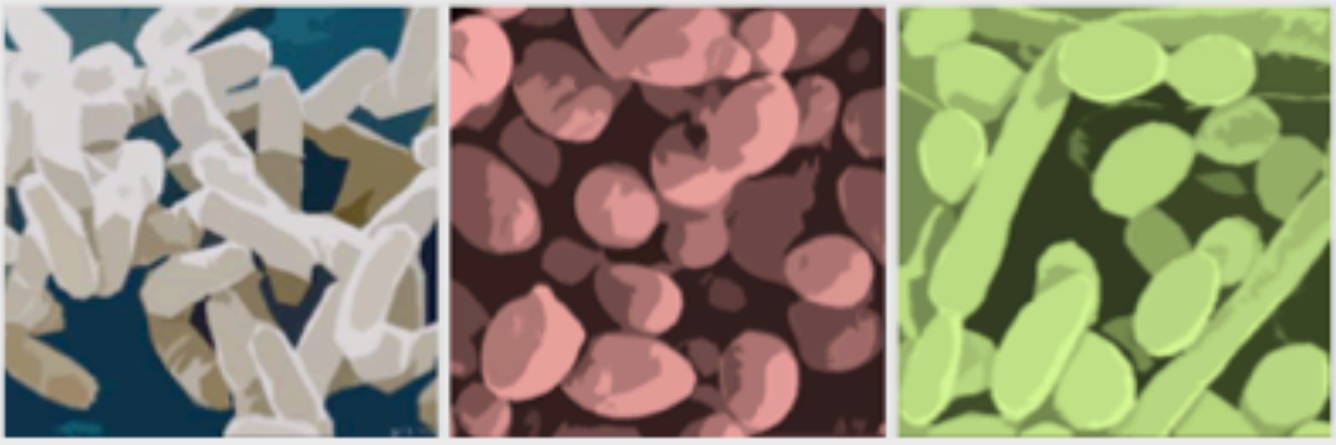
UNIVERSITAT ROVIRA i VIRGILI

Characterization of non-*Saccharomyces* wine yeasts during desiccation stress imposition

Gemma Roca Domènech



DOCTORAL THESIS
2018

Gemma Roca Domènech

Characterization of non-*Saccharomyces* wine yeasts during desiccation stress imposition

Doctoral thesis directed by:

Dr. Ricardo Cordero Otero and Dr. Nicolas Rozès

Department of Biochemistry and Biotechnology

Faculty of Oenology

University Rovira i Virgili

UNIVERSITAT ROVIRA i VIRGILI

Tarragona, 2018

This doctoral thesis was carried out from 2014 to 2018 in the Food Biotechnology Microbiology research group, at the Department of Biochemistry and Biotechnology in the Faculty of Oenology at the University Rovira i Virgili. The doctoral thesis was supervised by professors Ricardo Cordero and Nicolas Rozès.

The research was financially supported by a Spanish Government project (AGL2012-40018).

The doctoral research was support by the University Rovira i Virgili with a doctoral fellowship (2014PMF-PIPF-47). The researcher's three months stay at Changins College for Viticulture and Oenology (Nyon, Switzerland) was funded by a MOU International Student Exchange Programmes from the University Rovira i Virgili and by a Swiss-European Mobility Programme.

Editing of the thesis book was supported by Postgraduate and Doctoral School of the University Rovira i Virgili.

UNIVERSITAT ROVIRA i VIRGILI

Department of Biochemistry and Biotechnology

Faculty of Oenology

Marcel·lí Domingo, 1

43007 - Tarragona

Spain

We STATE that the present study, entitled "**Characterization of non-*Saccharomyces* wine yeasts during desiccation stress imposition**", presented by **Gemma Roca Domènech** for the award of the degree of Doctor with International Mention, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university.

Tarragona, 4th July 2018

Doctoral Thesis Supervisors

Dr. Ricardo Cordero Otero

Dr. Nicolas Rozès

ACKNOWLEDGEMENTS

Una etapa increïble acaba ara, sembla ahir que començava aquest camí que ha durat gairebé 4 anys i que m'ha aportat tantes i tantes experiències, en l'àmbit professional ha estat impressionant, l'oportunitat d'augmentar el meu coneixement crític i raonament científic ha estat fantàstic. Però, el que mai m'hagués pogut imaginar és la quantitat de persones que m'enduc d'aquesta experiència que mai podré oblidar; persones que mica en mica van anar entrant a la meva vida i que sense adonar-me'n s'han anat tornant imprescindibles per mi.

Per començar agrair als meus directors de tesis, al Dr. Ricardo Cordero i al Dr. Nicolas Rozès per brindar-me l'oportunitat de formar part del grup Biotecnologia Microbiana dels Aliments (BMA). Gràcies per guiar-me durant tot aquest procés, per tantes reunions i discussions científiques que han fet créixer el meu criteri científic i per ajudar-me a enfocar cadascun dels resultats obtinguts, tot i que alguns d'ells no fossin els esperats, per ensenyar-me a buscar alternatives i sobre tot per deixar-me gaudir tant amb allò que m'agrada, la investigació. Per tot això i més, només puc donar-vos les gràcies per tots els moments viscuts al llarg de tots aquests anys. A la Dra. Montse Poblet, també et vull agrair tots els bons consells que m'has donat durant tot aquest temps, sempre disposada a buscar solucions als entrebancs que podien anar sorgint al llarg de tota la part experimental, i per comptar amb mi per donar cursos i lligar el món de la ciència al món de la docència. També vull fer especial menció a tots els membres del tribunal de la tesis i als revisors externs.

Moreover, I would like to thank Dr. Roland Riesen and Dr. Ramón Mira de Orduña the opportunity that they gave to me working with them and their hospitality during my stay in the Changins College for Viticulture and Oenology of Nyon, in Switzerland. It was a pleasure for me. Thanks!

Un altre pilar fonamental en aquesta etapa heu estat vosaltres, Papa i Mama!! Però això ja ve de lluny, des de que tinc us de raó heu estat al meu costat, fent-me costat en tot, tant si era bo o dolent, divertit o trist, fàcil o difícil. I el més important... MAI heu dubtat de mi, fins i tot quan jo mateixa en dubtava. Sempre heu tingut fe cega en mi i que allò que em proposés ho podria arribar a aconseguir. Així que només puc agrair-vos per tant, per estar allí quan us necessitava, i per seguir-hi estant quan potser creia que no, perquè... sabeu què? SEMPRE sereu imprescindibles en la meva vida. Us estimo moltíssim! Equip RD!!!! Sabeu que sempre em tindreu pel que necessiteu!

Avis, aquesta tesis també és una miqueta vostra, perquè sense vosaltres i cadascun dels criteris que em vau ensenyar jo no seria qui sóc. Perquè sé que cadascú de vosaltres em cuida i vetlla per a que aconsegueixi els meus somnis. Estigueu on estigueu, aquí amb mi o al cel, moltes gràcies per tot. Us estimo!

I ara... un altre factor importantíssim, els amics!! Sembla mentida com les amistats poden fer que moments difícils es tornin més fàcils, com un mal dia en pot acabar sent un de bo i com la vida compartida amb gent que estimes pot ser meravellosa!

Per qui començar... ho tinc difícil... però crec que què millor que començar per aquelles persones que estaven quan vaig iniciar aquesta aventura? Així que, Gema i Anna, què dir-vos que no sapigueu? Recordo arribar al despatx de Ricardo l'últim curs de carrera amb moltíssimes ganes d'aprendre gràcies a l'oportunitat que m'havia ofert i poder descobrir si treballar en ciència era el que em feia feliç. I... les primeres persones que vaig conèixer vau ser vosaltres i vau fer que em sentís part del vostre equip (perquè sí, juntes éreu un equip imparable!), recordo els aniversaris on sempre teníeu detalls increïbles, hores de feina fins altes hores que acabaven amb riures i estones màgiques, els nostres "cafès" que sempre acabaven sent "chocolatitos", i infinitat de moments més. Gema, gràcies per la paciència i per tot el temps que vas invertir, gràcies a tu vaig aprendre moltíssim amb poc temps i gran part del que sé avui és gràcies a tu. Anna, gràcies per seguir al meu costat fins a dia d'avui, i que tot i que portem vides carregades de responsabilitats sempre ens tenim pel que faci falta. Més tard va arribar l'Eugènia que va acabar de completar el grup, ella també va ser un pilar fonamental i sempre tenia un somriure per a tothom, fent que les hores de feina passessin més ràpides. Durant els meus inicis també hi va haver molta més gent que va tenir un paper molt important, Eunice, Jenny, Laura, Manu, Mar, Òscar; Moltes Gràcies!

Després va arribar l'època en què deixava de ser aprenent per convertir-me en "mestre", de persones increïbles que sempre van demostrar moltíssimes ganes d'aprendre i treballar. El primer vas ser tu Víctor, recordo el primer dia... crec que no cal que mencioni cadascuna de les anècdotes, que en tan sols un dia ja es van crear... ni totes les següents... Però sí que et vull donar les gràcies per totes les estones que vam passar colze amb colze al laboratori i per tots els riures que vam passar junts (que n'hi va haver i molts!). El millor de tot, és que d'aquell temps me'n vaig endur un gran amic i a sobre vas madurar una miqueta, i mira't ara, tot un noi responsable! Estic molt orgullosa de tu. Moltes gràcies per tot!

A continuació, van arribar la Laura i la Carmen, dues noies increïbles, molt intel·ligents i amb una gran capacitat d'aprenentatge i ganes de treballar. Guiar els seus treballs de fi de grau i màster va ser tot un luxe i m'ho van posar molt molt fàcil! Gràcies per tants bons moments!

I mentre tot això anava succeint... les tres personetes més magnífiques que podrien haver aparegut a la meva vida van aparèixer com per art de màgia, Helena, Mercè i Núria!!! Elles han fet que acabar la tesis fos més fàcil, ja se sap que el tram final d'una tesis sempre és el més dur, però al seu costat tot ha sigut més lleuger. Milions de gràcies per ser com sou, si us plau no canvieu mai, sou increïbles i qualsevol persona que us pugui tenir al costat serà molt afortunada de tenir-vos. Hem passat mil moments, moments de riures infinits o moments en què semblava que el món s'acabés, però sempre juntes! Helena! Gràcies per tot, perquè vam tenir molta sort de que et donessin la plaça de tècnic i que ara et puguis quedar fent el doctorat! Perquè sempre tens un detall amb tothom en els moments importants. Mercè! Què dir-te... doncs que en res ens vam fer molt amigues, i sempre ens hem ajudat en qualsevol moment, ens hem visitat en les estades que hem fet i estic segura que seguirem ajudant-nos en tot allò que faci falta! Núria! Hem passat moments de tot els últims temps, però sempre ens hem ajudat i donat suport. Va ser una gran sort que t'oferissin fer el doctorat i que acceptessis! Ara espero que siguis molt feliç en la nova etapa que inicies! Moltes gràcies a les tres per ser com sou i perquè juntes formem un gran equip! Espero que aquest equip segueixi per molt de temps!

I mentrestant moltes altres persones també han sigut importants durant tot aquest recorregut; Adeline, Adrià, Aga, Aitor, Alba O., Alba, Bea, Chunxiao, Dalia, David, Flor, Jèssica, Jordi, Judit, Iñigo, Laura, Laura, Maria M., Maria M., Maria, Mariona, Mari Ángeles, Maria José, Micaela, Michael, Núria B., Olga, Pere, Stéphanie, Xiaolin.

També agrair tota l'ajuda i suport rebut per tots els tècnics, per tots els moments en què els he necessitat, gràcies a la Braulio, Isa, Niurka, Rosa, Santiago.

De la meva estada a Suïssa també m'enduc a altres persones increïbles, tècnics que em van ajudar molt quan vaig arribar i encara no coneixia gaire com funcionaven les coses allí. A Oswaldo por todos los consejos y tardes de charlas, además de prepararnos comida típica de México. And... Cintia, what can I say? I was very lucky to find you there. Thank you so much for all your help, your English classes and all the trips that we did. It was an amazing time and it was better because of you! I miss you, but I know that we will keep in contact!

Tampoc vull ni puc oblidar a molts d'altres amics, que tot i no estar vinculats directament amb el doctorat, han estat al meu costat durant tot aquest temps, aconsellant-me en certs moments, ajudant-me a sortir de la rutina i alegrant-se de que hagi pogut arribar fins on he arribat a dia d'avui, per això moltíssimes gràcies a les meves amigues de sempre, Cristina, Míriam i Sara! Al igual que a molts dels meus amics Salsixteros, com nombrar-vos a tots i no deixar-me'n cap? Vosaltres sabeu qui sou! I a les meves nenes d'orquestra, que sempre han confiat amb mi; Gràcies Laura i Montse!

Gràcies a tots vosaltres i molts d'altres que segur em deixo, ara comença una nova etapa, una etapa que arranco amb molta il·lusió però també amb certa malenconia per deixar tanta gent important enrere, però estic segura que l'amistat perdurarà.

"What is a scientist after all? It is a curious person looking through a keyhole, the keyhole of nature, trying to know what is going on".

Jacques Yves Cousteau, 1910-1997

Als meus pares,

exemple de constància, dedicació i fortalesa

CONTENTS

Objectives and Outline of the thesis 23

Chapter I 29

Literature review

Chapter II 83

Enhancing the tolerance of the *Starmerella bacillaris* wine strain to dehydration stress

Chapter III 107

Viability enhancement of *Schizosaccharomyces pombe* cells during desiccation stress

Chapter IV 131

Magnesium enhances dehydration tolerance in *Schizosaccharomyces pombe* by promoting intracellular 5'-methylthioadenosine accumulation

Chapter V 165

Metabolism of *Schizosaccharomyces pombe* under reduced osmotic stress conditions afforded by fed-batch alcoholic fermentation of white grape must

Chapter VI 183

General Discussion

Chapter VII 195

General Conclusions

List of Figures

Chapter I _____

1. Release of primary aroma compounds by yeasts 33
2. Yeast energy metabolism 37
3. Yeast nitrogen metabolism 39
4. Mechanisms of nutrient uptake in yeasts 43
5. Model for cellular acquisition and transport of metal ions 44
6. Potassium homeostasis in *Saccharomyces cerevisiae* 45
7. Calcium and magnesium homeostasis in *Saccharomyces cerevisiae* 48
8. Zinc and manganese homeostasis in *Saccharomyces cerevisiae* 50
9. Iron and copper homeostasis in *Saccharomyces cerevisiae* 52
10. Responses of yeast to stress factors 54
11. Schematic outline of the major pathways for phospholipid biosynthesis in yeast 56
12. Characterization of different phospholipids according to their structure factor . 57
13. A schematic diagram of the HOG pathway in *Saccharomyces cerevisiae* 60
14. Generation of Reactive Oxygen Species (ROS) in cells 62
15. Enzymatic defence systems involved in the control of the redox state and ROS detoxification in yeast 63
16. Signalling pathways activated by hydrogen peroxide (H_2O_2) in *Saccharomyces cerevisiae* 64
17. Antioxidant enzymes 66

Chapter II _____

18. *Starmerella bacillaris* cell viability based on the cellular physiological state 91
19. Evaluation the effect of temperature and tme on *Starmerella bacillaris* Sb3 viability following rehydration 91
20. Effect of trehalose treatment on *Starmerella bacillaris* Sb3 viability following air drying and rehydration 92
21. Effect of rehydration treatments on *Starmerella bacillaris* cell viability 94
22. Effect of rehydration treatments on *Starmerella bacillaris* cell viability 95
23. Effect of rehydration treatments on *Starmerella bacillaris* cell vitality 96

Chapter III

24. *Schizosaccharomyces pombe* cell viability based on the cellular physiological state 114
25. Effect of trehalose treatment on *Schizosaccharomyces pombe* cell viability following air-drying and rehydration 116
26. Effect of rehydration treatments on cell viability 117
27. Effect of rehydration treatments on cell viability 119
28. Quantification of the accumulation of ROS and growth curves after yeast cell rehydration 122
29. Consumption of sugars and malic acid during alcoholic fermentation 123

Chapter IV

30. Effect of rehydration treatments on the viability and quantification of intracellular magnesium of *Schizosaccharomyces pombe* strains 143
31. Quantification of ATP and pyruvate of *Schizosaccharomyces pombe* strains . . . 145
32. Outline of the biosynthetic pathways of methionine, β-alanine/pantothenate, glutathione, and phospholipids 147
33. Metabolites from methionine salvage pathway and related pathways detected by UHPLC-(ESI^+)-QqQ/MS of *Schizosaccharomyces pombe* strains 150
34. Relation between phospholipids and phosphatidylcholine intermediaries of *Schizosaccharomyces pombe* strains 153
35. Quantification of glutathione of *Schizosaccharomyces pombe* strains 154
36. Viability and quantification of ROS accumulation of *Schizosaccharomyces pombe* strains after rehydration 157
37. Magnesium and spermidine effect in *Schizosaccharomyces pombe* lifespan cells 159

Chapter V

38. Consumption of sugar and production of ethanol during traditional batch and fed -batch fermentations 171
39. Time course of total sugars, glucose and fructose during traditional batch and fed-batch fermentations 172
40. Time course of malic and lactic acid during traditional batch and fed-batch fermentations 173
41. Time course of sugar induced osmotic stress associated metabolites glycerol, acetic acid and acetaldehyde during traditional and fed-batch fermentations . .174

List of Tables

Chapter I

1. Diversity of the fermentation products of certain yeasts 31
2. Examples of studies of wine fermentation inoculated with selected mixtures of yeast species to improve primary and secondary wine aroma 35
3. Metals most required for yeast cell growth and metabolic functions 41

Chapter II

4. List of *Starmerella bacillaris* strains evaluated in chapter II 87
5. Concentration of volatile compounds produced at the end of all fermentation processes 98

Chapter III

6. List of *Schizosaccharomyces pombe* strains evaluated in chapter III 111
7. Concentration of volatile compounds produced at the end of all fermentation processes 124

Chapter IV

8. Analytical parameters of qTOF 137
9. Analytical parameters of UHPLC-(ESI^+)-QqQ 139
10. Number of metabolites identified with analysis in positive (ESI^+) and negative (ESI^-) ionization 146

Chapter V

11. Final concentrations of various fermentation parameters 172

OBJECTIVES and OUTLINE OF THE THESIS

The main target of this study has been the physiological characterization of non-*Saccharomyces* wine yeasts under desiccation stress imposition. Although *Saccharomyces cerevisiae* has been considered the primary yeast involved in the winemaking process, non-*Saccharomyces* wine yeasts are being increasingly investigated with regards to their grape winemaking potential. In the last few decades, active dry wine yeasts (ADWY) are the most widely used in winemaking as starters, ensuring the strain identity and genetic stability at room temperature, and reducing transport and storage costs. Most isolated or laboratory wine strains lose viability during the drying process, which is characterized by a dehydration of the yeast biomass to a final product with a residual moisture percentage of below 8%. Consequently, this drying process includes an imposition of several stresses (e.g. heat, osmotic and oxidative), which induce a number of stress responses such as, antioxidants, osmoregulation or ion homeostasis. Otherwise, ADWY must be previously rehydrated before its employment. Our group developed several biochemical and biophysical features focused on the evaluation of *Saccharomyces cerevisiae* as ADWY, while the presence of magnesium during the rehydration process enhance cell resistance to resume cell cycle. Within this framework, the working hypothesis of this thesis was:

Magnesium prevents dehydration cell-damage in non-*Saccharomyces* wine yeasts.

To validate this hypothesis, the general aim was to study the possible role of several metabolites in order to enhance the tolerance after stress imposition of different non-*Saccharomyces* wine yeasts. To achieve this general aim, the following specific objectives were attained:

OBJECTIVE 1. **Development of ADWY protocol for *Starmerella bacillaris* and *Schizosaccharomyces pombe* strains.** Results are reported and discussed in **Chapters II** and **III**.

Thanks to the availability of wine yeast strains as Active Dry Wine Yeast (ADWY), winemakers are able to achieve sequential inoculation of grape must to improve wine complexity. In chapter II, compound features were analyzed during the dehydration-rehydration process for *S. bacillaris* strains isolated from related environments of

alcoholic beverages. In chapter III, the features of metabolites were analyzed during the dehydration-rehydration process for different *Schiz. pombe* strains to determine whether these metabolites might play a positive role in ensuring cell viability before inoculation into the must. Moreover, in both chapters, fermentation performance and organoleptic evaluation were developed to characterize the ADWY version of these yeast strains.

OBJECTIVE 2. **Metabolomics characterization of magnesium during the rehydration process in *Schizosaccharomyces pombe* strains**. Results are reported and discussed in **Chapter IV**.

We analysed metabolite features during the dehydration-rehydration process for two *Schiz. pombe* strains with different viability rate, in order to determine whether metabolite contents were affected by the presence of magnesium during cell rehydration, which might play a role in cell viability. The qualitative changes of the intracellular metabolites of both strains were determined by comparing the metabolic profiles of cells before dehydration, after rehydration in water and magnesium solution, and after 2 h inoculation of both kinds of rehydrated cells in complete medium.

OBJECTIVE 3. **Study the metabolic response of *Schizosaccharomyces pombe* under reduced osmotic stress conditions attained by a novel automated fed-batch technique.** Results are reported and discussed in **Chapter V**.

Strains of *Schizosaccharomyces pombe* are being increasingly investigated with regards to their grape winemaking potential either in combination with the typical production yeast, *Saccharomyces cerevisiae*, or in monoseptic fermentations. Their ethanol tolerance and ability to degrade L-malic acid is oenologically convenient but contrasts with the comparatively high acetic acid and acetaldehyde formation potential which is considered undesirable, especially in white winemaking. We investigated the performance of a selected *Schiz. pombe* strain in monoseptic traditional batch fermentations and in automated fed-batch fermentations. Because of its known effect on growth and ethanol tolerance, the effect of Mg was also tested.

Thus, these three objectives were developed in the following chapters:

Chapter II. **Enhancing the tolerance of the *Starmerella bacillaris* wine strain to dehydration stress.** Results submitted to *Annals of Microbiology*.

Chapter III. **Viability enhancement of *Schizosaccharomyces pombe* cells during desiccation stress.** *Journal of Microbiology Research* (2016) 6: 82-91.

Chapter IV. **Magnesium enhances dehydration tolerance in *Schizosaccharomyces pombe* by promoting intracellular 5'-methylthioadenosine accumulation.** Results submitted to *Yeast.*

Chapter V. **Metabolism of *Schizosaccharomyces pombe* under reduced osmotic stress conditions afforded by fed-batch alcoholic fermentation of white grape must.** Results accepted in *Food Research International.*

CHAPTER I

Literature review

1. Yeast 31
1.1. Yeast and Wine Production 31
2. Yeast Metabolism 35
2.1. Carbon metabolism 36
2.2. Nitrogen metabolism 37
2.3. Vitamins 39
2.4. Minerals 40
2.4.1. Potassium 44
2.4.2. Magnesium 45
2.4.3. Calcium 47
2.4.4. Zinc 49
2.4.5. Manganese 49
2.4.6. Iron 50
2.4.7. Copper 51
3. Yeast Stress Factors and Responses 53
3.1. Cell membrane structural modification 55
3.2. Heat Shock Protein Biosynthesis 58
3.3. Trehalose and Glycogen Synthesis 59
3.4. Antioxidant defenses 62
3.4.1. Non-Enzymatic Antioxidant Defense Systems 64
3.4.2. Enzymatic Antioxidant Defense Systems 65
4. References 69

1. Yeast

Yeasts have been used for making bread, beer and wine since ancient times. In the second half of the 19th century, Louis Pasteur recognized the role of yeasts in the wine fermentation process, while Hansen and Müller obtained the first pure cultures (starters) of brewer's and wine yeast, respectively. Since then, the application of yeast cells as starters has become a common practice in industrial fermentation, not only for food and beverages, but also for a huge variety of other significant products in modern biotechnology, such as the production of heterologous enzymes, vaccines, hormones and biofuels (Table 1) (Walker, 2004; Barnett & Barnett, 2011; Mateo & Maicas, 2016).

Table 1. Diversity of the fermentation products of certain yeasts (adapted from Walker, 2004).

Yeast species	Industrial applications of yeasts
Saccharomyces cerevisiae	Beer, wine, distilled spirits, bioethanol, baked foods, probiotics/animal food supplement, organic chemical reductions, hepatitis B vaccine, human insulin, human serum albumin
Schizosaccharomyces pombe	Bioethanol, rum, wine-deacidification, indigenous fermented beverages, recombinant proteins
Kluyveromyces spp.	Cheese whey fermentations, biomass protein, pectinases, recombinant chymosin
Komagataella pastoris (Pichia pastoris)	Recombinant proteins
***Ogataea polymorpha* (*Hansenula polymorpha*)**	Recombinant proteins
Yarrowia lipolytica	Recombinant proteins
Xanthophyllomyces dendrorhous (Phaffia rhodozyma)	Food and feed pigment (astaxanthin)
Candida utilis (Cyberlindnera jadinii)	Biomass protein
Zygosaccharomyces rouxii	Traditional oriental fermented food (e.g., soy sauce, miso)

1.1. Yeast and wine production

Until about the 1980s, the contribution of yeasts to wine production was seen as a relatively simple concept. However, wine fermentation is a complex microbiological process in which yeasts play a fundamental role, where a succession of different yeast species are involved in the spontaneous alcoholic fermentation of grape juice. Yeast cells are responsible for converting grape sugars into ethanol, carbon dioxide and several other secondary products in alcoholic fermentations (Pretorius *et al.,* 1999; Fleet, 2008).

Over the last few decades, major advances have been made in terms of understanding the ecology, biochemistry, physiology and molecular biology of the yeasts involved in wine production, and how these yeasts affect the chemistry and sensory properties of the final wine, such as the aroma, taste, colour, clarity, mouthfeel and astringency (Fleet, 2008; Pinu *et al.,* 2014). One of the most important technological advances in winemaking has been the inoculation of grape juice with selected cultures of *Saccharomyces cerevisiae* (Comitini *et al.,* 2011). Nowadays, active dry wine yeasts (ADWY) are the most widely used in winemaking as starters, ensuring the strain identity and genetic stability at room temperature, and reducing costs of transportation and storage. However, the yeast ecology of the fermentations has been found to be much more complex than the assumed dominance of the inoculated strain of *S. cerevisiae*, which is the quintessential wine yeast because of its fermentation behaviour, its important role in the release of aroma precursors and the formation of secondary aroma. Many other species of yeasts belonging to various non-*Saccharomyces* genera occur in grape juice (Fleet, 2008; Padilla *et al.,* 2016). Several studies have reported that some genera of non-*Saccharomyces* wine yeasts, such as *Candida, Hanseniaspora*, *Metschnikowia*, *Komagataella* and *Torulaspora,* may dominate the early stages of traditional alcoholic fermentations, whereas, in later stages, they are replaced by *S. cerevisiae* yeasts, which complete the fermentations (Mills *et al.,* 2002; Zott *et al.,* 2008). Occasionally, species of other genera such as *Schizosaccharomyces and Zygosaccharomyces* may also be present in the early fermentation stages (Fleet, 2008; Medina *et al.,* 2012).

Moreover, while non-*Saccharomyces* yeasts have usually been considered responsible for organoleptic defects in wine and associated with high volatile acidity, and wine spoilage, several studies have now revealed the relevant role of selected non-*Saccharomyces* species in relation to the final analytical composition and sensorial characteristics of wine (Fleet *et al.,* 1984; Loureiro & Malfeito-Ferreira, 2003; Ciani *et al.,* 2006; Viana *et al.,* 2008). These sensorial characteristics may be attributable to the ability to secrete enzymes and produce several secondary metabolites such as glycerol, organic acids, higher alcohols, esters, acetates, aldehydes, ketones, amines and sulphur volatiles, leading to an enhancement of wine aromatic complexity and flavour diversity (Fleet *et al.,* 1984; Domizio *et al.,* 2011; Mateo & Maicas, 2016).

Aroma is one of the most important characteristics that contribute to the quality of wine and can be classified into three groups: the primary aroma, determined by the grape variety; the secondary aroma, coming from the fermentation process; and the tertiary aroma resulting from the transformation of aromas during aging.

For example, genera such as *Candida*, *Hanseniaspora, Metschnikowia, Komagataella, Schizosaccharomyces* and *Zygosaccharomyces* are strong producers of glycosidases, which are the enzymes responsible for the hydrolysis of non-volatile glycosylated precursors of the grape into volatile compounds, resulting in characteristic fruity, estery, spicy and vegetative aromas with an impact on the wine's final character (Charoenchai *et al.,* 2008; Lleixà *et al.,* 2016). Otherwise, *Starmerella bacillaris* (synonym of *Candida zemplinina*) and *Komagataella* can produce concentrations of volatile thiols comparable to those produced by *S. cerevisiae.* Volatile thiols, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), are generated from cysteinylated precursors by the action of carbon-sulfur-lyases, and contribute to positive fragrances such as tropical and passion fruit (Figure 1). *Candida* and *Komagataella* also present enzymes with proteolytic activity that hydrolyses the peptide linkages between amino acid units of proteins, improving the clarification process in wine and protein stability (Esteve-Zarzoso *et al.,* 1998; Lleixà *et al.,* 2016).

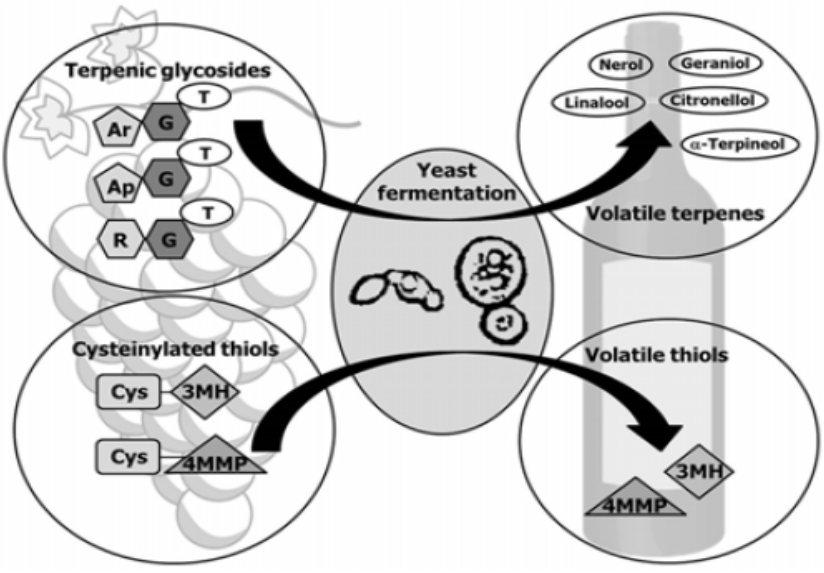


Figure 1. Release of primary aroma compounds by yeasts (Padilla *et al.,* 2016).

Higher alcohols (e.g. 2-phenylethanol, isoanyl alcohol) also contribute to the aromatic complexity of wine at concentrations below 300 mg/L. Specifically, increased production of 2-phenylethyl alcohol has been described as a characteristic of *Metschnikowia pulcherrima* and *S. bacillaris*. Otherwise, glycerol is involved in redox potential regulation in the yeast cells and it also contributes to smoothness, sweetness, and complexity in wines. *S. bacillaris* can produce high glycerol concentrations during wine fermentation. However, increased glycerol production may be correlated with increased acetic acid production, which can be detrimental to wine quality due to its flavour becoming unpleasant at concentrations higher than 1 g/L. *Hanseniaspora*, *Schizosaccharomyces* and *Zygosaccharomyces* have been commonly associated with high levels of acetic acid (Mateo & Maicas, 2016).

Furthermore, although *Schizosaccharomyces pombe* is commonly associated with wine spoilage organisms due to their strong negative organoleptic and chemical deviations (acetic acid, acetaldehyde, hydrogen sulphide (H_2S), acetoin, and ethyl acetate), *Schiz. pombe* shows a high capacity to deacidify wines by converting malic acid to ethanol and CO_2 (malic dehydrogenase activity), high autolytic polysaccharide release, gluconic acid reduction properties, and urease activity (Taillandier *et al.,* 1995; Peinado *et al.,* 2009; Domizio *et al.,* 2017). *Schiz. pombe* also has the capacity to exhibit high tolerance to low pH and high SO_2 levels. (Benito *et al.,* 2016; Silva *et al.,* 2003).

Otherwise, *S. bacillaris* is a psychrotolerant and osmotolerant yeast. These properties can be exploited in sweet wine production, which is characterized by high sugar concentration and low fermentation temperatures. Moreover, winemakers also have a special oenological interest in *S. bacillaris* because of its fructophilic character (Magyar & Tóth, 2011; Tofalo *et al.,* 2012). Fructose is approximately twice as sweet to taste as glucose, so excess fructose may be associated with undesirable sweetness in wines and could cause sluggish or stuck fermentations (Berthels *et al.,* 2008). Co-inoculation of *S. cerevisiae* (glucophilic yeast) and *S. bacillaris* (fructophilic yeast) may prevent fermentation stagnation due to the complementary use of fructose and glucose, allowing the consumption of all the sugar (Ciani & Ferraro, 1998).

Consequently, there has recently been growing interest in new and improved wine yeast strains for different types and styles of wines (Wang *et al.,* 2016). The use of

mixed starters of selected non-*Saccharomyces* yeasts with strains of *S. cerevisiae*, performed simultaneously or in sequential cultures, has become one of the main challenges faced by researchers and oenologists, taking advantage of the potentially positive role that non-*Saccharomyces* wine yeast species play in the organoleptic characteristics of wine (Table 2) (Ciani *et al.,* 2010; Comitini *et al.,* 2011; Rantsiou *et al.,* 2012; Padilla *et al.,* 2016). So, better yeast management during alcoholic fermentation enables winemakers to engineer wine character and obtain wines for a changing market, due to consumer demand for new wine styles (Fleet, 2008).

Table 2. Examples of studies of wine fermentation inoculated with selected mixtures of yeast species to improve primary and secondary wine aroma (adapted from Fleet, 2008; Padilla *et al.,* 2016).

Yeast species inoculated	Impact on wine aroma	References
Starmerella bacillaris / S. cerevisiae	3MH increase	Anfang *et al.,* 2009
	Acetic acid decrease	Ciani & Ferraro, 1998; Rantsiou *et al.,* 2012
	Ethyl acetate increase	Ciani & Ferraro, 1998; Jolly *et al.,* 2006
	Glycerol enhancement	Ciani & Comitini, 2006
Schizosaccharomyces pombe / S. cerevisiae	Wine deacidification	Silva *et al.,* 2003; Benito *et al.,* 2013, 2014
Hanseniaspora spp. */ S. cerevisiae*	Acetate ester increase	Moreira *et al.,* 2008; Andorrà *et al.,* 2010, 2012
	Acetate and ethyl ester increase	Viana *et al.,* 2009, 2011; Medina *et al.,* 2013
Metschnikowia pulcherrima / S. cerevisiae	α-Terpineol increase	Rodríguez *et al.,* 2010
	Acetic acid decrease	Comitini *et al.,* 2011
	Ethyl ester increase	Zohre & Erten, 2002; Rodríguez *et al.,* 2010
	Higher alcohol increase	Comitini *et al.,* 2011
Torulaspora delbrueckii / S. cerevisiae	Acetic acid decrease	Bely *et al.,* 2008; Comitini *et al.,* 2011
	Acetate and ethyl ester increase	Loira *et al.,* 2014, 2015; Renault *et al.,* 2015
	Higher alcohol increase	Comitini *et al.,* 2011; Azzolini *et al.,* 2012, 2015
Zygosaccharomyces bailii / S. cerevisiae	Ethyl ester increase	Garavaglia *et al.,* 2015

Next sections of literature review will be focused on the description of *S. cerevisiae* nutrition, metabolism, and its responses to stress factors. The amount of studies developed in the model organism *S. cerevisiae* provides the framework to understand and characterize non-*Saccharomyes* yeasts, even though non-*Saccharomyces* may present differences depending on the yeast species.

2. Yeast metabolism

Yeast cells are chemoorganotrophs, meaning that they require organic substrates as carbon and energy sources to sustain their growth (Walker, 2004). Yeast nutrition is an essential factor for successful fermentation. It refers to how yeast uptake water, essential organic acids and inorganic nutrients from their growth medium and the subsequent utilization of these essential sources. Yeasts have commonly been used in fermentations of cereals mashes, grape musts, and other naturally derived substrates, which provide rich sources of required nutrients such as; carbon/energy (e.g. glucose, fructose, galactose, mannose, sucrose, raffinose), nitrogen (e.g. ammonia, amino acids, nucleotide bases, peptides), vitamins (e.g. thiamine, biotin) and minerals (e.g. potassium, magnesium, calcium) (Walker, 2004).

The bioavailability of these key nutrients determines kinetics and completion of fermentation, as well as having an impact on the organoleptic profile of wine. Without proper nutrition, yeasts can undergo stress and produce undesirable characteristics such as: off-flavours, high bound SO_2, and stuck or sluggish fermentation (Broach, 2012). Moreover, all nutrients may limit the fermentation rate depending on their concentration. At low concentrations, the growth rate is proportional to concentration, but as the concentration increases, the growth rate enhances fast to reach a maximum value, which is maintained until the nutrient concentration reaches an inhibitory level, at which point the growth rate begins to fall again.

2.1. Carbon metabolism

Yeast cells can use several compounds as sources of energy and carbon-containing precursors of respiro-fermentative metabolism and biomass accumulation. However, yeast cells preferentially consume glucose or fructose before other mono-, di- and tri-saccharides, such as galactose, sucrose or raffinose. Yeast cells also prefer any fermentable carbon source over any source that has to be catabolized by oxidative phosphorylation (OXPHOS), such as glycerol, ethanol and acetate (Broach, 2012).

In detail, during respiration and fermentation, the carbon source (glucose) is metabolized via the glycolytic pathway and is converted into two molecules of pyruvate, CO_2, ATP, and NADH. Then, specifically in fermentation, the pyruvate is converted into acetaldehyde and reduced into ethanol by the action of an alcohol

dehydrogenase, which also recycles the NAD^+ consumed during glycolysis. However, in the presence of oxygen, respiration occurs, in which pyruvate is converted into CO_2 through the tricarboxylic (TCA) cycle and OXPHOS, thereby obtaining additional ATP for cellular biosynthesis (Figure 2) (Flores *et al.,* 2000; García *et al.,* 2016).

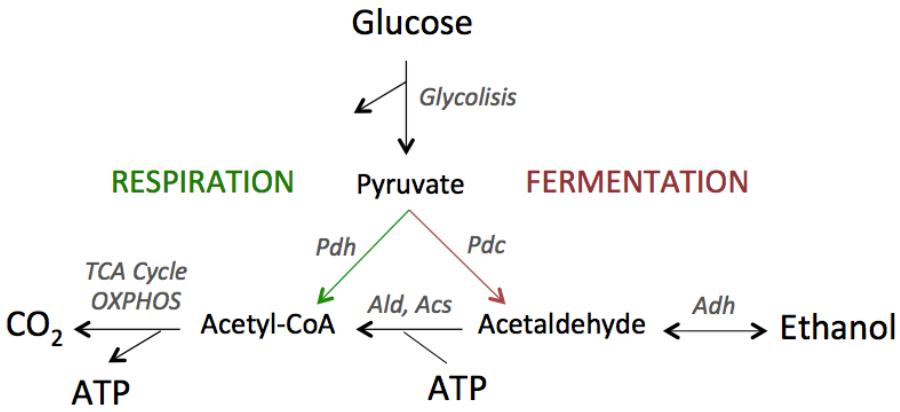


Figure 2. Yeast energy metabolism (adapted from Pfeiffer and Morley 2014).

Moreover, in respiro-fermentative metabolism of yeasts, a relevant mechanistic constraint seems to be the limited capacity of the respiration pathway to produce ATP. Specifically, when more sugar is available than can be processed by respiration, some yeast species can produce ethanol even under aerobic conditions. This phenomenon is called the Crabtree effect and these species are known as Crabtree positive yeasts (Pfeiffer & Morley, 2014). However, the biomass produced by Crabtree positive yeasts is lower than the biomass produced by Crabtree negative yeasts because a fraction of sugar produces ethanol instead of biomass. Nowadays, a glucose-limited feeding strategy may be used to overcome these constraints on yeast yield, obtaining higher biomass rates (Özadali & Özilgen, 1988; Vieira *et al.,* 2013).

2.2. Nitrogen metabolism

Nitrogen sources in the grape juice are essential for yeast metabolism, growth, and performance. Yeast cells are able to transport and catabolize several nitrogen sources and also to synthesize endogenously all essential nitrogen-containing compounds. Free amino acids and ammonium ions and small peptides are the major nitrogen-containing compounds in grape juice, with amino acids representing the largest proportion. All these compounds are called Yeast Assimilable Nitrogen (YAN) and a deficiency of them is one of the most important causes of stuck and sluggish fermentation (Gobert *et al.,* 2017). Moreover, the uptake and catabolism of nitrogen

sources also contributes to the production of secondary metabolites of oenological relevance, increasing wine flavour and quality. For example, higher alcohols and their associated fatty acids and esters are influenced by the quality and quantity of the nitrogen sources.

Furthermore, nitrogen demand is dependent on the yeast strain, sugar content and fermentation conditions, but at least 120-140 mg/L of YAN is required for satisfactory fermentation kinetics and final product quality. As mentioned earlier, yeast cells can use several nitrogen-containing compounds as nitrogen sources, but they have preferences for certain nitrogen sources due to their ability to use them more efficiently (Cooper, 1982; Barbosa *et al.,* 2012). Nitrogen Catabolite Repression (NCR) is a mechanism that allows yeast cells to discriminate between these preferential compounds and the less preferred nitrogen compounds, repressing the expression of genes required for uptake and catabolism of these less preferred nitrogen compounds (Broach, 2012). NCR is active at the beginning of the alcoholic fermentation, when the preferred nitrogen sources are present. However, when the amount of the preferred nitrogen sources decreases during fermentation, NCR decreases its action until a de-repressed state is reached. It is known that the preferential nitrogen sources for *S. cerevisiae* are ammonium, glutamine and asparagine, whereas there is not a lot of information about the nitrogen sources preferences of non-*Saccharomyces* yeasts (Gobert *et al.,* 2017).

Yeast cells react to the nitrogen content of the growth environment by controlling nitrogen source uptake and regulating catabolic and anabolic processes. Moreover, under conditions of nitrogen deficiency, yeasts can synthesize their own amino acids by supplementing with diammonium sulphate or phosphate. The nitrogen sources enter the yeast cells via permeases. Once inside the cells, the nitrogen sources are catabolized via deamination to generate ammonium ions or via transamination to generate glutamate. Moreover, the glutamate can also be produced from an ammonium ion and an α-ketoglutarate molecule, which is synthesized via TCA, catalysed by NADPH-dependent glutamate dehydrogenase (*GDH1*). However, the ammonium ions generated can be directly used in biosynthetic processes. Then, the condensation of glutamate and ammonium synthesizes glutamine through the action of glutamine synthetase (*GLN1*). Consequently, glutamate and glutamine are the two

major nitrogen donors in the biosynthesis reactions of purines, pyrimidines and several amino acids (Magasanik & Kaiser, 2002; Godard *et al.,* 2007; Ljungdahl & Daignan-Fornier, 2012).

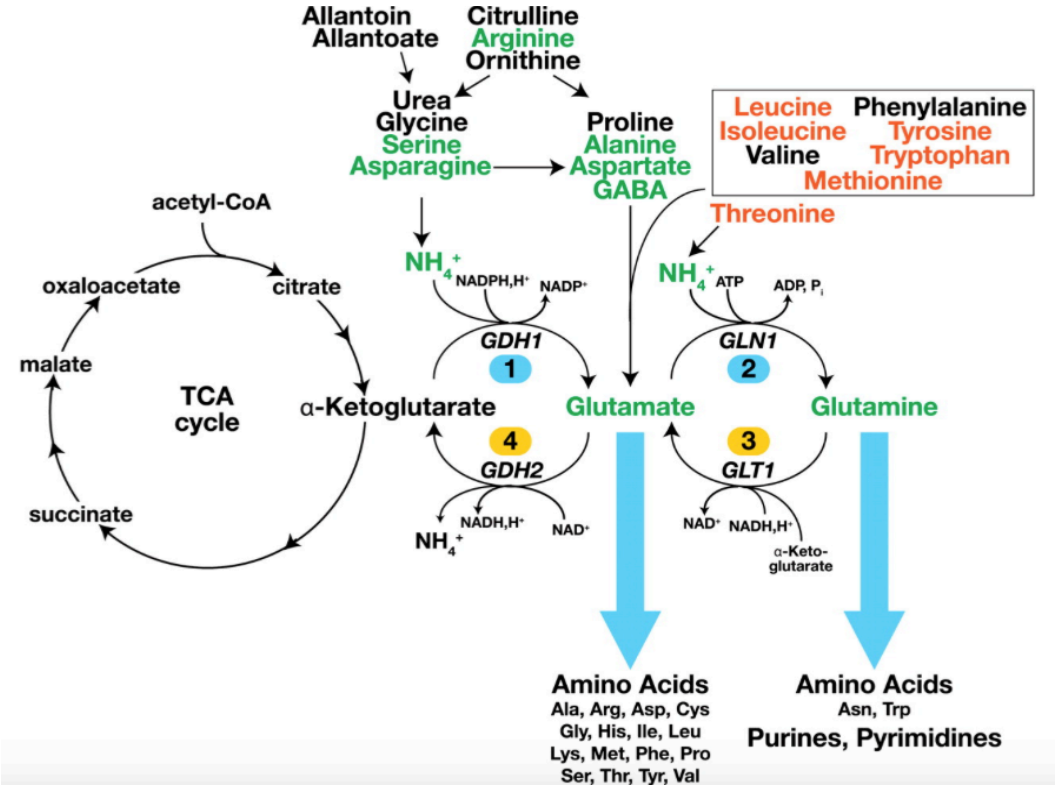


Figure 3. Yeast nitrogen metabolism. Diagram of the preferred (green), the non-preferred (red) nitrogen sources, and the nitrogenous compounds synthesized (blue arrows). Central anabolic reactions 1 and 2 are catalysed by NADPH-dependent glutamate dehydrogenase (GDH1) and glutamine synthetase (GLN1). Central catabolic reactions 3 and 4 are catalysed by NADH-dependent glutamate synthase (*GLT1*) and NAD^+-linked glutamate dehydrogenase (*GDH2*) (Ljungdahl & Daignan-Fornier, 2012).

2.3. Vitamins

Vitamins are growth factors, which are essential organic compounds required in very low concentrations (µM) for specific catalytic or structural roles in yeast, but which are not used as energy sources. In *S. cerevisiae*, vitamins most frequently required include **thiamine** (**vitamin B1**), **biotin**, **pantothenic acid** and **nicotinic acid**, with requirements of 5, 1, 6.25, and 5 mg/L, respectively (Berry & Brown, 1987). Thiamine is involved in decarboxylation reactions and influences several areas of yeast metabolism, such as respiratory competence, fatty acid, sterol and lipid formation, glycolysis and ethanol production. Biotin is essential for yeast growth and is involved in several areas of yeast metabolism including carboxylation of pyruvate, synthesis of pyridine nucleotides, nucleic acid synthesis, formation of purine and pyrimidine bases,

protein synthesis, polysaccharide synthesis and fatty acid synthesis. The functional form of pantothenic acid is coenzyme A, which is involved in *keto* acid oxidation reactions and other reactions such as fatty acid metabolism. Finally, nicotinic acid, which is required as nicotinamide, is involved in redox reactions and required for NAD^+ and $NADP^+$, which are essential for ATP generation (Berry & Brown, 1987; Walker, 1998).

2.4. Minerals

Minerals are required nutrients in some areas of yeast cell physiology and act as essential enzyme cofactors and also as environmental stress modulators. In the aqueous cellular environment, these elements exist as charged ions that, together with protons and hydroxide ions, facilitate biochemical reactions and establish the electrochemical gradients across membranes. Specifically, these metal ions are involved in both structural and signalling roles within cells. Yeast cells require a wide range of metals for maintaining the structural integrity of cells, organelles, proteins and phospholipids, as well as for cell-cell interactions (e.g. flocculation). However, metals are also needed for gene expression, cell division and growth, nutrient uptake mechanisms (e.g. Mg - ATPase), enzyme action in metabolism, osmoregulation and energy maintenance, and cell survival (Walker, 2004; Venkateshwar *et al.,* 2010; Cyert & Philpott, 2013). Consequently, mineral nutrition of yeast cells is important to winemakers because of its significant impact on the progress and efficiency of industrial fermentations. Metal ions influence some relevant parameters such as the increase of the fermentative capacity, the degree of final ethanol yields, the amount of yeast produced, cell viability, lipid metabolism, stress tolerance, yeast flocculation behaviour and the stability and dynamics of cell membranes (Walker, 2004). However, an excess content of some minerals may have a negative effect on the organoleptic properties of wine, such as final aroma, taste and even the colour of wine (Tariba, 2011).

Metal ions are classified depending on the concentrations required by yeasts. Bulk metals, such as potassium (K^+) and magnesium (Mg^{2+}), are required at millimolar (mM) concentrations, whereas trace metals, such as calcium (Ca^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+}), iron ($Fe^{2+,3+}$) and copper ($Cu^{+,2+}$), are required at micromolar (µM)

concentrations. These essential trace metals can become toxic in excess quantities, while other metal ions are intrinsically toxic, even at very low concentrations (µM), such as aluminium (Al^{3+}), arsenic (As^{3+}), cadmium (Cd^{2+}), chromium ($Cr^{3+,6+}$), mercury (Hg^{+}), and palladium (Pd^{2+}) (Table 3) (Nicola & Walker, 2009).

Table 3. Metals most required for yeast cell growth and metabolic functions (adapted from Walker, 2004).

Metal ions	Optimal concentrations in growth medium *	Main cellular functions
Bulk metals		
K^{+}	2 – 4 mM	Osmoregulation, enzyme activity
Mg^{2+}	2 – 4 mM	Enzyme activity, cell division
Trace metals		
Ca^{2+}	< µM	Second messenger, yeast flocculation
Zn^{2+}	4 – 8 µM	Enzyme activity, protein structure
Mn^{2+}	2 – 4 µM	Enzyme cofactor
$Fe^{2+,3+}$	1 – 3 µM	Haem-proteins, cytochromes
$Cu^{+,2+}$	1.5 µM	Redox pigments

* Figures relate to *S. cerevisiae* growth stimulation but are dependent on the yeast specie/strain and precise condition of growth. Culture media complexity, metallic ion interactions with organic compounds and with other metallic ions, pH, and the use of mutual metal transport systems by the microorganisms can hinder the precise determination of these concentrations (Gaensly *et al.,* 2014).

The rate of acquisition and utilization of these metal ions by the yeasts depends on the ion concentration in the grape juice and also on its bioavailability (Aleksander *et al.,* 2009). The bioavailability of these essential metal ions is an important factor in fermentation performance and it can be reduced by many factors, including metal ion deficiency or excess, processing conditions, chelating/absorbing material in the media and the presence of antagonistic or toxic metals (Walker, 2004; Nicola & Walker, 2009). To increase metal bioavailability from their growth environment, some yeast cells can secrete *siderophores*, which are low-molecular weight metal-sequestering compounds. However, an excess amount of certain metals may induce some undesirable consequences in the final wine. For example, K^{+} and Ca^{2+} can precipitate and cause spoilage of the wine, or $Fe^{2+,3+}$ can develop in unstable wines in the long term. Consequently, yeast cells have developed homeostatic mechanisms to regulate cellular metal ions levels and prevent toxicity, including mechanisms to acquire, utilize, sequester and store these ions, as well as mechanisms to detoxify yeast cells (Cyert & Philpott, 2013; Hosiner *et al.,* 2014).

- ***Metal ion transport across plasma membrane***

Membrane transport is responsible for the selection of nutrient acquisition and secretion through the interaction and communication of yeast cells with their growth environment (Walker, 1998). Yeast cells have developed a system of metal ion transporters in order to control environmental changes and survive to nutrient depletion or excess, temperature variation, metabolic inhibitors and absence of energy-yielding substrates. The membrane potential (ΔΨ), defined as the sum of all ionic gradients over a membrane of yeast cells, determines the uptake and efflux of ions and nutrients across the membrane (Cyert & Philpott, 2013). As such, ions transporters play a crucial role in maintaining the homeostasis of metal ions, controlling influx and efflux of free (ionized forms) metal ions (Stehlik-Tomas *et al.,* 2004). However, there are several factors, such as chelation, adsorption and binding, which can prevent metal ion acquisition by yeasts. Moreover, ions transporters have varying affinities for metals. High-affinity systems are very specific for certain ions to ensure that essential metals are accumulated under limiting conditions, while, in the case of an excess of metal ions, low-affinity systems control ion acquisition (Walker, 2004).

Acquisition of metal ions by the cell usually happens in two phases: a metabolism-independent (biosorption) and a metabolism-dependent (bioaccumulation) phase. Biosorption is a rapid and non-specific cell wall binding of metals to negatively charged cell wall moieties. In contrast, bioaccumulation occurs through a selective carrier that mediates the ion translocation across the plasma membrane to the cytosol, and can be influenced by factors such as temperature and the presence of metabolic inhibitors (Blackwell *et al.,* 1995). Although several mechanisms can be adopted to perform the last phase, such as free diffusion, facilitated diffusion, diffusion channels and active transport (Figure 4), the last of these mechanisms is the most common in yeasts, requiring energy-dependent (ATP-dependent) transport generated by the activity of the proton (H^+) and K^+ ions pumps (Figure 5) (Walker, 2004; Nicola & Walker, 2009).

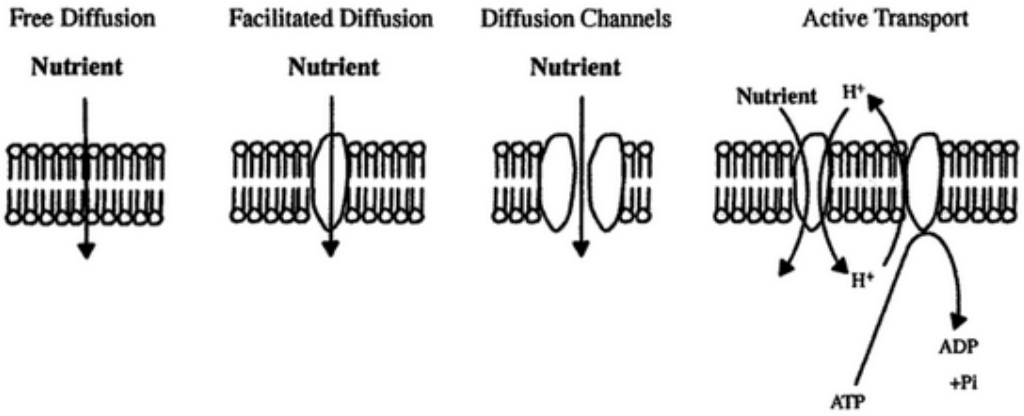


Figure 4: Mechanisms of nutrient uptake in yeasts (Walker, 1998).

- ***Intracellular metal ion location***

Once metal ions are transported through the plasma membrane into the yeast cells, they can be metabolized or stored by the cell. The distribution of ions to various regions of a cell is controlled at the cell membrane level (Aleksander *et al.,* 2009). Metal ions can remain free in the cytoplasm at very low concentrations (sub-µM), sequestered in the cytoplasm through intracellular chelators such as polyphosphates (e.g. ATP, ADP), metallothioneins (cysteine-rich polypeptides), calmodullin (calcium-specific protein) and polyamines (e.g. spermidine, spermine and putrescine), or compartmentalized in organelles such as the cell wall, vacuole, Golgi apparatus, mitochondria and nucleus. Specifically, vacuole plays a relevant role in ion homeostasis and in detoxification of toxic metal ions in yeast. Moreover, mitochondria and cell wall are also relevant in terms of ion accumulation and homeostasis in yeast cells. Selective ion transporters control the compartmentalization of metals in yeast cells, as in the plasma membrane ion transporters. In the case of the vacuole, there are H^+-pumping ATPases involved in metal ion transport (Figure 5) (Kosman, 1994; Walker, 1998; Walker, 2004; Nicola & Walker, 2009; Cyert & Philpott, 2013).

- ***Detoxification mechanisms of toxic metals***

As mentioned earlier, many metals are considered toxic to yeast cells and high amounts of trace metals can also become toxic. If toxic metals persist in the yeast cells, they have several effects on cellular oxidative stress development, enzyme and protein function, lipid peroxidation and DNA damage (Hosiner *et al.,* 2014). As such, the preservation of the balance between essential and toxic levels of certain metals is

crucial for yeast cells. Yeasts are able to perform heavy metal detoxification through several mechanisms; such as chemical transformation (e.g. by reduction, methylation and dealkylation), sequestration (e.g. by calmodullin, metallothioneins, and phytochelatins (D-glutamyl peptides derived from glutathione)), cell wall biosorption and immobilization (Walker, 2004).

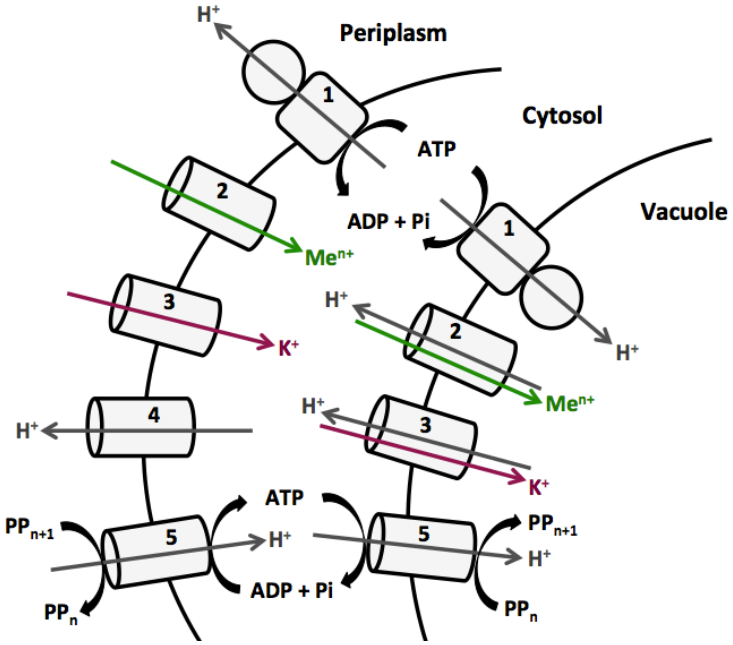


Figure 5. Model for cellular acquisition and transport of metal (Me^{n+}) ions with respect to the sources of energy required. 1. H^+-ATPase; 2. Metal ion transporter coupled directly or indirectly to H^+ and/or K^+ gradient; 3. K^+ transporter or K^+/H^+- antiporter; 4. H^+ efflux; 5. Polyphosphate (PP) kinase acting as a H^+ pump and for retaining metals in the vacuole (adapted from Kosman, 1994).

2.4.1. Potassium (K^+)

K^+ is the most abundant cellular essential cation in yeast, it constitutes around 1-2% of yeast cell dry weight. K^+ plays a significant role as a cofactor for enzymes involved in OXPHOS, protein biosynthesis and carbohydrate catabolism. K^+ is necessary for maximum utilization of glucose for yeast cells and it is rapidly exchanged across the membrane in active fermenting yeasts (Muntz, 1947). K^+ ions efficiently bind many molecules of H_2O, contributing to the cell size and turgor necessary for cell growth and division. Moreover, K^+ is also required for balancing charge across the plasma membrane, and thus contributes to maintaining both intracellular pH and ($\Delta\Psi$) (Walker, 2004; Kahm *et al.,* 2012; Borovikova *et al.,* 2013; Cyert & Philpott, 2013).

This charge balance is achieved through the activation of a plasma membrane H^+-ATPase (Pma1), when K^+ influx increases and under K^+ starvation conditions, and through a V-ATPase, which pumps H^+ to acidify the endomembrane systems (vacuole, Golgi, endosomal compartments and secretory vesicles). Moreover, yeast cells possess two plasma membrane proteins (Trk1 and Trk2), which are involved in K^+ influx, and a integral membrane protein (Tok1), which is involved in K^+ efflux and is activated by membrane depolarization. The plasma membrane proteins, Nha1 antiporter and Ena P-type ATPases (Ena1), also can efflux K^+ but play primary roles in Na^+ (competitive inhibitor of K^+) transport. Mdm38 and Mrs7 proteins modulate the transport of the K^+/H^+ exchange in mitochondria, whereas vacuole, Golgi and endosomes accumulate K^+ and Na^+ through H^+-dependent transport, such as Vnx1, Kha1 and Nhx2 proteins, respectively. Finally, Vhc1 is a K^+-Cl^- cotransporter that accumulates K^+ in the vacuole (Figure 6) (Kahm *et al.,* 2012; Cyert & Philpott, 2013).

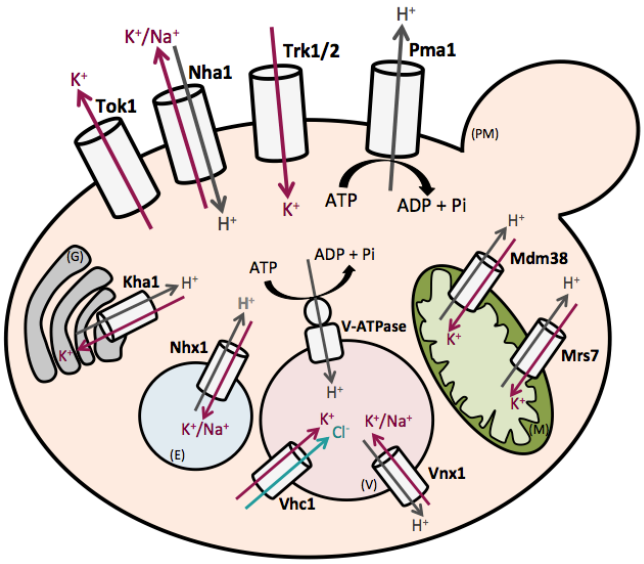


Figure 6: K^+ homeostasis in *S. cerevisiae*. Note that the V-ATPase also residues in endosome and late Golgi but was omitted from the figure due to space constraints. Subcellular locations are shown as: PM, plasma membrane; M, mitochondria; V, vacuole; E, endosome; G, Golgi (adapted from Cyert & Philpott, 2013).

2.4.2. Magnesium (Mg^{2+})

Mg^{2+} is the most abundant intracellular divalent cation in yeast cells and it constitutes around 0.3% (w/w) of yeast cell dry weight. Mg^{2+} essentially acts as a cofactor for many cellular enzymes and regulates cellular physiological processes such as growth, size and cell division. Specifically, magnesium is a cofactor for over 300

enzymes (e.g. synthetases, phosphatases, and kinases) in different bioenergetic and metabolic reactions, such as the synthesis of proteins and DNA, assembly and stabilization of cell membranes, environmental physical and chemical stress-protection (e.g. dehydration and rehydration, high ethanol exposure, thermal shock, reactive oxygen species (ROS) and metal toxicity), and respire-fermentative metabolism regulation. Mg^{2+} also acts as a cofactor of ATP molecules to stabilize phosphate groups used for phosphorylation and other $MgATP^{2-}$-dependent reactions like DNA polymerisation (Poreda & Tuszynski, 2007; Barros de Souza *et al.,* 2016).

Mg^{2+} displays a stronger tendency to become membrane-associated than to bind to soluble enzymes. The Mg^{2+} associated to the membrane promotes the charge stabilization of membrane phospholipids and the decrease of membrane fluidity, maintaining the membrane and organelle (e.g. mitochondria and ribosome) integrity, especially under stress conditions. For example, Mg^{2+} is able to alleviate the toxic effect of several heavy metals such as Al^{3+}or Cd^{2+} through membrane stabilization (e.g. charge neutralization of phospholipids) and competitive membrane binding in response to heavy metals (Walker *et al.,* 1982; Trofimova *et al.,* 2010). Moreover, Mg^{2+} ions decrease H^{+} and, especially, anion permeability by interacting with PLs, resulting in stabilization of the plasma membrane and decrease of membrane fluidity. This minimization of membrane fluidity fluctuations significantly increases yeast survival.

In contrast, the mitochondrial ATP-induced unselective channel (MUC) is a permeability transition pore. Opening the MUC mediates the increase in the unselective permeability to ions and small molecules across the inner mitochondrial membrane and can compromise cell homeostasis and trigger apoptosis in yeast cells. It has been seen that Mg^{2+} (such as Ca^{2+}, ADP and phosphate) can close the yeast MUC because it inhibits the ATP-induced MUC by binding free ATP, forming $MgATP^{2-}$, which does not stimulate MUC opening. Consequently, alternating between the open and closed states of MUC seems to protect cells from apoptosis, by alternatively increasing and decreasing Mg^{2+} concentrations (Bradshaw & Pfeiffer, 2006; Vianello *et al.,* 2012; Bradshaw & Pfeiffer, 2014; Cabrera-Orefice *et al.,* 2015).

Furthermore, it has been seen that incubation of yeast cells in low Mg^{2+} concentrations causes growth arrest, resulting in an increased percentage of cells in the G_0/G_1 phase, and a decreased percentage of cells in the S phase (Wolf *et al.,* 2008).

Consequently, Mg^{2+} bioavailability in grape juice is a relevant factor for efficient industrial fermentations. Mg^{2+} is required for the activation of several glycolytic enzymes (e.g. pyruvate carboxylase) influencing the rate of sugar consumption and ethanol production in Mg^{2+}-limited conditions, the conversion of sugar to alcohol may be suppressed, resulting in sluggish or incomplete fermentations, whereas an increase in free Mg^{2+} in grape juice seems to stimulate alcohol production. Mg^{2+} is a metabolic regulator of pyruvate metabolism and dictates the switch between fermentation and respiration depending on Mg^{2+} bioavailability (Barros de Souza *et al.,* 2016).

Most of the Mg^{2+} present in yeast cells is bound to phospholipids, proteins and nucleotides. Only a small fraction is free within the cells. Intracellular free Mg^{2+} is usually around 0.5-1 mM. For cellular Mg^{2+} transport and regulation of the cytosolic Mg^{2+} homeostasis by yeast cells, there are several plasma membrane transporters that belong to the metal ion transporter (MIT) superfamily. Alr1 and Alr2 membrane proteins are responsible for Mg^{2+} influx, cytosolic Mg^{2+} homeostasis and enhancement of Al^{3+} tolerance by yeast cells. Mg^{2+} is accumulated in the mitochondria and vacuole, playing an important role in regulating intracellular Mg^{2+} homeostasis and storage. Mrs2 and Lpe10 are proteins of the inner mitochondrial membrane that control mitochondrial Mg^{2+} influx, while Mnr2 controls the utilization of vacuolar Mg^{2+} stores. Finally, vacuole accumulates Mg^{2+} through a Mg^{2+}/H^{+} exchanger (Figure 7) (Walker, 1998; Graschopf *et al.,* 2001; Liu *et al.,* 2002; Walker 2004; Da Costa *et al.,* 2007; Pisat *et al.,* 2009; Har Lim *et al.,* 2011; Cyert & Philpott, 2013).

2.4.3. Calcium (Ca^{2+})

Ca^{2+} has structural, enzymatic and signalling roles within yeast cells. Ca^{2+} is essential and acts as a protector of certain secreted proteins (e.g. hydrolytic enzymes such as α-amylase). It also protects the membrane structure and facilitates yeast-yeast interaction. Specifically, Ca^{2+} is involved in yeast flocculation, which is a reversible process, due to its action in the activation of cell wall α-mannan residues, thus allowing lectin proteins to facilitate adhesion between adjacent yeast cells (Miki *et al.,* 1982). Consequently, Ca^{2+} is actively excluded from the yeast cells because of its relevant extracellular roles in yeast fermentation processes (Rees & Stewart, 1999). Moreover, Mg^{2+} can block Ca^{2+} binding to cell surface receptors due to its antagonistic

role against Ca^{2+}, whereas an excess of Ca^{2+} in fermentation media can also inhibit yeast growth and fermentation activity through this antagonism with Mg^{2+} and other essential cations (Walker, 2004).

Thus, yeast cells maintain cytosolic Ca^{2+} at very low levels (sub-µM) by active efflux of Ca^{2+} through the plasma membrane Ca^{2+} transporters (e.g. Ca^{2+}-H^{+} antiporter), sequestration with specific Ca^{2+} binding proteins (e.g. calmodulin) and transporting Ca^{2+} into the vacuole, which is the main organelle involved in regulating Ca^{2+} homeostasis in yeast (Rees & Stewart, 1997; Walker, 1998). However, yeast cells possess a high affinity Ca^{2+} system (Mid1, Cch1 and Ecm7) and a low affinity Ca^{2+} system for Ca^{2+} influx across the plasma membrane. There are additional Ca^{2+} influx pathways, such as the glucose-induce Ca^{2+} (GIC) system (Gpr1, Gpa2 and phospholipase C). Yeast cells also possess P-type ATPases (Pmr1 and Spf1/Cod1) for maintain Ca^{2+} homeostasis in the ER and Golgi, whereas an H^{+}/Ca^{2+} exchanger (Vcx1) and a P-type ATPase (Pmc1) are responsible of the vacuolar Ca^{2+} sequestration. Ca^{2+} can be released from vacuoles into the cytosol via the Yvc1 when yeast cells are exposed to osmotic shock (Figure 7). Finally, calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, increases Pmc1 and Pmr1 activity and inhibits the high affinity Ca^{2+} system and Vcx1 activity (Walker, 1998; Cyert & Philpott, 2013).

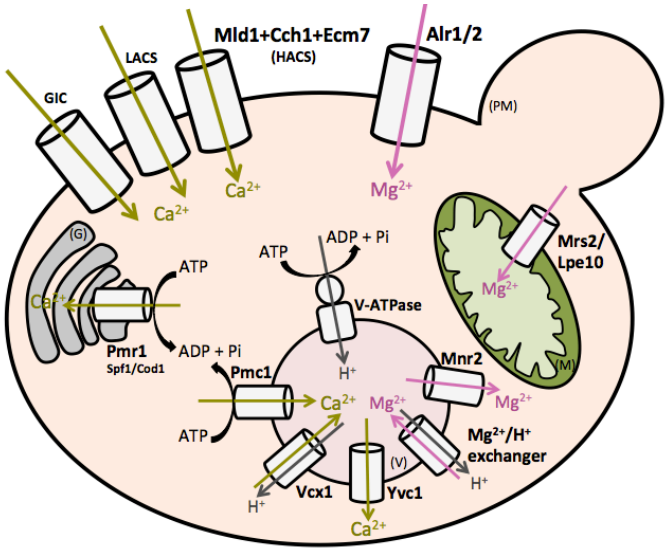


Figure 7: Ca^{2+} and Mg^{2+} homeostasis in *S. cerevisiae*. Note that the V-ATPase also residues in late Golgi but was omitted from the figure due to space constraints. Subcellular locations are shown as: PM, plasma membrane; M, mitochondria; V, vacuole; G, Golgi (adapted from Pisat *et al.*, 2009; Cyert & Philpott, 2013).

2.4.4. Zinc (Zn^{2+})

Zn^{2+} is important for yeast growth and metabolism, and for the cell surface integrity, promoting yeast flocculation (like Ca^{2+}) and stabilizing cellular membranes. Zn^{2+} also plays critical structural and functional roles in enzymes and many non-catalytic proteins (e.g. Zn-finger DNA binding proteins) and acts as a catalytic cofactor of nearly 300 enzymes (e.g. alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase, superoxide dismutase (SOD) and several carboxypeptidases). Zn^{2+}-deficiency causes oxidative stress, loss of SOD activity, increase of DNA damage and sluggish fermentations, whereas Zn^{2+}-excess promotes the increase of ROS levels disrupting mitochondrial function and inhibiting the activity of the electron transport chain. Moreover, Zn^{2+} plays a major role in yeast fermentative metabolism because it is essential for ethanol dehydrogenase activity, but it can also stimulate sugar uptake of maltose and maltotriose in yeast cells, thereby augmenting fermentation rates. (Walker, 1998; Walker, 2004; Stehlik-Tomas *et al.,* 2004; Poreda & Tuszynski, 2007; Nicola *et al.,* 2009; Nicola & Walker, 2009; Wu *et al.,* 2009).

Zap1 is the Zn^{2+} sensing transcription factor that controls the response to zinc deficiency in yeast, whereas in Zn^{2+} -replete cells Zap1 is inactivated. Zn^{2+} homeostasis is achieved through high and low affinity transporters of the ZIP family, Zrt1 and Zrt2, respectively. Cot1 and Zrc1 transporters are responsible for vacuolar Zn^{2+} storage, whereas Zrt3 transporter mobilizes vacuolar Zn^{2+} stores. In the lumen of the ER, there are several Zn^{2+} metalloenzymes that require Zn^{2+}, which is transferred from cytosol to the ER lumen by the activity of a heterodimeric transporter composed of Msc2 and Zrg17 (Figure 8) (Walker, 1998; Cyert & Philpott, 2013).

2.4.5. Manganese (Mn^{2+})

Mn^{2+} is also essential for yeast growth and metabolism. Mn^{2+} ions are accumulated to a greater extent than Ca^{2+} in yeasts, but to a much lesser extent than Mg^{2+}. Several metalloproteins, which are located in the nucleus, mitochondria, cytosol, Golgi and vacuole, require Mn^{2+} to function (e.g. oxidoreductases, dehydrogenases, peptidases, decarboxylases, sugar transferases and hydrolases). Mn^{2+} is also a protective anti-oxidant, especially as a back-up for superoxide dismutase (Sod2), which transforms toxic superoxide O_2^- into H_2O_2 and O_2, reducing mitochondrial ROS and

conferring protection against cell death. Under conditions of Mn^{2+}-deficiency, the activity of Mn^{2+}-dependent enzymes such as sugar transferases in the Golgi, Sod2 in the mitochondria and a cytosolic Mn-SOD, are attenuated (Stehlik-Tomas *et al.,* 2004; Reddi *et al.,* 2009).

For cellular Mn^{2+} homeostasis, there are two high-affinity H^{+}-coupled Mn^{2+} transporters, Smf1 and Smf2, which transport Mn^{2+} into the cytosol. Otherwise, yeast cells can influx Mn^{2+} through the high-affinity phosphate transporter Pho84, which takes up environmental $MnHPO_4$ under conditions of Mn^{2+} toxicity. Furthermore, Pmr1 plasma membrane transporter catalyses the ATP-dependent transfer of Mn^{2+} from the cytosol into the lumen of the Golgi. Finally, Ccc1 transporter is responsible for Mn^{2+} storage and detoxification, sequestering this transition metal ion in the vacuole (Figure 8) (Walker, 1998; Reddi *et al.,* 2009; Cyert & Philpott, 2013).

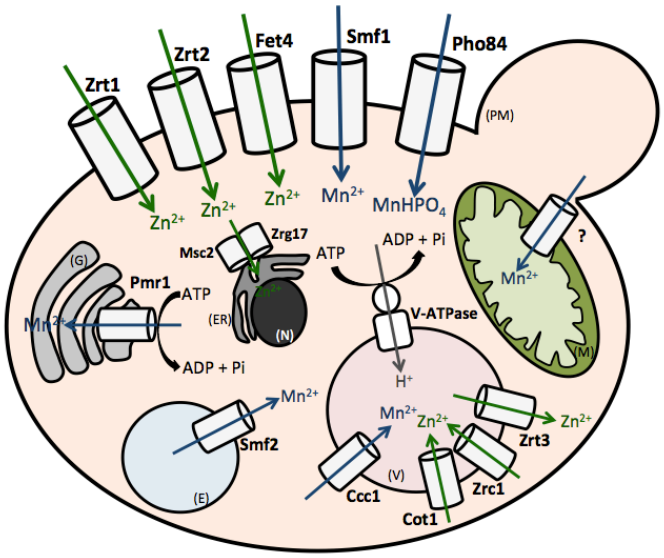


Figure 8: Zn^{2+} and Mn^{2+} homeostasis in *S. cerevisiae*. Note that the V-ATPase also residues in endosome and late Golgi but was omitted from the figure due to space constraints. Subcellular locations are shown as: PM, plasma membrane; N, nucleus; ER, endoplasmic reticulum; M, mitochondria; V, vacuole; E, endosome; G, Golgi (adapted from Eide, 2009; Cyert & Philpott, 2013).

2.4.6. Iron (Fe^{2+}, Fe^{3+})

Iron is an essential nutrient for the growth of yeast cells. It is required to facilitate the assembly of functional Fe-S cluster proteins, heme-binding proteins and ribonucleotide reductases (RNRs). These proteins have several functions in ribosome maturation, DNA replication and repair, and cell cycle control. Moreover, iron acts as a cofactor in several enzymes, such as the redox reaction of the respiratory chain, due to

its capacity to collect or lose electrons. Iron cofactors are also required for the synthesis of amino acids, proteins, sterols and fatty acids. Excess intracellular iron can result in the production of ROS causing cell damage, while iron deficiency may lead to the incorrect functioning of iron-dependent enzymes, Fe-S proteins and haemoproteins, as well as causing alterations in glucose metabolism and amino acid and lipid biosynthesis (Shakoury-Elizeh *et al.,* 2010; Zhang, 2014).

Iron exists in the aerobic environment in a higher valence state (Fe^{3+}) that is less stable and less soluble than the reduced form (Fe^{2+}) that may predominate in the yeast cells. The transcriptional activators, Aft1 and Aft2, activate the transcription of genes involved in the influx of iron into the yeast cells, iron vacuolar storage and the adaptation to iron-deficient conditions. Yeast cells obtain iron via the low-affinity plasma membrane transporter Fet4 or via two, reductive and non-reductive, high affinity systems. The reductive system reduces Fe^{3+} and siderophore (e.g. ferrichromes, ferrioxamine B and enterobactin) to Fe^{2+} through the *FRE* family of metalloreductases (Fre1-4), followed by a Fe^{2+} transport to the cytosol through a copper-dependent ferrous oxidase (Fet3) and an iron permease (Ftr1). The non-reductive system takes up Fe^{3+} siderophores chelates through transporters of the *ARN/SIT* family (Arn). Iron is stored in the vacuole through the Ccc1 transporter, whereas the efflux of Fe^{2+} from vacuole is performed by the reduction of Fe^{3+} through Fre6 and the transport of Fe^{2+} to the cytosol through Fet5 (oxidase)/Fth1 (permease) complex. Vacuolar efflux may also be activated through Smf3 (Figure 9) (Kosman, 1994; Cyert & Philpott, 2013; Zhang, 2014).

2.4.7. Copper (Cu^{+}, Cu^{2+})

Copper is an essential nutrient for the growth of yeast cells and it is also important for its participation in vital electron transfer reactions (redox reactions). It acts as a cofactor of some enzymes, such as Cu, Zn-superoxide dismutase (SOD) and cytochrome-c oxidase (CcO). Copper is also required for iron homeostasis in yeast cells. However, copper may also be toxic at high concentrations due to its ability to transfer and gain electrons from molecular oxygen and promote the formation of toxic ROS. As such, the maintenance of the cellular copper homeostasis is important, and yeast cells possess a complex regulatory mechanism. A copper metallothionein protein (e.g.

Cup1p) sequesters around 60% of the intracellular copper. Yeast cells may also prevent copper toxicity through copper biomineralization, accumulating CuS on the cell surface (Kosman, 1994; De Freitas *et al.,* 2003; Stehlik-Tomas *et al.,* 2004; Cyert & Philpott, 2013).

Transcriptional factors Mac1 and Ace1 are responsible of the regulation of copper influx and detoxification, respectively. As happens in the case of iron, copper homeostasis is achieved through plasma membrane reductases Fre1-2, which reduce Cu^{2+} to Cu^{+}, and then through Ctr1 and Ctr3 proteins Cu^{+} is transported into the cytosol. Moreover, the vacuolar membrane proteins Ctr2 and Fre6 mediate the Cu^{2+} reduction and Cu^{+} transport to the cytosol. Finally, Atx1 is a copper chaperone that binds cytosolic copper and delivers it to the P-type ATPase Ccc2 transporter, which transports copper ions from the cytosol to the lumen of Golgi, where the copper is inserted into Fet3 (Figure 9) (Kosman, 1994; De Freitas *et al.,* 2003; Cyert & Philpott, 2013).

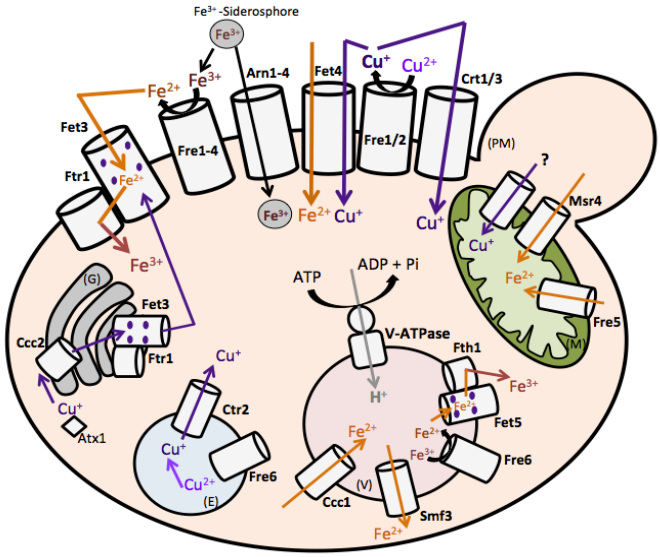


Figure 9: $Fe^{2+,3+}$ and $Cu^{+,2+}$ homeostasis in *S. cerevisiae*. Note that the V-ATPase also residues in endosome and late Golgi but was omitted from the figure due to space constraints. Subcellular locations are shown as: PM, plasma membrane; M, mitochondria; V, vacuole; E, endosome; G, Golgi (adapted from Philpott & Protchenko, 2008; Nevitt *et al.,* 2012).

3. Yeast stress responses

Winemaking yeast cells may be subjected to several physical, chemical or biological hostile conditions, which are collectively referred as “stress”, including heat shock, osmotic stress, oxidative stress, pH shock, ionic stress, ethanol toxicity, nutrient starvation, cell aging and desiccation. All these environmental stress factors may lead to poor yeast cell growth and metabolic activity. In general, cells in the stationary phase are significantly more tolerant to stress factors than cells in the exponential phase. Focusing on the desiccation stress, the production of selected active dry wine yeasts (ADWYs) involves desiccating the yeast biomass to a final product with a residual moisture percentage below 8% (Rodríguez-Porrata *et al.,* 2008). The term anhydrobiotic refers to organisms that are able to tolerate desiccation processes. Most studies on desiccation stress are performed with the rehydrated form of yeast, exhibiting the dehydrated phenotype. Removal of the cell water can cause cellular damage, and cell size and shape changes, resulting in a cellular response to minimize such adverse effects (Singh *et al.,* 2005). These effects include cell wall crenellation (Finn & Stewart, 2002), cytoplasmic crowding (Billi & Potts, 2002), DNA supercoiling (Shirkey *et al.,* 2003), membrane disruption (Beker *et al.,* 1984), phase transitions (Leslie *et al.,* 1995), and cell death. Additionally, desiccation undergoes several direct stress consequences at the same time, such as heat shock, osmotic stress and oxidative stress. Therefore, desiccation can be seen as a complex mixture of a number of stresses. Moreover, the cellular response to the sudden influx of water during rehydration is as complex as the response that occurs during the drying process itself (Gibson *et al.,* 2007). In order to counteract environmental stress factors, ensure yeast cell survival, protect essential cell components and permit a fast resumption of normal cellular activities, two major pathways regulate the general stress response in *S. cerevisiae*: the **heat shock response (HSR)**, which is primarily activated by sublethal heat stress, and the **environmental stress response (ESR)**, which can be activated by a number of environmental stresses in a nonspecific manner, such as starvation, pH, heat, osmotic and oxidative stresses. Essentially, the transcription factor Hsf1p is involved in the HSR pathway activating heat shock elements (HSE), whereas two zinc finger transcriptional factors Msn2p and Msn4p (Msn2/4p) are involved in the ESR pathway activating the stress response element (STRE:CCCCT) (Rodrigues-Pousada *et*

al., 2004; Gibson *et al.,* 2007; Castells-Roca *et al.,* 2011). As ESR may be induced by different stresses (heat, osmotic and oxidative stresses), it suggests cross-protection during the imposition of desiccation. For example, heat shock may also induce resistance to oxidative stress and vice versa. As such, yeast cells exposed to sublethal stress gain tolerance not only to the same stress, but also to other disparate environmental stresses. Moreover, the HSR can be considered a subset of the ESR, as all HSR genes are accounted for within the ESR regulon, whereas a number of ESR genes are not necessarily induced by heat shock. Since 2008, a phenomenon known as acquired stress resistance has been described, which shows that the ESR/HSR must be induced not only to survive the stimulating stress, but also to survive a subsequent stress. In *Schiz. pombe*, ESR is known as the core environmental stress response (CESR). ESR and CESR are largely conserved between the two evolutionary distant yeasts (Chen *et al.,* 2003).

Despite yeast's general stress responses, some stresses are also cross-protected by pathways that are activated by particular stresses. As a result of all these stress responses, yeast cells may display an induction of lipid membrane composition changes, an increase in the synthesis of heat shock protein (HSP), an increase in the synthesis of trehalose and glycogen and production of antioxidant defences (Figure 10) (Birch & Walker, 2000; Walker, 2004)**.** Understanding the causes and effects of the numerous types of yeast stress is useful for technological reasons, enabling improved methods to be established for yeast fermentation performance.

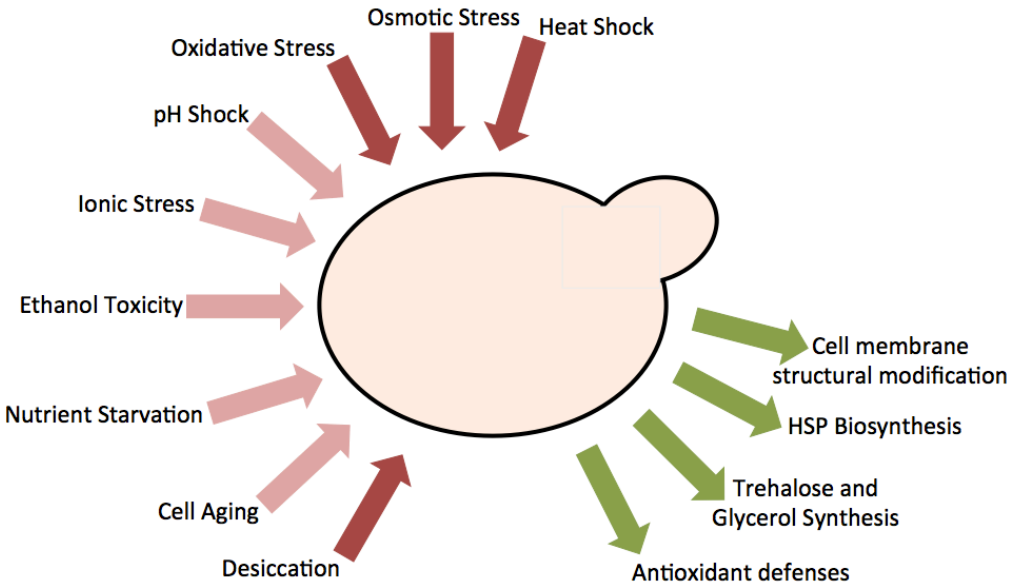


Figure 10: Responses of yeast to stress factors (adapted from Walker, 2004).

3.1. Cell membrane structural modification

Biological membranes consist of permeable lipid bilayers together with proteins and carbohydrates. Lipids include phospholipids (also named glycerophospholipids), sphingolipids and sterols.

Phospholipids (**PLs**) are the most abundant membrane lipids, which contribute to the structural definition of cells and participate in the regulation of many cellular processes. PLs consist of a diacylglycerol (DAG) as hydrophobic side attached to different polar head groups at the hydrophilic side through an ester bond. DAG is a glycerol backbone esterified in the *sn*-1 (saturated) and *sn*-2 (unsaturated) positions with fatty acids (FAs), which can influence the physical state of PLs and, consequently, membrane fluidity and permeability (Klug & Daum, 2014). FAs can be classified as saturated (SFAs), monosaturated (MUFAs) and polyunsaturated (PUFAs), containing no double bonds, one double bond and more than one double bond, respectively (Boyle 2005). The most common FAs in *S. cerevisiae* are oleic acid (C18:1) and palmitoleic acid (C16:1), followed by palmitic acid (C16:0), stearic acid (C18:0) and minor FA myristic acid (C14:0). *De novo* synthesis of FAs occurs in the cytosol and in mitochondria, followed by elongation and desaturation in the ER. The diversity of PLs is a consequence of the combination of possible FA in the DAG site. The major PLs in yeasts are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), while cardiolipin (CL) is an important minor PL (Klug and Daum 2014). Phosphatidic acid (PA) serves as a central metabolite in the *de novo* synthesis of PLs through the cytidine diphosphate-diacylglycerol (CDP-DAG) pathway. CDP-DAG is the precursor molecule for all major phospholipids and can be combined with serine, inositol or glycerol-3-phosphate (G3P), generating PS, PI and phosphatidylglycerophosphate (PGP), respectively. Moreover, PGP can be converted to CL by removing the phosphate group of PGP, PS can be decarboxylated to PE, and PE can be converted to PC by three methylation reactions (Figure 11) (Kodaki & Yamashita, 1987, 1989; Voelker, 1997). Furthermore, a second route for the synthesis of PE and PC exists. It is called Kennedy pathway or CDP-ethanolamine/choline pathway and uses exogenous ethanolamine and choline as the substrate for PE and PC synthesis. This pathway is highly active and essential when enzymes of the CDP-DAG pathway are blocked (Klug & Daum, 2014).

Sphingolipids (**SL**) and **sterols** also contribute to membrane architecture. **SL** are especially found in the plasma membrane and are constituted by a ceramide backbone which consists of an sphingoid long-chain base (LCB) linked through an amide bond to a FA, and an inositol phosphate that acts as a polar head group (Schneiter, 1999). Otherwise, **sterols** are nonpolar lipids, which are protected by SL head groups within the membrane. Ergosterol is the major sterol present in *S. cerevisiae,* and consists of a four-ring structure, an acyl side chain and a hydrophilic hydroxyl group, which facilitates insertion into membranes (Klug & Daum, 2014).

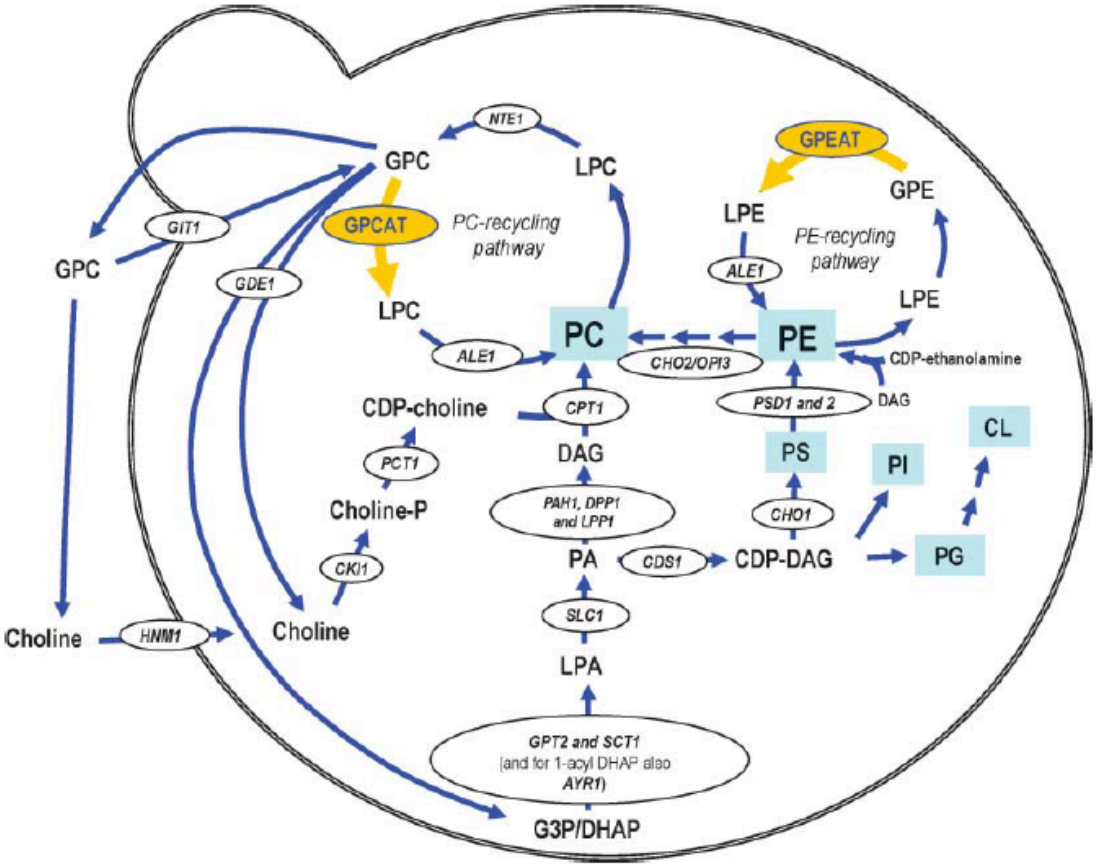


Figure 11: Schematic outline of the major pathways for phospholipid biosynthesis in yeast. DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol (Stalberg *et al.,* 2008).

Lipid composition determines the fluidity of the membranes. The PL composition influences the properties of membranes. PC, which usually contains an unsaturated FA (e.g. oleic acid (C18:1)), promotes a stable and fluid matrix for cellular membranes, while PE, PS and PI are determinants of the membrane surface charge, due to their

anionic head group. Moreover, negative curvature (cone shape) is induced by PLs with a small polar head/FA ratio (PE), neutral curvature (cylinder shape) is induced by the same head/FA ratio (PC) and positive curvature (inverted cone shape) is induced by bigger head/FA ratio (LPC) (Figure 12). Finally, sterols rigidify fluid membranes by reducing the flexibility of unsaturated acyl chains and increasing the membrane thickness and impermeability to solutes (Brown & London, 1998).

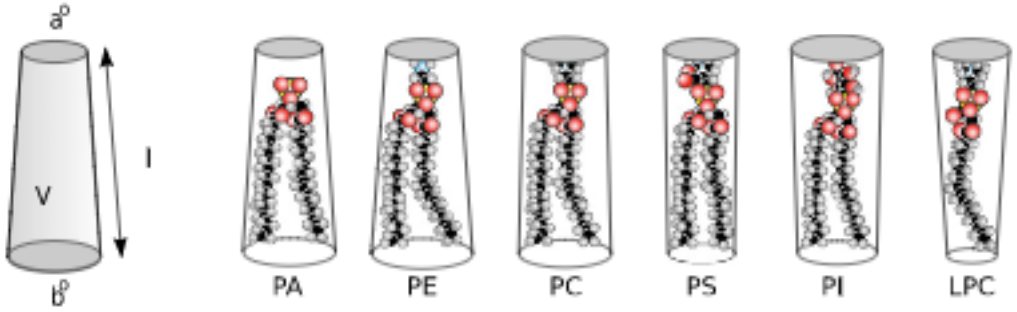


Figure 12: Characterization of different PLs according to their structure factor, $S = (1+b^0/a^0)/2$. $S > 1$ indicates cone like shape, whereas $S < 1$ describes lipids as inverted cones (adapted from Zanghellini *et al.,* 2010).

Changes in membrane structure may significantly disturb the membrane functions and alter the activity of membrane-associated enzymes and transporters. One of the main factors that determines the resistance of yeast cells to dehydration-rehydration is the maintenance of the structural integrity of the plasma membrane, which is one of targets damaged during this process (Trofimova *et al.,* 2010). The transfer of yeast cells into the state of anhydrobiosis promotes a significant decrease in cell volume, with a subsequent increase of the area/volume ratio and finally deformation. Then, cell volume is restored during the rehydration process (Beney *et al.,* 1998). As desiccation involves progressive cell H_2O loss, the internal and external yeast hydric potential have to be equilibrated by osmosis, leading to membrane reorganization and changing membrane permeability. Moreover, the packing density of the head groups of the PLs in membranes also increases, resulting in an increase of the membrane phospholipid temperature of phase transition (Tm). Correspondingly, when such 'dry' PLs are transferred into water (rehydration), they undergo a phase transition from gel to a liquid-crystalline phase. Consequently, PL bilayers of membranes temporarily become more permeable at such phase transitions (França *et al.,* 2007; Trofimova *et al.,* 2010). Moreover, plasma membrane is also a target site for thermal changes. High

temperatures also increase membrane fluidity and permeability to H^+ and other ions, resulting in the alteration of membrane composition. Furthermore, PLs are susceptible to the oxidative damage mediated by ROS, which cause lipid peroxidation. So, important variations in the membrane fluidity and structure may induce cellular damages and cell death. Consequently, under desiccation conditions, structural lipid changes may be displayed in the cell membranes in order to stabilize the plasma membrane and maintain its functions after exposure to stress (Beney & Gervais, 2001).

3.2. Heat Shock Protein Biosynthesis

Temperature is one of the most relevant parameters and it influences the growth and metabolism of yeast cells. Yeast is a psychrotrophic microorganism, which is able to develop at minimum temperatures of 1 - 3 ˚C and at a maximum temperature of 40 ˚C. However, its development is optimal between 25-30 ˚C. Heat shock could be induced by a temperature upshift or as a consequence of other stresses, such as desiccation (Birch & Walker, 2000; Bleoanca & Bahrim, 2013). Under these stress conditions, Hsf1 and Msn2/4 mediate the HSR by inducing the synthesis of **heat shock proteins (HSPs)**, being responsible for *S. cerevisiae* thermotolerance, which is the ability of yeast cells to survive to a lethal heat shock stress (Whitley *et al.,* 1999). However, as mentioned earlier, most of the stress responsive regulators have overlapping functions under some of the environmental stresses, such as heat shock, osmotic stress and oxidative stress. For example, the transcription factor Yap1p may induce Hsf1p-dependent expression promoting HSP induction under oxidative stress conditions (Liu & Thiele, 1996). Many of the HSPs act as chaperones, a ubiquitous group of proteins involved in the stabilization of new proteins to ensure correct folding and protein refolding in the case of those damaged by the cell stress. HSPs are termed according to their molecular masses. Hsp70, Hsp90, Hsp104 and small Hsp (sHsp) are the main chaperones activated by Hsf1p (Verghese *et al.,* 2012).

Hsp70s are found in many cellular compartments (e.g. mitochondria, ER and cytoplasm) and play major roles in damage protection and repair, and the translocation of proteins. Hsp70s ensure the proper folding of nascent or misfolded proteins. Hsp90s interact with a select group of substrates (e.g. many kinases and transcription factors) and are involved in the “final” maturation of proteins, assembly

of complex macromolecular structures, stabilization of misfolded proteins and interaction with regulatory signalling proteins. Moreover, the cytoplasmic Hsp104s are one of the few HSPs absolutely required for thermotolerance and are able to refold aggregate proteins. Finally, sHsps (e.g. Hsp26 and Hsp42) play an essential role in promoting protein solubility when heat or other stresses lead to general cytosolic protein unfolding. At the end of the heat shock response, Hsp70 and Hsp90 are involved in the repression of Hsf1p. Moreover, cAMP-protein kinase A (cAMP-PKA) indirectly also inhibits Hsf1 activity (Birch & Walker, 2000; Ferguson, 2004; Morano *et al.,* 2012; Verghese *et al.,* 2012).

3.3. Trehalose and Glycerol Synthesis

Yeast cells usually accumulate carbon and energy reserves to cope with starvation conditions. The accumulation of **trehalose** and **glycerol**, two of the main intracellular energy storage polymers, is sometimes crucial for overcoming general stress (Mahmud *et al.,* 2009).

Firstly, the disaccharide **trehalose** is an important stress protectant conferring stability to the plasma membrane and enzymes as well as ensuring the proper folding and repair of proteins, and acting as a carbon source during starvation. Trehalose works synergistically with Hsp104 to refold heat-damaged proteins in the cytosol and inside the ER lumen. Trehalose also plays a protective role for yeast cells against ROS (Gibson et al., 2007). However, trehalose may also inhibit some cellular defence mechanisms, including the activity of essential enzymes (e.g. glutathione reductase, glucose-6-phosphate dehydrogenase and cytosolic pyrophosphatase) and interfere with protein refolding by HSPs during recovery from stress. As such, trehalose accumulation should be a transient phenomenon in response to stress, and a fast degradation of trehalose is essential for the recovery of normal cellular activity (Sebollela *et al.,* 2004; Verghese *et al.,* 2012). STRE elements regulate the genes involved in trehalose synthesis (*TPS1*, *TPS2*, *TSL1*, *TPS3*) and degradation (*NTH1*, *NTH2*, *ATH1*) in *S. cerevisiae* (Mahmud *et al.,* 2009). In *Schiz. pombe,* an homologue of *TPS1* is also essential for trehalose synthesis. Certain conditions, such as heat shock, osmotic stress and oxidative stress, may up-regulate these genes. Many industries use these stress conditions as a strategy to increase the trehalose content of yeasts before

desiccation because trehalose may act as an anti-dehydration agent, due to water replacement and by forming glass structures, minimizing damaging effects during desiccation. Trehalose has a high glass transition temperature and leads to the formation of stable glasses during drying (França *et al.,* 2007). The formation of glass avoids the crystallization effects in the last step of desiccation due to its high viscosity, which maintain biomolecules in a form that allows them to return to their native structure and, therefore, be totally functional following rehydration (Buitink and Leprince, 2004). Moreover, trehalose may play a key role in protecting cellular constituents from oxidation, reducing oxidant-induced modifications of proteins during the exposure of yeast cells to H_2O_2 and reducing the levels of lipid peroxidation (Benaroudj *et al.,* 2001; França *et al.,* 2007). Trehalose may also play a role during rehydration, preventing phase transition events in the phospholipid bilayer and reducing membrane permeability, thereby protecting membranes from damage (Verghese *et al.,* 2012; Gibson *et al.,* 2007). In order to confer proper protection against desiccation, trehalose must be present on both sides of the lipid bilayer (França *et al.,* 2007).

However, **glycerol** acts as the most important osmoprotectant of yeast cells, maintaining the water balance and re-establishing the volume and turgor of the cells (Mahmud *et al.,* 2009). As mentioned earlier, hyperosmotic stress is also one of the stresses caused by dehydration, which induces the intracellular protective mechanisms involved in osmoregulation. Hyperosmotic stress promotes a massive efflux of cellular water, disassembly of the actin cytoskeleton and cell shrinkage, while osmoregulation is the cellular response for restoring and maintaining volume, turgor and normal biological activities of the cell. In *S. cerevisiae*, this osmoregulation is governed by the high osmolarity glycerol (HOG) pathway and also by general stress responses such as ESR (Mager & Varela, 1993; Gibson *et al.,* 2007; Saito & Posas, 2012). Once the HOG pathway is induced, a subsequent multistep of phosphorylation and activation of mitogen-activated protein kinase (MAPK) cascades occur. Specifically, the MAPKK Pbs2p activates Hog1p MAPK, which is the key element in the HOG pathway, while Pbs2p may be activated by two MAPKKK branches, Sln1p or Sho1p. After the activation of Hog1p, it is accumulated in the nucleus in order to induce transcriptional and translational responses, and accumulate osmolites (e.g. glycerol) to overcome the

stress imposed (Tamás & Hohmann, 2003; Saito & Tatebayashi, 2004; Saito & Posas, 2012). In fact, the key enzyme of glycerol formation is the cytosolic NAD-dependent glycerol-3-phosphate dehydrogenase (Gpd1p), which is strongly induced at transcriptional level through the HOG pathway (Figure 13) (Singh *et al.,* 2005). Finally, when the osmotic balance is re-established, Hog1p activity is reduced to basal levels and it is exported to the cytoplasm, the kinases are inactivated by dephosphorylation of Hog1p through Ptc1p phosphatase, resulting in the deactivation of the HOG pathway (Saito & Tatebayashi, 2004). In *Schiz. pombe*, the osmoregulation occurs through the Sty1/Spc1/Phh1p protein kinase pathway. Components of this MAPK cascade are homologous to components of the HOG pathway in *S. cerevisiae*. However, a range of stresses activates Sty1p, whereas the HOG pathway is specifically adapted to osmotic stress (Chen *et al.,* 2003).

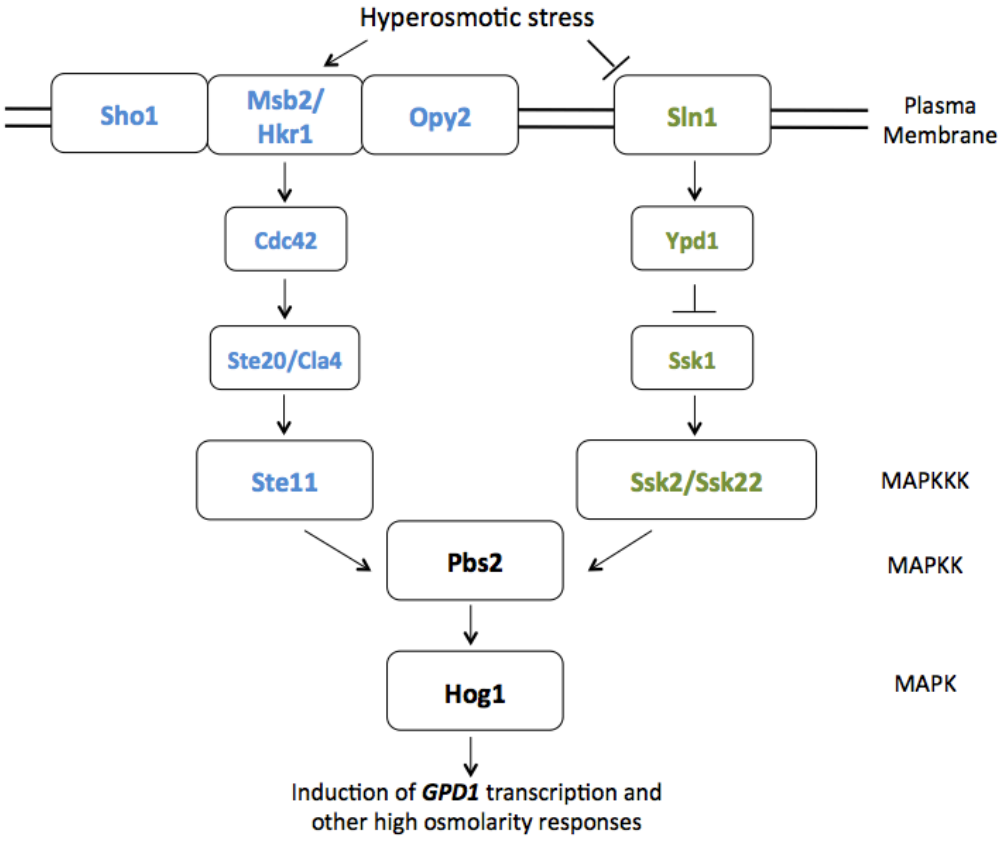


Figure 13: A schematic diagram of the HOG pathway in *S. cerevisiae.* The protein names separated by a thrash (/) are functionally redundant. Proteins that are specific to the Sln1 branch are green, those that are specific to the Sho1 branch are blue, and those that are common are black. Arrows indicate activation, whereas the T-shape bars represent inhibition (adapted from Nevoigt & Stahl, 1997; Saito & Posas, 2012).

3.4. Antioxidant defences

Oxygen is relatively unreactive and harmless in its ground state, but it can be partially reduced to form reactive oxygen species (ROS), such as the superoxide radical, (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) (González-Párraga *et al.,* 2003; Birben *et al.,* 2012; Morano *et al.,* 2012). Aerobic organisms use oxygen as an electron acceptor. However, during respiration, it can be partially reduced to form ROS (Figure 14).

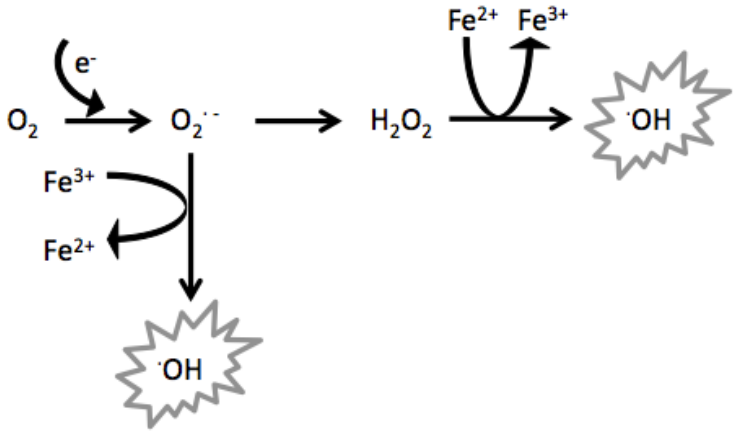


Figure 14. Generation of ROS in cells. O_2^- is the major ROS product from electron leakage and it is formed by the addition of 1 electron to the molecular O_2. H_2O_2 is generated by the breakdown of O_2^- and can be reduced by Fe^{2+} in the Fenton reaction to produce the highly reactive $^{\cdot}OH$. In the Haber-Weiss reaction, O_2^- can donate an electron to iron Fe^{3+}, generating $^{\cdot}OH$ and Fe^{2+}, which can further reduce H_2O_2 (adapted from Morano *et al.,* 2012).

Besides aerobic metabolism, numerous external agents and stress conditions may trigger ROS production. ROS are toxic agents that can damage cell components, contribute to cellular stress (oxidative stress) and aging, and lead to cell death. Specifically, ROS may contribute to lipid peroxidation, protein inactivation and nucleic acid damage (Moradas-Ferreira *et al.,* 1996; França *et al.,* 2007; Gibson *et al.,* 2007; Morano *et al.,* 2012). Although ROS are continuously produced and eliminated, if the balance between ROS production and elimination is disturbed, cells accumulate ROS, resulting in oxidative stress. Under these conditions, yeast cells have developed the means to protect their cellular components against reactive oxidants (Jamieson, 1998). Consequently, yeast cells possess antioxidant defence systems, which are present in different subcellular compartments and can be up-regulated in response to ROS exposure. These antioxidant defence systems are classified into non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidant defences consist of small

molecules that act as free radical scavengers, being oxidized by ROS and thereby removing oxidants from the solution, whereas enzymatic antioxidant defences remove oxygen radicals and contribute to the maintenance of the redox balance of the yeast cells (Figure 15) (Herrero *et al.,* 2008).

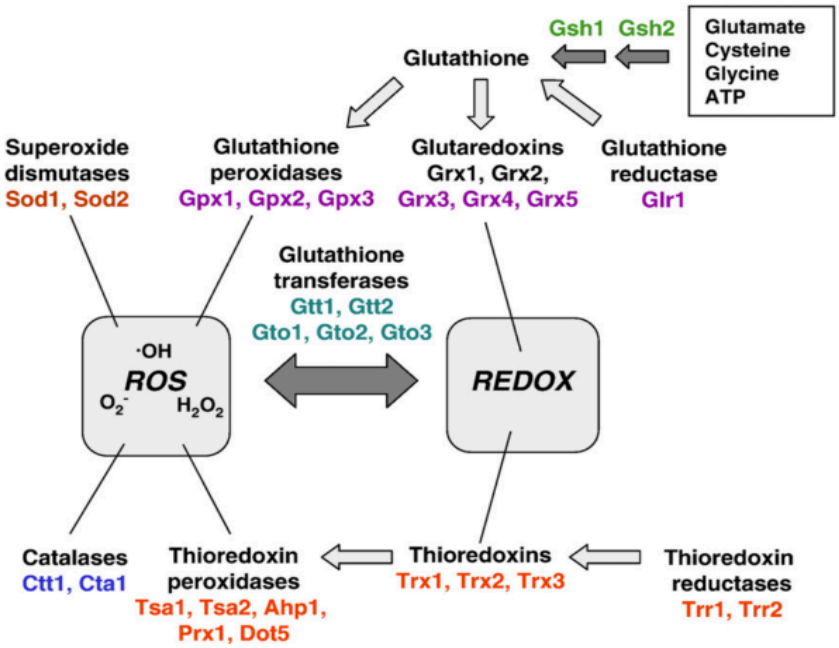


Figure 15: Enzymatic defence systems involved in the control of the redox state and ROS detoxification in yeast (Herrero *et al.,* 2008).

Oxidative stress is characterized by a disruption of cellular redox homeostasis due to the inability of the antioxidant defence systems and cellular survival mechanisms to cope with ROS production (Ribeiro *et al.,* 2015). Under desiccation, oxidative damage becomes one of the most deleterious effects of water depletion, with more than a 10-fold increase in oxidation after dehydration, confirming that the loss of water generates an oxidative stress. However, the origin of the free radicals and regulation of the antioxidant defences are not well-established during the desiccation process (França *et al.,* 2007). Moreover, although some ROS responses can be attributed to general stress, there are many ROS specific strategies with the increase of expression of specific genes under oxidative conditions. In *S. cerevisiae*, the most significant specific transcriptional factors are Yap1p, Skn7p and Hsf1p, whereas the ESR is mediated by the Msn2/4p transcription factor (Moradas-Ferreira *et al.,* 1996). Yap1p confers the ability to tolerate oxidants such as H_2O_2 and is also involved in the heat shock response through Hsf1p (mentioned earlier). Skn7p is associated with Yap1p and regulates proteins in response to H_2O_2 and is involved in heat shock response through

Hf1p (Figure 16) (Kuge & Jones, 1994; Izawa *et al.,* 1999; Costa & Moradas-Ferreira, 2001; He *et al.,* 2009). In addition, TOR and Ras-cAMP pathways also modulate desiccation tolerance through Sch9p and cAMP-dependent protein kinase A (PKA), respectively. However, while Ras-cAMP regulates nuclear exports, TOR prevents nuclear import of Msn2/4p (Verghese et al., 2012).

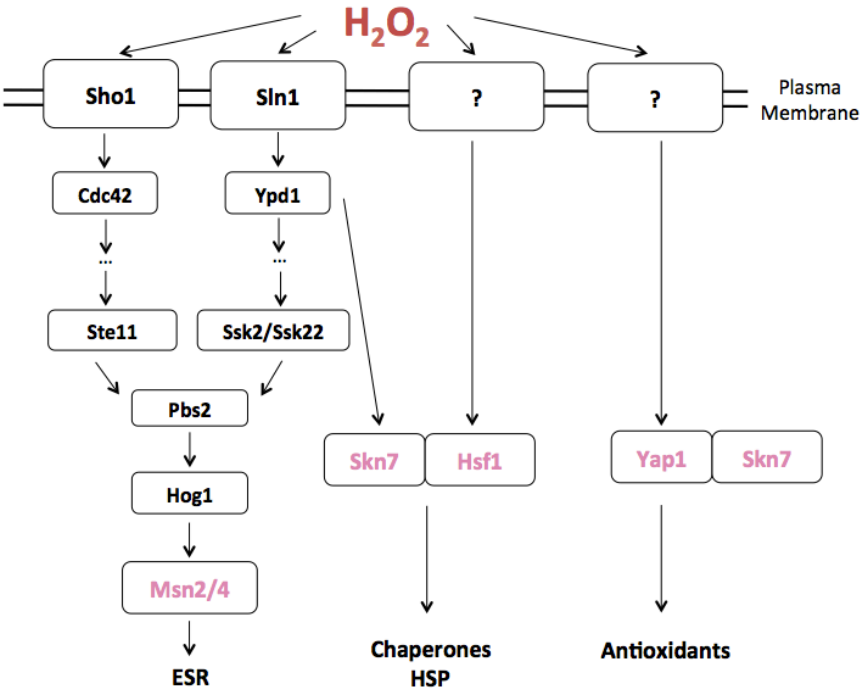


Figure 16. Signalling pathways activated by H_2O_2 in *S. cerevisiae* (adapted from Costa & Moradas-Ferreira, 2001).

3.4.1. Non-enzymatic antioxidant defence systems

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, **GSH**) seems to be the most abundant redox-scavenging molecule in cells. GSH is a low molecular-weight sulphydryl compound and acts as a radical scavenger with the redox active sulphydryl group reacting with ROS to produce oxidized disulphide glutathione (GSSG) (Jamieson, 1998). During dehydration, glutathione protects cell membranes and maintains redox homeostasis under conditions of water deficiency (Morano *et al.,* 2012). GSH synthesis is an ATP-dependent process and is performed through the action of two enzymes, γ-glutamylcysteine synthetase (Gsh1p) and the glutathione synthetase (Gsh2p). Gsh1p is responsible for the formation of the dipeptide γ-Glu-Cys (Glutamate-Cysteine), whereas Gsh2p ligates γ-Glu-Cys with glycine (Lisowsky *et al.,* 1993; Grant *et al.,* 1997). Glutathione-deficient mutants have been shown to be hypersensitive to oxidants such as H_2O_2.

Polyamines (e.g. spermine, spermidine and putrescine) are ubiquitous, polycationic, aliphatic amines that are involved in a variety of physiological processes, such as chromatic structure, gene expression, transcription, signal transduction, cell growth, cell cycle regulation, proliferation, cell attachment, cell migration, membrane stability, ion channels and cell signalling. Moreover, polyamines play a role in protecting yeasts from ROS. Yeast strains with a reduced content of polyamines are more sensitive to oxidative damage (Chattopadhyay *et al.,* 2006; Rider *et al.,* 2007).

Erythroascorbic acid is the yeast 5-carbon analogue of ascorbic acid, which is a H_2O soluble antioxidant. D-arabinono-1, 4-lactone oxidase (Alo1p) catalyses the last step of erythroascorbate biosynthesis and is induced by H_2O_2 and O_2^-, protecting yeast from ROS. However, its role as an antioxidant in yeasts is not clear, due to erythroascorbate being present at very low levels (Huh *et al.,* 1998; Jamieson 1998; Morano *et al.,* 2012).

3.4.2. Enzymatic antioxidant defence systems

Superoxide dismutases (**SODs**) are the primary defences against oxygen toxicity. SODs have an antioxidant function by catalysing the conversion of O_2^- to H_2O_2, which is further reduced to H_2O by catalases or peroxidases, and their activity requires redox active metal ions (Figure 17). Yeast possesses two intracellular SODs; Cu, Zn-SOD (Sod1) and Mn-SOD (Sod2), which are localized at the cytoplasm and the mitochondrial matrix, respectively (Jamieson, 1998). Moreover, Sod1 is the major enzyme involved in removing O_2^- from the cytoplasm and may also be localized in the mitochondrial intermembrane space (Herrero *et al.,* 2008). Although Sod1 and Sod2 are important for improving the survival of yeast cells, the absence of only one of them does not impair tolerance against desiccation due to its activity being compensated by an increase in the activity of the remaining Sod. However, desiccation tolerance increases through the overexpression of SOD1 or both (SOD1 and SOD2) enzymes, leading to a 5-fold and 8-fold tolerance increase, respectively (Jamieson, 1998; França *et al.,* 2007; Morano *et al.,* 2012).

Catalases catalyse the dismutation of H_2O_2 into H_2O and O_2 (Figure 17). Catalases also depend on the redox properties of the metal group associated with the enzyme. Yeast has two such enzymes: catalase A (Cta1p) and catalase T (Ctt1p). Cta1p is located in the peroxisome and has a specific role removing H_2O_2 produced by acyl-CoA oxidase during fatty acid β-oxidation, whereas Cct1p, which is located in the cytosol, seems to have a more general role induced by stress conditions such as heat, osmotic stress, oxidative stress and starvation (Martínez-Pastor *et al.,* 1996; Hiltunen *et al.,* 2003; Herrero *et al.,* 2008). Double catalase mutants are unable to induce an adaptive stress response to H_2O_2. Moreover, catalase increases its levels of activity and helps to maintain the intracellular redox balance during desiccation. Mutants lacking cytoplasmic catalase show higher sensitivity to water loss suggesting that tolerance to desiccation is dependent on catalase (Jamieson, 1998; França *et al.,* 2007; Morano *et al.,* 2012).

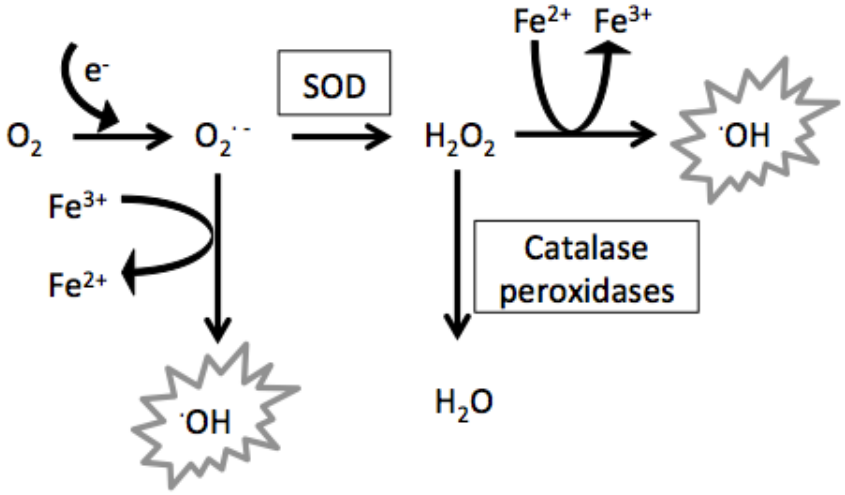


Figure 17. Antioxidant enzymes. SOD catalyses conversion of O_2^- to H_2O_2, whereas catalases and peroxidases detoxify H_2O_2 to prevent ROS generation (adapted from Morano *et al.,* 2008).

Reductases are responsible for maintaining the reduced-oxidized ratio inside the yeast cells. Reductases have been classified into 2 classes: **glutathione reductase** (**GLR**) and **thioredoxin reductases** (**TRR**). ROS decrease the GSH levels and increase the GSSG levels, so Glr1p (co-localized in the cytosol and mitochondria) reduces GSSG to GSH, maintaining the GSH/GSSG ratio in yeast cells in an NADPH-dependent process (Jamieson, 1998; Morano *et al.,* 2012).

Glutaredoxins (**GRX**s) are small heat-stable oxidoreductases with an active site containing two redox sensitive cysteines. Yeast has 8 GRXs (Grx1-8); Grx1 and Grx2 are active as GSH-dependent oxidoreductases, but they have different cellular functions during oxidative stress. Grx1p is responsible for the cells protection against O_2^- and H_2O_2, while Grx2p specializes in protecting cells against H_2O_2 (Luikenhuis *et al.,* 1998; Jamieson, 1998; Herrero *et al.,* 2008). Both are regulated in response to oxidative stress conditions through STRE elements. Otherwise, Grx3-5p regulate iron metabolism, but Grx3/4p are found in the nucleus, whereas Grx5p is found in the mitochondrial matrix. Moreover, Grx6/7p function in sulphydryl regulation in the early secretory pathway during stress conditions. Finally, Grx8p does not seem to play a role in the oxidative stress response (Eckers *et al.,* 2009; Morano *et al.,* 2012).

Thioredoxins (**TRX**s) are small sulphydryl-rich proteins and possess two redox-sensitive cysteines in their active sites. They play also roles in protection against oxidative stress induced by H_2O_2. Yeast has 2 cytosolic TRXs; Trx1p and Trx2p. While Trx2p is strongly up-regulated in response to oxidative stress conditions, Trx1p acts if Trx2p is not sufficient to provide antioxidant defence. Moreover, yeasts also present a mitochondrial TRX (Trx3p), which protects yeast cells against oxidative stress generated during respiratory metabolism (Jamieson, 1998; Herrero *et al.,* 2008; Morano *et al.,* 2012).

Peroxidases have been classified into 2 classes: **glutathione peroxidases** (**GPXs**) and **thioredoxin peroxidases** (**TRX**s) /**peroxiredoxins** (**PRX**s). GPXs are the major enzymatic defence against H_2O_2. Cytosolic GPXs employ the tripeptide GSH as a reductant. Although three cytosolic GPXs have been described (Gpx1p, Gpx2p, and Gpx3p), Gpx3p shows the highest peroxidase activity and, independently, it acts as a sensor of the oxidative response to H_2O_2 through the Yap1 transcription factor (Delaunay *et al.,* 2002). However, Gpx2 can also be up-regulated in an Skn7-dependent manner or through high Ca^{2+} concentrations but, in fact, it displays dependence on the TRX system. Otherwise, PRXs reduce peroxides and peroxinitrites with TRXs acting as the electron donor. Yeast presents five differently located PRXs; Tsa1p, Tsa2p, and Ahp1p (cytosol), Prx1p (mitochondria), and Dot5p (nucleus), and two cytosolic TRXs; Trx1p and Trx2p (Gan, 1991). Tsa1 and Tsa2 act together against oxidative stresses and this cooperation may extend to the other three PRXs.

Glutathione transferases (**GSTs**) are involved in the detoxification of many xenobiotic compounds through their removal via glutathione conjugate pumps. Yeast cells contain 2 functional GSTs (ER-Gtt1p and mitochondrial-Gtt2p) and their action is redundant with respect to the action of GRXs (Grx1p and Grx2p). Moreover, yeast cells also contain 3 omega class GSTs (peroxisomal-Gto1p, cytosolic-Gto2p, cytosolic-Gto3p) which are induced in response to oxidants under the control of Yap1 and STRE elements (Collison *et al.,* 2003; Barreto *et al.,* 2006; Garcerá *et al.,* 2006: Morano *et al.,* 2012).

Methionine sulphoxide reductases (**MSRs**) are oxidoreductases that catalyse thiol-dependent reduction of oxidized methionine (methionine sulphoxide) produced by ROS (Herrero *et al.,* 2008). MsrA and MsrB enzymes are responsible for reducing methionine-*S*-sulphoxide (Met-*S*-SO) and methionine-R-sulphoxide (Met-*R*-SO) residues in proteins, respectively. Additionally, a third MSR (fRMsr) reduces free Met-*R*-SO (Le *et al.,* 2009).

REFERENCES

Aleksander, P., Piotr, A., Tadeusz, T., & Makarewicz, M. (2009). Accumulation and release of metal ions by brewer's yeast during successive fermentations. *Journal of the Institute of Brewing*, *115,* 78-83.

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., & Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT Food Science and Technology, 49,* 8-13.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., & Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *European Food Research and Technology, 231,* 215-224.

Anfang, N., Brajkovich, M., & Goddard, M. R. (2009). Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. *Australian Journal of Grape and Wine Research, 15,* 1-8.

Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., & Zapparoli, G. (2012). Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. *European Food Research and Technology, 235,* 303-313.

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

Barbosa, C., Mendes-Faia, A., & Mendes-Ferreira, A. (2012). The nitrogen source impacts major volatile compounds released by *Saccharomyces cerevisiae* during alcoholic fermentation. *International Journal of Food Microbiology, 160,* 87-93.

Barnett, J. A., & Barnett, L. (2011). *Yeast* Research: a Historial Overview. ASM Press (1st ed.). Washington, USA.

Barreto, L., Galcerá, A., Jansson, K., Sunnerhagen, P., & Herrero, E. (2006). A peroxisomal glutathione transferase of *Saccharomyce cerevisiae* is functionally related to sulfur amino acid metabolism. *Eukaryotic Cell*, *5,* 1748-1759.

Barros de Souza, R., Karla Silva, R., Soares Ferreira, D., De Sá Leitao Paiva Junior, S., De Barros Pita, W., & De Morais Junior, M. A. (2016). Magnesium ions in yeast : setting free the metabolism from glucose catabolite repression. *Metallomics*, *8,* 1193-1203.

Beker, M. J., Blumbergs, J. E., Ventina, E. J., & Rapoport, A. I. (1984). Characteristics of cellular membranes at rehydration of dehydrated yeast *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *19,* 347-352.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii* - *Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology*, *122,* 312-320.

Benaroudj, N., Lee, D. H., & Goldberg, A. L. (2001). Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *Journal of Biological Chemistry*, *276,* 24261-24267.

Beney, L., & Gervais, P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Applied Microbiology and Biotechnology, 57,*34-42.

Beney, l., Linares, E., Ferret, E., & Gervais, P. (1998). Influence of the shape of phospholipid vesicles in the measurement of their size by photon correlation spectroscopy. *European Biophysics Journal, 27,* 567-574.

Benito, Á., Jeffares, D., Palomero, F., Calderón, F., Bai, F. Y., Bähler, J., & Benito, S. (2016). Selected *Schizosaccharomyces pombe* strains have characteristics that are beneficial for winemaking. *PLoS ONE*, 11, e0151102.

Benito, S., Palomero, F., Gálvez, L., Morata, A., Calderón, F., Palmero, D., & Suárez-Lepe, J. A. (2014). Quality and composition of red wine fermented with *Schizosaccharomyces pombe* as sole fermentative yeast, and in mixed and sequential fermentations with *Saccharomyces cerevisiae*. *Food Technology and Biotechnology, 52,* 376-382.

Benito, S., Palomero, F., Morata, A., Calderón, F., Palmero, D., & Suárez- Lepe, J. A. (2013). physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. *European Food Research and Technology, 236,* 29-36.

Berry, D. R., & Brown, C. (1987). Physiology of yeast growth. In: Berry, D. R., & Stewart, G. G. (ed). Yeast Biotechnology. pp 160-169.

Berthels, N. J., Cordero-Otero, R., Bauer, F. F., Pretorius, I. S., & Thevelein, J. M. (2008). Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast strains. *Applied Microbiology and Biotechnolology*, *77,* 1083-1091.

Billi, D., & Potts, M. (2002). Life and death of dried prokaryotes. *Research in Microbiology*, *153,* 7-12.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative Stress and antioxidant defense. *World Allergy Organization Journal, 5,* 9-19.

Birch, R. M., & Walker, G. M. (2000). Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology*, *26,* 678-687.

Blackwell, K. J., Singleton, I., & Tobin, J. M. (1995). Metal cation uptake by yeast. *Applied Microbiology and Biotechnology*, *43,* 579-584.

Bleoanca, I., & Bahrim, G. (2013). Overview on brewing yeast stress factors. *Romanian Biotechnological Letters, 18,* 8559-8572.

Borovikova, D., Herynkova, P., Rapoport, A., & Sychrova, H. (2013). Potassium uptake system Trk2 is crucial for yeast cell viability during anhydrobiosis. *FEMS Microbiology Letters*, *350,* 28-33.

Boyle, J. (2005). *Lehninger principles of biochemistry*. vol 33. (4th ed.): Nelson, D., & Cox, M. (eds). Freeman & Company. New York.

Bradshaw, P. C., & Pfeiffer, D. R. (2006). Release of Ca^{2+} and Mg^{2+} from yeast mitochondria is stimulated by increased ionic strength. *BMC Biochemistry*, *7,* 1-12.

Bradshaw, P. C., & Pfeiffer, D. R. (2014). Characterization of the respiration-induced yeast mitochondrial permeability transition pore. *Yeast*, *30,* 471-483.

Broach, J. R. (2012). Nutritional Control of growth and development in yeast. *Genetics*, *192,* 73-105.

Brown, D. A., & London, E. (1998). Membrane biology structure and origin of ordered lipid domains in biological membranes. *Journal of Membrane Biology, 164,* 103-114.

Buitink, J., & Leprince, O. (2004). Glass formation in plant anhydrobiotes : survival in the dry state. *Cryobiology*, *48,* 215-228.

Cabrera-Orefice, A., Ibarra-García-Padilla, R., Maldonado-Guzmán, R., Guerrero-Castillo, S., Luévano-Martínez, L. A., Pérez-Vázquez, V., Gutiérrez-Aguilar, M., & Uribe-Carvajal, S. (2015). The *Saccharomyces cerevisiae* mitochondrial unselective channel behaves as a physiological uncoupling system regulated by Ca^{2+}, Mg^{2+}, phosphate and ATP. *Journal of Bioenergetics and Biomembranes, 47,* 477-491.

Castells-Roca, L., García-Martínez, J., Moreno, J., Herrero, E., Bellı, G., & Pérez-Ortín, J. E. (2011). Heat shock response in yeast involves changes in both transcription rates and mRNA stabilities. *PLoS ONE*, *6,* 1–12.

Charoenchai, C., Fleet, G. H., Henschke, P. A., & Todd, B. E. N. (2008). Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape and Wine Research*, *3,* 2-8.

Chattopadhyay, M. K., White Tabor, C., & Tabor, H. (2006). Polyamine deficiency leads to accumulation of reactive oxygen species in a spe2 mutant of *Saccharomyces cerevisiae*. *Yeast*, *23,* 751-761.

Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., & Bähler, J. (2003). Global transcriptional responses of fission yeast to environmetal stress. *Molecular Biology of the Cell*, *14,* 214-229.

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. I*nternational Journal of Food Microbiology*, *108,* 239-245.

Ciani, M., & Comitini, F. (2006). Influence of temperature and oxygen concentration on the fermentation behavior of *Candida stellata* in mixed fermentation with *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology, 22,* 619-623.

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

Ciani, M., & Ferraro, L. (1998). Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *Journal of Applied Microbiology*, *85*, 247-254.

Collison, E. J., & Grant, C. M. (2003). Role of yeast glutaredoxins as glutathione S-transferases. *The Journal of Biological Chemistry*, *278,* 22492-22497.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., & Mannazzu, I. M. C. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology*, *28,* 874-882.

Cooper, T. G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J., Jones E. W., & Broach, J. B (eds). The molecular biology of the yeast *Saccharomyces*. Metabolism and Gene Expression. New York. pp 399-462.

Costa, V., & Moradas-Ferreira, P. (2001). Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. *Molecular Aspects of Medicine, 22,* 217-246.

Cyert, M. S., & Philpott, C. C. (2013). Regulation of cation balance in *Saccharomyces cerevisiae. Genetics*, *193,* 677-713.

Da Costa, B. M. T., Cornish, K., & Keasling, J. D. (2007). Manipulation of intracellular magnesium levels in *Saccharomyces cerevisiae* with deletion of magnesium transporters. *Applied Microbial and Cell Physiology*, *77*, 411-425.

De Freitas, J., Wintz, H., Hyoun Kim, J., Poynton, H., Fox, T., & Vulpe, C. (2003). Yeast, a model organism for iron and copper metabolism studies. *BioMetals*, *16,* 185-197.

Deak, T. (2008). *Handbook of food spoilage yeasts* (2nd ed.). In: Taylor & Francis Group. New York.

Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J., & Toledano, M. B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox transducer in gene activation. *Cell, 111,* 471-481.

Domizio, P., Liu, Y., Bisson, L. F., & Barile, D. (2017). Cell wall polysaccharides released during the alcoholic fermentation by *Schizosaccharomyces pombe* and *S. japonicus*: quantification and characterization. *Food Microbiology*, *61,* 136-149.

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

Eckers, E., Bien, M., Stroobant, V., Hermann, J. M., & Deponte, M. (2009). Biochemical characterization of dithiol glutaredoxin 8 from *Saccharomyces cerevisiae*: The catalytic redox mechanism redux. *Biochemistry, 48,* 1410-1423.

Eide, D. J. (2009). Homeostatic and adaptive responses to zinc deficiency in *Saccharomyces cerevisiae. Journal of Biological Chemistry, 284,* 18565-18569.

Esteve-Zarzoso, B., Manzanares, P., Ramón, D., & Querol, A. (1998). The role of non-*Saccharomyces* yeasts in industrial winemaking. *International Microbiololgy*, *1,* 143-148.

Ferguson, S. B. (2004). Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae. Genetics*, *169,* 1203-1214.

Finn, D. A., & Stewart, G. G. (2002). Fermentation characteristics of dried brewers yeast: effect of drying on flocculation and fermentation. *Journal of the American Society of Brewing Chemists*, *60,* 135-139.

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

Fleet, G. H., Lafon-Lafourcade, S., & Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied of Environmental Microbiology*, *48*, 1034-1038.

Flores, C. L., Rodríguez, C., Petit, T., & Gancedo, C. (2000). Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiology Reviews, 24,* 507-529.

França, M. B., Panek, A. D., & Eleutherio, E. C. A. (2007). Oxidative stress and its effects during dehydration. *Comparative Biochemistry and Physiology*, *146,* 621-631.

Gaensly, F., Picheth, G., Brand, D., & Bonfim, T. M. B. (2014). The uptake of different iron salts by the yeast *Saccharomyces cerevisiae*. *Brazilian Journal of Microbiology*, *45,* 491-494.

Gan, Z. R. (1991). Yeast thioredoxin genes. *Journal of Biological Chemistry, 266,* 1692-1696.

Garavaglia, J., De Cassia De Souza Schneider, R., Camargo Mendes, S.D., Welke, J.E., Alcaraz Zini, C., Bastos Caramão, E., & Valente, P. (2015). Evaluation of *Zygosaccharomyces bailii* BCV 08 as a co-starter in wine fermentation for the improvement of ethyl esters production. *Microbiological Research, 173,* 59-65.

Garcerá, A., Barreto, L., Piedrafita, L., Tamarit, J., & Herrero, E. (2006). *Saccharomyces cerevisiae* cells have three omega class glutathione S-transferases acting as 1-cys thiol transferases. *Biochemical Journal, 398,* 187-196.

García, M., Esteve-Zarzoso, B., & Arroyo, T. (2016). Non-*Saccharomyces* yeasts: biotechnological role for wine production. Grape and Wine Biotechnology, 249-272. (Chapter 11).

Grant, C. M., Maclver, F. H., & Dawes, I. W. (1997). Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Molecular biology of the cell*, *8,* 1699-1707.

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, *31*, 535-569.

Gobert, A., Tourdot-Maréchal, R., Morge, C., Sparrow, C., Liu, Y., Quintanilla-Casas, B., Vichi, S., & Alexandre, H. (2017). Non-*Saccharomyces* yeasts nitrogen source preferences : impact on sequential fermentation and wine volatile compounds profile. *Frontiers in Microbiology*, *8,* 1-13.

Godard, P., Urrestarazu, A., Kontos, K., Bontempi, G., Helden, J. V, & André, B. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, *27,* 3065-3086.

González-Párraga, P., Hernández, J. A., & Argüelles, J. C. (2003). Role of antioxidant enzymatic defences against oxidative stress (H_2O_2) and the acquisition of oxidative tolerance in *Candida albicans*. *Yeast, 20,* 1161-1169.

Graschopf, A., Stadler, J. A., Hoellerer, M. K., Eder, S., Sieghardt, M., Kohlwein, S. D., & Schweyen, R. J. (2001). The yeast plasma membrane protein Alr1 controls Mg^{2+}-homeostasis and is subject to Mg^{2+}-dependent control of its synthesis and degradation. *The Journal of Biological Chemistry*, *276,* 16216-16222.

Har Lim, P., Pisat, N. P., Gadhia, N., Pandey, A., Donovan, F. X., Stein, L., Salt, D. E., Eide, D. J., MacDiarmid, C. W. (2011). Regulation of Alr1 Mg transporter activity by intracellular magnesium. *PLoS ONE*, *6,* e20896.

He, H. J., Mulford, K. E., & Fassler, J. S. (2009). Oxidative stress function of the *Saccharomyces cerevisiae* Skn7 receiver domain. *Eukaryotic Cell, 8,* 768-778.

Herrero, E., Ros, J., Bellí, G., & Cabiscol, E. (2008). Redox control and oxidative stress in yeast cells. *Biochimica et Biophysica Acta*, *1780,* 1217-1235.

Hiltunen, J. K., Mursula, A. M., Rottensteiner, H., Wierenga, R. K., Kastaniotis, A. J., & Gurvitz, A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast *Saccharomyces cerevisiae. FEMS Microbiology Reviews*, *27,* 35-64.

Hosiner, D., Gerber, S., Lichtenberg-Frate, H., Glaser, W., Schüller, C., & Klipp, E. (2014). Impact of acute metal stress in *Saccharomyces cerevisiae. PLoS ONE*, *9,* e83330.

Huh, W. K., Lee, B. H., Kin, S. T., Kim, Y. R., Rhie, G. E., Baek, Y. W., Hwang, C. S., Lee, J. S., & Kang, S. O. (1998). D-Erythroascorbic acid is an important antioxidant molecule in *Saccharomyces cerevisiae*. *Molecular Microbiology*, *30,* 895-903.

Izawa, S., Maeda, K., Sugiyama, K.I., Mano, J., Inoue, I., & Kimura, A. (1999). Thioredoxin deficiency causes the constitutive activation of Yap1p, an AP-1-like transcription factor in *Saccharomyces cerevisiae. Journal of Biological Chemistry*, *274,* 28459-28465.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast*, *14,* 1511-1527.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture*, *27,* 15-39.

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

Kahm, M., Navarrete, C., Llopis-torregrosa, V., Herrera, R., Barreto, L., Yenush, L., Ariño, J., Ramons, J., & Kschischo, M. (2012). Potassium starvation in yeast : Mechanisms of homeostasis revealed by mathematical modeling. *PloS Computational Biology*, 8: e1002548.

Klug, L., & Daum, G. (2014). Yeast lipid metabolism at a glance. *FEMS Yeast Research*, *14,* 369-388.

Kodaki, T., & Yamashita, S. (1987). Yeast phosphatidylethanolamines methylation pathway. Cloning and characterization of two distinct methyltransferase genes. *Journal of Biological Chemistry, 262,* 15428-15435.

Kodaki, T., & Yamashita, S. (1989). Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. *European Journal of Biochemistry*, *185,* 243-251.

Kosman, D. J. (1994). Transition Metal Ion Uptake in Yeasts and Filamentous Fungi. In: Winkelman, G., & Winge, D. R. (eds). *Metal Ions in Fungi*. New York. pp 1-38.

Kuge, S., & Jones, N. (1994). *YAP1* dependent activation of *TRX2* in essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *The EMBO Journal*, *13,* 655-664.

Le, D. T., Lee, B. C., Marino, S. M., Zhang, Y., Fomenko, D. E., Kaya, A., Hacioglu, E., Kwak, G. H., Koc, A., Kim, H. Y., & Gladyshev, V. N. (2009). Functional analysis of Free Methionine-R-sulfoxide reductase from *Saccharomyces cerevisiae. The Journal of Biological Chemistry*, *284,* 4354-4364.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology, 61,* 3592-2597.

Liu, G. J., Martin, D. K., Gardner, R. C., & Ryan, P. R. (2002). Large Mg^{2+} -dependent currents are associated with the increased expression of ALR1 in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, *213,* 231-237.

Liu, X.D., & Thiele, D.J. (1996). Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. *Genes & Development*, *10,* 592-603.

Liwowky, T. (1993). A high copy number of yeast gamma-glutamylcysteine synthetase supresses a nuclear mutation affecting mitochondrial translocation. *Current genetics*, *23,* 408-413.

Ljungdahl, P. O., & Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. *Genetics*, *190,* 885–929.

Lleixà, J., Martín, V., Portillo, M. C., Carrau, F., Beltran, G., & Mas, A. (2016). Comparison of Fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae. Frontiers of Microbiology*, *7*, 1-12.

Loira, I., Morata, A., Comuzzo, P., Callejo, M. J., González, C., Calderón, F., & Suárez-Lepe, J. A. (2015). Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Research International,* 76, 325-333.

Loira, I., Vejarano, R., Bañuelos, M. A., Morata, A., Tesfaye, W., Uthurry, C., Villa, A., Cintora, I., & Suárez-Lepe, J. A. (2014). Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT Food Science and Technology, 59,* 915-922.

Loureiro, V., & Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*, *86,* 23-50.

Luikenhuis, S., Perrone, G., Dawes, I. W., & Grant, C. M. (1998). The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Molecular biology of the cell, 9,* 1081-1091.

Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. *Gene*, *290*, 1-18.

Mager, W. H., & Varela, J. C. (1993). Osmostress response of the yeast *Saccharomyces. Molecular Microbiology*, *10,* 253-258.

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

Mahmud, S. A., Nagahisa, K., Hirasawa, T., Yoshikawa, K., Ashitani, K., & Shimizu, H. (2009). Effect of trehalose accumulation on response to saline stress in *Saccharomyces cerevisiae*. *Yeast*, *26,* 17-30.

Martínez-Pastor, M. T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., & Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the Stress Response Element (STRE). *The EMBO Journal*, *15,* 2227-2235.

Mateo, J. J., & Maicas, S. (2016). Application of non-*Saccharomyces* yeasts to wine-making process. *Fermentation*, *2,* 1-13.

Medina, K., Boido, E., Fariña, L., Gioia, O., Gómez, M. E., Barquet, M., Gaggero, C., Dellacassa, E., & Carrau, F. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae. Food Chemistry, 141,* 2513-2521.

Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *International Journal of Food Microbiology*, *157,* 245-250.

Miki, B. L. A., Poon, N. H., James, A. P., & Seligy, V. L. (1982). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae. Journal of Applied Bacteriology, 150,* 878-889.

Mills, D. A., Johannsen, E. A., & Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Applied and Environmental Microbiology*, *68,* 4884-4893.

Moradas-Ferreira, P., Costa, V., Piper, P., & Mager, W. (1996). MicroReview: The molecular defences against reactive oxygen species in yeast. *Molecular Microbiology*, *19,* 651-658.

Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics*, *190,* 1157-1195.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *International Journal of Food Microbiology, 124,* 231-238.

Muntz, J. A. (1947). The role of potassium and ammonium ions in alcoholic fermentation. *Journal of Biological Chemistry*, *171,* 653-665.

Nevitt, T., Ohrvik, H., & Thiele, D. J. (2012). Charting the travels of copper in eukaryotes from yeast to mammals. *Biochimica et Biophysica Acta, 1823,* 1580-1593.

Nevoigt, E., & Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae. FEMS Microbiology Reviews*, *21,* 231-241.

Nicola, R., Hall, N., Melville, S. G., & Walker, G. M. (2009). Influence of zinc on distiller's yeast: Cellular accumulation of zinc and impact on spirit congeners. *Institute of Brewing & Distilling*, *115,* 265-271.

Nicola, R., & Walker, G. (2009). Interaction Between Yeasts and Zinc. In: Satyanarayana, T., & Kunze, G. (eds). *Yeast Biotechnology: Diversity and Applications.* Springer. pp 237-257.

Oro, L., Ciani, M., & Comitini, F. (2014). Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. *Journal of Applied Microbiology*, *116,* 1209-1217.

Özadali, F., & Özilgen, M. (1988). Microbial growth kinetics of fed-batch fermentations. *Applied Microbiology and Biotechnol*ogy, *29,* 203-207.

Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, *7,* 1-20.

Peinado, R. A., Maestre, O., Mauricio, J. C., Moreno, J. J. (2009). Use of a *Schizosaccharomyces pombe* mutant to reduce the content in gluconic acid of must obtained from rotten grapes. *Journal of Agricultural and Food Chemistry*, *57,* 2368-2377.

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences*, *1,* 1-6.

Philpott, C. C., & Protchenko, O. (2008). Response to iron deprivation in *Saccharomyces cerevisiae*. *Eucaryotic Cell*, *7,* 20-27.

Pinu, F. R., Edwards, P. J. B., Gardner, R. C., & Villas-Boas, S. G. (2014). Nitrogen and carbon assimilation by *Saccharomyces cerevisiae* during Sauvignon blanc juice fermentation. *FEMS Yeast Research*, *14,* 1206-1222.

Pisat, N. P., Pandey, A., & Macdiarmid, C. (2009). MNR2 regulates intracellular magnesium storage in *Saccharomyces cerevisiae. Genetics Society of America*, *183*, 873-884.

Poreda, A., & Tuszynski, T. (2007) Influence of magnesium and zinc ions on trehalose synthesis and fermentation activity in brewing yeast *Saccharomyces cerevisiae. Ecological Chemistry and Engineering*, 14, 197-207.

Pretorius, I. S., Van der Westhuizen, T. J., & Augustyn, O. P. H. (1999). Yeast biodiversity in vineyards and wineries and its importance to the south african wine industry. *South African Journal of Enology and Viticulture, 20,* 61-75.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., & Cocolin, L. (2012). *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology, 78,* 1987-94.

Reddi, A. R., Jensen, L. T., & Culotta, V. C. (2009). Manganese homeostasis in *Saccharomyces cerevisiae. Chemical Reviews*, *109,* 4722-4732.

Rees, E. M. R., & Stewart, G. G. (1997). The effects of increased magnesium and calcium concentrations on yeast fermentation performance in high gravity worts. *Journal of the Institute of Brewing*, *103,* 287-291.

Rees, E. M. R., & Stewart, G. G. (1999). Effects of magnesium, calcium and wort oxygenation on the fermentative performance of ale and lager strains fermenting normal and high gravity worts. *Journal of the Institute of Brewing*, *105,* 221-217.

Renault, P., Coulon, J., De Revel, G., Barbe, J. C., & Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii / S. cerevisiae* wine fermentation is linked to specific esters enhancement. I*nternational Journal of Food Microbiology, 207,* 40-48.

Ribeiro, T. P., Fernandes, C., Melo, K. V., Ferreira, S. S., Lessa, J. A., Franco, R. W. A., Schenk, G., Pereira, M. D., & Horn Jr, A. (2015). Iron, copper, and manganese complexes with *in vitro* superoxide dismutase and/or catalse activities that keep *Saccharomyces cerevisiae* cells alive under severe oxidative stress. *Free Radical Biology and Medicine*, *80,* 67-76.

Rider, J. E., Hacker, A., Mackintosh, C. A., Pegg, A. E., Woster, P. M., & Casero Jr, R. A. (2007). Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. *Amino Acids*, *33,* 231-240.

Rodríguez, M. E., Lopes, C. A., Barbagelata, R. J., Barda, N. B., & Caballero, A. C. (2010). Influence of *Candida pulcherrima* patagonian strain on alcoholic fermentation behaviour and wine aroma. *International Journal of Food Microbiology, 138,* 19-25.

Rodrigues-Pousada, C. A., Nevitt, T., Menezes, R., Azevedo, D., Pereira, J., & Amaral, C. (2004). Yeast activator proteins and stress response: an overview. *FEBS*, *567,* 80-85.

Rodríguez-Porrata, B., Novo, M., Guillamón, J., Rozès, N., Mas, A., & Cordero - Otero, R. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *International Journal of Food Microbiology*, *126,* 116-122.

Saito, H., & Posas, F. (2012). Response to hyperosmotic stress. *Genetics*, *192,* 289-318.

Saito, H., & Tatebayashi, K. (2004). Regulation of the osmoregulatory HOG MAPK cascade in yeast. *Journal of Biochemistry*, *136,* 267-272.

Schneiter, R. (1999). Brave little yeast, please guide us to Thebes: sphingolipid function in *S. cerevisiae*. *BioEssays.* 21: 1004-1010.

Sebollela, A., Roberto, P., Sola-Penna, M., Sarone-williams, V., Coelho-Sampaio, T., & Ferreira, S. T. (2004). Inhibition of yeast glutathione reductase by trehalose: possible implications in yeast survival and recovery from stress. *International Journal of Biochemistry & Cell Biology*, *36,* 900-908.

Shakoury-Elizeh, M., Protchenko, O., Berger, A., Cox, J., Gable, K., Dunn, T. M., Prinz, W. A., Bard, M., & Philpott, C. C. (2010). Metabolic response to iron deficiency in *Saccharomyces*. *Journal of Biological Chemistry, 285,* 14823-14833.

Shirkey, B., McMaster, N. J., Smith, S. C., Wright, D. J., Rodrigues, H., Jaruga, P., Birincioglu, M., Helm, R. F., & Potts, M. (2003). Genomic DNA of *Nostoc commune* (Cyanobacteria) becomes covalently modified during long-term (decades) desiccation but is protected from oxidative damage and degradation. *Nucleic Acids Research, 31,* 2995-3005.

Silva, S., Ramón-portugal, F., Andrade, P., Abreu, S., Texeira, M. D. F., & Strehaiano, P. (2003). Malic acid consumption by dry immobilized cells of *Schizosaccharomyces pombe*. *American Journal of Enology and Viticulture, 54,* 50-55.

Singh, J., Kumar, D., Ramakrishnan, N., Singhal, V., Jervis, J., Garst, J. F., Slaughter, S. M., De Santis, A. M., Potts, M., & Helm, R. F. (2005). Transcriptional response of *Saccharomyces cerevisiae* to desiccation and rehydration. *Applied and Environmental Microbiology, 71,* 8752-8763.

Stalberg, K., Neal, A. C., Ronne, H., & Stahl, U. (2008). Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*. *Journal of Lipid Research*, *49,* 1794-1806.

Stehlik-Tomas, V., Gulan Zeti, V., Stanzer, D., Grba, S., & Vahcic, N. (2004), Zinc, copper and manganese enrichment in yeast *Saccharomyces cerevisae. Food Technology and Biotechnol*ogy, *42,* 115-120.

Taillandier, P., Gilis, M., & Strehaiano, P. (1995). Deacidification by *Schizosaccharomyces*: interactions with *Saccharomyces*. *Journal of Biotechnology, 40,* 199-205.

Tamás, M. J., & Hohmann, S. (2003). The osmotic stress response of *Saccharomyces cereviasiae*. In: Hohmann, S., & Mager, W. H. (eds). Yeast Stress Responses. Heidelberg, Germany. pp 121-200.

Tariba, B. (2011). Metals in wine impact on wine quality and health outcomes. *Biological Trace Element Research*, *144,* 143-156.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., & Suzzi, G. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology*, *29,* 18-26.

Trofimova, Y., Walker, G., & Rapoport, A. (2010). Anhydrobiosis in yeast: Influence of calcium and magnesium ions on yeast resistance to dehydration-rehydration. *FEMS Microbiology Letters*, *308*, 55-61.

Venkateshwar, M., Chaitanya, K., Altaf, Md., Mahammad, E. J., Bee, H., & Reddy, G. (2010). Influence of micronutrients on yeast growth and β-D-fructofuranosidase production. *Indian Journal of Microbiology*, *50,* 325-331.

Verghese, J., Abrams, J., Wang, Y., & Morano, K. A. (2012). Biology of the heat shock response and protein chaperones : Budding yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiology and Molecular Biolology Reviews*, *76,* 115-158.

Viana, F., Belloch, C., Vallés, S., & Manzanares, P. (2011). Monitoring a mixed starter of *Hanseniaspora vineae - Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. *International Journal of Food Microbiology, 151,* 235-240.

Viana, F., Gil, J. V, Genovés, S., Vallés, S., & Manzanares, P. (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology*, *25,* 778-785.

Viana, F., Gil, J. V., Vallés, S., & Manzanares, P. (2009). Increasing the levels of 2- phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. I*nternational Journal of Food Microbiology*, *135,* 68-74.

Vianello, A., Casolo, V., Petrussa, E., Peresson, C., Patui, S., Bertolini, A., Passamonti, S., Braidot, E., & Zancani, M. (2012). The mitochondrial permeability transition pore (PTP) - An example of multiple molecular exaptation? *Biochimica et Biophysica Acta, 1817,* 2072-2086.

Vieira, E. D., Stupiello, M., & Andrietta, S. R. (2013). Yeast biomass production : a new approach in glucose-limited feeding strategy. *Brazilian Journal of Microbiology*, *44,* 551-558.

Voelker, D.R. (1997). Phosphatidylserine decarboxylase. *Biochimica et Biophysica Acta*, *1348,* 236-244.

Walker, G. M. (1998). Yeast Nutrition. In: Wiley, J., & Sons (eds). Yeast Physiology and Biotechnology. England. pp 51-93.

Walker, G. M. (2004). Metals in Yeast Fermentation Processes. *Advances in Applied Microbiology*, *54,* 197-229.

Walker, G. M., Birch-Andersen, A., Hamburger, K., & Kramhoft, B. (1982). Magnesium-induced mitochondrial polymorphism yeast, *Schizosaccharomyces pombe*. *Carlsberg Research Commun*ications, *47,* 205-214.

Wang, C., Mas, A., & Esteve-Zarzoso, B. (2016). The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Frontiers in Microbiology, 7,* 1-11.

Whitley, D., Goldberg, S. P., & Jordan, W. D. (1999). Heat shock proteins : A review of the molecular chaperones. *Journal of Vascular Surgery*, *29,* 748-751.

Wolf, F. I., Trapani, V., & Cittadini, A. (2008). Magnesium and control of cell proliferation: looking for a needle in a haystack. *Journal of the Institute of Brewing*, *21,* 83-91.

Wu, C. Y., Steffen, J., & Eide, D. J. (2009). Cytosolic superoxide dismutase (SOD1) is critical for tolerating the xidative stress of zinc deficiency in yeast. *PLoS ONE*, 4, e7061.

Zanghellini, J., Wodlei, F., & Von Grünberg, H. H. (2010). Phospholipid demixing and the birth of a lipid droplet. *Journal of Theorical Biology, 264,* 952-961.

Zhang, C. (2014). Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. *Protein & Cell*, 5, 750-760.

Zohre, D. E., & Erten, H. (2002). The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. *Process Biochemistry, 38,* 319-324.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., & Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. I*nternational Journal of Food Microbiology*, *125*, 197-203.

CHAPTER II

Enhancing the tolerance of the *Starmerella bacillaris* wine strain to dehydration stress

Gemma Roca-Domènech[1], Gema López Martínez[1], Eunice Barrera[1], Montse Poblet[1], Nicolas Rozès[1], Ricardo Cordero-Otero[1*]

[1] Department of Biochemistry and Biotechnology, Faculty of Oenology. University Rovira i Virgili, 43007- Tarragona, Catalonia, Spain

* Corresponding Author

Manuscript submitted to *Annals of Microbiology*

Abstract

Thanks to the availability of wine yeast strains as Active Dry Wine Yeast (ADWY), winemakers are able to achieve sequential inoculation of grape must to improve wine complexity. In the present study, compound features were analyzed during the dehydration-rehydration process for *Starmerella bacillaris* strains isolated from related environments of alcoholic beverages. Our results for one *S. bacillaris* strain show that the enhancement of viability might lead to a four-fold higher survival rate when cells are dried in the presence of 10% trehalose, followed by rehydration in 0.5% galactose solution. When co- and sequentially inoculated grape must fermentations with *Saccharomyces cerevisiae*, the laboratory scale wines obtained with *S. bacillaris* ADWY did not show major changes in terms of the main volatile compounds but there was an improvement in the fermentation performance behavior. The present study paves the way to develop a protocol for performing *S. bacillaris* as an ADWY.

Keywords: *Starmerella bacillaris*, Active Dry Wine Yeast, cell viability, wine

Introduction

Desiccation tolerance, also referred to as anhydrobiosis, is generally defined as the ability of an organism (anhydrobiont) to survive the removal of its cell water and then recover its normal functions after rehydration (Alpert, 2005). Desiccation is related to the extreme dehydration of cells and is considered as a state of suspended metabolism (Calahan *et al.,* 2011; Rodríguez-Porrata *et al.,* 2012a). Yeast cells are living organisms able to transition into a state of anhydrobiosis in unfavorable environmental conditions**.** This fact is frequently used in biotechnology for the production of active dry yeasts (ADYs), which are commonly employed in the food industry to produce beer, wine and bread (Rozenfelde & Rapoport, 2014). ADY production involves the dehydration of the yeast biomass to a final product with a residual amount of water below 8% (Dupont *et al.,* 2014). Moreover, almost all yeast-based food industries have expanded the use of ADY, due to its greater genetic stability at room temperature, consequently reducing transport and storage costs (Rodríguez-Porrata *et al.,* 2008). Therefore, the development of robust active dry wine yeasts (ADWYs) is an extended practice in wine industry in order to meet the demands of modern wine production processes. Inoculation with ADWYs replaces spontaneous grape must fermentations and they have traditionally been used in winemaking, in order to obtain more reproducible wines through greater control of the alcoholic fermentation (Ribereau-Gayon *et al.,* 2000). Some studies have investigated increasing the tolerance of wine yeast strains to the drying and rehydration process (Rodríguez-Porrata *et al.,* 2008; Vaudano *et al.,* 2014). In addition, being an excellent example for the study of eukaryotic cells, *Saccharomyces cerevisiae* is also able to overcome cell dehydration due to its anhydrobiotic qualities, making it an ideal model for gaining a better understanding of desiccation stress tolerance mechanisms (Pérez-Torrado *et al.,* 2005; López-Martínez *et al.,* 2012; Rodríguez-Porrata *et al.,* 2012b). The participation of different wine yeasts through the mixed inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* might be helpful in wine industry for optimizing fermentation processes and improving alcoholic beverages (Jolly *et al.,* 2014). The most common non-*Saccharomyces* yeasts present during alcoholic fermentation are *Starmerella bacillaris* and *Hanseniaspora uvarum* (Di Maio *et al.,* 2012). Winemakers are particularly

interested in *S. bacillaris* because of its strong fructophilic character, which resolves the fructose-glucose discrepancy during fermentation caused by the glucophilic character of *S. cerevisiae*, thereby preventing one of the causes of stuck fermentation. However, residual fructose is the main cause of undesirable sweetness in wines that are intended to be dry (Berthels *et al.,* 2008; Magyar & Tóth, 2011). To date, the non-*Saccharomyces Torulaspora delbrueckii* 291, *Metschnikowia pulcherrima* L1781 and *Schizosaccharomyces pombe* strains have been developed as ADWYs with sufficient quality to meet winemakers' expectations (Azzolini *et al.,* 2015; Roca-Domènech *et al.,* 2016). Therefore, the availability of ADWYs of the *S. bacillaris* strain could provide winemakers with a new biotechnological tool with the same effectiveness as commercial ADWYs for reducing the risk of sluggish fermentations or residual fructose.

In our study, a protocol was performed to obtain the *S. bacillaris* wine strain as an ADWY. Our results for a *S. bacillaris* strain isolated from wine showed that viability improves by up 50% if the yeast cells are in a specific physiological growth state before cell water deprivation and the rehydration process is performed in presence of galactose. Fermentation tests with the *S. bacillaris* ADWY displayed better fermentation efficiency, while the metabolic behavior was not affected.

Materials and Methods

Yeast Strains and Growth Conditions

The *S. bacillaris* strains used in this study (Table 4) were grown in shaker flasks at 120 rpm in SC medium (Synthetic Complete) at 28°C, inoculated with an overnight liquid culture at an initial OD_{600} of 0.2, and measured by microscope cell counting.

Table 4. List of *S. bacillaris* (syn. *Candida zemplinina*) strains evaluated in this study

Strain	Source	Origin
Sb1	[a]CBS 2649	Grape juice, France, Médoc
Sb2	CBS 157	Grapes, Germany
Sb3	CBS 1713	Wine, Italy
Sb4	CBS 9494	Wine, Tokay, Hungary
Sb5	CBS 1779	Fermenting Traditional English Ale beer

[a]Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/)

Desiccation-Rehydration Process

Growth curves were determined and 1 x 10^8 yeast cell suspensions, measured by microscope cell counting during the stationary phase, were desiccated in the presence of different trehalose concentrations (10, 20, 30, 40 and 50%) by exposure to dry air at 28°C for ~20 h (Rodríguez-Porrata *et al.,* 2012b). Cell rehydration was performed at several temperatures (30, 40 and 50°C) and times (10, 20 and 30 min). The viability of the *S. bacillaris* cells was determined for the following compounds: 0.5% and 1% proline; 0.5% and 1% glutamate; 0.5, 1, 2 and 5% raffinose; 1, 2, 4 and 6% glucose; 1.5 and 3 mM calcium; 2.5 and 5 mM magnesium; 0.25 and 1 mM ferrous (Fe^{+2}); 0.25 and 1 mM ferric (Fe^{+3}); 2.5, 5 and 10 mM ascorbic acid; 0.5 and 1 mM peroxide, 0.1 and 0.2 mM dimethyl sulphoxide (DMSO), 2.5 and 5 mM benzyl alcohol, 2.5 and 5 mM 2-phenyl-1-butanol, 2.5 and 5 mM 2-phenyl-1-propanol, 2.5 and 5 mM ethyl-hexanol, 2.5 and 5 mM p-cresol and 3.5 and 7 mM 2-ethyl-phenol. These compounds were tested individually in deionized water during the rehydration process at a final volume of 1mL (Rodríguez-Porrata *et al.,* 2008).

Determining Yeast Viability

After the rehydration process, the viable cell count was calculated by spreading cell dilutions using a Whitley Automatic Spiral Plater (AES Laboratoire, France) on YPD (Yeast Extract-Peptone-Dextrose) agar medium (Rodríguez-Porrata *et al.,* 2011). The plates were incubated at 28°C for 48 h and the colony-forming units (CFUs) were quantified using the ProtoCOL SR/HR counting system software, version 1.27, supplied by Symbiosis (Cambridge, UK).

Determination of Biological Parameters

Growth in microplate wells was monitored at 600 nm every 10 min after 20 s of shaking for 48 h at 28°C in a POLARstar OMEGA instrument (BMG Labtech, Germany). Microplate wells filled with YPD medium were inoculated with rehydrated cell inoculum measured by microscope cell counting to a final volume of 200 µL at an initial OD_{600} around 0.4, which is above the minimal detection limit previously established by calibration with Sb3 strain. Blanks were determined from five independent non-inoculated wells for each experimental 96-well plate. Three independent cultures of each strain were evaluated (six times each). Growth data from plate counts were

enumerated as log_{10} values. The biological parameters, duplication times (DT) and lag phase times (λ) were estimated by fitting the growth curves into the model using MicroFit software (Institute of Food Research, Norwich, UK) (Baranyi & Roberts, 1994).

Must Preparation, Fermentation and Sampling

Before fermentations, glucose and fructose were added to Tempranillo must to raise the final sugar content to 240 $g \cdot L^{-1}$ (100 $g \cdot L^{-1}$ and 140 $g \cdot L^{-1}$, respectively). In addition, the must with a corrected pH of 3.3 was supplemented with di-ammonium phosphate (250 $mg \cdot L^{-1}$), L-malic (8 $g \cdot L^{-1}$) and dimethyl bicarbonate (DMDC, 125 $mg \cdot L^{-1}$). After homogenization, the must was kept at 8 °C for 24 h to allow the DMDC to inhibit wild yeasts and lactic acid bacteria, which was verified by plate counting. Then, fermentations were performed in 500 mL fermentation flasks filled with 450 mL of must. The must was initially inoculated with $1 \cdot 10^7$ $cells \cdot mL^{-1}$ *S. cerevisiae* EC-1118 strain, determined by microscope cell counting, to ensure the complete fermentation of the sugars. Both, co- and sequential- fermentations were inoculated with $1 \cdot 10^8$ $cells \cdot mL^{-1}$ rehydrated *S. bacillaris* Sb3 strain. In the case of the sequential fermentations, the inoculation of EC-1118 was performed 72 h after the beginning of these fermentations. Moreover, all fermentations were performed in triplicate and conducted at 25 °C without agitation. Samples were taken to determine the end of the fermentations when the residual sugar was lower than 5 $g \cdot L^{-1}$. The final volatile compounds were analyzed by the Laboratory for Flavor Analysis and Oenology (Universidad de Zaragoza, Spain).

Statistical Analysis

Results were statistically analyzed by one-way ANOVA and the Scheffé test using the SPSS 15.1 statistical software package (SPPS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

Results

Cell viability during different growth phases

The desiccation tolerance capacity of a set of five *S. bacillaris* strains (Table 4) was assessed using a colony-counting assay as described by Rodríguez-Porrata *et al.,* (2011). The mean CFU·mL^{-1} (colony-forming units·mL^{-1}) value for cell viability after desiccation and rehydration with deionized water was calculated after taking into account the viability before drying. All strains showed viabilities lower than 2% (data not shown). Meanwhile, the *S. bacillaris* strains were dried in the presence of 10% trehalose and most of them showed viability above 5% (data not shown). Therefore, these five strains were pooled in three groups depending on their viability rate: Sb1 and Sb5, < 4% viability; Sb2 and Sb4, 5%-10% viability; and Sb3, > 10% viability (data not shown). The group with viability < 4% was discarded, because of its very low viability after imposition of stress. These strains did not show improvement in viability in the presence of trehalose. The cell viability of Sb2-Sb4 and Sb3 strains was evaluated in different cellular physiological states (Figure 18). In the case of three of them, the highest desiccation tolerance occurred during the stationary phase, between 20 h and 24 h. In this experiment, *S. bacillaris* strains were rehydrated by incubation at 37°C for 30 min. Further experiments were carried out with the Sb3 strain displaying the highest viability, around 13%.

Evaluation of cell rehydration temperature and time

Sb3 cell viability was assessed at temperatures between 30°C and 50°C and at durations of exposure ranging from 10 min to 30 min throughout the rehydration process (Figure 19). *S. bacillaris* cells were dried in presence of trehalose 10%. The incubation of cells at 50ºC resulted in a decrease in viability of more than 60%. No statistically significant differences were observed at 30°C and 40°C for times of 10 min and 20 min, respectively, or at 40°C for 30 min, which showed a viability of half of the highest recorded level of 18%. Therefore, for the remainder of the experiments *S. bacillaris* cells were rehydrated by incubation at 30°C for 30 min.

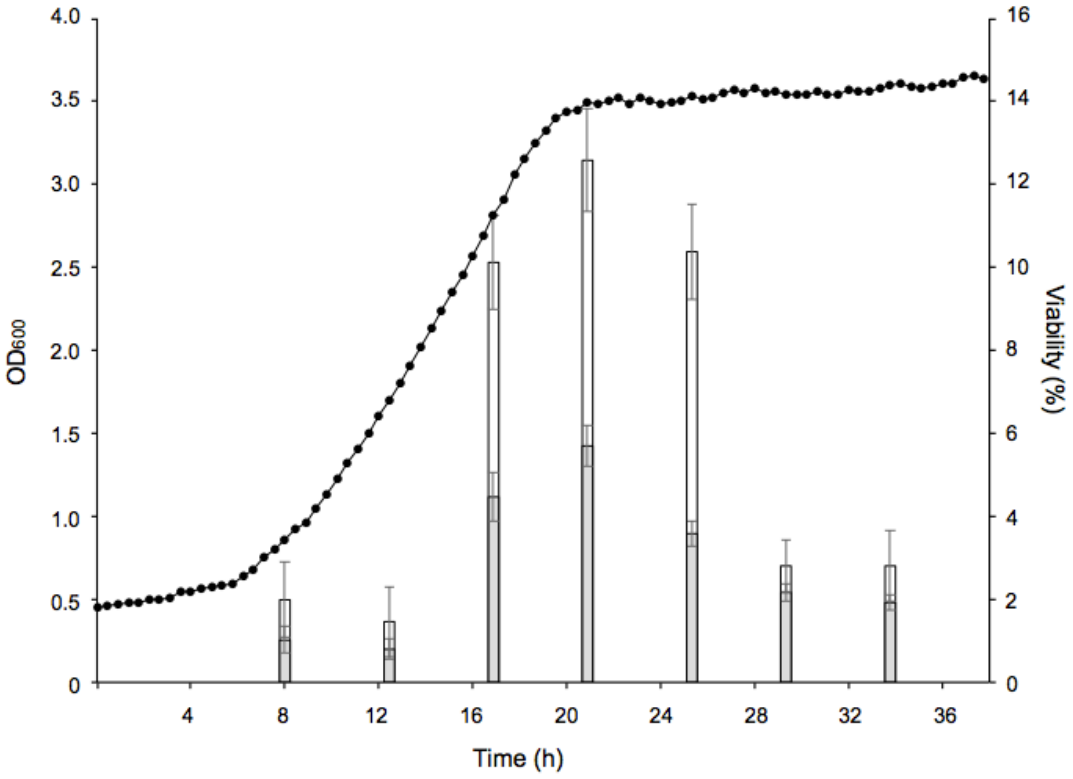


Figure 18. *S. bacillaris* cell viability based on the cellular physiological state. Both the Sb3 (•) strain growth curve and the viabilities (%) obtained at different time points for the Sb2 (grey bars) and Sb3 (white bars) strains are provided as a representative example of the evaluated strains. Values shown are means of at least n = 3 independent samples ± standard deviation (SD).

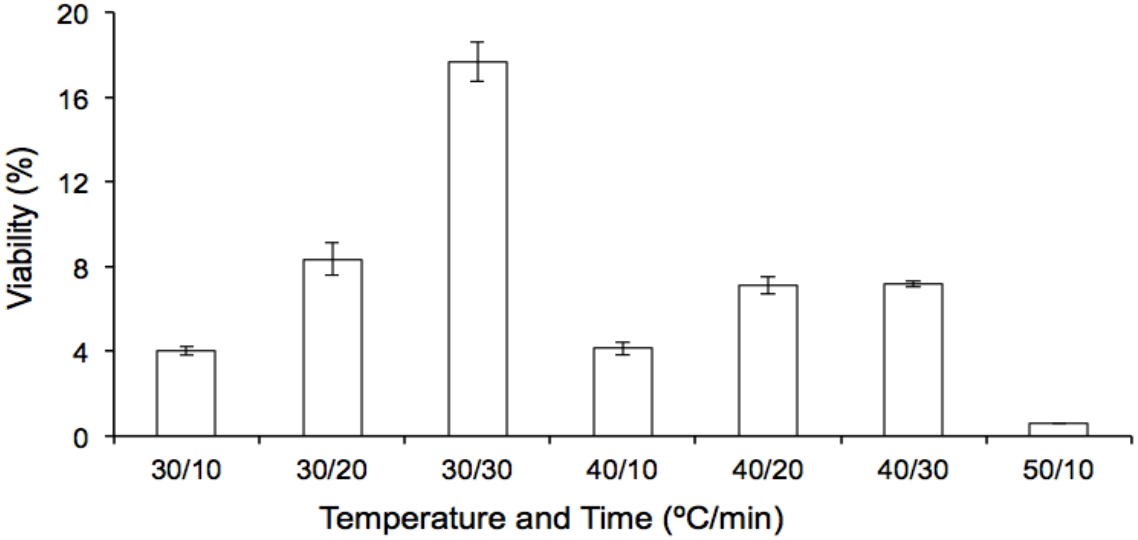


Figure 19. Evaluation the effect of temperature and time on *S. bacillaris* Sb3 viability following rehydration**.** Sb3 cells dried in the presence of 10% trehalose. The scale of viability (%) indicates the percentage of experimental values. Values shown are means of at least n = 3 independent samples ± SD. *Indicates $p < 0.05$ compared to 40°C/30 min.

Evaluation of the effect of trehalose on Sb3 viability after the imposition of stress

It was decided to ascertain whether the low viability rate of *S. bacillaris* Sb3 after the dehydration process was correlated with trehalose supplementation. The Sb3 cells were re-suspended prior to the drying process in 10, 20, 30, 40 and 50% trehalose. Deionized water was used as reference condition (Figure 20). The survival rate in the reference condition was lower than 2%, while in 20, 40 and 50% trehalose, the viability rates were around 10%. However, Sb3 cells showed a statistically significant increase in cell viability of 18% when re-suspended in 10% trehalose, compared with the pure water control. For the remainder of experiments, the Sb3 cells were re-suspended in 10% trehalose before drying.

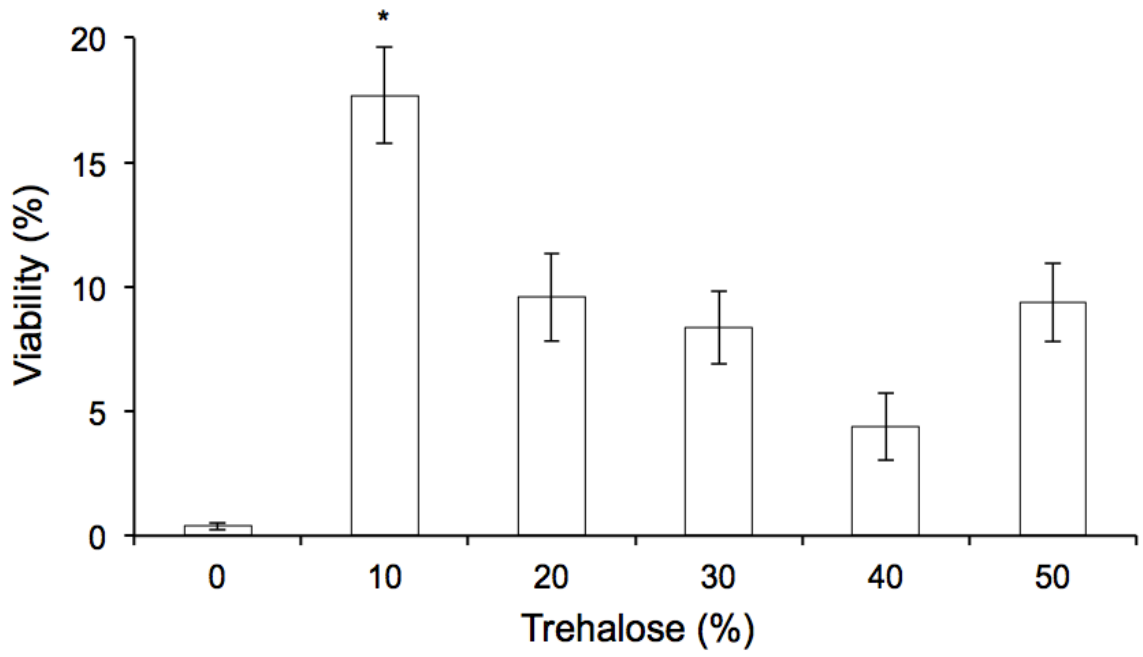


Figure 20. Effect of trehalose treatment on *S. bacillaris* Sb3 viability following air-drying and rehydration. The scale of viability (%) indicates the percentage of experimental values. Values shown are means of at least n = 3 independent samples ± SD. *Indicates $p < 0.01$ compared to H_2O and trehalose conditions.

Cell viability disparity with respect to rehydration conditions

Dried *S. bacillaris* Sb3 cells were tested in several types of rehydration media to assess the decrease in viability during this process. These media were divided into five groups: nitrogen, carbon, metallic ions, oxidant/antioxidant and membrane fluidity compounds. Figure 21 shows a representative example of the evaluated compounds (M&M). Takagi *et al.,* (2000) suggested that intracellular accumulation of nitrogen

sources such as proline could enhance dehydration tolerance in *S. cerevisiae.* The cells rehydrated in the presence of proline and monosodium glutamate supplementation showed no statistically significant differences with the rehydrated cell control (H_20). Previous studies have reported changes in sugar utilization during the rehydration process leading to cell gene regulation (Novo *et al.,* 2007). Therefore, the viability of rehydrated Sb3 cells with two carbon sources metabolized at different rates was assessed, with glucose being faster than raffinose. Rehydration with glucose did not provide any significant beneficial effects, whereas rehydration with raffinose showed a significant improvement in viability of up to 20%. During the drying process, cracks appeared in the cell membranes, which allow environmental ions and air to enter the cell, modifying the state of essential molecules for overcoming dehydration stress (Rodríguez-Porrata *et al.,* 2008). Consequently, the impact of the availability of metallic ions during the rehydration process was assessed in relation to the viability of Sb3 cells. Calcium and magnesium produced a negative effect, causing a significant statistical reduction in viability compared to the reference condition; however, ferric and ferrous ions (data not shown) did not affect cell viability. Moreover, the impact of the rehydration process was also evaluated in the presence of anti-oxidant (ascorbic acid) and oxidant (peroxide) agents, with no positive effect in cell viability being observed. In addition, peroxide resulted more detrimental than the ascorbic acid (6% and 12%, respectively). Furthermore, changes in membrane fluidity usually occur during the rehydration process. Some compounds are used to rigidify or fluidize membranes, such as dimethyl sulphoxide or benzyl alcohol, respectively (Sangwan *et al.,* 2002; Panadero *et al.,* 2006). Specifically, a number of membrane-fluidizing agents were evaluated, such as dimethyl sulphoxide, benzyl alcohol, 2-phenyl-1-butanol, 2-phenyl-1-propanol, ethyl hexanol, p-cresol and 2-ethyl-phenol. Benzyl alcohol, 2-phenyl-1-butanol, 2-phenyl-1-propanol and 2-ethyl-phenol showed a statistically significant decrease in the viability of the Sb3 cells, whereas ethyl-hexanol and p-cresol did not enhance Sb3 cell viability during the rehydration process.

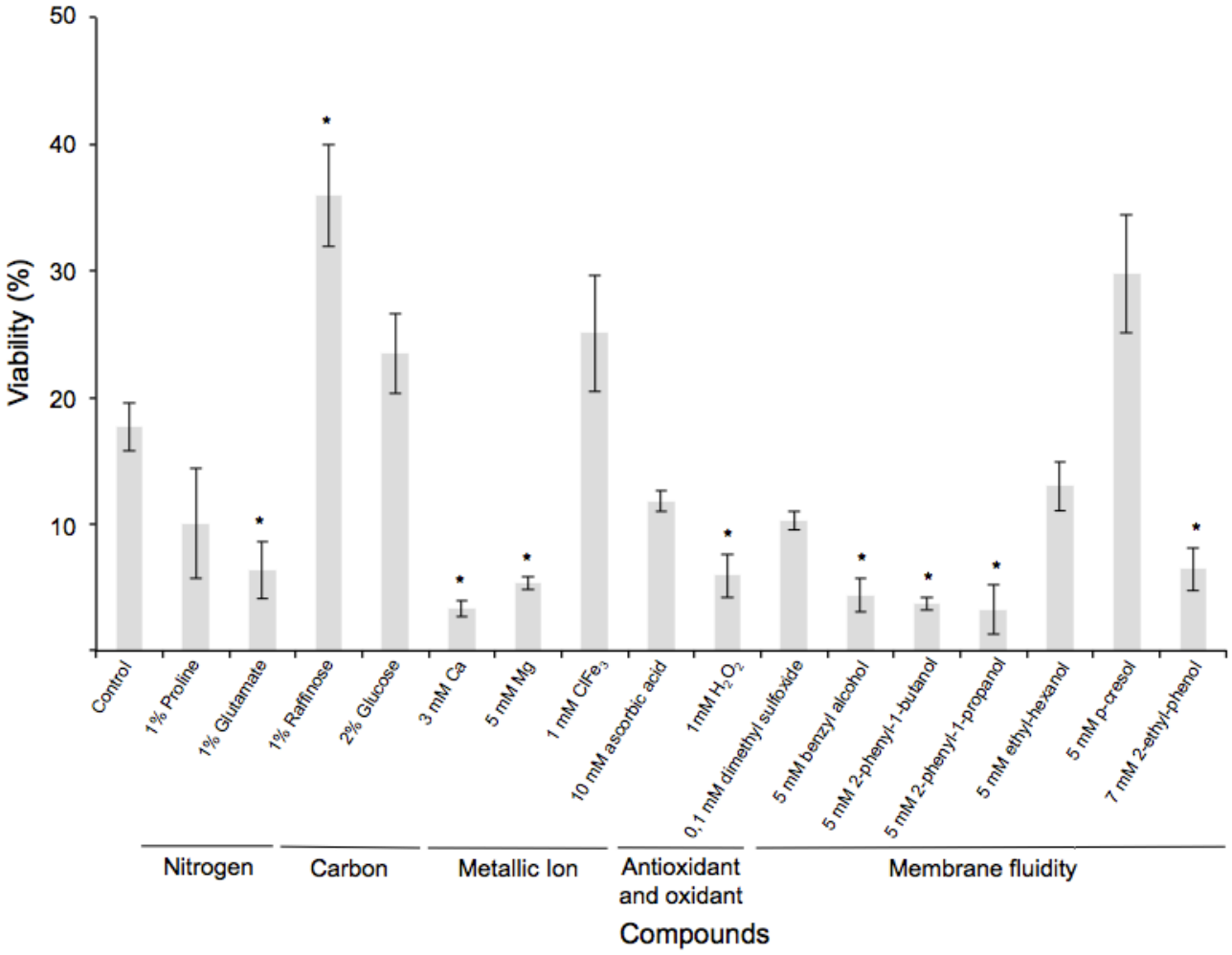


Figure 21. Effect of rehydration treatments on cell viability. Sb3 cells dried in the presence of 10% trehalose were incubated at 30°C for 30 min during rehydration in pure water or in the presence of nitrogen, carbon, metallic ions, oxidants/antioxidants and membrane fluidity compounds. The viabilities (%) provided are a representative example of the evaluated compounds. The values represent the means of three independent experiments ± SD. *Indicates $p < 0.05$ compared to the reference (H_2O).

Our results indicated that, in the presence of raffinose during the rehydration process, Sb3 cells showed a significant increase in cell viability after the imposition of stress. The trisaccharide raffinose is comprised of glucose, fructose and galactose. Therefore, it seemed important to establish whether the high viability rate of Sb3 was due to the raffinose or a monosaccharide effect. Consequently, Sb3 cells were dried in presence of 10% trehalose and rehydrated in different concentrations of raffinose and monosaccharides (Figure 22). Our results showed that 1 and 2% raffinose, and 1% fructose increase the viability rate to the same degree, up to 32%. Unexpectedly, 5%

raffinose and 2 and 5% fructose had a deleterious effect on the Sb3 cell viability. However, there was a similar statistically significant increase in cell viability when the medium was supplemented with 0.5 or 1% galactose. The values obtained for 2 and 5% galactose and glucose did not show significant differences in cell viability after the imposition of stress. The Sb3 cells rehydrated in combined preparations with 1% fructose and 0.5 or 1% galactose (data not shown) showed similar values of cell viability to those rehydrated with 0.5 and 1% galactose as a simple preparation. Therefore, our results indicate that *S. bacillaris* Sb3 cells dried in the presence of 10% trehalose and rehydrated in 0.5% galactose exhibit enhanced viability, which might directly influence the early vitality of over-active starter yeast.

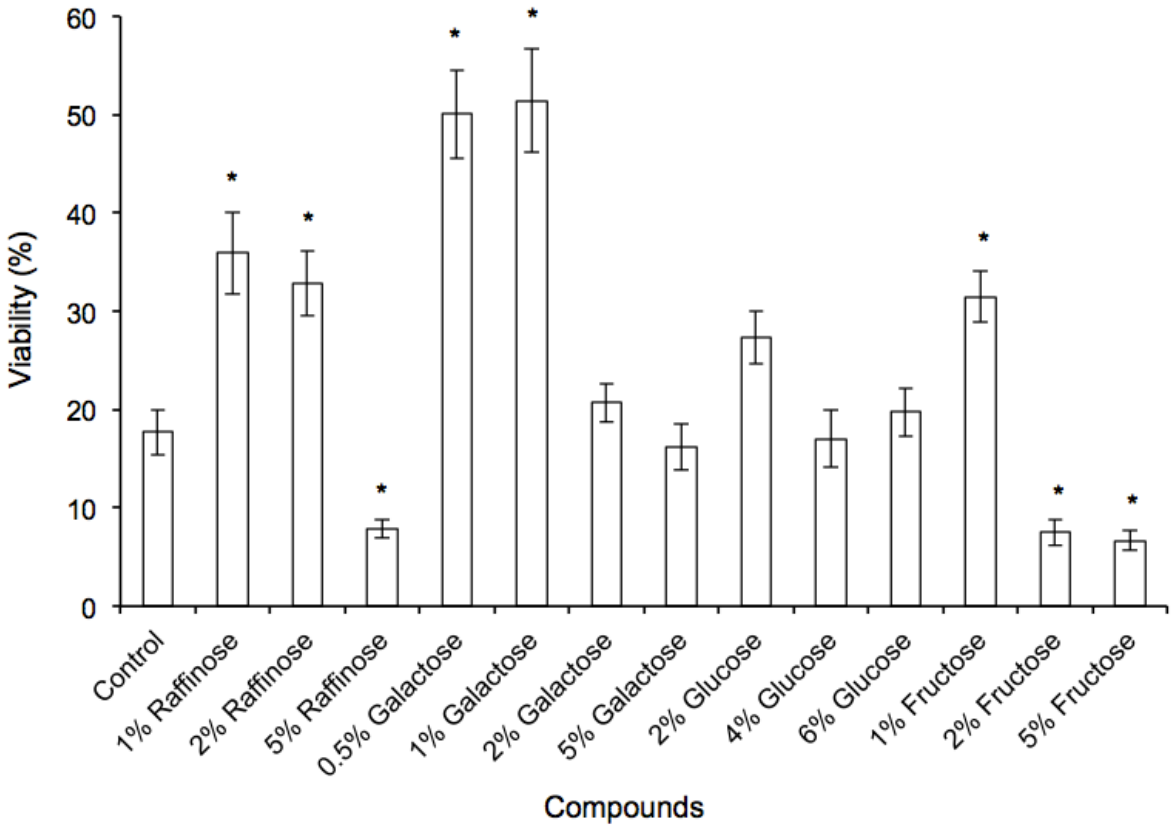


Figure 22. Effect of rehydration treatments on cell viability. Representative example of Sb3 cells dried in the presence of 10% trehalose and incubated at 30 °C for 30 min in pure water or in the presence of raffinose, galactose, glucose and fructose. The values are the means of at least three independent experiments ± SD. *Indicates $p < 0.05$ compared to the reference (H_2O).

Evaluation of cell vitality under different rehydration conditions

Next, our aim was to ascertain whether the rehydration conditions for relatively improved viability correlated with a shorter lag phase (λ) once the cells were inoculated in a complete medium compared to the reference condition (Figure 23). The Sb3 cells rehydrated in the presence of raffinose showed a λ that was 620 min longer than the control, whereas the lag time was 440 min, 420 min and 60 min shorter in cells rehydrated with fructose, glucose and galactose, respectively. Rehydration in galactose, glucose and raffinose resulted in a 1.32-fold increase in doubling time (DT), while, in fructose, it exhibited a 1.6-fold increase compared to the H_2O control, with a DT of 187 min.

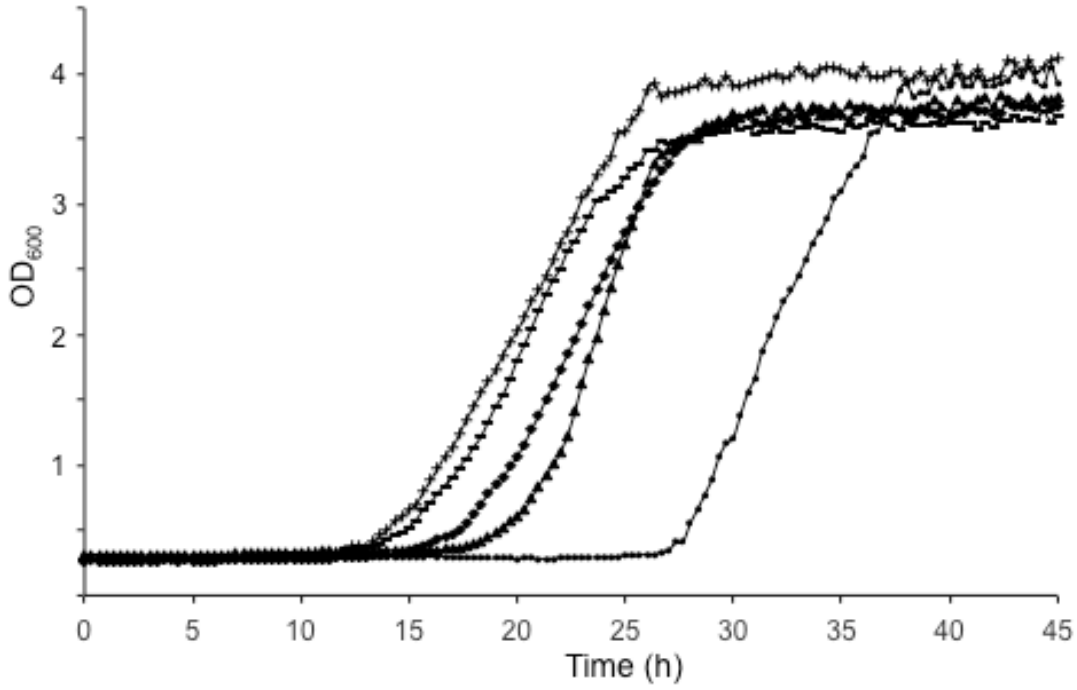


Figure 23. Effect of rehydration treatments on cell vitality. Growth curves after rehydration in the presence of H_2O (▲), 1% fructose (+), 1% galactose (♦), 2% glucose (-) and 1% raffinose (●). The curves are a representative example of growth experiments performed with yeast cells obtained from three independent compound-rehydration processes.

Fermentations

Of the three treatments performed, the *S. cerevisiae* EC-1118 treatment achieved values lower than 5 $g \cdot L^{-1}$ sugars in 6 days, whereas the co- and sequential- treatments (*S. bacillaris* ADWY-Sb3 and EC-1118) achieved values lower than 5 $g \cdot L^{-1}$ sugars in 5 days. Moreover, after 6 days, the single fermentation finished with an ethanol content of 12.3 ± 0.2%, whereas after 5 days, co- and sequential- fermentations finished with an ethanol content of 11.9 ± 0.5% and 11.4 ± 0.3%, respectively. At the end of day 2, prior to the addition of the rehydrated Sb3 cells to the sequential fermentations, the ethanol content was around 2%, v/v. At the end of fermentation in the co- and sequential- treatments, Sb3 cell viability was 2 and 10% respectively, while EC-1118 showed a viability of around 95% (data not shown).

***S. bacillaris* ADWY does not show negative alterations of metabolic behavior**

Treatments were performed to evaluate the metabolic behavior of Sb3 ADWY during fermentation by comparing the amounts of the principal volatile compounds determined in these experimental wines after the completion of alcoholic fermentations (Table 5). The total volatile compounds exhibited a two-fold increase for the co- and sequential- fermentations (EC-1118 + Sb3) compared to the EC-1118 control. In fact, 19 of 24 evaluated compounds showed some differences between fermentations, but only 13 of them exceeded the lowest odor detection value. Therefore, co- and sequential- inoculations showed statistically significant higher values for isoamyl acetate, ethyl butyrate, ethyl hexanoate, methionol, 2-phenylethanol, butyric acid, isovaleric acid, ethyl octanoate, ethyl hexanoate, hexanoic acid, isoamyl alcohol, ethyl acetate and octanoic acid in comparison to the pure (single) inoculation with EC-1118 yeast strain. However, of these 13 compounds, the last four listed above amounted to approximately 126 $mg \cdot L^{-1}$, which is the main reason for two-fold increase in total volatile compounds in mixed fermentation wines (around 400 $mg \cdot L^{-1}$) compared to the single treatment EC-1118 wine (177 $mg \cdot L^{-1}$). Nevertheless, it must be highlighted that all the tested compounds were detected at acceptable levels.

Table 5. Concentration of volatile compounds produced at the end of all fermentation processes

		Fermentation treatment ($mg \cdot L^{-1}$)		
Volatile compounds	**OTV**	**Join**	**Sequential**	**EC-1118**
Acetaldehyde	0.5	1.99 ± 0.68^{b}	2.21 ± 0.61^{b}	7.72 ± 0.91^{a}
Acetoin	150	4.82 ± 1.26^{b}	5.23 ± 1.37^{b}	83.46 ± 2.1^{a}
Ethyl acetate	12.3	40.64 ± 1.72^{b}	41.55 ± 7.69^{b}	4.29 ± 4.3^{a}
Isoamyl acetate	0.03	1.04 ± 0.1^{b}	0.98 ± 0.09^{b}	N.d.^{a}
Ethyl butyrate	0.12	0.21 ± 0.01^{b}	0.19 ± 0.01^{b}	N.d.^{a}
Ethyl hexanoate	0.06	0.41 ± 0.12^{c}	1.83 ± 0.03^{b}	N.d.^{a}
Ethyl octanoate	0.58	0.18 ± 0.16^{a}	0.81 ± 0.06^{b}	0.15 ± 0.03^{a}
Ethyl decanoate	0.2	0.02 ± 0.02^{a}	0.11 ± 0.01^{b}	0.03 ± 0.01^{a}
Isobutanol	40	28.72 ± 2.37^{b}	27.48 ± 3.22^{b}	11.59 ± 0.08^{a}
1-butanol	150	0.55 ± 0.03^{a}	0.57 ± 0.02^{a}	0.36 ± 0.01^{a}
Isoamyl alcohol	30	101.52 ± 3.47^{b}	100.49 ± 2.08^{b}	23.09 ± 0.42^{a}
1-hexanol	8	0.02 ± 0.01^{b}	0.02 ± 0.00^{b}	0.15 ± 0.01^{a}
Methionol	1	2.56 ± 0.1^{b}	2.31 ± 0.31^{b}	0.31 ± 0.01^{a}
Benzyl alcohol	200	0.01 ± 0.01^{a}	0.02 ± 0.00^{a}	0.01 ± 0.01^{a}
2-phenylethanol	14	16.78 ± 2.7^{b}	16.32 ± 3.21^{b}	8.22 ± 5.82^{a}
Ethyl lactate	154	1.01 ± 0.04^{b}	0.96 ± 0.04^{b}	0.44 ± 0.01^{a}
Diethyl succinate	200	0.11 ± 0.02^{b}	0.11 ± 0.01^{b}	0.07 ± 0.03^{a}
γ-Butyrolactone	35	1.05 ± 0.05^{a}	1.15 ± 0.04^{a}	0.71 ± 0.02^{a}
Acetic acid	300	179.67 ± 13.8^{b}	195.77 ± 13.4^{b}	33.12 ± 0.51^{a}
Butyric acid	0.17	0.78 ± 0.03^{b}	0.75 ± 0.01^{b}	0.21 ± 0.01^{a}
Isobutyric acid	2.3	1.12 ± 0.07^{a}	1.09 ± 0.05^{a}	1.54 ± 0.04^{a}
Isovaleric acid	0.03	0.73 ± 0.01^{b}	0.72 ± 0.02^{b}	0.18 ± 0.01^{a}
Hexanoic acid	0.42	3.34 ± 0.49^{b}	3.73 ± 0.41^{b}	0.32 ± 0.02^{a}
Octanoic acid	0.5	8.61 ± 0.21^{b}	9.47 ± 0.51^{b}	1.32 ± 0.13^{a}
Decanoic acid	1	0.57 ± 0.01^{b}	0.66 ± 0.11^{b}	0.13 ± 0.03^{a}
Total		396.42^{b}	414.509^{b}	177.374^{a}

OTV, Odor Threshold Value; N.d., no detected. Different letters indicate significant differences (95% confidence) between wine treatments.

Discussion

Organisms with the ability to cope with some type of extreme stress must be in an adequate physiological state to survive it. If the organism does not adjust to the stress to which it is exposed, the incurred injuries would seriously compromise its viability. Therefore, first of all, it was necessary to define the best physiological cell state to achieve the highest viability after dehydration and the imposition of stress. *S. bacillaris* cells obtained at the early stationary phase showed the highest desiccation tolerance. These results agreed with previous data observed for *Schizosaccharomyces pombe*, but differed from previous observations for *S. cerevisiae*, which reached its highest

desiccation tolerance capacity during the last stationary phase, before the decline phase (Rodríguez-Porrata *et al.,* 2012b; Dupont *et al.,* 2014; Roca-Domènech *et al.,* 2016).

Moreover, trehalose, a non-reducing disaccharide, was previously shown to act as a membrane protector by stabilizing and preventing the loss of cellular structural and functional integrity during the desiccation-rehydration process (Hottiger *et al.,* 1987). Two mechanisms that may explain the protective role of trehalose are: i) keeping the dried membranes in a fluid state by preventing the increase of membrane-transition temperature; ii) preventing the destruction of the cell structures by converting the cytoplasm in a highly viscous fluid (Potts 2001). *S. bacillaris* cells dried in presence of 10% trehalose show an increase of 18% in cell viability, the same improvement having previously been observed in cells of *S. cerevisiae* and *Schiz. pombe* exposed to dehydration stress (Rodríguez-Porrata *et al.,* 2011; Roca-Domènech *et al.,* 2016). Rehydration time and temperature above or below the optimal level generally leads to wrong timing in terms of phase transition, with the subsequent lethal damages (Hernández-García, 2011). The 30 min rehydration time was similar to the standard protocol used by winemakers for *S. cerevisiae* ADWY, which is 25 min longer than *Schiz. pombe* cells. The rehydration temperature was 7°C lower than *S. cerevisiae* and *Schiz. pombe* cells under similar conditions (Rodríguez-Porrata *et al.,* 2011; Roca-Domènech *et al.,* 2016). Yeasts could show different dehydration tolerance levels due to the presence of protectant peptides or heat shock proteins, or due to the unsaturated lipid composition of the cell membranes (Pérez-Torrado *et al.,* 2005; Rodríguez-Porrata *et al.,* 2011; López-Martínez *et al.,* 2012; Rodríguez-Porrata *et al.,* 2012b; Díaz-Hellín *et al.,* 2014). Therefore, a single factor correlation for explaining the lowest rehydration temperature of *S. bacillaris* is not accurate.

Moreover, the rehydration solution may also play a relevant role in dehydration stress tolerance. Our results with proline and monosodium glutamate during the rehydration process did not enhance the yeast survival rate. These results are in line with the results described for *S. cerevisiae* and *Schiz. pombe* cells, with intracellular nitrogen backup being preferred to extracellular sources at the time of the first rehydration to resume cell activity. The metal supplementation results for calcium and ferric ion are consistent with those described for *Schiz. pombe* and *S. cerevisiae*.

However, magnesium has a positive effect on *S. cerevisiae* and *Schiz. pombe* cell rehydration, but has a suppressive effect on *S. bacillaris* viability as a result of antagonism between the ferric and calcium ions (Rodríguez-Porrata *et al.,* 2008; Trofimova *et al.,* 2010; Roca-Domènech *et al.,* 2016). The modification of the supposed harmful effect by over-exposing intracellular constituents to environmental oxygen during the drying process showed similar results for the *S. cerevisiae* and *Schiz. pombe strains* (Rodríguez-Porrata *et al.,* 2011; Roca-Domènech *et al.,* 2016). These results suggest that the redox capacity of dry yeast cells may be shifted to a more oxidative state, becoming detrimental to cell viability. However, apparently, extracellular antioxidant agents cannot enhance the antioxidant capacity of cells in the early stationary state (López-Martínez *et al.,* 2012; Rodríguez-Porrata *et al.,* 2012a; Roca-Domènech *et al.,* 2016). The optimal achievement of membrane fluidity after cell rehydration allows the stress sensors of the membrane and cytoplasm to act and adapt the cell to the environmental conditions, in order to resume proliferation (Trott & Morano, 2003). Unexpectedly, the membrane-fluidizing agents benzyl alcohol, 2-phenyl-1-butanol, 2-phenyl-1-propanol and 2-ethyl-phenol showed significantly decreased cell viability in *S. bacillaris* during the rehydration process, which differs from the non-significant effect reported in the case of *S. cerevisiae* and *Schiz. pombe* strains under the same conditions (Rodríguez-Porrata *et al.,* 2008; Roca-Domènech *et al.,* 2016). The possible factors that could explain the different yeast response to the hydrophobic agents include the differences in lipid membrane composition between yeasts (Lee, 2004; Rodríguez-Porrata *et al.,* 2011; Díaz-Hellín *et al.,* 2014).

Of the carbon sources evaluated in the rehydration process, the trisaccharide raffinose and only one of its monomers (galactose) showed a statistically significant viability increase in *S. bacillaris* cells. Previous evaluations of these sugars in terms of *S. cerevisiae* and *Schiz. pombe* cell viability under the same conditions did not show any improvement (Rodríguez-Porrata *et al.,* 2008; Roca-Domènech *et al.,* 2016). However, it was observed that cells treated with both raffinose and galactose showed a similar DT in the presence of glucose as the sole carbon source, suggesting a non-toxic effect of these sugars on the cells. Nevertheless, the λ of the rehydrated cells treated with raffinose was 11.3 hours longer than the cells treated with galactose, which was 1 hour shorter than the control. *S. bacillaris* cells are able to assimilate raffinose very slowly,

but not galactose at all, suggesting that these sugars do not act as a metabolic booster in recovering cells from the damages caused during the dehydration/rehydration process (Sipiczki, 2003). It can be understood that the extended time of the λ phase of raffinose-treated cells is caused by the low metabolic assimilation ratio by *S. bacillaris*. A similar profile is observed in galactose-treated cells compared to the fructose- and glucose-treated cells. However, cells treated with raffinose and galactose show the best viability rates, which become unsatisfactory at higher concentrations (Figure 22). That enables us to hypothesize that the length of the λ phase mediated by these sugars might provide additional time for correct cell rescue activity and account for the increase in the viability rate. Therefore, the variation in the length of time for the cell to resume growth by using faster metabolized sugars or higher concentrations of viability-enhancer compounds has an undesirable effect on the cell. This observation was previously reported in *S. cerevisiae* and *Schiz. pombe* cells under similar exposure to stress (Rodríguez-Porrata *et al.,* 2008; Roca-Domènech *et al.,* 2016).

Moreover, it is well known that most yeasts, including *Saccharomyces* species, prefer fermenting glucose to fructose if both sugars are present in the medium (Magyar & Tóth, 2011). In contrast, *S. bacillaris* consumes fructose preferentially to glucose and is therefore considered as fructophilic yeast (Di Maio et al., 2012). Our results showed that co- and sequential- inoculations of *S. bacillaris / S. cerevisiae,* in the presence of an excess of fructose in the initial must, showed no residual sugar 1 day before the end of single *S. cerevisiae* fermentations. Therefore, the fructophilic character of *S. bacillaris* enhanced the kinetic fermentation, preventing stuck fermentation due to its fructophilic capacity, by fixing the fructose-glucose discrepancy present in the initial must, affecting *S. cerevisiae* with its strong glucophilic character. In addition, the fructophilic character allows the reduction of residual sugar in wines, which is mainly constituted by fructose (Berthels *et al.,* 2008; Jolly *et al.,* 2014). Furthermore, co- and sequential- fermentations showed a lower final ethanol content in comparison to single *S. cerevisiae* fermentations. The relatively low ethanol level is one of the most appealing features of *S. bacillaris* used to develop wines for a broad range of consumers (Magyar & Tóth, 2011; Di Maio *et al.,* 2012).

Finally, co- inoculations of non-*Saccharomyces* yeasts and *S. cerevisiae* have generated interest in the wine industry not only for technological reasons but also for

sensory reasons (Andorrà *et al.,* 2010). One example is that some non-*Saccharomyces* metabolites may act as intermediaries in aroma metabolic pathways (Jolly *et al.,* 2014). In particular, controversial results were published regarding the participation of *S. bacillaris* in the organoleptic profile of wines. Sadoudi *et al.,* (2012) reported that acetic acid, glycerol, aldehyde and acetate ester production decreases in comparison to single fermentations, and the opposite has also been reported (Andorrà *et al.,* 2010). Our results showed higher levels of esters, alcohols, acetic acid and lower levels of aldehyde in both co- and sequential- fermentations than in the case of single fermentation. Esters and acetates may have a positive effect on wine quality, as most of them contribute to the flowery and fruity aroma of wines. However, the main ester in our co- fermentations is ethyl acetate (around 41 g·L¯1), which, at 89 g·L¯1, produces an unpleasant aroma (pungent) to the detriment of the wine quality (Noble *et al.,* 1987). Isoamyl alcohol was identified as one of the main flavour constituents of banana and karanda fruit, and a concentration inferior of 300 $mg \cdot L^{-1}$ may contribute to the complexity of wine aroma (Lambrechts & Pretorius, 2000; Pino *et al.,* 2004). Therefore, the 5-fold higher isoamyl alcohol content in co- rather than single fermentations (20 $mg \cdot L^{-1}$) plays a role in enhancing the aroma profile. In contrast, higher concentrations of 20 mg·L¯1 of hexanoic and octanoic acid in wine were considered as unpleasant odorants, but they contribute significantly to the complexity of the overall flavor of the wine (Shinohara 1985). The content of these fusel alcohols together is 7 times higher in co- fermentations (around 12 mg·L¯1) than in single fermentations, but not at levels that are detrimental to the organoleptic wine profile. Therefore, the concentrations of volatile compounds produced at the end of co-fermentations are in line with previous reports, which showed the use of *S. bacillaris* for increasing organoleptic wine complexity (Sipiczki, 2003; Jolly *et al.,* 2014).

In conclusion, the present study paves the way to develop a protocol for developing *S. bacillaris* as an ADWY. The cell viability and vitality of three *S. bacillaris* strains after the desiccation-rehydration process has been characterized under several physiological conditions. Our results show that the presence of both trehalose during the drying process and raffinose/galactose during the rehydration process has a synergistic effect on the enhancement of cell viability. Furthermore, the wines obtained by combined treatments of *S. cerevisiae* and *S. bacillaris* did not show defects

in terms of the principal volatile compounds and better fermentation performance in respect to the single control fermentation. These findings indicate that the protocol applied to obtain a strain of *S. bacillaris* as an ADWY may have a broad industrial application.

Acknowledgements

This work was supported by grant AGL2012-40018 from the Spanish *Ministerio de Ciencia e Innovación*. The authors would like to thank the Universitat Rovira i Virgili for the doctoral fellowships FI-DGR 2012, AGAUR to GL and 2014PMF-PIPF-47 to GR.

References

Alpert, P. (2005). The limits and frontiers of desiccation-tolerant life. *Integrative and Comparative Biology, 45*, 685-695.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., & Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *European Food Research and Technology, 231*, 215-224.

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., & Zapparoli, G. (2015). Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology, 3*, 277-293.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology, 23*, 277-294.

Berthels, N. J., Cordero-Otero, R. R., Bauer, F. F., Pretorius, I. S., & Thevelein, J. M. (2008). Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast strains. A*pplied* Microbiology and Biotechnology, *77*, 1083-91.

Calahan, D., Dunham, M., Desevo, C., & Koshland, D. E. (2011). Genetic analysis of desiccation tolerance in *Sachharomyces cerevisiae*. *Genetics, 189*, 507-519.

Díaz-Hellín, P., Gómez-Alonso, S., Borrull, A., Rozès, N., Cordero-Otero, R., & Úbeda, J. (2014). Membrane lipid variability in *Saccharomyces cerevisiae* wine strains rehydrated in the presence of metabolic activators. *Journal of Agricultural and Food Chemistry, 62*, 8679-8685.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., & Oliva, D. (2012). Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. *South African Journal of Enology and Viticulture, 33*, 80-87.

Dupont, S., Rapoport, A., Gervais, P., & Beney, L. (2014). Survival kit of *Saccharomyces cerevisiae* for anhydrobiosis. A*pplied* Microbiology and Biotechnology, *98*, 8821-8834.

Hernández-García, A. (2011). Anhydrobiosis in bacteria: from physiology to applications. *Journal of Biosciences, 36*, 939-950.

Hottiger, T., Boller, T., & Wiemken, A. (1987). Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. *FEBS Letters, 220*, 113-115.

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

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeast and its importance to wine aroma. *South African Journal of Enology and Viticulture, 21*, 97-129.

Lee, A. G. (2004). How lipids affect the activities of integral membrane proteins. *Biochimica et Biophysica Acta, 1666*, 62-87.

López-Martínez, G., Rodríguez-Porrata, B., Margalef-Català, M., & Cordero-Otero, R. (2012). The STF2p hydrophilin from *Saccharomyces cerevisiae* is required for dehydration stress tolerance. *PLoS ONE, 7*, e33324.

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

Noble, A. C., Arnold, R. A., Buechsenstein, J., Leach, E. J., Schmidt, J. O., & Stern, P. M. (1987). Modification of a standardized system of wine aroma terminology. *American Journal of Enology and Viticulture, 38*, 143-146.

Novo, M., Beltran, G., Rozes, N., Guillamon, J. M., Sokol, S., Leberre, V., François, J., & Mas, A. (2007). Early transcriptional response of wine yeast after rehydration: osmotic shock and metabolic activation. *FEMS Yeast Research, 7*, 304-316.

Panadero, J., Pallotti, C., Rodríguez-Vargas, S., Randez-Gil, F., & Prieto, J. A. (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 281*, 4638-4645.

Pérez-Torrado, R., Bruno-Barcena, J. M., & Matallana, E. (2005). Monitoring stress-related genes during the process of biomass propagation of *Saccharomyces cerevisiae* strains used for wine making. *Applied and Environmental Microbiology, 71*, 6831-6837.

Pino, J. A., Marbot, R., & Vázquez, C. (2004). Volatile flavor constituents of Karanda (*Carissa carandas* L.) fruit. *Journal of Essential Oil Research, 16*, 432-434.

Potts, M. (2001). Desiccation tolerance: A simple process? *Trends in Microbiology, 9*, 553-559.

Ribereau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2000). *Handbook en Enology: The microbiology of wine and vinifications* (2nd ed.). UK.

Roca-Domènech, G., López-Martínez, G., Tirado, V., Borrull, A., Candelas, O., Rozès, N., & Cordero-Otero, R. (2016). Viability enhancement of *Schizosaccharomyces pombe* cells during desiccation stress. *Journal of Microbiology Research, 6*, 82-91.

Rodríguez-Porrata, B., Carmona-Gutierrez, D., López-Matínez, G., Reisenbichler, A., Bauer, M., Madeo, F., & Cordero-Otero, R. (2012a). Yeast cell death during the drying and rehydration process. In: Schmid I (ed) Flow Cytometry – Recent Perspectives, vol 1. UCLA, pp 119-132.

Rodríguez-Porrata, B., Carmona-Gutierrez, D., Reisenbichler, A., Bauer, M., López, G., Escoté, X., Mas, A., & Cordero-Otero, R. (2012b). Sip18 hydrophilin prevents yeast cell death during desiccation stress. *Journal of Applied Microbiology, 112*, 512-525.

Rodríguez-Porrata, B., López-Martínez, G., Redón, M., Sancho, M., Mas, A., Rozès, N., & Cordero-Otero, R. (2011). Enhancing yeast cell viability after dehydration by modification of the lipid profile. *World Journal of Microbiology and Biotechnology, 27*, 75-83.

Rodríguez-Porrata, B., Novo, M., Guillamón, J. M., Rozès, N., Mas, A., & Cordero-Otero, R. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *International Journal of Food Microbiology, 126*, 116-122.

Rozenfelde, L., & Rapoport, A. (2014). Anhydrobiosis in yeast: Is it possible to reach anhydrobiosis for yeast grown in conditions with severe oxygen limitation? *Antonie van Leeuwenhoek, 106*, 211-217.

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

Sangwan, V., Örvar, B. L., Beyerly, J., Hirt, H., & Dhindsa-Rajinder, S. (2002). Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *The Plant Journal, 31*, 629-638.

Shinohara, T. (1985). Gas chromatographic analysis of volatile fatty acids in wines. *Agricultural and Biological Chemistry, 49*, 2211-2212.

Sipiczki, M. (2003). *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *International Journal of Systematic and Evolutionary, 53*, 2079-2083.

Takagi, H., Sakai, K., Morida, K., & Nakamori, S. (2000). Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. *FEMS Microbiology Lett*ers, *184*, 103-108.

Trofimova, Y., Walker, G. M., & Rapoport, A. (2010). Anhydrobiosis in yeast: influence of calcium and magnesium ions on yeast resistance to dehydration-rehydration. *FEMS, 308*, 55-61.

Trott, A., & Morano, K. A. (2003). The yeast response to heat shock. In: Hohmann S & Mager PWH (Eds.*) Yeast Stress Responses*, vol 1. Springer, Heidelberg, pp 71-119.

Vaudano, E., Noti, O., Costantini, A., & García-Moruno, E. (2014). Effect of additives on the rehydration of *Saccharomyces cerevisiae* wine strains in active dry form: Influence on viability and performance in the early fermentation phase. *Journal of the Institute of Brewing, 120*, 71-76.

CHAPTER III

.

Viability enhancement of *Schizosaccharomyces pombe* cells during desiccation stress

Gemma Roca-Domènech[1], Gema López Martínez[1], Víctor Tirado[1], Anna Borrull[1], Óscar Candelas[1], Nicolas Rozès[1], Ricardo Cordero-Otero[1*]

[1] Department of Biochemistry and Biotechnology, Faculty of Oenology. University Rovira i Virgili, 43007- Tarragona, Catalonia, Spain

* Corresponding Author

Journal of Microbiology Research 2016, 6(4): 82-91

Abstract

In winemaking, the sequential inoculation of grape must with different oenological yeasts has been widely used due to the accessibility of different types of active dry yeast (ADY). *Schizosaccharomyces pombe* is of special interest for winemakers due to its ability to metabolize L-malic acid. Therefore, the availability of *Schiz. pombe* as an ADY will provide a new biotechnological tool with the same effectiveness as current commercially available oenological yeasts. In the present study, the features of metabolites were analyzed during the dehydration-rehydration process for different *Schiz. pombe* strains to determine whether these metabolites might play a positive role in ensuring cell viability before inoculation into the must. Our results show that the viability of cells dried in the presence of 10% trehalose and rehydrated in a solution complemented with 5 mM $MgSO_4$ was enhanced by up to 70% for certain strains. No significant change in fermentation behavior and main volatile compounds were detected in the wines obtained with *Schiz. pombe* ADY in sequential- and co-inoculated with *Saccharomyces cerevisiae* grape must fermentation at laboratory scale.

Keywords: *Schizosaccharomyces pombe*, Active Dry Yeast, cell viability, wine

Introduction

At present, most of the wine produced worldwide is made from musts inoculated from selected wine yeasts called *active dry wine yeasts* (ADWY) that are often traded in dried form. The ability of *Saccharomyces cerevisiae* to withstand dehydration and subsequent rehydration allows for the production of ADWY inocula, ensures that fermentation will start by increasing confidence in the strain identity and genetic stability at room temperature, and reduces the costs of transportation and storage (Dupont *et al.,* 2014). ADWY production involves dehydration of the yeast biomass to a final product with a residual moisture percentage of below 8% (Rodríguez-Porrata *et al.,* 2008). Many *S. cerevisiae* strains with an optimum oenological profile are excluded from commercial catalogues due to the sensitivity to the drying treatment. Studies have investigated the dehydration behavior exhibited by sensitive strains (Kraus *et al.,* 1981; Soubeyrand *et al.,* 2006). In contrast, few studies have investigated increases in dehydration tolerance to drying and rehydration by wine yeast strains; most of these studies were performed on representatives of the genus *Saccharomyces* (Rodríguez-Porrata *et al.,* 2008; Rodríguez-Porrata *et al.,* 2011; Vaudano *et al.,* 2014; Díaz-Hellín *et al.,* 2014; Rapoport *et al.,* 2014). Previously, only non-*Saccharomyces* yeast strains had been considered responsible for organoleptic defects in wine, but studies have reported that the sequential growth of different genera of non-*Saccharomyces* wine yeasts, such as *Hanseniaspora*, *Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces* and *Torulaspora,* may provide a greater aromatic complexity that is attributable to the production of secondary metabolites such as glycerol, 2-phenylethyl acetate and isoamyl acetate (Viana *et al.,* 2011; Fleet 2008; García *et al.,* 2002; Bely *et al.,* 2008). To date, a few non-*Saccharomyces* ADWYs, such as the *Torulaspora delbrueckii* 291 and *Metschnikowia pulcherrima* L1781 strains (Lallemand), have achieved sufficient quality to ensure good proliferation under wine-making conditions (Azzolini *et al.,* 2015; García *et al.,* 2010). Winemakers have a special interest in *Schiz. pombe* because of its high fermentative power, capacity to reduce the gluconic acid content of must and ability to efficiently metabolize L-malic acid into ethanol and carbon dioxide; these characteristics differentiate it from the other *Saccharomyces* strains (Taillandier *et al.,* 1991; Peinado *et al.,* 2009; Benito *et al.,*

2012). Additionally, co-inoculation with *S. cerevisiae* has been reported to prevent the off flavors generated by the production of H_2S, acetaldehyde, acetoin and ethyl acetate (Yokotsuka *et al.,* 1993; Benito *et al.,* 2013). In this process, fresh, immobilized *Schiz. pombe* cells are used for the partial or total consumption of L-malic acid before being removed to prevent off-flavor production (Silva *et al.,* 2003). The availability of active dry yeast of *Schiz. pombe* strains has provided winemakers with a new biotechnological tool with the same effectiveness as commercial ADWYs.

In the present study, a protocol that transforms dry and rehydrated *Schiz. pombe* strains into active dry yeast was developed. Our results showed that considering the cellular physiological state before drying and initiating the rehydration process in a physiological solution improved the viability of *Schiz. pombe* strains isolated from grape juice by up to 70%. The consequences of drying a *Schiz. pombe* strain for fermentative performance was investigated. The results obtained showed that the treatment to obtain ADWY of *Schiz. pombe* improved cell viability without affecting fermentation efficiency and metabolic behavior.

Materials and methods

Yeast Strains and Growth Conditions

Table 6 summarizes the *Schizosaccharomyces pombe* strains used in this study. The yeast strains were grown in shaker flasks at 120 rpm in EMM (Edinburgh Minimal Medium) at 32°C, inoculated with an overnight liquid culture at an initial OD_{600} of 0.25, and measured by microscope cell counting.

Table 6. *Schiz. pombe* strains used in this study

Strain	Source	Origin
Sp1	[a]CECT137	Grape must
Sp2	CECT11197	Sulphated grape juice
Sp3	CECT12622	Grape juice
Sp4	CECT12773	Castelli collection, Italy
Sp5	CECT12821	Concentrate grape juice
Sp6	CECT12918	Concentrate grape juice
Sp7	[b]CBS10395	Grape must
Sp8	CBS10498	Beverage industry
Sp9	CBS10500	Cape wines

[a]Spanish Type Culture Collection (http://www.cect.org/)
[b]Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/)

Desiccation-Rehydration Process

Growth curves were determined, and 5 x 10^7 yeast cell suspensions, measured by microscope cell counting during the stationary phase, were desiccated in the presence of several trehalose concentrations (5, 10, 20 and 30%) by exposure to dry air at 28°C for ~ 20 h (Rodríguez-Porrata *et al.,* 2012a). In all cases, 5 x 10^7 cells were rehydrated into a 1-ml final volume of water. Various rehydration temperatures (25, 30, 37 and 40 °C) and times (5, 15, 30 and 45 min) were tested using pure water as a control. The effect of rehydration solutions on dry *Schiz. pombe* cells after rehydration was studied by adding each compound individually to the pure water-based condition.

Cell viability was determined for the following compounds: 1% proline; 1% glutamate; 1% ammonium; 0.25 and 1% raffinose; 0.25, 0.5 and 1% galactose; 0.25 and 0.5% glucose; 0.25 and 0.5% fructose; 5, 10, 20 and 30% trehalose; 5 mM calcium; 1, 5 and 10 mM Mg; 0.25, 0.5 and 1 mM $FeSO_4$; 0.5 mM $FeCl_3$; 1 mM $FeSO_4$; 5 $mg{\cdot}l^{-1}$ sulfur dioxide; 5 mM ascorbic acid; 10 mg l^{-1} thiamine; 1 mM hydrogen peroxide; 0.1 mM dimethyl sulfoxide (DMSO); 5 mM benzyl alcohol; 5 mM 2-phenyl-1-butanol; 5 mM 2-phenyl-1-propanol; and 7 mM 2-ethyl-phenol (Rodríguez-Porrata *et al.,* 2008; Palabiyik *et al.,* 2014; Boutry *et al.,* 1977; Janda *et al.,* 1993).

Determining Yeast Viability

After the rehydration process, the viable cell count was calculated by spreading cell dilutions using a Whitley Automatic Spiral Plater (AES Laboratoire, France) on YPD agar medium. The plates were incubated at 32°C for 48 h, and the CFUs (colony-forming units) were quantified using the ProtoCOL SR/HR counting system software version 1.27 supplied by Symbiosis (Cambridge, UK).

Tests for Intracellular ROS Accumulation

Dihydroethidium (DHE) staining was performed as described in (López-Martínez *et al.,* 2012). The samples were analyzed by fluorescence microscopy. To determine the frequencies of the morphological phenotypes revealed by the DHE staining, a minimum of 500 cells were evaluated from three independent experiments using a Leica fluorescence microscope (DM4000B, Germany). A digital camera (Leica DFC300FX) and Leica IM50 software were used for the image acquisition.

Determination of Biological Parameters

Growth in microplate wells was monitored at 600 nm every 10 min after 20 s of shaking for 24 h at 32°C in a POLARstar OMEGA instrument (BMG Labtech, Germany). Microplate wells filled with 190 µl of YPD medium were inoculated with 10 µl of rehydrated cell inoculum measured by flow cytometry cell counting to obtain an OD_{600} of 0.6, which is above the minimal detection limit previously established by calibration. Blanks were determined from five independent non-inoculated wells for each experimental 96-wells plate. Three independent cultures of each strain were evaluated (six times each). Growth data from plate counts were enumerated as $\log_{10}$ values. The biological parameters, duplication times (DT) and lag phase times (λ) were estimated by fitting the growth curves into the model using MicroFit software (Institute of Food Research, Norwich, UK) (Baranyi & Roberts, 1994).

Must Preparation, Fermentation and Sampling

Sucrose was added to Tempranillo grape juice to raise the sugar content to 200 g/L. Before fermentations, the juice with a pH of 3.25 was complemented with diammonium phosphate (250 $mg \cdot l^{-1}$), L-malic (8 $g \cdot l^{-1}$) and 125 $mg \cdot l^{-1}$ dimethyl dicarbonate (DMDC). The juice was mixed and kept at 8°C for 24 h to give time for the DMDC to inhibit wild yeast and lactic acid bacteria. The effectiveness of this treatment was verified by plate counting. Fermentations were performed in 550 ml fermentation flasks filled with 500 ml of must. The must was initially inoculated with *S. cerevisiae* EC-1118 strain $1 \cdot 10^7$ $cells \cdot ml^{-1}$ -determined by using a Neubauer chamber- to ensure a complete fermentation of the sugars. The sequential- and co-inoculated must were inoculated with 1 ml of rehydrated *Schiz. pombe* Sp2 strain (± $1 \cdot 10^8$ $cfu \cdot ml^{-1}$) as determined by plate counting. All fermentations were performed in triplicated and conducted at 25 °C without agitation. Samples were taken every day to test for L-malic acid, to determine sugar concentration and yeast population sizes determined by plate counting on media YPD and the selective media lysine obtained by replica plating. Volatile compounds were analysed when fermentations arrived to a containing less than 5 $g \cdot l^{-1}$ of residual sugar. Major volatile wine compounds analyses were carried on by Lab for Flavour Analysis and Oenology (University of Zaragoza, Spain).

Statistical Analysis

Results were statistically analyzed by one-way ANOVA and the Scheffé test using the SPSS 15.1 statistical software package. The statistical significance was set at $p<0.05$.

Results and Discussion

Cell viability during different growth phases

The desiccation tolerance capacity of nine *Schiz. pombe* strains was assessed during growth using a colony-counting assay as described by (Rodríguez-Porrata *et al.,* 2011) (Figure 24). After re-suspending the cells in pure water, the CFU·ml^{-1} mean value for survival was calculated after taking into account the cells' viability before drying. Then, the 9 strains were ranked in ascending order based on the viability rate and divided them into three arrays: Sp3, Sp5 and Sp6, <8% viability; Sp1, Sp4, and Sp7, 8-17% viability; and Sp2, Sp8 and Sp9, >17% viability (Table 6). The viability timeframe evaluated for all strains showed that the highest tolerance to dehydration occurred during the early stationary phase, between 18 h and 20 h. These results differ from previous observations for *S. cerevisiae*, which reached its highest desiccation tolerance capacity during the late stationary phase, before the decline phase (Dupont *et al.,* 2014).

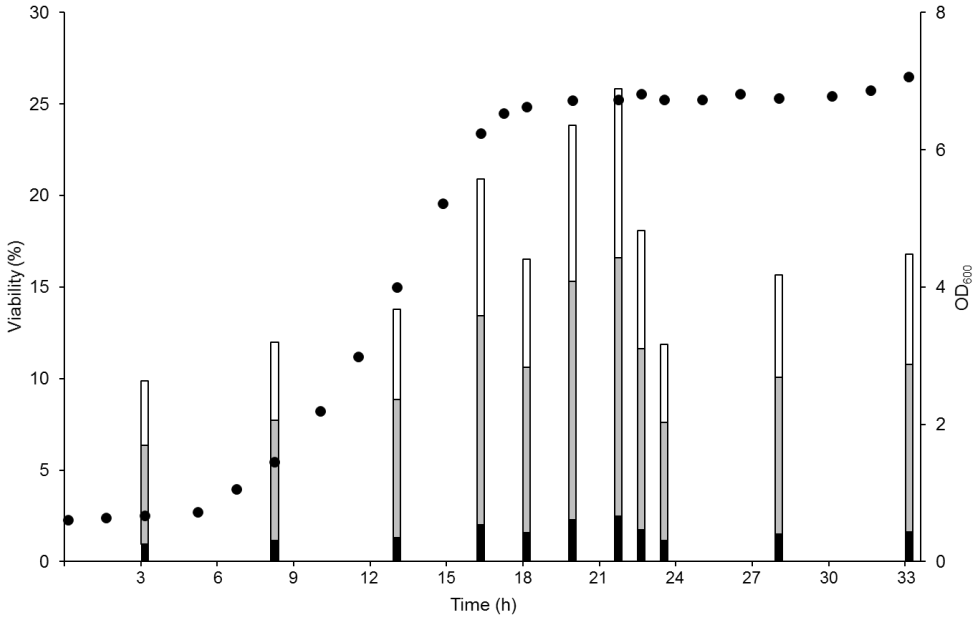


Figure 24. *Schiz. pombe* cell viability based on the cellular physiological state. Both the Sp2 (•) strain growth curve and the viabilities (%) obtained at different time points for the Sp3 (black bars), Sp4 (gray bars) and Sp2 (white bars) strains are provided as a representative example of the evaluated strains. Values shown are means of at least n=3 independent samples ± standard deviation (SD). The SD values were lower than 15%.

Evaluation of temperature and time during cell rehydration

The temperature and the time course have a direct effect on cell viability during rehydration. During this process, *S. cerevisiae* cells have shown a loss of up to 30% of soluble compounds due to the non-functionality of the cell membrane (Rapoport *et al.,* 1995; Rodríguez-Porrata *et al.,* 2008). Therefore, a more rapid functionality of the membrane may be beneficial for the viability of the rehydrated yeast cells. The temperature and time required to complete the rehydration process were correlated with desiccation tolerance. Cell viability was determined using a colony-counting assay for three *Schiz. pombe* strains (Sp2, Sp3 and Sp4) that demonstrated different viability rates. The viability at temperatures between 20°C and 60°C after 30 min of exposure to each temperature and at durations of exposure ranging from 5 min to 45 min at 37°C were assessed (data not shown). The incubation of cells at 55 °C and 60 °C resulted in a decrease of more than 80% for all strains, but no significant difference was observed between strains at temperatures between 20°C and 50°C. No statistically significant differences in the viability rate were observed for the three evaluated *Schiz*. *pombe* strains at any of the time points during incubation at 37°C. These results suggest that *Schiz. pombe* strains reach their best values for desiccation tolerance 10 min earlier than *S. cerevisiae* under similar conditions (Rodríguez-Porrata *et al.,* 2008). For the remainder of experiments, *Schiz. pombe* cells were rehydrated the by incubating them at 37°C for 5 min.

Evaluation of cell viability under several rehydration conditions

The first attempt to enhance dehydration stress tolerance was performed by re-suspending the cells for each *Schiz. pombe* strain prior to the drying process in 5, 10, 20 and 30% trehalose; deionized water was used as the reference condition. The survival rates of the Sp2, Sp3 and Sp4 strains in water and in 20% and 30% trehalose were very low, with none of the strains exhibiting greater than 30% viability (Figure 25). However, the Sp2 and Sp4 strains showed a significant increase in cell viability of approximately 30% and 20% when re-suspended in 5% and 10% trehalose, respectively, compared with the pure water control.

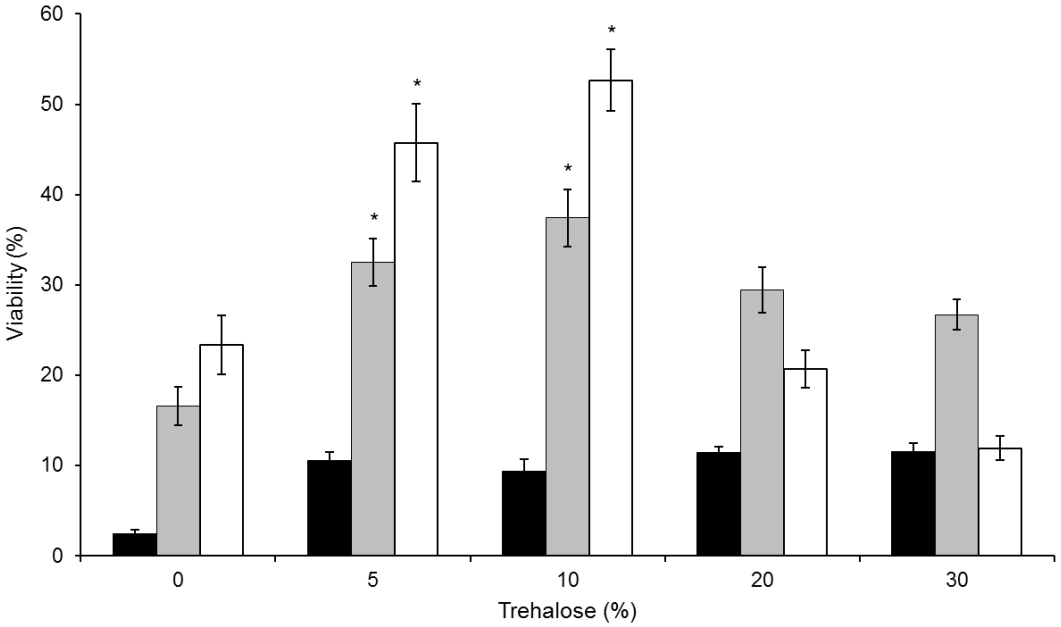


Figure 25. Effect of trehalose treatment on *Schiz. pombe* cell viability following air-drying and rehydration. The scale of viability (%) indicates the percentage of experimental values for the Sp3 (dark bars), Sp4 (gray bars) and Sp2 (white bars) strains. Values shown are means of at least n = 3 independent samples ± SD. *Indicates $p < 0.01$ compared to the 0% trehalose condition for each strain.

Trehalose, which is present during yeast drying, has been shown to act as a membrane protector that reduces the membrane phase-transition temperature during the rehydration process (Leslie *et al.,* 1994). Our data coincide with previous observations for two different *S. cerevisiae* strains (Rodríguez-Porrata *et al.,* 2011). For the remainder of the experiments, the *Schiz. pombe* cells were re-suspended in 10% trehalose before drying. To overcome the drop in yeast viability during this process, several types of rehydration media were tested. Five groups of media were used for dried Sp2 cells: carbon, nitrogen, metallic ions, oxidants/antioxidants, and membrane fluidity compounds (Figure 26). It was suggested that doubling the intracellular proline levels of desiccated *S. cerevisiae* might lead to a five-fold increase in the survival rate (Takagi *et al.,* 2000); however, this report does not agree with our findings for proline rehydration. Proline rehydration in the present study showed similar mean values of $CFU \cdot ml^{-1}$ compared to the control cells and the cells rehydrated in the presence of ammonium and monosodium glutamate. Our results are in agreement with previous studies in which supplementation during the rehydration process in the presence of nitrogen compounds did not improve *S. cerevisiae* survival rates (Rodríguez-Porrata *et al.,* 2008).

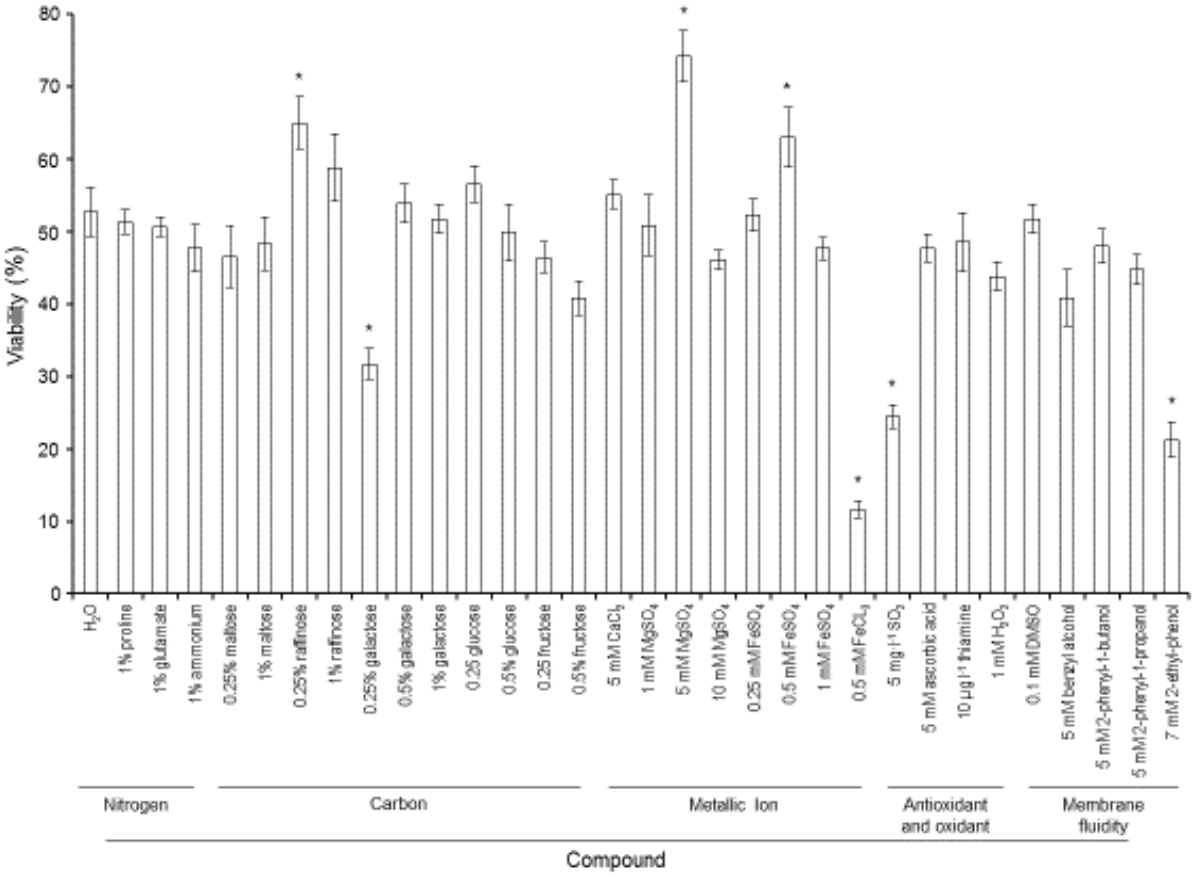


Figure 26. Effect of rehydration treatments on cell viability. Sp2 cells dried in the presence of 10% trehalose were incubated at 37°C for 5 min in pure water or in the presence of nitrogen, carbon, metallic ions, oxidants/antioxidants and membrane fluidity compounds. The values represent the means of three independent experiments ± SD. *Indicates $p < 0.05$ compared to the reference condition (H_2O).

Other studies that have investigated whether the response to rehydration involves regulation at the level of transcription and/or translation reported changes in sugar utilization (Novo *et al.,* 2003). The viability capacity of rehydrated Sp2 cells has been assessed in the presence of carbon sources that are metabolized more quickly (glucose and fructose) and more slowly (raffinose and maltose) than trehalose (Moreno *et al.,* 1991). Raffinose is a trisaccharide composed of glucose, fructose and galactose, and increasing its concentration by 0.25% resulted in an increased cell viability of 15% (Figure 26). Considering that increasing the galactose, glucose and fructose concentrations did not provide any beneficial effects, this finding may suggest that raffinose plays a role as a carbon store rather than as a faster energy supplier. The availability of certain metal ions in the fermentation media has been reported to be a key factor in the performance of *Schiz. pombe* (Walker, 2004). To assess any impact on yeast viability, the availability of several ions during the rehydration process were

evaluated (Figure 26). The ferric (Fe^{+3}) ion had a harmful effect on yeast viability; however, there was a beneficial increase in cell viability when the medium was complemented with 5 mM magnesium and 0.5 mM ferrous (Fe^{+2}) ion (74% and 65%, respectively, compared to the pure water control). Rehydrating conditions co-complemented with 5 and 10 mM calcium, 1 and 10 mM magnesium, or 0.25 and 1 mM ferrous ion exhibited profiles that were broadly similar to complementation with 1 and 10 mM calcium (data not shown). These results suggest that the ferric ion suppresses yeast viability via an antagonistic mechanism (Anderson *et al.,* 1994). In Walker (2004) was revealed that magnesium was essential for re-establishing cell cycle progression in *Schiz. pombe* cells.

Additionally, magnesium and iron are involved in numerous essential functions in yeast physiology, such as respirofermentive metabolism and the response to environmental stress (Birch & Walker, 2000; Hu *et al.,* 2003). Industrial biomass production occurs in a high concentration of molasses and aeration, which induces *Schiz. pombe* to initiate an adaptive response to osmotic and oxidative stress (Gasch, 2007). However, the drying process makes the membranes porous, thus exposing the cell components to the harmful effect of environmental oxygen (Beker & Rapoport, 1987). To address this issue, several rehydration solutions complemented with oxidant or antioxidant agents have been designed to evaluate the effects on cell viability (Palabiyik *et al.,* 2014). Ascorbic acid, thiamine, and hydrogen peroxide showed no significant effects on cell viability; however, sulfur dioxide had a detrimental effect, reducing cell viability to 25%. A relationship between higher stress sensitivity of cells in the exponential phase compared to cells in the stationary phase was previously reported in (Rodríguez-Porrata *et al.,* 2008). This finding may explain the high tolerance of non-growing cells to hydrogen peroxide but not the harmful effect of sulfur dioxide. Additionally, a rehydrating mix containing 1 mM hydrogen peroxide and 0.5% glucose increased cell death to 70% (data not shown). This high sensitivity of *Schiz. pombe* cells to hydrogen peroxide in the presence of fermentable carbon sources is in agreement with a previous report (Janda *et al.,* 1993). In Yang (1975) was reported that in fermentation conditions *Schiz. pombe* strains showed a three-fold increase in sulfur dioxide survival rate compared to *S. cerevisiae*. Our results differ from previous observations in *S. cerevisiae*, which did not show any change in its capacity for

desiccation tolerance under sulfur dioxide rehydration conditions (Rodríguez-Porrata *et al.,* 2008). The initial effects on the cell during the rehydration process are changes in membrane fluidity. Faster membrane activity allows the cells to proliferate after adapting to new conditions. Taking these observations into account, we explored whether changes in membrane fluidity had an effect on the viability of rehydrated *Schiz. pombe* cells (Figure 26). Dimethyl sulfoxide and benzyl alcohol are routinely used to rigidify and fluidize membranes, respectively (Sangwan *et al.,* 2002; Panadero *et al.,* 2006). Therefore, we evaluated membrane-fluidizer agents with hydrophobic properties, including 2-ethyl-phenol, 2-phenyl-1-propanol, and 2-phenyl-1-butanol. Our results showed that membrane fluidity agents did not enhance *Schiz. pombe* cell viability during the rehydration process. Moreover, in some cases complementation with agents such as in 2-ethyl-phenol could be detrimental; this finding is in agreement with previous results in *S. cerevisiae* (Rodríguez-Porrata *et al.,* 2008). Our results indicated that 0.25% raffinose, 5 mM magnesium and 0.5 mM ferrous ion showed a similar significant increase in cell viability after stress imposition.

Finally, the effect of these agents during the dehydration and rehydration processes as simple and combined preparations for the *Schiz. pombe* strains that showed different stress tolerances were evaluated. The viability of Sp2, Sp3 and Sp4 cells increased by more than 10% when dried in the presence of 10% trehalose and rehydrated in the presence of 5 mM $MgSO_4$. However, there was no significant improvement in the viability values obtained for the rehydrated preparations in 0.25% raffinose, 0.5 mM $FeSO_4$, 5 mM $MgSO_4$, 0.5% raffinose + 0.5 mM $FeSO_4$, 0.5% raffinose + 5 mM $MgSO_4$, 5 mM $MgSO_4$ + 0.5 mM $FeSO_4$ or 0.5% raffinose + 0.5 mM $FeSO_4$ + 5 mM $MgSO_4$ (Figure 27). Moreover, co-complementation of the Sp2, Sp3 and Sp4 cells with the above-mentioned preparations before the dehydration process was somewhat detrimental to cell viability in comparison with the application of the same solutions during the rehydration process (data not shown). Our results indicate that *Schiz. pombe* cells dried in the presence of 10% trehalose and rehydrated in 5 mM magnesium exhibited enhanced viability; thus, these results might directly influence early fermentation of an over-active starter yeast.

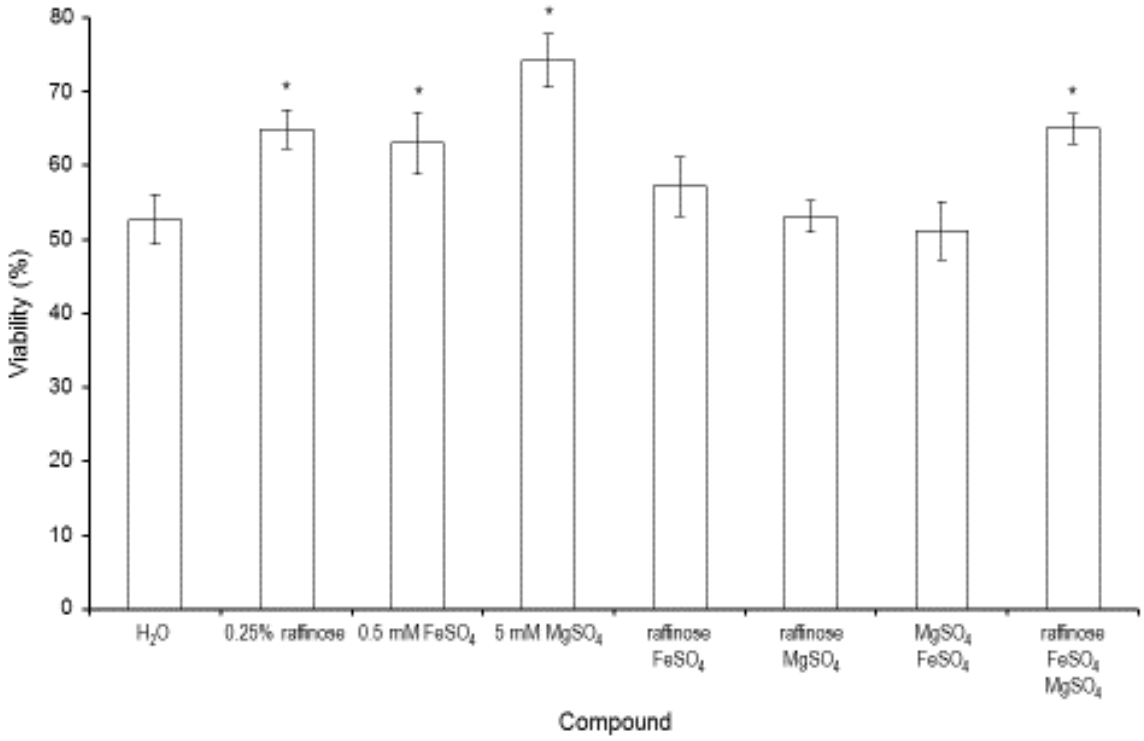


Figure 27. Effect of rehydration treatments on cell viability. Representative example of Sp2 cells dried in the presence of 10% trehalose and incubated at 37°C for 5 min in pure water or in the presence of 0.25% raffinose, 0.5 mM $FeSO_4$, 5 mM $MgSO_4$, 0.25% raffinose + 0.5 mM $FeSO_4$, 0.25% raffinose + 5 mM $MgSO_4$, 5 mM $MgSO_4$ + 0.5 mM $FeSO_4$ or 0.25% raffinose + 0.5 mM $FeSO_4$ + 5 mM $MgSO_4$. The values are the means of at least three independent experiments ± SD.*Indicates $p < 0.05$ compared to the reference condition (H_2O).

$MgSO_4$ prevents cellular ROS accumulation

Imposing dehydration stress by reducing intracellular ROS accumulation has been reported to enhance viability (López-Martínez *et al.,* 2012; Rodríguez-Porrata *et al.,* 2012b). Therefore, we investigated whether the higher viability rate of the cells rehydrated in the presence of $MgSO_4$ might be due to differences in ROS accumulation (Szeto *et al.,* 2007). After 10 min of rehydration in H_2O, 5 mM ascorbic acid or 5 mM $MgSO_4$, Sp2, Sp3 and Sp4 cells were inoculated into YPD medium. ROS-accumulating cells were evaluated over time using the dihydroethidium (DHE) assay (Figure 28A). After 1 h of incubation, the rehydrated cells in H_2O exhibited approximately 40% DHE-positive fluorescence; after 2 h of incubation, 10% fewer cells were detected under intense intracellular DHE staining. A similar significant reduction in ROS accumulating-cells was observed for cells rehydrated in the presence of ascorbic acid, although this effect was observed 1 h earlier than under H_2O conditions. Unexpectedly, *Schiz. pombe*

cells that were rehydrated in the presence of $MgSO_4$ did not show any changes in fluorescence between 5 min and 2 h, with similar percentages of DHE-positive cells compared to those incubated for 1 h in ascorbic acid and 2 h in H_2O. Considering the lower intracellular ROS levels of the cells rehydrated in the presence of $MgSO_4$, our results suggest that $MgSO_4$ allows the cells to prevent the accumulation of ROS; this process seems to occur during rehydration and very early after incubation in the YEPD medium. Therefore, the faster scavenging of ROS by cells rehydrated in $MgSO_4$ compared to ascorbic acid might explain the 18% differences observed in cell viability (Figure 28A).

Evaluation of cell vitality under several rehydration conditions

Next, the relatively improved rehydration conditions were correlated with a shorter lag phase (λ) once the cells were inoculated into complete medium compared to the controls. The cells rehydrated in $MgSO_4$ and in ascorbic acid + $MgSO_4$ showed a λ that was 208 min and 86 min longer than the controls, respectively, whereas the lag time was 220 min shorter in cells rehydrated in ascorbic acid (Figure 28B). The ascorbic acid conditions showed a 1.53-fold increase in doubling time DT compared to the controls, whereas the $MgSO_4$ condition showed 1.51-fold decrease in DT compared to the H_2O controls. However, rehydration in the presence of ascorbic acid + $MgSO_4$ did not result in any significant differences in DT. Cells rehydrated in raffinose and $FeSO_4$ did not exhibit any significant growth differences compared to the controls (data not shown). These results, together with the cell-accumulating ROS results, might confirm that there is no correlation between lower ROS values during stress imposition and rehydration conditions with a shorter λ phase (Figure 28A-B). Under magnesium conditions, there was an increase in the λ phase even though the accumulation of intracellular ROS stopped more quickly after stress induction. Similar variations in the λ phase were previously reported in (López-Martínez *et al.,* 2013), in which the reduction in the accumulation of intracellular ROS during dehydration stress was mediated by an over-expression of the encoding gene for *S. cerevisiae* hydrophilin.

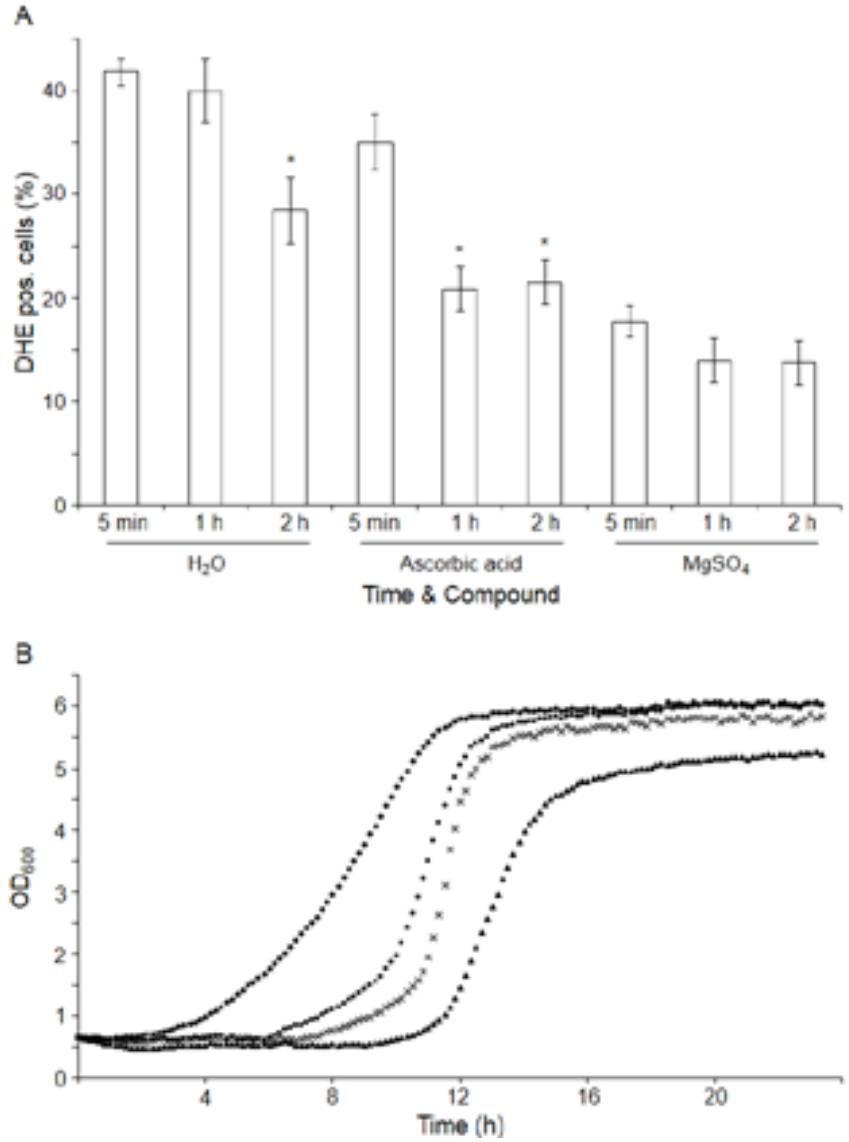


Figure 28. A) Quantification of the accumulation of ROS by yeast cells after cell rehydration using DHE staining. Values are the means of *n* = 4 determinations ± the SD. *Significant differences ($p \leq 0.05$) with respect to the 5 min step of each compound. B) Growth curves after rehydration in the presence of ascorbic acid (♦), H_2O (●), ascorbic acid + $MgSO_4$ (**x**) and $MgSO_4$ (▲). The curves are a representative example of growth experiments performed with yeast cells obtained from three independent compound-rehydration processes.

Fermentations

The three treatments began soon after inoculation. The *S. cerevisiae* EC-1118 and the join and the sequential treatments (*Schiz. pombe* ADY-Sp2 and EC-1118) the wines reached values lower than 2 $g \cdot l^{-1}$ sugar in 6 days (Figure 29A). After 9 days fermentation, both double- and single-fermentations finished with an ethanol content of 12.7 ± 0.2% and 11.9 ± 0.3% $v \cdot v^{-1}$ ethanol, respectively. On day 6, prior to the addition of the rehydrated Sp2 cells to the sequential fermentations, the ethanol

content was around 12%, v/v (data not shown). In the combined treatments, nearly 90% of the malic acid was metabolized 6 days after *Schz. pombe* inoculation (Figure 29B). Nevertheless, after day 9 in the EC-1118 single treatment only 12.5% of the total malic acid was metabolized. At the end of fermentation in the join and sequential treatments Sp2 cell viability was 2% and 10% respectively, while EC-1118 showed a viability of around 95% (data not shown). These data suggest that *Schiz. pombe* ADY is very efficient in malic acid utilization in sequential or join treatment, as previously observed using fresh inoculums (Benito *et al.,* 2013).

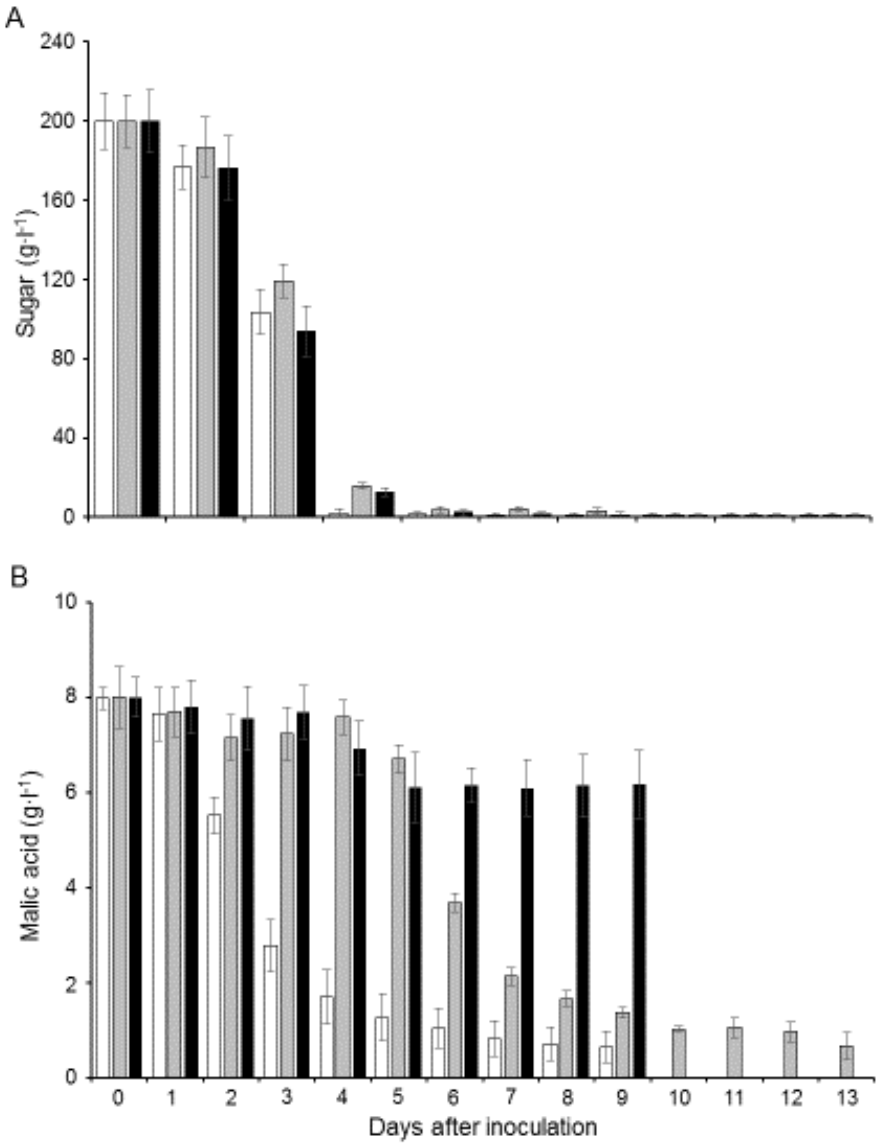


Figure 29. Consumption of sugars (A) and malic acid (B) during alcoholic fermentation in response to three yeast treatments: *S. cerevisiae* EC-1118 and *Schiz. pombe* Sp2 (white bars), EC-1118 + Sp2 at day 5 (grey bars) and EC-1118 (black bars). The values are the means of three independent experiments ± SD.

Schiz. pombe ADY metabolic behavior was not negatively affected

The metabolic behavior of Sp2 ADY during fermentation was evaluated by comparing the amounts of the principal volatile compounds determined in the experimental wines after completion of alcoholic fermentation (Table 7). The three treatments did not exhibit significant differences of total volatile compounds. In fact, only ten of the 24 evaluated compounds showed some differences among treatments. Furthermore, the join treatment (EC-1118 + Sp2) shows the higher values for most compounds such as ethyl acetate, isoamyl acetate, ethyl octanoate, isobutnol, butyric acid and isovaleric acid, suggesting a major fruited or floral profile.

Table 7. Concentration ($mg \cdot L^{-1}$) of volatile compounds produced at the end of all fermentation processes

Volatile compounds	OTV	Fermentation treatment ($mg \cdot L^{-1}$)		
		Join	Sequential	EC-1118
Acetaldehyde	0.5	8.98±1.19^{b}	7.90±1.30^{b}	11.37±5.36^{a}
Acetoin	150	1.80±0.13^{b}	1.83±0.32^{b}	3.20±0.18^{a}
Ethyl acetate	12.3	30.14±1.02^{c}	16.46±1.71^{b}	21.28±2.91^{a}
Isoamyl acetate	0.03	1.42±0.04^{c}	0.38±0.09^{b}	1.04±0.03^{a}
Ethyl butyrate	0.125	0.16 ± 0.01^{a}	0.13 ± 0.01^{a}	0.13±0.01^{a}
Ethyl hexanoate	0.062	0.16 ± 0.01^{a}	0.10 ± 0.01^{b}	0.16 ± 0.02^{a}
Ethyl octanoate	0.58	0.03 ± 0.01^{a}	0.10 ± 0.05^{b}	0.02 ± 0.01^{a}
Ethyl decanoate	0.2	0.04 ± 0.01^{b}	0.06 ± 0.02^{b}	0.13 ± 0.02^{a}
Isobutanol	40	54.83 ± 4.84^{a}	24.42 ± 2.02^{b}	45.79 ± 7.80^{a}
1-butanol	150	0.66 ± 0.06^{b}	0.62 ± 0.11^{b}	0.48 ± 0.14^{a}
Isoamyl alcohol	30	131.57±7.58^{a}	121.56 ± 3.72^{a}	142.77±38.81^{a}
1-hexanol	8	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}
Methionol	1	4.66 ± 0.18^{a}	5.15 ± 0.60^{a}	5.42 ± 1.08^{a}
2-phenylethanol	14	19.70 ± 0.15^{a}	27.40 ± 3.85^{b}	17.99 ± 6.56^{a}
Ethyl lactate	154	1.47 ± 0.01^{a}	0.64 ± 0.03^{b}	1.68 ± 0.16^{a}
Diethyl succinate	200	0.04 ± 0.01^{a}	0.07 ± 0.01^{b}	0.05 ± 0.02^{a}
γ-Butyrolactone	35	1.43 ± 0.07^{a}	1.24 ± 0.04^{a}	1.47 ± 0.29^{a}
Acetic acid	300	80.97 ± 1.58^{a}	98.83 ± 19.42^{b}	72.60 ± 18.94^{a}
Butyric acid	0.173	0.77 ± 0.02^{b}	0.69 ± 0.05^{b}	0.49 ± 0.03^{a}
Isobutyric acid	2.3	1.47 ± 0.05^{a}	1.69 ± 0.25^{b}	1.23 ± 0.15^{a}
Isovaleric acid	0.033	0.89 ± 0.01^{b}	0.67 ± 0.01^{a}	0.67 ± 0.17^{a}
Hexanoic acid	0.42	2.58 ± 0.03^{a}	2.26 ± 0.15^{a}	2.31 ± 0.11^{a}
Octanoic acid	0.5	7.39 ± 0.44^{a}	6.10 ± 0.49^{a}	7.60 ± 0.78^{a}
Decanoic acid	1	0.39 ± 0.11^{b}	0.31 ± 0.05^{b}	0.61 ± 0.02^{a}
Total		351.6 ± 31.8^{a}	318.6 ± 34.3^{a}	338.5 ± 48.7^{a}

The EC-1118 single fermentation produced 20% higher acetaldehyde concentration (11.37 $mg \cdot L^{-1}$) than the combined treatments, while the sequential treatment showed the highest value for the acetic acid (99 $mg \cdot L^{-1}$). However, it must be highlighted that all of the tested compounds were present at acceptable levels. Our results agree with previous data of grape must treated with *Schiz. pombe* presenting a more complex volatile profile when compared to the one obtained with single-culture fermentation involving *S. cerevisiae* (Benito *et al.,* 2012).

Conclusions

The desiccation tolerance of wine yeasts has enabled the wine industry to work with products with more stable microbiological characteristics. Nevertheless, such products exclude yeast strains that cannot cope with the cellular stress induced during dehydration and rehydration; this topic is of great interest for the beverage industry (Rodríguez-Porrata *et al.,* 2011). Non-*Saccharomyces* yeasts, especially those with interesting oenological properties, do not often survive under desiccation stress. This characteristic is a serious handicap for winemakers who are trained to use active dry yeasts. In the present study, different *Schiz. pombe* strains isolated from the beverage or concentration juice industries from different regions worldwide were characterized. Isolation of the *Schiz. pombe* strain occurred during spontaneous fermentations without prior selection based on oenological characteristics. Previous studies have aimed to improve the tolerance of *S. cerevisiae* to dehydration via compound supplementation before and after stress induction (Rodríguez-Porrata *et al.,* 2008; Jenkins *et al.,* 2011; Díaz-Hellín *et al.,* 2014; Vaudano *et al.,* 2014). This approach led us to characterize the cell viability and vitality of nine *Schiz. pombe* ADWYs under a set of physiological conditions. To the best of our knowledge, this is the first systematic study to establish a protocol for developing *Schiz. pombe* as an ADWY. Different techniques to evaluate the influence of the compounds during the dehydration-rehydration process in the studied strains were used. The 'fitness' of an ADWY is related to its ability to maintain cell viability and vitality during the yeast manufacture process, including desiccation and storage (Pretorius, 2000). In our study, yeast viability was assessed both directly - by determining the loss of cells after stress imposition and indirectly by assessing the impact of different compounds on ROS accumulation by the

cells, as previously reported in (Rodríguez-Porrata *et al.,* 2012b). The influence on cell vitality was assessed by determining the biological parameters of optimal growth conditions of the rehydrated cells. The various methods used enabled us to evaluate the effect of several rehydration conditions by uncoupling cell vitality and viability. In conclusion, the presence of both trehalose during the drying process and magnesium during the rehydration process has a synergistic effect on the enhancement of cell viability. Nevertheless, the longer lag phase promoted by the magnesium, which moves into the cell during the rehydration process, promotes sluggish cell activity. Furthermore, no defects were found between wines obtained by single *S. cerevisiae* and *Schiz. pombe* combined treatments as regard to the fermentative performance and volatile compounds influencing wine aroma. On the other hand, the malic acid was nearly completely metabolized by *Schiz. pombe* in combined treatments. These findings indicate that the applied protocol to obtain a *Schiz. pombe* as ADW yeast does not affect its oenological behavior.

Acknowledgements

This work was supported by grant AGL2012-40018 from the Spanish *Ministerio de Ciencia e Innovación*. The authors thank Rovira i Virgili University for the doctoral fellowships FI-DGR 2012, AGAUR to GL and 2014PMF-PIPF-47 to GR.

References

Anderson, G. J., Dancis, A., Roman, D. G., & Klausner, R. D. (1994). Ferric iron reduction and iron uptake in eucaryotes: studies with the yeasts. *Advances in Experimental Medicine and Biology, 356*, 81-89.

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

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *World Journal of Microbiology and Biotechnology, 23*, 277-294.

Beker, M. J., & Rapoport, A. I. (1987). Conservation of yeasts by dehydration. In: Adv. Biochem. Eng. Biot. vol 35. Fiechter, A., (ed.). pp. 128-171.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii* - *Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Mcrobiology, 122*, 312-320.

Benito, S., Palomero, F., Morata, A., Calderón, F., Palmero, D., & Suárez-Lepe, J. A. (2013). Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. *European Food Research and Technoligy*, *236*, 29-36.

Benito, S., Palomero, F., Morata, A., Calderón, F., & Suárez-Lepe, J. A. (2012). New applications for *Schizosaccharomyces pombe* in the alcoholic fermentation of red wines. *International Journal of Food Science & Technology, 47*, 2101-2108.

Birch, R. M., & Walker, G. M. (2000). Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology, 26*, 678-687.

Boutry, M., Foury, F., & Goffeau, A. (1977). Energy-dependent updake of calcium by the yeast *Saccharomyces pombe*. *Biochimica et Biophisica Acta, 464*, 602-612.

Díaz-Hellín, P., Gómez-Alonso, S., Borrull, A., Rozès, N., Cordero-Otero, R., & Úbeda, J. (2014). Membrane lipid variability in *Saccharomyces cerevisiae* wine strains rehydrated in the presence of metabolic activators. *Journal of Agricultural and Food Chemistry, 62*, 8679-8685.

Dupont, S., Rapoport, A., Gervais, P., & Beney, L. (2014). Survival kit of *Saccharomyces cerevisiae* for anhydrobiosis. *Applied Microbiology and Biotechnology, 98*, 8821-8834.

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

Garcia, A., Carcel, C., Dalau, L., Samson, A., Aguera, E., Agosin, E., & Gunata Z. (2002). Influence of a mixed culture with *Debaryomyces vanriji* and*Saccharomyces cerevisiae* on the volatiles in a Muscat wine*. Journal of Food Science, 67*, 1138-1143.

García, V., Vasquez, H., Fonseca, F., Manzanares, P., Viana, F., Martínez, C., & Ganga, M. A. (2010). Effect of the use of mixed wine yeasts cultures in the production of Chardonnay wines. *Revista Argentina de Microbiología, 42*, 226-229.

Gasch, A. P. (2007). Comparative genomics of the environmental stress response in ascomycete fungi. *Yeast, 24*, 961-976.

Hu, C. K., Bai, F. W., & An, L. J. (2003). Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} via reduction in plasma membrane permeability. *Biotechnology Letters, 25*, 1191-1194.

Janda, S., Gille, G., Sigler, K., & Höfer, M. (1993). Effect of hydrogen peroxide on sugar transport in *Schizosaccharomyces pombe*. Absence of membrane lipid peroxidation. *Folia Microbiologica, 38*, 135-140.

Jenkins, D. M., Powell, C. D., & Fischborn, T. (2011). Rehydration of active dry brewing yeast and its effect on cell viability. *Jounal of the Institute of Brewing, 117*, 377-382.

Kraus, J. K., Scopp, R., & Chen, S. L. (1981). Effect of rehydration on dry wine yeast activity. *American Journal of Enology and Viticulture, 32*, 132-134.

Leslie, S. B., Teter, S. A., Crowe, L. M., & Crowe, J. H. (1994). Trehalose lowers membrane phase transitions in dry yeast cells. *Biochimica et Biophysica Acta, 1192*, 7-13.

López-Martínez, G., Pietrafesa, R., Romano, P., Cordero-Otero, R., & Capece, A. (2013). Genetic improvement of *Saccharomyces cerevisiae* wine strains for enhancing cell viability after desiccation stress. *Yeast, 30*, 319-30.

López-Martínez, G., Rodríguez-Porrata, B., Margalef-Catalá, M., & Cordero-Otero, R. (2012). The STF2p hydrophilin from *Saccharomyces cerevisiae* is required for dehydration stress tolerance. *PLoS One*, *7*, e33324.

Moreno, S., Klar, A., & Nurse, P. (1991). Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods in Enzymology, 194*, 795-823.

Novo, M. T., Beltran, G., Torija, M. J., Poblet, M., Rozes, N., Guillamon, J. M., & Mas, A. (2003). Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. *International Journal of Food Microbiology, 86*, 153-161.

Palabiyik, B., Jafari Ghods, F., & Onay Ucar, E. (2014). A potential protective role for thiamine in glucose-driven oxidative stress. *Genetics and Molecular Research, 13*, 5582-5593.

Panadero, J., Palloti, C., Rodríguez-Vargas, S., Randez-Gil, F., & Prieto, J. A. (2006). A downshift in temperature activates the HOG pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 281*, 4638-4645.

Peinado, R. A., Maestre, O., Mauricio, J. C., & Moreno, J. (2009). Use of a *Schizosaccharomyces pombe* mutant to reduce the content in gluconic acid of must obtained from rotten grapes. *Journal of Agricultural and Food Chemistry, 57*, 2368-2377.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast, 16*, 675-729.

Rapoport, A., Rusakova, A., Khroustalyova, G., & Walker, G. (2014). Thermotolerance in *Saccharomyces cerevisiae* is linked to resistance to anhydrobiosis. *Process Bichemistry, 49*, 1889-1892.

Rapoport, A. I., Khrustaleva, G. M., Chamanis, G. Y., & Beker, M. E. (1995). Yeast anhydrobiosis: permeability of the plasma membrane. *Microbiology, 64*, 229-232.

Rodríguez-Porrata, B., Carmona-Butierrez, D., Reisenbichler, A., Bauer, M., Frank, M., & Cordero-Otero, R. (2012a). Yeast cell death during drying and rehydration process. In: Flow Cytometry, Vol 1. Indrid Schmid, INTECH (ed) UCLA, pp. 119-132.

Rodríguez-Porrata, B., Carmona-Gutierrez, D., Reisenbichler, A., Bauer, M., López, G., Escoté, X., Mas, A., Madeo, F., & Cordero-Otero, R. (2012b). Sip18 hydrophilin prevents yeast cell death during desiccation stress. *Journal of Applied Microbiology, 112*, 512-525.

Rodríguez-Porrata, B., Lopez, G., Redón, M., Sancho, M., Rozès, N., Mas, A., & Cordero-Otero, R. (2011). Effect of lipids on the desiccation tolerance of yeasts. *World Journal of Microbiology and Biotechnology, 27*, 75-83.

Rodríguez-Porrata, B., Novo, M., Guillamón, J., Rozès N., Mas, A., & Cordero-Otero R. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *Intrernational Journal of Food Microbiology, 126*, 116-122.

Sangwan, V., Örvar, B. L., Beyerly, J., Hirt, H., & Dhindsa, R. S. (2002). Oosite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *The Plant Journal, 31*, 629-638.

Silva, S., Ramon Portugal, F., Andrade, P., Texera, M., & Strehaino P. (2003). Malic acid consumption by dry immobilized cells of *Schizosaccharomyces pombe*. *American Journal of Enology and Viticulture, 54*, 50-55.

Soubeyrand, V., Julien, A., & Sablayrolles, J. M. (2006). Rehydration protocols for active dry wine yeasts and the search for early indicators of yeast activity. *American Journal of Enology and Viticulture, 57*, 474-480.

Szeto, S. S., Reinke, S. N., Sykes, B. D., & Lemire, B. D. (2007). Ubiquinone-binding site mutations in the *Saccharomyces cerevisiae* succinate dehydrogenase generate superoxide and lead to the accumulation of succinate. *Journal of Biological Chemistry, 282*, 27518-27526.

Taillandier, P., Riba, J. P., & Strehaiano, P. (1991). Malic degradation by *Schizosaccharomyces* yeasts included in alginate beads. *Bioprocess Engineering, 7*, 141-144.

Takagi, H., Sakai, K., Morida, K., & Nakamori, S. (2000). Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters, 184*, 103-108.

Vaudano, E., Noti, O., Costantini, A., & Garcia-Moruno, E. (2014). Effect of additives on the rehydration of *Saccharomyces cerevisiae* wine strains in active dry form: influence on viability and performance in the early fermentation phase. *Journal of the Institute of Brewing, 120*, 71-76.

Viana, F., Belloch, C., Vallés, S., & Manzanares, P. (2011). Monitoring a mixed starter of *Hanseniaspora vineae - Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. *International Journal of Food Microbiology, 151*, 235-240.

Walker, G. M. (2004). Metals in yeast fermentation processes. *Advances in Applied Microbiology, 54*, 197-229.

Yang, H. Y. (1975). Effect of sulfur dioxide on the activity of *Schizosaccharomyces pombe*. *American Journal of Enology and Viticulture, 26*, 1-4.

Yokotsuka, K., Otaki, A., & Naitoh, H. (1993). Controlled simultaneous deacidification and alcohol fermentation of a high-acid grape must using two immobilized yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *American Journal of Enology and Viticulture*, *44*, 371–377.

CHAPTER IV

Magnesium enhances dehydration tolerance in *Schizosaccharomyces pombe* by promoting intracellular 5'-methylthioadenosine accumulation

Gemma Roca-Domènech[1], Montse Poblet[1], Nicolas Rozès[1], Ricardo Cordero-Otero[1*]

[1] Department of Biochemistry and Biotechnology, Faculty of Oenology. University Rovira i Virgili, 43007- Tarragona, Catalonia, Spain

* Corresponding Author

Manuscript submitted to *Yeast*

Abstract

In the present study, we analysed metabolite features during the dehydration-rehydration process for two *Schizosaccharomyces pombe* strains with different viability rate, in order to determine whether metabolite contents were affected by the presence of magnesium during cell rehydration, which might play a role in cell viability. The qualitative changes of the intracellular metabolites of both strains were determined by comparing the metabolic profiles of cells before dehydration, after rehydration in water and magnesium solution, and after 2 h inoculation of both kinds of rehydrated cells in complete medium. Our results suggest that changes of metabolites from the methionine salvage pathway, in particular 5'methylthioadenosine, trigger by the increasing intracellular magnesium content may participate in the dehydration tolerance of Sp97 cells.

Keywords: *Schizosaccharomyces pombe*, Active Dry Yeast, magnesium, metabolomics, MTA, aging

Introduction

Increasing research interest in the role of metal ions in living cells has revealed intriguing and novel cellular properties, which are of biotechnological significance for the yeast-based industry. Specifically, the magnesium ion (Mg^{2+}) is an important and the most abundant divalent metal ion in yeast cells. Mg^{2+} constitutes around 0.3 % (w/w) of the dry weight of yeast (Walker, 2004). Most of the Mg^{2+} present in yeast cells is sequestered by intracellular polyphosphates, RNA, ATP and by the association to membranes to anchor soluble enzymes. Only a small fraction of Mg^{2+} is free within the cells and available for biochemical processes (Udeh *et al.,* 2013). Mg^{2+} homeostasis in yeast cells is regulated in the cytoplasm and intracellular organelles by a few specific membrane transport proteins. The ALR1p and ALR2p proteins belong to the group of metal ion transporters (MIT) and uptake Mg^{2+} from the media into the cytosol. In mitochondria, the inner membrane's MRS2p and MFM1p transporters are involved in maintaining mitochondrial Mg^{2+} concentrations, while MNR2p mediates vacuolar Mg^{2+} secretion to the cytosol and the Mg^{2+}/H^{+} exchanger, facilitating ion uptake (Liu *et al.,* 2002, Pisat *et al.,* 2009, Cyert & Philpott, 2013, Udeh *et al.,* 2013). In yeast cells, the ion Mg^{2+} activates over 300 enzymes in different metabolic reactions involved in processes such as the maintenance of cellular integrity, TCA cycle, β-oxidation, DNA and ATP synthesis, as well as the structural stabilization of nucleic acids, polysaccharides and lipids. The ion Mg^{2+} binds to ATP molecules at physiological pH, stabilizing phosphate groups used in ATP-dependent reactions, such as DNA polymerization (Barros de Souza *et al.,* 2016). The association to the phospholipids of membranes promotes membrane integrity by decreasing their fluidity, especially under stress conditions (Walker *et al.,* 1999). The Mg^{2+} ion also participates in yeast cell protection by activating detoxification processes for heavy metals (e.g. Al^{3+} or Cd^{2+}) and Reactive Oxygen Species (ROS) (Udeh *et al.,* 2013; Barros de Souza *et al.,* 2016). Moreover, growth of *Schizosaccharomyces pombe* has been resumed by adding Mg^{2+} to cells arrested with the metal chelator-ionophore A_{23187} (Walker, 2004). However, more studies are required to understand the role of Mg^{2+} in the molecular mechanism of cell division.

Several studies have investigated dehydration stress tolerance in wine yeast strains (Rodríguez-Porrata *et al.,* 2011, López-Martínez *et al.,* 2013) and, more specifically, the development of Active Dry Wine Yeast (ADWY), which must display a final moisture level below 8% (Dupont *et al.,* 2014). The use of ADWY ensures the strain identity and its genetic stability at room temperature and, consequently, transport and storage costs are reduced (Rodríguez-Porrata *et al.,* 2008). Previous studies have shown that Mg^{2+} enhances tolerance to the dehydration-rehydration process by maintaining the integrity of membranes in *Saccharomyces cerevisiae* and *Schiz. pombe* (Rodríguez-Porrata *et al*., 2008, Trofimova *et al.,* 2010, Roca-Domènech *et al.,* 2016).

In the present study, metabolomic and physiological features are analysed during the rehydration process of *Schiz. pombe* in the presence of magnesium, which enhances cell viability after stress imposition. In order to determine the putative metabolites involved in enhancing dehydration tolerance, a comparative metabolomic analysis of two *Schiz. pombe* strains has been performed, resulting in significantly different resistance to dehydration stress. The results obtained showed that the 5'methylthioadenosin (MTA) metabolite from the methionine salvage pathway participates in the enhancement of stress tolerance. However, the dehydration stress tolerance caused by the Mg^{2+} ion in *Schiz. pombe* is not a phenotype resulting from an individual metabolic pathway.

Materials and Methods

Yeast strains and growth conditions

The following *Schiz. pombe* strains from the Spanish Type Culture Collection (http://www.cect.org/) were used in this study: CECT11197 (Sp97), CECT12622 (Sp22). *Schiz. pombe* strains were growth in shaker flasks at 120 rpm for 40 h at 32°C in 50 mL of fresh YE medium (3.5% glucose and 0.5% yeast extract) with an initial OD_{600} of 0.25.

Desiccation-Rehydration process

Yeast cells from the stationary phase were desiccated by exposure to dry-air at 28°C for 24 h in the presence of trehalose 10%. Dried cells were rehydrated in 1 mL final volume of pure water, $MgSO_4$ 5 mM, methionine 40mM, spermidine 0.15 and 4 mM, spermine 0.15 mM, putrescine 0.15mM, 5'methylthioadenosine (MTA) 0.15 mM, SAM 0.15 mM, methylthiobutanoate 0.15 mM, β-alanine 0.15 mM, pantothenate 4 mM, phosphatidylcholine 50 mM and cholesteryl oleate 7.7 mM at 37ºC for 5 min. Some samples were also stored in YPD medium (2% glucose, 1% yeast extract, 2% peptone) without compound supplementation for 2 h after rehydration. These concentrations were based on previous studies to ensure yeast viability (Pösö *et al.,* 1975; Avila *et al.,* 2004; Eisenberg *et al.,* 2009; Rodríguez-Porrata *et al.,* 2011). The dry weight of cells was calculated after maintaining the cell-pellets at 60ºC for 2 days.

Determination of yeast viability

The viable cell count was calculated by spreading cell dilutions using a Whitley Automatic Spiral Plating Device (AES Laboratoire, France) on YPD medium agar (2% glucose, 1% yeast extract, 2% peptone, 2% agar). After incubation at 28°C for 48 h, the colony-forming units (CFUs) were quantified using the ProtoCOL SR/HR counting system software version 1.27, supplied by Symbiosis (Cambridge, UK).

Measurement of Intracellular magnesium by ICP-OES

After being washed with cold water, the cell pellets were stored at -20°C. The frozen cells were then re-suspended with 1 mL of 0.5% of nitric acid (HNO_3), transferred to a 15 mL glass screw-cap tube and incubated for 4 h at 120°C. Samples were stored at -20°C after adding pure water to a final volume of 10 mL. The magnesium was quantified using an inductively-coupled plasma optical emission spectrometer, ICP-OES (Spectro Arcos, Germany) and the Smart Analyzer Vision v. 4.02.0831 (Spectro, Germany) (Tariba, 2011). Calibration curves of magnesium and zinc were performed from 0 ppm to 80 ppm. Zinc was used as the internal control.

Determination of ATP and pyruvate concentrations

ATP content was assessed with the ATP Bioluminescence Assay Kit HSII (Roche Applied Science, Germany) and the cellular pyruvate concentration was determined using the Pyruvate Assay Kit (Biovision Research Products, USA). The quantification was carried out using a POLARstar Omega microplate reader equipped with two reagent injectors (BMG Labtech, USA).

Metabolite extraction

The extraction of intracellular metabolites was performed from $5 \cdot 10^8$ yeast cells. After washing with 1 mL of cold water, cells were quenched with 1 mL of cold methanol: water (1:1). In order to lyse the yeast cells, each sample was subjected three times to the French pressure cell press at 2.7 Kpsi. The cell debris obtained was appropriately diluted and images were captured using a DIC microscope. The pictures analysed showed a level of 90% of broken cells. The supernatants were obtained after centrifugation at 16,000 rpm for 10 min at 4°C, and stored at -80°C.

Untargeted global metabolomic analysis determined by UHPLC-ESI-qTOF

Metabolomic analysis of samples was carried out by the Centre for Omic Sciences (COS, Reus, Spain). The samples were analysed by UHPLC-(ESI)-Q-TOF (Agilent Technologies, Santa Clara, CA, USA) with a Zorbax SB-C8 guard column (30 x 2.1 mm, 1.8 µm) (Agilent Technologies, USA) and a Zorbax SB-Aq RR analytical column (50 x 2.1 mm, 1.8 µm) (Agilent Technologies, USA). The method used to analyse the samples consisted of a gradient elution using Milli-Q water (Solvent A) 0.2% (v/v) CH_3COOH and methanol (Solvent B) 0.2% (v/v) CH_3COOH as the mobile phase. The injection volume was 2 µL (4°C), at a temperature of 60°C and a column flow of 0.6 $mL \cdot min^{-1}$. Quality controls (QCs) were included by injecting sample controls every eight samples in order to monitor the adequacy of the system throughout all chromatographic analyses. Samples were analysed at random temperature. For detection in positive and negative mode (ESI^+ and ESI^-), the analyte was ionized using an electrospray source in line with the conditions described in Table 8.

Table 8. Analytical parameters of qTOF

Parameter	ESI^+	ESI^-
Gas Temperature	200ºC	200ºC
Drying Gas Flow	14 L/min	14 L/min
Sheath Gas Temperature	350ºC	350ºC
Sheath Gas Flow	11 L/min	11 L/min
Capillary Voltage	4000 V	3500 V
Nozzle Voltage	1000 V	1000 V
Fragmentor	140 V	140 V
Skimmer	65 V	65 V
OCT 1 RF Voltage	750 V	750 V
Scan Range	100-1200 m/z	100-1200 m/z
Ref. Mass	121.050873 922.009798	119.03632 980.016375

The instrument was calibrated before use and reference masses were used throughout the analysis. Data acquisition and processing was achieved using the Agilent Mass Hunter B.06.01 software. In order to carry out the statistical analysis, a pre-processing of the acquired data was required. This pre-processing consisted of deconvolving the obtained chromatograms to find the signals corresponding to the potential metabolites depending on their retention times and neutral mass, using the "Find by Molecular Feature" algorithm (Agilent MassHunter). Moreover, the Mass Profiler Professional v. 12.6 software (Agilent Technologies) was used to perform data alignment between the samples in order to find the same metabolites among the samples. Then, these metabolites were searched recursively in all samples using the "Find by Formula" algorithm (Agilent MassHunter), which takes into account the mass and retention time of the metabolites. Finally, the statistical analyses applied to compare the different sample conditions were the T-test and ANOVA with Bonferroni correction. The statistical significance was set at $p<0.01$. The Mass Profiler Professional/Gene Spring v.12.6 software (Agilent Technologies, USA) with the Pathway Architect extension was used for the metabolic pathway analysis of the identified metabolites. The *Schiz. pombe* metabolic pathways were identified using the Reactome database, which contained 363 metabolic pathways.

Targeted metabolomic analysis determined by UHPLC-(ESI^+)-QqQ/MS

The quantification of the metabolites (methionine, SAM, MTA spermine, pantothenate, choline, phosphocholine, L-α-glycerophosphocholine, cytidine-5'-diphosphocholine and betaine) was carried out by the Centre for Omic Sciences (COS, Reus, Spain). The samples were analysed by UHPLC-(ESI^+)-QqQ/MS (Agilent Technologies, Santa Clara, CA, USA) with a chromatographic column Kinetex 2.6 µm EVO C18 2.1x100 mm from Phenomenex. The method used to determine the metabolites consisted of a gradient elution using Milli-Q water 0.1% (v/v) CH_3COOH and 0.025% HFBA (solvent A) and acetonitrile (solvent B) as the mobile phase. The injection volume was 5 µL (4°C), temperature 25°C and the column flow 0.55 $mL \cdot min^{-1}$. QCs were included by injecting sample controls every seven samples in order to monitor the adequacy of the system throughout all chromatographic analyses. For the detection of metabolites in positive mode (ESI^+), the analyte was ionized using an electrospray source in line with the conditions described in Table 9. All the quantified metabolites showed regression coefficients ≥ 0.992, with an accuracy of 87%-113% and a precision of 1.19%-8.85%.

Table 9. Analytical Parameters of UHPLC-(ESI^+)-QqQ

Parameter	ESI^+
Gas Temperature	200ºC
Gas Flow	14 L/min
Sheath Gas Temperature	250ºC
Sheath Gas Flow	11 L/min
Nebulizer	20 psi
Capillary Voltage	3000 V
Nozzle Voltage	2000 V
Cell Acceleration Voltage	5 V

Lipid extraction from yeast cells

Prior to lipid extraction, a solution of 100 µL of cold methanol and 10 µL of EDTA 0.1 mM was added to the yeast cells with 1 g of glass beads (0.5 mm, BioSpec Products, Qiagen. USA) in an Eppendorf tube, and shaken for 5 min in a mini-bead-beater-8 (BioSpec Products, Qiagen, USA). The lipid extraction was carried out using a modified version of the method described by *Borrull et al.,* (2015). Lipid extraction was performed in four steps: the first two steps with 600 µL of chloroform/methanol (2:1, v/v, for 1 h), the third step with 600 µL of chloroform/methanol 1:1 (v/v, for 30 min)

and the fourth step with 600 µL of chloroform/methanol 1:2 (v/v, for 30 min). After each step, the supernatant was transferred into a 15 mL glass screw-cap tube and washed twice by adding KCl 0.88% (one-fourth of the total volume of the extract). After vortexing and cooling at 4°C for 10 min, the samples were centrifuged for 5 min at 1,000 rpm. The collected organic phase was evaporated under nitrogen. The residue was suspended with 100 µL of chloroform/methanol (2:1) and collected in a glass insert, introduced into a vial and stored at -80°C until TLC analysis.

Yeast phospholipid composition by high-performance thin-layer chromatography

The composition of the yeast phospholipids (PL) was determined using a modified version of the method described by Rodríguez-Porrata *et al.,* (2011). The yeast extract PLs were separated by 1-D high-performance thin-layer chromatography (HPTLC) on silica gel $60F_{254}$ plates (10 x 20 cm, 200 µm) (Merck, Germany) (Redon *et al.,* 2009). The plate application of samples and lipid standards was carried out with a semi-automatic injector (Linomat 5, Camag, Switzerland). Three successive migrations were performed with a semi-automatic developing chamber (ADC2, Camag, Switzerland): firstly with chloroform (13 mL), acetone (3 mL), methanol (2 mL), acetic acid (2 mL) and water (0.5 mL); secondly with hexane (8 mL), MTBE (2 mL) and acetic acid (0.2 mL), and thirdly with hexane (8 mL). The migration distance of the three steps was 65 mm, 75 mm and 85 mm, respectively. The plate was dried for 15 min between each migration step. The plate was then dipped in copper sulphate:phosphoric acid (10:3, v/v) solution for 1 min and heated at 170°C for 5 min on a TLC Plate Heater (Camag, Switzerland). PLs were identified using lipid standard solution spotted at different concentrations (1-4 $\mu g \cdot \mu L^{-1}$) on the plate. The plate with identified spots was acquired with an Image Scanner (Amersham, Bioscience). Each spot on the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by plotting the IODs of the lipid standard against the amount of lipid loaded.

Glutathione determination in yeast cells

Glutathione determination was performed using the method described by Borrull *et al.,* (2016). The GSH_{tot} and GSSG (oxidized) determinations were performed in a SPECTROstar Omega Instrument fluorescence plate reader (BMG Labtech), using 488 nm for excitation and 530 nm to collect fluorescence emission. Linear regression curves were performed using a reduced glutathione standard solution (GSH) made from GSH standard stock solution of 10 mM diluted with 5% SSA solution. GSH was calculated from GSH_{tot} and GSSG, and expressed as nM of glutathione per mg of dry weight.

Intracellular reactive oxygen species accumulation

DHE staining of $1 \cdot 10^7$ cells per sample was performed with dihydroethydium (DHE, Molecular Probes) and analysed by fluorescence microscopy as described by *López et al.,* (2012). To determine the frequencies of the morphological phenotypes revealed by the DHE staining, a minimum of 500 cells were evaluated from three independent experiments using a Leica fluorescence microscope (DM4000B, Germany). A digital camera (Leica DFC300FX) and Leica IM50 software were used for image acquisition.

Yeast chronological lifespan (CLS)

Chronological survival after 20 days incubation in the presence of $MgSO_4$ (5 mM), spermidine (0.15 mM) or $MgSO_4$ + spermidine (5 mM; 0.15 mM, respectively) was evaluated using standard the CLS method assay published by Longo *et al.,* (2012).

Statistical analysis

The results were statistically analysed by one-way ANOVA and the Scheffé test from the SPSS 15.1 statistical software package. The statistical significance was set at $p<0.01$.

Results & Discussion

The cell viability is enhanced by increasing intracellular magnesium in the rehydration process

In this study, the effect of the ion Mg^{2+} on cell viability under desiccation stress was evaluated on two strains of *Schiz. pombe,* Sp97 and Sp22, which display different resistance to dehydration (40% and 20% respectively), by adding the ion to the growth medium (before drying) and during the 5 min of cell rehydration. The Sp97 and Sp22 strains showed similar viability values to the control when they were supplemented with $MgSO_4$ before the stress imposition, at around 45% and 15%, respectively (Figure 30A). However, a statistically significant increase in viability was recorded for both strains when the rehydration solution was complemented with $MgSO_4$, at 80% and 30%, respectively. Then, the intracellular content of ion Mg^{2+} of strains was evaluated by ICP-OES during exposure to stress and in the subsequent lag phase of cells in YPD media after 2, 4 and 8 h of cultivation. Pellets of $5{\cdot}10^8$ cells were washed with cool water and stored at -20°C for further evaluation. The *Schiz. pombe* strains showed similar profiles in terms of intracellular magnesium variations. Therefore, for example, Figure 30B shows the values of intracellular Mg^{2+} content obtained from Sp97 cells. The *Schiz. pombe* strains did not show intracellular magnesium changes in comparison to the reference condition when supplementation occurred before exposure to stress (data not shown). At time 0, the rehydrated cells in the presence of magnesium showed more than a 2-fold increase (210 $\mu g{\cdot}10^8$ cells) in intracellular content compared to the control rehydrated in pure water (103 $\mu g{\cdot}10^8$ cells). Cells subjected to both sets of rehydration conditions showed a decrease in Mg^{2+} content from 0 h to 6 h in YPD media, and the values after 8 h were similar to those after 6 h incubation for both sets of rehydration conditions. However, for all the time points, the cells rehydrated in the presence of $MgSO_4$ showed at least 30 $\mu g{\cdot}10^8$ cells of Mg^{2+} more than the cells rehydrated in water. The intracellular increase of magnesium in cells rehydrated in the presence of the ion support the idea that the influx of ions through membranes, without selective permeability, is caused by the influx of rehydrating water. Therefore, the higher intracellular content of a divalent cation such as Mg^{2+} might block the mitochondrial unselective channel (MUC) by forming Mg-ATP^{2-},

promoting slow electron flux and leading to a reduction in the respiration rate, as described in the case of *S. cerevisiae* (Bradshaw & Pfeiffer, 2014, Cabrera-Orefice *et al.,* 2015). This reduction of the respiratory rate may explain the 208 min increase in the λ phase of *Schiz. pombe* cells rehydrated in the presence of $MgSO_4$ in comparison to cells rehydrated in pure water (Roca-Domènech *et al.,* 2016). The magnesium-rehydrated cells might require extra time to settle the intracellular magnesium to a permissible physiological level before resuming growth.

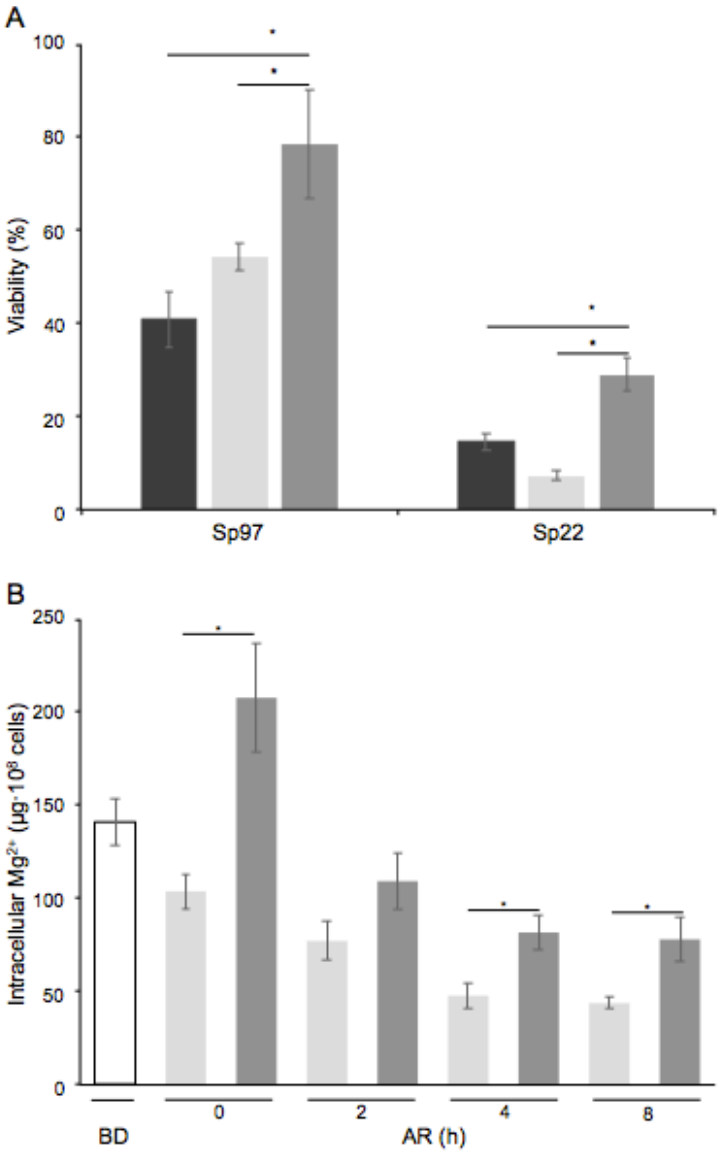


Figure 30. Effect of rehydration treatments on the viability (A) and quantification of intracellular Mg^{2+} (B) of *Schiz. pombe* strains (Sp97 and Sp22). Conditions evaluated were magnesium supplementation before drying + water rehydration (BDMg+ARH_2O, black bars), without magnesium supplementation before drying (BD, white bars), after water rehydration (ARH_2O, light gray bars), and after magnesium rehydration (ARMg, dark gray bars). Values shown are means of at least n = 3 independent samples ± standard deviation (SD). *Indicates p<0.01.

Enhancing intracellular magnesium reduces ATP and pyruvate cell levels

The putative blockage of the mitochondrial activity caused by magnesium might lead to changes in metabolites such as pyruvate and ATP. ATP is mostly synthesized in the mitochondria using pyruvic acid as the mine substrate when oxygen is present. Therefore, the ATP and pyruvate content of Sp97 and Sp22 cells were evaluated during stress imposition (Figure 31). The cells of Sp97 before dehydration showed more than twice the amount of ATP than Sp22 cells, 10 µmol·mg dry weight^{-1} and 2.3 µmol·mg dry weight^{-1}, respectively (Figure 31A). Sp97 cells rehydrated in water showed a reduction of 30% of ATP in comparison to cells before stress imposition. However, Sp22 cells did not show variations between these conditions. However, both strains showed a reduction of at least 80% of ATP when rehydration occurred in the presence of ion Mg^{2+} in comparison to cells rehydrated in water. The linkage of ATP to magnesium makes it bioavailable to the cell, depending on the magnesium concentration and the release of energy by ATP hydrolysis (Rubin, 2005). Therefore, our results suggest that the increase in intracellular magnesium promotes ATP hydrolysis, providing energy to repair and resume the activity of cells of both strains after stress imposition. The evaluation of pyruvate in both strains showed a reduction of around 50% between cells rehydrated in the presence of ion Mg^{2+} and cell rehydrated in water (Figure 31B). However, cells rehydrated in water did not show any significant change in comparison to cells before dehydration stress. As well as supplying energy to the cell through the citric acid cycle, pyruvate can also be converted back to carbohydrates (such as glucose) via gluconeogenesis, or to fatty acids by reacting with acetyl-CoA. In the case of both strains, ATP and pyruvate displayed the same reduction profile when the rehydrating solution was complemented with magnesium. The putative reduction in cell rate respiration in response to increasing intracellular ion Mg^{2+} suggests that the reduction in pyruvate is not metabolized to ATP for the most part. However, the reduction of both ATP and pyruvate might be correlated to an earlier activation of anabolic reactions mediated by ion Mg^{2+}.

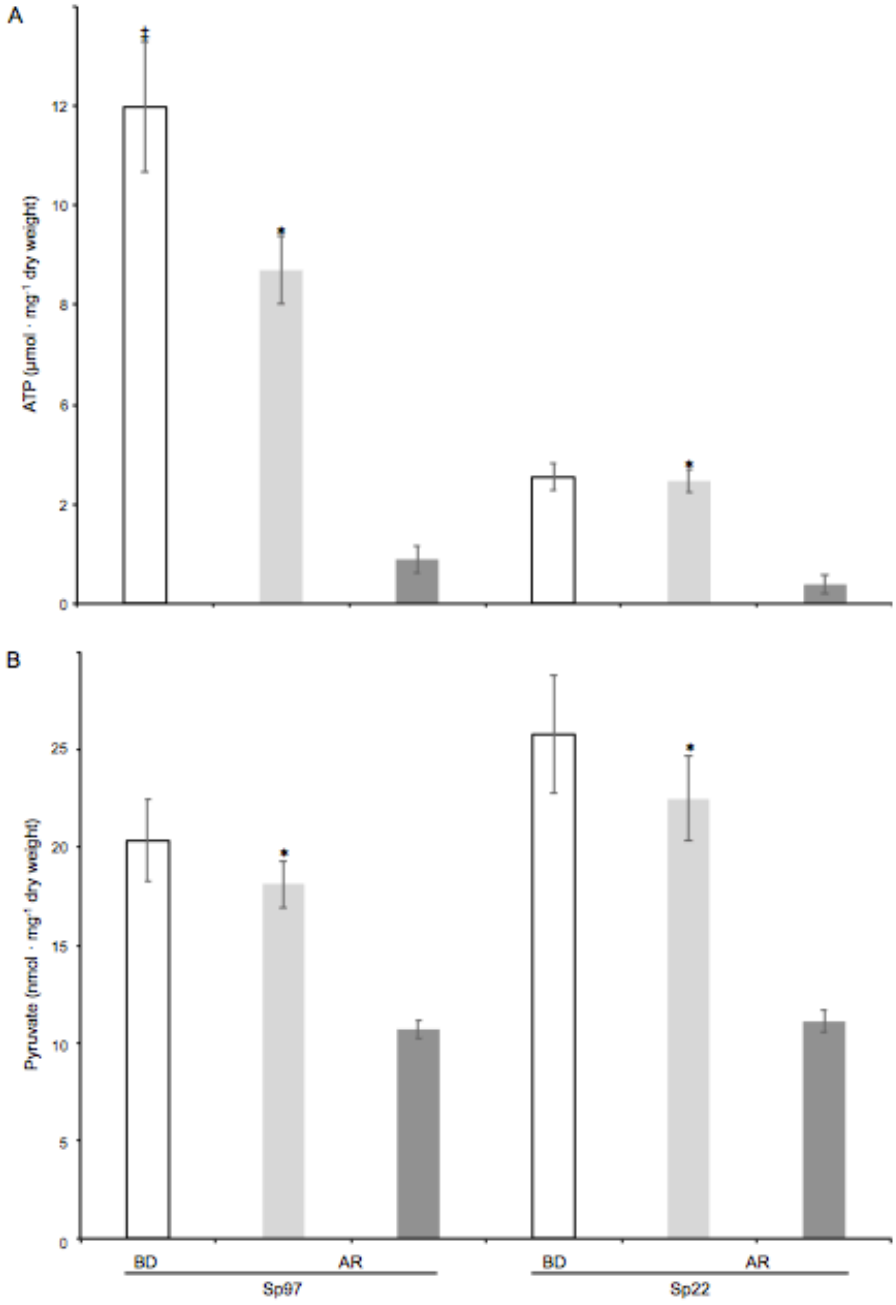


Figure 31. Quantification of ATP (A) and pyruvate (B) before drying (BD, white bars), after water rehydration (ARH_2O, light gray bars), and after magnesium rehydration (ARMg, dark gray bars) of *Schiz. pombe* strains (Sp97 and Sp22). Values shown are means of at least n = 3 independent samples ± standard deviation (SD). *Indicates $p < 0.01$ compared to ARMg. ‡Indicates $p < 0.01$ compared to ARH_2O and ARMg.

Untargeted metabolomics analysis during stress imposition

It was decided to ascertain whether the difference in the viability rate of the two strains after the dehydration process might be due to a difference in the general metabolite profile. To do so, using an Ultra-Performance Liquid Chromatography (UPLC), the qualitative changes of the intracellular metabolites of both SP97 and Sp22 strains were determined by comparing the metabolic profiles of cells before dehydration, after rehydration in water and magnesium solution, and after 2 h inoculation of both kinds of rehydrated cells in YPD medium. Table 10 summarizes the comparison of the evaluated conditions for both strains, which were based on data obtained from five independent replicates. The metabolites detected with ESI^+ and ESI^- were filtered: firstly, metabolites detected in all of the five replicates were selected; secondly, metabolites displaying qualitatively statistical differences between the studied conditions were retained; and finally, metabolites that match in the database were identified (Table 10).

Table 10. Number of metabolites identified in the evaluated groups with analysis in positive (ESI^+) and negative (ESI^-) ionization

Group Number	Group Evaluation	Database Hits	
		ESI^+	ESI^-
1	Sp97-BD vs. Sp22-BD	40	33
2	Sp97-BD vs. Sp97-ARH_2O vs. Sp97-ARH_2O2h	32	21
3	Sp97-BD vs. Sp97-ARMg vs. Sp97-ARMg2h	40	30
4	Sp22-BD vs. Sp22-ARH_2O2h	21	26
5	Sp22-BD vs. Sp22-ARMg2h	22	26
6	Sp97-ARH_2O2h vs. Sp22-ARH_2O2h	29	21
7	Sp97-ARMg2h vs. Sp22-ARMg2h	25	17

BD, before drying; AR, after rehydration; H_2O, water; Mg, magnesium; 2h, 2 hours in YPD medium

The identified metabolites showed changes in fifteen pathways. Groups 1, 2, 3, 6 and 7 displayed significant changes in the following metabolic pathways: glutathione synthesis and recycling, ROS detoxification and methionine salvage pathway. These metabolic pathways did not show variations in the case of groups 4 and 5. With respect to the three highlighted metabolic pathways, of the evaluated conditions, the metabolites of the methionine salvage pathway showed qualitative significant changes in methionine sulphoxide, 2-Oxo-4-methylthiobutanoic acid and 5'methylthioadenosine (MTA), in the Sp97 cells only. In group 2, it was identified that there was a higher content of methionine sulphoxide and 2-Oxo-4-methylthiobutanoic

acid in cells before drying (Sp97-BD) and after 2 h incubation in YPD media of cells rehydrated in water (Sp97-ARH_2O2h), than in the case of the cells rehydrated in water (Sp97-ARH_2O). However, a significantly higher content of the compound MTA in group 3 was recorded in cells rehydrated in the presence of $MgSO_4$ (Sp97-ARMg) than in Sp97-BD cells and the cells rehydrated in presence of $MgSO_4$ and incubated for 2 h in YPD medium (Sp97-ARMg2h). Methionine plays a central role in protecting cells from oxidative stress by acting as a scavenger of Reactive Oxygen Species (ROS) through the oxidation of its sulphur to sulphoxide (Campbell *et al*., 2016). These results suggest that metabolites from the methionine salvage pathway may participate in the dehydration tolerance of Sp97 cells.

Analysis of the targeted metabolomics during stress imposition

During desiccation, yeast cells are exposed to several stresses such as heat shock, osmotic, oxidative and cell aging (Rodríguez-Porrata *et al.,* 2011). The ability of cells to neutralize this multiple stress is fundamental for yeast survival. MTA is a product of S-adenosyl-1-methionine (SAM) catabolism during polyamine biosynthesis. SAM is synthetized via the methionine salvage pathway by *S*-adenosylmethionine synthetases from ATP and L-methionine (Figure 32) (Tabor & Tabor, 1984). In the synthesis of the pantothenate metabolic pathway, putrescine is converted to spermidine through the addition of an aminopropyl group by the spermidine synthase. The aminopropyl group is derived from SAM by S-adenosylmethionine decarboxylase that produces decarboxylated S-adenosylmethionine (dcSAM). Spermidine is converted to spermine through the addition of a second aminopropyl group by the spermine synthase. The spermine is converted to 3-amiopropanal by an amine oxidase, which is transformed by an aldehyde dehydrogenase to obtain beta-alanine. The pantothenate synthase will produce pantothenate using beta-alanine as a substrate. Moreover, SAM is a methyl donor in many biological processes and a key regulator compound in the homeostasis of the cellular metabolism of amino acids containing sulphur (Figure 32) (Shobayashi *et al.,* 2007).

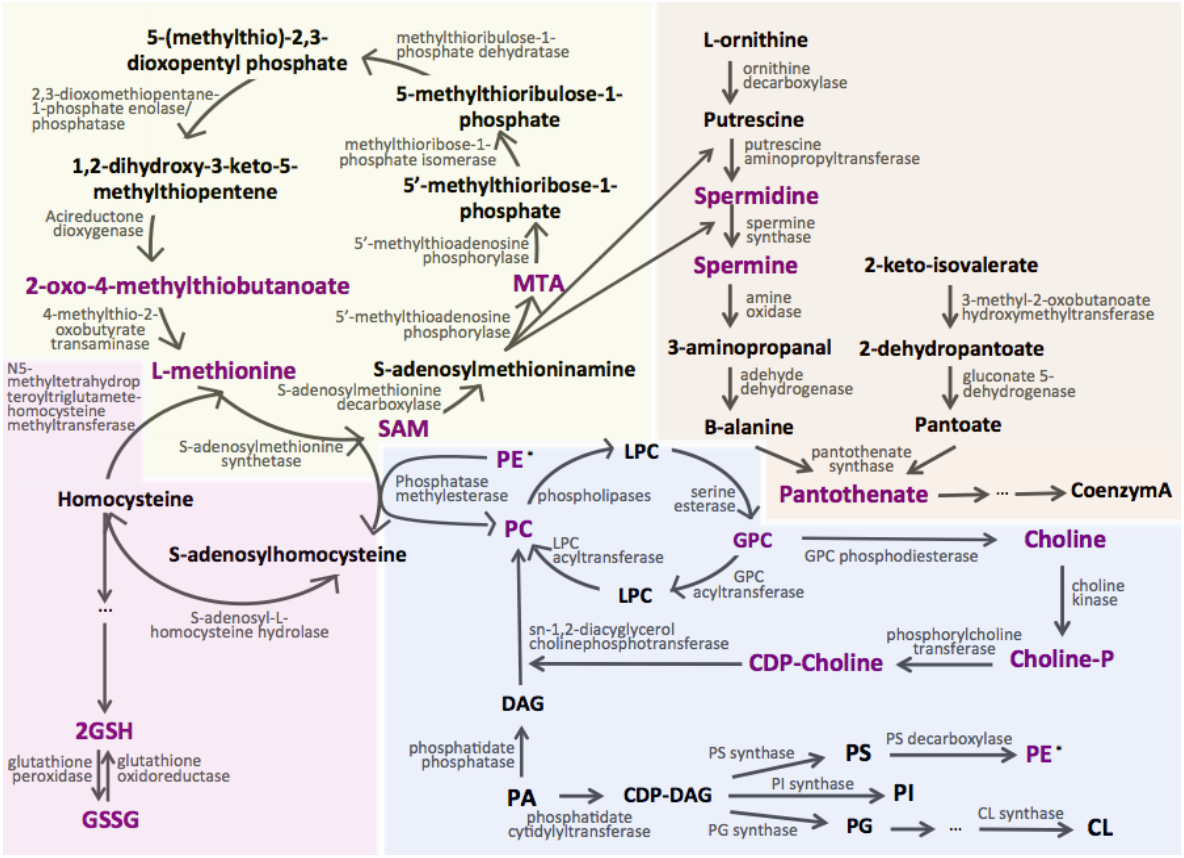


Figure 32. Outline of the biosynthetic pathways of methionine (yellow), β-alanine/pantothenate (orange), glutathione (pink) and phospholipids (blue). SAM, s-adenosyl-1-methionine; MTA, 5'methylthioadenosine; GSH, reduced glutathione; GSSG, oxidized glutathione; PA, phosphatidic acid; CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; GPC, glycerophosphocholine; LPC, lysophosphatidylcholine; CDP-choline, cytidine diphosphate choline. Metabolites evaluated in the study are in purple.

For further evaluations, a targeted UHPLC-ESI-QqQ analysis was developed to quantify the metabolites of the methionine salvage pathway (methionine; MTA; and S-adenosyl-1-methionine, SAM) and from related metabolic pathways: β-alanine/pantothenate pathway (spermine, pantothenate) and phospholipid pathway (choline, phosphocholine, L-α-glycerophosphocholine, cytidine-5'-diphosphocholine and betaine) for the same samples obtained for the untargeted metabolomic analysis. Figure 33 and 34 show the metabolite ratio between BD and AR in the reference condition and the samples complemented with magnesium. The samples with 2 h incubation in YPD medium showed a similar profile to AR (data not shown). The qualitative differences observed in methionine cell content between the strains in the

untargeted metabolomic analysis could not be confirmed by the targeted metabolomic analysis. Both strains showed around 870 $\mu g \cdot L^{-1}$ before dehydration and after rehydration in both conditions, increasing to around 1,000 $\mu g \cdot L^{-1}$ (Figure 33). Sp97 cells ARMg2h showed a statistically significant increase in SAM (31,015 $\mu g \cdot L^{-1}$) and MTA (407 $\mu g \cdot L^{-1}$) in comparison to BD (11,414 $\mu g \cdot L^{-1}$ and 114 $\mu g \cdot L^{-1}$, respectively) and ARH_2O2h (29,178 $\mu g \cdot L^{-1}$ and 319.1 $\mu g \cdot L^{-1}$, respectively). These significant differences were also recorded in the case of Sp97-ARMg cells (data not shown). However, Sp22 cells displayed similar SAM and MTA content values BD and AR2h conditions. SP97 and Sp22 cells showed a statistically significant increase in spermine AR2h in water (37.9$\mu g \cdot L^{-1}$ and 94.2 $\mu g \cdot L^{-1}$, respectively) and in magnesium (182.6 $\mu g \cdot L^{-1}$ and 199.8 $\mu g \cdot L^{-1}$, respectively) in comparison to the content of cells BD (6.5 $\mu g \cdot L^{-1}$ and 7.4 $\mu g \cdot L^{-1}$, respectively). However, the spermine level AR was at least 2.5-fold lower than in the case of cells 2 h YPD incubation from both sets of rehydration conditions (data not shown). It has been reported that the intracellular accumulation of MTA promotes the inhibition of polyamine biosynthesis. In particular, the spermine synthase in *S. cerevisiae* is highly inhibited by MTA (Garcea *et al.,* 1987; Chattopadhyay *et al.,* 2006; Valdés-Santiago & Ruiz-Herrera, 2014). Our results are in concordance with these previous findings, showing that Sp97 with the higher amount of MTA in cells BD had lower spermine values ARH_2O, while the opposite is true for Sp22. However, ARMg Sp97 cells have higher MTA and spermine concentration. Polyamines such as putrescine, spermidine and spermine play regulatory roles in cell growth and improve ionic equilibrium by modifying the plasma membrane to overcome osmotic stress and protecting cells from oxidative stress damage (Uemura *et al*., 2009). However, the similar values of spermine shown by Sp97 and Sp22 cells BD and the statistical significant highest values of ARH_2O Sp22 cells than ARH_2O Sp97 cells do not provide evidence that polyamine spermine plays a significant role in promoting cell dehydration tolerance in *Schiz. pombe*.

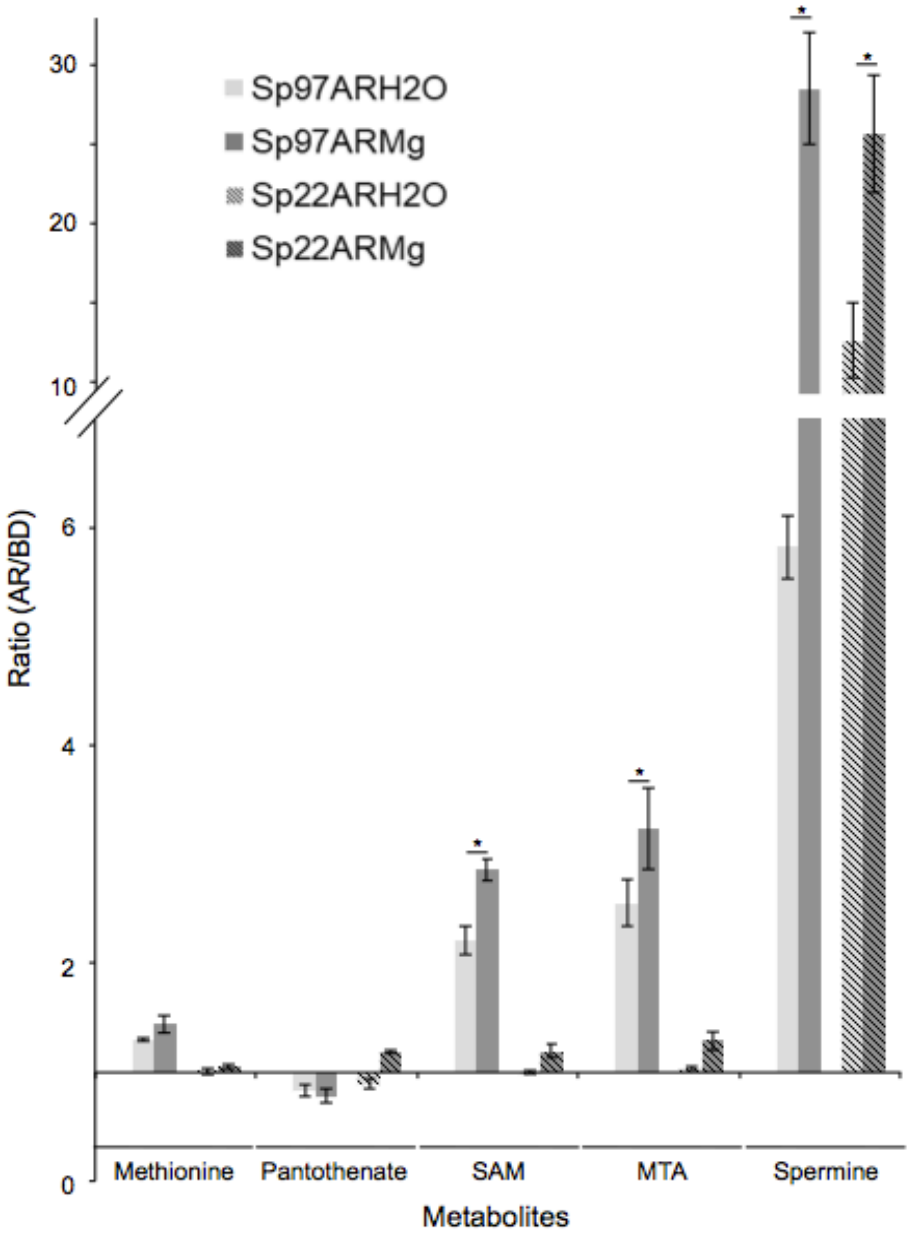


Figure 33. Metabolites from methionine salvage pathway and related pathways detected by UHPLC-(ESI$^+$)-QqQ/MS of *Schiz. pombe* strains. The metabolite ratios represented were obtained between before dehydration and after rehydration conditions for *Schiz. pombe* strains. BD, before drying; ARH_2O, after water rehydration; ARMg, after magnesium rehydration; SAM, S-adenosyl-1-methionine; MTA, 5'methylthioadenosine. Values shown are means of n = 5 independent samples ± standard deviation (SD). * Indicates $p < 0.01$ compared between rehydration condition.

On the contrary, the spermine results allow us to suggest a putative deleterious effect on Sp22 cells during stress imposition. Unexpectedly, the increase of spermine in Sp97 and Sp22 cells did not cause statistically significant changes in pantothenate

content AR in water (23 $\mu g \cdot L^{-1}$ and 30 $\mu g \cdot L^{-1}$, respectively) or in 5 mM magnesium (21.5 $\mu g \cdot L^{-1}$ and 40 $\mu g \cdot L^{-1}$, respectively) in comparison to cells BD (27.5 $\mu g \cdot L^{-1}$ and 33.5 $\mu g \cdot L^{-1}$, respectively). This unchanged contain in pantothenate by both *Schiz. pombe* cells before and after stress imposition was maintained in the 2 h incubated cells (data not shown). After several transformations that require cysteine and ATP, pantothenate can generate Coenzyme A, which plays a significant role in the synthesis and oxidation of fatty acids, and oxidation of pyruvate in the citric acid cycle. The difference in dehydration rate viability caused by cell rehydration in the presence of magnesium does not correlate with the unchanged cellular content of pantothenate, suggesting that magnesium does not have a significant effect on the pantothenate pathway during stress imposition. The transfer of a group methyl from the polyamine SAM to phosphatidylethanolamine (PE) mediated by a phosphatase methylesterase produces phosphatidylcholine (PC) and S-adenosylhomocysteine (SAH) on the phospholipid biosynthesis pathways (Stalberg *et al.,* 2018). SAH may be hydrolysed to obtain homocysteine, which is a precursor to cysteine synthesis and, therefore, the production of glutathione (Figure 32). With this in mind, cell phospholipid composition (phosphatidic acid, PA; phosphatidylethanolamine + cardiolipin, PE + CL; phosphatidylcholine, PC; phosphatidylinositol, PI; phosphatidylserine, PS) was assessed by high-performance thin-layer chromatography. With respect to the evaluated phospholipids, PC was the only one to show a statistically significant reduction in the Sp97 and Sp22 cells in comparison to the BD process (7.62 $\mu g \cdot 10^8$cells and 12.82 $\mu g \cdot 10^8$cells, respectively) with respect to the ARH_2O condition (1.78 $\mu g \cdot 10^8$cells and 2.84 $\mu g \cdot 10^8$cells, respectively), with the ARMg conditions being more important (0.3 $\mu g \cdot 10^8$cells and 0.99 $\mu g \cdot 10^8$cells, respectively). After 2 h incubation in YPD, the ARMg Sp97 cells showed a six-fold increase in PC content (1.75 $\mu g \cdot 10^8$cells), although the ARMg Sp22 cells after 2 h incubation in YPD showed 2.5-fold decrease in PC in comparison to AR cells (0.38 $\mu g \cdot 10^8$cells) (Figure 34A). These significant reductions in PC mediated by the presence of magnesium AR in Sp22 and Sp97 cells agree with previous results that showed a 50% reduction in PC after dehydration stress, while the PE + CL value remained constant during stress imposition. Therefore, this reduction in PC in *S. bayanus* correlates with a decrease in 75% in viability, which does not agree with the increase in the viability values shown by the *Schiz. pombe* strains after

rehydration in the presence of magnesium (Rodríguez-Porrata *et al.,* 2011). In *S. cerevisiae*, it has been reported that the absence of extracellular choline induces the turnover of PE-derived PC mediated by phospholipases yields choline for use in the CDP-choline pathway (McMaster & Bell, 1994). Therefore, it was hypothesized that the loss of PC in AR-cells in pure water and with magnesium supports phospholipase activity, resulting in a reduction in PC and increasing choline cell contents, or via the PC recycling pathway, yielding an increase in glycerophosphocholine (GPC) (review by Kroon, 2007). Therefore, with Ultra-Performance Liquid Chromatography (UPLC), the quantitative changes of the intracellular phosphatidylcholine turnover pathway metabolite -L-α-glycerophosphocholine (GPC) was determined, as well as the intermediary metabolites of GPC as the choline source (choline, phosphocholine (choline-P) and cytidine-5'-diphosphocholine (CDP-choline)) in the synthase of PC, and the choline derivative, betaine, was developed for the same samples. Betaine is also important because of its role in the donation of methyl groups to homocysteine to form methionine (Finkelstein *et al.,* 1972). Unexpectedly, all the evaluated metabolites showed a similar ratio profile at BD and AR stages under both sets of conditions. The significant differences between strains were shown by the accumulation of choline and choline-P in Sp97 cells after both sets of rehydration conditions in comparison to BD cells. In addition, choline-P displayed a statistically significant increase after 2 h incubation in YPD. However, the constant value of betaine shown by the AR cells suggests that the statistically significant decrease in PC content of the ARMg cells suggests the yielded choline was not channelled into betaine synthesis (Figure 34B). In *Schiz. pombe* the phospholipase B (PLase B) hydrolyses both fatty acyl chains of glycerophospholipids and is activated *in vitro* by the presence of magnesium (Hideki *et al.*, 1996). Our results agree with the data observed *in vitro*, that the presence of magnesium during the rehydration process enhanced intracellular PC reduction. However, the similar enhancement profile of choline and choline-P under the two sets of rehydration conditions does not clarify the role of the ion Mg^{2+} any further in relation to the differential in PC values.

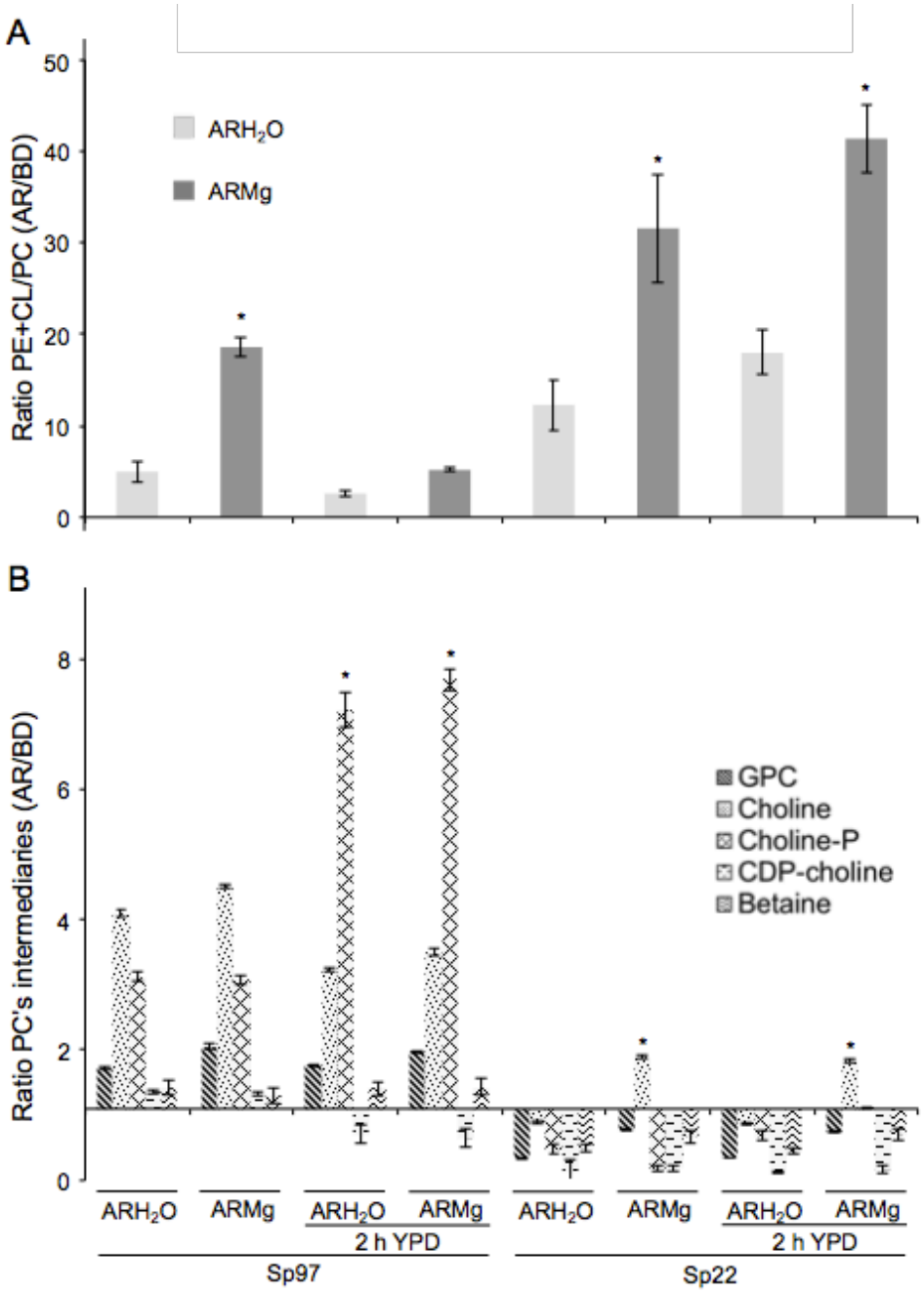


Figure 34. Relation between PE+CL / PC (A) and PC's intermediaries (B) AR / BD of *Schiz. pombe* strains. Ratios between conditions and *Schiz. pombe* strains were represented with coloured bars following legend. BD, before drying; ARH_2O, after H_2O rehydration; ARMg, after magnesium rehydration; GPC, glycerophosphocholine; CDP-choline, cytidine diphosphate-choline. Values shown are means of n = 5 independent samples ± standard deviation (SD). *Indicates $p < 0.01$ compared to ARH_2O.

The yeast antioxidant molecular system is composed of glutathione, thioredoxin system, superoxide dismutase and catalase. The increase of SAM for rehydrated cells in the presence of magnesium may increase the content of glutathione cell to

contribute towards enhancing oxidative stress resistance. Therefore, Sp97 cells were evaluated for glutathione content (Figure 35). Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is a tripeptide with low molecular weight and acts as a radical scavenger because its redox active sulphydryl group react with ROS to produce oxidized disulphide glutathione (GSSG) (Jamieson, 1998). The *Schiz. pombe* Sp97 cells BD displayed a statistically significant difference between GSSG and GSH (2.27 nmol·mg dry weight^{-1} and 0.14 nmol·mg dry weight^{-1}, respectively), but the cells from ARH_2O (0.9 nmol·mg dry weight^{-1} and 0.76 nmol·mg dry weight^{-1}, respectively) and ARMg (1.23 nmol·mg dry weight^{-1} and 0.95 nmol·mg dry weight^{-1}, respectively) showed similar values. However, total glutathione in ARMg Sp97 cells showed similar values to BD Sp97 cells, while ARH_2O showed a statistically significant 25% decrease. Glutathione (GSH) is synthesized in two sequential reactions involving glutamylcysteine synthetase (GCS), followed by glutathione synthetase (GS), in which magnesium is the GCS cofactor. Our results suggest that the enhancement of intracellular magnesium (Figure 30A) promotes the maintenance of total glutathione content during dehydration stress imposition.

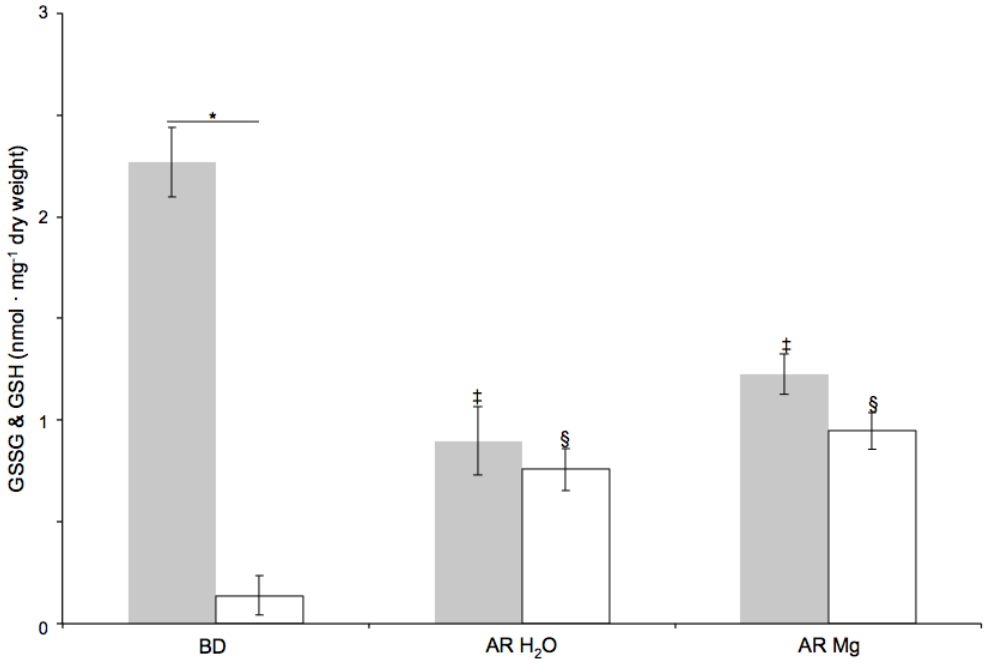


Figure 35. Quantification in Sp97 strain of glutathione oxidized (GSSG, gray bars) and reduced (GSH, white bars); before drying (BD), after water rehydration (ARH_2O), and after magnesium rehydration (ARMg). Values shown are means of at least n = 3 independent samples ± standard deviation (SD). *Indicate $p < 0.01$ compared between GSSG and GSH. §Indicate $p < 0.01$ compared to GSH-BD. ‡Indicates $p < 0.01$ compared to GSSG-BD.

Effect of several rehydration conditions on cell viability and DHE

It was decided to ascertain whether the increase in the viability rate of the Sp97 strain, after rehydration in the presence of magnesium, may be mimicked by the presence of any of the metabolites that showed intracellular variations during dehydration stress imposition. The rehydration media used in this study can be divided into two groups: supplemented with single compounds (spermidine, spermine, MTA, SAM, methylthiobutanoate or pantothenate) and co-supplemented with a single compound and magnesium. The cells rehydrated in the presence of MTA shown similar viability rates to the cells rehydrated in pure water (61% and 54%. respectively). However, the MTA + Mg^{2+} rehydration conditions showed similar values to the cells rehydrated in the presence of magnesium (85% and 78%, respectively). Moreover, the rehydrated cells supplemented with methionine (36%) and methionine + Mg^{2+} (56%) showed a reduction of 20% viability in comparison to the reference conditions of pure water and magnesium, respectively (Figure 36A). Therefore, the presence of methionine during cell rehydration has a deleterious effect on the viability rate and the Mg^{2+} ion partially inhibits that effect. The viability values obtained for the cells supplemented with spermidine, spermine (data not shown), SAM, methylthiobutanoate and pantothenate did not show a statistically significant change in viability after stress imposition in comparison to the cells co-supplemented with these metabolites + magnesium (Figure 36A). This allows us to suggest that the effect of magnesium during cell rehydration is moderated by the supplementation of any of these compounds. However, MTA did not show any harmful effect of viability and the effect of magnesium is not offset by the presence of MTA.

It has previously been reported that the reduction in intracellular ROS accumulation during the imposition of desiccation stress enhances viability (López-Martínez *et al.,* 2012; Rodríguez-Porrata *et al.,* 2012). Therefore, the relationship between the variations in viability rate and differences in accumulating ROS cells was evaluated (Figure 36B). Cells from the stationary phase before desiccation and after rehydration under several sets of conditions were analysed for the accumulation of reactive oxygen species (ROS). Before dehydration, the Sp97 cells showed 66.5% fluorescence after DHE incubation, while, after cell rehydration in the presence of magnesium, spermidine, MTA and pantothenate, less DHE cells were present (24%,

23%, 28% and 24%, respectively) than in cells rehydrated in pure water (40%) (Figure 36B). The antioxidant effect of spermidine shown by this data is in line with previous observations in *E. coli* and *S. cerevisiae* mutants, which have a reduced spermidine content, becoming more sensitive to oxidative damage (Chattopadhyay *et al.,* 2003a-b; Rider *et al.,* 2007; Jung *et al*., 2003). In addition, magnesium can enhance polyamine accumulation in the cells (Maruyama *et al.,* 1994). Despite this, after rehydration in the presence of methionine, SAM and methylthiobutanoate Sp97 cells show the same values for DHE as ARH_20 cells. The reduction of DHE cells in Sp97 rehydrated in the presence of pantothenate (24%) is in line with previous data, which show that, by incorporating pantothenate into Coenzyme A, cells are protected against peroxidative damage by increasing the level of glutathione (Hunter *et al.,* 2001). However, with respect to the cells rehydrated with co-supplemented solutions, only MTA + Mg^{2+} cells showed a statistically significant reduction of DHE positive cells in comparison to AR cells in MTA (14% and 28%, respectively). The reduction of DHE positive cells correlates with the enhancement of the viability rate (86% and 62%, respectively) in comparison to AR cells in pure water and magnesium (54% and 78%), suggesting a synergetic effect between magnesium and MTA. However, the cells rehydrated in the presence of spermidine or spermidine + Mg showed a reduced value of DHE cells content (23% and 19%, respectively) in comparison to ARH_20 cells (40%), although the viability rates after stress imposition were similar. This may be explained by the competitive inhibition already described between polyamines and magnesium (Maruyama *et al.,* 1994). Taken together with our results, it seems that there is no simple correlation between ROS level and *Schiz. pombe* cell tolerance to desiccation. In summary, it can be concluded that the reduction of intracellular ROS seems to be necessary but not sufficient for desiccation tolerance in *yeasts.* Moreover, magnesium has an effect on other metabolic adjustments in Sp97 cells for overcoming desiccation stress.

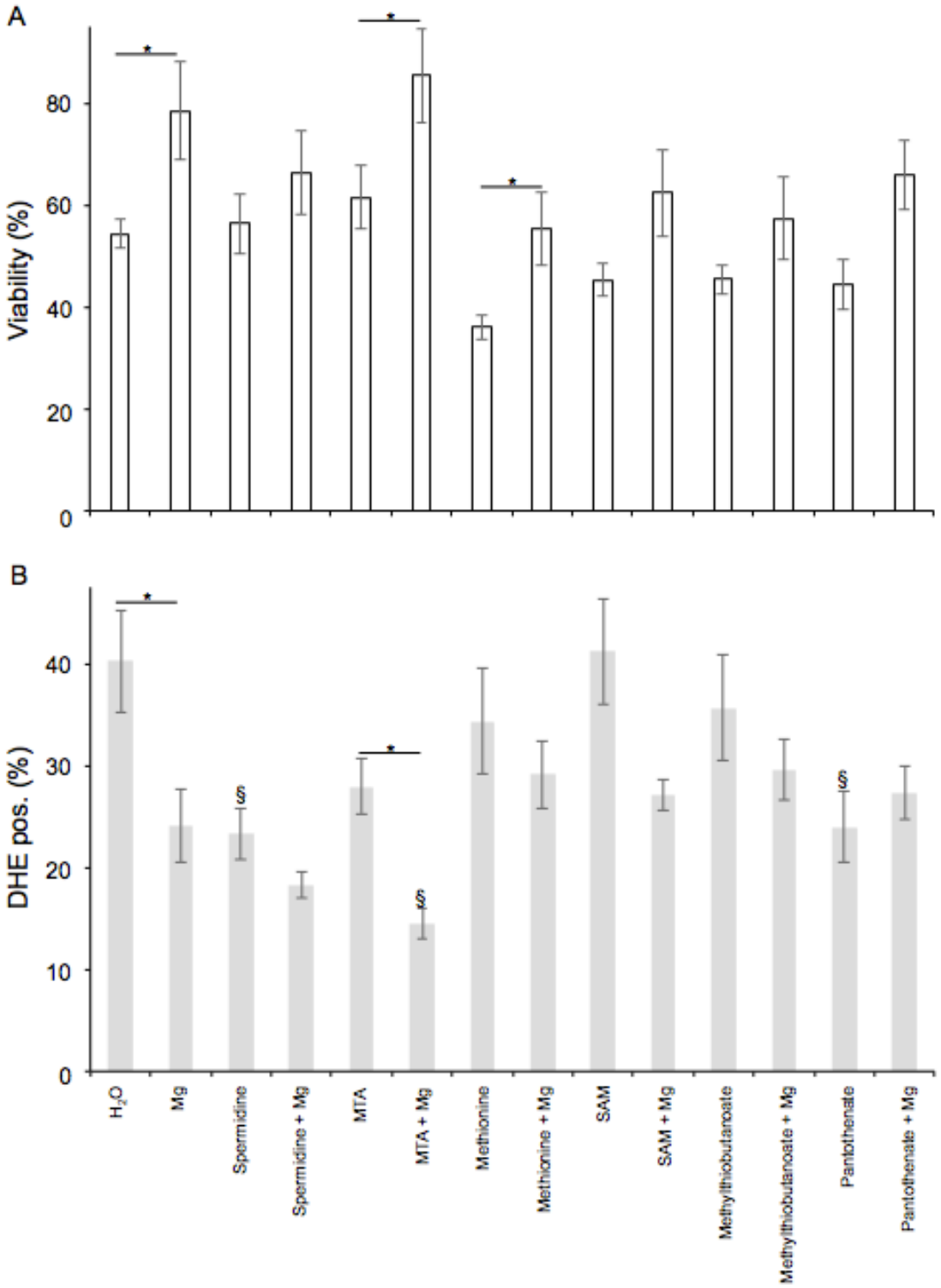


Figure 36. Sp97 strain viability (A) and DHE positive cells quantifiation (B) after rehydration (AR). Sp97 cells were dried in the presence of 10% trehalose and incubated at 37ºC for 5 min in: pure water, Mg, spermidine, spermidine + Mg, MTA, MTA + Mg, methionine, methionine + Mg, SAM, SAM + Mg, methylthiobutanoate, methyltiobutanoate + Mg, pantothenate, pantothenate + Mg. Values shown are means of at least n = 3 independent samples ± standard deviation (SD). *Indicates $p < 0.01$ compared to the same condition without Mg. §Indicates $p<0.01$ compared to the reference condition (ARH_2O).

Magnesium shortens the cell lifespan of Sp97 cells

The organisms attempting to overcome dehydration stress must address challenging issues such as cell age, longevity, the structural and biochemical properties of anhydrous cytoplasm and metabolic stasis (Potts, 2001). It was decided to ascertain whether the higher viability rate of Sp97 cells rehydrated in presence of magnesium compared to those rehydrated in pure water could be due to the differences in their life expectancy profile. However, it was shown that spermidine extends the lifespan in various organisms through epigenetic modifications, autophagy induction and suppression of necrosis (Eisenberg *et al.,* 2009). The chronological life span assay of the Sp97 strain was performed by growing yeast cells in YE medium and, at the stationary phase (72 h old), they were supplemented separately with: magnesium; spermidine, to lengthen their lifespan; spermidine + magnesium, to evaluate the putative interference between the compounds shown in figure 36A; and not supplemented, as a reference condition (Longo *et al.,* 2012). The viability was monitored every two days until day 20 (Figure 37A). Samples from day 20 were evaluated under the microscope and 1:10 serial dilutions were inoculated on YE plates (Figure 8B). The cells supplemented with spermidine showed a 2-fold increase in the survival rate compared to the reference condition (80% and 40%, respectively). However, the cells kept in the presence of Mg^{2+} or spermidine + Mg^{2+} showed a similarly significant reduction in survival cells (3% and 10%, respectively). This data suggests that magnesium accelerates the aging process. However, the low viability obtained for spermidine + Mg^{2+} cells might be explained by the competitive inhibition action of Mg^{2+} in relation to polyamine uptake, as shown previously for spermine transport in *S. cerevisiae*, overriding the deleterious effect of Mg^{2+} (Maruyama *et al.,* 1994).

The differential interference contrast images obtained with the microscope reveal a large number of cells damaged under Mg^{2+} and spermidine + Mg^{2+} conditions, which correlates with the lower growth show in those serial dilutions (Figure 37B). The serial dilutions developed from day 1 to day 10 for all the conditions exhibit similar profiles (data not shown). The premature chronological aging induced by magnesium in stationary phase cells suggests the activation of the cell metabolism, as observed in *S. cerevisiae* during fermentation (Walker, 2004). However, this enhancement of the

metabolic activity mistuned Sp97 cells in the exhausted nutrient medium, triggering early cellular aging.

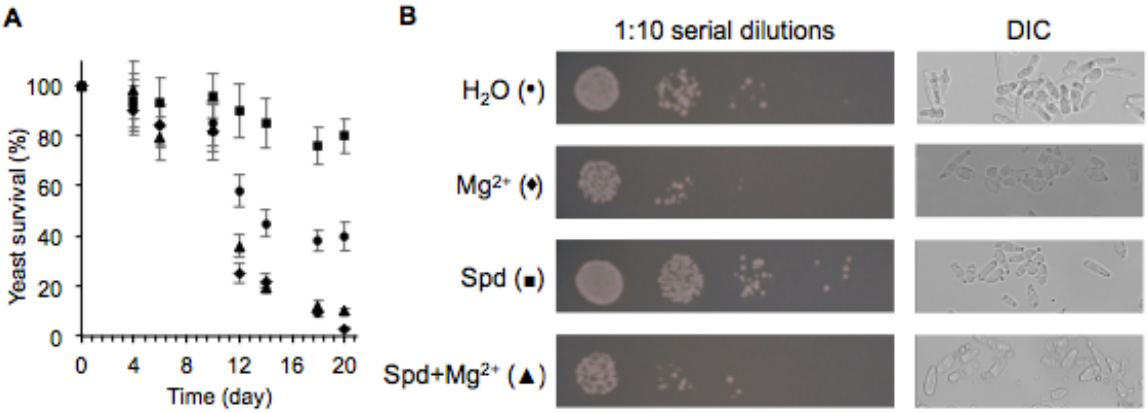


Figure 37. Magnesium (Mg^{2+}) and spermidine (Spd) effect in Sp97 lifespan cells. A) Sp97 cell survival until the day 20. B) Serial dilutions and differential interference contrast (DIC) images of the cultured yeast cells at day 20 after supplementation (B). Conditions evaluated were H_2O (•), magnesium(♦), spermidine (■), spermidine + magnesium (▲). Values shown are means of at least n = 3 independent samples ± standard deviation (SD).

Conclusions

The ability of cells to counteract the several stresses that occur during the desiccation process is fundamental for yeast survival. It has been observed that, when magnesium is present during the rehydration process, it enters the *Schiz. pombe* cells, enhancing dehydration stress tolerance. Global metabolic analysis highlights the putative significance of the methionine salvage pathway for overcoming stress imposition. Moreover, the putative effect of metabolites of the methionine salvage pathway and related pathways were characterized. Polyamines apparently act as cations within the cell taking the internal place of Mg^{2+}. Moreover, polyamines may have a toxic effect on cells with limited Mg^{2+} content (Maruyama *et al.,* 1994). The increase in methionine sulphoxide and 2-Oxo-4-methylthiobutanoic in cells during the rehydration process in pure water might promote faster polyamine synthesis when cells resume growth. Our results suggest that the shift to MTA accumulation by the cells rehydrated in the presence of Mg^{2+} can prevent the putative toxicity caused by early polyamine synthase in cells restarting growth. However, increasing intracellular ion availability might prevent the competitive inhibition of spermine with respect to Mg^{2+}.

Acknowledgements

This work was supported by grant AGL2012-40018 from the Spanish *Ministerio de Ciencia e Innovación*. The authors would like to thank the Universitat Rovira i Virgili for the doctoral fellowships 2014PMF-PIPF-47 to GR.

References

Avila, M. A., García-Trevijano, E. R., Lu, S. C., Corrales, F. J., & Mato, J. M. (2004). Methylthioadenosine. *The International Journal of Biochemistry & Cell Biology, 36,* 2125-2130.

Barros de Souza, R., Karla Silva, R., Soares Ferreira, D., De Sá Leitao Paiva Junior, S., De Barros Pita, W., & De Morais Junior, M. A. (2016). Magnesium ions in yeast : setting free the metabolism from glucose catabolite repression. *Metallomics*, *8*, 1193-1203.

Borrull, A., López-Martínez, G., Poblet, M., Cordero-Otero, R., & Rozès, N. (2015). A simple method for the separation and quantification of neutral lípid species using GC-MS. *European Journal of Lipid Science and Technology*, *17*, 274-280.

Borrull, A., López-Matínez, G., Miró-Abell, E., Salvadó, Z., Poblet, M., Cordero-otero, R., & Rozès, N. (2016). New insights into the physiological state of Saccharomyces cerevisiae during ethanol acclimation for producing sparkling wines. *Food Microbiology*, *54*, 20-29.

Bradshaw, P. C., & Pfeiffer, D. R. (2014). Characterization of the respiration-induced yeast mitochondrial permeability transition pore. *Yeast*, *30*, 471-483.

Cabrera-Orefice, A., Ibarra-García-Padilla, R., Maldonado-Guzmán, R., Guerrero-Castillo, S., Luévano-Martínez, L. A., Pérez-Vázquez, V., Gutiérrez-Aguilar, M., & Uribe-Carvajal, S. (2015). The *Saccharomyces cerevisiae* mitochondrial unselective channel behaves as a physiological uncoupling system regulated by Ca^{2+} ,Mg^{2+}, phosphate and ATP. *Journal of Bioenergetics and Biomembranes*, 47, 477-491.

Campbell, K., Vowinckel, J., Keller, M. A., & Ralser, M. (2016). Resistance via the pentose phosphate pathway. *Antioxidants & Redox Signaling*, *24*, 543-547.

Chattopadhyay, M. K., Tabor, C. W., & Tabor, H. (2003a). Polyamines protect *Escherichia coli* cells from the toxic effect of oxygen. *Proceeding of the National Academy of Science of the United States of America, 100,* 2261-2265.

Chattopadhyay, M. K., Tabor, C. W., & Tabor, H. (2003b). Spermidine but not spermine is essential for hypusine biosynthesis and growth in *Saccharomyces cerevisiae*: spermine is converted to spermidine in vivo by the FMS1-amine oxidase. *Proceeding of the National Academy of Science of the United States of America*, *100,* 13869-13874.

Chattopadhyay, M. K., Tabor, C. ., & Tabor, H. (2006). Methylthioadenosine and polyamine biosynthesis in a *Saccharomyces cerevisiae* meu1delta mutant. *Biochemical and Biophysical Research Communications*, *343*, 203-207.

Cyert, M. S., & Philpott, C. C. (2013). Regulation of cation balance in *Saccharomyces cerevisiae*. *Genetics*, *193*, 677-713.

Dupont, S., Rapoport, A., Gervais, P., & Beney, L. (2014). Survival kit of *Saccharomyces cerevisiae* for anhydrobiosis. *Applied Microbiology and Biotechnology*, *98*, 8821-8834.

Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., *et al.,* (2009). Induction of autophagy by spermidine promotes longevity. *Nature cell biology, 11,* 1305-1314.

Finkelstein, J. D., Harris, B. J., & Kyle, W. E. (1972). Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase. *Archives of Biochemistry and Biophysics, 153,* 320-324.

Garcea, R., Pascale, R., Daino, L., Frassetto, S., Cozzolino, P., Ruggiu, M. E., Vannini, M. G., Gaspa, L., & Feo, F. (1987). Variations of ornithine decarboxylase activity and S-adenosyl- L-methionine and 5'-methylthioadenosine contents during the development of diethylnitrosamine-induced liver hyperplastic nodules and hepatocellular carcinoma. *Carcinogenesis*, *8*, 653-658.

Hideki, O., Shinji, T., Yasuo, W., & Youichi, T. (1996). Purification and some properties of phospholipase B from *Schizosaccharomyces pombe*. *Bioscience, Biotechnology, and Biochemistry, 60,* 1087-1092.

Hunter, J. J., & Ruffner, H. P. (2001). Assimilate transport in grapevines -effect of phloem disruption. *Australian Journal of Grape Wine Research, 7,* 118-126.

Jamieson, D. J. (1998). Oxidative Stress Responses of the yeast *Saccharomyces cerevisiae*. *Yeast*, *14*, 1511-1527.

Jung, I.L., Oh, T.J., & Kim, I.G. (2003). Abnormal growth of polyamine-deficient *Escherichia coli* mutant is partially caused by oxidative stress-induced damage. *Archives of Biochemistry and Biophysics, 418,* 125-132.

De Kroon, AI. (2007). Metabolism of phosphatidylcholine and its implications for lipid acyl chain composition in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta, 1771,* 343-352.

Liu, G. J., Martin, D. K., Gardner, R. C., & Ryan, P. R. (2002). Large Mg^{2+}-dependent currents are associated with the increased expression of ALR1 in *Saccharomyces cerevisiae. FEMS Microbiology Letters*, *213*, 231-237.

Longo, V. D., Shadel, G. S., Kaeberlein, M., & Kennedy, B. (2012). Replicative and chronological aging in *Saccharomyces Cerevisiae. Cell Metabolism, 16,* 18-31.

López-Martínez, G., Pietrafesa, R., Romano, P., Cordero-Otero, R., & Capece, A. (2013). Genetic improvement of *Saccharomyces cerevisiae* wine strains for enhancing cell viability after desiccation stress. *Yeast, 30,* 319-330.

López-Martínez, G., Rodríguez, B., Margalef-Català, M., & Cordero-Otero, R. (2012). The STF2p hydrophilin from *Saccharomyces cerevisiae* is required for dehydration stress tolerance. *PLoS ONE*, *7*, e33324.

Maruyama, T., Masuda, N., Kakinuma, Y., & Igarashi, K. (1994). Polyamine-sensitive magnesium transport in *Saccharomyces cerevisiae*. *Biomembranes*, *1194*, 289-295.

McMaster, C. R., & Bell, R. M. (1994). Phosphatidylcholine biosynthesis in *Saccharomyces cerevisiae*. Regulatory insights from studies employing null and chimeric sn-1, 2-diacylglycerol choline- and ethanolaminephosphotransferases. *The Journal of Biological Chemistry, 269,* 28010-28016.

Pisat, N. P., Pandey, A., & MacDiarmid, C. (2009). MNR2 regulates intracellular magnesium storage in *Saccharomyces cerevisiae. Genetics Society of America*, *183*, 873-884.

Pösö, H., Sinervirta, R., & Jänne, J. (1975). S-adenosylmethionine decarboxylase from baker's yeast. *Biochemical Journal, 151,* 67-73.

Potts, M. (2001). Desiccation tolerance: A simple process? *Trends in Microbiology, 9*, 553-559.

Redón, M., Guillamón, J. M., Mas, A., & Rozès, N. (2009). Effect of lípid supplementation upon *Saccharomyces cerevisiae* lipid composition and fermentation performance at low temperature. *European Food Research and Technology*, *228*, 833-840.

Roca-Domènech, G., López- Martínez, G., Tirado, V., Borrull, A., Candelas, Ó., Rozès, N., & Cordero-Otero, R. (2016). Viability Enhancement of Schizosaccharomyces pombe Cells During Desiccation Stress. *Journal of Microbiology Research*, *6*, 82-91.

Rodríguez-Porrata, B., Carmona-Gutierrez, D., Reisenbichler, A., Bauer, M., López, G., Escoté, X., Mas, A., Madeo, F., & Cordero-Otero, R., (2012). Sip18 hydrophilin prevents yeast cell death during desiccation stress. *Journal of Applied Microbiology, 112,* 512-525.

Rodríguez-Porrata, B., Lopez-Martinez, G., Redón, M., Sancho, M., Mas, A., Rozès, N., & Cordero-Otero, R. (2011). Enhancing yeast cell viability after dehydration by modification of the lipid profile. *World Journal of Microbiology and Biotechnology*, *27*, 75-83.

Rodríguez-Porrata, B., Novo, M., Guillamón, J., Rozès, N., Mas, A., & Cordero - Otero, R. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *International Journal of Food Microbiology*, *126*, 116-122.

Rubin, H. (2005). Magnesium: The missing element in molecular views of cell proliferation control. *BioEssays*, *27*, 311-320.

Shobayashi, M., Fujii, T., & Iefuji, H. (2007). Effects of accumulated S -Adenosylmethionine on growth of yeast cells. *Biosciince, Biotechnology, and Biochemistry*, *71*, 1595-1597.

Stalberg, K., Neal, A. C., Ronne, H., & Stahl, U. (2008). Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*. *Journal of Lipid Research*, *49,* 1794-1806.

Tabor, C. W., & Tabor, H. (1984). Polyamines. *Annual Review of Biochemistry*, *53*, 749-790.

Tariba, B. (2011). Metals in wine - impact on wine quality and health outcomes. *Biological Trace Element Research*, *144*, 143-156.

Trofimova, Y., Walker, G., & Rapoport, A. (2010). Anhydrobiosis in yeast: Influence of calcium and magnesium ions on yeast resistance to dehydration-rehydration. *FEMS Microbiology Letters*, *308*, 55-61.

Udeh, O., Udeh, H. O., & Kgatla, T. E. (2013). Role of magnesium ions on yeast performance during very high gravity fermentation. *Journal of Brewing and Distilling*, *4*, 19-45.

Uemura, T., Higashi, K., Takigawa, M., Toida, T., Kashiwagi, K., & Igarashi, K. (2009). Stimulation of COX4 synthesis by spermidine at the level of translation. *The International Journal of Biochemistry & Cell Biology*, *41*, 2538-2545.

Valdés-Santiago, L., & Ruiz-Herrera, J. (2014). Stress and polyamine metabolism in fungi. *Frontiers on Chemistry*, *1*, 1-10.

Walker, G. M. (1999). Biotechnological implications of the interactions between magnesium and calcium. *Magnesium Research*, *12*, 303-309.

Walker, G. M. (2004). Metals in yeast fermentation processes. *Advances in Applied Microbiology*, *54*, 197-229.

CHAPTER V

Metabolism of *Schizosaccharomyces pombe* under reduced osmotic stress conditions afforded by fed-batch alcoholic fermentation of white grape must

Gemma Roca-Domènech[1], Ricardo Cordero-Otero[1], Nicolas Rozès[1], Marilyn Cléroux[2], Arnaud Pernet[2], Ramón Mira de Orduña[2,*]

[1] Department of Biochemistry and Biotechnology, Faculty of Oenology. University Rovira i Virgili, 43007- Tarragona, Catalonia, Spain

[2] University of Western Switzerland, Changins College for Viticulture and Enology, Nyon, Switzerland

* Corresponding Author

Manuscript accepted in *Food Research International*

Abstract

Strains of *Schizosaccharomyces pombe* are being increasingly investigated with regards to their grape winemaking potential either in combination with the typical production yeast, *Saccharomyces cerevisiae*, or in monoseptic fermentations. Their ethanol tolerance and ability to degrade L-malic acid is oenologically convenient but contrasts with the comparatively high acetic acid and acetaldehyde formation potential which is considered undesirable, especially in white winemaking. The purpose of this work was to investigate the performance of a selected *Schiz. pombe* strain in monoseptic femerntations of white grape must. Traditional batch fermentations were compared with an innovative and automated fed-batch fermentation technique where sugar concentrations are kept low during fermentations to decrease sugar induced osmotic stress. Because of its known effect on growth and ethanol tolerance, the effect of Mg was also tested. While Mg supplementation was not shown to significantly influence residual values of sugars, ethanol, glycerol, organic acids and acetaldehyde, the application of the fed-batch technique led to a fundamental change in yeast physiology. While glycerol values were only slightly reduced, the fed-batch approach allowed obtaining wines devoid of acetic acid whose levels were considerable in wines produced by the traditional batch technique (0.6 g/L). The work demonstrates that the acetic acid metabolism of in *Schiz. pombe* is associated to sugar induced osmotic stress such as for *S. cerevisiae*, too, and may be controlled by application of suitable fermentation techniques for winemaking.

Keywords: *Schizosaccharomyces pombe*, grape wine, non-*Saccharomyces*, fed-batch, acetic acid

Introduction

Alcoholic fermentation (AF) is a complex biochemical process based on the microbial conversion of sugars to ethanol, carbon dioxide and fermentation by-products, such as glycerol, organic acids and carbonyls (Pretorius *et al.,* 1999). In winemaking, *Saccharomyces cerevisiae* is typically considered the main responsible for AF, whereas non-*Saccharomyces* yeasts have usually been considered as 'spoilage' yeasts because of their association with products presenting organoleptic defects (Fleet *et al.,* 1984; Ciani *et al.,* 2010; Contreras *et al.,* 2014). The excessive production of acetic acid, acetaldehyde, and ethyl acetate is among the potential undesirable metabolic activities of non-*Saccharomyces* wine yeasts (Ciani *et al.,* 2010).

However, growing anecdotal and experimental evidence obtained over the last decade suggests that the winemaking potential of non-*Saccharomyces* yeast has been undervalued and that selected strains may play a relevant role with regards to aroma complexity and final wine quality (Ciani & Maccarelli, 1998; Ciani *et al.*, 2010; Padilla *et al.,* 2016). *Schizosaccharomyces pombe* is of particular interest. Unlike most other non-*Saccharomyces*, *Schiz. pombe* can ferment grape musts to dryness because of its high alcohol and SO_2 tolerance (Koukou *et al.,* 1990; Queiroz & Pareilleux, 1990). In addition to its fermentation performance, it has several interesting metabolic capabilities, too. Notably, *Schiz. pombe* strains have been shown to convert L-malic acid into ethanol and CO_2, to reduce gluconic acid (Peinado *et al.,* 2009), and to express urease (Taillandier *et al.,* 1995; Silva *et al.,* 2003; Benito *et al.,* 2013, 2016) and to produce significant amounts of cell wall polysaccharides (Domizio *et al.,* 2017). Over the last years, several studies considering the nutrition, growth and metabolism of *Schiz. pombe* (Hoffman *et al.,* 2015; Petersen & Russell, 2016) have been presented. However, fundamental aspects of the metabolism of *Schiz. pombe* such as its response to osmotic stress remain unknown. Recently, it was shown that reducing sugar induced osmotic stress during alcoholic fermentation by application of a fed-batch approach led to fundamental changes in the metabolism of *S. cerevisiae* that may be favourable for wine quality (Frohman & Mira de Orduña, 2013). Notably, the formation of the osmotic stress associated metabolite acetic acid was greatly reduced.

The aim of the current work was to investigate the metabolic response of *Schiz. pombe* under reduced osmotic stress conditions attained by an automated fed-batch technique (Pernet *et al.,* 2015) allowing alcoholic fermentation at constant and reduced sugar levels. The kinetics of sugar, ethanol and several secondary metabolites were documented. In addition, the effect of medium magnesium supplementation was considered based on previous works showing that Mg plays a crucial role in metabolism (Walker *et al.,* 1982), cell division (Wolf *et al.,* 2008) and may reduce ethanol toxicity associated stress (Dombek & Ingram, 1986; Chun-Keng *et al.,* 2003). The *Schiz. pombe* strain used was selected in a previous study based on its ethanol resistance, fermentation performance and L-malic acid degradation capacity, as well as its resistance towards the dehydration conditions applied for starter preparation (Roca-Domènech *et al.,* 2016).

Materials and methods

Medium, microorganism and culture conditions

The same flash-pasteurized Riesling must from the Palatinate region of Germany was used for all batch and fed-batch fermentations. Its sugar concentration was 191.9 g/L combined glucose and fructose, the pH was 3.37, the titratable acidity was 4.72 g/L expressed as tartaric acid and the yeast assimilable nitrogen (YAN) concentration was 150 mg/l N. For nitrogen yeast nutrition, the must was supplemented with 0.3 g/L of a complex supplement (Uvavital D, Eaton, Germany) and 0.2 g/L of laboratory grade $(NH_4)_2HPO_4$ (ACS, Sigma Aldrich, EU). Mg supplemented must was produced by increasing the native Mg concentration of 45 mg/L with $MgSO_4$ to reach 167 mg/L Mg.

Schiz. pombe strain CECT11197 was obtained from the culture collection of the Department of Biochemistry and Biotechnology, University Rovira i Virgili, Tarragona, Spain and was pre-grown under aerobic conditions in YEPD (Yeast Extract Peptone Dextrose Medium, Fisher Scientific) in shaker flasks (150 rpm) for 40 h at 25°C to obtain a yeast starter for inoculations. Batch fermentations were conducted by adding the yeast starter to the entire amount of must in 5 L glass bottles (Schott Duran, Germany) sealed with suitable air locks to allow for fermentation gas release and to prevent air ingress. These fermentations were inoculated to a titer of $1x10^7$ cells/mL as determined by a Neubauer counting chamber with respect to the total must volume.

Fed-batch treatments were conducted according to (Pernet *et al.,* 2015). Briefly, a Fourier-Transform Near-Infrared (FT-NIR) Spectrophotometer (MPA, Bruker, Germany) equipped with a suitable relfectance *in-situ* probe (IN271F, Bruker Optics Germany) was used to quantify the concentrations of sugars and ethanol in real-time based on previously established prediction models using the equipment's chemometric software (OPUS, Bruker, Germany). Sugar concentration data was then used to automatically control a peristaltic pump (Reglo ICC Digital Peristaltic Pump, 3-channels, Ismatec, WA) that delivered the fresh Riesling must (kept at 0°C) to the fermentation at such rates as to keep sugar concentrations stable at approximately 65 g/L during fermentations. Fed-batch fermentations had a starting volume of 68 mL consisting of 45 mL of yeast starter and 23 mL of flash-pasteurized Riesling must to obtain an initial sugar concentration of approximately 65 g/L. Inoculation occurred at a titre of $1x10^7$ cells/mL as determined by a Neubauer counting chamber with respect to the total final must volume to be delivered (1.8 L). During the initial phase of fed-batch fermentations (up to a volume of 500 mL), the head space of fed-batch incubations was flushed with CO_2 in order to prevent oxidation. All fermentations were carried out at 22±1°C.

Sample taking, analytical methods and statistical analysis

Viable cell numbers expressed in colony forming units per mL were obtained by plating out and counting serial dilutions of samples on solid commercial YPD medium (YPD agar, FisherScience) and incubation at 28°C for 48 h. For chemical analyses, the biomass was removed by centrifugation (5 min, 15,000 *g*) and the supernatant stored frozen (-20°C) until analysis. YAN was measured by quantifying amino nitrogen according to the NOPA method (Dukes & Butzke, 1998) and inorganic nitrogen by means of an enzymatic method for ammonia (Megazyme, Ireland). Sugars, ethanol, glycerol, acetic acid, lactic acid and malic acid were analysed with a HPLC system (Agilent 1260 Infinity, EU) equipped with a 64225A degasser, a G1310B isocratic pump, a GT329B autosampler, a G1316A column oven, a G1314F UV detector and a refractive index detector (RI-102, Shodex). Data acquisition and analysis were performed with the instrument software provided (Agilent OpenLab CDS Chemstation v.A02.09). The mobile phase consisted of 0.65 mM H_2SO_4 and was filtered prior to utilization (0.22 μ m, nylon, Millipore, Ireland). 500 μ L of sample was mixed with 4.5 mL of the mobile

phase and cleaned up using a commercial solid phase extraction cartridge loaded with 200 mg sorbent (Waters Oasis HLB 6cc). 20 µL sample was then injected and separated at 80°C on a PS-DVB phase (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad) at a flow rate of 0.5 mL/min. Sugars, ethanol and acids were quantified by refractive index while acetic acid was quantified by UV spectroscopy at 210 nm. Acetaldehyde was measured enzymatically with a commercial test kit (Megazyme, UK). External calibration standards were used to validate all analyses.

All fermentations were carried out in triplicate and data representation and rate fittings were carried out using OriginLab Origin v7.0. Results were statistically analysed using the XLSTAT 19.5 statistical software package.

Results

Sugars and ethanol

Figure 38 shows the time course of sugar and ethanol concentrations in traditional batch (A) and fed-batch (B) fermentations. Traditional batch fermentations without and with Mg-supplementation reached dryness (<5g/L of sugar) after 149 h. In fed-batch fermentations, sugars were kept constant at approximately 65 g/L until all must was consumed and during this period (100 h), 6-7% (v/v) of ethanol were reached. After conclusion of the must-feeding phase, the residual sugar was also completely degraded after 149 h irrespective of Mg supplementation. All fermentations displayed a slight glucophilic sugar degradation behaviour that was not significantly influenced by the type of fermentation or Mg supplementation (Figure 39).

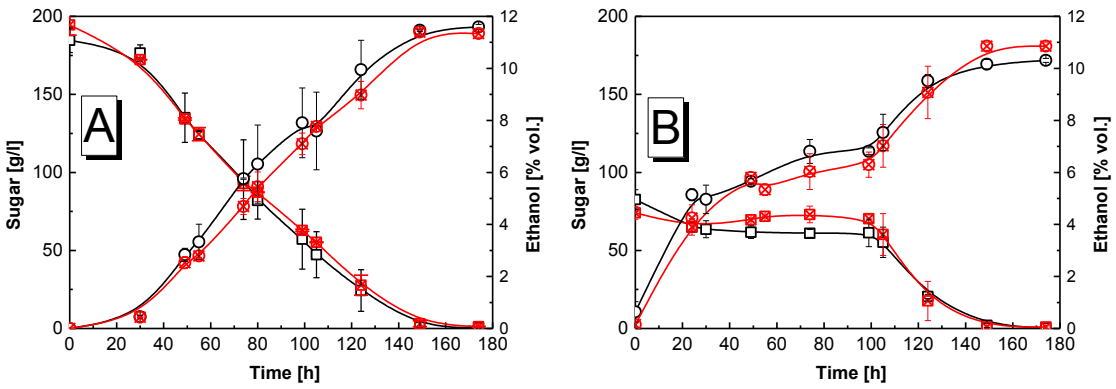


Figure 38. Consumption of sugar (□) and production of ethanol (○) during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by red symbols with crosses (sugar: □; ethanol: ○). Average values ± SD shown (n = 3).

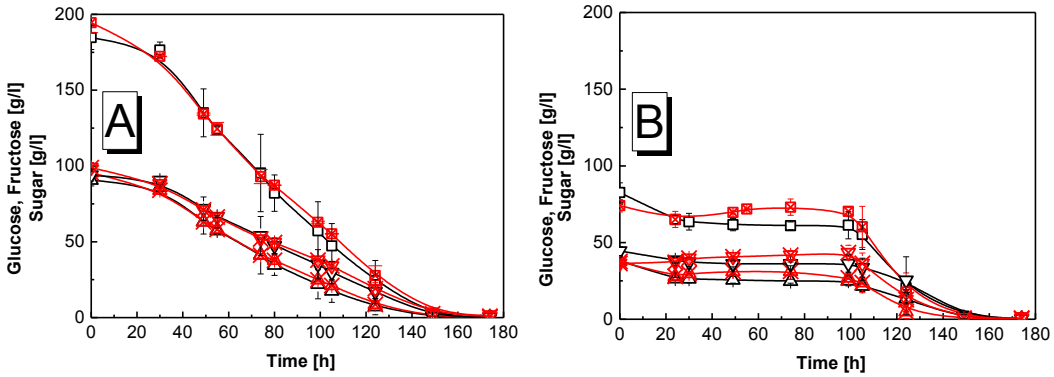


Figure 39. Time course of total sugars (glucose+fructose, □), glucose (Δ) and fructose (▽) during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by red symbols with crosses (total sugars: □; glucose: Δ; fructose: ▽). Average values ± SD shown (n = 3).

There was no statistically significant difference between the residual sugar concentrations of any of the treatments (Table 11). In spite of this, significant differences were found with regards to the final ethanol concentrations. Wines produced with the fed-batch technique had 0.55-1.3% (v/v) less ethanol (Table 11).

Table 11. Final concentrations of various fermentation parameters. Values displayed are means of triplicate experiments ± SD. Different superscript letters indicate statistical significance between averages at $p < 0.05$.

	Total Sugar (g/L)	Ethanol (% vol.)	Glycerol (g/L)	Acetic ac. (g/L)	Acetaldehyde (mg/L)	Malic ac. (g/L)	Lactic ac. (g/L)
Batch	0.81±0.08^{a}	11.47±0.10^{a}	11.96±1.11^{a}	0.55±0.07^{a}	67.54±26.45^{a}	n.d.	0.12±0.00^{a}
Batch+Mg	3.04±1.85^{a}	11.40±0.13^{ab}	11.64±0.05^{ab}	0.66±0.04^{a}	50.52±2.60^{a}	n.d.	0.12±0.00^{a}
Fed-Batch	1.94±1.33^{a}	10.16±0.18^{c}	9.52±0.90^{ab}	n.d.	35.95±1.32^{a}	n.d.	0.17±0.02^{a}
Fed-Batch+Mg	0.96±0.79^{a}	10.85±0.16^{b}	8.83±0.49^{b}	n.d.	39.94±2.5^{a}	n.d.	0.16±0.01^{a}

n.d.: not detected

Malic acid degradation

From an initial must concentration of 2.6 g/L, malic acid was rapidly degraded (<60 h) in traditional batch fermentations. In fed-batch incubations, malic acid concentrations never exceeded 0.6 g/L and remained below the LOD for most of the duration of incubations. The higher initial malic acid concentration in the fed-batch treatment with added Mg was attributable to differences in the process control (must

feeding rate). Except for this slight difference, Mg supplementation had no effect on the time course of malic or lactic acid levels, which stayed relatively constant throughout all fermentations. There was a slight trend towards higher final lactic acid concentrations in wines produced by the fed-batch technique, which was not statistically relevant (Figure 40).

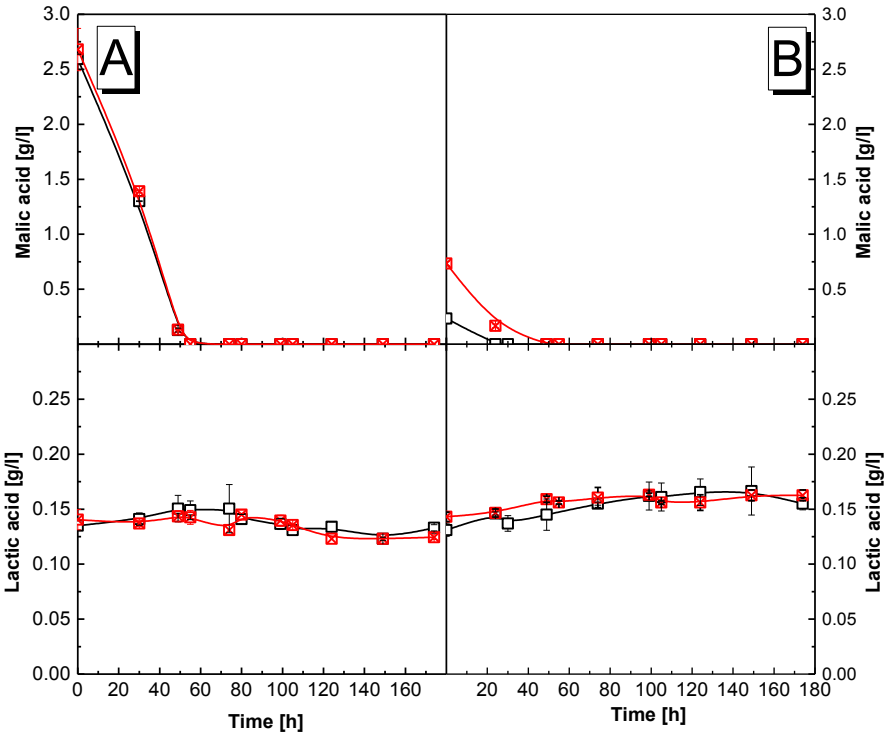


Figure 40. Time course of malic and lactic acid during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by symbols with crosses. Average values ± SD shown (n = 3).

Sugar induced osmotic stress response related metabolites

Figure 41 shows the kinetics of several sugar induced osmotic stress associated fermentation by-products in traditional batch and fed-batch fermentations. Supplementation with Mg led to slightly different kinetics of glycerol and acetic acid in some incubations. However, the effects were not consistent and Mg supplmentation had no statistically significant effect on the final values (Table 11) of any of the metabolites. In contrast, significant differences were observed with regards to the kinetics and the final values between traditional batch and fed-batch fermentations. In batch fermentations, glycerol levels increased rapidly up to 70-80 h of fermentation time and then stayed constant. In fed-batch incubations, glycerol formation already levelled off after 30 h and final glycerol concentrations were approximately 20% lower

(Table 11). The differences were striking with regards to acetic acid kinetics and final values (Figure 41, Table 11). A strong initial production of acetic acid in traditional batch fermentations transitioned to a slower production phase after approximately 90 h resulting in a mean final concentration of 600 mg/L acetic acid across all batch incubations. In contrast, acetic acid never exceeded 220 mg/L in fed-batch incubations (Figure 41) and no acetic acid was detectable in any of the fed-batch incubations after 25 h of incubation time (Table 11). The course of acetaldehyde concentrations followed typical kinetics with an initial increase followed by a partial reuptake (Jackowetz *et al.,* 2011). Peak and final acetaldehyde concentrations were similar across all incubations. There was a trend towards lower residual acetaldehyde concentrations (40% less) in wines produced by the fed-batch technique, which was not statistically significant.

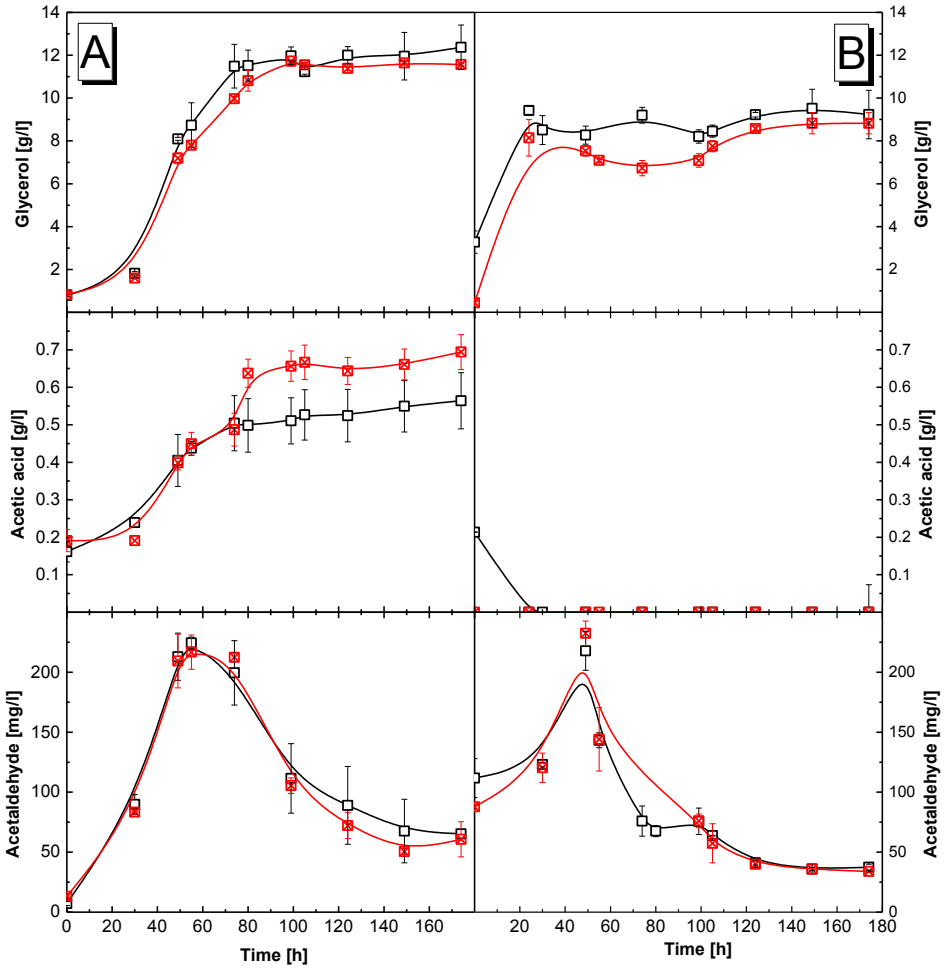


Figure 41. Time course of sugar induced osmotic stress associated metabolites glycerol, acetic acid and acetaldehyde during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by symbols with crosses. Average values ± SD shown (n = 3).

Discussion

Shizosaccharomyces pombe is a grape must fermentation associated non-*Saccharomyces* yeast with significant potential for winemaking (Benito *et al.,* 2016). *Schiz. pombe* strains have been shown to possess the ability to completely convert grape sugars to alcohol, and to degrade malic acid (Dharmadhikari & Wilker, 1998), which typically has to be achieved during a malolactic fermentation (Sumby *et al.,* 2014). In addition, its production of aldehyde compounds may be of interest for red wine colour development (Morata *et al.,* 2012; Mylona *et al.,* 2016).

In this work, its utilization as sole production organism was studied in monoseptic white grape must fermentations in traditional batch incubations, as well as fed-batch fermentations that have been shown to improve fermentation performance of *S. cerevisiae* and reduce the production of undesirable fermentation by-products (Frohman & Mira de Orduña, 2013). In addition, grape must supplementation with Mg was investigated. In *S. cerevisiae*, magnesium has been shown to act as a growth enhancer and to increase ethanol tolerance (Walker *et al.,* 1982). Mg supplementation has also been associated with improved growth and ethanol tolerance in *Schiz. pombe* (Hu *et al.,* 2003). However, in this study, Mg supplementation was not found to have a consistent effect on the time course or final values of any of the metabolites studied. Although the natural Mg concentration of the grape must used (45 mg/l Mg) was relatively low with regards to typical must Mg levels reported in the literature (50-200 mg/L Mg, Margalit, 2016), it is possible that it was still too high to cause a Mg deficiency. Since specific chelation for Mg sequestration is difficult, the effect of Mg may be better studied in synthetic grape must fermentations.

Overall, the differences caused by the fermentation mode were notable. While sugar degradation was complete in all treatments, final alcohol concentrations were slightly reduced in wines produced by the fed-batch technique. In studies with *S. cerevisiae*, fed-batch incubations did not lead to ethanol reductions compared with the traditional batch approach (Frohman & Mira de Orduña, 2013). As much as an ethanol reduction would be desirable considering the climate change associated increase in wine ethanol concentrations and its implications (Mira de Orduña, 2010), it is suggested that the results obtained in this study were an artefact of the technique applied rather than a physiological response to lower sugar concentrations.

Specifically, the CO_2 flushing applied to prevent oxidation in the small initial fed-batch fermentation volume is expected to have caused ethanol stripping.

Unlike malolactic fermentation (MLF) by lactic acid bacteria (Lonvaud-Funel, 1999), malic acid degradation by *Schiz. pombe* does not lead to lactic acid formation, which can lead to lactic notes from formation of ethyl lactate after esterification. Rather, malic acid metabolism by *Schiz. pombe* leads to formation of ethanol and CO_2 (Benito *et al.,* 2016). In both fermentation treatments, malic acid was degraded below the detection threshold of the method applied. Because of the high initial cell density present in fed-batch fermentations, must was essentially depleted from malic acid upon introduction to fed-batch fermentations. Lactic acid concentrations remained almost constant in all treatments, confirming the significant potential of *Schiz. pombe* for biological deacidification especially in high acidity musts.

The polyol glycerol is an alcoholic fermentation by-product and plays significant roles in yeast physiology such as maintaining cellular redox homeostasis and functioning as an osmoprotectant (Michnick *et al.,* 1997; Scanes *et al.,* 1998). Consequently, under high sugar induced osmotic pressure conditions, glycerol formation is increased and correlates with formation of acetic acid in *S. cerevisiae* (Pigeau & Inglis, 2007). A recent study investigating the alcoholic fermentation of *S. cerevisiae* showed that the reduced osmotic stress afforded by a fed-batch approach led to a 45% reduction in final glycerol and a 80% reduction in final acetic acid levels as compared with the traditional batch approach (Frohman & Mira de Orduña, 2013). In traditional batch fermentations, the *S. pombe* strain CECT11197 used in this study yielded acetic acid concentrations (0.55 g/L) that were similar to those reported by Mylona *et al.,* (2016) for another *S. pombe* strain (0.51 g/L for strains 7VA) but significantly lower than those reported by Miljić *et al.* (2017) and Mylona *et al.,* (2016, for strains 2139 and 938) which exceeded 1 g/L of acetic acid. Conversely, Mylona *et al*. (2016) and especially Du Plessis *et al.* (2017) reported much lower levels for *S. pombe* strains than ranged between 0.07 g/L (expressed as volatile acidity) and 0.35 g/L acetic acid. In this study, the fed-batch approach allowed obtaining wines devoid of acetic acid while glycerol levels still remained higher than those repoted by Mylona *et al.,* (2016). Accordingly, application of the fed-batch technique may allow to greatly reduce volatile acidity even with strains that produce significant amounts of acetic acid

in traditional batch culture, while maintaining relatively high levels of glycerol, which is associated with positive sensory notes in wine (Noble & Bursick, 1984; Lubbers *et al.,* 2001).

Acetaldehyde is the major carbonyl compound formed during alcoholic fermentation and has implications for sensory qualities and stability of wines as well as for the use of the wine preservative SO_2 (Jackowetz & Mira de Orduña, 2013). Compared with other wine associated yeast, *Schiz. pombe* produced relatively large amounts of acetaldehyde in resting cell and grape must incubations (Li & Mira de Orduña, 2011, 2017). While this metabolic property may be interesting in red wine fermentations where acetaldehyde contributes to the stabilization of red wine colours (Mylona *et al.,* 2016; Benito *et al.,* 2017), in white wines it is considered undesirable since acetaldehyde will bind signifcant quantities of SO_2 (Jackowetz *et al.,* 2011), a wine conservative whose levels are sought to be limited. In this study, there was a trend towards lower acetaldehyde levels in fed-batch treatments. In fact, the residual acetaldehyde concentrations of 40 mg/L in wines produced by the fed-batch technique in this study were congruent with the average concentrations measured in 127 commercial white wines in a recent study (Jackowetz & Mira de Orduña, 2013). Hence, in spite of the higher known acetaldehyde production potential of *Schiz. pombe* strains, application of *Schiz. pombe* strain CECT11197 with the fed-batch technique allowed obtaining wines with residual acetaldehyde levels comparable to current commercial standards. The acetaldehyde residues obtained with the *Schiz. pombe* strains selected by Mylona *et al.,* (2016) were even lower in batch fermenations. Accordingly, application of these superior strains with a fed-batch technique may allow to further reduce acetaldehyde levels.

Conclusions

The current work demonstrated the suitability of a selected *Shizosaccharomyces pombe* strain for monoseptic fermentations of white grape juice. Its ability to completely convert grape sugars to alcohol and its strong malic acid degradation capacity may be advantageous for a number of vinification challenges including acidic white musts. Applying a novel fed-batch technique, it was possible to avoid formation of acetic acid. The application of suitable strains reported elsewhere with the

innovative fed-batch fermentation approach may provide for an efficient utilization of *Schiz. pombe* in white winemaking.

Acknowledgements

This work was supported by grant AGL2012-40018 from the Spanish Ministry of Science and Innovation. G. Roca-Domènech acknowledges Rovira i Virgili University for the doctoral fellowship 2014PMF-PIPF-47.

References

Benito, Á., Calderón, F., & Benito, S. (2017). The combined use of *Schizosaccharomyces pombe* and *Lachancea thermotolerans*-effect on the anthocyanin wine composition. *Molecules*, *22(5)*, 739.

Benito, Á., Jeffares, D., Palomero, F., Calderón, F., Bai, F. Y., Bähler, J., & Benito, S. (2016). Selected *Schizosaccharomyces pombe* strains have characteristics that are beneficial for winemaking. *PLoS ONE, 11,* e0151102.

Benito, S., Palomero, F., Morata, A., Calderón, F., Palmero, D., & Suárez-Lepe, J. A. (2013). Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. *European Food Research and Technology, 236,* 29-36.

Chun-Keng, H., Feng-Wu, B., & Li-Jia, A. (2003). Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} via reduction in plasma membrane permeability. *Biotechnology Letters, 25,* 1191-1194.

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

Ciani, M., & Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14,* 199-203.

Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C., & Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Applied and Environmental Microbiology, 80,* 1670-1678.

Dharmadhikari, M. R., & Wilker, K. L. (1998). Deacidification of high malate must with *Schizosaccharomyces pombe*. *American Journal of Enology and Viticulture, 49,* 408-412.

Dombek, K. M., & Ingram, L. O. (1986). Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. *Applied and Environmental Microbiology, 52,* 975-981.

Domizio, P., Liu, Y., Bisson, L. F., & Barile, D. (2017). Cell wall polysaccharides released during the alcoholic fermentation by *Schizosaccharomyces pombe* and *S. japonicus*: quantification and characterization. *Food Microbiology, 61*, 136-149.

Dukes, B. C., & Butzke, C. E. (1998). Rapid determination of primary amino acids in grape juice using an *o*-phtalaldehyde/N-acetyl-L-cysteine spectrophotometric assay. *American Journal of Enology and Viticulture, 49,* 127-134.

Du Plessis, H., Du Toit, M., Hoff, J., Hart, R., Ndimba, B. & Jolly, N. (2017). Characterisation of Non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. *South African Journal of Enology and Viticulture*, *38,* 46-63.

Fleet, G. H., Lafon-Lafourcade, S., & Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology, 48,* 1034–1038.

Frohman, C. A., & Mira de Orduña, R. (2013). Cellular viability and kinetics of osmotic stress associated metabolites of *Saccharomyces cerevisiae* during traditional batch and fed-batch alcoholic fermentations at constant sugar concentrations. *Food Research International, 53,* 551–555.

Hoffman, C. S., Wood, V., & Fantes, P. A. (2015). An ancient yeast for young geneticists: a primer on the *Schizosaccharomyces pombe* model system. *Genetics, 201,* 403-423.

Hu, C. K., Bai, F. W., & An, L. J. (2003). Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} via reduction in plasma membrane permeability. *Biotechnology Letters, 25,* 1191-1194.

Jackowetz, J. N., Dierschke, S. E., & Mira de Orduña, R. (2011). Multifactorial analysis of acetaldehyde kinetics during alcoholic fermentation by *Saccharomyces cerevisiae*. *Food Research International, 44,* 310-316.

Jackowetz, J. N., & Mira de Orduña, R. (2013). Survey of SO_2 binding carbonyls in 237 red and white table wines. *Food Control, 32,* 687-692.

Koukou, A. I., Tsoukatos, D., & Drainas, C. (1990). Effect of ethanol on the phospholipid and fatty acid content of *Schizosaccharomyces pombe* membranes. *Journal of General Microbiology, 136,* 1271-1277.

Li, E., & Mira de Orduña, R. (2011). Evaluation of the acetaldehyde production and degradation potential of 26 enological *Saccharomyces* and non-*Saccharomyces* yeast strains in a resting cell model system. *Journal of Industrial Microbiology & Biotechnology, 38,* 1391-1398.

Li, E., & Mira de Orduña, R. (2017). Acetaldehyde kinetics of enological yeast during alcoholic fermentation in grape must. *Journal of Industrial Microbiology & Biotechnology, 44,* 229-236.

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek, 76,* 317-331.

Lubbers, S., Verret, C., & Voulley, A. (2001). The effect of glycerol on the perceived aroma of a model wine and a white wine. *Lebensmittel-Wissenschaft & Technologie, 34,* 262-265.

Margalit, Y. (2016). *Concepts in wine chemistry*. Board & Bench Publishing.

Michnick, S., Roustan, J. L., Remize, F., & Barre, P. (1997). Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase. *Yeast, 13,* 783-793.

Miljić, U., Puškaš, V., Vučurović, V. & Muzalevski, A. (2017). Fermentation characteristics and aromatic profile of plum wines produced with indigenous microbiota and pure cultures of selected yeast. *Journal of Food Science, 82,* 1443-1450.

Mira de Orduña, R. (2010). Climate change associated effects on grape and wine quality and production. *Food Research International*, *43,* 1844-1855.

Morata, A., Benito, S., Loira, I., Palomero, F., Gonzalez, M. C., & Suarez-Lepe, J. A. (2012). Formation of pyranoanthocyanins by *Schizosaccharomyces pombe* during the fermentation of red must. *International Journal of Food Microbiology, 159,* 47-53.

Mylona, A. E., Del Fresno, J. M., Palomero, F., Loira, I., Bañuelos, M. A., Morata, A., Calderón, F., Benito, S., & Suárez-Lepe, J. A. (2016). Use of *Schizosaccharomyces* strains for wine fermentation: Effect on the wine composition and food safety. *International Journal of Food Microbiology, 232,* 63-72.

Noble, A. C., & Bursick, G. F. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture, 35,* 110-112.

Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non- *Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology, 7,* 1-20.

Pernet, A., Mira de Orduña, R., Widmer, D., Riedo, A., Mertenat, M., Cléroux, M. Bourgeois, J-P., Vvorlet, O., & Frohman, C. (2015). Dosage en continu des sucres et de l'alcool pendant les fermentations par spectroscopie proche infrarouge - Une opportunité pour l'automatisation des vinifications et les défis œnologiques actuels. *Revue Suisse de Viticulture Arboriculture Horticulture, 47,* 192-197.

Peinado, R. A., Maestre, O., Mauricio, J. C., Moreno, J. J. (2009). Use of a *Schizosaccharomyces pombe* mutant to reduce the content in gluconic acid of must obtained from rotten grapes. *Journal of Agricultural and Food Chemistry*, *57,* 2368-2377.

Petersen, J., & Russell, P. (2016). Growth and the Environment of *Schizosaccharomyces pombe. Cold Spring Harbor Protocols, 3,* 1-17.

Pigeau, G. M., & Inglis, D. L. (2007). Response of wine yeast *Saccharomyces cerevisiae* aldehyde dehydrogenases to acetaldehyde stress during Icewine fermentation. *Journal of Applied Microbiology, 103,* 1576-1586.

Pretorius, I. S., Van der Westhuizen, T. J., & Augustyn, O. P. H. (1999). Yeast biodiversity in vineyards and wineries and its importance to the South African Wine Industry: a review. *South African Journal of Enology and Viticulture, 2,* 61-75.

Queiroz, H., & Pareilleux, A. (1990). Applied Microbiology Biotechnology Growth kinetics of *Schizosaccharomyces pombe* under various culture conditions: influence of pH, malate, ethanol and oxygenation. *Applied Microbiology and Biotechnology, 33,* 578-581.

Roca-Domènech, G., López- Martínez, G., Tirado, V., Borrull, A., Candelas, Ó., Rozès, N., & Cordero-Otero, R. (2016). Viability enhancement of *Schizosaccharomyces pombe* cells during desiccation stress. *Journal of Microbiology Research, 6,* 82-91.

Scanes, K. T., Hohmann, S., & Priori, B. A. (1998). Glycerol Production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. *South African Journal of Enology and Viticulture, 19,* 17-24.

Silva, S., Ramón-Portugal, F., Andrade, P., Abreu, S., Texeira, M. D. F., & Strehaiano, P. (2003). Malic acid consumption by dry immobilized cells of *Schizosaccharomyces* pombe. *American Journal of Enology and Viticulture, 54,* 50-55.

Sumby, K. M., Grbin, P. R., & Jiranek, V. (2014). Implications of new research and technologies for malolactic fermentation in wine. *Applied Microbiology and Biotechnology, 98,* 8111-8132.

Taillandier, P., Gilis, M., & Strehaiano, P. (1995). Deacidification by *Schizosaccharomyces*: interactions with *Saccharomyces*. *Journal of Biotechnology, 40,* 199-205.

Walker, G. M., Birch-Andersen, A., Hamburger, K., & Kramhoft, B. (1982). Magnesium-induced mitochondrial polymorphism and changes in respiratory metabolism in the fission yeast, *Schizosaccharomyces pombe*. *Carlsberg Research Communications, 47,* 205-214.

Wolf, F. I., Trapani, V., & Cittadini, A. (2008). Magnesium and control of cell proliferation: looking for a needle in a haystack. *Journal of the Institute of Brewing*, *21,* 83-91.

CHAPTER VI

.

General Discussion

The useful physiological properties of yeasts have led to their use in the field of biotechnology. Fermentation of sugars by yeasts is the oldest and largest application of this technology. Although *Saccharomyces cerevisiae* has been considered the primary yeast used in the winemaking process, non-*Saccharomyces* yeast strains can also be detected throughout wine fermentation. Non-*Saccharomyces* yeasts can therefore influence the course of fermentation and also the organoleptic characteristics of the resultant wine (Jolly *et al.,* 2006). Specifically, *Starmerella bacillaris* and *Schizosaccharomyces pombe* are two non-*Saccharomyces* yeasts with significant potential for winemaking. *S. bacillaris* strains are psychrotolerant, osmotolerant and fructophilic yeasts, while *Schiz. pombe* strains have the ability to completely convert sugars to alcohol, because of their high alcohol and SO_2 tolerance, and to degrade malic acid (Dharmadhikari & Wilker, 1998; Magyar & Tóth, 2011; Tofalo *et al.,* 2012). However, the winemaking process is a hostile environment for yeasts, especially for non-*Saccharomyces* yeasts.

Nowadays, active dry wine yeasts (ADWY) are the most widely used in winemaking, ensuring the strain identity and genetic stability at room temperature, and reducing costs of transportation and storage. However, the drying and rehydration processes induce several stress situations (e.g. heat, osmotic and oxidative) with the consequent loss of cell viability (Rodríguez-Porrata *et al.,* 2011). Previously research in our group worked in an ADWY protocol for *S. cerevisiae*, defining optimized conditions for yeast drying and rehydration processes to improve its viability and vitality (Rodríguez-Porrata *et al.,* 2008). In the present doctoral thesis we developed improved ADWY protocols for *S. bacillaris* and *Schiz. pombe* wine yeasts (see chapters II and III), followed by metabolomics and physiological analyses during desiccation and under reduced osmotic stress conditions of *Schiz. pombe* (see chapters IV and V).

We defined the best physiological cell state to achieve the highest viability after desiccation stress. Both non-*Saccharomyces* yeasts presented the highest desiccation tolerance at the early stationary phase, while previous studies with *S. cerevisiae* showed the highest tolerance during the last stationary phase. Otherwise, dehydration and rehydration processes play a relevant role in desiccation stress tolerance (Rodríguez-Porrata *et al.,* 2008). Both *S. bacillaris* and *Schiz. pombe* strains presented an increase of cell viability in the presence of 10% trehalose during dehydration. These

results agree with results obtained with *S. cerevisiae* (Rodríguez-Porrata, *et al.,* 2011), showing that trehalose may stabilize and prevent the loss of cellular structural and functional integrity during the desiccation-rehydration process. However, both non-*Saccharomyces* yeasts presented different optimal times and temperatures of rehydration between them, suggesting that yeasts present different desiccation tolerance levels (see chapters II and III).

On the other hand, providing all nutritional requirements during fermentation results in physiologically cells able to overcome desiccation stress. Moreover, the most relevant stress responses adapted by yeasts during desiccation are related to osmotic and oxidative stress, whereas one of the main factors that determine the resistance of yeast cells to dehydration-rehydration is the maintenance of the structural integrity of the plasma membrane. Consequently, the rehydration medium was classified depending on different additive compounds: carbon and nitrogen sources, metallic ions, oxidant and antioxidants, and membrane fluidity compounds.

Unexpectedly, in both non-*Saccharomyces* yeast strains, the rehydration with nitrogen sources, oxidant and antioxidant agents, and compounds that enhance the rigidity or fluidity of the membrane did not act as yeast viability enhancers. These results led us to suggest that yeasts prefer nitrogen backup than extracellular sources to resume cell activity during rehydration. Moreover, it also seems that the presence of extracellular antioxidant agents does not enhance the antioxidant capacity of the cells, while the presence of membrane fluidizing agents does not promote any significant effect on the cell viability or it is detrimental.

With regard to the carbon sources, *S. bacillaris* showed a statistically significant viability increase with raffinose and galactose rehydration, whereas *Schiz. pombe*, coinciding with *S. cerevisiae*, did not show any viability enhancement (Rodríguez-Porrata *et al.,* 2008). In addition, the longer λ phase, mediated by raffinose, may provide additional time for correct cell rescue activity and account for the increase in the viability rate (see chapter II).

Besides, metallic ion rehydration showed a deleterious effect on yeast viability in both non-*Saccharomyces* yeast strains, with the exception of the viability enhancement, λ phase extension, and Reactive Oxygen Species (ROS) accumulation reduction of *Schiz. pombe* as a result of the magnesium rehydration (see chapters III

and IV). In *S. cerevisiae,* our group observed similar results between magnesium rehydration and viability enhancement (Rodríguez-Porrata *et al.,* 2008). Magnesium ion is the most abundant divalent metal ion in yeast cells. It exerts a large variety of biological functions, ranging from structural to catalytic roles, and regulatory roles by modulating cell proliferation, cell cycle progression, differentiation and apoptosis (Hartwig, 2000; Walker, 2004).

Bradshaw & Pfeiffer (2014) described that the higher intracellular content of magnesium ion might block mitochondrial unselective channel (MUC), promoting a slow electron flux imparting a reduction in the respiration rate, which may explain the increase of the λ phase after magnesium rehydration. Our results suggest that cells rehydrated with magnesium might require extra to settle the intracellular magnesium to a permissible physiological level before resuming growth.

Moreover, the phenotypic response to dehydration and rehydration processes of *Schiz. pombe* led us to perform a comparative metabolomics analysis of two *Schiz. pombe* strains, CECT11197 (Sp97), CECT12622 (Sp22), in order to determine putative metabolites implicated in the increase of desiccation tolerance in presence of magnesium (see chapter IV).

We evaluated the metabolic profiles before drying (BD), and after water and magnesium rehydration (ARH_2O and ARMg, respectively) of both strains, Sp97 and Sp22, to determine the qualitative intracellular metabolite changes. Especially, Sp97 showed significant changes on the metabolic pathways: glutathione synthesis and recycling, ROS detoxification, and methionine salvage pathway. From the last pathway, methionine sulfoxide, 2-oxo-4-methylthiobutanoic acid and 5'methylthioadenosine (MTA) showed qualitative significant differences between conditions evaluated (BD, ARH_2O and ARMg). Our results led us to suggest that the methionine salvage pathway might have a relevant impact to overcome desiccation stress imposition. Consequently, we evaluated specific metabolites of the methionine salvage pathways, and from related pathways: polyamines pathway, and phospholipid pathway (see chapter IV).

Methionine plays a central role in stress resistance, because it is a direct target of ROS, acting as scavenger and protecting cells from oxidative stress (Campbell *et al.,* 2016). Moreover, it has been reported that products from the methionine salvage

pathway regulate polyamines biosynthesis. MTA is a product of S-adenosyl-1-methionine (SAM), which is synthetized at the methionine salvage pathway. Specifically, intracellular accumulation of MTA has an inhibitory effect over the polyamines biosynthesis, which have regulatory roles in cell growth and in improving ionic equilibrium protecting cells from osmotic and oxidative stresses (Garcea *et al.,* 1987; Chattopadhyay *et al.,* 2006; Uemura *et al.,* 2009; Valdés-Santiago & Ruiz-Herrera, 2014). However, our results after magnesium rehydration does not agree with the MTA inhibitory effect over the polyamine biosynthesis, showing a significant increase of spermine levels after magnesium rehydration in comparison to water rehydration.

Moreover, we also observed a statistically significant increase of SAM, which transfers a group methyl to phsophatidylethanolamine (PE) producing phosphatidylcholine (PC). PC is the major structural component of yeast membranes, and an important source for signalling molecules (Pollard *et al.,* 2017). Accordingly with previous studies (Rodríguez-Porrata et al., 2008), our results showed that the main difference between phospholipids composition was given by a significant decrease of PC contained in both *Schiz. pombe* yeast strains after dehydration and rehydration, whereas the contents of choline and phosphocholine (choline-P) significantly increased after water and magnesium rehydration (see chapter IV). In addition, magnesium rehydration induced a higher significant decrease of PC than water rehydration. Nevertheless, the similar increase of choline and choline-P in both rehydration conditions does not reveal the ion magnesium role in the differential PC values.

Otherwise, SAM is also a precursor of the synthesis of the antioxidant molecular system, glutathione. Glutathione (GSH) plays a role in protection against oxidative stress and is a non-enzymatic scavenger of ROS (Jamieson, 1998). Our findings indicated that the reduced GSH increases after water and magnesium rehydration, while the oxidized GSH decreases. However, only after magnesium rehydration the total GSH was similar to the total GSH before drying, suggesting that the magnesium rehydration promotes the maintenance of total GSH content during desiccation stress imposition (see chapter IV).

Although our previous metabolomics analyses suggested that magnesium rehydration might promote the increase of some metabolites (e.g. methionine, MTA, SAM, spermine), our findings showed that the rehydration with these metabolites does not have the same effect as magnesium on Sp97 viability and ROS accumulation. Moreover, we also observed that magnesium effect on the Sp97 viability during cell rehydration is counterbalanced by the supplementation of any of these compounds, excepting the co-supplementation of MTA and magnesium. A synergistic effect between magnesium and MTA was observed resulting with an enhancement of Sp97 viability and in a reduction of ROS accumulation in comparison to after water and magnesium rehydration (see chapter IV). These findings led us to conclude that there is not a simple correlation between ROS levels and Sp97 cell survival desiccation.

With respect to the lifespan, it is known that the desiccation stress could induce a premature aging due to all the stresses to which the cells are subjected during the process (Potts, 2001). It is known that spermidine acts extending lifespan. Our results showed that magnesium induces a *premature chronological aging in stationary phase* of Sp97 yeast cells and also can inhibit the effect of the spermidine uptake by a described competitive inhibition of magnesium over polyamines uptake (see chapter IV) (Maruyama *et al.,* 1994). We might conclude that magnesium promotes an activation of Sp97 cell metabolism with the consequent exhaustion of nutrients triggering early cellular aging.

On the other hand, as mentioned earlier, yeasts also have to resist several stresses such ethanol, osmotic or oxidative stresses during the alcoholic fermentation (Pretorius, 2000). We developed a study of the metabolic response of *Schiz. pombe* under reduced sugar induced osmotic stress conditions attained by an automated fed-batch technique (Pernet *et al.,* 2015). Since we had been observed that magnesium rehydration increases the *Schiz. pombe* viability after a desiccation stress imposition, we investigated the effect of magnesium in traditional and fed-batch fermentations, evaluating the possible magnesium supplementation effects on the fermentation performance. In *S. cerevisiae* and in *Schiz. pombe*, magnesium has been shown to act as a growth and ethanol tolerance enhancer (Walker *et al.,* 1982; Hu *et al.,* 2003). However, our results showed that magnesium does not have any effect of the fermentation performance in Sp97 in our grape must. The effect of magnesium may be

better studied in synthetic grape must fermentations (see chapter V). However, our results confirmed that *Schiz. pombe* has a significant potential for biological deacidification through the degradation of malic acid without leading lactic acid formation. Moreover, in *S. cerevisiae*, fed-batch fermentations improve fermentation performance and reduce the production of undesirable fermentation by-products (Frohman & Mira de Orduña, 2013). Our findings showed that the application of the fed-batch techniques allows a drastically reduction of the residual acetaldehyde and acetic acid levels maintaining relatively high levels of glycerol (see chapter V), which is associated with positive sensory notes in wine (Noble & Bursick, 1984; Lubbers *et al.,* 2001).

In summary, we can conclude that the effects of magnesium in yeast cells are strain and stress dependent. Magnesium presents certain effects in *S. cerevisiae* and *Schiz. pombe* after desiccation stress, while this effect can not be seen after desiccation in *S. bacillaris* or over the fermentation performance in *Schiz. pombe*. It seems that MTA metabolite accumulation by magnesium rehydration participates in the enhancement of *Schiz. pombe* desiccation stress tolerance. However, the desiccation stress tolerance driven by the magnesium ion in *Schiz. pombe* is not a phenotype resulting from an individual metabolic pathway. Consequently, we can conclude that magnesium has incidence on several metabolic adjustments in *Schiz. pombe* yeast cells to overcome desiccation stress. However, further research is required to evaluate the different roles of magnesium after desiccation stress. Since it has been described the competitive inhibition between magnesium and spermine in *S. cerevisiae* (Maruyama *et al.,* 1994), it would be worth to evaluate the expression of enzymes involved in the polyamine pathway in order to determine if magnesium could interfere in the activity of any of these enzymes, promoting metabolites accumulation.

REFERENCES

Bradshaw, P. C., & Pfeiffer, D. R. (2014). Characterization of the respiration-induced yeast mitochondrial permeability transition pore. *Yeast*, *30,* 471-483.

Campbell, K., Vowinckel, J., Keller, M. A., & Ralser, M. (2016). Resistance via the pentose phosphate pathway. *Antioxidants & Redox Signaling*, *24*, 543-547.

Chattopadhyay, M. K., White Tabor, C., & Tabor, H. (2006). Methylthioadenosine and polyamine biosynthesis in a *Saccharomyces cerevisiae* meu1delta mutant. *Biochemical and Biophysical Research Communications*, *343*, 203-207.

Dharmadhikari, M. R., & Wilker, K. L. (1998). Deacidification of high malate must with *Schizosaccharomyces pombe*. *American Journal of Enology and Viticulture, 49,* 408-412.

Frohman, C. A., & Mira de Orduña, R. (2013). Cellular viability and kinetics of osmotic stress associated metabolites of *Saccharomyces cerevisiae* during traditional batch and fed-batch alcoholic fermentations at constant sugar concentrations. *Food Research International, 53,* 551–555.

Garcea, R., Pascale, R., Daino, L., Frassetto, S., Cozzolino, P., Ruggiu, M. E., Vannini, M. G., Gaspa, L., & Feo, F. (1987). Variations of ornithine decarboxylase activity and S-adenosyl- L-methionine and 5'-methylthioadenosine contents during the development of diethylnitrosamine-induced liver hyperplastic nodules and hepatocellular carcinoma. *Carcinogenesis*, *8*, 653-658.

Hartwig, A. (2000). Recent advances in metal carcinogenicity. *Pure and Applied Chemistry, 72*, 1007-1014.

Hu, C. K., Bai, F. W., & An, L. J. (2003). Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} via reduction in plasma membrane permeability. *Biotechnology Letters, 25*, 1191-1194.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast*, *14,* 1511-1527.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture*, *27,* 15-39.

Lubbers, S., Verret, C., & Voulley, A. (2001). The effect of glycerol on the perceived aroma of a model wine and a white wine. *Lebensmittel-Wissenschaft & Technologie, 34,* 262-265.

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

Maruyama, T., Masuda, N., Kakinuma, Y., & Igarashi, K. (1994). Polyamine-sensitive magnesium transport in *Saccharomyces cerevisiae*. *Biomembranes*, *1194*, 289-295.

Noble, A. C., & Bursick, G. F. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture, 35,* 110-112.

Pernet, A., Mira de Orduña, R., Widmer, D., Riedo, A., Mertenat, M., Cléroux, M. Bourgeois, J-P., Vvorlet, O., & Frohman, C. (2015). Dosage en continu des sucres et de l'alcool pendant les fermentations par spectroscopie proche infrarouge - Une opportunité pour l'automatisation des vinifications et les défis œnologiques actuels. *Revue Suisse de Viticulture Arboriculture Horticulture, 47,* 192-197.

Pollard, T. D., Earnshaw, W. C., Lippincott-Schwartz, J., & Johnson, G. T. (2017). Second Messengers (Chapter 26). *Cell Biology* (3rd ed.). pp 443-462.

Potts, M. (2001). Desiccation tolerance: A simple process? *Trends in Microbiology, 9*, 553-559.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast, 16*, 675-729.

Rodríguez-Porrata, B., López-Martínez, G., Redón, M., Sancho, M., Mas, A., Rozès, N., & Cordero-Otero, R. (2011). Enhancing yeast cell viability after dehydration by modification of the lipid profile. *World Journal of Microbiology and Biotechnology, 27*, 75-83.

Rodríguez-Porrata, B., Novo, M., Guillamón, J. M., Rozès, N., Mas, A., & Cordero-Otero, R. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *International Journal of Food Microbiology, 126*, 116-122.

Stalberg, K., Neal, A. C., Ronne, H., & Stahl, U. (2008). Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*. *Journal of Lipid Research*, *49,* 1794-1806.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., & Suzzi, G. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology*, *29,* 18-26.

Uemura, T., Higashi, K., Takigawa, M., Toida, T., Kashiwagi, K., & Igarashi, K. (2009). Polyamine modulon in yeast - Stimulation of COX4 synthesis by spermidine at the level of translation. *The International Journal of Biochemistry & Cell Biology*, *41*, 2538-2545.

Valdés-Santiago, L., & Ruiz-Herrera, J. (2014). Stress and polyamine metabolism in fungi. *Frontiers on Chemistry*, *1*, 1-10.

Walker, G. M. (2004). Metals in yeast fermentation processes. *Advances in Applied Microbiology, 54*, 197-229.

Walker, G. M., Birch-Andersen, A., Hamburger, K., & Kramhoft, B. (1982). Magnesium-induced mitochondrial polymorphism yeast, *Schizosaccharomyces pombe*. *Carlsberg Research Commun*ications, *47,* 205-214.

CHAPTER VII

General Conclusions

Active dry wine yeasts (ADWY) are the most widely used in winemaking, ensuring the strain identity and genetic stability at room temperature, and reducing costs of transportation and storage. Nevertheless, the drying and rehydration processes induce several stress situations, such as heat, osmotic, and oxidative, with the consequent loss of cell viability. We developed ADWY protocols for *Starmerella bacillaris* and *Schizosaccharomyces pombe* strains, enhancing their viability without affecting their enological behavior when cells are dried in the presence of trehalose, followed by a rehydration in the presence of raffinose/galactose or magnesium, respectively.

The metabolomics characterization of the magnesium effect during the *Schiz. pombe* rehydration showed that methionine salvage pathway has a relevant incidence to overcome desiccation stress imposition. Specifically, 5'methylthioadenosine accumulation in rehydrated cells with magnesium, participates in the enhancement of *Schiz. pombe* desiccation stress tolerance and prevents the polyamines toxicity in resuming cell growth. Moreover, the reduction of intracellular ROS seems to be necessary but not sufficient for desiccation tolerance in *Schiz. pombe*. However, magnesium induces a premature chronological aging in stationary phase of *Schiz. pombe cells* suggesting an activation of cell metabolism. We can conclude that magnesium has incidence on several metabolic adjustments in yeast cells to overcome desiccation stress.

During the alcoholic fermentation, yeasts also have to resist several stresses such ethanol, osmotic or oxidative stresses. Since magnesium rehydration increases the *Schiz. pombe* viability after a desiccation stress, the effect of magnesium supplementation in traditional and in fed-batch fermentation was investigated. Although magnesium supplementation did not show any effect on *Schiz. pombe* fermentation performance, the application of the fed-batch technique allows obtaining wines with reduced volatile acidity, with relatively high levels of glycerol, and with similar acetaldehyde levels in comparison to commercial wines.

"S'ha de perseverar i, sobre tot, tenir confiança en un mateix."

Marie Curie, 1867-1934


UNIVERSITAT
ROVIRA i VIRGILI