Amino acids as modulators of the production of hydrogen sulfide in problematic wine fermentations

MARIA JOSE CAROLINA PAREDES MARTINEZ

2019

Pontificia Universidad Católica de Chile
Facultad de Agronomía e Ingeniería Forestal

Amino acids as modulators of the production of hydrogen sulfide in problematic wine fermentations

María José Carolina Paredes Martínez

Thesis
to obtain the degree of

Doctor
en Ciencias de la Agricultura

Santiago, Chile, September 2019

Thesis presented as part of the requirements for the degree of Doctor in Ciencias de la Agricultura, approved by the

Thesis Committee

Dr. Edmundo Bordeu S., Advisor

Dr. Liliana Godoy O., Co-advisor

Dr. Eduardo Agosin T.

Dr. Alejandra Urtubia U.

Santiago, September 2019

To all of those who have walked this bumpy path alongside with me.

This work was supported by CONICYT “Beca formación de Capital Humano 21120714/2012. We recognize the collaboration of FONDECYT National Research Funding project N°1120679. Thanks to Santa Emma Vineyard for providing grape must *Cabernet Sauvignon.* Special thanks to Inmunology laboratory (Dra Patricia Luz) at Universidad de Los Andes to provide a place to finish this work.

Acknowledgements

Es difícil plasmar en palabras ,sobretodo escrita, lo importantes que han sido personas tan especiales en este largo camino.

Primero quisiera agradecer a Edmundo Bordeu, quien sin conocerme me acepto como su alumna de doctorado. Gracias por haberme dado la oportunidad y la paciencia de trabajar conmigo.

A Liliana Godoy, recién llegada a la Universidad y tiene que lidiar con este pastelito. Sin duda sentí su apoyo, tenacidad y esfuerzo para lograr llegar al final, gracias!

Obvio, como no agradecer a Alejandra Zuñiga , que siempre pude contar con lo necesitara en el laboratorio y con ella, para todos mis cachitos. Y jamás voy a olvidar a Leslie Mora, que me ayudo a la distancia, incluso antes de una cirugía. Eres una persona maravillosa.

Tampoco puedo dejar de agradecer a Consuelo Olivares, que si no fuera por ella nunca habría podido tener los estándares de aminoácidos para mi HPLC. Y a Orlando Contreras, que hizo todo lo que pudo para que lográramos extraer RNA de mis levaduras.

A las increíbles Claudia y Marcela, las secas secretarias del DIP y a Wendy, que me dieron siempre lindas palabras de apoyo y fuerza, en los momentos más oscuros.

No habría llegado aquí tampoco si no fuera por Pati Luz, que me abrió las puertas no solo de su laboratorio. Me dio la oportunidad de trabajar mientras lograba terminar con la tesis y tuvo una paciencia infinita en todo lo que fue este proceso. Gracias totales.

Aquí es donde viene la parte mas difícil, ha sido un largo tiempo y con ello, lleno de cambios. Partiré por el principo,gracias Fabian, por alentarme a entrar al doctorado, por las aventuras que logramos construir y las que no también. Junto a Papel , Rorro , Claudito, Josecin y los pollitos fueron años de momentos increíbles y de las amistades que nunca se olvidan. Gracias por todo su cariño y apoyo , las juntas SOS , las conversaciones sin fin y la ayuda para los momentos en que las cosas se veían color hormiga.

Sergin Benavides, que hubiera sido de mí si no te hubiera conocido. Salimos adelante juntos y así seguiremos, armando proyectos locos y encapsulando todo, porque la amistad y el cariño que forjamos es de los buenos.

Familia querida, con uds siempre voy a quedar corta en como lograr agradecer todo lo que han hecho por mí. Siempre han dicho que soy una eterna estudiante, por suerte no fue así y logré terminar. Mi mayor felicidad fue ver sus caras de felicidad cuando todo acabo (aunque no hayan entendido mucho). Soy lo que soy gracias a uds, y estoy donde estoy gracias a todo lo que han hecho por mí y conmigo.

Felipe, llegaste a mi vida en lo que yo pensé era el final de este recorrido llamado doctorado pero mas que un final fuiste y eres el principio de un todo. Sin tu entrada avallasante en mi vida todavía estaría aletargada. Contigo retome la riendas y no podría estar mas feliz de poder llamarla ahora nuestra vida, que además incluye una Motita. Los tres lomitos en la aventura de la vida.

Contents

Chapter 1

ABSTRACT

All stages of winemaking can present problems, but alcoholic fermentation is, in particular, the stage with most complications. During wine fermentation some conditions can result in sluggish or stuck fermentations. The information provided by routine measurements has not been sufficient to detect and diagnose the state of the fermentation, and in case of paralization, rescue it on time. Hence, real-time monitoring of key variables, using advanced instrumentation, would anticipate problematic situations. Nitrogen is one of the most studied variables for sluggish or stuck fermentations. However, only a fraction of the available nitrogen is assimilated by the yeasts, constituted by ammonium ions and free α-amino acids. Nitrogen compounds have also been associated to be key factors in volatile compounds formation, including hydrogen sulfide (H_2S).

Sulfide release during winemaking is a longstanding and serious problem. H_2S is a malodorous compound with a low sensory threshold. It's produced as metabolic requirement of yeast for synthesizing sulphur compounds. Its formation is carried out by the sulfate reduction pathway starting from organic and inorganic sources, such as sulfites, cysteine, and glutathione. The role of amino acids and ammonium on H_2S formation has shown a high variability. Individual yeast nitrogen requirements, as well as its assimilation capacity of nitrogen compounds, together with the time nitrogen is supplemented during fermentation, are oenological factors that influence sulfide formation. Furthermore, yeast variability in sulfide metabolic

regulation has also a major impact on H_2S formation. Genetic variability, in the form of differential allelic expression controlling sulfide reduction pathway or adjacent routes, has been found to be a decisive trait that affects yeast capacity to produce H_2S. However, this information hasn't been able to help predict and control sulfide formation through alcoholic fermentation.

Considering the above, in this research nitrogen, specifically its organic sources, is studied, as regulator of H_2S formation. For this, the evolution of amino acids profile during wine fermentation of *Cabernet Sauvignon* must was measured. Two problematic fermentations conditions were evaluated, high initial sugar concentration and low initial assimilable nitrogen content, besides the standard winery condition, with four commercial wine yeast. Samples were collected at 24 hours intervals until the maximum amount of H_2S was produced. Amino acids were evaluated by HPLC (*High Performance Liquid Chromatography*), allowing their quantification. A colorimetric method was exclusively developed to accurately quantify H_2S production. Depending on the initial condition of the fermentation some amino acid showed a characteristic evolution profile, distinctive to each yeast. Most importantly, a unique H2S release patron was established for each wine yeast in relation to the starting conditions of the must fermentation.

INTRODUCTION

All stages of winemaking can present problems, but alcoholic fermentation is, in particular, the stage with most complications. During fermentation of grape must, yeasts are subject to multiple stress factors, including high osmotic pressure, acidity, nutrient deprivation, and high alcohol concentration (Rossignol et al. 2003a). In spite of the improvement in the control of the fermentation, paralyzed fermentations remain a great wine problem (Blateyron and Sablayrolles 2001). A fermentation is identified as problematic only when the sugar consumption rate slows or stops completely (Bisson 1999b). The detention in the fermentation rate leads to a high level of residual sugar which becomes a risk, mainly due to the threat of development of lactic acid bacteria, which can affect the quality of wines. The main mechanisms that have been considered responsible for this problem are nitrogen deficiency, extreme temperatures, high initial sugar content, anaerobic conditions, high ethanol content, toxicity of short and medium-chain fatty acids, incorrect oenological practices (Blateyron and Sablayrolles 2001); (Alexandre and Charpentier 1998).

Nitrogen is one of the most studied variables as a cause of paralyzation (A. Mendes-Ferreira, Mendes-Faia, and Leao 2004). It is the second most abundant macronutrient in wine fermentation and is essential for the metabolism and growth of yeast (Cristian Varela, Pizarro, and Agosin 2004), as well as for the metabolism of flavors and the aromatic profile of wine (Henschke P.A. and Jiranek V. 1993; Bell and Henschke 2005a).

The nitrogen content in the must is highly variable, not only in quantity but also in the types of compounds present (Henschke P.A. and Jiranek V. 1993). The fraction of nitrogen that is usable by yeasts is usually called yeast assimilable nitrogen (YAN) and is constituted on average by ammonium ions, and free α-amino acids (Crépin et al. 2012). The primary amino acids are an abundant source of YAN for yeast but can vary in their efficiency as a nitrogen source (Henschke and Jiranek, 1993). It is the assimilable portion of amino acids that is of oenological interest, that is, the total free amino acids, except for proline and hydroxyproline. Amino acids composition, as well as its total concentration, is determined in the vineyard and varies according to the cultivar, location, climatic conditions, level of maturity, and management of the vineyard (Bell and Henschke, 2005a).

The analytical measurement of assimilable nitrogen in the must have become a fundamental practice for the control of fermentation. This affects not only the kinetics of the fermentation but also the residual nitrogen content, the accumulation of urea, the production of biogenic amines, and even the microbiological and physical stability of the finished wine. Thus, when the nitrogen status is sub-optimal, it is possible to manipulate it by adding nitrogen to the crushed grapes, usually in the form of diammonium phosphate (DAP). Although it is not a common practice to add amino acids to the must, there are commercial products that provide complex forms of nitrogen, albeit in a limited way.

a. Nitrogen nutrition in *Saccharomyce cerevisiae*

It has been established that yeasts use certain sources of nitrogen and that the pattern of consumption depends both on the composition of the nitrogenous

compound and on its concentration (Bell and Henschke, 2005). In the absence of other factors considered limiting, the YAN of the must determines the biomass of yeast, together with the fermentation rate and the duration of the latter. Biomass formation is linearly related to the initial content of YAN, although the final biomass amount will be affected by the specific nitrogen requirements of the yeast strain, fermentation temperature and oxygen availability among others (Blateyron and Sablayrolles 2001). According to Varela et al. (2004), with higher biomass concentration, quicker fermentation rates are achieved. While the fermentation time response is exponential, biomass yield is the determining factor in the rate of fermentation.

On the other hand, the complexity of mixtures of nitrogen compounds also affects the growth rate of the yeast, and therefore, the rate and duration of fermentation (Jiranek, Langridge, and Henschke 1995a). Mixtures of amino acids or amino acids plus DAP deliver higher growth rates than individual nitrogen sources (Beltran et al. 2004). The nitrogenous compounds that favor high growth rates are those when metabolized, allow a fast synthesis of glutamate, glutamine, or ammonium. Glutamine, glutamate, asparagine, and ammonium are considered preferred sources of nitrogen over arginine, alanine, aspartate, and glycine, while proline, urea and allantoin are considered poor sources (Crepin et al., 2012). The aromatic and branched amino acids, although they do not support high growth rates, are consumed early during fermentation (Martínez-Moreno et al. 2012a). To mediate the selection among nitrogen sources, *S.cerevisiae* uses a mechanism known as Nitrogen catabolite repression (NCR), in which to favor good sources of nitrogen, it represses the transcription of genes involved in the use of the poorer sources, and

inactivates and degrades the corresponding products (ter Schure, van Riel, and Verrips 2000).

The nitrogen, and the carbon skeleton, obtained from the degradation of amino acids and ammonium is used for the synthesis of glutamate and glutamine, constituting the dynamic nitrogen pool of the yeast. These acidic amino acids are precursors in the synthesis of other amino acids, purines, and pyrimidines according to the metabolic needs of the yeast. The glutamate of this pool contributes 85% of the nitrogen requirements of the yeast (Magasanik and Kaiser 2002).

Under normal vinification conditions, nitrogen additions are made empirically, without taking into account neither the nitrogen requirements nor the time of addition, less the type of nitrogenous compound (Beltran et al. 2005b). Both the time of addition of nitrogen and the nature of the nitrogen added affect not only the kinetics of fermentation but also affects the pattern of consumption thereof. The additions of nitrogen in the form of DAP during the growth phase of yeast increases the cell population, while additions during the stationary phase showed a decrease in the duration of the fermentation. That is, the higher the availability of nitrogen, the higher the fermentation rate. In both cases, an increase in ethanol production was observed (A. Mendes-Ferreira, Mendes-Faia, and Leao 2004). It was also observed that the combination of amino acids as a mixed source of nitrogen generates higher amounts of biomass, highlighting the importance of the nature of the nitrogen source (Martínez-Moreno et al. 2012b). The higher efficiency presented by the amino-acids mixture has been associated with the ability of yeast to directly incorporate these amino acids into proteins, which would lead to

decrease the excess of NADH from the synthesis the novo amino acids. However, the type of nitrogen source also affects the formation of metabolites derived from glucose, especially glycerol. When an *S.cereviciae* strain of the bakery industry is grown at 20g /L glucose in the presence of a mixture of amino-acids, a minimal excess of NADH is produced, which is re-oxidized by the formation of glycerol, increasing the yield of ethanol. In contrast, when this yeast is grown in ammonium as the sole nitrogen source, the de novo synthesis of amino acids results in excess of NADH, increasing glycerol production and reducing potential ethanol formation by up to 14% (E Albers et al. 1996).

The mechanism that favors the consumption of one source of nitrogen over another is unclear. Nitrogen sources have been only classified based on the time and amount that they are consumed during fermentation (Jiranek, Langridge, and Henschke 1995a). Considering that, some compounds constantly vary in how they are classified. Crepin et al. (2012) showed that, although the time required to consume the different sources of nitrogen depends on the strain of *S.cerevisiae*, all of them present the same order of assimilation, and that this sequence is independent of the concentration of the compound, except for arginine. Therefore, the ability of the yeast to first assimilate a particular nitrogen source can be a key factor for the development of the fermentation.

During yeast growth, fermentative metabolism leads to the formation of a variety of volatile compounds that include higher alcohols, esters, sulfur compounds and fatty acids that contribute both to the aroma and palate of the wine. Nitrogen compounds contribute enormously to the formation of these compounds, especially alcohols and esters, and the regulation of the formation of hydrogen sulfide (H_2S),

thiols, mercaptans, and monoterpenes (Henschke and Jiranek, 1993). Therefore, any factor that influences the growth and metabolism of yeast will impact the composition of wine and its organoleptic properties, being one of these factors, the composition of amino acids in the grape must.

b. Formation of hydrogen sulfide in Saccharomyces cerevisiae

The release of H_2S during vinification is a big problem as it imparts an unpleasant aroma to the wine even at very low concentrations (≤1.6 µg/L) (Ana Mendes-Ferreira et al. 2009a; Ugliano, Kolouchova, and Henschke 2011a). The formation of H_2S occurs in response to yeast metabolic requirements imposed by growth, specifically by the need for sulfur compounds. Metabolically, H_2S is produced from inorganic sulfur compounds such as sulfates and sulphites, or from organic sulfur compounds such as cysteine, glutathione or methionine (Henschke and Jiranek, 1993).

Hydrogen sulfide is a product of the sulphate reduction pathway called sulphate reduction sequence (SRS) (Figure 1). It is derived from the HS- ion, which is a metabolic intermediate in the reduction of sulfates and sulphites necessary for the synthesis of all sulfur compounds (Figure 2), including S-adenosylmethionine (Swiegers and Pretorius 2007b; Lambrechts and Pretorius 2000a). In a series of stages, the sulphate is transported and reduced via 3'-phosphoadenosyl 5'-phosphosulfate (PAPS) reductase, where it is subsequently enzymatically combined with carbon and nitrogen precursors by the action of sulfurylase (Apostolos Spiropoulos et al. 2000; Lambrechts and Pretorius 2000a). Both the

transport and the action of the reductases and sulfurylase are inhibited by methionine and S-adenosylmethionine (Spiropoulus et al., 2000). If during the fermentation these reactions proceed in the presence of insufficient sources of nitrogen, and consequently, with deficiencies of methionine or cysteine, the SRS pathway will be uninhibited, producing the accumulation of free H_2S that will diffuse to the must (Swiegers and Pretorius 2007b).

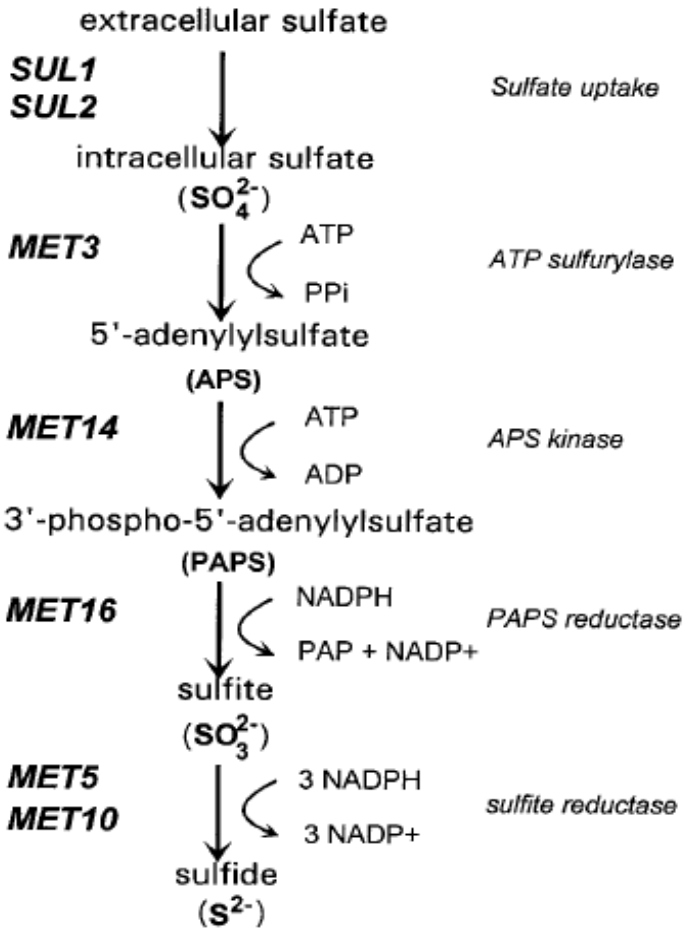


Figure 1. Sulphate Reduction Pathway (Thomas and Surdin-Kerjan, 1997).

Different factors have been associated with the production of H_2S under vinification conditions, which include levels of elemental sulfur, the presence of SO_2 and sulfur

organic compounds (Mendes-Ferreira et al, 2009). However, these factors are highly dependent on the gene load of the yeast. Even though, (Jiranek, Langridge, and Henschke 1995c) showed that under vinification conditions, H_2S release occurs in response to nitrogen depletion and is driven by inorganic sulfur. This relationship would be due to the joint activation of the Sulphate Reduction Sequence (SRS) and the depletion of sulfur amino acid precursors, which would lead to an inefficient incorporation of sulfide and therefore to the release of H_2S (Henschke and Jiranek, 1993).

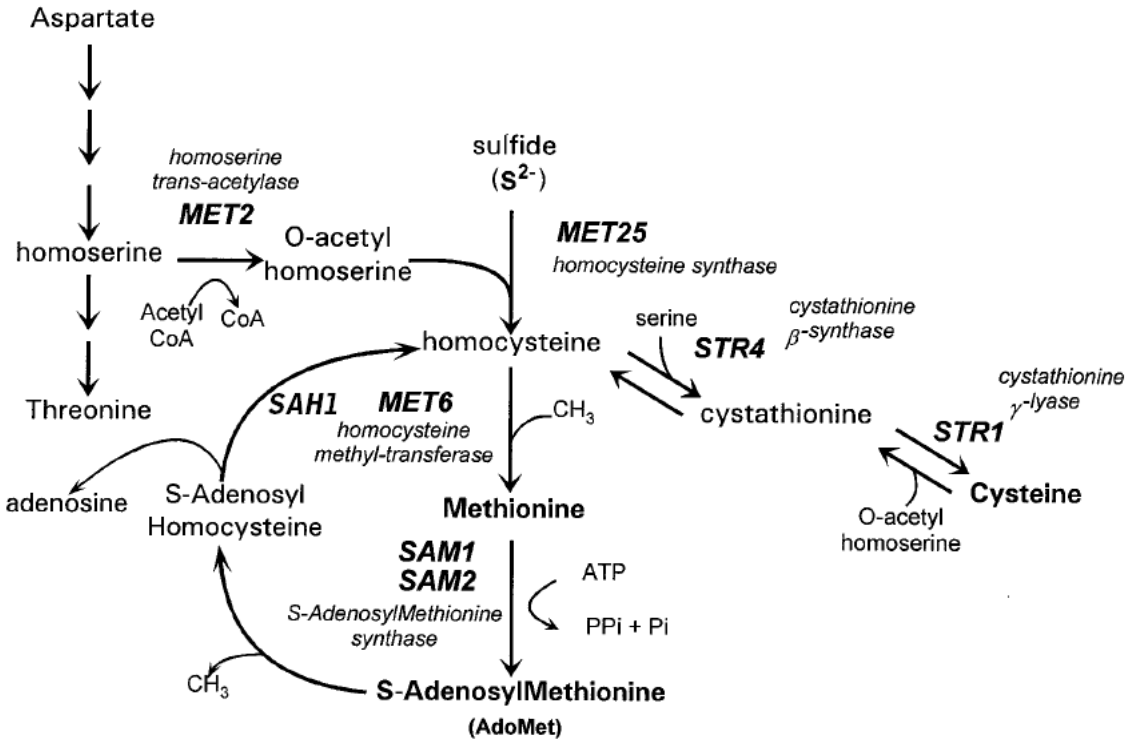


Figure 2. Route of synthesis of sulfur amino acids and adjacent pathways. (modified from Thomas and Surdin-Kerjan, 1997).

Subsequently, Mendes-Ferriera et al. (2009) showed that in nitrogen-limiting conditions, yeast strains produced a lower amount of H_2S, attributing it to the fact

that sulfide formation, being a highly demanding process, is diminished, allowing cellular survival. However, the amount of H_2S produced and the level of response to the addition of nitrogen depends on the *S.cereviciae* strain used. The strain EC118 responded vaguely to the different concentrations of nitrogen, administered as DAP, being almost undetectable the production of H_2S. In contrast, for strains, UCD522 and PYCC4072 nitrogen depletion triggered the release of H_2S. While under conditions of excess nitrogen, only strain UCD522 produced considerable amounts of sulfide.

One factor that was subsequently taken into consideration was the time during the fermentation in which it was supplemented with nitrogen. It was shown that both the wine yeast strain and the time in which DAP is added, either in a dose at the beginning of the fermentation or in divided doses, strongly affects the release of H_2S (a Mendes-Ferreira et al. 2010). For strains PYCC4072 and EC1118, the release of H_2S was lower when the DAP was added prior to fermentation, however, for strain UCD522, the release of H_2S decreased when it was added 72 hrs after the start of fermentation. Both Jiranek et al. (1995b) as Mendes-Ferreira et al. (2010) and (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012b)) also showed that by replacing the DAP supplementation with an amino acid or a mixture of amino acids as nitrogen sources, a decrease in H_2S release associated with nitrogen limitation was observed and also the strain differences seen in the pattern of sulfide release were suppressed between yeast strains.

According to (Apostolos Spiropoulos et al. 2000)), sufficient methionine would inhibit the SRS pathway, suppressing the release of H_2S, however, he observed that the methionine content does not correlate correctly with H_2S release.

Fermentations performed with a supplementation based on a mixture of amino acids that excluded methionine showed that for strains UCD522 and PYCC4072 the lack of methionine increased the release of H_2S, independently of the nitrogen source. While, for strain EC1118, when the nitrogen source was the amino-acids mixture without methionine, was observed an increase in H_2S production, corroborating what was previously suggested by Mendes-Ferreira et al, 2012, a strain dependency in relation to the H_2S produced.

Up to this point, the differences observed between yeast strains in the formation of H_2S have not been conclusively explained by the type of nitrogen compound that is added to the must, or by the time that nitrogen is added during fermentation, nor by the presence of elemental sulfur. Hence, it was studied the possibility that the difference in the expression or activity of the enzymes involved in the reduction of sulfur could account for the differences observed in the formation of H2S, attributed to the different strains.

Bisson et al. (2000) postulated that the variation in H_2S release was due to the ability of yeast to incorporate reduced sulfur, suggesting that differences in the regulation and enzymatic activity of O-acetylserine/O-acetylhomoserine sulfhydrylase (OAS/ OAH SHLase) affects the production of H_2S. Therefore, the inefficiency of this enzyme to incorporate sulfur into organic compounds would result in the "dripping" of sulfide from the assimilation pathway and the formation of H_2S. The *MET17* gene (*MET15* or *MET25*), which encodes the OAS / OAH SHLase enzyme, also known as homocysteine synthase, was overexpressed in commercial strains UCD522 and UCD713, which did not show a reduction in H_2S formation. On the contrary, a slight increase in its production was observed by the

transformed strains, in addition to reducing the rate of fermentation and growth in strain UCD713 (Spiropoulos and Bisson, 2000)

It has been seen that the genetic background of the *S.cerevisiae* wine strains has a marked influence on the production of H_2S. It is then, that according to Linderholm, et. al. (2006) it is possible that these differences are due to gene alterations of the expression of enzymes downstream of the enzyme Sulphite Reductase, therefore, modulating enzymes activity. To assess this possibility, this group cloned and sequenced the genes of the three enzymes immediately after Sulfite Reductase. The enzymes were: homocysteine synthase, encoded by the *MET17* gene, homocysteine methyltransferase, encoded by the *MET6* gene and cystathionine-β-synthase, encoded by *CYS4* or *SRT4*. They found that there were allelic variations in both *CYS4* and *MET6* genes concerning the laboratory strain S288C, but not in *MET17*. However, the transformation with vectors of the *CYS4* and *MET6* alleles of native strains highly producer of H_2S did not have any impact on the natural ability of the strains to produce sulfide (Linderholm et al. 2006). On the other hand, in a uniform genetic background and knock-out for each gene, it was observed that the strain with the deleted *CYS4* gene produced an higher amount of H_2S than the mutant strain for *MET6*. Likewise, the subsequent transformation of the mutant strain ***Δcys4*** with the variant allele of *CYS4*, increased the fermentation rate of the transformed strain, suggesting that *MET6* gene does not influence the formation of H_2S (Linderholm et al. 2006).

In a final approach, the Linderholm and Bisson group performed a screening of a set of mutant yeasts, with single deletions for each genome of *S.cerevisiae*, in order to identify those that could be affecting the production of H_2S. Of the 4,828

strains evaluated, 16 mutants were positively affecting the formation of H_2S, 8 of them resulting in high levels of H_2S production. Of these positive strains, 5 were identified as defective for genes encoding enzymes involved in the synthesis of sulfur amino acids or their precursors and were associated with the sulfur assimilation pathway, *CYS4*, *HOM2*, *HOM6* and *MET17* (Linderholm et al. 2008).

The nitrogen demand of yeast, the capacity and the assimilation pattern of nitrogen compounds and the time of supplementation of nitrogen, are relevant enological factors since the consequent production and release of H_2S depends on their interaction. However, the genetic background of yeast strains and their intrinsic variability in gene expression and allelic recombinations is the most influential factor in H_2S formation. But how enological factors directly impact the pathways leading to the formation of H_2S in wine yeast strains is still unknown. Today there is still no clear oenological management to control the formation of H_2S, its production remains unpredictable and highly variable. Hence the importance of identifying the correct ways to minimize or eliminate the formation of this compound through winery practices.

HYPOTHESIS

The sulfur assimilation pathway is the source of hydrogen sulfide formation, which can be modulated by the amino acids present in the grape must, in a yeast strain-dependent manner.

OBJETIVES

a. General objective

To establish the influence of amino acid consumption in the production of H_2S during problematic wine fermentation in four strains of *Saccharomyces cerevisiae.*

b. Specific Objectives

1. Obtain the aminoacids profile consumption during control and problematic conditions for four wine strain yeast.

2. Establish a timeline formation and quantification of H_2S during control and problematic fermentations for four wine strain yeast.

3. Identify the key or keys amino acid profiles to associate to H_2S formation, for each fermentation condition and yeast strain.

REFERENCES

Aceituno, F. F., Orellana, M., Torres, J., Mendoza, S., Slater, A. W., Melo, F., & Agosin, E. (2012). Oxygen response of the wine yeast Saccharomyces cerevisiae EC1118 grown under carbon-sufficient, nitrogen-limited enological conditions. *Applied and environmental microbiology*, *78*(23), 8340–52. doi:10.1128/AEM.02305-12

Albers, E., Larsson, C., Lidén, G., Niklasson, C., & Gustafsson, L. (1996). Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation. *Applied and environmental microbiology*, *62*(9), 3187–95.

Alexandre, H., & Charpentier, C. (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. *Journal of Industrial Microbiology and Biotechnology*, *20*(1), 20–27.

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*(2), 87–93.

Bell, S.-J., & Henschke, P. a. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research*, *11*(3), 242–295.

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of agricultural and food chemistry*, *53*(4), 996–1002.

Beltran, G., Novo, M., Rozès, N., Mas, A., & Guillamón, J. M. (2004). Nitrogen catabolite repression in Saccharomyces cerevisiae during wine fermentations. *FEMS yeast research*, *4*(6), 625–32.

Bisson, L. F. (1999). Stuck and Sluggish Fermentations. *American Journal of Enology and Viticulture*, *50*(1), 107–119.

Blateyron, L., & Sablayrolles, J. M. (2001). Stuck and slow fermentations in enology: statistical study of causes and effectiveness of combined additions of oxygen and diammonium phosphate. *Journal of bioscience and bioengineering*, *91*(2), 184–9.

Chicón, R., Hermosín-Gutiérrez, I., & Cabezudo, M. (2001). Chicon201.EMMDEaminoacidos.pdf. *Tecnologia del Vino [España]*, *1*(1), 95–100.

Crépin, L., Nidelet, T., Sanchez, I., Dequin, S., & Camarasa, C. (2012). Sequential use of nitrogen compounds by Saccharomyces cerevisiae during wine fermentation: a model based on kinetic and regulation characteristics of nitrogen permeases. *Applied and environmental microbiology*, *78*(22), 8102–11. doi:10.1128/AEM.02294-12

Dukes, B., & Butzke, C. (1998). Rapid Determination of Primary Amino Acids in Grape Juice Using Spectrophotometric Assay. *American Journal of Enology and Viticulture*, *49*(2), 125–134.

Gietz, R. D., & Woods, R. a. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in enzymology*, *350*(2001), 87–96.

Hermosín-Gutiérrez, I., Gómez-Alonso, S., & García-Romero, E. (2007). AMONIO MEDIANTE DERIVATIZACIÓN CON ETOXIMETILENMALONATO DE DIETILO (EMMDE). *Journal of agricultural and food chemistry*, *55*(3), 608–613.

Jiranek, V., Langridge, P., & Henschke, P. a. (1995a). Amino Acid and Ammonium Utilization by Saccharomyces cerevisiae Wine Yeasts From a Chemically Defined Medium. *American Journal of Enology and Viticulture*, *46*(1), 75–83.

Jiranek, V., Langridge, P., & Henschke, P. a. (1995b). Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. *Applied and environmental microbiology*, *61*(2), 461–7.

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeast and its Importance to Wine Aroma - A Review. *South African Journal of Enology and Viticulture*, *21*(June), 97–129.

Magasanik, B., & Kaiser, C. a. (2002). Nitrogen regulation in Saccharomyces cerevisiae. *Gene*, *290*(1-2), 1–18.

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., & Beltran, G. (2012). Biomass production and alcoholic fermentation performance of Saccharomyces cerevisiae as a function of nitrogen source. *FEMS yeast research*, *12*(4), 477–85.

Mendes-Ferreira, a, Barbosa, C., Inês, A., & Mendes-Faia, A. (2010). The timing of diammonium phosphate supplementation of wine must affects subsequent H2S release during fermentation. *Journal of applied microbiology*, *108*(2), 540–9.

Mendes-Ferreira, a, Mendes-Faia, A., & Leão, C. (2004). Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. *Journal of applied microbiology*, *97*(3), 540–5.

Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., & Mendes-Faia, A. (2009). The production of hydrogen sulphide and other aroma compounds by wine strains of Saccharomyces cerevisiae in synthetic media with different nitrogen concentrations. *Journal of industrial microbiology & biotechnology*, *36*(4), 571–83. doi:10.1007/s10295-009-0527-x

Montenegro-Montero, A. (2010). Smart cloning. *Labtimes*, (24), 66–67.

Rossignol, T., Dulau, L., Julien, A., & Blondin, B. (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast (Chichester, England)*, *20*(16), 1369–85.

Spiropoulos, A., Tanaka, J., Flerianos, I., & Bisson, L. F. (2000). Characterization of Hydrogen Sulfide Formation in Commercial and Natural Wine Isolates of Saccharomyces. *American Journal of Enology and Viticulture*, *51*(3), 233–248.

Swiegers, J. H., & Pretorius, I. S. (2007). Modulation of volatile sulfur compounds by wine yeast. *Applied microbiology and biotechnology*, *74*(5), 954–60. doi:10.1007/s00253-006-0828-1

Ter Schure, E. G., van Riel, N. a, & Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. *FEMS microbiology reviews*, *24*(1), 67–83.

Ugliano, M., Kolouchova, R., & Henschke, P. a. (2011). Occurrence of hydrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen. *Journal of industrial microbiology & biotechnology*, *38*(3), 423–9. doi:10.1007/s10295-010-0786-6

Varela, C., Pizarro, F., & Agosin, E. (2004). Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. *Applied and environmental microbiology*, *70*(6), 3392–3400.

Chapter 2

Sulfide formation during problematic natural must fermentations in four commercial wine yeast.

Maria Jose C. Paredes[a,b], Sergio Benavides[c], Liliana Godoy[a] and Edmundo Bordeu[a].

[a]Department of Fruticulture and Oenology, Faculty of Agriculture and Forestry, Pontificia Universidad Católica de Chile, P.O. Box 306, Santiago 6904411, Chile.
[b] Center of Biomedical Investigation, Faculty of Medicine, Universidad de Los Andes, Santiago 7620157, Chile.
[c]Faculty of Engineering and Business, Universidad Adventista de Chile, Camino a las Mariposas km 12, Chillán 3780000, Chile.

Abstract. Under vinification conditions, stress factors can influence yeast metabolism and therefore, vary their basal H_2S release. Nitrogen is one of the most studied variables for yeast to be able to complete fermentation, for the formation of the aromatic profile compounds and the regulation in the formation of hydrogen sulfide (H_2S). Hydrogen sulfide is an important concern due to its adverse effects and as a precursor of other compounds with undesirable sensory characteristics. The ability of most commercial yeast to produce H_2S has been tested in model grape juices, in conditions completely different from the ones found in wine fermentations. Therefore, the ability of four commercial yeast to produce H_2S was investigated in natural grape juice considering the impact of oenological conditions: high initial sugar content and low initial assimilable nitrogen. Results showed that in high sugar concentrations and no limited nitrogen conditions, yeast produces higher amounts of H_2S than in control and low nitrogen

fermentations. Also, a new strain was characterized as a high sulfide producer. Thus, despite the natural sulfide producing background of the yeast strain, its H_2S release can be influenced by the initial enological conditions of the fermenting must. The initial sugar level is one more condition that can increase sulfide release by high producing yeasts.

Keywords: yeast, sulfide, fermentation, sugar concentration.

Introduction

All stages of winemaking present problems, but alcoholic fermentation is, in particular, the stage with most complications. During growth, yeasts are subject to multiple stress factors, including high osmotic pressure, acidity, nutrient deprivation and high alcohol concentration (Rossignol et al. 2003a). Fermentative metabolism also leads to the formation of a variety of volatile compounds that include higher alcohols, esters, sulfur compounds and fatty acids that contribute both to the aroma and palate of wine. Nitrogen compounds contribute enormously to the formation of these compounds, especially alcohols and esters, and the regulation in the formation of hydrogen sulfide (H_2S). Nitrogen is one of the most studied variables for yeast to be able to complete the fermentation process and for the formation of the aromatic profile compounds in finished wines. Low concentration of yeasts assimilable nitrogen (YAN) has been associated with reduced biomass, loss of fermentation activity, and formation of H_2S (Henschke & Jiranek, 1993; Gardner et al., 2002; Jiranek et al., 1995). Hydrogen sulfide is a major concern due to the negative effect of its rotten egg odor (Ana Mendes-Ferreira et al. 2009b) and is also a precursor of other compounds with undesirable sensory characteristics (Yoshida et al. 2011) known as “reductive” off-flavors.

The addition of nitrogen as diammonium phosphate (DAP) is a well-established and common practice to prevent problematic fermentations and H_2S release (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a). Separate studies showed that in nitrogen-limiting conditions, yeast strains produced lower amounts of H_2S

(Ana Mendes-Ferreira et al. 2009b). However, the amount of H_2S produced and the level of response to the addition of nitrogen depends on the *S.cereviciae* strain used. More so, some studies suggest that nitrogen addition can even exacerbate H_2S formation, especially in moderate DAP additions (Ugliano, Kolouchova, and Henschke 2011b). Results from Mendes-Ferreira, 2010 also showed that the amount of sulfide released in response to nitrogen availability was significantly strain-dependent, meaning that the genetic background of the yeast makes it difficult to elucidate the bare effect of the conditions tested in the formation of H_2S. Another work by Mendes-Ferreira et al., 2010b evidenced that unlike growth and fermentation rate, both yeast strain and the timing of DAP supplementation strongly affected H_2S and replacement of DAP by an aminoacid mixture suppressed the genotypic differences. Genetic approaches have also been used for elucidating the effect of nitrogen supplementation in the production of H_2S, knowing that yeast strains respond differently to physiological factors (A. L. Linderholm et al. 2008). Nevertheless, the ability of most commercial yeast to produce H_2S has been tested in model grape juices, in conditions completely different from the ones found in wine fermentations (Ugliano, Kolouchova, and Henschke 2011b). The influence that initial sugar content and grape must nature could have in the response of yeast strain to nitrogen and therefore to sulfide release has not been considered strong enough. Accordingly, the production of H_2S in natural grape juice was investigated. A low nitrogen *Cabernet Sauvignon* was used, that was or wasn't supplemented with DAP to a consensus concentration for yeasts to complete fermentation, and with a mixture of glucose:fructose to simulate standar ripiness or high initial sugar content (over ripe grapes). H_2S released was quantified in all fermentations conditions for four commercial yeast with distint natural capacities to produce sulfide. So, it was possible to explore the real influence of more accurate enological conditions in the profile of sulfide realese by commercial wine yeast.

Materials and Methods

Yeast Strain and Wine Fermentations

Rehydrated active dry yeasts were used for inoculation were RED STAR Montrachet (Lessafre Yeast Corporation, USA), Lalvin ICV K1 MarquéeTM (Danstar Ferment AG, Denmark), Lalvin ICV OKAY® (Lallemand, Australia) and VIVACE (Renaissance Yeast Inc. Vancouver, Canada). Rehydrated active dry yeasts were hydrated and inoculated according to manufacturer instructions. 0,3g/L of dry yeast was mixed with sterile distilled water at 37-40°C and let for 20min at room temperature to rehydrate. The four commercial yeast strains were inoculated each in 200mL of grape must (21°Brix) in a 500mL two-neck flat bottom flask (SCHOTT/ILMABOR). Using *Cabernet Sauvignon* must three conditions with different levels of nitrogen and sugar concentration were prepared (Table 1).

Table 1. *Cabernet Sauvignon* initial conditions to achieve three different enological fermentation starters.

.

	Lack	**Control**	**Excess**
Initial Nitrogen content	≤ 150mg/L	250-300mg/L	250-300mg/L
Initial Sugar content	203g/L (21°B)	203g/L (21°B)*	250g/L (25°B)*

* Brix

Red grapes *cv.* Cabernet Sauvignon from Santa Emma vineyard, harvest 2016, was pasteurized (70°C for 1 minute) and kept in aliquots of 5L at -80°C. Alcoholic fermentations were carried out in

a laboratory-scale in 500mL two-neck 29/32 flat bottom flask (SCHOTT/ILMABOR) with constant agitation (100rpm) at 25°C, in semi-anaerobic conditions. Nitrogen supplementation was done with ammonium diphosphate (Merck, Germany); sugar

addition was made with a 1:1 mix glucose (Merck, Germany) and fructose (Merck, Germany)

H_2S quantification

A polymeric matrix was designed for the quantification of H_2S, based on entrapment of S^{2-} anion through molecular interactions with lead (manuscript in progress). The matrix was prepared following the modified method described by (Benavides et al. 2016)) and was used to fill glass columns inserted into the hole of one of the rubber stopper placed in one of the necks of the 500mL flask. The calibration curve was constructed by measuring the length of the blackened band in the matrix and plotting versus the corresponding concentration of H_2S using a standard solution. 0,5g of sodium sulfide nonahydrate (Aldrich, Milwaukee, WI, U.S.A.) was solubilized in 500mL of deionized water. A 25 ml aliquot was added to a glass beaker containing 125 ml of deionized water, 10 ml of 0.1 N iodine solution (Winkler, Chile), and 1 ml of dilute HCl (1+3). The excess iodine was titrated with 0.1 N sodium thiosulfate solution (Winkler, Chile). Increasing aliquots of stock solution were then added to a 2-neck flask to a volume of 200mL and sealed with a rubber stopper which had the polymeric matrix/column inserted. Nitrogen gas was bubbled up and the solution stirred continuously by a magnetic stirrer to force $H_2S(g)$ through the column (Park Seung-Kook 2008). The reproducibility of the tubes was calculated from five consecutive measurements of the same aliquot of the standard solution. The limit of detection was determined by measuring H_2S in serial dilutions.

Results and Discussion

Although most yeasts are able to produce and release sulfide, there is a wide variety among yeasts and the intrinsic amount of H_2S they produce. Under vinification conditions, stress factors can influence yeast metabolism and therefore, vary their basal H_2S release. In this work, we evaluated the impact that individual oenological conditions had on the production of H_2S on four commercial wine yeast

strains. Over-mature grapes are frequently harvested and become detrimental for successful fermentation (Malherbe et al., 2016). Therefore, to better understand how yeast H_2S metabolism is altered by an increase in sugar level, natural grape must, *var. Cabernet Sauvignon* was supplemented with glucose: fructose in a 1:1 proportion to a final concentration of 250g/L of initial sugar content. A control fermentation, to determine the basal H_2S production was carried out with an initial sugar content of 200g/L. Nitrogen supplementation was also incorporated, as it is also an everyday practice in wine cellars. Thus control and high sugar fermentation were supplemented with DAP to reach 250-300mg assimilable per liter, and low nitrogen fermentation was set at ≤150mg N/L.

The H_2S evolution in control fermentation showed strain-dependent differences (Figure 1), confirming previous studies (Mendes-Ferreira, 2009a, 2009b, 2010, Barbosa, 2012). It can be clearly observed two types of tendency, high H_2S producers and low to no producers. Montrachet (UCD522) has been reported earlier to be a high producer (A. Spiropoulos and Bisson 2000) but K1 hadn´t been reported yet. It appears that K1 produces lower amounts of H_2S compared to Montrachet when daily production rate is observed, but when quantifying the total released by each strain it can be observed a high difference between the two strains (Figure 4), resulting K1 a higher producer than Montrachet. High H_2S production of these yeasts, in the regular vinification conditions, could be attributed to similar fermentation metabolism, referred to as nitrogen requirements. To date, references report ranges from 120mg/L of yeast assimilable nitrogen (YAN) to 270mg/L (Bely, Sablayrolles, and Barre 1990) and it has been associated with better fermentation performance and lower H_2S production. However, recently, it has been seen that no DAP supplemented grape must was associated to lower H_2S release in high producers yeast strains (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; A. Mendes-Ferreira et al. 2010; Ana Mendes-Ferreira et al. 2009b) as it was seen for Montrachet and K1 (Figure 1, Figure 2, Figure 4). On the other hand, yeast Okay (Lallemand) and Vivace (Renaissance), presented low and none H_2S production respectively. Moreover, for Vivace, the production of sulfide was undetectable in all fermentation conditions tested.

In no DAP supplemented must, having around 120-150mg/L of initial assimilable nitrogen, and 200g/L of sugar concentration, yeasts showed different behaviors in their sulfide production compared to control fermentation. Sulfide production started earlier for Montrachet as for K1. The difference was the level of production these two yeasts reached. When fermentations were carried out with 200g/L sugar concentration and 300mg N/L (DAP supplementation), sulfide levels were around 400-500mg/L. Instead, when no DAP was added, the highest level of sulfide registered, produced by Montrachet, didn't go any higher than 100mg/L. On the other hand, Okay that didn't produce sulfide greater than 40mg/L, but in low nitrogen fermentations almost doubled its production. Clearly, nitrogen metabolism is closely related to sulfide formation and liberation but in a different way than previously thought. Low assimilable nitrogen was associated with higher sulfide liberation because it was thought that if low nitrogen was available, low precursors to bind sulfide would be available and therefore, sulfide would be released (Fleet 2003; Jiranek, Langridge, and Henschke 1995b; A. Spiropoulos and Bisson 2000; Swiegers and Pretorius 2007a). Instead, at low available nitrogen, sulfide released decreased especially in high producing yeast strains, while low producers, enhance their production (Figure 2; Figure 4), and even though the increase in sulfide release in low producers might not be statistically significant, could cause a sensorial impact anyway. As nitrogen availability impacts on sulfide release, studies had been done where different nitrogen sources had been tested (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Beltran et al. 2005a; Butzke 2011; Gutiérrez et al. 2012; Jiranek, Langridge, and Henschke 1996; Ugliano, Kolouchova, and Henschke 2011b). It was observed that nitrogen supplemented as a mix of amino acids reduced sulfide production in all yeast strains, independently of its genetic backgrounds while the exclusive addition of DAP imbalances the ratio of nitrogen composition affecting the nitrogen uptake balance and in consequence the production of H_2S (Beltran et al. 2005a; Gutiérrez et al. 2012). Additionally, early works by Jiranek et al. 1995 found that most amino acids are equally effective as suppressants of sulfide formation in nitrogen-deficient fermentations, But those which support high growth rates, such as serine,

glutamine, ammonium, aspartate, arginine, and asparagine are most potent amino acid suppressants of H_2S liberation. the contrary happens with amino acids with regulatory roles of the SRS pathway or involved in sulfur amino acids metabolism or which contain sulfur. Supplementation of fermentations with cysteine or in a mix with methionine, led to increased rates of H_2S liberation (Jiranek, Langridge, and Henschke 1995b).

As sugar concentrations gets higher, yeast nitrogen requirements change as well as the nitrogen availability in the must (Jones et al. 2005). One aspect that has not been considered in sulfide production is nitrogen requirements, as DAP supplementation, related to must sugar concentration. For many years, sugar concentrations were moderate, but due to global warming and phenolic ripeness, sugar concentrations have been increasing over the years to higher values (Davis, Robert E. 2000; Martínez-Moreno et al. 2012b). In fermentations performed under high initial sugar levels (250g/L), the only strain that showed a higher sulfide production was Montrachet. (Figure 3; Figure 4). This yeast increased significantly its sulfide release in around 100mg/L, a level that is sensorially detrimental. K1, on the other hand, showed a similar behavior as it did in the control fermentation conditions, displaying no differences in its sulfide production under higher sugar content. The same behavior was observed for the low producer strains. Its basal H_2S release was not impacted by the high sugar concentration in the fermentation must. Montrachet (UCD522) has been extensively used as a “model” yeast to study nitrogen supplementation and sulfide production, due to the high amount of S^{2-} it produces. These studies have identified factors, such as pantothenic acid deficiency, sulphur amino acid deficiency, rehydration nutrients, relating nitrogen status and supplementation with H_2S release but in synthetic grape must or grape juice with sugar concentration no higher than 200g/L (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Winter et al. 2011; Ugliano, Kolouchova, and Henschke 2011b; Giudici 1994). Even more, genes identified to affect sulfide formation were identified in most of the cases in low sugar fermentations, ≤160g/L (Bartra et al. 2010; C. Huang, Roncoroni, and Gardner 2014; A. L. Linderholm et al. 2008; A. Mendes-Ferreira 2010; Yoshida et al. 2011). Few reports have been made in which

high sugar concentration was considered a factor (Taillandier et al. 2007) and moreover UCD522 was the high producing sulfide yeast (X. D. Wang, Bohlscheid, and Edwards 2003). The group found that in high sugar fermentations, at normal nitrogen availability (250mg N/L), pantothenic acid lowered the amount of H_2S released by Montrachet. This vitamin is required for CoA synthesis, a known coenzyme essential in the decarboxylation step to obtain pyruvate and the precursors that fix S^{2-} to a carbon skeleton, O-acetyl-homoserine and O-acetyl-serine. K1 described here as a high S^{2-} producer was no affected by the elevated sugar concentration, as well as Okay or Vivace. This raises the option that it could be the central carbon metabolism that is most affected by a higher sugar flux, and the response depends on how "resistant" each strain is to the glucose overflow and where it re-routes this excessive flux, without affecting its survival. But, in this work, all yeasts rehydrations as well as the fermentations were carried out without additional nutrient supplementation, to be able to adequately explain yeast sulfide release of the strains under the same conditions of wine cellars. In this context, some studies have observed that a discrepancy exists between yeast strain and their glucose and fructose consumption, and that nitrogen supplementation enhanced fructose utilization as well as fermentation temperature (Berthels et al. 2004; Tronchoni et al. 2009).

Many genetic approaches have tried to detect the crucial gene responsible for sulfide production (Bartra et al. 2010; C. Huang, Roncoroni, and Gardner 2014; C.-W. Huang et al. 2016; A. L. Linderholm et al. 2008; Angela L Linderholm et al. 2006; A. Mendes-Ferreira 2010; Yoshida et al. 2011). Among them, the work by Linderholm and Bisson, after a whole-genome screening from deletant mutants, found a pool of genes that when deleted increased or lowered H_2S formation. Genetic crosses identified the gene leading to reduced H_2S formation as an allele of *MET10* (*MET10-932*), which encodes a catalytic subunit of sulfite reductase (A. Linderholm et al. 2010). Further investigations, and new genetic approaches, as next-generation sequencing (NGS) or quantitative trait loci (QTL), have proven to be efficient strategies for linking the genetic polymorphism that explain phenotypic differences (Zimmer et al. 2014). That is how the group of Blondin B. could identify

in an isolate of a low sulfide producing yeast strain used commercially, two variants of the *MET2* and *SKP2* genes that control the sulfur assimilation pathway and the production of H_2S (Noble, Sanchez, and Blondin 2015). Mutants in all of these genes have been then utilized to develop novel commercial yeast strains with reduced production of H_2S during wine fermentation (Bisson LF., Linderholm A., Dietkel D. 2010; Kinzurik et al. 2016; Sanchez 2017). However, some of these mutations have resulted in yeast strains that have difficulties in completing fermentation in winery conditions. Moreover, it is known that not all yeasts deliver the same aromatic profiles even if fermenting the same must. So, more determinant than generating new wine yeast strains that do not produce sulfide, it would be to decipher the strategy of how yeast can re-route excessive flux of carbon sources that allows them not to lose their fermentative capacities, but at the same time avoid the routes that generate undesirable flavors in finished wines.

Conclusion

Today there is still little information regarding how yeasts respond to high sugar concentration during fermentation in winemaking related to sulfide release. The majority of studies have been conducted under sugar concentration around 21°B (200g/L soluble solids), an optimal parameter accepted by enologists in most wine regions of the word, or low sugar levels. Here, we present four commercial yeast strains, Montrachet, K1 Okay and Vivace, that share standard nitrogen requirements, but their reaction related to sulfide production with high sugar level fermentations differs from their response under typical fermentation conditions. Many factors have been associated with high sulfide production, but sugar level was not considered before a condition that could influence H_2S release. The isolation of natural mutants with low levels of H_2S and genetic approaches have helped decipher in a more precise way which are the genes and metabolic routes that govern the formation of sulfide. Here, results showed that in high sugar concentrations and no limited nitrogen conditions, yeast produces higher amounts

of H_2S. Also, a new strain was characterized as a high sulfide producer, K1, which produce even higher concentration than the classic strain Montrachet. Therefore, more imperative than to select and develop new yeast strains with low sulfide capacity, it would be to find how key factors that can effectively alter the response of the desired yeast to regulate its sulfide production and release but without losing the positive fermentative attributes of the strain.

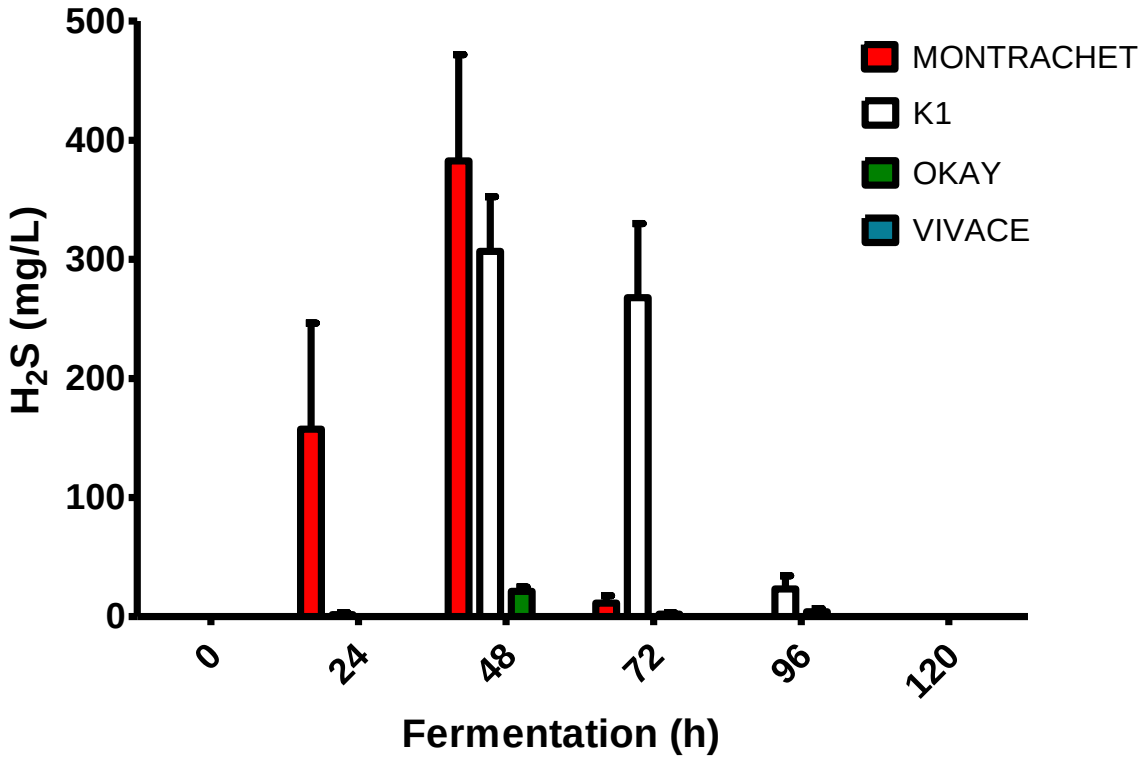


FIGURE 1. Evolution of H_2S in fermentations with standard initial sugar level and nitrogen content. *S. cerevisiae* UCD522 (Montrachet), K1 Maquée, ICV Okay and Vivace fermentation in 200g/L sugar concentration and 250mg N /L. Bars showed daily H_2S quantifications ±SD.

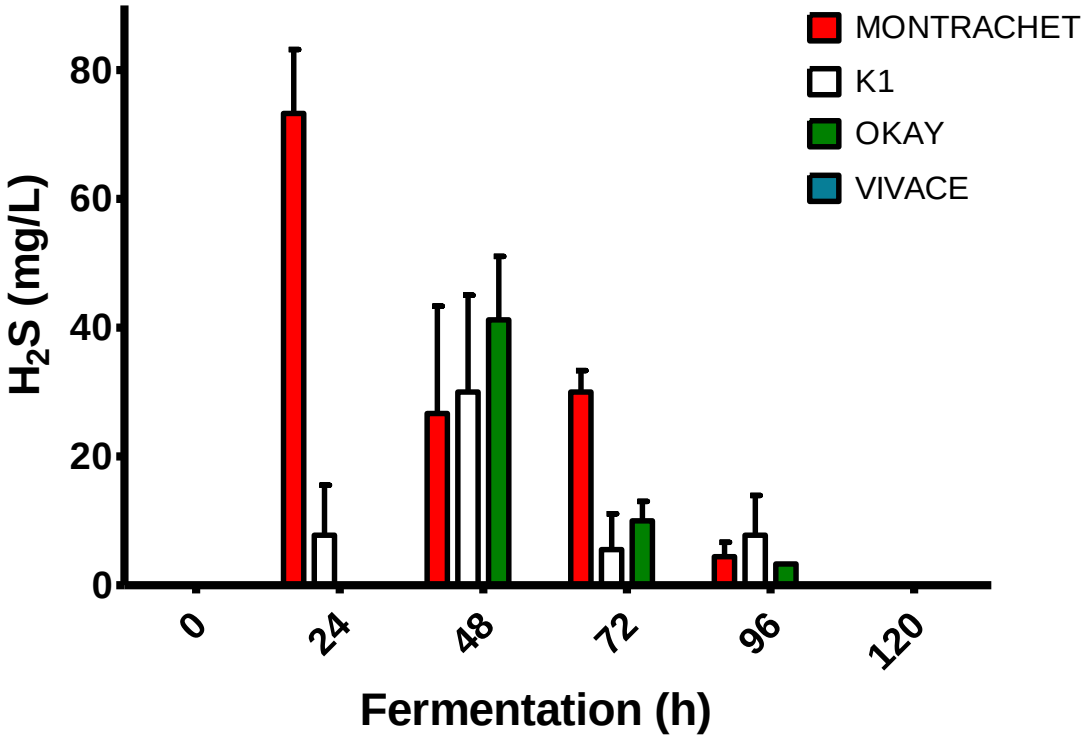


FIGURE 2. Evolution of H_2S in fermentations with low initial nitrogen content and standard sugar level. *S. cerevisiae* UCD522 (Montrachet), K1 Maquée, ICV Okay and Vivace fermentation in 200g/L sugar concentration and 120-150mg N /L. Bars showed daily H_2S quantifications ±SD.

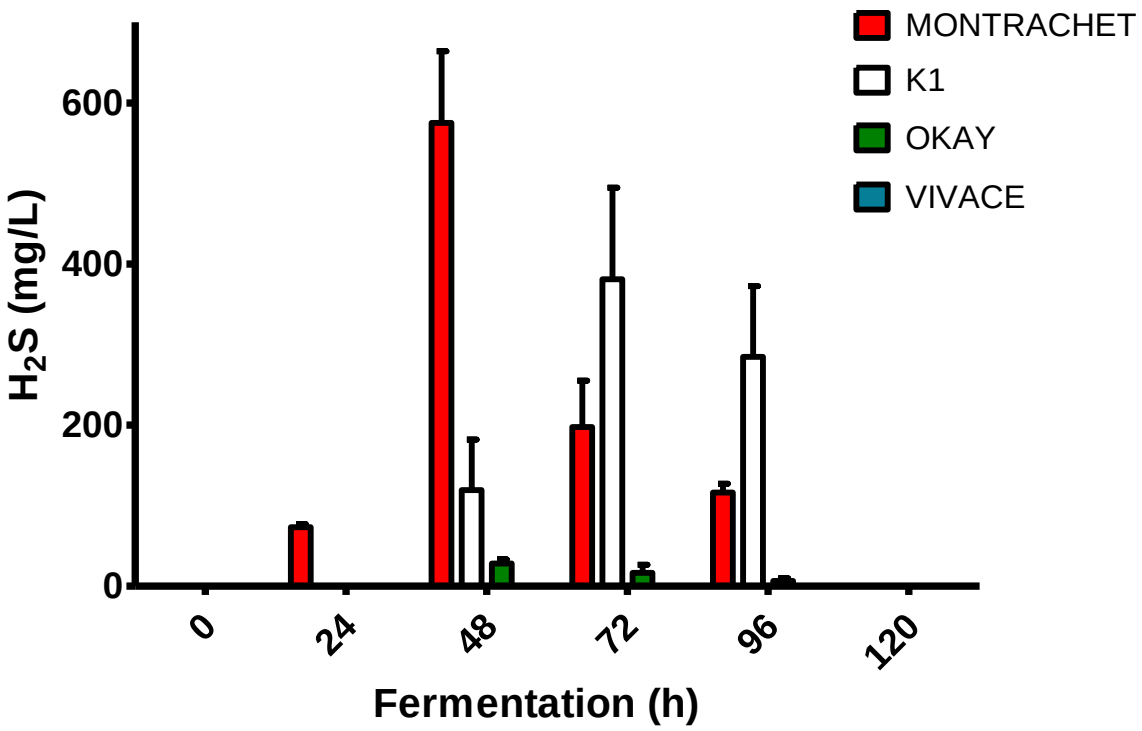


FIGURE 3. Evolution of H_2S in fermentations with high initial sugar level and standard nitrogen content. *S. cerevisiae* UCD522 (Montrachet), K1 Maquée, ICV Okay and Vivace fermentation in 250g/L sugar concentration and 250mg N /L. Bars showed daily H_2S quantifications ±SD.

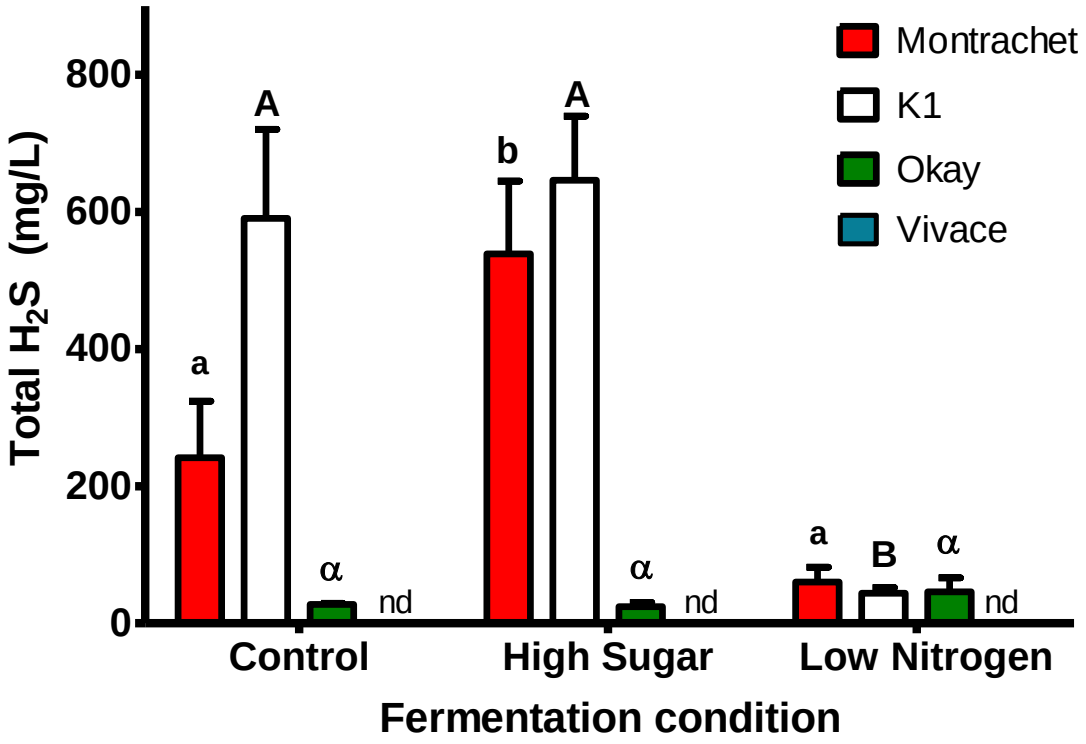


FIGURE 4. Total H_2S production in three distinct oenological fermentations conditions. *S. cerevisiae* UCD522 (Montrachet), K1 Marquée, ICV Okay and Vivace fermentation in **CTL**: 200g/L sugar concentration and 250mg N /L; **S+**: 250g/L sugar concentration and 250mg N /L; **N-**: 200g/L sugar concentration and 120-150mg N /L. Bars show H_2S quantification ±SD. **b**, $p < 0,05$; **B**, $p < 0,01$.

References

Barbosa, C., Mendes-Faia, A., Mendes-Ferreira, A., 2012. The nitrogen source impacts major volatile compounds released by Saccharomyces cerevisiae during alcoholic fermentation. Int. J. Food Microbiol. 160, 87–93. https://doi.org/10.1016/j.ijfoodmicro.2012.10.003

Bartra, E., Casado, M., Carro, D., CampamÃ, C., PiÃ±a, B., 2010. Differential expression of thiamine biosynthetic genes in yeast strains with high and low production of hydrogen sulfide during wine fermentation. J. Appl. Microbiol. https://doi.org/10.1111/j.1365-2672.2009.04652.x

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., Guillamón, J.M., 2005. Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption. J. Agric. Food Chem. 53, 996–1002. https://doi.org/10.1021/jf0487001

Bely, M., Sablayrolles, J.-M., Barre, P., 1990. Automatic Detection of Assimilable Nitrogen Deficiencies during Alcoholic Fermentation in Oenological Conditions 7.

Benavides, S., Cortés, P., Parada, J., Franco, W., 2016. Development of alginate microspheres containing thyme essential oil using ionic gelation. Food Chem. 204, 77–83. https://doi.org/10.1016/j.foodchem.2016.02.104

Berthels, N., Corderootero, R., Bauer, F., Thevelein, J., Pretorius, I., 2004. Discrepancy in glucose and fructose utilisation during fermentation by wine yeast strains. FEMS Yeast Res. 4, 683–689. https://doi.org/10.1016/j.femsyr.2004.02.005

Bisson LF., Linderholm A., Dietkel D., 2010. Compositions and Methods for Rreducing H2S levels in fermented beverages. US20100143536A1.

Butzke, C.E., 2011. Impact of Fermentation Rate Changes on Potential Hydrogen Sulfide Concentrations in Wine. J. Microbiol. Biotechnol. 21, 519–524. https://doi.org/10.4014/jmb.1010.10058

Davis, Robert E., J., Greory V., 2000. 2000: Characterization of Hydrogen Sulfide Formation in Commercial and Natural Wine Isolates of Saccharomyces 51, 16.

Fleet, G., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9

Gardner, J.M., Poole, K., Jiranek, V., 2002. Practical significance of relative assimilable nitrogen requirements of yeast: a preliminary study of fermentation performance and liberation of H_2S. Aust. J. Grape Wine Res. 8, 175–179. https://doi.org/10.1111/j.1755-0238.2002.tb00253.x

Giudici, P., 1994. The Effect of Nitrogen Deficiency and Sulfur-Containing Amino Acids on the Reduction of Sulfate to Hydrogen Sulfide by Wine Yeasts 45, 6.

Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F.N., Guillamon, J.M., 2012. Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. Food Microbiol. 31, 25–32. https://doi.org/10.1016/j.fm.2012.02.012

Huang, C., Roncoroni, M., Gardner, R.C., 2014. MET2 affects production of hydrogen sulfide during wine fermentation. Appl. Microbiol. Biotechnol. 98, 7125–7135. https://doi.org/10.1007/s00253-014-5789-1

Huang, C.-W., Walker, M.E., Fedrizzi, B., Roncoroni, M., Gardner, R.C., Jiranek, V., 2016. The yeast TUM1 affects production of hydrogen sulfide from cysteine treatment during fermentation. FEMS Yeast Res. 16, fow100. https://doi.org/10.1093/femsyr/fow100

Jiranek, V., Langridge, P., Henschke, P.A., 1996. Determination of sulphite reductase activity and its response to assimilable nitrogen status in a commercial Saccharomyces cerevisiae wine yeast. J. Appl. Bacteriol. 81, 329–336. https://doi.org/10.1111/j.1365-2672.1996.tb04335.x

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of Hydrogen Sulfide Liberation in Wine-Producing Saccharomyces cerevisiae Strains by Assimilable Nitrogen. APPL Env. MICROBIOL 61, 7.

Jones, G.V., White, M.A., Cooper, O.R., Storchmann, K., 2005. Climate Change and Global Wine Quality. Clim. Change 73, 319–343. https://doi.org/10.1007/s10584-005-4704-2

Kinzurik, M.I., Herbst-Johnstone, M., Gardner, R.C., Fedrizzi, B., 2016. Hydrogen sulfide production during yeast fermentation causes the accumulation of ethanethiol, S-ethyl thioacetate and diethyl disulfide. Food Chem. 209, 341–347. https://doi.org/10.1016/j.foodchem.2016.04.094

Linderholm, A., Dietzel, K., Hirst, M., Bisson, L.F., 2010. Identification of MET10-932 and Characterization as an Allele Reducing Hydrogen Sulfide Formation in Wine Strains of Saccharomyces cerevisiae. Appl. Environ. Microbiol. 76, 7699–7707. https://doi.org/10.1128/AEM.01666-10

Linderholm, A.L., Findleton, C.L., Kumar, G., Hong, Y., Bisson, L.F., 2008. Identification of Genes Affecting Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Appl. Environ. Microbiol. 74, 1418–1427. https://doi.org/10.1128/AEM.01758-07

Linderholm, A.L., Olineka, T.L., Hong, Y., Bisson, L.F., 2006. Allele Diversity among Genes of the Sulfate Reduction Pathway in Wine Strains of Saccharomyces cerevisiae 10.

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., Beltran, G., 2012. Biomass production and alcoholic fermentation performance of Saccharomyces cerevisiae as a function of nitrogen source. FEMS Yeast Res. 12, 477–485. https://doi.org/10.1111/j.1567-1364.2012.00802.x

Mendes-Ferreira, A., 2010. The Wine Yeast Strain-Dependent Expression of Genes Implicated in Sulfide Production in Response to Nitrogen Availability. J. Microbiol. Biotechnol. 20, 1314–1321. https://doi.org/10.4014/jmb.1003.03039

Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., Mendes-Faia, A., 2009. The production of hydrogen sulphide and other aroma compounds by wine strains of Saccharomyces cerevisiae in synthetic media with different nitrogen concentrations. J. Ind. Microbiol. Biotechnol. 36, 571–583. https://doi.org/10.1007/s10295-009-0527-x

Mendes-Ferreira, A., Barbosa, C., InÃªs, A., Mendes-Faia, A., 2010. The timing of diammonium phosphate supplementation of wine must affects subsequent H 2 S release during fermentation. J. Appl. Microbiol. 108, 540–549. https://doi.org/10.1111/j.1365-2672.2009.04457.x

Noble, J., Sanchez, I., Blondin, B., 2015. Identification of new Saccharomyces cerevisiae variants of the MET2 and SKP2 genes controlling the sulfur assimilation pathway and the production of undesirable sulfur compounds during alcoholic fermentation. Microb. Cell Factories 14. https://doi.org/10.1186/s12934-015-0245-1

Park Seung-Kook, P.S.-K., 2008. Development of a method to measure hydrogen sulfide in wine fermentation. 2008 18, 5.

Rossignol, T., Dulau, L., Julien, A., Blondin, B., 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast Chichester Engl. 20, 1369–85.

Sanchez, I., 2017. Montpellier (FR) (72) Inventors: Bruno Blondin, Montpellier (FR); Jessica Noble, Montpellier (FR): 15.

Spiropoulos, A., Bisson, L.F., 2000. MET17 and Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Appl. Environ. Microbiol. 66, 4421–4426. https://doi.org/10.1128/AEM.66.10.4421-4426.2000

Swiegers, J.H., Pretorius, I.S., 2007. Modulation of volatile sulfur compounds by wine yeast. Appl. Microbiol. Biotechnol. 74, 954–960. https://doi.org/10.1007/s00253-006-0828-1

Taillandier, P., Ramonportugal, F., Fuster, A., Strehaiano, P., 2007. Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food Microbiol. 24, 95–100. https://doi.org/10.1016/j.fm.2006.04.002

Tronchoni, J., Gamero, A., Arroyo-López, F.N., Barrio, E., Querol, A., 2009. Differences in the glucose and fructose consumption profiles in diverse Saccharomyces wine species and their hybrids during grape juice fermentation. Int. J. Food Microbiol. 134, 237–243. https://doi.org/10.1016/j.ijfoodmicro.2009.07.004

Ugliano, M., Kolouchova, R., Henschke, P.A., 2011. Occurrence of hydrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen. J. Ind. Microbiol. Biotechnol. 38, 423–429. https://doi.org/10.1007/s10295-010-0786-6

Wang, X.D., Bohlscheid, J.C., Edwards, C.G., 2003. Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. J. Appl. Microbiol. 94, 349–359. https://doi.org/10.1046/j.1365-2672.2003.01827.x

Winter, G., Henschke, P.A., Higgins, V.J., Ugliano, M., Curtin, C.D., 2011. Effects of rehydration nutrients on H2S metabolism and formation of volatile sulfur compounds by the wine yeast VL3. AMB Express 1, 36. https://doi.org/10.1186/2191-0855-1-36

Yoshida, S., Imoto, J., Minato, T., Oouchi, R., Kamada, Y., Tomita, M., Soga, T., Yoshimoto, H., 2011. A novel mechanism regulates H2S and SO2 production in Saccharomyces cerevisiae. Yeast 28, 109–121. https://doi.org/10.1002/yea.1823

Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D.J., Marullo, P., 2014. QTL Dissection of Lag Phase in Wine Fermentation Reveals a New Translocation Responsible for Saccharomyces cerevisiae Adaptation to Sulfite. PLoS ONE 9, e86298. https://doi.org/10.1371/journal.pone.0086298

Chapter 3

Aminoacids profiles during problematic natural must fermentations in four commercial wine yeast.

Laboratorio de Enología, Departamento de Fruticultura y Enología, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica, Av. Vicuña Mackenna 4860, Santiago, Chile

Abstract. During the evolution of alcoholic fermentation, problems can occur, resulting in sluggish or stuck fermentations. The information provided by routine measurements is not sufficient to detect and diagnose the state of the fermentation, and in case of stuck fermentations, rescue them on time. Nitrogen ammonium and free amino acids are one of the most studied variables involved in stuck or sluggish fermentations. This work aimed to study the evolution of amino acids during wine fermentation of *Cabernet Sauvignon* must as a tool to diagnose problematic fermentations. Fermentations were carried out, with different initial sugar concentrations and initial nitrogen content. Samples were collected at 24 hours intervals and amino acids were evaluated by HPLC (*High-Performance Liquid Chromatography*). In all fermentations, it was possible to detect and quantify the 18 amino acids allowing their quantification.

Depending on the initial condition of the fermentation (high initial sugar, low nitrogen) each amino acid showed a specific evolution profile, except for proline. These results support the potential use of amino acid profiles, to diagnose problematic fermentations and to predict the behavior of yeast in these conditions.

Keywords: Aminoacids, wine yeast, natural must, high sugar content, low nitrogen, fermentation.

Introduction

During the evolution of fermentation, problems may occur that result in sluggish or stuck fermentations. Typical problems causing stuck and sluggish fermentations are associated with extreme temperatures or pH, lack of nutrients, such as nitrogen or oxygen, high initial sugar content, ethanol toxicity, short- and medium-chain fatty acids, and incorrect enological practices (Rossignol et al. 2003a). Fermentations are identified as problematic at a late stage when the rate of sugar consumption has already slowed down, or it is wholly detained (Bisson 1999b). The information provided by routine measurements is not sufficient to detect and diagnose the state of the fermentation, and in case of stuck fermentations, rescue them on time. Instead, advanced monitoring can assist in the early identification of problematic fermentations.

Nitrogen is one of the most studied variables involved in stuck or sluggish fermentations (Mendes-Ferreira et al., 2004). It is the second most abundant

macronutrient in wine fermentation and is essential for the metabolism and growth of yeast (C. Varela, Pizarro, and Agosin 2004). The nitrogen content in the must is highly variable, not only in quantity but also in the types of compounds present. The fraction of available nitrogen is constituted on average by 51 to 92% of α-amino acids and 40% of ammonium (Bell and Henschke 2005b; Crépin et al. 2012). Mixes of ammonium plus amino acids can be a better source of nitrogen than ammonium alone. It was established that yeasts use specific sources of nitrogen and that the pattern of consumption depends both on the composition of the nitrogenous compound and in some cases on its concentration (F. Ferreira Monteiro and Linda F. Bisson 1992).

Under winemaking conditions, nitrogenous compounds are taken up and degraded in a specific order depending on environmental, physiological, and strain-specific factors (Salmon and Barre 1998). Arginine, glutamine, lysine, serine, threonine, and ammonium are assimilated very quickly. Proline, however, is only partially assimilated (Crépin et al. 2012; Jiranek, Langridge, and Henschke 1995a; Pszczólkowski et al. 2001).

The importance of assimilable nitrogen to yeast during fermentation does not reside only on yeast growth and biomass formation, and it is also a key factor for the metabolism of flavors and the aromatic profile of wine (Bell and Henschke 2005b). There are some studies relating yeast nitrogen demand to the profile of aroma compounds in wines (Carrau et al. 2008). One of the group of compounds that form the wine aroma are the higher alcohols. Fusel or higher alcohols are produced by yeasts during alcoholic fermentation through the conversion of the branched-chain amino acids present in the medium: valine, leucine, isoleucine,

threonine, and phenylalanine as well as de novo from sugar substrates (Lambrechts and Pretorius 2000b). The amino acids in must are among the most important factors influencing fusel alcohol formation. They are able to alter the yield of higher alcohols by its contribution to the total nitrogen content, due to the anabolic pathway depends on the nitrogen level (Äyräpää T. 1968).

The most negative aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides, and thiols (Lambrechts and Pretorius 2000b). The release of H_2S during vinification is a big problem as it contributes negatively to wine quality and it is considered a wine fault even at very low concentrations (≤1.6 µg/L) (Bekker et al. 2016; Ugliano, Kolouchova, and Henschke 2011b) as it imparts an unpleasant aroma to rotten eggs. Amino acids have also been implicated in the regulation of sulfide release. Jiranek et al., 1995b showed most amino acids had similar suppressive capacity to that of ammonia, except for proline, cysteine, and threonine. Therefore, one routine practice to limit the formation of H_2S is to provide yeast assimilable nitrogen(YAN) by the addition of diammonium phosphate, to ensure sufficient availability of amino acid, although this practice had become questionable (Bell and Henschke 2005b; Ugliano, Kolouchova, and Henschke 2011b). The aim of this work was then, to study the evolution of amino acids during wine fermentation of *Cabernet Sauvignon* must for four commercial wine yeast, as a tool to help predict faulty fermentations as well as to prevent the formation of undesirable off-flavors such as hydrogen sulfide.

2 Results & Discussion

2.1 Factors for problematic fermentations

To evaluate the amino acid profile, 2 major factors that can cause problems during wine fermentation were selected (Bisson 1999b). These conditions were nitrogen deficiency and initial sugar content. Therefore, a combination of normal and problematic grape musts was developed, without mixing these factors. This resulted in 3 types of fermentation depending on the excess, lack or normal level for each of the different factors. The control levels were set at 203g/L of initial sugar content, 250mg/L of YAN at the beginning of the fermentation; high initial sugar was stablished at 250g/L with initial YAN of 250mg/L; and nitrogen deficiency was YAN ≤ 150mg/L (120mg/L) with an initial sugar concentration of 203g/L. Temperature was set to 25°C to carry fermentations. According to Jiranek et al., (1995), the minimum nitrogen requirement corresponds to the concentration of assimilable nitrogen below which time for completion of fermentation is unsatisfactory. The minimum requirement for nitrogen has been reported between 120 to 140 mg N/L. Therefore, to assure that fermentations were carried out, a concentration equal or below to 150mg N/L was chosen. With respect to high initial sugar, sugar concentrations has been increasing over the years to higher values (Davis, Robert E. 2000; Martínez-Moreno et al. 2012b), establishing normal sugar values around 230g/L or higher (25°B).

2.2 Optimization of HPLC analysis

In order to identify and later quantify each amino acid along fermentations, it was necessary to determine their exact retention time in the chromatogram. To achieve this, the chromatographic method proposed by Chicón R., Hermosín I.,Cabeduzo M D., 2001 was implemented and even though it could detect 16 amino acid, some of the picks were overlapped and couldn't be completely separated from one another. This was the case of amino acids histidine, glycine and threonine. To get better individual picks, the initial gradient together with the flow rate was modified. Oven temperature together with elution time was returned to values established by Alaiz et al., 1992 and Wang et al., 2014. Hence, the final chromatographic program was set as shown in Table 1, with an oven temperature of 18°C and a total elution recording time of 41min, ideal to detect primordially free amino acids. Therefore, the selected conditions used were a concession between chromatographic separation of as many amino acids as possible, with quality resolution, and a relatively rapid analysis of samples.

Table 1. HPLC method binary gradient used to measure aminoacids in alcoholic fermentations

Time (min)	Acetate 25mM 0,02% azide pH 5,8 (%)	Acetonitrile:Methanol 80:20 (%)	Flux (mL/min)
0	94	6	0,8
13	84	16	0,8
14,5	82	18	0,8
18	82	18	0,8
21	78	22	0,8
33	68	32	0,8
38	63	37	0,8

2.3 Aminoacid standards, calibration curves, and method validation

For accurate quantification of amino acids in the fermentation samples, a commercial standard of 17 amino acids plus ammonium solution (Sigma, USA) was evaluated along with individual samples of each amino acid. These standards, together with the internal standard (2-aminoadipic acid) of the method, allowed setting the chromatographic conditions established in the point above. A Chromatogram of the standard solution is shown in Figure 1. Almost all amino acids are represented, the only amino acids not showing are Tryptophan (Trp), Asparagine (Asn) and Glutamine (Gln), because the standard solution did not

include them. Later on, when individual standard solutions of each amino acid were tested, four aminoacids were not able to be separated. The amino acid serine co-eluted with asparagine, and leucine co-eluted with tryptophan (Fig. 2) as it was corroborated when individual standard of each of these amino acids was run and the chromatograms of both amino acids compared against each other overlapped. These results agree with those of Gómez-Alonso *et.al.* (2007) even though the peaks for these amino acids in his chromatograms were almost overlapped, in comparison to the chromatogram obtained by Alaiz et al., 1992, that didn't consider these amino acids (Asn, Gln and Trp). In this case, the chromatographic separation made by Wang and coworkers accomplished a better separation, being able to distinguish Ser from Asn and Leu from Trp, that this study couldn't do. The structural similarities or behaviors between the derivatized amino acids that coeluted could be a reason why we weren't able to separate them, and a more polar gradient with lower flux would have been needed as the one used by Wang et al., 2014. Unfortunately, this compromised the resolution of the peaks at the beginning of the elution. But clearly, a High-Throughput (HT) chromatographic column has primarily a higher performance and resolution. Finally, the one amino acid that couldn´t be detected was glutamine. When tested in pure samples, for identification and calibration, it didn´t co-elute with any of the other amino acids and it wasn´t possible to identify the peak. For example, in some samples, with low proline content, it looked like the Gln peak appeared in the same elution times window that proline did, but in other cases, the peak appeared between the peaks of serine and histidine.

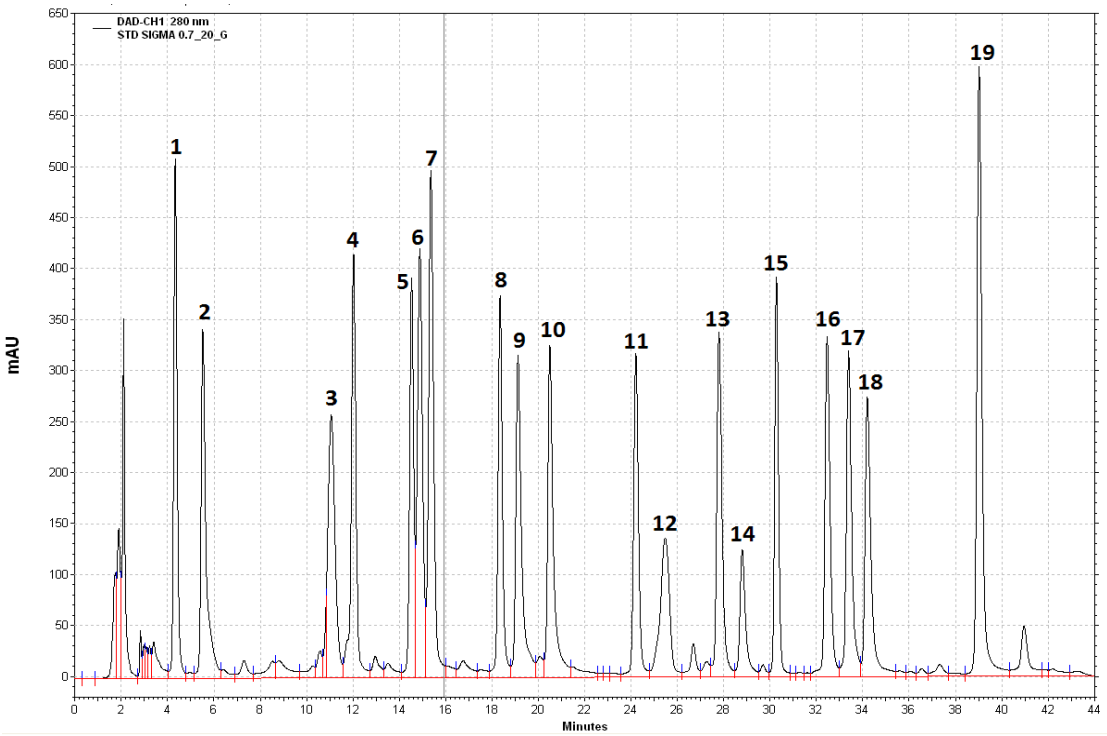


Figure 1. Chromatogram of a mixed standard solution of amino acids (Sigma). The profile of 18 amino acid plus internal standard (PI) was obtained by HPLC described in this study. 1. Aspartic acid; 2. Glutamic acid; 3. Aminoadipic acid (PI); 4. Serine; 5. Histidine; 6. Glycine; 7. Threonine; 8. Arginine; 9. Alanine; 10. Proline; 11. Tyrosine; 12. NH_4^+; 13. Valine; 14. Methionine; 15. Cysteine; 16. Isoleucine; 17. Leucine; 18. Phenilalanine; 19. Lysine.

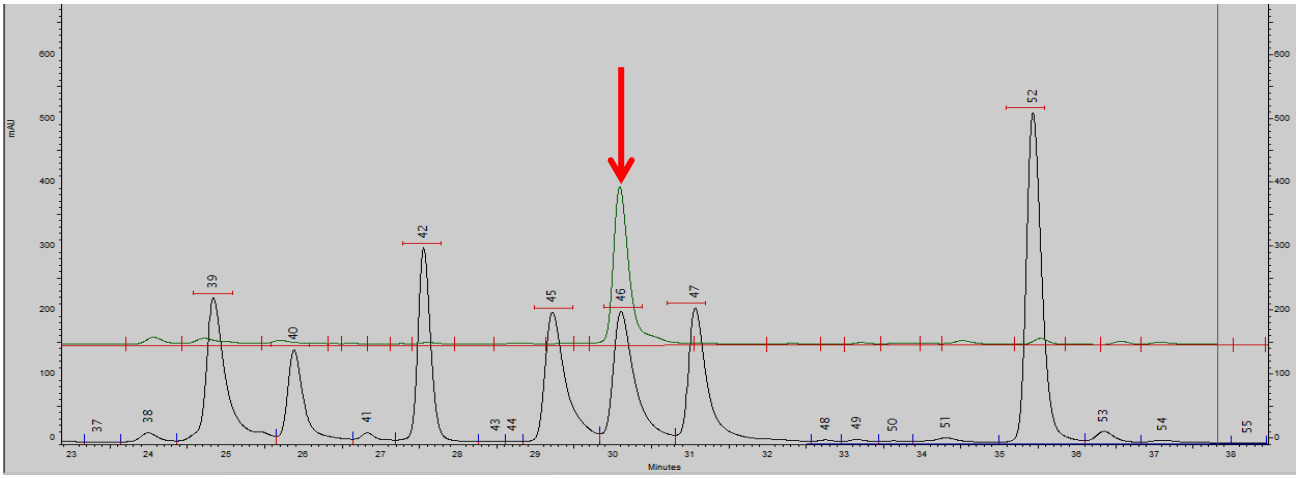


Figure 2. Close-up cromatogram for aminoacids: 39. Valina; 40. Metionina; 42. Cisteína, 45. Isoleucina; 46. Leucina+Triptofano; 47. Fenilalanina; 52. Lisina. It can be obserbed that a pure standar of Trp eluted at the same time as Leucina in the standar mix. In fermentations samples these two amino acids couldn't be separated to identify each unique peak.

Once the method was established and the elution time of each amino acid defined, the column specific behavior was evaluated. The linearity, detection limits and recovery of the method was evaluated, using the same standards solution mix and individual amino acids **(Table 3).** Specificity was evaluated by choosing two representative amino acids, methionine and cysteine **(Table 4)**, and lysine was chosen randomly to further evaluate recovery of the method **(Table 5)**. Cysteine and methionine were selected due to their polemic role as one of the possible factors that can trigger H_2S formation during alcoholic fermentation in wine yeast. All compounds showed good linearity over the range tested but not all amino acids could be quantified satisfactorily in must and wine samples. In those cases, another variable was used to obtain the concentration of the amino acids out of range. The relative response factor (RRF) was calculated for cysteine, using the internal standard concentration and its peak area. Proline, even though it was successfully detected and identified, it could not be quantified. In the majority of samples, the peak of proline was oversaturated, making it difficult to produce a calibration curve to quantify.

The repeatability of the method didn´t seem to be very precise to quantify ammonium according to the RSD obtained (Table 3). This problem must have been due to the proportion of the peak area detected. Along with proline, it was one of the largest peak areas detected, especially when evaluated in supplemented grape must. Therefore, apparently, the method is not very sensitive for detecting subtle changes in concentration when the peak area is sizable. Another point to consider is RSD calculation. While it can be obtained using peak area of the samples, it can also be derivate from recovery percentages. These may

be a more accurate measure to determine how precise the experimental data is. In general, area peaks don´t appear to differ greatly from one run to another, but subtle changes in area can result in significant changes in concentration. For example, recovery was calculated from 5 consecutive runs of the same standard lysine sample. Peak area (normalized versus internal standard area), range was 0,756 to 0,741 which was translated to a recovery ranging from 108% to 111%. This small variability was better detected by RSD obtained from recovery measurements than peak areas, which could reflect the intrinsic variability of the method and the particular chromatographic characteristics of the column used.

TABLE 3. Parameters of chromatographic method for 17 aminoacids identified plus ammonium. Retention times (RT), calibration, detection limit (LOD), quantification (LOQ) and repeatability.

	RT (min)	r^2	Linear range	LOD	LOQ	Repeatability *
Asp	6,92	1	1-20	0,21	0,71	3,13
Glu	9,06	0,9959	1-20	0,85	2,84	5,65
Ser+ Asn	13,74	0,9983	1-20	1,14	3,81	10,89
His	15,05	0,9984	1-20	1,63	5,42	13,31
Gly	16,20	0,9978	0,5-15	0,92	3,06	14,11
Thr	16,99	0,9984	1-20	1,26	4,20	5,28
Arg	18,37	0,999	1-25	0,47	1,56	3,79
Ala	20,02	0,9997	1-10	0,39	1,29	6,88
Tyr	25,22	0,9993	2-25	1,29	4,28	10,11
NH4	27,26	0,9587	1-200	1,53	5,11	23,31
Val	28,92	0,9956	1-20	0,16	0,54	8,16
Met	30,11	0,9923	1-20	3,43	11,42	13,99
Cys	31,20	0,9931	2-20	1,08	3,60	5,23
Ile	33,55	0,9996	1-20	0,65	2,18	7,15
Leu+Trp	34,58	0,9997	1-20	0,63	2,11	6,98
Phe	35,47	0,9995	1-20	0,96	3,19	7,42
Lys	39,37	0,9995	1-20	0,83	2,78	3,89

*RSD of 3 runs of the same standard in 3 different levels.

TABLE 4. Specificity/Selectivity for two representative aminoacids, cysteine (Cys) and methionine (Met). Standards samples were diluted in two matrixes, water and wine.

	Cys 14ppm				
matrix	**peak area[a]**	**mg/L**	**%Recovery**	**RSD[b]**	**RSD[c]**
water	0,342	18.968	135,486	0,072	0,076
wine	0,299	15.864	113,312	1,538	1,959

	Met 14ppm				
matrix	**peak area[a]**	**mg/L**	**%Recovery**	**RSD[b]**	**RSD[c]**
water	0,300	15.367	109,762	0,190	0,185
wine	0,131	6.477	46,262	0,955	0,897

a. peak area sample vs peak area internal standard.
b. RSD of %recovery from 5 consecutive runs.
c. RSD of peak area from 5 consecutive runs.

TABLE 5. Recovery of the method tested in a randomly chosen aminoacid, lysine (lys).

Lys

Addition	peak area[a]	mg/L	%recovery	RSD[b]	RSD[c]
12ppm	0,727	12.320	106,545	3,893	3,452

a. peak area sample vs peak area internal standard.
b. RSD of %recovery from 5 consecutive runs.
c. RSD of peak area from 5 consecutive runs.

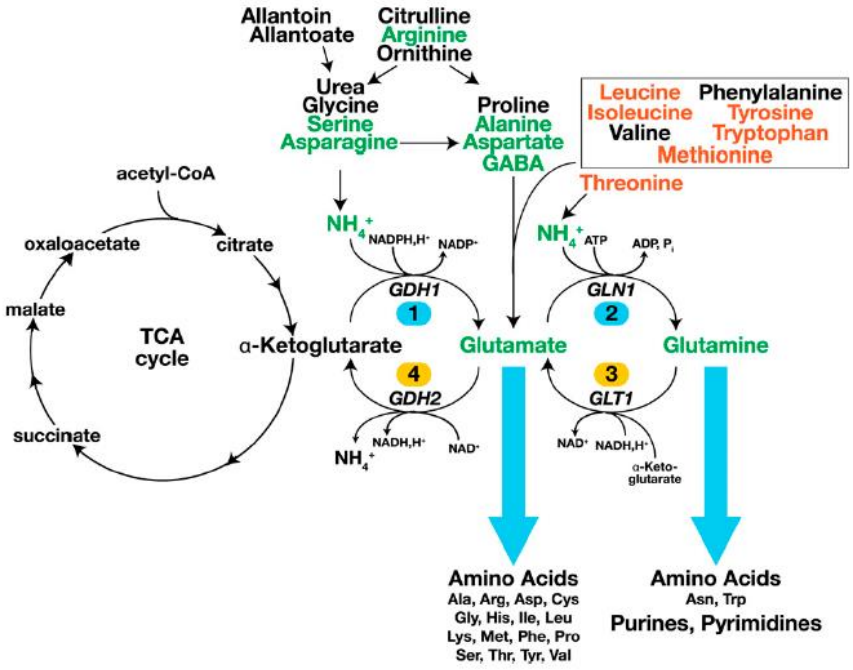
2.4 Evolution of amino acid profiles during *Cabernet Sauvignon* fermentations

2.4.1 Control fermentations

A total of 4 fermentations and their replicates were set up as controls with the established parameters (Table 2) for all 4-wine yeast (K1, Okay, Montrachet and Vivace). In doing these, we obtained the profile of amino acids in control conditions, which were used as a baseline to compare the change of the amino acids when the parameters were altered. In general, all the amino acids exhibited a rapid consumption at the beginning of fermentation, after 24- or 48-hours post-inoculation, remaining at low levels until the end (<50%), with some exceptions depending on the yeast strain (Fig.4). K1 was the only strain that consumed all

amino acids below 50% together. With Okay, the only exceptions were tyrosine and cysteine. For Montrachet, 5 of the 16 measured amino acids were left with residual percentages above 50% (Fig.4, Fig. 5) and with Vivace 6 amino acids had less than 50% consumption. What was interesting was these last two strains shared the same behavior for amino acids Hist, Gly and Cys (Fig. 4, Fig. 5), and for cysteine, they share this profile with Okay. Wine yeasts prefer to utilize certain aminoacids before others (Jiranek et al., 1995). Ammonium is a preferred nitrogen source and when present inhibits the uptake of arginine and alanine and stimulates the uptake of branched-chain and aromatic amino acids (Beltran et al. 2005a). Glutamine and asparagine are also considered preferred nitrogen sources, whereas arginine, alanine, aspartate, and glutamate are less preferred (Gutiérrez et al. 2013; Magasanik and Kaiser 2002). In conditions without ammonium; arginine, serine, glutamate, threonine, aspartate and lysine are generally most heavily utilized, contributing between 70.6% and 79.0% of the total nitrogen requirement (Jiranek, Langridge, and Henschke 1995a). As pointed out earlier, two yeast strain draw attention, Montrachet and Vivace (Fig. 4, Fig. 5). Montrachet or UCD522, is a known wine yeast used in many studies (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Bartra et al. 2010; Carrau et al. 2008; Giudici 1994; Giudici P., Zambonelli C., Kunkee R.E. 1993; A. Mendes-Ferreira 2010; A. Mendes-Ferreira et al. 2010; Ana Mendes-Ferreira et al. 2009b; Singh' and Kunkee 1976; X. D. Wang, Bohlscheid, and Edwards 2003), specially for its high ability to produce sulfide (S^{2-}). On the contrary, Vivace is a relatively recent commercialized yeast obtained by Bisson and her group after a whole-genome screening for genes that when deleted lowered H_2S formation (Bisson LF.,

Linderholm A., Dietkel D. 2012; A. Linderholm et al. 2010; A. L. Linderholm et al. 2008). Therefore, even these two yeasts showed different behaviors regarding its sulfide capacity, they share a similar response in how they consume/release histidine, glycine, and cysteine during must fermentations. Okay, is also known as a low sulfide producer but its cysteine consumption profile is very similar to that of Montrachet. According to Jiranek (1995), glycine is often excreted during fermentation, being especially high for Montrachet. Tyrosine is known to be a non-preferred nitrogen source and Okay didn't consume this amino acid above 20%. (Fig. 4). Only for K1 a residual percentage below 50% was observed for tyrosine. L-proline as it is not utilized during fermentation conditions (Jiranek, Langridge, and Henschke 1995a; Valero et al. 2003) was not studied, but it was found in large quantities (data not shown). According to Valero et al., 2003, there are other amino acids excreted under most fermentation conditions as leucine, tryptophan, methionine, and cysteine. This group found that cysteine release could be in part dependent on the oxygen presence. Moreover, anaerobiosis resulted in the consumption of fewer amino acids.



.

Figure 3. Schematic diagram of the main pathways of nitrogen metabolism. The entry routes of several nitrogen sources into the central core reactions are shown. The class A preferred sources are in green and class B nonpreferred sources are in red. Ljungdahl & Daignan-Fornier *et.al.*,2011

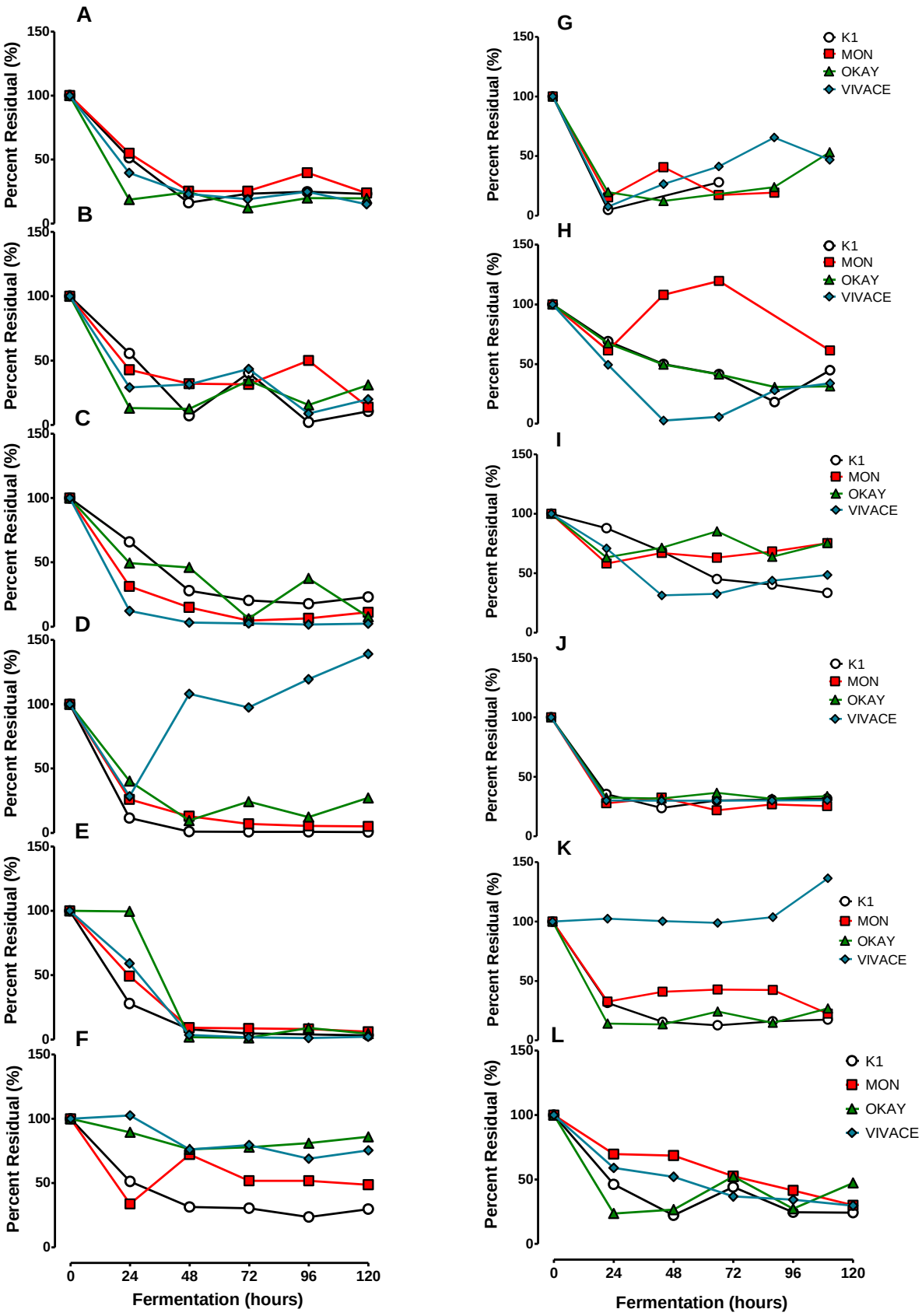


Figure 4. Porcent residual of aminoacids for yeast K1, Montrachet, Okay and Vivace in fermentation of *Cabernet Sauvignon* with initial conditions of 203g/L of sugar level and 250mg/L assimilable nitrogen. **A.** Aspartate; **B.** Glutamate; **C.** Threonine; **D.** Arginine; **E.** Ammonium; **F.** Tyrosine; **G.** Valine; **H.** Methionine; **I.** Cysteine; **J.** Isoleucine; **K.** Phenilalanine; **L.** Lysine

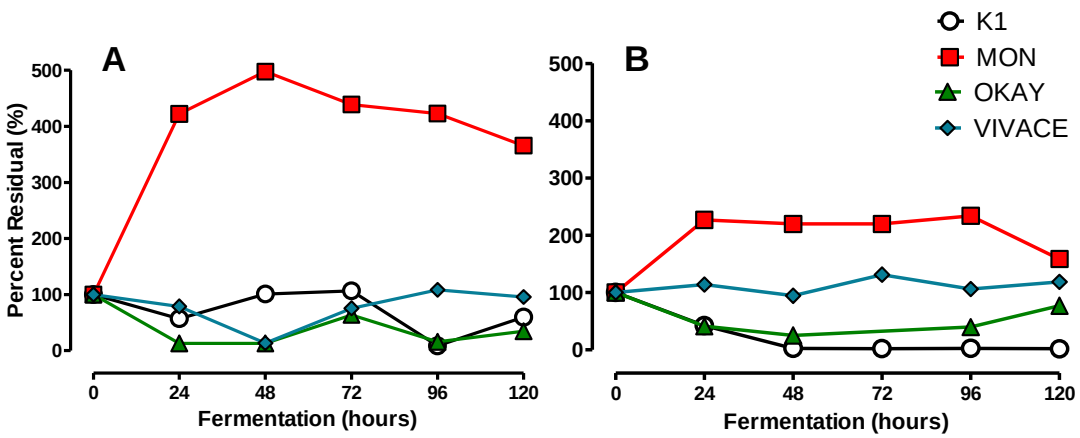


Figure 5. Percent residual of aminoacids Histidine and Glycine for yeast K1, Montrachet, Okay and Vivace in fermentation of *Cabernet Sauvignon* with initial conditions of 203g/L of sugar level and 250mg/L assimilable nitrogen. **A.** Histidine; **B.** Glycine.

2.4.2 Problematic fermentation

A total of 8 different fermentations plus their replicates were carried out for the four yeast with the factors chosen to cause problematic fermentations: high initial sugar concentration and low initial assimilable nitrogen (Fig. 6 and Fig. 7, respectively). The fermentations evaluated showed profiles altered compared with those in control conditions and it is a clearly strain-dependent consumption. Various studies have investigated the effect of low assimilable nitrogen in different variables such as fermentation rate, biomass production, volatile compounds (Arias-Gil, Garde-Cerdán, and Ancín-Azpilicueta 2007; Beltran et al. 2005a; Bely, Rinaldi, and Dubourdieu 2003; Clement et al. 2013; Henschke P.A. and Jiranek V. 1993; A. Mendes-Ferreira, Mendes-Faia, and Leao 2004; Taillandier et al. 2007; Vilanova et al. 2007). When amino acids consumption has been evaluated, synthetic media, must or model wines are supplemented with ammonium and/or with extra amino

acids and samples are usually taken at the beginning and/or end of fermentation, with just a few works where they follow the evolution of amino acids between those times (Arias-Gil, Garde-Cerdán, and Ancín-Azpilicueta 2007; Gómez-Alonso, Hermosín-Gutiérrez, and García-Romero 2007; Hernández-Orte et al. 2012; Martínez-Gil et al. 2012; Pozo-Bayón et al. 2009; Valero et al. 2003; Y.-Q. Wang et al. 2014). In this study, the amino acid profiles were followed through every 24 hours sampling for 5 days of fermentation, time when nitrogen was mostly consumed for all yeast (Fig 3E, 6G, and 7E). Once again, profiles that draw most attention were the ones by Vivace (Fig.6 A, E, I) and Montrachet (Fig 6 B, C, I), although in low nitrogen condition, K1 also showed an interesting behavior, that may suggest being a more efficient consumption of amino acids in general (Fig 3 and Fig. 6). In the deficient nitrogen condition, only glycine was released to the medium but to a much lesser extent than in control fermentation (Fig 5B, Fig 6G), and it was Montrachet the strain responsible. Histidine, unlike Glycine, was consumed by almost all strains, except for Montrachet (Fig 5A, Fig. 6C). Vivace consumed glycine (≤ 30% residual amount in must) but high amounts of residual arginine were found in fermentations with this yeast (Fig. 6E). Tyrosine was not consumed by Montrachet or Vivace and now Okay didn't consume it either (Fig. 6F). One amino acid that called extra attention was aspartate. It is considered one of the nitrogen sources that is heavily utilized, irrespective of the yeast strain (Jiranek, Langridge, and Henschke 1995a) and being in a low assimilable nitrogen condition (no ammonium supplementation), it would be expected to be consumed entirely (Fig 6A), as K1 did. Arginine also belongs to this group of amino acids, but

as it was seen, Vivace did not consume it in control fermentation nor deficient nitrogen (Fig 3D, Fig. 6E).

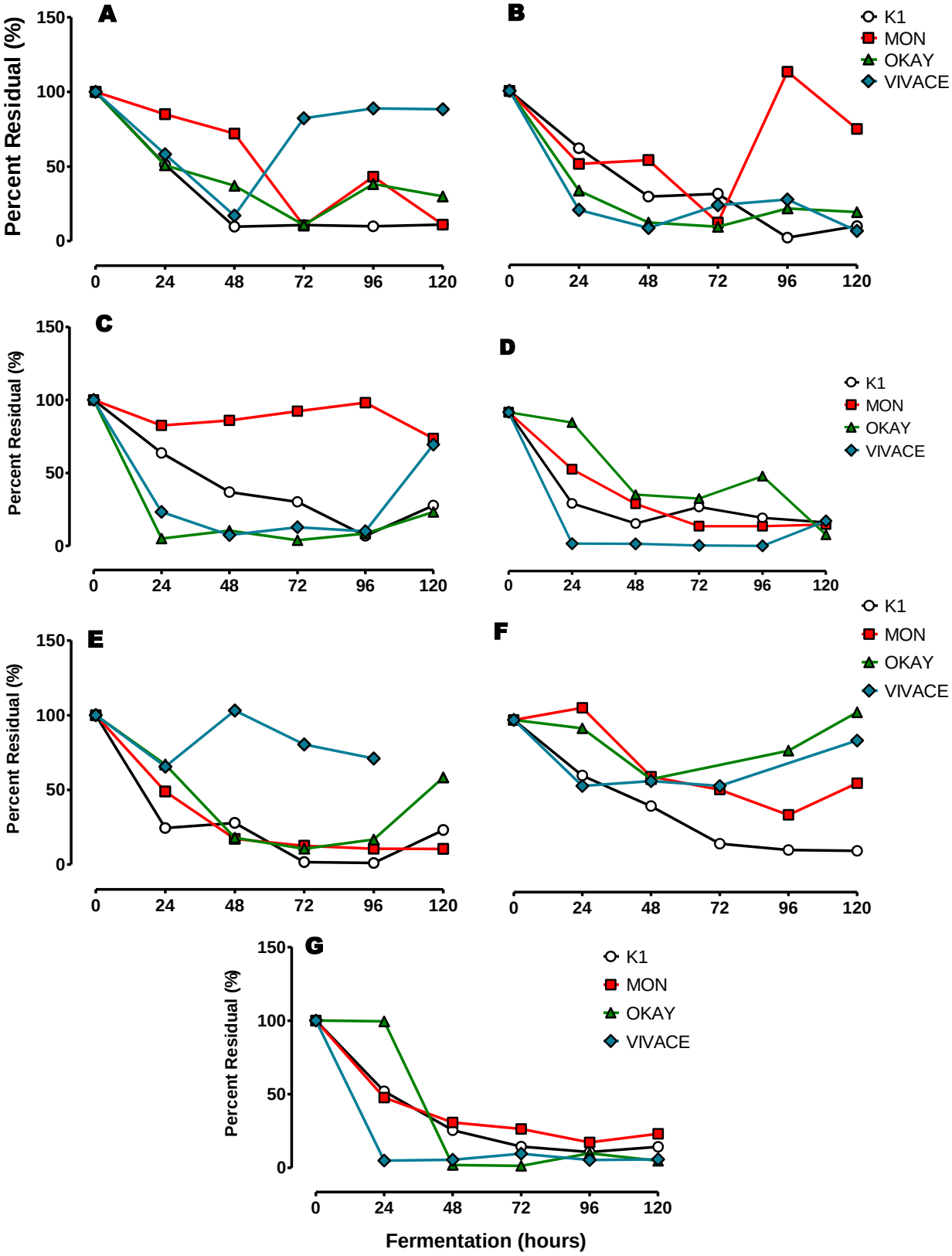
Finally, the last aspect that has not been considered in aminoacids profile consumption is DAP supplementation, in must with high initial sugar concentration. Taillandier et al., (2007) address the issue of fermentation of wine yeast in high sugar content, but they focused only on ammonium nitrogen consumption. Again, it can be seen the same amino acids that are not consumed by the same yeast strains (Fig. 7). Montrachet didn't use glutamate nor glycine or lysine. Vivace didn't absorb aspartate or arginine. K1 this time did not consume arginine nor histidine, being Montrachet that was showing no consumption for histidine in the control and no nitrogen conditions. For tyrosine, the profile was now equivalent for all yeast strains, neither of them consumed it. According to Valero et al., 2003 much of the ammonium ion and arginine are utilized by yeast during alcoholic fermentation, in particular in fermentations with high sugar concentrations (250g/L). The unique behavior showed by K1 and Vivace regarding arginine uptake (Fig. 7D) could be related to the possible utilization of another nitrogen source under high sugar concentration in anaerobiosis. What it is interesting is these two yeasts are counterparts when it comes to its classification as high and low H_2S producers, being Vivace the one cataloged as a no producer. In this matter, Jiranek et al., 1995b, proposed that amino acids that support high growth rates are the ones that better suppress H_2S formation, being arginine one of them. This greatly support the idea that it is not one key amino acid that can explain a particular behavior but rather the general trend for all amino acids in a particular condition.

Finally, to further study if the aminoacids that modified its uptake profile for each yeast could be associated with more than one strain, PCA analysis was performed, with its associated heatmap. Principal component 1 is yeast strains, and principal component 2 is amino acid consumption. The PCA was carried-ou for each of the times that amino-acids samples were measured. At 48hours post-inoculation, and fermentation began, yeast showed different behavior in how they grouped in high initial sugar, low nitrogen content in contrast to control conditions (Figure 8). In control conditions, K1 and Okay cluster together, suggesting that in this circumstance, these two yeast share a similarity in how they respond to amino-acid in the must and the way they consume them. On the other hand, Montrachet and Vivace are far away from each other and the other two yeast. It seems that Vivace does not have a strain-specific response to how it consumes the amino acids in normal fermentation conditions since none of the components analyzed explains its consumption profile. However, the situation completely changes when low nitrogen and high sugar is analyzed (Figure 8B and 8C). In low nitrogen supplementation, Vivace now is grouped with Okay, and Montrachet is the one not having a behavior based on a strain-dependent manner. This type of conduct could be explained in the way that is the concentration of amino acid that runs the profile of how this yeast consumed them, but clearly other factors are involved, since PC2 only explains approximately 20% of the variability of the data. K1 oppositely, respond to low nitrogen in a strain-specific way that can explain over 50% of the variability of the consumption profile. In a high sugar content, all four yeast function in their specific way, but is Vivace, who is most influenced in a strain-dependent manner regarding its consumption profile than the others yeast. Again, Montrachet

cluster does not respond to any of the variables, and Vivace response almost entirely strain-dependant. K1 and Okay, once more, showed a response that is a combination of both variables but with most substantial influence of the strain component. Subsequent, the heatmaps results showed that some yeast share uptake profiles from amino acids in the different fermentation conditions and that they also show a hierarchical clustering among them regarding amino acid uptake (Fig 9).

In all three conditions for the four yeast strains, three amino acids that commonly do not get much attention showed interesting behaviors throughout the fermentation. Threonine, as well as valine, are amino acids well known to be precursors of fusel alcohols trough the Ehrlich pathway (E. C.-H. Chen 1978). Threonine is consumed by all four yeast in every condition (Fig.3C, Fig 6D, Fig. 7C) but is specially taken up by Vivace and Montrachet. On the other side, valine is excreted by yeast Vivace and Okay in control conditions (Fig. 3C), while K1 and Montrachet consume it entirely. Montrachet in low nitrogen levels took up all valine present after only 48hours after inoculation (Fig. 6H). This behavior can also be seen for Vivace but only in high sugar fermentation and with a rapid consumption rate in the first 24hours. These two amino acids are responsible for the production of the fusel alcohols n-propanol, and isobutanol. It has been seen that the formation for both of these alcohols is inversely proportional to the concentration of amino acids added to low nitrogen must (Garde-Cerdán and Ancín-Azpilicueta 2008). Moreover, higher alcohols can also be produced from sugar catabolism, accounting for most of alcohols produced during fermentation containing a mixture

of ammonium and amino acids as nitrogen sources (Vilanova et al. 2007). In this context, a link to further look could be the increased n-propanol formation in some yeast strain with the ability to have a reduced formation of H_2S, as seen by Giudici P., Zambonelli C., Kunkee R.E., (1993). This group characterized two strains they already had classified as non H2S producers, finding that they were highly n-propanol producers. The average production of these strain accounted for almost 45% of total higher alcohols, while for the other strains tested it was between 5-11%, showing a strong inverse relation between *n-propanol* formation and the ability to form H_2S. One of the yeast used in this study was UCD522 (Montrachet), and as a high sulfide producer showed low *n-propanol* formation. As pointed out earlier, threonine and valine are precursors of higher alcohols and can play a role in regulating the formation of its corresponding higher alcohol. Threonine is the direct precursor of n-propanol, and it could be a key to regulate or re-route the formation of H_2S to a higher alcohol instead.


A
B
C
D
E
F
G
K1
MON
OKAY
VIVACE
Percent Residual (%)
150
100
50
0
0
24
48
72
96
120
Fermentation (hours)

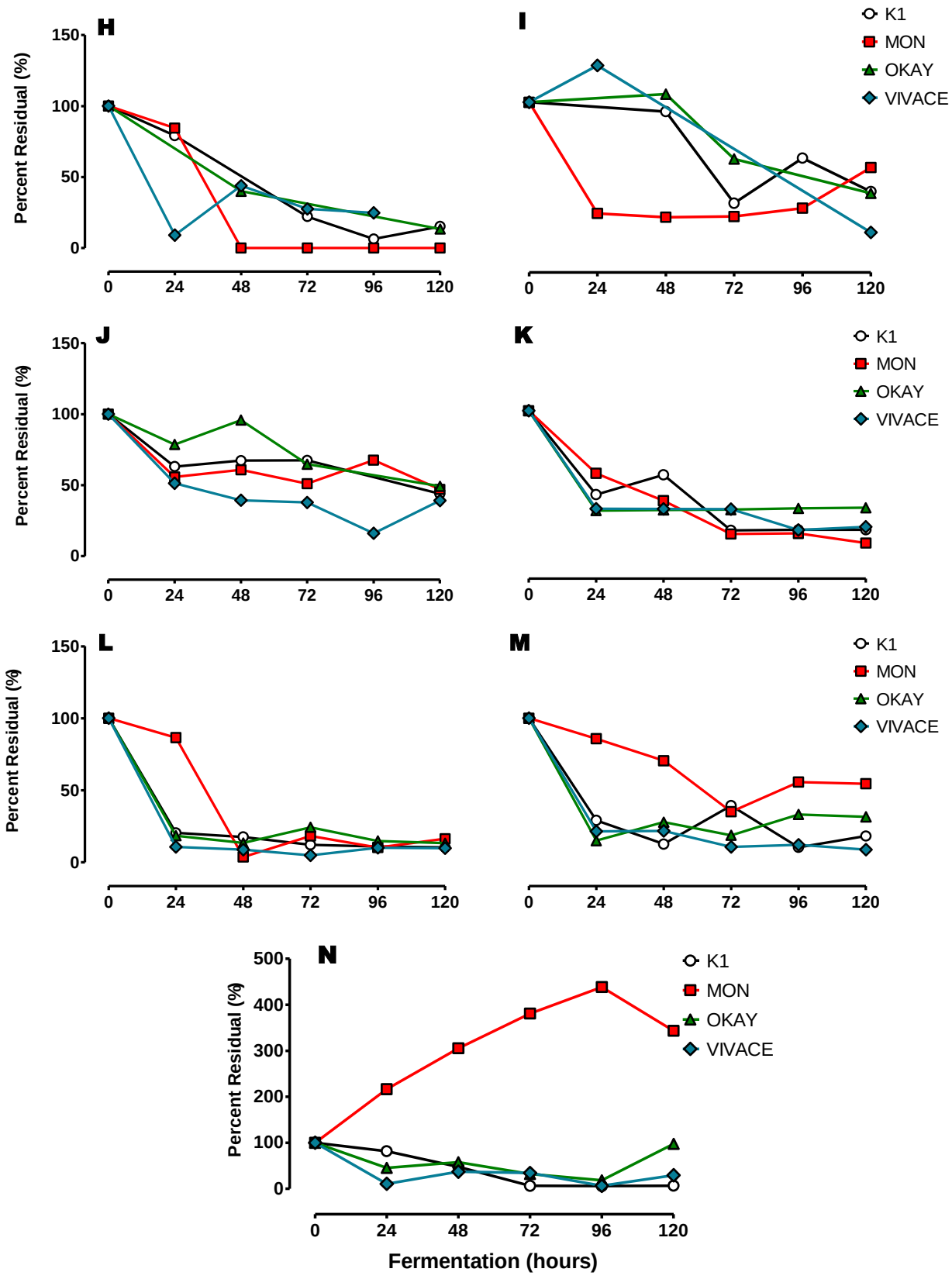
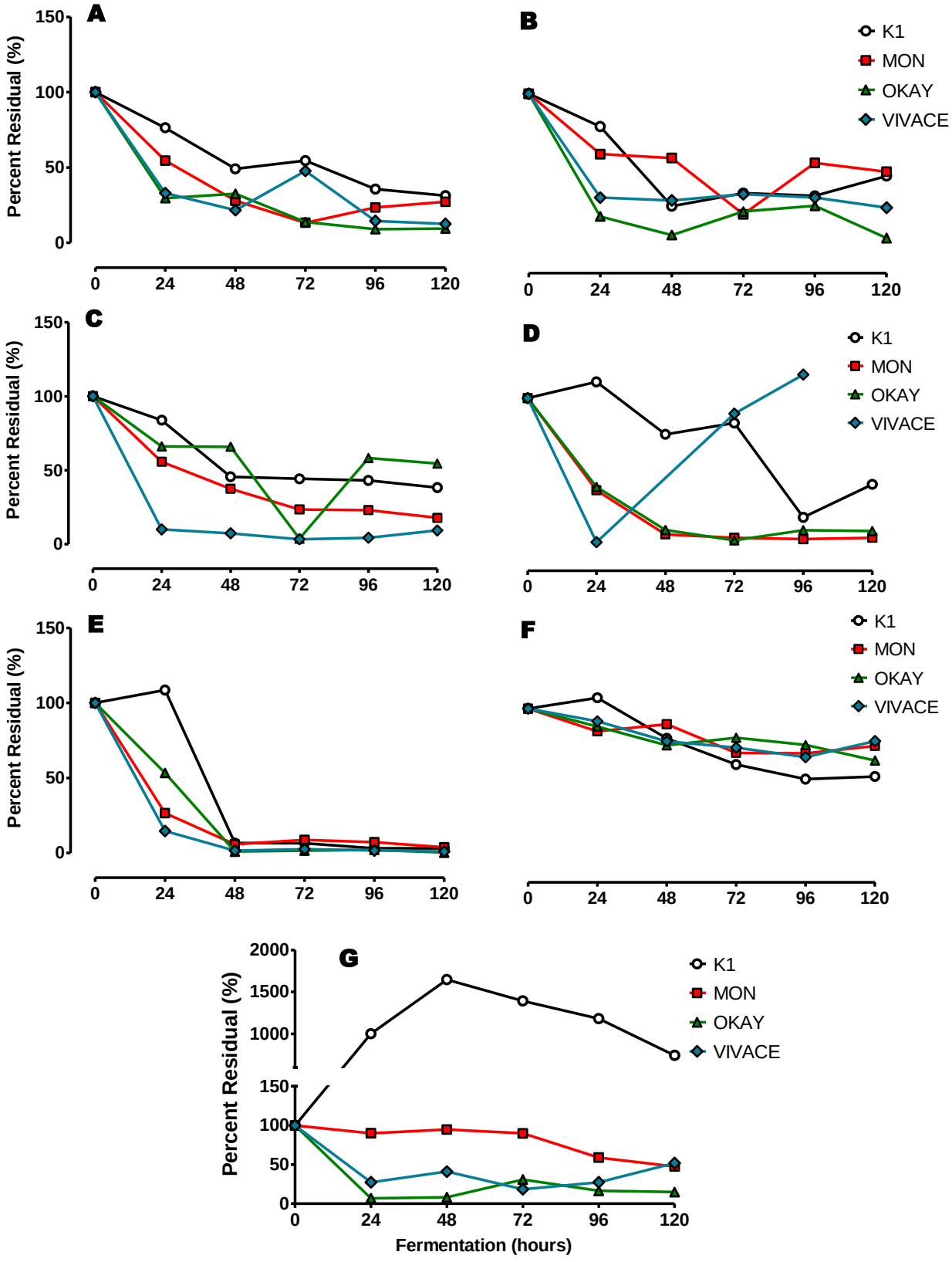


Figure 6. **Residual percentage of amino acids in fermentations with low initial assimilable nitrogen** for yeast K1, Vivace, Montrachet and Okay in *Cabernet Sauvignon* with 203g/L of sugar level and 120mg/L assimilable nitrogen. **A.** Aspartate; **B.** Glutamate; **C.** Histidine; **D.** Threonine; **E.** Arginine; **F.** Tyrosine **G.** Ammonium; **H.** Valine; **I.** Methionine; **J.** Cysteine; **K.** Isoleucine; **L.** Phenilalanine; **M.** Lysine; **N.** Glycine. Temperature was held constant at 25°C.


A
B
C
D
E
F
G
Percent Residual (%)
Fermentation (hours)
K1
MON
OKAY
VIVACE
0
24
48
72
96
120
50
100
150
1000
1500
2000

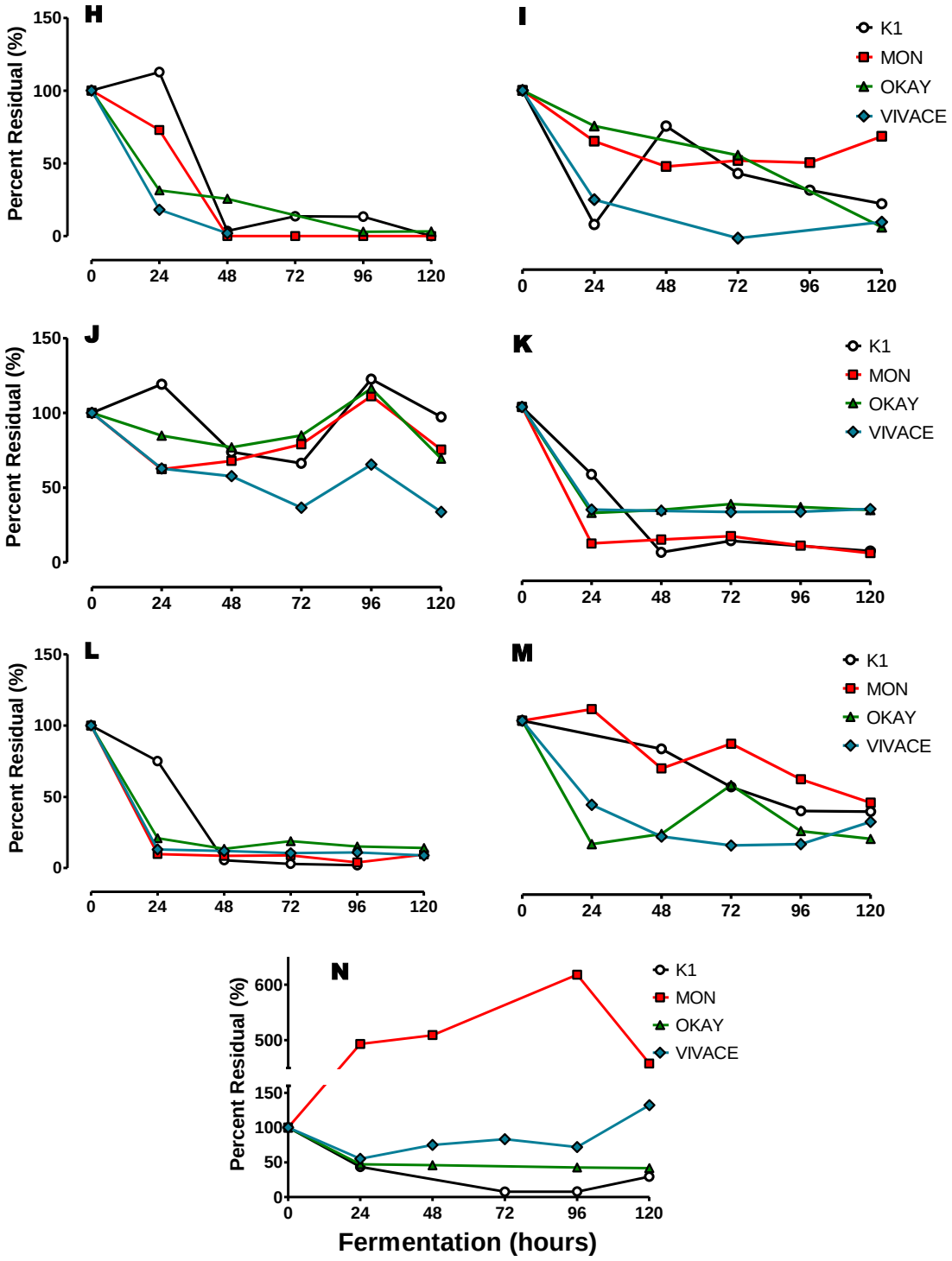


Figure 7. Residual percentage of aminoacid in fermentations with high initial sugar levels of *Cabernet Sauvignon* for yeast Montrachet, Vivace, Okay and K1. Initial conditions were 250mg/L of assimilablel nitrogen and 250g/L sugar content. Fermentation temperature was constant at 25°C. **A.** Aspartate; **B.** Glutamate; **C.** Threonine; **D.** Arginine; **E.** Ammonium; **F.** Tyrosine; **G.** Histidine; **H.** Valine; **I.** Methionine; **J.** Cysteine; **K.** Isoleucine; **L.** Phenilalanine; **M.** Lysine; **N.** Glycine.

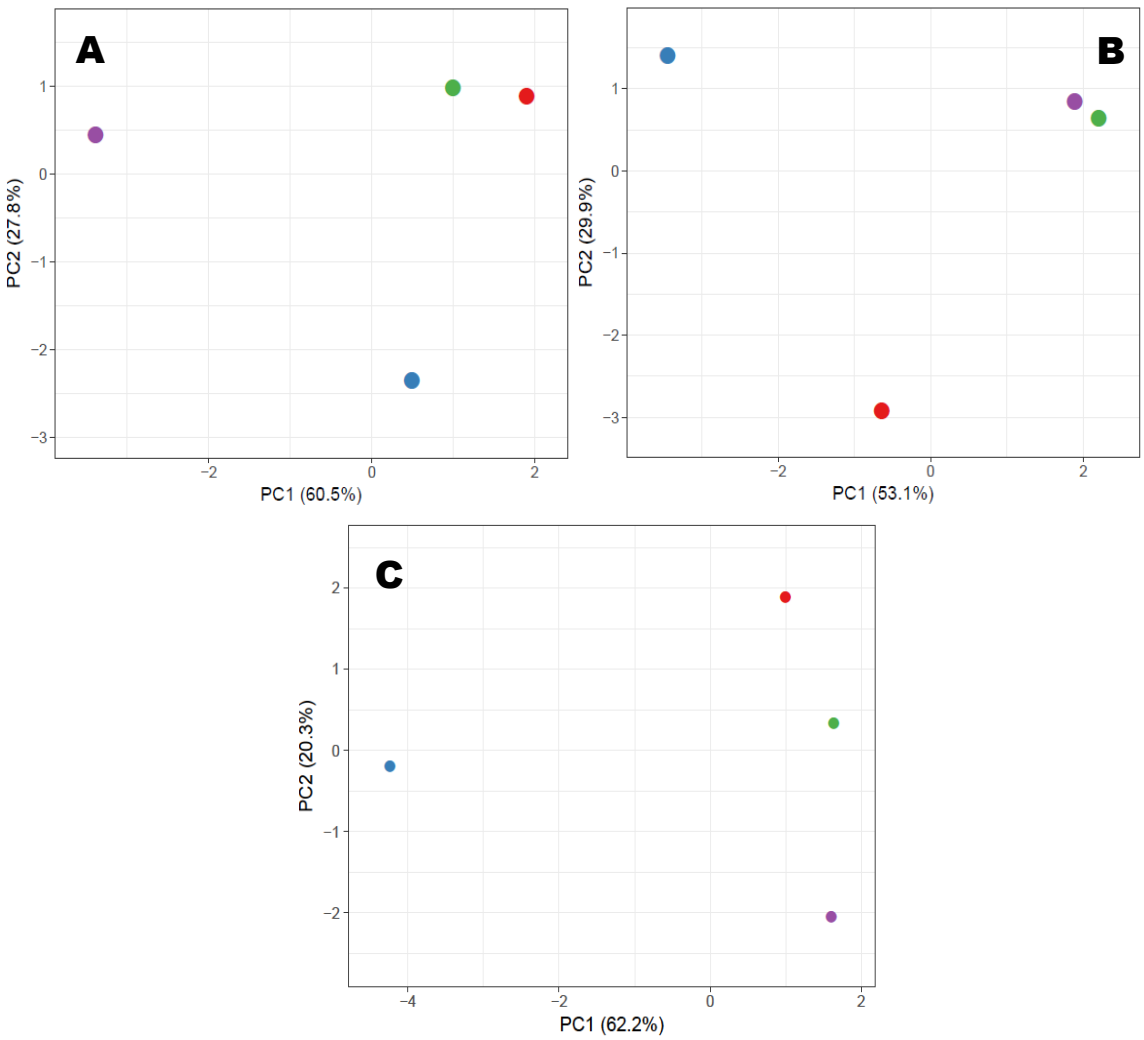


Figure 8. PCA analysis for three fermentation conditions and four yeast strains. A. Control fermentation (Ctl). **B.** High sugar content fermentation (S+). **C.** Low initial nitrogen fermentation (N-). Montrachet, Vivace, Okay, K1.

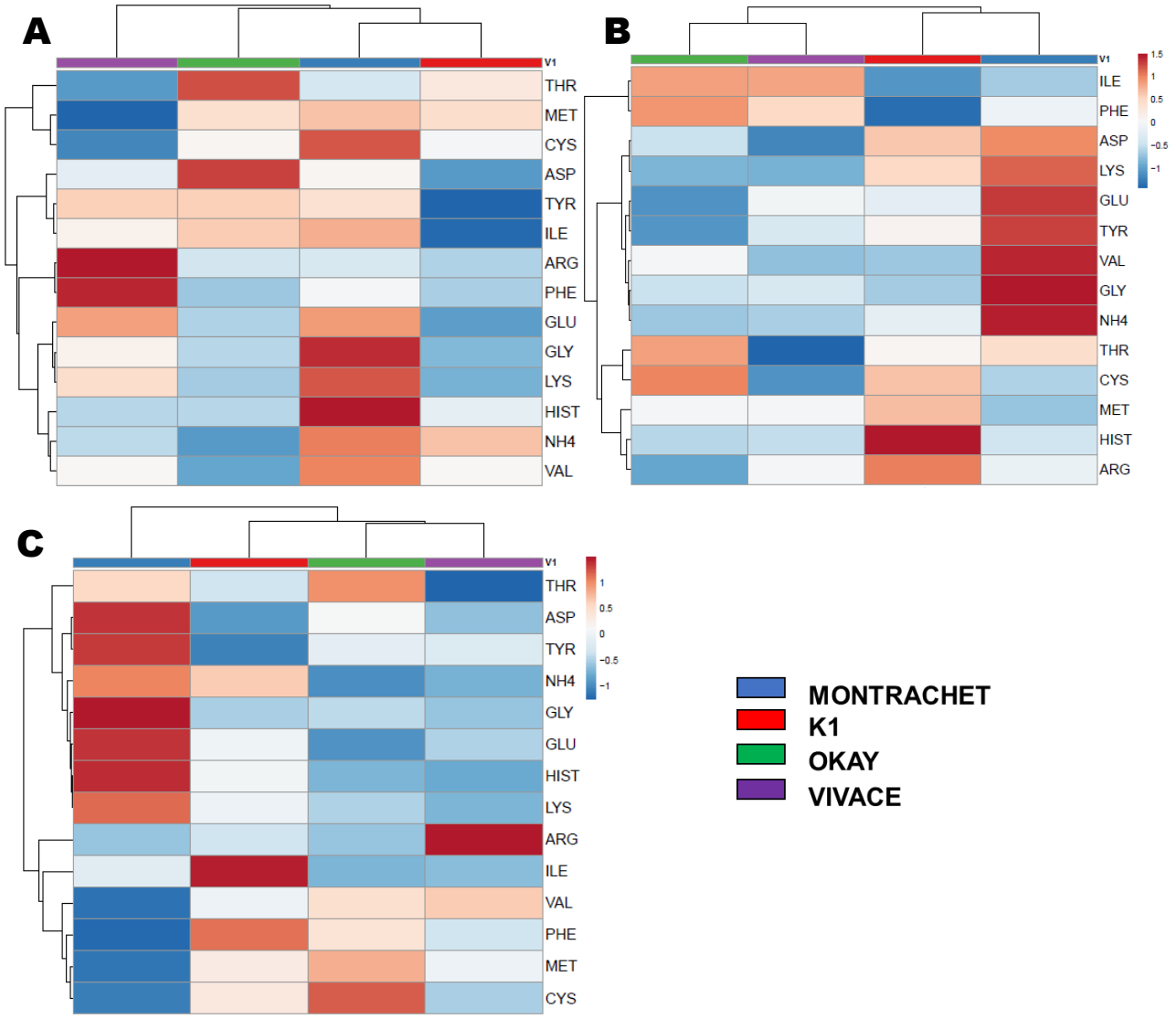


Figure 9. Heatmap percentage of residual amino acids during fermentations and clustering of yeast strains. A. Control fermentation (Ctl). **B.** High sugar content fermentation (S+). **C.** Low initial nitrogen fermentation (N-).

3. Conclusion

It is clear that under high sugar and low nitrogen f11 conditions, amino acid profiles do not fit the consumption profiles obtain form model grape solutions. It is then that these results greatly support the idea of using amino acid profiles as a tool to diagnose the status of a fermentation. Even though individual amino acids may have not special importance besides their role as nitrogen sources, amino acid profiles that change their behavior in response to specific problems can deliver the necessary information to correct the problem on time. Additionally, the same profiles could not only be helping to anticipate problematic fermentation but also the development of undesirable aromatic compounds that can impact the final wine composition.

4. Materials and methods

4.1 Yeast Strain and Wine Fermentations

Yeasts *Saccharomyces cerevisiae* RED STAR Montrachet (Lessafre Yeast Corporation, USA), Lalvin ICV K1 Marquée™ (Danstar Ferment AG, Denmark), Lalvin ICV OKAY® (Lallemand, Australia) and VIVACE (Renaissance Yeast Inc. Vancouver, Canada) were hydrated and inoculated according to manufacturer instructions. 0,3g/L of dry yeast was mix with sterile distilled water at 37-40°C and left for 20min at room temperature. The four commercial yeast strains were

inoculated in 200mL of grape must in 500mL 2-neck flat bottom flasks. Red *Cabernet Sauvignon* must from Santa Emma vineyard, harvest 2016, was pasteurized and kept in aliquots of 5L at -80°C. Alcoholic fermentations were carried out in 500mL 2-neck 29/32 flat bottom flasks (SCHOTT/ILMABOR). Nitrogen supplementation was ammonium diphosphate (Sigma-Aldrich); sugar addition was made with a 1:1 mix glucose (Merck) and fructose (Merck), as described in Table 2. Fermentations were carried out on a small laboratory scale, with constant agitation (100rpm) at 25°C, in semi-anaerobic conditions due to sample acquisitions.

4.1 Samples

1mL samples were taken during the course of alcoholic fermentation every 24hours until day 5. Samples were kept at -80°C for later HPLC analysis. All samples were diluted 1:2 in ultrapure (Milli-Q) water. A 0,5mL aliquot of diluted samples was used for derivatization and HPLC analysis.

4.2 Derivatization reaction

In a borosilicate test tube 0.875mL of borate buffer 1M, pH=9, 0.375mL methanol (MERCK,CHL), 0.010mL L-2-aminoadipic acid (1mg/L), 1mL of fermentation sample and 0.015mL of EMMDE (Sigma-Aldrich, USA) were mixed together. Derivatization was carried out for 30 minutes in a sonic bath. Subsequently,

samples were taken to a thermoregulated water bath at 70°C for 2 hours. Finally, samples were filtered through a 0.45µm PVDF membrane into HPLC vials (modified from Chicón R., Hermosín I.,Cabeduzo M D., 2001).

4.3 Chromatographic conditions

The analysis was carried out in an HPLC system LaChrom Elite® formed by a Hitachi L-2130pump, a Hitachi L-2200 Autosampler, a Hitachi L-2350 Column oven, a Hitachi L-2455 Diode detector, and the software EzChrom Elite Manager. The chromatography separation was performed in a reverse-phase column Nova Pak (Waters) C18, 4µm 3,9*300mm (Part N°WAT011695) thermostated at 18°C. The binary gradient (Table 1) was composed of a mobile phase A (acetate buffer 25mM pH 5,8- 0,02% sodium azide) and a mobile phase B (Acetonitrile: methanol 80:20). The detection was performed by an UV-detector, monitoring at 280nm during 41minutes (modified from Chicón R., Hermosín I.,Cabeduzo M D., 2001).

References

Alaiz, M., Navarro, J.L., Girón, J., Vioque, E., 1992. Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate. J. Chromatogr. A 591, 181–186. https://doi.org/10.1016/0021-9673(92)80236-N

Arias-Gil, M., Garde-Cerdán, T., Ancín-Azpilicueta, C., 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. Food Chem. 103, 1312–1318. https://doi.org/10.1016/j.foodchem.2006.10.037

Äyräpää T., 1968. FORMATION OF HIGHER ALCOHOLS BY VARIOUS YEASTS. J. Inst. Brew. 74, 169–178. https://doi.org/10.1002/j.2050-0416.1968.tb03111.x

Barbosa, C., Mendes-Faia, A., Mendes-Ferreira, A., 2012. The nitrogen source impacts major volatile compounds released by Saccharomyces cerevisiae during alcoholic fermentation. Int. J. Food Microbiol. 160, 87–93. https://doi.org/10.1016/j.ijfoodmicro.2012.10.003

Bartra, E., Casado, M., Carro, D., CampamÃ, C., PiÃ±a, B., 2010. Differential expression of thiamine biosynthetic genes in yeast strains with high and low production of hydrogen sulfide during wine fermentation. J. Appl. Microbiol. https://doi.org/10.1111/j.1365-2672.2009.04652.x

Bekker, M., Smith, M., Smith, P., Wilkes, E., 2016. Formation of Hydrogen Sulfide in Wine: Interactions between Copper and Sulfur Dioxide. Molecules 21, 1214. https://doi.org/10.3390/molecules21091214

Bell, S.-J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295. https://doi.org/10.1111/j.1755-0238.2005.tb00028.x

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., Guillamón, J.M., 2005. Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen Consumption. J. Agric. Food Chem. 53, 996–1002. https://doi.org/10.1021/jf0487001

Bely, M., Rinaldi, A., Dubourdieu, D., 2003. Influence of Assimilable Nitrogen on Volatile Acidity Production by Saccharomyces cerevisiae during High Sugar Fermentation. J BIOSCI BIOENG 96, 6.

Bisson, L.F., 1999. Stuck and Sluggish Fermentations. Am. J. Enol. Vitic. 50, 107–119.

Bisson LF., Linderholm A., Dietkel D., 2012. COMPOSITIONS AND METHODS FOR REDUCING HS LEVELS IN FERMENTED BEVERAGES. US008216803B2.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Res. 8, 1196–1207. https://doi.org/10.1111/j.1567-1364.2008.00412.x

Chen, E.C.-H., 1978. The Relative Contribution of Ehrlich and Biosynthetic Pathways to the Formation of Fusel Alcohols. J. Am. Soc. Brew. Chem. 36, 39–43. https://doi.org/10.1094/ASBCJ-36-0039

Chicón R., Hermosín I.,Cabeduzo M D., 2001. Método de Análisis de los aminoácidos libres y del ion amonio en vinos y mostos por HPLC tras derivatización con etoximetilenmalonato de dietilo (EMMDE). MayoJunio 2001 6.

Clement, T., Perez, M., Mouret, J.R., Sanchez, I., Sablayrolles, J.M., Camarasa, C., 2013. Metabolic Responses of Saccharomyces cerevisiae to Valine and Ammonium Pulses during Four-Stage Continuous Wine Fermentations. Appl. Environ. Microbiol. 79, 2749–2758. https://doi.org/10.1128/AEM.02853-12

Crépin, L., Nidelet, T., Sanchez, I., Dequin, S., Camarasa, C., 2012. Sequential use of nitrogen compounds by Saccharomyces cerevisiae during wine fermentation: a model based on kinetic and regulation characteristics of nitrogen permeases. Appl. Environ. Microbiol. 78, 8102–11. https://doi.org/10.1128/AEM.02294-12

Davis, Robert E., J., Greory V., 2000. 2000: Characterization of Hydrogen Sulfide Formation in Commercial and Natural Wine Isolates of Saccharomyces 51, 16.

F. Ferreira Monteiro and Linda F. Bisson, 1992. 1992: Nitrogen Supplementation of Grape Juice. I. Effect on Amino Acid Utilization During Fermentation. Am. J. Enol. Vitic. 43, 10.

Garde-Cerdán, T., Ancín-Azpilicueta, C., 2008. Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. LWT - Food Sci. Technol. 41, 501–510. https://doi.org/10.1016/j.lwt.2007.03.018

Giudici, P., 1994. The Effect of Nitrogen Deficiency and Sulfur-Containing Amino Acids on the Reduction of Sulfate to Hydrogen Sulfide by Wine Yeasts 45, 6.

Giudici P., Zambonelli C., Kunkee R.E., 1993. Increased Production of n-Propanol in Wine by Yeast Strains Having an Impaired Ability to Form Hydrogen Sulfide. Am. J. Enol. Vitic. 44, 5.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., García-Romero, E., 2007. Simultaneous HPLC Analysis of Biogenic Amines, Amino Acids, and Ammonium Ion as Aminoenone Derivatives in Wine and Beer Samples. J. Agric. Food Chem. 55, 608–613. https://doi.org/10.1021/jf062820m

Gutiérrez, A., Beltran, G., Warringer, J., Guillamón, J.M., 2013. Genetic Basis of Variations in Nitrogen Source Utilization in Four Wine Commercial Yeast Strains. PLoS ONE 8, e67166. https://doi.org/10.1371/journal.pone.0067166

Henschke P.A. and Jiranek V., 1993. Yeast-Metabolism of Nitrogen Compounds, in: Wine Microbiology and Biotechnology. Harwood Academic Publishers, pp. 77–164.

Hernández-Orte, P., Peña, A., Pardo, I., Cacho, J., Ferreira, V., 2012. Amino acids and volatile compounds in wines from *Cabernet Sauvignon* and *Tempranillo* varieties subjected to malolactic fermentation in barrels. Food Sci. Technol. Int. 18, 103–112. https://doi.org/10.1177/1082013211414762

Jiranek, V., Langridge, P., Henschke, P. a, 1995a. Amino Acid and Ammonium Utilization by Saccharomyces cerevisiae Wine Yeasts From a Chemically Defined Medium. Am. J. Enol. Vitic. 46, 75–83.

Jiranek, V., Langridge, P., Henschke, P.A., 1995b. Regulation of Hydrogen Sulfide Liberation in Wine-Producing Saccharomyces cerevisiae Strains by Assimilable Nitrogen. APPL Env. MICROBIOL 61, 7.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its Importance to Wine Aroma - A Review. Afr J Enol Vitic 21, 33.

Linderholm, A., Dietzel, K., Hirst, M., Bisson, L.F., 2010. Identification of MET10-932 and Characterization as an Allele Reducing Hydrogen Sulfide Formation in Wine Strains of Saccharomyces cerevisiae. Appl. Environ. Microbiol. 76, 7699–7707. https://doi.org/10.1128/AEM.01666-10

Linderholm, A.L., Findleton, C.L., Kumar, G., Hong, Y., Bisson, L.F., 2008. Identification of Genes Affecting Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Appl. Environ. Microbiol. 74, 1418–1427. https://doi.org/10.1128/AEM.01758-07

Magasanik, B., Kaiser, C.A., 2002. Nitrogen regulation in Saccharomyces cerevisiae. Gene 290, 1–18. https://doi.org/10.1016/S0378-1119(02)00558-9

Martínez-Gil, A.M., Garde-Cerdán, T., Lorenzo, C., Félix Lara, J., Pardo, F., Rosario Salinas, M., 2012. Volatile Compounds Formation in Alcoholic Fermentation from Grapes Collected at 2 Maturation Stages: Influence of Nitrogen Compounds and Grape Variety. J. Food Sci. 77, C71–C79. https://doi.org/10.1111/j.1750-3841.2011.02441.x

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., Beltran, G., 2012. Biomass production and alcoholic fermentation performance of Saccharomyces cerevisiae as a function of nitrogen source. FEMS Yeast Res. 12, 477–485. https://doi.org/10.1111/j.1567-1364.2012.00802.x

Mendes-Ferreira, a, Mendes-Faia, A., Leão, C., 2004. Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium

concentrations and its implications in winemaking industry. J. Appl. Microbiol. 97, 540–5.

Mendes-Ferreira, A., 2010. The Wine Yeast Strain-Dependent Expression of Genes Implicated in Sulfide Production in Response to Nitrogen Availability. J. Microbiol. Biotechnol. 20, 1314–1321. https://doi.org/10.4014/jmb.1003.03039

Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., Mendes-Faia, A., 2009. The production of hydrogen sulphide and other aroma compounds by wine strains of Saccharomyces cerevisiae in synthetic media with different nitrogen concentrations. J. Ind. Microbiol. Biotechnol. 36, 571–583. https://doi.org/10.1007/s10295-009-0527-x

Mendes-Ferreira, A., Barbosa, C., InÃªs, A., Mendes-Faia, A., 2010. The timing of diammonium phosphate supplementation of wine must affects subsequent H 2 S release during fermentation. J. Appl. Microbiol. 108, 540–549. https://doi.org/10.1111/j.1365-2672.2009.04457.x

Mendes-Ferreira, A., Mendes-Faia, A., Leao, C., 2004. Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. J. Appl. Microbiol. 97, 540–545. https://doi.org/10.1111/j.1365-2672.2004.02331.x

Pozo-Bayón, M.Á., Andujar-Ortiz, I., Alcaide-Hidalgo, J.M., Martín-Álvarez, P.J., Moreno-Arribas, M.V., 2009. Characterization of Commercial Inactive Dry Yeast Preparations for Enological Use Based on Their Ability To Release Soluble Compounds and Their Behavior toward Aroma Compounds in Model Wines. J. Agric. Food Chem. 57, 10784–10792. https://doi.org/10.1021/jf900904x

Pszczólkowski, P., Carriles, P., Cumsille, M., Maklouf, M., 2001. Reflexiones sobre la madurez de Cosecha y las condiciones de vinificación, con relación a la Problemática de Fermentaciones Alcohólicas Lentas y/o paralizante, en Chile. Fac. Agron. Pontif. Univ. Católica Chile Chile.

Rossignol, T., Dulau, L., Julien, A., Blondin, B., 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast Chichester Engl. 20, 1369–85.

Salmon, J.-M., Barre, P., 1998. Improvement of Nitrogen Assimilation and Fermentation Kinetics under Enological Conditions by Derepression of Alternative Nitrogen-Assimilatory Pathways in an Industrial Saccharomyces cerevisiae Strain. APPL Env. MICROBIOL 64, 7.

Singh', R., Kunkee, R.E., 1976. Alcohol Dehydrogenase Activities of Wine Yeasts in Relation to Higher Alcohol Formation. APPL Env. MICROBIOL 32, 5.

Taillandier, P., Ramonportugal, F., Fuster, A., Strehaiano, P., 2007. Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food Microbiol. 24, 95–100. https://doi.org/10.1016/j.fm.2006.04.002

Ugliano, M., Kolouchova, R., Henschke, P.A., 2011. Occurrence of hydrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen. J. Ind. Microbiol. Biotechnol. 38, 423–429. https://doi.org/10.1007/s10295-010-0786-6

Valero, E., Millán, C., Ortega, J.M., Mauricio, J.C., 2003. Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains: Amino acids in wine after fermentation. J. Sci. Food Agric. 83, 830–835. https://doi.org/10.1002/jsfa.1417

Varela, C., Pizarro, F., Agosin, E., 2004. Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. Appl. Environ. Microbiol. 70, 3392–3400. https://doi.org/10.1128/AEM.70.6.3392-3400.2004

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by Saccharomyces cerevisiae wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157. https://doi.org/10.1007/s00253-007-1145-z

Wang, X.D., Bohlscheid, J.C., Edwards, C.G., 2003. Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic

grape juice media deficient in assimilable nitrogen and/or pantothenic acid. J. Appl. Microbiol. 94, 349–359. https://doi.org/10.1046/j.1365-2672.2003.01827.x

Wang, Y.-Q., Ye, D.-Q., Zhu, B.-Q., Wu, G.-F., Duan, C.-Q., 2014. Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. Food Chem. 163, 6–15. https://doi.org/10.1016/j.foodchem.2014.04.064

Chapter 4

Plumbum microsphere columns as a new method to measure H2S release during fermentation

Maria Jose Paredes M[a,b] and Sergio Benavides V[c].

[a]Department of Fruticulture and Oenology, Faculty of Agriculture and Forestry, Pontificia Universidad Católica de Chile, P.O. Box 306, Santiago 6904411, Chile.
[b] Center of Biomedical Investigation, Faculty of Medicine, Universidad de Los Andes, Santiago 7620157, Chile.
[c]Faculty of Engineering and Business, Universidad Adventista de Chile, Camino a las Mariposas km 12, Chillán 3780000, Chile.

Abstract

Hydrogen sulfide (H_2S) is produced naturally by *Saccharomyces cerevisiae* during wine fermentations but it can impart undesirable reductive odors. The control in the production of H2S allows it to maintain its concentration under the sensorial perception umbral. However, its measurement is complicated to handle and generally associated with toxic compounds like lead. Although using lead-based methodologies allow the appropriate measurement of the sulfur produced, it is too risky for the operator, since lead is a heavy metal, which is toxic in most of its forms. An alternative is to immobilize the lead on a polymeric matrix, maintaining its functionality, but avoiding air suspension. A natural polymer, low cost, which allows the fixation of lead is alginate. Alginate is an anionic polymer that can generate cross-links with some poli-cations, like lead, immobilizing it within the alginate chains, but leaving free bonds to interact covalently with sulfide, S^{2-} from H_2S, and forme PbS. The objective of this research was to develop alginate microparticles loaded with lead, by the method of ionic gelation, as an alternative to measure H_2S formation in wine fermentations. Results show that microsphere-lead columns are highly effective to quantify H_2S. Moreover, an intense change of color,

yellow to dark black, was a clear indication that PbS was formed. In the conditions tested, the columns could discriminate without problems between four types of yeast and their capacity to produce H_2S. being especially accurate for high concentrations of sulfide.

1. Introduction

During the evolution of fermentation, problems may occur that result in off-flavors and are considered undesirable in wine. In this scenario, volatile-thiol compounds are important players in wine aroma, being hydrogen sulfide (H_2S) the key responsible for unpleasant aromas. H_2S is produced mostly by yeast metabolism during fermentation but the information provided by routine measurements doesn't allow to keep its production under observation and to assist the winemaker in early solutions. The ability to detect and quantify H_2S formation in fermenting wines is important in order to identify and correct its causes.

At present, there are instrumental methods for the qualitative and quantitative analyses of volatile sulphur compounds in wines, such as gas chromatography with flame photometric or sulfur chemiluminescence detection but these methods require expensive instrumentation and skilled personnel. A colorimetric method has also been used, however, this method requires a time-consuming set-up, and labor-intensive sampling procedures among other disadvantages (Park Seung-Kook 2008)

Diverse colorimetric methods are based in binding sulphur to any heavy metal, like lead (II) whose interaction forms a black, insoluble precipitate (lead-sulfide). The method consists of filling out glass columns with lead acetate using cellulose as a solid support. These columns are installed in any available exit of the fermentor, where the fermentation gases are released (CO_2 mostly), carrying H_2S (g) with it. Even though the method is effective as a qualitative way to determine H_2S formation, it implies a health risk to the operator, due to manipulation of a heavy metal, like lead, so it needs extra security measures. On the other hand, the

packing of columns is difficult resulting in irregularities, with empty/air zones and others overloaded and tightly compact what makes it difficult to accomplish a uniform distribution of the lead acetate throughout the column. All these problems make this economic method laborious to handle and not effective for quantifying the formation of H_2S during ongoing fermentations.

One solution to this problem is to adhere the lead (II) acetate to a polymeric matrix that allows to entrap and better manipulate the lead (II). Diverse biopolymers have the capacity to fix and retain heavy metals. Many of these polymers have been used as absorbents in purification of contaminated waters. These biopolymers are water-soluble macromolecules and have been typically used in numerous applications in the field of medicine, pharmaceutical industry, food industry and agriculture (Huamani-Palomino et al. 2018). These biopolymers can be highly efficient, reusable, biodegradable, and cost-effective (Hu et al. 2018) but especially attractive for their properties towards the uptake of heavy metals. Among these biopolymers, cellulose, chitosan and pectin have shown poor settling and low affinity towards metal ions. But alginate has emerged as a new alternative because of its wide availability as a major product of brown algae and their high affinity toward heavy metals via gelation (F. Wang, Lu, and Li 2016). Alginate is composed of two monomeric structures; 1-4β- D-mannuronic (M) acid and α-L-guluronic (G) (Wan et al. 2008). Additionally, it is a non-toxic and low-cost polymer (Baimark and Srisuwan 2014; Paques et al. 2014a). Alginate can form microstructures, by interaction between G groups with polyvalent cations, forming a polymeric network (Biom, Umr, and Roberval 2009).

The polymeric network with lead has been utilized with different technological purposes, most of which have their application in decontamination of water or other lead/heavy metals contaminated fluids. In addition, it is well known that lead interacts with sulfhydryl groups and it has been tested to be the divalent ion with higher affinity towards alginate (Huamani-Palomino et al. 2018; Mørch, Donati, and Strand 2006). Based on the above, the objective of this study was to develop an easy, fast, effective and real-time method to accurately measure in situ H_2S

production, using wine fermentation as a model. The method is based on ionic interactions to capture sulfide in lead adsorbed in alginate microspheres.

2. Materials and methods

2.1 Microspheres Preparation

The microspheres were prepared following the method described by Benavides et al., (2016). Briefly, an alginate solution (1% w/v) was extruded with a syringe and dripped into a plumbum acetate gelling solution (2.5% w/v) to form microspheres. The gelling solution was gently stirred with a magnetic stirrer at room temperature (20±1°C). The microspheres were kept in the gelling bath for 1 hour to foster suitable surface crosslinking. Subsequently, the obtained microspheres were washed with distilled water and dried in an oven (Memmert, model IN110, Schwabach, Germany) for 3 hours at 40° C.

2.2 Column Preparation and Calibration

The alginate-Pb microspheres were packed uniformly into transparent glass tubes. The size of the detecting tube was 2.5 mm i.d. with 230 mm length, used for monitoring the production of H_2S during fermentation in 200mL grape must on a 500mL 2-neck flat bottom flask. The calibration curve was constructed by measuring the length of the blackened band in the detecting tube and plotting versus the corresponding concentration of H_2S using a standard solution. 0.5 g of sodium sulfide nonahydrate (Aldrich, Milwaukee, WI, U.S.A.) in 500 mL of deionized water. A 25 mL aliquot was added to a glass beaker containing 125 mL of deionized water, 10 ml of 0.1 N iodine solution and 1 mL of dilute HCl (1+3). The excess iodine was titrated with 0.1 N sodium thiosulfate solution (Merck, Germany). An aliquot of stock solution was then added to a 2-neck flask to a volume of 200 mL and sealed with a rubber stopper with a Pb-column inserted. Nitrogen gas was used as the external gas source and the standard solution was

continuously stirred by a magnetic stirrer (modified from Park, 2008). The reproducibility of the tubes from the standard solution was calculated from five consecutive measurements. The limit of detection was also determined by measuring H_2S in the solutions with serial dilutions.

2.3 Scanning electron microscopy (SEM)

For Scanning electron microscopy (SEM) images, microspheres samples were freeze-dried, gold coated and observed using an accelerating voltage of 15 kV arranged in the electronic microscope (SEM-Quanta FEG 250). Images obtained were stored in digital *TIF format. The d*etermination of microspheres' elements composition was determined through elemental analysis (SEME-EDX) (Hosseini et al. 2013).

2.4 Yeast Strain and Wine Fermentations

The strains used to evaluate the Pb-microspheres were two commercial yeast RED STAR Montrachet (Lessafre Yeast Corporation, USA) and Lalvin ICV K1 Marquée™ (Danstar Ferment AG, Denmark). Red *Cabernet Sauvignon* juice from Santa Emma vineyard was pasteurized and fermented in a small laboratory scale. 200mL of juice (21°Brix) per 500mL of 2-neck 29/32 flat bottom flask (SCHOTT/ILMABOR) was inoculated with active dry yeast according to manufacturer instructions. The sulfide column was inserted into the hole of a rubber stopper, which was then placed into the 32-neck opening of the flask. The second opening was also closed with a rubber stopper so that all carbon dioxide produced during fermentation passed through the column.

3. Results and Discussion

In order to obtain a quantifiable measurement of hydrogen sulfide (H_2S) produced during the alcoholic fermentation, a colorimetric method was developed

based on the use of absorbents of naturally occurring biopolymers activated by metals cations. These natural polymers can be highly efficient, reusable, biodegradable, and cost-effective (Zhao-Hong *et.al.* 2018) but especially attractive for their properties towards the uptake of heavy metals. In general, biopolymers are water-soluble macromolecules and have been typically used in numerous applications in the field of medicine, pharmaceutical industry, food industry and agriculture (Huamani-Palomino *et.al.* 2018). Among these biopolymers, cellulose, chitosan and pectin have shown poor settling and low affinity towards metal ions. But alginate has emerged as a new alternative because of its wide availability as a major product of brown algae and its high affinity toward heavy metals polycations via gelation (Wang *et.al.* 2016). Lead is well known to interact with sulfhydryl groups, and it has been tested to be the divalent ion with higher affinity towards alginate (Huamani-Palomino *et.al.* 2018; Mørch *et.al.* 2006). In this matter, Huamani-Palomino at.al.2018 showed higher capacity of removal of Pb^{2+} in aqueous solution thanks to the synthesis of a modified alginate-cysteine biopolymer. Furthermore, addition of hydrogen sulfide or sulfide salts to a solution containing a lead salt, such as PbX_2, gives a black precipitate of lead sulfide (PbS), so insoluble and stable that it is probably one of the less toxic forms of lead. With this background, alginate-Pb columns were confectioned.

3.1 Lead-alginate Microspheres

To obtain the microspheres, an alginate solution was dripped to a lead (II)-acetate gelation solution (Fig. 1a). The resulting plumbum-alginate microsphere is a matrix consisting of a polymer of high molecular weight in cross-linking with a divalent cation (Fig. 1b). The microspheres were oven dried and let to sit at room temperature previous to be used. During drying, the lead-rich layer on the surface increases its density by water elimination, allowing the development of a cover (Benavides et al. 2016) that can be seen later in the SEM photographs. Prior columns for measure H_2S formation, qualitatively, were based on acetate-lead solutions embed in a cellulose matrix, forming a kind of sludge solution that needed

to be dried for using it. During this process, lead that is not adsorbed can remain in suspension in the air, and lead in most of its forms is toxic. The difference with gelification is that during microsphere formation, when lead was no longer available, the alginate droplet did not form a microsphere anymore. In this way, it was assured that all dissolved lead was absorbed and none of it was volatilized during the drying process.

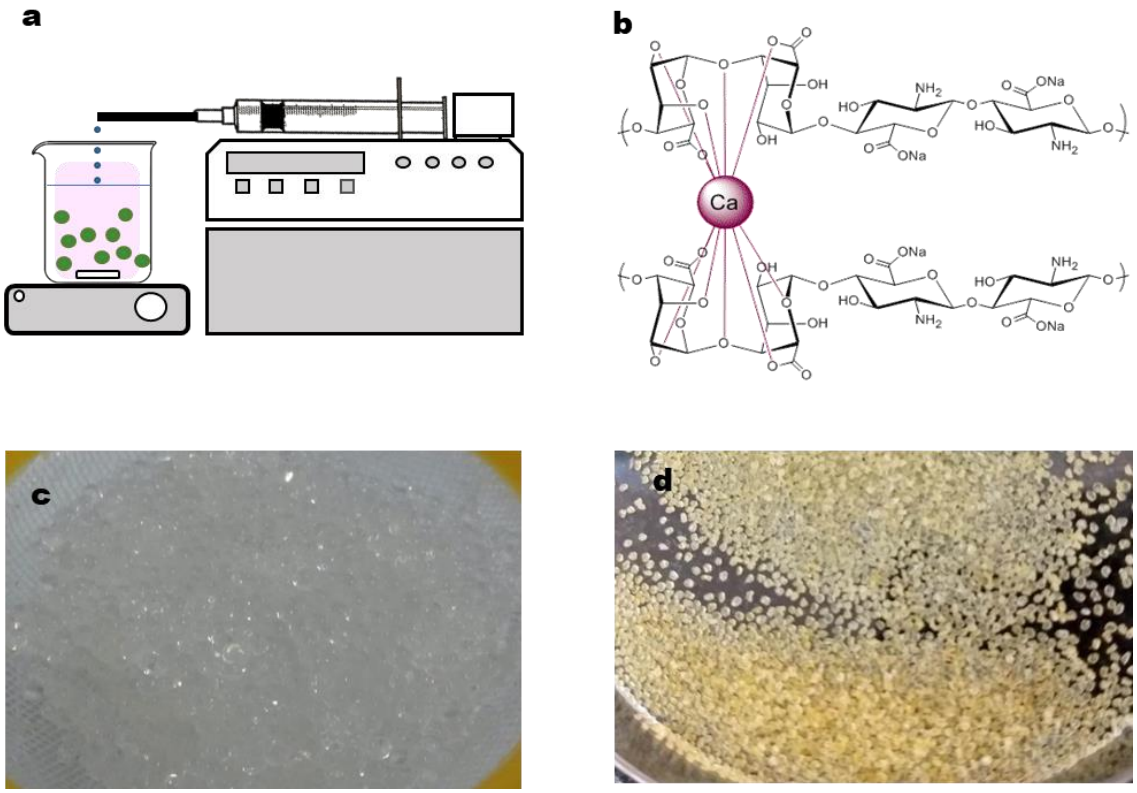


FIGURE 1. Lead-alginate microspheres obtention. **a**. Experimental set-up for microsphere elaboration; **b**. Squematic cross-linking that would form between Pb(II) and alginate (Ca for cation); **e.** Wet microsphere **d**. Dry lead microspheres.

3.2 Lead Columns

A comparison between lead columns was made to test the performance of the Pb-alginate microspheres over the cellulose matrix columns. Lab-scale fermentations

were carry-out in the same initial conditions. After 120 hours (5 days) of inoculation, a clear colorimetric difference could be observed (Figure 2). In Pb-cellulose column was not easy to observe and estimate the amount of H_2S produced. In the cellulose columns the color change was subtle, a light brown instead of the dark black expected by the formation of PbS, and the color was not evenly distributed throughout the column. This poor coloration could be due to the tight packaging of the cellulose in the column, not allowing a proper reaction between Pb^{2+} and H_2S passing with the CO_2 released. On the other hand, the microspheres column was able to detect H_2S in a notorious way. The color change from yellow to deep black, as expected for PbS, was evident and the blackening was uniform along the column. This dramatic color change gives the microsphere column a great advantage to detect even small quantities of sulfide being produced during fermentation.

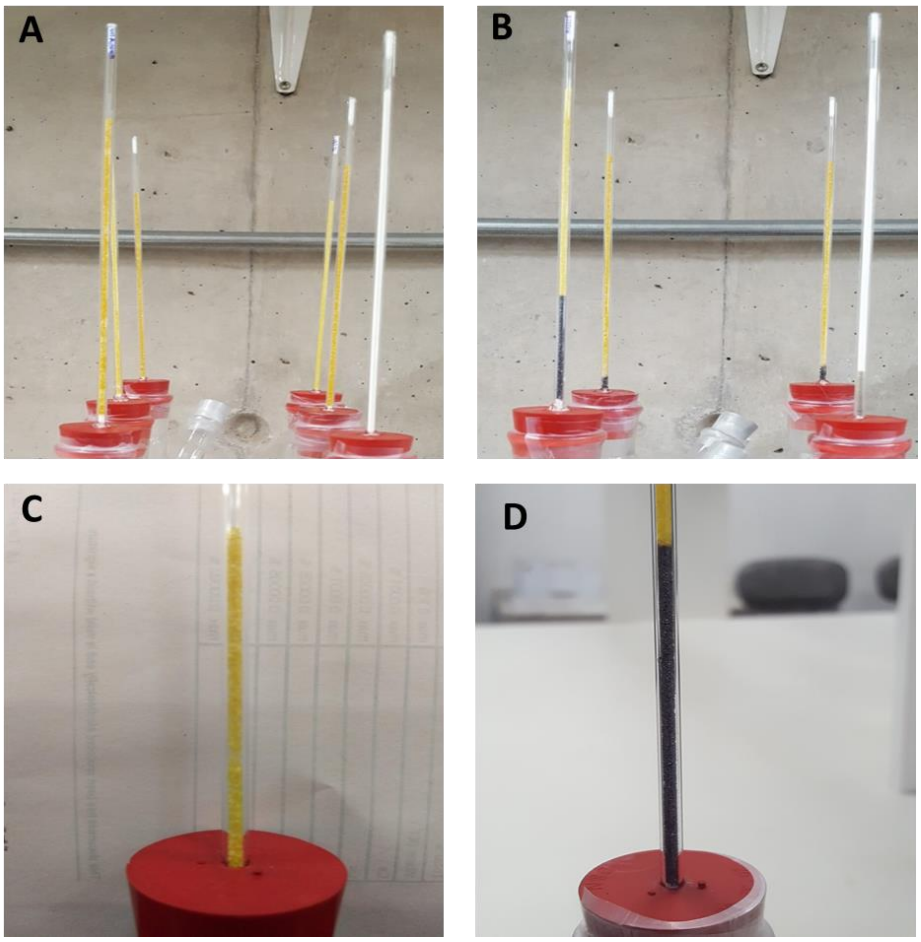


Figure 2. **Columns for measuring H_2S during alcoholic fermentation.** Yellow columns are packed with lead-alginate microsphere and white column with lead-cellulose. **A.** Beginning of fermentation, inoculation of Montrachet yeast. **B.** Five days after inoculation, the columns are colored with the formation of PbS. **C.** Column packed with lead-alginate microsphere. **D.** Column with the lead-alginate microsphere blackened by the formation of lead-sulfide (PbS).

3.3 Calibration and repeatability of microsphere column

Lead microsphere columns were calibrated in relation to the length, in centimeters, of the color band. To develop the calibration curve, a solution of Na_2S was standardized by iodine/thiosulfate titration. Known aliquots of the solution (as described in methodology) were diluted in Milli-Q water and using nitrogen as a gas source with constant agitation, the columns were verified. Fresh solution of Na_2S was prepared and standardized each time due to the volatile nature of the S^{2-} in aqueous solution, thus a true concentration of the stock solution was assured. The results showed a good linear correlation (r^2=0,995) between color band length and concentration of different standardized solutions tested. The detection limit of the columns is higher (≤0,5cm, which is equivalent to 16mg/L H_2S) compared to others been developed (Park Seung-Kook 2008), possibly due to the size and higher load capacity of the Pb-microsphere. The length and diameter of the glass support for the microspheres and columns construction were based on previous works, so if the diameter of the glass column is reduced to a size in which the microsphere still could fit, a smaller concentration of H_2S could be detected and the sensitivity of the columns could be higher.

3.4 Quantification of H_2S in alcoholic fermentations

The production of H_2S during alcoholic fermentation of 4 wine yeasts was quantified in three different initial must conditions: control, low available nitrogen,

and high sugar content. Grape must was from natural juice obtained from Santa Emma vineyard. Initial grape conditions were supplementation with ammonium phosphate to reach 250-300mg/L of assimilable nitrogen and addition of glucose: fructose mixture to reach 250g/L (25°B) of initial sugar content. Grape must, without supplementation, contained less than 150mg/L of nitrogen and 203g/L (21°B) sugar content. One of the yeast, Vivace, didn't produce any H_2S, no matter the initial must conditions. Montrachet and K1 produced relative higher amounts of H_2S, (Figure 3). Okay, as it is described by the manufacturer, is a low H_2S producer, almost to undetectable levels. One result that wasn´t excepted was the higher production of H_2S by K1 over Montrachet. UCD522 (Montrachet) strain is known to be a high H_2S producer and has been used in several studies to investigate the factors that influence the sulfide production (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Butzke 2011; Jiranek, Langridge, and Henschke 1995b; A. L. Linderholm et al. 2008; A. Mendes-Ferreira et al. 2010; Ana Mendes-Ferreira et al. 2009b). When measuring the length of black-band for Okay, even though it was possible to measure its length, the calibration curve was out of limits and for a great number of measurings, H_2S could not be quantified. A lower range of concentrations should have been used to calibrate the small band changes (< 1cm) observed with this yeast. In this regard, the higher Pb load capacity of the microspheres gives to the column a greater efficiency, meaning it can be able to detect even the smallest quantities of sulfide in the medium (gaseous). But for this model, in particular, raises the need to be not just efficient but also sensitive, especially on a laboratory scale. Having a large load capacity of Pb of the microspheres allows them to trap every single sulfide molecule that can go through the column along with the fermentation gases, making the column highly efficient. But, the efficiency of the microspheres compromised the sensitivity of the columns, as large amounts of sulfide are retained as PbS, the color length does not advanced until all the microsphere are saturated. Therefore, giving a visually narrower band, that can be difficult to measure if it is smaller than 1cm. One way to improve this downside of the columns would be to reduce the inside diameter of the glass tube. On average the microspheres have a size of 650µm, then a

diameter between 1-1.5mm should enhance the sensitivity of the columns by allowing a wider black color band for measure. Along with this high efficiency, the columns also showed remarkable specificity. Tests were run for columns embedded in a solution of sodium sulfide and there was no color change. These support that the Pb in the microsphere only reacts with sulfide in its gaseous form (H_2S). In solution, sulfide acts as a strong base, HS-, and in this state, there is no reaction with the Pb.

Fermentations were carried out in another common oenological condition, high initial sugar content, over 25°B to start fermentation, to keep studying the behavior of the yeast strains and the column capacity to quantify H_2S (Figure 3). In this condition, Montrachet produced higher amounts of H_2S. Compared with moderate sugar levels (21°B), its production is greatly enhanced, around 50% higher, a behavior expected for the elevated fermentation demand as elevated sugar levels. On the other hand, K1 showed a behavior that was not expected, it sulfide production remained among the same range of concentration as in control conditions, despite the fermentations demands**.** Okay, as a low producer according to manufacturer descriptions, kept similar production, as the one observed in control fermentations. Most studies had not considered sulfide production related to must sugar concentration, they have focused primarily on the influence of nitrogen requirements. A work by Wang et al., 2003 carried out experimental fermentations with 240g/L of glucose: fructose and used yeast strain Montrachet. They showed that H_2S production increased with increased YAN, but they only used 60mg/L of nitrogen and 250mg/L, both with high sugar levels. Therefore, it has not been possible to further corroborate these findings.

The last condition studied was low assimilable initial nitrogen. Under this condition, Montrachet was the strain with the highest production, but still, the amounts produced were at least one-third less than the production observed in control conditions (Figure 3). K1 almost didn't produce any H_2S and Okay maintained its low production behavior. Low initial nitrogen has been described as a problematic condition to begin fermentation, and also as one of the factors to enhance H_2S production (Henschke P.A. and Jiranek V. 1993). Previous works by Mendes-

Ferreira et al., 2010, 2009 also worked with Montrachet as high producing sulfide strain when fermenting in low nitrogen conditions, H_2S production was about 30% lower. Here, it can be seen the same tendency for the same yeast strain, Montrachet (Fig. 3), as well as for K1, with H_2S productions as much as 70% lower. In regard to the concentrations of H_2S registered in this work, it is known that hydrogen sulfide has a rotten egg odor and a low detection threshold, between 1,6 and 80 µg/L (Ugliano, Kolouchova, and Henschke 2011b; C. Wang et al. 2018). Clearly the concentration reported here and in most of the studies exceeds this range of concentrations, and according to Rauhult (König, Unden, and Fröhlich 2009) this is detrimental to wine aroma. One major difference with previous studies is the scale of the concentrations registered for the production of H_2S. Most of them showed concentrations in µg/L or total µg produced. Here, concentrations are expressed in mg/L. Just recently, a work by (Li et al. 2019) reported concentration H_2S formation in ranges of mg/L (ppm), reaching even one thousand ppm. A possible and clear explanation for the difference in concentrations could be the low sensibility and efficiency of previous methods to detect and quantified H_2S. Colorimetric methods based on spectrophotometric measurements (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Y. Chen et al. 2012; X. D. Wang, Bohlscheid, and Edwards 2003) were very useful when no other ways were available to study sulfide production, and with this techniques, concentrations of H_2S were established as reference. Now, even though there are better techniques it availability is restricted, either because better methodologies are too expensive or difficult to set and handle. As pointed out earlier, the microspheres are very specific and efficient in trapping sulfide, therefore the concentration reported here could be more accurate and real concentration, closer to the ones during real-time ongoing fermentation. In finished wines, H_2S concentration is in a much lower range, but still could be measured with the microsphere columns, using an inert gas as a vehicle to carry over H2S through the columns. Microspheres are easy to obtain in everyday laboratory work, and the columns easy to put together. They also can be calibrated without the need of having $H_2S(g)$, just a standard solution of Na_2S.

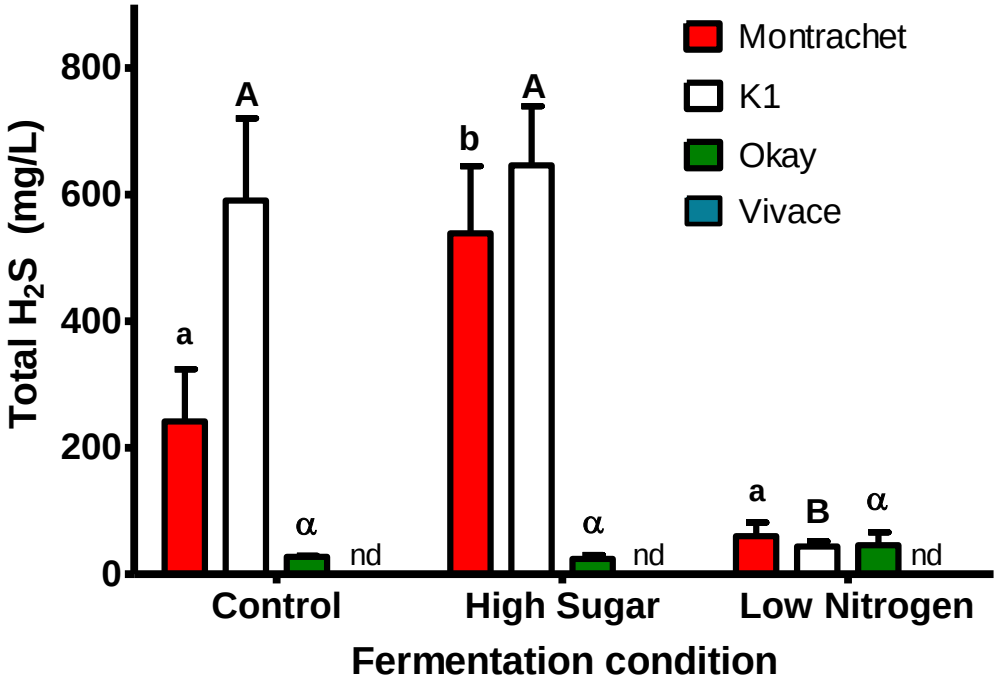


FIGURE 4. Total H_2S production in three distinct oenological fermentations conditions. *S. cerevisiae* UCD522 (Montrachet), K1 Marquée, ICV Okay and Vivace fermentation in **CTL**: 200g/L sugar concentration and 250mg N /L; **S+**: 250g/L sugar concentration and 250mg N /L; **N-**: 200g/L sugar concentration and 120-150mg N /L. Bars show H_2S quantification ±SD. **b** with $p < 0,05$; **B** with $p < 0,01$; **α** no significant differences.

3.5 SEM images and composition analysis of microspheres.

The SEM images showed that the microspheres had an average size of 655 ± 124 µm (Fig. 4). On the other hand, the microspheres presented an irregular shape due to the drying process. This process is carried out with hot air, on wet microspheres that maintain a spherical shape. The hot air generates a heterogenous drying effect on the surface of the microsphere. This particularity results in an irregular drying, with the subsequent loss of sphericity. However, this irregularity in the shape can be beneficial, because it can improve the flux of gases

inside the tube filled with microspheres and increases the contact surface of the sulfur gases with the surface activated by lead in the microspheres.

Another aspect that becomes evident in the microphotographs, is the striated surface of the microspheres. This would be due to the crosslinking of the alginate chains with the lead polycations. The chains are arranged in parallel, conjugated in their extension by the lead, generating the "Egg Box" effect, typical of the alginate with polycations in solution. This effect allows the generation of an "activated zone" that allows the fixation of sulfur ions to the free bonds of the lead polycations(Karthik and Meenakshi 2015; Paques et al. 2014b).

The SEM microscopy system allowed also the elementary evaluation of constitutive components of the microsphere, known as SEM-EDX (Scanning Electron Microscopy - Energy Dispersive X-ray spectroscopy)(Ozay and Ozay 2014). The lead: sulfur ratio in the microsphere at the end of the fermentation process is approximately 6: 1 and was determined in terms of the molecular weight of the microsphere and the analysis of its components, matching perfectly with the ratio obtained by the chemical weight and formula Lead Sulfide (PbS; MW 239,3 g/mol).

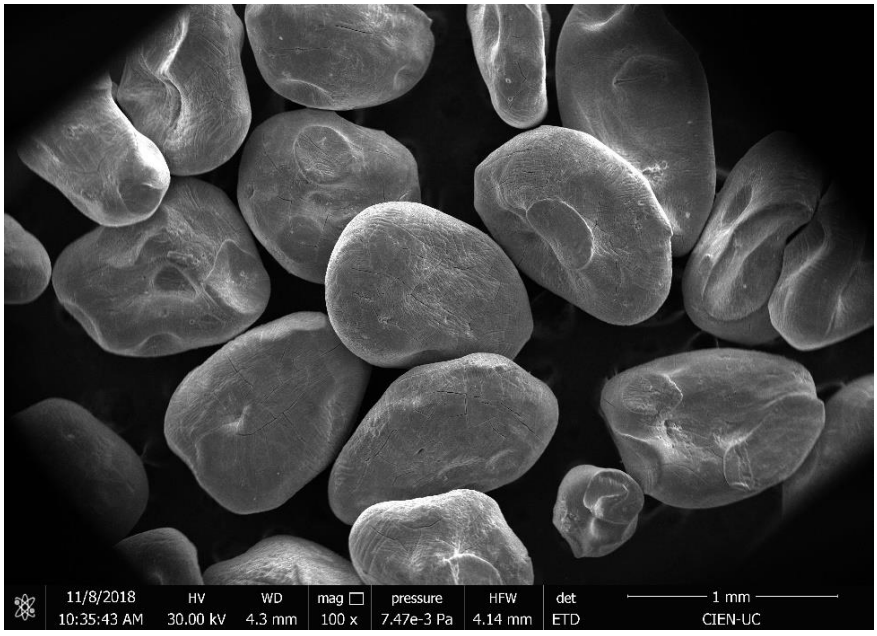


Figure 4. SEM microscopy of lead alginate microspheres, loaded with sulfur, after the alcoholic fermentation process.

References

Baimark, Y., Srisuwan, Y., 2014. Preparation of alginate microspheres by water-in-oil emulsion method for drug delivery: Effect of Ca2+ post-cross-linking. Adv. Powder Technol. 25, 1541–1546. https://doi.org/10.1016/j.apt.2014.05.001

Barbosa, C., Mendes-Faia, A., Mendes-Ferreira, A., 2012. The nitrogen source impacts major volatile compounds released by Saccharomyces cerevisiae during alcoholic fermentation. Int. J. Food Microbiol. 160, 87–93. https://doi.org/10.1016/j.ijfoodmicro.2012.10.003

Benavides, S., Cortés, P., Parada, J., Franco, W., 2016. Development of alginate microspheres containing thyme essential oil using ionic gelation. Food Chem. 204, 77–83. https://doi.org/10.1016/j.foodchem.2016.02.104

Biom, L., Umr, C., Roberval, L., 2009. Measurement of mechanical properties of alginate beads using ultrasound Abstract : Mots clefs : 19 Eme Congr. Francais Mec. 1–6.

Butzke, C.E., 2011. Impact of Fermentation Rate Changes on Potential Hydrogen Sulfide Concentrations in Wine. J. Microbiol. Biotechnol. 21, 519–524. https://doi.org/10.4014/jmb.1010.10058

Chen, Y., Yang, X., Zhang, S., Wang, X., Guo, C., Guo, X., Xiao, D., 2012. Development of Saccharomyces cerevisiae Producing Higher Levels of Sulfur Dioxide and Glutathione to Improve Beer Flavor Stability. Appl. Biochem. Biotechnol. 166, 402–413. https://doi.org/10.1007/s12010-011-9436-3

Henschke P.A. and Jiranek V., 1993. Yeast-Metabolism of Nitrogen Compounds, in: Wine Microbiology and Biotechnology. Harwood Academic Publishers, pp. 77–164.

Hosseini, S.F., Zandi, M., Rezaei, M., Farahmandghavi, F., 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study. Carbohydr. Polym. 95, 50–56. https://doi.org/10.1016/j.carbpol.2013.02.031

Hu, Z.-H., Omer, A.M., Ouyang, X., Yu, D., 2018. Fabrication of carboxylated cellulose nanocrystal/sodium alginate hydrogel beads for adsorption of Pb(II) from aqueous solution. Int. J. Biol. Macromol. 108, 149–157. https://doi.org/10.1016/j.ijbiomac.2017.11.171

Huamani-Palomino, R.G., Jacinto, C.R., Alarcón, H., Mejía, I.M., López, R.C., Silva, D. de O., Cavalheiro, E.T.G., Venâncio, T., Dávalos, J.Z., Valderrama, A.C., 2018. Chemical modification of alginate with cysteine and its application for the removal of Pb(II) from aqueous solutions. Int. J. Biol. Macromol. https://doi.org/10.1016/j.ijbiomac.2018.09.096

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of Hydrogen Sulfide Liberation in Wine-Producing Saccharomyces cerevisiae Strains by Assimilable Nitrogen. APPL Env. MICROBIOL 61, 7.

Karthik, R., Meenakshi, S., 2015. Removal of Pb(II) and Cd(II) ions from aqueous solution using polyaniline grafted chitosan. Chem. Eng. J. 263, 168–177. https://doi.org/10.1016/j.cej.2014.11.015

König, H., Unden, G., Fröhlich, J. (Eds.), 2009. Biology of microorganisms on grapes, in must and in wine. Springer, Berlin.
Li, Y., Zhang, Y., Liu, M., Qin, Y., Liu, Y., 2019. Saccharomyces cerevisiae isolates with extreme hydrogen sulfide production showed different oxidative stress resistances responses during wine fermentation by RNA sequencing analysis. Food Microbiol. 79, 147–155. https://doi.org/10.1016/j.fm.2018.10.021
Linderholm, A.L., Findleton, C.L., Kumar, G., Hong, Y., Bisson, L.F., 2008. Identification of Genes Affecting Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Appl. Environ. Microbiol. 74, 1418–1427. https://doi.org/10.1128/AEM.01758-07
Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., Mendes-Faia, A., 2009. The production of hydrogen sulphide and other aroma compounds by wine strains of Saccharomyces cerevisiae in synthetic media with different nitrogen concentrations. J. Ind. Microbiol. Biotechnol. 36, 571–583. https://doi.org/10.1007/s10295-009-0527-x
Mendes-Ferreira, A., Barbosa, C., InÃªs, A., Mendes-Faia, A., 2010. The timing of diammonium phosphate supplementation of wine must affects subsequent H_2S release during fermentation. J. Appl. Microbiol. 108, 540–549. https://doi.org/10.1111/j.1365-2672.2009.04457.x
Mørch, Ý.A., Donati, I., Strand, B.L., 2006. Effect of Ca^{2+}, Ba^{2+}, and Sr^{2+} on Alginate Microbeads. Biomacromolecules 7, 1471–1480. https://doi.org/10.1021/bm060010d
Ozay, H., Ozay, O., 2014. Synthesis and characterization of drug microspheres containing phosphazene for biomedical applications. Colloids Surf. Physicochem. Eng. Asp. 450, 99–105. https://doi.org/10.1016/j.colsurfa.2014.03.022
Paques, J.P., Sagis, L.M.C., van Rijn, C.J.M., van der Linden, E., 2014a. Nanospheres of alginate prepared through w/o emulsification and internal gelation with nanoparticles of CaCO3. Food Hydrocoll. 40, 182–188. https://doi.org/10.1016/j.foodhyd.2014.02.024
Paques, J.P., Sagis, L.M.C., van Rijn, C.J.M., van der Linden, E., 2014b. Nanospheres of alginate prepared through w/o emulsification and internal gelation with nanoparticles of CaCO3. Food Hydrocoll. 40, 182–188. https://doi.org/10.1016/j.foodhyd.2014.02.024
Park Seung-Kook, P.S.-K., 2008. Development of a method to measure hydrogen sulfide in wine fermentation. 2008 18, 5.
Ugliano, M., Kolouchova, R., Henschke, P.A., 2011. Occurrence of hydrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen. J. Ind. Microbiol. Biotechnol. 38, 423–429. https://doi.org/10.1007/s10295-010-0786-6
Wan, L.Q., Jiang, J., Arnold, D.E., Guo, X.E., Lu, H.H., Mow, V.C., 2008. Calcium Concentration Effects on the Mechanical and Biochemical Properties of Chondrocyte-Alginate Constructs. Cell. Mol. Bioeng. 1, 93–102. https://doi.org/10.1007/s12195-008-0014-x
Wang, C., Liu, M., Li, Y., Zhang, Y., Yao, M., Qin, Y., Liu, Y., 2018. Hydrogen sulfide synthesis in native Saccharomyces cerevisiae strains during

alcoholic fermentations. Food Microbiol. 70, 206–213. https://doi.org/10.1016/j.fm.2017.10.006

Wang, F., Lu, X., Li, X., 2016. Selective removals of heavy metals (Pb 2+ , Cu 2+ , and Cd 2+) from wastewater by gelation with alginate for effective metal recovery. J. Hazard. Mater. 308, 75–83. https://doi.org/10.1016/j.jhazmat.2016.01.021

Wang, X.D., Bohlscheid, J.C., Edwards, C.G., 2003. Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. J. Appl. Microbiol. 94, 349–359. https://doi.org/10.1046/j.1365-2672.2003.01827.x

Chapter 5

Discussion

Fermentation is the stage during winemaking that presents most problems and obstacles to overcome. Yeast are subject to multiple stress factors, including high osmotic pressure, acidity, nutrient deprivation, and high alcohol concentration (Rossignol et al. 2003b). Among the critical macronutrients during fermentation, nitrogen is one most studied. It is responsible for yeast growth and metabolism (C. Varela, Pizarro, and Agosin 2004), but most imperative has been linked with the aromatic and flavor profile of finished wines (Bell and Henschke 2005b; Carrau et al. 2008). The fraction of nitrogen that is usable by yeasts is composed on average by ammonium ions, 40%, and free α-amino acids, 51-92%. The amount of amino acids is highly dependent on the grape cultivar, location, and vineyard practices. Branched-chain, aromatic, and sulfur-containing amino acids that are available in grape must are significant precursors for flavor formation (Hazelwood et al. 2008). One group that constitutes wine aroma, and that is associated with amino acids is the production of higher alcohols or fusel alcohols during fermentation. The most negative aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides, and thiols, but especially attention takes hydrogen sulfide. Although most yeasts can produce and release sulfide, there is a wide variety among yeasts and the intrinsic amount of H_2S they produce. A common problematic oenological condition, as it is low initial assimilable nitrogen, modulates the amount of sulfide that yeast produces (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a; Jiranek, Langridge, and Henschke 1996; A. Mendes-Ferreira et al.

2010). The presence of sulphur amino acids, specially cysteine, has also been associated to sulfide release.

In this study, we evaluate different conventional oenological conditions to find other factors that could influence the production of sulfide during wine fermentation, specifically amino acid profiles, which were the target of study. For this purpose, four commercial yeast strains were selected according to their capacity to liberate H_2S. Montrachet, also known as UCD522, is utilized since approximately the early nineties, when it was recognized as a high sulfide producer (Giudici, Zambonelli, and Kunkee 1993) and it has been used not only for wine fermentation but also beer. Vivace is a commercial strain developed from a mutant strain in which the gene leading to reduced H_2S formation is an allele of MET10 (MET10-932) identified by Cordente et al. 2009 and later on by A. Linderholm et al. 2010, which encodes a catalytic subunit of sulfite reductase (US Patent 20100143536A1)and now is commercialized by Renassiance Yeast company. Okay and K1 were recently commercialized, and according to manufacturer instructions, Okay is a very low H_2S producing yeast. Actually, the group of Noble, Sanchez, and Blondin 2015 found variants of the genes SKP2 (previously identified by Yoshida et al. 2011) and MET 2 (studied also by the group of (C. Huang, Roncoroni, and Gardner 2014), that resulted in the generation of a new yeast strain that not only produce low yields of H_2S but also of SO_2, acetaldehyde and propanol.

To evaluate the amino acid profile then, two significant factors that can cause problems during wine fermentation were selected, nitrogen deficiency and high initial sugar content (Bisson 1999a).

In control conditions, all the amino acids exhibited a rapid consumption at the beginning of fermentation, after 24- or 48-hours post-inoculation, remaining at low levels until the end (<50%). Exceptions were Montrachet and Vivace; these two strains shared the same behavior for amino acids Hist, Gly and Cys, consuming less than 50% of the initial amount. One amino acid that is associated with higher H_2S production is methionine (Barbosa, Mendes-Faia, and Mendes-Ferreira 2012a), but its profile was not different from any of the strains, suggesting that it may not have such as significant impact as previously thought. The H_2S evolution in control fermentation showed strain-dependent differences confirming previous studies (Mendes-Ferreira, 2009a, 2009b, 2010, Barbosa, 2012). K1 has not been reported regarding its sulfide production, so when quantifying the total H_2S released by each strain, K1 showed being a higher producer than Montrachet. On the other hand, yeast Okay (Lallemand) and Vivace (Renaissance), presented low and none H_2S production respectively, supporting the information regarding Okay in its technical data. Moreover, for Vivace, the production of sulfide was undetectable in all fermentation conditions tested.

In no DAP supplemented must, having around 120-150mg/L of initial assimilable nitrogen, and 200g/L of sugar concentration, yeasts showed a diminished formation of sulfide, supporting that nitrogen metabolism is closely related to sulfide formation and liberation but differently than previously thought. Low assimilable nitrogen was associated with higher sulfide liberation because if no nitrogen were available less precursors would bind sulfide (Fleet 2003; Jiranek, Langridge, and Henschke 1996). Amino acid profiles that draw most attention in this condition were again the ones by Vivace and Montrachet, although in low

nitrogen condition, K1 also shows a behavior that may suggest that it is a more efficient amino acids consumer. Early works by Jiranek et al., (1995) found that most amino acids are equally effective as suppressants of sulfide formation in nitrogen-deficient fermentations. Those amino acids which support high growth rates, such as serine, glutamine, ammonium, aspartate, arginine, and asparagine are most potent amino acid suppressants of H_2S liberation. The contrary happens with amino acids with regulatory roles of the SRS pathway or involved in sulfur amino acids metabolism or which contain sulfur. Supplementation of fermentations with cysteine or in a mix with methionine, led to increased rates of H2S liberation (Jiranek, Langridge, and Henschke 1995b).

As sugar concentrations gets higher, yeast nitrogen requirements change as well as the nitrogen availability in the must (Jones et al. 2005). In fermentations performed under high initial sugar levels (250g/L), the only strain that showed a higher sulfide production was Montrachet. K1, on the other hand, showed a similar behavior as it did in the control fermentation conditions, as well as Okay and Vivace, their release of H_2S during high sugar concentration in the fermentation must was not different from the concentration in control conditions. An option for this behavior is that the central carbon metabolism is the most affected by the higher sugar flux, and the response of how much sulfide the yeast strains release, depends on how “resistant” each the strain to the glucose overflow. Montrachet (UCD522) has been extensively used as a “model” yeast to study nitrogen supplementation and sulfide production, due to the high amount of S^{2-} it produces, but just a few reports were made in which high sugar concentration was considered a factor (Taillandier et al. 2007). More so, many other factors and

genes, such as vitamin B5, thiamine,TUM1, affecting H2S were identified in low sugar level fermentations (Bartra et al. 2010; C.-W. Huang et al. 2016; X. D. Wang, Bohlscheid, and Edwards 2003).

In all three conditions for the four yeast strains, three amino acids that commonly do not get much attention draw attention. Threonine as well as valine are amino acids well known to be precursors of fusel alcohols trough the Ehrlich pathway (Chen, 1978). Threonine is consumed by all four yeast in every condition but is specially taken up by Vivace and Montrachet. On the other side, valine is excreted by yeast Vivace and Okay in control conditions, while K1 and Montrachet consume it completely. In low nitrogen levels, Montrachet took up all valine present, while Vivace did the same in high sugar fermentation. These two amino acids are responsible for the production of the fusel alcohols *n*-propanol, and isobutanol. It has been seen that the formation for both of these alcohols is inversely proportional to the concentration of amino acids added to low nitrogen must (Garde-Cerdán and Ancín-Azpilicueta, 2008). Moreover, higher alcohols can also be produced from sugar catabolism, accounting for most of alcohols produced during fermentation (Vilanova et al., 2007). In this context, a link could be made to further look at the increased *n*-propanol formation in some yeast strain with the ability to have a reduced formation of H_2S, as seen by Giudici P., Zambonelli C., Kunkee R.E., (1993). The two strains they studied had an average production of 45% of total higher alcohols (mostly n-propanol) while for the other strain tested was between 5-11%, showing a strong inverse relation between n-propanol formation H_2S. One of the yeast used was UCD522 (Montrachet), and as a high sulfide producer showed low n-propanol formation. In fact, threonine is the direct

precursor of n-propanol, and it could be a key to regulate or re-route the formation of H_2S to a higher alcohol instead. Additionally, pulses of Valina as nitrogen source, increased pyruvate formation as well as *n*-propanol formation, undoubtedly due to the excessive formation of threonine (Clement et al. 2013).

One recent finding associated with H_2S formation is that high sulfide formation can confer oxidative stress resistance during wine fermentation (Li et al. 2019). This group found that the thiamine synthesis pathway was one of the most altered between two yeast strains, high and low H_2S producers. Thiamine is a vitamin which function primarily as co-factor of several enzymes, including two routes related to isoleucine and valine synthesis in the Ehrlich pathway. Another path this group found to be related to stress resistance in yeast is the SAM synthesis pathway. *S-adenosyl/methionine synthase* (SAM) is the first enzyme, downriver of SRS pathways, that leads to the synthesis of adenosine and regenerates the homocysteine pool. DNA in all organism is sensitive to oxidate stress; therefore, it fits to find a mechanism that provides sufficient precursors to synthesized new purine bases, in an over oxidative environment.

Another source that contributes to the redox balance is NAD/NADH pools. The NAD consumption during the formation of amino acids may contribute to the imbalance between the NAD/NADH (Verduyn et al. 1990) and the excess of NADH, is for most part regenerated by the formation of glycerol (Eva Albers, Larsson, and Gustafsson 1996).

Every day, new information regarding how yeasts respond to winemaking conditions is reported. New genetic approaches, as next-generation sequencing (NGS) or quantitative trait loci (QTL), and RNA sequencing, have proven to be

efficient strategies for linking genes or gene expressions to explain in a more detail manner phenotypic differences between high and low sulfide producer strains. Nonetheless, most importantly would be to find how key factors can effectively alter the response of genes and routes that command sulfide production and release, so positive fermentative attributes of the yeast strain do not be jeopardized.

It is known that the formation of H_2S occurs in response to yeast metabolic requirements imposed by growth, specifically by the need for sulfur compounds. Metabolically, H_2S is produced from inorganic sulfur compounds such as sulfates and sulphites, or organic sulfur compounds such as cysteine, glutathione or methionine (Henschke P.A. and Jiranek V. 1993).

However, sulfide is not just a by product of sulfur metabolism. Genes in the SRS exhibit various functions under different genetic backgrounds. The amount of H_2S produced by high producer strains varies greatly in different fermentation conditions, whereas non-H_2S producers do not, as it coulb be seen in this work and others earlier (A. Linderholm et al. 2010; A. Spiropoulos and Bisson 2000).

H_2S has been studied in mammals and bacteria, with physiological functions such as synaptic transmission, vascular tone, angiogenesis, protecting cells from oxidative stress (Kimura 2014), and stress resistance (Li et al. 2019; Mironov et al. 2017). According to Kimura *et al.* 2014, the idea that H_2S could have physiological roles began with the discovery of endogenous sulfide in the brain and the relation as when Nitric oxide (NO) was identified as and endothelium-relaxing factor.

Therefore, pathways with cellular roles beyond sulfur amino acids metabolism may also affect H_2S liberation in yeast. In high sugar fermentations excess glucose

overflux could affect homeostatic potential of the cell, and increased H_2S release might be the response to overcome redox imbalance and allow cell survival.

In conclusion, mechanisms of H_2S production in yeast related to cellular roles of H_2S may be affecting H_2S liberation during fermentation.

Refereces

Albers, E., Larsson, C., Gustafsson, L., 1996. Influence of the Nitrogen Source on Saccharomyces cerevisiae Anaerobic Growth and Product Formation. APPL. ENVIRON. MICROBIOL. 62, 9.

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. https://doi.org/10.1016/j.ijfoodmicro.2012.10.003

Bartra, E., Casado, M., Carro, D., CampamÃ, C., PiÃ±a, B., 2010. Differential expression of thiamine biosynthetic genes in yeast strains with high and low production of hydrogen sulfide during wine fermentation. Journal of Applied Microbiology. https://doi.org/10.1111/j.1365-2672.2009.04652.x

Bell, S.-J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Australian Journal of Grape and Wine Research 11, 242–295. https://doi.org/10.1111/j.1755-0238.2005.tb00028.x

Bisson, L.F., 1999. 1999: Stuck and Sluggish Fermentations 50, 13.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Research 8, 1196–1207. https://doi.org/10.1111/j.1567-1364.2008.00412.x

Chen, E.C.-H., 1978. The Relative Contribution of Ehrlich and Biosynthetic Pathways to the Formation of Fusel Alcohols. Journal of the American Society of Brewing Chemists 36, 39–43. https://doi.org/10.1094/ASBCJ-36-0039

Clement, T., Perez, M., Mouret, J.R., Sanchez, I., Sablayrolles, J.M., Camarasa, C., 2013. Metabolic Responses of Saccharomyces cerevisiae to Valine and Ammonium Pulses during Four-Stage Continuous Wine Fermentations. Applied and Environmental Microbiology 79, 2749–2758. https://doi.org/10.1128/AEM.02853-12

Fleet, G., 2003. Yeast interactions and wine flavour. International Journal of Food Microbiology 86, 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9

Giudici, P., Zambonelli, C., Kunkee, R.E., 1993. Increased Production of n-Propanol in Wine by Yeast Strains Having an Impaired Ability to Form Hydrogen Sulfide. Am. J. Enol. Vitic. 44, 17.

Hazelwood, L.A., Daran, J.-M., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on Saccharomyces cerevisiae Metabolism. Applied and Environmental Microbiology 74, 2259–2266. https://doi.org/10.1128/AEM.02625-07

Huang, C.-W., Walker, M.E., Fedrizzi, B., Roncoroni, M., Gardner, R.C., Jiranek, V., 2016. The yeast TUM1 affects production of hydrogen sulfide from cysteine treatment during fermentation. FEMS Yeast Research 16, fow100. https://doi.org/10.1093/femsyr/fow100

Jiranek, V., Langridge, P., Henschke, P.A., 1996. Determination of sulphite reductase activity and its response to assimilable nitrogen status in a commercial Saccharomyces cerevisiae wine yeast. Journal of Applied Bacteriology 81, 329–336. https://doi.org/10.1111/j.1365-2672.1996.tb04335.x

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of Hydrogen Sulfide Liberation in Wine-Producing Saccharomyces cerevisiae Strains by Assimilable Nitrogen. APPL. ENVIRON. MICROBIOL. 61, 7.

Jones, G.V., White, M.A., Cooper, O.R., Storchmann, K., 2005. Climate Change and Global Wine Quality. Climatic Change 73, 319–343. https://doi.org/10.1007/s10584-005-4704-2

Kimura, H., 2014. Production and Physiological Effects of Hydrogen Sulfide. Antioxidants & Redox Signaling 20, 783–793. https://doi.org/10.1089/ars.2013.5309

Li, Y., Zhang, Y., Liu, M., Qin, Y., Liu, Y., 2019. Saccharomyces cerevisiae isolates with extreme hydrogen sulfide production showed different oxidative stress resistances responses during wine fermentation by RNA sequencing analysis. Food Microbiology 79, 147–155. https://doi.org/10.1016/j.fm.2018.10.021

Linderholm, A., Dietzel, K., Hirst, M., Bisson, L.F., 2010. Identification of MET10-932 and Characterization as an Allele Reducing Hydrogen Sulfide Formation in Wine Strains of Saccharomyces cerevisiae. Applied and Environmental Microbiology 76, 7699–7707. https://doi.org/10.1128/AEM.01666-10

Mendes-Ferreira, A., Barbosa, C., InÃªs, A., Mendes-Faia, A., 2010. The timing of diammonium phosphate supplementation of wine must affects subsequent H_2S release during fermentation. Journal of Applied Microbiology 108, 540–549. https://doi.org/10.1111/j.1365-2672.2009.04457.x

Rossignol, T., Dulau, L., Julien, A., Blondin, B., 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 20, 1369–1385. https://doi.org/10.1002/yea.1046

Spiropoulos, A., Bisson, L.F., 2000. MET17 and Hydrogen Sulfide Formation in Saccharomyces cerevisiae. Applied and Environmental Microbiology 66, 4421–4426. https://doi.org/10.1128/AEM.66.10.4421-4426.2000

Taillandier, P., Ramonportugal, F., Fuster, A., Strehaiano, P., 2007. Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food Microbiology 24, 95–100. https://doi.org/10.1016/j.fm.2006.04.002

Varela, C., Pizarro, F., Agosin, E., 2004. Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. Applied and Environmental Microbiology 70, 3392–3400. https://doi.org/10.1128/AEM.70.6.3392-3400.2004

Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1990. Physiology of Saccharomyces Cerevisiae in Anaerobic Glucose-Limited Chemostat Culturesx. Journal of General Microbiology 136, 395–403. https://doi.org/10.1099/00221287-136-3-395

Wang, X.D., Bohlscheid, J.C., Edwards, C.G., 2003. Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. Journal of Applied Microbiology 94, 349–359. https://doi.org/10.1046/j.1365-2672.2003.01827.x