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Study of glycerol and respiro-fermentative metabolism diversity among *Saccharomyces* yeasts

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STUDY OF GLYCEROL AND RESPIRO-FERMENTATIVE METABOLISM DIVERSITY AMONG *SACCHAROMYCES* YEASTS

Tesis doctoral presentada por:

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Para optar al grado de doctor en Biotecnología por la Universidad de Valencia

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CERTIFICAN que la presente memoria "STUDY OF GLYCEROL AND RESPIRO-FERMENTATIVE METABOLISM DIVERSITY AMONG *SACCHAROMYCES* YEASTS" constituye la tesis doctoral de Don. Bruno Motta Oliveira para optar al grado de doctor en Biotecnología por la Universitat de València. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

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'Dios nos hizo perfectos y no escoge a los capacitados,
sino que capacita a los escogidos. Hacer o no hacer
algo, sólo depende de nuestra voluntad y perseverancia’.
(Albert Einstein)

Para mis padres Leonel e Margareth
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I - RESUMEN AMPLIO

Introducción

Las levaduras son responsables de numerosos procesos biotecnológicos tales como la producción de bebidas y alimentos fermentados. Las levaduras de interés biotecnológico son organismos muy especializados que han evolucionado bajo restrictivas condiciones ambientales en distintos ambientes manipulados por el hombre. Durante el proceso de adaptación a estos ambientes manipulados por el hombre, distintas cepas y especies del género *Saccharomyces* se han visto sometidas a procesos selectivos generados por su uso inconsciente en la fermentación alcohólica (Querol et al., 2003), lo que también ha generado diferencias adaptativas entre ellas (Barrio et al., 2006). Aunque la especie más frecuente en fermentaciones vínicas, y objeto principal de la mayoría de los estudios es *S. cerevisiae*, otras especies pertenecientes al género *Saccharomyces* tales como *S. kudriavzevii, S. paradoxus, S. bayanus* y *S. eubayanus,* una nueva especie aislada en Argentina (Libkind et al. 2011), así como los híbridos de estas especies, han sido objetivo de estudio en los últimos, mostrando importantes diferencias genéticas y fisiológicas.

De los resultados obtenido de trabajos previos, cabe destacar que en comparación con cepas comerciales de *S. cerevisiae*, las cepas de las especies *S. uvarum* y *S. kudriavzevii* son capaces de fermentar mostos produciendo un menor grado alcohólico y una mayor concentración de glicerol, sin que ello conlleve un incremento de la cantidad de ácido acético (González et al. 2007, Tosi et al. 2009, Gamero et al. 2013), característica de gran interés para paliar los efectos del cambio climático en enología. También se ha observado que cepas de *S. uvarum* y *S. kudriavzevii* crecen mejor a bajas temperaturas que las cepas de *S. cerevisiae* en distintas condiciones fermentativas, tanto en mosto natural de uva (Tronchoni et al., 2009) como en mosto sintético (Arroyo-López et al., 2010). Sin embargo, pocos datos se conocen sobre la regulación de la síntesis de glicerol en *S. kudriavzevii* y en el resto de especies del género

Saccharomyces.

Estos datos revelan grandes diferencias entre las especies indicando una gran disparidad en el metabolismo central de carbono, probablemente debido a las diferencias de adaptación a diferentes condiciones ambientales. A la vista de estos datos se hace necesaria una mejor comprensión de los distintos mecanismos reguladores de la síntesis de glicerol entre estas especies. Las especies del género *Saccharomyces* presentan dos genes GPD1 y GPD2, que codifican las glicerol-3-fosfato deshidrogenasas, enzimas más importantes de la ruta de síntesis de glicerol. En *S. cerevisiae* se conoce muy bien la expresión de estos genes relacionada con la respuesta al estrés osmótico (Albertyn et al., 1994) y en condiciones de fermentación vínica (Pérez-Torrado et al., 2002). En cambio, GPD2 se induce en anaerobiosis para mantener el equilibrio redox celular mediante la reoxidación del NADH que se produce durante la síntesis del glicerol (Ansell et al. 1997), por lo que en el caso de la fermentación vínica la inducción se produce al pasar a condiciones anaeróbicas durante la fase de crecimiento exponencial. Sin embargo, poco se sabe en las otras especies del género *Saccharomyces*. En la primera parte de la tesis se analiza la acumulación de glicerol intracelular y se caracteriza la actividad enzimática de las dos Gpd1p en *S. kudriavzevii* y se compara con S. cerevisiae.

La producción y el balance de glicerol son esenciales para la supervivencia de las levaduras sometidas a condiciones estresantes como puede ser el estrés osmótico o por frío, situaciones que son habituales durante procesos industriales como son las vinificaciones. Las respuestas a estos estreses son bien conocidas en *S. cerevisiae*, mientras que se sabe poco en otras especies del género *Saccharomyces* próximas filogenéticamente y asociadas a los ambientes naturales o de fermentación, tales como *S. uvarum*, *S. paradoxus* o *S. kudriavzevii*. En la industria fermentativa, especialmente en la fabricación del vino, se exigen

actualmente dos características para utilizar una cepa de levadura: la resistencia a estrés osmótico y la capacidad para crecer a bajas temperaturas (Pretorius et al., 2012). Se sabe que *S. cerevisae* trata de aumentar los niveles de glicerol intracelular cuando está sometida a estrés osmótico o por frío en condiciones de vinificación o de crecimiento normal en laboratorio (Panadero et al., 2006; Petelenz-Kurdziel et al., 2013). Esta acumulación es muy importante para mantener el equilibrio durante la primera fase de la fermentación y el glicerol actúa como un agente crio-protector clave para la adaptación a los ambientes fríos, lo que permite la viabilidad celular determinante de un buen rendimiento fermentativo (Remize et al., 2001; Tulha et al., 2010). Por otro lado, se sabe muy poco sobre la relación del balance y producción del glicerol en respuesta a estos estreses en otras especies del género *Saccharomyces* asociadas también a ambientes fermentativos. Teniendo en cuenta que muchas de estas especies son de interés biotecnológico, se hace necesario un mejor entendimiento de sus características fisiológicas y moleculares en relación al balance de glicerol y que es objetivo del segundo capítulo de esta tesis.

Cabe destacar que cualquier cambio de expresión en genes implicados en la ruta del metabolismo central o respiro-fermentativo, como es el caso de genes de la síntesis de glicerol, también puede influir directamente en las características enológicas deseables en las cepas vínicas de levaduras posiblemente por alterar sus rendimientos en etanol, glicerol y ácido acético (Cordente et al., 2013; Pretorius et al., 2012; Varela et al., 2012). Actualmente todos los estudios metabólicos se centran en cepas de *S. cerevisiae* de interés industrial o de laboratorio, pero poco se conoce sobre cepas de *S. cerevisiae* de diferentes orígenes o de otras especies del género *Saccharomyces*. El estudio de las posibles variaciones en el metabolismo central o del efecto *Crabtree* en condiciones fermentativas entre cepas y especies del género *Saccharomyces* puede ser de gran interés. Con esta finalidad, se ha llevado a cabo un amplio estudio

genético y fermentativo con 94 cepas de *S. cerevisiae* de diferentes orígenes agrupadas como que vínicas, mosaico y no vínica según según los datos de Liti et al., (2009). Se ha analizado bajo distintas condiciones fermentativas, la producción de los metabolitos primarios y secundarios y los resultados han revelado importantes y significativas diferencias metabólicas entre cepas vínicas y no vínicas de *S. cerevisiae*.

Objetivos y Metodología

Esta tesis se centra en el estudio comparativo de la síntesis de glicerol incluyendo cambios en la regulación y en el estudio de cambios en el metabolismo fermentativo de levaduras del género *Saccharomyces* y otras especies tales como *S. uvarum* y *S. kudriavzevii* que son usualmente utilizadas en procesos fermentativos industriales sobre todo en vinificación como alternativas a *S. cerevisiae* en fermentaciones industriales.

Los principales objetivos y la metodología utilizada se enumeran a continuación:

1. Comprender los mecanismos moleculares reguladores de la elevada síntesis de glicerol por cepas de la especie *S. kudriavzevii* en condiciones de vinificación. Para explicar esta observación a nivel molecular se ha estudiado la expresión de los genes implicados en la ruta de síntesis de glicerol y se ha observado una expresión más alta de *GPD1* en *S. kudriavzevii* en comparación con *S. cerevisiae* en condiciones de micro-vinificación. También se ha detectado una mayor actividad enzimática de Gpd1p en *S. kudriavzevii* en respuesta a estrés por frío y osmótico y se ha demostrado que la enzima de esta especie presenta una mayor actividad catalítica que contribuye al aumento de la producción de glicerol. Finalmente, también se ha comparado la producción de glicerol entre las enzimas de ambas especies y con una variedad recombinante entre ambas, con el mismo fondo genético y se ha encontrado que la enzima de *S. kudriavzevii* produce mayores niveles de glicerol en distintas temperaturas de crecimiento, 12 o 28

°C. Para este estudio se emplearon las cepas de *S. cerevisiae* T73 y EC1118, usadas como modelos de cepas vínicas, además de la cepa vínica comercial FCry (Fermol Cryophile; AEB group), seleccionada por su adaptación a bajas temperaturas y buena producción de glicerol. La cepa diploide BY4743 se ha empleado como cepa de laboratorio. Las cepas de *S. kudriavzevii* estudiadas en este objetivo fueron la IFO1802 (tipo) y las naturales ZP591, ZP594 y ZP629, aisladas en Portugal, y CR85, CR89, CR90 y CA111, aisladas en España. Se llevaron a cabo micro-vinificaciones con la variedad de mosto Bobal o con mosto sintético MS300, que simula el mosto natural de uva. Para los experimentos de estrés, tras crecimiento durante una noche en medio GPY, las células se transfirieron a GPY con sorbitol 1M o a GPY previamente atemperado a 12 °C. Los plásmidos que expresan el gen GPD1 de *S. cerevisiae* o *S. kudriavzevii*, bajo el control del promotor GAL, se construyeron usando pYES2.1 TOPO TA Expression Kit (Invitrogen). Los plásmidos que expresan GPD1 de ambas especies bajo su proprio promotor se construyeron usando pGREG526 por recombinación homóloga en levaduras. También se construyó la versión con el promotor de *GPD1* de *S. cerevisiae* y la secuencia codificadora de ambas especies (pGREG526-$GPD1_{Scer\text{-}Skud}$). La modelización de la estructura de la enzima Gpd1p se realizó por medio del programa MODWED online basado en el software Modeller. Los niveles de glicerol se determinaron enzimáticamente por medio de un kit comercial (AMS-SYSTEA) adaptado a un instrumento automatizado ECHO y también por medio de HPLC acoplado a un detector de índice de refracción (Thermo Fisher Scientific, Waltham, MA). La expresión de los genes *GPD1*, *GPD2*, *GPP1* y *GPP2* se cuantificó por qRT-PCR (quantitative real-time PCR) usando Light Cycler FastStart DNA MasterPLUS SYBR green (Roche Applied Science, Germany) con el aparato LightCycler® 2.0 System (Roche Applied Science, Germany). La actividad de Gpd1p citoplasmática se determinó en extractos celulares brutos. El contenido proteico total se estimó con Bio-Rad Protein y albumina de suero bovino

como patrón. Las medidas de actividad obtenidas con las distintas concentraciones de sustrato se representaron y se ajustaron a la ecuación de Michaelis-Menten por regresión no lineal, usando GraphPad Prism 6.0 Software Enzyme Kinetics package, que permite calcular directamente la V_{max} y K_m. Todos los experimentos se realizaron por triplicado.

2. Comparar el balance de glicerol entre distintas especies del genero *Saccharomyces* durante estrés osmótico y por frío.

Para llevar a cabo este objetivo se estudió la expresión de cuatro genes clave para el balance del glicerol en cuatro especies con potencial biotecnológico (*S. cerevisiae; S. paradoxus; S. uvarum* and *S. kudriavzevii*). También se estudió la función del transportador de glicerol Stl1p en la supervivencia a cambios osmóticos y viabilidad celular en ambientes de vinificación. Se usaron dos cepas distintas de cada especie. En *S. cerevisiae*, la cepa vínica modelo T73 y la comercial Fermol Cryophile FCry (AEB group). Las cepas 108 y Chr16.2, aisladas de ambientes naturales, se usaron como representativas de la especie *S. paradoxus*. En *S. uvarum*, se emplearon las cepas 12600 y BMV58 aisladas en ambientes de vinificación en España. La cepa BMV58 fue patentada (patente ES2330709 B1) e implementada por Lallemand® Inc. por su alta producción de glicerol y buenas propiedades fermentativas. En la especie *S. kudriavzevii*, se usó la cepa tipo IFO1802 y la salvaje CR85 aislada en España. La cepa BY4741Δ*hog1*Δ*stl1* fue usada como cepa de laboratorio para la expresión de los genes *STL1* de las distintas especies y comparación de la función de sus productos bajo estrés osmótico. Las vinificaciones se realizaron en botellas de 250 mL con mosto sintético MS300 a 12 °C y 100 rpm. La tolerancia al estrés osmótico se evaluó diariamente por drop tests en placas con YPD; YPD + 0.8 M NaCl; YPD + 1.25 M KCl, incubadas a 12 °C y 25 °C. Para comparar el crecimiento de las distintas especies se usaron cultivos en placa con YPD con 2 M sorbitol o 2 M KCl y suplementado o no con glicerol 1mM. Para investigar las diferencias

funcionales de Stl1p, el crecimiento de las células de BY4741Δ*hog*1Δ*stl1* transformadas con plásmidos adecuados se siguió en placas con SC-ura en presencia de 0.7 M sorbitol o 0.3 M KCl. Las placas contenían o no glicerol 10 mM. Los plásmidos que expresan el gen *STL1* de las cepas T73 de *S. cerevisiae*, BMV58 de *S. uvarum* e IFO1802 de *S. kudriavzevii* bajo el control del promotor del gen *NHA1* se construyeron por sustitución de la secuencia codificadora del gen NHA1-985 (derivado del plásmido YEp352) por medio de recombinación homóloga. Las concentraciones de glicerol y azúcares residuales (glucosa y fructosa) se determinaron en las muestras de mosto y medio por HPLC acoplado a un detector de índice de refracción (Thermo Fisher Scientific, Waltham, MA). La expresión de los genes *GPD1*, *GPD2*, *STL1* y *FPS1* se cuantificó por qRT-PCR (quantitative real-time PCR) como se ha descrito más arriba.

3. Conocer las diferencias metabólicas entre cepas vínicas y no vínicas de *S. cerevisiae* genéticamente definidas bajo distintas condiciones de fermentación.

En este apartado se estudiaron 94 cepas de *S. cerevisiae* tanto a nivel de secuencias como en relación a los rendimientos de sus metabolitos fermentativos. Otras especies del género *Saccharomyces* (*S. uvarum*, *S. eubayanus*, *S. kudriavzevii*, *S. arboriculus*, *S. mikatae* and *S. paradoxus*) se estudiaron también metabólicamente en una primera condición fermentativa. Otras 19 cepas previamente caracterizadas genéticamente por Liti et al. (2009) apenas tuvieron sus secuencias de DNA usadas como referencia genética en el estudio de filogenia. 58 de todas esas cepas de *S. cerevisiae* se eligieron al azar y se estudió su metabolismo en varias condiciones de crecimiento. Las micro-fermentaciones se hicieron a 25 °C en 1.8 mL de medio YPD o SC en micro-placas (New Greiner Bio-one 96-well Masterblock, 2.4mL Polypro) cubiertas de manera no hermética con tapa de placas de micro titulaciones (Fischer Scientific). Las cuatro condiciones

fermentativas empleadas fueron: (1) Con medio YPD; (2) con YPD en ausencia de oxígeno; (3) con medio mínimo SC; (4) con medio SC en ausencia de aminoácidos. Las concentraciones de glucosa, etanol y ácidos orgánicos (pirúvico, acético, succínico y láctico) se determinaron en las muestras al final de las fermentaciones (niveles de glucosa ≤ 0.5g/L) por HPLC (Thermo Fisher Scientific, Waltham, MA) con un detector de índice de refracción. El DNA de las levaduras se aisló siguiendo el protocolo descrito por Querol et al. (1992) y las amplificaciones por PCR de los genes nucleares *EGT2*, *CAT8*, *BRE5* y *GAL4* se realizaron con la enzima Taq Phusion DNA polimerasa de Finnzymes®. Las reacciones de secuenciación se hicieron con el kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Las secuencias de DNA codificadoras de los cuatro genes y representativas de las 113 cepas de *S. cerevisiae* fueron obtenidas y analizadas. Los alineamientos de secuencia se hicieron por medio del programa MEGA5 y para cada cepa se concatenaron los 4 genes en una única secuencia para la construcción de un árbol filogenético por medio del programa SpliTstree4 v.4.13.1 (Huson y Bryant, 2006) con los parámetros Jukes Cantor characters, NeighborNet, EqualAngle.

Conclusiones

1. Comprender los mecanismos moleculares reguladores de la elevada síntesis de glicerol por cepas de la especie *S. kudriavzevii* en condiciones de vinificación.

En primer lugar nos centramos en comprender los mecanismos moleculares reguladores de la elevada síntesis de glicerol por cepas de la especie *S. kudriavzevii* en condiciones de vinificación. La especie *S. kudriavzevii* es capaz de producir altos niveles de glicerol y crecer a bajas temperaturas. Para explicar esta observación a nivel molecular se ha estudiado la expresión de los genes implicados en la ruta de síntesis de glicerol (*GPD1*, *GPD2*, *GPP1* y *GPP2*) y se ha observado una expresión más

alta de *GPD1* en *S. kudriavzevii* en comparación con *S. cerevisiae* en condiciones de micro-vinificación. También se ha detectado una mayor actividad enzimática de Gpd1p en *S. kudriavzevii* en respuesta a estrés por frío y osmótico y se ha demostrado que la enzima de esta especie presenta una mayor actividad catalítica que contribuye al aumento de la producción de glicerol. Finalmente, también se ha comparado la producción de glicerol entre las enzimas de ambas especies y con una variedad recombinante entre ambas, con el mismo fondo genético y se ha encontrado que la enzima de *S. kudriavzevii* produce mayores niveles de glicerol en distintas temperaturas de crecimiento, 12 o 28 °C. Los datos obtenidos en este trabajo destacan que la adaptación de esta especie se ha debido principalmente a la actividad enzimática de Gpd1p. Por otro lado, la adaptación de *S. cerevisiae* está más relacionada con el aumento de la regulación de la expresión de genes implicados en la ruta de síntesis de glicerol.

2. Comparar el balance de glicerol entre distintas especies del genero *Saccharomyces* durante estrés osmótico y por frio.

Comparamos el balance de glicerol entre distintas especies del genero *Saccharomyces* durante estrés osmótico y frío. Para llevar a cabo este objetivo se estudió la expresión de los cuatro genes clave para el balance del glicerol en las especies *S. cerevisiae*; *S. paradoxus*; *S. uvarum* and *S. kudriavzevii*. También se estudió la función del transportador de glicerol Stl1p en la supervivencia a cambios osmóticos y viabilidad celular en ambientes de vinificación, mostrando diferencias significativas de expresión para S. *uvarum*, indicando que este podría ser la característica por la que esta especie es más osmotolerante y produce más glicerol, la capacidad de regular mejor la concentración de glicerol intracelular. Las cuatro cepas estudiadas exhiben distintas estrategias de supervivencia bajo condiciones de estrés osmótico u osmótico-frío. En todas las especies, el balance de glicerol intracelular, que depende de su producción, salida,

entrada y de otros factores minoritarios, se altera dando lugar a un aumento de sus niveles. Por otro lado, mientras que en la especie *S. cerevisiae* hay más alteraciones en los niveles de producción, las demás tienden a depender más de la variación del transporte de glicerol al interior celular como ocurre en *S. uvarum* o *S. kudriavzevii*. Esta última conclusión se ha demostrado mediante la clonación de los alelos del gen *STL1* de las 3 especies en un mismo fondo genético, y pudimos comprobar que cuando la cepa tenía el alelo de *S. uvarum* o *S. kudriavzevii* aumentaba su capacidad de crecer en altos estreses osmóticos, así como incrementaba la producción de glicerol y la cantidad de glicerol intracelular.

3. Conocer las diferencias metabólicas entre cepas vínicas y no vínicas de *S. cerevisiae* genéticamente definidas bajo distintas condiciones de fermentación.

Durante la ejecución de esta parte del proyecto incluimos cepas de la especie *S. cerevisiae* aisladas de otros procesos fermentativos (no vínicos y naturales) como controles y observamos que el metabolismo de estas cepas era más próximo a las especies no-*cerevisiae* que a las cepas vínicas; este resultado nos pareció muy interesante, ya que confirmábamos nuestra hipótesis, que las cepas vínicas han sufrido una especiación. Por este motivo el estudio se ha realizado con 104 cepas que incluyen representantes de las especies *S. uvarum*, *S. eubayanus*, *S. kudriavzevii* y *S. paradoxus*, así como cepas de la especie *S. cerevisiae* vínicas y no vínicas.

Las cepas de *S. cerevisiae* genéticamente caracterizadas como cepas vínicas forman un grupo homogéneo y filogenéticamente distante a las otras cepas caracterizadas como grupos puros no vínicos, que muestran más alta variabilidad genética y metabólica entre ellas mismas. Las cepas de *S. cerevisiae* vínicas y las no vínicas se diferencian por sus perfiles respiro-fermentativos. Los análisis de rendimiento metabólico del etanol y glicerol juntamente con los ácidos orgánicos indican que las cepas no vínicas, al

igual que las especies diferentes a "*cerevisiae*" poseen una mayor capacidad respiratoria que las cepas vínicas. Los resultados indican que las cepas vínicas tienen capacidad de respiración aerobia más limitada y su metabolismo central es prioritariamente fermentativo. Estas cepas se han adaptado para el aumento de la eficiencia de la estrategia de vida conocida como "make-accumulate-consume". Por otro lado, las cepas no vínicas demuestran un metabolismo respiro-fermentativo más equilibrado y más eficientes en su adaptación a rápidos cambios ambientales, debido a que pueden balancear la intensidad del flujo metabólico entre las rutas respiratoria y fermentativa.

En el análisis metabólico-fermentativo realizado para llevar a cabo este objetivo, destaca la aplicabilidad biotecnológica de algunas cepas aquí estudiadas. La mayor parte de ellas serían cepas no vínicas con eficiencia fermentativa similar a las cepas vínicas, pero también pueden tener aplicación biotecnológica las cepas vínicas con altos rendimientos en glicerol y bajos en ácido acético. Además de estas cepas de *S. cerevisiae* con potencial de utilización directa como iniciadoras en procesos de vinificación, otras con propiedades metabólicas específicas pueden estudiarse mejor por medio de evolución adaptativa o técnicas de ingeniería metabólica con vistas a mejorar e expandir su aplicabilidad biotecnológica.

II – GENERAL INTRODUCTION

1 The genus *Saccharomyces* yeast.

1.1 General characteristics and life cycle.

Yeast are generally defined and recognized as unicellular fungi of the phyla *Ascomycetes*, *Basidiomycetes* and the imperfect fungi *Deuteromycetes*, characterized by their sexual reproduction mode or lack of it (*Deuteromycetes*). They can multiply asexually through budding, only *Schyzosaccharomyces* genera reproduce by binary fission. The yeast found on the surface of the grape and in wine belongs to *Ascomycetes* and Deuteromycetes. *Sacharomyces cerevisiae* belongs to *Ascomycetes*, so it can multiply either asexually by vegetative multiplication or sexually by forming ascospores. Under optimal nutritional and cultural conditions *S. cerevisiae* doubles its mass every 90 min. The cell division cycle in vegetative multiplication consists in four phases: G1 (period preceding DNA synthesis), S (DNA synthesis), G2 (period preceding the mitosis) and M (mitosis). During asexual reproduction, a bud is forming by the mother cell during S phase. This bud grows into a viable daughter cell throughout the rest of the cell cycle and then separates from the mother at the end of the M phase. Budding is asymmetric in *S. cerevisiae*, in contrast to other eukaryotic cells, and the resulting daughter cell is smaller than its mother cell (Figure 1). Sexual reproduction involves the formation of four haploid ascospores (two MATa and two MATα within an ascus after meiosis and is induced during nutrient starvation, specifically nitrogen and fermentable carbon sources (Taxis, 2005). MATa spores can only mate with MATα and vice versa, resulting in the fusion of two cells to form a diploid cell (zygote) (Jackson and Hartwell, 1990). Spore released from the ascus can also continue asexual reproduction as haploids for many generations, and are termed heterotallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homotallic (Figure 2).

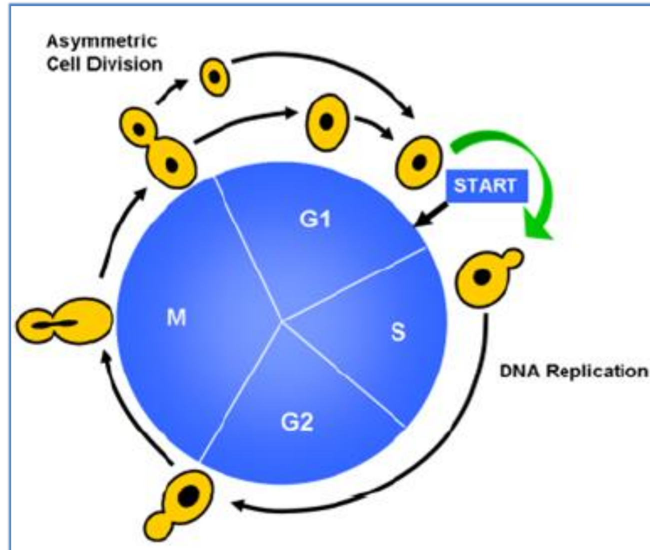

Figure 1. A schematic representation of the cell cycle of *S. cerevisiae*. (López-Malo, 2013).

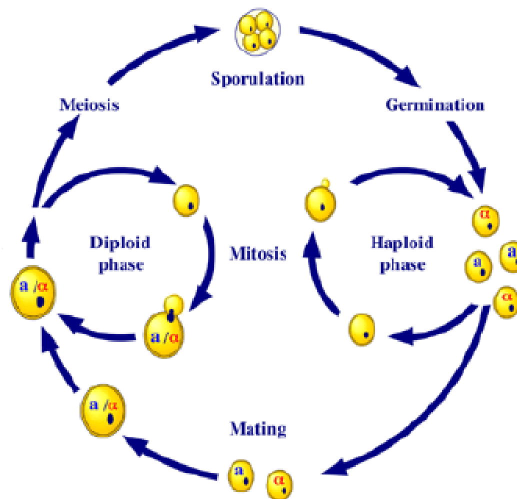

Figure 2. A schematic representation of the life cycle of *S. cerevisiae*. (López-Malo, 2013).

Yeast was probably domesticated during the Neolithic times and has for thousands of years been used for its fermenting properties for food

preservation and alcohol production. Before Louis Pasteur discovered the role of brewer's yeast in alcoholic fermentation, it was probably only known from its activity, and most manmade fermentations were spontaneous. The peculiar trait of the budding yeast *S. cerevisiae* to ferment even under aerobic conditions has made it the preferred organism for bread, alcoholic beverage and also lately for industrial bioethanol production (Oshoma et al., 2015; Ruyters et al., 2014; Sakihama et al., 2014). Given their above properties, the definition of yeast species becomes a complex question, as is often the case in organisms in which clonal propagation dominates sexual genetic exchanges. In the *Saccharomyces* genus clade, in which this question has been most thoroughly addressed, the reduced meiotic fertility of heterospecific hybrids fulfils the most classical criterion used to define species. However, several mechanisms combine to create this postzygotic barrier, not all of which are applicable to other yeast clades.

The polyploidy nature, the capability of exchanging genetic material, the high genetic variability and the complexity of evolution in *Saccharomyces* yeasts, make species definition very troublesome. Thus, the complex diversity of the genus *Saccharomyces*, including pure, hybrid and introgressed strains, makes species definition difficult and classification controversial. According to the fifth edition of 'The Yeast, a taxonomic study' (Vaughan-martini and Martini, 2010), the genus *Saccharomyces* is composed of eight species: *S. arboricolus, S. bayanus, S. cariocanus*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. pastorianus*. Although several studies have shown that *S. pastorianus* comprises a group of alloploid hybrid strains that originated from *S. cerevisiae* and a cryotolerant species similar to *S. bayanus*, the last systematic revision maintained the species status for *S. pastorianus*. In a recent study, Libkind et al., 2011 isolated and characterized a new *Saccharomyces* species, named *S. eubayanus,* and associated it with *Nothofagus* spp. trees in Patagonia (Argentina). As the draft genome sequence of this species was closely related to the non *S.*

cerevisiae portion of *S. pastorianus* (average divergence of 0.44%), the authors proposed *S. eubayanus* to be the previously mentioned *S. bayanus*-like donor of this subgenome in *S. pastorianus* hybrids.

The ecology of *Saccharomyces* species is diverse. Several species of this genus have been found only in natural environments, which is the case of *S. mikatae* (in partially decayed leaves), *S. kudriavzevii* (decayed leaves, soils and oaks), *S. arboricolus* (oak trees), *S. cariocanus* (*Drosophila* sp.) and *S. eubayanus* (bark); whereas *S. cerevisiae*, *S. paradoxus* and *S. bayanus* (including the actual *S. uvarum*) have been found associated to both natural and biotechnological environments. *S. cerevisiae* is the predominant species in most industrial fermentative processes such as dough production, brewing, winemaking, cider production, sake, cachaça, and also in traditional fermented beverages around the world (pulque, masato, chicha, sorghum beer, palm wine, etc.) (Ibáñez et al., 2014). The metabolic activities of *S. cerevisiae* have been exploited by men since agriculture developed, and, from the economic point of view, it can be considered the most important microorganism.

Although *S. cerevisiae* is the most important species from a biotechnological viewpoint, additional genomic variation can arise from interspecific hybridization, which can occur between two or more *Saccharomyces* species (Barrio et al., 2006; Dujon, 2010). Some examples are *S. cerevisiae*-*S. kudriavzevii* hybrid wine and brewing yeasts(González et al., 2008; Lopandic et al., 2007; Peris et al., 2012a, 2012b), *S. cerevisiae*-*S. uvarum* hybrid cider and brewing yeast (Masneuf et al., 1998; Rainieri et al., 2006), and the most well-known hybrid, the lager yeast *S. pastorianus*, which is an interspecific hybrid between *S. cerevisiae* and the recently described *S. eubayanus* (Libkind et al., 2011). Moreover other species like *S. paradoxus* and *S. bayanus* and natural yeast hybrids between species of the genus *Saccharomyces,* such as *S. cerevisiae* x *S. kudriavzevii* (González et al., 2008, 2006; Peris et al., 2012b) and *S. cerevisiae* x *S. bayanus* var. *Uvarum* (Demuyter

et al., 2004; Zhang et al., 2015), have also been associated with biotechnological environments (Figure 3).

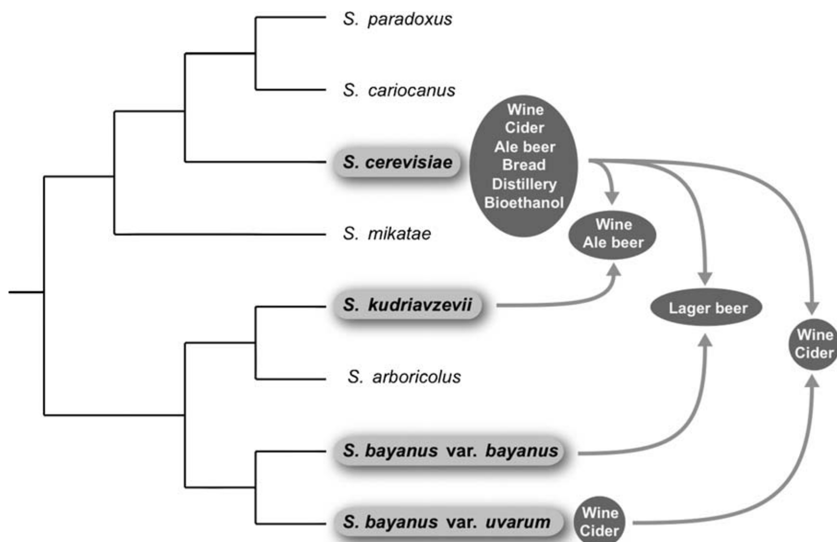

Figure 3. Schematic diagram of the phylogenetic relationships between the Saccharomyces species and their industrial specialization. The species involved in industrial processes and/or in hybrids are boxed in light grey. The products of industrial processes involving the hybrids and non-hybrids are boxed in dark grey. The arrows correspond to hybrids (Dequin and Casaregola, 2011).

1.2 The genus *Saccharomyces*: Biotechnological and alcoholic fermentation interest.

The molecular evidence for the production of fermented beverages dates back to 7000 BC from the Neolithic village of Jiahu in China (McGovern et al., 2004). However, Pasteur was the first to recognize yeast as a living organism (Figure 4) that actively converts sugars into ethanol and carbon dioxide under concomitant formation of acetate, succinate and glycerol (Pasteur, 1858). Few years later, Kühne in 1878 emphasized that enzymes were inseparably associated with living cells, which at the time were called

'ferments'.

Figure 4. Yeast globules as depicted in 1680 by Antonie van Leeuwenhoek. (Hazelwood, 2009).

Alcoholic fermentation is the principal metabolic process in winemaking and is defined as the biotransformation of grape sugars, including glucose and fructose, into ethanol and carbon dioxide (CO_2). The principal responsible for this transformation is the yeast. Yeasts are strongly inclined to perform alcoholic fermentation under aerobic and anaerobic conditions (van Dijken et al., 1993). Interestingly the alcoholic fermentation can occur in the presence of oxygen when there is a high concentration of sugars, above 20 g/L, because aerobic respiration is blocked (Jack T. Pronk, 1996). Yeast species belonging to the *Saccharomyces* genus have a number of unique characters not found in other yeast genera. While a majority of yeast cannot grow in the absence of oxygen, the majority of *Saccharomyces* species can survive without any oxygen by using the fermentation process (Sicard and Legras, 2011). This phenomenon is named Crabtree effect. So, for instance in *S. cerevisiae*, glucose and fructose are metabolized to pyruvate via glycolitic pathway. Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol. One molecule of glucose

or fructose yields two molecules of ethanol and CO_2, theoretically 180 g sugar are converted into 92 g ethanol and 88 g CO_2. However, this only could be expected in absence of any yeast growth, production of other metabolites and loss of ethanol as vapor.

From the genus *Saccharomyces* only the species *S. cerevisiae*, *S. bayanus-S. uvarum* and *S. pastorianus* are associated with anthropic environments, whereas the rest are mostly isolated from natural environments. In the next paragraph we will analyses the most relevant species in biotechnology and alcoholic fermentations.

The species ***S. cerevisiae*** is the predominant species responsible for alcoholic fermentation in the production of wine, ale-brewing, sake and different traditional fermented beverages. It is the yeast best adapted to grow at high temperatures within the *Saccharomyces* genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures (Salvadó et al., 2011). Also is the species with the highest ethanol resistance (Arroyo-López et al., 2010). Besides its traditional role in alcoholic fermentation, among the genus *Saccharomyces* species *S. cerevisiae* is also the most used for the production of bioethanol from hexoses, having had regularly employed highly fermenting strains (Greetham et al., 2014; Wimalasena et al., 2014). In the next paragraph we will analyses those other species which have been used as alternatives to the species S. cerevisiae in biotechnological applications.

The species ***S. paradoxus*** is also being used for fermentation of Croatian wines (Redžepović et al., 2002) and is the closest relative to *S. cerevisiae*, according to phylogenetic reconstructions (Scannell et al., 2011). This wild yeast is natural specie worldwide distributed with a fortuitous presence in fermentation processes although their strains have been isolated from natural environments usually associated with tree exudates (Naumov et al., 2000).

S. kudriavzevii species has been mainly isolated in natural

environments, like decaying leaves (Naumov et al., 2000) or oak barks (Lopes et al., 2010; Sampaio and Gonçalves, 2008). This specie participates in hybrid formation with *S. cerevisiae* and *S. bayanus* species, which are present in industrial fermentations in central Europe (Belloch et al., 2009; González et al., 2008, 2007, 2006; Lopandic et al., 2007; Masneuf et al., 1998; Peris et al., 2012a, 2012b; Sipiczki, 2008). Physiological characterization of *S. kudriavzevii* strains has showed up its cryotolerance, growing quite well at low temperatures (10-15ºC) (Belloch et al., 2008; Salvadó et al., 2011; Tronchoni et al., 2014).

The cryophilic ***S. bayanus***, although has been found in natural habitat in Far East Asia together with strains of *S. cerevisiae* and *S. paradoxus*, also appears associated to different fermentation processes: winemaking (Demuyter et al., 2004; Le Jeune et al., 2007), cider production (Coton et al., 2006), brewing, and as grape must contaminants. The type strain of this specie, originally isolated from beer, has been described as a hybrid possessing also nuclear genome from *S. cerevisiae* (De Barros Lopes et al., 2002; Nguyen and Gaillardin, 2005), which led to the proposal of the reinstatement of ***S. uvarum***, a former taxon included in *S. bayanus*, as a distinct specie (Nguyen and Gaillardin, 2005) or as a different variety within *S. bayanus* (Naumov et al., 2000a). In recent study Pérez-Través et al. (2014) showed that the 'uvarum' group presents high intraspecific homogeneity and that *S. bayanus* strains has different levels of homozygosity, hybridization and introgression. All *S. bayanus* stains are hybrids between *S. uvarum* and *S. eubayanus* and no pure *S. bayanus* var. *bayanus* strain was identified.

Comparison between ***S. uvarum*** and *S. cerevisiae* reveals that the former is more cryotolerant, produces smaller acetic acid quantities, lower amounts of amyl alcohols, but higher amounts of glycerol, succinic acid, malic acid, isobutyl alcohol, isoamyl alcohol and numerous secondary compounds (Sipiczki, 2008). Wines produced by *S. uvarum* strains have a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al., 2006). On the other hand, *S. uvarum* is less common and appears mainly

in fermentations at low temperatures (Demuyter et al., 2004; Masneuf-Pomarède et al., 2010).

In the wild, as well as in brewing and wine-making, both homoploid and allopolyploid hybrid yeast have been isolated whose genomes are wholly or partly derived from two or more different members of the *Saccharomyces* genus as we described before (see Figure 3). These *Saccharomyces* species can also be mated in the lab to create de novo interspecific hybrids (Dunn et al., 2013; Pérez-Través et al., 2012). Natural **hybrids of *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii*** conducting wine fermentations have been characterized by genetic approaches (Belloch et al., 2009; González et al., 2008, 2006; Naumov et al., 2000; Sipiczki, 2008). Hybrids between *S. cerevisiae* and *S. kudriavzevii* can ferment musts at low temperatures with especially good fermentation performance at 14, 18 and 22 °C (González et al., 2007). Physiological data suggest that *Saccharomyces* hybrids might have inherited the ability to grow at high temperatures (30-37°C) and ethanol tolerance from their *S. cerevisiae* parental and ability to grow at low temperatures (10-16°C) from their *S. bayanus* and *S. kudriavzevii* parental. Similar studies have been done with **hybrid between *S. cerevisiae* and *S. uvarum*** indicated that they have the cryotolerant capability of *S. uvarum* and the ethanol tolerance of *S. cerevisiae*.

The rest of the species are not associated with fermentative environments. *S. arboricolus* was found associated with the bark of two tree species of the family *Fagaceae* in different regions of China (Wang and Bai, 2008), *S. cariocanus* was isolated from a fruit fly (Drosophila sp.) in Brazil (Naumov et al., 2000b), *S. mikatae* was isolated from soil and decaying leaves in Japan (Naumov et al., 2000b) and *S. eubayanus* was found in in Nothofagus (Southern beech) forests in Patagonia (Libkind et al., 2011).

2. Yeasts and winemaking

The earliest reports about grapevine domestication date from 7000-4000 BC from a region between the Black Sea and Iran and the first evidence of winemaking is associated with Mesopotamia 5400-5000 BC from there vineyards and wine production expanded around the world (Chambers and Pretorius, 2010; Sicard and Legras, 2011). Wine fermentation technologies expanded from Mesopotamia towards Europe and subsequently spread to the "New World" and over time, wine has influenced geography, economics, archeology, history, mythologies and religions, arts and traditions, law and medicine. Today, this beverage has a unique place in most societies, with tremendous economic and cultural value (McGovern et al., 2004).

The grape microbiota varies according to the variety; temperature, rainfall and other climatic influences; soil, fertilization, irrigation and viticultural practices. Species of the genus *Hanseniaspora spp.* are predominant on the surface of grape berries, accounting for 50 – 75% of the total of the yeast population. Numerically less prevalent than these apiculate yeasts are species of *Candida* (e.g. *C. zemplinina, C. stellata*, *C. pulcherrima*), *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* (Fleet, 2008; Querol and Fleet, 2006). Contrary to popular belief, fermentative species of the genus *Saccharomyces* predominantly *S. cerevisiae*, occur at extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries (< 0.1%) and vineyards soils, while damaged grapes are believed to be an important source, providing inocula of 10^2 – 10^3 cells/mL (Mortimer and Polsinelli, 1999). While the fermentation process is occurring, the establishment of anaerobic conditions, the antimicrobial activity of sulphur dioxide added, the depletion of nutrients and the increasing levels of ethanol enlarge the selectivity of the medium. The non-*Saccharomyces* yeasts present in grape juice described before, such as *Hanseniaspora* (*Kloeckera*), *Candida*, *Pichia*, *Kluyveromyces* and *Metschnikowia* could proliferate to final populations of about 106–107 cfu/ml,

and started to decline by mid-fermentation, when the ethanol production by *S. cerevisiae* exceeds 5–7%. Ethanol production by *S. cerevisiae* is the major factor affecting the growth of non-*Saccharomyces* yeasts (Heard and Fleet, 1988). Besides *S. cerevisiae* is the most important species of the genus *Saccharomyces*, other species of this genus have also an important role during wine making as we described before, like *S. uva*rum and *S. kudriavzevii* as part of hybrids.

During the alcoholic fermentation and the industrial preparation for winemaking, *S. cerevisiae* and the other species of the genus *Saccharomyces* are subjected to a number of adverse conditions, the most important being osmotic and ethanol stresses (Pretorius and Høj, 2004; Pretorius, 2000; Rainieri and Pretorius, 2000). Other important stresses come from the aerobic fed-batch growth for biomass production where exposed to oxidative stress (derived mainly through the aerobic metabolism of yeasts), hyperosmotic stress, ionic stress, raised temperatures, nutrient limitation and starvation. At the end of this stage, cells are affected by starvation, hypo-osmotic stress and desiccation to obtain dry active yeast to be inoculated into the must. At this point, cells are affected by rehydration and by hyperosmotic stress due to the high sugar content in the must. Extreme conditions like these lead to a reduction in growth speed and survival rate, and therefore tend to reduce fermentation efficiency, these effects depending on the severity of the vinification procedures. The better and faster yeast strain is able to adapt to changes in the environment, the higher the probability of being the dominant strain during the winemaking process.

A typical wine fermentation (Figure 5) comprises a lag phase, which lasts for several hours, a short growth phase of 24–36 h, followed by a stationary phase, during which most of the sugar (between 50 and 80%) is fermented. During this phase, yeast activity continually decreases, although the viability levels remain high, generally over 90%, until the sugar is exhausted. The most desirable traits of wine yeasts include the rapid and

complete degradation of sugars into ethanol and CO_2 to provide metabolites and aroma compounds that positively impact the sensory balance of wine, without producing undesirable compounds (Dequin and Casaregola, 2011; Pretorius, 2000). Numerous fermentative by products (glycerol, carboxylic acids, aldehydes, higher alcohols, esters, carbonyl compounds, sulfur com- pounds, etc.) are derived from the degradation of sugars, amino acids and fatty acids, and yeasts can also transform grape precursors to release varietal aromas (monoterpenes and thiols) (Swiegers et al., 2005).

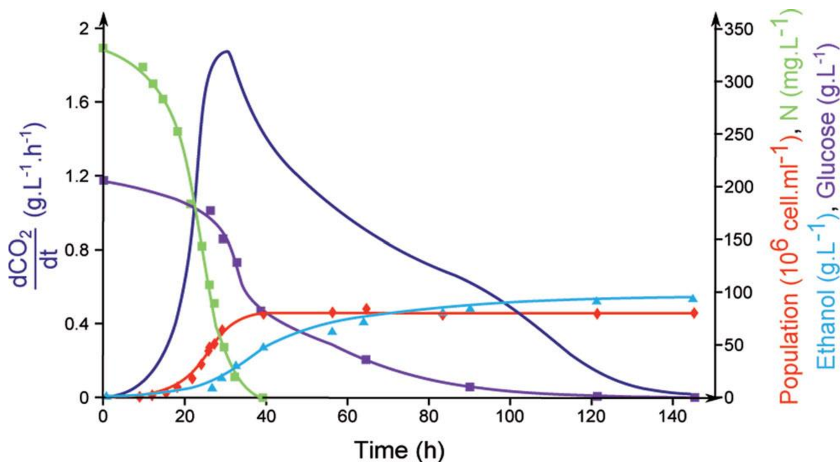

Figure 5. Main phases of wine fermentation. Evolution of the main fermentation parameters during wine fermentation on a synthetic medium containing 200 g L−1 glucose/fructose and 330 mg L−1 assimilable nitrogen, with the commercial wine strain EC1118 at 24◦C. Dark blue: fermentation rate; light blue: ethanol; red: cell number; green: nitrogen; and purple: sugars. (Marsit and Dequin, 2015).

The fermentative yeasts are able to grow on substrates characterized by high sugar and ethanol content, low pH, high sulphur dioxide concentrations and remains of fungicides. Other important aspect during wine fermentations is the temperature during the fermentation. Actually, thanks to the control of fermentation temperature by the wine industry, low

temperature alcoholic fermentations are becoming more common due to the aim to produce white and "rosé" wines with more pronounced aromatic profile. Wines produced at low temperatures (10-15ºC) are known to develop certain characteristics of taste and aroma (Gamero et al., 2013; Novo, 2003). Low temperatures increase not only the retention but also the production of some volatile compounds. Another interesting aspect is that low temperatures notably reduce the growth of acetic and lactic acid bacteria and they can facilitate the control of alcoholic fermentation (Ribéreau-Gayon et al., 2006). However, the optimal growth temperature of the wine yeast *S. cerevisiae* is far greater than these temperatures. Thus, 12ºC is restrictive and increases the risk of stuck and sluggish fermentations. Low temperature fermentations have some notably disadvantages i.e. increase the duration of alcoholic fermentation, decrease the rate of yeast growth and modify the ecology of wine fermentation (Torija et al., 2003), as well as produce longer lag-phases with the risk of prevailing non-*Saccharomyces* strains, modify the metabolic activity of yeast, with different production of secondary metabolites and modify the lipid membranes, with the consequent modification in the transport of compounds.

Nowadays, wine companies are looking for new fermenting yeast strains that besides to be able to perform winemaking at low temperatures, generate low alcohol amount while increasing glycerol concentration thus solving the astringency problem. Moreover, new yeast strains are also required to provide more aromatic wines (Pretorius et al., 2012). As we explain before, other species like *S. uvarum* and hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* are well adapted to ferment at low temperature, produce higher amounts of glycerol, less acetic acid and higher amounts of higher alcohols with regard to reference strains of *S. cerevisiae* (Arroyo-López et al., 2010, 2009; Gamero et al., 2013; González et al., 2007; Tronchoni et al., 2009).

Glycerol is the most important by-product of alcoholic fermentation after ethanol and carbon dioxide. It is produced in wine at concentrations of 2–11 g/l, depending on yeast strain; grape must composition and fermentation conditions. In *S. cerevisiae*, this polyol plays two major roles in physiological processes: it controls intracellular redox balance and combats osmotic stress (Hohmann et al., 2007). It has also been related to low-temperature tolerance in yeasts (Izawa et al., 2004). Glycerol is the principal solute accumulating in yeast cells exposed to hyperosmotic stress, and this accumulation helps to increase internal osmolarity, thereby preventing the rapid diffusion of water from the cell into the surrounding medium. The oenological importance of glycerol lies in its contribution to wine quality by providing slight sweetness, smoothness and fullness, reducing wine astringency (Remize et al., 1999).

3. Genetic diversity in *Saccharomyces cerevisiae*.

Although the basic principles underlying genetic variation are known, the extent of genetic biodiversity in *S. cerevisiae* strains has only recently been elucidated. Strains of *S. cerevisiae* collected from ecologically and geographically diverse sources typically demonstrate genetic divergence associated with habitat type rather than geographic origin (Fay and Benavides, 2005b; Legras et al., 2007; Liti et al., 2009; Novo et al., 2009; Schacherer et al., 2009). Strains of *S. cerevisiae* associated with vineyards and wine production referred to as ‘wine’ strains, often form a genetically differentiated group that is separate from ‘wild’ strains isolated from soil and oak tree habitats, and strains from other fermentations, such as palm wine and sake (Fay and Benavides, 2005b; Legras et al., 2007; Liti et al., 2009; Schacherer et al., 2009). The genetic divergence between wine and non-wine strains combined with an observed reduction in genetic diversity within

wine strains suggests that wine strains were domesticated from wild *S. cerevisiae* (Fay and Benavides, 2005b).

Legras et al., (2007) analyzed the genetic diversity among 651 strains from 56 different geographical origins, worldwide. Their genotyping at 12 microsatellite loci revealed 575 distinct genotypes organized in sub- groups of yeast types, i.e. bread, beer, wine, sake. Almost 95% of wine yeast strains are found in the upper part of the tree (Figure 6), which also includes cider strains.

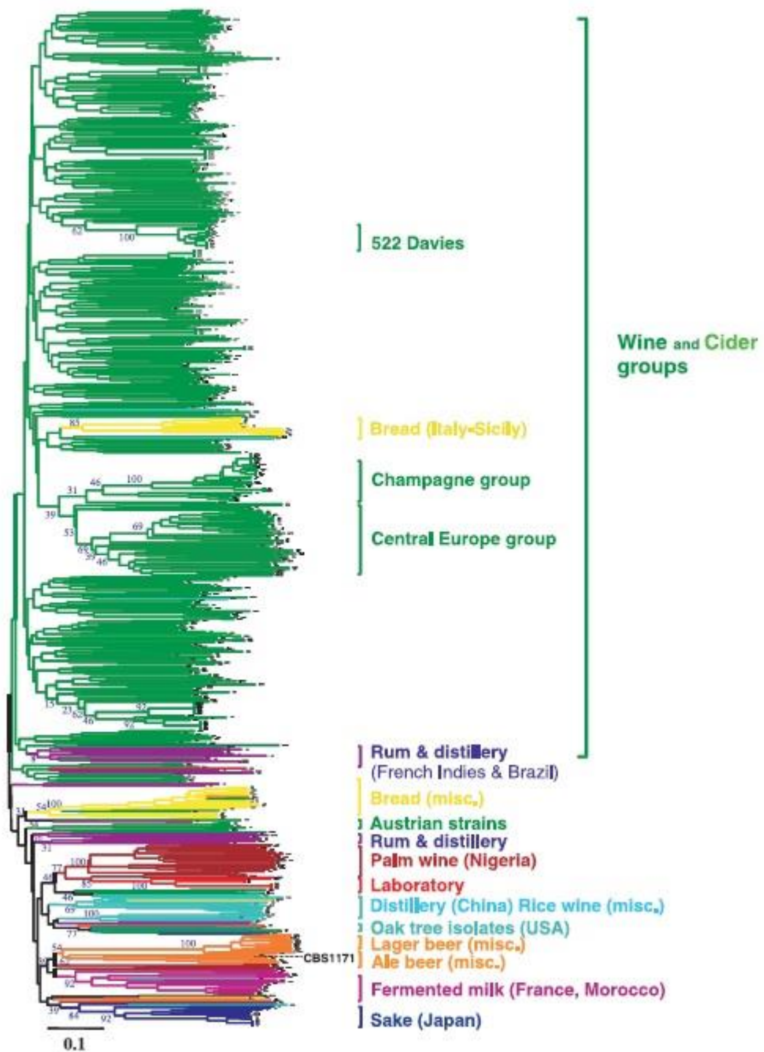

Figure 6. Neighbour-joining tree showing the clustering of 651 yeast strains isolated from different sources. (Legras et al., 2007)

Industrial or grape strains are scattered all over this clade so that it is not possible to differentiate them from other wine strains. These results suggest intimate association between man and wine yeast across centuries and that yeast followed man and vine migrations as a commensal member of grapevine flora. In another large study, Liti et al., (2009) investigated one to

four folds or more coverage of the genome sequences of over seventy isolates of *S. cerevisiae* and its closest species, *S. paradoxus*. *S. cerevisiae* isolates showed less differentiation and were comparable to a single *S. paradoxus* population. Then, was demonstrated that the population structure of *S. cerevisiae* consists of a few well-defined geographically isolated lineages and many different mosaics of these lineages (Figure 7), supporting the idea that human influence provided the opportunity for cross-breeding and production of new combinations of pre-existing variation.

From a set of *S. cerevisiae* isolates with worldwide origin, five distinct lineages were revealed based on their technological and geographic origin (West African, Malaysian, North American, Sake and European/wine), and many strains with mosaic genomes resulting from crosses between these well-defined lineages were identified (Figure 7).

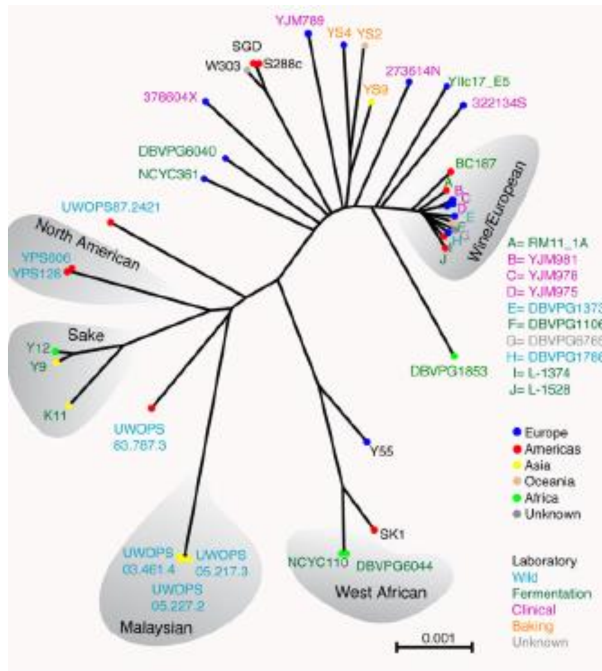

Figure 7. *S. cerevisiae* strains with clean lineages highlighted in grey, with color indicating source (name) and geographic origin (dots). (Liti et al., 2009).

Schacherer et al., (2009) reports a nucleotide-level survey of genomic variation in a diverse collection of 63 *S. cerevisiae* strains sampled from different ecological niches (beer, bread, vineyards, immunocompromised individuals, various fermentations and nature) and from locations on different continents. Analyses showed at least three distinct subgroups based on the source from which the strains were isolated (Figure 8). In this work once again most of the wine strains appeared as members of a single well-defined subpopulation. The wine strains show the lowest level of polymorphism among the groups, as well as an excess of low-frequency SNPs, consistent with a bottleneck during domestication. This subpopulation also included a number of strains collected from distilleries, nature (soil, cocoa beans, prickly pear and *Tuber magnatum*) and clinical sources, indicating that these strains derived from domesticated wine strains, which transited out of this group to

other human-associated fermentations as well as back into nature and therefore escaped their human-manufactured environment. Because these wine strains were collected from dispersed locations, this observation provided evidence of a single domestication event of yeast for winemaking, followed by human-associated migration of wine yeast all over the world (Schacherer et al., 2009).

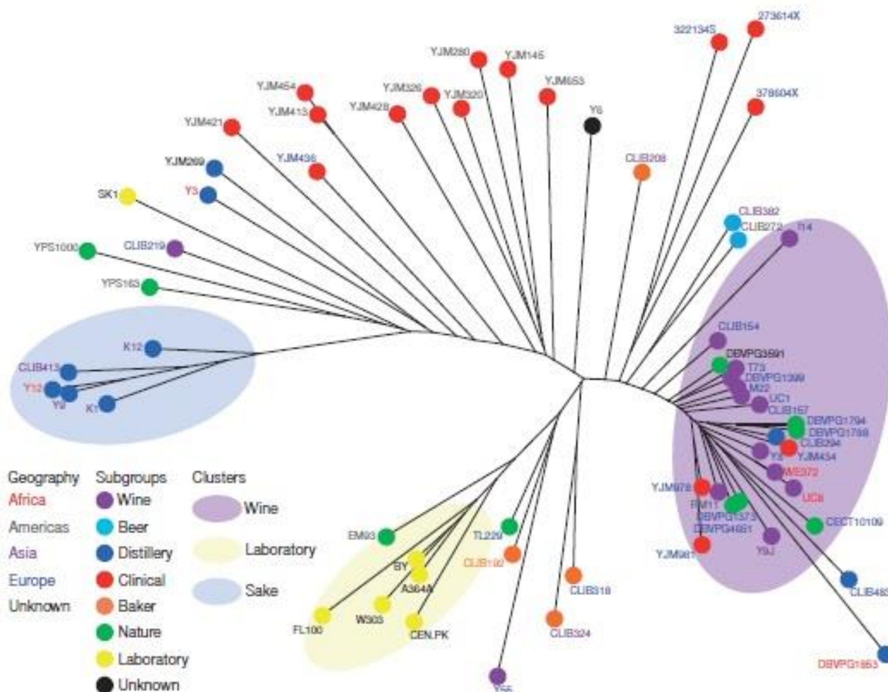

Figure 8. Neighbour-joining tree of 63 *S. cerevisiae* strains. Font color of strain name denotes geographical origin and circle color denotes ecological niche as specified in the key. (Schacherer et al., 2009).

Recently genome sequencing studies shows that half of the *S. cerevisiae* strains sequenced fell into a number of distinct lineages (Wang et al., 2012) (Figure 9). Genetic variants within these lineages are mostly unique

to a sub-population and absent in others and evenly distributed across the genome. These strains do not strictly follow geographic boundaries, for example, wine strains from Europe, Australia, Chile and New Zealand share recent ancestry and reflect human migration history (Legras et al., 2007; Liti et al., 2009). Although full genome information is not yet available for Chinese isolates of *S. cerevisiae*, they appear to exhibit strong population structure, with essentially double the combined amount of genetic variation identified in *S. cerevisiae* isolates sampled from the rest of the world (Wang et al., 2012).

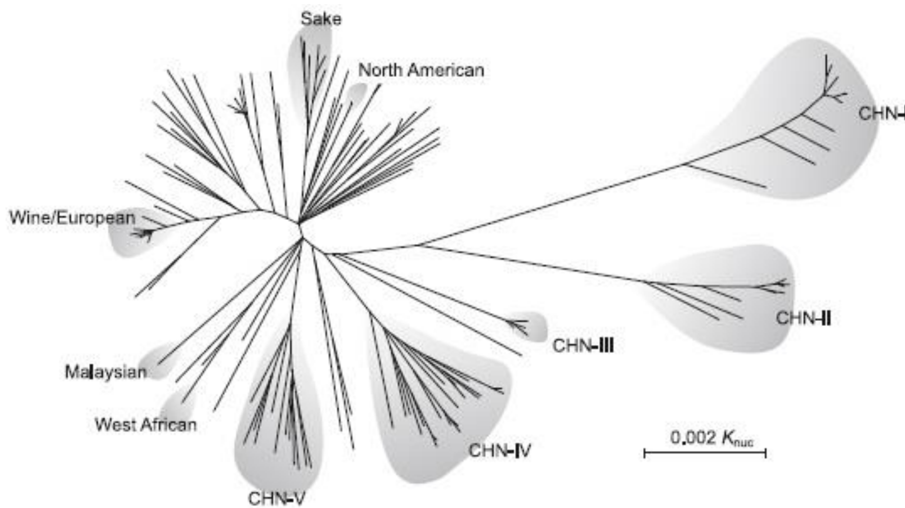

Figure 9. A phylogenetic tree of *S. cerevisiae* isolates. The main worldwide and Chinese lineages (denoted, CHN I-V) are highlighted. (Wang et al., 2012).

These genomic diversity studies show that specifically, wine strains form a distinct phylogenetic group with low diversity. However, diversity within *S. cerevisiae*, the main yeast associated with human activity, was at least partly linked to their industrial application and these studies showed that *S. cerevisiae* as a whole is not domesticated and that the population structure of this species, at least partially, reflects different ecological niches (Wang et

al., 2012). Recently it has been identified the source of the natural wild *S. cerevisiae* strains phylogenetically closer to the wine yeasts (Almeida et al., 2015). This wild population is associated with oak trees in Europe and surprisingly was not responsible for transmitting to the wine yeast some genes associated to winemaking, which were obtained from other species through horizontal transfer.

Moreover, genetic analysis of these wild and industrial strains revealed that the genetic diversity within industrial strains is rather limited compared to the full spectrum of natural biodiversity. Together, these observations suggest that the fermentation industry currently relies on only a very small fraction of the available genetic diversity of *S. cerevisiae*, ignoring a huge pool of unexplored wild strains. To exploit this huge natural diversity, it is necessary to deeply explore diverse wild yeast collections for industrially relevant traits. Indeed, continuous evolution and adaptation of indigenous yeast strains to their environment have equipped these strains with phenotypes valuable for production of wine (Steensels et al., 2014; Tosi et al., 2009).

4. Glycerol metabolism in yeast.

4.1 Glycerol and wine.

The wine industry is very interested in the development of wine yeasts that generate wines with improved organoleptic properties (Pretorius, 2000; Rainieri and Pretorius, 2000). Among other phenotypes, yeast strains with enhanced glycerol production are in demand. Glycerol is also associated with smoothness, sweetness and complexity in wines, but the variety of grapes and the wine style will determine the extent to which glycerol impacts in these properties (Jolly et al., 2014).

However, in the actually one of the most important contributions of the glycerol in winemaking is associated with the global climate change. The global climate change has different effects on vine grapes, which include lower acidity, altered phenolic maturation and tannin content, and notably higher sugar levels by the time of harvest, especially in warm climates. Early harvest is not a good alternative to avoid high sugar content in grape must, since it would prevent the optimal phenolic maturity and aromatic complexity required to produce the well-structured and full body wines currently demanded by consumers. Besides, excess ethanol compromises 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, can reduce wine alcohol levels and/or astringency increasing the higher glycerol yields.

4.2 Overview of glycerol metabolism.

Under anaerobic conditions, *S. cerevisiae* generates the free energy (ATP) that is needed in assimilation by fermentation of glucose to ethanol. One of the functions of glycerol in *S. cerevisiae* is to establish an intracellular redox balance. Alcoholic fermentation in itself is redox-neutral, as glycolytically formed NADH is re-oxidized via the reduction of acetaldehyde to ethanol (Bakker et al., 2001). However, assimilatory processes result in a net formation of NADH, which requires glycerol formation to maintain a redox balance. The availability of intracellular NADH can therefore influence the formation of glycerol (Figure 10).

The main genes responsible of the glycerol synthesis are *GPD1* and *GPD2*. Although the two isoenzymes encoded by the *GPD1* and *GPD2* genes catalyze the same reaction they have different metabolic functions. Gpd1p functions during osmotic stress induced glycerol production (Albertyn et al., 1994; Ansell et al., 1997) and appears to be responsible for the

dehydrogenase activity in the glycerol-3-phosphate shuttle (Larsson et al., 1998). The primary role of Gpd2p is redox mediated dihydroxyacetone phosphate (DHAP) reduction during anaerobicity or (in)directly inhibited respiratory activity (Ansell et al., 1997; Björkqvist et al., 1997; Epstein et al., 2001).

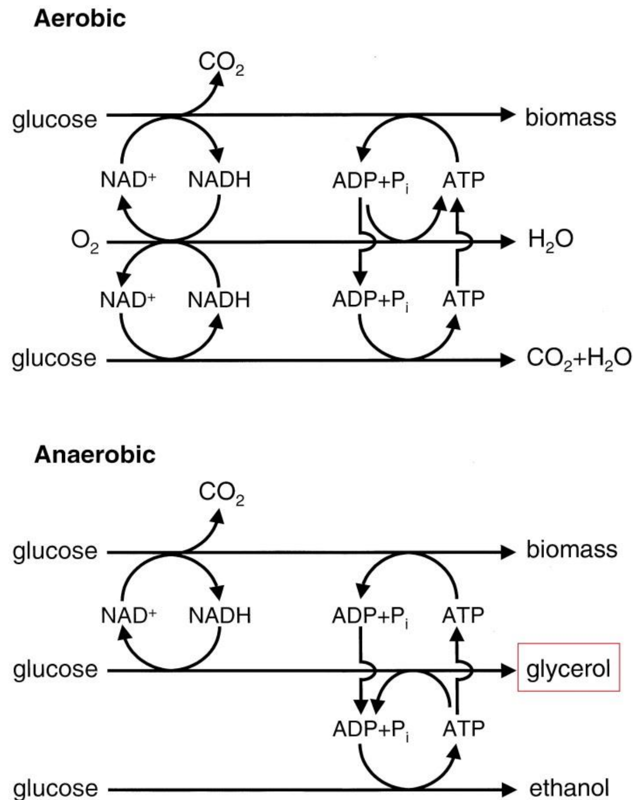

Figure 10. Schematic overview of NAD/NADH turnover in respiring (top) and fermentative (bottom) cultures of Saccharomyces cerevisiae. Depending on the concentrations of sugar and oxygen, intermediate situations are possible. In addition to biomass formation, production of low molecular mass metabolites, such as acetate, pyruvate, acetaldehyde or succinate, may affect turn-over of NAD/NADH. (Bakker et al., 2001)

Under anaerobic or respiratory deficient conditions, Gpd2p is proposed to help establish mitochondrial redox balance by driving the ethanol-acetaldehyde shuttle through oxidation of NADH in the

mitochondrial intermembrane space and the distinct intracellular localization of Gpd1p and Gpd2p is compatible with a different contribution to redox-driven glycerol production (Valadi et al., 2004). However, increased Gpd activity also led to increased amounts of acetic acid in the fermentation product. This was probably owing to rectification by one or more of the five aldehyde dehydrogenase isozymes of a redox imbalance that resulted from excessive Gpd driven oxidation of NADH. Aldehyde dehydrogenase isozymes drive the oxidation of acetaldehyde to acetic acid with concomitant reduction of co enzymes NAD+ or NADP, depending on which isozyme is involved (Eglinton et al., 2002). This might be good for a yeast cell struggling with an imposed redox imbalance, but an increase in acetic acid production is not good news for winemakers because the excess is not desirable in wine. This problem has been alleviated by knocking out one of the five aldehyde de hydrogenase isozymes, ALD6 (Eglinton et al., 2002).

Some authors discusses several metabolic engineering strategies that have been explored to generate wine yeasts that can divert some carbon metabolism away from ethanol production (Kutyna et al., 2010), with the aim of decreasing ethanol yields during vinification. Candidate genes that are likely to influence ethanol yields can be identified from a range of sources and then manipulated and cloned as required. Several works have targeted the glycerol-3-phosphate dehydrogenase isozymes GPD1 and GPD2, which divert carbon from glycolysis to glycerol production (Lopes et al., 2000; Michnick et al., 1997; Remize et al., 1999). However, while redirecting yeast metabolism towards increased glycerol production using GM approaches is relatively easy to achieve, the use of GM strains for winemaking in many parts of the world is not permitted, and consumer acceptance of GMOs remains a contentious issue (Grossmann et al., 2010). Thus, no-GMO approaches, such as adaptive evolution, must be relied upon (Kutyna et al., 2012). Adaptive evolution, based on long-term adaptation of yeast under environmental or metabolic constraints, has been used to improve yeast

strains for biotechnological applications, including winemaking (Kutyna et al., 2012; Mcbryde et al., 2006; Stanley et al., 2010; Wisselink et al., 2009). However, there has not previously been any description of an evolutionary approach that successfully generated strains with substantially reduced ethanol yield.

Several studies highlighted the interest in non-*Saccharomyces* yeasts to produce wine with a lower ethanol concentration, when used in a sequential inoculation regimen with an *S. cerevisiae* wine strain (Contreras et al., 2014; González-Royo et al., 2015; Morales et al., 2015; Wang et al., 2015, 2014). Therefore, despite the recent interest in non-*Saccharomyces* yeasts to reduce the ethanol content of wines and to improve their aromas, most of these species are aerobic and are easily replaced by *S. cerevisiae* during wine fermentations. But probably their major disadvantage is the high production of acetic acid, an undesirable property in wines and more studies are required.

On the other hand, other species of the *Saccharomyces* genus (*S. uvarum* and *S. kudriavzevii*) and hybrids among species exhibit interesting physiological properties, such as adaptation to lower fermentation temperatures, lower ethanol production and **increased glycerol production** without an increase in the acetic acid levels of wines, thus being of interest to solve the new winemaking challenges.

4.3 Glycerol transport and osmoregulation.

Transmembrane transport of glycerol is the first step for glycerol utilization, but is also very important for other things, like the efflux of glycerol after hypo-osmotic shock, or the capture of glycerol from the medium for osmotic balance purposes (see Figure 11). A ubiquitous strategy in osmoregulation probably used by all cells is the accumulation of one or several compatible solutes to control the osmolarity of the cytosol. The solutes

used are species specific and range from ions to amino acids and their derivatives, as well as sugars and sugar alcohols. The unicellular eukaryotic model organism (baker's yeast) uses glycerol (Brown, 1976). Thus, was identified that in yeasts glycerol serves as a compatible solute in situations of high extracellular osmolarity (Nevoigt and Stahl, 1997).

The diffusion of glycerol through the plasma membrane is relatively slow and for a long time it was believed that the extent of diffusion depends on the composition of membrane fatty acids and the concentration gradient (Watanabe, Y. and Takakuwa, 1987). Recently it was revealed that translocation of glycerol across the plasma membrane is mediated by a newly identified channel protein (encoded by YFL054c) (Beese-sims et al., 2011; Oliveira et al., 2003). Facilitator channels are an important factor in the ability to grow at high osmotic pressure. In *S. cerevisiae*, such a glycerol channel protein is embedded in the plasma membrane (Fps1p) (Beese-sims et al., 2011). On one hand the glycerol facilitator channel closes rapidly in response to high external osmolarity, contributing to the accumulation of the intracellular glycerol (Tamás et al., 1999) and on the other hand, it allows for regulated release of glycerol under osmotic downshift (Luyten et al., 1995). Fps1p also mediates glycerol uptake, albeit at a lower rate than export (Oliveira et al., 2003). A member of the major facilitator family (Stl1p), amongst which are sugar transporters, was found (Ferreira et al., 2005) to enable proton symport of glycerol into cells during an immediate response to osmotic shock. Thus considering, a full understanding of the glycerol transport and how it is involved in diverse yeast species and strains of importance biotechnological is also very important.

In summary the glycerol accumulation in the cell occurs when active Hog1 via translocation to the nucleus, induces the transcription of *GPD1* activating its production and regulating the expression of other genes. Additionally, the accumulation is facilitated by closure of the Fps1 channel protein upon stress. These mechanisms have been extensively studied and

are summarized in Figure 11.

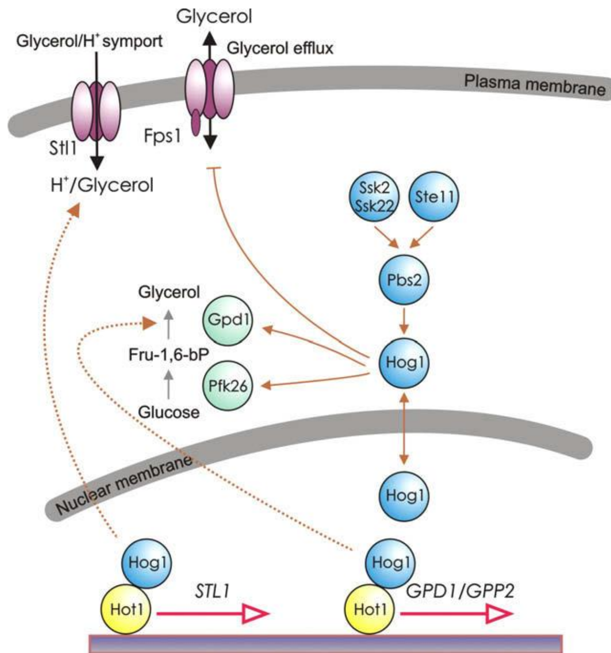

Figure 11. At the level of gene expression the capacity for glycerol uptake and production are increased by Hog1 and may participate in the control of the glycerol efflux channel Fps1. (Hohmann, 2009).

Fps1 is a member of the major intrinsic protein (MIP) family of channel proteins. Previous studies provide both genetic and biochemical evidences that aquaglyceroporins Fps1 functions as a homotetramer to regulate glycerol transport in yeast. Tamás et al. (1999) showed that increased external osmolarity induces Fps1 closure, whereas decreased osmolarity causes channel opening, both within seconds of the change in external osmolarity. This channel is required for survival of a hypo-osmotic shock when yeast cells have to export glycerol rapidly to prevent bursting and is also required for controlling turgor pressure during fusion of mating yeast cells (Luyten et al., 1995).

In addition, yeast possesses a glycerol-H+ symport system for active uptake of glycerol, Stl1, which is required for utilization of glycerol as carbon source as well as for glycerol uptake from the surrounding medium during adaptation to osmotic stress (Ferreira et al., 2005) and cold temperatures (Tulha et al., 2010). Thus, glycerol is actively transported into *S. cerevisiae* cells by an H+ symporter (Lages, F. & Lucas, 1997) encoded by *STL1*. The Stl1p belongs to the HXT family of sugar transporters (Nelissen et al., 1997) and STL1 expression is induced by salt and osmotic shock in a Hog1p- and Hot1p-dependent manner (Gasch et al., 2000). In induction conditions, it plays an important role for the fast accumulation of glycerol (Ferreira and Lucas, 2007; Ferreira et al., 2005; Swinnen et al., 2013; Tulha et al., 2010). The contribution of the glycerol/H+ symporter Stl1p for the accumulation of intracellular glycerol and consequent improved tolerance to cold/near-freeze/freeze stress is crucial in *S. cerevisiae* (Tulha et al., 2010). Based in a biotechnological perspective Tulha et al., (2010) claim that any *S. cerevisiae* strain already in use can be converted into a more resistant strain to freeze and near-freeze stress and therefore become even more interesting for industrial uses, just by simply adding glycerol to the broth.

Recently was also demonstrated that the osmotolerant yeast *Zygosaccharomyces rouxii* has two genes, Zr*STL1* and Zr*STL2*, encoding transporters mediating the active uptake of glycerol in symport with protons, contributing to cell osmotolerance and intracellular pH homeostasis (Dušková et al., 2015). This study also proves again that the accumulation of glycerol is essential for yeast viability upon hyperosmotic stress similar to what occurs in vinifications for example. The differences between different strains of *S. cerevisiae* and other important biotechnological species of the genus *Saccharomyces*, in relation to the glycerol metabolism and its implications in the osmotic strength, need to be better studied to provide knowledge and improve the application of these yeasts in biotechnological processes as winemaking.

5. Respiro-fermentative metabolism in yeast.

5.1 Crabtree effect and "make-accumulate-consume" life strategy.

The ability of fast ethanol accumulation and ethanol tolerance was first exploited by *Saccharomyces* yeasts to inhibit the growth of competing organisms, and then the accumulated ethanol could be 'digested' (make-accumulate-consume life strategy). The most common industrial yeast *S. cerevisiae* can convert simple sugars into ethanol even under fully aerobic conditions and by producing ethanol it can outcompete other microorganisms poisoning them with high ethanol concentrations (Piskur et al., 2006; Rozpędowska et al., 2011). Yeasts, which accumulate ethanol even in the presence of oxygen, such as *S. cerevisiae* and *Schizosaccharomyces pombe*, are called Crabtree-positive yeasts; whereas those that degrade sugars to CO_2, such as *Kluyveromyces lactis* and *Candida albicans*, are designated as Crabtree-negative yeasts (De Deken, 1966; Jack T. Pronk, 1996).

The Crabtree effect, which origin coincides with the origin of the first modern fruits approximately 125 million years ago (Hagman et al., 2014, 2013) represents the background for the 'make-accumulate-consume' life strategy, which helps the yeast species, especially *S. cerevisiae* and its closest relatives, to outcompete other microorganisms (Dashko et al., 2014; Piskur et al., 2006; Thomson et al., 2005). Other event underlying the 'make-accumulate-consume' life strategy is the whole genome duplication (WGD) (Wolfe and Shields, 1997). The most important consequences of this WGD are that an extra copy of the genome allowed a global rewiring of the yeast transcriptional network and gave the duplicated genes a chance to mutate and gain new or adapted functions compared to the original genes (Piskur

et al., 2006). For example, Thomson et al., (2005) showed that for the *ADH* genes the ancestral enzyme favored the forward reaction, being optimized to make ethanol, and Adh2 is the duplicated copy that acquired a new function, which is important for ethanol resistance as well as for its consumption and tolerance.

S. cerevisiae are regarded as facultative anaerobes, meaning they display both the respiratory and fermentative metabolism (Merico et al., 2007; Rozpędowska et al., 2011) and can spontaneously generate respiratory deficient mutants, also called petite mutants, which are characterized by a loss of the functional mitochondrial genome (mtDNA) (McArthur and Clark-Walker, 1983; Procházka et al., 2010) indicating the active respiratory chain is not necessary for the survival. For the development of the "make-accumulate-consume" strategy (MAC), the *Saccharomyces* yeast probably adapted their transcriptional network by a promoter rewiring in genes associated with respiration (Rozpędowska et al., 2011). It has been shown that after WGD, *S. cerevisiae* used a global promoter rewiring to change the gene expression of respiratory-associated genes, resulting in ethanol accumulation and thereby supporting the development of the MAC strategy. A specific cis-regulatory motif (AATTT) is present in *S. cerevisiae* at a conserved position in genes associated with rapid growth, but missing in respiratory-associated genes. The emergence of anaerobic growth in yeast is associated with the loss of the regulatory motif from the MRP (mitochondrial ribosomal protein) gene promoters (Ihmels et al., 2005).

5.2 Aerobic fermentation and energy metabolism.

Yeasts can typically use two different pathways to produce ATP from sugars, namely respiration and fermentation. While respiration results in a high yield of ATP (in *S. cerevisiae* approximately 18 ATP per glucose), fermentation

has a much lower ATP yield (2 ATP per glucose) but does not require oxygen (Figure 12). At high levels of sugar and oxygen, yeasts can produce ATP via respiration, fermentation, or a concurrent use of both pathways. Two strategies are commonly observed and relate to the well-known Crabtree effect (De Deken, 1966): the exclusive use of respiration in Crabtree-negative yeasts, and the simultaneous use of fermentation and respiration in Crabtree-positive yeasts.

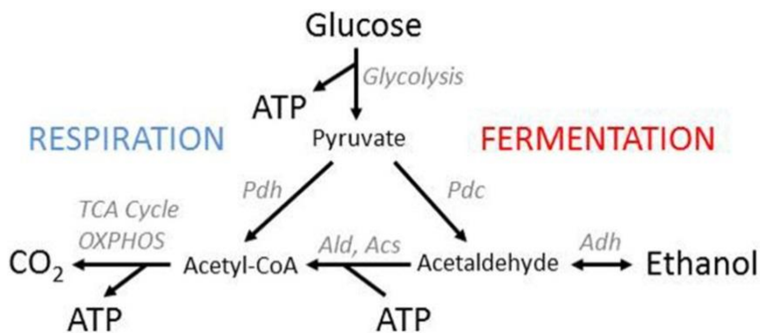

Figure 12. Yeast energy metabolism. Yeasts have two pathways for ATP production from glucose, respiration, and fermentation. Both pathways start with glycolysis, which results in the production of two molecules of pyruvate and ATP per glucose. Crabtree-positive yeasts, at sufficient levels of oxygen and glucose, use fermentation and respiration simultaneously. (Pfeiffer and Morley, 2014)

Batch culture experiments allow for the establishment of the glucose concentration at which fermentation sets in (Verduyn et al., 1984). For *S. cerevisiae* this has been shown to happen at a glucose concentration of about 150 mg/l (for comparison, the uptake rate of glucose starts saturating around 500 mg/l), though this might vary from species to species or strains to strains and depend on the specific conditions. The sharp drop in biomass yield associated with fermentation raises the question of why Crabtree-positive yeasts use the “wasteful” fermentation pathway if they could in principle rely solely on respiration for ATP production. An alternative view to

make-accumulate-consuming strategy, regarding the advantages and disadvantages of aerobic fermentation is based on trade-offs that emerge between rate and yield of ATP production, and consequently between growth rate and yield of an organism. This view has conceptually been outlined by Pfeiffer et al. (2001) and is referred to as the rate/yield trade-off hypothesis (RYT). While the ATP yield is the amount of ATP produced per unit of substrate, the rate of ATP production is the amount of ATP produced per unit of time. A trade-off between ATP rate and yield means that ATP can either be produced fast (i.e., at high rate and low yield) or efficiently (i.e., at low rate and high yield).

The *S. cerevisiae* wine yeasts have uniquely combined several properties including fast growth, efficient glucose repression, good ability to produce and consume ethanol, and a tolerance for several environmental stresses, such as high ethanol concentration and low oxygen levels providing a crucial competitive 'advantage' when production strains are selected for industrial fermentations in wineries (Steensels et al., 2014). Oxygen is discretely added during winemaking to avoid sluggish and stuck fermentations. The wide range of resulting dissolved oxygen concentrations has a deep impact on the physiology of wine yeast cells, improving the yeast fermentative rate as well as yeast viability. The impact of different levels of dissolved oxygen on the physiology of an industrial strain of *S. cerevisiae* under enological conditions was recently studied (Aceituno et al., 2012) and from a winemaking perspective, metabolic flux analysis and gene expression data suggest that elevated dissolved oxygen concentrations could affect yeast performance during and after fermentation. The results indicate that it is advisable not to keep wine yeast cells at certain oxygen levels for an extended period of time and that low levels could be beneficial for winemaking. For instance, with 1.2 μM dissolved oxygen, ethanol production is similar to anaerobiosis, with no acetic acid production, which is beneficial since acetic acid is a common "off-flavor" in wine. The viability and stress

resistance of the wine yeast might also increase, as the specific ergosterol content, a protective compound against stresses in wine fermentation, increases to its maximum (Aceituno et al., 2012).

Other studies have also demonstrated the importance of the role of oxygen and respiratory rates of yeast in industrial fermentations. Recently shown that ethanol and glycerol yields in winemaking can be reduced by taking advantage of the respiratory metabolism of some no-*Saccharomyces* yeast species (Morales et al., 2015; Quirós et al., 2014; Rodrigues et al., 2016). In these works, the effect of different aeration conditions and different co-inoculation ratios of *S. cerevisiae* and other yeasts strains selected has been evaluated with the aim of achieving a significant reduction of the alcohol level of wine and limiting volatile acidity production. The results indicated, as expected for Crabtree-negative yeasts, the extent of this reduction was higher for no-*Saccharomyces* yeast species. The potential of sugar respiration by no-*Saccharomyces* yeasts to help reduce alcohol levels in wine has been demonstrated and by using different strains of different species and simultaneous inoculation, was showed the crucial role of oxygen availability and respiratory metabolism in order to reduce alcohol levels by up to 3.7 % (v/v) by the end of fermentation of a natural grape must (Gonzalez et al., 2013). However, was also found a strong and significantly negative correlation between air concentration and the final yield of ethanol for both *S. cerevisiae* and mixed cultures (Morales et al., 2015). Recently an integrative parameter, Efficacy (efficacy for alcohol level reduction) was designed to simplify comparisons between strains or growth conditions and it integrates sugar consumption, ethanol yield, and acetic acid production data. Once again increasing oxygen supply shows a negative impact on ethanol yield, also compatible with the expected impact of oxygen availability on respiratory metabolism, for all strains tested (Rodrigues et al., 2016). Moreover, the current knowledge of the metabolic features of these alternative yeast species is limited, including the two main parameters to be considered for an

effective alcohol content reduction in wine, alcohol and acetic acid yields on sugar.

Currently, notably one realizes that there are studies that show the action of the respire-fermentative metabolism among different species and genus of yeast and that defined the negative and positive-Crabtree yeast (Hagman et al., 2014, 2013) but there are no metabolic studies between different strains of *S. cerevisiae* which shows the variation of this metabolism intra-specific manner. Obtaining the fermentative metabolic profiles of the strains and the association with their genetic bases can provide useful information for understanding the use of aerobic fermentation and its real benefits as life strategy as well as application in industrial fermentations like winemaking.

III – JUSTIFICATION & OBJECTIVES

Yeasts belonging to the genus *Saccharomyces* play a dominant role in different industrial fermentation processes, as well as the ecological characteristics as regards the composition of the microbiota as for the production of determinants fermentative metabolites on the quality of the final product (Querol and Bond, 2009). The process of domestication of these yeasts by man over the development of technological activities (production of wine, bread, beer and various traditional alcoholic beverages) (Baker et al., 2015; Sicard and Legras, 2011) made the different species of this group very well-known at genetic level, the whole genome, and also at metabolic level mainly concerning the biochemical pathways associated with fermentative processes (Arroyo-López et al., 2010; Demuyter et al., 2004; Ibáñez et al., 2014; López-Malo et al., 2013; Stribny et al., 2015; Tronchoni et al., 2014).

However, there is a constant dynamics of the biotech market relative to changing demand for new strains of yeast in order to meet the new needs and requirements of the fermentation industry. For example, in winemaking, global warming alters the content of sugars in the grape requiring the application of yeast strains that with a lower ethanol and higher glycerol yields and produced at low fermentation temperatures (Chambers and Pretorius, 2010; Pretorius, 2000). All these changes imply directly on the fermentation metabolism of yeast (Rodrigues et al., 2016). Faced with this dynamic, although current techniques and innovative methodologies such as adaptive evolution, reverse metabolic engineering and hybridization (Alexander et al., 2015; Kutyna et al., 2012; Medina, 2013; Oud et al., 2012; Pérez-Través et al., 2015, 2014) can help provide solutions and new yeast to the biotech market, also other species of the genus *Saccharomyces* like *S. uvarum* and hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* can be apply to resolve the new demands of the wine industry (González et al., 2008; Pérez-Torrado et al., 2015; Pérez-Través et al., 2012; Peris et al., 2012b).

Besides the interest to use no-*S. cerevisiae* strains in the sector, there is a relative lack of studies on the glycerol metabolism to compare under the same conditions different species and strains of biotechnological importance, focusing on yeast of the *Saccharomyces* genus like *S. cerevisiae, S. paradoxos, S. uvarum,* and *S. kudriavzevii* that can contribute to improve the wine quality. Based on this, the objectives 1 and 2 of this thesis were defined. In a first approach in order to meet the first objective (Chapter 1), we performed a transcriptomic analysis; a studied of the regulation of the main genes involved in glycerol synthesis; as well as the characterization the enzyme activity of the Gpd1p enzyme involved in the glycerol synthesis in *S. kudriavzevii* at different temperatures and under winemaking compared with *S. cerevisiae*. In a second approach (Chapter 2) glycerol metabolism was studied on the balance (synthesis and intracellular and extracellular content) in critical stress conditions for fermentation (osmotic stress and cold). In this approach, as well as *S. cerevisiae* and *S. kudriavzevii*, yeast strains of *S. paradoxus* and *S. uvarum* due to little information available about the mechanisms that determine the glycerol balance in all of them, were also studied.

There are many studies that have provided extensive knowledge about the overall genetic diversity of the genomes of different strains of *S. cerevisiae* from the most diverse environments (fermentation, wild, clinical and laboratory) (Fay and Benavides, 2005a; Fay, 2012; Liti et al., 2009; Sicard and Legras, 2011; Wang et al., 2012). Moreover, recent studies found marked differences at DNA sequence level that differentiate the genome of this species in wine and non-wine strains considering the domestication process of this species along migratory activities of mankind (Almeida et al., 2015, 2014; Eberlein et al., 2015; Pérez-Torrado et al., 2016). However, in general there is very little knowledge available that can determine different metabolic profiles of *S. cerevisiae* strains and associate them with different strains groups well characterized and genetically defined. Thus, to achieve

the third objective of this thesis (Chapter 3) was performed to search for metabolic differences that allow the separation of wine and non-wine strains genetically well-defined and characterized.

According to the background and justification presented, the objectives of this thesis were defined:

1 – Understand regulatory molecular mechanisms of high glycerol synthesis by *S. kudriavzevii* in winemaking conditions.

2 – To compare the glycerol balance among different species of the *Saccharomyces* genus during osmotic and cold stresses.

3 – To know metabolic differences between wine and non-wine *S. cerevisiae* strains genetically defined under different conditions of fermentation.

IV – MATERIAL & METHODS

1 Yeast strains, growth conditions and tests

The yeasts used in the present thesis belong to different species of the genus *Saccharomyces* as well as diverse *Saccharomyces cerevisiae* strains isolated from diverse environments. Besides the *Saccharomyces cerevisiae* constructed mutant strains were used distinct strains isolated from laboratory, natural, fermentations (wine, sake, beer, bioethanol, bread and traditional beverages) and clinical environments from different geographic regions of the planet (Table 1).

On our first approach (Chapter 1) *Saccharomyces cerevisiae* strain T73 and EC1118 were used as a wine yeast model (Pérez-Torrado et al., 2009; Querol et al., 1994). Fermol Cryophile (FCry) is a *S. cerevisiae* commercial wine yeast (AEB Group) isolated from wine fermentations, selected as a high glycerol producing strain adapted to low temperature conditions. Diploid strain BY4743 was used as a *S. cerevisiae* laboratory strain in certain experiments. Type strain IFO1802 was used as the *S. kudriavzevii* representative strain. ZP591, ZP594 and ZP629 were isolated in Portugal (Sampaio and Gonçalves, 2008) whereas CR85, CR89, CR90 and CA111 are natural *S. kudriavzevii* strains isolated in Spain (Lopes et al., 2010). Already in Chapter 2 two different strains of each species were studied. For *S. cerevisiae*, T73 model wine strain (Lopes et al., 2010; Querol et al., 1994) and the commercial wine strain Fermol Cryophile FCry (AEB Group); selected as adapted to low temperature (Gamero et al., 2013) were chosen. The 108 and Chr 16.2 strains isolated from natural environment were used as representatives of *S. paradoxus*. For *S. uvarum,* the 12600 and BMV58 strains isolated from wine in Spain were studied. BMV58 was commercially implanted (Lallemand Inc) because of its high glycerol production and good fermentative properties (patent ES2330709 B1).

Table 1. Yeast strains used in the present thesis, geographical origin and source of isolation, genetic description of the mutants, collections and chapter in which they were employed.

Yeast reference†	Species	Origin/Description	Provided by	Used in
T 73	*S. cerevisiae*	Wine strain, Spain	AQ 29	Chapter 1, 2, 3
FCry	*S. cerevisiae*	Wine strain, commercial	AEB, France	Chapter 1, 2
EC 1118	*S. cerevisiae*	Wine strain, commercial	Lalvin	Chapter 1, 3
ZP591	*S. kudriavzevii*	Wild strain, Portugal	Sampaio et al. (2008)	Chapter 1
ZP594	*S. kudriavzevii*	Wild strain, Portugal	Sampaio et al. (2008)	Chapter 1
ZP629	*S. kudriavzevii*	Wild strain, Portugal	Sampaio et al. (2008)	Chapter 1
CR85	*S. kudriavzevii*	Wild strain, Oak bark, Spain	AQ	Chapter 1,2
CR89	*S. kudriavzevii*	Wild strain, Spain	AQ	Chapter 1
CR90	*S. kudriavzevii*	Wild strain, Spain	AQ	Chapter 1
CA111	*S. kudriavzevii*	Wild strain, Spain	AQ	Chapter 1
IFO1802	*S. kudriavzevii*	Type strain, Soil, Japan	NBRC	Chapter 1,2
BY4743	*S. cerevisiae*	MATa/a his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0	ATCC	Chapter 1
BY4743*gpd1Δ*	*S. cerevisiae*	MATa/a his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 gpd1Δ/gpd1Δ	This thesis	Chapter 1
BY4741	*S. cerevisiae*	MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ0/ ura3Δ0	ATCC	Chapter 1
BY4741*gpd1Δ*	*S. cerevisiae*	MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 gpd1Δ	EUROSCARF	Chapter 1

BYpYES*GPD1*-Sc	*S. cerevisiae*	BY4741*gpd1Δ* pYES-*GPD1*$_{Scer}$	This thesis	Chapter 1
BYpYES*GPD1*-Sk	*S. cerevisiae*	BY4741*gpd1Δ* pYES-*GPD1*$_{Skud}$	This thesis	Chapter 1
BYp	*S. cerevisiae*	BY4741*gpd1Δ* pGREG526	This thesis	Chapter 1
BYp*GPD1*$_{Scer}$	*S. cerevisiae*	BY4741*gpd1Δ* pGREG526 -*GPD1*$_{Scer}$	This thesis	Chapter 1
BYp*GPD1*$_{Skud}$	*S. cerevisiae*	BY4741*gpd1Δ* pGREG526 -*GPD1*$_{Skud}$	This thesis	Chapter 1
BYp*GPD1*$_{Sce-Skud}$	*S. cerevisiae*	BY4741gpd1Δ pGREG526 -*GPD1*$_{Scer-Skud}$	This thesis	Chapter 1
Chr 16.2	*S. paradoxus*	Wild strain, Oak bark, Hungary	AQ	Chapter 2
108	*S. paradoxus*	Wild strain, Croatia	AQ	Chapter 2
BMV58	*S. uvarum*	Wine, Spain	AQ1580	Chapter 2, 3
12600	*S. uvarum*	Sweet wine, Spain	CECT12600	Chapter 2
BY4741*hog1Δstl1Δ*	*S. cerevisiae*	Lab strain	Dušková et al. (2015)	Chapter 2
BY-hs-YEp352	*S. cerevisiae*	BY4741*hog1Δstl1Δ*YEp352	This thesis	Chapter 2
BY-hs-p*STL1*-T73	*S. cerevisiae*	BY4741*hog1Δstl1Δ*YEp352-*STL1*$_{T73}$	This thesis	Chapter 2
BY-hs-p*STL1*-BMV58	*S. cerevisiae*	BY4741*hog1Δstl1Δ*YEp352-*STL1*$_{BMV58}$	This thesis	Chapter 2
BY-hs-p*STL1*-IFO1802	*S. cerevisiae*	BY4741*hog1Δstl1Δ*YEp352-*STL1*$_{IFO1802}$	This thesis	Chapter 2
CBS 1460	*S. cerevisiae*	Fermenting fruit, Indonesia	AQ 1084	Chapter 3
CBS 2087	*S. cerevisiae*	flower of lychee, China	AQ 1086	Chapter 3
NCAIM Y00678	*S. cerevisiae*	Fermented drink Hungary	AQ 995	Chapter 3
GB4 3	*S. cerevisiae*	Wine, Spain	CECT11761	Chapter 3
ZA 29	*S. cerevisiae*	Wine, South Africa	AQ 1327	Chapter 3
L 1005	*S. cerevisiae*	Wine, Argentina	AQ 1330	Chapter 3
QA 23	*S. cerevisiae*	wine	AQ 1346	Chapter 3
Vin 13	*S. cerevisiae*	wine	AQ 1331	Chapter 3

CECT 1477	*S. cerevisiae*	Sparkling wine, France	AQ 1347	Chapter 3
ZA 9	*S. cerevisiae*	Wine, South Africa	AQ 1357	Chapter 3
ZA 13	*S. cerevisiae*	Dry yeast wine, South Africa	AQ 1358	Chapter 3
L 7	*S. cerevisiae*	Wine, Chile	AQ 1369	Chapter 3
ZA 14	*S. cerevisiae*	Wine, South Africa	AQ 1387	Chapter 3
L 16	*S. cerevisiae*	Wine, Chile	AQ 1370	Chapter 3
L 246	*S. cerevisiae*	Wine, Chile	AQ 1327	Chapter 3
L 269	*S. cerevisiae*	Wine, Chile	AQ 1330	Chapter 3
L 958	*S. cerevisiae*	Wine, Argentina	AQ 1346	Chapter 3
L 1335	*S. cerevisiae*	Wine, France	AQ 1331	Chapter 3
L 960	*S. cerevisiae*	Wine, Argentina	AQ 1347	Chapter 3
L 1325	*S. cerevisiae*	Wine, Chile	AQ 1381	Chapter 3
L 1343	*S. cerevisiae*	Wine, Chile	AQ 1389	Chapter 3
L 962	*S. cerevisiae*	Wine, Argentina	AQ 1371	Chapter 3
CECT 1883	*S. cerevisiae*	Wine, Spain	AQ 924	Chapter 3
CECT 1479	*S. cerevisiae*	Wine, Hungary	AQ 1160	Chapter 3
CECT 1882	*S. cerevisiae*	Sherry wine, Spain	AQ 94	Chapter 3
CECT 11032	*S. cerevisiae*	Fermented must, Italy	AQ 626	Chapter 3
CECT 10557	*S. cerevisiae*	Grape must, Spain	AQ 629	Chapter 3
CECT 11827	*S. cerevisiae*	Dry yeast wine, Swiss	AQ 632	Chapter 3
CECT 11833	*S. cerevisiae*	Wine, Swiss	AQ 634	Chapter 3
CECT 11834	*S. cerevisiae*	Red wine, Spain	AQ 635	Chapter 3
GB Flor C	*S. cerevisiae*	Jerez wine, Spain	AQ 2492	Chapter 3

D14 n.14	*S. cerevisiae*	Dietetic complement	AQ 2587	Chapter 3
CECT 10120	*S. cerevisiae*	Fruit of *Arbutus unedo*	AQ 99	Chapter 3
CLIB 215	*S. cerevisiae*	Bakery	INRA, Paris	Chapter 3
CECT 10692	*S. cerevisiae*	Fermented grapes, champagne	AQ 625	Chapter 3
BC187††	*S. cerevisiae*	Barrel fermentation, USA	AQ 2504	Chapter 3
L-1374††	*S. cerevisiae*	Ferment from must País, Chile	AQ 2505	Chapter 3
L-1528††	*S. cerevisiae*	Ferment from must Cabernet, Chile	AQ 1391	Chapter 3
DBVPG1788††	*S. cerevisiae*	Soil Turku, Finland	Liti et al (2009)	Chapter 3
DBVPG6765††	*S. cerevisiae*	Unknown	Liti et al (2009)	Chapter 3
YJM 975††	*S. cerevisiae*	Clinic, Italy	Liti et al (2009)	Chapter 3
YJM 978††	*S. cerevisiae*	Clinic, Italy	Liti et al (2009)	Chapter 3
YJM 981††	*S. cerevisiae*	Clinic, Italy	Liti et al (2009)	Chapter 3
1.3 LM (9)	*S. cerevisiae*	Masato, Perú	AQ 876	Chapter 3
4 y M2	*S. cerevisiae*	Masato, Perú	AQ 880	Chapter 3
VI L7D	*S. cerevisiae*	Chicha de Jora, Perú	AQ 954	Chapter 3
CBS 2421	*S. cerevisiae*	Japanese kefyr grains	AQ 1087	Chapter 3
Chr 96.2	*S. cerevisiae*	*Quercus faginea*, Spain	AQ 2163	Chapter 3
CBS 6412	*S. cerevisiae*	Sake Kyokai no. 7, Japan	AQ 1314	Chapter 3
CBS 2992	*S. cerevisiae*	Palm wine, Pakistan	AQ 1088	Chapter 3
CBS 1591	*S. cerevisiae*	Fermenting cacao, Indonesia	AQ 1085	Chapter 3
YJM269	*S. cerevisiae*	Portuguese grapes	Liti et al (2009)	Chapter 3
CPE7	*S. cerevisiae*	Cachaça fermentation, Brazil	AQ 2494	Chapter 3
15M	*S. cerevisiae*	Agave culture, Mexico	AQ 2579	Chapter 3

Y12[††]	*S. cerevisiae*	Palm wine strain, Ivory Coast	AQ 633	Chapter 3
YPS128[††]	*S. cerevisiae*	Soil, *Quercus alba*, USA	Liti et al (2009)	Chapter 3
DBVPG 6044[††]	*S. cerevisiae*	Bili wine, West Africa	AQ 638	Chapter 3
UWOPS 03.461.4[††]	*S. cerevisiae*	Wild, Malasia	Liti et al (2009)	Chapter 3
UWOPS 05.227.2[††]	*S. cerevisiae*	Wild, Malasia	Liti et al (2009)	Chapter 3
UWOPS 05.217.3[††]	*S. cerevisiae*	Wild, Malasia	Liti et al (2009)	Chapter 3
CBS 8292	*S. cerevisiae*	Water, Sweden	AQ 1301	Chapter 3
CBS 8858	*S. cerevisiae*	Sorghum beer, Burkina Faso	AQ 1303	Chapter 3
2 y M2 (12)	*S. cerevisiae*	Masato, Perú	AQ 879	Chapter 3
CBS 8855	*S. cerevisiae*	Sorghum beer, Ghana	AQ 1305	Chapter 3
CBS 4455	*S. cerevisiae*	Kaffir beer, South Africa	AQ 1306	Chapter 3
CBS 8857	*S. cerevisiae*	sorghum beer, Burkina Faso	AQ 1323	Chapter 3
CBS 7764	*S. cerevisiae*	Fish, Sweden	AQ 1313	Chapter 3
GU4	*S. cerevisiae*	Agave, Mexico	AQ 904	Chapter 3
G1	*S. cerevisiae*	Beer, Belgium	AQ 843	Chapter 3
CECT 10131	*S. cerevisiae*	Flower of *Centaurea alba*, Spain	AQ 100	Chapter 3
Ch3 BL2	*S. cerevisiae*	Chicha de Jora, Perú	AQ 946	Chapter 3
CH1-L1	*S. cerevisiae*	Chicha de Jora, Perú	AQ 1006	Chapter 3
CH1-L2	*S. cerevisiae*	Chicha de Jora, Perú	AQ 1007	Chapter 3
Chr 9	*S. cerevisiae*	Forest soil, Hungary	AQ 2162	Chapter 3
Chr 7	*S. cerevisiae*	Forest soil, Hungary	AQ 2187	Chapter 3
LA 3M (4)	*S. cerevisiae*	Masato, Perú	AQ 871	Chapter 3
NCAIM Y00925	*S. cerevisiae*	Apricot pulp, Hungary	AQ 997	Chapter 3

YJM 326	*S. cerevisiae*	Clinic, J mcCuster lab	Liti et al (2009)	Chapter 3
Temoaya MI26	*S. cerevisiae*	Agave fermentation, Mexico	AQ 2493	Chapter 3
YJM 320	*S. cerevisiae*	Clinic, J mcCuster lab	Liti et al (2009)	Chapter 3
ZA 26	*S. cerevisiae*	Wine, South Africa	AQ 1336	Chapter 3
CECT 1384	*S. cerevisiae*	Beer	AQ 1164	Chapter 3
CECT 10392	*S. cerevisiae*	Fetid liquid olives, Spain	AQ 125	Chapter 3
CECT 11838	*S. cerevisiae*	Grape, Russia	AQ 639	Chapter 3
PE54 CJ	*S. cerevisiae*	Chicha de Jora, Peru	AQ 2332	Chapter 3
PE35M	*S. cerevisiae*	Masato, Peru	AQ 2356	Chapter 3
112M	*S. cerevisiae*	Mead, Mexico	AQ 2591	Chapter 3
NCYC361††	*S. cerevisiae*	Beer spoilage strain from wort, Ireland	AQ 1164	Chapter 3
273614N††	*S. cerevisiae*	Clinic, Newcastle, UK	Liti et al (2009)	Chapter 3
322134S††	*S. cerevisiae*	Clinic, Newcastle, UK	AQ 2502	Chapter 3
DBVPG6040††	*S. cerevisiae*	Fermenting fruit juice, Netherlands	Liti et al (2009)	Chapter 3
UWOPS87-2421††	*S. cerevisiae*	Cladode, Hawaii	Liti et al (2009)	Chapter 3
1124	*S. uvarum*	Mistela, Spain	AQ 1124	Chapter 3
1194	*S. uvarum*	Blackcurrant juice, Holland	AQ 1194	Chapter 3
NPCC1282	*S. eubayanus*	*Araucaria araucana*, Argentina	NPCC	Chapter 3
NPCC1286	*S. eubayanus*	*Araucaria araucana*, Argentina	NPCC	Chapter 3
2156	*S. paradoxus*	Mead, Mexico	AQ 2156	Chapter 3
2159	*S. paradoxus*	Grape surface, Croatia	AQ 2159	Chapter 3
2151	*S. kudriavzevii*	*Quercus*, Spain	AQ 2151	Chapter 3
2491	*S. arboriculus*	RVA	AQ 2491	Chapter 3

620	*S. mikatae*	NCYC 2888	AQ 620	Chapter 3

† - Reference identification used in this thesis.
†† - Yeasts used as genetic reference for wine (W), non-wine (nW) and mosaic (Mc) strains (Liti et al., 2009).
AQ - Identification number from collection Amparo Querol laboratory.

For *S. kudriavzevii* species, IFO1802 (type strain) and the CR85 wild strain isolated in Spain (Lopes et al., 2010) were used. The *S. cerevisiae* BY4741Δ*hog*1Δ*stl1* (Duskova et al., 2015) was used as a laboratory strain for the expression of *STL1* genes and comparison of the function of their products under hyperosmotic-stress conditions. However, in our latter approach (Chapter 3) 94 strains of *S. cerevisiae* were studied genetically at sequences level and regarding the production of fermentative metabolites. Six other species of the genus *Saccharomyces* (*S. uvarum*, *S. eubayanus*, *S. kudriavzevii*, *S. arboriculus*, *S. mikatae*, *S. paradoxus*) were also studied metabolically in a fermentative condition. The genome sequence of 19 *S. cerevisiae* strains previously characterized genetically by Liti et al., (2009) were used as genetic reference. Among all *S. cerevisiae* strains, 58 were chosen and metabolically also evaluated in 3 other fermentation conditions.

Yeast cells, wild or auxotrophic mutants to uracil, were maintained and grown in YPD, SC (Synthetic complete) or SC-Ura (Synthetic complete without uracil) medium at 28 °C for the *S. cerevisiae* and *S. paradoxus* species and 25 °C for *S. kudriavzevii* and *S. uvarum* species before all the fermentations and experiments.

1.1 Wine fermentation.

Micro-vinifications were performed in the Chapters 1 and 2 with synthetic must simulating standard grape juice natural or in Bobal variety must in some experiments (Rossignol et al., 2003). The cells from overnight precultures grown in YPD, after wash care with sterile distilled water (centrifuged for 1 minute at 3000 rpm), were inoculated at 5.0×10^5 - 5.0×10^6 cells/ml density.

In the chapter 1, besides the synthetic must, the natural Bobal variety must also was used in 100 ml bottles with gas interchange filled with must. The vinifications performed in the chapters 2 and 3 were performed in 250 ml

bottles filled with synthetic must. However all the batch fermentations were performed at 12 °C with gentle agitation (100-120 rpm) in triplicate.

1.2 Glycerol evaluation in mutants.

To test glycerol production of BYp, BYp*GPD1* $_{Scer}$, BYp*GPD1* $_{Skud}$ and BYp*GPD1* $_{Sce\text{-}Skud}$ mutant strains carried out in the Chapter 1, the media used was SC-Ura with 10 % of glucose because with standard glucose concentrations (2%) it was not possible to detect significant differences among glycerol or any metabolite produced (data not shown). The same medium and glucose concentration was used for the intracellular glycerol measurements in Stl1 mutants (Chapter 2). The cells were growth in 250 ml flasks with SC-ura with 10 % of glucose at 28 °C with agitation (150 rpm) until the glucose concentration achieve < 2 g/l. Experiments were performed in triplicate.

1.3 Osmotic and cold stress conditions.

For stress experiments performed in the Chapter 1, yeast were grown overnight in YPD media and the cells were diluted to OD_{600} = 0.2 and cultured at 28 °C until OD_{600}=1. Then, cells were transferred to 1 M sorbitol YPD or to 12 °C pre-cold YPD. In this chapter, the anaerobic condition also was studied between *S. cerevisiae* and *S. kudriavzevii* strains and for this purpose the cells were injected into bottles without O_2 (N_2 bubbled until saturation). To study the expression of genes related to glycerol balance under hyperosmotic stress between different species of the genus *Saccharomyces* (Chapter 2), the cells from exponentially growing precultures were carefully washed with sterile distilled (centrifuged at 3000 rpm for 1minute) water and transferred to YP (2% Bacto peptone and 1% yeast extract) with 2% glucose or 2% mannitol as a source of carbon (control), to the same medium supplemented with 1

M sorbitol (hyperosmotic stress), which is not assimilable for any of the species studied, or to H_2O (hypoosmotic stress). This experiment was performed in 2 L flasks in triplicate at 25 °C and 150 rpm.

1.4 Drop tests.

These tests evaluated the cell growth in solid medium (plate) of Stl1 transporter mutants and distinct *Saccharomyces* species under different osmotic stress conditions (Chapter 2). Then, the tolerance to hyperosmotic stress was evaluated by drop tests. Yeasts were grown overnight in YPD or SC-ura medium (Stl1 mutants), then cultures were diluted to OD_{600} = 0.2 and cells were allowed to grow in the same media until OD_{600} = 1. Then, serial dilutions of cells were transferred to plates with YPD; YPD + 0.8 M NaCl; YPD + 1.25 M KCl, incubated at 12 °C and 25 °C and evaluated each day. The growth of *Saccharomyces* species was also compared in plates with YPD containing 2 M sorbitol or 2 M KCl and supplemented or not with 1 mM glycerol. To investigate the functional differences of Stl1, the growth of BY4741Δ*hog*1Δ*stl1* cells transformed with appropriate plasmids was monitored on plates with SC-ura containing 0.7 M sorbitol, which or 0.3 M KCl and with or without 10 mM glycerol. Experiments were performed in triplicate

1.5 Micro-fermentations conditions.

To investigate the main fermentative metabolites of 94 *S. cerevisiae* strains and 10 strains from other six *Saccharomyces* species we defined micro-fermentations conditions on plates (Chapter 3). The fermentations were performed at 25 °C in 1.8 mL of distinct standardized medium contained in wells of microplates (New Greiner Bio-one 96-well Masterblock, 2.4mL Polypro) not covered hermetically with lid for microtitre plate (Fisher Scientific). All fermentations were conducted in triplicate and overnight

precultures were inoculated at 5.0×10^6 cells/ml density.

Four different fermentation conditions were performed: Using YPD medium (1), YPD in the absence of oxygen (2), SC medium (3) and SC in the absence of amino acids (4). For the condition of absence of oxygen, the plates were placed in an anaerobiosis bench chamber containing a flame burning. After inoculating the cells, the chamber was closed hermetically and the internal oxygen was consumed in less than 2 minutes putting out the flame. Before the inoculation the cells were carefully washed (gently centrifuged at 3000 rpm for 1 min.), the old medium discarded and the cells resuspended in 1.8 mL of fresh medium for fermentation and then transferred to each well of the plate. All plates were incubated at 25 ° C without agitation and for each strain the glucose consumption was monitored regularly until the concentration in the medium to achieve average values ≤ 0.5 g/L. Then, 1 mL of the supernatant was taken and frozen for subsequent analysis in HPLC. All the cells in each well of the plate were recovered to determine the dry weight (Cell biomass).

2 Culture media.

YPD or YEPD (Yeast Extract Peptone Dextrose) medium:

*Glucose	20 g
Peptone	20 g
Yeast extract	10 g
**Agar	15 g
H_2O (distilled) amount sufficient to	1000 mL

Autoclave at 120° C for 15 minutes.

* For the micro-fermentations carried out in Chapter 3, were added 100

g of glucose (10%).

** For solid medium preparation: Follow the same technique to make solid agar plates (media contains 20 g/l agar). Once the media has cooled to about 70° C, it can be poured directly from the bottle into sterile plastic petri dishes in sterile conditions. Fill petri dishes to about 1/3 capacity.

Minimal medium. Synthetic complete drop out (SC) medium:

Difco Yeast Nitrogen Base (w/o amino acids)	6.7 g
*Glucose	20 g
*Drop out mix (w/o Uracil, Formedium)	1.92 g
H_2O (distilled) amount sufficient to	1L
Agar (for solid media preparation)	15 g

Autoclave at 120° C for 15 minutes and after cooling the uracil or drop out mix solution, previously filtered in 0,22µm membrane filters, was added to the medium.

* For the micro-fermentations carried out in Chapter 3 and some experiments (Chapter 1), were added 100 g of glucose (10%). For the conditions in the Chapter 3 also uracil (76mg/L) was supplemented to the medium.

Synthetic complete drop out (SC) medium without amino acids:

Difco Yeast Nitrogen Base (w/o amino acids)	6.7 g
Glucose	100 g
*Adenine	18 mg
*Uracil	76 mg
**para*-Aminobenzoic Acid	8 mg
**myo*-Inositol	76 mg
H_2O (distilled)	1L

Autoclave at 120° C for 15 minutes and after cooling the solution with adenine, uracil, *para*-Aminobenzoic Acid and *myo*-Inositol, previously filtered

in 0,22µm filters, was added to the medium.

*Added so that only the lack of amino acids is the difference in the composition when compared to SC complete medium in micro-fermentations carried out in Chapter 3.

Synthetic must media:

Synthetic must media reproduces a standard natural must composition. This media is very useful to make lab micro-vinifications in a reproducible manner. Adapted from (Bely et al., 2003). Micro-fermentations were done at controlled temperature (25ºC or 12ºC).

Media composition for 1L consist in:

Sugars

Glucose	100 g
Fructose	100 g

Organic acids

Malic	5 g
Citric	0.5 g

Minerals

KH_2PO_4	0.75 g
K_2SO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
$CaCl_2.2H_2O$	0.155 g
NaCl	0.2 g
NH_4Cl	0.46 g

Weight the different substances and add distilled water (amount

sufficient to 1 L).

Autoclave at 121° C for 20 minutes.

Add the previous prepared stocking solution of:

Amino acids	13,09 mL
Oligoelements	1 mL
Vitamins	10 mL

pH = 3.3 with pellets of NaOH

Filter the whole volume using a 0.22µm filter.

Aminoacids stocking solution (1 L)

Tyrosine (Tyr)	1.5 g
Tryptophan (Trp)	13.4 g
Isoleucine (Ile)	2.5 g
Aspartic Acid (Asp)	3.4 g
Glutamic Acid (Glu)	9.2 g
Arginine (Arg)	28.3 g
Leucine (Leu)	3.7 g
Threonine (Thr)	5.8 g
Glycine (Gly)	1.4 g
Glutamine (Gln)	38.4 g
Alanine (Ala)	11.2 g
Valine (Val)	3.4 g
Methionine (Met)	2.4 g
Phenylalanine (Phe)	2.9 g
Serine (Ser)	6 g
Histidine (His)	2.6 g
Lysine (Lys)	1.3 g

Cysteine (Cys) 1.5 g

Proline (Pro) 46.1 g

Keep at -20 °C

Vitamins stocking solution (1 L)

Myo-inositol 12 g

Calcium pantothenate 15 g

Thiamine hydrochloride 0.025 g

Nicotinic acid 0.2 g

Pyridoxine 0.025 g

* Biotin 3 mL

*(stocking biotin solution 100 mg/L)

Keep at -20 °C

Oligoelements stocking solution (1 L)

$MnSO_4.H_20$ 4 g

$Zn\ SO_4.7H_2O$ 4 g

$CuSO_4.5H_2O$ 1 g

KI 1 g

$CoCl_2.6H_2O$ 0.4 g

H_3BO_3 1 g

$(NH_4)6Mo_7O_{24}$ 1 g

Keep at -20 °C

Natural must media:

Some Micro-vinifications were performed (Chapter 1) with natural Bobal variety must. The must was previously clarified by sedimentation for 24

h at 4°C to separate the clear juice from the sediment in presence of 60 mg/L of sulphur dioxide. After filtration the sugars concentrations were determined by HPLC and glucose and fructose were added to reach a concentration of 200 g/L. The amount of total sugars was selected to match with the experiments done with synthetic must. Finally, the must was sterilized by adding 1 ml/l of dimethyldicarbonate (Fluka, Switzerland).

3 Analytical determinations.

3.1 Sugars, Glycerol, Ethanol and Organic acids determinations.

For vinifications performed in Chapter 1 glycerol and sugar contents (glucose and fructose) in must samples were determined enzymatically using a commercial kit (AMS-SYSTEA) adapted to an automated ECHO instrument (Logotech), following the manufacturer's instructions. However for all other experiments and fermentations the concentrations of glucose, glycerol, ethanol, and organic acids (pyruvic, acetic, succinic and lactic) were determined by HPLC (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector. The column employed was a HyperREZTM XP Carbohydrate H+ 8µm (Thermo Fisher Scientific) and the conditions used in the analysis were as follows: eluent, 1.5 mM H_2SO_4; flux, 0.6 ml/min; and oven temperature, 50 °C. The samples were diluted 1/3, filtered through a 0.22-µm nylon filter (Symta, Madrid, Spain) and injected in duplicate. The average values in g/L were normalized for concentration of glucose consumed and yields are expressed as g/g except for the organic acids, expressed as mg/g.

To follow the development of each micro-fermentation carried out in Chapter 3, due to the small volume, the various glucose concentrations measured in the medium were performed by the DNS method (Robyt and Whelan, 1972) for chemical determination of reducing sugars added a few

modifications. Thus, aliquots of 20 µL were sufficient to determine the glucose levels at each time period and the results were very similar to those obtained by HPLC. The first aliquot was taken after 24 hours the start of fermentation and the other every 4, 6 or 8 hours, depending on the rate of glucose consumption of each yeast strain.

3.2 Intracellular glycerol determinations.

The Intracellular glycerol levels were determined in experiments performed in chapters 1 and 2 and their levels were normalized by dry weight of the same yeast cells collected. In the micro-fermentations performed on plates in chapter 3 the cell dry weight was also determined to indicate the biomass yield per consumed glucose. To determine intracellular glycerol content in experiments carried out in Chapter 1, overnight grown YPD yeast cells were diluted to OD_{600}=0.2 and cultured at 28 °C until OD_{600}=1. Then 10 OD_{600} units were harvested by filtration whereas in the chapter 2, 5 OD_{600} units were directly collected at different times from wine fermentations at 12 °C. In both cases after filtration the cells were quickly washed with 5 ml of water and transferred to a tube containing 1 ml of cold water. The yeast suspension was boiled for 10 min, cooled on ice, and centrifuged at 15.300 × *g* for 10 min at 4 °C. The supernatant was collected, filtered and analyzed by HPLC. To determine dry cell weight a second sample (5 or 10 OD_{600} units) was harvested by filtration in cellulose membrane, 25 mm pore size 0.45 µm (MF-Milipore®) previously dried in the oven at 80 °C for 24 h or in the microwave at 350W for 20 minutes and weighed. Then, the cells in the membrane were carefully washed with 1 ml of water, dried under the same conditions and weighed. The values obtained are expressed as µg of glycerol per mg of yeast cells. Experiments were performed in triplicate.

To determine dry weight (biomass) at the end of micro-fermentations performed in chapter 3 all the cells were recovered from the bottom plate

wells using suspension with a pipette then filtered, dried and weighed as described above. The mean values obtained were normalized and are expressed as g of biomass per g of consumed glucose.

4 Molecular techniques.

4.1 Primers and sequences.

The primers used are shown in Table 2. Primer pairs were designed using the Web Oligo Calc tool (http://www.basic.northwestern.edu/biotools/oligocalc.html) to calculate the appropriate parameters for the reactions and the web primer3 (v.0.4.0) tool for the RT-PCR primers design (http://bioinfo.ut.ee/primer3-0.4.0/). The sequences were sent to Invitrogen to be synthesized and the primers were used to amplify DNA in PCR and RT-PCR. To study gene diversity and design the primers, the genome sequences were obtained from *Saccharomyces* Genome Database (Cherry et al., 2012), and from (http://www.saccharomycessensustricto.org) (Scannell et al., 2011) and the specific web site (https://payengbrowse.gs.washington.edu/gb2/gbrowse/Port/) for some *S. kudriavzevii* strains.

Table 2. Primers used in this thesis, its objectives, species that were used and wherein chapters were employed.

Name	Sequence	Purpose	Species	Used in
GPD1-F	TGTGGTGCTTTGAAGAACG	qPCR and sequencing	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
GPD1-R	GTTTCTTCTCTAGATTCTGG	qPCR and sequencing	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
GPD2-F	GTTCCACAGACCWTACTTCC	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
GPD2-R	CCATCCCATACCTTCTACG	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
RHR2-F	CTTTCGATTTGGACTTCTTG	qPCR	*S. c., S. k*	Chapter 1
RHR2-R	GATTCGTGGTTCTTGACAAT	qPCR	*S. c., S. k*	Chapter 1
HOR2-F	YGCTCCAGCWGGTATTGC	qPCR	*S. c., S. k*	Chapter 1
HOR2-R	CRACTTCRTCTGTTTCGGC	qPCR	*S. c., S. k*	Chapter 1
ACT1-F	CATGTTCCCAGGTATTGCCG	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
ACT1-R	GCCAAAGCGGTGATTTCCT	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 1, 2
GPD1cl-C-F	ATGTCTGCTGCTGCTGATAG	Cloning pYES2.1 TOPO	*S. cerevisiae*	Chapter 1
GPD1cl-C-R	CTAATCTTCATGTAGATCTAA	Cloning pYES2.1 TOPO	*S. cerevisiae*	Chapter 1
GPD1cl-K-F	ATGTCTGCTGCTGCTGATAG	Cloning pYES2.1 TOPO	*S. kudriavzevii*	Chapter 1
GPD1cl-K-R	CTAATCTTCGTGTAGATCTAG	Cloning pYES2.1 TOPO	*S. kudriavzevii*	Chapter 1
GPD1sq-K-F	TCCGTTATAAGTTATTCTCACC	Sequencing	*S. kudriavzevii*	Chapter 1

GPD1sq-K-R	GCGCAAGAGCACGAGTTAAAC	Sequencing	*S. kudriavzevii*	Chapter 1
F-ProGPD1sc	CCTAGTACGGATTAGAAGCCGCCGAGCGGG TGACATTCGATTCCGGACTCGTCC	Cloning pGREG526	*S. cerevisiae*	Chapter 1
R-EndGPD1sc	GCGTGACATAACTAATTACATGACTCGAGGTC GACTGCGGAAGAGGTGTACAGC	Cloning pGREG526	*S. cerevisiae*	Chapter 1
F-ProGPD1kd	CCTAGTACGGATTAGAAGCCGCCGAGCGGG TGACAGGTTCGATTCCGGACTCG	Cloning pGREG526	*S. kudriavzevii*	Chapter 1
R-EndGPD1kd	GCGTGACATAACTAATTACATGACTCGAGGTC GACACATCGCGCAAGAGCACG	Cloning pGREG526	*S. kudriavzevii*	Chapter 1
F-OrfGPD1kd	CCCCCTCCACAAACACAAATATTGATAATATA AAGATGTCTGCTGCTGCTGATAG	Cloning pGREG526	*S. kudriavzevii*	Chapter 1
R-XhoGPD1kd	TCGGTTAGAGCGGATGTGG	Cloning pGREG526	*S. kudriavzevii*	Chapter 1
FPS1-F	GTTTTGYGTTTTTCCAAAGC	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 2
FPS1-R	TGATAAGCCATRGARGCATT	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 2
STL1-F	GCTTATTGGATTGATTTTGGG	qPCR	*S.c., S.u., S.p.*	Chapter 2
STL1-R	TGTTAACAGCATCGTGAAGC	qPCR	*S.c., S.u., S.p.*	Chapter 2
STL1-F	ACAGCATCGTGAAGCATAGC	qPCR	*S. kudriavzevii*	Chapter 2
STL1-R	TGGCTGATTTCTCAAAGTCG	qPCR	*S. kudriavzevii*	Chapter 2
18S-F	TTGCGATAACGAACGAGACC	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 2
18S-R	CATCGGCTTGAAACCGATAG	qPCR	*S.c., S.u., S.p., S.k.*	Chapter 2
P-NHA1	CAACTCTGTGTGATATAG	verification	*S. cerevisiae*	Chapter 2
ScSTL1 - R2	CAACCCTGTTCCAACACC	verification	*S. cerevisiae*	Chapter 2
ScSTL1 - F2	GGACAGTCCGGTTGGGGTTG	verification	*S. cerevisiae*	Chapter 2
SbSTL1 - F2	CTACCCTGAAACTGCTGG	verification	*S. uvarum*	Chapter 2

SbSTL1 - R2	GCCCAGTAGTCACGGAAAGC	verification	*S. uvarum*	Chapter 2
SkSTL1 - F2	CCCTGAAACCGCTGGTAG	verification	*S. kudriavzevii*	Chapter 2
SkSTL1 - R2	GCCTTGGACATTTCGGAC	verification	*S. kudriavzevii*	Chapter 2
YEp352-R	GGGGATGTGCTGCAAGGCG	verification	-	Chapter 2
YEp-SbSTL1-F	GTACATTATAAAAAAAAATCCTGAACTTAGCTA GATATTATGAAGGAATCAAAAGTATCTAAG	Cloning YEp352	*S. uvarum*	Chapter 2
YEp-SbSTL1-R	CACGACGTTGTAAAACGACGGCCAGTGCCA AGCTTGCATGTTACTTTTCAGAGCTGTTTTCAT	Cloning YEp352	*S. uvarum*	Chapter 2
YEp-ScSTL1-F	GTACATTATAAAAAAAAATCCTGAACTTAGCTA GATATTATGAAGGATTTAAAATTATCG	Cloning YEp352	*S. cerevisiae*	Chapter 2
YEp-ScSTL1-R	CACGACGTTGTAAAACGACGGCCAGTGCCA AGCTTGCATGTCAACCCTCAAAATTTGCTT	Cloning YEp352	*S. cerevisiae*	Chapter 2
YEp-SkSTL1-F	GTACATTATAAAAAAAAATCCTGAACTTAGCTA GATATTATGAGGAAATCAAAAGTATC	Cloning YEp352	*S. kudriavzevii*	Chapter 2
YEp-SkSTL1-R	CACGACGTTGTAAAACGACGGCCAGTGCCA AGCTTGCATGCTAGTTTTCGGAATTTGGTTTC	Cloning YEp352	*S. kudriavzevii*	Chapter 2
CAT8-F	AAGAGCAACTATAGYCTGACAAARYTAATGAG	PCR and verification	*S. cerevisiae*	Chapter 3
CAT8-R	CTACTTGGCRTTTTGCCAYTGRAA	PCR and verification	*S. cerevisiae*	Chapter 3
GAL4-F	GCTACTCTCCCAAGACCAARAGGTC	PCR and verification	*S. cerevisiae*	Chapter 3
GAL4-R	GCGATTTCAATCTGATTATTRTACARCATCAT	PCR and verification	*S. cerevisiae*	Chapter 3
BRE5-F	TTCATTCATCAACTTTGAGGCCCATGTCAT	PCR and verification	*S. cerevisiae*	Chapter 3
BRE5-R	TTCATTCATCAACTTTGAGGCCCATGTCAT	PCR and verification	*S. cerevisiae*	Chapter 3

EGT2-F	CAGATCATTGGTTCATAATAGAAGGKCAAYTGT	PCR and verification	*S. cerevisiae*	Chapter 3
EGT2-R	CCAGGCGGTRTTATTAGTTTTGTATATRCCACC	PCR and verification	*S. cerevisiae*	Chapter 3
SP6	ATTTAGGTGACACTATAG	Sequencing	-	Chapter 3
T7	TAATACGACTCACTATAGGG	Sequencing	-	Chapter 3

4.2 Yeast Plasmids: extractions and constructions.

For the yeast plasmid extraction a modified protocol described by Robzyk and Kassir, (1992) was used. Cells were growing in 5 mL overnight culture in YPD with G418 Geneticin at 0.2 g/L concentration in order to maintain the plasmid. 1.5 mL of the culture was transferred to new tube and centrifuged at 6000 rpm for 5 min. Supernatant was removed and then cells were resuspended in 100 µL of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl pH 8, 50 mM EDTA). 0.2 g glass beads (0.45 mm) were added, and then samples were mixed with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Another 100 µL of STET were added, samples were mixed briefly and were incubated in boiling water for 3 min. The samples were cooled in ice and then were centrifuged at 12000 rpm for 10 min at 4 °C. 100 µL of the supernatant were transferred to new tube and 50 µL of ammonium acetate 7.5 M was added. Samples were centrifuged at 12000 rpm for 10 min at 4 °C. 100 µL of supernatant was added to 200 µL of absolute ethanol, and then it was precipitated for 1h at – 20 °C. Samples were centrifuged at 12000 rpm for 15 min at 4 °C. Pellets were washed with ethanol 70% and samples were centrifuged 5 min at 12000 rpm at 4 °C. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA plasmid was re-suspended with 20 µL distilled water.

The plasmids expressing the *S. cerevisiae* or *S. kudriavzevii GPD1* gene under GAL promoter were constructed using pYES2.1 TOPO® TA Expression Kit (Invitrogen) following manufactures' instructions whereas plasmids expressing the *S. cerevisiae* or *S. kudriavzevii GPD1* gene under its own promoter were constructed using pGREG526 by homologous recombination in yeast (Jansen et al., 2005). To construct a version with *GDP1* promoter from *S. cerevisiae* and a recombinant *GPD1* coding sequence from *S. cerevisiae* and *S. kudriavzevii* (pGREG526-*GPD1*$_{Scer\text{-}Skud}$), the plasmid pGREG526-

*GPD1*Scer, linearized with *Xho*I and *Aat*II, was co-transformed with a PCR product containing *GPD1*Skud. All constructions were confirmed by sequencing. Plasmids expressing the *S. cerevisiae* T73, *S. bayanus* BMV58 and *S. kudriavzevii* IFO1802 *STL1* genes under *NHA1* gene promoter were constructed by exchanging the *NHA1* coding sequence in pNHA1-985 (YEp352 derivative, (Kinclova-Zimmermannova and Sychrova, 2006)) by homologous recombination. All constructions were confirmed by diagnostic PCR and sequencing. The primers, listed in Table 2, were used to amplify the DNA fragments (from genomic DNAs) with suitable flanking regions for homologous recombination and confirmation.

4.3 Enzyme activity measurements.

Cytoplasmic Gpd1p activity in crude extracts was assayed as described previously (Ansell et al., 1997) with minimal modifications. Samples were harvested by centrifugation, washed twice with cold isosmotic media and concentrated in 0.1 M potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$, 1 mM DTT and 1 mM EDTA. Cells were disrupted in FastPrep (MP Biomedicals) device at 4.5 m/s with glass beads in 30 s intervals over a total period of 2 min and disruption was confirmed by microscopy. Unbroken cells and debris were removed by centrifugation for 10 min at 10000 g. The supernatant was immediately used to assay Gpd1p activity. The enzymatic reaction (2 ml) contains: 20 mM imidazol–HCl (pH 7.0), 1 mM $MgCl_2$, 1 mM DTT, 0.67 mM dihydroxyacetone phosphate (DHAP) and 0.09 mM NADH. One unit (U) is defined as the amount of enzyme catalyzing the conversion of 1.0 µmol of DHAP to glycerol-3-phosphate per minute at 25 °C. Specific activity is expressed as units per mg of protein ($U \cdot mg^{-1}$). Total protein content was estimated by the Bio-Rad Protein Assay with bovine serum albumin as a standard. To determine the kinetic parameters, DHAP and NADH varied within the concentration range 0.2–4 mM and 0.02-2 mM, respectively.

Activity measurements obtained with the different substrate concentrations were represented and non-linear regression was adjusted to Michaelis-Menten equation using GraphPad Prism 6.0 Software Enzyme Kinetics package, which directly calculates V_{max} and K_m. Experiments were performed in triplicate. Gpd1p enzyme structure models were built using MODWED online server based on Modeller software (Eswar, 2003). Three individual statistical scores (e-value, z-Dope and GA341) where used to check the model quality and the models of the two species where considered reliable. Structures were visualized with Pymol viewer (DeLano, 2002).

4.4 Gene expression determinations and qRT-PCR Technics.

This technic allows us to amplify and simultaneously quantify targeted RNA molecules. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. In this work we have used a relative quantification method and the general methodology is described below:

RNA extraction

Yeast RNA was extracted from frozen cell samples (10^8 cell/mL), harvested from fermentation, using the method described by Sierkstra et al., (1992). Previous to freeze the samples, cells were centrifuged at 4 °C and washed with distilled water. The supernatant was removed and then the pellet was frozen with liquid nitrogen and then harvested at – 80 °C until the RNA extraction.

NOTE: all steps should be using RNase free labware and solutions. All the solutions and glass beads, with the exception of Tris- HCl, were treated with DEPC (Diethyl pyrocarbonate) to eliminate the possible RNases. For each 50 mL of solution 50 µL of DEPC was added, leaving work overnight and then

was autoclaved (121 °C for 20 min) to inactivate traces of DEPC.

Harvested cells were defrosted in ice, wash with free-sterile RNA dH_2O and then pelleted by centrifugation at 4,000 g for 5 minutes. The pelleted cells were resuspended in RNase free 2 ml microcentrifuge tube in the following way:

Adding 500 μL of LETS (LiCl 0,1M; EDTA 10mM; SDS 0,2%, Tris.HCl 10mM pH=7,4), 500 μL phenol-tris for RNA (RNA free) and 500 μL of glass-beads.

Cells are broken using a Mini Beadbeater, 6 x 30 seconds with placing the cells on ice 30 seconds between shakings.

Pellet the cells in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C.

Remove supernatant and repeat the phenol-tris extraction. Vortex.

Pellet the cells in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C.

Remove supernatant and extract by using phenol/chloroform/isoamyl alcohol (25:24:1) and vortex.

Repeat the extraction 3 times more by using chloroform/isoamyl alcohol (24:1) in last round.

Pellet the cells in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C.

Collect the supernatant and precipitate with one volume of LiCl 5M at -20 °C for 12 hours.

Pellet the cells in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C.

Wash with ethanol 500 μL (70%) and after pellet the cells, dry the ethanol.

Precipitate with 1/10 volume of NaOAc 3M and 2 volumes of EtOH (100 %), at –80°C for 2-3 hours.

Wash with ethanol 500 μL (70%) and after pellet the cells, dry the ethanol.

Pellet the cells in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C.

Add 50 to 100 μL of free-sterile RNA dH_2O and resuspend.

RNA purification.

RNA extracted was purified by using a commercial kit. RNA is digested

with a DNAse to eliminate the extra DNA from our samples (Ambion, Applied Biosystems).

RNA quantification.

Total RNA concentrations were quantified using a NanoDrop-1000 spectrophotometer (NanoDrop). Dilute the RNA to 1 µg/µL. RNA quality with verify by running the RNA electrophoresis in an agarose gel.

cDNA synthesis.

We use a negative control to verify that there is no DNA contamination in the samples (non-RT control) without reverse transcriptase.

To each tube:

Sample	1.0 µL (RNA 1µg/µL)
Oligo-dT 12-18 Primer (500 ng)	0.5 µL
Free-sterile RNA dH_2O	10.5 µL

Incubate in thermocycler 5 minutes at 65 °C.

1 minute on ice.

To each tube:

Buffer 5X (Invitrogen)	4 µL
DTT (0.1M)	1 µL
RNAse out	1 µL
dNTP (10nM)	1 µL

Incubate 5 minutes at 42 °C.

Add 1µL of Superscript III Rnase Reverse Transcriptase (Invitrogen).

Incubate 50 minutes at 42 °C. Incubate 15 minutes at 70 °C.

Real time quantitative PCR

Real-Time Quantitative PCR was performed using LightCycler® 480

SYBR Green I Master (Roche, Germany). All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and the most precise quantification. The SYBR PCR reactions contained 2.5 µM of each PCR primer, 5 µl cDNA and 10 µL of SYBR Green I Master (Roche, Germany) in a 20 µL reaction.

PCR conditions

All PCR reactions were mixed in LightCycler® 480 Multiwell Plate 96 (Roche, Germany) and cycled in a LightCycler® 480 Instrument II, 96 well thermal cycler (Roche, Germany) using the following conditions: 95 °C for 5 min, and 45 cycles at 95 °C for 10 sec, at 55 °C for 10 sec and 72 °C 10 sec. All samples were analyzed in triplicate with LightCycler® 480 Software, version 1.5 (Roche, Germany) and the expression values were averaged. Gene expression levels are shown as the concentration of the studied gene normalized to the concentration of the housekeeping genes, only ACT1 or ACT1 and RDN18.2, referenced to the control.

There were small differences in RNA levels determinations carried out in chapters 1 and 2 which are detailed below:

In the Chapter 1, the purified RNA was converted to cDNA and the expression of *GPD1*, *GPD2*, *GPP1* and *GPP2* genes was quantified by qRT-PCR. This reaction was performed with gene-specific primers (200 nM) (Table 2) in a 20 µl reaction, using the Light Cycler FastStart DNA MasterPLUS SYBR green (Roche Applied Science, Germany) in a LightCycler® 2.0 System (Roche Applied Science, Germany). All samples were processed for melting curve analysis, amplification efficiency and DNA concentration determination. A mixture of all samples and serial dilutions (10^{-1} to 10^{-5}) was used as standard curve. The constitutive *ACT1* gene expression was used to normalize the amount of mRNA and absolute values were represented.

To determinations performed in chapter 2 the expression of *GPD1*,

GPD2, *STL1* and *FPS1* genes was quantified and qRT-PCR was performed with gene-specific primers (200 nM) designed for each specie (Table 2) from sequences consensus between the different strains in a 10 µl reaction, using the Light Cycler FastStart DNA MasterPLUS SYBR green (Roche Applied Science, Germany) in a LightCycler® 2.0 System (Roche Applied Science, Germany). Also all samples were processed for melting curve analysis, amplification efficiency and DNA concentration determination and a mixture of all samples and serial dilutions (10^{-1} to 10^{-5}) was used as standard curve. However, two different constitutive reference genes were used (*ACT1* and *RDN18-1*) to normalize the amount of mRNA and ensure accuracy, correct interpretation and repeatability. The results were normalized by using the normalization factor obtained from geNorm VBA applet (Vandesompele et al., 2002). All reactions were performed in triplicate.

4.5 Yeast transformation.

Yeast transformation was performed using a lithium acetate protocol describe by Gietz and Schiestl, (1991). Transformation procedure was used to construct deletion and overexpressing strains. To construct the deletion strains was transformed with the deletion cassette, obtained by PCR. For the construction of overexpressing strains it's was co-transformed with the overexpression cassette, obtained by PCR, and the plasmids previously linearized by specific restriction enzyme each to avoid sticky ends and to make the recombination process easier.

Yeast cells were inoculated in 50 mL YPD, overnight at 30 °C and 200 rpm. This culture was used to inoculate an OD of 0.2 in 50 mL YPD and was incubated at 30 °C and 200 rpm until the cells were completed at least 3 divisions (OD of approximately 0.8). Then 5 mL culture was transferred to a new tube and centrifuged at 5000 rpm for 5 min. The media was poured off and cells were resuspended in 2.5 mL of sterile water and centrifuged

again. The water was removed and cells were resuspended in 100 µL of 0.1 M LiAc. Cells were pellet a top speed for 15 sec and LiAc was removed with a micropipette. Cells were resuspended in 40 µL of 0.1 M LiAc, centrifuged and LiAc was removed again with a micropipette. Then transformation mix was added in this order: 240 µL of PEG (50% w/v), 36 µL of 1 M LiAc, 50 µL of ss-DNA 2 mg/mL (previously boiled for 5 min and quickly chilled in ice) and 34 µL of deletion cassette or 26 µL of overexpression cassette + 8 µL of the plasmid. Each tube was vigorously vortexed until the cell pellet had been completely mixed. Tubes were incubated at 30 °C for 30 min, then 42 °C for 15-30 min. Tubes were centrifuged at 7000 rpm for 15 sec and the transformation mix was removed with a micropipette. Into each tube 1 mL of water was added and pellet was resuspended by pipetting it up and down gently. Then tubes were centrifuged at 7000 rpm for 15 sec and water was removed. Pellet was resuspended in 500 µL YPD and incubated at 30 °C for 3 h with a gentle shaking at 125 rpm. 200 µL (twice for each transformation) were plated onto selection plates (YPD with Geneticin G418, 0.2 g/L) and incubated at 30 °C until colonies appeared (2-3 days).

4.6 DNA extraction, PCR amplification and Sequencing.

The extraction of yeast DNA was carried out from 3 mL overnight culture in YPD at 30 °C, using the method described by Querol et al., (1992). 1.5 mL of the cell culture was centrifuged at 10000 rpm for 2 min and the YPD was removed. The cell pellet was washed with 1 mL of distilled water and centrifuged at 10000 rpm for 2 min to remove the water. 0.5 mL of Buffer 1 (sorbitol 0.9 M, EDTA 0.1 M pH 7.5) and 30 µL of Zymolyase (Seikagaku Corporation, Japan) were added. The samples were vortexed and the tubes were incubated at 37 °C for 20 min. Samples were centrifuged at 10000 rpm for 2 min to remove the supernatant. Protoplasts

were re-suspended in 0.5 mL of Buffer 2 (Tris 50 mM pH 7.4, EDTA 20 mM). Afterwards 13 µL of SDS 10% was added and the tubes were incubated at 65 °C for 5 min. After the incubation, 0.2 mL of potassium acetate was added, the samples were mixed and incubated in ice for 5 min. Samples were centrifuged 15 min at 12000 rpm at 4 °C to ensure the elimination of SDS. Supernatant was added to 0.7 mL of isopropanol (v/v) and incubated at room temperature for 5 min. Samples were centrifuged 10 min at 12000 rpm at 4 °C. Supernatant was removed and 0.5 mL of ethanol 70% was added. Samples were centrifuged 5 min at 12000 rpm at 4 °C. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA was re-suspended in 40 µL of TE (Tris 10 mM ph 7.4, EDTA 1 mM pH 8.0).

Standard DNA amplification was performed via PCR in a GenAmp PCR System 2700 (Applied Biosystems, USA) to synthesis the deletion cassettes, to check the deletion and for amplifying genes of interest from the genome for cloning and sequencing reactions. A typical 50 µL reaction was performed, contained 1-5 µL, 0.1 – 100 ng DNA, 2 µL 200 µM dNTPs, 5 µL 10 µM primers, 5 µL 10 x PCR buffer, 3-5 µL 50 mM MgCl2, 1 µL of Taq polymerase (rTaq, Takara), 0.03 U/µL and water to complete the 50 µL. PCR products were stored at – 20 °C until required.

PCR amplifications of the nuclear genes *EGT2*, *CAT8*, *BRE5* and *GAL4* were performed using the primers (Table 2) chosen based on the nucleotide diversity levels unveiled by Arias, 2008. The available sequences of the *S. cerevisiae* strains in the Saccharomyces Genome Database (Cherry et al., 2012) were compared for primer design. The PCR reactions were performed with four microlitre of DNA diluted to 1–50 ng μL^{-1} using 1 unit of Phusion high-fidelity DNA polymerase (Finnzymes, Finland) and its products were purified using the High Pure PCR product purification Kit (Roche v.16, Mannheim Germany) following the manufacturer's instructions. The purified and amplified gene segments were previously cloned into the pGEM®-T easy vector systems (Promega). Then, the ligation reaction was confirmed by PCR

(primers in table 2) and subsequently the sequencing reactions were performed for both strands of DNA from the vector: with the T7 primer to sequence the direct strand (5`-3`) and with the SP6 primer for sequencing the reverse strand (3`-5`).

The sequencing reactions employed a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. This reactions were run on a Techgene Thermal Cycler (Techne) programmed as follows: an initial denaturation at 94 °C for 3 min, followed by 99 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and polymerization at 60 °C for 4 min. Sequencing was performed on an Applied Biosystems automatic sequencer model ABI 3730 (Applied Biosystems) by the sequencing service of the University of Valencia.

4.7 Phylogenetic analysis.

The DNA sequences coding of four nuclear genes (*CAT8*, *GAL4*, *EGT2* e *BRE5*) from 94 *S. cerevisiae* strains were obtained and analyzed (Chapter 3). Among them are the sequences from 19 strains genetically characterized by Liti et al. (2009) and that served as a genetic reference in this approach. These sequences were retrieved from *Saccharomyces* Genome Resequencing Project (SGRP) of Wellcome Trust Sanger Institute database. Other sequences from the strains studied here were obtained from the work performed by Arias et al. (2008) where each allele studied in his work it was possible to be separated into wine or non-wine. Thus, we also perform an alignment with the program MEGA5 (Tamura et al., 2011) with all sequences of each gene separately which allowed us to group alleles from all *S. cerevisiae* strains studied in this approach as wine (W) or non-wine (nW). This allowed us to pre-classify as wine strain (W) that has all the four wine alleles, as non-wine strain (nW) with all no wine alleles and mosaic strain (Mc) one that has at least one distinct allele.

However, to reinforce this classification it was necessary to define a phylogenetic relationship between different strains. For this, the nucleotide sequences corresponding to the four distinct genes were concatenated into a single sequence for each strain. Thus, we perform the alignment using MEGA5 and then use these concatenated sequences for construction of a phylogenetic tree by SpliTstree4 program (v.4.13.1) (Huson and Bryant, 2006), with the parameters Jukes Cantor characters, Neighbor Net, Equal Angle.

V - RESULTS & DISCUSSION

CHAPTER 1. Enhanced enzymatic activity of glycerol-3-phosphate dehydrogenase from the cryophilic *Saccharomyces kudriavzevii*.

This chapter includes the following published article, detailed below, and make up this thesis with the prior authorization of all authors.

OPEN ACCESS Freely available online

Enhanced Enzymatic Activity of Glycerol 3 Phosphate Dehydrogenase from the Cryophilic *Saccharomyces kudriavzevii*

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Saccharomyces kudriavzevii is a species classified within the *Saccharomyces* genus, which is phylogenetically closely related to *Saccharomyces cerevisiae*. The similarity between these two species prompted the formation of natural interspecific hybrids, present in wine (González et al., 2007) and brewing environments (González et al., 2008). Initially, a few strains of *S. kudriavzevii* were described isolated in decayed leaves and soil in Japan (G I Naumov et al., 2000) but recently, several Iberian populations have been described (Lopes et al., 2010; Sampaio and Gonçalves, 2008). In fact, these isolation events were made possible due to the decrease in isolation temperatures, revealing one of the most interesting characteristics of *S. kudriavzevii* species, its adaptation to low temperature. Previous works have shown *S. kudriavzevii* to outperform *S. cerevisiae* strains in different low temperature conditions in natural grape juice fermentations (Tronchoni et al., 2009) or synthetic media growth (Arroyo-López et al., 2010; Belloch et al., 2008). Another important difference between the two species is that *S. kudriavzevii* produces higher amounts of glycerol during low temperature fermentations than *S. cerevisiae* (González et al., 2007).

Several features have been related to low temperature adaptation of yeasts, including membrane lipid composition, synthesis of ribosomal proteins and trehalose content (Aguilera et al., 2007). More recently, evidence has been found of the role played by glycerol production in cold stress (Tulha et al., 2010), via a regulatory mechanism involving the HOG (High Osmolarity Glycerol) pathway. Intracellular glycerol content was linked to the *S. cerevisiae* cell survival in fermentations after freezing and at low temperatures (Tulha et al., 2010). In this respect, intracellular glycerol accumulation was observed in response to cold stress, indicating the involvement of this molecule when cells face low or near freezing temperature conditions. Furthermore, intracellular glycerol is also involved in resistance to freeze/thawing stress (Izawa et al., 2004).

During growth in glucose, cryoprotectanct glycerol is synthesized by

a short branch of glycolysis, which involves two steps (Ansell et al., 1997; Pahlman et al., 2001). *Saccharomyces* yeasts have two isoenzymes for each step: GPD for glycerol-3-phosphate dehydrogenases (Gpd1p and Gpd2p) and GPP for glycerol-3-phosphatases (Gpp1p/Rhr1p and Gpp2p/Hor2p). Metabolic control analysis values calculated by flux modeling of glycerol synthesis indicate that the glycerol-3-phosphate dehydrogenase-catalyzed reaction has a flux control coefficient of approximately 0.85 and exercises the majority of flux control through this pathway in *S. cerevisiae* (Remize et al., 2001). Moreover, *GPD1* gene overexpression increases the glycerol levels produced while the overexpression of the other three enzymes does not (Nevoigt and Stahl, 1996; Pahlman et al., 2001; Remize et al., 2001) whereas reduction of *GPD1* leads to a reduced flux towards glycerol (Hubmann et al., 2011; Nevoigt and Stahl, 1996). *GPD1* and *GPP2* genes are is essential for growth under osmotic stress and their expression is regulated by the high-osmolarity glycerol response pathway (Albertyn et al., 1994), whereas *GPD2* and *GPP1* are activated to equilibrate the redox balance by regenerating NADH associated with biomass production (Ansell R, Granath K, Hohmann S, Thevelein JM, 1997). Furthermore, *GPD1* is activated in response to cold stress (Panadero et al., 2006).

The study presented here looks into different regulatory mechanisms of glycerol synthesis in *S. kudriavzevii*. We observed that an increased accumulation during low temperature micro-vinifications is present in many *S. kudriavzevii* strains isolated from different regions. An effort to understand this difference at the molecular level, as compared to *S. cerevisiae*, revealed increased *GPD1* gene expression levels in *S kudriavzevii* during alcoholic fermentation and a different expression pattern for the *GPD2* gene. Furthermore, we observed increased activity and suggest that it can be explained due to increased V_{max} of the Gpd1p enzyme, which also explain the increased amounts of glycerol produced by *S. kudriavzevii*. Finally, we evaluated the glycerol accumulation with *S. cerevisiae, S. kudriavzevii* or a

recombinant Gpd1p variant in the same background and observed that the *S. kudriavzevii* enzyme produced increased glycerol levels at 12 or 28 °C.

RESULTS

2.1 Increased extracellular glycerol accumulation during low temperature micro-vinifications.

Micro-vinification experiments in Bobal natural must were performed with the *S. cerevisiae* wine strain T73 and with the *S. kudriavzevii* type strain IFO1802 at 12 °C and fermentation performance was monitored by glucose consumption. Must samples were taken throughout the micro-vinification experiments and were used to measure extracellular sugars (Figure 13A) and extracellular glycerol concentrations (Figure 13B). As shown in Figure 13, the *S. kudriavzevii* IFO1802 strain was able to complete must fermentation at low temperature 9 days faster than the reference wine strain T73 (Figure 13A), revealing the better adaptation of *S. kudriavzevii* to cold environments.

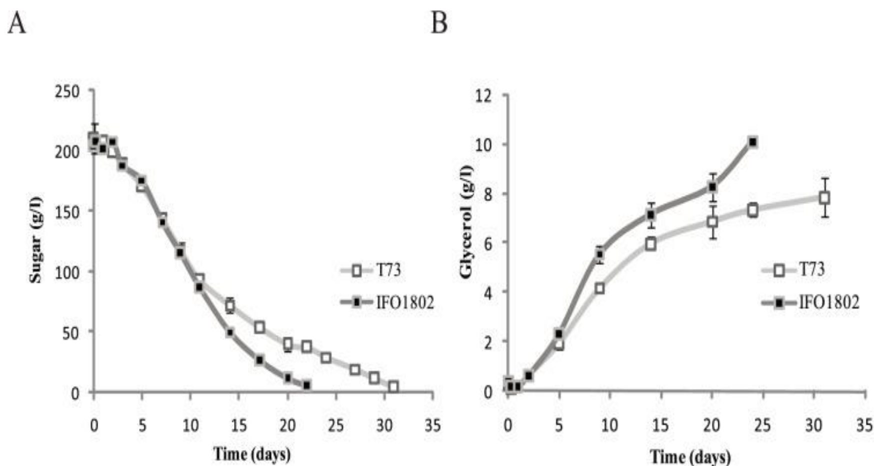

Figure 13. Micro-vinification experiments in natural must at low temperature with *S. cerevisiae* (T73) and *S. kudriavzevii* species (IFO1802). Precultured cells were inoculated in

Bobal natural must at 12 °C and samples were taken along the fermentation to determine sugars (glucose and fructose) (A) or glycerol (B) content for each species. Three independent micro-vinification bottles were used for each strain and average ± standard deviation is shown.

The Figure 13B also shows extracellular glycerol content measured throughout the winemaking process. For both strains, the extracellular glycerol accumulation pattern can be divided into two phases: a first step of high production that ends in the interval of 5-15 days and a second period of moderate extracellular glycerol accumulation lasting until the end of the process (Figure 13B). A clear difference between both strains can be observed after 5 days, indicating IFO1802 tends to produce higher extracellular glycerol amounts than T73 strain (Figure 13B). It is interesting to note that IFO1802 strain exhibits a higher extracellular glycerol accumulation than *S. cerevisiae* T73, even when glucose consumption is very similar (between days 5 and 10 of fermentation) (Figure 13A). At the end of the micro-vinification, IFO1802 accumulated 10.1 g/l of extracellular glycerol whilst T73 only reached 7.4 g/l.

To elucidate whether this difference was species specific or if it was only due to strain variability, micro-vinification experiments were carried out in synthetic must at 12 °C with several strains of both species. The amounts of accumulated extracellular glycerol are presented in Figure 13. FCry and EC1118, two commercial wine strains showing good performance in low temperature fermentations were selected as representative of *S. cerevisiae* wine strains. Moreover, seven additional *S. kudriavzevii* strains were assayed, since this species has been less studied in fermentations than *S. cerevisiae*. Normally dry wines produced by *S. cerevisiae* contain about 5 g/l of glycerol (Ribéreau-Gayon P, Dubourdieu D, Donèche B, 2006) and metabolic studies have observed decreases in extracellular glycerol production as temperature drops below 26 °C (Arroyo-López et al., 2010).

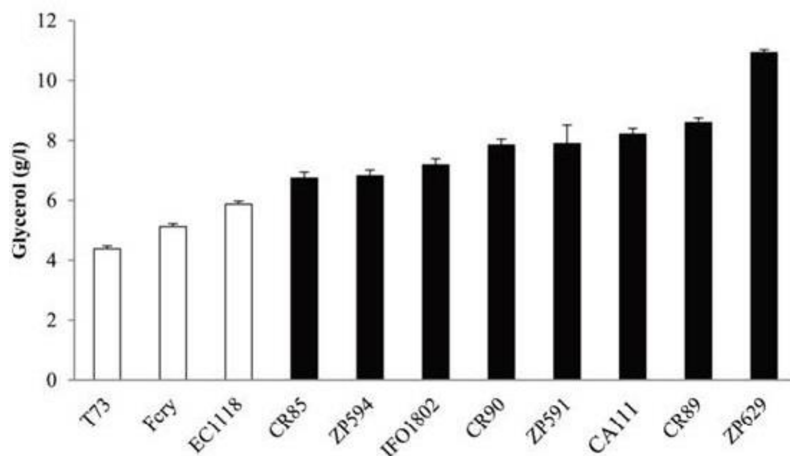

Figure 14. Micro-vinification experiments in synthetic must at low temperature with *S. kudriavzevii* (black bars) and *S. cerevisiae* (white bars) strains. Eight strains of *S. kudriavzevii* isolated in Japan (IFO1802), Portugal (ZP591, ZP594, ZP629) and Spain (CR85, CR89, CR90, CR111) were selected to compare with three *S. cerevisiae* (white bars) strains. Precultured cells were inoculated in synthetic must at 12 °C and samples were taken after sugar exhaustion to determine glycerol content for each species. Three independent micro-vinification bottles were used for each strain and average ± standard deviation is shown.

S. cerevisiae strains produced low amount of extracellular glycerol, between 4.4 and 5.8 g/l, in concordance to published levels (Ribéreau-Gayon P, Dubourdieu D, Donèche B, 2006) whereas *S. kudriavzevii* strains produced high levels of extracellular glycerol, between 7.0 and 10.9 g/l. The T73 strain produced the lowest amount of extracellular glycerol (4.4 ± 0.1) while *S. kudriavzevii* ZP629 presented the highest value (10.9 ± 0.6).

To study the relation between the increased glycerol accumulation observed in *S. kudriavzevii* strains and yeast cell resistance to osmotic stress we performed a drop test for two *S. cerevisiae* strains (T73 and EC1118) and four *S. kudriavzevii* strains (CA111, CR85, CR89 and ZP629) either at 28 or 12 °C in several osmotic stress conditions (sorbitol 1.5 and 1.8 M, KCl 1.25 M and NaCl 1.0 M). The results (Figure 15) suggest that increased extracellular glycerol levels do not necessarily mean increased osmotolerance since

strains CR89 and especially strain CA111, that present high levels of glycerol accumulation (Figure 14), showed lower osmotic stress resistance than *S. cerevisiae* strains, especially in KCl 1.25 M and in Sorbitol 1.8 M at 28 °C. On the other hand, ZP629, the strain with the highest glycerol production, showed the highest osmotolerance in most of the conditions tested (Figure 15). Combination of osmotic stress and growth at 12 °C produced a drastic reduction of yeast survival for all strains. In the mild osmotic stress KCl 1.25 M, only *S. kudriavzevii* strain CR85 was able to grow to some extent. Thus we can conclude that *S. kudriavzevii* strains accumulate higher amounts of glycerol that *S. cerevisiae* strains but this does not necessarily increase their osmotolerance.

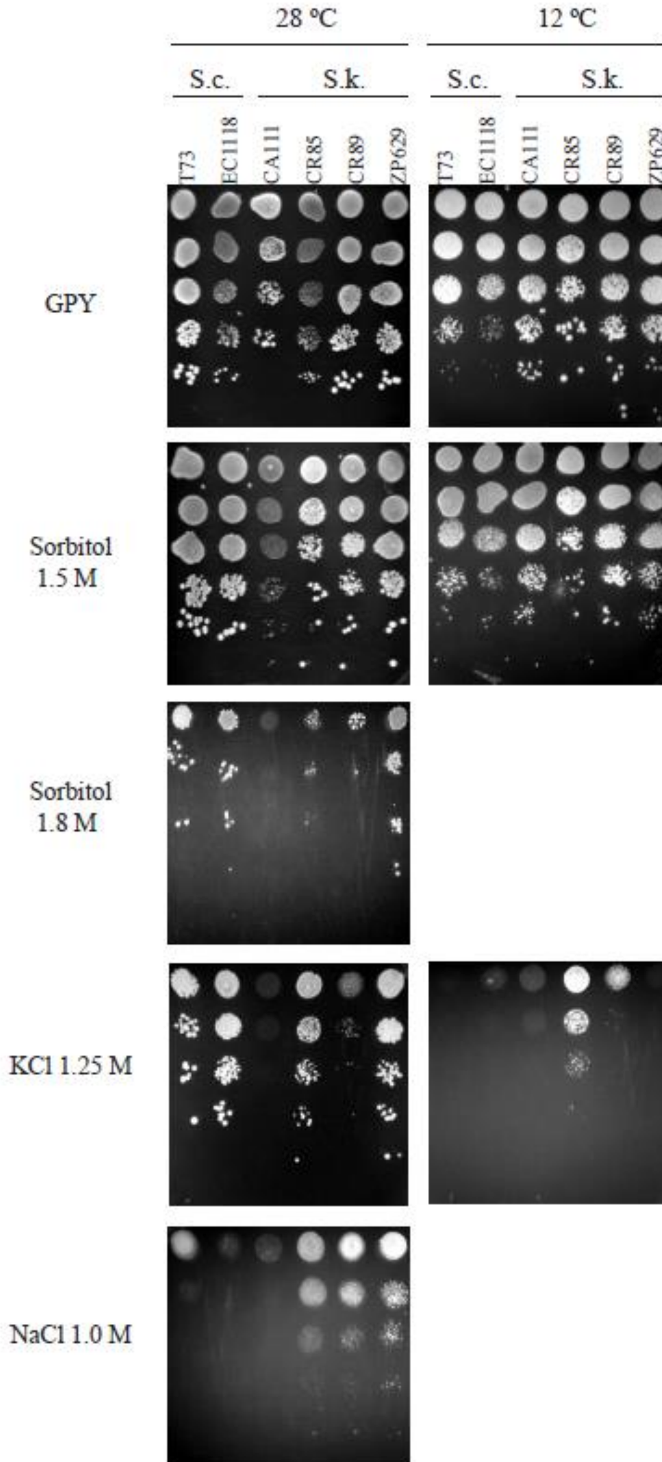

Figure 15. Evaluation of osmotolerance of different *S. cerevisiae* and *S. kudriavzevii* strains. After adjusting all strains to OD_{600}=0.3 of YPD batch cultures, 6 serial dilutions (1/5) of *S. cerevisiae* (T73 and EC1118) and *S. kudriavzevii* strains (CA111, IFO1802, CR85, CR89 and ZP629) where spotted on YPD with glucose or mannitol as a carbon source and with different osmotic stressors (Sorbitol 1.5 or 1.8 M; KCl 1.25 M; NaCl 1.0 M). No image is presented in the conditions where no growth was observed. Plates were incubated at 28 °C or 12 °C.

2.2 Variation of intracellular glycerol content with temperature.

To determine whether the higher amounts of extracellular glycerol produced by *S. kudriavzevii* strains reflect an increase in the production of this metabolite inside the cells in standard growth conditions, we measured the intracellular glycerol content in batch cultures at 28 or 12 °C in YPD at OD_{600}=1 (Figure 16).

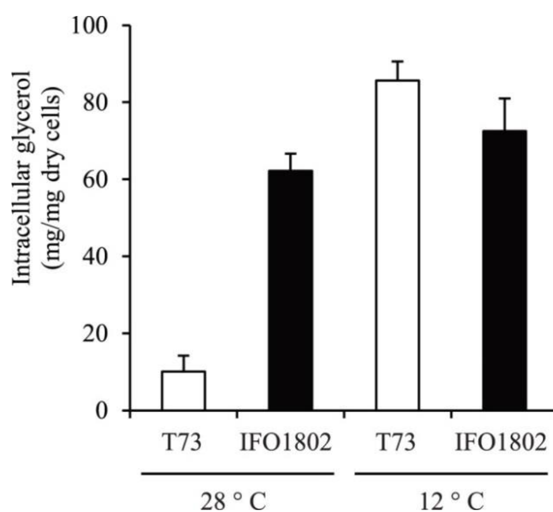

Figure 16. Intracellular glycerol determination for *S. kudriavzevii* (black bars) and *S. cerevisiae* (white bars) strains. A strain of *S. kudriavzevii* (IFO1802) was selected to compare with a *S. cerevisiae* (T73) strain the intracellular content of glycerol. Batch cultures at 12 or 28 °C in YPD medium were performed until OD_{600}=1. Then, cells were recovered by filtration, washed and glycerol was measured in the cell extracts. Three independent batches were used for each strain and averages ± standard deviation are normalized against *S. cerevisiae* value and expressed as µg of glycerol per mg of yeast cells, dry weight.

The IFO1802 intracellular glycerol content was 6.2 times higher than T73 strain at 28 °C. However no significant differences were observed at 12 °C between the two strains. *S. kudriavzevii* maintain elevated intracellular glycerol levels at both temperatures whereas *S. cerevisiae* strain is able to

increase intracellular glycerol content in response to cold conditions as was previously described (Panadero et al., 2006).

2.3 Gene expression of glycerol synthesis related genes.

In order to test whether glycerol accumulation in *S. kudriavzevii* was related to gene expression, we studied genes related to glycerol synthesis by the qPCR technique during the first days of synthetic must fermentation at 12 ºC. We focused our analysis on the two genes encoding glycerol-3-phosphate dehydrogenase (GPDH) isoforms *GPD1* and *GPD2,* and in the two genes encoding glycerol-3-phosphatase (GPP) isoforms: *GPP1*/*RHR2* and *GPP2*/*HOR2*. As can be seen in Figure 17, *GPD2, GPP2* and *GPP1* presented similar or reduced mRNA levels in some time points in strain IFO1802 compared to T73. In contrast, the expression of *GPD1* exhibited an increased level (between 3.1-3.8 fold at 0, 48 or 72 h time points) in the *S. kudriavzevii* strain IFO1802 (Figure 17).

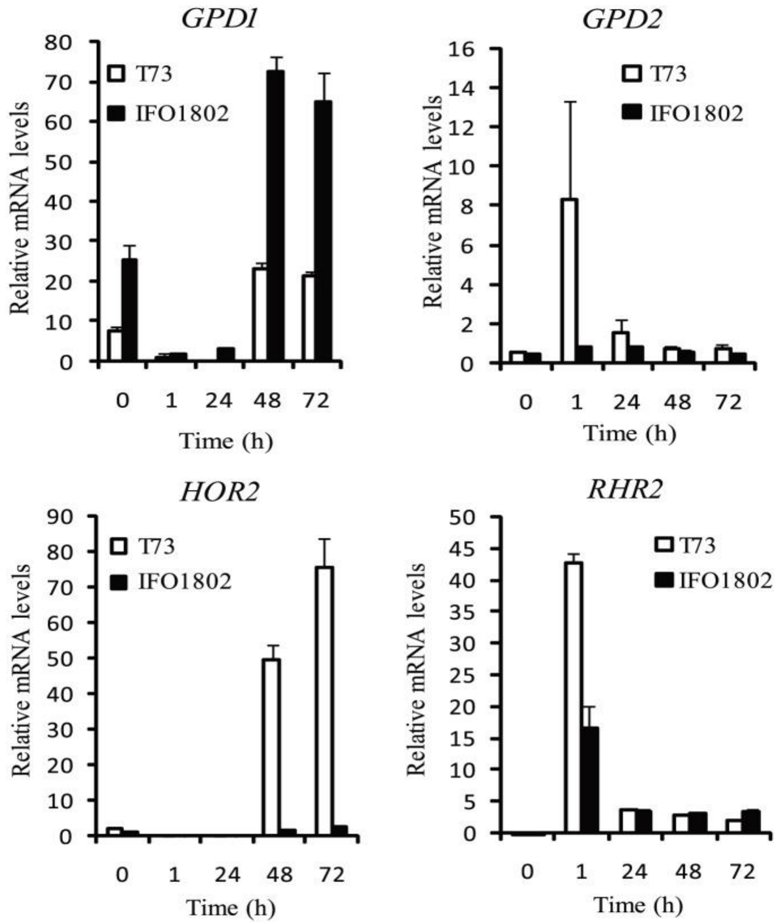

Figure 17. Expression of glycerol biosynthetic genes during first hours of low temperature micro-vinifications in synthetic must for *S. kudriavzevii* (black bars) and *S. cerevisiae* (white bars). *S. kudriavzevii* (IFO1802) was compared to *S. cerevisiae* (T73) in genes responsible for the first (*GPD1*, *GPD2*) and second (*GPP1, GPP2*) steps of specific glycerol biosynthetic pathway. Samples were taken in the first part of synthetic must micro-vinifications at 12 °C. After RNA extraction, expression of the different genes was determined by qPCR and values were normalised with *ACT1* constitutive gene and absolute levels are shown. Three independent micro-vinification bottles were used for each strain and averages ± standard deviation are shown.

In the micro-vinification experiments, cells are subjected to anaerobiosis and different stress conditions, mainly osmotic and cold stress (Ribéreau-Gayon P, Dubourdieu D, Donèche B, 2006). All these conditions

have been described to increase *GPD1* expression in *S. cerevisiae* (Albertyn et al., 1994; Ansell et al., 1997; Izawa et al., 2004; Panadero et al., 2006). To study *S. kudriavzevii GPD1* activation in response to classical stress induction experiments were performed to compare with *S. cerevisiae*. The *S. cerevisiae* laboratory diploid strain BY4743 background was used to take advantage of the deletion mutant collection and because it has been used as a laboratory model strain in many studies (Panadero et al., 2006). We performed batch cultures with BY4743 and IFO1802 strains and subjected the cells to osmotic stress (Figure 18A), cold stress (Figure 18B) or anaerobiosis (Figure 18C) in standard laboratory conditions. The results shown in Figure 23 indicated an increase in *GPD1* gene expression in response to osmotic (Figure 18A) and cold stress (Figure 18B) in the *S. cerevisiae* strain, as described previously (Ansell et al., 1997; Panadero et al., 2006). The *S. kudriavzevii* strain presented also an early activated expression (0-1 h) in response to osmotic stress but levels were decreased in the later time points (2-8 h) (Figure 18A). IFO1802 *GPD1* gene also showed lower mRNA levels in response to cold (Figure 18B) or anaerobic stress (Figure 18C) compared to *S. cerevisiae*. *GPD2* gene expression levels in response to anaerobiosis were also tested (Figure 18C). *S. cerevisiae* showed higher levels in response to anaerobiosis whereas *S. kudriavzevii* strain showed higher *GPD2* mRNA levels in osmotic stress compared to *S. cerevisiae*. All these data suggest that glycerol synthesis related genes are regulated differently in *S. cerevisiae* and *S. kudriavzevii.*

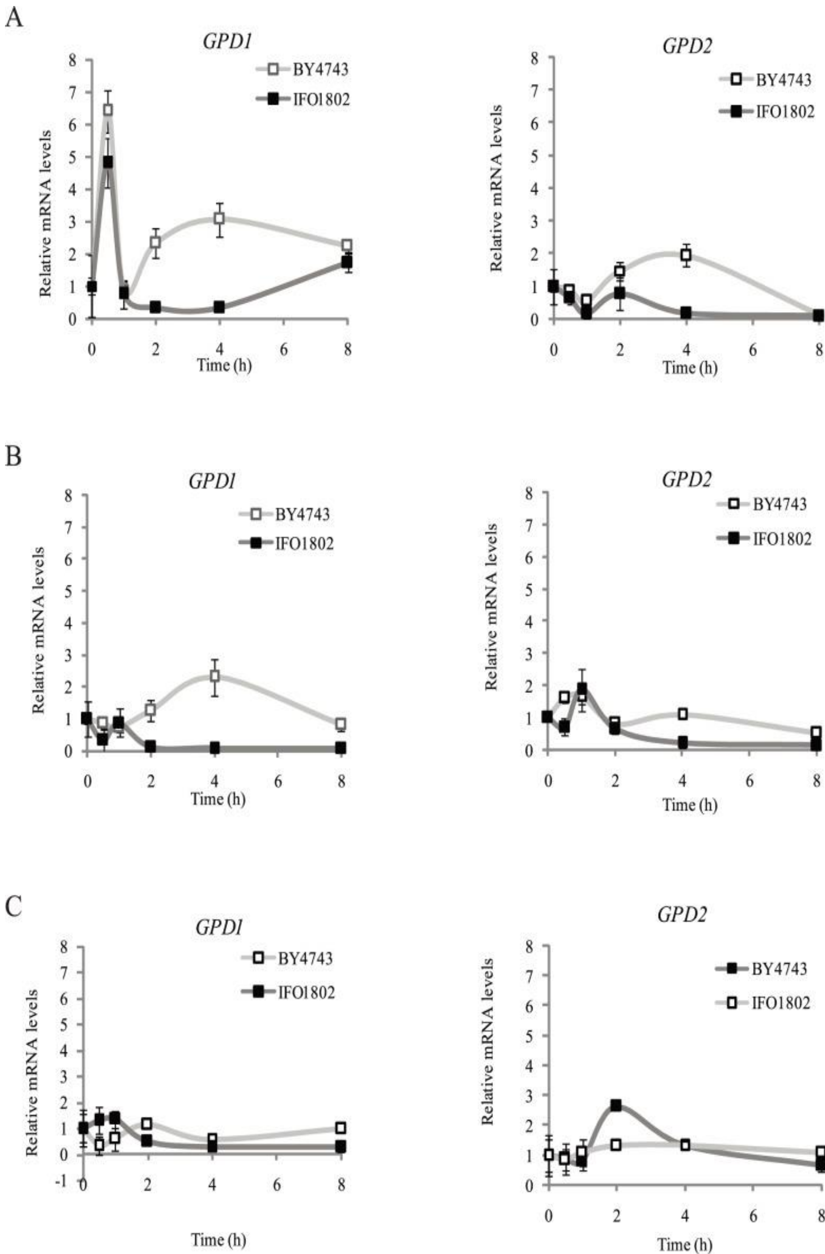

Figure 18. Expression of glycerol biosynthetic genes after osmotic (A), cold (B), and anaerobic stress (C) in laboratory conditions for *S. kudriavzevii* (black squares) and *S. cerevisiae* (white squares). A strain of *S. kudriavzevii* (IFO1802) was selected to compare with a *S. cerevisiae* (BY4743) strain the expression of the genes responsible for the first (*GPD1*, *GPD2*) steps of specific glycerol biosynthetic pathway. Batch cultures at 28 °C in YPD medium were grown until OD_{600}=1. Then, cells were transferred

to 1 M sorbitol YPD (A), to 12 ºC pre-cold YPD (B) or to YPD in bottles without O_2 (N_2 bubbled until saturation) (C) and samples were taken after 0, 0.5, 1, 2, 4 and 8 h. After RNA extraction, expression of the different genes was determined by qPCR and values were normalized with *ACT1* constitutive gene and relativized to time point 0 h. Three independent flask or bottles were used for each strain and averages ± standard deviation are shown.

2.4 Increased GPDH activity in *S. kudriavzevii*.

GPDH activity has been well correlated with glycerol production since this enzyme has a flux control coefficient of approximately 0.85 (Cronwright et al., 2002). Since higher glycerol production could be the result of increased activity of either isoform (Michnick et al., 1997), we were also interested in testing GPDH activity in response to specific stresses for both species. The results in Figure 20 show that IFO1802 exhibits significantly higher GPDH activity after osmotic (2.6, 5.2 and 3.6 fold after 2, 4 and 8 hour respectively) (Figure 20A) and cold stresses (9.7, 19.9 and 2.2 fold after 2, 4 and 8 hour respectively) (Figure 20B). It is worth noting that increased GPDH activity is observed in *S. kudriavzevii* in cold stress (Figure 20), whereas no *GPD1* mRNA increase was detected (Figure 18B).

To check Gpd2p contribution to the GPDH activity determination, we performed the same experiment with the BY4743gpd1Δ strain and no significant differences ($p<0.05$) were observed comparing to wild type strain after 2 or 4 h, although a possible contribution of Gpd2p cannot be completely discarded, especially after 8 h after osmotic stress and in cold stress samples. Increased GPDH activity in *S. kudriavzevii* can be a consequence of increased content of the Gpd1 protein or it can be due to enhanced kinetic properties of Gpd1p enzyme. New experiments were performed to explore this later possibility.

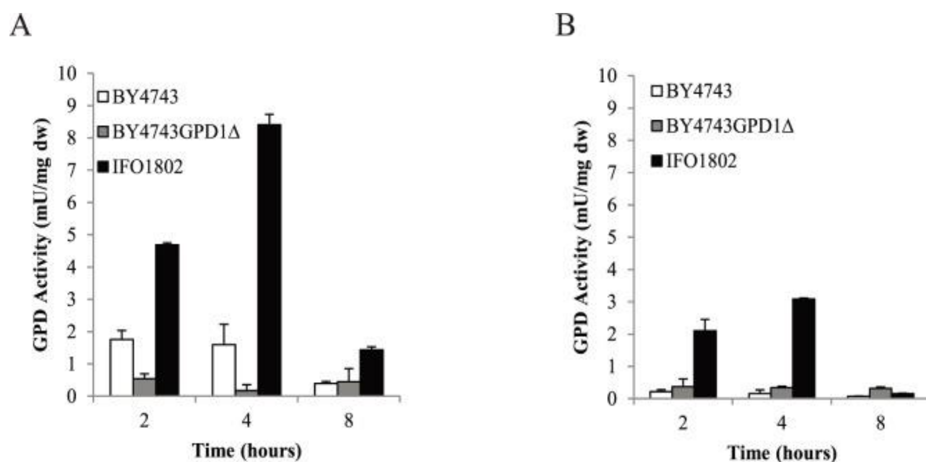

Figure 20. Determination of glycerol-3-phosphate 1 activity after osmotic (A) and cold stress (B) in laboratory conditions for *S. kudriavzevii* (black bars) and *S. cerevisiae* (white bars). A strain of *S. kudriavzevii* (IFO1802) was selected to compare with a *S. cerevisiae* (BY4743) strain the Gpd1p activity. A *S. cerevisiae* strain without *GPD1* gene (BY4743GPD1Δ, grey bars) was included to evaluate Gpd2p contribution to total activity. Batch cultures at 28 °C in YPD medium were performed until OD_{600}=1. Then, cells were transferred to 1 M sorbitol YPD YPD (A) or 12 °C pre-cold (B) and samples were taken after 2, 4 and 8 h. Gpd1p activity was determined in the cell extract and values were normalised with total protein content. Specific activity is expressed as miliunits per mg of proteins ($mU \cdot mg^{-1}$). Three independent batches were used for each strain were performed in triplicate and averages ± standard deviation are shown.

2.5 Gpd1p sequence and structure modeling

To test if the differences in Gpd1p activity between the two species were somehow related to differences in the protein sequence or structure we compared Gpd1p sequences of 24 *S. cerevisiae* strains obtained from the SGD database and three *S. kudriavzevii* strains, two obtained from the database (IFO1802 and ZP591) and one sequence obtained by us (Gene Bank accession number KF700356) from strain CR89. Intraspecific changes were not observed; however Gpd1p from *S. kudriavzevii* presented five conserved amino acid replacements compared to *S. cerevisiae* (Ala31Ile, Ile67leu, Glu76Asp, Asp142Asn and Ser143Pro) out of 391 total residues,

corresponding to an identity of 98.7 % (Figure 20). Two of these changes (Glu76Asp and Ser143Pro) were also observed in *S. bayanus*. This elevated identity was expected since this enzyme is highly conserved among yeast strains and even within eukaryotes. To determine whether any of the five changes affected the tertiary structure of the Gpd1p enzyme, we performed structure modeling and we compared the enzymes from the two species (Figure 21A).

```
#S._cerevisiae_S288C     MSAAADRLNL TSGHLNAGRK RSSSSVSLKA AEKPFKVTVI GSGNWGTTIA KVVAENCKGY
#S._paradoxus_Y-17217    .......... .......... .......... .......... .......... .......Q..
#S._bayanus_623-6c       .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_ZP591   .......... .......... .......... V......... .......... ..........
#S._kudriavzevii_CR89    .......... .......... .......... V......... .......... ..........
#S._kudriavzevii_IFO1802 .......... .......... .......... V......... .......... ..........

#S._cerevisiae_S288C     PEVFAPIVQM WVFEEEINGE KLTEIINTRH QNVKYLPGIT LPDNLVANPD LIDSVKDVDI
#S._paradoxus_Y-17217    ......T... .......... .......... .......... .......... ..........
#S._bayanus_623-6c       ......E... .....D.... .......... .......N.. .......... ..........
#S._kudriavzevii_ZP591   ......L... .....D.... .......... .......... .......... ..........
#S._kudriavzevii_CR89    ......L... .....D.... .......... .......... .......... ..........
#S._kudriavzevii_IFO1802 ......L... .....D.... .......... .......... .......... ..........

#S._cerevisiae_S288C     IVFNIPHQFL PRICSQLKGH VDSHVRAISC LKGFEVGAKG VQLLSSYITE ELGIQCGALS
#S._paradoxus_Y-17217    .......... .......... .......... .......... .......... ..........
#S._bayanus_623-6c       L......... ....G..... ..P....... .......... .......... ..........
#S._kudriavzevii_ZP591   .......... .......... .NP....... .......... .......... ..........
#S._kudriavzevii_CR89    .......... .......... .NP....... .......... .......... ..........
#S._kudriavzevii_IFO1802 ....L..... .......... .NP....... .......... .......... ..........

#S._cerevisiae_S288C     GANIATEVAQ EHWSETTVAY HIPKDFRGEG KDVDHKVLKA LFHRPYFHVS VIEDVAGISI
#S._paradoxus_Y-17217    .......... .......... .......... .......... .......... ..........
#S._bayanus_623-6c       .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_ZP591   .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_CR89    .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_IFO1802 .......... .......... .......... .......... .......... .........M

#S._cerevisiae_S288C     CGALKNVVAL GCGFVEGLGW GNNASAAIQR VGLGEIIRFG QMFFPESREE TYYQESAGVA
#S._paradoxus_Y-17217    .......... .......... .......... .......... .......... ..........
#S._bayanus_623-6c       .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_ZP591   .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_CR89    .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_IFO1802 .......... .......... .......... .......... .......... ..........

#S._cerevisiae_S288C     DLITTCAGGR NVKVARLMAT SGKDAWECEK ELLNGQSAQG LITCKEVHEW LETCGSVEDF
#S._paradoxus_Y-17217    .......... .......... .......... .......... .......... ..........
#S._bayanus_623-6c       .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_ZP591   .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_CR89    .......... .......... .......... .......... .......... ..........
#S._kudriavzevii_IFO1802 .......... .......... .......... .......... ...S...... ..........

#S._cerevisiae_S288C     PLFEAVYQIV YNNYPMKNLP DMIEELDLHE D*
#S._paradoxus_Y-17217    .......... .......... .......... ..
#S._bayanus_623-6c       .......... .......... .........G E.
#S._kudriavzevii_ZP591   .......... .......... .......... ..
#S._kudriavzevii_CR89    .......... .......... .......... ..
#S._kudriavzevii_IFO1802 .......... .......... .......... ..
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Figure 20. Alignment of Gpd1p sequences from different *Saccharomyces* species. The sequences of *S. cerevisiae* strain S288C, *S. paradoxus* strain Y-17217, *S. bayanus* strain 623-6c and *S. kudriavzevii* strains ZP591 and CR89 were aligned using MEGA software. Amino acidic variations comparing with S288C are shown.

Although both Gpd1p versions adopt very similar conformations, maintaining main secondary structures, several residues involved in the catalytic active site (Arg310, Asn246, Thr305, Lys245, Lys152, Asp205) showed positional differences ranging from 1.0 to 2.4 Å (supplementary Figure 21B). This study suggested that, among the five residues that are different between *S. cerevisiae* and *S. kudriavzevii*, the two residues that can have more influence in the catalytic properties are in positions 142 and 143, which are close to other residues involved in NAD binding (Phe129 and Lys152) (Figure 21C).

2.6 Gpd1p from *S. kudriavzevii* exhibits a higher V_{max}.

We studied whether the differences in glycerol accumulation observed between *S. kudriavzevii* and *S. cerevisiae* could be explained by differences in the catalytic properties of the Gpd1p enzyme. To that end, kinetic assays were performed to determine K_m and V_{max} of *S. cerevisiae* and *S. kudriavzevii* Gpd1p enzymes. The K_m and V_{max} for the two substrates, dihydroxyacetone phosphate (DHAP) and NADH, was performed. The assay provided reproducible and consistent data, since values are within the range previously described for the purified enzyme (Ansell et al., 1997).

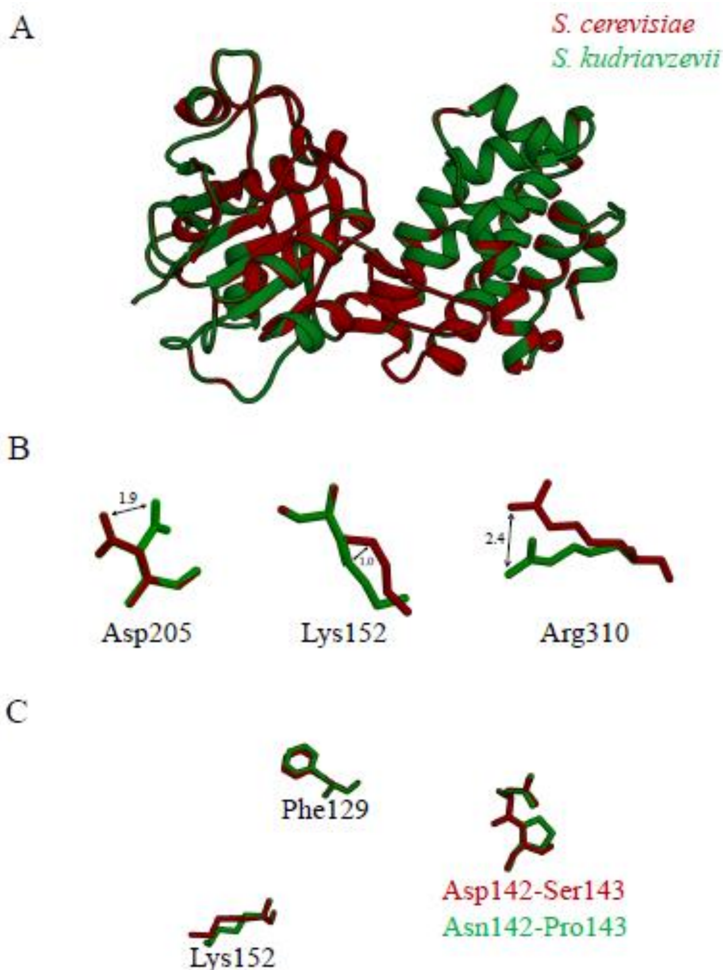

Figure 21. Comparison of *S. cerevisiae* and *S. kudriavzevii* Gpd1p three-dimensional structure models. The whole protein model is compared in panel A showing *S. cerevisiae* variant in red and *S. kudriavzevii* in green. Panel B compares side-chain differential position of Lys152, Asp205 and Arg310, three amino acids involved in the catalytic center. Panel C shows side-chains and relative position of dipeptides 142-143 respect to amino acids Phe129 and Lys152, involved in NADH binding. Models were built using MODWED online server based on Modeller software. Structures were visualized with Pymol viewer.

To perform the experiment BY4741gpd1Δ strain was complemented with multicopy expression plasmids containing (pYES) either the *S. cerevisiae* or *S. kudriavzevii GPD1* genes under the strong promoter pGAL1. Overnight precultures were used to inoculate exponential cultures in YPD galactose

medium. As a control we checked that no GPD activity was observed in the BY4741gpd1Δ strain without plasmid in the induction conditions. V_{max} and K_m values determined for both Gpd1p substrates were calculated (Table 3). V_{max}^{DHAP} determinations revealed significant differences between the two species whereas K_m^{NADH}, K_m^{DHAP} and V_{max}^{NADH} did not differ significantly. The higher catalytic rate of *S. kudriavzevii* Gpd1p, together with the overexpression of its encoding gene, may explain that *S. kudriavzevii* has a generally higher production of glycerol than *S. cerevisiae*. Taking into account that Gpd1p is the flux controlling enzyme in the pathway, the effect of increased V_{max}^{DHAP} on the glycerol accumulation could be highly significant, even if the increase is only around 20%.

Table 3. Gpd1p kinetic parameters.

	DHAP		NADH	
	K_m	V_{max}	K_m	V_{max}
BY4741-*GPD1*- *S. cerevisiae*	0,54±0,06	87,34±4,5	0,051±0,02	48,85±3,9
BY4741-*GPD1*- *S. kudriavzevii*	0,61±0,05	108,0±4,1*	0,11±0,02*	58,91±3,8

*$p < 0.05$ significant differences *S. cerevisiae* versus *S. kudriavzevii*.

2.7 *Gpd1p* from *S. kudriavzevii* produces more extracellular glycerol

In order to evaluate the metabolic effect of the presence of a *S. cerevisiae* or *S. kudriavzevii* Gpd1p enzyme, we expressed, with the low copy plasmid pGREG526, the *GPD1* gene of *S. cerevisiae* (BYp*GPD1*$_{Scer}$) or *S. kudriavzevii* (BYp*GPD1*$_{Skud}$) under their own promoter in the background strain BY4741gpd1Δ. These strains and the BY4741 wild type containing the empty vector (BYp), were inoculated in selective minimal media with 10 % glucose and the glycerol was measured after sugar exhaustion by HPLC. The experiment was performed at 12 and 28 °C. The results (Figure 22) showed that expression of either gene increased the amount of extracellular glycerol

produced at both temperatures. Interestingly, the strain containing *S. kudriavzevii* Gpd1p enzyme produced 22.8% more extracellular glycerol than the one with *S. cerevisiae* Gpd1p at 28 ºC and 24.9% more at 12 ºC (Figure 22), a significant increment comparable to the difference in V_{max}^{DHAP} observed between both enzymes. In order to elucidate if the increased extracellular glycerol accumulation was because of the changes in two residues (142 and 143) situated in the vicinity of the NAD binding site, we constructed a strain (BYp$GPD1_{Sce\text{-}Skud}$) containing the *S. cerevisiae GPD1* promoter next to a recombinant *S. cerevisiae-S. kudriavzevii GPD1* coding sequence containing the residues 142 and 143 from *S. kudriavzevii* (Figure 22) and performed the same experiment.

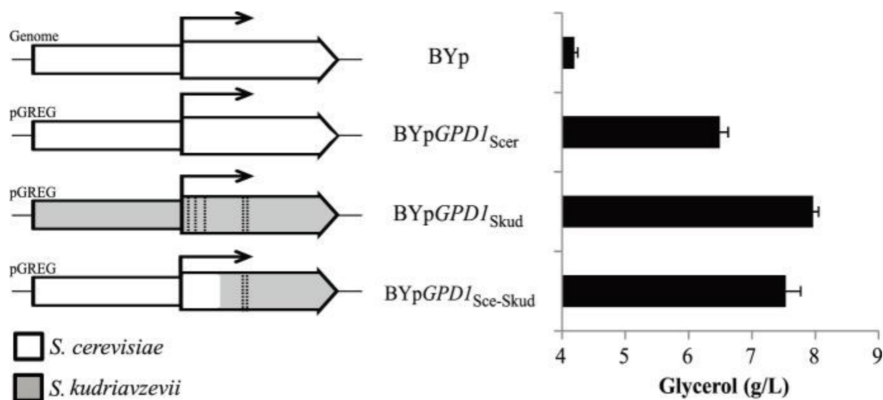

Figure 22. Glycerol production in strains with different versions of *GPD1*. Strains with wild type *GPD1* (BYp) or plasmid containing *GPD1* from *S. cerevisiae* (BYp$GPD1_{Scer}$), *S. kudriavzevii* (BYp$GPD1_{Skud}$) or a recombinant *S. cerevisiae - S. kudriavzevii* coding sequence (BYp$GPD1_{Sce\text{-}Skud}$), were grown in SC-Ura 10% glucose media until sugar exhaustion at 28 or 12 ºC. Aminoacidic changes observed in the *S. kudriavzevii* enzyme are represented as vertical black lines. Extracellular metabolites were determined with HPLC method. Biological triplicates were performed and averages ± standard deviation are shown.

The results (Figure 22) showed that BYp$GPD1_{Sce\text{-}Skud}$ also produced a significant ($p<0.001$) increase in glycerol accumulation (16.0%) respect to

BYp*GPD1*$_{Scer}$ at 28 °C. The glycerol produced with BYp*GPD1*$_{Sce-Skud}$ and BYp*GPD1*$_{Skud}$ at 28 °C was not significantly different suggesting that changes in residues 142 and 143 are sufficient to explain increased glycerol accumulation at this temperature. Also, the glycerol produced with BYp*GPD1*$_{Sce-Skud}$ at 12 °C was not significantly different to BYp*GPD1*$_{Scer}$. It is interesting to note, that no significant differences were observed in the ethanol production among the different strains (results not shown).

DISCUSSION

The industrial relevance of glycerol production by yeasts, especially for wine production, launched many studies that tried to increase it. Classic examples were the increase in *GPD1* expression and other genes to counteract the side-effect of higher acetate production (Cambon et al., 2006; Eglinton et al., 2002; Ehsani et al., 2009). This study was focused in *S. kudriavzevii* species which naturally produces increasing glycerol levels compared to *S. cerevisiae*. As our results showed, the increased extracellular glycerol synthesis at low temperatures is present in many *S. kudriavzevii* strains isolated from different regions and therefore it is a species-specific trait. The different metabolism in the two species can be explained by increased expression of metabolic genes underlying increased glycerol accumulation in *S. kudriavzevii*. In fact, we observed increased *GPD1* gene expression during must fermentation in *S. kudriavzevii* compared to *S. cerevisiae*. Unexpectedly, the genes *GPP1*, *GPP2* and *GPD2* showed decreased expression levels which may be explained by the evolution of *S. cerevisiae* to elevated levels of regulation of glycerol biosynthesis. However, we have also observed other differences at the protein level, which may contribute to the increase in glycerol production. *S. kudriavzevii* presents increased GPDH specific activity compared to *S. cerevisiae* in different laboratory conditions.

Tertiary structure prediction revealed certain differences suggesting potential disparity in enzymatic activity and in kinetic properties. Taking into account the structural differences detected, we observed an increased V_{max} in *S. kudriavzevii* Gpd1 enzyme compared to *S. cerevisiae*, which can explain the increased specific activity and therefore the increased glycerol levels. Also, previous work has described increased *S. kudriavzevii* GPDH activity respect to *S. cerevisiae,* especially in cold stress conditions (Arroyo-López et al., 2010), supporting our model. Finally, direct comparison of the two *GPD1* and a recombinant version in the same background let us determine that the changes in the residues 142 and 143 of *S. kudriavzevii* have a significant impact in the increased ability of the enzyme to produce glycerol, although residues 31, 67 and 76 are relevant for low temperature glycerol production. Intracellular glycerol accumulation and Gpd1p activity was high in *S. kudriavzevii* at 12 but also at 28 °C. This suggests that, as a consequence of *S. kudriavzevii* adaptation to low temperatures, this species has high flux to glycerol biosynthetic pathway even at high temperatures.

Glycerol production is a key process to resist osmotic stress in yeast cells (Albertyn et al., 1994; Ansell et al., 1997; Pahlman et al., 2001). Some *S. kudriavzevii* strains showed a correspondence between osmotolerance and glycerol levels produced after glucose fermentation but other strains did not (Figure 14), (Belloch et al., 2008). This may reflect that osmotolerance depends as well on other key players. For example, upon hyperosmotic shock, cells first mobilize a rapid rescue system that prevents excessive loss of ions and water. The potassium antiporters Nha1p and Nhx1p are implicated in response to cell shrinkage upon osmotic stress and their presence in cells is important for recovery from sudden exposure to hyperosmotic media (Kinclova-Zimmermannova and Sychrova, 2006; Nass and Rao, 1999). Thus, changes in efficiency of any of those players can explain why some *S. kudriavzevii* strains that show increased glycerol accumulation are not more osmotolerant than *S. cerevisiae* strains. So, new studies on the functional

differences in any of those proteins will be important to understand the glycerol level differences among the two species.

We have studied the regulation of glycerol synthesis in *S. kudriavzevii* compared to *S. cerevisiae* at low temperatures. The results reveal important differences between the two species, supporting a significant disparity in the central carbon metabolism, probably due to adaptation to specific environments. In this respect, we showed in a recent work (Salvadó et al., 2011) that temperature has influenced the evolution of the *Saccharomyces* genus, favoring the adaptation of some species to grow at either lower (*S. kudriavzevii*) or higher (especially *S. cerevisiae*) temperatures. We hypothesized that, in a first evolutionary event, *S. cerevisiae* and other species diverged from *S. kudriavzevii* and *S. uvarum,* which are better adapted to grow at low temperatures. In fact, all these diversifications were preceded by whole genome duplication (WGD), which increased glycolytic flux (Conant and Wolfe, 2007). They suggested that this bestows selective advantages on yeast when competing for resources to growth in conditions with high sugar levels. Not with standing, we hypothesize that *Saccharomyces* species developed two main strategies after the WGD to fine tune its metabolism and adapt to different niches. Some species, like *S. kudriavzevii*, derived this increased glycolytic flux towards the production of elevated levels of cryoprotectanct glycerol. This strategy has enabled them to adapt to low temperature environments and maintain the NAD^+/NADH ratio in alcoholic fermentations. However, other species like *S. cerevisiae* took advantage of the increased glycolytic flux and promoted increased levels of ethanol production. Other authors (Salvadó et al., 2011) have proposed that *S. cerevisiae* followed this evolutionary strategy to better compete with other microorganisms for resources. It is reasonable to suppose that *S. cerevisiae* has developed a much tighter regulation of the glycerol biosynthetic pathway to redirect the glycolytic flux from glycerol to ethanol and maximize its production. In fact our results reveal high complexity levels in *S. cerevisiae*

on the regulation of glycerol biosynthetic pathway genes. In *S. cerevisiae*, *GPD2, GPP1* and *GPP2* expression is induced during must fermentation at certain time points, whereas in *S. kudriavzevii* these genes show lower or no induction. The same occurs when we study stress response where lower or no inductions where observed for *GPD1* or *GPD2* genes.

In conclusion, the species *S. kudriavzevii* is able to produce high levels of glycerol and grow at low temperatures. The data obtained in this work place *S. kudriavzevii* adaptation mainly at the Gpd1p enzymatic level. By contrast, *S. cerevisiae* evolution is linked more closely to the increased gene expression regulation of glycerol synthetic pathway genes. The comparison of our data with data obtained for other *Saccharomyces* species will shed more light on the adaptive mechanisms of these yeasts. This work can have a relevant, practical, use, taking advantage of industrially relevant properties by using *S. kudriavzevii* or *S. cerevisiae - S. kudriavzevii* hybrids in wine production (González et al., 2008, 2007; Lopes et al., 2010; Sampaio and Gonçalves, 2008; Tronchoni et al., 2009).

CHAPTER 2. Alternative glycerol balance strategies among *Saccharomyces* species in response to stress.

This chapter includes the following article submitted for publication, detailed below, and make up this thesis with the prior authorization of all authors.

Microbial Cell Factories

Alternative glycerol balance strategies among Saccharomyces species in response to stress

--Manuscript Draft--

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Article Type:	Research
Section/Category:	Food microbiology
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Abstract:	Background Production and balance of glycerol is essential for the survival of yeast cells in certain stressful conditions as hyperosmotic or cold shock that occur during industrial processes as winemaking. These stress responses are well known in S. cerevisiae, however, little is known in other phylogenetically close related Saccharomyces species associated with natural or fermentation environments such as S. uvarum, S. paradoxus or S. kudriavzevii. Results In this work we have investigated the expression of four genes (GPD1, GPD2, STL1 and FPS1) crucial in the glycerol pool balance in the four species with a biotechnological potential (S. cerevisiae; S. paradoxus; S. uvarum and S. kudriavzevii), and the ability of strains to grow under osmotic and cold stresses. The results show different pattern and level of expression among the different species, especially for STL1. We also studied the function of Stl1 glycerol symporter in the survival to osmotic changes and cell viability in winemaking environments. These experiments also revealed a different functionality of the glycerol transporters among the different species studied. Conclusions All these data point to different strategies to handle glycerol accumulation in response to hyperosmotic or cold-hyperosmotic stresses in the different species, with variable emphasis in the production, influx or efflux of glycerol.
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In the fermentation industry, especially winemaking, two major features are required for a yeast strain as the resistance to osmotic stress and the ability to grow at low temperatures (Pretorius et al., 2012). It is known that *S. cerevisiae* seeks to increase intracellular glycerol content when subjected to osmotic stress or cold in vinification or standard laboratory growth conditions (Oliveira et al., 2014; Panadero et al., 2006; Petelenz-Kurdziel et al., 2013). This intracellular accumulation is very important for osmotic equilibrium during the first phase of fermentation and to act as key cryoprotector agent for adaptation to cold environments allowing cellular viability with implications in the fermentation yield (Remize et al., 2001; Tulha et al., 2010). A rapid and specific activation of the gene expression have been identified as an essential mechanism in the *S. cerevisiae* cells to respond to acute stresses, such as those associated with the different industrial fermentation processes (de Nadal et al., 2011). However, little is known about these stress responses in other *Saccharomyces* species associated with natural or fermentation environments such as *S. uvarum* (Demuyter et al., 2004; Naumov et al., 2002; Rementeria et al., 2003), *S. paradoxus*, isolated from Croatian vineyards (Redžepović et al., 2002) or natural yeast hybrids between species of the genus *Saccharomyces* such as *S. cerevisiae* x *S. kudriavzevii* (González et al., 2007) and *S. cerevisiae* x *S. uvarum* (Le Jeune et al., 2007; Pérez-Torrado et al., 2015) which may participate in the fermentative processes. *S. uvarum* and *S. kudriavzevii* present important physiological traits like the ability to grow at lower temperatures and produce more glycerol than *S. cerevisiae* (Gamero et al., 2013; González et al., 2007; Oliveira et al., 2014). However, *S. paradoxus*, besides being a widely distributed yeast species mainly associated with natural environments and not very relevant in fermentations, is physiologically more similar to *S. cerevisiae* (Tronchoni et al., 2009).

It is well known that *S. cerevisiae* and other yeast species are capable to modulate the glycerol synthesis and its intracellular content in accordance

with environmental osmotic changes (Hohmann et al., 2007; Hubmann et al., 2011). They can also control an active glycerol import from the extracellular medium in symport with protons via Stl1 transporter (Dušková et al., 2015; Tulha et al., 2010). Besides its important role in osmoregulation, the Stl1 function is directly related to cell survival and adaptation to cold stress in *S. cerevisiae* strains (Tulha et al., 2010). The yeast cells may also regulate their glycerol content by controlling its efflux via the Fps1 channel (Luyten et al., 1995). This channel can be quickly closed avoiding the glycerol efflux, and thus contributing to an efficient osmoregulation with direct implications on increasing the fermentation yield (Wei et al., 2013).

The understanding of the phylogenetic and physiological relationships between *S. cerevisiae* and other *Saccharomyces* species, as well as main ecological, environmental and human factors that have driven the emergence of phenotypic changes among species of *Saccharomyces* genus, have been cleared in many works (Landry et al., 2006; Peris et al., 2014). Several studies have focused in understanding the cryophilic character of *S. uvarum* and specially *S. kudriavzevii* at the molecular level, including transcriptomic and metabolomic studies (Combina et al., 2012; López-Malo et al., 2013). Some aspects of *S. kudriavzevii* have been highlighted in relation to cold resistance and winemaking as membrane composition (Tronchoni et al., 2012), or translation efficiency (Tronchoni et al., 2014). However, little information about these species and the glycerol synthesis is available. In the case of *S. kudriavzevii*, the increased cold tolerance has been related to elevated glycerol synthesis as a consequence of increased expression and activity of Gpd1p in winemaking conditions (Oliveira et al., 2014). For this reason a better understanding of *Saccharomyces* species physiological and molecular features with potential biotechnological interest is needed.

Hence, in this work we decided to investigate the expression of genes crucial to the balance of glycerol (*GPD1*, *GPD2*, *STL1* and *FPS1*) in two yeast

strains of each of the four species with a biotechnological potential (*S. cerevisiae*; *S. paradoxus*; *S. uvarum* and *S. kudriavzevii*). We also studied the function of Stl1 glycerol symporter, in the survival to osmotic changes and cell viability in winemaking environments.

RESULTS

2.1 *Saccharomyces* species differ in tolerance to hyperosmotic and cold stresses.

The behaviour of *S. cerevisiae* and other *Saccharomyces* species interesting for industrial applications was evaluated in response to wine fermentation relevant stresses. We selected hyperosmotic (NaCl 0.8 M and KCl 1.25 M) and a combination of hyperosmotic and cold stresses (12 °C), two frequent suboptimal conditions during winemaking. We performed a drop test with two strains of each species (*S. paradoxus, S. cerevisiae, S. kudriavzevii,* and *S. uvarum*) on complete media and compared the growth in the above mentioned conditions (Figure 23). The results revealed that the used stresses have a very different effect on yeast growth depending not only on the species but even on the strain. The stress with KCl 1.25 M is the condition that has less effect on the yeast viability, and the NaCl 0.8 M plus 12 °C the most severe stress. The conditions NaCl 0.8M hyperosmotic stress and KCl 1.25 M at 12 °C hyperosmotic-cold stress generated intermediate viability levels. The results showed clearly that the strains can cope better with a higher osmotic stress (KCl 1.25 M) than with the sodium toxicity (NaCl 0.8 M). In hyperosmotic stress mediated by NaCl 0.8 M we observed that *S. uvarum* strains are the ones presenting the highest tolerance to hyperosmotic and a similar observation can be made in the most severe condition (NaCl 0.8 M plus 12 °C).

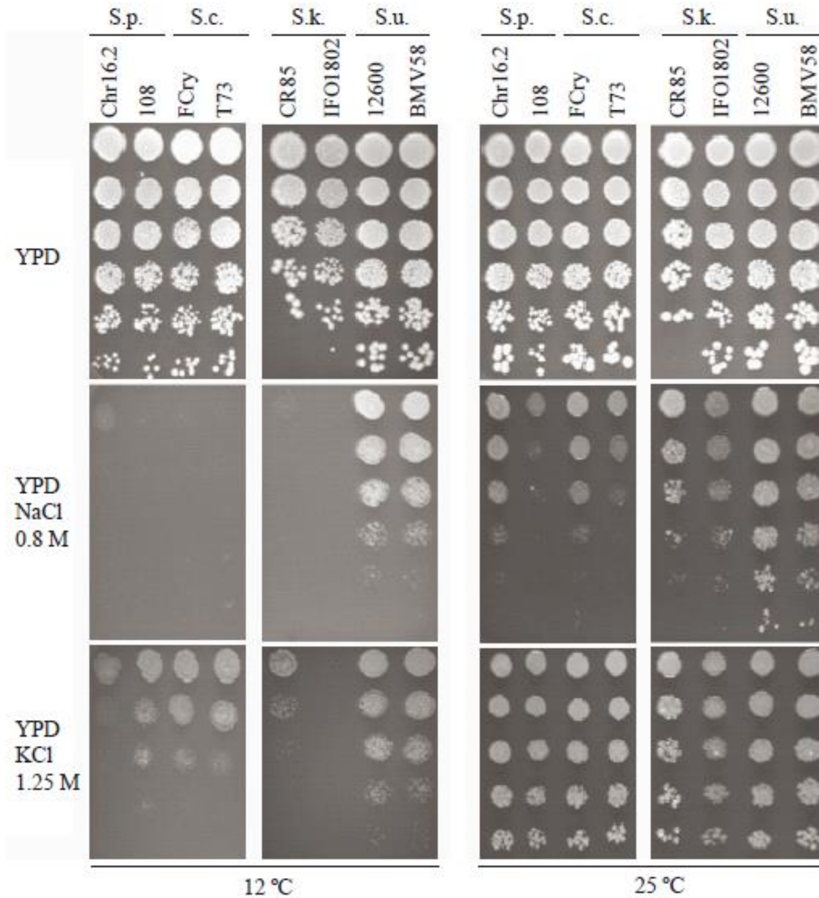


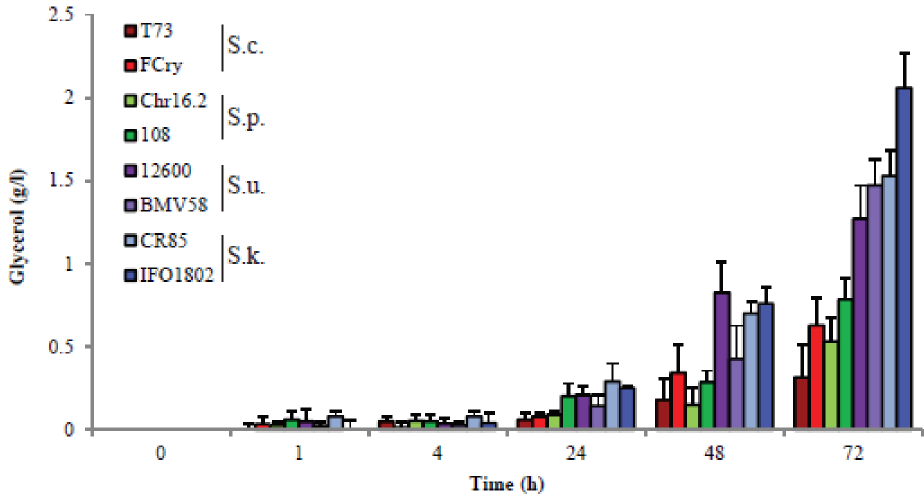
Figure 23. Osmotolerance of *S. paradoxus* (S.p., Chr16.2, 108), *S. cerevisiae* (S.c. FCry, T73), *S. kudriavzevii* (S.k., CR85, IFO1802) and *S. uvarum* (S.u.12600, BMV58) strains was estimated at 25 and 12 °C, respectively. Serial dilutions were plated in rich media with (YPD NaCl 0.8 M or KCl 1.25 M) or without (YPD) hyperosmotic stress. A representative image of biological triplicates is presented.

The other species showed similar behaviour although *S. kudriavzevii* strains showed low growth levels in cold stress condition, especially IFO1802 strain. *S. cerevisiae* and *S paradoxus* strains showed similar growth levels but strain 108 in NaCl 0.8 M and strain Chr16.2 in KCl 1.25 M presented lower growth levels than *S. cerevisiae* strains.

2.2 Glycerol levels during wine fermentation.

Since hyperosmotic and also cold stress responses are unequivocally related to glycerol accumulation we wanted to determine glycerol levels during hyperosmotic-cold stress in wine fermentations. Thus we performed wine fermentations in synthetic must with the studied *Saccharomyces* species and strains, and we measured intra- and extracellular amount of glycerol during the first hours and days of the fermentation. In the results presented in Figure 24 we observed two steps regarding glycerol accumulation in *S. cerevisiae* strains. In the first step, glycerol starts to accumulate inside the cell (Figure 24B) immediately after inoculating into the cold-hyperosmotic condition, reaching a maximal value after 24 h. Also, minimal glycerol levels are accumulated in extracellular media in the beginning of our experiment (Figure 24A). In the next two days, intracellular glycerol is reduced and tends to recover its original levels whereas extracellular glycerol increases with the time. In the case of *S. paradoxus* and *S. kudriavzevii*, maximal intracellular glycerol accumulation, which are approximately half of those in *S. cerevisiae* strains, occurs in the first hours and levels are maintained during 48 h. Analyzing the intracellular glycerol level (Figure 24B), it is interesting to note that, comparing with the other species, *S. cerevisiae* strains accumulated the higher levels of glycerol between 4 and 48 hours of incubation. The *S. uvarum* strains showed the lowest values of intracellular glycerol with a maximal level after 1 h in the case of BMV58 and after 48 h in the case of 12600. Regarding extracellular glycerol (Figure 24A), *S. paradoxus* presented similar levels and accumulation pattern as *S. cerevisiae* and *S. uvarum* and, in addition, *S. kudriavzevii* showed a similar pattern but higher accumulation levels (around 5 times more).

A



B

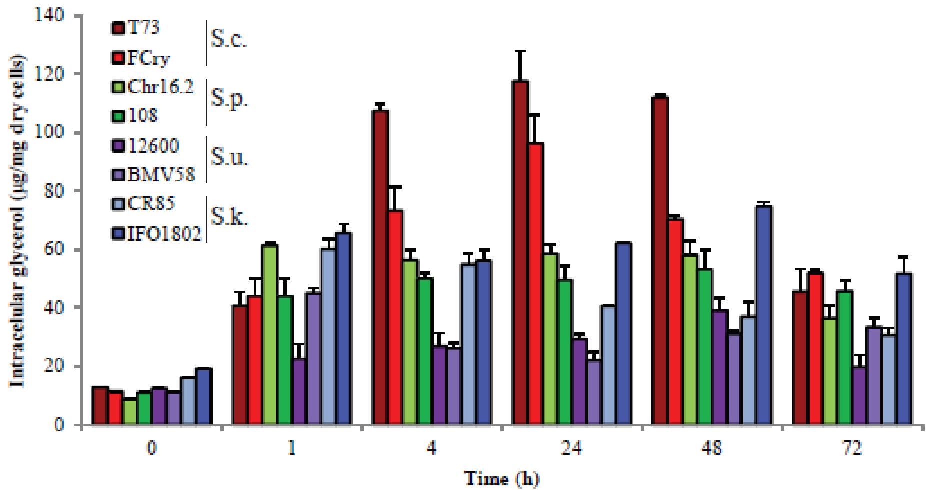


Figure 24. Microvinification experiments in synthetic must at low temperature with *S. cerevisiae* T73 (dark red) and FCry (light red), *S. paradoxus* Chr16.2 (light green) and 108 (dark green), *S. uvarum* 12600 (dark purple) and BMV58 (light purple) and *S. kudriavzevii* CR85 (light blue) and IFO1802 (dark blue) strains. Precultured cells were inoculated in synthetic must at 12 °C and samples were taken after 0, 1, 4, 24 and 48 hours to determine extra (A) and intracellular (B) glycerol content for each strain. Three independent microvinification bottles were used for each strain and average ± standard deviation is shown.

2.3 Changes in mRNA levels of genes related to glycerol balance during wine fermentation and hyperosmotic stress of different *Saccharomyces* species.

To gain insights on the regulation of glycerol pools balance we studied variation in mRNA levels of key genes related to glycerol biosynthesis (*GPD1* and *GPD2*), efflux (*FPS1*) and influx (*STL1*) in the same wine fermentation conditions described above and in the same strains and species. The results (Figure 25) clearly revealed different patterns and levels of gene expression among the species in all four genes studied. In the case of *GPD1*, all the strains showed a general pattern of induction after the first hour but with marked differences in the expression levels. *S. kudriavzevii* strains showed the highest mRNA levels, specially IFO1802 strain that presented elevated expression of *GPD1* before stress and even more after one hour of inoculation. For the *GPD2* gene, some of the strains presented an induction with maximal levels after four (*S. uvarum* strains, FCry, 108 and CR85) or 48 (T73) hours whereas other strains (Chr16.2 and IFO1802) seem to not activate this gene showing low mRNA levels. The *FPS1* gene expression peaked after one hour (108, CR85, *S. cerevisiae* and *S. uvarum* strains) or four hours (Fcry), with the *S. cerevisiae* and *S. uvarum* strains showing the highest levels. The IFO1802, Chr16.2 and *S. kudriavzevii* strains did not showed meaning activation of mRNA levels compared to the inoculum. Finally, the *SLT1* gene presented the most variable mRNA levels among the species showing highest values for the *S. uvarum* strains, especially BMV58, with a maximum after one hour. Other species showed a moderate amount of mRNA with maximum levels after one hour (*S. kudriavzevii* strains) or 4 hours (Fcry). *S. paradoxus* strains showed very low *SLT1* mRNA levels along the experiment. These results emphasize the importance of *GPD1* and *STL1* in *S. kudriavzevii* and *S. uvarum* respectively, regarding their increased glycerol accumulation.

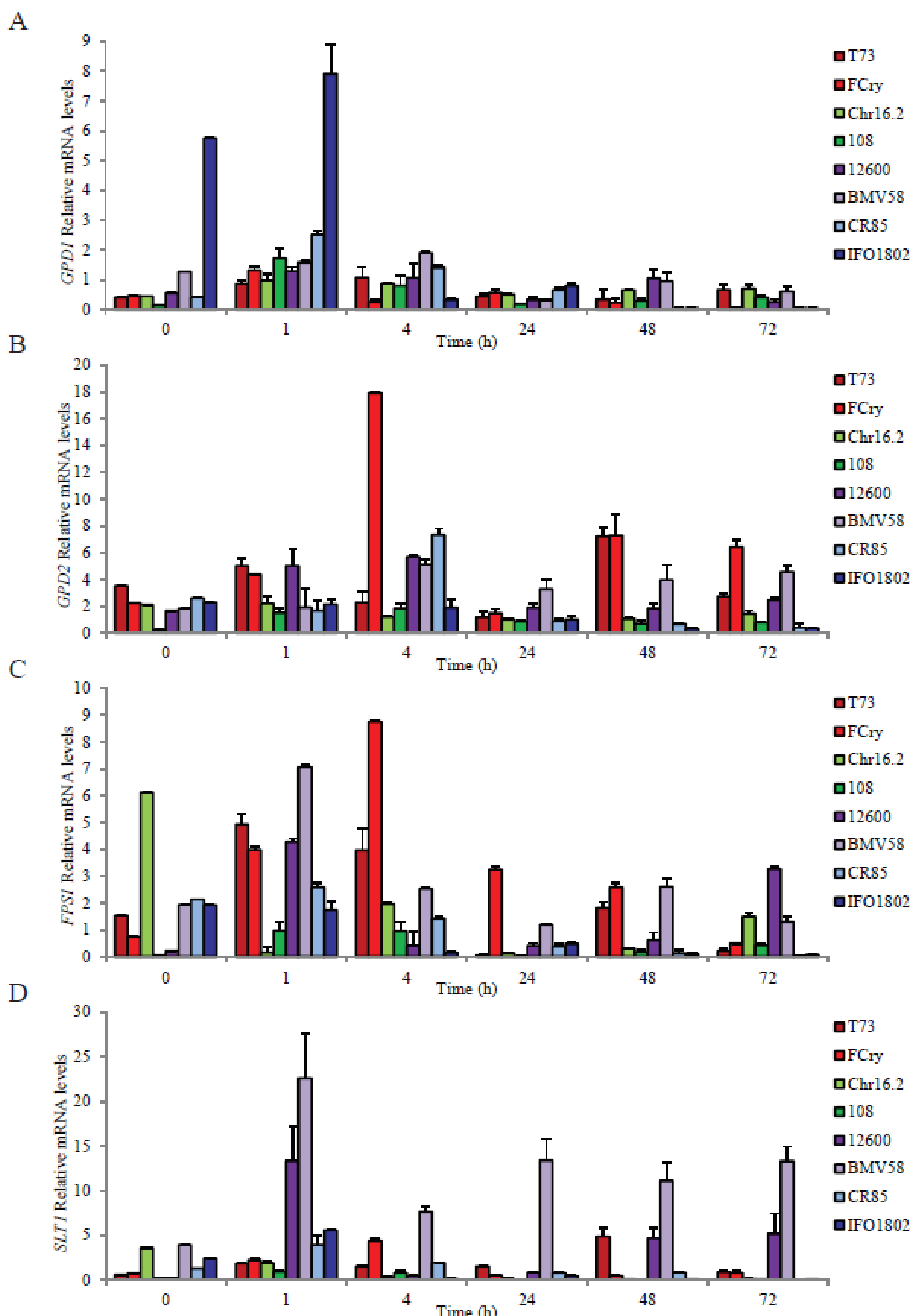


Figure 25. Expression of glycerol balance related genes during first hours of low temperature microvinifications in synthetic must for *S. cerevisiae* T73 (dark red) and FCry (light red), *S. paradoxus* Chr16.2 (light green) and 108 (dark green), *S. uvarum* 12600 (dark purple) and BMV58 (light purple) and *S. kudriavzevii* CR85 (light blue) and IFO1802 (dark blue) strains. The genes related to glycerol biosynthesis,*GDP1* (A) and *GPD2* (B), and glycerol export, *FPS1* (C), and import, *STL1* (D), were studied. Samples were taken in the first part (0, 1, 4, 24, 48 and 73 hours) of synthetic must microvinifications at 12 °C. After RNA extraction, expression of the different genes was determined by qPCR and values were normalised with *ACT1* and *RDN18-1* constitutive genes. Three independent microvinification bottles were used for each strain and averages ± standard deviation are shown.

To study the regulation of key genes related to intracellular glycerol balance under standard lab conditions (Figure 26) we used a representative strains of each species (T73, Chr16.2, BMV58 and IFO1802) and measured mRNA levels of *GPD1*, *STL1* and *FPS1* after half, one and two hours of transfer cells to a non-stress SC media (Figure 26A), hyperosmotic SC 1 M sorbitol (Figure 26B) or hypoosmotic (water) media (Figure 26C). In addition, another analog set of experiments were performed but using mannitol as a carbon source (Figure 26D-F), which is a non-fermentable carbon source that complicates the energy supply for cellular processes. We can observe that all strains, especially T73 and BMV58, activate *GPD1* 0.5-1 h after hyperosmotic stress (Figure 26B) but is not activated in non-stress conditions (Figure 26A) or hypoosmotic stress (Figure 26C).

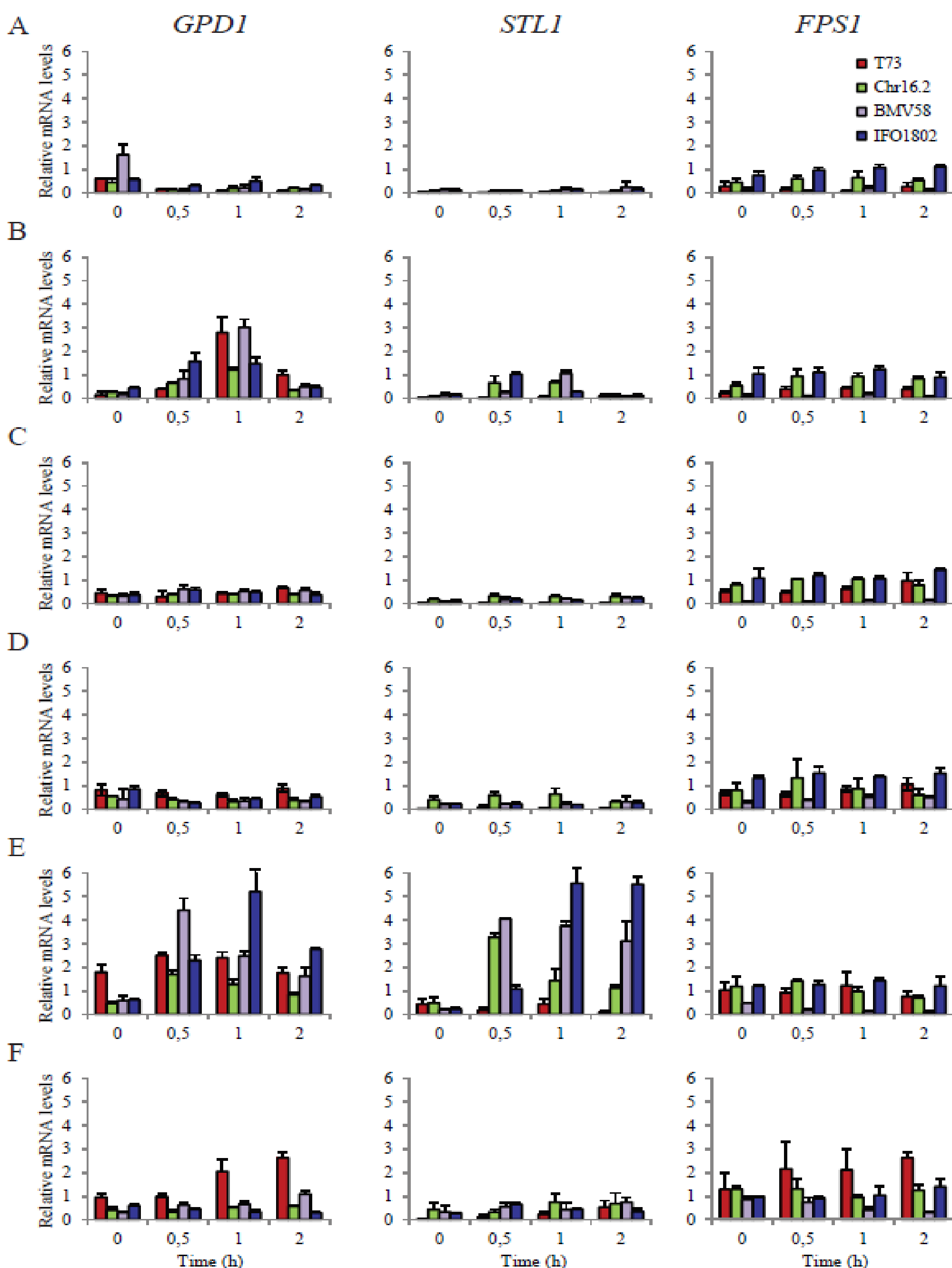


Figure 26. Expression of glycerol balance related genes of *S. cerevisiae* T73 (red), *S. paradoxus* Chr16.2 (green), *S. uvarum* BMV58 (purple) and *S. kudriavzevii* IFO1802 (blue) strains in various conditions. The genes related to glycerol biosynthesis (*GDP1*) and glycerol transport (*FPS1* and *STL1*) were studied. Samples from non-stress SC media (A,D), hyperosmotic SC 1 M sorbitol (B,E) or hypoosmotic (water) media (C,F) cultures were taken after 0, 0.5, 1 and 2 hours of the inoculation (from pre grown cultures in SC). The SC media were supplemented with 2 % glucose (A – C) or 2 % mannitol (D-F) as a carbon source. After RNA extraction, expression of the different genes was determined by qPCR and values were normalised with *ACT1* and *RDN18-1* constitutive genes. Three independent microvinification bottles were used for each strain and averages ± standard deviation are shown.

A similar situation but with higher mRNA levels is observed in presence of mannitol instead of glucose where hyperosmotic stress (Figure 26E) activates *GPD1* gene, especially for BMV58 and IFO1802. In this case, hypoosmotic stress (Figure 26F) does activate the *GPD1* gene in the case of T73 and BMV58. The *STL1* gene reacts with a similar patter as *GPD1* increasing mRNA levels in hyperosmotic stress (Figure 26B) but not upon hypoosmotic stress in the presence of glucose (Figure 26C). *STL1* shows also a similar patter as *GPD1* in presence of mannitol, increasing expression levels after hyperosmotic stress (Figure 26E), though to higher levels compared to glucose (Figure 26B and 25E). Interestingly, *S. cerevisiae* T73 strain shows very low *STL1* levels in any conditions and no significant activation (Figure 26F). On the contrary, the *FPS1* gene seems to be unresponsive to any condition in all the strains with except for the case of T73 growth in mannitol and hypoosmotic stress (Figure 26F). Similar levels are presented for all strains and conditions although BMV58 presented lower levels that the other strains. Altogether, it is the *STL1* gene whose expression shows the highest level of variation in different conditions and among the species and strains.

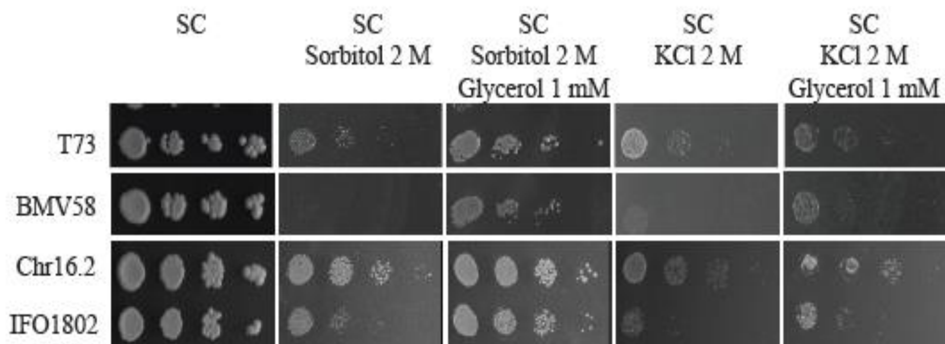
2.4 Stl1 functional differences in *Saccharomyces* species.

Since *STL1* gene presented important differences in mRNA levels in strains from different *Saccharomyces* species we wanted to study the possible functional differences of this glycerol importer. For that we first compared the growth of a representative strain of *S. cerevisiae* (T73), *S. uvarum* (BMV58), *S. paradoxus* (Chr16.2) and *S. kudriavzevii* (IFO1802) species in conditions where the activity of Stl1 is important (Figure 27A). A drop test with the four strains was performed in non-stress media (SC), in hyperosmotic stress media (SC with 2 M sorbitol or 2 M KCl) and in hyperosmotic stress media supplemented with a very low amount of glycerol (SC with 2 M sorbitol (or 2 M KCl) and 1 mM glycerol). In these conditions, if the cells are able to

efficiently import glycerol to the cytosol they have a growth advantage when extracellular glycerol is present, i.e. before they synthesize the necessary amount to counterbalance the external osmotic pressure. The results show that cell growth is affected by hyperosmotic stress conditions proportionally to the osmotic pressure, i.e. more in the presence of 2 M KCl than in the presence of 2 M sorbitol. We can observe that BMV58 is the strain with the lowest and Chr16.2 the highest survival level in both hyperosmotic stress conditions. Interestingly, as shown in the Fig. 31A, some strains, as IFO1802 and especially BMV58, benefit from the presence of glycerol in the medium more than others (e.g. T73 and Chr16.2). These results are indicative of different capacity to import glycerol in response to hyperosmotic stress among the studied strains.

To confirm these Stl1 functional differences we cloned the different *STL1* alleles from T73, BMV58 and IFO1802 strains in an *S. cerevisiae* multicopy plasmid behind a weak and constitutive promoter, and expressed them in a laboratory osmosensitive *S. cerevisiae* strain (BY4741*Δslt1Δhog1*). As a control, this strain was also transformed with the empty YEp352. Then, the growth of strains was tested in non-stress media (SC), in hyperosmotic-stress media (SC with 0.7 M sorbitol or 0.3 M KCl) and in hyperosmotic-stress media supplemented with extracellular glycerol (SC 0.7 M sorbitol or 0.3 M KCl, and 10 mM glycerol). The results (Figure 27B) showed that the strains with the BMV58 and IFO1802 *SLT1* allele are clearly able to recover growth when they have extracellular glycerol in the presence of a hyperosmotic stress. However, the strain containing the T73 *STL1* allele presented only a minor growth recovery when it can use extracellular glycerol in the presence of a hyperosmotic-stress.

A



B

C

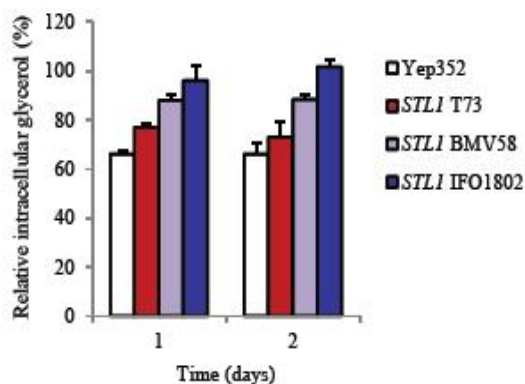


Figure 27. YEp352 and not Yep352 in B and C. Importance of glycerol import for osmotolerance of *S. cerevisiae* (T73), *S. uvarum* (BMV58) *S. paradoxus* (Chr16.2) and *S. kudriavzevii* (IFO1802) in drop test assays. (A) Serial dilutions of the different strains were plated in non-stress media (SC), in hyperosmotic stress media (SC with 2 M sorbitol or 2 M KCl) and in hyperosmotic stress media supplemented with glycerol (1 mM glycerol) (B) Growth of *S. cerevisiae* BY4741*Δstl1Δhog1* strain expressing *STL1* alleles from *S. cerevisiae* (T73), *S. uvarum* (BMV58) or *S. kudriavzevii* (IFO1802) was monitored in drop tests on non-stress media (SC), in hyperosmotic stress media (SC with 0.7 M sorbitol or 0.3 M KCl), and in hyperosmotic stress media supplemented with 10 mM glycerol. A representative image of biological triplicates is presented. (C) In the same strains used in (B), intracellular glycerol accumulation was measured collecting samples after 0, 1 or 2 days of growth in SC with 10% glucose. Cells precultured in the same media were inoculated (OD_{600} = 0.3) and incubated at 25 °C in 100 ml flasks. Data in time 0 for each strain was considered 100%. Three independent experiments were performed for each strain and averages ± standard deviation are shown.

We also evaluated the different Slt1 functionality by measuring the intracellular glycerol accumulation of the *S. cerevisiae* strains expressing different *STL1* genes after one and two days of growth in 10% glucose (Figure 27C) without any additional osmotic agents. The strain with IFO1802 Stl1 was able to recover the original intracellular glycerol levels by importing some of the diffused out glycerol. The strain with BMV58 Stl1 was able to recover more than 80 % of the original intracellular glycerol levels. On the contrary, after two days the strain with the T73 Stl1 showed intracellular glycerol levels recovery no significantly different than a strain without Slt1. This results points in the same direction of the previous experiments and suggest a low functionality of T73 Stl1 compared with BMV58 and IFO1802.

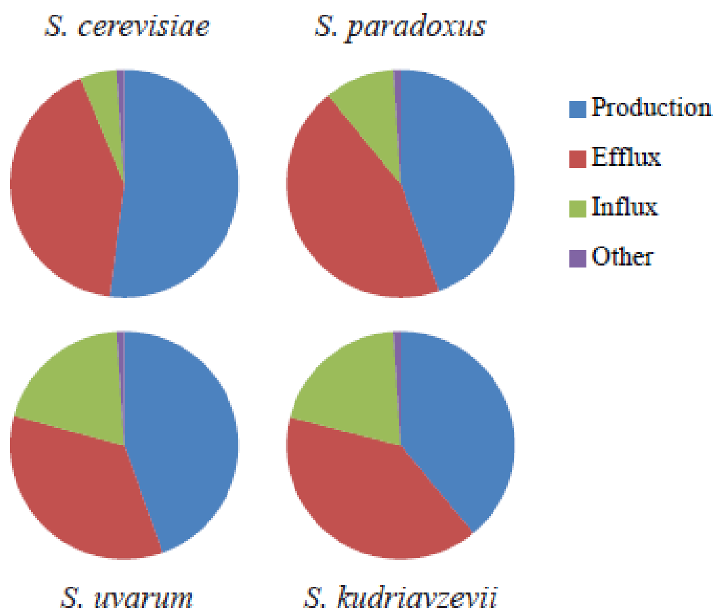


Figure 28. Schematic representation of the weight of glycerol production, efflux, influx or other actions regarding glycerol balance after hyperosmotic stress. This representation compares the dynamics of glycerol accumulation in response to hyperosmotic stress that has been quantitatively analyzed and modelled using physiologic, metabolic, enzymatic and transcriptomic data of the key actors in *S. cerevisiae* (Petelenz-Kurdziel et al. 2013). Here we compared with the other species (*S. paradoxus*, *S. uvarum* and *S. kudriavzevii*) using data provided in this work and others.

DISCUSSION

In this work we studied intracellular glycerol pool balance and regulation in response to stresses that occur upon inoculating wine-related yeast species in grape musts. We have analyzed strains belonging to four species that participate in winemaking directly (*S. cerevisiae*, *S. uvarum* and *S. paradoxus*) or thorough hybrids (*S. kudriavzevii*). A first approach was to compare survival of these species during hyperosmotic and cold-hyperosmotic stress. Other studies have found that *S. cerevisiae*, *S. uvarum* and *S. paradoxus* strains have similar tolerance to hyperosmotic stress

whereas *S. kudriavzevii* strains show a decreased survival in 15 % sorbitol at 30ºC (Wimalasena et al., 2014). However, this result is doubtful since *S. kudriavzevii* strains are sensitive to this temperature (Arroyo-López et al., 2010). In our results using 25 ºC, an optimal temperature for *S. kudriavzevii*, this species shows similar or slightly higher tolerance to some hyperosmotic conditions compared to *S. cerevisiae* and *S. paradoxus*. In contrast, we observed an increased hyperosmotic stress tolerance in *S. uvarum* strains that is even more evident in hyperosmotic-cold stress conditions, where glycerol balance is determinant for cell survival. These results argue in favor to a more efficient handling of intracellular glycerol in *S. uvarum* strains in this condition. On the contrary, hyperosmotic tolerances in *Saccharomyces* species seems to be dependent on the media since *S. uvarum* strain BMV58 shows the lowest hyperosmotic tolerance in minimal media (Figure 27A) instead of the highest tolerance in complete media (Figure 23). All these data point to different strategies in the different species to handle glycerol accumulation in response to hyperosmotic or cold-hyperosmotic stresses.

In winemaking conditions, cells suffer a hyperosmotic or cold hyperosmotic mild stress that does not affect viability in any *Saccharomyces* species (results not shown). However, significant differences can be observed in extra and intracellular glycerol levels and also in gene expression of key genes involved in glycerol homeostasis. These data suggest once again that the *Saccharomyces* species are using a different strategy to cope with alterations in the osmotic pressure and cold in the environment. In fact this argument is not that surprising since *Saccharomyces* species are genetically quite distance showing coding region identities such as the one showed when comparing human and mouse (85 %).

The dynamics of glycerol accumulation in response to hyperosmotic stress has been quantitatively analyzed and modelled using physiologic, metabolic, enzymatic and transcriptomic data of the key actors in *S. cerevisiae* (Petelenz-Kurdziel et al., 2013). The strategy of this species consists

in a transcriptional activation of *GPD1* to increase glycerol accumulation inside the cell by redirecting glycolytic flux. On the other hand, the glycerol efflux stops by the closing of Fps1channel. These are the principal mechanisms to balance glycerol after a hyperosmotic shock. Glycerol influx and other elements contribute in a minor fraction (Figure 28). From the results of this work and others, we can hypothesize that non-*cerevisiae Saccharomyces* species have changed the weight of the different elements involved in glycerol balance. Based on *STL1* gene activation and Stl1 functionality assays we speculate that *S. uvarum* and *S. kudriavzevii* rely more in the glycerol import to compensate the osmotic pressure (Figure 28). This strategy is not exclusive of these species, In fact, it has been shown that the most osmotolerant yeasts species present a very efficient glycerol-import capacity (Lages et al., 1999).

A possible explanation of the different strategies applied by the *Saccharomyces* species to balance glycerol in osmotically non-optimal environments could be amount of intracellular glycerol that cells need to accumulate. We observed that, in our winemaking conditions, *S. cerevisiae* accumulates the highest amount of glycerol in the cells. This promotes the supposition that the other species can partially compensate the osmotic pressure by other unknown means. This will allow them to diversify the mechanisms available to compensate water efflux by using more frequently other elements that can be inefficient in specific situations, for example the glycerol import, which can be useless if there is no glycerol outside the cel.

CHAPTER 3. Metabolic and genetic profiles differentiate wine and non-wine *S. cerevisiae* strains.

This chapter includes the detailed article below in preparation for publication.

1 In preparation

2 Metabolic and genetic profiles differentiate wine and no wine *S.*
3 *cerevisiae* strains

4

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Saccharomyces yeasts, mainly *S. cerevisiae*, are competitive in the production of ethanol and especially harnessed in industrial fermentation processes due to a combination of several properties including fast growth, efficient glucose repression, good ability to produce and consume ethanol, and a tolerance for several environmental stresses, such as high ethanol concentration and low oxygen levels (Piskur and Langkjaer, 2004). Depending on the degree of dependence of the yeast with oxygen during the life cycle, they are metabolically classified as obligate aerobes which develop exclusively aerobic respiration; facultative anaerobes that play a concomitant respiratory and fermentative metabolism and obligate anaerobes displaying exclusively fermentative metabolism (Merico et al., 2007). It is known that the growth of a yeast culture in a medium where there is minimum dissolved oxygen concentration is due solely to its ability to carry out fermentation. However S. cerevisiae develops preferably fermentative metabolism up to a certain threshold of available sugar, even under aerobic conditions where breathing enables increased energy efficiency through the use of oxygen to the ultimate electron acceptor. This phenomenon has been described as Crabtree effect (De Deken, 1966) and the yeasts expressing this trait are called Crabtree-positive. Therefore, enough ATP should be generated during glycolysis to support the yeast growth, and NADH generated during glycolysis gets re-oxidized. It has been believed that these yeasts remodeled their carbon metabolism to be able to accumulate ethanol under aerobic conditions and at the expense of decreasing biomass production developing a strategy described as make-accumulate-consume (Piskur et al., 2006). The duplication of glycolytic genes (Conant and Wolfe, 2007) as a consequence of whole genome duplication (WGD) event (Conant and Wolfe, 2007; Wolfe and Shields, 1997); a global rewiring of the transcriptional network (Ihmels et al., 2005) and the parallel gain of genes from other organisms, which were crucial to the growth capacity in anaerobiosis conditions (Gojković et al., 2004) are the events that has been

implicated in the appearance of several key physiological characteristics of *S. cerevisiae* contributing to high glycolytic capacity (Merico et al., 2007) and anaerobic life style in the *Saccharomyces* lineage. Several recent studies have been conducted to explain both the origin and the importance this strategy and molecular events to the establishment of these peculiar fermentative characteristics (Dashko et al., 2014; Hagman et al., 2013; Solis-Escalante et al., 2015).

In addition to differences concerning to the central and respiro-fermentative metabolism there is a marked genetic diversity among different *S. cerevisiae* strains associated with different environments. Several genetic approaches have shown that wine strains form a homogeneous phylogenetic group and indicate that these strains have undergone a domestication process (Legras et al., 2007; Liti et al., 2009; Schacherer et al., 2009; Wang et al., 2012). Recently it has been identified the source of the natural wild *S. cerevisiae* strains phylogenetically closer to the wine yeasts (Almeida et al., 2015). This wild population is associated with oak trees in Europe and surprisingly was not responsible for transmitting to the wine yeast some genes associated to winemaking, which were obtained from other species through horizontal transfer (Almeida et al., 2015). Specifically, these genes have been identified as being relevant components for growth in the wine fermentation environment (Novo et al., 2009). In parallel, the genomic regions more related to wine-specific traits have developed as a result of strong artificial selection inherent to the fermentative environments (Querol et al., 2003). However, interesting diverse determining genes for winemaking have recently gained from other yeast species (Eberlein et al., 2015).

It is important to note that any changes in genes related to the respiro-fermentative metabolism can interfere directly in oenological characteristics of the wine strains, e.g. with regard to the yields in ethanol, glycerol and acetic acid (Cordente et al., 2013; Pretorius et al., 2012; Varela et al., 2012). Currently, there is a lack in metabolic studies of genetically

distinct *S. cerevisiae* strains in order to better know them on possible variations regarding to the central metabolism. In this light, there is a need to deeply know the possible variation on the fermentative metabolism among genetically distinct *S. cerevisiae* strains in order to investigate whether there is a specific metabolic-fermentation profile able to define group of strains as the wine yeast strains that have been domesticated by man along the development of winemaking.

To this end, we conducted a genetic and fermentative study in 94 distinct *S. cerevisiae* strains which classify in wine, non-wine or mosaic strains according to their phylogenetic and metabolic relationships. Furthermore 58 different strains were used in four distinct fermentation conditions and analyzed as to the yields in glycerol, ethanol, biomass and organic acids (acetic, pyruvic, lactic and succinic). The results determined important and significant metabolic differences that have been assigned to the distinct wine and no wine genetic profiles; increasing knowledge of the fermentative metabolism of several *S. cerevisiae* strains.

RESULTS

2.1 Genetic characterization of *S. cerevisiae* strains and their classification in wine (W), non-wine (nW) and mosaic (Mc).

The phylogeny among 75 *S. cerevisiae* strains isolated from various sources (Table 2), and 38 *S. cerevisiae* strains (Table 4), genetically defined, belonging to groups previously established by Liti et al. (2009) was determined based on the sequence variability contained in four nuclear genes (*CAT8*; *GAL4*; *BRE5*; *EGT2*). Initially, through DNA sequence alignments, each allele in separate was classified in wine or non-wine in accordance with sequence variability and similarity to the genetic reference (GF) strains

alleles. Thus, it was possible to classify 75 strains in wine (W), non-wine (nW) and mosaic (Mc) according to the alleles present in each strain. Those strains that contain both W and nW alleles were classified as mosaic (Table 4). It is important to note that this classification does not always correspond to the strain isolation of origin, for example, the strains identified with the numbers 1, 2, 3, 34, 35 and 36 although are not isolated from the wine environment, the sequences studied classify them as wine strains.

Table 4. Strain classification in wine, non-wine and mosaic based on alignments performed with sequences of four different genes and 38 genetic reference (GR) strains.

Exp. Nº †	**Strain name**	**Source of isolation**	**Sequenced and aligned alleles**				**Strain classification**
			CAT8	***GAL4***	***EGT2***	***BRE5***	
1	CBS 1460	Fermenting fruit	**33**	**27**	**3**	**58**	Wine
2	CBS 2087	Flower of lychee	**33; 52**	**27**	**3**	**61**	Wine
3	NCAIM Y00678	Fermented drink	**33**	**27**	**3**	**31; 32**	Wine
4	GB4 3	Wine	**57; 33**	-	**3**	**63**	Wine
5	T 73	Wine	NCBI	NCBI	NCBI	NCBI	Wine
6	ZA 29	Wine	**32**	**27**	**3**	**32**	Wine
7	L 1005	Wine	**31**	**27**	**3**	**32**	Wine
8	QA 23	Wine	NCBI	NCBI	NCBI	NCBI	Wine
9	Vin 13	Wine	NCBI	NCBI	NCBI	NCBI	Wine
10	CECT 1477	Sparkling wine	**31**	**27**	**21**	**41**	Wine
11	ZA 9	Wine	**33**	**27**	**3**	**32**	Wine
12	ZA 13	Dry yeast wine	**33**	**27**	**3**	**82**	Wine
13	L 7	Wine	-	-	**3**	**32**	Wine
14	ZA 14	Wine	**33**	**1**	**3**	**27**	Wine
15	L 16	Wine	**85**	**27**	**3**	**32**	Wine
16	L 246	Wine	**33**	**27**	**3**	**32**	Wine

Exp. Nº †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	*GAL4*	*EGT2*	*BRE5*	
17	L 269	Wine	**31**	**27**	**3**	**89**	Wine
18	L 958	Wine	**33**	**84**	**3**	**32/49**	Wine
19	L 1335	Wine	**33**	**1**	**3**	**93**	Wine
20	L 960	Wine	**33/55**	**1**	**3**	**41/90**	Wine
21	EC 1118	Wine	NCBI	NCBI	NCBI	NCBI	Wine
22	L 1325	Wine	**52**	**27**	**3**	**41**	Wine
23	L 1343	Wine	**31**	**27**	**3**	**32**	Wine
24	L 962	Wine	**32**	**27**	**8**	**43**	Wine
25	CECT 1883	Wine	**56**	**27**	**3**	**64**	Wine
26	CECT 1479	Wine	**55**	**58**	**3**	**49/63**	Wine
27	CECT 1882	Sherrry wine	**57**	**1**	**3**	**63**	Wine
28	CECT 11032	Fermented must	**33**	**27**	**3**	**41**	Wine
29	CECT 10557	Grape must	**61**	**27**	**3**	**32**	Wine
30	CECT 11827	Dry yeast wine	**52**	**27**	**3**	**41/66**	wine
31	CECT 11833	Wine	**32**	**1**	**3**	**32/67**	wine
32	CECT 11834	Wine	**55**	**1**	**3**	**68**	wine
33	GB Flor C	Jerez wine	**55/32**	**1**	**3**	**63**	wine
34	D14 n.14	Dietetic complement	**31**	**27/79**	**3**	**33/41**	wine

Exp. Nº †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	*GAL4*	*EGT2*	*BRE5*	
35	CECT 10120	Fruit of *Arbutus unedo*	**58**	**55**	**3**	**41**	wine
36	CLIB 215	bakery	**72**	**1**	-	**32**	wine
37	CECT10692	Fermented grapes	**55**	**1**	**3**	**66**	wine
W 1	BC187	Barrel fermentation	GR	GR	GR	GR	wine
W 2	L-1374	Ferment from must	GR	GR	GR	GR	wine
W 3	L-1528	Ferment from must	GR	GR	GR	GR	wine
W 4	DBVPG 1788	Soil	GR	GR	GR	GR	wine
W 5	DBVPG 6765	Unknown	GR	GR	GR	GR	wine
W 6	YJM 975	Clinic	GR	GR	GR	GR	wine
W 7	YJM 978	Clinic	GR	GR	GR	GR	wine
W 8	YJM 981	Clinic	GR	GR	GR	GR	wine
-	RM11_1A††	wine	GR	GR	GR	GR	wine
-	DBVPG1373††	Soil	GR	GR	GR	GR	wine
-	DBVPG1106††	Grapes	GR	GR	GR	GR	wine
38	1.3 LM (9)	Masato	3/4	-	22	23/25	non-wine
39	4 y M2	Masato	10	-	22	23/25	non-wine
40	VI L7D	Chicha de Jora	3/4	13	23/1	85/86	non-wine
41	CBS 2421	kefyr grains	53	52/53	11	62	non-wine

Exp. N° †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	***GAL4***	***EGT2***	***BRE5***	
42	Chr 96.2	*Quercus faginea*	23/22	67/60	12/22	62	non-wine
43	CBS 6412	Sake (Kyokai no. 7)	21	67	11	79	non-wine
44	CBS 2992	Palm wine	50	45	22	53	non-wine
45	CBS 1591	Fermenting cacao	50/51	51	22	59/60	non-wine
46	YJM 269	Grapes	47	45/46	12	53	non-wine
47	CPE7	Cachaça fermention	21	65	12	59	non-wine
48	15M	Agave culture	43	1	29	44/45	non-wine
nW 1	Y12	Palm wine strain	GR	GR	GR	GR	non-wine
nW 2	YPS128	Soil beneath	GR	GR	GR	GR	non-wine
nW 3	DBVPG 6044	Bili wine	GR	GR	GR	GR	non-wine
nW 4	UWOPS 03.461.4	wild	GR	GR	GR	GR	non-wine
nW 5	UWOPS 05.227.2	wild	GR	GR	GR	GR	non-wine
nW 6	UWOPS 05.217.3	wild	GR	GR	GR	GR	non-wine
-	NCYC 110 ††	Ginger beer	GR	GR	GR	GR	non-wine
-	K11 ††	Shochu sake strain	GR	GR	GR	GR	non-wine
-	YPS606 ††	Bark of *Q. rubra*	GR	GR	GR	GR	non-wine
-	Y9 ††	Ragi sake wine	GR	GR	GR	GR	non-wine
49	CBS 8292	Water	**31**	**1**/69	**3**	**27/41**	mosaic

Exp. Nº †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	*GAL4*	*EGT2*	*BRE5*	
50	CBS 8858	Sorghum beer	**33**/76	**61**	12/13	**75/76**	mosaic
51	2 y M2 (12)	Masato	**3**;4	-	**3**	2;4	mosaic
52	CBS 8855	Sorghum beer	**33**/75	**61**	12;13	**75/76**	mosaic
53	CBS 4455	Kaffir beer	**73**	65/66	13	**41**	mosaic
54	CBS 8857	Sorghum beer	5	3	**3**/11	4/5	mosaic
55	CBS 7764	Fish	**31/33**	69	**3**	**31**/65	mosaic
56	GU4	Agave	25/**26**	**25**/26	11	23/24	mosaic
57	G1	Beer	10	3	3	4	mosaic
58	CECT 10131	Flower	21/59	**27**	11	**27**/65	mosaic
59	Ch3 BL2	Chicha de Jora	3/4	13/50	**3**	4/5	mosaic
60	CH1-L1	Chicha de Jora	3/4	13	**3**	4/5	mosaic
61	CH1-L2	Chicha de Jora	10	-	**3**	4/5	mosaic
62	Chr 9	Forest soil	**87/33**	**1/61**	**10/5**	**93/51**	mosaic
63	Chr 7	Forest soil	-	**1/61**	**3**	**31/62**	mosaic
64	LA 3M (4)	Masato	**3**/4	-	22	23/25	mosaic
65	NCAIM Y00925	Apricot pulp	27/40	34	1	**40**	mosaic
66	YJM 326	Clinic	**16**	37/38	**32**	**32**	mosaic
67	Temoaya MI26	Agave	29/24	-	**27**	23/24	mosaic

Exp. Nº †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	*GAL4*	*EGT2*	*BRE5*	
68	YJM 320	Clinic	77/79	**79/2**	**3/16**	**32**	mosaic
69	ZA 26	Wine	5	3	**3**/11	85/86	mosaic
70	CECT 1384	Beer	**64**	**58**	11	59/70	mosaic
71	CECT 10392	Fetid liquid olives	60	**27**	**19**	**31**	mosaic
72	CECT 11838	Grape	63	**19**	47	46	mosaic
73	PE54 CJ	Chicha de Jora	3/13	13/14	**3**/11	5	mosaic
74	PE 35M	Masato	3/9	4/5	13/**3**	2/4	mosaic
75	112 M	Mead	**33**/47	**1**/43	13	9/**31**	mosaic
Mc 1	NCYC 361	Beer spoilage	GR	GR	GR	GR	mosaic
Mc 2	273614N	Clinic	GR	GR	GR	GR	mosaic
Mc 3	322134S	Clinic	GR	GR	GR	GR	mosaic
Mc 4	DBVPG 6040	Fruit juice	GR	GR	GR	GR	mosaic
Mc 5	UWOPS 87-2421	Cladode	GR	GR	GR	GR	mosaic
-	UWOPS 83.787 ††	*Opuntia stricta*	GR	GR	GR	GR	mosaic
-	DBVPG 1853 ††	White Teff	GR	GR	GR	GR	mosaic
-	YS2 ††	Baking	GR	GR	GR	GR	mosaic
-	YS4 ††	Baking	GR	GR	GR	GR	mosaic
-	Y55 ††	Grape	GR	GR	GR	GR	mosaic

Exp. Nº †	Strain name	Source of isolation	Sequenced and aligned alleles				Strain classification
			CAT8	***GAL4***	***EGT2***	***BRE5***	
-	YS9 ††	Baking	GR	GR	GR	GR	mosaic
-	S288c ††	Lab. strain	GR	GR	GR	GR	mosaic
-	YJM789 ††	Clinic	GR	GR	GR	GR	mosaic
-	SK1 ††	Soil	GR	GR	GR	GR	mosaic
-	YIIc17_E5 ††	Wine	GR	GR	GR	GR	mosaic
-	378604X ††	Clinic	GR	GR	GR	GR	mosaic
-	W303 ††	Lab. strain	GR	GR	GR	GR	mosaic

Based on alignments performed for each allele of 113 *S. cerevisiae* strains, their DNA sequences were classified in wine or non-wine according to the degree of nucleotide similarity with the DNA sequence of reference strains used. Alleles classified as wine are highlighted in bold. The strains that showed wine and no wine alleles were classified as Mosaic. **NCBI** - gene sequences obtained from the NCBI database, not sequenced in this work. **GR –** *S. cerevisiae* strains (38) with previously characterized genome in pure groups by Liti et al.(2009). The DNA sequences of their genes were used as genetic reference (GR) in the alignments performed. **† -** Identification number of all *S. cerevisiae* strains (94) used in fermentation experiments. **†† -** Not used in fermentation experiments, only the DNA sequences of their genes were used as genetic reference in alignments and phylogeny.

However, although the sequence variability of the four alleles were able to differentiate *S. cerevisiae* strains in a previous work from our laboratory (Arias, 2008), to establish a more refined differentiation among the strains studied here, we concatenate the genes in a single sequence and conducted a phylogenetic analysis.

As a result of the analysis, the established phylogenetic tree (Figure 29) shows that all the reference strains used for defining pure genetic groups maintained an equal phylogenetic distribution compared to that obtained in the phylogeny performed by whole genome analysis (Liti et al., 2009). It is possible to identify the same five strains sub-groups (Malaysian, West African, North American, Sake and Wine/European) previously defined and that the 75 strains characterized in this study also distributed according to the classification given in Table 4. This result not only confirms the discriminating power of using these four nuclear genes in *S. cerevisiae* strains but also supports the classification conducted by separating the 75 strains in 37 W, 11 nW and 27 Mc. Another interesting characteristic that supports this phylogeny (Figure 29) is that in a similar manner to other studies (Liti et al., 2009; Schacherer et al., 2009; Wang et al., 2012) the wine strains form a completely homogenous group and separate from the mosaic strains which are distributed mainly in an intermediate position between the W and nW strains and permeating the pure groups of nW strains, but far from the compact group of European wine strains. Further, this analysis (Figure 29) shows a remarkable bipolar distribution among wine and non-wine strains, positioned at opposite ends of the tree.

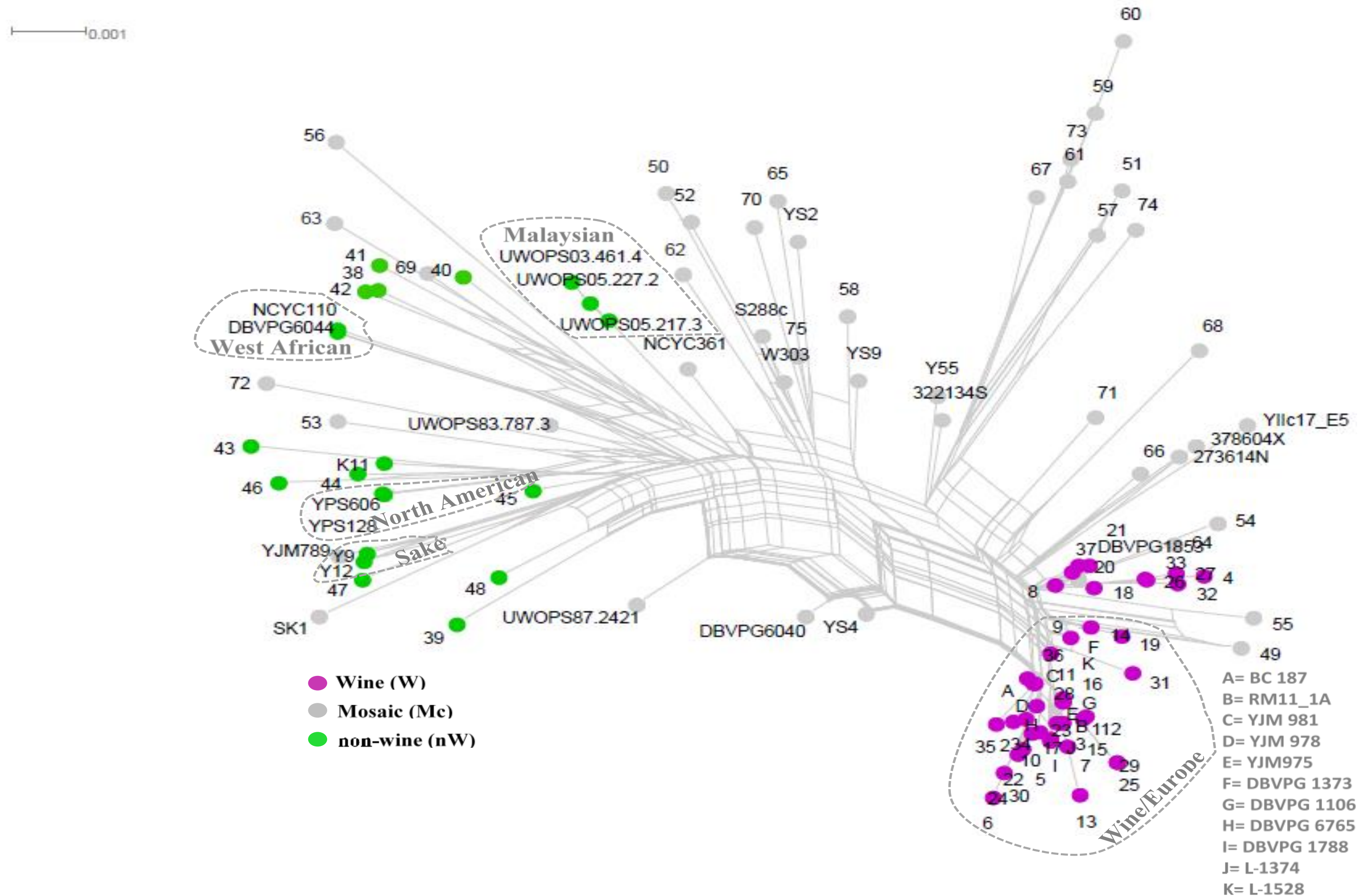


Figure 29. Phylogeny performed with 4 genes from 113 *S. cerevisiae* strains. 75 of them were classified in this study in wine, not wine and mosaic (Identification Numbers in table 4) and 38 strains were previously characterized by Liti et al. (2009) and are identified in the tree with their names. The phylogenetic analysis was performed based on a single DNA sequence for each strain with *CAT8, GAL4, EGT2* and *BRE5* alleles concatenated. The Splits Tree 4 (version 4.13.1) program was used with Characters; JukesCantor; NeighborNet; EqualAngle parameters.

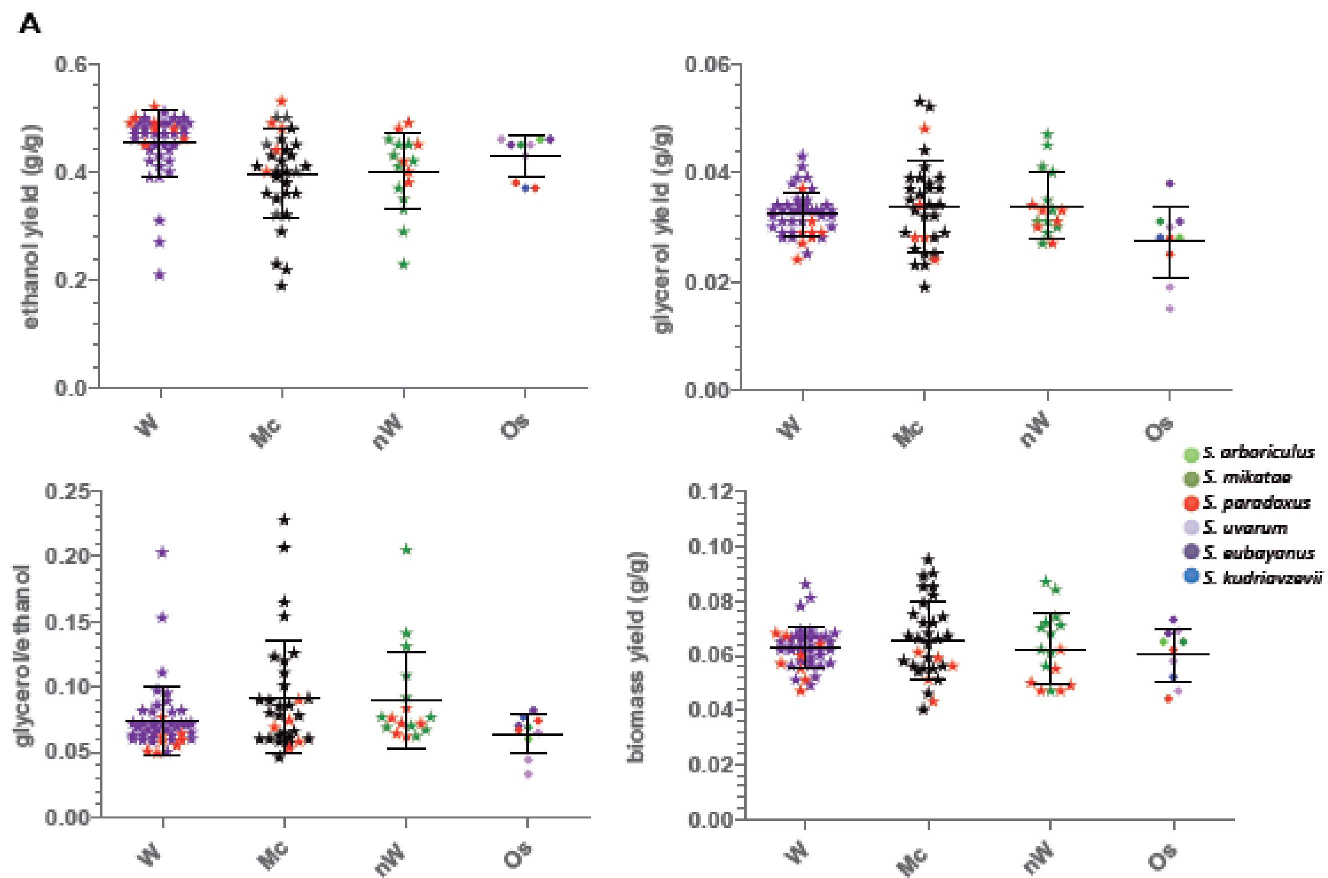
2.2 Fermentative screening of *S. cerevisiae* strains indicates metabolic differences among genetically distinct groups of strains.

To investigate differences in the fermentative metabolism among the different *S. cerevisiae* strains, we performed fermentations in micro-plate with YPD medium containing 10% glucose at 25 ° C in order to detect significant differences in biomass and metabolites (ethanol, glycerol and acetic, lactic, succinic acid and pyruvic). Then we analyze these yields to better understand the central metabolism in different yeast strains. To this end, we used 94 *S. cerevisiae* strains (45 W, 17 nW and 32 Mc) (Table 4) and also 10 strains belonging to other species of the genus *Saccharomyces* (*S. uvarum* (3); *S. eubayanus* (2); *S. paradoxus* (2); *S. kudriavzevii* (1); *S. arboriculus* (1) and *S. mikatae* (1)) (Table 2) in order to metabolically compare them to the *S. cerevisiae* strains. For these initial fermentations, in addition to 75 *S. cerevisiae* strains genetically characterized and classified in this work, 19 (8 W; 6 nW e 5 Mc) genetic reference (GF) strains, were also evaluated.

In general, in most comparisons of metabolite yields the wine strains showed a more compact and homogeneous group unlike the high dispersion and values variability shown by non-wine strains (Figure 30). Indeed, the wine and non-wine strains significantly differ in the ethanol (0.45 g/g Glu (W); 0.40 g/g Glu (nW); p value: 0.005); succinic (5.1 mg/g Glu (W); 7.7 mg/g Glu (nW); p value: 0.004) and pyruvic acids (0.61 mg/g Glu (W); 0.63 mg/g Glu (nW); p value: 0.02) average yields. The W strains showed higher average yield compared to nW only on ethanol (1.1 times more) and pyruvic acid (1.5 times more). The biomass average yields between the two groups were almost the same, 0.0625 g/g Glu for wine and 0.0626 g/g Glu for non-wine strains (p value: 0.96). The same applies to the representative lactic acid average values (0.61 mg/g Glu (W); 0.63 mg/g Glu (nW); p value: 0.65). The nW strains showed average values over the W strains in glycerol (0.032 g/g Glu (W); 0.034 g/g Glu (nW); 1.05 times more), acetic acid (3.9 mg/g Glu (W);

4.3 mg/g Glu (nW); 1.1 times more) and succinic (1.5 times more) yields and glycerol/ethanol ratio (1.2 times more). However, only for succinic acid these values were significantly different between the two groups. The Mc strains presented more similar values to the non-wine strains or intermediaries between both. It is interesting to note that in general, the distribution of distinct groups of strains shown in graphs (Figure 30) reflects their metabolic differences and somehow relate to the phylogenetic distribution profile of such strains on the analysis in Figure 29. In both analyses, the Mc strains show a distribution pattern intermediate to the W and nW and more similar to the nW strains. The Mc and nW strains show up more scattered indicating greater variability as compared to the wine group, more compact and homogeneous.

Regarding to the yields concerning the strains belonging to other species (OS) of the genus *Saccharomyces*, is interesting to note that similar to the nW group, there was high variability in the results reflected in the spread of strains and species in the chart (Figure 30). In general, for most compounds and analyzes, this group (OS) resembled more with nW *S. cerevisiae* strains. The most interesting analyses about the OS group occurred on succinic and pyruvic acids yields, where there were no statistically significant differences in the comparison to the nW group. However, these other species obtained succinic acid average yields higher to W *S. cerevisiae* group (5.1 mg/g Glu (W); 9.4 mg/g Glu (OS); p value < 0.0001) but smaller in pyruvic acid also to W *S. cerevisiae* group (0.54 mg/g Glu (W); 0.13 mg/g Glu (OS); p value < 0.0001). With regard to the acetic acid, the wine (3.9 mg/g Glu (W); 3.1 mg/g Glu (OS); p value: 0.02) and non-wine (4.3 mg/g Glu (nW); 3.1 mg/g Glu (OS); p value: 0.04) *S. cerevisiae* strains had higher yields compared to the OS group.


A
ethanol yield (g/g)
0.6
0.4
0.2
0.0
W
Mc
nW
Os
glycerol yield (g/g)
0.06
0.04
0.02
0.00
glycerol/ethanol
0.25
0.20
0.15
0.10
0.05
0.00
biomass yield (g/g)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
S. arboricolus
S. mikatae
S. paradoxus
S. uvarum
S. eubayanus
S. kudriavzevii

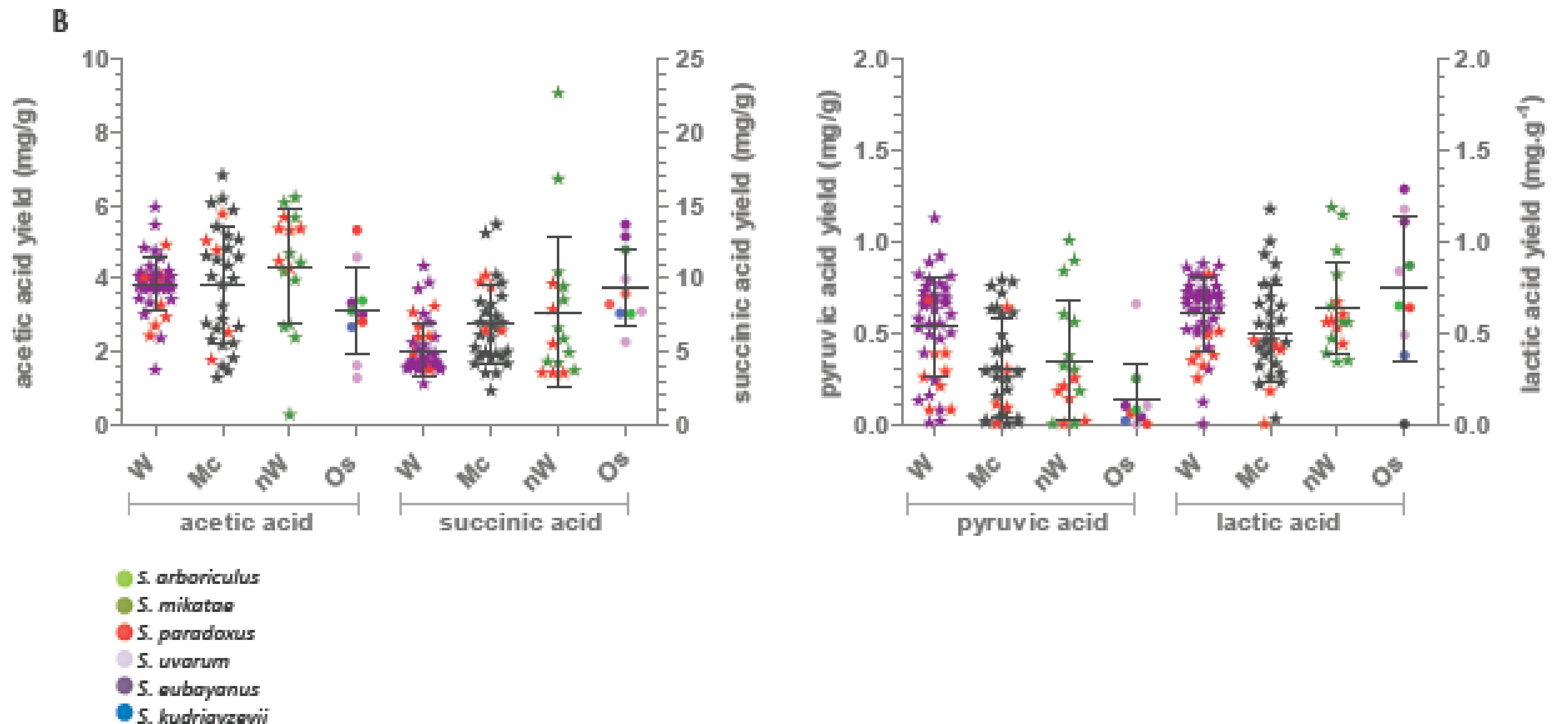


Figure 30. Fermentative metabolites and biomass yield of 104 yeasts strains (45 wine (W), 17 non-wine (nW), 32 mosaic (Mc) *S. cerevisiae* strains and 10 others species (Os) *Saccharomyces* sp strains: *S. uvarum* (3); *S. eubayanus* (2); *S. paradoxus* (2); *S. kudriavzevii* (1); *S. arboriculus* (1); *S. mikatae* (1)) after micro-fermentations, in triplicate, in YPD medium at 25 °C. For each *S. cerevisiae* strain, the average value of its triplicate is represented on charts in a star shape. The red stars represent the 19 strains used in this study as genetic references (Liti et al. (2009)) and identified in Table 4 as GR. The others species strains (Os) are represented by circles. **A** – Ethanol, glycerol and biomass yields expressed in grams per gram of glucose consumed. **B** - Organic acids yields, acetic, succinic, pyruvic and lactic expressed in micrograms per gram of glucose consumed.

The other species did not differ significantly in lactic acid yields although they showed higher average value compared to the W and NW. There were also no significant differences in ethanol and biomass yields produced by the OS group compared with the W and nW strains groups. Their average yields are similar in ethanol (0.45 g/g Glu (W); 0.42 g/g Glu (OS); 0.40 g/g Glu (nW)) and biomass (0.0626 g/g Glu (W); 0.0603 g/g Glu (OS); 0.0625 g/g Glu (nW)). The group of other species shows average values in the glycerol yields smaller than both *S. cerevisiae* groups. The relation between their glycerol/ethanol ratio average yields is slightly lower (p value: 0.049) in the nW and no significantly different compared to the W *S. cerevisiae* strains group.

To globally investigate the fermentative metabolic behavior of the yeasts studied here, we performed a PCA 3D analysis with the metabolites and biomass yields of all 114 yeast strains (Figure 31). The result shows a strains distribution pattern in which 79% of the variability related to the data is explained by three main components.

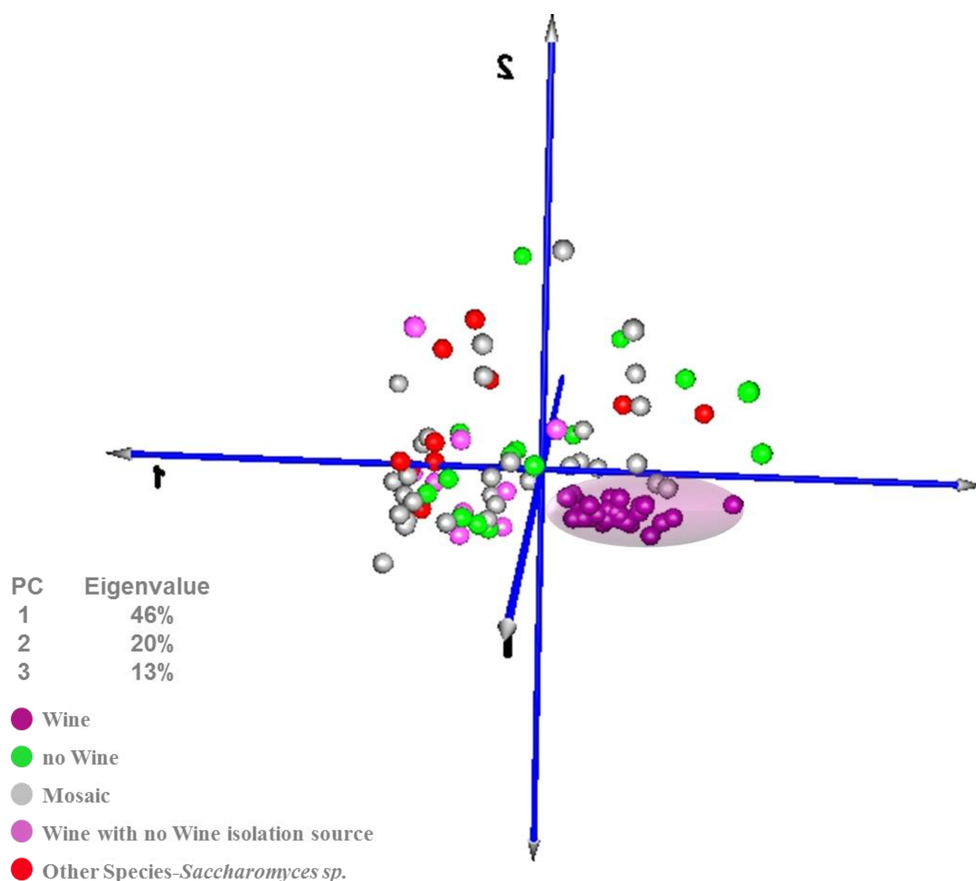


Figure 31. PCA-3D (Principal Component Analysis in 3D) performed using MeV (MultiExperiment Viewer) version 4.9.0. with the normalized values of all analyzed metabolites yields (8 variables, glycerol, ethanol, biomass, acetic acid, pyruvic, succinic and lactic and also glycerol/ethanol ratio) for the 104 yeast strains (94 *S. cerevisiae* strains and 10 others species *Saccharomyes* sp strains) subjected to fermentation in YPD at 25 °C. Each sphere is a yeast strain and its color corresponds to the prior classification defined in this work and according to the figure caption exposed. The different strains groups were imputed in MeV as gene clusters, strains as genes and their yields values represented as line expression of genes. Respectively, pyruvic and lactic acid yields were the most influential variables in determining the percentage of the principal component 1 (PC1), contributing to the distribution pattern of the strains along the axis 1. For PC 2 and 3, succinic acid yield and glycerol/ethanol ratio were respectively the most determinant variables. The yeast distribution on the chart explains 79% of the data variability among the strains.

This global analysis confirms the previous individual analysis and shows more clearly that the different yields referring to the main fermentative metabolites of wine and non-wine *S. cerevisiae* strains are sufficient to differentiate them metabolically in the standard fermentative conditions studied. The Figure 31 shows that wine strains form a more homogeneous group than non-wine strains who position themselves more dispersed, similarly to the mosaic strains. However, it is interesting to note that only nine wine yeast strains that scattered (CBS1460; CBS2087; NCAIMY00678; CECT10692; DBVPG1788; DBVPG1765; YJM975; YJM978; YJM981), highlighted in light violet, are also the only genetically characterized as wine (W), but isolated from no wine environment (Table 4). On the other hand, the 10 strains belonging to other species of the genus *Saccharomyces* (in red) although are a bit scattered as nW strains, are positioned over the left and on the opposite end of the W strains. This analysis summarizes the results previously presented and indicates that the group of *S. cerevisiae* yeasts presents metabolic differences able to distinguish them from other groups (nW and OS) and suggests different metabolic behaviors.

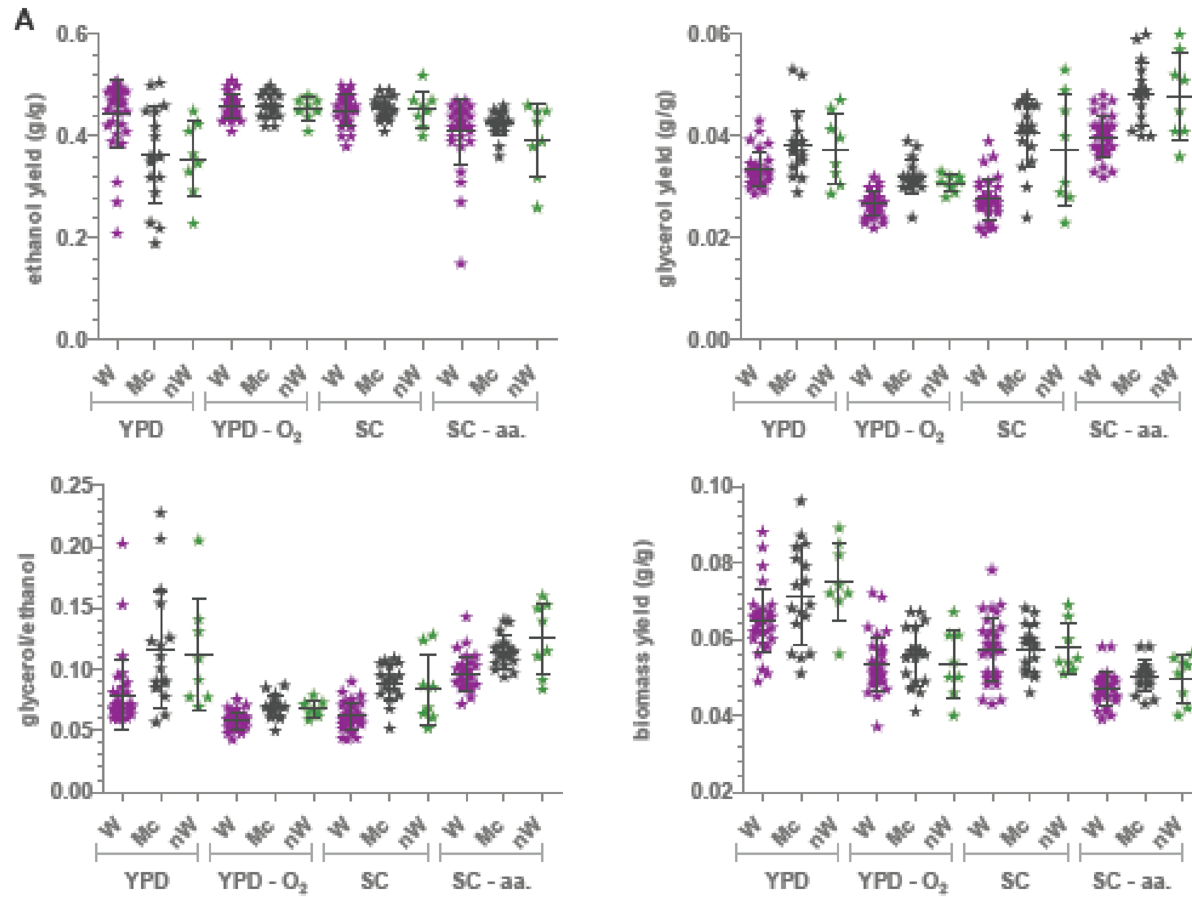
2.3 Metabolic characterization of *S. cerevisiae* strains under distinct fermentation conditions.

Given the previous results presented and to better understand the distinct metabolism fermentation of *S. cerevisiae* strains we decided to deepen these studies in a smaller group consisting of 58 of the 94 *S. cerevisiae* strains initially studied. So we randomly selected 58 strains, 33 W (1-32 and W1), 8 nW (38-45) and 17 Mc (49-65) (Table 4). To investigate whether the different *S. cerevisiae* strains also have distinct metabolic behavior in more restrictive fermentative conditions and understand metabolic changes, besides in YPD (condition 1), we analyzed the same fermentative yields in anaerobic condition (YPD without oxygen – condition 2), in minimal medium

(SC - condition 3) and in minimal medium without amino acids (condition 4).

Considering the W and nW groups, the results illustrated in Figure 32A show that the W strains had higher yield in ethanol (0.44 g/g Glu (W); 0.36 g/g Glu (nW); p value: 0.002) and lower yields in glycerol (0.033 g/g Glu (W); 0.037 g/g Glu (nW); p value: 0.02), glycerol/ethanol ratio (0.08 g/mL (W); 0.11 g/mL (nW); p value: 0.01) and biomass (0.06 g/g Glu (W); 0.07 g/g Glu (nW); p value: 0.01) than the nW strains. Concerning to the organic acids, higher succinic acid average yield (4.5 mg/g Glu (W); 8.5 mg/g Glu (nW); p value: 0.001) produced by nW strains was confirmed as well as higher pyruvic acid yields by W strains (0.65 mg/g Glu (W); 0.48 mg/g Glu (nW); 1.3 times more), although the latter does not show statistical significance. Again there were no significant differences between the W and nW groups for lactic acid and acetic yields. It is interesting to note that for all analyses the Mc strains have a similar yield to nW strains.

It is important to understand the yields changes among the yeasts groups when the strains are submitted to distinct fermentation environments imposed by the three other conditions: the absence of oxygen (2 - YPD-O2); fermentation in minimal medium (3 - SC) and absence of amino acids in a minimal medium (4 - SC - aa). Generally, in these others more restrictive conditions, especially anaerobiosis, the yields of the different strains become more homogeneous, leaving the strains less dispersed in the graphs (Figure 32A-B). This is noted especially among nW strains. Interestingly, the anaerobiosis increased by 1.3 times the ethanol average yield between nW strains, YPD (0.35g/g Glu) and YPD-O_2 (0.45g/g Glu), but did not change significantly among the W strains, YPD (0.44g/g Glu) and YPD-O_2 (0.45g/g Glu). This meant that there were no significant differences in average ethanol yield between the two groups in YPD – O_2.


A
ethanol yield (g/g)
0.6
0.4
0.2
0.0
glycerol yield (g/g)
0.06
0.04
0.02
0.00
glycerol/ethanol
0.25
0.20
0.15
0.10
0.05
0.00
biomass yield (g/g)
0.10
0.08
0.06
0.04
0.02
W
Mc
nW
YPD
YPD - O2
SC
SC - aa.

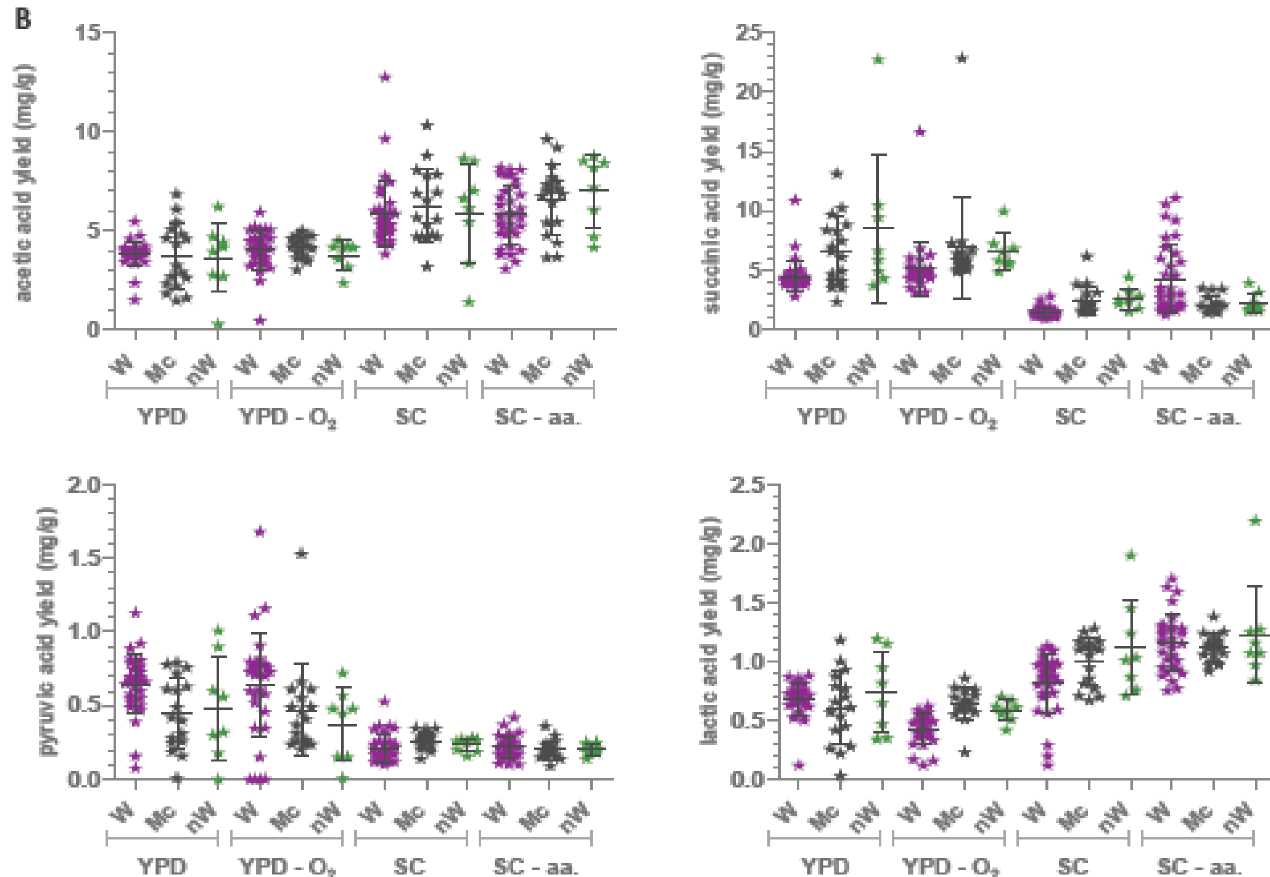


Figure 32. Fermentative metabolites and biomass yield of 58 *S. cerevisiae* strains (33 wine (W), 8 non-wine (nW) and 17 mosaic (Mc)) after micro-fermentations, in triplicate, and under different fermentation conditions shown in each graph (1-YPD, in YPD medium; 2-YPD-O_2, in YPD medium under anaerobiosis; 3-SC, in SC medium; 4-SC-aa, in minimal medium without amino acids). All the fermentations were performed at 25 °C until the glucose concentration achieved average values ≤ 0.5 g/L. For each *S. cerevisiae* strain, the average value of its triplicate is represented on charts in a star shape. **A** – Ethanol, glycerol and biomass yields are expressed in grams per gram of glucose consumed. **B** - Organic acids yields, acetic, succinic, pyruvic and lactic expressed in micrograms per gram of glucose consumed.

The anaerobiosis also provided an increased difference in average glycerol yield between W and nW strains (0.027 g/g Glu (W); 0.031 g/g Glu (nW); p value< 0.0001), so that also the glycerol/ethanol ratio (0.06 g/mL (W); 0.07 g/mL (nW); p value: 0.001) became higher for nW strains. The biomass yields were strongly reduced in both W and nW (0.053 g/g Glu (W); 0.054 g/g Glu (nW); p value: 0.9) strains under this condition, although more sharply among the nW strains (1.35 times more). The succinic (5.1 mg/g Glu (W); 6.6 mg/g Glu (nW); p value: 0.09) and pyruvic (0.64 mg/g Glu (W); 0.38 mg/g Glu (nW); p value: 0.05) acids yields were significantly affected only among nW strains, decreasing by 1.3 times. These modifications eliminated significant differences that previously existed between W and nW concerning to biomass and succinic acid yields. Also in anaerobiosis there were no significant changes or differences in acetic acid production between these strains groups. However, both had a reduction in their lactic acid yields and slightly higher among W strains, leading to significant differences between the two groups.

Another scenario is observed when the yeasts ferment in minimal medium (SC). As in the anaerobic condition, the ethanol yields are similar between W and nW (0.451 g/g Glu (W); 0.454 g/g Glu (nW); p value: 0.86) groups. However, nW strains have an steeper average glycerol (0.028 g/g Glu (W); 0.037 g/g Glu (nW); p value: 0.0002) yield, similar to those shown in the first condition, whereas the W strains show reduced yields, more similar to the anaerobic condition. Thus, the previous differences as regards the glycerol/ethanol (0.06 g/mL (W); 0.08 g/mL (nW); p value: 0.0009) ratio which had been recorded in the first fermentation condition (YPD), becomes even more pronounced. Although with slightly increased yields compared to anaerobic conditions, a strong reduction in biomass yields in both W and nW (0.057 g/g Glu (W); 0.057 g/g Glu (nW); p value: 0.9) groups was observed in minimal medium and especially among nW strains, resulting in very similar average yields. Significant increases in yields of acetic and lactic acid and

abrupt cuts for the pyruvic and succinic acids were striking. Acetic (5.88 mg/g Glu (W); 5.89 mg/g Glu (nW); p value: 0.98) and pyruvic (0.21 mg/g Glu (W); 0.23 mg/g Glu (nW); p value: 0.5) average yields were very similar between the different groups. Significant differences are noted regarding to the succinic (1.5 mg/g Glu (W); 2.6 mg/g Glu (nW); p value< 0.0001) and lactic acids (0.8 mg/g Glu (W); 1.1 mg/g Glu (nW); p value: 0.009), where nW strains show average yields of around 1.8 and 1.4 times higher, respectively, than those presented by W strains.

Comparing the fermentations carried out in SC medium and SC medium without amino acids (condition 4), we can observe that in this fourth condition the pattern of differentiation between W and n W strains for the compounds analyzed in Figure 32 (ethanol, glycerol and biomass) is maintained, only the average yields of each changes. For example, the main change relates to glycerol (0.040 g/g Glu (W); 0.048 g/g Glu (nW); p value: 0.0004) yields and it is remarkable that nW had higher yield than the W strains in both fermentative conditions (SC and SC-aa). However, in the absence of amino acids there is a significant increase in glycerol production in both groups, W (1.4 times) and nW (1.3-fold) reaching the highest average yields among all analyzed conditions. This result reflects in glycerol/ethanol (0.09 g/mL (W); 0.12 g/mL (nW); p value: 0.0002) ratio. Interestingly, although in this fourth condition the nW also present biomass yields slightly higher than W strains, the yields (0.047 g/g Glu (W); 0.050 g/g Glu (nW); p value: 0.1) were reduced by 1.2 times for both groups, and are the lowest values for all four conditions. Significant changes can also be identified as regards to the succinic (4.3 mg/g Glu (W); 2.2 mg/g Glu (nW); p value: 0.06) and lactic acid yields (Figure 32B). In the absence of amino acids W strains increased the average succinic acid yield in 2.9 times while nW strains maintained similar yields to those produced in complete SC medium. This change annulled the significant differences that existed between the two groups for this compound. Similarly, W strains also increased the lactic acid average yield

at 1.4 times eliminating the significant difference with the nW strains. As to the organic acids, nW strains were only affected as regards the acetic acid (5.8 mg/g Glu (W); 7.0 mg/g Glu (nW); p value: 0.06) yields which were elevated by 1.2 times while there was no change in W strains.

Although there was a clear distinction between the nW and W strains groups within each fermentative condition studied, due to yields variability for the different compounds analyzed there was the need for a joint analysis of data in order to make it possible to determine a robust differentiation between the W and nW *S. cerevisiae* strains.

2.4 Overall fermentative metabolism features different metabolic profiles for wine and non-wine *S. cerevisiae* strains.

In order to determine whether there is any relation between the previous genetic characterization, showing the W and nW *S. cerevisiae* yeast strains groups, and the metabolic differences presented between these different strains in different fermentative conditions, we performed a global statistical analysis with all metabolic data. The metabolite yields data analyzed for distinct fermentative conditions of the 58 yeast strains were included in a single PCA analysis in 3D (Figure 33). This analysis informs that the spatial distribution of yeasts along the three axes explains 54% of the variability of all data for the analyzed strains. It is interesting to note that with the exception of three wine strains (CBS 1460; CBS2087 and NCAIM Y00678), there is a separation between W and nW strains into two distinct groups, being the Mc strains distributed permeating nW strains. The three strains, stained with lighter violet and separated from the wine group, are the only among all 33 W strains analyzed in this group that were isolated from no wine sources (Tables 2 and 4).

This PCA-3D displays a result that reinforces the previous individual analyses on the metabolite yields, performed separately for each

fermentative condition, and that determined significant differences capable of differentiating W and nW yeast strains. Thus, after the joint analysis of all these data, the results indicate two distinct fermentative-metabolic behaviors that differentiate W and nW strains and therefore, correlate with different genetic profiles that previously classified them in W and nW yeasts.

Succinic acid under SC-aa condition and pyruvic acid under YPD-O_2, were the most influential variables in determining the percentage of the principal components 1 and 2 (PC1 and PC2) contributing to the distribution pattern of the strains along of their axes. After these variables, the glycerol/ethanol ratio obtained from YPD medium was the more decisive for PC1 and, the pyruvic acid yield in SC medium for PC2. The PC3 was determined mainly by succinic acid yields obtained from YPD-O_2 and from SC-aa.

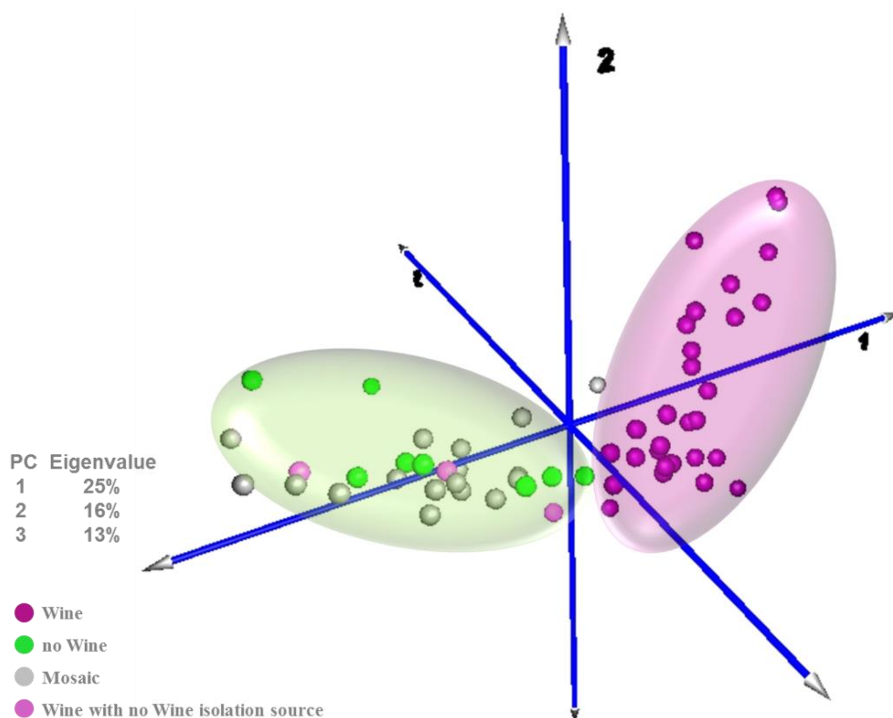


Figure 33. PCA-3D (Principal Component Analysis in 3D) performed using MeV (MultiExperiment Viewer) version 4.9.0. with the normalized values of all analyzed

metabolite yields (glycerol, ethanol, biomass, acetic acid, pyruvic, succinic and lactic and also glycerol/ethanol ratio) obtained from the four fermentative conditions (1-YPD, in YPD medium; 2-YPD-O_2, in YPD medium under anaerobiosis; 3-SC, in SC medium; 4-SC-aa, in minimal medium without amino acids), totaling 32 variables for each of the 58 *S. cerevisiae* strains. Each sphere is a yeast strain and its color corresponds to the prior classification defined in this work and according to the figure caption exposed.

DISCUSSION

In this work, we performed a broad genetic and fermentative study that allowed characterizing and classifying different *S. cerevisiae* strains. Thus it was possible to define two distinct groups, wine (W) and non-wine (nW), and also compare their metabolic profiles with the other species of the genus *Saccharomyces*. Recently it was identified the wild population closest to the wine yeast in the Mediterranean region (Almeida et al., 2015) and it was found that the genomic regions that are key to wine production today are not derived from their ancestral natural yeasts. Their results indicated that during *S. cerevisiae* wine strains domestication there was limited gene flow from wild to wine strains and that genes related to winemaking were gained from other yeast species by horizontal gene transfers. The genetic characterization and phylogeny that we conducted in this study with 113 different strains also show that wine strains strongly diverge from wild or natural strains (Figure 29). Moreover, we report that, although W strains have different geographical origins and sources of isolation, they establish a homogeneous and distinct group of the nW strains which are more dispersed. This homogeneity of W strains led us to think that the winemaking stressful conditions might be driving their genetic differentiation to make possible a better adaptation to the fermentative environment. Thus, this raises the hypothesis that these strains may also have developed a different fermentative metabolism. In fact, the metabolic differences that we find between different yeasts, shows that the respiro-fermentative metabolism of

S. cerevisiae wine strains is distinct from all other yeasts groups classified in this work (non-wine, mosaic and other species of the genus *Saccharomyces*).

These results, expressed by distinct metabolites yield, products and by-products from central carbon metabolism, are summarized in two PCA analyses (Figures 30 and 32). Our analyses show a similar distribution pattern between the different groups of yeast strains (W, nW and Mc) both in phylogenetic analyzes performed (Figure 29) as in the two PCA. In all three figures the wine strains group is isolated and more homogeneous than the others. This similarity between genetic and metabolic findings support the hypothesis that the genomic homogeneity of W strains also seen in many studies (Liti et al., 2009; Sicard and Legras, 2011; Wang et al., 2012), is reflected into their particular metabolic-fermentative features. Probably, these specificities are a result of the *S. cerevisiae* domestication process to winemaking conditions as a way of adapting to the fermentation conditions. The other species (OS) of the genus *Saccharomyces* showed, in general, a fermentative behavior relatively distant from the W strains, but more similar to nW strains. This result also suggests that there may be some metabolic peculiarities in the *S. cerevisiae* wine strains. It is interesting to understand the metabolites yield changes when we compare the fermentations performed in YPD-O_2 (2) with YPD (1); as well as SC-aa (4) with SC medium (3). Under anaerobiosis most of the observed differences cease to be significantly different due to the metabolites yields decrease among the nW strains. The same happened in SC medium without amino acids when compared to the SC medium, but due to the yields increase among the W strains. But still, the glycerol significantly differentiates the two groups in all four fermentative conditions (Figure 32). About ethanol yields, it is important to note that W strains remained almost constant among all conditions, while nW strains increased their yields in 1, 2 and 3 conditions, eliminating the significant differences that existed between W and nW strains groups. Accompanying this ethanol variation, the differences concerning biomass yields also

disappeared mainly due to the reduction among the nW strains. These results indicate that the main differences among W and nW strains relate to the respiro-fermentative metabolism.

Our data show that, compared to nW strains, the W strains are best producers of ethanol and indicate their preference for fermentative metabolism. These observations are based on the differences in fermentative metabolite yields, mainly higher yields of ethanol produced by strains W. In yeasts, the much higher ATP yield from respiratory sugar dissimilation is reflected in the biomass yields. The typical biomass yield on glucose of respiratory cultures is 0.5 g biomass per g glucose, whereas the biomass yield of anaerobic, fermentative cultures is typically 5-fold lower (Verduyn et al., 1991). Then, probably due to the lower ATP energy yield the W strains also produce less biomass than nW strains. However the biomass formation, amino acid synthesis and also the excretion of some oxidized metabolites as pyruvate, acetaldehyde or acetate is accompanied by a net production of reducing equivalents in the form of NADH which needs to be oxidized (Rigoulet et al., 2004). During respiratory growth, the excess NADH formed in glycolysis can be re-oxidized by mitochondrial respiration and thus contributes to meeting the overall ATP requirement for growth, whereas in fully anaerobic conditions, since alcoholic fermentation is itself a redox-neutral process, ethanol formation cannot account for the re-oxidation of assimilatory NADH and *S. cerevisiae* solve this redox problem by reducing glucose to glycerol (Bakker et al., 2001; Overkamp et al., 2002). Thus, comparing to the nW strains, the lower yields in glycerol produced by the W strains can be explained by its also lower biomass yield, consequently there will be no surplus NADH formation enough to be re-oxidized by glycerol synthesis.

It is interesting to remark that the lower glycerol yield from W strains could be also the consequence of a lower respiratory activity, justified by lower succinic acid yields. The residual TCA pathway activity is maintained at

certain levels during fermentation primarily to fuel required biosynthetic reactions and leads to the excretion of organic acids as succinate (Camarasa et al., 2003). In winemaking, depending on the dissolved oxygen levels, there may be an intermediate metabolic configuration characterized by a mixed respiro-fermentative metabolism. It was demonstrated in EC1118 wine yeast strain that above concentrations of 2.7 μM dissolved oxygen, the re-oxidization of cytosolic NADH through the mitochondrial redox shuttle significantly exceeded its re-oxidization by glycerol synthesis (Aceituno et al., 2012). In this study the main features of the respiro-fermentative metabolic configuration were significant respiratory activity, TCA cycle operation in its canonical direction, increased levels of succinic acid production, and a mitochondrial redox shuttle working as a significant cytosolic NADH sink. Compared to the fully anaerobic condition the results showed a large increase (approximately 10-fold) in the level of succinic acid production. Therefore, the highest succinic acid yields found in nW strains compared to the W strains (Figure 30B and 32B) indicates that the latter have fermentative metabolism priority respect to other pathways as respiration when compared to the nW strains. Our results suggest that within the Crabtree-positive *S. cerevisiae* yeasts, the wine strains specialize in the development of "make-accumulate" strategy as a way to better adapt to winemaking. The significant changes of ethanol, biomass and succinic acid yields in nW strains compared to W under anaerobiosis condition (Figure 31), reinforce our hypothesis. The metabolic change of these strains (nW) in response to lack of oxygen, demonstrated by strong increases in ethanol and a reduction of biomass and succinic acid yields, is indicative that there is more mitochondrial metabolic flux than in W strains, and that they have the metabolic capacity and control adjustment for adapting to anaerobic conditions.

In accordance with all this a transcriptional profiling study suggests that succinate formation is coupled to mitochondrial redox balancing, and

more specifically, reductive TCA cycle activity (Agren et al., 2013). This study also supports the higher respiratory efficiency of nW strains deduced from their higher succinic acid yield as a result of higher mitochondrial metabolic flux and necessity of redox balance. Moreover, the reduction of succinic acid yield detected in W and nW strains when comparing rich (YPD) to minimum medium (SC) is accompanied, at the same time, by increase in ethanol and a decrease in biomass yield (from condition 1 to 3, see Figure 31). This variation was significantly found among nW strains indicating ability of respiro-fermentative metabolism regulation, in this case, through decreasing the respiratory activity. In the same direction, it is observed that the highest yields in glycerol were achieved for both groups of strains when fermentations were carried out in SC medium without amino acid as a consequence to the excess in cytosolic NADH generated during amino acid biosynthesis (Bakker et al., 2001; Geertman et al., 2006). Surprisingly, in this condition there was also a significant increase of succinic acid yield among the W strains surpassing by 1.9-fold the nW strains yield. This can be explained because another feature of the respiro-fermentative metabolism in *S. cerevisiae* is the increase in the shuttling of redox equivalents from the cytoplasm to the mitochondria, in this case, possibly favoring the succinic acid formation. Then, probably due to metabolic specialization of W strains in primarily produce ethanol and lower relative ability to synthesize glycerol, producing succinic acid as an alternative to re-oxidize the NADH excess generated through intense amino acids synthesis. On the other hand, as the data suggests that nW strains exhibit a more flexible respiro-fermentative metabolism, its glycerol synthesis can supply the redox imbalance of this condition (SC-aa) not requiring high formation of succinic acid.

The acetic acid has a negative impact on yeast fermentative performance and affects the quality of most of fermented beverages such as wine, being undesirable in certain levels in the fermentative industry of food and beverages (Querol and Fleet, 2006; Vilela-Moura et al., 2011).

However, we did not detect significant differences in acetic acid yields between the distinct W and nW strains groups in any of the studied conditions. Only in the absence of amino acids the nW strains produce slightly higher yields than W, probably also for the redox balance required due to the high amino acid synthesis in this condition. In fact, acetate formation also play a physiological role in the regeneration of reducing equivalents (NADH and NADPH) that are essential for the maintenance of the redox balance (Saint-Prix et al. 2004; Remize et al. 2000). This information is also suggestive that nW strains are more efficient than W to maintain the redox balance.

Above all, the metabolic-fermentative screening performed in this work highlights the biotechnological applicability of some strains studied here. Mostly some no wine strains with similar fermentation capacity compared to wine strains, but also W strains with good glycerol yields and low acetic acid yields. In addition to these *S. cerevisiae* strains that can be used directly as starters in winemaking, others with specific metabolic properties can be enhanced employing adaptive evolution and metabolic engineering techniques in order to improve and expand its applicability in biotechnology.

VI - CONCLUSIONS

The general conclusions that can be derived from this thesis are detailed below:

1. The species *S. kudriavzevii* produce higher levels of glycerol than *S. cerevisiae*. These higher levels are determined mainly by the increased Gpd1p enzyme activity.

2. Gpd1p from *S. kudriavzevii* presented five conserved amino acid replacements compared to *S. cerevisiae* (Ala31Ile, Ile67leu, Glu76Asp, Asp142Asn and Ser143Pro). The two residues that can have more influence in the catalytic properties are in positions 142 and 143, which are close to other residues involved in NAD binding (Phe129 and Lys152). The changes these residues were sufficient to explain increased glycerol accumulation.

3. The glycerol synthesis related genes are regulated differently in *S. cerevisiae* and *S. kudriavzevii.* Unlike *S. kudriavzevii*, the glycerol produced by *S. cerevisiae* was closely linked to the increased gene expression regulation.

4. *S. kudriavzevii* maintain elevated intracellular glycerol levels at both temperatures (12 °C and 28 °C) whereas *S. cerevisiae* strain is able to increase intracellular glycerol content in response to cold conditions.

5. The species *S. cerevisiae*, *S. paradoxus*, *S. uvarum* and *S. kudriavzevii* show different strategies to survive under osmotic or cold-osmotic stressful conditions. In all species, the balance of intracellular glycerol, which depends on the production, efflux, influx and other minor elements is altered in order to increase its levels.

6. The species *S. cerevisiae* relays more in changes in the production to

balance the intracellular glycerol while others, mainly *S. uvarum* or *S. kudriavzevii,* tend to depend more on the variation of the influx.

7. The presence of the *S. uvarum* BMV58 and *S. kudriavzevii* IFO1802 *SLT1* alleles are clearly more able to promote growth recovery when there is extracellular glycerol in the presence of a hyperosmotic stress compare to S. cerevisiae wine strain T73. We also suggest that this is due a low functionality of T73 Stl1p.

8. The genetically characterized as wine *S. cerevisiae* strains (W) form a more homogeneous group and phylogenetically distant and opposite to other strains characterized as pure non-wine, which shows higher genetic variability among themselves.

9. Different respiro-fermentative behaviors distinguish the wine and non-wine *S. cerevisiae* strains. The results suggest that wine strains have limited aerobic respiration capacity and favored fermentation metabolic pathway. These strains adaptations promote an increase in the efficiency of life strategy "make-accumulate". On the other hand, non-wine strains show more balanced respiro-fermentative metabolism and are more efficient in adapting to abrupt environmental changes because they relay in a robust balance of respiratory and fermentative metabolic pathways.

VII - REFERENCES

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