



FILAMENTOUS GROWTH IN WINE YEAST: SIGNAL TRIGGERING AND GENETIC FACTORS INVOLVED

Beatriz González Sánchez

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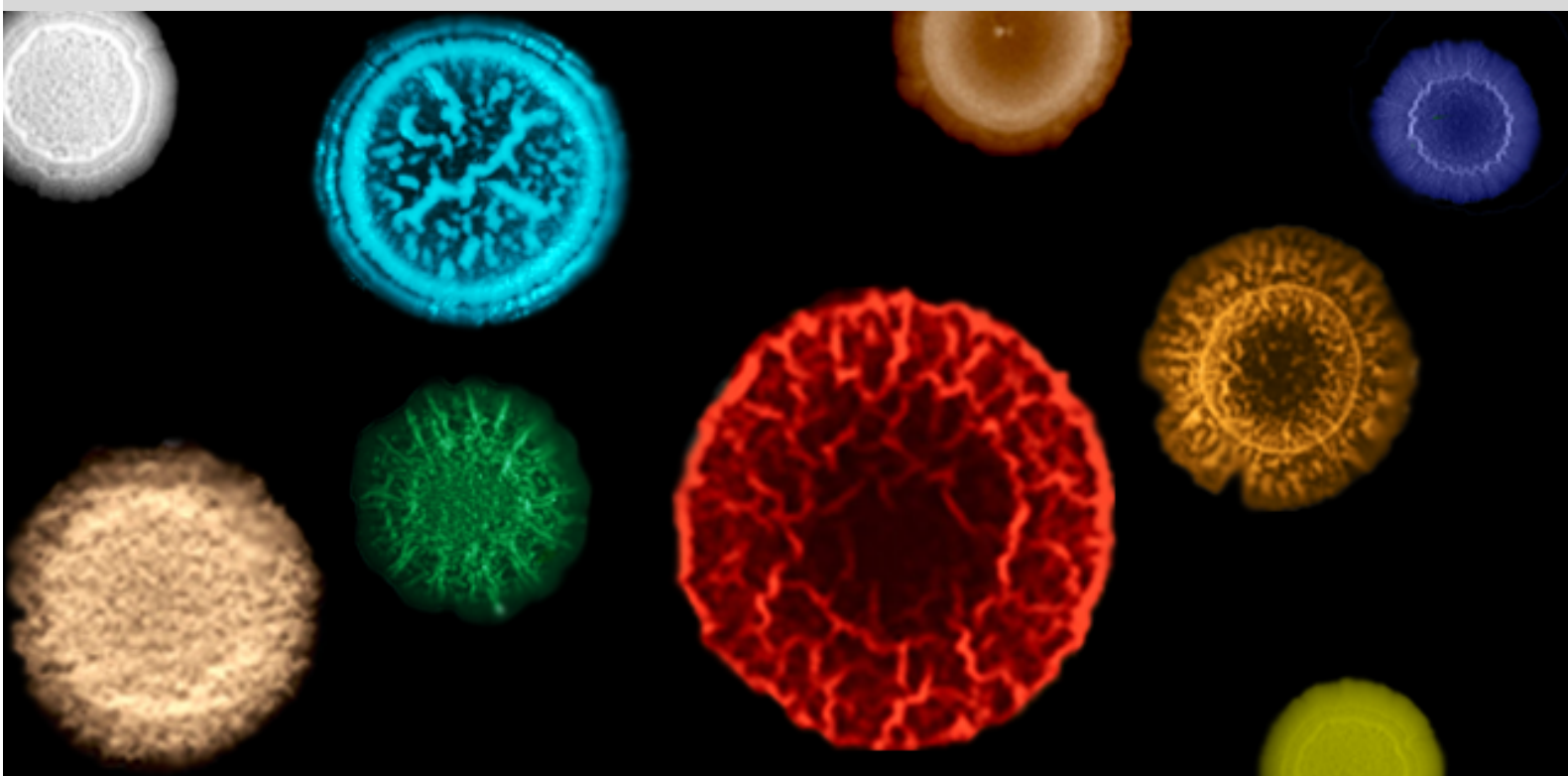
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Filamentous growth in wine yeast: signal triggering and genetic factors involved

BEATRIZ GONZÁLEZ SÁNCHEZ



DOCTORAL THESIS
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Filamentous growth in wine yeast: signal triggering and genetic factors involved



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WE STATE that the present study, entitled “**Filamentous growth in wine yeast: signal triggering and genetic factors involved**”, presented by **Beatriz González Sánchez** for the award of the degree of Doctor, has been carried out under our supervision at the Department Biochemistry and Biotechnology of this university.

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Tarragona, September 2017

A mis padres

A mi hermano

A Javi

The test of all knowledge is experiment.

*Experiment is the sole judge of scientific truth. **Richard P. Feynman***

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Objectives and outline of thesis

The present work in fulfilment of my PhD degree was carried out from 2013 to 2017 at the University Rovira i Virgili. This research was developed in the Oenological Biotechnology research group, Department of Biochemistry and Biotechnology. During these years, I held a pre-doctoral fellowship from the University Rovira i Virgili.

The work that I carried out was part of the project “Production of bioactive compounds derived from aromatic amino acids during alcoholic fermentation”, which was supported by the Ministry of Economy and Competitiveness of Spain (grant no. AGL2013-47300-C3). The main objective of the project was to understand the metabolism of aromatic amino acids to control the concentrations of bioactive compounds in fermented beverages. During alcoholic fermentation, yeasts are able to synthesize certain compounds derived from aromatic amino acids (tryptophan, phenylalanine and tyrosine), such as tryptophol, phenylethanol and tyrosol, which are associated with quorum sensing activities in yeast. Quorum sensing appears to control cell density and morphogenesis in yeast.

With this framework, the hypothesis was as follows: **Aromatic amino acid metabolism generates compounds with quorum sensing activity that modulate growth and morphological changes in wine yeast species.**

To demonstrate this hypothesis, the general objective was to study the production of compounds derived from aromatic amino acids during alcoholic fermentation as well as their effects on the physiological responses of different yeast species. This general objective was divided in the following specific objectives:

1. Objectives and outline of thesis

OBJECTIVE 1: To assess the production of aromatic alcohols during alcoholic fermentation under various nutrient conditions by different wine yeast species (Chapter 1).

Yeasts are able to synthesize aromatic alcohols derived from aromatic amino acids during alcoholic fermentation via the Ehrlich pathway. However, production of aromatic alcohols, which are associated with quorum sensing activities in yeast, has been poorly explored, and the regulation of this pathway is not completely understood.

We studied the production of tyrosol, 2-phenylethanol and tryptophol by five different species of oenological interest: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*. Fermentation was performed in synthetic media, with variations in carbon and nitrogen contents. First, production of these compounds was evaluated in standard synthetic must (SM-300 mg of N/L and 200 g/L sugars). To understand the synthesis patterns of the molecules of interest, two other synthetic musts with nitrogen (LNM-100 mg of N/L and 200 g/L sugars) and carbon (LGM-300 mg of N/L and 20 g/L sugars) limitation were used. In addition, under all conditions, two initial concentrations of aromatic amino acids were used: standard (1x) and five-fold higher (5x). To relate the synthesis of aromatic alcohols to growth phases, the intracellular and extracellular concentrations of these molecules were determined for the first 48 h in SM with precursors at a 5x concentration.

This objective is described in Chapter 1: **The production of aromatic alcohols in wine yeast is modulated by nutrient availability**. The results were submitted to the Journal of Agricultural and Food Chemistry.

OBJECTIVE 2: To study the effects of compounds derived from aromatic amino acids on the physiological responses of different wine yeast species in terms of growth and morphological changes (Chapters 2 and 3).

Aromatic alcohols and ethanol modulate quorum sensing activities. Quorum sensing is related to cell density and filamentous growth in yeast. Additionally, other molecules derived from tryptophan metabolism, such as melatonin, serotonin and tryptamine, are produced by yeast from tryptophan, but their roles remain unknown.

A collection of wine yeast comprising different species, including *S. cerevisiae*, *H. uvarum*, *St. bacillaris*, *M. pulcherrima* and *T. delbrueckii*, was used in this study. Two different experiments were carried out. First, yeasts were grown in synthetic medium supplemented with different concentrations of serotonin, melatonin, tryptamine, tyrosol, phenylethanol and tryptophol to evaluate their effects on yeast growth. Increasing concentrations of these compounds (from 50 ppm to 1000 ppm) were tested.

Second, the effects of aromatic alcohols and ethanol on the filamentous growth of non-*Saccharomyces* wine yeasts were evaluated. Three media with differing nutrient contents were used: SD-synthetic medium (rich media), SALG-synthetic medium with low glucose (glucose limitation) and SLAD-synthetic low ammonium dextrose medium (nitrogen limitation). Filamentous growth (invasive and pseudohyphal growth) was evaluated in the three media with and without tyrosol, phenylethanol, tryptophol and ethanol supplementation.

Chapter 2: **Aromatic amino acid-derived compounds induce morphological changes and inhibit the cell growth of wine yeast species.** The results were submitted to *Frontiers in Microbiology*.

Chapter 3: **Role of mitochondrial retrograde pathway in regulating ethanol-inducible filamentous growth in yeast.** *Frontiers in Physiology* (2017) 8, 148.

1. Objectives and outline of thesis

OBJECTIVE 3: To decipher the regulation and genetic basis of the filamentous growth of *S. cerevisiae*.

Previous data show that ethanol strongly induces filamentous growth to a greater degree than aromatic alcohols. How ethanol is sensed and how it triggers filamentous growth have been poorly studied. Signaling pathways known to regulate filamentous growth were tested to determine if they played a role in regulating ethanol-induced filamentous growth. A broader collection of mutants of the lab strain $\Sigma 1278$ comprising genes implicated in these pathways was employed. All mutants were spread on SLAD as a control and SLAD media supplemented with ethanol, and the filamentation properties were evaluated.

After evaluating the results obtained for Objective 2, we selected two wine strains of *S. cerevisiae* that exhibited clear differences in filamentous growth under low nitrogen conditions (SLAD media). To identify the genetic variations causing these differences in filamentation, we mapped QTLs (quantitative trait loci) in segregants from the two selected strains. Segregants belonging to the F13 population were pooled according to their phenotypic behaviour, and genomic DNA was extracted and subsequently sequenced by Illumina MiSeq.

Chapter 3: **Role of mitochondrial retrograde pathway in regulating ethanol-inducible filamentous growth in yeast.** *Frontiers in Physiology* (2017) 8, 148.

Chapter 4: **Genetic factors involved in the filamentous growth of wine yeast identified using QTL analysis.** Manuscript in preparation

Introduction

2.1 Yeast

Yeasts are eukaryotic and unicellular organisms that belong to the fungal kingdom. At the biochemical level, yeast cells are very similar to other eukaryotic cells. Due to their ease of cultivation and manipulation, yeasts, particularly *Saccharomyces cerevisiae*, have been used as model microorganisms to study mechanisms in eukaryotic cells. Since 1876, when Pasteur first identified yeast as the microorganism responsible for alcoholic fermentation, numerous studies utilizing these microorganisms have been performed, particularly given the importance of *S. cerevisiae* in multiple human activities, such as baking, brewing and winemaking.

2.1.1 Yeast physiology

Yeast is the simplest of eukaryotes. The cells of *S. cerevisiae* and other ascomycetes are spherical, oval or cylindrical, and cell division typically takes place by budding. Yeast cells contain a cell wall that envelops a plasma membrane that is separated by the periplasmic space. The cell wall represents 15-25 % of the dry weight of the cell (Fleet, 1991; Klis, 1994; Stratford, 1994) and is largely composed of polysaccharides, β -glucans and mannoproteins. The main function of the cell wall is to protect the cell. Additionally, the cell wall is the site of several cellular interactions. Furthermore, a large number of enzymes, generally hydrolases, are connected to the cell wall or situated in the periplasmic space. The shape of the cell wall constantly changes during cellular morphogenesis. The plasma membrane primarily consists of lipids (phospholipids and sterols) and glycoproteins; contains important proteins, such as adenosine triphosphatase (ATPase), sugar and amino acid transporters (e.g., GAPs), and enzymes; and

2. Introduction

is also involved in stress responses and sporulation.

Inside the cell, different organelles, such as the endoplasmic reticulum, Golgi apparatus, vacuole, mitochondria, nucleus and ribosomes, are located in the cytoplasm (Figure 1). The primary function of the endoplasmic reticulum is the biosynthesis of many membrane components, including membrane proteins and lipids, and this organelle is also involved in the transport and folding of proteins. The Golgi apparatus is responsible for processing and packaging macromolecules, such as proteins and lipids, to be reused within the cell or to be secreted. The vacuole is the largest organelle in yeast cells and is functionally equivalent to the mammalian lysosome. Its principal function is the storage of organic molecules, detoxification and proton and ion homeostasis. Ribosomes are highly conserved ribonucleoproteins that translate information in the genome to create the proteome in all cells.

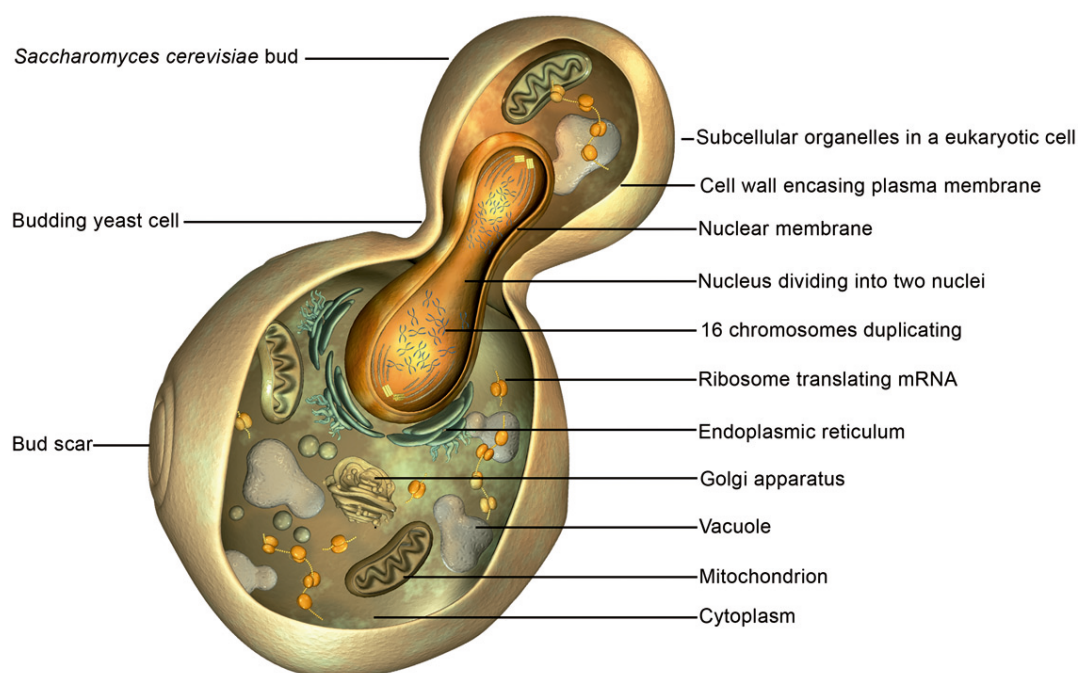


Figure 1: Architecture of a typical *Saccharomyces cerevisiae* cell (Pretorius, 2016).

Mitochondria are situated in the periphery of the cytoplasm. During aerobic processes, *S. cerevisiae* contains approximately fifty mitochondria; however, these organelles are degenerated during anaerobic metabolism. Mitochondria are essential organelles whose main function is the synthesis of ATP by oxidative phosphorylation. These organelles are also the site of many important metabolic and biosynthetic reactions, such as the tricarboxylic acid cycle (TCA) and amino acid and heme biosynthesis. The mitochondrial genome of *S. cerevisiae* comprises 85,779 bp but contains only 8 protein-encoding genes. Other than the genes that encode RNA and proteins, the genome of yeast mitochondria contains large

stretches of adenine/thymine (AT)-rich DNA that have no apparent function.

S. cerevisiae, like other eukaryotes, stores its genetic information on chromosomes that are sequestered from other cellular organelles by a double membrane, which forms the nucleus. The nuclear envelope consists of a double membrane attached to the endoplasmic reticulum. Elaborate molecular machinery forms large pores that traverse the double membrane and regulate the traffic of macromolecules. The nucleus also guarantees the proper expression, duplication, repair and segregation of DNA while ensuring proper processing and export of messenger and ribosomal RNA (Ribéreau-Gayon et al., 2006). *S. cerevisiae* has 16 chromosomes, and its genome is 12.1 Mbp in size, encoding approximately 6000 proteins, of which only 900 to 1200 are deemed essential for cell survival (Zhang and Ren, 2015). Relatively few of these protein-encoding genes contain introns.

2.1.2 Yeast growth

Yeast is non-motile, non-photosynthetic and, like all fungi, may reproduce sexually and/or asexually. Perfect fungi undergo both reproductive cycles, while imperfect fungi only reproduce asexually (by mitosis). However, the most common cell division in yeasts occurs by budding, as is the case for *S. cerevisiae*. This vegetative cell cycle involves a succession of events whereby a cell grows and divides into two daughter cells (the cell cycle explained in detail in Section 2.1.2.1). Conversely, some yeasts, such as *Schizosaccharomyces pombe*, reproduce by fission instead of budding.

Sexual reproduction in yeast takes place via conjugation of two cells of different mating types and subsequent sporulation by meiosis, but it is very rare in some yeast species. The life cycle of *S. cerevisiae* is shown in Figure 2. *S. cerevisiae* presents two mating types: a haploid a cell mates with a haploid α cell to form a diploid cell that undergoes vegetative multiplication by budding. However, under unfavourable conditions, *S. cerevisiae* diploid cells induce sporulation, producing four haploid spores by meiosis. *S. cerevisiae* exists both as a haploid and a diploid cell, and in presence of adequate nutrients, both haploids and diploids undergo repeated rounds of vegetative growth and mitosis.

2. Introduction

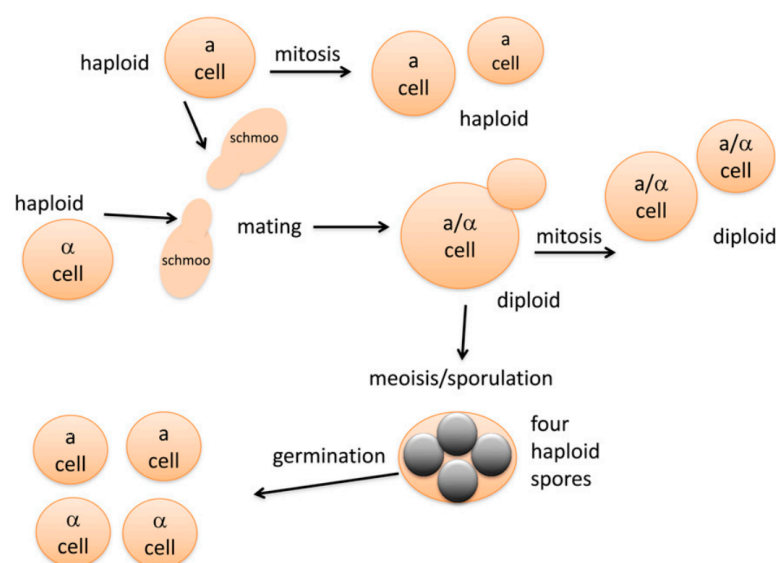


Figure 2: *Saccharomyces cerevisiae* life cycle (Duina et al., 2014).

2.1.2.1 Cell cycle

First, a yeast cell undergoes an expansion period, increasing its volume; then, bud emergence occurs. During bud formation, the total volume of the mother plus daughter bud cell is constant such that bud growth occurs as a consequence of cell mother depletion. Once separated, the new daughter cell and original mother cell grow and reach the same size at the same time; thus, the daughter grows faster than the mother (Posten and Cooney, 1993). Across two divisions, the genetic material must be replicated and segregated into the two daughter cells. DNA replication and sister chromatid separation occur in temporally distinct phases of the eukaryotic cell cycle, which takes place every 90 minutes under optimal conditions (Figure 3). The different phases are: S-phase (DNA synthesis) and M-phase (mitosis, in which first the nucleus and then the cytoplasm divide). These two phases are separated by two gaps: G1, in which cells grow and prepare the machinery for DNA replication, and G2, in which cells also grow and prepare for mitosis, beginning the reorganization of microtubules to form a spindle.

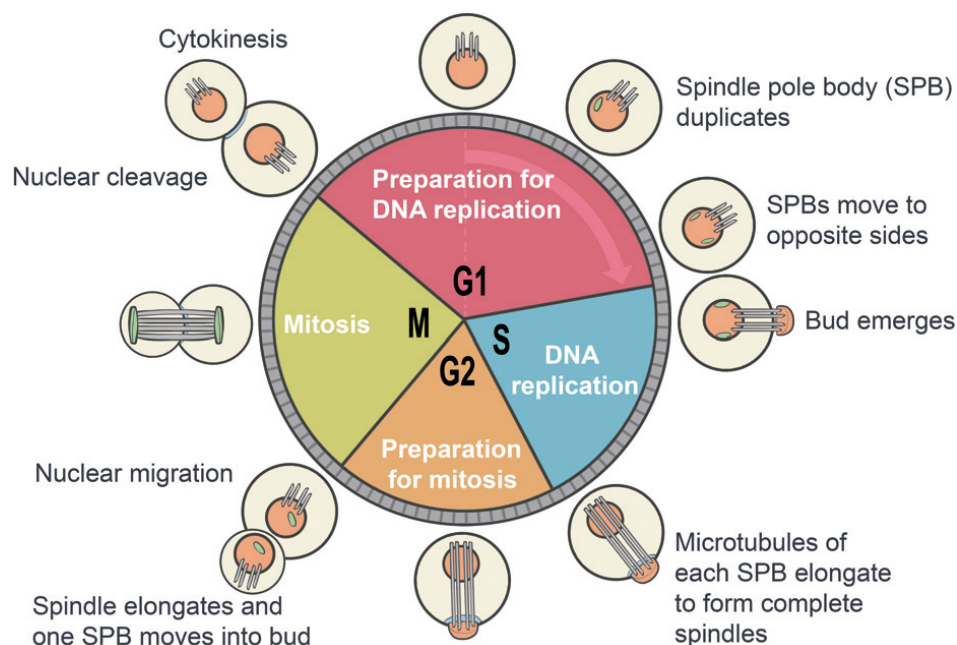


Figure 3: Cell cycle of the budding yeast *Saccharomyces cerevisiae* (Pretorius, 2016).

In *S. cerevisiae* vegetative cells, the budding pattern is controlled by cell type. Haploid cells (a and α cells) bud in an axial pattern, in which both mother and daughter cells bud adjacent to the preceding division site. Conversely, diploid cells (a/α) normally bud in a bipolar pattern, in which the daughter cell generally buds at the pole distal to the division site, and the mother cell buds near either pole (Figure 4) (Freifelder, 1960; Madden and Snyder, 1998; Sloat et al., 1981; Streiblová, 1970). Cells regulate their budding patterns using a GTPase; when this GTPase is mutated, cells bud in a random pattern (Bender and Pringle, 1989; Chant et al., 1991).

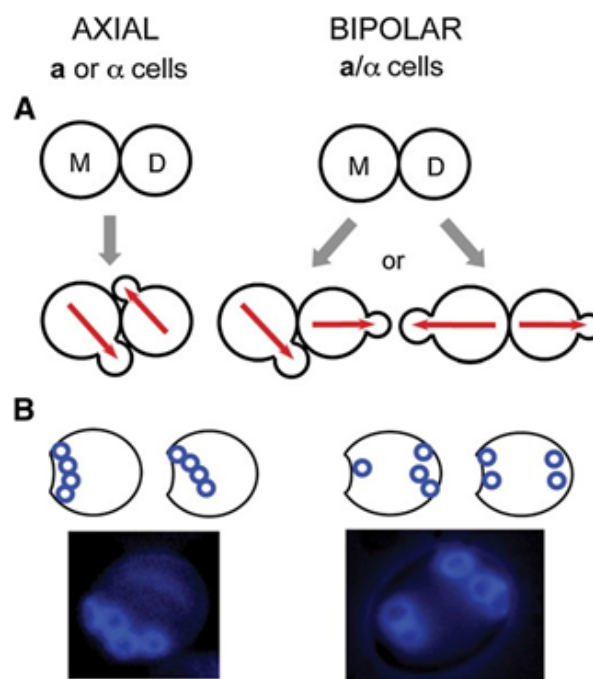


Figure 4: Budding patterns for haploid and diploid cells in *Saccharomyces cerevisiae* (Bi and Park, 2012).

2.1.2.2 Kinetics of yeast growth

Under optimal conditions, yeast cells demonstrate a typical microbial growth curve. This sigmoid curve is composed of different phases: lag phase, exponential phase, deceleration phase and stationary phase (saturation). Figure 5 depicts the phase patterns of population growth in a rich medium to which no additional nutrients are added. Initially, the lag phase occurs; during this time, yeast cells adapt to the new environment and the cell machinery prepares to grow at a higher rate. Population growth at this time is practically null. Afterwards, the exponential phase is the period during which optimal population growth occurs; at the end of this phase, the deceleration phase occurs, and subsequently, yeast cells achieve maximal growth. The time required to double the population is called the generation time and is approximately 90 minutes in length for *S. cerevisiae* under optimal conditions. Then, cells enter the stationary phase, in which cell numbers no longer increase after a certain time; then, dead cells begin to accumulate, causing the death phase. When cells do not grow in an optimal medium, the growth curve is affected. For a long time, growth rates have been used by the microbiology field to study phenotypic properties, which requires laborious techniques. The use of automatic microreaders has greatly simplified the measurement of cell kinetics. Growth rates have been employed in several areas of study, including the regulation of gene expression, phenotype quantification in environmental

studies and also fermentation performance (Gutiérrez et al., 2013b; Hall et al., 2014; López et al., 2004; Warringer and Blomberg, 2003).

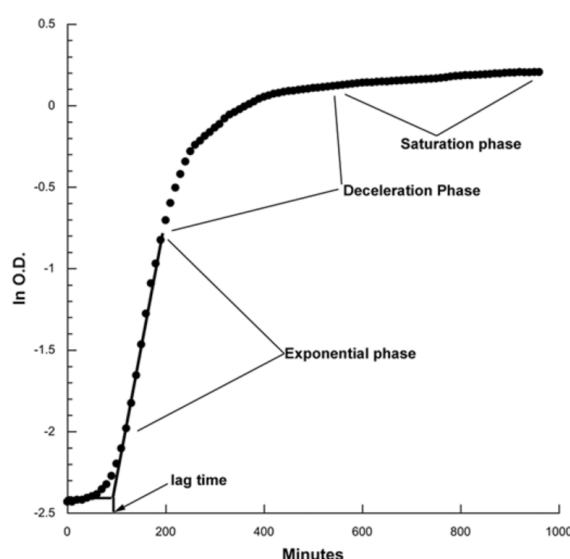


Figure 5: Yeast population growth curve (Hall et al., 2014).

2.2 Yeast Metabolism

2.2.1 Carbon metabolism

Yeasts are able to degrade sugars via two different metabolic pathways, which is determined by available oxygen. However, both pathways share glycolysis as a first step to breakdown glucose. Glycolysis is a metabolic pathway involving a sequence of ten enzyme-catalysed reactions, whereby a molecule of glucose is converted into two molecules of pyruvate in the cytosol. This process generates energy for cell activity in the form of high-energy molecules, adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Once pyruvate is formed, this metabolite is degraded either through the respiration pathway, which is an oxygen-dependent reaction, or through alcoholic fermentation, which occurs in the absence of oxygen (Figure 6). Respiration is energetically more favourable than fermentation.

2.2.1.1 Respiration

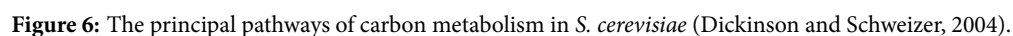
In the case of respiration, pyruvate is oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex, which is located in the mitochondria, resulting in one molecule of NADH and another of

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CO₂. Then, in presence of oxygen, acetyl-CoA is degraded by the citric acid cycle (TCA or Krebs cycle) into carbon dioxide and water. This cycle involves 8 steps that are catalysed by different enzymes and co-enzymes. Overall, the respiration pathway in eukaryotes produces up to 38 molecules of ATP from one molecule of glucose, including the two ATPs produced by glycolysis.

2.2.1.2 Alcoholic fermentation

After glycolysis, the alcoholic fermentation pathway consists of two enzymatic reactions. The first decarboxylates pyruvate into acetaldehyde, releasing a molecule of carbon dioxide. This reaction is catalysed by pyruvate decarboxylase (*PDC1*; *PDC5*). In the second step, acetaldehyde is reduced to ethanol by alcohol dehydrogenase (ADH). In this reaction, the NADH formed during glycolysis passes its electron to acetaldehyde, which acts as a terminal electron acceptor and regenerates NAD⁺ to keep glycolysis active. The net gain of ATP in alcoholic fermentation is 2 for each molecule of glucose degraded.



2.2.1.3 Crabtree effect

Some yeast species, such as *S. cerevisiae*, demonstrate facultative growth in response to oxygen, growing under both aerobic and anaerobic conditions. These yeasts have the ability to adapt their metabolism depending on glucose availability. The use of fermentation in the presence of oxygen and at high sugar concentrations (>9 g/L) is referred to as the Crabtree effect (De Deken, 1966). Yeasts that display the Crabtree effect are Crabtree-positive; yeasts that do not display the Crabtree effect are Crabtree-negative. The Crabtree-positive phenotype makes *S. cerevisiae* preferentially consume sugars by fermentation under almost any growth conditions, apart from carbon-limited chemostat that operates at low dilution rates (Ciani et al., 2016). This feature in *S. cerevisiae* appears to have played a key role in the adaptation of this yeast to sugar-rich environments (Piskur et al., 2006). Instead, the Crabtree-negative phenotype of some yeast species limits their metabolism under oxygen-deprived conditions (Alexander and Jeffries, 1990). Examples of Crabtree negative species include *H. uvarum* and *C. utilis* (Venturin et al., 1994; Verduyn, 1991); both of these genera related to vanish throughout the alcoholic fermentation (Ciani et al., 2010; Ribéreau-Gayon et al., 2006).

2.2.2 Nitrogen metabolism

S. cerevisiae use a wide variety of nitrogen sources, such as amino acids and ammonium, which are present in its natural environment (Cooper, 1982). Yeast reacts to the nitrogen content of the growth environment by controlling nitrogen uptake and regulating catabolic and anabolic processes. Yeast use a variety of nitrogenous compounds as sole sources of nitrogen for growth (Cooper, 1982). In fact, in most yeast strains, all L-amino acids, with the exception of lysine, histidine and cysteine, support growth as sole nitrogen sources. However, each amino acid supports yeast growth in a different manner; e.g., in a medium containing glucose as the main carbon source, the generation time can vary from 2 h (e.g., asparagine, glutamine and arginine) to more than 4 h (methionine and tryptophan) (Ljungdahl and Daignan-Fornier, 2012). The first step for cells to use amino acids is internalization; for this reason, cells possess multiple permeases to transport amino acids across the membrane. Once inside the cell, amino acids are directly used as building blocks in biosynthetic pathways and are deaminated to generate ammonium or transaminated to form glutamate (Godard et al., 2007). Additionally, the presence of external amino acids stimulates the expression of several broad-specificity permeases, allowing amino acids to induce their own uptake (Ljungdahl, 2009). Yeast cells must transform any nitrogen-

containing molecule to glutamate and glutamine; these two amino acids are used as nitrogen donors for all other nitrogen-containing compounds in the cell (Magasanik, 1992). Under glucose growth conditions, ammonium is assimilated by two reactions. The first reaction consists of glutamate synthesis from ammonium and α -ketoglutarate (which is produced by TCA); this reaction is catalysed by *GDH1* (NADPH-dependent glutamate dehydrogenase). The second reaction is the synthesis of glutamine from ammonium and glutamate by glutamine synthase (*GLN1*). Furthermore, when glutamine is the unique source of nitrogen, *GLT1* (NADH-dependent glutamate synthase) catalyses the synthesis of glutamate. The catabolic release of ammonium from glutamate is catalysed by the enzyme *GDH2* (NAD⁺ linked glutamate dehydrogenase) (Ljungdahl and Daignan-Fornier, 2012). These reactions are depicted in Figure 7. Nearly 85 % of general cellular nitrogen is incorporated via the amino nitrogen of glutamate, and the remainder is derived from the amide nitrogen of glutamine (Cooper, 1982).

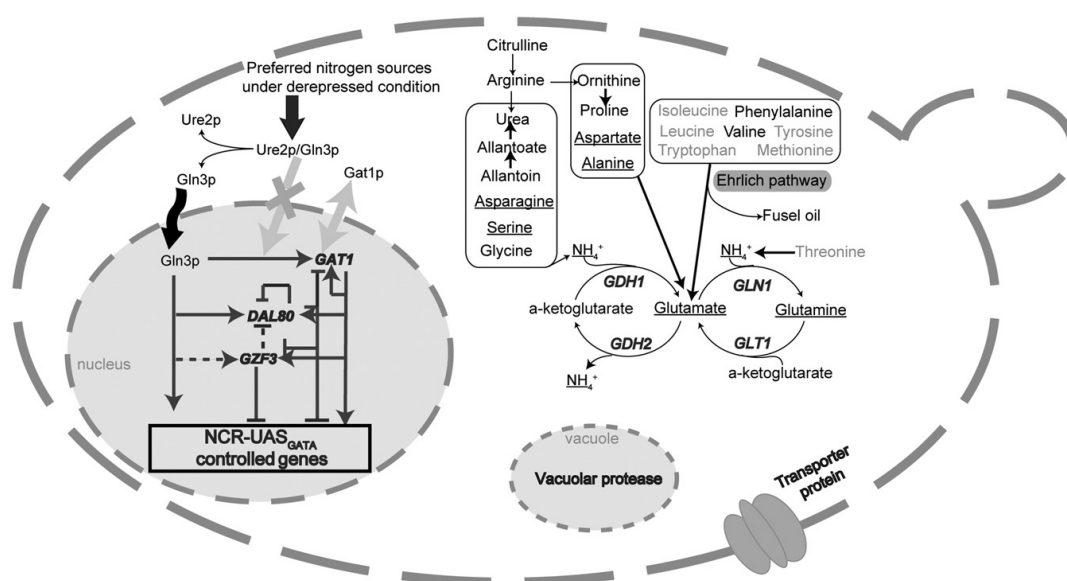


Figure 7: Schematic diagram of the NCR process. The preferred and non-preferred nitrogen sources are labeled in underline and light gray, respectively. In the nucleus, lines with arrows represent activation, while lines with bars represent repression. Genes, proteins, and reactions sensitive to NCR are in bold text and arrows (Zhao et al., 2014).

Nevertheless, not all nitrogen sources support growth equally well. Thus, yeast present higher growth rates in the presence of good nitrogen sources (preferred) rather than poor (non-preferred) nitrogen sources. Ammonium, glutamine and asparagine are classified as good nitrogen sources, whereas arginine, alanine, aspartate and glutamate are less preferred, and proline and urea are considered poor

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nitrogen sources (Figure 7). Branched-chain and aromatic amino acids do not support high growth rates but typically accumulate early in fermentation (Beltran et al., 2005, Henschke and Jiranek, 1993), thus breaking the assumed correlation between the nitrogen source growth rate and preference. This nitrogen source classification is based on two criteria: the ability of different nitrogen sources to support yeast growth as sole nitrogen sources and the ability of certain nitrogen sources to repress the utilization of other nitrogen sources. This phenomenon is regulated under a mechanism designated the nitrogen catabolite repression (NCR) system (Henschke and Jiranek, 1993; Magasanik and Kaiser, 2002; Minehart and Magasanik, 1992; ter Schure et al., 2000). However, classification varies depending on the strain, often in strains with different backgrounds (Magasanik and Kaiser, 2002). Growth on good nitrogen sources yields NCR, repressing the transcription of certain genes, such as permeases and catabolic enzymes, which are needed to utilize non-preferred nitrogen sources. This process permits *S. cerevisiae* to maximize nitrogen sources in the medium. The general amino acid permease *GAP1* is efficiently regulated by nitrogen availability; thus, during nitrogen limitation, the expression of this permease increases significantly (Minehart and Magasanik, 1992). In addition, two transcription factors, Dal80 and Deh1, inhibit the transcription of nitrogen-repressed genes, whereas Gln3 and Gat1 promote the derepression of such genes. All four transcription factors have been reported to cross-regulate each other to ensure the correct expression of nitrogen-repressed genes. In addition, under preferred nitrogen conditions, the transcriptional regulator Ure2 appears to be involved in retaining Gln3 in the cytoplasm (Figure 7) (Coffman et al., 1997; Hall and Beck, 1999; Rødkær and Færgeman, 2014).

Ammonium incorporation into yeast cells involves three permeases: Mep1, Mep2 and Mep3 (Marini et al., 1997). These permeases are only expressed under low concentrations of ammonium because they are not essential for growth under high concentrations of ammonium (> 20 mM) and are, in fact, repressed in the presence of good nitrogen sources due to the NCR. *MEP2* presents the highest affinity for NH_4^+ (Km 1-2 μ M), followed by Mep1 (Km 5-10 μ M) and Mep3, a lower-affinity system (Km 1.4-2.1 mM) (Marini et al., 1997). Triple permease mutants are unable to grow under low ammonium concentrations (<5 mM) using ammonium as the sole nitrogen source, but the presence of any of these permeases restores yeast growth. Alcoholic fermentation is a good example of NCR. Under non-limited nitrogen conditions, the expression of *GAP1* and *MEP2* is repressed, whereas under limited nitrogen conditions, the expression of both genes increases considerably (Beltran et al., 2004).

All previous findings have used *S. cerevisiae* as a model microorganism. With respect to non-

Saccharomyces yeasts, there has been little research regarding nitrogen requirements. A recent study (Kemsawasd et al., 2015) analysed how different nitrogen sources affect growth and fermentation performance under oxygen-limited conditions in *S. cerevisiae* and certain non-*Saccharomyces* species (*L. thermotolerans*, *H. uvarum*, *T. delbrueckii* and *M. pulcherrima*). These authors classified these nitrogen sources according to their specific growth rate (μ_{max}), cell viability at the beginning (N_{max}) and end (N_{end}) of stationary phase and glucose consumption (GluC) for all species (Table 1). The results showed the differential use of nitrogen sources by non-*Saccharomyces* species in relation to *Saccharomyces*, indicating that the influence of nitrogen sources on both growth and fermentation performance during alcoholic fermentation is species-dependent.

Table 1: Influence of nitrogen sources on oxygen-limited yeast growth and fermentation performance in wine yeast species. Preferred nitrogen sources are represented in green, non-preferred in red and intermediate in white. Grey denote nitrogen sources exhausted before the final day of fermentation (Kemsawasd et al., 2015).

N-source	<i>S. cerevisiae</i>					<i>L. thermotolerans</i>					<i>H. uvarum</i>					<i>M. pulcherrima</i>					<i>T. delbrueckii</i>				
	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP
Ala																									
Arg																									
Asn																									
Asp																									
Cys																									
Gln																									
Glu																									
Gly																									
His																									
Ile																									
Leu																									
Lys																									
Met																									
MixAA																									
MixNH ₄																									
Neg																									
NH ₄																									
Phe																									
Ser																									
Thr																									
Trp																									
Tyr																									
Val																									

2.2.3 Production of fusel alcohols

2.2.3.1 Ehrlich pathway

Amino acids represent the main source of assimilable nitrogen for wine yeast in grape must. Some amino acids (branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp)), and the sulphur-containing amino acid (methionine)) are primarily assimilated via the Ehrlich pathway to form a corresponding fusel acid or fusel alcohol. This pathway was first proposed a century ago by Ehrlich (Ehrlich, 1907). Amino acids that are catabolized via this pathway are taken up slowly throughout the fermentation process (Jones and Pierce, 1964). The Ehrlich pathway is used by yeast to acquire nitrogen from amino acids in the medium when preferred nitrogen sources, such as ammonium, are not available. Additionally, higher alcohols represent the largest group of volatile components, and their presence is essential for overall flavour quality (Fabre et al., 1998). Furthermore, higher alcohols may also be synthesized via an anabolic pathway from a sugar substrate (Figure 8).

Several reactions are needed to completely degrade an amino acid (Figure 8). The first reaction is a transamination, catalysed by the aromatic amino acid transferases encoded by *ARO8* and *ARO9* or by branched-chain amino acid transferases encoded by *BAT1* and *BAT2*; after this reaction, the resulting α -keto acid is not redirected into central carbon metabolism. The amino group resulting from this transamination is used to produce glutamate. The second reaction is a decarboxylation catalysed by one of several decarboxylases enzymes (*ARO10*, *PDC1*, *PDC5* and *PDC6*). This step results in a fusel aldehyde, which is reduced to a fusel alcohol or oxidized to a fusel acid. The balance between the oxidation and reduction of fusel aldehydes depends on cultivation conditions (Boer et al., 2007; Vuralhan et al., 2005). In glucose-grown batch cultures, amino acids are almost entirely converted to fusel alcohol, while oxidation to fusel acid plays a minor role. Hence, the reduction of a fusel aldehyde is catalysed by any one of the ethanol dehydrogenases (*ADH1*, *ADH2*, *ADH3*, *ADH4* and *ADH5*) or by a formaldehyde dehydrogenase (*SFA1*). In this reaction, a molecule of NADH is generated, whereby this pathway may provide an alternative, energy-efficient means for NADH regeneration. Once the fusel alcohol is formed, yeast cells export the compound to the external medium.

This catabolic pathway appears to be regulated by different factors, such as temperature and amino acid content, among others. Two genes that catalyse the first step, *BAT1* and *BAT2*, are expressed and function during the cell phase growth (Lilly et al., 2006). In the same study, the E3 ubiquitin-protein

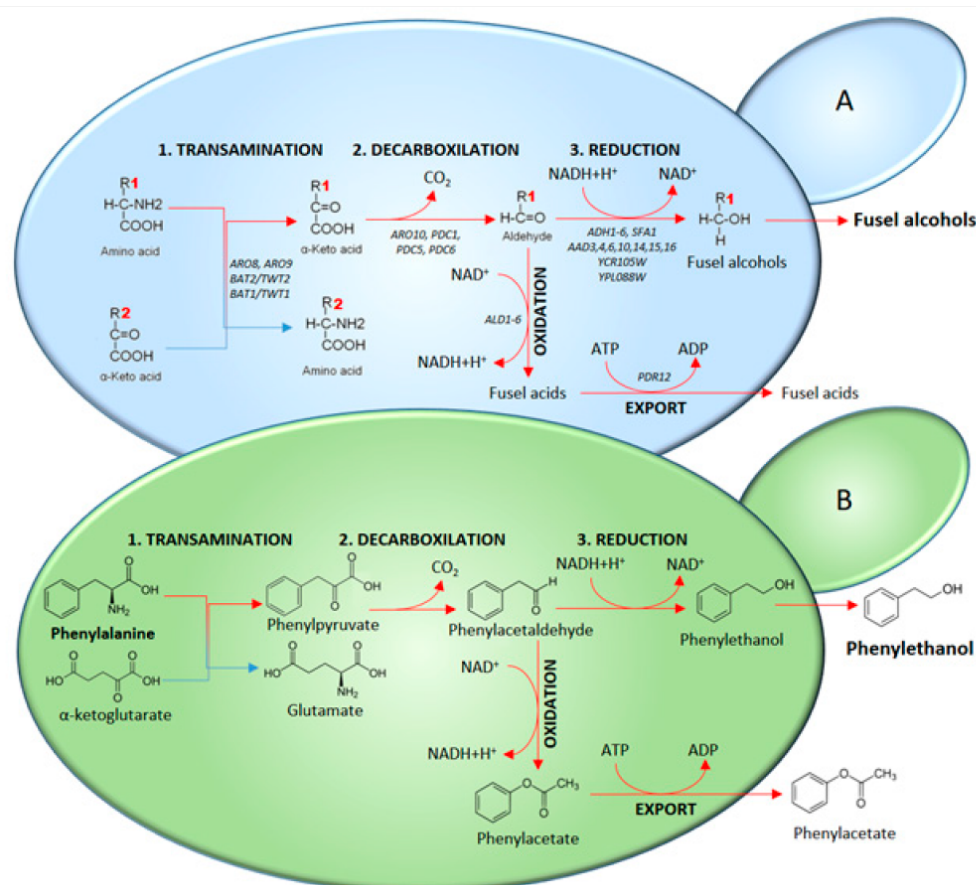


Figure 8: The Ehrlich pathway in *Saccharomyces cerevisiae*. (A) Genes encoding enzymes involved in the process. (B) Catabolism of Phe via Ehrlich pathway (Belda et al., 2017).

ligase Huw1 was shown to be involved in regulating the Ehrlich pathway; Huw1 increased the expression of the ARO9 and BAT1 genes. In addition, ARO80 (*Zn₂Cys₆*) was identified as a transcriptional activator of ARO9 and ARO10 in the presence of aromatic amino acids (Iraqi et al., 1999). As shown by ARO10 expression was regulated at the posttranscriptional level in a carbon and nitrogen source-dependent manner. In a study by Avbelj et al. (2015), the authors monitored the expression of these genes throughout alcoholic fermentation, showing that peak production rates of phenylethanol, tryptophol and tyrosol in *S. cerevisiae* correlated with peak expression of ARO8, ARO9 and ARO10. Despite the decrease in aromatic alcohol production rates after entry into stationary phase, the expression of these genes remained relatively high, suggesting positive feedback regulation by these alcohols, as aromatic amino acid concentrations were still high in the medium (Avbelj et al., 2015).

Moreover, several non-*Saccharomyces* wine species have been reported to produce all three aromatic alcohols; thus, the Ehrlich pathway is also present in non-*Saccharomyces* yeast. Zupan et al. (2013) demonstrated that wine species such as *H. uvarum*, *Z. bailli* and *T. pretoriensis* synthesized these aro-

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matic alcohols during alcoholic fermentation at different concentrations as their secretion was linked to yeast growth phases. However, other non-*Saccharomyces* species, specifically *C. zemplinina* (*S. bacillaris*) and *D. bruxellensis*, did not produce any aromatic alcohols after 28 h of fermentation. Other studies have also demonstrated the synthesis of these alcohols by non-*Saccharomyces* species: *Debaromyces hansenii* produces tryptophol, phenylethanol and tyrosol (Gori et al., 2011), and *Kluyveromyces marxianus* produces phenylethanol (Fabre et al., 1998). Additionally, the use of mixed inocula containing *Saccharomyces* non-*Saccharomyces* species changes the fusel alcohol profiles in wines (Comitini et al., 2011; Giudici et al., 1990; Jolly et al., 2014; Rankine, 1967).

The presence of fusel alcohols during the growth of certain yeast species may induce morphological changes. Indeed, one of the main functions of the Ehrlich pathway is to contribute to the adaptation of yeast cells to environmental conditions; this point will be discussed later.

2.3 Yeast ecology and its importance in alcoholic fermentation

The transformation of fermentable sugars into alcohols during fermentation is the basis for the production of multiple alcoholic beverages, such as wine, beer, cider and sake, among others. This metabolic process is very complex and encompasses many more transformations than ethanol production. *S. cerevisiae* presents the most efficient fermentative catabolism, and traditionally, this species is preferred for alcoholic fermentation and is often simply referred to as “the wine yeast” (Pretorius, 2000).

Wine is produced by the transformation of grape sugars into ethanol by yeast. The surfaces of grapes are primarily colonized by non-*Saccharomyces* yeasts, including *Hanseniaspora*, *Starmerella* (sym *Candida*), *Hansenula*, or *Metschnikowia*, among others. Indeed, yeasts belonging to the *Saccharomyces* genus are difficult to detect in must due to their low predominance on grapes. For this reason, non-*Saccharomyces* yeasts initiate spontaneous alcoholic fermentation and are replaced by *S. cerevisiae* throughout the process (Fleet, 2003a; Heard and Fleet, 1988; Ribéreau-Gayon et al., 2006). Traditionally, this succession of species during wine fermentation is associated with the low ethanol tolerance of non-*Saccharomyces* yeasts; strains of *Saccharomyces* better adapt to must conditions. Moreover, non-*Saccharomyces* yeasts were originally seen as responsible for microbe-related problems in wines due to their isolation from spoiled grapes and wines. However, it was recently shown that non-*Saccharomyces* species, largely present during the early steps of alcoholic fermentation, significantly contribute to improve the sensory qualities of wines. In fact, some of these species have

been described to secrete important aroma compounds, such as aromatic alcohols, ethyl esters and acetate esters, in higher amounts, enhancing the wine flavour. (Belda et al., 2017; García et al. 2010; Jolly et al., 2014; Romano et al., 2003). Consequently, the role of non-*Saccharomyces* species in wine production has been debated extensively (Fleet, 2003; Jolly et al., 2014; Pretorius, 2000; Varela, 2016). In the last decade, studies have focused on the use of these yeasts as starter cultures for sequential or simultaneous inoculation with *S. cerevisiae* (Table 2 (Ciani et al., 2010; Jolly et al., 2014). However, the basic physiology of non-*Saccharomyces* species has been poorly explored in terms of their growth and fermentation performance (Kemsawasd et al., 2015).

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Table 2: Influence of non-*Saccharomyces* yeast in recent studies (Adapted from Varela, 2016).

Non- <i>Saccharomyces</i> yeast	Impact on physical properties, chemical composition or sensory attributes	References
<i>Torulaspora delbrueckii</i>	Increased acetate esters and medium-chain fatty acids (MCFAs)	(Cordero-Bueso et al., 2013)
	Increased concentration of thiols, 3-sulfanylhexas-1-ol (3SH) and 3-sulfanylhexasyl acetate (3SHA)	(Renault et al., 2016)
	Increased concentration of terpenes α -terpineol and linalool	(Cus and Jenko, 2013)
	Increased overall wine quality	(Azzolini et al., 2015)
	Increased 'aroma intensity', 'complexity' and 'persistence'	(Belda et al., 2016)
	Increased 'colour intensity', 'overall impression' and 'aroma quality'	(Renault et al., 2015)
	Increased 'complexity' and 'fruity' attributes	(González-Royo et al., 2015)
<i>Hanseniaspora uvarum</i>	Increased foamability and foam persistence Preferred by sensory panel	(González-Royo et al., 2015)
	Increased concentrations of several C13-norisoprenoids and some terpenes Increased 'tropical fruit', 'berry', 'floral', and 'nut aroma' characters	(Hu et al., 2016)
<i>Hanseniaspora vineae</i>	Increased concentration of acetate esters, esters of MCFAs, isoamyl alcohol, 2-phenylethanol and α -terpineol	(Garofalo et al., 2016)
	Increased 'white prune', 'pear', 'citric fruits', and 'honey' attributes	(Medina et al., 2013)
<i>Starmerella bacillaris</i>	Increased concentration of phenyl-ethyl acetate, ethyl lactate and α -terpineol Increased 'floral' attribute and panel preferences	(Lleixà et al., 2016)
	Increased glycerol concentration, greater volatile complexity	(Englezos et al., 2016)
	Increased glycerol content, 'jam', 'softness', 'structure' and 'persistence' attributes, and decreased 'bitter' characters	(Zara et al., 2014)
<i>Metschnikowia pulcherrima</i>	Increased 'persistence' and decreased 'reduced' characters	(Tofalo et al., 2016)
	Increased concentration of mannoproteins	(Domizio et al., 2014)
	Increased 'overall impression', 'citrus/grape fruit' and 'pear' attributes	(Benito et al., 2015)
	Increased foam persistence and 'smoky' and 'flowery' attributes	(González-Royo et al., 2015)

2.3.1 Yeast Interactions

A large variety of microorganism interactions occur during alcoholic fermentation: bacteria-bacteria, yeast-yeast and yeast-bacteria. Thus, alcoholic fermentation is an ideal process to study interactions and communicative behaviours between different microorganisms.

Due to increasing interest in the use of non-*Saccharomyces* yeast in the winemaking process, reports studying the interactions between yeasts during fermentation have increased considerably. Diverse factors (abiotic and biotic) cause growth inhibition of certain strains and species (Figure 9) and consequently increase the population of others that are better adapted to the medium. Several studies have reported that abiotic factors, such as fermentation temperature (Torija et al., 2003), competition for nutrients, nitrogen concentration or ethanol tolerance (Gao and Fleet, 1988), play important roles in selecting *S. cerevisiae* strains during alcoholic fermentation. Additionally, all of these factors affect non-*Saccharomyces* species growth (Ciani et al., 2016).

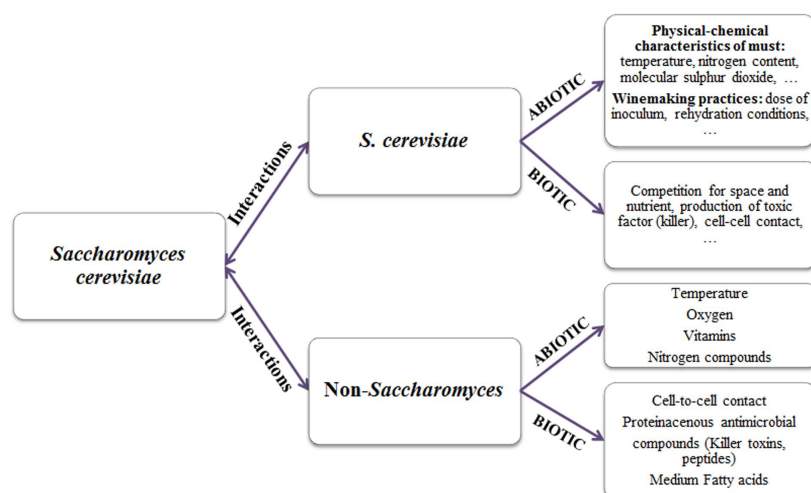


Figure 9: Interactions between yeast species during wine-making process (Ciani et al., 2016).

On the other hand, during alcoholic fermentation, yeasts synthesize certain compounds that, depending on the concentration achieved, inhibit their own growth or that of other yeast species. In addition to ethanol, compounds such as short-to-medium-chain fatty acids (e.g., acetic, hexanoic, octanoic and decanoic acids) and yeast killer toxins induce cell lysis of certain yeast species, including some strains of *S. cerevisiae* (Pérez et al., 2001). Moreover, direct microbial interactions (i.e., through physical contact) have been reported to be involved in the early death of non-*Saccharomyces* yeast, although such death mechanisms are dependent on cell density when cultures are competing for space (Nissen et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013). Recently, interactions between species have

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also been associated with the secretion of specific compounds, known as quorum sensing molecules, by yeast during alcoholic fermentation (Albergaria and Arneborg, 2016; Ciani and Comitini, 2015, Wang et al, 2015, 2016).

2.3.1.1 Quorum sensing

Microorganisms live together in a large variety of complex ecosystems, thus requiring communication with each other. This communicative behaviour is present in many living systems and is called quorum sensing. In 1970, Nealson et al. described the bioluminescence activity of the bacteria *Vibrio fischeri* under the term “autoinduction”; some years later, this activity was renamed quorum sensing (Fuqua et al., 1994). Quorum sensing activity is the ability of certain microorganisms to secrete molecules that are recognized by others, which modify their behaviour accordingly; thus, quorum sensing is an intercellular and coordinated process. The signal molecules are secreted at basal levels during growth and accumulate in the environment. When the accumulation of these molecules achieves a threshold concentration, indicating that the population is sufficiently dense to make a behavioural group-based decision, specific gene expression is triggered. In addition to bacteria, several studies have reported this type of communication in fungal species, particularly in yeast; these molecules are related to the control of cell density, morphological changes, biofilm formation and pathogenesis.

The dimorphic fungal human pathogen *C. albicans* is able to regulate its morphological transition using quorum sensing molecules. Specifically, in this species, farnesol and tyrosol induce morphogenetic changes, although the opposite response is triggered. While farnesol blocks hyphae formation at high cell densities (Hornby et al., 2001), tyrosol shortens the lag phase and induces hyphae formation (Chen et al., 2004). Like other fungal species, the budding yeast *S. cerevisiae* undergoes morphological changes from yeast to a filamentous form in response to environmental cues (Gimeno et al., 1992). Recently, the production of autosignalling alcohols was related to the induction of pseudohyphal growth in *S. cerevisiae* (Chen and Fink, 2006; Dickinson et al., 2003). All fusel alcohols derived from aromatic amino acids appear to have signalling roles affecting yeast growth, phenylethanol and tryptophol in *S. cerevisiae* and tyrosol in *C. albicans* (Chen et al., 2004; Chen and Fink, 2006). Additionally, other alcohols derived from amino acid catabolism, specifically 2-methyl-1-butanol (the end-product of isoleucine catabolism), 1-pentanol (‘n-amyl alcohol’; from threonine breakdown) and 2-methyl-1-propanol (the end-product of valine catabolism), have also been described to induce filament formation in *S. cerevisiae* in rich complex medium (Dickinson, 1996). All of these alcohols are the final

products of the catabolism of non-preferred amino acids, which accumulate when nutrients become limited. Thus, the effects of these alcohols on *S. cerevisiae* morphology suggest that yeast respond to their own metabolic by-products (Dickinson, 2008). In *S. cerevisiae*, the production of phenylethanol and tryptophol is controlled by cell density and nitrogen availability. Moreover, Chen and Fink (2006) suggested that the production of tryptophol is autostimulatory and that the addition of this alcohol induces *ARO9* and *ARO10* expression. Indeed, an *aro8Δ/aro9Δ* mutant exhibited greatly decreased filamentous growth, pinpointing a clear relationship between the Ehrlich pathway and morphogenesis regulation in *S. cerevisiae*. According to Fabre et al. (1998), the production of aromatic alcohols is associated with growth phases in *K. marxianus*, affirming that the addition of phenylalanine after the exponential growth phase results in no formation of phenylethanol during the stationary phase. Phenylethanol and tyrosol were also detected in *Debaromyces hansenii* depending on the cell density and environmental conditions (Gori et al., 2011). Moreover, Pu et al., (2014) showed that phenylethanol promotes adhesion and biofilm formation in *Kloeckera apiculata* (teleomorph *H. uvarum*).

2.4 Filamentous Growth

Filamentous growth is a fungal behaviour that occurs in response to extracellular stimuli. The major stimulus that triggers filamentous growth is nutrient limitation; thus, filamentous growth is thought to represent a fungal scavenging response (Cullen and Sprague, 2012). The term filamentous growth has been applied to describe two different phenomena: invasive growth and pseudohyphal growth. Yeasts are able to invade solid substrates, such agar; this process is designated invasive growth. During pseudohyphal growth, cells become elongated and grow in connected chains, but yeast are unable to form true hyphae. In this case, these elongated cells remain attached to each other by proteins in the cell wall after cytokinesis.

Filamentous growth involves at least three major changes (Cullen and Sprague, 2012):

1. Increase in the cell length. This is mediated by an extension of the G2 phase of the cell cycle (Figure 10) (Kron et al., 1994), resulting in the mother and daughter cells achieving the same size during mitosis. In this case, the mother and daughter initiate bud formation at the same time. Simultaneously, the cells induce and increase polarized growth via a Cdc42p-dependent mechanism that involves the polarisome (Cullen and Sprague, 2002).

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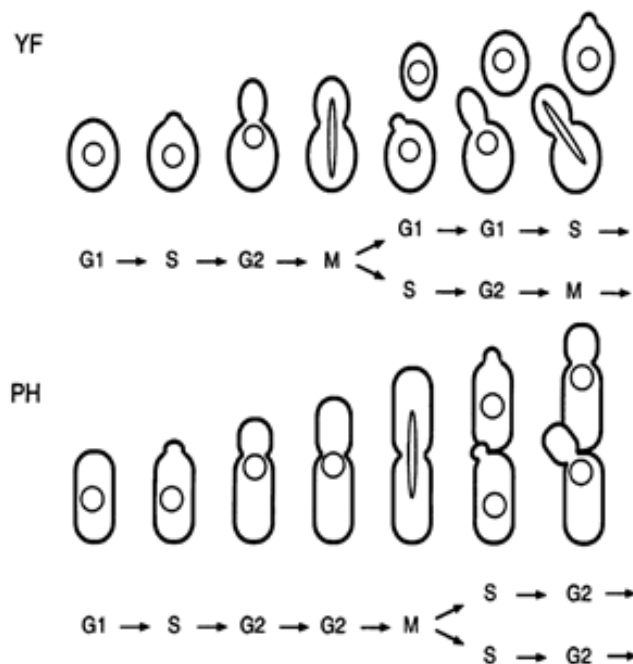


Figure 10: A model comparing cell cycles in normal budding growth (YF) and pseudohyphal growth (PH) (Kron et al., 1994).

2. Reorganization of cell polarity. Under nutrient-rich conditions, haploid cells bud axially, while diploid cells bud in a bipolar pattern. Under nutrient limitation, both type of cells shift cell polarity to distal-unipolar budding. In distal-unipolar growth, the new cell emerges away from the mother cell, resulting in the creation of new territories in connected cells and indicating important alterations to the cell surface (Figure 11). This altered polarity occurs via a Bud8-dependent mechanism (Cullen and Sprague, 2002; Gimeno et al., 1992).

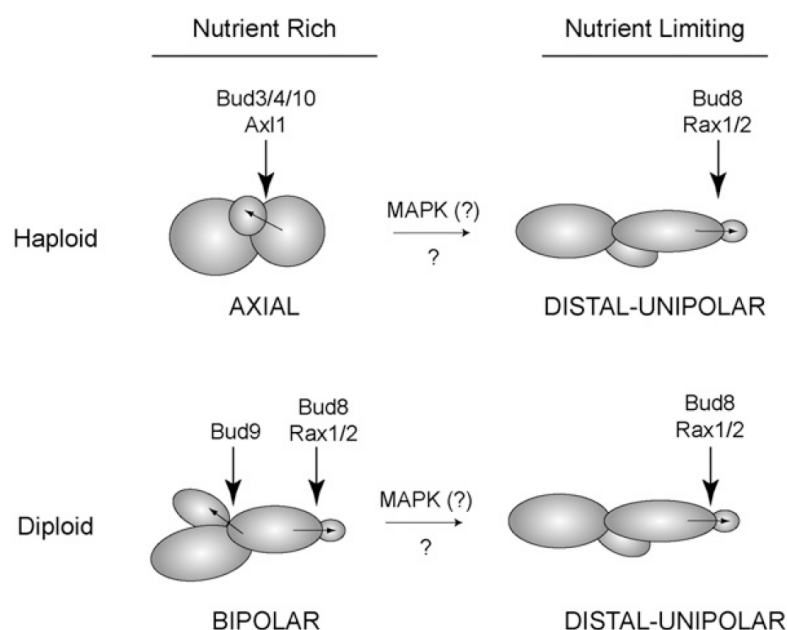


Figure 11: Change in cell polarity during filamentous growth (Cullen and Sprague, 2012).

- Enhanced cell-cell adhesion via the expression of *FLO11*. The *FLO11* gene encodes the major cell adhesion molecule, the flocculin *FLO11*, which controls filamentous growth. *FLO11* is a glycosylphosphatidylinositol (GPI)-linked glycoprotein with a structure and properties similar to the adhesins of other fungi; additionally, *FLO11* is the most intensively studied fungal adhesion molecule. *FLO11* belong to the FLO gene family (*FLO1*, *FLO5*, *FLO9* -*FLO11*), which encodes specific lectins involved in flocculation (Teunissen and Steensma, 1995). *FLO11* is an essential protein for invasive and pseudohyphal growth and is also required for biofilm formation (Cullen and Sprague, 2000; Lo and Dranginis, 1998; Reynolds and Fink, 2001).

Most signal pathways that have been described to regulate filamentous growth converge in *FLO11* and have been associated with the regulation of filamentous growth in *S. cerevisiae* (Figure 12), including the AMP-dependent kinase AMPK Snf1 (Cullen and Sprague, 2000; McCartney and Schmidt, 2001); the RAS-cAMP-protein kinase A (PKA) pathway (Gimeno et al., 1992; Liu et al., 1993; Toda et al., 1985); a mitogen-activated protein kinase pathway called the filamentous growth (fMAPK) pathway (Adhikari et al., 2015; Adhikari and Cullen, 2014; Karunanithi and Cullen, 2012; Rupp et al., 1999; Saito, 2010); the target of rapamycin (TOR) pathway, which senses nitrogen availability (Hall and Beck, 1999a; Heitman et al., 1991; Kingsbury et al., 2015); and the retrograde pathway (RTG) (Chavel et al., 2010). (Figure

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12).

Most signal pathways that have been described to regulate filamentous growth, converge in *FLO11*, including the AMP-dependent kinase AMPK Snf1p (Cullen and Sprague, 2000; McCartney and Schmidt, 2001), the RAS-cAMP- protein kinase A (PKA) pathway (Gimeno et al., 1992; Liu et al., 1993; Toda et al., 1985) and a mitogen-activated protein kinase pathway called the filamentous growth (fMAPK) pathway (Adhikari et al., 2015; Adhikari and Cullen, 2014; Karunanithi and Cullen, 2012; Rupp et al., 1999; Saito, 2010), the target of rapamycin (TOR) pathway , which sense nitrogen availability (Hall and Beck, 1999; Heitman et al., 1991; Kingsbury et al., 2015), and Retrograde pathway (RTG) (Chavel et al., 2010) have been related to regulate filamentous growth in *S. cerevisiae*.

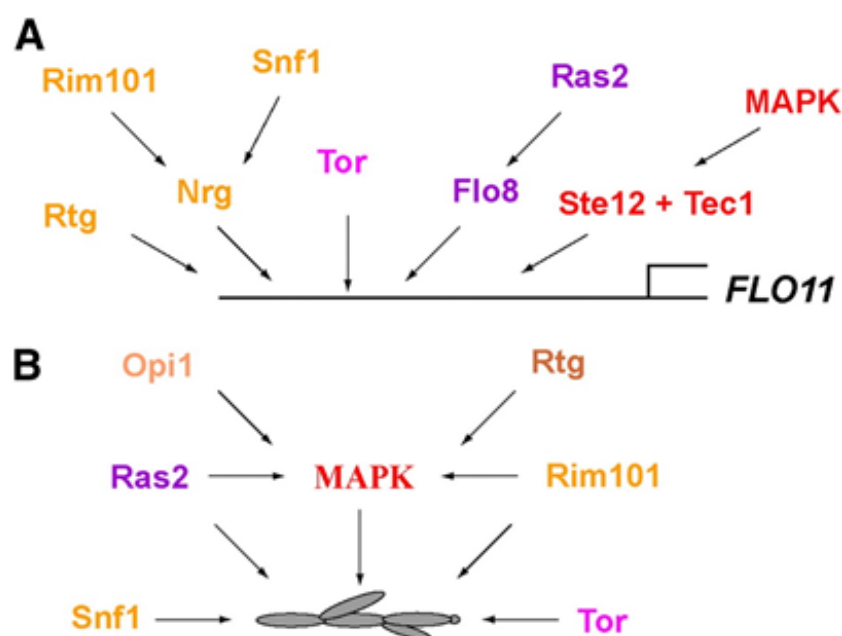


Figure 12: Signal integrations among regulatory pathways that control filamentous growth. (A) Multiples signaling pathways converge on *FLO11* gene. (B) Several signaling pathways regulate filamentous MAPK pathway (Cullen and Sprague, 2012).

2.4.1 Filamentous MAPK pathway

MAPKs are evolutionary conserved kinases that are involved in multiple responses in eukaryotes, such as haploid mating or pheromone responses, the high osmolality glycerol response (HOG) pathway and the filamentous growth pathway. Elements of the pheromone response pathway are expressed in diploid cells when these cells do not mate, demonstrating the roles of these kinases in filamentous growth. Indeed, Liu et al. (1993) postulated that mating in haploids and filamentous growth in diploids share the same “core module”: p21-activated (PAK) kinase Ste20, MAPKKK Ste11, the MAPKK Ste7 and the transcription factor Ste12 (Figure 13). The filamentous growth MAPK pathway is composed of three plasma membrane sensors: Msb2, Sho1 and Opy2 (Cullen et al., 2004; Cullen and Sprague, 2012; Wu et al., 2006; Yamamoto et al., 2010). That are connected by the Cdc42p GTPase to the “core module” (Bi and Park, 2012). Therefore, the following model has been postulated to explain filamentous growth through the MAPK pathway: nutrient limitation induces expression of Yps1 (encoding the aspartyl protease), which processes the extracellular domain of the signalling mucin Msb2, required for MAPK activation. Processed Msb2 forms a complex with Sho1 and with the Rho GTPase Cdc42 (Cullen et al., 2004). Activated Cdc42 binds effector proteins, including the PAK Ste20, which, when activated, phosphorylates the MAPKKK Ste11, thereby activating the MAPK cascade. Thus, both proteins (Msb2 and Sho1) initiate the filamentation MAPK pathway. Additionally, the downstream filamentous growth pathway is activated under glucose depletion conditions and controls cell adhesion, elongation and the reorganization of cell polarity through Kss1-mediated activation of the transcription factors Ste12 and Tec1 (Cullen and Sprague, 2000, 2012; Saito, 2010).

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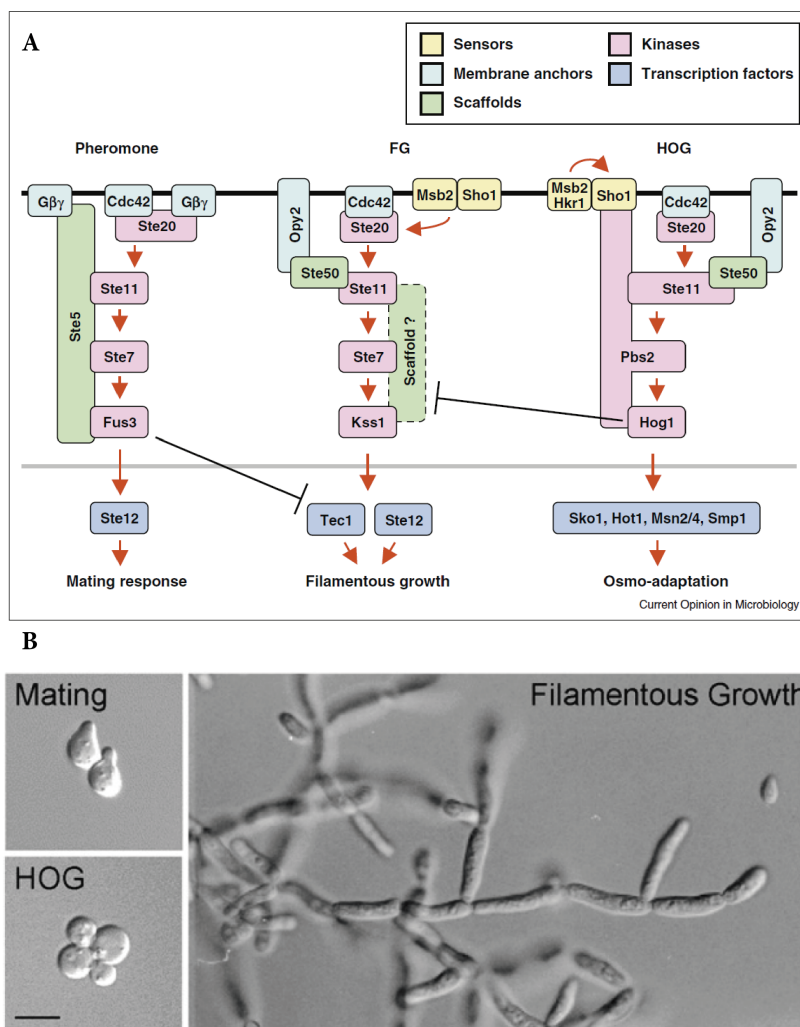


Figure 13: Three MAPK pathways in yeast. (A) Three MAPK ways control mating response, filamentous growth and osmo-adaptation. The scaffold protein in the FG pathway is hypothetical. Red arrows indicate signal flow, whereas black T-shaped bars indicate inhibition. Black horizontal line represents the plasma membrane and gray line the nuclear membrane. (Role of phosphatidylinositol phosphate signaling in the regulation of the filamentous-growth mitogen-activated protein kinase pathway) (Saito, 2010). (B) Morphogenesis induced by MAPK in yeast (Cullen and Sprague, 2012).

2.4.2 Ras2/cAMP-PKA pathway

Cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) lies at the heart of an important signal transduction pathway in *S. cerevisiae*. PKA activity influences many of the physiological responses of cells, including glucose-mediated regulation of gene expression, the cell division cycle, polarized growth and glycogen synthesis. cAMP is produced by the *S. cerevisiae* adenylyl cyclase (Cyr1p), which in turn requires two mammalian ras-related guanine-nucleotide binding proteins (Ras1, Ras2) for its activity (Dickinson and Schweizer, 2004). cAMP levels are proposed to be critical in determining whether cells undergo filamentous growth (Mosch et al., 1996) (Figure 14). The RAS/PKA pathway

contains the sensing protein Gpr1, an upstream regulator (Lorenz et al., 2000; Tamaki et al., 2000). This receptor is coupled to a heterotrimeric G protein α -subunit, Gpa2, which is also required for pseudo-hyphal differentiation and exerts a role in nutrient sensing (Kübler et al., 1997). cAMP regulates the activity of three protein kinases in *S. cerevisiae*: *TPK1*, *TPK2* and *TPK3* (Toda et al., 1985). Moreover, each *TPK* gene exerts different changes in filamentous growth; for example, *TPK2* inhibits filamentous growth, whereas *TPK3* promotes hyperfilamentation (Pan and Heitman, 1999; Robertson and Fink, 1998). *TPK2* activates the *FLO11* gene through the transcription factor Flo8 (Pan and Heitman, 1999). Additionally, cAMP regulates *FLO11* expression via *TPK1*. This protein kinase is a negative regulator of Yak1, a dual-specificity tyrosine-regulated kinase. *TPK1* inactivates Yak1 via phosphorylation; however, in its nonphosphorylated form, Yak1 is active and regulates the expression of *FLO11* through the transcription factors Sko2 and Phd1 (Malcher et al., 2011). In *S. cerevisiae*, aromatic alcohols, which are signalling molecules that induce morphogenetic changes, are thought to exert their effects through a mechanism that primarily involves the PKA but not the MAPK pathway. These alcohols, produced at high concentrations under low nitrogen conditions, are sensed via *TPK2* and the downstream effector Flo8, which induces the *FLO11* gene, resulting in morphological changes in cells (Chen and Fink, 2006).

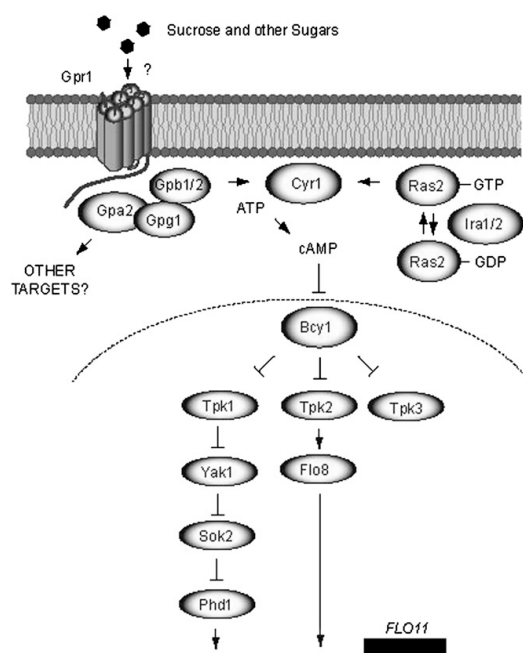


Figure 14: RAS2/PKA pathway role in filamentous growth (Cullen and Sprague, 2012).

2.4.3 The TOR pathway

The target of rapamycin signaling (TOR) pathway was first identified in yeast and represents a highly conserved mechanism in eukaryotes, through which cells respond to nutrients. The TOR pathway controls the transcription of NCR-sensitive genes (*GAP1*, *GAT1*, *DAL5*), retrograde response pathway (*CIT2*, *DLD3*, *DH1/2*) and is also involved in filamentous growth regulation. The regulation of filamentous growth by TOR is apparently independent of the RAS/PKA and MAPK pathways. TOR kinase is the main serine/threonine kinase with regard to nitrogen sensing, cell growth and in many stressful situations (Wullschleger et al., 2006). TOR kinase functions in two different multiprotein complexes, TORC1 (complex 1) and TORC2 (complex 2). TORC1 regulates transcription of the *FLO11* gene via the transcription factor Gcn4 (Braus et al., 2003). The activation of Gcn4 depends on the eukaryotic translational initiation factor 2 ($eIF2\alpha$); the phosphorylation of $eIF2\alpha$ reduces general protein biosynthesis by promoting *GCN4* translation (Hinnebusch, 2005). The transcriptional activator Gcn4, found in yeast and other fungi, induces expression of most amino acid biosynthetic enzymes in response to starvation for any amino acid in the cross-pathway, or to exert general amino acid control (Broach et al., 2012). Furthermore, the TOR pathway is a major regulator of the retrograde to nucleus pathway (RTG), whose gene expression is not directly regulated by the nitrogen source but by the product of its catabolism. Thus, nitrogen sources that yield glutamate down-regulate RTG gene expression, whereas the expression of these gene is up-regulated in response to sources yielding ammonia (Tate et al., 2002).

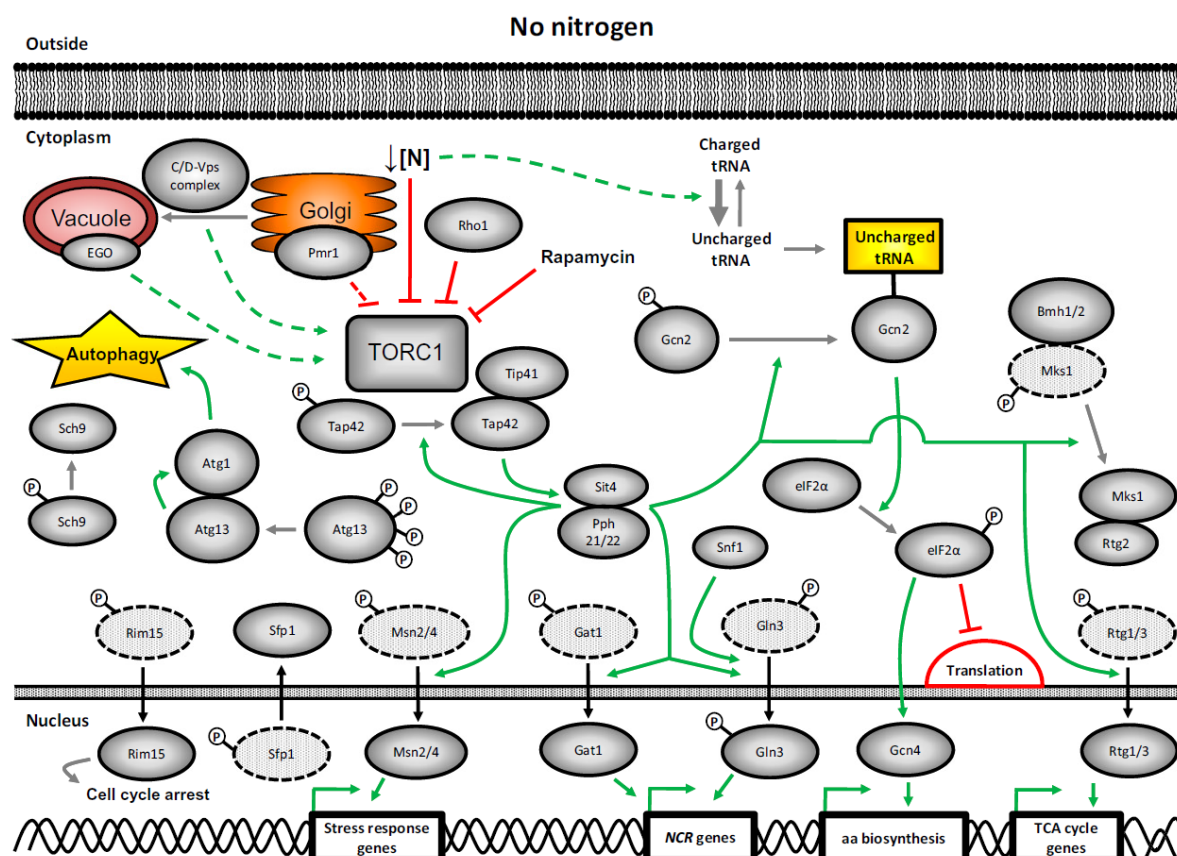


Figure 15: The nitrogen signaling pathway during nitrogen limitations. When yeast cells starve from nitrogen (N), TORC1 activity is reduced. Additionally, TORC1 can also be inhibited by rapamycin, the protein kinase Rho1, and possibly also by the $\text{Ca}^{2+}/\text{Mn}^{2+}$ -ATPase, Pmr1. When the activity of TORC1 is low, the phosphatases Sit4 and Pph21/22 dissociate from Tap42 and thus become active. Consequently, Tap42 is further dephosphorylated and associates with Tip41. Sit4 and Pph21/22 activities result in dephosphorylation and thereby stimulation of Gcn2 among others. Meanwhile, nitrogen starvation also increases the amount of uncharged tRNAs, which directly bind to Gcn2. Collectively, these events activate Gcn2, which phosphorylates eIF2 α resulting in stimulation of Gcn4 and inhibition of translation. Subsequently, Gcn4 promotes transcription of more than 500 genes (e.g. genes involved in amino acid (aa) biosynthesis). Sit4 and/or Pph21/22 also dephosphorylate Gln3 and Gat1 leading to derepression of NCR genes (dephosphorylation is also mediated by Pph3). Snf1 aids this derepression by phosphorylating Gln3. Moreover, Sit4 and/or Pph21/22 promote the nuclear localization of Msn2/4 resulting in initiation of the yeast stress response. The retrograde response pathway is likewise affected by Sit4 and/or Pph21/22, which dephosphorylate RTG1/3 and Mks1. Dephosphorylated RTG1/3 enters the nucleus, and dephosphorylated Mks1 interacts with RTG2, and consequently, Mks1 relieves its inhibitory effect on RTG1/3 translocation into the nucleus. Finally, low TORC1 activity promotes the dephosphorylated state of Rim15, which thereby localizes in the nucleus and induces cell cycle arrest. Atg13 is likewise dephosphorylated leading to promotion of autophagy, whereas the dephosphorylation of the protein kinase Sch9 results in inactivation. A green arrow represents activation, a red line or half circle represents inhibition, a gray arrow indicates a metabolic reaction, and a black arrow points to a relocation event (Rødkaer and Færgeman, 2014).

2.4.4 RTG pathway

The RTG pathway, an intracellular signalling pathway that indicates mitochondrial dysfunction, was discovered in 1987 (Parikh et al., 1987). The RTG pathway is induced by cytoplasmic, mitochondrial and peroxisomal proteins, among others (Epstein et al., 2001). The general process of mitochondrial signalling is conserved from yeast to humans; however, the molecular mechanisms do not appear to be conserved across species (Liu and Butow, 2006). The *CIT2* gene, a target of the RTG pathway, encodes a peroxisomal isoform of citrate synthase, whose expression is significantly increased in cells demonstrating mitochondrial dysfunction (ρ^0 petites) (Liao et al., 1991). Analysis of this target gene (*CIT2*) has revealed multiple positive and negative regulators of the RTG pathway. The *RTG1*, *RTG2*, *RTG3* and *GRR1* genes encode proteins that are positive regulators of the RTG pathway, whereas Mks1 and Lst8 are negative regulators of this pathway (Liu et al., 2003a; Sekito et al., 2002).

RTG senses the functional state of mitochondria via the level of glutamate (Epstein et al., 2001; Liu and Butow, 2006; Sekito et al., 2002), a potent repressor of RTG-dependent gene expression. Therefore, RTG is likely to be involved in glutamate homeostasis. Indeed, RTG genes reportedly control the expression of *CIT1*, *ACO1*, *IDH1*, and *IDH2* (genes encoding the enzymes that catalyse the first three steps in the TCA cycle, leading to the production of α -ketoglutarate, the direct precursor of glutamate) in cells with compromised or dysfunctional mitochondria (Liu and Butow, 2006). (Figure 16)

Moreover, oxygen seems to be a signal for filamentous growth, since haploid cells were demonstrated to reduce their ability to form pseudohyphae under anaerobic conditions (Wright et al., 1993). This fact suggests an important role of mitochondria to regulate signaling pathways, which emerge in filamentous growth development. Indeed, the ρ^0 petites lose the capacity to undergo pseudohyphae (Jin et al., 2008). Recently, RTG pathway has been shown to regulate the filamentation response to butanol (Starovoytova et al., 2013). However, the mechanism that RTG pathway uses to transduce the signal that induces filamentous growth has been poorly explored.

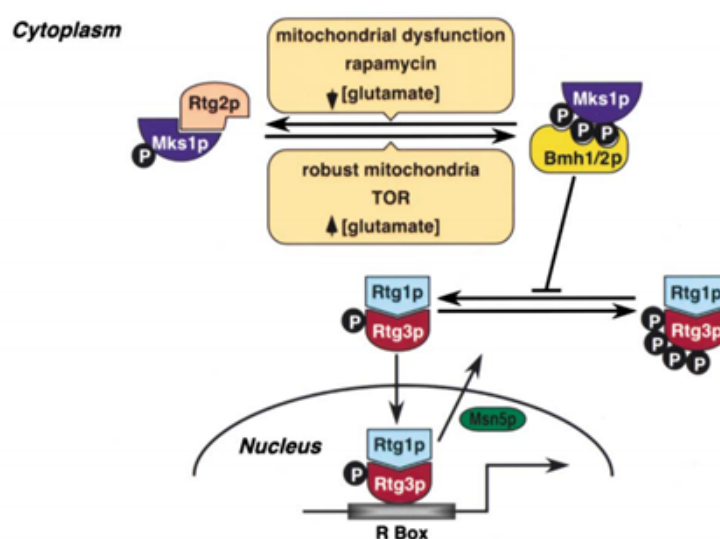


Figure 16: RTG pathway regulation. (Liu et al., 2003)

2.4.5 Other sensing pathways

The depletion of fermentable sugars also triggers filamentous growth. Snf1, a protein kinase important for growth on carbon sources that are less preferred than glucose, regulates *FLO11* in response to glucose limitation by counteracting the actions of Nrg1 and Nrg2, which are repressors of *FLO11* (Kuchin et al., 2002). Recently, Snf1 was reported to play a different role in regulating filamentous growth depending on whether carbon or nitrogen is the limiting nutrient (Orlova et al., 2010). In this study, the authors postulated that Sak1, a protein kinase that phosphorylates Snf1, is involved in pseudohyphal growth because it affects Snf1 activation. In addition, Nrg1 and Nrg2 are also controlled by the Rim101 pathway (pH sense), which is involved in filamentous growth (Barrales et al., 2008; Lamb and Mitchell, 2003). Other regulators of filamentous growth include the chromatin remodeling complex Rpd3(L) (Barrales et al., 2008; Carrozza et al., 2005; Ryan et al., 2012), the tRNA modification complex elongator (Krogan and Greenblatt, 2001), and the Ph80p-Pho85 cyclin and cyclin-dependent kinase (Chavel et al., 2014). Most of these pathways interact amongst themselves, exerting a coordinated response to filamentous growth (Figure 17).

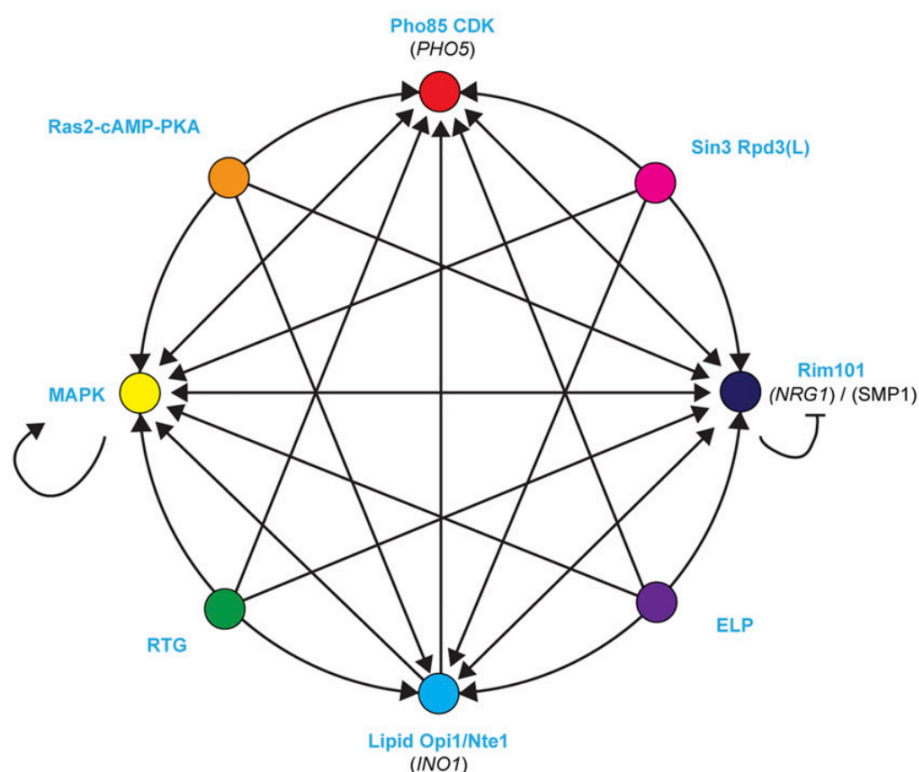


Figure 17: Interactions among filamentous growth inducer pathways (Chavel et al. 2014).

2.4.6 Technical approaches

Filamentous growth was first described in the $\Sigma 1278b$ strain (Gimeno et al., 1992). Several technology-based approaches have been employed to elucidate genes, proteins and pathways that comprise this yeast response. Strains transformed via plasmids, knockout mutants, immunoblot analysis, secretion profiling, construction of a genome-wide set of disrupted genes and transcriptomic analysis are examples of genetic techniques employed for this purpose (Birkaya et al., 2009; Chavel et al., 2014; Cullen and Sprague, 2000; Gimeno and Fink, 1994; Granek et al., 2010; Jin et al., 2008; Pan and Heitman, 2002). Additionally, green fluorescent protein (GFP), real-time PCR (QPCR) and LacZ analysis based on β -galactosidase assays have been performed to study the expression of related genes such as *FLO11* (Chavel et al., 2010; Chen and Fink, 2006). Microscopy techniques have played a key role in filamentous growth studies, allowing the observation of morphological changes, (Figure 18) (Basu et al., 2016; Cullen et al., 2004; Lorenz et al., 2000; Madden and Snyder, 1998; Malcher et al., 2011).

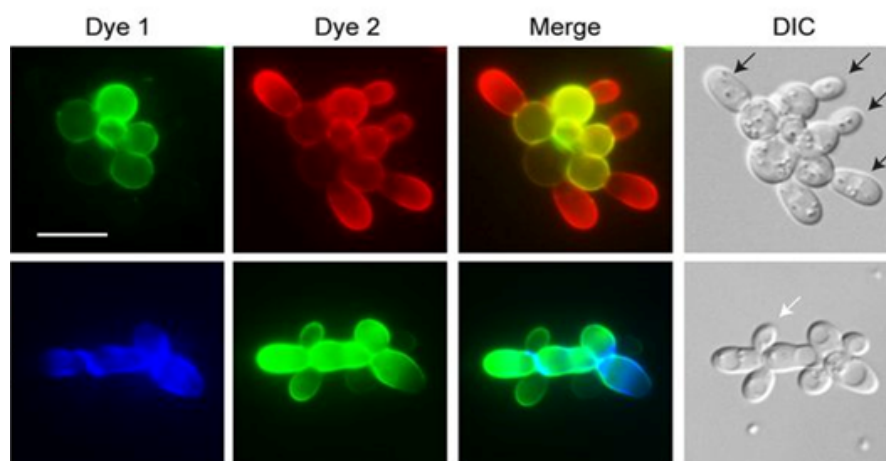


Figure 18: Examples of FITC-ConA/TRITC-ConA double-labeling (Upper) and CFW/FITC-ConA double-labeling (Lower) of cells from the 5-h time point. Distal buds are marked with black arrows. The axial bud in the lower panel is marked with a white arrow (Scale bar, 5 μ m) (Basu et al., 2016).

2.5 Yeast Genetic variation

Wild yeasts colonize natural habitats, implying little opportunity for sexual reproduction, resulting in low genetic segregation and recombination (Tibayrenc et al., 1991). Lab strains and strains used in food microbiology are derived from wild isolates that have been adapted to grow in the laboratory, so the evolutionary rates of these strains exceed those of certain wild isolates (Ronald et al., 2006). In addition, the process of picking single colonies favours the accumulation of genetic variations, such as point mutations, indels (insertion and deletions), and changes in the ploidy level, among others (Wills, 2007). For example, the reference strain S288c, which is commonly used in research laboratories (Mortimer and Johnston, 1986), acquired a mutation in transcription factor Flo8 that rendered it unable to trigger filamentous growth (Dowell et al., 2010; Liu et al., 1993). Recently, the genomes of different isolates of *S. cerevisiae* from diverse origins were examined. This analysis revealed a human role in genetic variability because *S. cerevisiae* strains did not pool according to their origin or isolation source, and yet, strains were separated based on human activity, such as fermentation or baking, among others, indicating partial domestication (Liti et al., 2009) (Figure 19). In the same study, natural isolates of *S. paradoxus*, which are not related to human activity, were grouped according to their geographical origin.

Through experiments, we are able to determine the phenotypes exhibited by different strains under specific conditions. These traits are related to the DNA that causes them; for this reason, it is essential to develop appropriate genetic technologies. The availability of the complete *S. cerevisiae* genome (Gof-

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feau et al., 1996) together with growing advances in genomics, transcriptomics and metabolomics have helped to elucidate the genetic factors that control different traits. Moreover, yeast genetic variation has been analysed to identify key genes that are useful for improving the alcoholic fermentation process, such as nutrient limitation, ethanol stress or nitrogen requirements (Beltran et al., 2005; Dimitrov et al., 2009; Tesnière et al., 2013; Zhang et al., 2017).

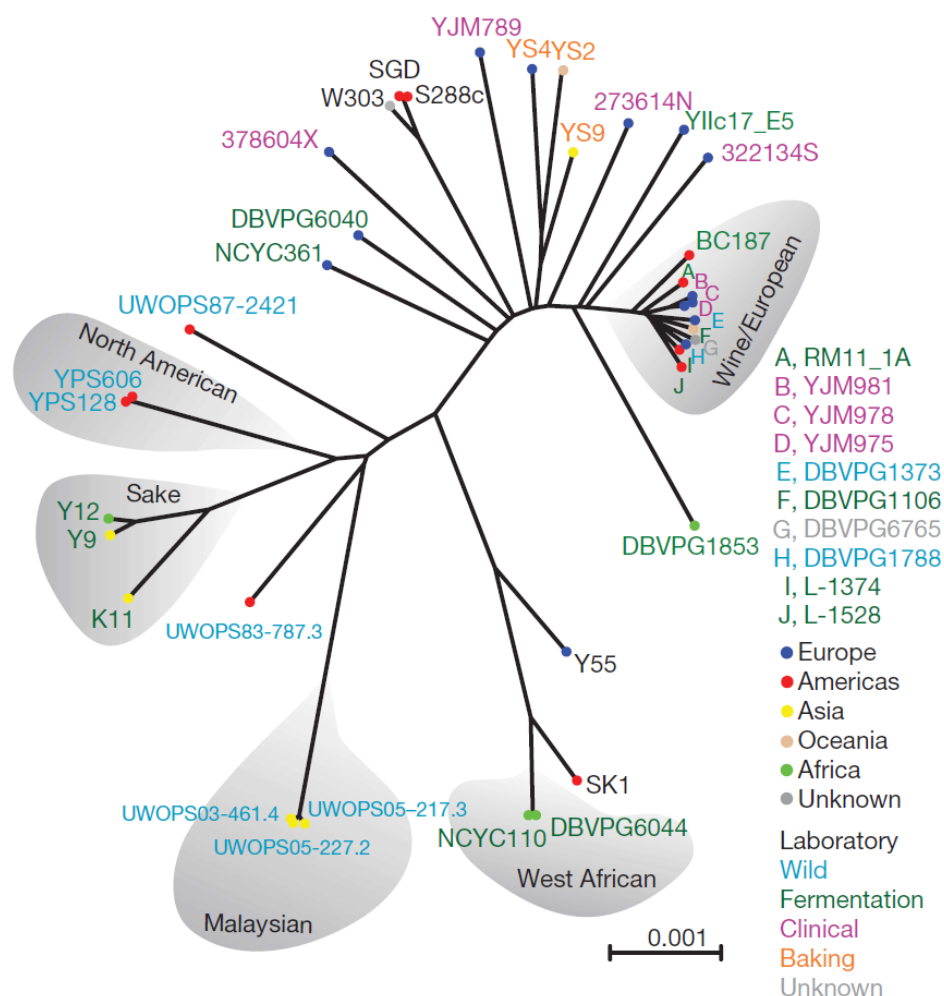


Figure 19: *S. cerevisiae* strains with clean lineages highlighted in grey, with color indicating source (name) and geographic origin (dots) (Liti et al., 2009).

2.5.1 Phenotype to genotype

More recently, insight into genotype to phenotype (reverse) approaches has increased based on knowledge gained from linkage studies using modern genotyping techniques. Conversely, forward genomic approaches involve the association of a determinate trait with the genetic mechanism that causes it (from phenotype to genotype). In yeast, the genetic bases for several traits, such as resistance to antibiotics, chemicals, carbon source utilization and certain oenological phenotypes, are complex and have been identified based on multiple Quantitative Trait Loci (QTL) (Cubillos, 2016; Warringer et al., 2011). Additionally, genetic variation in yeast has been queried to identify QTLs, which play roles in ethanol tolerance, acetic acid production or spontaneous mitochondrial genome instability (Dimitrov et al., 2009; Foss et al., 2007; Marullo et al., 2007). This technique has also been applied to study genetic factors that are involved in filamentous growth (Song et al., 2014b), identifying new genetic determinants of yeast invasive growth and indicating the coevolution of proteins within complexes to drive phenotypes.

2.5.2 QTL mapping

The first step in QTL mapping is to cross two parental strains that differ genetically for a trait of interest to generate a segregant population. The most common molecular markers are nucleotide polymorphisms (SNPs), polymorphic insertions or deletions (indels) and simple sequence repeats (microsatellites). QTLs are mapped in the segregation progeny of crosses between genetically divergent strains (linkage mapping); thus, QTLs may be genes or DNA regions that are involved in complex traits at different levels. As a result of crossing, heterosis (hybrid vigour) is observed in segregant strains (Steinmetz et al., 2002).

Briefly, different methods are available to map QTLs (Figure 20). One method is bulk segregant analysis (BSA), which involves selective genotyping of either two phenotypically distinct sub-population (bulks) of segregants (Michelmore et al., 1991) or two distinct populations, one grown under control conditions and the other under environmental pressure (Bloom et al., 2013; Parts et al., 2011; Segrè et al., 2006; Wilkenning et al., 2014). A second method is individual segregant analysis (ISA), in this method, each segregant is individually phenotypically and genotypically characterized to detect genomic regions (Foss et al., 2007; Martí-Raga et al., 2017; Marullo et al., 2007; Steinmetz et al., 2002). The third method is reciprocal hemizygosity scanning (RHS), in which a hemizygous deletion in a hybrid genetic background is constructed (Wilkenning et al., 2014). In this last study, the three approaches were used for

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QTL mapping on *Saccharomyces cerevisiae*.

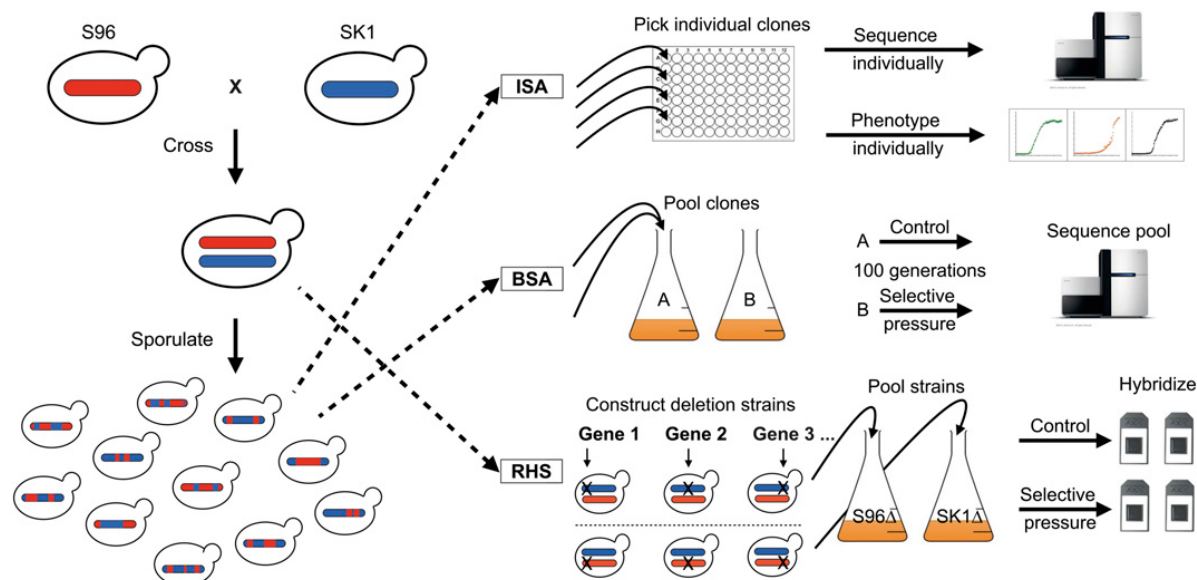


Figure 20: Different strategies to map QTL (Wilkening et al., 2014).

One strategy broadly used to identify genes responsible for natural phenotypic variation in complex traits is linkage analysis. Linkage analysis is based on a statistical approach, whereby the probability that a gene involved in a trait is linked to a genetic marker (commonly SNPs) is measured. After identifying a QTL, the critical DNA interval size must be reduced to select good candidate genes. The final step involves the validation of these genes by reciprocal hemizygosity, allele swapping or mutational analysis (Steinmetz et al., 2002) (Figure 21).

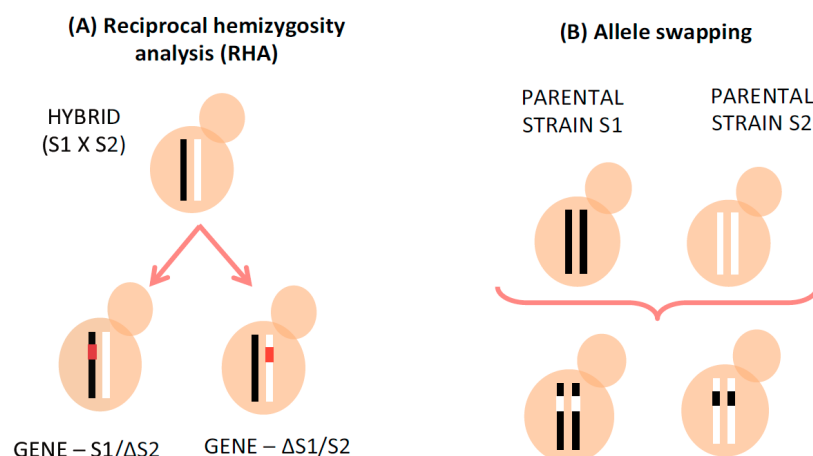


Figure 21: QTL validation methods (Martí-Raga, M., doctoral thesis, 2015).

QTL mapping has been successfully applied in oenology to identify genes related to nitrogen requirements (Gutiérrez et al., 2013; Jara et al., 2014), fermentation performance (Salinas et al., 2012; Zimmer et al., 2014), yeast adaptation to second fermentation in sparkling wine (Martí-Raga et al., 2017) and low-temperature fermentation (García-Ríos et al., 2017).

In summary, QTL mapping is useful both to increase basic knowledge of the yeast genetic architecture and to identify industrially relevant alleles to select new strains (Marullo and Dubourdieu, 2010; Dufour et al. 2013).

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Results

CHAPTER 1

The production of aromatic alcohols in wine yeast is modulated by nutrient availability

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Abstract

Aromatic alcohols are produced by yeast during alcoholic fermentation via the Ehrlich pathway. Production of aromatic alcohols by non-*Saccharomyces* wine yeasts has been poorly studied. Additionally, regulation of this pathway in *S. cerevisiae* is not completely understood. Variations in nutrient availability have been associated with changes in the synthesis of these compounds by yeast. Here, we studied how different wine yeast species modulate the synthesis patterns of aromatic alcohol production depending on glucose, nitrogen and aromatic amino acid availability. Nitrogen limitation strongly promoted the production of aromatic alcohols in all strains, whereas low glucose generally inhibited it. Increased aromatic amino acid concentrations stimulated the production of aromatic alcohols in all of the strains and conditions tested. Thus, there was a clear association between the nutrient conditions and production of aromatic alcohols in most of the wine yeast species analysed.

Key Words: Phenylethanol, tyrosol, tryptophol, alcoholic fermentation, Ehrlich pathway

Introduction

S. cerevisiae is a model microorganism used in many industrial applications due to its ability to synthesize ethanol from diverse sugar sources. For this reason, this budding yeast is commonly employed in several technological processes such as brewing, baking and winemaking. In winemaking, alcoholic fermentation involves the succession and coexistence of a large diversity of yeast genera and species. At the beginning of fermentation, non-*Saccharomyces* yeast populations are high, and genera such as *Hanseniaspora*, *Issatchenkia*, *Starmerella* (sym. *Candida*), *Torulaspora* and *Metschnikowia* are commonly found. During fermentation, non-*Saccharomyces* species are replaced by *S. cerevisiae* due to its superior performance during this process and its ability to produce high ethanol concentrations (Fleet, 2003; Heard and Fleet, 1988). However, several studies recently confirmed the positive contributions of these non-*Saccharomyces* yeasts to wine: they are able to produce certain additional aromatic compounds that improve the flavour and bouquet (Fleet, 2003; Jolly et al., 2014; Lleixà et al., 2016). Although ethanol is the major by-product of alcoholic fermentation, yeasts also produce other alcohols such as aromatic (fusel) alcohols derived from the aromatic amino acids that are present in grapes. Tyrosine (Tyr), tryptophan (Trp) and 2-phenylalanine (Phe) are converted to tyrosol (TyrOH), tryptophol (TrpOH) and 2-phenylethanol (PheOH), respectively, via yeast metabolism through the Ehrlich pathway, particularly under nitrogen-limiting conditions (Ehrlich, 1907; Hazelwood et al., 2008). Those alcohols are largely used as additives in foods and beverages, and PheOH specifically is widely used in the cosmetics industry due to its rose-like scent (Fabre et al., 1998). Fusel alcohols positively influence the flavour and bouquet of wines and are also of interest due to their potential bioactivity for humans. TyrOH and TrpOH have been described as an antioxidant and a sleep inducer, respectively (Cornford et al., 1981; Giovannini et al., 1999). Moreover, recent studies in yeasts have demonstrated that these three aromatic alcohols are involved in growth regulation (Avbelj et al., 2015; Zupan et al., 2013) and are suggested to be quorum sensing molecules (Dickinson, 1996; Lorenz et al., 2000; Chen and Fink 2006). Many microbes use quorum-sensing communication to transmit information about population density and environmental conditions (Bassler, 2002; Fuqua et al., 1994); in yeast, filamentous growth has also been associated with these quorum-sensing molecules (Hornby et al., 2001; Kruppa, 2009; Sprague and Winans, 2006).

In some yeast species, such as *S. cerevisiae*, the aromatic alcohols secreted into the extracellular medium are recognized by other cells which are able to modulate their behaviour accordingly. More-

over, as mentioned above, nitrogen-limiting conditions increase the production of aromatic alcohols, leading to stronger filamentous growth in *S. cerevisiae*. In a recent study (González et al., 2017), we studied the filamentous growth behaviour of a collection of *S. cerevisiae* strains in the presence of different alcohols and observed higher effects for ethanol than for aromatic alcohols in terms of its ability to promote stronger filamentous growth under nitrogen-limiting conditions. On the other hand, studies performed with non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Pichia kudriavzevii* and *Pichia fabianii*, have reported that these yeasts also show changes in their growth mode under nutrient-limited conditions (nitrogen or carbon) or other stress factors (Pu et al., 2014; van Rijswijk et al., 2015). Therefore, the finding that aromatic alcohols exert different responses on morphogenesis in a yeast-dependent manner shows that these molecular signals may be species-specific (Chen and Fink, 2006).

The aim of this study was to evaluate the production of aromatic alcohols (TyrOH, TrpOH and PheOH) by four wild non-*Saccharomyces* wine strains in different media (containing different concentrations of nitrogen and carbon) and compare them to a “model” *S. cerevisiae* wine strain.

Materials And Methods

Yeast strains and inoculum preparation

The following wine yeast species were used in this study: commercial *Saccharomyces cerevisiae* QA23 (Lallemand, Canada) and four wild non-*Saccharomyces* isolates from the winemaking Priorat region of Spain, specifically Hv4 (CECT 13130, *Hanseniaspora uvarum*), Cz4 (CECT 13129, *Starmerella bacillaris* (sym. *Candida zemplinina*)), Mpp (CECT 13131, *Metschnikowia pulcherrima*) and Tdp (CECT 13135, *Torulaspora delbrueckii*) (Padilla et al., 2016). Yeast strains were taken from stocks preserved at -80 °C in glycerol and grown on YPD plates (2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose and 2 % (w/v) agar) for 48-72 h at 28 °C. Then, cells were cultured for 24 h in 50 mL of YPD medium at 28 °C and 120 rpm and transferred into 250 mL of fresh minimal medium (1X Yeast Nitrogen Base without amino acids or ammonia, 2 % (w/v) glucose, 10 mM $(NH_4)_2SO_4$) and cultured for 2 days at 28 °C and 120 rpm.

Fermentation conditions

Fermentation was performed in three different media based on synthetic must (Beltran et al., 2004), containing modified sugar and nitrogen concentrations: standard synthetic must (SM: 200 g/L sugars and 300 mg of N/L of yeast assimilable nitrogen (YAN)), low glucose must (LGM: 20 gglucose/L and 300 mgN/L of YAN) and low nitrogen must (LNM: 200 gsugars/L and 100 mgN/L for YAN) (Table 1.1). Fermentations were inoculated using precultures in minimal medium to obtain an initial population of 2×10^6 cells/mL. To study the production of aromatic alcohols (TyrOH, TrpOH and PheOH) in each must, aromatic amino acids (Tyr, Trp and Phe), which are precursors of those alcohols, were added at two concentrations (1x and 5x, see Table 1.1). The increase of the concentrations of aromatic amino acids was at expense of the rest of the amino acids, maintaining the same final concentration of nitrogen. Fermentations were performed in triplicate at 28 °C with continuous orbital shaking (120 rpm). Cell populations were evaluated daily by measuring the optical density (OD_{A600nm}), and 1 mL of wine was stored to quantify the extracellular concentrations of TyrOH, TrpOH and PheOH by UHPLC after five days of fermentation for LGM and after seven days for SM and LNM.

Another set of alcoholic fermentations in SM and 5x aromatic amino acids was performed to associate the synthesis and secretion of aromatic alcohols with growth phases. Then, intracellular and extracellular concentrations of aromatic alcohols were analysed during the first 48 h (0 h, 12 h, 24 h, 48 h).

Table 1.1: Reducing sugar, nitrogen and aromatic amino acid contents of the different synthetic musts used in this study.

Synthetic Must	[Aromatic amino acid]	Nitrogen concentration					
		Sugar concentration (g/L)		Aromatic amino acid (g/l)			Yeast assimilable nitrogen (YAN-mg nitrogen/L)
		Glucose	Fructose	Tyrosine	Tryptophan	Phenylalanine	
Synthetic Must (SM)	1x	100	100	0,020	0,174	0,038	300
	5x	100	100	0,098	0,871	0,189	300
Synthetic Low Glucose Must (LGM)	1x	20	0	0,020	0,174	0,038	300
	5x	20	0	0,098	0,871	0,189	300
Synthetic Low Nitrogen Must (LNM)	1x	100	100	0,007	0,058	0,013	100
	5x	100	100	0,033	0,290	0,063	100

Aromatic alcohol analysis

Aromatic alcohols were detected and quantified by performing liquid chromatography triple quadrupole mass spectrometry (LCQqQ). For intracellular samples, prior extraction using boiling buffered ethanol (Gonzalez et al., 1997) was necessary. Briefly, a volume of culture corresponding to 10^7 cells was centrifuged at 5000 rpm for 10 min, and 1 mL of boiling absolute ethanol buffered with 2 mL of 1 M Hepes (pH 7.5) was added directly into the tube. Subsequently, the mix was incubated for 3 min at 80 °C, and the supernatant was evaporated at 45 °C using a SpeedVac (Thermo Fisher Scientific, USA). The residue was resuspended in 1 mL of sterile MilliQ water. Then, 100 µL of this extract or of the extracellular sample was diluted 1:10 in MeOH (≥ 99.7 % (LC-MS), Sigma Aldrich, USA), centrifuged at 15000 rpm and maintained at 4 °C for 10 min. Samples were then serially diluted in MeOH to a final dilution of 1:100 for intracellular samples and 1:1000 for extracellular samples. LCQqQ was performed to analyse diluted extracts.

A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, USA) was used to evaluate TyrOH, TrpOH and PheOH. The chromatographic column was an Xbridge Shield RP18 (150 x 2.1 mm i.d., 3.5-µm particle size) (Waters). The mobile phases were 0.2 % acetic acid (≥ 99.7 %, Sigma-Aldrich, USA) in water (MilliQ system, Millipore) (solvent A) and ACN (solvent B). The flow rate was 0.6 mL/min. To validate our quantitative method, calibration curves, linearity, precision, accuracy and the limits of detection and quantification were determined by analysing serial standard dilutions prepared in ultrapure LC-MS water and pooled samples spiked with standard solutions. The obtained validation parameters for the method for TyrOH (≥ 99 % Sigma-Aldrich, USA), PheOH (≥ 98 %, Sigma-Aldrich, USA) and TrpOH (≥ 99 %, Sigma-Aldrich, USA), permitted the quantification of studied compounds in extract samples.

Aromatic amino acid analysis

Final concentrations of Tyr, Phe and Trp were determined using an Agilent 1100 Series High-performance Liquid Chromatograph (Agilent Technologies, Germany) (Gómez-Alonso et al., 2007). In brief, fifty microlitres from each sample was used in a derivatization reaction with DEEMM. Separation was performed in an ACE HPLC column (C18-HL) with a particle size of 5 µm (250 x 4.6 mm) that was controlled thermostatically at 20 °C. Two eluents were used: eluent A contained 25 mM acetate buffer (pH = 5.8) with 0.02 % sodium azide, and eluent B contained an 80:20 mixture of acetonitrile and

methanol. The flow rate was 0.9 ml/min. The concentration of each compound was calculated using internal (L-2-aminoadipic acids, 1 g/l) and external standards.

Statistical analysis

Three biological replicates were performed for each experiment. Tukey's test was used to generate p-values. To better understand how different measured parameters influenced the production of aromatic alcohols, Principal Component Analysis (PCA) was performed using XLSTAT Software.

Results

Production of aromatic alcohols by different wine yeast species

In addition to aromatic alcohols, residual sugar and maximal growth were determined at the end of fermentation (Table 1.2). In LGM, all sugars were consumed by yeast. At high sugar concentrations (SM and LNM), only *S. cerevisiae* was able to consume all sugars present in the must, followed by *T. delbrueckii*. High sugar concentrations were present at the end of the process for the other three species, particularly under nitrogen restriction (LNM). In general, the increase in aromatic alcohol precursors affected the maximal growth and sugar consumption.

In SM, all strains tested were able to produce the three aromatic alcohols, although *S. bacillaris* produced much lower levels (Fig 1.1A). Under the 1x condition, *S. cerevisiae* QA23 exhibited the maximum production of aromatic alcohols, together with the highest growth and total consumption of sugars (Table 1.2). TrpOH was the primary aromatic alcohol produced, followed by PheOH and TyrOH. Notably, synthetic must contains higher concentrations of Trp compared to Phe or Tyr (see Table 1.1). On the other hand, non-*Saccharomyces* species synthesized lower levels of aromatic alcohols; specifically, *S. bacillaris* produced the lowest concentrations (below 5 μ M TyrOH and PheOH, and no detectable TrpOH). Among non-*Saccharomyces* species, *T. delbrueckii* presented the highest growth and sugar consumption (although more than 20 g/l residual sugars was present after seven days of fermentation), *S. bacillaris* demonstrated the lowest growth, and *M. pulcherrima* demonstrated the lowest sugar consumption.

Higher concentrations of aromatic amino acids in the media (5x) resulted in higher production of aromatic alcohols, despite the lack of proportional increases in alcohols with increases in precursors (Fig 1.1A). Under the 1x condition, TrpOH was the major aromatic alcohol produced with the exception of

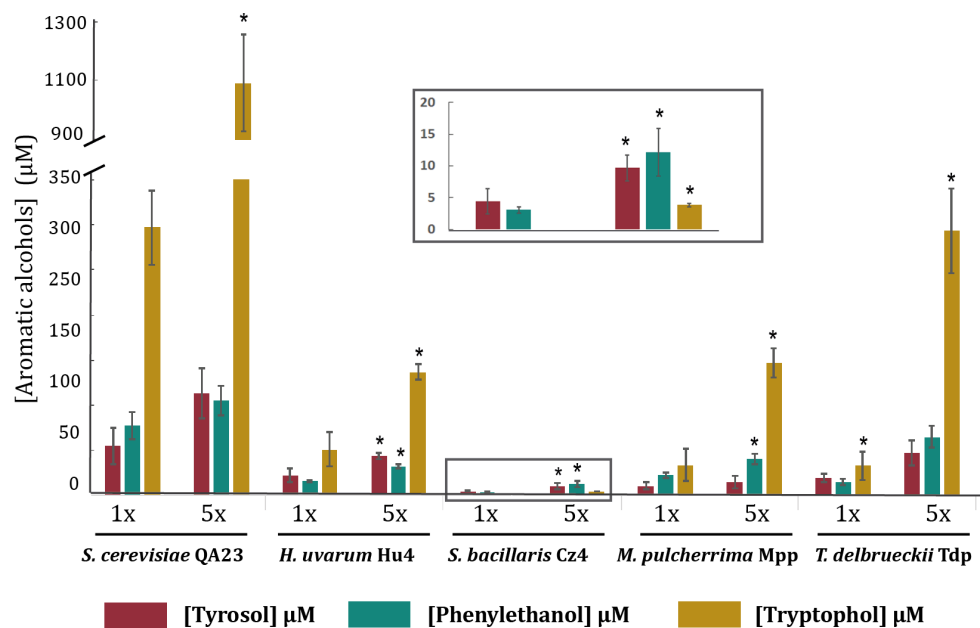
S. bacillaris, which synthesized more PheOH, but at much lower levels. Although not always significant, TyrOH was produced at lower concentrations. Moreover, although the differences were not statistically significant, the presence of higher concentrations of aromatic amino acids affected cell growth in all species: growth was lower for *S. cerevisiae*, *H. uvarum*, and *T. delbrueckii* but higher for *S. bacillaris* and *M. pulcherrima* (Table 1.2).

To better understand how different variables affected the strains, PCA was performed (Fig 1.1B). This PCA analysis accounted for 90.17 % of the variance: all variables were primarily explained by component F1 (74.86 %). Triplicates appeared together in the biplot, and PCA clearly separated *S. cerevisiae* and *T. delbrueckii* from the other species, which grouped together, primarily due to their superior growth and higher consumption of sugars. In those strains, samples from media containing different aromatic amino acid contents grouped separately based on the higher production of aromatic alcohols under the 5x condition. Two additional PCA were performed using only the data from the 1x and 5x conditions (Fig S1.1). This PCA explains 92.23 % and 91.7 % of the variance under the 1x and 5x conditions, respectively. As expected, PCA generated two clusters for *S. cerevisiae* and *T. delbrueckii*, individually, and a third cluster associated with and the rest of the non-*Saccharomyces* strains.

Table 1.2: Maximal growth and sugar consumption achieved by each yeast species and in each medium. The results are expressed as the average; the standard deviation (SD) was calculated from three biological replicates. Statistical significance was calculated by comparing three different musts within the same strain; letters indicate significant differences (p-value < 0.05).

Yeast strain	Must	[Aromatic amino acid]	Maximal growth		Consumed Sugar	
			600nm	SD	g/l	SD
<i>S. cerevisiae</i> QA23	SM	1x	11,47 ^c	1,32	199,95 ^b	0,00
		5x	9,22 ^c	1,01	194,60 ^b	1,60
	LNM	1x	7,64 ^b	1,66	198,58 ^b	1,00
		5x	7,25 ^b	0,97	197,26 ^b	1,75
	LGM	1x	4,80 ^a	0,37	19,97 ^a	0,04
		5x	4,20 ^a	0,31	19,49 ^a	0,35
<i>H. uvarum</i> Hu4	SM	1x	4,72 ^c	0,14	145,40 ^c	10,23
		5x	4,33 ^c	0,24	136,75 ^c	3,42
	LNM	1x	1,95 ^b	0,38	78,43 ^b	6,45
		5x	2,29 ^b	0,36	81,55 ^b	6,00
	LGM	1x	3,89 ^a	0,46	19,93 ^a	0,11
		5x	3,76 ^a	0,12	19,90 ^a	0,00
<i>S. bacillaris</i> Cz4	SM	1x	3,65 ^a	0,33	123,66 ^c	3,49
		5x	4,44 ^c	0,25	127,40 ^c	2,59
	LNM	1x	2,45 ^b	0,35	71,85 ^b	2,36
		5x	2,71 ^b	0,32	81,35 ^b	8,61
	LGM	1x	3,74 ^a	0,16	19,913 ^a	0,12
		5x	2,38 ^b	0,39	19,653 ^a	0,26
<i>M. pulcherrima</i> Mp4	SM	1x	3,67 ^b	1,08	106,18 ^d	0,06
		5x	6,57 ^c	0,65	100,20 ^d	7,80
	LNM	1x	3,36 ^b	0,38	37,66 ^b	4,88
		5x	5,36 ^a	1,02	70,39 ^c	15,29
	LGM	1x	4,49 ^a	0,56	19,99 ^a	0,00
		5x	5,72 ^a	0,20	19,82 ^a	0,26
<i>T. delbrueckii</i> Tdp	SM	1x	10,31 ^c	0,40	176,34 ^c	2,72
		5x	8,35 ^c	1,58	177,82 ^c	2,00
	LNM	1x	5,95 ^b	0,66	107,81 ^b	6,03
		5x	5,00 ^b	0,79	93,03 ^b	10,89
	LGM	1x	3,06 ^a	0,50	19,99 ^a	0,00
		5x	4,28 ^d	0,08	19,95 ^a	0,04

A



B

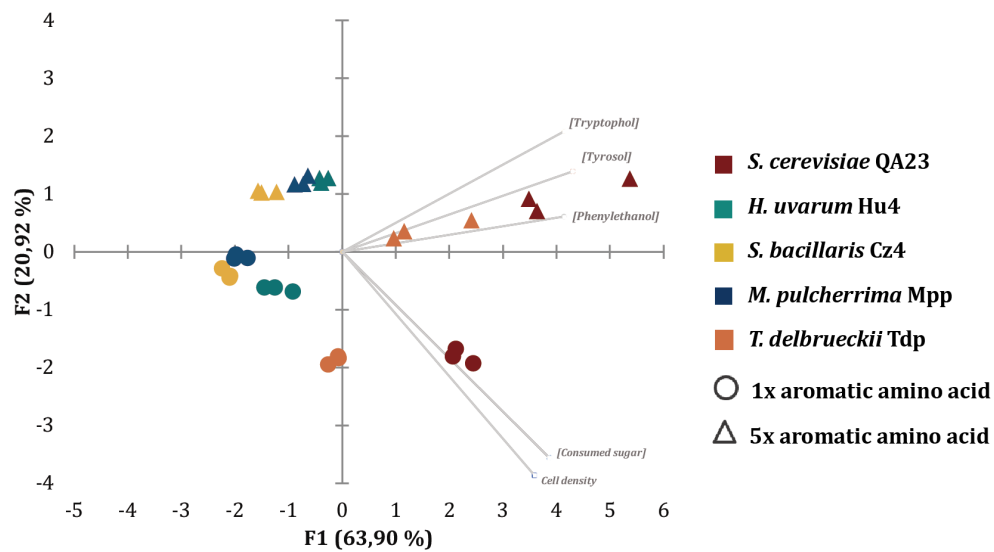


Figure 1.1: Production of aromatic alcohols by different yeast species in standard must (SM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in SM (300 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05. (B) Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation.

Synthesis of aromatic alcohols under low nitrogen conditions

To analyse the effects of nitrogen concentration on the synthesis of aromatic alcohols, fermentations were carried out using low nitrogen synthetic must (100 mg of N/L) at two different concentrations of aromatic amino acids (1x and 5x). In LNM, all strains grew poorly and, in general, fermented less sugar than in SM (Table 1.2), with the exception of *S. cerevisiae*, which consumed practically all sugars present in the media despite nitrogen limitation. As in SM, TrpOH was the main fusel alcohol synthesized, except in *S. bacillaris*. This strain did not secrete TrpOH under SM conditions and produced the other aromatic alcohols at very low concentrations, despite consuming almost all of the precursors. The second aromatic alcohol most frequently secreted by non-*Saccharomyces* species was TyrOH, unlike *Saccharomyces*, which secreted PheOH (Fig 1.2).

When the precursors of these aromatic alcohols were five-fold higher, growth and sugar consumption during fermentation were not significantly affected, with the exception of Mpp. In this case, higher concentrations of aromatic amino acids in the medium resulted in a clear increase in growth, and sugar consumption was doubled in comparison to 1x condition. In terms of the production of aromatic alcohols, a significant increase in their concentrations was observed after the addition of precursors. However, TrpOH was the most relevant because the concentration of this alcohol was between 6 and 24 times greater than that under the 1x condition. In both cases, aromatic amino acids were practically exhausted, but production rates were higher under the 5x condition (Table 1.3 and Supplementary Table S1.1 for 5X).

PCA (Fig 1.2B) indicated a variance of 87.29 % (F1: 56.53 %; F2:27.76 %), and the variables were primarily explained by component 1. *S. cerevisiae* was clearly separated from non-*Saccharomyces* species largely due to its ability to generate a higher biomass and its elevated sugar consumption. Moreover, within this strain, samples from media containing different aromatic amino acid contents grouped separately due to the higher production of TrpOH and PheOH in 5x medium. In general, non-*Saccharomyces* species were also separated based on the concentration of aromatic amino acids (1x and 5x), primarily due to the higher amounts of TrpOH and TyrOH under the 5x condition, with the exception of the *S. bacillaris* Cz4 strain at 5x, which presented similar concentrations under both conditions.

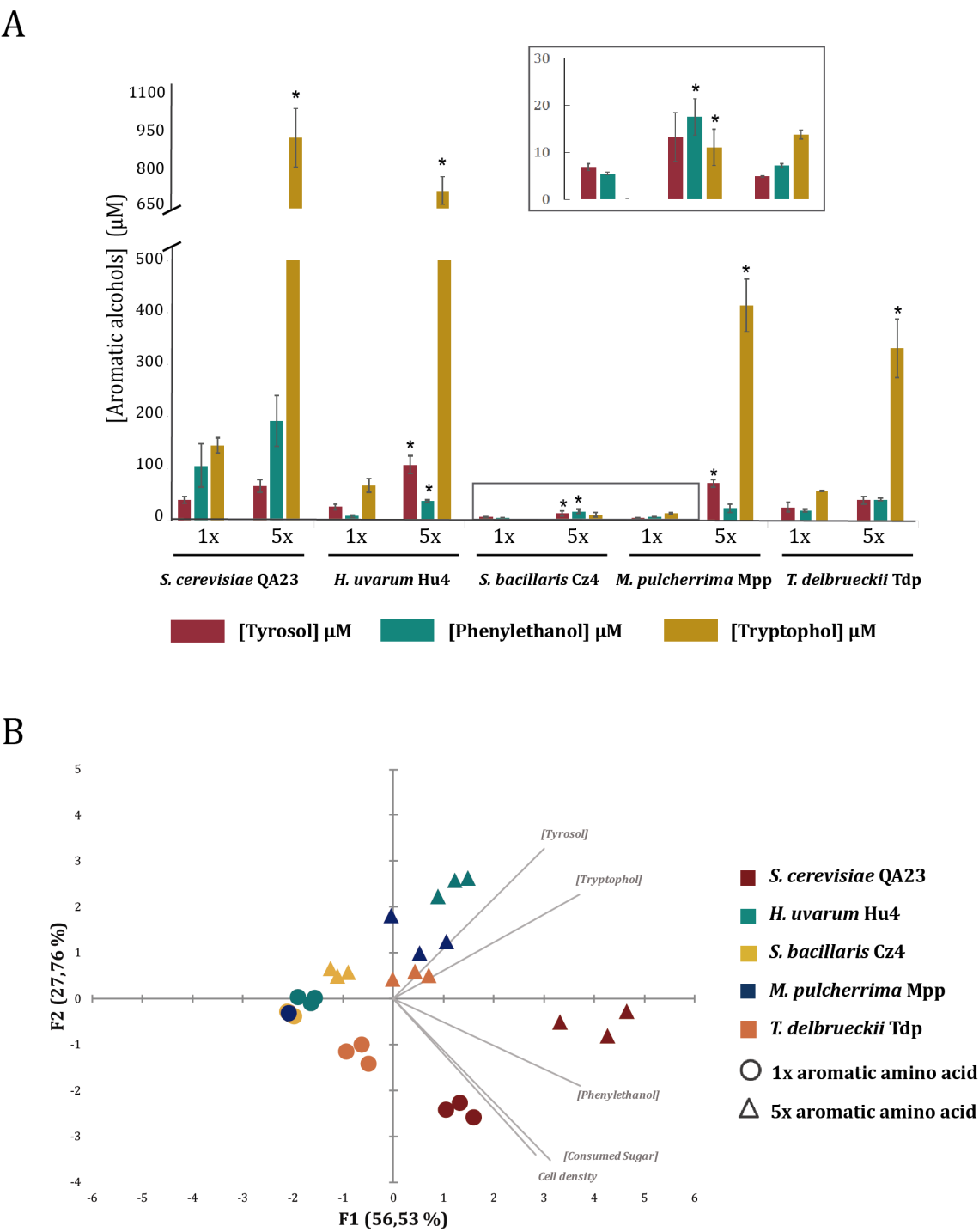


Figure 1.2: Production of aromatic alcohols by different yeast species under nitrogen limitation (LNM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LNM (100 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x). (B) Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

LNМ media contained the same amount of sugars as SM, but the concentrations of YAN and aromatic amino acids were three-fold lower. Despite these lower amounts of precursors, the production of aromatic alcohols was similar in both media, indicating a higher yield of alcohol in LNМ. Thus, the ratio between each aromatic amino acid and its respective aromatic alcohol was calculated (Table 1.3). For all strains, with the exception of *M. pulcherrima*, this rate of transformation was significantly higher in LNМ than in SM, explaining why the final concentration of aromatic alcohols was similar despite the presence of fewer precursors. In *S. cerevisiae* QA23 grown in LNМ under the 1x condition, all Tyr and Phe present in the medium were converted into their corresponding alcohols (ratios of approximately 1). In non-*Saccharomyces* species, the largest increase in this ratio occurred for TrpOH and TyrOH, corresponding to the higher production of these two aromatic alcohols by non-*Saccharomyces* species as opposed to *S. cerevisiae*, which synthesized more PheOH than TyrOH. When a PCA was performed using the data from SM and LNМ, 90.21 % of the variance was explained (F1: 50.2 %, F2: 40.01 %), which corresponded to the amounts of aromatic amino acids consumed; the Tyr/TyrOH ratio was largely explained by F1, and the remainder of the variables were explained by F2. The uptake of aromatic amino acids was negatively correlated with alcohol transformation ratios, confirming that higher precursor uptake resulted in lower aromatic alcohol production ratios. Due to the differences in aromatic amino acid uptake, as well as growth and sugar consumption, the PCA clearly separated both media, and the latter two variables, together with aromatic alcohol production, were responsible for *Saccharomyces* forming a separate cluster versus non-*Saccharomyces* species.

Table 1.3: Production yield of aromatic alcohols in SM and LNM. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account three biological replicates. In all cases, comparisons are within the same species; letters indicate significant differences (p-value < 0.05).

Yeast strain	Must	[TyOH] / [Tyr]		[PheOH] / [Phe]		[TrpOH] / [Trp]	
		($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD
<i>S. cerevisiae</i> QA23	SM	0,50 ^a	0,19	0,33 ^a	0,09	0,34 ^a	0,08
	LNM	1,01 ^b	0,18	1,03 ^b	0,06	0,50 ^b	0,05
<i>H. uvarum</i> Hu4	SM	0,20 ^a	0,07	0,06 ^a	0,00	0,05 ^a	0,03
	LNM	0,72 ^b	0,14	0,10 ^b	0,03	0,23 ^b	0,05
<i>S. bacillaris</i> Cz4	SM	0,04 ^a	0,01	0,01 ^a	0,00	ND	-
	LNM	0,19 ^b	0,02	0,07 ^b	0,00	ND	-
<i>M. pulcherrima</i> Mpp	SM	0,09 ^a	0,04	0,09 ^a	0,01	0,03 ^a	0,02
	LNM	0,13 ^b	0,01	0,09 ^a	0,01	0,04 ^a	0,00
<i>T. delbrueckii</i> Tdp	SM	0,17 ^a	0,04	0,06 ^a	0,02	0,03 ^a	0,02
	LNM	0,71 ^b	0,26	0,25 ^b	0,04	0,19 ^b	0,00

Synthesis of aromatic alcohols under low sugar conditions

To analyse the effects of sugar concentration on aromatic alcohol synthesis, the same experiment was performed using a medium containing the same nitrogen concentration as SM but with low glucose content (20 g/l). All species depleted the sugars in LGM media (Table 1.2), and all were able to produce the three aromatic alcohols (Fig 1.3A) but at much lower concentrations than those obtained with SM or LNM. Increased amounts of precursors (5x condition) also induced a significant increase in aromatic alcohol production for all species, particularly *T. delbrueckii*, which produced almost no aromatic alcohols under the 1x condition but exhibited higher synthesis of aromatic alcohols under the 5x condition, as well as significantly better growth (Table 1.2). Interestingly, in this medium, *T. delbrueckii* produced PheOH as the main aromatic alcohol, and *M. pulcherrima* produced the highest amount of TrpOH obtained under either AAA condition, even higher than *S. cerevisiae*. Notably, *M. pulcherrima* reached the highest cell density in this low glucose media, primarily under the 5x condition. Once again, *S. bacillaris* produced very low amounts of aromatic alcohols and only produced TrpOH under the 5x condition, despite exhibiting poor growth (Fig 1.3A; Table 1.2).

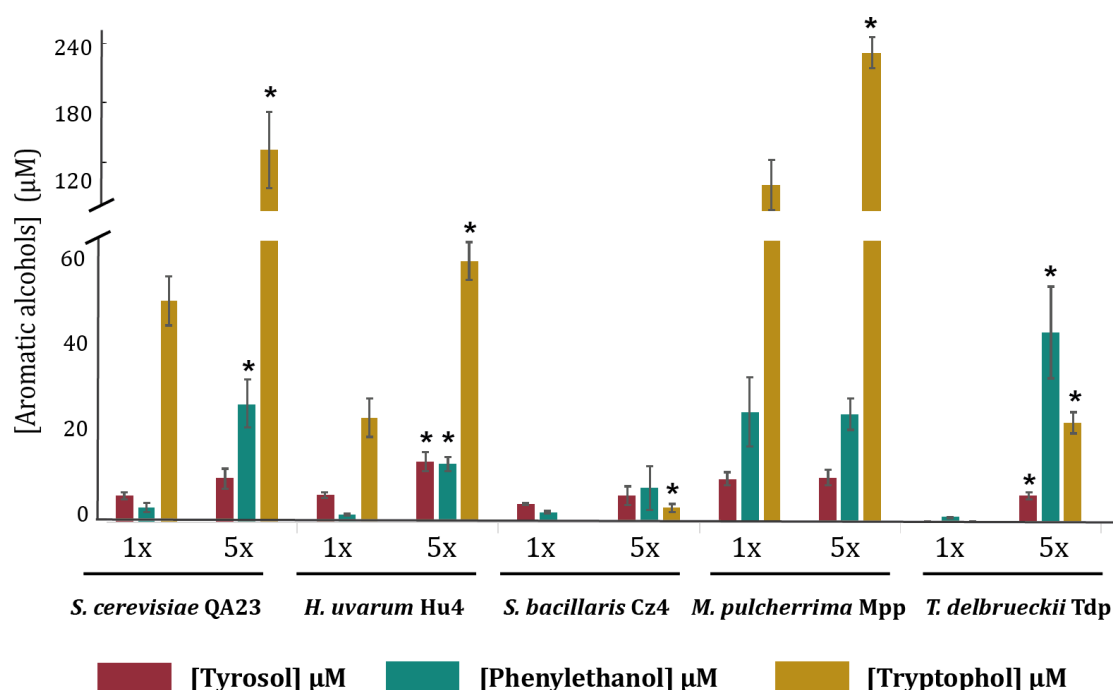


Figure 1.3: Production of aromatic alcohols by different yeast species in low glucose must (LGM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LGM (300 mg/l nitrogen and 20 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

PCA accounted for a variance of 71.02 % (F1: 49.30 %, F2: 21.72 %) (Fig S1.2). All factors were primarily explained by the F1 component, with the exception of sugar consumed, which was associated with F2. In general, the 1x and 5x conditions clustered separately, but in LGM, *S. cerevisiae* was not separated from non-*Saccharomyces* species. Instead, *M. pulcherrima* grouped separately from the others due to its high growth and aromatic alcohol production, while *S. bacillaris* under the 5x condition was in the other cluster.

Synthesis and secretion of aromatic alcohols during fermentation

To study the synthesis and secretion of aromatic alcohols in different species during alcoholic fermentation and growth, fermentations in SM medium with 5X aromatic amino acids were repeated, and samples were obtained to analyse the intracellular and extracellular concentrations of these molecules during the first 48 h (Fig 1.4). Initially, cells contained basal intracellular concentrations of fusel alcohols (from preculture growth), and then fusel alcohols increased considerably at 12 h, matching the early exponential phase. Then, during the period between the end of the exponential phase and the beginning of stationary phase, the intracellular concentration of aromatic alcohols decreased drasti-

cally, particularly for TrpOH and PheOH, likely due to their secretion into external media. Once in stationary phase, the intracellular levels of those alcohols maintained basal concentrations, similar to those observed at the beginning of the process. This decrease in intracellular levels coincided with the increase in aromatic alcohols observed in the extracellular medium, which slowed down when the cells were in mid-stationary phase. This profile was observed in *S. cerevisiae* (Fig 1.4A), *H. uvarum* (Fig 1.4B) and *M. pulcherrima* (Fig 1.4D). However, in *T. delbrueckii*, PheOH demonstrated a peak at 12 h as in the other species, but TrpOH peaked at 24 h (Fig 1.4E), likely due to the low population of this strain (1×10^6 cells/mL) in early exponential phase compared to the other strains ($>5 \times 10^6$ cells/mL) in mid-exponential phase. Therefore, the synthesis of PheOH coincided with early exponential phase, and that of TrpOH coincided with the mid-late exponential phase. Due to this delay in growth and thus aromatic alcohol synthesis, accumulation in the extracellular medium was also delayed, resulting in the detection of very low amounts of aromatic alcohols at 24 h outside the cells. Finally, in *S. bacillaris* (Fig 1.4C), the intracellular accumulation of aromatic alcohols was very low (as expected given the low concentrations detected in the extracellular medium) and delayed due to the longer lag phase of this strain compared with the other strains. Moreover, in this strain, PheOH was the major aromatic alcohol that accumulated both inside and outside of the cell, unlike the other species.

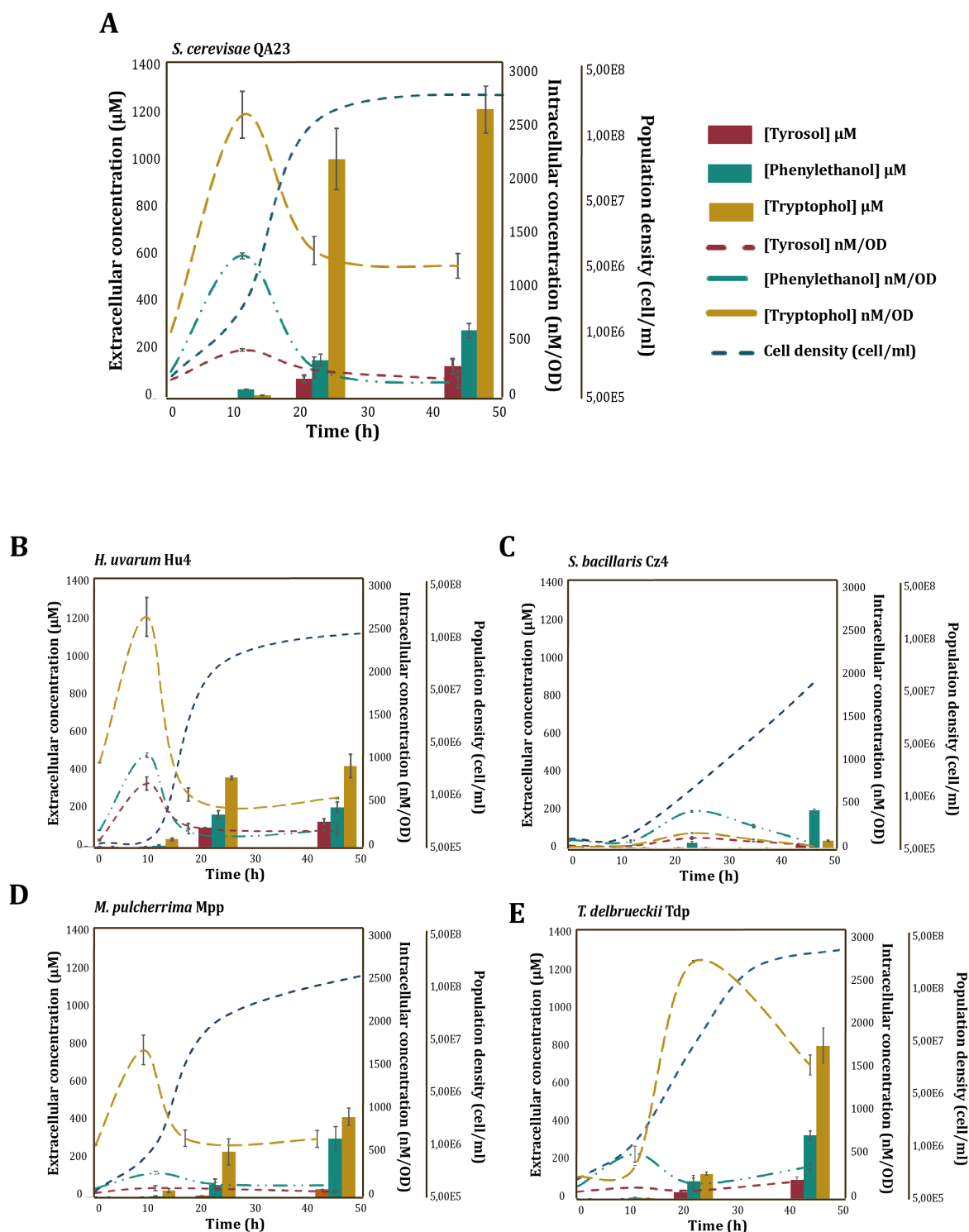


Figure 1.4: Monitoring the intra- and extracellular production of aromatic alcohols and cell populations during fermentation with different yeast species. Fermentations were carried out for 48 h at 28 °C in standard must (SM) supplemented with a five-fold increase in aromatic amino acids (5X). (A) *S. cerevisiae* QA23, (B) *H. uvarum* Hu4, (C) *S. bacillaris* Cz4 (D) *M. pulcherrima* Mpp, and (E) *T. delbrueckii* Tdp. Aromatic alcohol concentrations are expressed as the average of biological triplicates.

Discussion

In this study, we analysed the synthesis of aromatic alcohols (TrpOH, PheOH and TyrOH), which may act as quorum sensing molecules, by different wine yeast species during alcoholic fermentation. Additionally, we studied how the concentrations of aromatic amino acids, yeast assimilable nitrogen and sugar affected the production of these aromatic alcohols.

S. cerevisiae QA23 produced higher concentrations of aromatic alcohols, and its production yield was highest in must with nitrogen restriction. These findings agree with previous studies that showed an inverse correlation between initial nitrogen concentration and the production of fusel alcohols (Beltran et al., 2005; Carrau et al., 2008; Jiménez-Martí, E. and del Olmo, 2008; Mouret et al., 2014), indicating that the closer the nitrogen concentration is to growth-limiting levels, the higher the yield of fusel alcohols. In fact, it has been considered that the Ehrlich pathway is activated to provide nitrogen from amino acids, such as the aromatic amino acids, which have a limited requirement for protein synthesis and growth. Thus, higher levels of these alcohols may be a positive signal for growth regulation in yeasts (Mas et al., 2014). Indeed, Chen and Fink (2006) reported that the production of these aut signalling alcohols is regulated by nitrogen; specifically, they are activated under nitrogen-poor conditions. On the other hand, the same authors stated that aromatic alcohol production is not affected by low concentrations of glucose; however, according to our results (Fig 1.4 and Table S1.1), *S. cerevisiae* produced significantly lower concentrations of fusel alcohols in LGM than in SM, despite the same total nitrogen content in both media. There are different potential explanations for this low production. First, it may be related to low cell density (the OD_{600nm} value in LGM was 4.80 ± 0.37 vs. 11.47 ± 1.32 and 7.64 ± 1.66 in SM and LNM, respectively). Additionally, cells at low density required lower protein synthesis, which may explain why cells consume fewer aromatic amino acids. Recent studies have associated the production of these alcohols with cell density, suggesting that high population density stimulates the synthesis of aromatic alcohols (Avbelj et al., 2015; Chen and Fink, 2006; Sprague and Winans, 2006; Wuster and Babu, 2009). However, in the case of low nitrogen (LNM), the production yield (with respect to the amount of precursor consumed) was higher despite a lower cell density than that in SM, indicating that cell density is not the sole variable that determines the synthesis of these alcohols. Second, the more rapid depletion of glucose in LGM may also be responsible for the low amounts of aromatic alcohols produced, as explained by Vidal et al. (2014). In their experiments, these authors observed that the accumulation of isoamyl alcohol halted after glucose exhaustion, despite the presence

of residual leucine, its precursor, suggesting that leucine degradation through the Ehrlich pathway was strongly dependent on the cytosolic availability of NADH and/or of the amino acceptor α -ketoglutarate. This was confirmed in glucose pulse experiments in the stationary phase that re-established the synthesis of the higher alcohol. Finally, differences in respiro-fermentative metabolism may also be partly responsible for this low accumulation; in *C. albicans*, the production of aromatic alcohols is higher under anaerobic growth than aerobic growth (Ghosh et al., 2008). In LNM and SM, the metabolism of *S. cerevisiae* is anaerobic due to the Crabtree effect; however, in LGM, a medium containing significantly lower amounts of sugar, *S. cerevisiae* metabolism is partially aerobic, which may result in decreased aromatic alcohol synthesis.

Aromatic alcohols are quantitatively the largest group of volatile components, and their presence, particularly phenylethanol, is essential for overall flavour quality. The Ehrlich pathway is active in other yeast such as *Kluyveromyces marxianus* and *Yarrowia lipolytica* (Celińska et al., 2013; Fabre et al., 1998). Additionally, other non-conventional wine yeast, such as *H. uvarum*, *Zygosaccharomyces bailii*, *T. pretoriensis* (Zupan et al., 2013), *C. zemplinina*, *M. pulcherrima* and *T. delbrueckii*, which we reported here, are able to produce aromatic alcohols. However, in all cases, non-*Saccharomyces* species produce lower quantities than *S. cerevisiae*, indicating that the Ehrlich pathway may not be as active in non-*Saccharomyces* species as in *Saccharomyces* under nitrogen-limiting conditions.

TrpOH was the major aromatic alcohol synthesized by most species. This may be attributable to the fact that Trp was present in higher concentrations than the other two aromatic amino acids in the media; however, the production yield of TrpOH was also higher for most species. Additionally, although Trp is a poor source of nitrogen, cells uptake this amino acid early during fermentation (Beltran et al., 2005; Henschke and Jiranek, 1993), which may favour the synthesis of TrpOH. The increase in precursors resulted in a general increase in the production of aromatic alcohols, which was consistent with previous studies (Ghosh et al., 2008; Gori et al., 2011), although the increases in alcohols were not truly proportional to the increases in precursors. Crépin et al. (2017) claimed that modulation of the production of targeted fermentative aromas was achieved by modifying the availability of exogenous amino acids; the authors reported a low contribution of the carbon skeletons of consumed amino acids to the production of volatile compounds. However, these authors studied the synthesis of higher alcohols derived from branched chain amino acids, which primarily originate from sugar catabolism (Crépin et al., 2017), whereas aromatic alcohols are synthesized when their precursors are added to the medium (Ehrlich, 1907; Webb et al., 1963). Moreover, in *S. cerevisiae*, certain reactions in the aromatic amino

acid biosynthesis pathway from glucose are subject to feedback inhibition (Helmstaedt et al., 2005). On the other hand, in *Saccharomyces*, the increase in alcohol precursors resulted in a lower cell density, which may be related to the high content of Trp in this medium, as the growth of *S. cerevisiae* is negatively affected when Trp is used as a unique nitrogen source (Gutiérrez et al., 2013).

The production of aromatic alcohols has been previously studied in other wine yeast species, such as *H. uvarum*, *S. bacillaris* (Sym *C. zemplanina*), *T. pretoriensis* and *Z. bailii*, in a medium similar to SM (Zupan et al., 2013). However, this production has not yet been studied in other yeast species of increasing interest for wine fermentation. In general, non-*Saccharomyces* species synthesize lower amounts of aromatic alcohols than *S. cerevisiae*. This may occur because non-*Saccharomyces* species differ from *S. cerevisiae* in the distribution of metabolic flux during fermentation and therefore differ in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Tóth, 2011; Milanovic et al., 2012; Tofalo et al., 2012).

Moreover, these species experience an elevated contribution of respiration to their metabolism; this is the case even for *T. delbrueckii*, which has been described as Crabtree-positive (Alves-Araujo et al., 2007). Indeed, Quirós et al., (2014) investigated the extent of respiration-fermentative metabolism in different yeast strains and reported that some species commonly found in oenological environments, such as *M. pulcherrima*, *Starmerella bombicola* and *T. delbrueckii*, respire between 40-100 % of consumed sugar under suitable aeration conditions. Thus, an elevated respiratory metabolism may underlie lower aromatic alcohol production, as described for *C. albicans* (Ghosh et al., 2008). In fact, in this study, *C. albicans* cells grown anaerobically at 30 °C produced roughly twice as much PheOH, TrpOH and TyrOH as they do under aerobic conditions. As in *Saccharomyces*, the higher production of aromatic alcohols was detected under nitrogen-limiting conditions in non-*Saccharomyces* species. Another interesting aspect of our study is the finding that *S. bacillaris* produces all three aromatic alcohols, although at very low concentrations, unlike the observations of Zupan et al., (2013); one explanation for this may be the lower detection limits used in our method. Under the conditions used by Zupan et al., (2013), no aromatic alcohol synthesis was detected during alcoholic fermentation by *S. bacillaris*. This species belonged to the *Candida* genus until 2012 (Duarte et al., 2012), and although it is currently included in another genus, it continues to share many features with *Candida*. Low levels of aromatic alcohols were secreted by *S. bacillaris*, but these were similar to the concentrations detected in *C. albicans* in SD medium (3 µM TyrOH; Chen et al., 2004). Regarding *M. pulcherrima*, it is interesting that the synthesis of aromatic alcohols appeared to be favoured under low glucose condition. Furthermore, this

species grew and underwent significantly better fermentation when the aromatic amino acid contents were increased, unlike the other species. In a recent study performed using single nitrogen sources in media, Kemsawasd et al. (2015) observed that aromatic amino acids did not support well neither growth or fermentation performance, and they were particularly deleterious for sugar consumption, including that of *M. pulcherrima*, although another species was also investigated. After *S. cerevisiae*, *T. delbrueckii* presents a better oenological profile; higher concentrations of aromatic alcohols in SM medium were also observed for other non-*Saccharomyces* yeasts. *T. delbrueckii* synthesized the fewest aromatic alcohols, with the exception of *S. bacillaris*, in other media containing low nitrogen and low glucose. Therefore, *T. delbrueckii* appears to be affected by nutrient limitation. Moreover, in LGM, the profile of accumulated aromatic alcohols changed, and PheOH was the major alcohol formed by *T. delbrueckii*.

When the intracellular synthesis of these aromatic alcohols was studied during SM fermentation, similar profiles were observed for *S. cerevisiae*, *H. uvarum* and *M. pulcherrima*. These yeasts synthesized aromatic alcohols during the early exponential phase, when the yeast population was increasing and the need for nitrogen was higher; therefore, the deamination and transamination of amino acids were essential steps to fulfil the nitrogen demands of cells. The transamination reaction, which is the first step in the Ehrlich pathway, is followed by decarboxylation, which is thermodynamically favoured (Henry et al., 2007). This decarboxylation pulls transamination toward complete utilization of the nitrogen-donating amino acid, resulting in the formation of the fusel alcohol or fusel acid, depending on the redox state of the cell (Hazelwood et al., 2008). We have observed that cells secreted those aromatic alcohols into the medium during the shift from exponential to stationary phase, when mechanisms required for the starvation period are induced, which is consistent with previous studies (Chen and Fink, 2006; Gori et al., 2011; Zupan et al., 2013). The secretion of these molecules at the end of the exponential phase indicates a connection to cell density, suggesting that aromatic alcohols are potential QS molecules in these species, as previously suggested for *C. albicans*, *S. cerevisiae* and *D. hansenii* (Chen et al., 2004; Chen and Fink, 2006; Gori et al., 2011). According to Fabre et al. (1998), if Phe is added after the exponential growth phase, no bioconversion to PheOH is observed during stationary phase in *Kluyveromyces marxianus*. Compared to other yeasts investigated in this study, *T. delbrueckii* alone reached its highest levels of PheOH and TrpOH at two different times. PheOH may exert an auto-stimulatory effect on TrpOH production, as synergies between both alcohols have been observed in *S. cerevisiae* (Chen and Fink, 2006). Based on this result, *T. delbrueckii* undertakes growth phase-dependent differential reg-

ulation to synthesize these aromatic alcohols. This delay in synthesis, together with a low population during the first 12 h, also resulted in a delay in the extracellular secretion of the aromatic alcohols. In the case of *S. bacillaris*, very low intracellular accumulation and extracellular secretion were related to the deficient growth of this species.

In this study, we have demonstrated that an increase in precursor levels resulted in the higher accumulation of the resulting alcohols; therefore, these compounds may be increased under oenological conditions. Moreover, aromatic alcohols have been reported to possess quorum sensing activity, and their effects, together with ethanol, on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). However, there is limited knowledge of the effects of these QS molecules in non-*Saccharomyces* wine yeasts. Thus, further studies investigating the roles of those aromatic alcohols in non-*Saccharomyces* yeast species will be necessary to understand yeast interactions and quorum sensing mechanisms in wine yeasts.

To summarize, we show that, in addition to the well-studied *S. cerevisiae*, the Ehrlich pathway is active in other yeasts that produce aromatic alcohols during alcoholic fermentation, although their regulation appears to be somewhat different than that of *S. cerevisiae*. *S. bacillaris* was the lowest producer of aromatic alcohols, whereas *S. cerevisiae* was the highest producer. Carbon and nitrogen availability as well as precursors influence the production of these alcohols; in particular, nitrogen depletion induced notable levels of aromatic alcohols. To date, the synthesis of these molecules by wine yeasts demonstrates high oenological potential, as *M. pulcherrima* and *T. delbrueckii* had not previously been evaluated. Here, these two species were able to synthesize considerable amounts of alcohols, which may regulate their growth. Aromatic alcohols also have many important biotechnological applications, and relevant concentrations of these compounds positively affect wine. In this study, we demonstrated that varying nutrient concentrations in must result in adjustments to the synthesis patterns of aromatic alcohols for all yeast species studied.

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Supplementary figures

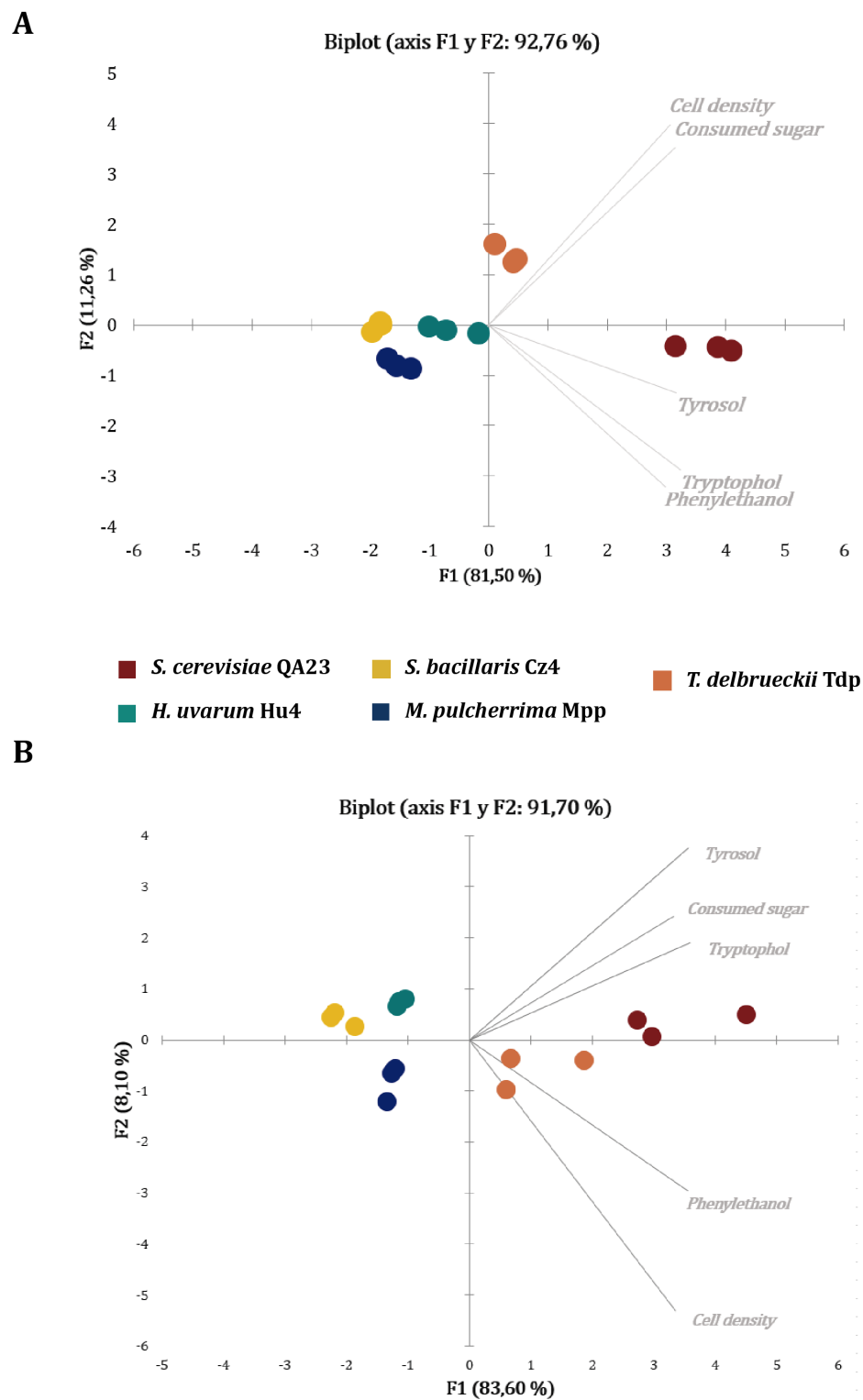


Figure S1.1: Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation in SM 1X (A) and 5x (B) of aromatic amino acid content.

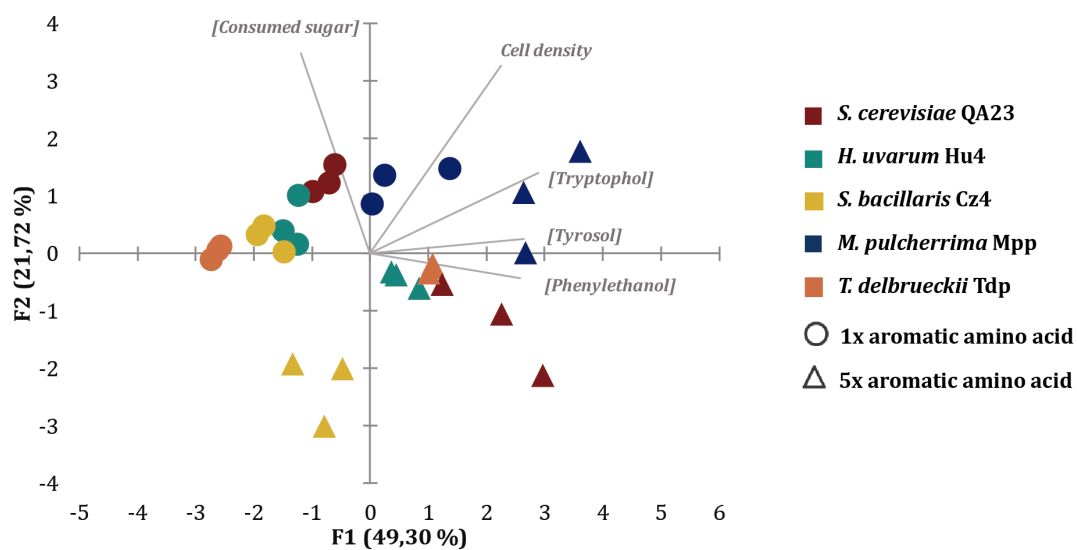


Figure S1.2: Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation in LGM.

Table S1.1: Production yield of aromatic alcohols in SM and LNM at 5x of aromatic amino acid content. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account three biological replicates. In all cases, comparisons are within the same species; letters indicate significant differences (p-value < 0.05), aaa;aromatic amino acid.

Yeast strain	Must	[aaa]	[TyOH]/[Tyr]		[PheOH]/[Phe]		[TrpOH]/[Trp]	
			($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD
<i>S. cerevisiae</i> QA23	SM	5x	0,62 ^a	0,15	0,04 ^a	0,01	1,05 ^a	0,08
	LNM	5x	0,37 ^b	0,07	0,50 ^b	0,13	0,70 ^b	0,10
<i>H. uvarum</i> Hu4	SM	5x	0,24 ^a	0,02	0,01 ^a	0,00	0,44 ^a	0,03
	LNM	5x	0,59 ^b	0,09	0,09 ^a	0,01	0,53 ^b	0,05
<i>S. bacillaris</i> Cz4	SM	5x	0,05 ^a	0,02	0,01 ^a	0,00	0,01 ^a	0,00
	LNM	5x	0,07 ^a	0,03	0,04 ^a	0,01	0,01 ^a	0,01
<i>M. pulcherrima</i> Mpp	SM	5x	0,08 ^a	0,04	0,02 ^a	0,00	0,48 ^a	0,05
	LNM	5x	0,40 ^b	0,04	0,06 ^a	0,02	0,29 ^b	0,07
<i>T. delbrueckii</i> Tdp	SM	5x	0,26 ^a	0,14	0,04 ^a	0,01	0,95 ^a	0,80
	LNM	5x	0,21 ^a	0,04	0,11 ^b	0,01	0,23 ^b	0,09

Table S1.2: Production yield of aromatic alcohols in LGM. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account two biological replicates, aaa;aromatic amino acid.

Yeast strain	[aaa]	[TyOH]/[Tyr]		[PheOH]/[Phe]		[TrpOH]/[Trp]	
		($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD	($\mu\text{M}/\mu\text{M}$)	SD
<i>S. cerevisiae</i> QA23	1x	0,16	0,15	0,14	0,01	0,09	0,00
	5x	0,15	0,00	0,09	0,04	0,03	0,00
<i>H. uvarum</i> Hu4	1x	0,13	0,00	0,03	0,00	0,02	0,00
	5x	0,41	0,01	0,08	0,00	0,16	0,01
<i>S. bacillaris</i> Cz4	1x	0,04	0,00	0,00	0,00	0,01	0,00
	5x	0,03	0,01	0,00	0,00	0,00	0,00
<i>M. pulcherrima</i> Mpp	1x	0,09	0,00	0,11	0,12	0,09	0,01
	5x	0,05	0,02	0,26	0,00	0,00	0,00
<i>T. delbrueckii</i> Tdp	1x	0,00	0,00	0,00	0,00	0,00	0,00
	5x	0,03	0,01	0,03	0,01	0,01	0,00

CHAPTER 2

Aromatic amino acid-derived compounds induce morphological changes and inhibit the cell growth of wine yeast species

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Abstract

Yeasts secrete a large diversity of compounds during alcoholic fermentation, which affect growth rates and developmental processes, like filamentous/invasive growth. Several compounds are produced during aromatic amino acid metabolism, including aromatic alcohols, serotonin, melatonin and tryptamine. We evaluated the effects of these compounds on growth parameters in 16 different wine yeasts, including non-*Saccharomyces* wine strains, for which the effects of these compounds have not been well-defined. Serotonin, tryptamine and tryptophol negatively impacted growth. Phenylethanol and tyrosol specifically affected cell growth in non-*Saccharomyces* strains. We demonstrated that the compounds are able to affect to invasive and pseudohyphal growth in a manner dependent on nutrient availability. Many of the compounds also showed species-specific effects. These findings add to the understanding of the fermentation process and illustrate the diversity of metabolic communication that may occur among related species during metabolic processes.

Key Words: Aromatic alcohols, serotonin, tryptamine, quorum sensing, pseudohyphal growth, non-*Saccharomyces*, invasive growth

Introduction

Wine is produced by alcoholic fermentation, in which grape sugars are metabolized into ethanol by yeast. During grape ripening, the surfaces of berries are primarily colonized by non-*Saccharomyces* yeast, such as *Hanseniaspora*, *Starmerella* (sym *Candida*), *Hansenula*, or *Metschnikowia*. Microorganisms belonging to the *Saccharomyces* genus are present in low abundance and are difficult to detect in initial must (Ribéreau-Gayon et al., 2006). For this reason, during spontaneous fermentation, non-*Saccharomyces* yeasts are responsible for initiating alcoholic fermentation and are then out-competed by *S. cerevisiae* throughout fermentation (Fleet, 2003; Heard and Fleet, 1988; Ribéreau-Gayon et al., 2006). Traditionally, the low ethanol tolerance and competitiveness of non-*Saccharomyces* yeasts compared to *Saccharomyces* species (Ribéreau-Gayon et al., 2006) has resulted in a lack of interest in these yeast species for many years. However, recently, the importance of non-*Saccharomyces* strains in alcoholic fermentation has become appreciated, particularly in terms of their contribution to wine aroma, during the early steps of fermentation. Indeed, these species have been reported to positively impact winemaking via the production of high amounts of aromatic compounds, such as aromatic alcohols, ethyl esters and acetate esters (Belda et al., 2017; García et al. 2010; Jolly et al., 2014; Romano et al., 2003). Furthermore, these strains appear to be present throughout much of the fermentation process, although this finding has been neglected because such strains have been difficult to culture (Millet and Lonvaud-Funel, 2000; Wang et al., 2015a, 2016).

Budding yeast of the species *Saccharomyces cerevisiae* are unicellular fungi that reproduce asexually by budding and are able to undergo filamentous growth to scavenge for nutrients (Cullen and Sprague, 2012; Verstrepen and Klis, 2006; Wendland and Philippsen, 2001). Filamentous growth includes morphological changes that involve the global reorganization of cellular processes to produce a new cell type. Cells alter their budding pattern, becoming more elongated and remaining attached to each other through the formation of pseudohyphae. Moreover, under certain conditions, yeast cells penetrate surfaces through a process known as invasive growth (Roberts and Fink, 1994). Although much of the genetic characterization of this response has been performed in *S. cerevisiae* strains on the $\Sigma 1278b$ background (Cullen and Sprague, 2000; Gimeno et al., 1992), the response has also been studied in many strains and genera (Gimeno and Fink, 1994; Lo and Dranginis, 1998; San-blas et al., 1997). For example, the human pathogen *Candida albicans* (Biswas et al., 2007; Chen et al., 2004; Hornby et al., 2001; Kruppa, 2009) undergoes pseudohyphal and hyphal growth (pathogenic form), which confers the

ability to infect human tissues (Leberer et al., 2001; Lo et al., 1997; Rocha et al., 2001).

Filamentous growth in yeasts has been reported to occur in response to cell density. Several molecules, such as aromatic alcohols and ethanol, have been identified as stimuli that induce these morphological changes (Dickinson, 1996; Gimeno et al., 1992; González et al., 2017; Lorenz et al., 2000). Indeed, aromatic alcohols, tyrosol (TyrOH), tryptophol (TrpOH) and phenylethanol (PheOH), which are derived from the amino acids tyrosine, tryptophan and phenylalanine, respectively, have been suggested to act as quorum sensing molecules (QSM) in yeasts, regulating cell density and evoking morphogenetic transitions (Chen et al., 2004; Chen and Fink, 2006). Moreover, nitrogen limitation results in the increased production of aromatic alcohols, leading to elevated filamentous growth in *S. cerevisiae*. In this species, PheOH and TrpOH act as inducers of morphogenesis, while TyrOH has no detectable effects. However, in *C. albicans*, these alcohols exhibit the opposite behaviour: TyrOH promotes pseudohyphal growth, whereas PheOH and TrpOH inhibit it. The finding that different aromatic alcohols exert different responses on morphogenesis depending on the yeast species implicates these molecules as inducers of species-specific effects (Chen and Fink, 2006). In a recent study (González et al., 2017), ethanol specifically induced filamentous growth under nitrogen-limiting conditions, whereas aromatic alcohols did not. Thus, environmental conditions impact the efficacy of these compounds. Non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Pichia kudriavzevii* and *Pichia fabianii*, undergo filamentous growth under nutrient-limited conditions (nitrogen or carbon) or in the presence of other stress factors (Pu et al., 2014; van Rijswijck et al., 2015), but the roles of these alcohols have not been extensively explored.

During alcoholic fermentation, yeast synthesize compounds, that, depending on the concentration, can be inhibitory to their own growth or the growth of other yeast species. A primary example is ethanol, which is a potent inhibitory compound for growth. Other metabolites, such as short-to-medium-chain fatty acids (e.g., acetic, hexanoic, octanoic and decanoic acids) and yeast killer toxins, also inhibit growth and even induce the death of certain yeast species, including strains of *S. cerevisiae* (Pérez et al., 2001). Other compounds derived from tryptophan metabolism during wine fermentation also appear to display antifungal activity (Lass-Flörl et al., 2003; Lass-Flörl et al., 2002). Moreover, direct microbial interactions (i.e., through physical contact) are reportedly involved in the growth inhibition of non-*Saccharomyces* yeast, although such mechanisms are dependent on cell density, when cultures are competing for space. (Nissen et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013). Recently, interactions between species were shown to be impacted by the secretion of com-

pounds by yeast during alcoholic fermentation (Albergaria and Arneborg, 2016; Ciani and Comitini, 2015; Wang et al., 2015b). To our knowledge, there have been no studies investigating the effects of aromatic alcohols or other QSMs synthesized during alcoholic fermentation on the growth and vitality of yeasts. Moreover, the effects of aromatic alcohols on the filamentous growth of non-*Saccharomyces* wine yeast species have not been explored. The investigation of these areas might help begin to unravel the possible roles of QSMs in the interactions between yeasts during alcoholic fermentation. Additionally, through tryptophan metabolism, yeasts also produce other metabolites that are related to indoles, such as serotonin, melatonin or tryptamine. Serotonin and melatonin are of special relevance for their bioactivity in higher organisms, including humans. Rodriguez-Naranjo et al., (2012) demonstrated that melatonin is produced during alcoholic fermentation by yeast, and different strains and species synthesize this compound at different concentrations. The role of melatonin in yeasts is still unclear, although a recent paper showed that the compound demonstrated possible antioxidant activity in response to oxidative damage by hydrogen peroxide in *S. cerevisiae* (Vázquez et al., 2017). On the other hand, serotonin has also been detected in red wines at mg/L concentrations after malolactic fermentation (Wang et al., 2014). This compound appears to exert antifungal activity against *Candida* and *Aspergillus* spp. in vitro (Lass-Flörl et al., 2003; Lass-Flörl et al., 2002). Thus, the objective of this study was to evaluate the effects of different aromatic compounds derived from amino acid metabolism and produced during alcoholic fermentation on the growth and physiology of different wine yeast species. We first describe an analysis of the growth parameters of different yeast strains and species in the presence of increasing concentrations of specific compounds of interest. Then, the effects of aromatic alcohols and ethanol, which are well-known morphogenesis inducers in *S. cerevisiae*, were examined for their impact on the filamentous growth of different non-*Saccharomyces* wine species.

Materials And Methods

Effects On Yeast Growth

Strains and growth media

Eight strains from *Saccharomyces* species and two strains from four species of non-*Saccharomyces* yeast were used in the study. The *S. cerevisiae* strains included the laboratory strain Σ 1278b, the wine strains SB, QA23, T73, P5 and P24, the animal nutrition strain Sc20 and the hybrid *S. kudriavzevii*/*S. cerevisiae* Vin7. The non-*Saccharomyces* yeast were *C. zemplinina* (Cz4-CECT13129, Cz11), *H. uvarum* (Hu4-CECT13130, Hu11), *M. pulcherrima* (Mpp-CECT 13131, FLAVIA) and *T. delbrueckii* (Tdp-CECT 13135, BIODIVA). FLAVIA and BIODIVA are commercial strains (Lallemand, Inc.) whereas the other non-*Saccharomyces* strains were isolated from grapes/wine media (Padilla et al., 2016). Yeasts were typically grown on YPD [2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose and 2 % (w/v) agar] at 28 °C. To evaluate the effects of nitrogen concentration, yeasts were grown on minimal medium [(MM) 1x Yeast Nitrogen Base (YNB) without (w/o) amino acids (aa) or ammonium, 2 % (w/v) glucose and 10 mM $(NH_4)_2SO_4$] and on low nitrogen medium [(LNM) 1x Yeast Nitrogen Base (YNB) w/o aa or ammonium, 2 % (w/v) glucose and 1 mM $(NH_4)_2SO_4$]. Media were supplemented with increasing concentrations of melatonin (Mel), tryptamine (Trpm), serotonin (Ser), tyrosol (TyrOH), phenylethanol (PheOH) and tryptophol (TrpOH), ranging from 0 to 1000 ppm.

Growth monitoring

Yeasts were pre-cultured for 48 h on MM at 28 °C and then inoculated into each medium, adjusting the initial optical density (OD_{A600nm}) to 0.2. All assays were performed using a POLARstar Omega microplate reader (BMG LABTECH, Germany) and were performed in triplicate at 28 °C for 48 h. Microplate wells were filled with 250 μ L of inoculated media. A control well containing medium without inoculum was used to determine the background signal. Measurements were taken every 30 min after pre-shaking the microplate for 30 sec at 500 rpm. For each growth curve, the variable generation time (GT), growth efficiency (OD max) (Warringer and Blomberg, 2003) and lag phase (Hall et al., 2014) were measured.

Statistical data processing

All experiments were performed in triplicate, and p-values for the growth parameters were generated ($p < 0.5$). For each compound, relative values were calculated using the condition in the absence of added compound (0 ppm) as a control. To better understand the interactions between the calculated parameters and their effects on yeast growth, Principal Component Analysis (PCA) was performed using XLSTAT Software at a concentration of 1000 ppm for each compound and under both nitrogen conditions (MM with 10 mM and LNM with 1 mM) for all strains tested.

Filamentous Growth Assays

Yeast strains, media and growth conditions

For the filamentous growth assay, two strains of each non-*Saccharomyces* species were tested, using the strain QA23 (*S. cerevisiae*) as a control (González et al., 2017). Yeasts were grown on minimal medium [MM - 1x Yeast Nitrogen Base (YNB) w/o aa or ammonium, 2 % (w/v) glucose, and 10 mM $(NH_4)_2SO_4$] with agitation (120 rpm) for 16 h at 28 °C before seeding on plates for filamentation analysis. To evaluate invasive and pseudohyphal growth, three different media were used, with variations in glucose and nitrogen concentrations: SD - synthetic medium [1x YNB w/o aa or ammonium, 2 % (w/v) glucose and 37 mM $(NH_4)_2SO_4$] and 2 % (w/v) agar], SALG - synthetic medium with low glucose [1x YNB w/o aa or ammonium, 0.5 % (w/v) glucose and 37 mM $(NH_4)_2SO_4$] and 2 % (w/v) agar] and SLAD - synthetic low-ammonium dextrose medium [SLAD - 1x YNB w/o aa or ammonium, 2 % (w/v) glucose and 50 μ M $(NH_4)_2SO_4$] and 2 % (w/v) agar]. To test the effects of aromatic alcohols, the above media were supplemented with TyrOH, TrpOH or PheOH at 500 μ M or 2 % (v/v) ethanol.

Invasive and pseudohyphal growth assays

Cells pre-grown in MM for 16 h were harvested by centrifugation, washed once in sterile water and adjusted to an OD_{600nm} of 2.0. Subsequently, 10 μ l of cells were spotted in triplicate on semisolid agar media. Plates were incubated at 30 °C for 3 days, 5 days and 7 days depending on the experiment. Invasive growth was determined in a plate washing assay (Roberts and Fink, 1994). Colonies were photographed before and after the plates were washed in a stream of water, after which the colonies were rubbed from the surface with a gloved finger. ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to quantitate invasive growth in the plate-washing assay. The background intensity was determined

for each spot and subtracted from the densitometry of the invasive area. Densitometry analysis was performed on invasive patches over multiple days. To obtain p-values, Tukey's test was used. The examination of pseudohyphae was determined as described by Gimeno et al. (1992). Before washing the plates, the colony periphery was observed and photographed each day under microscopy (Raman FT-IR).

Results

Effects of the presence of aromatic amino acids on yeast growth

To evaluate the effects of amino acid-derived compounds on yeast growth, five strains of *S. cerevisiae* and one strain of each non-*Saccharomyces* species were grown in the presence of 1000 ppm of Mel, Ser, Trpm, TyrOH, PheOH or TrpOH. As these molecules are derived from nitrogen metabolism, and QSMs are produced during nutrient limitation, we tested their effects under two different nitrogen conditions: 1 and 10 mM $(NH_4)_2SO_4$ (Fig 2.1). As an example, the growth curves obtained with *S. cerevisiae* QA23 (Fig 2.1A) and *S. bacillaris* Cz4 (Fig 2.1B) in the presence of 1000 ppm of the different compounds and 10 mM $(NH_4)_2SO_4$ are shown. In the QA23 strain, Ser completely inhibited cell growth. In addition to this dramatic phenotype, other subtle phenotypes were observed. TrpOH caused a reduction in growth rate and maximal growth, and Trpm increased the lag phase. The other compounds tested did not significantly affect the growth profile. In comparison, the growth of strain Cz4 was reduced by TrpOH and Trpm but not by the other compounds. Therefore, different compounds cause the growth inhibition of different species.

We next investigated what impact these compounds had on other strains. Nine strains were examined. The relative values of OD max (Fig 2.1C) and generation time (Fig 2.1D) were calculated for each compound, under conditions lacking additives as a control. Overall, the addition of these compounds (with the exception of Mel) exerted negative impacts on the maximal growth obtained for most of the tested strains (Fig 2.1C). Because nitrogen levels can impact the efficacy of these compounds, we examined their effects in the presence of high and low nitrogen levels. Ser decreased the OD max in all yeast species, particularly under low nitrogen conditions, while Trpm and aromatic alcohols had a major impact in non-*Saccharomyces* strains under both nitrogen conditions. On the other hand, Ser caused growth reduction in all strains, increasing their GT (Fig 2.1D). In general, this increase was significant for *Saccharomyces* strains under both nitrogen conditions but only under low nitrogen con-

ditions for most non-*Saccharomyces* strains. Increases in GT were also observed when the medium was supplemented with TrpOH in all the non-*Saccharomyces* strains under both nitrogen conditions. The other two aromatic alcohols, PheOH and TyrOH, exerted no effects in *Saccharomyces* strains, and only the Tdp strain was affected by PheOH and *S. bacillaris* by TyrOH at 1 mM $(NH_4)_2SO_4$ among non-*Saccharomyces* strains. In general, the relative OD max or GT presented a similar trend under both nitrogen conditions; the most relevant differences consisted of greater effects from Ser in the non-*Saccharomyces* strains under low nitrogen conditions. Based on these results, many compounds have deleterious effects on the growth of many strains. These effects can be impacted by exogenous nitrogen levels. Importantly, different strains exert different effects.

PCA analysis classifies samples in terms of their similarity to each other. The PCA plot constructed for the high nitrogen concentration (10 mM $(NH_4)_2SO_4$) explained 58 % of variance (Fig 2.2A). Yeast species were clustered into two groups, separating *Saccharomyces* and non-*Saccharomyces* strains, according Component 1; this was primarily attributable to the greater reduction in the OD max in non-*Saccharomyces* strains based on the presence of aromatic alcohols and Trpm. A PCA was also constructed with growth data obtained under low nitrogen conditions (Fig 2.2B). In this case, the variance explained by the PCA was 53 %, and all strains of *Saccharomyces* were included in the same cluster. Non-*Saccharomyces* were plotted into two groups because *T. delbrueckii* clustered separately from the other non-*Saccharomyces* species due to the high GT in PheOH.

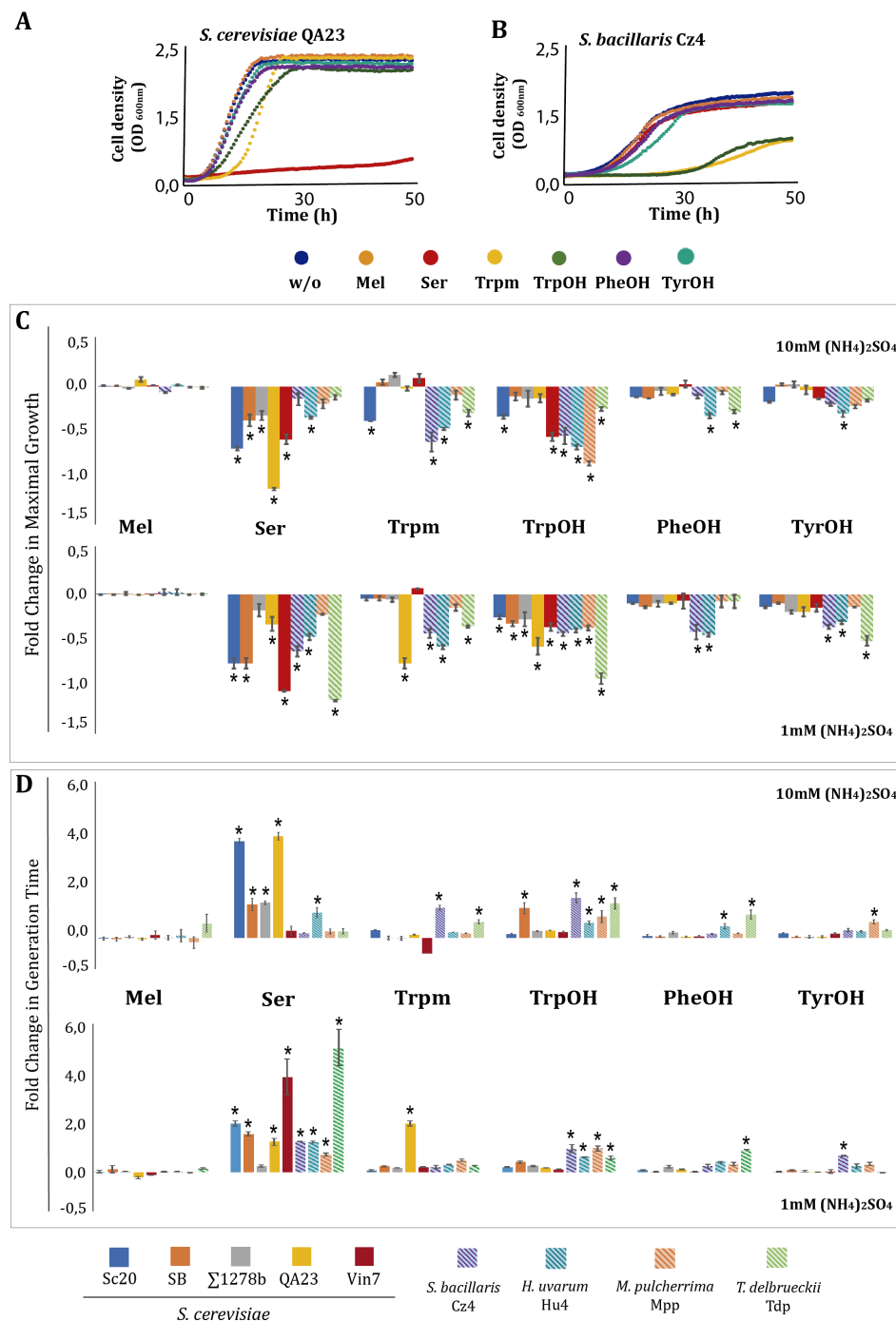


Figure 2.1: Effects of aromatic amino acid-derived compounds on the growth of wine yeast species at different nitrogen concentrations. The effects of Mel, Ser, Trpm, TrpOH, PheOH and TyrOH on the growth of four strains of *S. cerevisiae* and four non-*Saccharomyces* were determined. Yeast were grown for 48 h at 28 °C in minimal medium with two different nitrogen concentrations (10 mM or 1 mM (NH₄)₂SO₄) and supplemented with 1000 ppm of each compound. Non-supplemented cultures were used as controls. Experiments were carried out in triplicate. Growth curves of *S. cerevisiae* QA23 (A) and *S. bacillaris* Cz4 (B), with the different compounds at 10 mM (NH₄)₂SO₄ are shown. For each nitrogen condition and compound, maximal growth (C) and generation time (D) were calculated. The fold-change for each growth parameter was determined in relation to its control condition. Statistical analysis was performed by comparing the effects of each compound in the different strains; p-value < 0.05.

PCA plot showing the separation of *Saccharomyces* and *Non-Saccharomyces* yeasts based on metabolite profiles. The x-axis represents Component 1 (39,32 %) and the y-axis represents Component 2 (19,19 %). *Saccharomyces* yeasts (Qa23, SC20, Vin7) are clustered on the left, while *Non-Saccharomyces* yeasts (Hu4, Cz4, Mpp) are clustered on the right. Various metabolites are labeled with vectors pointing from the origin, such as Tryptophol OD, Tyrosol OD, and Melatonin Gt.

PCA plot showing the separation of metabolite profiles for *Saccharomyces* (green dashed box) and *Non-Saccharomyces* (blue dashed box) yeasts. The x-axis represents Component 1 (29.54%) and the y-axis represents Component 2 (24.06%).

Saccharomyces (green dashed box) is associated with metabolites such as Tyrosol OD, Melanilin OD, Phenylethanol OD, and various other compounds like Vin7, SC20, SB, Sigma, Qa23, and Mpp.

Non-Saccharomyces (blue dashed box) is associated with metabolites such as Tdp, Tdp, Tdp, Phenylethanol Gt, Tyrosol Gt, and various other compounds like Hu4, Cz4, and Mpp.

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Effects of the concentrations of aromatic amino acid-derived compounds on yeast growth

According to our previous results, the effects of certain aromatic amino acid-derived compounds were slightly greater under low nitrogen conditions than under high nitrogen conditions. For this reason, we investigated how the concentrations of these compounds affect the growth of a broad collection of wine yeast in nitrogen-limiting conditions.

We tested the effects of increasing concentrations of aromatic amino acid-derived compounds (from 50 to 1000 ppm) on the growth of eight *S. cerevisiae* strains and two strains each of *S. bacillaris*, *H. uvarum*, *M. pulcherrima* and *T. delbrueckii* on nitrogen-limiting media in triplicate. Among the metabolites tested, Ser (Fig 2.3 and S2.1), TrpOH (Fig 2.4 and S2.1) and Trpm (Fig 2.5 and S2.1) exerted higher impacts on the cell growth of different yeast strains, as observed in the previous section at a concentration of 1000 ppm for all compounds. As expected, decreasing the concentration of Mel had no effect on cell growth, whereas only non-*Saccharomyces* strains were affected by TyrOH and PheOH (Fig S2.2), which reduced the maximal growth; PheOH exhibited the greatest effects, including at a low concentrations (50 ppm).

The effects of different Ser concentrations on the *S. cerevisiae* Sc20 strain are shown as an example (Fig 2.3A). Clear inhibition of yeast growth was observed at concentrations of Ser above 500 ppm, increasing GT and decreasing the OD max. Interestingly, GT and OD max values obtained in the presence of Ser were strongly correlated (R^2 0.8204), indicating that this compound influenced both growth parameters for most strains (Fig 2.3B). As shown in Fig 2.3C, all strains were affected by the presence of Ser in the medium, and the increase in GT was directly proportional to the Ser concentration, illustrating a dose-dependent effect starting at 250 ppm through higher concentrations. Most wine *S. cerevisiae* strains showed growth inhibition above 250 ppm of Ser, resulting in a high increase in GT, primarily in Vin7, T73, P5 and P24. Conversely, the non-*Saccharomyces* strains of *S. bacillaris*, *H. uvarum* and *M. pulcherrima* appeared to be more tolerant to the presence of this compound, exhibiting lower GT; additionally, the growth of some strains was only significantly affected above 500 ppm. Indeed, the lab strain Σ 1278b was least affected by Ser, with a slight increase in GT only at the highest dose (1000 ppm). *T. delbrueckii* presented a specific profile for both analysed strains; growth was only affected above 750 ppm of Ser, and both strains exhibited the highest growth inhibition at 1000 ppm compared to other species. The effects of Ser on the relative OD max of the strains showed a profile similar to GT

(Fig S2.1).

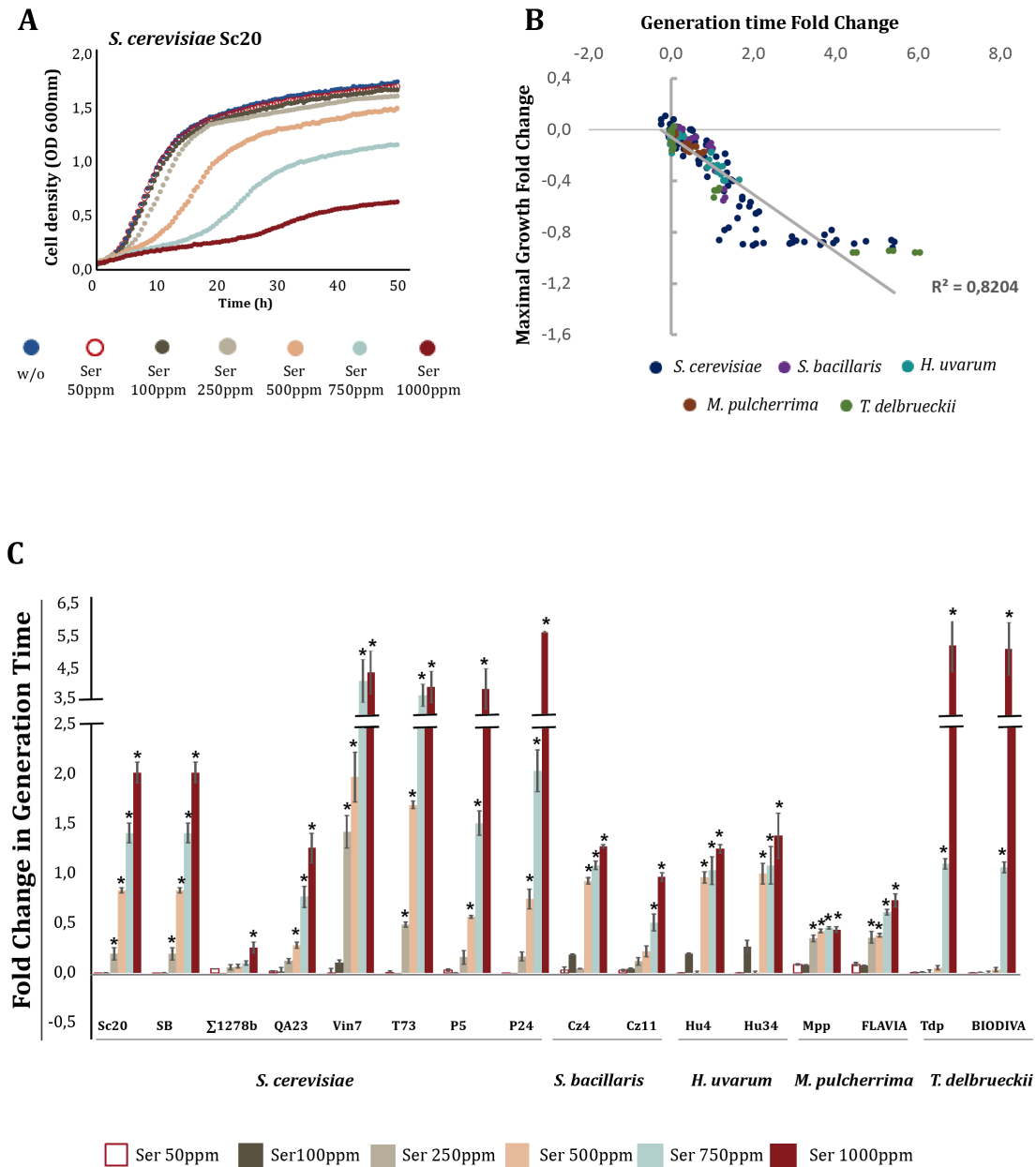


Figure 2.3: Effects of increasing serotonin (Ser) concentrations on yeast growth. Ser was added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000 ppm). (A) Growth curves obtained with *S. cerevisiae* Sc20. (B) Correlation between the generation time and maximal growth fold-changes obtained with different yeast species. (C) Generation time fold-change for each strain at different Ser concentrations. Statistical analysis was performed comparing the effects of Ser concentrations in each strain; p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (without Ser).

For most strains, the addition of TrpOH caused a decrease in the OD max in a dose-dependent manner (see Fig 2.4A and S2.1). The correlation between GT and OD max values showed that the presence of TrpOH had a greater impact on GT than on maximal growth (Fig 2.4B). Indeed, high concentrations of TrpOH caused a significant increase in GT, particularly in non-*Saccharomyces* strains (Fig 2.4C), and significantly decreased the OD max in all strains analysed (Fig S2.1). Among the non-*Saccharomyces* strains, the strain most tolerant to the presence of TrpOH was FLAVIA, belonging to *M. pulcherrima*, which was not affected by TrpOH in terms of GT values at any of the concentrations tested and was only affected doses higher than 750 ppm for the OD max. Conversely, the other *M. pulcherrima* strain, Mpp, was one of the most heavily affected by TrpOH in the GT, indicating that sensitivity to TrpOH is strain-dependent. In general, *S. cerevisiae* strains presented similar behaviour in terms of GT, exhibiting only slight modifications, and experienced greater effects in terms of OD; QA23 was the strain most affected by this compound.

The effects of Trpm on yeast growth were strain dependent, which may have resulted in increases in the lag phase or in the GT, decreases in the OD max, or perhaps no inhibitory effects at all (see two examples in Fig 2.5A). Thus, within the same species, we observed different responses to the presence of Trpm. For example, among *S. cerevisiae* strains, Vin7 only showed an increase during the lag phase, and there were no significant effects on the other growth parameters. QA23 primarily increased its generation time and decreased the OD max, while the other *S. cerevisiae* strains were barely affected by Trpm (Fig 2.5B, 2.5C and S2.1). On the other hand, in general non-*Saccharomyces* strains were more affected by the presence of this biogenic amine, even at low concentrations; most strains exhibited a significant decrease in the OD max, an increase in the GT, and in some cases an increase in the lag phase. Interestingly, in *M. pulcherrima* strains, the effects of Trp on the OD max and GT were not dose-dependent, demonstrating similar inhibition from 100 ppm to 1000 ppm (Fig 2.5 and S2.1).

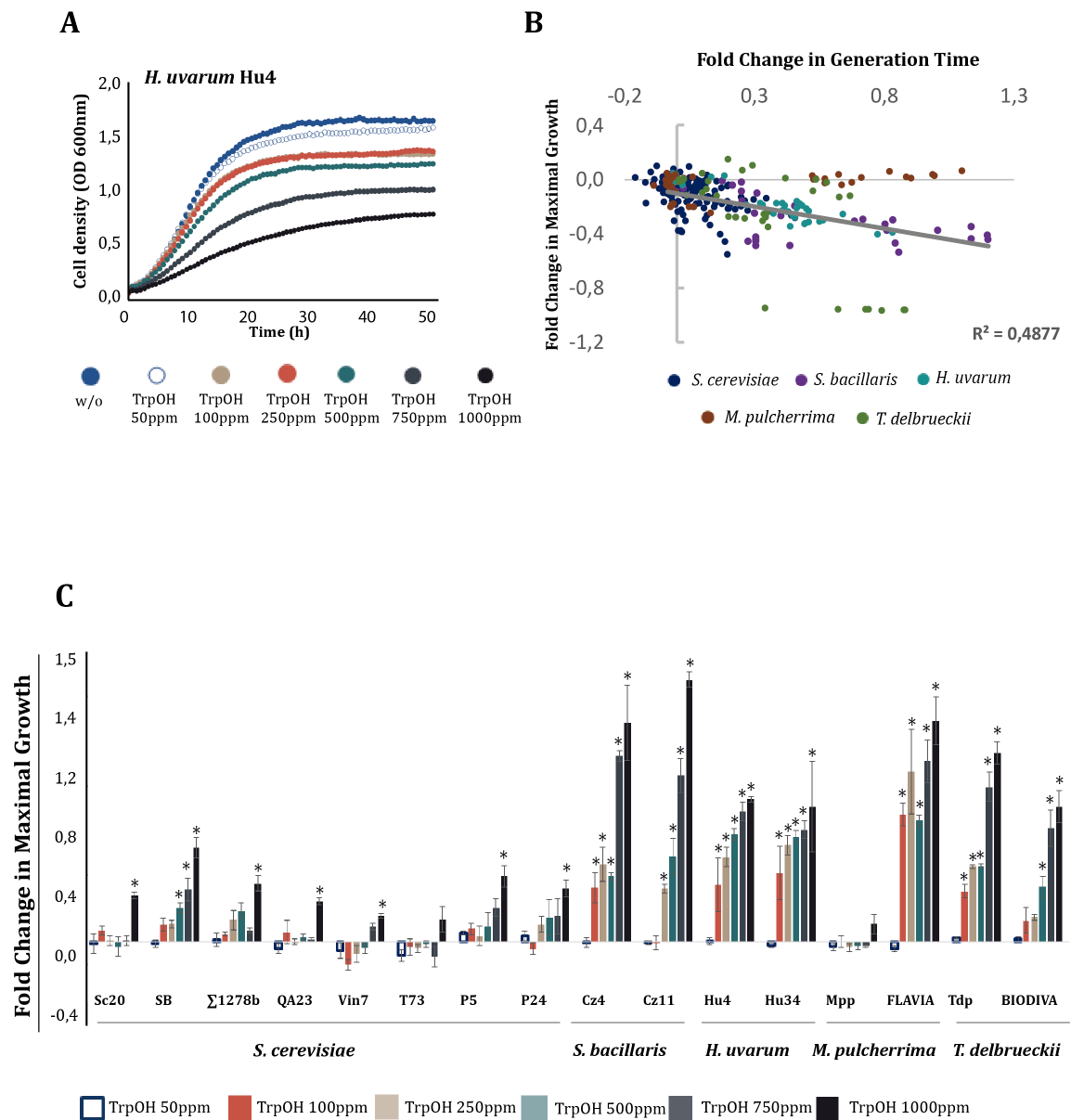


Figure 2.4: Effects of increasing tryptophol (TrpOH) concentrations on yeast growth. TrpOH was added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000 ppm). (A) Growth curves obtained with *H. uvarum* Hu4. (B) Correlation between the generation time and maximal growth fold-changes obtained with different yeast species. (C) Maximal growth fold-change for each strain at different TrpOH concentrations. Statistical analysis was performed comparing the effects of TrpOH concentrations in each strain; p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (without TrpOH).

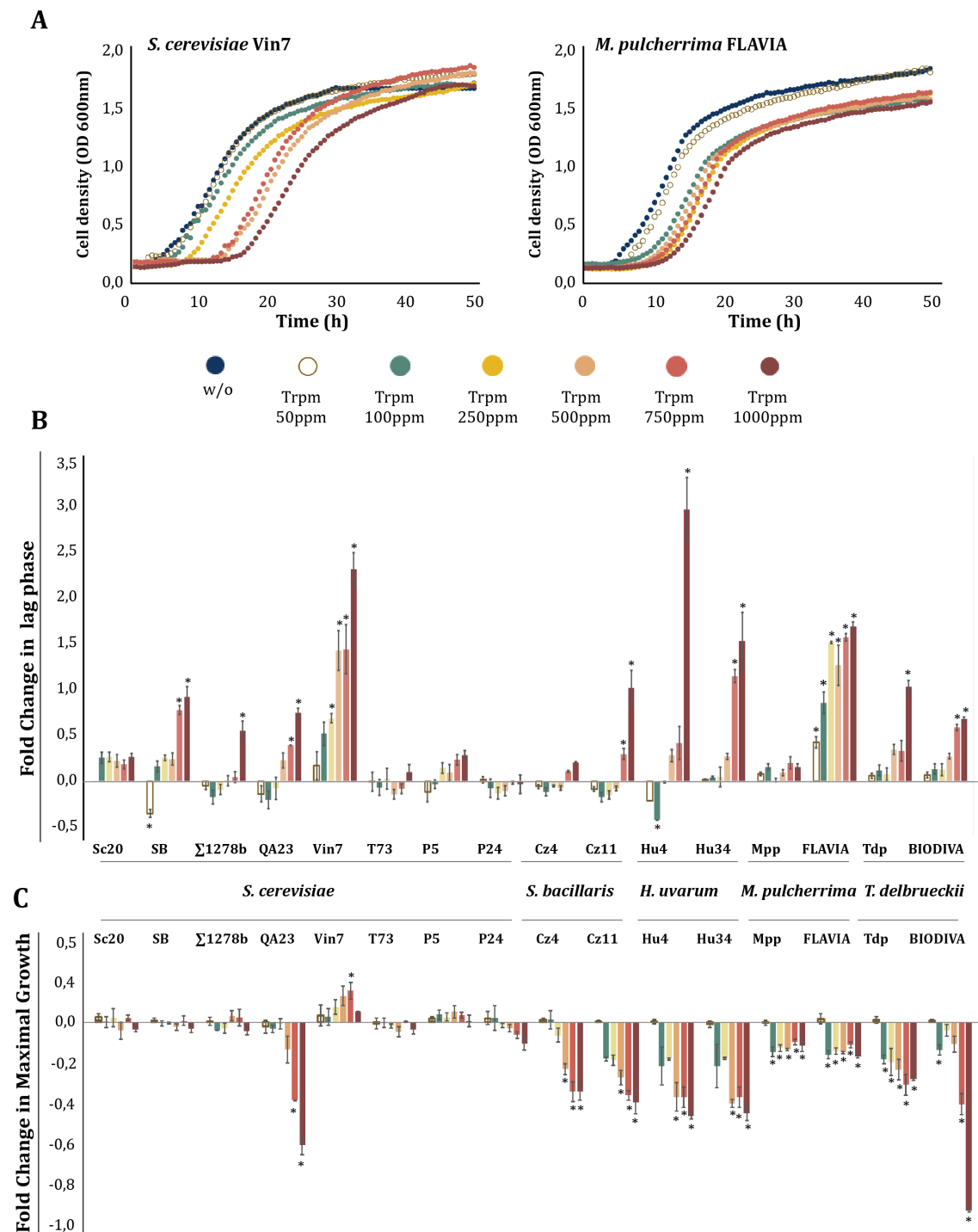


Figure 2.5: Effects of increasing tryptamine (Trpm) concentrations on yeast growth. Trpm was added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000 ppm). (A) Growth curves obtained with *S. cerevisiae* Sc20 and *M. pulcherrima* FLAVIA. (B) Lag phase fold-change for each strain at different Trpm concentrations. (C) Maximal growth fold-change for each strain at different Trpm concentrations. Statistical analysis was performed comparing the effects of Trpm concentrations in each strain; p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (without Trpm).

Effects of culture medium composition on filamentous growth in non-*Saccharomyces* species

The aromatic alcohols and ethanol have been described as molecules signalling morphological changes in different yeasts, primarily in *S. cerevisiae* and *C. albicans*; therefore, we analysed their effects on the non-*Saccharomyces* strains used in the prior study. Before ascertaining the roles of these alcohols in morphogenesis, the filamentous growth of these strains in different media was evaluated. We first studied invasive growth, which represents one aspect of filamentous growth and is evaluated in a plate-washing assay (PWA) by measuring the penetration of cells into substrates. Invasive growth was tested on rich (SD) and nutrient-limiting [glucose (SALG) and nitrogen (SLAD)] media for all strains (Fig 2.6) using *S. cerevisiae* QA23 as a control, as previously shown by González et al. (2017). Interestingly, all strains exhibited a certain degree of invasive growth. Moreover, media limited for glucose or nitrogen resulted in enhanced invasive growth for most strains. Specifically, in SLAD plates, most strains showed invasive growth that was significantly higher than on SD, with the exception of *H. uvarum* strains. *M. pulcherrima* and *T. delbrueckii* strains were the most invasive non-*Saccharomyces* yeasts in the absence of nitrogen. Carbon source limitation (SALG) had a similar effect as nitrogen; most strains presented significant invasive growth compared to rich media, with the exception of the two *T. delbrueckii* strains.

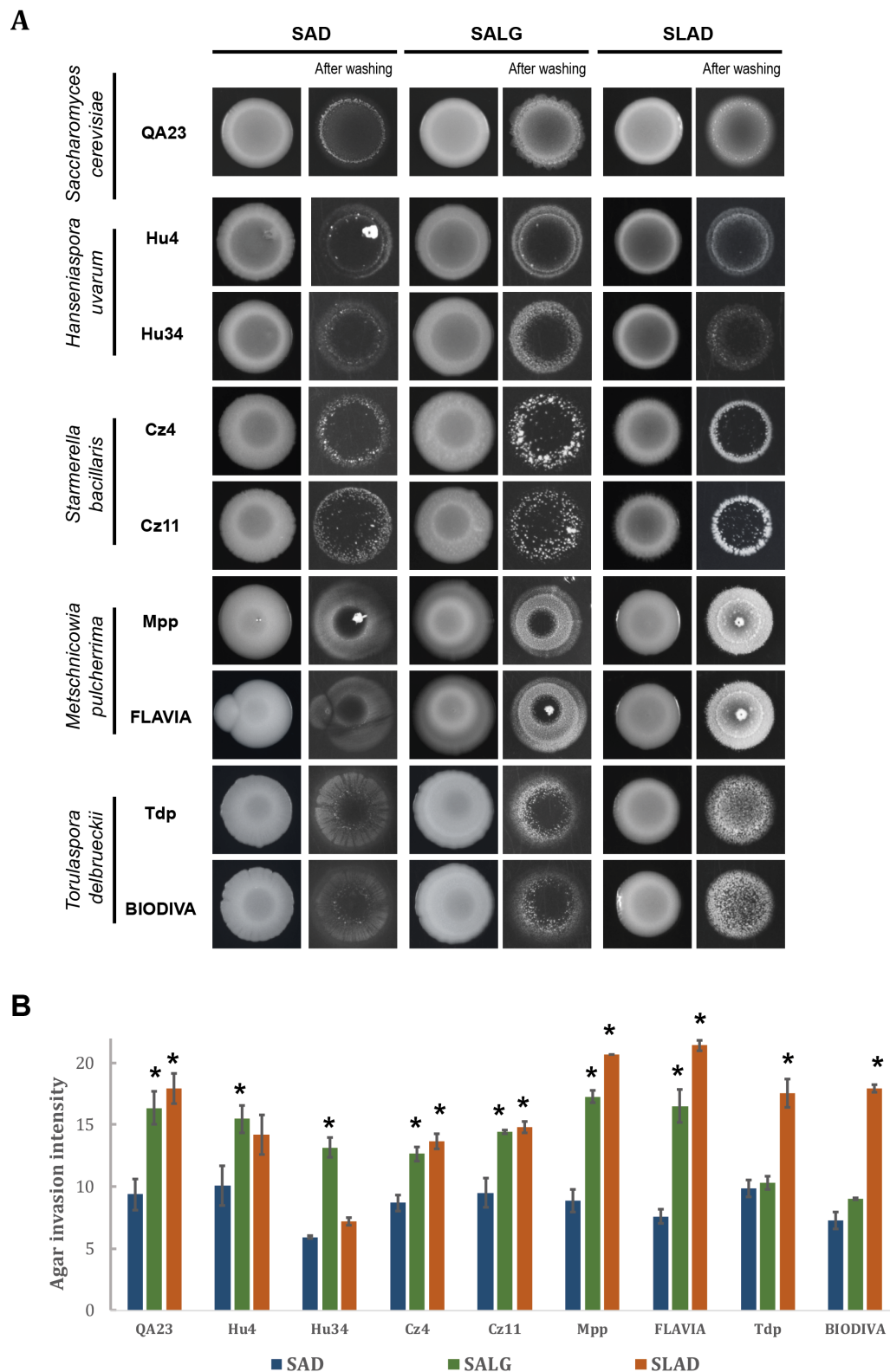


Figure 2.6: Invasive growth phenotypes of different wine yeast species. (A) In a plate washing assay (PWA), equal concentrations of cells were spread on media with different nutrient contents and incubated for 5 days at 28 °C. (B) Quantification of invasive growth was performed after washing the plate via densitometry analysis. Cells were spotted in triplicate, and the average values are shown. Statistical analysis was carried out by comparing each strain with respect to rich media; p-value < 0.05.

We also determined the ability of these yeasts to form pseudohyphae by analysing the morphology of their colonies in SD, SLAD and SALG media. The colonies were observed daily under a microscope for 10 days. Figure 2.7 shows the morphology of the colony peripheries at day 7. Pseudohyphae patterns varied among strains, but in some cases, such as *H. uvarum* and *M. pulcherrima*, there was a correlation between nitrogen limitation and pseudohyphae formation. This indicated that ammonium starvation also induces pseudohyphal growth, particularly in *H. uvarum*, which formed many filaments. On rich agar, only *H. uvarum* was able to produce pseudohyphae. The unique yeast species that did not undergo filamentous growth under any conditions was *T. delbrueckii*. None of the non-*Saccharomyces* strains formed pseudohyphae in SALG plates. The lack of glucose was not a limiting factor to trigger this aspect of the filamentous growth response in non-*Saccharomyces* yeast.

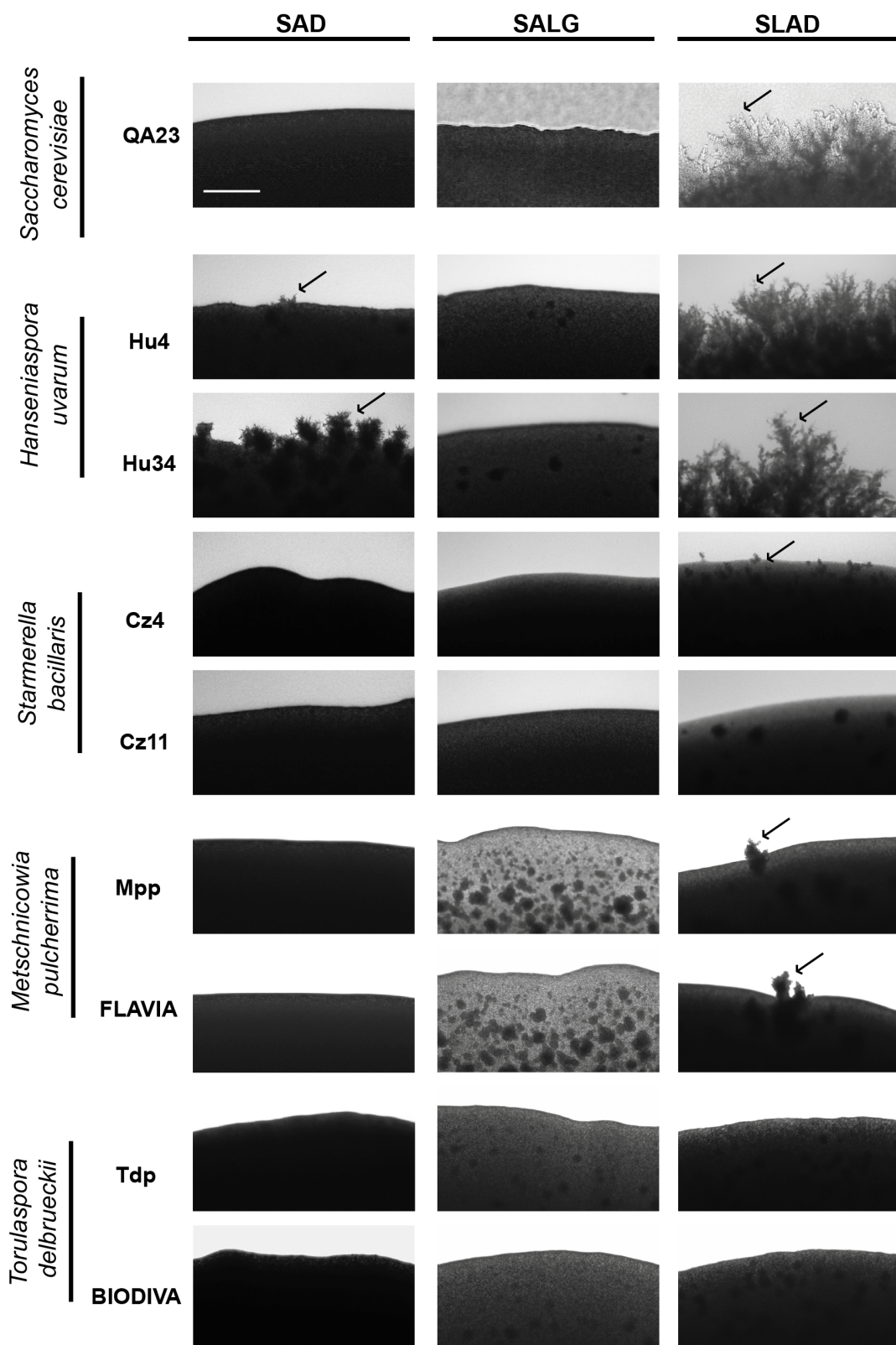


Figure 2.7: Pseudohyphal growth phenotypes of different wine yeast species. Cells were spotted on rich medium (SAD) and nutrient limitation media (SALG and SLAD). Colony peripheries were photographed after incubation for 5 days at 28 °C. Scale bar is 50 μm.

Effects of alcohols on filamentous growth in non-*Saccharomyces* species

Ethanol and other alcohols stimulate filamentous growth (Chen and Fink, 2006; Dickinson, 1996; Lorenz et al., 2000). The effects of alcohols on invasive growth was assayed on SD, SALG and SLAD plates, both with and without supplementation with different alcohols.

Plates were incubated for 3 days to observe whether alcohols induced filamentous growth, similar to ethanol in *S. cerevisiae* (González et al., 2017). In general, the effects of alcohols varied depending on the medium and the species (Fig 8A). On SD medium (Fig 8B), TrpOH and PheOH promoted invasive growth in the *S. cerevisiae* strain, as expected. Among non-*Saccharomyces* species, PheOH only promoted invasive growth in *H. uvarum*, while ethanol and TrpOH only promoted invasive growth in *T. delbrueckii*. Furthermore, no significant effects were observed in *S. bacillaris* or in *M. pulcherrima*. On SALG plates (Fig 8C), aromatic alcohols significantly decreased the invasive growth of the commercial QA23 strain. Among non-*Saccharomyces* strains, TrpOH and PheOH significantly stimulated invasive growth by *H. uvarum* Hu4 and *T. delbrueckii* Tdp, respectively. Ethanol appeared to strengthen invasive growth in *S. bacillaris*, *M. pulcherrima* and *T. delbrueckii*, while TyrOH induced similar effects in the two strains of *M. pulcherrima* and in the commercial *T. delbrueckii* BIODIVA strain. On SLAD plates (Fig 8D), ethanol induced invasive growth in the QA23 strain, as well as in both strains of *S. bacillaris* and *T. delbrueckii*. *H. uvarum* and *S. bacillaris* increased their invasive growth in the presence of PheOH. On the other hand, TyrOH significantly reduced the invasive growth of *M. pulcherrima* strains.

Chapter 2

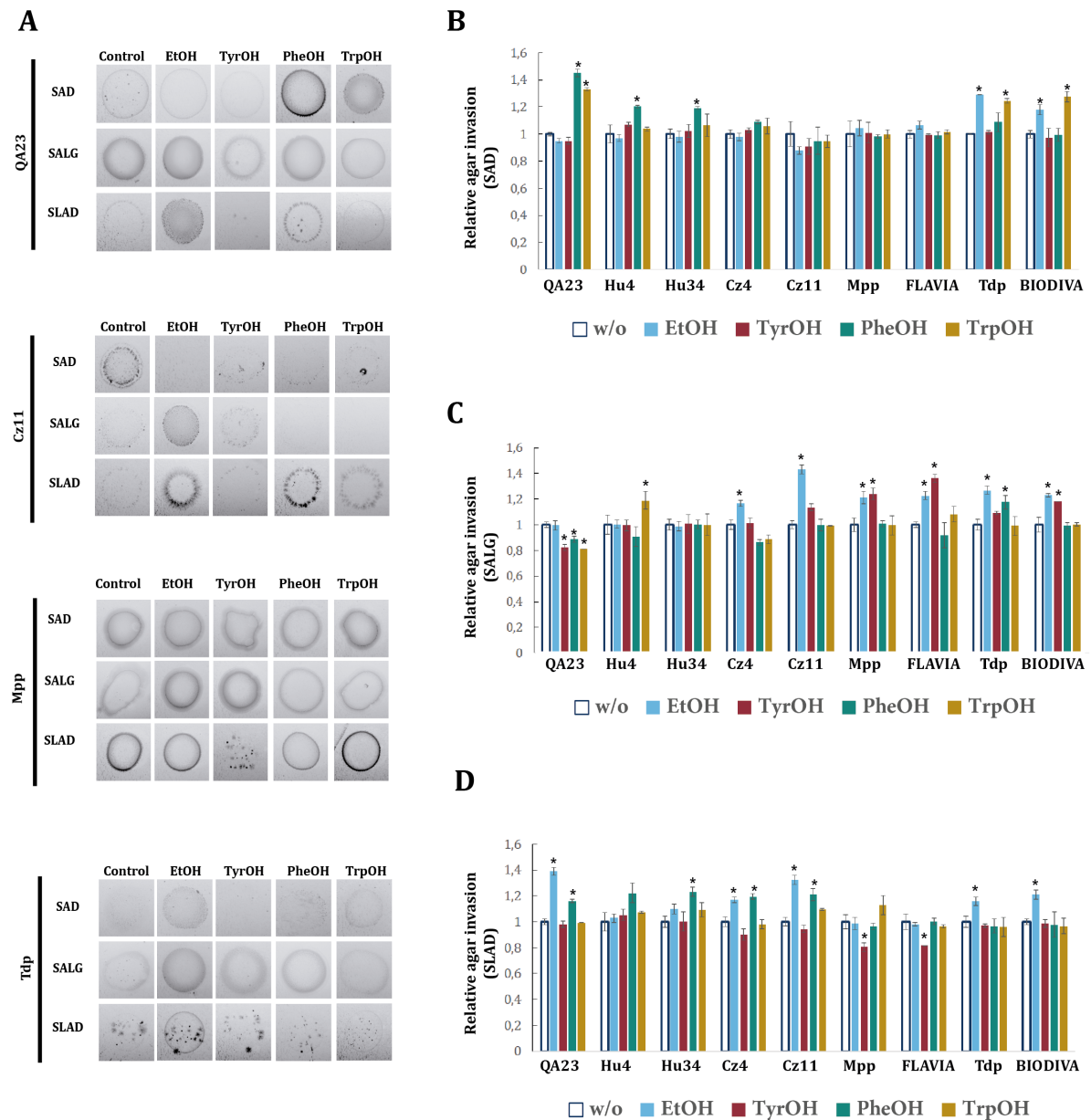


Figure 2.8: Invasive growth of wine yeast species in the presence of aromatic alcohols and ethanol. (A) In a plate washing assay (PWA), equal concentrations of cells were spread on SAD, SALG and SLAD media in the presence of the aromatic alcohol (TyrOH, PheOH or TrpOH) at 500 μ M or 2 % (v/v) EtOH and incubated for 3 days at 28 °C. Panel A shows the results from the washed plate. The invasive growth obtained with different wine yeast species in SAD (B), SALG (C) and SLAD (D) was obtained via densitometry. Asterisks denote a p-value < 0.05 for samples relative to each strain's invasion in the media without added alcohol.

To study the effects of alcohols in pseudohyphal growth, we focused on SLAD medium (Fig 2.9). Ethanol and PheOH stimulated pseudohyphal formation in *S. cerevisiae*. However, the addition of alcohols to agar plates resulted in a reduction in filamentation in both strains of *H. uvarum*. Similar to *S. cerevisiae*, ethanol changed growth patterns to a more filamentous form in *S. bacillaris*, but the aromatic alcohols tested did not affect pseudohyphae development. TyrOH considerably increased filament formation in *M. pulcherrima*. Moreover, the two strains of *T. delbrueckii* tested did not form pseudohyphae when starved for nitrogen in the presence of any alcohol tested.

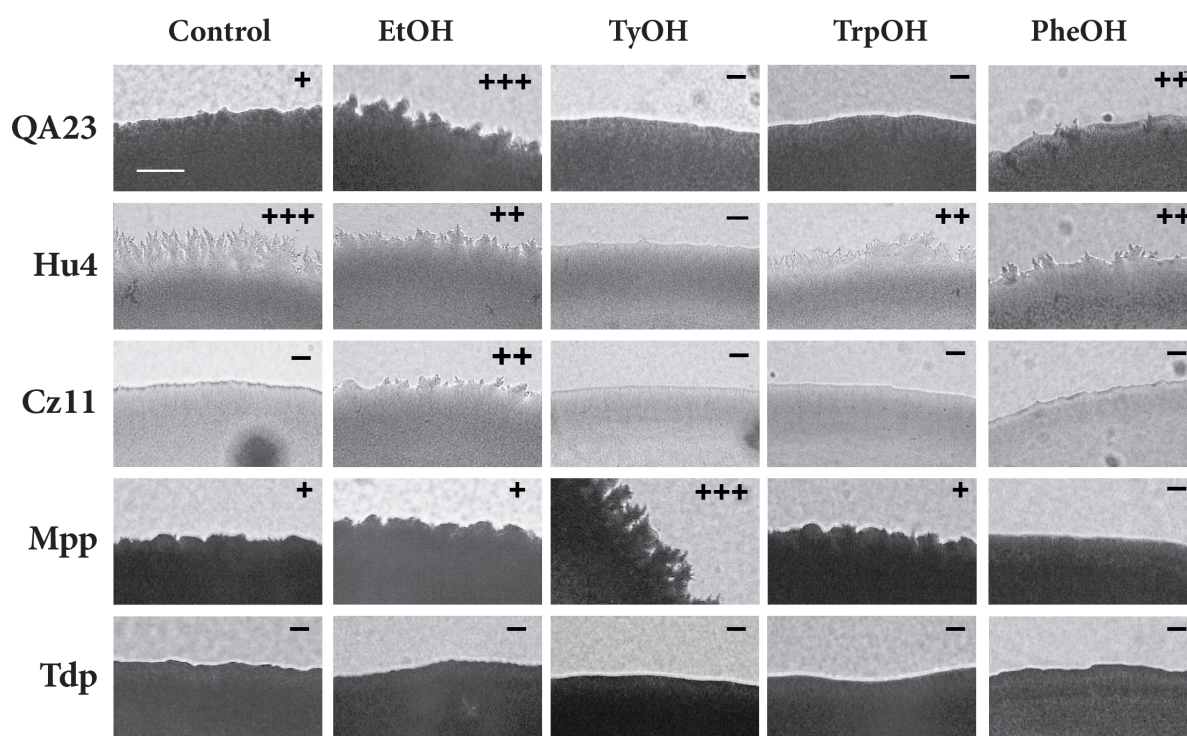


Figure 2.9: Pseudohyphal growth phenotypes of different wine yeast species in response to ethanol and aromatic alcohols. Cells were spotted on SLAD medium. Colony peripheries were photographed after incubation for 3 days at 28 °C. Scale bar is 50 µm.

Discussion

No organisms exist in isolation. All species share common environments and compete for nutrients. Interactions between organisms are commonplace and may be diverse. Although there are many examples of cooperation and symbiotic relationships among organisms, many interactions are combative, with one species profiting from another's detriment. An excellent example of this can be seen on rotting fruit, where yeast and other microorganisms compete for sugar food sources. Although budding yeast becomes the major organism in fruit must, it is interesting to study the behaviours of other organisms that share this environment. Non-*Saccharomyces* yeasts are predominant in grape must, even during the first stages of spontaneous fermentations, but are rapidly replaced by *S. cerevisiae*, which completes the process (Fleet, 2003). This population change is traditionally associated with the diminished cultivability of non-*Saccharomyces* strains due to multiple factors, such as high ethanol concentrations, nitrogen or oxygen limitation, and others (Holm Hansen et al., 2001; Monteiro and Bisson, 1991; Ribéreau-Gayon et al., 2006). Recently, some findings have associated interactions between species with the secretion of certain compounds by yeast during alcoholic fermentation (Albergaria and Arneborg, 2016; Ciani and Comitini, 2015, Wang et al, 2015a). Some alcohols are produced at high density by *S. cerevisiae* (Zupan et al., 2013). Our results showed that only TrpOH was able to reduce growth in *S. cerevisiae* strains. However, in non-*Saccharomyces* species, the three fusel alcohols exerted negative effects on GT and maximal growth in some strains, even at low concentrations (100-250 ppm). These aromatic alcohols are produced by yeast during alcoholic fermentation and are associated with the quorum sensing (QS) phenomenon by regulating cell density, promoting the shift of cells into the stationary phase and inducing morphogenetic transitions (Chen et al., 2004; Chen and Fink, 2006; Sprague and Winans, 2006; Zupan et al., 2013). The production of these auto-signalling alcohols is regulated by nitrogen and is activated under nitrogen-poor conditions (Chen and Fink, 2006).

Non-*Saccharomyces* strains also produce these aromatic alcohols but at lower concentrations (Zupan et al, 2013, González, 2017). The concentrations found in fermented beverages range from 4-197 mg/L PheOH, 100-450 mg/L TrpOH, and 5-40 mg/L TyrOH (Swiegers et al., 2005; Zupan et al., 2013; González et al., manuscript in preparation). However, certain natural strains produce huge amounts of these aromatic alcohols during fermentation; for example, strain 96581 produces phenylethanol, tyrosol, and tryptophol at concentrations as high as 434 mg/kg, 365 mg/kg, and 129 mg/kg, respectively, during beer fermentation (Banach and Ooi, 2014). Thus, the production of aromatic alcohols may play

a role in certain yeast interactions, inhibiting the growth of non-*Saccharomyces* strains and even directing the replacement of non-*Saccharomyces* species during alcoholic fermentation. Indeed, the inhibitory effects of high levels of PheOH on yeast growth were previously reported (Banach and Ooi, 2014). In this study, we tested the effects of these alcohols individually. Mixtures of these alcohols may greater impacts on yeast growth.

Mel is synthesized from tryptophan and exhibits various biological activities in humans, such as antioxidant activity (Anisimov et al., 2006; Reiter et al., 2001). Yeasts generate low concentrations of Mel during alcoholic fermentation; however, its role in yeast regulation is still unknown. Mel is able to reduce oxidative damage induced by hydrogen peroxide in *S. cerevisiae* (Vázquez et al., 2017). In our study, the presence of Mel in the media did not affect the growth of the yeast strains tested. Other compounds derived from tryptophan metabolism were tested, specifically the precursor of Mel, serotonin (5-hydroxytryptamine), and the biogenic amine tryptamine. In contrast to Mel, Ser considerably reduced the maximal growth and doubling time of all strains tested and was the most potent compound tested, which indicates that Ser has toxic effects in yeast. Nevertheless, Trpm was most effective during the lag phase, reducing growth at low concentrations but increasing growth at high concentrations. Ser is a biogenic amine that is among the most common neurotransmitters in nature. Serotonin has previously shown antifungal activity against *Candida* and *Aspergillus* spp. in vitro (Lass-Flörl et al., 2003; Lass-Flörl et al., 2002). In addition to being fungicidal at higher concentrations, Ser exerts inhibitory effects on *C. albicans* even at low concentrations by inhibiting phospholipase activity and secreted aspartyl proteinases production (Mayer et al., 2013). Although Ser is a precursor of Mel synthesis in yeast, it is not abundant during alcoholic fermentation (Fernández-Cruz et al., 2017). Indeed, serotonin is found in wines at levels ranging from 2 to 23 mg/L, primarily due to malolactic fermentation (Domizio et al., 2014; Manfroi et al., 2009). Biogenic amines are basic nitrogenous compounds produced in wine through the decarboxylation of amino acids by yeasts or lactic acid bacteria, although some are also present in grape musts. The concentration of biogenic amines that are potentially produced in wine largely depends on the abundance of amino acid precursors in the medium, the presence of decarboxylase-positive microorganisms, and wine parameters such as pH, alcohol, and sulphur dioxide, which will impact the microbial growth (Moreno-Arribas et al., 2010; Smit et al., 2008). The biogenic amines commonly found in wines are histamine, tyramine, putrescine and cadaverine. Trpm levels in wines are usually very low (0.02 – 0.2 mg/l), and its synthesis largely depends on fermentation temperature but not on supplementation with its precursor amino acid (Lorenzo et al., 2017). There-

fore, although Trpm appears to significantly affect different growth parameters, this does not occur at concentrations usually found in wines.

Growth arrest mediated through cell-to-cell contact is a specific case illustrating the QS phenomenon. Recently, the death of non-*Saccharomyces* yeasts in mixed fermentation with *S. cerevisiae* was associated with mechanisms mediated through cell-to-cell contact as well as high cell densities (Nissen et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013). However, the role of cell-to-cell communication through QSM in inhibiting the growth of certain yeast strains during mixed-culture fermentation remains unclear (Avbelj et al., 2016, Wang et al., 2015b). QS in yeasts involves a morphological transition from a filamentous to a yeast form, or vice versa (Sprague and Winans, 2006). Yeasts undergo this transition from a unicellular to a filamentous form in response to environmental cues, which may arise from alterations in nutrient concentrations or in the presence of auto-inductive molecules that are secreted by cells (Chen and Fink, 2006). Stimuli that trigger filamentous growth include nitrogen limitation (Gimeno et al., 1992) and glucose limitation (Cullen and Sprague, 2000). Filamentation is well established in *Saccharomyces* (Chen and Fink, 2006; Cullen and Sprague, 2012) and the dimorphic fungal human pathogen *C. albicans* (Chen et al., 2004; Hornby et al., 2001), but little is known about this type of growth in other genera and species of yeasts (Gori et al., 2011; Pu et al., 2014; van Rijswijk et al., 2015). In our study, we tested two strains each of the major genera involved during wine fermentation to test their ability to penetrate surfaces (invasive growth) or to form pseudohyphae. All strains tested were wild yeasts isolated from wine environments and were able to invade, even in rich media. Indeed, natural yeast isolates exhibit high levels of invasion (Casalone et al., 2005), allowing them to colonize natural niches, such as grapes. According to Pitoniak et al., (2009), yeasts require the filamentous growth pathway and *FLO11* to be able to full colonize this environment. Nutrient limitation also promotes increased invasive growth in non-*Saccharomyces* species. The *S. bacillaris* and *M. pulcherrima* strains increased their invasive growth both under glucose and nitrogen limitation, but they only formed small pseudohyphae with nitrogen limitation. This filamentous growth is considered a fungal scavenging response. Indeed, the ability to form pseudohyphae and invade agar upon nutrient deprivation provides a selective advantage to yeast cells, facilitating foraging for scarce nutrients at a distance from their initial position (Casalone et al., 2005). On the other hand, *H. uvarum* exhibited a striking behaviour because its cells primarily invade the agar under glucose limitation but form a large number of pseudohyphae under nitrogen limitation and, to a lesser extent, in rich media. The ability of these strains to form

pseudohyphae in rich media may be an advantage to colonize fruits by adhesion and a possible reason for the wide distribution of this species on natural fruit surfaces; in some studies, *H. uvarum* is the main species found in grape habitats (Cadez et al., 2002; Ocón et al., 2010; Padilla et al., 2016; Pretorius, 2000). Finally, *T. delbrueckii* was the only species that did not form pseudohyphae in any of the tested media. Nevertheless, this species was able to invade under nitrogen limitation. This suggests the differential regulation of both phenotypes in this species. A possible explanation for this lack of pseudohyphal growth may be related to its ability to flocculate in liquid medium, especially in YPD medium. Both phenotypic traits are mediated by the same gene, *FLO11*, and a recent study demonstrated that variations in the amino acid sequence of the adhesion domain of *FLO11p* causes different flocculation activities (Barua et al., 2016). Moreover, one yeast strain may possess the ability to either flocculate or form pseudohyphae or express both of these two phenotypes (Wilcocks, 1995). The extent of flocculation, pseudohyphal development and invasive growth are dependent on growth conditions, medium composition (nitrogen content and carbon source) and the ploidy of the cells (Blacketer et al., 1995; Bowen and Ventham, 1994; Gimeno et al., 1992; Lambrechts et al., 1996; Smit et al., 1992).

Overall, the two strains of each species tested presented similar behaviours, indicating that filamentous growth is a similar trait in several species. The morphological transition in many fungi is also controlled by auto-regulatory molecules through QS mechanisms (Chen et al., 2004; Hornby et al., 2001; Kügler et al., 2000). Aromatic alcohols have been reported to possess QS activity, and their effects together with ethanol on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). However, filamentous growth properties in non-*Saccharomyces* wine yeasts have been less well-studied. In this study, the effects of aromatic alcohols and ethanol were analysed in three different media, which differed in their glucose and ammonium content. As previously described, PheOH and TrpOH exerted effects on filamentous growth in *S. cerevisiae*. However, the results are not completely in concordance with Chen and Fink (2006), since they observed that PheOH and TrpOH both exerted effects on pseudohyphal growth but only PheOH affected invasive growth, and in our study we observed the opposite. Moreover, we also observed inhibitory effects on pseudohyphae with all aromatic alcohols in low glucose medium. In *H. uvarum*, the sole aromatic alcohol that promoted invasive growth was PheOH, both in rich and nitrogen-limiting media. A reduction in pseudohyphae formation was observed in the presence of aromatic alcohols, which also occurred with farnesol in *C. albicans* (Hornby et al., 2001). In a recent

study, Pu et al. (2014) described the involvement of PheOH in filamentous growth, adhesion and biofilm formation in this species. TyrOH increased pseudohyphae formation but reduced invasive growth in *M. pulcherrima* in SLAD medium. However, this alcohol promoted invasive growth in low glucose medium. TyrOH has also been described as an inducer of filamentous growth in *C. albicans* (Chen et al., 2004). In our study, TyrOH did not significantly impact *S. bacillaris* growth under any conditions tested. This might be expected because this organism is closely related to *C. albicans*. Only PheOH exerted any effects in this species, and these effects only occurred only in SLAD medium. This species produces very low concentrations of aromatic alcohols; a previous study detected no synthesis (Zupan et al., 2013). Therefore, in this species, other molecules may be the signals that initiate changes in morphogenesis, similar to *C. albicans* with farnesol (Kruppa, 2009).

Ethanol has been extensively reported to stimulate pseudohyphal growth in *S. cerevisiae* (González et al., 2017; Lorenz et al., 2000). In our study, ethanol affected all species to varying degrees, with the exception of *T. delbrueckii*. However, even in this strain, ethanol promoted invasive growth under all tested conditions. As we have previously shown, *T. delbrueckii* did not undergo pseudohyphal growth under any of the tested conditions, but these strains presented flocculent growth in liquid media, which may suppress filamentation, as both responses are controlled by the same gene family. Therefore, the aromatic alcohols appear to be species-specific signalling molecules because different species manifest different responses to these auto-regulatory molecules. This finding was previously observed for *S. cerevisiae* and *C. albicans*: Chen and Fink (2006) demonstrated that different aromatic alcohols exert different effects on the morphogenesis of these two yeast species.

To conclude, we demonstrated that aromatic amino acid-derived compounds produced during alcoholic fermentation by yeast, and at the concentrations found in fermented beverages, influence the growth of certain yeast species. Among these compounds, aromatic alcohols appear to be the most interesting because yeasts synthesize these compounds at levels that have physiological effects. Our study reinforces the idea that these molecules play roles as QSM. They appear to be able to induce or repress filamentous growth and also function as possible modulators of growth. Moreover, different effects of these aromatic alcohols were observed in non-*Saccharomyces* yeast species, suggesting these compounds promote different responses depending on the yeast species.

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Chapter 2

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Supplementary figures

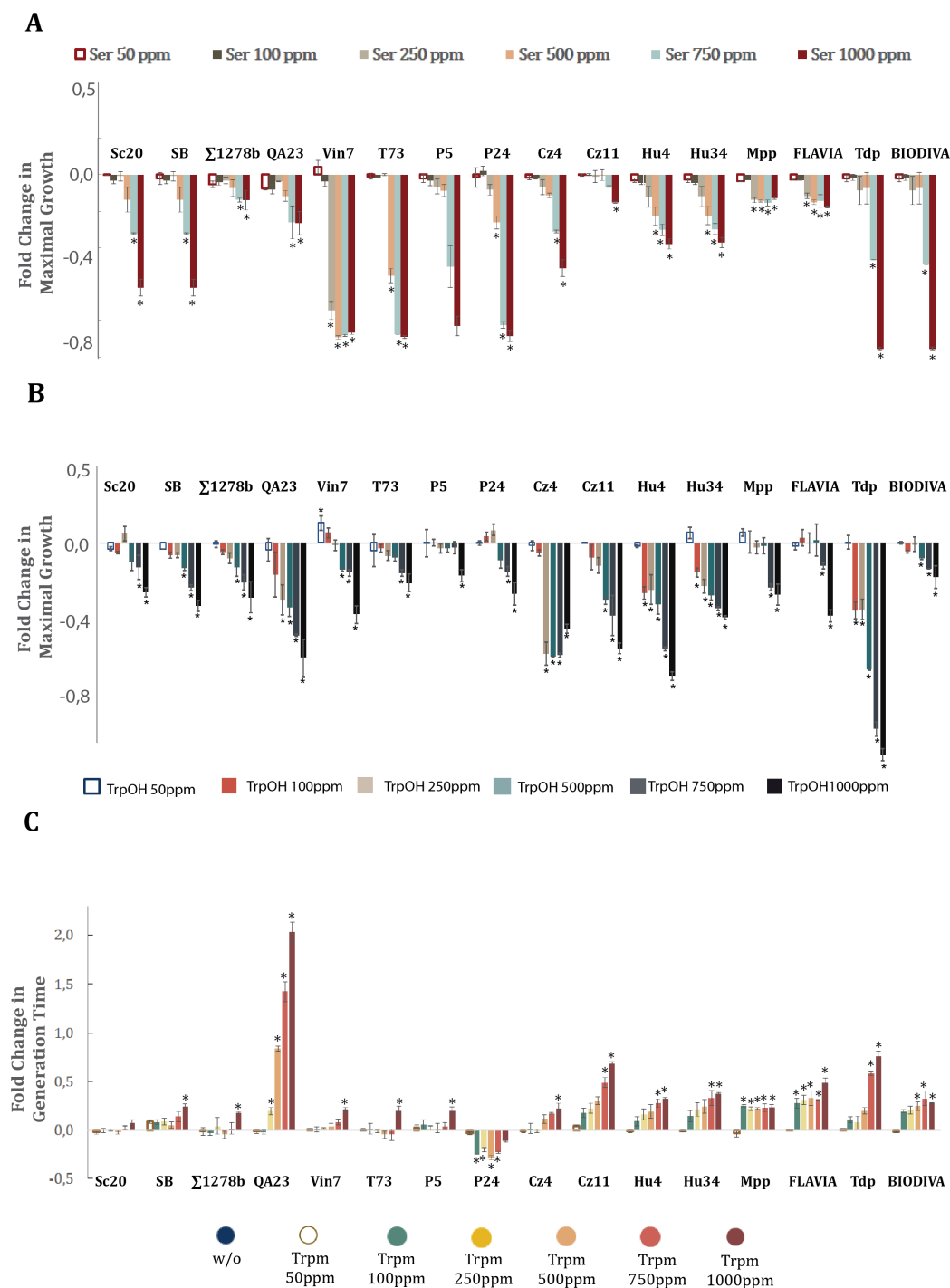


Figure S2.1: Effects of increasing compound, Ser(A), TrpOH (B), Trpm (C), concentrations on yeast growth. different compounds were added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000 ppm). Statistical analysis was performed comparing the effects of compound concentrations in each strain; p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (without compounds).

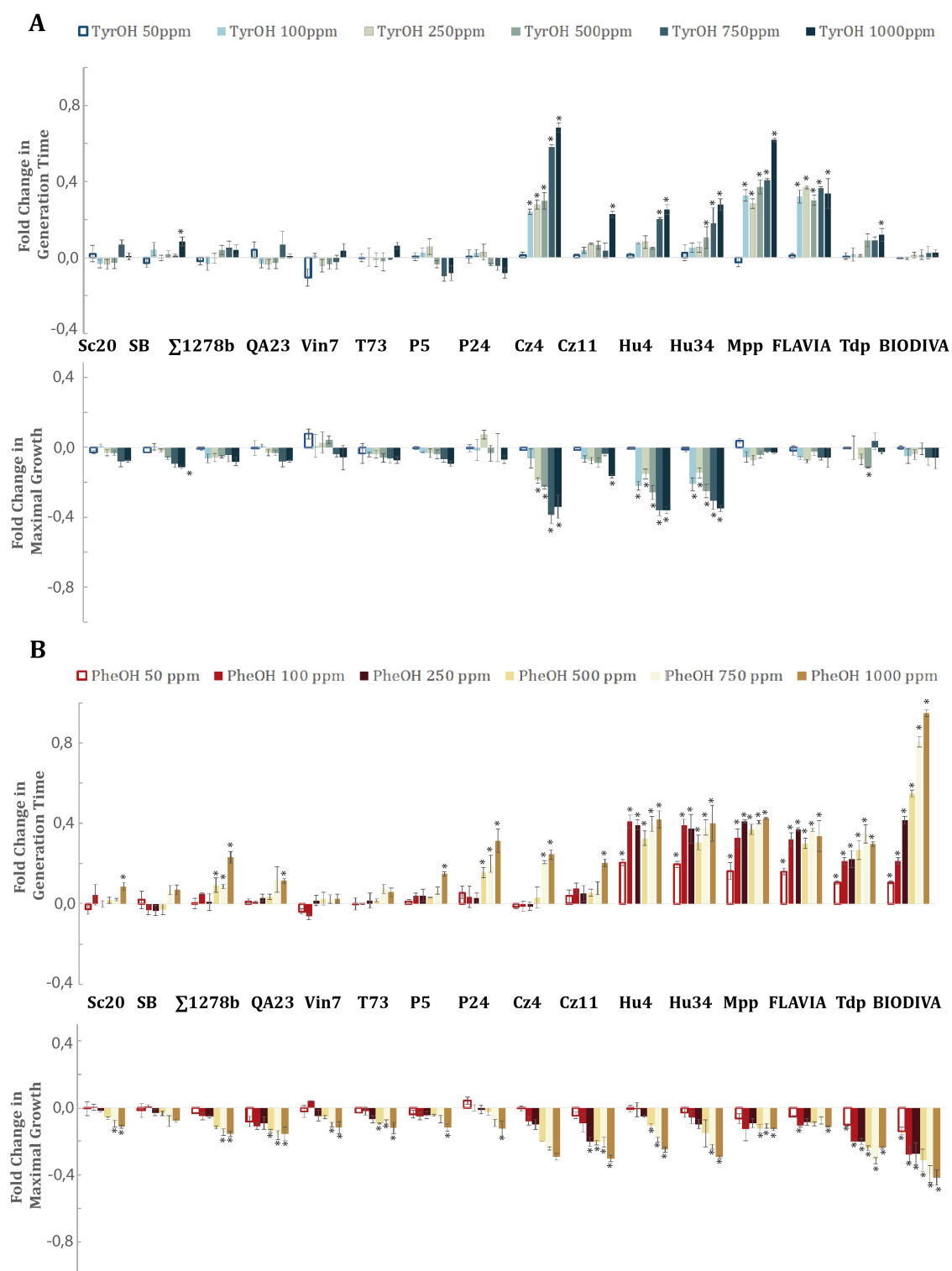


Figure S2.2: Effects of increasing aromatic alcohols, TyrOH (A) and PheOH (B), concentrations on yeast growth. different compounds were added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000 ppm). Statistical analysis was performed comparing the effects of alcohol concentrations in each strain; p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (without compounds).

CHAPTER 3

Role of mitochondrial retrograde pathway in regulating ethanol-inducible filamentous growth in yeast

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Abstract

In yeast, ethanol is produced as a by-product of fermentation through glycolysis. Ethanol also stimulates a developmental foraging response called filamentous growth and is thought to act as a quorum-sensing molecule. Ethanol-inducible filamentous growth was examined in a small collection of wine/European strains, which validated ethanol as an inducer of filamentous growth. Wine strains also showed variability in their filamentation responses, which illustrates the striking phenotypic differences that can occur among individuals. Ethanol-inducible filamentous growth in $\sum 1278b$ strains was independent of several of the major filamentation regulatory pathways [including fMAPK, RAS-cAMP, Snf1, Rpd3(L) and Rim101] but required the mitochondrial retrograde (RTG) pathway, an inter-organellar signalling pathway that controls the nuclear response to defects in mitochondrial function. The RTG pathway regulated ethanol-dependent filamentous growth by maintaining flux through the TCA cycle. The ethanol-dependent invasive growth response required the polarisome and transcriptional induction of the cell adhesion molecule Flo11p. Our results validate established stimuli that trigger filamentous growth and show how stimuli can trigger highly specific responses among individuals. Our results also connect an inter-organellar pathway to a quorum sensing response in fungi.

Key Words: filamentous growth, pseudohyphal growth, quorum sensing, mitochondria-to-nucleus pathway, Krebs cycle

Introduction

Fungal species represent a diverse group of microorganisms. Most fungal species exist in the wild. Other species live in commensal or pathogenic relationships with host organisms, while others still have been domesticated for food and technological benefits. *Saccharomyces sensu stricto* represents a group of highly related yeasts (Borneman and Pretorius 2015). *Saccharomyces cerevisiae* and its relatives are commonly used in research laboratories and a variety of industrial processes. The ability of *Saccharomyces* to produce ethanol from several sugar sources makes it an essential component of the brewing and wine-making industries. Yeast not only produces ethanol as the major by-product of the alcoholic fermentation of sugars but also produces minor compounds such as aromatic (or fusel) alcohols that impart flavour and bouquet to wines. These properties have been studied to improve ethanol production and to understand the molecular basis of nutrient sensing and regulatory mechanisms in eukaryotes (Fleet and Heard 1993; Ribéreau-Gayon et al. 2000; Beltran et al. 2004; Alper et al. 2006; Beltran et al. 2008; Zaman et al. 2008).

Many fungal species, including yeasts, can undergo filamentous growth. Filamentous growth in yeast is a developmental foraging response, where cells become elongated and grow in connected chains (Gimeno et al. 1992; Kron et al. 1994). In some settings, cells can penetrate surfaces, which is known as invasive growth (Roberts and Fink 1994). Some fungal species grow as multinucleate hyphae. Other species, like *S. cerevisiae*, produce pseudohyphae where cells undergo cytokinesis at each cell division. Filamentous growth has been extensively studied in yeast and other species, particularly pathogens, which require filamentous growth for virulence (Madhani and Fink 1998; Lengeler et al. 2000; Polvi et al. 2015). Such studies have led to insights into the triggers, signalling pathways and transcriptional targets that control developmental responses in fungi and other eukaryotes.

One inducer of filamentous growth is nitrogen limitation (Gimeno et al. 1992). Another is the limitation of fermentable sugars like glucose (Cullen and Sprague 2000). The morphogenetic response to limiting glucose is mediated by several pathways, including a mitogen-activated protein kinase pathway called the filamentous growth (fMAPK) pathway (Saito 2010; Karunanithi and Cullen 2012; Adhikari and Cullen 2014; Adhikari et al. 2015), the AMP-dependent kinase AMPK Snf1p (Celenza and Carlson 1989; Woods et al. 1994; Lesage et al. 1996; Cullen and Sprague 2000; McCartney and Schmidt 2001; Kuchin et al. 2002), and the RAS-cAMP-protein kinase A (PKA) pathway (Toda et al. 1985; Gimeno et al. 1992; Mosch et al. 1996; Colombo et al. 1998; Robertson and Fink 1998b; Robertson and Fink

1998a; Mosch et al. 1999; Rupp et al. 1999b; Robertson et al. 2000; Pan and Heitman 2002). Filamentous growth is also regulated by the Rim101 pathway, which regulates the response to pH (Lamb et al. 2001; Lamb and Mitchell 2003; Barrales et al. 2008). Other regulators include the chromatin remodeling complex Rpd3(L) (Carrozza et al. 2005; Barrales et al. 2008; Ryan et al. 2012), the tRNA modification complex Elongator (Krogan and Greenblatt 2001; Winkler et al. 2001; Petrakis et al. 2004; Li et al. 2007; Svejstrup 2007), and the Pho80p-Pho85p cyclin and cyclin-dependent kinase (Measday et al. 1997; Huang et al. 2002; Shemer et al. 2002; Moffat and Andrews 2004; Huang et al. 2007; Chavel et al. 2014). In addition to these pathways, genetic (Lorenz and Heitman 1998; Palecek et al. 2000), genomic and proteomic screens (Jin et al. 2008; Xu et al. 2010; Ryan et al. 2012) have identified many other proteins and pathways that impact filamentous growth. Thus, filamentous growth resembles cell differentiation in metazoans, where global reorganization of cellular processes results in the construction of a new cell type.

Fungal species also utilize small molecules to interpret information about their environment. Like many other microbial species (Miller and Bassler 2001; Parsek and Greenberg 2005; Rumbaugh et al. 2009), *S. cerevisiae* exhibits quorum-sensing responses (Hlavacek et al. 2009; Prunuske et al. 2012). Yeast can sense and respond to ammonia (Palkova et al. 1997), aromatic (fusel) alcohols (Chen and Fink 2006), and ethanol (Dickinson 1994; Dickinson 1996; Lorenz et al. 2000). By products of the Ehrlich reactions (Hazelwood et al. 2008), fusel alcohols are formed by conversion of several amino acids into glutamate as a nitrogen source under nitrogen-limiting conditions (Ljungdahl and Daignan-Fornier 2012). Fusel alcohols are produced at higher levels in nitrogen-limiting medium and sensed in a density-dependent manner by a PKA-dependent mechanism to regulate filamentous growth (Chen and Fink 2006). Multiple fungal species produce and sense a variety of aromatic alcohols, which may impart selectivity in this type of cellular communication (Chen et al. 2004; Chen and Fink 2006; Sprague and Winans 2006; Kruppa 2008; Langford et al. 2013). Recent efforts have expanded the diversity alcohols that can be sensed and measured their impact on fungal behavioural responses (Ghosh et al. 2008; Wuster and Babu 2010; Sharma and Prasad 2011; Albuquerque and Casadevall 2012; Bojsen et al. 2012; Avbelj et al. 2015; Williams et al. 2015). An open question has been to identify the regulatory pathways that control alcohol-mediated morphogenesis and understand how cells detect and respond to these stimuli. Addressing this problem has a practical benefit, as industrial manipulation of yeast may be accelerated by understanding density-dependent growth and behavioural responses (Westman and Franzen 2015).

To better understand common and unique elements of the filamentous growth response, a diverse collection of strains was examined from the ‘wine/European’ group (Goffeau et al. 1996; Wei et al. 2007; Borneman et al. 2008; Argueso et al. 2009; Liti et al. 2009; Novo et al. 2009; Borneman et al. 2011). Most strains tested underwent filamentous growth in response to limiting glucose, limiting nitrogen, or the presence of ethanol or fusel alcohols. A specific role for the mitochondrial retrograde (RTG) pathway, which controls the response to compromised mitochondrial function (Liu and Butow 2006) and is known to regulate filamentous growth (Jin et al. 2008; Chavel et al. 2010; Aun et al. 2013; Starovoytova et al. 2013; Chavel et al. 2014), was identified as a specific regulator of ethanol-inducible invasive growth. RTG regulated TCA cycle flux in response to ethanol to modulate filamentous growth. Thus, the study connects an inter-organellar signalling pathway to a quorum-sensing morphogenetic response in fungi.

Materials And Methods

Yeast Strains, Media and Growth Conditions

Yeast strains are described in Table 3.1. Standard media was used (Rose 1990). Yeast strains were generated by polymerase chain reaction (PCR)-based homologous recombination techniques using auxotrophic or antibiotic resistant markers (Goldstein and McCusker 1999). Yeast were grown on YPD (2 % peptone, 1 % yeast extract, 2 % glucose, and 2 % agar), minimal medium [(MM) 1X Yeast Nitrogen Base (YNB) without amino acids or ammonia, 2 % glucose, and 10 mM $(NH_4)_2SO_4$, synthetic media [(SD) 1 X YNB, 2 % glucose, and 37 mM $(NH_4)_2SO_4$] with ammonium and dextrose [(SAD) 1X YNB, 1 % glucose, and 37 mM $(NH_4)_2SO_4$], or with ammonium and low glucose [(SALG) 1X YNB, 0.5 % glucose, and 37 mM $(NH_4)_2SO_4$]. To evaluate pseudohyphal growth, yeast were grown on synthetic medium with dextrose and low-ammonium [(SLAD) 1X YNB, 2 % glucose, 50 μ M $(NH_4)_2SO_4$, and 2 % agar (Gimeno et al. 1992)]. Media was supplemented with uracil for auxotrophic mutants. For some experiments, SD and SLAD media were supplemented with 500 μ M tryptophol, tyrosol, or phenylethanol and 2 % (v/v) ethanol. The *CIT2-lacZ* plasmid has been described (Liu and Butow 1999) and was provided by Dr. Zhengchang Liu (Louisiana State University, New Orleans).

Table 3.1: Yeast strains used in the study.

Strain	Genotype	Reference
S288c	MAT α SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6	Mortimer and Johnston 1986
Nsa (a)	MATa / MAT α	Wang et al. 2014
S1 (b)	MATa/ MAT α	Padilla et al. 2015
QA23 (c)	MATa/ MAT α	Borneman et al. 2011
T73 (d)	MATa/ MAT α	Querol et al., 1992
SB	HO/HO, asp1-H142/asp1-H142	Marullo et al. 2007
P5 (e)	MATa/ MAT α	García-Rios et al. 2014
P24 (c)	MATa / MAT α	García-Rios et al, 2014
VIN7 (f)	Triploid allohybrid <i>S. cerevisiae</i> x <i>S. kudriavzevii</i>	Borneman et al., 2012
W27 (c)	Hybrid <i>S.cerevisiae</i> x <i>S. kudriavzevii</i>	Schütz and Gafner, 1994
PC312 (g)	MATa ura3-52	Liu et al. 1993
PC313	MATa ura3-52	Liu et al. 1993
PC318	MATa ura3-52 rho0	Chavel et al. 2010
PC344	MATa/ MAT α ura3-52 / ura3-52	Cullen and Sprague 2000
PC443	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 snf1::URA3	Cullen and Sprague 2000
PC471	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud6::KIURA3 (h)	Cullen and Sprague 2002
PC538	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52	Cullen et al. 2004
PC539	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KLURA3	Cullen et al. 2004
PC549	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3	Cullen and Sprague 2000
PC563	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud8::KIURA3	Cullen and Sprague 2002
PC611	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::URA3	Cullen et al. 2004
PC999	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA	Cullen et al. 2004
PC2549	MATa ura3-52 ras2::KIURA3	Chavel et al. 2010
PC2584	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 tpk1::NAT	Chavel et al. 2010
PC2763	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 elp2::KIURA3	Abdullah and Cullen 2009
PC2953	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rim101::ura3	Chavel et al. 2010
PC3030	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sin3::NAT	Chavel et al. 2010
PC3035	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mks1::NAT	Chavel et al. 2010
PC3097	MAT α ura3-52 leu2 pex3::HYG	This study
PC3363	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA nrg1::KLURA3	Chavel et al. 2010
PC3642	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg3::NAT	Chavel et al. 2010
PC3643	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tco89::NAT	Chavel et al. 2014
PC3652	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT	Chavel et al. 2010
PC3654	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tor1::NAT	Chavel et al. 2010
PC3695	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg1::NAT	Chavel et al. 2014
PC3909	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KLURA3 mks1::NAT	This study
PC3910	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3 mks1::NAT	This study
PC3911	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::URA3 mks1::NAT	This study
PC4041	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT ssk1::KIURA3	This study
PC4141	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 tpk2::URA3	Chavel et al. 2014
PC5059	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig2::HYG	This study
PC5084	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 Msb2-HA tpk3::NAT	Chavel et al. 2014
PC5582	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::KanMX6	This study
PC5594	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KIURA3	This study
PC5864	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sch9::KIURA3	This study
PC6017 (i)	MAT α can1 Δ ::Ste2pr-spHIS5 lyp1 Δ ::Ste3pr-LEU2 his3::hisG leu2 Δ 0 ura3 Δ 0	Ryan et al. 2014
PC6018	MATa/ MAT α can1 Δ ::Ste2pr-spHIS5/can1 Δ ::Ste2pr-spHIS5 lyp1 Δ ::Ste3pr-LEU2/lyp1 Δ ::Ste3pr-LEU2 his3::hisG/his3::hisG leu2 Δ 0/leu2 Δ 0 ura3 Δ 0/ura3 Δ 0	Ryan et al. 2014

- (a) Natural isolate from wine
- (b) Natural isolate from wine (CECT 13132)
- (c) Natural isolate from wine (CECT 13132)
- (d) Commercial wine yeast Lalvin® Lallemend
- (e) Commercial wine yeast Lalvin® Lallemend (CECT1894)
- (f) Commercial wine yeast Lalvin® ICVGRE Lallemend
- (g) Commercial wine yeast AWRI1539®
- (h) All PC strains are in the S1278b strain background
- (i) KIURA3 refers to the *Kluyveromyces lactis* URA3 gene cassette
- (j) Mutants derived from this strain were constructed in a genomic collection and were also tested in the study

Pseudohyphal Growth Assays

Examination of pseudohyphae was determined as described (Gimeno et al. 1992). Strains were grown for 16 h at 28°C in MM and harvested by centrifugation (1000 rpm for 3 min). To obtain single colonies, cells were diluted by a factor of 106 in sterile water, and 100µL of cells were spread onto media (SAD, SALG, and SLAD). Plates were incubated at 28°C and observed daily for 10 d by microscopy for colony morphology.

Invasive Growth Assays

Strains were grown for 16 h at 30°C in MM, harvested by centrifugation (10000 rpm for 3min) at an optical density (O.D. A_{600}) of 2.0, washed once in sterile water and resuspended in sterile water. Ten microliters of cells were spotted on semisolid agar media. Plates were incubated at 28°C. Invasive growth was determined by the plate-washing assay (Roberts and Fink 1994). Colonies were photographed before and after washing over a 10 d period. Plates were washed in a stream of water (soft wash) and colonies were rubbed from the surface with a gloved finger (hard wash). ImageJ (<http://rsb.info.nih.gov/ij/>) was used to quantitate invasive growth (Zupan and Raspor 2008). Background intensity was determined for each spot and subtracted from the densitometry of the area of invaded cells. Densitometric analysis was performed on invasive patches over multiple days. Tukey's t-test was used to determine statistical significance and generate p-values. The Shapiro-Wilk and Jarque-Bera normality tests showed that the data fit a normal distribution. A non-parametric statistics test (Wilcoxon test) showed the same results as the Tukey's t-test.

Quantitative Polymerase Chain Reaction (qPCR) Analysis

Quantitative PCR was performed as described (Beltran et al. 2004). Ethanol addition stimulated the expression of *FLO11* at all-time points except 24 h. Strains were grown in MM for 24h at 28°C, washed with MiliQ sterile water (Millipore Q-PODTM Advantage A10) and resuspended in the indicated media at an O.D. A_{600} of 2.0. Cells were inoculated in SLAD media and in SAD media, and samples were taken at 2h. To study the effect of nitrogen concentration in *FLO11* expression, strains were grown in MM for 24h at 28 °C, washed with MiliQ sterile water (Millipore Q-PODTM Advantage A10) and resuspended in SAD and SLAD media at an O.D. A_{600} of 2.0. Samples were taken at 2h to analyze the *FLO11* expression. To study the effect of ethanol in *FLO11* expression, cells were inoculated at

an O.D. A_{600} of 2.0 in SLAD medium with or without ethanol (2 % v/v) Samples were taken at 45 min, 2 h, 8 h and 24 h. RNA extraction was performed using an RNeasy Mini Kit (Qiagen). RNA concentration was adjusted to 320 ng/ μ L. Reverse transcription was performed using SuperScript® III Reverse Transcriptase (Invitrogen) and Oligo (dt) 20 Primer (Invitrogen).

Real time PCR (RT-PCR) was performed using an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, USA). SyberGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, USA). Reactions contained 25 μ L sample (5 μ L cDNA, 1 μ M each primer, 10 μ L SyberGreen master mix, H₂O q.s.p. 25 μ L). The starting quantity of genes was normalized with *ACT1* (Chavel et al. 2010). Relative gene expression was calculated using the $2^{-\Delta C_t}$ formula, where C_t is defined as the cycle at which fluorescence was determined to be statistically significant above background; ΔC_t is the difference in C_t of the *FLO11* gene and housekeeping gene (*ACT1*). The primers used were *FLO11* forward (5'-CACTTTTGAAGTTTATGCCACACAAG-3') and *FLO11* reverse (5'-CTTGCATATTGAGCGGCACTAC-3') based on (Chen and Fink 2006), and *ACT1* forward (5'-TGGATTCCGGTGATGGTGTT-3') and *ACT1* reverse (5'-CGGCCAAATCGATTCTCAA-3').

Microscopy

Differential-interference-contrast (DIC) and bright-field microscopy was performed using an Axioptan 2 fluorescent microscope (Zeiss) with a PLAN-APOCHROMAT 100X/1.4 (oil) objective (N.A. 0.17). Digital images were obtained with the Axioacam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis and for rendering 3D Z-stack images. Images were further analyzed in Adobe Photoshop, where adjustments of brightness and contrast were made.

Results

Exploring Filamentous Growth in a Collection of Wild and Industrial Yeast Strains

To understand the common and unique features of filamentous growth in yeast, a collection of wild and industrial yeast strains used in wine making was examined (Table 3.1). Strains were compared to Σ 1278b, a well-characterized strain background that undergoes filamentous growth (Gimeno et al. 1992), and S288c, which is commonly used in research laboratories (Mortimer and Johnston 1986) but has acquired mutations due to genetic manipulation that render it unable to undergo filamentous growth (Liu et al. 1996; Dowell et al. 2010; Chin et al. 2012).

One aspect of filamentous growth is invasive growth, which can be assessed by the plate-washing assay (PWA), and which measures penetration of filamentous cells into surfaces (Roberts and Fink 1994). Invasive growth in nutrient-rich (SAG) conditions was compared to conditions that induce filamentous growth, nitrogen limitation [SLAD, (Gimeno et al. 1992)] and glucose limitation [SALG (Cullen and Sprague 2000)] as shown in Fig 3.1A. The results were quantitated by densitometric analysis (Fig 3.1B). As expected, S288c did not undergo invasive growth, and Σ 1278b underwent invasive growth that was higher in media lacking glucose or nitrogen (Fig 3.1A, washed and Fig 3.1B). Most wine strains underwent invasive growth, which was stimulated in nitrogen- and glucose-limited medium (including VIN7, W27, QA23, T73, SB and S1; Fig 3.1, A and B). Three strains showed a different trend: P5 invaded equally well in glucose-rich and glucose-limiting media, P24 did not invade nitrogen-limiting medium, and Nsa showed constitutive invasion. Moreover, the pattern of invasive growth varied widely among strains (Fig 3.1A).

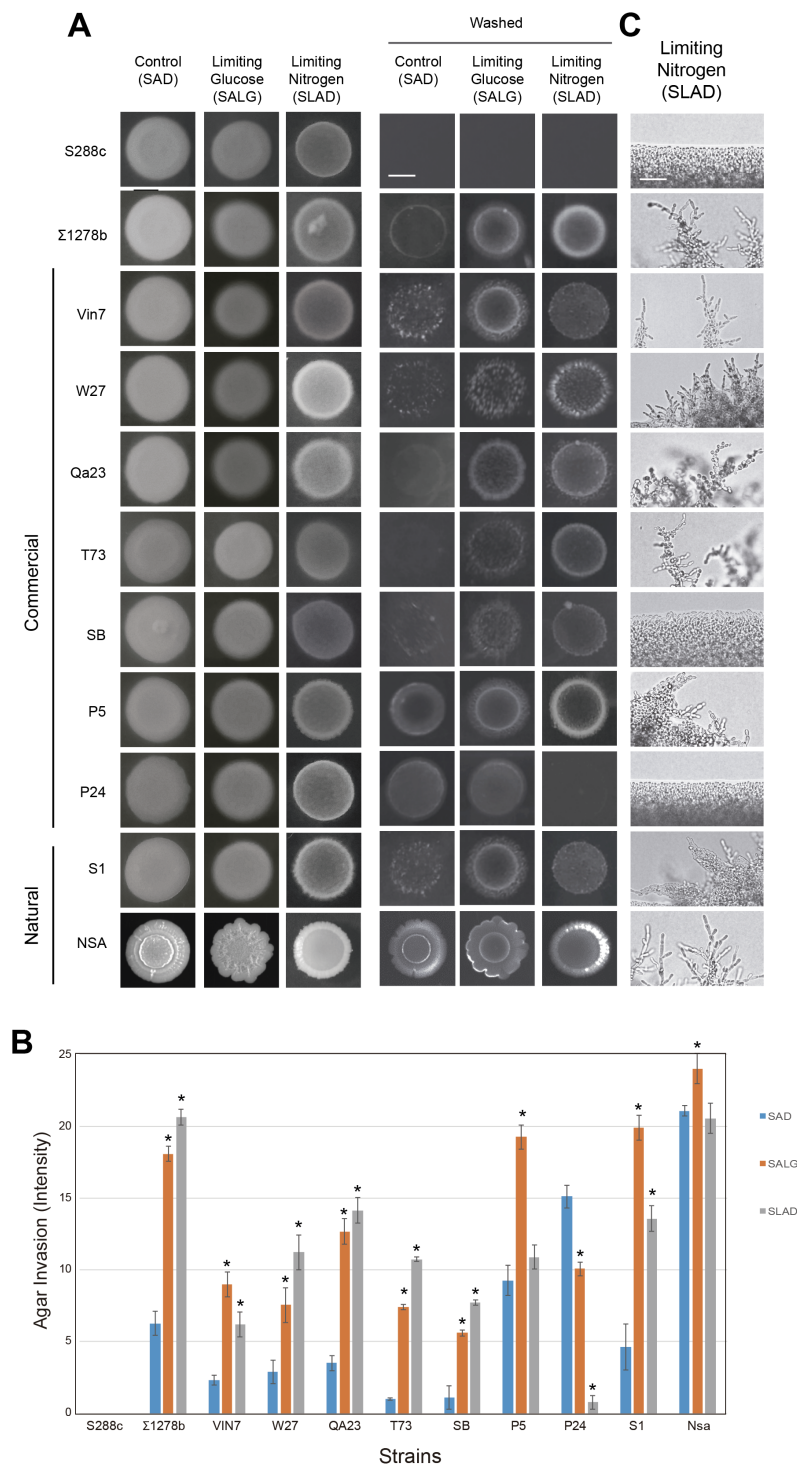


Figure 3.1: Filamentous growth phenotypes of wine strains. (A) Plate-washing assay (PWA). Equal concentrations of cells ($ODA_{600nm} = 2$) were spotted in 10 μ L aliquots onto the indicated media. Plates were incubated for 5 days at 30 °C and washed in a stream of water. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SAD. (C) Pseudohyphal growth of micro-colonies. Cells were grown for 3 days in minimal medium (MM) at 30 °C, diluted by a factor of 10^6 and spotted onto SLAD media. Plates were incubated for 5 days. Colonies were examined by microscopy at 40X magnification. A representative image is shown. Bar, 25 μ m. Arrows mark examples of pseudohyphae.

Another aspect of filamentous growth is pseudohyphal growth, which can be measured by microscopic examination of colony peripheries (Gimeno et al. 1992). As expected, S288c did not form pseudohyphae, and $\Sigma 1278b$ formed pseudohyphae in nitrogen-limiting medium (Fig 3.1C, SLAD). Most strains formed pseudohyphae in nitrogen-limiting media (Fig 3.1C, including VIN7, W27, QA23, T73, SB, P5, S1, and Nsa), except SB, which did not form pseudohyphae until day 16 (for Fig 3.1C, day 5 is shown) and P24, which did not form pseudohyphae by day 20 when the experiment was terminated. The pattern of pseudohyphae varied among strains. With the exception of Nsa, which formed pseudohyphae in glucose- (Fig S3.1A, Nsa SALG, arrow) and nitrogen-limiting media, all other strains formed pseudohyphae exclusively under nitrogen-limiting conditions. Invasive and pseudohyphal growth require cell adhesion mediated by the flocculin Flo11p (Lambrechts et al. 1996; Lo and Dranginis 1996; Guo et al. 2000). *FLO11* expression is induced during filamentous growth (Rupp et al. 1999a). A subset of wine strains that were tested all showed induction of *FLO11* expression under nitrogen-limiting conditions (Fig S3.1B). Therefore, above results agree with the widely accepted notion that glucose and nitrogen limitation are general inducers of filamentous growth. Ethanol also stimulates filamentous growth (Dickinson 1994; Dickinson 1996; Lorenz et al. 2000). Ethanol induced filamentous growth specifically in nitrogen-limiting medium (Fig S3.2A) and showed a maximal effect at a concentration of 2 % (Fig S3.2B). At this concentration, ethanol did not impact growth [Fig S3.2C; yeast can survive in 12 % ethanol (Lleixa et al. 2016)]. Thus, tests were performed at 2 % ethanol in nitrogen-limiting media. As expected, S288c did not show invasive growth by the addition of ethanol (Fig 3.2, A-C), and $\Sigma 1278b$ showed ethanol-inducible invasive growth (Fig 3.2A-B). In particular, cells invaded the agar more robustly (Fig 3.2A-B), and pseudohyphae formed at earlier time points (Fig 3.2C, colonies were grown for 2 days compared to 5 days in Fig 3.1C). With the exception of P24 and Nsa, most strains showed increased invasive growth in response to ethanol (Fig 3.2, A-C including VIN7, W27, QA23, T73, SB, S1, and P5). By these criteria, ethanol can also be viewed as a general inducer of filamentous growth. The fusel alcohol tryptophol stimulates filamentous growth in $\Sigma 1278b$ strains [Fig S3.3, (Chen and Fink 2006)]. Tryptophol stimulated invasive growth of most wine strains in nitrogen-rich (SAD) but not nitrogen-limiting (SLAD) medium (Fig S3.3, including VIN7, W27, QA23, T73, and S3.1). Thus, in line with previous studies, fusel alcohols like tryptophol are general inducers of filamentous growth.

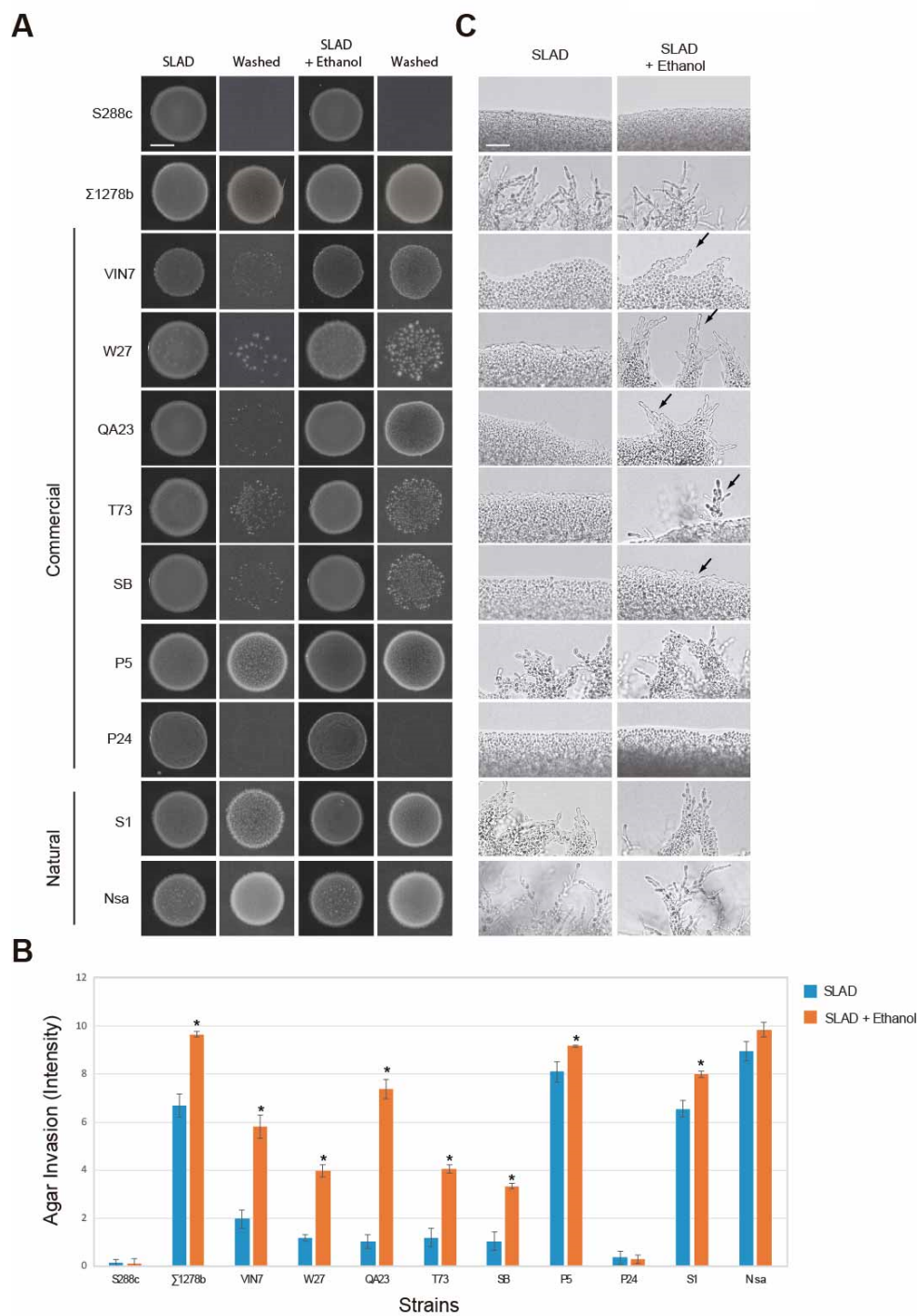


Figure 3.2: Response of wine strains to ethanol. (A) PWA of cells spotted onto nitrogen-limited medium (SLAD) with or without ethanol (2 % v/v). Plates were incubated for 2 days at 30°C and washed in a stream of water. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry, performed as described in Figure 1B. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. (C) Microscopy of colony perimeters with or without ethanol at 40X magnification. Bar, 25 μm. Arrows mark examples of pseudohyphae.

Major Filamentation Regulatory Pathways Are Not Required for Ethanol-Inducible Filamentous Growth

We focused on ethanol-inducible filamentous growth because ethanol was a stronger inducer of filamentous growth than fusel alcohols. How ethanol is sensed and triggers filamentous growth has not been extensively studied. The ethanol response occurred in diploid (3.2) and haploid (3.3) strains of the Σ 1278b background, which facilitated genetic analysis of the response.

Signaling pathways known to regulate filamentous growth were tested for a role in regulating ethanol-inducible filamentous growth. Specifically, mutants were tested that lack key regulators of fMAPK (*ste11* Δ ; Ste11p is the MAPKKK), Ras2p-cAMP-PKA (*ras2* Δ) and PKA (Tpk in yeast) subunits Tpk1p, Tpk2p, and Tpk3p (*tpk1* Δ , *tpk2* Δ , and *tpk3* Δ), Snf1p (*snf1* Δ), Rim101p (*rim101* Δ), Rpd3p(L) (*sin3* Δ), Elongator (*elp2* Δ), and Pho85p (*pho85* Δ). Surprisingly, all of the mutants showed enhanced invasive growth in media containing ethanol (Fig S3.3, A and B). The examination of colony perimeters generally bore this out, either showing enhanced filament formation or clumpiness (Fig 3.3C, arrows), which is indicative of elevated cell-cell adhesion. Colony perimeters did not show a change for the *ste11* Δ and *rim101* Δ mutants. Thus, fMAPK and Rim101 pathways may play some role in mediating ethanol-dependent filamentous growth. In summary these results show that ethanol exerts its effect on filamentous growth independent of several of the major regulatory pathways that control filamentous growth.

Unexpectedly, several mutants did not show an invasive growth defect in SLAD media. Specifically, the *rim101* Δ , *sin3* Δ , *snf1* Δ , *elp2* Δ , and *pho85* Δ mutants invaded the agar as well as or better than wild-type cells [Fig 3.3, A and B; *tpk3* Δ is not defective for invasive growth (Robertson and Fink 1998a; Robertson et al. 2000; Chavel et al. 2010)]. We have previously shown that the *rim101* Δ (Chavel et al. 2014), *sin3* Δ (Chavel et al. 2010), *snf1* Δ (Cullen and Sprague 2000), *elp2* Δ (Abdullah and Cullen 2009), and *pho85* Δ (Chavel et al. 2014) mutants have an invasive growth defect on rich media, and we verified that phenotype here (Fig S3.4A; YPD). Thus, there may be differences in the roles these pathways play in regulating invasive growth depending on growth on YPD or SLAD. This hypothesis is consistent with the fact that mutants scored for pseudohyphal and invasive growth do not completely overlap in a genome-wide screen (Ryan et al. 2012) and with the fact that several pathways, like Snf1p, play different roles in response to carbon and nitrogen limitation (Orlova et al. 2010).

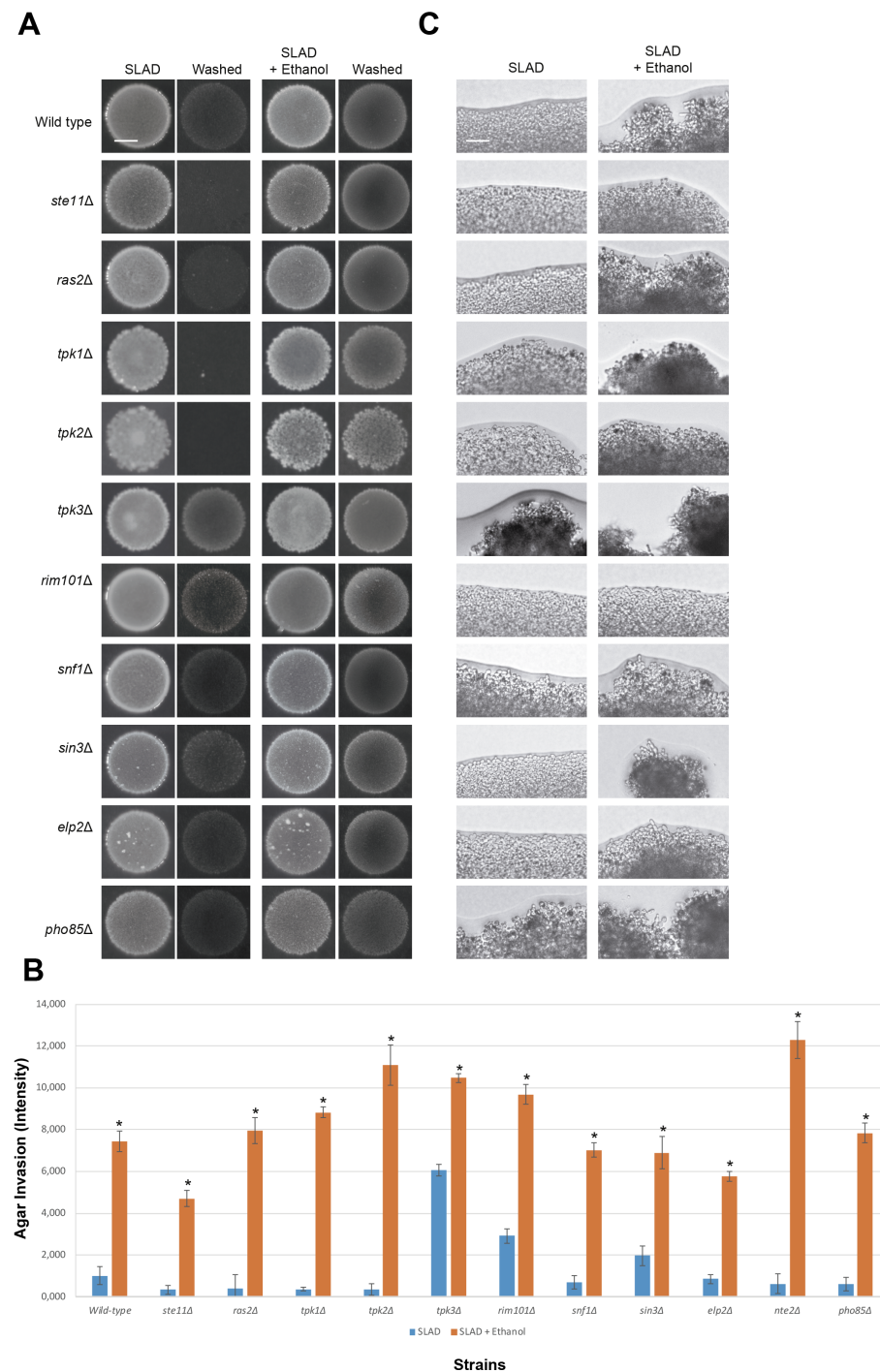


Figure 3.3: Evaluating mutants lacking established filamentation regulatory pathways for ethanol-inducible invasion. (A) Wild-type cells (PC538, Σ 1278b *MATa* haploid) and the indicated isogenic mutants were spotted onto nitrogen-limited medium (SLAD) with or without 2 % ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry, performed as described in the legend for Figure 1B. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. (C) Colony peripheries from the plates in panel (A) were examined at 20X magnification. Bar, 50 μ m. Arrows mark examples of pseudohyphae.

Mitochondrial Retrograde Pathway Is Required for Ethanol-Inducible Invasive Growth

Other proteins and pathways regulate filamentous growth than those tested above (Ryan et al. 2012). A broader collection of genes implicated in filamentous growth regulation was examined. One of these is the mitochondrial retrograde pathway [or RTG pathway (Sekito et al. 2002; Liu et al. 2003; Liu and Butow 2006; Kleine and Leister 2016)], which senses changes in metabolic respiration (Aun et al. 2013) to regulate filamentous growth. The RTG pathway has recently been shown to regulate the filamentation response to the alcohol butanol (Starovoytova et al. 2013). Rtg2p is a positive regulator of the retrograde pathway (Ferreira Junior et al. 2005). The *rtg2* Δ mutant was defective for ethanol-dependent invasive growth (Fig 3.4, A-C). The RTG pathway is composed of two other regulators, the basic helix-loop-helix leucine zipper transcription factors Rtg1p and Rtg3p, which hetero-dimerize to regulate transcription (Jia et al. 1997). The *rtg1* Δ and *rtg3* Δ mutants were also defective for invasive growth (Fig 3.4, A-C).

The RTG pathway controls expression of genes that function to ameliorate defects in mitochondrial function (Epstein et al. 2001). The activity of the RTG pathway can be assessed by examining the expression of the *CIT2* gene, which is a target of the retrograde pathway (Liao and Butow 1993; Chelstowska and Butow 1995; Kos et al. 1995; Jia et al. 1997; Liu and Butow 1999) that encodes peroxisome citrate synthase (Kim et al. 1986).

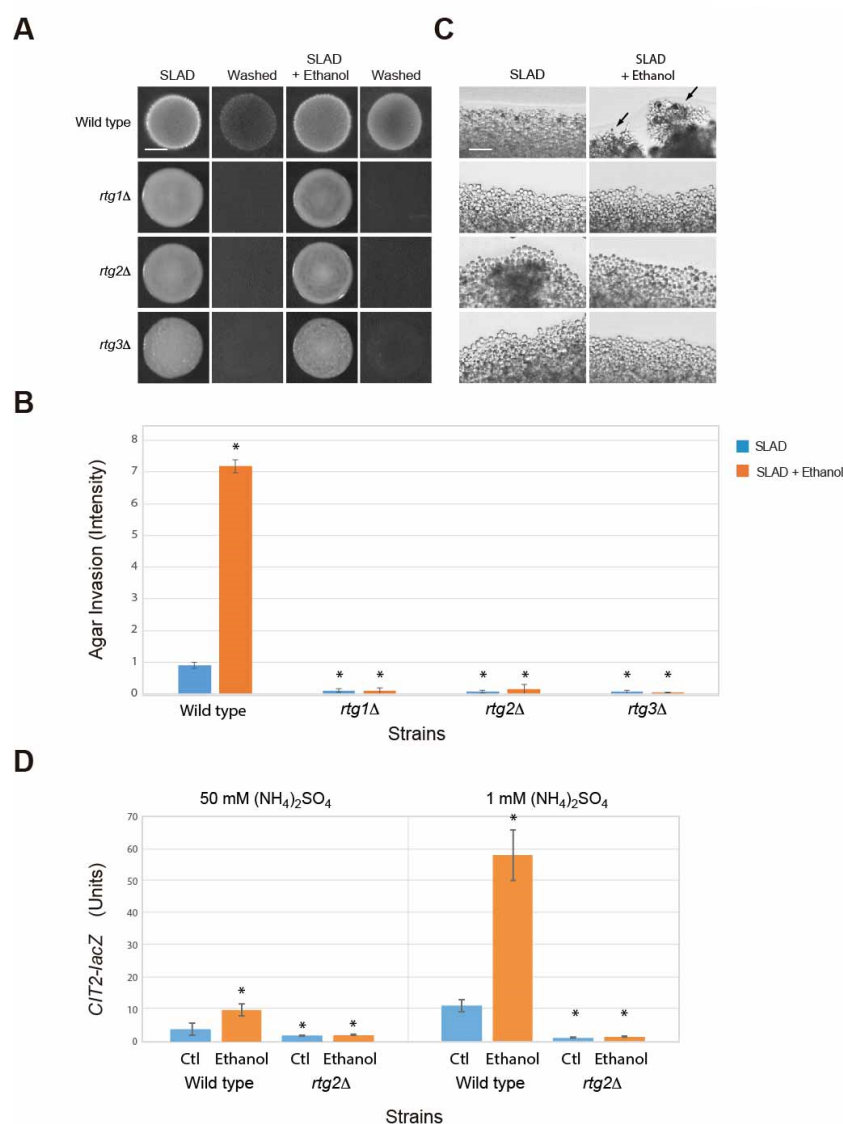


Figure 3.4: Role of the RTG pathway in regulating ethanol-inducible invasive growth. (A) Wild-type cells (PC538, Σ 1278b *MATa* haploid) and the indicated isogenic mutants were spotted onto nitrogen limiting medium (SLAD) with or without 2 % ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry, performed as described in the legend for Figure 1B. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to wild type in SLAD. (C) Colony peripheries from the plates in panel (A) were examined by microscopy at 20X magnification. Bar, 50 μ m. Arrows mark examples of pseudohyphae. (D) Beta-galactosidase activity of the *CIT2-lacZ* reporter in wild-type cells and the *rtg2Δ* mutant grown in 1 or 50 mM (NH₄)₂SO₄ with or without 2 % ethanol (v/v). Experiments were performed in triplicate from independent inductions. Error bars represent the standard deviation between experiments. Asterisk denotes a $p < 0.01$ for samples relative to wild type in media lacking ethanol (Ctl).

Ethanol stimulated the activity of a *CIT2-lacZ* transcriptional reporter (Fig 3.4D) in a manner that was dependent on Rtg2p (Fig 3.4D). Interestingly, the data indicates that ethanol induces the RTG pathway. One possibility is that nitrogen and ethanol both activate the RTG pathway. The addition of ethanol to cells grown in nitrogen-limiting media showed an additional stimulation (Fig 3.4D). Thus, nitrogen limitation and ethanol both contribute to RTG pathway activity. Therefore, the mitochondrial retrograde pathway regulates ethanol-inducible filamentous growth.

Mitochondrial Retrograde Pathway Regulates Ethanol-Inducible Filamentous Growth Independent of fMAPK, TOR and HOG Pathways

To define how the RTG pathway connects to the ethanol response, known regulators of that pathway were examined. Mks1p is a negative regulator of multiple pathways, including Rtg2p in the mitochondrial retrograde pathway (Dilova et al. 2004; Ferreira Junior et al. 2005). Mks1p was not required for invasive growth in response to ethanol (Fig 3.5, A-C), which indicates that another negative regulator of the pathway might function in this context. The RTG pathway can regulate the fMAPK pathway (Chavel et al. 2010), as part of a highly coordinated transcriptional sensing and signaling circuit among the pathways that regulate filamentous growth (Borneman et al. 2006; Bharucha et al. 2008; Chavel et al. 2014). We tested whether cells with an up-regulated RTG pathway functioned through fMAPK. An *mks1Δ ste11Δ* double mutant, which has an up-regulated retrograde pathway and lacks the MAPKKK for the fMAPK pathway (Ste11p), showed ethanol-inducible invasive growth. This result aligns with the abovementioned results that fMAPK does not regulate ethanol-dependent filamentous growth and indicates that the mitochondrial retrograde pathway does not control filamentation through fMAPK (Fig 3.5, A-B). As shown above, the *mks1Δ ste11Δ* double mutant did not show an increase in filamentation at colony peripheries (Fig 3.5C).

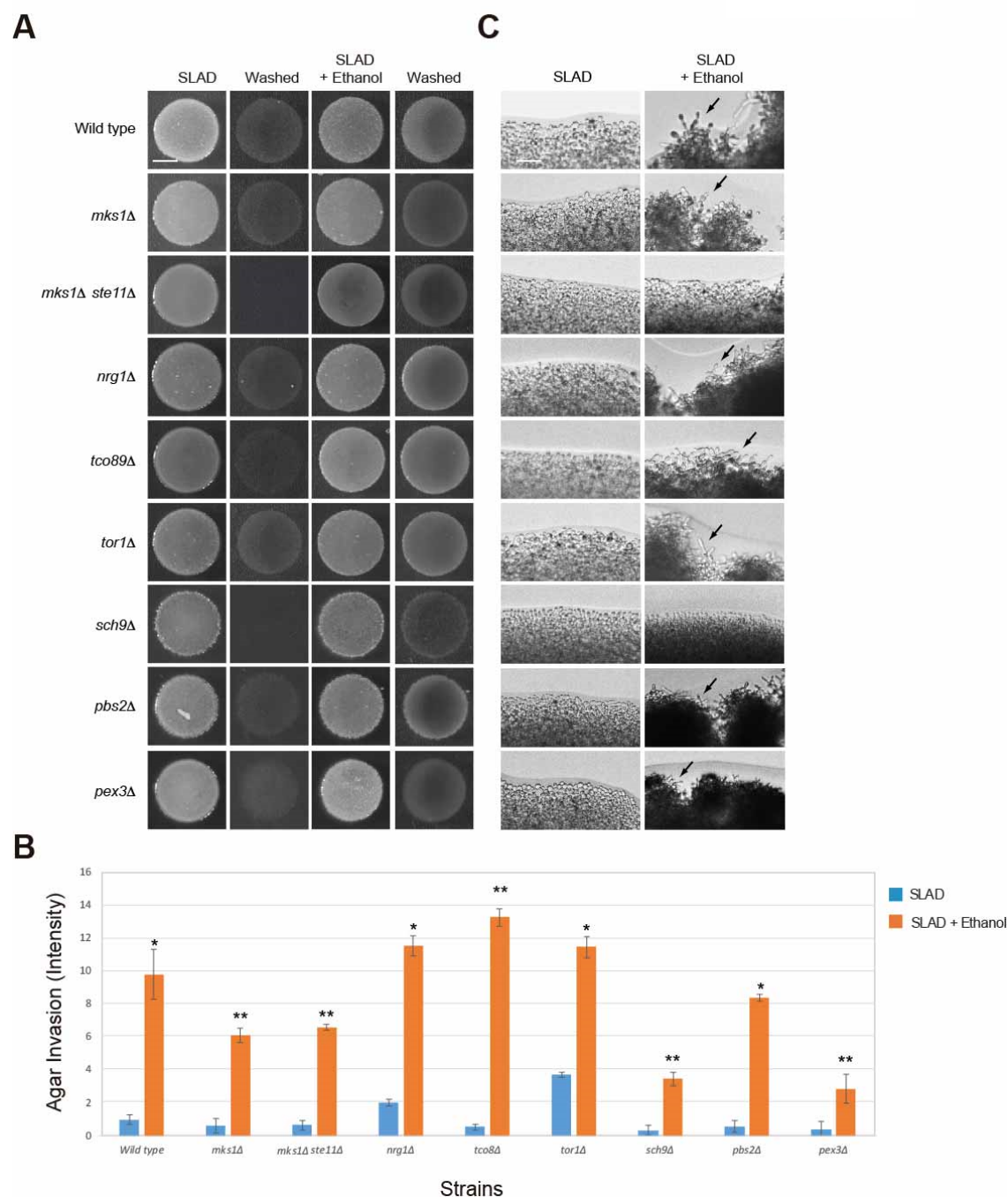


Figure 3.5: Role of RTG pathway regulators in controlling ethanol-inducible invasive growth. (A) Wild-type cells (PC538, $\Sigma 1278b$ *MATa* haploid) and the indicated isogenic mutants were spotted onto nitrogen-limiting medium (SLAD) with or without 2 % ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry, performed as described in the legend for Figure 1B. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. Double asterisk refers to a $p < 0.01$ for samples relative to each strain's invasion in SLAD compared to wild-type in SLAD with ethanol. (C) Colony peripheries from the plates in panel (A) were examined by microscopy at 20X magnification. Bar, 50 μ m. Arrows mark examples of pseudohyphae.

Another major regulator of the mitochondrial retrograde pathway is the TOR pathway, which is a ubiquitous nutrient-regulatory pathway in eukaryotes (Bar-Peled and Sabatini 2014). TOR plays an important role in nutrient-regulated responses in yeast (Heitman et al. 1991) and is a master regulator of nitrogen control (Beck and Hall 1999; Cardenas et al. 1999; Bruckner et al. 2011; Kingsbury et al. 2015). TOR signaling also links nitrogen quality to the activity of the Rtg1p and Rtg3p transcription factors (Komeili et al. 2000).

TOR specifically regulates the expression of genes encoding RTG pathway components (Crespo et al. 2002; Dilova et al. 2004). We found that the TOR pathway was not required for ethanol-inducible filamentous growth (Fig 3.5, A-C; *tor1Δ*, *tco89Δ*). In addition, the AGC-type kinase Sch9p, which is phosphorylated by and is a major target of TORC1, and which contributes to TORC1-mediated regulation of ribosome biogenesis (Urban et al. 2007; Wei and Zheng 2009), was not required for ethanol-dependent invasion (*sch9Δ* Fig 3.5, A-B, although it was required for filamentation at colony perimeters Fig 3.5C). These results may not be entirely surprising, because although TOR and the mitochondrial retrograde pathway are functionally connected, the retrograde response to mitochondrial dysfunction is not dependent on TOR1-dependent regulation of retrograde gene expression (Giannattasio et al. 2005). Therefore, the mitochondrial retrograde pathway controls ethanol-inducible filamentous growth independent of TOR and at least partly independently of Sch9p.

In addition to TOR, the SAP- or p38-type high osmolarity glycerol response (HOG) MAP kinase pathway, which controls the response to osmotic and other stresses (Westfall et al. 2004; Saito 2010), also regulates the RTG pathway (Ruiz-Roig et al. 2012). The HOG pathway was not required for ethanol-inducible filamentous growth (Fig 3.5, A-C, *pbs2Δ*). Another function of the RTG pathway is to stimulate peroxisome biogenesis in periods of mitochondrial stress (Liao and Butow 1993; Chelstowska and Butow 1995; Kos et al. 1995; Epstein et al. 2001). Peroxisomes, which control elements of metabolism and can be regulated by the RTG pathway (Chelstowska and Butow 1995), may impact ethanol-dependent filamentous growth. A mutant lacking peroxisomes was not required for ethanol-dependent filamentous growth, indicating that this is not the case (Fig 3.5, A-C, *pex3Δ*). However, the *pex3Δ* (Fig 3.5B) and Cit2p, which is a target of RTG, was induced by ethanol (Fig 3.4D). These proteins regulate the glyoxylate cycle (Jazwinski 2013) and it is possible that that metabolic pathway plays a role in regulating ethanol-inducible filamentous growth.

Therefore, the mitochondrial retrograde pathway regulates ethanol-inducible filamentous growth in a manner that is separate from TOR, fMAPK, and HOG, and partly independent of peroxisome

function.

Regulation of the TCA Cycle Underlies the Role of the Mitochondrial Retrograde Pathway In Controlling Ethanol-Induced Filamentous Growth

The tricarboxylic acid (TCA or citric acid / Krebs) cycle functions through a series of reactions to generate ATP and produce reducing agents necessary for mitochondrial electron transport and energy generation. The TCA cycle is compromised in cells experiencing mitochondrial defects, but flux through the pathway can be maintained by the action of the RTG pathway (Liu and Butow 1999; Lin et al. 2011), which is a major function of the RTG pathway (Butow and Avadhani 2004). Glutamate can suppress the requirement for the retrograde pathway in the TCA cycle by increasing metabolic flux (Liu and Butow 1999). Glutamate suppressed the defect in ethanol-inducible filamentous growth of the *rtg1* Δ , *rtg2* Δ , and *rtg3* Δ mutants (Fig 3.6, A-C). The role of the RTG pathway in regulating ethanol-inducible invasion suggests that mitochondrial respiration is important for ethanol-dependent invasive growth. Consistent with this possibility, *rho*⁰ cells, which lack a functional mitochondria, were defective for ethanol-inducible invasive growth (Fig 3.6, A-C). Thus, one function of the RTG pathway in ethanol-dependent filamentous growth is to stimulate flux through the TCA cycle. Glutamate did not suppress the invasive growth defect of the *flo11* Δ mutant (Fig 3.6, A-C). Given that Flo11p is the main cell adhesion molecule that regulates filamentous growth, these results suggest that glutamate-dependent invasive growth in *rtg* mutants is mediated (in some manner) through Flo11p.

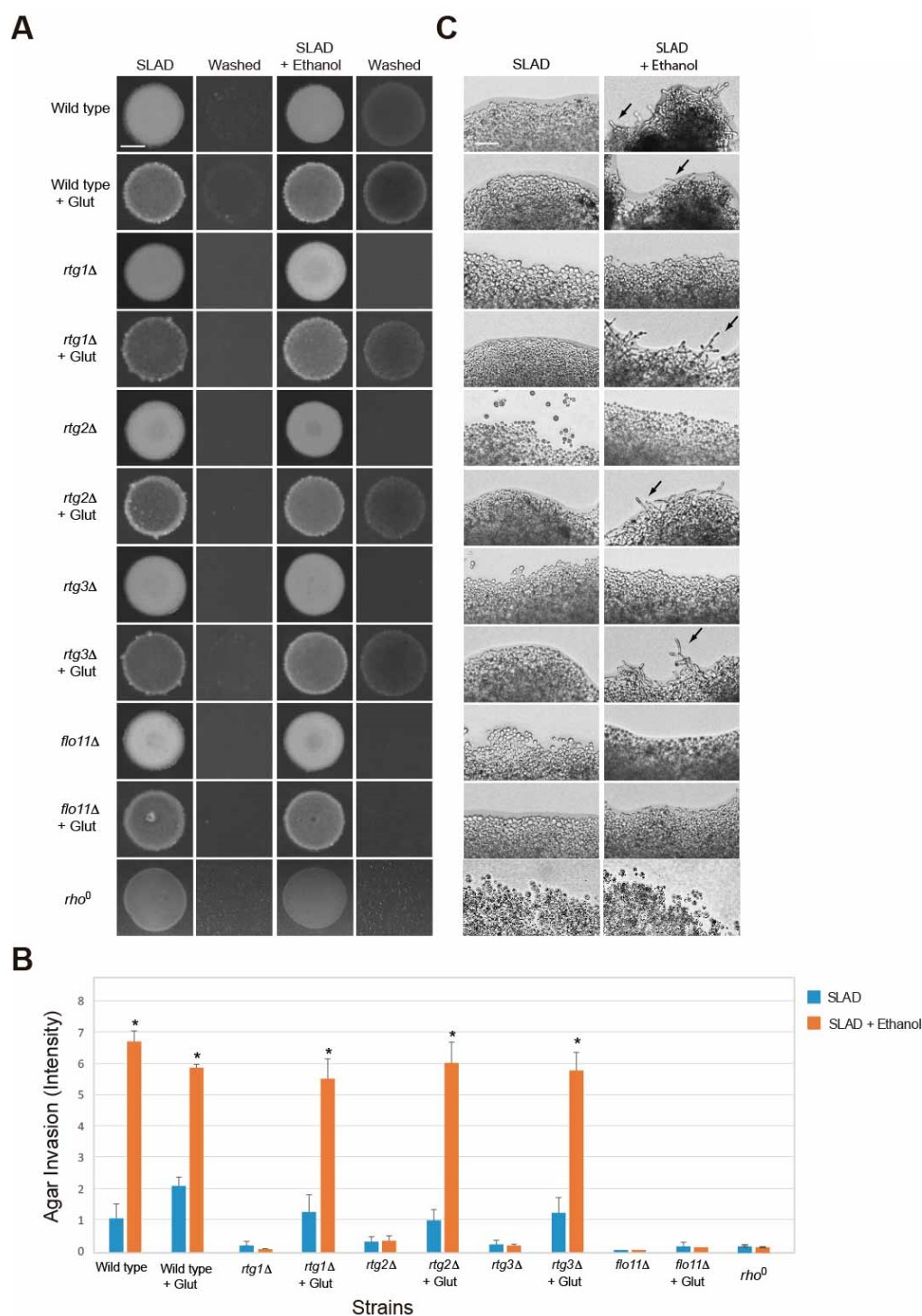


Figure 3.6: Impact of glutamate on ethanol-inducible invasive growth defect of *rtg* mutants. (A) Wild-type cells (PC538, Σ 1278b *MATa* haploid) and the indicated isogenic mutants were spotted onto nitrogen limiting medium (SLAD) with or without 2 % ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. Glutamate was added at a concentration of 200 μ M. (B) Quantitation of invasive growth in panel (A) by densitometry. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.05$ for samples relative to each strain's invasion in SLAD. (C) Colony peripheries from the plates in panel (A) were examined by microscopy at 20X magnification. Bar, 50 μ m. Arrows mark examples of pseudohyphae.

The mitochondrial retrograde pathway has also been shown to regulate deoxyribonucleotide pools by impacting the rate of threonine metabolism (Hartman 2007). Hydroxyurea induces a cell-cycle delay (Adams and Lindsay 1967) and reduces the rate of DNA synthesis (Niu et al. 2008), and accordingly triggers a filamentation-like response (Jiang and Kang 2003). Ethanol may impact threonine levels and DNA synthesis rates and induce retrograde-dependent filamentation. However, hydroxyurea, unlike ethanol, did not cause invasive growth in SLAD medium (Fig S3.4B). Moreover, the elongated cell morphology induced by hydroxyurea was retrograde-independent (Fig S3.4C). Therefore, the mitochondrial retrograde pathway probably does not regulate ethanol-dependent filamentous growth by influencing the rate of threonine metabolism.

Several other mutants that are defective in pathways surrounding the TCA cycle, ethanol uptake and metabolism, signaling, and the cell cycle were examined for a role in ethanol-inducible filamentous growth (Fig S3.5). Most of the mutants examined showed a detectable reduction in ethanol-inducible invasive growth (Fig S3.5). Two mutants stood out. One lacked *Adh2p*, which might be expected as that protein catalyzes the conversion of ethanol to acetaldehyde (Bennetzen and Hall 1982; Young and Pilgrim 1985; Dickinson et al. 2003). The other lacked *Csf1p* (Fig S3.5), a protein that is required for fermentation at low temperatures (Tokai et al. 2000). Notably, the wine yeast P24, which does not invade the agar in SLAD medium, is defective for growth at low temperatures (Garcia-Rios et al. 2014). Thus, the regulators of ethanol-inducible filamentous growth may encompass a more diverse collection of proteins than has been defined here.

Ethanol-Inducible Filamentous Growth Requires the Polarisome and Occurs Through Induction of *FLO11* Expression

Filamentous growth involves at least three major regulatory changes. One is an increase in cell length, which is mediated by a delay in the cell cycle (Kron et al. 1994) and by an increase in polarized growth by a *Cdc42p*-dependent mechanism that involves the polarisome (Cullen and Sprague 2002). The formin *Bni1p* (Evangelista et al. 1997) and accessory proteins *Bud6p*, *Pea2p*, and *Spa2p* comprise the polarisome (Amberg et al. 1997; Sagot et al. 2002; Graziano et al. 2011; Tu et al. 2012). Another change is a switch in polarity to distal-unipolar budding that requires the distal-pole landmark *Bud8p* (Gimeno et al. 1992; Cullen and Sprague 2002). *Bud8p* is a distal-pole marker that localizes to the distal pole of the cell (Harkins et al. 2001). The third change, as discussed above, is an increase in

adhesion mediated by the cell adhesion molecule Flo11p (Lambrechts et al. 1996; Lo and Dranginis 1996; Guo et al. 2000). The different aspects of filamentous growth are genetically separable and can be examined by mutants that specifically compromise each aspect of the response (Cullen and Sprague 2002). Mutants were examined that were specifically defective for polarized growth (*bud6*Δ), polarity reorganization (*bud8*Δ), or cell adhesion (*flo11*Δ). Ethanol-inducible filamentous growth occurred in cells lacking Bud8 (Fig 3.7, A-C, *bud8*Δ), which indicates that ethanol does not function mainly through the switch in polarity. Ethanol-inducible filamentous growth was reduced in cells lacking the polarisome component Bud6p (Fig 3.7, A-C, *bud6*Δ). Thus, ethanol induces filamentous growth by a mechanism that is partly dependent on the increase in polarized growth driven by the polarisome. This is consistent with studies of fusel alcohols, which induce dramatic changes in cell length (Dickinson 1996; Lorenz et al. 2000). Ethanol-inducible filamentous growth was also dependent on Flo11p (Fig 3.7, A-C, *flo11*Δ). Consistent with this result, ethanol stimulated the expression of the *FLO11* gene (Fig 3.7D). Therefore, ethanol-inducible filamentous growth, which is controlled by the RTG pathway, requires polarisome function and occurs by a mechanism that involves Flo11p-dependent transcriptional induction.

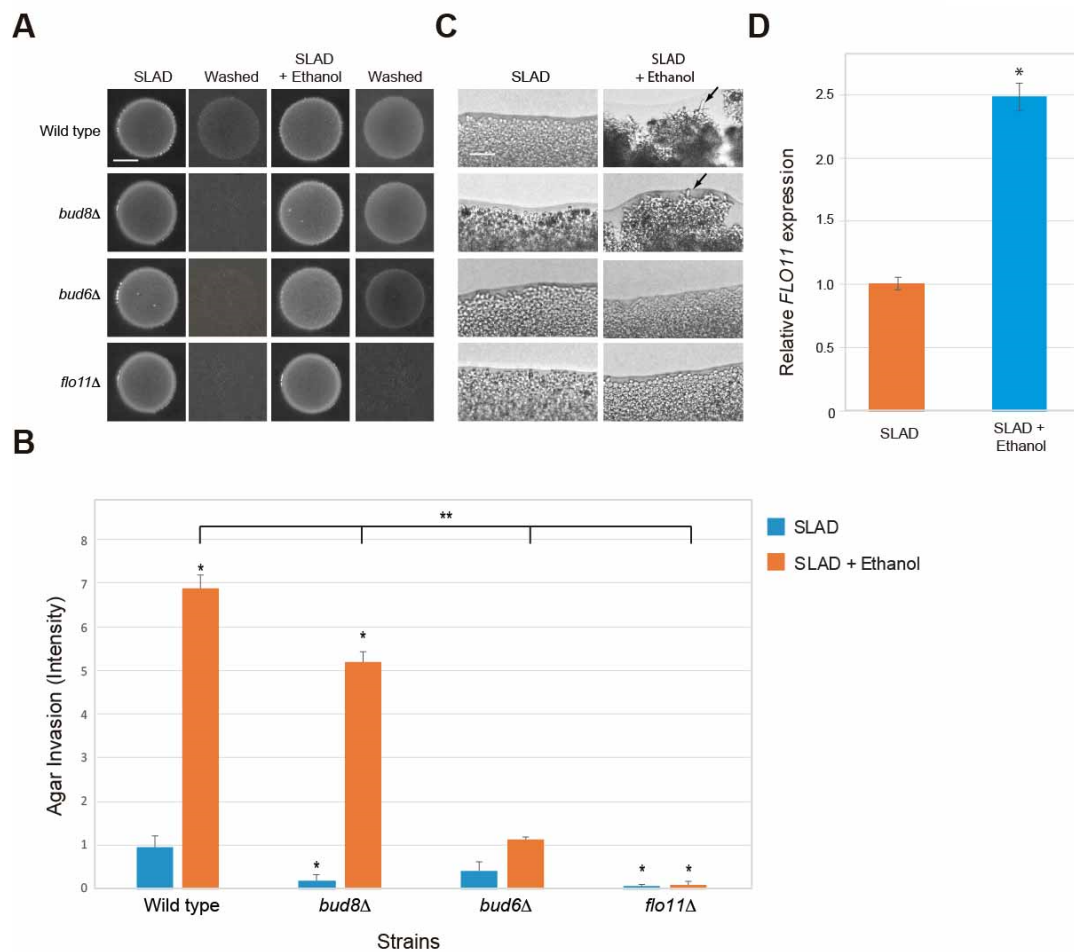


Figure 3.7: Requirement for Bud8, Bud6, and *FLO11* in mediating ethanol-inducible invasive growth. (A) Wild-type cells (PC538, Σ 1278b *MATa* haploid) and the indicated isogenic mutants were spotted onto nitrogen-limiting medium (SLAD) with or without ethanol (2 %v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. (B) Quantitation of invasive growth in panel (A) by densitometry, performed as described in the legend for Figure 1B. Asterisk denotes a $p < 0.01$ for samples relative to wild type invasion in SLAD. Double asterisk denotes a $p < 0.01$ for samples relative to wild type invasion in SLAD with ethanol. (C) Colony peripheries from the plates in panel (A) were examined by microscopy 20X magnification. Bar, 50 μ m. (D) Ethanol stimulates *FLO11* expression in SLAD medium. Cells were incubated in SLAD (orange bar) or SLAD with ethanol (blue bar). Gene expression was examined by qPCR at time 45 min and normalized to a control transcript (*ACT1*). Error bar represents standard difference between samples. Asterisk denotes a $p < 0.01$.

Discussion

Filamentous growth in yeast has been mainly studied in one strain background $\sum 1278b$, (Gimeno et al. 1992)], in part because most laboratory strains have lost filamentation properties due to genetic manipulation in the laboratory (Liu et al. 1996; Dowell et al. 2010; Chin et al. 2012). Although filamentous growth is common among ‘wild’ *S. cerevisiae* strains (Carstens 1998; Sidari et al. 2014), the triggers of filamentous growth have not been extensively characterized in other backgrounds. By examining a collection of wine yeast, we show that most wine strains undergo filamentous growth. The strains also showed a high degree of phenotypic variation. Phenotypic variation is common among individual strains (Dowell et al. 2010) and may not be surprising given that these strains have undergone selection based on flavor, cold-sensitivity, alcohol tolerance, and flocculation (Suzzi et al. 1984; Fleet 2003; Borneman et al. 2011).

We show here that nitrogen limitation and carbon limitation induce filamentous growth in most strains. This is consistent with previous claims that nitrogen limitation (Gimeno and Fink 1994) and carbon limitation (Cullen and Sprague 2000) trigger the filamentation response. We also show that ethanol and fusel alcohols induce filamentous growth. Ethanol (Dickinson 1994; Lorenz et al. 2000) and fusel alcohols (Dickinson 1996; Chen and Fink 2006) are known to stimulate filamentous growth. Fusel alcohols induced filamentous growth under nutrient-replete conditions, and ethanol stimulated filamentous growth under nitrogen-limiting conditions. Ethanol is a by-product of glycolysis, whereas fusel alcohols are by-products of Ehrlich reactions. Thus, the two types of alcohols may provide information about different nutritional states. During alcoholic fermentation, *S. cerevisiae* produces ethanol when it has reached a maximum population density that corresponds with consumption of nitrogen (Beltran et al. 2005). Because nitrogen limitation is itself a trigger for filamentous growth, ethanol may be a coincidence detector of nitrogen levels and TCA compromise. Alternatively, glucose uptake correlates with the rate of the TCA cycle (Heyland et al. 2009). We also identify a potential role for the glyoxylate cycle in regulating ethanol-dependent filamentous growth. Thus, ethanol production may be a readout of nitrogen or glucose availability.

The cellular response to mitochondrial stress is important for biological responses in many systems. Generally speaking, cellular responses to mitochondrial dysfunction have been implicated in cancer (Guha and Avadhani 2013), aging (Friis et al. 2014; da Cunha et al. 2015; Jazwinski 2015), development (Berkowitz et al. 2016), and inter-organellar homeostasis (Liu and Butow 2006). Here, we show

that the fungal-specific RTG pathway controls ethanol-inducible invasive growth in yeast. Lorenz and Heitman argued that the fMAPK pathway mediates the response to alcohols (Lorenz et al. 2000), and we show that it may play a minor role. Here we establish the RTG pathway as a key pathway in the response. How does the RTG pathway control ethanol-dependent filamentous growth without involving other major filamentation regulatory pathways? One possibility is that the RTG pathway is part of the sensing/signaling mechanism that controls the rate of flux through the TCA cycle (Liu and Butow 1999; Lin et al. 2011). TCA cycle rate is dependent on carbon and nitrogen levels, which are key inducers of filamentous growth in yeast and other fungal species. Canonical metabolic regulatory pathways that control filamentous growth also control TCA cycle flux including Snf1 (Hedbacker and Carlson 2008) and TOR (Komeili et al. 2000); thus, TCA cycle activity may be a nexus for monitoring nutritional health. The connection between TCA cycle flux and filamentous growth may be relevant from the perspective of pathogenicity. TCA cycle flux has been connected to the evolution of pathogenicity in filamentous fungi (Hogan et al. 2015) and apicomplexan parasites (Oppenheim et al. 2014). TCA cycle reprogramming is becoming increasingly tied to developmental transitions in pathogens ranging from *C. albicans* (Askew et al. 2009; Guedouari et al. 2014; Grahl et al. 2015), to *Plasmodium falciparum* (Ke et al. 2015) to *Yersinia pseudotuberculosis* (Bucker et al. 2014). The boost in TCA cycle flux is critical for phagosomal escape of the bacterial pathogen *Francisella* (Ramond et al. 2014). Moreover, the fungal RTG pathway is responsible for evasion of programmed cell death in yeast cells growing on non-repressing carbon sources (Guaragnella et al. 2013). Both the RTG pathway and relief of carbon catabolite repression are required for programmed cell death resistance. Evasion of programmed cell death and filamentous growth may be two hallmarks that fungi must acquire to become pathogenic. Our study therefore connects TCA cycle flux, as regulated by the RTG pathway, to an aspect of filamentous growth. Perhaps TCA flux controls developmental and morphogenetic responses in other eukaryotic systems.

Abbreviations

AMPK, AMP-dependent protein kinase; DIC, differential-interference-contrast; fMAPK, filamentous growth mitogen activated protein kinase; HOG, high osmolarity glycerol pathway; MM, minimal medium; OD, optical density; PWA, plate-washing assay; PKA, protein kinase A; qPCR, quantitative polymerase chain reaction; SAD, synthetic medium with ammonium and dextrose; SALG, synthetic medium with ammonium and low glucose; SLAD, synthetic medium with dextrose and low-ammonium; TOR, target of rapamycin; TCA, tricarboxylic acid; Trp-OH, tryptophol; v/v, volume-to-volume percent; YNB, yeast nitrogen base; and YPD, yeast peptone dextrose.

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Chapter 3

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Supplementary figures

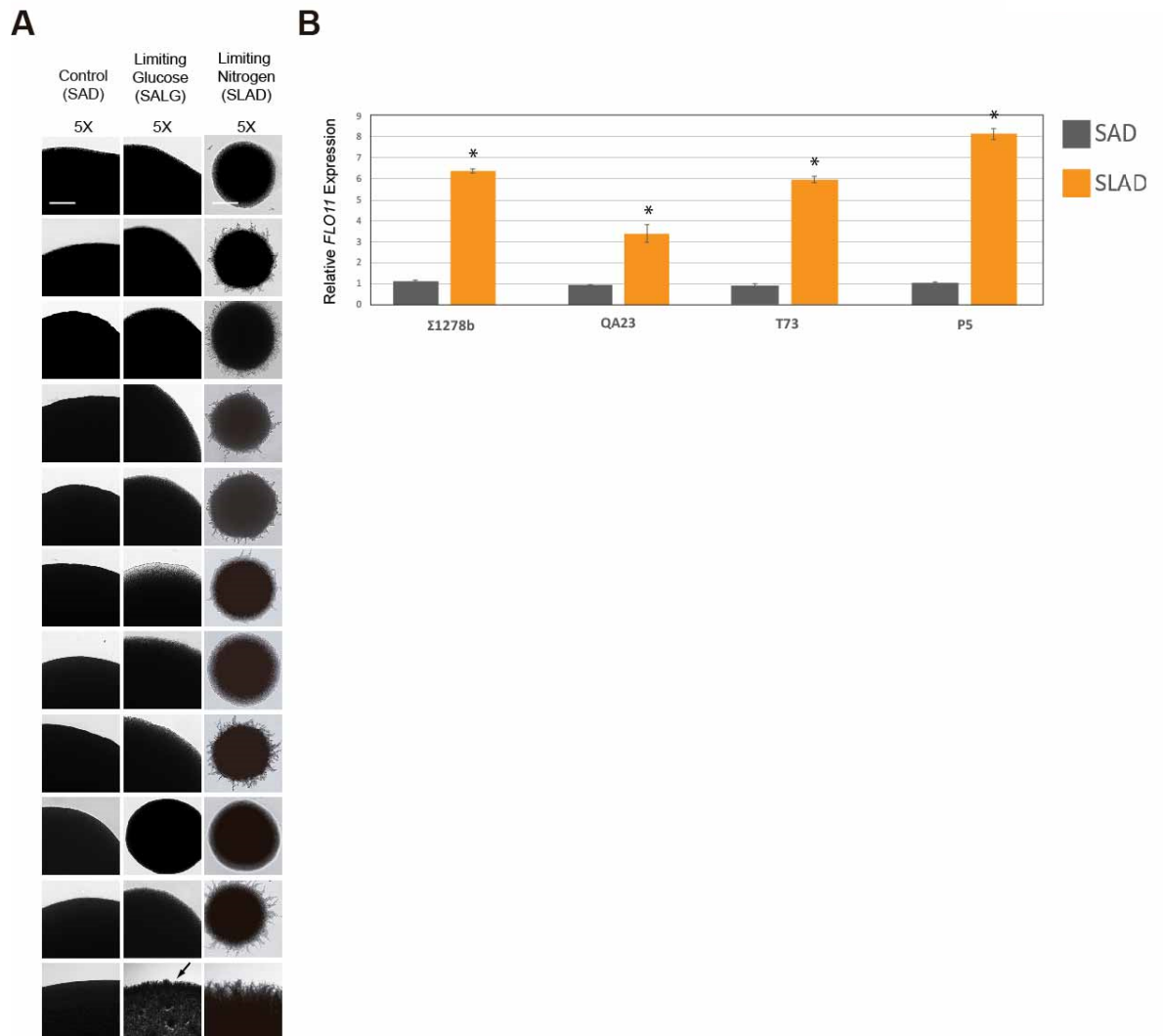


Figure S3.1: Pseudohyphal growth and *FLO11* expression in conditions with different nitrogen concentrations. A) Pseudohyphal growth of micro-colonies. Cells were grown for 3 days in minimal medium (MM) at 30°C, diluted by a factor of 10^6 and spotted onto SAD, SLAD, and SALG media. Plates were incubated for 5 days. Colonies were examined by microscopy at 5X magnification. Representative images are shown. Bars, 200 microns. B) The expression of *FLO11* was examined by qPCR in four strains Σ1278b, QA23, T73 and P5. Cells were grown in SAD (grey bars) and in SLAD (orange bars) for 2h. Error bars represent the standard error between samples. Asterisk denotes a p-value of <0.01.

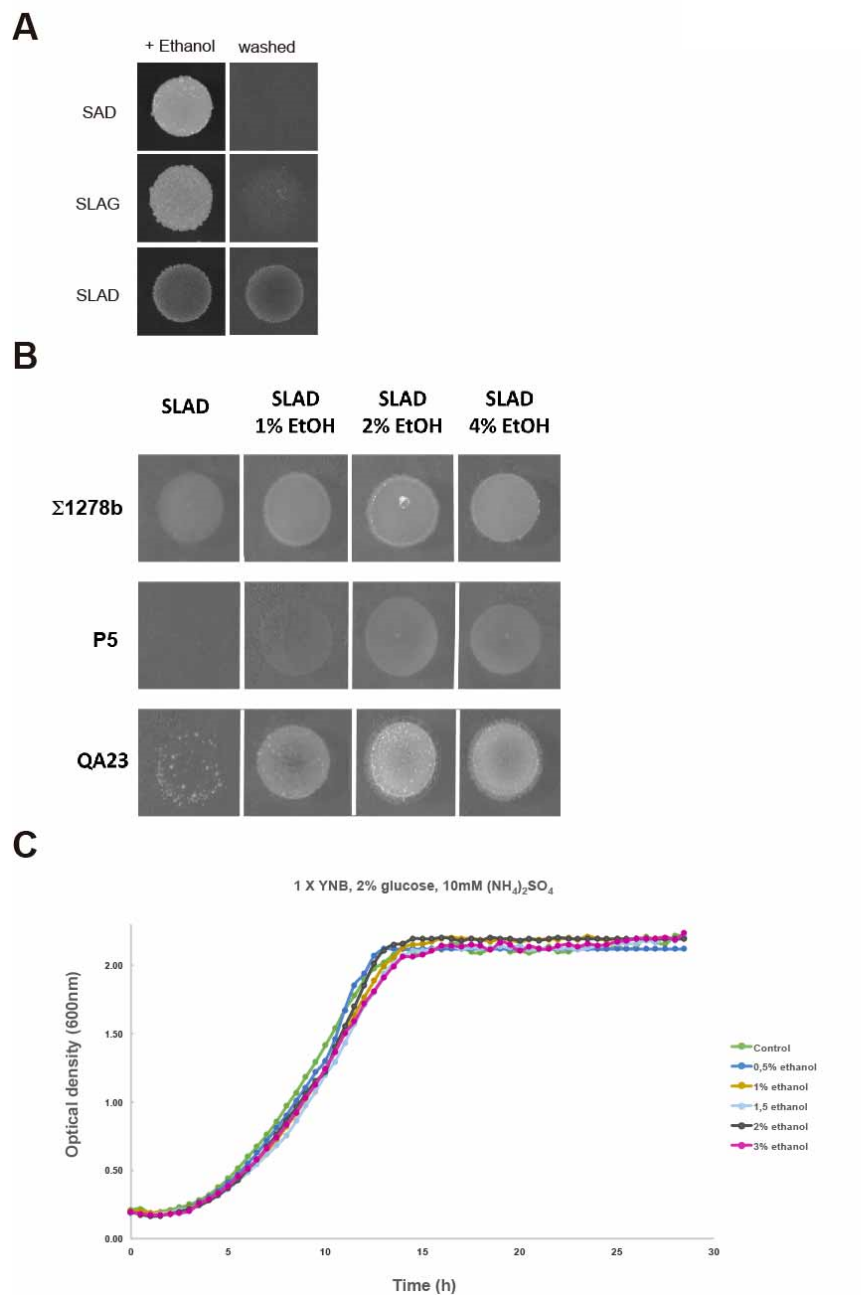


Figure S3.2: Characterization of ethanol concentration on filamentous growth and growth rate. A) The effect of ethanol on invasive growth of cells grown on different media in the QA23 strain. B) Ethanol concentration and invasive growth in SLAD media for the indicated yeast strains. C) Impact of different concentrations of ethanol on growth of wild-type MATa / MAT α $\Sigma 1278b$ cells (PC344) over a time-course experiment.

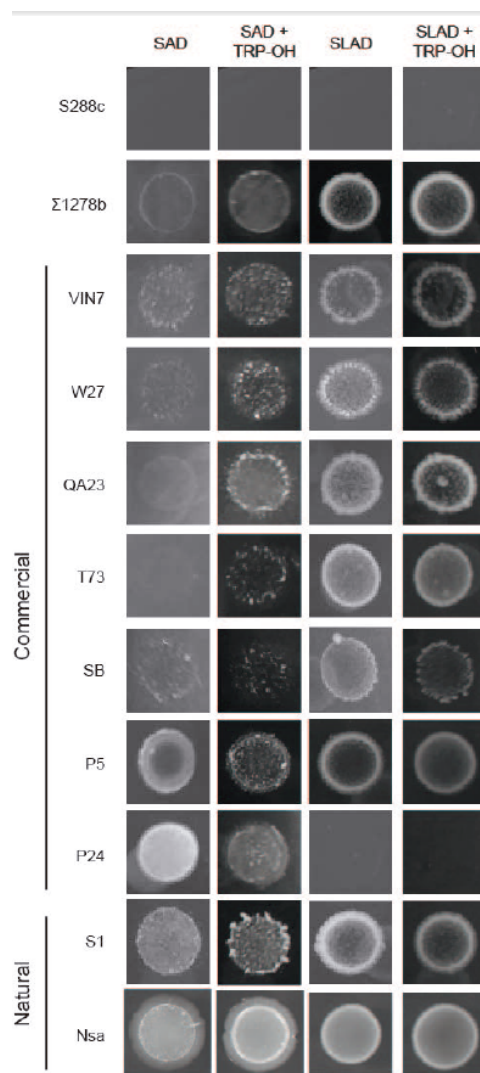


Figure S3.3: Impact of the fusel alcohol tryptophol on filamentous growth of wine yeast. Equal concentrations of cells (OD_{600nm} 2) were spotted in 10 μ L aliquots onto SAD and SLAD media with or without 500 μ M tryptophol (TRP-OH). Plates were incubated for 5 days at 30°C and washed in a stream of water. Colonies before washing grew to approximately equal levels and were similar in appearance (not shown).

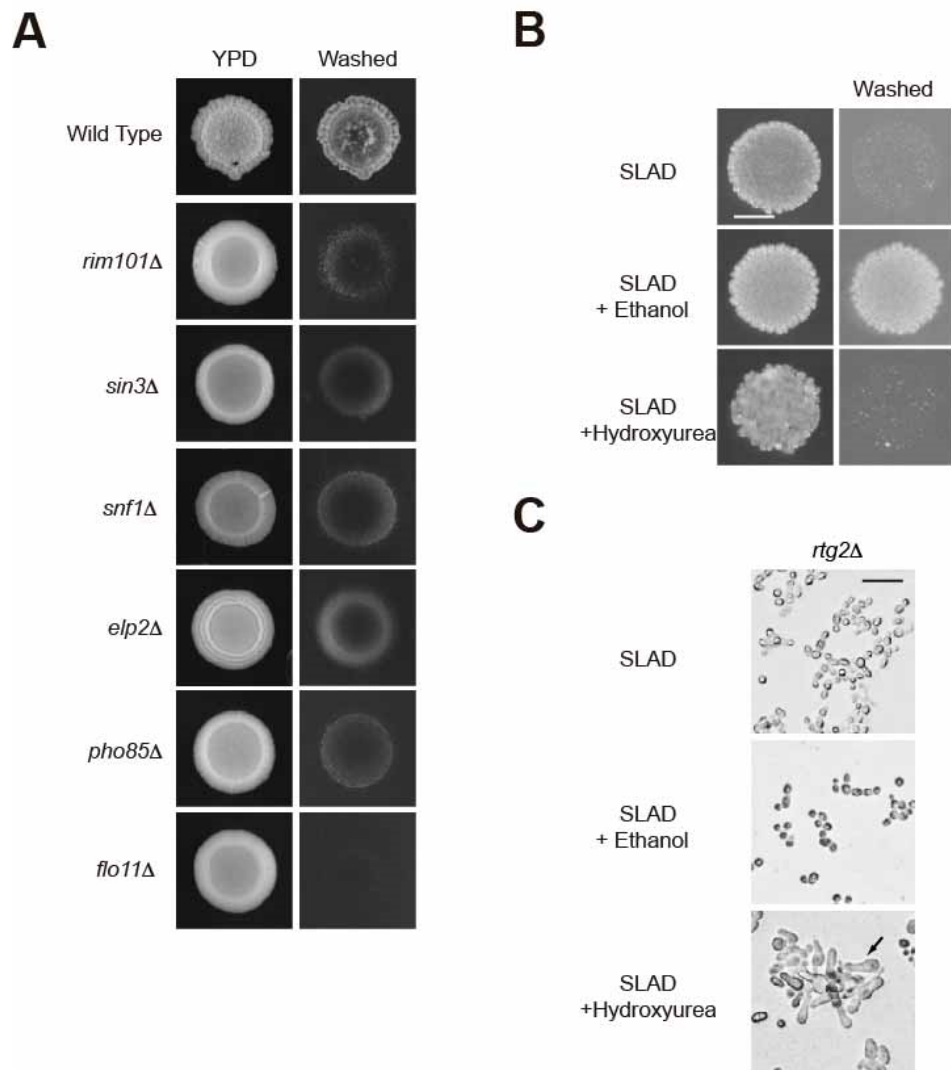


Figure S3.4: Invasive growth phenotypes of signaling mutants. A) Cells were spotted onto YPD media. Plates were incubated for 2 days at 30°C and washed in a stream of water. (B) Plate-washing assay of colonies grown in SLAD medium or SLAD medium supplemented with ethanol (2 % v/v) or hydroxyurea (100 mM). (C) Cells lacking Rtg2p grown under the same conditions as panel B. Bar, 20 microns.

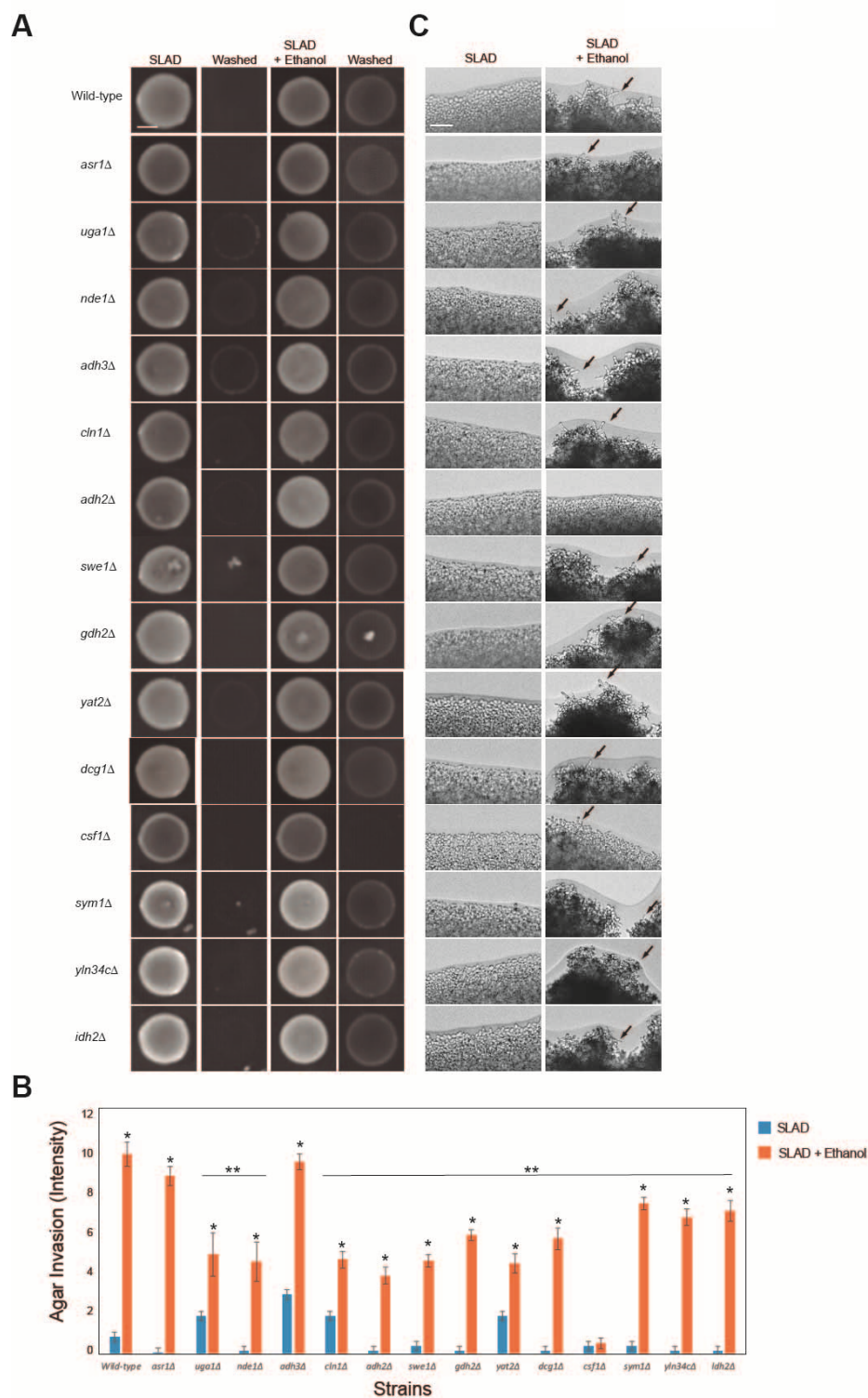


Figure S3.5: Phenotypes of a collection of mutants involved in metabolism were tested for ethanol-dependent filamentous growth. (A) Wild-type cells and the indicated mutants were spotted onto nitrogen-limited medium (SLAD) with or without 2% ethanol (v/v). Plates were photographed, washed in stream of water, and photographed again. Bar, 5 millimeters. (B) Quantitation of invasive growth in panel A by densitometry. The average values of three independent replicates are shown. Error bar represents standard difference between samples. Single asterisk denotes the p-value of <0.01 compared to the no ethanol control (blue to orange bars). Double asterisk denotes p-value of <0.01 compared to wild-type ethanol control. (C) Colony peripheries from the plates in panel A were photographed at 20X magnification. Bar, 25 microns. Arrows mark examples of pseudohyphae.

CHAPTER 4

Genetic factors involved in filamentous growth of wine yeast identified using QTL analysis

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Abstract

S. cerevisiae has the ability to change its morphology when concentrations of nutrients in its environment vary. In particular, nitrogen starvation has been shown to control morphogenesis in this species. The aim of this study was to decipher the genetic basis underlying filamentous growth differences between two wine strains using a Quantitative Trait Loci (QTL) mapping approach. For this, we analyzed a F13 offspring recombinant population obtained from a round of intercrosses between the two strains exhibiting opposite filamentous growth phenotype, and mapped QTL of this phenotype. The segregants exhibited both phenotypes, but, unexpectedly, the segregation was not even in both cases (only 4% of the progeny underwent filamentous growth). The study revealed a wide set of obvious candidate genes involved in this trait, such as *MEP2*, *NGR1*, *ELM1*, *PDH1* and *SRV2*, however our study was also able to report some previously unlinked loci with this trait. Future experiments are necessary to corroborate the contribution of these candidate genes to the filamentous growth phenotype.

Key Words: *MEP2*, invasive growth, pseudohyphal growth, *Saccharomyces cerevisiae*

Introduction

The budding yeast, *Saccharomyces cerevisiae*, is commonly described as a unicellular organism, however, nutrients limitation can alter its normal growth pattern and trigger several responses that evoke morphology changes. In response to limitation conditions, such as, nitrogen deprivation and fermentable sugar limitation, *S. cerevisiae* undergo filamentous growth to scavenge for nutrients, being characterized by elongated cells and unipolar and invasive growth (Cullen and Sprague, 2000; Gimeno et al., 1992). Furthermore, under these limiting conditions, yeast cells generate several responses, including the activation of specific pathways, resulting in pseudohyphal growth (Cullen and Sprague, 2012). The major pathways that are involved in filamentous yeast growth are the Kss1p MAPK pathway (fMAPK) (Adhikari et al., 2015; Karunanithi and Cullen, 2012; Saito, 2010), the global nutrient regulatory RAS-cAMP-protein kinase A (PKA) pathway (Gimeno et al., 1992; Pan and Heitman, 2002; Toda et al., 1985) and the Snf1p kinase pathway, which is required for glucose derepression (Cullen and Sprague, 2000; Kuchin et al., 2002). Additionally, this type of foraging growth is regulated by other pathways, such as the tRNA modification complex Elongator (Krogan and Greenblatt, 2001; Li et al., 2007), the Rim101 pathway (Lamb and Mitchell, 2003) and the TOR pathway, which is a regulator of nitrogen control (Crespo et al., 2002; Hall and Beck, 1999), among others (Chavel et al., 2014; Cullen and Sprague, 2012).

Filamentous growth is associated with responses including quorum sensing mechanisms in yeast (Chen et al., 2004; Dickinson et al., 2003; Sprague and Winans, 2006). This response is mediated by molecules such as aromatic alcohols and ethanol (Chen et al., 2004; Chen and Fink, 2006; Dickinson, 1996; Lorenz et al., 2000), which are induced by different pathways. Aromatic alcohols are produced at high concentrations under nitrogen limitation conditions and modulate filamentous growth in a PKA-dependent manner (Chen and Fink, 2006). Additionally, ethanol strongly induces filamentous growth under nitrogen deprivation via the RTG pathway (González et al., 2017). Both mechanisms converge in *FLO11* expression, which is essential to undergo invasive and pseudohyphal growth (Lo and Dranginis, 1998; Reynolds and Fink, 2001). All of these signalling pathways have been described on the $\Sigma 1278b$ genetic background, however, wine yeasts are also able to undergo filamentous growth (Carstens et al., 1998; González et al., 2017). In this context, the genetic factors that could cause cell differentiation are likely different across genetic background. For example, the S288c strain is unable to undergo filamentous growth due to a mutation in a transcription factor of *FLO11*, *flo8* (Liu et al., 1996). However, Chin et al., (2012) compared S288c and the $\Sigma 1278b$ adhesion mutant and revealed that the fMAPK pathway

is required for adhesion in $\Sigma 1278b$ but not in S288c. This finding indicates that the regulation of filamentous growth likely involves alternative pathways in the different genetic backgrounds (Dowell et al., 2010).

Yeast has recently emerged as a prime model organism for quantitative genetics, particularly through Quantitative Trait Loci (QTL) mapping (Liti and Louis, 2012). QTL analysis has been applied in several studies to decipher the genetic bases associated with quantitative oenological phenotypes in wine yeast (Ambroset et al 2011; Cubillos et al., 2017; García-Ríos et al., 2017; Gutiérrez et al., 2013; Jara et al., 2014; Martí-Raga et al., 2017, Salinas et al 2012). Additionally, QTL approaches have also been used to identify genes involved in morphogenesis in *S. cerevisiae* (Granek et al., 2010; Song et al., 2014). In a previous study exploring filamentation properties in a collection of wine strains, we observed two opposing filamentous growth phenotypes in two strains (P5 and P24) under nitrogen-limited conditions (González et al., 2017). Those two strains clustered into the Wine/European lineage with a bootstrap value of 1 (García-Ríos et al., 2017; Liti et al., 2009) and have been previously studied to elucidate their differential adaptation at low temperatures (García-Ríos et al., 2017). Thus, to further analyse the genetic basis of pseudohyphal growth, we applied a QTL mapping approach using a set of F13 segregants derived from a cross between these two wine strains, which exhibited divergent phenotypes in terms of filamentous growth but had very similar genotypes (García-Ríos et al., 2017). This method allowed to identify candidate genes which are involved in signaling pathways, related to filamentous growth response.

Materials And Methods

Strains and segregant formation

The two parental strains used for the generation of the studied progeny were P5 and P24. P5 corresponds to the commercial strain Lalvin®ICVGRE (Lallemand), and P24 has no commercial name because it is currently undergoing development. The F13 offspring were generated in a previous work (García-Ríos et al., 2017), in which the process to obtain the segregants is described in detail. In brief, first P5 and P24 haploid and auxotrophic strains to the *URA3* and *LYS2* genes, respectively, were constructed; then, two parental strains selected for their opposite mating types, specifically mat α for P5 and mat a for P24, were crossed overnight in YPD medium. This population was replica-plated in minimal medium (SD) to select diploid F1 hybrids. Two F1 hybrids were sporulated on KAc (>90% efficiency), collected and treated first with ether to selectively kill unsporulated cells and after with zymoliasse to remove the ascus, mixed, and then plated at high density to begin a second round of mating and meiosis (F2). The process of intercrossing was repeated until F13 offspring were obtained, providing a large pool of segregants for QTL mapping (See Fig 4.1).

Yeast invasive and pseudohyphal growth assays

Invasive growth and pseudohyphal formation was determined based on Gimeno et al. (1992). Strains were grown for 16 h at 28 °C on minimal medium (1% Yeast Nitrogen Base without amino acids or ammonium, 2% glucose and 10 mM $(NH_4)_2SO_4$), adjusting the optical density to 2.0 (OD_{A600nm}) after centrifugation. Then, ten microliters of cells were spotted on synthetic medium containing dextrose and low ammonium (SLAD) (Gimeno et al., 1992). A plate washing assay (PWA) was performed to assess the ability to penetrate the agar by the yeast, and colony peripheries were observed under a microscope to evaluate filamentation. Additionally, in parallel, strains grown in minimal media were diluted by a factor of 10^6 to obtain single colonies, which were spread onto SLAD medium. SLAD plates were incubated at 28 °C for three days, and ImageJ software was employed to measure invasive growth via densitometry analysis.

Pool segregant selection

Individual segregants belonging to the F13 population were selected to analyse filamentous growth properties. Initially, a pool of F13 segregants was spread on solid YPD medium (1% yeast extract, 2% bacteriological peptone, 2% glucose and 2% of agar) and grown at 28 °C for 48 h. Then, 200 single colonies were isolated and cultured in YPD. Those 200 colonies were individually tested for invasive growth and filament formation. According to the results of these tests, each segregant was assigned to the invasive-filament pool (Pool-Si) or to the non-invasive-non-filament pool (Pool-No). To ensure equal representation of all segregants in the final population, each was independently grown in liquid YPD for 24 h at 28 °C and then mixed in the corresponding pool in equal proportions ($OD_{A_{600nm}}=1.0$ for each segregant) (See Fig 4.1).

Yeast DNA was extracted from 3 mL of an overnight culture in YPD at 30 °C using the method described by Querol et al., (1992). The genomic DNA of the parental strains and each pool was extracted in triplicate.

Gene polymorphism analysis

Twelve genes involved in filamentous growth were sequenced in the P5 and P24 strains by Macrogen Inc. The sequences obtained were compared to evaluate their polymorphisms (mutfunc, EMBL-EBI). The selected genes were *FLO11*, *FLO8*, *PEA2*, *SFL1*, *MDM32*, *STE12*, *TEC1*, *RAS2*, *TPK2*, *SPE1*, *VPS20* and *RIM101*. Additionally, non-synonymous SNPs detected between parental P5 and P24 strains in these genes were analysed in six segregant strains for each pool. Primers were designed using Primer Express (Table S4.1).

Sequencing and variant calling

The genomic DNA of parental strains and each pool was sequenced by Miseq (Illumina, California, USA). Genomic libraries were prepared following the manufacturer's standard instructions. Sequencing was carried out by 300 nt paired-end read following the manufacturer's standard protocols. Reads were trimmed to improve quality using sickle v1.33 (<https://github.com/najoshi/sickle>) with a length parameter $-l$ 180 and q parameter 30. The trimmed reads were mapped on the reference genome S288c (downloaded from SGD website 25/11/2016) by using bowtie2 v2.2.9 (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>) with default parameters.

SNPs corresponding to the parental strains (P5 and P24) were identified by processing variants with SAMtools mpileup (-q 1) and VarScan 2.4.0 (somatic -min_coverage_tumor 8 -strand_filter 1) (Koboldt et al., 2009), with P5 considered as normal and P24 considered as tumor. Bam files corresponding to Pool-Si-1, Pool-Si-2, Pool-Si-3 samples and Pool-No-1, Pool-No-2, Pool-No-3 samples, were merged into single mapping files (Si-pool and No-pool, respectively) by using SAMtools (merge). Merged bam files were subsequently processed by SAMtools, after which high confidence single nucleotide polymorphisms were called using VarScan, with the No-pool considered as normal and the Si-pool considered as tumor. The output file was filtered to intersect the variants that were previously called in P5 and P24 by using the compare tool of VarScan. The putative effects of each variant were predicted with snpEff 4.0 (Cingolani et al., 2012). Differential SNP frequency was estimated through a Fisher test using R as previously described (Cubillos et al., 2017). A Manhattan plot was made in R using the manhattanly script.

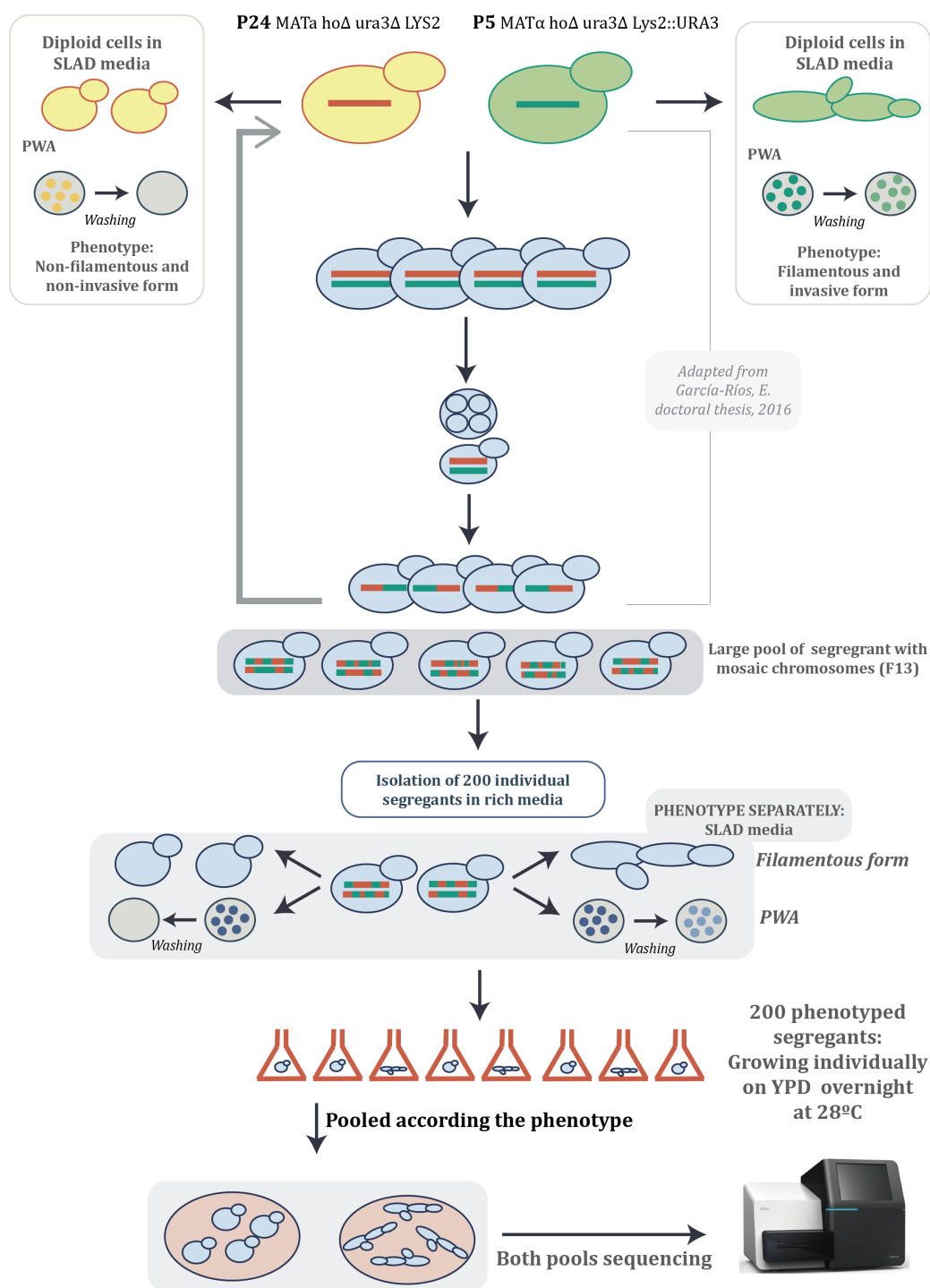


Figure 4.1: Overall strategy of the offspring population construction and phenotyping. Filamentous strain P5 and non-filamentous strain P24 were crossed during multiple rounds of random mating and sporulation in order to create F13 progeny. The resulting segregants were individually characterized and pooled according to the phenotype. The whole-genome of two outcome groups was sequenced.

Results

Phenotype analysis of the parental and F13 populations for filamentous growth

The parental strains P5 and P24 showed opposite phenotypes for invasive and pseudohyphal growth under nitrogen starvation (Fig 4.2, data previously published in González et al. 2017). P5 invaded strongly the agar in SLAD medium, whereas P24 was able to grow in this medium but not undergo invasion (Fig 4.2A). Additionally, colonies of both strains showed opposite phenotypes: P5 underwent robust filament formation, while P24 did not form pseudohyphae under nitrogen limitation (Fig 4.2B).

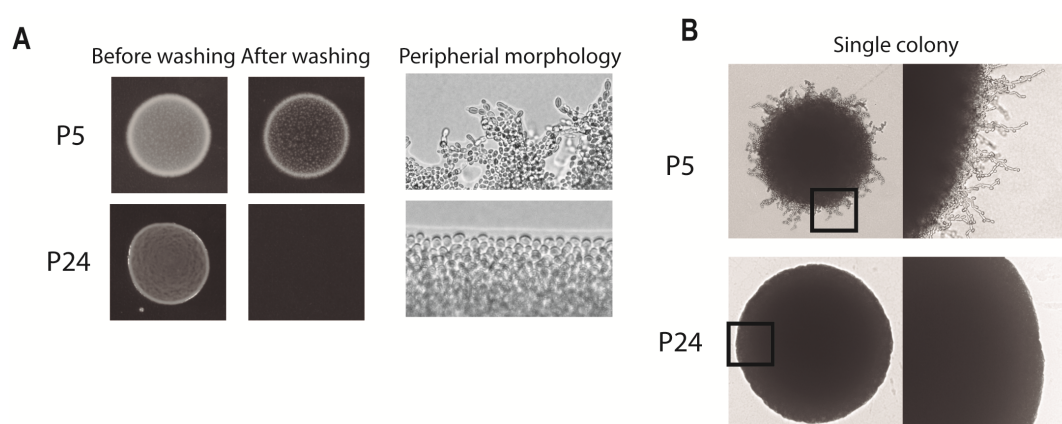


Figure 4.2: Overview of parental strain phenotypes. (A) Plate washing assay (PWA) of P5 and P24 and their peripheral morphology (Bar 50 μ m). (B) Morphology of P5 and P24 colonies in SLAD medium, examined by microscopy at 20x and 40x magnification, Bar 50 μ m, 25 μ m.

In order to reduce linkage block sizes, offspring populations were generated by intercrossing both strains for 13 generations (F13) (García-Ríos et al., 2017; Parts et al., 2011). Initially, the pool from F13 was spread on SLAD medium to observe the morphology of single colonies. Two different phenotypes for pseudohyphal growth were observed, but these did not occur at the same proportion, as only few colonies formed pseudohyphae, hyperfilamentating after longer incubation times (Fig 4.3A). To evaluate phenotype segregation, we determined the filamentous growth phenotype of the progeny by analysing 200 single colonies belonging to the F13 population. First, we studied the ability of single diploid segregants to invade the agar by performing a PWA. Two different and opposite phenotypes for invasive growth were observed, where most of the colonies did not invade the agar (Fig 4.3B). In addition, the colony periphery was observed to analyse the formation of pseudohyphae (Fig 4.3B). All segregants that exhibited invasive growth also formed pseudohyphae, and segregants that did not invade the agar did not exhibit pseudohyphal growth. Agar penetration was measured by performing densit-

ometry analysis from biological duplicates (Fig 4.3C). Some segregants exhibited both phenotypes, but unexpectedly phenotype segregation was not even in both cases. Out of 200 segregants, only eight were able to invade the agar and exhibited pseudohyphae formation at the colony periphery. As expected, colonies spread in SLAD media revealed the same results: 8 segregants exhibited pseudohyphal growth, whereas the other 192 did not. Thus, segregation for both phenotypes was different, where only 4% of progeny underwent filamentous growth. The 8 selected segregants showed a similar invasive growth compared to P5 strain. Given that one of the two parental stains displayed invasive growth, the other not, the expected (mean) segregating pattern in a completely neutral, additive model would be 100:100. The observed result 8:192 is extremely unlikely (cumulative binomial distribution, 3.6×10^{-47}) under these assumptions. Indeed, the observed inheritance ratio predicts at least 4.64 underlying genetic loci ($1/25 = 1/2^{4.64}$) (Steinmetz et al., 2002).

Thus, 192 and 8 segregants were pooled for non-invasive and invasive phenotype, respectively and subsequently sequencing and linkage analysis was performed to establish the genetic basis underlying these phenotypic differences.

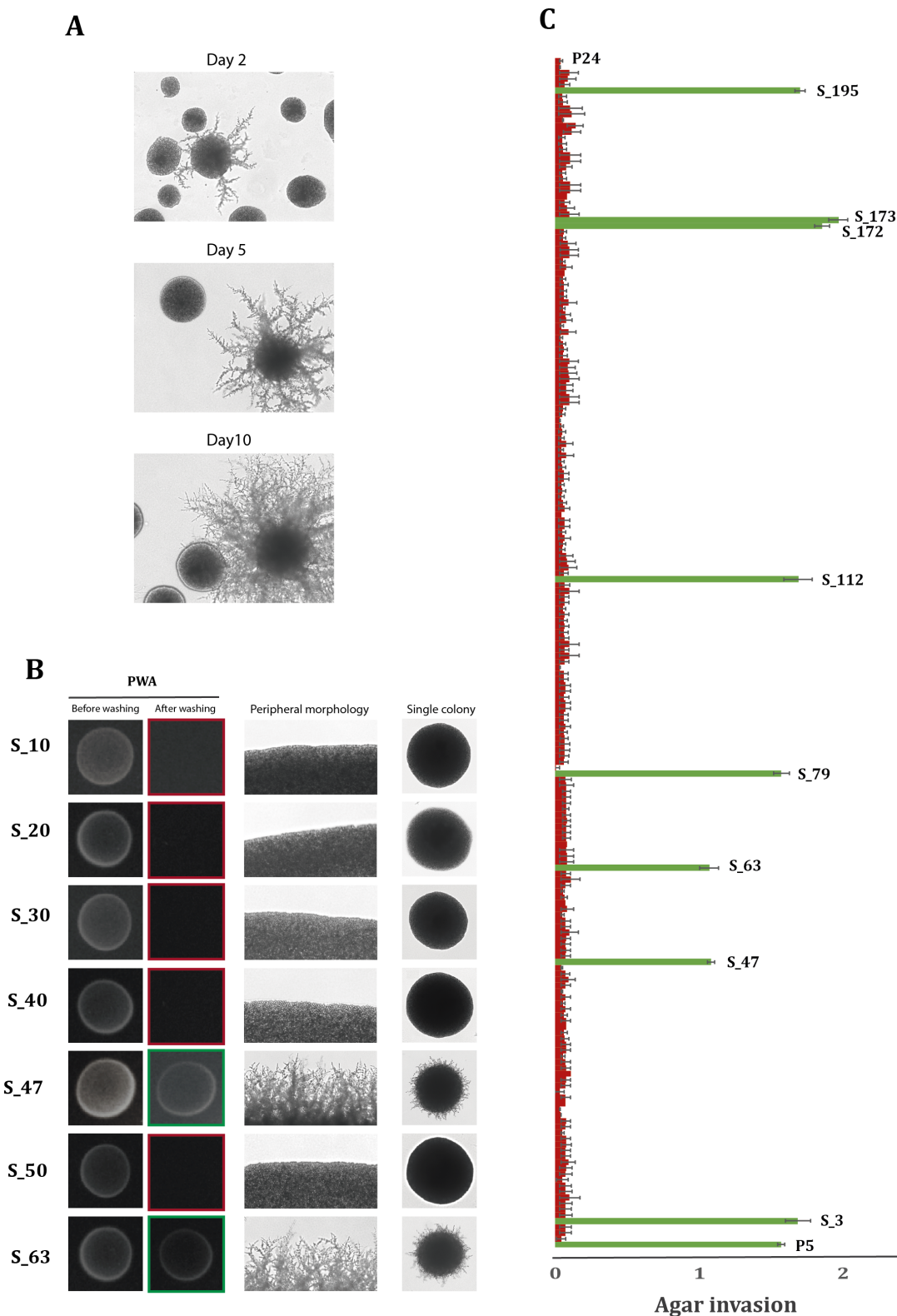


Figure 4.3: Analysis of filamentous growth segregation in F13 progeny. (A) Pseudohyphal formation in SLAD medium of F13 pool. Colonies were examined by microscopy at 10x magnification. (B) Plate washing assay (PWA), peripheral morphology (Bar, 50µm) and pseudohyphal growth of colonies (Bar 50µm) of some individual F13 segregants in SLAD medium. (C) Quantification of invasive growth of the 200 individual F13 segregants by densitometry. Error bars indicate standard deviations of two independent measurements. Green and red color denote invasive and non-invasive growth, respectively.

Genetic characterization of parental and segregant populations associated with their filamentous phenotypes

Although the genomes of the two parental wine strains had been previously sequenced and compared to the S288c reference strain (García-Rios et al 2017), isolates were resequenced in this study. The comparison of the two genomes revealed gene content differences. For example, *MEP2* which encodes for a high affinity ammonium transporter whose expression is controlled by Nitrogen Catabolite Repression (NCR) system (Marini et al., 1997) and likely required in filamentous growth (Lorenz and Heitman, 1998), was absent in P24 strain, but present in P5. We further analyzed the presence of this gene in the F13 population, and our results showed that the presence or absence of this gene correlated with filamentous growth, i.e. no-invasive pool presented deletion of *MEP2*, whereas invasive pool maintained the sequence of this gene (Fig S4.1).

Table 4.1: Identification of non-synonymous SNPs between P24 and P5 strains in filamentous growth related genes. The table lists specific nucleotide and amino acid changes between both strains.

Gene	Systematic name	Chr	Position (nt)	Polymorphism (nt allele change P24 to P5)	Amino acid change (P24 to P5)
<i>FLO11</i>	YIR019C	IX	393074	C → T	C201Y (TGT → TAT)
		IX	393156	C → T	D174N (GAT → AAT)
		IX	393338	T → C	Y113C (TAC → TGC)
		IX	393395	C → G	W94S (TGG → TCG)
		IX	393403	C → A	K91N (AAG → AAT)
<i>FLO8</i>	YER109C	V	376843	C → T	A258T (GCA → ACA)
<i>STE12</i>	YHR084W	VIII	274295	A → G	D41G (GAC → GGC)
<i>SPE1</i>	YKL184W	XI	97327	C → A	Q191K (CAG → AAG)
<i>VPS20</i>	YMR077C	XIII	421780	T → C	N124D (AAT → GAT)

The sequences of twelve genes related to filamentous growth were compared between the two parental strains. The genes that presented non-synonymous SNPs were *FLO11*, *FLO8*, *SPE1*, *STE12*, and *VPS20* (Table 4.1). *FLO11* is an essential gene for pseudohyphal growth (Lo and Dranginis, 1998), *FLO8* encodes the transcription factor *FLO8*, which positively regulates *FLO11*, *STE12* cooperates with Tec1 to regulate filamentous growth via MAPK (Saito, 2010), *Spe1* and *VPS20* are also related to filamentous growth response (Ryan et al., 2012). The five genes presented different degree of

polymorphism comparing P5 and P24 genotype. *FLO11* presented five non-synonymous SNPs and a deletion of 36bp in P24 strain. In addition, *FLO8*, *STE1*, *SPE1* and *VPS20*, presented one non-synonymous SNP each when comparing both sequences. To corroborate if the polymorphism of the parental strains was segregated to F13 population, six segregants of each pool were analysed. The results of the sequence analysis for those genes are represented in Fig 4.4. The *STE12* and *VPS20* sequences in the twelve segregants analysed were equal to P24, indicating that these genes are not involved in the different filamentous growth of P5 and P24 strains. The other sequences analysed presented different polymorphisms, in some cases being similar to P5 strain, and in others to P24 strain. However, not all strains of each pool conserved the polymorphism presented by the corresponding parental strain, according with their phenotype. Thus, these genes did not seem to be critical for the differences between P5 and P24 strains to undergo filamentous growth.

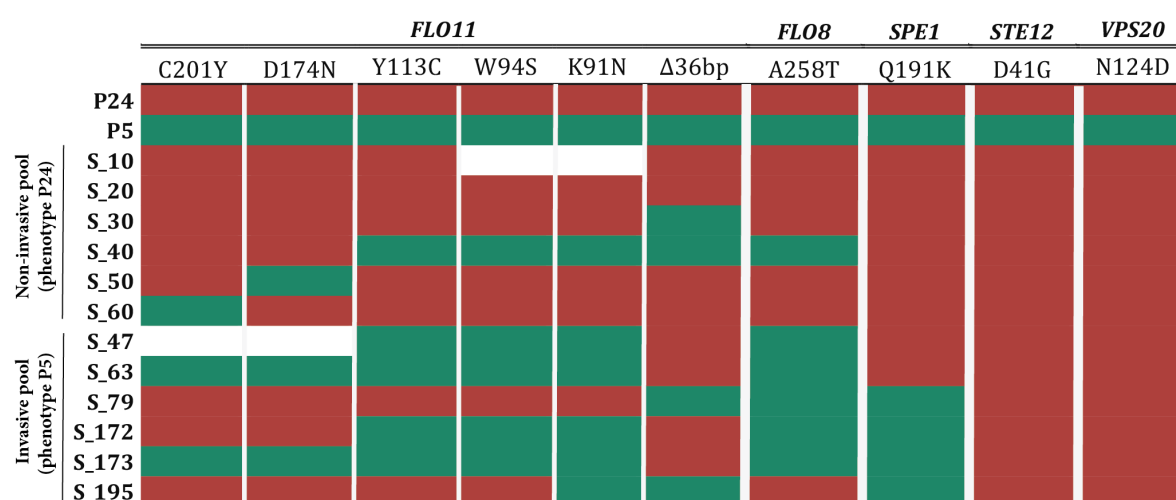


Figure 4.4: Identification of amino acid changes exhibited in some filamentous growth related proteins in the parental strains and in six individual F13 segregants of each phenotype. Green and red color indicate similarity to P24 and P5 parental strain, respectively. White color indicates no information for this amino acid sequence.

Linkage analysis

The two pools including populations with opposite phenotype for filamentous growth were sequenced, genotyped and compared with the whole genome sequence of the parental strains. The allele frequency analysis allowed us to map QTL intervals responsible for the phenotypic variation in the F13 population. A total of 7,982 markers were used (average SNP distance: 1500 kb) and allele frequencies were compared across the genome between both pools. We found several regions located in different chromosomes that exhibited high significance (Fig 4.5, $-\log_{10} p\text{-value} > 25$). Overall, we were able to identify 9 QTLs distributed along the genome, in chromosomes II, IV, IX, XI, XII, XIV and XV. In order to identify causative loci for the trait of interest, we examined the genomic regions containing QTLs, using gene function descriptions from SGD and selected possible candidate genes. Table 4.3 provides a list of genes close to the mapped QTLs regions, which could be good candidates for the phenotypic variation of the strains.

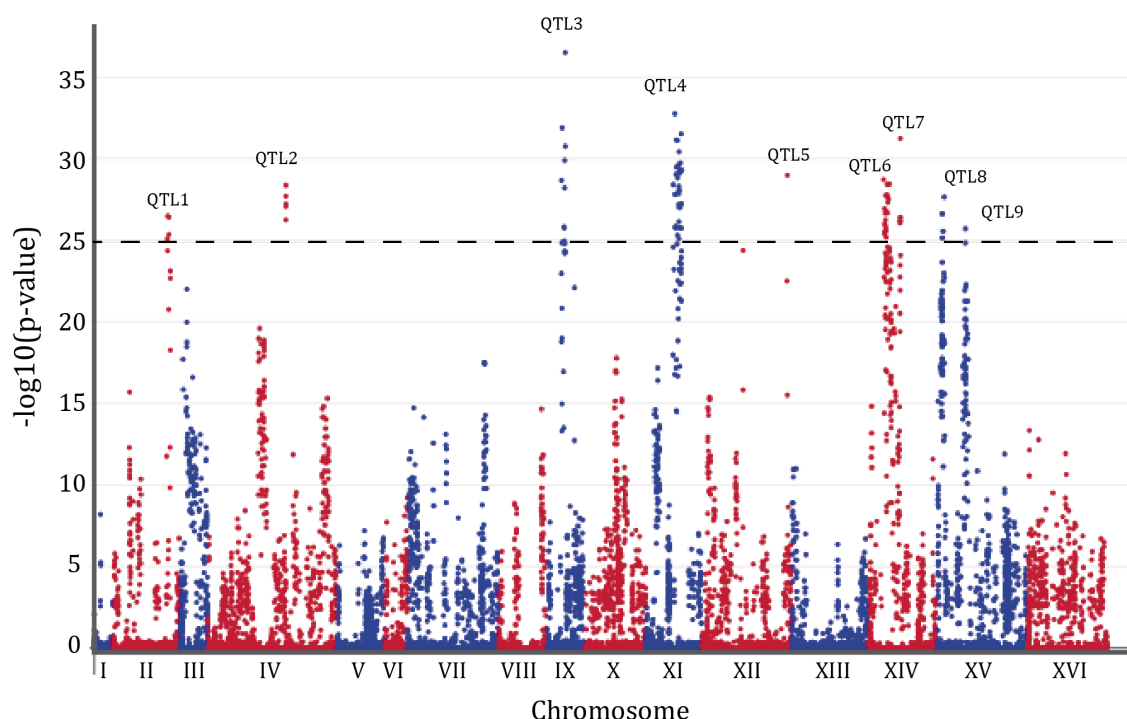


Figure 4.5: Manhattan plot of the genome-wide $-\log_{10}(\text{P-value})$ for SNP allelic frequency differences between the two selected pools. The linkage analysis revealed nine QTL regions ($-\log p\text{ value} > 25$).

NGR1, *AME1*, *PDB1* and *YBR221W-A* were identified on Chr II. *NGR1* is associated with filamentous growth in a Snf1 pathway dependent manner (Kuchin et al., 2002), *PDB1* encodes a pyruvate dehydrogenase involved in acetyl-coA biosynthesis (Pronk et al., 1996), and the other two genes have no known functions. QTL2 was mapped in Chr IV, downstream of *COX20*, which is a cytochrome c oxidase (Hell et al., 2000). QTL3 contained a SNP that demonstrated the highest significance in the *SER33* gene, which encodes a dehydrogenase that catalyses the first step in serine and glycine biosynthesis from α -ketoglutarate and whose expression is regulated in relation to available nitrogen sources (Albers et al., 2003). Eight other genes appeared in QTL3 (Chr IX), some with unknown function (*PKR1* and *SPO22*), as well as *FYV10* and *SGA1*, which are involved in glycogen catabolism (François and Parrou, 2001; Menssen et al., 2012). Moreover, linkage analysis revealed a notable polymorphism on Chr XI (QTL4), which involved more than twenty genes. Among them, four with indeterminate function (*TTI1*, *YKL023C-A*, *YKL023W*, *YKL050C*), and five (*CDC16*, *PAN3*, *PUT3*, *SPT23* and *URB1*) related to nucleotide processing. Interestingly, two genes in this region previously associated with filamentous growth were identified: *ELM1* and *PHD1*. On Chr XII (QTL5) only the gene *SIR3*, which encodes a chromatin binding protein, was found (Johnson et al., 1990). A high diversity of genes was present on Chr XIV (QTL6 and QTL7). None of genes included in the QTL6 region were previously reported to be involved in filamentous growth. However, *SRV2* and *YNL234W* encode proteins that participate in the Ras pathway and in glucose signal regulation, respectively (Fedor-Chaiken et al., 1990; Kaniak et al., 2004). QTL7 on chromosome XIV mapped 8 kb away from *MEP2*, an ammonium permease essential for filamentous growth. Finally, in the QTLs mapped to Chr XV, few genes were identified; a unique gene encoding an mRNA binding protein was detected in QTL8, whereas no genes related to filamentation were detected in QTL9. However, certain genes coding unknown proteins were found in these regions. Some of these genes, which are provided in the list, exhibit linkage due to their proximity to a determinant allelic region. Overall, seven genes were considered to be potentially relevant to filamentous growth: *NGR1*, *ELM1*, *PDH1*, *SER33*, *MEP2*, *SVR2* and *YNL234W*.

Table 4.2: List of genes identified in each QTL region

QTL position	GENES
QTL1 (Chr II)	<i>AME1, YBR221W-A, PDB1, NGR1</i>
QTL2 (Chr IV)	<i>COX20</i>
QTL3 (Chr IX)	<i>SER33, PRK1, SPO22, SEC28, SGA1, AIR1, THS1, FYV10, YIL077C</i>
QTL4 (Chr XI)	<i>YKL063C, YKL050C, ELM1, DCW1, PDH1, NFU1, TTI1, CDC16, URB1, GPX1, SPT23, LAC1, YKL018C-A, PUT3, URA6, PAN3, YKL023C-A, YKL023W, SPC42, VPS24, UFD4, TUL1, CCE1</i>
QTL5 (Chr XII)	<i>SIR3</i>
QTL6 (Chr XIV)	<i>SIP3, NRD1, MRPL17, RAD50, MPA43, YNL247W, SLA2, ATG2, KEX2, SIN4, YNL234W, ADE12, ALG9, ATG4, CSL4, YNL226W</i>
QTL7 (Chr XIV)	<i>MEP2, SRV2, YSF3</i>
QTL8 (Chr XV)	<i>PAP2, YGK3, HRP1</i>
QTL9 (Chr XV)	<i>YOL128C, YOR011W-A, IRC11</i>

The sequences of *NGR1*, *ELM1*, *PDH1*, *SER33*, *SVR2* and *YNL234W* were compared among parental strains. Most of them presented non-synonymous SNPs (Table 4.3), with the exception of the *SER33* gene. The results confirm that these selected genes are good candidates to validate by Reciprocal Hemizyosity Analysis (RHA).

Table 4.3: Candidate genes selected based on their function and the presence of non-synonymous SNP in the parental strain sequences

Gene	Systematic name	Chr	Position (nt)	Polymorphism (nt allele change P24 to P5)	Amino acid change (P24 to P5)
<i>NGR1</i>	YBR212W	II	151	A → G	I388V
<i>ELM1</i>	YKL048C	XI	1162	G → C	A51P
<i>PDH1</i>	YPR002W	XI	1512	G → A	M504I
<i>SRV2</i>	YNL138W	XIV	1225	A → G	I409V
<i>YNL234W</i>	YNL234W	XIV	296	A → G	Q99R

Discussion

Several genes and signaling pathways have been related to control filamentous growth response in yeast (Cullen and Sprague, 2002, 2012; Saito, 2010; Vinod et al., 2008). However, these studies are usually performed using lab strains, which share, in most cases, the genetic background. In fact, genetic backgrounds have been reported to be able to influence in the expression and function of genes related to filamentous growth (Chin et al., 2012). For this reason, we applied QTL analysis to explore genetic factors involved in this phenotype using two wine yeasts as parental strains. Although most natural wine strains undergo filamentous growth (Carstens et al., 1998; González et al., 2017), nevertheless, only 4% of the progeny studied in this work formed invasive and pseudohyphal growth. This result could emerge under non-additivity if inheritance of multiple epistatic alleles are required for invasive growth to manifest. Indeed, the segregation pattern observed (1:25) predicted that four or five alleles must co-exist in the offspring genomes, if alleles are unlinked. These could be correlated to the number of QTLs observed in this study. The linkage analysis presented in this work detected a broad set of genes as possible candidates contributing to yeast pseudohyphal growth (Table 4.3). One of the candidate genes, *MEP2*, an ammonium permease, was naturally absent in P24 genetic background. The role of this high-affinity ammonium permease is to transport ammonium into the cell under low nitrogen conditions. *MEP2* has been shown to be required for filamentous growth (Lorenz and Heitman, 1998; Rutherford et al., 2008). *S. cerevisiae* cells lacking *MEP2* are unable to undergo filamentous growth in SLAD medium (Lorenz and Heitman, 1998; Rutherford et al., 2008). The filamentation defect in *MEP2* does not appear to be caused by a defect in ammonium transport, because there are other permeases, Mep1 and Mep3, that perform this function in the cell. *MEP2* has a specific role in signaling filamentous growth through a mechanism not well understood via MAPK pathway (Rutherford et al., 2008). In our study, the variation in invasive growth phenotype seemed to be connected to the presence of *MEP2*, as the absence of this gene segregates perfectly with the invasive phenotypes (all 192 offspring without invasive growth lack the *MEP2*, as P24 strain). Therefore, it is easy to associate this deletion with the inability of P24 to undergo filamentous growth in SLAD medium. In this case, the segregation pattern of *MEP2* would need to be not neutral during the construction of the F13 pool. However, despite this deletion, linkage analysis revealed a notable polymorphism involving a large number of determinant loci that may also be involved in filamentous growth.

The *NGR1* gene encodes an RNA binding protein that negatively regulates the growth rate; specif-

ically, *NGR1* regulates glucose catabolism under nitrogen-limited conditions (Gardner et al., 2005). Moreover, *NGR1* negatively regulates invasive growth by repressing *FLO11* expression through the Snf1-dependent pathway under glucose-limited conditions (Kuchin et al., 2002). P24 strongly invades agar under non-glucose-limited conditions (González et al., 2017). This finding may explain why P24 exhibits different regulation in rich medium than P5; however, further analysis is needed to corroborate this hypothesis. The protein *SER33* is located in the cytoplasm and catalyses the conversion to D-2-hydroxyglutarate from α -ketoglutarate (Albers et al., 2003). However, SNPs did not produce a residue change in this protein. The *PHD1* gene encodes the transcription factor *PHD1*, which is involved in pseudohyphal growth development (Gimeno and Fink, 1994). This gene has also been analysed in the pathogen *C. albicans* and is essential for pseudohyphal growth (Lo et al., 1997). *ELM1* is a serine/threonine protein kinase (Blacketer et al., 1995) that promotes proper septin assembly with the Gin4 and Cla4 proteins (Mortensen et al., 2002) and is involved in budding (Kang et al., 2016). Septin-defective cells promote Swe1 accumulation; as a result, cellular mechanisms fail to switch from apical growth to isotropic growth, leading to the formation of elongation buds (Kim et al., 2015). *PHD1* positively regulates *FLO11* expression downstream of the PKA pathway, and the overexpression of this transcriptional factor triggers hyperfilamentation (Gimeno and Fink, 1994; Malcher et al., 2011). In this case, a non-synonymous mutation was detected between parental strains. P5 forms large amounts of pseudohyphae and demonstrates the highest invasive growth phenotype in SLAD medium compared to other commercial wine yeasts (González et al., 2017). Defective regulation of *FLO11* by *NGR1* may explain the robust filamentation of the P5 strain. On the other hand, the gene *SVR2* encodes an adenylylate cyclase-binding protein, which is reportedly involved in filamentous growth induced by nitrogen starvation (Mösch and Fink, 1997) as well as the Ras signalling pathway (Fedor-Chaiken et al., 1990). Moreover, its deletion and overexpression cause defective filamentous growth and hyperfilamentation, respectively, in Σ 1278b (Wu and Jiang, 2005). Finally, the *YNL234W* gene encodes a protein of unknown function. However, this protein is regulated by Rgt1 and is likely involved in glucose signalling or metabolism (Kaniak et al., 2004).

To conclude, using QTL approach, we identified several genes that are potential candidates to regulate filamentous growth in *S. cerevisiae*. Some of these genes were previously reported to be regulators of this foraging response, while others were not previously associated with filamentous growth. Reciprocal hemizygosity and allele swapping analysis are needed to validate these candidate genes.

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Supplementary figures

Table S4.1: Primers used in this study. Primers were designed using Primer Express software (ThermoFisher Scientific).

Primer	Sequence	Reference
FLO8M-F	5'-CCAGTAACGTTTGTGACTCCGATT-3'	This study
FLO8M-R	5'-GCGGTTCAAATGCGCAAT-3'	This study
SPE1M-F	5'-GATGAACATCTCGCCGGATAG-3'	This study
SPE1M-R	5'-GAACCGACGTGGAAAGAAACA-3'	This study
STE12M-F	5'-GAAAAGATAACAGGTAAGCACTGAAGAC-3'	This study
STE12M-R	5'-CAAAGCCTTGTCCTACTATTCAGATAG-3'	This study
VP20M-F	5'-CCTTGCGCGCTAAATAGATGAA-3'	This study
VP20M-R	5'-TCCCCTCCTAAATGCAGTACATAGT-3'	This study
Flo11P1-F	5'-GTGTAGCAGGAGCGGTGGTT-3'	This study
Flo11P1-R	5'-CTTTGGATGTGACTTCCGTTTCT-3'	This study
FLo11P2-F	5'-CTTGCATATTGAGCGGCACTAC-3'	This study
FLo11P2-R	5'-ACGCACACTATGCAAAGACCAT-3'	This study
MDM32-F	5'- AAAAAAGGCTAAATGTCAGGGATGA -3'	This study
MDM32-R	5'- TCTCCGTCTTTTGATCTCCTTGA -3'	This study
Flo11Zd-F	5'-GTACATGGAGTGGTGTCTGTGATG-3'	This study
Flo11Zd-R	5'-TTTGAAGTTTATGCCACACAAGATG-3'	This study

Chapter 4

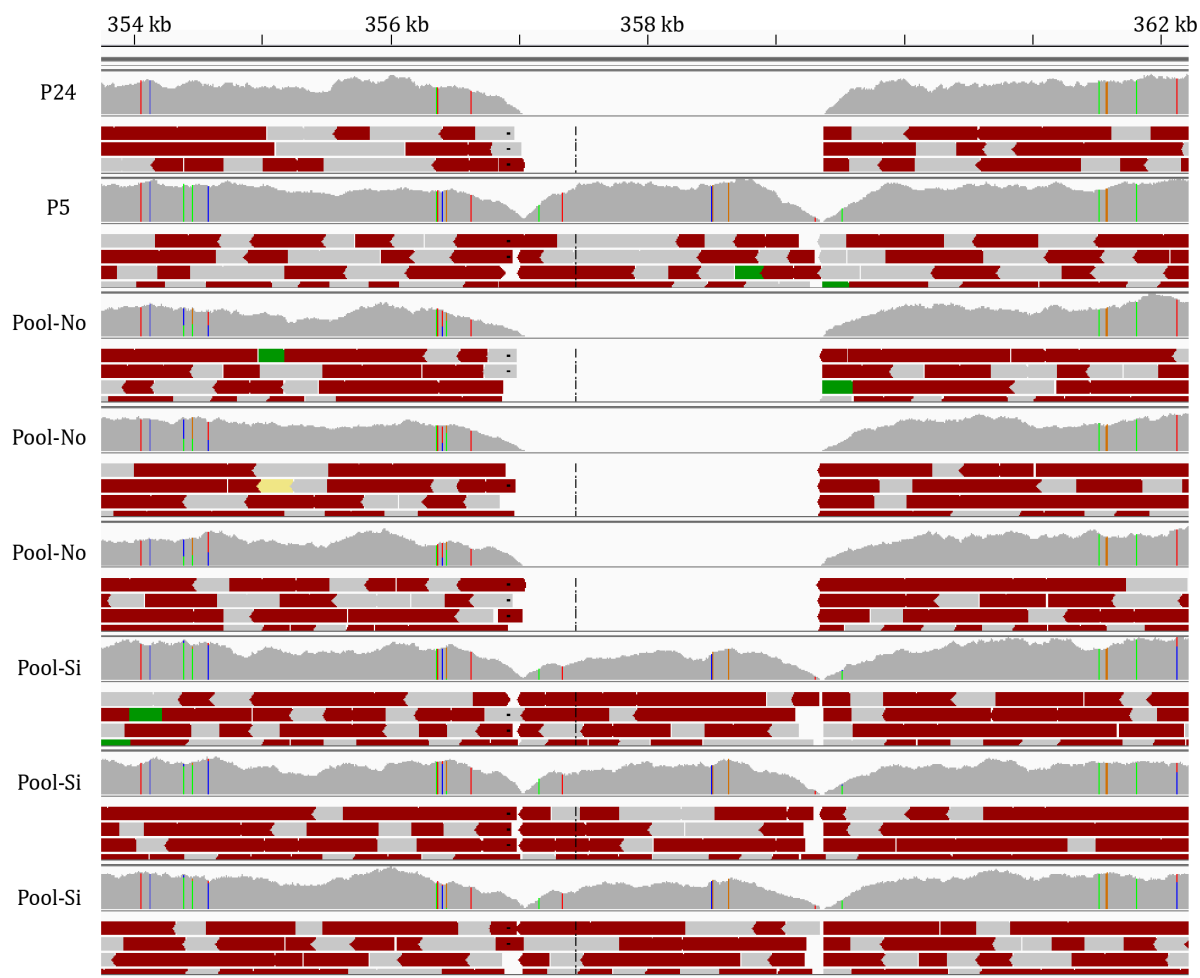


Figure S4.1: Alignment of *MEP2* sequences of parental strains (P24 and P5) and invasive (Pool-Si) and non- invasive (Pool-No) pools.

General Discussion

The winemaking process involves microorganism succession and interaction. Non-*Saccharomyces* yeast are found during the first stages of the process; however, throughout alcoholic fermentation, various factors, such as ethanol, anaerobic conditions or acid pH, make the environment more favourable for *S. cerevisiae*, which eventually overwhelms the microbial population to the detriment of other yeast species. Microbiological interactions through physical contact have been shown to induce the early death of non-*Saccharomyces* species through a mechanism dependent on cell density. Recently, interactions between species have also been associated with the secretion of specific compounds during alcoholic fermentation, known as quorum sensing molecules (Albergaria and Arneborg, 2016; Ciani and Comitini, 2015, Wang et al, 2015, 2016).

During alcoholic fermentation, similar to other bioprocesses, a myriad of compounds are generated via microorganism metabolism. Ethanol is the main by-product of alcoholic fermentation; however, grape must contains various precursors that are used as substrates by yeast. Clear examples are the aromatic amino acids tyrosine, tryptophan and phenylalanine, which are converted to tyrosol, tryptophol and phenylethanol, respectively, by yeast. These alcohols, classified as Quorum Sensing (QS) molecules, are linked to the generation of several responses in yeast, such as the regulation of cell density and the induction of filamentous growth. These morphology changes have been extensively studied in the pathogen *C. albicans* and *S. cerevisiae* lab strain Σ 1278b (Chen and Fink, 2006; Kruppa, 2009). However, little research has been carried out in other yeast genera or species. Thus, the aim of this thesis is to study the production and effects of those QS compounds on wine yeasts and to determine the genetic factors involved in their filamentous growth.

Aromatic alcohol production

Aromatic alcohol production has been poorly studied in non-*Saccharomyces* species, and its regulation in *Saccharomyces* remains unclear, although it appears that nutrient availability may play a key role. Thus, our first objective was to evaluate the production of aromatic alcohols by different wine yeasts (*S. cerevisiae*, *H. uvarum*, *S. bacillaris*, *M. pulcherrima* and *T. delbrueckii*) under different nutrient conditions (different carbon, nitrogen and aromatic amino acid concentrations) (Chapter 1). We observed that all species tested were able to produce all three aromatic alcohols, but their concentrations differed depending on yeast strain and nutrient (and precursor) availability. *S. cerevisiae* was the strain that produced highest concentrations of these compounds. Overall, nitrogen starvation increased the production yield, whereas glucose deprivation reduced it. Previous studies (Chen and Fink, 2006) performed with another *S. cerevisiae* strain, Σ 1278b, also demonstrated that nitrogen limitation activated aromatic alcohol synthesis, especially that of tryptophol and phenylethanol. These authors suggested that the production of these autosignaling aromatic alcohols was regulated by nitrogen (activated in nitrogen-poor conditions), and also controlled by cell density (being much higher in high-density cultures, than that in low-density cultures), and subjected to positive feedback regulation. Thus, the lower production of aromatic alcohols in low glucose may be explained by lower yeast cell density in those conditions. However, despite yeast exhibiting lower growth under nitrogen limitation, higher concentrations of aromatic alcohols were detected in nitrogen-limited must rather than in standard must, indicating that cell density is not the sole variable that determines the synthesis of these alcohols. In addition, the increase in corresponding precursors (aromatic amino acids) was positively correlated with aromatic alcohol production in all cases.

The fact that all species tested were able to synthesize these aromatic alcohols indicates that the Ehrlich pathway is active in other genera and species than *S. cerevisiae*; however, the lower aromatic alcohol secretion of the non-*Saccharomyces* yeasts, compared to *S. cerevisiae*, may indicate that this pathway presents a lower efficiency in non-*Saccharomyces* species, perhaps due to the poor management of nitrogen sources by them. Moreover, unlike *S. cerevisiae*, some wine yeast species, such as *M. pulcherrima*, *S. bombicola* and *T. delbrueckii*, are able to respire between 40-100% of sugars under aeration conditions (Quirós et al., 2014), which has been described, in case of (*C. albicans*, to generate lower aromatic alcohols, compared to anaerobic growth (Ghosh et al., 2008). Thus, the prevalence of respiratory catabolism in non-*Saccharomyces* yeasts is likely related to reduced aromatic alcohol production.

Aromatic alcohols have been associated with cell growth regulation, *S. cerevisiae* produce higher concentrations of aromatic alcohols at a specific cell density (Chen and Fink, 2006; Avbelj et al., 2015). In this study we observed that in *S. cerevisiae*, cells produced aromatic alcohols during the early exponential phase, and these compounds were secreted into the medium during the shift from the exponential to stationary phase, when the induction of mechanisms required for the starvation period occurs. These findings are consistent with previous studies (Chen and Fink, 2006; Zupan et al., 2013). Similar observations were made in non-*Saccharomyces* yeast. Secretion of these molecules at the end of exponential phase indicates a connection to cell density, suggesting that aromatic alcohols are potential QS molecules in all the tested species, as previously suggested for *C. albicans*, *S. cerevisiae* and *D. hansenii* (Chen et al., 2004; Chen and Fink, 2006; Gori et al., 2011). To conclude this part, we demonstrated that all of the wine yeasts tested were able to produce the three aromatic alcohols evaluated, although their synthesis patterns were modulated by nutrient availability (being activated by nitrogen limitation and inhibited by glucose limitation), and were also partially dependent on yeast strain and growth phases.

Effects of aromatic alcohols on wine yeast

In *S. cerevisiae*, filament formation is induced by nitrogen or fermentable carbon starvation (Cullen and Sprague, 2000; Gimeno et al., 1992; Roberts and Fink, 1994), as well as by low concentrations of aromatic alcohols (Chen and Fink, 2006; Dickinson, 1996; Lorenz et al., 2000). *S. cerevisiae* is a non-motile microorganism and is unable to scavenge for nutrients; therefore, under nutrient limitation, filamentous growth is explained as a foraging response to explore the surroundings (Gimeno et al., 1992). On the other hand, the presence of fusel alcohols may signal the depletion of preferred nitrogen sources and the catabolism of less-preferred ones (Magasanik and Kaiser, 2002), such as aromatic amino acids, which are precursors of these alcohols (Chen and Fink, 2006; Dickinson, 2008). In Chapter 2 and Chapter 3, we evaluated the effects of nutrient limitation, fusel alcohols and ethanol on the filamentation properties of wine yeasts. The triggers for filamentous growth have not been extensively characterized in other backgrounds apart from lab strain $\Sigma 1278b$. By examining a collection of wine yeasts, we showed that most wine strains undergo filamentous growth in response to nutrient limitation, as observed in previous reports (Cullen and Sprague, 2000; Gimeno et al., 1992), with the exception of the commercial wine strain P24, which did not trigger any filamentous growth in nitrogen limiting medium (SLAD). Additionally, we observed that yeast natural isolates produced stronger filament formation compared

4. General Discussion

to commercial wine strains. This finding may be explained by previous studies that demonstrated the influence of human activity on “domesticated” strains in terms of cross-breeding and the production of new combinations (Liti et al., 2009), likely resulting in different phenotypes. Apart from *S. cerevisiae*, few studies have investigated this type of growth in other yeast genera and species, as Pu et al., (2014) who showed that phenylethanol promote pseudohyphae formation in *H. uvarum*. Thus, we analysed the ability of non-*Saccharomyces* strains involved in wine fermentation to penetrate surfaces (invasive growth) or to form pseudohyphae. All of the tested species strongly invaded the agar, and this invasion increased under nutrient depletion. However, the only two strains of *H. uvarum* exhibited robust pseudohyphal growth in rich media. Indeed, this species are frequently found in ecology studies, colonizing the grape surface (Padilla et al., 2016; Pretorius, 2000). The ability to form robust filament cells likely confers advantages over other yeast genera or species. Aromatic alcohols and ethanol modulated filamentous and invasive growth in a nutrient-dependent manner. In *S. cerevisiae*, ethanol only induced filamentous growth under nitrogen limitation, whereas in *T. delbrueckii*, ethanol promoted invasive growth in all cases tested. Among the other species, the effects of ethanol depended on the medium employed. Tyrosol, tryptophol and phenylethanol resulted in the modification of filamentation patterns for all species, similar to ethanol, but again, this response was conditioned by the availability of carbon or nitrogen sources. Finally, we also examined the effects of aromatic alcohols in the cell growth. As a result, the growth of *Saccharomyces* strains was significantly reduced only by tryptophol, however, the growth of all tested non-*Saccharomyces* species was negatively affected by all three aromatic alcohols. Tyrosol and phenylethanol reduced the generation time and maximal growth in non-*Saccharomyces* species even at low concentrations, which are easily found during wine alcoholic fermentation, nevertheless, same concentrations did not affect to *S. cerevisiae* strains. The finding that aromatic alcohols exert higher inhibitory effects in non-*Saccharomyces*, compared to *Saccharomyces*, as well as the ability of *S. cerevisiae* to produce them in higher quantities during alcoholic fermentation (Chapter 1), suggests that these alcohols may play a role in yeast interactions during the winemaking process, especially in the predominance of *S. cerevisiae* over non-*Saccharomyces*.

It has to be noticed that we tested the effect of these alcohols individually, however, during the alcoholic fermentation yeast can produce these alcohols simultaneously, and therefore, mixtures of these alcohols could have a greater impact on the growth and morphology of yeast. Hence, further analyses are needed to corroborate this hypothesis.

In summary, we demonstrated that both the synthesis of aromatic alcohols and their effect on wine yeast

growth and morphology are modulated by nutrient availability in the environment. Moreover, the signals triggered by aromatic alcohols appear to be species-specific. In general, yeasts respond to quorum sensing molecules in several ways during adaptation to the environment, inducing the entrance into the stationary phase, as a sign that nutrients are depleted, and simultaneously scavenging to search for nutrients through filamentous growth.

Genetic factors involved in filamentous growth

The genetic factors involved in the different phenotypes exhibited by non-*Saccharomyces* are difficult to study because the genomes of these yeasts are not all available. Thus, we focused our study on the genetic factors involved in the filamentous growth of *S. cerevisiae*. The mechanistic pathway through which tryptophol and phenylethanol trigger filamentous growth is well established in *S. cerevisiae* (Chen and Fink, 2006), however, this mechanism has not been extensively studied for ethanol. Our results showed that ethanol is a stronger inducer of filamentation than aromatic alcohols under nitrogen starvation conditions. For these reasons, we decided to study the genetic factors and pathways implicated in ethanol-inducible filamentous growth.

Different pathways are involved in the filamentous growth response in *S. cerevisiae*. We tested mutants for the major filamentation regulatory pathways, including fMAPK (Adhikari and Cullen, 2014; Saito, 2010), Ras2-cAMP-PKA (Gimeno et al., 1992; Toda et al., 1985), Snf1 (Cullen and Sprague, 2000), and TOR (Hall and Beck, 1999), among others. Unexpectedly, most mutants did not show growth defects in presence of ethanol, indicating that this molecule exerts its effects on filamentous growth independent of the major regulatory pathways that control this phenotype. Recently, other pathways has been described as regulators of filamentous growth, one of them is the mitochondrial retrograde to nucleus (RTG) pathway, which sense changes in metabolic respiration (Aun et al., 2013). Yeast cells respond to mitochondrial dysfunction by altering the expression of a subset of nuclear genes through this pathway. Additionally, an important role of the RTG signalling pathway is maintenance of intracellular glutamate supplies in yeast cells with dysfunctional mitochondria (Sekito et al., 2002). In our study, we found that cells that presented inactivation of the RTG pathway were defective for ethanol-induced filamentous growth and that both nitrogen limitation and ethanol induced this pathway. Moreover, the tricarboxylic acid (TCA) cycle is compromised in cells experiencing mitochondrial defects (Liu et al., 2003), but TCA flux is maintained by the actions of the RTG pathway. However, glutamate

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suppresses the requirements for the RTG pathway in the TCA cycle by increasing metabolic flux (Liu and Butow, 2006). Indeed, we demonstrated that glutamate supplementation eliminated the defects in ethanol-inducible filamentous growth in the RTG pathway mutants. Additionally, cells with deficient mitochondria (ρ^0 petites) did not undergo filamentous growth under nitrogen starvation. Thus, we established that the RTG pathway is essential to ethanol-dependent filamentous growth, being its main function to stimulate the rate of flux through the TCA cycle.

Furthermore, filamentous growth involves three major regulatory changes: increase in cell length, which is mediated by a delay in the cell cycle (Kron et al., 1994), reorganization of cell polarity that involved the polarisome (Cullen and Sprague, 2002) and enhance cell-cell adhesion via the expression of the flocculin Flo11, which is essential for filamentous growth (Lo and Dranginis, 1998). Utilizing mutants lacking genes involved in these pathways, we detected that ethanol induces filamentous growth, which is controlled by the RTG pathway as above-mentioned, by a mechanism that is partly dependent on the polarized growth driven by the polarisome, fully dependent on the Flo11 induction. Phenotypic variation is common among individual strains, in fact, different genetic backgrounds are able to influence the expression and function of genes related to filamentous growth (Dowell et al., 2010). In addition, most studies are usually performed using lab strains, which frequently share the genetic background. In our study, we observed that two yeast strains (P5 and P24) exhibited an opposite filamentation phenotype in SLAD medium (Chapter 3). Thus, another aim of this thesis was to decipher the genetic factors involved in filamentous growth in wine yeast strains. To infer the natural genetic variation causing the differences detected among these two strains, a program to detect Quantitative Trait Loci (QTL) was performed (Chapter 4).

We used Bulk Segregant Analysis (BSA) to map QTL, this strategy is based on selective genotyping of two phenotypically distinct sub-population (bulks) of segregants (Michelmore and Kesseli, 1991). The two parental strains were crossed during rounds of random mating and sporulation, in order to create a F13 progeny, which was phenotyped for filamentation growth. Unexpectedly, a low segregation of invasive growth phenotype was observed (4%) in the offspring population. This result could indicate non-additivity, and inheritance of multiple epistatic alleles that can be required for filamentous growth phenotype in these strains. Considering these segregation results, four or five unlinked allele loci seem to be implicated in this phenotype. Through QTL approach, we detected nine regions which correlated with the variation in the phenotype. Different genes were located in these regions, and taking into account the function of each gene and the polymorphism exhibited between the parental strains, six of

them were chosen as potential candidates to contribute to this phenotype: *NGR1*, *ELM1*, *MEP2*, *PDH1*, *SVR2*, *YNL234W*. Most of them have been previously linked to participate in filamentous response. *Ngr1* negatively regulates invasive growth by repressing *FLO11* expression, through *Snf1*-dependent pathway, under glucose limited conditions (Kuchin et al., 2002). The deletion of *EML1* gene confers elongated cell morphology and constitutive pseudohyphal growth (Blacketer et al., 1993). *PHD1* gene encodes for a transcription factor that participates in pseudohyphal growth development (Gimeno and Fink, 1994). The gene *SVR2* encodes a adenylate cyclase-binding, which has been reported to be involved in filamentous growth induced by nitrogen starvation (Mösch and Fink, 1997), and also in Ras signaling pathway (Fedor-Chaiken et al., 1990). Finally, the gene *YNL234W* that encodes a protein of unknown function, but appears to be involved in the glucose metabolism (Kaniak et al., 2004). Nevertheless, as mentioned above, these are candidate genes, and reciprocal hemizygosity analysis is needed to determine if they are really implicated in this phenotype. Additionally, when the genome of the parental strains was compared, a deletion in the chromosome XIV of the P24 strain was revealed. This deletion includes the gene *MEP2*, which encodes an ammonium permease controlled by *NCR*, and also involved in pseudohyphal development (Lorenz and Heitman, 1998; Rutherford et al., 2008). In our segregant population, *MEP2* absence segregates perfectly with the invasive phenotype, as all non-invasive segregants lack the *MEP2*, as P24, and vice-versa. This agrees perfectly with the reported strong effect of *MEP2* on pseudohyphal and invasive growth in lab strains (Lorenz and Heitman, 1998). Thus, the segregation pattern observed in these strains could be due to the underlying segregation of *MEP2*. If this was the case, and *MEP2* could be the unique gene responsible for the differences in this phenotype between both strains, the expected segregating pattern would be 100:100. However, the 8:192 observed could only be explained by *MEP2* segregation not being neutral during the construction of the F13 pool. Pool construction involved mating, sporulation, germination, and vegetative growth, so probably *MEP2* is likely to affect one of these processes in the environment and the genetic contexts used. In order to corroborate if *MEP2* is the unique gene responsible for the defective filamentous growth in P24, new experiments using allele swapping techniques are needed. This approach is based on exchanging the alleles of the interest gene between the parental strains. By this way, we could observe if the insertion of *MEP2* from P5 reverts the phenotype in P24 strain, or still other of the candidate genes contribute to the non-invasive phenotype of this strain. In conclusion, both hypothesis (one or several genes responsible for this opposite phenotype) must be validated using the different approaches mentioned above.

4. General Discussion

In summary, during the different experiments performed in this thesis, we demonstrated that nutrient limitation can modulate the synthesis of aromatic alcohols and these compounds also are able to affect the cell growth and induce morphological changes in wine yeast, in species-specific way, verifying our postulated hypothesis: aromatic amino acid metabolism generates compounds with quorum sensing activity that modulates growth and morphological changes in wine yeast species. In addition, we also observed that the main by-product of alcoholic fermentation, ethanol, induces a strong morphogenesis in wine yeast, and established that the RTG pathway is the key to regulate this ethanol-inducible filamentous growth in nitrogen starvation conditions. Finally, we have selected solid candidate genes to be responsible for the opposite filamentous growth phenotype observed in two wine yeasts, although further analysis should be performed to elucidate the role of these genes.

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4. General Discussion

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General Conclusions

1. The synthesis patterns of tryptophol, phenylethanol and tyrosol in *S. cerevisiae* are controlled by nutrient availability:
 - (a) Nitrogen deprivation triggers elevated aromatic alcohol production, whereas sugar limitation has the opposite effect.
2. The tested non-*Saccharomyces* wine yeasts are able to produce the three aromatic alcohols, tryptophol, phenylethanol and tyrosol, although at lower concentrations than those produced by *S. cerevisiae*
 - (a) Nutrient starvation controls aromatic alcohol production.
 - (b) *S. bacillaris* produces very low levels of tryptophol, phenylethanol and tyrosol compared to other strains.
3. Compounds derived from aromatic amino acids differentially affect growth in the tested species:
 - (a) Tryptamine and serotonin only inhibit yeast growth at high concentrations, which are not common in wine.
 - (b) Aromatic alcohols are able to affect yeast growth at levels commonly found in wines:
 - i. Tryptophol reduces growth in all of the tested yeast species.
 - ii. Phenylethanol and tyrosol reduce yeast growth but primarily in non-*Saccharomyces* species

5. General Conclusions

4. Aromatic alcohols and ethanol have signalling roles, affecting yeast morphology in both *Saccharomyces* and non-*Saccharomyces*. However, these effects are strain-specific and depend on nutrient availability.
5. In *S. cerevisiae*, ethanol-inducible filamentous growth is controlled by the RTG pathway, which requires polarisome function and occurs via Flo11-dependent transcriptional induction.
6. Six genes have been identified as candidates to modulate filamentous growth in two natural wine strains: *MEP2*, *NGR1*, *PHD1*, *SRV2*, *ELM1*, *YNL234W*. All of these genes are involved in regulatory sensing pathways.

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CULTURE MEDIA

YPD: (Yeast Peptone Dextrose Medium)

Glucose	20 g/l
Yeast Extract	10 g/l
Bacteriological Peptone	20 g/l
Agar (Solid medium)	17 g/l

MM: (Minimal Medium)

Glucose	20 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
Ammonium Sulfate	10 mM

LNM: (Low Nitrogen Medium)

Glucose	20 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
Ammonium Sulfate	1 mM

SAD: (Synthetic Ammonium and Dextrose Medium)

Glucose	20 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
Ammonium Sulfate	37 mM
Agar (Solid medium)	10 g/l

SALG: (Synthetic Ammonium Low Glucose Medium)

Glucose	0.5 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
Ammonium Sulfate	37 mM
Agar (Solid medium)	10 g/l

SLAD: (Synthetic Low Ammonium Dextrose Medium)

Glucose	20 g/l
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/l
Ammonium Sulfate	50 µM
Agar (Solid medium)	10 g/l

SD: (Synthetic Defined Medium)

Glucose	20 g/l
Yeast Nitrogen Base without amino acid	6.7 g/l
Amino acids:	
Histidine	20 mg/l
Leucine	120 mg/l
Lysine	60 mg/l
Arginine	20 mg/l
Tryptophan	20 mg/l
Tyrosine	20 mg/l
Threonine	40 mg/l
Methionine	20 mg/l
Phenylalanine	50 mg/l
Uracil	20 mg/l
Adenine	20 mg/l
Agar in case of Solid Medium	20 g/l

SM: Synthetic Must (1x)

Glucose	100 g/l
Fructose	100 g/l
Citric acid	5 g/l
Malic acid	0.50 g/l
Tartaric acid	3 g/l
KH ₂ PO ₄	0.75 g/l
K ₂ SO ₄	0.50 g/l
MgSO ₂ 7H ₂ O	0.25 g/l
CaCl ₂ 2H ₂ O	0.15 g/l
NaCl	0.20 g/l
NH ₄ Cl	0,460 g/l
Total NH₄Cl	120 mg/l
YAN (mg N/l)	
<u>Amino acids: Add 10mL from stock:</u>	
STOCK Solution:	
Tyrosine	1.95 g/l
Tryptophan	17.42 g/l
Isoleucine	3.25 g/l
Aspartic acid	4.42 g/l
Glutamic acid	11.96 g/l
Arginine	36.79 g/l
Leucine	4.81 g/l
Threonine	7.54 g/l
Glycine	1.82 g/l
Glutamine	49.92 g/l
Alanine	14.56 g/l
Valine	4.42 g/l
Methionine	3.12 g/l
Phenylalanine	3.77 g/l
Serine	7.8 g/l
Histidine	3.38 g/l
Lysine	1.69 g/l
Cysteine	2.08 g/l
Proline	59.93 g/l
Total amino acids (YAN-mg N/l)	179.66 mg/l

Total YAN (mg N/l)	299.66 mg/l
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Vitamins: Add 10 ml from stock solution

STOCK 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 (from stock 100mg/l)

Oligo-elements: Add 1ml from stock solution

STOCK solution:

MnSO ₄ · H ₂ O	4 g/l
ZnSO ₄ · 7 H ₂ O	4 g/l
CuSO ₄ · 5 H ₂ O	1 g/l
KI	1 g/l
CoCl ₂ · 6 H ₂ O	0.4 g/l
H ₃ BO ₃	1 g/l
(NH ₄) ₆ Mo ₇ O ₂₄	1 g/l

**YAN: Yeast Assimilable Nitrogen*

LNM: Low Nitrogen Must (1x)

*SM modified by reducing Nitrogen content to 100 mg/l of YAN

NH ₄ Cl	0.154 g/l (YAN 40 mg/l)
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Add 3.33 ml from amino acid STOCK	3.33 ml (YAN 59.89 mg/l)
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Total YAN (mg N/l)	99.89 mg/l
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LGM: Low Glucose Must (1x)

*SM modified by reducing Sugar content to 20 g/l

Glucose	20 g/l
---------	--------

Total sugars content (mg N/l)	20 g/l
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SM_5x: Synthetic Must (5x)

*SM modified by increasing fivefold the content of aromatic amino acids (5x)
but without modifying the YAN

Amino acids: Add 10mL from stock solution:

STOCK solution of aromatic amino acids

(aaa):	
Tyrosine	17.5 g/l
Tryptophan	3.8 g/l
Phenylalanine	1.95 g/l

STOCK solution of other amino acids (aa):

Isoleucine	3.25 g/l
Aspartic acid	4.42 g/l
Glutamic acid	11.96 g/l
Arginine	36.79 g/l
Leucine	4.81 g/l
Threonine	7.54 g/l
Glycine	1.82 g/l
Glutamine	49.92 g/l
Alanine	14.56 g/l
Valine	4.42 g/l
Methionine	3.12 g/l
Serine	7.8 g/l
Histidine	3.38 g/l
Lysine	1.69 g/l
Cysteine	2.08 g/l
Proline	59.93 g/l

Add 50 ml from aaa stock solution

Add 7.55 ml from aa stock solution

Total amino acids (YAN-mg N/l)	179.66 mg/l
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Total YAN (mg N/l)	299.66 mg/l
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LNM: Low Nitrogen Must (5x)

*SM_5x modified by reducing the Nitrogen content to 100 mg/l of YAN

NH ₄ Cl	0.154 g/l (YAN 40 mg/l)
Add from aaa STOCK Solution	16.67 ml
Add from aa STOCK Solution	2.52 ml
Total YAN (mg N/l)	99.89 mg/l

LGM: Low Glucose Must (5x)

*SM_5x modified by reducing the Sugar content to 20 g/l

Glucose	20 g/l
Total sugars content (mg N/l)	20 g/l

PROTOCOLS

Yeast Transformation

Based on (Gietz and Schiestl, 2007)

LiOAcTE solution: 1.5 ml 1M LiOAc. 1.5 ml 10xTE. 12 ml H₂O

PEGLiOAcTE solution: 1.5 ml 1M LiOAc. 1.5 ml 10xTE. 12 ml PEG (50%)

1. Scrape visible amount of yeast from a plate into 5 ml YPD broth
2. Incubate at 30°C overnight with shaking
3. Transfer 400 µl of overnight culture to 10 ml fresh YPD
4. Incubate at 30°C for 4.5 hours with shaking
5. Transfer to centrifuge tubes and spin for 5 minutes
6. Drain off soup. Add 1 ml H₂O and resuspend. Spin and drain soup
7. Repeat step 6 one time.
8. Add 1 ml LiOAcTE. Resuspend. Spin for 30 seconds. Drain soup.
9. Add 180 µl LiOAcTE. Resuspend. Let sit at 30°C for 15 minutes.
10. Each tube (200 µl) is good for 4 transformations (50 µl each)
 - a. Add 1.25 µl ssDNA to each transformation
 - b. Label new tubes for each transformation
 - c. Add 50 µl of yeast/ssDNA mixture to each tube
 - d. Add 300 µl PEGLiOAcTE to each tube
11. Place tubes at 30°C for 30 minutes
12. Heat shock at 42° for 20 minutes
13. Centrifuge for 20 seconds. Remove soup
14. Resuspend in 400 µl H₂O
15. Plate (SD + aux.). Grow plate in 30° for 3 days.

The Plate Washing Assay (PWA)

Adapted from Cullen. (2015)

1. Inoculate yeast from YPD plates to minimal media [1x Yeast Nitrogen Base. 2% glucose. 10mM (NH₄)₂SO₄]
2. Incubate at 28 °C for 48 hours
3. Remove 1 OD (600nm) of cells from the precultures. Pellet the cells by centrifugation at 10000r.p.m. for 2 minutes. Resuspend the cells in sterile H₂O q.s.p. 1 OD (600nm).
4. Plate a drop of 10 µL of culture in the plate.
5. Incubate plates for 3-10 days at 28 °C in the corresponding plate (SAD. SLAD. SALG).
6. Photograph the entire colony. Examine the periphery under the microscope at different magnification and photograph.
7. Wash the plates in a stream of water. Use the same flow rate and water temperature for all plates- Wash each colony for the same period of time; do not wash more invasive colonies longer.
8. Photograph the plates by transmitted light. using a light box. to reveal details of the invasive pattern.
9. Examine the invaded cells by microscopy at different magnification.
10. Quantitative agar invasion. See Zupan and Raspor (2008).
 - a. Analyze photographs of colonies and washed plates by densitometry using ImageJ software (<https://imagej.nih.gov/ij/>). Use the invert function and subtract background values.
 - b. Examine multiple replicates and perform statistical analysis to assess whether differences in agar invasion are statistical significant.

HPLC ANALYSIS

Aromatic Alcohol Determination

o Chemicals and reagents

Acetonitrile LC-MS grade, methanol LC-MS grade and acetic acid $\geq 99.7\%$ were purchased to Sigma-Aldrich (St. Louis. U.S.A.). Ultrapure LC-MS water was obtained from a Milli-Q system (Millipore). Analytical standards of Tyrosol (TyrOH, $\geq 99\%$ Sigma-Aldrich. USA), 2-phenylethanol (PheOH, $\geq 98\%$, Sigma-Aldrich. USA) and Tryptophol (TrpOH, $\geq 99\%$, Sigma-Aldrich. USA) were used.

o Instrumental conditions

A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies. Palo Alto, U.S.A.) was used for TyrOH. TrpOH and PheOH determination. The chromatographic column was a Xbridge Shield RP18 (150mm x 2.1mm i.d., 3.5 μ m particle size) (Waters). Mobile phases were 0.2% acetic acid in water (solvent A) and ACN (solvent B). Flow rate was 0.6 mL/min. Elution gradient was 0-0.5 min 10 % B isocratic, 0.5-6 min 10-43 % B. 6-8 min 43-100 % B. 8-10 min 100 % B isocratic and 10-12 min 100-10 % B. A post run of 3 min was applied. Injected sample volume was of 2 μ L.

ESI conditions were drying gas temperature and flow of 200°C and 17 L/min respectively, nebulizer gas pressure of 35 psi. sheath temperature and flow of 350°C and 10 L/min respectively. capillary voltage of 3500V and nozzle voltage of 2000V. Acquisition was done in negative polarity for TyrOH and TrpOH and in positive polarity for PheOH. QqQ operated in multiple reaction monitoring (MRM) mode, applying a fragmentor voltage of 380V and a cell accelerator voltage of 3V. Quantitative and qualitative transitions and the corresponding collision energy are showed in Table 1 for all studied compounds.

Table 1. MRM transitions and collision energy (CE) for the studied compounds.

TYROSOL		2-PHENYLETHANOL		TRYPTOPHOL	
Transition*	CE (V)	Transition	CE (V)	Transition	CE (V)
137.06->119.1	12	105->77	20	160.07->130	12
137.06->106	12	105->103	20	160.07->142	16
137.06->93.2	20	105->51	30	160.07->128	20
-	-	-	-	160.07->116.1	16

* Quantitative transitions are in bold

o Sample preparation:

The obtained sample extracts were diluted 1:10 in MeOH. centrifuged at 15000 rpm and kept at 4°C for 10 min. Then they were serially diluted in MeOH to a final dilution rate of 1:100 for intracellular samples and 1:1000 for extracellular samples. Diluted extracts were analyzed by LCQqQ.

o Method validation and sample quantification

For the quantitative method validation. calibration curves. Linearity, precision, accuracy and limits of detection and quantification were studied. by analysis of a standard dilutions serial prepared in ultrapure LC-MS water and pooled samples spiked with standard solutions. Reproducibility was determined from relative standard deviation (RSD%) in the analysis of a spiked pooled sample. Limit of detection (LoD) was defined as the concentration corresponding to three times the signal/noise rate, and limit of quantification (LoQ) was defined as the lowest concentration giving a linear response. The obtained validation parameters of the method, which are showed in Table 2 for TyrOH, PheOH and TrpOH, allowed the quantification of studied compounds in the extract samples.

Table 2. Retention time (Rt) and method validation parameters of reproducibility, method detection and quantification limits, linearity, accuracy and determination coefficient (R^2) for tyrosol, tryptophol and 2-phenylethanol.

	Rt (min)	Reprod (%RSD. $n=10$)	LoD (mg/L)	LoQ (mg/L)	Lrity (mg/L)	Accuracy (%)	R^2
TYROSOL	2.14	4.6	0.0001	0.0003	0.5	100.5	0.9994
2-PHENYLETHANOL	3.97	5.0	0.0008	0.0025	0.5	125.4	0.9997
TRYPTOPHOL	4.52	4.1	0.0001	0.0003	1.1	99.4	0.9996

Amino Acid Determination

Aromatic amino acids were detected by the method of Gómez-Alonso et al.. (2007).

Sample preparation

- o 400 μ L of sample
- o 15 μ L of diethylethoxymethylenemalonate (Fluka, Germany)
- o 700 μ L of borate buffer (1M. pH 9)
- o 300 μ L of MeOH
- o 10 μ L of L-2-aminoadipic acid (Internal standard. 1 g/L)

Incubation at 80 °C for 2h.

Chromatograph specifications: Agilent 1100 Series HPLC (Agilent Technologies, Germany). Quaternary pump, an autosampler and a multiple wavelength detector at 269. 280. 300 nm. Nitrogen compound separation of sample (50 μ L) was carried out using a 4.6 x 250 mm 5 μ m ACE C18-HL column (Symta. Spain) with a guard column (ACE5 C18-HL) through a binary gradient:

- o Phase A: 25 mM acetate buffer pH 5 with 0.02% sodium azide
- o Phase B: 80: 20 mixture of acetonitrile and MeOH
- o Flow: 0.9 mL/min.

The target nitrogen compounds were identified according to the retention time of corresponding standards and were quantified using the internal standard method.

GROWTH DETERMINATION BY MICROPLATE READER

Pre-culture: 48 h at 28 °C in MM.

Stock of all the compounds: Melatonin, Serotonin, Tryptamine, Tryptophol, Tyrosol and Phenylethanol. were prepared at 10.000 ppm (10 g/L). From this stock solution. serially dilutions were performed to obtain the tested concentrations: 1000 ppm. 750 ppm. 500 ppm. 250 ppm. 100 ppm. 50 ppm.

1. Tryptophol, Tryptamine, Melatonin and Serotonin were pre-heated for 30 min at 70 °C.
2. After complete dissolution. the standard solutions were filtered (0.22 µm).
3. 1mL of MM (supplemented with the target compound) was inoculated. adjusting the optical density to 0.2 (OD_{600nm})
4. Microplate wells were filled with 250 µL of the inoculated medium. Avoid using the most external wells due to evaporation. Different colors in the plate indicate different compounds.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0 ppm (control)			50 ppm			100 ppm			Blank	
C		250 ppm			500 ppm			750 ppm				
D		1000 ppm			50 ppm			100 ppm				
E		250 ppm			500 ppm			750 ppm			1000 ppm	
F		1000 ppm			50 ppm			100 ppm				
G		250 ppm			500 ppm			750 ppm				
H												

5. Read microplate in POLARstar Omega microplate reader (BMG LABTECH. Germany) at 28 °C for 48h. A control well. containing medium without inoculum. was used to determine the background signal. Measurements were taken every 30 min after pre-shaking the microplate for 30 sec at 500 rpm. For each growth curve. the variable generation time (GT). growth efficiency (OD max) (Warringer and Blomberg. 2003) and lag phase (Hall et al.. 2014) were determined.

MOLECULAR TECHNIQUES

Detection of single nucleotide polymorphisms

Yeast strains were growth on liquid YPD medium for 24 h at 28 °C. DNA was extracted using the DNeasy plant minikit (Qiagen, USA):

DNA was extracted following manufacturer’s instruction which contained cell lysis, removal of RNA and other cell constituents (cell debris, protein and polysaccharide), DNA cleaning and collection. Cell lysis was performed in a mini bead-beater (Biospec Products Inc., Okla) with presence of 700 µL buffer AP1 and 1 g glass beads (0.5 mm diameter) using three times of 1 min at maximum velocity with intervals of 1 minute on ice. RNA treatment was performed at 65 °C for 10 min with 4 µL RNase A in cell suspension and other cell constituents were placed in a QIAshredder Mini spin column using 130 µL buffer AP2 in cell suspension at 4 °C. Then DNA was collected in DNeasy Mini spin column, washed by 675 µL buffer AP3 and 1 mL buffer AW and finally dissolved in 100 µL AE buffer.

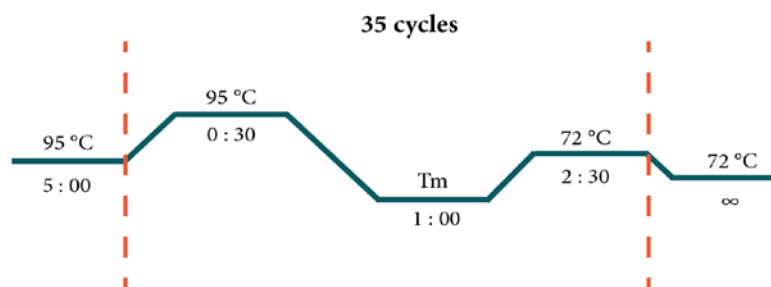
Table 3. Primers used in this thesis).

Primer	Sequence	Tm (°C)
FLO8M-F	5'-CCAGTAACGTTTGTACTCCGATT-3'	57
FLO8M-R	5'-GCGGTTCAAATGCGCAAT-3'	57
SPE1M-F	5'-GATGAACATCTCGCCGGATAG-3'	55
SPE1M-R	5'-GAACCGACGTGGAAAGAAACA-3'	55
STE12M-F	5'-GAAAAGATAACAGGTAAGCACTGAAGAC-3'	55
STE12M-R	5'-CAAAGCCTTGTCCACTATTCAGATAG-3'	55
VP20M-F	5'-CCTTGGCGGCTAAATAGATGAA-3'	58
VP20M-R	5'-TCCCCTCCTAAATGCAGTACATAGT-3'	58
Flo11P1-F	5'-GTGTAGCAGGAGCGGTGGTT-3'	55
Flo11P1-R	5'-CTTTGGATGTGACTTCCGTTTCT-3'	55
FLo11P2-F	5'-CTTGCATATTGAGCGGCACTAC-3'	55
FLo11P2-R	5'-ACGCACACTATGCAAAGACCAT-3'	55
Flo11Zd-F	5'-GTACATGGAGTGGTGTCTGATG-3'	55
Flo11Zd-R	5'-TTTGAAGTTTATGCCACACAAGATG-3'	55

The PCR mix was prepared as follows:

- o 1.5 µL of MgCl₂ (50 mM)
- o 1 µL of each primer
- o 2.5 µL buffer (10x w/o Mg²⁺)
- o 2 µL mix of dNTPs (10mM each dNTP)
- o 1.25 U Taq DNA polymerase
- o 1 µL of DNA
- o H₂O milliQ sterile water. q.s.p 25 µL

o PCR program:



Gene expression by Real time-PCR (qPCR):

The expression of *FLO11* gene was determined by qPCR. using ACT as housekeeping gene. The methodology used was:

- o The RNA extraction was performed using the commercial kit RNeasy Mini Kit (Qiagen). according to the manufacturer's instructions:

1x10⁷ cells were resuspended in 350 µL of buffer RLT, and centrifuged for 3 min at 14.000 rpm. 700 µL of ethanol (70% v/v) were added and mixed by pipetting. 700 µL of the sample were transferred to an RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged for 15 sec at 10000 rpm. 700 µL of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 sec at 1000 rpm. 500 µL of buffer RPE were added to the RNeasy spin column and centrifuged 2 min at 10000 rpm. 30 µL of RNase-free water were added directly to the spin column membrane and centrifuged 1 min at 10000 rpm. The final concentration of RNA was measured in NanoDrop (ThermoFisher Scientific, USA).

- o RNA concentrations was adjusted to 320 ng/µL.
- o Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen) and Oligo (dt) 20 Primer (Invitrogen). according to the manufacturer's instructions (Applied Biosystems. USA):

1 µL of oligo-dT was added and incubated 5 min at 65 °C, and placed 1 min at 4 °C. 7 µL of the following mix were used:

- o 4 µL of buffer
- o 1 µL of dNTPs
- o 2 µL of DTT

The mix was incubated at 42 °C for 5 min, after that, 1 µL of RT enzyme was added. Final solution was incubated first at 42 °C for 50 min, after another incubation at 70 °C for 15 min.

qPCR was used for yeast quantification (Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, USA))

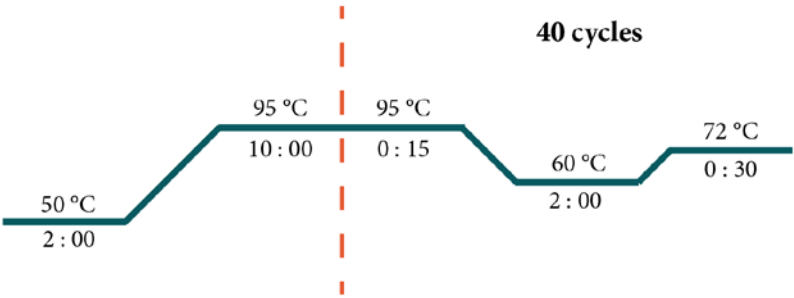
The PCR mix was prepared as follows:

- o 5 µL cDNA
- o 1 µM of each primer
- o 10 µL SyberGreen master mix (Applied Biosystems. USA)
- o H₂O q.s.p. 25 µL

Table 4. Primers used.

Primer	Sequence
FLO11-F	5'-CACTTTTGAAGTTTATGCCACACAAG-3'
FLO11-R	5'-CTTGCATATTGAGCGGCACTAC-3'
ACT1-F	5'-TGGATTCCGGTGATGGTGTT-3'
ACT1-R	5'-CGGCCAAATCGATTCTCAA-3'

PCR program:



- o The expression of *FLO11* was normalized with *ACT1*.
- o Relative gene expression was determined with the following formula: $2^{-\Delta C_t}$, where C_t of the cycle which fluorescence was determined to be statistically significant above background.

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