Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC)

Departamento de Biotecnología

Grupo de Microbiología Molecular de Levaduras Industriales

Nitrogen metabolism in wine yeast during alcoholic fermentation: effect on growth, fermentation activity and aroma production

Memoria presentada por

Alicia Gutiérrez Linares

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Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC)

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HAGO CONSTAR que la presente memoria titulada "**Nitrogen metabolism in wine yeast during alcoholic fermentation: effect on growth, fermentation activity and aroma production**" presentada por la **Srta. Alicia Gutiérrez Linares** para optar al grado de Doctor en Ciencia, Tecnología y Gestión Alimentaria por la Universidad Politécnica de Valencia, ha sido realizada bajo nuestra dirección y supervisión, reuniendo las condiciones necesarias para ser defendida por su autora.

Valencia, 20 de Junio de 2013

Dr. José Manuel Guillamón Navarro Dra. Rosa Ana Chiva Tomás

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ABSTRACT

Nitrogen deficiency has been associated with major problems encountered in contemporary winemaking, especially those related to slow and incomplete fermentations. Under wine-making conditions, initial low levels of nitrogen act by limiting growth and biomass, resulting in a reduced fermentation rate. Nitrogen compounds present in the must have also influence on the production of volatile and non-volatile metabolites which regulate sensorial profile and quality of wine. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen. However, the effect of these additions is regulated by specific nitrogen requirements of each yeast strain and the conditions of fermentation.

This doctoral thesis studies the individual nitrogen metabolism of four commercial wine strains, widely used in Spanish wine industry, especially regarding cell growth and fermentation activity, as well as metabolites production. This study has focused on the importance of nitrogen quantity and quality presented in the must, in order to achieve optimal fermentation performance. Through this work the effectiveness of different markers has been tested to find a biosensor that can be used to detect nitrogen limitation conditions under fermentation process. This work has been performed using biochemical, analytical and molecular approaches. Thus, some genetic variations involved in nitrogen utilization have been detected, allowing in the future the possibility to enhance fermentation performance of these strains.

Metabolismo del nitrógeno en levaduras vínicas durante la fermentación alcohólica: efecto en el crecimiento, actividad fermentativa y producción de aromas

RESUMEN

La carencia de nitrógeno es uno de los principales problemas encontrados en la elaboración del vino, especialmente relacionados con fermentaciones lentas e incompletas. En condiciones de vinificación, bajos niveles iniciales de nitrógeno limitan el crecimiento y la producción de biomasa, provocando una baja tasa de fermentación. Los compuestos nitrogenados presentes en el mosto también influyen en la producción de metabolitos volátiles y no volátiles que son los que regulan el perfil sensorial y la calidad del vino. Actualmente, el método más común utilizado para tratar carencias de nitrógeno en la fermentación, es la adición de nitrógeno. Sin embargo, el efecto de estas adiciones se rige por los requerimientos específicos nitrogenados de cada cepa y de las condiciones de fermentación.

Esta tesis doctoral estudia el metabolismo nitrogenado de cuatro cepas vínicas comerciales, ampliamente utilizadas en la industria vínica española, especialmente en lo referente al crecimiento celular y la actividad fermentativa, así como la producción de metabolitos. Este estudio se ha centrado en la importancia de la cantidad y la calidad de nitrógeno presente en el mosto, con el fin de lograr un rendimiento óptimo de la fermentación. A través de este trabajo la eficiencia de diferentes marcadores ha sido probada con el fin de encontrar un biosensor que pueda ser utilizado en la detección de condiciones limitantes de nitrógeno durante el proceso de fermentación. Este trabajo se ha realizado utilizando diferentes técnicas bioquímicas, analíticas y moleculares. Así, algunas variaciones genéticas implicadas en la utilización del nitrógeno se han detectado, permitiendo en el futuro la posibilidad de mejorar el rendimiento fermentativo de estas cepas.

Metabolisme del nitrogen en llevats vínics durant la fermentació alcohólica: efecte en el creixement, activitat fermentativa i producció d'aromes

RESUM

La carència de nitrogen és un dels principals problemes associtas a l'elaboració del vi, especialment relacionats amb fermentacions lentes i incompletes. En condicions de vinificació, baixos nivells inicials de nitrogen limiten el creixement i la producció de biomassa, provocant una baixa taxa de fermentació. Els compostos nitrogenats presents en el most també influeixen en la producció de metabòlits volàtils i no volàtils que són els que regulen el perfil sensorial i la qualitat del vi. Actualment, el mètode més comú utilitzat per tractar carències de nitrogen en la fermentació, és l'addició de nitrogen. No obstant, l'efecte d'aquestes addicions es regeix pels requeriments específics nitrogenats de cada soca i de les condicions de fermentació.

Aquesta tesi doctoral estudia el metabolisme nitrogenat de quatre soques víniques comercials, àmpliament utilitzades en la indústria vínica espanyola, especialment sobre el creixement cel · lular i l'activitat fermentativa, així com la producció de metabòlits. Aquest estudi s'ha centrat en la importància de la quantitat i la qualitat de nitrogen present en el most, a fi d'aconseguir un rendiment òptim de la fermentació. A través d'aquest treball l'eficiència de diferents marcadors ha sigut provada amb la finalitat de trobar un biosensor que puguera ser utilitzat en la detecció de condicions limitants de nitrogen durant el procés de fermentació. Aquest treball s'ha realitzat utilitzant diferents tècniques bioquímiques, analítiques i moleculars. Així, algunes variacions genètiques implicades en la utilització del nitrogen s'han detectat, permetent en el futur la possibilitat de millorar el rendiment fermentatiu d'aquestes soques.

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"*La ciencia es el alma de la prosperidad de las naciones y la fuente de vida de todo progreso*"

Louis Pasteur

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INTRODUCTION

1. YEAST AND ALCOHOLIC FERMENTATION

1.1. Historical notes on yeast and fermentation research

Over the course of human history, and using a system of trial, error, and careful observation, different cultures began producing fermented beverages (around 1700-1100 BC). At that time, people knew that leaving fruits and grains in covered containers for a long time produced wine and beer, but no one fully understood why the recipe worked. The process was named fermentation, from the Latin word *fervere*, which means "to boil". At the time, no one knew that the alcohol produced during fermentation was produced by one celled eukaryotic fungus: yeast. It took several hundred years before researchers observed these microorganisms.

In the seventeenth century, **Antoni van Leeuwenhoek** developed high-quality lenses and was able to observe yeast for the first time. In the eighteenth and nineteenth centuries, chemists worked hard to decipher the nature of alcoholic fermentation through analytical chemistry and chemical nomenclature. Our modern understanding of the fermentation process comes from the work of the chemist **Louis Pasteur**. Pasteur was the first to demonstrate experimentally that fermented beverages result from the action of living yeast transforming glucose into ethanol. Moreover, Pasteur demonstrated that only microorganisms are capable of converting sugars into alcohol from grape juice, and that the process occurs in the absence of oxygen (Pasteur, 1876; Barnett, 2000).

Today, wine making is huge, enormously profitable agricultural industries. This industry has been developed from ancient and empirical knowledge from many different cultures around the world, and now it has been combined with basic and applied science toward modern production processes. All of this is the result of the laborious work of hundreds of scientists who were curious about how things work.

1.2. General characteristics of *S. cerevisae*

1.2.1. The *Saccharomyces* genus

The *Saccharomyces* genus (previously called *Saccharomyces sensu stricto*) currently includes the species *S. cerevisiae*, *S. paradoxus*, *S. bayanus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* (Naumov *et al*., 2000) and *S. arboricolus* (Naumov *et al*., 2010). *S. bayanus* includes two varieties: *uvarum* and *bayanus* (Rainieri *et al*., 2006). *Saccharomyces* species are used to produce a range of fermented beverages, including wine, cider and lager beer. The polyploid nature, the capability of exchanging genetic material, the high genetic variability and the complexity of evolution in *Saccharomyces* yeasts, make species definition very troublesome. Additional genomic variation can arise from interspecific hybridization, which can occur between two or more *Saccharomyces* species (Barrio *et al*., 2006; Dujon, 2010). Some examples, *S. cerevisiae*-*S. kudriavzevii* hybrid wine and brewing yeasts (González *et al*., 2008), *S. cerevisiae*-*S. uvarum* hybrid cider and brewing yeasts (Rainieri *et al*., 2006), and the most well-known hybrid, the lager yeast *S. pastorianus*, which is an interspecific hybrid between *S. cerevisiae* and the recently described *S. eubayanus* (Libkind *et al*., 2011).

1.2.2. Life cycle

S. cerevisiae can multiply either asexually by vegetative multiplication or sexually by forming ascospores (Figure 1.1). Under optimal nutritional and cultural conditions *S. cerevisiae* uses the most common and typical mode of reproduction of *Ascomycetes* yeasts, vegetative growth. The cell division cycle in **vegetative multiplication** involves: G_1 (period preceding DNA synthesis), S (DNA synthesis), G_2 (period preceding the mitosis) and M (mitosis and cytokinesis). Once mitosis is complete and the bud nucleus and other organelles have migrated

into the bud, cytokinesis commences and a septum is formed in the isthmus between mother and daughter.

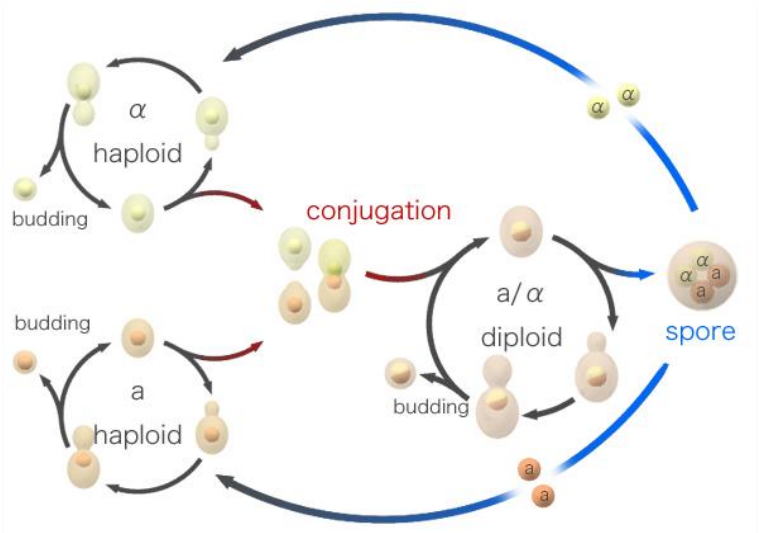

Figure 1.1. A schematic representation of the life cycle of yeasts

Though vegetative growth is the major way of yeast reproduction, **sexual reproduction** is an alternative when nutrient supplies fall short. Meiosis is induced and leads to sporulation and finally to the propagation of four haploid spores (two *MATa* and two *MATα*) encapsulated by an ascus. The latter process involves the conjugation of cells of opposite mating type (***a*** and ***α***), which synchronize their cell cycles to start at the same time in response to their mating factors (Shimoda, 2004). Strains that can be maintained stably for many generations as haploid are termed **heterothallic**. Strains in which sex reversals, cell fusion and diploid formation occur are termed **homothallic**. Wine strains are generally homothallic.

1.2.3. Kinetics of yeast growth during fermentation

Yeast population growth is the result of cell division and the progression through the cell cycle. Under optimal growth conditions, yeast growth kinetic follows the typical microbial growth curve, comprising three main phases: lag phase, exponential phase and stationary phase (Figure 1.2). The **lag phase** reflects the time required for yeast cells to adapt to their new environment by synthesizing ribosomes and enzymes needed to establish growth at a higher rate. The duration of this phase depends on firstly the initial population size and secondly environmental conditions. Once the cell starts actively metabolizing, they begin DNA replication and shortly after the cells divide. This begins the second phase of growth called the **exponential phase** of growth. This is the period in which the cells reproduce at maximum specific growth rate (μ_{max}). The time it takes the population to double is called generation time. Yeast strain, growth medium, and temperature are important factors in determining the generation time. Industrial fermentations aim to extend this phase for maximizing the output of biomass and metabolites production (López *et al*., 2004). The third phase in yeast growth **is stationary phase**, a period of no growth when metabolism slows and cell division is stopped. The factors that cause cells to enter stationary phase are related to change in the environment, such as nutrient deprivation, toxic metabolites and high temperatures. After prolonged periods in stationary phase, cells may die and autolysate.

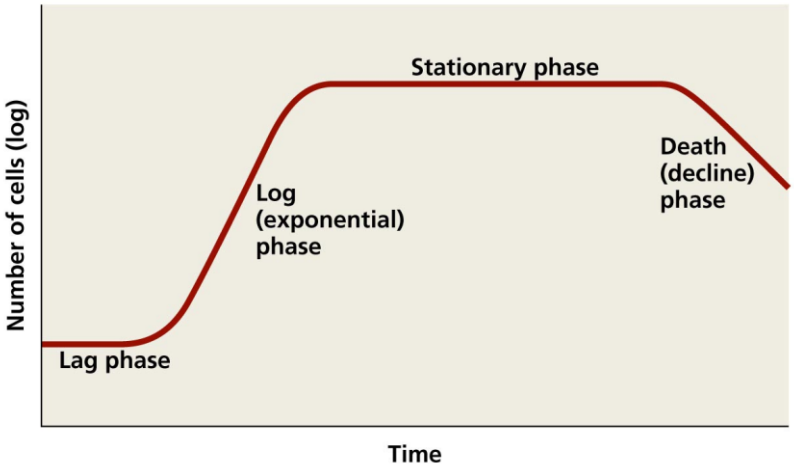

Figure 1.2. Typical yeast growth curve

1.2.4. Genetic constitution of wine yeast: lab *vs* industrial strains

The traditional yeast species *Saccharomyces cerevisiae* is an exceptional yeast. It is the best characterized of all eukaryotes and therefore acts as a model, since the basic functions of eukaryotic cells appear to be highly evolutionarily conserved (Hadfield *et al.*, 1993). *S. cerevisiae* has a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns (Pretorius, 2000). Haploid strains contain approximately 12-13 Mb of nuclear DNA, distributed along 16 linear chromosomes ranging from 200-2200 Kb in length. The genome of a laboratory strain of *S. cerevisiae* was the first eukaryote completely sequenced, containing roughly 6000 protein-encoding genes (Goffeau *et al.*, 1996).

The genome sequence deeply transformed yeast research, with technical improvements and development of methods that greatly accelerated research. However, the use of limited number of laboratory yeast strains represents limited genetic and phenotypic diversity, and is furthermore significantly different from the strains that are used for industrial and commercial purposes. Industrial *S. cerevisiae* strains are highly specialized organisms, which have evolved to grow in

the different environments or ecological niches that have been provided by human activity. These environments constitute much of the evolutionary framework of the species in the past centuries, and many genes that appear not to be associated with a specific function in laboratory strains may be responsible for specific phenotypes in industrial strains. They have a large capacity for genome reorganization through chromosome rearrangements (Bidenne *et al*., 1992; Rachidi *et al*., 1999; Puig *et al*., 2000), promoting rapid adaptation to environmental changes. This specialization has been associated with some genome characteristics which cover a wide range of phenotypic traits, such as diploid genome with the presence of aneuploidies or polyploidies, high level of chromosome length polymorphism, homotallism, high heterozygosis, genome renewal and allopolyploid/hybrid genomes (Mortimer *et al.*, 1994; Querol *et al.*, 2003). Furthermore, industrial yeast strains are notoriously harder to sporulate than lab yeast, and often produce non-viable spores (Bakalinsky and Snow, 1990). It has been proposed that the ploidy of the wine yeasts may confer advantages in adapting to variable external environments or increasing the dosage of some genes important for fermentation (Bakalinsky and Snow 1990; Salmon 1997). Recently, genome sequencing of a *S. cerevisiae* wine yeast have revealed the presence of horizontal gene transfers that could be involved in adaptation to industrial environment (Novo *et al.*, 2009). In addition, wine strains have been characterized by the presence of a set of duplication and depletion genes referred as "commercial wine yeast signature" (Dunn *et al.*, 2005; Carreto *et al.*, 2008).

1.2.5. *S. cerevisiae* ecology

The budding yeast, *Saccharomyces cerevisiae*, was for a long time considered a domesticated unicellular organism distinct from any of its counterparts in natural habitats (Ciani *et al*. 2004). This view emerged in part because *S. cerevisiae* had been used for tens of centuries in baking, brewing, distilling, and wine-making. Several lines of evidence suggest that *S. cerevisiae* existed as a distinct species in

natural environments long before it was utilized in food production (Landry *et al*., 2006). It has increasingly become clear that *S. cerevisiae* occupies numerous habitats that are not necessarily associated with human activities. Common habitats include soil, plant exudates, animal tissues, and surfaces within vineyards and wineries. The diversity, composition and distribution of yeast in association with grapes and musts are influenced by a variety of environmental and technological factors: climate and geographical location (Longo *et al.,* 1991; Versavaud *et al.,* 1995; Beltran *et al.,* 2002; Schuller *et al.,* 2005), grape variety (Martini *et al.,* 1980), presence of yeast starters (Heard and Fleet, 1985; Valero *et al.,* 2007), and the fermentation temperature used (Torija *et al.,* 2003a).

Natural wine fermentation involves a multitude of biochemical and ecological interactions between many microbial species (Pretorius, 2000). Early stages of wine fermentation involve many different microorganisms, including bacteria of various kinds as well as molds and yeasts of the genera *Kloeckera*, *Metschnikowia*, *Torulaspora*, *Candida*, *Kluyveromyces*, *Pichia*, *Brettanomyces*, *Dekkera*, *Zygosaccharomyces*, and *Saccharomyces* (Fleet and Heard, 1993). Although *S.cerevisiae* only represents a small fraction of this population (approximately 10-100 CFU/mL), this wine yeast dominates the final stages of fermentation (Figure 1.3). During the fermentation progress, *S.cerevisiae* displaces the non-*Saccharomyces* species and occupies their fermentative niche. A strong selective pressure favors the *Saccharomyces*'s strategy of making life difficult for other microorganisms by rapidly converting the available sugars into ethanol, a toxic compound, which can be later respired by *Saccharomyces* (Thomson *et al*., 2005; Woolfit and Wolfe, 2005; Piskur *et al*., 2006). Ethanol concentration creates strong pressure which limits growth of non-*Saccharomyces*, consequently, they can not compete with the *Saccharomyces*, which could generally stand higher ethanol levels (10-15% v/v) (Fleet and Heard, 1993; Rainieri and Pretorius 2000). Moreover, the temperature increase produced by *Saccharomyces* during

fermentation provides these species with a clear advantage over non-*Saccharomyces* species (Salvadó *et al*., 2011).

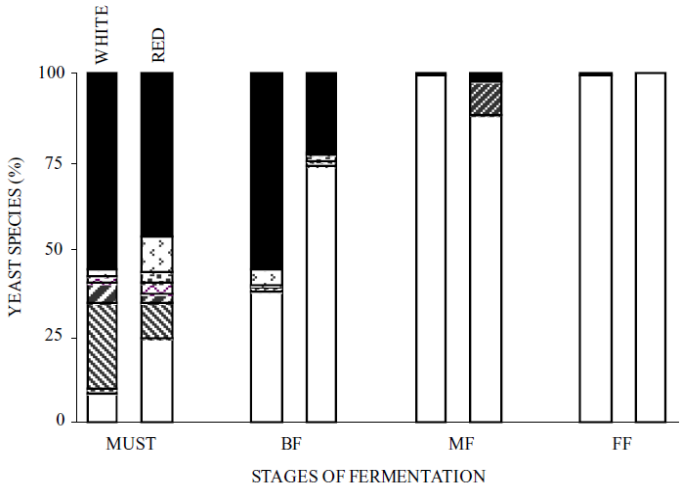

Figure 1.3. Biodiversity of yeast species during alcoholic fermentation of white wine and red wine. BF: Beginning of fermentation, MF: Mid of fermentation, FF: Final of fermentation. □*Saccharomyces,* ■*Candida stellata*, ▧*Hanseniaspora uvarum*, ⁙*Candida colliculosa*, ◪*Metschnikowia pulcherrima*, ▨*Schizosaccharomyces* spp., ⁘*Zygosaccharomyces bailii*, ⊠*Kluyveromyces thermotolerans*, ⊡non identified (Torija *et al*., 2001)

1.3. Alcoholic fermentation

Alcoholic fermentation is the anaerobic transformation of the principal grape sugars, glucose and fructose, into ethanol and carbon dioxide (Zamora, 2009). It can be summarized by this overall reaction:

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

In addition, other important compounds are produced throughout alcoholic fermentation, such as glycerol, organic acids, acetaldehyde, alcohols and esters. These metabolites are very important in the final quality of wine.

The must largely consists of sugars, mainly glucose and fructose. Organic acids are also important in must composition: in particular tartaric and malic acids, citric acid to a lesser extent, and also succinic and keto acids. Furthermore, it contains nitrogen compounds, other mineral salts and vitamins (Henschke and Jiranek, 1993). Finally, phenolic compounds and aromas contribute to wine aroma, although they do not play an essential role in fermentation kinetics.

Yeasts can degrade sugars by two metabolic pathways: fermentative and oxidative. Glycolysis is the common route for both these processes (Figure 1.4). The sugars in grape juice are metabolized to pyruvate by the enzymes of the glycolytic pathway. Afterwards, in the fermentative one, the pyruvate is decarboxylated to acetaldehyde, which is reduced to ethanol, whereas, in the respirative pathway, the pyruvate that arose from glycolysis undergoes an oxidative decarboxylation in the presence of coenzyme A inside the mitochondria. The respiration can take place at a low sugar concentration and in the presence of oxygen. However, for high glucose concentrations (above 9 g/L approximately), yeasts only metabolize sugars by the fermentative pathway. Even in the presence of oxygen, respiration is blocked. This phenomenon is known as the Crabtree effect, catabolic repression by glucose or the Pasteur contrary effect (Ribéreau-Gayon *et al*., 2006). The high sugar concentration of the grape must makes the fermentative pathway the main sugar catabolic route. In a standard fermentation, one molecule of sugar (glucose/fructose) yields two molecules of ethanol and carbon dioxide. However, only 90-95% of the sugar is converted into ethanol and carbon dioxide, 1-2% into cellular material and 4-9% into other secondary metabolites such as glycerol, succinic and acetic acids, fusel alcohols and esters (Boulton *et al*., 1996). Fermentation activity decreases under stressful fermentation conditions, such as

nutrient limitation, low pH, lack of oxygen, extreme temperatures, and the presence of toxic substances.

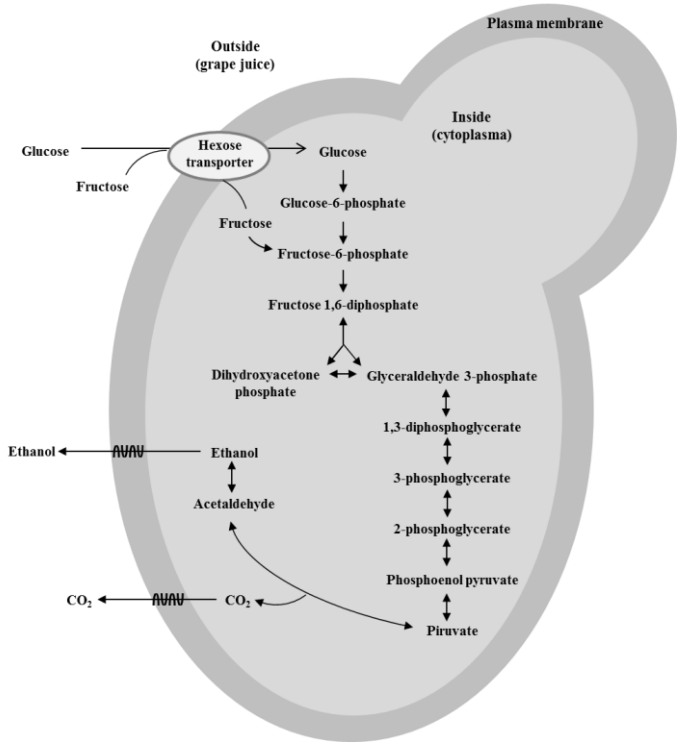

Figure 1.4. Glycolitic pathway in wine yeast (adapted from Pretorius, 2000)

1.3.1. Spontaneous and inoculated fermentations

Grape juice can support the growth of a multitude of microorganisms. However, its low pH (3 to 3.5) and high sugar content (average 200 g/L) exert a selective pressure on microbial populations. Wine has traditionally been produced by allowing the microorganisms naturally present on grapes to grow. Yeasts, fungi, acetic acid bacteria and lactic acid bacteria are the main microorganisms able to grow in grape juice. Once fermentation is under way, the anaerobic conditions that are created contribute to the selective pressure, and microorganisms incapable of

fermentative metabolism (such as fungi and acetic acid bacteria) are inhibited. As the fermentation progresses nutrients are depleted and ethanol concentration increases, thereby inhibiting ethanol-sensitive species (Henschke, 1997).

In a **spontaneous** fermentation the growth of many microorganisms that are present on grapes or in the cellar predominates in the early phase (Fleet and Heard, 1993; Fleet *et al*., 1998). Although these yeasts are vigorous, *S. cerevisiae* becomes the dominant species when the ethanol content reaches 5% (v/v) (Fleet and Heard, 1993; Mortimer *et al*., 1994) and temperature increases (Salvadó *et al*., 2011). *S. cerevisiae* plays the main role in the spontaneous fermentation of grape juice.

After the importance of yeasts for wine quality was established, a technique to isolate yeasts derived from a single cell was developed. In 1890, Muller-Thurgau produced wine by inoculating grape juice with a pure yeast culture (Kunkee and Amerine, 1970). Yeast strains have since been selected and commercialized to be used as starter cultures in fermentation. The **inoculation** with commercially produced active dried wine yeast (ADWY) strain tends to dominate the fermentation from the beginning. Since the commercial strains are well characterized, such inoculation provides winemakers with a better control of the process, and a better ability to achieve a specific desired outcome. This inoculation strategy using starters decreases the lag phase, minimizes the influence of wild yeast by imposition of the inoculated yeast strain, ensures rapid and complete grape must fermentation and, as a result, improves the reproducibility of the wine production (Fleet and Heard, 1993; Bauer and Pretorius, 2000). The main critics of the practice of guided fermentation (using starter cultures) dislike the fact that the commercial wine strains, despite being numerous, possess very ordinary characteristics. Commercial yeast strains produce wines with average qualities and do not enhance the aromatic traits that characterize the strains and species diversity of the spontaneous fermentation.

The natural availability of *S. cerevisiae* strains possessing an ideal combination of technological and qualitative traits is highly improbable. Enological strains have to

posses the ability to efficiently ferment the must under stress conditions and adapt quickly when the environment changes (transcriptional and post-transcriptional mechanisms). Nevertheless, wine strain characteristics can be improved and ideal combinations of oenological traits can be artificially achieved by means of genetic and molecular techniques. The selection process is, however, a fundamental step in any yeast improvement program. For this reason, it is important the identification and selection of yeasts that can ferment grape juice efficiently and produce good quality wines. Analysis of several traits related with the process has been proposed for selecting the strains (Table 1.1).

Table 1.1. Oenological characteristics to be considered in the selection of wine strains (adapted from Schuller and Casal, 2005)

Oenological characteristics	Comment	Desirable
Fermentation vigour	Maximum amount of ethanol (%, v/v) produced at the end of fermentation.	Good ethanol production and tolerance. Complete fermentation of sugars.
Fermentation rate	Grams of CO_2 produced during the first 48 h of fermentation.	Prompt fermentation initiation. Minimization of lag phase.
Mode of growth in liquid medium	Dispersed or flocculent growth. Sedimentation speed.	Dispersed yeast growth during, but sedimentation at the end of fermentation.
Foam production	Height of foam produced during fermentation.	Increased foam production.
Optimum fermentation temperature	Thermotolerance and cryotolerance is related to oenological properties.	Optimum fermentation temperature ranges between 18 and 28 ºC. Capacity of fermentation at low temperatures.

Volatile acidity	Mainly produced by acetic acid.	Strains should not release more than 100-400 mg/L during fermentation.
Malic acid degradation or production		Depends on the characteristics of the must.
Glycerol production	Contributing to wine sweetness, body and fullness.	Production between 5-8 g/L.
Esters, higher alcohols and volatile compounds	Contribute positively to global sensorial characteristics.	Desirable metabolites which influence wine flavour and depend on the presence of precursors.
SO_2 tolerance and production	Antioxidant and antimicrobial agent.	High fermentation capacity in the presence of SO_2. Low production.
H_2S production	Detrimental to wine quality with very low threshold value.	No production.
Cooper resistance	High cooper concentrations may cause stuck fermentations.	High cooper resistance and the ability to reduce the cooper content.
Stress resistance	Several stress during fermentation.	Tolerance to different stress in the same strain.

1.3.2. Causes of stuck or sluggish fermentations

One of the very important objectives during most winemaking processes is the achievement of complete alcoholic fermentation, so that the residual fermentable sugar in the wine is less than 2-4 g/L. The completion of fermentation then allows the wine to be stored under conditions of restricted contact with air, avoiding the destructive oxidation reactions. Furthermore, complete alcoholic fermentation may

help avoid problems not only with acetic acid bacteria but also with lactic acid bacteria, which could metabolize residual sugars to increase volatile acidity and also in the formation of abnormal esters and perhaps alter the pattern of diacetyl formation (O'Connor-Cox and Ingledew, 1991).

The study of the factors responsible for the occurrence of stuck and sluggish fermentations is receiving increased interest because of the economic impact of fermentation problems. Wine fermentations termed 'stuck' are those which fail to reach completion and contain high levels of residual sugars (> 2-5 g/L) (Fleet, 2003). 'Sluggish' fermentations are those which take longer than normal to finish (Pizarro *et al.,* 2007). Many factors such as nutritional deficiencies, presence of inhibitory substances or enological practices may be involved. The effects related with these factors are numerous and include decrease in pH, inhibition of key enzymes activity, and alteration of plasma membrane. These may induce decrease in the metabolism of the yeast cell and consequently decreases in biomass production, cell viability and fermentation rate. Furthermore, occurrence of stuck and sluggish fermentation could be the result of interaction of these factors (Alexandre and Charpentier, 1998).

1.3.2.1. Nutritional deficiencies

In some cases stuck or sluggish fermentation would appear to be caused by insufficient levels of assimilable **nitrogen** (Ingledew and Kunkee, 1985). A low initial level of nitrogen acts by limiting growth rate and biomass formation of yeast, resulting in a low rate of sugar catabolism (Monteiro and Bisson, 1991a). This sugar transport inactivation is triggered by the arrest in protein synthesis linked to the lack of nitrogen source. To avoid glucose transport inactivation, it is necessary to maintain a high rate of protein synthesis which can be supported by an ample ammonium supply, before the depletion of assimilable nitrogen. Metabolism of yeast is also dependent on the presence of dissolved **oxygen** at the beginning of the fermentation. Decrease in oxygen availability results in an inhibition of fatty

acid and sterol biosynthesis in the yeast (David and Kirsop, 1973; Aries and Kirsop, 1978) and consequently a decrease in biomass production, rate of glycolysis and yeast viability. Some sluggish fermentations also appear to be associated with insufficient availability of **vitamins** (Ough *et al*., 1989) and **minerals** like magnesium which is important for many metabolic and physiological functions in yeast (Walker, 1994).

1.3.2.2. Inhibitory substances

Accumulation of some substances such as ethanol, medium chain fatty acids (MFCA) and acetic acid can produce toxic effect in the cell. **Ethanol** during the fermentation can lead to inhibition of the process and cause other unfavorable effects in yeast cells that decrease yeast growth and viability. Moreover, it inhibits the amino acid permeases and the glucose transport system (Mauricio and Salmon, 1992). The presence of ethanol also affects the plasma membrane, resulting in a modification of membrane organization and permeability (Lloyd *et al*., 1993). **Medium chain fatty acids** (MCFA) and **acetic acid** activity is highly dependent on the pH of the medium: toxicity increases as pH decreases, affecting fermentation rate (Viegas *et al*., 1989; Pampulha and Loueiro-Dias, 1990).

1.3.2.3. Enological practices

Excessive must clarification, addition of sulphite and extreme temperatures can often cause sluggish fermentation. Addition of **SO_2** to the must should be tightly controlled; the dose used should inhibit the growth of undesired species but allow the development of fermentation yeast. It is well known that yeast resistance to SO_2 varies with the species. High concentration of SO_2 can produce stuck fermentation, since once inside the cell, sulphites cause a rapid decrease of the intracellular ATP level, and the depletion in ATP is the decisive event causing cell death (Hinze and Holzer, 1986). **Must clarification** affects the assimilation of nitrogen compounds,

reduces nutrients and eliminates fatty acids, sterols and macromolecules (Alexandre and Charpentier, 1998). Extreme **temperatures** during fermentation can severely affect yeast growth and metabolism (Specht, 2003). The principal effect of the extreme temperatures is produced in the plasma membrane (Watson, 1987; Suutari *et al*., 1990). At higher temperatures, the cell membrane fluidity increases and ethanol can enter the cell more quickly, adversely affecting metabolism and cell viability. Cooler temperatures may enhance ethanol resistance by increasing sterol levels in yeast cell membranes (Suutari *et al*., 1990; Torija *et al*., 2003b) resulting in lower accumulation of intracellular ethanol (Lucero *et al*., 2000). However, this low temperature also produces problems with nutrient transport (excess of rigidity in the plasma membrane) and a low enzymatic kinetic which impairs crucial metabolic functions of the cell, such as protein synthesis, ATP formation, stress response, etc.

1.4. Yeast and the post-genomic era

The first complete sequence of a eukaryotic genome (Goffeau *et al*., 1996) produced a dramatic transformation of yeast research. The transformation began with technical improvements that greatly accelerated research, especially any research involving identification of pieces of DNA cloned, and technologies unimaginable before, such as DNA microarrays containing each and every yeast gene, became commonplace. The availability of the entire genome sequence has made possible the asking of new kinds of research questions, questions that can be answered only when one has truly comprehensive information about an organism.

The current 'genomic revolution' is generating large amounts of valuable information in the form of genome sequences. This new knowledge has to be accompanied by post-genomic studies based on new advanced methods, strategies and technologies which have to be continuously developed and improved. The most advanced post-genomic strategies are directed to the elucidation of new genes, their function and mechanisms of regulation, using new techniques at

different levels of study: genome, transcriptome, proteome and metabolome, in an integrative or systems biology perspective (Delneri *et al*., 2001; Kitano, 2002; Oliver, 2002a; Oliver *et al*., 2002b).

The importance of *S.cerevisae* in food industry, particularly wine sector, has produced a huge interest for the genetic improvement of these commercial strains. Traditionally, the genetic manipulation strategies of wine yeasts to produce better new strains exploit different strategies, which included the selection of natural and inducted mutants to sexual recombination methods (Zambonelli, 1988). Nevertheless, the enormous amount of genomic information currently available makes the application of the recombinant DNA technologies more feasible for industrial yeasts genetic engineering. The knowledge of the genetic nature of the desired trait is essential to perform the appropriate choice among different genetic improvement approaches, as well as, to understand the adaptation of yeast to industrial stressful environments and will facilitate strain improvement through breeding and genetic engineering. Usually the most important oenological traits, such as fermentative vigour, ethanol yield and tolerance, and growth temperature profile, depend on a multitude of loci (QTLs) (Giudici *et al*., 2005). Quantitative trait locus (QTL) mapping is a proven approach to map the genetic variation responsible for quantitative traits in *S. cerevisiae*. The basic principles of QTL mapping are quite simple and involve examining the associations between molecular markers, such as microsatellites or restriction fragment length polymorphisms (RFLPs), and the values for phenotypic traits; the closer a marker is to a QTL, the greater the expected level of association (Christians and Keightley, 2002). Although, it has proven extremely difficult to refine their position to sufficiently high resolution in order to identify the molecular basis of their effect, it has been successfully applied to high-temperature growth (Steinmetz *et al.*, 2002; Sinha *et al.*, 2006; Sinha *et al.*, 2008), sporulation (Deutschbauer and Davis, 2005; Ben-Ari *et al.*, 2006; Gerke *et al.*, 2006; Katou *et al.*, 2009), cell morphology (Nogami *et al.*, 2007), drug sensitivity (Kim and Fay, 2007), ethanol tolerance and

growth (Hu *et al.*, 2007; Smith and Kruglyak, 2008; Katou *et al.*, 2009), and flocculation (Brauer *et al.*, 2006). Therefore, once the QTL have been identified, it still must be down-scaled to the gene and/or nucleotide level by a combination of traditional methods such as sequence analysis, candidate gene prediction, and functional complementation (Abiola *et al.*, 2003). One of these approaches, reciprocal hemizygosity analysis (RHA) (Steinmetz *et al.*, 2002), evaluates all genes in a QTL for relevance in establishing the trait of interest. It is based on the construction of two isogenic strains in the hybrid diploid background from both parent strains that differ genetically only in the alleles of one copy of a specific candidate gene (Figure 1.5). By comparing the phenotypes of the two strains, it will be revealed whether an allele from one genetic background is advantageous over that from the other. As RHA analyzes the contribution of each allele in the hybrid diploid background, it takes into account the possible requirement for interactions with other mutant alleles from the parental backgrounds to confer the phenotype under study.

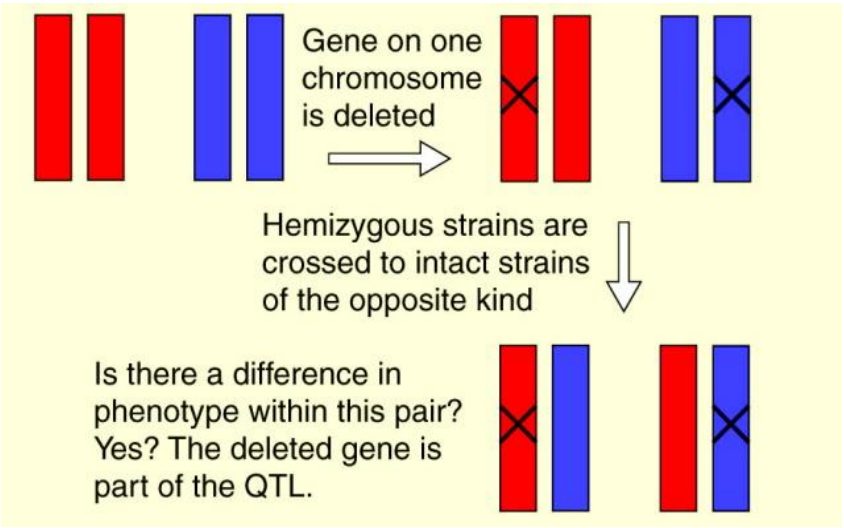

Figure 1.5. Reciprocal hemizygosity analysis

Although quantitative traits of *S. cerevisiae* strains have great importance for industrial use, few QTL studies have been carried out in commercial strains (Marullo *et al*., 2007; Katou *et al*., 2009). Industrial strains or natural isolates pose a much greater challenge than laboratory strains for QTL mapping.

2. NITROGEN METABOLISM

2.1. Central nitrogen metabolism

Yeast can use distinct nitrogen containing compounds, including amino acids, urea, ammonium, nitrogen bases, and purine derivatives as a source of nitrogen (Figure 2.1). These molecules enter cells via permeases and can be immediately used as building blocks in biosynthesis, converted to a related compound and utilized in biosynthesis or catabolized to release nitrogen in the form of ammonium (via deamination), glutamate (via transamination), or both (Magasanik and Kaiser, 2002).

The yeast cell converts these molecules into ammonium, glutamate and glutamine, which function as amino donor in all other biosynthetic reactions. All reactions that convert ammonium and glutamine into glutamate are referred to as the Central Nitrogen Metabolism (CNM) (Cooper, 1982; Magasanik, 1992). These reactions showed in Figure 2.2 allow the synthesis of glutamate from α-ketoglutarate and ammonium and for the synthesis of glutamine from glutamate and ammonium. By using different sets of enzymes, these core reactions allow the interconversion of ammonium, glutamate and glutamine. From these two amino acids all other nitrogen containing compounds in the cell are produced.

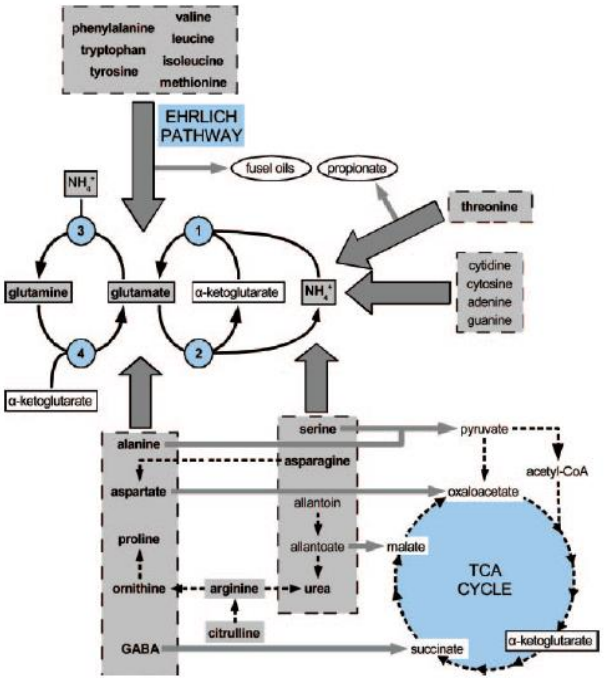

Figure 2.1. Main reactions involved in the utilization of nitrogen sources in the yeast *S. cerevisiae* (Godard *et al*., 2007)

When cells have an abundant source of ammonium, either by conversion of other nitrogen sources to ammonium or by growth on ammonium itself, the $NADP^+$-glutamate dehydrogenase, encoded by *GDH1*, is responsible for the synthesis of glutamate by combining ammonium with the citric acid cycle intermediate α-ketoglutarate. Glutamate can then combine with ammonium in a reaction catalyzed by glutamine synthetase (GS), the product of *GLN1*. When glutamate is the sole nitrogen source, the NAD^+-glutamate dehydrogenase, encoded by *GDH2*, is responsible for the release of ammonium required for the synthesis of glutamine from glutamate. Glutamate synthetase (GOGAT), the product of *GLT1*, catalyzes the synthesis of glutamate from glutamine.

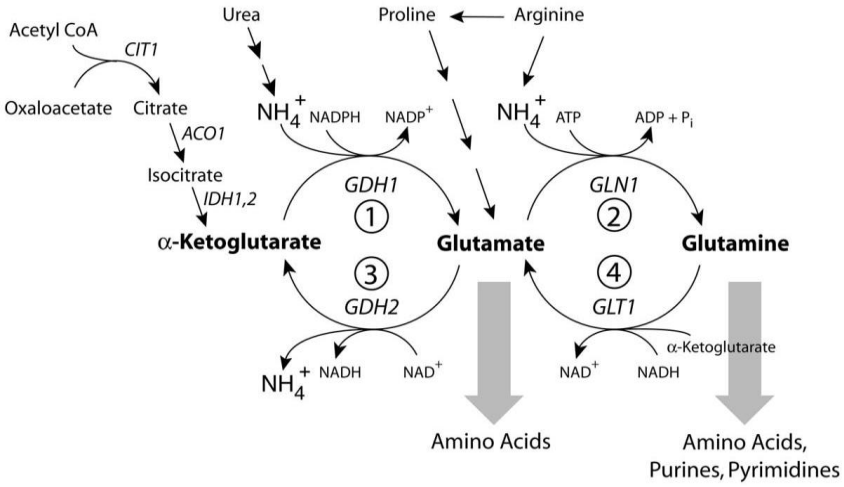

Figure 2.2. Central pathways for nitrogen metabolism (numbers indicate the gene responsible of the production of the enzyme in this step of the pathway) (Magasanik and Kaiser, 2002)

2.2. Nitrogen catabolite repression (NCR)

Saccharomyces cerevisiae can use different nitrogen sources for growth but not all of them support its growth equally well. It selects nitrogen sources that enable best growth through a mechanism called Nitrogen Catabolite Repression (NCR). This nitrogen regulation is the mechanism designed to prevent or reduce the unnecessary transcription of genes encoding enzymes and permeases for the utilization of poorer nitrogen sources when a preferred source is available such as glutamine, asparagine and ammonium (Magasanik, 1992). The transcription repression and the selective inactivation and subsequent degradation of permeases upon addition of good nitrogen sources in the growth medium prevents uptake of the poorer ones (Figure 2.3). For instance, the general amino acid permease Gap1 and the proline permease Put4 are regulated by the nitrogen source present in the medium. However, permeases like the histidine permease Hip1, the lysine

permease Lyp1 or the tryptophan permease Tat2 are expressed constitutively, regardless of the nitrogen source and concentration (ter Schure *et al*., 2000).

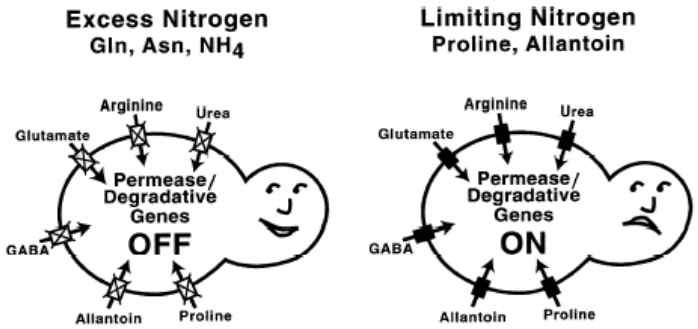

Figure 2.3. Representation of Nitrogen Catabolite Repression (NCR) mechanism in different conditions of nitrogen availability (Cooper, 2002)

a. Uptake regulation

The first step in the regulation of nitrogen utilization is at the level of uptake via more or less specific permeases. Most amino acids present in the external medium are detected by yeast cells via a membrane-associated sensor complex (Ssy1-Ptr3-Ssy5 [SPS]) (Forsberg and Ljung-dahl, 2001). The main function of this sensor complex is to respond to the presence of external amino acids by inducing expression of several genes encoding amino acid and peptide permeases via transcription factors (Stp1, Stp2 and Uga35/Dal81). Among these are the *AGP1*, *BAP2*, and *BAP3* genes (encoding wide range specificity amino acid permeases) and the ditripeptide permease gene *PTR2* (Barnes *et al*., 1998; Iraqui *et al*., 1999; Regenberg *et al*., 1999). Once the permeases are properly expressed they become activated e.g. by phosphorylation and inactivated by dephosphorylation, followed by degradation via the ubiquitin pathway (Stanbrough and Magasanik, 2005).

b. Operation at the protein level: inactivation, internalization and degradation of permeases

When good nitrogen sources are present in the medium, the principal mechanism of post-transcriptional regulation is inactivation of permeases which transport poor nitrogen sources, such as Gap1, Put4, Dal4, and their degradation in the vacuole. Specific kinases/phosphatases produce the phosphorylation/dephosphorylation of the permeases. In the case of Gap1, the inactivation of Npr1 (encoding a protein kinase) or the activation of unidentified phosphatase, produce dephosphorylation of the permease when in the medium there are good nitrogen sources. Subsequently dephosphoryled permease becomes a target for Npi1 which ubiquitinates the permease triggering its degradation in the vacuole (Figure 2.4). It is suggested that activation/inactivation by phosphorylation/dephosphorylation is a common process among nitrogen permeases.

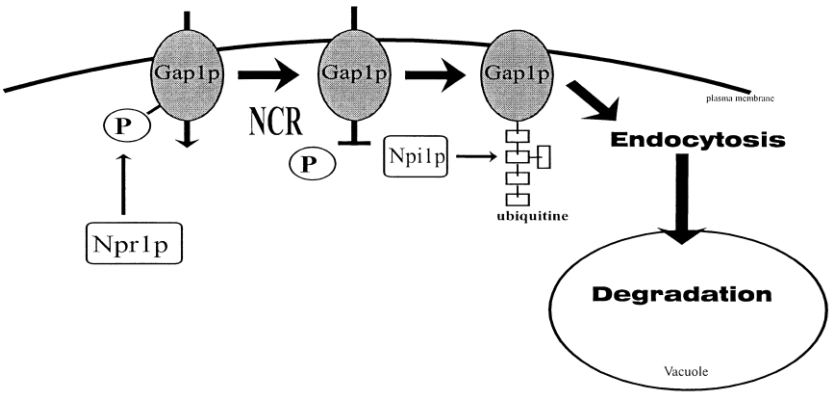

Figure 2.4. Post-translational regulation of the general amino acid permease Gap1 (ter Schure *et al*., 2000)

c. Transcription repression

Nitrogen catabolite repression has been shown to be mediated by inhibition of, among others, transcription factors that recognize UAS_{NTR} sites. UAS_{NTR} is necessary and sufficient for NCR regulation. The nitrogen regulated activation sequence UAS_{NTR} consists of two separate dodecanucleotide sites with the sequence GAT(T/A)A at their core.

Until now, five key players have been identified to participate in NCR at the level of transcription. These include four GATA transcription factors, two positive (activators): Gln3, Gat1/Nil1 and two negative (repressors): Dal80/Uga43, Deh1/Gzf3/Nil2 (Figure 2.5). The four transcription factors share a characteristic zinc finger region which permits them to bind and recognize a region of DNA with GATA sequences of the genes subject to nitrogen regulation. The fifth player is the regulatory protein Ure2, which forms a cytoplasmic complex with Gln3 and Gat1 (Cooper, 2002).

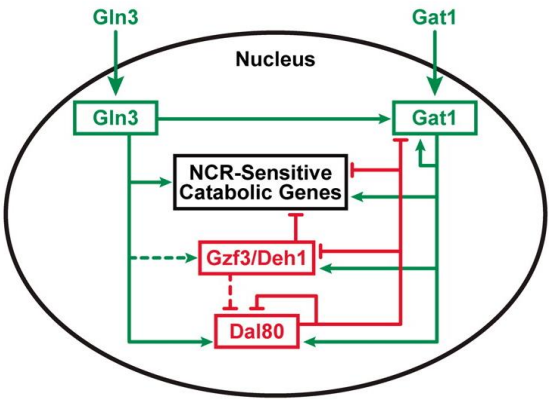

Figure 2.5. Regulation of GATA transcription factors. Green lines and arrows indicate positive regulation, whereas red lines and bars indicate negative regulation. Dashed lines indicate weak regulatory relationships (Georis *et al*., 2009)

Gln3 and Gat1 factors are most active under limiting nitrogen conditions (e.g., when cells grow on poor nitrogen sources like urea and proline) and are also transiently activated upon the addition of rapamycin to nitrogen-rich media. Rapamycin inhibits the Tor proteins (central controllers of cell growth), which are proposed to govern the inhibition of Gln3 and Gat1 under good nitrogen supply conditions. The Tor-dependent inhibition of Gln3 involves the Ure2 protein, whereas the repression of Gat1-dependent expression under good nitrogen supply conditions is also dependent of Deh1/Gzf3/Nil2.

Under nitrogen-rich conditions, Gln3 and Gat1 are present in the cytoplasm in phosphorylated form complexes with Ure2, which retains them in the cytoplasm and avoid their entrance in the nucleus (Figure 2.6). Under nitrogen-limiting conditions or in poorer nitrogen sources, Gln3 and Gat1 are dephosphorylated and release from Ure2, move from the cytoplasm to the nucleus, and increase expression of NCR-sensitive gene. With the aid of the nuclear import factor Srp1, Gln3 can then enter the nucleus where it can activate transcription of nitrogen-regulated genes. *GAT1* is self-regulated, added to the NCR regulation, increasing their transcription when Gln3 and/or Gat1 are in the nucleus.

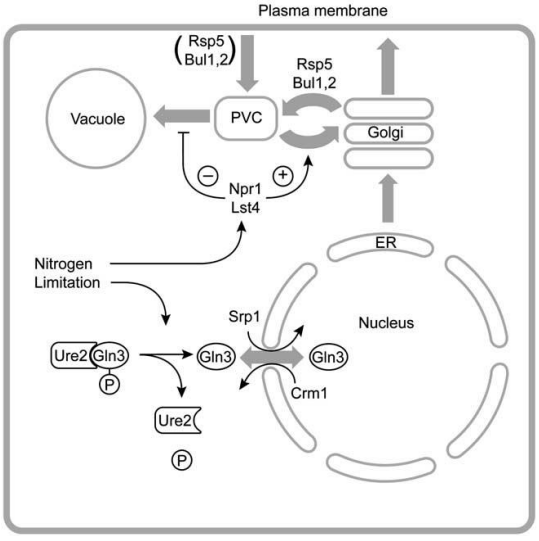

Figure 2.6. The role of intracellular trafficking pathways in nitrogen regulation (Magasanik and Kaiser, 2002)

In excess nitrogen conditions, the nitrogen-regulated permease Gap1 is transported through the secretory pathway to the Golgi complex. In the Golgi, ubiquitination of Gap1 by the E3 ubiquitin ligase complex consisting of Rsp5, Bul1 and Bul2 causes Gap1 to be diverted to the prevacuolar compartment (PVC) and the vacuole. Conditions of nitrogen limitation allow Gap1 to be recycled to the Golgi and to the plasma membrane. Recycling may involve the action of the Npr1 kinase and the membrane protein Lst4 (Figure 2.6).

2.3. Classification of nitrogen sources

Different nitrogen sources can be assimilated by *S. cerevisiae*, but not all sources support growth equally well and produce the same response in the cell. It is very important to know the nitrogen concentration and the type and quality of these sources in the grape must used in the alcoholic fermentation.

Two different criteria can be used to judge the quality of a particular nitrogen source. First is the growth rate that can be supported by a source of nitrogen. The second one is based on the level to which systems for use of alternative nitrogen sources are derepressed during growth on a particular nitrogen source (Magasanik and Kaiser, 2002). Thus, nitrogen sources that do not derepress the pathways for utilization of alternative nitrogen sources are generally considered to be preferred nitrogen sources, whereas nitrogen sources that do lead to derepression of the alternative pathways are considered to be non-preferred. Thus, taking into account the growth rate and the different transcriptional controls on genes involved in nitrogen metabolism, Godard *et al*., 2007 classified nitrogen sources in three groups (Figure 2.7):

Group A comprises asparagine, glutamine, serine, ammonium, aspartate, alanine, arginine and glutamate. On these nitrogen sources, growth is rapid and NCR occurs. However, the level of NCR does not correlate exactly with the generation time. For instance, NCR is partially relieved on glutamate and aspartate, despite rapid growth.

Group B involves leucine, isoleucine, methionine, threonine, tryptophan, and tyrosine. On these nitrogen sources, growth is particularly slow and NCR does not occur. Furthermore, transcriptional control of amino acid biosynthesis (GAAC) is activated in these nitrogen sources.

Finally the classification of the **remaining nitrogen sources**: valine, phenylalanine, ornithine, proline, GABA and citrulline, that are assimilated when groups A and B are depleted.

A major difference between the two groups of nitrogen sources is that the NCR is active and GAAC is inactive in group A, whereas the oppossite is in group B compounds. This result is in agreement with the concept that NCR is active on good and inactive on poor nitrogen sources (Cooper, 2002; Magasanik and Kaiser, 2002). Another difference between both groups is the carbon derivatives resulting from the catabolism of these compounds. Whereas the transamination or deamination of group A compounds yields derivatives directly assimilable by the cell metabolism, the transamination of group B nitrogen sources leads to keto acids undergoing decarboxylation to aldehydes which are in turn converted by dehydrogenases into long-chain or complex alcohols (Godard *et al*., 2007). The amino acid derivatives generated by Ehrlich pathway are toxic and thus excreted by the cell, and this contributes to the formation of fusel oils.

Glycine, lysine, histidine and the pyrimidines can not be utilized by most strains of *Saccharomyces* as a source of nitrogen, but they can readily be taken up directly as biosynthetic precursors (Boulton, 1998). However, the preference of utilization of nitrogen containing compounds may change depending upon environmental, physiological and strain-specific factors.

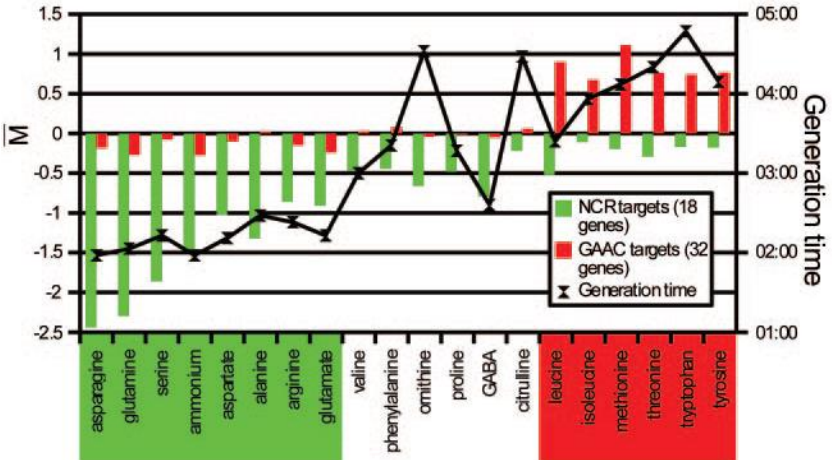

Figure 2.7. Classification of nitrogen sources depending on the generation time of yeast cells and on the average expression of genes subject to NCR or to GAAC (Godard *et al*., 2007)

2.4. Transport of nitrogen compounds

In *S. cerevisiae*, the plasma membrane is not freely permeable to nitrogen compounds. Therefore, the first step in their utilization is the transport across the plasma membrane. The number of transport systems is surprisingly high. In many cases a given substrate is transported by several permeases with different substrates affinities, specificities, capacities and regulations.

a) Ammonium transport

S. cerevisiae has three specific ammonium transporters, Mep1, Mep2 and Mep3. These permeases are expressed when low ammonium concentrations are present in the growth media. Mep2 displays the highest affinity for NH_4^+ (Km 1- 2 µM), followed closely by Mep1 (Km 5 - 10 µM) and finally by Mep3, whose affinity is much lower (Km 1.4 - 2.1 mM). The Mep transporters seem to be required to retain NH_4^+ inside the cells during growth on some nitrogen sources other than NH_4^+. The

MEP genes are subjected to nitrogen control. At high ammonium concentrations or in presence of a good nitrogen source the *MEP* genes expression becomes repressed. On a poor nitrogen source, *MEP2* expression is much higher than *MEP1* and *MEP3* (Marini *et al*., 1997).

b) Amino acid transport

S. cerevisiae encodes several amino acid permeases (Table 2.1). The amino acid permeases are integral membrane proteins with 12 predicted transmembrane domains which are delivered by the secretory pathway to the plasma membrane where they function to take up amino acids for protein synthesis and for use as sources of nitrogen. These permeases can be divided into two classes of mechanism for transporting amino acids across the plasma membrane, which are different in their regulation and function. The nitrogen-regulated permeases include Gap1 (Jauniaux and Grenson, 1990), which transports all naturally occurring amino acids, Put4 which transport only proline (Lasko and Brandiss, 1981) and Agp1 (Abdel-Sater *et al*., 2004). Bap2, Bap3 and Tat2 are also regulated by the presence of amino acids in the growth media. These members, which are called **adaptive or inducible**, are derepressed during growth on poor nitrogen sources and developed under conditions where they may be both necessary and sufficient for cell growth or survival. In addition, *S. cerevisiae* can synthesize a range of at least eleven transport systems each of which is specific for just one or a small number of amino acids. The regulation of these transport systems is such that only some are permanently present. These are called **constitutive** permeases, which are expressed even when cells are grown on a preferred source of nitrogen and are ready to transport amino acids for protein synthesis at any time. Most of the transported amino acids are accumulated inside the yeast cells against a concentration gradient. When amino acids are to be used as a general source of nitrogen, this concentration is crucial because most enzymes which catalyse the first reaction of the catabolic pathways have a low affinity for their substrates (Grenson, 1992).

Table 2.1. Amino acid permeases family (Yeast Transport Protein database: YTPdb) (Andre, 1995)

Gene	Description	Substrates
AGP1 Schreve *et al.* (1998)	Broad-specificity, relatively-low-affinity, amino-acid permease - inducible by most neutral amino acids	L-alanine, L-asparagine, L-cysteine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine
BAP2 Grauslund *et al.* (1995)	Broad-specificity amino-acid permease - inducible by most neutral amino acids	L-cysteine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, L-valine
BAP3 Regenberg *et al.* (1999)	Broad-specificity amino-acid permease - inducible by most neutral amino acids	L-alanine, L-cysteine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-valine
GNP1 Zhu *et al.* (1996)	Broad-specificity amino-acid permease	L-asparagine, L-cysteine, L-glutamine, L-leucine, L-methionine, L-proline, L-serine, L-threonine
TAT1 Schmidt *et al.* (1994)	Broad-specificity amino-acid permease	L-tryptophan, L-tyrosine, L-valine
TAT2 Schmidt *et al.* (1994)	Broad-specificity amino-acid permease - inducible by most neutral amino acids	L-phenylalanine, L-tryptophan, L-tyrosine
GAP1 Jauniaux and Grenson (1990)	General amino acid permease	Canavanine, D-histidine, gamma-aminobutyrate, L-alanine, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-cysteine, L-glutamate, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, putrescine, spermidine, spermine, beta-alanine
HIP1 Tanaka and Fink (1985)	Histidine permease	D-histidine, L-histidine, manganese
MMP1 Rouillon *et al.* (1999)	s-methylmethionine permease	S-methylmethionine
SAM3 Rouillon *et al.* (1999)	S-adenosylmethionine permease	Putrescine, S-adenosylmethionine, spermidine
APL1 Regenberg *et al.* (1999)	Protein of the amino acid permease family associated with low uptake activity for basic amino acids	L-arginine, L-lysine
CAN1 Broach *et al.* (1979)	Arginine permease	Canavanine, L-arginine

LYP1 Sychrova and Chevallier (1993)	Lysine permease	L-lysine
AGP2 Van Roermund *et al.* (1999)	Plasma membrane protein with proposed role in carnitine, polyamine and amino acid transport	Carnitine, L-isoleucine, L-leucine, L-phenylalanine, L-threonine, L-valine, putrescine, spermidine, bleomycin
AGP3 Schreve and Garrett (2004)	Protein of the amino acid permease family with proposed role in low-affinity uptake of branched-chain and aromatic amino acids	L-asparagine, L-isoleucine, L-leucine, L-phenylalanine, L-threonine, L-valine
PUT4 Jauniaux *et al.* (1987)	Proline permease	Gamma-aminobutyrate, L-alanine, L-glycine, L-proline, sarcosine
DIP5 Regenberg *et al.* (1998)	Glutamate and aspartate permease - able to mediate transport of other amino acids	L-alanine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-glycine, L-serine, alpha-aminoadipate
SSY1 Klasson *et al.* (1999)	Sensor of extracellular amino acids - member of the amino acid permease family	L-alanine, L-asparagine, L-citrulline, L-cysteine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-ornithine, L-phenylalanine, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine

2.4.1. The general amino acid permease (Gap1)

The general amino acid permease, with its broad specificity, its large capacity, and its regulation according to nitrogen availability, is well adapted for taking up any available amino acid as a source of nitrogen. These nitrogen compounds are used directly in the biosynthesis of amino acids, cofactors, vitamins, or degraded via the nitrogen catabolic pathways to serve as building blocks in the anabolic system. This general amino acid permease is encoded by the *GAP1* gene and it is 601 amino acids long. Control of Gap1 activity appears to be very complex. One reason that Gap1 activity is so tightly regulated is that the lack of Gap1 activity results in secondary regulatory effects due to inducer exclusion, thus preventing induction of the catabolic pathways needed to utilize poor nitrogen sources (Magasanik, 1992). There are three systems involved in the Gap1 activity: transcriptional repression of permease synthesis, reversible permease inactivation, and feedback inhibition of Gap1 induced by various amino acids. The global nitrogen permease

inactivation/degradation system is mediated by two proteins encoded by *NPI1* and *NPI2* (Nitrogen Permease Inactivator) (Grenson and Acheroy, 1982). Npi1 is shown to be an ubiquitin-protein ligase also taking part in the Gap1 degradation. The permease activity can be reactivated by a protein encoded by *NPR1* (Nitrogen Permease Reactivator) when only poor nitrogen sources are present (Grenson, 1983). The predicted amino acid sequence presents strong similarity to the catalytic domain of protein kinases suggesting that regulation of permease activity involves protein phosphorylation (Vandelbol *et al*., 1990). Inactivation of Gap1 correlates well with Gap1 dephosphorylation (Stanbrough and Magasanik, 1995). Npr1 contains several PEST regions (the amino acids P, E, S, and T). These PEST sequences are known to promote self-degradation of the proteins that contains them. *NPR1* expression is not repressed on ammonium (Vandelbol *et al*., 1987). A specific Gap1 activator, *AUA1* (amino acid uptake activator), has been found to work on poor growth media. The *AUA1* gene is repressed by ammonium and activated on glutamate and proline.

Regulation of *GAP1* expression

The transcriptional activity of *GAP1* is repressed in a medium containing ammonium, asparagine, or glutamine. As poor nitrogen sources induce *GAP1* transcription, it is sensitive to NCR (Janiaux and Grenson, 1990; Stanbrough and Magasanik, 1996). Inspection of the promotor of *GAP1* reveals the presence of five single 5' GATAA 3' sequences. Feedback inhibition of Gap1 function is the way of saving energy and metabolites. In the presence of overwhelming amounts of any amino acid, Gap1 function is repressed because there is no point in transporting amino acids if there is sufficient present inside the cell.

2.5. Nitrogen starvation response

During vinification, nitrogen deficient musts produce stress conditions to yeast cells and their survival depend on their ability to adapt quickly to the changing environment. There is a direct correlation between fermentation efficiency and stress resistance, which refers to the ability of a yeast strain to adapt efficiently to a changing environment and unfavourable growth conditions (Ivorra *et al*., 1999; Gasch, 2002).

The cellular machinery for controlling these stress conditions involves the rapid synthesis of protective molecules and the activation of signal transduction systems that induce the activation of enzyme activities and the transcription of genes encoding factors with protective function.

2.5.1. Role of trehalose

Trehalose is present in the yeast cell as a reserve carbohydrate and as a stress protectant. One of the main functions of trehalose is to protect cells against the denaturation and aggregation of proteins during periods of stress. Thus, trehalose has remarkable properties which may determine the survival response of yeasts under extreme environmental conditions. Yeast cells accumulate trehalose when exposed to adverse conditions, such as nutrient depletion (Van Vaeck *et al*., 2001). Accumulation of trehalose started almost simultaneously with the end of the exponential phase, which suggested a link between the inhibition of growth and the triggering of accumulation (Parrou *et al.,* 1999).

Several studies show the metabolism of trehalose is affected by nitrogen availability. When nitrogen is consumed, yeast cells start to accumulate intracellular trehalose (Parrou, 1999; Novo *et al*., 2005; Hazelwood *et al*., 2009). The triggering of this synthesis happened simultaneously with total ammonium consumption. Parrou *et al*. (1999) have already stated that nitrogen limitation appears to be the primary condition for triggering the activation of trehalose

anabolism. Winemaking fermentations are considered to be limited by nitrogen, with ammonium as the primarily nitrogen source and easily consumed by yeasts. Therefore, ammonium concentration would be a key metabolite for triggering trehalose synthesis in *S. cerevisiae* in wine fermentations, although other sources of nitrogen may still be available.

2.5.2. Arginase activity

Before yeasts start growing in must, the biosynthetic pools of amino acids are filled, so most of the amino acids in the medium are consumed during the initial hours of fermentation. Arginine pool is located in the vacuole as a reserve, and it is used when nitrogen is depleted from the medium. When cells are growing in ammonium as nitrogen source, about 93% of arginine is located in the vacuole. Nevertheless, under nitrogen starvation conditions, high quantities of arginine are released from the vacuole into the cytoplasm, leading to the activation of its degradative enzymes and allowing arginine recycling to sustain biosynthetic processes (Figure 2.8).

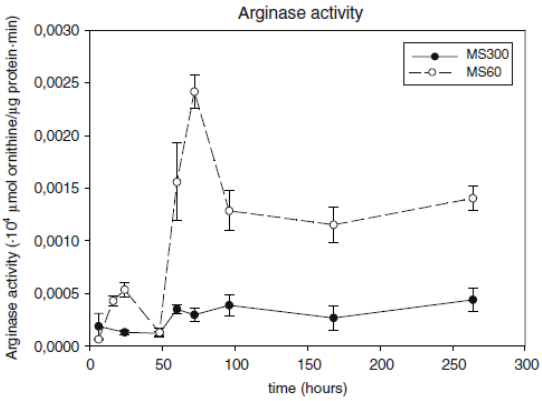

Figure 2.8. Arginase activity profile under vinifications conditions carried out with different nitrogen concentration: 300 mg N/L as control condition, and 60 mg N/L as limited condition (Jiménez-Martí *et al*., 2007)

The first reaction in arginine degradation is the conversion of L-arginine into L-ornitine and urea (Jauniaux *et al*., 1978). This reaction is catalyzed by the cytoplasmatic enzyme arginase. Its activity is regulated by NCR, arginine concentration and post-transcriptional events (Bossinger and Cooper, 1977; Messenguy and Dubois, 1983).

2.5.3. Gene response

There is a continuous adjustment of yeast cells to stressful conditions. Nitrogen concentration has a decisive effect on gene expression during fermentation. Mendes-Ferreira *et al*. (2007) studied the transcriptional profile of *S. cerevisiae* in different nitrogen concentrations. They observed that under low nitrogen conditions, yeast cells were characterized by the induction of genes involved in oxidative glucose metabolism. In contrast, high nitrogen conditions increase the expression of genes involved in biosynthesis (Bisson *et al*., 2001). On the other hand, the yeast cells have the capacity to reprogram the gene expression after nitrogen addition. These reprogramming affected genes involved in glycolysis and energy pathways, which enabled the cells to overcome the nitrogen stress produced and restart the process. For instance, the addition of glutamine to cells growing on a less preferred nitrogen source results in repression of genes involved in nitrogen catabolism as well as induction of ribosomal biogenesis and ribosomal protein genes (Zaman *et al*., 2008).

3. NITROGEN AND WINE FERMENTATION

Many variables are involved to fulfill wine fermentation performance and quality. However, nitrogen availability in grape must is one of the most important factors. Nitrogen plays an important role in the fermentative process since this macronutrient increases biomass production and stimulates the sugar utilization, avoiding stuck and sluggish fermentations (Bisson, 1999). Nitrogen also regulates

the organoleptic quality of the final product, synthetizing different compounds contributed in the flavour of wine. Some problems related to the nitrogen composition of grape must include the formation of reduced-sulfur compounds, in particular hydrogen sulfide (Henschke and Jiranek, 1991), and the potential formation of urea, the major precursor of the carcinogen ethyl carbamate (Ough, 1991).

In summary, the main effects of nitrogen composition during wine fermentation are:

- Effect on biomass production
- Influence in the rate of sugar consumption
- Modification in the metabolic activity of yeast, with different production of secondary metabolites
- Change in the aromatic profile
- Effect in the pattern of the nitrogen consumption of each strain
- Influence on yeast strain behavior in these specific nitrogen conditions

3.1. Nitrogen grape composition

A wide variety of nitrogen-containing compounds are present in grape juice. These depends on the variety of grape and the time of harvest, as well as upon several aspects of vineyard management, including nitrogen fertilization, berry maturation, vine water status, soil type and fungal infection (Henschke and Jiranek, 1992).
Nitrogen composition of grape must is highly variable, not only in concentration, but also in the types of nitrogen compounds present (Henschke and Jiranek, 1993).
Ammonium, nitrates, amines, amino acids, peptides, proteins and vitamins are presented in grape must (Margalit, 1997). Nevertheless, only some of the main nitrogen compounds of grape are important to yeast. The nitrogen fraction that can be assimilated to yeast is called Yeast Assimilable Nitrogen (YAN). *S. cerevisiae*

preferentially uses simple nitrogen sources such as ammonium ions and free alpha amino nitrogen compounds, present in the form of primary amino acids (Cooper, 1982; Monteiro and Bisson 1991b; Henschke and Jiranek 1993; Jiranek *et al*. 1995a). These compounds are rapidly accumulated by yeast in the early stages of fermentation, during which they fill the biosynthetic pools of amino acids needed for protein synthesis and growth, while the surplus is stored in the cell vacuole (Bisson, 1991; Salmon, 1996). Low, but not high, molecular weight peptides can also be used, but grape proteins can not be used as a source of nitrogen since *S. cerevisiae* lacks significant extracellular proteolytic activity.

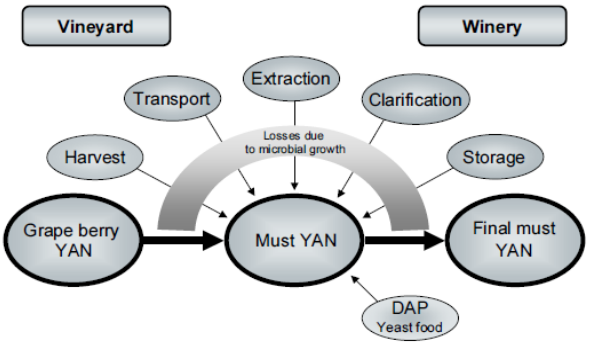

Figure 3.1. The major processes that determine the final must concentration and composition of yeast assimilable nitrogen (YAN) (Bell and Henschke, 2005)

Ammonium is an important component of must YAN and, being one of the most preferred nitrogen sources by yeast, it is readily assimilated (Cooper, 1982; Bisson, 1991; Henschke and Jiranek, 1993; Jiranek *et al*. 1995a). However, this rapid assimilation has been recently questioned by Crépin *et al*. (2012) which ranked ammonium as a late consumed nitrogen source. These discrepancies between studies may be the result of a complex pattern of assimilation, which depend on conditions that differ between studies.

The concentration of ammonium varies widely in grapes and has been reported to range between 5–325 mg N/L (Ough, 1969; Ough and Kriel, 1985; Bely *et al*., 1991; Henschke and Jiranek, 1993; Butzke, 1998).

The primary **amino acids** constitute a major source of YAN for yeast, however, they vary in their efficiency as nitrogen sources (Cooper, 1982; Jiranek *et al*., 1995a,b). Some amino acids are also precursors to some of the volatile compounds formed during fermentation, e.g. fusel or higher alcohols (Henschke and Jiranek, 1993; Rapp and Versini, 1996).

L-arginine and L-proline generally make up the greatest proportion of the total amino acid concentration present in the grape (Kliewer, 1970; Ough and Bell 1980; Bell, 1994; Stines *et al*., 2000). Arginine, the second most abundant amino acid, is a less readily utilized source of nitrogen. This makes it available for uptake during active fermentation as well as during stationary phase (Bisson and Butzke, 2000). Although arginine is often the most available amino acid in grapes, only in three of its four nitrogen atoms are assimilated by *S. cerevisiae* during winemaking. The fourth is incorporated into proline, which can not be used as a nitrogen source in the absence of oxygen (Martin, 2003). This lack of proline assimilation by yeast during fermentation is thought to be due, firstly, to inhibition of the yeast proline uptake system, proline permease, by other amino acids. Secondly, the enzyme required for proline catabolism in yeast, proline oxidase, requires oxygen for catalytic activity.

Table 3.1. Amino acid composition and concentration found in the whole grape and/or juice at harvest. (adapted from Bell and Henschke, 2005)

Amino acid	Concentration range (mg/L)
Alanine	10 – 227
Arginine	20 – 2322
Asparagine	1 – 171
Aspartic acid	10 – 138
Citrulline	0.1 – 83
Cysteine	1 – 8.2
Glutamine	9 – 4499
Glutamic acid	27 – 454
Glycine	1 – 20
Histidine	5 – 197
Isoleucine	1 – 117
Leucine	2 – 160
Lysine	0.7 – 45
Methionine	1 – 33
Ornithine	0.1 – 27.2
Phenylalanine	2.8 – 138
Proline	9 – 2257
Serine	13-330
Threonine	9-284
Tryptophan	0.2-11
Tyrosine	2-33
Valine	7-116

3.2. Influence of nitrogen on fermentation kinetic

Metabolism of nitrogen compounds by *S. cerevisiae* governs the efficiency of alcoholic fermentation. It is well established that, in the absence of other growth

and fermentation limiting factors, YAN concentration in the grape must largely determines yeast cell population or biomass yield, and fermentation rate and duration (Henschke and Jiranek, 1993). Maximum fermentation rates are correlated with high availability of nitrogen in the medium. Consequently, a lack of nitrogen results in slow and stuck fermentations.

Fermentation kinetic is exponentially related to initial YAN, but the quantitative relationship depends on many factors, including yeast demand for nitrogen and nature of nitrogen source. Nitrogen composition affects yeast growth rate with complex mixtures favouring higher rates than single compounds. Thus, mixtures of amino acids give higher rates of growth than the most preferred single nitrogen sources (i.e. ammonium, glutamine and asparagine), which in turn give higher rates than most individual amino acids (Bell and Henschke, 2005). The greater efficiency of amino acid mixtures is linked to the ability of yeast to directly incorporate amino acids into protein, thereby minimizing the need to maintain an energetically expensive amino acid synthesis capability.

In fact, nitrogen impacts yeast cells in two ways, one by increasing the biomass production and the other by stimulating the rate of sugar utilization (Varela *et al*., 2004). Several studies have shown that under nitrogen deficiency conditions, the biomass produced is lower than in optimum nitrogen concentration, there is a slow sugar consumption even leaving residual sugar that can affect the stability of the final wine, and therefore the synthesis of ethanol is decreased (Table 3.2; Figure 3.2). Other significant compounds, like glycerol, and succinic and acetic acids, also modified their synthesis depending on the nitrogen composition of the must (Albers *et al*., 1996).

Table 3.2. Final fermentation values of normal and sluggish fermentations (Varela *et al*., 2004)

	Fermentation time (h)	Cell dry wt (g/L)	Fermentation rate (g/L*day)	Ethanol (%, v/v)	Glycerol (g/L)	Succinic acid (g/L)	Acetic acid (g/L)
Normal	170 ± 12	5.8 ± 0.1	33.5 ± 2.6	12.7 ± 0.9	7.8 ± 0.6	1.8 ± 0.2	1.0 ± 0.1
Sluggish	700 ± 10	1.5 ± 0.1	7.6 ± 0.3	9.5 ± 0.4	7.2 ± 0.3	1.0 ± 0.1	1.0 ± 0.1

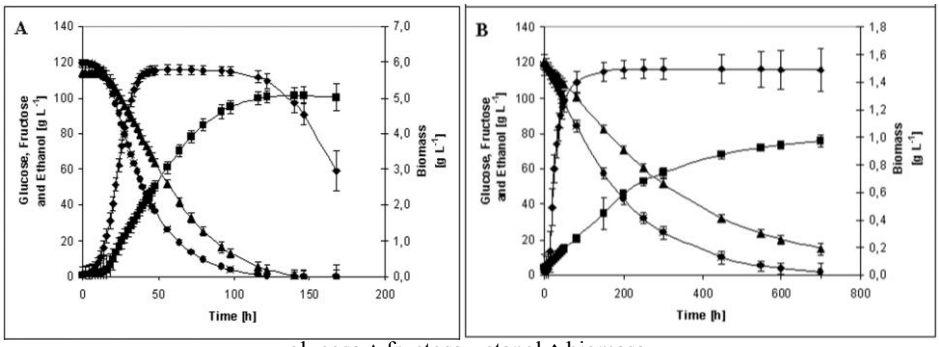

Figure 3.2. Fermentation profile of A) normal (300 mg N/L) and B) sluggish (50 mg N/L) nitrogen conditions (Varela *et al*., 2004)

Several studies have been performed to find the optimal nitrogen concentration in must to guarantee complete fermentation (Agenbach, 1977; Bely *et al*., 1991; Mendes-Ferreira *et al*., 2004). The absolute minimum amount of nitrogen required for alcoholic fermentation is very difficult to determine. In fact, ranges from 120 to 267 mg YAN/L (Mendes-Ferreira *et al*., 2004) have been recommended, although this concentration is affected by numerous factors (yeast strain, nitrogen quality, temperature, oxygen) (Valero *et al*., 2003).

It is clear, that nitrogen concentration and quality improve the alcoholic fermentation. However, which factors are mostly responsible of this improvement?

1- Fermentative capacity of individual cell: glycolytic pathway of each cell is enhanced for the high amount of nitrogen involved in the synthesis of enzymes and transporters.
2- Size of cell population: the availability of nitrogen produces a higher production of biomass. This increase in number of cells that can participate in the fermentation, increase the fermentation kinetic.

Varela *et al*. (2004) tried to distinguish both effects by performing fermentations with different nitrogen concentrations and with different initial biomass concentration. They conclude that the increase in viable cell concentrations positively is correlated with the increase in fermentations rates, and fermentations ended more quickly, even when cells were grown under conditions of severe nitrogen shortage.

3.2.1. Effect on fermentation rate

Fermentation rate, measured as either sugar catabolic rate or CO_2 evolution rate, is related with yeast biomass, but becomes uncoupled from growth during the latter stages of fermentation after growth has ceased. It is exponentially related to initial nitrogen concentration, and a direct relationship exists between fermentation rate and the amount of nitrogen utilized by yeast (Agenbach, 1977).
Under limited nitrogen conditions, the fermentation rate of the cells decreases and incomplete fermentation can be produced (Figure 3.3). Incomplete fermentation occurs in part because nitrogen depletion irreversibly arrests hexose transport and affects the level of glycolytic enzymes.

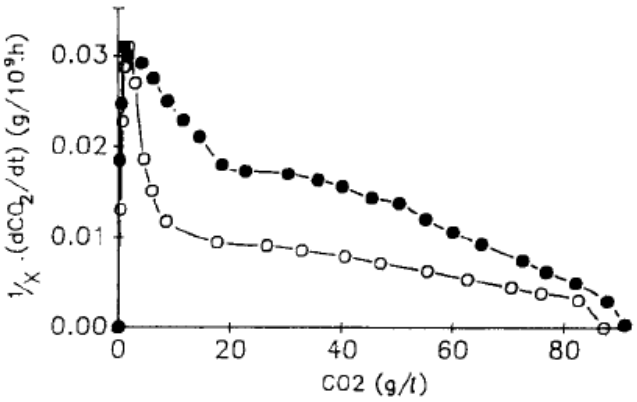

Figure 3.3. Evolution of the specific CO_2 production rate during the fermentation. Initial nitrogen concentration in the must: ● 327 mg N/L, ○ 94 mg N/L (Bely *et al*., 1990)

The rate of hexose transportation is determined by both the activity and the number of hexose transporters in the plasma membrane. Upon nitrogen starvation or the arrest of protein biosynthesis, several sugar permeases are specifically inactivated (Busturia and Lagunas, 1986; Salmon *et al*., 1993; Luyten *et al*., 2002). This inactivation is thought to be due to the stimulation of general protein turnover upon nitrogen starvation and results from degradation of the proteins in the vacuole after endocytosis-mediated internalization. In addition, the activity of the individual transporters declines as a result of noncompetitive inhibition by ethanol produced during alcoholic fermentation (Salmon *et al*., 1993).

3.2.2. Effect on cell growth

Nitrogen availability is directly related to biomass production during the yeast exponential growth phase at early stages of alcoholic fermentation. It increases with the amount of nitrogen available in the must, but it is also dependent on the nature of the nitrogen source (Godard *et al*., 2007).

Taking into account that the amount of biomass governs the fermentation rates in musts, some possible solutions have been described to deal with nitrogen-deficient musts (Varela *et al*., 2004). One of these alternatives is involved in early nitrogen supplementation of the must. Knowledge of the optimum concentration of nitrogen required to achieve the maximum cell growth is very important to winemakers. Consequently, it is highly recommended that the wine producers understand the nitrogen demands of each strain and routinely evaluate the quantity and quality of assimilable nitrogen in the must. Moreover, these additions can modify nitrogen uptake, metabolism response and final aroma of wine (Beltran *et al*., 2005). Varela *et al*. (2004) alternatively propose the addition of viable biomass from other fermentation tanks in nitrogen-limited must to increase the biomass size during wine fermentation.

3.3. Nitrogen supplementation

Nitrogen content of grape must has been identified as being suboptimal in many viticultural regions surveyed world-wide (Ough and Amerine, 1988b; Gockowiak and Henschke, 1992; Henschke and Jiranek, 1993; Butzke, 1998). Grape juices with nitrogen levels below 140 mg N/L, mostly considered the threshold concentration, have a high probability of becoming problematic in fermentation. There are two basic strategies to circumvent problems linked to nitrogen deficiency: (i) prevention by optimizing vineyard fertility, and more commonly, (ii) supplementation with ammonium salts, such as diammonium phosphate (DAP). More recently, organic nitrogen sources, such as commercial preparations that contain inactivated yeast or yeast products, which also contain lipids and sometimes other nutrients, have become commercially available (Munoz and Ingledew, 1990; Belviso *et al*., 2004).

Supplementation of musts with ammonium salts, decrease the risk of slow or stuck fermentations and may also decrease the formation of undesirable volatile sulfur compounds (Marks *et al*., 2003). Moreover, it can regulate the formation of yeast

volatile and non-volatile metabolites, which contribute to wine flavor (Beltran *et al*., 2005).

The timing of the nitrogen additions influenced the biomass yield, the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites. During the early stages of fermentation, the main sources of assimilable nitrogen present in the must are rapidly accumulated by yeast. Thus, fulfilling the biosynthetic requirements for amino acids needed for protein synthesis and growth, and any surplus is then stored in the cell vacuole. Nitrogen additions during the period of cell growth have resulted in maximum cell populations, and ensure that fermentation proceeds without excessive release of H_2S (Mendes-Ferreira *et al*., 2009). Latter additions during the stationary phase have had no effect on the cell population but have increased the specific fermentation rate, thus reducing the length of fermentation (Manginot *et al*., 1997; 1998).

Furthermore, nitrogen supplementation affects the pattern of nitrogen uptake. The addition of ammonium salts, a preferred nitrogen source, represses the uptake and degradation of other nitrogen sources (Marks *et al*., 2003). This situation determines which nitrogen sources are selected to assimilate, and is modified the synthesis of aroma compounds (Beltran *et al*., 2005). Several studies have demonstrated that nitrogen addition at the one-third to mid-point stage of fermentation is generally most effective to resolve aberrant fermentation kinetics for musts initially low in YAN (Bely *et al*., 1990; Manginot *et al*., 1998; Hernández-Orte *et al*., 2006).

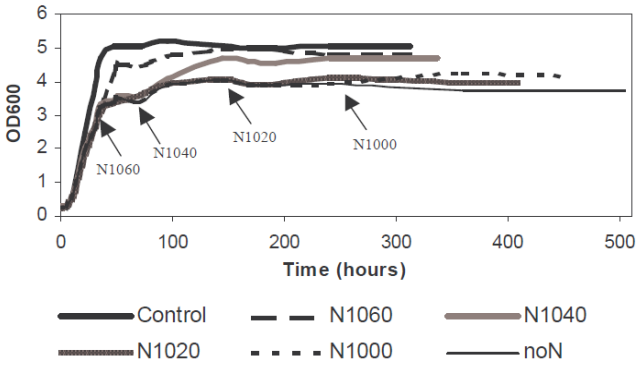

Figure 3.4. Effect of the timing of nitrogen additions on fermentation kinetic (Beltran *et al*., 2005). Initial nitrogen concentration: Control (300 mg N/L), N (60 mg N/L). Nitrogen was added at different points when density of the must was: 1060 g/L, 1040 g/L, 1020 g/L, and 1000 g/L. NoN was not supplemented

These additions are generally made empirically in wine cellars. Excessive addition of inorganic nitrogen often results in excessive levels of residual nitrogen, leading to microbial instability and ethyl carbamate accumulation in wine (Ough, 1991). This implies that knowledge of the nitrogen content of grape juice and the requirement for nitrogen by yeast are important considerations for optimal fermentation performance and the production of wines that comply with the demands of regulatory authorities and consumers. Over the past two decades, the questions of how much nitrogen and when to add it have been addressed.
Moreover, current climate change situation leads to grapes presenting higher sugar levels and increased grape maturity than those desired, producing a change in the nitrogen availability in the medium and yeast nitrogen requirements.

3.3.1. Residual nitrogen and microbial instability

Grapes with a high nitrogen content or excessive addition of DAP during fermentation can lead to significant residual nitrogen which, under some

winemaking circumstances, could encourage microbial instability and lead to loss of wine quality. Residual nitrogen is also thought to be an important contributor to problems arising from the presence of *Brettanomyces* in wine. Other yeasts and lactic acid bacteria can grow when the medium contains essential nutrients (residual nitrogen).

3.3.2. Urea and ethyl carbamate

Ethyl carbamate has been identified as a mild human carcinogen and is thus an undesirable component of wine. Generally, only low concentrations (1.2–4.3 μg/L) of ethyl carbamate have been found in table wine produced from grapes (Ough, 1991).

The main precursors of ethyl carbamate in wine are urea and ethanol, although citrulline can also participate (Ough *et al.*, 1988a). The production of urea during fermentations is a direct consequence of the metabolism of arginine. Arginine is degraded into ornithine and urea by arginase, encoded by the *CAR1* gene. Since urea becomes toxic to yeast cells at higher concentrations, yeast cells excrete urea into the wine where it spontaneously reacts with ethanol. It depends on the different factors, like temperature and strain used. When better nitrogen sources are depleted, urea can be transported back into the cell and degraded to ammonium by urea amydolase (encoded by the *DUR1,2* gene), which can be used for the synthesis of new nitrogenous compounds.

Addition of DAP to grape must results in the repression of genes that encode enzymes which metabolize poorer nitrogen sources, like urea (Marks *et al.*, 2003). The repression of these genes produces a secretion of urea into wine, which may lead to the production of high levels of ethyl carbamate in wine.

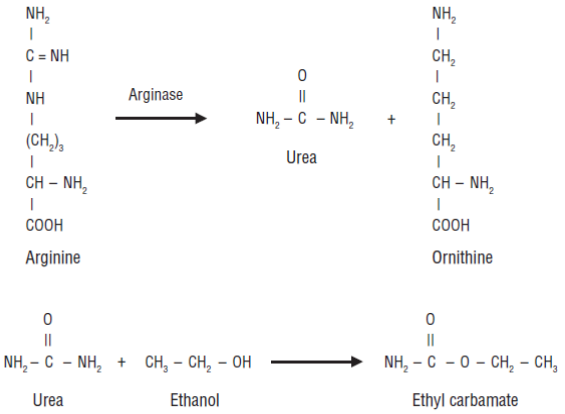

Figure 3.5. Formation of urea from arginine, and its chemical reaction with ethanol to form ethyl carbamate (Bell and Henschke, 2005)

3.4. Influence of nitrogen on yeast metabolism

The aroma profile of wines is dominated by those components that are formed and retained mostly during fermentation. During fermentation, the glycolytic and associated pathways form volatile and non-volatile metabolites that contribute to wine flavor. The yeast strain, fermentation conditions and nutrient content of the must modulate the production of these compounds, and consequently, a wine's flavor profile.

Nitrogen content affects biochemical activities of yeast and, as a result, the production of ethanol, secondary metabolites such as glycerol, acetic acid, succinic acid, and aromatic compounds such as fusel alcohols, acetate and ethyl esters and S-flavor compounds (Figure 3.6).

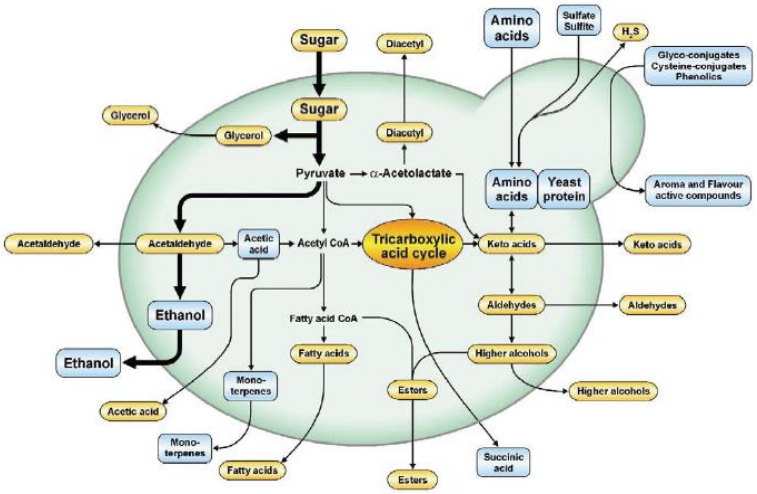

Figure 3.6. A schematic representation of the formation of different compounds by yeast during alcoholic fermentation (Swiegers and Pretorius, 2005a)

3.4.1. Effect on major wine compounds synthesis

The non-volatile compounds, glycerol and the carboxylic acids malic acid, α-ketoglutaric acid and succinic acid, have all been reported to vary according to nitrogen source and concentration (Radler, 1993; Albers *et al*., 1996; Camarasa *et al*., 2003).

3.4.1.1. Ethanol

Ethanol concentration affects the sensory perception of wine flavour-active compounds (Guth and Sies, 2002) but is also an issue of growing importance to the consumer (Swiegers *et al*., 2005b).

It is well established that the choice of nitrogen source can significantly modulate ethanol yield by altering metabolite formation, especially glycerol and organic acids (Albers *et al*., 1996). Growth of yeast on amino acids, as the main nitrogen

source, limits the need for amino acid biosynthesis, and hence little associated NADH is generated. This limits the need for NADH reoxidation, which is coupled to glycerol production. On the other hand, growth on ammonium salts requires de novo amino acid synthesis, which results in significant glycerol production in order to maintain redox balance. The increase in glycerol results in a lower ethanol production. However this decrease is not generally considerable in the total ethanol produced.

3.4.1.2. Glycerol

Glycerol production provides a major route for the reoxidation of NADH generated from cellular anabolic reactions under anaerobic growth conditions. It is also a compatible intracellular solute generated to offset osmotic stress. Glycerol is derived from the glycolytic intermediate, dihydroxyacetonephosphate, by reduction to glycerol-3-phosphate followed by dephosphorylation to glycerol.

Glycerol production is modulated by fermentation conditions, especially those that affect growth or physiological stress. Nutrient availability appears to affect glycerol mainly through redox maintenance mechanisms. Unlike aerobic growth which use O_2 as the terminal electron acceptor, anaerobic growth depends on glycerol production to restore NAD^+:NADH balance (Verduyn *et al*., 1990). Nitrogen availability generally stimulates biomass formation and hence NADH production, although the effects are strain dependent (Albers *et al*., 1998; Vilanova *et al*., 2007). When yeast cells are growing on amino acids little surplus NADH is generated from protein synthesis and growth, resulting in minimal loss of ethanol formation due to minimized glycerol formation. However, growth on ammonium, requires the cell to synthetize all of the amino acids, which result in increased NADH formation and, thus in higher glycerol synthesis.

3.4.1.3. Organic acids

Acidity and pH constitute fundamental importance to the sensory perception of wine, essentially defining its structure and balance. Excessive acidity increases the perception of sourness whereas low acidity decreases flavour harmony. The perception of sourness and astringency of organic acids depends on concentration, pH and anion species (Sowalsky and Noble, 1998). Wine acidity is largely determined by the major grape acids L-tartaric and L-malic, with smaller contributions from citric acid. Furthermore, wine also contains various non-volatile acids (succinic, pyruvic and α-ketoglutaric acids).

Some strains produce **malic acid**. L-malate is modulated by environmental conditions, like low nitrogen availability that favored its production (Radler, 1993).

A variety of fermentation conditions affect **succinic acid** accumulation, including assimilable nitrogen. Abnormal amounts of succinic acid result from high levels of γ -amino butyric acid, which can form in grape must under certain conditions (Bach *et al*., 2004).

Keto acids can form from sugar catabolism and from their respective amino acids, by the Ehrlich pathway. Its formation is further modulated by assimilable nitrogen composition of must.

However the organic acid with a great impact on aroma is **acetic acid**. There is clear evidence in the literature of an inverse correlation between nitrogen availability and acetic acid formation at low or moderate nitrogen levels and a direct relationship at higher levels (Bely *et al*., 2003; Hernández-Orte *et al*., 2006; Barbosa *et al*., 2009). These latter authors (Barbosa *et al*., 2009) explained the decrease in acetic acid because the availability and addition of nitrogen stimulates yeast growth and this growth increases the production of NADH, reducing the need for the cell to generate NADH through other redox reactions, such as in the oxidative formation of acetic acid from acetaldehyde. Another explanation for this reduction could be that high demands of acetyl-CoA for lipids synthesis under such

stimulated yeast growth conditions could account for less acetic acid formation (Ugliano *et al*., 2008).

3.4.2. Effect on aroma compounds

Aromatic compounds synthesized by yeasts during alcoholic fermentation constituted the secondary aroma. The most important compounds within fermentative aroma are higher alcohols, acetate esters and ethyl esters (Mountounet, 1969; Rapp and Mandery, 1986). However, other low sensory impact compounds such as aldehydes (acetaldehyde), ketones and organic acids (acetone and acetic acid), volatile phenols and sulfurous compounds (hydrogen sulfide) are also important (Lambrechts and Pretorius, 2000). Many of the yeast volatile compounds that contribute to wine aroma are affected by type and/or concentration of nitrogen (Bell and Henschke 2005).

3.4.2.1. Higher alcohols

Higher alcohols, sometimes referred to as fusel alcohols, are, from a quantitative point of view, the most important group of volatile compounds produced by yeast during alcoholic fermentation (Table 3.3). They are characterised by containing more than two carbon atoms and include the branched-chain alcohols isobutanol, hexanol, amyl alcohol, and isoamyl alcohol, and the aromatic alcohols 2-phenylethanol and tyrosol. According to Rapp and Versini (1996) concentrations of higher alcohols below 300 mg/L add desirable complexity to wine, whereas higher concentrations can be detrimental to wine quality. Conversely, 2-phenylethanol is generally a positive contributor to wine aroma, being characterized by a pleasant rose-like aroma (Swiegers *et al*., 2005b). The concentrations of each higher alcohol that act positively or negatively on wine aroma depend on aroma intensity and style of wine. In spite of having aroma themselves, the main oenological importance of

higher alcohols lies in the fact that they are precursors of acetate esters (Soles *et al*., 1982).

Table 3.3. Some higher alcohols produced by yeast in wine (Lambrechts and Pretorius, 2000)

Compound	Amino acid	Concentration (mg/L)	Threshold value (mg/L)	Odour
Propanol	Threonine/GABA	9-68;125	500[†];800[‡]	Stupefying
Butanol	-	0.5-8.5		Fusel odour
Isobutyl alcohol	Valine	9-28(100);140	500[†];75[*];200[‡]	Alcoholic
Active amyl alcohol	Isoleucine	15-150	65[‡]	Marzipan
Isoamyl alcohol	Leucine	45-490	300[†];7.0[*];70[‡]	Marzipan
Hexanol	-	0.3-12	5.2[*];4[‡]	
Tyrosol	Tyrosine			Bees wax, honey-like
Tryptophol	Tryptophan			
Phenilethyl alcohol	Phenylalanine	10-180	7.5[*];125[‡]	Floral, rose

[*]Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w)

[†]In a wine solution; [‡]In beer

Higher alcohols are synthesized by the Ehrlich pathway from branched-chain amino acids, leucine, valine and isoleucine, aromatic amino acids, phenylalanine, tyrosine and trytophan, and the sulfur-containing amino acid methionine. In this metabolic pathway the amino acids are transaminated to the corresponding α-ketoacid, followed by decarboxylation to aldehydes. Finally, these aldehydes are reduced to higher alcohols, whereas NADH is oxidized NAD^+. These chemical reactions are carried out by amino acid permeases, transaminases, decarboxylases

and dehydrogenases (Figure 3.7). Higher alcohols can be also produced *de novo* through carbohydrate metabolism (Äyräpää, 1971).

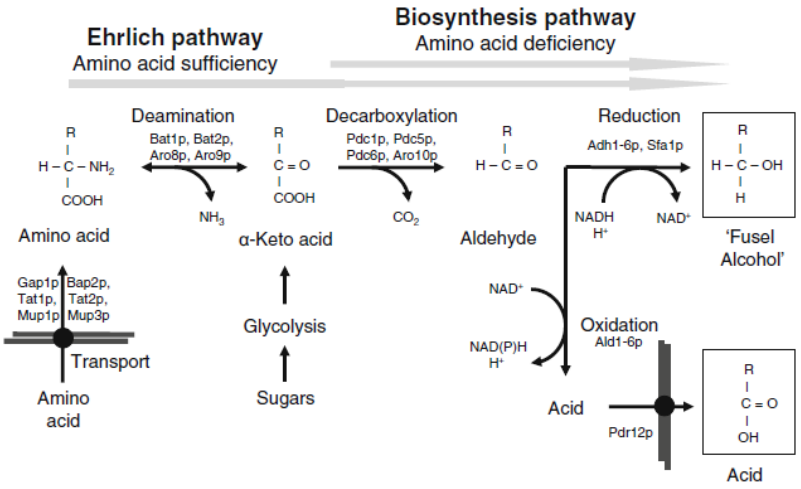

Figure 3.7. Formation of higher alcohols from sugars and amino acids by the Ehrlich pathway (Ugliano and Henschke, 2009)

The assimilable nitrogen composition of grape juice has a strong influence on the production of higher alcohols during fermentation. Low initial nitrogen concentrations results in greater concentrations of higher alcohols (Vilanova *et al*., 2007; Carrau *et al*., 2008). According to Oshita *et al*. (1995), under these conditions, surplus keto acids can not be converted into amino acids, due to the lack of available nitrogen, and are therefore excreted as higher alcohols. Conversely, at high initial juice nitrogen concentrations, increased availability of nitrogen causes a reduction in higher alcohol production because most of the keto acids produced are directly converted into the corresponding amino acids. Many other factors affect the levels of higher alcohols in wine, such as yeast strain.

3.4.2.2. Acetate esters

Acetate esters such as ethyl acetate (at low concentrations), isoamyl acetate, isobutyl acetate, 2-phenylethyl acetate, hexyl acetate and benzyl acetate give desirable fruity and floral aromas to the wines (Lambrechts and Pretorius, 2000; Swiegers e*t al*., 2005b).

Acetate esters are formed through the condensation between higher alcohols and acetyl CoA, catalyzed in the cell by alcohol acetyltransferase (Figure 3.8). The final concentration of these compounds is the result of the balance between alcohol acetyltransferase promoting their synthesis, and esterase enzymes promoting their hydrolysis.

Ethyl acetate is the most common ester in wine, being the product of condensation of acetic acid and ethanol generated during fermentation. The aroma of ethyl acetate is most vivid in younger wines and contributes towards the general perception of "fruitiness", although excessive amounts of this acetate ester, from 150 to 200 mg/L, are considered a wine fault (Amerine and Gruess, 1960; Corison *et al*., 1979).

The nitrogen composition of must can strongly affect the accumulation of volatile esters during fermentation. Total and individual esters tend to increase with increasing must amino nitrogen, although the responses of the various esters to individual amino acids is not yet clear (Hernández-Orte *et al*., 2002). Addition of ammonium salts, a common practice in the wine industry, strongly stimulates the production of esters.

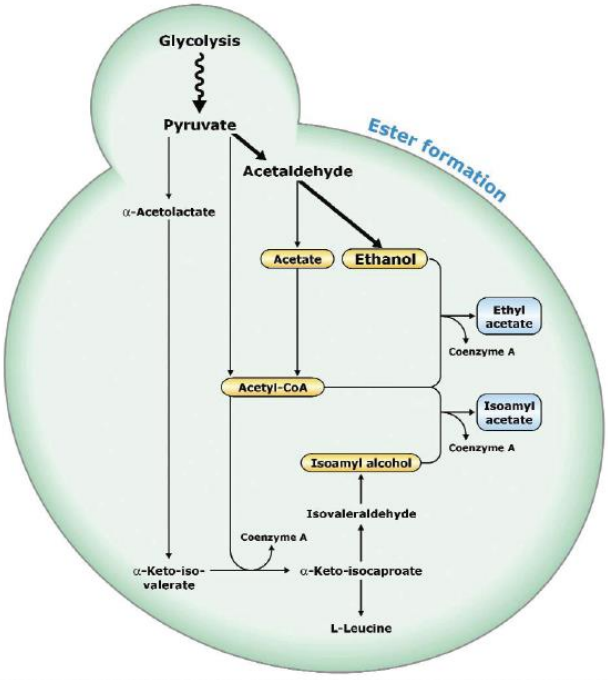

Figure 3.8. Schematic representation of the formation of ethyl acetate and isoamyl acetate in wine yeast (Swiegers and Pretorius, 2005a)

3.4.2.3. Ethyl esters

Ethyl esters such as ethyl propanoate, ethyl butanoate, ethyl hexanoate (ethyl caprylate), ethyl octanoate (ethyl caproate), ethyl decanoate (ethyl caprate) and ethyl lactate give desirable fruity and flowery aroma to the wine (Table 3.4). They are produced by condensation between ethanol and acyl-CoA, reaction mediated by acyltransferases (Figure 3.9).

Nitrogen concentration also affects the formation of ethyl esters, as the same way that in acetate esters.

Table 3.4. Some esters produced by yeast (Boulton *et al*., 1995)

Compound	Concentration (mg/L)	Threshold value (mg/L)	Odour
Ethyl acetate		17.62*;12.3	Varnish, nail polish, fruity
Isoamyl acetate	0.03-8.1	0.26*	Banana, pear
2-Phenilethyl acetate	0.01-4.5		Rose, honey, fruity, flowery
Ethyl isovalerate	ND-0.7		Apple, fruity
Isobutyl acetate	0.01-0.8		Banana
Ethyl butanoate	0.01-1.8	0.4	Floral, fruity
Ethyl 2-methyl-butanoate	ND-0.9		Strawberry, pineapple
Hexyl acetate			
Ethyl hexanoate	Trace-3.4	0.08	Apple, banana, violets
Ethyl octanoate	0.05-3.8	0.58;0.258*	Pineapple, pear
Ethyl decanoate	Trace-2.1	0.5	Floral

*Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w)

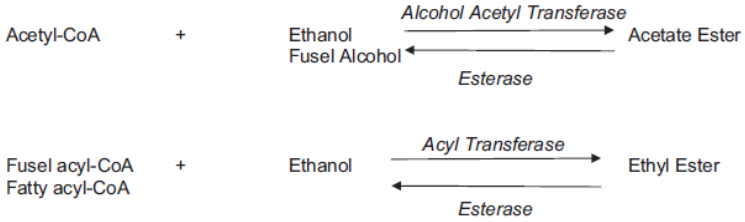

Figure 3.9. Synthesis of esters in *Saccharomyces cerevisae*. Two classes of esters are formed by *Saccharomyces*: those that contain acetate as the acyl compound and those that contain ethanol as the alcohol (Bisson and Karpel, 2010)

3.4.2.4. Volatile fatty acids

Volatile fatty acids have an important impact on wine quality, of which acetic acid accounts for more than 90% of the total wine volatile acidity. Acetic acid is formed as a metabolic intermediate in the synthesis of acetyl-CoA from pyruvic acid.
Inverse relationship exists between acetic acid concentration in wine and initial nitrogen concentration, except at high nitrogen concentrations exceeding 450 mg N/L, when high concentrations of volatile acidity are formed (Bely *et al*., 2003; Torrea and Henschke, 2004).

3.4.2.5. Hydrogen sulfide (H_2S)

Hydrogen sulfide is a highly volatile compound with a very low odour threshold and an objectionable 'rotten-egg' odour. It is well established that H_2S is formed metabolically by yeast from either inorganic sulfur compounds, sulfate and sulfite, or organic sulfur compounds, cysteine and glutathione (Henschke and Jiranek, 1993; Rauhut, 1993).
Sulfate is usually present in excess amounts (up to 700 mg/L). It is accumulated by specific transporters, and reduced to H_2S by the sulfate reductive assimilation pathway. The last step of this pathway reduces sulfite to H_2S, catalyzed by sulfite reductase, which is encoded by the *MET5* and *MET10* genes (Figure 3.10).
Sulfite is often added as an antioxidant and antimicrobial compound. When it is present in the must, enters the cell by diffusion across the plasma membrane and can be directly reduced to sulfide. Sulfite is a favored source of sulfur in many yeast and can lead to abundant H_2S production because its uptake is essentially unregulated.
H_2S is usually formed in response to a metabolic requirements, such as that induced by growth, for organic sulfur compounds. Some strains of yeast, however, appear to form unregulated amounts of H_2S and presumably represent metabolic defects.

Grape must is typically deficient in organic sulfur compounds (less than 10 mg/L). Deficiency of organic sulfur compounds in the must signals yeast to synthetize organic sulfur compounds from inorganic sources, which are typically plentiful in grape must. H_2S is, therefore, a metabolic intermediate in the reduction of sulfate or sulfite needed for the synthesis of organic sulfur compounds. When these reactions proceed in the presence of a suitable nitrogen supply, H_2S is sequestered by *O*-acetyl homoserine, which is derived from nitrogen metabolism, forming the sulfur amino acids methionine and cysteine. However, under some conditions, when insufficient or unsuitable nitrogen sources are available, free H_2S can accumulate in the cell and diffuse into the fermenting must (Henschke and Jiranek, 1991).

H_2S that is formed during the early to middle stages of grape must fermentation is associated with yeast growth and typically responds to nutrient addition, especially DAP (Marks *et al*., 2003).

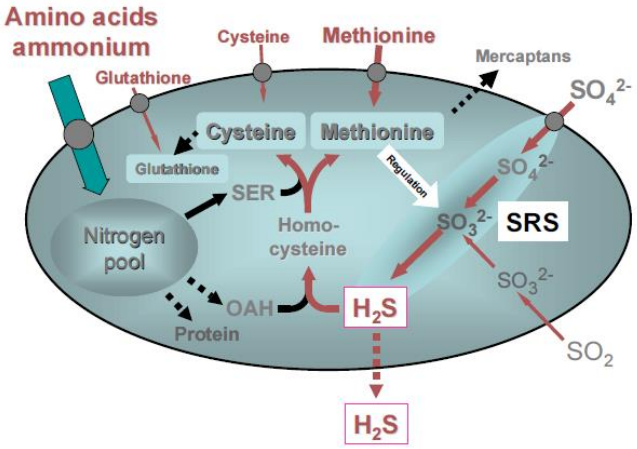

Figure 3.10. Sulfur metabolism in wine yeast (Bell and Henschke, 2005)

3.5. Factors affecting nitrogen assimilation

Many factors affect the assimilation of nitrogen during the fermentation. These include process conditions, medium composition and yeast strain used.

The presence and relative abundance of **good nitrogen sources** as ammonium affect the order of amino acid consumption, repressing the utilization of the poorer nitrogen sources by NCR (Henschke and Jiranek, 1992; Beltran *et al*., 2004).

Another aspect to be considered is the **must sugar concentration** that are also related with nitrogen requirements of yeast (Martínez-Moreno *et al*., 2012). During the last decades, increase of sugar content has been observed because of global warming. The higher the sugar concentration, the higher nitrogen concentration is needed to complete the alcoholic fermentation.

Industrial **wine yeast strains** show a variation in the demand of assimilable nitrogen. Strains not only vary in the proportions of different amino acids utilized, but the total amount of nitrogen used also change. Strains show different efficiencies of fermentation when nitrogen becomes limiting. These differences are strain specific and mostly appear during the stationary phase (Jiranek *et al*., 1991; Manginot *et al*., 1998). This variability in nitrogen requirements of strains is relevant and confirms the importance to select nitrogen-effective strains for the best control of fermentation process.

Fermentation **temperature** also affects the nitrogen transport and uptake. The membrane permeases are temperature-dependent, producing conformational changes in these transporters. Low temperatures decrease the membrane fluidity, affect the activity of membrane-associated enzymes, and is produced a major reduction in membrane transport. Moreover, low temperatures result in a lower rate of amino acid assimilation, consistent with lower rates of fermentation and yeast growth. Both nitrogen and temperature have an influence on the fermentation kinetics. Under high nitrogen content in the medium and higher temperature, higher fermentation rates are obtained.

Oxygen has the most important effect on nitrogen assimilation. Yeasts have long been known to use considerably more nitrogen in the presence of oxygen or aerated fermentations (Ribéreau-Gayon *et al*., 1975).

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JUSTIFICATION & OBJECTIVES

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen. Nitrogen additions improve fermentation activity and increase the production of biomass. Likewise, nitrogen availability can also affect many aspects of yeast metabolism, including the formation of compounds that are important for the organoleptic quality of wine. However, this strategy is not always successful, because excessive levels of these compounds may have negative consequences, such as microbial contamination or ethyl carbamate formation. Moreover, not only is nitrogen concentration important but also the nitrogen source used for supplementing grape musts. Nitrogen quality present in the must affects uptake profile and determines the final composition of wine. On the other hand, the nitrogen requirements and preferences are shown to be strain dependent. Each yeast strain responds differently to nitrogen conditions present in the environment.

For this reason, this doctoral thesis aims to determine the specific nitrogen demands and analyze nitrogen metabolism of four commercial yeast strains, widely used in the Spanish wine industry, in order to achieve optimal fermentation performance and so wine production meets the demands set by regulatory authorities and consumers. This research is focused on the oenological industry and specifically in nitrogen metabolism during winemaking fermentations. This project was supported by AGROVIN Company and by the "Centro para el Desarrollo Tecnológico Industrial (CDTI; Ministerio de Industria)" into the context of the Project CENIT DEMETER and the Spanish Government (project AGL2010-22001-C02-01, awarded to José M. Guillamón). The project CENIT DEMETER has dealt with the effects of climate change on viticulture and oenology. This project has been a collaborated industrial project in which have participated 25 companies of the wine industry and 17 public researching centers. The consortium was led by the winery Miguel Torres S.A. Our group in the "Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) has led the study of

nitrogen metabolism during alcoholic wine fermentation and the adaptation to the new context of climate change. Most of the objectives of this project overlap with the objectives of this thesis work. To get this main goal, we established the following objectives:

1. Analysis of the growth behavior in different nitrogen conditions of the four commercial wine strains

We analyzed yeast growth in a synthetic grape must with different nitrogen concentrations and nitrogen sources. We tested the concentration effect to determine the different nitrogen requirements of the particular wine strains and ensure the maximum population during exponential growth phase, in order to avoid excessive supplementation carried out by winemakers, which can lead to microbial instability and production of off-flavors of the final wines. Although, ammonium salts are the main source used in supplementation, we also tested glutamine and arginine which are two of the major amino acids of the assimilable organic nitrogen. We also analyzed the growth in different nitrogen sources, such as amino acids, ammonium, urea and purine derivatives, to detect those which have some problems or advantages to be metabolized in the industrial strains.

The results are reported in Chapter 1 and Chapter 2.

2. Detection of the genetic bases for wine yeast variations in nitrogen source utilization

The knowledge of the genetic bases (mutations, allele polymorphism, etc.) underlying phenotypic differences in nitrogen utilization, is very important to understand the adaptation of yeast to nitrogen environment and facilitate strain and process improvement. Thus, we aimed to identify genes responsible for phenotypic

variation through Reciprocal Hemizygosity Analysis (RHA), based on the construction of two strains which differ genetically only in one allele of a specific gene. The comparison of both phenotypes allows identify defects from wine strains that could be genetically improved in the future, allowing better fermentation performance.

The results are presented in Chapter 2.

3. Development a biomarker for detecting nitrogen limitation during wine fermentation

We aimed to test the effectiveness of different biochemical and molecular markers to find a biosensor that can be used to detect nitrogen limitation conditions under fermentation process. We analyzed the accumulation of intracellular trehalose, the arginase activity and the expression of several genes related to nitrogen metabolism (transport, stress targets, catabolism and anabolism). The identification of two promoters specific of nitrogen starvation were used to construct a reporter-strain with green fluorescent protein (GFP).

The results are presented in Chapter 3.

4. Impact of the different nitrogen sources on the nitrogen catabolite repression (NCR): Correlation with growth and nitrogen consumption

Nitrogen catabolite repression (NCR) is the mechanism responsible to regulate nitrogen assimilation during the wine fermentation. We wanted to analyze the NCR response at each individual nitrogen source (ammonium and amino acids), as indicator of the availability and nature of nitrogen during wine fermentations. The expression of the general amino acid permease (*GAP1*), which is subjected to

NCR, was used as biomarker to indicate the response to different nitrogen sources in the four commercial wine strains. We also aimed to correlate the repression effect of an individual nitrogen source with the capacity to support rapid growth and assimilation and suitable metabolites synthesis.

The results are shown in Chapter 4.

5. Study of nitrogen requirements during stationary phase to obtain the maximum fermentation rate and suitable aroma production

We analyzed the influence of nitrogen addition during stationary phase, when cell growth is not affected but nitrogen availability can improve the fermentation rate by promoting optimal protein turnover in the cell. We tested two nitrogen sources: ammonium, the main inorganic source used in the industry, and arginine, the major assimilable amino acid of grape must. The relationship between nitrogen source and concentration with volatile and non-volatile compounds produced were studied during these fermentations, to judge the final quality of the product.

The results are shown in Chapter 5 and Chapter 6.

6. Determination of nitrogen requirements in grape must with high sugar concentration

As consequence of climate change and overriping of grapes, the grape must present greater sugar concentrations which jeopardize final metabolization by yeasts. Because nitrogen requirements are also related to the sugar concentration in the must, we determined how nitrogen needs in the stationary phase changed in grape must with higher sugar content. We also carried out different additions in form of

organic and inorganic nitrogen, and tested the effect in the fermentation progress, aroma and other metabolites production.

The results are reported in Chapter 5.

CHAPTER 1

Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must

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Abstract

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). However, it is important to know the specific nitrogen requirement of each strain, to avoid excessive addition that can lead to microbial instability and ethyl carbamate accumulation. In this study, we aimed to determine the effect of increasing nitrogen concentrations of three different nitrogen sources on growth and fermentation performance in four industrial wine yeast strains. This task was carried out using statistical modeling techniques. The strains PDM and RVA showed higher growth rate and maximum population size and consumed nitrogen much more quickly than strains ARM and TTA. Likewise, the strains PDM and RVA were also the greatest nitrogen demanders. Thus, we can conclude that these differences in nitrogen demand positively correlated with higher growth rate and higher nitrogen uptake rate. The most direct effect of employing an adequate nitrogen concentration is the increase in biomass, which involves a higher fermentation rate. However, the impact of nitrogen on fermentation rate is not exclusively due to the increase in biomass because the strain TTA, which showed the worst growth behavior, had the best fermentation activity. Some strains may adapt a strategy whereby fewer cells with higher metabolic activity are produced. Regarding the nitrogen source used, all the strains showed the better and worse fermentation performance with arginine and ammonium, respectively.

***Keywords**:* yeast assimilable nitrogen, *Saccharomyces*, cell growth, fermentation rate, alcoholic fermentation, microbial modeling

1. Introduction

The use of active dry wine yeasts (ADWY) is a widespread practice in wine technology. These ADWY are supplied to oenologists in dehydrated form and must be rehydrated prior to inoculation in grape must. Subsequently, wine yeasts go through the typical growth phases of a microbial culture: lag, exponential and stationary phases. Lag phase represents the adaptation of the ADWY to the new medium, involving transcriptional (Novo *et al*., 2007), proteomic (Salvadó *et al*., 2008) and metabolic changes (Bauer and Pretorius, 2000). Successful adaptation leads to the growth or exponential phase in which the culture increases from 10^6 CFU/mL (inoculated population) to approximately 10^8 CFU/mL. Although, in principle, grape must contains all the nutrients needed for yeast to develop, yeast cell growth under enological conditions is often considered to be nitrogen limited, and nitrogen deficiency is a major cause of stuck or sluggish fermentations (Bisson, 1999; Bell and Henschke, 2005). Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). Nitrogen addition can affect fermentation rate either by enhancing fermentation rate per cell (increase fermentation activity) or by enhancing the number of cells per population. Varela *et al*. (2004) tried to distinguish both effects by performing fermentations with different nitrogen concentrations and with different initial biomass concentrations. They concluded that the increase in viable cell concentrations positively correlated with the increase in fermentation rates, and fermentations ended more quickly, even when cells were grown under conditions of severe nitrogen shortage. From an industrial perspective, they proposed two alternatives to deal with nitrogen-deficient musts. The first alternative involves providing the inoculated yeast with the required nitrogen, allowing it to reach maximum population size and ensuring a normal fermentation profile. The second alternative involves adding viable biomass from other fermentation tanks or by inoculating a higher population size.

Biomass transfer from fermentation tanks is technologically difficult and might involve contamination problems. Moreover, it has been proven that continuous anaerobic growth of the same culture during many generations decreases fermentation fitness of cells as a consequence of inefficient synthesis of unsaturated fatty acids and ergosterol (Rosenfeld *et al*., 2003) and cell-wall properties (Powell *et al*., 2003). Another way to increase initial biomass is by inoculating a higher population of ADWY in the grape must. This practice would increase winery costs for commercial yeasts and decrease the number of yeast generations during the alcoholic fermentation. The limitation of growth generations could decrease secondary metabolite synthesis, thereby affecting wine aroma and flavor profiles (Carrau *et al*., 2010). Thus, the other alternative to ensure a regular fermentation profile is by providing the inoculated strain with a suitable nitrogen concentration.

The assimilable nitrogen in grape juice is constituted by ammonium and amino acids in similar proportions (Henschke and Jiranek, 1993). However, most nitrogen supplementation is carried out with ammonium salts (sulfate or phosphate). The exclusive addition of ammonium imbalances the natural ratio of inorganic/organic nitrogen composition and affects the amino acid uptake pattern (Beltran *et al*., 2005). Ammonium is a preferred yeast nitrogen source, and when plentiful, it represses the expression of catabolic pathways which use other nitrogenous compounds. This mechanism, called nitrogen catabolite repression (NCR), has recently been studied during wine fermentations (Beltran *et al*., 2004; 2005). Ammonium addition inhibits arginine and alanine uptake and stimulates the consumption of glutamine, branched-chain and aromatic amino acids. Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds (particularly hydrogen sulfide) and the amount of urea, which is the major precursor of the carcinogen ethyl carbamate (Adams and Van Vuuren, 2010; Mendes Ferreira *et al*., 2009; Torrea *et al*., 2003; 2011; Vilanova and Martínez, 2007). In many cases, winemakers proactively add nitrogen to the must, even

without knowing its initial nitrogen status, which may cause high nitrogen levels that exceed the minimum necessary to complete fermentation. Moreover, minimal nitrogen amount and preferences are shown to be strain dependent (Manginot *et al*., 1998; Taillandier *et al*., 2007). Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, leading to microbial instability and ethyl carbamate accumulation in wine (Ough, 1991). Therefore, it is important to know the nitrogen content of grape juice and the nitrogen requirement for each specific yeast strain, in order to achieve optimal fermentation performance and so wine production meets the demands set by regulatory authorities and consumers.

In this context, this study aims to determine the specific nitrogen demands of four commercial yeast strains, widely used in the Spanish wine industry. Our objective was to determine the minimum nitrogen amount required by each commercial strain to ensure the maximum population during growth or exponential phase in a synthetic grape must. However, as explained above, not only is nitrogen concentration important but also the nitrogen source used for supplementing grape musts. To this end, we have analyzed yeast growth in a synthetic grape must with different nitrogen concentrations and nitrogen sources. As sole nitrogen sources, we have used ammonium, arginine and glutamine. These amino acids are two of the major ones of the assimilable organic nitrogen (proline is also very abundant but it is not metabolized during wine fermentations) (Henschke and Jiranek, 1993), however, their utilization by the cell is very different. Glutamine, such as ammonium, is considered a preferred nitrogen source and is firstly consumed by *Saccharomyces cerevisiae*. Conversely, arginine is strongly subjected to NCR and is hardly consumed when there is an excess of the "so-called" good nitrogen sources (Beltran *et al*., 2004; 2005). We used a microplate-based method that allowed real-time and high-throughput monitoring of the wine yeasts growth curves. These data were then modeled to quantitatively describe the behavior of the different wine yeasts as a function of nitrogen concentration and source. In the

second part of this study, we analyzed the effect of nitrogen source and concentration on fermentation performance. The results obtained in this work are important for winemakers because nitrogen can be added taking into account the different nitrogen needs of the particular wine strain used and thus avoid excessive preventive supplementation, which can lead to microbial instability of the final wines and production of off-flavors or unhealthy compounds.

2. Materials and methods

2.1. Yeast strains and inocula preparation

The four commercial strains used in this study were provided by Agrovin Company (Ciudad Real, Spain). Three of these strains belong to species *Saccharomyces cerevisiae var. cerevisiae* (PDM, RVA and TTA), while another strain is commercialized as *Saccharomyces cerevisiae var. uvarum* (ARM).

Inocula for both growth curves and fermentation experiments were prepared by rehydrating the dry yeasts in water following the manufacturer's recommendations (30 min at 37 ºC). Subsequent to microscope counting, the appropriate dilution of the rehydrated wine yeast was inoculated in a synthetic grape must (henceforth SM) to obtain an initial cell concentration of ~2 x 10^6 cells/mL. This SM was prepared according to Riou *et al*. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L. The following mineral salts were used: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L. The following vitamins were used: myo-inositol 20 mg/L, calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L.

Nitrogen content and nitrogen source were modified for the different fermentations as described below. The final pH of the SM was adjusted to 3.3 with NaOH.

2.2. Microvinification trials

To test the effect of nitrogen concentration on fermentation performance, SM was modified with a mixture of ammonium and amino acids at: 60 mg N/L (18 mg N/L as ammonium and 42 mg N/L in amino acid form), 140 mg N/L (42 mg N/L as ammonium and 98 mg N/L in amino acid form), 200 mg N/L (60 mg N/L as ammonium and 140 mg N/L in amino acid form), and 300 mg N/L (90 mg N/L as ammonium and 210 mg N/L in amino acid form). The proportion of each amino acid was administered as previously proposed by Riou *et al*. (1997). After this nitrogen administration, the pH of the SM was adjusted to 3.3 and filtered to sterility. To test the effect of nitrogen source on fermentation rate, ammonium, glutamine and arginine were used as sole nitrogen sources at a final concentration of 140 mg N/L. This concentration was chosen as it is the minimal amount of nitrogen to avoid stuck fermentations according to previous reports (Bely *et al*., 1990). Nitrogen concentrations were calculated considering all nitrogen atoms assimilable by yeast in fermentative conditions: one in ammonium, two in glutamine, and three in arginine.

Fermentations were performed in 250 mL glass bottles containing 200 mL of SM and capped with closures than enabled the carbon dioxide to escape and the samples to be removed. Thus, fermentations were performed in semi anaerobic conditions, since limited aeration was necessary in order to harvest samples for subsequent analysis. Fermentations were done in triplicate at 28 ºC with continuous orbital shaking (150 rpm). They were monitored by the medium density using a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Yeast cell growth was also determined by absorbance at 600 nm. Fermentation was considered to be complete when density was below 998 g/L. The supernatant of these samples was

stored at -20 ºC for HPLC analysis of the content of sugars, glycerol, ethanol and acetic acid (see below).

2.3. Modeling trials

Yeast growth curves were carried out in a microtiter plate reader model POLARstar Optima (BGM Labtech, Offenburg, Germany). Growth was monitored by optical density (OD) changes at a wavelength of 595 nm. Measurements were every 30 min for 45 h at 28 ºC (until yeast cells reached the stationary phase), after a pre-shaking of 20 s. The growth medium was SM modified with different nitrogen concentrations (arginine, glutamine and ammonium), ranging from 5 up to 200 mg of N/L. Regarding this nitrogen concentration, it was also taken into account that glutamine and arginine contain two and three assimilable molecules of N, respectively (arginine contains four molecules of N, but only three are used by yeast cells in fermentation conditions; Martin *et al*., 2003). A total of 432 growth curves (3 nitrogen sources x 12 nitrogen concentrations x 4 yeast strains x 3 replicates) were obtained and analyzed.

Biological growth parameters were deduced from each growth curve by directly fitting OD measurements *versus* time to the reparameterized Gompertz equation proposed by Zwietering *et al*. (1990), which has the following expression:

$$y = D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_\infty/OD_0)$ is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h). OD/time data were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function $(\text{observed-predicted})^2$. This primary modeling was accomplished

using the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option.

The area obtained under the OD *versus* time curves was also used in this work as a valuable procedure to estimate the effects of nitrogen source and content on overall yeast growth because its relationship with the biological growth parameters (Arroyo-López *et al*., 2009). This parameter was obtained by integration using the OriginPro 7.5 software (OriginLab Corporation, Northampton, USA).

For secondary modeling, the Monod's growth model was used. This model was initially proposed as an empirical approach to describe microbial growth as a function of the concentration of a limiting nutrient with the following equation:

$$y = (V*S)/(K+S)$$

where *y* is the microbial growth parameter deduced from primary modeling (in our case μ_{max} or the area under OD/time curve) for the diverse nitrogen concentrations (S), V is the theoretical maximum value obtained for the growth parameters, and K is the substrate concentration which supports half-V. An important feature of this model is that the growth parameter is zero when there is no substrate but it tends to an upper limit when the substrate is greatly in excess. The link between these two extreme conditions is described by a hyperbola (Lobry *et al*., 1992). As in the previous case, the fit was carried out by a non-linear regression procedure using Statistica 7.0 software.

2.4. Fitness advantage

The concept of "fitness advantage" (*m*) between two microorganisms can be defined by the following expression: $m = r1 - r2$, where *m* corresponds to the μ_{max} difference of two strains (r1 and r2) for specific environmental conditions (Goddard, 2008; Salvado *et al*., 2011). In this study, we have obtained the *m* value

of the PDM strain (used as reference) compared with the other three commercial yeasts as a function of the nitrogen source and concentration. In this way, we aim to theoretically determine the strain better adapted to nitrogen variations.

2.5. HPLC analysis

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed in all the samples at the end of the fermentation process. Analytical HPLC was carried out on a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and UV-Visible detector. Prior to injection, samples were centrifuged at 13.300 rpm for 5 min, supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Spain) and diluted 5 or 10-folds. A total volume of 25 μl was injected into a HyperREZ^{TM}XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 ml/min and a column temperature of 50 ºC. The concentration of each metabolite was calculated using external standards. Each sample was analyzed in duplicate.

2.6. Analysis of Variance

An analysis of variance was performed by means of the factorial ANOVA module of Statistica software version 7.0, using "yeast strain" and "nitrogen source" as categorical predictor variables. Dependent variables introduced for the analysis were the Monod's parameters obtained for μ_{max} ($V_{\mu max}$ and $K_{\mu max}$) and the area under OD/time (V_{area} and K_{area}). Briefly, ANOVA tests differences in means among-groups variables by analyzing their variances. Thus, when statistical significance is obtained ($p \leq 0.05$), we can reject the null hypothesis that no differences between means exist, and accept the alternative hypothesis that the means differ from each other. With this criterion in mind, we can use a *post-hoc*

comparison test to check for significant differences among treatments and to form homogenous groups. This task was carried out by means of the Scheffé test, which is considered one of the most conservative *post-hoc* tests (Winer, 1962). An alternative advantage of the Scheffé test is that it can also be used with unequal sample sizes.

3. Results

3.1. Influence of nitrogen on yeast growth

We have studied and compared the effect of three nitrogen sources at different concentrations on the growth of four commercial wine yeast strains (PDM, ARM, RVA and TTA). Different concentrations, which ranged from 5 up to 200 mg of N/L, of ammonium, glutamine and arginine were tested as sole nitrogen sources in SM. For this purpose, yeasts were monitored by means of OD measurements, and their respective biological growth parameters (μ_{max} and area under OD/time curve) estimated for each nitrogen condition. Then, the Monod's model was used as a secondary empirical model to explain the effect of nitrogen concentration on yeast growth (Figures 1 and 2). Tables 1 and 2 show the parameters obtained for this fit, while Figure 3 graphically shows the ANOVA with the significant differences among yeasts for these parameters. As expected, increased nitrogen levels augmented growth rate and the area under the curve of the different strains up to a certain concentration for which the maximum value was reached and kept constant (Figures 1 and 2).

K of these two parameters indicates the nitrogen concentration for which half of the maximum value was reached (see Tables 1 and 2). The K of μ_{max} was much lower than the K of area. Thus, when maximum growth rate is reached, much more nitrogen is still required to attain the maximum area value. Regardless of nitrogen source, the different strains showed different μ_{max}. These strains could be ordered

from lowest to highest μ_{max} as TTA < ARM < RVA < PDM (Table 1 and Figure 3). The TTA and ARM did not show significant differences in the μ_{max} for the three nitrogen sources. The μ_{max} of the RVA significantly increased when grown in glutamine. The PDM showed the highest values of μ_{max} in all nitrogen sources assayed. Comparing growth of this strain in the different sources, ammonium yielded the highest μ_{max} with 0.175 h^{-1}. This growth rate practically represented twice that of TTA μ_{max} in the same medium.

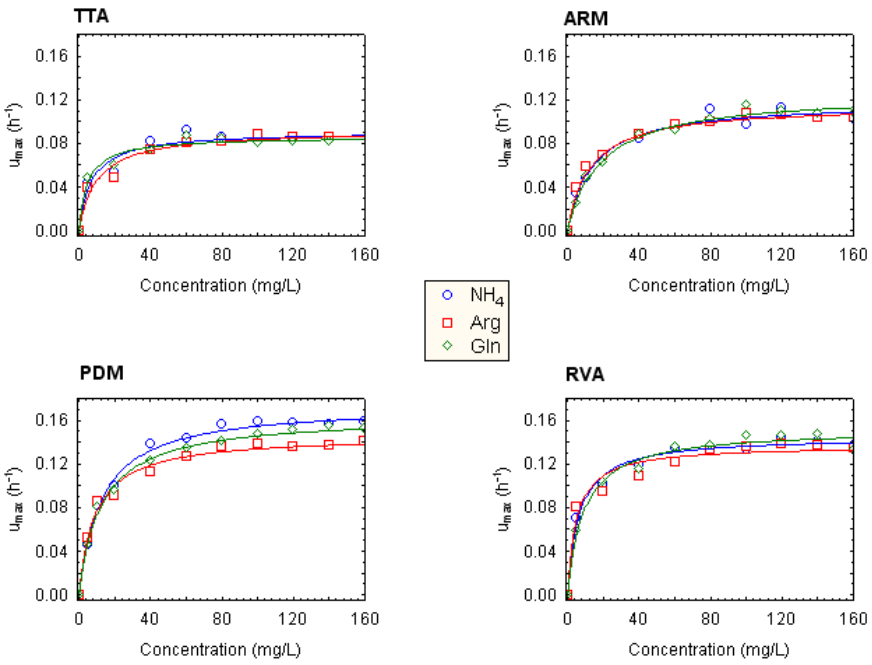


Figure 1. Effect of nitrogen source and concentration on the specific growth rate (μ_{max}) of the assayed commercial wine yeast strains

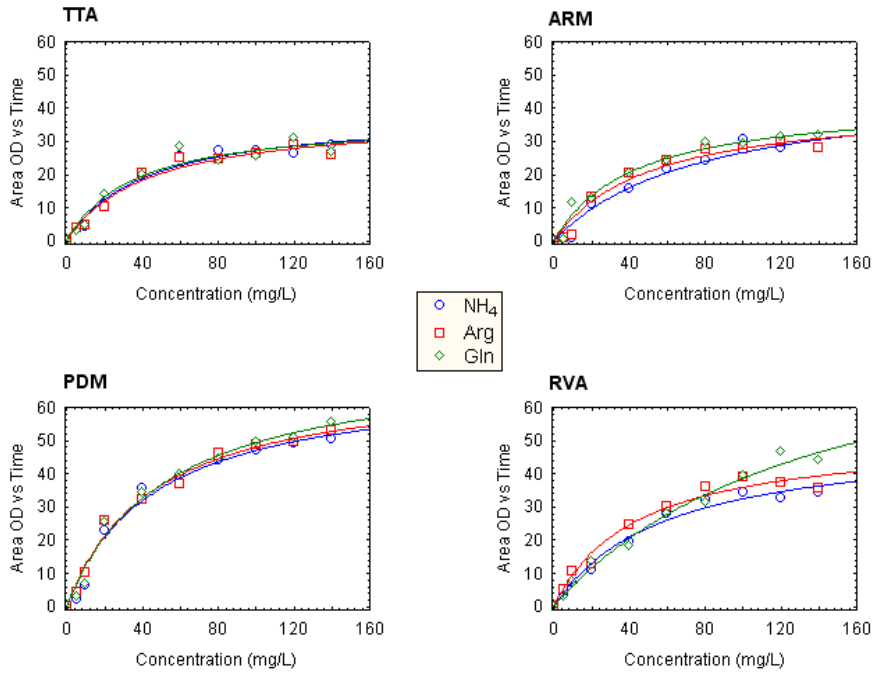


Figure 2. Effect of nitrogen source and concentration on the area under OD *vs* time curve of the assayed commercial wine yeast strains

A similar trend was observed when the area under the growth curve was analyzed (see Table 2 and Figure 3). This parameter represents the overall maximum growth obtained in a specific condition and includes all the lag, log and stationary phases. Comparing all the strains, PDM also showed the highest area values growing in ammonium and arginine while RVA obtained the highest value in glutamine. These area values did not significantly change for the different nitrogen sources with the exception of the above mentioned growth of the RVA in glutamine.

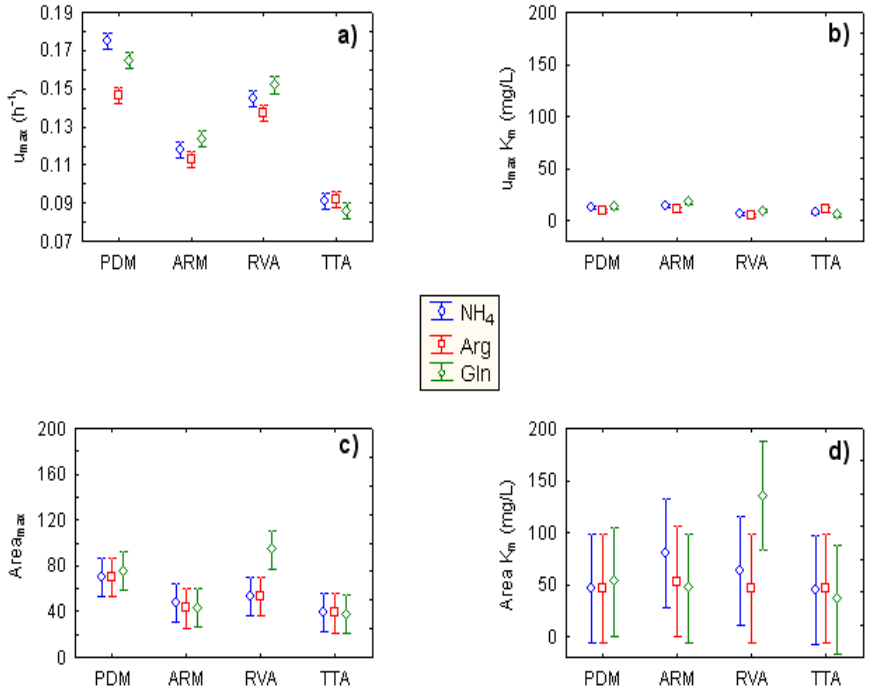


Figure 3. ANOVA for the parameters obtained by means of the Monod's model. a) maximum value obtained for μ_{max} , b) nitrogen concentration where the half of the maximum value for μmax is reached, c) maximum value obtained for the area under OD *vs* time curve, d) nitrogen concentration where the half of the maximum value for area is reached

Table 1. Parameters of the Monod's model, V (maximum value obtained) and K (concentration where the half of the maximum value is reached) for the fit of μ_{max} as a function of different nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments

	Monod's parameter V (h^{-1})			Monod's parameter K (mg/L)		
Yeast	**NH_4**	**Arg**	**Gln**	**NH_4**	**Arg**	**Gln**
TTA	0.091 (0.006) [a]	0.091 (0.001) [a]	0.086 (0.002) [a]	7.27 (0.42) [a,b]	10.25 (0.92) [a,b,c]	5.20 (2.25) [a]
ARM	0.117 (0.002) [b]	0.112 (0.004) [b]	0.123 (0.002) [b,e]	13.58 (1.81) [c,d]	10.18 (1.29) [a,b,c]	17.24 (1.36) [d]
PDM	0.175 (0.007) [g]	0.146 (0.002) [c,d]	0.164 (0.003) [f,g]	12.86 (2.44) [b,c,d]	9.12 (0.10) [a,b,c]	12.67 (1.05) [b,c,d]
RVA	0.144 (0.002) [c,d]	0.137 (0.001) [c,e]	0.152 (0.002) [d,f]	6.56 (1.37) [a]	5.09 (0.61) [a]	9.18 (1.53) [a,b,c]

Note: Values followed by different superscript letters, within the same Monod's parameter, are significantly different according to a Scheffe's post-hoc comparison Test

Table 2. Parameters of the Monod's model, V (maximum value obtained) and K (concentration where the half of the maximum value is reached) for the fit of the area under the OD *vs* time curve as a function of different nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments

	Monod's parameter V			Monod's parameter K (mg/L)		
Yeast	**NH_4**	**Arg**	**Gln**	**NH_4**	**Arg**	**Gln**
TTA	38.98 (2.91) [a]	38.51 (3.23) [a]	37.51 (2.05) [a]	44.10 (11.60) [a]	45.77 (13.77) [a]	35.78 (5.98) [a]
ARM	47.88 (3.58) [a]	42.75 (4.90) [a]	43.46 (10.15) [a]	80.39 (10.25) [a]	53.13 (14.61) [a]	46.68 (30.98) [a]
PDM	69.85 (5.60) [a,b]	70.20 (1.32) [a,b]	75.40 (7.11) [a,b]	46.43 (1.86) [a]	46.21 (5.71) [a]	52.66 (13.07) [a]
RVA	52.96 (9.72) [a]	52.86 (4.11) [a]	93.82 (25.23) [b]	63.28 (26.11) [a]	45.87 (25.02) [a]	135.12 (41.68) [a]

Note: Values followed by different superscript letters, within the same Monod's parameter, are significantly different according to a Scheffe's post-hoc comparison Test

Growth rate values were used to calculate the fitness advantage of PDM strain compared to the other strains (Figure 4). The *m* value was always positive for pairs PDM vs. ARM and PDM vs. TTA. This result indicates that PDM had better fitness than ARM and TTA for all the nitrogen sources and concentrations assayed. However, the *m* value was close to zero, or even negative for low nitrogen concentrations (below 40 mg of N/L), for the pair PDM vs. RVA. Both strains presented similar fitness for arginine and glutamine and only PDM was more competitive at high ammonium concentrations.

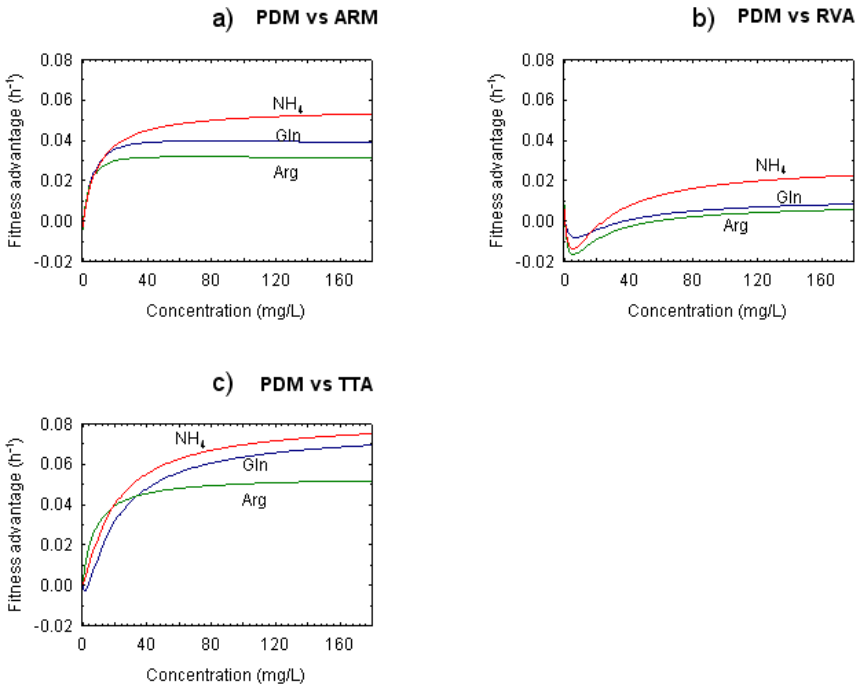


Figure 4. Model predictions for the fitness advantage (m, h^{-1}) of the PDM strain *vs* a) ARM, b) RVA and c) TTA yeast strains as a function of the nitrogen concentration

3.2. Influence of nitrogen on the fermentation performance

In this section, our aim was to test the effect of nitrogen source and nitrogen concentration on fermentation activity. The three nitrogen sources (ammonium, arginine and glutamine) were used at the same concentration (140 mg N/L) in SM. To test the effect of nitrogen concentration, we used a mixture of ammonium and amino acids at 60, 140, 200 and 300 mg N/L. Fermentation performance was monitored by density reduction of the grape must and, in each case, the area under must density *versus* time curve. Conversely to the area under the growth curve, higher values of the area mean slower fermentation activity. These values of area for the effect of nitrogen source and the effect of nitrogen concentration are graphically represented in Figures 5 and 6, respectively. Table 3 shows the results of the ANOVA carried out for this parameter. The ARM showed the highest area values, that is, the slowest fermentation activity regardless of nitrogen source (Figure 3). The strains RVA and PDM showed similar fermentative behavior and TTA presented the quickest fermentation activity for all three nitrogen sources tested. Curiously, the TTA strain exhibited the best fermentation activity and the worst growth rate in all three nitrogen sources. Depending on the nitrogen source, the different strains showed slight, but significant, differences in fermentation activity (Table 3). SM with arginine was fermented more quickly by all four strains whereas the use of ammonium as sole nitrogen source yielded the slowest fermentation activities.

Regarding the effect of concentration on the fermentation performance of the strains under study, increasing nitrogen concentration from 60 up to 140 mg N/L significantly increased the fermentation activity of all the strains (Figure 5). However, no significant differences were observed for fermentation activity when the subsequent nitrogen concentration increased (200 and 300 mg N/L; Table 3). In these fermentations, using a mixture of ammonium and amino acids decreased the differences in fermentation activity among strains. In fact, in this case, no

significant differences were detected in fermentation activity among strains for the same nitrogen concentration.

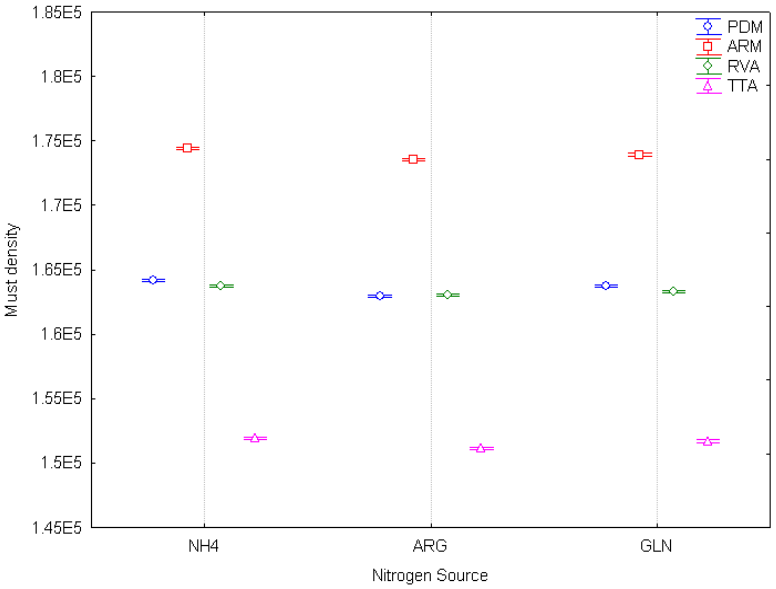


Figure 5. Effect of the nitrogen source (at 140 mg/L) on the area under must density reduction *vs* time curve for the assayed commercial wine yeast strains

For a better understanding, fermentation activity was also expressed as the time required to reduce 5 (T5), 50 (T50) and 100% (T100) of must density (1080 g/L) to the density of the wine (998 g/L) (Table 4). The T100 of the fermentations with 60 mg N/L was not calculated because none of the strains could finish these fermentations. The T100 values confirmed that fermentations with arginine and ammonium were the quickest and slowest ones respectively. However, conversely to the area curve values, the increase in nitrogen concentration yielded significant decreases in fermentation time for all the strains.

The kinetic fermentation values were also confirmed by analyzing residual sugars, glycerol, ethanol and acetic acid of the final wines (see Table 5 in supplementary

material). The fermentations with arginine and ammonium had the lowest and highest sugar concentration in the final wines, respectively (with the exception of RVA with glutamine). Regarding nitrogen concentration, the higher the nitrogen concentration was the lower the residual sugars in the wine.

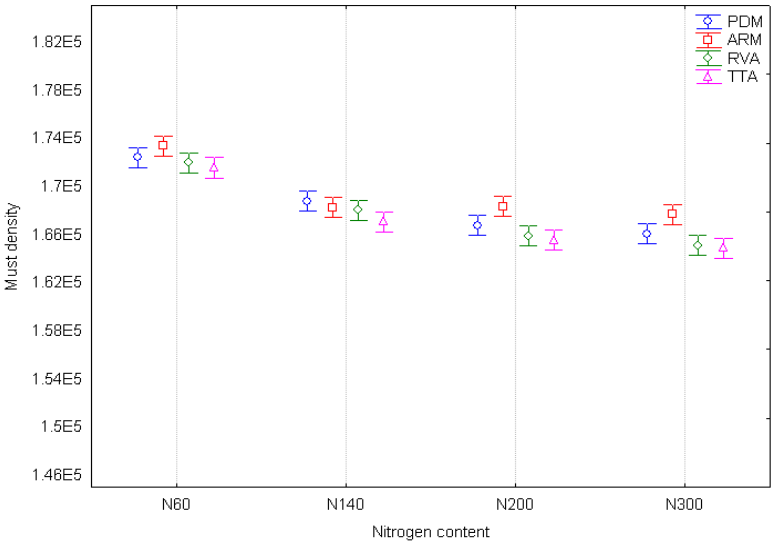


Figure 6. Effect of nitrogen content on the area under must density reduction *vs* time curve for the assayed commercial wine yeast strains

Table 3. ANOVA analysis for the area under the must density *vs* time curve as a function of different concentrations and nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments

	Area Density (x 10^5) vs. Time			Area Density (x 10^5) vs. Time			
Yeast	**NH_4**	**Arg**	**Gln**	**N60**	**N140**	**N200**	**N300**
TTA	1.519 (0.000) [a]	1.511 (0.001) [e]	1.517 (0.001) [a]	1.715 (0.000) [e,f]	1.670(0.001)[a,b,c,d]	1.655 (0.001) [a,b,c]	1.649 (0.000) [a]
ARM	1.744 (0.000) [i]	1.735 (0.001) [g]	1.739 (0.001) [h]	1.734 (0.001) [f]	1.682 (0.028)[b,c,d]	1.683 (0.001) [c,d]	1.676 (0.335) [a,b,c,d]
PDM	1.642 (0.000) [f]	1.630 (0.001) [b]	1.637 (0.001) [d]	1.724 (0.000) [f]	1.687 (0.001) [d,e]	1.667(0.001)[a,b,c,d]	1.660 (0.001) [a,b,c,d]
RVA	1.637 (0.002) [d]	1.630 (0.001)[b, c]	1.633 (0.001) [c]	1.719 (0.001) [f]	1.680(0.001)[a,b,c,d]	1.658(0.001)[a,b,c,d]	1.651 (0.001) [a,b]

Note: Values followed by different superscript letters are significantly different according to a Scheffe's post-hoc comparison Test

Table 4. Time, expressed in hours, required for the assayed yeast strains to reduce 5% (T5), 50% (T50) and 100% (T100) of must density (1080 g/L) to the density of the wine (998 g/L). These values are the mean ± SD of three independent experiments

Yeast strain			T5	T50	T100
PDM	Nitrogen source	NH_4	17.45±0.90	61.72±0.78	199.61±0.39
		Arg	17.97±0.78	49.22±0.78[a]	149.22±0.00[a]
		Gln	16.15±0.45	55.99±0.45[a]	167.97±1.10[a]
	Nitrogen concentration	60	16.6±1.00	151.04±3.11[b]	-
		140	16.60±0.00	66.73±0.56	197.76±0.69
		200	18.51±0.41[b]	47.58±0.41[b]	159.26±8.45[b]
		300	19.22±0.41[b]	41.25±0.41[b]	115.67±2.49[b]
ARM	Nitrogen source	NH_4	21.09±1.17[c]	75.39±0.68[c]	277.34±2.43[c]
		Arg	20.31±0.68[c]	62.11±0.00[a,c]	217.97±13.26[a,c]
		Gln	19.53±0.68[c]	67.22±1.38[a,c]	246.09±6.52[a,c]
	Nitrogen concentration	60	29.30±1.00[b,c]	248.5±0.71[b,c]	-
		140	26.69±0.56[c]	80.39±1.14[c]	248.05±2.76
		200	28.36±1.07[c]	63.52±1.07[b,c]	173.85±8.420[b]
		300	29.53±0.70[b,c]	58.83±0.81[b,c]	140.63±5.07[b]
RVA	Nitrogen source	NH_4	15.27±1.15	54.40±1.94[c]	203.56±0.51[c]
		Arg	15.47±0.70[c]	47.11±0.70[a,c]	171.92±6.51[a,c]
		Gln	13.02±0.90[c]	45.97±1.58[a,c]	190.50±2.12[a,c]
	Nitrogen concentration	60	10.17±0.01[b,c]	132.05±1.06[b,c]	-
		140	9.77±0.00[c]	53.71±0.00[c]	220.00±0.00
		200	10.02±0.63[c]	37.55±1.14[b,c]	111.01±5.71[b,c]
		300	11.20±0.23[b,c]	31.51±0.23[b,c]	82.55±5.21[b,c]
TTA	Nitrogen source	NH_4	12.92±2.19[c]	47.09±0.95[c]	153.17±3.88[c]
		Arg	13.54±1.44[c]	40.63±0.63[a,c]	117.71±0.36[a,c]
		Gln	11.67±0.72[c]	44.59±0.36[a,c]	134.37±10.60[a,c]
	Nitrogen concentration	60	11.46±0.91[c]	118.75±1.56[b,c]	-
		140	10.68±0.45[c]	49.22±0.78[c]	168.00±8.23[c]
		200	11.07±0.23[c]	36.59±0.59[b,c]	85.55±2.80[b,c]
		300	11.85±0.23[b,c]	29.82±0.23[b,c]	73.05±0.78[b,c]

T100 =time to reach a density ≤ 998 g/L
[a] Significant differences in nitrogen sources and compared with ammonium (control source)
[b] Significant differences in nitrogen concentration and compared with 140 mg N/L (control concentration)
[c] Significant differences between strains and compared with PDM (control strain)
- Unfinished fermentation

Table 5. Basic composition of the wines obtained after fermentations with different nitrogen sources and concentrations

Yeast strain			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic acid (g/L)
PDM	Nitrogen source	NH_4	1.67±0.033	11.47±0.219	6.74±0.191	9.78±0.337	0.79±0.079
		Arg	0±0[a]	1.85±0.091[a]	6.62±0.071	10.14±0.205[a]	1.02±0.034[a]
		Gln	0.94±0.44[a]	8.27±2.673[a]	6.33±0.06[a]	10.12±0.273	0.89±0.049[a]
	Nitrogen concentration	60	16.52±2.762[b]	38.60±3.951[b]	5.5±0.417[b]	5.83±0.148[b]	0.30±0.005[b]
		140	0.22±0.381	0.57±0.993	6.88±0.358	10.62±0.545	0.64±0.198
		200	0±0	0±0	6.67±0.179	10.18±0.452	1.30±0.165[b]
		300	0.09±0.150	0±0	6.98±0.401	9.69±0.42[b]	2.55±0.119[b]
ARM	Nitrogen source	NH_4	0.72±0.105[c]	6.16±0.738[c]	6.98±0.095[c]	9.51±0.205	1.43±0.056[c]
		Arg	0±0[a]	0.97±1.677[a]	6.68±0.065[a]	8.89±0.22[a.c]	1.06±0.017[a.c]
		Gln	0.27±0.238[a.c]	3.47±0.702[a.c]	6.47±0.189[a]	9.07±0.87[c]	1.12±0.100[a.c]
	Nitrogen concentration	60	31.86±1.009[b.c]	51.80±1.187[b.c]	5.35±0.205[b]	4.56±0.476[b.c]	0.30±0.041[b]
		140	0.30±0.264	4.01±2.219[c]	6.05±0.473[c]	9.60±0.901[c]	0.54±0.179
		200	0.12±0.214	0±0[b]	6.22±0.046[c]	9.95±0.045	1.14±0.094[b]
		300	0±0[b]	0±0[b]	6.33±0.151[c]	9.84±0.272	1.84±0.203[b.c]
RVA	Nitrogen source	NH_4	1.34±0.463	13.55±2.918	6.91±0.040[c]	9.58±0.11[c]	1.02±0.052[c]
		Arg	0.52±0.58[a.c]	8.50±4.424[a.c]	5.97±0.358[a.c]	9.62±0.496[c]	0.93±0.046[a.c]
		Gln	0.15±0.263[a.c]	5.5±1.384[a.c]	5.62±0.11[a.c]	9.38±0.452[c]	0.94±0.034[a]
	Nitrogen concentration	60	15.08±1.8[b]	38.87±3.092[b]	3.56±0.324[b.c]	5.31±0.467[b.c]	0.67±0.101[b.c]
		140	0.47±0.059	6.89±1.369[c]	4.90±0.384[c]	9.03±0.649[c]	0.92±0.078[c]
		200	0±0[b]	0±0[b]	6.56±0.241[b]	11.11±0.258[b.c]	1.51±0.419[b]
		300	0±0[b]	0±0[b]	6.96±0.410[b]	10.48±0.810[b.c]	1.95±0.805[b]
TTA	Nitrogen source	NH_4	0.44±0.033[c]	7.19±1.258[c]	6.93±0.170	9.53±0.27	1.17±0.077[c]
		Arg	0±0[a]	1.71±0.183[a]	6.14±0.055[a.c]	9.54±0.36[c]	0.95±0.061[a]
		Gln	0±0[a.c]	6.15±0.870	5.72±0.368[a.c]	9.67±0.293[c]	0.92±0.115[a]
	Nitrogen concentration	60	8.16±0.4[b.c]	37.41±1.536[b]	5.62±0.227[b]	6.73±0.361[c]	0.67±0.048[b.c]
		140	0±0	0±0	6.71±0.141	8.89±3.514	1.03±0.227[c]
		200	0±0	0±0	6.69±0.38	10.75±0.833	1.15±0.044
		300	0.21±0.075[b]	0±0	7.28±0.341[b]	10.85±0.331[c]	1.37±0.207[b.c]

[a] Significant differences in nitrogen sources and compared with ammonium (control source)
[b] Significant differences in nitrogen concentration and compared with 140 mg N/L (control concentration)
[c] Significant differences between strains and compared with PDM (control strain)

4. Discussion

Higher fermentation rates can be achieved either by enhancing the glycolytic flux per cell or by increasing the yeast population. Recently Albertin *et al*. (2011) have compared the fermentative behaviors of different yeast strains in three main fermentative processes (winemaking, brewing and baking), concluding that the maximum fermentation rate is driven by population size rather than by specific flux per cell. Thus, the positive correlation found between population size and fermentation rate suggests that increasing maximum population would also increase fermentative ability. Previously, Varela *et al*. (2004) also stated that cell concentration (biomass) governs fermentation rate in nitrogen deficient wine musts. Nitrogen is the most important growth-limiting substrate during wine fermentation (Varela *et al*., 2004), and its deficiency represents one of the main causes of stuck or sluggish fermentations (Bisson, 1999). In this study, we aimed to determine the effect of increasing nitrogen concentrations on growth and fermentation performance in four industrial wine yeast strains. The other question dealt with in this study is the nature of the added nitrogen. Mostly inorganic nitrogen (ammonium salts) is used for supplementing nitrogen-deficient grape musts. However, there is a growing interest in the use of nutrient complements enriched in organic nitrogen during wine fermentations. For this reason, we have also compared the effect on growth and fermentation activity of ammonium, arginine and glutamine used as sole nitrogen sources in a synthetic grape must.

4.1. Effect of nitrogen concentration

To determine the effect of nitrogen concentration on yeast growth we have calculated two key parameters: the maximum growth rate (μ_{max}) and the maximum area under OD *vs* time curve. The μ_{max} denotes the maximum rate of cell division, which is related to the generation time ($GT= \ln(2)/ \mu_{max}$). The second parameter in

yeasts is related to all the biological growth parameters (maximum population size, lag phase and μ_{max}) (Arroyo-López *et al*., 2009). Several authors have also used this value as a valuable parameter to estimate the effects of diverse inhibitory and stimulating substances (sugars, chloride salts, weak acids) on microbial growth (Lambert and Pearson 2000; Bautista-Gallego *et al*., 2008; Arroyo-López *et al*., 2009). As the amount of inhibitor increases, the effect on organism growth also increases, and this inhibitory effect on growth is reflected by a reduction in the area under the OD/time curve. However, if the substance has a growth stimulating effect, an increase in the area under the OD/time curve is observed by increasing the maximum population level and μ_{max} (Bautista-Gallego *et al*., 2008; Arroyo-López *et al*., 2009).

The nitrogen limiting concentration required to reach the μ_{max} value was much lower ($K_{\mu max}$ ranging from 5 up to 18 mg N/L) than the nitrogen concentration to obtain the maximum area ($K_{areamax}$ ranging from 35 up to 135 mg N/L). It seems logical to think that, up to the μ_{max} nitrogen limiting concentration threshold, cells use this nitrogen to reproduce quickly. Nitrogen concentrations above this limit allow the cell to reproduce at maximum speed and keep growing until nitrogen depletion (or shortly after, as they can use nitrogen stored inside cellular compartments), thus reaching higher population sizes with higher nitrogen available. Above certain concentrations, nitrogen is no longer the limiting factor for cell growth, and increases in nitrogen do not have an effect on μ_{max} or population size. This would be the nitrogen limiting concentration for a certain strain or nitrogen source. However, regardless of nitrogen concentration, there must be a genetic component which determines different μ_{max} for the different strains studied. A direct correlation was observed between the two growth parameters analyzed because the strains which presented a higher growth rate also corresponded to a higher area (correlated to the maximum OD obtained). Although the genetic bases controlling growth-rate are not fully understood, phenotypic variation was detected among strains in terms of nitrogen consumption rate, which

might partially explain the differences in growth behavior. Strains PDM and RVA consumed nitrogen much more quickly than strains ARM and TTA, especially as there was an abundance of nitrogen in the medium (Figure 7). Recently, Wang *et al*. (2011) have proved that growth rate and maximum population size can be modified by increasing glucose uptake as a consequence of changing gene dosage in three glycolytic enzyme genes. This possible correlation in wine yeasts between nitrogen uptake rate and growth traits should be dealt with in depth in future studies. However, our study also provides evidence that the strains with higher areas are also the greatest nitrogen demanders. This result is contrary to the popular belief among the winemakers relating scarce biomass yield during winemaking with high nitrogen demanding strains. Another proof that the strains with better growth have a higher nitrogen demand is that the fitness advantage of PDM decreased as nitrogen decreased (Figure 4), because the differences in growth rate compared with other strains also decreased.

It is very difficult to distinguish clearly between the effect of nitrogen on fermentation activity and the effect of nitrogen on biomass yield as both effects are interdependent. Varela *et al*. (2004) used metabolic flux balancing and biomass concentration experiments to evaluate whether the direct correlation between fermentation rate and nitrogen concentration is due to an increase in cellular metabolic activity or to biomass increase. They showed that the higher the biomass concentration, the quicker the fermentation was completed, even when using cells grown with a severe nitrogen shortage. Thus, the main effect of nitrogen concentration on fermentation performance was mainly due to the increase in biomass. However, our results do not fully support this conclusion because the strain which showed the worst growth behavior had the best fermentation activity. Conversely, the correlation between biomass production and fermentation activity was confirmed for strain ARM, which showed the worst fermentation performance. Therefore, the effect of nitrogen concentration on metabolic activity cannot be ruled out and, like growth behavior, it seems to be strain-dependent. At the same

nitrogen concentration, strain TTA yielded a lower area than PDM; however, these cells have higher metabolic activity. Nevertheless, it should be kept in mind that growth and fermentation activity of these strains were obtained by very different experimental conditions (volume, aeration, etc.) which might limit the comparison between both parameters.

Another result supporting the effect of nitrogen on cellular metabolic activity was that the nitrogen concentration boosting fermentation rate is much higher than the nitrogen concentration promoting increased growth (Table 4). Manginot *et al*. (1998) and Beltran *et al*. (2005) already highlighted the importance of nitrogen during the non-proliferating or stationary phase of wine fermentations. However, as already reported Jiranek *et al*. (1991), strain differences in fermentation rate were more important at concentrations in which nitrogen became limiting for growth.

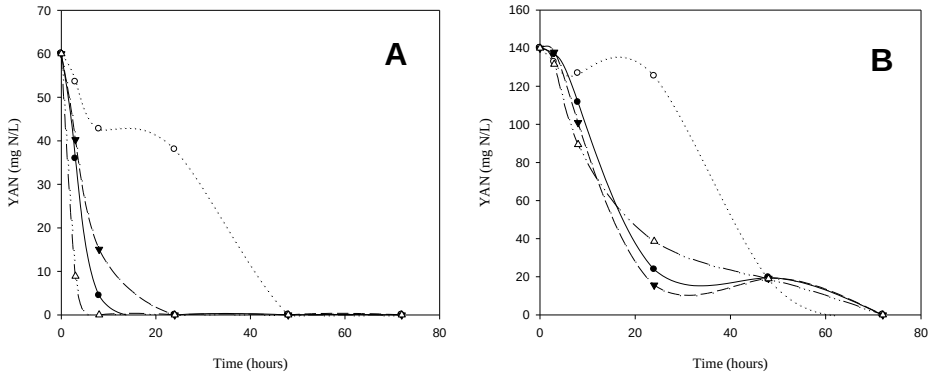
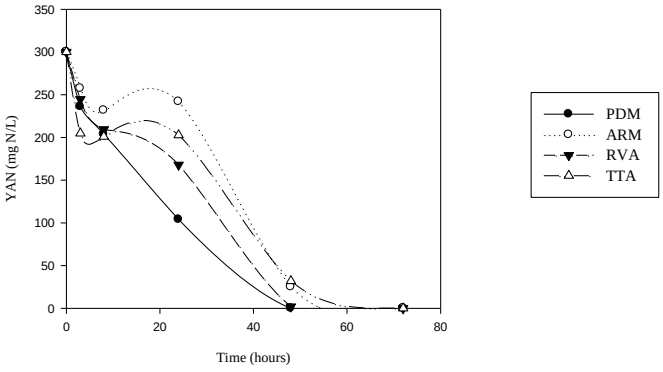


Figure 7. Nitrogen consumption throughout alcoholic fermentation with different nitrogen concentrations: 60 mg N/L (**A**), 140 mg N/L (**B**) and 300 mg N/L (**C**)



4.2. Effect of nitrogen source

To our knowledge, there are no prior studies into the effect of individual nitrogen sources on yeast growth and fermentation activity. The first conclusion of this study is that the effect of nitrogen source on growth parameters is much more limited than the concentration of nitrogen. The four strains showed similar growth parameters for all three nitrogen sources, with few exceptions. The RVA strain improved its growth in glutamine whereas PDM showed the highest μ_{max} in ammonium. Consequently, this strain always showed better fitness in ammonium than in arginine and glutamine (Figure 4). Regarding effects on fermentation activity, differences were also limited but all the strains showed the best and worst fermentation performances with arginine and ammonium, respectively (Tables 3 and 4). These findings were also confirmed by analysis of residual sugars in the final wines (Table 5).

5. Conclusions

Results obtained in this survey confirm that industrial yeast strains have significantly different nitrogen requirements and these nitrogen needs are strongly strain-dependent. We observed that these differences in nitrogen demand positively correlated with higher growth rate and higher nitrogen uptake rate. This correlation should be confirmed because these phenotypic traits should be important selection criteria for wine yeasts. The most direct effect of employing an adequate nitrogen concentration is the increase in biomass, which involves a higher fermentation activity. However, the impact of nitrogen on fermentation is not exclusively due to the increase in biomass. Some strains may adapt a strategy whereby fewer cells with higher metabolic activity are produced.

Currently, the most common method for dealing with nitrogen-deficient fermentations is to add ammonium salts (usually ammonium phosphate), however,

the wine industry may consider using other supplements based on organic nitrogen (amino acids and mostly arginine). The comparison of wine fermentations supplemented either with inorganic or organic nitrogen sources has shown that nitrogen concentration rather than nitrogen source drove the increase in fermentation rate and biomass yield. However, some significant differences were observed in terms of fermentation performance. The use of arginine as sole nitrogen source showed the best fermentation performance among all the strains studied.

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CHAPTER 2

Genetic basis of variations in nitrogen source utilization in four wine commercial yeast strains

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Abstract

The capacity of wine yeast to utilize the nitrogen available in grape must directly correlates with the fermentation and growth rates of all wine yeast fermentation stages and is, thus, of critical importance for wine production. Here we precisely quantified the ability of low complexity nitrogen compounds to support fast, efficient and rapidly initiated growth of four commercially important wine strains. Nitrogen substrate abundance in grape must failed to correlate with the rate or the efficiency of nitrogen source utilization, but well predicted lag phase length. Thus, human domestication of yeast for grape must growth has had, at the most, a marginal impact on wine yeast growth rates and efficiencies, but may have left a surprising imprint on the time required to adjust metabolism from non growth to growth. Wine yeast nitrogen source utilization deviated from that of the lab strain experimentation, but also varied between wine strains. Each wine yeast lineage harbored nitrogen source utilization defects that were private to that strain. By a massive hemizygote analysis, we traced the genetic basis of the most glaring of these defects, near inability of the PDM wine strain to utilize methionine, as consequence of mutations in its *ARO8*, *ADE5,7* and *VBA3* alleles. We also identified candidate causative mutations in these genes. The methionine defect of PDM is potentially very interesting as the strain can, in some circumstances, overproduce foul tasting H_2S, a trait which likely stems from insufficient methionine catabolization. The poor adaptation of wine yeast to the grape must nitrogen environment, and the presence of defects in each lineage, open up wine strain optimization through biotechnological endeavors.

Keywords: nitrogen utilization, methionine, wine yeast, *ARO8*, *VBA3*, *ADE5,7*

1. Introduction

Inoculation of selected yeast into wine must, rather than relying on spontaneous fermentation, is an established oenological practice that allows better control of organoleptic wine characteristics and guarantees the homogeneity of successive fermentations. Nowadays, most commercial wine production is based on such commercial starter wine yeasts, which were originally selected mainly from natural varieties of the Wine/European genetic clade (Liti *et al*., 2009), given their superior fermentation properties. However, the overall suitability of wine yeasts to grape wine production, which imposes demands for a large number of genetically complex traits, has not been stringently evaluated. The vast variability among natural yeasts (Warringer *et al*., 2011), in combination with widespread antagonistic pleiotropy, suggests that any one strain selected from a natural stock is unlikely to possess an ideal combination of oenological characteristics. It is also unclear as to what extent wine strains have adapted to wine production conditions; for example, many wine strains are poor at utilizing proline, the predominant nitrogen source in grape wine, despite undergoing nitrogen limitation during wine fermentation (Salmon and Barre, 1998). Thus, it is easy to envision a substantial potential for optimization of existing wine yeasts.

Nitrogen source utilization has a substantial impact on alcoholic fermentation, influencing both the fermentative process and wine quality (Bisson, 1999; Bely *et al*., 1990). Nitrogen deficiency can produce sluggish or stuck fermentations, and both nitrogen deficiency and incomplete nitrogen utilization can confer poor organoleptical properties. Conversely, excessively high nitrogen levels may have negative effects, such as microbial contamination, production of off-flavors (Jiranek *et al*., 1995) or ethyl carbamate formation, which is a suspected carcinogen (Ough, 1991). Thus, there is particular interest in optimizing wine yeast nitrogen utilization in a way that ensures that all the nitrogen compounds present in the grape must are utilized completely and efficiently. Common lab strains of

Saccharomyces cerevisiae can catabolize a variety of low complexity organic nitrogen sources, such as most amino acids, the animal secretion products urea and allantoin, the arginine derivative citrulline, some nitrogen bases and one inorganic nitrogen source, ammonium (Cooper, 1982). These compounds enter cells via permeases and are rapidly used as building blocks in biosynthesis or are catabolized to yield the internal nitrogen currencies ammonium or glutamate (Magasanik, 1992). In complex mixtures of nitrogen compounds, wine yeast prefers utilizing certain sources before others, and this pattern of nitrogen compound uptake depends on both nitrogen and sugar composition (Beltran *et al*., 2005; Martínez-Moreno *et al*., 2012). In the presence of a single nitrogen source, neither nitrogen source preference nor the achieved growth rate has been exhaustively mapped across wine strains. Nevertheless, based on lab strain experiments, it is commonly assumed that preferred nitrogen sources allow higher growth rates. In lab strains, nitrogen source preference is mediated by the nitrogen catabolite repression (NCR) system by stimulating the expression of permeases for the preferred nitrogen source and the degradation of permeases of non preferred sources (Magasanik and Kaiser, 2002). Ammonium, glutamine and asparagine are preferred nitrogen sources whereas arginine, alanine, aspartate and glutamate are less preferred, and urea and proline non preferred (ter Schure *et al*., 2000; Magasanik and Kaiser, 2002). Branched-chain and aromatic amino acids do not support high growth rates, but typically accumulate early in fermentation (Henscke and Jiranek, 1993; Beltran *et al*., 2005), thus breaking the assumed correlation between the nitrogen source growth rate and preference.

A primary challenge for the human-induced improvement of nitrogen-associated properties of wine yeast is the dissection of the genetic architectures underlying variations in the capacity to utilize nitrogen sources between commercially established wine strains. The yeast universal reference strain S288c, and its relative Σ1278b, on which much molecular understanding of the nitrogen metabolism is based, are phenotypically much diverged from wine yeasts (Warringer *et al*.,

2011). Thus, only a limited extrapolation of knowledge from lab strain experimentation is possible, and the associations between variation in nitrogen utilization traits and genetic variation have to be established in wine strains without prior assumptions. Yeast has recently emerged as a prime model organism for quantitative genetics in general and for Quantitative Trait Loci (QTL) mapping in particular (Liti and Louis, 2012). Variations in high temperature growth (Steinmetz *et al*., 2002; Parts *et al*., 2011), sporulation efficiency (Deutschbauer *et al*., 2005), drug response (Perlstein *et al*., 2006; Cubillos *et al*., 2011), telomere homeostasis (Liti *et al*., 2009), cell morphology (Nogami *et al*., 2007), ethanol tolerance (Hu *et al*., 2007) and acetic acid production (Marullo *et al*., 2007) have all been mapped to individual genes. More recently, Salinas *et al.* (2012) and Ambroset *et al*. (2011) identified QTLs of oenological phenotypes. However, the genetic basis of trait variations in commercially relevant strains have been dissected only in a very small number of cases (Marullo *et al*., 2007; Katou *et al*., 2008; Ambroset *et al*., 2011).

Here, we precisely and exhaustively quantified variations in the ability of four widely used commercial wine strains in Spanish wineries to utilize the complete palette of low complexity nitrogen sources that is normally accessible to yeast. We report extensive growth differences between nitrogen sources and different wine strains. Some of these differences in growth are present in all the lineages, while other variations are nitrogen defects that are private to each strain. We traced the genetic origin of the incapacity of the PDM wine strain to utilize methionine to defects in its *ARO8*, *ADE5,7* and *VBA3* alleles, which is of particular interest as these defects may contribute to an excessive production of foul tasting H_2S in this strain. Finally, we suggest specific nucleotides that can be targeted in efforts to alleviate this deficiency.

2. Materials and methods

2.1. Yeast strains and media

The yeast strains used in this study are the following: PDM, ARM, RVA and TTA; all of which were provided by Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com). A taxonomic description of these strains was carried out by the RFLPs of the ITS/5.8S region (Guillamón *et al*., 1998). Strains PDM (Pasteur Prise de Mousse), RVA and TTA belong to species *Saccharomyces cerevisiae*, while we identified strain ARM as a hybrid between *S. cerevisiae* and *S. kudriavzevii*, following the procedure proposed by González *et al*. (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has recently been confirmed by Dunn *et al*. (2012). These wine strains were grown at 30ºC on plates of YPD medium (2% glucose, 1% yeast extract, 1% peptone and 2% agar).

The synthetic wine must (SWM) was prepared according to Riou *et al*. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). Only the nitrogen content changed. Each medium was prepared with a single nitrogen source, except for the control condition (SWMc), which was composed of a mixture of ammonium and amino acids (40% of ammonium + 60% of amino acids), as described in Beltran *et al*. (2004). The nitrogen sources used were: adenine, allantoin, ammonium, cytosine, GABA, L-alanine, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, L-urea. The tested concentrations were 30 mg N/L as a highly nitrogen deficient condition and 140 mg N/L as a control condition, which resembles the more realistic nitrogen concentrations in wine must.

2.2. Measure of growth variables

Two consecutive pre-cultures of 72 hours were performed by incubating cells at 30ºC in 350 µL of SWM medium with 30 mg/L of ammonium as the sole nitrogen source in 100-well micro-cultivation plates. This low concentration is required for all the strains to deplete their nitrogen reserves; thus enabling the test of their utilization of different nitrogen sources, starting from the same initial cellular state. Asexual reproduction was monitored at 600 nm in a Bioscreen analyser C (Thermo Labsystems Oy, Finland). Pre-cultures were inoculated at an initial OD of approximately 0.1 (inoculum level of 10^6 CFU/mL) in the SWM with different nitrogen concentrations and sources. Incubation was maintained at 30ºC (10 min preheating time). Microcultivation plates were subjected to shaking at the highest shaking intensity with 60 s of shaking every other minute. OD measurements were taken every 20 min over a 72-hour period. This time allows yeast cells to reach the stationary phase in all but the worst nitrogen environments. All the conditions were run in duplicate at both nitrogen concentrations. In all, 384 growth curves (24 nitrogen sources x 2 nitrogen concentrations x 4 yeast strains x 2 replicates) were obtained and analyzed. For each growth curve, the variables lag phase, doubling time and growth efficiency were extracted as described (Warringer and Blomberg, 2003). Briefly, the lag phase was estimated using the slope calculation from every eight consecutive data values along the curve (corresponding to a time span of 2.5h). An intercept between every slope and a straight line corresponding to the initial OD was calculated. A mean of the two highest calculated intercepts was taken as the lag phase. Generation time was calculated by taking into account the slopes between every third consecutive measurements for the whole growth curve. Of the seven highest slopes, the highest two were discarded to provide a safety margin, and a mean was calculated for the remaining five. The generation time was obtained as ln 2 divided by the mean of the slopes. Growth efficiency was

calculated based on the six last time points in the measurement. The difference between end OD and initial OD was taken as the stationary phase OD increment.

2.3. Construction of haploid strains and mating type determination

To carry out the construction of derivative haploid wine strains, the *HO* gene was deleted in the PDM diploid strain using the short flanking homology method reported by Güldener *et al*. (1996). This method replaces one copy of the open reading frame of *HO* gene with the *nat*MX4 cassette. The deletion cassette was obtained by PCR using the pAG25 plasmid that contains nourseothricin resistance. The primers used, HO-S1 and HO-C2 (Table 1), have 50 nucleotide extensions corresponding to the regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). PDM strain was transformed by the lithium acetate procedure (Gietz and Woods, 2002). Transformants were selected by resistance to nourseothricin and correct integration of the deletion cassette was confirmed by diagnostic PCR using the primers upstream and downstream of the deleted region (Table 1).

Sporulation was induced by incubating cells on acetate medium (1% potassium acetate and 2% agar) for 5 days at 30ºC. Following the preliminary digestion of ascus walls with 2 mg/ml glucuronidase (Sigma), spores were dissected using micromanipulation (Singer instruments, United Kingdom). In all cases, >50% of spores were viable. Finally, monosporic cultures were grown on YPD plates in the presence of nourseothricin. To test the mating type of each haploid strain selected, PCR against the *MAT* locus was performed using *MAT**a*** and *MAT*α primers (Huxley *et al*., 1990) (Table 1). Both the 544bp haploid *MAT**a*** and the 404bp haploid *MAT*α bands were observed in the diploid strains. PCR was done under the following conditions: 94ºC for 5 min, 30 cycles at 94ºC for 1 min, 58ºC for 2 min and 72ºC for 2 min, and 72ºC for 7 min. Haploid strains were grown under the same nitrogen conditions as their diploid parent strains.

Table 1. Primers used in this study

Gene	Name	Oligonucleotide sequence (5'-3' end)
HO disruption cassette *	HO-S1	AGACATCGCAAACGTCACGGCTAACTCTTACGTTATGT GCGCAGATGGCTCGTACGCTGCAGGTCGACA
	HO-C2	ACTCTTATGAGGCCCGCGGACAGCATGAAACTGTAAG ATTCCGCCACATTACTAGTGGATCTGATATC
HO PCR verification	HOc-F	GAGGTTTGCAGAAGCTTGTTGA
	HOc-R	TTGGCGTATTTCTACTCCAGCAT
Mating type verification	MAT	AGTCACATCAAGATCGGTTATGG
	MATF	GCACGGAATATGGGACTACTTCG
	MATa	ACTCCACTTCAAGTAAGAGTTTG
ARO8 disruption cassette *	ARO8-Ft	AACCCTGCAGTTGATACAGACATTGAATAGGACAACC GATCGTTACTATCCGTACGCTGCAGGTCGACG
	ARO8-Rt	CGTACGTCCTTTTTTCACCTTATATATATTCTTCCAACG TATTTACCTCTACTAGTGGATCTGATAT
BAT2 disruption cassette *	BAT2-Ft	AAAATTTTAGAAATTTAAGGGAAAGCATCTCCACGAG TTTTAAGAACGATCGTACGCTGCAGGTCGACG
	BAT2-Rt	AGTTTTATTCTTTTTAACTTTTAATTACTTTACGTAGCA ATAGCGATACTACTAGTGGATCTGATAT
ADE5,7 disruption cassette *	ADE5,7-Ft	TATTACTTTCTTAATCATAGCTTAAGAGAACCATTCTC CCTCCCCTCACACGTACGCTGCAGGTCGACG
	ADE5,7-Rt	TAATATATGTACGCGCATATATGAATCTATTATAAAGT TAATATTGTTGAACTAGTGGATCTGATAT
VBA3 disruption cassette *	VBA3-Ft	CAATACTTATTTTTGAAGCCGGATCCCTAATTGCTGCC CTTGCCTCTTCACGTACGCTGCAGGTCGACG
	VBA3-Rt	GTTGAAGTCTGTATAAAAGGCGAAAAAAATAAAATGA AAATAAGAAAATAACTAGTGGATCTGATAT
ARO8 PCR verification	ARO8-Fc	TGGCTCATATACACCATCCA
	ARO8-Rc	GGAGATTCATGGTACCAGACA
BAT2 PCR verification	BAT2-Fc	TCACGACCTAGCATACCACTA
	BAT2-Rc	GATAGGCCAGCACTAGATGA

ADE5,7 PCR verification	ADE5,7-Fc	CCGTTGTTCATACCGTGACTA
	ADE5,7-Rc	GCCAGTAAAACCTGCTGAAA
VBA3 PCR verification	VBA3-Fc	TACCTAGAACTGATCAGACCA
	VBA3-Rc	CTTATTGTGAAAGGTGTTTGA
ARO8 sequence	ARO8-Fs1	CGTTACTATCATGACTTTACC
	ARO8-Rs1	TACCTCTCTATTTGGAAATAC
	ARO8-Fs2	CTGGTGCTCCTAAACCAAAGT
	ARO8-Rs2	TCATGCAAACTAAGTAAGGC
BAT2 sequence	BAT2-Fs1	GCATCTCCACGAGTTTTAAG
	BAT2-Rs1	CGTAGCAATAGCGATACTTCA
	BAT2-Fs2	TATATGTCATTTGCTGCCCTG
	BAT2-Rs2	TTCATGGTGCCGACTTCAGTA
ADE5,7 sequence	ADE5,7-Fs1	TAAGAGAACCATTCTCCCTC
	ADE5,7-Rs1	GATTAGTAAAGCTTAGTTCCG
	ADE5,7-Fs2	GGCAAGCAGGTTGTGATAGAA
	ADE5,7-Rs2	GAATTTTGGAAATGGACGCA
	ADE5,7-Fs3	GCCACCGATTCTTTATTGACC
	ADE5,7-Rs3	TTAATACAGCACCAACAGCGG
VBA3 sequence	VBA3-Fs1	GCCTCTTCAATGAATATGCTC
	VBA3-Rs1	TAACTGCGCTACTTGTCTTCT
	VBA3-Fs2	CGATAAATTCAACCCGGA
	VBA3-Rs2	GTGGTTTAACTAAACCGAGC

* Underlining indicates homology to the *nat1* cassette from plasmid pAG25. The remaining sequences of the primers are homologous to the flanking region of the *HO* open-reading frame.
PCR, polymerase chain reaction.

2.4. Hemizygosity analysis

To identify the alleles contributing to variations in nitrogen source utilization, 228 hemizygote hybrids, each resulting from a cross between a derivative haploid PDM strain and a BY4741 derivative lacking one of the 228 nitrogen utilization genes, were constructed. The haploid of the PDM wine strain (*MAT***α**; *ho*Δ) was crossed with the deletion mutants from the BY4741 deletion collection (*MAT***a**; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*). Table 1 (Annex I – Materials and Methods) lists all the deletion strains used. A heterozygote hybrid strain, which also maintained the BY

gene, was also constructed and used as a control. Constructions were performed as follows: haploid strains were grown in 96-well plates with liquid YPD media for 24 h at 30ºC. The wine and BY strain cultures were spotted onto the same positions on solid YPD medium in 96-well format dishes using a benchtop RoToR HDA robot (Singer Instruments, United Kingdom) with default settings. After 48 h at 30ºC, colonies were re-pinned onto similar YPD 96-well format dishes supplemented with 0.2 mg/mL geneticin and 0.05 mg/mL nourseothricin resistance to select the diploid hybrids from successful matings. Strains were manually transferred to 100-well bioscreen microcultivation and plates, and were grown in SWM with selected nitrogen sources. Growth was quantified as indicated above and comparing each hemizygote to the heterozygote diploid control (n=5).

2.5. Construction of the haploid deletion mutants in the PDM wine strain

ARO8, BAT2, ADE5,7, VBA3 were independently deleted in the haploid derivative of the PDM wine strain using a short flanking homology (Güldener *et al*., 1996). The deletion cassette contained hygromycin B resistance, amplified from plasmid pAG32 with the primers shown in Table 1. Primers had 50-nucleotide extensions corresponding to the regions upstream and downstream of the target ORF and were transformed into the haploid wine strain following the lithium acetate procedure (Gietz and Woods, 2002). For each construct, three transformants resistant to hygromycin B were analyzed by diagnostic PCR and were used as independent repeats (n=3). Wine deletion strains were crossed to the BY4741 strain on YPD, and diploids were selected on the medium containing 0.2 mg/mL geneticin and 0.3 mg/mL hygromycin B. Once again, these hemizygotes were grown in SWM with selected nitrogen sources, as described above, and were compared to each respective reciprocal hemizygote missing the BY allele. The growth of these hemizygotes was also performed in 50 mL tubes to check their phenotype under conditions more similar to real wine production.

2.6 Sequence analysis

Four genes (*ARO8*, *ADE5,7*, *BAT2* and *VBA3*) were sequenced in the wild-type strain PDM by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The primers designed for PCR amplification are shown in Table 1.

2.7 Clustering methods and statistical analysis

A two-tailed Student's t-test with equal variance assumption was used for the two-group comparisons. The cut-off level of significance was set to $\alpha \leq 0.05$. Hierarchical clustering was performed using MeV MultiExperiment Viewer, and Pearson correlation metrics and group clustering based on group averages (average linkage). Pearson correlation coefficients were employed for the correlation analysis. Significance of the correlations was calculated using a Student's t-distribution, $t = r\sqrt{\frac{n-2}{1-r^2}}$, where r = Pearson correlation coefficient and n = number of nitrogen sources. Degrees of freedom = n - 2.

3. Results

3.1 Different wine yeast growth measures provide complementary views of nitrogen source suitability

To quantify variations in the nitrogen source utilization among wine strains, strains PDM, ARM, RVA and TTA were microcultivated at low and intermediate concentrations (30 and 140 mg N/L) of 23 individual nitrogen substrates. Together, these substrates covered the entire width of the low complexity nitrogen compounds utilizable as sole nitrogen sources by the yeast lab strain S288c. High

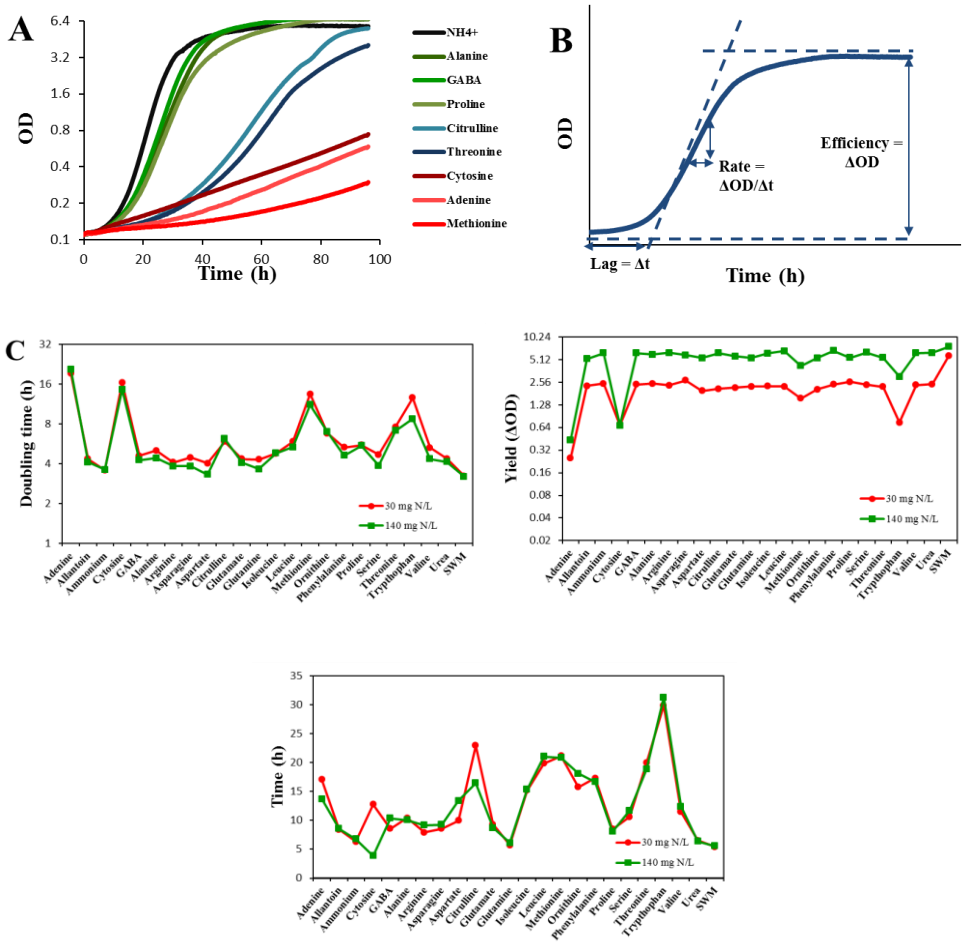
density mitotic growth curves revealed vast variations in the capacity of the wine strains to utilize different nitrogen sources (Fig. 1A), which did not immediately agree with the established wisdom on nitrogen source preference. To obtain quantitative measures of mitotic performance and to allow a stringent evaluation, lag phase length (lag), the exponential growth rate (doubling time) and growth efficiency (total change in density) were extracted (Fig 1B). Together, these measures encapsulate yeast mitotic fitness. Overall, the tested (low or intermediate) nitrogen concentrations were found irrelevant for the lag and rate of wine strain mitotic proliferation (Fig. 1C). In contrast, the efficiency of all the nitrogen sources, excluding the very poor adenine and cytosine, which were not exhausted within the experimental time frame, were very strongly affected by nitrogen availability (Fig. 1C). Thus, nitrogen availability was limiting for the biomass yield, but not for the rate or the lag of biomass production. This result is in agreement with recent findings (Gutiérrez *et al*., 2012). The mean difference in efficiency between nitrogen concentration (excluding adenine and cytosine) ranged from 3.85 (PDM) to 3.57 (TTA), which is reasonably close to the theoretical expectation of (4.66-fold) by assuming strictly additive effects of nitrogen increase on yield within the concentration range considered.

To analyze the effect of the different nitrogen sources on the three mitotic fitness measures in wine strains, the average growth data of the four strains were used to establish a ranking of these nitrogen compounds in terms of their rate, efficiency (yield) and lag (Fig. 1D). The relative capacity of different nitrogen sources to support wine yeast proliferation diverged from the accepted view of nitrogen source suitability for lab strains. Furthermore, the different fitness measures only partially overlapped (Godard *et al*., 2007). As expected, a ranking of the nitrogen sources based on the mean growth rate showed that: nucleotide bases were very poor nitrogen sources; aromatic and branched amino acids, together with ornithine and citrulline, were poor nitrogen sources; the nitrogen sources traditionally classified as preferred supported fast or very fast growth (average generation time

~ 4 h) (Fig. 1D). Surprisingly however, methionine supported only very slow growth in wine strains, whereas urea and allantoin promoted fast reproduction. The two latter compounds have been traditionally classified as poor sources that do not exert an NCR effect (Godard *et al*., 2007). In contrast, the efficiency measure, arguably the most relevant for wine production, revealed that several nitrogen sources traditionally regarded as poor, notably phenylalanine, leucine and citrulline, were very efficiently utilized, whereas nitrogen sources traditionally classified as good, such as aspartate, glutamine and glutamate, were less optimal in utilization efficiency terms. Finally, the ranking of lag phase lengths revealed that urea, proline, ammonium and glutamine were metabolized with a short delay, whereas tryptophan, leucine, methionine and citrulline required almost one full re-adjustment day before allowing proliferation to take off. Thus, the different growth measures provided complementary views of nitrogen source suitability, which deviated from what has been formerly been established using lab strains. This underscored the importance of weighing different aspects of mitotic growth when judging nitrogen source suitability as well as the limitations of extrapolations from lab to wine strains.

The capacity of a particular nitrogen source to support fast or efficient yeast growth showed no correlation whatsoever to the abundance of this nitrogen compound in grape must (Fig. 1E). For example, wine strains were excellent at utilizing urea and allantoin, which are absent in grape must, but proved to be slower and less efficient in utilizing very abundant nitrogen compounds, such as proline. This casts doubts on the assumption that yeast in general, and wine yeast in particular, are well-adapted to grape must. Surprisingly, lag phase length, a trait which has received little attention in wine production and in yeast research, showed a strong inverse correlation (Pearson, $r = -0.69$, $p=0.01$) to nitrogen abundance. That is, lag phase length was much shorter when wine yeasts were adjusting their metabolism to the nitrogen compounds that are abundant in grape must. Taken together, human domestication of yeast for wine production appears to leave the rate and efficiency

of nitrogen source utilization unaffected, but may substantially shorten the lag before this utilization takes off.



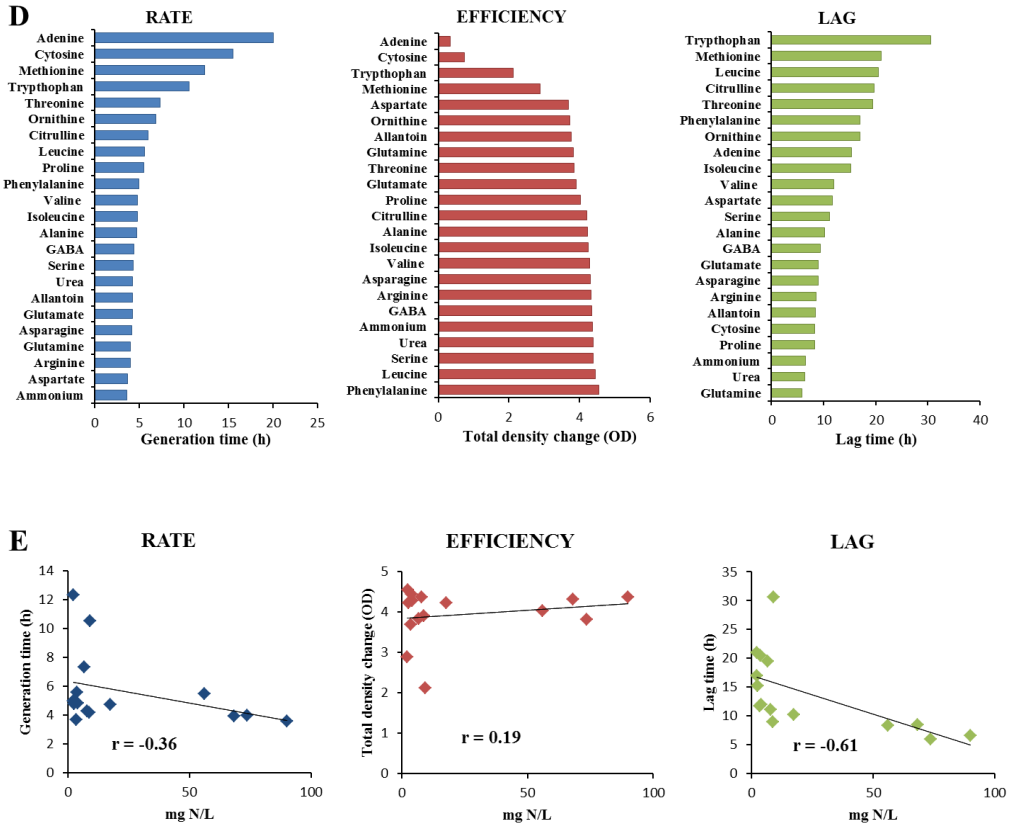


Figure 1. **Large variations between wine yeasts in nitrogen source utilization capacity**. The capacity of four wine yeasts to utilize low complexity nitrogen compounds as sole nitrogen sources was quantified using microcultivation and extraction of asexual fitness components from high density mitotic growth curves. A palette of 24 nitrogen sources was tested in low (30 mg N/mL) and intermediate (140 mg N/mL) concentration. A) Sample mitotic growth curves of the PDM strain in a subset of nitrogen sources. B) The asexual fitness components lag phase (time to initiate asexual proliferation), rate (asexual generation time) and efficiency (total change in population density during asexual growth) were extracted from each high density growth curve. C) Effect of the concentration of nitrogen on the mean (n=2) of the asexual fitness components rate, efficiency and lag of the four wine strains. D) The mean of each fitness component measure (n=2 for each strain), over all four wine strains, was calculated. Nitrogen sources were then ranked according to mean performance separately for each fitness component. E) The mean in performance between the four wine strains was plotted against amount of nitrogen of each

particular source present at SWM. Linear regression (black line) is displayed. The squared Pearson correlation coefficient (r) is given in the figure.

3.2. Wine strains differ in their capacity to utilize different nitrogen sources

To control for the general differences between strains, growth measures were $\log_2$-transformed and normalized to the corresponding fitness measure in a medium containing a complex mixture of nitrogen sources. By visualizing the relative measures of nitrogen utilization ability, we found marked differences between the wine strains in terms of their capacity to utilize different nitrogen sources (Fig. 2A). As in the ranking described in Figure 1D, grouping the nitrogen sources based on similarities in their suitability for different strains revealed three distinct clades. Clade 1 contained the consistently very poor adenine and cytosine; Clade 2 included the branched-chain and aromatic amino acids, together with arginine intermediates ornithine and citrulline and serine and threonine; Clade 3 comprised the generally good nitrogen sources asparagine, aspartate, arginine, GABA, alanine, glutamate, glutamine, ammonium, together with the animal secretion products allantoin and urea. Methionine, proline and tryptophan constituted the outliers in clustering, which imply that their profiles over all the strains and variables did not substantially resemble any other nitrogen source profile. This suggests that private mutations with little influence on other nitrogen catabolic processes underlie the variations in the utilizations of these nitrogen sources.

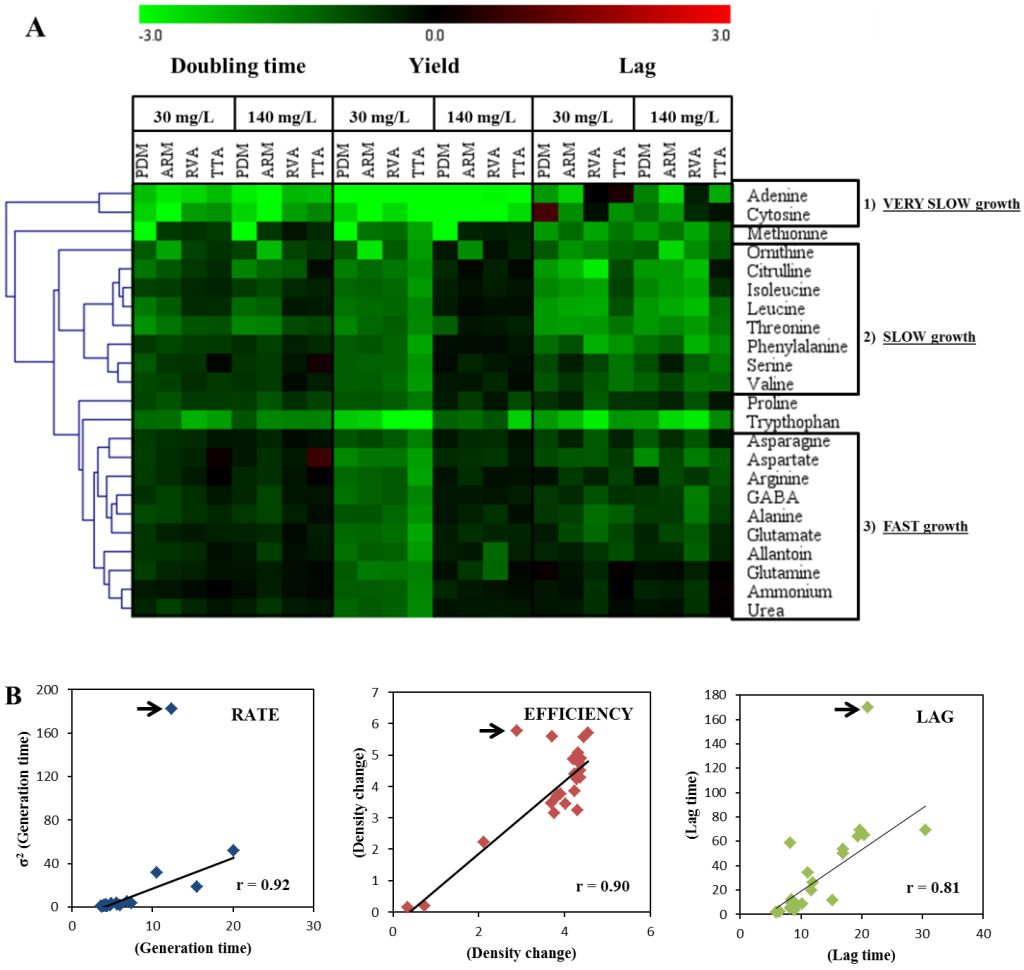
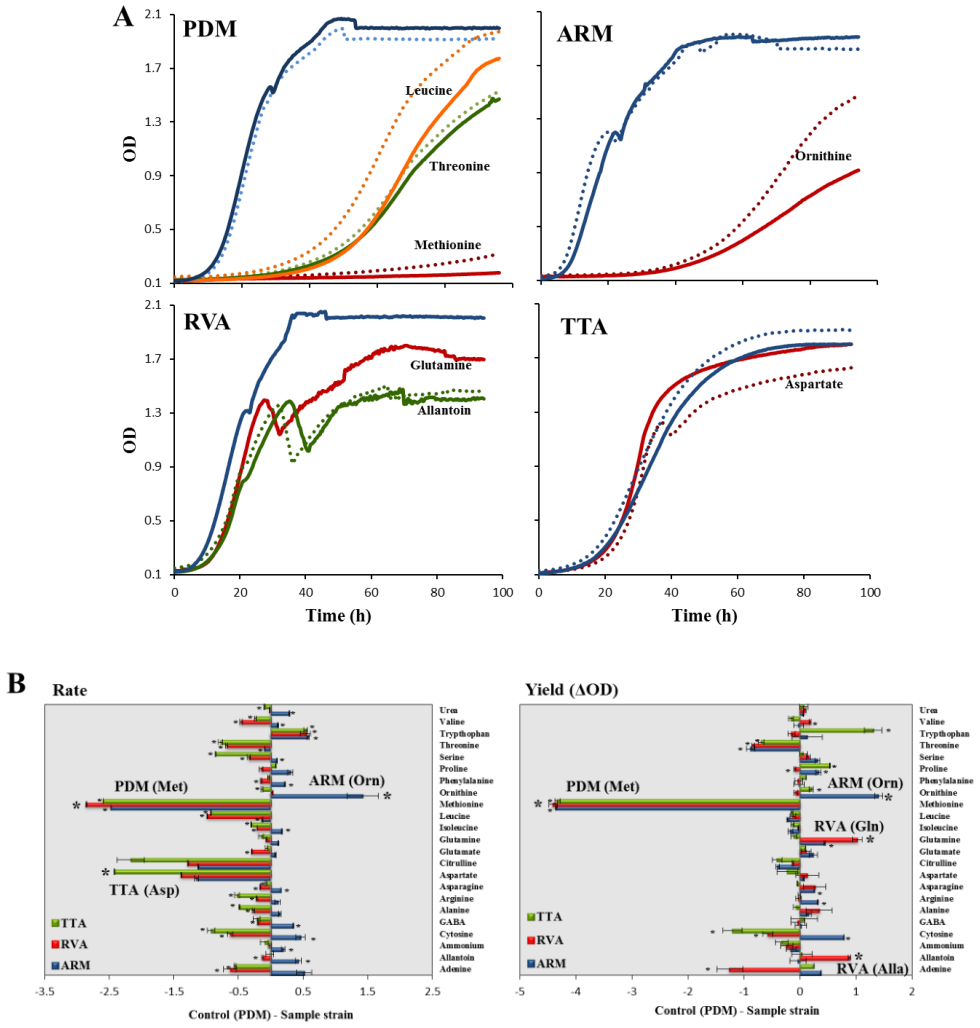


Figure 2. Individual Capacity of four wine strains to utilize different nitrogen sources. A) Hierarchical clustering of nitrogen sources based on the asexual fitness parameters of four wine strains. Each asexual fitness component estimate was log(2) transformed, a mean estimate was obtained (n=2) and this mean was normalized to the corresponding estimate (n=2) of that strain in synthetic wine must (SWM). The heatmap color reflects the normalized fitness component measure: green = inferior, red = superior and black = equal performance using a particular nitrogen source relative performance in SWM. Based on overall performance considering all fitness measures of all four strains, nitrogen sources was classified in discrete categories as "fast growth", "slow growth" and "very slow growth". Clustering of nitrogen sources were performed on the basis of all

fitness measures and using a Pearson correlation coefficient. Groups were clustered using group means. B) The variance in performance between the four wine strains was plotted against the mean performance, considering nitrogen source and each fitness component separately. Linear regression (black line) is displayed. The squared Pearson correlation coefficient (r) is given in the figure. Methionine (marked with an arrow), which was a clear outlier due to inability of PDM to utilize this nitrogen source, was excluded. Including methionine, squared Pearson correlation coefficients were: rate = 0.56, efficiency=0.81, lag=0.56.

Interestingly, the between-strain variations in nitrogen source suitability strongly and inversely correlated with mean suitability (Pearson $R>0.8$, $p<0.001$) (Fig. 2B); i.e., the most pronounced between-strains difference were observed for the worst nitrogen sources. The remarkable exception to this rule was methionine, which supported only marginal PDM growth, but emerged as an intermediate nitrogen source for other strains (Fig. 3A-B). Additionally, PDM achieved only very an inefficient utilization of threonine, and a remarkably slow utilization of leucine. Other notable strain-specific nitrogen phenotypes revealed a surprisingly poor growth of the ARM (both rate and yield) in ornithine, and diminished efficiency of RVA in glutamine and allantoin and, the fast growth of TTA using aspartate. In fact, the proliferation rate of TTA on aspartate as a sole nitrogen source exceeded the growth rate reached using the complex nitrogen mixture (Fig. 3A). Thus, aspartate was a rare exception to the widely accepted assumption of superior yeast performance in a complex mixture of nitrogen sources. Taken together, we highlight a remarkable variation in nitrogen source suitability among wine strains, with each strain harboring clear nitrogen utilization deficiencies that are potentially curable by molecular genetics.



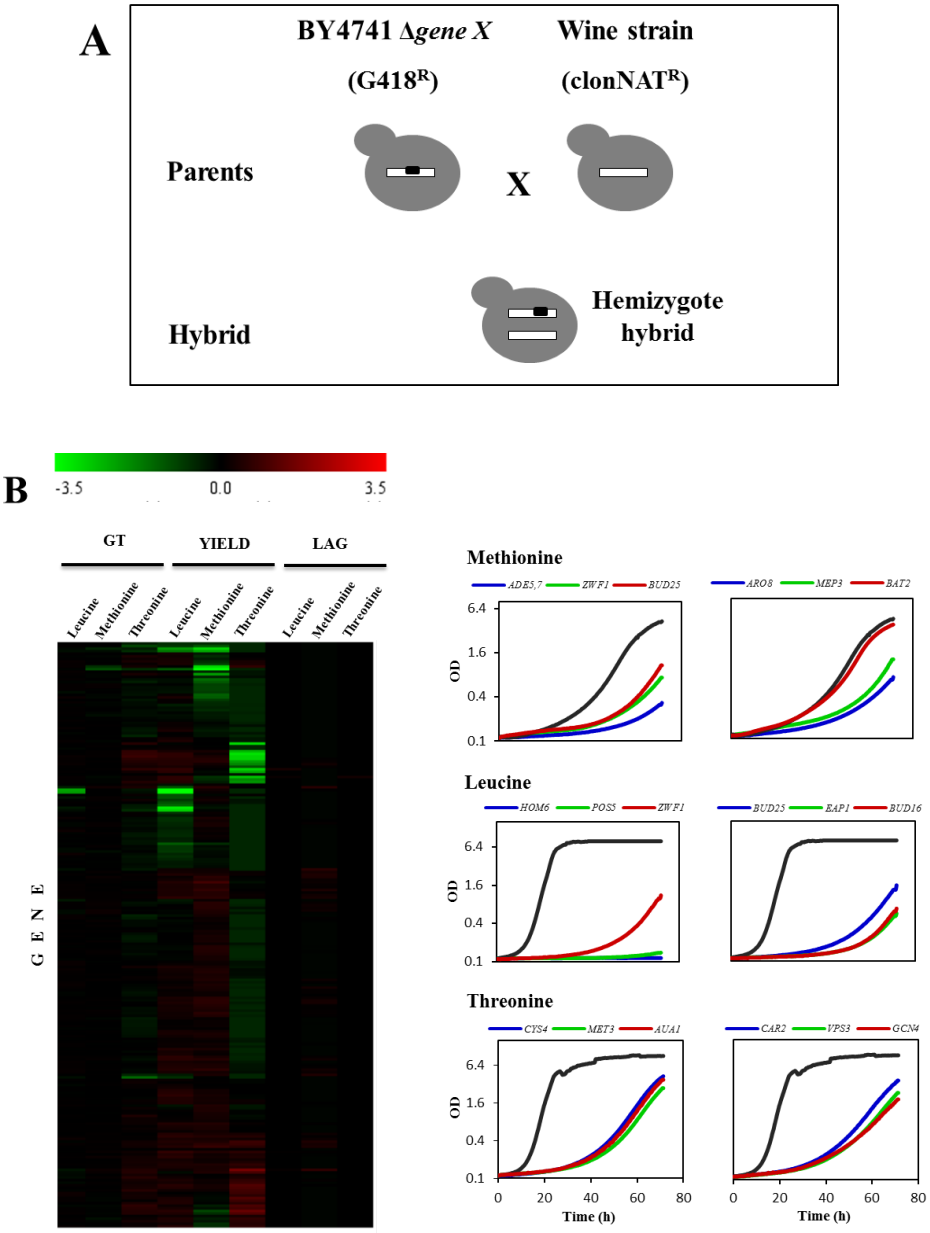
* Significance from t- Student with P≤0.05 compared with the control condition (PDM strain)

Figure 3. **Wine strains differ in the ability to use a variety of nitrogen sources.** A) Growth curves of the four wine strains in those nitrogen sources more affected with respect SWM control (blue line). Both replicates are displayed, as full and broken lines of the same colour. B) The log(2) asexual fitness component measures (generation time and yield) of TTA, RVA and ARM utilizing individual nitrogen sources were compared to the corresponding measures of PDM, $\log_2$(PDM) - $\log_2$(strain). Negative values indicate worse performance of the PDM strain, positive values indicate better performance of the PDM strain. Error bars = SEM (n=2).

3.3. Wine-lab strain hybrids hemizygotic for individual nitrogen utilization genes, show mostly unperturbed proliferation during nitrogen restriction

To identify the potential candidate genes harboring variations that underlie differences in nitrogen source utilization, we performed a large-scale hemizygosity analysis. To this end, 228 genes involved in nitrogen transport, catabolism, storage or regulation were selected (Table 1 – Annex I). The haploid single gene deletion strains in lab strain BY4741 were crossed to a haploid derivative of the PDM wine strain by a robotized procedure, and the diploid hybrids were automatically selected based on dual antibiotics resistance (Fig. 4A). The capacity of the resulting hemizygotes, which contain only the wine strain allele of each individual candidate gene, to utilize the nitrogen sources unsuitable for the wine parent was finally compared to the corresponding capacity of the heterozygote hybrid with both parental alleles intact.

The vast majority of hemizygotes closely resembled the control heterozygote (Fig. 4B). This means that retention of a single allele in the diploid hybrid was almost always sufficient to maintain an unperturbed nitrogen utilization capacity. Essentially no overlap in hemizygote defects between different nitrogen sources was observed (Fig. 4C), meaning that the effects of impairing individual nitrogen utilization functionalities had only nitrogen source-specific effects. Among the 36 hemizygotic genes showing impaired growth in methinonine, leucine and threonine (Fig. 4C), most belonged to amino acid metabolism (14 genes) and to nucleotide/nucleoside/nucleobase metabolism (5 genes). In amino acid metabolism, most alleles belonged to the sulfur amino acid metabolism (9 genes), the metabolism of the aspartate family (6) and the metabolism of glutamate (4 genes).


A
BY4741 Δgene X
(G418R)
Wine strain
(clonNATR)
Parents
X
Hybrid
Hemizygote hybrid
B
-3.5
0.0
3.5
GT
YIELD
LAG
Leucine
Methionine
Threonine
GENE
Methionine
ADE5,7
ZWF1
BUD25
ARO8
MEP3
BAT2
Leucine
HOM6
POS5
ZWF1
BUD25
EAP1
BUD16
Threonine
CYS4
MET3
AUA1
CAR2
VPS3
GCN4
OD
6.4
1.6
0.4
0.1
0
20
40
60
80
Time (h)

C

N source	Hemizygotic genes
Leucine	*HOM6, POS5, EAP1, BNA6, TNA1, BUD16, BUD25, NPL6, CPA1*
Methionine	*ZWF1, ADE5,7, ARO8, AVT6, ADE4, CHA1, TRP4, BUD25, BAT2, VBA3, AGP3, MEP3*
Threonine	*MUP1, CYS4, MET3, AUA1, CAR2, VPS65, VPS3, GCN4, HOM3, RNR1, CGR1, VPS45, ADE6, ECM29, QCR10, ARG4, TED1, MET18*

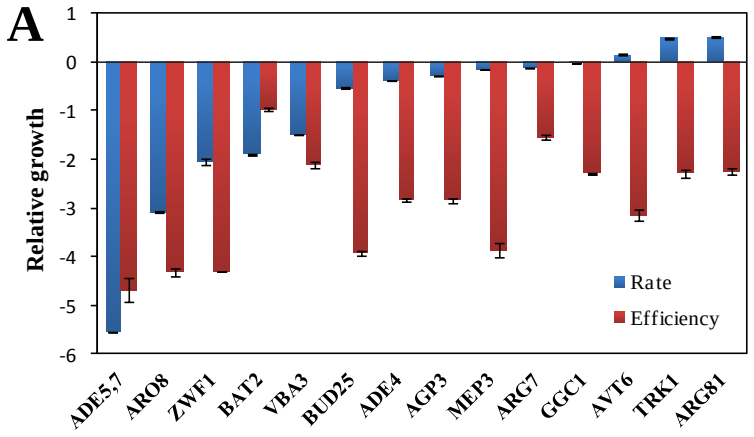
Figure 4. **Tracing the genetic basis of wine strain variations in nitrogen utilization through designed hemizygosity in diploid hybrids.** A) Principles for hemizygosity analysis. Gene deletions corresponding to individual genes annotated based on lab strain experimentation as involved in nitrogen utilization (Table 1 – Annex I) was obtained from the BY4741 gene deletion collection. These were individually crossed, using an automated procedure, to haploid versions of each wine strain and diploid hemizygote hybrids were selected using reciprocal antibiotics selection. The hemizygotes, carrying only the wine strain allele of each individual targeted gene, was cultivated in nitrogen conditions of interest, and compared to a heterozygotic diploid hybrid control carrying both the BY and wine strain allele. Deviations identify cases of haplosufficiency/haploproficiency as well as wine strains alleles encoding inferior or superior nitrogen utilization. B) Capacity of PDM x BY4741 diploid hybrids hemizygotic for individual nitrogen utilization genes (n=228) to utilize a variety of nitrogen sources. Log_2 of the asexual fitness components (generation time, yield and lag phase) were normalized to the corresponding measure of the heterozygotic diploid hybrid control to produce a relative measure of nitrogen utilization capacity. Heatmap colour indicates performance: green = inferior, red = superior and black = equal performance of the hemizygote to the heterozygotic control. The right panel shows sample growth curves of hemizygotic hybrids that are affected with respect the heterozygotic diploid hybrid control (black line). Gene names indicate hemizygotic genes. C) List of wine alleles (genes) which showed impaired growth in hemizygosity

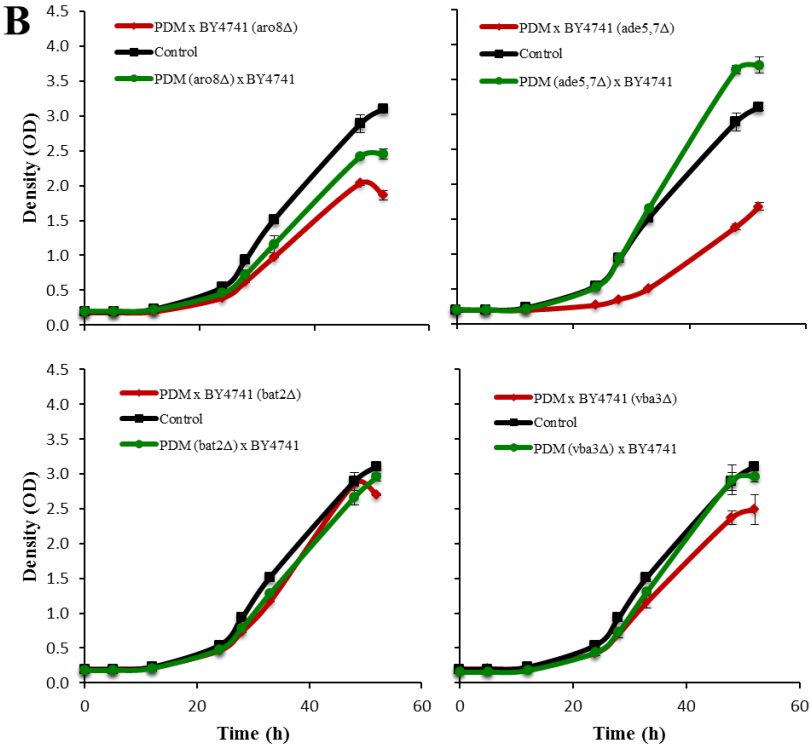
3.4. Incapacity of the PDM wine strain to utilize methionine is due to defects in *ARO8, ADE5,7* and *VBA3*

The genes underlying the hemizygozity associated defects in the wine-lab strain hybrid may be due to haploinsufficiency, which implies that one gene copy is not enough to maintain proliferation. Although interesting, such cases have no direct implications for wine yeast optimization for wine production. However, hemizygote defects may also be because the wine strain allele encodes an inferior gene product. Such impaired alleles are candidates for the molecular genetics-mediated optimization of wine yeast to enhance nitrogen utilization. It is possible to distinguish between haploinsufficiency and wine strain allele defects using reciprocal hemizygosity; i.e., comparing two hemizygotic diploid hybrids in which the two parental alleles of a candidate gene have been reciprocally deleted. To identify the genetic defects underlying the incapacity of the PDM wine strain to utilize methionine, we constructed reciprocal hemizygotes for four of the most promising candidate genes (Fig. 5A). The PDM methionine utilization defect was of specific interest as the aberrant utilization of methionine may contribute to the accumulation of the foul tasting and smelling sulfur intermediates in this strain [39], which may limit its value in wine production. Of the four candidate genes, *ARO8*, *ADE5,7*, *BAT2* and *VBA3*, two (*ARO8* and *BAT2)* encode the aminotransferases in the methionine salvage pathway, *ADE5,7* encodes an enzyme involved in purine biosynthesis, and *VBA3* is an amino acid transporter which facilitates the uptake of amino acids into the vacuole.

Despite comparing the methionine-based growth of the reciprocal hemizygotes in 50mL cultures, to better mimic real wine production conditions, we were unable to confirm the defect of the *BAT2* hemizygote, identifying it as a micro-cultivation specific effect (Fig. 5B). Micro-cultivation differs from 50mL E-flask cultures in several aspects, including lower oxygenation in the wells, potential acidification due to restricted CO_2 efflux and restrictions on nutrient dispersion, all of which is

reflected in strong activation of the general stress response system (Warringer and Blomberg, 2003). This may explain the absence of the *BAT2* phenotype in 50mL E-flasks. In contrast, for *ARO8*, *ADE5,7* and *VBA3*, the hemizygote defects were conserved, but less pronounced than in the micro-cultivation set up. In all these cases, the hemizygotes carrying the wine strain allele performed worse than the hemizygotes carrying the lab strain allele (Fig. 5B). This established a direct causality between the PDM alleles of *ARO8*, *ADE5,7* and *VBA3* and the incapacity of PDM to utilize methionine. To identify the SNPs which potentially underlie these allelic defects, the PDM alleles were sequenced and aligned to published lab and wine strain sequences. PDM *ARO8* were found to contain six SNPs, of which only one was non synonymous, *aro8*(K7R) (Fig. 5C). *aro8*(K7R) was also present in the genetically similar wine strain QA23, but was absent in other sequenced wine strains. Of the two SNP's in the PDM *ADE5,7* allele, A107V was non synonymous and private to PDM (Fig. 5C). The PDM *VBA3* allele was sequence identical to all the other analyzed strains, implying cis-regulatory elements underlying PDM defects. In summary, we implicated PDM *ARO8*, *ADE5,7* and *VBA3* to be causally linked to the incapacity of PDM to utilize methionine. We also identified non synonymous SNPs in *ARO8* and *ADE5,7* as good candidate targets for biotechnological efforts to alleviate the methionine defect.





C

	ADE5,7	ARO8
S288c	100 KAAQLE**A**SKA 110	1 MTLPES**K**DFSYLFSD 15
PDM	100 KAAQLE**V**SKA 110	1 MTLPES**R**DFSYLFSD 15
QA23	100 KAAQLE**X**SKA 110	1 MTLPES**R**DFSYLFSD 15
T7	100 KAAQLE**A**SKA 110	1 MTLPES**K**DFSYLFSD 15
T73	100 KAAQLE**A**SKA 110	1 MTLPES**K**DFSYLFSD 15
Kyokai	100 KAAQLE**A**SKA 110	1 MTLPES**K**DFSYLFSD 15

Figure 5. Inability of the PDM wine strain to utilize methionine is due to defects in the *ARO8, ADE5,7* and *VBA3* genes. A) Log_2 rate and efficiency of PDM x BY4741(ΔX) hemizygotes with deficient methionine utilization. Log_2 measures were normalized due to the corresponding measures of the heterozygotic PDM x BY4741 hybrid control. Negative values indicate poor performance. The mean of 3 independent replicates are displayed. B) To separate haploinsufficiency effects from defects in PDM alleles, reciprocal hemizygotes of the four candidate genes *ARO8*, *ADE5,7*, *BAT2* and *VBA3* were constructed. PDM gene copies were removed by targeted deletions and the nitrogen utilization capacity of PDM x BY4741(ΔX) and PDM (ΔX) x BY4741 were directly compared. The heterozygote hybrid control is included as a reference. Change in optical density was measured manually. Error bars = SEM. C) Non-synonymous mutations distinguishing the PDM alleles of *ADE5,7* and *ARO8* from their S288c counterparts and different wine strains. Variations were revealed by targeted sequencing of each locus.

4. Discussion

4.1. Nitrogen source utilization differs systematically between wine yeast and the lab strain

The fermentation rate and growth rate at all the wine yeast fermentation stages positively correlated with both the nitrogen uptake rate and the total amount of assimilated nitrogen (Ambroset *et al*., 2011; Gutiérrez *et al*., 2012). Thus, the selection, evolution or breeding of yeast strains that are able to utilize all the available nitrogen sources with a maximum rate and efficiency and a minimum lag has the potential to substantially increase the fermentation capacity in wine production. In this work, we developed a strategy to assess the ability of four commercial wine strains to utilize all the low complexity nitrogen sources supporting yeast growth and we observed a substantial quantitative variation between both strains and sources. At the most superficial level, and when only considering the growth rate, source variations approximately agreed with a crude classification into “slow”, “intermediate” and “fast” nitrogen sources, as previously

established when considering lab strain Σ1278b (Godard *et al*., 2007). The main determinant of this categorization is believed to be the carbon derivatives resulting from the catabolism of these compounds (Godard *et al*., 2007). Whereas transamination or deamination of "fast" sources produces C-compounds directly assimilable by metabolism, the transamination of "slow" sources leads to keto-acids, which are converted into complex alcohols. However, this division into discrete categories is clearly artificial as the nitrogen sources followed an uninterrupted continuum in terms of their ability to support fast reproduction. Furthermore, a close look at the data revealed marked differences between the wine strains and the lab strain, and the most outstanding deviation was the excellent ability of urea and allantoin to support fast wine strain growth. This is somewhat surprising given that urea and allantoin are not present in grape must, but are the two main nitrogen secretion products of animals. Together with the fact that wine strains are also poorly adapted to utilize proline, the most prevalent nitrogen source in wine (Salmon and Barre, 1998), this casts doubts about the extent to which wine yeasts are actually adapted to wine must. In general, yeast phenotypes tend to follow a population structure rather than the classifications based on source environment from which yeast is isolated (Warringer *et al*., 2011). This suggests that they are a consequence of either a genetic drift or a selection in ecological contexts other than the niches they currently occupy. The life history of yeast, with outcrossing being rare, and with frequent and narrow population bottlenecks, may indeed mean that it is especially prone to accumulate population-specific alleles through a genetic drift (Zörgö *et al*., 2012). When considering the efficiency of wine strain nitrogen source utilization, the established picture of nitrogen source suitability offers even less predictive power. Many slow nitrogen sources, notably phenylalanine, leucine and citrulline, were efficiently utilized, whereas fast nitrogen sources, such as aspartate, glutamine and glutamate, were less efficiently employed. The distinction between rate and efficiency is important because, in wine production, the growth rate is typically of less importance than the final yield

achieved (Varela *et al*., 2004). Wine yeasts are supplied to oenologists in a dehydrated form and must be rehydrated prior to inoculation in grape must. Considering the lag time before growth takes off essentially reflects the time required to leave the latent state after rehydration and to produce sufficient metabolic and ribosomal proteins to sustain growth, which further complicates the picture. Urea, proline, ammonium and glutamine were metabolized with slight delay, whereas tryptophan, leucine, methionine and citrulline required almost one full re-adjustment day. Although the lag time in wine production has received little attention (Novo *et al*., 2003), it may be of substantial importance because every time delay in yeast growth is an opportunity for competing microorganisms to take over and spoil fermentation.

4.2. Natural variation in nitrogen utilization is strain-dependent and linked with casual mutations

The individual analysis of growth also revealed anomalous behaviors in different strains. The most remarkable strain-specific difference was detected in the PDM strain, which was almost completely incapable of utilizing methionine, a nitrogen source which offers otherwise better suitability to the other wine strains. Methionine is a key player of intermediary metabolism which is not only involved in protein synthesis, but is also an essential determinant of the one-carbon metabolism. Indeed in its activated form, *S*-adenosylmethionine (AdoMet) acts as the methyl donor in hundreds of transmethylation reactions of nucleic acids, proteins or lipids (Thomas *et al*., 2000). Thus, a defect in methionine utilization, leading to elevated intracellular pools of methionine and AdoMets, can potentially affect a large number of reactions. PDM showed several other nitrogen sources utilization defects, including inefficient utilization of threonine, and remarkably, the slow utilization of leucine. The threonine metabolism is interconnected with the methionine metabolism by the common intermediate O-acetyl L-homoserine, and

several enzymes of the biosynthesis pathways are regulated by methionine or its derivatives (Thomas and Surdin-Kerjan, 1997). Thus, it is not unreasonable to speculate that the methionine and threonine defects may be genetically and molecularly linked. In fact, most hemizygote strains which showed a growth defect in either of these nitrogen sources harbored wine single alleles of the sulfur amino acid metabolism (Fig. 4C). The PDM strain, and its commercial derivatives, is one of the most important genotypes in the wine industry despite its high H_2S production in certain wine fermentation circumstances. H_2S is a necessary intermediary in the synthesis of sulfur amino acids from sulfate. However, if not catabolized, it probably becomes a major wine production problem because of its poor organoleptical properties. Cordente *et al*. (2009) obtained low H_2S-producing strains deriving from the commercial PDM by random mutagenesis. These low H_2S-producing strains harbored specific mutations in the *MET10* and *MET5*, which encoded the catalytic α - and β - subunits of the sulfite reductase enzyme, and they were auxotroph for methionine. Besides the deficiencies of the PDM strain, we also found nitrogen source utilization defects in all three commercial strains considered. Thus, there are ample opportunities for optimizing the nitrogen source utilization capacity of all these strains to potentially improve their suitability for industrial wine fermentation. Defects were private to each strain, meaning that they are unlikely to be the products of adaptations to the industrial process *per se*. This is important because their correction should not elicit any immediate negative influences on other phenotypes of industrial importance through the antagonistic pleiotropy relating to the gene products involved. It also suggests a possible way forward to construct commercial strains that lack these deficiencies to help face the challenges of GMO restrictions that preclude targeted genetic manipulations. As defects are caused by recessive loss-of-function mutations, which appear to be the source of the vast majority of phenotypic variations in yeast (Zörgö *et al*., 2012), the hybridization of haploid derivatives of different commercial strains to yield fully heterozygotic diploids should compensate for the respective genetic defects

through reciprocal masking. Strains readily sporulated, and the mixing of spores from two different backgrounds should result in a fraction of hybrid diploids that can be selected for their phenotypic superiority in terms of nitrogen traits.

4.3. Defects in the PDM alleles of ARO8, ADE5,7 and VBA3 underlie the inability to utilize methionine

However, any effort into strain optimization for wine production would benefit from prior knowledge of the underlying genetics. The wine strains herein investigated do not easily lend themselves to QTL mapping; instead, we utilized a naive, large-scale hemizygote approach to test whether any of 228 genes known to be involved in nitrogen utilization can harbor wine strain polymorphisms causing phenotypic deficiencies. Although this approach allows for the rapid generation of large sets of hemizygotes, through the mating of the BY deletion collection to a haploid wine strain derivative, it does not immediately distinguish between defects to haploinsufficiency, i.e., retention of only a single gene copy, and defects due to polymorphisms. However, given the scarcity of hemizygote defects, <5% (10 of 228) in the case of the PDM methionine defects, despite the gene products being directly involved in the molecular process targeted, this should be of less concern. We conclude that even in challenging nitrogen-limited environments, retention of a single copy of nitrogen metabolism-related genes is almost always enough to maintain nitrogen-dependent functionalities unperturbed. This has several important implications. First, it supports and extends the observations from lab strains in optimal environments (Deutschbauer *et al*. 2003) that haploinsufficiency is remarkably rare. This also agrees with functional alleles tending to completely dominate non functional alleles in yeast hybrids (Zörgö *et al*., 2012). This is not because of the compensatory induction of the remaining gene copy, but because half the normal production of a gene product suffices to support proliferation (Springer *et al*., 2010). It also suggests that such a naive approach to understanding

genetic variation, circumventing QTL mapping and fine mapping of QTLs, and focusing directly on allele phenotype interactions, are a viable alternative in yeast genetics. This shifts the burden of work from strain construction to phenotypic screening.

We selected 4 of the 14 gene candidates for causing the methionine defect, and found that 3 of these corresponded to true defects in wine strain alleles. Of the three alleles, *ARO8*, *ADE5,7* and *VBA3*, herein identified as contributing to the methionine utilization deficiency of PDM, *ARO8* is the only one with a clear, direct connection to methionine metabolism. This gene encodes a transaminase of the methionine salvage pathway, together with Bat2, herein initially identified, but not confirmed as a candidate, and Aro9 and Bat1. At low methionine concentrations, these enzymes transfer the amino group from an amino acid to 2-oxo-4-methylthiobutanoate, resulting in the production of a ketoacid and methionine (Pirkov *et al*., 2008). The methionine salvage pathway comprises a set of complex reactions that allows the direct synthesis of methionine from 5'-methylthioadenosine (MTA) (Albers, 2009). Moreover, the first step of this pathway also releases adenine in the metabolism of which the other defective allele, *ADE5,7*, is also involved. Although the salvage pathway has primarily been studied in the methionine synthesis context, it is likely that the use of methionine as a sole nitrogen source, and therefore as a sole amino donor, reverses the flow of this reaction. *ARO8* would then be a key amino transferase by shifting nitrogen from methionine to keto acids in the first step of the methionine catabolic pathway. In this case, the other three transaminases catalyzing this reaction (Aro9, Bat1 and Bat2) are apparently unable to compensate for the *ARO8* defect, potentially because of the various affinities for different ketoacids. It is quite plausible that the *ARO8* defect correlates to excess H_2S production. Dysfunction in the conversion of methionine into other amino acids should increase intracellular methionine. An elevated pool of internal methionine leads to increased homocysteine, and parts of

this excess can be converted into O-acetyl-serine via Met17, with H_2S emerging as a secondary product of this reaction.

The connection of *VBA3* and *ADE5,7* to methionine utilization defects are less clear. *VBA3* is a vacuolar transporter of basic amino acids lysine, histidine and arginine (Shimazu *et al*., 2005), but likely not of methionine, although it has not been tested. If facilitates the vacuolar storing of these amino acids at high concentrations; e.g. >20x the cytoplasmic concentration in terms of arginine (Kitamoto *et al*., 1988), when they are in excess, but it is not known whether it can catalyze the mobilization of these storages when deficiencies emerge. It can be speculated that a deficiency in such vacuolar mobilization of these aminoacids, when their cytoplasmic production from methionine is impaired, can be the mechanistic cause of the wine strain Vba3 allele's contribution to poor methionine growth. Ade5,7 is a bifunctional enzyme that facilitates nucleotide biosynthesis when sufficient nucleotides are not supplied externally. The methionine salvage pathway is also a supplier of purines. Thus, an irregular function of this pathway can increase the synthesis requirement through the *de novo* biosynthesis of purine nucleotides in which the mutated *ADE5,7* allele operates. Interestingly, Ade5,7 deletion strains are also highly sensitive to the sulfite-like metal ion tellurite, a phenotype that is otherwise strongly associated with defects in the methionine metabolism (Ottosson *et al*., 2010). This supports the existence of links to this metabolic pathway.

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CHAPTER 3

Biomarkers for detecting nitrogen deficiency during alcoholic fermentation in different commercial wine yeast strains

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Abstract

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. Several putative biomarkers were tested in order to analyze their appropriateness to detect nitrogen stress in the yeast. To this aim, four commercial wine strains (PDM, ARM, RVA and TTA) were grown in a synthetic grape must with different nitrogen concentrations. Trehalose accumulation, arginase activity and the expression of eleven genes were tested in these wine strains, known to have different nitrogen requirements. The overall response of the four strains was similar, with differences in response intensity (PDM and RVA with higher intensity) and response time (which was also related with nitrogen consumption time). Trehalose response was mostly related to entry into the stationary phase, whereas arginase activity was responsive to nitrogen depletion, although its measurement is too complicated to be used for routine monitoring during winemaking. The expression of the genes *DAL4*, *DAL5*, *DUR3* and *GAP1* was clearly related to nitrogen depletion and thus, *GAP1* and *DAL4* were selected as markers of nitrogen deficiency. In order to adapt expression analysis to winemaking conditions, the original strains were transformed into reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4*. The transformants had a similar fermentative capacity to the parental strains and were able to detect alterations in yeast physiological status due to nitrogen limitations.

***Keywords**:* nitrogen starvation, arginase activity, gene expression, general amino acid permease, GFP-biosensor, fluorescence reporter genes, yeast assimilable nitrogen

1. Introduction

Nitrogen composition of grape juice is important to ensure good wine fermentation performance. However, yeast cell growth under enological conditions is often considered to be nitrogen limited, which would cause stuck or sluggish fermentations. A low initial nitrogen concentration acts by limiting growth rate and biomass formation of yeast, resulting in a low rate of sugar catabolism (Bisson, 1991). These problematic fermentations have economic relevance because wines with higher residual sugar negatively impact on the quality of the final product. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). However excessive levels of these compounds may have negative consequences, such as microbial contamination or ethyl carbamate formation (Monteiro *et al*., 1989; Ough *et al*., 1988) and undesirable aroma production (Bell and Henschke, 2005). Therefore, it is important to know the nitrogen content of grape juice and the nitrogen requirement for each specific yeast strain.

A mechanism known as Nitrogen Catabolite Repression (NCR) allows the selection of the best nitrogen source for yeast growth. Good nitrogen sources (glutamine, asparagine and ammonium) decrease the level of enzymes and permeases required to use the poor nitrogen sources (Magasanik, 1992). This repression is mainly obtained by inhibiting the transcription of genes required for the use of poor nitrogen sources (Magasanik and Kaiser, 2002). Although NCR regulation during growth on many different nitrogen sources has been investigated in depth (Godard *et al*., 2007), the regulation of this process during fermentation is not well characterized (Deed *et al*., 2011). During wine fermentation, a nitrogen-repressed condition changes to a nitrogen-de-repressed condition as nitrogen is consumed (Beltran *et al*., 2004). This shift in the NCR regulation could be used as a marker to detect nitrogen limitation during grape must fermentation by monitoring transcriptional activity of these NCR genes, mainly comprised by core

nitrogen metabolism genes (*GDH1*, *GDH2*), permease genes (*AGP1*, *DUR3*, *DAL4*, *DAL5*, *GAP1*) and catabolic pathway genes, including those for utilization of proline, arginine and urea (*CAR1*, *PUT2*).

As mentioned above, most of the intracellular pools of amino acids are located in the vacuole (Wiemkem and Durr, 1974), and this is especially significant in the case of arginine. The first reaction in arginine degradation is the conversion of L-arginine into L-ornithine and urea. This reaction is catalyzed by the cytoplasmic enzyme arginase (Jauniaux *et al*., 1978), encoded by the *CAR1* gene (Jauniaux *et al*., 1982). Arginase is also regulated by NCR, arginine concentration and post-transcriptional events (Bossinger and Cooper, 1977; Messenguy and Dubois, 1983). Arginase activity analysis is very useful as it responds to arginine mobilization from the vacuole under nitrogen-limiting conditions (Sumrada and Cooper, 1978). Previous studies (Carrasco *et al*., 2003; Beltran *et al*., 2004; Jiménez-Martí *et al*., 2007) have reported that arginase activity could be a good marker to detect nitrogen depletion in grape must fermentation.

A plethora of stress conditions for yeast cells take place during wine fermentation. The cellular mechanism for controlling these stress situations involves the rapid synthesis of protective molecules and the activation of signal transduction systems that induce the activation of enzyme activities and the transcription of genes encoding factors with protective functions. Of these, trehalose has remarkable stress protection properties and may determine the survival response of yeasts under extreme environmental condition. Several studies have shown the metabolism of trehalose is affected by nitrogen availability; therefore, once the nitrogen in the growth medium is consumed, yeast cells start to accumulate intracellular trehalose (Parrou *et al*., 1999; Novo *et al*., 2005; Hazelwood *et al*., 2009). Thus, trehalose accumulation could also be an indicator of nitrogen limitation conditions during yeast growth during fermentation.

In a recent work (Gutiérrez *et al.*, 2012), we determined the minimum nitrogen amount required by four commercial yeast strains to ensure the maximum

population in the growth or exponential phase in a synthetic grape must. These strains showed different nitrogen requirements, growth and nitrogen uptake rates. In this work, we have tested two biochemical markers such as intracellular trehalose content and arginase activity and a molecular marker based on gene regulation of NCR genes in the same four commercial strains. These wine strains were inoculated in a synthetic grape must with different nitrogen concentrations. Our aim was to select the most suitable marker or sensor for detecting nitrogen limitation by cells growing during wine fermentation. This marker would go beyond determining nitrogen levels in fermenting must, revealing real yeast nitrogen needs and the correct timing and concentration for nitrogen supplementation during wine-making fermentations. Several studies have shown the importance of adding the correct nitrogen supplementation to avoid fermentation problems or off-flavor production as a consequence of both nitrogen shortage and excess (Mendes-Ferreira *et al*., 2009; Torrea *et al*., 2011). We also aimed to develop a very simple and handy method for detecting nitrogen limitation in other wine strains, which could be used in the future to avoid stuck fermentation or inadequate addition in the wine industry.

2. Materials and methods

2.1. Yeast strains and growth conditions

The yeast strains used in this study are the following: PDM, ARM, RVA and TTA, all of them provided by Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com). A taxonomic description of these strains was carried out by RFLPs of the ITS/5.8S region (Guillamón *et al*., 1998). The strains PDM (Pasteur Prise de Mousse), RVA and TTA belonged to species *Saccharomyces cerevisiae*, while we identified the strain ARM as a hybrid between *S. cerevisiae*

and *S. kudriavzevii*, following the procedure proposed by Gonzalez *et al*. (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has been confirmed by Dunn *et al*. (2012). These wine strains were used at an initial population of 2 x 10^6 cell/mL of active dry yeast rehydrated in warm water prior to inoculation, according to the manufacturer's instructions (37ºC for 30 min). Microvinification experiments were carried out using synthetic grape must (SM). This SM was prepared according to Riou *et al*. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). Only the nitrogen content changed in the different concentrations: 140 mg N/L as control condition, 60 mg N/L as limiting condition and 300 mg N/L as non-limiting condition. SM with a very high excess of nitrogen (1200 mg N/L) was also prepared for the selection of the NCR genes. The proportion of ammonium and different amino acids was as described by Beltran *et al*. (2004) and this proportion was maintained for the different nitrogen concentrations.

Fermentations were performed in 250 mL glass bottles containing 200 mL of SM and capped with closures than enabled carbon dioxide to escape and samples to be removed. Fermentations were done in triplicate at 28 ºC with continuous orbital shaking (150 rpm). Cell samples were collected throughout the fermentation at different time points. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at -80ºC. Supernatant was also stored at -20 ºC to analyze the concentration of YAN at different fermentation times. In order to monitor the induction of fluorescence emission, fermentations in Falcon tubes of 50 ml with 40 ml of SM were also performed to carry out a more intensive sampling during the first hours of N-limiting (60 mg N/L) and control (140 mg N/L) fermentations.

2.2. Nitrogen content analysis

Ammonia concentration was measured with a kit using an enzymatic method (Roche Applied Science, Germany). The concentration of free amino acid nitrogen was determined using the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen/mL. The addition of ammonium and amino acids represents the yeast assimilable nitrogen (YAN).

2.3. Trehalose content determination

Intracellular trehalose contents of the four yeast strains were determined following the method described by Parrou and François (1997).

2.4. Arginase activity determination

Crude protein extracts were obtained from the different samples and arginase activity was measured as described in Carrasco *et al*. (2003).

2.5. Gene expression analysis by real-time quantitative PCR

Total RNA was isolated from yeast samples as previously described by Siekstra *et al*. (1992) and resuspended in 50 µL diethylpyrocarbonate (DEPC)-treated water.
To purify total RNA suspensions, contaminant genomic DNA was removed using a High Pure Isolation kit (Roche, Mannheim, Germany) in accordance with the manufacturer's protocol. Purified RNA concentrations were determined using a Nanodrop (ND-1000 Spectrophotometer) and verified electrophoretically on 0.8% agarose gels.

cDNA was synthesised from total RNA using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 9700 (Applied Biosystem); 0.5 µL oligo $(dT)_{12-18}$ primer (Invitrogen) was used with 0.8 µg total RNA as template in a reaction volume of 20 µL. Following the manufacter's protocol, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min. Finally, the reaction was stopped at 70 ºC for 15 min.

Gene expression was determined using the real-time quantitative PCR technique. The primers used in this study are listed in Table 1.

The real-time quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). In the PCR reaction, the final volume was 25 µL, contained 300 nM of each primer, together with 1 µL of the cDNA previously synthesized from total RNA. All PCR reactions were mixed in 96-well optical plates and cycled in a Step One Plus Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles at 95 ºC for 15 s and at 60 ºC for 60 s.

Each sample had two controls, which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔCt is the difference in Ct of the gene of interest and Ct of the houskeeping gene (*ACT1*); and ΔΔCt is the difference in ΔCt at time = t and ΔCt at time = 3 hours in the non-limiting condition (300 mg N/L). All samples were analyzed in triplicate and the expression values were the average calculated by the analysis software (Applied Biosystems, USA).

Table 1. Primers used in this study

Gene	Name	Oligonucleotide sequence (5'-3' end)
GAP1 disruption cassette *	GAP1m-F	ACAGACCAAGGACAGCAACATTTATAAGAAACAAA AAAAAGAAATAAAAAATGTCTAAAGGTGAAGAATT
	GAP1m-R	TATTATGATTATCTAAAAAATAAAGTCTTTTTTTGTC GTTGTTCGATTCACTAGTGGATCTGATATCATC
GAP1 PCR verification	GAP1c-F	GATTGTTAAATGTCAGTTTGG
	GAP1c-R	TTGAAGCTCACACAGATTAGT
DAL4 disruption cassette *	DAL4m-F	CTTTTATATTCATCTACATCTTGTGATATAAAACATC AACAAAGACGAGAATGTCTAAAGGTGAAGAATT
	DAL4m-R	TATCAATTCTCCTGATCACTACTGGTAAGAGAATAA ATTGGGATTTTTATCTAGTGGATCTGATATCATC
DAL4 PCR verification	DAL4c-F	ACATTTGCGCCTATTCGATG
	DAL4c-R	TATTCAGACGGGAATGCATG
ACT1 primer real-time PCR	ACT-F	TGGATTCCGGTGATGGTGTT
	ACT-R	CGGCCAAATCGATTCTCAA
AGP1 primer real-time PCR	AGP1-F	CGCCATATGTCATTGCTGTTG
	AGP1-R	CATGGACAGCACGGAAAGTAGA
CAR1 primer real-time PCR	CAR1-F	TGGGTATCGCCGCCTTT
	CAR1-R	TGACAGCGTTGATGCCGTAT
DAL4 primer real-time PCR	DAL4-F	GGCTCCTCATAAAATCAGGCATT
	DAL4-R	CCGTGCGATTTCTTCAAAGC
DAL5 primer real-time PCR	DAL5-F	AGCATGTCTTGCGGTGGAA
	DAL5-R	GGAATTCGCACTGATATTGGAAA
DUR3 primer real-time PCR	DUR3-F	AGCATGTCTTGCGGTGGAA
	DUR3-R	TTTGCCTGGAACGAAGTAAGTG
GAP1 primer real-time PCR	GAP1-F	CTGTGGATGCTGCTGCTTCA
	GAP1-R	CAACACTTGGCAAACCCTTGA
GDH1 primer real-time PCR	GDH1-F	CACCATCTCTGGTAGTGGTAACGT
	GDH1-R	ACGACAGTACCACCTAGCTCAATAAC
GDH2 primer real-time PCR	GDH2-F	TTGTGGAGGGTGCCAATCTATT
	GDH2-R	AGCCATGTTCCTCCAAAGCA
HSP26 primer real-time PCR	HSP26-R	CCTTGCCAGTAGAATCCTTTGC

	HSP26-F	GGGTGAAGGCGGCTTAAGAG
PUT2 primer real-time PCR	PUT2-R	CGCTTTTCCCGCAGACTAAC
	PUT2-F	GCTTGTGTGACGTTTGCCAGTA
RTN2 primer real-time PCR	RTN2-R	CGGACCTTGTTGGCGTCTT
	RTN2-F	CGCAGGCAGACGATCCA

* Underlining indicates homology to the yEGFP-kanMX4 cassette from plasmid pKT-127. The remaining sequences of the primers are homologous to the flanking region of the *GAP1* or *DAL4* open-reading frame.

2.6. Construction of Green Fluorescence Protein (GFP) reporters

One copy of the open reading frame (ORF) of the genes *GAP1* and *DAL4* were replaced in the four strains by the deletion cassette GFP-*KanMX4* by using the short flanking homology (SFH) method (Güldener *et al*., 1996). The plasmid pKT127 (Sheff and Thorn, 2004) was used as template to obtain this deletion cassette (primers shown in Table 1). *S. cerevisiae* transformation was carried out using the lithium acetate method (Gietz and Woods, 2002). Transformants were selected by resistance to geneticin. The correct integration of the deletion cassette was confirmed by PCR using primers upstream and downstream of the cloning site. Moreover, the fluorescence emission of the transformants was also tested after an overnight culture in SD medium (glucose 20 g/L, yeast nitrogen base YNB 1.7 g/L, ammonium sulphate 5 g/L).

2.7. Fluorescence microscopy and flow cytometry

Cells were harvested during fermentation at different time points to detect fluorescence by microscope and flow cytometry.

Fluorescence images were acquired with a Nikon Eclipse 90i epifluorescence microscope by using a 40X objective with a GFP filter (B-2E/C). Bright-field images were used to focus on the cells and the Nikon Digital Sight DS-5Mc captured the images. NIS-Elements software was used to control the camera and image acquisition and to analyze images.

For flow cytometry, samples were harvested by centrifugation, frozen in liquid nitrogen and stored at -80ºC until analysis. The cells were rinsed with sterile phosphate-buffered saline and measured in the cytometer (Beckman Coulter Epics XL Flow Cytometer, Minessota). A total of 20000 cells of the sample were measured at a 700V voltage in FL1 FITC, revealing the number and percentage of fluorescent cells and fluorescence intensity. The EXPO 32 ADC software was used for these measurements. The freezing process during cell storage did not affect their fluorescence emission (data not shown). The parameters measured with the cytometer were the number of fluorescent cells and the average of fluorescence intensity. The arbitrary value of fluorescence used to compare among strains and nitrogen conditions was obtained by applying the formula: (number of fluorescent cells x the average of fluorescence intensity)/1000.

3. Results

The aim of this study was to determine the most appropriate biomarker to be used during alcoholic fermentation to indicate the state of the cells in relation with the quantity of nitrogen. Furthermore, it is interesting to know how these strains respond to progressive nitrogen depletion, when they detect insufficient nitrogen and activate a mechanism to combat this problem. Our approach was to monitor trehalose content, arginase activity and gene expression throughout the alcoholic fermentation in a synthetic grape juice mimicking an enological environment. Fermentations with three nitrogen concentrations were used: a limiting concentration (60 mg N/L) which leads to a sluggish fermentation, the minimum concentration (140 mg N/L) required by the strains to complete fermentation (control fermentation) and a non-limiting nitrogen concentration (300 mg N/L).

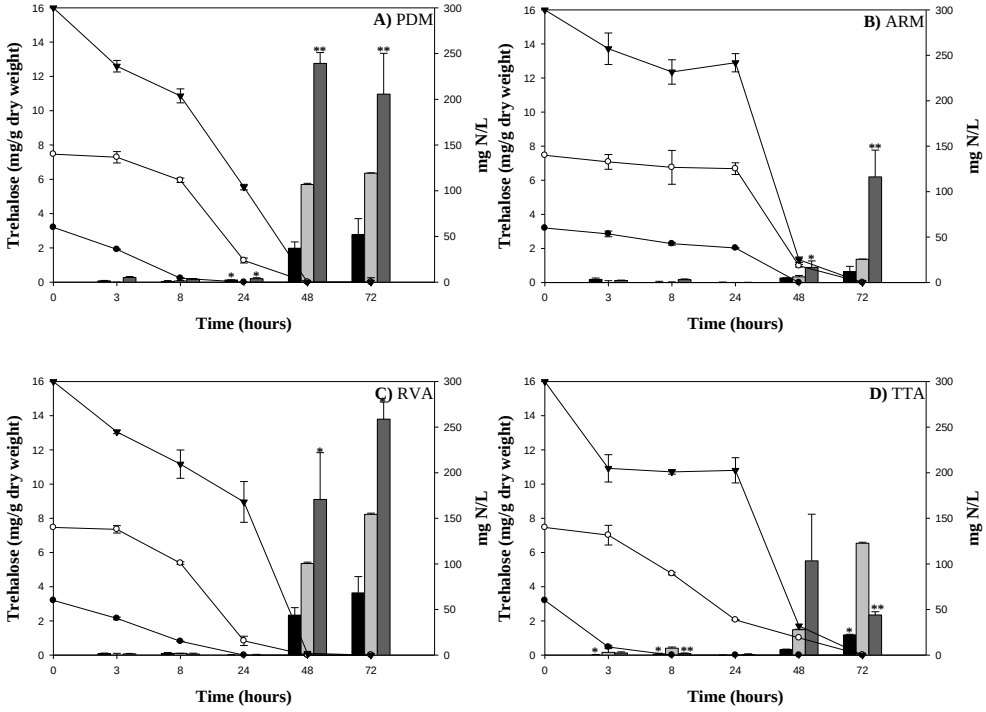
3.1. Biochemical markers

Time course of intracellular trehalose content (Fig. 1) and arginase activity (Fig. 2) of the four commercial wine strains were determined in the first days of SM fermentation. Nitrogen (YAN) uptake for the different fermentations is also shown in these figures (Fig. 1 and 2). Both PDM and RVA strains needed 24 and 48 hours for nitrogen consumption in the N-limiting and control fermentations respectively. Total nitrogen was also exhausted in 48 hours in the non-limiting or excess condition. The ARM strain showed a slower uptake, requiring 48 hours for nitrogen depletion in the N-limiting condition and 72 hours for the control and non-limiting fermentations. Intriguingly, the TTA strain was the quickest to consume nitrogen in the limiting condition (8 hours) but also needed 72 hours for nitrogen depletion in the control and non-limiting fermentations.

Regardless of the nitrogen concentration, trehalose accumulation started 48 hours after yeast inoculation (Fig. 1). Generally speaking, trehalose synthesis matched with the entrance into the stationary phase (data not shown) and, unexpectedly, trehalose concentration was directly correlated with nitrogen content in the SM. Thus, the higher the nitrogen content, the more trehalose accumulated. However, some differences were detected among strains in the trehalose concentration reached in the studied time-lapse. The PDM and RVA strains reached a trehalose concentration twice that of the ARM and TTA strains.

Conversely to trehalose content, arginase activity was inversely proportional to nitrogen concentration. With some exceptions, this activity started before in the N-limiting condition, mainly when ammonium was consumed (data not shown), and reached higher values than in the control or non-limiting conditions (Fig. 2). In fact, very scarce or null activity was detected for the samples taken from the non-limiting condition, with the exception of the PDM strain. Inter-strain comparison showed that RVA and ARM strains reached the highest values of arginase activity after 48 hours of yeast inoculation in the limiting and control fermentation

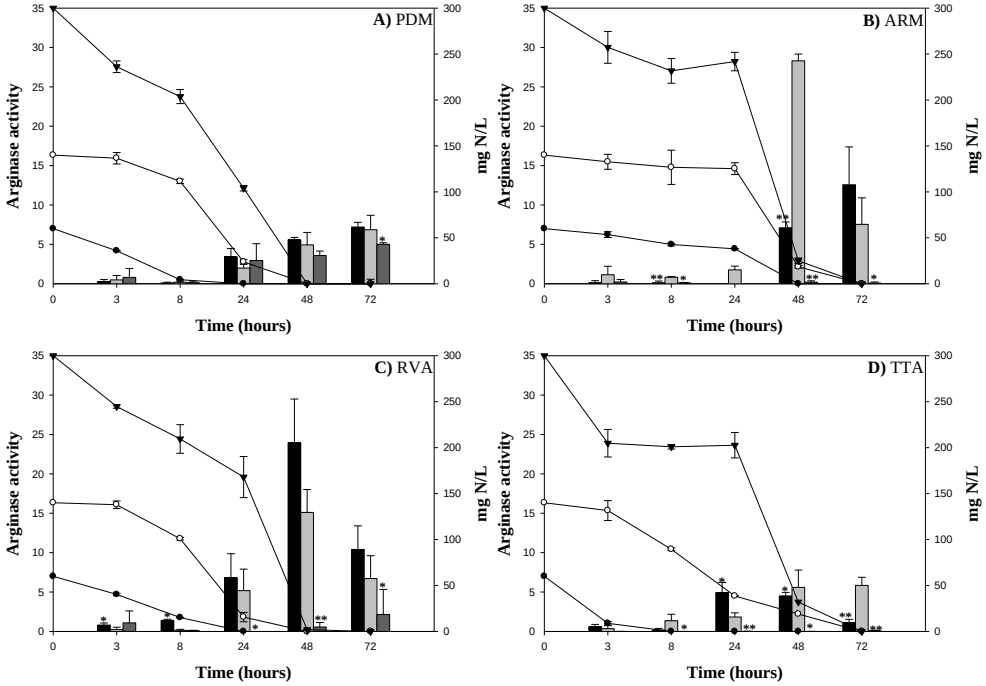
respectively. On the contrary, PDM showed no significant differences in activity at the different time-points or conditions.



***** Significance from t- Student with P≤0.05 compared with the control condition (140 mg N/L)

******Significance from t- Student with P≤0.01 compared with the control condition (140 mg N/L)

Figure 1. Intracellular content of trehalose and yeast assimilable nitrogen (YAN) consumption of four yeast strains: **A**) PDM, **B**) ARM, **C**) RVA and **D**) TTA, during fermentation with different nitrogen concentration: 60 mg N/L (*black bars*/*filled circles*), 140 mg N/L (*grey light bars*/*open circles*) and 300 mg N/L (*grey dark bars*/*filled triangles*). The error bars indicate the standard deviation of three replicates



***** Significance from t- Student with P≤0.05 compared with the control condition (140 mg N/L)

******Significance from t- Student with P≤0.01 compared with the control condition (140 mg N/L)

Figure 2. Arginase activity profile and nitrogen consumption of four yeast strains: **A)** PDM, **B)** ARM, **C)** RVA and **D)** TTA, throughout 60 mg N/L (*black bars*/*filled circles*), 140 mg N/L (*grey light bars*/*open circles*) and 300 mg N/L (*grey dark bars*/*filled triangles*) fermentations. Arginase activity was expressed as: nmol ornithine/µg protein x min

3.2. Molecular markers

An initial screening was performed of putative genes, which may predict nitrogen deficiency during wine fermentation. Most of these genes have been classified as NCR sensitive genes (Godard *et al*., 2007; Deed *et al*., 2011) or, without being directly related to nitrogen metabolism, show significant up-regulation under nitrogen limitation and starvation (Mendes-Ferreira *et al*., 2007; Jiménez-Martí *et*

al., 2007). Gene activity was monitored at several time-points after yeast inoculation (PDM strain) under two fermentation conditions: one N-limiting (60 mg N/L) and one with very high excess nitrogen (1200 mg N/L), which exerted a clear repression of the nitrogen sensitive genes (Fig. 3).

The array of screened genes comprised genes involved in central nitrogen metabolism (*GDH1*, *GDH2*), permease genes (*AGP1, DUR3, DAL4, DAL5, GAP1*), genes involved in utilization of poor nitrogen sources such as arginine and proline (*CAR1*, *PUT2*) and two genes involved in environmental stress response (ESR) (*HSP26* and *RTN2*) (Gasch *et al*., 2000). The ESR genes did not show a clear correlation with nitrogen concentration. *HSP26* was strongly up-regulated after 24-48 hours of yeast inoculation under both nitrogen conditions and this increase could be related with entry into the stationary phase, as previously described (Zuzuarregui and del Olmo, 2004). The central nitrogen metabolism genes *GDH1* and *GDH2* or the proline and arginine utilization genes *PUT2* and *CAR1* did not show any clear change in expression in response to the low nitrogen concentration. Lastly, the permeases *DAL4*, *DAL5*, *DUR3* and *GAP1* seemed to be good candidates for predicting nitrogen deficiency because they were strongly up-regulated in response to nitrogen starvation and were kept repressed in the presence of excess nitrogen in the fermentation medium. *AGP1* was the only analyzed permease gene that did not clearly respond to nitrogen concentration. Finally, we selected the *DAL4* (allantoin permease) and *GAP1* (general amino acid permease) because they displayed the highest induction values (ranging from 100 to 400 fold). The other criterion for selecting these genes is based on the fact they belong to the two categories of NCR sensitive genes, according to the classification by Godard *et al*. (2007): genes whose transcription is exclusively regulated by NCR such as *GAP1* and genes which are subjected to other transcriptional regulation in addition to NCR such as *DAL4*, which is also inducible by allophanate (a product of urea degradation).

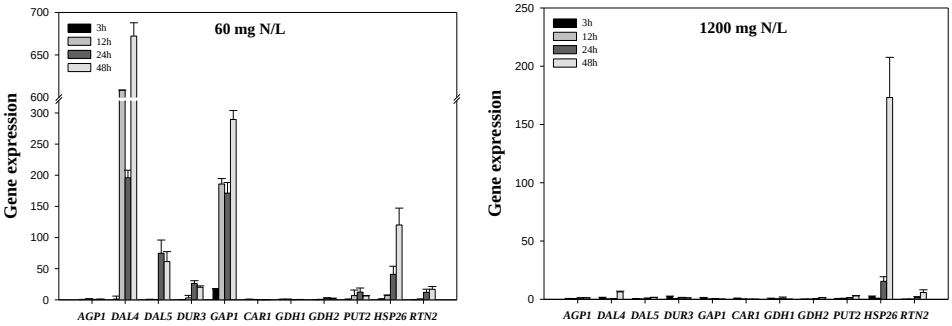
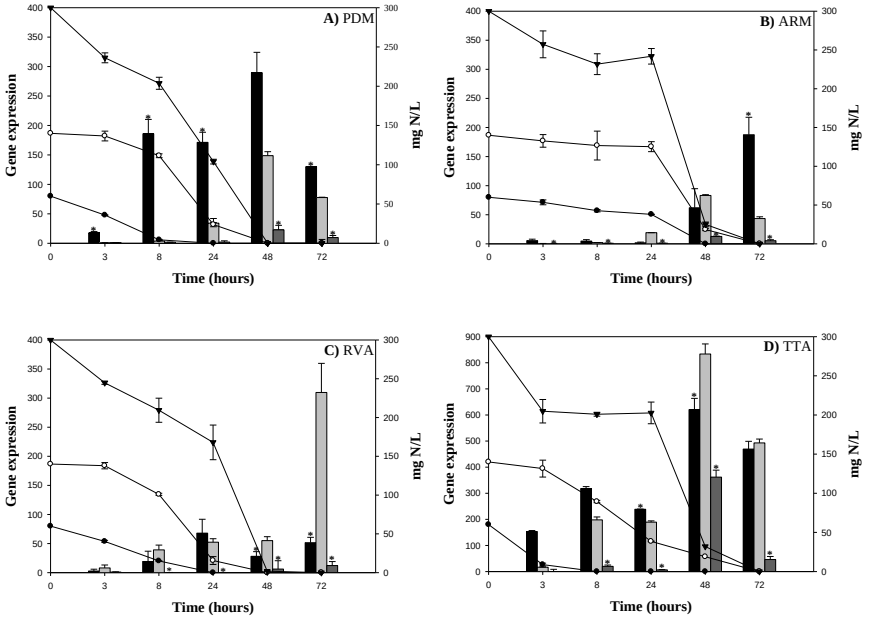


Figure 3. Changes in gene expression of the PDM strain throughout alcoholic fermentation in two nitrogen concentrations: 60 mg N/L and 1200 mg N/L. The genes considered are: *AGP1* (amino acid permease with high glutamine affinity), *DAL4* (allantoin permease), *DAL5* (allantoate permease), *DUR3* (transporter of urea), *GAP1 (*general amino acid permease), *CAR1* (arginase enzyme), *GDH1* ($NADP^+$ dependent glutamate dehydrogenase), *GDH2* (NAD^+ dependent glutamate dehydrogenase), *PUT2* (proline permease), *HSP26* (heat shock protein) and *RTN2* (reticulum protein involved in ER morphology). The values are expressed relative to their expression at the time of lowest expression (1200 mg N/L fermentation at 3h), and normalized with the concentration of the housekeeping gene (*ACT1*)

Thereafter, we analyzed the transcriptional activity of the selected genes in the four commercial strains and under the same nitrogen conditions as those used for the biochemical markers (Fig. 4). All the transcriptional changes are expressed relative to the point with lowest expression (3 h after yeast inoculation in the 300 mg N/L condition). The four strains showed a transcriptional profile for both genes correlated to nitrogen concentration. Both genes were activated/de-repressed in limited and control fermentations when ammonium was depleted, even though the YAN concentration in the media was still important.

A) *GAP1*


A) PDM
B) ARM
C) RVA
D) TTA
Gene expression
mg N/L
Time (hours)

B) *DAL4*

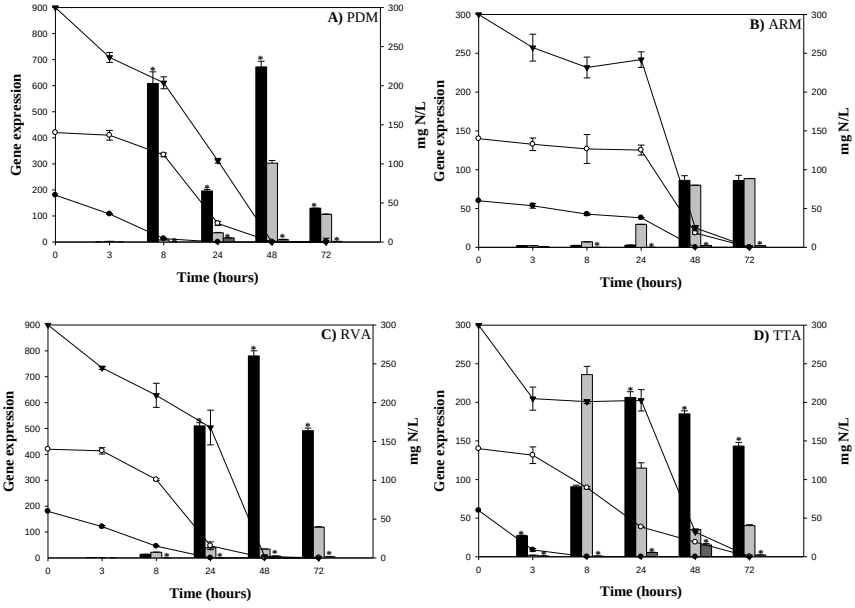

A) PDM
B) ARM
C) RVA
D) TTA
Gene expression
mg N/L
Time (hours)

Figure 4. Changes in gene expression of *GAP1 (*general amino acid permease) and *DAL4* (allantoin permease), during fermentations with different nitrogen concentrations: 60 mg N/L (*black bars*), 140 mg N/L (*grey light bars*) and 300 mg N/L (*grey dark bars*). The values are expressed relative to their expression at the time of lowest expression (300 mg N/L fermentation at 3h), and normalized with the concentration of the housekeeping gene (*ACT1*). YAN consumption throughout the fermentations is also indicated (60 mg N/L *filled circles*; 140 mg N/L *open circles*; 300 mg N/L *filled triangles*). *****Significance from t- Student with $P \leq 0.01$ compared with the control condition (140 mg N/L)

Generally, *GAP1* was first up-regulated in the N-limiting condition, in line with ammonium consumption, whereas scarce or null transcriptional activity was detected in the non-limiting condition. These induction values steeply increased after total nitrogen depletion. It is worth mentioning that, as in the case of the biochemical markers, the ARM strain showed a delay in the transcriptional induction as a consequence of slower nitrogen consumption. Regarding *DAL4*, similar conclusions can be drawn. This gene was also induced earlier in the N-limiting condition and was kept repressed in the non-limiting condition during the time-lapse studied. Again the ARM strain showed delayed transcriptional induction with smaller values than the other strains. Generally speaking, nitrogen consumption correlates well with gene induction.

3.3. Rapid method to detect nitrogen deficiency

Once the transcriptional activity of *GAP1* and *DAL4* was revealed as a good tool to predict nitrogen deficiency, we also aimed to simplify this method to convert it into a rapid, cheap and accurate test. We constructed reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4*.

One allele of these genes was replaced by the GFP gene in the four commercial strains. No differences were detected in terms of growth, fermentation activity and nitrogen consumption in these reporter strains versus the commercial strains (data not shown).

The reporter strains were inoculated in SM with the same nitrogen concentrations previously used. Samples from these fermentations were taken in the first hours of fermentation for further fluorescence analysis. In order to validate the correct construction of the reporter strains, we observed the emission under fluorescence microscopy after 48 hours of yeast inoculation. As an example, Figure 5 shows bright-field and fluorescence images of the ARM strain under the different fermentation conditions. As expected, the lower the concentration of nitrogen, the greater the number of fluorescent cells and the stronger the fluorescence intensity. Quantitatively, this result was corroborated by the flow cytometry histograms, also shown in Figure 5.

However, the fluorescent intensities were different in the two reporter genes studied. The *GAP1* reporter yielded stronger intensity, indicating higher protein production than *DAL4*, which showed duller fluorescence. Therefore, as the transcriptional behavior was very similar between both permeases, only the results obtained with the *GAP1* reporter in the four strains are shown (Fig. 6 and Fig. 7). The transcriptional (Fig. 4) and the fluorescence profiles (GFP synthesis) (Fig. 6) in the same fermentation conditions were very similar. Nevertheless, the induction of fluorescence was observed later than the induction of gene activity. This delay represents the time-lapse between transcription and translation. However, this reasoning did not hold for the ARM strain, which showed an earlier induction of fluorescence. Another surprising result between transcription and GFP synthesis was the lack of fluorescence observed in the control condition (140 mg N/L) of the TTA strain, because *GAP1* gene showed early induction.

These results led us to design a new experiment to monitor the induction of fluorescence emission when cells of each strain detected nitrogen deficiency in

their environment. We carried out more intensive sampling during the first hours of N-limiting and control fermentations. These data of fluorescence in each individual strain are plotted against the nitrogen consumption (Fig. 7). In comparison with data obtained in the former experiment (Fig. 6), although common trends in fluorescence induction and intensity can be observed, clear differences were detected for the same strain and N condition. The different fermentation conditions and the intensive sampling changed the fermentation kinetics, specially the nitrogen uptake, which may impact on the expression of this reporter gene. In the N-limiting fermentation, strains RVA and TTA emitted fluorescence 10 h after yeast inoculation whereas strains PDM and ARM increased this fluorescence after 14 and 15 h respectively. In terms of intensity, PDM showed a much higher intensity than the other strains. In the control condition, the earliest fluorescence emission was detected in RVA and PDM strains. As in previous results, strain TTA did not emit fluorescence in the first 32 h of fermentation.

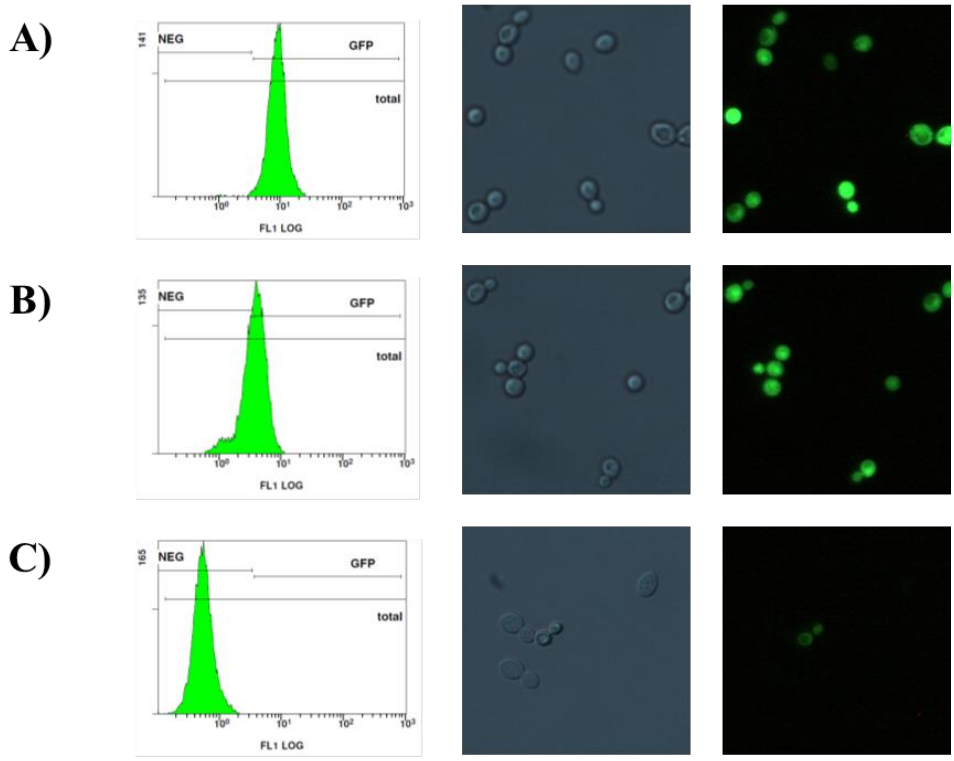
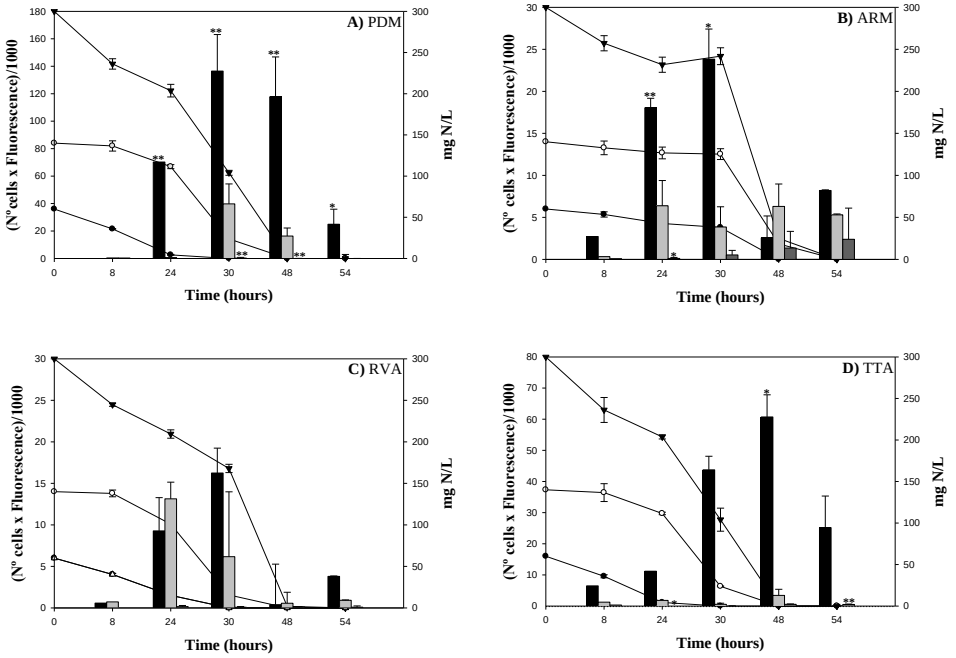


Figure 5. Fluorescence images of ARM cells, expressing GFP from the *GAP1* promoter throughout the different fermentations: **A)** 60 mg N/L; **B)** 140 mg N/L and **C)** 300 mg N/L. Representative flow cytometry histograms, bright-field and epifluorescence images of the *GAP1*-GFP strain growing in different nitrogen concentrations 48h post inoculation



***** Significance from t- Student with P≤0.05 compared with the control condition (140 mg N/L)
******Significance from t- Student with P≤0.01 compared with the control condition (140 mg N/L)

Fig. 6 Changes in fluorescence ((number of cells x fluorescence intensity)/1000) during fermentations with wine strains expressing GFP from *GAP1* promoter **A)** PDM, **B)** ARM, **C)** RVA and **D)** TTA). Fluorescence was measured at different nitrogen concentrations: 60 mg N/L (*black bars*), 140 mg N/L (*grey light bars*) and 300 mg N/L (*grey dark bars*). The consumption of nitrogen was also calculated throughout the process: 60 mg N/L (*filled circles*), 140 mg N/L (*open circles*) and 300 mg N/L (*filled triangles*)

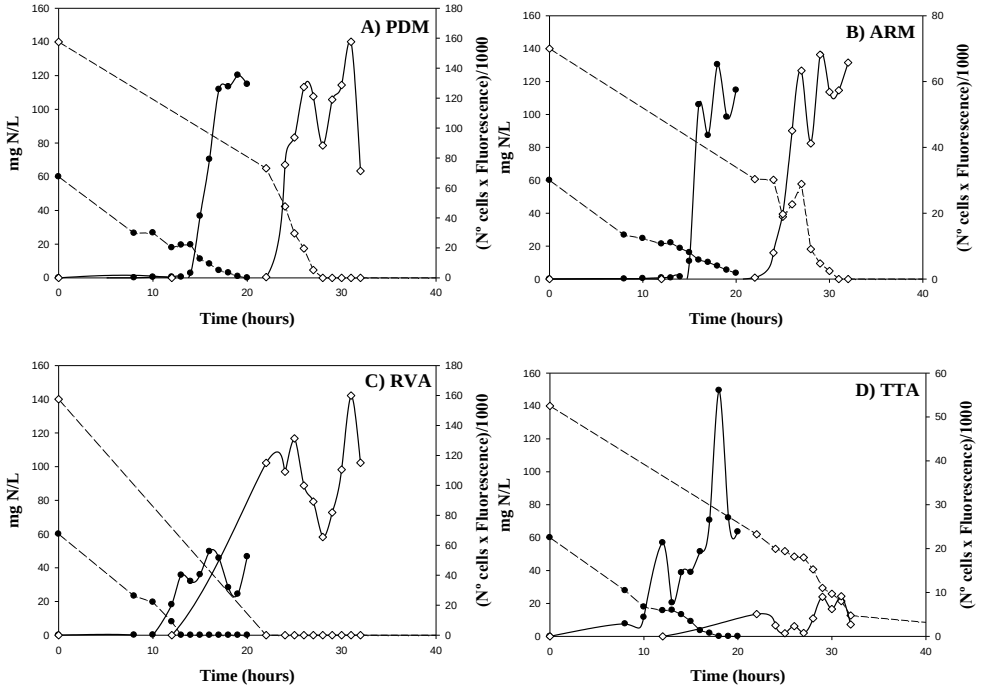


Figure 7. Nitrogen consumption (dotted line) and changes in fluorescence (solid line) in each individual strain during fermentations with different nitrogen concentrations: 60 mg N/L (*filled circles*) and 140 mg N/L (*open diamonds*)

4. Discussion

In the present study, the main goal was to identify biomarkers showing robust changes in their activity levels associated with nitrogen starvation, which could be potential candidates as biosensors for predicting sluggish or stuck fermentations. We analyzed the biochemical and molecular responses under several nitrogen concentrations (limited, control and non-limiting condition). There have been previous reports about the use of biomarkers to predict nitrogen deficiency during alcoholic fermentation (Carrasco *et al*., 2003; Beltran *et al*., 2004; Jiménez-Martí *et al*., 2007; Mendes-Ferreira *et al*., 2007); however, this is the first systematic study

comparing several molecular markers and several commercial strains under different nitrogen conditions. The selected molecular marker should be very sensitive to the cellular nitrogen limitation and should be useful for all strains, regardless of their physiological characteristics (nitrogen consumption, growth rate, fermentation capacity, etc.).

Previous studies have suggested that reserve carbohydrate accumulation starts in response to nitrogen limitation (François and Parrou, 2001; Hazelwood *et al*., 2009; Albers and Larsson, 2009) and, likewise, we also observed trehalose accumulation when most of the nitrogen was consumed and there were still sugars in the media. However, Hazelwood *et al*. (2009) reported that trehalose levels were specifically higher in nitrogen-limited chemostat cultures, whereas we did not observe this negative correlation between trehalose accumulation and nitrogen concentration of the medium. Conversely, we observed a lower trehalose content in the nitrogen-starved cells, which can be explained by a lower protein level which, in consequence, involves a lower amount of glucose transporters (lower glycolytic flux) and trehalose synthesis enzymes (Varela *et al*., 2004; Albers *et al*., 2007). However this lower accumulation of trehalose can also be explained because these cells, growing in high glucose and nitrogen limitation, overproduce ATP (excess energy). This excess ATP can be counteracted by a futile cycle between trehalose synthesis and degradation (Hottiger *et al*., 1987; Thevelein and Hohmann, 1995). In any case, trehalose accumulation cannot be considered as a useful biomarker for nitrogen limitation because it is not directly linked to the level of nitrogen shortage, and the beginning of its synthesis mainly occurs at the onset of the stationary phase. Novo *et al*. (2005) previously studied trehalose accumulation in a wine yeast and reached the same conclusion: trehalose accumulation began when cells entered into the stationary phase, regardless of the nitrogen content in the medium.

A more interesting result is the difference in trehalose accumulation among strains. In a recent study, we analyzed the specific nitrogen requirements of these four commercial strains during wine fermentation (Gutiérrez *et al*., 2012). In this study,

strains PDM and RVA showed higher growth rate and maximum population size and were also the greatest nitrogen demanders. Likewise these strains accumulated 2-fold more trehalose than strains ARM and TTA. Therefore trehalose accumulation could be interpreted as an indicator of growth behavior and fermentation activity. Varela *et al*. (2004) previously found a strong correlation between trehalose accumulation and ethanol production.

Conversely, the positive correlation between arginase activity and nitrogen deficiency can be ascribed to most of the strains and, as previously reported (Carrasco *et al*., 2003; Beltran *et al*., 2004; Jiménez-Martí *et al*., 2007), it could be a good marker to detect nitrogen depletion in grape must fermentation. However, arginase activity determination is tedious, time-consuming and difficult to simplify, thus we decided to look for molecular markers related with gene activity in terms of the quantity of the nitrogen of the grape must. With the aim of selecting the most suitable genes for detecting nitrogen deficiency, we analyzed the transcriptional activity of genes, which were previously reported to be regulated by nitrogen concentration of the medium (Beltran *et al*., 2004; Godard *et al*., 2007; Jiménez-Martí *et al*., 2007; Mendes-Ferreira *et al*., 2007; Deed *et al*., 2011) in N-limited and N-excess media. In a genome-wide analysis of wine yeast, Mendes-Ferreira *et al*. (2007) identified 36 genes that are strongly expressed under conditions of low or absent nitrogen, in comparison with a nitrogen-excess condition. *RTN2* was one of the 36 genes that showed a clear response under nitrogen limitation or starvation. However, we did not detect a strong up-regulation of this gene comparing the N-limited with the N-excess condition. Furthermore, in the case of *HSP26*, growth rate decrease (entrance into the stationary phase) seems to be a greater determinant in its regulation than nitrogen concentration. Despite the data obtained for arginase activity, regulation of *CAR1* expression was not dependent on the nitrogen concentration in the medium. This result highlights the importance of post-transcriptional and post-translational mechanisms in the regulation of arginase activity. Under our experimental conditions, permeases *DAL4*, *DAL5*, *DUR3* and

GAP1 showed the strongest up-regulation in the nitrogen-limited condition in comparison with the null or scarce activity in the nitrogen-excess condition. As explained above, the *DAL4* and *GAP1* genes were selected because they showed the strongest activation and, according to Godard *et al*. (2007), their regulation as NCR genes was different. According to our data, the activation of both genes was dependent on the depletion of nitrogen in the growth medium, which was likewise determined by the initial concentration and uptake rate of the wine yeast strain. Thus, at the same nitrogen concentration, a strain with a slower uptake rate, such as ARM, showed later gene induction than another strain which consumes nitrogen more rapidly. The nitrogen metabolism depends heavily on its uptake through the different nitrogen permeases (Beltran *et al*., 2005). In our previous work with the same commercial strains (Gutiérrez *et al*., 2012), we observed a positive correlation among growth and nitrogen uptake rates. The strains with better growth had a higher nitrogen demand. In this study, the biomarkers proposed were able to detect nitrogen limitation earlier in the strains which consumed nitrogen faster.

Regardless of these differences among the studied strains, the transcriptional activity of both genes fulfilled all the requirements for them to be considered good molecular biomarkers to detect nitrogen deficiency. However, transcriptional activity measured by quantitative PCR or Northern blots represents neither a simple nor a rapid method for use as a biomarker. An ideal biomarker should provide a rapid and cheap reading of the environmental conditions.

Green fluorescence protein (GFP) has become an increasingly popular protein tag for determining protein localization and abundance (Sheff and Thorn, 2004). The use of gene promoters to control reporter genes which contain green fluorescent protein is a successful innovation for gene expression studies (Niedenthal *et al*., 1996). The use of the GFP as reporter of *GAP1* and *DAL4* transcriptional activity has yielded similar results to the data obtained by quantitative PCR. The detection of the fluorescent cells and the intensity of this fluorescence allowed rapid measurement of the nutritional status of the cells regarding nitrogen availability

during wine fermentation conditions. The simplicity of the analysis means many time-points can be monitored during fermentation, thereby detecting the moment at which the yeast needs nitrogen supplementation during vinification, which would depend on the wine strain used. In our opinion, the biomarker or biosensor proposed here has great potential use to study the different nitrogen requirements of most commercial strains currently available on the market, thus providing very useful information for winemakers.

5. Conclusions

Knowledge of the nutritional state of cells during alcoholic fermentation is necessary to avoid sluggish and stuck vinifications and low quality of the final product. We show how the use of diverse biochemical or molecular markers related to nitrogen metabolism could provide useful information for the wine industry. Arginase activity and expression of *GAP1* and *DAL4* genes in the cells are good indicators of nitrogen nutritional state. We report the creation and characterization of a yeast-based biosensor using the *GAP1* promoter and a GFP reporter to detect nitrogen deficiency. These markers can be useful to explore the relationship between nitrogen availability and nitrogen requirements of commercial yeast strains in greater depth.

6. Acknowledgements

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CHAPTER 4

Impact of nitrogen sources on growth, consumption rate and nitrogen catabolite repression in different yeast strains during wine fermentations

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Abstract

Nitrogen quality presents in the medium governs cell growth, consumption profile and synthesis of metabolites with high relevance in the quality of wine. Nitrogen catabolite repression is the mechanism responsible to regulate nitrogen assimilation during the process. The expression of the general amino acid permease (*GAP1*), which is subjected to NCR, was used as biomarker to indicate the response to different nitrogen sources in four commercial wine strains. It was demonstrated that *GAP1* is very effective to differentiate the impact of nitrogen sources in several yeast strains. As a consequence, each nitrogen source produces particular profile of growth, uptake rate and product formation. Preferred nitrogen sources are able to support rapid growth and assimilation, high repressor effect and suitable metabolites synthesis. On the contrary, non-preferred nitrogen sources are characterized by very slow growth related with some problems in consumption and a derepressor effect. Furthermore, the four strains studied showed some differences in assimilation of particular N sources. This knowledge should take into account in the process to obtain the best performance and adequate wine profile.

***Keywords*:** nitrogen quality, *GAP1*, uptake, repression, NCR, metabolite synthesis, cell growth

1. Introduction

The yeast *Saccharomyces cerevisiae* is able to use a wide variety of nitrogen sources that they find in their natural environment, such as amino acids, ammonium, urea, nitrogen bases and purine derivatives. During fermentation, some of these nitrogen compounds are present in grape juice, and are taken up through first part of the growth phase. They are incorporated into the cells by active transport systems, which involve a general amino acid permease (Gap1) and other specific permeases with a variable degree of specificity for particular sets of amino acids (Cooper, 1982). Once inside the cell, nitrogen sources can either be incorporated directly into the protein synthesis or be degraded to ammonium and glutamate, entering at the beginning of the central nitrogen metabolism. Remaining nitrogen compounds are directly stored into the vacuole until needed.

Nitrogen sources are degraded in a specific order depending on environmental, physiological, and strain-specific factors (Jiranek *et al*., 1990). Yeast cells are capable of selectively using good nitrogen sources (glutamine and ammonium) in preference to poor sources (proline and urea). The regulatory mechanism that governs this hierarchy is known as nitrogen catabolite repression (NCR), that inhibits expression of the genes required for the uptake and degradation of poor nitrogen sources when cells are provided with a good nitrogen source. When the good nitrogen source is exhausted, or a poor nitrogen source is provided in its place, NCR is relieved and the expression of NCR-sensitive genes increases (Magasanik and Kaiser, 2002). Regulation of gene expression is mediated by an interplay of four GATA family zinc-finger transcription factors: two transcription activators, Gln3 and Gat1 (also known as Nil1, Mep80) and two repressors, Dal80 and Gzf3 (Deh1, Nil2), as well as the regulatory protein Ure2 (Cooper, 2002). In the presence of good nitrogen sources in the media, is produced the activation of Ure2, which binds with Gln3, retaining it in the cytoplasm and preventing the activation of nitrogen-regulated genes. During growth on poor nitrogen sources,

Gln3 activates transcription of GATA target genes involved in central nitrogen metabolism (*GDH1*, *GDH2*), transport (*AGP1*, *DUR3*, *DAL4*, *GAP1*), and catabolic pathway, including those for utilization of proline, arginine and urea (*CAR1*, *PUT2*) which are induced (Godard *et al*., 2007). In a recent report, Gutiérrez *et al*. (2013) analyzed the expression of these genes in different nitrogen concentrations during fermentation conditions. Two genes were characterized for their high activation showed when cells detected nitrogen limitation in the medium. One of these genes was *GAP1* that encodes for the general amino acid permease and transports all biological amino acids across the plasma membrane. It is regulated at the transcriptional level by Gln3 and Ure2 and is inactivated by dephosphorylation in the presence of glutamate and glutamine (Stanbrough and Magasanik, 1995). It means that NCR is the main transcriptional regulation. The second gene selected was *DAL4* that encodes for the allantoin permease, and is subjected to other transcriptional regulation in addition to NCR, since it is also inducible by allophanate (a product of urea degradation). Thus, reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4* were constructed as biosensors to detect nitrogen deficiency during alcoholic fermentation.

The availability of nitrogen compounds in the must play a significant role in the fermentation progress. It is well documented, that elevated nitrogen concentrations stimulate the biomass production and decrease the fermentation time. In fact, nitrogen deficiencies are considered to be the main cause of stuck and sluggish fermentations (Kunkee, 1991). However, not only nitrogen amount influences the process, nature and quality is an important point that must be considered. The assimilation and degradation of these compounds is not equal for all of them and depends on the transport efficiency, the possibility of conversion into ammonia and glutamate without releasing toxic compounds, and on the requirements of energy and cofactors (Bisson, 1991).

However, the metabolism of nitrogen sources is not only important for providing precursors for protein biosynthesis. Most of the amino acids are aromatic precursors which determine the organoleptic properties of the resulting wine. Albers *et al*. (1996) studied the influence of different nitrogen sources on product formation of an industrial strain. They observed a change in the glycerol and ethanol concentration when amino acids were used instead of ammonium. The glycerol yield was reduced and ethanol was increased. Moreover, nitrogen quality in the must influences cell status from nitrogen- repressed to derepressed situations that determine the different patterns of nitrogen consumption (Beltran *et al*., 2004). Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds, and the amount of urea, the major precursor to the carcinogen ethyl carbamate (Marks *et al*., 2003).

Several studies have investigated in depth the influence of different nitrogen sources: transcriptional response, growth effect and NCR regulation during growth on many different nitrogen sources (Boer *et al*., 2007; Godard *et al*., 2007), the effect of nitrogen additions in NCR, uptake and aroma production (Marks *et al*., 2003; Beltran *et al*., 2005; Jiménez-Martí and del Olmo, 2008; Deed *et al*., 2011), the influence of different nitrogen sources in metabolites and aroma production (Albers *et al*., 1996; Torrea *et al*., 2011), even a clear understanding of how cell sense the quality of a nitrogen source (Chen and Kaiser, 2002). In this study, we propose to study the influence of different nitrogen sources on growth, consumption, metabolite production and NCR, and determine the relationship between these factors in four commercial wine strains. To do this, we carry out a new approach, by using strain reporters constructed in our previous study (Gutiérrez *et al*., 2012) which showed efficient response to nitrogen concentration, detecting limited nitrogen condition in the must. Now, we want to analyze their response at nitrogen quality, as indicator of the availability and nature of nitrogen during wine fermentations, and determine repression/derepression cell status originated of each nitrogen source.

2. Material and methods

2.1 Yeast strains and inoculum preparation

The yeast strains used in this study are the following: PDM, ARM, RVA and TTA, all of them are diploids and provided by Agrovin Company (Ciudad Real, Spain). A taxonomic description of these strains was carried out by RFLPs of the ITS/5.8S region (Guillamón *et al*., 1998). The strains PDM, RVA and TTA belonged to species *Saccharomyces cerevisiae*, while we identified the strain ARM as a hybrid between *S. cerevisiae* and *S. kudriavzevii*, following the procedure proposed by Gonzalez *et al*. (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has been confirmed by Dunn *et al*. (2012). These wine strains were grown at 30ºC on plates of YPD medium (2% glucose, 1% yeast extract, 1% peptone and 2% agar).

Inoculation cultures were taken from cultures that had been inoculated from loop-fulls of cells from agar plates and incubated during 132h at 30ºC in 10 mL of YNB medium (1.7% YNB w/w nitrogen and 2% glucose) with 0.5% of ammonium as sole nitrogen source. This preculture was so longer incubated because we determined the time needed for not detecting fluorescence in these wine strains.

2.2. Culture media

The synthetic grape-must (SM) used in this experiment was prepared according to Riou *et al*. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). Only the nitrogen content changed. Each medium was prepared with a sole nitrogen source. The nitrogen sources used were: adenine, ammonium, cytosine, GABA, L-alanine, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-

serine, L-threonine, L-tryptophan, L-valine, L-urea. The concentrations tested were 140 mg N/L as control condition. We also prepared a complete SM composed with a mixture of ammonium and amino acids (40% of ammonium + 60% of amino acids) as control.

2.3. Growth conditions

The precultures were inoculated in SM at an initial population of 0.2 units of OD_{600nm}. SM fermentations took place at 28ºC with continuous orbital shaking (150 rpm). They were performed in 50 mL falcon tubes containing 40 mL of the SM in semi-anaerobic conditions since limited aeration was necessary to harvest samples for the subsequent analysis. Cell growth was monitored by optical density (OD) changes at 600 nm, and the supernatant was recollected and stored at -20ºC at different times. Biological growth parameters were deduced from each growth curve by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering *et al*. (1990), which has the following expression:

$$y = D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_\infty/OD_0)$ is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h). OD/time data were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model. This modeling was accomplished using the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA).

2.4. Nitrogen content analysis

Ammonium and urea concentrations were measured by a kit using an enzymatic method (Roche Applied Science, Germany). The concentration of free amino acid nitrogen was determined by following the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The absorbance values at 260 and 280 nm were used to calculate the nucleotide equivalents = (0.063·A260)–(0.036·A280) (Herbert *et al.*, 1971). The results were expressed as mg nitrogen (N)/mL. The sum of ammonium and amino acids represented yeast assimilable nitrogen (YAN).

2.5. Metabolites (High Performance Liquid Chromatography)

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed in all the samples at the end of the exponential phase (46h) and at stationary phase (146h). Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, MA, USA), equipped with a refraction index detector, autosampler and UV-Visible detector. Prior to injection, samples were centrifuged at 13.300 rpm for 5 min, supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Spain) and diluted 5- or 10-fold. A total volume of 25 μL was injected into a HyperREZ^{TM}XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50ºC. The concentration of each metabolite was calculated using external standards. Each sample was analysed in duplicate.

2.6. Flow cytometry

Cells were harvested during growth at different time points and rinsed with PBS, and measured flourescence using Becton Dickinson FACSAria flow cytometer (BD Bioscience, San Diego, CA). A total of 10000 cells of the sample were measured at GFP-A filter, revealing the number of fluorescent cells and fluorescence mean of these cells.

2.7 Statistical analysis

Hierarchical clustering was performed using a centered Pearson correlation metric and average linkage mapping. A principal component analysis (PCA) was used to reduce the dimensionality of the data and to find the best differentiation among samples. The PCA was carried out with Statistica 7.0 software.

3. Results

In order to understand and separate the differential effects of several nitrogen sources, we used synthetic grape must (SM) that mimics natural grape juice, but only containing a specific nitrogen source, except the control SM medium which contained a mixture of ammonium and amino acids. We analyzed the influence of the nitrogen source on growth, nitrogen uptake rate and production of important secondary metabolites. We also monitored NCR during growth, and how this mechanism regulates different processes.

3.1 Impact of particular nitrogen sources on cell growth and nitrogen consumption

Growth effect of 22 nitrogen sources was analyzed in four commercial strains. Figure 1A shows the growth profile in every condition studied (22 nitrogen sources x 4 strains x 8 time points). Color range displayed at the heat map, represents the evolution between no growth (black) and growth (green) with different intensities, depending on the growth level reached in each condition. Growth values were used to establish a classification of the tested nitrogen sources. Thus, we applied hierarchical clustering to obtain a tree based in these profiles. Three clusters were differentiated: good (A), medium (B) and bad (C) nitrogen sources. Cluster A includes aspartate, glutamate, GABA, alanine, arginine, asparagine, ammonium and glutamine. Maximum growth values were reached after 36-47h. These sources support rapid growth with the highest specific growth rates (μ_{max}), and maximum yield values (Fig. 1B). Alanine and arginine are the unique sources that have lowest μ_{max} in comparison with the other sources of the same group, obtaining similar yield values. Cluster B includes ornithine, proline, tryptophan, serine, phenylalanine, valine and urea. This group is characterized for yield values similar to the group A, even higher (except proline that displays lower OD_{max}), but lower growth rates. Phenylalanine, serine, valine and urea are more similar to group A (OD_{max} reached above 36-47h), and remaining sources, ornithine, proline and tryptophan need more time to obtain maximum cell growth. Finally, cluster C includes cytosine, citrulline, adenine, methionine, threonine, isoleucine and leucine. These sources support slow, even very slow growth determined for lowest growth rates, producing lower biomass. Interestingly, isoleucine and leucine which showed medium growth also appear in this group. It is important to indicate, that the classification is based on behavior of four strains. Some sources can be classified in groups not really fitting, caused by strain-specific responses to the same N source. For instance, PDM and ARM showed very slow growth in

methionine, whereas other two strains presented better growth in this source. Strain responses to nitrogen sources analyzed are not identical, even when control conditions (SM) were compared (Fig. 2). PDM and TTA strains had the highest values of OD_{max} and specific growth rate respectively, while ARM displayed the lowest values. In comparison with SM, main sources showed a decrease in fitness parameters, except some nitrogen sources, such as aspartate, asparagine, glutamate, glutamine and ammonium, which improved growth. TTA was the unique strain in which no one nitrogen source got greater growth than the complete SM. Nitrogen consumption was also determined at different points for all conditions. Figure 3 shows the evolution of nitrogen consumption at the same time points in which growth was also measured. In the same way, heat map displays color range that means not consumption (black) and total consumption (green). Again, values were used to classify nitrogen sources depending on uptake profile in four strains. Nitrogen sources can be grouped into two groups: quick (1) and slow (2) uptake rate. Group 1 includes main nitrogen sources that were consumed after 86h (right part). Although strains showed similar behavior, only PDM and TTA assimilated all nitrogen present in the medium of this group around 65h. Consumption time was delayed in ARM (~72h), with incomplete assimilation of asparagine and urea. RVA strain was unable to total consumption in these preculture conditions.

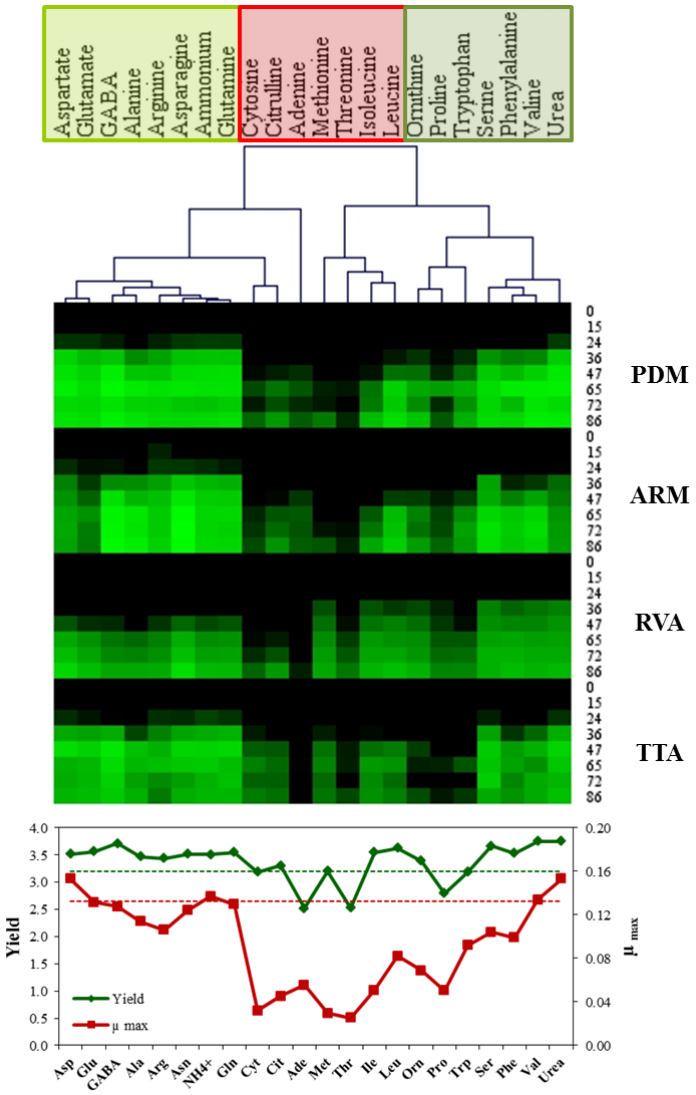


Figure 1. Growth profile of four commercial wine strains growing in 22 nitrogen sources. Hierarchical clustering of nitrogen sources based on profile, performed using Pearson correlation metric and average linkage mapping. Black = no growth, Green = growth. At the bottom part is showed specific growth rate (μ_{max}) and yield values of PDM strain. Dashed lines are SWM

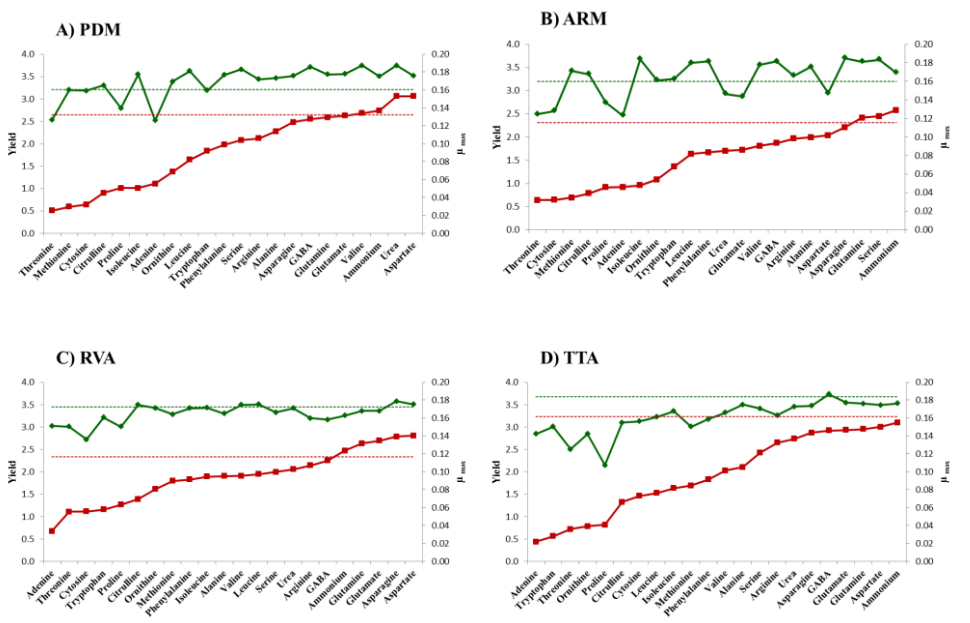


Figure 2. Specific growth rate (μ_{max}) and yield values of four wine strains growing under different nitrogen sources. Nitrogen sources are ranked according specific growth rate (low to high)

Group 2, that includes adenine, citrulline, isoleucine, methionine, proline, threonine, cytosine and tryptophan (most of them belong to group C of fig. 1), were much less consumed during the time-lapse studied. Nucleotide bases, proline and tryptophan were not consumed in any case. Citrulline and isoleucine were totally consumed in TTA and PDM, while TTA was the only strain able to assimilate methionine in this time-lapse. These sources were not totally consumed in RVA strain. Growth curves and consumption kinetic of the four strains in SWM medium were shown in Figure 4. PDM and TTA strains have similar profile. They reach 7 units of OD_{600nm} and consumed all the available nitrogen around 20-30h. ARM strain needs more time to assimilate nitrogen, and biomass yield is lower.

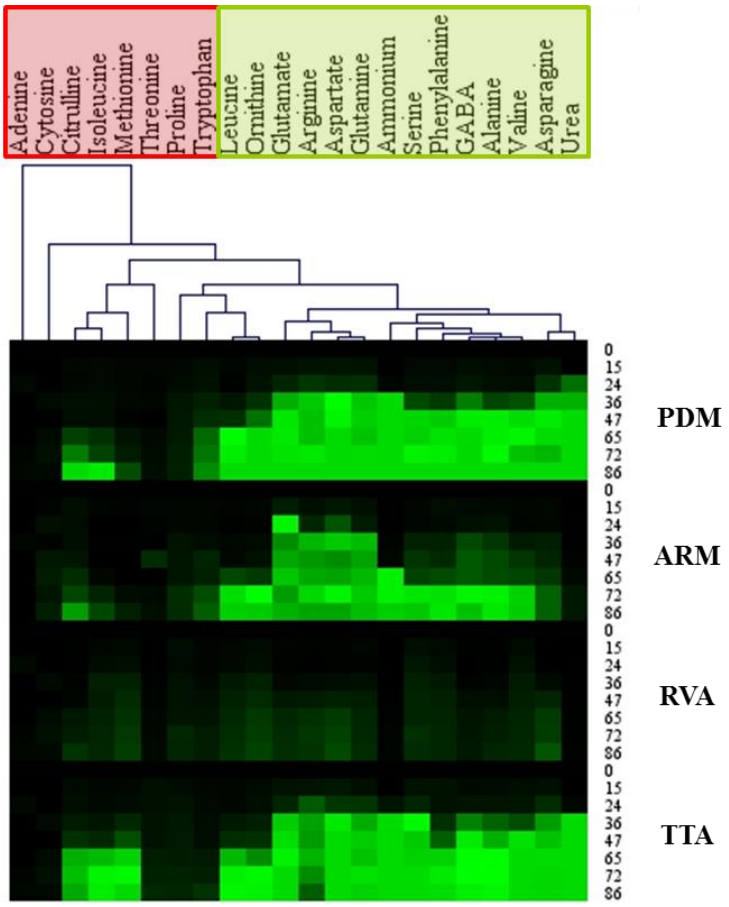


Figure 3. Consumption profile of four commercial wine strains growing in 22 nitrogen sources. Hierarchical clustering of nitrogen sources based on profile, performed using Pearson correlation metric and average linkage mapping. Black = no consumption, Green = consumption

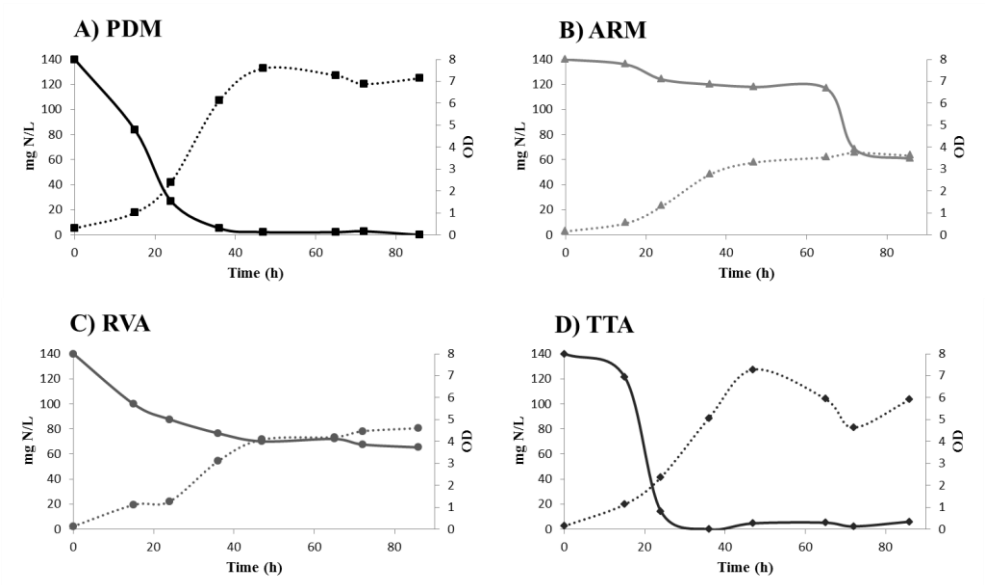


Figure 4. Nitrogen consumption and growth of four strains: ■ PDM, ▲ ARM, ● RVA and ♦ TTA, in synthetic wine must (SWM)

However, RVA strain seems to have problems for uptaking nitrogen in this particular preculture condition. Growth and nitrogen consumption data reveal an inverse correlation between these parameters, producing an exponential increase in the growth when nitrogen is quickly assimilated by the yeasts. Similarly, this direct correlation between nitrogen consumption and growth rate was also observed when the four strains were grown in an unique nitrogen source (Fig. 5). At this point of the process (86h), the majority of sources that have been more consumed, are the same that reached higher values of biomass production. Other sources difficult to assimilate, such as adenine, cytosine, methionine, threonine and proline, reached lower cell growth. This correlation growth-N consumption was even more direct at the end of the exponential phase (47 h; Fig. 6). Figure 5 also clearly revealed the general uptake problem of the RVA strain with all the nitrogen sources and the slowness consumption rate of the ARM strain with most of these compounds.

There are some exceptions to this general trend. Urea seems a particular case, because this source supported higher growth rate with fewer N uptake in this strain.

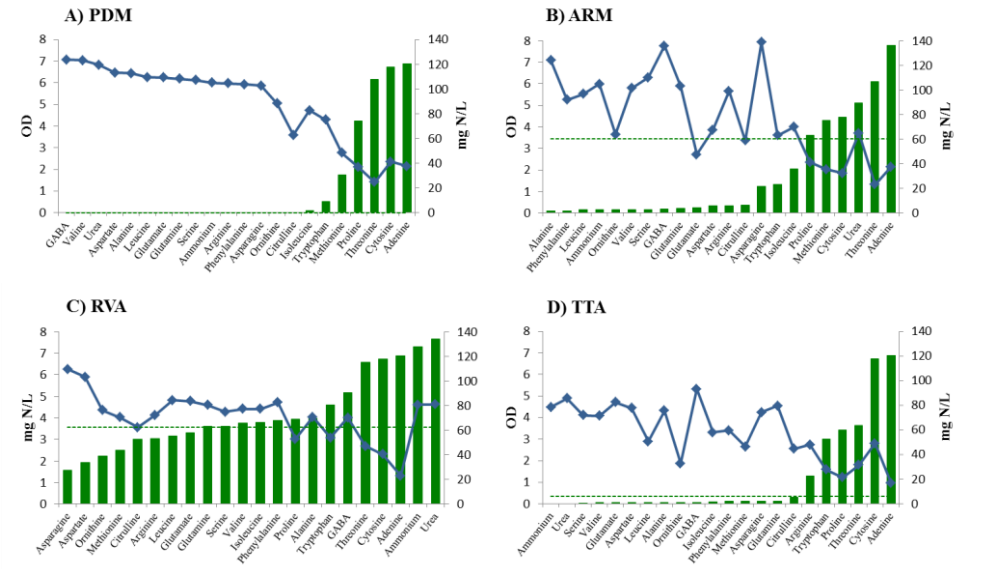


Figure 5. Maximum growth and residual nitrogen concentration of four commercial strains growing in different nitrogen sources at 86h. Nitrogen sources are ranked according to nitrogen uptake rate (high to low). Line represents nitrogen concentration in SWM.

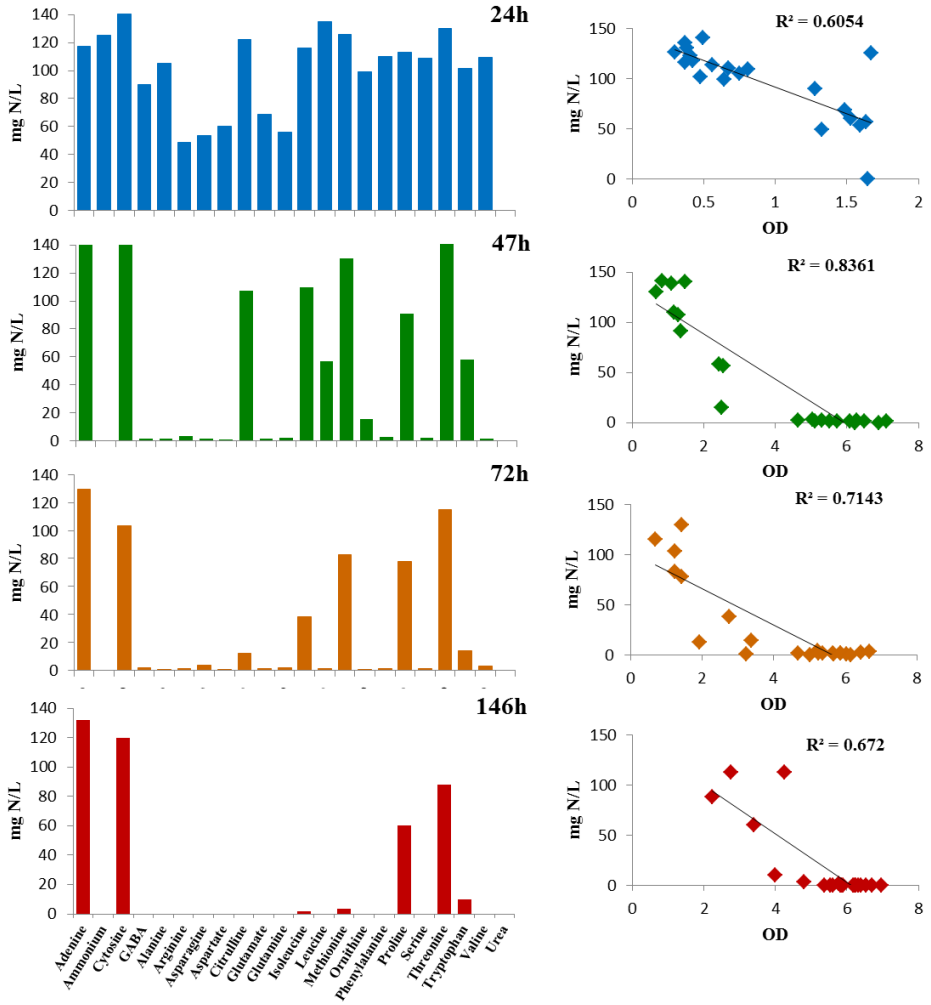


Figure 6. Nitrogen consumption profile at different times (24h, 47h, 72h and 146h) of each nitrogen source in PDM strain. Right part shows direct correlation between nitrogen consumption and growth in each condition

3.2 Influence of the nitrogen source on *GAP1* and *DAL4* expression through GFP-reporter

The transcriptional response of the nitrogen transporters *GAP1* and *DAL4* was analyzed during growth in SM with a sole nitrogen source. For it, we used reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4* (Gutiérrez *et al*., 2013). One allele of these genes was replaced by the GFP gene in the four commercial wine strains, obtaining 8 reporter strains (4 strains x 2 genes). Preculture media was used to analyze strain responses at the same initial condition. Both genes were repressed (without fluorescence) at the inoculation time in each specific medium. Figure 7 shows the fluorescence evolution during growth of the reporter strains under different nitrogen conditions. *GAP1* was repressed in the first hours after inoculation. This gene started to be activated/derepressed after 36h, when nitrogen was almost depleted. In the RVA strain starting point was around 65h, delay observed in the same way in growth and consumption. The expression increased continuously through time period measured, reaching different intensities depending on N source tested and the strain. However, this direct correlation between N consumption and fluorescence activation was not similar for all the N sources. Some of them did not get a full repression of *GAP1* when there was an important amount of N left in the medium. These differences were important to rank the different N sources from most to less repressor sources. In relation with SM, expression of GFP was increased when nitrogen was totally depleted in PDM and TTA strains, whereas RVA and ARM strains showed the highest peak of fluorescence around 65h, when the medium already presented residual nitrogen (data not shown). On the other hand, *DAL4* promotor showed a lower level of induction than *GAP1*. *DAL4* also kept the repression condition after the inoculation and few nitrogen sources showed induction of this gene in the strains PDM, ARM and RVA in the time-lapse studied

(cytosine, arginine, citrulline and urea were the most prominent in PDM and RVA).

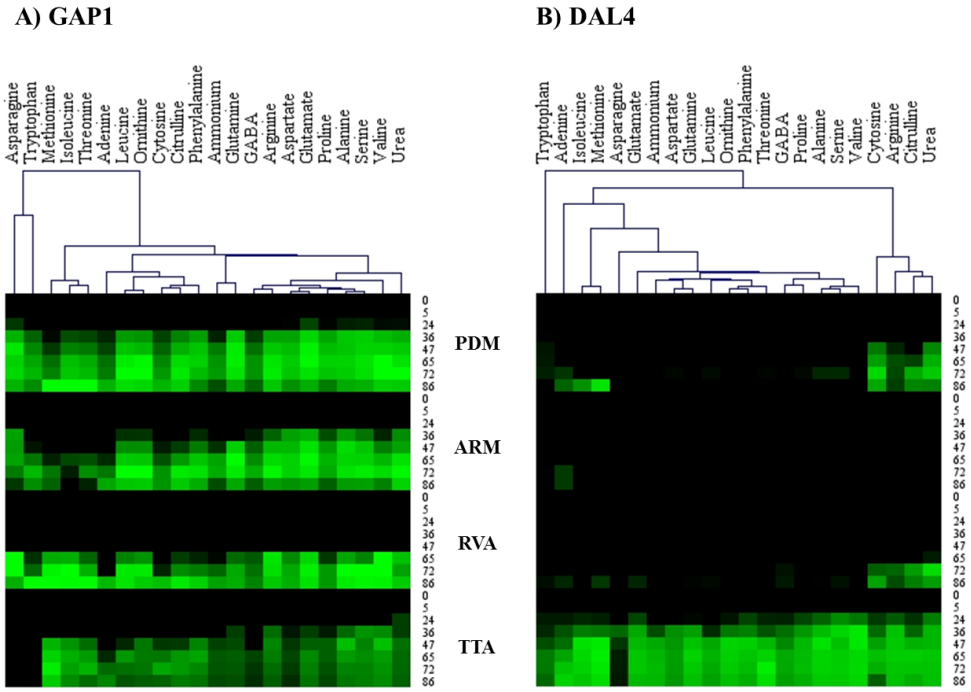


Figure 7. Fluorescence profile of four commercial wine strains growing in 22 nitrogen sources. A) GAP1 reporter strains, B) DAL4 reporter strains. Hierarchical clustering of nitrogen sources based on profile, performed using Pearson correlation metric and average linkage mapping. Black = no fluorescence, Green = fluorescence

Only the TTA strain produced similar profile in *DAL4* and *GAP1*, being even observed an earlier induction in *DAL4*. This strain also showed *DAL4* induction after 36h in the complete nitrogen medium (SM), when nitrogen was exhausted. Curiously the other strains did not show any fluorescence activity at any time in

SM (data not shown). As *GAP1* induction marked clearer differences among the strains and nitrogen sources, we followed-up the study by using this gene as molecular marker of NCR.

In order to detect which sources exerted a strong nitrogen catabolite repression, fluorescence evolution and nitrogen consumption was correlated in the PDM strain (Fig. 8). This correlation enabled us to classify the nitrogen sources in function of their repression capacity. The fluorescence activity was not detected meanwhile the nitrogen was not consumed in the sources with a higher repression capacity. This is the case of ammonium, glutamine and urea. Conversely, fluorescence was earlier detected in the cells growing in the sources with weaker repression activity, when residual nitrogen was still higher in the medium. Clear examples were the adenine, cytosine, threonine, proline and methionine. Thus, the less repressive the source was the quickest the fluorescence induction was and nitrogen depletion is not necessary for this activation.

NCR profile response was similar in the four strains, but some strain-specific differences were detected. In order to highlight these differences, nitrogen sources were classified from the strongest to the weakest with NCR capacity in the PDM strain and compared with fluorescence evolution in the other strains in some of these sources (Fig. 9). TTA profile was very similar to control strain, but with lower fluorescence expression. Asparagine did not show any activation when nitrogen was exhausted. ARM was also expressed much lower in asparagine that control strain, and urea showed lower repressor effect. RVA showed the strangest behavior, since was unable to total assimilation. For instance, ammonium and urea the most repressor sources in PDM strain, showed slow consumption, and increase of *GAP1* expression when high nitrogen concentration was in the medium.

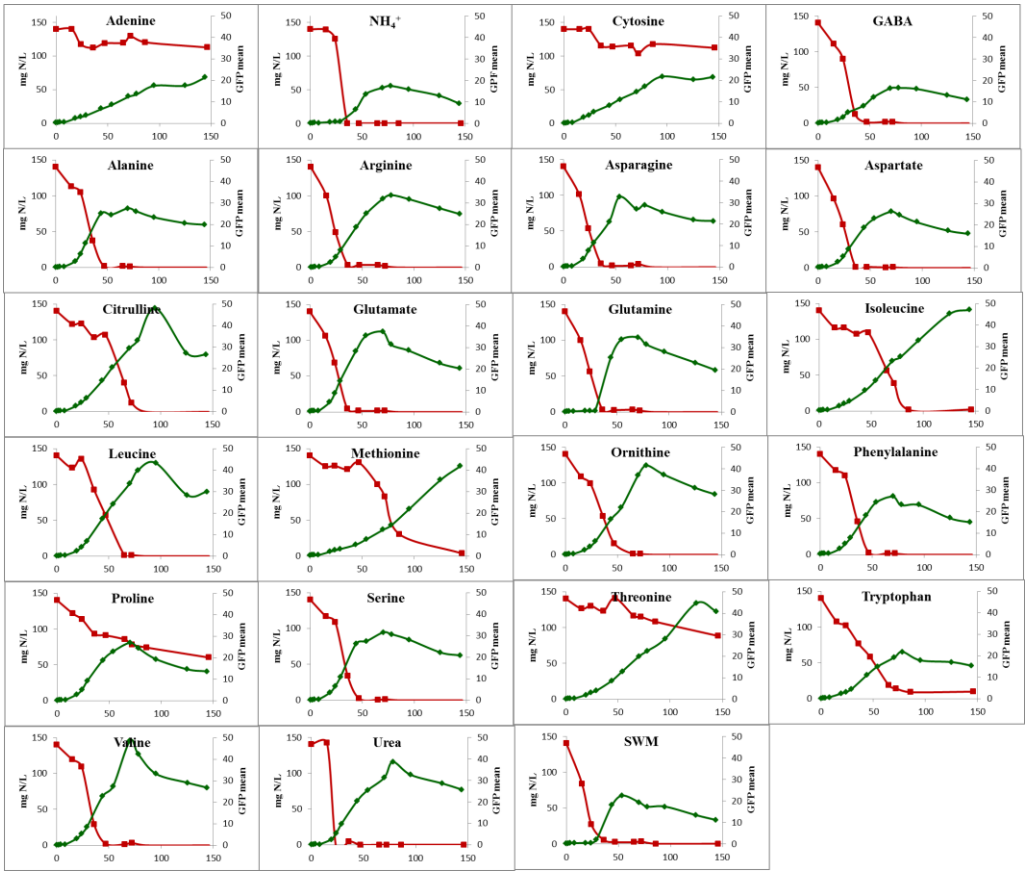


Figure 8. Fluorescence evolution and nitrogen consumption of PDM-*GAP1* reporter strain under different nitrogen source content (22 nitrogen sources and SWM).

Arginine and asparagine also expressed *GAP1* when residual nitrogen was presented in the medium. In these three strains, methionine and threonine did not produce the same effect that in PDM, where was detected a problem to assimilate both nitrogen sources.

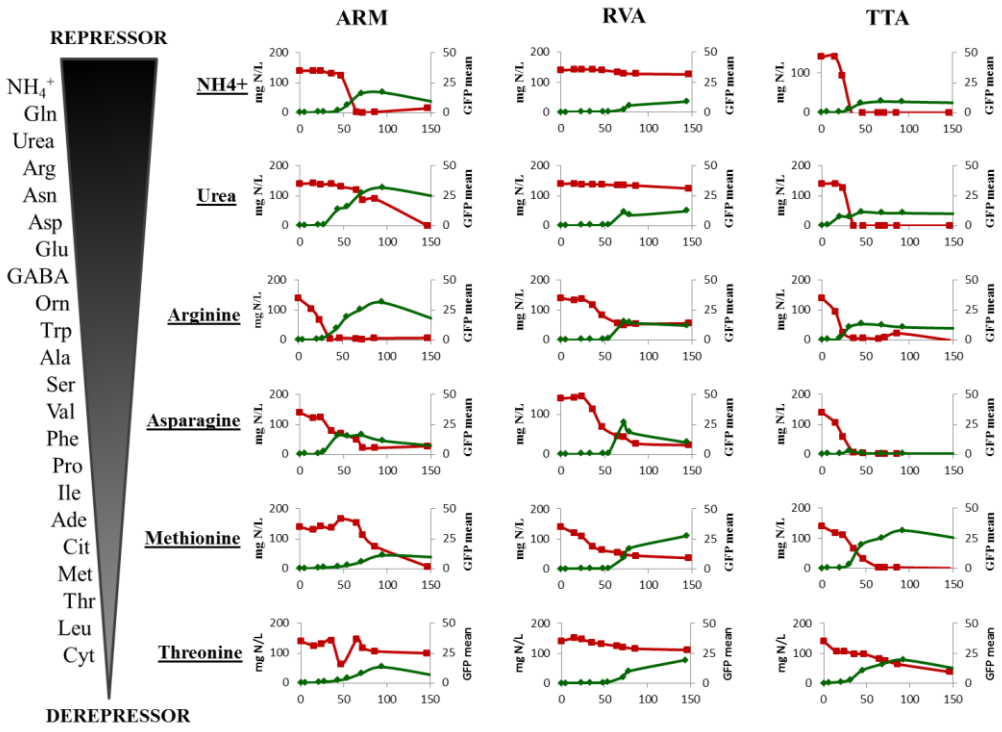


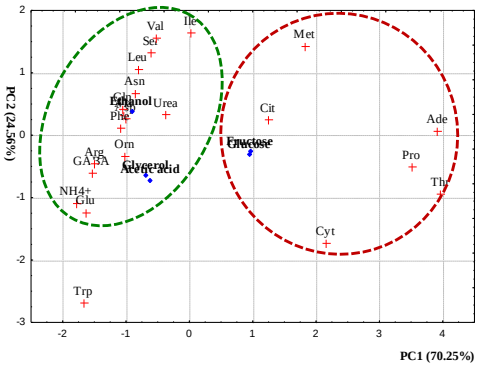
Figure 9. Nitrogen sources ranking according repressor/derepressor effect in PDM strain. Graphs represent consumption and *GAP1* fluorescence in N sources with certain differences with respect PDM

3.3 Effect in metabolites synthesis

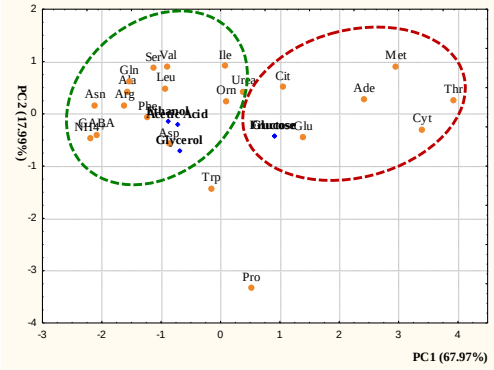
We analyzed the residual sugars, ethanol, glycerol and acetic acid obtained from the different conditions at stationary phase (146h) (Table 1-4). The nitrogen source affected to the fermentation activity and the formation of the main fermentative metabolites. As expected, the sources which supported worse growth also showed lower fermentation activity. For cultures grown in adenine, cytosine, citrulline, proline, threonine and methionine as unique nitrogen source, ethanol and glycerol were significantly lower compared with the other sources. Glycerol concentration

was higher when GABA was used as the nitrogen source. Ammonium and tryptophan produced the highest concentrations of acetic acid (except in the RVA strain). To reduce the huge amount of data and to reveal the most determinant differences among conditions and strains, we carried out a principal component analysis (PCA) of each yeast strain (Fig. 10). In the PDM strain, the analysis accounted for 94.81% of total variance. PC1 was characterized by glucose and fructose with a positive loading, while ethanol, glycerol and acetic acid exhibited negative loadings. For PC2, ethanol was the only compound with a positive loading. This PCA classified nitrogen sources in two groups: the right part with sources with higher residual sugar concentration, and obviously the left part with sources that produce more amount of ethanol, glycerol and acetic acid. Leucine, serine, valine and isoleucine showed the highest values of ethanol. Tryptophan condition was clearly separated from the other nitrogen sources in the bottom part, denoting particular effect in metabolite synthesis. Surprisingly, this source reached the highest amounts of ethanol and acetic acid but was unable to consume completely sugars presented in the media. ARM analysis showed similar profile of PDM. Two components were used to explain 85.96% of the total variance. Sources were very similar grouped to the PDM, even tryptophan with high amount of glycerol. Remarkably, glutamate left a much higher concentration of residual sugars than the other strains. In the RVA analysis accounted for 89.28% of the total variation, was difficult to classify nitrogen sources, since at this time point this strain is still consumed sugars. All conditions contained high sugar concentration, except asparagine as unique source with low sugar concentration and high ethanol and glycerol yield. Finally, TTA analysis with the same profile that control strain, but curiously cytosine condition has particular behavior in this strain, since sugars were almost consumed. If compare results obtained of the four strains in SWM, we observed that PDM and TTA were able to consume all sugars and reach high amount of ethanol, glycerol and acetic acid, whereas ARM and RVA seem unable to degrade sugars in the lapse-time measured.

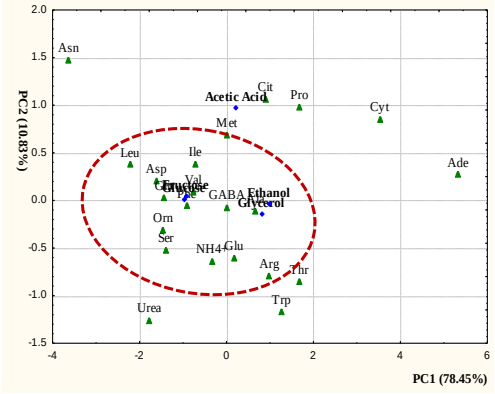
A) PDM



B) ARM



C) RVA



D) TTA

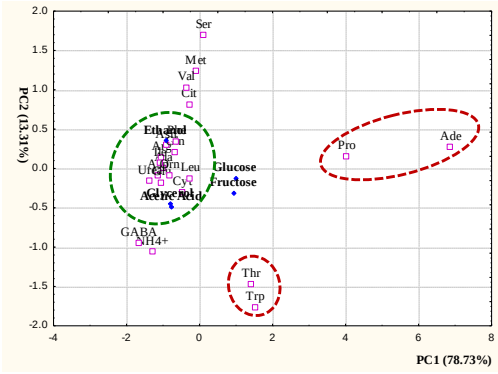


Figure 10. Results of the principal component analysis carried out on the metabolite products in four strains. Every graph represents scores of the strain under different nitrogen sources and loadings of the variables (glucose, fructose, glycerol, ethanol and acetic acid) on the first two principal components

Table 1. Concentration of metabolites obtained with PDM strain under different nitrogen sources

	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic Acid (g/L)
Adenine	20.50±0.02	35.30±0.02	4.29±0.01	5.21±0.00	0.32±0.00
Ammonium	0.00±0.00	0.00±0.00	7.26±0.01	7.70±0.03	1.28±0.25
Cytosine	17.83±0.02	28.81±0.07	6.14±0.13	5.52±0.01	0.94±0.03
GABA	0.00±0.00	0.00±0.00	8.07±0.01	7.65±0.01	0.77±0.00
Alanine	0.00±0.00	0.00±0.00	6.30±0.01	7.76±0.01	0.79±0.14
Arginine	0.00±0.00	0.00±0.00	6.58±0.09	7.83±0.00	1.15±0.12
Asparagine	0.00±0.00	0.00±0.00	6.20±0.04	7.78±0.01	0.63±0.06
Aspartate	0.00±0.00	0.00±0.00	6.82±0.00	7.61±0.01	0.65±0.09
Citrulline	7.19±0.00	20.50±0.00	5.09±0.00	6.68±0.00	0.65±0.03
Glutamate	0.35±0.00	1.99±0.01	7.29±0.01	7.54±0.00	1.30±0.03
Glutamine	0.00±0.00	0.00±0.00	5.86±0.01	7.89±0.00	0.88±0.17
Isoleucine	0.67±0.01	6.32±0.04	5.00±0.11	7.85±0.05	0.38±0.04
Leucine	0.00±0.00	0.00±0.00	5.88±0.09	8.04±0.01	0.56±0.04
Methionine	6.42±0.16	19.77±0.31	4.67±0.16	6.63±0.15	0.13±0.03
Ornithine	0.79±0.12	5.59±0.26	6.53±0.18	7.69±0.02	1.01±0.20
Phenylalanine	0.00±0.00	0.00±0.00	6.89±0.01	7.65±0.01	0.71±0.03
Proline	21.37±0.02	33.60±0.07	5.75±0.28	5.23±0.01	0.22±0.03
Serine	0.00±0.00	0.00±0.00	5.44±0.01	7.96±0.03	0.53±0.07
Threonine	24.83±0.04	35.92±0.04	5.02±0.02	4.84±0.05	0.50±0.02
Tryptophan	2.55±0.01	8.21±0.06	9.12±0.13	7.00±0.02	1.37±0.02
Valine	0.00±0.00	0.00±0.00	5.62±0.06	8.02±0.01	0.36±0.01
Urea	0.67±0.00	5.30±0.01	6.13±0.11	7.24±0.02	0.67±0.01
SWM	0.00±0.00	0.00±0.00	6.70±0.01	7.71±0.01	1.06±0.00

Table 2. Concentration of metabolites obtained with ARM strain under different nitrogen sources

	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic Acid (g/L)
Adenine	24.68±0.00	37.94±0.01	4.04±0.02	4.74±0.01	0.30±0.00
Ammonium	0.38±0.00	2.26±0.01	7.31±0.06	6.91±0.02	0.90±0.01
Cytosine	36.77±0.02	45.24±0.05	4.11±0.01	3.35±0.01	0.35±0.00
GABA	0.00±0.00	0.00±0.00	7.67±0.12	7.49±0.00	0.65±0.01
Alanine	0.00±0.00	0.00±0.00	6.26±0.01	7.57±0.07	0.56±0.01
Arginine	0.83±0.01	5.85±0.00	6.15±0.01	7.48±0.01	0.73±0.00
Asparagine	0.00±0.00	0.00±0.00	6.24±0.09	7.75±0.01	0.83±0.01
Aspartate	6.81±0.00	19.44±0.01	6.56±0.00	6.41±0.01	0.77±0.02
Citrulline	12.62±0.00	26.60±0.00	4.64±0.00	5.53±0.00	0.39±0.00
Glutamate	21.95±0.00	35.46±2.28	5.15±0.03	4.92±0.01	0.57±0.00
Glutamine	0.41±0.00	2.90±0.00	5.44±0.01	7.35±0.00	0.75±0.15
Isoleucine	4.59±0.01	16.28±0.01	4.71±0.01	6.63±0.13	0.40±0.02
Leucine	2.04±0.01	10.22±0.01	5.61±0.00	7.39±0.01	0.54±0.01
Methionine	22.06±0.33	34.78±0.49	3.71±0.32	3.90±0.29	0.08±0.00
Ornithine	9.24±0.21	19.85±0.40	5.34±0.18	6.1±0.03	0.54±0.01
Phenylalanine	1.77±0.01	10.05±0.02	6.62±0.02	7.10±0.03	0.60±0.00
Proline	33.10±0.00	49.16±0.04	8.96±1.10	8.40±0.00	0.35±0.00
Serine	0.00±0.00	0.00±0.00	5.84±0.85	8.06±0.01	0.29±0.00
Threonine	36.90±0.03	44.06±0.00	3.37±0.00	2.77±0.01	0.25±0.00
Tryptophan	18.35±0.01	27.00±0.01	7.06±0.01	5.14±0.01	0.89±0.00
Valine	0.74±0.00	5.98±0.01	5.16±0.01	7.01±0.01	0.55±0.07
Urea	11.50±0.01	22.31±0.03	4.61±0.13	5.82±0.01	0.62±0.03
SWM	19.66±0.00	28.11±0.02	4.53±0.10	5.23±0.01	0.79±0.00

Table 3. Concentration of metabolites obtained with RVA strain under different nitrogen sources

	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic Acid (g/L)
Adenine	51.79±0.02	58.29±0.06	1.78±0.01	1.84±0.01	0.17±0.00
Ammonium	21.03±0.01	37.04±0.00	3.98±0.01	4.78±0.01	0.67±0.01
Cytosine	38.64±0.01	49.99±0.06	2.52±0.15	3.015±0.01	0.15±0.00
GABA	22.13±0.01	38.02±0.01	4.13±0.01	4.67±0.01	0.50±0.00
Alanine	25.60±0.01	40.93±0.06	3.80±0.16	4.32±0.03	0.48±0.00
Arginine	28.19±0.01	43.29±0.05	3.50±0.01	3.92±0.01	0.62±0.00
Asparagine	0.34±0.00	4.63±0.01	4.33±0.01	7.32±0.01	0.60±0.00
Aspartate	9.61±0.00	27.85±0.01	4.17±0.02	5.77±0.00	0.62±0.00
Citrulline	19.93±0.00	37.92±0.00	3.08±0.00	4.73±0.00	0.32±0.00
Glutamate	23.14±0.01	38.58±0.03	3.61±0.01	4.56±0.01	0.66±0.00
Glutamine	7.43±1.44	24.46±4.68	3.65±0.49	5.23±0.74	0.71±0.10
Isoleucine	13.37±0.00	32.66±0.00	3.91±0.01	5.32±0.09	0.52±0.00
Leucine	5.76±0.02	23.88±0.04	4.21±0.02	6.30±0.01	0.65±0.00
Methionine	18.04±0.43	37.03±0.64	4.15±0.70	4.83±0.06	0.35±0.00
Ornithine	10.10±0.15	29.39±0.26	3.93±0.13	5.48±0.01	0.74±0.02
Phenylalanine	14.05±0.01	33.98±0.02	4.39±0.01	5.06±0.00	0.56±0.00
Proline	27.52±0.02	43.11±0.00	3.56±0.03	3.94±0.00	0.19±0.03
Serine	11.63±0.01	29.83±0.06	3.81±0.01	5.48±0.04	0.40±0.00
Threonine	34.03±0.01	46.98±0.02	3.59±0.02	3.35±0.03	0.54±0.03
Tryptophan	32.59±0.04	45.74±0.08	3.75±0.18	3.42±0.03	0.63±0.02
Valine	7.75±0.03	21.56±0.07	2.93±0.04	4.43±0.01	0.73±0.10
Urea	12.28±0.01	31.09±0.01	4.15±0.01	5.46±0.02	0.93±0.04
SWM	9.35±0.01	27.58±0.01	4.14±0.00	5.72±0.01	0.85±0.02

Table 4. Concentration of metabolites obtained with TTA strain under different nitrogen sources

	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic Acid (g/L)
Adenine	34.92±0.01	52.74±0.02	2.95±0.01	3.52±0.06	0.15±0.01
Ammonium	0.00±0.00	0.00±0.00	7.06±0.11	7.37±0.01	0.81±0.05
Cytosine	0.66±0.00	11.42±0.02	6.18±0.10	7.85±0.04	0.69±0.00
GABA	0.00±0.00	0.00±0.00	8.45±0.09	8.03±0.02	0.63±0.05
Alanine	0.24±0.01	1.71±0.01	6.31±0.11	8.30±0.04	0.69±0.04
Arginine	0.00±0.00	0.00±0.00	6.40±0.01	8.39±0.01	0.66±0.00
Asparagine	0.00±0.00	0.00±0.00	6.11±0.00	8.37±0.04	0.66±0.00
Aspartate	0.00±0.00	0.00±0.00	7.22±0.13	8.22±0.02	0.58±0.06
Citrulline	0.38±0.00	5.15±0.00	6.32±0.00	8.28±0.00	0.43±0.00
Glutamate	0.00±0.00	0.00±0.00	6.67±0.10	8.01±0.01	0.67±0.01
Glutamine	0.00±0.00	0.00±0.00	5.83±0.10	7.84±0.00	0.68±0.01
Isoleucine	0.00±0.00	1.28±0.01	6.71±0.13	8.44±0.17	0.63±0.01
Leucine	0.48±0.00	10.54±0.00	6.47±0.00	7.58±0.03	0.58±0.00
Methionine	0.22±0.01	3.24±0.14	5.56±0.10	8.35±0.13	0.45±0.00
Ornithine	0.26±0.04	5.21±0.19	6.95±0.16	8.13±0.01	0.58±0.00
Phenylalanine	0.00±0.00	1.62±0.01	6.35±0.02	8.03±0.02	0.56±0.00
Proline	17.27±0.03	41.58±0.08	5.18±0.29	5.11±0.01	0.15±0.02
Serine	0.00±0.00	0.00±0.00	5.48±0.04	8.27±0.06	0.34±0.05
Threonine	9.17±0.01	32.00±0.01	6.98±0.02	5.88±0.06	0.53±0.00
Tryptophan	9.22±0.01	34.34±0.04	6.06±0.01	5.58±0.07	0.71±0.01
Valine	0.24±0.00	1.76±0.01	5.73±0.02	8.44±0.01	0.50±0.01
Urea	0.00±0.00	0.00±0.00	6.79±0.19	8.58±0.01	0.71±0.02
SWM	0.00±0.00	0.00±0.00	6.87±0.12	8.49±0.00	0.92±0.08

4. Discussion

Yeast cells evaluate and respond to the nature and amount of available nitrogen-containing compounds. Although *Saccharomyces* can use a variety of nitrogen sources as sole source of all cellular nitrogen, it shows some preferences in its utilization. These preferences in nitrogen source are manifested either quantitatively by an enhanced growth rate in media containing the preferred source or qualitatively by the ability of the preferred sources to induce repression of genes required for catabolism of other nitrogen sources (Magasanik and Kaiser, 2002). These two different criteria can be used to judge the quality of a particular nitrogen source. Although the growth rate is the simplest criterion, in some cases it is difficult to make clear distinctions between sources. For this reason, the second criterion is used, which consider preferred nitrogen sources as those repressing the pathways for utilization of alternative (or poorer) nitrogen sources, and, conversely, non-preferred sources as those leading to derepression of the alternative pathways. The stimulus responsible to arrest the expression of nitrogen-regulated genes is the intracytoplasmic concentration of glutamine and glutamate (Magasanik and Kaiser, 2002). These sources are present in the central nitrogen metabolism and are the final products of the pathways for the utilization of non-preferred nitrogen sources. Because of the importance of nitrogen in wine fermentations, we investigated the effect of different nitrogen sources in four parameters: yeast growth, consumption, fermentative metabolite synthesis and NCR on four commercial wine strains, and the relationship among them. To this end, we used the expression of two genes, *GAP1* and *DAL4*, which are induced during growth on poor nitrogen conditions. To perform simpler analysis, we used reporter strains based on the GFP expression under promotor control of *GAP1* or *DAL4*. This determination has the advantage that different nitrogen sources cause easily observable differences in the expression of these genes involved in the use of alternative nitrogen sources. In a previous study (Gutiérrez *et al*., 2013) we

observed that both permeases were repressed at the beginning of wine fermentation, when nitrogen in the medium is high, and derepressed as this nitrogen (specially ammonium) is consumed. The present study confirms the effective response of *GAP1* to nitrogen quality, since, in these conditions, *DAL4* expression was almost negligible in most cases and was not shown differences between nitrogen sources. Henceforth, *GAP1* was used as reporter of how four strains sense nitrogen quality in the fermentation media.

Preferred nitrogen sources response

Main of the pathways for the utilization of nitrogen sources feed into a central nitrogen metabolism. It is formed for the synthesis of glutamate from α-ketoglutarate and ammonia and for the synthesis of glutamine from glutamate and ammonia, catalyzed by a set of enzymes. These enzymes are responsible to adjust glutamate and glutamine concentration. The nitrogen sources permitting the most rapid and highest growth are glutamine, asparagine, ammonium, GABA, aspartate and glutamate. These results are in accordance with previous studies (Hofman-Bang, 1999; Godard *et al*., 2007). These sources either are directly involved in the heart of nitrogen metabolism (glutamine, ammonium and glutamate) or can be easily degraded to these ones (asparagine and aspartate). Moreover, they have specific permeases to make possible the fast uptake of these sources. Although glutamate is involved in central nitrogen metabolism, in some occasions it does not seem as good as glutamine and ammonium supporting cell growth. It is probably because of lower transport rate. Anyway, these nitrogen sources support rapid growth and are rapidly assimilated, exerting a strong NCR. The expression of *GAP1* is only shown when the medium is either totally or almost depleted. However NCR did not always correlate with cell growth (Godard *et al*., 2007), and there are certain differences between yeast strains. Ammonium and glutamine are the most repressor sources in the PDM strain, since *GAP1* expression begins when

media was exhausted. In ARM, aspartate and glutamate are also considered repressor sources. RVA showed strange behavior due to preculture conditions; it was unable to consume ammonium, and then was not repressor. Surprisingly, GABA considered as alternative source, also showed preferred N source profile showing a high cell growth and repressor effect. Alanine and arginine were also included in this group. They reached similar maximum growth (yield) to the other sources, but growth rates were reduced (mainly in arginine). It is converted into ornithine and urea and subsequently degraded to glutamate and ammonium. Probably this source is slower due to time required for degradation. In both cases are shown a derepressor effect when N source is still available. It is curious that some nitrogen sources like arginine, alanine, glutamate and aspartate were hardly consumed when there are an excess of nitrogen in a complex media composed by ammonium and different amino acids (Beltran *et al*., 2005), and have a high repressor effect when they are as sole N source. It should be pointed out that behavior of these N sources is also caused because transamination or deamination of these compounds produce pyruvate or Krebs cycle intermediates (α-ketoglutarate, oxaloacetate), directly assimilable by cell metabolism. Therefore sugar degradation is faster and synthesis of metabolites relevant to the final quality of wine is increased (Albers *et al*., 1996).

Response triggered by arginine intermediates, branched-chain and aromatic amino acids

Any nitrogen compound that can be transported into the cell and metabolized into glutamate or ammonium can be used as a sole nitrogen source. Arginine intermediates (urea, ornithine, citrulline), branched-chain amino acids (leucine, isoleucine and valine) and some aromatic amino acids (phenylalanine and serine) are an example of this behavior. They are transported inside the cell with several specific transporters and are catabolized through several steps to nitrogen

compounds which can be used directly by cell metabolism. These sources are able to reach similar growth yield that preferred nitrogen sources. However, they require more time to achieve the maximum growth and, therefore, their growth rates are lower. The consumption is also slower, but nitrogen sources are totally depleted in each specific media (except in RVA strain). Furthermore, the transamination of some of these N sources leads to keto acids and aldehydes which are converted into long-chain or complex alcohols, whose accumulatin are toxic for the cells. It means that not all products obtained in catabolism of these N sources are directly assimilated by the cell. In this group, NCR occurs but is not as strong as in preferred N sources. Gene activation was also shown strain-dependent (higher values in PDM than TTA). These sources, described as alternative nitrogen sources, in a complex media are used after depletion of preferred N sources. Trend showed in the metabolite production of this group (mainly branched-chain and aromatic amino acids) was the higher synthesis of ethanol, but, contrary to the preferred sources, with lower final concentration of glycerol and acetic acid. Glycerol synthesis requires ATP and is energetically unfavorable. However, this production is necessary to reduce NADH excess produced during fermentation, and to maintain the cellular redox balance. Therefore, these sources use Ehrlich pathway to synthetize higher alcohols from amino acid catabolism, and provide an alternative, energy-efficient pathway for NADH regeneration (Hazelwood *et al*., 2008).

Non-preferred nitrogen sources response

Not all nitrogen sources are assimilated equally well by the cell. Some sources support slow growth and hardly assimilation. This group includes adenine, cytosine, methionine, threonine, proline and tryptophan. They supported the lowest specific growth rates (half rate in comparison with preferred N sources), and achieved lower biomass yield. They are poor nitrogen sources in terms of the

energy required and the relative difficulty of converting them to a usable nitrogen compound. Moreover, catabolism of these compounds produces nonmetabolizable products that the cells excrete in form of higher alcohols. On these nitrogen sources NCR does not occur. They revealed a soft repressor effect, since *GAP1* fluorescence reached the highest values and started when available nitrogen was above half of the initial N concentration. Cells of these conditions detect poor quality of the nitrogen when the media still contains nitrogen. They respond to this stress with the activation of *GAP1*expression with the objective to assimilate more N.

Proline is one of the most abundant nitrogen sources in the wine must. It is catabolized within the mithocondrion in a two steps conversion to glutamate. The first enzyme, proline oxidase (*PUT1*) is oxygen dependent and then cells are unable to assimilate this source when the process is carried out in anaerobic conditions. In this study, were used semi-anaerobic conditions that were required for sampling that could justify the hardly assimilation. However, before it can be catabolized, proline is transported into the cell through Gap1 and the specific permease Put4, and we showed that medium contain proline and it was hardly uptake. It is known, that these permeases are only repressed when the media contains rich N sources, and in this case, proline is the unique source presents in the media. Probably, preculture conditions in ammonium excess produce a repression in transport of this source, and later proline is hardly catabolized for the low amount of oxygen.

Nucleotides adenine and cytosine showed the worst assimilation profile and high *GAP1* expression. It is required high energy amount to transport these compounds which are ineffective as nitrogen source. The high expression of *GAP1* was required when cells detect a poor quality of nitrogen in the media.

Methionine was a particular case, whose effect depended on the strain. Although it was characterized as poor source, some strains were showed more affected (mainly PDM). Methionine is involved in sulfur metabolism, especially the sulfate

reduction sequence, which generates hydrogen sulfide. This compound generates unpleasant "rotten egg" odour in wine, and for this reason it is important to know the response of strains used in the wine production to methionine assimilation to take control in hydrogen sulfide production.

Sugar degradation is slower in these sources because nitrogen availability is related with glycolisis, due concentration of metabolites is also lower. Tryptophan showed a particular behavior in glycerol synthesis in PDM and ARM strains, producing more glycerol amount that the preferred nitrogen sources, even when sugar not is totally degraded.

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CHAPTER 5

Nitrogen needs of a commercial wine yeast strain in the stationary phase: effect of sugar concentration and impact on aroma production

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Abstract

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. The wine industry uses different nitrogen preparations to avoid nitrogen deficiency in wine fermentations. However, it is important to know each strain's specific nitrogen requirement in order to avoid excessive addition. In a previous study, the minimum nitrogen amount required by commercial yeast strain PDM was determined to ensure maximum populations during growth or in the exponential phase. In this work, the nitrogen demands in the stationary phase of this strain used worldwide were established. Nitrogen availability in this phase stimulated the fermentation rate. Nevertheless, the amount of nitrogen required in this phase was much lower than that required in the exponential phase. Likewise, nitrogen concentration and source had a huge impact on aroma synthesis. Thus higher nitrogen concentrations, mainly in the form of arginine, enhanced the synthesis of the main fermentative aromas. This yeast nitrogen demand also depended on the sugar concentration of grape must. The results indicate that different nitrogen sources can be used to produce faster fermentations and wines with divergent aroma profiles in order to meet consumer demands for diversified products.

Keywords: nitrogen additions, arginine, aroma compounds, fermentation rate, stationary phase, high sugar concentration

1. Introduction

Nitrogen composition plays an important role in the fermentative process since this macronutrient increases biomass production and stimulates the sugar utilization rate. Nitrogen deficiency has been associated with major winemaking problems, especially those related to slow, incomplete fermentation (Bisson, 1991). Nowadays, the wine industry avoids these problems by adding nutritional supplements, usually inorganic forms of nitrogen such as ammonium salts, to grape must before fermentation (Monteiro and Bisson, 1992). Organic nitrogen sources, such as commercial preparations containing inactivated yeast or yeast products, are also commercially available. In many cases, winemakers proactively add nitrogen to must without knowing its initial nitrogen status, which may lead to high nitrogen levels that exceed the minimum amount required to complete fermentation. Excessively high nitrogen levels may have negative effects, such as microbial contamination, production of off-flavors (Jiranek *et al*., 1995) or ethyl carbamate formation, which is a suspected carcinogen (Ough, 1991). In fact, yeasts' nitrogen requirements depend not only on the strain (Jiranek *et al*., 1995), but also on the fermentation conditions (sugar concentration, temperature, presence of oxygen, etc.) (Valero *et al*., 2003). The timing of nitrogen additions also influences fermentation performance. If nitrogen addition takes place during the cell growth period, the achieved cell populations peak. Later additions in the stationary phase have no effect on cell populations, but raise the specific fermentation rate, thus reducing fermentation duration (Bely *et al*., 1990; Manginot *et al*., 1997; Manginot *et al*., 1998; Mendes-Ferreira *et al*., 2004; Beltran *et al*., 2005; Hernández-Orte *et al*., 2006).

Nitrogen availability can also affect many aspects of yeast metabolism, including the formation of volatile compounds, which are important for the organoleptic qualities of wine ("fermentation bouquet"). Many are affected by nitrogen type and concentration (Bell and Henschke, 2005) since ammonium and amino acids

determine the intracellular nitrogen pool which regulates the metabolic pathways of aroma compounds formation. Higher alcohols (e.g., isobutanol, isoamyl alcohol and 2-phenylethanol) and esters (e.g., ethyl acetate, isoamyl acetate, ethyl caproate, ethyl caprate, ethyl caprylate and phenylethyl acetate, among others) are the main groups of flavor compounds originating from yeast metabolism. Higher alcohols are generated from α-ketoacids, which derive mainly from sugars and through anabolic reactions from branched-chain amino acids, which occur by the Ehrlich pathway and involve transamination, followed by decarboxylation and reduction steps (Eden *et al*., 2001). Higher alcohols can impart fusel or solvent odors, or floral ones (e.g., 2-phenylethanol) (Lambrechts and Pretorius, 2000). They appear in wine at variable concentrations; at a moderate concentration, they confer a desirable complexity to the aroma, while they can deteriorate aroma at higher concentrations. An inverse relationship is generally observed between the initial nitrogen concentration and higher alcohols, except at very low nitrogen levels (Carrau *et al*., 2008). Moreover, esters are produced by the condensation of an alcohol and a coenzyme-A-activated acid (acyl-CoA) catalyzed by an acyltransferase (Sumby *et al*., 2010). Specifically, acetate esters result from the combination of acetyl-CoA and an alcohol by the action of alcohol acetyl transferases, whereas ethyl esters are biochemically generated from acyl-CoA and ethanol by the action of acyltransferases. Ethyl and acetate esters are important for wine quality because they elicit pleasant aromas.

As previously mentioned, nitrogen requirements are also related to the sugar concentration in must. The current climate change situation often leads to grapes presenting higher sugar levels and increased grape maturity than those desired. The extremely high sugar concentrations reached at harvest, especially in warm climates, are often related with wanting to optimize polyphenolic and aromatic maturity. Although grapes can be harvested earlier, it is necessary to wait for appropriate phenolic ripeness. When this occurs, grapes become overripe, and

yeast nitrogen requirements and nitrogen availability in the medium change (Malherbe *et al*., 2007; Mira de Orduña, 2010; Martínez-Moreno *et al*., 2012).

In this context, determining the specific nitrogen demands of wine yeasts is of paramount importance for proper wine fermentations management. A previous study (Gutiérrez *et al.*, 2012) determined the minimum nitrogen amount required by this commercial yeast strain to ensure maximum populations during the exponential growth phase in a synthetic grape must. However, Manginot *et al*. (1998) observed that the main differences in nitrogen requirements among wine yeast strains appear mainly in the stationary phase. These nitrogen needs are certainly related to the ability of yeast to maintain protein synthesis for various functions such as sugar transport. Thus once we knew the specific nitrogen requirement to obtain the maximum biomass (exponential phase), the present study aimed to determine the specific nitrogen requirement in the non proliferating or stationary phase in order to accomplish the maximum fermentation rate and an impact on aroma production. Very few works have addressed nitrogen needs in the stationary phase of fermentation, and the effectiveness of additions of different nitrogen sources during this period. To this end, the effect of different nitrogen concentrations of ammonium and arginine was analyzed, which were used as sole nitrogen sources, on both the fermentation rate and aroma production. Ammonium and arginine respectively represent the major inorganic and organic nitrogen sources in grape must. Ammonium is the more preferred nitrogen source, whereas arginine has shown positive effects on cell growth and fermentation (Gutiérrez *et al*., 2012). In order to separately study the exponential and stationary phases, a synthetic grape-must fermentation, with the initial nitrogen concentration to obtain the maximum biomass (Gutiérrez *et al.*, 2012), was divided at the end of the exponential phase into different fermentors, which were supplemented with distinct nitrogen concentrations. Based on the direct correlation between nitrogen demand and sugar concentration in grape musts previously reported (Taillandier *et al*.,

2007), this study also aimed to know how nitrogen requirements in the stationary phase change in grape must with a high sugar content.

2. Materials and methods

2.1. Yeast strain and inocula preparation

The commercial strain used in this study was provided by the Agrovin Company (Ciudad Real, Spain), and was obtained as an active dry wine yeast (ADWY). The PDM strain belongs to the species *Saccharomyces cerevisiae*.

For inoculation, active dry yeast cells were rehydrated in water following the manufacturer's instructions (30 min at 37ºC). After microscope counting, the appropriate dilution of the rehydrated wine yeast was inoculated in a synthetic grape must (SM) to obtain an initial cell concentration of ~2 x 10^6 cells/mL.

2.2. Culture media

The synthetic grape must (SM) was prepared according to Riou *et al*., (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L; tartaric acid 3 g/L. The mineral salts that follow were utilized: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L. These vitamins were employed: myo-inositol 20 mg/L, calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L. The yeast assimilable nitrogen (YAN) content in the synthetic grape must was 140 mg N/L: 42 mg N/L as ammonium nitrogen (NH_4Cl) and 98 mg N/L as an amino acids form. The proportion of each amino

acid was administered as previously proposed by Riou *et al*., (1997). This amount of YAN was established as the minimum concentration required to obtain maximum growth in the strain used in this experiment (Gutiérrez *et al.*, 2012). The final pH of the SM was adjusted to 3.3 with NaOH.

The effect of sugar concentration on nitrogen demand was also tested by running fermentations with 240 g/L and 280 g/L. The initial nitrogen concentration of the SM was also modified to 160 mg N/L (48 mg N/L as ammonium and 112 mg N/L as amino acids) and 180 mg N/L (54 mg N/L as ammonium and 126 mg N/L as amino acids) for the fermentations with 240 g/L and 280 g/L, respectively (Martínez-Moreno *et al.*, 2012).

2.3. Fermentation conditions

Fermentations took place in the exponential phase at 28ºC with continuous orbital shaking (150 rpm). They were performed in 1 L glass bottles containing 900 mL of the SM under semi-anaerobic conditions since limited aeration was needed to harvest samples for the subsequent analysis. Cell growth was measured by OD at 600 nm. The supernatant was recollected and stored at -20ºC at different fermentation times to analyze sugar and nitrogen consumption. After achieving the stationary phase and stopping cell growth, each medium was subdivided into other smaller flasks (250 mL bottles filled with 150 mL). Nitrogen was added at different concentrations as either ammonium (40 mg N/L, 80 mg N/L and 200 mg N/L) or arginine (40 mg N/L, 80 mg N/L, 120 mg N/L and 200 mg N/L). Only the control condition was not supplemented with nitrogen. These conditions were used in triplicate at 28ºC with permanent agitation (150 rpm).

2.4. Monitoring fermentation

Prior to nitrogen addition, the alcoholic fermentation progress was monitored by the medium density using a Densito 30 PX densitometer (Mettler Toledo, Switzerland) and by sugar consumption. After the subdivision into different bottles and nitrogen supplementation, the ANKOM^{RF} Gas Production System (ANKOM Technology, NY, U.S.A.) was used to monitor CO_2 production. The wireless system consisted of: radio-frequency (RF) pressure sensor modules connected to bottles (fermentors); a zero remote module that measured ambient pressure; a computer interface base coordinator and operational software. The pressure of each bottle was measured in psi at 30-minute intervals. Pressure measurements were recorded on an Excel spreadsheet and gas production curves were generated. Fermentations were stopped when successive increments of CO_2 production were lower than 0.05 psi. The supernatant was taken to analyze the concentrations of YAN, metabolites and volatile compounds.

2.5. Yeast assimilable nitrogen determination

Ammonium and urea concentrations were measured by an enzymatic kit method (Roche Applied Science, Germany). The free amino acid nitrogen concentration was determined by following the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen(N)/mL. The sum of ammonium and amino acids represented yeast assimilable nitrogen (YAN).

2.6. Metabolite analysis (HPLC)

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed in all the samples at the end of the exponential phase (change point) and at the end of

fermentation. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, MA, USA) equipped with a refraction index detector, autosampler and UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min. Then supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Spain) and diluted 5- or 10-fold. A total volume of 25 μL was injected into a HyperREZ^{TM}XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its corresponding guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50ºC. The concentration of each metabolite was calculated using external standards. Each sample was analyzed in duplicate.

2.7. Volatile aroma compounds analysis

Higher alcohols and esters were analyzed based on a headspace solid phase microextraction (SPME) technique using a 100 μm poly-dimetylsiloxane (PDMS) fiber (Supelco, Sigma-Aldrich, Spain). Aliquots of 1.5 mL of sample were placed into 15 mL vials and 0.35 g of NaCl and 20 μL of 2-heptanone (0.005%) were added as an internal standard. Vials were closed with screwed caps and 13 mm silicone septa. Solutions were stirred for 2 h to obtain the required headspace-liquid equilibrium. Fibers were injected through the vial septum, exposed to the headspace for 7 min and desorbed for 4 min in a gas chromatograph (TRACE GC Ultra, Thermo Scientific) with a flame ionization detector (FID), equipped with an HP INNOWax 30 m x 0.25 mm capillary column coated with a 0.25-m layer of cross-linked polyethylene glycol (Agilent Technologies). The carrier gas was helium (1mL/min) and the oven temperature program was: 5 min at 35ºC, 2ºC/min to 150ºC, 20ºC/min to 250ºC and 2 min at 250ºC. The injector and detector temperatures were maintained at 220ºC and 300ºC, respectively. A chromatographic signal was recorded by the ChromQuest program. Volatile compounds were identified by comparing the retention time for reference

compounds. Volatile compound concentrations were determined using the calibration graphs of the corresponding standard volatile compounds. 2-heptanone (0.005% w/v) was used as an internal standard.

2.8. Statistical analysis

All the experiments were repeated at least three times, and the data were reported as the mean value ± SD. Significant differences were determined by *t*-tests (the SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$. A principal component analysis (PCA) was used to reduce the dimensionality of the data and to find the best differentiation among samples. The PCA was carried out with the Statistica 7.0 software.

3. Results

The main purpose of the present work was to determine not only the specific nitrogen needs in the stationary phase of a worldwide used wine strain, but also the impact on fermentation rate and aroma production. To this end, fermentation was started in a synthetic must (SM) with an adequate nitrogen concentration to ensure optimal growth (Gutiérrez *et al*., 2012). When growth was stopped, the fermentation medium was divided and different ammonium or arginine concentrations were added as the sole nitrogen source. The use of a novel system to monitor CO_2 production allowed us to establish the effect of the different nitrogen conditions on fermentation activity.

3.1. Influence of nitrogen addition in the stationary phase on fermentation activity

After 48 h of inoculation, PDM reached the stationary phase (no growth). Sugar, ethanol, ammonium and amino acid concentrations were determined at the end of the exponential phase (Table 1). At this point, half the sugars (around 90 g/L), and practically all the available nitrogen, had almost been consumed in the exponential phase. Then, fermentations were subdivided into smaller bottles and were supplemented with the different nitrogen conditions: ammonium (40 mg N/L, 80 mg N/L and 200 mg N/L) and arginine (40 mg N/L, 80 mg N/L, 120 mg N/L and 200 mg N/L). Significant increases in the CO_2 production and maximum sugar fermentation rates (V_{max}) were displayed for all the nitrogen additions as compared with the control condition (Figure 1 and Table 1). Moreover, significant decreases were detected when the residual sugar was analyzed at the end of the various fermentations (Table 1). However, no significant dose response was detected under some nitrogen conditions. A linear correlation between the higher nitrogen concentrations and the fermentation (V_{max}) rate was observed only up to 40 mg N/L for the ammonium and 80 mg N/L for the arginine additions (Table 1 and Figure 2). Cell growth was also measured at the end of fermentation. No changes in OD were detected if compared to the end of the exponential phase, thus confirming that nitrogen additions affected only the fermentation rate.

The added nitrogen was not completely consumed under any condition. The larger the nitrogen addition, the more residual nitrogen remained at the end of fermentation. As expected, the addition of arginine increased residual amino acids, but not ammonium. Nonetheless, addition of ammonium increased both inorganic and organic nitrogen. Interestingly, the amino acid concentration also increased under the control condition, to which nitrogen was not added. Evidently, therefore, this fraction of residual amino acids originated from the cellular lysis. Despite total consumption not taking place, the added arginine was more consumed in the

stationary phase than ammonium. Except for the condition with 200 mg N/L of arginine, the residual assimilable nitrogen in the other fermentations supplemented with this amino acid was similar to the control condition (no addition). The urea concentration in the final wines was also measured as it has been related with arginine catabolism and as precursor of carcinogenic ethylcarbamate. However, urea was not present under any fermentation conditions (data not shown).

Table 1. Maximum fermentation rate (V_{max}) and final composition of the wines fermented with different concentrations and sources (ammonium and arginine) of nitrogen. The change point refers to the end of the exponential phase and the time of nitrogen addition

	V_{max} (g/L/h sugars)	**Sugar (g/L)**	**Ethanol (%)**	**NH_4^+ (mg/L)**	**Amino Acids (mg/L)**
Change point (48h)	-	113.00±0.03	4.08±0.07	2.72±0.04	5.00±1.24
Control	0.99±0.03	10.27±0.70	10.00±0.12	1.00±0.20	17.94±5.43
40 NH_4^+	1.22±0.05[a,b]	5.04±0.83[a,b]	10.81±0.13[a,b]	13.00±0.51[a,b]	20.37±3.37
80 NH_4^+	1.14±0.02[a,b]	6.73±2.20[a]	10.59±0.55	38.2±4.70[a]	21.72±6.30
200 NH_4^+	1.18±0.05[a]	3.27±1.36[a,b]	9.98±0.73	86.22±2.21[a,b]	35.09±2.00[a,b]
40 Arg	1.01±0.18	5.25±1.64[a,b]	10.57±0.29[a,b]	2.77±0.91	17.99±1.69
80 Arg	1.19±0.01[a]	5.72±2.76[a]	10.71±0.33[a]	3.02±0.26	26.14±1.26[b]
120 Arg	1.16±0.05[a]	6.46±1.21[a]	9.68±0.66[b]	4.08±1.14	17.72±3.78[b]
200 Arg	1.05±0.01[a,b]	2.47±0.01[a,b]	10.46±0.00[a,b]	0.91±0.14[b]	83.12±2.43[a,b]

[a] Significant differences compared with control (no nitrogen addition)
[b] Significant differences compared with the previous concentration of the same nitrogen source

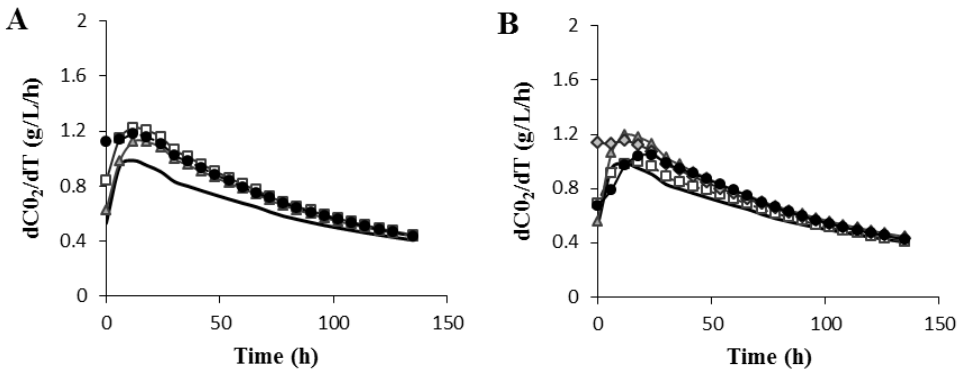


Figure 1. Evolution of the CO_2 production rate in the stationary phase with different concentrations (40 □, 80 ▲, 120 ♦ and 200 ● mg N/L) and sources (ammonium (A) and arginine (B)) of nitrogen. The control condition was not supplemented with nitrogen (─)

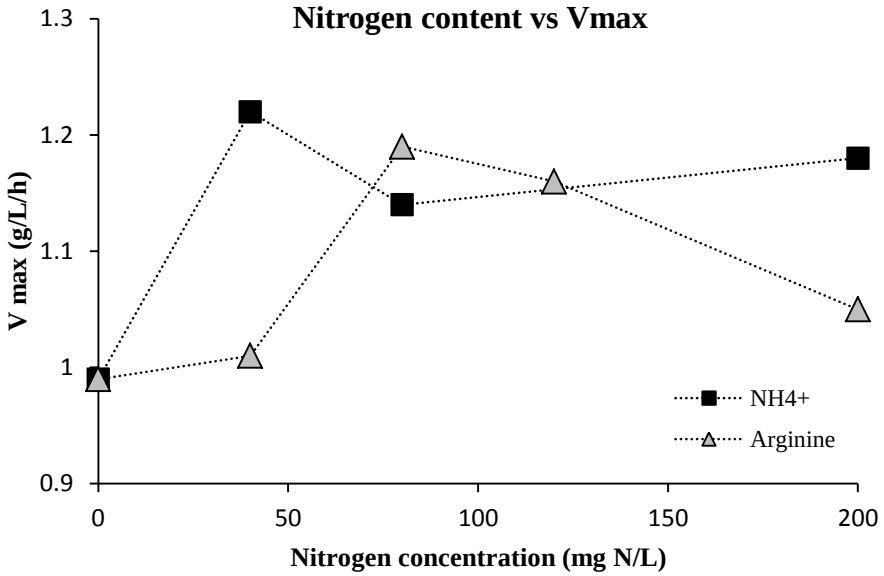


Figure 2. Correlation between nitrogen concentration and source added at the beginning and the maximum fermentation rate (V_{max}) during the stationary phase

3.2. Changes in aroma composition in response to nitrogen addition in the stationary phase

This study also aimed to analyze the effect of nitrogen concentration and source on the synthesis of major volatile compounds. Higher alcohols, acetate esters and ethyl esters were determined for all the conditions tested (Table 2). Additions of nitrogen at the beginning of the stationary phase increased the concentrations of the acetate esters and ethyl esters. Ethyl acetate and phenylethyl acetate were the acetate esters which increased the most under the majority of nitrogen conditions assayed.

Table 2. Concentration of aroma compounds of the wines fermented with different concentrations and sources (ammonium and arginine) of nitrogen

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	40Arg	80 Arg	120 Arg	200 Arg
Ethyl acetate	24.260±0.52	33.635±1.50**	30.075±1.99*	24.953±0.44	29.083±0.73**	29.075±2.75	22.708±2.36	31.192±0.80**
Isobutyl acetate	0.220±0.01	0.269±0.07	0.209±0.05	0.811±0.20*	0.194±0.06	0.256±0.06	0.866±0.07**	0.201±0.03
Isobutanol	24.729±2.76	16.878±5.91	19.258±2.23	22.090±0.29	23.518±7.27	19.395±5.24	25.180±2.03	24.446±0.50
Isoamyl acetate	0.766±0.11	1.009±0.05*	0.976±0.05	0.624±0.04	0.960±0.13	0.875±0.17	0.652±0.05	1.078±0.04
Isoamyl alcohol	54.110±6.38	59.351±11.33	40.009±1.98	48.511±1.21	64.894±13.08	53.185±14.34	56.750±2.85	50.837±1.05
Ethyl caproate	0.504±0.06	0.432±0.17	0.562±0.21	0.404±0.02	0.550±0.21	0.535±0.22	0.443±0.04	0.676±0.05
Ethyl caprylate	0.179±0.03	0.155±0.08	0.208±0.07	0.291±0.03*	0.296±0.13	0.240±0.08	0.304±0.03*	0.401±0.04**
Ethyl caprate	0.056±0.01	0.075±0.04	0.096±0.03	0.161±0.01**	0.136±0.06	0.090±0.02	0.139±0.01**	0.191±0.01**
Phenylethyl acetate	0.355±0.04	0.420±0.00*	0.434±0.06	0.581±0.01**	0.548±0.14	0.404±0.07	0.563±0.03**	0.477±0.01*
2-phenylethanol	19.785±2.09	17.761±0.77	16.199±5.51	21.382±1.05	21.237±3.50	17.309±3.14	21.783±5.76	20.138±0.80
∑ Higher alcohols	98.624±9.14	93.990±2.82	75.465±6.27	91.983±1.69	109.649±9.77	89.889±8.28	103.713±8.78	95.421±6.50
∑ Acetate esters	25.601±1.78	35.332±1.57*	31.694±1.99*	26.989±0.32	30.784±0.77*	30.610±2.95	24.789±2.37*	32.948±0.80*
∑ Ethyl esters	0.739±0.01	0.662±0.04*	0.866±0.00	0.856±0.04	0.981±0.12	0.864±0.13	0.886±0.08	1.269±0.01*

Significant differences compared with control (no nitrogen addition) *p<0.05; **p<0.01

Regarding ethyl esters, some nitrogen conditions produced significantly more ethyl caprylate and ethyl caprate, mainly with the arginine additions. No significant differences were observed with the levels of higher alcohols.

As an overview of the results, a PCA was done to highlight the relevance of nitrogen concentration and nitrogen source in aroma compounds production (Figure 3). The first principal component (PC1) accounted for 46.69% of total variance, while PC2 explained 27.12%. Two components with large eigenvalues were used to explain 73.81% of total variance. PC1 was characterized by ethyl acetate and isoamyl acetate, with positive loadings and 2-phenylethanol with negative values. For PC2, ethyl esters showed positive loadings, while isobutyl acetate exhibited negative ones. Samples were ordered according to the nitrogen source and concentration added. The arginine addition brought about a wider separation in the PCA plot in relation to the control condition, except for 80 mg N/L. The most distant groupings found were $200NH_4^+$/120Arg and 200 Arg, corresponding to the higher concentration of isobutyl acetate and ethyl esters, respectively. Moreover, the main aroma compounds showed higher concentrations for the arginine additions.

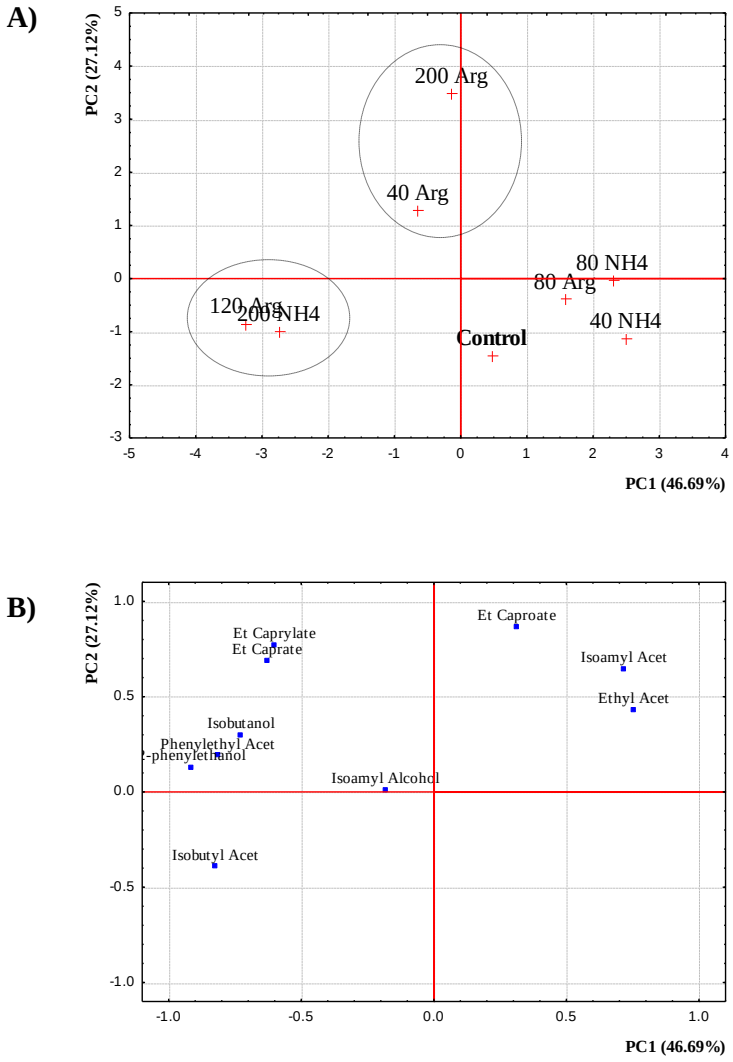


Figure 3. Results of the principal component analysis of volatile compounds. A) Scores obtained under different nitrogen conditions on the plane formed by the two first principal components. B) Loadings of the variables on the first two principal components

3.3. Impact of sugar concentration on nitrogen demand, fermentation rate and aroma synthesis

This section follows the same experimental design as that explained above, but the sugar concentrations of the initial grape musts was increased to 240 g/L (24%) and 280 g/L (28%). The initial nitrogen available in grape must was also increased to 160 mg N/L and 180 mg N/L, respectively, to obtain a maximum biomass yield in the exponential phase (Martínez-Moreno *et al.*, 2012). As before, when cell growth ceased, the fermentation medium was subdivided into smaller bottles and was supplemented with similar nitrogen concentrations and sources. The time required to reach the stationary phase was longer in these fermentations (90 h and 144 h, respectively). The sugar concentration at the change point was approximately half the initial amount (Table 3). Regarding the residual nitrogen at the end of the exponential phase, ammonium was practically exhausted, but a substantial amount of amino acids remained (57 mg N/L) in the fermentation with 24% of sugars. In relation to the higher sugar content fermentation (28%), both ammonium and amino acids were left at the change point.

The CO_2 production and the maximum sugar fermentation (V_{max}) rates under these conditions are provided in Figure 4 and Table 3. The higher sugar and ethanol concentrations at the beginning of the stationary phase lowered the CO_2 production rate and V_{max} to practically half, if compared with the fermentations performed at 200 g/L (Figure 1 and Table 1). Nitrogen addition hardly affected the V_{max}, although most additions improved the CO_2 production rate and reduced the residual sugars of the final wines. Arginine additions showed less residual sugars at the end of fermentations than ammonium additions.

Table 3. Maximum fermentation rate (V_{max}) and final composition of the wines fermented with different sugar and nitrogen concentrations and sources (ammonium and arginine). The change point refers to the end of the exponential phase and the time of nitrogen addition

Fermentation		V_{max} (g/L/h sugars)	Sugar (g/L)	Ethanol (%)	NH_4^+ (mg/L)	Amino Acids (mg/L)
24%	**Change point** (90h)	-	114.23±2.79	6.70±0.14	0.83±0.32	57.21±1.52
	Control	0.51±0.19	39.75±2.51	11.08±0.61	5.69±1.11	56.48±3.86
	40 NH_4^+	0.52±0.07	42.51±8.51	11.06±1.10	44.85±4.41[a,b]	62.62±5.62
	80 NH_4^+	0.50±0.04	43.80±4.08	10.77±1.10	64.68±7.86[a,b]	63.95±6.89
	200 NH_4^+	0.53±0.01	32.43±5.37	11.54±0.41	82.85±0.31[a,b]	73.57±2.02[a]
	80 Arg	0.42±0.04	20.67±1.04[a,b]	12.38±0.39[a,b]	5.08±1.37	91.38±0.33[a,b]
	120 Arg	0.48±0.01	12.80±1.73[a,b]	12.80±0.10[a,b]	4.13±0.31	112.05±1.75[a,b]
	200 Arg	0.49±0.00	10.84±1.02[a,b]	12.87±0.14[a]	3.37±1.01	127.10±9.43[a]
28%	**Change point** (144h)	-	155.85±0.17	6.34±0.01	19.03±0.02	98.43±0.06
	Control	0.33±0.13	94.78±0.03	9.36±0.96	26.98±3.03	83.29±3.18
	40 NH_4^+	0.27±0.12	86.04±0.04	10.61±0.72[a,b]	78.82±1.63[a,b]	86.19±5.01
	80 NH_4^+	0.35±0.01	87.42±5.78	10.28±1.09	80.48±0.66[a]	87.14±5.23
	200 NH_4^+	0.39±0.01[b]	84.02±2.02	10.41±0.66	81.19±0.53[a]	96.05±3.72[a]
	80 Arg	0.34±0.01	57.15±3.40[a,b]	12.15±0.11[a,b]	17.31±0.49[a,b]	112.19±2.15[a,b]
	120 Arg	0.24±0.04[b]	58.68±0.82[a]	12.04±0.13[a]	16.01±0.54[a,b]	135.86±6.78[a,b]
	200 Arg	0.35±0.00[b]	58.11±5.08[a]	12.11±0.33[a]	16.61±1.29[a]	145.71±7.30[a]

[a] Significant differences compared with control (no nitrogen addition)
[b] Significant differences compared with the previous concentration of the same nitrogen source

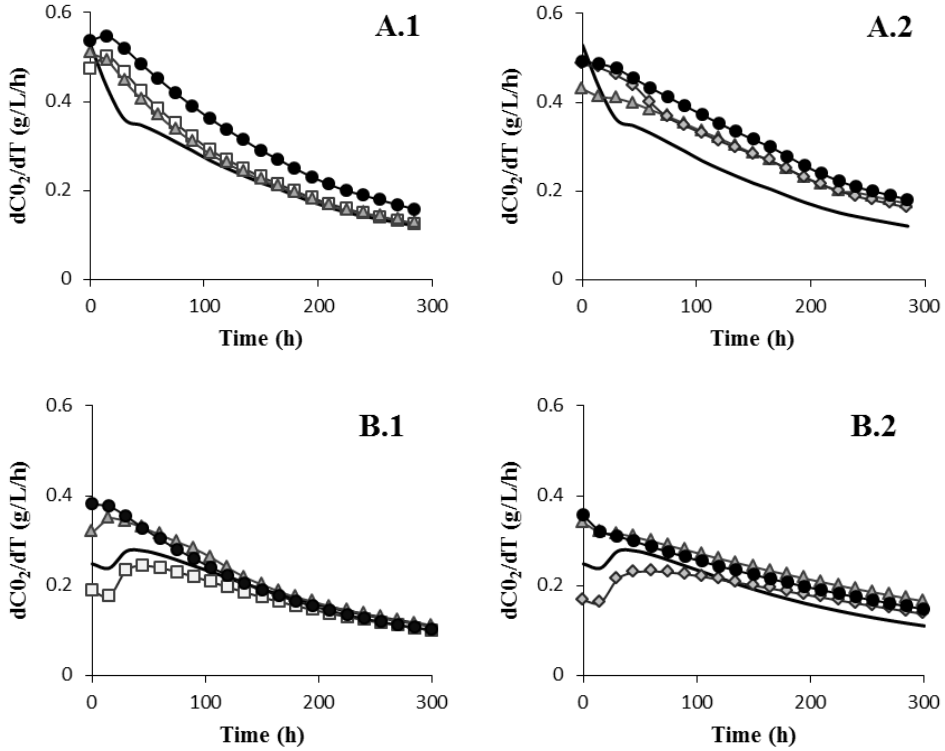
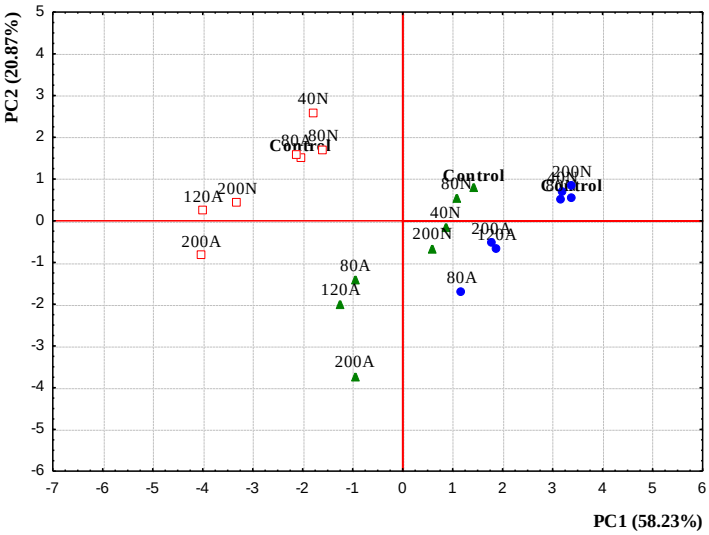


Figure 4. Evolution of the CO_2 production rate in the stationary phase with different concentrations (40 □, 80 ▲, 120 ♦ and 200 ● mg N/L) and sources (ammonium (1) and arginine (2)) of nitrogen. A) Fermentations with 24% of initial sugar, B) fermentations with 28% of initial sugar. The control condition was not supplemented with nitrogen (─)

A PCA was done to identify the aromas that best discriminated among the wines fermented with different sugar concentrations and nitrogen additions (Figure 5). The concentration of these aromas for the fermentations at 24% and 28% of sugars is shown in the Tables 4 and 5. This PCA integrated 3 sugar concentrations x 7 nitrogen additions x 3 triplicates. The scores (A) reveal the distribution of the conditions studied in relation to the loadings (B) displaying the volatile

compounds. The PCA accounted for 79.1% of total variance (58.23% and 20.87%, respectively). The first component (PC1) was characterized by higher levels of ethyl acetate and by negative loadings of phenylethyl acetate and 2-phenylethanol. The second principal component (PC2) attributes ethyl caprate, isobutanol and ethyl caprylate gave the highest negative values, while isoamyl acetate displayed a positive value. The effect of sugar concentration on aroma production was more important than nitrogen availability, as revealed in the PCA distribution (Figure 5). The separation along PC1 was associated with the amount of sugars. Those samples fermented with 28% of sugars had a higher ethyl acetate concentration, thus they were distributed mainly on the positive part of the graph. Furthermore, 20% fermentation was composed of acetate esters (except ethyl acetate), ethyl caproate and 2-phenylethanol. In addition, 24% of fermentation was distributed in the medium between the other fermentations. Along PC2, samples were separated according to the nitrogen source used in the addition. Most arginine additions were situated in the lower quadrants, whereas the control and ammonium conditions were always distributed in the upper graph quadrants. Arginine samples were characterized by higher concentrations of ethyl caprylate and ethyl caprate.

A)



B)

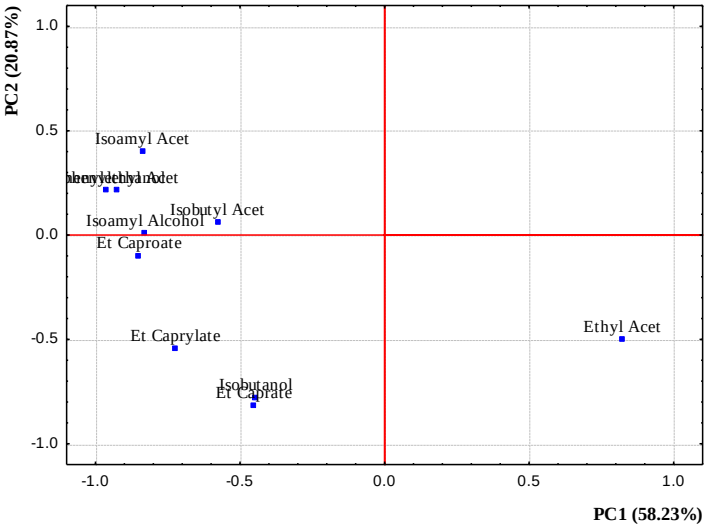


Figure 5. Results of the principal component analysis carried out on the volatile fermentation products of grape musts with different sugar concentrations (20% □, 24% ▲, 28% ●). A) Scores obtained under different initial sugar and nitrogen conditions (3 sugars x 7 nitrogen x 3 replicates) on the plane formed by the two first principal components. B) Loadings of the variables on the first two principal components. N (NH_4^+), A (arginine)

Table 4. Concentration of aroma compounds of the wines fermented with PDM strain with different concentrations and sources (ammonium and arginine) of nitrogen in synthetic must with 24% of sugar concentration

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	80 Arg	120 Arg	200 Arg
Ethyl acetate	50.988±6.72	60.539±5.08	51.086±5.22	64.671±2.62	60.853±1.70	61.085±2.01	67.307±5.96
Isobutyl acetate	0.153±0.08	0.234±0.04	0.238±0.07	0.217±0.03	0.258±0.14	0.235±0.05	0.186±0.07
Isobutanol	20.582±1.46	23.776±1.50	21.190±2.60	24.549±2.22	28.241±1.47	28.458±1.89	28.881±1.09*
Isoamyl acetate	0.261±0.06	0.292±0.05	0.258±0.05	0.336±0.06	0.347±0.06	0.350±0.03	0.311±0.05
Isoamyl alcohol	38.193±6.40	45.778±3.84	42.095±1.01	42.250±1.27	56.151±3.38	55.694±1.19*	42.999±1.43
Ethyl caproate	0.287±0.08	0.304±0.03	0.283±0.04	0.345±0.04	0.493±0.08*	0.479±0.06*	0.437±0.02*
Ethyl caprylate	0.123±0.03	0.133±0.02	0.108±0.03	0.149±0.04	0.182±0.05	0.291±0.09*	0.384±0.11*
Ethyl caprate	0.086±0.01	0.109±0.02	0.106±0.02	0.132±0.04	0.145±0.03*	0.156±0.03*	0.250±0.05*
Phenylethyl acetate	0.258±0.08	0.242±0.04	0.244±0.05	0.244±0.03	0.268±0.03	0.269±0.08	0.259±0.01
2-phenylethanol	10.616±0.40	10.801±0.29	11.658±0.30	11.356±2.44	13.592±0.37	13.590±2.38	12.345±0.74
∑ Higher alcohols	69.391±13.36	80.355±13.42	74.943±14.57	81.155±5.58	98.164±11.15*	97.742±8.20*	84.225±18.17*
∑ Acetate esters	51.660±12.32	61.308±11.03	51.825±9.48	65.468±2.65	61.726±5.81	61.939±4.88	68.062±6.86
∑ Ethyl esters	0.496±0.13	0.546±0.07	0.494±0.09	0.626±0.10	0.820±0.13**	0.926±0.19*	1.070±0.14

Significant differences compared with control (no nitrogen addition) *p<0.05; **p<0.01

Table 5. Concentration of aroma compounds of the wines fermented with PDM strain with different concentrations and sources (ammonium and arginine) of nitrogen in synthetic must with 28% of sugar concentration

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	80 Arg	120 Arg	200 Arg
Ethyl acetate	77.853±2.89	63.804±4.01	65.794±0.92*	70.016±6.26	74.394±5.28	60.526±3.54	61.562±7.12
Isobutyl acetate	0.140±0.01	0.146±0.04	0.111±0.01	0.176±0.06	0.204±0.07	0.365±0.05**	0.207±0.07
Isobutanol	17.768±0.85	17.790±0.15	19.402±2.89	18.697±2.49	24.320±2.93*	21.045±1.04*	20.810±3.02
Isoamyl acetate	0.136±0.00	0.130±0.01	0.132±0.02	0.138±0.03	0.199±0.02**	0.089±0.01**	0.084±0.00**
Isoamyl alcohol	32.682±0.00	34.212±0.28	35.821±0.52	34.971±4.08	43.596±2.34*	38.353±3.01*	38.452±2.09*
Ethyl caproate	0.266±0.05	0.243±0.03	0.229±0.06	0.213±0.06	0.461±0.04**	0.271±0.02	0.246±0.01
Ethyl caprylate	0.010±0.04	0.123±0.05	0.061±0.02	0.043±0.02	0.274±0.02**	0.238±0.03**	0.217±0.05*
Ethyl caprate	0.075±0.01	0.073±0.01	0.089±0.02	0.072±0.00	0.126±0.02*	0.117±0.01*	0.125±0.04*
Phenylethyl acetate	0.039±0.07	0.000±0.00	0.000±0.00	0.046±0.08	0.000±0.00	0.000±0.00	0.155±0.02
2-phenylethanol	6.164±0.79	5.412±0.82	7.049±1.52	5.889±0.70	6.673±2.24	6.669±1.61	8.304±0.54
∑ Higher alcohols	56.614±0.28	57.418±3.18	62.271±7.21	59.557±6.83	74.589±8.58*	66.068±5.46*	67.566±7.83*
∑ Acetate esters	78.169±14.99	64.080±4.00	66.037±8.09	70.376±10.11	74.796±8.71	60.981±4.87	62.007±5.12
∑ Ethyl esters	0.441±0.07	0.439±0.04	0.379±0.07	0.329±0.07	0.861±0.08**	0.625±0.07*	0.587±0.09

Significant differences compared with control (no nitrogen addition) *p<0.05; **p<0.0

4. Discussion

The nature and availability of nitrogen in grape must affect yeast cell growth, and the fermentation rate and secondary aroma production during wine fermentation. A recent work (Gutiérrez *et al.*, 2012) determined the minimum nitrogen amount required to ensure the maximum population in the growth or the exponential phase in a synthetic grape must. The present study aimed to establish the nitrogen requirement of the PDM strain in the non proliferating or stationary phase when most sugars are consumed. PDM consumed all the nitrogen in the exponential growth, but needed more nitrogen in the stationary phase. The nitrogen supplementation in this stage improved the fermentation rate and produced faster sugar consumption. However, the nitrogen requirements in the stationary phase were much lower than those needed for the exponential phase. The nitrogen-limiting concentration to reach the maximum fermentation rate ranged from 40 mg N/L to 80 mg N/L for ammonium and arginine, respectively (Figure 2). Moreover, higher nitrogen concentrations left a considerable amount of residual nitrogen, which can jeopardize the microbial stabilization of wine or the synthesis of unhealthy compounds (ethyl carbamate and biogenic amines). In any case, and conversely to the nitrogen consumption profile described by Beltran *et al*. (2004) during wine fermentation, arginine was more efficiently consumed in the stationary phase than ammonium. Moreover, this arginine consumption did not involve urea synthesis, a precursor of carcinogenic ethylcarbamate.

Although the higher nitrogen concentrations added showed no further increases in the fermentation rate, all the nitrogen conditions had a much clearer impact on aroma production. The use of a defined grape must allowed the quantification of the volatile compounds synthesized only by the yeast strain metabolism in the absence of varietal aromas and precursors. Thus, significant differences in most quantified aromas were detected among the different nitrogen concentrations and sources. In this case, higher nitrogen concentrations positively correlated with

higher concentrations of acetate and ethyl esters, the main compounds conferring wines fruity and floral notes. Previous studies have observed this correlation between nitrogen concentration and ester production (Carrau *et al.*, 2008; Garde-Cerdán and Ancín-Azpilicueta, 2008; Torrea *et al.*, 2011). Regarding the effect of the nitrogen source available in the stationary phase on aroma synthesis, our results evidence that arginine additions also present higher levels of acetate esters and ethyl esters. This finding is in line with previous studies which compared the effect of inorganic and organic nitrogen supplementations on the volatile composition (Torrea *et al.*, 2011; Barbosa *et al.*, 2012).

In the current climate change context, grape musts arrive at cellars with higher sugar contents than they did some years ago. Thus, the present study also aimed to study how nitrogen requirements change in the stationary phase with higher sugar and ethanol concentrations. Recently, Martínez-Moreno *et al.* (2012) reported that biomass yield is dependent on the amount of available nitrogen, the nature of the nitrogen source and the sugar concentration in the grape must. Thus, if the sugar concentration determines the nitrogen requirements in the exponential phase, a similar behavior in the non proliferating phase can be expected. Our results indicate that increased sugar content significantly affected the fermentation rate, which leads to stuck fermentations, regardless of the nature and the amount of nitrogen source. The presence of residual amino acids at the end of the exponential phase also limited the impact of different nitrogen additions. However, arginine additions presented lower residual sugars at the end of the process than ammonium additions. In line with this result, Martínez-Moreno *et al.* (2012) also reported maximum sugar consumption when a mixture of five amino acids was used as the nitrogen source in a high sugar content fermentation.

The PCA analysis that integrated sugar concentration and nitrogen conditions also revealed a major impact of sugar concentration on aroma synthesis. High sugar content promoted ethyl acetate synthesis, the only ester that is related with an unpleasant glue or solvent odor at high concentrations (Swiegers and Pretorius,

2005). It has been previously reported that yeast cells subjected to high osmotic stress during wine fermentation can enhance the synthesis of acetic acid and its ester ethyl acetate (Bely *et al*., 2003). The PCA also showed that the most interesting wine aromas are distributed by matching samples and the lowest sugar concentration (20%), and those whose nitrogen source in stationary phase is arginine.

5. Conclusions

Our results indicate that nitrogen availability in the stationary phase stimulates the fermentation rate. However, the nitrogen concentration to reach the maximum fermentation rate is much lower than that required in the exponential phase, when yeast cells grow (Gutiérrez *et al.*, 2012). Nevertheless, presence of nitrogen in sufficient amounts to avoid becoming a limiting nutrient does not guarantee successful fermentation if other stresses are also present. A high sugar concentration in grape must produces a sluggish, stuck fermentation, regardless of the nature and amount of nitrogen involved. Conversely, nitrogen concentration and source have a huge impact on aroma synthesis. Both the increase in concentration and presence of arginine as the sole nitrogen source enhance the synthesis of the main fermentative aromas. Volatile compounds formation is probably related to greater nitrogen consumption by wine strains as these nutrients act as precursors during the synthesis of esters and regulate their production (Swiegers and Pretorius, 2005). The obtained results support the idea that different nitrogen sources and concentrations can be used to produce wines with divergent aroma profiles, which helps meet consumer demands for diversified products. Inorganic nitrogen salts, and organic nitrogen preparations to an ever increasing extent, are widely used to ameliorate nitrogen deficiency during wine fermentation. On the basis of the results obtained in our previous study (Gutiérrez *et al.*, 2012) and in the present work, providing yeast cells with enough nitrogen to obtain a

maximum biomass at the beginning of fermentation is recommended; and if necessary, the further addition, mainly in the form of organic nitrogen, at the beginning of the stationary phase can also be done. This procedure should ensure a good fermentation rate and an optimal aroma profile in the absence of other nutritional or environmental stresses.

6. Acknowledgements

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CHAPTER 6

Arginine addition in the stationary phase increases fermentation activity and aroma synthesis in different wine strains

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Abstract

During fermentation, the nature and amount of nitrogen added into grape must affect the fermentation rate and secondary aroma production. Timing of addition is also relevant for the resultant wine. The wine industry uses different nitrogen preparations to avoid nitrogen deficiency in wine fermentations. However, it is important to know the proper way to carry out additions. In this work, we studied the effect of nitrogen addition to three commercial wine strains in the stationary phase. We analyzed the impact of the concentration and source of nitrogen on fermentation activity and aroma production. Nitrogen addition stimulates the fermentation rate, mainly in N-deficient medium. Moreover, aroma synthesis changes with nitrogen additions. The use of ammonium and arginine as additives produces wines with different aroma profiles. The results indicate that different wine strains and nitrogen sources can be used to produce wines with divergent aroma profiles to meet consumer demands for diversified products.

Keywords: nitrogen preparations, arginine, addition timing, aromas, fermentation activity, acetate and ethyl esters

1. Introduction

Nitrogen deficiency is commonly observed in many viticultural regions and is often considered a growth-limiting factor (Pretorius, 2000). The importance of nitrogen for growth and fermentation activity is well-established (Bisson, 1999). Low nitrogen in grape must leads to low yeast populations and poor fermentation vigor, increased risk of sluggish or stuck fermentations, and undesirable thiols, such as hydrogen sulfide. Conversely, a high nitrogen concentration leads to increased biomass and stimulates the sugar utilization rate. Furthermore, changes in must nitrogen composition can positively or negatively affect the quality of the final wine (Bell and Henschke, 2005). Currently, addition of nitrogen to must is a usual practice in winemaking to avoid nitrogen limitation-related problems and it has a significant effect on wine flavor. Inorganic supplements, such as diammonium phosphate (DAP), are widely used to prevent these problems. Ammonium is the preferred source of nitrogen by yeast and is firstly consumed by repressing the assimilation of other nitrogen compounds. More recently, organic nitrogen sources, such as commercial preparations containing inactivated yeast or yeast products, have become commercially available and a common practice during fermentation. Winemakers sometimes proactively add nitrogen to must, even without knowing its initial nitrogen status and the consequences related with specific yeast strains used in the process. The addition of nitrogen compounds to must should follow certain criteria to prevent a large quantity of residual nitrogen, which may have negative consequences, such as microbial contamination and formation of ethyl carbamate (Ough, 1991; Araque *et al*., 2013).

Determination of optimum supplementation timing has also been studied (Bely *et al*., 1990; Manginot *et al*., 1997; 1998; Hernández-Orte *et al*., 2006). Nitrogen addition in the yeast growth phase increases the size of the yeast population and, conversely, additions in later fermentation stages have little effect on population size. Regarding the fermentation rate, nitrogen addition is equally effective

throughout fermentation, with response kinetics decreasing in later stages. The stationary phase has a high technological impact on enology since most sugar is metabolized after growth has ceased.

Nitrogen availability can also affect many yeast metabolism aspects, including the formation of volatile and non volatile compounds that are important for the organoleptic quality of wine (Albers *et al*., 1996; Bell and Henschke, 2005). A higher glycerol yield in a synthetic medium has been observed when ammonium was used as the sole nitrogen source instead of a mixture of ammonium and amino acids (Albers *et al*., 1996). Nitrogen supplementation has also been seen to stimulate the production of some volatile compounds, such as ethyl and acetate esters, and it reduces others, such as higher alcohols (Bell and Henschke, 2005). These compounds are the main groups of flavor compounds originating from yeast metabolism. Higher alcohols are generated from α-ketoacids, which derive mainly from sugars and branched-chain amino acids, which occur by the Ehrlich pathway (Eden *et al*., 2001). Higher alcohols can impart fusel or solvent odors, or floral ones in the case of 2-phenylethanol (Lambrechts and Pretorius, 2000). They appear in variable concentrations in wine: they add a desirable complexity to the aroma at moderate concentrations, while they can deteriorate aroma at higher concentrations. Esters are produced by the condensation of an alcohol and a coenzyme-A activated acid (acyl-CoA), catalyzed by an acyltransferase (Sumby *et al*., 2010). Ethyl esters are important for wine quality because they elicit pleasant aromas.

As mentioned above, most nitrogen additions are done empirically in winemaking, and they do not take into account the yeast strain's different nitrogen requirements during wine fermentation, the proper timing of these additions or the nitrogen source added. Nowadays, research is being conducted into new supplements; therefore, it is of interest to know how nitrogen source addition affects the fermentation rate, metabolite synthesis, and the production of higher alcohols and their corresponding esters.

In this context, this study aims to determine the effect of the addition of different nitrogen sources in the stationary phase in three commercial wine yeasts, which are widely used in the Spanish wine industry. Nitrogen addition is performed in the stationary phase to distinguish the impact on fermentation rate with no growth cell factor. To this end, a synthetic grape must with the suitable initial nitrogen concentration to ensure maximum population was provided (Gutiérrez *et al*., 2012), and the must was divided into different bottles and supplemented with distinct nitrogen concentrations and sources when the stationary phase was reached. Ammonium and arginine were used as nitrogen supplements. Although the effect of ammonium and a mixture of amino acids have been well-studied (Ancín-Azpilicueta *et al*., 2010; Hernández-Orte *et al*., 2006; Mendes-Ferreira *et al*., 2009), we aimed to focus on arginine as a sole source, which is one of the most common nitrogenous compounds in grape juice. In our previous work (Gutiérrez *et al*., 2012), we observed the positive effect of this amino acid on biomass production and fermentation activity. Our new approach was to determine whether the effect is also shown when the addition is carried out in the stationary phase or if it occurs only when cells are proliferated. Moreover, our objective was to analyze the impact on volatiles and non volatiles metabolites which contribute to wine quality.

2. Materials and methods

2.1. Yeast strains and inoculum preparation

The yeast strains used in this study were the following: ARM, RVA and TTA, all of which were provided by the Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com). A taxonomic description of these strains was carried out by the RFLPs of the ITS/5.8S region (Guillamón *et al*., 1998). Strains RVA and

TTA belonged to the species *Saccharomyces cerevisiae*, while strain ARM was identified as a hybrid between *S. cerevisiae* and *S. kudriavzevii*, following the procedure proposed by Gonzalez *et al*., (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has been confirmed by Dunn *et al*., (2012). These wine strains were used at an initial population of 2 x 10^6 cell/mL of active dry yeast (ADY) rehydrated in warm water prior to inoculation, according to the manufacturer's instructions (37ºC for 30 min).

2.2. Fermentation media

The synthetic grape must (SM) was prepared according to Riou *et al*., (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and with no anaerobic factors (Beltran *et al*., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L. The following mineral salts were utilized: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L. The following vitamins were employed: myo-inositol 20 mg/L, calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L. The yeast assimilable nitrogen (YAN) content in the synthetic grape must was 140 mg N/L: 42 mg N/L as ammonium nitrogen (NH_4Cl) and 98 mg N/L as the amino acids form. The proportion of each amino acid was administered as previously proposed by Riou *et al*., (1997). This amount of YAN was established as the minimum concentration required to obtain maximum growth in the strains used in this experiment (Gutiérrez *et al.*, 2012). The final pH of the SM was adjusted to 3.3 with NaOH.

2.3. Fermentation conditions

The first stage of the fermentation (exponential phase) was carried out at 28ºC with continuous orbital shaking (150 rpm). They were performed in 1 L glass bottles containing 900 mL of the SM under semi-anaerobic conditions since limited aeration was required to harvest samples for the subsequent analysis. The supernatant was recollected and stored at -20ºC at different fermentation times to analyze sugar and nitrogen consumption. When cell growth (measured by OD at 600 nm) was stopped, the fermentation medium was divided into other smaller flasks (250 mL bottles filled with 150 mL) and supplemented with different ammonium (40, 80 and 200 mg N/L) or arginine (40, 80, 120 and 200 mg N/L) concentrations. Only the control condition was not supplemented with nitrogen. After these additions, the ANKOM^{RF} Gas Production System (ANKOM Technology, NY, USA) was used to monitor CO_2 production. The wireless system consisted of a Radio-Frequency (RF) pressure sensor modules connected with the bottles (fermentors), a zero remote module that measured ambient pressure, a computer interface base coordinator and operational software. The pressure of each bottle was measured at 30-minute intervals in psi. Pressure measurements were recorded on an Excel spreadsheet and gas production curves were generated. Fermentations were stopped when successive increments of CO_2 production were lower than 0.05 psi. The supernatant was taken to analyze the concentration of YAN, metabolites and volatile compounds. These conditions were used in triplicate at 28ºC and with permanent agitation (150 rpm).

2.4. Yeast assimilable nitrogen determination

Ammonium and urea concentrations were measured by a kit using an enzymatic method (Roche Applied Science, Germany). The free amino acid nitrogen concentration was determined by following the σ-phthaldehyde/N-acetyl-L-

cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen(N)/mL. The sum of ammonium and amino acids represented yeast assimilable nitrogen (YAN).

2.5. Metabolite analysis (HPLC)

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed in all the samples at the end of the exponential phase (change point) and at the end of fermentation. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, MA, USA), equipped with a refraction index detector, autosampler and UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min, and supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analítica, Spain) and were diluted 5- or 10-fold. A total volume of 25 μL was injected into a HyperREZ^{TM}XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50ºC. The concentration of each metabolite was calculated using external standards. Each sample was analyzed in duplicate.

2.6. Volatile aroma compounds analysis

Higher alcohols and esters were analyzed based on a headspace solid phase microextraction (SPME) technique using a 100 μm poly-dimetylsiloxane (PDMS) fiber (Supelco, Sigma-Aldrich, Spain). Aliquots of 1.5 mL of the sample were placed into 15 mL vials and 0.35 g of NaCl and 20 μL of 2-heptanone (0.005%) as an internal standard was added. Vials were closed with screwed caps and 13 mm silicone septa. Solutions were stirred for 2 h to obtain the required headspace-liquid equilibrium. Fibers were injected through the vial septum and exposed to the headspace for 7 min and were then desorbed for 4 min in a gas chromatograph

(TRACE GC Ultra, Thermo Scientific) with a flame ionization detector (FID), equipped with an HP INNOWax 30 m x 0.25 mm capillary column coated with a 0.25-m layer of cross-linked polyethylene glycol (Agilent Technologies). The carrier gas was helium (1mL/min) and the oven temperature program utilized was: 5 min at 35ºC, 2ºC/min to 150ºC, 20ºC/min to 250ºC and 2 min at 250ºC. The injector and detector temperatures were maintained at 220ºC and 300ºC, respectively. A chromatographic signal was registered by the ChromQuest program. Volatile compounds were identified by comparing the retention time for reference compounds. Volatile compound concentrations were determined using the calibration graphs of the corresponding standard volatile compounds. 2-heptanone (0.005% w/v) was used as an internal standard.

2.7. Statistical analysis

All the experiments were repeated at least three times, and the data are reported as the mean value ± SD. Significant differences were determined by *t*-tests (the SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$. A principal component analysis (PCA) was used to reduce the dimensionality of the data and to find the best differentiation among samples. Factorial design experiments were used to analyze the influence of nitrogen (x8) and yeast strain (x3) on aroma compounds formation. The PCA and two-way analysis of variance (ANOVA) were carried out with the Statistica 7.0 software.

3. Results

Synthetic must with an adequate initial concentration was provided to determine the impact of nitrogen additions in the stationary phase of the three commercial wine strains (ARM, RVA, TTA), as well as the effect on fermentation rate and aroma production. When growth was stopped, the fermentation medium was

divided and supplemented with different ammonium or arginine concentrations as the sole nitrogen sources. The use of a novel system to monitor CO_2 production allowed us to determine the effect of the different nitrogen conditions on fermentation activity. The impact of the different nitrogen conditions on the synthesis of the major non volatile and volatile compounds contributing to wine aroma was also analyzed.

3.1. Impact of nitrogen additions on fermentation activity

As previously reported (Gutiérrez *et al.*, 2012), the three commercial strains showed a different behavior in the growth exponential phase. Strain RVA grew faster and reached the stationary phase (no growth) after 48 h of inoculation, whereas strains ARM and TTA needed 72 h before their growth stopped. The sugar, ammonium and amino acid concentrations were determined at the end of the exponential phase (change point; Table 1). At this point, all the strains had consumed almost half the sugars (around 90 g/L), except the ARM strain, which consumed only 50 g/L. Strain RVA practically consumed all the available nitrogen in the exponential phase, whereas the ARM and TTA fermentations contained an important concentration of amino acids (no ammonium) at the end of this phase.

Afterward, fermentations were subdivided into smaller bottles and were supplemented with the different nitrogen concentrations as ammonium (40, 80 and 200 mg N/L) or as arginine (40, 80, 120 and 200 mg N/L). The fermentation rate of each strain and nitrogen condition was measured by CO_2 production in the stationary phase (Figure 1). The addition of nitrogen improved the fermentation rate under some conditions, but no significant response was detected in any of the tested strains. Yeast strains showed different CO_2 production profiles, with the lowest fermentation activity noted in the ARM strain. The presence of residual amino acids at the change point in the ARM and TTA fermentations avoided the fermentation rate from significantly increasing under the nitrogen conditions

studied. Conversely the RVA strain, which exhausted the majority of initial nitrogen in the exponential phase, was more reactive to nitrogen additions. Arginine addition had the greatest impact on this strain. However when the residual sugar was analyzed at the end of the different fermentations (Table 1), significant decreases were noted under most nitrogen addition conditions. This positive effect was produced totally by enhanced cell fermentation activity since population size did not increase in the stationary phase (data not shown).

Added nitrogen was not completely consumed under any condition. Additions of higher nitrogen concentrations left more residual nitrogen at the end of fermentation. As expected, the addition of arginine increased the residual amino acids, but not ammonium. Nonetheless, addition of ammonium increased both inorganic and organic nitrogen. Interestingly, the amino acid concentration also increased under the control condition, to which nitrogen was not added. Evidently, therefore, this fraction of residual amino acids originated from the cellular lysis. The RVA strain showed the quickest nitrogen consumption in this stage, as had already happened in the exponential phase. Interestingly ammonium, the preferred and fastest nitrogen source consumed by yeast, was hardly assimilated at later stages if compared with arginine. The urea concentration was also determined at the end of fermentation and its presence was not observed under any of the fermentation conditions (data not shown). Glycerol, ethanol and acetic acid did not show significant differences amongst nitrogen conditions studied (data not shown).

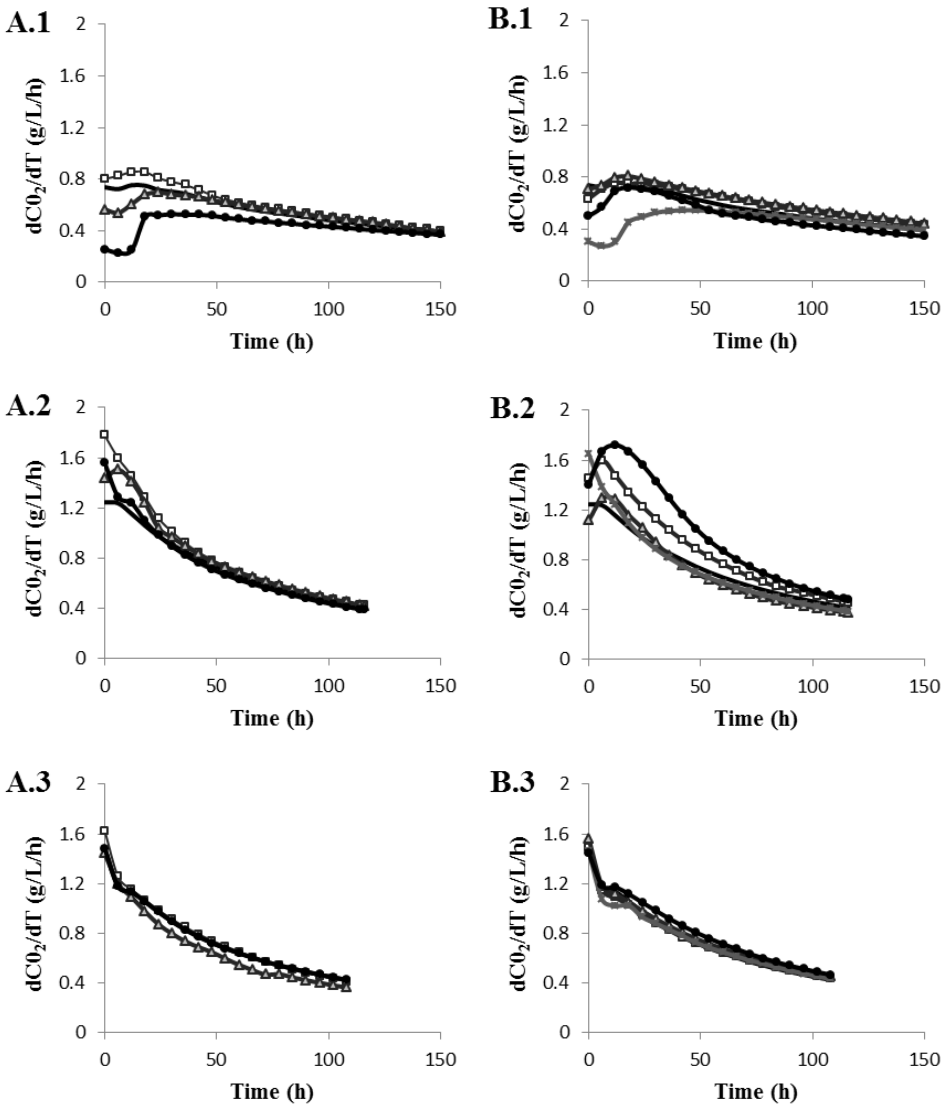

Figure 1. Evolution of the CO_2 production rate after nitrogen addition in three wine strains ARM (1), RVA (2), TTA (3). Additions tested with different concentrations (40 □, 80 ▲, 120 ♦ and 200 ● mg N/L) and sources (ammonium (A) and arginine (B)) of nitrogen. The control condition was not supplemented with nitrogen (─)

Table 1. Final composition of the wines supplemented with different concentrations and sources (ammonium and arginine) of nitrogen at the beginning of stationary phase in ARM, RVA and TTA strains. The change point refers to the time of nitrogen addition

Yeast strain	ARM			RVA			TTA		
	Sugar (g/L)	NH_4^+ (mg N/L)	AAs (mg N/L)	Sugar (g/L)	NH_4^+ (mg N/L)	AAs (mg N/L)	Sugar (g/L)	NH_4^+ (mg N/L)	AAs (mg N/L)
Change point	150.00±1.29	0.00±0.00	38.00±1.05	108.79±1.06	4.30±0.57	1.64±0.64	111.70±0.02	1.06±0.023	29.47±0.03
Control	35.53±1.85	1.11±0.83	22.31±1.51	10.34±0.38	3.27±1.48	9.56±1.97	21.32±4.12	3.02±1.24	36.41±1.35
40 NH_4^+	25.89±5.30	2.42±1.93	41.46±5.32[a,b]	14.00±2.22[a,b]	3.93±0.21	17.67±1.08[a,b]	23.42±6.26	27.23±2.03[a,b]	50.24±2.03[a,b]
80 NH_4^+	21.68±4.86[a]	49.83±11.91[a,b]	50.06±3.12[a]	6.26±0.55[a,b]	43.94±2.03[a,b]	20.60±1.92[a]	21.10±3.14	62.51±3.42[a,b]	51.41±3.45[a]
200 NH_4^+	27.11±1.98[a,b]	85.37±0.23[a,b]	83.66±3.11[a,b]	8.64±1.61[a,b]	86.27±0.23[a,b]	36.55±3.92[a,b]	8.74±0.61[a,b]	84.36±0.86[a,b]	41.23±1.33[a,b]
40 Arg	19.77±1.56[a,b]	2.42±1.74	41.99±3.95[a,b]	4.86±0.57[a,b]	2.00±1.00	18.26±0.82[a,b]	17.61±4.11	1.36±0.80[a]	52.04±1.77[a,b]
80 Arg	17.31±0.31[a,b]	0.30±0.15	54.52±2.84[a,b]	10.05±1.44[b]	3.62±0.99	32.85±1.37[a,b]	13.58±0.75[a,b]	2.47±1.26	66.41±2.17[a,b]
120 Arg	30.48±1.53[b]	1.56±1.54	76.59±5.04[a,b]	7.68±1.28[a,b]	4.33±1.15	33.39±1.24[a]	11.42±0.69[a,b]	0.35±1.27	32.90±1.10[a,b]
200 Arg	17.57±5.27[a,b]	0.00±0.00[a]	64.43±6.69[a]	3.35±0.00[a,b]	4.98±1.03	80.55±3.03[a,b]	13.73±2.94[a]	1.51±1.14	118.53±2.48[a,b]

[a] Significant differences compared with control (no nitrogen addition)
[b] Significant differences compared with the previous concentration of the same nitrogen source

3.2. Effect of nitrogen addition on aroma compounds

We determined the aroma compounds as the higher alcohols, acetate esters and ethyl esters obtained from the fermentations with different nitrogen additions (Table 2-4). As an overview of the results, a PCA was used to highlight the relevance of nitrogen concentration, nitrogen source and strains in aroma compounds production (Figure 2). The first principal component (PC1) accounted for 58.20% of total variance, while PC2 explained 25.14%. Two components with large eigenvalues were used to explain 83.34% of total variance. PC1 was characterized by phenylethyl acetate with a negative loading, while isobutyl acetate was the only compound with a positive loading. For PC2, ethyl acetate and isoamyl acetate showed positive loadings, while 2-phenylethanol and isoamyl alcohol exhibited negative ones. The relevance of each yeast strain in the aroma profile obtained during fermentation was greater than the influence of the nitrogen concentration or source in the stationary phase. All the conditions of the same strain were grouped together at different positions in the PCA plot. The ARM strain was clearly separated from the other strains in the right quadrant, denoting the most specific aroma profile. The musts fermented with this strain were the richest in isobutyl acetate, while they were the poorest in isoamyl acetate, phenylethyl acetate, ethyl caprylate, isoamyl alcohol and 2-phenylethanol. RVA was characterized by producing the lowest amount of isobutyl acetate and high concentrations of isoamyl alcohol and 2-phenylethanol. The grape musts fermented by TTA were rich in isobutanol, ethyl acetate, isoamyl acetate and phenylethyl acetate. In summary, TTA and RVA were the richest in acetate esters and higher alcohols, respectively.

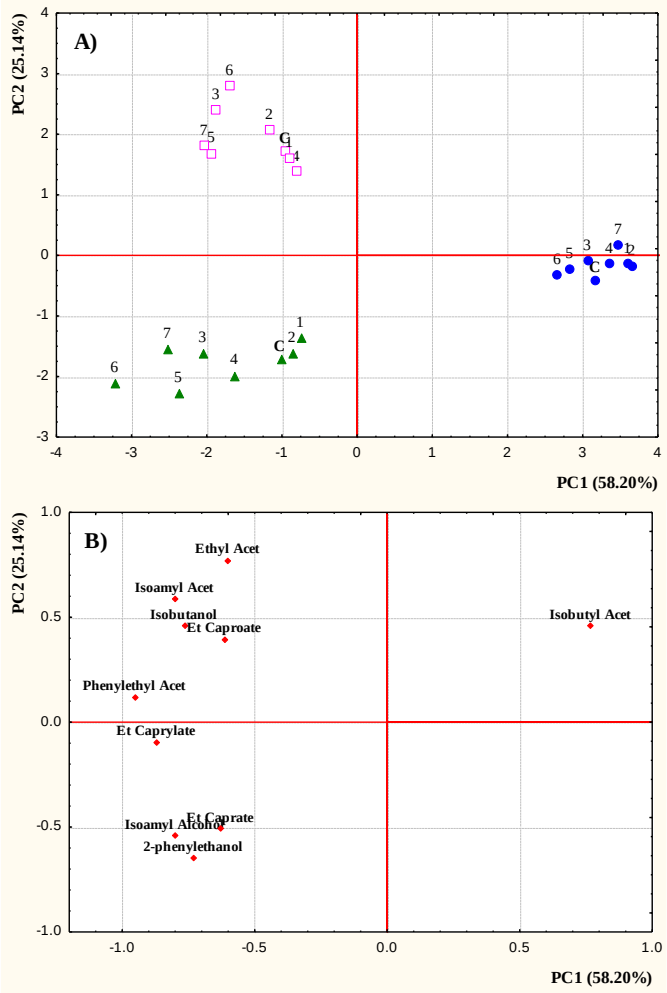

Figure 2. Results of the principal component analysis of volatile compounds carried out in three strains. A) Scores of the three strains obtained under different nitrogen conditions on the plane formed by the two first principal components. B) Loadings of the variables on the first two principal components. Symbols refer to yeast strain: ARM ●, RVA ▲, TTA □. The number that follows refers to the nitrogen concentration and source: (C) Control, (1) 40 NH_4^+, (2) 80 NH_4^+, (3) 200 NH_4^+, (4) 40 Arg, (5) 80 Arg, (6) 120 Arg, (7) 200 Arg

Addition of nitrogen in the stationary phase significantly affected the production of yeast-derived fermentation aroma (Tables 2-4). Different PCA analyses were carried out to identify the volatile compounds that best discriminated among the nitrogen fermentation conditions in each strain (Figure 3). As a first approach, samples were ordered according to the nitrogen source added. Arginine addition generally produced a broader separation in the PCA plot in relation to the control condition. The 200 mg N/L of ammonium condition came closer to the arginine samples than to the other ammonium samples. PCA plots showed an overall trend in the three strains: the greater the nitrogen availability in the stationary phase, the higher the concentration of ethyl and acetate esters in the final wine. Moreover, the main aroma compounds showed higher concentrations in the arginine additions. By way of example, PC1 in the ARM PCA plot (Fig. 3 A.1-B.1) exhibited ethyl acetate with positive loadings and higher alcohols with negative values. PC2 was characterized by negative values for ethyl esters. The most distant groupings found in relation to the control were 200NH_4^+ and 120 Arg, corresponding to the higher concentration of acetate and ethyl esters.

In order to determine the statistical significance and respective hierarchy of the factors (yeast strain and/or nitrogen) on aroma compounds production, a two-way ANOVA analysis was performed. As shown in Table 5, both the yeast strain and nitrogen had a major impact. The "yeast strain" factor had a statistically significant effect on all the quantified compounds, whereas the "nitrogen" factor affected the same compounds, except 2-phenylethanol, phenylethyl acetate, isobutyl acetate and ethyl acetate which were affected by only the yeast strain.

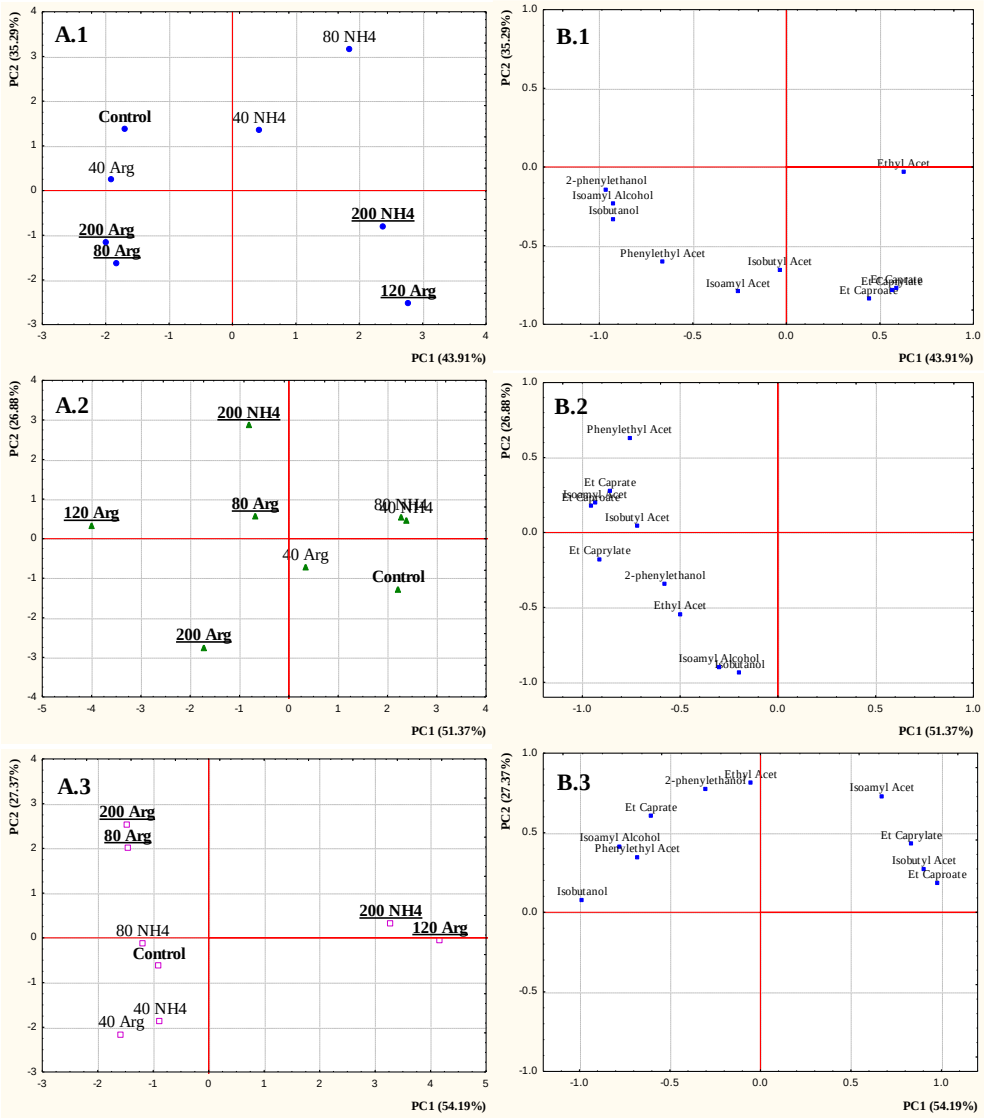

Figure 3. Results of the principal component analysis of volatile compounds. A) Scores of the three strains separately under different nitrogen conditions on the plane formed by the two first principal components. B) Loadings of the variables on the first two principal components. Symbols refer to yeast strain: ARM ●, RVA ▲, TTA □. Underlined conditions are the most aroma compounds production

Table 2. Concentration of aroma compounds of the wines fermented with ARM strain with different concentrations and sources (ammonium and arginine) of nitrogen

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	40Arg	80 Arg	120 Arg	200 Arg
Ethyl acetate	16.07±1.39	17.45±1.10	18.51±0.97	18.69±0.32*	17.04±0.99	17.45±1.76	17.56±0.83	17.82±2.61
Isobutyl acetate	0.48±0.32	0.75±0.17	0.53±0.29	0.82±0.21	0.85±0.20	0.66±0.27	0.91±0.23	1.12±0.56
Isobutanol	29.84±3.96	21.74±2.42*	20.10±1.12*	21.87±0.94*	30.74±2.23	32.16±0.92	22.52±1.06*	32.03±3.95
Isoamyl acetate	0.17±0.04	0.16±0.03	0.16±0.01	0.18±0.00	0.16±0.03	0.22±0.01	0.19±0.01	0.20±0.06
Isoamyl alcohol	34.04±3.97	25.02±3.18*	22.86±1.50**	27.25±0.87*	34.97±3.34	37.06±2.21	22.25±9.18	36.07±4.24
Ethyl caproate	0.17±0.03	0.16±0.03	0.16±0.02	0.23±0.01*	0.17±0.02	0.22±0.01*	0.25±0.02*	0.18±0.04
Ethyl caprylate	0.06±0.01	0.06±0.01	0.04±0.01	0.13±0.01**	0.07±0.01	0.09±0.01*	0.18±0.02**	0.07±0.02
Ethyl caprate	0.07±0.01	0.08±0.02	0.06±0.01	0.11±0.01**	0.08±0.01	0.09±0.01	0.15±0.03**	0.08±0.04
Phenylethyl acetate	0.11±0.03	0.11±0.01	0.04±0.07	0.08±0.07	0.12±0.02	0.13±0.04	0.11±0.01	0.12±0.01
2-phenylethanol	7.34±0.66	6.35±0.71	5.52±1.61	5.18±1.10*	8.75±1.34	8.48±3.16	5.06±1.19*	9.10±2.36
Higher alcohols	71.22±8.57	53.11±5.03*	48.47±4.10*	54.30±2.27*	74.46±4.36	77.70±4.45	49.83±11.24	77.19±10.38
Acetate esters	16.82±1.72	18.47±1.27	19.23±0.74	19.77±0.44*	18.16±1.04	18.46±2.00	18.77±0.63	19.26±2.20
Ethyl esters	0.30±0.04	0.30±0.04	0.26±0.04	0.47±0.01**	0.32±0.02	0.40±0.01*	0.59±0.06**	0.34±0.09

Significant differences compared with control (no nitrogen addition) *p<0.05; **p<0.01

Table 3. Concentration of aroma compounds of the wines fermented with RVA strain with different concentrations and sources (ammonium and arginine) of nitrogen

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	40Arg	80 Arg	120 Arg	200 Arg
Ethyl acetate	19.71±1.00	22.43±2.78	21.06±0.49	20.11±2.77	20.94±0.69	21.20±3.01	23.07±2.30	25.47±1.61*
Isobutyl acetate	0.07±0.03	0.06±0.03	0.04±0.00	0.06±0.01	0.09±0.02	0.06±0.02	0.20±0.00	0.06±0.02
Isobutanol	59.45±3.14	50.83±0.25**	50.51±1.50*	47.01±2.14**	54.19±1.72	53.04±6.03	55.09±6.16	61.90±1.95
Isoamyl acetate	0.56±0.11	0.57±0.09	0.57±0.09	0.89±0.07*	0.63±0.03	0.68±0.11	0.97±0.07**	0.83±0.06*
Isoamyl alcohol	95.10±7.59	81.35±0.73*	82.29±2.95	76.93±4.47*	89.52±2.48	87.43±8.77	91.48±8.67	98.06±2.42
Ethyl caproate	0.25±0.02	0.25±0.03	0.24±0.03	0.33±0.02**	0.28±0.01	0.29±0.05	0.35±0.07	0.32±0.01*
Ethyl caprylate	0.21±0.01	0.20±0.06	0.19±0.03	0.28±0.03*	0.26±0.06	0.31±0.05*	0.32±0.09	0.34±0.02**
Ethyl caprate	0.13±0.04	0.12±0.03	0.11±0.04	0.19±0.04	0.15±0.04	0.24±0.07*	0.27±0.06*	0.15±0.03
Phenylethyl acetate	0.60±0.04	0.65±0.02	0.73±0.12	0.95±0.05**	0.67±0.06	0.84±0.11**	0.96±0.08**	0.71±0.01*
2-phenylethanol	36.73±6.86	36.63±6.74	43.64±4.44	39.80±3.27	49.61±6.77	42.29±5.62	46.68±6.99	47.30±1.26
Higher alcohols	191.28±9.94	168.81±7.14	176.44±10.68	163.74±2.78	193.32±7.32	182.76±5.07	193.24±12.52	207.26±7.38
Acetate esters	20.93±1.17	23.71±1.10	22.40±0.37	22.01±1.29	22.33±0.50	22.78±1.49	25.21±3.16	27.06±1.20*
Ethyl esters	0.59±0.01	0.57±0.18	0.54±0.11	0.80±0.10	0.69±0.15	0.84±0.09	0.94±0.12	0.81±0.10**

Significant differences compared with control (no nitrogen addition) *$p<0.05$; **$p<0.01$

Table 4. Concentration of aroma compounds of the wines fermented with TTA strain with different concentrations and sources (ammonium and arginine) of nitrogen

Volatile compounds (mg/L)	Control	40 NH_4^+	80 NH_4^+	200 NH_4^+	40Arg	80 Arg	120 Arg	200 Arg
Ethyl acetate	37.84±2.49	35.14±6.76	38.69±2.92	36.93±4.21	33.34±4.94	37.90±4.33	36.69±2.33	39.33±5.08
Isobutyl acetate	0.39±0.08	0.37±0.10	0.41±0.01	0.51±0.33	0.33±0.08	0.41±0.04	0.66±0.61	0.43±0.03
Isobutanol	83.06±8.03	83.06±10.66	86.08±7.34	58.55±4.90*	85.21±5.16	87.79±10.57	60.88±5.13*	88.27±11.19
Isoamyl acetate	1.25±0.34	1.19±0.35	1.31±0.04	1.50±0.15	1.12±0.21	1.38±0.09	1.53±0.10	1.47±0.14
Isoamyl alcohol	55.19±4.28	50.06±6.95	50.93±4.29	47.16±4.59	54.91±3.18	57.10±5.53	48.27±6.01	58.12±7.88
Ethyl caproate	0.28±0.06	0.26±0.08	0.28±0.01	0.59±0.07**	0.25±0.03	0.30±0.01	0.63±0.04**	0.31±0.02
Ethyl caprylate	0.16±0.03	0.17±0.10	0.14±0.02	0.33±0.07*	0.16±0.04	0.25±0.02*	0.33±0.02**	0.23±0.05
Ethyl caprate	0.12±0.02	0.12±0.05	0.10±0.01	0.10±0.03	0.10±0.01	0.12±0.01	0.08±0.00*	0.15±0.01
Phenylethyl acetate	0.71±0.05	0.83±0.29	0.99±0.14*	0.72±0.03	0.82±0.09	0.93±0.14	0.71±0.11	0.90±0.12
2-phenylethanol	14.52±0.90	12.83±1.07	15.31±5.90	16.04±3.03	15.82±2.22	21.75±6.19	14.26±1.96	18.35±3.23
Higher alcohols	152.77±11.42	145.94±8.30	152.32±10.99	121.75±8.56*	155.94±7.49	166.64±7.68	123.41±5.67*	164.74±7.29
Acetate esters	40.20±2.85	37.53±7.35	41.39±3.08	39.66±4.33	35.61±5.22	40.62±4.59	39.59±3.06	42.13±5.37
Ethyl esters	0.55±0.03	0.52±0.24	0.52±0.01	1.03±0.16**	0.50±0.07	0.67±0.02	1.04±0.05**	0.69±0.04*

Significant differences compared with control (no nitrogen addition) *p<0.05; **p<0.01

Table 5. *F*-values and significant differences for volatile compounds obtained by a two-way ANOVA

	Sources of variation		
Compound	**Strain**	**Nitrogen**	**Strain*Nitrogen**
Ethyl acetate	329.59***	1.29	0.76
Isobutyl acetate	57.65***	1.32	0.93
Isobutanol	668.57***	14,37***	4.59***
Isoamyl acetate	509.37***	6.10***	1.47
Isoamyl alcohol	926.31***	11.71***	1.52
Ethyl caproate	131.58***	38.06***	12.11***
Ethyl caprilate	111.31***	15.68***	1.79
Ethyl caprate	51.43***	7.72***	4.30***
Phenylethyl acetate	436.89***	2.09	4.24***
2-phenylethanol	261.33***	1.57	0.89
DF	2	7	14

*p<0.05; **p<0.01; ***p<0.001

4. Discussion

Nitrogen addition is widely used by winemakers, mainly as a precaution against fermentation problems. The present study aims to determine the proper conditions to supplement wine fermentations and to obtain the desirable organoleptic profile. In a previous work (Gutiérrez *et al.*, 2012), we determined the minimum nitrogen concentration required by the same three commercial yeast strains to ensure the maximum population in growth in a synthetic grape must. In this study, our aim was to determine the effect of nitrogen addition in the stationary phase when each strain reached the maximum biomass production. At this time, nitrogen can improve the fermentation process by enhancing fermentation activity per cell.

Fermentation activity, metabolites production and volatile compound profile were obtained. Ammonium and arginine were chosen as the nitrogen supplement. Although ammonium salts are the most frequent nutrient used in wine fermentations, new nutritional supplements enriched in amino acids, especially arginine, are available nowadays. These new products are based mainly on fully lysed yeasts.

In a recent study, which aimed to decipher the molecular basis of wine yeast fermentation traits, Ambroset *et al*. (2011) observed that the fermentation rate at all stages positively correlated with assimilated nitrogen. Our data corroborate that the cell capacity to use nitrogen in all the fermentation phases has a strong impact on the fermentation rate. In fact, the strain with the highest nitrogen demand also shows the greatest fermentative activation after nitrogen additions. Thus, a valid conclusion drawn from our data is that nitrogen addition after growth cessation (tumultuous fermentation) is recommendable when residual nitrogen is very low; otherwise the effect of addition on the fermentation rate is imperceptible. Moreover, excessive residual nitrogen in final wines may cause microbial stabilization problems or the synthesis of unhealthy compounds (ethyl carbamate and biogenic amines) by yeast or lactic acid bacteria (Ough, 1991; Aredes Fernández *et al*., 2010; Araque *et al*., 2013). Regarding the nitrogen source, it should be highlighted that arginine additions in the stationary phase have a much bigger impact in terms of the CO_2 production rate and the residual sugar concentration than ammonium additions.

The use of a defined grape must allow the quantification of volatile compounds synthesized only by the yeast strain metabolism in the absence of varietal aromas and precursors. The ANOVA analysis reveals that the "yeast strain" factor has a greater impact on aroma production than the "nitrogen" factor. This result may be explained by most of the aroma being produced in the exponential phase and by the different nitrogen conditions operating only in the stationary phase. However, significant differences in most quantified aromas are also detected among different

nitrogen concentrations and sources. Previous studies have shown an inverse correlation between the initial nitrogen content in grape musts and higher alcohols in wine (Carrau *et al*., 2008; Vilanova *et al*., 2012). We observe a similar trend with a lower concentration of higher alcohols when N addition is carried out. Although both nitrogen sources follow a similar profile, ammonium shows more marked decreases than arginine additions. Regarding ester compounds, higher nitrogen concentrations positively correlate with higher concentrations of acetate and ethyl esters, these being the main compounds conferring fruity and floral notes to wines. This correlation between nitrogen concentration and ester production has been observed in previous studies (Carrau *et al.*, 2008; Garde-Cerdán and Ancín-Azpilicueta, 2008; Torrea *et al.*, 2011). Interestingly, an arginine source presents higher levels of acetate and ethyl esters. This result is in line with previous studies which comparing the effect of inorganic and organic nitrogen supplementations on volatile composition (Torrea *et al.*, 2011; Barbosa *et al*., 2012).

In conclusion, our results indicate that the timing of nitrogen addition determines the fermentation performance and aroma characteristics of wines. Nitrogen addition availability in the stationary phase stimulates the fermentation rate, although it depends on the different wine strain and nitrogen status of the fermentation. Moreover, nitrogen concentration and source have a huge impact on aroma synthesis. Both the increase in nitrogen concentration and the presence of arginine as the sole nitrogen source enhance the synthesis of the main fermentative aromas. Enhancement becomes more evident when addition is carried out in N-deficient must. As a final point, we recommend using nitrogen timing as a tool to modulate aroma compound formation during wine production. We also suggest providing different nitrogen sources to obtain the desirable aroma profile. Therefore, ammonium can be used to produce wines with small amounts of higher alcohols, whereas arginine can be utilized to produce fruitier and more floral aroma profiles. Combinations of different wine strains with nitrogen concentrations and sources can be used to obtain new and original aroma profiles.

5. Acknowledgements

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GENERAL DISCUSSION

The nitrogen composition of grape juice is important to ensure good wine fermentation performance and quality wine. The nitrogen content of grape must depends on the variety of grape and the time of harvest, and also on several vineyard management aspects, and is essential for yeast metabolism and growth, and for the synthesis of aroma compounds. However, yeast cell growth under enological conditions is often considered nitrogen-limited, which could produce stuck or sluggish fermentation. A low initial nitrogen concentration acts by limiting the growth rate and the biomass formation of yeast, resulting in a low sugar catabolism rate (Bisson, 1991). These problematic fermentations are of economic importance because wines with higher residual sugar contents have a negative impact on the quality of the end product. Currently, the most widely used method to deal with N-deficient fermentations is the addition of supplementary nitrogen, usually in the form of ammonium phosphate salts. In many cases, winemakers proactively add nitrogen to grape must, even without knowing its initial nitrogen status, which may cause high nitrogen levels that exceed the minimum required to complete fermentation. Excessive nitrogen additions may lead to the presence of non assimilated residual nitrogen at the end of fermentation, causing microbial instability and ethyl carbamate accumulation in wine (Ough, 1991). Moreover, minimal nitrogen requirements and preferences are shown to be strain-dependent (Manginot *et al*., 1998; Taillandier *et al*., 2007).

The overall aim of this Doctoral Thesis is to analyze the nitrogen metabolism of four commercial yeast strains which are widely used in the Spanish wine industry in order to know the nitrogen requirement for each specific yeast strain to achieve optimal growth and fermentation performance so that wine production meets the demands set by the Regulatory Authorities and consumers. It is very important to consider the nitrogen demand of yeast in the selection criteria of commercial strains. This main objective has been fulfilled by carrying out the fermentations of four strains under different nitrogen conditions (concentrations and sources) using different biochemical and molecular approaches, and by determining the capacity

of growth, fermentation and product formation. Likewise, different biomarkers and new strategies have been developed to detect not only nitrogen starvation in must during fermentation, but also the genes responsible for variations in the assimilation of some nitrogen sources in particular yeast strains.

Nitrogen assimilation is essential for yeast because it is required to synthesize the proteins and enzymes involved in cell growth and metabolism. Yeast strains can be surveyed in a wide variety of nitrogen conditions present in the environment. However, the quantity and quality of these conditions are crucial and decisive to trigger the most favorable response in growth terms.

Nitrogen concentration determines the specific growth rate and biomass production; higher nitrogen concentrations produce faster growth and larger cell populations. However, the nitrogen impact is more relevant in biomass production because the nitrogen concentration required to reach the maximum growth rate is much lower than the nitrogen concentration to obtain the maximum population size. First cells reach the maximum growth rate. Then they use this nitrogen to reproduce quickly and continue growing until depletion. Thus, it is possible to obtain larger cell populations with greater nitrogen availability, up to the so-called N-limiting concentration. Above this concentration, nitrogen is no longer the limiting factor, and increases of nitrogen have no effect on the growth rate and biomass. An N-limiting concentration was seen to be strain-dependent. PDM and RVA strains needed a higher N concentration to achieve their maximum growth rate and population size values. Moreover, these strains consumed nitrogen faster than the other two strains, especially when the media contained a high nitrogen concentration. The growth rate, biomass production and nitrogen consumption seem to be interlinked since the strains that reach the highest growth rate and the largest populations were the same as those which assimilated nitrogen more quickly.

A wide variety of nitrogen compounds with different natures can be assimilated by yeast through different enzymatic reactions. Nitrogen is obtained from either an organic source or an inorganic source, but is ultimately converted into ammonia and glutamate. Thus, not all sources cause the same effect in the cell and depend on transport efficiency, the possibility of conversion into easy assimilated sources, and energy requirements (Bisson, 1991). Therefore, they are classified according to the response triggered in growth: nucleotide bases which support very slow growth are considered poor sources, whereas the sources supporting fast growth, which are mainly involved in the central nitrogen metabolism or can be easily degraded to these, are considered good nitrogen sources (e.g., asparagine, glutamine, ammonium). "Fast" sources achieve the highest specific growth rates (μ_{max}) and maximum yield values. Arginine intermediates and branched-chain and aromatic amino acids support medium growth. Thus, the main determinant of this categorization is believed to be the carbon derivatives resulting from the catabolism of these compounds (Godard *et al*., 2007). Whereas the transamination or deamination of "fast" sources produces C-compounds that are directly assimilated by metabolism, the transamination of "slow" sources leads to keto acids, which are converted into complex alcohols. As it is difficult to make clear distinctions in some cases between the quality of a particular nitrogen source by contemplating only the growth rate, the ability of nitrogen sources to induce the repression of the genes required for the catabolism of other nitrogen sources is also used to judge this quality (Magasanik and Kaiser, 2002). This criterion considers preferred nitrogen sources to be those which repress the pathways for the utilization of alternative nitrogen sources, and conversely, non preferred sources are those which lead to the derepression of alternative pathways. The construction of reporter strains based on the GFP expression under the control of promoter *GAP1* is useful to determine which sources are more affected by NCR and their repressor/derepressor effect. Thus, nitrogen sources which support rapid growth are the same as those that exert a strong NCR, whereas non preferred nitrogen sources

do not produce a repressor effect. Obviously, when good nitrogen sources are presented in the media, yeast first needs to assimilate those sources, which support better growth by repressing the expression of the genes involved in poor nitrogen assimilation. The use of poor sources proves more inefficient for growth, requires more energy, and then they are assimilated when good nitrogen sources are depleted. Moreover, the efficiency of nitrogen assimilation correlates with the growth rate and NCR intervention.

Although the majority of nitrogen sources produce a similar growth response in different yeast strains, nitrogen utilization is strain-dependent. First, industrial strains differ in nitrogen utilization from lab strains. The exponential growth rate, growth efficiency and lag phase length measures provide complementary views of nitrogen source suitability, which deviate from lab strains utilization. Marked differences should be taken into account for suitable performance during industrial processes. Furthermore the growth analysis of the four strains also revealed variations among them, with particular defects noted in the use of some nitrogen sources. The most remarkable strain-specific difference was detected in the PDM strain, which was almost completely incapable of utilizing methionine, a nitrogen source that offers better growth in other wine strains. The genetic basis underlying this particular phenotype is most important, particularly because this strain is widely used in wineries, and it produces large amounts of H_2S, which is undesirable in the end product. Recently, the quantitative trait locus (QTL) approach has been used to map the genetic variation responsible for different traits, such as oenological properties (Ambroset *et al*., 2011; Salinas *et al*., 2012). These regions contain hundreds of genes and it is sometimes difficult to find the particular genes explaining the phenotype. Thus, a genetic basis can be dissected by different approaches, one of which is the reciprocal hemizygosity analysis (RHA), which compares the phenotypes of two strains that differ only in terms of the allele of one copy of the candidate gene. Differences in phenotype can reveal

defects in the gene which can be responsible for the phenotype, or can show haploinsufficiency when one copy of the gene is not enough to maintain normal nitrogen utilization. The majority of the hemizygotes obtained with the PDM strain in our study did not show haploinsufficiency, which means that this strain can support its nitrogen utilization capacity, even with only one copy of the nitrogen metabolism-related genes. Nevertheless, the phenotype of some hemizygotes has been shown to be greatly affected, and some of them have been found to be involved in methionine as a faulty use of this strain (*ARO8*, *ADE5,7*, *VBA3*). We suggest that the RHA approach can prove to be a powerful tool for improving industrial strains. In fact, this approach is capable of linking interesting phenotypes to genetic determinants, and thereby allows the study of the desirable or undesirable phenotypes present in different yeast strains to help optimize industrial strains.

The study of the nitrogen metabolism of the four commercial wine strains used in this thesis proves most useful to help understand their fermentative profile, and to then apply this knowledge to improve the process as regards biomass production, the fermentative rate and flavor synthesis aspects.

Nitrogen impacts yeast cells in two ways: by increasing the numbers of cells per population; by stimulating the sugar utilization rate. Distinguishing between both effects is no easy task. Previous studies (Varela *et al*., 2004; Albertin *et al*., 2011) have concluded that the maximum fermentation rate is driven by population size. Thus, the positive correlation found between population size and the fermentation rate suggests that increasing the maximum population can also enhance fermentative ability. For this reason, we determined the minimum nitrogen quantity required by each commercial strain to ensure the maximum biomass in the exponential phase. Nitrogen concentration directly correlates with population sizes until the N-limiting concentration is reached. Above this concentration, nitrogen

addition has no effect on cell growth. Therefore, knowledge of this concentration is very important in winemaking to support the suitable fermentation of each particular wine strain, and to avoid the shortage of nitrogen in the must or excessive nitrogen additions, which can have negative effects. Regarding the strains used in this work, the yeast strains which reach the largest population sizes are the same as those which show greater nitrogen requirements and the quickest fermentation (PDM and RVA). This result is contrary to winemakers' general belief in relating a scarce yield biomass during the process with strains' high nitrogen requirements. Surprisingly, strain TTA did not show this behavior, and was the fastest fermenting strain with a low biomass in N-deficient media. This result contradicts the statement of Varela *et al*. (2004), which states that biomass governs the fermentation rate. Our results prove that each strain's metabolic activity can sometimes determine the process. Another result to support the effect of nitrogen on cellular metabolic activity is that the nitrogen concentration which boosts the fermentation rate is much higher than the nitrogen concentration which promotes increased growth. Given this result, we aimed to determine the nitrogen requirements of these strains in the non growing stationary phase. In wine fermentations, most sugars are consumed in this phase, in which nitrogen availability is used directly to stimulate the fermentation rate without biomass production. The nitrogen requirements in the stationary phase were also strain-dependent, but were much lower than for the growth phase. PDM and RVA, which achieved the best cell growth and showed the quickest consumption, were also the highest nitrogen demanders in this phase. Once again, we corroborated the fact that the yeast strains characterized by slow growth and long fermentation times are not the highest nitrogen demanders. Although no clear correlation was found between nitrogen concentration and the fermentation rate, the final residual sugar concentration correlated inversely with the nitrogen concentration (even in the lowest nitrogen-demanding strains).

The exclusive use of ammonium salts as a nitrogen supplement can also produce anomalies during alcoholic fermentation. Assimilable nitrogen in must is composed of ammonium, like inorganic nitrogen, and by a mixture of amino acids. The addition of ammonium produces an imbalance in the natural inorganic/organic nitrogen composition ratio and modifies the nitrogen uptake profile (Beltran *et al*., 2005). These changes affect the production of aroma, spoilage compounds and carcinogen ethyl carbamate (Adams and Van Vuuren, 2010). Nowadays, organic nitrogen sources, such as commercial preparations containing inactivated yeast, have become commercially available. Thus, we analyzed the effect of ammonium and two amino acids, glutamine and arginine, which are the major components of assimilable organic nitrogen in the medium. Glutamine is considered a good nitrogen source, whereas arginine is subjected to NCR and is hardly consumed when preferred nitrogen sources are present in the media. Although these sources showed no significant differences in growth terms, both amino acids produced faster fermentation activity, especially arginine. Additions of arginine in the stationary phase also reduced the fermentation length more efficiently than ammonium. These organic sources can be considered suitable for commercial preparations of nitrogen supplements which can be added at different time points during fermentation.

Nitrogen availability can also affect many yeast metabolism aspects, including the formation of volatile compounds, which are important for wine organoleptic qualities. Yeast strains have a major impact on the aroma profile, and are characterized by the production of different volatile compounds. However, most quantified aromas are also susceptible to nitrogen additions, with notable differences occurring depending on the concentration and source used as additions. Higher nitrogen concentrations and the use of arginine as a supplement provide higher concentrations of acetate and ethyl esters, which confer fruity and floral notes to wines.

Another aspect that should be considered when studying nitrogen requirements is the grape must sugar concentration. In recent years, climate change has often led to grapes presenting higher sugar levels and increased grape maturity than those desired. Higher sugar concentrations in must diminish fermentation activity, leading to sluggish and stuck fermentations for the higher ethanol production reached at the beginning of the stationary phase.

By taking into account the results obtained in the exponential and stationary phases, we propose providing yeast cells with enough nitrogen to accomplish maximum biomass production at the beginning of fermentation (120-140 mg N/L), which means supplementing must before fermentation when the nitrogen concentration is inadequate. This concentration achieves a maximum population size, but does not ensure maximum fermentation activity. Then at the end of the exponential phase, or when entering the stationary phase, we recommend supplementing the medium with lower concentrations than those used in the exponential phase, at around 40 mg N/L. The nitrogen status of must addition should be low to ensure the best effectiveness, otherwise the effect of addition is imperceptible. All in all, nitrogen concentrations should be estimated in accordance with the yeast strain and the must sugar content. Regarding the nitrogen source, we suggest using ammonium (or a mixture of ammonium and amino acids) in the exponential phase, and arginine for later additions. Ammonium supports rapid growth, efficient assimilation and a stronger repression of utilization of the non preferred N sources in the proliferating phase. However, ammonium is hardly consumed in the stationary phase. Therefore, organic sources such as arginine should be added to reduce the fermentation length and to obtain wines with a major aroma complexity. Different nitrogen combinations of organic and inorganic nitrogen can be used to obtain divergent and desirable aroma profiles.

Winemakers sometimes have difficulties in applying the procedure explained above because they lack the information and facilities to know the exact nitrogen

requirements of the yeast strains they use. Nitrogen markers are required in current wineries to guarantee additions with suitable performance. Therefore, biomarkers might show robust changes in their activity in association with nitrogen shortage, can allow rapid and simple monitoring, and are also useful for all conditions and strains, regardless of their physiological characteristics (e.g., nitrogen consumption, the growth rate, fermentation capacity). We have developed a biosensor based on the *GAP1* expression which displays fluorescence through fermentation when yeast cells detect N-deficient concentrations in the media. As nitrogen availability depends on the quantity and quality present in media, we also tested its effectiveness with different nitrogen sources. The determination of the cell nitrogen status in the wine process and the possibility of analyzing different times to detect the actual moment when yeast needs nitrogen supplementation prove to be very useful knowledge for winemakers, and can be applied in different strains and environments.

The information provided by this thesis is most useful for wineries, especially those using these commercial strains. Good process management and bearing in mind all the considerations will help ensure suitable fermentation and wine production. Moreover, global knowledge on the nitrogen metabolism and molecular approaches used in this study can be applied in other yeast strains and processes. One of today's biotechnological challenges is to unravel the molecular basis of wine yeast fermentation traits (Ambroset *et al*., 2011). Studying different very important strains for industrial use and traits with a high economic value (fermentation capacity, stress tolerance, flocculation, substrate range and compounds production) should be dealt with in the near future. The knowledge and understanding of the genes underlying specific features can help us to carry out much more rational strain selections and to genetically improve the current commercial strains which, despite being highly competitive, can harbor genetic defects which produce undesirable industrial properties (i.e., H_2S production of the

PDM strain). Although tremendous progress has been made in genetic engineering to improve wine yeasts, genetically modified organisms (GMO) are still controversial. The use of molecular breeding techniques for unconsidered GMOs, such as intra- and inter-specific hybridization (Pérez-Través *et al*., 2012) or the classical backcross approach (Marullo *et al*., 2009), can prove very useful for introducing quantitative trait loci into a commercial strain background or for curing a genetic mutation.

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CONCLUSIONS

1. Industrial yeast strains have vastly different nitrogen requirements. Differences in nitrogen demands positively correlate with a higher growth rate and higher nitrogen uptake.

2. Nitrogen concentration also positively correlates with the specific growth rate and biomass production. However for the nitrogen amount range available in the grape must, the effect of nitrogen concentration is more relevant on biomass production than on the growth rate. Irrespectively of the nitrogen concentration, there must be a genetic component which determines different μ_{max} and a maximum population size for different wine strains.

3. The impact of nitrogen on fermentation is not exclusively due to increased biomass. Some strains may adapt a strategy whereby fewer cells with greater metabolic activity are produced.

4. Nitrogen availability in the non proliferating or the stationary phase of wine fermentation also boosts the fermentation rate. However, the nitrogen requirements in the stationary phase are much lower than those required for the exponential phase.

5. During wine fermentation, yeast cells with enough nitrogen to obtain a maximum biomass at the beginning of fermentation should be provided, and if necessary, the further addition, mainly in the form of organic nitrogen, at the beginning of the stationary phase can also be performed. This procedure should ensure a good fermentation rate and an optimal aroma profile in the absence of other nutritional or environmental stresses.

6. Different nitrogen sources determine the growth rate, the maximum population and the lag period, with marked differences between wine strains and the lab strain.

The use of these sources also reveals that wine yeasts are not fully adapted to the nitrogen composition of the grape must.

7. The reciprocal hemizygosity analysis (RHA) is a suitable approach to study the genetic basis underlying particular oenological phenotypes. The RHA of the PDM strain detected that some genes (*ARO8*, *ADE5,7* and *VBA3*) contribute to the methionine utilization deficiency. This information is very useful for the genetic improvement of this strain.

8. This RHA analysis also revealed that the retention of a single copy of nitrogen metabolism-related genes almost always suffices for nitrogen-dependent functionalities to remain unperturbed. Thus, haploinsufficiency is remarkably rare, at least for this group of genes.

9. The use of diverse biochemical or molecular markers (sensors) to obtain accurate knowledge about the nutritional state of cells during alcoholic fermentation provides useful information for the wine industry. Arginase activity and the expressions of the *GAP1* and *DAL4* genes in cells are good indicators of the nitrogen nutritional state. These markers can be useful to explore the relationship between nitrogen availability and nitrogen requirements of commercial yeast strains in much more detail.

10. The use of *GAP1* activity, as an indicator of the nitrogen nutritional state of cells, revealed that the growth rate, nitrogen uptake and the strong repression of the genes controlled by NCR directly correlate. Good nitrogen sources are characterized by supporting fast growth as they are involved in central nitrogen metabolism or can be easily degraded to these, and by rapid assimilation and the repression of the genes involved in poor nitrogen utilization.

11. Nitrogen concentration and source have a huge impact on aroma synthesis. Both the increased concentration and the presence of arginine as the sole nitrogen source enhance the synthesis of the main fermentative aromas. Thus, different nitrogen sources and concentrations can be used to produce wines with divergent aroma profiles, which help meet consumer demands for diversified products.

12. The sugar concentration of the grape must also determines nitrogen requirements, and should be taken into account in order to avoid stuck and sluggish fermentations.

ANNEX I

Materials and Methods

1. Strains

1.1. Commercial yeast strains

The yeast strains used in this study are the following: PDM, ARM, RVA and TTA, all of them provided by Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com).

1.2. Laboratory yeast strains

BY4741 (MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0) and set of deletion strains (haploid MATa strains) were used to carry out Reciprocal Hemizygosity Analysis (RHA). All of them were provided by Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). Table 1 shows the distribution in GO-functional categories of genes used to construct hybrid strains.

1.3. Plasmids

The plasmids used in this work were: pKT127, pAG25, pAG32.

pKT127 contains the EGFP and *kanMX4 module* as transformation marker for selecting *Saccharomyces cerevisiae* G418 resistant transformants (Sheff and Thorn, 2004).

pAG25 contains the *nat1*gene from *Streptomyces noursei* encoding nourseothricin N-acetyl-transferase and confers resistance to the antibiotic nourseothricin of transformed yeasts (Goldstein and McCusker, 1999).

pAG32 contains the *hph*gene from *Klebsiella pneumoniae* encoding hygromycin B phosphotransferase and confers resistance to the antibiotic hygromycin B of transformed yeasts (Goldstein and McCusker, 1999).

Annex 1

Table 1. Distribution in GO-functional categories of genes used to construct hybrid strains.

Functional categories	Genes
METABOLISM	
Amino acid metabolism	
Glutamine	*CPA2, ZWF1, CPA1*
Glutamate	*GDH3, GLT1, GDH2, PUT2, AAT1, MEU1, AAT2, PUT1, ACO1, GAD1, GDH1*
Arginine	*VBA3, ARG82, ARG5,6, ARG4, ARG2, ARG3, CPA2, PUT1, CAR2, ARG81, ARG80, VBA1, ARG1, ORT1, CPA1, CAR1, ARG7*
Aspartate	*ASP1, ARG4, AAT1, AAT2, ARG1*
Threonine	*CHA1, THR4, HOM2, HOM3, ILV1, THR1, HOM6*
Methionine	*HOM2, SAM2, HOM3, MET6, MET10, CYS4, THR1, MET18, MET3, CBF1, ECM17, HOM6, MET14, MER1, SAM1, MET17, ADI1, MET2, YPR118w, MET16, MET5*
Lysine	*LYS2, VBA3, LYS5, HOM6, VBA1*
Serine	*CHA1, HOM2, HOM3, SER3, ILV1, SER2, THR1, SER33, HOM6, MET2, SER1*
Phenylalanine	*TYR1, ARO1, ARO10, ARO2, ARO8, ARO9, PHA2, ARO7*
Tryptophan	*TYR1, TRP1, ARO1, TRP4, TRP2, TRP5, ARO2, ARO8, ARO9, BNA2, TRP3, BNA5, ARO7*
Pyruvate family	*ILV6, ARO10, ILV1, THR1, BAT2*
Regulation of amino acid metabolism	*ARG82, GCN4, AUA1, ARO8, ARO9, MET28, DAL81, CBF1, ARG81, ARG80*
Nitrogen metabolism	*DUR1,2, DAL1, DAL2, DAL7, DAL3, DAL82, CPS1, ALT1*
Regulation of nitrogen metabolism	*MET8, UGA3, NPR2, GLN3, AUA1, GAT1, VID30, UGA1, MET28, DAL81, DCG1, GZF3, CBF1, DAL80, ARG81, ARG80, MKS1, NPR1, URE2, CAR1, NPR3*
Purin nucleotide metabolism	*ADE1, DUR1,2, HIS4, HPT1, ADE8, ADE5,7, ADE6, ADE3, DAL1, DAL2, DAL7, DAL3, MEU1, ADE16, APT1, AMD1, ADE17, ADE4, ADE12, ADE2, SER1, ADY2, RNR1, DAL81, MET3, MKS1, DAL82, FCY1*
Phosphate metabolism	*HIS4, KCS1, PPH3, ARO1, ARG82, HOM3, ARG5,6, FAB1, SER2, THR1, TOR1, MET14, VPS34, CTK3, NPR1, POS5*
Carbohydrate metabolism	*ADH5, ARO4, ILV6, ADY2, SFA1, ARO3, ARO1, ARO10, ADE8, SAM2, YEA4, GSY1, ADH4, UGA1, PDC6, ADE3, GND1, DAL7, GON7, ADE16, PDC1, PDC5, SAM1, ACD1, ADH3, ADE17, ADH2, MKS1, ZWF1, ATO2, TKL1, YPR118W*
Lipid metabolism	*PDX3, KCS1, ARG82, DPL1, FAB1, TOR1, OPI3, VPS34, ANT1*
Vitamins metabolism	*PDX3, THR4, THI3, BNA6, ARO2, LYS5, ADE3, BNA1, BNA2, MET1, BNA5, RIB4, SER1, MET7, POS5*
TRANSPORT	*AVT5, TAT1, VMA2, AGP1, GGC1, UGA4, PEP7, ATO3, VPS3, GNP1, CAN1, FCY22, AVT6, AGP3, LOC1, VPS45, MUP1, MEP1, BTN2, TNA1, DUR3, MUP3, DAL4, TRK1, AVT1, DAL5, AVT3, GAP1, VPS9, NPL6, AVT4, MEP2, ALP1, TAT2, PUT4, FRE3, FIT2, VPS28, DIP5, AGC1, VMA13, MEP3, OPT2*
ENERGY	*QCR10*
TRANSCRIPTION	*STP2, ISY1, TIF2, ROX3, TRM1, CDC40, CGR1, PRP18, STB5*
CELL RSUE AND DEFENSE	*SOD1, ROX3, HMF1*
PROTEIN FATE	*NTA1, VAM6, VMA1, HPA3, ECM29*
PROTEIN SYNTHESIS	*EAP1, CAF20, TIF2, YOR302W*
CELL CYCLE AND PROCESSING	*MSC1, BUR2, CDC40, HPA3*
BIOGENESIS OF CELLULAR COMPOUNDS	*ECM25, VAM6, HPA3, CGR1, ECM29*
CELL TYPE DIFFERENTIATION	*BUD16*
Unclassified proteins	*YER091C-A, YER137C, APT2*
	IBA57, MRI1, VPS65, YDR008C, YDR442W, BUD25, YER068C-A,TED1

1.4. *Escherichia coli*

Escherichia coli DH5α was used during plasmids cloning.

2. Culture media

2.1. Yeast growth in laboratory conditions

2.1.1. YPD

Yeast Extract Peptone Dextrose. General medium to grown yeast.

Glucose	20 g/L distilled water
Peptone	20 g/L
Yeast extract	10 g/L

This medium could be liquid or solid adding 20 g/L of agar.
Transformants were selected with different antibiotic resistance. Thus media was supplemented in each case with antibiotics required at these concentrations: 0.2 mg/mL Geneticin, 0.05 mg/mL Nourseothricin and 0.3 mg/mL Hygromicin B.

2.1.2. SD

Minimum medium.

Glucose	20 g/L distilled water
Yeast nitrogen base (YNB)	1.7 g/L
Ammonium sulphate	5 g/L

2.1.3. Potassium Acetate (KAc)

Sporulation medium.

Glucose	0.5 g/L distilled water
Potassium acetate	10 g/L
Yeast extract	1 g/L
Agar	20 g/L

2.2. Yeast growth in fermentation conditions

2.2.1. Inocula preparation

Active Dry Wine Yeasts (ADWY) was prepared by rehydrating in water following the manufacturer's recommendations (30 min at 37 ºC). Subsequent to microscope counting, the appropriate dilution of the rehydrated wine yeast was inoculated in a synthetic grape must to obtain an initial cell concentration of ~2 x 10^6 cells/mL.

2.2.2. Synthetic must (SM)

Synthetic must was prepared according to Riou et al. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran et al., 2004). Nitrogen content and nitrogen source were modified for the different fermentations.

Glucose	100 g/L distilled water
Fructose	100 g/L
Malic acid	5 g/L
Citric acid	0.5 g/L
Tartaric acid	3 g/L

KH_2PO_4	0.75 g/L
K_2SO_4	0.5 g/L
$MgSO_4$ $7H_2O$	0.25 g/L
$CaCl_2$ $2H_2O$	0.16 g/L
NaCl	0.2 g/L
NH_4Cl* (300 mg N/L)	0.35 g/L

Autoclaved at 121ºC for 15 min

Once autoclaved add the next solution previous sterilized:

Amino acid solution* (300 mg N/L)	9.82 ml/L
Oligoelements solution	1 ml/L
Vitamines solution	10 ml/L

Adjust pH 3.3 (with NaOH 10 M)

Nitrogen proportions in follow final concentrations:

300 mg N/L: 90 mg N/L NH_4^+ + 210 mg N/L Aas
200 mg N/L: 60 mg N/L NH_4^+ + 140 mg N/L Aas
140 mg N/L: 42 mg N/L NH_4^+ + 98 mg N/L Aas
60 mg N/L: 18 mg N/L NH_4^+ + 42 mg N/L Aas

<u>Amino acid solution</u>

Tyrosine (Tyr)	1.5 g/L (heat at 100ºC)
Tryptophan (Trp)	13.4 g/L (70ºC)
Isoleucine (Ile)	2.5 g/L (70ºC)
Aspartic acid (Asp)	3.4 g/L (degas CO_2)
Glutamic acid (Glu)	9.2 g/L (degas CO_2)

Arginine (Arg)	28.3 g/L
Leucine (Leu)	3.7 g/L
Threonine (Thr)	5.8 g/L
Glycine (Gly)	1.4 g/L
Glutamine (Gln)	38.4 g/L
Alanine (Ala)	11.2 g/L
Valine (Val)	3.4 g/L
Methionine (Met)	2.4 g/L
Phenylalanine (Phe)	2.9 g/L
Serine (Ser)	6.0 g/L
Histidine (His)	2.6 g/L
Lysine (Lys)	1.3 g/L
Cysteine (Cys)	1.5 g/L
Proline (Pro)	46.1 g/L

Sterilization by filtration, divided into aliquota and keep at -20ºC.

Oligoelements solution

$MnSO_4$ H_2O	4 g/L
$ZnSO_4$ $7H_2O$	4 g/L
$CuSO_4$ $5H_2O$	1 g/L
KI	1 g/L
$CoCl_2$ $6H_2O$	0.4 g/L
H_3BO_3	1 g/L
$(NH_4)6Mo_7O_{24}$	1 g/L

Sterilization by filtration, divided into aliquota and keep at 4ºC.

Vitamines solution

Myo-inositol	2 g/L
Pantothenate calcium	0.15 g/L
Thiamine hydrochloride	0.025 g/L
Nicotinic acid	0.2 g/L
Pyridoxine	0.025 g/L
Biotine	3 ml/L from 100 mg/mL

Sterilization by filtration, divided into aliquota and keep at -20ºC.

2.3. *E.coli* growth

2.3.1. LB

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L

This medium could be liquid or solid adding 20 g/L of agar.

Transformants were selected with antibiotic resistance. Thus media was supplemented with 0.05 mg/mL Ampicillin.

2.3.2. SOC

It is used after electroporation to cell recovery.

Tryptone	200 g/L
Yeast extract	25 g/L
NaCl	50 g/L
Solution	10 mL

Solution: Glucose 2M, $MgCl_2$ 1M, $MgSO_4$ 1M

3. Monitoring wine fermentations

3.1. Microscope counting

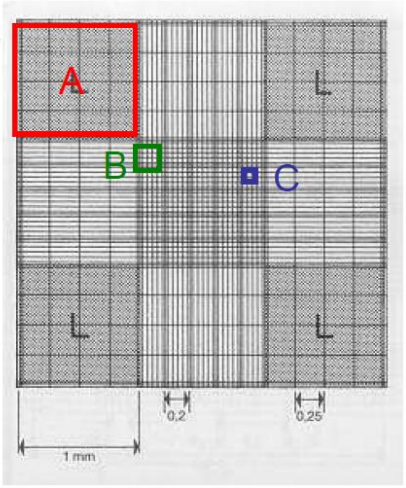
The number of total microorganisms was determined counting the cells with a Neubauer chamber under optical microscope. Following there is a representation of a Neubauer chamber, with the different squares which can be used to count the cells.

Relation among different squares

A = 16 B

B = 25 C

Profundity = 0.1 mm



Volume **A** = 1 x 1 x 0.1 = 0.1 mm^3 = 10^{-4} mL

Volume **B** = 0.25 x 0.25 x 0.1 x 10^{-3} = $6.25 \cdot 10^{-5}$ mL

Volume **C** = 0.05 x 0.05 x 0.1 x 10^{-3} = $2.5 \cdot 10^{-6}$ mL

$$\frac{Cells\ number}{(Square\ number\ x\ Square\ volume\ x\ Dilution)}$$

3.2. Optical density

The spectrophotometer measure the absorbance at 600 nm. This absorbance is directly proportional to the biomass formed by microorganisms. The cells in a medium have the capability to disperse the light, this make to the medium change

their turbidity, being more turbid. The absorbance can be directly related to the biomass although standard curves have to be created for each microorganism. Absorbance value was used to determine growth.

Yeast growth curves were carried out in a microtiter plate reader model POLARstar Optima (BGM Labtech, Offenburg, Germany) and Bioscreen analyser C (Thermic Labsystems Oy, Finland).

3.3. Plate counting

Strains were plated in different medium and incubated during specific time and conditions. After this incubation each cell forms a colony, colonies were counted to obtain the number of colony form units (UFC/mL).

3.4. Density

During wine fermentation yeasts metabolize sugars that are directly proportional to the decrease of density. This is the method most used to follow the alcoholic fermentation, because of the simplicity of this technique. Medium density was monitored using a Densito 30 PX densitometer.

3.5. ANKOM^{RF} Gas Production System

This system provides an easy to use method for monitoring and measuring gas production during fermentation. It consists of RF pressure sensor modules, a "zero Remote" that measures ambient pressure, a computer interface base coordinator and operational software. Fermentation bottles with sensor modules were placed in an incubator, and pressure generated was registered to obtain the gas production curves of each condition.

4. DNA and RNA isolation

4.1. Isolation of yeast genomic DNA (Querol *et al*., 1992)

1. Grow yeast cells in an overnight culture of 5 mL YPD at 30ºC.
2. Centrifuge cells at 3500 rpm.
3. Discard supernatant and wash cells with 1 mL sterile water.
4. Centrifuge cells at 3500 rpm.
5. Discard supernatant and adds 500 µL Buffer 1 (0.9M sorbitol, 0.1M EDTA, pH 7.5).
6. Then they are transferred to a 1.5-mL tube, to which 30 µL of a solution of Zymolyase 60000 (1.5 mg in 1300 µL B1) is added. Incubate tubes at 37°C for 60 min.
7. Centrifuge for 3 min at 12000 rpm, and removes supernatant.
8. Resuspend cells in 500 µL of Buffer 2 (50 mM Tris, 20 mM EDTA, pH 7.4).
9. After suspension, 13 µL of 10% sodium dodecyl sulfate (SDS) is added.
10. The mixture is incubated at 65°C for 5 min.
11. Immediately thereafter, adds 200 µL of 5 M potassium acetate, mix by inversion, and the tubes are placed on ice for 10 min.
12. Centrifuge at maximum speed for 15 min at 4ºC.

13. Supernatant is transferred to a new tube, and the DNA is precipitated by adding 700 µL of isopropanol.
14. After incubation at room temperature for 10 min, centrifuge the tubes at 4ºC for 15 min at 12000 rpm.
15. Removes supernatant and washes the DNA with 500 µL 70% ethanol.
16. Centrifuge at 4ºC for 3 min at 12000 rpm, and remove supernatant.
17. Dry pellet with Speedy Vac, and dissolve in 40 µL of TE (10 mM Tris, 1 mM EDTA, pH 8).

4.2. Isolation of total RNA (Sierkstra *et al.*, 1992)

1. Cells are centrifuged, washed in sterile water, and froze in liquid nitrogen. Afterwards cells could keep at -80ºC until RNA extraction.
2. Wash pellet with 1 mL Extraction buffer (100 mM Tris-HCl pH 7.4, 100 mM LiCl, 0.1 mM EDTA).
3. Centrifuge at 4ºC at 10000 rpm for 5 min.
4. Discard supernatant, and resuspend pellet in 0.5 mL Vortex buffer (100 mM LiCl, 10 mM EDTA, 0.5 % SDS, pH 7.4).
5. The solution is transferred into 2 mL tubes with 1 g of glass beads.
6. The wall of cells is broken with a high agitation in the mini bead-beater (Biospec Products Inc., Bartlesville, Okla.). This agitation is conducted during 30 sec a maxima velocity then 30 sec in ice, and this is repeated 3 times, to avoid an overheating of the sample.
7. The upper phase is extracted with phenol/chloroform/isoamyl alcohol (25:24:1). Repeat this step 3 times.
8. Centrifuge at 4ºC for 5 min at 10000 rpm.
9. Finally, the upper phase is extracted with chloroform/isoamyl alcohol (24:1). Centrifuge to obtain upper phase.

10. Add 3M sodium acetate ph 5.6 (1/10 v/v) and 96% ethanol (2.5 v). Precipitate supernatant for 15 min at -80ºC.
11. Centrifuge for 30 min at 4ºC, and wash pellet with 70% ethanol.
12. Dry pellet with Speedy Vac, and resuspend in 100 µL DEPC-treated water.
13. Purify total RNA suspensions with High Pure RNA Isolation Kit (Roche, Mannheim, Germany) in accordance with the manufacturer's protocol. Determine concentration and purity of RNA with Nanodrop (ND-1000 Spectrophotometer) and 0.8% agarose gels.

Each solution is treated with diethylpyrocarbonate (DEPC) to remove RNases (except Tris-HCl). 50 µL of DEPC is added in 50 mL of solution.

4.3. Plasmid DNA isolation from *E.coli*

1. Inoculate in 3 mL LB supplemented with 3 µL Ampicillin at 37ºC overnight, in a shaker at 200 rpm.
2. Spin down 1.5 mL culture for 10 min at maxima speed. Remove supernatant.
3. Resuspend the pellet in 100 µL Lysis buffer by vortexing.
4. Add 200 µL of 0.2M NaOH/1% SDS, and mix gently by inversion.
5. Add 150 µL 3M KAc pH 5.5, and mix by inversion.
6. Spin down for 15 min at 4ºC.
7. Precipitate the supernatant with 250 µL 2-propanol (-20ºC), and spin down for 15 min at 4ºC.
8. Wash the pellet with 500 µL of 70% ethanol, and dry in the Speedy Vac.
9. Resuspend the pellet in 40 µL TE.

5. Molecular techniques

5.1. Polymerase Chain Reaction (PCR) and Electrophoresis

PCR was used to obtain different deletion cassettes and to confirm their correct integration using primers upstream and downstream from the deleted region. The mix for the amplification was done in 25 µL or 50 µL of total volume containing:

Primer-F (10 µM)
Primer-R (10 µM)
Buffer Taq 10x (Ecogen)
$MgCl_2$ (50 mM) (Ecogen)
dNTPs (10 mM)
Taq DNA polymerase (Ecotaq, Ecogen) (5 U/µL)
DNA 10 (ng/µL)

The PCR conditions were changed depending on primers used and size of amplified fragment. The amplified fragment was tested using a gel electrophoresis (1%). For it, was used TBE buffer (1x) containing (Tris base 89 mM, boric acid 89 mM, EDTA 2 mM, pH 8). Samples were mixed with loading buffer which was composed by bromophenol blue (0.25%), xylene cyanol (0.025%), and glycerol (30%). Voltage used was 70-80V. Finally, gel was tinted with Red Safe (0.05 µl/ml) and observed through a transiluminator UV system. Different molecular weight markers were used to determine the size bands.

5.2. Gene expression analysis by Real-Time PCR

This technique is used to quantify the expression of several genes in specific conditions. RT-PCR is based in isolation of mRNA and reverse transcription to obtain cDNA which is quantified.

cDNA was synthesised from total RNA using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 9700 (Applied Biosystem); 0.5 µL oligo $(dT)_{12-18}$ primer (Invitrogen) was used with 0.8 µg total RNA as template in a reaction volume of 20 µL. Following the manufacter's protocol, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min. Finally, the reaction was stopped at 70 ºC for 15 min.

The real-time quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). In the PCR reaction, the final volume was 25 µL, contained 300 nM of each primer, together with 1 µL of the cDNA previously synthesized from total RNA. All PCR reactions were mixed in 96-well optical plates and cycled in a Step One Plus Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles at 95 ºC for 15 s and at 60 ºC for 60 s.

Each sample had two controls, which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔCt is the difference in Ct of the gene of interest and Ct of the houskeeping gene (*ACT1*); and ΔΔCt is the difference in ΔCt at time = t and ΔCt at time = control. All samples were analyzed in triplicate and the expression values were the average calculated by the analysis software (Applied Biosystems, USA).

5.3. RFLP rDNA (Guillamón *et al*., 1998)

This technique was applied to identify yeasts species. Consist first in an amplification of the region comprised between the 18S and 26S rDNA gene. Later

is produced a digestion with restriction enzyme. The mix for the amplification was done in 50 μL of total volume containing:

Primer ITS1 (10 μM) 1 μL
Primer ITS4 (10 μM) 1 μL
Buffer Taq 10x (Ecogen) 5 μL
MgCl2 (50 mM) (Ecogen) 3 μL
dNTPs (40 mM) 4 μL
Taq DNA polymerasa (Ecotaq, Ecogen) (5 U/ μL) 0.5 μL
H2O milli-Q 33 μL
DNA 2.5 μL

The PCR conditions were: 5 min at 95 ºC followed by 35 cycles of 30 sec at 95ºC, 1 min at 52ºC and 1 min at 72ºC and a finishing step of 7 min at 72ºC. Once obtained the amplified fragment, the fragment can be tested using a gel electrophoresis (1% p/v), after it is done the digestion. In this work was used *Hae*III (Roche Diagnostics). The mix used to conduct the digestion is in 20 μL of total volume. This reaction was conducted overnight at 37ºC. Finally the product was carried in an eletrophoresis gel (3% p/v) to conduct the separation of the digestion bands. Each species is characterized by specific restriction patterns.

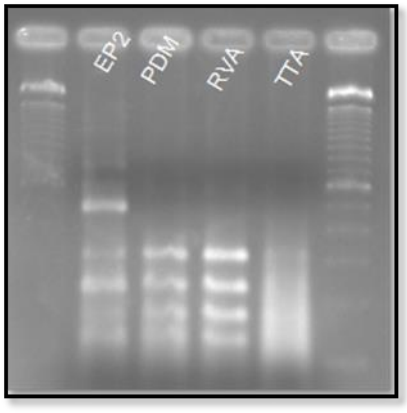


Figure 1. Restriction patterns of rDNA of four commercial strains.

5.4. RFLP mDNA (Querol *et al*., 1992)

This technique was used to differentiate yeast strains.
DNA digestion was carried out with HinfI enzyme in 20 µL final volume.

Buffer 2 µL
RNase 1 µL
HinfI 1 µL
H_20 Milli-Q 6 µL
DNA 10 µL

Reaction was conducted for 3 h at 37ºC. This enzyme recognize a large number of sites in the yeast nuclear DNA but few sites in the mDNA. The product was separated in an eletrophoresis gel (0.8% p/v) with voltage 20V overnight. Each strain is characterized by specific restriction patterns.

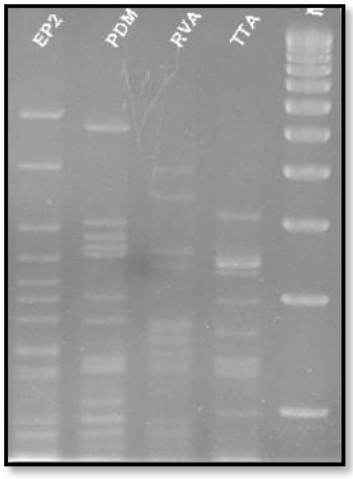


Figure 2. Restriction patterns of mDNA of four commercial strains.

5.5. Delta elements (Legras and Karst, 2003)

This technique was also used to differentiate yeast strains.
PCR amplification was carried out in 50 µL reaction volume, containing:

Primer δ12 (50 µM) 1 µL
Primer δ21 (50 µM) 1 µL
Buffer Taq 10x (Ecogen) 5 µL
BSA 1 µL
MgCl2 (50 mM) (Ecogen) 2.5 µL
dNTPs (40 mM) 1 µL
Taq DNA polymerasa (Ecotaq, Ecogen) (5 U/ µL) 0.2 µL
H2O milli-Q 33.3 µL
DNA 5 Ml

Amplification reaction was performed using the following program: 4 min at 95 ºC followed by 35 cycles of 30 sec at 95ºC, 30 sec at 46ºC and 90 sec at 72ºC and a finishing step of 10 min at 72ºC. Amplification products were separated by electrophoresis 0.8% agarose gels submitted to 100V.

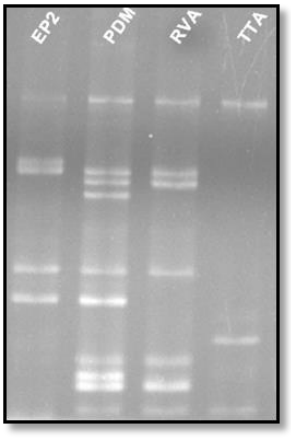


Figure 3. Restriction patterns of delta elements of four commercial strains.

5.5. Spores and mating type

Potassium acetate medium was used to induce the sporulation of strains. The plates were incubated at 30ºC during 5 days. After this time, we tested that the yeast cells have sporulated by examining them under optical microscope. Yeast strains differed in their efficiency and the required time in sporulation. When we can see the tetrad of spores, we continued with the microdissection. Took a small bit of cells of the sporulation plate with a toothpick and mixed with 5 µL glucuronidase in an eppendorf tube. Following the digestion of the ascus wall, the spores of the tetrad can be separated and grown. This solution was incubated for 5 minutes at 30ºC. To stop the reaction was added 5 µL sorbitol 1M, and the tube was placed on ice. The dissection of spores was done with micromanipulator equipment (Singer instruments, United Kingdom) in YPD plates. The viability of the spores was above 50%. After, the spores were grown in YPD plates with antibiotic resistance.
To test the mating type of each spore, we carried out a PCR with MAT, MATa and MATα primers (Huxley et al., 1990). The specific primers produce a characteristic PCR product that differentiates if the strain is a/α haploid or diploid. The size of the band is 544bp (haploid a) or 404bp (haploid α). If it is a diploid we observed both bands. PCR was done under the following conditions: 94ºC for 5 min, 30 cycles at 94ºC for 1 min, 58ºC for 2 min and 72ºC for 2 min, and 72ºC for 7 min.

6. Transformation

6.1. *E. coli* transformation

6.1.1. Electrocompetents preparation

Transformation of *E. coli* in electrocompetent cells was performed as described Dower *et al*., 1988.

1. Inoculate 5 ml LB with a fresh single colony of DH5α at 37ºC overnight with vigorous shaking.
2. Inoculate 1 mL *E. coli* in 200 mL LB and incubate at 37ºC until DO 0.5-0.6.
3. Keep on ice during 15 min.
4. Transfer to falcons of 50 mL. Centrifuge 10 min at 5000 rpm at 4ºC.
5. Discard the supernatant and resuspend the pellet in 2 mL of cold H_2O.
6. Centrifuge 10 min at 5000 rpm at 4ºC. Discard the supernatant.
7. Repeat the steps 5 and 6.
8. Resuspend the pellet in 10 mL of cold glycerol 10%.
9. Centrifuge 10 min at 5000 rpm at 4ºC.
10. Add the same volume of the glycerol 10% than the pellet, around 350 µL.
11. Aliquota 45 µL of *E. coli* in eppendorf tubes.
12. Frozen in N_2 liquid and maintain at -80ºC.

6.1.2. Electroporation (Dower *et al*., 1988)

Previous preparation:

- Defrost electrocompetent cells in ice just before use.
- Sterilize the cuvettes twice 3 min in the UV and maintain in ice until use.
- Maintain the SOC media in ice.

Transformation the plasmid inside the *E.coli* through electroporation:

1. Add 2-3 µl of the plasmid into *E. coli* electrocompetents.
2. Transfer to electroporation cuvette.
3. Electroporate at 1700 Volts for 4 sec with an Electroporator 2510 (Eppendorf, USA).
4. Resuspend the *E.coli* with 500 µL of SOC medium and transfer to eppendorf tube.
5. Maintain 45 min at 37ºC.

6. Plate the solution into LB-Ampicillin plates.

6.2. *S. cerevisiae* transformation (Gietz and Woods, 2002)

1. Inoculate 50 mL of YPD and incubate overnight with shaking at 30ºC.
2. Inoculate 50 mL of warm YPD to a cell density of 2 x 10^6 cells/mL.
3. Incubate the culture at 30ºC on a shaker at 200 rpm until reach OD 0.8-1. This will take 3 to 5 h. It is important to allow the cells to complete 2 to 4 cell divisions to obtain maxima efficiency in transformation.
4. Centrifuge 5 ml of the culture at 5000 rpm for 5 min.
5. Discard supernatant and resuspend the cells in 2.5 mL of sterile water. Centrifuge again.
6. Discard supernatant and resuspend the cells in 100 µL 0.1M LiAc. Transfer the suspension to a 1.5 mL tube.
7. Centrifuge at maxima speed for 15 sec and remove supernatant with micropipette.
8. Resuspend the cells to a final volume of 50 µl (2 x 109 cells/ml). Add about 40 µl of 0.1M LiAc.
9. Boil ss-DNA for 5 min (add when water is boiling) and quickly chill in ice. It is not desirable to boil the carrier DNA every time. Keep a small aliquot in freezer box and boil after 3-4 thaws. Keep on ice when out.
10. Centrifuge at maxima speed for 15 sec and remove LiAc with micropipette.
11. Add the transformation mix in the order listed, that consists of:

240 µl PEG (50% w/v)
36 µl 1M LiAc
50 µl ss-DNA (2 mg/ml)
34 µl PCR product/plasmid (0.1-10 µg)
Total volume=360 µl

12. Vortex each tube vigorously until the cell pellet has been completely mixed. Usually takes about 1 min.
13. Incubate 30 min at 30ºC.
14. Heat shock in a water bath at 42ºC for 30 min. The optimum time can vary for different yeast strains. Please test this if you need high efficiency from your transformations.
15. Centrifuge for 15 sec at 7000 rpm and remove the transformation mix with a micropipette.
16. Wash the cells with 1ml of sterile water. Resuspend the pellet gently at this step if high efficiency is important.
17. Centrifuge and discard the supernatant.
18. Resuspend the cells with 1 ml of YPD and maintain for 2-3 h at 30ºC with shaking at 350 rpm for recovering.
19. Spread yeast inoculum onto plates of YPD with adequate antibiotic. Incubate plates for 2-4 days at 30ºC.

7. Analytic techniques

7.1. Metabolites (High-performance liquid chromatography)

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed. Analytical HPLC was carried out on a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and UV-Visible detector. Prior to injection, samples were centrifuged at 13.300 rpm for 5 min, supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Spain) and diluted 5 or 10-folds. A total volume of 25 μl was injected into a HyperREZ^{TM}XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 ml/min and a column temperature of 50 ºC. The

concentration of each metabolite was calculated using external standards. Each sample was analyzed in duplicate.

7.2. Nitrogen content

Ammonia concentration was measured with a kit using an enzymatic method (Roche Applied Science, Germany). The concentration of free amino acid nitrogen was determined using the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen/mL.

7.3. Aromas (Gas Chromatography)

Higher alcohols and esters were analysed based on a headspace solid phase microextraction (SPME) technique using a 100 µm poly-dimetylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Spain). Aliquots of 1.5 mL of the sample were placed into 15 mL phials and 0.35 g of NaCl and 20 µL of 2-heptanone (0.005%) as internal standard was added. Phials were closed with screwed caps and 13 mm silicone septa. Solutions were stirred for 2 h to obtain the required headspace-liquid equilibrium. Fibres were injected through the phial septum and exposed to the headspace for 7 min and were then desorbed for 4 min in a gas chromatograph (TRACE GC Ultra, Thermo Scientific) with a flame ionization detector (FID), equipped with an HP INNOWax 30 m x 0.25 mm capillary column coated with a 0.25-m layer of cross-linked polyethylene glycol (Agilent Technologies). The carrier gas was helium (1mL/min) and the oven temperature program utilised was: 5 min at 35ºC, 2ºC/min to 150ºC, 20ºC/min to 250ºC and 2 min at 250ºC. The injector and detector temperatures were maintained at 220 and 300ºC, respectively. A chromatographic signal was registered by the ChromQuest program. Volatile compounds were identified by comparing the retention time for reference compounds. Volatile compound concentrations were determined using the

calibration graphs of the corresponding standard volatile compounds. 2-heptanone (0.005% w/v) was used as an internal standard.

7.4. Trehalose content

Intracellular trehalose contents of the four yeast strains were determined following the method described by Parrou and François (1997).

1. Resuspend cells ($2\text{-}5\cdot10^8$ cells) in 250 µL Na_2CO_3 (0.25M).
2. Incubate for 4 h at 95º C.
3. Add 150 µL acetic acid (1M) and 600 µL sodium acetate (0.2M).
4. Incubate half part of solution with trehalase (0.05 U/mL) overnight at 37º C. Add 40 µL of trehalase solution (0.64 U/mL).
5. Centrifuge for 3 min at 7500 rpm.
6. Determine glucose concentration using glucose enzymatic kit (Boehringer Mannheim).
7. Trehalose content is calculated taking into account dry weight at the same sampling time (mg trehalose/dry weight).

7.5. Arginase activity

Crude protein extracts were obtained from the different samples and arginase activity was measured as described in Carrasco et al. (2003).

$$\text{L-arginine} + H_2O \rightarrow \text{L-ornithine} + \text{Urea}$$

A. Protein extraction

1. Homogenize 200 mg of cells in 500 µL Tris buffer and 1 g glass beads.
2. Vortex for 30 s and incubate in ice for 30 s (repeat 4-8 times).
3. Centrifuge for 5 min at 5000 g 4º C.
4. Transfer the supernatant (protein extract) to an eppendorf tube.

B. Protein quantification (Bradford)

1. Create standard curve with Albumin (Sigma, A-4503): 0, 5, 10, 15, 20, 30, 40 µg/mL (stock solution 400 µg BSA/mL).
2. Prepare sample with adequate solution.
3. Mix 800 µL sample with 200 µL Bradford reagent in the cuvette.
4. Wait 5 min and measure absorbance at 595 nm.
5. Calculate protein concentration with standard curve.

C. Enzymatic activity trial

1. Mix 25 µL protein extract (2-40 µg) and 25 µL $MnCl_2$ in 2 mL eppendorf tube. Incubate for 20 min at 55º C (number of tubes equally number of stop reactions).
2. Incubate for 4 min at room temperature.
3. Add 150 µL carbonate buffer (0.1M, pH 9.5) tempered at 37º C.
4. Start reaction with substrate addition (50 µL arginine 0.1M).
5. Incubate at 37º C.
6. Stop reaction with addition 750 µL glacial acetic acid at different times: 0, 3, 6…min (one tube for each time).

D. Ninhidrine determination

1. Add 250 µL ninhidrine reagent (140 mM ninhidrine in 60% acetic acid, 40% phosphoric acid 6M in methanol).
2. Boil the sample at 100º C for 30 min.
3. Measure at 515 nm and calculate ornithine concentration with ornithine standard curve (0-100 nM).
4. Prepare blank without protein extract and other without arginine for each sample.
5. Calculate ornithine concentration in every time and obtain slope value (units). Arginase activity is obtained in nmols Orn/min*µg protein.

Arginase activity = (Units * Dilution)/ [Protein]*Volume (0.025 mL)

8. Online sources

Saccharomyces Genome Base (SGD)

http://www.yeastgenome.org/

Basic Local Alignment Search Tool (BLAST)

http://blast.ncbi.nlm.nih.gov/

Reverse Complement – Bioinformatics

http://www.bioinformatics.org/sms/rev_comp.html

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ANNEX II

Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must

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Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must

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ABSTRACT

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). However, it is important to know the specific nitrogen requirement of each strain, to avoid excessive addition that can lead to microbial instability and ethyl carbamate accumulation. In this study, we aimed to determine the effect of increasing nitrogen concentrations of three different nitrogen sources on growth and fermentation performance in four industrial wine yeast strains. This task was carried out using statistical modeling techniques. The strains PDM and RVA showed higher growth-rate and maximum population size and consumed nitrogen much more quickly than strains ARM and TTA. Likewise, the strains PDM and RVA were also the greatest nitrogen demanders. Thus, we can conclude that these differences in nitrogen demand positively correlated with higher growth rate and higher nitrogen uptake rate. The most direct effect of employing an adequate nitrogen concentration is the increase in biomass, which involves a higher fermentation rate. However, the impact of nitrogen on fermentation rate is not exclusively due to the increase in biomass because the strain TTA, which showed the worst growth behavior, had the best fermentation activity. Some strains may adapt a strategy whereby fewer cells with higher metabolic activity are produced. Regarding the nitrogen source used, all the strains showed the better and worse fermentation performance with arginine and ammonium, respectively.

1. Introduction

The use of active dry wine yeasts (ADWY) is a widespread practice in wine technology. These ADWY are supplied to oenologists in dehydrated form and must be rehydrated prior to inoculation in grape must. Subsequently, wine yeasts go through the typical growth phases of a microbial culture: lag, exponential and stationary phases. Lag phase represents the adaptation of the ADWY to the new medium, involving transcriptional (Novo et al., 2007), proteomic (Salvadó et al., 2008) and metabolic changes (Bauer and Pretorius, 2000). Successful adaptation leads to the growth or exponential phase in which the culture increases from 10^6 CFU/mL (inoculated population) to approximately 10^8 CFU/mL. Although, in principle, grape must contains all the nutrients needed for yeast to develop, yeast cell growth under enological conditions is often considered to be nitrogen limited, and nitrogen deficiency is a major cause of stuck or sluggish fermentations (Bisson, 1999; Bell and Henschke, 2005). Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). Nitrogen addition can affect fermentation rate either by enhancing fermentation rate per cell (increase fermentation activity) or by enhancing the number of cells per population. Varela et al. (2004) tried to distinguish both effects by performing fermentations with different nitrogen concentrations and with different initial biomass concentrations. They concluded that the increase in viable cell concentrations positively correlated with the increase in fermentation rates, and fermentations ended more quickly, even when cells were grown under conditions of severe nitrogen shortage. From an industrial perspective, they proposed two alternatives to deal with nitrogen-deficient musts. The first alternative involves

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providing the inoculated yeast with the required nitrogen, allowing it to reach maximum population size and ensuring a normal fermentation profile. The second alternative involves adding viable biomass from other fermentation tanks or by inoculating a higher population size.

Biomass transfer from fermentation tanks is technologically difficult and might involve contamination problems. Moreover, it has been proven that continuous anaerobic growth of the same culture during many generations decreases fermentation fitness of cells as a consequence of inefficient synthesis of unsaturated fatty acids and ergosterol (Rosenfeld et al., 2003) and cell-wall properties (Powell et al., 2003). Another way to increase initial biomass is by inoculating a higher population of ADWY in the grape must. This practice would increase winery costs for commercial yeasts and decrease the number of yeast generations during the alcoholic fermentation. The limitation of growth generations could decrease secondary metabolite synthesis, thereby affecting wine aroma and flavor profiles (Carrau et al., 2010). Thus, the other alternative to ensure a regular fermentation profile is by providing the inoculated strain with a suitable nitrogen concentration.

The assimilable nitrogen in grape juice is constituted by ammonium and amino acids in similar proportions (Henschke and Jiranek, 1993). However, most nitrogen supplementation is carried out with ammonium salts (sulfate or phosphate). The exclusive addition of ammonium imbalances the natural ratio of inorganic/organic nitrogen composition and affects the amino acid uptake pattern (Beltran et al., 2005). Ammonium is a preferred yeast nitrogen source, and when plentiful, it represses the expression of catabolic pathways which use other nitrogenous compounds. This mechanism, called nitrogen catabolite repression (NCR), has recently been studied during wine fermentations (Beltran et al., 2004, 2005). Ammonium addition inhibits arginine and alanine uptake and stimulates the consumption of glutamine, branched-chain and aromatic amino acids. Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds (particularly hydrogen sulfide) and the amount of urea, which is the major precursor of the carcinogen ethyl carbamate (Adams and Van Vuuren, 2010; Mendes-Ferreira et al., 2009; Torrea et al., 2003, 2011; Vilanova and Martínez, 2007). In many cases, winemakers proactively add nitrogen to the must, even without knowing its initial nitrogen status, which may cause high nitrogen levels that exceed the minimum necessary to complete fermentation. Moreover, minimal nitrogen amount and preferences are shown to be strain-dependent (Manginot et al., 1998; Taillandier et al., 2007). Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, leading to microbial instability and ethyl carbamate accumulation in wine (Ough and Amerine, 1988). Therefore, it is important to know the nitrogen content of grape juice and the nitrogen requirement for each specific yeast strain, in order to achieve optimal fermentation performance and so wine production meets the demands set by regulatory authorities and consumers.

In this context, this study aims to determine the specific nitrogen demands of four commercial yeast strains, widely used in the Spanish wine industry. Our objective was to determine the minimum nitrogen amount required by each commercial strain to ensure the maximum population during growth or exponential phase in a synthetic grape must. However, as explained above, not only is nitrogen concentration important but also the nitrogen source used for supplementing grape musts. To this end, we have analyzed yeast growth in a synthetic grape must with different nitrogen concentrations and nitrogen sources. As sole nitrogen sources, we have used ammonium, arginine and glutamine. These amino acids are two of the major ones of the assimilable organic nitrogen (proline is also very abundant but it is not metabolized during wine fermentations) (Henschke and Jiranek, 1993), however, their utilization by the cell is very different. Glutamine, such as ammonium, is considered a preferred nitrogen source and is firstly consumed by *Saccharomyces cerevisiae*. Conversely, arginine is strongly subjected to NCR and is hardly consumed when there is an excess of the "so-called" good nitrogen sources (Beltran et al., 2004, 2005). We used a microplate-based method that allowed real-time and high-throughput monitoring of the wine yeasts growth curves. These data were then modeled to quantitatively describe the behavior of the different wine yeasts as a function of nitrogen concentration and source. In the second part of this study, we analyzed the effect of nitrogen source and concentration on fermentation performance. The results obtained in this work are important for winemakers because nitrogen can be added taking into account the different nitrogen needs of the particular wine strain used and thus avoid excessive preventive supplementation, which can lead to microbial instability of the final wines and production of off-flavors or unhealthy compounds.

2. Materials and methods

2.1. Yeast strains and inocula preparation

The four commercial strains used in this study were provided by Agrovin Company (Ciudad Real, Spain). Three of these strains belong to species *Saccharomyces cerevisiae var. cerevisiae* (PDM, RVA and TTA), while another strain is commercialized as *Saccharomyces cerevisiae var. uvarum* (ARM).

Inocula for both growth curves and fermentation experiments were prepared by rehydrating the dry yeasts in water following the manufacturer's recommendations (30 min at 37 °C). Subsequent to microscope counting, the appropriate dilution of the rehydrated wine yeast was inoculated in a synthetic grape must (henceforth SM) to obtain an initial cell concentration of $\sim 2 \times 10^6$ cells/mL. This SM was prepared according to Riou et al. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran et al., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L. The following mineral salts were used: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L. The following vitamins were used: myo-inositol 20 mg/L, calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L. Nitrogen content and nitrogen source were modified for the different fermentations as described below. The final pH of the SM was adjusted to 3.3 with NaOH.

2.2. Microvinification trials

To test the effect of nitrogen concentration on fermentation performance, SM was modified with a mixture of ammonium and amino acids at: 60 mg N/L (18 mg N/L as ammonium and 42 mg N/L in amino acid form), 140 mg N/L (42 mg N/L as ammonium and 98 mg N/L in amino acid form), 200 mg N/L (60 mg N/L as ammonium and 140 mg N/L in amino acid form), and 300 mg N/L (90 mg N/L as ammonium and 210 mg N/L in amino acid form). The proportion of each amino acid was administered as previously proposed by Riou et al. (1997). After this nitrogen administration, the pH of the SM was adjusted to 3.3 and filtered to sterility. To test the effect of nitrogen source on fermentation rate, ammonium, glutamine and arginine were used as sole nitrogen sources at a final concentration of 140 mg N/L. This concentration was chosen as it is the minimal amount of nitrogen to avoid stuck fermentations

according to previous reports (Bely et al., 1990). Nitrogen concentrations were calculated considering all nitrogen atoms assimilable by yeast in fermentative conditions: one in ammonium, two in glutamine, and three in arginine.

Fermentations were performed in 250 mL glass bottles containing 200 mL of SM and capped with closures than enabled the carbon dioxide to escape and the samples to be removed. Thus, fermentations were performed in semi anaerobic conditions, since limited aeration was necessary in order to harvest samples for subsequent analysis. Fermentations were done in triplicate at 28 °C with continuous orbital shaking (150 rpm). They were monitored by the medium density using a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Yeast cell growth was also determined by absorbance at 600 nm. Fermentation was considered to be complete when density was below 998 g/L. The supernatant of these samples was stored at −20 °C for HPLC analysis of the content of sugars, glycerol, ethanol and acetic acid (see below).

2.3. Modeling trials

Yeast growth curves were carried out in a microtiter plate reader model POLARstar Optima (BGM Labtech, Offenburg, Germany). Growth was monitored by optical density (OD) changes at a wavelength of 595 nm. Measurements were every 30 min for 45 h at 28 °C (until yeast cells reached the stationary phase), after a pre-shaking of 20 s. The growth medium was SM modified with different nitrogen concentrations (arginine, glutamine and ammonium), ranging from 5 up to 200 mg of N/L. Regarding this nitrogen concentration, it was also taken into account that glutamine and arginine contain two and three assimilable molecules of N, respectively (arginine contains four molecules of N, but only three are used by yeast cells in fermentation conditions; Martin et al., 2003). A total of 432 growth curves (3 nitrogen sources × 12 nitrogen concentrations × 4 yeast strains × 3 replicates) were obtained and analyzed.

Biological growth parameters were deduced from each growth curve by directly fitting OD measurements *versus* time to the reparameterized Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

$$y = D^{*}\exp\{-\exp[((\mu_{max}{}^{*}e)/D)^{*}(\lambda - t)) + 1]\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_\infty/OD_0)$ is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h). OD/time data were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function (observed-predicted)^2. This primary modeling was accomplished using the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option.

The area obtained under the OD *versus* time curves was also used in this work as a valuable procedure to estimate the effects of nitrogen source and content on overall yeast growth because its relationship with the biological growth parameters (Arroyo-López et al., 2009). This parameter was obtained by integration using the OriginPro 7.5 software (OriginLab Corporation, Northampton, USA).

For secondary modeling, the Monod's growth model was used. This model was initially proposed as an empirical approach to describe microbial growth as a function of the concentration of a limiting nutrient with the following equation:

$$y = (V^{*}S)/(K + S)$$

where y is the microbial growth parameter deduced from primary modeling (in our case μ_{max} or the area under OD/time curve) for the diverse nitrogen concentrations (S), V is the theoretical maximum value obtained for the growth parameters, and K is the substrate concentration which supports half-V. An important feature of this model is that the growth parameter is zero when there is no substrate but it tends to an upper limit when the substrate is greatly in excess. The link between these two extreme conditions is described by a hyperbola (Lobry et al., 1992). As in the previous case, the fit was carried out by a non-linear regression procedure using Statistica 7.0 software.

2.4. Fitness advantage

The concept of "fitness advantage" (m) between two microorganisms can be defined by the following expression: $m = r1 - r2$, where m corresponds to the μ_{max} difference of two strains (r1 and r2) for specific environmental conditions (Goddard, 2008; Salvadó et al., 2011). In this study, we have obtained the m value of the PDM strain (used as reference) compared with the other three commercial yeasts as a function of the nitrogen source and concentration. In this way, we aim to theoretically determine the strain better adapted to nitrogen variations.

2.5. HPLC analysis

Glucose, fructose, glycerol, ethanol and acetic acid were analyzed in all the samples at the end of the fermentation process. Analytical HPLC was carried out on a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and UV–Visible detector. Prior to injection, samples were centrifuged at 13.300 rpm for 5 min, supernatants were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Spain) and diluted 5 or 10-folds. A total volume of 25 μl was injected into a HyperREZ™XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 ml/min and a column temperature of 50 °C. The concentration of each metabolite was calculated using external standards. Each sample was analyzed in duplicate.

2.6. Analysis of variance

An analysis of variance was performed by means of the factorial ANOVA module of Statistica software version 7.0, using "yeast strain" and "nitrogen source" as categorical predictor variables. Dependent variables introduced for the analysis were the Monod's parameters obtained for μ_{max} ($V_{\mu max}$ and $K_{\mu max}$) and the area under OD/time (V_{area} and K_{area}). Briefly, ANOVA tests differences in means among-groups variables by analyzing their variances. Thus, when statistical significance is obtained ($p \leq 0.05$), we can reject the null hypothesis that no differences between means exist, and accept the alternative hypothesis that the means differ from each other. With this criterion in mind, we can use a *post-hoc* comparison test to check for significant differences among treatments and to form homogenous groups. This task was carried out by means of the Scheffé test, which is considered one of the most conservative *post-hoc* tests (Winer, 1962). An alternative advantage of the Scheffé test is that it can also be used with unequal sample sizes.

3. Results

3.1. Influence of nitrogen on yeast growth

We have studied and compared the effect of three nitrogen sources at different concentrations on the growth of four commercial wine yeast strains (PDM, ARM, RVA and TTA). Different

concentrations, which ranged from 5 up to 200 mg of N/L, of ammonium, glutamine and arginine were tested as sole nitrogen sources in SM. For this purpose, yeasts were monitored by means of OD measurements, and their respective biological growth parameters (μ_{max} and area under OD/time curve) estimated for each nitrogen condition. Then, the Monod's model was used as a secondary empirical model to explain the effect of nitrogen concentration on yeast growth (Figures S1 and S2 in supplementary material). Tables 1 and 2 show the parameters obtained for this fit, while Fig. 1 graphically shows the ANOVA with the significant differences among yeasts for these parameters.

As expected, increased nitrogen levels augmented growth rate and the area under the curve of the different strains up to a certain concentration for which the maximum value was reached and kept constant (Figures S1 and S2). *K* of these two parameters indicates the nitrogen concentration for which half of the maximum value was reached (see Tables 1 and 2). The *K* of μ_{max} was much lower than the *K* of area. Thus, when maximum growth rate is reached, much more nitrogen is still required to attain the maximum area value. Regardless of nitrogen source, the different strains showed different μ_{max}. These strains could be ordered from lowest to highest μ_{max} as TTA < ARM < RVA < PDM (Table 1 and Fig. 1). The TTA and ARM did not show significant differences in the μ_{max} for the three nitrogen sources. The μ_{max} of the RVA significantly increased when grown in glutamine. The PDM showed the highest values of μ_{max} in all nitrogen sources assayed. Comparing growth of this strain in the different sources, ammonium yielded the highest μ_{max} with 0.175 h^{-1}. This growth rate practically represented twice that of TTA μ_{max} in the same medium.

A similar trend was observed when the area under the growth curve was analyzed (see Table 2 and Fig. 1). This parameter represents the overall maximum growth obtained in a specific condition and includes all the lag, log and stationary phases. Comparing all the strains, PDM also showed the highest area values growing in ammonium and arginine while RVA obtained the highest value in glutamine. These area values did not significantly change for the different nitrogen sources with the exception of the above mentioned growth of the RVA in glutamine.

Growth rate values were used to calculate the fitness advantage of PDM strain compared to the other strains (Fig. 2). The *m* value was always positive for pairs PDM vs. ARM and PDM vs. TTA. This result indicates that PDM had better fitness than ARM and TTA for all the nitrogen sources and concentrations assayed. However, the *m* value was close to zero, or even negative for low nitrogen concentrations (below 40 mg of N/L), for the pair PDM vs. RVA. Both strains presented similar fitness for arginine and glutamine and only PDM was more competitive at high ammonium concentrations.

3.2. Influence of nitrogen on the fermentation performance

In this section, our aim was to test the effect of nitrogen source and nitrogen concentration on fermentation activity. The three nitrogen sources (ammonium, arginine and glutamine) were used at the same concentration (140 mg N/L) in SM. To test the effect of nitrogen concentration, we used a mixture of ammonium and amino acids at 60, 140, 200 and 300 mg N/L. Fermentation performance was monitored by density reduction of the grape must and, in each case, the area under must density *versus* time curve. Conversely to the area under the growth curve, higher values of the area mean slower fermentation activity. These values of area for the effect of nitrogen source and the effect of nitrogen concentration are graphically represented in Figs. 3 and 4, respectively. Table 3 shows the results of the ANOVA carried out for this parameter. The ARM showed the highest area values, that is, the slowest fermentation activity regardless of nitrogen source (Fig. 3). The strains RVA and PDM showed similar fermentative behavior and TTA presented the quickest fermentation activity for all three nitrogen sources tested. Curiously, the TTA strain exhibited the best fermentation activity and the worst growth rate in all three nitrogen sources. Depending on the nitrogen source, the different strains showed slight, but significant, differences in fermentation activity (Table 3). SM with arginine was fermented more quickly by all four strains whereas the use of ammonium as sole nitrogen source yielded the slowest fermentation activities.

Regarding the effect of concentration on the fermentation performance of the strains under study, increasing nitrogen concentration from 60 up to 140 mg N/L significantly increased the fermentation activity of all the strains (Fig. 4). However, no significant differences were observed for fermentation activity when the subsequent nitrogen concentration increased (200 and 300 mg N/L; Table 3). In these fermentations, using a mixture of ammonium and amino acids decreased the differences in fermentation activity among strains. In fact, in this case, no significant differences were detected in fermentation activity among strains for the same nitrogen concentration.

For a better understanding, fermentation activity was also expressed as the time required to reduce 5 (T5), 50 (T50) and 100% (T100) of must density (1080 g/L) to the density of the wine (998 g/L) (Table 4). The T100 of the fermentations with 60 mg N/L was not calculated because none of the strains could finish these fermentations. The T100 values confirmed that fermentations with arginine and ammonium were the quickest and slowest ones respectively. However, conversely to the area curve values, the increase in nitrogen concentration yielded significant decreases in fermentation time for all the strains.

The kinetic fermentation values were also confirmed by analyzing residual sugars, glycerol, ethanol and acetic acid of the final wines (see Table S1 in supplementary material). The fermentations with arginine and ammonium had the lowest and highest sugar concentration in the final wines, respectively (with the exception of RVA with glutamine). Regarding nitrogen concentration, the higher the nitrogen concentration was the lower the residual sugars in the wine.

4. Discussion

Higher fermentation rates can be achieved either by enhancing the glycolytic flux per cell or by increasing the yeast population.

Table 1
Parameters of the Monod's model, *V* (maximum value obtained) and *K* (concentration where the half of the maximum value is reached) for the fit of μ_{max} as a function of different nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments.

	Monod's parameter *V* (h^{-1})			Monod's parameter *K* (mg/L)		
Yeast	NH_4	Arg	Gln	NH_4	Arg	Gln
TTA	0.091 (0.006)^{a}	0.091 (0.001)^{a}	0.086 (0.002)^{a}	7.27 (0.42)^{a,b}	10.25 (0.92)^{a,b,c}	5.20 (2.25)^{a}
ARM	0.117 (0.002)^{b}	0.112 (0.004)^{b}	0.123 (0.002)^{b,e}	13.58 (1.81)^{c,d}	10.18 (1.29)^{a,b,c}	17.24 (1.36)^{d}
PDM	0.175 (0.007)^{g}	0.146 (0.002)^{c,d}	0.164 (0.003)^{f,g}	12.86 (2.44)^{b,c,d}	9.12 (0.10)^{a,b,c}	12.67 (1.05)^{b,c,d}
RVA	0.144 (0.002)^{c,d}	0.137 (0.001)^{c,e}	0.152 (0.002)^{d,f}	6.56 (1.37)^{a}	5.09 (0.61)^{a}	9.18 (1.53)^{a,b,c}

Note: Values followed by different superscript letters, within the same Monod's parameter, are significantly different according to a Scheffe's *post-hoc* comparison Test.

Table 2
Parameters of the Monod's model, *V* (maximum value obtained) and *K* (concentration where the half of the maximum value is reached) for the fit of the area under the OD *versus* time curve as a function of different nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments.

	Monod's parameter *V*			Monod's parameter *K* (mg/L)		
Yeast	NH_4	Arg	Gln	NH_4	Arg	Gln
TTA	38.98 (2.91)[a]	38.51 (3.23)[a]	37.51 (2.05)[a]	44.10 (11.60)[a]	45.77 (13.77)[a]	35.78 (5.98)[a]
ARM	47.88 (3.58)[a]	42.75 (4.90)[a]	43.46 (10.15)[a]	80.39 (10.25)[a]	53.13 (14.61)[a]	46.68 (30.98)[a]
PDM	69.85 (5.60)[a,b]	70.20 (1.32)[a,b]	75.40 (7.11)[a,b]	46.43 (1.86)[a]	46.21 (5.71)[a]	52.66 (13.07)[a]
RVA	52.96 (9.72)[a]	52.86 (4.11)[a]	93.82 (25.23)[b]	63.28 (26.11)[a]	45.87 (25.02)[a]	135.12 (41.68)[a]

Note: Values followed by different superscript letters, within the same Monod's parameter, are significantly different according to a Scheffe's *post-hoc* comparison Test.

Recently Albertin et al. (2011) have compared the fermentative behaviors of different yeast strains in three main fermentative processes (winemaking, brewing and baking), concluding that the maximum fermentation rate is driven by population size rather than by specific flux per cell. Thus, the positive correlation found between population size and fermentation rate suggests that increasing maximum population would also increase fermentative ability. Previously, Varela et al. (2004) also stated that cell concentration (biomass) governs fermentation rate in nitrogen-deficient wine musts. Nitrogen is the most important growth-limiting substrate during wine fermentation (Varela et al., 2004), and its deficiency represents one of the main causes of stuck or sluggish fermentations (Bisson, 1999). In this study, we aimed to determine the effect of increasing nitrogen concentrations on growth and fermentation performance in four industrial wine yeast strains. The other question dealt with in this study is the nature of the added nitrogen. Mostly inorganic nitrogen (ammonium salts) is used for supplementing nitrogen-deficient grape musts. However, there is a growing interest in the use of nutrient complements enriched in organic nitrogen during wine fermentations. For this reason, we have also compared the effect on growth and fermentation activity of ammonium, arginine and glutamine used as sole nitrogen sources in a synthetic grape must.

4.1. Effect of nitrogen concentration

To determine the effect of nitrogen concentration on yeast growth we have calculated two key parameters: the maximum growth rate (μ_{max}) and the maximum area under OD *versus* time curve. The μ_{max} denotes the maximum rate of cell division, which is related to the generation time (GT = ln (2)/μ_{max}). The second parameter in yeasts is related to all the biological growth parameters (maximum population size, lag phase and μ_{max}) (Arroyo-López et al., 2009). Several authors have also used this value as a valuable parameter to estimate the effects of diverse inhibitory and stimulating substances (sugars, chloride salts, weak acids) on microbial growth (Lambert and Pearson, 2000; Bautista-Gallego et al., 2008; Arroyo-López et al., 2009). As the amount of inhibitor increases, the effect on organism growth also increases, and this inhibitory effect on growth is reflected by a reduction in the area under the OD/time curve. However, if the substance has a growth stimulating effect, an increase in the area under the OD/time curve is observed by increasing the maximum population level and μ_{max} (Bautista-Gallego et al., 2008; Arroyo-López et al., 2009).

The nitrogen limiting concentration required to reach the μ_{max} value was much lower ($K_{\mu max}$ ranging from 5 up to 18 mg N/L) than

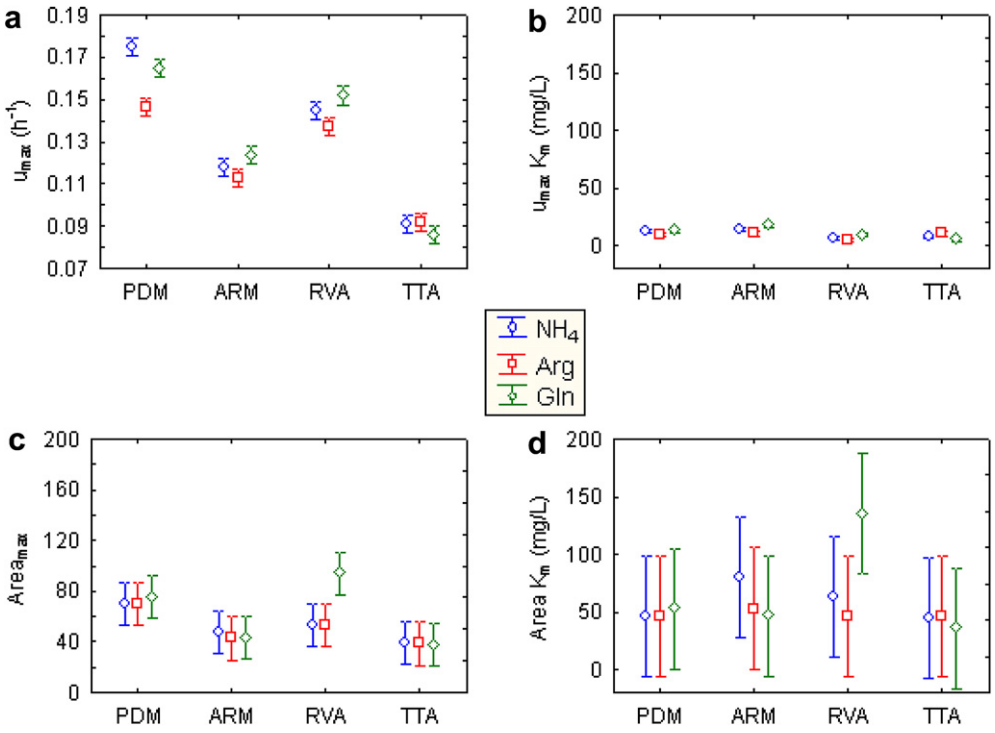

Fig. 1. ANOVA for the parameters obtained by means of the Monod's model. a) maximum value obtained for μ_{max}, b) nitrogen concentration where the half of the maximum value for μ_{max} is reached, c) maximum value obtained for the area under OD *versus* time curve, d) nitrogen concentration where the half of the maximum value for area is reached.

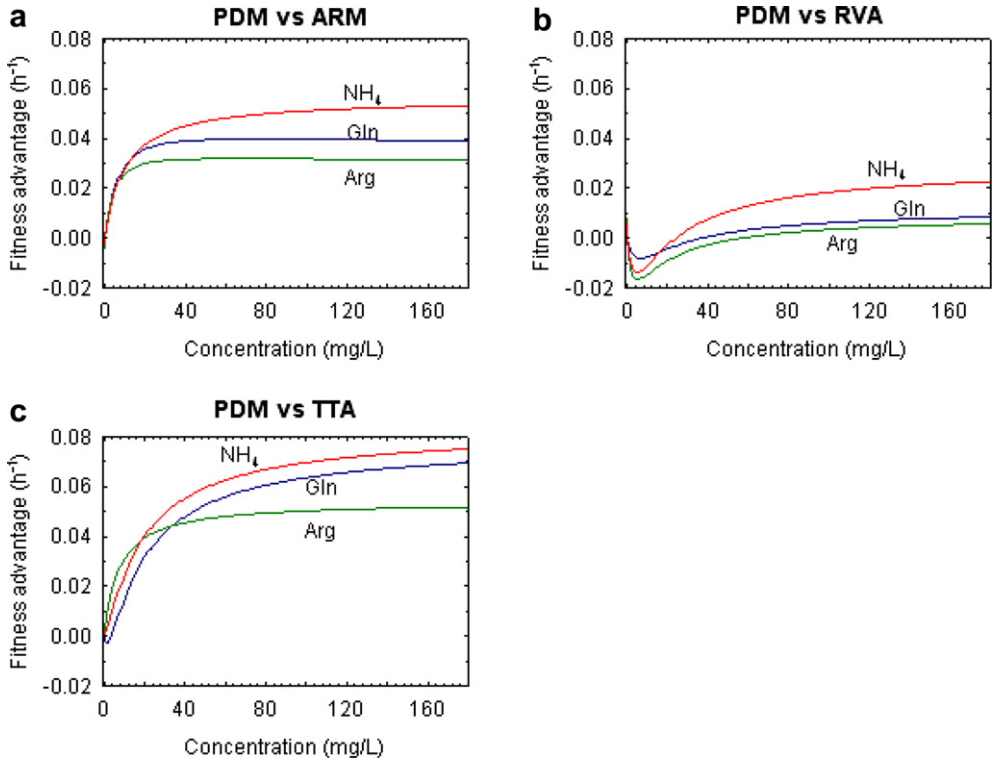

Fig. 2. Model predictions for the fitness advantage (m, h^{-1}) of the PDM strain *versus* a) ARM, b) RVA and c) TTA yeast strains as a function of the nitrogen concentration.

the nitrogen concentration to obtain the maximum area ($K_{areamax}$ ranging from 35 up to 135 mg N/L). It seems logical to think that, up to the μ_{max} nitrogen limiting concentration threshold, cells use this nitrogen to reproduce quickly. Nitrogen concentrations above this limit allow the cell to reproduce at maximum speed and keep growing until nitrogen depletion (or shortly after, as they can use nitrogen stored inside cellular compartments), thus reaching higher population sizes with higher nitrogen available. Above certain concentrations, nitrogen is no longer the limiting factor for cell growth, and increases in nitrogen do not have an effect on μ_{max} or population size. This would be the nitrogen limiting concentration for a certain strain or nitrogen source. However, regardless of nitrogen concentration, there must be a genetic component which determines different μ_{max} for the different strains studied. A direct correlation was observed between the two growth parameters analyzed because the strains which presented a higher growth rate also corresponded to a higher area (correlated to the maximum OD obtained). Although the genetic bases controlling growth-rate are not fully understood, phenotypic variation was detected among strains in terms of nitrogen consumption rate, which might partially explain the differences in growth behavior. Strains PDM and RVA consumed nitrogen much more quickly than strains ARM and TTA, especially as there was an abundance of nitrogen in the medium (Figure S3). Recently, Wang et al. (2011) have proved that growth rate and maximum population size can be modified by increasing glucose uptake as a consequence of changing gene dosage in three glycolytic enzyme genes. This possible correlation

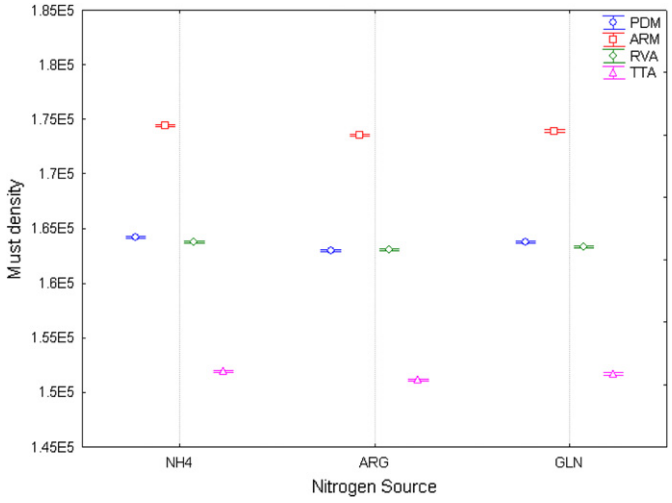

Fig. 3. Effect of the nitrogen source (at 140 mg/L) on the area under must density reduction *versus* time curve for the assayed commercial wine yeast strains.

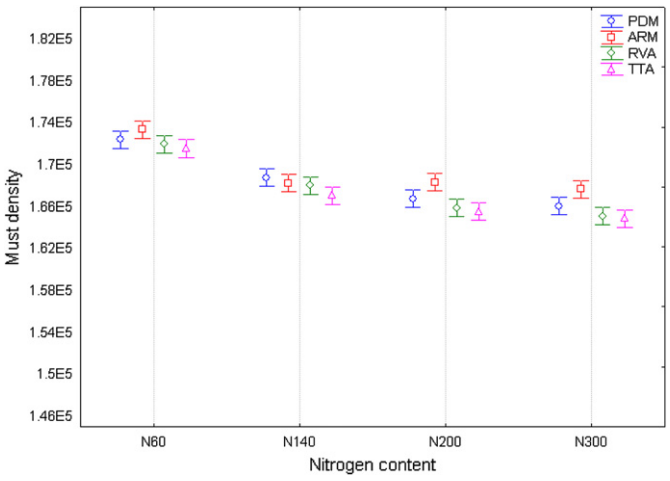

Fig. 4. Effect of nitrogen content on the area under must density reduction *versus* time curve for the assayed commercial wine yeast strains.

Table 3
ANOVA analysis for the area under the must density *versus* time curve as a function of different concentrations and nitrogen sources. Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments.

	Area Density ($\times 10^5$) vs. Time			Area Density ($\times 10^5$) vs. Time			
Yeast	NH_4	Arg	Gln	N60	N140	N200	N300
TTA	1.519 (0.000)^{a}	1.511 (0.001)^{e}	1.517 (0.001)^{a}	1.715 (0.000)^{e,f}	1.670 (0.001)^{a,b,c,d}	1.655 (0.001)^{a,b,c}	1.649 (0.000)^{a}
ARM	1.744 (0.000)^{i}	1.735 (0.001)^{g}	1.739 (0.001)^{h}	1.734 (0.001)^{f}	1.682 (0.028)^{b,c,d}	1.683 (0.001)^{c,d}	1.676 (0.335)^{a,b,c,d}
PDM	1.642 (0.000)^{f}	1.630 (0.001)^{b}	1.637 (0.001)^{d}	1.724 (0.000)^{f}	1.687 (0.001)^{d,e}	1.667 (0.001)^{a,b,c,d}	1.660 (0.001)^{a,b,c,d}
RVA	1.637 (0.002)^{d}	1.630 (0.001)^{b,c}	1.633 (0.001)^{c}	1.719 (0.001)^{f}	1.680 (0.001)^{a,b,c,d}	1.658 (0.001)^{a,b,c,d}	1.651 (0.001)^{a,b}

Note: Values followed by different superscript letters are significantly different according to a Scheffe's *post-hoc* comparison Test.

in wine yeasts between nitrogen uptake rate and growth traits should be dealt with in depth in future studies. However, our study also provides evidence that the strains with higher areas are also the greatest nitrogen demanders. This result is contrary to the popular belief among the winemakers relating scarce biomass yield during winemaking with high nitrogen demanding strains. Another proof that the strains with better growth have a higher nitrogen demand is that the fitness advantage of PDM decreased as nitrogen decreased (Fig. 2), because the differences in growth rate compared with other strains also decreased.

It is very difficult to distinguish clearly between the effect of nitrogen on fermentation activity and the effect of nitrogen on biomass yield as both effects are interdependent. Varela et al. (2004) used metabolic flux balancing and biomass concentration experiments to evaluate whether the direct correlation between fermentation rate and nitrogen concentration is due to an increase in cellular metabolic activity or to biomass increase. They showed that the higher the biomass concentration, the quicker the fermentation was completed, even when using cells grown with a severe nitrogen shortage. Thus, the main effect of nitrogen concentration on fermentation performance was mainly due to the increase in biomass. However, our results do not fully support this conclusion because the strain which showed the worst growth behavior had the best fermentation activity. Conversely, the correlation between biomass production and fermentation activity was confirmed for strain ARM, which showed the worst fermentation performance. Therefore, the effect of nitrogen concentration on metabolic activity cannot be ruled out and, like growth behavior, it seems to be strain-dependent. At the same nitrogen concentration, strain TTA yielded a lower area than PDM; however, these cells have higher metabolic activity. Nevertheless, it should be kept in mind that growth and fermentation activity of these strains were obtained by very different experimental conditions (volume, aeration, etc.) which might limit the comparison between both parameters.

Another result supporting the effect of nitrogen on cellular metabolic activity was that the nitrogen concentration boosting fermentation rate is much higher than the nitrogen concentration promoting increased growth (Table 4). Manginot et al. (1998) and Beltran et al. (2005) already highlighted the importance of nitrogen during the non-proliferating or stationary phase of wine fermentations. However, as already reported Jiranek et al. (1991), strain differences in fermentation rate were more important at concentrations in which nitrogen became limiting for growth.

Table 4
Time, expressed in hours, required for the assayed yeast strains to reduce 5% (T5), 50% (T50) and 100% (T100) of must density (1080 g/L) to the density of the wine (998 g/L). These values are the mean ± SD of three independent experiments.

Yeast strain			T5	T50	T100
PDM	Nitrogen source	NH_4	17.45 ± 0.90	61.72 ± 0.78	199.61 ± 0.39
		Arg	17.97 ± 0.78	49.22 ± 0.78^{a}	149.22 ± 0.00^{a}
		Gln	16.15 ± 0.45	55.99 ± 0.45^{a}	167.97 ± 1.10^{a}
	Nitrogen concentration	60	16.6 ± 1.00	151.04 ± 3.11^{b}	–
		140	16.60 ± 0.00	66.73 ± 0.56	197.76 ± 0.69
		200	18.51 ± 0.41^{b}	47.58 ± 0.41^{b}	159.26 ± 8.45^{b}
		300	19.22 ± 0.41^{b}	41.25 ± 0.41^{b}	115.67 ± 2.49^{b}
ARM	Nitrogen source	NH_4	21.09 ± 1.17^{c}	75.39 ± 0.68^{c}	277.34 ± 2.43^{c}
		Arg	20.31 ± 0.68^{c}	62.11 ± 0.00^{a,c}	217.97 ± 13.26^{a,c}
		Gln	19.53 ± 0.68^{c}	67.22 ± 1.38^{a,c}	246.09 ± 6.52^{a,c}
	Nitrogen concentration	60	29.30 ± 1.00^{b,c}	248.5 ± 0.71^{b,c}	–
		140	26.69 ± 0.56^{c}	80.39 ± 1.14^{c}	248.05 ± 2.76
		200	28.36 ± 1.07^{c}	63.52 ± 1.07^{b,c}	173.85 ± 8.420^{b}
		300	29.53 ± 0.70^{b,c}	58.83 ± 0.81^{b,c}	140.63 ± 5.07^{b}
RVA	Nitrogen source	NH_4	15.27 ± 1.15	54.40 ± 1.94^{c}	203.56 ± 0.51^{c}
		Arg	15.47 ± 0.70^{c}	47.11 ± 0.70^{a,c}	171.92 ± 6.51^{a,c}
		Gln	13.02 ± 0.90^{c}	45.97 ± 1.58^{a,c}	190.50 ± 2.12^{a,c}
	Nitrogen concentration	60	10.17 ± 0.01^{b,c}	132.05 ± 1.06^{b,c}	–
		140	9.77 ± 0.00^{c}	53.71 ± 0.00^{c}	220.00 ± 0.00
		200	10.02 ± 0.63^{c}	37.55 ± 1.14^{b,c}	111.01 ± 5.71^{b,c}
		300	11.20 ± 0.23^{b,c}	31.51 ± 0.23^{b,c}	82.55 ± 5.21^{b,c}
TTA	Nitrogen source	NH_4	12.92 ± 2.19^{c}	47.09 ± 0.95^{c}	153.17 ± 3.88^{c}
		Arg	13.54 ± 1.44^{c}	40.63 ± 0.63^{a,c}	117.71 ± 0.36^{a,c}
		Gln	11.67 ± 0.72^{c}	44.59 ± 0.36^{a,c}	134.37 ± 10.60^{a,c}
	Nitrogen concentration	60	11.46 ± 0.91^{c}	118.75 ± 1.56^{b,c}	–
		140	10.68 ± 0.45^{c}	49.22 ± 0.78^{c}	168.00 ± 8.23^{c}
		200	11.07 ± 0.23^{c}	36.59 ± 0.59^{b,c}	85.55 ± 2.80^{b,c}
		300	11.85 ± 0.23^{b,c}	29.82 ± 0.23^{b,c}	73.05 ± 0.78^{b,c}

T100 = time to reach a density ≤998 g/L.
– Unfinished fermentation.
[a] Significant differences in nitrogen sources and compared with ammonium (control source).
[b] Significant differences in nitrogen concentration and compared with 140 mg N/L (control concentration).
[c] Significant differences between strains and compared with PDM (control strain).

4.2. Effect of nitrogen source

To our knowledge, there are no prior studies into the effect of individual nitrogen sources on yeast growth and fermentation activity. The first conclusion of this study is that the effect of nitrogen source on growth parameters is much more limited than the concentration of nitrogen. The four strains showed similar growth parameters for all three nitrogen sources, with few exceptions. The RVA strain improved its growth in glutamine whereas PDM showed the highest μ_{max} in ammonium. Consequently, this strain always showed better fitness in ammonium than in arginine and glutamine (Fig. 2). Regarding effects on fermentation activity, differences were also limited but all the strains showed the best and worst fermentation performances with arginine and ammonium, respectively (Tables 3 and 4). These findings were also confirmed by analysis of residual sugars in the final wines (Table S1).

5. Conclusions

Results obtained in this survey confirm that industrial yeast strains have significantly different nitrogen requirements and these nitrogen needs are strongly strain-dependent. We observed that these differences in nitrogen demand positively correlated with higher growth rate and higher nitrogen uptake rate. This correlation should be confirmed because these phenotypic traits should be

important selection criteria for wine yeasts. The most direct effect of employing an adequate nitrogen concentration is the increase in biomass, which involves a higher fermentation activity. However, the impact of nitrogen on fermentation is not exclusively due to the increase in biomass. Some strains may adapt a strategy whereby fewer cells with higher metabolic activity are produced.

Currently, the most common method for dealing with nitrogen-deficient fermentations is to add ammonium salts (usually ammonium phosphate), however, the wine industry may consider using other supplements based on organic nitrogen (amino acids and mostly arginine). The comparison of wine fermentations supplemented either with inorganic or organic nitrogen sources has shown that nitrogen concentration rather than nitrogen source drove the increase in fermentation rate and biomass yield. However, some significant differences were observed in terms of fermentation performance. The use of arginine as sole nitrogen source showed the best fermentation performance among all the strains studied.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fm.2012.02.012.

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ANNEX III

Biomarkers for detecting nitrogen deficiency during alcoholic fermentation in different commercial wine yeast strains

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Biomarkers for detecting nitrogen deficiency during alcoholic fermentation in different commercial wine yeast strains

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ABSTRACT

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. Several putative biomarkers were tested in order to analyze their appropriateness to detect nitrogen stress in the yeast. To this aim, four commercial wine strains (PDM, ARM, RVA and TTA) were grown in a synthetic grape must with different nitrogen concentrations. Trehalose accumulation, arginase activity and the expression of eleven genes were tested in these wine strains, known to have different nitrogen requirements. The overall response of the four strains was similar, with differences in response intensity (PDM and RVA with higher intensity) and response time (which was also related with nitrogen consumption time). Trehalose response was mostly related to entry into the stationary phase, whereas arginase activity was responsive to nitrogen depletion, although its measurement is too complicated to be used for routine monitoring during winemaking. The expression of the genes *DAL4*, *DAL5*, *DUR3* and *GAP1* was clearly related to nitrogen depletion and thus, *GAP1* and *DAL4* were selected as markers of nitrogen deficiency. In order to adapt expression analysis to winemaking conditions, the original strains were transformed into reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4*. The transformants had a similar fermentative capacity to the parental strains and were able to detect alterations in yeast physiological status due to nitrogen limitations.

1. Introduction

Nitrogen composition of grape juice is important to ensure good wine fermentation performance. However, yeast cell growth under enological conditions is often considered to be nitrogen limited, which would cause stuck or sluggish fermentations. A low initial nitrogen concentration acts by limiting growth rate and biomass formation of yeast, resulting in a low rate of sugar catabolism (Bisson, 1991). These problematic fermentations have economic relevance because wines with higher residual sugar negatively impact on the quality of the final product. Currently, the most common method for dealing with nitrogen-deficient fermentations is adding supplementary nitrogen (usually ammonium phosphate). However excessive levels of these compounds may have negative consequences, such as microbial contamination or ethyl carbamate formation (Monteiro et al., 1989; Ough et al., 1988) and undesirable aroma production (Bell and Henschke, 2005). Therefore, it is important to know the nitrogen content of grape juice and the nitrogen requirement for each specific yeast strain.

A mechanism known as Nitrogen Catabolite Repression (NCR) allows the selection of the best nitrogen source for yeast growth. Good nitrogen sources (glutamine, asparagine and ammonium) decrease the level of enzymes and permeases required to use the poor nitrogen sources (Magasanik, 1992). This repression is mainly obtained by inhibiting the transcription of genes required for the use of poor nitrogen sources (Magasanik and Kaiser, 2002). Although NCR regulation during growth on many different nitrogen sources has been investigated in depth (Godard et al., 2007), the regulation of this process during fermentation is not well characterized (Deed et al., 2011). During wine fermentation, a nitrogen-repressed condition changes to a nitrogen-de-repressed condition as nitrogen is consumed (Beltran et al., 2004). This shift in the NCR regulation could be used as a marker to detect nitrogen limitation during grape must fermentation by monitoring transcriptional activity of these NCR genes, mainly comprised by core nitrogen

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metabolism genes (*GDH1*, *GDH2*), permease genes (*AGP1*, *DUR3*, *DAL4*, *DAL5*, *GAP1*) and catabolic pathway genes, including those for utilization of proline, arginine and urea (*CAR1*, *PUT2*).

As mentioned above, most of the intracellular pools of amino acids are located in the vacuole (Wiemken and Durr, 1974), and this is especially significant in the case of arginine. The first reaction in arginine degradation is the conversion of L-arginine into L-ornithine and urea. This reaction is catalyzed by the cytoplasmic enzyme arginase (Jauniaux et al., 1978), encoded by the *CAR1* gene (Jauniaux et al., 1982). Arginase is also regulated by NCR, arginine concentration and post-transcriptional events (Bossinger and Cooper, 1977; Messenguy and Dubois, 1983). Arginase activity analysis is very useful as it responds to arginine mobilization from the vacuole under nitrogen-limiting conditions (Sumrada and Cooper, 1978). Previous studies (Carrasco et al., 2003; Beltran et al., 2004; Jiménez-Martí et al., 2007) have reported that arginase activity could be a good marker to detect nitrogen depletion in grape must fermentation.

A plethora of stress conditions for yeast cells take place during wine fermentation. The cellular mechanism for controlling these stress situations involves the rapid synthesis of protective molecules and the activation of signal transduction systems that induce the activation of enzyme activities and the transcription of genes encoding factors with protective functions. Of these, trehalose has remarkable stress protection properties and may determine the survival response of yeasts under extreme environmental condition. Several studies have shown the metabolism of trehalose is affected by nitrogen availability; therefore, once the nitrogen in the growth medium is consumed, yeast cells start to accumulate intracellular trehalose (Parrou et al., 1999; Novo et al., 2005; Hazelwood et al., 2009). Thus, trehalose accumulation could also be an indicator of nitrogen limitation conditions during yeast growth during fermentation.

In a recent work (Gutiérrez et al., 2012), we determined the minimum nitrogen amount required by four commercial yeast strains to ensure the maximum population in the growth or exponential phase in a synthetic grape must. These strains showed different nitrogen requirements, growth and nitrogen uptake rates. In this work, we have tested two biochemical markers such as intracellular trehalose content and arginase activity and a molecular marker based on gene regulation of NCR genes in the same four commercial strains. These wine strains were inoculated in a synthetic grape must with different nitrogen concentrations. Our aim was to select the most suitable marker or sensor for detecting nitrogen limitation by cells growing during wine fermentation. This marker would go beyond determining nitrogen levels in fermenting must, revealing real yeast nitrogen needs and the correct timing and concentration for nitrogen supplementation during winemaking fermentations. Several studies have shown the importance of adding the correct nitrogen supplementation to avoid fermentation problems or off-flavor production as a consequence of both nitrogen shortage and excess (Mendes-Ferreira et al., 2009; Torrea et al., 2011). We also aimed to develop a very simple and handy method for detecting nitrogen limitation in other wine strains, which could be used in the future to avoid stuck fermentation or inadequate addition in the wine industry.

2. Materials and methods

2.1. Yeast strains and growth conditions

The yeast strains used in this study are the following: PDM, ARM, RVA and TTA, all of them provided by Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com). A taxonomic description of these strains was carried out by RFLPs of the ITS/5.8S region (Guillamón et al., 1998). The strains PDM (Pasteur Prise de Mousse), RVA and TTA belonged to species *Saccharomyces cerevisiae*, while we identified the strain ARM as a hybrid between *S. cerevisiae* and *Saccharomyces kudriavzevii*, following the procedure proposed by Gonzalez et al. (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has been confirmed by Dunn et al. (2012). These wine strains were used at an initial population of 2×10^6 cell/mL of active dry yeast rehydrated in warm water prior to inoculation, according to the manufacturer's instructions (37 °C for 30 min). Microvinification experiments were carried out using synthetic grape must (SM). This SM was prepared according to Riou et al. (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran et al., 2004). Only the nitrogen content changed in the different concentrations: 140 mg N/L as control condition, 60 mg N/L as limiting condition and 300 mg N/L as non-limiting condition. SM with a very high excess of nitrogen (1200 mg N/L) was also prepared for the selection of the NCR genes. The proportion of ammonium and different amino acids was as described by Beltran et al. (2004) and this proportion was maintained for the different nitrogen concentrations.

Fermentations were performed in 250 mL glass bottles containing 200 mL of SM and capped with closures than enabled carbon dioxide to escape and samples to be removed. Fermentations were done in triplicate at 28 °C with continuous orbital shaking (150 rpm). Cell samples were collected throughout the fermentation at different time points. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80 °C. Supernatant was also stored at −20 °C to analyze the concentration of YAN at different fermentation times. In order to monitor the induction of fluorescence emission, fermentations in Falcon tubes of 50 mL with 40 mL of SM were also performed to carry out a more intensive sampling during the first hours of N-limiting (60 mg N/L) and control (140 mg N/L) fermentations.

2.2. Nitrogen content analysis

Ammonia concentration was measured with a kit using an enzymatic method (Roche Applied Science, Germany). The concentration of free amino acid nitrogen was determined using the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen/mL. The addition of ammonium and amino acids represents the yeast assimilable nitrogen (YAN).

2.3. Trehalose content determination

Intracellular trehalose contents of the four yeast strains were determined following the method described by Parrou and François (1997).

2.4. Arginase activity determination

Crude protein extracts were obtained from the different samples and arginase activity was measured as described in Carrasco et al. (2003).

2.5. Gene expression analysis by real-time quantitative PCR

Total RNA was isolated from yeast samples as previously described by Siekstra et al. (1992) and resuspended in 50 μL diethylpyrocarbonate (DEPC)-treated water.

To purify total RNA suspensions, contaminant genomic DNA was removed using a High Pure Isolation kit (Roche, Mannheim, Germany) in accordance with the manufacturer's protocol. Purified RNA concentrations were determined using a Nanodrop (ND-1000 Spectrophotometer) and verified electrophoretically on 0.8% agarose gels.

cDNA was synthesized from total RNA using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 9700 (Applied Biosystem); 0.5 μL oligo $(dT)_{12\text{-}18}$ primer (Invitrogen) was used with 0.8 μg total RNA as template in a reaction volume of 20 μL. Following the manufacturer's protocol, after denaturation at 70 °C for 10 min, cDNA was synthesized at 42 °C for 50 min. Finally, the reaction was stopped at 70 °C for 15 min.

Gene expression was determined using the real-time quantitative PCR technique. The primers used in this study are listed in Table 1. The real-time quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). In the PCR reaction, the final volume was 25 μL, contained 300 nM of each primer, together with 1 μL of the cDNA previously synthesized from total RNA. All PCR reactions were mixed in 96-well optical plates and cycled in a Step One Plus Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s.

Each sample had two controls, which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔCt is the difference in Ct of the gene of interest and Ct of the housekeeping gene (*ACT1*); and ΔΔCt is the difference in ΔCt at time = *t* and ΔCt at time = 3 h in the non-limiting condition (300 mg N/L). All samples were analyzed in triplicate and the expression values were the average calculated by the analysis software (Applied Biosystems, USA).

2.6. Construction of green fluorescence protein (GFP) reporters

One copy of the open reading frame (ORF) of the genes *GAP1* and *DAL4* were replaced in the four strains by the deletion cassette GFP-*KanMX4* by using the short flanking homology (SFH) method (Güldener et al., 1996). The plasmid pKT127 (Sheff and Thorn, 2004) was used as template to obtain this deletion cassette (primers shown in Table 1). *S. cerevisiae* transformation was carried out using the lithium acetate method (Gietz and Woods, 2002). Transformants were selected by resistance to geneticin. The correct integration of the deletion cassette was confirmed by PCR using primers upstream and downstream of the cloning site. Moreover, the fluorescence emission of the transformants was also tested after an overnight culture in SD medium (glucose 20 g/L, yeast nitrogen base YNB 1.7 g/L, ammonium sulfate 5 g/L).

2.7. Fluorescence microscopy and flow cytometry

Cells were harvested during fermentation at different time points to detect fluorescence by microscope and flow cytometry.

Fluorescence images were acquired with a Nikon Eclipse 90i epifluorescence microscope by using a 40× objective with a GFP filter (B-2E/C). Bright-field images were used to focus on the cells and the Nikon Digital Sight DS-5Mc captured the images. NIS-

Table 1
Primers used in this study.

Gene	Name	Oligonucleotide sequence (5′–3′ end)
GAP1 disruption cassette[a]	GAP1m-F	ACAGACCAAGGACAGCAACATTTATAAGAAACAAAAAAAAAGAAATAAAAAATGTCTAAAGGTGAAGAATT
	GAP1m-R	TATTATGATTATCTAAAAAATAAAGTCTTTTTTTGTCGTTGTTCGATTCACTAGTGGATCTGATATCATC
GAP1 PCR verification	GAP1c-F	GATTGTTAAATGTCAGTTTGG
	GAP1c-R	TTGAAGCTCACACAGATTAGT
DAL4 disruption cassette[a]	DAL4m-F	CTTTTATATTCATCTACATCTTGTGATATAAAACATCAACAAAGACGAGAATGTCTAAAGGTGAAGAATT
	DAL4m-R	TATCAATTCTCCTGATCACTACTGGTAAGAGAATAAATTGGGATTTTTATCTAGTGGATCTGATATCATC
DAL4 PCR verification	DAL4c-F	ACATTTGCGCCTATTCGATG
	DAL4c-R	TATTCAGACGGGAATGCATG
ACT1 primer real-time PCR	ACT-F	TGGATTCCGGTGATGGTGTT
	ACT-R	CGGCCAAATCGATTCTCAA
AGP1 primer real-time PCR	AGP1-F	CGCCATATGTCATTGCTGTTG
	AGP1-R	CATGGACAGCACGGAAAGTAGA
CAR1 primer real-time PCR	CAR1-F	TGGGTATCGCCGCCTTT
	CAR1-R	TGACAGCGTTGATGCCGTAT
DAL4 primer real-time PCR	DAL4-F	GGCTCCTCATAAAATCAGGCATT
	DAL4-R	CCGTGCGATTTCTTCAAAGC
DAL5 primer real-time PCR	DAL5-F	AGCATGTCTTGCGGTGGAA
	DAL5-R	GGAATTCGCACTGATATTGGAAA
DUR3 primer real-time PCR	DUR3-F	AGCATGTCTTGCGGTGGAA
	DUR3-R	TTTGCCTGGAACGAAGTAAGTG
GAP1 primer real-time PCR	GAP1-F	CTGTGGATGCTGCTGCTTCA
	GAP1-R	CAACACTTGGCAAACCCTTGA
GDH1 primer real-time PCR	GDH1-F	CACCATCTCTGGTAGTGGTAACGT
	GDH1-R	ACGACAGTACCACCTAGCTCAATAAC
GDH2 primer real-time PCR	GDH2-F	TTGTGGAGGGTGCCAATCTATT
	GDH2-R	AGCCATGTTCCTCCAAAGCA
HSP26 primer real-time PCR	HSP26-R	CCTTGCCAGTAGAATCCTTTGC
	HSP26-F	GGGTGAAGGCGGCTTAAGAG
PUT2 primer real-time PCR	PUT2-R	CGCTTTTCCCGCAGACTAAC
	PUT2-F	GCTTGTGTGACGTTTGCCAGTA
RTN2 primer real-time PCR	RTN2-R	CGGACCTTGTTGGCGTCTT
	RTN2-F	CGCAGGCAGACGATCCA

[a] Underlining indicates homology to the yEGFP-kanMX4 cassette from plasmid pKT-127. The remaining sequences of the primers are homologous to the flanking region of the *GAP1* or *DAL4* open-reading frame.

Elements software was used to control the camera and image acquisition and to analyze images.

For flow cytometry, samples were harvested by centrifugation, frozen in liquid nitrogen and stored at −80 °C until analysis. The cells were rinsed with sterile phosphate-buffered saline and measured in the cytometer (Beckman Coulter Epics XL Flow Cytometer, Minessota). A total of 20,000 cells of the sample were measured at a 700 V voltage in FL1 FITC, revealing the number and percentage of fluorescent cells and fluorescence intensity. The EXPO 32 ADC software was used for these measurements. The freezing process during cell storage did not affect their fluorescence emission (data not shown). The parameters measured with the cytometer were the number of fluorescent cells and the average of fluorescence intensity. The arbitrary value of fluorescence used to compare among strains and nitrogen conditions was obtained by applying the formula: (number of fluorescent cells × the average of fluorescence intensity)/1000.

3. Results

The aim of this study was to determine the most appropriate biomarker to be used during alcoholic fermentation to indicate the state of the cells in relation with the quantity of nitrogen. Furthermore, it is interesting to know how these strains respond to progressive nitrogen depletion, when they detect insufficient nitrogen and activate a mechanism to combat this problem. Our approach was to monitor trehalose content, arginase activity and gene expression throughout the alcoholic fermentation in a synthetic grape juice mimicking an enological environment. Fermentations with three nitrogen concentrations were used: a limiting concentration (60 mg N/L) which leads to a sluggish fermentation, the minimum concentration (140 mg N/L) required by the strains to complete fermentation (control fermentation) and a non-limiting nitrogen concentration (300 mg N/L).

3.1. Biochemical markers

Time course of intracellular trehalose content (Fig. 1) and arginase activity (Fig. 2) of the four commercial wine strains were determined in the first days of SM fermentation. Nitrogen (YAN) uptake for the different fermentations is also shown in these figures (Figs. 1 and 2). Both PDM and RVA strains needed 24 and 48 h for nitrogen consumption in the N-limiting and control fermentations respectively. Total nitrogen was also exhausted in 48 h in the non-limiting or excess condition. The ARM strain showed a slower uptake, requiring 48 h for nitrogen depletion in the N-limiting condition and 72 h for the control and non-limiting fermentations. Intriguingly, the TTA strain was the quickest to consume nitrogen in the limiting condition (8 h) but also needed 72 h for nitrogen depletion in the control and non-limiting fermentations.

Regardless of the nitrogen concentration, trehalose accumulation started 48 h after yeast inoculation (Fig. 1). Generally speaking, trehalose synthesis matched with the entrance into the stationary phase (data not shown) and, unexpectedly, trehalose concentration was directly correlated with nitrogen content in the SM. Thus, the higher the nitrogen content, the more trehalose accumulated. However, some differences were detected among strains in the trehalose concentration reached in the studied time-lapse. The

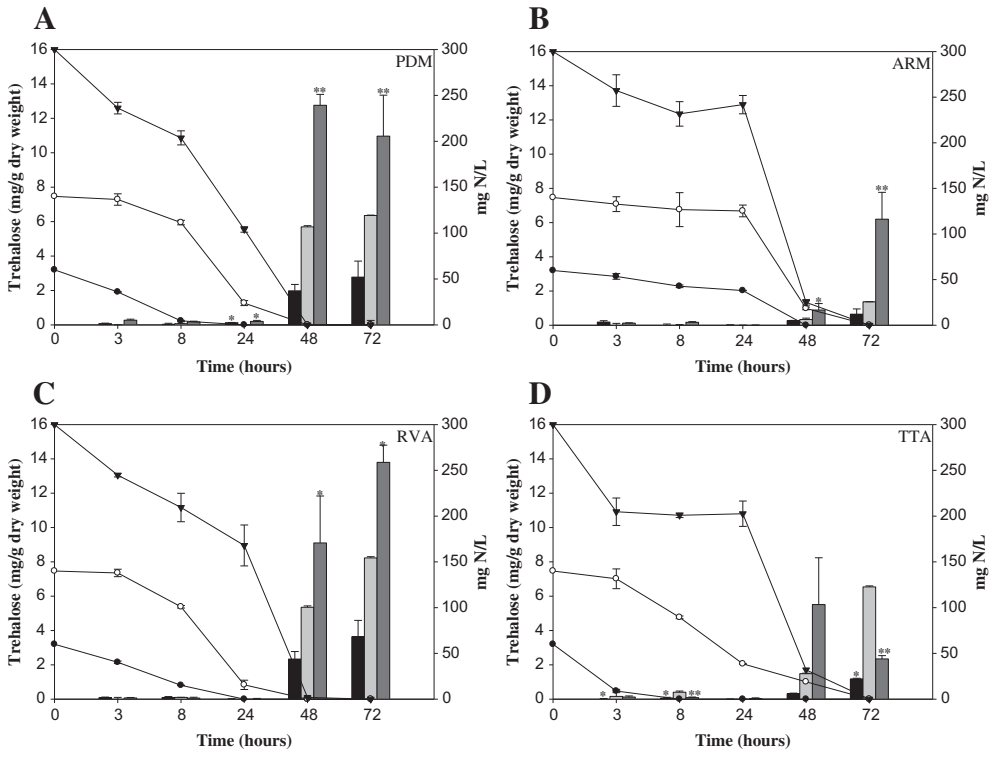

Fig. 1. Intracellular content of trehalose and yeast assimilable nitrogen (YAN) consumption of four yeast strains: **A)** PDM, **B)** ARM, **C)** RVA and **D)** TTA, during fermentation with different nitrogen concentration: 60 mg N/L (*black bars*/*filled circles*), 140 mg N/L (*gray light bars*/*open circles*) and 300 mg N/L (*gray dark bars*/*filled triangles*). The error bars indicate the standard deviation of three replicates.

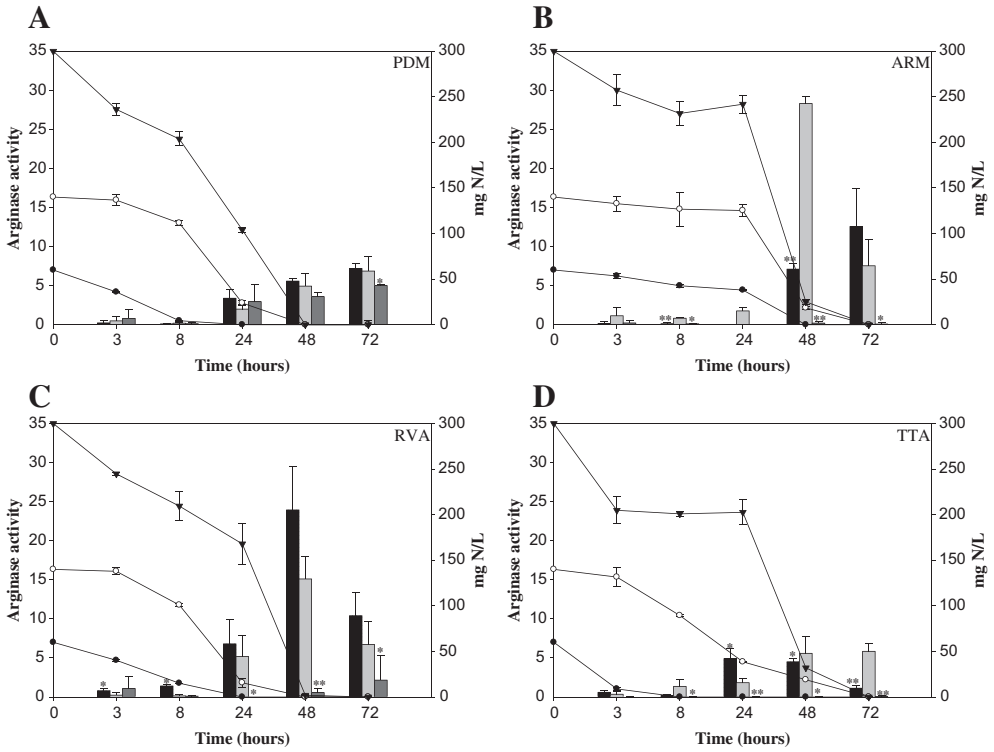

Fig. 2. Arginase activity profile and nitrogen consumption of four yeast strains: **A)** PDM, **B)** ARM, **C)** RVA and **D)** TTA, throughout 60 mg N/L (*black bars*/*filled circles*), 140 mg N/L (*gray light bars*/*open circles*) and 300 mg N/L (*gray dark bars*/*filled triangles*) fermentations. Arginase activity was expressed as: nmol ornithine/μg protein × min.

PDM and RVA strains reached a trehalose concentration twice that of the ARM and TTA strains.

Conversely to trehalose content, arginase activity was inversely proportional to nitrogen concentration. With some exceptions, this activity started before in the N-limiting condition, mainly when ammonium was consumed (data not shown), and reached higher values than in the control or non-limiting conditions (Fig. 2). In fact, very scarce or null activity was detected for the samples taken from the non-limiting condition, with the exception of the PDM strain. Inter-strain comparison showed that RVA and ARM strains reached the highest values of arginase activity after 48 h of yeast inoculation in the limiting and control fermentation respectively. On the contrary, PDM showed no significant differences in activity at the different time-points or conditions.

3.2. Molecular markers

An initial screening was performed of putative genes, which may predict nitrogen deficiency during wine fermentation. Most of these genes have been classified as NCR sensitive genes (Godard et al., 2007; Deed et al., 2011) or, without being directly related to nitrogen metabolism, show significant up-regulation under nitrogen limitation and starvation (Mendes-Ferreira et al., 2007; Jiménez-Martí et al., 2007). Gene activity was monitored at several time-points after yeast inoculation (PDM strain) under two fermentation conditions: one N-limiting (60 mg N/L) and one with very high excess nitrogen (1200 mg N/L), which exerted a clear repression of the nitrogen sensitive genes (Fig. 3). The array of screened genes comprised genes involved in central nitrogen metabolism (*GDH1*, *GDH2*), permease genes (*AGP1*, *DUR3*, *DAL4*, *DAL5*, *GAP1*), genes involved in utilization of poor nitrogen sources such as arginine and proline (*CAR1*, *PUT2*) and two genes involved in environmental stress response (ESR) (*HSP26* and *RTN2*) (Gasch et al., 2000). The ESR genes did not show a clear correlation with nitrogen concentration. *HSP26* was strongly up-regulated after 24–48 h of yeast inoculation under both nitrogen conditions and this increase could be related with entry into the stationary phase, as previously described (Zuzuarregui and del Olmo, 2004). The central nitrogen metabolism genes *GDH1* and *GDH2* or the proline and arginine utilization genes *PUT2* and *CAR1* did not show any clear change in expression in response to the low nitrogen concentration. Lastly, the permeases *DAL4*, *DAL5*, *DUR3* and *GAP1* seemed to be good candidates for predicting nitrogen deficiency because they were strongly up-regulated in response to nitrogen starvation and were kept repressed in the presence of excess nitrogen in the fermentation medium. *AGP1* was the only analyzed permease gene that did not clearly respond to nitrogen concentration. Finally, we selected the *DAL4* (allantoin permease) and *GAP1* (general amino acid permease) because they displayed the highest induction values (ranging from 100 to 400 fold). The other criterion for selecting these genes is based on the fact they belong to the two categories of NCR sensitive genes, according to the classification by Godard et al. (2007): genes whose transcription is exclusively regulated by NCR such as *GAP1* and genes which are subjected to other transcriptional regulation in addition to NCR such as *DAL4*, which is also inducible by allophanate (a product of urea degradation).

Thereafter, we analyzed the transcriptional activity of the selected genes in the four commercial strains and under the same nitrogen conditions as those used for the biochemical markers

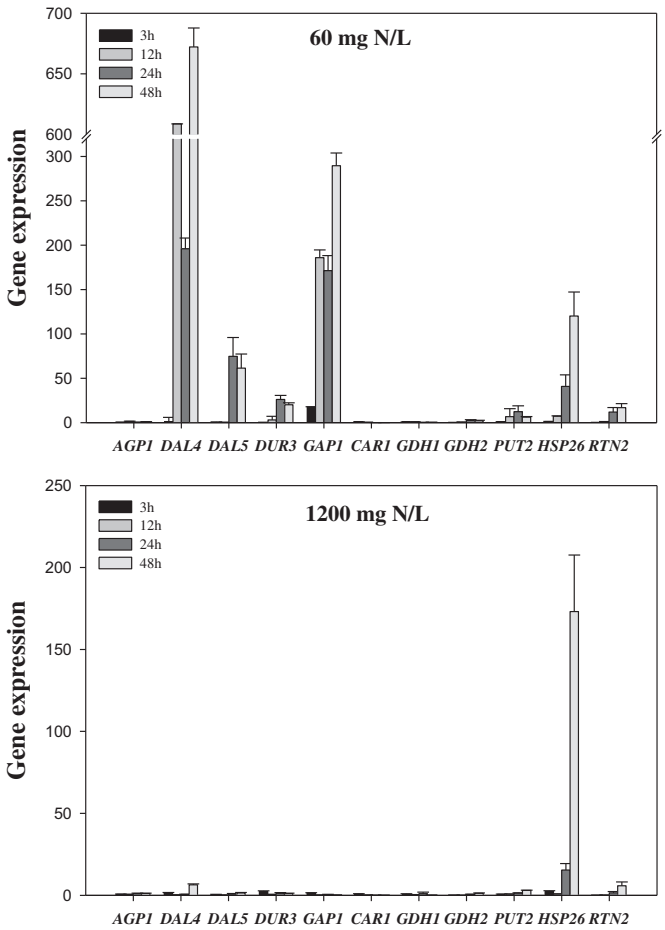

Fig. 3. Changes in gene expression of the PDM strain throughout alcoholic fermentation in two nitrogen concentrations: 60 mg N/L and 1200 mg N/L. The genes considered are: *AGP1* (amino acid permease with high glutamine affinity), *DAL4* (allantoin permease), *DAL5* (allantoate permease), *DUR3* (transporter of urea), *GAP1* (general amino acid permease), *CAR1* (arginase enzyme), *GDH1* ($NADP^+$ dependent glutamate dehydrogenase), *GDH2* (NAD^+ dependent glutamate dehydrogenase), *PUT2* (proline permease), *HSP26* (heat shock protein) and *RTN2* (reticulum protein involved in ER morphology). The values are expressed relative to their expression at the time of lowest expression (1200 mg N/L fermentation at 3 h), and normalized with the concentration of the housekeeping gene (*ACT1*).

(Fig. 4). All the transcriptional changes are expressed relative to the point with lowest expression (3 h after yeast inoculation in the 300 mg N/L condition). The four strains showed a transcriptional profile for both genes correlated to nitrogen concentration. Both genes were activated/de-repressed in limited and control fermentations when ammonium was depleted, even though the YAN concentration in the media was still important. Generally, *GAP1* was first up-regulated in the N-limiting condition, in line with ammonium consumption, whereas scarce or null transcriptional activity was detected in the non-limiting condition. These induction values steeply increased after total nitrogen depletion. It is worth mentioning that, as in the case of the biochemical markers, the ARM strain showed a delay in the transcriptional induction as a consequence of slower nitrogen consumption. Regarding *DAL4*, similar conclusions can be drawn. This gene was also induced earlier in the N-limiting condition and was kept repressed in the non-limiting condition during the time-lapse studied. Again the ARM strain showed delayed transcriptional induction with smaller values than the other strains. Generally speaking, nitrogen consumption correlates well with gene induction.

3.3. Rapid method to detect nitrogen deficiency

Once the transcriptional activity of *GAP1* and *DAL4* was revealed as a good tool to predict nitrogen deficiency, we also aimed to simplify this method to convert it into a rapid, cheap and accurate test. We constructed reporter strains based on the expression of green fluorescent protein (GFP) under control of the promoters for *GAP1* and *DAL4*.

One allele of these genes was replaced by the GFP gene in the four commercial strains. No differences were detected in terms of growth, fermentation activity and nitrogen consumption in these reporter strains versus the commercial strains (data not shown).

The reporter strains were inoculated in SM with the same nitrogen concentrations previously used. Samples from these fermentations were taken in the first hours of fermentation for further fluorescence analysis. In order to validate the correct construction of the reporter strains, we observed the emission under fluorescence microscopy after 48 h of yeast inoculation. As an example, Fig. 5 shows bright-field and fluorescence images of the ARM strain under the different fermentation conditions. As expected, the lower the concentration of nitrogen, the greater the number of fluorescent cells and the stronger the fluorescence intensity. Quantitatively, this result was corroborated by the flow cytometry histograms, also shown in Fig. 5.

However, the fluorescent intensities were different in the two reporter genes studied. The *GAP1* reporter yielded stronger intensity, indicating higher protein production than *DAL4*, which showed duller fluorescence. Therefore, as the transcriptional behavior was very similar between both permeases, only the results obtained with the *GAP1* reporter in the four strains are shown (Figs. 6 and 7). The transcriptional (Fig. 4) and the fluorescence profiles (GFP synthesis) (Fig. 6) in the same fermentation conditions were very similar. Nevertheless, the induction of fluorescence was observed later than the induction of gene activity. This delay represents the time-lapse between transcription and translation. However, this reasoning did not hold for the ARM strain, which showed an earlier induction of fluorescence. Another surprising result between transcription and GFP synthesis was the lack of fluorescence observed in the control condition (140 mg N/L) of the TTA strain, because *GAP1* gene showed early induction.

These results led us to design a new experiment to monitor the induction of fluorescence emission when cells of each strain detected nitrogen deficiency in their environment. We carried out more intensive sampling during the first hours of N-limiting and control fermentations. These data of fluorescence in each individual strain are plotted against the nitrogen consumption (Fig. 7). In comparison with data obtained in the former experiment (Fig. 6), although common trends in fluorescence induction and intensity can be observed, clear differences were detected for the same strain and N condition. The different fermentation conditions and the intensive sampling changed the fermentation kinetics, specially the nitrogen uptake, which may impact on the expression of this reporter gene. In the N-limiting fermentation, strains RVA and TTA emitted fluorescence 10 h after yeast inoculation whereas strains PDM and ARM increased this fluorescence after 14 and 15 h respectively. In terms of intensity, PDM showed a much higher intensity than the other strains. In the control condition, the earliest fluorescence emission was detected in RVA and PDM strains. As in previous results, strain TTA did not emit fluorescence in the first 32 h of fermentation.

4. Discussion

In the present study, the main goal was to identify biomarkers showing robust changes in their activity levels associated with

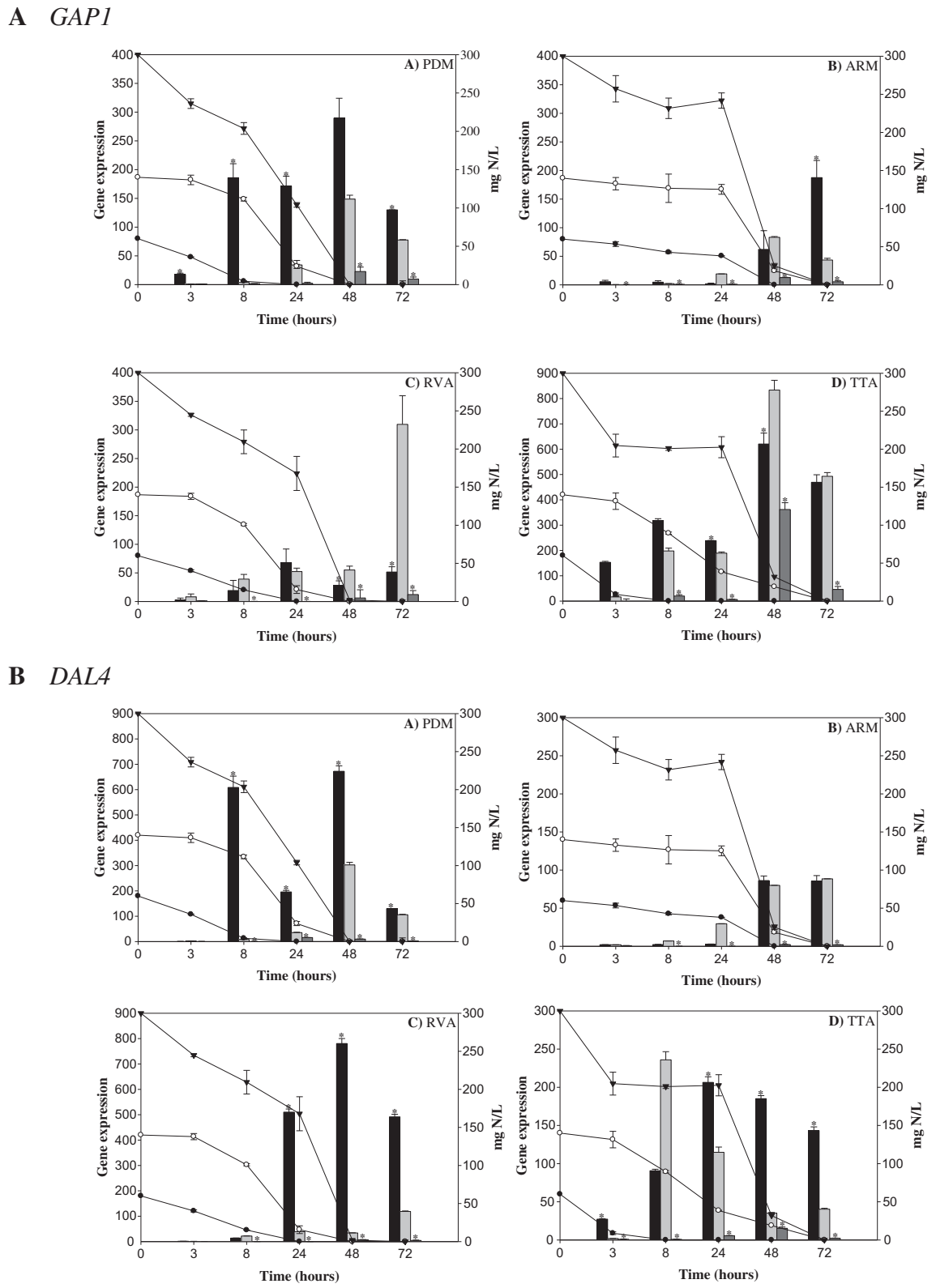

Fig. 4. Changes in gene expression of *GAP1* (general amino acid permease) and *DAL4* (allantoin permease), during fermentations with different nitrogen concentrations: 60 mg N/L (*black bars*), 140 mg N/L (*gray light bars*) and 300 mg N/L (*gray dark bars*). The values are expressed relative to their expression at the time of lowest expression (300 mg N/L fermentation at 3 h), and normalized with the concentration of the housekeeping gene (*ACT1*). YAN consumption throughout the fermentations is also indicated (60 mg N/L *filled circles*; 140 mg N/L *open circles*; 300 mg N/L *filled triangles*). *Significance from *t*-Student with $P \leq 0.01$ compared with the control condition (140 mg N/L).

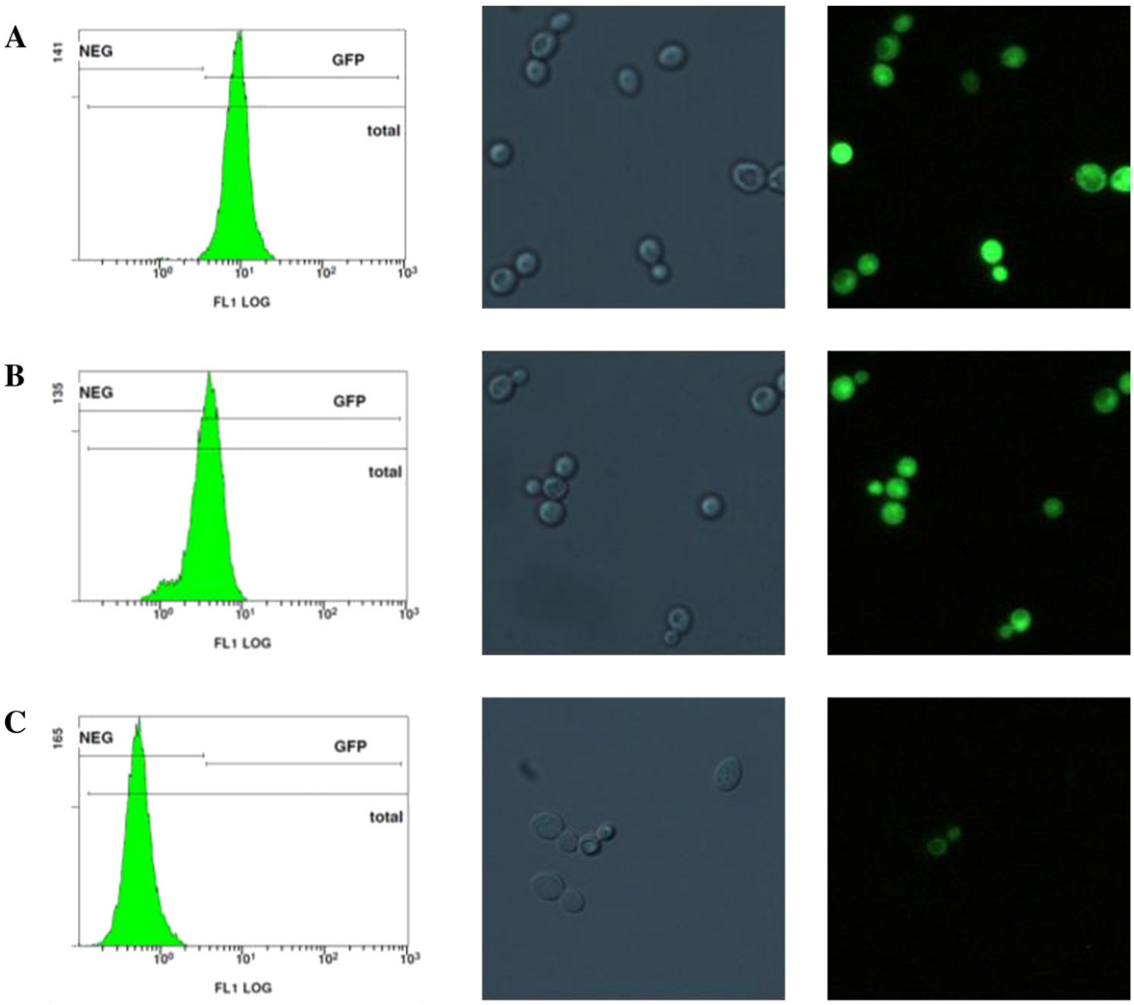

Fig. 5. Fluorescence images of ARM cells, expressing GFP from the *GAP1* promoter throughout the different fermentations: **A)** 60 mg N/L; **B)** 140 mg N/L and **C)** 300 mg N/L. Representative flow cytometry histograms, bright-field and epifluorescence images of the *GAP1*-GFP strain growing in different nitrogen concentrations 48 h post inoculation.

nitrogen starvation, which could be potential candidates as biosensors for predicting sluggish or stuck fermentations. We analyzed the biochemical and molecular responses under several nitrogen concentrations (limited, control and non-limiting condition). There have been previous reports about the use of biomarkers to predict nitrogen deficiency during alcoholic fermentation (Carrasco et al., 2003; Beltran et al., 2004; Jiménez-Martí et al., 2007; Mendes-Ferreira et al., 2007); however, this is the first systematic study comparing several molecular markers and several commercial strains under different nitrogen conditions. The selected molecular marker should be very sensitive to the cellular nitrogen limitation and should be useful for all strains, regardless of their physiological characteristics (nitrogen consumption, growth rate, fermentation capacity, etc.).

Previous studies have suggested that reserve carbohydrate accumulation starts in response to nitrogen limitation (François and Parrou, 2001; Hazelwood et al., 2009; Albers and Larsson, 2009) and, likewise, we also observed trehalose accumulation when most of the nitrogen was consumed and there were still sugars in the media. However, Hazelwood et al. (2009) reported that trehalose levels were specifically higher in nitrogen-limited chemostat cultures, whereas we did not observe this negative correlation between trehalose accumulation and nitrogen concentration of the medium. Conversely, we observed a lower trehalose content in the nitrogen-starved cells, which can be explained by a lower protein level which, in consequence, involves a lower amount of glucose transporters (lower glycolytic flux) and trehalose synthesis enzymes (Varela et al., 2004; Albers et al., 2007). However this lower accumulation of trehalose can also be explained because these cells, growing in high glucose and nitrogen limitation, overproduce ATP (excess energy). This excess ATP can be counteracted by a futile cycle between trehalose synthesis and degradation (Hottiger et al., 1987; Thevelein and Hohmann, 1995). In any case, trehalose accumulation cannot be considered as a useful biomarker for nitrogen limitation because it is not directly linked to the level of nitrogen shortage, and the beginning of its synthesis mainly occurs at the onset of the stationary phase. Novo et al. (2005) previously studied trehalose accumulation in a wine yeast and reached the same conclusion: trehalose accumulation began when cells entered into the stationary phase, regardless of the nitrogen content in the medium.

A more interesting result is the difference in trehalose accumulation among strains. In a recent study, we analyzed the specific nitrogen requirements of these four commercial strains during wine fermentation (Gutiérrez et al., 2012). In this study, strains PDM and RVA showed higher growth rate and maximum population size and were also the greatest nitrogen demanders. Likewise these strains accumulated 2-fold more trehalose than strains ARM and TTA. Therefore trehalose accumulation could be interpreted as an indicator of growth behavior and fermentation

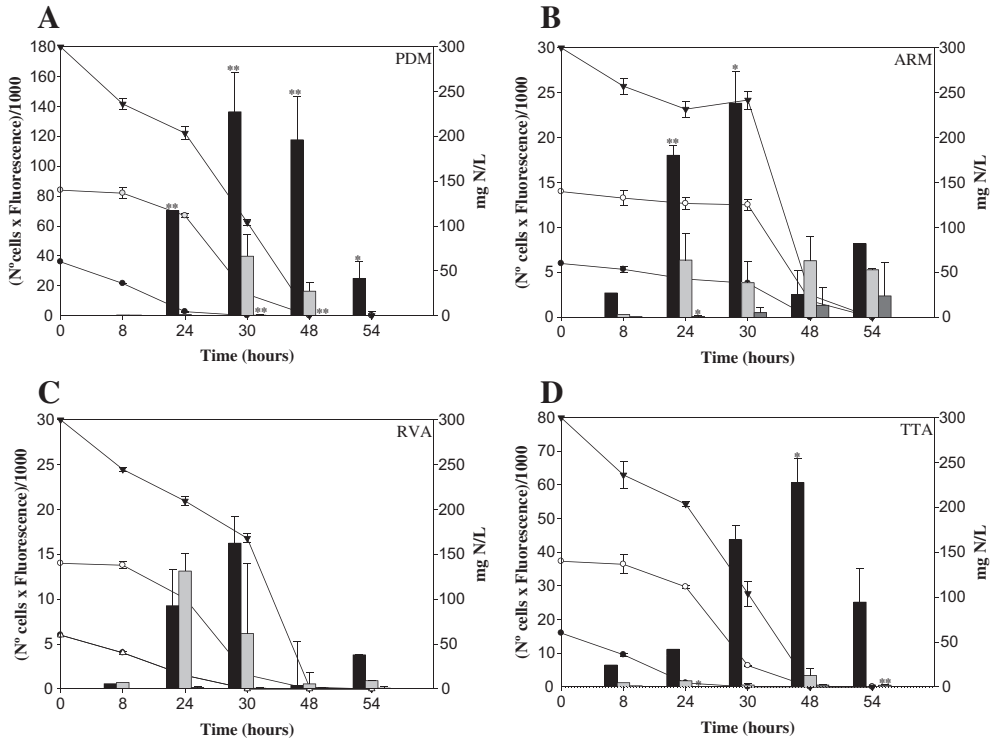

Fig. 6. Changes in fluorescence ((number of cells × fluorescence intensity)/1000) during fermentations with wine strains expressing GFP from *GAP1* promoter **A)** PDM, **B)** ARM, **C)** RVA and **D)** TTA). Fluorescence was measured at different nitrogen concentrations: 60 mg N/L (*black bars*), 140 mg N/L (*gray light bars*) and 300 mg N/L (*gray dark bars*). The consumption of nitrogen was also calculated throughout the process: 60 mg N/L (*filled circles*), 140 mg N/L (*open circles*) and 300 mg N/L (*filled triangles*).

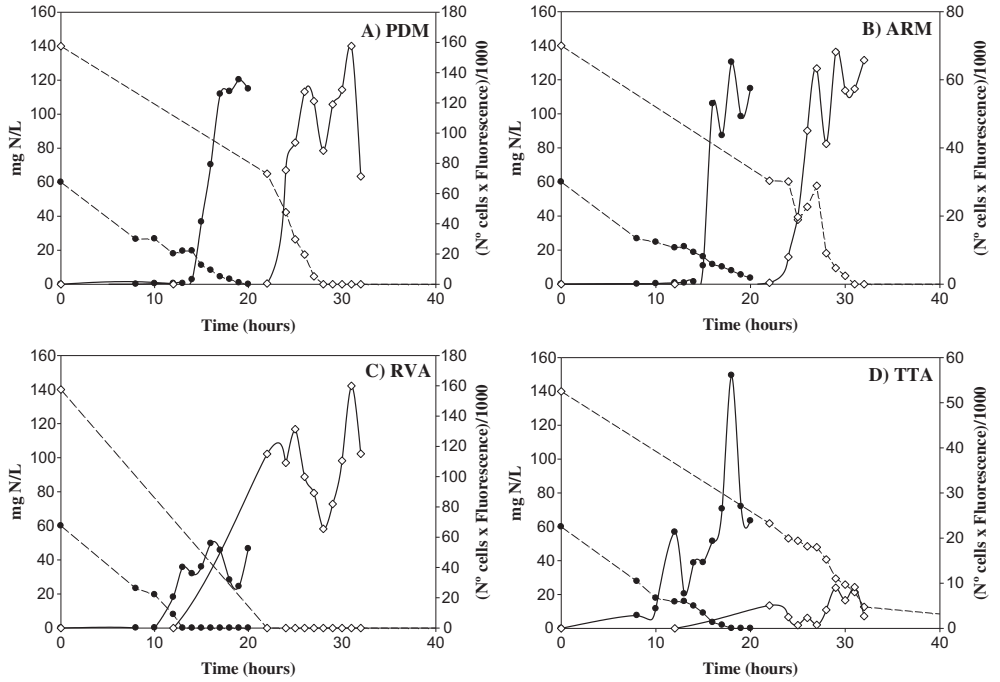

Fig. 7. Nitrogen consumption (dotted line) and changes in fluorescence (solid line) in each individual strain during fermentations with different nitrogen concentrations: 60 mg N/L (*filled circles*) and 140 mg N/L (*open diamonds*).

activity. Varela et al. (2004) previously found a strong correlation between trehalose accumulation and ethanol production.

Conversely, the positive correlation between arginase activity and nitrogen deficiency can be ascribed to most of the strains and, as previously reported (Carrasco et al., 2003; Beltran et al., 2004; Jiménez-Martí et al., 2007), it could be a good marker to detect nitrogen depletion in grape must fermentation. However, arginase activity determination is tedious, time-consuming and difficult to simplify, thus we decided to look for molecular markers related with gene activity in terms of the quantity of the nitrogen of the grape must. With the aim of selecting the most suitable genes for detecting nitrogen deficiency, we analyzed the transcriptional activity of genes, which were previously reported to be regulated by nitrogen concentration of the medium (Beltran et al., 2004; Godard et al., 2007; Jiménez-Martí et al., 2007; Mendes-Ferreira et al., 2007; Deed et al., 2011) in N-limited and N-excess media. In a genome-wide analysis of wine yeast, Mendes-Ferreira et al. (2007) identified 36 genes that are strongly expressed under conditions of low or absent nitrogen, in comparison with a nitrogen-excess condition. *RTN2* was one of the 36 genes that showed a clear response under nitrogen limitation or starvation. However, we did not detect a strong up-regulation of this gene comparing the N-limited with the N-excess condition. Furthermore, in the case of *HSP26*, growth rate decrease (entrance into the stationary phase) seems to be a greater determinant in its regulation than nitrogen concentration. Despite the data obtained for arginase activity, regulation of *CAR1* expression was not dependent on the nitrogen concentration in the medium. This result highlights the importance of post-transcriptional and post-translational mechanisms in the regulation of arginase activity. Under our experimental conditions, permeases *DAL4*, *DAL5*, *DUR3* and *GAP1* showed the strongest up-regulation in the nitrogen-limited condition in comparison with the null or scarce activity in the nitrogen-excess condition. As explained above, the *DAL4* and *GAP1* genes were selected because they showed the strongest activation and, according to Godard et al. (2007), their regulation as NCR genes was different. According to our data, the activation of both genes was dependent on the depletion of nitrogen in the growth medium, which was likewise determined by the initial concentration and uptake rate of the wine yeast strain. Thus, at the same nitrogen concentration, a strain with a slower uptake rate, such as ARM, showed later gene induction than another strain which consumes nitrogen more rapidly. The nitrogen metabolism depends heavily on its uptake through the different nitrogen permeases (Beltran et al., 2005). In our previous work with the same commercial strains (Gutiérrez et al., 2012), we observed a positive correlation among growth and nitrogen uptake rates. The strains with better growth had a higher nitrogen demand. In this study, the biomarkers proposed were able to detect nitrogen limitation earlier in the strains which consumed nitrogen faster.

Regardless of these differences among the studied strains, the transcriptional activity of both genes fulfilled all the requirements for them to be considered good molecular biomarkers to detect nitrogen deficiency. However, transcriptional activity measured by quantitative PCR or Northern blots represents neither a simple nor a rapid method for use as a biomarker. An ideal biomarker should provide a rapid and cheap reading of the environmental conditions.

Green fluorescence protein (GFP) has become an increasingly popular protein tag for determining protein localization and abundance (Sheff and Thorn, 2004). The use of gene promoters to control reporter genes which contain green fluorescent protein is a successful innovation for gene expression studies (Niedenthal et al., 1996). The use of the GFP as reporter of *GAP1* and *DAL4* transcriptional activity has yielded similar results to the data obtained by quantitative PCR. The detection of the fluorescent cells and the intensity of this fluorescence allowed rapid measurement of the nutritional status of the cells regarding nitrogen availability during wine fermentation conditions. The simplicity of the analysis means many time-points can be monitored during fermentation, thereby detecting the moment at which the yeast needs nitrogen supplementation during vinification, which would depend on the wine strain used. In our opinion, the biomarker or biosensor proposed here has great potential use to study the different nitrogen requirements of most commercial strains currently available on the market, thus providing very useful information for winemakers.

5. Conclusions

Knowledge of the nutritional state of cells during alcoholic fermentation is necessary to avoid sluggish and stuck vinifications and low quality of the final product. We show how the use of diverse biochemical or molecular markers related to nitrogen metabolism could provide useful information for the wine industry. Arginase activity and expression of *GAP1* and *DAL4* genes in the cells are good indicators of nitrogen nutritional state. We report the creation and characterization of a yeast-based biosensor using the *GAP1* promoter and a GFP reporter to detect nitrogen deficiency. These markers can be useful to explore the relationship between nitrogen availability and nitrogen requirements of commercial yeast strains in greater depth.

Acknowledgments

This project was supported by AGROVIN Company and by the Centro para el Desarrollo Tecnológico Industrial (CDTI) (Project CENIT DEMETER) and the Spanish Government (project AGL2009-07331, awarded to GB). AG also wants to thank to CSIC for her “JAE-PRE” grant.

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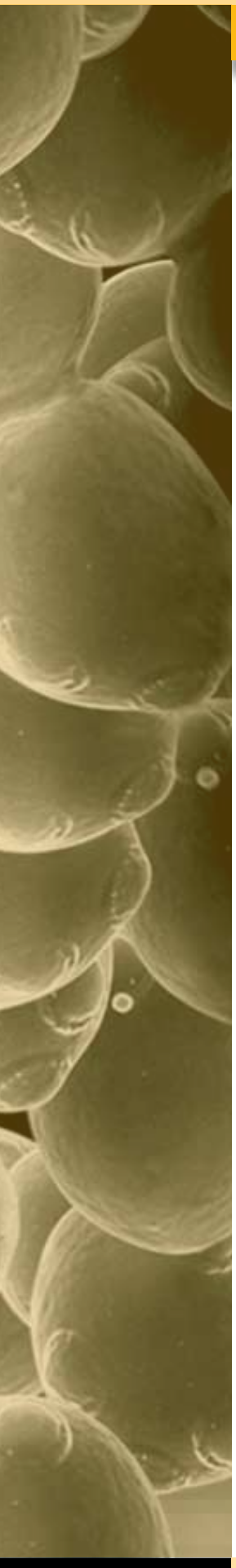
ANNEX IV

Commercial specifications of yeast strains

PDM

Especificaciones técnicas



Descripción
Levadura pura, seca, activa, seleccionada para la elaboración de vino por su seguridad fermentativa.

Tipo
Saccharomyces cerevisiae [var. bayanus].

Origen
Cepa "Prise de Mousse" o cepa Pasteur. Procedente de la región de Champagne, Francia.

Tasa de fermentación
PDM resulta indicada para la fermentación a bajas temperaturas, debido a su vigor inherente. Tiene una corta fase de latencia y una cinética fermentativa constante a bajas temperaturas (12-15°C). Cinética fermentativa rápida y vigorosa a temperaturas superiores a 18°C.

Requerimiento de nitrógeno
PDM tiene unos bajos requerimientos nutricionales. La fermentación a altas temperaturas puede agotar rápidamente el nitrógeno fácilmente asimilable en el mosto.

Tolerancia alcohólica
Maurivin PDM tiene una elevada resistencia al etanol en el rango de 15-17% (v/v).

Acidez volátil
Generalmente menos de 0,3 g/l.

Formación de Espuma
Es una cepa de moderada formación de espuma.

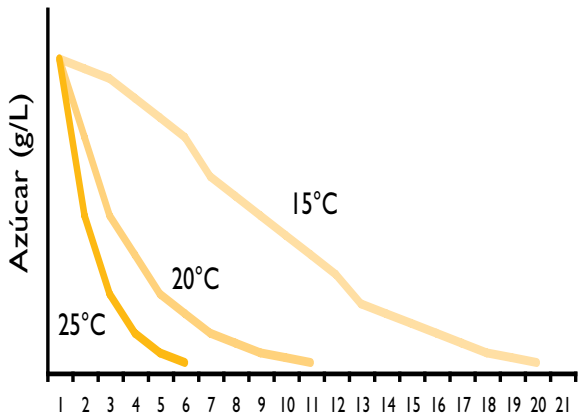
Floculación
PDM tiene propiedades de sedimentación excelentes tras fermentación alcohólica.

Producción total de SO_2
PDM produce SO_2 de forma moderada(hasta 40 mg/L SO_2 total).

Contribución al vino
La cepa PDM respeta la tipicidad varietal de los vinos. Escasa producción de compuestos aromáticos.

Aplicaciones
Es una cepa de levadura recomendada para la elaboración de vinos blancos y tintos, particularmente en casos donde se desee asegurar los finales de fermentación. PDM también resulta excelente para la toma de espuma de vinos espumosos y sidras.

Tasas de fermentación de PDM a diferentes temperaturas de fermentación.



DOSIS

Vinificación	20-30 g/HL
Paradas de fermentacion	30-50 g/HL

MODO DE EMPLEO

Para obtener los mejores resultados es indispensable asegurar la buena implantación de la cepa en el medio, por lo tanto es importante:

- Mantener una buena higiene en la bodega.
- Añadir la levadura lo antes posible.
- Respetar la dosis prescrita.
- Rehidratar bien la levadura.

Rehidratación

①Espolvorear lentamente las levaduras secas activas en 10 veces su peso en agua pone a a 35-40°C (5 litros de agua por 500 g de levadura).

②Dejar reposar las levaduras durante 15 minutos, SIN MEZCLAR. Transcurrido este tiempo, homogeneizar.

③En uva o mostos muy fríos, añadir poco a poco fracciones de mosto hasta unos 10 litros aproximadamente (exento de SO_2) de tal forma que la temperatura descienda de 5°C en 5°C.

④Transcurridos 10 minutos, inocular el depósito a fermentar, respetando una diferencia de 5°C entre la solución de rehidratación y el mosto a inocular.

Precauciones de trabajo

➢En cualquier caso, la levadura no deberá estar rehidratándose más de 30 minutos en ausencia de azúcares.
➢El respeto del tiempo, temperatura y modo de empleo descrito garantizan la máxima viabilidad de la levadura hidratada.
➢Se desaconseja el pie de cuba por favorecer las contaminaciones por microorganismos oxidativos indeseables.

ASPECTO FISICO

Gránulos de color tostado, desprovistos de polvo.

PRESENTACIÓN

Paquetes de 500g envasados al vacío en envuelta multilaminar de aluminio en cajas de 10 Kg.

PROPIEDADES FÍSICO-QUÍMICAS Y MICROBIOLÓGICAS

Sólidos	> 92,5 (%)
Actividad Fermentativa (clinitest)	< 2.0 hrs
Cultivo Puro Seleccionado	> 2 x 10^{10} UFC/g
Bacterias Putrefactivas	< 5 x 10^{4} UFC/g
Bacterias Lácticas	< 8 x 10^{3} UFC/g
No Saccharomyces ssp	< 5 x 10^{2} UFC/g
Coliformes	< 10 UFC/g

CONSERVACIÓN

El producto conforme a los estándares cualitativos se conserva en su envase sellado al vacío durante un periodo de tres años en cámara refrigerada entre 4 y 10°C.

Eventuales exposiciones prolongadas a temperaturas superiores a 35°C y/o con humedad reducen su eficacia.

REGISTRO

R.G.S.A: 31.00391/CR

Producto conforme el Codex Enológico Internacional y el Reglamento CE 606/2009.

AGROVIN

EP2

Especificaciones técnicas

Descripción
Levadura pura, seca, activa, seleccionada para la elaboración de vino por su capacidad de incrementar la intensidad aromática.

Tipo
Saccharomyces cerevisiae

Origen
Colección del Australian Wine Research Institute. Procedente de la región Épernay, Francia.

Tasa de fermentación
La fermentación tiene un rango óptimo de temperatura de 18-25°C. EP2 tiene una fase de latencia media y una cinética de fermentación moderada. EP2 es sensible a temperaturas más bajas, siendo recomendable fermentar a partir de 17°C siempre que se quiera agotar el consumo de azúcares en el mosto.

Requerimiento de nitrógeno
EP2 tiene unas necesidades nutricionales altas. Cuando se fermentan mostos muy clarificados o a temperatura inferior a 20°C, se recomienda cuidar la nutrición mediante la utilización de nutrientes de fermentación.

Tolerancia alcohólica
EP2 tiene tolerancia alcohólica de hasta 13% (v/v).

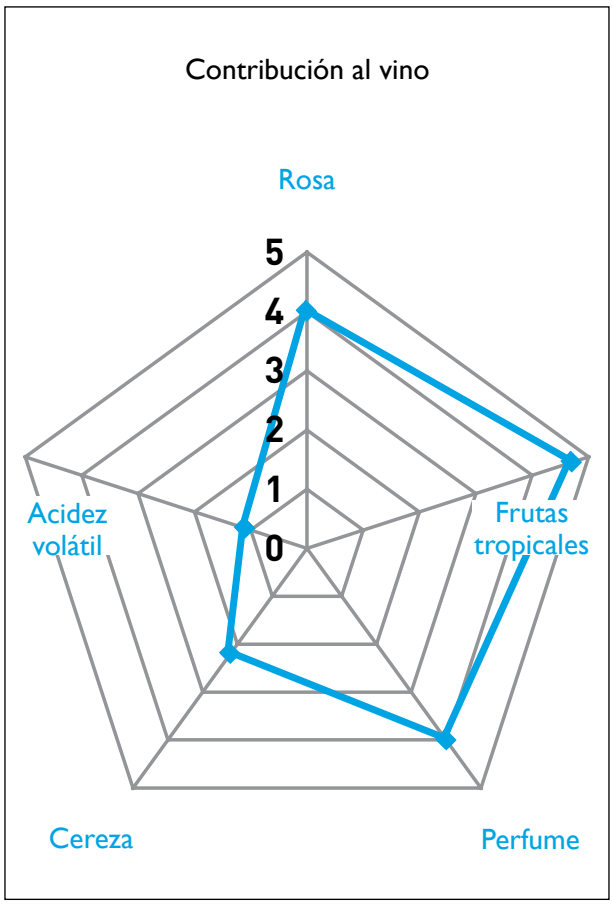
Acidez volátil
Generalmente menos de 0,3 g/l.

Floculación
EP2 tiene buenas propiedades de sedimentación tras la fermentación alcohólica.

Formación de Espuma
EP2 es una cepa de baja a moderada formación de espuma.

Contribución al vino
EP2 es una levadura productora de aromas floral (rosa) y frutales (pera) muy intensos y perfumados. Estos aromas son de naturaleza sutil, lo que puede combinarse con la expresión de la variedad de uva.

Aplicaciones
En vinos blancos de variedades neutras como Airén y Macabeo incrementa la intensidad aromática de tipo manzana y pera. La cepa resulta idónea para la elaboración de vinos dulces: Su sensibilidad a las bajas temperaturas y sulfuroso, permitiendo la detención de la fermentación en el momento deseado.



DOSIS

Vinificación	20-30 g/HL

MODO DE EMPLEO

Para obtener los mejores resultados es indispensable asegurar la buena implantación de la cepa en el medio, por lo tanto es importante:

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- Añadir la levadura lo antes posible.
- Respetar la dosis prescrita.
- Rehidratar bien la levadura.

Rehidratación

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②Dejar reposar las levaduras durante 15 minutos, SIN MEZCLAR. Transcurrido este tiempo, homogeneizar.

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④Transcurridos 10 minutos, inocular el depósito a fermentar, respetando una diferencia de 5°C entre la solución de rehidratación y el mosto a inocular.

Precauciones de trabajo

➢En cualquier caso, la levadura no deberá estar rehidratándose más de 30 minutos en ausencia de azúcares.
➢El respeto del tiempo, temperatura y modo de empleo descrito garantizan la máxima viabilidad de la levadura hidratada.
➢Se desaconseja el pie de cuba por favorecer las contaminaciones por microorganismos oxidativos indeseables.

ASPECTO FISICO

Gránulos de color tostado, desprovistos de polvo.

PRESENTACIÓN

Paquetes de 500g envasados al vacío en envuelta multilaminar de aluminio en cajas de 10 Kg.

PROPIEDADES FÍSICO-QUÍMICAS Y MICROBIOLÓGICAS

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Coliformes	< 10 UFC/g

CONSERVACIÓN

El producto conforme a los estándares cualitativos se conserva en su envase sellado al vacío durante un periodo de tres años en cámara refrigerada entre 4 y 10°C.

Eventuales exposiciones prolongadas a temperaturas superiores a 35°C y/o con humedad reducen su eficacia.

REGISTRO

R.G.S.A: 31.00391/CR

Producto conforme el Codex Enológico Internacional y el Reglamento CE 606/2009.

AGROVIN

RVA viniferm

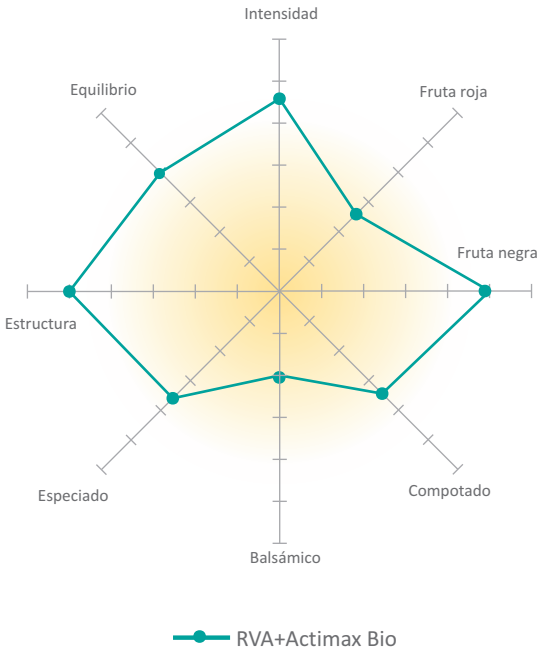
Cepa seleccionada para la elaboración de vinos tintos de maceraciones largas

Características

Viniferm RVA es una levadura especialmente recomendada para vinos tintos de elevada tolerancia al etanol, capaz de respetar los aromas propios de la variedad. Permite las maceraciones prolongadas y realizar maceración prefermentativa en frío.

Propiedades enológicas

- Inicio de la fermentación rápido con extracción temprana de polifenoles.
- Curso medio y final de la fermentación moderado, permite prolongar la maceración del líquido con la pasta.
- Carácter killer: (K2) favorece su predominancia en el mosto.
- Tolerancia al alcohol hasta 16% vol.
- Buen rendimiento azúcar/etanol.
- Exigencia nutricional: media. Es aconsejable la corrección de nitrógeno asimilable, especialmente en mostos de elevado grado alcohólico potencial y procedentes de uva sobremadura.
- Temperas óptimas de fermentación: 20-30ºC.
- Producción importante de glicerol.
- Poca afinidad de la pared celular por la materia colorante, lías menos coloreadas y respeto de la concentración polifenólica.



*Perfil aromático de **Viniferm RVA** (Variedad Tinto Fino DO Ribera de Duero, 14 % vol con adición de nutriente orgánico **Actimax Bio**.*

Cualidades organolépticas

Respeto de las características aromáticas varietales. **Incrementa la complejidad aromática de carácter especiado y balsámico**, interesante para la crianza en madera.

Al ser una cepa muy glicérica, **aporta cuerpo y sensación de volumen en boca**.

Indicada para todo tipo de **variedades tintas destinadas a envejecer en madera**: Tinto fino (tempranillo), Merlot, Cabernet-Sauvignon, Shiraz, Monastrell, Tinta Roriz.

Tinto	Crianza	factor competitivo	temperatura de trabajo	rendimiento alcohólico	tolerancia al etanol	necesidades nutricionales	efecto sensorial
+++	+++	killer	20-30 ºC	bajo	16	elevado	cuerpo y estructura

Aplicación

- Indicada para la elaboración de vinos tintos con maceraciones largas: su cinética de fermentación pausada permite prolongar el tiempo de contacto con los hollejos. Permite proteger e incrementar la intensidad colorante en vinificación en tinto.

Origen

Saccharomyces cerevisiae var. cerevisiae. Colección Agrovin. Cepa procedente de viñedos de Ribera del Duero (España).

Dosis

Vinificación **20-30 g/hl**

Modo de empleo

Para obtener los mejores resultados es indispensable asegurar la buena implantación de la cepa en el medio, por lo tanto es importante:

- Mantener una buena higiene en la bodega.
- Añadir la levadura lo antes posible.
- Respetar la dosis prescrita.
- Rehidratar bien la levadura.

Rehidratación:

1.- Añadir las levaduras secas en 10 veces su peso en agua a 35 - 40ºC (10 litros de agua por 1 Kg de levadura).

2.- Esperar 10 minutos.

3.- Agitar la mezcla.

4.- Esperar 10 minutos e incorporar al mosto, procurando que no haya una diferencia de más de 10ºC entre el medio rehidratado y el mosto.

Precauciones de trabajo:

- En cualquier caso, la levadura no deberá estar rehidratandose más de 30 minutos en ausencia de azúcares.
- El respeto del tiempo, temperatura y modo de empleo descrito garantizan la máxima viabilidad de la levadura hidratada.
- Se desaconseja el pié de cuba por favorecer las contaminaciones por microorganismos oxidativos indeseables.

Aspecto físico

Gránulos de color tostado, desprovistos de polvo.

Presentación

Paquetes de 500g envasados al vacío en envuelta multilaminar de aluminio, en cajas de 10 kg.

Propiedades fisico-químicas y microbiológicas

Sólidos [%]	92 - 95
Bacterias totales [UFC/g]	$< 10^6$
Levaduras contaminantes [UFC/g]	$< 10^5$
Células vivas [UFC/g]	$> 2 \cdot 10^{10}$
Actividad fermentativa [h]	< 2:00

Conservación

El producto conforme a los estándares cualitativos se conserva en su envase sellado al vacío durante un periodo de tres años en cámara refrigerada entre 4 y 10ºC.

Eventuales exposiciones prolongadas a temperaturas superiores a 35ºC y/o con humedad reducen su eficacia.

REGISTRO: R.G.S.A: 31.00391/CR

Producto conforme con el Codex Enológico Internacional y el Reglamento CE 606/2009.

Av. de los Vinos, s/n - P.I.Alces - 13600 Alcázar de San Juan (Ciudad Real) - Tel. **+34 926 55 02 00** · Fax **+34 926 54 62 54** **www.agrovin.com**

TTA viniferm

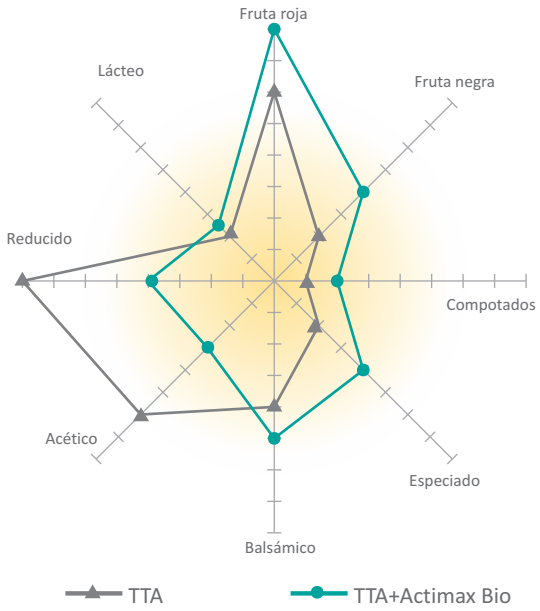
Tintos jóvenes aromáticos y rosados de carácter frutal

Características

Viniferm TTA es una levadura capaz de formar ésteres en fermentación ideal para fermentar vinos tintos jóvenes, maceraciones carbónicas y rosados. Elevada producción de aromas fermentativos.

Propiedades enológicas

- Fermentación regular y completa.
- Buen rendimiento alcohólico.
- Producción elevada de glicerina, que contribuye a la fijación de compuestos aromáticos.
- Muy baja formación de acidez volátil.
- Exigencias nutricionales: medias. En mostos deficitarios en nitrógeno o de elevada graduación alcohólica (superior a 12,5% vol) se aconseja la adición de nutrientes.
- Temperatura de trabajo: 18-28ºC.
- Escasa formación de espuma, debido a su fuerte actividad proteolítica.



*Perfil aromático de **Viniferm TTA** (Variedad Tempranillo, 12,5 % vol) tras fermentar un mosto con 190 mg/l de NFA (TTA) y con adición de 30 g/hl de nutriente orgánico **Actimax Bio** (TTA+Actimax Bio).*

Cualidades organolépticas

Cepa idónea para resaltar el **carácter frutal** de las elaboraciones de uva tinta. Permite la expresión característica de los aromas varietales (frutos rojos: cereza, grosella, fresa) la vez que aporta mayor espectro aromático y sensaciones de untuosidad en boca.

Aplicación

- Elaboración de vinos **tintos jóvenes de alta calidad**. Se obtienen aromas frescos, limpios y con notas aromáticas intensas.
- Vinos tintos de **maceración carbónica**.
- Proporciona complejidad y estructura en la elaboración de **vinos rosados de corte afrutado**.

Rosado	Tinto	factor competitivo	temperatura de trabajo	rendimiento alcohólico	tolerancia al etanol	necesidades nutricionales	efecto sensorial
+++	+++	neutro	18-28 ºC	medio	14	elevada	produce ésteres

Origen

Saccharomyces cerevisiae var. cerevisiae. Colección Agrovin.

Dosis

Vinificación **20-30 g/hl**

Modo de empleo

Para obtener los mejores resultados es indispensable asegurar la buena implantación de la cepa en el medio, por lo tanto es importante:

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Células vivas [UFC/g]	$> 2 \cdot 10^{10}$
Actividad fermentativa [h]	< 1:45

Conservación

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Eventuales exposiciones prolongadas a temperaturas superiores a 35ºC y/o con humedad reducen su eficacia.

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