Analysis of genetic and physiological determinants involved in the production of aromatic amino acid derived compounds (AADC) in *Saccharomyces cerevisiae*

Ricardo Bisquert Alcaraz

Supervised by
Dr. José Manuel Guillamón Navarro

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PhD Thesis

Ricardo Bisquert Alcaraz

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supervised by

Dr. José Manuel Guillamón Navarro

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JOSÉ MANUEL GUILLAMÓN NAVARRO, Doctor en Ciencias Biológicas y Profesor de Investigación del Instituto de Agroquímica y Tecnología de los Alimentos (IATA) del Consejo Superior de Investigaciones Científicas (CSIC),

INFORMA:

Que Ricardo Bisquert Alcaraz, graduado en Biología por la Universidad de Valencia, ha realizado bajo mi dirección el trabajo de Tesis Doctoral que lleva por título "Analysis of genetic and physiological determinants involved in the production of aromatic amino acid derived compounds (AADC) in *Saccharomyces cerevisiae*". Revisado el presente trabajo, expreso mi conformidad para la presentación del mismo por considerar que reúne los requisitos necesarios para ser sometido a discusión ante el Tribunal correspondiente, para optar al título de Doctor en Biomedicina y Biotecnología por la Universitat de València.

Firmado: José M. Guillamón

Profesor de Investigación del Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC)

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INTRODUCTION

General characteristics of yeast *Saccharomyces cerevisiae*

Yeasts are eukaryotic unicellular fungi that are widely distributed in natural environments, and they have been found in association with plants, animals and insects. So far, around 1500 species of yeasts belonging to over 100 genera have been described, and this information is estimated to cover only around 1% of extant species of yeasts on earth. Among the vast diversity of yeasts *Saccharomyces cerevisiae*, also known as “baker’s” yeast, stands out for its historical relation with human activities such as elaboration of fermented food and beverages. *S. cerevisiae* belongs to *Ascomycota* phylum, as it can form ascospores to sexually reproduce, the *Saccharomycotina* subphylum, the class *Saccharomycetes*, the order *Saccharomycetales* and the family *Saccharomycetaceae*. The *Saccharomyces* genus comprises eight known species, *S. paradoxus, S. cerevisiae, S. mikatae, S. jurei, S. kudriavzevii, S. arboricola, S. eubayanus* and *S. uvarum* (Kurtzman, 2003; Peris et al., 2012; Boyton et al., 2014; Dujon et al., 2017). Furthermore, hybrids with contributions from two to four of these species have been isolated and characterized (González et al., 2008; Querol et al., 2009; Peris et al., 2012; Langdon et al., 2019).

Interspecific hybridization is a source of diversification that allows adaptation to different environments, for example, hybrids between *S. cerevisiae* and *S. uvarum* have been isolated from wine; hybrids from *S. cerevisiae* and *S. kudriavzevii* have also been isolated from wine, cider and beer; and *S. cerevisiae* x *S. eubayanus*, which is known as *S. pastorianus* have been found in lager beer fermentation (Figure I.1). Yeast cells can be pushed to undergo hybridization under controlled lab conditions to obtain new strains that combine desired traits from parental strains. These artificial hybrids can be of special interest as they can offer a non-GMO way to obtain robustness and different desired traits in an industrial environment, such as cryotolerance, good ethanol tolerance and unique metabolite production profiles that impact on flavor, texture and bouquet in wine. Such hybrids are often infertile and they only reproduce by budding (Belloch et al., 2008; Gibson et al., 2013; Boyton et al., 2014; Peris et al., 2014; García-Ríos et al., 2019; Su et al., 2019; Lairón-Peris et al., 2020).

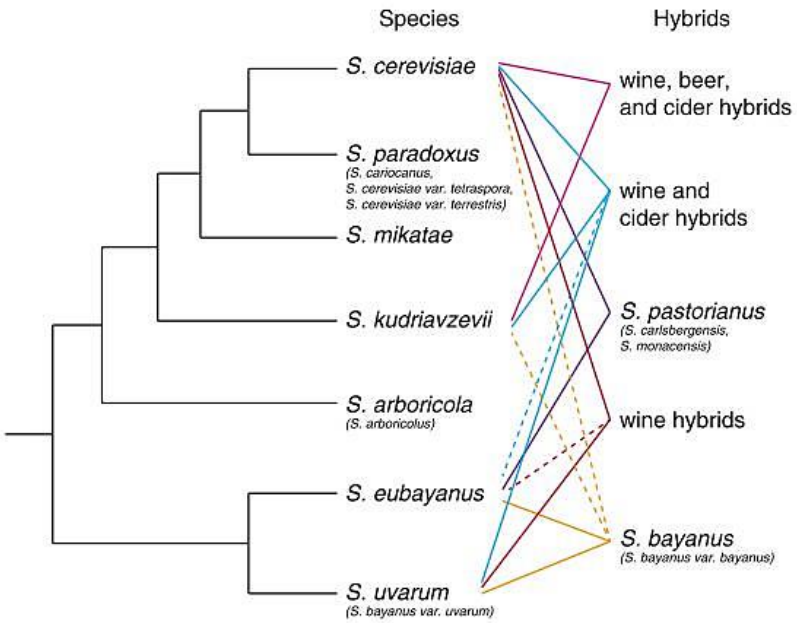

Figure I.1. Phylogenic relationship among Saccharomyces species and frequently isolated hybrids**.** Dashed lines represent introgressions from a third or fourth species into a hybrid. Synonyms are given in parentheses below species names (Boyton and Greig, 2014).

Life cycle of *Saccharomyces cerevisiae*

S. cerevisiae can present two different mating types in haploid form depending on their mating locus (*MAT*) genotype, mating type **a** and **α**. Both haploid cell types can reproduce asexually through vegetative multiplication as they are only capable to mate with the opposite cell type. Each mating type encodes a G protein-coupled receptor which recognizes the pheromones from the other mating type when they coexist in the same environment, being Ste2 the receptor coded by the *MAT*a cells and Ste3 the one coded by the *MAT*α cells (Madhani, 2007) (Figure I.2). Recognition of pheromones by their respective receptor triggers the formation of “schmoos” and mating occurs resulting in a diploid *MAT*a/*MAT*α cell (Duina et al., 2014). Diploid *MAT*a/*MAT*α cells can reproduce asexually by forming buds, but under starvation conditions they undergo meiosis and sporulation, which originate an ascus containing four spores, two *MAT*a and two *MAT*α, and these spores will germinate in four haploid yeast cells, and therefore closing the life cycle.

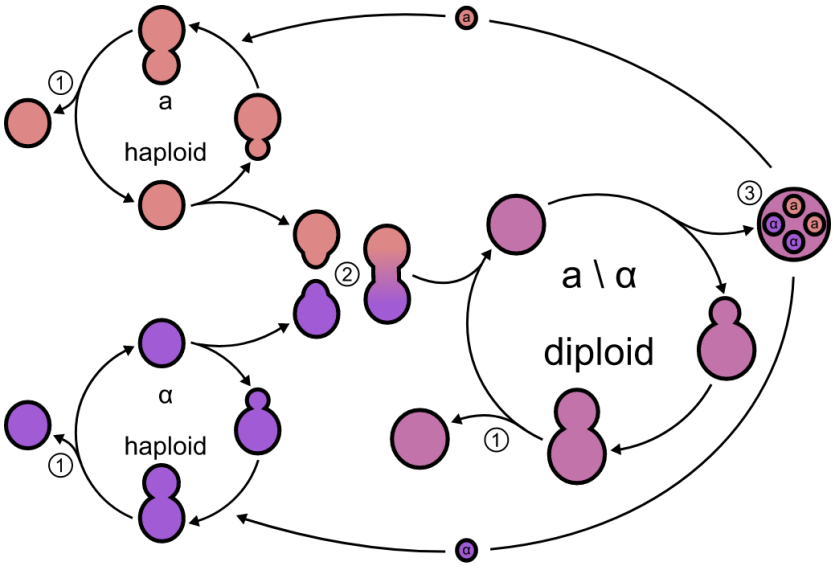

Figure I.2. A budding yeast cell cycle representation. Haploid cells (*MAT*a or *MAT*α) undergo mitotic division through budding (1). The two cell types release pheromones, initiating the formation of schmoos and subsequent mating (2), resulting ultimately in a stable diploid *MAT*a/*MAT*α (a/α cell). Diploid cells can also undergo mitotic division producing genetically identical daughter cells or, under nutrient limiting conditions, undergo meiosis forming asci (3) containing four haploid spores, which can germinate into two *MAT*a cells and two *MAT*α cells.

In *S. cerevisiae*, *MAT* locus is flanked by two other loci *HML* and *HMR* that bear a transcriptionally silent version of *MAT*α and *MAT*a genes, respectively, and all three loci are flanked by identical sequence regions called X and Z. When endonuclease *HO* is expressed, it cleaves the *MAT* locus creating a double strand break (DSB) and, guided by homology at X and Z regions, the cell uses *HML* or *HMR* as a template for DNA repair with a clear preference for the silent locus with the opposite mating type to the current *MAT* genotype (Figure I.3). This process is known as mating-type switch and allows a haploid population to become diploid by mating with their own mitotic daughter cells (Hanson et al., 2017). This process can only take place in *HO* expressing strains, also known as homothallic strains, alternatively, heterothallic strains don't have a functional *HO* gene so they cannot switch the mating type and become diploid, but they're still able to mate with cells with the opposite mating type when mixed together (Nasmyth, 1982; Katz Ezov et al., 2010). This is the case of most laboratory strains and many natural isolates that remain haploid after culturing them.

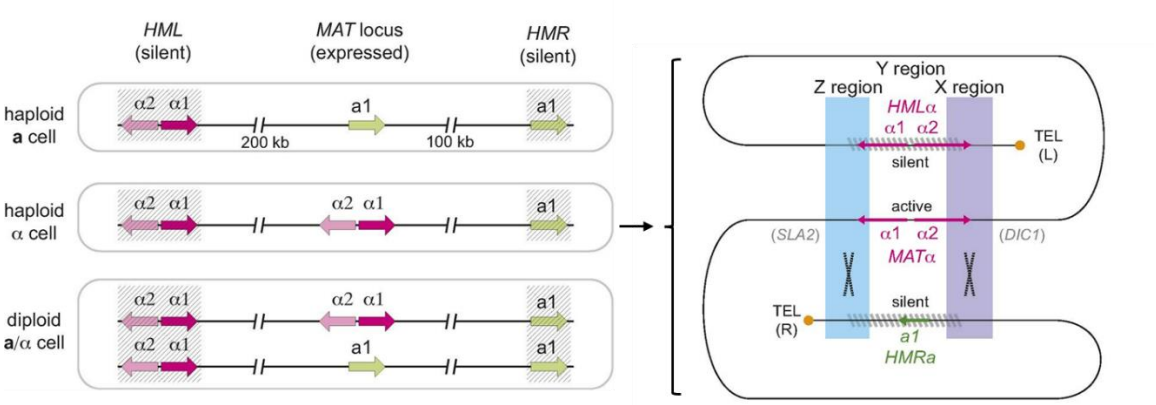

Figure I.3. MAT locus organization and mating-type switching in *S. cerevisiae* from a haploid MATα cell to MATa where HO endonuclease is required to initiate the process. During this event the genetic homology at X and Z regions allow homologous recombination to repair the double-strand break caused by HO endonuclease. Information at HMRa is used to replace the MATα allele at the active locus with the MATa allele. (Hanson et al., 2014)

As a budding yeast *S. cerevisiae* uses the vegetative life cycle when environmental conditions are favorable, regardless of its ploidy. In each round of division, mother cells bud to give rise to a new daughter cell in a nonsymmetrical division where mother cell remains 2/3 times larger than daughter cell. This budding process is tightly linked to cellular phase as unbudded cells are in G1 phase (before DNA replication), cells with small buds are in S phase (DNA replication), and cells with large buds can be in G2 or mitosis (Piekarska et al., 2010; Hanson, 2018). Only when cells are under severe nutrient limitation (nitrogen starvation and non-fermentable carbon sources) diploid cells start meiosis and sporulation process. Sporulation is a complex process in which expression of more than 1000 genes are modified trough a transcriptional cascade (Piekarska et al., 2010; Neiman, 2011).

Yeast growth kinetics

S. cerevisiae's population growth as the result of cellular division will take place in a specific fashion regarding cell density across time, following five differentiated phases: lag phase (λ), exponential phase, diauxic shift, stationary phase and death (Figure I.4). Yeasts will follow these growth kinetics under favorable conditions in which energetic carbon source is the limiting factor. As a facultative anaerobe, *S. cerevisiae* is able to grow in absence of oxygen (O_2), but only in a

nutrient rich growth medium that also comprises sterols and unsaturated fatty acids (anaerobiosis factors) which require O_2 for their synthesis.

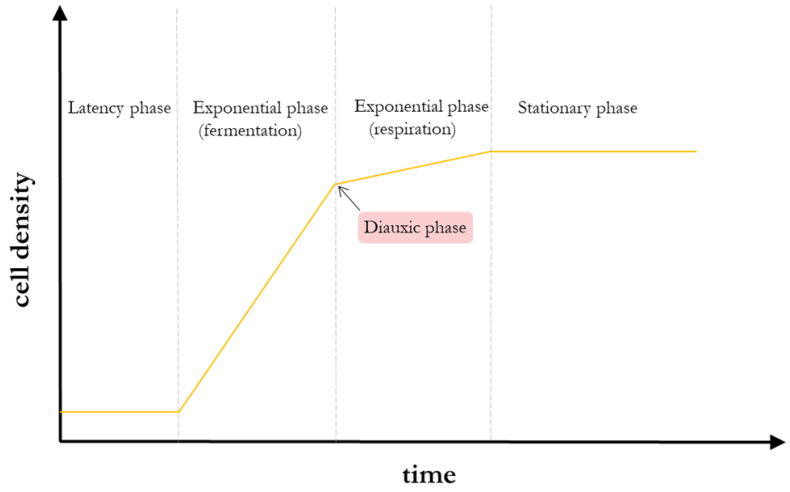

Figure I.4. Representation of a S. cerevisiae yeast growth curve.

The lag phase corresponds to an acclimation process to a fresh growth medium where cells, although metabolically active, they are not yet generally multiplying but preparing enzymatic machinery for growth and vegetative division. Its duration may vary depending on the initial cell density, growth media composition, the strain's characteristics and other growth conditions such as temperature (Maier, 2009).

The exponential or log phase covers the period in which population reaches its maximum specific growth rate (μ_{max}). The growth rate is an inverse function of generation time (GT), which is the time a culture needs to double, and provides information about how fast a population can grow. Together with other parameters it is useful to determine phenotypic properties of a strain (Hall et al., 2014). Growth rate (μ) depends on the strain, but also in the surrounding environment, *i.e.* media composition and growth conditions such as temperature or aeration conditions. Yeast growth can be modelized by the equation:

$$N = N_0 e^{\mu t}$$

where N represents the number of cells at any time (t), and N_0 represents the initial population (Maier, 2009).

During population growth, when concentration of glucose decreases and O_2 is available, cells may utilize the ethanol produced during glucose fermentation as

a carbon source, and they oxidize it to carbon dioxide. This change from a fermentative metabolism to an aerobic use of ethanol is called diauxic shift.

When the carbon source is insufficient to support population growth, cells enter in a stationary phase. In this phase cells divide at the same rate that they die, so population becomes dynamically stable. Cells can survive in this phase for long periods of time using other lysed cells as nutrients (endogenous metabolism) (Maier, 2009; Galdieri et al., 2010). When this source of nutrients is exhausted population enter a death phase in which they gradually reduce cell density.

Yeast as a model organism and its industrial importance

In the field of scientific research yeast serves as a model organism to study eukaryotic cell properties as it shares many common key cellular processes with human cells. Its relatively small genome was the first eukaryotic genome to be fully sequenced (Goffeau et al., 1996) which inspired the concept of the *Saccharomyces* Genome Deletion Project and, with great cooperative effort, it was carried out with the objective of elaborating a genome-wide yeast mutant collection in which every open reading frame (ORF) was individually substituted by a “deletion cassette” that contained a drug resistance gene marker and synthetic genetic “bar codes” (Winzeler et al., 1999). The availability of gene deletion and overexpression libraries, and research infrastructures such as *Saccharomyces* Genome Database (SGD), favor the extended use of this organism as a model (Botstein et al., 2011). Early analyses of the genomic sequence revealed a high proportion of human homologs in *S. cerevisiae*. In fact, it presents conserved genetic traits that allowed to replace 47% of the yeast coding sequences with human orthologs, showing more than 80% of replaceability for genes related to lipids, cofactors and vitamins, carbohydrate and amino acid metabolism (Mell et al., 2003; Kachroo et al., 2015). Conservation of many key transduction processes between yeast and human also indicated conserved protein-protein interactions, regulation hierarchies and signal crosstalk (Nielsen, 2019a; Dahiya et al., 2020). *S. cerevisiae* can also be useful in the discovering of target and off-target effects of specific compounds in the area of drug discovery and development. It is also used as a model for fungal pathogenesis and as a valuable test bed to develop treatments, despite of not

being a threatening pathogen (Goldstein et al., 2001; Parsons et al., 2006; Hanson, 2018).

Aside from being the driving force behind the traditional elaboration of alcoholic beverages and fermented foodstuffs (e.g., wine, beer, sake, cider, and bread), and constituting an important model organism for the study of other eukaryotic organisms, it has also proven to be a suitable industrial platform for the production of a large portfolio of value-added chemical building blocks, bioethanol, vaccines, and therapeutic proteins (Otero et al., 2013). This is due to a series of characteristics such as a rapid growth, easy cultivation, the ease of genetically engineering and the abundance of previous data on genetic sequences and its physiology and metabolism. Regarding the ease of genetically manipulate yeast, its preference for homologous recombination (HR) over non-homologous end joining (NHEJ) for double stranded break (DSB) repair allows for site-specific installation of genetic material and genomic editions (Nielsen et al., 2013; Hanson, 2018). Furthermore, compared to *E. coli*, *S. cerevisiae* has Generally Recognized as Safe (GRAS) designation from the U.S. Food and Drug Administration (FDA) and is a robust, endotoxin-free microbial host strain (Guo et al., 2019). These advantages make yeast a good platform to industrially produce multitude of compounds such as higher alcohols, enzymes, fatty acids and amino acids, among others from a sustainable approach as the use of different fermentable feedstocks from by-products of other processes or from simple and inexpensive media substrates (Hansen et al., 2009; Nandy et al., 2018; Yu et al., 2018 a; Cordente et al., 2019; Hu et al., 2019; Levisson et al., 2019; Nielsen, 2019 b).

Yeast metabolism in alcoholic fermentation

One of the most notorious examples of how yeast historically affected food industry is through the elaboration of fermented beverages, and particularly wine. It can be traced back to at least year 5000 BC, but it was not until 1860 that Louis Pasteur detailed yeast implication in the process of alcoholic fermentation in wine. In this complex process grape must is transformed into wine as the result of microbial metabolism in which alcoholic fermentation (AF) by yeast plays a major role. As a biochemical process, winemaking consists in the conversion of sugars

present in must into ethanol, carbon dioxide and other metabolites, but the process comprises the sequential development of microbial species of fungi, yeasts, lactic acid bacteria and acetic acid bacteria. Microbiome composition of wine during fermentation has different sources, and it changes throughout the process until *Saccharomyces cerevisiae* invariably dominates the final stages due to its fermentative behavior, ethanol tolerance and sulfur dioxide (SO_2) resistance (Pretorius et al., 1999; Fleet, 2008; Bagheri et al., 2015). At the initial stage of the AF the sudden availability of nutrients from the crushing or pressing of the grape favors epiphytic microbiota of the grape berries to thrive along microorganisms already present in the equipment and winery environment. Medium conditions rapidly become anaerobic due to the characteristics of fermenting containers. Regarding nutrient composition of must, there is a high sugar availability, and it also contains various sources of nitrogen in the form of amino acids and ammonium in addition to lipids, minerals and vitamins, which are essential for yeast growth but they are more limiting, as it is the case of thiamine (Labuschagne et al., 2021). To circumvent this problem, winemakers often add specific yeast nutrients to avoid stuck or sluggish fermentation and SO_2 to prevent the excessive development of grapes' indigenous microbiota. Wine yeasts are able to metabolize more than 20 substances as unique sources of nitrogen for growth. However, yeasts prefer some nitrogen sources over others under conditions of alcoholic fermentation, and hierarchies for their utilization have been proposed. Ammonium, glutamine, glutamate, and asparagine are the sole nitrogen sources supporting a high specific growth rate and are considered good or preferred nitrogen sources (Gobert et al., 2019; Gutierrez et al., 2016; Su et al., 2020).

During the AF process yeast undergoes the different growth phases described above and the fermentation activity can be monitored as the rate of release of CO_2, yeast assimilable nitrogen (YAN) and sugars consumption and cell viability ratio. In the lag phase, that can last from a few hours to several days depending on the different conditions of temperature, oxygen availability or the initial inoculum, cells deal with the stress conditions they are recently subjected to, such as acid and osmotic stress. The fermentative activity is low in this stage and cells essentially synthesize the ribosomes and enzymes necessary for the next steps

while consuming very few nutrients. In the next growth phase population grows exponentially and consume nutrients at a high rate until nitrogen depletion causes the growth arrest and thus entering stationary phase (Tesnière et al., 2015) although an early growth arrest can also occur by lipid or vitamin deficiencies. At the end of the exponential phase about 1/3 of the total sugar has been consumed and the fermentation rate has reached its maximum. The rest of the sugar fermentation occurs during the stationary phase at a slower pace, making the survival capacity and the vitality of yeast during these stages a crucial for successful winemaking (Aranda et al., 2019).

In the stationary phase autophagic processes are triggered by nitrogen starvation. Such events cause the release of cell constituents, providing a last source of nutrients for maintaining energy levels and ensuring amino acids homeostasis (Alvers et al., 2009; Huang et al., 2015). Although glycerol synthesis, reactive oxygen species (ROS) detoxification, the degradation of damaged proteins promote cell longevity, the accumulated concentration of ethanol, acetate, acetaldehyde and ROS in the final stage of fermentation shorten the life span of yeast cells forcing them to enter into the death phase (Orozco et al., 2012a; 2012b).

After the AF in wine making a malolactic fermentation (MLF) usually takes place, which is carried out by lactic acid bacteria (LAB), which are microaerophilic Gram-positive bacteria that decarboxylate L-malic acid to L- lactic acid. MLF takes place mostly in red wines but it is not common in white wines, except for the production of certain styles of Chardonnay and sparkling wines. MLF confers wine stability (lowering the risk of gas production in bottled wine due to unexpected microbiological activity), as well as more aroma, flavor and complexity and it lowers acidity (Pretorius, 2016). Wine fermentation is a special complex mix of chemistry, biology and culture, where microorganism composition plays a critical role (Belda et al., 2017). The use of selected yeast in combination with selected LAB is a great resource to promote homogeneity in the wine production over the different seasons and to avoid an incomplete AF or MLF that can originate defects in the final product such as, sugar residues, spoilage of wines due to residual malic acid, off-flavors, off-odors and the production of unwanted biogenic amines, that would translate in significant economic losses in wine industry products

(Betteridge et al., 2015; Pretorius, 2016; Berbegal et al., 2018). In this context, the cultivation, selection and purification of different yeast strains with particular and unique characteristics and their use as starter inocula in winemaking, modern bakery and brewing provide the microbiological tools to achieve quality and homogeneous productions. Thus, the inoculation of starter strains of *S. cerevisiae*, in the form of active dry yeast (ADY), is a common practice widely used in industrial fermentations.

The influence of microbiological composition to wine production does not only affect LAB and *S. cerevisiae* but also non-*Saccharomyces* strains. Originally considered as an unimportant or even spoilage yeasts during wine production, many positive contributions to desirable properties in wine, such as the decrease in final ethanol content, the increase of aroma complexity and the control of the growth of spoilage microorganisms have been attributed to some non-*Saccharomyces* strains (Comitini et al., 2011; Jolly et al., 2014; Quirós et al., 2014; Ciani et al., 2015; Alonso-del-Real et al., 2017; Bellut et al., 2019). The characterization and design of mixed yeast, or yeast and bacteria starter cultures to directly impact the final product features is a hot research topic with a great interest from the wine industry, however the determination of aromatic profiles of wines using mixed-starter cultures calls for a complete understanding of the metabolic interactions between the microorganisms in the consortia for it to be effective (Liu et al., 2017). The obtention of new yeast strains for winemaking and the design of micro-organisms consortia emerge as a response to the needs of winemaking industry as it greatly affects and alleviates the main issues encountered during winemaking, such as the excessive alcohol content concentration, low acidity and lack of aroma complexity.

As a result of AF the major components found in wine are water 86%; ethanol, 12%; glycerol and polysaccharides or other trace elements, 1%; different types of acids, 0.5%; and volatile compounds, 0.5% (Markoski et al., 2016). Wine also contains a great diversity of minor compounds derived from yeast metabolism that confer important features that directly impact on wine’s properties such as aroma, flavor, stability and texture.

Carbon metabolism in yeast

As facultative anaerobic microorganisms *Saccharomyces* yeast can grow through a fermentative metabolism by reducing sugars to ethanol, or through a respiratory metabolism in which oxygen is used as a final electron acceptor in the mitochondrial respiratory chain. Under aerobic conditions, respiration is possible with O_2 as the final electron acceptor, but *S. cerevisiae* still exhibits alcoholic fermentation until the sugar is depleted from the medium. This behavior is referred to as the Crabtree effect (Crabtree, 1928; De Deken, 1966) and the yeast expressing this trait are called Crabtree-positive yeasts. *S. cerevisiae* is a good representative of a Crabtree-positive yeast. Yeast under this category tend to channel most of the carbon flux towards the fermentative metabolism of sugar, regardless of oxygen availability. This fermentative metabolism yields two molecules of adenosine triphosphate (ATP) per molecule of glucose (Figure I.5). There are several mechanisms contributing to the Crabtree effect in this species (Barnett et al., 2005), including carbon catabolite transcriptional repression of genes required for aerobic respiration. It has been reported that slowing down the rate of sugar consumption results in a Crabtree effect alleviation (Otterstedt et al., 2004), although under high sugar concentration a high sugar uptake rate result in an excess of metabolic flux through glycolysis to pyruvate (Pronk et al., 1996). Under such overflow towards pyruvate most of the pyruvate is metabolized to acetaldehyde by the activity of pyruvate decarboxylase (*PDC1*, *PDC5*, *PDC6*). The reducing power of the NADH generated through glycolysis is then transferred to acetaldehyde as a final electron acceptor by alcohol dehydrogenases (*ADH1* and *ADH2*) to form ethanol (Pronk et al., 1996; Piškur et al., 2006).

During AF, glucose and fructose used as a carbon source are converted into alcohol and CO_2. Approximately 90 ~ 95% of sugars present in the must are converted into ethanol and CO_2, about 4 ~ 5% are converted into secondary metabolites such as higher alcohols, acids, esters and glycerol, and finally 1 ~ 2% is used for cell growth and maintenance.

As a contrast to this fermentative metabolism, “Crabtree-negative” yeasts lack fermentative products, and under aerobic conditions they generate biomass and carbon dioxide are the sole products. Crabtree-positive yeasts exhibit lower biomass production because most of the sugar is converted into ethanol. In order

to produce amounts of biomass comparable to Crabtree-negative yeasts, they would need to consume more glucose due to the lower yield in free energy, and therefore, the lower proportion of sugar destined to biomass in the process of fermentation when compared to the respiration metabolism. This difference in energetic efficiency between Crabtree-negative and -positive yeasts could result in lower growth rates in Crabtree-positive yeasts that would be outcompeted by non-fermenting ones and other microorganisms. Nevertheless, the Crabtree-positive yeasts compensate the low efficiency in energy production with a high glucose uptake rate and ethanol production. This characteristics allow them to generate a severe environmental pressure that prevents other competitor microorganisms to tolerate such stress and thrive, thus revealing an evolutionary trait controlling proliferation of possible competitors (Piškur et al., 2006; Dashko et al., 2014).

Respiration offers more energetic efficiency than alcoholic fermentation. When sugar is used by the respiratory pathway, the pyruvate originated from glycolysis undergoes an oxidative decarboxylation in the presence of coenzyme A (CoA) and NAD^+ originating acetyl-CoA and NADH and enters the Krebs cycle, also called the tricarboxylic acid cycle (TCA), where a total of 38 ATP molecules originate from a molecule of glucose. In this cycle ATP molecules and essential precursors are formed, such as α-ketoglutarate, oxaloacetate, and succinyl-CoA, required for the synthesis of amino acids and the prosthetic group heme (Noor et al., 2010; Pfeiffer et al., 2014). In this aerobic process an oxidative phosphorylation of adenosine diphosphate (ADP) is linked to the transport of electrons to an oxygen molecule that acts as final electron acceptor in the cytochromic respiratory chain in the mitochondria.

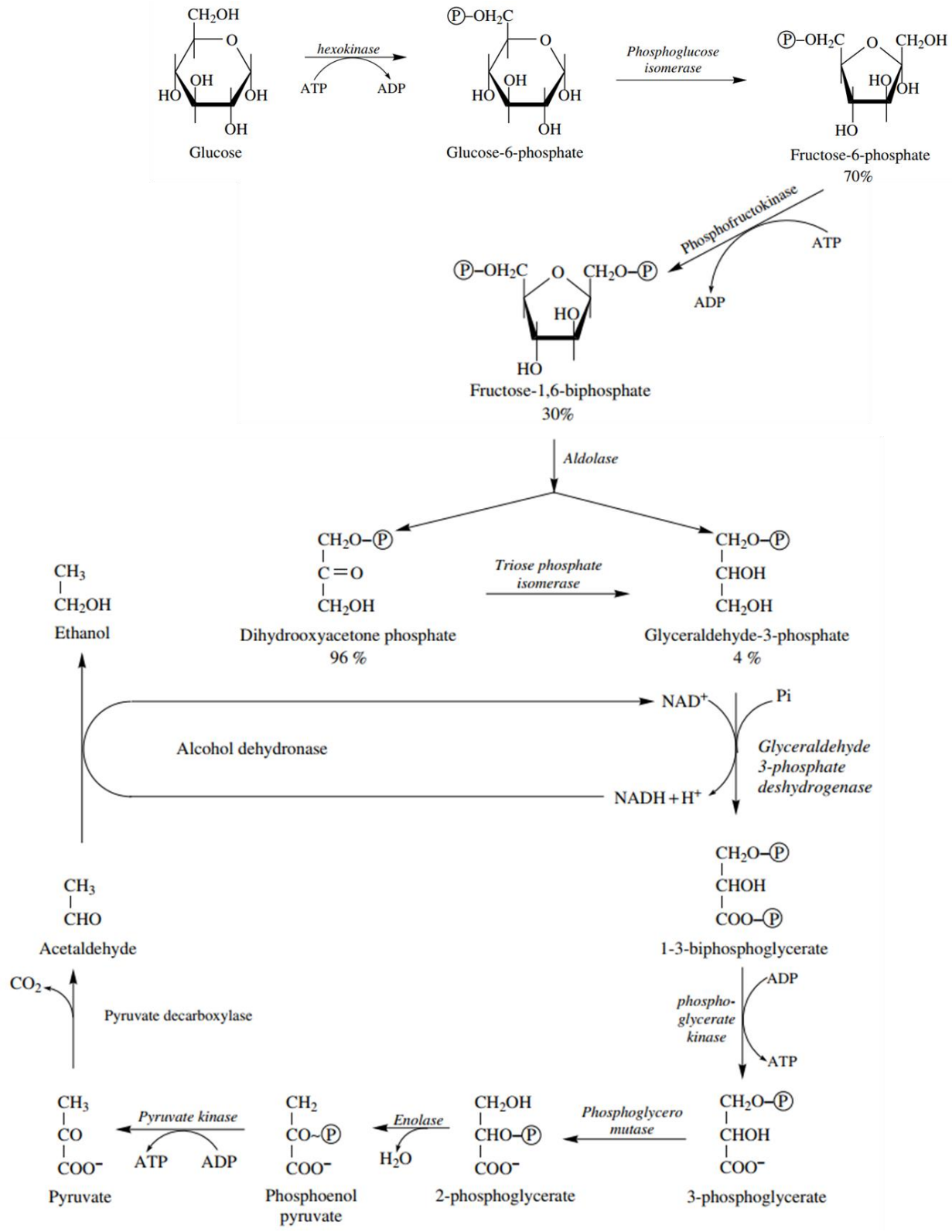

Figure I.5. Glycolysis and alcoholic fermentation pathway.

When exposed to O_2 and under a low glycolytic flux originated by the intracellular accumulation of hexoses, yeasts are subjected to the Pasteur effect, in which alcoholic fermentation is suppressed, favoring respiration. The lower affinity of pyruvate decarboxylase than pyruvate dehydrogenase for their substrate pyruvate and the inhibition of enzyme phosphofructokinase by the ATP generated during respiration are behind the occurrence of this phenomenon. Pasteur effect only affects *S. cerevisiae* under well aerated and sugar-limited chemostat cultures, or in resting cell suspensions where the sugar consumption rate is very low (Ishtar Snoek et al., 2007).

Although alcoholic fermentation and respiration are the most important metabolic pathways from an energetic point of view, they are also important as they provide the cell with the main precursor metabolites that will be used to assemble more complex molecules such as lipids, proteins and nucleic acids. To this regard the central carbon metabolism has a third biosynthetic pathway, the pentose phosphate pathway (PPP) that produces important precursors and cofactors such as nicotinamide-adenine dinucleotide phosphate, erythrose-4-phosphate and ribose-5-phosphate, which are involved in the synthesis of amino acids and nucleic acids, among others.

Yeast Nitrogen metabolism

Nitrogen as a nutrient is of paramount importance in yeast metabolism during a fermentative process, as it exerts a crucial role in a final wine's quality but also drives the pace of an alcoholic fermentation as a limiting factor. It influences the total biomass obtained and the duration of the whole fermentation process as nitrogen availability is important to avoid an early stuck or sluggish fermentation (Bell & Henschke, 2005; Gobert et al., 2019).

The main sources of yeast assimilable nitrogen (YAN) for *S. cerevisiae* are ammonium, and amino acids. Among such nitrogen sources some can be classified as "preferential" or good nitrogen sources and some others classify as "non-preferential" or "poor nitrogen sources" according to their capacity of support growth and fermentation activity although this classification may vary depending on the used strain and the growth conditions (Gobert et al., 2019). An inadequate

availability of these sources in the grape must can lead to stuck or sluggish fermentations (Varela et al., 2004; Bell et al., 2005).

Yeast has several mechanisms to import YAN present in the medium. Ammonium transporters Mep1p, Mep2p and Mep3p are uniport systems for ammonium uptake, being Mep2p the one with the highest affinity (Marini et al., 1997). Amino acids uptake occurs with different selectivity, by the action of different amino acid permeases that are active transporters. A general amino acid permease Gap1p enables the import of any amino acid whereas other permeases show more specificity. *S. cerevisiae* has developed the ability to select the nitrogen source with specificity and regulate broad-specificity permeases for a better growth through two mechanisms. The first one is the nitrogen catabolite repression (NCR) involving nutrient sensing and different transcriptional and post-transcriptional control mechanisms by which the preferred nitrogen sources can repress the uptake of non-preferred ones (Zhang et al., 2018). Ammonium permeases are not essential when high concentration of ammonium is available. They are also affected by NCR, and they are only expressed under low ammonium concentration (Marini et al., 1997). Multiple amino acid permeases (*GAP1*, *CAN1*, *PUT4*, *DIP5* and *UGA4*) and ammonium permeases (*MEP1*, *MEP2*, *MEP3*) are known to be regulated by this system (Ljungdahl et al., 2012). Expression of certain amino acid permeases can also be triggered by the response to the membrane Ssy1-Ptr3-Ssy5 (SPS)-sensor (Ljungdahl, 2009; Ljungdahl et al., 2012).

Although the imported amino acids can be used directly in biosynthetic processes yeast can also synthesize most of the amino acids necessary for constructing its proteins by fixing an ammonium ion to a carbon skeleton derived from the metabolism of sugars, e.g. deaminating them to form ammonium, or to use them as substrates for a transamination reaction that transfer an amino group to α-ketoglutarate to produce glutamate. This node in the anabolism and catabolism of nitrogen that overlaps the central carbon metabolism is constituted by α-ketoglutarate, ammonium, glutamate and/or glutamine, and they constitute the central nitrogen metabolism (Ter Schure et al., 2000; Magasanik et al., 2002). The reaction of incorporating ammonium to α-ketoglutarate with NADPH as a cofactor and generating glutamate is performed by glutamate dehydrogenase

(*GDH1*) and the incorporation of ammonium to glutamate to reach glutamine is performed by glutamine synthase (*GLN1*). The opposite direction in which glutamate is synthesized from glutamine takes place when glutamine is the only nitrogen source by the role of glutamate synthase (*GLT1*). *GLT1* accepts a molecule of glutamine and a molecule of α-ketoglutarate as a substrate to produce two molecules of glutamate by oxidizing NADH. Ammonium can also be released from glutamate to form α-ketoglutarate and NADH by a glutamate dehydrogenase (*GDH2*). These reactions of the central nitrogen metabolism endow the cell with the ability to synthesize the rest of amino acids from the amino acid glutamine or glutamate and other metabolites derived from the central carbon metabolism. The amino acids can be classified into six biosynthetic families depending on their nature and their carbon precursor (Figure I.6).

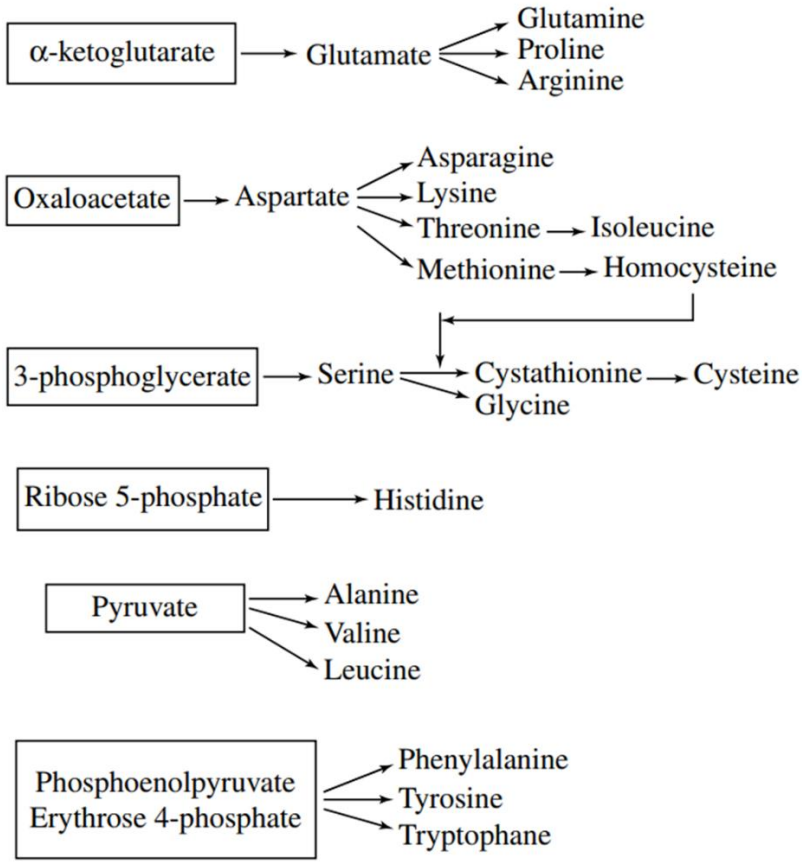

Figure I.6. General synthesis pathways of amino acids classified according their precursor.

The nitrogen metabolism on wine quality and its importance on winemaking is essential to understand the different fermentative aroma precursors in which nitrogen is involved (Styger et al., 2011). Amino acid composition of the initial

must is determinant in the flavor and aroma of the final wine, but yeast also contributes to the final aroma in different ways. There are flavor-neutral compounds in must that yeast metabolize into aromatic and flavor compounds apart from those aromatic compounds synthesized *de novo*. Ethyl and acetate esters, volatile fatty acids, higher alcohols, monoterpenoids and volatile sulfur compounds have been reported as yeast-produced aromatic compounds (Cordente et al., 2012; Gobert et al., 2019). In order to rationally modulate wine flavor and aroma, the relation between nitrogen metabolism and volatile compounds must be studied. To this regard, many different experimental conditions, including matrix, initial sugar and nitrogen concentration, yeast strain employed and yeast culture conditions before inoculation, among others, need to be tested in a standardized way to be able to observe a useful trend (Rollero et al., 2018; Gobert et al., 2019). However, there's evidence that the metabolic fate of nitrogen additions during fermentation is not proportionally related to the increase of volatile compounds such as fusel alcohols and acids and their derivatives, but rather to de novo synthesis of proteinogenic compounds (Crépin et al., 2017). Furthermore, the main α-keto acid precursors required for the synthesis of these volatile compounds derivate from the catabolism of sugars rather than the anabolism of consumed amino acids.

Yeast metabolism of aromatic amino acids during alcoholic fermentation

Aromatic amino acids (AAA), namely tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), that are part of YAN, have been reported to contribute to the formation of volatile compounds in both *Saccharomyces* and non-*Saccharomyces* yeasts (Vilanova et al., 2007; Barbosa et al., 2012; Gobert et al., 2017, 2019).

Yeast metabolism of AAA can be harnessed in order to modified the aroma and flavor of fermented products (Cordente et al. 2019). Of these aroma compounds, higher alcohols, also known as fusel alcohols, and esters are the most abundant group. There are three classes of amino acid derived higher alcohols, branched-chain, sulphur-containing and aromatic. Tryptophol (TOL), tyrosol and 2-phenylethanol (2PE) derive from the AAA L-tryptophan, tyrosine and phenylalanine respectively (Figure I.7). The metabolic process by which yeast

synthesizes these higher alcohols from AAAs entails three successive steps: transamination, decarboxylation and reduction. This degradative pathway converting an amino acid into a higher alcohol is the Ehrlich pathway (Ehrlich, 1907).

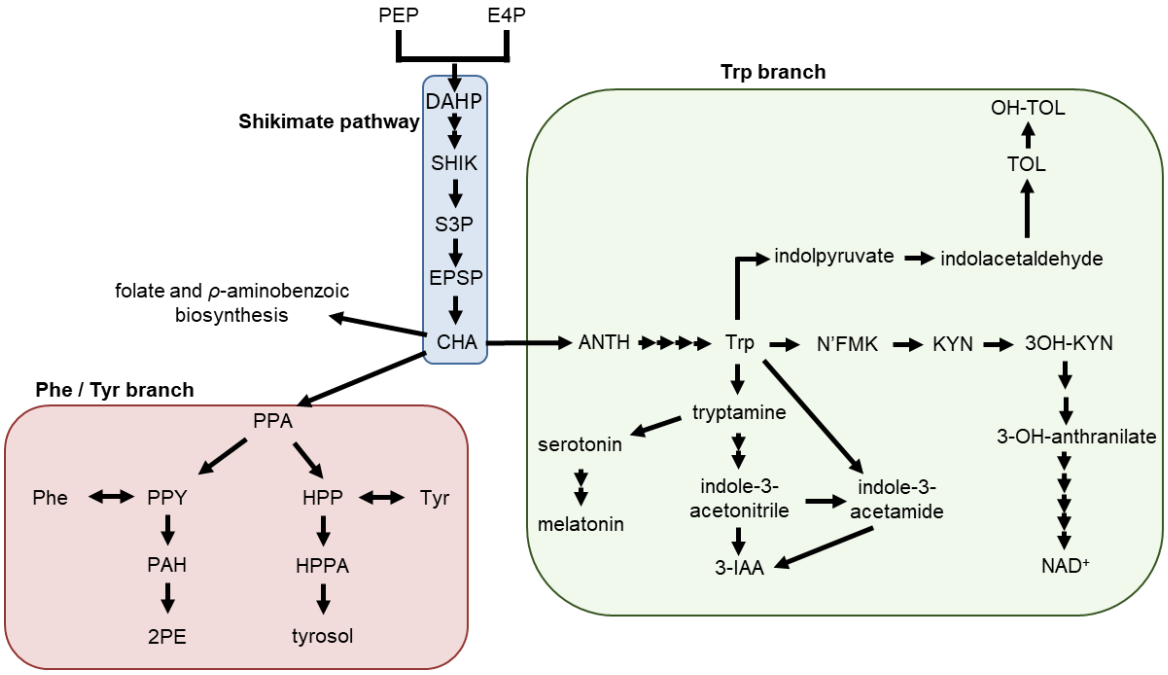

Figure I.7. Pathways associated with the metabolism of aromatic amino acids in *S. cerevisiae*. The amino acids possessing an aromatic cycle (tyrosine, phenylalanine, tryptophan) are derived from erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). These two compounds are intermediaries of the pentose cycle and glycolysis, respectively. Their condensation forms shikimate. The condensation of this compound with another molecule of phosphoenolpyruvate produces chorismate (CHA), a precursor of aromatic amino acids.

Following Ehrlich pathway reactions from the three aromatic amino acids, the first deamination reaction is accomplished by amino acid transferases (*ARO8*, *ARO9*) to generate the α-keto acids that will give raise to volatile compounds. In the second reaction a decarboxylation occurs to produce the fusel aldehydes by the action of pyruvate decarboxylase isozymes (*PDC1*, *PDC5* and *PDC6*) and the phenylpyruvate decarboxylase *ARO10*. Then, as a final step, alcohol dehydrogenases (*ADH1-6*) and the formaldehyde dehydrogenase *SFA1* reduce the aldehydes to alcohols to form 2-phenylethanol, tyrosol and tryptophol.

Synthesis *de novo* of the α-keto acid precursors can also be achieved from the metabolism of sugars, sharing the same pathway from the α-keto acid

decarboxylation step described above. The same Ehrlich reaction occurs with the other two types of amino acid-derived higher alcohols: the sulphur-containing and branched-chain. The same metabolic pathway reaching the α-keto acid precursors is also employed by yeast to synthesize *de novo* aromatic amino acids. In this pathway, central carbon metabolism connects with the shikimate pathway with the condensation of phosphoenol pyruvate (PEP), from the glycolytic pathway, and the erythrose-4-phosphate (E4P), from the non-oxidative branch of the pentose phosphate cycle, to generate 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP), which is the first of six consecutive reactions in the shikimate pathway to form chorismate (CHA), in which point the pathway diverges towards the tryptophan branch through anthranilate (ANTH) or to the phenylalanine and tyrosine branch through prephenate (PPA) (Figure I.7) (Gientka et al., 2009).

The metabolism of AAA in wine fermentation is important due to the production of aromatic fusel alcohols, which can impact the stability and aroma profile of a wine, but also due to the generation of aromatic amino acid-derived molecules that are produced in very small amounts with interesting properties and benefits when consumed (Mas et al., 2014). These molecules are known as bioactive compounds, and some of the most important yeast-derived bioactive molecules are originated from tryptophan and tyrosine.

Bioactive compounds derived from aromatic amino acids in fermented beverages

Bioactive compounds are nutrients and non-nutrients present in the food matrix that can produce physiological effects beyond their classical nutritional properties, even when present at low concentrations. Interestingly, some of the effects attributed to these molecules include protection against some of the most prevalent diseases in industrialized countries, such as cardiovascular diseases, diabetes and some types of cancer (Liu, 2013; Maruca et al., 2019).

Nevertheless, not all bioactive compounds are desirable in a fermented product for human consumption such as wine or beer as they can modify the organoleptic properties and quality. For example the presence of high concentrations of

biogenic amines, which originate from the metabolism of microorganisms through the different stages of wine production, can result in undesirable and hazardous effects such as nausea and tachycardia (Aredes-Fernandez et al., 2016; Restuccia et al., 2018).

In the complex matrix that is wine, there are hundreds of molecules present at different concentrations and among them, there are health-promoting bioactive compounds. An important amount of these interesting compounds derives directly from the grapes, and research topics have been focused mainly on polyphenols, as these bioactive compounds are released into the fermented product. Nevertheless, the role of yeast metabolism in the production of aromatic and bioactive compounds cannot be ignored. The great advances in modern analytical techniques has allowed the identification of molecules present in low concentrations and since then new detected phenolic and indolic compounds such as melatonin, serotonin or hydroxytyrosol in wines have been described and attributed to yeast metabolism during alcoholic fermentation (Rodriguez-Naranjo et al., 2011 a; Fernández-Pachón et al., 2014; Aredes-Fernandez et al., 2016; Varoni et al., 2018; Fernández-Cruz et al., 2019; Gonzalez-Ramirez et al., 2023). Furthermore, it has been demonstrated that melatonin and hydroxytyrosol present in wines are superior to polyphenols of wine in their capacity to protect DNA against oxidative stress in human central nervous system (Marhuenda et al., 2016, 2017; Gallardo-Fernández et al., 2019, 2020).

The growing knowledge about alcoholic fermentation and the microbiological and ecological aspect of wine making has allowed the improvement of the AF process to obtain wines with desired features such as lower ethanol concentration, higher organoleptic complexity, higher amounts of phenolic compounds and improving bioactive compounds concentration (Barcenilla et al., 1989; Moreno-Arribas et al., 2009; Banach et al., 2014; Contreras et al., 2014; Cordente et al., 2019).

Furthermore, the properties that emerge from yeast-derived metabolites and their impact on human health broadens their applications beyond winemaking, to functional nutrition and pharmaceutical. These yeast-derived compounds are still not fully understood, regarding their mechanisms of synthesis in yeast or their physiological determinants and importance for the producer organism, among others. The need of expanding the aromatic amino acid-derived compounds in

yeast, especially those with a demonstrated bioactive capacity, will enable the increase and modulation of these bioactive compounds in a fermentative context, but also to develop production systems to use these compounds in other applications.

Tryptophan derived compounds

Tryptophan is a non-polar aromatic amino acid that is part of proteins, and unlike mammals, yeast have the ability to synthesize it from glycolysis and PPP precursors through the shikimate pathway, as mentioned above. Its metabolism is linked to the production of a wide variety of compounds, among them, neurologically active compounds such as melatonin, serotonin, indole acetic acid, kynurenic acid and kynurenine, but also tryptophan ethyl ester, tryptophol and indole-3-acetic acid (IAA), all of which have important biological activities and health implications, and they have been detected in wine (Maslov et al., 2011; Davis et al., 2015; Tudela et al., 2016; Fernández-Cruz et al., 2017; Antonia Álvarez-Fernández et al., 2019; Palmieri, 2019; Mukherjee et al., 2022).

To the bioactivity of some of these compounds, the organoleptic impact of some compounds derived from them must be considered. For example, IAA and tryptophan are the main precursors of the off-flavor associated 2-aminoacetophenone compound in wines and beer (Palamand et al., 1974; Maslov et al., 2011; Engin, 2015).

Other properties characterize compounds like IAA and tryptophol as signaling molecules, capable to modify gene expression and drive invasivity, flocculation and pseudo-hyphal growth in yeast cells. They have been identified as quorum-sensing molecules (QSM), as their synthesis and accumulation have been observed in a cell density-dependent manner in different *S. cerevisiae* strains, although they have also been related to induction of pseudohyphae formation and increased invasive growth in non-*Saccharomyces* species. The synthesis of this autosignaling alcohols in yeast seems to be regulated by high population density, but also by nutrient availability like nitrogen limitation and/or elevated glucose (Chen et al., 2006; González et al., 2018a).

Regarding tyrosol, a negative growth affectation has been observed in a dose-dependent manner when added externally to a minimal medium or synthetic grape must in concentrations commonly found in wines revealing the relevance of this QSM to shape population dynamics in winemaking (Fernández-Cruz et al., 2018; González et al., 2018 b; Valera et al., 2019).

As a tryptophan-derived bioactive compound, tryptamine is the biogenic amine formed by the decarboxylation of tryptophan and its presence in wine has also been reported (Anl et al., 2009). The presence of biogenic amines draw attention in a consumable product as they can present undesirable toxic effects for consumers when absorbed at too high concentration (Ancín-Azpilicueta et al., 2008), although tryptamine from fermented rice bran has also been associated to exert anti-inflammatory effect (Agista et al., 2022). The synthesis of this compound has been attributed to yeasts and LAB during wine fermentation, and its metabolization into *N*-acetyltryptamine has been described for yeasts *S. cerevisiae* and *Zygosaccharomyces priorianus* (Rosazza et al., 1973).

Other indolic biogenic monoamine, 5-methoxytryptamine, can be synthesized from serotonin, 5-methoxytryptophan and melatonin. In mammals it is catabolyzed by monoamine oxidase (MAO) into 5-methoxyindole-3-acetaldehyde, which in turn can either be converted to 5-methoxyindole-3-acetic acid by an aldehyde dehydrogenase or into 5-methoxytryptophol by an alcohol dehydrogenase (Hardeland, 2010). These compounds are present in the pineal gland of mammals, and their biological activity and properties as antioxidants and anti-tumor compounds have been assessed. In fact, 5-methoxytryptamine is reportedly more powerful as a tumor growth inhibitor than melatonin or other indoles and it improves melatonin anti-cancer properties when used together (Sze et al., 1993; Lissoni, 2006). In yeast, the presence of 5-methoxytryptamine has been reported both as a metabolite product of melatonin by *N*-acetylation and as a substrate for its production, being the preferred substrate over *N*-acetilserotonin (Sprenger et al., 1999a; 1999b; Ganguly et al., 2001; Muñiz-Calvo et al., 2019).

One of the 5-methoxytryptamine precursors is serotonin, a key neurotransmitter that is involved in the modulation of several functions in humans and acts as a precursor of melatonin, through *N*-acetylserotonin synthesis, in response to

signals from the circadian clock (Engin, 2015; Olivier, 2015; Hornedo-Ortega et al., 2016, 2018 a). Serotonin presence in fermented beverages such as beers and wines have been detected as a result of LAB metabolism, but the contribution of yeast to serotonin synthesis has also been reported (Manfroi et al., 2009; Wang et al., 2014; Fernández-Cruz et al., 2019). Its synthesis in yeast has been linked to a response against UV radiation (Strakhovskaia et al., 1983), and it possesses antimicrobial properties as demonstrated previously *in vitro* (Lass-flörl et al., 2002; Lass-Flörl et al., 2003). The importance of the above-mentioned *N*-acetylserotonin, also called normelatonin, is not limited to its consideration as a precursor of melatonin as it possess its own properties according to different patterns of brain distribution in mammals (Jang et al., 2010; Sompol et al., 2011). Antioxidant and antiaging properties have been attributed to this compound together with the ability to improve cognition and protect β-amyloid-induced neurotoxicity, preserving optimal fluidity of the biological membranes (Oxenkrug et al., 2001; García et al., 2014; Álvarez-Diduk et al., 2015). It also constitutes a biomarker for autism spectrum disorders detection as the disruption of the metabolic pathway involving the reactions involved in melatonin synthesis from serotonin has been related to these disorders.

The detection of *N*-acetylserotonin in wine as a result of yeast metabolism has previously been reported (Sprenger et al., 1999 a; Fernández-Cruz et al., 2019) but there's still uncertainty about the physiological effect in yeast.

Melatonin

Melatonin, also named *N*-acetyl-5-methoxytryptamine, was originally considered a neurohormone as it was isolated for the first time in 1958 from bovine pineal gland (Lerner et al., 1958, 1959). Although the early consideration as an exclusive neurohormone in mammals, nowadays it is considered an ubiquitous molecule, with representation in organisms across many different taxa including (Tan et al., 2010; Reiter et al., 2016). Melatonin's origin can be traced back mor than 2 billion years to photosynthetic bacteria and other primitive unicellular organisms. Since this bacteria are the precursors of mitochondria and chloroplasts these are the organelles where primary synthesis of melatonin occurs in animals and plants. There are evidences that point out the initial and primary function of melatonin was to serve as a as a mechanism to cope with toxic O_2 derivatives produced

during photosynthesis (Tan et al., 2014 a; Manchester et al., 2015; Reiter et al., 2017).

Melatonin is a methoxyindole, it possesses an indole ring with two functional groups: a methoxy group at position 5 and an *N*-acetylaminoethyl group at position 3, these two side chains are decisive in the specificity of receptor binding as well as for the amphipathic character of the molecule. (Figure I.8).

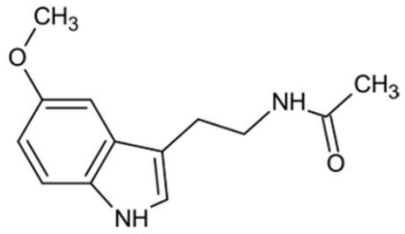

Figure I.8. Chemical structure of melatonin.

Melatonin's primitive function as an antioxidant is retained to present days as its ability to control oxidative stress response reported in multiple in vitro and *in vivo* studies (J. Reiter et al., 2000; Bonnefont-Rousselot et al., 2010; Galano et al., 2011; Pan et al., 2015). It also acts as a free radical scavenger and exerts a detoxifying action against reactive oxygen and nitrogen species.

It can also contribute to an antioxidant effect by promoting the expression of antioxidant enzymes, by synergistically improve the action of other antioxidants and increasing the efficiency of the mitochondrial electron transport chain (Antolín et al., 1996; Urata et al., 1999; Martín et al., 2000; Gitto et al., 2001; López-Burillo et al., 2003 a; Rodriguez et al., 2004; López et al., 2009). Furthermore, some of the melatonin catabolites, resulting from the oxidation of melatonin also show biological activity an antioxidant properties (López-Burillo et al., 2003 b; Tan et al., 2015; Lee et al., 2016; Galano et al., 2018; Pérez-González et al., 2018; Zhao et al., 2019).

Different other functions have been attributed to melatonin for a variety of higher organisms in animal and plant kingdoms. The importance of melatonin in the regulation of circadian rythms, cellular aging, inflammation and immunoregulation in pathological conditions and anti-tumoral effects for some type of cancer in animals have been widely demonstrated (Mediavilla et al., 2011; Escrivá et al.,

2016; Fathizadeh et al., 2019). Due to the capacity of melatonin to control innate immune system in mammals and reduce inflammation it has been proposed as a therapy for viral infections such as SARS, MERS, COVID-19, Ebola and avian flu (Tan et al., 2014 b; Anderson et al., 2015; Bahrampour Juybari et al., 2020; Reiter et al., 2020; Tan et al., 2020). In plants melatonin exerts a regulatory role in germination, rooting, growth, fruit development, maturation, parthenocarpy and senescence; and it can also act as a protection against several stresses such as drought, ultraviolet (UV) light, salinity and environmental toxins (Arnao et al., 2015, 2018, 2020; Pelagio-Flores et al., 2016; Bhattacharjee, 2018).

Production of melatonin has also been reported in unicellular organisms like dinoflagellate *Gonyaulax polyedra* where it regulates cyst formation according to photoperiodicity (Hardeland et al., 1995). *S. cerevisiae* ability to produce melatonin was first demonstrated by Sprenger et al. (1999) under laboratory conditions. Melatonin formation during winemaking process was also demonstrated as an increase in melatonin concentration was assessed during the progress of the yeast-conducted fermentation of musts with no initial melatonin in them (Rodriguez-Naranjo et al., 2012).

In yeast, although circadian behavior and physiological rhythms in response to external stimuli have been reported (Eelderink-Chen et al., 2010; Molin et al., 2020), the role of melatonin in these processes is unclear. In *S. cerevisiae*, several studies demonstrated melatonin significantly alleviates the oxidative stress generated during stationary phase as well as by different stimulators of reactive oxygen species (ROS) production such as menadione or hydrogen peroxide (H_2O_2) (Vázquez et al., 2017, 2018; Bisquert et al., 2018; Zampol et al., 2018).

It is also reported melatonin's ability to modulate cell fatty acids composition raising the ratio of unsaturated / saturated fatty acids, which is directly related to a greater tolerance against oxidative stress and may play a role in low temperature adaptation (Owsiak et al., 2010; Guillamon et al., 2011; Vázquez et al., 2018).

During winemaking process yeast are exposed to a number of stressors, each with the potential to cause cellular damage and impair fermentation performance, including sugar substrates-induced high osmolarity, increased ethanol

concentration, oxygen metabolism-derived ROS, and elevated/low temperature (Gibson et al., 2007; Auesukaree, 2017). Given that the origin of melatonin indicates that its main function is strongly related to the defense against oxidative stress, it makes sense that melatonin in yeasts, as has been seen in other organisms, could maintain this function.

Otherwise, a screening of Saccharomyces and non-Saccharomyces strains revealed that melatonin synthesis took place at the end of the exponential growth phase, and its presence during fermentation followed a zigzag pattern that appeared and disappeared (Fernández-Cruz et al., 2017; Morcillo-Parra et al., 2019 a). These results were also corroborated during grape must fermentation (Álvarez-Fernández et al., 2018). The interaction of melatonin with glycolytic proteins in yeast indicates a possible role in melatonin transport through membranes, a feature that has also been reported in mammals, where the relation between melatonin and glucose transporters and glucose metabolism was assessed (Hevia et al., 2015). These findings also support a possible role as a growth signal molecule and its production have been correlated with a yeast-growth phase (Valera et al., 2019).

Melatonin biosynthesis

Melatonin synthesis from tryptophan consists on four sequential enzymatic reactions: a hydroxylation, a decarboxylation, an *N*-acetylation and an *O*-methylation. In the mammalian model the pathway has been intensively investigated and fully characterized and the four sequential enzymes consist of: tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC), serotonin *N*-acetyltransferase (SNAT), and *N*-acetylserotonin *O*-methyltransferase (ASMT), that act in that specific order to achieve melatonin production from tryptophan following the sequence tryptophan/serotonin/*N*-acetyl serotonin/melatonin (Tan et al., 2014 a, 2016).

In plants however, up to six different enzymes are known to be involved in the conversion of tryptophan to melatonin, thus implying the existence of multiple pathways. The enzymes involved are the same four as in animals, but tryptamine 5-hydroxylase (T5H) and caffeic acid *O*-methyltransferase (COMT), would undertake the conversion of tryptamine to serotonin in the case of T5H and

serotonin to 5-methoxytryptamine or *N*-acetylserotonin to melatonin in the case of COMT (Back et al., 2016).

There is a high degree of conservation of the enzymatic reactions that lead to melatonin synthesis from tryptophan. It is the order of these reactions what characterizes the biosynthetic route in yeasts. *S. cerevisiae* seems to convert tryptophan to tryptamine in a first decarboxylation step, followed by a hydroxylation to form serotonin (Figure I.9). Then melatonin is formed from serotonin by *N*-acetylation followed by *O*-methylation of *N*-acetylserotonin or, alternatively, by an *O*-methylation of serotonin to form 5-methoxytryptamine followed by its *N*-acetylation (Muñiz-Calvo et al., 2019).

Although genes and enzymes are well characterized in higher organisms, there's still uncertainty around the responsible genes in yeast. Only the homolog of the mammalian aralkylamine *N*-acetyltransferase (AANAT), has been proposed as responsible for the N-acetylation steps in yeast melatonin biosynthetic pathway (Ganguly et al., 2001). This gene encodes a polyamine acetyltransferase named *PAA1*, with the *in vivo* capacity of acetylating spermine, spermidine and putrescine (Liu et al., 2005). Evidences supporting its role on melatonin synthesis have been reported as kinetic studies on its substrate specificity *in vitro* against multiple substrates, obtaining a higher affinity for 5-methoxytryptamine than for serotonin (Tan et al., 2016).

These results are in agreement to those obtained by Muñiz-Calvo et al. (2019), where *in vivo* bioconversion assays were conducted and melatonin production, although in low concentrations, was observed from serotonin but more intensively from 5-methoxytryptamine. The assays conducted on *PAA1* revealed its ability to accept melatonin precursors for *N*-acetylation, but this has only been observed under *in vitro* conditions, while *in vivo* function has not been unequivocally demonstrated. Further study on the role of *PAA1* is required to explain its contribution to melatonin biosynthesis in yeast under *in vivo* conditions, but also on the search of yeast gene candidates for the rest of the steps of the biosynthetic route.

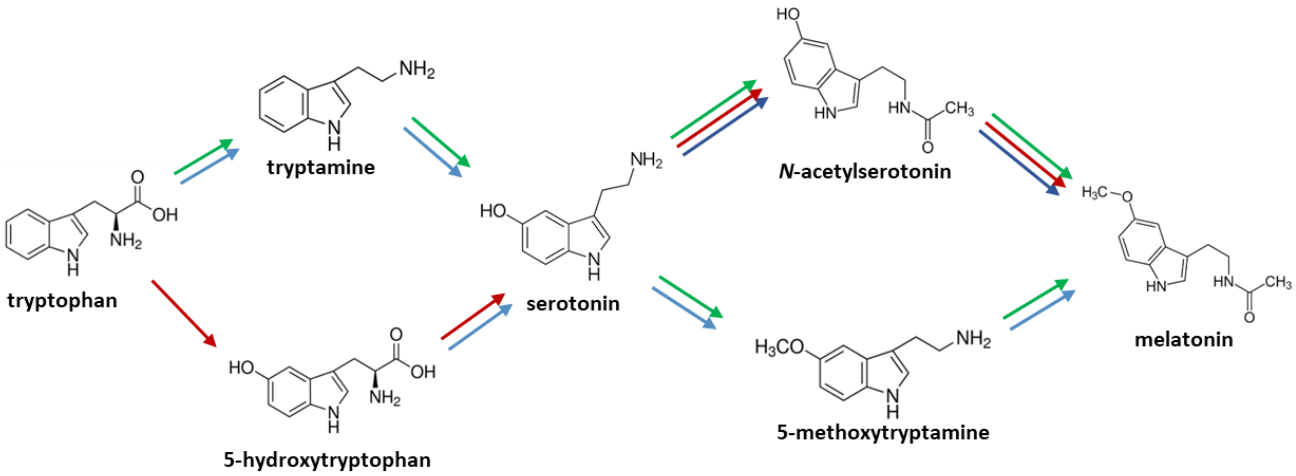

Figure I.9. Melatonin biosynthetic pathways showing formation of different indolic compounds, described for mammals (red arrows), plants (green arrows) and yeast (blue arrows).

Tyrosine derived compounds

Tyrosine (4-hydroxyphenylalanine) is one of the phenolic aromatic amino acids that influence bioactive compounds production by yeast *S. cerevisiae*. It is a proteinogenic amino acid, especially important in phosphorylation-mediated signal transduction processes as its hydroxyl group in the phenol ring is susceptible of being phosphorylated, and therefore altering the protein function in this process. In mammals, tyrosine hydroxylation results in the synthesis of L-DOPA, the precursor of dopamine, one of the most important neurotransmitters. In yeast, tyrosine is synthesized from precursors derived from the metabolism of sugars E4P and PEP. They enter the shikimate pathway generating DAHP reaching prephenate synthesis (PPA), then a prephenate dehydrogenase (*TYR1*) catalyzes the conversion of prephenate to 4-hydroxyphenylpyruvic acid (HPP), the α-keto acid that serves as the immediate precursor of tyrosine in an aminotransferase reaction catalyzed by *ARO8* or *ARO9*. As a precursor of higher alcohols, tyrosine can undergo Ehrlich degradative pathway to produce the higher alcohol tyrosol, as described above (Figure I.7). Tyrosol is produced by yeast using tyrosine as a substrate in a directly proportional manner (Garde-Cerdán et al., 2008), in turn has also be reported to undergo a hydroxylation

process to originate hydroxytyrosol as a result of yeast metabolism (Álvarez-Fernández et al., 2018).

These phenolic compounds, although not exclusively, are known to influence important characteristics in wine such as color, astringency and bitterness, and their bioactive health-promoting properties include antitumoral, antibacterial and antioxidant properties (Renaud et al., 1992; Tripoli et al., 2005; Sabel et al., 2017). Tyrosol and hydroxytyrosol are the most important bioactive phenolic compounds derived from tyrosine, and their presence in wine has been widely reported, and their synthesis in relation to yeast and other factors such as temperature and alcoholic degree have been established (Di Tommaso et al., 1998; Silva et al., 2005; Piñeiro et al., 2011; Bordiga et al., 2016; Álvarez-Fernández et al., 2018; Guerrini et al., 2018).

Tyrosol and hydroxytyrosol

Tyrosol or 2-(4-hydroxyphenyl)ethanol, is a bioactive compound with antioxidant properties when accumulated in cells although it is more stable than other polyphenols and it is less prone to autooxidation (Marković et al., 2019). Tyrosol has been described as a cardioprotective, antioxidant and anti-inflammatory agent (Muriana et al., 2017), and it was proven effective in inhibiting oxidative damage of muscle cells by inhibiting H_2O_2-induced cell death in part through regulation of extracellular kinase-mediated signal transduction and by increasing ATP production (Lee et al., 2018). It has been detected in fermented beverages such as beer, wine and sake, and related to influence final aroma and taste in such products (Silva et al., 2005; Soejima et al., 2012). It constitutes in yeast one of the QSM, together with 2-phenylethanol and tryptophol as it promotes pseudo-hyphal growth in *S. cerevisiae* (González et al., 2018 a). Tyrosol hydroxylation to produce hydroxytyrosol is achieved by the action of proteins from cytochrome p450 family in mammals (Rodríguez-Morató et al., 2017), but for yeast, although synthesis of hydroxytyrosol has been observed, the responsible proteins or genes encoding them are still unknown.

Hydroxytyrosol or 2-(3,4-dihydroxyphenyl)ethanol is naturally present in high concentrations in olive tree (*Olea europaea L.*) leaves and fruits. It is synthesized from its precursor oleuropein after a hydrolysis process that results in elenolic acid together with the hydroxytyrosol formation (Figure I.10) (Rodríguez-Gutiérrez et al., 2011). This bioactive molecule is incorporated and dose dependently absorbed in humans through diet, primarily due to the consumption of olives and olive oil (Visioli et al., 2000).

Hydroxytyrosol has been detected in wines, in much lower concentrations than those observed in olive oils, due to the microorganisms activity, but also as a result of the enzyme polyphenol oxidase present in grapes (García-García et al., 2013; Bordiga et al., 2016; Álvarez-Fernández et al., 2018; Rebollo-Romero et al., 2020). Although later studies have also reported yeast hydroxytyrosol production during fermentation (Romboli et al., 2015; Bordiga et al., 2016; Álvarez-Fernández et al., 2018; Guerrini et al., 2018; Rebollo-Romero et al., 2020).

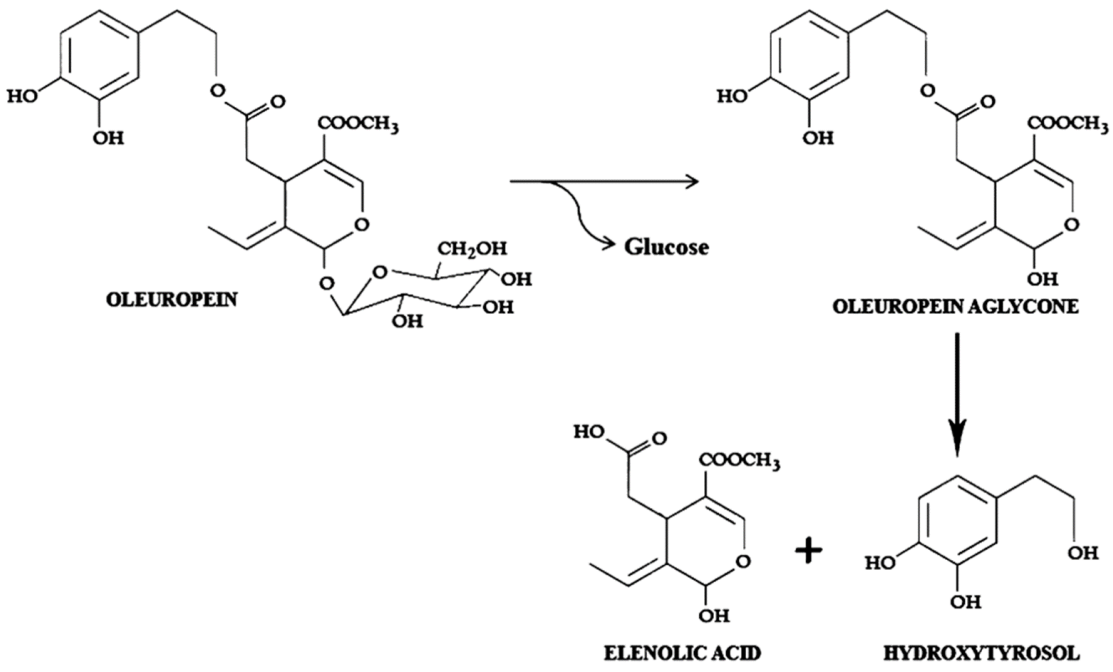

Figure I.10. Biosynthesis of hydroxytyrosol in *Olea europaea L*. (Granados-Principal et al., 2010)

Hydroxytyrosol is one of the most potent dietary antioxidants, its beneficial effects on human health encompass its great antioxidant capacity as a ROS scavenger

but also as a modulator of the stress response. It also plays an important role in anti-inflammatory processes in respiratory diseases, as a neuroprotector, skin protector, antimicrobial and anti-tumor, among others. (Boronat et al., 2018; Hornedo-Ortega et al., 2018 b; Robles-Almazan et al., 2018; Gallardo-Fernández et al., 2020). Inclusion of both tyrosol and hydroxytyrosol in the diet is recommended due to their beneficial biological activity and a high degree of bioavailability. Furthermore, a positive opinion on hydroxytyrosol consumption has been issued by EFSA, establishing a safe daily intake under 50 $mg \cdot kg^{-1}$ body weight (EFSA 2017). Most researches on the desired effect of this bioactive compound have been conducted employing a purified form of hydroxytyrosol. These commercially available format is only used for research purposes and its production prices are really high when compared to hydroxytyrosol-rich extracts obtained from olive leaves (Achmon et al., 2015), but these extracts, obtained from the by-products of olive oil industry, are far from being pure and they can vary their hydroxytyrosol or other co-extracted compound concentrations with a clear effect on any further application. Microbial produced hydroxytyrosol, and specifically yeast-produced hydroxytyrosol, raises as an interesting way to explore a cleaner, sustainable and cost-effective production of this molecule.

The presence of hydroxytyrosol in wines is not only interesting for the numerous beneficial and health-promoting properties but also for its antimicrobial effect. The possible application in the winemaking process to exploit such antibacterial and antioxidant properties rise up in a context where the use of SO_2 as a common bacteriostatic and antioxidant is widely employed to control growth of undesired bacteria during the process while preventing an excessive oxidation of wine (Santos et al., 2012). The rise in allergy-related cases caused by SO_2-derived compounds (sulfites) has led to a reduction of the maximum legal concentration of SO_2 allowed in wines and food products (Santos et al., 2012; Lisanti et al., 2019). Under these premises, hydroxytyrosol could result an interesting candidate as a food preservative with the added value of conferring healthier properties to fermented products. Under this area of application, wines have been previously treated with hydroxytyrosol extracts as a measure to reduce SO_2 concentration and obtained promising results as the hydroxytyrosol enriched extract showed a great potential to control undesired microbial growth although it

didn’t allow the complete elimination of SO_2 from the process. Despite the promising results in studies where hydroxytyrosol is employed to partially substitute SO2, especially when using hydroxytyrosol extracted from olive industry byproducts, the addition of these extracts caused a negative alteration in the olfactometric and sensory profile (Raposo et al., 2015, 2016). More studies on the antioxidant activity of hydroxytyrosol in a real wine environment are needed (Lisanti et al., 2019), as well as the use of yeast-produced hydroxytyrosol, originated by tyrosol hydroxylation rather than oleuropein hydrolyzation, and how it impacts the quality in a final wine is an exciting topic that may lead to the reduction or even elimination of SO_2 without losing its effect nor affecting the final quality.

Detection methods of bioactive amino acid-derived compounds

Tryptophan-derived bioactive compounds such as melatonin and their related metabolites and their detection from fermented foods, beverages or even yeast growth media poses a challenge as they are usually present in such sample matrices in really low concentrations, usually micrograms and nanograms per liter, while others such as tryptophol can be detected in a $mg \cdot L^{-1}$ range. Moreover, melatonin’s amphipathic nature, together with its tendency to react with other oxidant components difficult its analysis (Arnao et al., 2009; Stege et al., 2010).

The most successful techniques for melatonin detection in food are derived from separative methods. Those include gas chromatography coupled with mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) coupled with electrochemical, UV, fluorescence or diode array (DAD) detectors (Iriti et al., 2006; Mercolini et al., 2008, 2012; Muszyńska et al., 2012). Currently the most powerful method for determining not only melatonin’s concentration, but several different indolic compounds under the same run, is based on ultra-high performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS). This technology allowed to lower LOD and LOQ to unprecedented values ($LOD < 0.5\ pg \cdot L^{-1}$) for multiple melatonin-related

indolic compounds, analyzed from culture media and fermented products (Fernández-Cruz et al., 2016).

Tyrosine-derived bioactive compounds such as shikimic acid-related compounds or aromatic alcohols such as tyrosol, hydroxytyrosol or 2-phenylethanol have also been detected from fermented beverages such as wine or beer, from supernatants of growth medium and intracellular samples by the use of separative methods. The most widely used are HPLC with DAD, fluorescence or UV detectors (Proestos et al., 2005; Li et al., 2008; Piñeiro et al., 2011; Zupan et al., 2013; Lai et al., 2017; Guerrini et al., 2018; Chen et al., 2019). Although a higher stability of tyrosol and hydroxytyrosol allowed their successful detection by GC-MS after sample derivatization (Di Tommaso et al., 1998; Boselli et al., 2006; Minuti et al., 2006).

All these separative methods offer the detection of multiple compounds and the advantage of their high sensitivity when dealing with samples in which a low concentration of metabolite is expected. Nevertheless, they also bring important trade-offs. The sample preparation entails at least a chemical extraction, but it is commonly advised to prepare the samples using solid-phase extraction (SPE) cartridges prior to HPLC analyses of indoles (Fracassetti et al., 2019). The importance of choosing an adequate extraction method is crucial to be consistent on the resulting concentration inferred from the analysis, and the adaptation of the extraction method to the analyzed matrix should be considered as the use of SPE extraction might vary the results in a matrix-dependent manner (Garcia-Parrilla et al., 2009; Duportet et al., 2012; Federico et al., 2016; Vitalini et al., 2020).

In regard to phenolic compounds extraction procedures, different approaches have been followed. For liquid samples most authors use centrifugation and sample filtration before HPLC (Li et al., 2008; Piñeiro et al., 2011; Zupan et al., 2013; Bordiga et al., 2016; Marhuenda et al., 2016), although methanol (González et al., 2018 a) or ethyl acetate and diethyl ether dilution (Romboli et al., 2015; Guerrini et al., 2018) and SPE cartridges have also been employed (Di Tommaso et al., 1998; Álvarez-Fernández et al., 2018).

Other analytical methods, based on a faster, simpler and easier-to-adapt routine technique than those mentioned above draw special interest to widely detect yeast-derived samples when aiming for adapting a reliable detection and quantitation method to facilities with less access to the expensive analytical devices that require specialized facilities and trained specialists to operate them, such as research labs or wineries.

As one of the alternative rapid methods for melatonin and indolic compounds detection, a method based on voltammetry of immobilized particles (VIMP) applied directly to yeast cells constitute an important milestone achieved in the recent years (Muñiz-Calvo et al. 2017). This method has been successfully applied to monitor melatonin content in different yeast strains as well as the detection of intermediates of melatonin synthetic route. Nevertheless, sample preparation requires preparing dry yeast prior to analysis, as the measurements take place directly from dry yeast cells and its discriminating power between analytes can't compare to other chromatographic techniques.

This type of techniques also cover fast spectrofluorimetric approaches that have been applied for melatonin detection from pistachio nuts (Oladi et al., 2014). After a solid-liquid extraction assisted by ultrasound the fluorescent signal received correlates with melatonin concentration as corroborated by GC-MS. Although reaching a good detection limit (3.6 $\mu g.L^{-1}$), the major inconvenient resides in the low discriminating power of this method, allowing the co-detection of any similar fluorescent molecule. Other examples of alternative techniques which pose a high discriminating power, in contrast to the previous spectrofluorimetric-based technique, are based on the specificity of an inmunoreaction, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (Poeggeler et al., 1994; Rodriguez-Naranjo et al., 2011b). Nevertheless, RIA resulted in cases of false positives and a general overestimation of melatonin concentration when compared with a separative method such as GC-MS. Regarding ELISA, kits for melatonin detection are available, they are adapted for human biological samples, but for using them in fermented beverages, they need adaptation and further optimization (Garcia-Parrilla et al., 2009; Rodriguez-Naranjo et al., 2011 b).

Other more recent examples of rapid detection methods also rely in protein interaction to provide specificity, they specifically use human melatonin receptors to provoke a physiologically measurable response in a living cell biosensor. A method involving mammalian melatonin receptor MTNR1B and human cell lines as a sensor in a whole-cell bioassay has been recently developed. It relies on the release of a Gal4-VP16 transcription factor upon melatonin binding by the receptor that binds the upstream activation sequence (UAS) of a beta-lactamase reporter gene. When reporter gene is active, it cleaves a FRET (Förster resonance energy transfer)-enabled substrate that changes its fluorescent properties giving an unequivocal positive signal (Morcillo-Parra et al., 2019b). This method showed an extraordinary high sensitivity, even comparable to HPLC-MS/MS results reported in previous studies (Rodríguez-Naranjo et al., 2011; Vigentini et al., 2015). Nevertheless, the whole-cell bioassay requires a daily good maintenance of the cell lines before the assay and it comprises long periods of exposure to ligand. Although this method addresses the issue of the sample preparation it might not be an ideal quick, easy or inexpensive method to implement as a routine analysis.

The second method using melatonin receptors is a yeast-based biosensor. In this case a yeast cell chassis express the MTNR1A receptor and the reporter system, based on the expression of a “superfolder” green fluorescent protein (sfGFP) in response to melatonin binding. This is an attractive and promising method, as yeast confer a robust, easy to maintain or manipulate and easy to cryogenicize and revive when needed. This method harness the similarity of yeast pheromone receptors to MTNR1A as they are both G-protein coupled receptors (GPCR) and presents a modular GPCR signal transduction system implemented into a heavily engineered yeast strain to isolate the response to melatonin concentration in the surrounding media from endogenous metabolism and thus providing a response in a dose-dependent manner (Shaw et al., 2019). This method fulfills all desirable features when aiming to a rapid, cost-effective and easy to implement detection method, including a great selectivity for the substrate and no sample pre-processing. However, the limit of detection is far from what yeast endogenous metabolism is usually able to reach in terms of melatonin concentration.

Regarding hydroxytyrosol detection, two different methods for high-throughput screening of tyrosol hydroxylation have been reported (Chen et al., 2019; Yao et al., 2020). The first method is based on spectrophotometry and uses sodium periodate, an oxidant chemically converting o-diphenol to o-quinone (Weidman et al., 1966) and easily measurable by optic spectroscopy. This method offers a quick and really easy-to-adapt method for hydroxytyrosol detection with the conspicuous inconvenient of its high LOD, reaching 3.8 $mg \cdot L^{-1}$. This LOD makes this method unsuitable for detecting spontaneous yeast hydroxytyrosol production as it ranges around 0.106 mg/L (Álvarez-Fernández et al., 2018), although it can be very convenient when analyzing genetically modified hydroxytyrosol producer strains. A second hydroxytyrosol detection method consists in a bacterial whole-cell biosensor (Yao et al., 2020). This method is based on the adaption of a vanillic acid-responsive regulatory protein (VanR) from *Corynebacterium glutamicum* to hydroxytyrosol stimulus. The authors switched the induction specificity of VanR from vanillic acid to hydroxytyrosol. Modified VanR unlocks the transcription of a reporter red fluorescent protein (RFP) upon hydroxytyrosol binding. With the development of this biosensor, the authors design a strategy for a high-throughput screening tool. This method may not be applied to yeast samples without major optimization primarily due to the high LOD (>0.16 mg/L).

Genetic improvement of *Saccharomyces cerevisiae*

Yeast strains development towards industrial applications is a topic that has been fervently pursued since the use of yeast as fermentation starters in the late XVIII century. Making yeasts more capable of delivering new properties in a fermented product without having to resort to mixed ferments necessarily involves a good understanding on yeast physiology and genetics, to be able to detect and modify genetic features to obtain an improved product. This objective gained importance since the full yeast genome sequencing allowed the comprehension of genetic mechanisms underlying important cellular processes (cell division, mating, senescence, etc.) and enabled more sophisticated techniques to modify genetic characteristics in a very specific manner (Verstrepen et al., 2006).

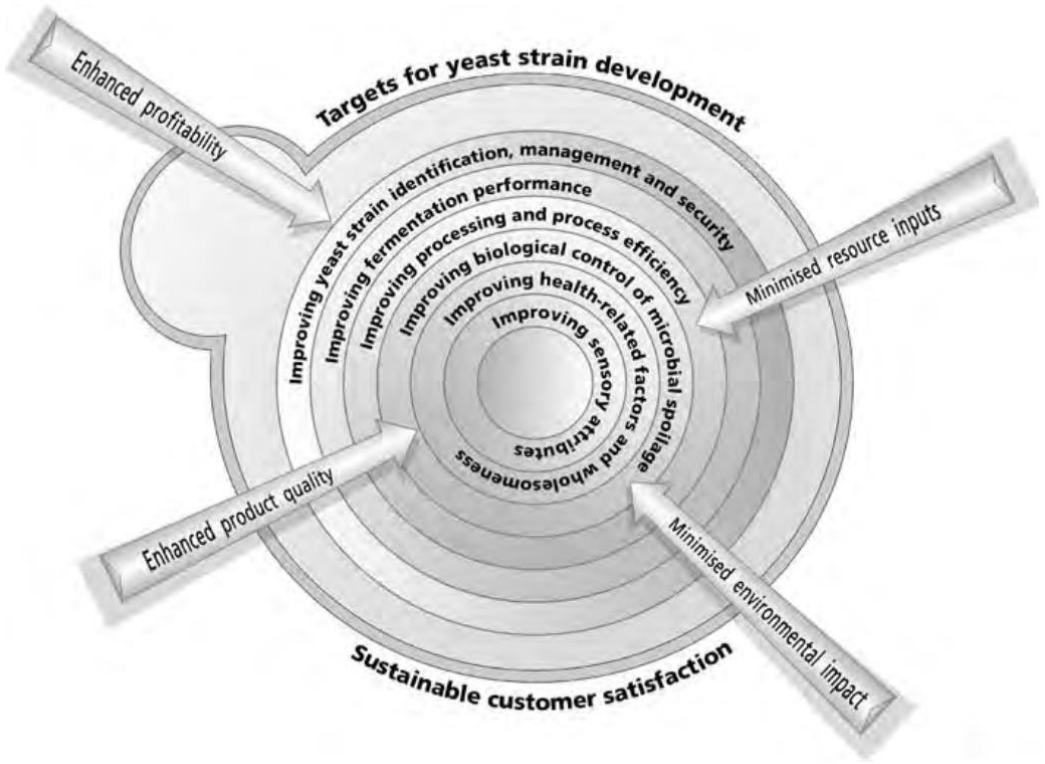

Figure I.11. Improvement objectives in industrial yeast offer a multifactorial approach where GMO and non-GMO techniques can be implemented.

Many different improvements can be made that affect yeast performance and products quality in industrial applications by means of genetic modifications (Figure I.11). From a political point of view a clear differentiation must be observed between those modifications that confer the genetically modified organism (GMO) status when applied, from those that are not considered GMO techniques. Nowadays there's a generally negative perception of a GMO-related product, and especially in Europe, the situation towards GMO utilization in food industry is very stringent. For that reason the differentiation between GMO and non-GMO approaches in yeast genetic improvements has to be considered.

Non-GMO approaches

According to European legislation (Directive 2001/18/EC), a genetically modified organism (GMO) is considered that whose genetic material has been altered in a way that can't occur naturally through mating or natural recombination using genetic engineering techniques to insert or remove genetic material from an organism. Under this classification the use of GMO is strictly regulated and its utilization faces a practical unviability in food industry in Europe.

Non-GMO techniques are employed in industrial yeast to develop new strains to confer desired properties to a final fermented product. Although methods that are not based on yeast genetic modification such as selection of strains, co-cultivations, rational nutrient supplementation, the use of SO_2, and fermentation process optimization, are commonly employed to improve the fermentation performance, the microbiological control, process efficiency and other aspects, there are other techniques directed to impact genetic and therefore phenotypic characteristics of yeast industrial strains. These techniques include random mutagenesis, intra- and interspecific hybridization and adaptive laboratory evolution (ALE), also known as directed evolution (Gonzalez et al., 2022). Hybridization have been extensively used in industrial yeasts as a non-GMO approach to combine the advantages of two different strains and often obtaining more robust characteristics in the hybrids than in the parent strains (Belloch et al., 2008; Gibson et al., 2013; Boyton et al., 2014; Peris et al., 2014; García-Ríos et al., 2019; Su et al., 2019; Lairón-Peris et al., 2020).

As another non-GMO method, ALE is also extensively used to improve yeast strains and it consists on subjecting yeast cultures to a selective pressure and over the generations increase this pressure gradually to eventually cause an adaptation. This method may or may not include causing an additional random mutagenesis by the use of mutagens such as ethylmethanesulphonate or ultra-violet light . The adaptation occurs as genomic mutations that confer a differential fitness towards a specific environment, such as growing in the presence of the selective pressure employed, accumulate and allow the gradual selection of those surviving cells when increasing the intensity of the selective pressure. Finally these genetic traits become stable and entail key phenotypical differences that may make the new strain more suitable for a specific industrial process (Aarnio et al., 1991; Matsutani et al., 1992; Takagi et al., 1997; Novo et al., 2014; García-Ríos et al., 2022; Holyavkin et al., 2023).

GMO approaches

Existing tools to modify yeast genome involves the inclusion of external DNA into the cell by a transformation process. This DNA can be delivered in linear form or cloned into a vector “backbone” that serves the purpose of expressing and

maintaining any desired genetic feature. The simplest type of DNA modification is based on the *S. cerevisiae* convenient ability to undergo homologous recombination when facing a DNA double-strand break, but this event also takes place randomly. When provided with a linear DNA fragment flanked by sequences that are homologous to the region where the edition must be made, it will be inserted in that specific region as a random event. Using this approach every ORF in the genome has been substituted by a selection marker cassette conferring antibiotic resistance in the *Saccharomyces* Genome Deletion Project (Giaever et al., 2014).

In *S. cerevisiae* there are multiple plasmids used to express heterologous genes where a DNA fragment encoding a gene is cloned into the vector by any of the different available cloning methods. These plasmids, which are commonly maintained and replicated in *Escherichia coli* and finally transformed into *S. cerevisiae* are called “shuttle vectors”. According to their mechanism of replication and their fate into the yeast cell plasmids can be classified as yeast episomal plasmids (YEp), yeast centromeric plasmids (YCp) and yeast integrating plasmids (YIp) (Mumberg et al., 1995; Da Silva et al., 2012; Gnügge et al., 2017). YCp and YEp are autonomously maintained in the cell in low and high-copy numbers, respectively. For their self-replication, YCp contain the autonomously replicating sequences (ARS) and centromeric (CEN) sequences whereas YEp contain a replication origin (ORI) and a *cis*-acting sequence (STB) which are derived from the natural yeast 2μ plasmid (Gnügge et al., 2017). YIp, on the contrary, don’t possess a replication origin and therefore cannot be autonomously maintained unless they integrate into chromosomes, and once integrated they are replicated and transmitted to successor cells as part of a chromosome (Da Silva et al., 2012; Gnügge et al., 2017).

The great variety of available plasmids has allowed the development of multiple ways to vary a gene’s expression, for example. Depending on the final objective a type of plasmid might be more convenient than other as varying the copy number of an expressed gene will affect the intensity of the expression, being generally more intense as the copy number raises, but this approach can yield less expression stability (Jensen et al., 2014; Ryan et al., 2014; Lee et al., 2015). On the other hand, the most stable expression is obtained with genomic

integration of the desired gene. However, to obtain higher levels of expression with genomic integrations, it is necessary to integrate multiple copies of each gene. To achieve this goal, the integration of the gene of interest targeted to genome repeated sequences such as rDNA, Ty and δ sequences can provide a multiple integration of the desired sequence in a single transformation to achieve a stable and high expression (Lopes et al., 1989; Parekh et al., 1996; Maury et al., 2016; Semkiv et al., 2016).

Based on this technology, current genome-editing tools such as CRISPR-Cas9, has enabled the seamless and completely directed yeast genome editing. This technology is based on the expression of programmable nucleases. As examples of programmable nucleases, zinc finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs) also stand out, but CRISPR-Cas9 is the most used editing tool due to its versatility and the fully customizable targets (Kim, 2016; Vigentini et al., 2019). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is based on the anti-viral defense mechanism from bacteria *Streptococcus pyogenes* where a ribonucleoprotein targets and cleaves the recognized DNA inducing a double-strand break (DSB). This ribonucleoprotein is formed by a guide-RNA, bearing a “target” sequence also named protospacer followed by a constant and short spacer (together named crRNA) and its complementary “tracrRNA”, and a Cas9 nuclease. In yeast, the expression system of a single guide RNA (sgRNA) is usually transcribed as a single sequence bearing crRNA + tracrRNA. When assembled and expressed, Cas9 binds to guide-RNA which hybridizes with the complementary target sequence in the genome and provokes a DSB 3 nucleotides upstream of the 3’ end of the target sequence. This target sequence (protospacer) always locates next to the protospacer adjacent motif (PAM), consisting of 3 nucleotides immediately downstream of the 3’ end of the strain that doesn’t hybridize with the guide-RNA (Figure I.12). The PAM sequence is nuclease-dependent, being NGG in the case of Cas9.

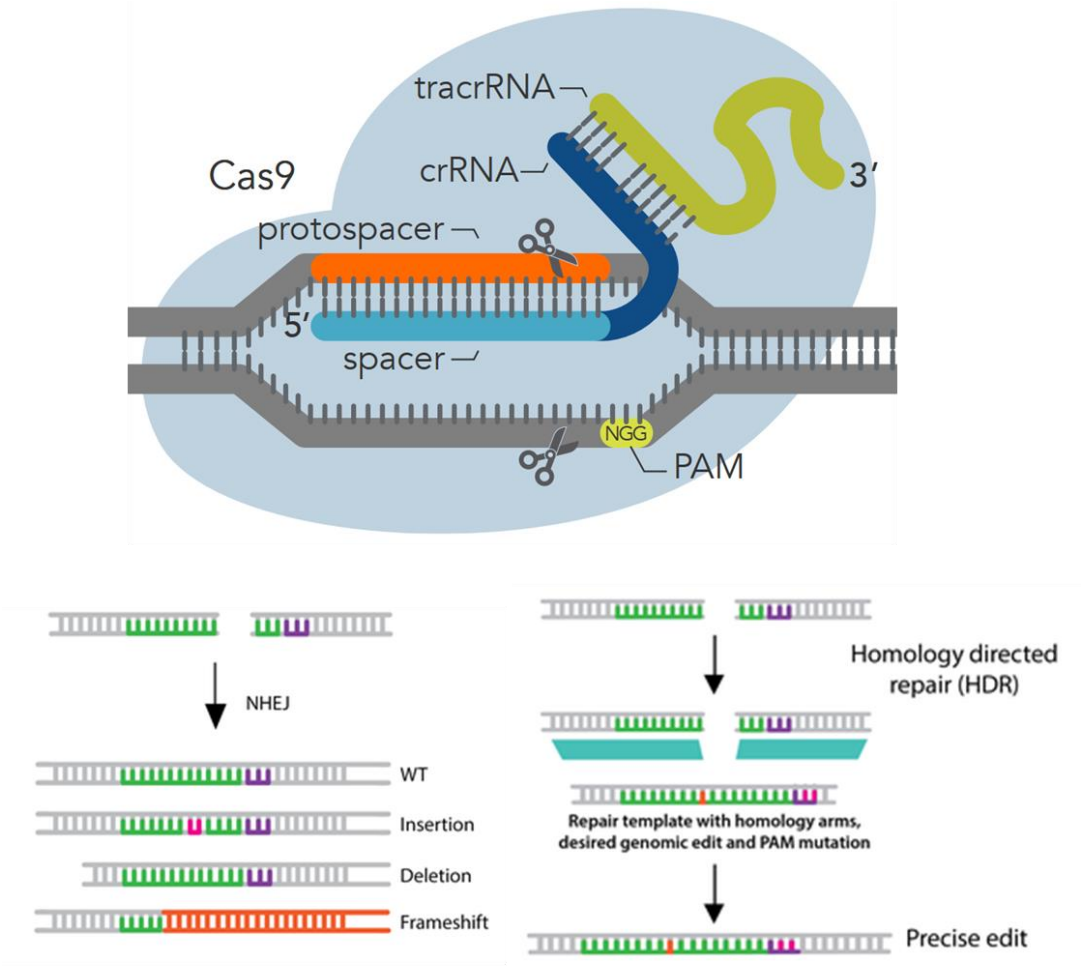

Figure I.12. CRISPR-Cas9 operating principles. The two possible strategies after generating a double-strand break are summarized as non-homologous end joining (NHEJ) and homology directed repair (HDR).

When a DSB is generated, the yeast cell repair mechanisms activate, and without a repair template yeast will join both ends via non-homologous end joining (NHEJ), which is error prone and it will likely provoke indels resulting in a frameshift causing a non-functional gene. The first great potential of this editing technique resides in its versatility as assembling different target sequences will change the site where the DSB is formed, and when a repair DNA with homology upstream and downstream the DSB is provided to the cell, it will use the repair template to “fix” the DSB and thus inserting or deleting virtually any desired sequence. Based on this principle, multiple CRISPR tools and protocols have been developed, not only to permanently edit the genome but to promote gene activation or repression, to provoke site-directed methylations, to favor precision

DNA immunoprecipitation and to perform extensive screenings of bulk activation/repression of multiple genes (Xu et al., 2019) (Figure I.13). All these applications are based on the use of a “nuclease-dead” structurally fused to an effector.

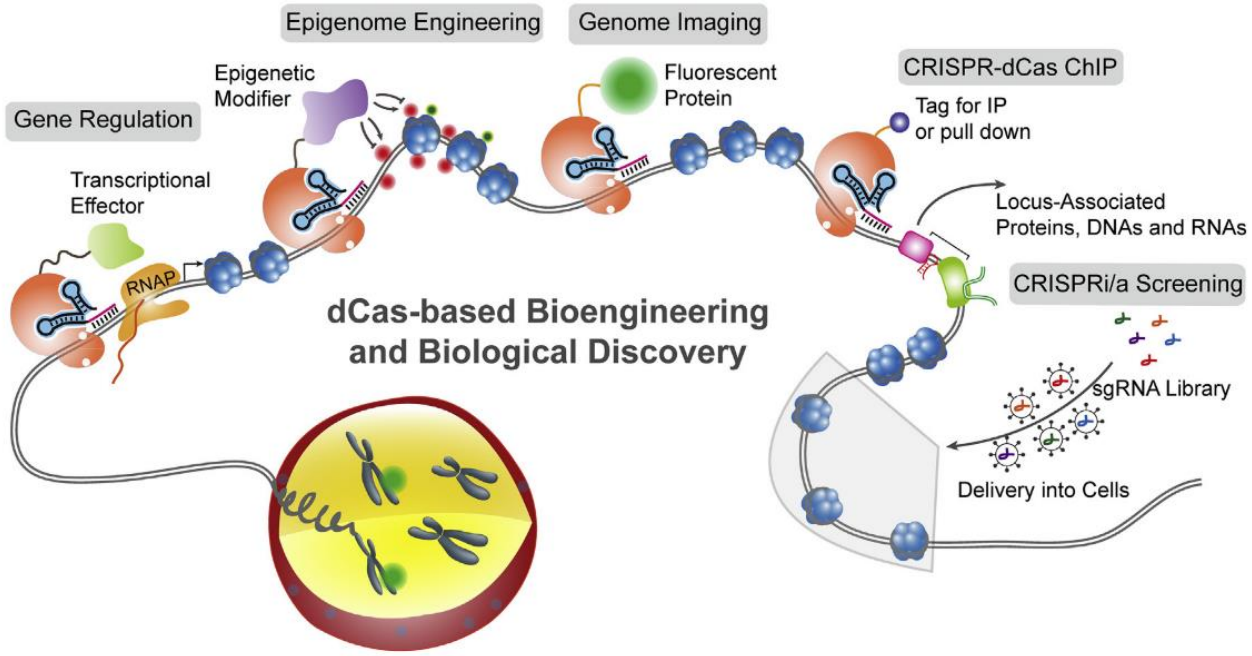

Figure I.13. Applications of CRISPR–dCas9 technologies

Synthetic biology

Synthetic biology intends to engineer the biological systems for various applications of human needs. It applies engineering principles to integrate biology, mathematics, chemistry, biophysics, and automation, to construct synthetic enzymes, circuits, pathways, chromosomes and organisms in a systematic, modular and standardized fashion following a design-build-test-learn dynamic. This field of science presents two general approaches, bottom-up and top-down to achieve its purpose. The bottom-up approach attempts to assemble a minimal functional live organism by characterizing single individual modules (*e.g.* promoters, ribosome binding sites, etc.) to study and characterize them in isolation and then assemble them in a live organism. Top-down approach, on the other hand, focuses on reengineering preexisting natural living systems for

desired purposes. This approach addresses the questions regard desirable and undesirable potential interactions between the constructed subsystem or genetic circuit and its biological context. It also focuses on development of strategies for harnessing or compensating for these influences. A perfect example of a synthetic biology approach in yeast is the yeast biosensor system engineered by Shaw et al. (2019) mentioned above. In that study a synthetic transcription factor and promoter together with an chimeric bacterial operator were employed and up to 15 genes involved in the yeast native GPCR signal transduction were deleted to isolate the core signal transduction mechanism and thus avoiding undesired interactions with the native response to pheromones (Shaw et al., 2019; Access et al., 2020; Liu et al., 2022 b).

The multiple currently available cloning technologies, especially those based on the creation of fully customizable overhangs for fragments assembly like Gibson Assembly® (New England Biolabs), Golden Gate® (New England Biolabs) and uracil-specific excision reaction (USER)-based cloning (New England Biolabs) (Lopes et al., 1989; Hartley et al., 2000; Nour-Eldin et al., 2006; Geu-Flores et al., 2007; Gibson et al., 2009) have led to the emergence of standardized modular systems such as the MoClo yeast toolkit (YTK) (Lee et al., 2015). This molecular toolkit enables the synthesis of vectors with virtually any desired feature in a standardized use of customizable DNA overhangs for DNA assembly using type II-S restriction enzymes (Golden Gate®). This cloning system was employed in the development of useful plasmid collections such as EasyClone® (Stovicek et al., 2015; Jessop-Fabre et al., 2016; Maury et al., 2016). This collection offers pre-assembled integrative vectors bearing a USER cloning site and predesigned primer sets to easily assemble of one or two transcriptional units per plasmid. Most of the plasmids in the collection are oriented to genome integration of the desired genes of interest, to that end plasmids contain upstream and downstream homology arms with different stable and characterized integration sites across the genome, covering single and multiple integrations with recyclable selection markers or without them. After the assembly of the different genes and promoters combination, these plasmids need to be linearized to generate an integration fragment that will integrate the genome by the random event of homologous recombination. The “marker-free” version of this collection

relies on the use of a CRISPR-Cas9 approach to generate a DSB and ensure the integration of the desired fragment.

Metabolic engineering and systems biology

The efforts on trying to elucidate the biological function of each gene after the first genome sequencing, such as the *Saccharomyces* Genome Deletion Project, and the great increase of available data in databases such as *Saccharomyces* Genome Database (SGD) from many yeast strains and experimental conditions brought a new and broader way to study DNA sequence, gene expression, protein function, study of non-coding regions and protein-DNA interaction by using a genome-wide approach, considering the combination of available data, in contrast to the classical gene-by-gene approach and thus giving way to the field of functional genomics. Together, these data are used to model interactive and dynamic networks that regulate gene expression, cell differentiation, cell cycle progression and metabolic flux prediction (Bunnik et al., 2013; Lu et al., 2019; Domenzain et al., 2023; Henriques et al., 2023).

With the combination of data from multiple whole-genome and classical sources and computational modeling, a new level of biological research arises as a scientific field termed “systems biology” (Borneman et al., 2007; Nielsen, 2017; Yu et al., 2019). Metabolic engineering and synthetic biology offer synergies as both fields feed back to offer interesting applications. Metabolic engineering focuses on the optimization of cellular processes, endemic to a specific organism, to produce a compound of interest from a substrate (García-Granados et al., 2019). The combined approach of metabolic engineering and synthetic biology becomes of special relevance when developing a yeast cell factory for use in industrial biology (Nielsen et al., 2011).

Cell factory

S. cerevisiae offers a good chassis platform for a cell factory development as it presents a number of advantageous properties such as its rapid growth, which contributes to cost-effectiveness, robustness, temperature, pH and salt concentration tolerance, a good genetic tractability and the great abundance of available data on yeast genome and physiology, among others (Nielsen et al., 2013; Guo et al., 2019). Also it is a “generally recognized as safe” (GRAS)

organism as stated by the U.S. Food and Drug Administration (FDA), which facilitates the use of it in labs and production plants without posing a risk of handling. All these advantages have led to using of yeast for the production of a multitude of compounds such as second generation alcohols, aromatic chemicals, enzymes, fatty acids, amino acids and derived compounds, among others using renewable feedstocks in fermentation (Hansen et al., 2009; Nandy et al., 2018; Yu et al., 2018 a; Cordente et al., 2019; Hu et al., 2019; Levisson et al., 2019; Nielsen, 2019).

This cell factory approach has been addressed for the production of amino acid-derived bioactive compounds like hydroxytyrosol in bacterial platforms, in which a whole-cell biocatalyst has been used to maximize this phenolic compound production from both engineered and non-engineered cells (Allouche et al., 2005; Liebgott et al., 2007, 2009; Brouk et al., 2009; Satoh et al., 2012; Li et al., 2018, 2019; Chen et al., 2019; Trantas et al., 2019; Yao et al., 2020). Using a cell factory entails a series of advantages as it allows the production of compounds through multi-step reactions, with cofactor regeneration, with high region- and stereo-selectivity, under mild operational and environment-friendly conditions.

OUTLINE OF THESIS & OBJECTIVES

The work presented in this thesis is oriented to the obtention of a PhD degree. It reflects the formation acquired and the work developed in José Manuel Guillamón laboratory from 2018 to 2023. It took place in the research group “Systems Biology in Yeast of Biotechnological Interest (SBYBI)”, englobed in the Food Biotechnology department of the Institute of Agrochemistry and Food Technology (IATA) of the Spanish National Research Council (CSIC), Valencia (Spain) (https://www.iata.csic.es/es). I received a predoctoral contract financed by the Spanish Ministry of Economy, Industry and Competitiveness through the State Talent Promotion Program (BES-2017-079640) as a part of the funding of the national project grant AGL2016-77505-C3-1-R awarded to José Manuel Guillamón. In addition, this grant covered additional funding that allowed me to stay for three months in the Systems and Synthetic Biology division at Chalmers University of Technology (Sweden) under the supervision of professor Yun Chen, (PhD senior researcher and lab leader).

This thesis takes place under the scope of the SYNBIOFERM and CONSORWINE projects, entitled “Analysis of the molecular and physiological determinants in the synthesis of indole compounds by *S. cerevisiae*” and “Use of the genetic and metabolic diversity within wine yeasts to improve the stability and bioactivity of wines" and they have been funded by the Spanish Ministry of Science and Innovation (AGL2016-77505-C3-1-R and PID2019-108722RB-C31).

The recently established relation between the metabolism of aromatic amino acids and the synthesis of bioactive molecules such as melatonin, serotonin and hydroxytyrosol in *Saccharomyces cerevisiae* brings interesting applications in the field of food and pharmacological industry but also poses a great challenge. The synthesis of these molecules is attributed to yeast during the fermentation process but the underlying mechanisms or the responsible genes remain unknown.

In this context, the main hypothesis in this thesis is: Through the study of the synthesis of bioactive aromatic amino acid-derived compounds and their underlying mechanisms in *S. cerevisiae* it is possible to promote an increase of these metabolites in fermented foods and beverages as a result of yeast metabolism, but it is also possible to rationally tune the production of these

compounds of interest for a massive and yet sustainable production in a yeast cell factory.

From this approach, the main objective of this thesis is to broaden the knowledge about the molecular determinants responsible for the synthesis of bioactive compounds derived from aromatic amino acids, especially melatonin and hydroxytyrosol, and explore yeasts overproducing capacity of these metabolites by different genetic improvement approaches, such as adaptive laboratory evolution (ALE), metabolic engineering and synthetic biology.

This general objective contained the following specific objectives:

Objective 1. **To adapt a rapid, sensitive and cost-effective method to detect and quantitate melatonin directly from growth media samples.**

One of the challenges of studying melatonin biosynthesis by yeast resides in the usually low concentrations of this compound that can be detected in fermented products or yeast growth media as a result of yeast metabolisms. The unequivocally determination of this compound in a matrix such as spent media or fermented must poses a great analytical challenge due to the presence of chemically similar compounds in the matrix and many efforts in this field led to the use of powerful analytical tools such as UHPLC-MS/MS, which is the current reference method due to its low limit of detection (LOD) and quantitation (LOQ). Nevertheless, the use of this method entails previous sample manipulation as sample extraction and preparation are required, besides the use of high energy-consuming and expensive devices, as well as specialized trained operators are needed to operate them and perform the analysis. We believed an alternative method to detect and quantify melatonin from yeast growth samples in a fast, simple and inexpensive manner was a necessary step to take that would enable us to easily analyze large batches of samples and characterize the melatonin synthesis ability of multiple yeast strains, among other applications.

To that end, we adapted a biosensor yeast strain, based on the high specificity melatonin G-protein coupled receptor from mammals, to our growth and detection system. We applied genetic modifications to adapt it to the characteristics of the intended samples taking into account three important parameters: sensitivity,

basal activity and maximum output signal. We evaluated the new modifications and, as a proof of concept, we analyzed 101 samples from different yeast strains obtaining differences in melatonin concentrations among them, using an inexpensive, quick and cost-effective method and eliminating any pre-treatment of the samples.

The results of this objective are described in:

Chapter 1. Adapting yeast biosensor to detect and quantify extracellular yeast-secreted melatonin.

Objective 2. **To reveal the genes responsible for the synthesis of melatonin in *S. cerevisiae*.**

Melatonin production in fermented beverages as a result of yeast's metabolism has been previously demonstrated, and many advances on deciphering the novel metabolic pathway of melatonin synthesis from tryptophan have been achieved. In fact, in the recent years we have proposed a melatonin metabolic pathway for yeast that substantially differs from the mammalian model. Although progress on the knowledge of this novel pathway has been achieved, the responsible genes for the four necessary reactions for melatonin synthesis still remain unknown. Only one gene, *PAA1*, has been proposed as a homolog of the mammalian arylalkylamine N-acetyltransferase (AANAT) as responsible for the *N*-acetylation steps in yeast pathway. This polyamine acetyltransferase has the capacity to accept serotonin or 5-methoxytryptamine as a substrate to convert them to *N*-acetylserotonin and melatonin, respectively. We assess the implication of this gene to contribute to melatonin biosynthesis in yeast using *in vivo* bioconversion assays. We conclude *PAA1* implication to melatonin synthesis is very limited and not exclusive, and using a combined approach involving analysis of global gene expression during melatonin synthesis and the predictive search for acetylase domains allowed us to propose and test a new candidate, *HPA2,* with the capacity of acetylating 5-methoxytryptamine to produce melatonin. None of the described yeast candidates, however, has the arylalkylamine *N*-acetyltransferase as their main *in vivo* activity in yeast as they are considered moonlighting proteins.

The results of this objective are described in:

Chapter 2. The role of *PAA1* gene on melatonin biosynthesis in *Saccharomyces cerevisiae*: a search of new arylalkylamine *N*-acetyltransferases.

Objective 3. **To increase yeast's ability to produce bioactive aromatic amino acid-derived compounds.**

Bioactive aromatic amino acid-derived compounds such as melatonin, serotonin, hydroxytyrosol (HT) and other related metabolites present health-promoting benefits and can also contribute to the quality and stability of fermented foods and drinks. There is a growing interest on increasing these compounds concentrations in fermented beverages such as wine. Taking melatonin as a model, due to its relation to light stimuli in higher organisms and its UV protection effect also in yeast cells, we conducted a novel adaptive laboratory evolution using white light as selective pressure on a *S. cerevisiae* strain, isolated from a winery environment, to obtain an evolved strain (EVO) with the capacity of overproducing melatonin-related and other bioactive metabolites, especially when challenged with photo-oxidative stress.

On the other hand, overproduction of other bioactive compounds such as hydroxytyrosol attracts special interest for its potential applications in food, feed, supplement and pharmaceutical industries as its main source for extracting this compound derives from the olive oil production process or as a costly alternative, the chemical synthesis. We approached the biotechnological production of this interesting compound using a yeast platform as a cell factory. To do so, we overexpressed exogenous genes to perform the final step in the synthetic pathway to convert the naturally produced tyrosol into HT, and then undertook a series of genetic modifications involving knocking out, knocking down and over-expressing different genes across the metabolic pathway to increase and favor the glucose metabolic flux towards tyrosol and hence HT. Our resulting HT producer strain was capable to produce high titers of HT from a clean, non-supplemented growth media with glucose as the sole carbon source and main precursor.

The results of this objective are described in:

Chapter 3. Light-induced adaptive laboratory evolution to increase bioactive aromatic amino acid-derived compounds in a wine strain of *Saccharomyces cerevisiae*

Chapter 4. Metabolic engineering of *Saccharomyces cerevisiae* for hydroxytyrosol production directly from glucose.

MATERIAL & METHODS

Culture media

YPD (Yeast peptone dextrose medium)

Glucose ..... 20 $g \cdot L^{-1}$
Bacteriological peptone ..... 20 $g \cdot L^{-1}$
Yeast extract..... 10 $g \cdot L^{-1}$
Agar (omitted in liquid medium) ..... 16 $g \cdot L^{-1}$

YPG (Yeast peptone glycerol medium)

Glycerol ..... 30 $g \cdot L^{-1}$
Bacteriological peptone ..... 20 $g \cdot L^{-1}$
Yeast extract..... 10 $g \cdot L^{-1}$

YNB80 (300 mg of YAN from L-Trp and NH_4^+ in equal proportion)

Glucose ..... 80 $g \cdot L^{-1}$
YNB ..... 3.7 $g \cdot L^{-1}$
NH_4Cl..... 0.57 $g \cdot L^{-1}$
L-tryptophan ..... 2.19 $g \cdot L^{-1}$

SD (Synthetic defined medium)

Glucose ..... 20 $g \cdot L^{-1}$
Difco Yeast Nitrogen Base (w/o amino acids and ammonium sufate) ..... 1.7 $g \cdot L^{-1}$
$(NH_4)_2SO_4$..... 5 $g \cdot L^{-1}$
Agar (omitted in liquid medium) ..... 16 $g \cdot L^{-1}$
*Note: SD can be supplemented to satisfy auxotrophic requirements and prepared at different glucose concentrations (Chapter 4)

SC (Synthetic complete medium)

Glucose ..... 20 $g \cdot L^{-1}$
Difco Yeast Nitrogen Base (w/o amino acids and ammonium sufate) ..... 1.7 $g \cdot L^{-1}$
$(NH_4)_2SO_4$..... 5 $g \cdot L^{-1}$
Kaiser complete SC drop-out mixture (Formedium)* ..... 2 $g \cdot L^{-1}$
Agar (omitted in liquid medium) ..... 16 $g \cdot L^{-1}$
*Note: SC with single, double or triple drop-out was used according to manufacturer to supplement auxotrophies.

SMM (synthetic minimal medium)

Glucose ..... 7.5 $g \cdot L^{-1}$
$(NH_4)_2SO_4$..... 5 $g \cdot L^{-1}$
KH_2PO_4..... 3 $g \cdot L^{-1}$
$MgSO_4$..... 0.5 $g \cdot L^{-1}$
Vitamins..... 1 $mL \cdot L^{-1}$ (from stock)
Oligo-elements ..... 1 $mL \cdot L^{-1}$ (from stock)
***** Adjust to pH 6 with NaOH

SM (Synthetic must) adapted from Riou et al., (1997)

Sugars:

Glucose ..... 100 $g \cdot L^{-1}$
Fructose ..... 100 $g \cdot L^{-1}$
Organic acids:
Citric acid ..... 5 $g \cdot L^{-1}$
Malic acid ..... 0.5 $g \cdot L^{-1}$
Tartaric acid ..... 3 $g \cdot L^{-1}$
Mineral salts:
KH_2PO_4 ..... 0.75 $g \cdot L^{-1}$
K_2SO_4 ..... 0.5 $g \cdot L^{-1}$
$MgSO_4 \cdot 7\ H_2O$ ..... 0.25 $g \cdot L^{-1}$
$CaCl_2 \cdot 2\ H_2O$ ..... 0.15 $g \cdot L^{-1}$
NaCl ..... 0.20 $g \cdot L^{-1}$
NH_4Cl ..... 0.46 $g \cdot L^{-1}$
Amino acids ..... 13.09 $mL \cdot L^{-1}$ (from stock)
Vitamins ..... 10 $mL \cdot L^{-1}$ (from stock)
Oligo-elements ..... 1 $mL \cdot L^{-1}$ (from stock)
* Adjust to pH 3.3 with NaOH

YAN from NH_4Cl ..... 120 $mg \cdot L^{-1}$
YAN from amino acids ..... 180 $mg \cdot L^{-1}$
Total YAN ..... 300 $mg \cdot L^{-1}$

STOCK solutions
Amino acids stock:
Tyrosine ..... 1.5 $g \cdot L^{-1}$
Tryptophan ..... 13.4 $g \cdot L^{-1}$
Isoleucine ..... 2.5 $g \cdot L^{-1}$
Aspartic Acid ..... 3.4 $g \cdot L^{-1}$
Glutamic Acid ..... 9.2 $g \cdot L^{-1}$
Arginine ..... 28.3 $g \cdot L^{-1}$
Leucine ..... 3.7 $g \cdot L^{-1}$
Threonine ..... 5.8 $g \cdot L^{-1}$
Glycine ..... 1.4 $g \cdot L^{-1}$
Glutamine ..... 38.4 $g \cdot L^{-1}$
Alanine ..... 11.2 $g \cdot L^{-1}$
Valine ..... 3.4 $g \cdot L^{-1}$
Methionine ..... 2.4 $g \cdot L^{-1}$
Phenylalanine ..... 2.9 $g \cdot L^{-1}$
Serine ..... 6 $g \cdot L^{-1}$
Histidine ..... 2.6 $g \cdot L^{-1}$
Lysine ..... 1.3 $g \cdot L^{-1}$
Cysteine ..... 1.5 $g \cdot L^{-1}$
Proline ..... 46.1 $g \cdot L^{-1}$
Vitamins stock:
Myo-inositol ..... 2 $g \cdot L^{-1}$
Pantothenate calcium ..... 0.15 $g \cdot L^{-1}$
Thiamine hydrochloride ..... 0.025 $g \cdot L^{-1}$

Nicotinic acid ..... 0.2 $g \cdot L^{-1}$
Pyridoxine ..... 0.025 $g \cdot L^{-1}$
Biotin ..... 3 ml (from a 100 $mg \cdot L^{-1}$ stock)
Oligo-elements stock:
$MnSO_4 \cdot H_2O$ ..... 4 $g \cdot L^{-1}$
$ZnSO_4 \cdot 7\ H_2O$ ..... 4 $g \cdot L^{-1}$
$CuSO_4 \cdot 5\ H_2O$ ..... 1 $g \cdot L^{-1}$
KI ..... 1 $g \cdot L^{-1}$
$CoCl_2 \cdot 6\ H2O$ ..... 0.4 $g \cdot L^{-1}$
H_3BO_3 ..... 1 $g \cdot L^{-1}$
$(NH_4)_6Mo_7O_{24}$ ..... 1 $g \cdot L^{-1}$

LB (Luria Bertani medium)
Tryptone ..... 10 $g \cdot L^{-1}$
Yeast extract ..... 5 $g \cdot L^{-1}$
NaCl ..... 5 $g \cdot L^{-1}$
Agar (for solid media preparation) ..... 16 $g \cdot L^{-1}$

2x TY medium with 1% glucose
Tryptone ..... 16 $g \cdot L^{-1}$
Yeast extract ..... 16 $g \cdot L^{-1}$
NaCl ..... 5 $g \cdot L^{-1}$
Glucose ..... 10 $g \cdot L^{-1}$

S.O.C (Super Optimal broth with Catabolite repression)
Tryptone ..... 20 $g \cdot L^{-1}$
Yeast extract ..... 5 $g \cdot L^{-1}$
NaCl ..... 0.5 $g \cdot L^{-1}$
KCl ..... 0.186 $g \cdot L^{-1}$
$MgCl_2$ ..... 0.952 $g \cdot L^{-1}$
$MgSO_4 \cdot 7H2O$ ..... 2.465 $g \cdot L^{-1}$
Glucose ..... 3.603 $g \cdot L^{-1}$

PBS (Phosphate-buffered saline medium)
NaCl ..... 8 $g \cdot L^{-1}$
KCl ..... 0.2 $g \cdot L^{-1}$
Na_2HPO_4 ..... 1.42 $g \cdot L^{-1}$
KH_2PO_4 ..... 0.24 $g \cdot L^{-1}$

Strains and plasmids

Table M.1. List of yeast strains used in this thesis

Yeast Strain	Relevant relevant information / genotype	Chapter	Source
BY4743	*MATa/α his3Δ1/his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2/lys2*Δ0 *met15*Δ0/*MET15 ura3*Δ0/*ura3*Δ0	1, 2 and 4	EUROSCARF
yWS1544	(*S. cerevisiae*, BY4741 derivative) *sst2*Δ0 *far1*Δ0 *bar1*Δ0 *ste2*Δ0 *ste12*Δ0 *gpa1*Δ0 *ste3*Δ0 *mf(alpha)1*Δ0 *mf(alpha)2*Δ0 *mfa1*Δ0 *mfa2*Δ0 *gpr1*Δ0 *gpa2*Δ0 + LexO(6x)-p*LEU2*m-sf*GFP*-t*TDH1* + p*PGK1-GPA1*-t*ENO2*-p*RAD27*-LexA-PRD-t*ENO1-URA3* + *HHF2*p-MTNR1A-*TDH1*t-*LEU2*	1	Shaw et al. (2019)
yWS1544ura$^-$	yWS1544 auxotroph derivative	1	This work
1544 +GPCR	yWS1544ura$^-$ + Ty1::*CCW12*p-MTNR1A-*CYC1*t-*URA3*	1	This work
1544 +GPCR+rep	yWS1544ura$^-$ + Ty1::*CCW12*p-MTNR1A-*CYC1*t-*URA3* + pX-4::LexO(6x)-p*LEU2*m-sf*GFP*-t*TDH1*-Spacer2-p*PGK1-GPA1*-t*ENO2*-p*RAD27*-LexA-PRD-t*CYC1-HIS5*	1	This work
1544 +GPCR+rep(multi)	yWS1544ura$^-$ + Ty1::CCW12p-MTNR1A-CYC1t-URA3 + Ty2::LexO(6x)-pLEU2m-sfGFP-tTDH1-Spacer2-pPGK1-GPA1-tENO2-pRAD27-LexA-PRD-tCYC1-HIS5	1	This work
BY4743Δpaa1	BY4743 *paa1*::kanMX4	2	EUROSCARF
BY4743Δfms1	BY4743 *fms1*::kanMX4	2	EUROSCARF
BY4743 PAA1	BY4743 with plasmid p426GPD*PAA1* (*URA3*)	2	This work
BY4743 AANAT	BY4743 with plasmid p426GPD *AANAT* (*URA3*)	2	This work
B28	*S. cerevisiae* winery strain isolate	3	Bodegas Murviedro
EVO	*S. cerevisiae*, B28 derivative	3	This work
BY4741	MATa; *his3*Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0 met15Δ0	4	EUROSCARF
BY4741 trp1-	BY4741 *trp1*::loxP	4	This work

BY4743Δtrp2	BY4743 *trp2*::kanMX4	4	EUROSCARF
BY4743Δabz1	BY4743 *abz1*::kanMX4	4	EUROSCARF
BY4743Δpha2	BY4743 *pha2*::kanMX4	4	EUROSCARF
BY4743 HpaB	BY4743 with plasmid p426GPD*hpaB*	4	Muñiz-Calvo et al. (2020)
BY4743 HpaC	BY4743 with plasmid p425GPD*hpaC*	4	Muñiz-Calvo et al. (2020)
BY4743 HpaB + HpaC	BY4743 with plasmid p426GPD*hpaB* and p425GPD*hpaC*	4	Muñiz-Calvo et al. (2020)
BY4743 HpaBC	BY4743 with *TEF1hpaC PGK1hpaB* integrated into genome (*Ty1Cons2*)	4	This work
BY4743 + ARO10	BY4743 with plasmid p425GPD *ARO10*	4	This work
BY4743 ARO3	BY4743 HpaBC with plasmid p423GPD *ARO3*	4	This work
BY4743 ARO4	BY4743 HpaBC with plasmid p423GPD *ARO4*	4	This work
BY4743 ARO7	BY4743 HpaBC with plasmid p423GPD *ARO7*	4	This work
BY4743 HpaBC + ARO10	BY4743 HpaBC with plasmid p425GPD *ARO10*	4	This work
BY4743 ARO3K222L	BY4743 HpaBC with plasmid p423GPD *ARO3K222L*	4	This work
BY4743 ARO4K229L	BY4743 HpaBC with plasmid p423GPD *ARO4K229L*	4	This work
BY4743 ARO7G141S	BY4743 HpaBC with plasmid p423GPD *ARO7G141S*	4	This work
BY4741 trp1- HpaBC	BY4741 *trp1*-::*loxP* with *TEF1hpaC PGK1hpaB* into genome (*Ty1Cons2*)	4	This work
BY4741 HpaBC ARO3 ARO4 ARO10	BY4741 trp1- HpaBC with plasmids p423GPD *ARO3* p424GPD *ARO4* p425GPD *ARO10*	4	This work

BY4741 HpaBC ARO3 ARO4* ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4K229L p425GPDARO10	4	This work
BY4741 HpaBC ARO3* ARO4 ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 K222L p424GPD ARO4 p425GPDARO10	4	This work
BY4741 HpaBC ARO3* ARO4* ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3K222L p424GPD ARO4K229L p425GPDARO10	4	This work
BY4741 HpaBC ARO3 ARO4 ARO10 ARO7	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3* p424GPD *ARO4* p425GPD *ARO10* p426GPD *ARO7*	4	This work
BY4741 HpaBC ARO3 ARO4* ARO10 ARO7	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3* p424GPD ARO4K229L p425GPD *ARO10* p426GPD *ARO7*	4	This work
BY4741 HpaBC ARO3* ARO4 ARO10 ARO7	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3K222L* p424GPD *ARO4* p425GPD *ARO10* p426GPD *ARO7*	4	This work
BY4741 HpaBC ARO3* ARO4* ARO10 ARO7	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3K222L* p424GPD *ARO4K229L* p425GPD *ARO10* p426GPD *ARO7*	4	This work
BY4741 HpaBC ARO3 ARO4 ARO10 ARO7*	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3* p424GPD *ARO4* p425GPD *ARO10* p426GPD *ARO7G141S*	4	This work
BY4741 HpaBC ARO3 ARO4* ARO10 ARO7*	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3* p424GPD *ARO4K229L* p425GPD *ARO10* p426GPD *ARO7G141S*	4	This work
BY4741 HpaBC ARO3* ARO4 ARO10 ARO7*	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3* K222L p424GPD *ARO4* p425GPD *ARO10* p426GPD *ARO7G141S*	4	This work
BY4741 HpaBC ARO3* ARO4* ARO10 ARO7*	BY4741 *trp1*- HpaBC with plasmids p423GPD *ARO3K222L* p424GPD *ARO4K229L* p425GPD *ARO10* p426GPD *ARO7G141S*	4	This work

Table M.2. List of *E. coli* strains used in this thesis

Bacterial Strain	Relevant genotype / plasmid	Chapter	Source
NZYα	fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	2	NZYTech
Rosetta (DE3)™	F- ompT hsdSB(rB- mB-) gal dcm (DE3) pRARE (CamR)	2	Novagen
Rosetta pGEX5X1	Rosetta transformed with pGEX-5X-1	2	This work
Rosetta PAA1	Rosetta transformed with pGEX-5X-1 *PAA1*	2	This work
Rosetta AANAT	Rosetta transformed with pGEX-5X-1 *AANAT*	2	This work
Rosetta ARD1	Rosetta transformed with pGEX-5X-1 *ARD1*	2	This work
Rosetta NAT4	Rosetta transformed with pGEX-5X-1 *NAT4*	2	This work
Rosetta GNA1	Rosetta transformed with pGEX-5X-1 *GNA1*	2	This work
Rosetta YIR042C	Rosetta transformed with pGEX-5X-1 *YIR042C*	2	This work

Rosetta HPA2	Rosetta transformed with pGEX-5X-1 *HPA2*	2	This work
Rosetta NAT3	Rosetta transformed with pGEX-5X-1 *NAT3*	2	This work
Rosetta HAT1	Rosetta transformed with pGEX-5X-1 *HAT1*	2	This work

Table M.3. List of yeast strains used in chapter 1 screening.

Strain code	Species	Origin
A01	*S. cerevisiae*	Olives (Manzanilla-Aloreña)
A02	*S. cerevisiae*	Olives (Manzanilla-Aloreña)
A03	*S. cerevisiae*	Cachaça
A04	*S. cerevisiae*	Cachaça
A05	*S. cerevisiae*	Chicha
A06	*S. cerevisiae*	Huayruro cork
A07	*S. cerevisiae*	Environmental isolate
A08	*S. cerevisiae*	winery isolate
A09	*S. cerevisiae*	Environmental isolate
A10	*S. cerevisiae*	Flor
A11	*S. cerevisiae*	Environmental isolate
A12	*S. cerevisiae*	Masato
A14	*S. cerevisiae*	Mezcal
A15	*S. cerevisiae*	Tequila
A16	*S. paradoxus*	Environmental isolate
A17	*S. paradoxus*	Environmental isolate
A18	*S. paradoxus*	Environmental isolate
A19	*S. paradoxus*	Environmental isolate
A21	*S. kudriavzevii*	Quercus
A22	*S. kudriavzevii*	Quercus
A23	*S. kudriavzevii*	Quercus
A24	*S. uvarum*	Environmental isolate
A26	*S. uvarum*	Quercus
A27	*S. uvarum*	Environmental isolate
A28	*S. uvarum*	Environmental isolate
A29	*S. bayanus*	Environmental isolate
A30	*S. bayanus*	Environmental isolate
A31	*S. eubayanus*	Environmental isolate
A32	*S. eubayanus*	Environmental isolate
B28	*S. cerevisiae*	Winery isolate (MURVIEDRO)
BMV58	*S. uvarum*	Lallemand™
E19	*S. cerevisiae (lab)*	Lab strain
G12	*S. cerevisiae (lab)*	Lab strain
S01	*S. cerevisiae*	Lallemand™
S02	*S. cerevisiae*	Lallemand™
S03	*S. cerevisiae*	Lallemand™
S04	*S. cerevisiae*	Lallemand™
S05	*S. cerevisiae*	Lallemand™

S06	*S. cerevisiae*	Lallemand™
S07	*S. cerevisiae*	Lallemand™
S08	*S. cerevisiae*	Lallemand™
S09	*S. cerevisiae*	Lallemand™
S10	*S. cerevisiae*	Lallemand™
S11	*S. cerevisiae*	Lallemand™
S12	*S. cerevisiae*	Lallemand™
S13	*S. cerevisiae*	Lallemand™
S14	*S. cerevisiae*	Lallemand™
S16	*S. cerevisiae*	Lallemand™
S17	*S. cerevisiae*	Lallemand™
S18	*S. cerevisiae*	Lallemand™
S19	*S. cerevisiae*	Lallemand™
S20	*S. cerevisiae*	Lallemand™
S21	*S. cerevisiae*	Lallemand™
S22	*S. cerevisiae*	Lallemand™
S28	*Metschnikowia pulcherrima*	Lallemand™
S29	*Lachancea thermotolerans*	Lallemand™
S30	*Torulaspora delbrueckii*	Lallemand™
S35	*S. cerevisiae*	Environmental isolate
S36	*S. cerevisiae*	Environmental isolate
S37	*S. cerevisiae*	Environmental isolate
S38	*S. cerevisiae*	Environmental isolate
S39	*S. cerevisiae*	Environmental isolate
S40	*S. cerevisiae*	Environmental isolate
S41	*S. cerevisiae*	Environmental isolate
S42	*S. cerevisiae*	Environmental isolate
S44	*S. cerevisiae*	Environmental isolate
S45	*S. cerevisiae*	Environmental isolate
S46	*S. cerevisiae*	Environmental isolate
S47	*S. cerevisiae*	Environmental isolate
S49	*Zygosaccharomyces bailii*	Winery isolate (MURVIEDRO)
S50	*Pichia kudriavzevii*	Winery isolate (MURVIEDRO)
S51	*Pichia kudriavzevii*	Winery isolate (MURVIEDRO)
S52	*Torulaspora delbrueckii*	Winery isolate (MURVIEDRO)
S53	*Torulaspora delbrueckii*	Winery isolate (MURVIEDRO)
S56	*Wickerhamiella pararugosa*	Winery isolate (MURVIEDRO)
S60	*Lodderomyces elongisporus*	Winery isolate (MURVIEDRO)
S61	*Lodderomyces elongisporus*	Winery isolate (MURVIEDRO)
S67	*Starmerella sp.*	Winery isolate (MURVIEDRO)
S69	*S. uvarum*	Environmental isolate
S70	*S. eubayanus*	Environmental isolate
S71	*S. cerevisiae*	Environmental isolate
S72	*Zygosaccharomyces bailii*	Environmental isolate
S73	*S. uvarum*	Environmental isolate
S74	*Dekkera bruxellensis*	Environmental isolate

S76	*S. cerevisiae*	Environmental isolate
S15	*S. cerevisiae*	Lallemand™
S43	*S. cerevisiae*	Environmental isolate
S48	*Zygosaccharomyces bailii*	Winery isolate (MURVIEDRO)
S54	*Pichia membranifaciens*	Winery isolate (MURVIEDRO)
S55	*Pichia membranifaciens*	Winery isolate (MURVIEDRO)
S57	*Wickerhamiella pararugosa*	Winery isolate (MURVIEDRO)
S62	*Metschnikowia sp.*	Winery isolate (MURVIEDRO)
S63	*Metschnikowia sp.*	Winery isolate (MURVIEDRO)
S64	*Candida cantarelli*	Winery isolate (MURVIEDRO)
S65	*Candida cantarelli*	Winery isolate (MURVIEDRO)
S66	*Starmerella sp.*	Winery isolate (MURVIEDRO)
S68	*Filobasidium capsuligenum*	Winery isolate (MURVIEDRO)
S78	*S. cerevisiae*	Cocoa
A13	*S. cerevisiae*	Beer
A20	*S. kudriavzevii*	Quercus
A25	*S. cerevisiae*	Beer

Table M.4. List of oligonucleotides used in this thesis.

Nº	Name	Sequence 5’ to 3’
1	URA3 target F	GACTttATTGGATGTTCGTACCACCA
2	URA3 target R	AAACTGGTGGTACGAACATCCAATaa
3	URA3 STOP F	TATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTAAAACTTGCGCTCAATTCC
4	URA3 STOP R	CATGTGTTTTTAGTAAACAAATTTTGGGACCTAATGCTTCGGAATTGAGCGCAAGTTTTA
5	PV2F (CCW12p)	CGTGCGAUcacccatgaaccacacggtta
6	GV2R(MTNR1A)	CACGCGAUAACGGAGTCAACCTTGACAACA
7	PV2F (LBD 6Xp)	CGTGCGAUTGAATTCGCATCTAGACTGA
8	GV2R (PRD)	CACGCGAUGGTTGCATCTGGAAGGTTTTT
9	PAA1 F BamHI	AGGTCGTGGGATCCCCATGGCCTCCTCAAGTAGCA
10	PAA1 R XhoI	TGCGGCCGCTCGAGCTAGTTGTCGTATTCTTCCTTAAT
11	AANAT F BamHI	AGGTCGTGGGATCCCCATGAGCACCCCGAGCAT
12	AANAT R XhoI	TGCGGCCGCTCGAGTTAACGATCGCTATTACGACGCA
13	GPD Pro F	CGGTAGGTATTGATTGTAATTCTG
14	CYC1-R	GCGTGAATGTAAGCGTGAC
15	pGEX seq F	GGGCTGGCAAGCCACGTTTGGTG
16	pGEX seq R	CCGGGAGCTGCATGTGTCAGAGG
17	HAT1 EcoRI F	CGGAATTCATGTCTGCCAATGATTTCAA
18	HAT1 XhoI R	CCGCTCGAGTTAACCTTGAGATTTATTTA
19	GNA1 BamHI F	GTCGTGGGATCCTTATGAGCTTACCCGATGGATT
20	GNA1 XhoI R	CCGCTCGAGCTATTTTCTAATTTGCATTT
21	ARD1 EcoRI F	CGGAATTCATGCCTATTAATATTCGCAG
22	ARD1 XhoI R	CCGCTCGAGTTATACAATGATATCATTTA
23	NAT4 BamHI F	GTCGTGGGATCCTTATGCGTTCTTCGGTATATAG
24	NAT4 XhoI R	CCGCTCGAGTCACGGCATGTGAAGGCAAT
25	NAT3 BamHI F	GTCGTGGGATCCTTATGACAACAATTCAGCCTTT

26	NAT3 XhoI R	CCGCTCGAGTTAAAATCTTACATCATGTG
27	HPA2 BamHI F	GTCGTGGGATCCTTATGTCCAACACTAGCGAAGA
28	HPA2 XhoI R	CCGCTCGAGTTAATATCCCTTCCTCTTGT
29	YIR042C BamHI F	GTGGGATCCCCATGGCGAATTTAAATATATTTGGGC
30	YIR042C XhoI R	GCCGCTCGAGTCAGTTTGACAAGCTTTCTCT
31	PAA1 (qPCR) F	GGTTTCCCACCAAACGAAAG
32	PAA1 (qPCR) R	ACTTCTTTGCCCTCGATCTC
33	ACT1 (qPCR) F	GCAAACCGCTGCTCAATCTTC
34	ACT1 (qPCR) R	TGGGGCTCTGAATCTTTCGT
35	PV2F (GPDp)	CGTGCGAUAGTTTATCATTATCAATACTCGCC
36	GV2R (ARO4)	CACGCGAUCTATTTCTTGTTAACTTCTCTTCTTTGTCT
37	HHF2p F (with TEF2pF)	AGTAATTGTTTACATTGATCTTGACCTATCAATGTGGAGTGTTTGCTTGGATTC
38	HHF2p R	TATTTTATTGTATTGATTGTTGTTTTTGCTACTCT
39	TEF2p F	TTGATAGGTCAAGATCAATGTAAACAATTACT
40	TEF2p R	GTTTAGTTAATTATAGTTCGTTGACCGTATATTCTA
41	PG1R (HHF2p)	ACCTGCACUTATTTTATTGTATTGATTGTTGTTTTTGCTACTCT
42	PG2R (TEF2p)	ATGACAGAUGTTTAGTTAATTATAGTTCGTTGACCGTATATTCTA
43	GP2F (TOM β)	ATCTGTCAUAAAACAATGGGTGAATGGGACAAATTATTGGA
44	GV2R (TOM β)	CACGCGAUTCACAAACCGACTTTGTTCATTCT
45	GV1R (TOM α)	CGTGCGAUTCACGCAGCTTCAGATTCCTTA
46	GP1F (TOM α)	AGTGCAGGUAAAACAATGGCTGCTACCAAGAAGATGT
47	PHA2p us F	TGTATGATGACTTCCCTAGCAGAATACAAAC
48	PHA2p us R	CTGCGTATTATATTCATCTACTAAAATTTTTATTTTC
49	PHA2p ds F	ATGGCCAGCAAGACTTTGAG
50	PHA2p ds R	GAAGAACAATCCAATCTTATAAAGGTGACCTGC
51	REV1p F (with PHA2p us R)	GAAAATAAAAATTTTAGTAGATGAATATAATACGCAGGTGTTGTTATCCGATACAAC
52	REV1p R (with PHA2p ds F)	CTCAAAGTCTTGCTGGCCATCGCTGGATATGCCTAGAAA
53	PHA2 target F	GACTttCACTAGTATAGATTATTCAG
54	PHA2 target R	AAACCTGAATAATCTATACTAGTGaa
55	ARO10 F BamHI	AGGTCGTGGGATCCCC ATGGCACCTGTTACAATT
56	ARO10 R XhoI	TGCGGCCGCTCGAGCTATTTTTTATTTCTTTTAAGTG
57	hpaB F BamHI	AGGTCGTGGGATCCCCATGAAACCAGAAGATTTCCG
58	hpaB R XhoI	CCGCTCGAGTTATTTCAGCAGCTTATCCAGC
59	hpaC F BamHI	AGGTCGTGGGATCCCCATGCAATTAGATGAACAACGC
60	hpaC R XhoI	CCGCTCGAGTTAAATCGCAGCTTCCATTTCC
61	ARO3 F BamHI	GTCGTGGGATCCTTATGTTCATTAAAAACGATCACGCCGGTG
62	ARO3 K222L R	GCAGTGACACCTGGCaaTGTGACAGAAAGG
63	ARO3 K222LF	CCTTTCTGTCACAttGCCAGGTGTCACTGC
64	ARO3 R XhoI	CCGCTCGAGCTATTTTTTCAAGGCCTTTCTTCTGTTTCTAACACC
65	ARO4 F BamHI	GTCGTGGGATCCAAATGAGTGAATCTCCAATGTTCGCTGCCAAC
66	ARO4 K229L R	GCAGCAACACCATGCaaAGTAACACCCATG
67	ARO4 R XhoI	CCGCTCGAGCTATTTCTTGTTAACTTCTCTTCTTTGTCTGACAGC
68	ARO4 K229L F	CATGGGTGTTACTttGCATGGTGTTGCTGC
69	ARO7 F BamHI	GTCGTGGGATCCATATGGATTTCACAAAACCAGAAACTGTTT

70	ARO7 G141S R	CTAGTGGCAACAGAAGAGAAGTTATTCTTA
71	ARO7 G141S F	TAAGAATAACTTCTCTTCTGTTGCCACTAG
72	ARO7 R XhoI	CCGCTCGAGTTACTCTTCCAACCTTCTTAGCAAGTATTCC
73	GV1R (HpaC)	CGTGCGAUTTAAATCGCAGCTTCCATTTCC
74	GP1F (HpaC)	AGTGCAGGUAAAACAATGCAATTAGATGAACAACGC
75	PG1R(TEF1p)	ACCTGCACUTTTGTAATTAAAACTTAGATTAGATTGCTAT
76	PG2R (PGK1p)	ATGACAGAUTTGTTTTATATTTGTTGTAAAAAGTAGATAATT
77	GP2F (HpaB)	ATCTGTCAUAAAACAATGAAACCAGAAGATTTCCG
78	GV2R(HpaB)	CACGCGAUTTATTTCAGCAGCTTATCCAGC
79	p426GPD seq R	GACCTAGACTTCAGGTTGTC
80	PaHpaB XhoI R	CCGCTCGAGTCATTGGCGGATGCGATCGAG
81	SeHpaB BamHI F	AGGTCGTGGGATCCCCATGAAACCTGAAGATTTTCGT
82	HpaB mid R	AACGAACATCAGTGCGAAGT
83	PaHpaC BamHI F	AGGTCGTGGGATCCCCATGTCCCAGCTCGAACCCAGGCAG
84	PaHpaC XhoI R	CCGCTCGAGTCAGGCCGCCCGCCGGGGGCA
85	SeHpaC BamHI F	AGGTCGTGGGATCCCCATGCAAGTAGATGAACAACG
86	SeHpaC XhoI R	CCGCTCGAGTTAAACAGGCGCTTCCATCTC
87	GV2R(PaHpaB)	CACGCGAUTCATTGGCGGATGCGATC

Table M.5. List of plasmids used in this thesis.

Plasmid name	Relevant characteristics	Integration site/ replicon	Marker	Source
pCfB2797HIS3	modified USER cloning cassette-*ADH1&CYC1* terminators	TY2Cons	*HIS3*	This work
pCfB2988	USER cloning cassette-*ADH1&CYC1* terminators	TY1Cons2	*URA3*	Maury et al. (2016)
pCfB258	USER cloning cassette-*ADH1&CYC1* terminators	pX-4	*HIS5*	Jensen et al. (2014)
pCfB2797-reporter	LexO(6x)-p*LEU2*m-sfGFP-t*TDH1*+p*PGK1-GPA1*-t*ENO2*-p*RAD27*-LexA-PRD	TY4Cons	*LEU2*	This work
pCfB2988-MTNR1A	*CCW12*p-*MTNR1A*	TY1Cons2	*URA3*	This work
pCfB258-reporter	*LexO*(6x)-*pLEU2m-sfGFP-tTDH1+pPGK1-GPA1-tENO2-pRAD27-LexA-PRD*	pX-4	*HIS5*	This work
pWS172	Cas9 and sgRNA expression backbone (sfGFP dropout)	2µ	*HIS3*	Shaw et al. (2019)
pGEX-5X-1	Taq promoter-GST-MCS	pBR322	AmpR	GE Healthcare
p426GPD	*TDH3*p-MCS-*CYC1t*	2µ	*URA3*	Mumberg et al. (1995)
p426GPD-PAA1	*TDH3p-PAA1-CYC1t*	2µ	*URA3*	This work
pGEX-5X-1 PAA1	Taq promoter-GST-*PAA1*	-	AmpR	This work
p426GPD-AANAT	*TDH3p-AANAT-CYC1t*	2µ	*URA3*	This work
pGEX-5X-1 AANAT	Taq promoter-GST-*AANAT*	-	AmpR	This work

pGEX-5X-1 ARD1	Taq promoter-GST-*ARD1*	-	AmpR	This work
pGEX-5X-1 NAT4	Taq promoter-GST-*NAT4*	-	AmpR	This work
pGEX-5X-1 GNA1	Taq promoter-GST-*GNA1*	-	AmpR	This work
pGEX-5X-1 YIR042C	Taq promoter-GST-*YIR042C*	-	AmpR	This work
pGEX-5X-1 HPA2	Taq promoter-GST-*HPA2*	-	AmpR	This work
pGEX-5X-1 NAT3	Taq promoter-GST-*NAT3*	-	AmpR	This work
pGEX-5X-1 HAT1	Taq promoter-GST-*HAT1*	-	AmpR	This work
pWS174	Cas9 and sgRNA expression backbone (sfGFP dropout)	2µ	NatR	Shaw et al. (2019)
p423GPD	*TDH3p*-MCS-*CYC1t*	2µ	*HIS3*	Mumberg et al. (1995)
p424GPD	*TDH3p*-MCS-*CYC1t*	2µ	*TRP1*	Mumberg et al. (1995)
p425GPD	*TDH3p*-MCS-*CYC1t*	2µ	*LEU2*	Mumberg et al. (1995)
p427GPD	*TDH3p*-MCS-*CYC1t*	2µ	*MET15*	This work
p425GPD-hpaC	*TDH3p-hpaC-CYC1t*	2µ	*LEU2*	This work
p426GPD-hpaB	*TDH3p-hpaB-CYC1t*	2µ	*URA3*	This work
p423GPD-ARO3	*TDH3p-ARO3-CYC1t*	2µ	*HIS3*	This work
p423GPD-ARO3K222L	*TDH3p-ARO3K222L-CYC1t*	2µ	*HIS3*	This work
p423GPD-ARO4	*TDH3p-ARO4-CYC1t*	2µ	*HIS3*	This work
p423GPD-ARO4K229L	*TDH3p-ARO4K229L-CYC1t*	2µ	*HIS3*	This work
p424GPD-ARO4	*TDH3p-ARO4-CYC1t*	2µ	*TRP1*	This work
p424GPD-ARO4K229L	*TDH3p-ARO4K229L-CYC1t*	2µ	*TRP1*	This work
p425GPD-ARO10	*TDH3p-ARO10-CYC1t*	2µ	*LEU2*	This work
p423GPD-ARO10	*TDH3p-ARO10-CYC1t*	2µ	*HIS3*	This work
p426GPD-ARO7	*TDH3p-ARO7-CYC1t*	2µ	*URA3*	This work
p426GPD-ARO7G141S	*TDH3p-ARO7G141S-CYC1t*	2µ	*URA3*	This work
p423GPD-ARO7	*TDH3p-ARO7-CYC1t*	2µ	*HIS3*	This work
p423GPD-ARO7G141S	*TDH3p-ARO7G414S-CYC1t*	2µ	*HIS3*	This work
pCfB2803	USER cloning cassette-*ADH1&CYC1* terminators	TY4Cons	*LEU2*	Maury et al. (2016)
pCfB2988 EcHpaBC	*TEF1p-EcHpaC + PGK1p+EcHpaB*	TY1Cons2	*URA3*	This work

pCfB2988 PaHpaB-EcHpaC	*TEF1p-EcHpaC + PGK1p+PaHpaB*	TY1Cons2	*URA3*	This work
pCfB2803 ARO4*	*TDH3p-ARO4K229L*	TY4Cons	*LEU2*	This work
pCfB2797 AmTOM	*HHF2p-AmTOMα + TEF2p-AmTOMβ*	TY2Cons	*HIS3*	This work

Microbiological methods

All SD cultures mentioned onwards were only supplemented with 76 $mg \cdot L^{-1}$ of uracil and histidine, and with 380 $mg \cdot L^{-1}$ of leucine when needed to complement auxotrophies. Likewise, all SC cultures mentioned were prepared with SC-single, double or triple dropouts according to manufacturer when needed to maintain plasmid selection.

Melatonin ligand sensing protocol

All sensor strains were picked into 5 mL of SC media and grown in 12 mL tubes at 28 °C with constant shaking overnight. The next day, saturated strains were pelleted and then diluted with fresh SC medium to OD600 1 and distributed 220 µL per well in a black 96-well plate with clear flat bottom (Corning, Glendale, AZ, USA). After 4 h of incubation the strains were induced with 30 µL of melatonin ligand or sample and incubated for 4 h at 28°C. After incubation fluorescence intensity was measured in a CLARIOstar® (BGM Labtech, Offenburg, Germany) microplate reader with the GFP preset values for excitation and emission parameters. Measures were obtained from the bottom of the plate at a focal distance of 4.6 mm. A melatonin concentration curve covering concentrations from 1 to 10^5 nM served as calibration for interpolation purposes. Fluorescence values were normalized by subtracting background fluorescence and fitting all values between the maximum and minimum detected fluorescence (100 and 0 %, respectively). Each sample was grown and measured repeatedly in at least 3 different wells.

Yeast growth for melatonin screening

Strains in table M.3 were grown at 28°C until saturated, 5 µL of grown precultures were inoculated in 96-well plates with 245 µL of fresh YNB80 and incubated for

72 h at 28°C with constant orbital shaking. To collect the samples, plates were centrifuged at 3000 × g for 4 min in in a benchtop 5804-R centrifuge with a rotor for plates (Eppendorf, Leipzig, Germany) and supernatants were collected for further analysis.

Spot assays

After growth on SC at 28°C until stationary phase, cells were harvested by centrifugation, washed with sterile water, resuspended in sterile water to an OD600 value of 0.5, and followed by serial dilution. From each dilution, 3.5 µL were spotted onto SC-ura with or without pantothenate agar plates. Plates were incubated at 28°C for 2 and 5 days.

Bioconversion assays

To overexpress heterologous genes in *E. coli*, the plasmids pGEX-5X-1 constructed with the different candidate genes were transformed into Rosetta™ (DE3) competent cells (Novagen), empty vector was also transformed and used as a negative control. Transformants were grown under continuous shaking at 37° C overnight in 15 mL tubes with 5 mL of LB medium, supplemented with 100 $\mu g \cdot L^{-1}$ ampicillin and 34 µg·L-1 chloramphenicol. Next day, 15 µL of grown preculture were inoculated into 1.5 mL of 2xTY medium with 1% glucose, supplemented with ampicillin and chloramphenicol at the same concentrations mentioned above. Cultures were grown at 37° C until OD_{600} reached 0.6, after that, 0.25 mM of IPTG and 1 mM of the desired precursor was added to the culture. The culture was further grown for 24 h, at 28°C under 300 rpm orbital shaking, then samples were taken and stored at -20°C until extraction and HPLC analysis.

To overexpress the different genes in *S. cerevisiae*, a vector-based constitutive overexpression system was employed. To overexpress the genes of interest, high-copy number vector p426GPD, which enables strong constitutive expression by using GPD (*TDH3*) promoter, was used to clone the candidate genes and transformed into BY4743 yeast strain (EUROSCARF). Empty vector was also transformed and used as a negative control in the assays. Individual transformants were grown overnight in 1.5 mL tubes containing 0.8 mL of SC

without uracil (SC-ura) medium at 28° C under continuous shaking at 150 rpm, then 30 µL of the grown pre-inoculum was inoculated into 1.5 mL of fresh SC-ura medium in a 24 well microtiter plate with 2 mL well capacity. Plates were incubated at 28° C under constant shaking in a microplate orbital shaker at 300 rpm and 1 mM of precursor was added to the culture when late exponential phase (0.6 to 0.8 OD_{600}) was reached. Samples were taken after 50 h and stored at -20° C until extraction and HPLC analysis.

Adaptive laboratory evolution under white light stress

Batch cultures were grown at 28°C in two 0.5 L reactors (MiniBio, Applikon Biotechnology) with a working volume of 0.4 L of SMM media. A temperature probe connected to a Peltier cryostat controlled the temperature cultures. The stirrer was set at 300 rpm. Both bioreactors were initially inoculated with approximately $2 \cdot 10^6$ cells·mL^{-1} of B28 strain from a preculture previously grown overnight in YPD medium. One of the bioreactors was faced with a white JARO led lamp (Brennenstuhl®, Tübingen, Germany) at an initial irradiance of 317 µmol·m^{-2}·s^{-1}, corresponding to a focal distance of 16 cm from the vessel. The other bioreactor was set to the same growth conditions except for the light stimulus and after the experiment B28 from the unchallenged bioreactor was kept as a control. The bioreactors were depleted of ~90% of the spent media and refilled with fresh media after cultures reached stationary phase. OD600 was registered at the beginning and end of each batch to estimate the number of generations according to g = (log($final_{OD}$)-log($initial_{OD}$)) / log(2). Selective pressure was increased gradually until irradiance reached 3377 µmol·m-2·s-1 which corresponded to a distance of 4 cm from the vessel, while allowing cell growth. After approximately 170 generations cells were collected and glycerinated and experiment was concluded.

Growth curve analysis

All the presented growth curves and their integration to obtain area under the curve (AUC) were obtained in a 96-well microtiter plate platform. Each well was filled with 250 µL of the different media and condition and an initial OD_{600} of 0.1 was used to inoculate them from previously grown overnight precultures. Growth

curves were monitored by recording the increase of optical density (OD) at wavelength 600 nm. The microtiter plates were incubated in SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 30°C with 500 rpm double-orbital shaking. The optical density of each well was measured every 30 minutes until the growth reached the stationary phase. The growth character of each strain under different media/condition can be calculated by directly fitting OD measurements versus time to the Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

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

Where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_\infty/OD_0)$ is the OD value reached with OD_∞ as the asymptotic maximum, μmax is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). *R* software (v4.1.3) was used to fit growth data to Gompertz equation and infer growth parameters.

The Area Under the Curve (AUC), which represents the three main growth parameters: lag phase, μmax and maximum population (yield), was calculated using Origin Pro 2019.

Intracellular ROS measuring by flow cytometry

After an overnight growth in a rich YPD medium, the intracellular ROS levels of the freshly inoculated yeast cells were measured as described in Ballester-Tomás et al., (2015). Briefly, grown cell cultures of both B28 and EVO strains were pelleted and resuspended (OD_{600} = 0.25) in sterile PBS. Then dihydrorhodamine 123 (DHR 123, Sigma) was added at 5 μg mL^{-1} of cell culture from a 2.5 mg mL^{-1} stock solution in ethanol. Cells were incubated in the dark for 90 min at 28°C. Finally, cells were harvested, washed, resuspended in PBS and analyzed using a flow cytometer MACSQuant® Analyzer (Miltenyi Biotec, Madrid, Spain). The settings were adjusted using negative (DHR 123-untreated cells) and positive (4 mM H_2O_2/60 min-stressed cells treated with DHR 123) controls. Data were expressed as the percentage of cells that show DHR 123-positive staining.

Screening for HT production

Single yeast colonies were inoculated from solid media into 800 µL of liquid media. SC medium was used for screen colonies with HpaBC integration, while SD was used when evaluating null mutants for *TRP2* (ΔΔ*trp2*), *ABZ1* (ΔΔ*abz1*) and *PHA2* (ΔΔ*pha2*). When the cultures were grown to OD ~ 6, 30 µL were transferred to flat-bottomed 24-multiwell plates with 1.5 mL of fresh medium, plus 1 mM of tyrosol. Cultures were incubated for 72 h. The final OD_{600} was measured and HT levels were determined by liquid chromatography (HPLC-PDA).

Evaluation of ARO genes overexpression in tyrosol and HT production

Precultures of each strain were grown over night at 28°C with orbital shaking at 150 rpm in 1.5 mL tubes with 800 µL of SD medium. The next day, 30 µL of the grown culture were inoculated in 1.5 mL of fresh SD medium in 24-well plates (2 mL capacity). This culture was incubated with constant shaking (300 rpm) at 28°C for 72 h. The final OD_{600} was measured, and tyrosol and HT levels were determined by HPLC-PDA.

Evaluation of the HT production of HpaBC versions from other organisms.

HpaB and HpaC homologs from *Salmonella enterica*, *Pseudomonas aeruginosa* and *Escherichia coli* were overexpressed in episomal vectors (Table M. 5) in BY4743. Precultures of each strain were grown over night at 28°C with orbital shaking at 150 rpm in 1.5 mL tubes with 800 µL of SD medium. 1.5 mL of fresh SD medium with 160 $g \cdot L^{-1}$ of glucose was inoculated in 24-well plates (2 mL capacity). This culture was incubated with constant shaking (300 rpm) at 28°C for 120 h.

HT and aromatic higher alcohols production in shake flasks

In order to evaluate the effect of sampling time and glucose concentration on HT production, the ARO4* strain was inoculated in 1 mL of SD and grown o/n at 28°C with shaking. The culture was further inoculated in 250 mL flasks containing 50 mL of the same fresh SD medium at two different glucose concentrations (20 or 160 g/L). This culture was incubated with constant shaking at 150 rpm and 28°C. Samples were taken after 120, 144, 168, 197, 223, 247 and 295 h of growth. The

final OD_{600} was measured, and aromatic higher alcohols and HT levels were determined by HPLC-PDA.

Transformation of *S. cerevisiae* cells

Yeast transformation was carried out by the PEG / lithium acetate method (Gietz and Woods 2002). Cells were incubated in the presence of lithium acetate, polyethylene glycol, single strand DNA "carrier" and the DNA of interest to transform (plasmid or linear fragment) for 30 min at 30°C and subjected to a thermal shock of 42°C for 30 minutes. After this thermal shock, cells were washed with sterile water and plated in selective solid media. When the selection of transformants involved expressing antibiotic resistance, the cells were incubated in YPD for at least two hours under shaking after the heat shock. Subsequently, the cells were spread in solid media with due antibiotic and incubated at 30°C for 2 or 3 days.

When using pCfB integrative plasmids, prior to transformation, the integrative plasmids were digested by *Not*I and column-purified (NZYGelpure, NZYTech). At least 1 µg DNA (single integration site) or 1.5 µg (multi-copy integrative plasmids) were transformed into yeast cells whereas approximately 0.2-0.4 µg were used for 2µ plasmids.

Transformation of *E. coli* cells

Different bacterial strains were used for different purposes. Usually, NZY5α were used for cloning, reproducing and maintaining plasmids, while Rosetta (DE3) were used for protein expression in the bacterial bioconversion assays. For transformation, 50 µl of competent cells were used. Plasmid DNA (10-400 ng) or up to 5 µl of ligation-reaction product were added to the cells and incubated on ice for 10 min. Heat shock at 42°C for 90 s was applied following an ice incubation for 2 min. Subsequently, 150 µl of liquid S.O.C medium was added and cells were incubated at 37°C for 30 min. Then cells were plated (only 50 µl in the case of plasmid transformation) onto a pre-heated LB plate with the selective antibiotic. The plates were incubated at 37°C for overnight (12-16 h).

Molecular methods

Yeast genomic DNA extraction

Yeast genomic DNA was extracted from 1-5 mL cultures grown normally in YPD according to the method described in Querol et al. (1992). Briefly, cells from grown precultures were pelleted and washed twice with sterile water, then resuspended in 0.5 mL of Buffer 1 (0.9 M sorbitol, 0.1 M EDTA pH 7.5) and 30 µL of Zimolyase (amsbio) were added for a mild mixing and incubation at 37°C for 30 min. Spheroplasts were centrifuged 3 min at 15000 × g and resuspended in Buffer 2 (50mM Tris pH 7.4, 20mM EDTA). Then 13 µL of 10% SDS were added and incubated during 5 min. at 65°C. After incubation 200 µL of 5M potassium acetate were added, mildly mixed by inversion and kept on ice for 10 min. After this, tubes were centrifuged at 4°C for 15 min. at 15000 × g to remove the SDS by precipitation. The supernatants were transferred to new tubes and 0.7 mL of isopropanol were added prior to a brief 10 min incubation at room temperature. After incubation, tubes were centrifuged at 4°C for 15 min at 15000 × g and supernatants were discarded. Pellets were then washed with 0.5 mL of 70% ethanol. After centrifugation for 3 min 15000 × g supernatants were eliminated and pellets were dried under vacuum and re-suspended in TE (10 mM Tris, 1mM EDTA pH 8.0) or Milli-Q water.

Plasmid construction

Different approaches have been carried out for assembling DNA into plasmids, depending on the final application or the available technology:

Traditional cloning

Oligonucleotides in table M.4 with a 5’ recognition site for a restriction enzyme were used to amplify genes of interest (*e. g. PAA1* and *HPA2,* among others). This introduces restriction sites at the ends of the gene of interest. Next, the insert and the vector backbone were digested with due restriction enzymes according to manufacturer’s protocol, and their ligation was performed using T4 DNA ligase provided in the Rapid DNA ligation kit (Thermo Fisher Scientific). Ligation reaction is stopped by incubation at 65°C for 10 min or at 70°C for 5 min. The ligation

product was then used directly to transform NZY5α *E. coli* cells as mentioned above.

USER cloning

The DNA sequences to be cloned (Biobricks) were amplified by PCR with primers containing uracils (Table M.4) by using Phusion U Hot Start DNA (Thermo). In parallel, the vectors containing USER cloning sites (pCfB vectors from EasyClone collection) (Table M.5) were prepared by digesting them sequentially with the enzymes *Asi*SI (*Sfa*AI) (Thermo Fisher Scientific) and *Bsm*I (New England Biolabs). After purification, the prepared vectors and the biobricks were mixed and treated with USER™ enzyme and the reaction product was used for bacterial transformation.

Golden Gate

Plasmids from Shaw et al. (2019), bearing Cas9 and sgRNA (single-guide RNA) for CRISPR-Cas9 applications (pWS vectors) (Table M.5) were assembled by using type II-S restriction enzymes, which recognize asymmetric DNA sequences and cleave outside its recognition site. This enables overhang customization by using less variety of enzymes. Oligos F and R with CRISPR “target” sequences (Table M.4) are phosphorylated with T4 polynucleotide kinase and annealed. Then they are incubated in a one-tube reaction with the plasmid, the enzyme *Bsm*BI (NEB) and the T4 DNA ligase (Thermo). When reaction is complete sfGFP sequence in pWS plasmid is substituted by the fragment bearing the target sequence. With the ligation product, NZY5α *E. coli* cells were transformed, and the absence of fluorescence in the colonies indicated correct assembly.

To verify the exact sequence of the insert, the cloned inserts were sequenced using Sanger sequencing (Eurofins genomics) and sequencing primers (Table M.4).

Plasmid extraction from *E. coli*

Plasmid DNA was extracted and purified from *E. coli* transformed cells using the NZYMiniprep kit following the manufacturer’s protocol. Overnight bacterial liquid

cultures grown in 5 mL of LB with the required antibiotic to maintain the plasmids at 37°C in a roller shaker are used as starting material.

Polymerase chain reaction

Different DNA amplicons were generated for multiple purposes and accordingly different polymerases have been used: NZYTaq II DNA polymerase (NZYTech) was used for routine verifications and Phusion High-Fidelity (ThermoFisher Scientific) or Phanta Flash (Vazyme) were used for high fidelity amplicon generation due to error-proof 3'-5' exonuclease activity. Phusion U Hot Start DNA Polymerase (ThermoFisher Scientific) was used to generate biobricks for USER cloning due to its functionality when uracil is involved. High fidelity PCR was performed for amplification of fragments prior cloning or generating DNA donor to edit yeast genome. The mixture of oligonucleotides complementary to the template DNA sequence, dNTPs, buffer solutions and the DNA that acts as a template was performed as indicated by the manufacturer for each of the enzymes. Tm Calculator from NEB was used to estimate the annealing temperature in the PCR reaction according to the polymerases and buffers used. Oligonucleotides serving as primers were ordered to Integrated DNA Technologies (IDT).

Apart from the above-mentioned common applications of PCR there are two cases in which PCR was used for a different goal in this work:

Fusion PCR

The generation of a linear DNA to use as a donor in a yeast transformation can be achieved without the use of *E. coli* and cloning procedures. This approach was used for the synthesis of a donor DNA to change the promoter region of PHA2 gene in chapter 4. It consists on the amplification of two or more fragments that share an overlapping region between them. In a first PCR fragments are amplificated separately, and in a second PCR reaction, the fragment products from the first reaction are used as a template and oligonucleotides annealing on both ends of the final fused product are used as primers (Figure M.1). In chapter 4, primers nº 47-52 (Table M.4) were used to amplify 3 fragments with overlapping regions between them.

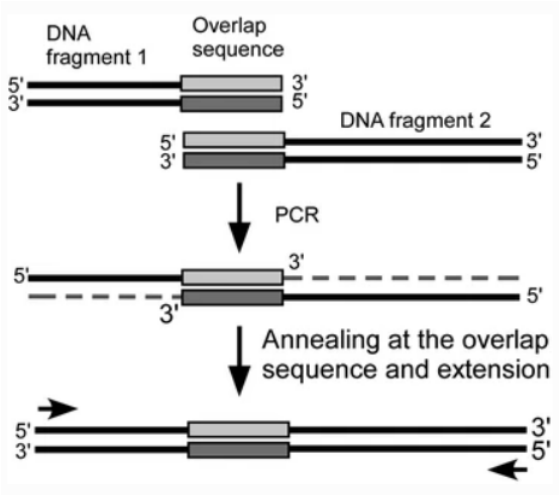

Figure M.1. Fusion PCR by overlap extension. DNA fragment 1 and 2 contain an overlap sequence at the 3′ and 5′ end, respectively. PCR allows annealing of the sequence that overlaps between fragment 1 and 2, followed by DNA polymerase extension from the 3′ end of the overlap sequence. Primers (arrows) at the both ends of the fused fragment produce fusion DNA after the extension (Cha-aim et al., 2012).

Site-directed mutagenesis

Two target gene segments were amplified from genomic template DNA by a first PCR in a similar fashion of fusion PCR, four primers were used, including two flanking primers containing restriction enzyme sites and two mutagenic primers. Mutagenic primers constitute the overlapping area mentioned above, in this case it bears a point mutation to produce the allelic variants ARO3*, ARO4* and ARO7*, the primers used in this case are primers nº 62, 63, 66, 68, 70 and 71 (Table M.4). After the initial PCR, overlapping gene segments were generated that were subsequently used as a DNA template for a second PCR driven by only the flanking primers, generating the full-length product with the mutation of interest (Figure M.1). These fragments were created to be cloned into a vector by flanking them with the restriction enzymes recognition sequence for traditional cloning.

Yeast RNA extraction and expression analysis

To study global gene expression under melatonin synthesis conditions, preinocula of BY4743 were grown overnight in SC medium and inoculated in 250 mL shake flasks containing 100 mL of SC medium to an initial OD_{600} of 0.15 and incubated at 28°C with 150 rpm orbital shaking. When cells reached OD_{600} of 0.8, a supplementation of 5-methoxytryptamine was added to a final concentration of

1 mM. Non-supplemented cultures were used as negative controls and 12.5 mL of each culture were taken as samples 15 and 45 min after supplementation. For each sample, cells were pelleted and snap frozen with liquid nitrogen and stored at -80° C for further RNA extraction. Supernatant was also collected to determine melatonin production under these experimental conditions by UHPLC-MS/MS.

To assess the overexpression of *PAA1* in yeast, we performed quantitative PCR (qPCR) on cells bearing plasmid p426GPD-PAA1 and the wild type control. Cells were grown overnight in SC-ura and SC media, respectively, then inoculated in shake flasks as described above. When they reached an OD_{600} of 0.8 cells were pelleted, snap frozen and stored at -80° C until RNA extraction.

To extract RNA, cell pellets were resuspended in 0.4 mL of LETS buffer [0.1 M LiCl, 0.01 M EDTA, pH 8.0, 0.01 MTris-HCl, pH 7.4, and 0.2% (wt/vol) SDS] and added to 2 mL screw tubes containing 0.4 mL of phenol (pH 4.5)-chloroform (5:1) and 0.3 mL of glass beads. Then cells were ruptured with a Tehtnica MillMix 20 homogenizer (Tehtnica, Slovenia). Supernatants were extracted with phenol–chloroform (5:1) and chloroform-isoamyl alcohol (24:1). RNA was precipitated twice overnight at -20° C, first by adding 2.5 volumes of 96% ethanol and 0.1 volume of 5 M LiCl, and secondly by adding 2.5 volumes of 96% ethanol and 0.1 volume of 3 M sodium acetate. RNA was finally resuspended in RNase-free MilliQ water and the concentration was determined in a NanoDrop spectrophotometer (Thermo Scientific, United States).

RNA sequencing (RNAseq)

RNA sequencing was performed by SCSIE (Central Support Service for Experimental Research of the University of Valencia). Briefly, RNA quality was determined with a Bioanalyzer 2100 (Agilent) and concentration measured with a Qubit®2.0 (Life Technologies, USA), yielding RNA integrity numbers (RIN) between 9.4 and 9.7, thus indicating nearly intact RNA, and concentrations ranged between 400 and 900 ng/mL across all samples. Libraries for RNA-Seq were prepared with TruSeq® stranded mRNA Kit (Illumina, USA) following the manufacturer's protocol and subsequently sequenced on an Illumina HiSeq 2500 with 2×75-bp paired-end reads. Adapters from raw reads were trimmed with

trimmomatic. Adapter content and read quality were assessed with *FastQC*, then reads were mapped to the S288c genome with *Bowtie2* and counts tables were obtained with *htseq-count*. The gene expression abundance was normalized by log2-CPM (counts per million, corrected for the different library sizes, expressed in a log2 scale) using *edgeR* (v3.36.0). *limma* package (v3.50.3) was used to estimate log2-CPM mean variance, establish the different contrasts of hypothesis and an empirical Bayes moderation of the standard errors was applied to increase statistical power of differentially expressed genes (DEGs). Significance ($p < 0.05$) was accounted on the corrected *p*-value obtained from the tests applied by *eBayes* module (limma package). Whole statistical computing was run on R software (v4.1.3).

Enrichment of GO terms and gene clustering was analyzed based on the identified DEGs. Specific gene functions and biological pathways were annotated according to SGD (http://www.yeastgenome.org) and UniProt (http://www.uniprot.org/). The inter-action networks of DEGs were obtained using the STRING v11.5 database (http://string-db.org/).

Quantitative PCR (qPCR)

RNA used in qPCR analysis was treated for 15 min at 25°C with DNase I RNase-free (Roche, Switzerland) according to the manufacturer's recommendations using 1 µg of total RNA from each sample. NZY First-Strand cDNA Synthesis kit (NZTtech, Portugal) was used to synthesize cDNA from the DNase I-treated RNA following the manufacturer's protocol. Quantitative real-time PCR was performed in a Light Cycler 480 II (Roche) using the SYBR Premix Ex Taq kit (TaKaRa, Japan) for fluorescent labeling. For this purpose, 2.5 µL cDNA were added to each reaction at a final volume of 10 µL. The real-time PCRs were performed using 0.2 µM of the corresponding oligonucleotides under the following conditions: 95°C for 10 s, followed by 40 cycles of 10 s at 95°C and 15 s at 55°C. At the end of the amplification cycles, a melting-curve analysis was conducted to verify the specificity of the reaction. A standard curve was made with serial dilutions of the cDNA sample ($2{\cdot}10^{-1}$, $1{\cdot}10^{-1}$, $2{\cdot}10^{-2}$, $1{\cdot}10^{-2}$, $2{\cdot}10^{-3}$, $1{\cdot}10^{-3}$). The primers used to determine the transcript levels are represented in Table M.4.

Analytical methods

UHPLC-MS/MS analyses were performed in the mass spectrometry section of the Central Support Service for Experimental Research of the University of Valencia (SCSIE). Samples were diluted 1:1 with methanol, centrifuged at 7500 × g for 10 min at 4°C and filtered through a 0.22 µm nylon filter before UHPLC-MS/MS analysis.

Melatonin and related compounds determination by UHPLC-MS/MS (Chapter 2)

Liquid chromatography was performed in an Acquity ultra-high-performance liquid chromatography (U-HPLC) (Waters, USA) in an Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) column (Waters, USA) with mobile phases A (0.5 % formic acid in water) and B (acetonitrile). The flow rate was 0.4 mL/ min, and the injection volume was 5 µL. The gradient program was as follows: 0–0.5 min, 95:5 % (v/v), 0.5–3.5 min 0:100 % (v/v), and 3.5–7 min 95:5 % (v/v). The column temperature was set at 30°C.

An ACQUITY® TQD triple quadrupole mass spectrometer equipped with a Z-spray electrospray ionization source was used for detection purposes. Spectra were acquired in the positive ionization mode using multiple-reaction monitoring method employing an interchannel delay of 0.07 s. The multiple-reaction method transitions were m/z 177 → 132.21 and 177 → 160.03 for serotonin, m/z 219 → 159.96 and m/z 219 → 202.00 for N-acetylserotonin, m/z 233 → 174.10 and 233 → 216.10 for melatonin, and m/z 162.82→117.20 and 162.82→144.10 for tryptophol.

Metabolomic analysis from extracellular media directed to tryptophan related metabolites (Chapter 3).

Samples were thawed at room temperature, vortexed for 15 s and centrifuged at 10000 × *g* for 15 min at 4°C. 50 µL of samples were placed into 1.5 mL eppendorf tubes and mixed with 50 µL of an internal standard solution containing hydroxytryptophan-D_4, L-Kynurenine-D_4, indole-D_5-3-acetamide, 4-chloro-kynurenine-$^{13}C_2$,^{15}N, 6-hydroxymelatonin-D_4, kynurenic acid-D_5, PAGN-D_5,

phenylalanine-D_5, serotonin-D_4, tryptamine-D_4, tryptophan-D_5, xanthurenic acid-D_4 and phenylalanine-D_5 (900 nM each). Samples are mixed (vortex, 15 s) and transferred to a 96-well plate for UPLC-MSMS analysis. The quantification of the set of metabolites including tryptophan and phenylalanine metabolites was carried out on an Acquity-Xevo TQS (Waters, Milford, USA) system equipped with an electrospray ionization source using a previously validated method (Lario et al., 2017) using the MRM conditions summarized in **Table M.6**.

Samples were analysed using an Acquity HSS T3 C_{18} (100 × 2.1 mm, 1.8 µm) column. Mobile phases were H_2O (0.1% v/v HCOOH) (A) and (0.1% v/v HCOOH) CH_3CN (B). The gradient elution was as follows: phase B was held 2% from 0 to 0.5 min, then increased linearly to 45% over the following 5 min. Then phase B was increased to 90% in 0.2 min followed by a fast return to initial conditions between 5.7 and 6 min, which were held for 1.5 min for column re-equilibration. Injection volume, flow rate and column temperature were set at 3 µL, 550 µL/min and 55°C, respectively. Autosampler temperature was set at 6°C during sample analysis. Electrospray ionization was carried out using the following conditions: capillary 2.9 kV, cone 25 V, source temperature 120°C, desolvation temperature 395°C, N_2 cone and desolvation gas flow rates were 150 and 800 L/h, respectively.

Table M.6. MRM parameters for the quantification of Trp pathway metabolites and other endogenous metabolites in directed analysis from chapter 3.

	MRM
SAM	298.9 > 136
Guanine	152 > 110
SAHC	385.1 > 134
Hypoxanthine	137>110
Aminophenol	110.14 > 92
p-Tyrosine	182.1 > 136.1
Guanosine	284 > 152
m-Tyrosine	182.1 > 136.1
3-Hydroxy Kynurenine	225.1 > 110
5-Hydroxy-Tryptophan	221.1 > 162.2
5-Hydroxy-Tryptophan (D4)	225 > 208
Serotonin (D4)	181 > 164
Serotonin	177 > 115
o-Tyrosine	182.1 > 136.1
Kynurenine (D4)	213 > 98

Kynurenine	209 > 94
5HIAA	192.1 > 91.1
Phenylalanine (D5)	171 > 125
N-Formyl-Kynurenine	237.1 > 136
Phenylalanine	166.1 > 91
3-Hydroxy anthranilic acid	153.9 > 80
Tryptophan (D5)	210 > 193
Tryptophan	205 > 118
Xanthurenic acid (D4)	210 > 164
Xanthurenic Acid	206.1 > 132
Kynurenic acid	190 > 89
Kynurenic acid (D5)	195 > 149
Tryptamine (D4)	165 > 148
Tryptamine	161 > 122
N-Acetyl-5-Hydroxy-Tryptamine	219.2 > 115
5-Methoxytryptamine	191 > 143
4-Chloro-Kynurenine	243.1 > 128
4-Chloro-Kynurenine (12C2, 15N)	246 > 228
PAGN (D5)	268 > 145
PAGN	263 > 127
6-Hydroxymelatonin (D4)	253 > 194
Anthranilic acid	137.89 > 120
2,3-Pyridinedicarboxylic acid	168 > 124
Indole-3-acetamide (D5)	180 > 133
Indole-3-acetamide	175-1 > 103.4
For.-N-Ac.-Methoxy-Kynurenine	265.2 > 114
Indolelactic acid	204 > 116
Melatonin (D4)	237 > 178
Melatonin	233.17 > 159
3-Indoleacetic acid	174 > 130
Tryptophol	162.1 > 103
3-Indoleacetonitrile	157.1 > 64

Untargeted metabolomic analysis from extracellular media (Chapter 3)

Samples were thawed at room temperature, vortexed for 15 s and centrifuged at 10000 × *g* for 15 min at 4°C. 100 µL of samples were placed into 1.5 mL eppendorf tubes and mixed with 60 µL of H_2O and 60 µL of a formic acid solution (0.1% v/v) in acetonitrile. Samples are mixed (vortex, 15 s) and transferred to a 96-well plate for UPLC-MSMS analysis. Sample analysis was carried out in an Agilent 1290 Infinity UPLC chromatograph using a UPLC BEH C18 column (100 × 2.1 mm2, 1.7 µm, Waters, Wexford, Ireland). Autosampler and column temperatures were set to 4 and 40°C, respectively, and the injection volume was 4 µL. Gradient elution was performed at a flow rate of 400 µL/min as follows: initial conditions of 98% of mobile phase A (H_2O (0.1% v/v HCOOH)) were kept for 0.5 min, followed by a linear gradient from 2 to 25% of mobile phase B (CH3CN (0.1% v/v HCOOH)) in 3 min and from 25 to 98% B in 4.5 min. Conditions of 98% B were held for 1 min; then, a 0.25 min gradient was used to return to the initial conditions, which were held for 2 min.

All solvents were of LC-MS grade and were purchased from Scharlau (Barcelona, Spain). Ultrapure water was generated with a Milli-Q water purification system (Merck Millipore, Darmstadt, Germany).

Formic acid (≥95%) was obtained from Sigma-Aldrich Quiḿica SA. Full-scan MS data from 100 to 1700 m/z with a scan frequency of 5 Hz were collected on an iFunnel quadrupole time-of-flight (QTOF) Agilent 6550 spectrometer (Agilent Technologies, CA, USA) in the TOF MS mode. The following electrospray ionization parameters were selected in positive and negative modes: gas T, 200°C; drying gas, 14 L/min; nebulizer, 35 psig; sheath gas T, 350°C; sheath gas flow, 11 L/min. Automated UPLC-ESI(+/-)-QqTOF (MS/MS) analysis of the QC was carried out to support compound identification using two 20 V as collision energy.

Data dependent MSMS acquisition and metabolite annotation are described in Ten-Doménech et al. (2020). System conditioning and data clean-up was carried out as described in (Martínez-Sena et al., 2019).

Peak table generation was carried out using XCMS software. The *centWave* method was used for peak detection with the following parameters: mass accuracy: 20 ppm, peak width: (5,25), snthresh: 12, prefilter: (5,5000). A minimum difference in *m*/*z* of 7.5 mDa was selected for overlapping peaks. Intensity weighted *m*/*z* values of each feature were calculated using the *wMean* function. Peak limits used for integration were found through descent on the Mexican hat filtered data. Grouping before and after RT correction was carried out using the *nearest* method and 9 s as *rtCheck* argument. Finally, missing data points were filled by reintegrating the raw data files in the regions of the missing peaks using the *fillPeaks* method. Peak area, RT and peak widths, calculated as the difference between the end and start of the integration points, were extracted from XCMS data for each sample and UPLC-MS feature.

Data clean-up was carried out after within-batch effect correction using the set of blank samples for each batch independently. UPLC-MS features were classified as 'informative' and retained for further analysis if the ratio between the minimum values in biological samples and the maximum value in blanks is >3. Due to the limited number of analyzed samples, QCs were injected at the beginning and end of the sequence, but no within-batch effect correction was required (Sánchez-Illana et al., 2018).

Hydroxytyrosol and tyrosol determination by UHPLC-MS/MS (Chapter 4)

Tyrosol and HT were analyzed using an Acquity ultra-high performance liquid chromatography (U-HPLC) system (Waters), equipped with a Kinetex XB-C18 100Å column (2.1 x 100 mm, 1.7 µm; Phenomenex) when expected concentrations were below 200 $\mu g \cdot mL^{-1}$. Buffer A (milliQ water with 1 mM ammonium acetate, pH 5) and buffer B (acetonitrile) were used as a mobile phase. Analytes were eluted at 40°C with a flow rate of 0.4 ml/min and the injection volume was 5 µL. The gradient program was as follows: 0 – 2.5 min 97:3 % (v/v), 2.5 – 3.5 min, 10:90 % (v/v), 3.6 – 8 min, 97:3 % (v/v). Eluted compounds were detected using an ACQUITY® TQD triple quadrupole mass spectrometer (Waters) equipped with a Z-spray electrospray ionization source. Spectra were acquired in negative ionization multiple reaction monitoring mode employing an

interchannel delay of 0.1 s. The multiple-reaction method transitions were m/z 137 → 93 and 137 → 106 for tyrosol and m/z 153 → 123 for HT.

Hydroxytyrosol and other phenolic compounds determination by HPLC-PDA (Chapter 4)

Extracellular HT and aromatic higher alcohols (tyrosol, 2-phenylethanol and tryptophol) were detected by HPLC on an Acquity ARC sytem core (Waters, USA) equipped with a photodiode array wavelength detector (Waters 2998 PDA), a quaternary pump, an autosampler and an online degasser. Chromatographic separation was carried out in an Accucore™ C18 (4.6 × 150 mm, 2.6 µm) column (Thermo Fisher Scientific) with mobile phases A (0.01 % TFA acid in water) and B (acetonitrile). The flow rate was 1 mL/min and the injection volume was 10 µL. The gradient program was as follows: 0–18 min, 100 % A (0 % B), 18-19 min 90 % A (10 % B), 19-28 min 75 % A (25 % B), 28-31 min 0% A (100 % B) and 31-39 min 100% A (0 % B). The column temperature was set at 30°C and samples were left at 10°C. The PDA detector was set at λ = 210 nm. The identification of all the aromatic higher alcohols and HT was based on their retention times, determined by injecting the reference standards individually and as a mixture. The calibration curves of each analyte, i.e., peak area *vs*. concentration, were linear and data were fitted by the least-squares method. Linearity was assessed by the least squares fitting of the independent six-point calibration curves. The retention time for HT, tyrosol, 2-phenylethanol and tryptophol was 9.366 min, 13.112 min, 21.958 min and 22.769 min, respectively.

Colorimetric assay for rapid hydroxytyrosol determination (Chapter4)

The colorimetric assay for hydroxytyrosol determination referred to in chapter 4 is described in Chen et al. (2019) and it is based on the oxidation of the *o*-diphenol group to *o*-quinone by the action of the sodium periodate, this reaction causes a color change to yellow in the case of HT and dopamine, but not for tyrosol, tyrosine or tyramine and the colorimetric difference can be quantified measuring OD_{400}. Briefly, 180 µL of the supernatant of previously centrifuged samples was added into 20 µL of 100 mM sodium periodate in a transparent 96-well plate, and

OD_{400} of the reaction mixture was determined in a SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany).

Statistical analysis

All the experiments carried out in this thesis were performed at least in triplicate, with the exception of the ALE in chapter 3. Data are expressed as the mean values ± standard deviation when possible. Experimental results were analyzed and compared by statistical analyses such as *t*-tests, ANOVA and linear and non-linear regression models, using R and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) software. A confidence level of at least 95% was considered for significance.

RESULTS

CHAPTER 1

Adapting yeast biosensor to detect and quantify extracellular yeast-secreted melatonin.

1.1. Introduction

The recently described relation between aromatic amino acids metabolism and melatonin synthesis by *S. cerevisiae* raises interest from different fronts. The ability to synthesize melatonin is nowadays established in many yeasts, as well as in multiple representatives from every kingdom, although specific genes responsible for this process are still not described in yeast. To study melatonin production ability across yeasts from different clades and environments can give a great insight on melatonin function and origin in yeast. From an industrial point of view, melatonin turns out to be a desirable bioactive compound to be present in fermented foods and beverages, and the use of yeasts with the ability to produce and increase melatonin's concentration is always an aim to enrich fermented products due to melatonin's antioxidant properties. From a biotechnological approach, the use of yeast as a cell factory to produce different interesting compounds such as melatonin stands out as one of the best production systems, as it unites different important requirements that make *S. cerevisiae* suitable for it, namely ease of engineering, robustness, a good substrate spectrum and a great knowledge base, among others (Nielsen, 2019).

The study of melatonin production by yeast poses a great challenge that resides on the generally low amount of melatonin naturally produced by them. This fact shows that extremely sensitive and reliable analytical techniques are required for its study, which allow melatonin to be detected and quantified with confidence and robustness. Currently, the most powerful technique in terms of low limit of detection (LOD) and limit of quantitation (LOQ) is the ultrahigh performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS), nonetheless, this technique often requires an extraction step before chromatography such as solid-phase extraction (SPE) or liquid-liquid extraction. Besides of sample preparation, this technique demands costly infrastructures and equipment and highly trained operators. In a context of determining potential melatonin producer strains from a large pool of natural or industrial strains, having a quick, reliable and inexpensive method for detecting and quantifying melatonin directly from supernatant of yeast cultures is of great interest. As alternative rapid detection methods for melatonin there are important

milestones achieved in the recent years such as voltammetry of immobilized particles method (VIMP) which can be applied directly to yeast cells (Muñiz-Calvo et al., 2017). This method has been successfully applied to monitor melatonin content in different yeast strains but its discriminating power is not comparable to chromatographic techniques. Other more specific methods rely on the specificity of monoclonal antibodies, such as radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) (Poeggeler et al., 1994; Rodriguez-Naranjo et al., 2011 b). RIA method is reported to overestimate melatonin concentration when compared to gas chromatography mass spectrometry tandem (GC-MS) and to yield false positive results. And for ELISA methods, there are available kits for biological samples like urine, but their use in other matrices like fermented food, drinks or yeast growth still needs to be optimized. The development of new monoclonal antibodies suitable for these complex matrices is critical for a correct detection and quantitation. A more recent study uses the mammalian melatonin receptor MTNR1B expressed in mammalian cells in a whole-cell bioassay where melatonin is detected by receptor MTNR1B, and it activates β-lactamase enzyme (BLA) that cleaves a FRET (Förster resonance energy transfer)-based substrate (CCF2/4) that presents green fluorescence when intact but blue fluorescein activity when cleaved (Morcillo-Parra et al., 2019 b). This method showed an extraordinary high sensitivity, even comparable to HPLC-MS/MS results reported in previous studies (Rodríguez-Naranjo et al., 2011; Vigentini et al., 2015), but the whole-cell bioassay requires a daily good maintenance of the cell lines before the assay and it comprises long periods of exposure to ligand, making the whole process not ideal when aiming for a quick, inexpensive and practical method.

Yeast-based biosensors are attractive and promising tools for this objective, as they offer a practical solution to address a cost-effective way to rapidly analyze a high sample number, as long as they are sensitive and specific enough to discriminate and quantitate melatonin from an unprocessed yeast growth media supernatant. Many advances have been made towards yeast biosensors development, and particularly to melatonin detection and quantitation. Recently, Shaw et al. (2019) engineered a tunable and modular G-protein coupled receptor (GPCR) signal transduction system and applied it to five different receptors to respond to different peptides, metabolites and hormones relevant to human

health, being melatonin one of them. GPCRs are a major sensing mechanism in eukaryotes, and so they are for yeast, as they are responsible for the detection of mating type hormones that drive the activation of different mating genes. By deleting yeast native GPCR receptor for α-factor and up to 15 genes involved in the signal transduction they isolated the core signal transduction mechanism to avoid any gene activation/repression not related with the melatonin sensing function. To this refactored pathway three important modifications have been integrated into the genome using linear cassettes. This modifications consist in the overexpression of a human GPCR receptor for melatonin (MTNR1A), the overexpression of the Gα subunit encoded by *GPA1*, expression of a green fluorescent protein as a reporter gene (*sfGFP*) preceded by a synthetic promoter bearing *LexO* bacterial upstream activating sequences (UAS) and a synthetic transcription factor consisting of the pheromone response domain (PRD) from native gene *STE12* fused to the DNA-binding domain from bacterial operator *LexA* (Figure 1.1). These last three modifications were based on three key design principles: to increase sensitivity by increasing GPCR numbers, to reduce basal activity by increasing Gα protein (Gpa1) and thus reducing free Gβ (Ste18) and Gγ (Ste4) and to modulate the pathway output by using synthetic transcription factors and promoters. Regarding the operational range of the biosensor system, defined as the concentration span between 5% and 95% of the activated response, it is a characteristic that is mainly determined by the ligand-binding properties of the receptor. This operational range can be narrowed to obtain a switch-like behavior of the biosensor, preferably by the use of a two-cell system approach, where there's an amplifier cell that senses through MTNR1A and responds secreting α-factor, and a second cell that senses α-factor and responds with *sfGFP* gene expression. Operational range can also be widened using a consortia of biosensors with different sensitivities to the ligand in equal proportions, resulting in a more linear output signal but usually at the expense of a LOD raise. Although narrowing the operational range and gaining sensitivity can be interesting for obtaining a digital response in samples with extremely low concentrations of melatonin, the scope of this chapter is to increase sensitivity in a single-population approach to still be able to successfully quantify melatonin in a low range but maintaining simplicity during the assay procedure.

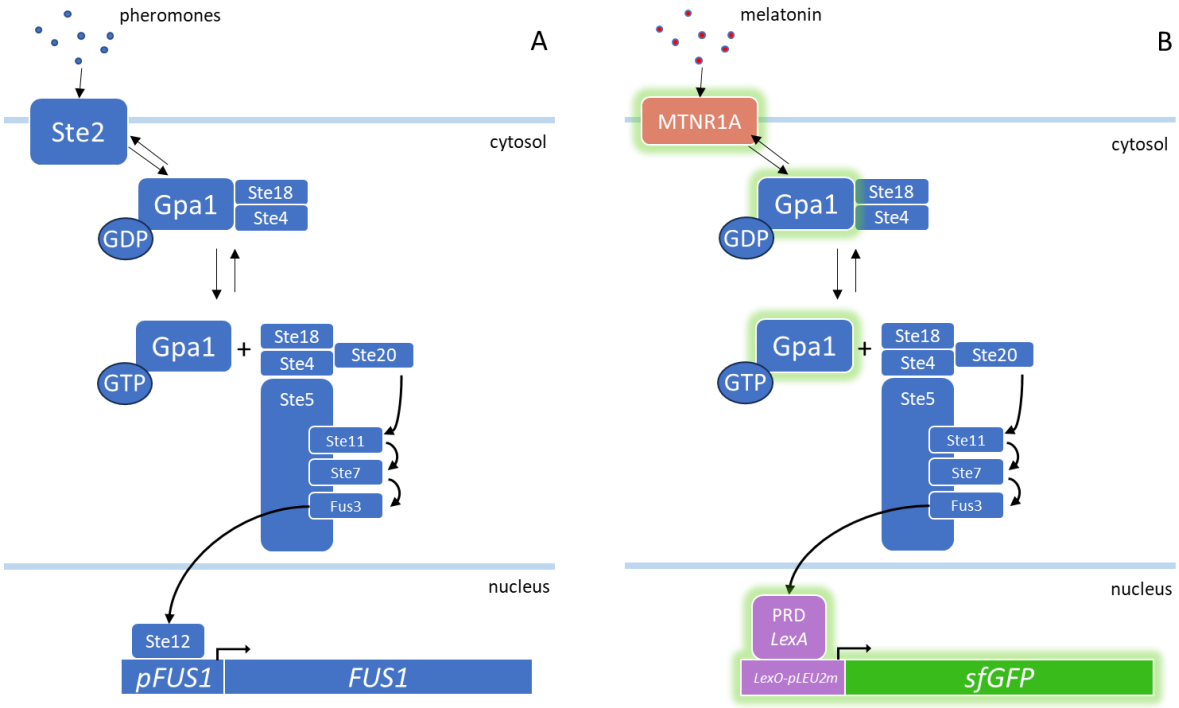

Figure 1.1 Yeast α-factor signaling pathway for FUS1 gene activation (A), binding of pheromones to the specific GPCR Ste2 provokes GDP-GTP exchange on Gα subunit (Gpa1) and the release of the dimer Gβγ (Ste18 and Ste4). This recruits the MAP-kinase cascade inducing a transcriptional response via Ste12 transcription factor. In the strain used in this study (B) native GPCR have been replaced by MTNR1A melatonin receptor, native *GPA1* gene is overexpressed and transcription factor Ste12 is substituted by synthetic transcription factor using native PRD and *LexA* DNA binding domain to decouple pheromone response pathway from pheromone response elements (PRE)-containing genes. *LexA*-PRD targets a synthetic promoter *LexO(6x)-pLEU2m* that drives *sfGFP* expression as a reporter gene. Modifications addressed in this study are highlighted in green.

We adapted one of the heavy engineered melatonin biosensor strain from Shaw et al. (2019) to a small volume 96-well plate system and we initially replicated previous results in melatonin detection. Then we significantly reduced sensitivity to make the system suitable for melatonin detection from yeast growth media supernatant. For this purpose, in an initial stage, MTNR1A expression numbers are increased by integrating multiple copies into the genome. We also addressed modifications downstream of the signaling pathway by integrating extra copies of the reporter system to evaluate a possible increase in the output signal and the Gα subunit to maintain a reduced basal activity and a good signal to noise ratio.

We evaluated the curve fitting of the different modifications towards melatonin concentration. Then we tested the sensing ability of the different engineered strains when ligand is diluted in the growth media YNB80, which has tryptophan and ammonium as nitrogen sources, and we performed recovery assays from this matrix with known concentrations of melatonin to determine accuracy and precision of the method. Finally, as a proof of concept, we analyzed 101 yeast strains from different origins and species after growth in a simple tryptophan-enriched laboratory medium and assessed their melatonin production potential.

In this chapter, a detection and quantitation method based on a melatonin GPCR yeast biosensor strain is tested and adapted to the context of the search for yeast strains with the ability to naturally produce melatonin from the tryptophan present in the growth media. After modifying key features on the original design of the strain we were able to perform a rapid screening of 101 strains grown under the same conditions, allowing us to select good candidates to further characterize and establish their melatonin production efficiency and suitability for industrial fermentations and other applications.

1.2. Results and Discussion

1.2.1. Evaluating original biosensor strain potential in our growth and detection systems.

The yeast biosensor strain used in this study for implementing modifications is derived from BY4741 and it bears a melatonin receptor MTNR1A under the control of *HHF2p* and expresses a reporter system consisting on the expression of *GPA1* under the control of strong promoter *PGK1p*, a synthetic transcription factor (*LexA-PRD*) controlled by *RAD27p* and the superfolder green fluorescent protein (*sfGFP*) immediately preceded by the last 125 nucleotides of *LEU2p* and controlled by six bacterial UAS sequences *LexO* (Figure 1.2). In order to establish a starting point and evaluate the suitability of this strain for our purposes we assayed different melatonin concentrations ranging from 100 µM to 0.1 nM (-4 to -10 log[M]), similarly to the concentrations assayed originally by Shaw et al. (2019) but adapting the assay to a small volume 96-well plates using just 220 µL

of culture and ligand was delivered by adding 30 µL to the desired final concentration as described in Materials and Methods section. This adjustment in the assay allowed us to use CLARIOstar® reader directly on the incubation plates and set a volume proportion between samples and biosensor cell culture that avoid excessive dilution of the samples. Initial results showed a curve fitting with an EC50 of 117 nM, and an operational range of 3.5 orders of magnitude (Figure 1.2C). Similar results were also observed in the original study (Shaw et al., 2019).

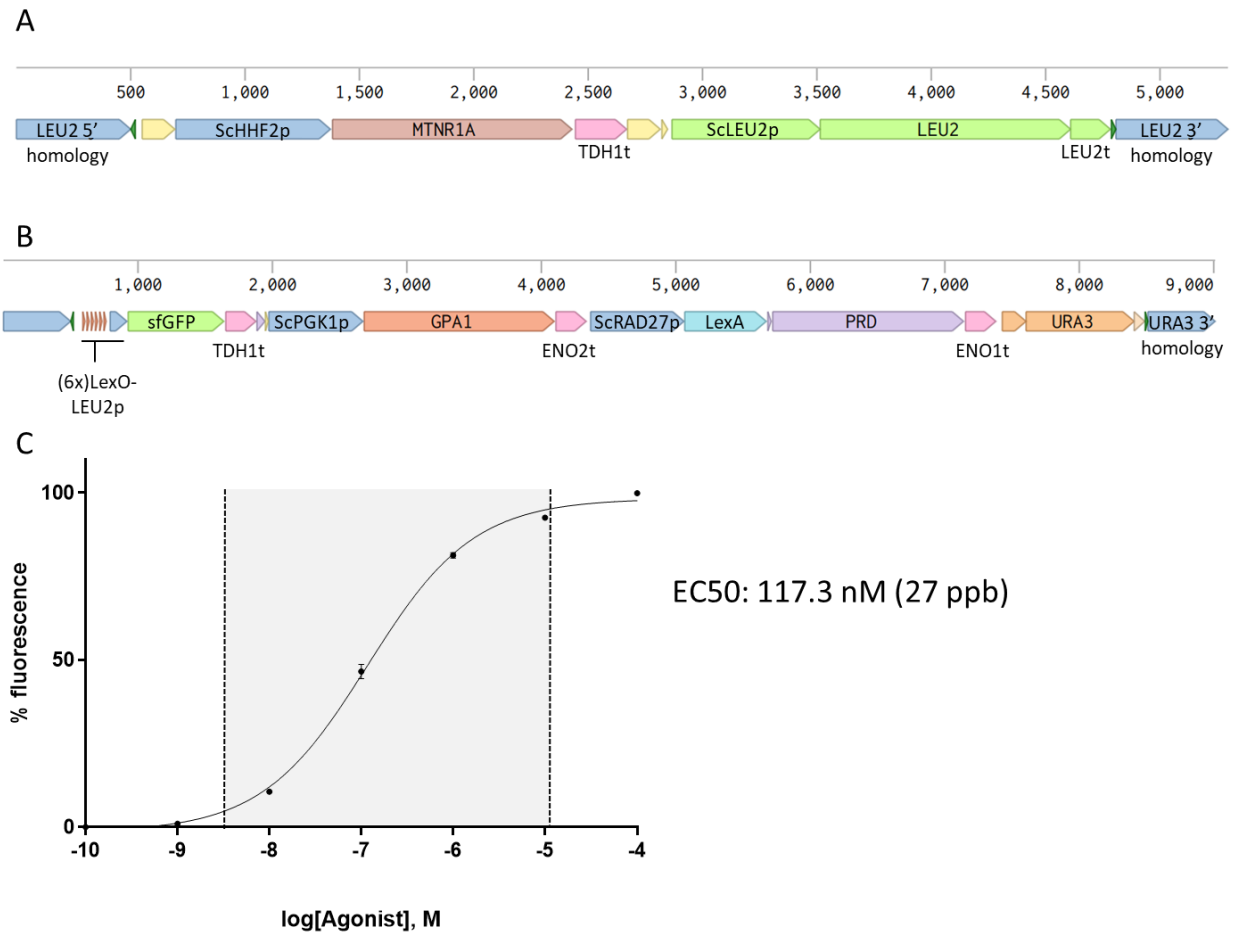

Figure 1.2 Original biosensor features. (A) GPCR integration cassette. This strain has MTNR1A integrated in *LEU2* locus. (B) Reporter integration cassette bearing a synthetic transcription factor (*LexA-PRD*), a synthetic promoter driving the expression of the reporter gene *sfGFP* and a Gα (*GPA1*) overexpression. This cassette is integrated in *URA3* locus. (C) Curve fitting of yWS1544 strain derived from Shaw et al. (2019). Measurements are normalized sfGFP fluorescence levels and shown as mean ± SD of triplicates. Curve fitting and parameters were obtained using GraphPad Prism 9.5.1 variable slope (four parameter) nonlinear regression fit.

Due to the conditions of the assay, a 117 nM concentration would be equivalent to 227 ng·mL^{-1} or 977 nM of melatonin in the original sample. Currently, the highest amounts of melatonin detected as a result of natural yeast metabolism do not exceed 160 ng/mL in wine samples (Rodriguez-Naranjo et al., 2011 b; Marhuenda et al., 2016), and, in these type of samples that rarely achieve more than 100 ng/mL, the matrix complexity must be observed when using a fluorescent based biosensor strain as the richness of polyphenols and other aromatic compounds with optical properties may affect final readings and interpretation. As the scope in this chapter encompasses the adaption of the existing biosensor to a natural yeast-produced melatonin detection and quantitation, the improvement of sensitivity becomes a priority. So far, the operational range and sensitivity of this strain and the possibility to use a 3/25 sample dilution are promising features in that direction.

1.2.2. Enhancing of biosensor capacity

An integration cassette bearing MTNR1A receptor controlled by the constitutive strong promoter *CCW12p* and *URA3* selection marker flanked by sequences for homology recombination with *Ty1* transposable element was constructed using pCfB2988 plasmid as a backbone (Maury et al., 2016) and integrated into yWS1544 strain (original biosensor strain described in previous section). To do so, first *URA3* gene was disrupted to generate strain yWS1544ura$^-$ targeting the active *URA3* gene by cloning a 20-bp target sequence into pWS172 CRISPR-Cas9 plasmid and using the sequence resulting from the annealing of oligonucleotides nº 3 and 4 (Table M.4) as a DNA repair template and then introducing an early stop codon and disrupting the gene. After integration of MTNR1A cassette 36 colonies were grown in SC media and subjected to a concentration of 1 mM of melatonin to perform a fast screening and the most fluorescent colony was selected.

Selected candidate (“+GPCR” strain) was assayed under the same melatonin concentrations described above, obtaining an EC50 of 22.4 nM and a similar operational range as the original strain (Figure 1.3A). Despite the significant improvement in sensitivity we wanted to explore if a further enhancement was

possible following the similar approach of inserting more copies of the reporter system to obtain a better signal over background output, especially at low concentrations. After integrating the elements in the reporter cassette (Figure 1.2B) into previously characterized genomic locations *Chr. X-4* and *Ty2Cons* (Jensen et al., 2014; Maury et al., 2016) to generate "+GPCR+rep" and "+GPCR+rep(multi)" strains, respectively.

The strain with an extra copy of the reporter system showed a reduced EC50 when compared to the original strain, but also when compared to "+GPCR" strain. Surprisingly "+GPCR+rep" strain showed a narrower operational range than the two previous strains, covering nearly 2.2 orders of magnitude. When we integrated more than one extra copy of the reporter system into the "+GPCR" modified strain, we observed a further improvement of EC50 value, but at the expense of a great reduction in the maximum output signal, and a drastic reduction of the operational range when compared to original and "+GPCR" strain, only covering 2 orders of magnitude (Figure 1.3).

The integration of extra copies of the reporter cassette notably changed the dose-response curves implying a shortening of the dynamic range, offering a somewhat digital response. A similar effect has been previously achieved by implementing positive feedback loops into the MAPK cascade that magnify the response, bringing it closer to a 0-1 digital response (Ingolia et al., 2007). But, in our case, using a synthetic transcriptional factor is preventing any autoregulatory feedback that Ste12 may exert through its native promoter. This observed change in the dose-response is more likely to occur due to an imbalance between Gα and Gβγ subunits when Gpa1 is overexpressed to such levels. Varying G protein signaling component stoichiometries has been demonstrated to alter the maximum output as an incorrect trafficking of Gβγ in the absence of free Gα may decrease GFP signal in the original biosensor strain (Shaw et al., 2019). Although in the last modifications we exaggerated the expression of Gα (*GPA1*), and therefore an excess of free Gα is expected, to integrate one or more extra copies of *GPA1* under a strong constitutive promoter such as *PGK1p* can lead to a certain metabolic burden, protein misfolding or altered protein turnover rates that appears to be directly impacting signal transduction behavior.

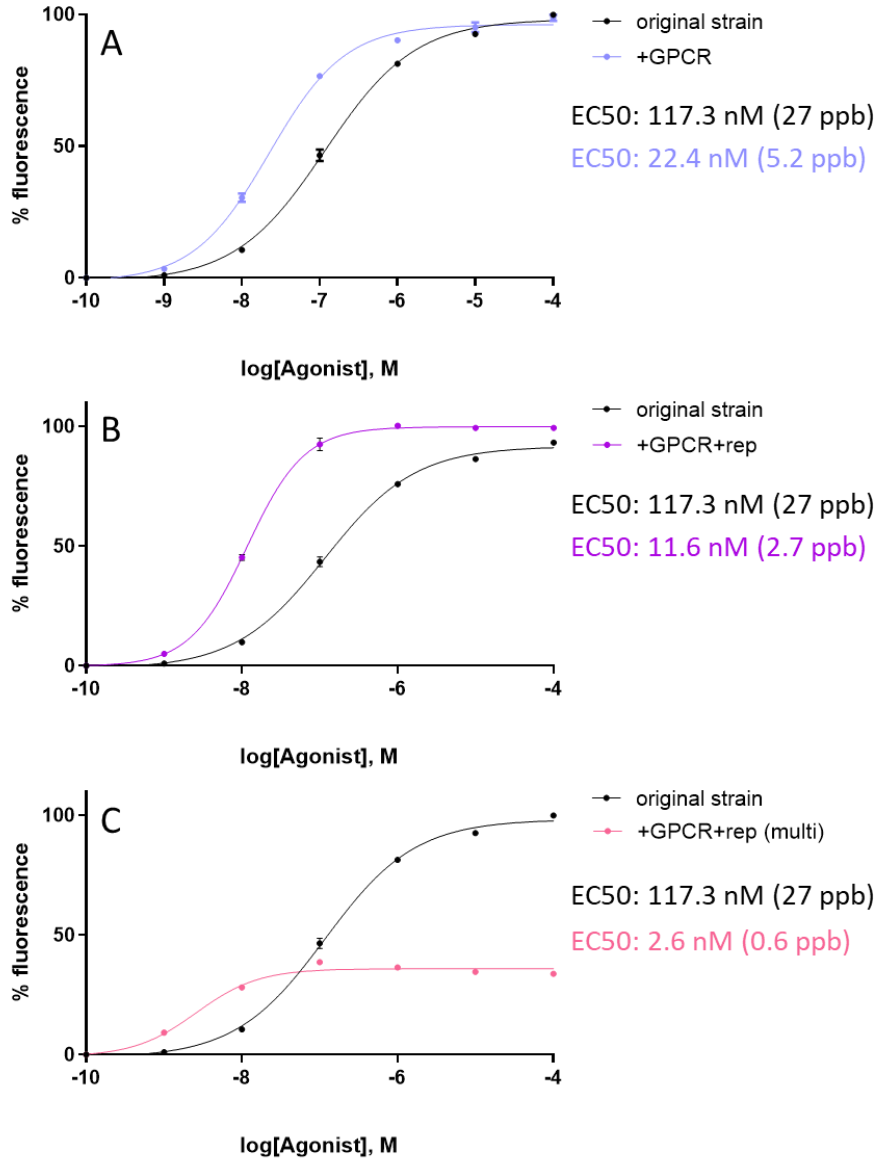

Figure 1.3 Modifications on original strain affecting melatonin sensing properties. A comparison of the dose-response between original biosensor strain and after increasing MTNR1A receptor number ("+GPCR") (A), after increasing MTNR1A and adding one extra copy of the reporter cassette ("+GPCR+rep") (B) and after increasing MTNR1A and integrating reporter cassette multiple times ("+GPCR+rep(multi)") (C). Measurements are normalized sfGFP fluorescence levels and shown as mean ± SD of triplicates. Curve fitting and parameters were obtained using GraphPad Prism 9.5.1 variable slope (four parameter) nonlinear regression fit.

Biosensor strain with the integration of a single extra copy of the reporter system "+GPCR+rep" displayed a higher output signal and lower sensitivity when compared to original strain (Figure 1.3B). Despite of showing a narrower operational range, the EC value of 11.6 nM offers a great potential for our screening purposes as this value equals a 22.5 ng/mL concentration in a

hypothetical sample, which is a concentration value that can be naturally produced by yeast. Regarding strain “+GPCR+rep(multi)” (Figure 1.3C), including more copies of the reporter system resulted in a strikingly lower output signal. This early GFP saturation at low intensities can difficult a quality reading that can result in false positives, and although it might be of interest when looking for a bimodal response rather than a quantitative method to interpolate fluorescence intensity values, we did not consider this strain for later quantitative purposes.

1.2.3. Biosensor evaluation

We believe the use of a defined synthetic media is adequate to start testing a variety of strains for melatonin production in this biosensor system due to its simplicity, as more complex matrixes may negatively impact our detection system, and as in previous studies other synthetic media with tryptophan supplementation have been also used for melatonin production purposes (Vigentini et al., 2015; Germann et al., 2016). Growth medium YNB80 is enriched with tryptophan, the basic precursor for melatonin synthesis, and we used is as a preferred medium for melatonin production, therefore we decided to test the capacity of the biosensor strain “+GPCR+rep” compared to the original yWS1544 in this matrix. In order to evaluate biosensor strains in this media we first tested their response to melatonin when it is dissolved in this media to determine the matrix effect of it, *i.e.* how it affects the dose-response curve when the ligand is dissolved in it instead of distilled water. As expected, the dose-response curves were affected by the sample media, causing a loss of sensitivity and decrease of the maximum signal intensities, especially noticeable in the original strain (Figure 1.4). Although strain with multiple integrations of the reporter system was not considered for quantitation purposes, we also assayed melatonin concentrations ranging from 100 nM to 0.1 nM in YNB80 in this strain and the early output signal saturation at low intensities when compared to the other strains was still maintained (Figure 1.4C), however, we did not observe a strong matrix effect, even resulting in a slightly better dose-response curve.

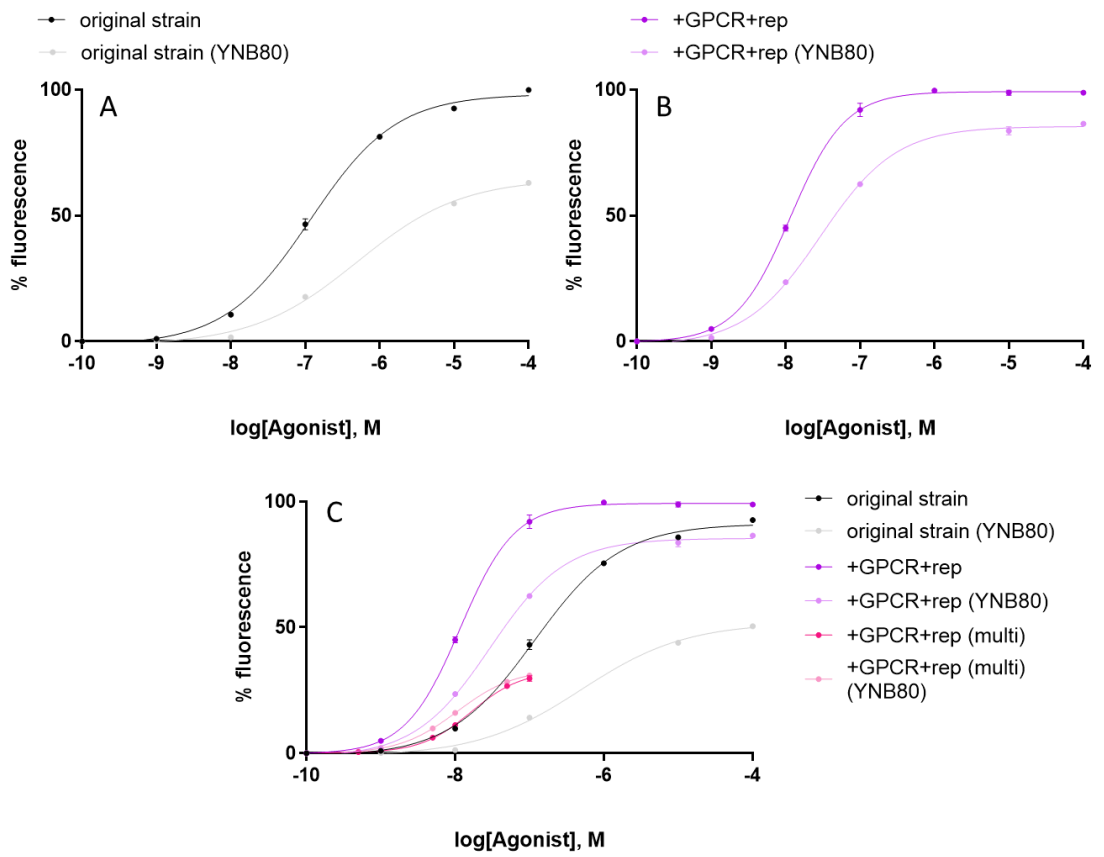

Figure 1.4. Modified yeast biosensor dose-response was evaluated for melatonin detection from growth media YNB80. Original strain showed a substantial decrease of maximum signal and an increase in EC50 from 117.2 to 498.9 nM (A). Our selected strain "+GPCR+rep" displayed less conspicuous changes, although the increase in EC50 raised from 11.6 to 29.4 nM (B). Maximum signal of +GPCR+rep strain almost doubles that from original strain when using YNB80 as a matrix. Strain with multiple integrations is also depicted for comparative purposes (C).

In order to assess accuracy and precision of the selected modified strain "+GPCR+rep" we performed recovery assays from samples of YNB80 spiked with known concentrations of melatonin in a low range that resemble the expected concentrations on positive melatonin natural producers. Melatonin concentrations in these assays ranged from 3 to 30 nM, and recovery rates showed this method offers a good accuracy (80 to 120%) between the assayed values, although precision can be affected when using this growth media and considerable errors can be expected (Table 1.1). With these established parameters we believe it is feasible to determine differences in melatonin concentration in YNB80 media due to yeast metabolism since enough variability

in melatonin production among different species and even among different strains in the same species is expected under the same growth conditions.

Table 1.1. Recovery assays from known concentrations of melatonin spiked in YNB80 media. Each recovery assay was performed in triplicates and repeated three times in three different days. Recovery rates (R) are expressed in percentage of melatonin detected in relation to the real known concentration. Coefficient of variation (CV) agglutinates the errors between samples and between assays performed on different days and thus reflecting the repeatability of the assay when similar matrix and concentrations are expected.

Spiked melatonin (nM)	Sample dilution	YNB80 +trp	
		R (%)	CV (%)
3	30/250	92	26
10	30/250	93	25
30	30/250	113	12

1.2.4. Rapid screening of yeast melatonin production from 101 different strains.

With the advantages achieved by our selected strain, and after assessing the potential and limitations of its use on YNB80 media, as a proof-of-concept we decided to analyze a collection of 101 yeast strains from diverse origins, *i.e.* isolates from natural environment, from different brewing or winemaking environments and also commercial strains (Table M.3). In the same screening we included a control sample consisting of YNB80 media with a known concentration of melatonin (25 nM) and the detected concentration was 21.4 nM (data not shown) which indicated a recovery of 85.6 ± 0.1%. As another positive control, two genetically modified laboratory strains with a BY4743 background, one with the ability to overproduce serotonin and another strain with melatonin production ability, were included in the analysis (strains E19 and G12, respectively). In the case of G12 (melatonin overproducer), we detected 0.15 mg/L of melatonin (664 nM) which is an amount that clearly stands out from the rest of analyzed samples, as expected, while melatonin detected levels for E19 (serotonin overproducer) is in the same range than for other strains (Figure 1.5),

which shows that the overproduced serotonin is not converted into more melatonin in this engineered strain. As expected, most of the strains remained indistinguishable between them in terms of melatonin concentration since spontaneous natural production of melatonin by yeasts occurs in a very inconspicuous manner. Nonetheless, for some of the assayed strains, a clear distinctive melatonin concentration was observed, allowing us to perfectly distinguish those with more melatonin production ability under the tested conditions. Melatonin production of environmental isolates S47 and A11, or commercial strain S14 seem to stand out in *S. cerevisiae* group, while other representatives from *S. bayanus* (A29), *S. kudriavzevii* (A23) or *S. uvarum* (A28 and BMV58) also show melatonin levels clearly above the group's median.

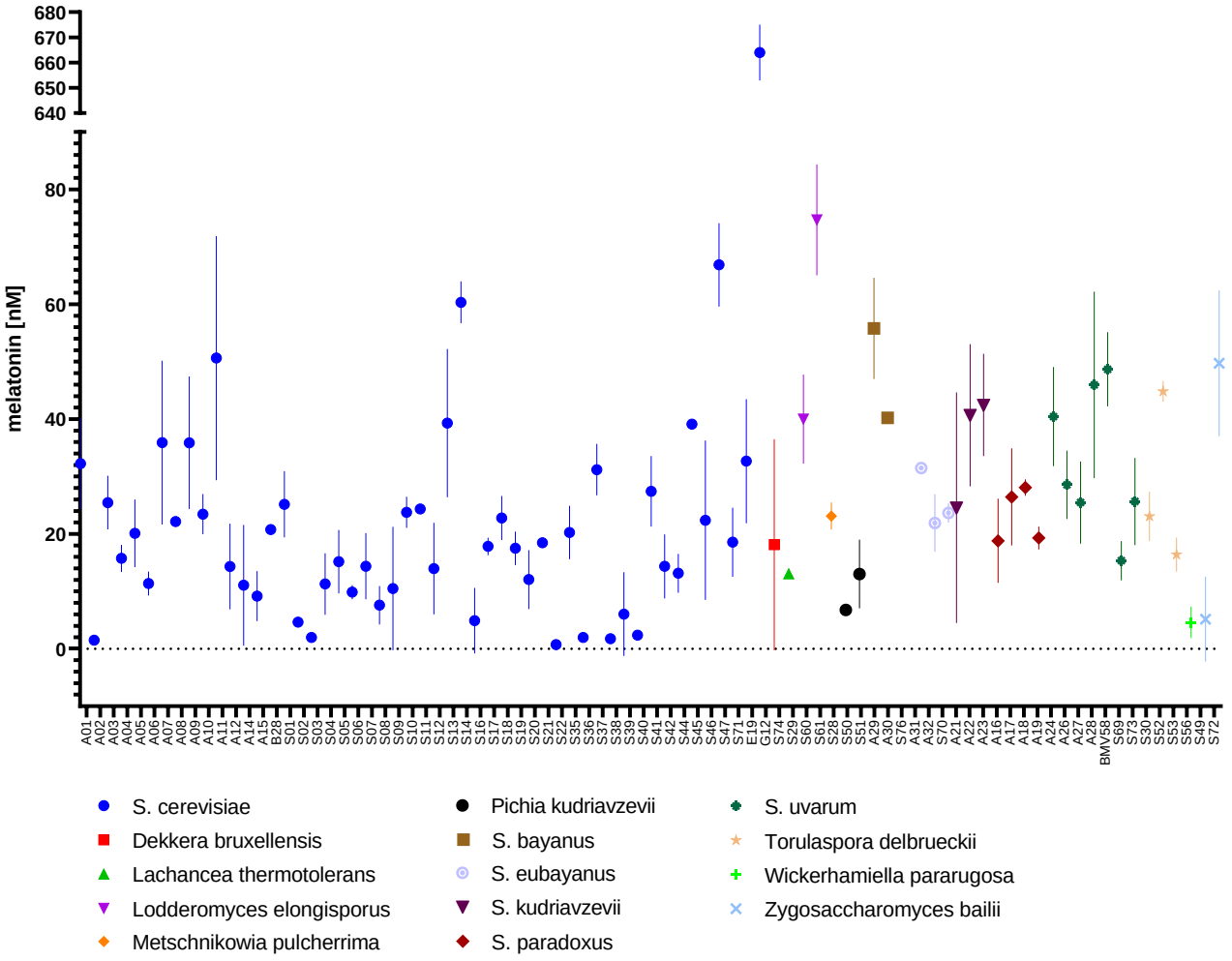

Figure 1.5. Screening of 101 yeast strains. Yeast biosensor strain "+GPCR+rep" was employed in this assay. sfGFP fluorescence for each sample was interpolated in the fitted calibration curve

for melatonin using GraphPad Prism 9.5.1 standard curve interpolation (four parameter) tool. Melatonin concentration values are shown as mean ± SD of triplicates. 17 of the 101 strains assayed were excluded from interpolation due to lack of enough signal.

1.3. Conclusions

In this chapter we address the challenge of improving a heavy engineered yeast biosensor strain by tweaking genetic features that directly affect their sensitivity, maximum signal and signal to noise ratio, to use it as a simple, inexpensive and quick method that allows us to detect and quantify melatonin directly from supernatants of yeast growth media, and thus enabling us to perform extensive screenings of a variety of yeasts grown in monoculture to detect their melatonin biosynthesis potential.

The integration of multiple copies of the melatonin receptor together with the integration of an extra single copy of the reporter system involving the synthetic transcriptional factor, the overexpression of native α subunit of G protein and the reporter gene controlled by a synthetic promoter resulted in a higher maximum output signal, a better signal to noise ratio and a great increase of sensitivity, but it slightly narrows the operational range to perform good quantifications. Although multiple integrations of both GPCR and the reporter system showed a drastic decrease in maximum signal, it also resulted in an early response at low concentrations of melatonin and therefore it presents a great potential to use as a bimodal response system that may allow us to determine presence or absence of melatonin. This is especially interesting, for example, for detecting melatonin from complex matrixes like fermented beverages, where detecting presence or absence of this metabolite may be a priority. But for that purpose, optimization of copy numbers of each genetic modification, and extensive matrices evaluation must be carried out. The genetic improvements we carried out and tested, together with an assay setting that allows us to expose yeast biosensor culture to a significant volume of sample supernatant are crucial to establish a practical and effective method to quickly analyze samples with such low expected concentrations of melatonin.

CHAPTER 2

The role of *PAA1* gene on melatonin biosynthesis in *Saccharomyces cerevisiae*: a search of new arylalkylamine *N*-acetyltransferases.

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2.1. Introduction

After the discovery of melatonin outside the animal kingdom, the research of melatonin in other clades emerged. Thus, melatonin was found to be a ubiquitous phylogenetically ancient molecule in almost every organism, from primitive photosynthetic bacteria to humans (Dun Xian Tan et al. 2016). For melatonin synthesis, the majority of studies have been performed in vertebrates, particularly in mammals, and more recently in plants (Dun Xian Tan et al. 2015). Occurrence of melatonin in yeast was described for the first time by Sprenger *et al*. [3] as a product of the metabolism of precursors such as tryptophan, serotonin, *N*-acetylserotonin, and 5-methoxytryptamine. These results have been later confirmed and extended by numerous studies demonstrating yeast is responsible for the biosynthesis of this molecule in a fermentative context, and bringing the challenge of studying the function of melatonin in yeast and the mechanisms underlying its synthesis (Fernández-Pachón et al. 2014; Garcia-Moreno, Calvo, and Maldonado 2013; Rodríguez-Naranjo et al. 2011; Vigentini et al. 2015). Melatonin exerts multiple physiological roles on different organisms, from regulating biorythms and aging, modulating immune system response, inhibiting tumor growth and protect from UV light, among others (Russel J. Reiter et al. 2018; Rosales-Corral et al. 2012; Dun Xian Tan et al. 2010). In the case of *Saccharomyces cerevisiae*, it has been empirically demonstrated that melatonin has a protective role against oxidizing agents and UV light (Bisquert, Muñiz-Calvo, and Guillamón 2018; Vázquez et al. 2017; 2018). Regarding melatonin biosynthetic pathway, there is a high degree of conservation of the enzymatic reactions that lead to melatonin synthesis from tryptophan. It is the order of these reactions what characterizes the biosynthetic route in yeasts. *S. cerevisiae* seems to convert tryptophan to tryptamine in a first decarboxylation step, followed by a hydroxylation to form serotonin. Then melatonin is formed from serotonin by *N*-acetylation followed by O-methylation of *N*-acetylserotonin or, alternatively, by an O-methylation of serotonin to form 5-methoxytryptamine followed by its *N*-acetylation, which is the preferred alternative for *S. cerevisiae*, although further evidence suggest more branches on the pathway in which the tryptophan as a precursor is (Muñiz-Calvo et al. 2019). Therefore, the classical melatonin pathway model for vertebrates does not seem to apply for yeast.

Despite all the advances on melatonin biosynthesis in yeast, there is still uncertainty around the specific genes involved in the route. Only one gene has been described and characterized as involved in melatonin production, *PAA1*, a polyamine acetyltransferease, homolog of the vertebrate's aralkylamine N-acetyltransferase (*AANAT*), which can acetylate serotonin to *N*-acetylserotonin and 5-methoxytryptamine to melatonin (Ganguly et al. 2001), while the remaining genes and enzymes of the route are still unknown. Therefore, the search for genes homologous to those described in vertebrates and plants in *S. cerevisiae* represents a challenging goal and a key point to improve the synthesis of these molecules during fermentation processes in which *S. cerevisiae* participates.

In this study, we assessed the *in vivo* function of *PAA1* by evaluating the bioconversion of the different possible substrates, such as 5-methoxytryptamine, tryptamine and serotonin using different protein expression platforms. To that aim, we overexpressed *PAA1* gene, and the aralkylamine *N*-acetyltransferase of *Bos taurus* (Bt*AANAT*) as a positive control, in *S. cerevisiae* and *Escherichia coli*, and we measured production of acetylated metabolites after a precursor pulse into the media. As the results evidenced the presence of alternative enzymes with AANAT activity in *S. cerevisiae*, we expanded the search for *N*-acetyltransferase candidates by combining a global transcriptional expression analysis (RNAseq), under melatonin synthesis conditions, and the use of powerful bioinformatic tools. This strategy has allowed us to propose new candidates to explain melatonin-related acetylation activity in yeast.

2.2. Results and discussion

Ganguly *et al*. (2001) cloned and overexpressed the *S. cerevisiae PAA1* gene in *E. coli* and, after its purification, characterized its enzymatic activity *in vitro*. These authors concluded that this enzyme have activity generally typical for AANAT family members, although the substrate preference pattern was somewhat broader, the specific activity was lower, and the pH optimum was higher than the reference mammalian AANAT. Complementary to this previous study, we aim to characterize the *in vivo* function of *PAA1* by overexpressing this gene in *S. cerevisiae* and determine the impact of this overexpression in the acetylated products of the melatonin biosynthesis pathway.

2.2.1. Overexpression of *PAA1* in *S. cerevisiae*

Conversely to our expectations, the overexpression of *PAA1* in *S. cerevisiae*, in a medium supplemented with 5-methoxytryptamine, as this precursor has been described as the best amine substrate for *PAA1* (Ganguly et al. 2001), did not show significant differences in melatonin production in comparison to the wild type strain transformed with the empty vector (control strain). Nonetheless, Bt*AANAT* overexpression produced a 25-fold higher melatonin concentration than the control and the *PAA1* overexpressing strain (PAA1) (Figure 2.1A). In view of this unexpected result, we also tested the acetylase capacity of PAA1 on other substrates (tryptamine and serotonin). In this assay, we also included the null mutant strain (paa1) to evaluate a possible loss of acetylating function for any of the substrates. Again, no significant differences in acetylated products from any substrate were detected among these strains. Null mutant still showed same levels of acetylated products as the control or the overexpressed strains (Figure 2.1B). This result contradicts to the previous Ganguly *et al*. (2001) study which reported a reduced arylalkylamine acetylation in crude homogenates of the paa1 mutant strain in comparison with the wild type.

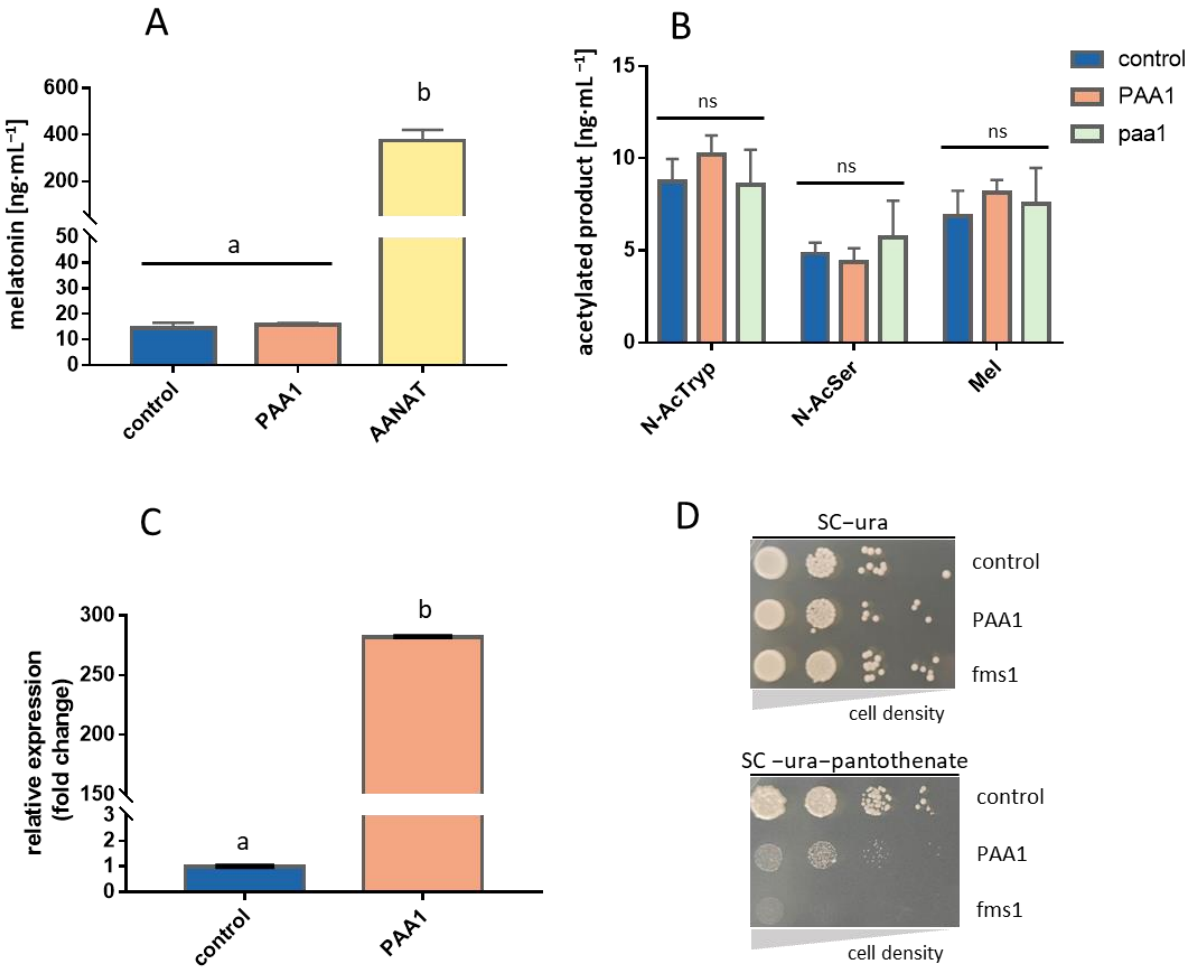

Figure 2.1. *In vivo* testing of PAA1 function in yeast strain BY4743. (A) Bioconversion assay for melatonin production on overexpressing strains. (B) Yeast *in vivo* production of N-acetyltryptamine (N-AcTryp), N-acetylserotonin (N-AcSer) and melatonin (Mel) was achieved by supplementing precursors tryptamine, serotonin and 5-methoxytryptamine, respectively, either in overexpressing (PAA1) or null mutant (paa1) strain. (C) An effective overexpression of *PAA1* resulted in a great increase of mRNA levels when compared to control strain. (D) *PAA1* is functional as it increases the consumption of polyamines as substrates and provokes a growth defect when no pantothenate is available. This growth defect reflects a depletion of polyamine precursors of pantothenate due to the increased action of Paa1p. p426GPD backbone with no cloned gene was used as a control in all cases. Different letters “a, b” indicate groups that are significantly different ($p < 0.05$). “ns” reflects no significant difference when comparing the mean values below.

In order to assure that the induction of *PAA1* in the overexpressing strain had been achieved, we determined the gene transcriptional activity of both the control and the overexpressing PAA1 strain by qPCR. *PAA1* was found to be expressed over 270 time-fold in the PAA1 strain compared to the control strain, which

indicates that overexpression had been correctly achieved (Figure 2.1C). As the lack of increases in acetylation activity was not connected with the transcriptional activity, we tested if functional overexpressed proteins were also obtained. Liu et al. (2005) reported that *PAA1* overexpression caused partial growth inhibition in a medium without pantothenate, but not in a rich medium. An increase in intracellular Paa1p led to an excess of acetylated polyamines, such as putrescine, spermidine, and spermine, which are the immediate precursors in the synthesis of pantothenate (Figure 2.2). A shortage in these polyamines turned out in a reduction of intracellular pantothenate and a growth defect in the absence of this vitamin in the growth medium. In turn, *FMS1* gene encodes a polyamine oxidase that converts spermine into 3-aminopropanal, which is then converted to β-alanine. The β-alanine is a precursor of pantothenate, which is in turn a precursor of coenzyme A (Figure 2.2). Based on these previous metabolic data, we performed a drop test in an SC medium with and without pantothenate to indirectly verify if we had functionally overexpressed *PAA1*. We also tested the *FMS1* null mutant strain (fms1) which is unable to grow in the absence of pantothenate. These drop tests showed a growth defect in the PAA1 strain and a total inhibition in the fms1 strain, as previously reported (Liu et al., 2005). This result evidenced that *PAA1* was not only overexpressed but also translated, into functional proteins (Figure 2.1D).

In light of these results, the absence of significant arylalkylamine acetylation in the *PAA1* overexpressing strain could be explained because arylalkylamines are not the main *in vivo* substrate of Paa1p, although it conserves a lower specific activity than that of the mammalian enzyme (Ganguly et al. 2001). Liu et al. (2005) also provided strong evidence that spermine was the main *in vivo* substrate of Paa1p. However, the fact that we also detected acetylated activity in the null paa1 mutant suggests that there may be other *N*-acetyltransferases in *S. cerevisiae*. Another explanation of the obtained results is that, in spite of the overexpression, this higher transcriptional activity in *PAA1* is not enough to increase the concentration of acetylated arylalkylamines. As it is well known that *E. coli* produces higher expression levels of recombinant proteins than *S. cerevisiae*, and several previously uncharacterized members of the yeast *N*-acetyl transferases were expressed in *E. coli*, and the recombinant proteins were

purified and assayed for their acetylation activity (Neuwald and Landsman 1997; Ganguly et al. 2001), we decided to overexpress *PAA1* in *E. coli* and determine the acetylation activity in different arylalkylamines, in a similar *in vivo* bioconversion assay to the one performed in *S. cerevisiae*.

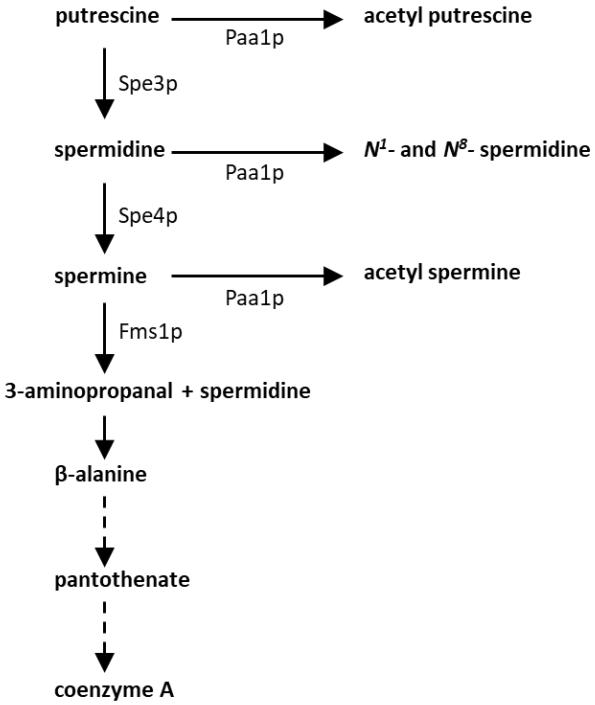

Figure 2.2. Biosynthetic pathway for the synthesis of coenzyme A in yeast. Adapted from Liu *et al*. 2005.

2.2.2. Overexpression of *PAA1* in *E. coli*

In order to assess *PAA1* activity in *E. coli*, an *in vivo* bioconversion of 5-methoxytryptamine into melatonin was performed in an inducible overexpression system and, conversely to the overexpression in *S. cerevisiae*, a significant increase in melatonin production was detected in comparison to the wild-type strain (Figure 2.3A). As previously, the Bt*AANAT* was also overexpressed as a positive control and this system yielded a much higher titers of melatonin than the strain overexpressing *PAA1* (more than 20-folds), evidencing a much higher specific activity of the mammalian enzyme. Once the overexpression of *PAA1* showed positive bioconversion results, we tested Paa1p acetylase activity for other possible substrates related to the melatonin pathway, namely tryptamine and serotonin, to test *PAA1* specificity and preference of substrate by measuring

the corresponding acetylated products *N*-acetyltryptamine and *N*-acetylserotonin. Interestingly, we observed *in vivo* acetylase activity of *PAA1* using tryptamine, 5-methoxytryptamine, and serotonin as a substrate, being tryptamine the preferred substrate that yielded approximately 1 $\mu g \cdot mL^{-1}$ of *N*-acetyltryptamine, while serotonin produced the lowest amount of acetylated product with 50 $ng \cdot mL^{-1}$, which was still significant when compared to the wild-type strain (Figure 2.3B).

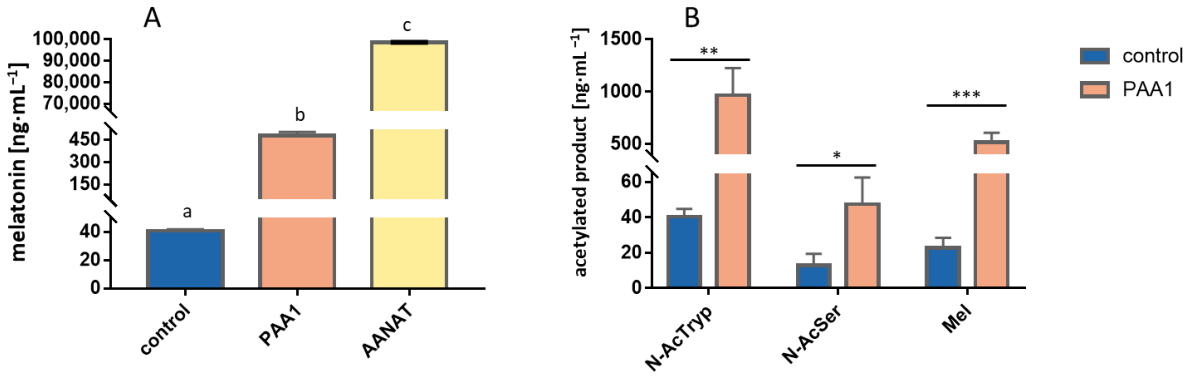

Figure 2.3. *In vivo* testing of PAA1 function in E. coli by bioconversion assays showing a significant acetylation activity for melatonin production when supplemented with 5-methoxytryptamine (A), but also for N-AcTryp and N-AcSer when supplemented with tryptamine and serotonin, respectively, demonstrating a broad substrate scope of this gene (B). pGEX-5X-1 backbone without any cloned gene was transformed into E. coli and used as a control in these assays**.** Different letters "a–c" indicate variables that are significantly different from each other ($p < 0.05$). Asterisks show significant differences in relation to their control ($p < 0.05$ (*), $p < 0.005$ (**), and $p < 0.001$ (***)).

With all the evidence obtained with the overexpression of *PAA1* in both host organisms, we reasoned *PAA1* might be involved in the melatonin biosynthetic pathway in yeast, but it is not essential for an *in vivo* significant production of this compound. This makes clear there are still other candidates to consider as responsible for the acetylase step in the melatonin pathway, and probably, as well as for *PAA1*, they may have other main functions. The fact melatonin is produced in such small amounts in yeast, and the enzymes responsible for the different necessary reactions are not exclusive of this pathway, highlights the difficulty of the search for candidates using yeast cells for the bioconversion assays. Thus we concluded bioconversion assays for testing possible candidates

should be carried out using the bacterial expression system because they produce an outsized effect of the tested protein, revealing any possible acetylating activity, even when it happens in a residual way in yeast.

2.2.3. Search for new *N*-acetyltransferases in *S. cerevisiae*

Melatonin biosynthesis in yeast is not a conspicuous trait in terms of the amount of generated product, but its importance relies on melatonin's free radical scavenging activity and its modulation of gene expression, even when it is present at low concentrations (Reiter et al. 2016; Rodriguez et al. 2004). All gathered evidence around *PAA1* gene, together with the usual low concentrations of melatonin detected as a result of spontaneous biosynthesis, led us to infer that enzymes involved in this biosynthetic route, and especially *PAA1*, may not be exclusive for this route but they rather have other main functions instead, even though they can eventually contribute to melatonin biosynthesis in a leaky and inefficient manner. The search for these possible gene candidates involved in melatonin synthesis becomes a complicated and subtle labor in which expression levels of specific genes can bring out their relevance in this process. As we understand, a global transcriptome analysis during melatonin production can provide an interesting starting point in the search for candidate genes, especially those involved in the acetyltransferase activity, if a melatonin synthesis situation triggers a differential expression of the genes involved in it. Despite multiple pieces of evidence of melatonin function in *S. cerevisiae*, the external conditions that are capable of inducing its production are difficult to establish as no media and growth conditions have been unequivocally associated to spontaneous increase of detectable melatonin levels. For this reason, following our previous approach for a reproducible melatonin synthesis (Muñiz-Calvo et al. 2019), we directly induced this synthesis by supplementing the growth media with an immediate precursor such as 5-methoxytryptamine to observe melatonin production (Figure 2.4A).

We explored transcriptional response after 15 and 45 min of the precursor supplementation and, under our strict criteria for significance, only 13 and 8 genes were significantly upregulated at time 15 and 45 min, respectively. We

observed an intense activation of genes related to iron and copper homeostasis and specific genes related to transmembrane transporter activity, especially after 15 min of the precursor addition, when we compared supplemented culture with its non-supplemented control (Figure 2.4B). A reasonable explanation for this result is that melatonin and some precursors such as 5-methoxytryptamine can act as iron and copper chelators, as previously pointed out (Galano et al. 2015; Reiter et al. 2016; Yu et al. 2018). Therefore, a change in metal ions availability in the growth medium may occur when using 5-methoxytryptamine as a supplement to produce melatonin. Differences in media composition regarding heavy metals between our supplemented and control cultures need to be taken into account when analyzing expression patterns as a certain grade of the transcriptional response to metal deficiency is inevitably expected. For a time of 45 min, the overexpressed genes belong to the “protein folding, and protein targeting to ER” network cluster (STRING), indicating the response to chelation occurred in a short period of time, and the main overexpression at 45 min is related to a replicative and translational stress response (Figure 2.4C).

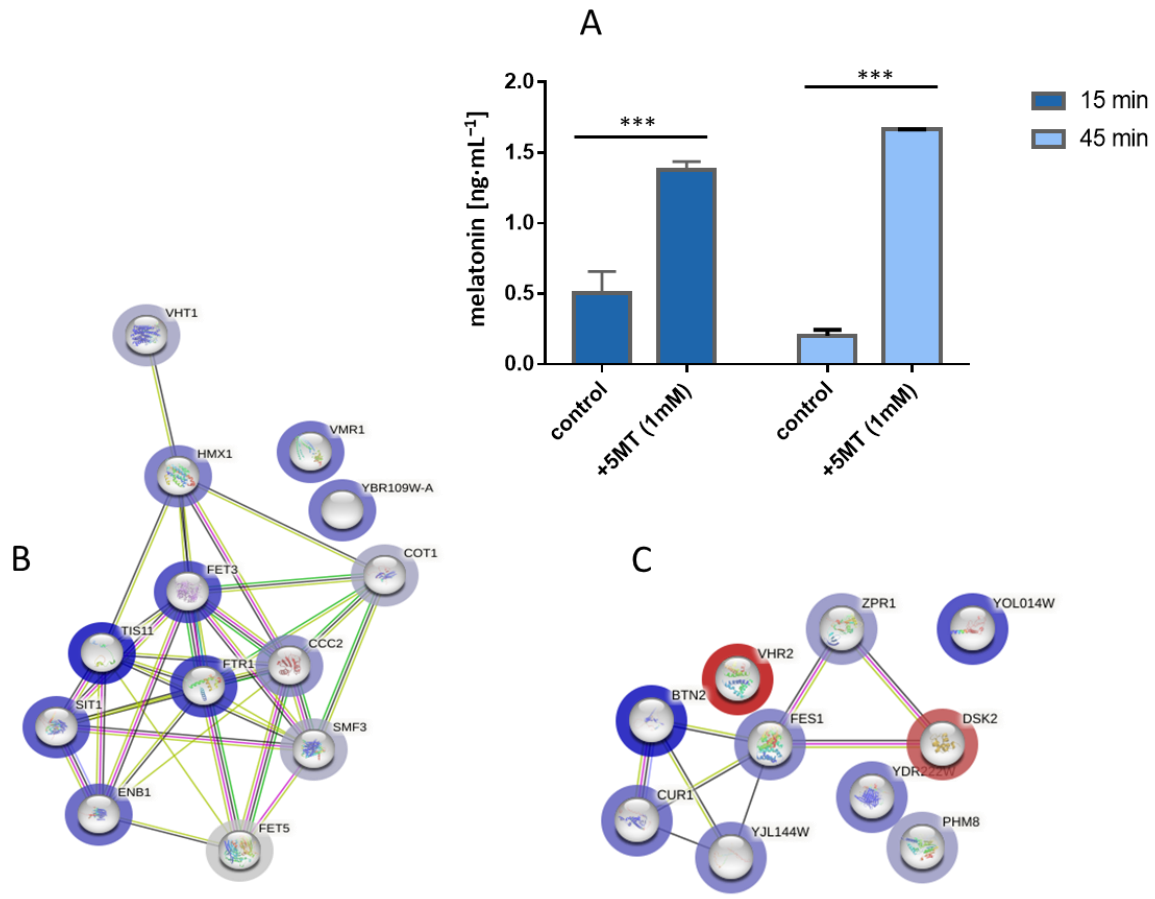

Figure 2.4. Melatonin production modulated yeast transcriptional response. (A) Melatonin production achieved by supplementing the media with precursor 5-methoxytryptamine (5MT). Samples taken at 15 and 45 min after the 5MT addition are shown. Transcriptional response is depicted as a network of predicted interaction between the different genes (nodes) showing whether they are overexpressed (blue rim) or repressed (red rim) relative to control at time 15 min (B) or 45 min (C). Asterisks show significant differences in relation to their control ($p < 0.001$ (***)).

We believe the response to the chelating effect of the studied compounds has apparently masked any less conspicuous gene expression and hindered their significance. Therefore, in order to be able to detect other activated genes in response to 5-methoxytryptamine supplementation or melatonin production, we looked further down in the list of overexpressed genes which are structurally related to *AANAT* and cautiously considered them as possible candidates. We used RNAseq information as a guide and crossed it with the InterPro database (Mulder et al. 2008) to search for protein candidates among *S. cerevisiae* with a functional homology with the GNAT domain (IPR000182) of the reference gene *AANAT*. We found eight coincidences between the family domain search results and overexpressed genes from the RNAseq results, one of them expectedly being *PAA1,* so we considered seven new gene candidates with a GNAT domain for testing for melatonin production.

2.2.4. Detection of AANAT activity in the new gene candidates by overexpression in *E. coli*

The seven selected candidates were overexpressed in *E. coli* as described above (Table M.5). Six out of these seven genes did not show any significant difference in the melatonin yielded in comparison with the wild type. Thus, the involvement of these genes in the putative production of melatonin or in the acetylation of other compounds of the route can be ruled out. Nonetheless, we detected that the overexpression of the gene *HPA2* resulted in significant higher titers of melatonin, in an amount similar to the previously reported for *PAA1* and, again, in lower concentration than the production of the overexpression of Bt*AANAT* (Figure 2.5). The new positive candidate *HPA2* was early overexpressed at a time of 15 min, while *PAA1* overexpression was detected only in the next time point analyzed at 45 min (Figure 2.6). The sequential transcription of different genes involved in melatonin synthesis can explain the lack of a strong transcriptional

response of one single responsible gene, reinforcing the theory of a common function from multiple genes when it comes to melatonin biosynthesis. *HPA2* is a tetrameric histone acetyltransferase, a member of the Gcn5 acetyltransferase family, which acetylates histones H3 and H4 *in vitro* and also acetylates polyamines. However, this is the first report that clearly proves the involvement of this enzyme in the acetylation of arylalkylamines, such as 5-methoxytryptamine, and, therefore, in the synthesis of melatonin in yeasts. As it was the case for *PAA1*, the substrate specificity and the specific activity was significantly lower than the mammalian enzyme, but, likely, as a moonlighting protein, Hpa2 was able to significantly convert 5-methoxytryptamine into melatonin.

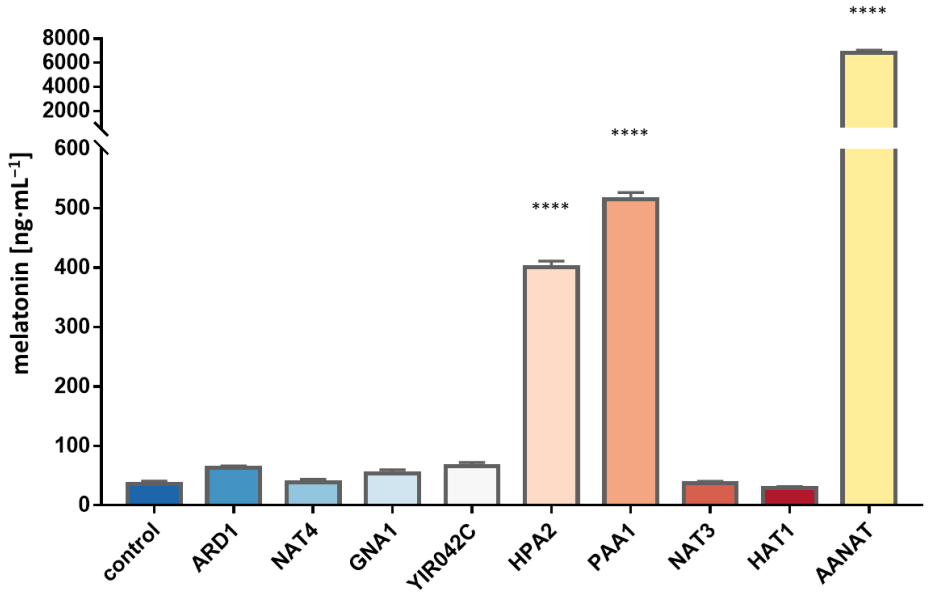

Figure 2.5. Melatonin production from 5-methoxytryptamine of the different gene candidates extracted from RNAseq results and homology domain search results. Genes were overexpressed in *E. coli* and pGEX-5X-1 backbone with no cloned gene was used as a control. Asterisks show significant differences in relation to the control ($p < 0.0001$ (****)).

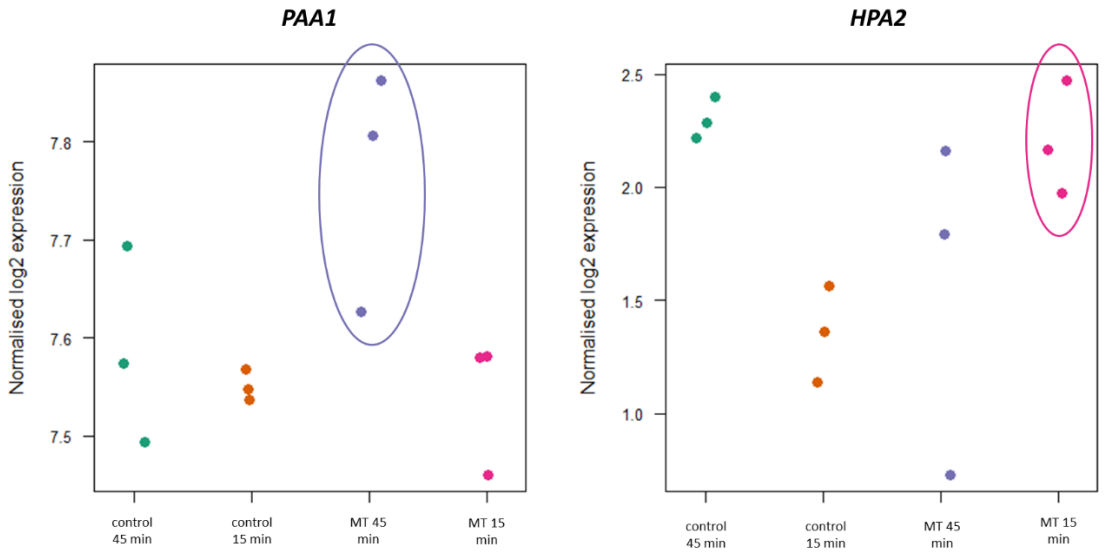

Figure 2.6. Secuential overexpression of HPA2 and PAA1 under melatonin synthesis conditions. RNAseq results showed expression levels of *HPA2* were higher than control at time 15 min (*p*-value: 0.0185), while *PAA1* showed higher transcript levels at time 45 min (*p*-value: 0.0319).

2.3. Conclusions

So far, only the *PAA1* gene has been correlated with the unknown pathway of melatonin biosynthesis in yeasts. In this chapter we aimed to characterize the enzymatic activity of this gene by a whole-cell biotransformation. To this end, we overexpressed *PAA1* in its own host, *S. cerevisiae*, and in *E. coli*. However, we did not detect significant acetylation activity in *S. cerevisiae* whereas this higher activity was evident in *E. coli*. Therefore, a clear conclusion for the future search for new enzymes of the route is that the overexpression in *E. coli* reveals higher differences in the enzymatic activity. Our results also evidenced that *PAA1* was not the only enzyme with AANAT activity in *S. cerevisiae*. The combination of criteria from transcriptomics and structure prediction to find similar domains let us narrow down the list of gene candidates to test in an *in vivo* assay that resulted in the proposal of a new gene candidate. We can conclude that *HPA2*, a histone acetyltransferase also related to polyamine acetylation, was able to significantly convert 5-methoxytryptamine to melatonin. Consequently, together with Paa1, Hpa2 should be also considered an arylalkylamine *N*-acetyltransferase in *S. cerevisiae*. However, both enzymes should be considered moonlighting proteins,

and taking into account the yield of acetylated arylalkylamine in comparison with the mammalian enzyme, this AANAT activity does not seem to be the main acetylation activity. Therefore, the presence of an AANAT enzyme with a higher arylalkylamine substrate specificity in *S. cerevisiae* cannot be ruled out.

CHAPTER 3

Light-induced adaptive laboratory evolution to increase bioactive aromatic amino acid-derived compounds in a wine strain of *Saccharomyces cerevisiae*

3.1. Introduction

The presence of bioactive molecules in fermented food and beverages is often linked to microbial metabolism and particularly yeast stands out as a major responsible of the synthesis of these health-promoting compounds (Marhuenda et al., 2016; Rodriguez-Naranjo et al., 2011; Vilela, 2019). Among them, aromatic amino acid-derived compounds (AADCs) such as tryptophol (TOL), 2-phenylethanol (2PE), and tyrosol, as well as the indolic compounds derived from tryptophan (Trp), such as melatonin (Mel) , serotonin and 3-indoleacetic acid (3IAA) are specially relevant as they can contribute to the quality and stability of fermented beverages like wine (Cordente et al., 2019; Fernández-Cruz et al., 2016). In this context, obtaining yeast strains that are capable of increasing the amount of these compounds in the final product is of great interest to the wine industry that, despite the presence of a toxic metabolite such as ethanol in their product, the antioxidant, antimicrobial, preservative and health-promoting benefits from bioactive AADCs are highly desirable. The generation of strains with these desirable traits faces two main obstacles: To effectively, and accurately modify the yeast metabolism to obtain higher amounts of a certain metabolite its biosynthetic route needs to be fully understood in terms of involved precursors, cofactors, responsible genes and proteins, synthesis conditions, possible bottlenecks and leakage points. Such details remain still unraveled for some of the most interesting bioactive AADCs like melatonin, serotonin or hydroxytyrosol (HT) in yeast. On the other hand, even with all due information about their biosynthesis, rational genetic manipulation in food industry is highly restricted by the European regulation on genetically modified organisms (GMOs). The quest for genetically improving industrial yeast strains without the commercial and regulatory constraints of being considered a GMO gave rise to a series of methods to achieve a genetic diversity to get to the desired phenotypical traits. These methods include random mutagenesis, intra- and interspecific hybridization and adaptive laboratory evolution (ALE), also known as directed evolution (Gonzalez & Morales, 2022). Among them, ALE has been successfully conducted to improve yeast tolerance to freeze-thaw, salt, acetic acid, temperature and ethanol in industrial yeast strains (Aarnio et al., 1991; García-Ríos et al., 2022; Matsutani et al., 1992; Novo et al., 2014; Takagi et al., 1997).

This approach is based on the natural genetic and phenotypic diversity of yeast, that is iteratively subjected to a selective pressure through multiple generations that allows population to fix genetic and phenotypic traits that are different from the initial parental strain. Although the experimental design can be relatively straight-forward when improving traits such as ethanol tolerance and the others above mentioned, in other cases indirect selection strategies are required, *e.g.* the use of a selective pressure such as osmotic stress to select strains with higher glycerol production (Tilloy & Ortiz-julien, 2014). In this work we conducted a novel directed evolution experiment in a *Saccharomyces cerevisiae* strain, isolated from a winery environment, using a very particular selective pressure, such as white light. Bioactive AADCs such as hydroxytyrosol and especially tryptophan derived ones such as serotonin, tryptamine and melatonin, among others, have an antioxidant effect on yeast at different levels, acting as free-radical scavengers but also by inducing expression of oxidative response genes (Bisquert et al., 2018; Vázquez et al., 2017). Taking melatonin as a model of a potent antioxidant indolic compound and its relation to light stimuli in higher organisms and its UV protection effect also in yeast cells, we decided to use a physical ROS-generating selective pressure such as white light in our ALE experiment. Our hypothesis was cell's primary oxidative stress responses would be challenged in a directed evolution context when generating a photooxidation by illuminating the growth vessel with a white light LED lamp which covered mainly photosynthetically active radiation (PAR) wavelengths ranging from 400 nm to 700 nm. Among these primary oxidative stress defense systems, the increase of non-enzymatic forms of antioxidant response such as the synthesis of melatonin or other indolic and phenolic compounds was therefore a possible and expectable outcome. In this chapter we conducted a light-induced ALE on a wine yeast, assessed genotypical and phenotypical differences between evolved and parental strain, analyzed their complete extracellular metabolome and that allowed us to propose interesting relations between specific mutations and the increase of bioactive AADCs. Furthermore, our results revealed the potential of white light as a severe selective pressure, and even as a mutagen to be used in the generation of new strains from a non-GMO approach.

3.2 Results and discussion

Evolution under light-induced stress

Saccharomyces cerevisiae strain B28 was subjected to white light stress during growth in 400 mL of a defined synthetic minimal medium at 28 °C under 300 rpm stirring inside a 0.5 L bioreactor in batch mode. The initial irradiance was set to 317 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by adjusting the distance of the LED lamp. It is interesting to note that above the initial irradiance value B28 strain was not able to start growth, staying in a lag phase until the stress was eliminated or reduced. At the initial irradiance the growth was severely affected and therefore we decided to apply the radiation in intervals of 17 h, allowing cells to reach stationary phase. The batch refreshments consisted in the depletion of >90% of the grown culture and the addition of fresh media to 400 mL when growth reached at least early stationary phase. At the same time, through multiple generations, irradiance was progressively increased to a maximum of 3377 $\mu mol \cdot m^{-2} \cdot s^{-1}$, which represented more than 10-fold the radiation at the beginning of ALE, with the evolved strain growing under stress similar to the non-stressed control growing in parallel in a non-irradiated bioreactor. As a track of the progress across the ALE experiment, we recorded the generations occurring in each batch and the total radiation received in each batch (Figure 3.1). After 170 generations, the ability of the evolved B28 (henceforth EVO) strain of growing under an irradiance 10 times higher than the initial, and the fact of the parental B28 was unable to grow under the same condition, were clearly indicating our EVO strain would hold enough genetic differences with the parental strain to explain the endurance against this unusual stress.

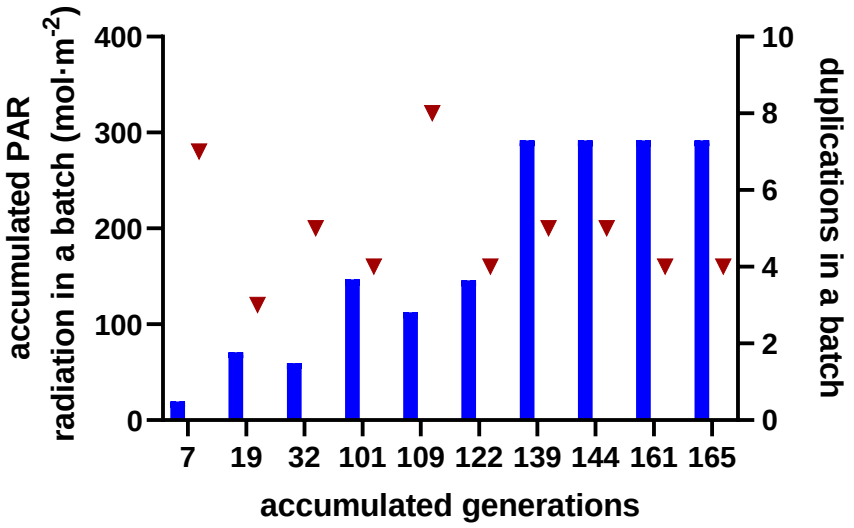

Figure 3.1. Evolution process of B28 strain across multiple generations showed as the amount of duplication happening in a batch (red triangles) in relation to the stress severity, shown as accumulated PAR radiation in the same batch. Irradiance describes the amount of received energy per unit of time, to better reflect the total dose received by the bioreactor the time of each batch was taken into account to calculate the accumulated PAR radiation (blue bars). As observed in early stages of ALE, cells enduring ~70 mol of photons per square meter duplicated three to five times during a batch while in final stages they duplicated four to five times but endured ~290 mol·m^{-2} of accumulated radiation.

Phenotypic traits resulting from ALE

A clear resistance to white light achieved throughout ALE allowed EVO strain to grow under radiation conditions that are incompatible with cell growth for the parental B28. Our interest was to assess further phenotypical differential traits, especially those related to the EVO metabolic profile compared to the parental strain but also to any other affecting growth parameters, oxidative stress resistance and fermentative performance. Light-induced stress is known to produce an increase of intracellular reactive oxygen species (ROS) and it can affect the expression of genes involved in oxidative stress response (Robertson et al., 2013). Thus, an improvement of growth performance under oxidative stress as a direct result of light adaptation may be an expectable association. We grew both B28 and EVO strains in 96-well plates under different media and conditions to compare their growth performance (Figure 3.2). When the strains were grown in the same medium and temperature used in the directed evolution batches, EVO strain performed similar to the parental strain, but with a significant delay,

as observed in the growth curves and indirectly reflected in the area under the curve (AUC) parameter (Figure 3.2AB). Contrary to what we expected, we did not observe any improvement in EVO growth compared to B28, when cells were challenged with 3 mM of hydrogen peroxide to increase oxidative stress. In fact, EVO strain showed a greater sensitivity to H_2O_2 and reflected it as a very pronounced delay in growth when compared to parental strain (Figure 3.2BC). With the aim of partially delimiting the causes of this slower growth in EVO strain we measured intracellular ROS levels by determining the abundance of DHR123-positive cells in non-stressed cultures using a flow cytometer (Figure 3.2D). This dye enters and accumulates in living cells and display a green fluorescence when oxidized by ROS. Surprisingly EVO strain exhibited a significantly higher proportion of DHR123-positive cells than B28 under the same unstressed condition. These differences in the default intracellular ROS levels may be the reason behind the slower growth for EVO strain. Evolved cells are coping with a basal oxidative stress that parental strain does not have, and the situation is exacerbated when they are challenged with more oxidative stress. These results appear to disagree with an expected adaptation to oxidative stress after the evolution experiment. Although white light resulted a severe growth-impairing stress for parental strain and despite its known effect on generating ROS, evolved strain didn't seem more resistant to oxidative stress but more sensitive. It is noteworthy to point out this slower growth occurred consistently throughout other performed growth assays, *i.e.* different media such as SD, SC and YPG, and different temperatures such as 28, 20 and 12 **°**C (data not shown), nonetheless further growth tests under different stresses could help establishing a possible adaptive advantage of EVO strain beyond of the evidenced endurance to visible light stress.

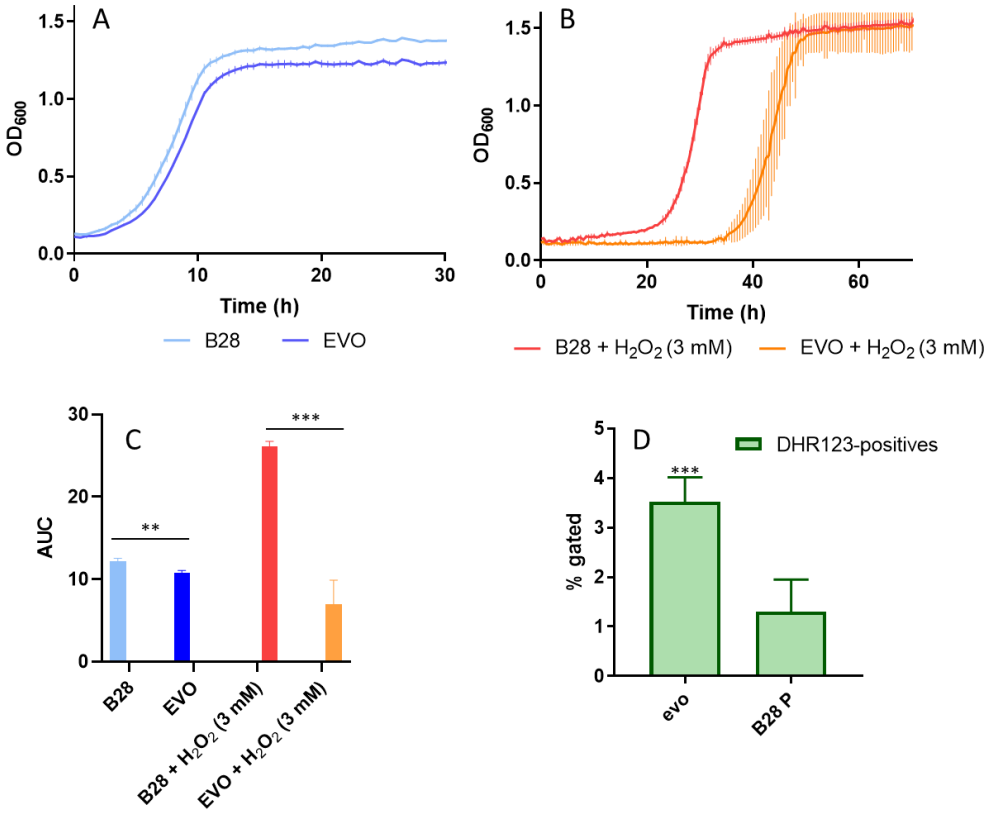

Figure 3.2. Growth curves of B28 and EVO strain reflected a slight decrease in growth for the evolved strain when grown at 28 **°**C (A) and an increased sensibility against hydrogen peroxide as it aggravates the growth delay effect in EVO strain in comparison to B28 when growing at 28 **°**C in the presence of this stress (B). AUC was calculated at a time where all curves reached stationary phase, being 18 h for unstressed cultures, and 48 h for cultures challenged with H_2O_2 (C). Intracellular ROS levels was determined by the percentage of positive DHR123 stain detected events in a flow cytometer where 10000 cells were measured in each run. EVO cells were statistically more oxidized than B28 under no stress condition (D). All results show median values from biological triplicates where error bars represent standard deviation. Asterisks denote significance level from Student's *t*-test pairwise comparisons ($p < 0.001$ (***), $p < 0.01$ (**)).

Given the mild affectation of EVO strain's growth performance we decided to test their ability to successfully ferment a synthetic must, due to its origin from a winemaking context. To do so B28 and EVO strains were inoculated in 80 mL of a synthetic must (SM), derived from Riou et al. (1989), to mimic a grape must fermentation, and grown in fermentation flasks capped with airlock valves. Fermentations were monitored by following weight loss until sugars were finally consumed and, although EVO strain was still able to finish the must fermentation,

it showed a slower performance than parental B28 (Figure 3.3). It is important to note despite all these phenotypical differences, fermentation capacity of the strain remained competent for an eventual application in the winemaking industry.

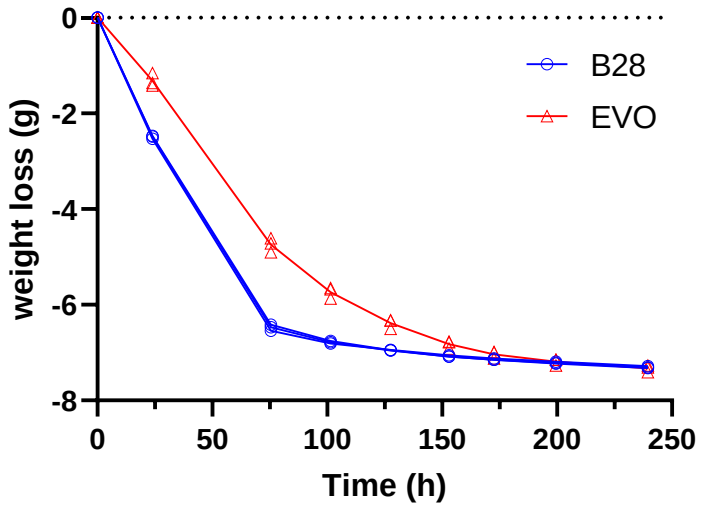

Figure 3.3. Fermentation kinetics represented as total weight loss of evolved strain (EVO) compared with the parental strain (B28) in a synthetic must containing 20 % of sugars (fructose + glucose) reveal a slight delay in EVO strain fermentation. Fermentations were performed in triplicates and sampling points for all of them are plotted in the graph.

Besides the described effects resulting from light-induced ALE, we observed EVO strain cultures showed curious visual characteristics when grown under light stress conditions. Such differences encompass a visible red to brown pigmentation of the cells when grown under light stress and cells in liquid culture seem partially aggregated (Figure 3.4). These two phenotypical traits were only exhibited after growth under light-induced stress and pigmentation seemed to be retained intracellularly.

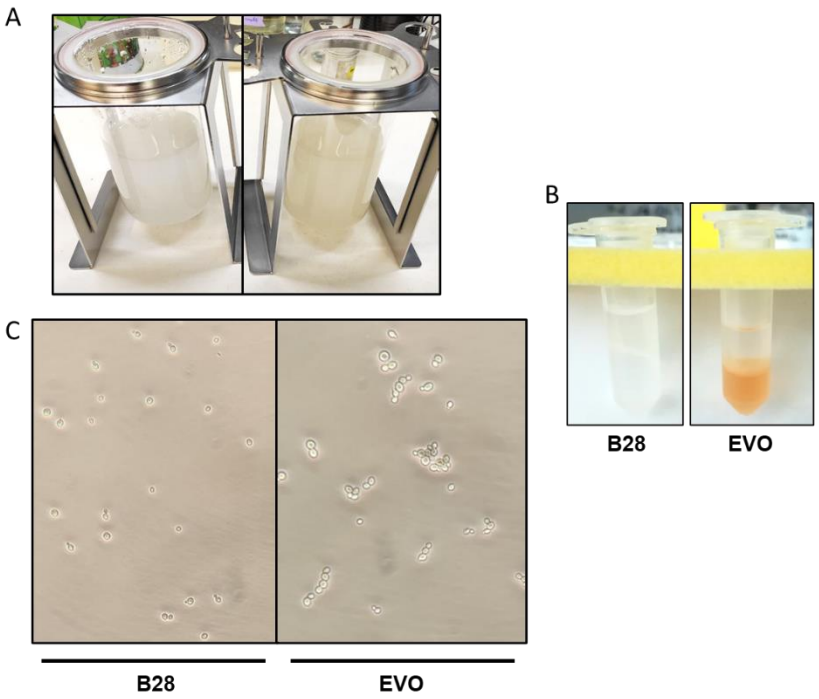

Figure 3.4. Visual characteristics of B28 and EVO cultures in the synthetic minimal medium. Bioreactor vessels with 400 mL of grown culture displayed a darker coloration in the case of the stress treated cultures (A). The observed coloration was due to a coloring agent retained in the cells. After cell disruption and liquid extraction with dichloromethane, pigmentation is retained in the organic phase (B). Medium without cells remained transparent under the same light conditions. Optical microphotography revealed certain grade of cell aggregation in EVO cultures after light exposure that contrast to the typical unaggregated cell culture of parental B28 (C).

High throughput metabolome analysis

The lack of a clear advantageous trait in EVO strain against the tested growth conditions and the unexpected phenotype of cell aggregation and pigmentation prompted us to analyze the metabolites produced and exported to the media by each strain. To that aim both EVO and B28 strains were grown in the same defined medium at 28 **°**C and constant shaking under the same conditions in the dark. In parallel we grew three more replicas of EVO strain, but challenged with constant white illumination. Lamp was set to exert 3377 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR radiation to the shake flasks for 72 h, B28 strain could not grow under these conditions. Supernatants of the three groups of samples, B28, EVO and EVO grown under white light stress (henceforth “LIGHT”), were collected and an untargeted metabolome analysis was performed using a reverse-phase UPLC-ESI(+/-)-QqTOF-MS/MS. Metabolites were separated and fragmented,

generating a total of 3958 ions in each sample, including positive and negative modes. Pairwise comparisons between extracted compounds and fragments were made between the three sample sets, and as expected, the comparison between B28 and EVO showed more differential metabolites than any other, yielding 1009 differential metabolites from which 934 are unique from this comparison. This result clearly indicates that despite the differences between evolved and parental strain are not very pronounced when comparing growth in different media, each strain displays a clear distinct metabolome after growth. These differential metabolites are not as evident when comparing EVO and "LIGHT" samples, as they are both generated by the same evolved strain (Figure 3.5).

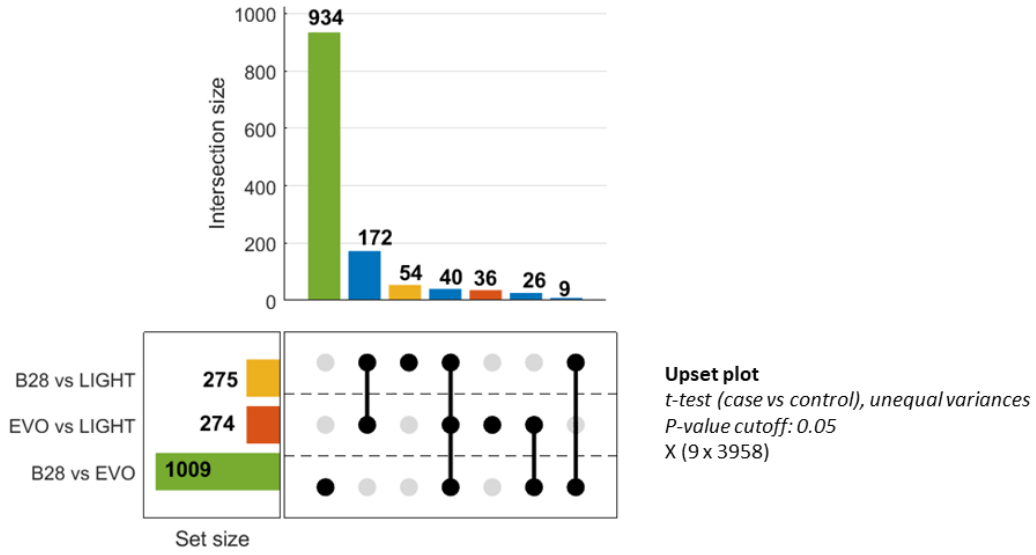

Figure 3.5. Upset plot showing the number of differential metabolites between each sample group pairwise comparison (horizontal bars inside "Set size" block) and the number of differential metabolites overlapping with every other comparison, individually or combined. Among the 1009 differential metabolites from B28 vs EVO comparison, 934 are exclusive from this comparison, while 40 are shared between all comparisons, 26 are coincident in comparisons EVO vs LIGHT and B28 vs EVO and 9 are coincident between B28 vs LIGHT and B28 vs EVO. Samples from biological triplicates of each group were used and a total pool of 3958 ions were detected and compared.

When analyzing the intersective portions between the comparisons, we observed most of the differential metabolites between B28 and EVO belong only to that comparison, while in the 274 differential metabolites from the comparison between sample groups EVO and "LIGHT" 172 metabolites are shared between

that comparison and that between B28 and “LIGHT” samples. This reveals that EVO and “LIGHT” samples have less differential metabolites when comparing them, as expected, but also parental B28 metabolome has less differential metabolites when compared to evolved strain’s metabolome when EVO is grown under light stress condition (LIGHT) than when compared to EVO grown in darkness.

Taking into account all annotated and unannotated metabolites and ions and their relative abundance in the resulting comparison between B28 and EVO sample groups, a *mummichog* functional analysis was performed to predict those metabolic pathways that are primarily involved in such differences (Li et al., 2013). Surprisingly, among the differential metabolites in the comparison between B28 and EVO metabolomes, 12% of them were clustered under “Indoles and derivatives” category, and “Tryptophan metabolism” and “glutathione metabolism” were clearly overrepresented in the functional analysis prediction (Figure 3.6A). These general results point to a possible relation between the synthesis of indoles and protection against light-induced stress as previously reported for melatonin (Bisquert et al., 2018). To better understand this change in tryptophan metabolism and propose hypothesis on the cause of the ability to grow under light stress more detailed information on specific compounds was obtained. The same analysis was also performed for the comparison between EVO and “LIGHT” sample groups to explore how metabolome was shaped by this stress condition and, although the number of differential metabolites was much lower in this comparison, tryptophan and glutathione metabolism appear once more overrepresented. Glutathione metabolism is a differential category in both comparisons. Curiously enough, the relative amount of reduced glutathione (GSH), which is a central metabolite in the response against oxidative stress, is diminished in EVO strain when grown in darkness. The relative amount detected in this sample group represented only 48% of that detected in B28. Nevertheless, this tendency is completely reverted in the LIGHT sample group, showing a 14-fold increase in glutathione amount in LIGHT sample group when compared to B28, or more than 30-fold increase when compared to EVO. Metabolites clustered under lysine, arginine and proline metabolism categories were also differential in the EVO versus LIGHT comparison (Figure 3.6B). Comparison

between EVO and LIGHT sample groups provided us with information about the evolved strain's ability to produce higher concentrations of certain metabolites when subjected to stress, such as S-adenosylmethionine (SAM), kynurenine, tryptophan, hydroxyindole acetate, 3-hydroxyanthranilate or indole-5,6-quinone, among others.

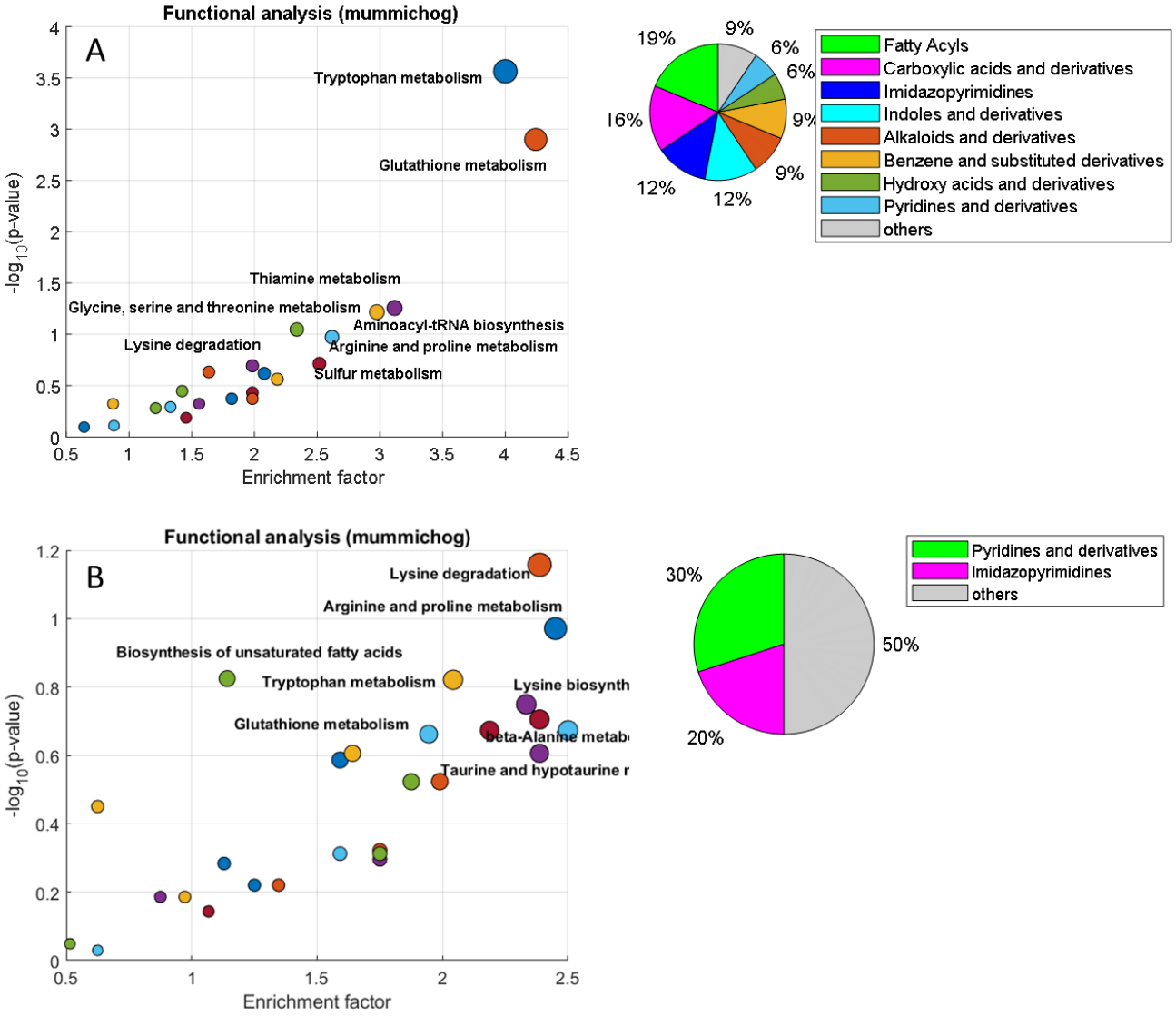

Figure 3.6. A functional analysis was performed on those significant differential metabolites in the sample groups comparisons B28 vs EVO (A) and EVO vs LIGHT (B). Bubble plots show the differential metabolites clustered by pathway using the information from KEGG database, while pie charts cluster the differential metabolites of each comparison by the chemical type according to the human metabolome database (HMDB).

Directed analysis of tryptophan-related metabolites.

To complete the untargeted metabolome analysis, we further analyzed the same samples using a multiple reaction monitoring (MRM) in the UHPLC-MS/MS that targeted and quantified up to 34 tryptophan metabolites (Table M.6). Among these metabolites, the overproduction of tryptophan, tyrosine and phenylalanine in EVO strain stood out with concentrations of 14 nM, 5.5 mM and 14.6 µM, respectively. LIGHT sample group showed a striking increase in the concentration of most of the analyzed compounds in the directed analysis, reaching 1.9 µM, 1.2 M and 1.1 mM for tryptophan, tyrosine and phenylalanine, respectively, revealing an increase of several orders of magnitude for each compound. We could not relate this great increase in aromatic amino acids with a higher accumulation of precursors from PPP or glycolysis, although we observed accumulation of other different products.

With the combination of concentrations data from the targeted method and relative signal intensities from the untargeted approach we were able to detect an increase of a collection of aromatic amino acid-derived compounds in evolved strain when compared to parental B28 strain (Table 3.1). Tryptophan-related compounds such as melatonin and serotonin did not increase in EVO strain when compared to B28, nevertheless, other known precursors and catabolites like tryptophan, tryptamine, 5-hydroxyindoleacetic acid or coumaroylserotonin were found in higher concentrations in EVO strain. Interestingly some overproduced compounds such as tryptamine, 5-hydroxytryptophan, indole-3-acetonitrile, 3-indolacetic acid, 3-hydroxyanthranilic acid, 3-hydroxyindolacetic acid or hydroxytryptophol depend on catalytic activities that are still not annotated in yeast. The increase of hydroxylated products suggests a higher monooxygenase activity in EVO strain, this activity is reported in yeast but none of the above-mentioned hydroxylated metabolites is related to any annotated yeast gene to date. Furthermore, not only EVO strain has gained the ability to excessively produce important antioxidant metabolites, but it synthesizes them triggered by light stimulus. A better understanding on the genetic changes that EVO strain has undergone can provide starting hypothesis on the genetic determinants of

aromatic amino acid-derived bioactive compound production and reveal the mechanisms behind some of the observed phenotypes.

Table 3.1. Relative amounts of the selected differential metabolites detected in the extracellular media in B28, EVO and LIGHT sample groups. Amounts are expressed as the mean ratio of the comparisons indicated in the first row, thus indicating fold change of the detected amount. For 5-hydroxytryptophan no relative amount could be expressed as no metabolite was detected in B28, thus a plus (+) sign indicates an increase.

	EVO / B28	LIGHT / B28	LIGHT / EVO
3-Indoleacetonitrile	0.89 ± 0.11	2.45 ± 0.34	2.75 ± 0.38
5-Hydroxyindoleacetic acid	1.2 ± 0.18	17.78 ± 5.72	14.83 ± 4.77
5-Hydroxytryptophan	+	+	40.77 ± 20.34
5-Hydroxytryptophol	0.76 ± 0.24	144.62 ± 68.69	189.9 ± 90.2
Anthranilate	2.41 ± 0.79	911.82 ± 257.74	378.15 ± 106.89
Glutathione	0.43 ± 0.06	23.99 ± 14.8	55.31 ± 34.12
Hydroxyanthranilate	2 ± 0.37	1.09 ± 0.53	0.54 ± 0.27
Hypoxanthine	1.42 ± 0.07	9.36 ± 0.03	6.58 ± 0.02
Indole-5,6-quinone	0.89 ± 0.13	363.03 ± 102.51	409.75 ± 115.71
Indoleacetaldehyde	0.81 ± 0.24	256 ± 202.17	315.96 ± 249.52
Kynurenine	1.41 ± 0.46	279.33 ± 24.35	197.77 ± 17.24
Phenylalanine	2.04 ± 0.42	236.1 ± 48.29	115.8 ± 23.69
Tyramine	1.61 ± 0.29	6.01 ± 3.16	3.73 ± 1.96
Coumaroylserotonin	0.89 ± 0.24	1787.59 ± 728.91	2014.54 ± 821.45
N-Formylkynurenine	1.39 ± 0.04	124.87 ± 99.99	89.91 ± 72
Tyrosine	2.15 ± 0.64	1352.22 ± 774.32	628.55 ± 359.93
Tryptamine	0.91 ± 0.84	8.18 ± 6.35	9.01 ± 7
Tryptophan	1.37 ± 0.49	248.53 ± 88.84	181.85 ± 65
Tryptophol	1.32 ± 0.65	21.57 ± 16.27	16.36 ± 12.34
Xanthine	1.94 ± 0.15	2.13 ± 0.03	1.1 ± 0.01

Genetic traits of evolved strain

The ALE process produced a vast amount of changes at a metabolome level. In order to establish possible relations of observed phenotypes with the genetic characteristics of the evolved strain we sequenced the genome of both parental B28 and evolved EVO strain and extracted the differential sequence polymorphisms due to ALE process. Copy number variation was established for both strains using S288c genome as a reference and general differences in duplications and deletions size between EVO and B28 were calculated for each chromosome. Evolved strain accumulated larger deletion regions than B28 in

chromosome I, III, VI and IX, while it duplicated approximately a 60 % of chromosome VII (Figure 3.7). Genes related with tryptophan metabolism such as *TRP5* or NMA2, but also flavin-dependent monooxygenase COQ6, purine metabolism-related ADE3 and ADE6 or pyruvate decarboxylase PDC6 were present in the duplication regions of chromosome VII. Regarding single nucleotide variants (SNV) and insertions or deletions of larger fragments in EVO strain compared to B28 we found more than 2400 mutations affecting over 800 open reading frames (ORFs). We did not clearly identify gene clusters that were specially affected by the mutations and assumed great part of the mutations occurred to some extent indiscriminately. We believe white light stress is not only a severe stress for its photo-oxidative effect but it also exerted a mutagenic effect in the yeast genome and it might be used to generate a great number of genome mutations in different ALE with multiple purposes of genetic improvement.

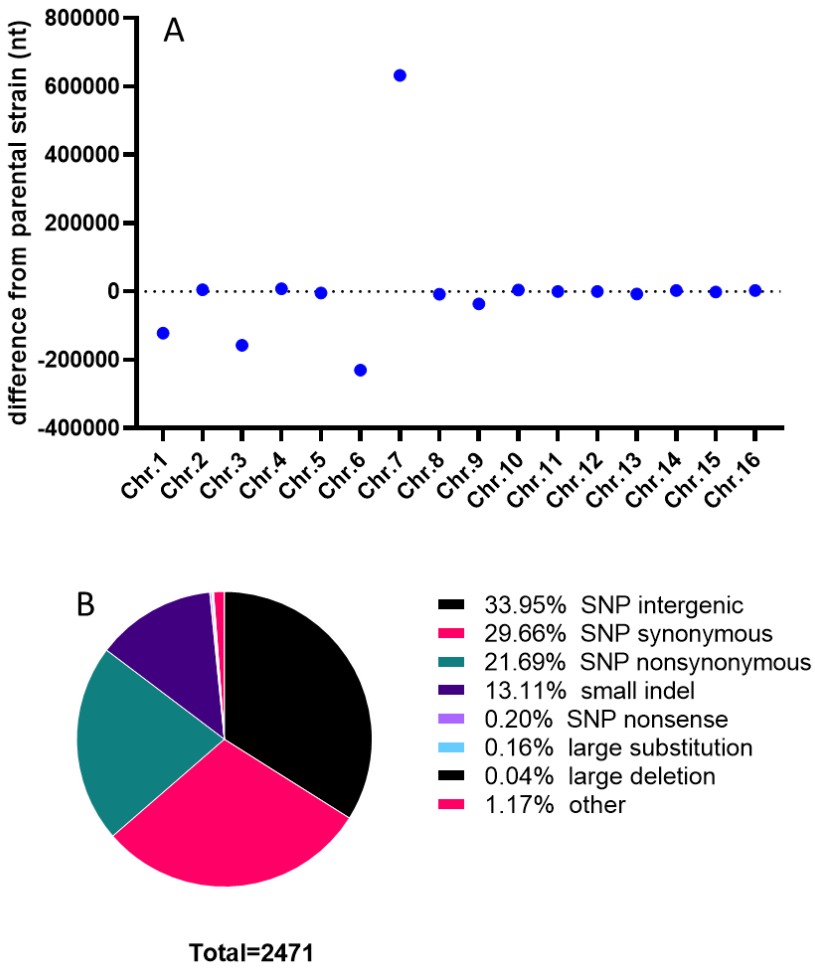

Figure 3.7. Large chromosomal duplications and deletions were detected. When comparing the size of every deletion or duplication for each chromosome between B28 and EVO, we found EVO strain presented large deletions regions in chromosome I, III and VI, while it duplicated more than 630 kb of chromosome VII (A). Genome sequencing of B28 and EVO strains revealed 2471 mutations in EVO strain as a result of the adaptive evolution. Pie chart shows the distribution of the different types of mutation detected in evolved strain (B).

Due to the wide distribution of SNV's across multiple ORFs we initially focused on establishing potential relations between newly detected genetic characteristics and the most conspicuous phenotypical traits such as the high production of aromatic amino acid-derived metabolites (Figure 3.8), the high production of xanthine and hypoxanthine, the apparent increase in cell adhesion and pigmentation after light treatment and the ability to grow under light stress condition. All the detected mutations we propose as potentially related to the observed phenotypes are due to be empirically tested in isolation, which exceeds the scope of this chapter.

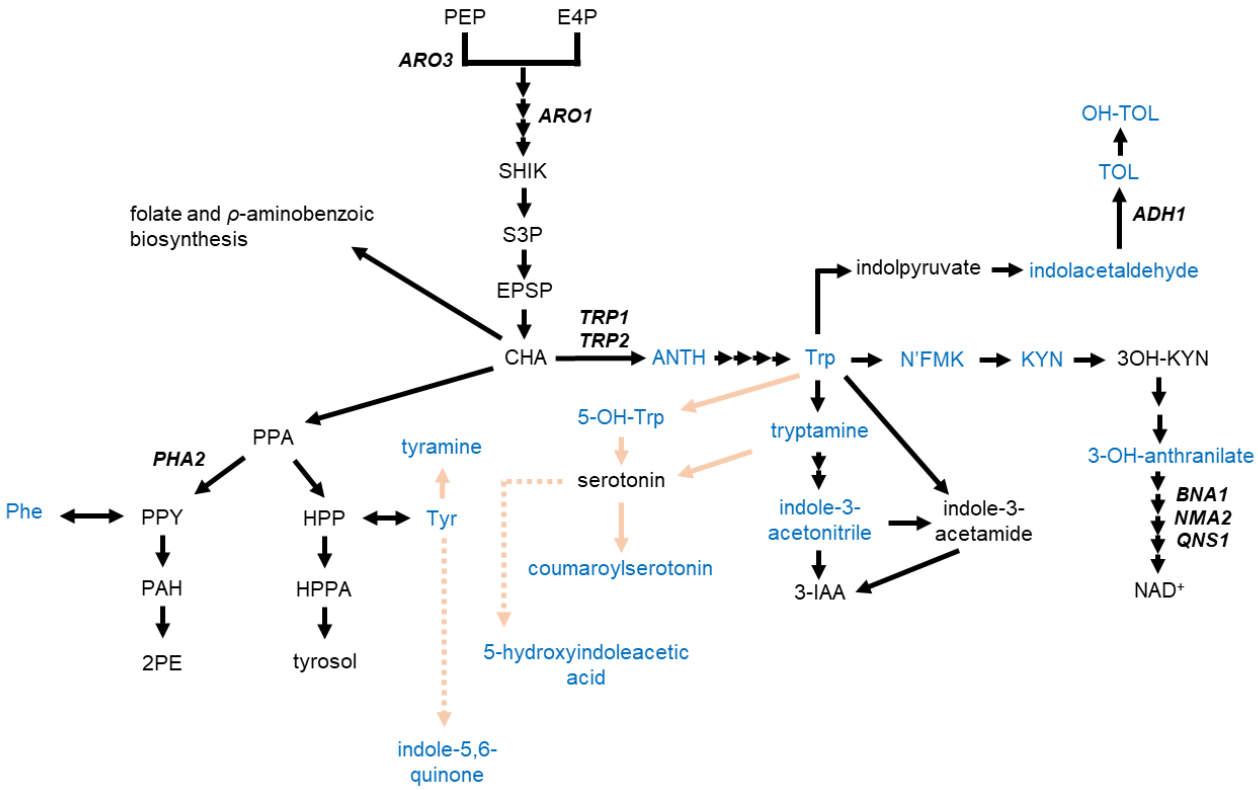

Figure 3.8. Evolved strain aromatic amino acids phenylalanine, tyrosine and tryptophan metabolic pathways. Related metabolites that were increased in EVO strain are shown in blue. Black arrows represent reactions previously reported in yeast while yellow arrows indicated

reactions still not established in yeast. Multiple step reactions are shown as dotted lines and all genes named in the figure presented some kind of mutation.

The increased relative amount of aromatic amino acid-derivatives found in EVO strain as a result of adaptation to light-induced stress was surprising as for some of the detected interesting metabolites there are still no associated genes in yeast, namely tyramine, indole-5,6-quinone, 5-hydroxytryptphan, 5-hydroxyindoleacetic acid and coumaroylserotonin.

Single nucleotide polymorphisms were found in EVO strain for nine genes related with the synthesis of aromatic amino acid-derived compounds highlighted in figure 3.8, and they could play a role in the increase of these metabolites. We propose a deregulation or an alteration in prephenate dehydratase (*PHA2*) expression, which could explain the observed increase in phenylalanine, reaching a 2-fold increase in EVO strain compared to parental B28, or over 100-fold increase in evolved strain under light condition, compared to EVO grown in darkness. Two SNPs were detected for this gene in EVO strain, one of them at the promoter level and the other producing an amino acid point mutation (I113L). Tyrosine and its derivative tyramine were also produced in higher amounts (2.1 and 1.6-fold, respectively) in EVO strain, and indole-5,6-quinone was more than 200-fold overproduced only under light stress condition. No genes in yeast are still associated to the decarboxylation of tyrosine to form tyramine, and no record of endogenous indol-5,6-quinone was found for yeast to date. Mutations upstream this biosynthetic route were found in 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (*ARO3*) as a point mutation in its promoter but also in shikimate dehydrogenase *ARO1*, which accumulated two point mutations in the coding region (E219A and L1148P). Regarding the tryptophan-derived compounds we found SNPs on 6 genes directly involved in the pathway. Anthranilate synthase (*TRP2*) presented a point mutation in K225E as well as a single nucleotide mutation in the promoter region while other single nucleotide mutation in the promoter region was also found for *TRP1* gene. The overproduction of at least thirteen metabolites downstream these genes occurred in EVO strain cultures, among them, only 5-hydroxytryptophan, N-formylkynurenine, anthranilate and hydroxyanthranilate were consistently detected in higher amounts in EVO strain grown in darkness but also under light-

induced stress. On the other hand, tryptamine, 5-hydroxyindoleacetic acid, tryptophan, kynurenine, coumaroylserotonin, 5-hydroxytryptophol, tryptophol, indoleacetaldehyde and indoleacetonitrile were only conspicuously overproduced under light stress condition (Table 3.1). No allelic variants or other SNVs were found for annotated genes involved in this part of the metabolic pathway, but mutations in genes related to NAD^+ synthesis from hydroxyanthranilate such as *BNA1*, *NMA2* and *QNS1* were found. These modifications found in evolved strain comprise up to eight SNPs in the promoter region of *BNA1*, an amino acid change (V112G) in *NMA2* and the premature stop codon in position Y227* in *QNS1* gene. Furthermore, NMA2 gene is located in one of the large duplicated regions of chromosome VII. The effect of a loss of function of *QNS1*, but also *BNA1* or *NMA2* at this point of the NAD^+ biosynthesis can favor a bottleneck in the metabolic flux at this point and promote accumulation of precursors upstream, which corresponds to some of the overproduced metabolites in evolved strain. The last modified gene that may have an impact on this metabolic pathway is *ADH1*, an alcohol dehydrogenase which plays a central role in the metabolism of ethanol and higher alcohols. In this case, it is not the only responsible of tryptophol synthesis, but up to four SNPs were detected, three of them causing changes of amino acid in the coding region (V59I, V59A and I314V) and one SNP located in the promoter region, and, therefore, their effect should be empirically established. Higher concentrations of tryptophol have been related to a limited growth in a wine fermentation context, although this effect can be reversed by co-culturing with non-*Saccharomyces* strains (Valera et al., 2019).

Tryptophol and other higher alcohols have also been identified as signaling molecules that affect expression of genes such as *TPK2*, *FLO8* and *FLO11*, favoring cell aggregation and filamentous growth. The aggregation of cells observed in EVO strain cultures can be a mild result of higher alcohols quorum sensing properties (González et al., 2018). But there are other genetic characteristics in the EVO strain that may be contributing to this cell aggregation, for example *AGA2* gene, encoding the adhesion subunit of a-agglutinin, accumulated up to six SNPs in the promoter region, and a nonsynonymous mutation in the coding region (I9V). *AGA2* gene has also more copies in EVO

strain as it is located in the same large duplicated region of chromosome VII as *NMA2*.

Purine metabolism was also altered in EVO strain, xanthine and hypoxanthine were overproduced in EVO when comparing to parental B28, especially under light stress, being hypoxanthine the most overproduced among them, up to a 9-fold increase in the LIGHT sample group (Table 3.1). Adenine deaminase *AAH1* is the responsible of the synthesis of hypoxanthine from adenine and involved in the salvage pathway of hypoxanthine and purine-containing compounds. This gene concentrated an unusual amount of mutations in the promoter region in EVO strain, up to 23 mutations in a span of 167 nucleotides located 76 nucleotides upstream the start codon. An upregulation of *AAH1* gene can favor the overproduction of hypoxanthine and indirectly xanthine. Accumulation of xanthine and hypoxanthine has been related to an increase of mutagenic deaminated nucleobases as a result of alterations in purine metabolism. If this is the case of EVO strain, to some extent, alterations in purine metabolism can be contributing to alter the strain's fitness (Pang et al., 2012; Wuenschell et al., 2003), although further genetic alterations in this pathway were only found as a SNP in the promoter region of *ADO1* gene and three SNPs in the promoter region of *ISN1* (Figure 3.9).

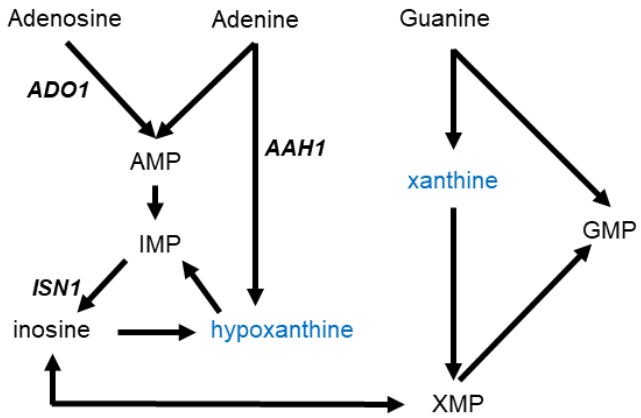

Figure 3.9. Metabolism of purines (partial) where xanthine and hypoxanthine were overproduced (blue) in EVO strain. Gene names indicated in the figure (*ADO1*, *AAH1* and *ISN1*) showed mutations in their ORFs or promoters.

From the metabolites overproduced under light-induced stress condition, those derived from serotonin metabolism such as 5-hydroxyindoleacetic acid or

coumaroylserotonin are reported to possess a potent antioxidant capacity (Lazari et al., 2017; Mercolini et al., 2012) and the ability to synthesize them can confer yeast cells an adaptive advantage against oxidative stress, nonetheless this synthesis is apparently triggered by light and, therefore, this antioxidant effect is not observed in darkness as we previously verified by growth experiments with or without H_2O_2. This production of antioxidants as a response to light-induced stress follows the same trend as in the case of the increased glutathione production mentioned above and it partially explains that basal ROS levels in EVO strain are not lower than the parental strain when grown in darkness. The reason behind ROS levels being higher in EVO strain and its generally slower growth may represent a metabolic tradeoff of the strain's capacity to specifically respond to stress by light by inducing the synthesis of a set of metabolites that were not being synthesized in the absence of light. The metabolic cost of exclusively specializing to respond to this particular stress and the fact ALE process also produced mutations in an indiscriminate manner that could affect genes unrelated to the adaption against the stress, also provides a reasonable explanation of the slight general loss of fitness when grown in darkness.

In a similar fashion, indol-5,6-quinone is only overproduced under light condition. The mere occurrence of this metabolite in yeast is unprecedented, it is a tyrosine derivate that depends on tyrosinase activity in mammals for its synthesis. It is a known precursor of melanin and it is synthesized from tyrosine by hydroxylation to dihydroxyphenylalanine (DOPA) and subsequent oxidation to dopaquinone. Then it is cyclized to dopachrome and oxidized to indol-5,6-quinone, one of the monomers involved in the eumelanin structure. As it has been recently described, this compound by itself possess hallmark properties of eumelanin such as a broadband wavelength absorption between 200-1000 nm and antioxidant properties, among others (Wang et al., 2023). The synthesis of this metabolite requires the reactions of amine decarboxylases and monooxygenases that still remain unknown in yeast. The ability to produce indole-5,6-quinone as a response to a light stress is a great adaptive advantage that explain the capacity of EVO strain to grow under such radiation intensities where parental strain is not able to grow and opens a possible interpretation to the observed pigmentation of

cells, as indole-5,6-quinone can be spontaneously oxidized and polymerized to form bigger structures and crystals with optical properties similar to melanin.

3.3. Conclusions

The obtention of new yeast strains with the capacity of increasing bioactive compounds in wine is of great interest. Recently the presence of AADCs such as serotonin and melatonin or their derivatives in wines and their attribution to yeast metabolism has prompted the search of effective methods to increase yeast ability to increase the amount of these interesting compounds. The biosynthesis of some of the most interesting compounds such as hydroxytyrosol, melatonin or serotonin still remain unknown in yeast, and the use of a non-GMO approach contributes to the arduousness of selecting a stimulus to push yeast metabolism towards their synthesis. In this chapter we perform an adaptive laboratory evolution experiment on a wine yeast using a novel selective pressure based on white light irradiation to generate a stress by photooxidation. After 170 generations, yeast strain subjected to directed evolution was able to proficiently grow under a PAR radiation dose 10 times higher than the dose which inhibits growth of the parental strain, revealing a clear adaptation to this stress. Contrary to what we expected, adaption to photooxidation doesn't seem to improve the strain's fitness against oxidative stress when challenged with hydrogen peroxide. The strain resulting from the evolution experiment manifested a slight delay in growth, a higher sensitivity to oxidants and default higher levels of intracellular ROS under no stress conditions. EVO strain is still capable to grow and fully ferment in synthetic must and therefore still holds a potential use for vinification purposes.

An initial metabolomic analysis was performed on parental strain and evolved strain grown in darkness and under light conditions, revealing great differences between B28 and EVO strains. Some of the most significant differences involved an increase in tryptophan-related metabolites such as indolic compounds, finding interesting bioactive or bioactive precursors increased due to EVO metabolism, such as 5-hydroxytryptophan, N-formylkynurenine, anthranilate and hydroxyanthranilate. We also detected that growth under light stress provoked a distinctive phenotype involving a mild cell aggregation and pigmentation, and it

also triggered the synthesis of more antioxidant metabolites in EVO strain such as glutathione, serotonin derivates and aromatic amino acids phenylalanine, tyrosine and tryptophan. Among the light-triggered overproduced metabolites, indole-5,6-quinone is of special relevance, due to its antioxidant and photoprotective properties, which are shared with those of melanin in mammals. This finding as a phenotypic trait of EVO strain is of great importance as it can provide a novel biosynthetic route for a new pigment that conferes photoprotection and antioxidant properties.

The sequencing of both parental and evolved strain allowed us to detect genetic variations on genes involved in the metabolic pathways of the increased metabolites and propose new hypothesis to explain the mechanisms behind the observed phenotypes. Further genomic data mining and the assessment of the described mutations will be addressed in our next work.

CHAPTER 4

Metabolic engineering of *Saccharomyces cerevisiae* for hydroxytyrosol production directly from glucose.

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In addition, this work resulted in a technology registered as a patent in the Spanish Patent and Trademark Office with number **P202031186**, with consideration for international application and it can be found in Annex I of this thesis.

4.1. Introduction

Hydroxytyrosol (HT) is a natural antioxidant considered to be one of the main ingredients that promotes health and a bioactive component in Mediterranean diet characterized by regular olive oil intake (Daniele et al., 2017). Several studies have demonstrated extensive biological HT properties with both *in vitro* and *in vivo* models (D'Angelo et al., 2020). The ability to cross the brain barrier and its high bioavailability and degree of absorption, together with the health claim approved by EFSA (EFSA Panel on Dietetic Products, 2017), highlight the importance of this polyphenol for food, feed, supplement and pharmaceutical industries (Britton et al., 2019). However, the price of commercially available pure HT forms can be high, which makes its use in the food industry economically unviable (Achmon et al., 2015).

The main ways to obtain HT are plant extraction or chemical synthesis. As olive tree derivatives are the most accessible source, the majority of HT products come from the extraction of olives or olive oil waste streams, of which the latter is a favorable source because it originates from a by-product. However, HT extraction from any of these sources is a lengthy process that yields low recovery rates, which can vary seasonally from batch to batch. Chemical synthesis methods usually involve non environmentally friendly solvents and expensive starting substrates, which sometimes make it unsuitable for large-scale industrial production (Zhang et al. 2012; Achmon and Fishman 2015; Britton, Davis, and O'Connor 2019). Therefore, biotechnological HT production can potentially be the dominant production process for the future. Baker's yeast, *Saccharomyces cerevisiae*, is a promising cell factory for producing recombinant HT thanks to a number of advantages, such as robust growth on simple media, feasibility in genetic manipulations and it is "generally regarded as safe" (GRAS) (Nielsen et al., 2013; Guo et al., 2019).

We previously succeeded in performing the heterologous overexpression of genes *hpaB* and *hpaC* from *Escherichia coli* in *S. cerevisiae* by using episomal plasmids in our previous work (Muñiz-Calvo et al., 2020). Through this overexpression, we achieved titers ranging from 1.15 to 4.6 $mg \cdot L^{-1}$ in a minimal medium in which either 1 mM tyrosine or 1 mM tyrosol was respectively added, with tyrosol being the preferred starting material. Nevertheless, the price of tyrosol and tyrosine is around 6250- and 600-fold higher than that of glucose,

which makes this monosaccharide a more appealing source to produce HT for its low cost. In this chapter we engineer an HT-overproducing yeast strain directly from a simple carbon source like glucose in *S. cerevisiae*. For that, we first constructed a plasmid-free yeast strain harboring the HpaBC complex integrated into multiple copies and then we tested different genetic modifications to increase the metabolic flux towards the main immediate precursor tyrosol following a metabolic engineering approach and we obtained the highest concentration of hydroxytyrosol produced by yeast to date. After achieving this milestone we focused our efforts towards the genetic improvement of the HT producer strain to prioritize productivity over maximum concentration of HT by using other versions of HpaBC complex, by regulating the expression of a native gene to favor a metabolic flux towards HT and by overexpressing a novel potential fungal hydroxylase complex to convert remaining precursors such as tyrosol or 2-phenylethanol into HT.

4.2. Results and discussion

Genetic modifications to improve HT synthesis

A HT producer strain BY4743 HpaB+HpaC was previously constructed, which was able to produce a maximum concentration of 4.6 ± 0.9 $mg \cdot L^{-1}$ of HT in a minimal medium with a tyrosol supplementation (Muñiz-Calvo et al., 2020). After this achievement, our interest focused on improving maximum HT concentrations due to yeast metabolism, but using glucose as a substrate. We first decided to integrate both copies of HpaBC complex into the yeast genome before any further modification to achieve greater stability in the expression of those genes compared to the overexpression with episomal plasmids carried out so far. We constructed an integrative cassette which contained *hpaC* with the *TEF1* promoter and *hpaB* with the *PGK1* promoter using pCfB2988 plasmid from EasyCloneMulti series (Maury et al., 2016) for the cassette assembly. Among its features, this vector contains homologous sequences to Ty1 transposable elements, allowing the multiple integration of the cloned genes into the yeast genome. After integrating pCfB2988 cassette in BY4743 we tested 24 transformants for HT production when grown in SC with 1 mM of tyrosol and selected the strain with a higher production (Figure 4.1). A wide range of HT concentration was achieved by the different tested transformants, ranging from

0.2 to more than 25 mg·L^{-1} and thus obtaining a good number of transformants exhibiting better HT titers than the plasmid-based overproducer strain. The better results regarding overexpression when comparing multiple integration and episomal plasmid have been previously reported (Maury et al., 2016), as only a small fraction of the cell population seem to exhibit a high expression of the gene of interest when it is overexpressed with an episomal plasmid due to segregational instability (Jensen et al., 2014). And the big differences in the HT production between transformants can be explained by the heterogeneity in the number of integrated copies between them. The best producer strain selected from this assay, henceforth HpaBC strain, was taken as a chassis for further genetic modifications.

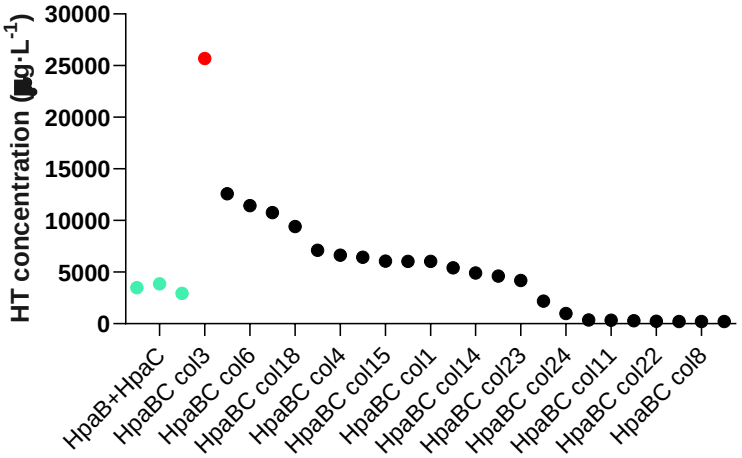

Figure 4.1. Screening for hydroxytyrosol production at 72 h in SC medium supplemented with tyrosol by the different BY4743 transformants harboring HpaBC complex integrated into the genome in several copies (black dots). The most productive strain was selected and used in further studies and is colored in red. Strain BY4743+p426GPD-*hpaB* +p425GPD-*hpaC* (HpaB + HpaC) is depicted as green dots for comparative purposes.

Metabolic engineering of the shikimate and Ehrlich pathways to increase tyrosol and HT production

In *S. cerevisiae*, tyrosol can be obtained from tyrosine through the Ehrlich pathway (Ehrlich, 1907; Sentheshanmuganathan et al., 1958) (Figure 4.2). Indeed the yeast genes involved in these reactions have been heterologously expressed in *E. coli* to overproduce tyrosol and HT (Xue et al. 2017a; Li et al. 2018b). However, a recent study has shown that α-keto acid precursors, required for the *de novo* synthesis of aromatic higher alcohols, come mainly from the

catabolism of sugars through the shikimate pathway, with a limited contribution from the anabolism of consumed amino acids (Crépin et al., 2017).

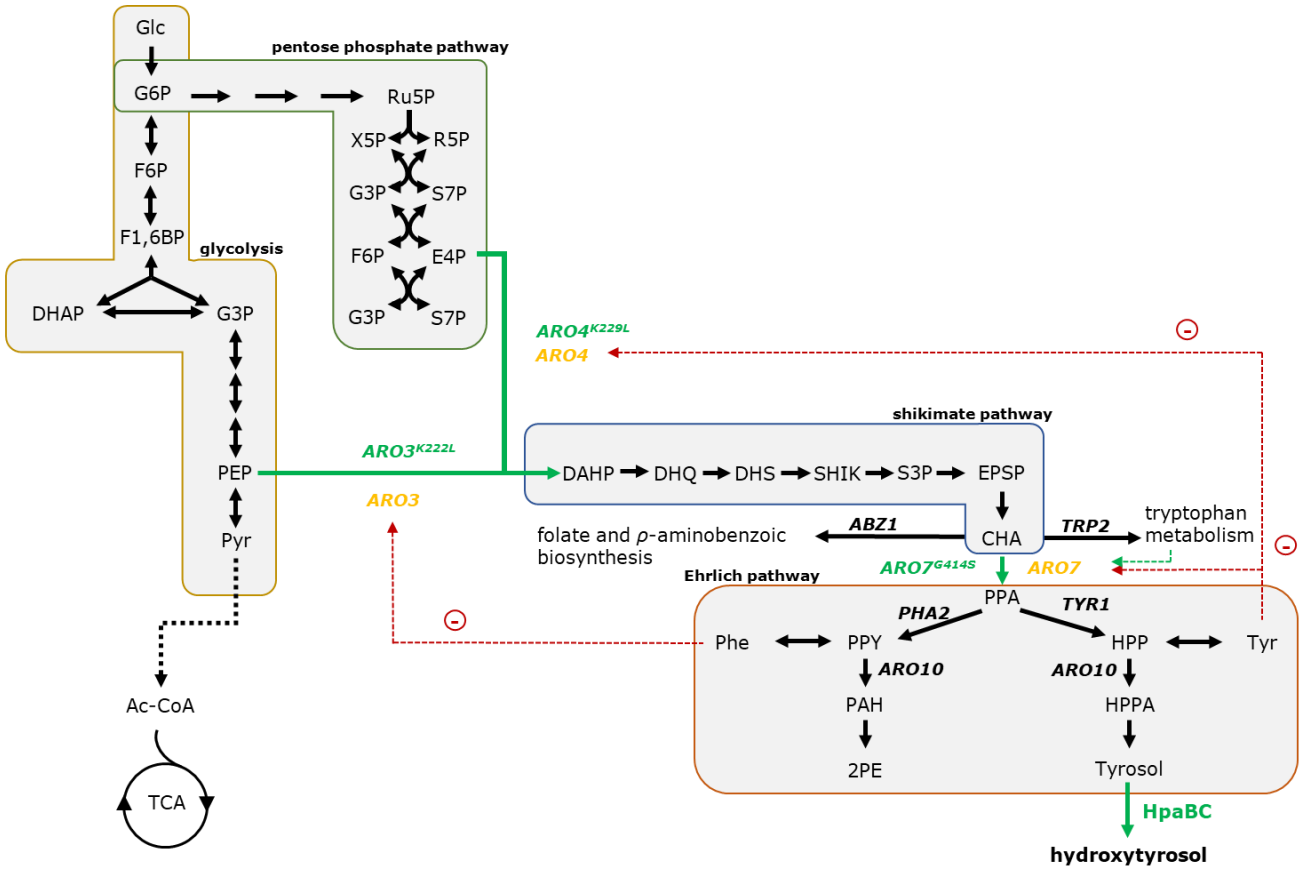

Figure 4.2. Overview of the aromatic amino acid metabolism in *Saccharomyces cerevisiae*. Hydroxytyrosol was heterologously produced from glucose through the shikimate and Ehrlich pathways, together with the hydroxylase complex (HpaBC). The overexpression of the native or engineered enzymes is indicated in yellow and green respectively. The dotted red lines indicate allosteric inhibition by the phenylalanine to *ARO3* and by the tyrosine to *ARO4* and *ARO7,* whereas the green dotted line denotes *ARO7* activation by tryptophan.

To promote an increase of the metabolic flux from sugar catabolism towards tyrosol we first decided to limit flux towards competitive pathways such as tryptophan, *p*-aminobenzoic acid and phenylalanine biosynthesis. For that purpose we measured the tyrosol produced by our control strain BY4743 and its deleterious mutants for genes *TRP2*, *ABZ1* and *PHA2*, which are responsible for the initial step of their respective pathways using chorismate and prephenate as a substrate (Figure 4.2). *TRP2* encodes an anthranilate synthase, which catalyzes the initial tryptophan biosynthesis step (Fantes et al., 1976), *ABZ1* encodes a *para*-aminobenzoate synthase involved in the synthesis of *p*-aminobenzoic acid (PABA) from chorismate, but has also been related to 2-

phenylethanol production (Edman et al., 1993; Steyer et al., 2012), and *PHA2* encodes prephenate dehydratase, which consumes prephenate in the phenylalanine biosynthesis pathway (Maftahi et al., 1995). Unexpectedly the deletion of these genes did not increase the chorismate flow towards tyrosol production as no increase was observed in the *trp2*$^{-}$ strain, and even a decrease was detected for mutant strains *abz1*$^{-}$ and *pha2*$^{-}$ (Figure 4.3) compared to the wild-type strain BY4743. Given that the single deletion of those genes was not an optimal strategy to increase tyrosol production in *S. cerevisiae*, we decided to follow a different strategy. Our next steps focused on the overexpression of different ARO genes (native or deregulated versions), either directly involved in the Ehrlich pathway for the synthesis of tyrosol or upstream this pathway in the Shikimate pathway.

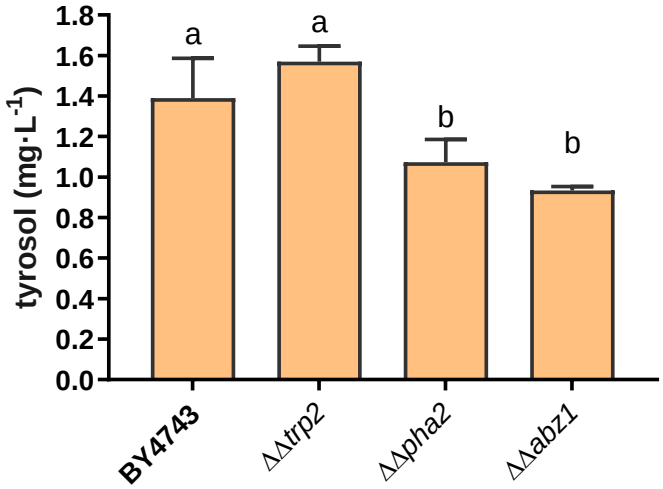

Figure 4.3. Effect of knockouts TRP2, PHA2 or ABZ1 on tyrosol production in the BY4743 background. Tyrosol levels produced by the BY4743 wild-type strain (control) and their knockout mutants for *TRP2* (ΔΔ*trp2*), *ABZ1* (ΔΔ*abz1*) and *PHA2* (ΔΔ*pha2*) were determined after growing in SD medium for 72 h. The tyrosol concentration was determined from the supernatant extracted with methanol and subjected to UHPLC-MS/MS. Error bars represent the standard deviations calculated from biological triplicates. The values under the same letter are not significantly different according to the Tukey HSD test.

Effect of individual ARO genes overexpression on tyrosol and HT production

Phenylpyruvate decarboxylase *ARO10* catalyzes a major reaction in the Ehrlich pathway of aromatic amino acids tyrosine and phenylalanine, converting 4-hydroxyphenylpyruvate (4HPP) into 4-hydroxyphenylacetaldehyde (4HPAA), in parallel it also catalyzes the conversion of phenylpyruvate into

phenylacetaldehyde in the phenylalanine branch (Figure 4.2). As a strategy to produce high concentrations of tyrosol, *ARO10* overexpression has been previously reported in *E. coli* (Xue et al., 2017; Xu et al., 2020) and we decided to overexpress this gene using episomal plasmid p423GPD in our BY4743 HpaBC selected strain and evaluate its effect on tyrosol and HT production. *ARO10* overexpression increased 45.5-fold the tyrosol levels in SD +leu medium when comparing to the control BY4743 HpaBC strain bearing an empty vector. This increase in tyrosol levels resulted in 40-fold higher HT production for this strain compared to the control (Figure 4.4) (Table 4.1). These results highlighted the potential of *ARO10* to yield a synergistic effect together with the overexpression of HpaBC complex to successfully increase HT concentration.

Nonetheless, we wanted to test other key genes upstream of the Ehrlich pathway, to favour more flux from glucose metabolism through the shikimate pathway and increase the pool of HT precursors. The first precursor of the shikimate pathway is 3-amino-D-arabino-heptulosonate-7-phosphate (DAHP) and it is formed from phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) from the glycolysis and the pentose phosphate pathways, respectively, and this step is catalyzed by the two DAHP synthase isozymes *ARO3* and *ARO4* (Teshiba et al., 1986). Other important precursor upstream of the Ehrlich pathway that is a crossroad from which the branches of tryptophan metabolism, and folate and PABA synthesis pathways diverge is the chorismate, and the chorismate mutase, encoded by *ARO7*, catalyzes the conversion of chorismate to prephenate (Ball et al., 1986), the last common precursor to both phenylalanine and tyrosine biosynthesis. It is well known that *ARO3* is allosterically inhibited by phenylalanine and *ARO4* by tyrosine (Braus, 1991), and *ARO7* is also sensitive to allosteric regulation, being inhibited by tyrosine and stimulated by tryptophan (Schmidheini et al., 1989). Regarding allosteric inhibition in these genes, allelic variants with a single nucleotide mutation have been previously described for all three of them, and overexpression of $ARO3^{K222L}$(*ARO3**), $ARO4^{K229L}$(*ARO4**) and $ARO7^{G141S}$(*ARO7**) have been used to successfully increase their product bypassing the negative feedback loop that operates in the wild-type variants (Schmidheini et al. 1989; Fukuda et al. 1991; Reifenrath and Boles 2018; Luttik et al. 2008; Curran et al. 2013; Gottardi et al. 2017; Liu et al. 2019) so we followed the same approach as for *ARO10* and we individually overexpressed *ARO3*,

ARO4 and *ARO7*, as well as their deregulated versions *ARO3**, *ARO4** and *ARO7** in BY4743 HpaBC strain to examine if there was any measurable effect on tyrosol and HT biosynthesis.

Table 4.1. Tyrosol, hydroxytyrosol (HT), 2-phenylethanol (2-PE) and tryptophol (TOL) production by HpaBC strain transformed with the empty p423GPD vector and the same strain, but overexpressing in the same plasmid one of the following genes: *ARO3*, *ARO4*, *ARO7*, *ARO10*, $ARO3^{K222L}$, $ARO4^{K229L}$ and $ARO7^{G141S}$ after growing in SD medium for 72 h. Values are represented as $mg \cdot L^{-1}$ ± SD. Asterisk indicates the overexpression of the mutant variant of the gene (*ARO3**, *ARO4** and *ARO7** to indicate $ARO3^{K222L}$, $ARO4^{K229L}$ and $ARO7^{G141S}$, respectively)

	Tyrosol	**HT**	**2-PE**	**TOL**
HpaBC	0.75 ± 0.42	0.015 ± 0.005	2.49 ± 0.71	0.22 ± 0.03
ARO3	24.82 ± 0.30	0.59 ± 0.27	28.26 ± 0.93	2.87 ± 0.08
ARO4	11.32 ± 0.09	0.58 ± 0.05	16.34 ± 0.24	1.28 ± 0.01
ARO7	0.64 ± 0.01	0.03 ± 0.00	2.54 ± 0.11	0.17 ± 0.01
ARO10	24.27 ± 0.52	0.55 ± 0.08	54.13 ± 0.81	1.04 ± 0.02
ARO3*	26.85 ± 0.99	1.34 ± 0.15	33.08 ± 1.57	3.26 ± 0.15
ARO4*	57.24 ± 2.15	2.08 ± 0.27	74.91 ± 2.48	8.31 ± 0.34
ARO7*	1.05 ± 0.14	0.03 ± 0.003	2.80 ± 1.09	0.19 ± 0.02

Our results showed that the individual overexpression of either *ARO3* or *ARO4* was successful for raising tyrosol levels, and achieved an increase of about 50- and 21-fold, respectively. The single overexpression of $ARO3^{*}$ and $ARO4^{*}$ also resulted in higher tyrosol levels, and tyrosine feedback-resistant $ARO4^{*}$ overexpression had the strongest impact (~110-fold). Regarding HT, the overexpression of *ARO3* or *ARO4* also increased the production of this molecule, similarly to the concentration achieved by *ARO10* overexpression, while deregulated versions displayed better results than the wild-type ones and the largest amount of tyrosol was also the highest titer of the HT concentration (2.08±0.27 $mg \cdot L^{-1}$) achieved by $ARO4^{*}$ overexpression. This concentration represented an increase in HT of 150-fold (Figure 4.4). No significant increase was observed for *ARO7* or *ARO7** overexpression in tyrosol or HT concentrations under the same conditions. It is worth mentioning that the overexpression of the same genes also turned out in a significant increase in the other aromatic higher alcohols, mainly in the case o 2-phenyl ethanol, denoting that this overexpression was clearly increasing the metabolic flux into the shikimate pathway.

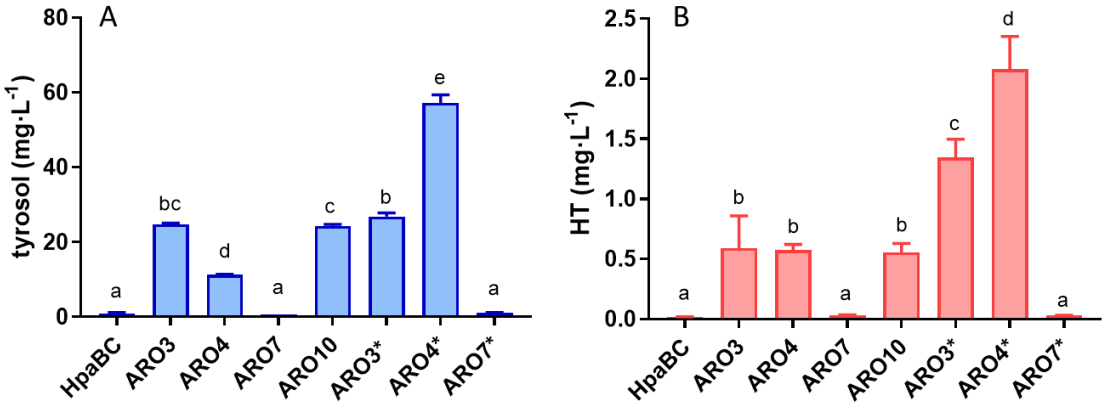

Figure 4.4. Effect of the single overexpression of several genes involved in amino acid metabolism on tyrosol (A) and hydroxytyrosol (B) production. HpaBC strain was transformed with the empty p423GPD plasmid (control) or with p423GPD containing one of the following genes: *ARO3*, *ARO4*, *ARO7*, *ARO10*, *ARO3**, *ARO4** and *ARO7**. Each strain harbouring one plasmid was cultured for 72 h at 30 °C in SD+leu. Tyrosol and hydroxytyrosol were determined from the supernatant extracted with methanol and analysed by UHPLC-MS/MS. The values under the same letter are not significantly different according to the Tukey HSD test.

Effect of the combined overexpression of the native and deregulated versions of ARO genes on tyrosol and HT production.

To determine if a possible additive effect on HT production could result from combining the overexpression of some previous genes, up to four ARO genes were overexpressed in the BY4741 Δ*trp1* strain background. The reason for switching from the diploid (BY4743) to the haploid version (BY4741) was that a larger number of auxotrophies is required to overexpress up to four ARO genes in the same strain. Haploid BY4741 harbors four auxotrophic markers (Δ*ura3*, Δ*leu2*, Δ*his3*, Δ*met15*), and we additionally deleted the *TRP1* gene to generate a new auxotrophy (Δ*trp1*) and avoid leakage of metbolic flux towards the tryptophan branch. Logically, we also constructed strains HpaBC and *ARO4** in the BY4741 genetic background, which were used as control strains.

In this experiment, together with tyrosol, we also measured the other aromatic higher alcohols (tryptophol and 2-phenylethanol) to better understand the carbon flux redirection. Figure 4.5 shows the production of the three aromatic higher alcohols generated by the different engineered strains. Remarkably, the simultaneous overexpression of different ARO genes led aromatic higher alcohols titers to vastly increase compared to control strain HpaBC. Of the three

different aromatic higher alcohols, 2-phenylethanol was produced more abundantly, followed by tyrosol and finally by tryptophol.

We detected up to 90 mg·L^{-1} of 2-phenylethanol in one clone of the strain that harbored the combinatorial *ARO3*, *ARO4*, ARO10* and *ARO7** overexpression. Similar concentrations have been reported by Shen et al. (2016) in a yeast strain that overexpressed *ARO10* and *ADH1* combined with the knockout of the *ARO8* gene in the BY4741 background. The tyrosol level ranged from 20 to 45 mg·L^{-1} for the different engineered strains (Figure 4.4). Interestingly, the strain that led to the highest 2-phenylethanol levels (*ARO3*, *ARO4*, ARO10* and *ARO7**) was the same as that which generated the highest tyrosol concentration, which reflects a good correlation observed between tyrosol and 2-phenylethanol production (r = 0.9824). As mentioned above, we do not observe an increase in tyrosol levels with the single overexpression of the *ARO7* wild-type allele or feedback-insensitive form *ARO7** (Figure 4.3). Nonetheless, when this allele, especially *ARO7**, was co-overexpressed with other modifications, including *ARO3*/*ARO3**, *ARO4** and *ARO10*, tyrosol production increased by about 20-fold compared to the control strain (Figure 4.5). Previous works have already observed a similar phenotype regarding *ARO7* overexpression in glucose-limited chemostat cultures (Luttik et al., 2008).

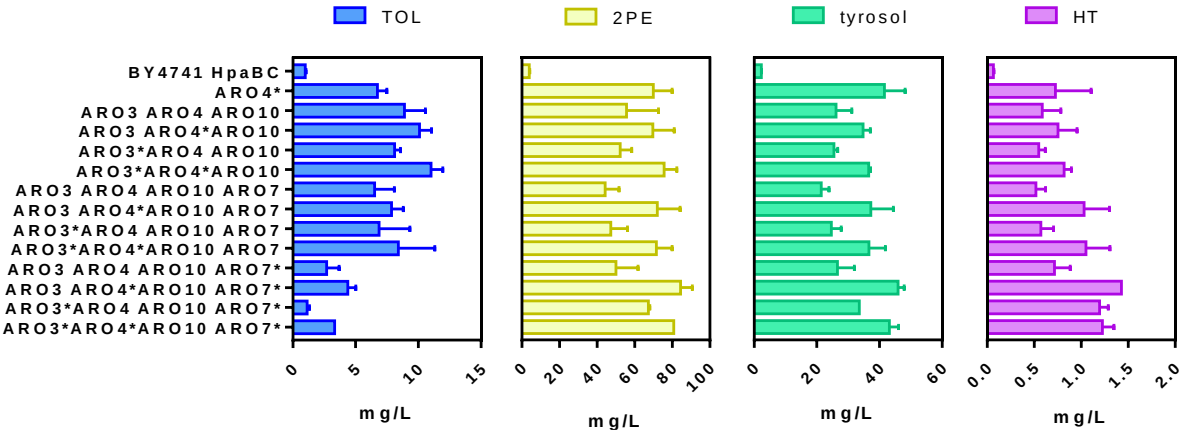

Figure 4.5. Effect of the combined overexpression of the several genes involved in aromatic amino acid metabolism on aromatic higher alcohols production. BY4741 HpaBC was transformed with different plasmids, each containing a *S. cerevisiae* gene for its overexpression (*ARO3, ARO4*, *ARO7*, *ARO10* and feedback-resistant derivatives *ARO3**, *ARO4** and *ARO7**). The different strains were cultured for 72 h at 30 °C in SD. The tryptophol (TOL), 2-phenylethanol (2PE) and tyrosol concentrations were determined from the supernatant extracted with methanol, and analyzed by HPLC-PDA.

Finally, tryptophol levels fell within ranges from 1 to 11 mg·L^{-1}, and the strain that co-overexpressed *ARO3**, *ARO4** and *ARO10* was the best producer. Curiously when the modified strains co-overexpressed *ARO7** together with other modifications, tryptophol levels also considerably lowered (Figure 4.5). For instance, *ARO7** overexpression in the highest tryptophol-producing strains (*ARO3**, *ARO4** and *ARO10*) actually lowered tryptophol levels from 11.05±0.95 to 3.33±0.27 mg·L^{-1} (Figure 4.5). This result can be explained because the tryptophol pathway competes with the pathways of tyrosol and 2-phenylethanol at the chorismate node. Therefore when *ARO7* is overexpressed, chorismate is used preferentially to produce prephenate and its derivatives 2-phenylethanol and tyrosol, which results in less chorismate availability for the synthesis of the precursor of tryptophol, namely anthranilate. Insensitive-feedback allele *ARO7** overexpression further enhanced this metabolic flux toward tyrosol and 2-phenylethanol synthesis. Regarding HT levels, the different overexpression combinations had a positive impact compared to the control strain HpaBC, and the strain overexpressing *ARO3*, *ARO4**, *ARO10* and *ARO7** was the highest producer with 1.5 mg·L^{-1} of HT, which is practically twice the amount produced by the strain that only overexpressed *ARO4** in the haploid version. However, when we compared the single overexpression of *ARO4** in the haploid and diploid background, BY4743 *ARO4** synthesized more than 2-fold HT (2.08 mg·L^{-1}) than BY4741 *ARO4** (0.73 mg·L^{-1}). Moreover, the single overexpression of *ARO4** in BY4743 resulted in higher HT titers than the better combination of the ARO genes in BY4741. As previously reported by Suástegui et al. (2016), the production capacity of aromatic compounds can display a wide range of variability in a strain-dependent manner, even when the same exact genetic modifications are conducted in different strains. These different capacities are closely related to each strain's ability to adjust copy numbers of episomal vectors, but are also related to differences in pathway balancing to channel carbon metabolism in aromatic amino acid pathways. We finally selected the overexpression of *ARO4** as a major key modification to overproduce HT, together with the HpaBC complex, and aimed to produce the maximum HT concentration using this strain. Although the co-overexpression of *ARO4** and *ARO10*, *ARO3** or *ARO7** are also currently being implemented on the best HT producer strain to maximize metabolic flux to tyrosol from the genetic modifications approach.

Effect of sugar concentration in the HT production.

In order to establish suitable medium conditions for HT synthesis, we tested different glucose concentrations in the media to increase HT production. For this purpose, we cultured engineered strains BY4743 *Ec*HpaBC (strain HpaBC) and our best HT producer strain at the moment, BY4743 *Ec*HpaBC + *ARO4** (strain ARO4*), in SD at six glucose concentrations (20, 80, 160, 200, 250 y 300 $g \cdot L^{-1}$). HT production was steadily increasing from 20 up to 160 $g \cdot L^{-1}$ glucose concentration. Further glucose availability in the growth medium did not result in a significant increase in HT concentration (data not shown), therefore 160 $g \cdot L^{-1}$ of glucose was taken as a reference for a high production medium when we further compared the HT biosynthesis between different strains. As expected, a remarkable effect of raising the initial precursor glucose was observed when comparing HT production of ARO4* strain in both media (Figure 4.6A), samples were taken from 120 h of incubation in order to establish a time of incubation when the maximum HT concentration is reached. The maximum production was obtained from a sampling time of 223 h with no significant increases in further samplings. However, this HT accumulation in growth medium only occurred in SD160, and not in SD20, which showed a constant concentration for all the sampling times, and therefore sampling was not continued after 197 h in this case. HT concentration was 84-fold higher in the ARO4* strain in SD160 when compared to the same strain in SD20 under the same incubation time (Figure 4.6B).

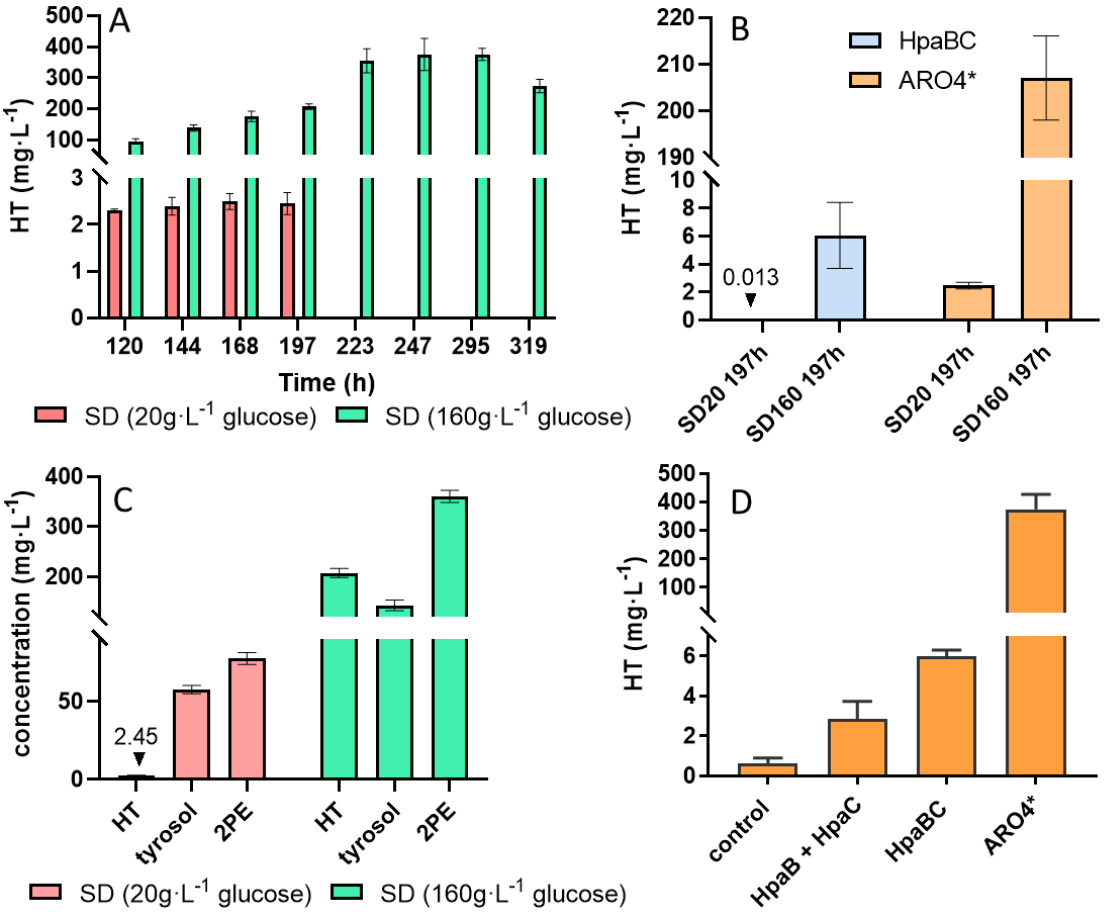

Figure 4.6. Effect of glucose concentration on hydroxytyrosol production over time (A). Strains BY4743 *Ec*HpaBC (HpaBC) and *Ec*HpaBC+*ARO4** (ARO4*) were cultured at 30 °C in shake flasks filled with SD containing 20 and 160 g·L^{-1} of glucose (SD20 and SD160, respectively). A comparison between strains HpaBC and ARO4* showed a great relevance of *ARO4** modification, especially when an excess of glucose is available (B). Strain ARO4* reached high HT titers after 197h of incubation, surpassing those from tyrosol in SD160 but not in SD20 (C). The HT concentration reached by ARO4* strain at time 240 h represented a 130-fold increase when comparing to the plasmid-based BY4743+p426GPD-*hpaB* +p425GPD-*hpaC* strain (HpaB + HpaC) (D). HT concentration was determined from the supernatant and analyzed by HPLC-PDA. The comparisons between strains were significantly different in all cases according to the Student's t-test (P value ≤ 0.05).

Considering HT levels in SD20 remained constant in these late sampling times, if we assumed the same concentration at the time 223 h, the HT concentration in SD160 would represent an increase of almost 150-fold. When comparing production of other higher alcohols like tyrosol and 2PE at time 197 h we observe they were similarly increased in SD160 when compared to SD20, but in a different fashion in ARO4* strain and HpaBC strain. Apparently, in ARO4* strain there's a synergetic effect of both genetic modifications that result in a more efficient

conversion of tyrosol into HT. In the ARO4* strain concentration of HT surpassed that of tyrosol for the first time, which reaffirms *ARO4** modification as an important trait to maximize the conversion of tyrosol to HT (Figure 4.6C). Another conclusion reached with this result is that the HT produced and secreted to culture medium remained stable and was either not subsequently metabolized or metabolized at the same rate it was produced, reaching an equilibrium state. In fact, that allowed us to obtain the greatest amount of HT so far, generated directly by glucose, at the late sampling time of 240 h, in which we obtained 375 $mg \cdot L^{-1}$ of HT as a result of the ARO4* strain metabolism (Figure 4.6D), representing a 130-fold increase over the producer strain from our previous work, which overexpressed HpaBC complex in episomal plasmids.

Improving HT production by selecting the optimum bacterial monooxygenase complex

The great improvement in HT biosynthesis achieved so far when overexpressing *ARO4** and HpaBC complex from *E. coli*, and the fact that precursors like tyrosol or 2PE are also generated in high amounts made us think further genetic improvements should be addressed, especially at the hydroxylating step in which HpaBC is involved. The high concentration of remaining tyrosol pointed out the occurence of a bottleneck in HT production in this final step. A more efficient hydroxylase activity at this point could increase HT levels under the same conditions by lowering final tyrosol concentration. As previously reported, subunits of HpaBC from other microorganisms such as *Pseudomonas aeruginosa* (*Pa*) or *Salmonella enterica* (*Se*) have been heterologously expressed in yeast and successfully replaced, and improved the function of those from *E. coli* (Liu et al. 2019). Hydroxylase subunits *hpaB* and *hpaC* from *Ec*, *Pa* and *Se* were amplified and cloned in episomal vectors p426GPD and p425GPD, respectively. Yeast strain BY4743 was transformed with the different versions of HpaBC and cells were grown for 120 h at 30 °C in SD160 with 76 $mg \cdot L^{-1}$ histidine supplementation to fulfill the strains growth requirements, and higher alcohols HT, tyrosol, 2PE and TOL were quantified (Figure 4.7A).

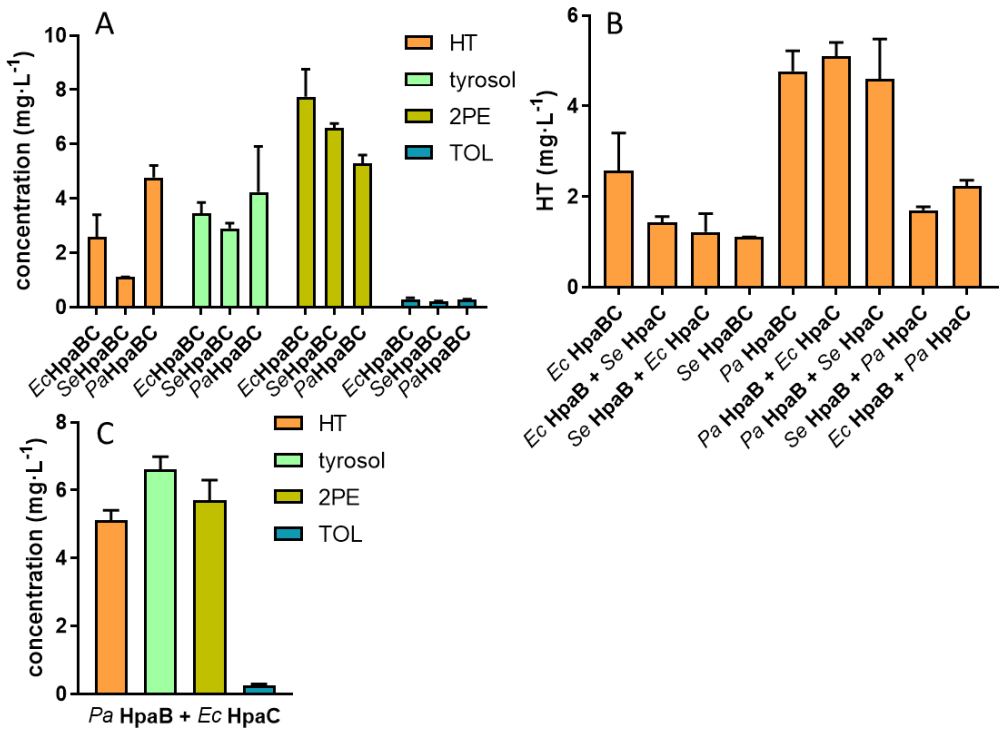

Figure 4.7. Overexpression of HpaBC complex from *Escherichia coli* (*Ec*), *Salmonella enterica* (*Se*) and *Pseudomonas aeruginosa* (*Pa*) yielded different concentrations of HT when overexpressed in yeast (A). Higher HT concentrations were consistently obtained when overexpression of *Pa*HpaB was involved for every possible combination between HpaB and HpaC subunits (B). Overexpression of the combination *Pa*HpaB + *Ec*HpaC also presented high concentrations of tyrosol and 2PE (C).

Among the HpaBC complexes from different organisms, the one from *Pseudomonas aeruginosa* stood out as it reached higher HT concentration. We also tested different combinations of *hpaB* and *hpaC* from the three different microorganisms and we observed a clear increase in HT concentration in every combination involving the hydroxylase subunit *Pa hpaB* (Figure 4.7B). Apart from the highest production of HT (5.11 mg·L^{-1} in 72 h), the specific combination of *Pa hpaB* and *Ec hpaC* also showed more tyrosol accumulation (Figure 4.7C), and therefore a higher precursor concentration for more HT production. We then selected this HpaBC combination and carried out the multiple integration of them into the yeast genome, in Ty1 loci in a similar way we did before for the full *E. coli* version, but this time, we also included an integration cassette, targeting *Ty2* loci, and harboring *ARO4** under the control of the strong constitutive *TDH3* promoter. By applying these modifications we reproduced those genetic traits present in the previous HT producer ARO4* strain but with two main distinctive features, the

use of a new hydroxylase subunit from *P. aeruginosa* (*Pa hpaB*) and the overexpression of *ARO4** by multiple integration into the genome, achieving a plasmid-free strain with more stable genome integrations. A new selected strain harboring these modifications (strain A1.1) was obtained and HT concentration was determined after growth in SD160 at 28 ºC (Figure 4.8).

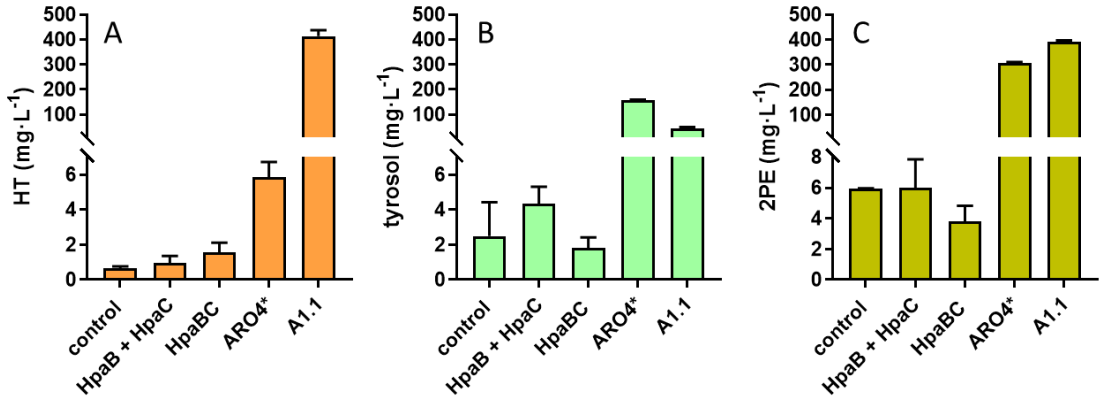

Figure 4.8. Highest concentration of HT was reached by A1.1 strain after 120 h of growth which halved the time required to reach a maximum HT concentration compared to the previous strains (A). A1.1 strain showed better tyrosol hydroxylation than ARO4* strain, despite this, high concentration of tyrosol remained unmetabolized (B). Both ARO4* and A1.1 strains displayed a great 2PE synthesis ability, evidencing a clear potential to further redirect metabolic flux toward HT (C).

Surprisingly, strain A1.1 not only reached the highest titer of HT so far (430 mg·L^{-1}), but it achieved such concentration in half the incubation time (120 h), which is a significative improvement in terms of efficiency that resulted specially striking when comparing HT concentration reached by the previous producer strains at the same sampling time, being more than 70 times higher than the previous ARO4* strain, or 280 times greater than the HpaBC strain (Figure 4.8). Although we achieved the highest HT concentration by reducing to 120 h the incubation time and using SD160 as a growth medium in shake flasks with constant shaking, sampling of A1.1 at longer sampling times didn't yield higher HT concentration (data not shown). This great increase in HT in A1.1 occurred at the expense of tyrosol concentration, which was significantly lowered to less than one third of that produced by the ARO4* strain. This indicates the hydroxylase subunit from *P. aeruginosa* partially alleviated the bottleneck at this final step, but an increase in 2PE was also observed, indicating the metabolic flux toward HT has an

important leakage point at the prephenate level, that diverges towards the phenylalanine branch and culminates in an accumulation of 2PE (Figure 4.8B and C). After achieving HT titers close to 0.5 $g \cdot L^{-1}$ we focused on relieving the accumulation of precursor tyrosol, and the catabolite from the phenylalanine branch 2PE by applying two more genetic modifications on A1.1 strain.

Improving HT synthesis capacity by complementing with a hydroxylase complex of fungal origin.

Our previous goal was to achieve a maximum HT concentration in a genetically modified strain that under the above determined conditions reached high HT titers. In a next step, we focused on the improvement of this HT ability regardless of the maximum final HT concentration. For this reason, the next assays were primarily designed to compare new genetic modifications to the previous preferred strain A1.1 to determine a modification that positively contributed to either increase HT concentration or decrease 2PE to favor tyrosol or HT synthesis, so growth conditions were adjusted to smaller volumes, and occasionally shorter incubation times to gain practicality. We proposed two strategies regarding genetic modifications: the first one involved the overexpression of a new heterologous hydroxylase complex and the second strategy consisted in decreasing the metabolic flow towards 2PE by knocking down gene *PHA2*, responsible of the prephenate shunt towards the phenylalanine branch. At this point, the use of a fast, reliable and inexpensive method of HT detection and quantitation was of special interest. To this regard, a colorimetric method for a rapid HT determination have been previously reported (Chen et al. 2019), which is based on the oxidation of the *o*-diphenol group to *o*-quinone by the action of the sodium periodate, this reaction causes a color change to yellow in the case of HT and dopamine, but not for tyrosol, tyrosine or tyramine and the colorimetric difference can be quantified measuring OD_{400}. The use sodium periodate as a HT indicator in a fast-colorimetric assay offers a good alternative to quantitate HT concentration in a liquid sample or to compare several similar samples and determine their relative amount of HT.

The first approach involves the overexpression of a toluene monooxygenase (TMO) to perform successive double hydroxylation of 2PE, which consists in a first hydroxylation reaction to produce tyrosol, and the second reaction producing

HT from tyrosol. TMOs have been previously used for HT production in a bacterial platform, tweaking their regiospecificity and activity by applying single amino acid mutations in the hydrophobic cavity surrounding the di-iron binding site of their hydroxylase *α* subunit (Brouk et al., 2009). Particularly we took as a model the toluene *ortho*-monooxygenase complex (TOM) from *Burkholderia cepacia* (*Bc*) which natively hydroxylates toluene at the *ortho* position to form *o*-cresol (Canada et al., 2002). This bacterial complex consists of six subunits (A0 to A5), where A3, A1 and A4 correspond to subunits *αβɣ* of the hydroxylase component; A2 acts as an effector component that enhances the catalytic rate of the enzyme exerting a regulatory function, A5 is a reductase component that provides electrons from NADH and A0, which is involved in the electron transfer between reductase and hydroxylase components. Due to the complexity of expressing such a large bacterial multi-component enzyme in yeast, we decided to search for a fungal homolog, which could have the same hydroxylating function in a fungal organism, closer to yeast. To do so we performed a search for a possible fungal homolog of hydroxylase components using the domain enhanced lookup time accelerated BLAST® (DELTA-BLAST) (Boratyn et al., 2012). The alignment yielded two uncharacterized protein sequences from *Aureobasidium melanogenum* (*Am*), with GenBank® accession numbers KAH0432424.1 and KAH0432426.1 (henceforth *Am*TOM*α and Am*TOM*β,* respectively) as homolog candidates for the hydroxylase subunits *α* and *β*, while no results with significant homology were obtained for the rest of the subunits. Residue V106 in *α* subunit of *Bc*TOM has a major influence on the specificity and activity of this enzyme, and this particular region, along with more than 60% of the whole sequence, was perfectly conserved in *Am*TOM*α* (Figure 4.9). Homology of *Am*TOM*β* with *Bc*TOM beta subunit reached 48% of perfect identities and this prompted us to consider it a plausible candidate to test. According to Brouk and Fishman (2009), the point mutation V106E in *α* subunit of *Bc*TOM yielded the highest activity among the different tested mutants of this enzyme, which was able to perform both hydroxylations, from 2PE to tyrosol, and from tyrosol to HT. We extracted the *Am*TOM sequences from GenBank® database, optimized them for yeast codon usage and purchased both ready-to-clone synthetic genes. In the design of the synthetic genes we observed V106 residue in *Bc*TOM*α* corresponded to

V103 in *Am*TOM*α* according to the alignment (Figure 4.9) and we ordered the desired point mutation V103E in order to mimic *Bc*TOM*α* specificity.

Figure 4.9. Protein alignment of BcTOMα subunit (TomA3) with the A. melanogenum homolog (KAH0432424.1). Identical coincidences are highlighted in green, and residue V106 from BcTOM*α* is squared in red, together with the identical counterpart from *A. melanogenum*.

Both synthetic genes, *Am*TOM*α and Am*TOM*β*, were cloned into a modified pCfB2797 plasmid together with a strong constitutive dual promoter *pHHF2-pTEF2* and an integrative cassette targeting *Ty2* loci was generated after digestion of the plasmid. We transformed A1.1 strain with this construct and generated a new strain bearing multiple copies of *pHHF2-AmTOMα* and *pTEF2-AmTOMβ* summed to the previous modifications (henceforth TOM strain). We selected the strain with higher HT signal in a rapid screening using the above-mentioned sodium periodate assay in a 96-well plate (Figure 4.10).

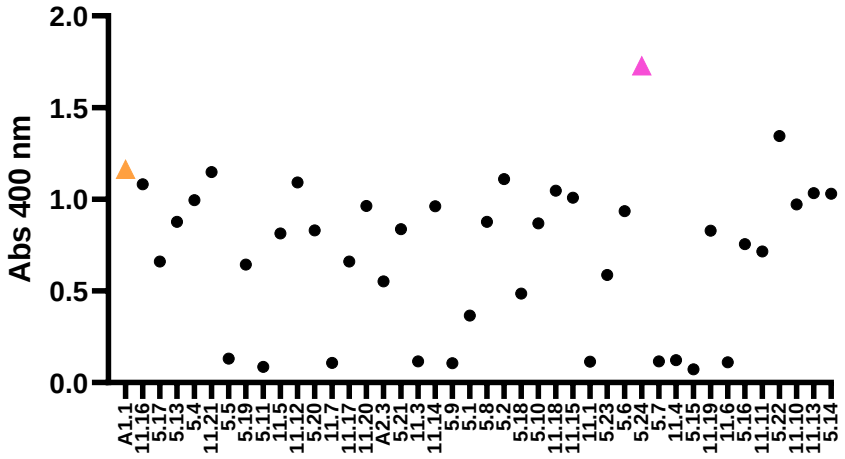

Figure 4.10. Screening for hydroxytyrosol production at 120 h in SD160 medium of the different A1.1 transformants harboring *Am*TOMαβ integrated into the genome in several copies (black dots labelled with numbers). The most productive strain was selected and used in further assays and it is depicted as a pink triangle. Control strain A1.1 is depicted as an orange triangle for comparative purposes.

Our next approach for alleviating the metabolic flux towards 2PE consisted in introducing a change of the native promoter *pPHA2* for a weak constitutive one to avoid causing a phenylalanine auxotrophy while minimizing the conversion of prephenate to phenylpyruvate in the phenylalanine branch. Based on the characterization of constitutive promoters developed in Lee et al. (2015) (Figure 4.11) we selected *pREV1* due to its low expression levels to perform our edit on *PHA2* promoter region. To apply this change of promoter we originated a blunt double-strand break using a CRISPR-Cas9 approach and provided a repair DNA sequence with the new promoter (*pREV1*) flanked by 500 bp homology arms for homologous recombination. Briefly, we designed a single-guide RNA to target the promoter region of *PHA2* and expressed it in a Cas9-expressing plasmid (pWS174), then we transformed A1.1 strain and TOM strain with the plasmid and the repair DNA. Then, transformants were validated by PCR to ensure edit was made in both alleles of the diploid strain.

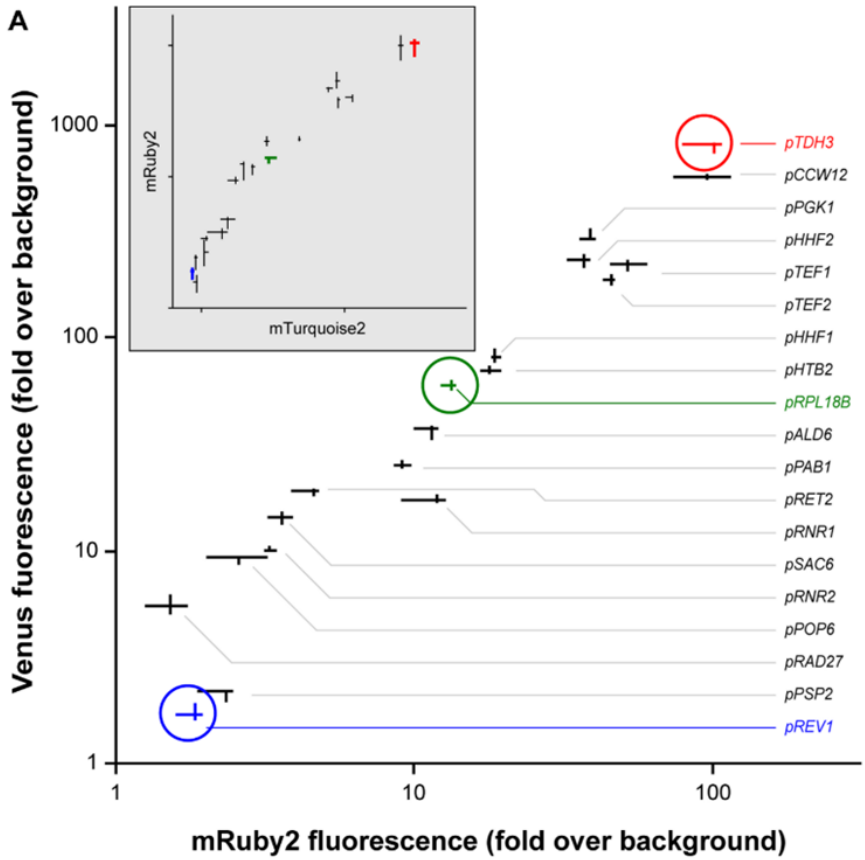

Figure 4.11. Characterization of 19 constitutive promoters. To examine the strength of each promoter, they were cloned upstream of a fluorescent reporter (mRuby2 and Venus) and measured bulk fluorescence on a plate reader.

The four resultant strains, namely A1.1, A1.1 *pREV1-PHA2*, TOM and TOM *pREV1-PHA2*, were incubated for 120h in SD160 and HT from supernatant was determined by the sodium periodate method, but HT concentrations were also validated with our reference method (HPLC-DAD). Sodium periodate method responded to changes in HT concentrations in a linear fashion when high concentrations were expected (Figure 4.12A) and results from supernatant analysis of the four strains presented a Pearson correlation above 0.99 between absorbance obtained by sodium periodate and concentration determined by HPLC-DAD. It is of special relevance the strains harboring *Am*TOM*αβ* presented a higher HT production ability than the reference producer strain A1.1. Taking into account that the ORF sequences from *Aureobasidium melanogenum* are still not characterized, and they don't have any associated function yet, this is the first report linking these two genes with a hydroxylation activity of aromatic higher alcohols (Figure 4.12B). Although the hydroxylation of aromatic compounds in

fungi is known to be catalyzed by P-450 monooxygenases (Van Den Brink et al., 1998), and the native function of these homologs still need to be tested, the great similarity with hydroxylase *Bc*TOM subunits may indicate a similarity of this hydroxylation function in the fungal host *A. melanogenum*.

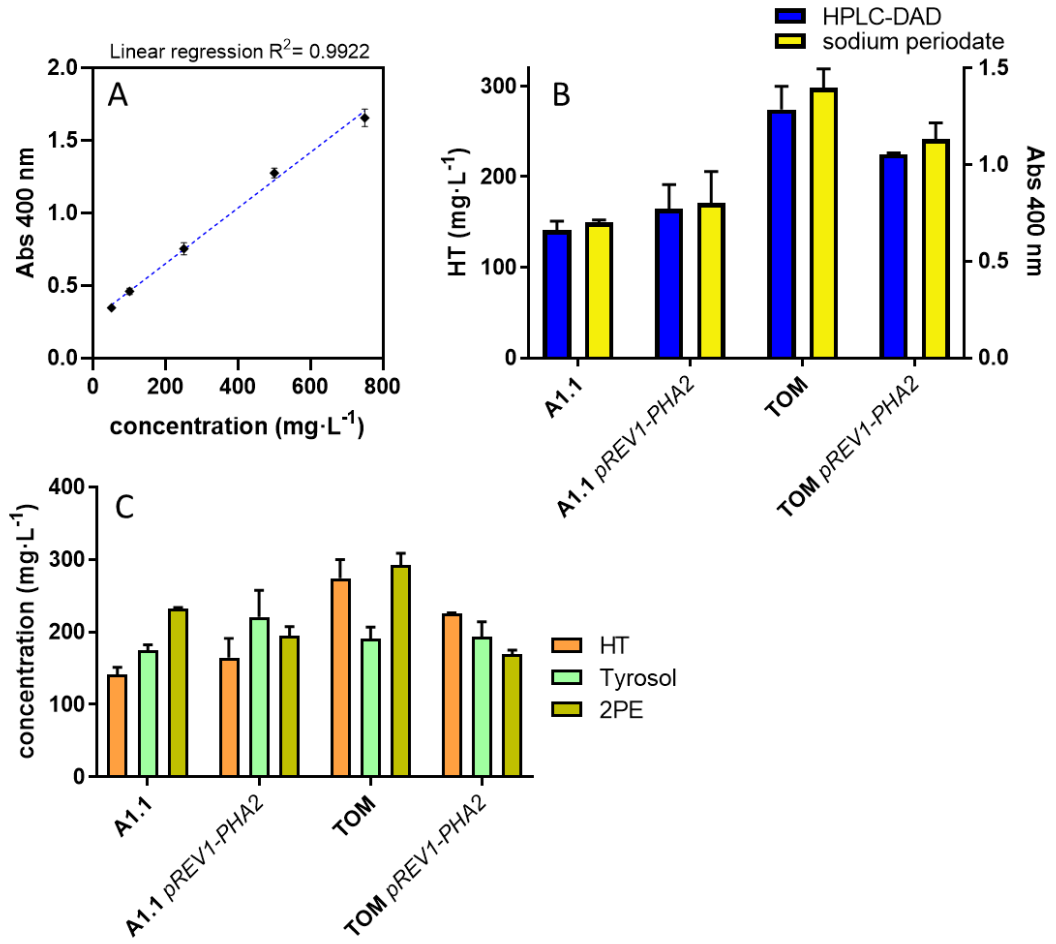

Figure 4.12. Sodium periodate assay reflects the real HT concentration in the supernatant as a measure of absorbance at wavelength of 400 nm, which shows linear dependency with real HT concentrations between 50 to 750 mg·L^{-1} (A). TOM strain produced higher concentration of HT than A1.1 strain but *PHA2* knock-down didn't increase HT biosynthesis in any strain (B). The reduction of 2PE in strains with a knocked down *PHA2* reflects the effect of the change promoter while the decrease in accumulated tyrosol in TOM strain shows *Am*TOM hydroxylating capacity (C).

Interestingly, no significant increase in HT levels was observed when *PHA2* was knocked down in A1.1 strain and even a decrease of HT concentration occurred in the TOM *pREV1-PHA2* strain when comparing it to the TOM strain (Figure 4.12B and C). Regarding *Am*TOM overexpression, we observed tyrosol hydroxylation activity but it was not possible to determine the use of 2PE as a substrate as no decrease in 2PE concentration was observed. Taking A1.1 as a

reference, we observed that under this incubation conditions the accumulation of tyrosol and 2PE was greater than that of HT, but 2PE levels decreased when *PHA2* was knocked down. This same trend is more evident in the case of the TOM *pREV1-PHA2* strain and TOM, where the effect of the knock down can be observed as a decrease of 2PE levels (Figure 4.12C), however, it does not translate into an increase of HT concentration in any of the strains. The fact knocking down *PHA2* resulted in a decrease of 2PE but not in an increase of HT suggests although the metabolic flux is more limited towards the phenylalanine branch as a result of the change of the *PHA2* promoter, efficiency in HT production is being affected either by a bottleneck at the prephenate to 4-hydroxyphenylpyruvate step, or at any other downstream step in the tyrosol branch. In order to detect a possible bottleneck in this part of the pathway, a broad study covering more metabolites should be carried out to detect accumulation of any of the precursors upstream HT. The substitution of yeast's native prephenate dehydrogenase Tyr1 for other heterologous deregulated version of this enzyme has also been demonstrated to improve the biosynthesis of metabolites downstream this step due to Tyr1 is natively regulated by tyrosine concentration (Liu et al. 2019). Other possible points of leakage in this biosynthetic route should be also considered when broadening the analyzed metabolites to optimize this process, such as the amination of 4-hydroxyphenylpyruvate to synthesize tyrosine by the action of aromatic amino acid aminotransferase II (Aro9), or the oxidation of the immediate precursor of tyrosol, 4-hydroxyphenylacetaldehyde to generate 4-hydroxyphenylacetate by the action of an aromatic aldehyde dehydrogenase.

Regarding HT productivity, and as previously stated, overproduction of HT entails a high consumption of $FADH_2$ and NADH by HpaB and HpaC, respectively, and a clear growth affectation is always observed in any yeast strain expressing this enzymatic complex when compared to a wild-type strain, especially lengthening the latency, but also lowering the maximum growth rate (Muñiz-Calvo et al., 2020). When comparing growth curves of our best HT producer strains we observed A1.1 strain showed a slower growth performance when knocking down *PHA2* gene (Figure 4.13A) while surprisingly, TOM *pREV1-PHA2* strain performed faster than TOM strain, showing an opposite growth tendency (Figure 4.13B). Although certain growth defect is expected due to a redox imbalance

caused by the great consumption of $FADH_2$ and NADH by the hydroxylase complexes overexpressed in these strains, the biosynthesis of other compounds derived from prephenate in the tyrosol branch, may also explain the reason behind the better performance of TOM *pREV1-PHA2* when compared to TOM strain. The notorious decrease of 2PE in TOM *pREV1-PHA2* could explain a better growth performance by itself as this compound can increase membrane fluidity and then cause a reduced uptake of nutrients (Seward et al., 1996) but other interesting related metabolites such as 4-hydroxyphenylacetate and other tyrosine derivates should be further studied.

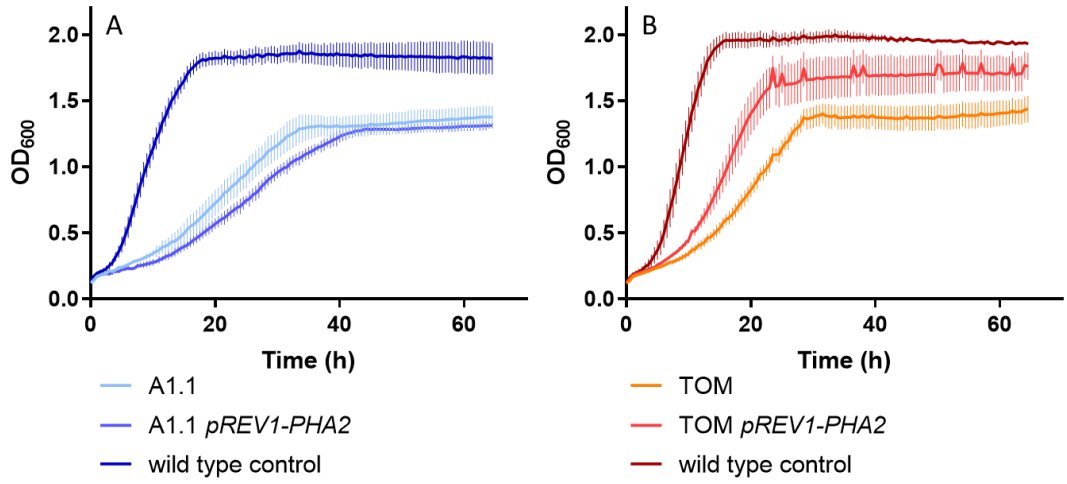

Figure 4.13. Yeast growth dynamics is affected in HT overproducer strains. A1.1 strain's growth was partially hindered by knocking down of *PHA2* gene (A) while TOM strain showed a significant improvement when *PHA2* was weakly expressed by *pREV1* (B).

Despite this growth affectation, high concentrations of HT were reached in 120 h from a medium with an excess of the primary substrate glucose (SD160) from which less than a half of glucose amount (75 $g \cdot L^{-1}$) is consumed by yeast to metabolize, grow and multiply. Since HT is not being produced in high amounts until late exponential / early stationary phase, the next logical step to increase productivity should be to determine the best cultivation and growth conditions, especially regarding glucose concentration, to maximize the ratio of HT produced / glucose consumed. We are currently developing strategies to shorten times of HT production as a parallel approach to improve productivity from two perspectives, both dedicated to attain high cell densities as fast as possible. It is our goal to establish growth methods more suitable for industrial production of

this interesting metabolite such as controlled fed-batch systems that allow us to modulate the carbon flux between fermentative or respiratory metabolism to obtain a high cell density. In parallel we aim to decouple the growth phase and biomass production from HT production phase from a synthetic biology approach, by the use of metabolic valves driving the expression of the hydroxylase complex HpaBC and externally controlled by light stimuli as previously described (Zhao et al., 2018; Figueroa et al., 2021). This approach will allow us to achieve a rapid biomass production, characteristic of the wild-type strain, while expression of HpaBC remain switched down, for a late massive activation of all HpaBC when early stationary phase is reached and thus obtaining significant HT production under 120 h and consuming most of the main precursor, glucose.

4.3. Conclusions

Given its antioxidant and beneficial properties, much interest is shown in HT for its use in functional foods, and on pharmaceutical or nutraceutical markets. Unlike chemical synthesis or extraction from natural sources, biotechnological approaches followed to produce lower cost pure HT are very appealing. Unlike our previous work, we metabolically engineered *S. cerevisiae* for high-level HT production from a simple inexpensive carbon source by first integrating the HpaBC complex into the genome and overexpressing several aromatic amino acid pathway-related genes. Among these modifications, the single tyrosine insensitive *ARO4* allele overexpression (*ARO4**) had the strongest effect in the diploid background (BY4743). Nonetheless, the combinatorial overexpression of several ARO genes in the haploid strain (BY4741) enhanced HT production compared to the single *ARO4** overexpression. These combinations of overexpressed ARO genes should be taken into account as effective modifications to transfer to the diploid background when a further increase of tyrosol is intended. We established a medium and growth conditions to maximize final HT titers of our producer strains and after 240 h of growth in 50 mL shake-flasks with SD160 medium we obtained a 130-fold increase in HT concentration when comparing to the producer strain from our previous work. To maximize HT production we constructed a new strain, A1.1, bearing multiple copies of ARO4* gene and a new combination of HpaBC complex where hydroxylase subunit derived from *P. aeruginosa* while reductase subunit derived from *E. coli*, and we

obtained the highest HT titers so far, over 400 $mg \cdot L^{-1}$, which although it represented a slight concentration improvement, we achieved it in just half the time, in 120 h under the same other conditions. After reaching this milestone we focused on improving precursor conversion into HT and we applied two genetic modifications on A1.1 strain, a knock-down of a native gene and an overexpression of a novel fungal monooxygenase (*Am*TOM) with putative hydroxylating activity. These modifications produced a redirection of metabolic flux towards HT synthesis, although the study of other related compounds would provide a better understanding of the process, especially of the effect of *PHA2* knock-down.

This work lays down the first steps to overproduce HT in yeasts form glucose by a metabolic engineering approach and to further develop a yeast cell factory for HT production. To the aim to increase product yields and to ensure consistent product quality, our next goals are to optimize the cultivation and growth conditions of our producer strain, and to decouple the biomass production phase and HT synthesis to increase the production rate.

GENERAL DISCUSSION

Aromatic amino acids are important precursors to secondary metabolites which influence many properties of fermented food and beverages. Among them, bioactive health-promoting compounds such as melatonin, serotonin, hydroxytyrosol and other related metabolites raise great interest in food industry as a dietary intake of these bioactive compounds exert beneficial effects on human health (Fathizadeh et al., 2019; Fernández-Mar et al., 2012; Hornedo-Ortega, Da Costa, et al., 2018; Marković et al., 2019; Sugiyama et al., 2022). Yeast, as the main responsible of the alcoholic fermentation, also stands out as the responsible of the synthesis of these compounds during fermentative process (Álvarez-Fernández et al., 2018; Guerrini et al., 2018; Rodriguez-Naranjo et al., 2011). Although these bioactive compounds are usually found in very small concentrations in fermented products, the modern analytical techniques permitted their detection and quantitation from complex matrices such as wine, but such techniques usually entail sample preprocessing and dependance on the availability of potent and costly equipment, which introduces a bottleneck in the analytical process, especially when performing a screening of a large number of samples. Yeast plays a central role in the synthesis of these compounds but essential biosynthetic routes of compounds like melatonin or hydroxytyrosol are still not well established in this organism. The knowledge on the implicated intermediary metabolites, the physiological conditions to promote their synthesis, the enzymes responsible for the necessary reactions or the genes encoding them is still really limited in yeast. All these factors, summed to the analytical challenge of the commonly low concentration of melatonin, serotonin or hydroxytyrosol in samples, exceptionally difficult the study of these metabolites over other more commonly studied compounds. Limitations concerning analytical methods are addressed in chapter 1 of this thesis.

Rapid detection method to detect and quantitate melatonin directly from growth media.

Many analytical techniques have been used to determine melatonin from food, fermented beverages and yeast samples, most of them are separative methods based on chromatography. The most powerful technique among the separative methods is ultra-high performance liquid chromatography coupled with high-

resolution tandem mass spectrometry (UHPLC-HRMS/MS). This method achieves the lowest limit of detection (LOD) and quantitation (LOQ) while allowing the simultaneous analysis of multiple metabolites, showing a high discriminating power. The use of this potent method for melatonin analysis requires expensive and specialized facilities and equipment and sample characteristics usually need a previous preparation that are an important tradeoff to consider as melatonin concentration in yeast growth media is usually very low and analyses of a high number of samples can also cause important delays in the process. Thus, the development of rapid methods to detect melatonin with a minimal sample preparation and in an easy cost-effective manner becomes a primary goal when studying melatonin synthesis by yeast.

We chose the adaption of a melatonin yeast biosensor, which was recently developed by Shaw et al. (2019) as a proof of concept of their tunable GPCR-signaling model as it offered a high selectivity to melatonin and a potential for sensitivity improvements from a synthetic biology approach. The use of a yeast biosensor provided a series of advantages over the chromatographic methods such as avoiding the pre-treatment of the samples. The reported presence of melatonin in fermented products as a result of yeast metabolism is necessarily linked to the excretion of this metabolite to surrounding media, either due to active transport or passive diffusion, and, with this procedure, the biosensor yeast cells are directly subjected to the supernatant of potential melatonin-containing spent media, avoiding any previous steps of concentration or chemical extraction from the sample. A reduction in the pre-processing steps eliminates accumulated error or analyte loss across the procedure. Another advantage is its high selectivity to melatonin, as minimal or null activity of reporter for 100 µM of ligand was reported when similar melatonin-related ligands were used (Shaw et al., 2019). A clear disadvantage of this method when compared to the powerful reference method UHPLC-HRMS/MS is the multiplexing capacity, as one of the yeast biosensor's main virtues relies in its specificity and affinity to melatonin over any other metabolite. Sample matrix should also be taken into account as a possible limitation of the biosensor strain. The convenience of avoiding a previous sample extraction is subjected to an acceptable and low matrix effect. When performing recovery assays from a given matrix or observing the effect of diluting the ligand

calibration curve in the tested matrix, a loss of sensitivity or changes in linearity of interpolation area may be observed in a matrix-dependent manner, therefore adjustments in the biosensor strain may need to be addressed when a severe matrix effect is observed. Synthetic biology offers a wide range of possibilities to adapt the biosensor to the requirements of different matrices, for example, with the prospect of using this biosensor strain for melatonin detection in red wines changing the reporter fluorophore from sfGFP to a red fluorescent protein would be an interesting approach as, due to its optical properties, red wine can absorb wavelengths close to sfGFP emission maximum (Vijan & Giosanu, 2010), and thus partially quenching the output signal. The use of synthetic DNA regulatory parts and modern genome editing techniques such as CRISPR-Cas allowed the modularization of the different components of the GPCR signal transduction and reporter in the biosensor strain to prevent endogenous metabolism to interfere in it and tune the biosensor properties.

The strategy of using a yeast platform to develop GPCR-based biosensors is also being applied to other metabolites related to melatonin such as serotonin (Lengger et al., 2022) due to the versatility of expressing yeast/mammalian G-protein alpha subunit chimeras for coupling a mammalian receptor into the yeast mating pathway (Brown et al., 2000). In fact, the results obtained from the melatonin biosensor strain prompted us to expand the use of this system and adapt it to other metabolites of interest for our lines of research, such as hydroxytyrosol. One of many benefits of hydroxytyrosol is related to its ability to promote apoptosis in breast cancer cells, as a described mechanism to this feature, the ability to bind the human G-protein coupled receptor GPR30 (GPER) have been reported (Chimento et al., 2014). This GPER can activate breast cancer cells proliferation upon estrogen binding but, in turn, when binding hydroxytyrosol or oleuropein an inverse agonist effect is produced by this bioactive compound. The finding of a well described GPCR of hydroxytyrosol brings the opportunity for the design and synthesis of chimeric GPCRs, harboring the transmembrane and intracellular domains of the receptor conserved in yeast but expressing the specific amino acids in the active site related to hydroxytyrosol binding which are derived from in silico predictions and in vitro ligand-binding assays (Brown et al., 2000; Chimento et al., 2014).

Other different efforts towards obtaining a rapid detection and quantitation method for melatonin have been addressed, such as the use of voltammetry of immobilized particles method (VIMP), which represented a great advance in terms of speed of analysis and it avoided sample extraction, taking measurements directly from cells. But it presented a major drawback as its discriminating power was not comparable to that of separative methods and only intracellular content could be analyzed with this method (Muñiz-Calvo et al., 2017). The use of mammalian melatonin receptors coupled to a fluorescent reporter system has been previously proven to provide a highly sensitive and specific method, suitable for melatonin detection from some fermented samples. The system was expressed in a human cell line platform, which introduced complexity to the method due to the maintenance of the cells, the longtime of exposure to analytes and the cells sensitivity to some analyzed matrices (Morcillo-Parra, Beltran, et al., 2019).

The adaption and modification of this yeast GPCR-based biosensor have been tested in chapter 1 to perform a screening of 101 samples of supernatants from different yeast strains, and we detected a differential melatonin synthesis across the samples. From this screening we selected a newly winery-isolated strain of *S. cerevisiae* (B28), with a capacity of melatonin synthesis slightly above the average from the rest of analyzed *S. cerevisiae* strains, and subjected it to an adaptive laboratory evolution to modify its bioactive production profile from a non-GMO approach, as it is addressed in chapter 3.

From our perspective, this analytical method, based on the use of a genetically modified yeast laboratory strain, with enough sensitivity to differentiate spontaneous melatonin production among different strains was of paramount importance, as it enabled us to perform extensive screenings of yeast strains from different species and it is currently being used for the design of yeast-yeast and yeast-bacteria consortia to enhance melatonin and related metabolites concentrations in fermented beverages.

Biosynthetic pathway of melatonin in yeast

The study of melatonin in yeast have become a subject of great interest in the last decade. Although nowadays it is considered an ubiquitous molecule and its presence have been reported in a wide variety of organisms, the melatonin increase in fermented food and beverages as a result of yeast metabolism raised questions about its role in yeast, but also about its biosynthetic route, as the genes and enzymes responsible for the synthesis of melatonin still remain unknown. The challenge of establishing a biosynthetic route for melatonin in yeast is only partially addressed so far. There is a high degree of conservation regarding the metabolic steps needed to synthesize melatonin from tryptophan. It consists of four enzymatic reactions: decarboxylation, hydroxylation, *N*-acetylation and *O*-methylation. The order of these reactions differ among organisms from different clades. For instance, in the mammalian biosynthetic pathway, tryptophan is firstly hydroxylated to 5-hydroxytryptophan, then decarboxylated to serotonin followed by its *N*-acetylation to form *N*-acetylserotonin and finally a methylation reaction forms melatonin. On the other hand, plants possess the enzymatic machinery to undertake the same order of reactions but also present alternative steps like the decarboxylation of tryptophan to tryptamine as a first reaction, then the hydroxylation of tryptamine to form serotonin and the *O*-methylation of serotonin to produce 5-methoxytryptamine followed by its *N*-acetylation to form melatonin (Yu et al., 2018).

In a previous study, we proposed a new biosynthetic route occurring in yeast based on its ability to convert the different precursors in their respective products, in which unlike plants and animals, it seems that the synthesis of 5-hydroxytryptophan by the hydroxylation of tryptophan does not occur in yeasts. Instead tryptophan can be decarboxylated into tryptamine as the first step of the route, and serotonin can be *N*-acetylated or *O*-methylated to produce *N*-acetylserotonin or 5-methoxytryptamine, respectively, with an apparent preference for the latter reaction (Muñiz-Calvo et al., 2019).

Despite the advances on describing the biosynthetic route, there are no genes associated to all reactions. In fact, only one gene *PAA1* have been described as a candidate to carry out the *N*-acetylation step in melatonin synthesis in yeast.

This gene is a polyamine acetyltransferase with reported aralkylamine *N*-acetyltransferase (AANAT) activity in enzymatic assays using 5-methoxytriptamine, tryptamine and serotonin, among other substrates (Ganguly et al., 2001). Chapter 2 is focused on determining the role of *PAA1* gene in melatonin biosynthesis in the context of the search of new candidate genes involving *N*-acetylation reaction in melatonin biosynthesis. The first part of the study aims to establish the importance of the only candidate gene described as involved in melatonin synthesis in yeast. To that aim bioconversion assays were performed on yeast overexpressing *PAA1* gene, but also on knockout strains for that gene and on cells overexpressing the mammalian reference gene Bt*AANAT* as a positive control. No differences in the production of acetylated compounds were found between cells overexpressing *PAA1* or those with the knockout mutation which led to hypothesize although *PAA1* holds the potential of performing such reactions *in vivo*, it is not possible it is the only responsible gene for the *N*-acetylation during melatonin production. Data base search based on sequence homology was originally performed using vertebrate AANAT as a reference. *PAA1* resulted a candidate with a high similarity but limited primarily to the catalytic core of vertebrate AANAT bearing the conserved sequence motifs A and B of the GNAT superfamily. As a candidate gene, *PAA1* ability to contribute to melatonin biosynthetic pathway has been demonstrated (Ganguly et al., 2001). Nevertheless, Liu et al. (2005) have pointed out spermine as the main *in vivo* substrate for Paa1p, and we could verify its function as polyamine acetyltransferase, as well as demonstrate *PAA1* overexpression in yeast was occurring when intended although the impact on acetylated product was not significant. An important remark on bioconversion assays emerged as we detected an increase of N-acetylated products in an *in vivo* assay when overexpressing *PAA1* in a bacterial platform. This findings made us consider the use of bacterial overexpression system for further candidate evaluations. Motivated by yeast ability to produce melatonin, even when *PAA1* gene was knocked out, we set for the search of new candidates following an approach from two fronts. On the one hand, gene expression was analyzed under melatonin synthesis conditions, but those significantly overexpressed genes were overrepresenting a strong transcriptional response to iron and copper deficiency, due to the chelating properties of the compounds involved in the bioconversion

assay, namely 5-methoxytryptamine and melatonin. Genes related to metal ion homeostasis were filtered out and information on overexpressed genes was cautiously considered for further analysis. On the other hand, a new database search was conducted using *AANAT* as a reference, not limiting the search to sequence homology but taking into account GNAT family to predict and find similar functional domains using the comprehensive database InterPro. This approach greatly differed from that used before by Ganguly et al. (2001) as only sequence homology was taken into account, and GNAT superfamily was then characterized by amino acid motifs A and B, which constitute conserved sequences established several years before three-dimensional structural information became available (Coon et al., 1995; Tercero et al., 1992). With the information from both transcriptomic and *in silico* candidate search based on structure prediction we selected those candidates resulting from the database search that were also overexpressed during melatonin synthesis condition, and narrowed the list to eight candidates. The fact *PAA1* was one of them supported our hypothesis when using this system to find new candidates, although our approach relied on the transcriptional response to an excess of precursor and therefore we assumed a possible loss of information as not all the genes related to this function are necessarily upregulated by an increase of substrate, or their response at the selected times might not be optimal. All of them were tested for melatonin synthesis in a bioconversion assay using a bacterial system and obtained a new candidate, *HPA2*, which was able to convert 5-methoxytryptamine to melatonin, apart from its other previously attributed functions as a histone acetyltransferase or its ability to also acetylate polyamines.

One of the most important obstacle when studying melatonin biosynthetic pathway in yeast resides in the lack of exclusivity of function of its responsible enzymes. Melatonin production occurs in a marginal manner in terms of concentration in yeast, we believe this production is carried out due to enzymes with other primary functions for which they are more specific and efficient while having a leaky contribution to melatonin biosynthetic pathway due to a broad substrate specificity. In the case of the validated candidates Paa1p and Hpa2p, their main function and the function contributing to melatonin biosynthesis do not drastically differ as they perform an acetylation but they accept structural

differences in the substrate. This is the case of “moonlighting” proteins, according to *The Compact Oxford English Dictionary*, one meaning of moonlighting is “to do (paid) work, (usually at night), in addition to one’s regular employment,” and the word has been used in this sense since the late 1950s. This term was coined by Jeffery (1999) to designate these shared genes. To maintain a minor secondary function of an enzyme coded under the same ORF can reflect a key aspect of molecular evolution as it allows adaption to simultaneous selective pressures, or in the case of melatonin they may represent an evolutionary vestige of a primitive mechanism to cope with oxidative stress (Piatigorsky & Wistow, 1999; Dun Xian Tan, Zheng, et al., 2014). The *N*-acetyltransferase reaction in melatonin biosynthetic pathway should yield more candidates with an effective bioconversion of melatonin’s substrates as there is a high number of genes related to this function that may have moonlighting activity, and, as an ongoing research, we are exploring new candidates using predictive tools also among the unannotated genes across yeast genome.

The work carried out in chapter two partially establishes a roadmap to the prospect of revealing candidates for the three other reactions in the melatonin pathway in yeast, especially concerning the bioconversion assays for testing the candidates’ ability to perform the reaction and the use of predictive models to find possible homologs. Nonetheless, more information on the spontaneous melatonin or its precursors’ synthesis conditions are needed to better understand the occurrence of these interesting compounds in yeast, and narrow the siege in the search for new candidates.

Detailed information about a biosynthetic route is of great importance when addressing genetic modifications on yeast’s genome to improve the synthesis of bioactive compounds. Although chapter 2 contributed to broaden our knowledge about an unknown biosynthetic pathway, it is still far from being completely elucidated, and other interesting bioactive compounds, resulting from the metabolism of tryptophan and tryptamine, such as tryptophol, tyrosol and hydroxytyrosol among others, are equally interesting from an industrial point of view as the increase of some of these compounds in a food product as a result of yeast metabolism can be desirable, but also from a basic research approach,

as for compounds like hydroxytyrosol, melatonin or their derivatives, there is still no information about the responsible genes involved. Chapters 3 and 4 are focused on the genetic modifications that allow yeast to overproduce such interesting compounds but from two completely different approaches and applications. Chapter 3 is focused on the obtention of a new yeast strain by means of adaptive laboratory evolution (ALE) to a selective pressure that allows to select a specific resulting phenotype as a result of the adaptation process. This makes it impossible for this approach to be completely directed to improve the production of a specific metabolite, but the resulting strain may be used in food industry, if deemed appropriate, as GMO regulations will not apply to such strain. On the other hand, chapter 4 pursues the objective of increasing the amount of a single metabolite, hydroxytyrosol, but based on a metabolic engineering and synthetic biology approach.

Improving yeast bioactive compounds from a non-GMO approach

Natural and spontaneous synthesis of bioactive amino acid-derived compounds by yeast and the transfer of this feature to industrial strains represent an interesting goal for food industry as it would allow the production of fermented foods and beverages with a high added value due to the increase in bioactive compounds present in the final product. Nevertheless, the information about interesting yeast-produced bioactive compounds is limited in terms of the conditions which can trigger a higher production or the knowledge about their biosynthetic pathways. Furthermore, genetically improving a yeast strain for food industry applications demands a compliance with GMO regulations that prevents the use of any rational design for genetic manipulation. In chapter 3 we took advantage on the previously studied physiological effects of melatonin in yeast, especially its antioxidant properties and more importantly, its photoprotective effect described in our own previous work (Bisquert et al., 2018), to design a novel ALE experiment using white light as the selective pressure. The directed evolution approach can bring a double benefit, on the one hand, obtaining a new strain with a differential metabolic profile which may be interesting and transferable to the food industry, and, in the other hand, if the initially intended phenotype is achieved, *i. e.* a higher production of melatonin, a reverse-

engineering approach can reveal the genetic determinants for that phenotype. The initial goal of using this selective pressure was to generate a photo-oxidation of yeast cells by subjecting them to white light PAR radiation provoking a selection towards those cells exhibiting a better oxidative stress response. The strain selected for the ALE experiment derives from the melatonin screening performed in chapter 1, where B28 strain is a *S. cerevisiae* isolated by our group from a winery environment and it holds a mild melatonin production ability, above the average value of the rest of *S. cerevisiae* screened strains. We considered B28 for its potential to produce melatonin and, presumably, other related metabolites, offering room for improvement on melatonin synthesis. White light has been proven to be an effective selective pressure, milder than the use of ultraviolet light but capable of causing random mutations across the genome. Evolved strain (EVO) offered a completely different extracellular metabolome, and most of the differences involved the tryptophan metabolism and indolic profile. Although melatonin was not a differential metabolite when comparing parental and evolved strain, other related indoles such as kynurenine and formylkynurenine, or precursor anthranilate were clearly overproduced in EVO strain. One of the most surprising results was the ability to greatly alter the concentrations of aromatic amino acid derived compounds as a response mechanism to light induced stress. The mechanism through which the cell reacts to this radiation is not clear, although according to previous studies most of the overexpressed metabolites in EVO strain possess radiation absorbing properties (Filho et al., 2022; Molin et al., 2020). Indole rings can be excited by the shorter and more energetic wavelengths of the radiation employed and in tryptophan present in proteins, indole excitation may convert proteins to photosensitizers and produce ROS that can cause cellular disfunctions and mutations as previously reported (Learmonth, 2006), which would explain the surprisingly high random mutations found across the genome. The ability of EVO strain to respond against the specific stress of white light radiation suggests an underlying regulatory mechanism based on light sensing, which is an unprecedented feature in *Saccharomyces cerevisiae* since no photoreceptors are described in this species, although evidence on the impact of visible light on *S. cerevisiae* have been reported (Idnurum et al., 2011; Molin et al., 2020). Our next step to detect the underlying regulatory mechanisms that drive response against light stress is to

sequence the RNA transcripts of parental and evolved strain under the same conditions used in metabolomics in chapter 3, but establishing several time sampling points for each sample group. This information will help to detect and understand the response of EVO strain against white light and, following a similar approach to that used in chapter 2, also explore the genes responsible for the synthesis of unusual compounds like indole-5,6-quinone, 5-hydroxyindoleacetic acid, coumaroylserotonin or 5-hydroxytryptophan. Exploring the latter four compounds will also contribute to the elucidation of melatonin's biosynthetic pathway in yeast, as 5-hidroxyindoleacetic acid and coumaroylserotonin are derivatives of serotonin, and 5-hydroxytryptophan formation from serotonin as a substrate has been reported, but not from tryptophan (Muñiz-Calvo et al., 2019).

EVO strain's ability to successfully ferment a synthetic must has been demonstrated, although it showed a delay when comparing to the parental strain B28. As the use of controlled multi-starter fermentation using selected cultures of *Saccharomyces* and non-*Saccharomyces* strains has been encouraged in the last two decades (Ciani & Comitini, 2015; Valera et al., 2019), with the aim of enhancing the composition and aroma profile of the final wine, we believe EVO strain is a good candidate to perform different co-inoculations with both *Saccharomyces* and non-*Saccharomyces* strains. From an applied point of view, the interesting changes in aromatic compounds metabolic profile in evolved strain, especially those involving the increase of phenylalanine, tyrosine or tryptophan precursor anthranilate and hydroxyanthranilate, can impact a final wine's aroma profile in such co-cultures, as they can result in an enhancement of desired higher alcohols (Fairbairn et al., 2017; Fleet, 2003; Sadoudi et al., 2012). However, more importantly, wine can be enriched in a plethora of compounds that can contribute to improve the bioactivity and stability of the final product.

As a third future prospect on EVO strain, the intracellular metabolome analysis is imminently addressed to shed light on two main unknowns, especially on the LIGHT sample group. The identification of an unknown pigment that seems to accumulate in cells rather than in the growth media can derive in new hypothesis to this regard and allow us to explain its occurrence as a possible new anti-photooxidant defense. On the other hand, a specific nutrient intracellular

accumulation profile, especially regarding amino acids and glutathione, is of great interest for the industry of organic nutrition for winemaking processes. Hydrolyzed yeast is used as a fermentation activating nutrient during winemaking process and the different proportions of the accumulated nutrients offer a wide variety of benefits in a final product, allowing to add a boost of nutrients directed to increase intensity, freshness and complexity in wine.

Chapter 3 yielded a surprising amount of results that are being currently addressed with great interest in our lab according to the three main research lines discussed above: Supporting basic research on aromatic amino acid derived compounds to find genetic determinants for the synthesis of melatonin and other interesting compounds in yeast, exploring the fermentation capacity for establishing a yeast-yeast consortia for improving aromatic profile in winemaking and finally determining the possible application of this new strain as a biological source of nutrients to be used in a hydrolyzed format in winemaking industry.

Metabolic engineering for hydroxytyrosol production in yeast

In chapter 4, a completely different approach is taken with the objective of increasing the concentration of a single bioactive metabolite by means of metabolic engineering. This time our efforts were focused on the tyrosine-derived metabolite hydroxytyrosol (HT). This is one of the most potent dietary antioxidants, with a reported high antioxidant activity (Boronat et al., 2018; Hu et al., 2014). Hydroxytyrosol is naturally present at high concentrations in olive tree (*Olea europaea* L.) and its main human dietary intake is almost exclusively through olives and olive oil consumption, and the general HT intake is estimated to be low (Gallardo-Fernández et al., 2022). There are numerous studies that demonstrate the beneficial effects of HT on human health, it exerts beneficial roles in cardiovascular and respiratory diseases, metabolic syndrome, neuro- and skin-protection, antitumor formation, as well as anti-inflammatory effects (D'Angelo et al., 2020; Gallardo-Fernández et al., 2020; Robles-Almazan et al., 2018). The beneficial effects of incorporating this compound to the diet and its consideration as a novel food ingredient prompted us to pursue a sustainable, clean and inexpensive HT production platform, distinct from the usual plant extraction methods or chemical synthesis and based on a yeast cell factory. Other

previous works pursuing the same objective utilize a bacterial cell factory where yeast genes such as *ARO10* or *ADH6* need to be overexpressed to achieve synthesis of the precursor tyrosol (Li et al., 2018). But, similarly to melatonin, HT synthesis ability has also been attributed to yeast (Álvarez-Fernández et al., 2018) as a result of grape-must fermentation. Yeast is often the preferred cell factory for the production of food-grade products due to its long use in the food industry, the ease of genetically engineering it, its tolerance to different temperatures, pH or salts and its consideration as GRAS (Guo et al., 2019; Nielsen et al., 2013). All above-mentioned properties and the fact yeast is naturally capable of synthesizing high concentrations of immediate precursors, and even low concentrations of HT, we chose it as our target bioactive metabolite in chapter 4, and focused on applying the necessary genetic modifications to reach a high production of this metabolite.

Our approach was based on two strategies, the overexpression of an exogenous bacterial hydroxylase complex needed for the one-step reaction of tyrosol hydroxylation, and the redirection of the metabolic flux from glucose to increase precursors phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) from glycolysis and pentose phosphate pathways, respectively.

For the objective of overexpressing the bacterial hydroxylase complex (HpaBC) we opted for the integration of multiple copies into the genome and tested its hydroxylation capacity in an *in vivo* bioconversion assay where hydroxytyrosol was measured after precursor supplementation. The goal of redirecting the glucose metabolic flux toward tyrosol was central in our yeast platform, as achieving a high concentration of HT without involving any precursor supplementation other than glucose represented a major increase in the value of our cell factory. These modifications towards redirecting metabolic flux involved a wide set of bioconversion assays, with deletions of genes competing for the substrate across the tyrosol biosynthetic pathway such as *TRP2, ABZ1* and *PHA2,* and overexpression of genes that directly favor tyrosol synthesis. During the construction of the HT producer strain we found the minimal necessary modifications that caused a sudden increase of HT titers in the assays, the overexpression of HpaBC complex together with the overexpression of

ARO4^{K229L} allelic variant, which overruns the negative feedback loop that drives its regulation in relation to tyrosine concentration. This surprising finding allowed us to register a patent (patent number P202031186; Annex I) on the HT production method using a yeast cell factory.

Further genetic improvements on the patented strain were carried out, as we adapted a combination of HpaBC from *Pseudomonas aeruginosa* and *Escherichia coli* to improve productivity and reduce the time to obtain a maximum HT production. This combination, together with some of the previous modifications has also been recently reported successful for the synthesis of HT in yeast (Liu et al., 2022). The access to CRISPR-Cas9 editing technology or the chemical synthesis on demand of large DNA fragments covering whole genes enabled us to perform the knock-down of *PHA2* gene as well as ordering a synthetic, codon-optimized Am*TOM* gene that offered promising results regarding HT production potential.

Chapter 4 offers an approach to overproducing a metabolite of interest, in this case hydroxytyrosol, using a genetically engineered yeast as a cell factory. This approach automatically prevents this HT producer strain from being used in food according to European regulation, as it is a GMO, but it holds great relevance in animal feed, or after a purification process to generate supplements and nutraceuticals (Britton et al., 2019). Nevertheless the cell factory developed in this chapter is currently undergoing an optimization process, especially regarding growth media optimization.

As future prospects, three immediate lines derived from this work. It is our goal to establish an optimal HT yield in relation to glucose and time. To that end, different concentrations of glucose and nitrogen sources are being tested to maximize HT productivity. Given the growth affectation of the developed HT producer strain, the decoupling of growth and HT production phases is being addressed. A novel inducible expression system based on optogenetics is being adapted in our lab to drive the expression of Pa*HpaB*, the hydroxilase subunit that has been associated to the growth defect observed in the HT producer strain. This approach consist on expressing Pa*HpaB* under the control of a 5xGAL1 synthetic promoter, while expressing the FUN-LOV (FUNgal Light-Oxygen-

Voltage) carrying the fungal blue light photoreceptors WC1 and VVD from *Neurospora crassa* (Figueroa et al., 2022; Salinas et al., 2018) and keeping the rest of genetic modifications unaltered. Under these conditions, while growing in darkness Pa*HpaB* will remain in a “switched off” state and, thus, allowing normal growth, to subsequently trigger its overexpression when a high biomass is achieved by light induction.

Parallel to the further optimization of the HT producer strain, a redesign of the cell factory is also being addressed. During the whole process, a series of genetic modifications have been made based on the tyrosol and, therefore, HT biosynthetic pathway, either to knock out, knock down or overexpress related genes. Although effective modifications were achieved, we believe a rational design based on a wider metabolic network can reveal some unexpected, yet effective, target genes to modify in order to maximize HT production but also considering the minimal loss of fitness. We are currently using a newly described computational predictive model (Domenzain et al., 2023) to target key genes to maximize tyrosol synthesis in a lab strain with the objective of expressing the HpaBC complex as a final step.

The potential of *S. cerevisiae* as a cell factory, together with the newly acquired access to technologies that allow us to predict, synthesize and edit virtually any genetic sequence, has resulted in a major line of research in our lab. In fact, the use of a similar predictive model to maximize the synthesis of indole compounds like serotonin and melatonin will be one of the next year’s prospects in our lab.

CONCLUSIONS

The main conclusions that emerge from the work carried out in this thesis are the following:

1. Genetic modifications such as increasing the number of melatonin membrane receptors and tuning up the expression of yeast G protein α subunit and its fluorescent reporter system, allowed a significant gain of sensitivity and an increase of maximum output signal and signal to noise ratio in a yeast GPCR-based biosensor of melatonin.

2. A heavily engineered yeast biosensor has proven to be an effective tool in the detection and quantitation of melatonin from supernatants of yeast culture medium as it allows to discriminate between yeast-produced melatonin in a simultaneous screening of multiple samples in a quick, easy and inexpensive manner.

3. Although *PAA1* gene can contribute to the melatonin biosynthetic pathway in *S. cerevisiae* its role is not essential for the synthesis of this compound, pointing out a “moonlighting” activity and revealing more genes are involved in the *N*-acetylation steps of the route.

4. The combined use of gene expression data and *in silico* domain structure prediction followed by *in vivo* bioconversion assays in *E. coli* enabled the proposal and test of new *N*-acetyltransferase gene candidates leading to the newly proposed *HPA2* gene as involved in this biosynthetic route in yeast.

5. White light constitutes a severe adaptive pressure for yeast and a good mutagen as it is capable of causing indiscriminate mutations throughout the whole genome in an ALE context.

6. Evolved industrial wine strain EVO significantly changed its metabolomic profile compared to its parent strain B28. These changes affect nitrogen metabolism and indolic compound production, and reveal the ability to produce bioactive compounds and antioxidants, especially under light stressing conditions.

7. The analysis of genomic sequences of the light stress-adapted EVO strain and its parental strain B28 revealed a notable accumulation of chromosome duplications in chromosome VII in EVO strain, and point mutations in genes involved in the biosynthesis of differentially produced metabolites such as phenylalanine, hypoxanthine and bioactive tryptophan-related compounds including 5-hydroxytryptamine, 5-indoleacetic acid, tryptamine or tryptophol, among others.

8. Adaption to white light stress conferred EVO strain the ability to respond to white light stimulus by producing an unknown pigment as well as a novel antioxidant and photoprotective metabolite, indole-5,6-quinone, which is closely related to eumelanin and shares common properties.

9. The production of a high value molecule such as hydroxytyrosol (HT) can be achieved from a yeast cell factory in high concentrations (>400 $mg \cdot L^{-1}$) using a simple and inexpensive growth medium and using glucose as the main substrate.

10. The multiple integration into the genome of overexpression cassettes containing hydroxylase *hpaB* from *P. aeruginosa*, the reductase *hpaC* from *E. coli* and the deregulated version of *S. cerevisiae* gene *ARO4* resulted in the maximum HT concentration obtained by a yeast strain from a simple growth medium.

11. The expression of unknown genes from *A. melanogenum* with conserved homology domains with toluene ortho-monooxygenase from *B. cepacia* in a yeast cell factory contributes to increase HT production and, by extension, it reveals a possible monooxygenase function in its host organism.

RESUMEN EN ESPAÑOL

Saccharomyces cerevisiae es un hongo unicelular eucariota perteneciente al filo *Ascomycota*, ya que puede formar ascosporas para reproducirse sexualmente. Esta levadura también es conocida como levadura "panadera" por su relación histórica con actividades humanas como la elaboración de alimentos y bebidas fermentadas.

En el campo de la investigación científica, la levadura sirve como organismo modelo para estudiar las propiedades de las células eucariotas, ya que comparte muchos procesos celulares clave comunes con las células humanas. Su genoma, relativamente pequeño, fue el primer genoma eucariota que se secuenció por completo (Goffeau et al., 1996). La disponibilidad de colecciones de mutantes, información sobre expresión génica, y de infraestructuras de investigación como la *Saccharomyces* Genome Database (SGD) favorecen el uso extendido de este organismo como modelo (Botstein et al., 2011). Los primeros análisis de la secuencia genómica revelaron una alta proporción de homólogos humanos en *S. cerevisiae*, de hecho, presenta rasgos genéticos conservados que permitieron reemplazar el 47% de las secuencias codificantes de la levadura por ortólogos humanos, mostrando más del 80% de reemplazabilidad para genes relacionados con lípidos, cofactores y vitaminas, metabolismo de carbohidratos y aminoácidos (Mell et al., 2003; Kachroo et al., 2015). Procesos conservados entre levaduras y humanos como la transducción de señales propiciarion el estudio de interacciones proteína-proteína conservadas, jerarquías de regulación y sistemas de señalización cruzada (Nielsen, 2019 a; Dahiya et al., 2020). *S. cerevisiae* también puede ser útil en el descubrimiento del efecto diana de compuestos específicos en el área del desarrollo de fármacos. También se utiliza como modelo de patogénesis fúngica y como valioso banco de pruebas para desarrollar tratamientos, a pesar de no ser un patógeno peligroso (Goldstein et al., 2001; Parsons et al., 2006; Hanson, 2018).

Además de ser el motor básico en la elaboración tradicional de bebidas alcohólicas y alimentos fermentados (por ejemplo, vino, cerveza, sake, sidra y pan), y de constituir un importante organismo modelo para el estudio de otros organismos eucariotas, también ha demostrado ser una plataforma industrial adecuada para la producción de una amplia gama de componentes químicos de

valor añadido, bioetanol, vacunas y proteínas terapéuticas (Otero et al., 2013). Esto se debe a una serie de características como su rápido crecimiento, la facilidad de cultivo, la facilidad de manipulación genética y la abundancia de datos previos sobre secuencias genéticas, así como su fisiología y metabolismo. En cuanto a la facilidad de la levadura para ser manipulada genéticamente, su preferencia por la recombinación homóloga (HR) frente a la unión de extremos no homólogos (NHEJ) para la reparación de roturas de doble cadena (DSB) en su ADN permite la instalación de material genético en sitios específicos y otras ediciones genómicas (Nielsen et al., 2013; Hanson, 2018). Además, en comparación con *E. coli*, *S. cerevisiae* tiene la designación de generalmente reconocida como segura (GRAS) de la agencia para la administración de alimentos y fármacos de los Estados Unidos (FDA) y es una cepa huésped robusta y libre de endotoxinas (Guo et al., 2019). Estas ventajas hacen de la levadura una buena plataforma para producir industrialmente multitud de compuestos como alcoholes superiores, enzimas, ácidos grasos y aminoácidos, entre otros desde un enfoque sostenible como es el uso de diferentes materias primas fermentables a partir de subproductos de otros procesos o de medios de crecimiento sencillos y económicos (Hansen et al., 2009; Nandy et al., 2018; Yu et al., 2018 a; Cordente et al., 2019; Hu et al., 2019; Levisson et al., 2019; Nielsen, 2019 b).

La levadura también tiene una importancia fundamental en el complejo proceso de vinificación, el mosto de uva se transforma en vino como resultado del metabolismo microbiano, en el que la fermentación alcohólica (FA) llevada a cabo principalmente por levaduras desempeña un papel crucial. Como proceso bioquímico, la vinificación consiste en la conversión de los azúcares presentes en el mosto en etanol, dióxido de carbono y otros metabolitos, pero el proceso comprende el desarrollo secuencial de especies microbianas de hongos, levaduras, bacterias lácticas y bacterias acéticas.

Como resultado de esta FA los principales componentes que se encuentran en el vino son agua 86%; etanol, 12%; glicerol y polisacáridos u otros oligoelementos, 1%; diferentes tipos de ácidos, 0,5%; y compuestos volátiles, 0,5% (Markoski et al., 2016). El vino también contiene una gran diversidad de compuestos menores derivados del metabolismo de las levaduras que confieren

características importantes que repercuten directamente en las propiedades del vino, como el aroma, el sabor, la estabilidad y la textura.

Como microorganismo anaerobio facultativo, la levadura *Saccharomyces* puede crecer a través de un metabolismo fermentativo mediante la reducción de azúcares a etanol, o a través de un metabolismo respiratorio en el que el oxígeno se utiliza como aceptor final de electrones en la cadena respiratoria mitocondrial. En condiciones aeróbicas, la respiración es posible con O2 como aceptor final de electrones, pero *S. cerevisiae* sigue mostrando fermentación alcohólica hasta que se agota el azúcar del medio. Este comportamiento se conoce como efecto Crabtree (Crabtree, 1928; De Deken, 1966) y las levaduras que expresan este rasgo se denominan levaduras Crabtree-positivas. *S. cerevisiae* es un buen representante de las levaduras Crabtree-positivas. Las levaduras de esta categoría tienden a canalizar la mayor parte del flujo de carbono hacia el metabolismo fermentativo del azúcar, independientemente de la disponibilidad de oxígeno. En contraste con este metabolismo fermentativo, las levaduras "Crabtree-negativas" en condiciones aerobias generan biomasa y dióxido de carbono como únicos productos. Las levaduras "Crabtree-positivas" presentan una menor producción de biomasa porque la mayor parte del azúcar se convierte en etanol. Para producir cantidades de biomasa comparables a las levaduras Crabtree-negativas necesitarían consumir más glucosa debido al menor rendimiento en energía libre, y por tanto, a la menor proporción de azúcar destinada a biomasa en el proceso de fermentación en comparación con el metabolismo de la respiración.

Durante la FA, la glucosa y la fructosa utilizadas como fuente de carbono se convierten en alcohol y CO2. Aproximadamente el 90 ~ 95% de los azúcares presentes en el mosto se convierten en etanol y CO2, alrededor del 4 ~ 5% se convierten en metabolitos secundarios como alcoholes superiores, ácidos, ésteres y glicerol, y finalmente el 1 ~ 2% se utiliza para el crecimiento y mantenimiento celular. Aunque la fermentación alcohólica y la respiración son las vías metabólicas más importantes desde el punto de vista energético, también lo son porque proporcionan a la célula los principales metabolitos precursores que se utilizarán para ensamblar moléculas más complejas como lípidos, proteínas y ácidos nucleicos. En este sentido, el metabolismo central del

carbono cuenta con una tercera vía biosintética, la vía de las pentosas fosfato (PPP) que produce importantes precursores y cofactores como la nicotinamida-adenina dinucleótido fosfato, la eritrosa-4-fosfato y la ribosa-5-fosfato, que intervienen en la síntesis de aminoácidos y ácidos nucleicos, entre otros.

La síntesis y degradación de aminoácidos forma parte del metabolismo del nitrógeno y es de suma importancia durante un proceso fermentativo, ya que ejerce un papel crucial en la calidad final del vino, pero también controla el ritmo de una fermentación alcohólica como factor limitante, influye en la biomasa total obtenida y en la duración de todo el proceso de fermentación, ya que la disponibilidad de nitrógeno es un factor crítico para evitar una fermentación lenta o un paro en la fermentación (Bell & Henschke, 2005; Gobert et al., 2019). Las principales fuentes de nitrógeno asimilable por la levadura (NFA) para *S. cerevisiae* son el amonio y los aminoácidos. Entre dichas fuentes de nitrógeno, algunas pueden clasificarse como "preferentes" o buenas fuentes de nitrógeno y otras como "no preferentes" según su capacidad para promover el crecimiento y la actividad fermentativa, aunque esta clasificación puede variar dependiendo de la cepa utilizada y de las condiciones de crecimiento (Gobert et al., 2019). Aunque los aminoácidos pueden ser asimilados a partir del medio de crecimiento la levadura también puede sintetizar la mayoría de los aminoácidos necesarios para construir sus proteínas fijando un ion amonio a un esqueleto de carbono derivado del metabolismo de los azúcares, por ejemplo, desaminándolos para formar amonio o utilizarlos como sustratos de una reacción de transaminación que transfiere un grupo amino a α-cetoglutarato para producir glutamato. Este nodo en el anabolismo y catabolismo del nitrógeno que se cruza con el metabolismo central del carbono implica reacciones entre α-cetoglutarato, amonio, glutamato y/o glutamina, y constituye el metabolismo central del nitrógeno (Ter Schure et al., 2000; Magasanik et al., 2002). Las reacciones del metabolismo central del nitrógeno dotan a la célula de la capacidad de sintetizar el resto de aminoácidos a partir del aminoácido glutamina o glutamato y de otros precursores derivados del metabolismo de los azúcares.

Durante la vinificación, la composición de aminoácidos del mosto inicial es determinante en el sabor y aroma del vino final, pero las levaduras también contribuyen al aroma final de diferentes maneras. Existen compuestos de sabor

neutro en el mosto que la levadura metaboliza en compuestos que aportan aroma y sabor aparte de aquellos compuestos aromáticos sintetizados *de novo*, entre ellos, ésteres de acetatos y de etilos, ácidos grasos volátiles, alcoholes superiores, monoterpenoides y compuestos sulfurosos volátiles (Cordente et al., 2012; Gobert et al., 2019). Para influir sobre el sabor y el aroma del vino desde el punto de vista microbiológico, se debe estudiar la relación entre el metabolismo del nitrógeno y los compuestos volátiles. Se ha descrito que los aminoácidos aromáticos (AAA), concretamente el triptófano (Trp), la tirosina (Tyr) y la fenilalanina (Phe), que forman parte del NFA, contribuyen a la formación de compuestos volátiles tanto en levaduras *Saccharomyces* como en levaduras no *Saccharomyces* (Vilanova et al., 2007; Barbosa et al., 2012; Gobert et al., 2017, 2019).

El metabolismo de los AAA en la fermentación del vino es importante en tanto que la producción de alcoholes superiores puede impactar en la estabilidad y perfil aromático de un vino, pero también debido a la generación de moléculas aromáticas derivadas de aminoácidos que se producen en cantidades muy pequeñas con interesantes propiedades y beneficios cuando se consumen (Mas et al., 2014), estas moléculas se conocen como compuestos bioactivos, y algunas de las moléculas bioactivas más importantes producidas por levaduras se originan a partir del triptófano y la tirosina. En el vino hay cientos de moléculas presentes en diferentes concentraciones y entre ellas, se encuentra una variedad de compuestos bioactivos con propiedades beneficiosas para la salud. Una cantidad importante de estos interesantes compuestos procede directamente de las uvas, sin embargo el papel del metabolismo de las levaduras en la producción de compuestos aromáticos y bioactivos se ha revelado muy importante en los últimos diez años. Los grandes avances en las técnicas analíticas modernas han permitido la identificación de moléculas presentes en bajas concentraciones y desde entonces se han descrito nuevos compuestos fenólicos e indólicos presentes en bebidas fermentadas como melatonina, serotonina o hidroxitirosol y atribuidos al metabolismo de las levaduras durante la fermentación alcohólica (Rodríguez-Naranjo et al., 2011 a; Fernández-Pachón et al., 2014; Aredes-Fernández et al., 2016; Varoni et al., 2018; Fernández-Cruz et al., 2019; González-Ramírez et al., 2023). Las propiedades que se desprenden de estos

metabolitos derivados de la levadura y su repercusión en la salud humana amplían sus aplicaciones más allá de la elaboración del vino, con interés en el campo de la nutrición funcional y las aplicaciones farmacéuticas. Estos compuestos producidos por la levadura aún no son del todo conocidos en cuanto a sus mecanismos de síntesis en la levadura o sus determinantes fisiológicos e importancia para este organismo. La necesidad de ampliar el conocimiento sobre los compuestos derivados de aminoácidos aromáticos en levadura (AADC), especialmente aquellos con capacidad bioactiva demostrada, permitirá la modulación e incremento de estos compuestos bioactivos en un contexto fermentativo, pero también permitirá desarrollar sistemas de producción para utilizar estos compuestos en otras aplicaciones.

La relación recientemente establecida entre el metabolismo de los aminoácidos aromáticos y la síntesis de moléculas bioactivas como la melatonina, la serotonina y el hidroxitirosol en *Saccharomyces cerevisiae* aporta interesantes aplicaciones en el campo de la industria alimentaria y farmacológica, pero también plantea un gran desafío. La síntesis de estas moléculas se atribuye a la levadura durante el proceso de fermentación, pero los mecanismos subyacentes o los genes responsables siguen siendo desconocidos. En este contexto, planteamos la hipótesis de que a través del estudio de la síntesis de compuestos bioactivos derivados de aminoácidos aromáticos y sus mecanismos subyacentes en *S. cerevisiae* es posible promover un incremento de estos metabolitos en alimentos y bebidas fermentadas como resultado del metabolismo de la levadura, pero también es posible modular racionalmente la producción de estos compuestos de interés para una producción masiva y a la vez sostenible utilizando la levadura como factoría celular.

Desde este enfoque, el objetivo principal de esta tesis es **ampliar el conocimiento sobre los determinantes moleculares responsables de la síntesis de compuestos bioactivos derivados de aminoácidos aromáticos, especialmente melatonina e hidroxitirosol, para explorar la capacidad de sobreproducción de estos metabolitos por parte de las levaduras**.

Este objetivo general se desglosa en los siguientes objetivos específicos:

Objetivo 1. Adaptar un método rápido, sensible y de bajo coste para detectar y cuantificar la melatonina directamente a partir de muestras de medios de crecimiento.

Uno de los retos del estudio de la biosíntesis de melatonina por las levaduras reside en las concentraciones habitualmente bajas de este compuesto que pueden detectarse en los productos fermentados o en los medios de crecimiento de las levaduras como resultado de su metabolismo. La determinación inequívoca de este compuesto en una matriz como medios de cultivo o mosto fermentado plantea un gran reto analítico debido a la presencia de compuestos químicamente similares en la matriz y la investigación en este campo ha llevado al desarrollo de potentes herramientas analíticas como la UHPLC-MS/MS, que es el método de referencia actual debido a su bajo límite de detección (LOD) y cuantificación (LOQ). Sin embargo, el uso de este método conlleva una manipulación previa de la muestra ya que se requiere la extracción y preparación de la misma, además del uso de aparatos de alto consumo energético y elevado coste, así como la necesidad de técnicos especializados y entrenados para su manejo análisis. En un contexto de determinación de potenciales cepas productoras de melatonina a partir de un gran conjunto de cepas ambientales o industriales, disponer de un método rápido, fiable y barato para detectar y cuantificar la melatonina directamente a partir del sobrenadante de cultivos de levadura resulta de gran interés. Los biosensores basados en levaduras son herramientas atractivas y prometedoras para este objetivo, ya que ofrecen una solución práctica para abordar de forma rentable el análisis rápido de un elevado número de muestras, siempre que sean lo suficientemente sensibles y específicos como para discriminar y cuantificar la melatonina a partir de un sobrenadante de medios de crecimiento de levaduras sin procesar. En un trabajo reciente, Shaw et al. (2019) diseñaron levaduras biosensoras basadas en una transducción de señales mediante receptores acoplados a proteínas G (GPCR). Los GPCR son un mecanismo de detección importante en eucariotas, y también lo son para la levadura, ya que son responsables de la detección de hormonas sexuales que impulsan la activación de diferentes genes de involucrados en la reproducción sexual. Al suprimir el receptor GPCR nativo de la levadura para el factor α y hasta 15 genes implicados en la transducción de la señal, aislaron el

mecanismo central de transducción de la señal para evitar cualquier activación/represión génica no relacionada con la función de detección de la melatonina. A esta vía refactorizada le integraron tres modificaciones importantes en el genoma en forma de fragmentos lineales. Estas modificaciones consisten en la sobreexpresión de un receptor GPCR humano para la melatonina (MTNR1A), la sobreexpresión de la subunidad Gα codificada por *GPA1*, la expresión de una proteína verde fluorescente como gen reportero (*sfGFP*) precedida por un promotor sintético portador de secuencias activadoras ascendentes (UAS) bacterianas *LexO* y un factor de transcripción sintético consistente en el dominio de respuesta a feromonas (*PRD*) del gen nativo *STE12* fusionado al dominio de unión al ADN del operador bacteriano *LexA*.

Nuestro objetivo era adaptar una de las cepas biosensoras de melatonina de Shaw et al. (2019) a un sistema de placas de 96 pocillos de pequeño volumen e inicialmente replicamos los resultados anteriores en la detección de melatonina. A continuación, redujimos significativamente la sensibilidad para que el sistema fuera adecuado para la detección de melatonina a partir del sobrenadante del medio de crecimiento de levadura. Para ello, en una etapa inicial se incrementamos la expresión de MTNR1A mediante la integración de un mayor número de copias en el genoma. También abordamos modificaciones aguas abajo de la vía de señalización integrando copias extra del sistema reportero para evaluar un posible aumento de la señal de salida, y de la subunidad Gα para mantener una actividad basal reducida y una buena relación señal/ruido. Evaluamos y ajustamos la respuesta generada en función de la concentración de melatonina en las diferentes modificaciones. Después probamos la capacidad de detección de las diferentes cepas al diluir el ligando en el medio de crecimiento YNB80, que tiene triptófano y amonio como fuentes de nitrógeno, y realizamos ensayos de recuperación a partir de esta matriz con concentraciones conocidas de melatonina para determinar la exactitud y precisión del método. Finalmente, como prueba de concepto, analizamos 101 cepas de levadura de diferentes orígenes y especies tras su crecimiento en un medio de laboratorio sencillo enriquecido con triptófano y logramos obtener diferencias en las concentraciones de melatonina entre las distintas muestras utilizando un método barato, rápido y de bajo coste, a partir de muestras sin ningún pretratamiento.

Objetivo 2. Desvelar los genes responsables de la síntesis de melatonina en *S. cerevisiae*.

La producción de melatonina en bebidas fermentadas como resultado del metabolismo de la levadura se ha demostrado previamente, y se han logrado muchos avances en el desciframiento de la nueva vía metabólica de la síntesis de melatonina a partir del triptófano. De hecho, en los últimos años hemos propuesto una vía metabólica de la melatonina para la levadura que difiere sustancialmente del modelo de mamíferos, ya que *S. cerevisiae* parece convertir el triptófano en triptamina en un primer paso de descarboxilación, seguido de una hidroxilación para formar serotonina. A continuación, la melatonina se forma a partir de la serotonina por *N*-acetilación seguida de *O*-metilación de la *N*-acetilserotonina o, alternativamente, por una *O*-metilación de la serotonina para formar 5-metoxitriptamina seguida de su *N*-acetilación, que es la alternativa preferida por *S. cerevisiae*, aunque nuevas evidencias sugieren más ramificaciones en la vía en la que se encuentra el triptófano como precursor (Muñiz-Calvo et al. 2019).

Aunque se ha avanzado en el conocimiento de esta vía de síntesis, aún se desconocen los genes responsables de las cuatro reacciones necesarias para la síntesis de melatonina. Sólo un gen ha sido propuesto como homólogo de la arilalquilamina *N*-acetiltransferasa de mamíferos (*AANAT*) como responsable de los pasos de *N*-acetilación en la vía de la levadura, *PAA1*. Esta poliamina acetiltransferasa tiene la capacidad de aceptar serotonina o 5-metoxitriptamina como sustrato para convertirlas en *N*-acetilserotonina y melatonina, respectivamente.

Evaluamos la función *in vivo* de *PAA1* valorando la bioconversión de los diferentes sustratos posibles, como la 5-metoxitriptamina, la triptamina y la serotonina utilizando diferentes plataformas de expresión de proteínas. Para ello, sobreexpresamos el gen *PAA1*, y la aralquilamina *N*-acetiltransferasa de *Bos taurus* (Bt*AANAT*) como control positivo, en *S. cerevisiae* y *Escherichia coli*, y medimos la producción de metabolitos acetilados tras un pulso de precursor en el medio. Como los resultados evidenciaron la presencia de enzimas alternativas con actividad AANAT en *S. cerevisiae*, ampliamos la búsqueda de *N*-

acetiltransferasas candidatas combinando un análisis global de expresión transcripcional (RNAseq) en condiciones de síntesis de melatonina, y el uso de herramientas predictivas *in silico*. Esta estrategia nos ha permitido proponer nuevos candidatos para explicar la actividad de acetilación relacionada con la melatonina en levadura.

Concluimos que la implicación de *PAA1* en la síntesis de melatonina es muy limitada y no exclusiva, y utilizando un enfoque combinado que incluye el análisis de la expresión génica global durante la síntesis de melatonina y la búsqueda predictiva de dominios acetilasa nos permitió proponer y probar un nuevo candidato, *HPA2*, con capacidad de acetilar 5-metoxitriptamina para producir melatonina. Sin embargo, ninguna de las candidatas descritas tiene la *N*-acetiltransferasa de arilalquilamina como principal actividad *in vivo* en levaduras, ya que se consideran proteínas “moonlighting”, es decir, poseen actividades alternativas a su función más habitual y efectiva..

Objetivo 3. Aumentar la capacidad de la levadura para producir compuestos bioactivos derivados de aminoácidos aromáticos.

Los compuestos bioactivos derivados de aminoácidos aromáticos como la melatonina, la serotonina, el hidroxitirosol (HT) y otros metabolitos relacionados presentan beneficios que promueven la salud y también pueden contribuir a la calidad y estabilidad de los alimentos y bebidas fermentados. Existe un interés creciente en aumentar las concentraciones de estos compuestos en bebidas fermentadas como el vino, ya que pueden contribuir a la calidad y estabilidad del vino fermentado (Cordente et al., 2019; Edwin Fernández-Cruz et al., 2016). En este contexto, la obtención de cepas de levadura capaces de aumentar la cantidad de estos compuestos en el producto final es de gran interés para la industria del vino, pero la generación de cepas de levadura con estos rasgos deseables se enfrenta a dos obstáculos principales: Para modificar de forma eficaz y precisa el metabolismo de la levadura con el fin de obtener mayores cantidades de un determinado metabolito es necesario conocer a fondo su ruta biosintética en términos de precursores implicados, cofactores, genes y proteínas responsables, condiciones de síntesis, posibles cuellos de botella y puntos de fuga. Estos detalles siguen sin conocerse en el caso de algunos de

los AADC bioactivos más interesantes, como la melatonina, la serotonina o el hidroxitirosol (HT) en levadura. Y por otro lado, incluso con toda la información necesaria sobre su biosíntesis, la manipulación genética racional en la industria alimentaria está muy restringida por la normativa europea sobre organismos modificados genéticamente (OMG).

Tomando la melatonina como modelo, debido a su relación con los estímulos lumínicos en organismos superiores y a su efecto protector frente a radiación UV, también en células de levadura, llevamos a cabo una novedosa evolución adaptativa de laboratorio utilizando la luz blanca como presión selectiva sobre una cepa de *S. cerevisiae*, aislada del entorno de una bodega, para obtener una cepa evolucionada (EVO) con capacidad de sobreproducción de metabolitos relacionados con la melatonina y otros metabolitos bioactivos, especialmente cuando se ve sometida a estrés fotooxidativo. Evaluamos las diferencias genotípicas y fenotípicas entre la cepa evolucionada y la parental, analizamos su metaboloma extracelular completo y eso nos permitió proponer interesantes relaciones entre mutaciones específicas y el aumento de AADCs bioactivos. Además, nuestros resultados revelaron el potencial de la luz blanca como una presión selectiva muy potente, e incluso como un mutágeno para ser utilizado en la generación de nuevas cepas desde un enfoque no transgénico.

Por otro lado, exploramos alternativas a los enfoques no transgénicos para la síntesis de compuestos bioactivos. A este respecto, la sobreproducción de otros compuestos bioactivos, como el hidroxitirosol, suscita especial interés por sus posibles aplicaciones en la industria alimentaria, de piensos, de suplementos y farmacéutica. Las principales formas de obtener HT son la extracción a partir de material vegetal o la síntesis química. Dado que los derivados del olivo son la fuente más accesible, la mayoría de los productos de HT proceden de la extracción de aceitunas o de subproductos de la elaboración del aceite de oliva, de las cuales esta última es una fuente favorable porque permite revalorizar un subproducto. Sin embargo, la extracción de HT de cualquiera de estas fuentes es un proceso largo que arroja bajos índices de recuperación y pureza, que pueden variar estacionalmente de un lote a otro. Los métodos de síntesis química suelen implicar disolventes no respetuosos con el medio ambiente y sustratos de partida caros, lo que a veces los hace inadecuados para la

producción industrial a gran escala (Z. L. Zhang et al. 2012; Achmon y Fishman 2015; Britton, Davis y O'Connor 2019). Por lo tanto, la producción biotecnológica de HT tiene potencial para convertirse en el proceso de producción dominante en el futuro. La levadura *Saccharomyces cerevisiae*, resulta una prometedora factoría celular para producir HT gracias a una serie de ventajas, como el crecimiento robusto en medios simples, la viabilidad en las manipulaciones genéticas y que es "generalmente considerada segura" (GRAS) (Guo et al. 2019; Nielsen et al. 2013).

Para ello, sobreexpresamos genes exógenos para llevar a cabo el paso final en la vía sintética para convertir el tirosol producido naturalmente en HT, y luego emprendimos una serie de modificaciones genéticas que implicaban eliminar, desregular y sobreexpresar diferentes genes a lo largo de la vía metabólica para aumentar y favorecer el flujo metabólico de glucosa hacia tirosol y, por lo tanto hacia HT. Anteriormente habíamos conseguido realizar la sobreexpresión heteróloga de los genes *hpaB* y *hpaC* de *E. coli* en *S. cerevisiae* mediante el uso de plásmidos episomales en nuestro trabajo previo, en el que obtuvimos producción de HT cuando suplementamos el medio de crecimiento con precursores (Muñiz-Calvo et al. 2020). En este trabajo diseñamos una cepa de levadura sobreproductora de HT directamente a partir de una fuente de carbono simple como la glucosa en *S. cerevisiae*. Para ello primero construimos una cepa de levadura libre de plásmidos que albergaba el complejo HpaBC integrado en múltiples copias y después probamos diferentes modificaciones genéticas para aumentar el flujo metabólico hacia el principal precursor inmediato, tirosol, siguiendo un enfoque de ingeniería metabólica y obtuvimos la mayor concentración de hidroxitirosol producida por levadura hasta la fecha. Tras alcanzar este hito, centramos nuestros esfuerzos en la mejora genética de la cepa productora de HT para priorizar la productividad en HT mediante el uso de otras versiones del complejo HpaBC, la regulación de la expresión de un gen nativo para favorecer un flujo metabólico hacia el HT y la sobreexpresión de un nuevo complejo potencial de hidroxilasa fúngica para convertir en HT precursores restantes como el tirosol o el 2-feniletanol. Estos resultados establecen los primeros pasos para sobreproducir HT en levaduras a partir de

glucosa mediante un enfoque de ingeniería metabólica y para seguir desarrollando una fábrica de células de levadura para la producción de HT.

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ANNEX I: PUBLICATIONS

microorganisms

MDPI

Article

The Role of the *PAA1* Gene on Melatonin Biosynthesis in *Saccharomyces cerevisiae*: A Search of New Arylalkylamine *N*-Acetyltransferases

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Abstract: Recently, the presence of melatonin in fermented beverages has been correlated with yeast metabolism during alcoholic fermentation. Melatonin, originally considered a unique product of the pineal gland of vertebrates, has been also identified in a wide range of invertebrates, plants, bacteria, and fungi in the last two decades. These findings bring the challenge of studying the function of melatonin in yeasts and the mechanisms underlying its synthesis. However, the necessary information to improve the selection and production of this interesting molecule in fermented beverages is to disclose the genes involved in the metabolic pathway. So far, only one gene has been proposed as involved in melatonin production in *Saccharomyces cerevisiae*, *PAA1*, a polyamine acetyltransferase, a homolog of the vertebrate's aralkylamine *N*-acetyltransferase (AANAT). In this study, we assessed the in vivo function of *PAA1* by evaluating the bioconversion of the different possible substrates, such as 5-methoxytryptamine, tryptamine, and serotonin, using different protein expression platforms. Moreover, we expanded the search for new *N*-acetyltransferase candidates by combining a global transcriptome analysis and the use of powerful bioinformatic tools to predict similar domains to AANAT in *S. cerevisiae*. The AANAT activity of the candidate genes was validated by their overexpression in *E. coli* because, curiously, this system evidenced higher differences than the overexpression in their own host *S. cerevisiae*. Our results confirm that *PAA1* possesses the ability to acetylate different aralkylamines, but AANAT activity does not seem to be the main acetylation activity. Moreover, we also prove that Paa1p is not the only enzyme with this AANAT activity. Our search of new genes detected *HPA2* as a new arylalkylamine *N*-acetyltransferase in *S. cerevisiae*. This is the first report that clearly proves the involvement of this enzyme in AANAT activity.

Keywords: yeast; *E. coli*; melatonin; tryptophan metabolism; *N*-acetylserotonin; 5-methoxytryptamine; polyamine acetyltransferase; *HPA2*; AANAT activity

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1. Introduction

After the discovery of melatonin outside the animal kingdom, research on melatonin in other clades emerged. Thus, melatonin was found to be a ubiquitous phylogenetically ancient molecule in almost every organism, from primitive photosynthetic bacteria to humans [1]. For melatonin synthesis, the majority of studies have been performed in vertebrates, particularly in mammals, and more recently in plants [2]. The occurrence of melatonin in yeast was described for the first time by Sprenger et al. [3] as a product of the metabolism of precursors such as tryptophan, serotonin, *N*-acetylserotonin, and 5-methoxytryptamine. These results have been later confirmed and extended by numerous

microbial biotechnology

Open Access

Metabolic engineering of *Saccharomyces cerevisiae* for hydroxytyrosol overproduction directly from glucose

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Summary

Hydroxytyrosol (HT) is one of the most powerful dietary antioxidants with numerous applications in different areas, including cosmetics, nutraceuticals and food. In the present work, heterologous hydroxylase complex HpaBC from *Escherichia coli* was integrated into the *Saccharomyces cerevisiae* genome in multiple copies. HT productivity was increased by redirecting the metabolic flux towards tyrosol synthesis to avoid exogenous tyrosol or tyrosine supplementation. After evaluating the potential of our selected strain as an HT producer from glucose, we adjusted the medium composition for HT production. The combination of the selected modifications in our engineered strain, combined with culture conditions optimization, resulted in a titre of approximately 375 mg l^{-1} of HT obtained from shake-flask fermentation using a minimal synthetic-defined medium with 160 g l^{-1} glucose as the sole carbon source. To the best of our knowledge, this is the highest HT concentration produced by an engineered *S. cerevisiae* strain.

Introduction

Both virgin olive oil and wine are rich in polyphenols, including tyrosol, oleuropein, hydroxytyrosol (HT), elenolic acid and resveratrol (Carrasco-Pancorbo *et al.*, 2005; Fernández-Mar *et al.*, 2012). Of these, HT is a natural antioxidant considered to be one of the main ingredients that promotes health and a bioactive component in Mediterranean diet characterized by regular olive oil intake (Daniele *et al.*, 2017). Several studies have demonstrated extensive biological HT properties with both *in-vitro* and *in-vivo* models (D'Angelo *et al.*, 2020). The ability to cross the brain barrier and its high bioavailability and degree of absorption, together with the health claim approved by EFSA (Turck *et al.*, 2017), highlight the importance of this polyphenol for food, feed, supplement and pharmaceutical industries (Britton and Davis, 2019). However, the price of commercially available pure HT forms can be high, which makes its use in the food industry economically unviable (Achmon and Fishman, 2015).

The main ways to obtain HT are plant extraction or chemical synthesis. As olive tree derivatives are the most accessible source, the majority of HT products come from the extraction of olives or olive oil waste streams, of which the latter is a favourable source because it originates from a by-product. However, HT extraction from any of these sources is a lengthy process that yields low recovery rates, which can vary seasonally from batch to batch. Chemical synthesis methods usually involve non environmentally friendly solvents and expensive starting substrates, which sometimes make it unsuitable for large-scale industrial production (Zhang *et al.*, 2012; Achmon and Fishman, 2015; Britton *et al.*, 2019). Therefore, biotechnological HT production can potentially be the dominant production process for the future. Whole-cell catalysts for HT biosynthesis have been used with different bacterial microorganisms (Liebgott *et al.*, 2009; Bouallagui and Sayadi, 2018; Li *et al.*, 2018; Hassing *et al.*, 2019; Yao *et al.*, 2020). Specially remarkable is the use of *Escherichia coli*, in which the co-expression of yeast *ARO10* and *ADH6* genes, and the overexpression of HpaBC, produced important amounts of HT (Chung and Kim, 2017; Li *et al.*, 2018). Recently, through structure-guided modelling and directed evolution, the HpaBC complex was used as tyrosine hydroxylase instead of tyrosol hydroxylase, leading to a 95% conversion rate of tyrosine to L-DOPA. This strategy yields a remarkably high HT production using tyrosine as a first substrate via L-DOPA decarboxylase, tyramine oxidase (TYO) and alcohol dehydrogenase using an *in vivo* evolved TYO (Yao

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TÍTULO
DE
PATENTE DE INVENCIÓN

Cumplidos los requisitos previstos en la vigente Ley 24/2015, de 24 de julio, de Patentes, se expide el presente TÍTULO, acreditativo de la concesión de la Patente de Invención. La solicitud ha sido tramitada y concedida con realización del Informe sobre el Estado de la Técnica y **con Examen Sustantivo** de los requisitos de patentabilidad establecidos en la Ley.

Se otorga al titular un derecho de exclusiva en todo el territorio nacional, bajo las condiciones y con las limitaciones previstas en la Ley de Patentes. La duración de la patente será de **veinte años** contados a partir de la fecha de presentación de la solicitud (26/11/2020).

La patente se concede sin perjuicio de tercero y sin garantía del Estado en cuanto a la validez y a la utilidad del objeto sobre el que recae.

Para mantener en vigor la patente concedida, deberán abonarse las tasas anuales establecidas, que se pagarán por años adelantados. Asimismo, deberá explotarse el objeto de la invención, bien por su titular o por medio de persona autorizada de acuerdo con el sistema de licencias previsto legalmente, dentro del plazo de cuatro años a partir de la fecha de presentación de la solicitud de patente, o de tres años desde la publicación de la concesión en el Boletín Oficial de la Propiedad Industrial, aplicándose el plazo que expire más tarde.

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— *con la parte de lista de secuencias de la descripción (Regla 5.2(a))*

(54) **Title:** RECOMBINANT SACCHAROMYCES CEREVISIAE FOR THE PRODUCTION OF HYDROXYTYROSOL

(54) **Título:** SACCHAROMYCES CEREVISIAE RECOMBINANTE PARA LA PRODUCCIÓN DE HIDROXITIROSOL

(57) **Abstract:** The present invention relates to a wine-making yeast, specifically Saccharomyces cerevisiae, genetically modified by multiple integration of the enzymatic hydroxylase complex HpaBC from Escherichia coli, and the enzyme ARO4 from Saccharomyces cerevisiae with a point mutation, specifically the substitution of K (lysine) by L (leucine) at position 229 in the amino acid sequence