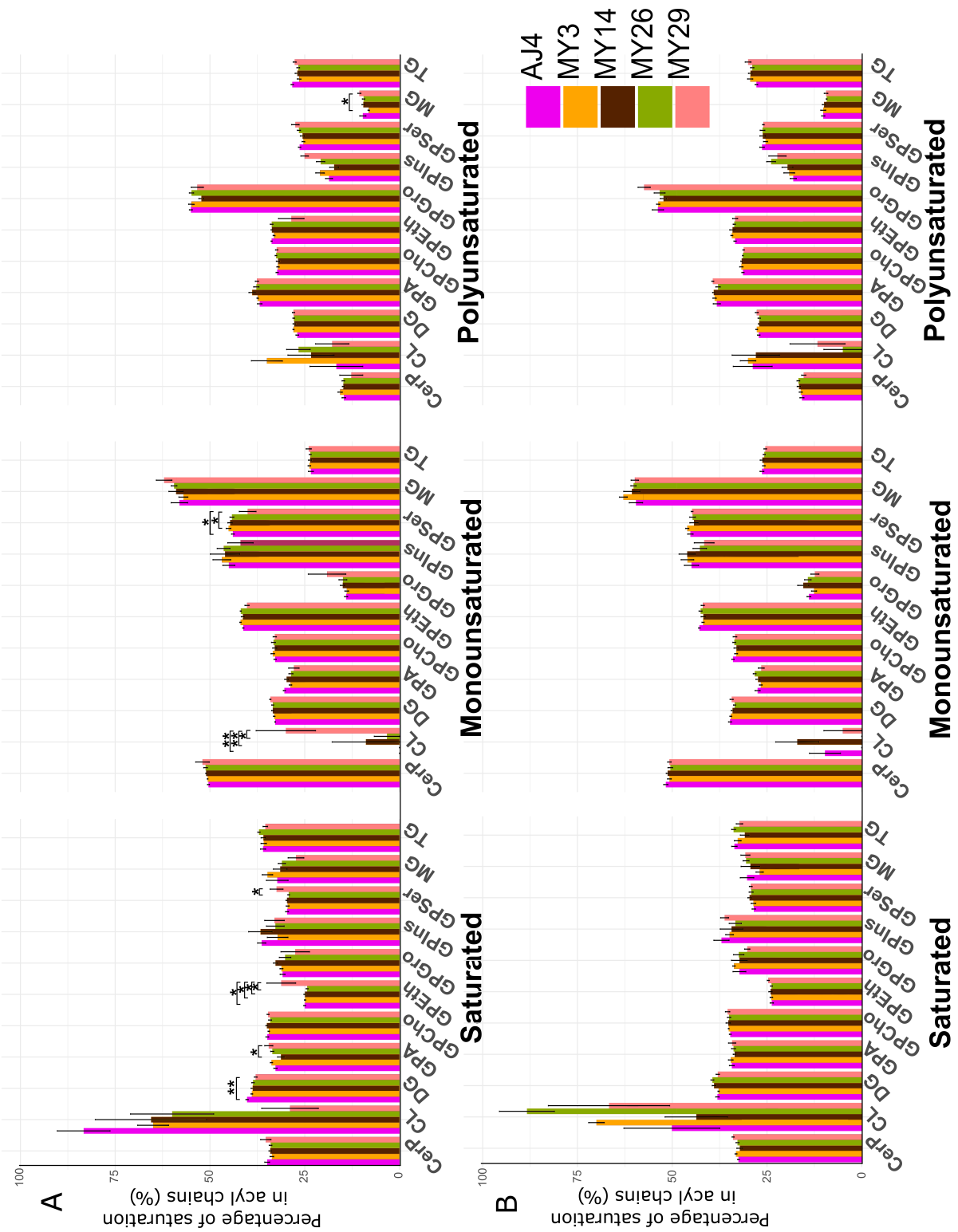


FIGURE 1.7 Percentage of saturated, monounsaturated and polyunsaturated chains by lipid class showing significant changes. A) AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 0% ethanol, and B) AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 6% ethanol. Lipids were extracted and analysed by LC-MS in positive and negative ion mode (n = 5).

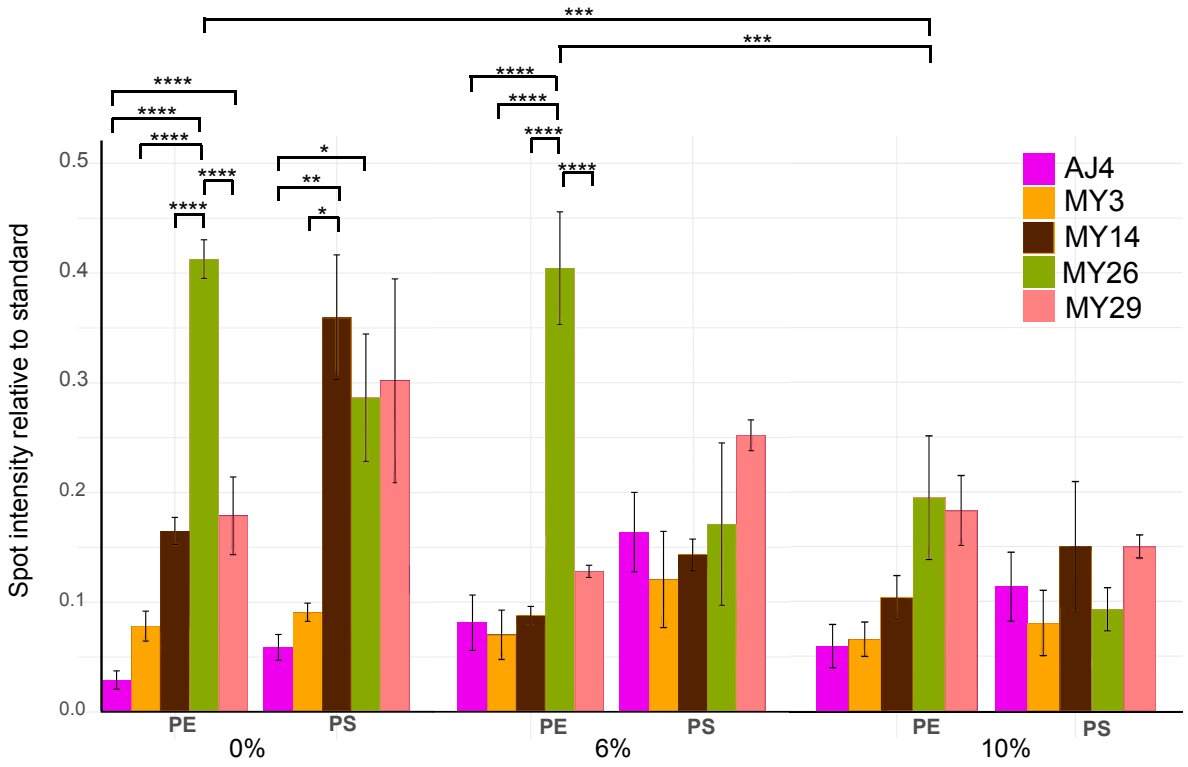


FIGURE 1.8 TLC analysis of PE and PS abundance for AJ4, MY3, MY14, MY26 and MY29 strains. Samples were loaded in triplicate and spot intensity was analyzed using ImageJ. Spot intensity is plotted relative to phospholipid standards loaded onto each plate.

percentage saturated acyl chains in MY29 compared to AJ4 (37.95 ± 0.35 and 40.22 ± 0.30; $P < 0.01$). There was a significantly higher percentage of monounsaturated CL species in MY29 (30 ± 7.83) compared to AJ4 and MY3 (0 ± 0.0 in both cases; $P < 0.01$), and MY26 (3.33 ± 3.33; $P < 0.05$). For GPA, there was a significantly higher percentage saturated chains in MY29 (34.51 ± 1.07) compared to MY14 (31.30 ± 0.88); $P < 0.05$. For GPEth, there were more saturated chains in MY29 compared to AJ4, MY3, MY14, and MY26 (31.21 ± 3.79; 25.30 ± 0.24; 24.92 ± 0.16; 24.96 ± 0.26; 24.38 ± 0.26; $P < 0.05$ in each case). There was a significantly greater number of saturated GPSer species in MY29 compared to MY26 (32.44 ± 1.70 and 29.24 ± 0.22; $P < 0.05$) and a lower number of monounsaturated species in MY29 (40.07 ± 2.20) compared to MY3 and MY14 (45.11 ± 0.62 and 44.7 ± 0.59; $P < 0.05$). Lastly, there was a significantly higher percentage of MG species containing two unsaturations in MY29 (10.59 ± 0.40) compared to MY3 (8.14 ± 0.17) ($P < 0.05$). Once again, MY29 is the most different in terms of saturated species at 0% ethanol and remodels its membrane to be more similar to the other strains at 6%.

There were no significant differences observed between strains for 6% ethanol samples (Figure 1.7B), but there were between strains grown in 0% compared to 6% ethanol. There was a significantly higher percentage of saturated DG species for AJ4 at 0% than 6% ethanol (40.22 ± 0.30 and 38.08 ± 0.44), and a lower percentage of monounsaturated species for AJ4 (32.80 ± 0.09 and 34.75 ± 0.38; $P < 0.001$) and MY3 (33.06 ± 0.21 and 34.54 ± 0.25; $P < 0.05$) at 0% compared to 6% ethanol. For saturated GPEth species, there was a significantly higher percentage in 0% MY29 than 6% MY29 (31.21 ± 3.79 and 24.65 ± 0.26; $P < 0.05$), and significantly fewer monounsaturated species in 0% MY29 compared to 6% (40.23 ± 0.55 and 41.94 ± 0.42; $P < 0.05$). There were significantly more monounsaturated GPGro species in MY29 at 0% compared to 6% ethanol (19.12 ± 4.95 and 12.37± 1.05). In addition, there were significantly fewer monounsaturated GPSer species in 0% MY29 than in 6%. Lastly, for TG species, there were significantly more saturated species in MY14 at 0% ethanol than in MY14 at 6% (35.94 ± 0.58 and 30.86 ± 1.16; $P < 0.001$), more monounsaturated species in AJ4 6% (26.33 ± 0.503; $P < 0.01$), MY14 6% (6.24 ± 0.55; $P < 0.01$), and MY26 6% (25.73 ± 0.26; $P < 0.05$) compared to the 0% samples (23.40 ± 0.64; 23.60 ± 0.40 and 23.55 ± 0.25 respectively), and fewer species containing two unsaturations in MY3 (26.50

± 0.47; P < 0.01) and MY14 at 0% (26.98 ± 0.55; P < 0.05) compared to 6% (29.43 ± 0.68 and 29.39 ± 0.48) samples.

To assess variation in overall lipid unsaturation the unsaturation index (UI) was calculated at the lipid level by lipid class for species identified in each strain at 0% and 6% ethanol (Table 1.3) using the percentage of lipids weighted by the number of unsaturated bonds: UI = % with one unsaturation + (2 x % with two unsaturations) + (3 x % with three unsaturations) + (4 x % with four unsaturations). The UI for DG was significantly lower for AJ4 compared to MY29 at 0% ethanol (86.76 ± 0.64 and 90.03 ± 0.61, P < 0.01) and higher for GPEth species in the 0% AJ4, MY14, MY26 strains compared to MY29 (108.72 ± 0.35, 108.72 ± 0.28, 109.36 ± 0.60 and 97.36 ± 7.13 respectively, where P < 0.05 in each case). The UI for MY29 at 0% was also significantly lower than at 6% ethanol (108.73 ± 0.92, P < 0.05). Lastly, the UI for MG species at 0% ethanol was significantly lower for MY3 compared to MY29 (73.30 ± 16.58 and 83.27 ± 18.95, P < 0.05), and the UI for MY29 at 0% ethanol was significantly higher compared to 6% MY29 (83.27 ± 18.95 and 78.74 ± 1.52, P < 0.05).

Due to changes observed in PE and PS species diversity in Figure 1.6, we undertook quantitative TLC analysis of these lipids. This showed significant differences in the abundance of PE in MY26 grown in 0% ethanol (0.41 ± 0.02), where the abundance was higher compared to AJ4 (0.03 ± 0.01 ; P < 0.0001), MY3 (0.08 ± 0.01; P < 0.0001), MY14 (0.17 ± 0.01; P < 0.0001) and MY29 (0.18 ± 0.04; P < 0.0001) grown in 0% ethanol as illustrated by Figure 1.8).

There was also a significantly greater abundance of PE in 6% MY26 (0.41 ± 0.05) compared to 6% AJ4 (0.08 ± 0.03; P < 0.05), MY3 (0.07 ± 0.02; P < 0.0001), MY14 (0.09 ± 0.01; P < 0.0001) and MY29 (0.13 ± 0.01; P < 0.0001). In addition, there was a lower abundance of PE in MY26 at 10% ethanol (0.20 ± 0.06) compared to MY26 at both 0% (0.41 ± 0.02) and 6% ethanol (0.41 ± 0.051); P < 0.001). There was a significantly lower abundance of PS in AJ4 at 0% ethanol (0.06 ± 0.01) compared to MY14 and MY29 (0.36 ± 0.06 and 0.30 ± 0.09; P < 0.01 and P < 0.05, respectively). There was also a significantly lower abundance of PS in MY3 compared to MY14 at 0% ethanol (0.09 ± 0.01 and 0.36 ± 0.06; P < 0.05). It is notable that MY26, the least

TABLE 1.3 Unsaturation index (UI) for lipids identified in each strain was calculated using the percentage of lipids with each number of unsaturated bonds: one unsaturation + (2 x two unsaturations) + (3 x three unsaturations) + (4 x four unsaturations). Statistically significant differences between strains and ethanol conditions are highlighted in bold (two-way anova and Tukey's multiple comparisons test). Errors (SD) are shown in brackets, n = 5.

	0% ethanol					6% ethanol				
Lipid species	**AJ4**	**MY3**	**MY14**	**MY26**	**MY29**	**AJ4**	**MY3**	**MY14**	**MY26**	**MY29**
CerP	41.38 (± 1.09)	42.50 (± 1.01)	41.86 (± 0.54)	41.90 (± 0.50)	40.23 (± 4.34)	83.25 (± 0.58)	82.93 (± 0.41)	84.39 (± 1.17)	83.93 (± 0.43)	83.25 (± 0.58)
CL	33.33 (± 13.92)	70.00 (± 8.15)	62.22 (± 23.36)	90.00 (± 31.83)	88.00 (± 35.91)	107.24 (± 34.56)	60.00 (± 4.08)	111.79 (± 32.31)	30.00 (± 19.96)	84.33 (± 32.53)
DG	**86.76 (± 0.64)**	89.02 (± 0.29)	88.99 (± 0.62)	89.25 (± 0.48)	**90.03 (± 0.61)**	89.10 (± 4.08)	89.91 (± 0.31)	88.31 (± 0.63)	87.65 (± 0.64)	89.71 (± 0.69)
GPA	104.16 (± 0.98)	103.65 (± 0.34)	107.60 (± 1.63)	104.22 (± 1.01)	103.12 (± 0.87)	103.96 (± 23.31)	104.26 (± 1.00)	105.53 (± 0.34)	104.08 (± 1.02)	105.09 (± 1.13)
GPCho	50.20 (± 0.57)	50.29 (± 0.17)	50.03 (± 0.43)	50.72 (± 0.32)	50.36 (± 0.35)	96.64 (± 19.96)	96.66 (± 0.49)	96.47 (± 0.59)	96.35 (± 0.50)	95.77 (± 0.71)
GPEth	**108.72 (± 0.35)**	108.20 (± 0.35)	**108.72 (± 0.28)**	**109.36 (± 0.60)**	**97.36 (± 7.13)**	109.69 (± 32.53)	110.43 (± 0.50)	110.04 (± 1.06)	109.81 (± 0.36)	**108.73 (± 0.92)**
GPGro	124.20 (± 0.97)	123.76 (± 1.02)	119.56 (± 1.01)	124.69 (± 2.03)	125.84 (± 5.31)	121.53 (± 3.14)	120.00 (± 0.31)	120.03 (± 2.85)	120.87 (± 2.67)	127.20 (± 2.24)
GPIns	82.24 (± 1.21)	88.80 (± 3.42)	80.55 (± 2.65)	88.05 (± 3.25)	92.06 (± 2.02)	81.06 (± 2.40)	84.26 (± 1.81)	85.24 (± 4.14)	90.56 (± 2.16)	86.07 (± 2.39)
GPSer	96.68 (± 0.59)	95.85 (± 0.33)	95.92 (± 0.62)	97.30 (± 0.43)	95.06 (± 1.70)	97.83 (± 22.32)	96.80 (± 22.09)	96.52 (± 22.01)	97.00 (± 22.13)	96.68 (± 22.05)
MG	77.42 (± 17.68)	**73.30 (± 16.58)**	78.09 (± 17.71)	78.43 (± 17.77)	**83.27 (± 18.95)**	79.90 (± 1.88)	83.21 (± 1.26)	80.59 (± 2.78)	78.74 (± 0.90)	**78.74 (± 1.52)**
TG	116.88 (± 1.35)	118.43 (± 2.36)	118.00 (1.16)	114.93 (± 0.62)	118.19 (± 0.54)	118.75 (± 1.18)	120.76 (± 1.65)	125.55 (± 2.90)	118.22 (± 0.91)	122.29 (± 1.91)

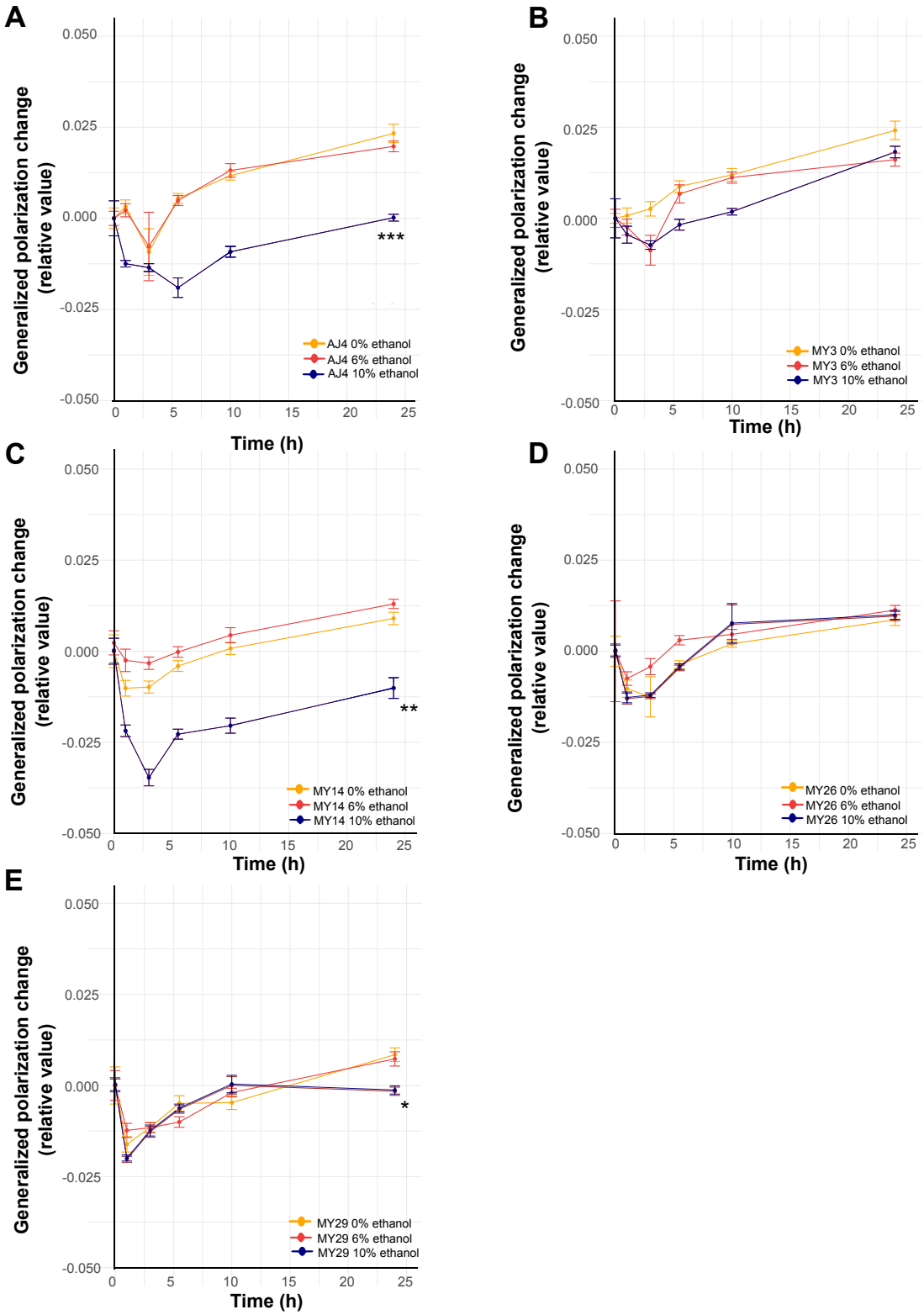


FIGURE 1.9 The effects of ethanol upon the fluidity of live yeast throughout the fermentation, measured by changes to Laurdan GP.

tolerant strain, is the most different at 0% and 6% ethanol, but has a similar composition to the other strains at 10%. Qualitatively, the amount of PE in the membrane at 0% ethanol correlates well (MY26>MY29>MY14>MY3>AJ4; Figure 1.8) with the NIC (MY29<MY26<MY14<MY3<AJ4; Figure 1.4).

We next examined the effect of ethanol upon the fluidity of the yeast membranes as they grew in cultures with and without ethanol. We utilized the fluorescent dye, Laurdan, which has been used to study phase properties of membranes as it is sensitive to the polarity of the membrane environment (Learmonth and Gratton, 2011). GP (Generalized Polarization) values, which inversely correlate with fluidity, were calculated at six timepoints during the growth of AJ4, MY3, MY14, MY26 and MY29 strains in GPY, GPY containing 6% ethanol and GPY containing 10% ethanol. The assay suggests that the fluidity of the yeast membranes decreases with culture time as shown by the increase in GP (Figure 1.9).

AJ4 and MY14 strains demonstrated large changes in fluidity when treated with 10% ethanol (AJ4 showed a GP value change of -0.0002 ± 0.0009 at 10% and a GP value change of 0.0233 ± 0.0025 at 0% and MY14 showed a GP value change of -0.0101 ± 0.002 at 10% and a GP value change of 0.009 ± 0.002 at 0%) ($P < 0.001$ and $P < 0.01$, respectively). MY29 also became significantly more fluid at 10% ethanol (GP value change of -0.0016 ± 0.0011 at 10% and a GP value change of 0.0084 ± 0.0019 at 0%) ($P < 0.05$) . However, these strains did not show any increases in fluidity with 6% ethanol. The other strains showed no significant differences to fluidity with ethanol treatment. It is notable that the most tolerant strains show the largest increases in membrane fluidity in response to ethanol exposure.

To examine membrane permeability, we investigated the integrity of liposomes composed of lipids extracted from each of the strains and loaded with carboxyfluorescein (CF) dye. The liposomes were challenged with increasing concentrations of exogenous ethanol, and the fluorescence increase from CF dye release was measured. The data in Figure 1.10 shows that the liposomes containing lipids extracted from AJ4 demonstrated a signigficantly greater increase in fluorescence at high ethanol concentrations than those composed of lipids from the other strains

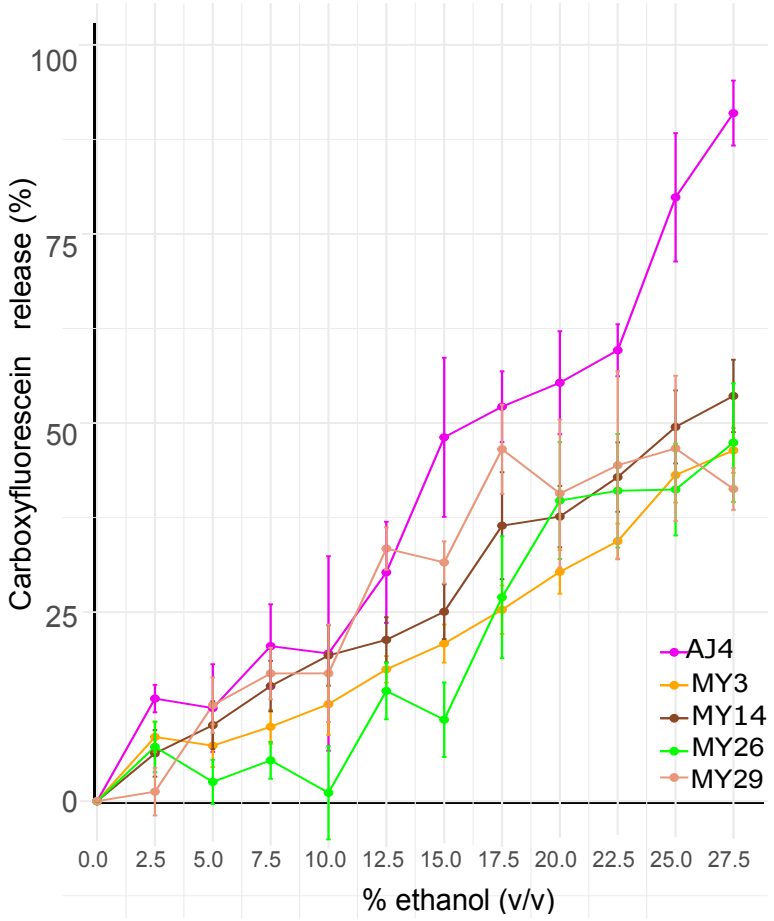


FIGURE 1.10 The effects of ethanol upon liposomes composed of lipids extracted from AJ4, MY3, MY14, MY26 and MY29 strains normalized to the maximum amount of dye released upon treatment with 5% Triton X-100.

(ANOVA and Tukey's multiple comparisons test (90.98 ± 4.29 fluorescence increase; $P < 0.001$). MY3 and MY26 liposomes were less "leaky" overall (46.38 ± 2.97 and 47.41 ± 7.84 of fluorescence increase). This increase in fluorescence indicates increased "leakiness" of the membranes.

1.3.6 Principal component analysis of the 5 strains

With the aim of grouping the 5 selected strains based on their lipid composition and their ethanol tolerance, the data obtained in the previous sections was used to perform a PCA (Figure 1.11). The data from the variables NIC, MIC, and the drop test growth value at 14% and 16% of ethanol in the plates, related to the ethanol tolerance were used. For the lipid composition, the data of the carboxyfluorescein release at the last time point; the data from the Laurdan experiments of the differential GP value at 10% of ethanol and when no ethanol is present in the last time point, and the PE abundance at 0% and 6% of ethanol in the media was used. The two commercial wine strains MY3 and MY14 group together, and MY26 (the most sensitive to ethanol) and AJ4 (the most tolerant) are the two strains that show more differences among them. It is interesting to note that MY26 is associated in the PCA with an accumulation of PE in the membrane at low ethanol concentration and a higher membrane rigidity, and the most tolerant stain, AJ4, associated with a high membrane fluidity in the presence of ethanol.

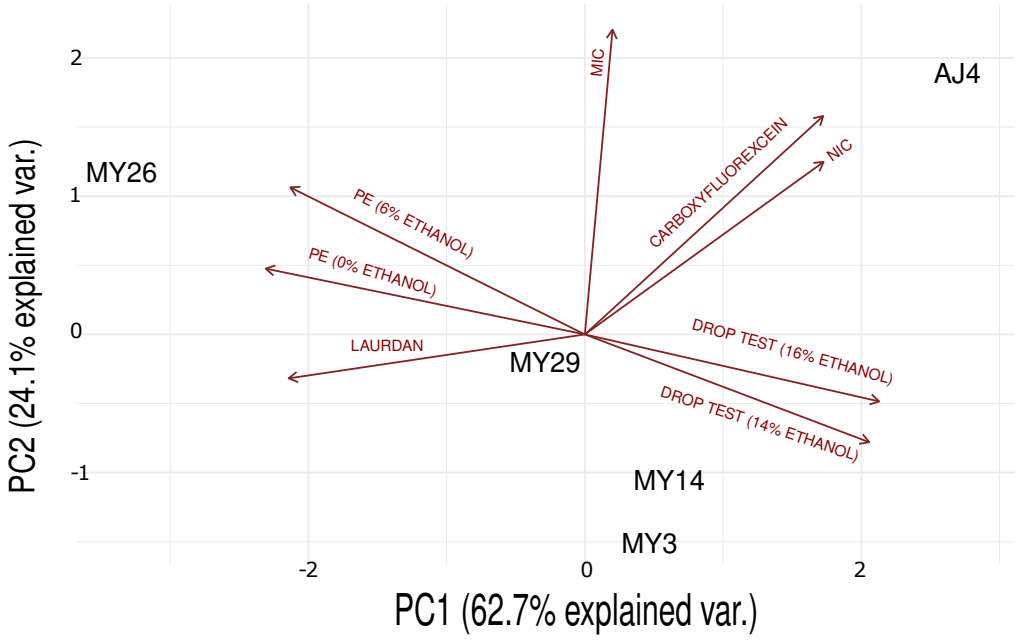


FIGURE 1.11 PCA of the five *S. cerevisiae* selected strains regarding their lipid composition and their ethanol tolerance.

1.4 Discussion

In this study, we investigated the membrane properties of the selected yeast strains to try to understand their different levels of ethanol tolerance. The mass spectrometry analysis of the lipid composition of each strain in the absence of ethanol highlighted differences in particular between MY29 and the other strains, not only in the variety of species observed for the lipid classes but also in their saturation. MY29 is a flor yeast. These yeasts constitute a separate phylogenetic group within *S. cerevisiae* species. They are characterized by forming a layer on top of wine known as flor, which allows them to access the oxygen during the fermentation of sherry wines, so they show different behavior and thus, physiological characteristics to wine yeast. Moreover, they have been reported to survive under extreme conditions (ethanol content over 15%) (Aguilera et al., 2006; Naumov, 2017) which could relate to their membrane structure.

Upon treatment with 6% ethanol, the lipid composition of MY29 underwent significant changes;

the composition was then found to be more similar to that of the other strains, suggesting that the membrane of MY29 underwent more drastic changes than the other strains in response to ethanol. The lack of significant differences at 6% ethanol suggests that each of the strains move towards a more common lipid composition in response to ethanol. However, despite the fewer differences to lipid composition at 6% ethanol between the strains, MY29 dominated the fermentation at this concentration. In addition, the lipid composition of AJ4 was not significantly different from the other strains at 6% ethanol, although it is the most tolerant to ethanol. It is possible that there may be further adaptation of the membrane at higher ethanol concentrations than were investigated in this study, but it is likely that other factors contribute to the ethanol tolerance of these strains. Indeed, this has been suggested by other studies, where the relationship between H+-ATPase activity and ergosterol content as well as the sterol to phospholipid and protein to phospholipid ratios are important (Aguilera et al., 2006; Alexandre et al., 1994; Shobayashi et al., 2005). Ethanol tolerance is a complex phenotype, and different mechanisms may lead to improved tolerance. Fluidisation of the yeast membranes by ethanol is also known to activate the unfolded protein response (UPR), and it is speculated that a better response could lead to greater tolerance (Navarro-Tapia et al., 2018). Moreover, yeast cells can increase their tolerance to ethanol by other mechanisms, such as the increase the biosynthesis of some amino acids, as tryptophan (Yoshikawa et al., 2009) and trehalose accumulation (Bandara et al., 2009).

Nevertheless, it is striking that yeast species with different membrane compositions in the absence of ethanol, become more similar upon exposure, suggesting a common, or limited number, of membrane compositions that maximize tolerance to ethanol.

Incorporation of longer acyl chains and a decrease in shorter chains has previously been shown to occur in yeast in response to ethanol (Chi and Arneborg, 1999a; You et al., 2003); however, we did not observe any significant changes in chain length. Our study does suggest that there were significant differences in saturation between the species upon ethanol treatment. These changes occurred in GPGro and GPEth in MY29, and occurred predominantly in DG and TG for the other strains, with shifts towards increased saturation for AJ4 and increased unsaturation for MY3 and MY14. These changes appear to be complex and specific to each strain. Documented

changes to the membrane of yeast upon ethanol challenge are conflicting (Henderson and Block, 2014); while some studies have shown that increased levels of unsaturated fatty acids are linked to improved ethanol tolerance (Chi and Arneborg, 1999a), changes to the unsaturation index may not necessarily be associated with improved tolerance, or lead to the expected changes in membrane fluidity and it is rather the potential of the cell to alter its composition (Alexandre et al., 1994; Huffer et al., 2011). The lipid membrane is a highly complex environment and multiple factors can influence membrane fluidity and permeability. Further study of these strains is required to determine if their different compositions have similar biophysical properties.

We investigated the fluidity of the membranes and the Laurdan assay demonstrated that the fluidity of the membranes for each strain decreased over the duration of the fermentation, which has been observed previously (Ishmayana et al., 2017), and may be linked to nutrient depletion and changes in the growth rate of the cells. In our study, the most tolerant strain, AJ4, underwent the largest changes in fluidity, where the membranes were significantly more fluid at 10% ethanol than in the other conditions. AJ4 lipid-containing liposomes were also the “leakiest” when compared to the other strains. This strain may therefore be better able to tolerate the fluidising effects of ethanol upon the membrane or to modulate its membrane composition to lead to an increase in fluidity; this more fluid composition may allow more efficient movement of ethanol across the membrane. The membranes of one of the least tolerant strains, MY26, did not alter in fluidity in any of the conditions and liposomes comprised of MY26 lipids were less leaky when challenged with ethanol. In addition, our analysis of PE abundance shows that MY26 contained significantly more PE than the other strains in both 0% and 6% ethanol, while the most tolerant strain, AJ4, contained less PE in general than other strains. PE has a small headgroup and can form hydrogen bonds with adjacent PE molecules (Murzyn et al., 2005). It influences lipid packing and therefore membrane fluidity, where increased PE content results in less fluid membranes (Ballweg et al., 2020; Dawaliby et al., 2016), consistent with our hypthesis. Lower PE content in relation to PC has been correlated with more tolerant strains (Chi and Arneborg, 1999a; Jurešić et al., 2009). These findings suggest that more tolerant strains are more fluid and permeable, while less tolerant strains are more rigid and less permeable. Several studies have correlated membrane fluidity and

ethanol tolerance, and many of these point to increased fluidity being associated with more tolerant strains (Alexandre et al., 1994; Huffer et al., 2011), although another study suggests that less fluid membranes are associated with more tolerant strains (Ishmayana et al., 2017). In this study we provide further support for the concept that a low PE content is beneficial for ethanol tolerance. This result can guide engineering to improve ethanol tolerance towards the reduction of PE synthesis. This compound is produced by four separate pathways, but the Psd pathway, which utilizes PS as a substrate is the predominant in *S. cerevisiae* (Birner et al., 2001; Bürgermeister et al., 2004), so future works can be addressed in this direction.

In summary, the lipid composition of most of the yeast strains in this study were comparable but there were significant differences between these and the MY29 strain. Upon ethanol treatment, this composition changed significantly and a more similar composition was reached, suggesting an adaptation mechanism in common with the other strains. Changes in saturation were observed for each of the strains upon ethanol treatment, but it is not clear if these changes have a direct impact upon fluidity and tolerance, and it is likely that other factors beyond the scope of this study play a critical role and further investigation is needed. The PE abundance of the least tolerant strain, MY26, was significantly higher than in the other strains. Our investigation therefore suggests that the membranes of more tolerant strains are more fluid and contain less PE. Overall, our results point to a reduced set of desirable membrane compositions and features that promote ethanol tolerance with increased fluidity and permeability appearing to be key.

CHAPTER 2

Transcriptome analysis in *S. cerevisiae* strains under ethanol stress reveals different specific responses related to the synthesis of membrane lipids

2.1 Introduction

During fermentation processes, yeasts have to cope with a wide variety of environmental stresses, including low and high temperatures (Cardona et al., 2007), high sugar concentrations (Charoenchai et al., 1998), oxidation (Sha et al., 2013), and ethanol accumulation (Bauer and Pretorius, 2000). Among them, ethanol toxicity is considered the primary factor limiting the fermentation process (Kasavi et al., 2016; Lam et al., 2014). Ethanol reduces cell growth, limits cell cycle and also alters many functions in microorganisms, such as lipid and amino acid metabolism, trehalose biosynthesis, and mitochondrial function (Snoek et al., 2016; Navarro Tapia, 2016). Moreover, cell membranes are primary targets of ethanol presence, and they are particularly affected by its presence (Alexandre et al., 1994; Beaven et al., 1982).

Due to the relevant and limiting role of ethanol toxicity during fermentations, many studies have addressed how this compound affects the behavior of industrial yeast strains of the *Saccharomyces* genus. The response of yeast to ethanol stress is associated with general stress response mechanisms (Alexandre et al., 2001; Stanley et al., 2010a). However, it is possible that apart from general responses shared by this genus, each *Saccharomyces* strain has its strategy to cope with ethanol present in the media (Kasavi et al., 2016).

At the same time, the fatty acid compositions of lipid membranes have been associated with the ethanol tolerance of different *Saccharomyces* strains (Ghareib et al., 1988; Mishra and Prasad, 1989; Sajbidor et al., 1995; You et al., 2003). Even though, the underlying mechanisms for these associations remain unclear and more evidences are needed to understand how the ethanol presence can change gene expression of these involved in lipid biosynthesis and thus lipid membrane composition.

Over the past decade, transcriptomic analysis are gaining popularity as they provide a precise and comprehensive technique to measure levels of transcripts in a determined biological context (Li et al., 2017; Stark et al., 2019). The advent of next generation sequencing (NGS) technologies has developed RNA sequencing (RNA-seq), which is an indispensable tool for transcriptome

wide analysis in the form of differential gene expression which enable the comparison between conditions and strains (Li et al., 2017; Stark et al., 2019). Many transcriptomic analyses after the exposure of selected *S. cerevisiae* strains to ethanol stress conditions have been carried out, revealing particularities for each strain in response to ethanol (Alexandre et al., 2001; Li et al., 2010, 2017; Navarro-Tapia et al., 2016; Stanley et al., 2010b).

The aim of this study was to further investigate molecular mechanisms and pathways leading to different ethanol tolerances in three *S. cerevisiae* strains, especially those that affect membrane composition. The novelty of this study is that these *S. cerevisiae* strains have an industrial interest and that they have been previously characterized for their ethanol tolerance and membrane composition. For this purpose, the genome-wide transcriptional responses of three *S. cerevisiae* strains selected in the previous chapter (MY3, MY26, and AJ4) were investigated in the absence and in the presence of ethanol at three different time points.

2.2 Materials and Methods

2.2.1 Strains' selection

Three strains: AJ4, MY26, and MY3, which show different ethanol tolerances and whose lipid compositions have been characterized in the previous chapter (Lairón-Peris et al., submitted) were used. AJ4 is a strain from Lallemand used in fermentations and it is a high ethanol tolerant strain; MY3 is a wine strain from Lallemand, mainly used in the fermentation of rosé and red wines and it is a tolerant strain to ethanol; and MY26 is a strain used in the Agave fermentation, and it was described as a low tolerant strain. The three strains are diploid, with no aneuploidies on their genomes.

2.2.2 Growth conditions and experimental design for acquiring the biological samples

The experimental design consisted in growing the three yeast strains in GPY media with 0%, 6%, and 10% of ethanol. The growth of the strains was followed by OD measurements and samples were extracted in triplicate for posterior transcriptomic analyses.

First, strains were inoculated in GPY media without ethanol, and after a short acclimatization phase of 1h, ethanol was added in some cultures to reach a final ethanol concentration of 6% and 10% respectively. The remaining cultures in which no ethanol was added were used as a 0% ethanol growing condition (control without ethanol). Samples were retrieved at four different time points: t0 (before the ethanol addition, which is a control time point), t1 (early exponential phase or EEP), time t2 (late exponential phase or LEP), and t3 (stationary phase or SP).

A high cell quantity was needed in the samples for extracting a sufficient amount of RNA from these cells. An overnight preinocula for each one of the three strains was done in 500 mL of GPY (OD_{600}=0.2). The next day, 1 L Erlenmenyer flasks containing 750 mL of GPY media were inoculated with an OD_{600}=0.2.

We prepared a total number of 63 flasks, 21 flasks per strain: AJ4, MY3, and MY26 (21 x 3) and 7 flasks per ethanol condition: 0%, 6% and 10% of ethanol (7 x 3). Fermentations were carried out at 28ºC with orbital agitation at 150 rpm. Strain's growth at the three ethanol conditions were followed by measuring the OD_{600} at different time points.

At t0 we extracted the total volume of three of the flasks per strain. These samples were treated as control samples. Then, we added ethanol to the media in the other 18 flasks per strain (6% in 9 of the flasks and 10% in the other 9, respectively). Cell samples were retrieved at three more time points: t1 using the entire volume of a flask; and time t2 and t3 using the volume of the other flask. Cells were harvested by centrifuging and then stored at −80ºC. A scheme of the retrieved samples can be seen in Figure 2.1.

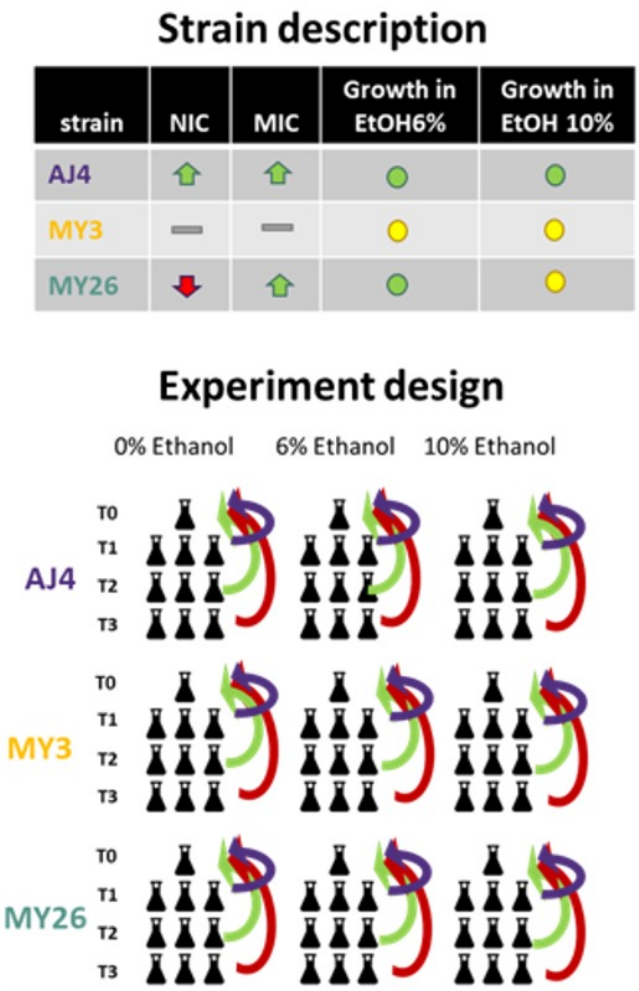


FIGURE 2.1 Experiment design of the samples retrieved during the growth of the three strains under ethanol stress conditions. AJ4, MY3, and MY26 *Saccharomyces* strains were grown under ethanol and non-ethanol media and samples were retrieved at different time points: t0, t1, t2, and t3. The NIC and MIC parameters were calculated in a previous work(Lairón-Peris et al., submitted, Chapter 1.3.3)

2.2.3 RNA-seq analysis

Extractions of total RNA were carried out following a protocol based on phenol chloroform as in Lairón-Peris et al. (2020). It consisted in an initial step of washing the cells with DEPC-treated water; subsequently, cells were treated with phenol-tris, phenol-chloroform (5:1), and chloroform-isoamyl alcohol (24:1). The two final steps consisted in two precipitations with LiCl and ethanol with sodium acetate respectively. The obtained RNA was sequenced (Illumina Hiseq 2000, paired end reads 75 bases long).

The obtained sequenced reads were quality trimmed using sickle (length 50, quality 23) and aligned to the *S. cerevisiae* pangenome from Peter et al. (2018) reference using bowtie2. The mapped reads were subsequently counted using htseq-count (union mode) (Anders et al., 2015). We used a new pipeline to generate these counts using a pangenome and a nucleotide blast search for the detection of non-reference ORF (Alonso-del-Real and Morard, in preparation). The pangenome consists of 7,796 ORFs collected from sequencing 1,011 strains. After the mapping of the sample reads using this pipeline, each strain presented a different number of expressed ORFs: 5,495 AJ4; 5,655 MY26, and 5,304 mapped MY3.

The R software was used for statistical analyses (R Core Team, 2013). The data was imported, processed, and normalized by removing low expressed genes and using the variance stabilizing transformation method implemented in DESeq2 (Love et al., 2014). The data sets were then transformed using the limma package (v.3.32.2) (Ritchie et al., 2015). Limma voom was used to transforms counts to log-cpm and then, differential expression analysis was performed using limma. Differentially expressed genes with an adjusted P value lower than 0.05 (Benjamini Hochberg correction) (Benjamini and Hochberg, 1995) were used to GO terms enrichment search by using Funspec (Robinson et al., 2002) (p-value < 0.05, Benjamini Hochberg correction).

2.3 Results

2.3.1 Differential growth in ethanol of the three strains

AJ4, MY3, and MY26 are three *S. cerevisiae* strains from isolation sources related to fermentative environments, that were previously characterized as high, moderately and slightly tolerant to ethanol, respectively (Lairón-Peris et al., submitted). They were cultivated in rich media at 0%, 6%, and 10% ethanol, and their growth was followed by OD_{600} measurements (Figure 2.2).

After their growth in GPY + ethanol, different behaviors were observed. AJ4 growth was similar in GPY without ethanol and in GPY+6% of ethanol. MY26 growth showed the same behavior, but at GPY+10% of ethanol, its growth was dramatically reduced. Regarding MY3, it was the most affected strain by the presence of ethanol at a concentration of 6%, but it showed better growth than MY26 at a concentration of 10% of this compound.

The three sampling points were selected based on these growth curves, to capture samples in a similar growth phase instead of strictly refer to the same time point. However, exact times in hours are depicted in Table 2.1.

TABLE 2.1 Time points in which samples from the three *S. cerevisiae* strains growth in ethanol were collected

	t1	t2	t3
AJ4 0%	4h	9h	24h
AJ4 6%	4h	9h	24h
AJ4 10%	5h	23h	30h
MY3 0%	4h	8.5h	24h
MY3 6%	4h	9.5h	24h
MY3 10%	5h	23.5h	29.5h
MY26 0%	4h	8h	24h
MY26 6%	5h	8h	23h
MY26 10%	4h	28h	43h

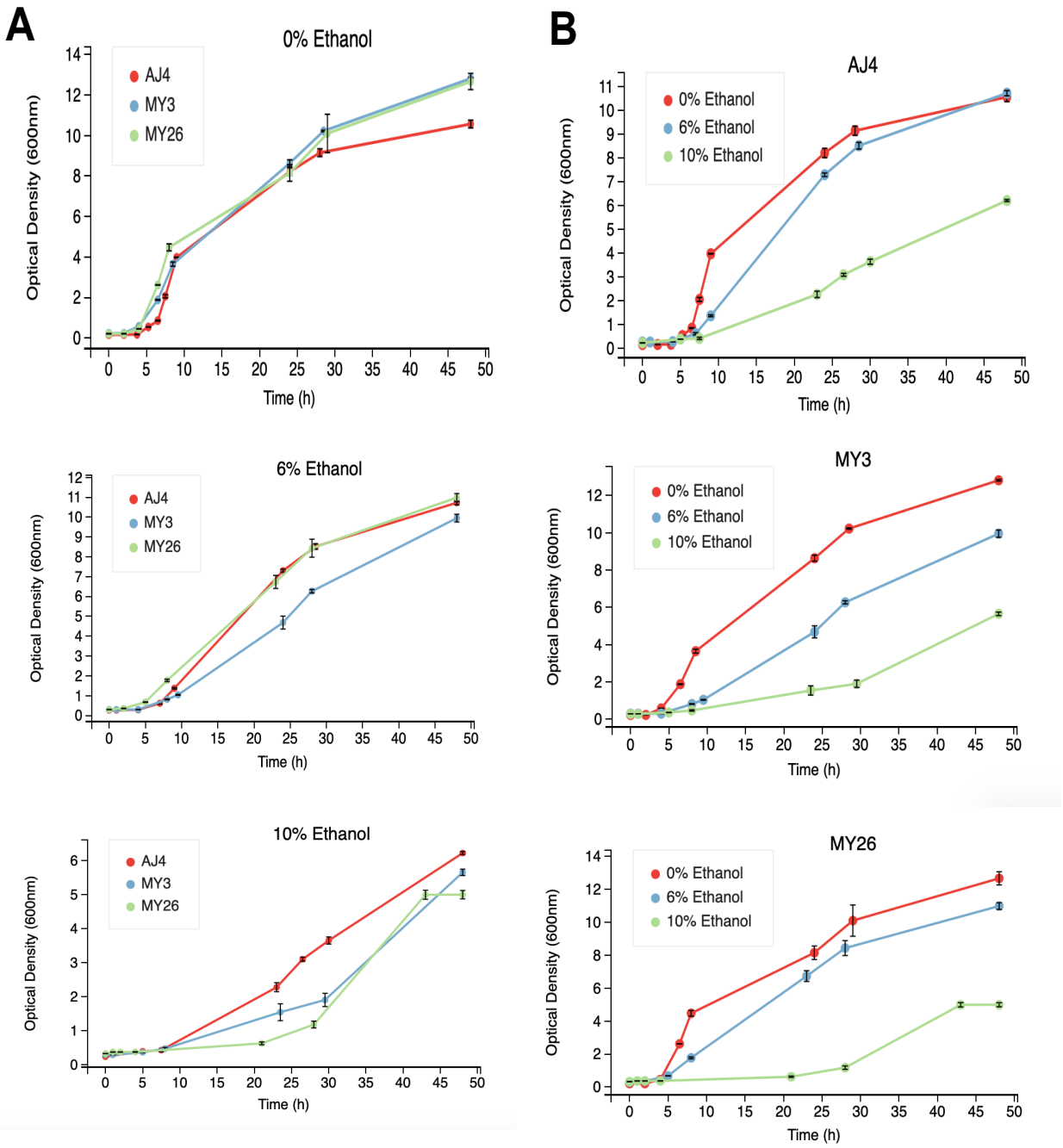


FIGURE 2.2 Growth analysis of strains AJ4, MY3, and MY26 in GPY media with 0%, 6% and 10% of ethanol. Samples were grouped by ethanol concentration (panel A) and by strain (panel B) for its easier visualization.

2.3.2 Transcriptomic analysis of AJ4, MY3, and MY26 during their growth in ethanol media

To elucidate the differential transcriptomics response to ethanol that might explain the range of sensitivities to ethanol, samples were retrieved during different time points for their RNA-seq analysis. The first sampling was carried out before ethanol addition (t0). Further cell samples were taken at the early exponential phase (t1), late exponential phase (t2) and stationary phase (t3) (Figure 2.1).

Thus, establishing t0 as the reference in our experimental design, we focused on the genes that were differentially expressed (DE) at one time point and ethanol condition exclusively in a strain with respect to another. The large amount of strain exclusive genes is indicative of highly variable transcriptome profiles among the studied strain (Figure 2.3). In addition, these exclusive genes are variable depending on the ethanol presence.

Genes related to lipid metabolism and membrane homeostasis were investigated with special attention by filtering out all the genes not present in the functional categories plasma membrane (GO:0005886) or lipid metabolism (GO:0044255) from to Gene Ontology (Ashburner et al., 2001). The lists of the functional enrichment analyses can be seen in Table 2.2 and Table 2.3.

Interestingly, in ethanol 6% and 10% conditions, protein-coding genes involved in ergosterol biosynthesis were repressed at every time point in the case of MY26 and MY3 strains, but not in AJ4. In contrast, the expression of these genes seemed to be similar for all strains in absence of ethanol (Figure 2.4A).

Looking at the expression of every single gene in the pathway, it is clear that most of them were repressed in the cases above mentioned, rather than one or few genes standing out (Figure 2.4B). Following the same tendency, *ERG20* and *ERG1* genes were strongly overexpressed in AJ4 in ethanol presence, moderately overexpressed in MY3 in some cases, and not overexpressed at all in MY26 (Figure 2.4C).

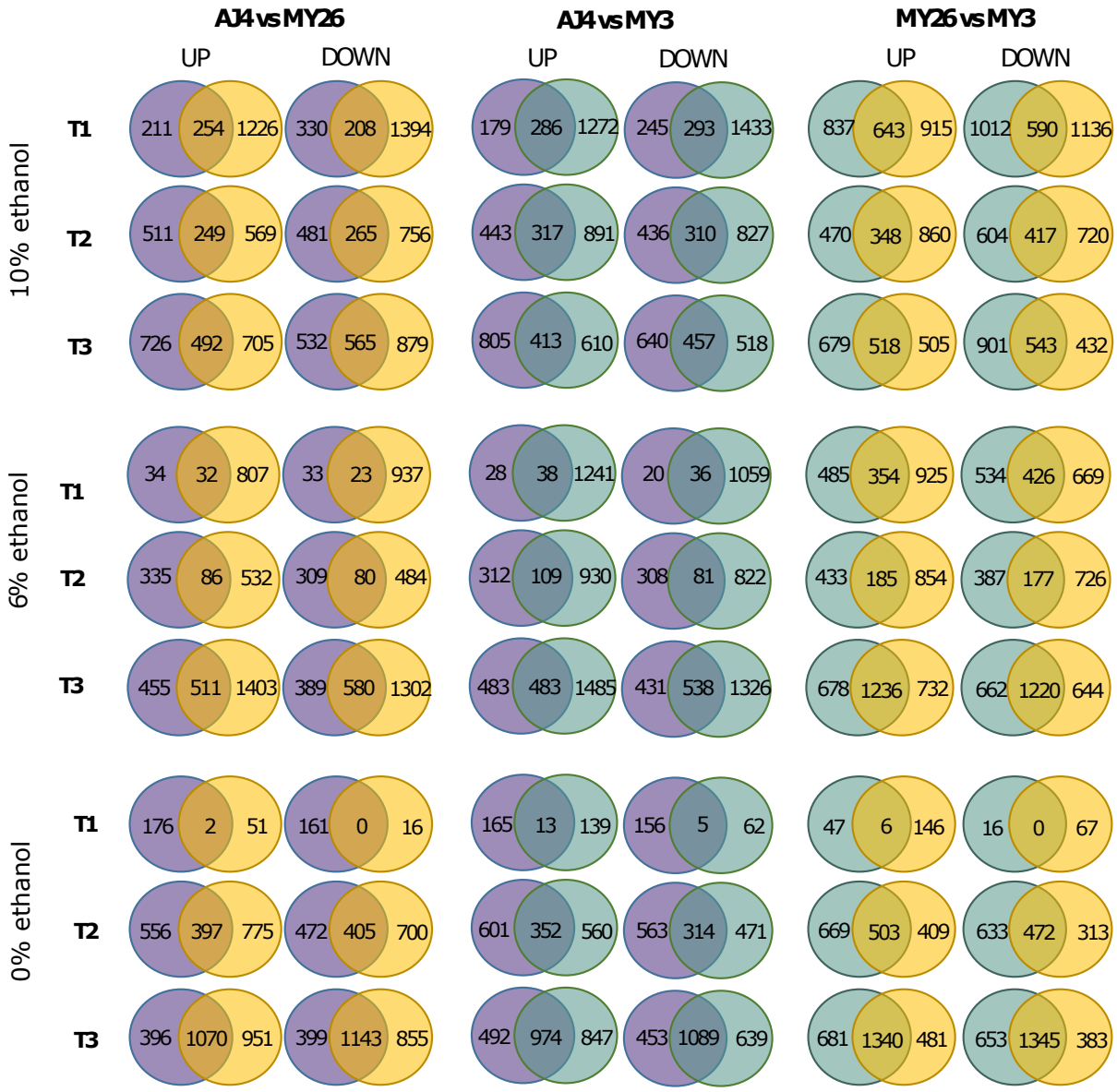


FIGURE 2.3 Venn diagrams with the number of exclusively up-regulated and down-regulated genes for AJ4, MY3, and MY26 strains. These samples were retrieved at 3 time points (t3, t2, t1) and compared with the t0 gene expression at the three ethanol conditions (0, 6 and 10% of ethanol in GPY media). Genes were retrieved after carrying out the DE analysis if their adjusted p-value (BH correction) was lower than 0.05.

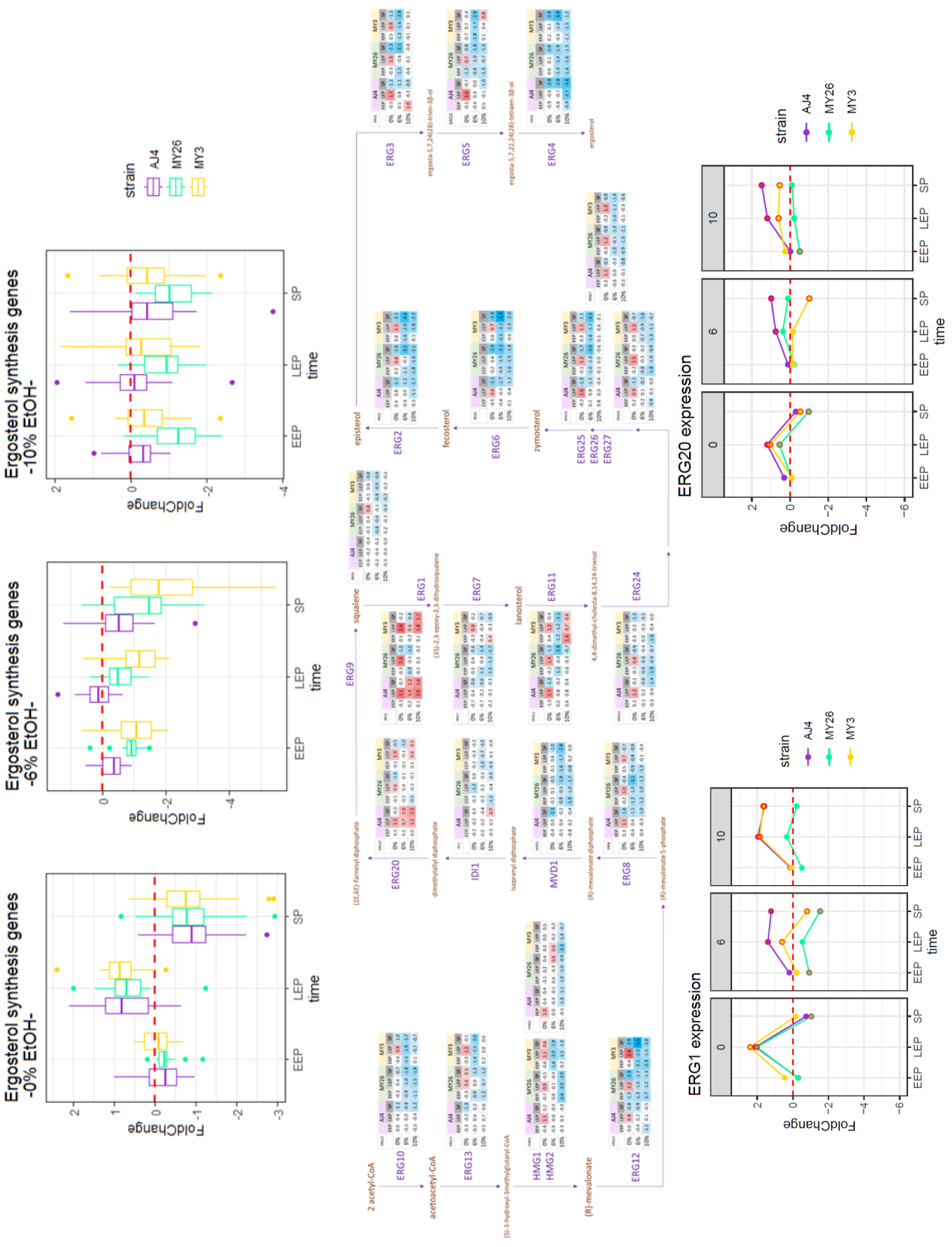


FIGURE 2.4 Differential expression analysis of genes involved in ergosterol synthesis. Fold change value from the global analysis of ergosterol synthesis genes and of each ergosterol gene is depicted, when compared with each strain (AJ4, MY3 and MY26) at three ethanol concentrations and time points with the basal level of expression at t0 with no ethanol added in the media.

Among the main biosynthesis processes of membrane lipids, there were others with genes that presented a differential behavior among strains. It is the case of *HMN1*, the transporter of phospholipid precursors such as choline and ethanolamine, and *EKI1*, the first enzyme in the transformation of ethanolamine into PE (phosphatidylethanolamine). Both genes presented similar expression dynamics in absence of ethanol for the three studied strains. However, we observed that their expression changed in the presence of ethanol, being AJ4 the one keeping higher transcriptional levels for both genes (Figure 2.5A). Besides, *OLE1*, responsible for the desaturation step in the synthesis of oleic and palmitoleic acid, was repressed in MY26 under alcoholic conditions. In contrast, AJ4 and MY3 showed higher expression levels (Figure 2.5B).

Interestingly, all these genes are regulated by the transcription factor Ino2p according to Yeastract (DNA binding and expression evidence) (Monteiro et al., 2020). A multiple alignment of the Ino2p protein sequence from our three strains revealed two variations in AJ4 with respect to the other strains: H86R and V263I, but the totality of the amino acidic changes is marked as conservative by Clustal Omega (Sievers and Higgins, 2014). We carried out a multiple alignment of sequences of the protein Ino2p from 979 different strains (Peter et al., 2018) plus our three sequences in order to assess the presence of the same mutations observed in AJ4 in other strains. V263I is present only in other 9 strains, and H86R can be found in these same 9 strains as well as in other 5 strains. To question the possible impact of these variants on ethanol tolerance further data by Peter et al. (2018) work was used. This work included a phenotypic assay under multiple conditions for all the strains we mentioned, including growth in a medium containing 15% ethanol. The strains presenting exclusively the H86R mutation have relatively moderate ethanol tolerance, however, the strains presenting both mutations are clearly above the median (Figure 2.6B). Ino2p C-terminal domain has been proved to interact with Ino4p (Schwank et al., 1995), forming a dimer required for derepression of inositol-choline-regulated genes involved in phospholipid synthesis.

When selecting Yeastract ”without expression evidence” option for the same group of input genes, Gnc4p appeared as the first hit. This activator of transcription presents changes in its sequence for AJ4 compared to MY3 and MY26: D91A, D196E and N275K. Again, multiple alignment analysis with the 979 strains was performed. A91, A196, and N275 in AJ4 are the most

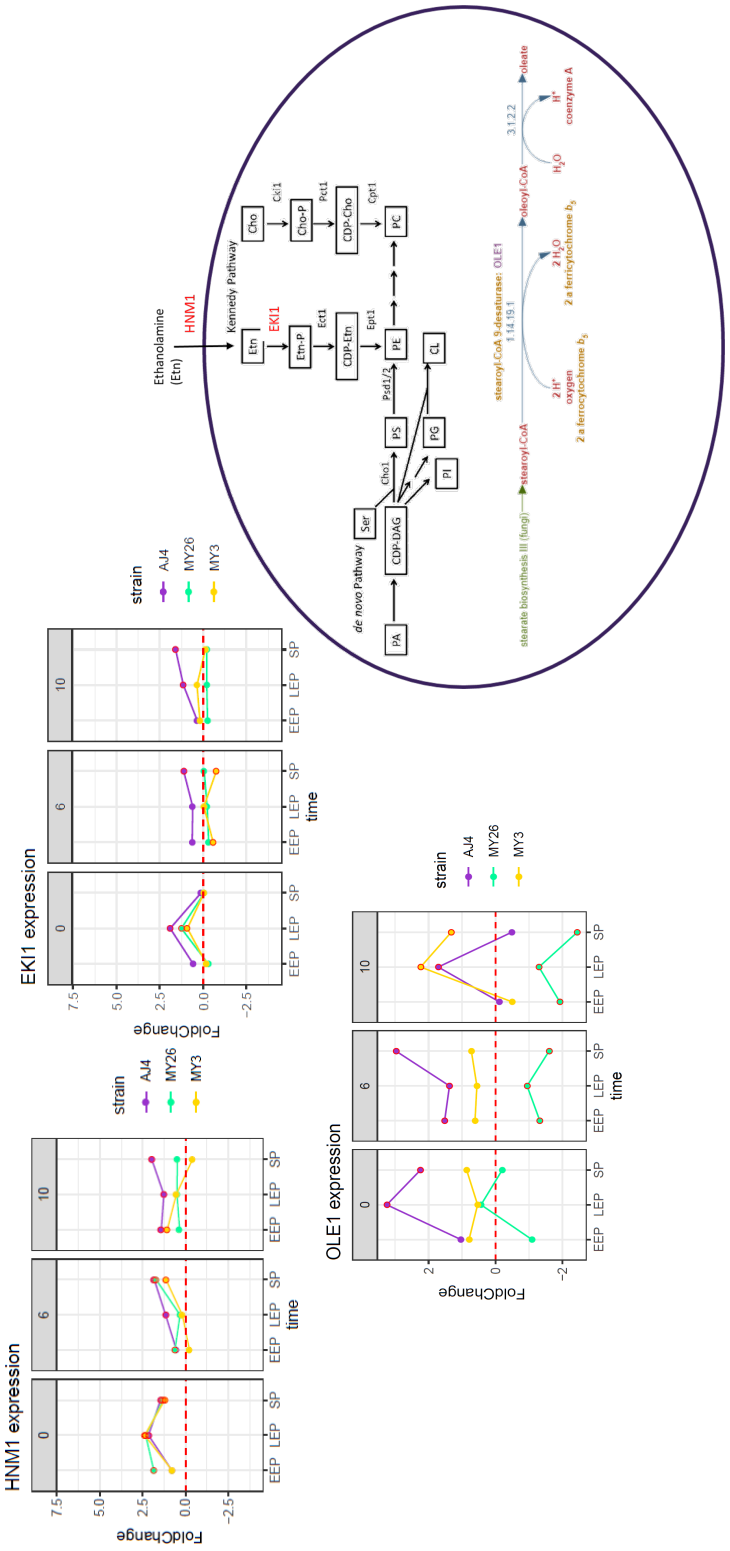


FIGURE 2.5 Differential expression of genes *HMN1*, *EKI1* and *OLE1* in AJ4, MY3 and MY26. Samples were retrieved at three time points EEP, LEP and SP corresponding to early exponential phase, late exponential phase, and stationary phase respectively, and at three ethanol concentrations and compared with the expression at the latency time point without ethanol. In the right part of the figure, a scheme representing the synthesis rout of different phospholipids in which *HMN1* and *EKI1* genes are involved is depicted too.

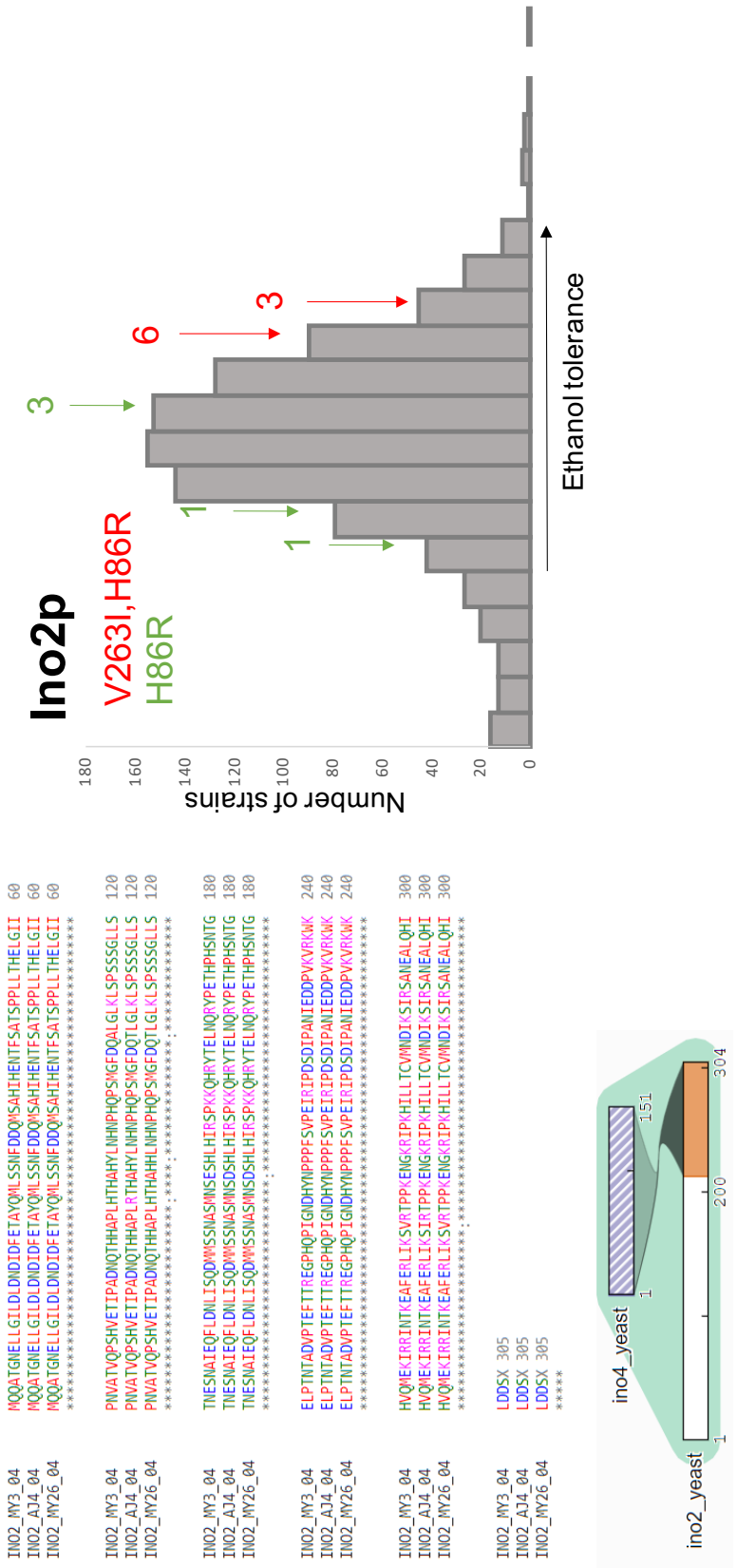


FIGURE 2.6 Changes in Ino2p transcription factor. Alignment of Ino2p sequence of AJ4, MY3 and MY26 strains (A) and representation of the ethanol tolerance of 979 strains (Peter et al., 2018), indicating the presence of mutations in V263I and in H86R in Ino2p (B)

common amino acids for these positions (83%, 80% and 98% of the total amount of compared strains, respectively). However, *GCN4* expression could have a role, since it was found to be overexpressed in AJ4 in several of the studied conditions (Figure 2.7).

2.4 Discussion

Multiomic approaches are largely contributing to the understanding of the mechanisms underlying differential yeast tolerance to different stresses. Thanks to these approaches, a better comprehension of yeast behavior under certain stressing conditions -high and low temperature, ethanol, availability of nitrogen, etc.- is nowadays possible.

Ethanol toxicity is the condition which more remarkably affects yeast cells used in industrial biotechnology. For that reason, elucidation of ethanol tolerance mechanisms in yeast is essential for understanding the role of specific genes and therefore apply more directed methodologies to improve strains which can provide more sustainable processes.

In previous studies, ethanol has been associated with different molecular changes. Recently, ethanol stress has been correlated with the activation of the unfolded protein response (UPR) in *S. cerevisiae* strains (Navarro-Tapia et al., 2016). The UPR is a conserved signalling pathway which is activated to counteract stresses, thus activating homeostasis mechanisms in yeast cells.

Moreover, the presence of ethanol has been associated with membrane lipidic changes. In Navarro-Tapia et al. (2017), the UPR was further studied revealing that when inositol levels are low this pathway is also activated. Inositol is an important component of the structural lipids, and in Navarro-Tapia et al. (2018) it was observed that after ethanol stress, key genes involved in lipid metabolism, like *INO1* were up-regulated at the same time that the UPR was activated. These authors proposed that membrane fluidification -caused by either ethanol or other agents- activate the UPR (Navarro-Tapia et al., 2018) and that this activation leads to changes to counteract and better resist environmental changes in yeast cells.

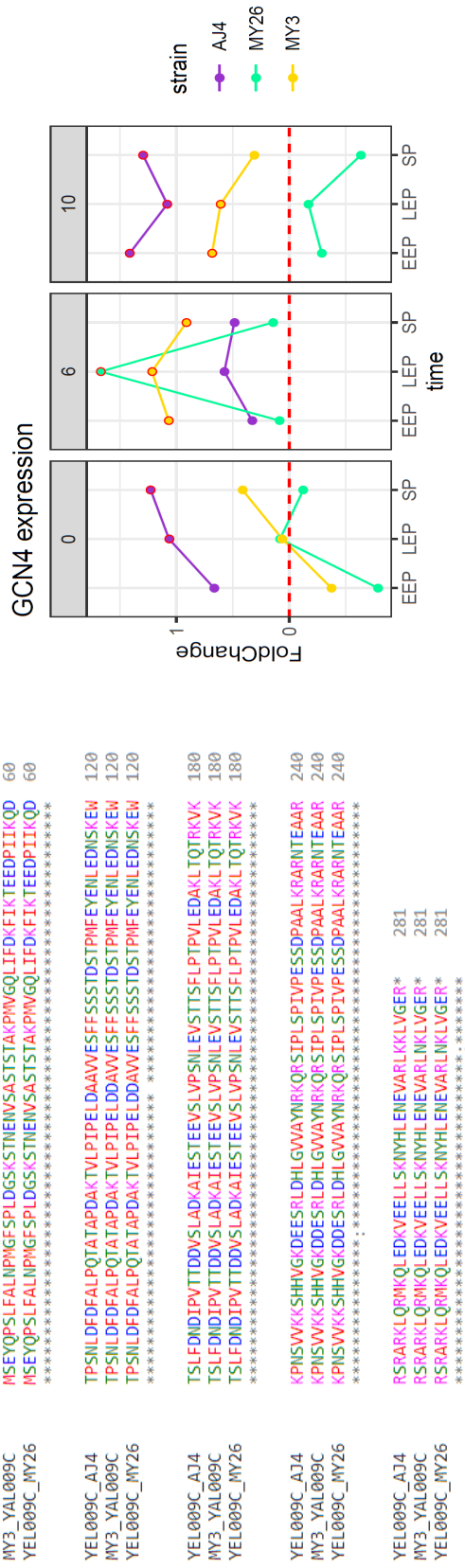


FIGURE 2.7 Alignment of YEL009C (*GCN4*) gene sequence of AJ4, MY3 and MY26 strains (A) and representation of its log fold change expression (B). Three time points (EEP, LEP and SP) and three ethanol conditions (0%, 6% and 10%) were used for this analysis.)

In this work, we have focused our analysis in gene expression in three *S. cerevisiae* strains under different ethanol concentrations and stages, paying special attention to differential changes which affect the lipid yeast membranes.

Different genes involved in ergosterol synthesis were more up-regulated in AJ4 strain under ethanol concentrations in comparison with MY3 and MY26 strains. Ergosterol is the predominant sterol in yeast, and it plays an important role in modulating the lipid membrane, especially in the presence of high ethanol (Bisson, 1999; Ma and Liu, 2010; Vanegas et al., 2012; You et al., 2003). As AJ4 is the most ethanol tolerant strain of the three strains evaluated, we propose that its differential expression of ergosterol synthesis genes could have changed the lipid composition of the membrane of this strain under ethanol growth, thus providing a better tolerance to ethanol.

We also observed up-regulation in genes related with the biosynthesis of membrane phospholipids, such as *HMN1* and *EKI1* in AJ4 strain under ethanol growth. These genes have a consensus sequence (5'-CATGTGAAAT-3') in their promoter region, which is known as the inositol-sensitive upstream activation sequence (UAS_{INO}) (Bachhawat et al., 1995; Wimalarathna et al., 2011). Ino2p protein activates their transcription in response to inositol depletion. When inositol levels are low, Ino2p and Ino4p proteins interact and form an heterodimer which binds to this promotor fragment (UAS_{INO}), activating the transcription of genes related with phospholipids biosynthesis (Bachhawat et al., 1995; Jesch et al., 2005; Kasavi et al., 2016).

The characterization of Ino2p sequence in the three strains, AJ4, MY3 and MY26 revealed that AJ4 Ino2p had two mutations in comparison with the other two strains. The ethanol tolerance analysis of 979 strains performed in (Peter et al., 2018) showed that strains containing these two mutations in Ino2p are above the median on its ethanol tolerance. These differences in the regulation of key membrane genes suggest important regulation mechanisms in which both the ethanol and the inositol are involved and whose response vary among *S. cerevisiae* strains and are in accordance with previous works (Navarro-Tapia et al., 2016, 2017, 2018).

Moreover, genes up-regulated in AJ4 under growth in ethanol conditions are also regulated by the transcription factor *GCN4*, which also presents changes in its sequence for AJ4 compared

to MY3 and MY26. Although Gcn4p has been described as a regulator of gene expression during amino acid starvation in yeast (Natarajan et al., 2001), its expression is also induced under other stress conditions besides, including growth on the non-fermentable carbon source ethanol (Hinnebusch and Natarajan, 2002; Yang et al., 2000). Taken together, our results showed that genes involved in the biosynthesis of membrane phospholipids had different expressions in the three selected *S. cerevisiae* strains when they were grown in ethanol media. This suggests that molecular regulation mechanisms involved in lipid biosynthesis are different in *S. cerevisiae* strains and could be the reason for the different ethanol tolerances found in the strains of this species.

However, further studies need to be done in order to confirm the function of the specific genes here analyzed, such as the transcription factor Ino2p and the *GCN4* gene. It would be necessary to perform specific allele changes in these strains, and then test the phenotypic effects of the changes. The available approaches to do that include reciprocal hemizygosity and allele swapping (Biot-Pelletier and Martin, 2016; Glazier et al., 2002; Kessi-Pérez et al., 2016; Mans et al., 2015; Parts et al., 2011; Salinas et al., 2016; Su et al., 2021; Tapia et al., 2018).

Despite this, ethanol tolerance in *S. cerevisiae* is regulated by several complicated and sophisticated systems. Thus, the ethanol tolerance can be expected to be a result from the collaboration of a group or many groups of genes. A further in-depth analysis should be conducted to decipher the general genetic pattern that determines yeast ethanol tolerance and its relation with membrane properties.

TABLE 2.2 List of GO retrieved from the list of unique differentially expressed genes in AJ4 and MY26. U means that the GO term genes are up-regulated and D that are down-regulated.

Ethanol	Time	MY26	AJ4
10%	1	**Ergosterol biosynthetic process (D) [GO:0006696]**: *ERG28 ERG26 ERG4 ERG7 ERG6 HMG1 ERG13 ERG5 ERG2 ERG8 ERG24 ERG10* **Isoprenoid biosynthetic process (D) [GO:0008299]**: *HMG1 ERG13 ERG8 MVD1 IDI1* **Fatty acid biosynthetic process (D) [GO:0006633]** *FEN1 OLE1 PHS1 ELO1 SUR4* **Carbohydrate transport (U) [GO:0008643]** *RGT2 MPH2 HXT7 HXT3 HXT10 HXT1 HXT14* **Integral to membrane (U) [GO:0016021]** *TAT1 ADP1 HSP30 ARE1 RGT2 MPH2 HXT7 HXT3 PDR15 PUG1 HXT10 YFR012W YGL010W SCS3 MTL1 FHN1 CHO2 SNG1 DUR3 HXT1 PDR11 ASG7 GEX2 ORM2 VID22 SCS7 CPT1 PRM1 HXT14 KRE1 GAS5 RSB1 YOR059C CRC1 MUM3 PDR10 YOR365C VTC3 KRE6*	**Amino acid transmembrane transport(D) [GO:0015171]** *AGP2 AGP1 CAN1 GAP1 DIP5* **Integral to membrane (D/U) [GO:0016021]** **D**: YAR028W *AGP2 PHO89 AGP1 SNQ2 ENA5* YDR089W YDR090C *GPI19 CAN1 PMA1 FLC3 MEP1 SFK1 GAP1 PTR2 GAL2 PUN1 GPI12 MEP2 YNL194C FRE3 SUR1 PDR12 DIP5* YPR003C *SGE1* **U**: *ITR1 FTR1 DNF1 HNM1 YSR3 VBA5 FPS1 GAS2 BUD8 NTE1 PHO84 GAS4 MCH5 SSU1*
10%	2	**Ergosterol biosynthetic process (D) [GO:0006696]**: *ERG7 ERG27 ERG6 HMG1 ERG2 ERG12 ERG10* **Integral to membrane (D) [GO:0016021]** YBR219C YBR220C *GNP1 ZRT1 TNA1 ELO1 ZRT2 HMG1 HXT2 VBA1 ERG2 TCB2 SLA2 ARE2 FRE4 ENB1 NRT1 TPO4 ERI1 ANT1*	**Transmembrane transport (U) [GO:0055085]** *FLC2 VBA2 SUL1 PCA1 ATO3 ITR1 STL1 ALR2 PMC1 HNM1 DUR3 PHO90 TPO5 STE6 AQR1 ALP1 THI72* YOR378W **Fatty acid biosynthetic process (U) [GO:0006633]** *OLE1 HTD2 OAR1 FAS1 FAS2* **Integral to membrane (U) [GO:0016021]** *FLC2 GPI18 CDS1 VBA2 SUL1 PCA1* YCR007C *IPT1 ATO3 PDR15 ITR1 STL1 STE2 ALR2 PMC1 OLE1 HNM1 BUD9* YGR149W *ERG1 DUR3 PHO90* YJR054W *TPO5 STE6 PUN1 NTE1 FKS3 GAS1 AQR1 ALP1 GAS4 THI72 PDR10* YOR378W *FRE3 FRE5 PMA2 SUR1 FLC1*
6%	1	**Ergosterol biosynthetic process (D) [GO:0006696]**: *ERG4 ERG11 ERG7 ERG3 ERG27 ERG12* **Fatty acid metabolic process (U) [GO:0006631]** *AGP2 FAA2 OAR1 CAT2 CRC1 MCT1* **Transport(U) [GO:0006810]** *TAT1 AGP2 PHO89 ADY2 KIN82 OSH2 SNF3 HXT7 PDR15 JEN1 YCT1 CAT2 LST8 YPT53 BIO5 PDR18 HXT17 RSB1 CRC1*	**Transport (D) [GO:0006810]** *ITR1 OLE1 HNM1 TPO2 VBA5 THI7 PHO84 AQR1 NRT1 MCH5*
6%	2	**Ergosterol biosynthetic process (D) [GO:0006696]**: *ERG4 ERG6 ERG12 ERG10* **Transport (U)** *TAT1 OSH2 DNF1 HXT10 ARN1 YHK8 OPT1 ALP1 HOL1 BIO5 RSB1 PDR10 FRE3*	**Transmembrane transport (U) [GO:0055085]** *FLR1 ENA5 BAP3 HXT3 HXT10 YGL114W MUP1 VHT1 MEP1 TPO2 MUP3 ARN2 DAL4 OPT1 TPO5 TE6 LYP1 THI72 TPO4 DIP5 SAM3 MEP3 SGE1*
6%	3	**Ergosterol biosynthetic process (D) [GO:0006696]** *ERG25 ERG11 ERG7 ERG13 ERG5 ERG12 ERG10* **Transmembrane transport (D) [GO:0055085]** *FLC2 RGT2 VBA4 GNP1 SPF1 FCY21 FTR1 FLC3 ZRT1 HXT4 PHO90 FPS1 ZRT2 NHA1 VBA1 ITR2 ENB1 NRT1 SSU1* **Integral to membrane (D) [GO:0016021]** *FLC2 CHS3 RGT2 MRH1 LCB2 VBA4 GPI8 GNP1 PMP2 SPF1 FCY21 SHO1 FTR1 FLC3 ZRT1 ERG25 ERG11 HXT4 DFG10 AXL2 MGA2 PHO90* YJR054W *MCD4 SAC1 GPI13 FPS1 FRE6 RAX2 ZRT2 NHA1 FKS1 SUR4 VID22 YLR413W PGA3 VBA1 SSO2 LCB1*	**Integral to membrane (D/U) [GO:0016021]** *GPI18 FLR1 FUS1 ARE1 ENA5 BAP3 HXT3 PFA5 ISC1 HXT10 STE2 YGL114W GSC2 MUP1 VHT1 MEP1 TPO2 MUP3 ARN1 ARN2 SYG1 DAL4 OPT1 TPO5 STE6 GAS2 PUN1 PGA1 LYP1 ARE2 FRE4 THI72 TPO4 MUM3 PDR10 FRE3 FLC1 DIP5 SAM3 MEP3 KRE6 SGE1* **Amino acid transmembrane transport (U) [GO:0015171]** *BAP3 MUP1 MUP3 TPO5 LYP1 DIP5 SAM3* **Siderophore transport (U) [GO:0015891]** *ARN1 ARN2 FRE4 FRE3*

TABLE 2.3 List of the GO retrieved from the list of unique genes with differential expression in AJ4 and MY3. U means that the GO term was retrieved from the up-regulated genes and D from the down-regulated genes.

Ethanol	Time	MY3	AJ4
10%	1	**Phospholipid biosynthetic process (D) [GO:0008654]** *PGS1 YDR018C CHO1 EPT1 URA8 PAH1 PDR17 INO4 PIS1* **Endocytosis (D) [GO:0006897]** *SNC1 OSH2 SAC6 GTS1 PIL1 YAP1802* *YSC84 YPK1 YPT53 LSP1 KES1* **Lipid biosynthetic process (D) [GO:0008610]** *TSC13 ERG28 ERG26 PHS1 ELO1 HMG2 MVD1 KES1*	**Transmembrane transport (D) [GO:0055085]** *PHO89 AGP1 PMA1 FLC3 TAM41 MEP1* *GAL2 DIP5* YPR003C *OPT2 SGE1*
10%	2	**Esterol biosynthetic process (D) [GO:0016126]** *ECM22 ERG6 HMG1 ERG2*	**Nucleobase, nucleoside, nucleotide and nucleic acid transport (D) [GO:0015931]** *FCY21 FCY22 TPN1 SNG1* **Transmembrane transport (U) [GO:0055085]** *FLC2 VBA2 SUL1 PCA1 ATO3 ITR1* *STL1 ALR2 PMC1 HNM1 TPO2 DUR3 PHO90* *TPO5 STE6 GEX2 AQR1 ALP1 THI72 MCH5* YOR378W
10%	3	-	**Gluconeogenesis (D) [GO:0006094]** *PGI1 PGK1 TPI1 TDH3 ENO2 FBA1* **Nucleobase transport (D) [GO:0015851]** *FUI1 FCY2 FCY21 FCY22 TPN1* **Transmembrane transport (D) [GO:0055085]** *FUI1 UGA4 ATO3 FCY2 FCY21 FCY22 TPN1* *HIP1 QDR2 GAP1 ZRT2 PHO84 VBA1 TAT2 SSU1 OPT2* **Signal transducer activity (U) [GO:0004871]** *GPB2 GPR1 STE2 GPA1 RHO2 RGS2*
6%	1	**Ergosterol biosynthetic process (D) [GO:0006696]** *ERG25 ERG11 ERG3 ERG6 HMG1* *ERG13 ERG5 ERG2 ERG8 ERG10* **Isoprenoid biosynthetic process (D) [GO:0008299]** *HMG1 ERG13 ERG8 MVD1 IDI1* **Lipid catabolic process (U) [GO:0016042]** *TGL2 TGL1 NTE1 PLB2 SPO1*	**ATP catabolic process (D) [GO:0006200]** *SNQ2 AUS1 PDR5 PDR12*
6%	2	**Tetracyclic and pentacyclic triterpenes (cholesterin, steroids and hopanoids) metabolism (D) [01.06.06.11]** *ERG6 ERG10 KES1*	**Steroid biosynthetic process (D) [GO:0006694]** *ERG3 ERG27 ERG6 HMG1* *ERG13 ERG5 ERG2 ERG8 KES1* **Transmembrane transport (D) [GO:0055085]** *GNP1 MUP1 MEP1 ARN2 GAL2* *HXT2 ALR1 TPO4 DIP5 SGE1*
6%	3	**Steroid biosynthetic process (D) [GO:0006694]** *ERG25 ERG11 ERG13 ERG5 MVD1 KES1* **Cellular cell wall organization (D) [GO:0007047]** *CHS3 ECM33 EXG2 CIS3 YLR194C FKS1 DFG5 GAS1 CHS1* **Fatty acid beta-oxidation (U) [GO:0006635]** *MDH3 POT1 TES1 FOX2 ECI1 IDP3* **Cardiolipin metabolic process (U) [GO:0032048]** *CLD1 UPS2 UPS1 TAZ1* **Phospholipid biosynthetic process (U) [GO:0008654]** *CDS1 YDR018C GPT2 CKI1 INO4 TAZ1*	**Nucleobase transport (D [GO:0015205]** *FCY2 FCY22 TPN1* **Amino acid transmembrane transport (U) [GO:0015171]** *BAP3 HNM1 MUP1 MUP3 TPO5 LYP1* **Transmembrane transport (U) [GO:0055085]** *BAP3 HXT10 ALR2 HNM1 MUP1 VHT1* *TPO2 MUP3 TPO5 LYP1 THI72 SGE1*

CHAPTER 3

Differential contribution of the parental genomes to a *S. cerevisiae* × *S. uvarum* hybrid, inferred by phenomic, genomic, and transcriptomic analyses, at different industrial stress conditions

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3.1 Introduction

Wine fermentation is a complex process in which yeasts have the most predominant role (Cavalieri et al., 2003). Traditionally, yeasts present on grapes spontaneously convert sugars into ethanol and carbon dioxide, as well as other metabolites, such as glycerol, acetate, succinate, pyruvate, higher alcohols, and esters (Pretorius and Lambrechts, 2000). *Saccharomyces cerevisiae* is the predominant yeast in most wine fermentations (Pretorius, 2000), however, in cold areas, it is frequently replaced by *S. uvarum* (Origone et al., 2017; Rainieri, 1999), or its hybrids with *S. kudriavzevii* and *S. uvarum* (Antunovics et al., 2005; Demuyter et al., 2004; Erny et al., 2012; González et al., 2007; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998; Peris et al., 2012c; Sipiczki, 2008).

Wine *S. cerevisiae* and *S. uvarum* strains are adapted to grow in wine fermentation environments, characterized by high sugar contents, low pH, and high sulfur dioxide concentrations (Alonso-del Real et al., 2019; Morard et al., 2019; Pérez-Torrado et al., 2015, 2018; Querol et al., 2018). However, each *Saccharomyces* species exhibits unique physiological properties that give the final wine different characteristics. The most important differences between these two species are ethanol tolerance and optimal growth temperature. *S. cerevisiae* exhibits a higher optimum growth temperature and higher ethanol resistance (up to 15%) (Arroyo-López et al., 2010b; Belloch et al., 2008; Salvadó et al., 2011a), which explains its dominance at high fermentation temperatures.

The present challenges in the wine industry are related to the effects of global climate change on winemaking and to consumer's preferences. The global climate change has different effects on grapevines, which include a lower acidity, an altered phenolic maturation, a different tannin content, and notably, higher sugar levels by the time of harvest, especially in warm climates (Jones et al., 2005; Mozell and Thachn, 2014). At the same time, consumers prefer wines with less ethanol content and fruitier aromas. The excess of ethanol compromises the perception of wine aromatic complexity, as well as rejection by health-conscious consumers, road safety considerations, or

trade barriers and taxes. To face these challenges, yeasts may have an important role. Thus, a new trend to respond to the wine industry demands is the selection of yeasts which reunite different characteristics, such as a lower ethanol yield, a higher glycerol production- to mask astringency due to unripe tannins- and which exhibit a more complex aromatic profile (Querol et al., 2018). However, these properties are not so frequent among wine *S. cerevisiae* strains, because they were unconsciously selected for millennia by humans to produce increasing amounts of ethanol in the warm climate regions, Fertile Crescent and Mediterranean basin, where vines were domesticated and winemaking was developed (This et al., 2006).

A possible solution to fulfill the wine industry demands comes from the use of wine *S. uvarum* strains, which exhibit interesting enological properties. *S. uvarum* is considered a cryotolerant yeast (Salvadó et al., 2011b) with several enological advantages over *S. cerevisiae*, such as lower ethanol and acetic acid productions, and higher glycerol and succinic acid synthesis (Bertolini et al., 1996). This species also produces high levels of a larger variety of fermentative volatiles, e.g. phenyl ethanol and phenylacetate (Gamero et al., 2013; Masneuf-Pomarède et al., 2010; Stribny et al., 2015). Nonetheless, the most important limitation of *S. uvarum* as a starter to conduct wine fermentation is its lower ethanol tolerance (Arroyo-López et al., 2010b), which explains why it is outcompeted by *S. cerevisiae* in wine fermentations performed at temperatures > 20ºC (Alonso-del Real et al., 2017b), as in the production of red wines. Therefore, an ethanol tolerance improvement in *S. uvarum* would be an important achievement for its beneficial use in the wine industries.

Ethanol tolerance is a quantitative trait determined by > 200 genes involved in many different cellular processes affected by ethanol (Snoek et al., 2016). Although many efforts have been made, mechanisms of ethanol tolerance are hardly understood yet.

Hybridization between *Saccharomyces* species has been proposed as an adaptation mechanism to different stresses (Sipiczki, 2008). As mentioned, natural hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* are present in, and even dominate, wine fermentations at low temperatures in regions of Continental or Oceanic climates (Erny et al., 2012; González et al., 2007; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998; Peris et al., 2012c).

The physiological and enological characterization of these hybrids showed that they inherited the ethanol tolerance and a good fermentation performance from *S. cerevisiae*, and adaptation to grow at low temperatures from *S. uvarum* and *S. kudriavzevii* (Pérez-Torrado et al., 2018; Querol et al., 2018). This observation prompted artificial hybridization as a good approach to improve industrial yeasts (Steensels et al., 2014b). This way, in previous works, *S. cerevisiae* × *S. uvarum* hybrids were generated, by different methods (Origone et al., 2018; Sipiczki, 2008), to improve cryotolorance in wine *S. cerevisiae* strains (García-Ríos et al., 2019c; Kishimoto, 1994; Origone et al., 2018; Sebastiani et al., 2002; Solieri et al., 2005).

In the present study, we used artificial hybridization of a commercial wine *S. uvarum* strain with a *S. cerevisiae* strain to improve its ethanol tolerance. This commercial *S. uvarum* strain, Velluto BMV58^{TM}, is characterized by its low ethanol yield and high glycerol production in wines at the industrial level, improving the roundness, and a soft mid-palate mouthfeel. It also produces richer secondary aromas, which confer floral and fruity notes to wines. Although this strain possesses all these interesting properties, which fulfill the consumers' demands, its ethanol tolerance during wine fermentation is low. To improve its ethanol tolerance, we selected a highly alcohol-tolerant *S. cerevisiae* strain to obtain an interspecies hybrid with the properties of both parents. Hybrids were obtained by rare-mating and subsequently sporulated to obtain diverse hybrid derivatives. The rare-mating hybrids, their spore derivatives and the parental strains were physiologically characterized, and one spore-derivative hybrid, H14A7, was selected because it shows the best fermentative profile, an improved ethanol tolerance, and a higher glycerol yield. The genomes of this spore-derivative hybrid, as well as those of the parental *S. uvarum* and *S. cerevisiae* strains, were sequenced to determine which is the genome composition of the hybrid compared to its parents. Finally, we also analyzed the transcriptomic response of the spore-derivative hybrid during wine fermentations performed at two different temperatures, 15 and 25ºC, to be compared with its parental strains under the same fermentation conditions.

3.2 Materials and Methods

3.2.1 Strains and sequencing

The strains used in the present work were the *S. uvarum* wine strain BMV58 (Velluto BMV58^{TM} from Lallemand), a commercial wine strain that was selected in our laboratory, and three wine *S. cerevisiae* strains, AJ4, AJB, and AJW, provided by Lallemand Inc.

3.2.2 Sporulation assays

Yeast cells were incubated on acetate medium (1% sodium acetate, 0.1% glucose, 0.125% yeast extract, and 2% agar) for 5–7 days at 25ºC to induce sporulation. 16 asci were collected for each strain when they were present. Ascus wall was digested with β 1,3-glucuronidase (Sigma) adjusted to 2 mg mL^{-1}, and spores were then dissected in GPY agar plates with a Singer MSM manual micromanipulator. Spores were incubated at 28ºC for 3–5 days, and then, their viability was measured as the percentage of spores able to form colonies.

3.2.3 *MAT* locus analysis

DNA from each strain was extracted according to Querol et al. (1992). The *MAT* locus was amplified with the same '*MAT*α' (5'- GCACGGAATATGGGACTACTTCG -3') primer described for *S. cerevisiae* by Huxley et al. (1990), but with degenerated '*MAT*α' (5'-ACTCCRCTTCAAGAGTYTG -3'), and '*MAT* common' primers (5'- AGTCACATCAAGATCRTTTATG -3') to also allow the amplification of the *MAT* locus from *S. uvarum*. PCR reactions were performed in 100 µl final volume following the NZYTAqII DNA polymerase supplier instructions, under the following conditions: initial denaturing at 94ºC for 5 min, then 30 PCR cycles with the following steps: denaturing at 94ºC for 30 s, annealing at 58ºC for 30 s and extension at 72ºC for 30 s; and a final extension at 72ºC for 7 min. The *S. cerevisiae* and *S. uvarum MAT* locus were differentiated

by restriction analysis with endonuclease *MseI*. Simple digestions of the PCR products with *MseI* (FastDigest SaqAI, Thermo Scientific) were performed with 15 µl of amplified DNA to a final volume of 20 µl at 37ºC according to supplier's instructions. Restrictions fragments were separated on 3% agarose gel in 0.5× TBE buffer and a mixture of 50-bp 100-bp DNA ladder markers (Roche Molecular Biochemicals, Mannheim, Germany) served as size standards.

3.2.4 Evaluation of ethanol tolerance

Ethanol tolerance of the strains was evaluated by performing growing tests in synthetic must (SM) with 10 g L^{-1} glucose, 20 g L^{-1} fructose, and 60 mg L^{-1} potassium metabisulfite, and increasing ethanol concentrations [0 to 10, 12, 15, and 20% (v/v)]. Strain growth was monitored by measuring absorbance at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). The wells of the microplate were filled with 0.25 mL of SM and inoculated with 1×10^6cells mL^{-1} for each strain and ethanol concentration. The experiments were performed at 15 and 25ºC. Uninoculated wells were included in every plaque as a negative control to establish a threshold to discard OD_{600} values due to background noise. Measurements were taken every 30 min during over 3 days, after a pre-shaking of 20 s. The overall yeast growth was estimated as the area under the OD vs. time curve using Origin Pro 8.0 software (OriginLab Corp., Northampton MA), and the NIC and MIC parameters were obtained as described elsewhere (Arroyo-López et al., 2010b). The most ethanol tolerant *S. cerevisiae* strain was subsequently used for the hybridization experiments.

3.2.5 Hybridization by rare-mating

For the selection of natural auxotrophic markers, cells were grown on 15 mL of GPY medium (% w/v: 0.5 yeast extract, 0.5 peptone, 2 glucose) for 5 days at 28ºC. One milliliter of each culture was seeded in 15 mL of fresh GPY medium and incubated again in the same conditions. This process was repeated 10 times. At the 5th and subsequent repetitions, aliquots of each culture were seeded

onto α-aminoadipic (α-AA), 5-fluoroanthranilic acid (5-FAA) and 5-fluoroorotic acid (5-FOA) agar plates to select natural lys−, trp−, and ura− mutant colonies, respectively (Boeke et al., 1987; Toyn et al., 2000; Zaret and Sherman, 1985). Colonies were grown on α-AA, 5-FAA or 5-FOA plates and picked again on a new α-AA, 5-FAA or 5-FOA plate, respectively. Auxotrophies were confirmed by spotting a cell suspension onto GPY-A (GPY medium with 2% w/v agar-agar), minimal medium (MM; 0.17% Yeast Nitrogen Base without amino acids, 2% glucose and 2% agar) and MM supplemented with proline (1 g L^{-1}), and uracil (10 mg L^{-1}), lysine (30 mg L^{-1}) or tryptophan (30 mg L^{-1}), depending on the auxotrophy. Plates were incubated for 5 days at 28ºC.

Auxotrophic colonies were grown separately in 25 mL GPY broth for 48 h at 28ºC. Cells were recovered by centrifugation and suspended in the residual supernatant. Pairs of yeast cultures to be hybridized were placed together in the same tube and aliquots of these mixed strains were inoculated in 2 mL of fresh GPY medium. After 5–10 days of static incubation at 28ºC in a slanted position, cells were recovered by centrifugation, washed in sterile water, suspended in 1 mL of starvation medium and incubated for 2 h.

The parental strains AJ4 and BMV58 were assayed for sporulation in the rich GPY medium used for the rare-mating. In the case of AJ4, no sign of sporulation was detected after more than ten days, however, sporulation efficiency for BMV58 was very low and difficult to observe in this medium, but a few asci were present.

A concentrated suspension of the mixed culture was spread on MM plates and incubated at 28ºC. Prototrophic colonies usually appeared after 3–5 days. These colonies were isolated and purified by restreaming on the same medium (Pérez-Través et al., 2012). The hybrid nature of the colonies selected in MM was confirmed by PCR-RFLP of the genes *UGA3* and *GSY1* to confirm that they showed hybrid profiles (Pérez-Través et al., 2014b).

3.2.6 Test of stability

Two strategies were carried out to determine the stability of hybrids: adaptive stabilization by vegetative growth without sporulation and adaptive stabilization by vegetative growth after sporulation. The stability test by vegetative growth was done as described elsewhere (Pérez-Través et al., 2012), with some modifications. The media was a synthetic must with 40 g L^{-1} of glucose, 45 g L^{-1} of fructose, 2.5% of EtOH and 60 mg L^{-1} of potassium metabisulfite, and the experiment was incubated at 28ºC. A single colony of each hybrid strain was individually inoculated into 20 mL of this must and they grew in those conditions for 10 days. At that moment, 200 µL of each fermentation, were inoculated in a new fresh media at the same conditions. The process was repeated 5 times. Once the fifth fermentation ended, for each one of the hybrids 10 colonies were tested for their molecular characterization by mtDNA-RFLP and delta elements analysis to be compared with the original hybrid and among them. We considered a genetically stable hybrid when all colonies recovered after individual growths maintained the same molecular pattern than the original culture. Only hybrids that maintained the same molecular pattern in its 10 colonies at the end of the process were considered for the artificial hybrid selection (next section). Only one of the ten colonies of each stable hybrid was randomly selected as a representative for subsequent artificial hybrid selection.

The test of adaptive stability by vegetative growth after sporulation was performed by incubating the hybrids in acetate- agar plates as described in the 'sporulation assays' section. For each hybrid, 10 spores were selected and characterized by PCR- RFLP analysis of 4 nuclear genes (*APM3*, *UGA3*, *GSY1*, and *BRE5*) and the internal transcribed spacer (ITS) region to confirm that they still showed a hybrid profile in at least one gene region. These genome regions were tested pairwise and when a hybrid pattern was obtained with the first pair, the next ones were not analyzed. The colonies showing a hybrid profile in at least one region (out of 5) were used for the same adaptive stability test described above for the adaptive stability without sporulation. At the end of the last fermentation, 10 colonies were isolated and they were also tested by mtDNA-RFLP (Querol et al., 1992) and delta elements analysis (Legras and Karst, 2003). Again, only hybrids that showed

identical molecular patterns in the 10 derivative colonies at the end of the process were considered for the artificial hybrid selection.

3.2.7 Artificial hybrid selection

Those strains exhibiting a hybrid pattern, according to the different molecular markers used, and that were stable during vegetative growth in fermentation without or after sporulation were considered to screen its phenotype for selection. Their growth in the presence of ethanol was monitored by measuring absorbance at 600 nm in a SPECTROstar Omega in SM with 10 g L^{-1} glucose, 20 g L^{-1} fructose, 60 mg L^{-1} potassium metabisulfite and 6.5% of ethanol. Growth conditions and the statistical analysis were performed as described above.

The same ethanol tolerance assay, described above, was performed using these selected hybrid strains, as well as the two parental strains, both at 15 and 25ºC. For the enological characterization of the selected artificial hybrids, triplicate fermentations were conducted in 250 mL bottles, closed with Müller valves, containing 200 mL of Verdejo natural must, supplemented with 0.3 g L^{-1} of nutrients, and incubated with shaking (100 rpm) at two different temperatures, 15 and 25ºC. The parental strains AJ4 and BMV58 were also included for comparative purposes. Fermentations were followed by weight loss as in Pérez-Través et al. (2014a). At the end of fermentation, supernatant samples were analyzed by HPLC to determine the amount of residual sugar (glucose and fructose), glycerol, ethanol, and organic acids. For this purpose, a Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA), equipped with a refraction index detector, was used. The column employed was a HyperREZTM XP Carbohydrate H + 8µm (Thermo Fisher Scientific) and it was protected by a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). The conditions used in the analysis were as follows: eluent, 1.5 mM sulfuric acid; flux, 0.6 mL min^{-1}; and oven temperature, 50ºC. Samples were 5-fold diluted, filtered through a 0.22-µm nylon filter (Symta, Madrid) and injected in duplicate.

Weight loss data was corrected with respect to the percentage of consumed sugars (Pérez-Través et al., 2014a). Percentages of consumed sugars over time were fitted to a Gompertz

equation (Zwietering et al., 1990), and the following kinetic parameters were calculated from the adjusted curves: m, maximum sugar consumption rate (g L^{-1} h^{-1}); l, latency or lag phase period (h); and t50 and t90, time to consume 50% and 90% of sugars (h), respectively. All the data were tested to find significant differences among them by using the one-way ANOVA module of the Statistica 7.0 software (StatSoft, Tulsa, OK, United States). Means were grouped using the Tukey HSD test ($\alpha = 0.05$).

3.2.8 Genome sequencing, assemblage, and annotation

Total DNA from the artificial hybrid strain and from the *S. cerevisiae* parental strain AJ4 were extracted according to Querol et al. (1992) and sequenced using the Illumina Miseq system, with paired-end reads of 250 bp (NCBI accession number SRP148850). The genome of Velluto BMV58^{TM}, the other parental strain, was already sequenced, assembled, and annotated in a previous study from our lab (Macías et al., in preparation).

Sequencing reads were trimmed and quality filtered using Sickle (Joshi and Fass, 2011), and then assembled following a semiautomatic pipeline (Macías et al., 2019; Morard et al., 2019) that uses programs Velvet (Zerbino and Birney, 2008), Sopra (Dayarian et al., 2010), SSPACE (Boetzer et al., 2011), Gapfiller (Boetzer et al., 2012) and MUMMER (Kurtz et al., 2004). The assembly was confirmed by comparison with that of the reference *S. cerevisiae* strain S288C genome (version R64-2-1, *Saccharomyces* genome database, http://www.yeastgenome.org).

Genes were annotated combining the *ab initio* method with Augustus (Stanke and Morgenstern, 2005) and annotation transfer method with RATT (Otto et al., 2011). Genes were manually curated using Artemis (Rutherford et al., 2000), NCBI BLAST web interphase (Johnson et al., 2008) and the SGD Database (Macías et al., 2019; Morard et al., 2019).

3.2.9 Flow cytometry analysis

The DNA contents of the selected hybrid and the parental strains were assessed by flow cytometry using a FACSVerseTM flow cytometer (BD Biosciences). Cells were grown overnight in GPY and 1 OD_{600} of each culture was harvested by centrifugation. DNA staining was performed using dye SYTOX Green (Haase and Reed, 2002) . Fluorescence intensity was compared with a haploid (S288C) and diploid (FY1679) reference *S. cerevisiae* strains.

3.2.10 Copy Number Variation analysis

The *S. cerevisiae* reads were mapped against the reference genome of S288c using Bowtie2 version 2.3.2 (Langmead and Salzberg, 2012). Genome annotations were visualized using Artemis (Rutherford et al., 2000) with the mapped reads to predict deletions and duplications present in the *S. cerevisiae* parental. Artificial hybrid reads were mapped to a combination of the *S. cerevisiae* and *S. uvarum* parental consensus sequences, including mitochondrial genomes, by using bowtie2 version 2.3.2 (Langmead and Salzberg, 2012), with the default settings.

SppIDer (Langdon et al., 2018) was used to visualize the genome composition of the selected hybrid. By using this tool, the reads of the hybrid genome were mapped to the reference genome of its parental *S. cerevisiae* and *S. uvarum* strains.

Bedtools (Quinlan and Hall, 2010) was used to obtain the coverage “per base”. These coverage files were processed to reduce the noise using a sliding windows method with a window size of 1000 positions. As a complementary approach, CNVnator was used for copy number variation discovery (Abyzov et al., 2011).

3.2.11 Variant calling analysis

The gdtools command installed as part of breseq (Barrick et al., 2014; Deatherage and Barrick, 2014) was used to identify single nucleotide polymorphisms (SNPs) on Genome Diff files. The

minimum polymorphism frequency to call an SNP using breseq was set to 0.20. To calculate heterozygosity levels, the SNP number was divided by the genome size of each strain. We subtracted and annotated SNP regions that were not present in the parental genomes but present in the hybrid genome, with an in-house python script.

3.2.12 RNA-Seq analysis

The RNA-seq analysis was performed using the cells collected from the Verdejo must micro vinifications, described above. We used white natural must to avoid RNA degradation due to the oxidation of polyphenols present in red musts. Fermentations were followed by weight loss; kinetic parameters were analyzed as explained above.

Cell samples were collected at two different fermentation time points: at the lag phase (4 h) and at the mid-exponential growth phase (24 h at 25ºC and 48 h at 15ºC respectively). Cells were harvested by centrifugation and then stored at −80ºC. Total RNA was extracted following a protocol based on an initial step of washing with DEPC-treated water and subsequent treatments with phenol-Tris, phenol-chloroform (5:1) and chloroform- isoamyl alcohol (24:1), and finally, a first precipitation with LiCl, and a second with sodium acetate and ethanol. After RNA extraction, we combined equal proportions of RNAs from the two parental strains in the same sample to reduce the number of libraries to sequence. Instead of 36 original RNA extracted samples (3 strains ×2 temperatures ×2 time points × triplicate), we had 24 samples to sequence. These samples were sequenced using the Illumina Hiseq 2000, paired-end reads 75 bases long (NCBI accession number PRJNA473074). Sequence reads were trimmed and quality filtered using Sickle (Joshi and Fass, 2011) (length 50, quality 23) and aligned to a combined concatenated reference of both genomes (AJ4 and BMV58) using bowtie2 version 2.3.2 (Langmead and Salzberg, 2012). Read counts for each gene were obtained using HTSeq-count version 0.9.0 (Anders et al., 2015), with a combination of BMV58 and AJ4 annotations and the mapping files ordered by names. The mapping reads with a quality score lower than 2 or those that aligned in more than one genome position were discarded.

All the samples were split into two files: One containing the *S. cerevisiae* gene counts and the other with the *S. uvarum* gene counts, as we had half of the sample containing hybrid reads and the other half with the merged sequences, which corresponded to the *S. cerevisiae* and to the *S. uvarum* parental strains during fermentation. The data was analyzed by a principal component analysis (PCA) among samples using the DESeq2 package (Anders and Huber, 2010). Read counts for each one of the 48 files were extracted and used for differential expression analyses with the EdgeR package (Robinson et al., 2009). Normalization factors were calculated among reads to scale the raw library sizes, the negative binomial conditional common likelihoods were maximized to estimate a common dispersion value across all genes, and finally, the tagwise dispersion values were estimated by an empirical Bayes method based on weighted conditional maximum likelihood.

Finally, genewise exact tests were computed for differences in the means between two groups of negative-binomially distributed counts, only retrieving a gene if the number of counts in all samples is > 1. Differential expression levels (relative RNA counts) between the different conditions were considered as significantly different with a false discovery rate (FDR) (Benjamini and Hochberg, 1995) at a threshold of 5%. Venn Diagrams were constructed with the number of differential expressed genes for each assayed condition and Gene Ontology (GO) terms were attributed using SGD. Statistical overrepresentation tests for the differentially expressed genes were also performed using Panther Version 14.1 (released 2019-03-12) with default settings (Mi et al., 2019). We retrieved p-values and fold enrichment for each GO term. Fold enrichment indicates if the observed gene number for each category in the list is higher than the expected, based on the number of uploaded genes. If > 1, it indicates that the category is overrepresented in our experiment. The p-values indicate the probability that the number of genes observed in this category occurred by chance, as determined by our reference list.

3.3 Results

3.3.1 *S. cerevisiae* parental strain selection according to ethanol tolerance

The main objective of the present work is to improve ethanol tolerance in a wine *S. uvarum* strain, Velluto BMV58^{TM} (Lallemand Inc.), by interspecific hybridization. First, we characterized and selected a *S. cerevisiae* parental strain that can complement BMV58 with its high ethanol tolerance. For this, we analyzed the growth in several ethanol concentrations of three industrial *S. cerevisiae* strains, previously selected by Lallemand for its tolerance to ethanol in industrial processes. Accordingly, we confirm that *S. uvarum* strain BMV58^{TM} is the one with the lower non-inhibitory concentration (NIC) and minimum inhibitory concentration (MIC) values, being unable to grow in concentrations that did not affect the growth of the *S. cerevisiae* strains (Table 3.1). The *S. cerevisiae* strain AJ4 was selected for hybridization because it exhibits the highest NIC and MIC values (11.6% and 18.6%, respectively). The parental strains AJ4 and BMV58 were assayed for their mating competence, with an analysis of their *MAT* locus and both were heterozygous *MAT*a/*MAT*α. Their sporulation efficiency and spore viability was tested both on acetate plates and in the rich GPY liquid medium used for rare mating. As mentioned, no sign of sporulation for *S. cerevisiae* AJ4 was detected in GPY after more than ten days. However, sporulation efficiency in GPY was very low and difficult to observe for BMV58, but a few asci were present. On acetate plates, both strains sporulated with spore viabilities of 75% for AJ4 and > 95% for BMV58. Several dissected spores were also assayed for the *MAT* locus and were heterozygous *MAT*a/*MAT*α, indicating that both parental strains are homothallic (data not shown).

3.3.2 Hybrid generation and characterization

Selection procedures of hybrids based on auxotrophic complementation of parental strains is difficult since industrial strains are prototrophic. For this reason, spontaneous auxotrophic mutants for AJ4 (lys−) and BMV58 (trp−) were selected by growth on α-AA and 5-FAA agar plates,

TABLE 3.1 One-way ANOVA analysis for the NIC and MIC parameters of 4 different *Saccharomyces* strains. NIC and MIC parameters were obtained for the *S. uvarum* BMV58 and *S. cerevisiae* AJ4, AJB, and AJW strains in SM + ethanol media at 28ºC. Standard deviations were obtained from triplicate experiments. Values followed by different superscript letters, within the same column, are significantly different according to an ANOVA and Tukey HSD tests, α = 0.05.

Strain	NIC (%)	MIC (%)
AJ4	11.65 ± 0.32^{d}	18.56 ± 1.48^{c}
AJB	10.03 ± 0.16^{c}	13.76 ± 0.19^{b}
AJW	8.63 ± 0.45^{b}	14.94 ± 0.49^{b}
BMV58	5.69 ± 0.9^{a}	10.8 ± 1.19^{a}

respectively. However, no ura− auxotrophs were isolated on 5-FOA plates (Pérez-Través et al., 2012).

A rare-mating approach was used to obtain putative allotetraploid hybrids with the whole-genome content of both parents (Pérez-Través et al., 2012). After the rare-mating process, 15 prototrophic colonies were recovered in the selection media. Eight of them were confirmed to be hybrids by PCR amplification and restriction analysis of *UGA3* and *GSY1* gene regions (Pérez-Través et al., 2014b). Seven out of eight colonies (H3 to H5, H8, H12, H14, and H15) showed a hybrid profile in both genes (data not shown).

These 7 hybrids were subjected to a test of stability by vegetative growth during fermentation. Each hybrid was inoculated into synthetic must during five passages. Once the fifth fermentation ended, we isolated colonies and 10 of them were randomly selected for each hybrid. These colonies were molecularly characterized by mtDNA-RFLP and delta element analysis. The analysis of the hybrids revealed that only the 10 colonies from hybrid H8 showed different delta profiles. For the subsequent phenotypic characterization, one of these 10 colonies of each hybrid, showing the same molecular patter, was randomly selected for each hybrid. From now on, these vegetative stabilized hybrids will be named H3, H4, H5, H12, H14 and H15.

Three of the original hybrids (H3, H4, and H14) were able to sporulate with a sporulation efficiency > 95%. Therefore, they were sporulated and more than 16 asci were dissected. Hybrid spore viabilities were: 76.7%, 53.6% and 39% for H3, H4, and H14, respectively. However, only 10

viable spores were selected for each hybrid. These monosporic derivatives were named after the original hybrid name (H3, H4, or H14) followed by a letter and a number indicating the dissection plate coordinates.

The hybrid nature of the monosporic-derivative strains was analyzed by PCR amplification and subsequent restriction analysis of six gene regions to determine the presence of at least one hybrid pattern. According to this analysis, only 9 monosporic strains, all of them recovered from hybrid H14, showed an interspecific hybrid pattern for at least one the genes assayed. These monosporic derivative hybrids were also subjected to a test of stability by performing fermentation in synthetic must at 25ºC. In the end, 10 colonies from each fermentation were isolated and the genome stability was confirmed using δ elements and mtDNA-RFLP patterns (Legras and Karst, 2003; Querol et al., 1992). All these hybrid monosporic derivatives were stable in their patterns along the fermentation.

3.3.3 Phenotypic and enological characterization of the artificial hybrids for the selection of the best suitable strain

The strains that showed to be stable during vegetative growth without and after sporulation, along with the two parental strains AJ4 and BMV58, were evaluated for growth in SM (30 g L^{-1} of glucose) supplemented with 6.5% ethanol (Figure 3.2). We observed that the maximum growth rate varied between the different artificial hybrids and spore derivatives. It is interesting to point out that the monosporic derivative H14A7 showed a higher growth rate, even better than *S. cerevisiae* AJ4. The kinetic parameters for the other strains were intermediate between those of their parents, except H14B1 and H14A6, which show lower maximum growth rates (μmax). Accordingly, we selected the hybrid spore derivative H14A7 because showed a μmax higher than both parents did Figure 3.1.

H14A7 was an isolate from a three-spored ascus obtained of H14. Only two of the spores from this ascus were viable (H14A6 and H14A7), being one of them, H14A7 the selected strain.

Ethanol tolerance assays of the derivative hybrid were performed at 15 and 25ºC using the two

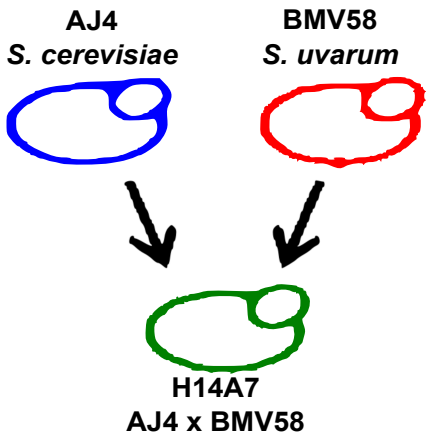


FIGURE 3.1 Scheme of H14A7 obtainment. AJ4 and BMV58 strains were selected to obtain different *S. cerevisiae* x *S. cerevisiae* hybrids. Finally, one of the hybrids, H14A7 was selected.

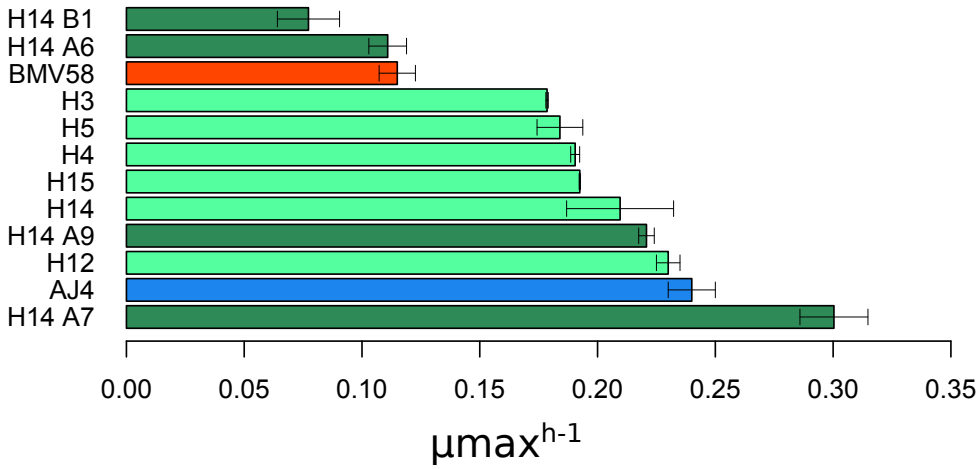


FIGURE 3.2 Maximum growth rate (µ max) of the different colonies after stabilization by vegetative growth and sporulation. µ max data is represented as the mean value of three replicates with its standard deviation. The data was retrieved after growing the colonies in SM with 30 g/L of sugars and 6.5%(v/v) of exogenous ethanol. Colonies stabilized by vegetative growth are filled in light green color, and those stabilized by sporulation in dark green; Parental AJ4 is shown in blue, and BMV58 in red.

TABLE 3.2 One-way ANOVA analysis for the NIC and MIC parameters of *S. uvarum* BMV58 and H14A7 strains at 15 and 25ºC. NIC and MIC parameters were obtained for the *S. uvarum* BMV58, *S. cerevisiae* AJ4 and H14A7 *S. cerevisiae* × *S. uvarum* strains in SM + ethanol media at 25 and 15ºC. Standard deviations were obtained from triplicate experiments. Values followed by different superscript letters, within the same column, are significantly different according to an ANOVA and Tukey HSD tests, α = 0.05.

Strain	NIC		MIC	
	15ºC	25ºC	15ºC	25ºC
BMV58	8.93 ± 0.67^{a,b}	6.05 ± 0.26^{a}	11.86 ± 0.86^{a,b}	9.52 ± 0.14^{a}
AJ4	6.84 ± 1.26^{a,b}	7.97 ± 0, 62^{a,b}	17.45± 1.16^{c}	16.73 ± 0.18^{c}
H14A7	9.19 ± 0.63^{b}	7.56 ± 0.49^{a,b}	12.16 ± 0.22^{b}	11.44 ± 0.30^{b}

parental strains AJ4 and BMV58 as controls. Their NIC and MIC values at both temperatures can be seen in Table 3.2. H14A7 NIC value at 15ºC is the highest, and its MIC values are between both parents at both temperatures.

Enological properties of the hybrid monosporic derivative H14A7 and the parental strains AJ4 and BMV58 were evaluated by performing fermentations in Verdejo grape musts at 15 and 25ºC. Their sugar consumption profiles, kinetic parameters, and metabolite production are shown in Figure 3.3 and Table 3.3. Sugars (glucose and fructose) of the Verdejo musts were practically exhausted at the end of all fermentations performed at both temperatures.

Glycerol production was higher at 25ºC in the H14A7 strain and the *S. uvarum* parental, whereas at 15ºC the hybrid derivative showed an intermediate profile of glycerol production, higher than AJ4 but lower than BMV58. The analysis of the production of organic acids showed that parental AJ4 and the hybrid monosporic derivative produce higher amounts of lactic acid compared to BMV58. It is worth to note that H14A7 presented a longer latency phase at both temperatures compared to its parents but, during the exponential phase, exhibited the maximum sugar consumption rate and fermentation speed at 25ºC, and an intermediate sugar consumption rate between those of AJ4 and BMV58 at 15ºC. Therefore, we can conclude that the hybrid derivative strain inherited the good fermentation performance and the higher production of organic acids from the *S. cerevisiae* AJ4 parent, and the higher synthesis of glycerol from BMV58 (Table 3.3).

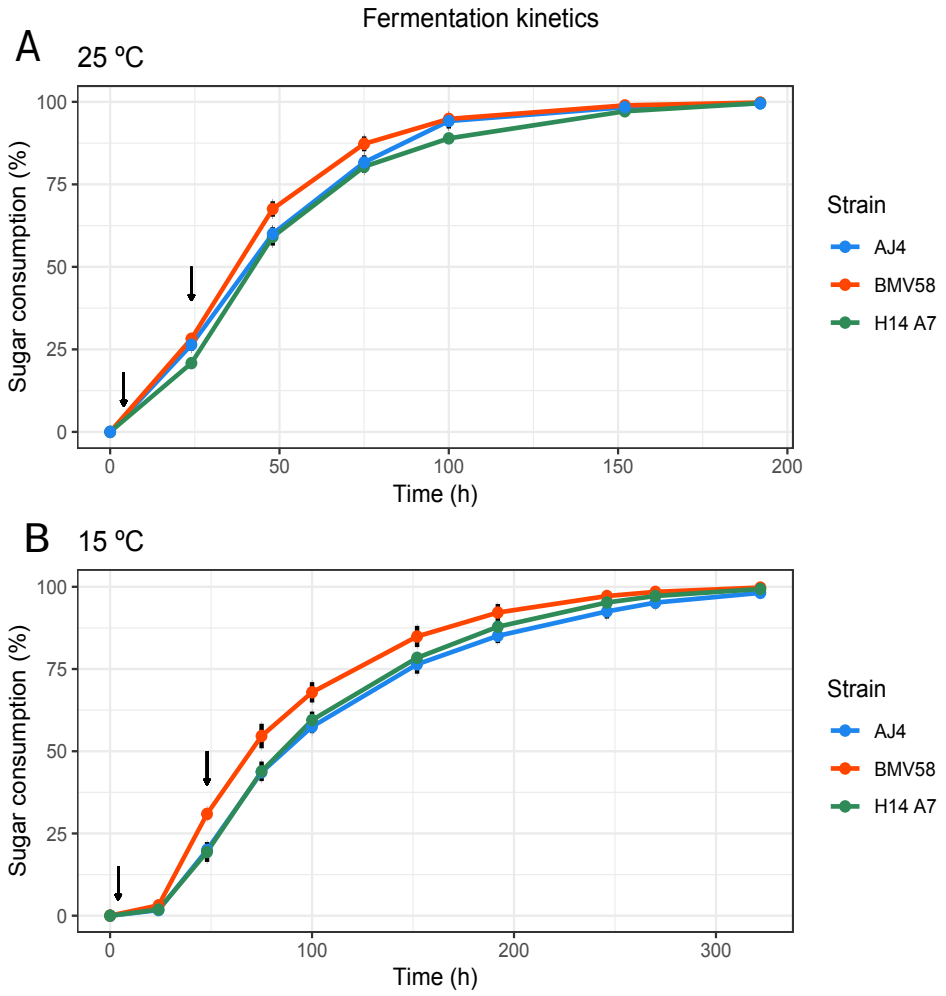


FIGURE 3.3 Main analytical and kinetic parameters of the fermentation carried out with both parental strains and the obtained hybrid in Verdejo must at 15 and 25ºC. Sugar consumption represents the percentage of sugars consumed at different time points of the fermentation for AJ4 (blue), BMV58(red) and H14A7 (green), at 25ºC (A) and 15ºC (B). Arrows indicate the time points when samples for transcriptomic analysis were taken (t = 4 h and t = 24 h at 25ºC, and t = 4 h and t = 48 h at 15ºC).

TABLE 3.3 Kinetic parameters and chemical composition of fermentation in Verdejo must inoculated with AJ4, BMV58 and H14A7 strains at 15 and 25ºC. m is the maximum sugar consumption rate, l is the fermentation lag phase duration, and t50, t90 are the times employed to consume 50% and 90% of the sugars present in the must. These values were obtained after adjustment to Gompertz equation of three biological replicates. Values are a mean of the three biological replicates followed by their standard deviation. Different superindexes in the same row and belonging to the same temperature are significantly different according to the Tukey HSD test (α = 0.05).

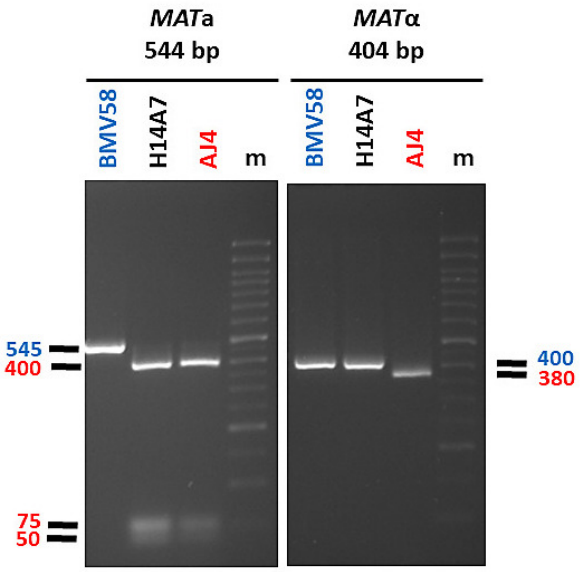
	25ºC fermentations			15ºC fermentations		
	H14A7	**AJ4**	**BMV58**	**H14A7**	**AJ4**	**BMV58**
	Final must composition			**Final must composition**		
Glucose (g/L)	0.02 ±0.02^{a}	0.00 ± 0.00^{a}	0.03 ± 0.04^{a}	0.00 ± 0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
Fructose (g/L)	0.77 ±0.16^{a}	1.01 ± 0.44^{a}	0.39 ± 0.1^{a}	1.41 ± 0.53^{a}	2.39 ±1.01^{a}	0.47 ±0.82^{a}
Glycerol (g/L)	11.23 ± 0.13^{a}	10.22 ± 0.51^{b}	11.66 ± 0.43^{a}	8.70 ± 0.09^{b}	7.52 ± 0.23^{a}	11.07 ± 0.29^{c}
Ethanol (%)	12.72 ±0.36^{a}	12.83 ±0.51a	12.38 ± 0.13^{a}	12.86 ± 0.12^{c}	12.35 ±0.1^{b}	11.69 ± 0.02^{a}
Citric acid (g/L)	0.39 ± 0.01^{b}	0.27 ±0.02^{a}	0.23 ± 0.02a	0.28 ±0.05^{a}	0.29 ±0.02^{a}	0.3 ± 0.01^{a}
Tartaric acid (g/L)	2.4 ± 0.12^{a}	2.22 ± 0.09^{a}	2.19 ± 0.12^{a}	1.92 ± 0.09^{a}	2.23 ±0, 23^{a}	1.88 ±0.12^{a}
Malic acid (g/L)	1.96 ± 0.14^{b}	2.68 ±0.26^{a}	1.94 ± 0.22^{b}	1.79 ±0.07a	2.66 ±0.78^{a}	1.92 ± 0.11^{a}
L- Lactic acid (g/L)	1.02 ± 0.14b	1.95 ± 0.31^{a}	0.73 ± 0.03^{b}	0.38 ± 0.03^{a}	0.32 ±0.02^{a}	0.26 ±0.06^{a}
	Kinetic parameters			**Kinetic parameters**		
m (g L^{-1} h^{-1})	1.761 ± 0.0985b	1.485 ± 0.0706a	1.513 ± 0.114^{a}	0.78 ± 0.0265^{ab}	0.75 ±0.05^{a}	0.924 ±0.089^{b}
l (h)	9.84 ± 0.80194^{b}	6.96 ± 0.551^{a}	8.081 ± 0.54^{a}	23.95 ± 2.17^{b}	22.071 ± 1.89^{ab}	18.37 ± 0.97^{a}
t50 (h)	43.20 ± 1.61^{b}	40.88 ± 1.80^{b}	36.72 ± 1.37^{a} 77.91	87.91 ± 4.13^{b}	89.89 ±4.43^{b}	73.21 ±4.13^{a}
t90 (h)	93.62 ± 4.44^{b}	88.84 ± 4.47^{a,b}	77.91 ±4.29^{a}	186.96 ±7.61^{a,b}	207.206 ± 3.22^{b}	157.26 ± 3.96^{a}
Glycerol/sugar yield (g/g)	0.056 ± 0.0005^{a}	0.0512 ± 0.0004^{b}	0.058 ±0.0005^{a}	0.044 ±0.0002^{b}	0.038 ±0.0004a	0.055 ±0.0004^{bc}

3.3.4 Comparative genome analysis between the best artificial hybrid and its parents

To determine the genome constitution of the artificial hybrid and those changes that occurred during the process of rare-mating hybridization and the subsequent sporulation, a comparative genome analysis between the artificial hybrid derivative and its parents was performed. For this purpose, we sequenced, assembled and annotated the whole genome of monosporic derivative H14A7 and the *S. cerevisiae* AJ4 parental strain. The BMV58 genome sequence and annotation were already available in our laboratory (Macías et al. unpublished data).

A total of 6182 genes of AJ4 were annotated and manually revised. The retrieved BMV58 annotation consists of a set of 5710 manually revised genes. A total of 5393 gene sequences were well annotated and shared between AJ4 and BMV58.

The H14A7 sequence reads were mapped against the genomes of AJ4 and BMV58 strains to unveil its genome constitution by means of an analysis of copy number variations (CNV) in its chromosomes. It is interesting to note that the artificial hybrid H14 and its spore derivative H14A7 inherited the *S. cerevisiae* mitochondrial genome. This genome constitution analysis was complemented with an analysis of ploidy by flow cytometry. Strikingly, although both parents are diploid strains (AJ4, 2.28 ± 0.01; and BMV58, 2.28 ± 0.01), H14A7 is allotriploid (2.98 ± 0.02), and not allodiploid as expected after sporulation of a putative allotetraploid. The analysis of genome sequences confirmed these results and provided more information on the H14A7 genome composition. Average read depths across the *S. cerevisiae* subgenome were twice of the *S. uvarum* subgenome (Figure 3.4). Together with the flow cytometry results, this suggests that the monosporic derivative H14A7 is allotriploid with a diploid *S. cerevisiae* subgenome and a haploid *S. uvarum* subgenome. An exception was observed for chromosome III, which in both subgenomes appeared in only one copy. These observations were also confirmed by the CNVnator analysis. When we searched for deletions and duplications of small regions with CNVNator, no significant changes were detected.



SUPPLEMENTARY FIGURE 3.1 Agarose gel electrophoresis showing the *MAT* locus restriction patterns of the artificial hybrid spore derivative H14A7 and the *S. cerevisiae* AJ4 and *S. uvarum* BMV58 parental strains (indicated in red and blue, respectively). PCR fragments were amplified with *MAT*a (amplicon length 544 bp) and *MAT*α (404 bp) specific primers and digested with endonuclease *MseI* to differentiate the *MAT* alleles of the parental species. The length of the diagnostic bands, specific of *S. cerevisiae* and *S. uvarum*, are indicated in red and blue, respectively. Restriction fragments were separated on 3% agarose gel in 0.5× TBE buffer and a mixture of 50-bp 100-bp DNA ladder markers was used as size standards (m).

The *MAT* locus was also tested for strain H14A7, containing a *MATa* allele in the monosomic *S. cerevisiae* chromosome III and a *MAT*α allele in the haploid *S. uvarum* subgenome (Supplementary Figure 3.1).

To better understand how the selected spore-derivative hybrid could be originated, we compared single nucleotide polymorphisms SNPs in H14A7, AJ4, and BMV58. The heterozygosity levels are higher in the *S. cerevisiae* parental strain (0.067% SNPs in the genome) than in the *S. uvarum* one (0.022% SNPs). The hybrid *S. cerevisiae* subgenome maintains the same levels of heterozygosity than AJ4 for each chromosome pair, except for the homozygous chromosome III, due to the single copy maintained in the hybrid. Apart from the SNPs located in chromosome III, H14A7 only showed the fixation of three non-synonymous homozygous SNPs, present in its parental *S. cerevisiae* strain in the form of heterozygous sites, likely by a loss of heterozygosity mechanism. These three homozygous SNPs occurred in gene *TRK2* (YKR050W), located on chromosome XI, which is part of the Trk1p-Trk2p potassium transport system.

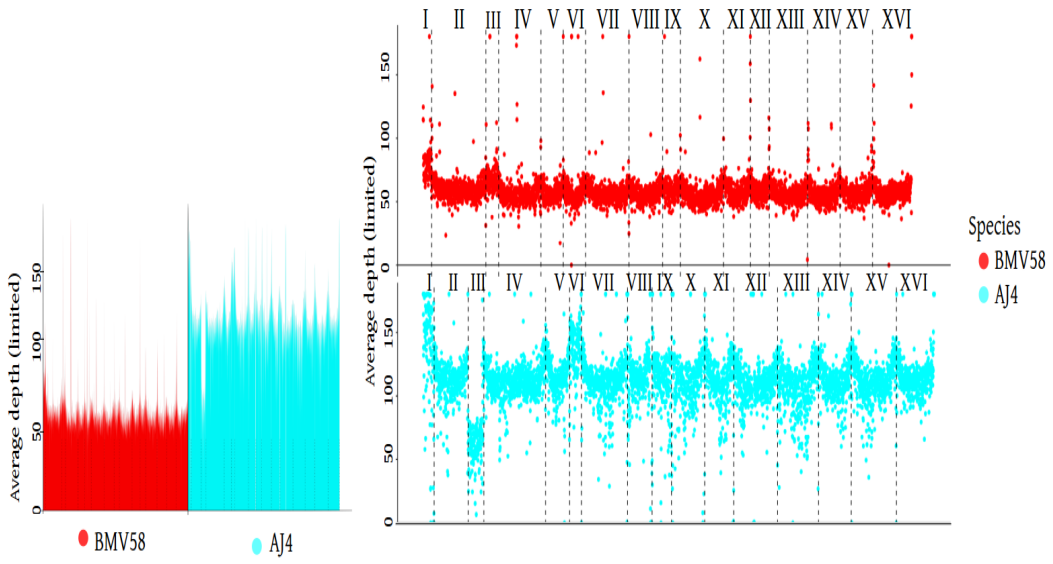


FIGURE 3.4 Graphic representation of the hybrid H14A7 genome composition. Obtained with sppIDer (https://github.com/GLBRC/sppIDer), a pipeline that uses bwa –mem to map the reads of the hybrid genome to the reference genome of its parental strains, BMV58 and AJ4.

3.3.5 Comparative expression analysis during wine fermentation

To better understand the properties acquired by the hybrid respect to both parents, we performed a comparative study of gene expression during Verdejo must fermentations. A total number of 36 samples (3 strains × 2 times × 2 temperatures × triplicates) of RNA were retrieved during the fermentations and sequenced. In the case of the artificial monosporic derivative H14A7 samples, transcriptomic data of the *S. uvarum* and *S. cerevisiae* genes were treated separately.

A principal component analysis (PCA) with the DESeq2 package was performed. Figure 3.5 showed that triplicates group together and that the greater variance (61%) in the samples correspond to the fermentation phase variable. The first principal component (PC1) separated samples from latency and exponential growth phases. The second component (PC2), which explains 24% of the variance, is the subgenome (*S. cerevisiae* or *S. uvarum*). The PCA also showed clustering of samples into 4 separate groups: samples belonging to *S. cerevisiae* gene expression in the exponential phase; *S. cerevisiae* gene expression in the latency phase; *S. uvarum* gene expression in exponential phase; and *S. uvarum* gene expression in latency phase.

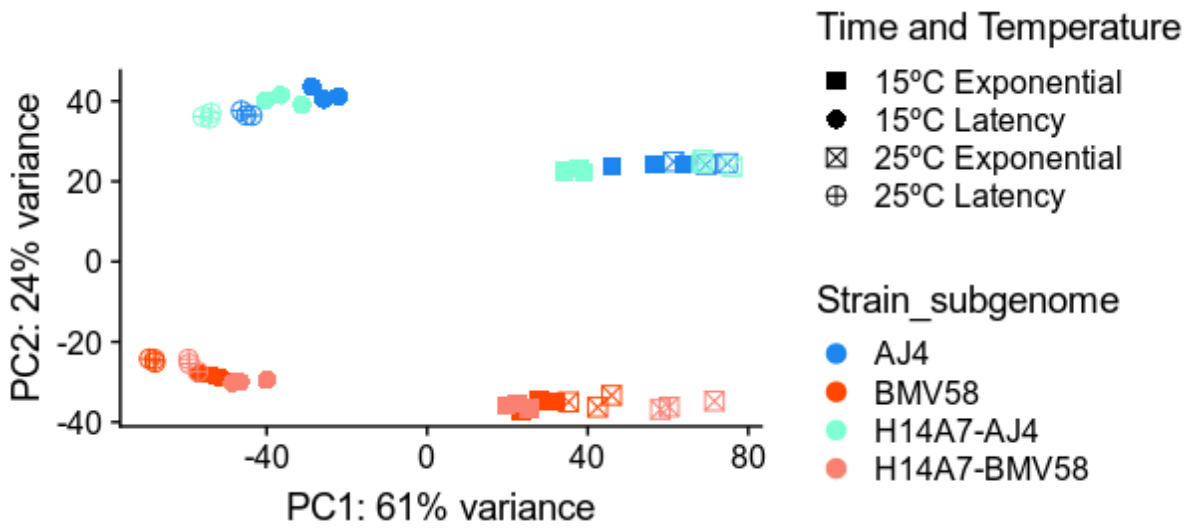


FIGURE 3.5 Principal component analysis of the transcriptome variation in *S. uvarum* BMV58, *S. cerevisiae* AJ4, and the *S. uvarum* and *S. cerevisiae* subgenomes of the artificial hybrid under two different temperatures and fermentation phases. All sequenced transcriptomic samples were plotted in this PCA. The PCA plot shows the greater variation in the fermentation phase and in the species gene expression. Triplicates are shown in the same color and shape, as follows: blue, AJ4; red, BMV58; orange, H14A7-uvarum; turquoise, H14A7-cerevisiae; squares, exponential phase; circles, latency phase; filled, 15ºC; a cross, 25ºC.

We conducted a first differential expression analysis using only the samples corresponding to H14A7 fermentations to compare *S. cerevisiae* and *S. uvarum* gene-specific expressions in this hybrid. We performed simple assays comparing gene expression between the hybrid subgenome genes in 4 conditions (the latency phase at 15 and at 25ºC, and the exponential phase at 15 and at 25ºC), with adjusted p-values < 0.01 (FDR). We normalized *S. cerevisiae* and *S. uvarum* subgenome expressions according to the number of copies of each gene present in the hybrid. Gene- specific overexpression differences can be seen in Figure 3.6. At 15ºC, the number of differentially expressed DE genes was higher in the latency phase than in the exponential stage, whereas at 25ºC both phases showed a similar number of differentially expressed genes in the hybrid. A GO term enrichment analysis was performed, and the 5 GO terms with a lower p-value for each condition are represented against their fold-enrichment in Figure 3.7 and Figure 3.8.

It is remarkable that the fatty acid catabolic process and short-chain fatty acid metabolic process are overrepresented terms in the *S. uvarum* subgenome when compared with the *S. cerevisiae* subgenome during the exponential phase at 25ºC. These two GO terms could be related to the

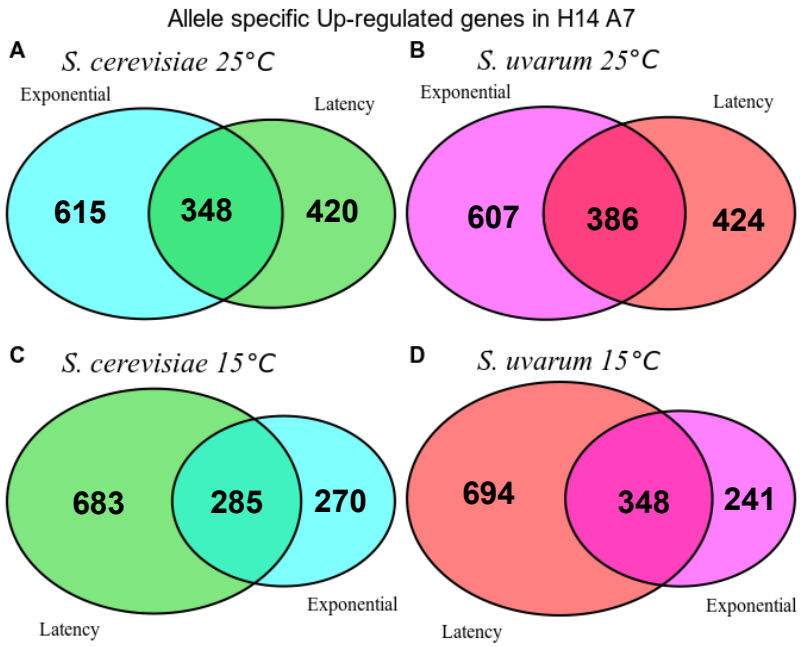


FIGURE 3.6 Venn diagrams with the number of differentially expressed genes when performing differential expression analysis between the *S. cerevisiae* and *S. uvarum* subgenomes of the H14A7 monosporic derivative. Up-regulated genes in *S. cerevisiae* genome against *S. uvarum* subgenome at 25◦C at two phases (A), Up-regulated genes in *S. uvarum* genome against *S. cerevisiae* subgenome at 25ºC at two phases (B), up-regulated genes in *S. cerevisiae* genome against *S. uvarum* subgenome at 15◦C at two phases (C), up-regulated genes in *S. uvarum* genome against *S. cerevisiae* subgenome at 15ºC at two phases (D).

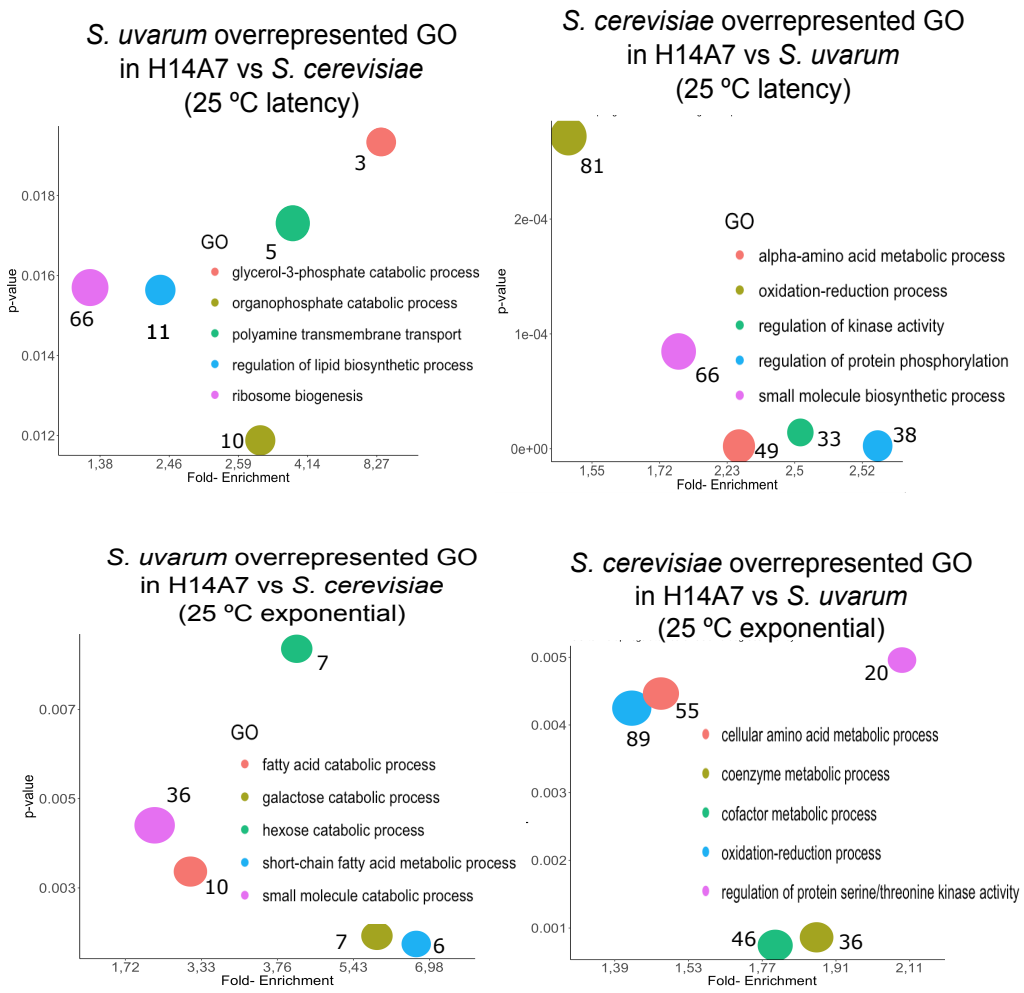


FIGURE 3.7 Top 5 significant GO terms retrieved from the differentially expressed genes between the *S. cerevisiae* and *S. uvarum* subgenomes in the H14A7 monosporic derivative at 25ºC. For each of the 4 graphs (*S. uvarum* latency overrepresented, *S. cerevisiae* latency overrepresented, *S. uvarum* exponential overrepresented and *S. cerevisiae* exponential overrepresented) the x-axis represents de fold-enrichment and the y-axis the p-value, retrieved from Panther Gene List Analysis. The sizes of the circles represent the number of terms that are included in each GO.

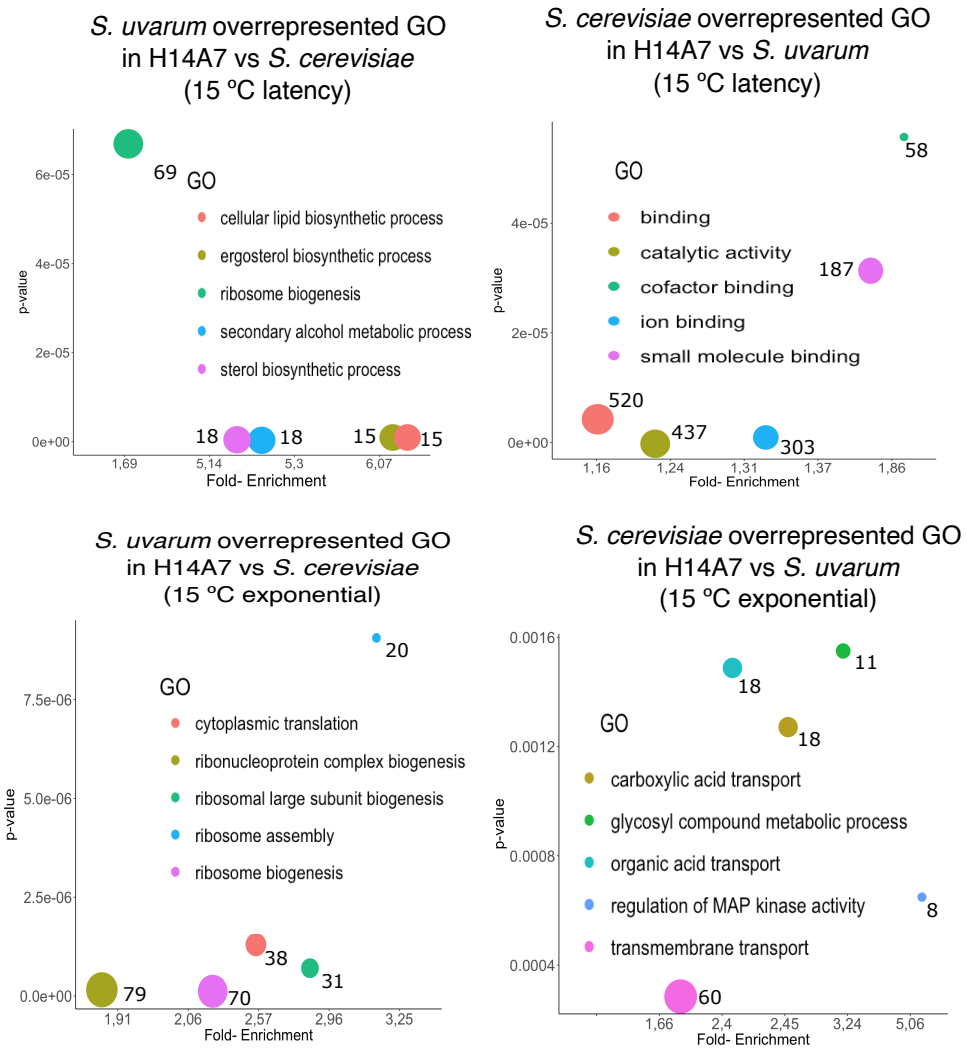


FIGURE 3.8 Top 5 significant GO terms retrieved from the differentially expressed genes amongst *S. cerevisiae* and *S. uvarum* subgenomes in the H14A7 monosporic derivative at 15ºC. For each one of the 4 graphs (*S. uvarum* latency overrepresented, *S. cerevisiae* latency overrepresented, *S. uvarum* exponential overrepresented and *S. cerevisiae* exponential overrepresented) the x-axis represents de fold-enrichment and the y-axis the p-value, retrieved from Panther Gene List Analysis. The sizes of the circles represent the number of terms that are included in each GO.

H14A7 behavior during alcoholic fermentation, as they are related to membrane composition of yeast cells and, thus, to ethanol tolerance.

In the subsequent differential expression analyses, we compared gene expression during fermentation of the *S. cerevisiae* genes of H14A7 monosporic derivative and those from the parental AJ4, and of the *S. uvarum* genes of H14A7 and those from the parental BMV58. We analyzed H14A7 differentially expressed genes against AJ4 (adjusted p-values of 0.05) and only found 5 up-regulated genes, including *FSH1*, encoding a serine hydrolase, and *ARG1*, involved in the arginine biosynthesis pathway. Of the 66 down-regulated genes, 36 of them are located on chromosome III, present as a single copy in the hybrid. It is important to remark this under-expression is significant considering that expression levels were corrected according to the number of copies of the genes. Other underexpressed genes in the hybrid are *GPX2*, encoding a glutathione peroxidase; *ARE1*, an acyl-coenzyme A; *NDE2*, a NADH dehydrogenase; and *ADH2*, alcohol dehydrogenase II, which catalyzes the conversion of ethanol to acetaldehyde.

RNA seq analysis of *S. uvarum* allele expression between H14A7 and BMV58 showed that there were 33 differentially expressed genes (adjusted p-values of 0.05): 17 of them are up-regulated in H14A7 and 16 up-regulated in BMV58. It is worth noticing that the gene *ADH5*, encoding an alcohol dehydrogenase involved in ethanol synthesis, is overexpressed in the hybrid derivative H14A7. The function of *ADH5* is uncharacterized, though it has been proposed to share a common ancestor with *ADH1*/*ADH2*, from which it appeared to have diverged as part of a whole-genome duplication occurred in the ancestor of the *Saccharomyces* lineage (Wolfe and Shields, 1997).

As a complementary approach to compare AJ4 and BMV58 parental strains with H14A7 gene expression, we also compared each gene expression of the parental (AJ4 and BMV58, respectively) with the total addition of the *S. cerevisiae* and *S. uvarum* alleles expression of the hybrid. With this approach, we could compare the whole genome expression of the hybrid with the expressions of each parent. We found more significant differentially expressed genes than in the subgenome comparisons. A PCA analysis that groups samples according to their gene

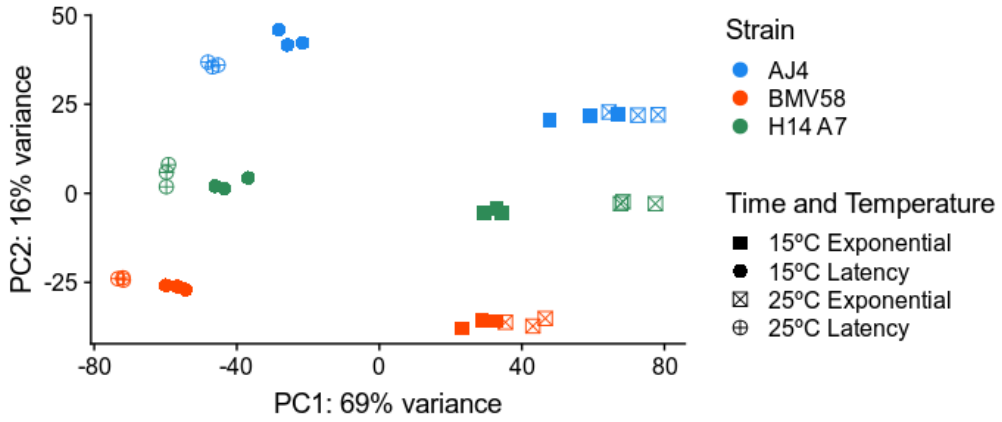


FIGURE 3.9 Principal Component Analysis (PCA) of the transcriptome variation in *S. uvarum* BMV58, *S. cerevisiae* AJ4, and the monosporic derivative H14A7 genome (*S. uvarum* + *S. cerevisiae* subgenomes) under two different temperatures and fermentation phases. All sequenced transcriptomic samples were plotted in this PCA (3 strains × 2 phases × 2 temperatures × triplicates). The PCA plot shows the greater variation in expression levels in the fermentation phase and in the species-specific gene expression. Triplicates are shown in the same color and shape, as follows: blue, AJ4; red, BMV58; green, H14A7; squares, exponential phase; circles, latency phase; filled, 15ºC; a cross, 25ºC.

expressions can be seen in Figure 3.9.

At the latency phase of fermentations at 25ºC, the hybrid showed up-regulation in amino acid biosynthesis when compared with both AJ4 and BMV58 strains, in 46 and 43 genes, respectively.

Genes *ARO1*, *ARO80*, and *HIS2* are more expressed in H14A7 than in BMV58, *CYS3*, *MET8*, and *TRP2* are more expressed in H14A7 with respect to AJ4, and *HIS1*, *MET6*, and *ARO8* are up-regulated in comparison to both parents.

At the exponential phase during fermentations at 25ºC, H14A7 showed an up-regulation in oxidative-reduction processes with respect to BMV58, and in glycogen biosynthesis, galactose degradation and hexose catabolism in comparison with AJ4. At the latency phase during fermentations at 15ºC, the hybrid derivative overexpressed the ribosome biosynthesis genes in comparison with AJ4, and transmembrane transport genes and genes that respond to oxidative stress with respect to BMV58. Finally, at the exponential phase at 15ºC, the hybrid overexpressed alpha-amino acid metabolism genes in comparison to BMV58 and ergosterol and

sterol biosynthesis genes in comparison to AJ4.

It has to be mentioned that, during the exponential phase, the GO terms: positive regulation of ergosterol biosynthetic process, positive regulation of steroid biosynthetic process, positive regulation of steroid metabolic process, and positive regulation of sterol biosynthetic process, are over-represented in the genome expression of H14A7 against AJ4 at 15ºC, and theGO term: positive regulation ofalcohol biosynthetic process, at 25ºC. At 15ºC during the exponential phase, the GO terms: alpha-aminoacid metabolic process and cellular amino acid metabolic process are among the overrepresented GO terms from the differentially expressed genes between H14A7 and BMV58.

As a short summary, *S. cerevisiae* and *S. uvarum* alleles are differentially expressed in H14A7. This differential expression among alleles is very evident in the latency stage at 15ºC, genes involved in the ergosterol biosynthetic process and in cellular lipid biosynthetic process are overexpressed in the *S. uvarum* subgenome, whereas the *S. cerevisiae* subgenome overexpressed genes are involved in catalytic activities, among others. When comparing H14A7 total genes against AJ4 and BMV58 parents, the most interesting result is the differential expression of genes related to amino acid biosynthesis.

3.4 Discussion

In the last decade, a great effort has been devoted to the study of natural *Saccharomyces* hybrids present in industrial fermentations (Kodama et al., 2005; Peris et al., 2018). These *Saccharomyces* hybrids have mainly been isolated from fermentative environments in European regions with Continental and Oceanic climates, and they were originated by spontaneous hybridization between *S. cerevisiae* and a cryophilic species: *S. eubayanus*, *S. kudriavzevii*, or *S. uvarum* (Boynton and Greig, 2014). The best-known example is the lager yeasts S. pastorianus, a hybrid between *S. cerevisiae* and *S. eubayanus* (Monerawela and Bond, 2017).

The physiological characterization of natural hybrids demonstrated that they inherited the good

fermentation performance and ethanol tolerance of *S. cerevisiae* and the ability to grow at lower temperatures of the *S.* non-*cerevisiae* partner, as well as other properties of enological interest (Pérez-Torrado et al., 2018). These interesting properties contributed by the *Saccharomyces* non-*cerevisiae* species prompted the development of artificial interspecific hybrids for industrial applications. The main purpose was the generation of new hybrids to increase diversity, such as in the case of lager yeasts (Hebly et al., 2015; Mertens et al., 2015), or to improve low-temperature tolerance to wine strains (García-Ríos et al., 2019c; Kishimoto, 1994; Origone et al., 2018). However, the main purpose of this study is to obtain an artificial *S. cerevisiae* × *S. uvarum* hybrid conjugating the interesting enological properties of a commercial wine *S. uvarum* strain, and the high ethanol tolerance of a *S. cerevisiae* strain, able to grow at ethanol concentrations in which most of the *Saccharomyces* yeasts are not able to grow (Arroyo-López et al., 2010b).

It has been reported that increased genome size on the hybrids can confer adaptive flexibility fermenting under different conditions (Miklos and Sipiczki, 1991; Pfliegler et al., 2014) and in the case of our hybrid derivative strain, that proved to be true.

Ploidy of hybrids influences fermentation performance, a triploid strain, as in our case, is improving fermentation when compared with diploid strains (Krogerus et al., 2016). This effect was more remarkable when fermentation took place at 25ºC, in which maximum growth rate of the hybrid was higher than the parental rates, but also at 15ºC, in which the hybrid showed an intermediate behavior, as described for other *S. cerevisiae* × *S. uvarum* hybrids (Coloretti et al., 2006).

Artificial hybrids are usually obtained by ‘canonical’ mating between haploid cells/spores of opposite mating types, either by spore-to-spore crosses or by mass mating between haploid spores/cells (Antunovics et al., 2005; Caridi et al., 2002; Zambonelli et al., 1997). However, the genomic characterization of natural *S. cerevisiae* × *S. kudriavzevii* hybrids (Morard et al., 2020a) suggests that, in these hybrids, the most probable mechanism of hybridization is ‘rare’ mating, although not the only one. Diploid *Saccharomyces* cells can become mating competent by a mating-type conversion to a homozygous genotype (Gunge and Nakatomi, 1972), being able

to cross with another mating-competent haploid or diploid cell. This technique, known as rare mating, is less common because hybridization frequency is lower ('rare') than those obtained by spore-to-spore or mass-mating conjugations. However, as hybrid genomic architectures will differ according to the mating involved in the hybridization, rare mating has the advantage of maintaining the heterozygosity levels of the parents in all the initial putative allotetraploid hybrids (Bellon et al., 2015; Pérez-Través et al., 2012). The first step required for rare mating is the selection of natural auxotrophic markers in the strains to hybridize, so the prototrophic recovery technique can be used to select the hybrids.

Theoretically, when diploid strains are used to obtain hybrids, as in the case of our *S. cerevisiae* and *S. uvarum* selected parental strains, allotetraploids with the same putative genomic constitution of the parents are obtained. If a hybrid strain is going to be transferred to the industry, it is necessary to ensure its genomic stability. Then, an adaptive stability test needs to be performed. In our case, it was carried out by vegetative growth in fermentative conditions, mimicking the winemaking process, for hybrids and spore-derivative hybrids. During the mitotic or meiotic divisions, different genomic rearrangements or chromosome segregations can be produced, giving rise to a variety of derived allopolyploids (during vegetative growth) and allodiploids (after sporulation) and even mosaic strains with potential physiological differences ofinterest. An autotetraploid produces autodiploid spores possessing two complete sets of chromosomes, but malsegregation of the octavalent chromosomes during meiosis usually results in aneuploidies. An allotetraploid also produces diploids but these are not autodiploids but allodiploids due to the phenomenon referred to as autodiploidization of the allotetraploid meiosis (Karanyicz et al., 2017). If we take into account all the obtained hybrids, the different behavior and genome composition can be due to different factors considered above, and on the other hand, during the stabilization process, we did not use a high selective pressure, so chromosome losses and stabilization can occur in different ways by chance.

Artificial interspecific hybrids are often disadvantageous compared with their parental species because of their potential reduced viability (Mercer et al., 2007; Piotrowski et al., 2012). However, one of the hybrid monosporic derivatives, H14A7, showed hybrid vigor (Lippman and Zamir, 2007).

Thus, H14A7 performed wine fermentations at 25ºC faster than its parents and the other derived hybrid, and at lower temperatures showed a better behavior than the *S. cerevisiae* parental strain.

As a monosporic derivative of a putative allotetraploid hybrid generated by rare mating, strain H14A7 was expected to be an allodiploid hybrid. However, a combination of flow cytometry and genome sequencing data indicated that H14A7 strain is an almost perfect allotriploid, with one copy of the *S. uvarum* genome, and two heterozygous copies of each *S. cerevisiae* chromosome, except chromosome III, which is present in one copy. Moreover, the levels of heterozygosity of the *S. cerevisiae* subgenome of the hybrid, except for the monosomic chromosome III, were identical to those of the parental *S. cerevisiae* genome. This indicates that the hybrid maintains the two homologous copies ofthe *S. cerevisiae* parental chromosomes, with the exception of chromosome III.

There are two possible explanations for the genome composition of this monosporic-derivative hybrid H14A7. In the first, the original hybrid H14 could be a perfect allotetraploid, and the missegregation of the homologous *S. cerevisiae* chromosomes during the meiotic division I generated the H14A7 allotriploid. The different meiotic behavior of the subgenomes is consistent with the autodiploidization ofthe allotetraploid meiosis Sipiczki (2018). This scenario is supported by previous studies with artificial *S. cerevisiae* × *S. uvarum* hybrids (García-Ríos et al., 2019c). Allotetraploids are more prone to malsegregation than the autotetraploids, supposedly due to occasional allosyndetic pairing between homeologous chromosomes of the subgenomes (Sipiczki, 2018). In H14A7, it could be hypothesized that the *S. cerevisiae* subgenome, as a whole, failed to perform normal meiosis I. Another scenario, which could have produced an allotriploid from an allotetraploid, is the loss of the *S. uvarum* part of the hybrid during the course of successive meiotic divisions (Antunovics et al., 2005), a process termed genome autoreduction in meiosis (GARMe) (Sipiczki, 2018). This scenario is less relevant here because it takes place after the breakdown of the sterility barrier and cannot result in a one-step malsegregation of all chromosomes of the *S. uvarum* subgenome.

In the second hypothesis about the origin of the H14A7 monosporic derivative, the original

hybrid H14 could be originated by a rare mating event between a mating-competent *S. cerevisiae* diploid cell and a *S. uvarum* haploid cell. The subsequent sporulation of this allotriploid, the two *S. cerevisiae* homologous chromosomes and the *S. uvarum* homeologous one should move together during the wrong meiosis I division. In this case, two spores would be viable and the other two non-viable, which is congruent with the tetrad composition from which the H14A7 spore was dissected. This scenario is supported by a previous study in our laboratory, in which an artificial *S. cerevisiae* × *S. kudriavzevii* hybrid was generated by rare mating (Morard et al., 2020a). This *S. cerevisiae* × *S. kudriavzevii* hybrid showed the same genome composition than H14A7, it was an allotriploid with one copy of the non-cerevisiae (in this case, *S. kudriavzevii*) genome and two heterozygous copies of each *S. cerevisiae* chromosome (the same than its *S. cerevisiae* parental strain T73), except a monosomic chromosome III.

Both parental *S. kudriavzevii* (CR85) and *S. uvarum* (BMV58) strains were able to sporulate in the rare-mating rich media, although the first much more efficiently than the latter. The most important difference between both studies is the fact that the artificial *S. cerevisiae* × *S. uvarum* hybrid H14 was subjected to sporulation to generate H14A7, but the artificial *S. cerevisiae* × *S. kudriavzevii* hybrid not, but in both cases converged to analogous genome compositions.

Therefore, the genome composition of H14A7 indicates that the original hybrid H14 could be the result of a 'rare mating' event involving a mating-competent *S. cerevisiae* AJ4 diploid cell and a *S. uvarum* BMV58 haploid or mating-competent diploid cell with the opposite mating type.

However, our spore-derivative hybrid resulted to be an aneuploid allotriploid with one *S. uvarum* genome copy, and two heterozygous copies of each *S. cerevisiae* chromosome, with the exception of a single copy of chromosome III, which contains the *MAT* locus. This result opens the possibility that the parental diploid *S. cerevisiae* strain acquired mating-competence, not by becoming homozygous for the *MAT* locus due to gene conversion, but because of the loss of one of the chromosomes III. A mating-competent diploid *S. cerevisiae* cell, monosomic for chromosome III with the *MATa* allele, could conjugate with a *MAT*α haploid or *MAT*α/*MAT*α diploid cell of *S. uvarum* to generate H14. This scenario is supported by the fact that the artificial *S. cerevisiae*

× *S. kudriavzevii* hybrid generated by rare mating (Morard et al., 2020a), but not subjected to sporulation, also was an allotrianeuploid with one copy of the *S. kudriavzevii* genome and two highly heterozygous copies of each *S. cerevisiae* chromosome, except a monosomic chromosome III. This is also congruent with the fact that the *S. cerevisiae* chromosome III, one of the smallest, shows the highest loss frequency (Kumaran et al., 2013), and the fact that the presence of a single copy of chromosome III in diploid hybrid sub-genomes is common in rare mated hybrids (Krogerus et al., 2016).

However, as the genome sequence of the original hybrid H14 is not available, we cannot completely discard that the rare mating, originating H14, could involve a *S. cerevisiae* euploid cell competent for mating due to gene conversion. In that case, the presence of only one *S. cerevisiae* chromosome III copy in H14A7 could be the result of a chromosome loss during the meiotic division of the H14 hybrid, as chromosome III is one of the least stable chromosomes also in alloploid hybrid genomes (Kumaran et al., 2013). In other words, as the genome composition of H14 is unknown, we cannot determine if the lack ofone copy ofthe *S. cerevisiae* chromosome III in H14A7 is due to a prezygotic (occurring in AJ4, the *S. cerevisiae* parent, before the hybridization) or to a postzygotic (taking place during the meiotic division of the hybrid cell) event.

The availability of artificial hybrids, in addition to their biotechnological interest, offers new challenges to study how two genomes, two transcriptomes, two proteomes, and two metabolomes interact to merge into a single system in the hybrid, and what are the consequences of this fusion to generate functional innovations for the adaptation to wine fermentation environments. In our case, we analyzed transcriptomic data obtained during fermentation at two temperatures, 15ºC typical for white and rosé wines, and 25ºC for red wines. Multivariate analysis showed that the first two principal components, corresponding to the fermentation phase and species, respectively, described 84% of the variability. This result corroborates that strain behavior depends strongly on the wine fermentation phase (Marks et al., 2008; Varela et al., 2005; Zuzuarregui et al., 2006) and on the properties of each strain (James et al., 2003; Tronchoni et al., 2014, 2017). The third factor that affected gene expression was the temperature, mainly due to cold stress response (Tronchoni et al., 2014, 2017).

In the comparative expression analysis between hybrid subgenomes, previous studies (Duval et al., 2010; Pfliegler et al., 2014) reported that each parental fraction act differentially during fermentation; being the *S. cerevisiae* subgenome more efficient in fermentation performance and the *S. uvarum* in temperature adaptation. In our case, we observed the most significant differences in the fermentation latency phase, when yeasts have to cope with the new stress conditions of the beginning of fermentation, such as high osmolarity due to increased sugar concentrations, high sulfite levels, acid stress, and low temperature, in the case of fermentation at 15ºC. At this temperature, whilst the *S. cerevisiae* hybrid subgenome focuses on catalytic activity and nutrient uptake (cofactor, ion, and vitamin binding), congruent with its better nutrient uptake efficiency (Alonso-del Real et al., 2019), *S. uvarum* fraction of the hybrid shows a higher expression in ribosome biogenesis, involved in the translation machinery necessary for growth and division, as well as in the metabolism of ergosterol, a membrane compound required for membrane protein trafficking at low temperature (Abe and Minegishi, 2008; Parks et al., 1995). An analysis of the differential expression between *S. cerevisiae* and *S. kudriavzevii*, another cryophilic species, during fermentation at low temperature, concluded that *S. kudriavzevii*, under cold stress, enhances translation efficiency by synthesizing ribosomes to overcome the alteration in the stability of functional RNAs (Tronchoni et al., 2014). This response to low temperature was also observed in a transcriptome analysis of natural *S. cerevisiae* × *S. kudriavzevii* hybrids (Tronchoni et al., 2017), in which, as occurs in our *S. cerevisiae* × *S. uvarum* hybrid, the most remarkable group of upregulated genes corresponded to the translation machinery category and membrane composition due to the response of the non-*cerevisiae* subgenome to cope with the cold shock.

In the latency phase of the fermentation at 25ºC, the *S. uvarum* subgenome showed two up-regulated genes, *GPD1* and *GPD2*, of great importance because they encode glycerol-3-phosphate dehydrogenases involved in glycerol synthesis. The higher production of glycerol, typical of cryophilic species such as *S. uvarum* and *S. kudriavzevii*, has been proposed as a mechanism to adapt to low-temperatures, high osmolarity, and also to maintain the NAD + /NADH redox balance during fermentation (Oliveira et al., 2014; Pérez-Torrado et al., 2016). According to these results, we can conclude that the interactions between the two subgenomes in the hybrid

improve those differential species-specific adaptations to the wine fermentation environments, already present in the parental species.

Regarding the ethanol tolerance of H14A7, which proved to be higher than BMV58 but lower than AJ4 at the tested temperatures, it is difficult to analyze specific gene expression, as yeast answer to ethanol stress is complex and not fully understood yet (Mager and Moradas Ferreira, 1993). However, there are some traits that have been related to ethanol tolerance answer: changes in membrane composition, as unsaturated fatty acid and ergosterol content (Mishra and Prasad, 1989; Vanegas et al., 2012), and different amino acid presence in media (Hirasawa et al., 2007).

When we compared GO term over-representation in *S. uvarum* and *S. cerevisiae* subgenomes of the hybrid that could be related to ethanol tolerance, we focused on transcriptomic data obtained in the exponential phase because, during the latency phase, the amount of ethanol in the media is low. In H14A7, some of the GO terms of genes that are differentially regulated in the species subgenomes of the hybrid, are fatty acid catabolic process and short-chain fatty acid metabolic process (*S. uvarum* vs. *S. cerevisiae* exponential 25ºC) as well as cellular amino acid metabolic process (*S. cerevisiae* vs. *S. uvarum* exponential 25ºC). The two first processes are related to membrane composition modification as a response to the effect of the ethanol on membrane fluidity (Ma and Liu, 2010). Our results suggest that H14A7 is combining *S. cerevisiae* and *S. uvarum* strategies to respond to ethanol stress.

Nevertheless,this transcriptomic analysis is an attempt to determine the relative contribution ofeach subgenome in H14A7, but the equilibrium acquired between both subgenomes in the hybrid is the result of complex processes, and some up-regulated genome-specific alleles may be under the control of regulators of the other species (Tronchoni et al., 2017).

CHAPTER 4

Adaptive response to wine selective pressures shapes the genome of a *Saccharomyces* interspecies hybrid

This chapter is accepted for publication in *Microbial Genomics*.

4.1 Introduction

Winemaking is one of the human fermentation practices in which yeast species play an important role, by converting sugar present in the grape must into ethanol, CO_2, and different metabolites. Yeast cells undergo different stresses during fermentation: osmotic pressure due to the high sugar concentration in grape musts at the beginning of the process, ethanol accumulation that can represent a percentage up to a 16% in the media, low pH, SO_2 presence, etc. (Alexandre and Charpentier, 1998; Arroyo-López et al., 2009; Belloch et al., 2008; Charoenchai et al., 1998; Fleet and Heard, 1993; Margalit, 1997).

S. cerevisiae is the *Saccharomyces* species most widely used in fermentation, as it can overcome these stressful conditions, especially ethanol stress conditions (Arroyo-López et al., 2010b), during the fermentation process. *S. uvarum* is a cryotolerant species that produce more glycerol and less acetic acid than *S. cerevisiae* as well as presenting rich aroma profiles (Castellari et al., 1994; Giudici et al., 1995; González et al., 2007). *S. cerevisiae* x *S. uvarum* hybrids (Masneuf et al., 1998) are also found in natural habitats and mainly in alcoholic fermentation environments (González et al., 2006). It has been stated that they can present an advantage in winemaking, especially for white wines, which are fermented at low temperatures (González et al., 2007).

Hybrid genomes are known to fix mutations under selective pressure and undergo adaptive evolution through genome re-organization (Gorter de Vries et al., 2019; Pérez-Través et al., 2014a; Peris et al., 2017). This way, *Saccharomyces* interspecies hybrids can be used as model organisms for studying adaptation to stressful environments and better understand the interactions of their subgenomes in the adaptation to these conditions (Lopandic, 2018).

Although the study of how genome adaptation occurs is an interesting area of study, adaptation strategies have also been carried out with the aim to improve yeasts at industrial level. The use of sequential batch fermentations with selected strains has proved to increase the fitness of *Saccharomyces* hybrids in sulfate limitation conditions and in lager-brewing conditions (Gorter de Vries et al., 2019; Sanchez et al., 2017).

As for wine strains, it is possible to use a media simulating wine fermentation and its stresses to adapt strains to that particular must (McBryde et al., 2006) . Adaptation to wine must is interesting as this media contains a high ethanol concentration and sulfite, which are toxic compounds for yeast cells. In response to ethanol exposure, yeasts incorporate this molecule into the membrane, which causes an increase in the membrane fluidity and an alteration in the lipid composition of membranes (Jones and Greenfield, 1987; Lloyd et al., 1993). Sulfite (SO_3^{2-}), is usually added in the form of potassium metabisulfite ($K_2S_2O_5$) and it is used because it inhibits the presence of other undesirable microorganisms, but it also affects yeast cells (Ribéreau-Gayon et al., 2006).

In a previous study, we obtained a *S. cerevisiae* x *S. uvarum* hybrid, H14A7 (Lairón-Peris et al., 2020), which showed a high glycerol production during natural musts fermentations in comparison to *S. cerevisiae* parental strain and a higher ethanol tolerance than the *S. uvarum* parental strain, which are interesting traits for wine strains. This hybrid was stabilized by vegetative growth in fermentative conditions.

In the present work, we aimed to characterize what happens in H14A7 hybrid genome when we perform a laboratory adaptation strategy by mimicking a must media similar to that present in wine fermentations advanced stages, when a high sulfite content and reduced levels of sugars and increasing levels of ethanol are present. H14A7 has two subgenomes: *S. cerevisiae*, conferring ethanol tolerance, and *S. uvarum*, conferring higher glycerol production and capacity to grow at low temperatures (Lairón-Peris et al., 2020); so the analysis of how adaptation to this stressful media affects each subgenome is one of the main goals of this work.

The adapted strain genome was sequenced and wine fermentations at 15ºC and 25ºC were performed. Analysis of the transcriptomic and the lipidomic profiles of the newly generated hybrid during the fermentation was carried out to compare the expression and the membrane composition of the adapted hybrid with the original H14A7 strain.

4.2 Materials and Methods

4.2.1 Growth on modified synthetic must

Adaptation to a stressful media of the *S. cerevisiae* AJ4 x *S. uvarum* BMV58 hybrid H14A7, obtained in Lairón-Peris et al. (2020) was performed using batch cultures in triplicate, in bottles of 100 mL with 60 mL of modified synthetic must (M-SM) (Rossignol et al., 2003). Different M-SM compositions were used with different sugar and ethanol concentrations, which are specified in Table 4.1. In all conditions 100 mg/L of metabisulfite, $K_2S_2O_5$ were added to M-SM. The inoculated cell population in each bottle was approximately 2 x10^6, and once stationary phase was achieved the culture was transferred in fresh media and cultivated the same way. The initial ethanol concentration was 2.5% (v/v). The media was refreshed approximately every 7 days. Ethanol concentration was increased every two or three weeks depending on the latency period and the time the cultures took to reach the stationary phase. All the adaptation processes were performed at an incubation temperature of 28ºC and orbital continuous shaking at 100 rpm. When an ethanol concentration of 9% in the media was reached, a pool of colonies was selected and named H14A7-etoh (Figure 4.1).

TABLE 4.1 Composition of the modified synthetic must (M-SM) used during the adaptive laboratory evolution. Besides the compounds described in (Rossignol et al., 2003) for synthetic must, different ethanol percentages were added and sugar content was modified. In all conditions 100mg/L of metabisulfite, $K_2S_2O_5$, were added to M-SM.

Condition	Ethanol % (v/v)	Glucose (g/L)	Fructose (g/L)
0	2.5	75	75
1	5	50	50
2	6	40	45
3	6.5	35	40
4	7	30	40
5	7.5	25	35
6	8	20	35
7	9	20	35

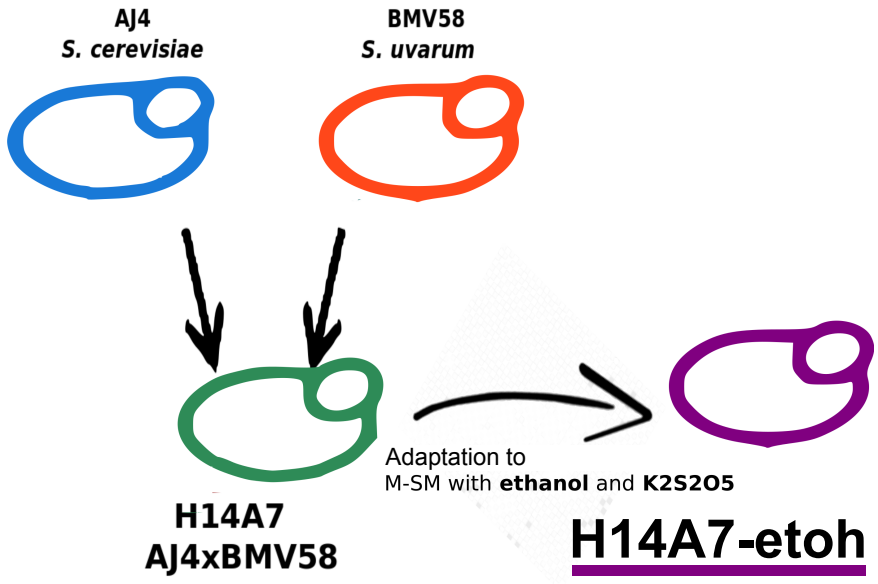


FIGURE 4.1 Scheme of H14A7-etoh obtainment.

4.2.2 Yeast growth media conditions.

Tolerance to ethanol was determined by growing H14A7 and H14A7-etoh strains in YNB with increasing ethanol concentrations (0; 1; 2.5; 4; 6; 8; 10; 12.5; 14; 15.5; 17; 18; 20 (%)) in microtitter plates. The overall yeast growth was estimated as the area under the OD vs. time curve using GCAT (Bukhman et al., 2015), and the NIC and MIC parameters, which are ethanol-tolerance indicators, were obtained as described elsewhere (Arroyo-López et al., 2010b).

Tolerance to sulfite stress was evaluated by drop tests. Sulfite plates were prepared by using YEPD+TA (tartaric acid) agar plates and supplementing them with different $K_2S_2O_5$ concentrations. YEPD+TA plates were prepared as described in Park et al. (1999) (YEPD:2 % glucose, 2% peptone and 1% yeast extract; 75 mM L-tartaric acid buffered at pH 3.5). YEPD+TA+$K_2S_2O_5$, sulfite plates, were prepared by pouring and spreading freshly prepared $K_2S_2O_5$ to each YEPD+TA solid plate to reach the following concentrations of metabisulfite: 0; 0.5; 1; 1.5; 2; 2.5; 3; 3.5 and 4 mM. Yeast precultures were grown overnight in GPY (peptone 0.5%, yeast extract 0.5%, glucose 2%) medium. Cell cultures were diluted to OD_{600} = 0.1. Then, serial dilutions of cells were transferred

to the plates and incubated at 25ºC for a week.

4.2.3 Genome sequencing, copy number analysis, and SNPs fixation analysis

The sequenced reads of H14A7 original hybrid and the genome assemblies and genome annotation files of BMV58 and AJ4 strains were available from a previous work (Lairón-Peris et al., 2020). H14A7-etoh DNA was extracted according to Querol et al. (1992) and sequenced using the Illumina Miseq system, with paired end reads of 250 pair bases. These reads were trimmed and quality filtered to a quality of 28 and a length of 180 using Sickle (Joshi and Fass, 2011) (NCBI accession number PRJNA604709). H14A7-etoh reads were mapped to the *S. cerevisiae* and *S. uvarum* parentals AJ4 and BMV58 concatenated sequences using BOWTIE2 with the default settings. Bedtools was used to obtain the coverage "per base". These coverage files were processed to reduce the noise using sliding windows with a windows size of 1000 positions. As a complementary approach, CNVnator was used for the discovery of copy number variation (CNV) (Abyzov et al., 2011). We used sppIDer (https://github.com/GLBRC/sppIDer) to plot chromosomes'coverage.

The gdtools command installed as part of breseq (version 0.27.1) was used to identify single nucleotide polymorphisms (SNPs) in H14A7-etoh genome which were not present in neither the parental genomes (AJ4 and BMV58) nor in the hybrid H14A7. We used H14A7-etoh read files and the annotation files of AJ4 and BMV58 as a reference, with option-p –polymorphism-frequency-cutoff of 0.20. The same procedure was performed with H14A7, AJ4 and BMV58 reads to only retain variants which are only present in H14A7-etoh. We manually curated the SNPs present in non synonymous positions using the software Tablet (Milne et al., 2013), by visualizing the reads of H14A7 and H14A7-etoh against the assemblies of AJ4 and BMV58 parentals, to only take into account SNPs that were fixed in the adapted hybrid. Only indels and SNPs which were supported by more than 20 reads in a region were taken into account.

4.2.4 Flow cytometry analysis

The DNA content of the adapted hybrid was assessed by flow cytometry using a FACSVerse™ flow cytometer (BD Biosciences). Cells were grown overnight in GPY and 1 OD_{600} of each culture was harvested by centrifugation. DNA staining was performed using dye SYTOX Green as previously described (Haase and Reed, 2002). Haploid (S288c) and diploid (FY1679) reference *S. cerevisiae* strains were used to compare the fluorescence intensity.

4.2.5 Microfermentations in Verdejo must and transcriptomic analysis

Microvinifications were conducted in triplicate in Verdejo must with the strain H14A7-etoh at two different temperatures: 25ºC and 15ºC, as described previously (Lairón-Peris et al., 2020) for *S. cerevisiae* AJ4, *S. uvarum* BMV58, the hybrid H14A7 strains. Final metabolites were measured by HPLC in the last stage of fermentation. Weight loss data was followed during the fermentations and corrected to the percentage of consumed sugar as described previously (Pérez-Través et al., 2015). Data on the percentage of consumed sugars was fitted to the Gompertz equation (Zwietering et al., 1990). Kinetic parameters D, maximum sugar consumption value reached (the asymptotic maximum, (%)), m (maximum sugar consumption rate, (g L^{-1} h^{-1})), l (lag phase period, (h)) were calculated. These data were tested to find significant differences among them by using the one-way ANOVA module of the Statistica 7.0 software (StatSoft, Tulsa, OK, USA). Means were grouped using the Tukey HSD test (α=0.05).

Samples for RNA-seq were collected at two different time points: lag phase (which corresponded to 4h of fermentation at both temperatures) and mid-exponential growth phase (which corresponded to 24h of fermentation at 25ºC and to 48h of fermentation at 15ºC respectively) and were analyzed as in (Lairón-Peris et al., 2020). Reads were sequenced using the Illumina Hiseq 2000, paired end reads of 75 bases long were generated and submitted to NCBI SRA (accession number PRJNA604708). These reads were quality trimmed using sickle (length 50, quality 23) and aligned to the fasta reference using bowtie2. We used HTSeq-count

(Anders et al., 2015) with both annotated file and the mapping files ordered by names and generated the counts table. The mapping reads with a quality score lower than 2 and with more than one alignment were discarded. Data were analyzed using the EdgeR package to look for differential expression genes (Robinson et al., 2009). We calculated normalization factors to scale the raw library sizes and then we tested for differential expression between two groups of count libraries. Differential expression levels (relative RNA counts) between the different conditions were considered significantly different with a false discovery rate (FDR) (Benjamini and Hochberg, 1995) at a threshold of 5%. Gene Ontology (GO) terms were attributed to the lists of differentially expressed genes by using YeastMine from SGD Database (https://yeastmine.yeastgenome.org/). GO terms enrichment were retrieved with p-values < 0.05 after computing the Benjamini and Hochberg correction for multiple hypotheses.

4.2.6 Mass spectrometry of lipids present in the membrane of the strains

Four yeast strains (AJ4, BMV58, H14A7 and H14A7-etoh) were grown in 25 mL GPY media, with five flasks set up per strain. After 4h of growth, the cultures were harvested and total lipids were extracted using a modified Bligh and Dyer protocol (Spickett et al., 2001). The lipids were reconstituted in 100 µL chloroform and then diluted 1 in 50 in solvent A (50:50 acetonitrile:H_2O, 5 mM ammonium formate and 0.1% v/v formic acid). Analysis of 10 µL samples was performed by LCMS. LC was performed on a U3000 UPLC system (Thermo scientific, Hemel Hempstead) using a Kinetex C18 reversed phase column (Phenomenex, 2.6 µm particle size, 2.1 mm x 150 mm), at a flow rate of 200 µL/min with a gradient from 10% solvent A to 100% solvent B (85:10:5 isopropanol: acetonitrile: H_2O, 5 mM ammonium formate and 0.1% v/v formic acid) with the following profile: t=0 10% A, t=20 86%A, t=22 96%A, t=26 95%A. MS analysis was carried out in positive and negative ionization mode on a Sciex 5600 Triple TOF. Source parameters were optimized on infused standards. Survey scans were collected in the mass range 250-1250 Da for 250 ms. MSMS data was collected using top 5 information dependent acquisition and dynamic exclusion for 5 s, using a fixed collision energy of 35V and a collision energy spread of 10V for 200 ms per

scan. ProgenesisQI® was used for quantification and LipidBlast for identification. All data were manually verified and curated.

4.2.7 Lipid quantification by ammonium ferrothiocyanate assay

To quantify the lipids, 10 µL sample was taken from the above 100 µL reconstituted lipids in chloroform and added to 2 mL chloroform with 1 mL of assay reagent (0.1M $FeCl_3.6H_2O$, 0.4 M ammonium thiocyanate) in a 15 mL glass tube. Samples were vortexed for 1 min and centrifuged at 14 500 g for 5 mins. The lower layer was collected into quartz cuvettes. The absorbance was measured at 488 nm, and the concentration of lipid was determined by comparison with a standard curve of a mixture of phospholipid standards (POPC, POPE and POPG) (Sigma).

4.2.8 TLC analysis

Yeast lipids extracted as above after 24 h growth were analyzed by TLC. Briefly, 20 µg of lipid sample and 10 µg phospholipid lipid standards (POPC, POPE and POPS) (Sigma) were loaded onto silica gel TLC plates (Sigma) and separated using chloroform/methanol/acetic acid/water 25:15:4:2. The plates were air dried and either sprayed with molybdenum blue reagent (1.3 % molybdenum oxide in 4.2 M sulphuric acid) (Sigma), or sp ninhydrin reagent (0.2% ninhydrin in ethanol) (Sigma) and charred at 100°C for 5 mins. Spot intensity was determined using ImageJ software.

4.2.9 Laurdan membrane fluidity assay

Yeast precultures of each one of the four selected strains (AJ4, BMV58, H14A7 and H14A7-etoh) were first propagated overnight in 25 mL of GPY media at 200 rpm and 28ºC. Then, 10 mL of GPY media in 15 mL falcon tubes was inoculated to an OD_{595} of 0.4 and incubated at 200 rpm, 28ºC. Samples were taken after 24 h and live yeast were diluted to an OD_{595} of 0.4

in GPY and incubated with 5 μM laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) for 1 h. Fluorescence emission of these cells stained with Laurdan was taken using a microplate reader (Mithras, Berthold) with the following filters; λex=460 λem=535. Generalized Polarization (GP), derived from fluorescence intensities at critical wavelengths, can be considered as an index of membrane fluidity and is calculated as $GP = (I_{460} - I_{535}) / (I_{460} + I_{535})$

4.3 Results

4.3.1 Characterization of the hybrid after the adaptation process.

Hybrid H14A7 was subjected to adaptation to ethanol in liquid media. A series of synthetic musts with increasing ethanol content mimicking different stages of the fermentation process were used to that end, maintaining a high concentration of metabisulfite in all cases (Table 4.1). H14A7 was exposed to these media conditions for approximately 200 generations and the obtained strain was named H14A7-etoh.

After that process, we carried out drop tests in plates containing different concentrations of sulfite to see if the presence of metabisulfite in our adaptation media had an effect on sulfite tolerance in the adapted strain. Interestingly, the phenotypes of the different tested strains (H14A7, H14A7-etoh, BMV58, and AJ4) showed remarkable differences, with H14A7-etoh being the most resistant (Figure 4.2).

We tested the ethanol tolerance of H14A7-etoh and H14A7 strains to see if the addition of ethanol had an impact on the phenotype too. The NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) which are two parameters that respectively indicate which ethanol concentration affects a strain and at which ethanol concentration the strain is not able to grow, were calculated. H14A7 NIC and MIC values were 8.51±0.27 (%) and 14.5±0.354 (%); whereas H14A7-etoh NIC and MIC values were 7.93±0.26 (%) and 15.3±0.143 (%). The adapted hybrid showed slightly more ethanol tolerance than H14A7 in its MIC value, ($P < 0.1$, ANOVA and

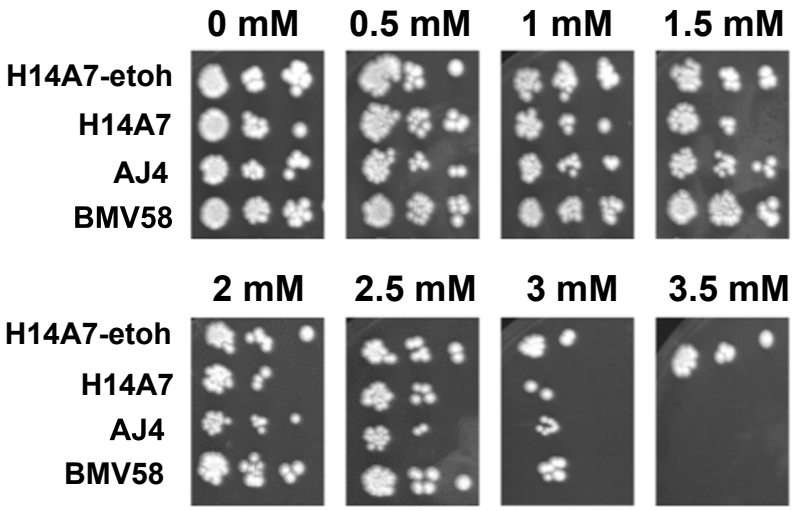


FIGURE 4.2 Sulfite tolerance analysis of BMV58, AJ4, H14A7 and H14A7-etoh *Saccharomyces* strains. Plates' media is YEPD + TA + different $K_2S_2O_5$ concentrations. Images were taken after seven days of growth at 25ºC.

Tukey's test) being NIC values non-significant ($P < 0.05$, ANOVA and Tukey's test).

4.3.2 H14A7-etoh shows different signals of adaptation to the selection media on its genome: CCNV

This part of the study aimed to detect genomic differences of the adapted hybrid in comparison to the original strain. After obtaining the sequenced reads of the H14A7-etoh genome, they were quality trimmed and reduced to a total number of 6767268 reads, which represents a coverage of approximately 67.5x. With these reads, we performed analyses to identify differences from the original H14A7 hybrid genome.

From a previous work, (Lairón-Peris et al., 2020) we knew that H14A7 had two copies of each *S. cerevisiae* chromosome, except for chromosome III, which was present only in one copy; and one copy of each *S. uvarum* chromosome. After analysis with sppIDer and CNVnator, we noticed large modifications in the chromosomes of the adapted strain H14A7-etoh compared to the hybrid H14A7 genome; *S. cerevisiae* (III-cer) and *S. uvarum* chromosome VII-XVI (VII-XVI-uva) had been duplicated. Moreover, a chromosomal loss of *S. uvarum* chromosome I (I-uva) had taken place.

Analysis by flow cytometry revealed that the ploidy of H14A7-etoh is 3.27±0.1, whereas H14A7 had a ploidy of 2.98±0.02 (Lairón-Peris et al., 2020). This increased ploidy can be explained because of the aneuploidies mentioned above, especially *S. uvarum* VII-XVI duplication. *S. cerevisiae* chromosome III and *S. uvarum* chromosome I are small chromosomes, and their contribution to the ploidy is smaller than *S. uvarum* VII-XVI. In Figure 4.3A and Figure 4.3B, a representation of the chromosome copy number of H14A7 and H14A7-etoh can be seen. III-cer and VII-XVI-uva aneuploidies could have a relevant role in the adapted hybrid. VII-XVI-uva is a chromosome with a translocation in BMV58 parental strain which confers sulfite resistance to BMV58 strain, as it recombines *FZF1t* transcription factor (present in chromosome VII) with *SSU1* gene involved in sulfite metabolism (present in chromosome XVI) (Macias et al ., submitted). The presence of an extra copy of this chromosome in H14A7-etoh is the most reasonable explanation of H14A7-etoh high resistance to sulfite. III-cer aneuploidies have been correlated with ethanol tolerance in *S. cerevisiae* strains (Morard et al., 2019).

4.3.3 SNPs, duplications, and deletions in H14A7-etoh genome

To better understand genetic variation in the adapted strain, we retrieved single nucleotide polymorphisms (SNPs) in H14A7-etoh strain. The total number of SNPs present in codifying positions of H14A7-etoh strain which were not present in H14A7 were: 200 in non synonymous positions and 256 in synonymous positions of genes. Of these SNPs, we manually retrieved those in which the adapted hybrid has fixed a variant, that is, they were present in the hybrid as a heterozygous nucleotidic base and now their frequency is 1 or they have changed the nucleotidic base present in H14A7 genome. There are 4 positions in *S. cerevisiae* chromosome I with fixed positions: they are in genes YAL016C-A (dubious open reading frame), YAL010C (a subunit of both the ERMES and the SAM complex), YAR019C (a protein kinase of the mitotic exit network) and YAR035W (an outer mitochondrial carnitine acetyltransferase) (Figure 4.3C). None of these changes is generating loss of function genes. Moreover, we observed that the heterozygosity present in S. cerevisiae chromosome I is lost all over this chromosome, as a loss of heterozygosity

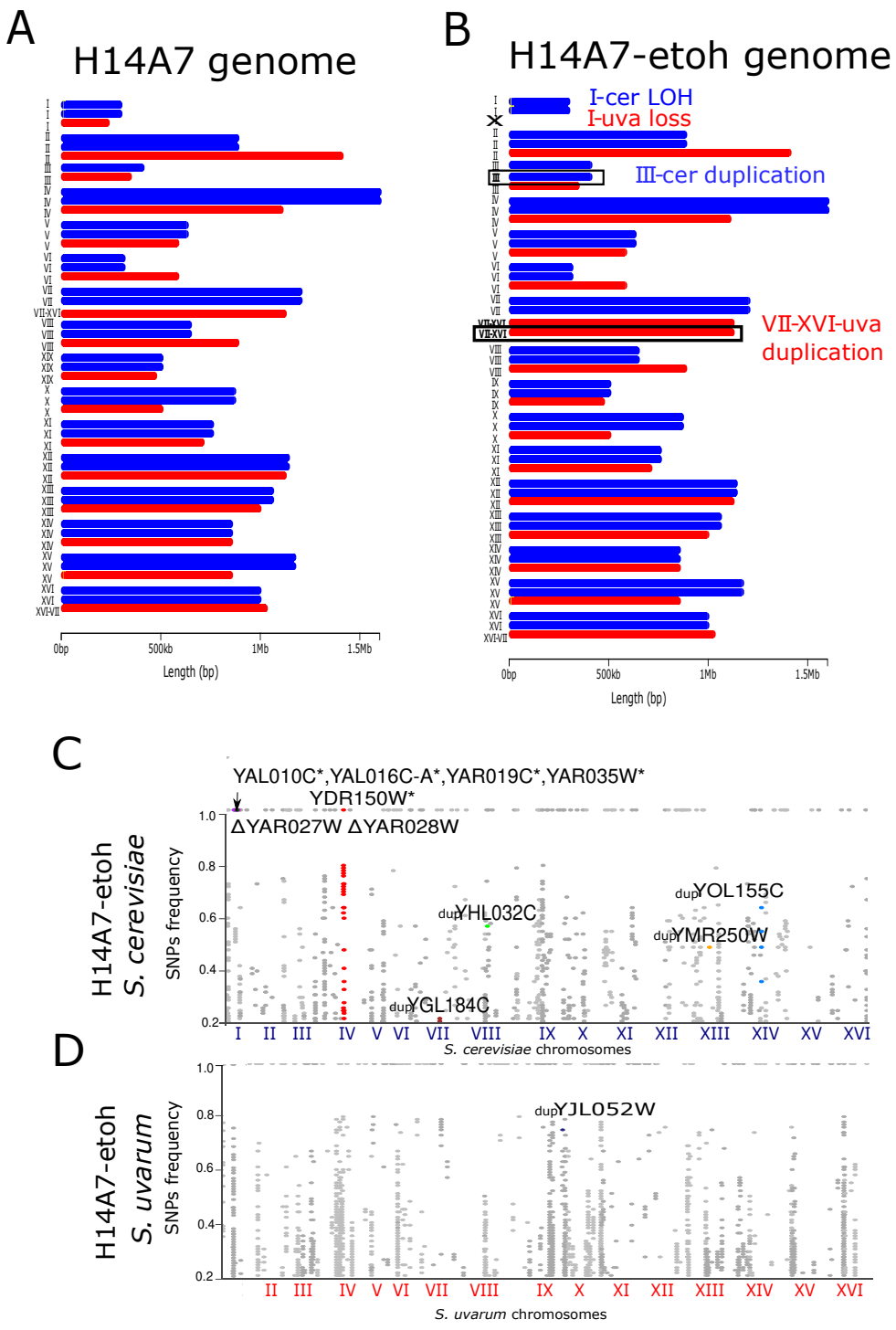


FIGURE 4.3 Genome-wide representation of H14A7-etoh strain. The chromosomes of H14A7 (A) and H14A7-etoh adapted strain (B) were represented after the analysis with sppIDer and CNVnator by using chromoMap R package. Chromosomes' length is based on AJ4 *S. cerevisiae* and BMV58 *S. uvarum* reference genomes. SNPs present in *S. cerevisiae* (C) and *S. uvarum* (D) chromosomes of H14A7-etoh strain are represented. The SNPs whose frequency is 1 and whose change affects a non synonymous position of a gene are marked with a *, confirmed duplications are marked as dup and confirmed deletions as Δ.

(LOH) event took place during the adaptation.

The possible duplications and deletions of different chromosome regions that were obtained by using CNVnator on H14A7-etoh were compared with H14A7 CNVnator coverage values and were visualized by using the mapped reads of H14A7 and H14A7-etoh against AJ4-BMV58 parental genomes. CNVnator normalizes the coverage values to 1, if the resulting number of coverage has deviated from these values, there is a putative deletion or duplication in the region.

In an H14A7-etoh *S. cerevisiae* chromosome I there is a region which comprises 6.2 KB whose coverage value is 0.2983 instead of 1. In this region, two genes are deleted in the adapted hybrid: YAR028W and YAR027W, putative integral membrane proteins of unknown function; members of *DUP240* gene family. This region, has two flanking Ty1 elements in the original hybrid genome, so a Ty1-Ty1 recombination event could have taken place and provoked a deletion (Figure 4.3C). In H14A7-etoh *S. uvarum* subgenome the gene *TDH1*/YJL052W seems to be duplicated (coverage ratio H14A7-etoh/H14A7 is 2.14). This is a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved in glycolysis and gluconeogenesis which is located next to ARS1011 duplication origin. In H14A7-etoh AJ4 subgenome there are 4 genes that showed a coverage ratio (cr) H14A7-etoh/H14A7 greater than 1.5 indicating 4 possible duplications: *GAD1* / YMR250W (cr = 1.65); *GUT1* / YHL032C (cr = 1.58); *STR3* / YGL184C (cr = 1.52) and *HPF1*/YOL155C (cr = 1.68) (Figure 4.3D). *GAD1*/YMR250W is a glutamate decarboxylase which converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism and that is involved in response to oxidative stress (Coleman et al., 2001). It is located between the Ty2 LTR and ARS1328. *GUT1* / YHL032C is a glycerol kinase; it converts glycerol to glycerol-3-phosphate; *STR3* / YGL184C is a peroxisomal cystathionine beta-lyase which converts cystathionine into homocysteine; and YOL155C/ *HPF1* a haze-protective mannoprotein. None of the SNPs, nor small duplications and deletions detected seem to have a special role in the adaptation to the hybrid adapted yeast to the M-SM used. Instead, it is important to point out that detected CNVs are near ARS and Ty elements.

TABLE 4.2 Kinetic parameters of the fermentations performed at 25ºC and 15ºC in Verdejo must. Parameters were obtained through an adjustment to Gompertz equation (Zwietering et al., 1990). D represents maximum sugar consumption value reached (%), m the maximum sugar consumption rate, (g L^{-1} h^{-1}) and l the lag phase period(h). Values are given as mean± standard deviation of three biological replicates. An ANOVA analysis was carried out and values followed by different superindexes are significantly different according to the Tukey HSD test (α=0.05).

	D %	m (g L^{-1} h^{-1})	l (h)
H14A7-25ºC	97.51±0.28^{a}	1.761±0.0985^{a}	9.84±0.080^{a}
H14A7-etoh-25ºC	98.02±0.49^{a}	1.79±0.028^{a}	11.30±0.95^{a}
	D %	**m (g L^{-1} h^{-1})**	**l (h)**
H14A7-15ºC	96.96±0.78^{a}	0.78±0.026^{a}	23.96±2.20^{a}
H14A7-etoh-15ºC	94.65±0.64^{b}	0.77±0.069^{a}	25.08±5.10^{a}

4.3.4 H14A7-etoh performance during Verdejo fermentations.

H14A7 and H14A7-etoh were used as starters of fermentations in Verdejo that were carried at 15ºC and 25ºC, conditions that mimic wine industrial conditions. The fermentation kinetics were similar between H14A7 and H14A7-etoh strains and showed no statistical differences in the calculated parameters except the maximum sugar consumption rate value, which was higher for H14A7 at 15ºC (Table 4.2). Final wine composition varied between H14A7 and H14A7-etoh (Table 3). H14A7-etoh left fructose in the fermentations at both temperatures. The amounts of fructose left behind were significantly higher than that of H14A7 (5.55 g/L at 25ºC and 5.44 g/L at 15ºC; whereas H14A7 left 0.77 g/L and 1.44 g/L respectively) (Table 4.3). One of the three biological replicas of H14A7-etoh fermentation at 15ºC was slightly delayed in comparison with the other two biological replicas (data not shown).

Ethanol and glycerol percentages were similar for both strains if we compare the final must concentrations for these compounds at the same temperature. Surprisingly, glycerol production was higher at 25ºC that at 15ºC. Acetic acid production was higher for the H14A7-etoh strain at both temperatures and the rest of the acids (tartaric, malic, citric, and L-lactic) showed no statistical differences in their content between the two strains.

TABLE 4.3 Chemical composition of the Verdejo Fermentations at 25ºC and 15ºC respectively at final point. Each component amount was measured by HPLC. Values are given as mean± standard deviation of three biological replicates. An ANOVA analysis was carried out and values followed by different superindexes are significantly different according to the Tukey HSD test (α=0.05).

	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic acid (mg/L)	Citric acid (mg/L)	Tartaric acid (mg/L)	Malic acid (mg/L)	L-Lactic acid (mg/L)
H14A7-25ºC	0.02±0.02^{a}	0.77±0.16^{a}	11.23±0.13^{a}	12.72±0.36^{a}	0.46±0.07^{a}	0.39±0.01^{a}	2.4±0.12^{a}	1.96±0.14^{a}	1.02±0.14^{a}
H14A7-etoh-25ºC	0.02±0.00^{a}	5.55±1.04^{b}	11.11±0.54^{a}	12.78±0.58^{a}	0.907±0.07^{b}	0.39±0.05^{a}	2.36±0.17^{a}	2.36±0.24^{a}	0.79±0.06^{a}
	Glucose (g/L)	**Fructose (g/L)**	**Glycerol (g/L)**	**Ethanol (%)**	**Acetic acid (mg/L)**	**Citric acid (mg/L)**	**Tartaric acid (mg/L)**	**Malic acid (mg/L)**	**L-Lactic acid (mg/L)**
H14A7-15ºC	0.00±0.00^{a}	1.41±0.53^{a}	8.70±0.09^{a}	12.86±0.12^{a}	0.58±0.07^{a}	0.28±0.05^{a}	1.92±0.09^{a}	1.79±0.07^{a}	0.38±0.03^{a}
H14A7-etoh-15ºC	0.03±0.01^{b}	5.44±0.57^{b}	8.82±0.53^{a}	12.53±0.18^{a}	1.06±0.15^{b}	0.24±0.02^{a}	2.19±0.18^{a}	1.77±0.06^{a}	0.40±0.15^{a}

4.3.5 Transcriptomic analysis of the adapted hybrid is correlated with its phenotype.

To better understand the properties acquired in the adapted hybrid (H14A7-etoh) compared to the initial strain (H14A7), we performed a comparative study of gene expression of the adapted hybrid and H14A7 during Verdejo fermentations. We retrieved a total number of 24 samples (2 strains x 2 times x 2 temperatures x three replicates) that were obtained during the Verdejo fermentations and processed to obtain RNA and transcriptomic data. We first subdivided this samples into 48 subfiles with gene counts for each species subgenome of the two strains (a file with the expression of *S. uvarum* alleles and a file with the expression of *S. cerevisiae* alleles). We observed that samples belonging to the third replicate of H14A7-etoh fermentation at 15ºC were outliers, so we excluded them from the subsequent analyses. This replicate corresponds to the one whose growth was delayed during the fermentation.

The first step of our analyses consisted of carrying a principal component analysis (PCA), that clustered the remaining 44 subfiles depending on the variance among their gene expression. We used the normalized gene count data of 5392 genes that were shared between *S. cerevisiae* and *S. uvarum* parental annotations. In this first PCA analysis, the first component, which represented 58% of the variance, is the stage of the fermentation, latency and exponential phase and temperature; the second component PC2 depended on the species subgenome analyzed (*S. cerevisiae* or *S. uvarum* part) (26%) (Figure 4.4A).

This plot showed that the most important condition that separates samples is the stage of growth. As we wanted to use the total number of ORFs annotated in both H14A7 and H14A7-etoh (*S. cerevisiae* and *S. uvarum* alleles expression), and the species subgenome analyzed corresponded to PC2, we constructed another PCA based on 22 samples corresponding to H14A7 and H14A7-etoh fermentations. They correspond to the samples that were retrieved from fermentations excluding the two samples belonging to the third replicate of H14A7-etoh fermentation at 15ºC. The RNA reads obtained of these 22 samples were mapped against a

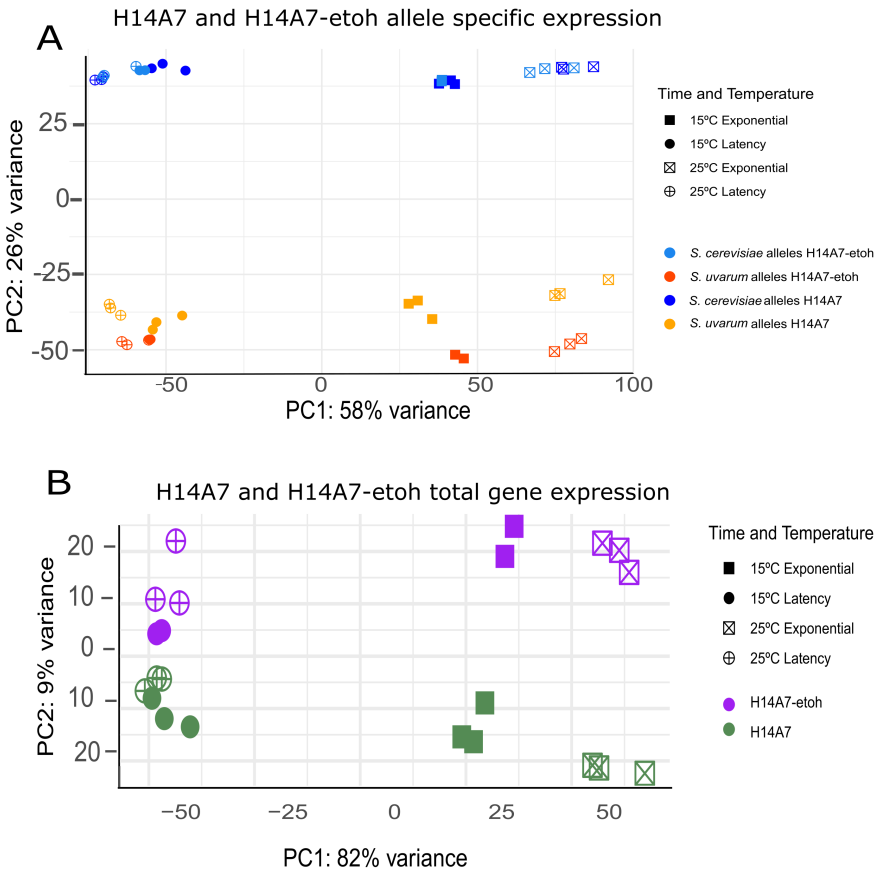


FIGURE 4.4 PCA of the transcriptome variation in H14A7-etoh and H14A7 strains. ORF expression variation of *S. cerevisiae* and *S. uvarum* alleles per separate is represented in A; and total ORF expression (*S. cerevisiae* + *S. uvarum* alleles) is represented in B. H17A7-etoh and H14A7 strains' transcriptomic samples are splitted into two different temperatures and fermentation stages.

concatenated file which contains 6182 CDS that were annotated in AJ4 parental genome and 5714 CDS that were annotated in BMV58 genome; 11896 ORFs in total. Figure 4.4B shows that the first PC (PC1), which accounts for the greater variance among samples depends on the stage phase and temperature (82% of variation in the gene expression can be explained based on these two parameters). It has to be noted that 15ºC samples and 25ºC samples are more separated in the exponential phase than in the latency phase. PC2 accounts only for 9% of sample variability and it clearly corresponds to variation between strains (H14A7 or H14A7-etoh), and again, exponential samples are more separated regarding PC2.

We, therefore, performed one differential expression test for each one of the four conditions: exponential 25ºC; latency 25ºC; exponential 15ºC and latency 15ºC, to compare H14A7-etoh differential gene expression against H14A7. We first retrieved the ORFs with less than 0.5 counts per million (CPM) in one of the conditions in order not to use low expression genes for the differential expression analysis, or genes that are not expressed in one of the samples because these genes are not present in the genome of the strains. H14A7-etoh strain has an aneuploidy in *S. uvarum* chromosome I (the copy of this chromosome was lost during the adaptive process) so we expected that the genes present in *S. uvarum* chromosome I would be excluded and no transcription of H14A7-etoh Su chrI strain was expected to take place. Transcriptomic samples of H14A7-etoh showed two exceptions, as there were reads that mapped with genes Su-YAL038W and Su-YAR035W. This happened because these two AJ4 (*S. cerevisiae* reference) genes are partially annotated and the equivalent *S. uvarum* genes are very similar to their *S. cerevisiae* variant. After excluding the genes with low expression, a total number of 10589 ORFs were used in the differential analysis, with 5411 *S. cerevisiae* ORFs and 5177 *S. uvarum* ORFs.

We performed the comparison between H14A7-etoh and H14A7 for the four conditions by using edgeR R package and kept the differentially expressed (DE) genes whose Benjamini Hochberg p-value were lower than 0.05.

As we knew that *S. uvarum* chromosome VII-XVI (VII-XVI-uva) and *S. cerevisiae* chromosome III (III-cer) have two copies in H14A7-etoh and one copy in H14A7, we first plotted the logFC, which

represent if a gene is more expressed in H14A7 or in H14A7-etoh, for each one of the genes (not only the differentially expressed) against its calculated gene ratio (H14A7-etoh vs H14A7) (Figure 4.5).

The calculated gene ratios are based on the coverage files that were obtained for each of the strains using the sliding windows approach. In Figure 4.5 it can be observed that gene expression is significantly related to the number of copies of each gene. Genes belonging to III-cer and VII-XVI-uva have mainly negative logFC for each one of the four conditions, that in our comparison (H14A7 vs H14A7-etoh) indicated that these genes are more expressed in the adapted hybrid than in the hybrid. This tendency can be particularly observed in the exponential stage at 25ºC.

We further analyzed and represented the LogFC (log2 FC) of the H14A7 vs H14A7-etoh transcriptome comparison for all of the genes grouped by chromosome for the four conditions (latency at 25ºC, exponential 25ºC, latency 15ºC, and exponential 15ºC) (Supplementary Figure 4.1).

The objective of carrying out this analysis was to observe if any other chromosome had its genes expressed differentially in the H14A7 or H14A7-etoh strains under any of the experimental conditions. Those genes which are present in the chromosomes which have an extra copy are more expressed in H14A7-etoh in all of the 4 conditions, with only one exception: chromosome VII-XVI-uva at latency stage at 15ºC, which seems to have its genes more expressed than the other chromosomes. In the case of the exponential stage at 25ºC, apart from VII-XVI-uva and III-cer, *S. uvarum* chromosome III (III-uva) genes seem to be more expressed than the genes of the rest of chromosomes.

The bar chart in Supplementary Figure 4.2 represents the number of statistically DE genes for each condition and strain in comparison with the other, identifying which of the genes belong to the *S. uvarum* and *S. cerevisiae* subgenome and which genes belong to III-cer or VII−XVI−uva.

In the four conditions, H14A7-etoh overexpressed more genes that belong to III-cer and VII-XVI-uva than H14A7, and H14A7 shows virtually no overexpression of any gene that belong to these two chromosomes. The number of up-regulated genes in H14A7-etoh compared to H14A7

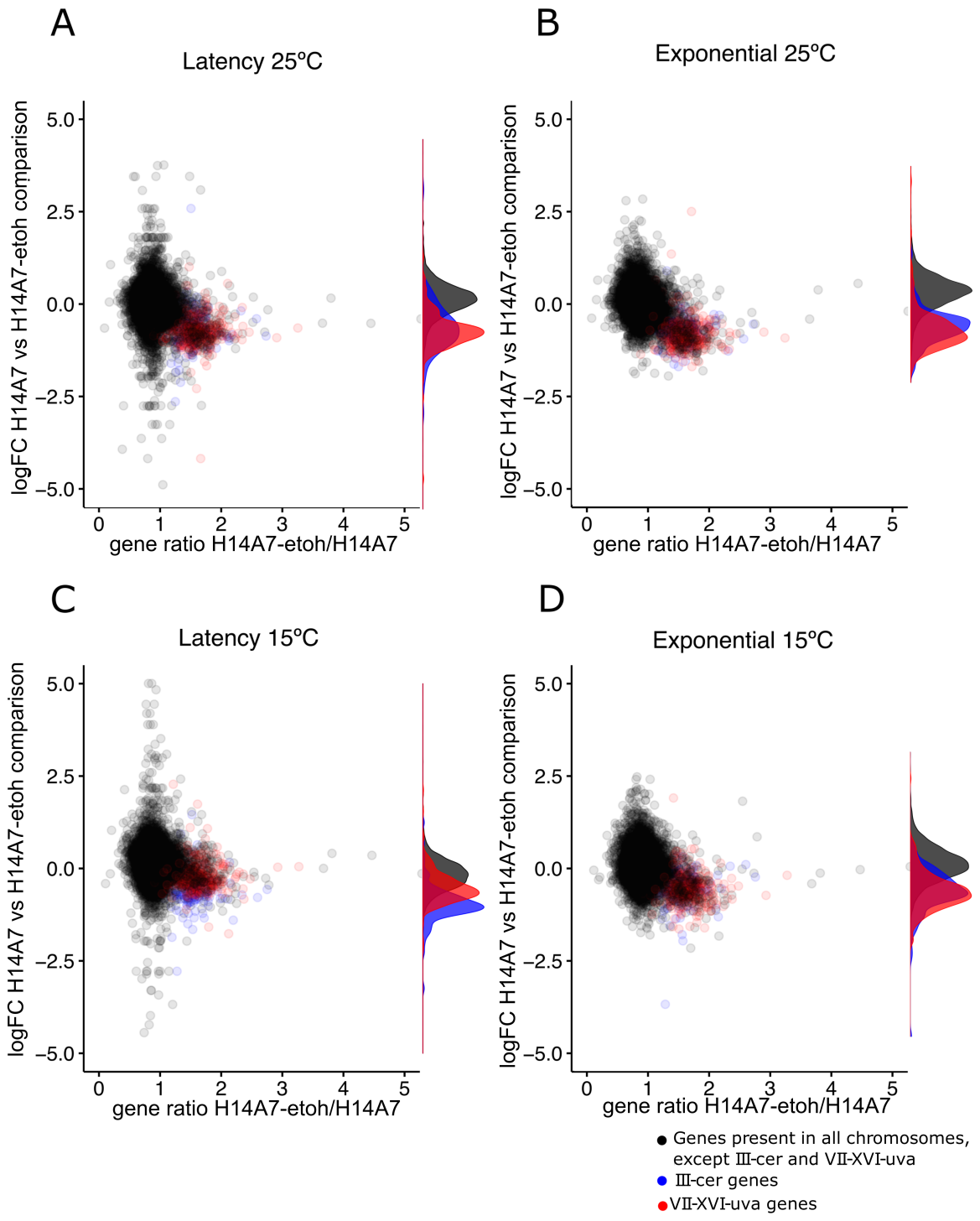
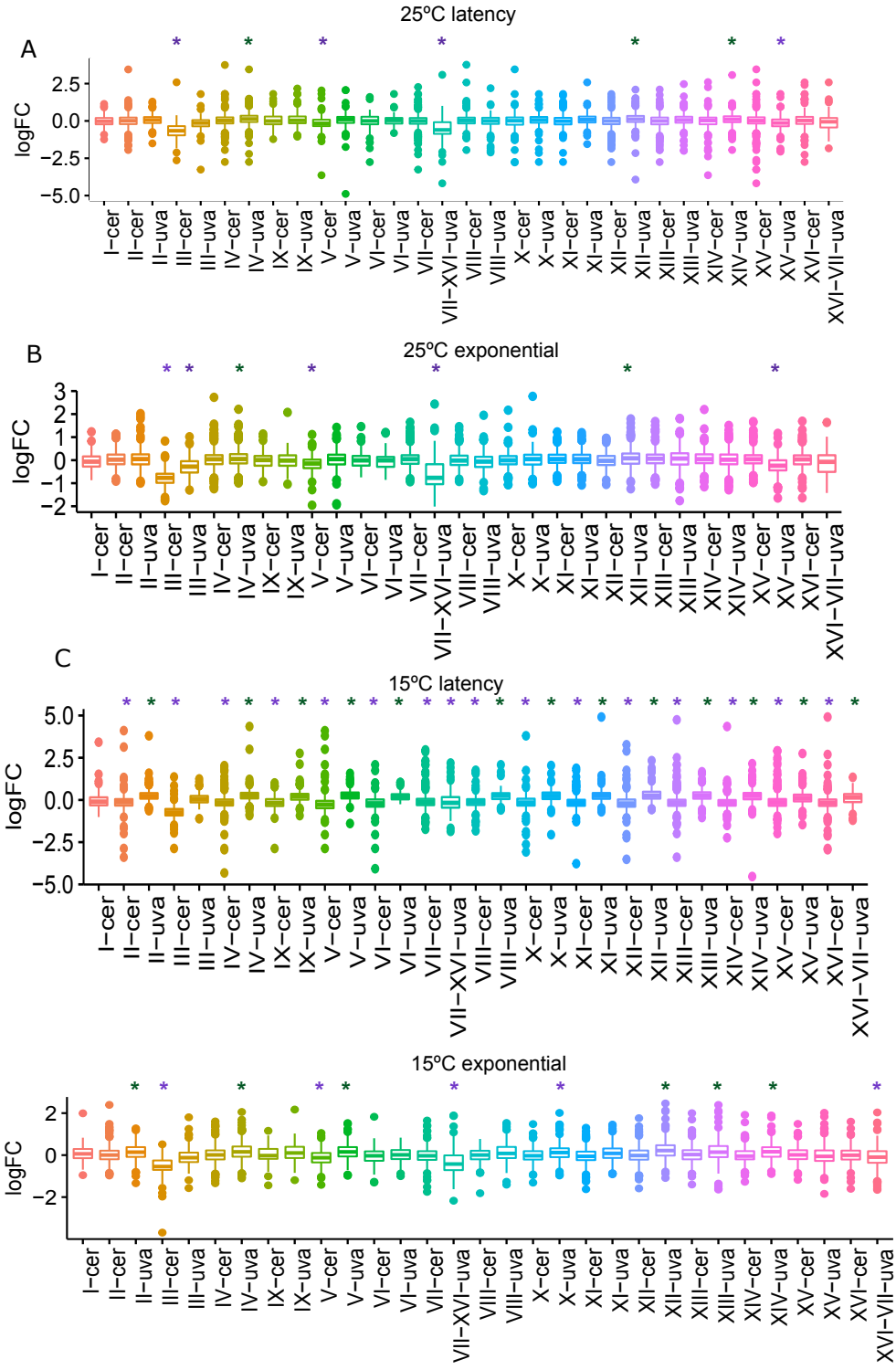
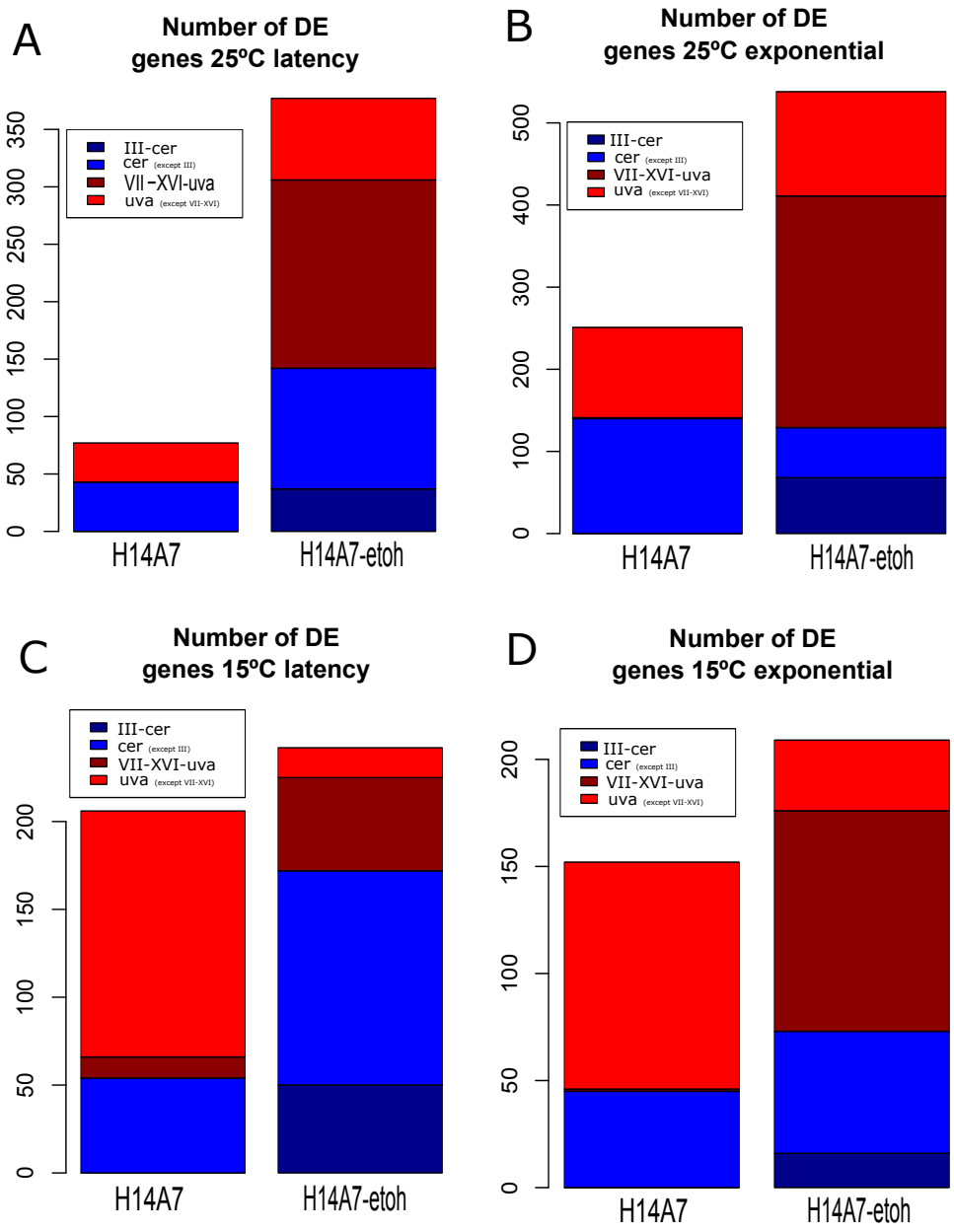


FIGURE 4.5 Representation of the logFC (log 2 FC) of the H14A7 vs H14A7-etoh transcriptomic comparison against the H14A7-etoh vs H14A7 gene coverage for every gene present in both strains. Negative values indicate that the genes are more expressed in H14A7-etoh and positive values that these genes are more expressed in H14A7. Genes belonging to *S. cerevisiae* chromosome III are colored in blue, genes belonging to *S. uvarum* chromosome VII-XVI are colored in red, and genes belonging to the rest of chromosomes are colored in black.



SUPPLEMENTARY FIGURE 4.1 logFC (log 2 FC) of the H14A7 vs H14A7-etoh transcriptome comparison for all of the genes grouped by chromosome for the four conditions: latency at 25ºC, exponential 25ºC, latency 15ºC and exponential 15ºC. Negative values indicate that the genes are more expressed in H14A7-etoh. Significance symbols (Wilcoxon test, $p < 0.0001$) are colored in purple if the chromosome is upregulated in H14A7-etoh and in green if it is downregulated.



SUPPLEMENTARY FIGURE 4.2 Number of differentially expressed genes when performing differential expression analysis between H14A7 and H14A7-etoh strains. The light blue bar represents the *S. cerevisiae* alleles except those present in chromosome III (represented in dark blue) and the light red bar represents the *S. uvarum* alleles except those present in chromosome VII-XVI (represented in dark red). The DE tests were performed at 4 conditions: latency phase at 25ºC (A), exponential phase at 25ºC (B). latency phase at 15ºC (C) and exponential phase at 15ºC (D).

is higher than the number of H14A7 compared to H14A7-etoh at 25ºC. At this temperature at both time points (latency and exponential growth phase), the overexpressed genes in H14A7-etoh belong to the *S. uvarum* sub-genome of the adapted hybrid, and especially to the chromosome VII-XVI. Moreover, no GO terms could be retrieved from these lists of *S. uvarum* overexpressed genes. In the latency stage at 25ºC H14A7-etoh *S. cerevisiae* subgenome overexpressed genes related to protein folding and catabolic process, and in exponential stage at 25ºC H14A7-etoh *S. cerevisiae* subgenome overexpressed genes related with the GO term alpha-amino acid metabolic process (GO:1901605).

At 15ºC, for both time points the number of differentially expressed genes between the strains is lower than at 25ºC, and the number of up-regulated genes in H14A7-etoh and the number of up-regulated genes in H14A7 is very similar. One behavior that should be considered is that at 15ºC *S. uvarum* alleles show more up-regulation in H14A7 compared to H14A7-etoh than the *S. cerevisiae* alleles. In the latency stage at 15ºC H14A7-etoh overexpressed more *S. cerevisiae* than *S. uvarum* alleles. This behavior could be of interest as S. cerevisiae and *S. uvarum* species show differential behavior at low temperatures. The 5 GO terms that were enriched in this list of overexpressed *S. cerevisiae* alleles in H14A7-etoh are protein folding (GO:0006457), protein refolding (GO:0042026), regulation of ATPase activity (GO:0043462), positive regulation of ATPase activity (GO:0032781) and response to heat (GO:0009408).

No GO terms could be identified from the list of *S. uvarum* overexpressed alleles in H14A7-etoh at the 15ºC latency stage. Nevertheless, many GO terms that were overrepresented in H14A7 in comparison with H14A7-etoh at 15ºC latency stage could be identified. *S. cerevisiae* specific GO terms are related to energy reserve metabolic processes: glycogen biosynthesis (GO:0005978), glycogen metabolism (GO:0005977) and oxidation-reduction (GO:0055114). The *S. uvarum* GO terms that are overexpressed in H14A7 in comparison with H14A7-etoh at latency stage at 15ºC are secondary alcohol biosynthesis (GO:1902653), ergosterol metabolism (GO:0008204) and cellular alcohol metabolism (GO:0044107).

Since we determined that H14A7-etoh was more sulfite resistant than H14A7, we examined

the expression of genes YGL254W (transcription factor *FZF1*) and YPL092W (*SSU1* sulfite pump) in the four conditions and species alleles expression. The *SSU1 S. uvarum* allele is overexpressed in H14A7-etoh for 3 out of 4 conditions: 25ºC exponential, 25ºC latency, and 15ºC exponential. *S. uvarum* transcription factor *FZF1t* is overexpressed in 2 out of 4 conditions: 25ºC exponential and 25ºC latency. None of the *S. cerevisiae FZF1t* and *SSU1* alleles showed differential expression. We therefore identified a tendency for the overexpression of *S. uvarum SSU1* and *FZF1* alleles in H14A7-etoh when compared with H14A7 strain at 25ºC latency and exponential stages.

4.3.6 Membrane lipid composition of the strains

Modulation of membrane lipid composition is a key mechanism by which yeast increase ethanol tolerance (Alexandre et al., 1994; Beaven et al., 1982; Henderson and Block, 2014). However, the homeoviscous response is complex (Ernst et al., 2016) and the effect of altered gene expression on membrane composition may not be intuitive. Therefore, we compared the membrane properties of the adapted strain with the initial strains. We used mass spectrometry MS and thin layer chromatography TLC to characterize the membrane composition of AJ4, BMV58, H14A7, and H14A7-etoh strains, and a Laurdan dye assay as an indication of the relative fluidity of the membranes. As a surrogate for the general abundance of lipid classes, the number of species for each class of lipid between the strains is shown in Figure 4.6A; there were significant differences for phosphatidylcholine (GPCho), with more species observed for both AJ4 and H14A7-etoh compared to BMV58 ($P < 0.05$ and $P < 0.01$ respectively, ANOVA and Tukey's multiple comparison test).

For phosphatidylserine species (GPSer), there were significantly more species identified in AJ4 compared to BMV58 and H14A7 ($P < 0.001$ and $P < 0.05$) and more in H14A7-etoh than BMV58 ($P < 0.01$). There were significantly more triacylgycerol (TG) species identified in AJ4 compared to H14A7-etoh ($P < 0.05$).

Membrane fluidity is affected by the presence of short chain alcohols, and two key lipids characteristics that influence membrane fluidity are acyl chain length and saturation. The average

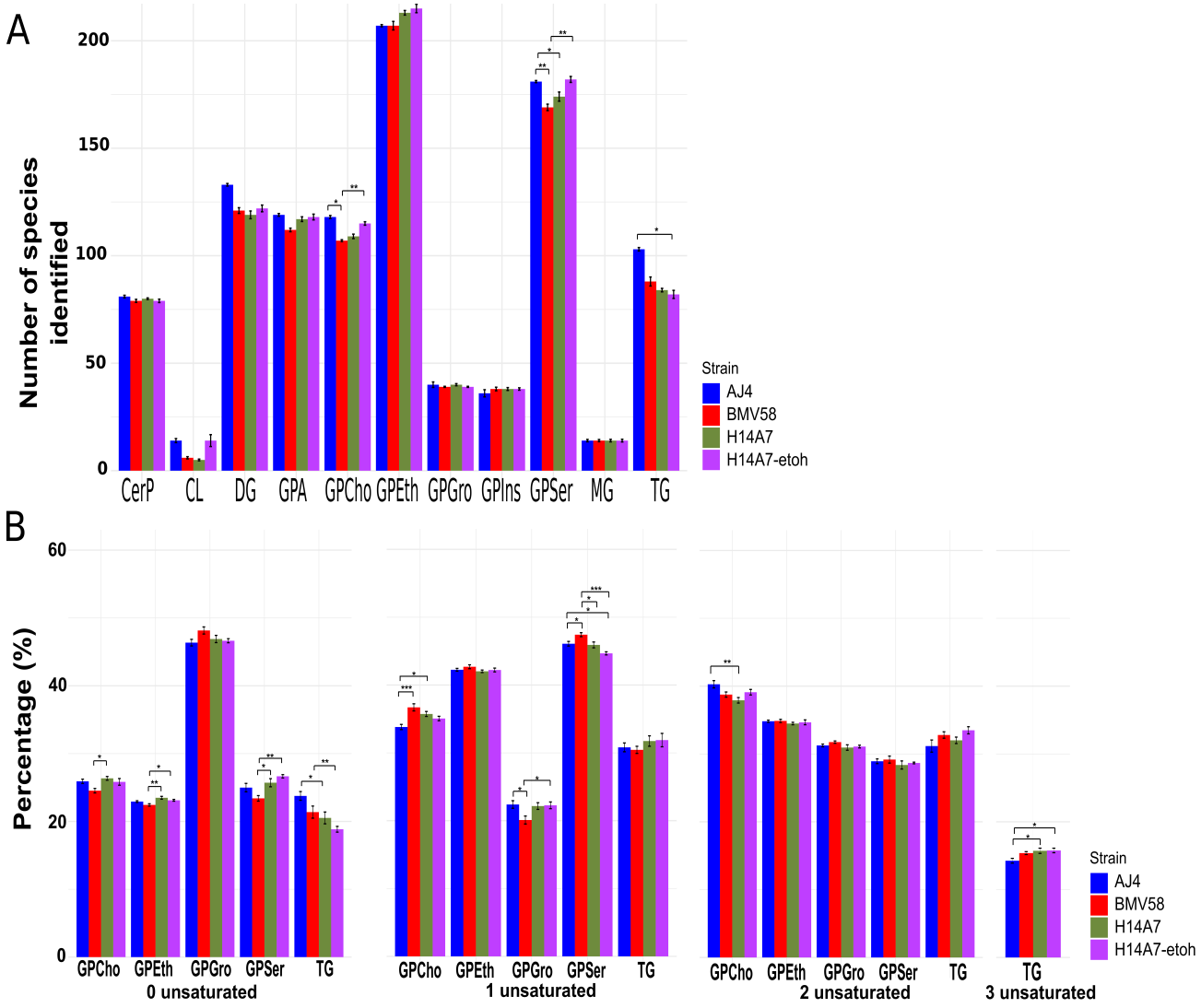
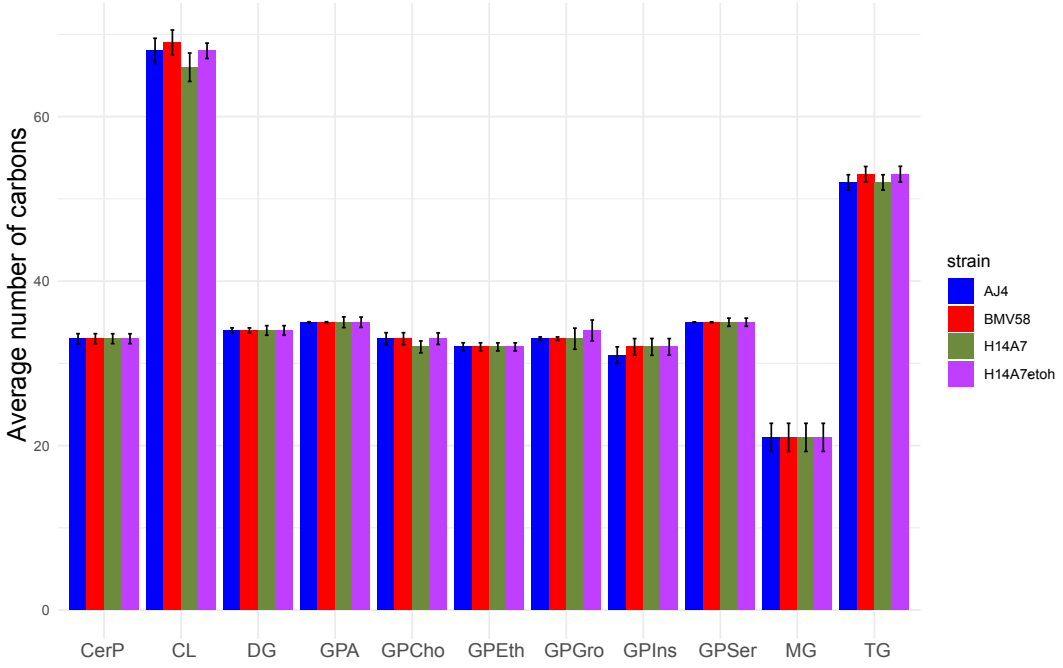


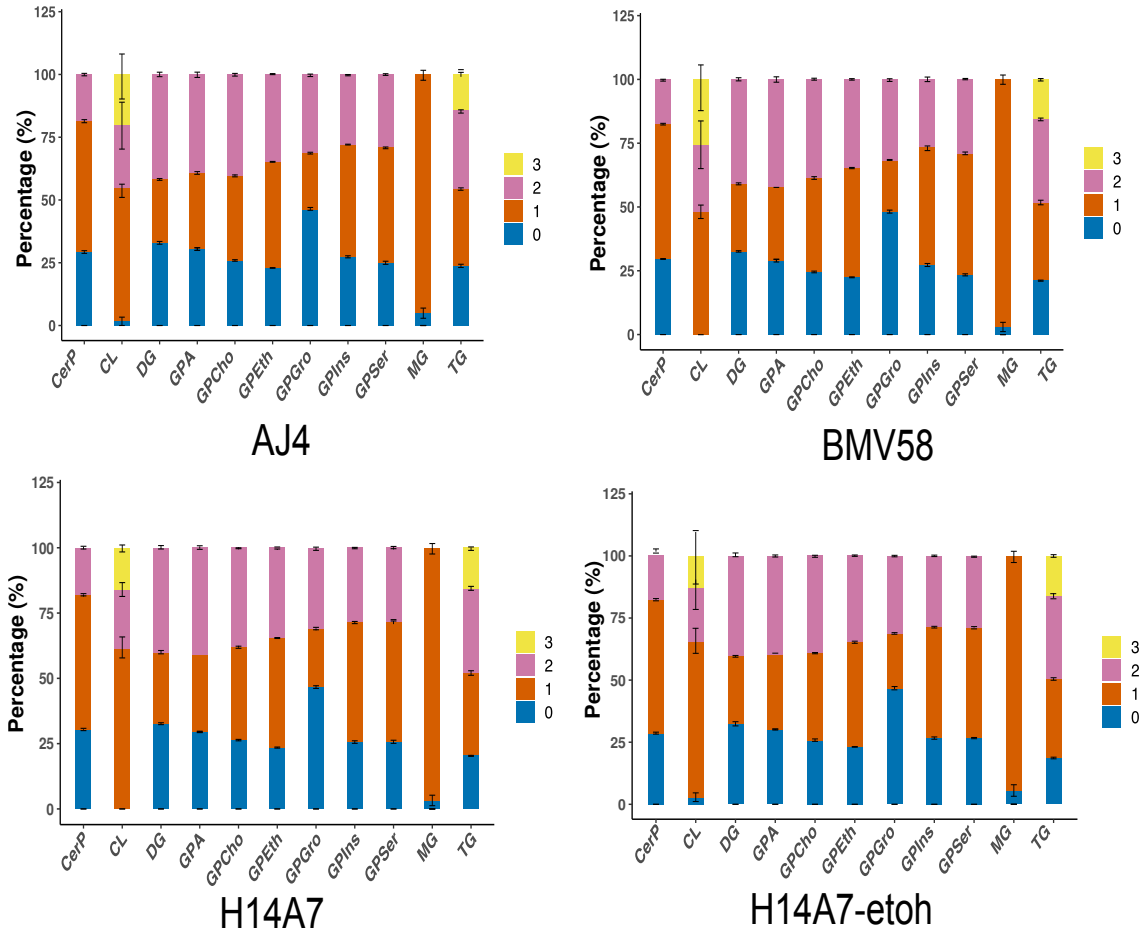
FIGURE 4.6 Number and percentage of different lipid species identified in AJ4, BMV58, H14A7 and H14A7-etoh strains. Number of species identified by lipid class. Lipids were extracted in quintuplicate and analyzed by LC-MS in positive and negative ion mode (A). Percentage of saturated, monounsaturated and polyunsaturated chains by lipid class showing significant changes (B). Significant differences among are indicated as *, ** and ***, when the probabilities are P <0.05, P <0.005 and P < 0.001 respectively, using ANOVA and Tukey's multiple comparison test.



SUPPLEMENTARY FIGURE 4.3 Number of species identified by lipid class for AJ4, BMV58, H14A7 and H14A7-etoh strains. Lipids were extracted and analyzed by LC-MS in positive and negative ion mode.

number of carbons in the acyl chains was not significantly different between the different strains (Supplementary Figure 4.3, Supplementary Figure 4.4). The two main genes related with sphingolipids synthesis are *LCB1* and *ELO2*. Since we have available the list of differentially expressed (DE) genes among H14A7-etoh and H14A7, we inspected this table and found that *ELO2* (YCR034W) is more expressed in H14A7-etoh at two conditions: latency at 15ºC (the *S. cerevisiae* allele of the adapted hybrid) and exponential at 25ºC (the *S. uvarum* allele of the adapted hybrid). *ELO2* is involved in biosynthesis of very long chain fatty acids but we saw no evidence for an increase in average chain length, suggesting a complex phenotype.

Figure 4.6B illustrates the lipid species where significant changes to saturation between the strains were observed; significant differences were found for GPCho, with a significantly higher percentage of saturated species found in BMV58 compared to H14A7 ($P < 0.05$), a lower percentage of monounsaturated species in AJ4 compared to BMV58 and H14A7 ($P < 0.001$ and $P < 0.05$), and a higher percentage of polyunsaturated lipids found in AJ4 compared to H14A7 ($P < 0.01$). For phosphatidylethanolamine (GPEth), a lower percentage of saturated species was observed for BMV58 than in H14A7 and H14A7-etoh ($P > 0.01$ and $P < 0.05$). For



SUPPLEMENTARY FIGURE 4.4 Percentage of saturated, monounsaturated and polyunsaturated chains by lipid class for AJ4, BMV58, H14A7 and H14A7-etoh strains.

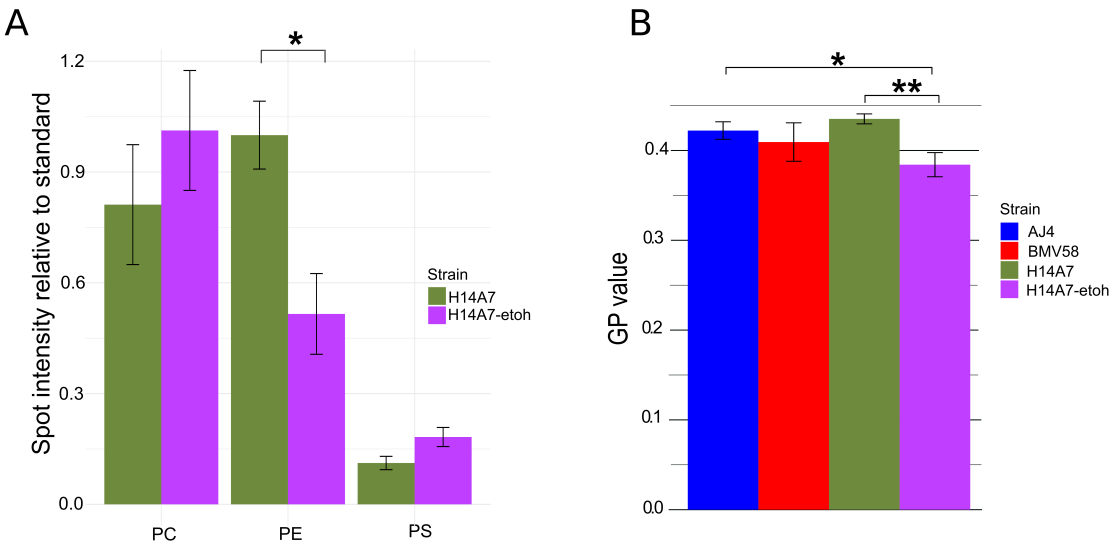


FIGURE 4.7 TLC and Laurdan assay of the analyzed strains. Thin layer chromatography analysis of phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and Phosphatidylserine (PS) abundance for H14A7 and H14A7-etoh strains. Samples were loaded in triplicate and spot intensity was analyzed using ImageJ. Spot intensity is plotted relative to phospholipid standards loaded onto each plate (A). Laurdan assay to compare the state of the membranes of AJ4, BMV58, H14A7 and H14A7-etoh strains. The relative GP was determined after 24 h growth in GPY media (B). Significant differences among are indicated as *, ** and ***, when the probabilities are $P < 0.05$, $P < 0.005$ and $P < 0.001$ respectively, using ANOVA and Tukey's multiple comparison test.

phosphatidylglycerol (GPGro), higher percentages of monounsaturated lipids were seen in AJ4 and H14A7-etoh compared to BMV58 ($P < 0.05$). Significanlty greater percentages of monounsaturated species were observed for GPSer in BMV58 compared to the AJ4, H14A7 and H14A7-etoh ($P < 0.5$, $P < 0.5$ and $P < 0.001$) and less saturated species in BMV58 compared to H14A7 and H14A7-etoh ($P < 0.05$ and $P < 0.01$). For TG, the percentage of saturated species was greater for AJ4 compared to H14A7 ($P < 0.05$) and for BMV58 compared to H14A7-etoh ($P < 0.01$), while a higher percentage of polyunsaturated species were observed for AJ4 compared to both H14A7 and H14A7-etoh ($P < 0.05$).

A further important contributor to membrane characteristics is the nature of the phospholipid headgroup. Quantitative TLC analysis of the abundance of PC, PE and PS in the H14A7 and H14A7-etoh samples (Figure 4.7A) shows that there is significantly less PE in the H14A7-etoh strain, while the abundance of PC and PS was not significantly different.

Ethanol has been demonstrated to affect membrane fluidity, resulting in toxicity. Laurdan is

sensitive to the polarity of the membrane environment and has been used to study membrane fluidity (Learmonth and Gratton, 2011). We utilized this to compare the state of the membrane for each of the strains (Figure 4.7B). The data shows that H14A7-etoh has a significantly lower GP compared to H14A7 ($P < 0.01$) and AJ4 ($P < 0.05$). This indicates that the membrane is less ordered and more fluid in H14A7-etoh, while H14A7 possessed the most ordered membrane.

4.4 Discussion

In a previous work, a *S. cerevisiae* x *S. uvarum* hybrid, H14A7, was obtained in our laboratory (Lairón-Peris et al., 2020). The objective of that initial work was to improve the ethanol tolerance of BMV58 (*S. uvarum* strain) by hybridization with a high ethanol tolerant *S. cerevisiae* strain (AJ4). Indeed, we clearly improved the ethanol tolerance of the *S. uvarum* parental, as well as other fermentative properties.

In this work, we wanted to study if this interspecific hybrid, H14A7, shows genomic instability after its growth in stressful wine media conditions, and if the possible genomic changes affect its phenotype. We carried an adaptation strategy in a media that mimics the conditions during industrial wine fermentations at late stages. This media contained a high sulfite concentration, and increasing ethanol concentrations while decreasing sugars concentrations. The obtained strain was named H14A7-etoh and both a physiological and a genomics characterization on this strain was performed.

Using this adaptation strategy, ethanol tolerance is only slightly improved. However, we clearly improved H14A7-etoh sulfite tolerance with respect to H14A7. The added compound, metabisulfite, is not stable in aqueous solutions and quickly converts to sulfite, so the adaptation of H14A7 was directed to sulfite resistance (Weil and Sandler, 1983). The adapted hybrid proved to be more sulfite tolerant than both BMV58 and H14A7. This phenotype improvement can be correlated with the genomic composition of H14A7-etoh. H14A7-etoh has duplicated *S. uvarum* chromosome VII-XVI. *S. uvarum* chromosome VII-XVI, carries the *FZF1-SSU1* recombination

whose gene expression confers sulfite resistance (Macias et al ., submitted).

SSU1 is a gene involved in the sulfite efflux from the cell by the membrane pump, which is one of the strategies that use yeasts to cope with sulfite toxicity (Casalone et al., 1992; Nadai et al., 2016; Park and Bakalinsky, 2000). It has been reported that *SSU1* gene expression is generally constitutive, and that its expression level is strain-dependent and is not regulated by sulfite presence (Divol et al., 2012; Nadai et al., 2016; Park and Hwang, 2008). In the fermentation media from which we retrieved the transcriptomic samples, no metabisulfite was added, and in 3 out of 4 conditions, H14A7-etoh expressed more *S. uvarum-SSU1* gene than H14A7 did, indicating the constitutive expression of this allele.

Wineries widely use sulfite (SO_2) as a preservative to avoid contamination by spoil microorganisms (Ripper, 1892), but it also can result in toxicity to *Saccharomyces* yeasts (Divol et al., 2012; Ingram, 1948). Thus, sulfite tolerance improvement of H14A7 strain is interesting for the wine industry.

However, the adapted hybrid showed a trade-off on its behavior, as it clearly left more fructose in Verdejo must fermentation than the original strain. The modified synthetic must had lower sugar concentration than a natural must in the moment of inoculation in the winery because it was designed to simulate more advanced stages of fermentation. Thus, the hybrid could have lost fermenting capacity, as it was not obliged to ferment the regular amount of sugars during that adaptation process, but rather to cope with the ethanol and sulfites present in the media. Despite this, the obtained end point sugars concentration was still within the limits that wineries consider acceptable in the final product.

In other works which used experimental evolution on *Saccharomyces* strains, fitness trade-offs also occurred (Aguilera et al., 2010; Kutyna et al., 2012; Wenger et al., 2011), demonstrating that when applying adaptive evolution strategies to generate new microbial strains with desirable traits, side effects may also appear.

Adaptation during evolution experiments generates structural variants, as deletions, amplifications, and translocations in different yeast populations (Dunham et al., 2002; Fisher

et al., 2018; Gresham et al., 2008). It is interesting to note that when we first obtained the hybrid (Lairón-Peris et al., 2020) its genome seemed to be stable, and no significant deletions, duplications, or rearrangements were reported, except some SNPs in *S. cerevisiae* chromosome III. Here, we have concluded that under adaptation to an stressful environment, as well as SNP fixation; deletions and duplications occurred in the H14A7-etoh genome due to the selective media employed during the experiment.

Sub-genomes of the yeast interspecies hybrid H14A7 adapted differently during the process. A small region of the *S. cerevisiae* genome was deleted, which contains two genes encoding for putative integral membrane proteins of the DUP240 family. This region could be eliminated from the genome as it is surrounded by Ty1-Ty1 retrotransposon sites, and it has been described that a recombination event under environmental stress can take place between these two elements (Libuda and Winston, 2010). Moreover, some fixation of SNPs and small duplications in concrete genes may have taken place in this *S. cerevisiae* part, as well as a LOH event in *S. cerevisiae* chromosome I. LOH events are usual during adaptive selection processes in *S. cerevisiae* yeasts (James et al., 2019) and these events also drive adaptation in hybrid yeasts (Smukowski Heil et al., 2017).

Nevertheless, one large aneuploidy occurred in the *S. cerevisiae* subgenome: the duplication of chromosome III. As H14A7 was an aneuploid allotriploid with one *S. uvarum* genome copy, and two heterozygous copies of each *S. cerevisiae* chromosome except for a single copy of chromosome III, *S. cerevisiae* chromosome III duplication could be the result to a restoration of diploidy in all *S. cerevisiae* chromosomes, or because chromosome III affects ethanol tolerance.

Previously, it had been reported that yeast cells favour restoration of euploidy for chromosomes (Waghmare and Bruschi, 2005). Moreover, it has been hypothesized that tolerance to aneuploidy occurs at the chromosome level, perhaps through the action of DNA cis-acting elements, or selection for the restoration of euploidy of the previously aneuploid chromosome.

It has also been reported that chromosome III is one of the chromosomes which undergoes gains in strains under stress conditions, such as ethanol present in the media (Adamczyk et al.,

2016). Morard et al. (2019) also observed that *S. cerevisiae* chromosome III aneuploidy appears frequently in the most ethanol tolerant strains. *S. cerevisiae* chromosome III duplication could be a result of an adaptation of H14A7 strain to the ethanol media present during the adaptation, as this chromosome III duplication could confer an advantage when growing in high ethanol concentrations.

S. uvarum H14A7-etoh sub-genome only seems to have one gene duplicated, YJL052W, but this subgenome was modified in the form of chromosomal losses (chromosome I) and gains (chromosome VII-XVI). *S. uvarum* chromosome I is the smallest chromosome, and it has been reported that chromosome losses often affect the smaller chromosomes (Deregowska et al., 2015).

The most interesting changes in H14A7-etoh compared with H14A7 are these whole chromosome duplications an losses. Yeasts have this ability to increase and maintain individual chromosomal copy number, as these aneuploidies are well tolerated and stable (Waghmare and Bruschi, 2005). Previous studies growing *S. cerevisiae* yeasts under stress conditions have demonstrated that hyperploidy of concrete chromosomes can spontaneously occur. In Whittaker et al. (1988), a *S. cerevisiae* culture was grown in a copper-rich environment. These yeasts increased the copy number of chromosome VIII, which carries *CUP1-1* and *CUP1-2* genes, related to resistance to high copper concentrations. The duplication of *S. uvarum* chromosome VII-XVI in H14A7-etoh may be related with strain adaptation to a medium with an elevated concentration of sulfites.

The change in the copy number of chromosomes is one accessible way to change expression levels of specific key genes (Voordeckers et al., 2015a). In the case of H14A7-etoh, this appeared to occur, as transcriptomic analysis revealed that, in general terms, III-cer, VII-XVI-uva genes are up-regulated in H14A7-etoh in comparison with H14A7 strain under the same condition.

The transcriptomic analysis of both H14A7 and H14A7-etoh strains also revealed that H14A7 could be more efficient fermenting wine must at low temperatures than H14A7-etoh adapted strain. Enrichment in GO terms related to secondary alcohol biosynthetic process (GO:1902653) and ergosterol metabolic process (GO:0008204) were found for H14A7 in latency stage at 15ºC. An

increase in ergosterol metabolism has been previously associated with low temperature tolerance in *Saccharomyces* (Hemmi et al., 1995; Abe and Minegishi, 2008) and the production of higher alcohol production is correlated with the usage of *S. uvarum* strains and low temperatures at fermentations (Gamero et al., 2013; Masneuf-Pomarède et al., 2010; Stribny et al., 2015).

These same GO terms were obtained in our previous work ((Lairón-Peris et al., 2020)) when comparing H14A7 hybrid with its parental strains. Thus, the capability of growth and fermentation under low temperature conditions could be related to these processes, and the lack of expression of the genes related to them could be caused by the absence of that selective pressure during H14A7-etoh development. That would also explain why the strain has more difficulties for complete sugar consumption.

Improvements to ethanol tolerance were observed for H14A7-etoh, and we investigated changes to the membrane which may have occurred as a mechanism of ethanol tolerance. The differences in the lipidome of the yeast strains, such as number of species identified for each class and the unsaturation status of the acyl chains, appears to be complex, and the overall effect upon the membrane is difficult to predict. Several studies have found a correlation between chain length, membrane fluidity and ethanol tolerance, with the incorporation of longer chains at the expense of short chains to counteract the fluidising effect of ethanol upon the membrane (Chi and Arneborg, 1999a; You et al., 2003). The ability of cells to change the unsaturation index has been suggested as an ethanol adaptation response. Furthermore, cholesterol acts to modulate membrane fluidity and it is possible that the transcriptomic changes seen within the ergosterol metabolic process genes is responsible for the increased fluidity of H147a-etoh membranes.

S. cerevisiae has been demonstrated to increase unsaturated lipids in response to ethanol (Alexandre et al., 1994; Beaven et al., 1982; Chi and Arneborg, 1999a), and this has been associated with more tolerant strains (Alexandre et al., 1994). However, another study found that unsaturation had no correlation with membrane fluidity and ethanol tolerance (Huffer et al., 2011).

It has been suggested that membrane fluidity alone cannot not fully account for ethanol tolerance in some microorganisms, and that mechanisms of adaptation varies between strains and

between organisms (Alexandre et al., 1994; Huffer et al., 2011). Our analyses suggest that, whilst changes in saturation may occur within the hybrid strains, this alone is unlikely to fully account for the observed increase in ethanol tolerance.

In our study, we observed a significantly lower abundance of PE in the H14A7-etoh strain compared to H14A7; this could be an adaptive response to ethanol stress. PE is known to play a role in the regulation of membrane fluidity (Dawaliby et al., 2016), and membranes containing PE have been demonstrated to be less fluid than those containing PC alone, possibly because PE increases lipid packing (Ballweg et al., 2020).

The Laurdan experiments suggested that the membranes of H14A7-etoh were more fluid compared to those of the H14A7 strain; this is consistent with the TLC data and a decrease of PE, which could be expected to result in an increase in membrane fluidity. A study by Chi and Arneborg (1999a) compared two yeast strains with different abilities to tolerate ethanol, and found that the more tolerant strain contained a greater proportion of PC and a lower proportion of PE. Another study demonstrated increased mass fractions of PC and less PE in recycled yeast exposed to fermentation stress compared to non stressed started yeast cultures (Jurešić et al., 2009).

Recently, a *S. cerevisiae* strain was adapted under osmotic stress, and different complex sphingolipids changed their abundance (Zhu et al., 2020). Moreover, it has been stated that sphingolipids are abundant in highly ordered membrane regions with sterol, and that ergosterol seem to interact preferentially with PCs (de Almeida and Joly, 2014; Khmelinskaia et al., 2020).

These results are consistent with our findings, suggesting one possible conserved mechanism of increasing membrane tolerance to ethanol. Reported membrane changes upon ethanol production / exposure remain conflicting (Henderson and Block, 2014). This is likely due to differences in the experimental conditions. Yeast are known to incorporate exogenous polyunsaturated fatty acids (Lou et al., 2018; Tyurina et al., 2017), and this can be influenced by the composition of the growth media. In addition, there may be multiple alternative cellular strategies for mitigating ethanol tolerance. Due to the sampling in our experiments we are likely looking at the “basal” membrane condition before significant ethanol challenge and further remodelling may

occur with increased ethanol concentrations.

Overall, our results show that when a *S. uvarum* x *S. cerevisiae* strain is adapted under a media which mimics wine pressures during fermentation -ethanol and sulfites-, its genome is unstable and show different genomic changes with have an effect on its phenotype. Both subgenomes adapt differently to this media, and the characteristic that was clearly improved was the sulfite tolerance. The way to improve it was with the duplication of *S. uvarum* chromosome VII-XVI, which has an impact on gene expression of this entire chromosome. Ethanol tolerance seem to be improved too, and *S. cerevisiae* chromosome III duplication, could have been the cause of this improvement. Membrane fluidity of the adapted hybrid is increased and, could be a potential mechanism by which the ethanol tolerance is higher for H14A7-etoh. A trade-off is present in this adapted hybrid, as its speed to ferment sugars is reduced.

CHAPTER 5

Adaptive evolution of *S. kudriavzevii* and *S. uvarum* strains under ethanol stress

5.1 Introduction

Adaptive evolution is a natural process by which the beneficial alleles present in a population increase and the deleterious alleles decrease due to selective pressures. In nature, the environment is constantly changing and the individuals showing the best phenotypes for a determined condition are selected and the corresponding genotypes are fixed in a population (Taddei et al., 1997).

Yeast cells are unicellular fungi that are widely distributed in the natural environment. Therefore, they use several mechanisms to respond to environmental challenges thus adapting and evolving (Conrad et al., 2011; Dragosits and Mattanovich, 2013). One of the main ecological conditions under which yeast cells have to grow and reproduce in nature is in the presence of high levels of ethanol (Bisson, 1999). Although ethanol is produced by yeasts, this compound is toxic for them and compromises their ability to survive and proliferate (Bisson, 1999).

Yeast strains showing a better ethanol-tolerance phenotype have a fitness advantage over less ethanol-tolerant strains (Arroyo-López et al., 2010b; Voordeckers et al., 2015a). It is not surprising that in industrial environments with high ethanol concentrations, as in wine, beer, and bioethanol producing companies, the predominant yeasts are those exhibiting these phenotypes (Voordeckers et al., 2015a).

Wine fermentation represents, together with beer fermentation, the main industrial process in which yeasts have been unconsciously selected and utilized for centuries, thus allowing the evolution of these organisms towards a more favorable ethanol-tolerant phenotype (Conant and Wolfe, 2007; Dashko et al., 2014; Cubillos, 2016; Legras et al., 2007; Sicard and Legras, 2011). Nowadays, wineries select and use pure ethanol-tolerant *Saccharomyces* yeast strains and add them to the grape must to carry out the wine fermentation. The use of these starters is essential to have a reproducible process and to maintain a high final product quality (Querol et al., 2018).

S. cerevisiae is the preferred yeast species to initiate the fermentation process as it is the most ethanol tolerant species in the *Saccharomyces* genus and its use reduces the risk of sluggish

or stuck fermentations due to ethanol presence (Alexandre and Charpentier, 1998; Arroyo-López et al., 2010b; Jolly et al., 2014). Nevertheless, non-conventional *Saccharomyces* species, such as *S. uvarum* and *S. kudriavzevii*, are good candidates to be used in the wine industry as they exhibit good fermentation properties at low temperatures and produce wines with lower alcohol and higher glycerol content than *S. cerevisiae* (Bertolini et al., 1996; Demuyter et al., 2004; Giudici et al., 1995; González et al., 2007; Pérez-Torrado et al., 2018; Peris et al., 2016; Salvadó et al., 2011a; Tronchoni et al., 2012). Despite their potential, these species cannot compete on an industrial level with *S. cerevisiae* industrial strains, which in general terms have greater resistance to ethanol and the ability to ferment at higher temperatures (Belloch et al., 2008).

One scientific approach towards the improvement of yeast strains with interesting properties but with low ethanol tolerance is the use of adaptive laboratory evolution (also known as directed evolution or ALE) on these yeasts. ALE is based on the principle that cell populations adapt to their environment over time by means of natural selection. Therefore, under changing environmental conditions, the fittest phenotypes are selected and their corresponding genotypes fixed in the population, thus allowing for the perpetuation of those organisms in the new environment (Çakar et al., 2005; Chambers et al., 2009; Zeyl, 2005). Phenotypic changes obtained in the evolved strains can be associated with the growth environment used during the ALE strategy. If the evolved and the original strains genomes are obtained via whole genome sequencing (WGS), phenotype and genotype can also be correlated (Dragosits and Mattanovich, 2013; Fay, 2013; Solieri et al., 2013).

The strains obtained by using ALE are not considered GMO, which is essential for the utilization of the obtained strains at the industrial level due to the complex legislation and poor consumer acceptance (Wunderlich and Gatto, 2015). Moreover, since ethanol tolerance is a quantitative phenotype that depends on a large number of genes (QTLs), performing ALE seems a good approach to improve *S. uvarum* and *S. kudriavzevii* strains (Sanchez et al., 2017).

In a previous work we obtained the H14A7 hybrid (Lairón-Peris et al. (2020), Chapter 3) and we performed an adaptation of this strain in a media mimicking wine fermentation conditions until

a 9% of ethanol was reached (Lairón-Peris et al., under revision, Chapter 4). The obtained strain was named H14A7-etoh and its ethanol tolerance was slightly improved by using this adaptation strategy on a hybrid that already showed a good ethanol tolerance.

The aim of the present work was to use a similar strategy to adapt and evolve four low ethanol tolerant strains: BMV58 and CECT 12600 (*S. uvarum* strains) and CR85 and CA111 (*S. kudriavzevii* strains). To achieve this, we first adapted BMV58, CECT 12600, CR85 and CA111 to a media with 8-9% of ethanol. Then, we started a second evolution strategy with these 4 strains and applied colony selection by using a bottleneck strategy in ethanol media. The genomes of the evolved strains were sequenced to correlate the changes present in the strains with their adaptation to the used ethanol media and the lipid composition of the strains was also analyzed.

5.2 Materials and Methods

5.2.1 Yeast strains

For this study, two *S. uvarum* strains BMV58 (Velluto BMV58^{TM} from Lallemand) and CECT 12600 (isolated from mistela in Alicante, Spain) and two *S. kudriavzevii* strains: CR85 (isolated from oak tree bark in Ciudad Real, Spain) and CA111 (isolated from oak tree bark in Castellón, Spain) were selected (Lopes et al., 2010).

5.2.2 Adaptive laboratory evolution. Part I

Directed evolution of the strains was performed using batch cultures in triplicate, in bottles of 100 mL with 60 mL of modified synthetic must M-SM, (Rossignol et al., 2003), decreasing the amount of sugars and increasing the percentage of exogenous added ethanol as in the previous chapter (Section 4.2.1, Table 4.1). Some of the strains were evolved until an ethanol percentage of 8% was reached and others until an ethanol percentage of 9%, according to the ethanol tolerance.

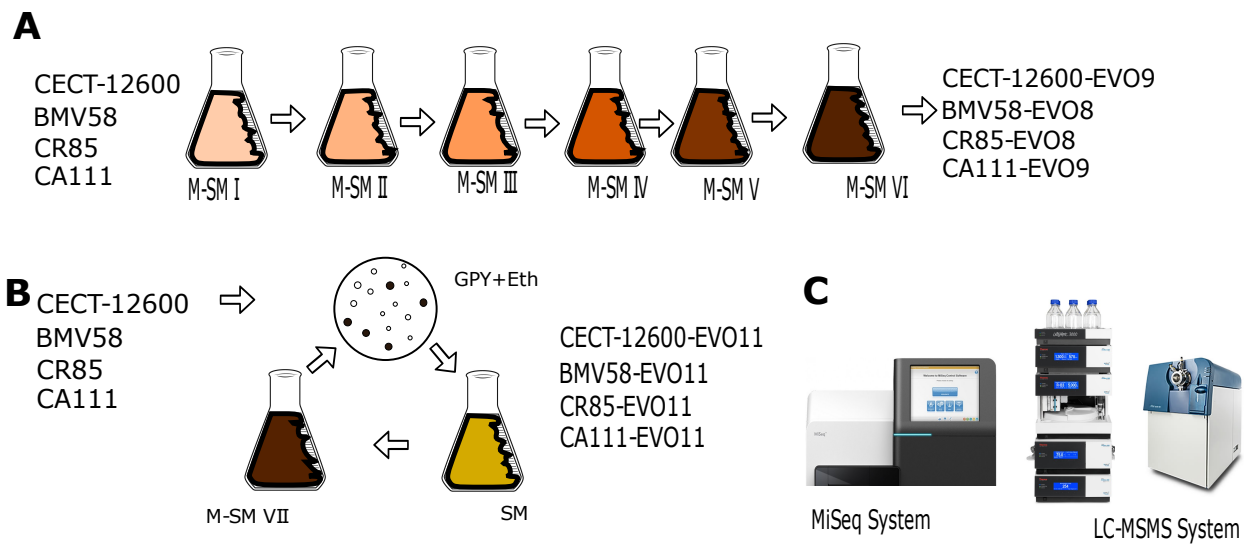


FIGURE 5.1 Scheme of the evolution of the 4 *Saccharomyces* strains. First, the *Saccharomyces* strains were evolved using M-SM media with increasing ethanol concentrations (A). Thereafter, the obtained evolved strains were evolved by following adaptation rounds in M-SM, in GPY+ethanol and in SM (B). The evolved strains were sequenced using an Illumina MiSeq System and its lipid composition was analyzed using LC-MSMS (C).

In all conditions 100 mg/L of metabisulfite, $K_2S_2O_5$ were added to SM.

5.2.3 Adaptive laboratory evolution. Part II

When fermentations reached a concentration of 8% or 9% of ethanol in the media, the ALE strategy was modified. First, a round with modified SM, which contained 60 g/L of sugars (20 g/L glucose and 40 g/L of fructose) and a percentage of ethanol that varied between 9 and 10 percent was carried. After this step, colonies were recovered in GPY plates at 25ºC containing 16% of ethanol OD=$1E^{-4}$ and the 5 bigger ones were selected. Then, colonies were recovered in GPY media, and fermentations were performed using a standard SM media with 200 g/L of sugars (100 g/L glucose and 100 g/L of fructose) to continue again with the step 1 (Figure 5.1).

5.2.4 Mitochondrial DNA (mtDNA) restriction analysis

To detect the possible contamination problems during the evolution experiment, a mitochondrial DNA (mtDNA) restriction analysis of each one of the colonies obtained after their selection in GPY+ethanol plates was performed as in López et al. (2001). Yeast DNA was digested with *HinfI* restriction enzyme (Roche, Mannheim, Germany) and the fragments were separated in 1% agarose gels in 1X TAE (tris-acetic acid-EDTA) buffer at 90 V. Gels were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRION Biotech) and fragments were visualized under UV light. The restriction fragment sizes were compared with lambda *Pst I* restriction enzyme (Roche, Mannheim, Germany).

5.2.5 Estimation of the ethanol tolerance by drop tests

Tolerance to ethanol stress was evaluated by drop tests. Ethanol plates were prepared by autoclaving GPY (%w/v: yeast extract 0.5, peptone, 0.5, glucose 2, agar 2) and when this media was about to solidify, ethanol was added in the media in the following percentages: 0, 6, 8, 10, 12, 14 and 16 (%). Yeast precultures were grown overnight in GPY (peptone 0.5%, yeast extract 0.5%, glucose 2%) medium. Cell cultures were diluted to OD_{600} = 0.1. Then, 4 serial dilutions of cells were transferred to the plates and incubated at 25ºC for a week.

5.2.6 Genome sequencing of the *Saccharomyces* strains

The DNA from of the evolved strains was extracted and strains were sequenced on the Illumina Miseq sequencing platform using 2 × 300 bp paired-end chemistry.

TABLE 5.1 Genomic sequences used in this work.

Strain	Species	Reference	Percentage of ethanol reached in ALE process
CR85	*S. kudriavzevii*	Macías et al. (2019)	-
CR85-EVO8	*S. kudriavzevii*	This study	8.00 %
CR85-EVO11	*S. kudriavzevii*	This study	11.00 %
CA111	*S. kudriavzevii*	Macías et al. (2019)	-
CA111-EVO9	*S. kudriavzevii*	This study	9.00 %
CA111-EVO11	*S. kudriavzevii*	This study	11.00 %
CECT-12600	*S. uvarum*	Macías et al., in preparation	-
CECT-12600-EVO9	*S. uvarum*	This study	9.00 %
CECT-12600-EVO11	*S. uvarum*	This study	11.00 %
BMV58	*S. uvarum*	Macías et al., in preparation	-
BMV58-EVO8	*S. uvarum*	This study	8.00 %
BMV58-EVO11	*S. uvarum*	This study	11.00 %

5.2.7 Flow cytometry analysis

A FACSVerse™ flow cytometer (BD Biosciences) instrument was used to measure the ploidy of the original and evolved yeast strains. Briefly, cells were grown in GPY and 1 OD_{600} of each culture was harvested and cells were stained using SYTOX dye Green as previously described (Haase and Reed (2002), Section 4.2.4.). The fluorescence intensity of each strain was compared with a haploid strain (S288c) and with a diploid strain (FY1679).

5.2.8 CCNV and SNPs analysis of the evolved strains

The sequenced reads of the evolved strains were aligned to the parental strains' assemblies. Briefly, Bowtie2 v2.3.0 (Langmead and Salzberg, 2012) was used with default parameters to map the paired-end reads to the reference genomes. This generated files with a SAM format for each strain which were converted to BAM files. Thereafter, Bedtools v2.17.0 (Quinlan and Hall, 2010) was used to obtain the coverage of the reads "per base" in the genome. The obtained files were processed to obtain the consensus coverage for each 1000 positions.

A median coverage value per chromosome was obtained for every strain and the chromosome coverage per 1000 positions were visualized by using ggplot2 R package to detect CCNV. CNVnator was used for the discovery of copy number variation (CNV) (Abyzov et al., 2011) and to confirm CCNV. Visualization of the CNV was done using Artemis (Rutherford et al., 2000).

For SNP calling, the gdtools command installed as part of breseq (version 0.27.1) was used (Deatherage et al., 2015). We used the read files of the evolved strains and the annotation files of the original strains as a reference, with option -p –polymorphism -frequency-cutoff of 0.20. The Genome Diff files obtained were manipulated by using the following commands: gdtools SUBTRACT (to compare 1st evolution point to original strain; 2nd evolution point to 1st evolution point; and 2nd evolution point to original) and gdtools ANNOTATE with the reference gene bank annotations. Finally, the SNPs present in codifying regions were curated and we annotated their positions in IGV alignments (sorted-bam) against the reference (Robinson et al., 2011).

5.2.9 Laurdan assay

Fluorescence emission of cells stained with Laurdan is an indirect method to know the fluidity of lipidic membranes. The same methodology as in Chapter 1 was used to measure the membrane fluidity of the original and evolved yeast strains. Briefly, yeast cultures were incubated in GPY media overnight, and the next day 25 mL of GPY media containing 0% ethanol and 10% ethanol was inoculated to an OD_{595} of 0.5. Samples were taken 24 hours after the fermentation, and live yeast were diluted to an OD_{595} of 0.4 in GPY and incubated with 5 μM Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) for 1 h. Fluorescence emission of these cells stained with Laurdan was taken using a microplate reader (Mithras, Berthold) with the following filters; λex=350 λem=460 and 535. Generalized Polarization (GP), derived from fluorescence intensities at critical wavelengths was calculated.

5.2.10 Mass spectrometry of the lipids present in the strains

Lipid composition for each one of the strains was analyzed by mass spectrometry. The same methodology as in 1.2.6 was used to extract the lipids present in each strains. Briefly, cells were propagated in GPY media at 25ºC for 24h, and total lipids were extracted using a modified Bligh and Dyer protocol (Spickett et al., 2011). Then, the lipids were quantified by using an ammonium ferrothiocyanate assay. The quantity of lipids was then adjusted to contain 5 µg/µL lipid in 100 µL of chloroform. These samples were treated as in 1.2.7. Briefly, LC was performed on a U3000 UPLC system (Thermo scientific, Hemel Hempstead) using a Kinetex C18 reversed phase column and MS analysis was carried out in positive and negative ionization mode on a Sciex 5600 Triple TOF. ProgenesisQI® was used for quantification and LipidBlast (https://fiehnlab.ucdavis.edu/projects/LipidBlast) for identification. All data were manually verified and curated.

5.2.11 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's significant differences were performed by using the R package rstatix. Non parametric Wilcoxon test was performed using the R package stats.

5.3 Results

5.3.1 Ethanol screening of the *Saccharomyces* strains

The adaptive laboratory evolution process was performed with the strains for approximately 200 generations using the methodology of Section 5.2.2. The obtained strains were named CR85-EVO8, CA111-EVO9, CECT-12600-EVO9, and BMV58-EVO8. Ethanol is a volatile compound, and we used growth in solid plates with ethanol to infer the growth of the strains in

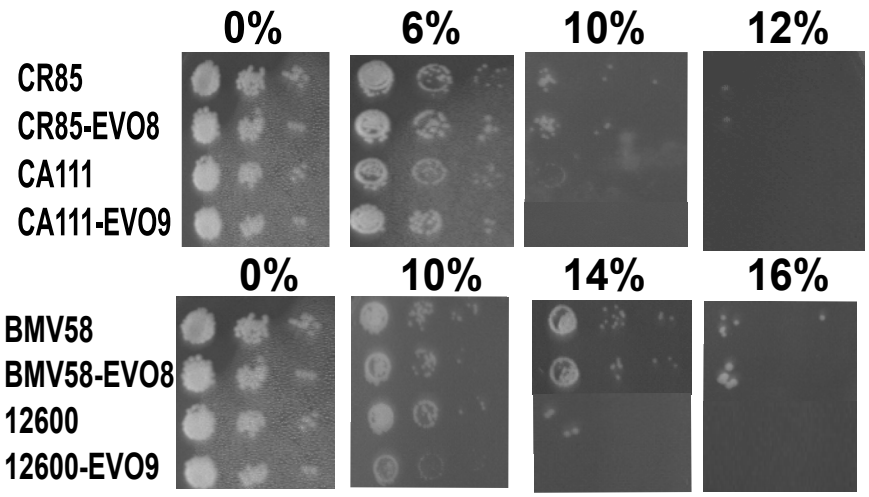


FIGURE 5.2 Drop test of the evolved and original *Saccharomyces* strains after using ALE Part I. CR85, CA111, CECT 12600, and BMV58 strains were grown in GPY plates with different ethanol percentages (0-16% depending on the strain) in quadruplicate. One replicate of the plates and strains is shown.

this media. The growth of the strains at this evolution point was evaluated, but none of them showed improvement in comparison with the original strains (Supplementary Figure 5.2).

Then, the methodology specified in Section 5.2.3 was applied until a percentage of 11% of ethanol in the media was reached and strains CR85-EVO11, CA111-EVO11, CECT-12600-EVO11, and BMV58-EVO11 were obtained (Table 5.1). In Figure 5.3 can be seen the growth of the original and the final evolved strains. It is important to remark that the same number of culture (OD_{600}=0.1 to 1E-4) was used for each strain.

All of the evolved strains, except for BMV58, have increased their ethanol tolerance after the ALE process using the bottleneck strategy and higher ethanol percentages. The strain CA111-EVO11 was the one which showed the major differences in comparison with CA111 original strain in its growth in the GPY + ethanol plates.

5.3.2 CCNV analysis in the evolved strains

To quantify the cellular DNA content and infer the overall ploidy of each one of the evolved and original strains, we used flow cytometry (Table 5.2). At the same time, the coverage values (cv)

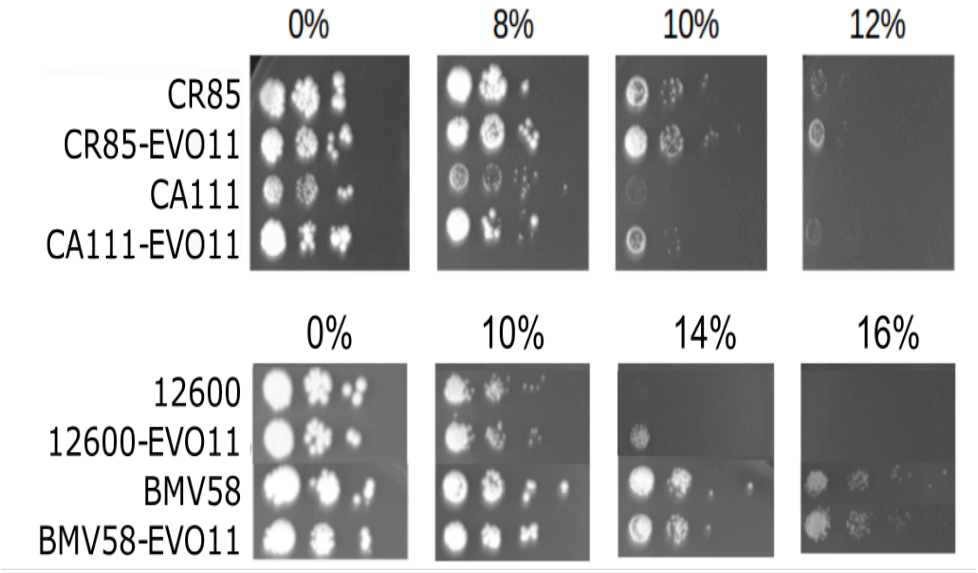


FIGURE 5.3 Drop test of the evolved *Saccharomyces* strains after using ALE Part II. CR85, CA111, CECT 12600, and BMV58 strains were grown in GPY plates with different ethanol percentages (0-16% depending on the strain) in quadruplicate. One replicate of the plates and strains is shown.

obtained after the mapping of the sequenced reads of each evolved strain to the genome sequence of the original strains (cv_{EvsO}) were processed and represented. The mean cv_{EvsO} for each 1000 pb positions were normalized using a log2 transformation against the mean cv_{EvsO} in all the regions of that strain and represented for each chromosome (Figure 5.4).

Two of the strains have duplicated some of the chromosomes all over the evolution process: CA111 and CR85 (Wilcoxon test p-value < 0.05, using the mean chromosome coverage values). The CCNVs acquired during the evolution of CA111 and CR85 *S. kudriavzevii* strains were studied in depth.

The genes present in duplicated chromosomes of CA111 and CR85 were retrieved and their function obtained by using SGD. Chromosome VIII of CA111 encomprises a total number of 251 genes. Some of these genes are very related with ethanol tolerance. This is the case of *ETP1* (YHL010C) gene, which encodes a protein required for growth on ethanol (Snowdon et al., 2009) and of *EPT1* (YHR123W) which is related with phospholipid biosynthesis (Hjelmstad and Bell, 1988).

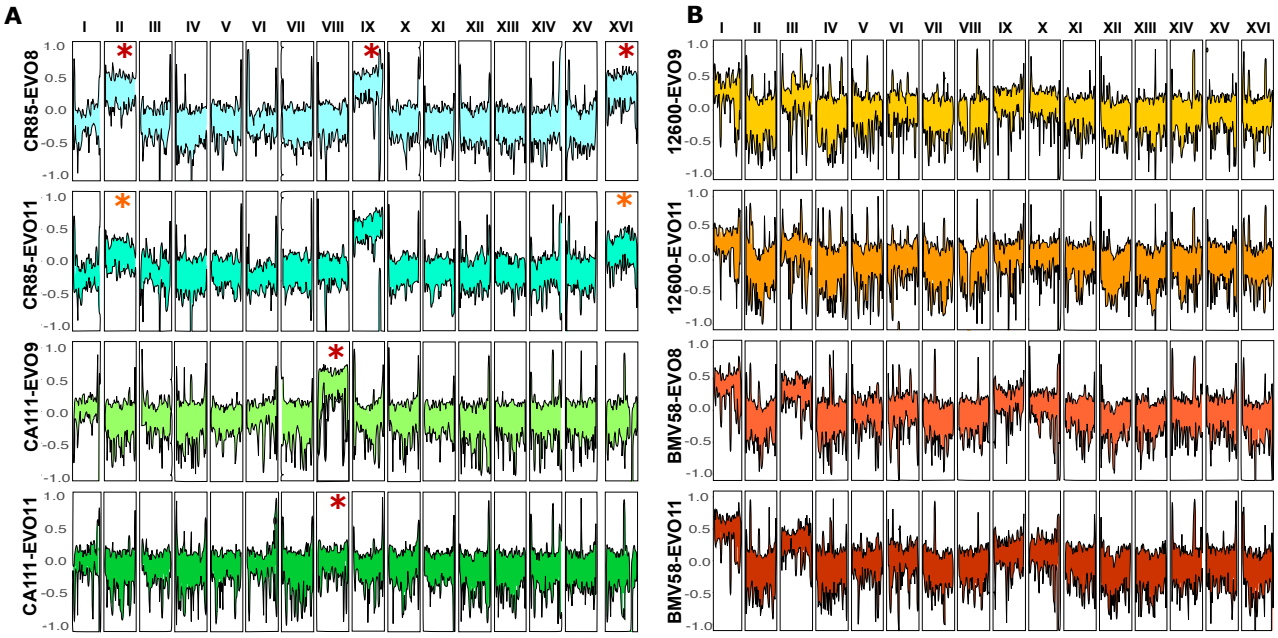


FIGURE 5.4 Coverage per chromosome of the *Saccharomyces* strains. *S. kudriavzevii strains* (A) and *S. uvarum* strains (B) at both evolution points. The plots were created using ggplot package in R (Wickham, 2009) with the coverage values per sliding windows of 1000 pb. Red asterisks indicate a deviation in the medium CCNV of that chromosome in relation with the other chromosomes present in the genome and in relation with the same chromosome in a previous evolution stage (Wilcoxon test); orange asterisks indicate that that chromosome presents a partial CCNV in the evolved population.

The gene function of those genes present in the chromosomes II, IX and XVI of CR85 strain were also subtracted. The chromosome II of CR85 encomprises a total number of 411 genes. Among them the following genes are present: *LDH1* (YBR204C) which has a proposed role in lipid homeostasis (Debelyy et al., 2011); *HSP26* (YBR072W), a stress-responsive heat shock gene which encode proteins related to protein folding; and *SSE2* (YBR169C) an stress-responsive heat shock gene, which encodes proteins related to protein folding. The chromosome IX of CR85 encomprises a total number of 201 genes. Gene *SOA1*, related with sulfite transport; gene *SDP1* related with oxidative stress resistance, and gene *CCT2* related with protein folding are present in this chromosome. The chromosome XVI of CR85, which includes 454 genes, contains the genes *SSU1*, related with sulfite tolerance, *HSP82* (YPL240C), a heat shock protein, and *SSE1*, an ATPase component of the heat shock protein Hsp90.

TABLE 5.2 Ploidy values obtained after flow cytometry and CCNV inferred from sequenced reads of each strain.

	Ploidy value	CCNV
CR85	2.19± 0.02	Original euploid strain
CR85-EVO8	2.58 ± 0.02	+ chr II, IX, XVI
CR85-EVO11	2.35 ± 0.07	- (some colonies lost chr II & XVI)
CECT-12600	1.9 ± 0.0	Original euploid strain
CECT-12600-EVO9	2.14 ± 0.01	-
CECT-12600-EVO11	1.93 ± 0.01	-
BMV58	2.28 ± 0.01	Original euploid strain
BMV58-EVO8	2.09 ± 0.01	-
BMV58-EVO11	1.74 ± 0.01	-
CA111	2.21 ± 0.01	Original euploid strain
CA111-EVO9	2.29 ± 0.03	+ chr VIII
CA111-EVO11	2.23 ± 0.05	- chr VIII

5.3.3 SNPs, deletion and duplications acquired during the evolution process

CNVnator usage confirmed the CCNV mentioned in the above section. Moreover, this software pinpointed at some specific genes with differences on their copy number during the evolution process in the strains. We used Artemis to put together the annotated chromosomes of the original strains with the reads alignment of the original and the two evolved strains to check if the coverage values changes were sequencing artifacts (a gain or a loss of coverage is found in all of the strains) or if the coverage values could indicate the presence of a duplication or deletion. In Table 5.3 can be observed the duplications and deletions found in the evolved strains.

Regarding the SNPs present in the evolved strains, their number varied depending on the strain (Figure 5.5). CECT 12600 was the strain with more SNPs changes on its genomes with a total number of SNPs present in codifying regions of 417. Strains CR85, CA111 and BMV58 acquired a total number of 22, 24, and 21 SNPs respectively. The type of SNPs also varied among strains. The main class of change in 12600 was the acquisition of a new variant, that is, homozygous positions became heterozygous. Instead, in BMV58, CA111 and CR85 changes were from heterozygous positions to homozygous positions.

TABLE 5.3 Duplicated and deleted gene copies in the evolved strains. These genes were selected after the usage of CNVnator and their visualization in Artemis.

Strain	Chr	Pos.	Class	Gene	Molecular Function	Reference
BMV58	VII		Gene duplication BMV58-EVO11	*PIB2*	Phosphoinositide 3-kinase, regulator of receptor signaling cascade	Burd and Emr (1998)
BMV58	VIII		Gene duplication BMV58-EVO8 and BMV58-EVO11	*HST3*	Involved in short-chain fatty acid metabolism	Starai et al. (2003)
BMV58	VIII		Gene duplication BMV58-EVO8 and BMV58-EVO11	*PUT4*	Proline permease	Courchesne and Magasanik (1983)
BMV58	VIII		Gene duplication BMV58-EVO8 and BMV58-EVO11	*CIN1*	Involved in protein folding	Tian et al. (1996)
BMV58	IX		Gene duplication BMV58-EVO8 and BMV58-EVO11	*IMP2*	Enables transcription coactivator activity	Masson and Ramotar (1998)
BMV58	XVI		Gene loss of a copy	*HSP82*	Enables unfolded protein binding	Picard (2002)
12600	VIII		Gene duplication 12600-EVO9 and 12600-EVO12	YOR186W	Expression during heat stress; sphingolipid-dependent	Cowart et al. (2003)

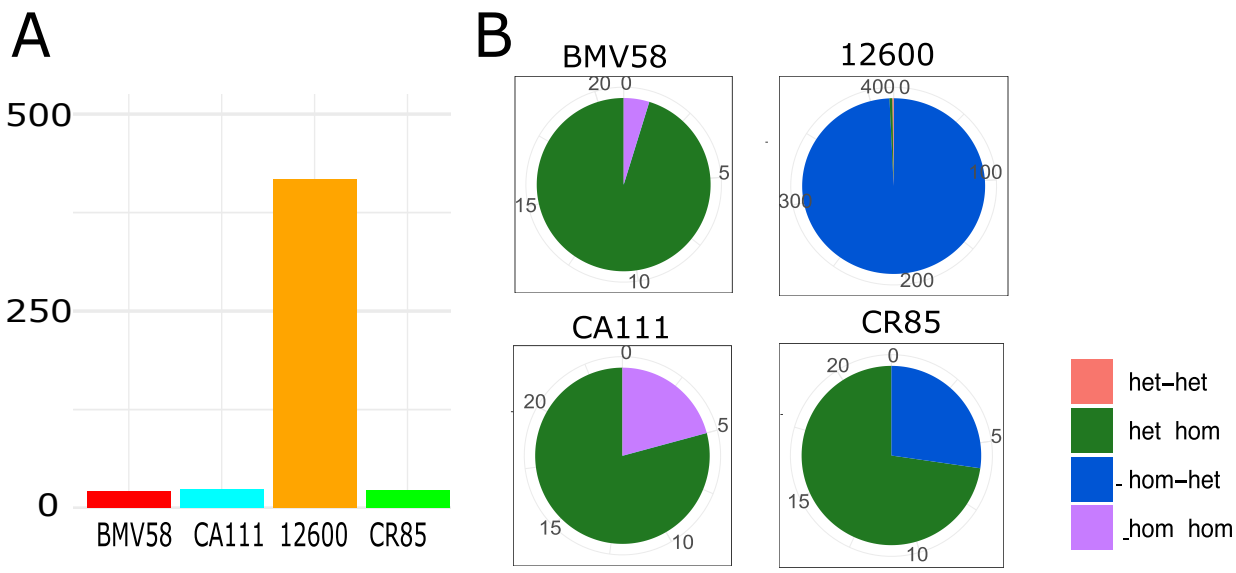


FIGURE 5.5 SNPs acquired during the evolution of the *Saccharomyces* strains. In Panel A, the total number of SNPs is represented for each strain, whereas in Panel B a pie chart showing the percentage of changes from each class is represented.

The functions of the genes in which the presence of a SNP led to an amino acid change were retrieved. A selection of the SNPs present in codifying positions whose change lead to a non-synonymous amino acid of a protein which could be of relevance for the conditions of the evolution can be seen in Table 5.4.

5.3.4 Laurdan assay

Laurdan assays were carried with cells retrieved after the growth of the strains in GPY media with 0% and 10% of ethanol after 24 h growth. The most relevant changes were observed in the media containing a 10% of ethanol, as 3 out of 5 evolved strains showed a lower GP value than the original strains: BMV58, CA111 and CR85. This fact indicated that the membrane of these evolved strains had become more fluid in the presence of ethanol (Figure 5.6).

TABLE 5.4 SNPs present in codifying regions of the evolved strains with non synonymous changes. The list of the SNPs whose presence could be of interest due to the evolution conditions are depicted in this table. The exact positions (pos.) in the chromosomes (chr) of the original strains are indicated, as well as the nucleotide change (mut.) and the molecular function of the gene.

Strain	Chr.	Pos.	Ref.	Mut.	Gene	Molecular function	Change	Reference
12600	VI	224209	A	A/T	*TDH1*	Enables glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity	Y/*	Delgado et al. (2001)
12600	VI	441683	C	C/G	*HSP150*	Enables structural constituent of cell wall	S/T	Russo et al. (1992, 1993)
12600	VI	441695	A	A/G	*HSP150*	Enables structural constituent of cell wall	L/S	Russo et al. (1992, 1993)
12600	XI	516436	T	T/A	*YSR3*	Enables sphingosine-1-phosphate phosphatase activity	Y/F	Mandala et al. (1998)
12600	XIII	593545	G	G/A	*DDR48*	Enables ATPase activity	D/N	Tkach et al. (2012)
12600	XIII	593548	A	A/G	*DDR48*	Enables ATPase activity	T/A	Tkach et al. (2012)
12600	XV	663824	G	G/T	*ENO2*	Enables phosphopyruvate hydratase activity	A/S	Entian et al. (1987)
12600	XV	663903	T	T/A	*ENO2*	Enables phosphopyruvate hydratase activity	F/Y	Entian et al. (1987)
12600	XV	664268	G	G/C	*ENO2*	Enables phosphopyruvate hydratase activity	E/Q	Entian et al. (1987)
BMV58	IV	481537	G	A/G	*SNQ2*	Enables ATPase-coupled xenobiotic transmembrane transporter activity	V/I	Decottignies et al. (1995)
CA111	VII	886999	T/A	A	*TDH3*	Enables glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity	M/*	Delgado et al. (2001)

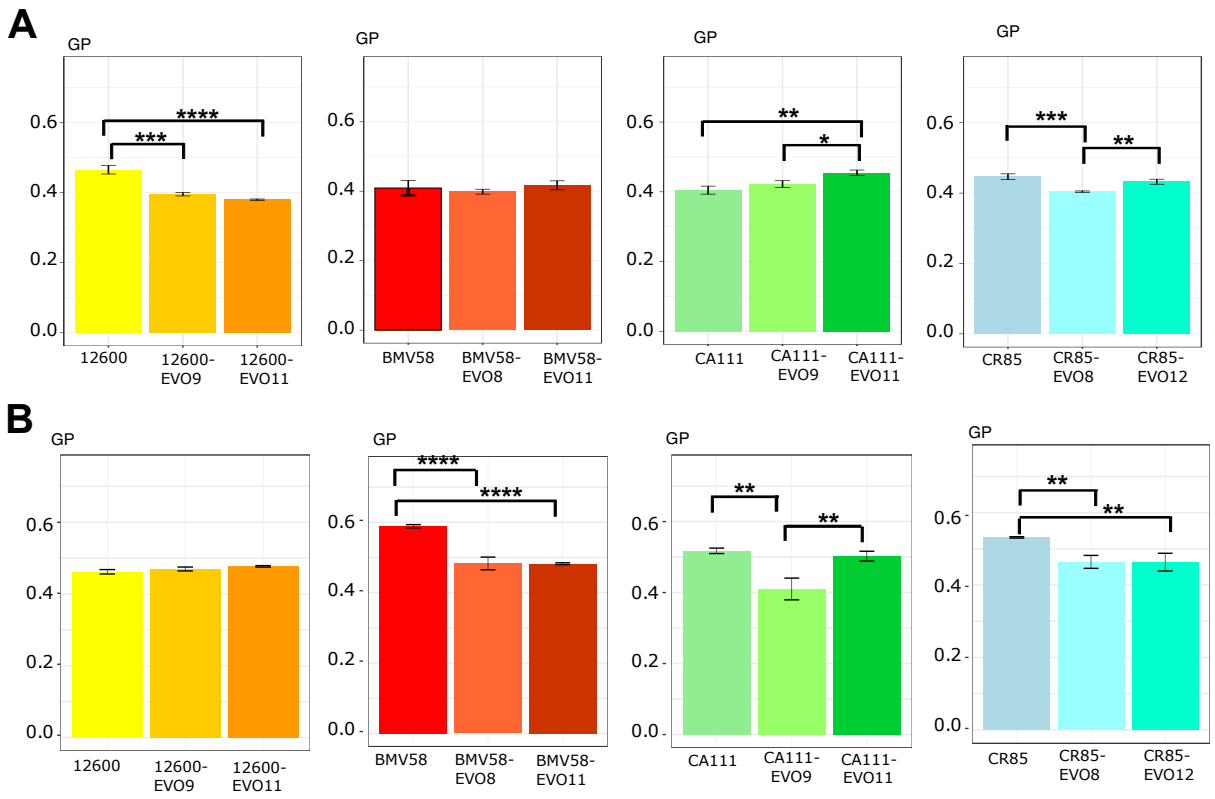


FIGURE 5.6 Laurdan assay to compare the state of the membranes of 12600, BMV58, CA111 and CR85 original and evolved strains. Cells were grown on GPY+0% ethanol (A) GPY+10% ethanol media for 24h and then, membranes were extracted and Laurdan assays performed. Anova (Analysis of variance) and Tukey HSD (Tukey Honest Significant Differences) tests were performed * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) and **** ($P < 0.0001$).

5.3.5 Lipid composition and membrane properties

We determined the total lipid composition of each of the evolved and original strains, together with AJ4 (*S. cerevisiae* strain) by mass spectrometry. The number of species identified for major lipid classes for each strains after their growth in GPY is shown in Figure 5.7. There are differences mainly for AJ4 for the total numbers of species identified and also for CA111-EVO9 compared to the rest of the strains. Concretely, strains AJ4 and CA111-EVO9 possess more lipid species for the following lipid classes: GPA, GPEth, GPSer and PE. Moreover, 12600−EVO11 is the strain with a higher number of sulfatide GPIns and DG. The differences in the number of average carbons in the acyl chains are less variable among strains and can be observed in Figure 5.8.

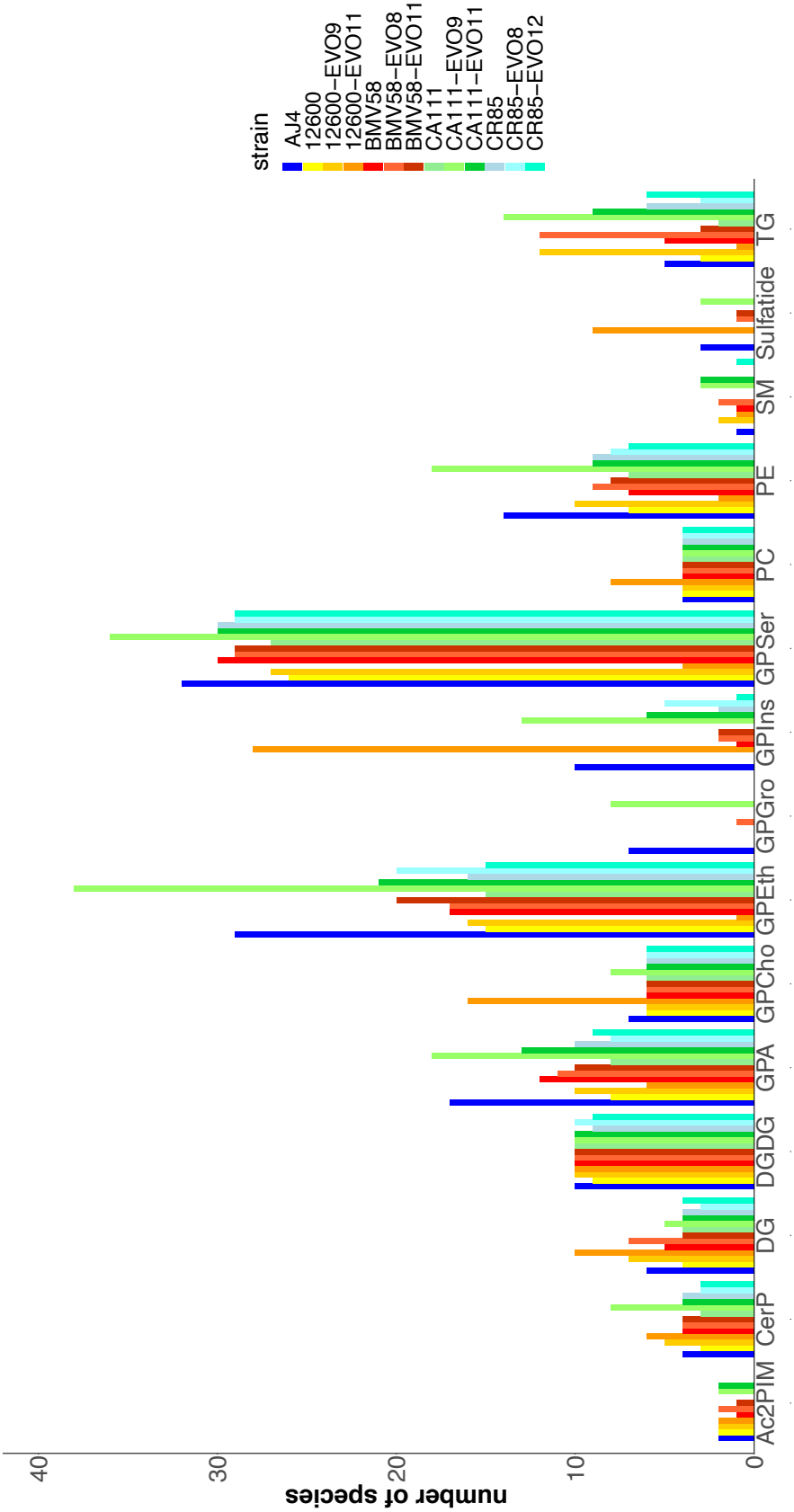


FIGURE 5.7 Number of lipid species in each *Saccharomyces* strain. Lipids were extracted and analyzed by LC-MS in positive and negative ion mode (n = 1).

FIGURE 5.8 Average number of carbons in each *Saccharomyces* strain. Lipids were extracted and analyzed by LC-MS in positive and negative ion mode (n = 1).

5.4 Discussion

Non-*cerevisiae* strains are gaining attention in oenology because they positively modify wine composition (Querol et al., 2018). *S. uvarum* and *S. kudriavzevii* strains produce wines with a high quantity of aroma-active higher alcohols and glycerol, and a low ethanol concentration (Gamero et al., 2013; González et al., 2007; Oliveira et al., 2014; Stribny et al., 2015; Pérez-Torrado et al., 2016). Moreover, these species have the ability to carry out fermentations at low temperatures (Salvadó et al., 2011a). These yeast traits are very appreciated by winemakers, but apart from them, high ethanol tolerant strains are required, which is a characteristic that *S. uvarum* and *S. kudriavzevii* strains do not possess. Among the approaches that are feasible to reunite these characteristics together -good aromatic profile and high ethanol tolerance- adaptive laboratory evolution can be performed on selected *S. uvarum* and *S. kudriavzevii* strains.

In this study, we used a media that mimicked wine fermentation conditions at different stages to led to an evolutionary adaptation of *S. kudriavzevii* and *S. uvarum* strains to this media. We wanted to study the results of using this strategy with different *Saccharomyces* species in both their genomes and phenotypes. The adaptive laboratory evolution allowed the fixation of mutations by genetic drift (Warringer et al., 2011; Zörgö et al., 2012). As far as we know, this is the first time that *S. kudriavzevii* strains are exposed to ALE. In other works, *Saccharomyces* hybrids, including *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*, were evolved for different traits (Lopandic et al., 2016; Sanchez et al., 2017).

In Sanchez et al. (2017) *S. uvarum* populations, together with other *Saccharomyces* species were evolved in sulfate-limited conditions. The aim of that work was to investigate if the genetic background of different *Saccharomyces* species influence the evolutionary outcomes when they were under sulfate limitation. Their results showed that *SUL1* locus was amplified in *S. cerevisiae*, *S. paradoxus*, and *S. mikatae* populations, but in *S. uvarum SUL2* locus amplification was identified instead (Sanchez et al., 2017) .

In this work, by using ALE, one *S. uvarum* strain (CECT 12600) and two *S. kudriavzevii* strains

(CR85 and CA111) improved their ethanol tolerance. However, after exploring the genome of the evolved strains, we concluded that each strain showed different patterns of evolutionary dynamics, even when they are exposed to the same conditions, as happened in Sanchez et al. (2017).

The two *S. kudriavzevii* strains CA111 and CR85 were the ones which showed CCNV in their genomes during the two steps of the evolution. In the case of CA111 strain, chromosome VIII was duplicated in the first stage of the evolution, but then, in the next evolution steps, this extra copy of the chromosome was lost. CR85 strain at the first evolution step gained an extra copy of chromosomes II, IX and XVI; but then, in the following evolution steps some colonies lost chromosomes II and XVI. The two *S. kudriavzevii* strains have highly homozygosity levels and a low ascospore viability, thus, asexual reproduction is predominant (González et al., 2008; Lopes et al., 2010). Their genomic instability at the level of whole chromosome duplications and losses and their selection during harsh conditions for these strains could be produced during the mitotic phase.

It is interesting to remark two aspects: The two *S. kudriavzevii* strains gained chromosomes during the ALE process. Chromosome copy number variation was also observed in the previous chapter with the *S. cerevisiae* x *S. uvarum* hybrid (Lairón-Peris et al., under revision, Chapter 4). However, CECT 12600 was the strain which presented a bigger number of SNPs on its genome during the evolution. BMV58, the strain whose ethanol improvement was not observed after the drop tests in ethanol media, only showed some SNPs during the evolution process and some duplicated genes. Thus, during the growth cycles under the media conditions, the spontaneous mutations that occurred randomly in the strains and were later selected varied depending on the strain, being point mutations the major change in CECT 12600 strain and chromosome duplications in CR85 and CA111 strains.

Ethanol tolerance is a complex trait to evolve as there are numerous causative genes involved in high ethanol tolerance in yeasts (Fujita et al., 2006; Swinnen et al., 2012; van Voorst et al., 2006). In other works, its improvement has been addressed by using genetically engineered strains (Chandler et al., 2004; van Voorst et al., 2006), implementing adaptive laboratory evolution

strategies (Aguilera et al., 2010; Novo et al., 2014), or by obtaining artificial hybrids (Lairón-Peris et al., 2020; Serra et al., 2005). Also previously, the presence of a more fluid lipidic membrane has been correlated with more ethanol tolerant strains (Chi and Arneborg, 1999a; Jurešić et al., 2009). Moreover, the presence of determined phospholipid species, as PE in membrane has been associated with this higher ethanol tolerant strains (Murzyn et al., 2005).

In this work we have observed that in the two evolved *S. kudriavzevii* strains the fluidity of its membranes in the presence of ethanol is higher than the fluidity of the original strains. Moreover, CA111-EVO9 *S. kudriavzevii* strain is the one which possess major differences in the lipid classes present in comparison with the other strains. The number of lipid species in this evolved CA111-EVO9 strain is similar to the composition of a tolerant *S. cerevisiae* strain, possessing more GPA, GPEth, GPSer and PE. This results support the fact that during ALE some of the mutations fixed in the population -because they confer an advantage under selection on ethanol media- are related with genomic characteristics that affect membrane composition.

One of our main findings in the present work is that it is possible to adapt and evolve *Saccharomyces* non-*cerevisiae* strains in the laboratory and obtain strains with improved phenotypes for the ethanol tolerance. The strain which showed more relevant changes was CA111, *S. kudriavzevii* strain, at both genomic and lipidomic level. This strain, CA111, was the less ethanol tolerant strain of the *Saccharomyces* strains used in the adaptive evolution. Although the genomic mechanisms leading to that improvement are not completely understood, we propose that the gain and loss of the extra copy of chromosome VIII had provoked a global transcriptional variation in this strain.

General Discussion

Saccharomyces yeasts are of great importance in the winemaking industry as they conduct the alcoholic fermentation process in an efficient way (Sicard and Legras, 2011). For millenia, they have unconsciously been used and selected, which make of yeasts “domesticated” organisms with genetic particularities (Steensels et al., 2019). Nowadays, it is possible to investigate and characterize both the phenotype of a strain and its genome using NGS, allowing to determine phenotype-genotype correlations (Dragosits and Mattanovich, 2013; Solieri et al., 2013). These techniques permit the selection of the most suitable strain to be used in a determined industrial process, and also to understand the underlying molecular mechanisms involved in its properties of interest (Dequin and Casaregola, 2011; Marsit et al., 2017).

Different strategies have proved to be useful to obtain new *Saccharomyces* yeast strains with the desired phenotype that fulfill both the industry demands and the preferences of wine consumers. Among them, artificial hybridization and experimental adaptive evolution are two of the most popular techniques (Çakar et al., 2005; Pérez-Través et al., 2015). The reason is that the new strains generated with these approaches are not considered GMO (Wunderlich and Gatto, 2015).

In this doctoral thesis, we aimed to understand and improve the ethanol tolerance of different *Saccharomyces* yeast strains, especially those of interest for its use the wine industry. In the first moment, we used different methods to classify existing *Saccharomyces* strains regarding their ethanol tolerance. We also used “omic” techniques to decipher which traits differentiate the ethanol

tolerant strains from the non-tolerant ones. The knowledge generated was used to improve strains with low ethanol tolerance with other interesting traits for its use in enology, such as *S. uvarum* and *S. kudriavzevii* strains. We used different strategies to obtain a new strain: rare mating for the obtaining of a *S. cerevisiae* x *S. uvarum* hybrid yeast and adaptive laboratory evolution with different *Saccharomyces* strains.

Among the *Saccharomyces* yeast species, the most well studied and used in industrial environments species is *S. cerevisiae* (Moyad, 2007). This species is present in a wide range of habitats and niches, and as a consequence, each strain shows particularities regarding its ethanol tolerance. In the first chapter, we focused on finding differences among different *S. cerevisiae* yeasts concerning their ethanol tolerance and their lipid composition. We selected and studied the ethanol tolerance of 61 *S. cerevisiae* strains that are present in different environmental sources and some *S. cerevisiae* strains that are used in the wine industry. We proved that *S. cerevisiae* strains can be evaluated and classified by analyzing their growth in both liquid media (containing different ethanol concentrations), and in solid media with ethanol. After characterizing the 61 strains, we concluded that the most ethanol tolerant strains belong to wine commercial *S. cerevisiae* strains; but also that strains belonging to the same isolation source can show different behaviors.

5 strains were selected to study more in-depth their lipid composition: AJ4, the most tolerant strain; MY29, a sherry wine strain, had an intermediate behavior in ethanol conditions; MY26, an agave strain, that was one of the least tolerant strain; and MY3 and MY14, two commercial wine strains tolerant to ethanol. Membranes, mainly formed by lipids, are the first barrier that yeast cells possess against ethanol. The study of lipid membrane composition was used to correlate different ethanol tolerances with different membrane compositions. The mass spectrometry analysis of the lipids present in those strains revealed that the most relevant differences are found among the lipid composition of MY29, the flor yeast, and the other strains. A significantly higher PE concentration was observed in the least tolerant strain, MY26, at 0% and 6% ethanol compared to the other strains. Besides, we observed that the most tolerant strain, AJ4 had a higher membrane fluidity, which could confer an advantage to this strain in the presence of ethanol.

Due to these results, we wanted to study more in-depth the transcriptomic response to ethanol of the three strains with intermediate phenotypes for this trait. In the second chapter, we selected *S. cerevisiae* strains MY3, MY26 and AJ4 and carried out the growth of these three strains under GPY media with three ethanol concentrations: 0%, 6%, and 10%. At different time points, we retrieved samples to conduct the transcriptomic analyses. The three strains showed differential changes that affect the lipid yeast membrane composition. Ergosterol synthesis genes were more up-regulated in AJ4 strain in the presence of ethanol than in MY3 and MY26 strains. Moreover, genes related to the biosynthesis of membrane phospholipids, such as *HMN1* and *EKI1* were up-regulated in AJ4 strain under ethanol growth. As these genes' transcription is activated by Ino2p, the sequence of this protein was analyzed and AJ4 showed two mutations in comparison with the other two strains, which could play a role in the differential activation of the genes. Some up-regulated genes in AJ4, grown in high ethanol conditions, are regulated by *GCN4*, whose sequence is different in AJ4 compared to MY3 and MY26. Together, these analyses suggested that these specific allele changes could play a role in ethanol tolerance regulation, but more analyses need to be performed.

With the previous knowledge that *S. cerevisiae* AJ4 strain is an ethanol tolerant strain, we decided to improve BMV58, an *S. uvarum* strain with interesting properties for its usage in oenological conditions, but with a lower ethanol resistance. In the third chapter, we used "rare mating" for obtaining a hybrid: H14A7, among AJ4 and BMV58. We proved that this a good technique to improve the characteristics of two strains, by merging the positive characteristics of both parentals. It has to be mentioned, that after crossing two strains with the desired phenotypes, it was necessary to characterize a set of the obtained hybrids to select the one that reunited the desired properties. In the case of H14A7, it performed wine fermentations at 25ºC faster than both parental strains, and at lower temperatures showed a better behavior than AJ4.

Another interesting question in biology is the study of how an interspecies hybrid behaves when it is put in a stressful environment. In the fourth chapter we aimed to study the adaptation of the *S. cerevisiae* x *S. uvarum* hybrid strain H14A7 to a must media similar to that present in wine fermentation at advanced stages. We used a modified synthetic must (M-SM) containing high ethanol and low sugar concentrations, which also contained metabisulfite, a preservative that

is used during wine fermentation as it converts to sulfite. After the adaptation process under these selected environmental stressful conditions, the tolerance of the adapted strain (H14A7-etoh) to sulfite and ethanol was investigated, revealing that the adapted hybrid is more resistant to sulfite if we compare it with H14A7 strain, whereas ethanol improvement was slight. However, a trade-off in the adapted hybrid was present, as it had lost the capacity to ferment sugars.

Different signals of adaptation in the H14A7-etoh genome were detected, confirming that the hybrid genome is unstable under these stressful conditions and that each subgenome present in the strain had adapted differently. Chromosome aneuploidies were present in *S. cerevisiae* chromosome III and in *S. uvarum* chromosome VII-XVI, which had been duplicated. Moreover, *S. uvarum* chromosome I was not present in H14A7-etoh and a loss of heterozygosity (LOH) event arose on *S. cerevisiae* chromosome I. The RNA-seq analysis showed differential gene expression between H14A7-etoh and H14A7, which can be easily correlated with the signals of adaptation that were found in the H14A7-etoh genome. Finally, we reported alterations in the lipid composition of the membrane, consistent with conserved tolerance mechanisms.

Although the adaptation of H14A7 to ethanol only showed a slight improvement in its ethanol tolerance in comparison with H14A7 tolerance, we wondered if other *Saccharomyces* strains, which are less ethanol tolerant, could be evolved by following a similar strategy. We were also interested in which genomic changes occur during the process. In the fifth chapter, we performed the strategy of an ”adaptive laboratory evolution” under ethanol conditions of *S. kudriavzevii* and *S. uvarum strains*. One interesting result is that the genomic adaptation to ethanol varied among the strains, even among strains belonging to the same species, thus revealing different evolutionary dynamics across the genome. The two *S. kudriavzevii* strains, CR85 and CA111, duplicated some of their chromosomes. CECT 12600 *S. uvarum* strain fixated new point mutations on its genome. The membrane fluidity assays revealed that in the presence of ethanol BMV58, CA111 and CR85 evolved strains have a more fluid membrane than the original strains. CECT 12600 evolved strain showed a more rigid membrane when no ethanol was present in the media, but when ethanol was present, there were no differences in membrane fluidity among CECT 12600 evolved strains and CECT 12600.

Conclusions

These are the most relevant conclusions drawn from the results obtained in the thesis:

1) Different *S. cerevisiae* strains from different origins were analyzed and classified by their ability to grow under ethanol stressful conditions. In general terms, the most ethanol tolerant strains belong to wine commercial *S. cerevisiae* strains and strains from the same isolation source showed different behaviors under ethanol stress.
2) Five strains were selected because they showed different ethanol behaviors: AJ4, a commercial strain, that resulted to be the most ethanol tolerant strain; MY29, the most tolerant sherry wine strain, that had an intermediate behavior in ethanol conditions; MY26, an agave strain, that was one of the least tolerant strains and MY3 and MY14, that are commercial wine strains tolerant to ethanol.
3) The mass spectrometry analysis of the lipid composition of each strain in the absence of ethanol highlighted that the most relevant differences are found among MY29, the flor yeast, and the other strains.
4) The most tolerant strain, AJ4, underwent the largest changes to fluidity. This strain resulted to be better able to tolerate the fluidizing effects of ethanol by modulating its membrane composition to lead to an increase in fluidity.
5) The membrane of one of the least tolerant strains, MY26, did not alter its fluidity in any of the conditions and liposomes comprised of MY26 lipids were less leaky when challenged with ethanol.

6) The membranes of the most tolerant *S. cerevisiae* strains are more fluid and contain less PE whereas the membrane of the least tolerant strains contain more PE.
7) Transcriptomic analysis carried out with AJ4, MY3 and MY26 strains under ethanol revealed that each strain differentially expresses different genes under ethanol indicating a high variable response among strains.
8) Genes related to ergosterol biosynthesis were repressed at every time point in the case of MY26 and MY3, but not in AJ4 under 6% and 10% conditions.
9) The most ethanol tolerant strain, AJ4, has amino acid mutations of the transcription factor Ino2p and *GCN4* genes. These factors activate the expression of *HMN1*, *EKI1*, and *OLE1*, genes related to phospholipid biosynthesis and oleic and palmitoleic acid production, respectively.
10) It is possible to obtain an artificial *S. cerevisiae* × *S. uvarum* hybrid via rare mating which combines both the interesting enological properties of a commercial wine *S. uvarum* strain: BMV58, and the high ethanol tolerance of a *S. cerevisiae* strain, AJ4.
11) H14A7 the obtained hybrid, showed hybrid vigor, performing wine fermentations at 25ºC faster than its parents, and at lower temperatures showed a better behavior than the *S. cerevisiae* parental strain.
12) H14A7 strain is an almost perfect allotriploid, with one copy of the *S. uvarum* genome, and two heterozygous copies of each *S. cerevisiae* chromosome, except chromosome III, which is present in one copy.
13) The comparative expression analysis (RNA-seq analysis) between hybrid subgenomes, reported that each parental fraction acted differentially during fermentation. In the fermentation latency phase, whilst the *S. cerevisiae* hybrid subgenome focused on catalytic activity and nutrient uptake, *S. uvarum* fraction of the hybrid showed a higher expression in ribosome biogenesis and ergosterol metabolism.
14) When we compared H14A7 total genome expression against AJ4 during the exponential at 15ºC and 25ºC, GO terms related to ergosterol regulation and alcohol biosynthetic process were over-represented. When we compared H14A7 total genome expression against

BMV58 GO terms related to the amino acid metabolic processes were over-represented.

15) The adaptation of H14A7 hybrid to a media mimicking the stresses present in wine fermentations at late stages, with great ethanol and sulfite, permitted the obtention of a strain: H14A7-etoh with an increased sulfite tolerance and slightly better adapted to high ethanol concentrations.
16) The characterization of H14A7-etoh fermentations in Verdejo must showed that this strain did not ferment all the sugars present in the must, suggesting that this strain has lost fermenting capacity compared to H14A7.
17) One large aneuploidy occurred in the *S. cerevisiae* subgenome of H14A7-etoh: the duplication of chromosome III. This duplication could be the result of either a restoration of diploidy in all *S. cerevisiae* chromosomes, or the result of an adaptation of H14A7 strain to the ethanol media present during the adaptation, as this chromosome III duplication could confer an advantage when growing in high ethanol concentration
18) The *S. uvarum* subgenome of H14A7-etoh was modified in the form of one chromosomal loss (chromosome I) and one chromosome gain (chromosome VII-XVI). *S. uvarum* chromosome I is the smallest chromosome and the translocated chromosome VII-XVI, carries the *FZF1-SSU1* recombination whose gene expression confers sulfite resistance.
19) Transcriptomic analysis of H14A7-etoh revealed that, in general terms, III-cer and VII-XVI-uva genes are up-regulated in H14A7-etoh in comparison with H14A7 strain under the same condition, showing that one way to increase expression of a gene is to increase its copy number in a strain.
20) *SSU1 S. uvarum* allele was differentially expressed in H14A7-etoh, even when no metabisulfite was added, as its expression is constitutive.
21) A significantly lower abundance of PE in the H14A7-etoh strain when compared to H14A7 was observed. Laurdan experiments suggested that the membranes of H14A7-etoh cells were more fluid compared to those of the H14A7 strain; which is consistent with a decrease of PE.
22) Adaptive evolution of the five strains: CR85 and CA111 (*S. kudriavzevii*), 12600 and BMV58

(*S. uvarum*) and H14A7 (*S. cerevisiae* x *S. uvarum*) improved the ethanol tolerance of all of the strains except for BMV58 (*S. uvarum*).

23) Genomic changes occurred in all of the strains, being aneuploidies present in the two *S. kudriavzevii* strains and in the hybrid; small deletions and duplications in BMV58 and SNPs in 12600 strain.

24) The evolved BMV58, CA111 and CR85 strains showed a higher fluidity in their membranes than the original strains when they were grown under ethanol media. The number of some lipid species present in the evolved CA111-EVO9 and 12600−EVO11 strains was higher in comparison with the original strains.

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Annex I-Publications

The following publications are included in this thesis:

Lairón-Peris et al. 2020. *Front Bioeng Biotechnol.* 8:1–20. Chapter 3 of this thesis.

Lairón-Peris et al. 2021. *Appl Environ Microbiol.* 87:26. Chapter 1 of this thesis.

Lairón-Peris et al. 2021. *Microb Genomics*. Accepted for publication. Chapter 4 of this thesis.

Throughout this thesis, I have also contributed in the following works:

Alonso del Real et al. 2017. *Front Microbiol.* 8:150

Morard et al. 2019. *Front Genet.* 10:82

García-Ríos et al. 2021. *Int J Food Microbiol.* 342:10977

Minebois et al. Published Online. *Environ Microbiol.*

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BIOTECHNOLOGY

Lipid Composition Analysis Reveals Mechanisms of Ethanol Tolerance in the Model Yeast Saccharomyces cerevisiae

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ABSTRACT Saccharomyces cerevisiae is an important unicellular yeast species within the biotechnological and the food and beverage industries. A significant application of this species is the production of ethanol, where concentrations are limited by cellular toxicity, often at the level of the cell membrane. Here, we characterize 61 S. cerevisiae strains for ethanol tolerance and further analyze five representatives with various ethanol tolerances. The most tolerant strain, AJ4, was dominant in coculture at 0 and 10% ethanol. Unexpectedly, although it does not have the highest noninhibitory concentration or MIC, MY29 was the dominant strain in coculture at 6% ethanol, which may be linked to differences in its basal lipidome. Although relatively few lipidomic differences were observed between strains, a significantly higher phosphatidylethanolamine concentration was observed in the least tolerant strain, MY26, at 0 and 6% ethanol compared to the other strains that became more similar at 10%, indicating potential involvement of this lipid with ethanol sensitivity. Our findings reveal that AJ4 is best able to adapt its membrane to become more fluid in the presence of ethanol and that lipid extracts from AJ4 also form the most permeable membranes. Furthermore, MY26 is least able to modulate fluidity in response to ethanol, and membranes formed from extracted lipids are least leaky at physiological ethanol concentrations. Overall, these results reveal a potential mechanism of ethanol tolerance and suggest a limited set of membrane compositions that diverse yeast species use to achieve this.

IMPORTANCE Many microbial processes are not implemented at the industrial level because the product yield is poorer and more expensive than can be achieved by chemical synthesis. It is well established that microbes show stress responses during bioprocessing, and one reason for poor product output from cell factories is production conditions that are ultimately toxic to the cells. During fermentative processes, yeast cells encounter culture media with a high sugar content, which is later transformed into high ethanol concentrations. Thus, ethanol toxicity is one of the major stresses in traditional and more recent biotechnological processes. We have performed a multilayer phenotypic and lipidomic characterization of a large number of industrial and environmental strains of Saccharomyces to identify key resistant and nonresistant isolates for future applications.

KEYWORDS membrane properties, Saccharomyces cerevisiae, ethanol

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism that has been employed as a model organism to study diverse relevant phenomena in biology at

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Differential Contribution of the Parental Genomes to a *S. cerevisiae* × *S. uvarum* Hybrid, Inferred by Phenomic, Genomic, and Transcriptomic Analyses, at Different Industrial Stress Conditions

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In European regions of cold climate, *S. uvarum* can replace *S. cerevisiae* in wine fermentations performed at low temperatures. *S. uvarum* is a cryotolerant yeast that produces more glycerol, less acetic acid and exhibits a better aroma profile. However, this species exhibits a poor ethanol tolerance compared with *S. cerevisiae*. In the present study, we obtained by rare mating (non-GMO strategy), and a subsequent sporulation, an interspecific *S. cerevisiae* × *S. uvarum* spore-derivative hybrid that improves or maintains a combination of parental traits of interest for the wine industry, such as good fermentation performance, increased ethanol tolerance, and high glycerol and aroma productions. Genomic sequencing analysis showed that the artificial spore-derivative hybrid is an allotriploid, which is very common among natural hybrids. Its genome contains one genome copy from the *S. uvarum* parental genome and two heterozygous copies of the *S. cerevisiae* parental genome, with the exception of a monosomic *S. cerevisiae* chromosome III, where the sex-determining *MAT* locus is located. This genome constitution supports that the original hybrid from which the spore was obtained likely originated by a rare-mating event between a mating-competent *S. cerevisiae* diploid cell and either a diploid or a haploid *S. uvarum* cell of the opposite mating type. Moreover, a comparative transcriptomic analysis reveals that each spore-derivative hybrid subgenome is regulating different processes during the fermentation, in which each parental species has demonstrated to be more efficient. Therefore, interactions between the two subgenomes in the spore-derivative hybrid improve those differential species-specific adaptations to the wine fermentation environments, already present in the parental species.

Keywords: *Saccharomyces cerevisiae*, *S. uvarum*, artificial hybrid, wine fermentation, ethanol tolerance, genome sequencing, RNA-seq

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Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* Species against a *S. cerevisiae* Wine Strain in Wine Fermentation: Competition, Physiological Fitness, and Influence in Final Wine Composition

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Saccharomyces cerevisiae is the main microorganism responsible for the fermentation of wine. Nevertheless, in the last years wineries are facing new challenges due to current market demands and climate change effects on the wine quality. New yeast starters formed by non-conventional *Saccharomyces* species (such as *S. uvarum* or *S. kudriavzevii*) or their hybrids (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*) can contribute to solve some of these challenges. They exhibit good fermentative capabilities at low temperatures, producing wines with lower alcohol and higher glycerol amounts. However, *S. cerevisiae* can competitively displace other yeast species from wine fermentations, therefore the use of these new starters requires an analysis of their behavior during competition with *S. cerevisiae* during wine fermentation. In the present study we analyzed the survival capacity of non-*cerevisiae* strains in competition with *S. cerevisiae* during fermentation of synthetic wine must at different temperatures. First, we developed a new method, based on QPCR, to quantify the proportion of different *Saccharomyces* yeasts in mixed cultures. This method was used to assess the effect of competition on the growth fitness. In addition, fermentation kinetics parameters and final wine compositions were also analyzed. We observed that some cryotolerant *Saccharomyces* yeasts, particularly *S. uvarum*, seriously compromised *S. cerevisiae* fitness during competences at lower temperatures, which explains why *S. uvarum* can replace *S. cerevisiae* during wine fermentations in European regions with oceanic and continental climates. From an enological point of view, mixed co-cultures between *S. cerevisiae* and *S. paradoxus* or *S. eubayanus*, deteriorated fermentation parameters and the final product composition compared to single *S. cerevisiae* inoculation. However, in co-inoculated synthetic must in which *S. kudriavzevii* or *S. uvarum* coexisted with

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Aneuploidy and Ethanol Tolerance in *Saccharomyces cerevisiae*

Miguel Morard[1,2], Laura G. Macías[1,2], Ana C. Adam[2], María Lairón-Peris[2], Roberto Pérez-Torrado[2], Christina Toft[1,2†] and Eladio Barrio[1,2]*

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Response to environmental stresses is a key factor for microbial organism growth. One of the major stresses for yeasts in fermentative environments is ethanol. *Saccharomyces cerevisiae* is the most tolerant species in its genus, but intraspecific ethanol-tolerance variation exists. Although, much effort has been done in the last years to discover evolutionary paths to improve ethanol tolerance, this phenotype is still hardly understood. Here, we selected five strains with different ethanol tolerances, and used comparative genomics to determine the main factors that can explain these phenotypic differences. Surprisingly, the main genomic feature, shared only by the highest ethanol-tolerant strains, was a polysomic chromosome III. Transcriptomic data point out that chromosome III is important for the ethanol stress response, and this aneuploidy can be an advantage to respond rapidly to ethanol stress. We found that chromosome III copy numbers also explain differences in other strains. We show that removing the extra chromosome III copy in an ethanol-tolerant strain, returning to euploidy, strongly compromises its tolerance. Chromosome III aneuploidy appears frequently in ethanol-tolerance evolution experiments, and here, we show that aneuploidy is also used by natural strains to enhance their ethanol tolerance.

Keywords: *Saccharomyces cerevisiae*, wine yeasts, chromosome III, aneuploidy, comparative genomics, ethanol tolerance

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INTRODUCTION

The yeast *Saccharomyces cerevisiae* is among the most beneficial microorganisms for humans, especially industrial strains involved in the production of fermented products, such as bread, beer or wine. *S. cerevisiae*, as well as other *Saccharomyces* species, are characterized by their ability to ferment simple sugars into ethanol, even when oxygen is available for aerobic respiration (Crabtree effect), due to an overflow in the glycolysis pathway (Hagman and Piškur, 2015). Although, alcohol fermentation is energetically less efficient than respiration, it provides a selective advantage to these yeasts to out-compete other microorganisms. This way, sugar resources are consumed faster and the ethanol produced during fermentation, as well as high levels of heat and CO_2, can be harmful or less tolerated by their competitors. Once competitors are overcome, *Saccharomyces* yeasts can use the accumulated ethanol as a substrate for aerobic respiration in the presence of oxygen. This ecological strategy was named (ethanol) "make-accumulate-consume" (Thomson et al., 2005; Piškur et al., 2006).

With the advent of the human hunter-gatherer societies, *S. cerevisiae*, due to its fermentative capabilities, successfully occupied a new ecological niche in the crushed grape berries, collected by

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Thermo-adaptive evolution to generate improved *Saccharomyces cerevisiae* strains for cocoa pulp fermentations

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ABSTRACT

Cocoa pulp fermentation is a consequence of the succession of indigenous yeasts, lactic acid bacteria and acetic acid bacteria that not only produce a diversity of metabolites, but also cause the production of flavour precursors. However, as such spontaneous fermentations are less reproducible and contribute to produce variability, interest in a microbial starter culture is growing that could be used to inoculate cocoa pulp fermentations. This study aimed to generate robust *S. cerevisiae* strains by thermo-adaptive evolution that could be used in cocoa fermentation. We evolved a cocoa strain in a sugary defined medium at high temperature to improve both fermentation and growth capacity. Moreover, adaptive evolution at high temperature (40 °C) also enabled us to unveil the molecular basis underlying the improved phenotype by analysing the whole genome sequence of the evolved strain. Adaptation to high-temperature conditions occurred at different genomic levels, and promoted aneuploidies, segmental duplication, and SNVs in the evolved strain. The lipid profile analysis of the evolved strain also evidenced changes in the membrane composition that contribute to maintain an appropriate cell membrane state at high temperature. Our work demonstrates that experimental evolution is an effective approach to generate better-adapted yeast strains at high temperature for industrial processes.

1. Introduction

Thermotolerant microorganisms may be useful for industrial applications, such as a high-temperature growth yeast for bioethanol production (Mienda and Shamsir, 2013) or cocoa fermentation (Goddard, 2016). During these processes, cells have to face with high stress levels such as the temperature, which influences both growth and fermentation capacity (Morano et al., 2012). Industry spends a huge amount of energy cooling or heating fermentations to fine-tune temperature as closely as possible to the optimum growth temperature (Hamelinck et al., 2005; Stephen et al., 2012). In spite of this, this optimum temperature does not often very well match the final product's cost-effectiveness or quality. These problems can be avoided by providing better-adapted yeasts to ferment at non-optimal temperatures. However, we are far from either understanding the molecular and physiological mechanisms of adaptation at high temperatures or knowing what makes them thermotolerant.

Several genes have been related to thermotolerance in *S. cerevisiae*. Enzymes involved in membrane synthesis and composition have been linked to high thermotolerance, such as *ERG3* (Caspeta et al., 2014), a C-5 sterol desaturase; *ERG13* (Pinheiro et al., 2020), a protein involved in early ergosterol biosynthesis; chaperones like *HSP104* and *HSP12* (Sanchez et al., 1992); trehalose and glycogen genes *TPS1*, *TPS2*, *NTH1* (De Virgilio et al., 1994) and *GSY1* (Pinheiro et al., 2020); genes of RNA processing like *PRP42* and *SMD2* (Yang et al., 2013). Overexpression of *RSP5*, a ubiquitin ligase, also increases thermotolerance (Shahsavarani et al., 2012). Nevertheless, these genes have not yet been applied to genetically improve yeast strains for industrial processes. This could be because trade-offs occur with other properties that are important in industry, such as the fermentation, propagation, drying or storage of yeasts (Deparis et al., 2017; Matallana and Aranda, 2017; Walker et al., 2019).

Experimental evolution is an important tool for investigating adaptive shifts, clonal dynamics, competition and fitness, and the genetic

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Metabolic differences between a wild and a wine strain of *Saccharomyces cerevisiae* during fermentation unveiled by multi-omic analysis

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Summary

***Saccharomyces cerevisiae*, a widespread yeast present both in the wild and in fermentative processes, like winemaking. During the colonization of these human-associated fermentative environments, certain strains of *S. cerevisiae* acquired differential adaptive traits that enhanced their physiological properties to cope with the challenges imposed by these new ecological niches. The advent of omics technologies allowed unveiling some details of the molecular bases responsible for the peculiar traits of *S. cerevisiae* wine strains. However, the metabolic diversity within yeasts remained poorly explored, in particular that existing between wine and wild strains of *S. cerevisiae*. For this purpose, we performed a dual transcriptomic and metabolomic comparative analysis between a wild and a wine *S. cerevisiae* strains during wine fermentations performed at high and low temperatures. By using this approach, we could correlate the differential expression of genes involved in metabolic pathways, such as sulfur, arginine and thiamine metabolisms, with differences in the amounts of key metabolites that can explain some important differences in the fermentation performance between the wine and wild strains.**

Introduction

Saccharomyces cerevisiae is a widespread yeast species found both in the wild (Wang *et al*., 2012) and in fermentative processes, including winemaking (Legras *et al*., 2018). Natural isolates of *S. cerevisiae* have been isolated from highly diverse living environments, such as fruits, tree bark, rotten wood, cacti, soil and exudates of oak trees. Over the last few decades, the increasing availability of *S. cerevisiae* strains and their genomes has continuously consolidated the position of this species as a model organism in ecology and population genomics (Almeida *et al*., 2015; Gallone *et al*., 2016; Legras *et al*., 2018; Liti *et al*., 2009; Peter *et al*., 2018; Peter & Schacherer, 2016; Schacherer *et al*., 2009).

Among the available strains, increased attention has been paid to *S. cerevisiae* wine strains. Indeed, the repeated exposure of wine *S. cerevisiae* strains to the variety of stresses occurring during alcoholic fermentation (e.g. osmotic stress, ethanol content, nitrogen starvation, addition of sulfites), has led to their passive domestication and the emergence of differential adaptive traits of biotechnological interest (Querol *et al*., 2003; Barrio *et al*., 2006). In this aspect, different genomic changes of adaptive value, often referred to as 'footprints' of the domestication process have been reported in wine strains (Marsit and Dequin, 2015; Gallone *et al*., 2016, 2019; Gorter de Vries *et al*., 2017). Nucleotide variation (Schacherer *et al*., 2009; Eldarov *et al*., 2018), chromosomal rearrangements (Guijo *et al*., 1997; Pérez-Ortín *et al*., 2002; García-Ríos *et al*., 2019), gene copy number variation (Ibáñez *et al*., 2014; Peter *et al*., 2018), introgressions (Almeida *et al*., 2014), hybridization (Dunn *et al*., 2013; Morard *et al*., 2020), aneuploidy (Hose *et al*., 2015; Mangado *et al*., 2018; Morard *et al*., 2019) and horizontal gene transfer (HGT) (Marsit *et al*., 2015, 2016) are the highlighted genetic mechanisms described in the adaptation of *S. cerevisiae* wine strains to winemaking. For instance, the reciprocal translocation between chromosomes VII and XVI is a well-documented case of gross chromosomal rearrangement with the adaptive advantage of sulfite resistance, only present in wine strains of *S. cerevisiae* (Pérez-Ortín *et al*., 2002; Yuasa *et al*., 2004; García-Ríos *et al*., 2019). More recently, the genes of region C (Novo *et al*., 2009), which results from

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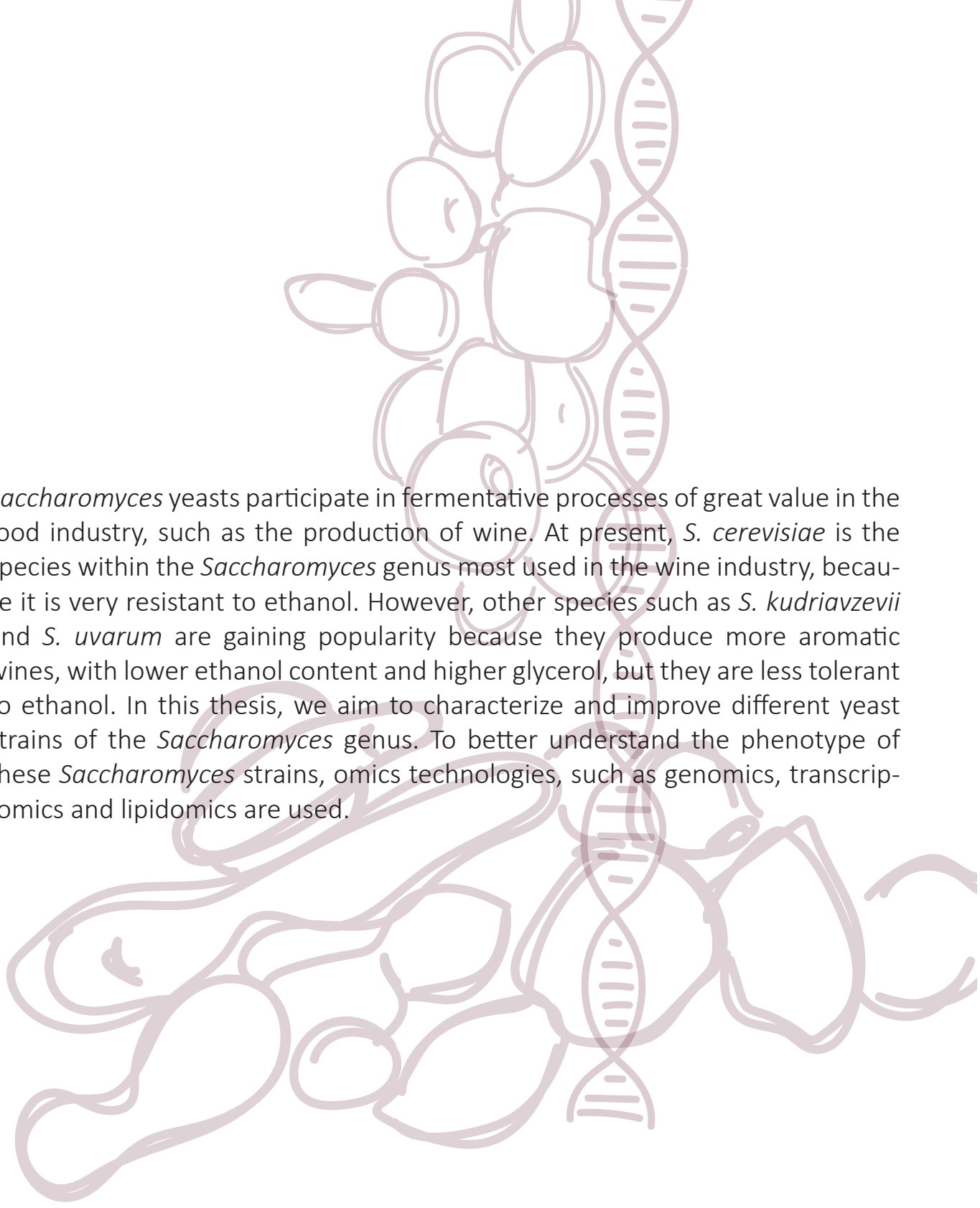

Saccharomyces yeasts participate in fermentative processes of great value in the food industry, such as the production of wine. At present, *S. cerevisiae* is the species within the *Saccharomyces* genus most used in the wine industry, because it is very resistant to ethanol. However, other species such as *S. kudriavzevii* and *S. uvarum* are gaining popularity because they produce more aromatic wines, with lower ethanol content and higher glycerol, but they are less tolerant to ethanol. In this thesis, we aim to characterize and improve different yeast strains of the *Saccharomyces* genus. To better understand the phenotype of these *Saccharomyces* strains, omics technologies, such as genomics, transcriptomics and lipidomics are used.

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