Biochemical and molecular basis involved in the synthesis of melatonin and other derivatives of aromatic amino acids in *Saccharomyces cerevisiae*

Sara Muñiz Calvo

Supervised by
Dr. José Manuel Guillamón Navarro

Valencia 2021

Biochemical and molecular basis involved in the synthesis of melatonin and other derivatives of aromatic amino acids in *Saccharomyces cerevisiae*

PhD Thesis

Sara Muñiz Calvo

December, 2020

supervised by

Dr. José Manuel Guillamón Navarro

Dpt. de Bioquímica y Biología Molecular

Doctorado en Biomedicina y Biotecnología

Paterna, 21 de diciembre de 2020

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

INFORMA:

Que Sara Muñiz Calvo, graduada en Biología por la Universidad de Valencia, ha realizado bajo mi dirección el trabajo de Tesis Doctoral que lleva por título "Biochemical and molecular basis involved in the synthesis of melatonin and other derivatives of aromatic amino acids 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 Doctora 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)

The present work has been carried out at the Department of Food Biotechnology of the IATA (CSIC). I received a predoctoral contract co-financed by the CSIC and the Valencian Ministry of Education, Research, Culture and Sports through the PROMETEO Program for research groups of excellence (PROMETEOII/2014/042). The Ministry of Economy and Competitiveness made possible the development of this thesis through the concession of the project AGL2013-47300-C3-3-R, and the project AGL2016-77505-C3-1-R. In addition, I was awarded with funding for a short fellowship from Valencian Ministry (BEFPI17) in the Department of Biomedicine of Bergen University (Norway), which was important for the development of this work.

AGRADECIMIENTOS

Por mucho que me esfuerce sé que no voy a encontrar las palabras que se acerquen a lo enormemente agradecida que me siento por la oportunidad de realizar esta tesis y por toda la gente a la que he tenido la suerte de conocer y tener a mi lado durante todos estos años. Extraordinarias personas que me han ayudado muchísimo y que sin ellas nada hubiese sido igual.

En primer lugar, me gustaría agradecer a mi director José Manuel Guillamón su confianza en mí desde un primer momento, y ofrecerme la posibilidad de incorporarme al laboratorio. Por compartir conmigo los resultados buenos y los no tan buenos, tu optimismo inagotable, tus palabras de ánimo, por tu cariño y por transmitirme tu entusiasmo por la investigación. Gracias también por dejarme ser tan independiente, porque incluso si eso ha significado fallar y equivocarme muchas veces, me ha ayudado a crecer como científica. Por último, gracias por fomentar un ambiente de trabajo tan bueno en el que ha sido realmente fácil realizar mi doctorado.

También me gustaría agradecer especialmente a Amparo Querol, la oportunidad brindada a través del contrato de investigación asociado al PROMETEO para la realización de esta tesis, así como a todos los investigadores del grupo de Biología de Sistemas en Levaduras de Interés Biotecnológico (SBYBI) su ayuda y consejos durante los seminarios de grupo. Gracias Amparo, Sergi, Eladio y Roberto también por los buenos momentos celebrados en las comidas de navidad, así como en las paellas del IATA (y fuera del IATA). También a otros investigadores externos al grupo, en especial al Dr. Antonio Abad y al Dr. Antonio Doménech. Gracias por toda vuestra ayuda, implicación y colaboración durante la realización de esta tesis.

Gracias a todos los miembros del equipo que forman o han formado parte del "*melatoteam*". Gracias a las doctoras Ana Mª Troncoso, María Carmen García, Eva Valero, María Jesús Torija, Gemma Beltrán y el Dr. Albert Más por vuestros consejos y sugerencias, por vuestro gran sentido del humor, así como por la gran hospitalidad brindada durante las reuniones en Sevilla y Tarragona. Gracias a todos los doctorandos (y muchos ya doctores) con los que he coincidido y podido colaborar durante el proyecto, Edwin, Mª Antonia, Marta, Jennifer, Bea, Mercé y en especial Mª Ángeles por introducirme (junto con Pedro y Víctor) en vuestro grupo en el máster y por todos todos los buenos momentos vividos.

I want to move to Norway for a moment, because my stay there was very important during the development of my thesis. First of all I would like to thank to Professor Aurora Martínez for having welcomed me to her group and for her affection and valuable discussions and suggestions during my short stay. I also want to send my sincere gratitude to the other members of the biorecognition group, Helene, Karina, Åge, Ming, Kunwar, Fredrik, Oscar, Sten, Knut and Lars for all their support and especially to Marte for your kind help and friendship. I would also like to thank Andrea and Stanley, because you were my great friends during my time there. Finally, I would also like

to include my thanks to Vivian Sollid, the best roommate I could have in Bergen and a great friend that I am fortunate to have around in the present.

Toca el momento de agradecer a todas las personas que más cerca he tenido cerca durante este tiempo. Empezando por los que conforman o han conformado el laboratorio 303. Gracias a todos por hacer que haya sido tan fácil formar parte del equipo. Fani o como tu dirías “amigui”, gracias por darme la oportunidad de empezar en el lab (aunque llevase zapatillas de ir por casa) y por contribuir a que me quedara, esta tesis no la hubiese conseguido si no fuera por ti. Gracias por escucharme y comprenderme, por tu integridad y por controlar todo y preocuparte por todos. También por engancharme al gym, por las risas y los lloros durante nuestras tesis y por todo lo que he aprendido de ti. Gracias por los grandes momentos vividos dentro y fuera del lab, por tus consejos y por querer hacer lo imposible para estar en la defensa. Ying, creo que tu nombre no podía ser más acertado. Gracias por llegar al laboratorio e iluminarlo con tu luz propia. Gracias por tu generosidad, tu sentido del humor y tu locura que te hacen única y que conquistan a quien te conoce. Gracias por apuntarte a un millón de aventuras con nosotros, por adaptarte e integrarte tan bien pese al nivel de inglés inicial (“*comprobe*”) y sobre todo por no desistir con todas las complicaciones burocráticas y los papeleos. Gracias por haber sido mi 孪生姐妹, ya sabes que siempre tendrás un lugar allí dónde estemos y también en Vila-real cuando quieras venir. Antonio y Judit, gracias por hacer que sea tan fácil compartir laboratorio con vosotros y por toda vuestra ayuda y compañerismo durante estos años. Andrés gracias por ayudarme con los experimentos finales cuando llegaste al lab y por todas las propuestas e ideas de mejora. Te deseo mucha suerte en tu tesis. Flor, gracias por ser tan detallista con todo, por querer escuchar, tu honestidad y por estar siempre dispuesta a echar una mano sin necesidad de pedirla. Gracias a todos los estudiantes o “nietos” del 303, Roberto, Marcos, Andrea, Elena y little Alba ¡Nos queda al menos una cena pendiente! També gràcies a Paula Peña per tota la teua ajuda durant el TFM al 303 i sobretot després amb tots els ELISA. Por último, gracias a las maravillosas personas que han venido de estancia a nuestro laboratorio y he tenido el privilegio de conocer y aprender de ellos. Gracias a Jenny, Florence, Tania, y en especial a Eduardo por compartir más que lab y seguir manteniendo la relación tras todos estos años. Por último, desde Tucumán, querida Daniela sabes que me siento muy afortunada de haberte conocido y sé que nuestra amistad, va a ser para siempre. Eres una persona increíble y gran amiga. Espero que nuestros caminos se encuentren de nuevo.

Saliendo del 303, es un poco complicado agrupar por laboratorios a la gente puesto que durante estos años ha habido cambios y reubicaciones, así que gracias a las personas que están o han estado en la tercera planta del IATA. Gràcies Vicky, per preocupar-te tant per cada un de nosaltres i per tot el teu afecte durant tots aquests anys. David gracias por los grandes momentos que hemos tenido y por tu apoyo. Laura Pérez, gracias por toda tu ayuda y conocimiento. Eli y Lidia, gracias por vuestro cariño y consejos. Jiří y Aurora, gracias por las risas aseguradas siempre que habéis estado presentes.

Walter, gracias por tener cada día un saludo simpático desde el primer día. Pero sobre todo por invitarme a uno de los viajes más auténticos que he vivido y por abrirnos las puertas de tu casa. Javi, gracias por tu sensatez junto con tu humor que te caracterizan, porque a lo tonto hemos compartido un montón de momentazos, por descubrirme el chuletón a la brasa y por acordarte de mí y tener detalles tan bonitos como unos auténticos mantecados, eres un gran amigo. Alba C gracias por invitarnos a la fiesta del embutido, por tu amistad y por esa sonrisa que hacen que se le olviden los problemas a uno, eres genial ¡no cambies nunca! Dolo gracias por esa energía y simpatía que te caracterizan, por tus consejos sobre gatos y por ser tan transparente. Romain, Laura y Seba, gracias por las invitaciones múltiples a vuestro gym, por las cenas y risas posteriores y también por vuestro apoyo durante la tesis. Sonia, gracias por ser ese soplo de aire fresco que todos necesitamos y por tu simpatía. Tania, gracias por las múltiples conversaciones, pruebas de clases de gym y la noche de los zombies. Ana Perea, Ana Cris y Pilar, muchas gracias por vuestra ayuda y porque siempre es super agradable encontraros y hablar de cualquier cosa con vosotras. Raquel, gràcies per les experiències compartides al congrés a Göteborg. Alba, sempre he tingut la sensació de que et conec de molts anys enrerre. Ja saps que et considere una persona fonamental en la meua vida i que ens tindrem per el que faça falta. Gràcies per ser tan generosa, especial, humil i bonica. Ceci, cuento los meses para que volváis a Valencia. Gracias por ser tan única, por tu forma de expresarte, tu pasión por todo lo que haces y por saber escuchar y aconsejar tan bien. Anto eres de esas personas que poquito a poco te llega al alma. Gracias por todo, por escucharme tanto, por las risas, por tu sinceridad, por tus consejos, por no juzgar y por siempre estar ahí. Gracias por acogernos en tu casita de Gothebörg y junto a Ceci enseñarnos lo mejorcito de esa ciudad. Laura, gracias por tu forma de ser tan grande ¡y a la vez tan humilde! Te admiro un montón. Gracias por tu humor, tu confianza y por todo tu apoyo. Tengo mil momentos vividos contigo (camping con y sin nevera, tardes de juego y gin tonic, clases de gym…) en los que con solo recordarlos me sale una sonrisa, y lo mejor es que sé que nos quedan muchos más por compartir, porque eres una amiga para siempre. Adri, gracias por tus consejos y por animar y darle un toque de humor a todo. Lucía, pese que al principio ninguna de las dos lo pensaría, has llegado a ser una persona super importante para mí. Gracias por tu amistad tan sincera, por todos los viajes a Haro en los que me he sentido muy bien acogida, por ser una compi de piso genial incluyendo tu ayuda para peinarme jeje. Gracias por tu gran generosidad y por siempre estar dispuesta para lo que sea. Peris, gràcies per la nit en que et vaig coneixer en la qual vam xarrar per hores David, tu i jo al carrer, en la que ja vaig presentir que series un bon amic. Eres molt gran, desitge que aconseguisques tot el que et proposes. Miguel, gracias por todo tu apoyo y tu cariño constante y por transmitir esa pasión y emoción por las cosas que te importan. María, gracias por estar siempre dispuesta a lo que sea, por invitarnos a vivir les falles desde dentro y por las tardes tanto de gym como de dulce de leche. Gracias a las personas que vinieron desde muy lejos para un tiempo pero que han dejado su huella en el IATA. En especial, Andrea Origone, Migue, Ceci Lerena y Nubia. He sido muy afortunada de poder conoceros y gracias por seguir manteniendo el contacto hoy en día.

Gracias a personas que de una forma u otra han estado unidas a mi durante el periodo de mi doctorado y que siempre es genial cuando nos volvemos a juntar. Gracias a Laura Montes, por los días de gym, por las cenas en tu terracita, por avisar siempre que vienes a Valencia y por todo el cariño que nos tenemos. Gracias a los mejores vecinos del mundo, Pablo y Ana, gracias por todos los vermuts y por vuestra ayuda con la portada de la tesis ¡Sois geniales! Gracias a los Carlos (Menor y Navarro) por sumar siempre, por vuestro excepcional sentido del humor y por los magníficos momentos compartidos. Gracias a Marcos Parras, por tu simpatía y afecto y por seguir manteniendo el contacto. Gracias a Cristina Sanabria por ser de esas personas de las que te sientes infinitamente afortunada de conocer, gracias por tu amor verdadero, por venir a visitarme a Bergen y por acogerme siempre tan bien todas las veces en Osnabrück, te quiero mucho.

Gracias a las "*biolocas*" (Anita, Marta, Mónica, Aida, Amparo, Julia y Alicia) por empezar en el camino de la ciencia juntas y hoy en día seguir estando presentes. Gracias por las cenitas de desconexión y días de playa (incluyendo a Estrella). Gracias Marlen por compartir los últimos años de carrera juntas y por convencerme para venir al IATA a colaborar. También por acogerme en tu casa de Berlín y por seguir manteniendo el contacto. Eres un poco responsable de esta tesis. También quiero agradecer el soporte brindado por Johanna Nater durante mis estudios de biología, ya que sin ello no se si hoy en día habría llegado tan lejos académicamente.

Gràcies també al grup de Biolegs & CIA, en especial a Marina, Víctor, Galo, Rafa i Patri. Gràcies per l'enorme amistat que tenim, també per escoltar-me tantes voltes les meues històries durant el doctorat, pel vostre suport incansable i per tots els grans moments que ens uneixen (viatges incloent la visita a Bergen, vesprades de jocs, descobriments gastronòmics, dies de cales o rius, paelles alternatives, bodes i oficiaments...). Sou genials i també la meua família a València, vos estime moltíssim.

Gràcies als meus amics de Vila-reaaal (i Asturies), per tot el seu suport i estima i per ser tan bona gent. Sabeu que "vos vuic" més que res en el món. Gràcies per escoltar-me i per estar sempre junts, pese a què ja no visquem tots a Vila. Aideta Cercós, gràcies per ser com eres una superbona persona amb eixe punt de locureta que et caracteritza, et considere una lluitadora. Gràcies per seguir mantenint una gran amistat, desde l'insti et vares convertir ja en la gran amiga que eres hui en dia. Albeta, gràcies per tots aquests anys en els quals no has deixat d'estar mai, per tots els dinarets en ta casa i pel teu afecte. Montse, gràcies per fer que siga tan fàcil estimar-te. Per la teua generositat i gran cor que et caracteritzen. També per fer sempre un esforç per veuren's i naturalment per la teua memòria eidètica que fan que no puguem esborrar els grans moments viscuts des de la infància. Lari, gràcies per ser la persona que forma part de molts dels millors moments que tinc en la ment. Per ser una amiga de les de per a tota la vida. Eres molt gran i t'admire i t'estime a parts iguals. Senc que no ens vejam i parlem tant com m'agradaria, però m'encanta quan fem les nostres quedaetes i mai canvia la nostra unió. T'estime bruixeta!

Oda, la meua germana. Gràcies per estar al meu costat des que tinc memòria, per créixer i aprendre juntes, per les hores i hores de telèfon, perquè només anant a voret fas que recarregue les piles al 100%. El teu suport per a mi ha sigut fonamental sempre i, per tant, també per a fer aquesta tesi. No seria qui soc sense tu, t'estime. Ele, gràcies per ser tan autèntica i especial com eres. Per preocupar-te per tots i cadascun de nosaltres. Per comprendrem tan bé i aplegar a ser una de les millors persones i amigues que tinc la sort de conèixer. T'estime t'estimo t'estim. Veruuu, muchísimas gracias por aparecer en nuestras vidas para ocupar un pedacito de nuestro corazón. Siempre pienso lo afortunados que fuimos todos cuando te introdujiste al grupo. En el relativo "poco" tiempo que nos conocemos has pasado a ser una persona crucial para mí. Te quiero mucho amiga. Bo xiquets ara vosaltres. Marc, gràcies per ser fill de Pacaloparte i per incorporar a Vero a la nostra vida (jeje), pero sobretot gràcies per ser un amic increïble. Eres un exemple de persona a seguir, de les que sempre veuen el got mig ple. Segur que tot el que et proposes ho aconseguiràs. Gràcies per estar sempre dispost a fer el que faça falta. Xorxe, gràcies per tots el moments viscuts (incloent viure junts i el viatge a l'illa de Man), i també per estar sempre disposat a ajudar en el que siga. Eres un gran amic, i et desitge tot el millor en el teu camí encara que implique allunyar-te. Senyor Pepe, gràcies pel teu ingeni e incansable humor per a tots. Gràcies per acollir-me sempre en la vostra casa i per formar part de molts moments genials i els que ens queden. Eres un crack, te "vuic".

Per últim gràcies a la meua família. Gràcies Bruno per escoltar-me mil vegades quan no he vist les coses clares, i també per fer-me, junt a Marta, tia d'una xiqueta tan preciosa com és la Leonor "te vuic molt". Gràcies a Mavi i Vicent, per tot el vostre suport durant tots aquests anys i per sempre estar per al que necessite. He tingut una sort immensa de poder ser família vostra junt amb Vicentica, Domingo, Vicent, Lourdes i el bonic de Guillem. Gracias mi querida tía Mª Carmen, por todo tu cariño y tu gran corazón, por transmitirme constantemente tu amor que me ha ayudado mucho en mi camino. No podia faltar l'agraïment a possiblement la millor persona que he conegut en la meua vida. Em sent enormement agraïda de haber pogut viure i créixer al seu costat, i de tot el que vaig aprendre d'ella. Gràcies pels millors records d'estius i pasqües a Estivella. Només sent afecte i gratitud de recordar-te, sense dubte aquesta tesi va dedicada a la meua estimada iaia.

I finalment, gràcies a la persona més important per a mi en aquest món. Al meu COMPANY. Company de laboratori, de projecte i, del més important, de la vida. Eres una persona excepcional i un gran científic. Gràcies per fer tan fàcil compartir-ho tot amb tu. Per aplegar a coneixer-me tant i estimar-me cada dia. Tot el teu suport ha sigut primordial per a poder afrontar aquesta tesi. Gràcies per la teua humiltat i sentit de l'humor. A més, gràcies per la teua paciència i ajuda amb el format de la tesi. Et desitge tot el millor en el teu camí i sempre estaré per ajudar-te. No imagine una vida sense tu. T'estime Rick.

“Without yeast, earth would be an alcohol-free planet

and every loaf of bread would be unleavened”

(Money 2018).

Table of contents

Abbreviations

AAA (Aromatic amino acids)

AAAH (Aromatic amino acid hydroxylases)

AADC (Aromatic amino acid decarboxylase)

AANAT (Arylalkylamine *N*-acetyltransferase)

ASMT (*N*-acetylserotonin *O*-methyltransferase)

AF (Alcoholic fermentation)

AFM (N1-acetyl-5-methoxykynuramine)

AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine)

ATP (Adenosine triphosphate)

AUC (Area under the curve)

BA (Biogenic amine)

BLA (β-lactamase enzyme)

CPRs (Cytochrome P450 reductases)

CRISPR (Clustered regularly interspaced short palindromic repeats)

COMT (Caffeic acid O-methyltransferase)

CoA (Coenzyme A)

CYPs (Cytochromes P450)

DBS (Double strand break)

DAHP (D-arabino-heptulosonate-7-phosphate)

EFSA (European food safety authority)

ELISA (Enzyme-linked immunosorbent assay)

E4P (Erythrose 4-phosphate)

ER (Endoplasmic reticulum)

F (Fluoresecence)

GC (Gas chromatography)

GCE (Glassy carbon working electrode)

GFP (Green fluorescent protein)

GMO (Genetically modified organism)

GT (Generation time)

HPLC (High-pressure liquid chromatography)

HR (Homologous recombination)

HRMS/MS (High-resolution tandem mass spectrometry)

HTE (Hydroxytyrosol-enriched extract)

IAA (Indole-3-acetic acid)

IC (Indolic compounds)

INDELS (insertions and deletions)

LAB (Lactic acid bacteria)

LC (Liquid chromatography)

LOD (Limit of detection)

LOQ (Limit of quantification)

MAO (Monoamine oxidase)

MAMS (microarrays for mass spectrometry)

MCS (Multiple cloning site)

MIs (Melatonin isomers)

MLF (Malolactic fermentation)

MS (Mass spectrometry)

NHJE (non-homologous end joining)

O2 (Oxygen)

OD (Optical density)

PAM (protospacer adjacent motif)

PC (Phenolic compounds)

PCR (Polymerase chain reaction)

PEP (Phosphoenolpyruvate)

QSM (Quorum-sensing molecule)

qPCR (Quantitative polymerase chain reaction)

RE (restriction endonucleases)

RGENs (RNA-guided endonucleases)

SAM (S-adenosyl-L-methionine)

sgRNA (single guide RNA)

SO2 (Sulfur dioxide)

SNAT (Serotonin N-acetyltransferase)

TALEN (transcription-activator-like effector nucleases)

T5H (Tryptamine-5-hydroxylase)

TDC (Tryptophan decarboxylase)

TPH (Tryptophan hydroxylase)

UHPLC (Ultra-high-pressure liquid chromatography)

USER (Uracil-specific excision reagent)

UV (Ultraviolet)

VIMP (Voltammetry of immobilized particles)

YCp (Yeast centromeric plasmid)

YEp (Yeast episomal plasmid)

YIp (Yeast integrating plasmids)

ZFN (zinc finger nucleases)

μ (Growth rate constant)

μmax (Maximum specific growth rate)

List of figures

List of tables

.

INTRODUCTION

Budding Yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, commonly known as “baker’s” or “brewer’s” yeast, is a unicellular eukaryotic organism that belongs to the *Fungi* kingdom and the phylum *Ascomycota* (due to ascospores formation for sexual reproduction) that mainly reproduces asexually by budding.

S. cerevisiae is a member of the genus *Saccharomyces* (previously included into the *Saccharomyces sensu stricto* complex) (Borneman and Pretorius 2015). This genus currently encompasses 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 and Greig 2014; Dujon and Louis 2017; Naseeb et al. 2017). Some of them with clear industrial importance, while others are confined to natural environments, such as wild forests devoid from human domestication activities (Alsammar and Delneri 2020). In addition to these species, numerous hybrids have been isolated and characterized by genome sequence analyses from different environments. These hybrids have complex genomes with contributions from two to four species of the clade (Figure I.1) (González et al. 2008; Querol and Bond 2009; Peris et al. 2012; Langdon et al. 2019).

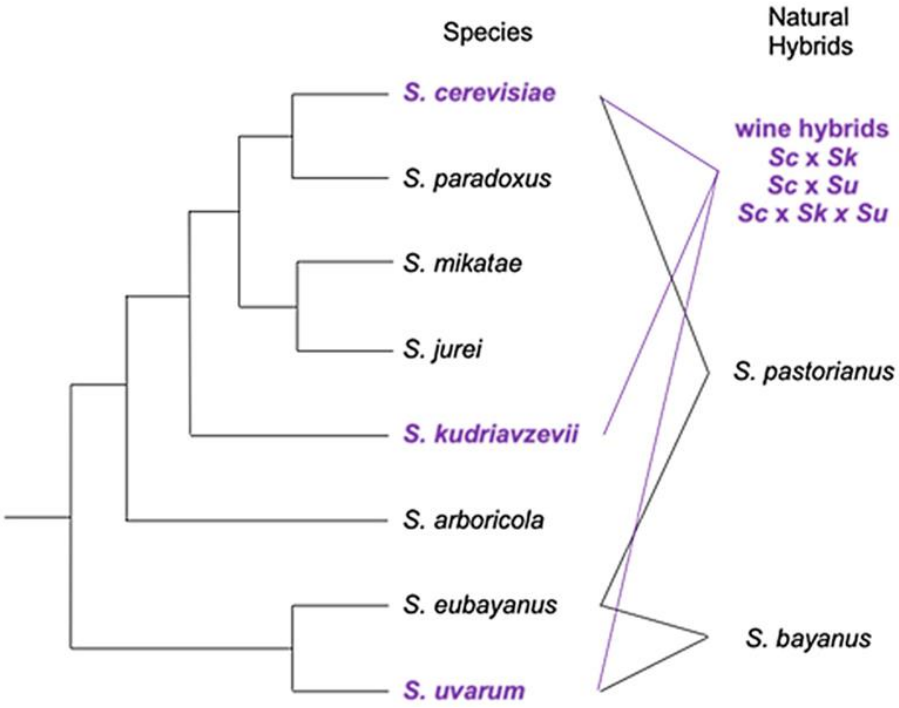

Figure I.1. Phylogenic relationship among *Saccharomyces* species and frequently isolated hybrids. Dashed lines represent the recognized hybrids and the hybrids that can be isolated from oenological environments (Vigentini et al. 2019).

Interspecific hybridization is an important and common route towards diversification and adaptation to novel niches (Gallone et al. 2019). Thus, artificial hybridization in a laboratory can be used as a suitable non GMO tool for generating new yeast strains that combines the capabilities of the parental species. The artificial hybrids obtained combine unique properties and often exhibit more robust characteristics over the parents including cryotolerance, high ethanol tolerance or unique flavor profiles in beer and wine (Belloch et al. 2008; Gibson et al. 2013; Boyton and Greig 2014; Peris et al. 2014; García-Ríos et al. 2019; Su et al. 2019; Lairón-Peris et al. 2020). The resulting hybrids are typically infertile but can reproduce asexually by budding (Gallone et al. 2019).

The lifecycle of *Saccharomyces cerevisiae*

The life cycle of *S. cerevisiae* (Figure I.2) can be composed of any of the following three different cell types: a diploid **a**/**α** or in any of the two different haploid forms, **a** or **α**. Since haploid **a** and **α** cell types can mate only with each other, cell types are also called “mating types”.

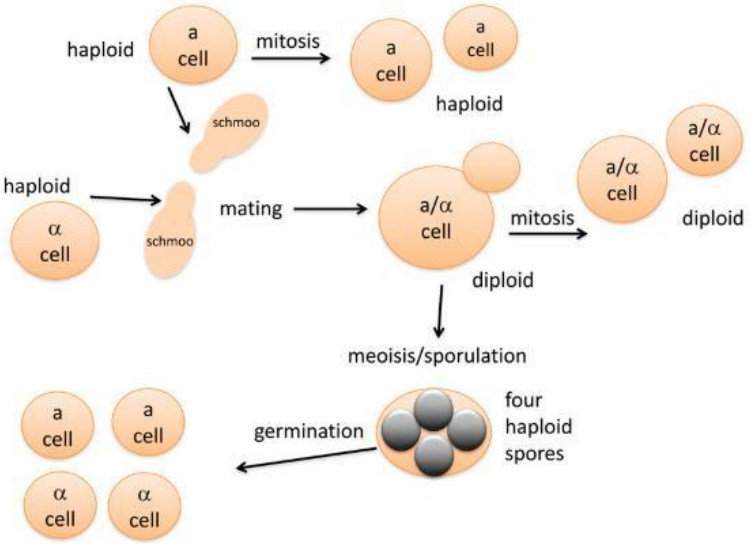

Figure I.2. **A simplified schematic representation of the life cycle of yeasts**. Haploid cells (*MAT*a or *MAT*α) undergo mitotic division through budding. The two cell types release pheromones, initiating the formation of schmoos and subsequent mating, resulting ultimately in a stable diploid *MAT*a/*MAT*α (a/α cell). Diploid cells can also undergo mitotic division producing genetically identical daughter cells or, under nutrient limiting conditions, undergo meiosis forming asci containing four haploid spores, which can germinate into two *MAT*a cells and two *MAT*α cells (Duina et al. 2014).

Each mating type **a** or **α**, encodes a G protein-coupled receptor that recognizes the pheromone released by the opposite mating type. Therefore, the **a** cells have the Ste2 receptor that recognizes the **α** factor and the **α** cells have the Ste3 receptor that recognizes the a factor (Madhani 2007). When the receptor recognizes the pheromone from the opposite mating type, the formation of "schmoos" starts and subsequent mating occurs, resulting in a diploid MAT**a**/MAT**α** (Duina et al. 2014). In addition, there is a process known as mating-type switching or cell-type switching, by which an **a** haploid cell can become an haploid **α** cell, by changing its genotype in the mating type locus (*MAT*) from *MAT***a** to *MAT***α**, or vice versa (Hanson and Wolfe 2017). In homothallic strains rapidly switching of the mating types to the opposite occurs and then the spores can fuse with the members of the population. This gene-conversion process is initiated by the *HO* gene, which encodes a site-specific endonuclease that generates a double strand DNA break and the *MAT* allele will be replaced with a copy from one of two silent loci, HMLα or HMRa (Figure I.3) (Katz Ezov et al. 2010)

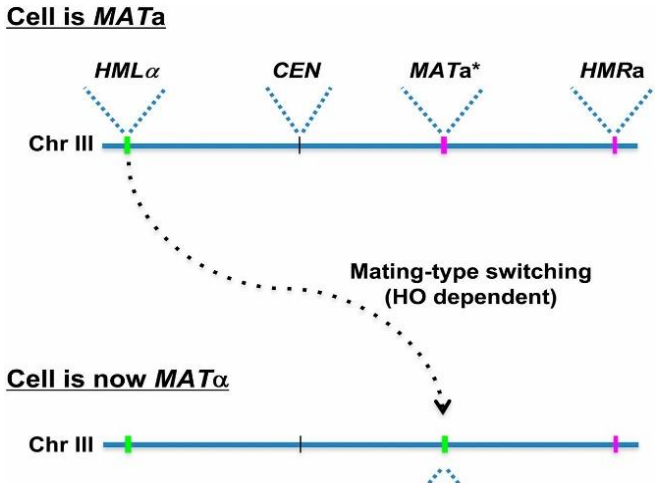

Figure I.3. Representation of mating-type switching event in *S. cerevisiae* from an haploid MATa cell to MATα where HO endonuclease is required to initiate the process. During this event the genetic information at HMLα is used to replace the MATa allele at the MAT locus with the MATα allele. The resulting chromosome III (bottom) expresses MATα information, which causes the cell to become phenotypically MAT**α**. The asterisks next to MATa and MATα highlight the fact that these alleles are actively expressed, as opposed to the alleles present at the HMLα and HMRa loci that are transcriptionally silent. (Duina et al., 2014)

In contrast, in heterothallic strains, for example most laboratory strains and many natural isolates, the two different haploid mating types remain haploid when cultured separately, due to absence of a functional *HO* gene (heterotallic strains), but can fuse when mixed together (Nasmyth 1982; Katz Ezov et al. 2010). When environmental conditions are favorable, regardless of their ploidy, the cells use the vegetative life cycle. In each round of the mitotic cycle, what is called the mother cell buds giving a new daughter cell and give rise to next generations of cells. Actually, the stage of the cycle in which the cell is found can be guessed according to the size and shape of the bud as follows: Unbudded cells are in G1 (period before DNA replication), cells with small buds are in S phase (DNA replication), and cells with large buds are in G2 or mitosis (Piekarska et al. 2010; Hanson 2018).

However, when cells are under nutrient limiting conditions (specifically starvation of nitrogen in the presence of a non-fermentable carbon source such as acetate), haploid cells enter a dormant stationary phase whereas diploid cells enter meiosis resulting in the formation of an ascus. Since sporulation requires the presence of

both *MAT***a** and *MAT***α** allele, only diploid cells whose products form the a1/α2 heterodimer, are able to sporulate (Piekarska et al. 2010). Each ascus will contain four meiotic spores that have two distinct mating types (**a** and **α**) and will germinate when favorable conditions are restored. The morphogenetic events of spore formation are due to an underlying transcriptional cascade that regulates the genes involved in specific sporulation processes (described more than 1000 regulated yeast genes during sporulation) (Piekarska et al. 2010; Neiman 2011).

Yeast kinetics growth

S. cerevisiae is a facultative anaerobe. In complete absence of oxygen (O_2), it will need the presence of anaerobiosis factors (sterols and unsaturated fatty acids) since O_2 is required for producing those compounds. The yeast growth curve in a rich medium and when the limiting nutrient is the carbon and energy source comprises five phases: lag phase (λ), exponential or log phase, diauxic phase, stationary phase and the death phase (Figure I.4).

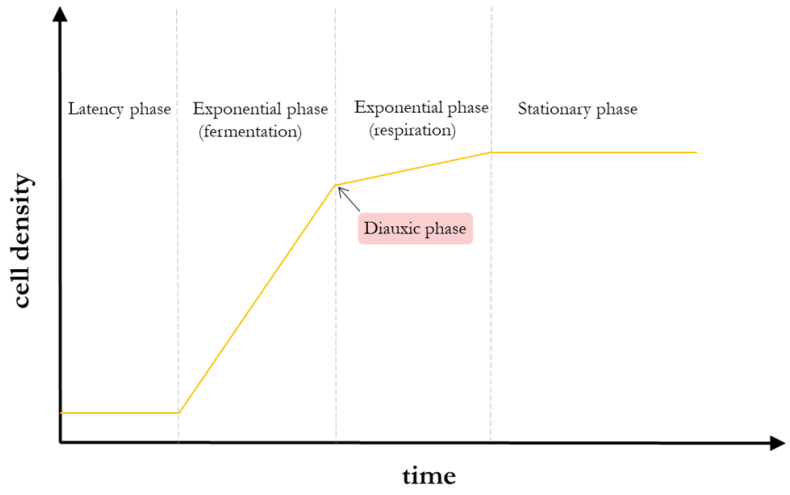

Figure I.4. Example of a typical *S. cerevisiae* yeast growth curve.

Firstly, when yeast cells are inoculated into a fresh growth medium, these enter a brief lag phase where they are biochemically active in preparation for the log phase, but there is no cell division and, therefore, no increase in cell number. The length of the lag phase can be controlled to some extent due to its dependence on the inoculum size, the growth conditions and the yeast strain characteristics (Maier 2009).

The second phase is the log or exponential phase. During exponential growth, cells reproduce at maximum specific growth rate (μ_{max}), so the rate of increase of cells in the culture is proportional to the number of cells present at a given time. The time it takes the culture to double is called generation time (GT) and it is expressed as generations/hour. The inverse of the generation time is the growth rate constant (μ), which is expressed as 1/time. In this representation, $\mu = \ln 2 / GT$.

The exponential growth of yeast can be described 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 number of cells at the beginning of the interval being analyzed (Maier 2009). Growth rates have long been used in microbiology to quantify phenotypic properties (Hall et al. 2014). The growth rate varies with respect to the yeast strain, the composition of the medium, the aeration conditions and the temperature.

In some circumstances, when glucose becomes limiting in the medium (decreasing below that needed to support μ_{max}) and O_2 is available, the cells start utilizing the ethanol, produced during the fermentation growth, which is completely oxidized to carbon dioxide. This is known as the diauxic phase or diauxic shift and it is characterized by a decreased growth rate and by changing the metabolism of glycolysis to the aerobic use of ethanol.

When the carbon source is depleted from the medium, cells enter in the stationary phase. During this phase, cells still grow and divide but there is no net growth because the number of cells that dying and dividing is balanced. The stationary phase is reached because, on one hand, carbon sources and energy have completely ended and, on the other hand, the accumulation of toxic metabolites inhibits growth. As cells approach to the stationary phase, they start accumulating storage molecules, such as glycogen, trehalose, triacylglycerols, and probably also polyphosphate, and induce autophagy. Growth on lysed cells as a source of carbon and energy is known as endogenous metabolism. With all these reserves, the cells can survive in stationary phase for long periods of time (Maier 2009;

Galdieri et al. 2010). Finally, during the death phase the population begins to fall progressively.

Functional genomics, systems biology and synthetic biology

In 1996, the first sequenced eukaryotic genome came with the publication of the genome sequence of *S. cerevisie* (Goffeau et al. 1996) and early analyses of the sequence revealed that 31% of its protein-encoding genes had clear homologs in the human genome (Mell and Burgess 2003). The next stage of the yeast genome project was to elucidate the biological function of each of the genes found. The *Saccharomyces* community organized a cooperative effort to eliminate each open reading frame (ORF) by homologous recombination and replaced each yeast gene with a "deletion cassette" that contained a drug resistance marker gene and synthetic "bar code" sequences (Winzeler et al. 1999). Other mutant libraries became available by expressing full-length, chromosomally tagged green fluorescent protein (GFP) fusion proteins (Lequin et al. 2003) as well as similar deletion library for fission yeast *Schizosaccharomyces pombe*, which also allowed for the first time the comparison of dispensability for ortholog pairs between the two yeast (Kim et al. 2010). Today the “Saccharomyces Genome Database” (SGD; http://www.yeastgenome.org/) provides information for the vast majority of yeast genes based not only on the literature, but also on the systematic study (Botstein and Fink 2011).

As important traits often arise from a combination of factors, the reductionist research of “one laboratory - one gene - one function” has reached its limits and have allowed biological research transition into the “whole-genome” era (Borneman et al. 2007). The field of “functional genomics” attempts to describe the functions and interactions of genes and proteins by making use of genome-wide approaches, in contrast to the gene-by-gene approach of classical molecular biology techniques. It combines data derived from the various processes related to DNA sequence, gene expression, and protein function, such as coding and non-coding transcription, protein translation, DNA/RNA/protein and its interactions. Together, these data are used to model interactive and dynamic networks that regulate gene expression, cell differentiation, and cell

cycle progression (Bunnik and Le Roch 2013). Thereby, by combining data from multiple whole-genome and classical sources with computational modelling, a new level of biological research has evolved as a scientific field termed “systems biology” (Borneman et al. 2007; Nielsen 2017; Yu and Nielsen 2019) (Figure 5). Finally, a new multifaceted discipline has emerged, it combines advanced biomolecular and computational sciences with information technology and engineering called “synthetic biology”. One definition of this emerging discipline is the design and construction of new biological parts (genes, promoters, terminators, transcriptional factors and their binding sequences…), devices (gene networks) and modules (biosynthetic pathways), and the redesign of biological systems (cells and organisms) for useful purposes; in addition to obtaining quantitative information for the creation of models that can predict the behavior of biological systems (Figure I.5) (Cameron et al. 2014; Pretorius and Boeke 2018; García-Granados et al. 2019). In short, synthetic biology is on a continuum with molecular biology and systems biology and entails the design and construction of novel biological systems, including complex “synthetic” organisms (Pretorius 2017). There are many synergies between metabolic engineering and synthetic biology, and the two fields need one another. Metabolic engineering emerged at the beginning of the decade of the 1990s and consists for the optimization of cellular processes, endemic to a specific organism, to produce a compound of interest from a substrate, preferably cheap and simple (Nielsen and Keasling 2011; Stephanopoulos 2012; García-Granados et al. 2019). Synthetic biology is an advanced definition of framework for design, conceptualization and manufacture of biological systems, so that genetic engineering, genomics and systems biology are used to make, control and program the biological device to create predictable properties and control the cell behavior which was previously not found in the system (Nandy and Srivastava 2018). Thus, in most cases, the design and construction of cell factories for use in industrial biology requires both synthetic biology and metabolic engineering (Figure I.5) (Nielsen and Keasling 2011).

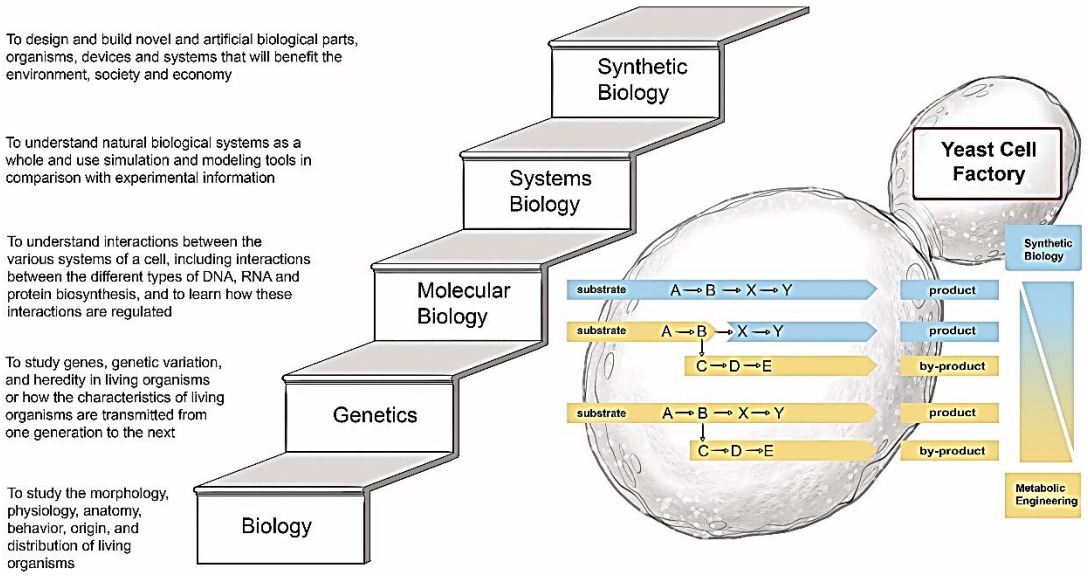

Figure I.5. The transition from biology, genetics, molecular biology, and systems biology into synthetic biology. In the right part, there is an illustration showing the overlap between synthetic biology (blue) and metabolic engineering (yellow) during the design and construction of a yeast cell factory. The first approach (totally yellow approach in the bottom) consists in a naturally producing yeast which is selected as a "cell factory" for the production of the desirable product. Typically, the flux toward the product is naturally low, but through the use of traditional, non-GM strain improvement or the use of directed genetic modifications – metabolic engineering – it is possible to increase the flux toward the product. In the second approach (when yellow and blue are combined) the yeast cell does not naturally produce the product of interest. By including a synthetically designed pathway into the yeast cell, the yeast acquires the ability to produce the product, often in small amounts initially. However, through pathway optimization the flux toward this synthetic pathway can be increased to ensure a high flux toward the product. So, this approach applies concepts from both metabolic engineering and synthetic biology. Finally, in the third approach (totally blue metabolic pathway in the diagram), a complete synthetic yeast cell could potentially be constructed such that it is dedicated to producing a desirable product. (Nielsen and Keasling, 2011; Pretorius, 2017)

Yeast as a model organism and as cell factory

Given its eukaryotic architecture yeast has several attractions as an experimental organism. Yeast is used as model organism for added knowledge about human cells. This may be possible due to the high degree of preservation of many key cellular processes between yeast and human cells (Nielsen 2019). As for yeast genes, nearly half (47%) of them can be replaced successfully by their human orthologs (Kachroo et al. 2015). Autophagy, protein translocation and secretion, degradation of proteins associated with the endoplasmic reticulum, actin-mediated cellular functions, thermal shock and protein folding and chaperone

functions are some examples of processes really suitable for study in yeasts (Nielsen 2019; Akram et al. 2020). Also *S. cerevisiae* can be useful for discovering target and off-targets effects of specifics compounds needed for drug discovery and development. Although *S. cerevisiae* is not a threatening pathogen, it has been used as a model for fungal pathogenesis as well as a valuable test bed to develop treatments (Goldstein and McCusker 2001; Parsons et al. 2006; Hanson 2018).

On the other hand, *S. cerevisiae* is a popular choice for conversion into a "cell factory". This is due to its rapid growth which contributes to its cost effectiveness and usability, together and an abundance of prior research on its physiology and metabolism. Also its excellent genetic tractability greatly facilitated by a convenient preference for homologous recombination (HR) over non-homologous end joining (NHEJ) for double stranded break (DSB) repair, which has enabled 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). All these advantages have led to the use 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 and Srivastava 2018; Yu et al. 2018; Cordente et al. 2019; Hu et al. 2019; Levisson et al. 2019; Nielsen 2019).

Genetic and molecular tools for yeast engineering

Development of strains for efficient production of chemicals and pharmaceuticals often requires multiple rounds of genetic engineering (Guirimand et al. 2020). For genetic manipulation of yeast, numerous selection marker genes have been employed, including prototrophic markers from each amino acid (e.g., *LEU2* , *TRP1*) or nucleotide base (e.g., *URA3* , *ADE2*), markers conferring drug resistance (*ble*, *hph*, *kan*), autoselection markers, and counterselectable markers (Siewers 2014) in order to maintain expression vectors for high-level production of native or heterologous proteins as well as to perform knock-out mutations or cassette insertions. *URA3* gene is the most frequently used marker and at the

same time counterselectable, which does not allow for growth in the presence of 5-fluoroorotic acid (Siewers 2014). However, the use of auxotrophic marker genes requires a host strain with a corresponding genotype. In this context it is important to highlight that laboratory strains with multiple selectable auxotrophies are available, for example BY4741 (*MATa*, *ura3*Δ*0*, *leu2*Δ*0*, m*et15*Δ*0*, *his3*Δ*1*). However, industrial strains are prototrophic, diploid, polyploid or even aneuploid and often exhibit low transformation efficiencies and lower levels of HR generally (Spencer and Spencer 1983; Stovicek et al. 2015; Gnügge and Rudolf 2017). Marker genes that confer resistance against antibiotics or other toxic compounds are best suited for use in industrial yeasts. However, the addition of these toxic compounds to the growth media prevents their use in large-scale processes due to the high price and toxicity (Pronk 2002). Besides, there are a limited number of selectable marker genes. A strategy to overcome this limitation is known as "marker recycling" and relies on flank the marker gene in order to eliminate it by recombination and it offers the possibility of reusing it, in an iterative gene deletion or gene integration approach, which extends the possibilities when it is necessary to introduce multiple genes into metabolic pathways (Alani et al. 1987; Güldener et al. 1996; Storici et al. 1999; Gueldener et al. 2002; Siddiqui et al. 2014; Choi and Kim 2018). The recombination of sequences between flippase recognition target (FRT) sites by the recombinase flippase (Flp) derived from the natural 2 µ plasmid from *S. cerevisiae* (Flp-FRTsystem) and between loxP sequences by Cre recombinase (loxP-Cre sytem) from bacteriophage P1 are the specific recombination methods most widely used (Storici et al. 1999; Gueldener et al. 2002). These systems use short sequences flanking the gene marker (34 bp) which increases the efficiency of recombination and are currently used in different approaches (Siewers 2014). For example, by Cre/loxP recombination, simultaneous and sequential integration of several genes in *S. cerevisiae* have been reported recently (Choi and Kim 2018). Despite these all advantages, this method still requires separate transformation of a plasmid encoding the galactose-inducible Cre expression cassette, induction of the Cre recombinase, replica plating to confirm excision, and curing of the Cre expression plasmid from the cells, thus extending the workflow (Siddiqui et al. 2014). In addition to being time consuming, marker removal can also cause genomic instability (Delneri et al. 2000).

In *S. cerevisiae* there are various plasmids used to introduce heterologous genes, which carry a multiple cloning site (MCS) for the insertion of expression cassette. When these plasmids can be constructed, maintained and analysed in both *E. coli* and *S. cerevisiae* are so called “shuttle vectors”. Even plasmids that can express the same open reading frame in *E. coli* and *S. cerevisiae* have been reported (Sinah et al. 2012). The vectors widely used for metabolic engineering applications are yeast episomal plasmids (YEp), yeast centromeric plasmids (YCp) and yeast integrating plasmids (YIp) (Mumberg et al. 1995; Da Silva and Srikrishnan 2012; Gnügge and Rudolf 2017). YCp and YEp are mantained extrachromosomally with few or a few dozen plasmids per cell, respectively. For their mantainace, 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 and Rudolf 2017).

YIp cannot be maintained in the cell unless they integrate into chromosomes since they lack a replication origin, and once integrated they are replicated and transmitted to successor cells as part of a chromosome (Da Silva and Srikrishnan 2012; Gnügge and Rudolf 2017). A variety of high (YEp) and low (YCp) copy as well as integrative shuttle plasmids from the pRS series (Sikorski and Philip 1989; Christianson et al. 1992), have been built and extensively used in gene function studies and in metabolic engineering and are still constantly being upgraded with additional features (Chee and Haase 2012; Gnügge et al. 2016). Depending on the final objective and as a method to regulate the general expression by controlling copy number, one or another type of plasmid will be chosen (Da Silva and Srikrishnan 2012). However, it is highly recommended to opt for integration into the genome for a more stable, robust and repeatable gene expression of interest (Jensen et al. 2014; Ryan et al. 2014; Lee et al. 2015). Nevertheless, to obtain higher levels of expression with genomic integrations, it is necessary to integrate multiple copies of each gene. To achieve this, repetitive rounds of genetic transformation into unique sites, which is time-consuming, or multiple insertions of the gene of interest by targeting for repeated sequences, such as rDNA, Ty and δ sequences, which occur naturally in the genome of the yeast, can be performed (Lopes et al. 1989; Parekh et al. 1996; Maury et al. 2016;

Semkiv et al. 2016). Recently, a new platform for multiple genes integration called "CASCADE" has been reported. (Strucko et al. 2017).The system uses a series of different yeast strains (GAS-X starter strains) which enables the integration up to 9 amplicons at defined chromosomal sites due to the preexistence of integration cassettes (GA cassettes) in their genome to be used as receptacles for these amplicons.

Since precise control of gene expression of multiple foreign or native genes is a crucial point in metabolic engineering, regulation through both, *cis*- (regions of non-coding DNA which regulate the transcription of neighboring genes) and *trans*- (DNA sequences that encode *trans*-acting factors which regulate the expression of distant genes) regulatory elements choice is another promising tool for this purpose (Da Silva and Srikrishnan 2012; Leavitt et al. 2016). Different works have demonstrated the importance of *cis* action (such as transcription promoters, 5 'and 3' untranslated regions in mRNA, and terminators) and of the *trans* action regulatory elements as endogenous (such as Aro80p) or by orthogonal implementation (such as optogenetic switches devices, or both together) (Blount et al. 2012; Da Silva and Srikrishnan 2012; Curran et al. 2015; Leavitt et al. 2016; Salinas et al. 2017, 2018; Shaw et al. 2019). Plasmids containing bidirectional promoters are a solution for efficient two gene co-expression (Vogl et al. 2018). Efforts in the characterization of these DNA sequences have contributed to obtain a catalog of standard biological parts, which are in continuous development, for yeast applications as a synthetic biology host, since it was initially for *E. coli* (Canton et al. 2008; Lee et al. 2015).

The DNA assembly and cloning phase is a key step in the pathway engineering. Traditionally cloning, involves restriction endonucleases (RE) to generate DNA fragments with specific complementary end sequences that can be joined together with a DNA ligase, prior to transformation. The main advantages of this cloning system are low cost, versatility due to different main vector options and that directional cloning can be done easily. However, it performs poorly when assembling large artificial genetic circuits because the sequential digestion–ligation–transformation–preparation steps are time-consuming and the number of available RE cleavage sites decreases with the increasing number and size of DNA sequences to be assembled (Schmid-Burgk et al. 2013). Several high-

throughput cloning methods have been developed to decrease time consumed compared to conventional cloning. For example Gateway™ cloning (Invitrogen), Gibson Assembly ® cloning (New England Biolabs), Golden Gate cloning, Biobrick Assembly ®(New England Biolabs), Infusion cloning (Clontech) 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). BioBrick Assembly involves the use of RE and BioBrick "parts". Different DNA fragments encoding proteins, promoters, ribosome binding sites, etc., have been standardized and are contained in a "parts" registry of plasmids with identical restriction sites flanking the "payload" of the part (Canton et al. 2008). All BioBrick parts are flanked in the 5′ and 3′ ends with standardized sequences for specific RE sites, which is carried by a vector. The standard sequences are known as prefix and suffix sequences and because of these sequences, it is possible to put together different BioBrick parts in order to create new parts with more complex functions than the constituents. Since RE sites with compatible ends that destroy the recognition site when joined together are chosen, the parts can be combined and the original flanking sites can be reused for the next round of assembly (Røkke et al. 2013). The main limitation of this method is the introduction of a sequence scar for each ligation event and the multiple rounds of assembly required to manufacture a device. Golden Gate Assembly exploit the ability of Type IIS RE (as *Bsa*I, *Bbs*I, *BsmB*I and *Esp3*I) to cleave DNA outside of the recognition sequence, allowing simultaneously and directionally assemble multiple DNA fragments into a single piece. Additionally, since the restriction site is eliminated from the ligated product the digestion and ligation can be carried out simultaneously, which highly decreases the time of work flow and, finally, no scar sequence is introduced (Engler et al. 2008). Gibson Assembly allows insertion of one or more overlapping DNA fragments into virtually any position of the linearized vector and does not rely on the presence of RE within a particular sequence to be synthesized or cloned. However, in order to achieve this, the technique requires, on one hand, DNA sequences to be joined together that overlap with each other, 20–150 bp. On the other hand, the following three enzymatic activities are needed: (i) T5 exonuclease, removes nucleotides from the 5′end, creating single-stranded DNA in the ends of all sequences and, thus, generates long overhangs (ii) polymerase

for filling in the gaps of the annealed single strand regions, and (iii) DNA ligase for sealing the nicks of the annealed and filled-in gap (Gibson et al. 2009; Røkke et al. 2013). Similarly, USER cloning methodology allows combining multiple PCR fragment assembly by only 6-10 bases of homology between the neighboring fragments (Figure I.6). Since the primers contain a single deoxyuracil residue (dU) flanking the 3', the method requires a proof-reading DNA polymerase able to tolerate uracils present in DNA templates during the PCR amplification. Subsequent treatment of the PCR products with USER™ enzyme mix (DNA glycosylase and DNA glycosylase-lyase Endo VIII) creates a single nucleotide gap at each location of dU resulting in PCR fragments flanked with single-stranded extensions that allow seamless and directional assembly of customized DNA molecules into a linearized vector (Geu-Flores et al. 2007).

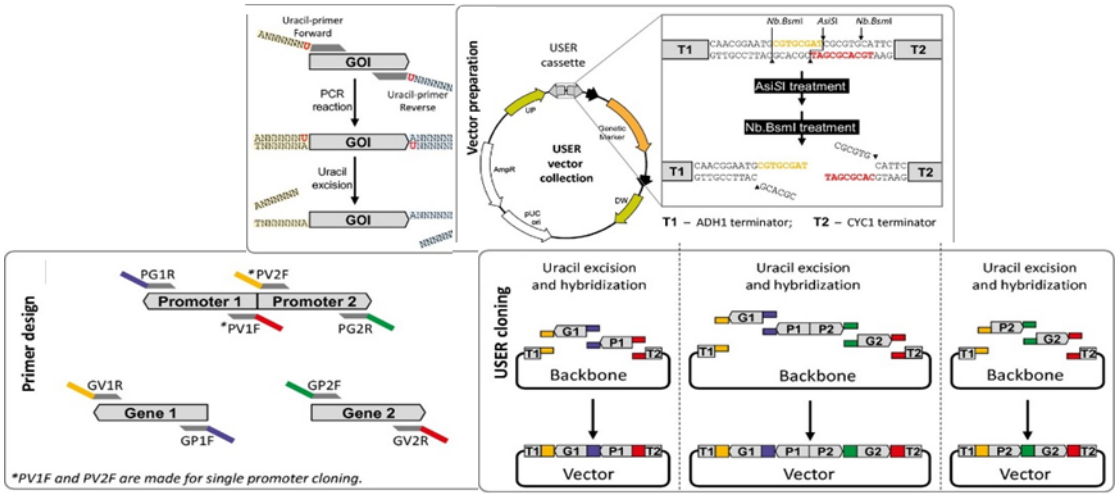

Figure I.6. Overview of the procedure for cloning with USER methodology. In this case the vector employed consist in a EasyClone vectors containing an USER cassette, but any vector can be prepared using uracil-containing primers as is used in this example for amplification of any gene of interest (GOI), or promotorer (adapted from Jensen et al. 2014).

Some of these assembly or cloning methods have been the basic technique for development of cloning vector sets for yeast that are really interesting to decrease the turnaround time in the metabolic engineering cycle. For example, cloning vectors containing USER cassettes for multiple and simultaneous chromosomal integrations in *S. cerevisiae* with a wide repertoire of LoxP-flanked selection markers has become available under name Easyclone, Easyclone 2.0 and EasycloneMulti (Jensen et al. 2014; Stovicek et al. 2015; Maury et al. 2016). Similarly, a Modular Cloning system, based on Golden Gate technology has been

developed under the name MoClo-YTK (https://www.addgene.org/kits/moclo-ytk/). This MoClo-derived assembly enables great versatility as it allows multiple fragment types assembly in few steps. Additionally, a set of promoters, terminators, as well as protein degradation tags, have been characterized for MoCLo kit (Lee et al. 2015).

All the limitations for genetic engineering seem to be left behind because of the applicability and use of different developed programmable nucleases. This includes, zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs) and cluster of regularly interspaced palindromic repeats-associated protein 9 (CRISPR–Cas9) RNA-guided endonucleases (RGENs) (Kim 2016). Among all these nucleases, CRISP-Cas9 RGENs is the most widely used editing tool due to its customizable ability by the replacement of guide RNAs (Kim 2016; Vigentini et al. 2019). Three types of CRISPR mechanisms have been identified, being type II bacterial CRISPR system the most studied (Figure I.7). In this system, the nuclease Cas9 is guided by single-guide RNA (sgRNA) to a cleavage site in the double-stranded DNA, which must contain a protospacer adjacent motif (PAM), which is composed of any of the four bases followed by two guanines (Mali et al. 2013). After cutting, this DNA damage can be repaired, through the double strand break (DSB) repairing mechanism, either via non-homologous end joining (NHEJ) or homologous repair (HR). If DSB are repaired via NHEJ, insertions and deletions (INDELs) may be introduced that can disrupt gene function since this repair pathway is error-prone. Alternatively, if a donor template with homology to the targeted locus is supplied, the DBS may be repaired through HR pathway. If the donor used is almost identical to the sequence in the proximity of the cutting site except for some nucleotides the kind of change obtained will be point mutations or non-sense mutations, to induce an amino acid change or to insert a stop codon. Instead, if the donor brings the gene of interest flanked by homologous sequences adjacent to the cutting site, the insertion of the gene of interest will be obtained (Vigentini et al. 2019).

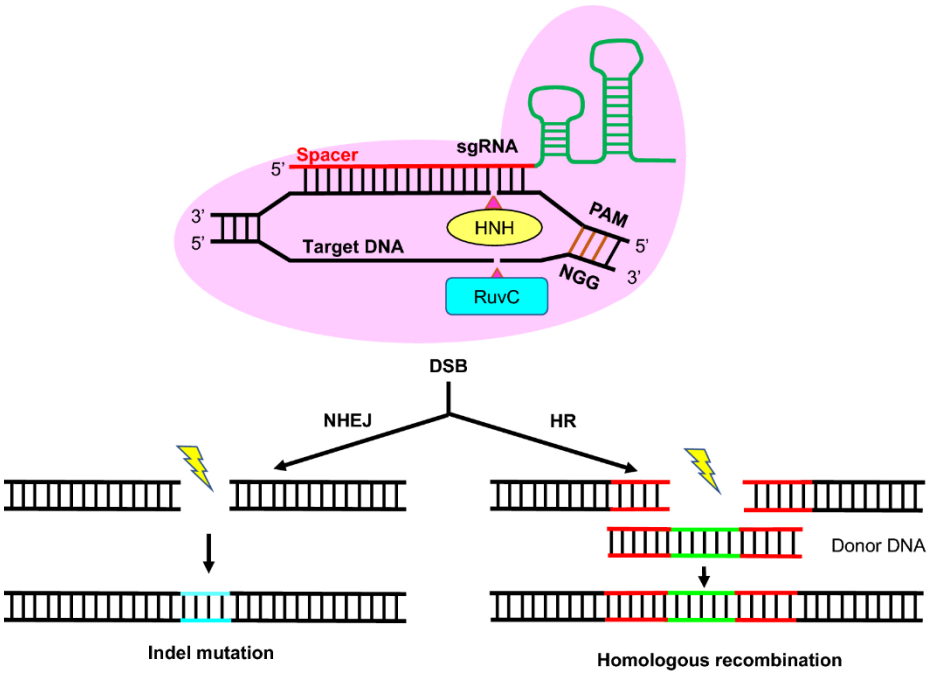

Figure I.7 Overview of the CRISPR–Cas9-mediated genome editing system. The Cas9 and sgRNA form a complex *in vivo* and then bind on the target DNA sequence upstream of PAM sequence. The Cas9 nuclease domain HNH then cleaves the target DNA sequence complementary to the 20 bp guide sequence, while RuvC domain cuts another DNA strand, forming a DSB. DSB must be repaired via either NHEJ or HR immediately to avoid cell death (Cai et al. 2019)

Since DiCarlo et al. (2013) reported the use of this system as a genome-editing tool in yeast, a diverse number of novel strategies have been developed for Cas9 and guide RNA expression, allowing highly efficient genome editing that can be multiplexed without the need for a marker (Stovicek et al. 2017). Design, expression and delivery of the sgRNA components are crucial parameters for successful CRISPR/Cas9 engineering whereas the form of Cas9 expression does not seem to be a critical parameter. Therefore CRISPR/Cas genome editing and transcriptional regulation are particularly suitable for developing yeast cell factories (Stovicek et al. 2017; Vanegas et al. 2017; Mitsui et al. 2019). Likewise, CRISPR-Cas9 is an excellent tool for developing of one step multiple-auxotrophic wine and industrial yeast strains, for further genetic modifications, as iterative gene targeting can then be performed without need for marker recycling (Bao et al. 2015; Lee et al. 2015; Jessop-Fabre et al. 2016; Liu et al. 2016) . It is worth mentioning that, despite being the most convenient and powerful tool, it is still in

development, with new strategies emerging to achieve maximum efficiency (Bao et al. 2015). This is crucial, for example, when working with a large number of genes to be modified and in strains that are not haploid.

Finally, it should be mentioned that only a small number of yeast strains (most often limited to *S. cerevisiae*) has been used for novel cell factories. Although this model organism is indeed advantageous for all the reasons discussed above, production yields can vary significantly within strains in the same species (Suástegui et al. 2016). Furthermore, sometimes they may not be suitable for a wide range of biotechnological applications that could easily be achieved through the use of an unconventional cellular host (Nora et al. 2019). Therefore, studies aiming to develop or implement molecular toolboxes for novel microbial chassis are emerging (Stovicek et al. 2017; Rantasalo et al. 2018; Raschmanová et al. 2018; Cai et al. 2019; Guirimand et al. 2020).

Wine fermentation

Wine fermentation is defined as the biotransformation of grape nutrients present in the must into ethanol, carbon dioxide (CO_2) and secondary metabolites (Figure I.8). This transformation of grape juice into wine is essentially a microbial process.

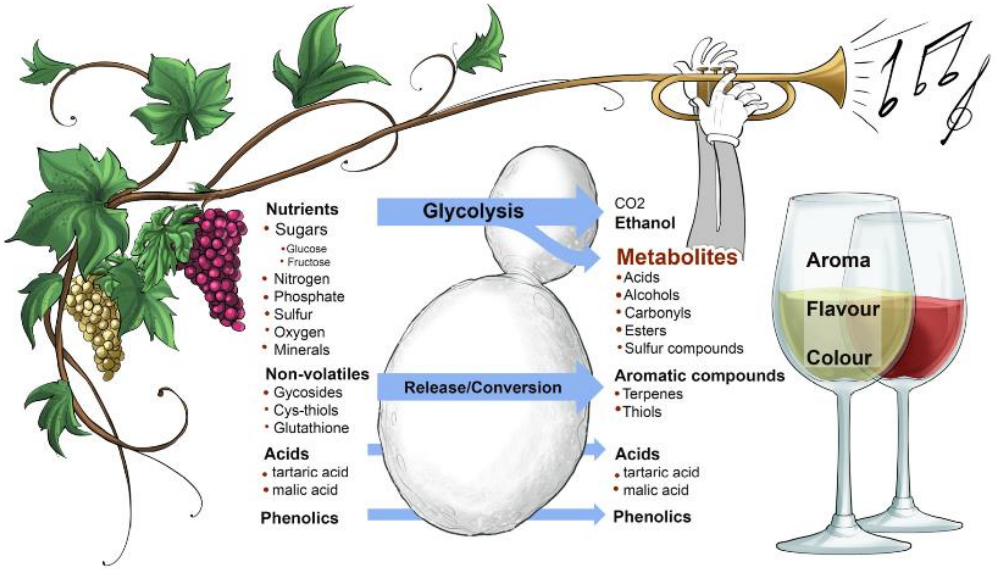

Figure I.8. The metabolic conversion of grape juice to wine by the action of wine yeast. Grape nutrients are converted into ethanol, carbon dioxide and secondary metabolites through the glycolytic metabolism of wine yeast (Pretorius, 2016).

In fact, wine fermentation is a special complex mix of chemistry, biology and culture, where microorganisms play a critical role (Belda et al. 2017). Over time, wine has influenced geography, economics, archeology, history, mythologies and religions, arts and traditions, law and medicine. Today, this beverage has a unique place in most societies, with tremendous cultural value and high commercial importance (Marsit and Dequin 2015).

Yeast diversity during wine fermentation and their interactions

It was not until the invention of the microscope followed by the pioneering scientific work of Louis Pasteur in the second half of the 19th century that yeast was identified as a living organism and the agent responsible for alcoholic fermentation (AF) and dough leavening (Pasteur 1858).

Although AF depends almost exclusively on yeast, other organisms, including bacteria and mold, are naturally present in grapes during harvest (Lonvaud-Funel 1999). Regarding to yeast, many species are present during the different stages of winemaking process (Figure I.9). The diversity of yeast encompasses more than 20 different genera that have been included in both Ascomycota and Basidiomycota phyla and are named as "non-*Saccharomyces*" yeast; *e.g*: *Aureobasidium*, *Auriculibuller*, *Brettanomyces*, *Bulleromyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Issatchenkia*, *Kazachstania*, *Kluyveromyces*, *Lachancea*, *Lipomyces*, *Metschnikowia*, *Phaemoniella*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Sporidiobolus*, *Sporobolomyces*, *Starmerella*, *Torulaspora*, *Wickerhamomyces*, *Yarrowia*, *Zygoascus*, and *Zygosaccharomyces* (Fleet 2003, 2008; Padilla et al. 2016). Most of the non-*Saccharomyces* species are present in vineyard and in the winery environment and the composition varies according to several factors such as location, climatic conditions, cultivar, presence of pesticides, agronomic practices, stage of ripening and health of the grapes, harvesting procedures, ability to form a biofilm on solid surfaces and specific environmental conditions in the must (Pretorius et al. 1999; Jolly et al. 2014; Bagheri et al. 2015; Varela and Borneman 2017; Abdo et al. 2020).

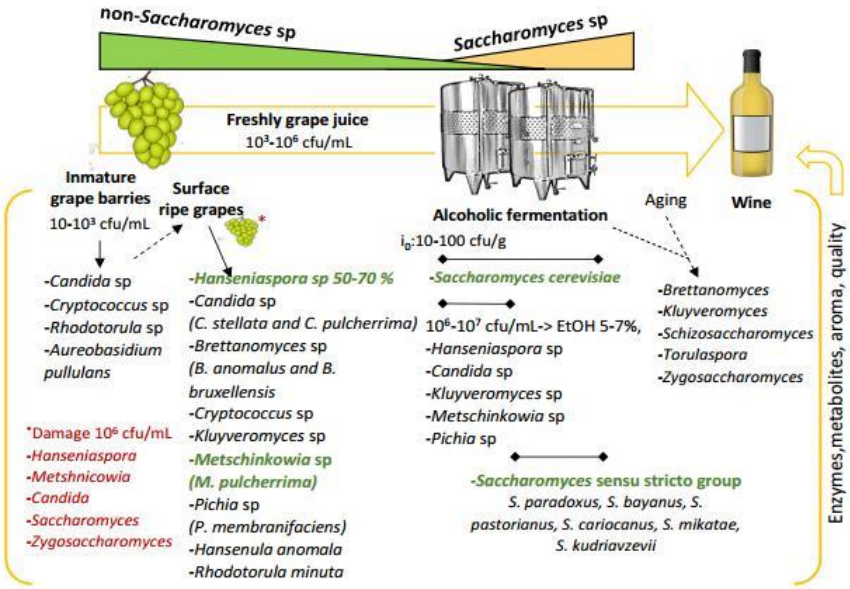

Figure I.9. Sequential presence of different yeast during the winemaking process.

Despite the complex wine microbial ecology in the initial stages of fermentation, *S. cerevisiae* is almost invariably the dominant yeast in the latter stages of natural wine fermentations due to its fermentative behavior, ethanol tolerance and sulfur dioxide (SO_2) resistance (Figure I.9) (Pretorius et al. 1999; Fleet 2008; Bagheri et al. 2015). After the AF a secondary fermentation, known as malolactic fermentation (MLF), which is carried out by lactic acid bacteria (LAB), is often undertaken. LAB are Gram positive, microaerophilic and characterized by a unique enzymatic decarboxylation of the L-malic acid to L-lactic acid. Usually, MLF is uniformly done for all red wine and, by contrast, it is uncommon in white wine making, except for the production of certain styles of Chardonnay and sparkling wines. Due to MLF, wine is microbiologically more stable (lowering the risk of gas production in bottled wine), as well as more aromatic, flavored and complex and less acidic (Pretorius 2016). The four genera usually found in wine are *Lactobacillus, Pediococcus, Leuconostoc, and Oenococcus. Oenococcus oeni* has special characteristics that make them highly relevant for vinification, specifically because of its ability to grow and perform in the low acidic wine environment (pH ≤ 3.5) and capacity to survive in high ethanol, SO_2, nutrient shortage and low temperature. Also, some *Lactobacillus plantarum* isolates have also displayed the ability to survive harsh wine conditions and there are

commercial cultures of this LAB species (Bravo-Ferrada et al. 2013; Bartowsky 2017). Despite this, even with starter cultures the MLF could be slow or incomplete. Residual malic acid present in wine carries a risk of spoilage microorganisms growing to produce haze, off-odors, and/or dissolved carbon dioxide (CO_2) in the bottle as well as biogenic amines (BAs) that can impose health risks to consumers of fermented products (Betteridge et al. 2015; Pretorius 2016). For example, the use of selected yeast combined with selected LAB can be presented as biological alternative to avoid the *B. bruxellensis* presence in wines, which leads to constant economics losses in wine industry (Berbegal et al. 2018).

Yeast strains used not only in winemaking but also in modern bakery and brewing, have been carefully cultivated, selected and purified and each strain has its own particular and unique characteristics. 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. This practice aims to obtain wines that are homogeneous in terms of quality and at the same time avoid problems of sluggish or stuck fermentation and spoilage of the final wine (Lleixà et al. 2016). However, winemaking with monocultures of pure yeast leads to a greater microbiological control but can generate wines which lack the complexity of flavor. Intra- or inter- specific hybridization, as mentioned above, is one of the tools that allow to expand the set of strains available to improve the diversity in the taste of the wine with respect to the parental species that form the hybrid (Belloch et al. 2008; Fleet 2008).

Non-*Saccharomyces* yeasts were considered initially to be of secondary significance or even spoilage yeasts during the wine production (Padilla et al. 2016). However, nowadays several studies have been shown that some non-*Saccharomyces* strains have positive contributions to desirable winemaking properties such as the aroma complexity, the decrease in the ethanol content and as controlling the growth of other wine spoilage microorganisms among others (Comitini et al. 2011; Jolly et al. 2014; Quirós et al. 2014; Ciani and Comitini 2015; Alonso-del-Real et al. 2017; Bellut and Arendt 2019). The realization that yeasts other than *Saccharomyces* species are ecologically and metabolically significant in the fermentation of wine, has brought a new research topic in which these new

species are studied as well as their co-inoculation and interaction with different yeast starter cultures. However, determining the aromatic profile of a wine using selected mixed-starter cultures of yeast or bacteria cannot be effective without understanding how microorganisms interact with each other (Liu et al. 2017). In this context, yeast interactions have a fundamental role to obtain the desired product characteristics since these interactions can affect the metabolite production and/or the microbial growth of the microorganism involved (Ciani and Comitini 2015; Rollero et al. 2018) (Figure I.10). To control and optimize the complex mixed fermentation, further knowledge about physiological and metabolic interactions between *Saccharomyces* and non- *Saccharomyces* wine yeast, as well as between yeast and LAB during MLF, is needed. Different yeast strains or species can be involved in several interactions through the production of toxic compounds, or as a result of competition for nutrients which can lead to determining variation in the gene expression and enzymatic activity (Pretorius 2000; Alexandre et al. 2004; Milanovic et al. 2012; Rantsiou et al. 2012; Rossouw et al. 2012; Branco et al. 2014; Curiel et al. 2017; Pérez-Torrado et al. 2017). Proteomic and transcriptomic patterns analyses as well as the analytical profiles of wines obtained during pure and co-cultures have been contributed to elucidate metabolic interactions in mixed fermentations (Barbosa et al. 2015; Curiel et al. 2017; Pérez-Torrado et al. 2017; Alonso-del-Real et al. 2019; Peng et al. 2019). Interestingly, cell aggregation (co-flocculation) between different yeast species has recently been proposed as, a complementary mechanism that could govern population dynamics during AF (Rossouw et al. 2015; Pérez-Torrado et al. 2017). Thereby, AF is an ideal process to study biochemical and ecological interactions between microorganisms. Untargeted metametabolomics revealed synergistic relationships, exchanges of metabolites and cell-to-cell signaling between species within a community (Raes and Bork 2008). Metametabolomics combined with wine microbiome analysis further allows the identification of yet unknown metabolite markers through networks-based approaches (Liu et al. 2017). Metabolite production modification by microbial role-players has been already shown by metabolic footprint of monoculture, mixed cultures or blended wines studies which has been shown clear differences (Howell et al. 2006; Peng et al. 2018; Rollero et al. 2018). With all the information obtained, winemakers will dispose of rational selection of mixed starters combining both non-

Saccharomyces and *Saccharomyces* and the conditions to positively interfere with the growth and fermentation behavior of the yeast species, allowing the introduction of the new desired characteristics in the final wine (Fleet 2003, 2008; Viana et al. 2008; Sadoudi et al. 2012; Ciani and Comitini 2015) (Figure I.10).

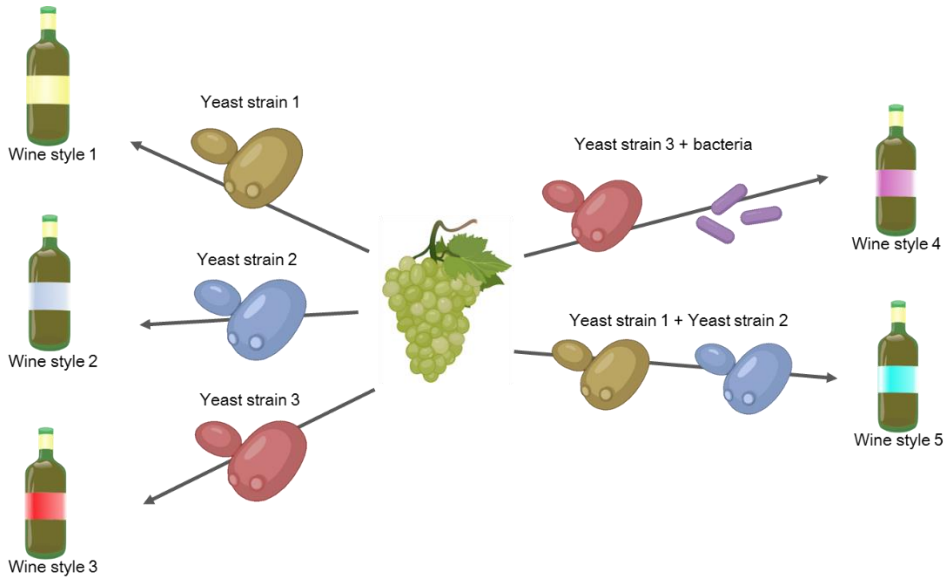

Figure I.10. Representation of the effect of the yeast strain used during the winemaking process on the final wine. As shown, different styles of wine can be obtained by fermentation of identical grapes but with different strains of yeast (shown as yeast strains 1, 2 and 3) and combined or not with bacteria.

Carbon metabolism in yeast

In terms of biochemistry, AF is the conversion of sugar, glucose and fructose, into alcohol and CO_2 at both anaerobic and aerobic conditions. Approximately 90 ~ 95% of sugars present in the must are converted into ethanol and CO_2, about 4 ~ 5% into secondary metabolites such as higher alcohols, acids, esters and glycerol, and finally 1 ~ 2% is used for cell growth and maintenance.

During AF, yeast metabolizes the six-carbon compounds to pyruvate via glycolysis. During this process, energy is produced at substrate-level phosphorylation generating only two adenosine triphosphate (ATP) molecules and NADH. Pyruvate is then decarboxylated to acetaldehyde through pyruvate decarboxylase (*PDC1*, *PDC5*, *PDC6*), which is reduced to ethanol by alcohol dehydrogenase (*ADH1* and *ADH2*). In this last step, acetaldehyde serves as

electron acceptor which allow the re-oxidation of the glycolytic NADH (Piškur et al. 2006).

Respiration consists in the oxidation of the pyruvate from the glycolysis into CO_2 by the tricarboxylic acid cycle, leading to the production of NADH, which is re-oxidized while generating ATP by oxidative phosphorylation, using O_2 as the terminal acceptor of electrons. As mentioned, fermentative metabolism generates only two ATPs per glucose molecule, compared to 38 ATPs during aerobic oxidation (Pfeiffer and Morley 2014).

When yeast cells are exposed to air (O_2), what is known as the “Pasteur effect”, glucose consumption is reduced by slowing the glycolytic flux. The regulation of this phenomenon is due to the fact that, on the one hand, pyruvate decarboxylase (involved in fermentation) has a lower affinity for pyruvate than the pyruvate dehydrogenase and, on the other hand, phosphofructokinase is inhibited by the ATP generated during respiration, and phosphorylated hexoses accumulate, which in turn decrease the transmembrane transport of sugars (Ribéreau-Gayon et al. 2006). In *S. cerevisiae*, the Pasteur effect occurs in aerobic sugar-limited chemostat cultures and in resting-cell suspensions because of low sugar consumption rates (Ishtar Snoek and Yde Steensma 2007).

A fundamental physiological characteristic of wine yeasts is their ability to ferment sugars at both anaerobic and aerobic conditions (Piškur et al. 2006). 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. In contrast, “Crabtree-negative” yeasts lack fermentative products, and under aerobic conditions, biomass and carbon dioxide are the sole products. Crabtree effect results in lower biomass production because most sugar is converted into ethanol. This means that more glucose has to be consumed to achieve the same yield of cells if comparing with Crabtree-negative yeasts. Because only a fraction of sugar is used for the biomass and energy production, this could theoretically result in lower growth rate in Crabtree-positive yeasts and these could then simply be out-competed by Crabtree-negative yeasts and other microorganisms. However, ethanol could be used as a tool to

slow down and control the proliferation of other competitive microorganisms (Dashko et al. 2014).

Yeast Nitrogen metabolism during alcoholic fermentation

After carbon compounds, nitrogen compounds are the second most important nutrient in wine. Thus, nitrogen is an essential nutrient for yeast during wine fermentation and it plays a predominant role during AF, influencing both the fermentative process and wine quality (Bell and Henschke 2005; Gobert et al. 2019). With regard to AF progress, nitrogen is a key limiting factor for biomass production, fermentation kinetics and fermentation duration. In *S. cerevisiae*, the main sources of yeast assimilable nitrogen (YAN) are ammonium (around 30-40%), which represents a significant proportion of nitrogen sources, and amino acids. Insufficient nitrogen source availability in must can lead to stuck or sluggish fermentations (Varela et al. 2004; Bell and Henschke 2005). Thereof, nitrogen sources have been classified in terms of supporting growth rates and fermentation activity as “preferential or good nitrogen sources” and “non-preferential or poor nitrogen sources” depending on the AF conditions, strain used and classification method (Gobert et al. 2019). Despite variations in the classification of nitrogen sources, ammonia, asparagine, and glutamine have been significantly more often classified as preferred sources of nitrogen (Gobert et al. 2019).

In *S. cerevisiae*, YAN is transported into the cell by various specific or non-specific permeases. Ammonia is transported by uniport systems consisting of three ammonium permeases with different affinities, with *Mep2p* having the highest affinity followed by *Mep1p* and *Mep3p* (Marini et al. 1997). Amino acid permeases are active symport systems with different selectivity for amino acids. The general amino acid permease, Gap1, allows the transport of all amino acids whereas others permeases transport only one or a group of amino acids. There are two mechanisms by which *S. cerevisiae* is able to select the nitrogen source for a better growth through the regulation of broad-specificity permeases. The first consists in an amino acid-signaling pathway mediated by the plasma membrane localized Ssy1-Ptr3-Ssy5 (SPS)-sensor (Ljungdahl 2009; Ljungdahl and Daignan-Fornier 2012). The second is called "Nitrogen Catabolite Repression" (NCR) (Wiame et al. 1985; Magasanik 1992). Currently, the amino

acid permeases: *GAP1*, *CAN1*, *PUT4*, *DIP5*, *UGA4*, and the ammonium permeases: *MEP1*, *MEP2*, *MEP3*, are known to be under the control of this regulatory system (Ljungdahl and Daignan-Fornier 2012). The ammonium permeases are only expressed under low concentrations of ammonium, because they are not essential for growth under high concentrations of ammonium (> 20 mM), and are repressed in the presence of good nitrogen sources due to the NCR (Marini et al. 1997). Conversely, general de-repression of the genes regulated by the NCR system leads the cell to nonspecifically use other sources of nitrogen in the absence of a preferential nitrogen source (Gobert et al. 2019). Some recent works have been shown a similarity on the nitrogen source uptake between *S. cerevisiae* and some non-*Saccharomyces* strains, suggesting that similar regulatory mechanisms like NCR and SPS could also be present in the these species (Lleixà et al. 2019; Su et al. 2020). In *S. cerevisiae* the phenotypic diversity concerning YAN preferences correlates with the presence of genetic variants (Gutiérrez et al. 2013; Cubillos et al. 2017).

Once transported through the respective permease and internalized, nitrogenous compounds can be used directly in biosynthetic processes, be deaminated to generate ammonium, or serve as substrates of transaminases that transfer amino groups to α-ketoglutarate to form glutamate (Ljungdahl and Daignan-Fornier 2012). Ammonia is incorporated during the formation of glutamate from α-ketoglutarate by NADPH-dependent glutamate dehydrogenase (*GDH1*), and glutamine from glutamate by glutamine synthetase (*GLN1*). *S. cerevisiae* is able to synthesize all L-amino acids from appropriate source of carbon and ammonium. Moreover, when glutamine is the only source of nitrogen, a glutamate synthase (*GLT1*) metabolizes glutamine in glutamate. From this glutamate, ammonium can be released by glutamate dehydrogenase (*GDH2*) (Ljungdahl and Daignan-Fornier 2012). All these reactions form the so-called Central Nitrogen Metabolism (CNM) (Ter Schure et al. 2000).

With regard to the role of nitrogen on wine quality, the catabolism of nitrogen sources is involved in the synthesis of fermentative aroma precursors in wine yeasts (Styger et al. 2011). Then, the amino acid composition in the wort as well as in the grape have an impact in the flavor of beer and wine (Hernández-Orte et al. 2002; Ferreira and Guido 2018). Yeast contribute to the wine aroma in different

ways, not only by *de novo* biosynthesis of aromatic compounds but also converting flavor-neutral grape compounds into active aromatic and flavor compounds, or regulating the growth of malolactic and spoilage bacteria (Fleet 2003; Styger et al. 2011). Among the aromatic compounds generated or released by yeast or its enzymes, ethyl and acetate esters, volatile fatty acids, higher alcohols, monoterpenoids and volatile sulfur compounds have been reported (Cordente et al. 2012; Gobert et al. 2019). Deciphering the link between nitrogen metabolism and volatile compound synthesis will provide useful information with potential means to modulate wine flavour and magnify the different sensory profiles (Vilanova et al. 2007; Gobert et al. 2019). To this aim, the experimental conditions in the different studies (matrix, initial sugar concentration, initial nitrogen concentration, yeast strain used, yeast culture conditions before inoculation...) must be standardized to be able to observe any trend (Rollero et al. 2018; Gobert et al. 2019). However, a recent study demonstrates that the nitrogen addition is mainly catabolized and involved in the *de novo* synthesis of proteinogenic compounds but not in the formation of volatile compounds (fusel organic acids, fusel alcohols, and their acetate-ester derivatives) (Crépin et al. 2017). Also, in this study the authors reported that α-keto acid precursors required for the *de novo* synthesis come from the catabolism of sugars, with a limited contribution from the anabolism of consumed amino acids (Crépin et al. 2017).

Yeast metabolism of aromatic amino acids during alcoholic fermentation

The aromatic amino acids (AAA) (L-tryptophan, tyrosine and phenylalanine) present in grape must are, together with other amino acids (except proline), ammonium ions and small peptides, sources of so-called YAN (Mas et al. 2014). Some YAN sources have been reported to be involved in the formation of volatile compounds in both *Saccharomyces* and non-*Saccharomyces* (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). The higher alcohols, also known as fusel alcohols, tryptophol, tyrosol and 2-phenylethanol derive from the AAA L-tryptophan, tyrosine and phenylalanine respectively (Figure I.11). The yeast synthesizes these aromatic alcohols via the degradative pathway of Ehrlich, which consists of

a metabolic conversion of the AAA through the three successive steps: transamination, decarboxylation and reduction (Figure I.12) (Ehrlich 1907).

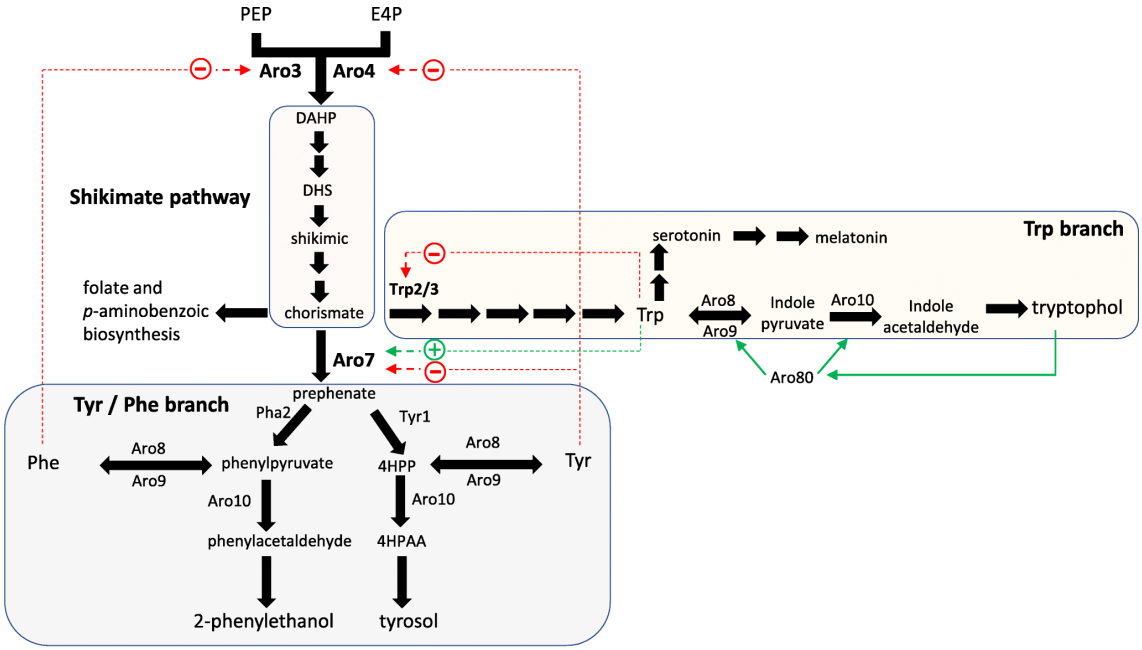

Figure I.11. Pathways associated with the metabolism of aromatic amino acids in *S. cerevisiae*. Dotted red lines indicate the major allosteric checkpoints in the pathway: inhibition of Aro3p by L-phenylalalnine (Phe) and Aro4p and Aro7p by L-tyrosine (Tyr). In addition, Aro7p is activated by L-tryptophan (Trp) (green dotted lines). The biosynthesis of aromatic amino acids starts with the condensation of two glucose-derived metabolites erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) and followed by a series of enzymatic reactions which lead to the formation of chorismate (shikimate pathway). Then, chorismate can enter the Trp biosynthesis branch or converted by Aro7p into prephenate, the last common precursor for Tyr/Phe biosynthesis. Tryptophol, tryptophan and cell density up- regulate the expression of *ARO9* and *ARO10*, which is dependent on the transcription factor Aro80p (green lines). DAHP: 3-deoxy-D-arabinoheptulosonate-7-phosphate; DHS: dehydroshikimate, 4HPP: 4-hydroxyphenylpyruvate, 4HPAA: 4-hydroxyphenylacetaldehyde. (adapted from Cordente et al., 2019)

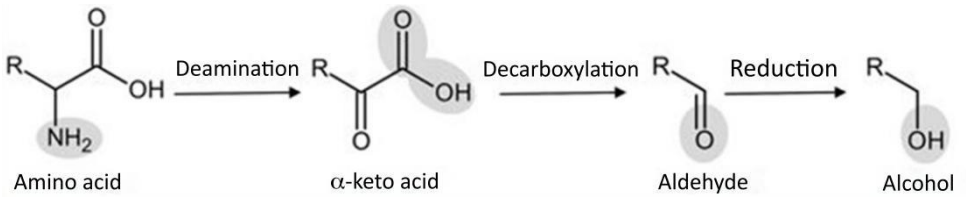

Figure I.12. Ehrlich pathway (El-Dalatony et al., 2019).

In the first stage of the route, these AAA are deaminated by amino acid transferases (*ARO8*, *ARO9*) generating the intermediate α-keto acids. Sugar catabolism is another way to generate *de novo* precursor, the α-keto acid, which contributes to the production of volatile compounds (Crépin et al. 2017). The decarboxylation of the α-keto acid to form a 'fusel aldehyde' in the second step of the pathway are conducted by the three pyruvate decarboxylase isozymes (*PDC1*/*5*/*6*) as well as by the phenylpyruvate decarboxylase (*ARO10*). Finally, aldehyde is reduced by six different alcohol dehydrogenases isozymes (*ADH1*-*6*) and by the bifunctional alcohol dehydrogenase and formaldehyde dehydrogenase (*SFA1*). In addition to the AAA, the branched-chain amino acids valine, leucine, isoleucine and the sulfur-containing amino acid methionine, are catabolized in the same way via the Ehrlich pathway. The amount of higher alcohols produced from fermentation depends on the quantity of available nutrients (nitrogen) and also on the genus, species, and strain (El-Dalatony et al. 2019). It should be mentioned that in aerobic glucose-limited chemostat cultures grown with several amino acids as sole nitrogen sources, the "fusel aldehyde" undergo oxidation, instead of reduction, forming fusel acids (Hazelwood et al. 2008).

With respect to the anabolism of AAA, yeasts are capable of producing all three AAA (L-tryptophan, phenylalanine and tyrosine) from the precursor chorismate through the shikimate pathway. The shikimate pathway consists of 7 sequential catalytic steps and begins connecting the shikimate route to the central carbon metabolism 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) (Figure I.11) (Gientka and Duszkiewicz-Reinhard 2009).

The importance and impact of AAA in wine fermentation is high. On the one hand, the precursors of higher alcohols through the Ehrlich pathway are generated. On the other hand, there are minority molecules also produced through AAA metabolism that are worth taking into account, as they could be bioactive molecules with interesting properties and benefits when consumed (Mas et al. 2014). These molecules are known as bioactive compounds and encompass the

tryptophan-derivate and tyrosine-derivative compounds, which are detailed in the following sections (Figure I.13).

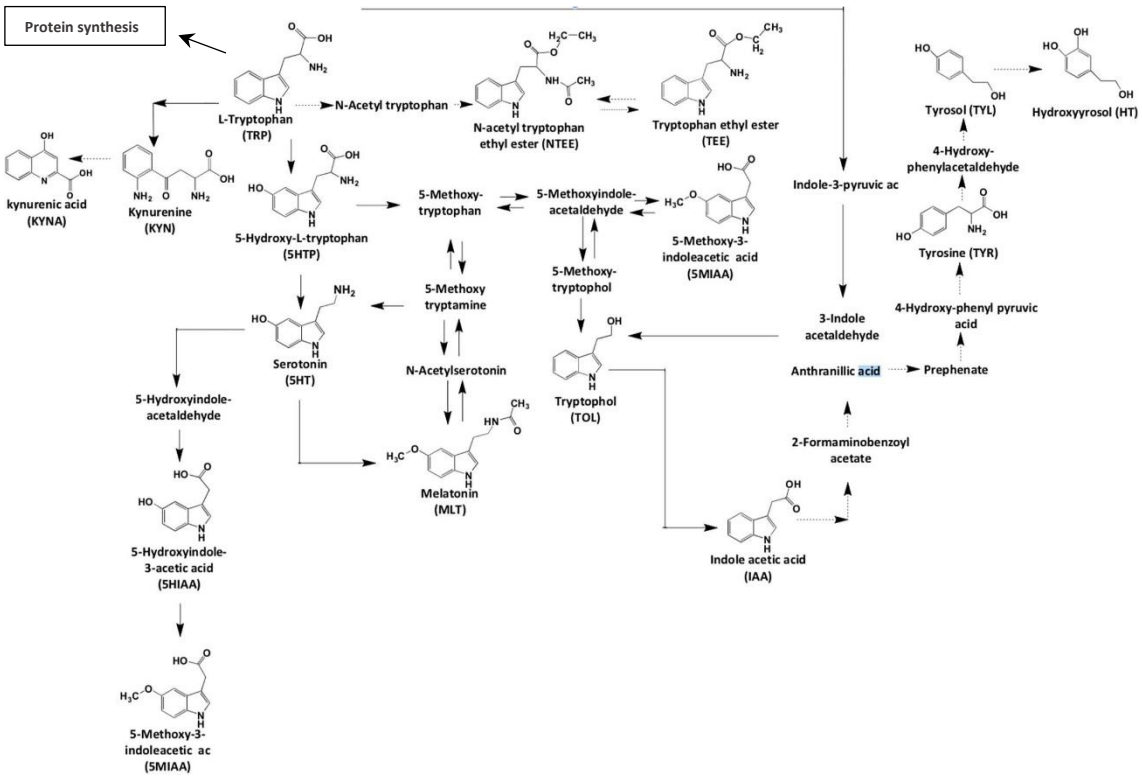

Figure I.13. Compounds related with tryptophan and tyrosine metabolism (adapted from Álvarez-Fernández, et al. 2019).

Bioactive compounds in wine

Regarding wine composition, the average concentrations of the major components 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). Therefore, ethanol, together with other metabolites from the glycolysis such as glycerol and organic acids, are quantitatively the main components in wine produced during the AF. However, the improvement of analytical techniques has allowed the scientific community to identify molecules in lower concentrations. In fact, wine is a complex mixture of more than hundreds of molecules present in different concentrations (Nisbet et al. 2014), some from grapes and some metabolic by-products of the activity of microorganisms during fermentation. Bioactive compounds are defined as components of food, even in

low concentrations, that influence physiological or cellular activities when consumed. Some of the activities exhibited by these compounds include protection against some of the most prevalent diseases in industrialized countries, such as cardiovascular disease, diabetes and some types of cancer (Liu 2013; Maruca et al. 2019). Nevertheless, some wine bioactive compounds generated under certain conditions can modify the organoleptic properties and quality of wines. Biogenic amines, for example, nitrogen compounds produced mainly by the metabolism of microorganisms associated to different stages of the wine production, can be hazard for humans if they are ingested at high concentrations (Aredes-Fernandez et al. 2016; Restuccia et al. 2018).

Moderate alcohol drinking based on wine, as part of a healthy diet is recognized among the major characteristics of the Mediterranean diet which constitutes a unique model, recommended by many specialists and several dietary guidelines in different countries. Furthermore, it could be partially responsible for health-promoting properties observed in the Mediterranean diet. The "French paradox" highlights the inverse relationship between moderate alcohol consumption and coronary heart disease among the French population, despite the fact that their diet is rich in saturated fatty acids (Renaud and Lorgeril 1992). Both white and red wines are considered as rich sources of antioxidant phenolic compounds. Since then, research on plausible metabolites to attribute the health promoting properties in wine has focused primarily on polyphenols. But more recently, new detected indole derivatives compounds such as serotonin, melatonin or hydroxytyrosol are receiving attention (Fernández-Mar et al. 2012; Iriti and Varoni 2014; Aredes-Fernandez et al. 2016; Varoni et al. 2018). Melatonin intake after wine melatonin-enriched have shown to counteract the physiological decrease of the endogenous melatonin in the serum (Varoni et al. 2018). Additionally, different studies both *in vivo* and *in vitro* shows that melatonin and hydroxytyrosol present in wines are more effective compounds than PP in DNA protection against oxidative attack and against oxidative stress related to the central nervous system (Marhuenda et al. 2016, 2017; Gallardo-Fernández et al. 2019).

Although both the fruit and the skin of the grape are sources of bioactive compounds in wine, the role of the metabolism of microorganisms in the production of aromatic and bioactive compounds cannot be ignored. Due to the

extensive knowledge of AF and yeast strains, there has been a great improvement in fermentation processes resulting in final wines with desired characteristics, including wines with lower ethanol concentration, greater organoleptic complexity, increasing phenolic compounds yield, and/or enriched in bioactive compounds (Barcenilla et al. 1989; Moreno-Arribas and Polo 2009; Banach and Ooi 2014; Contreras et al. 2014; Cordente et al. 2019). This is really interesting since consumers have been driven to take a more critical attitude about what they eat and drink as a requirement of modern life.

Recently, research on molecules derived from yeast metabolism has increased and could be relevant from different aspects related to yeast regulation and human health. The properties exerted by these compounds extend the applications from the use of nutritional supplements to functional ones. However, the role of these compounds in yeast is still far from being fully understood. Therefore, expanding research in the field of AAA metabolism in yeast would provide the theoretical basis for the ability to further modulate the production and emergence of bioactive compounds in fermented foods, as well as a wide range of applications in modern nutrition.

Tryptophan derived compounds

Tryptophan AAA, (2S) -2-amino-3- (1H-indole-3-yl) propanoic acid, is a non-polar amino acid characterized by containing a side chain indole ring, which gives it a strong hydrophobic character. L-tyrptohan form part of the proteins and its metabolism is linked to the synthesis of a variety of derived metabolites of which are involved in numerous anabolic pathways. Therefore, tryptophan is the precursor to numerous neurologically active compounds such as melatonin, serotonin, indole acetic acid, kynurenic acid, kynurenine, all of which have important biological activities and health implications (Davis and Liu 2015). The concentration of tryptophan in wines reported in bibliography range 3-80000 ng/mL (Fernández-Cruz et al. 2018). In contrast to mammalian cells, yeasts are able to produce tryptophan starting at central metabolic intermediates from pentose phosphate pathway and glycolysis through the shikimate pathway as it was mentioned previously. Thus, tryptophan is an essential component of the human diet, since humans do not possess the enzymes necessary for their *de novo* biosynthesis, but only those that perform some catabolic activities, such as

the breakdown of the indole nucleus of the molecule (Vitalini et al. 2020). In *S. cerevisiae*, tryptophol and indole-3-acetic acid (IAA) have been described as the major metabolites from tryptophan (Rosazza et al. 1973). Thus, despite tryptophan itself it is not a bioactive compound, it is the precursor to different compounds related to yeast metabolism such as, kynurerine, kynurenic acid, triptofol, IAA, tryptophan ethyl ester, serotonin and melatonin, all of which have important biological activities and health implications and have been detected in wines (Maslov et al. 2011; Davis and Liu 2015; Tudela et al. 2016; Fernández-Cruz et al. 2017; Antonia Álvarez-Fernández et al. 2019) (Figure I.13). Moreover, other tryptophan-derived molecules also impact on the organoleptical properties of wines.

IAA and tryptophan are the main precursors of 2-aminoacetophenone, an aroma compound that is responsible for “untypical ageing off-flavour” of wines and beer (Palamand and Grigsby 1974; Maslov et al. 2011; Engin 2015). IAA is known as the major auxin-type phytohormone that regulates many cellular processes and plant development. However, it has been shown that it can act as a signaling molecule in microorganisms, and it imparts tolerance to various toxic compounds and stress conditions in *E. coli* (Bianco et al. 2006), which means it is capable of regulating gene expression. Moreover, exogenous administration of IAA in *S. cerevisiae* leads to the conversion of vegetative cells into their filamentous form and promotes yeast invasion, and induces activation of *FLO11* (which encodes a GPI-anchored cell surface glycoprotein). This gene could be essential for nature yeast cells to infect wound sites in plants (Prusty et al. 2004). It has been also observed an IAA accumulation in a cell density-dependent manner, by different *S. cerevisiae* strains, which supports the role of IAA as a quorum-sensing molecule (QSM) in yeast. These findings are important since IAA also induces transition to hyphal growth (pathogenic form) in the human pathogen *Candida albicans*, regulating the virulence factor that confers the ability to infect human tissues (Fu et al. 2015).

Similarly to IAA, tryptophol, a fusel alcohol derived from tryptophan, produced by the Ehrlich pathway (and the other two higher alcohols tyrosol and 2-phenylethanol) stimulate pseudohyphal growth in *S. cerevisiae* and have been shown to be QSM under nitrogen limitation and to regulate the induction of

pseudohyphae formation according to the local population density (Chen and Fink 2006; Avbelj et al. 2015). Nutrient limitation also promotes induction of pseudohyphae formation and increased invasive growth in non-*Saccharomyces* species (González et al. 2018a). 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 and Fink 2006; González et al. 2018b). Finally, tryptophol addition on minimal medium and synthetic grape must have been found to cause a decrease in growth and affects fermentation kinetics during AF respectively, for both *Saccharomyces* and non-*Saccharomyces* (González et al. 2018a; Valera et al. 2019). The affectation on growth was observed in a dose-dependent manner and the sensitivity to tryptophol was strain-dependent, being *S. cerevisiae* strains the more resistant (González et al. 2018a). In wines and beers, tryptophol has been detected in mg/L range (Hornedo-Ortega et al. 2016; Fernández-Cruz et al. 2018).

Tryptamine is the BA generated by tryptophan decarboxylation and it has been detected in red wines at mg/L (Anl and Bayram 2009). BAs in wines have been studied extensively for 30 years and particularly over the last decade, as a consequence of the increasing attention to consumer protection (Restuccia et al. 2018), since they can present undesirable toxic effects for consumers when absorbed at too high concentration (Ancín-Azpilicueta et al. 2008). The formation of amines has been observed during AF due to the metabolism of different strains of yeast, but also due to the action of LAB during MLF (Lonvaud-Funel 2001; Garde-Cerdán and Ancín-Azpilicueta 2008; Manfroi et al. 2009; Fernández-Cruz et al. 2017; Restuccia et al. 2018). In yeast, the metabolization of tryptamine into *N*-acetyltryptamine have been described for both *Zygosaccharomyces priorianus* and *S. cerevisiae* (Rosazza et al. 1973).

In the pineal gland, 5-methoxytryptamine, which is one of the biogenic monoamines, can be formed from melatonin, 5-methoxytryptophan and serotonin and it is catabolized by monoamine oxidase (MAO) into 5-methoxyindole-3-acetaldehyde. This compound, can be either converted by aldehyde dehydrogenase to 5- methoxyindole-3-acetic acid, or by alcohol dehydrogenase to 5-methoxytryptophol (Hardeland 2010). 5-Metoxytryptamine together with 5-methoxytryptophol and 5-methoxy-indole acetic acid are active pineal indole

molecules with endocrine relevant effects (Lissoni et al. 1998, 2003). *In vitro* studies with cell lines have been shown that the 5-methoxytryptamine was more potent than melatonin in inhibiting growth of several tumor cell lines (Leone et al. 1988; Shellard et al. 1989; Sze et al. 1993). Sze et al. (1993) reported that the order of the inhibitory potency of the indoles was: 5-methoxytryptmine > melatonin, 5-methoxytryptophol, hydroxytyrosol and methoxyindoleacetic acid > serotonin and 5-hydroxyindoleacetic acid. Not only *in vitro*, but in biological clinical studies it was observed that 5-methoxytryptamine may further improve the efficacy of melatonin alone (Lissoni et al. 2000, 2001). Similar results were obtained in a study conducted with the simultaneous use of melatonin with 5-methoxytryptamine and 5-methoxytryptol in the modulation of anticancer (Lissoni 2006). In lower eukaryotic unicellular organisms, such as *Gonyaulax polyedra*, 5-methoxytryptamine is also produced (increasing the concentration when exposed to low temperatures (Fuhrberg et al. 1997)) and has been related with cyst formation (Balzer and Hardeland 1991a), bioluminescence stimulation (Balzer and Hardeland 1991b) and cytoplasmic acidification (Hardeland 2007). 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 (Sprenger and Hardeland 1999; Sprenger et al. 1999; Ganguly et al. 2001). In *G. polyedra*, 5-methoxytryptamine seems to represent primarily a product metabolite rather than a precursor of melatonin, however, if serotonin is available at high concentrations or when the activity of the melatonin-deacetylating aryl acylamidase is low, melatonin is then formed from 5-methoxytryptamine (Hardeland 1993).

Serotonin, is an ancient indoleamine which is a key neurotransmitter that modulates a wide variety of functions in humans and is the precursor of melatonin in response to signals from the circadian clock through *N*-acetylserotonin intermediate (Engin 2015; Olivier 2015; Hornedo-Ortega et al. 2016, 2018c). Serotonin is found in beers and wines reaching levels of mg/L, mainly as a result of MLF but also yeast can produce it since it has been quantified in the intracellular compartment (Manfroi et al. 2009; Wang et al. 2014; Fernandez-Cruz et al. 2019). Regarding the effect of serotonin in *S. cerevisiae*, some years ago it was discovered that serotonin produced by yeast was related to photo-induced

synthesis in response to UV radiation (Strakhovskaia et al. 1983). In a more recent study, a clear inhibition of yeast growth was observed at concentrations of serotonin above 500 mg/L (González et al. 2018a), which is in agreement with antifungal *in vitro* activity previously observed (Lass-flörl et al. 2002; Lass-Flörl et al. 2003). However, since the concentrations used in this study were much higher than those produced by *S. cerevisiae* under natural conditions, the effects observed are not physiologically relevant (Winters et al. 2019), and further research is required to explore the role of serotonin in yeast.

Traditionally *N*-acetylserotonin, also known as a normelatonin, was considered only a metabolite intermediate in melatonin biosynthesis from serotonin. However, due to different patterns of brain distribution as well as its own properties exhibited, *N*-acetylserotonin have been hypothesized to be more than just a melatonin precursor (Jang et al. 2010; Sompol et al. 2011). *N*-acteylserotonin has been reported to exhibit antioxidant and antiaging activities as well as to improve cognition and protect against β-amyloid-induced neurotoxicity, preserving optimal fluidity of the biological membranes (Oxenkrug et al. 2001; García et al. 2014; Álvarez-Diduk et al. 2015). Similar to 5-methoxytrypamine, *N*-acetylserotonin can be both a substrate (via *N*-acetylation of serotonin) and a catabolite of melatonin (Young et al. 1985). The disruption of serotonin/*N*-acetylserotonin/melatonin pathway has been proposed as a biomarker for autism spectrum disorders since is a very frequent trait in patients (Pagan et al. 2014). *N*-acetylserotonin has been detected in wine during AF (Tudela et al. 2016; Fernández-Cruz et al. 2018), produced by yeast (Sprenger et al. 1999), and in its intracellular compartment (Fernandez-Cruz et al. 2019). However, there is a paucity of information on the activity of *N*-acetylserotonin in yeast.

Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) was isolated and identified for the first time from the pineal gland in vertebrates by Lerner *et al*. and, therefore, was considered as neurohormone (Lerner et al. 1958, 1959). Historically, melatonin was thought to be uniquely of pineal gland origin. However, it is now clear that this is not the case, since only vertebrates have a pineal gland, while other animal species and plants lack even a homologous organ (Reiter et al. 2018). Nowadays,

melatonin has been detected in several phylogenetically distinct organisms including bacteria, fungi, nematode, plants and animals (Tan et al. 2016). Therefore, the exclusive concept of melatonin as neurohormone has become outdated. It is speculated that melatonin evolved in bacteria prior to the endosymbiotic process and its origins may be traced back to more than 3.0-2.5 billion years ago (Manchester et al. 2015).

As for its structure, melatonin is a biogenic indolamine that belongs to the family of methoxyindoles, with a molecular weight of 232 Daltons. It possesses an indolic-type ring with two functional groups, a methoxy group at position 5 and an *N*-acetylaminoethyl group at position 3 (Figure I.14.A).

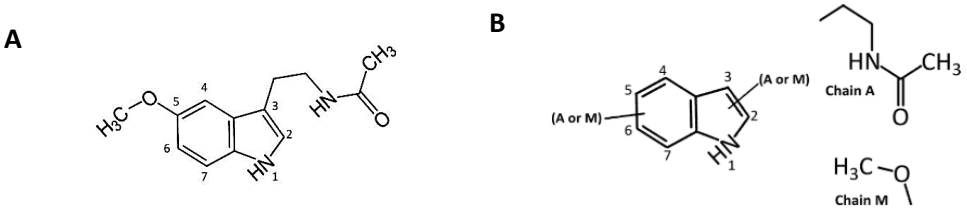

Figure I.14. Chemical structures of melatonin (A) and proposed melatonin isomers (B). The numbers identify the positions on the indole ring and the A or M represents the side chain A and side chain M, respectively (Tan et al., 2012).

Theoretically, either one of these two groups can be potentially relocated to any one of the seven positions in the indole ring to form melatonin isomers (MIs). Based on the possible positions of the two side chains on the indole nucleus of melatonin (only seven positions are available for occupation by the side chains), it has been calculated that as many as 42 melatonin isomers (MIs) (6 × 7) could exist (Tan et al. 2012, 2014b) (Figure I.14.B). These two side chains are decisive in the specificity of receptor binding as well as for the amphipathic character of the molecule.

The physicochemical properties of melatonin allow it to easily cross the lipid membranes of cells and the blood-brain barrier. This is important since considering the extended distribution of both cellular membrane receptors, the existence of nuclear binding sites/receptors as well as the fact that some of melatonin's actions are receptor independent, means that after administered

exogenously, melatonin probably works in every cell it comes in contact with (Reiter et al. 2000; Reiter et al. 2007).

Melatonin actions in different organisms

It has been hypothesized that melatonin emerged as an antioxidant and radical scavenger when appeared in early photosynthetic prokaryotic bacteria (Tan et al. 2013; Manchester et al. 2015; Zhao et al. 2019), in order to neutralize the toxic molecular O_2 derivatives that were produced during photosynthesis (Reiter et al. 2017), and the other secondary functions of melatonin came about much later in evolution (Manchester et al. 2015). Melatonin has retained, until the present time and in all organisms, its ability to control oxidative stress and many *in vitro* and *in vivo* studies have contributed to demonstrate this evidence (J. Reiter et al. 2000; Bonnefont-Rousselot and Collin 2010; Galano et al. 2011; Pan et al. 2015). Melatonin protects various biomolecules against damage caused by free radicals by acting as a direct scavenger to detoxify reactive oxygen and nitrogen species. Also indirectly, melatonin can reduce oxidative stress by increasing the activities of antioxidant defense systems; stimulating the expression and function of a series of antioxidant enzymes, as well as interacting synergistically with other antioxidants (e.g. glutathione); 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. 2003a; Rodriguez et al. 2004; López et al. 2009). The final oxidation products of these reactions are cyclic 3-hydroxy melatonin, N^1-acetyl-5-methoxykynuramine (AMK), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), and melatonin hydroxylated at either 2, 4, or 6 position (Hirata et al. 1974; Tan et al. 2001; Horstman et al. 2002; Hardeland 2017a). Some of these metabolites also possesses antioxidant activity like melatonin itself and sometimes it also possesses biological activities (López-Burillo et al. 2003b; Tan et al. 2015; Lee et al. 2016; Galano and Reiter 2018; Pérez-González et al. 2018; Zhao et al. 2019).

Melatonin’s additional functions have been widely studied in a variety of organisms in animal and plant kingdoms. A large number of studies have shown that melatonin plays an important role in sleep, chronobiotic and chronobiological regulation, in cellular aging and in age-related, anti-inflammatory and immunoregulatory diseases, as well as anticancer for some types of cancer in

animals (Mediavilla et al. 2011; Escrivá et al. 2016; Fathizadeh et al. 2019). Recently, it has been suggested that melatonin may be a complementary or even regular therapy for deadly viral infections including SARS, MERS, COVID-19, Ebola and avian flu, due to the ability to control the innate immune response and reduce inflammation derived from the infection (Tan et al. 2014a; Anderson et al. 2015; Bahrampour Juybari et al. 2020; Reiter et al. 2020; Tan and Hardeland 2020). The European Food Safety Authority (EFSA) has given a scientific opinion on the evidence of melatonin's health properties with sleep health, with a melatonin dose between 0.5 and 5 mg (EFSA 2010). Furthermore, it has been demonstrated that intake of a glass of melatonin-enriched red wine changed serum levels of the indoleamine, representing a dietary source (Varoni et al. 2018).

In plants, melatonin has been related to physiological aspects, such as germination, growth, rooting, fruiting, parthenocarpy, maturation, delayed senescence, and postharvest. But also, it has been recognized that melatonin can act as a protector and relief agent against stressors, both biotic and abiotic, such as ultraviolet (UV) light, drought, temperature variations, high salinity, cadmium salts and environmental toxins. (Arnao and Hernández-Ruiz 2015, 2018, 2020; Pelagio-Flores and López-Bucio 2016; Bhattacharjee 2018).

Physiological role of melatonin in yeast

Unicellular organisms like dinoflagellate *Gonyaulax polyedra* also produce melatonin, which regulate cyst formation. This physiological process occurs according to photoperiodicity, in which melatonin photooxidation plays an important role in metabolic regulation (Hardeland et al. 1995). In yeast, circadian behavior has also been reported as a systematic circadian metabolism in response to cyclic environment stimuli (Eelderink-Chen et al. 2010), but the role of melatonin in metabolic rhythms remain unclear. So, regarding physiological role of melatonin in yeast, it is still a novel topic based on the number of publications in recent years, which are still scarce compared to melatonin in relation to other organisms (Figure I.15), although some recent studies have been shown that melatonin can act as antioxidant in both *Saccharomyces* and non-conventional yeast (Vázquez et al. 2017, 2018; Bisquert et al. 2018). In *S. cerevisiae*, 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 and Barros 2018). Also, melatonin has been shown to regulate gene expression of genes related to antioxidant defense and increases peroxisome accumulation (Wang et al. 2015; Vázquez et al. 2018). Moreover, the presence of melatonin modulates cell fatty acids composition, increasing oleic and palmitoleic acids, leading to higher unsaturated fatty acids / saturated fatty acids (UFA/SFA) ratios, which have been previously related to greater H_2O_2 tolerance (Owsiak et al. 2010; Vázquez et al. 2018).

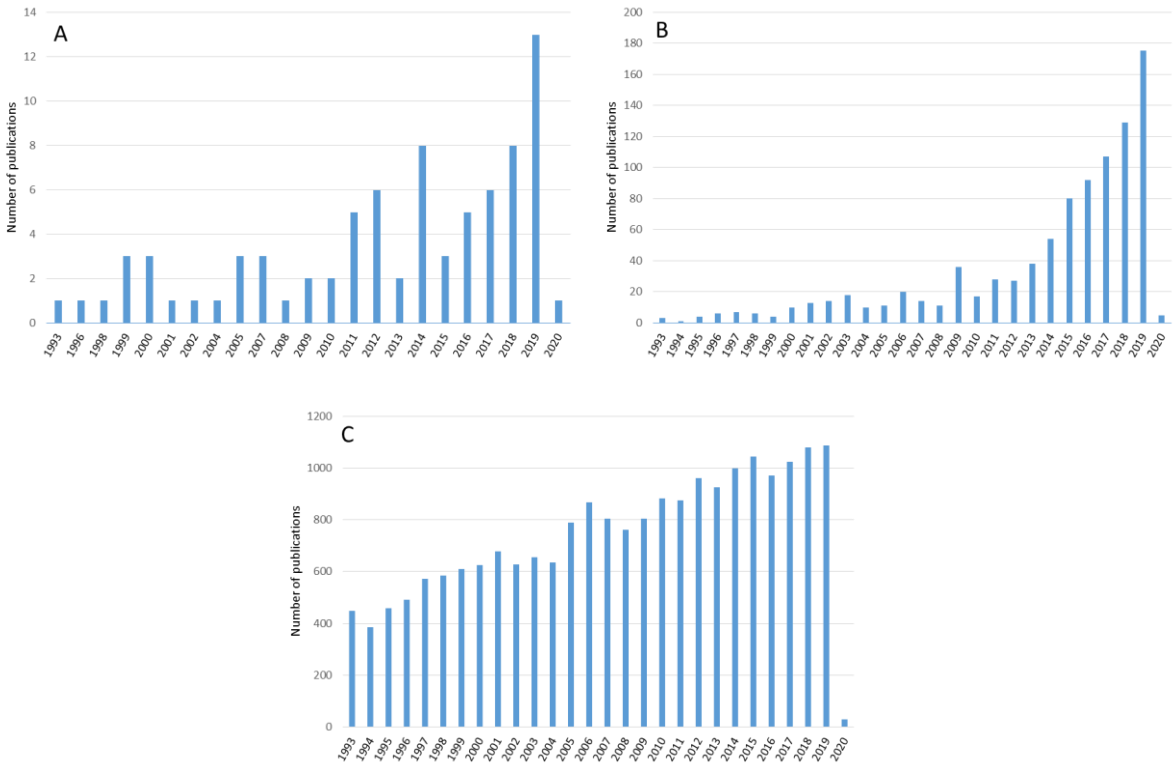

Figure I.15. Evolution of publications on topics "melatonin yeast" (A), "melatonin plants" (B) and "melatonin mammals" (C) since 1993. For 2020, the data correspond to the first month of the year.

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. 2019b). 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 biosynthetic pathway in animals and plants consists in four enzymes involved in the conversion of tryptophan to melatonin. In animals, 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) (official name in vertebrates: aralkylamine *N*-acetyltransferase (AANAT)), and *N*-acetylserotonin *O*-methyltransferase (ASMT) (formerly hydroxyindole-*O*-methyltransferase (HIOMT)), respectively. This pathway is also known as “classic melatonin pathway” and it allows the metabolite conversion as follows: tryptophan/serotonin/*N*-acetyl serotonin/melatonin (Tan et al. 2014b, 2016). In plants however, up to six different enzymes are known to be involved in the conversion of tryptophan to melatonin, suggesting the existence of multiple pathways (Back et al. 2016). The enzymes involved are the same four as in animals (TPH, AADC, AANAT/SNAT, ASMT/HIOMT), but tryptamine 5-hydroxylase (T5H) and caffeic acid O- methyltransferase (COMT), are related to

melatonin synthesis only in plants (Figure I.16). Furthermore, depending on the growth condition, two different main pathways based on enzyme kinetics have been proposed. Under normal growth conditions, the route that is followed is that of tryptophan/tryptamine/serotonin/*N*-acetyl serotonin/melatonin, and under certain conditions the alternative route is that of tryptophan/tryptamine/serotonin/5-methoxytryptamine/melatonin, which can occur when plants produce large amounts of serotonin, as in senescence (Back et al. 2016).

Whereas in plants and animals the route is well known, in yeast remains to be unidentified. It has been suggested that melatonin pathway in *S. cerevisiae* seems to be similar to the synthetic route and enzymes described in animals (Figure I.16), however 5-methoxytryptamine was shown to cause rises in melatonin levels (Sprenger et al. 1999). With regard to yeast genes and enzymes involved in melatonin synthesis, only the homolog of the mammalian AANAT has been described and characterized (Ganguly et al. 2001). This gene encodes for a polyamine acetyltransferase and is named *PAA1* (Liu et al. 2005). The ORF of this gene was cloned and kinetic studies on its substrate specificity *in vitro* were conducted. The K_ms of *PAA1* with 5- methoxytryptamine and serotonin were 2.7 ± 0.6 and 5.1 ± 0.4 mM, respectively. The calculated catalytic efficiency of the reaction with 5-methoxytryptamine was approximately seven-fold greater than that with serotonin (Tan et al. 2016). All these data suggest that, similar to what happens in plants, the production of melatonin from 5-methoxytryptamine through SNAT activity as a last step in the pathway could happen. However, further studies on *PAA1* are required, in order to determine the physiological significance of these route in yeast, as well as to find the remaining genes in the pathway.

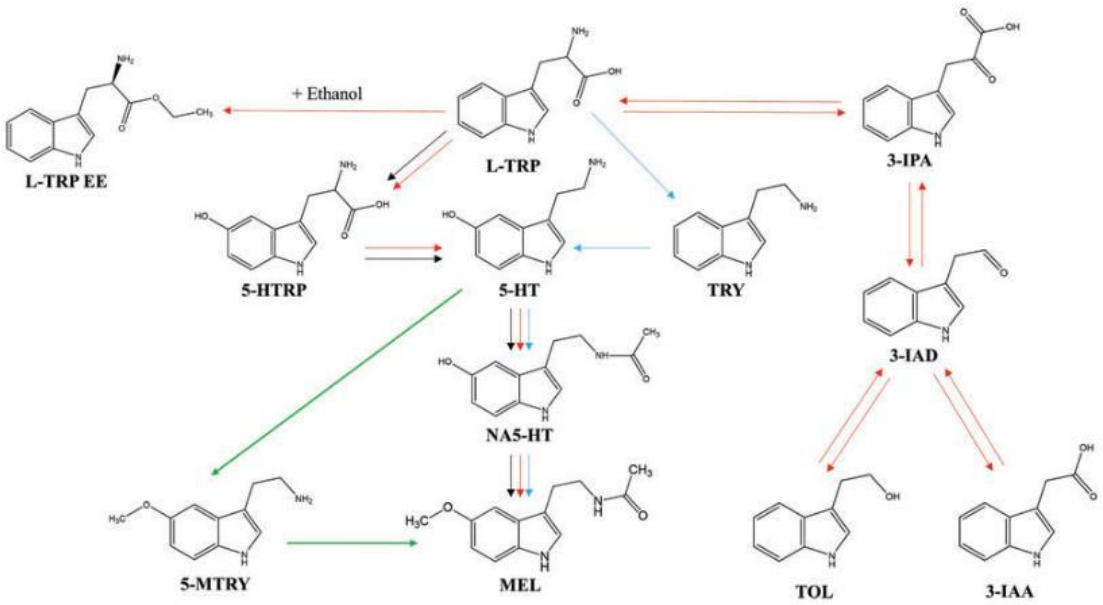

Figure I.16. Tryptophan conversion pathways showing formation of different indolic compounds, including melatonin, described for mammals (black arrows), plants (green arrows) and yeast (red arrows). 3-IAA, 3-indoleacetic acid; 3-IAD, 3-indole acetaldehyde; 3-IPA, 3-indolepyruvic acid; 5-HT, 5-hydroxytryptamine; 5-HTRP, 5- hydroxytryptophan; 5-MTRY, 5-methoxytryptamine; L-TRP, L-tryptophan; MEL, melatonin; NA5-HT, N- acetyl-5-hydroxytryptamine; TOL, tryptophol; TRY, tryptamine (Fernández-Cruz et al., 2017)

Melatonin and melatonin isomers occurrence in food, fermented beverages and yeast cells

Melatonin was first reported in grapes in 2006 and in wine in 2008, so the presence of melatonin in food products derived from fermentation is no exception as evidenced by various publications in the last decade (Table I.1) (Iriti 2009; Meng et al. 2017; Juhnevica-Radenkova et al. 2020).

Table I.1. Concentration of melatonin in various fermented food and beverages. Grape varieties represented are Sangiovese (Sg), Trebbiano (Tb), Chardonnay (Ch), Malbec (Mb), Cabernet Sauvignon (CS), Petit Verdot (PV), Syrah (Sh), Prieto Picudo (PP), Tempranillo (Tp), Merlot (Ml), Palomino Fino (PF), Tintilla de Rota (TR), Gropello (Gp), Alaban (Ab), Garnacha (Gc), Corredera (Cr), Moscatel (Ms), Sauvignon Blanc (SB), Vijiriega (Vj), Nebbiolo (Nb).

Fermented product	Melatonin concentration	Analytical method	Reference
Sg and Tb wine	0.4-0.5 ng/mL	SPE-HPLC-F	(Mercolini et al. 2008)
Ch, Mb, CS wine	0.16-0.32 ng/mL	CE+HPLC-UV	(Stege et al. 2010)
Beer	51.8-169.7 pg/mL	ELISA	(Maldonado et al. 2009)
PV, Sh, CS, PP, Tp	140.5-277.5 pg/mL (ELISA); 5.1-129.5 ng/mL (HPLC-MS/MS)	ELISA, HPLC-F and HPLC-ESI-MS/MS	(Rodriguez-Naranjo et al. 2011a)
CS, Ml, PF, Syrah, Tp, TR wine	74.13-423.01 ng/mL	HPLC-MS/MS	(Rodriguez-Naranjo et al. 2011b)
Gp, Ml wine	5.8-8.1 ng/mL	UHPLC-MS/MS	(Vitalini et al. 2011)
Ab, Sg, Tb wine	0.3–1.5 ng/mL	MEPS-HPLC-F	(Mercolini et al. 2012)
Pomegranate wine	0.54-5.50 ng/mL	HPLC-ESI-MS/MS	(Mena et al. 2012)
Red, white and dessert wines	0.05–0.62 ng/mL	UHPLC-MS/MS	(Vitalini et al. 2013)
Beer	58-169 pg/mL	ELISA	(Garcia-Moreno et al. 2013)
Fermented orange juice	3.15-21.80 ng/mL	UHPLC-QqQ-MS/MS	(Fernández-Pachón et al. 2014)
Beer	94.50 pg/mL	HPLC-MS/MS	(Kocadağli et al. 2014)
Bread	138.10-341 pg/g	HPLC-MS/MS	(Kocadağli et al. 2014)
Yogurt	126 pg/g	HPLC-MS/MS	(Kocadağli et al. 2014)
Mulberry wine	3.41-14.20 ng/mL	HPLC-ESI-MS/MS	(Wang et al. 2016)
Tp, Gc wine	0.03-161.83 ng/mL	UHPLC-QqQ-MS/MS	(Marhuenda et al. 2016)
Cr, Ch, Ms, PF, SB,Vj and Tp wine	0.07-322.70 ng/mL	UHPLC/HRMS	(Fernández-Cruz et al. 2018)
Nb wine	0.038-0.063 ng/mL	SPE-HPLC-FL UHPLC/ESI-QTRAP	(Fracassetti et al. 2019)

The fact that *S. cerevisiae* is the primary responsible for alcoholic fermentation could imply a role in melatonin synthesis by yeast. This hypothesis was unequivocally demonstrated by Rodriguez-Naranjo et al. (2011). These authors conducted the winemaking process and monitored melatonin synthesis during alcoholic fermentation. They observed melatonin production from musts that lacked melatonin initially, before being converted into wine. Subsequently, they evaluated the capacity of melatonin production in a synthetic medium and observed that the growth phase of the yeast and the composition of the medium, especially the concentrations of tryptophan and reducing sugars, affected the synthesis of melatonin in a different way depending on the yeast strain (Rodriguez-Naranjo et al. 2012). Although, the synthesis of melatonin by *S. cerevisiae* under laboratory conditions was previously demonstrated by Sprenger et al. (1999). After these initial works, different studies have focused on demonstrating the production of melatonin by both *Saccharomyces* and non-*Saccharomyces* in different media (Vigentini et al. 2015; Fernández-Cruz et al. 2016, 2017, 2018; Fernandez-Cruz et al. 2019). A very recently study was focused on the evaluation of the effects of different fermentation parameters in melatonin synthesis including sugar and nitrogen concentration, temperature or initial population (Morcillo-Parra et al. 2020a). In this study, sugar content and low temperature (12ºC) were the parameters with an impact on intracellular melatonin production profile, being low temperature the condition that led the higher production.

Tyrosine derived compounds

Tyrosine (4-hydroxyphenylalanine) is the main precursor of tyrosol, which is produced by yeast from the amino acid in a directly proportional manner (Garde-Cerdán and Ancín-Azpilicueta 2008), and hydroxytyrosol, which is formed by tyrosol hydroxylation (Figure I.17).

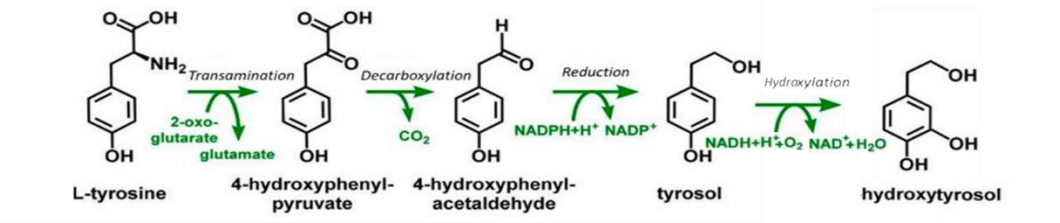

Figure I.17. Schematic representation of tyrosol formation from tyrosine through Ehrlich pathway in yeast and the hydroxylation which produces hydroxytyrosol (adapted from Vilela, 2019).

Both tyrosol and hydroxytyrosol belong to phenolic compounds (PC), molecules containing a benzene ring bearing one or more hydroxyl groups, and are the main PC present in olives, virgin olive oil and waste water obtained during the production of olive oil (Tripoli et al. 2005), but they are also present in wine. Red wine is known to contain more phenolic compounds than white wine (Aredes-Fernandez et al. 2016). The PC of wine are very important since they determine important sensorial characteristics, particularly color, mouthfeel, astringency and bitterness, and they are also involved in health promoting properties including antioxidant, anticarcinogenic, and antibacterial properties (Renaud and Lorgeril 1992; Tripoli et al. 2005; Sabel et al. 2017). Several studies have focused on the identification and quantification of hydroxytyrosol and tyrosol in wine (Chamkha et al. 2003; Proestos et al. 2005; Boselli et al. 2006; De La Torre et al. 2006; Piñeiro et al. 2011). Hydroxytyrosol was first detected in Italian wines (Di Tommaso et al. 1998). Different works have been also focused in quantifying the impact of yeast or different factors (temperature, alcoholic degree, etc…) on the production of such molecules (Silva et al. 2005; Bordiga et al. 2016; Álvarez-Fernández et al. 2018; Guerrini et al. 2018).

Tyrosol (*p*-hydroxy phenyl ethanol) has been detected in different alcoholic beverages such as red or white wine, beer or sake, and it has been found to play a role on the aroma and taste (Silva et al. 2005; Soejima et al. 2012). Bioavailability of tyrosol in humans from both virgin olive oil and red wine has been demonstrated (Covas et al. 2003; Boronat et al. 2018). Dietary tyrosol has been reported to be endogenously transformed into hydroxytyrosol through

cytochrome P450s (Rodríguez-Morató et al. 2017). Furthermore, tyrosol has been described as a cardioprotective, antioxidant and anti-inflammatory agent (Muriana et al. 2017), and its presence in wine has been related to some of the positive effects of moderate wine consumption (Vitalini et al. 2011). In yeast, along with tryptophol and 2-phenylthanol, as explained above, tyrosol is a QSM linked with the stimulation of population density-dependent pseudohyphal growth in *S. cerevisiae* (González et al. 2018b).

Hydroxytyrosol (3,4-dihydroxyphenyl ethanol) is one of the most potent dietary antioxidants (Boronat et al. 2018) which displays much more effective antioxidant activity than other PC (Hu et al. 2014). Bioavailability studies have demonstrated that hydroxytyrosol is dose dependently absorbed in humans after its ingestion (Visioli et al. 2000).

Hydroxytyrosol is present at high concentration in the leaves of the olive tree (*Olea europaea L.*) and in olive oil, where it is produced from the hydrolysis of oleuropein (the ester of elenolic acid with hydroxytyrosol) by an esterase (Rodríguez-Gutiérrez et al. 2011) (Figure I.18).

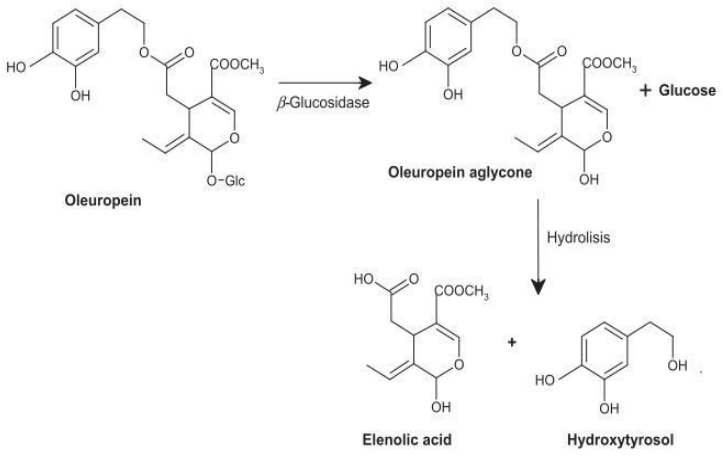

Figure I.18. **Biosynthesis of hydroxytyrosol in *Olea europea L*. and olive related food-stuff** (Fernández-Mar et al., 2012)

In wines, the hydroxytyrosol concentrations are always lower than they usually are in extra virgin olive oil or extracts from leaves. The presence of hydroxytyrosol

in wines has been related not only to microorganisms activity during alcoholic and malolactic fermentation, but also to the hydroxylation of tyrosol into hydroxytyrosol by the enzyme polyphenol oxidase present in grapes (EC 1.14.18.1) (García-García et al. 2013; Bordiga et al. 2016; Álvarez-Fernández et al. 2018; Rebollo-Romero et al. 2020). Regarding yeast production only few studies have been conducted and the results obtained suggest a strain-specific production and may depend on different fermentation variables (Romboli et al. 2015; Bordiga et al. 2016; Álvarez-Fernández et al. 2018; Guerrini et al. 2018; Rebollo-Romero et al. 2020)

Numerous studies have demonstrated the beneficial effects of hydroxytyrosol on human health and preventing diseases. The roles of hydroxytyrosol in cardiovascular and respiratory diseases, metabolic syndrome, neuro- and skin-protection, antitumor formation, as well as anti-inflammatory effects are well detailed in the review of Robles-Almazan and other publications (Hornedo-Ortega et al. 2018b; Robles-Almazan et al. 2018; Gallardo-fern et al. 2020). The biological activity of both tyrosol and hydroxytyrosol (Karkovic Markovic et al. 2019), along with their high degree of bioavailability, has led to consider the recommendation for inclusion in the diet and for use as nutraceuticals (García-García et al., 2013). The EFSA has given a positive opinion on the use of hydroxytyrosol up to 20 mg/kg/day (EFSA 2011), although dosage of 50 mg/kg body weight per day haven’t shown adverse effects (EFSA 2017 2017).

Despite the reported biological importance of hydroxytyrosol, it is worth mentioning that most of the desired health effects have been obtained in recent works by using the purified form. Pure forms are commercially available only for research purposes and can reach really high prices compared with extracts from olive leaves in an un-purified form (Achmon and Fishman 2015). For instance, 1g of hydroxytyrosol with ≥98% of purity can range from 8,240 to 13,600 € while 1 g of tyrosol from the same distributor does not reach 10 € (Sigma-Aldrich). This high price can be attributed to low yields obtained by the majority of the hydroxytyrosol production processes described in the literature. Since olive tree derivatives are the most accessible source, the majority hydroxytyrosol products comes from extraction from olives or olive oil waste streams (Figure I.19), being

the last a much favorable source due to the fact that it originates from a by-product. However, the product extraction from any of these sources is a long process and yields low recovery rates, which can vary seasonally from batch to batch.

Additionally, chemical synthesis methods have been reported, but they usually involve toxic solvents and expensive starting material making it sometimes unsuitable (Zhang et al. 2012; Achmon and Fishman 2015; Britton et al. 2019). Spanish company named “Seprox Biotech” (www.seprox.es) have been developing a synthetic process based on a precursor of the 2-(3,4-dihydroxyphenyl) acetic acid, which was reported leading 99% pure hydroxytyrosol granting the GRAS qualification by the FDA (Food and Drug Administration) (Gomez-Acebo 2015).

Finally, the third method for the synthesis of hydroxytyrosol consists in the biotechnological production of hydroxytyrosol. Both, “purified enzyme or cell-free extracts” and “whole cell” have been used as a biocatalyst for hydroxytyrosol production, and are very well reviewed by Britton et al. (2019). The principal advantage of using whole cells is that 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. Therefore, microbial products obtained can be labelled as ‘natural compounds’, making fragrances and food additives potentially be recognized as GRAS substances, increasing their value to increasingly health-conscious consumers (de Carvalho 2017). Hydroxytyrosol has been successfully obtained by bacterial whole cells from both engineered cells with an artificial pathway (Figure I.20), and non-engineered cells (Allouche et al. 2004; Allouche and Sayadi 2005; Bouallagui and Sayadi 2006; Brooks et al. 2006; Liebgott et al. 2009, 2007; Brouk and Fishman 2009; Santos et al. 2012b; Satoh et al. 2012; Xue et al. 2017a; Chung et al. 2017; Li et al. 2018, 2019; Trantas et al. 2019; Chen et al. 2019; Yao et al. 2020).

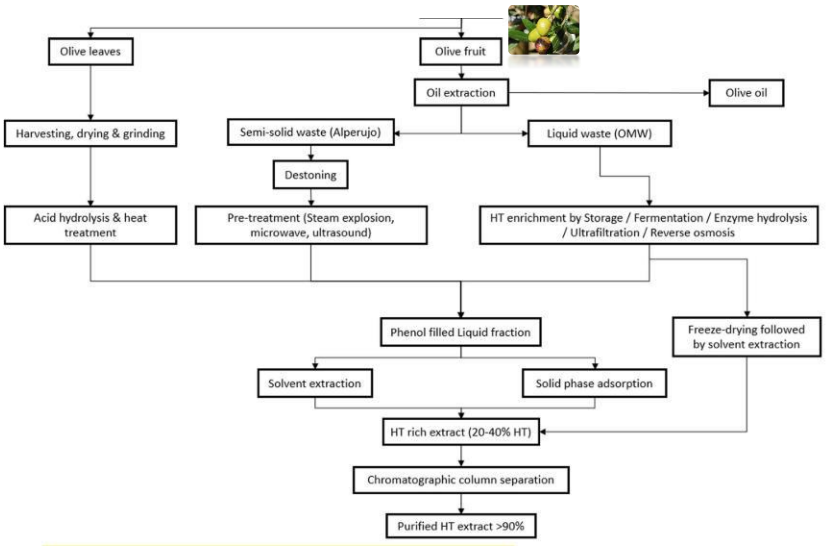

Figure I.19. Flow diagram overview of hydroxytyrosol extraction methods from olive tree derivatives (adapted from Briton et al., 2019)

All these works have been carried out using bacteria, however, *S. cerevisiae*, as a factory to produce hydroxytyrosol, has different advantages that make it a very interesting potential candidate for hydroxytyrosol overproduction. First, tyrosol is easily obtained in high concentrations by yeast from tyrosine (Sentheshanmuganathan and Elsden 1958). Indeed, *Saccharomyces* genes involved in these reactions have been heterologous expressed in *E. coli* to overproduce tyrosol and hydroxytyrosol (Xue et al. 2017a; Li et al. 2018). Additionally, through rational metabolic engineering, tyrosine and tyrosol yields from glucose have been significant improved (Gold et al. 2015; Gottardi et al. 2017; Averesch and Krömer 2018; Guo et al. 2019). Second, tyrosol and hydroxytyrosol have been shown toxicity on bacterial cultures, limiting the amount of hydroxytyrosol produced (Allouche et al. 2004; Liebgott et al. 2007) or needing an *in situ* removal of them from the culture media (Li et al. 2018). Conversely, tyrosol has no effect on the growth of *Saccharomyce*s strains. Although hydroxytyrosol can affect growth, further studies should be conducted due to different hydroxytyrosol concentrations are produced by different wine strains (Romboli et al. 2015; Álvarez-Fernández et al. 2018), and, thereof, the sensitivity could be also strain-dependent (González et al. 2018a; Canal et al. 2019). Thus,

choosing budding yeast *S. cerevisiae* as the host is a promising biotechnological way to increase the hydroxytyrosol levels during AF.

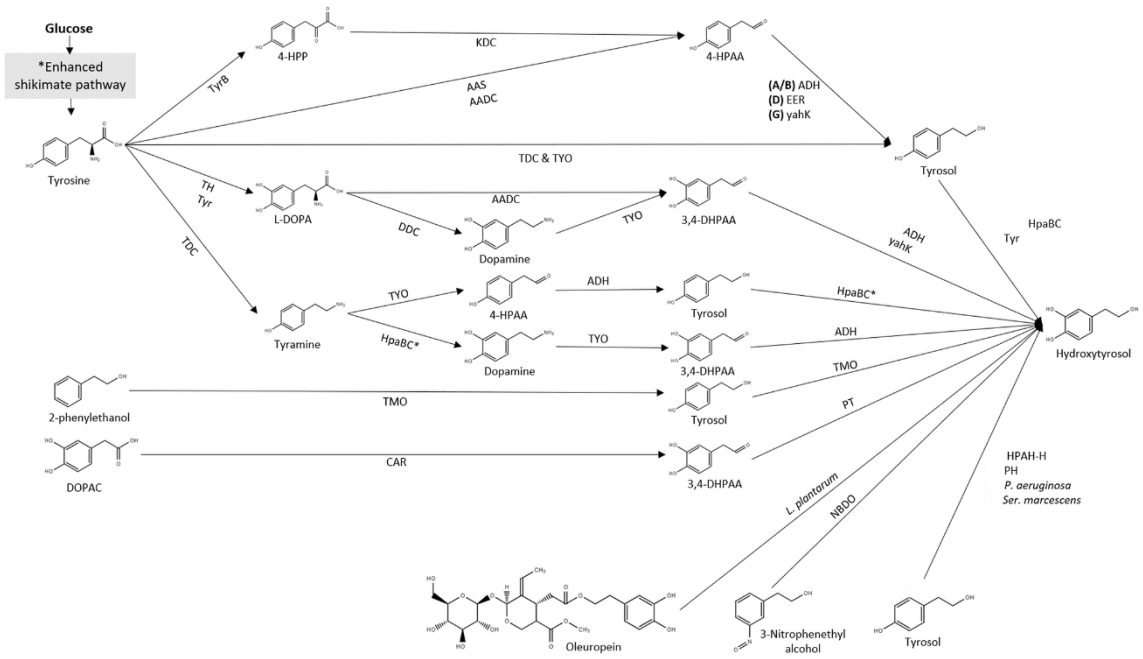

Figure I.20. Biotechnological whole-cell pathways for the production of hydroxytyrosol. Enzymes responsible for each reaction are abbreviated as follows: KDC, ketoacid decarboxylase; ADH, alcohol dehydrogenase; AAS, aromatic acetaldehyde synthase; HpaBC, 4-hydroxyphenylacetic acid 3-hydroxylase; HpaBC*, 4- hydroxyphenylacetic acid 3-hydroxylase with tyrosol and tyramine hydroxylase activity; TyrB, aromatic-amino-acid aminotransferase; TH, tyrosine hydroxylase; Tyr, tyrosinase; AADC, aromatic acetaldehyde de- carboxylase; yahK, aldehyde reductase; TDC; tyrosine decarboxylase; DDC, L-DOPA decarboxylase; TYO, tyramine oxidase; AAS, aromatic acetaldehyde synthase; EER, endogenous *E.coli* reductase; TMO, toluene monooxygenase; CAR, carboxylic acid reductase; PT, phosphopantetheinyl transferase; NBDO, nitrobenzene dioxygenase; HPAH-H, 4-HPA 3-hydroxylase; PH, phenol hydroxylase. (Adapted from Britton et al. 2019)

Hydroxytyrosol application in wine fermentation

Hydroxytyrosol is drawing attention for its possible application in the winemaking process due to its antioxidant and antibacterial properties. This is because the addition of SO_2 is widely used as a preservative in musts and wines during the winemaking (and in most food industries) to control the bacterial growth of undesirable LAB and acetic acid bacteria, as well as against wine oxidation (Santos et al., 2012). However, allergies caused by SO_2-derived compounds (sulfites) are becoming more frequent between sensitive individuals. Quantities

ranging from 20 to 50 mg can cause different symptoms including headaches, nausea, gastric irritation, dermatitis/urticaria, angioedema, breathing difficulties and anaphylaxis (Vally and Thompson 2003; Vally et al. 2009). Consequently, the legislated maximum concentration of SO_2 allowed in wines has been gradually reduced (Santos et al. 2012a) and food products, including wine, with SO_2 concentration higher than 10 mg/L or 10 mg/kg (Directive 2003/89/EC) must be labelled with the phrase “containing sulfites” (Lisanti et al. 2019). Therefore, there is increasing concern about the health risks connected with these enological additives and high interest in finding alternatives. The application of natural antioxidants as preservatives in food industry is an emerging practice that is gaining importance lately in order to allow reduction or even elimination of SO_2. In this context, hydroxytyrosol could be an interesting candidate as a novel food preservative due to its antioxidant capacity and its antimicrobial activity since is naturally found in wine and higher concentrations may provide a healthier wine with added-value. Ruiz-Moreno et al. (2015) were the first authors who tried to replace SO_2 in model wine system with hydroxytyrosol-enriched extract (HTE) from olive mill solid waste. The authors observed, for an equivalent hydroxytyrosol concentration about 80 mg/L, that the antimicrobial activity was comparable to that of the same dose of SO_2 against some wine microorganisms (*Hanseniaspora uvarum*, *Candida stellata*, *Lactobacillus plantarum*, *Pediococcus damnosus*, and *Acetobacter aceti*), while its efficacy was higher than SO_2 against *Oenococcus oeni* and lower against *Dekkera bruxellensis* and *Botryotinia fuckeliana*. So the authors concluded that HTE itself was not sufficient inhibiting the maximum number of spoilage microorganism in winemaking, but they suggested further studies on different SO_2/HTE combinations to enlarge the microbial spectra and to reduce the odor contribution from HTE (Ruiz-Moreno et al. 2015). In similar works, two different commercial products enriched in hydroxytyrosol (HTB coming from chemical synthesis and HTG coming from natural extract from olive by products) were evaluated in wines as a SO_2 alternative (Raposo et al. 2015, 2016). In white wine, no significant differences were found in enological parameters and volatile composition (esters, alcohols and acids). However, significant differences were observed in color related parameters, antioxidant capacity, sensory analysis and olfactometric profile (especially in HTG treated wines). The point in the winemaking process where

these significant differences were observed in SO_2-free wines was during bottle storage. For this reason, the authors recommended more studies on the joint use of hydroxytyrosol and with some other antioxidant additive (ascorbic acid, low concentration of SO_2, glutathione, tannins, etc.) in bottling (Raposo et al. 2015, 2016). Despite these results, studies on the antimicrobial activity of hydroxytyrosol (alone or in combination with other PC) in the real wine environment are lacking (Lisanti et al. 2019), as well as evaluation on the use of hydroxytyrosol produced by microorganisms (which it comes from tyrosol hydroxylation) instead of olive-derived hydroxytyrosol, which could have the undesirable effects on the sensory quality of the final wine.

Detection methods of bioactive compounds in food and yeast samples

Melatonin and other related indolic compunds detection

Analysis of melatonin in food represents a highly challenging task. The concentration varies over a wide range (from micrograms to picograms per gram). Melatonin reacts rapidly with other food components as a result of its antioxidant capacities and, finally, the difficult selection of the extraction solvent derived from the amphipathic feature of the molecule make difficult its analysis (Arnao and Hernández-Ruiz 2009; Stege et al. 2010).

The main analytical techniques followed to determine melatonin in food, fermented beverages and yeast cells derive from the development or adaptation of existing techniques to determine this molecule in biological tissues. Based on chromatography separation, melatonin has been successfully determined by gas chromatography coupled with mass spectometry (GC-MS) and by high-performance liquid chromatography (HPLC) coupled with electrochemical, UV and fluorescence (F) detectors (Iriti et al. 2006; Mercolini et al. 2008, 2012; Muszyńska and Sułkowska-Ziaja 2012). A combination HPLC with capillary electrochromatography (CE) technique has been proposed and used to determine melatonin in wine, grape skin and plant extracts (Stege et al. 2010). Thin-layer chromatography (TLC) coupled with densitometric detection has allowed the determination and quantification of five indole compounds, including melatonin, from methanolic extracts of shoot cultures (Muszyńska et al. 2014).

Currently, the most powerful technique for determining indolic compounds is based on ultra-high performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS). This technique has lowered the limit of detection (LOD) and the limit of quantification (LOQ) of these tryptophan-derived compounds present at very low concentrations. Regarding these objectives, Fernández-Cruz et al. (2016) developed and validated a new UHPLC/HRMS method with a LOD below 0.5 ng/mL for nine indolic compounds (tryptophan, tryptamine, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, melatonin, tryptophan ethyl ester, tryptophol and IAA) in both culture medium and fermented products (Fernández-Cruz et al. 2016). More recently Fraccasetti et al. (2019) highlighted sample preparation using solid-phase extraction (SPE) prior to chromatographic conditions to detect five melatonin isomers (MIs), simultaneously with melatonin and tryptophan ethyl ester in wine (Fracassetti et al. 2019). The detection of MIs was not previously possible by other techniques (Gomez et al. 2012; Fernández-Pachón et al. 2014; Kocadağli et al. 2014) and this could led to co-measure melatonin and its isomers, including tryptophan ethyl ester, a compound with the same molecular weight of melatonin and previously considered as a melatonin isomer. Unfortunately, MIs identification remains difficult due to different factors such as the lack of reference standards, low concentrations in the food samples that makes difficult its purification for the NMR analysis, and the large number of potential isomers. To date, only *N*-acetyl-3-(2-aminoethyl)- 6-methoxyindole is available as a standard (Gardana et al. 2014). Although the use of internal standards with an specific LC-MS/MS fragmentation are reported to give a more accurate detection and quantitation (Paroni et al. 2019). For this technique, several authors have reported the importance of the extraction method because inadequate choice could lead to poor melatonin recovery due to its amphipathic nature and solubility (Garcia-Parrilla et al. 2009; Duportet et al. 2012; Federico et al. 2016). Ethanol and methanol in different proportions are the generally preferred extraction solvents, but in-depth recovery studies should be conducted depending on the matrix (Poeggeler and Hardeland 1994; Escrivá et al. 2016).

Most metabolomic research relies on the analysis of yeast extracellular metabolites, while studies on intracellular metabolic changes are relatively less

abundant. The possibility of following the behavior of metabolites during the fermentation process, in parallel to both intracellular and extracellular media, is useful for gaining knowledge of signaling and the metabolomic reaction network. Álvarez-Fernández et al. (2019) evaluated three different procedures for the intracellular extraction of tryptophan and tyrosine-derived metabolites by UHPLC-HRMS using 3-nitrotyrosine solution as an internal standard. These authors suggested that low-temperature intracellular extraction methods were more suitable for studying melatonin and its related compounds in yeast. After each extraction method they recommended a clean-up and concentration procedure prior to sample injection in order to remove the phospholipids and proteins (Álvarez-Fernández et al. 2019). Conversely the use of SPE cartridges for this purpose are reported to severely affect analyte recovery rates (Vitalini et al. 2020). Similarly, Vitalini et al. (2020) tested four different extraction procedures and developed a multicomponent analytical method to measure and improve the recovery of 14 tryptophan derivatives in different plant matrices by UHPLC-MS/MS. They found water extraction at room temperature was the most suitable when working with plant foods samples, and they also remarked the importance of adapting the election of internal standards according to each compound class (amino acids, indoleamines, *N*-acetyl indoleamines, amino-benzoic, and pyridine derivatives) (Vitalini et al. 2020). These results are in agreement with a previous study in which temperature was the main factor determining the stability of melatonin (Erland et al. 2016).

Shikimate related phenolic compounds detection

Unlike indole compounds, detection of tyrosine and related shikimate compunds such as aromatic alcohols can be easily detected by gas or liquid chromatography (Table I.2). HPLC with photodiode array (PDA), FL or UV detectors have been widely used for the detection of higher alcohols and shikimic acid-related molecules, from both supernatant or intracellular yeast samples and also in fermented beverages such as wine or beer {Formatting Citation}.

Table I.2 Concentration of higher alcohols (tyrosol, tryptophol and 2-phenylethanol) and hydroxytyrosol in various fermented beverages and yeast.

Sample	Concentration (mg/L)				Analytical method	HT LOD (mg/L)	Reference
	2-phenylethanol	Trytpophol	Tyrosol	Hydroxytyrosol (HT)			
Red and white wines	nd	nd	1.42 – 4.80	1.72 – 4.20	GC-MS	0,015	(Di Tommaso et al. 1998)
Wine	nd	nd	up to 45.4	ni	HPLC-PDA-GC/MS	ni	(Boselli et al. 2006)
Synthetic must	nd	1.73 – 5.46	4.06 – 7.17	nf	HPLC-PDA	ni	(Guerrini et al. 2018)
Sangiovese wine	nd	6.3 – 25.9	4.7 – 40.0	2.5 – 25.7	ni	ni	(Romboli et al. 2013)
Red wine	nd	nd	20.38 – 44.46	0.28 – 5.02	HPLC-F and HPLC-PDA	0.023 (F) 0.256 (PDA)	(Piñeiro et al. 2011)
Beer	13.3 – 73.39	0.11 – 1.03	5.3 – 22.20	nd	HPLC-PDA	-	(Li et al. 2008)
Wine	nd	nd	nd	0.53 – 35.11	UHPLC - MS/MS	ni	(Marhuenda et al. 2016)
Wine	nd	1.8 –12.5	20 – 60	0.2 – 5.2	HPLC–MS/MS	0.001– 0.015	(Bordiga et al. 2016)
Wine and intracellular yeast content	nd	nd	nd	0.235 – 0.4 (wine), 0,086 –1,062 (yeast)	UHPLC–HRMS	0,035	(Álvarez-Fernández et al. 2018)
Intracellular yeast content	49.3 – 82.9 (µmol/gCDW)	0.8 – 1.2 (µmol/gCDW)	nq	nd	HPLC-DAD	-	(Lai et al. 2017)
Synthetic must	130.6 (fmol/cells/h)	123.9 (fmol/cells/h)	36.4 (fmol/cells/h)	nd	HPLC-F	-	(Zupan et al. 2013)
Different growth media	1.5 – 9	0.8 –177	0.78 – 11	nd	UHPLC-MS/MS	-	(González et al. 2018b)

nd: not determined; ni: not indicated; nq: not quantified

Hydroxytyrosol and tyrosol in wine have been successfully detected by GC-MS after sample derivatization (Di Tommaso et al. 1998; Boselli et al. 2006; Minuti et al. 2006). However, GC involves long extraction period and entails high temperatures during analysis that can facilitate the degradation of the analytes (Li et al. 2008; Fu 2016). For the quantification of hydroxytyrosol, MS front-end has been employed for to unequivocally identify and quantify this compound in order to elucidate the origin and evolution of HT in wines (Bordiga et al. 2016; Marhuenda et al. 2016; Álvarez-Fernández et al. 2018). Most of these methods have been developed for a small set of metabolites, with the exception of the method of Lai et al. (2017) that, does not include hydroxytyrosol. Despite this, due to the very low quantification/detection limit obtained (as low as ≈ 14,9 - 0, 0149 ng/L) it would be very interesting to configure a single method to control them all at once following their chromatographic conditions.Finally, with regard to the extraction procedures prior to HPLC analysis, 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. 2018b) or ethyl acetate and diethyl ether dilution (Romboli et al. 2015; Guerrini et al. 2018) and SPE with C18 cartridges (Di Tommaso et al. 1998; Álvarez-Fernández et al. 2018) extraction have also been applied. For intracellular extraction there are less reports, but different approaches have been followed. Alvárez-Fernández et al. (2018) used a cold glycerol solution for quenching yeast cells, followed by cycles of freeze-thaw and sonication using methanol-water solution (50% [v/v]). Lai et al. (2017) resuspended the cells with a solution of 75% [v/v] ethanol-water at 100 °C for 10 min and after centrifugation the supernatant was directly used for quantification. Similarly, González et al. (2018) resupended the cells with a boiling absolute ethanol solution buffered with HEPES (pH 7.5) and incubated for 3 min at 80 °C. Subsequently, the supernatant was evaporated and the residue was reconstituted and diluted with methanol. It has been reported that during the drying process, volatile aromatics (such as phenylethanol) are lost, so direct injection of the extracts would be preferable (Lai et al. 2017).

Development and set up of rapid methods for bioactive compounds detection

MS-based methods generally show advantages in higher resolution and a lower LOD. On the other hand, however, these methods also require special and expensive facilities and, depending on the platform, sample pretreatment as it was discussed before.

Thus, an important milestone reached in recent years for melatonin and other indolic compounds has been the development and setup of rapid detection methods. Even so, faster, simpler and easier-to-adapt routine technique detection methods to widely detect yeast-derived samples are still demanded. Of these techniques, a fluorescence-based method have been applied for melatonin determination in pistachio nuts after ultrasound-assisted solid-liquid extraction (Oladi et al. 2014). With the optimized conditions, regarding to the type and volume of solvent extraction, temperature, sonication time and pH, a linear dependence of fluorescence intensity on melatonin concentration was observed with a detection limit of 0.0036 $\mu g.mL^{-1}$. In addition, the results obtained showed good agreement with those obtained by GC-MS. Notwithstanding, spectrofluorimetric methods does not possess the high discriminating capacity of chromatographic techniques. Other alternative rapid techniques are based on the use of specific antibodies against melatonin as an antigen, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (Poeggeler and Hardeland 1994; Rodriguez-Naranjo et al. 2011a). In the case of RIA detection of melatonin, results indicate an overestimation of melatonin concentration when compared to that detected by GC-MS, and cases of false positives are also reported. ELISA kits for the detection of melatonin are available, especially for biological samples like urine, but their use in food samples like fermented products is still not suitable as they may need further optimization (Garcia-Parrilla et al. 2009; Rodriguez-Naranjo et al. 2011a). Development and validation of new antibodies for these complex matrices may be critical for ELISA method to be completely suitable. More recently, two different methods that employ mammalian melatonin receptors are worth mentioning. The first consists of engineered yeasts that act as melatonin sensors.

This is possible because human melatonin receptor MTNR1A is a G-protein coupled receptor (GPRC), just like the yeast pheromone receptor, and downstream signal transduction occurs when yeast GPRC is replaced by MTNR1A. Thus, by using a reporter gene at the end of the pheromone response pathway, a heterologous yeast strain was obtained that acted as a melatonin sensor. By employing the green fluorescent protein (GFP) as a reporter, the authors detected melatonin production by yeasts using a microplate reader without having to process the sample, but directly from the supernatant (Shaw et al. 2019). The second method was set up to detect melatonin in fermented beverages, and it involved mammalian melatonin receptor MTNR1B and cell lines as a sensor. In this case, the reporter system is different because it uses the β-lactamase enzyme (BLA) and a FRET (Förster resonance energy transfer)-based fluorescent substrate (CCF2/4) that presents green fluorescence when is intact. Thus, only when melatonin is present in the sample, the BLA enzyme cleaves CCF2/4 to generate blue fluorescence. The relation between blue and green fluorescence is what is used for melatonin quantification (Morcillo-Parra et al. 2019a). Methodologies like these offer the advantage of not requiring excessive sample processing; e.g. using C18 SepPak cartridges for each analysis, which cuts analysis costs and reduces the complexity of melatonin determination in large detection tests. This would allow to cost-effectively extend melatonin detection under many different conditions and by different strains of various yeast species. However, melatonin can be detected only by these methods, which reduces the understanding of tryptophan metabolism and its derivatives. When this is the study objective, HPLC-MS/MS is the most reliable method because it allows the simultaneous quantitation of such analytes.

Regarding hydroxytyrosol, two different methods for high-throughput screening of tyrosol hydroxylation has been reported recently (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 and Kaiser 1966), which only turns yellow when there is hydroxytyrosol or dopamine in the sample. Since no color change was observed with tyrosine, tyrosol, and tyramine, colorimetric difference can be quantified by measuring absorbance (OD_{400}) in a multiwell reader. The inability to discriminate

hydroxytyrosol from dopamine in principle is not a problem to apply this method in yeast as no dopamine production by yeast has been known. However, this method was developed for the detection of hydroxytyrosol from *E. coli* colonies that overexpressed the tyrosol hydroxylase complex and the minimum amount detected by the method was above 3.8 mg/L. This is a problem for the application of the method to the detection of endogenous hydroxytyrosol production by yeasts since the range of intracellular hydroxytyrosol production reported is around 0.106 mg/L (Álvarez-Fernández et al. 2018). Notwithstanding, further optimization of this method to achieve lower detection limit could be really interesting. The second method developed, consist in a whole-cell hydroytyrosol biosensor (Yao et al. 2020). The biosensor is based in a regulatory protein named VanR from *Corynebacterium glutamicum*. This protein is induced by vanillic acid and regulates the expression of the vanABK operon, which is responsible for the catabolism of vanillic acid. When vanillic acid is present, it binds to the VanR protein, inducing a conformational change which releases the promoter DNA (P_{vanABK}), thus activating the operon transcription (Heravi et al. 2015). What the authors did was to adapt a functional VanR regulatory system in *E. coli* by replacing the −10 region of promoter P_{vanABK} with tac promoter (P_{tac}) in order to constitutively expressed VanR. Furthermore, the VanR specificity for vanillic was altered in order to being regulated by hydroxytyrosol instead of the original inductor vanillic or other subtrates in the hydroxytyrosol engieneered pathway from these authors, such as tyrosine, L-DOPA, dopamine and 3,4-dihydroxyphenyl acetaldehyde. In order to analyze the specifity induction towards hydroxytyrosol during huge screenings, the promoter was coupled to the downstream reporter red fluorescent protein enconded by *rfp* gene, what allowed to use fluorescence-activated cell sorting (FACS). Finally, in order to apply the VanR mutant specifically responsive to hydroxytyrosol successfully selected to a hydroxytyrosol biosensor the authors used β-galactosidase enocoded by *lacZ* (instead of *rfp*) to facilitate the subsequent screening on agar plates. With this biosensor as an end product, the authors design a strategy for a hight throughput screening tool.

OUTLINE OF THESIS & OBJECTIVES

The aim of the present work was to obtain a PhD degree. To this end, I have been working in José Manuel Guillamón Navarro laboratory from 2015 to 2020, which it is one of the four laboratories forming the research group “Systems Biology in Yeast of Biotechnological Interest (SBYBI)” located at the Institute of Agrochemistry and Food Technology (IATA) of the Spanish National Research Council (CSIC), Valencia (Spain) (https://www.iata.csic.es/es). During this period, I had a predoctoral contract co-financed by the CSIC and the Valencian Ministry of Innovation, Universities, Science and Digital Society through the PROMETEO Program for research groups of excellence (PROMETEOII/2014/042). In addition, in 2017, I received a grant (BEFPI17) through the European Social Fund to stay three months in Dr. Martínez lab, located at the Department of Biomedicine (University of Bergen) under the supervision of Prof. Aurora Martínez (PhD leader of Biorecognition group).

The thesis was developed within the framework of the BIOACTIYEAST and SYNBIOFERM projects, entitled “Production and physiological effects of bioactive compounds derived from aromatic amino acids in yeast populations” and “Metabolism and protective effects of indole compounds in yeasts of food interest”, respectively. These two projects have been funded by the Spanish Ministry of Economy and Competitiveness (AGL2013-47300-C3-1-R and AGL2016-77505-C3-3-R, respectively) and has given continuity to the recently new existing project CONSORWINE entitled "design of microbial consortia to improve the stability and bioactivity of wines".

Recently, the metabolism of aromatic amino acids in yeasts has been linked to the synthesis of bioactive molecules (melatonin, serotonin, tyrosol, hydroxytyrosol, etc.) that could be relevant from different aspects related to both yeast regulation and human health. The activities of these compounds as a potent antioxidant and other aspects beneficial to consumer health makes it studying its synthesis really interesting. However, few information is known about the synthesis of these bioactive molecules by yeast because it is a very recent topic of study.

Therefore, the **working hypothesis** of the present thesis was: Through a better understanding of the aromatic amino acid metabolism routes in *S. cerevisie* yeast, we can increase the content of bioactive molecules in products of alcoholic

fermentation. Thus, yeast metabolism can increase the content of bioactive molecules in fermented beverages and foods, which could have an impact on the health of the consumer and which will undoubtedly increase the added value of these beverages, better positioning them in an increasingly competitive and global market.

In this context, the **main objective** of this thesis work was **to study the molecular and physiological mechanisms involved in the production of bioactive compounds derived from the aromatic amino acid metabolism, mainly tryptophan derived compounds such as melatonin and serotonin, in *Saccharomyces cerevisiae.***

This general objective was divided into the following four **specific objectives**:

Objective 1: To adapt and set up a simple, rapid, and low-cost technique for detecting the presence of melatonin in *S. cerevisiae* intracellular samples.

Since melatonin, as well as directly related compounds in yeasts, are sensitive to temperature changes and oxidizing agents, the concentrations of these molecules vary during their determination. The validated melatonin analysis method is HPLC-MS/MS. However, costly cleaning processes of the sample (such as solid phase extraction), as well as the availability of the equipment, are required, making the analysis more expensive. We set up a rapid and simple technique based on an electroanalytical method to rapidly monitor melatonin and related tryptophan derivative metabolites (such as 3-indoleacetic acid and tryptophol), using the variation of the electrochemical responses of extracts taken directly from dried yeast cells (and avoiding intracellular content extraction). This method is based on the fact that these indole compounds are oxidized electrochemically, which drives their determination by voltammetric techniques. We obtained the voltammetry responses in various *Saccharomyces* industrial strains spiked with tryptophan (QA23, BMV58, AROMA WHITE). For QA23 wine yeast strain, it was also tested 5-hydroxytryptophan and serotonin. Finally, we compare the synthesis of these compounds in two mutant strains with deletion in two key genes of tryptophan metabolism (*ARO10* and *TRP1*) in relation to their wild strain BY4743 when

tryptophan was added. Finally, the variations in the relative intensity of the anode peaks corresponding to the oxidation of the indolic molecules were compared with the HPLC-MS/MS method.

The results of this objective are described in:

Chapter 1: Detecting and monitoring the production of melatonin and other related indole compounds in different *Saccharomyces* strains by solid-state electrochemical techniques.

Objective 2: To determine the effect of melatonin on yeast *S. cerevisiae* as antioxidant and UV protector

The possible effect of melatonin was studied in the BY4743 laboratory strain in the presence and absence of H_2O_2 and UV radiation. For this purpose, we first enriched the cells with melatonin and measured the intracellular ROS levels. Next, yeast growth and viability were determined after exposure to stress for both non-enriched and melatonin-enriched cells. Finally, transcriptional analyses by real-time PCR (qPCR) for genes involved in antioxidant defense were performed with the Mel-treated and untreated cells challenged with H_2O_2 and UVC light.

The results of this objective are described in:

Chapter 2: Protective role of intracellular melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*.

Objective 3: To unveil the melatonin biosynthetic pathway in *S. cerevisiae*

The first work related to the biosynthesis of melatonin in yeasts demonstrated the ability to produce melatonin from tryptophan, as well as other potential substrates (also present in the melatonin pathways of animals and plants), in resting cells. We aimed to provide new empirical data about the melatonin production in yeasts following, but expanding, this previous work. To this end, we evaluated the products generated from different substrates of the route (L-tryptophan, 5-hydroxytryptophan,

serotonin, *N*-acetylserotonin, tryptamine, and 5-methoxytryptamine), in the wine yeast strain QA23 at different stages of growth by HPLC-MS / MS.

On the other hand, with regard to the genes and enzymes involved, although the biosynthetic pathway of melatonin in mammals and plants is well studied, very little is known about yeasts. Thus, in order to identify the genes that respond to the synthesis of melatonin in yeasts, we performed a BLAST analysis using the protein sequences of animals and plants. The genes selected as putative orthologs, as well as a positive gene as a control (from animals or plants), were overexpressed both in *S. cerevisiae* and *E. coli*.

The only gene that has been proposed as homolog of the arylalkylamine N-acetyltransferase (AANAT) of vertebrates is *PAA1.* However, the role of *PAA1* in the melatonin biosynthetic pathway still has to be elucidated. Previous work indicates the possibility that arylalkylamines (such as tryptamine, serototonin or 5-methoxytryptamine) are not *in vivo* substrates of *PAA1* in *Saccharomyces cerevisiae*, being the biogenic amines spermine, spermidine or putrescine their natural substrates. In an attempt to shed light on the role of *PAA1* in melatonin synthesis in yeast, we first evaluated the production of melatonin by acetylation of the substrate 5-metoxitriptamine in the laboratory strain BY4743 wild and *PAA1* deficient strain. We overexpressed *PAA1* and determined the acetylation capacity of this strain for different arylalkylamines compared to the AANAT vertebrate overexpression.

The results of this objective are described in the following two chapters:

Chapter 3: Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites.

Chapter 4: Overexpression of selected yeast genes as potential orthologs involved in melatonin biosynthesis. Role of yeast polyamine acetyltransferase (*PAA1*) in melatonin production.

Objective 4: To overproduce bioactive molecules in *S. cerevisiae* by genetic engineering

Hydroxytyrosol is considered one of the most potent antioxidants in nature. The majority of hydroxytyrosol currently available in the market comes from olive leaf extracts or residues from the olive industry (alpechin). However, the extracted hydroxytyrosol is part of a poorly purified fraction. On the contrary, pure hydroxytyrosol reaches very high market prices due to the low concentration in its natural sources, the low extraction yields and the difficulty of chemically synthesizing hydroxytyrosol. Therefore, it is of great interest to produce this compound through biotechnological approaches by using microorganisms to obtain yields that may be viable for industrial production. To produce greater amounts of hydroxytyrosol in *S. cerevisiae*, we constructed a strain modified by multiple integration into the genome of the *E. coli* hydroxylase HpaBC complex. This complex is responsible for the activity of 4-hydroxyphenylacetate monooxygenase which, due to its broad substrate specificity, is capable of transforming tyrosol into hydroxytyrosol. Subsequently, several changes in yeast metabolism have been made to increase the flow of the route to the synthesis of tyrosol, the higher alcohol derived from tyrosine catabolism. These modifications have consisted both in the overexpression of ARO genes, such as *ARO3*, *ARO4*, ARO7 and *ARO10*, as well as in the deregulation of catabolic repression in the metabolism of aromatic amino acids (tryptophan, phenylalanine and tyrosine) through specific mutations on some of these genes (*ARO3, ARO4* and *ARO7*). The concentrations of hydroxytyrosol, tyrosol, 2-phenylethanol, and tryptophol acid produced by the different strains were determined by HPLC-PDA.

The results of this objective are described in:

Chapter 5: **Metabolic engineering of *Saccharomyces cerevisiae* for enhance hydroxytyrosol production, and other metabolites related to shikimate, from glucose.**

MATERIAL & METHODS

Culture media

Culture media for yeast

YPD (Yeast peptone dextrose medium)

Glucose	20 g/L
Bacteriological peptone	20 g/L
Yeast extract	10 g/L
Agar (for solid media preparation)	16 g/L

MM (Minimal medium)

Glucose	20 g/L
Difco Yeast Nitrogen Base (without amino acids and ammonium sufate)	1.7 g/L
Ammonium sufate	0.283 g/L
Agar (for solid media preparation)	16 g/L

*****Note: MM can be supplemented to satisfy auxotrophic requirements of yeast strain

SD (Minimal synthetic defined medium)

Glucose	20 g/L
Difco Yeast Nitrogen Base (without amino acids and ammonium sufate)	1.7 g/L
Ammonium sufate	5 g/L
Agar (for solid media preparation)	16 g/L

*****Note: SD can be supplemented to satisfy auxotrophic requirements of yeast strain

SC (Synthetic complete defined Medium)

Glucose	20 g/L
Difco Yeast Nitrogen Base (without amino acids and ammonium sufate)	1.7 g/L
Ammonium sufate	5 g/L
Kaiser complete SC drop-out mixture (Formedium)*	2 g/L
Agar (for solid media preparation)	16 g/L

SC-ura (Synthetic complete defined medium without uracil)

Glucose	20 g/L
Difco Yeast Nitrogen Base (without amino acids and ammonium sufate)	1.7 g/L
Ammonium sufate	5 g/L
Kaiser SC single drop-out -URA (Formedium)*	1.93 g/L
Agar (for solid media preparation)	16 g/L

***Note:** For Kaiser other single, double or triple drop-out used to supplement encountered auxothropies, use the amount indicated by the manufacturer

SC-ura-pant (Synthetic complete defined medium without uracil and panthotenate)

Glucose	20 g/L
Ammonium sufate	5 g/L
Kaiser SC single drop-out -URA (Formedium)*	1.93 g/L
Agar (for solid media preparation)	16 g/L

*YNB without panthotenate:

Vitamins: Add 1 mL from 1000X and 10 mL from 100X stock solutions	
1000 X STOCK	
Biotin	0,002 g/L
Folic acid	0,002 g/L
100 X STOCK	
Inositol	0,2 g/L
Niacin	0,04 g/L
p-aminobenzoic acid	0,02 g/L
Pyridoxine hydrochloride	0,04 g/L
Ribofalvin	0,02 g/L
Thiamine hydrochloride	0,04 g/L
Compounds supplying trace elements: Add 10mL from a 100X stock solution	0.05 g/L
H_3BO_3	0.004 g/L
$CuSO_4 \cdot 5H_2O$	0.01 g/L
KI	0.02 g/L
$FeCl_3$	0.04 g/L
$MnSO_4$ xH_2O	0.02 g/L
Na_2MoO_4	0.04 g/L
$ZnSO_4$	
Salts	
KH_2PO_4	1 g/L
$MgSO_4$	0.5 g/L
NaCl	0.1 g/L
CaCl	0.1 g/L

PBS (Phosphate-buffered saline medium)

NaCl	8 g/L
KCl	0.2 g/L
Na_2HPO_4	1.42 g/L
KH_2PO_4	0.24 g/L

SLT (Salt medium)

NaCl	5 g/L
Na_2HPO_4	50 mmol/L

*Citric acid for adjust the pH 5.8

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

Sugars	
Glucose	100 g/L
Fructose	100 g/L
Organic acids	
Citric acid	5 g/L
Malic acid	0.5 g/L
Tartaric acid	3 g/L
Mineral salts	
KH_2PO_4	0.75 g/L
K_2SO_4	0.5 g/L
$MgSO_4\ 7H_2O$	0.25 g/L
$CaCl_2\ 2H_2O$	0.15 g/L
NaCl	0.20 g/L
NH_4Cl	0.46 g/L
Total NH_4Cl YAN (mg/l)	**120 mg/L**
Amino acids: Add 13.09 mL from stock	
STOCK Solution:	
Tyrosine	1.5 g/L
Tryptophan	13.4 g/L
Isoleucine	2.5 g/L
Aspartic Acid	3.4 g/L
Glutamic Acid	9.2 g/L
Arginine	28.3 g/L
Leucine	3.7 g/L
Threonine	5.8 g/L
Glycine	1.4 g/L
Glutamine	38.4 g/L
Alanine	11.2 g/L
Valine	3.4 g/L
Methionine	2.4 g/L
Phenylalanine	2.9 g/L
Serine	6 g/L
Histidine	2.6 g/L
Lysine	1.3 g/L
Cysteine	1.5 g/L
Proline	46.1 g/L
Total amino acids (YAN mg N/l)	**180 mg/L**
Total YAN (mg N/l)	**300 mg/L**
Vitamins: Add 10 mL from stock solution	
STOCK solution:	
Myo-inositol	2 g/L
Pantothenate calcium	0.15 g/L
Thiamine hydrochloride	0.025 g/L
Nicotinic acid	0.2 g/L

Pyridoxine	0.025 g/L
Biotine	3 ml (from a stock 100 mg/L)

Oligo-elements: Add 1mL from stock solution

STOCK solution:

$MnSO_4$ H_2O	4 g/L
$ZnSO_4$ 7 H_2O	4 g/L
$CuSO_4$ 5 H_2O	1 g/L
KI	1 g/L
$CoCl_2$ 6 H_2O	0.4 g/L
H_3BO_3	1 g/L
$(NH_4)_6Mo_7O_{24}$	1 g/L

***** pH = 3.3 was adjusted with pellets of NaOH

SM w/o AA (Synthetic must without amino acids)

*SM modified with 100mg/L YAN in $NHCl_4$ form

Culture media for bacteria

LB (Luria Bertani medium)

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L
Agar (for solid media preparation)	16 g/L

2x TY medium with 1% glucose

Tryptone	16 g/L
Yeast extract	16 g/L
NaCl	5 g/L
*Glucose	10 g/L

S.O.C (Super Optimal broth with Catabolite repression)

Tryptone	20 g/L
Yeast extract	5 g/L
NaCl	0.5 g/L
KCl	0.186 g/L
$MgCl_2$	0.952 g/L
$MgSO_4$ $7H_2O$	2.465 g/L
Glucose	3.603 g/L

* Complete SOC can be filter sterilized through a 0.22 μm filter

Microbiological techniques

Culture conditions

Batch cultivations

Melatonin intermediates depletion /restoration experiments by arrested cells (Sprenger et al. 1999) (Chapter 1 & Chapter 3)

Yeast cells were suspended at cell densities of ~10^8 cells/mL in the non-proliferative medium SLT and incubated in Erlenmeyer flasks with orbital agitation (150 rpm) at 28°C in complete darkness for 4 h for 4 hours. After the incubation period in SLT, indolic compounds of interest were added at a final concentration of 1 mM and cells were incubated. Sampling was done at different times (5, 10, 15 and/or 30 min) after adding the indolic compounds. For harvesting, 10 mL was centrifuged at 3220 × g for 3 minutes at 4°C. The obtained pellet (~10^9 cells) was washed twice with sterile distilled water. Pellets were frozen with liquid nitrogen and stored at −80°C, and the supernatants were stored at −20°C until further extractions.

Melatonin intermediates depletion /restoration experiments in yeast cells during exponential growth (Chapter 3)

Yeast cells, from an overnight preculture, were suspended at an optical density of 600 nm (OD_{600}) of 0.1 (~10^6 cells/mL) in 100mL flasks with 20 mL of fresh SC or SD medium. When cells were in the exponential growth stage (0.6~0.8), melatonin intermediates at the 1 mM final concentration were added. A volume of ~ 10 units of OD_{600} was sampled at 15 minutes and centrifuged at 4000 rpm for 3 minutes at 4°C. After centrifugation, the cell suspension was washed twice with sterile distilled water. Finally, the pellet was frozen with liquid nitrogen and stored at −80°C until intracellular extraction and the supernatant was stored at −20°C until further extraction.

Treatment for melatonin uptake and intracellular enrichment (Chapter 2)

Melatonin solution was added to the exponential-growing cultures in SC at three different concentrations (0.05, 0.1, and 20 mM) to establish a suitable dose for further assays. After 30 min, cells were pelleted and washed twice with sterile water to remove extracellular remains. The control cells were mock-treated with the same volume of ethanol as the melatonin-treated ones.

Growth conditions to test the activity of the putative orthologues yeast genes for the melatonin synthesis pathway (Chapter 4)

i) Heterologous overexpression of the selected genes in *E. coli*

For overexpression of heterologous genes in *E. coli*, the pGEX-5X-1 vectors containing one gene of interest (Table M.3) were introduced into Rosetta™ *E. coli* strain (Novagen). This strain enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli* (tRNAs for AGG, AGA, AUA, CUA, CCC and GGA on pRARE plasmid which confers resistance to chloramphenicol). Transformants were grown for o/n at 37º with shaking 15 mL tubes with 5 mL of LB supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Next day, 15 µL of the saturated cultures were inoculated in1.5 mL of 2XTY with 1% glucose medium supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol at 37 °C up to an OD_{600} nm of 0.6. When OD_{600} reached 0.6, 0.25 mM IPTG and 1mM of the indolic compound as a substrate was added to the culture. The culture was grown at 28 °C under constant shaking in a microplate shaker at 300 rpm, and after 24 h of growth a sample was taken to analyze by liquid chromatography and to recorded the OD_{600}.

ii) Overexpression of the selected genes in *S. cerevisiae*

BY4743 strain were transformed with p426GPD vectors containing one gene of interest (Table M.3). Single colonies of each transformant were grown for o/n at 28º with orbital shaking at 150 rpm in 1.5 mL tubes with 800 µL of SC-ura. Next day, 30 µL of the grown culture was inoculated in 1.5 mL of fresh SC-ura medium in 24-well

plates of 2 mL capacity. This culture was incubated under constant shaking at 28 ºC in a microplate shaker at 300 rpm. When OD_{600} reached 0.6 – 0.8, 1mM of the indolic compound as a substrate was added to the culture. After 50 h of growth sampling was conducted to analyze by liquid chromatography and to recorded the OD_{600}.

Growth conditions for hydroxytyrosol production characterization (Chapter 5)

i) Evaluation of the hydroxytyrosol and higher alcohols production on microplate system

Precultures of the corresponding strain were grown for o/n at 28º with orbital shaking at 150 rpm in 1.5 mL tubes with 800 µL of medium. Next day, 25 µL of the grown culture was inoculated in 1.5 mL of fresh medium in 24-well plates of 2 mL capacity. This culture was incubated under constant shaking at 28 ºC in a microplate shaker at 300 rpm, and after 72 h of growth a sample was taken to analyze by liquid chromatography and to recorded the OD_{600}.

ii) Time-profile of the hydroxytyrosol and higher alcohols production in shake-flask fermentation

To evaluate the effect of time and glucose concentration on the production of hydroxytyrosol, the laboratory strain with HpaBC integrated into its genome (BY4743 HpaBC) and this strain transformed with the plasmid p423GPD-ARO4K229L (BY4743 ARO4K229L) were inoculated in 1 mL of SD supplemented with leucine (380 mg/L) and grown overnight at 28ºC with agitation. The next morning, 500 µL of the grown culture were inoculated in 250-mL flasks containing 50 mL of fresh SD supplemented with leucine with 20 and 160 g/L of glucose. This culture was incubated under constant shaking at 150 rpm at 28ºC and after 120, 144, 168, 197, 223, 247 and 295 h of growth, samples were taken to analyze by HPLC-PDA and to measure OD_{600}.

Chemostat cultures (continuous cultures) (Chapter 3)

Continuous cultures were performance at 28 ºC in 0.5 L reactor (MiniBio, Applikon Biotechnology) with a working volume of 0.3 L. A temperature probe connected to a cryostat controlled the temperature cultures. pH was measured online and kept constant at 3.3 by the automatic addition of 1 N NaOH and 1 N HCl. The stirrer was set at 300 rpm. The population inoculated in the chemostat was approximately ∼2 × 10^6 cells/mL. The initial inoculum came from re-hydrating the active dry yeast (ADY) cells in sterile distilled water during 30 minutes at 37°C, and it was inoculated in the same media used for the indicated experiment. Previously to start the continuous culture, cells were allowed to grow in batch mode to achieve enough biomass. The continuous culture was connected when the batch culture entered the stationary phase. The dilution rate (D) was 0.15 h^{-1}. The indolic compound interest pulse in the culture was for a 0.5 mM final concentration and was performed only after all the continuous cultures had been running for at least five residence times, and the biomass values were constant. Once addition was done, a volume of ∼35 units of OD600 was sampled at 15 minutes and centrifuged at 4000 rpm for 3 minutes at 4°C. After centrifugation, the cell suspension was washed twice with sterile distilled water, and the pellet was frozen with liquid nitrogen and stored at −80°C until intracellular extractions. Finally, the supernatant was stored at −20°C until further extractions. Two independent chemostat cultures were run for each indolic pulse addition.

Oxidative and UV stress exposure to yeast cells and viable cell counting after stress exposure (Chapter 2)

Control and melatonin-intracellular enriched cells were resuspended in PBS and incubated for 1 h (30˚C, 300 rpm) with H_2O_2 to a final concentration of 10 mM.

For UV stress, 2–3 × 10^8 cells were resuspended in sterile PBS and dispersed on 90-mm Petri dishes at a depth of no more than 1 mm. Cells were irradiated with 106.5 and 248.5 J/m^2 UVC (254 nm) under a Vilber VL-6.C filtered lamp (Fisher

Biotec, Australia) using the lower radiation dose for the growth assays in liquid media, while the higher dose was used for the viability assays.

To calculate the viable yeast after stresses, both the control and melatonin-enriched yeast cells were washed twice and plated on solid media at an adequate dilution and incubated for 2 days at 28 ºC for counting the colony-forming units (CFU).

Intracellular ROS measurement by flow cytometry (Chapter 2)

After the treatment for melatonin enrichment, the intracellular ROS levels of the exponentially growing yeast cells were measured as described in Ballester-Tomás et al., (2015). Briefly, cells after 30 min treatment with melatonin and the untreated controls 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 the “Annexin V and Cell Death” channel of a flow cytometer Muse Cell Analyzer (Millipore, United States). 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.

Spot test (Chapter 4)

For the spot analysis, the cells, after grown on SC at 28 ºC up to the stationary phase were harvested by centrifugation, washed with sterile water, resuspended in sterile water to an OD_{600} value of 0.5, and followed by serial dilution. From each dilution, 3.5 µL were spotted onto SC-ura-panthotenate agar plates. Plates were incubated at 28 for 2 and 5 days.

Growth Curve Analysis (Chapter 5)

Yeast strain growth curves were screened by microtiter plate screening method. 96 well microtiter plates were used. Each well was filled with 250 µL of different media/condition and with 10^6 yeast cells/mL. 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 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 (ODt/OD0), OD0 is the initial OD and ODt is the OD at time t; D = ln (OD∞/OD0) 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 code (statistical software R, v.3.0) was used to facilitate calculating µmax and λ with Gompertz equation.

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

Yeast strains

The yeasts used in the present thesis are shown in Table M.1. All strains are belonging to genus *Saccharomyces* and to *S. cerevisiae* species, with the exception of VellutoBMV58 which belongs to *S. uvarum*.

Yeast strains were maintained in Petri plates with the solid appropriated medium at 4 °C for short-term conservation and in appropriated liquid medium:glycerol (65:35) at -80 °C for long-term conservation.

Transformation of *Saccharomyces* yeast cells

Yeast transformation was carried out by the lithium acetate/single-stranded carrier DNA/PEG method (Gietz and Woods 2002). Cells were incubated in the presence of lithium acetate, polyethylene glycol, DNA "carrier" and the plasmid or "cassette" of interest for 30 min at 30 ° C and subsequently are 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*. If the selection of transformants required the presence of antibiotics, the cells were incubated YPD for two or three hours under shaking after the heat shock. Subsequently, the cells were spread in the solid media with the antibiotic of interest and incubated at 30 ºC for 2 or 3 days.

For the construction of production strains, prior to transformation, the integrative plasmids were digested by *Not*I and column-purified (NZYGelpure, NZYTech). Approximately 1 µg DNA (single integrative) or 1.5 µg (multi-copy integrative, Ty2) was transformed into competent yeast cells whereas 0.3 µg were used for 2µ plasmids.

*Note: The selection of plasmids in yeast was based on the use of auxotrophic mutant strains that cannot grow without a specific media component (an amino acid, purine or pyrimidine). Transformation with a plasmid containing the mutated gene enables the transformant to grow on a medium lacking the required component.

Bacterial strains

Different bacterial strains were used for different purposes. Usually, DH5 Alpha cells were used to maintain and amplify plasmids. XL10-Gold ultracompetent cells and NZY5α were used for cloning large plasmids or ligated DNA. The information about the genotype of the different bacterial strains used and constructed in this thesis is in the table M.1.

Bacterial strains were maintained in Petri plates with LB solid medium with the appropriated antibiotics at 4 °C for short-term conservation and in water:glycerol (50:50) at -80 °C for long-term conservation.

Transformation of bacterial cells

The solid LB media plates containing the antibiotic of interest were pre-heated at 37 ºC during the transformation procedure. For transformation, 50 µl of competent cells were used. Plasmid DNA (10-100 ng) or up to 5 µl of ligation-reaction product were added to the cells and incubated on ice for 30 min. Heat shock at 42 ºC for 45 s were performed and then the cells were placed on ice for 2 min. Subsequently, 150 µl of liquid S.O.C medium was added and cells were incubated at 37 ºC for 45 min. After this period of time, cells were plated (only 50 µl in the case of plasmid transformation) onto the pre-heated LB plate with selective antibiotic. The plates were incubated at 37 ºC for overnight (12-16 h).

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

Yeast Strain	Relevant genotype/plasmid added	Chapter	Source
S288c	MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6	4, 5	(Mortimer and Johnston 1986)
BY4743	MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0	1, 2, 4, 5	EUROSCARF
BY4741	MATa; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0 met15Δ0	5	EUROSCARF
BY4741 trp1-	BY4741 trp1::loxP	5	This work
QA23	Prototrophic (HIS3, TRP1, LEU2, URA3)	1, 3	LALLEMAND
VELLUTOBMV58	Prototrophic (HIS3, TRP1, LEU2, URA3)	1	LALLEMAND
AROMA WHITE	Prototrophic (HIS3, TRP1, LEU2, URA3)	1	ENARTIS
BY4743Δaro10	BY4743 aro10::kanMX4	1	EUROSCARF
BY4743Δtrp1	BY4743 trp1::kanMX4	1	EUROSCARF
BY4743Δtrp2	BY4743 trp2::kanMX4	5	EUROSCARF
BY4743Δabz1	BY4743 abz1::kanMX4	5	EUROSCARF
BY4743Δpha2	BY4743 pha2::kanMX4	5	EUROSCARF
BY4743ΔΔadh3	BY4743 adh3::kanMX4	5	EUROSCARF
BY4743ΔΔnde1	BY4743 nde1::kanMX4	5	EUROSCARF

BY4743ΔΔnde2	BY4743 nde2::kanMX4	5	EUROSCARF
BY4743Δpaa1	BY4743 paa1::kanMX4	4	EUROSCARF
QA23Δtrp1	ho:: KanMX4; trp1::NatMX4	1	(Salvadó et al. 2012; López-Malo et al. 2014)
BY4743 DPL1	BY4743 with plasmid p426GPDDPL1 (URA3)	4	This work
BY4743 GAD1	BY4743 with plasmid p426GPDGAD1 (URA3)	4	This work
BY4743 DDC	BY4743 with plasmid p426GPDDDC (URA3)	4	This work
BY4743 PAA1	BY4743 with plasmid p426GPDPAA1 (URA3)	4	This work
BY4743 AANAT	BY4743 with plasmid p426GPD AANAT (URA3)	4	This work
BY4743 ERG6	BY4743 with plasmid p426GPDERG6 (URA3)	4	This work
BY4743 CRG1	BY4743 with plasmid p426GPDCRG1 (URA3)	4	This work
BY4743 ASMT	BY4743 with plasmid p426GPDASMT	4	This work
BY4743 DIT2 NCP1	BY4743 with plasmid p426GPDDIT2 and p425GPDNCP1	4	This work
BY4743 ERG5 NCP1	BY4743 with plasmid p426GPDERG5 and p425GPDNCP1	4	This work
BY4743 ERG11 NCP1	BY4743 with plasmid p426GPDERG11 and p425GPDNCP1	4	This work
BY4743 Δ37ERG11 NCP1	BY4743 with plasmid p426GPD Δ37ERG11 and p425GPDNCP1	4	This work
BY4743 Δ43ERG11 NCP1	BY4743 with plasmid p426GPD Δ43ERG11 and p425GPDNCP1	4	This work

BY4743 Δ51ERG11 NCP1	BY4743 with plasmid p426GPD Δ51ERG11 and p425GPDNCP1	4	This work
BY4743 Δ80ERG11 NCP1	BY4743 with plasmid p426GPD Δ80ERG11 and p425GPDNCP1	4	This work
BY4743 GST-Δ37T5H	BY4743 with plasmid p426GPDGST-Δ37T5H	4	This work
BY4743 HpaB	BY4743 with plasmid p426GPDhpaB	5	This work
BY4743 HpaC	BY4743 with plasmid p425GPDhpaC	5	This work
BY4743 HpaB + HpaC	BY4743 with plasmid p426GPDhpaB and p425GPDhpaC	5	This work
BY4743 HXT7p	BY4743 with plasmid p426 HXT7 and p425 HXT7	5	This work
BY4743 RTN2p	BY4743 with plasmid p426 RTN2 and p425 RTN2	5	This work
BY4743 HXT7 HpaB+HpaC	BY4743 with plasmid p426HXT7 hpaB and p425HXT7 hpaC	5	This work
BY4743 RTN2 HpaB+HpaC	BY4743 with plasmid p426RTN2 hpaB and p425 RTN2 hpaC	5	This work
BY4743 HpaB(A10) + HpaC	BY4743 with plasmid p426GPDhpaB(A10) and p425GPDhpaC	5	This work
BY4743 HpaBC	BY4743 with TEF1hpaC PGK1hpaB integrated into genome (Ty1Cons2)	5	This work
BY4743 + ARO10	BY4743 with plasmid p425GPD ARO10	5	This work
BY4743 ARO3	BY4743 HpaBC with plasmid p423GPD ARO3	5	This work
BY4743 ARO4	BY4743 HpaBC with plasmid p423GPD ARO4	5	This work
BY4743 ARO7	BY4743 HpaBC with plasmid p423GPD ARO7	5	This work
BY4743 HpaBC + ARO10	BY4743 HpaBC with plasmid p425GPD ARO10	5	This work

BY4743 ARO3K222L	BY4743 HpaBC with plasmid p423GPD ARO3K222L	5	This work
BY4743 ARO4K229L	BY4743 HpaBC with plasmid p423GPD ARO4K229L	5	This work
BY4743 ARO7G141S	BY4743 HpaBC with plasmid p423GPD ARO7G141S	5	This work
BY4741 trp1- HpaBC	BY4741 trp1-::loxP withTEF1hpaC PGK1hpaB into genome (Ty1Cons2)	5	This work
BY4741 HpaBC ARO3 ARO4 ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4 p425GPD ARO10	5	This work
BY4741 HpaBC ARO3 ARO4* ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4K229L p425GPDARO10	5	This work
BY4741 HpaBC ARO3* ARO4 ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 K222L p424GPD ARO4 p425GPDARO10	5	This work
BY4741 HpaBC ARO3* ARO4* ARO10	BY4741 trp1- HpaBC with plasmids p423GPD ARO3K222L p424GPD ARO4K229L p425GPDARO10	5	This work
BY4741 HpaBC ARO3 ARO4 ARO10 ARO7	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4 p425GPD ARO10 p426GPD ARO7	5	This work
BY4741 HpaBC ARO3 ARO4* ARO10 ARO7	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4K229L p425GPD ARO10 p426GPD ARO7	5	This work
BY4741 HpaBC ARO3* ARO4 ARO10 ARO7	BY4741 trp1- HpaBC with plasmids p423GPD ARO3K222L p424GPD ARO4 p425GPD ARO10 p426GPD ARO7	5	This work
BY4741 HpaBC ARO3* ARO4* ARO10 ARO7	BY4741 trp1- HpaBC with plasmids p423GPD ARO3K222L p424GPD ARO4K229L p425GPD ARO10 p426GPD ARO7	5	This work

BY4741 HpaBC ARO3 ARO4 ARO10 ARO7*	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4 p425GPD ARO10 p426GPD ARO7G141S	5	This work
BY4741 HpaBC ARO3 ARO4* ARO10 ARO7*	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 p424GPD ARO4K229L p425GPD ARO10 p426GPD ARO7G141S	5	This work
BY4741 HpaBC ARO3* ARO4 ARO10 ARO7*	BY4741 trp1- HpaBC with plasmids p423GPD ARO3 K222L p424GPD ARO4 p425GPD ARO10 p426GPD ARO7G141S	5	This work
BY4741 HpaBC ARO3* ARO4* ARO10 ARO7*	BY4741 trp1- p HpaBC with plasmids p423GPD ARO3 K222L p424GPD ARO4K229L p425GPD ARO10 p426GPD ARO7G141S	5	This work

Bacterial Strain	**Relevant genotype/plasmid added**	**Chapter**	**Source**
DH5α Subcloning Efficiency™	F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-	-	Thermo Fisher
NZYα	fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	-	NZYTech
One Shot TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG	-	Thermo Fisher
XL10-Gold	TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZDM15 Tn10 (Tetr) Amy Camr	-	Agilent
Rosetta (DE3)™	F- ompT hsdSB(rB- mB-) gal dcm (DE3) pRARE (CamR)	-	Novagen
Rosetta pGEX5X1	Rosetta transformed with pGEX-5X-1	4	This work
Rosetta DPL1	Rosetta transformed with pGEX-5X-1 DPL1	4	This work

Rosetta GAD1	Rosetta transformed with pGEX-5X-1 GAD1	4	This work
Rosetta DDC	Rosetta transformed with pGEX-5X-1 DDC	4	This work
Rosetta PAA1	Rosetta transformed with pGEX-5X-1 PAA1	4	This work
Rosetta AANAT	Rosetta transformed with pGEX-5X-1 AANAT	4	This work
Rosetta ERG6	Rosetta transformed with pGEX-5X-1 ERG6	4	This work
Rosetta CRG1	Rosetta transformed with pGEX-5X-1 CRG1	4	This work
Rosetta ASMT	Rosetta transformed with pGEX-5X-1 ASMT	4	This work
Rosetta DIT2	Rosetta transformed with pGEX-5X-1 DIT”	4	This work
Rosetta ERG5	Rosetta transformed with pGEX-5X-1 ERG5	4	This work
Rosetta ERG11	Rosetta transformed with pGEX-5X-1 ERG11	4	This work
Rosetta Δ37ERG11	Rosetta transformed with pGEX-5X-1 Δ37ERG11	4	This work
Rosetta Δ43ERG11	Rosetta transformed with pGEX-5X-1 Δ43ERG11	4	This work
Rosetta Δ51ERG11	Rosetta transformed with pGEX-5X-1 Δ51ERG11	4	This work
Rosetta Δ80ERG11	Rosetta transformed with pGEX-5X-1 Δ51ERG11	4	This work
Rosetta GST-Δ37T5H	Rosetta transformed with pETDuet1-GST-Δ37T5H	4	This work

Molecular biology techniques

Oligonucleotides

Table M.2. List of primers used in this thesis work

Name	Sequence 5’ to 3’	Chapter
ACT1 F	TCTGAGGTTGCTGCTTTGGT	2
ACT1 R	CCGACGATAGATGGGAAGACA	2
CTT1 F	ATTACATACGCCGCTCCATAC	2
CTT1 R	CAGTGTCTGGTGTACCACTTT	2
GPX2 F	CTTCACGCCGCAGTATAAAGA	2
GPX2 R	CCTGCTTCCCGAACTGATTAC	2
SOD1 F	TGCTGGTCCTCACTTCAATC	2
SOD1 R	TTCGTCCGTCTTTACGTTACC	2
TRX2 F	TTCTTCCATGCCTACCCTAATC	2
TRX2 R	GCAATAGCTTGCTTGATAGCAG	2
CTA1 F	TCTGCGGGTCTGCTATGTTT	2
CTA1 R	TCGGCACTACCTTTATCACCAC	2
GPX3 F	GAACCTGGCTCTGATGAAGAA	2
GPX3 R	GGTCCTCATTGCCACCATTA	2
SOD2 F	TGGGAACACGCCTACTACTT	2
SOD2 R	TCTTTCCAGTTGACCACATTCC	2
TRX3 F	GATGATGCAACCACACTTAACG	2
TRX3 R	GCCGTCACTTCACACTCTT	2
RAD18 F	AGGTTCATCGGACAGTTCAG	2
RAD18 R	TCGGTTCCCTGGTCACTTT	2
RAD52 F	TTCCAGCGAGTGTGCTAAAA	2

RAD52 R	TACTTGATTCCCAGCCCCTTC	2
DPL1 F BamHI	AGGTCGTGGGATCCCCATGAGTGGAGTATCAAATAAAACA	4
DPL1 R XhoI	TGCGGCCGCTCGAGCTACTTGGTGGCGGTATCCT	4
GAD1 F SalI	ACGCGTCGACGTCATGTTACACAGGCACGGTT	4
GAD1 R XhoI	TGCGGCCGCTCGAGTCAACATGTTCCTCTATAGTTTC	4
DDC F BamHI	AGGTCGTGGGATCCCCATGAATGCAAGCGAATTTCGT	4
DDC R XhoI	TGCGGCCGCTCGAGTTATTCACGTTCGGCACGCA	4
DIT2 F EcoRI	GGAATTCATGGAGTTGTTAAAGCTTCT	4
DIT2 R XhoI	CCGCTCGAGTTATTCCATTATATTCTCGTTAA	4
ERG5 F EcoRI	CCGATATCATGAGTTCTGTCGCAGAAAAT	4
ERG5 R XhoI	CCGCTCGAGTTATTCGAAGACTTCTCCAGTA	4
ERG11 F EcoRI	CCGATATCATGTCTGCTACCAAGTCAAT	4
ERG11Δ37 F FseI	TAAGGCCGGCCATGCCTTTCATTTACAATATTGT	4
ERG11Δ43 F FseI	TAAGGCCGGCCATGGTATGGCAATTACTATATTCTTTG	4
ERG11Δ51 F FseI	TAAGGCCGGCCATGAGAAAGGACCGTCCAC	4
ERG11Δ80 F FseI	TAAGGCCGGCCATGGAAGAATGTCAAAAGAAATA	4
ERG11 R XhoI	CCGCTCGAGTTAGATCTTTTGTTCTGGATT	4
Δ37 T5H F SmaI	GCCCGGGATGCTGCCGCCGTCGCCGCCG	4
GST-Δ37 T5H F XmaI	GCCCGGGATGTCCCCTATACTAGGTTATTG	4
Δ37 T5H R EcoRI	GGAATTCTTAAACCTCACTAAGCTCCTC	4
PAA1 F BamHI	AGGTCGTGGGATCCCCATGGCCTCCTCAAGTAGCA	4
PAA1 R XhoI	TGCGGCCGCTCGAGCTAGTTGTCGTATTCTTCCTTAAT	4
AANAT F BamHI	AGGTCGTGGGATCCCCATGAGCACCCCGAGCAT	4
AANAT R XhoI	TGCGGCCGCTCGAGTTAACGATCGCTATTACGACGCA	4
ERG6 F BamHI	AGGTCGTGGGATCCCCATGAGTGAAACAGAATTGAGA	4
ERG6 R XhoI	TGCGGCCGCTCGAGTTATTGAGTTGCTTCTTGGGA	4
CRG1 F EcoRI	CGGAATTCATGCCTAAAACTAGTTATTTAAAC	4

CRG1 R XhoI	CCGCTCGAGTCATTCTCTTTTCCTACATAAG	4
ASMT F BamHI	AGGTCGTGGGATCCCCATGGGTAGCAGCGAAGATCA	4
ASMT R XhoI	TGCGGCCGCTCGAGTTATTTACGTGCCAGGATTGCA	4
COMT BamHI	AGGTCGTGGGATCCCCATGGGTTCAACGGCAGAGACA	4
COMT R XhoI	TGCGGCCGCTCGAGTTAGAGCTTCTTGAGTAACTCAATAAGG	4
ARO10 F BamHI	AGGTCGTGGGATCCCC ATGGCACCTGTTACAATT	5
ARO10 R XhoI	TGCGGCCGCTCGAGCTATTTTTTATTTCTTTTAAGTG	5
hpaB F BamHI	AGGTCGTGGGATCCCCATGAAACCAGAAGATTTCCG	5
hpaB R XhoI	CCGCTCGAGTTATTTCAGCAGCTTATCCAGC	5
hpaC F BamHI	AGGTCGTGGGATCCCCATGCAATTAGATGAACAACGC	5
hpaC R XhoI	CCGCTCGAGTTAAATCGCAGCTTCCATTTCC	5
F A10 HpaB	CATGATTGGCTTCGGCACCCTGGAAGTAATGGGCGAAAA	5
R A10 HpaB	TTTTCGCCCATTACTTCCAGGGTGCCGAAGCCAATCATG	5
ARO3 F BamHI	GTCGTGGGATCCTTATGTTCATTAAAAACGATCACGCCGGTG	5
ARO3 K222L R	GCAGTGACACCTGGCaaTGTGACAGAAAGG	5
ARO3 K222LF	CCTTTCTGTCACAttGCCAGGTGTCACTGC	5
ARO3 R XhoI	CCGCTCGAGCTATTTTTTCAAGGCCTTTCTTCTGTTTCTAACACC	5
ARO4 F BamHI	GTCGTGGGATCCAAATGAGTGAATCTCCAATGTTCGCTGCCAAC	5
ARO4 K229L R	GCAGCAACACCATGCaaAGTAACACCCATG	5
ARO4 R XhoI	CCGCTCGAGCTATTTCTTGTTAACTTCTCTTCTTTGTCTGACAGC	5
ARO4 K229L F	CATGGGTGTTACTttGCATGGTGTTGCTGC	5
ARO7 F BamHI	GTCGTGGGATCCATATGGATTTCACAAAACCAGAAACTGTTT	5
ARO7 G141S R	CTAGTGGCAACAGAAGAGAAGTTATTCTTA	5
ARO7 G141S F	TAAGAATAACTTCTCTTCTGTTGCCACTAG	5
ARO7 R XhoI	CCGCTCGAGTTACTCTTCCAACCTTCTTAGCAAGTATTCC	5
GV1R (HpaC)	CGTGCGAUTTAAATCGCAGCTTCCATTTCC	5
GP1F (HpaC)	AGTGCAGGUAAAACAATGCAATTAGATGAACAACGC	5

PG1R(TEF1p)	ACCTGCACUTTTGTAATTAAAACTTAGATTAGATTGCTAT	5
PG2R (PGK1p)	ATGACAGAUTTGTTTTATATTTGTTGTAAAAAGTAGATAATT	5
GP2F (HpaB)	ATCTGTCAUAAAACAATGAAACCAGAAGATTTCCG	5
GV2R(HpaB)	CACGCGAUTTATTTCAGCAGCTTATCCAGC	5
GPDPro-F	CGGTAGGTATTGATTGTAATTCTG	4, 5
p426GPD seq R	GACCTAGACTTCAGGTTGTC	4, 5
CYC1-R	GCGTGAATGTAAGCGTGAC	4, 5
HpaB mid R	AACGAACATCAGTGCGAAGT	5
RTN2p F (SacI)	CCCGAGCTCTCCCCAGGGTCAATTGACAA	5
RTN2p R (BcuI)	CCCACTAGTGTTGATGATAGTTTGTGGCAAGTTG	5
HXT7p F (SacI)	CCCGAGCTCAACAAACATCTCATCTAGTT	5
HXT7p R (BcuI)	CCCACTAGTTTTTTGATTAAAATTAAAAAAAC	5

Plasmids

Table M.3 List of plasmids used in this thesis

Plasmid name	Relevant characteristics	Integration site/ replicon	Marker	Source	Chapter
pGEX-5X-1	Taq promoter-GST-MCS	pBR322	AmpR	GE Healthcare	4
p423GPD	TDH3p-MCS-CYC1t	2µ	HIS3	(Mumberg et al. 1995)	5
p424GPD	TDH3p-MCS-CYC1t	2µ	TRP1	(Mumberg et al. 1995)	5
p425GPD	TDH3p-MCS-CYC1t	2µ	LEU2	(Mumberg et al. 1995)	4, 5
p426GPD	TDH3p-MCS-CYC1t	2µ	URA3	(Mumberg et al. 1995)	4, 5
p427GPD	TDH3p-MCS-CYC1t	2µ	MET15	This work	5
p425RTN2	RTN2p-MCS-CYC1t	2µ	LEU2	This work	5
p425HXT7	HXT7p-MCS-CYC1t	2µ	LEU2	This work	5

p426RTN2	RTN2p-MCS-CYC1t	2µ	URA3	This work	5
p426HXT7	HXT7p-MCS-CYC1t	2µ	URA3	This work	5
pCfB2988	USER cloning cassette-ADH1&CYC1 t	TY1Cons2	LoxURA3	(Maury et al. 2016)	5
p426GPD DPL1	TDH3p-DPL1-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 DPL1	Taq promoter-GST-DPL1	-	AmpR	This work	4
p426GPD GAD1	TDH3p-GAD1-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 GAD1	Taq promoter-GST-GAD1	-	AmpR	This work	4
p426GPD DDC	TDH3p-DDC-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 DDC	Taq promoter-GST-DDC	-	AmpR	This work	4
p426GPD DIT2	TDH3p-DIT2-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 DIT2	Taq promoter-GST-DPL1	-	AmpR	This work	4
p426GPD ERG5	TDH3p-ERG5-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 ERG5	Taq promoter-GST-ERG5	-	AmpR	This work	4
p426GPD ERG11	TDH3p-*ERG11*-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 ERG11	Taq promoter-GST-ERG11	-	AmpR	This work	4
p426GPD T5H	TDH3p-GST∆37aaT5H-CYC1t	2µ	URA3	This work	4
pETDuet1-GST-∆37T5H	GST∆37aaT5H	-	AmpR	(Park et al. 2011)	4
p426GPD PAA1	TDH3p-PAA1-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 PAA1	Taq promoter-GST-PAA1	-	AmpR	This work	4
p426GPD AANAT	TDH3p-AANAT-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 AANAT	Taq promoter-GST-AANAT	-	AmpR	This work	4
p426GPD ERG6	TDH3p-ERG6-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 ERG6	Taq promoter-GST-ERG6	-	AmpR	This work	4
p426GPD CRG1	TDH3p-CRG1-CYC1t	2µ	URA3	This work	4
pGEX-5X-1 CRG1	Taq promoter-GST-CRG1	-	AmpR	This work	4

pCfB1252	TEF1p-HsASMT	XII-1	LoxLEU2	(Germann et al. 2016)	4
pCfB2628	TEFp-HsDDC, PGK1p-BtAANAT	XI-5	HIS5	(Germann et al. 2016)	4
p425GPDhpaC	TDH3p-hpaC-CYC1t	2µ	LEU2	This work	5
p426GPDhpaB	TDH3p-hpaB-CYC1t	2µ	URA3	This work	5
p426GPDhpaB(A10)	TDH3p-hpaB(S210T/A211L/Q212E) -CYC1t	2µ	URA3	This work	5
p425HXT7hpaC	HXT7p-hpaC-CYC1t	2µ	LEU2	This work	5
p425RTN2 hpaC	HXT7p-hpaC-CYC1t	2µ	LEU2	This work	5
p426HXT7hpaB	HXT7p-hpaB-CYC1t	2µ	URA3	This work	5
p426RTN2 hpaB	HXT7p-hpaB-CYC1t	2µ	URA3	This work	5
pCfB2988 HpaBC	TEF1p::hpaC PGK1p::hpaB	TY1Cons2	LoxURA3	This work	5
p423GPDARO3	TDH3p-ARO3-CYC1t	2µ	HIS3	This work	5
p423GPDARO3K222L	TDH3p-ARO3K222L-CYC1t	2µ	HIS3	This work	5
p423GPDARO4	TDH3p-ARO4-CYC1t	2µ	HIS3	This work	5
p423GPD-ARO4K229L	TDH3p-ARO4K229L-CYC1t	2µ	HIS3	This work	5
p424GPDARO4	TDH3p-ARO4-CYC1t	2µ	TRP1	This work	5
p424GPD-ARO4K229L	TDH3p-ARO4K229L-CYC1t	2µ	TRP1	This work	5
p425GPDARO10	TDH3p-ARO10-CYC1t	2µ	LEU2	This work	5
p423GPDARO10	TDH3p-ARO10-CYC1t	2µ	HIS3	This work	5
p426GPDARO7	TDH3p-ARO7-CYC1t	2µ	URA3	This work	5
p426GPDARO7G141S	TDH3p-ARO7G141S-CYC1t	2µ	URA3	This work	5
p423GPDARO7	TDH3p-ARO7-CYC1t	2µ	HIS3	This work	5
p423GPDAROG141S	TDH3p-ARO7G414S-CYC1t	2µ	HIS3	This work	5

Molecular techniques for the study and manipulation of DNA

Genomic DNA extraction from yeast

Obtaining yeast genomic DNA was normally carried out from 1-5 ml of culture in YPD medium following the isolation method described by Querol et al. (1992). Yeasts were grown in 5 mL YPD at 28 ° C for overnight and collected by centrifugation during 1 min at 4000 rpm. The supernatant was removed and the cells were washed twice with 1 mL of sterile water. The cell pellet was resuspended in 500 µL of Buffer 1 (0.9 M sorbitol, 0.1 M EDTA pH 7.5) and. 30 µL of Zimolyase (amsbio) was added, mixed by invertion and incubated for 30 min. at 37 ºC. After centrifugation of 3 min. at 12000 rpm, the supernatant was removed and the protoplasts were resuspended in 500 µL of Buffer 2 (50mM Tris pH 7.4, 20mM EDTA). Then 13 µL of 10% SDS was added and incubated during 5 min. at 65 ºC. After incubation, 200 µL of 5M potassium acetate was added, and the tubes were mixed by invertion and incubated on ice for a minimum of 10 min. After incubation, the tubes were centrifuged at 4ºC for 15 min. at 14000 rpm to remove the SDS completely and the supernatant was transferred to a new tube. 700 µL of isopropanol were added to the supernatant and the sample was incubated at room temperature for 10 minutes. After incubation, the tubes were centrifuged for 15 min. at 12000 rpm the cold and the supernatant was discarded. The precipitated pellet was washed with 500 µL of 70% ethanol. After centrifugation for 3 min. at 12000 rpm, the supernatant was eliminated and the pelled was dried under vacuum and re-suspended in TE (10 mM Tris, 1mM EDTA pH 8.0) or Milli-Q water.

Plasmid extraction from yeast cells

For plasmid extraction from yeast cells we adapted the method described by Singh and Weil, (2002) in which silica gel-based columns are used. This protocol is described using the NZYMiniprep from NZYTech. For plasmid extraction 2-5 mL of yeasts were grown on selective medium for overnight or alternatively ¼ of the surface (fully covered) from a fresh plate can be taken directly. Cells were re-suspended in 500 µL of Buffer 1 (from genomic DNA extraction protocol) and. 30 µL of Zimolyase (amsbio) was added, mixed by invertion and incubated for 1 h.

at 37 ºC. After centrifugation of 1 min. at 11000 rpm, the supernatant was removed and the protoplasts were re-suspended in 250 µL of the Buffer A1 and then 100 - 200 µL of glass beads of 0.5 mm diameter were added. The protoplasts were ruptured by glass bead beating by applying three shaking cycles at 30 s−1 for 30 s in a Tehtnica MillMix 20 homogenizer (Tehtnica, Slovenia) at 4◦C. 250 µL of lysis Buffer A2 was added and homogenization was performed by inversion 6-10 times. The mixture was incubated at room temperature for a maximum of 5 min and after this time, 300 µL of Buffer A3 was added and mixed by inversion 6-10 times to neutralize the lysis reaction. Immediately the mixture was centrifuged at maximum speed to clarify the lysate and after centrifugation the supernatant was transferred to a new 1.5 mL tube and centrifuged again for 1 min at maximum speed. Then, up to 750 µL of supernatant was transferred to a NZYTech spin column and centrifuged for 1 min at 11.000 g. The flow-through was discarded and the column was washed with 500 µL of Buffer AY. After centrifugation for 1 min at maximum speed, the flow-through was discarded and 600 µL of Buffer A4 was added onto the column. After centrifugation for 1 min at maximum speed, the NZYTech spin column was placed into a new empty 1.5 mL tube and centrifuged at maximum for 2 min in order to dry the silica membrane. Finally, the dried column was trasferred into a clean 1.5 mL microcentrifuge tube and 50 µL of Buffer AE was added into the center part of the column. After 1 min incubation at room temperature, the plasmidic DNA was eluted by centrifugation for 1 min at maximum speed.

Plasmid extraction from bacterial cultures

For plasmid isolation and purification from *E. coli* transformed cells, the NZYMiniprep or NZYMidiprep were used following the protocol provide by the manufacturer. Usually, overnight cultures coming from tubes with 5 mL of of LB with the antibiotic inoculated with a single colony, grown at 37 ºC in a roller shaker, were employed for NZYMiniprep.

Plasmid construction

The construction of plasmids was carried out following different strategies:

i) Traditional cloning:

The DNA sequence to be cloned (insert) was amplified by PCR using oligonucleotides (Table M2) that introduce restriction sites at the ends of the amplified fragment and that allow its insertion into the corresponding vector. Next, the insert and the vector were digested with the corresponding restriction enzymes and their ligation was performed using T4 DNA ligase provided in the Rapid DNA ligation kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. The ligation reaction mixture was directly used for bacterial transformation prior to heat inactivation of T4 DNA Ligase at 65 °C for 10 min or at 70 °C for 5 min.

ii) USER cloning:

The DNA sequences to be cloned (Biobricks) were amplified by PCR with primers containing uracils (Table M.2) by using Phusion U Hot Start DNA. In parallel, the EasyClone vectors containing USER cloning sites were prepared by sequentially treatment with the enzymes AsiSI (SfaAI) (Thermo Fisher Scientific) and BsmI (New England Biolabs). After purification, the prepared vectors and the biobricks were mixed and treated with USER^{TM} enzyme and after the reaction, the mixture was directly used for bacterial transformation.

Successful cloning in the different vectors was identified by PCR in *E. coli* colonies or by small-scale digestion of plasmid DNA, purified from recombinant clones after overnight cultivation, using restriction enzymes. To verify the exact sequence of the insert, the cloned inserts were sequenced using Sanger sequencing (Eurofins genomics) and sequencing primers (Table M2).

Polymerase chain reaction and DNA electrophoresis in agarose gels

For the amplification of DNA fragments from a genomic DNA or plasmid template, different DNA polymerases were used depending on the need for error correction activity: Biotaq (Bioline) or NZYTaq II DNA polymerase (NZYTech) without 3´→5´ exonuclease activity and Phusion High-Fidelity (ThermoFisher Scientific) with 3´→ 5´ exonuclease activity. Additionally, Phusion U Hot Start DNA Polymerase (ThermoFisher Scientific) was used in order to construct biobricks with primers containing uracils. 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. Primers were ordered from IDT. The T_m of the primers used and the appropriate annealing temperature for the different DNA polymerases was estimated with the on-line tool Tm Calculator from NEB (https://tmcalculator.neb.com/). MultiGene™ OptiMax Thermal Cycler (Labnet) was employed to synthesize the deletion/insertion cassettes, to check the deletion/insertion, to perform site-directed mutagenesis, colony PCR and for USER cloning. The usually conditions and cycling instructions for the different programs used were the folowing:

Deletion cassette amplification

The deletion cassette was amplified from different plasmids (Table M.3) or by amplifying the KanMX4 cassette and flanking regions (about 500-pb upstream and downstream) from the corresponding mutant strain in the BY4743 background. Primers used have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at the correct temperature for each plasmid for 30 sec and elongation at 72 ºC for 1 min/kb or 15-30 sec/kb when Biotaq (Bioline) or NZYTaq II (NZYTech) was used, respectively. The amplification terminated with an extended incubation at 72 ºC for 7 min and cooling to 16 ºC.

Deletion/cassette insertion verification

The genomic DNA of possible mutants was analyzed by PCR using primers upstream and downstream of the deleted/integrated region, and a combination with primers from the insert/deletion cassette introduced (Table M.2). Reactions were run as for deletion cassette amplification with an elongation time depending on the different PCR product length.

Site-directed mutagenesis by PCR

Two target gene segments were amplified from genomic template DNA by a first PCR using four primers, including two flanking primers containing restriction

enzyme sites and two mutagenic primers. 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). This fragment can be cloned into a vector since it is flanked by the restriction enzymes introduced by the external primers.

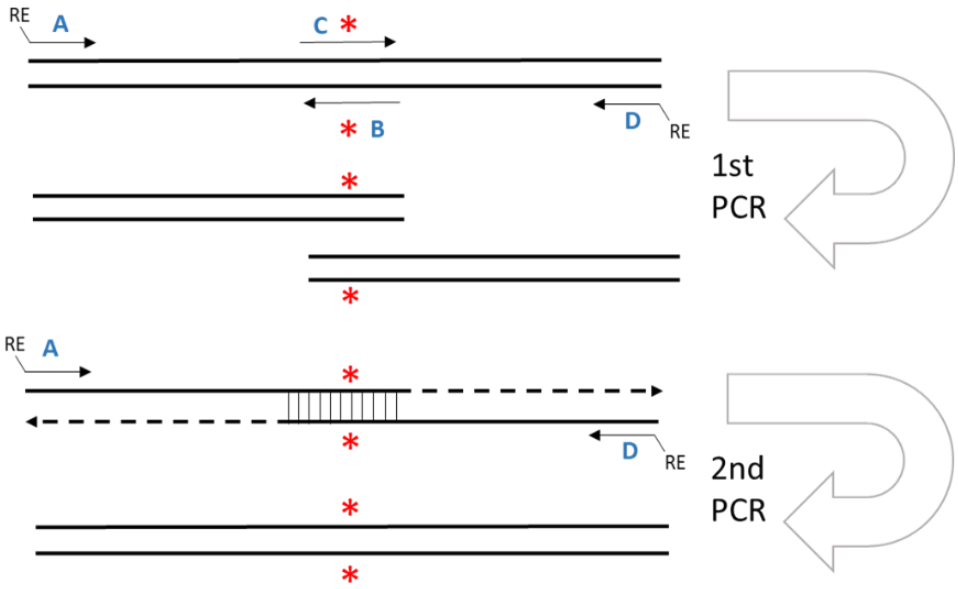

Figure M.1 Site-directed mutagenesis by PCR from yeast genomic DNA used in the present work. In the diagram, primers B and C contain the mutation of interest (mutagenic primers) whereas primers A and D contain a restriction enzyme site (flanking primers). The first round of PCR uses primers A-B and C-D to create two products with the mutated sequence. In the second PCR round, since primers B and C contain complementary sequences, the products from the first round will hybridize after they are denatured following the first PCR cycle. Primers A-D can then be used to amplify the full-length product that contains the desired mutation and the restriction enzyme sites for later cloning the mutated gene into a plasmid. Alterations to this method can also create deletions or longer additions.

Agarose gels were used to separate the DNA at different concentrations of agarose D1 Low EEO (Condalab), between 0.8 and 2%, depending on the size of the DNA fragments. The gels were prepared with 1X TAE buffer (Tris base pH 8,4 M glacial acetic acid, 1 mM EDTA) which was also used as electrophoresis buffer. To visualize the nucleic acids under UV light, RedSafe™ Nucleic acid

staining (iNtRON) was added to the agarose gels. The DNA was resuspended in loading buffer and as a standard of sizes to estimate the fragment of the DNA isolates, different ladders were used including GeneRuler 1Kb and GeneRuler 100 bp DNA Ladder (Thermo Scientific).

Molecular techniques for the study of RNA

Previous to RNA extraction, yeast cells were centrifuged at 4 ºC and washed with distilled water and frozen with liquid nitrogen and then stored at -80 ºC.

The cells were resuspended in 500uL of LETS buffer (0.1 M LiCl, 10 mM EDTA pH 8.0, 0.2% SDS, 10 mM Tris-HCl pH 7.4) and transferred to tubes containing glass beads and 500ul phenol: chloroform (5 :1). Cells were disrupted by cold 30 second shaking cycles using Millmix 20 bead beater (Tehtnica). The supernatant was transferred to another tube containing 1 volume of phenol: chloroform (5: 1) and subsequently to another with chloroform-isoamyl alcohol (25: 1). Finally, the RNA was precipitated by adding 2.5 volumes of cold 96% ethanol and 0.1 volume of 5 M LiCl. A second precipitation was performed by adding 2.5 volumes of 96% ethanol and 0.1 volume of 3 M sodium acetate. Subsequently, the RNA was resuspended in RNase-free milliQ water and its concentration and integrity were determined using the NanoDrop and agarose gel electrophoresis. Then, in order to eliminate the DNA, 1 µg of RNA was treated was treated for 15 min at 25◦C with DNase I RNase-free (Roche) according to the manufacturer's protocol. NZY First-Strand cDNA Synthesis Kit (NZyTech) was used to synthesize cDNA from the DNase I-treated RNA following the manufacturer's recommendations.

Quantitative real-time PCR was performed in a Light Cycler 480 II (Roche) using the TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) 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 (2×10^{-1}, 1×10^{-1}, 2×10^{-2}, 1×10^{-2}, 2×10^{-3}, 1×10^{-3}). The primers used to determine the transcript levels are in the table M.2.

Molecular techniques for the study of proteins

For check the maintenance of the reading frame between GTS and the corresponding protein as well as the better conditions for expression induction (IPTG concentration and temperature), aliquots of cells were collected by centrifugation before and after IPTG induction. Cell pellets were either sonicated on ice in minimal volumes of 50 mM Hepes pH 7.4 containing 0.1% Triton X-11 or directly resuspended in 75 µL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The sample for SDS-PAGE was heated at 95°C for 10 min. Typically 5 µL of this was used for electrophoresis.

Electrophoresis under denaturing conditions to separate proteins were performed in polyacrylamide gels (SDS-PAGE), varying the concentration of polyacrylamide as a function of the molecular weight of the study protein. The MiniProtean 3 system (BioRad) was used with 1X SDS protein electrophoresis buffer (25 mM Tris Base, 192 mM Glycine, 0.1% SDS, pH: 8.1 - 8.5) at a constant voltage of 100 V. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used as a protein standard and ladder.

In silico analysis

BLAST

Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) was used to identify putative melatonin biosynthetic genes in *S. cerevisiae* from orthologous sequences in other classes of organisms. More specifically BLASTp, which searches protein databases using a protein query combined with the domain enhanced lookup time accelerated BLAST (DELTA-BLAST) algorithm, which searches a database of pre-constructed position-specific score matrix (PSSMs) using the results of a Conserved Domain Database search before searching a protein-sequence database, to yield better homology detection (Boratyn et al. 2012).

Serial cloner

The molecular biology software Serial cloner was used for *in silico* DNA cloning, sequence analysis and visualization and sequence alignment. This software is free and can be downloaded from: http://serialbasics.free.fr/Serial_Cloner.html

Neighbour-joining tree construction

The phylogenetic tree, using different plant and vertebrate SNATs including the PAA1 sequence from *S. cerevisiae,* was constructed using the neighbor-joining method and a bootstrap test with 1000 iterations. The GenBank accession numbers of the sequences used for the aligment were: NP_442603 (cyanobacteria, Synechocystis sp. PCC 6803, SNAT), AK059369 (rice, *O. sativa*, SNAT1), AK068156 (rice, O. sativa, SNAT2) ABD19662 (Arabidopsis, *A. thaliana*, SNAT), NP_001009461 (sheep, *O. aries*, SNAT), NM_009591 (mouse, *M. musculus*, SNAT), KJ156532 (apple, *M. zumi*, SNAT3), KJ156533 (apple, *M. zumi*, SNAT4). The phylogenetic tree was constructed with the MEGA6 software (Tamura et al. 2011).

Analytical techniques

Voltammetry of immobilized particles (VIMP)

All the electrochemical determinations were carried out in the laboratory of Dr. Antonio Doménech Carbó in the Department of Analytical Chemistry located in the Faculty of Chemistry at the University of Valencia. Figure M.2 shown a schematic representation of experimental setup for the electrochemical measurements.

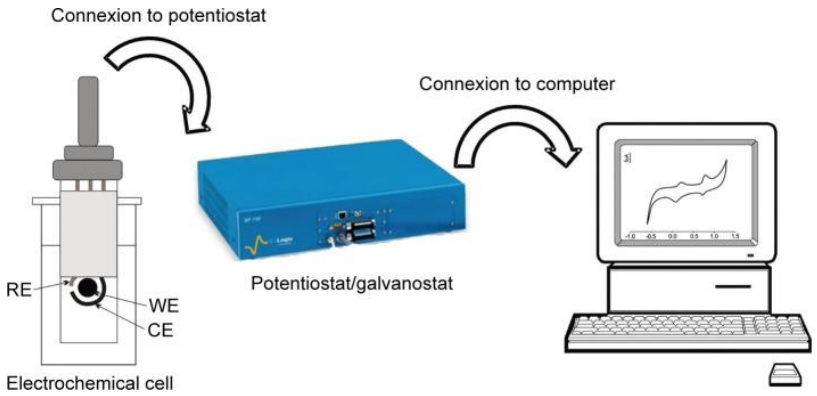

Figure M.2 Experimental setup of the electrochemical measurements for melatonin detection. electrochemical cell, potentiostat/galvanostat and personal computer. RE, reference electrode; WE, working electrode; CE: counter electrode (Apetrei and Apetrei, 2016).

Chemicals and solutions

Stock solutions of each indolic compound (tryptophan, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, 3-indoleacetic acid and tryptophol) were freshly prepared and diluted as required. All reagents used were of analytical grade and were purchased from Sigma Aldrich (Sigma-Aldrich, USA).

Supporting electrolytes used for electrochemical experiments were aqueous acetic acid/sodium acetate (HAc/NaAc) buffer solutions, concentration 0.25 M and 0.10 M, pH 4.75 (Panreac, Spain).

Electrochemical measurements directly on yeast cells

After incubation with the interest indolic compound, 10 mL of each culture was centrifuged (10 min at 4000 rpm). The obtained pellet (~10^9 cells) was washed twice with distilled water and transferred to a microcentrifuge tube to dry cells at 60 °C for around 16 h. 1 mg of dried cells was powdered with an agate mortar and pestle by adding 0.5 mL of ethanol (Panreac, Spain) for 2 min. Then, 50 µL of the resulting suspension was dropped onto the GCE surface. After solvent evaporation in air, the electrode was inserted into the electrochemical cell and electrochemical runs were performed. Linear potential scan (LSV), cyclic (CV), and square wave voltammetry (SWV) were used as the detection modes.

Instrumentation and voltammetric measurements

Electrochemical measuraments were taken at 298 ± 1 K in a CH cell using a laboratory CH I660 potentiostat (Cambria Scientific, Llwynhendy, Llanelli, Wales, UK). A BAS MF2012 glassy carbon working electrode (GCE) (geometrical area 0.071 cm^2), a platinum wire auxiliary electrode, and an Ag/AgCl (3 M NaCl) reference electrode were used in a conventional three-electrode arrangement (Figure M.1). Voltammetric measurements were taken in the solutions of an intracellular metabolite content at unmodified GCE and with freshly prepared, sample-modified GCEs immersed in aqueous acetate buffer. For electrode conditioning, ca. 1 mg of dried cells was powdered with an agate mortar and pestle by adding 0.5 mL of ethanol (Panreac, Spain) for 2 min. Then, 50 µL of the resulting suspension was dropped onto the GCE surface. After solvent evaporation in air, the electrode was inserted into the electrochemical cell and electrochemical runs were performed. Linear potential scan (LSV), cyclic (CV), and square wave voltammetry (SWV) were used as the detection modes.

High performance liquid chromatography (HPLC)

Intra- and extracellular extraction methods for HPLC analysis

Intracellular metabolites extraction with ethanol and glass beads for melatonin and melatonin intermediates analyses

1 mL of cold mixture ethanol/water 50% (v/v) to the cell pellets and 0.3 mL of glass beads. Cells were ruptured by a Tehtnica MillMix 20 homogenizer (Tehtnica) with three shaking cycles at 30 s−1 for 30 s at 4°C. Lysed cells were centrifuged at 5000 g and 4°C for 10 minutes to remove the insoluble particles. The supernatant was transferred to a new tube, and stored at −20°C until further analyzed by UHPLC-MS/MS.

Intracellular metabolites extraction by the boiling buffered ethanol (adapted from González et al., 1997)

Three mililiter of a solution of 75 % (v/v) boiling absolute ethanol that contained 70 mM (final concentration) of HEPES buffer (pH 7.5) was added to the cell pellet. This mixture was incubated for 3 min at 80 °C and for 3 min on ice. The extract

was concentrated by evaporation of the volume at 45 °C in a 5301 Concentrator plus/Vacufuge® plus (Eppendorf, Spain). The final intracellular content was resuspended in 1 mL of ultrapure Milli-Q water and centrifuged at 5000g for 10 min at 4 °C to remove insoluble particles. The supernatant was transferred to a new tube and stored at −20 °C until analysis.

Extracellular content treatment for melatonin and melatonin intermediates analyses

For the extracellular extraction, an equal amount of absolute ethanol was added to the culture. The mixture was stored at −20°C until analysis.

Extracellular content treatment for higher alcohols and hydroxytyrosol analyses

For the extracellular extraction, an equal amount of absolute methanol was added to the culture. The mixture was stored at −20°C until analysis.

Melatonin and other indolic compounds determination by UHPLC-MS/MS

UHPLC-MS/MS analysis were performed in the mass spectrometry section of the Central Support Service for Experimental Research of the University of Valencia (SCSIE).

All the samples and standard solutions were diluted as required and prior injection into HPLC the intra or extracellular samples were centrifuged at 7500 *g* for 5 min at 4 °C and the supernatant was filtered by a 0.22 µm nylon filter.

Standard solution preparation

The stock solutions of tryptophan, 5-hydroxytryptophan and serotonin were prepared with water whereas for tryptamine, *N*-acetylserotonin and melatonin the stock solutions were prepared in ethanol absolute (Panreac-AppliChem, Spain). The prepared solutions were stored at -20 °C when not in use for a maximum of one month. The patterns for HPLC calibration were prepared from stock solutions of 1 mg/mL of each indolic compound in methanol and diluted in ultrapure Milli-Q water. The final concentrations of the calibration standards were 0, 0.1, 1, 10, 100, and 1000 ng/mL, which were prepared freshly before use.

Instrumentation and chromatographic conditions

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 221 → 162.19 and 221 → 203.99 for 5-hydroxytryptophan, m/z 219 → 159.96 and m/z 219 → 202.00 for *N*-acetylserotonin, m/z 205 → 118.03 and m/z 205 → 145.85 for L-tryptophan, 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.

Higher alcohols and hydroxytyrosol determination

All the samples and standard solutions were diluted as required and prior injection into HPLC the intra or extracellular samples were centrifuged at 7500 *g* for 5 min at 4 °C and the supernatant was filtered by a 0.22 µm nylon filter.

Standard solution preparation

For standard solution preparation, individual stock solutions of shikimic, tyrosol, hydroxytyrosol, 2-phenylethanol and tryptophol were prepared by dissolving the compounds in methanol. The prepared solutions were stored at -20 °C. The patterns for HPLC calibration were prepared from stock solutions of each compound in methanol and diluted in ultrapure Milli-Q water. The final concentrations of the calibration standards were 0, 10,100, 500, 1000 and 5000 ng/mL, which were prepared freshly before use.

Instrumentation and chromatographic conditions

HPLC-PDA

The hydroxytyrosol and higer alcohols (tyrosol, 2-phenylethanol and tryptophol) were detected by HPLC on Acquity ARC sytem core (Waters, USA) equipped with a photodiode array wavelength detector (Waters 2998 PDA), a quaternary pump, autosampler and on-line degasser. Chromatographic separation was carried on 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, 100:0 % (v/v), 18–19 min 90:10 % (v/v), 19–28 min 75:25 % (v/v), 28 – 31 min 0:100 % (v/v) and 31 -39 min 100:0 % (v/v). The column temperature was set at 30 °C and the samples were kept at 10 ºC. PDA detector was set at **λ =** 210 nm. Identification of all the higher alcohols and hydroxytyrosol was based on their retention times, determined injecting the reference standards individually and as a mixture. The calibration curves of each analyte, i.e., peak area *versus* concentration, were linear and data were fitted by the least-squares method. Linearity was assessed by least squares fitting of independent six-points calibration curves. The retention time for hydroxytyrosol, tyrosol, 2-phenylethanol and tryptophol was 9.366 min, 13.112 min, respectively.

UHPLC-MS/MS

UHPLC-MS/MS analysis was performed in the mass spectrometry section of the Central Support Service for Experimental Research of the University of Valencia (SCSIE). Hydroxytyrosol, tyrosol and shikimic acid were detected by UHPLC on ACQUITY® TQD system (Waters, USA) equipped with a Kinetex XB-C18 100 Å column (2.1×100 mm, 1.7 µm; Phenomenex). Buffer A (milliQ water with 1mM 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 & 137→106 for tyrosol, m/z 153→123 for hydroxytyrosol and 173 > 93 & 173 > 111 for shikimic acid.

Statistical analysis

All the experiments were carried out at least in triplicate, with the exception of the chemostat cultures conducted in Chapter 3 which were performed in duplicates. Data were expressed as the mean values ± standard deviation. Experimental results were analyzed and compared by statistical analyses such as ANOVA, Tukey's honestly significant difference (HSD) and t-tests using R or GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) software. A significance level between 1 and 5% was chosen as the cut-off point.

RESULTS

CHAPTER 1

Detecting and monitoring the production of melatonin and other related indole compounds in different *Saccharomyces* strains by solid-state electrochemical techniques.

This chapter has been published in:

Food Analytical Methods (2017) 10:1408–1418 (doi:10.1007/s12161-016-0699-8)

1.1. Introduction

Originally, melatonin was seen as a unique product of the pineal gland of vertebrates and was called a neurohormone. However, in the last two decades, it has been identified within a wide range of invertebrates, plants, bacteria, and fungi. Therefore, today, melatonin is considered a ubiquitous molecule present in most living organisms (Tan et al. 2015). In particular, this indolamine has been significantly detected in many food plants, and consequently, it can now be considered a dietary component, even if its daily intake is very difficult to estimate (Iriti et al. 2010; Iriti and Varoni 2015). Among food plants, grape vine (*Vitis vinifera*) represents a source of melatonin that deserves being paid special attention because of wine production, an alcoholic beverage of economic relevance, with the presence of molecules with putative bioactivity**.**

Melatonin has been detected in wine, and despite the contribution of grapes to the final concentration of melatonin and other indolamines, significant increases in these molecules in the fermentation step of the wine-making process (Vigentini et al. 2015), indicating the pivotal role of yeasts in this production. A pioneering paper demonstrated that *S. cerevisiae* synthesizes melatonin and metabolizes it to other 5-methoxylated indoles (5-methoxytryptamine and 5-methoxytryptophol) (Sprenger et al. 1999). More recently, Rodriguez-Naranjo et al. (2012) proved the synthesis of melatonin in several wine yeast strains under conditions that mimicked grape must fermentation. These authors concluded that melatonin synthesis largely depends on the growth phase of yeasts and the concentration of L-tryptophan and sugars in the growth medium.

The synthesis by yeasts of this molecule and other derived indolic compounds, with potential health benefits, opens up a new exciting research field, which can lead to increase these bioactive molecules in food, in which yeasts take part. A key point to improve the synthesis of these molecules during the fermentation process is to elucidate the complete metabolic pathway involved in the biosynthesis of these indolic compounds. Although very little information on melatonin biosynthesis in organisms other than plants and vertebrates is available, in yeast, the pathway seems to be similar to the synthetic route and enzymes described in vertebrates (Sprenger et al. 1999). This synthesis route is

very simple, with four enzymes involved in the conversion of tryptophan into 5-hydroxytryptophan, this into serotonin and *N*-acetylserotonin intermediates, and finally into melatonin (Figure 1.1.A). However, the genes that encode the enzymes of this putative melatonin synthesis pathway are completely unknown.

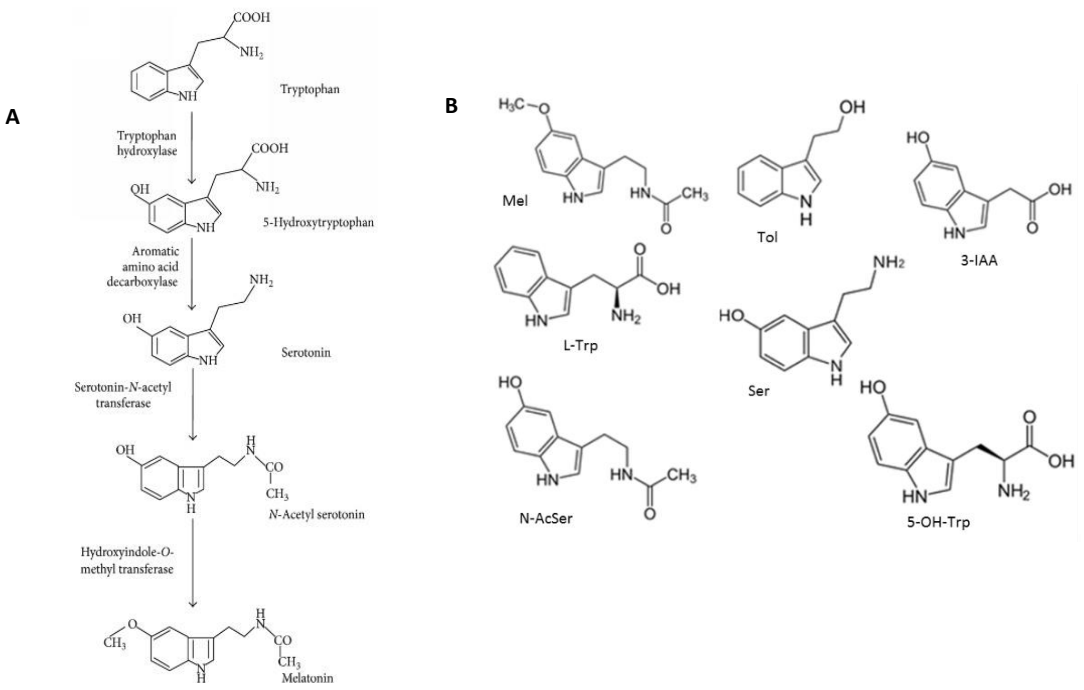

Figure 1.1. Synthesis of melatonin from trytophan (adapted from Mas et al. (2014)) **A**. Representation of the structures of melatonin (Mel), tryptophol (Tol), 3-indoleacetic acid (3-IAA), tryptophan (L-Trp), 5-hydroxytryptophan (5-OH-Trp), *N*-acetyl serotonin (N-AcSer), and serotonin (Ser) **B**.

We were very interested in elucidating this melatonin route by searching other homolog genes to those described in vertebrates and by assaying the enzymes in the conversion of different intermediates into the final melatonin product (results presented in chapter 3 and 4 of the present thesis). However, to achieve this aim, we needed a sensitive, easy, reliable, and economic method to measure intracellular melatonin. This molecule has been determined by HPLC with fluorescence detection (Mercolini et al. 2008; Yin et al. 2016) and/or MS detection (Yang et al. 2002; Iriti et al. 2006; Arnao and Hernández-Ruiz 2007; Kollmann et al. 2008; Fernández-Cruz et al. 2016), GC and MS (Best et al. 1993; Van Tassel et al. 2001), and ELISA (Pape and Lüning 2006; Kollmann et al. 2008; Maldonado et al. 2009). Nonetheless, given the low melatonin production levels by yeasts (Rodriguez-Naranjo et al. 2012), these methods are poorly sensitive or

technically complex to become a routine technique to detect melatonin production in yeasts. Thus, our aim was to adapt and set up a simple, rapid, and low-cost technique for detecting the presence of melatonin and its intermediates in *S. cerevisiae* intracellular samples.

In this context, conventional solution-phase electrochemical techniques have been used to determine different individual analytes (Yang et al. 2015), estimate antioxidant capacities (Karadag et al. 2009; Mülazimoğlu and Mülazimoğlu 2012), and evaluate quality parameters (Ziyatdinova et al. 2015) of foods. Given the electroactive character of melatonin ((Radi and Bekhiet 1998) and the peculiar nature of the involved samples, we tested the possibility of using solid-state electrochemical techniques to acquire analytical information based on the voltammetry of immobilized particles (VIMP) methodology.

This technique, developed by Scholz et al. in the late 1980s (see comprehensive reviews in Scholz and Meyer (1998) and Scholz et al. (2014), provides the electrochemical response of sparingly soluble solids attached to inert electrodes that come into contact with suitable electrolytes applied in a variety of fields (Doménech-Carbó et al. 2013). By means of this approach, the voltammetric response of the microparticulate deposits that result from evaporating ethanol, acetone, etc., extracts from the cellular strains on the surface of solid electrodes were measured in contact with aqueous electrolytes. Since melatonin as well as directly related compounds in yeasts are sensitive to temperature changes and oxidizing agents (Tan et al. 2007; Garcia-Parrilla et al. 2009), the proposed methodology, involving room temperature operations and “smooth” extraction pretreatment, offers in principle high representativity.

This allowed us to record the responses associated with selected electroactive species without having to determine their entire extract composition. Application for screening tomato fruits (Doménech-Carbó et al. 2015b) and tea (Liu et al. 2014; Domínguez and Doménech-Carbó 2015) varieties, studying the antioxidant capacity associated with the interaction with reactive oxygen species (Doménech-Carbó et al. 2015c), and monitoring plant defenses against external stressors (Doménech-Carbó et al. 2015a) have been described. In particular,

VIMP has been proposed to access chemotaxonomic information to yield phylogenetic trees (Doménech-Carbó et al. 2015d).

This methodology was applied here into cellular samples to screen the capability of melatonin biosynthesis by different industrial and laboratory *Saccharomyces* strains and to explore melatonin production by testing different possible pathways. This application exploits the fact that serotonin (Wrona et al. 1986; Wrona and Dryhurst 1987), melatonin (Crespi et al. 1994), tryptophan, and other directly related indolic compounds (Enache and Oliveira-brett 2011) are electrochemically oxidized, which prompt their determination by voltammetric techniques. Although a variety of modified electrodes have been reported for melatonin (Radi and Bekhiet 1998; Xiao-ping et al. 2002; Beltagi et al. 2003; Corujo-Antuña et al. 2003; Qu et al. 2005; Saber 2010; Wei et al. 2012; Caro et al. 2016) and tryptophan (Safavi and Momeni 2010; Liu et al. 2013; Beitollahi et al. 2015) determination in pharmaceutical formulations and biological fluids, their application to direct cellular yeast strains, where a complex matrix exists and several potential interferents are present, appears uncertain when such conventional solution-phase electrochemistry methodologies are employed.

Thus, in this chapter, we report the setup of a solid electrochemical method that allowed us to detect the synthesis of the main compound of the melatonin metabolic pathways and other compounds related to tryptophan metabolism, such as 3-indoleacetic acid and tryptophol. The application of this method was conducted directly on yeast cells from laboratory and industrial strains. Furthermore, the production of these compounds after the pulses of the initial substrate, tryptophan, but also intermediates, such as 5-hydroxytryptophan and serotonin has been assessed. Finally, we also tested the voltammetric changes in the mutant strains with deleted genes of tryptophan metabolism.

1.2. Results and Discussion

1.2.1. Electrochemistry of reference compounds

The electrochemistry of the relevant indolic compounds (see the structures in Figure 1.1b), serotonin, 5-hydroxytryptophan, *N*-acetylserotonin, melatonin, and tryptophan films on glassy carbon working electrode (GCE) from their ethanolic suspensions, was studied upon immersion into aqueous acetate buffer at pH 4.75. Under these conditions, the films remained stable for 10–15 min and thus allowed the determination of a well- defined electrochemical response, as illustrated in Figure 1.2. In all cases, an apparently irreversible oxidation wave was recorded, and the peak potential ranged between +0.45 and +1.05 V vs. Ag/AgCl. As we suspected a possible overlapping between melatonin and other major tryptophan metabolism compounds, we also included the analysis of films on GCE pre- pared by the evaporation of ethanolic solutions of tryptophol and 3-indoleacetic acid to define the electrochemistry response. As can see in Figure 1.2, the voltammetric signal for these three compounds was almost identical. In order to solve this overlapping signal for melatonin, tryptophol, and 3- indoleacetic acid, a generalized Tafel analysis and a modified Tafel analysis were applied as explained in “Discrimination among melatonin, tryptophol and 3- indoleacetic acid” section.

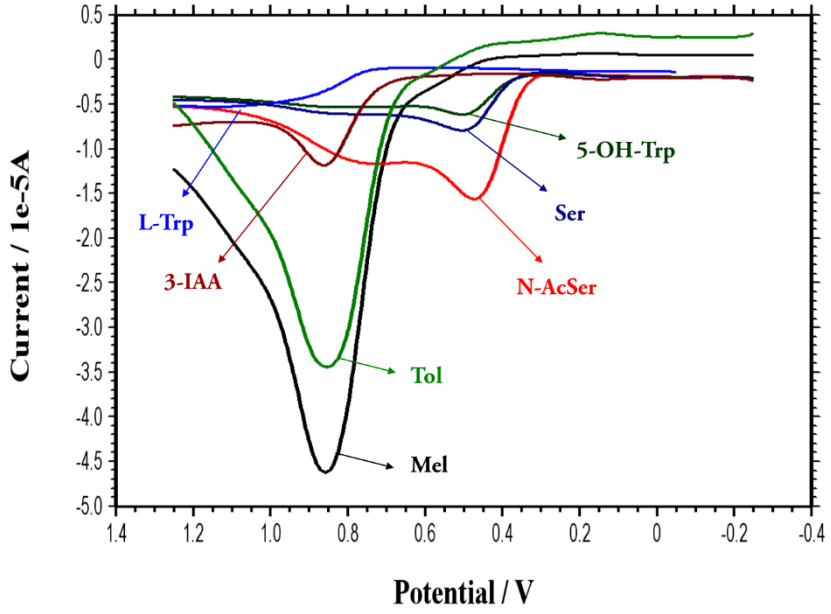

Figure 1.2 Square wave voltammograms of different indolic compunds. Films of melatonin (Mel), tryptophol (Tol), 3-indoleacetic acid (3-IAA), tryptophan (L-Trp), *N*-acetyl serotonin (N-AcSer), serotonin (Ser) and 5-hydroxytryptophan (5-OH-Trp) on glassy carbon electrode in contact with 0.25 M HAc/NaAc aqueous solution at pH 4.75. Potential scan initiated at -0.25 V in the positive direction, potential step increment 4 mV, square wave amplitude 25 mV, and frequency 5 Hz.

As reported by Wrona et al. (1986, 1987), oxidation of serotonin in aqueous electrolytes proceeds via an initial one-electron one-proton oxidation step that yields a phenoxyl radical that subsequently undergoes a second one-electron one-proton oxidative step to give a very reactive quinone imine. This species reacts with water to give 4,5-dihydroxytryptamine, which, in turn, undergoes a two-electron two-proton oxidation to a quinone, tryptamine-4,5-dione, which thus results in an overall four-electron process. The same pathway would be operative for 5-hydroxytryptophan and *N*-acetylserotonin, which, consistently, produced an oxidation peak at a potential essentially identical one to that of serotonin (Figure 1.2) +0.45 V.

The electrochemistry of 3-indoleacetic acid and tryptophan (the latter displayed a broad anodic wave at ca. +0.95 V) would consist in the following two steps: oxidation at position C2 on the pyrrole ring and hydroxylation at the C7 position of the benzene moiety (Safavi and Momeni 2010; Beitollahi et al. 2015). Melatonin and tryptophol produced an oxidation peak at +0.70 V, which can be

described, following Wrona et al. (1986,1987), and in two-electron oxidation terms, to form a quinoneimine, which is susceptible to nucleophilic attack to subsequently form dimers.

1.2.2. Discrimination among melatonin, tryptophol and 3-indoleacetic acid

In the context of melatonin determination, it is pertinent to note that voltammetric techniques can be considered in this context as complementary to chromatographic techniques. HPLC provides high discriminating capability and the concomitant unambiguous identification and quantification of individual species; however, the need of extraction or sample pretreatments diminishes to some extent the representativity of the sample. The voltammetric methodology described here, although having less discriminating ability, offers the possibility of analyzing samples with minimal pretreatment and fast measurement. As previously noted, discrimination remained an analytical problem because of the marked coincidence of the voltammetric signals. This can be seen in Figure 1.2, where the SWVs, after semi-derivative convolution, of the films on GCE, prepared by the evaporation of the solutions of tryptophol, melatonin, and 3-indoleacetic acid in water, are depicted. After taking five independent measurements, the peak potentials were 735 ± 5, 725 ± 5, and 755±5 mV, respectively, under our experimental conditions.

In order to discriminate among melatonin, tryptophol, and 3-indoleacetic acid, the generalized Tafel analysis and the modified Tafel analysis based on voltammetric measurements in the solution phase (Reinmuth 1960, 1962), and previously introduced to study solid samples (Doménech et al. 2011), were applied. This is possible because in both linear potential scan and square wave voltammetries, the peak current for reducing the surface-immobilized species can be approached by an expression of the type (Lovrić and Komorsky-Lovric 1988; Lovrić et al. 1991; Komorsky-Lovrić et al. 1992; Komorsky-Lovrić and Lovrić 1995).

$$i_p = H(\frac{\alpha n_a F}{RT})q_o \qquad (Eq.1)$$

Where *H* is an electrochemical coefficient of the response characteristic of the electrochemical process and the electrode area, and *v* is the potential scan rate (LSV) or the square wave frequency *f* (SQWV). By assuming that both the linear scan and square wave voltammograms behaved similarly, the current at the beginning of the voltammetric peak can be tentatively represented as (Doménech-Carbó et al. 2011).

$$i \approx q_o k_o \exp(-\frac{\alpha n_a F}{RT} E) \qquad (Eq.2)$$

where q_o represents the total charge involved in the complete reaction of the electroactive solid. By combining Eqs. (1) and (2), we obtain:

$$\ln(i / i_p) = \ln\left(\frac{k_o RT}{H \alpha n_a F} \right) - \frac{\alpha n_a F}{RT} E \qquad (Eq.3)$$

This equation (Eq. 3) characterizes the TA. Here both the generalized Tafel slope ($SL = \alpha n_a F/RT$) and the ordinate at the origin ($OO = ln(k_o RT/H\alpha n_a F)$) became characteristic of the solid analyte, regardless of the amount of sample deposited on the electrode.

The application of this formalism to the voltammograms of melatonin, tryptophol and 3- indoleacetic acid produced $\ln(i/i_p)$ *vs.* E-E_p plots, and exhibited excellent linearity in terms of the linear regression coefficient (Table 1.1).

Table 1.1 Statistical parameters for the Tafel analysis of the voltammetric curves for films melatonin, tryptophol and 3-indoleacet acid that came into contact with aqueous acetate buffer at pH 4.75.

Analyte	**Slope (mV^{-1})**	**Ordinate at the origin**	***r* (*N*)**
Melatonin	–0.029±0.0011	1.36±0.13	0.9990 (5)
Tryptophol	–0.033±0.0011	1.35±0.10	0.9990 (5)
3-Indoleacetic acid	–0.031±0.0004	1.50±0.04	0.9998 (5)

Although the voltammetric peaks were essentially coincident and the slopes of the three straight lines were essentially identical, the ordinates at the origin differed slightly, but consistently, which allowed the discrimination among these compounds, or even the identification of mixtures, where an intermediate ordinate at the origin should be obtained.

In the intermediate region of the voltammetric peak, where *E* came close to *E*º', the following approximate expression, which defined the MTA, can be taken (Doménech et al. 2011):

$$\ln\left(\frac{i_p - i}{i_p}\right) \approx \ln\left(A\frac{\alpha n_a F}{RT}\right) + \ln(E - E_p) \qquad (Eq.4)$$

For practical purposes, this equation offered greater applicability than Eq. (3) because minor interfering peaks frequently appeared at the beginning of voltammetric peak I. Application of the modified Tafel analysis to the blanks of melatonin and tryptophol and to the mixtures of such compounds provided straight lines, whose slope and ordinate differed at the origin for the pure compounds, whereas a sigmoidal plot was obtained with the mixtures, which approached the straight lines for tryptophol and melatonin in its extreme regions (Figure 1.3).

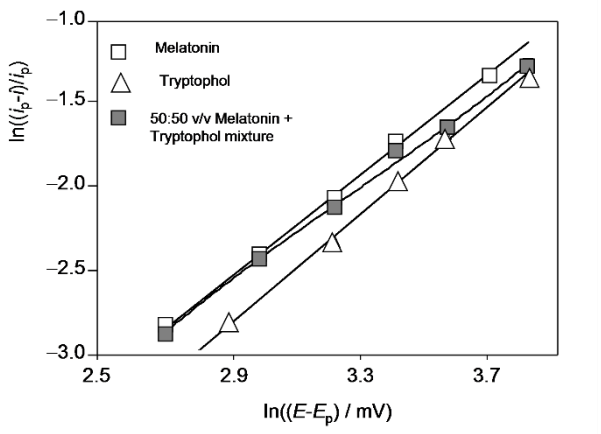

Figure 1.3 Application of the modified Tafel analysis to the central region of the voltammetric wave I for linear potential scans of microparticulate deposits on glassy carbon electrode of ethanol extracts of melatonin, tryptophol and a 50:50 mixture of both compounds. Electrolyte 0.25 M HAc/NaAc, pH 4.75. Potential scan rate 20 mV s-1.

The above analysis was consistent with the experimental data for the variation of the voltammetric response of the cellular specimens spiked with tryptophan during the incubation time.

Figure 1.4 illustrates the application of the modified Tafel analysis to the central region of the voltammetric wave I for the linear potential scan voltammograms of the microparticulate deposits on GCE of the ethanol extracts of samples QA23Δ*trp1* without (wo) tryptophan, QA23Δ*trp1* + tryptophan 30 min, QA23Δ*trp1* + tryptophan 45 min, and QA23Δ*trp1* + tryptophan 60 min. Here we can see that (i) sample QA23Δ*trp1* without tryptophan yielded a curved path, which suggests the existence of a mixture of components, and (ii) sample QA23Δ*trp1* + tryptophan 30 min provided a path with high linearity. It is reasonable to conjecture that here, melatonin largely predominated so that the Tafel plots looked like that which corresponded to a unique component (straight line); (iii) the Tafel plot for sample QA23Δ*trp1* without tryptophan tended to present large potentials to that for QA23Δ*trp1* + tryptophan 30 min (Figure 1.4a). This suggests that melatonin could be one of the components of the initial mixture in the sample without tryptophan; (iv) samples and QA23Δ*trp1* + tryptophan 60 min provided curved Tafel plots, which suggests that these samples were composed of a mixture of components. At low potentials, these curves tended to coincide with that for melatonin (Figure 1.4b). This indicated that a certain proportion of this compound remained in the QA23Δ*trp1* + tryptophan 45 min and QA23Δ*trp1* + tryptophan 60 min mixtures.

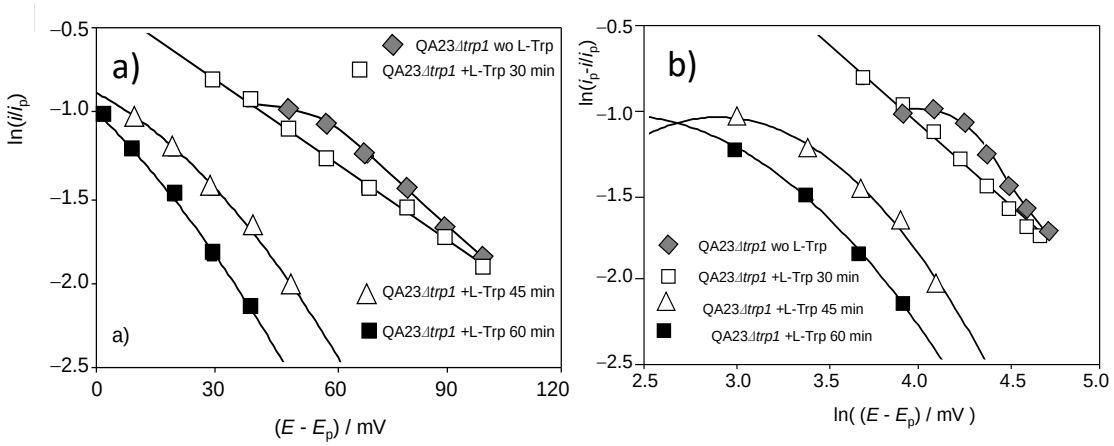

Figure 1.4 Application of the (a) Tafel analysis and (b) modified Tafel analysis methods to the voltammetric wave at ca. +0.70 V recorded for linear potential scan voltammograms of microparticulate deposits on glassy carbon electrode of ethanol extracts of QA23Δtrp1 samples. QA23Δtrp1 wo L-Trp, QA23Δtrp1 + L-Trp 30 min, QA23Δtrp1 + L-Trp 45 min, and QA23Δtrp1 + L-Trp 60 min. Electrolyte 0.25 M HAc/NaAc, pH 4.75. Potential scan rate 20 mV s^{-1}. Witout (wo); L-tryptophan (L-Trp).

1.2.3. Indolic compound production in a commercial wine *S. cerevisiae*

Once we determined the electrochemical response of different indolic compounds, we aimed to monitor the production of these metabolites after the pulse of the different intermediates of the presumptive metabolic pathway. Tryptophan, 5-hydroxytryptophan, and serotonin were added to the resting cells of wine strain QA23, incubated in a non proliferant medium (salt medium). After a time that ranged from 5 to 30 min (only 30 min for the tryptophan pulse), the intracellular content was extracted and used as aqueous samples for conventional electrochemical measuring. Before adding the indolic compounds, cells displayed an oxidation signal close to +1.0 V, which can be attributed mainly to the oxidation of water to oxygen (oxygen evolution reaction), overlapped to this one of the original tryptophan. This oxidation signal was accompanied by weak signals within the +0.4 to +0.8 potential range, attributed to the oxidation of the intracellular metabolites already present in cells without addition (note that some amounts of tryptophan, melatonin, etc., were in the original samples). The voltammograms of QA23 + tryptophan (Figure 1.5) displayed an intense anodic wave at +0.70 V, which was attributable to melatonin, and were eventually accompanied by tryptophol and 3-indoleacetic acid, which were entirely absent in the strain without tryptophan addition.

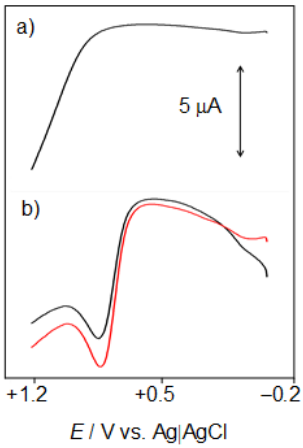

Figure 1.5 Square wave voltammograms at glassy carbon electrode of aqueous samples a) QA23 without tryptophan addition and b) QA23 + tryptophan addition (two replicate experiments). Potential scan initiated at -0.05 V in the positive direction, potential step increment 4 mV, square wave amplitude 25 mV, frequency 5 Hz.

This result was consistently repeated in the replicate experiments for all the tested samples, thus denoting excellent repeatability. Similar results were obtained upon the addition of 5-hydroxytryptophan and serotonin pulses and after examining the intracellular liquid content (Figure 1.6). This signal increased significantly after 15 min with both indolic coumpound additions, and maximum intensity of the melatonin peak was achieved at 30 min after the pulse for serotonin. At this time, the signal that corresponded to serotonin appeared at +0.45 V. At longer times, the melatonin signal was accompanied by signals at ca. +0.45 V, which denoted the formation of secondary products.

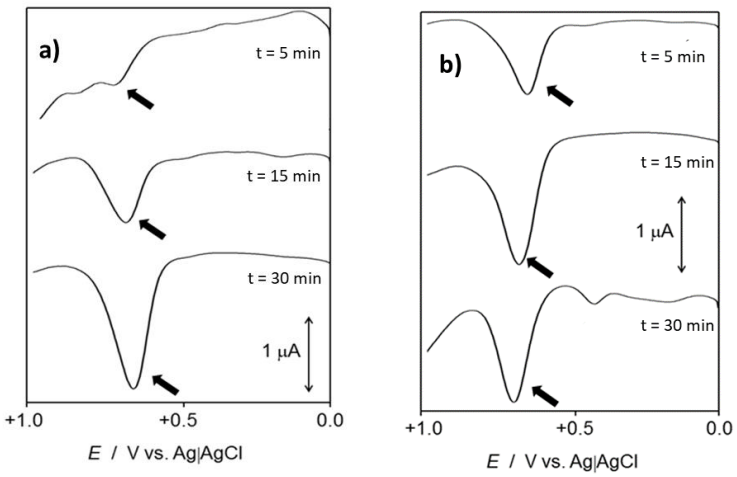

Figure 1.6 Monitoring the production of melatonin from its precursors using VIMP. Semi-derivative convolution of linear potential scan voltammograms of at glassy carbon electrode of QA23 liquid intracellular content at different times (5, 15 and 30 min) after addition of 5-hydroxytryptophan (a) and serotonin (b). Potential scan initiated at +0.00 V in the positive direction; potential scan rate 50 mV s-1. Oxidation signals of 5-hydroxytryptophan, serotonin and melatonin+ tryptophol+ 3-indoleacetic acid (arrow) are marked.

Interestingly, in all the experiments, by varying the incubation time, variations in the relative intensity of the anodic peaks that corresponded to the oxidation of the Mel group (melatonin+ tryptophol+ 3-indoleacetic acid) and Trp, IMelG/ITrp agreed well with those calculated from the HPLC data using the sum of the concentrations of melatonin, tryptophol, and 3-indoleacetic acid and the concentration of tryptophan. This can be seen in Figure 1.7 for QA23 + 5-hydroxytryptophan. As previously noted, although the proposed voltammetric methodology does not possess the high discriminating capacity of chromatographic techniques, quantifications of groups of compounds from this method were consistent with those obtained by HPLC, thus supporting the possibility of a complementary use of such techniques.

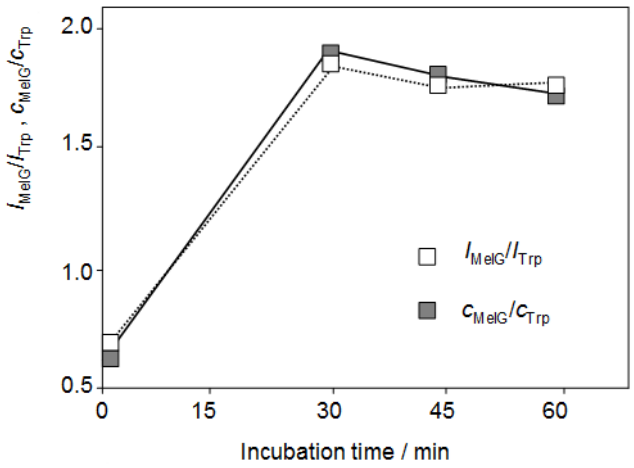

Figure 1.7 Comparison between HPLC and voltametric analysis data. Variation of the IMelG/ITrp ratio from voltammetric data and the melatonin+ tryptophol+ 3-indoleacetic acid/L-tryptophan concentration ratio determined from HPLC data (cMelG/cL-Trp) relative to the respective initial values on the incubation time for the QA23 + 5-hydroxytryptophan system.

We were very interested in developing a simple and rapid procedure to monitor the biosynthesis of these indolic compounds. To this end, intracellular metabolite extraction is a time- and resource-consuming procedure that should be avoided. Solid-state electrochemistry techniques can be applied to monitor compositional changes with high sensitivity using microparticulate films on glassy carbon electrodes, obtained upon the evaporation of the ethanolic extracts of dried cells. The detection of voltammetric changes directly from dried QA23 cells after adding the indolic compounds (5-hydroxytryptophan and serotonin) was also assessed at different times (5, 10, 15, and 30 min; Figure 1.8).

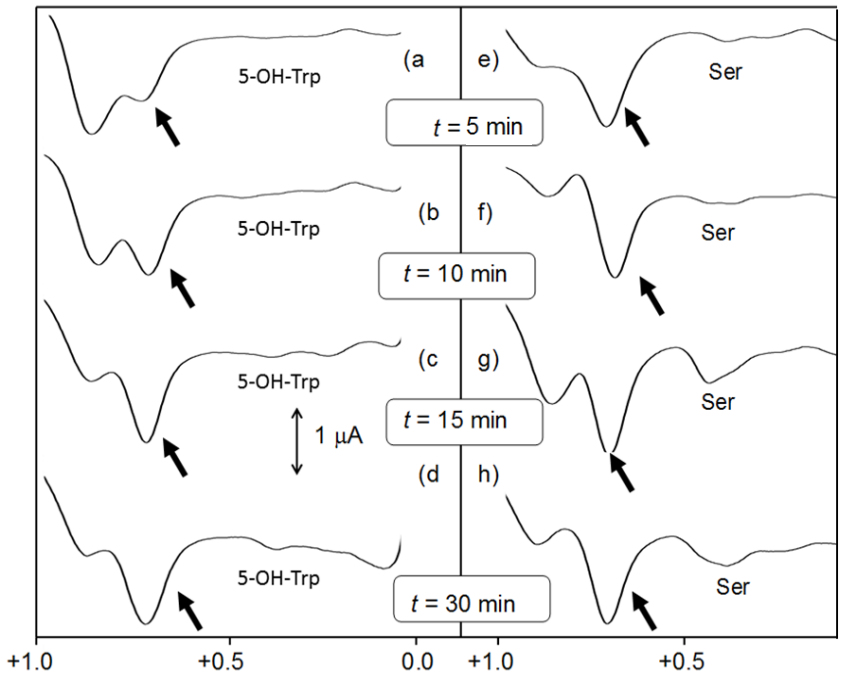

Figure 1.8 Electrochemical response in the QA23 strain in the melatonin group oxidation signal after the addition of 5-hydroxytryptophan and serotonin. Semi-derivative convolution of linear potential scan voltammograms of at glassy carbon electrode of QA23 films at different times (5, 10, 15 and 30 min) after addition of a (**a-d)** 5-hydroxytryptophan (5-OH-Trp) and (**e-h**) serotonin (Ser). Potential scan initiated at +0.00 V in the positive direction; potential scan rate 5 mV s^{-1}. Oxidation signals of melatonin + tryptophol + 3-indoleacetic acid (arrow) are marked.

In this figure, we can see that the oxidation signal of the melatonin group at +0.70 V (marked by an arrow) increased with time compared to the tryptophan signal at ca. +0.95 V, which peaked at times between 10 and 15 min and then slowly decreased. At longer times, the Mel signal was accompanied by signals at ca. +0.45 V, which denotes the formation of secondary products.

1.2.4. Screening different wine commercial strains

We set up a method to rapidly monitor metabolite changes using the variation of the electrochemical responses of extracts taken directly from dried yeast cells (and avoiding intracellular content extraction). For this purpose, the voltammetric responses of the microparticulate deposits on the GCE of the ethanolic extracts of the dried yeast cell samples were performed by procedures that have been previously described for screening vegetal varieties (Doménech-Carbó et al. 2015d, b). We monitored the voltammetric response of the films from the

ethanolic extracts of different *Saccharomyces* commercial wine strains and of lab strain BY4743. These voltammetric responses were dominated by a series of oxidation waves within the potential 0.0 and +1.2 V range, which can be attributed to different metabolites, such as flavonoids and indolic compounds. As shown in Figure. 1.9, the linear potential scan voltammetric profiles differed significantly from one *Saccharomyces* wine strain to another and before and after the tryptophan pulse. We can see that the response of strain BY4743 consisted in almost isolated peaks for melatonin and tryptophan oxidation, and the first increased at the expense of the second after the tryptophan pulse, whereas for the wine strains (Figure 1.9), the initial voltammogram consisted in a more or less complicated series of overlapping anodic signals, which yielded an increased melatonin signal after the tryptophan pulse. This last effect, however, was low for AROMA WHITE (Figure 1.9c, d), for which the relative enhancement of the melatonin signal became less marked.

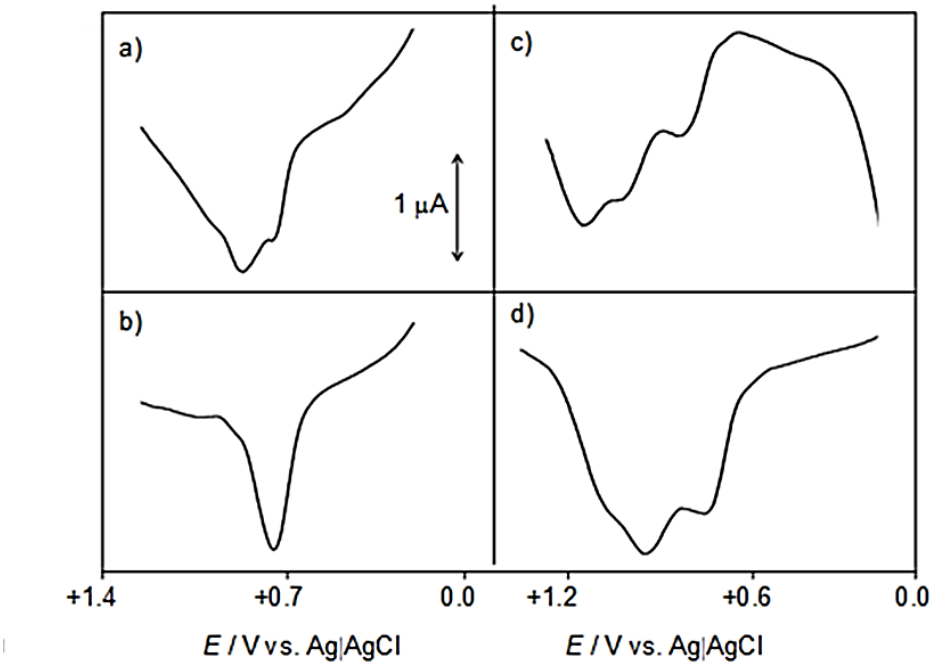

Figure 1.9 Electrochemical response in wine yeast strains, BMV58 and AROMA WHITE, with and without addition of tryptophan, in the melatonin oxidation signal. Linear potential scan voltammograms, after semi- derivative convolution, of films of ethanolic extracts of *Saccharomyces* strains of (**a**, **b**) BMV58 and (**c**, **d)** AROMA WHITE before (**a**, **c)** and after (**b**, **d**) pulse of L-tryptophan 1 mM, on glassy carbon electrode in contact with 0.25MHAc/NaAc, pH 4.75. Potential scan initiated at +0.0 V in the positive direction; potential scan rate 50 mV s^{-1}.

By taking into account the different linear potential scan voltammetric profiles observed for each *Saccharomyces* strain, this technique could also be applied for typing different strains. This application has been already used for discriminating other biological species and for establishing correlations with phylogenetic trees (Doménech-Carbó et al. 2015d, b). The availability of techniques that can discriminate at inter- and intra-specific levels is important for industry because they allow yeast populations and the contribution of inoculated strains to the process to be monitored. Many molecular methods have been developed for yeast strains typing; most of which are based on the DNA (Esteve-Zarzoso et al. 2010). Since previous results on plant leaves suggested the possibility of obtaining species-characteristic voltammetric profiles (Doménech-Carbó et al. 2015d), it would be possible to hypothesize that voltammetric data can be applied for a fast screening of yeast strains. Further studies should be done with more *Saccharomyces* strains for this purpose.

1.2.5. Effect of the deletion of tryptophan metabolism genes on the indolic compound profile

Tryptophan metabolism seems the key metabolic route for the synthesis of different indolic compounds. Thus, our aim was to compare the synthesis of these compounds in two mutant strains with deletion in two key genes of tryptophan metabolism (*ARO10* and *TRP1*) in relation to their wild strain BY4743. *ARO10* is a phenylpyruvate decarboxylase that catalyzes the first tryptophan catabolism steps (Kneen et al. 2011). Thus, a mutant strain of this gene should accumulate more intracellular tryptophan than the wild type and be more metabolically available for indolic compound synthesis. Figure 1.10 compares the voltammograms of strains BY4743 and BY4743Δ*aro10* before and after adding a pulse of tryptophan.

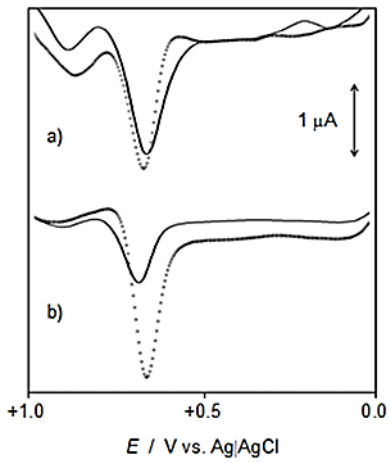

Figure 1.10 Electrochemical response in yeast strains, BY4743 and BY4743Δaro10 with and without addition of tryptophan, in the melatonin oxidation signal. Semi-derivative convolution of linear potential scan voltammograms at glassy carbon electrode in contact with 0.25 M HAc/NaAc, pH 4.75. Films of ethanolic extracts from (**a**) BY4743 and (**b**) BY4743Δaro10 strains before (black line) and after (dotted line) addition of 1 mM of L-tryptophan. Potential scan initiated at +0.0 V in the positive direction; potential scan rate 50 mV s−1

As this figure depicts, the voltammetric response to the exogenous tryptophan addition was clearly higher in the mutant that in its parental strain. This is likely because tryptophan catabolism, via the Ehrlich pathway, is lower or null in the mutant strain and maintains a higher intracellular concentration of this amino acid and more available substrate for the synthesis of different indolic compounds. Conversely, *TRP1* is a phosphoribosylanthranilate isomerase that catalyzes the third tryptophan biosynthesis step. The mutant strains in this gene are auxotroph for tryptophan because they are unable to synthesize this amino acid according to the exogenous supply in the culture medium. The variation in the voltammetric response of BY4743Δt*rp1* after adding the tryptophan pulse was similar to the *aro10* mutant results. Hence, the melatonin peak at +0.70 V increased compared to the tryptophan signal at more positive potentials. In this case, however, the relative peak enhancement was lower than in strain BY4743Δ*aro10*.

We also tested this mutant Δ*trp1* in wine strain QA23. Figure 1.11 compares the voltammograms of the wild strain and the mutant before and after the tryptophan pulse. Here, we can observe that the initial voltammograms of the wild and mutant strains significantly differed. After the tryptophan pulse, however, the signal for melatonin oxidation was dramatically enhanced in both cases compared to any other voltammetric peaks.

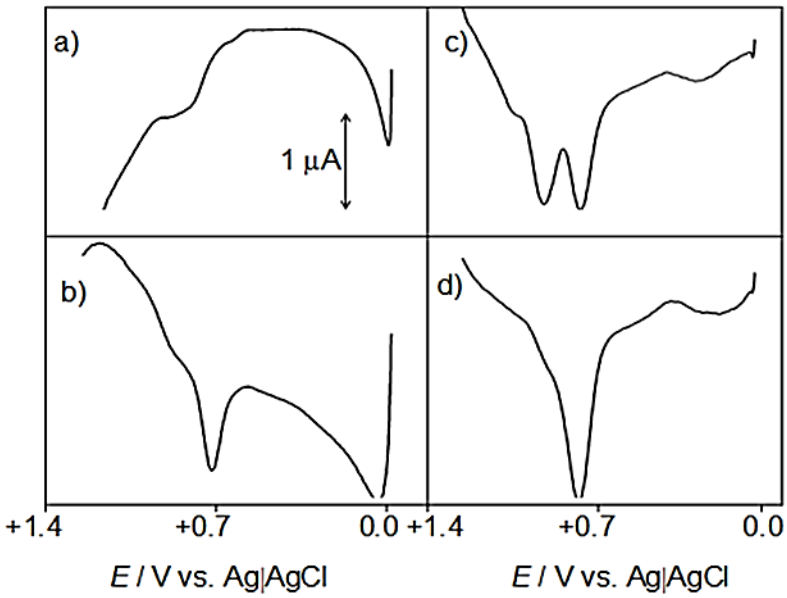

Figure 1.11 Linear potential scan voltammograms, after semi-derivative convolution, of films of ethanolic extracts of *Saccharomyces* strains. QA23 (**a**, **b**) and QA23Δ*trp1*(**c, d**), before (**a**, **c**) and after (**b**, **d**) pulse of tryptophan 1 mM, on glassy carbon electrode in contact with 0.25 M HAc/NaAc, pH 4.75. Potential scan initiated at +0.0 V in the positive direction; potential scan rate 50 mV s−1.

1.3. Conclusions

In this chapter, we report the setup of a solid electrochemical method that allowed us to detect the synthesis of the main compound of the melatonin metabolic pathways and other compounds related to tryptophan metabolism, such as 3-indoleacetic acid and tryptophol. The application of this method to different lab and industrial strains has revealed the production of not only these compounds after the pulses of the initial substrate, tryptophan, but also intermediates, such as 5-hydroxytryptophan and serotonin. The voltammograms obtained for the

different strains showed different profiles, which denotes a distinct concentration and distribution of the indolic compounds produced per strain. Moreover, these differences in the voltammetric response could be applied to type different commercial strains, which could be an important quality control tool for industry. Finally, we also tested the voltammetric changes in the mutant strains with the deleted genes of tryptophan metabolism. The deletion of a single gene of this metabolic pathway impacted the synthesis of melatonin and other indole compounds, which can be a promising strategy to improve the synthesis of these bioactive molecules and to increase their concentration in different fermented foods.

CHAPTER 2

Protective role of intracellular melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*.

This chapter has been published in:

Frontiers in Microbiology (2018) 9:318 (doi: 10.3389/fmicb.2018.00318)

2.1. Introduction

Melatonin has proved to be a highly efficient antioxidant molecule, capable of detoxifying various ROS (Antolín et al. 1997). However, its antioxidant capacity does not seem to be limited to its ability to scavenge free radicals. It has been also reported that its presence is capable of stimulating the synthesis of other important intracellular antioxidants, such as glutathione (Rodriguez et al., 2004), inducing antioxidant enzymes by suppressing pro-oxidant enzymes, and improving the mitochondrial function and thereby reducing free radical formation (Acuña Castroviejo et al., 2011; Zhang and Zhang, 2014; Paradies et al., 2015). Gene expression modulation by melatonin may underlie these functions, as described for mammalian copper zinc superoxide dismutase (CuZn SOD) and glutathione peroxidase (GPx), whose gene expression is modulated by melatonin in a dose-dependent manner (Mayo et al., 2002). However, the mechanisms by which melatonin modulate antioxidant enzyme activities have not been unequivocally identified (Reiter et al. 2018).

Melatonin has been significantly detected in many food plants, so consequently it can now be considered a dietary component, even if its daily intake is very difficult to estimate (Iriti and Varoni, 2015). In the last few years its presence has been reported in fermented drinks, such as wine or beer, as a result of melatonin content in the different vegetal sources used, but also notoriously as a result of yeast metabolism (Rodriguez-Naranjo et al., 2011, 2012). However, there is still uncertainty as to the conditions that trigger melatonin production, and its biosynthetic pathway still remains unknown. For an organism like yeast, unraveling melatonin production conditions and its effects is an exciting topic in the food science field because presence of melatonin in food due to yeast activity can confer numerous health benefits (Iriti and Varoni, 2015). Moreover, the use of melatonin-producing yeasts can provide other technological gains, such as application to post-harvest treatments as an antifungal control agent (Iriti and Varoni, 2014; Sun et al., 2015; Li et al., 2016; Ma et al., 2016; Xu et al., 2016).

Saccharomyces cerevisiae is the main yeast used in the winemaking process, where it is exposed to a number of stressors, each with the potential to cause cellular damage and impair fermentation performance (Gibson et al., 2007). One

of the main stressors described during wine fermentation is oxidative stress (Auesukaree, 2017) and, the synthesis of melatonin can be a response to cope with this stress, similarly to the role ascribed to other antioxidant molecules, e.g., glutathione, or a signaling molecule to trigger the molecular machinery of antioxidant response. Previous studies on the origins of melatonin indicate that its primary function is strongly related to defense against oxidative stress (Tan et al., 2014). This antioxidant function is highly conserved among the organisms that produce this compound, and antioxidant protection is indicated as a possible function in aerobic non-vertebrates (Hardeland and Poeggeler, 2003). Despite an undoubtedly powerful capacity to antagonize oxidative damage, the physiological significance of antioxidative protection is less clear. Very little evidence is available that shows an advantageous effect of endogenous melatonin at physiological concentrations in yeasts, especially in the first stages of growth. Above physiological concentrations, melatonin can even produce moderate growth inhibition, which is presumably dose-dependent as demonstrated before for mammal cells (Koziol et al., 2005; Owsiak et al., 2010; Zhang and Zhang, 2014). Since the ideal conditions for endogenous melatonin production remain unknown, we set up a melatonin-charging method to emulate endogenous production and to perform our different experiments with melatonin-charged cells after removing extracellular melatonin. Before the oxidative stress treatments, we tested the growth performance of melatonin-charged cells to assess growth improvement and we measured intracellular ROS levels by flow cytometry. As mentioned above, the presence of melatonin can modulate the synthesis of many other molecules and enzymes that play an antioxidant role. This general triggering of antioxidant response can rely on transcriptional regulation. Thus we selected a group of four genes that encode the enzymes directly related to antioxidant activity in the cytoplasm (*SOD1*, *TRX2*, *GPX2*, and *CTT1*) and their four representatives in mitochondria (*SOD2*, *TRX3*, *GPX3*, and *CTA1*) since melatonin has been considered as a mitochondria-targeted antioxidant due the action at mitochondrial level (Tan et al. 2016; Reiter et al. 2017, 2018). We perform gene expression analyses under H_2O_2 oxidative stress for both melatonin-treated and control cells with the genes selected. Oxidative stress is closely related to UV radiation since radiation is a significant source of ROS and DNA damage (Farrugia and Balzan, 2012). In plants, melatonin has been

reported on numerous species. The largest amounts of melatonin have been determined in oily seeds and highly UV-exposed plant organs, which suggests that melatonin also serves as a UV protector and promotes seed viability (Hardeland, 2016). Thus we aimed to test the effect of melatonin on UV-irradiated yeast as an interesting issue within the scope of melatonin's protective ability.Similarly to oxidative stress, we also performed expression analyses with two radiation-sensitive genes involved in DNA damage repair, namely *RAD18* and *RAD52*, using UVC light as a stress-triggering agent.

Recently, Vazquez et al. (2017) reported that melatonin supplementation to the culture medium alleviates the oxidative stress generated in the stationary phase and up-regulates the gene expression of the antioxidant defense-related genes in *S. cerevisiae*. Also the same authors, observed a decrease in ROS accumulation as well as in lipid peroxidation for both *Saccharomyces* and non-*Saccharomyces* when melatonin was present in the culture (Vázquez et al. 2018). In our study, in order to clearly assess the protective role of intracellular melatonin, we developed an intracellular melatonin-charging method. These melatonin-charged cells were then tested against oxidative and UV stress. Following this approach, we were able to prove that melatonin is uptaken by yeast cells and these melatonin-enriched cells improve its growth and tolerance to oxidative and UV stress by modulating the gene expression in both unstressed cells and during stress treatments. These results corroborate some of those previously reported by Vazquez et al. (2017) and provide new evidence for the protective role of this molecule against specific stresses in *S. cerevisiae*.

2.2. Results and discussion

2.2.1. Enrichment in intracellular melatonin and its impact on growth and intracellular ROS

It was only until recently that melatonin was shown to reduce oxidative stress damage to yeast cells when they grow in the presence of melatonin in the medium (Vázquez et al. 2017, 2018). In this study, we followed a different approach by using exponentially growing cells charged with melatonin to assess the protective role of this molecule. This was because when we started this study, we were

unable to induce reproducible melatonin synthesis in *S. cerevisiae.* Thus we decided to charge cells with melatonin by incubating them during a short period of time (30 min) in the presence of this molecule. The exponentially growing yeast cells were incubated in the presence of different melatonin concentrations in order to enrich them with this molecule and to assess its impact on growth. The added melatonin was uptaken by yeast cells, which showed a significant intracellular increase compared to the untreated control cells, with melatonin levels of 37.60, 66.23, and 13577.25 ng/10^8 cells for the melatonin-treated cells respectively for treatment doses 0.05, 0.1, and 20 mM (Figure 2.1).

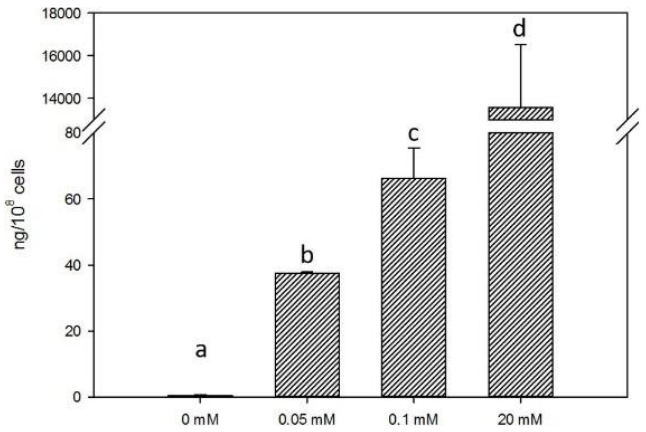

Figure 2.1 Yeast response to melatonin treatment. Intracellular melatonin detected in the control and melatonin-treated cells with doses of 0.05, 0.1, and 20 mM. Values with a different letter are significantly different with a $P < 0.05$.

Thus, analysis of melatonin-treated cells clearly demonstrated an increase in intracellular melatonin and, therefore, the ability of yeast to absorb this indole. Regarding the mechanism of melatonin intake in yeast is still unknown and there is no clear evidence of whether this molecule can be absorbed by passive diffusion or by active transport facilitated by any specific permease. Reiter and Tan (2013) have reported that, given its amphiphilic nature, melatonin can cross physiological barriers in both the lipid and aqueous environments of mammalian cells. Conversely, Hevia et al. (2015) have proved that members of the SLC2/GLUT family glucose transporters play a central role in melatonin uptake in mice cells. Another question posed is whether the intracellular concentration determined in the melatonin-treated cells is comparable to a physiological concentration of endogenous synthesis. Although a wide variation in the standard

physiological concentration of intracellular melatonin has been reported in yeasts, Reiter and Tan (2003) consider that physiological levels of melatonin can be acceptably variable, even when exceeding nanomolar ranges, which is consistent with the intracellular concentration detected in the melatonin-treated cells we used for the stress assays (~66 ng/10^8 cells). In fact, we are now able to induce endogenous melatonin synthesis by pulses of tryptophan, and other intermediates of the route, and can obtain similar concentrations to that obtained in this study (Chapter 3).

To assess whether enrichment in melatonin can confer cells better fitness, the melatonin-treated and untreated cells were grown in SC liquid media. These growth assays revealed a significant increase ($P < 0.05$) in the area under the curve (AUC) of the 0.1 mM melatonin-treated cells versus the untreated cells (Figure 2.2B), while a decrease was observed for the 20 mM melatonin-treated cells (Figure 2.2C) and no differences were found for the 0.05 mM treatment (Figure 2.2A). These differences in AUC were primarily due to changes in the lag phase of growth as there was no observable effect on the μmax or the maximum OD obtained by the different cultures.

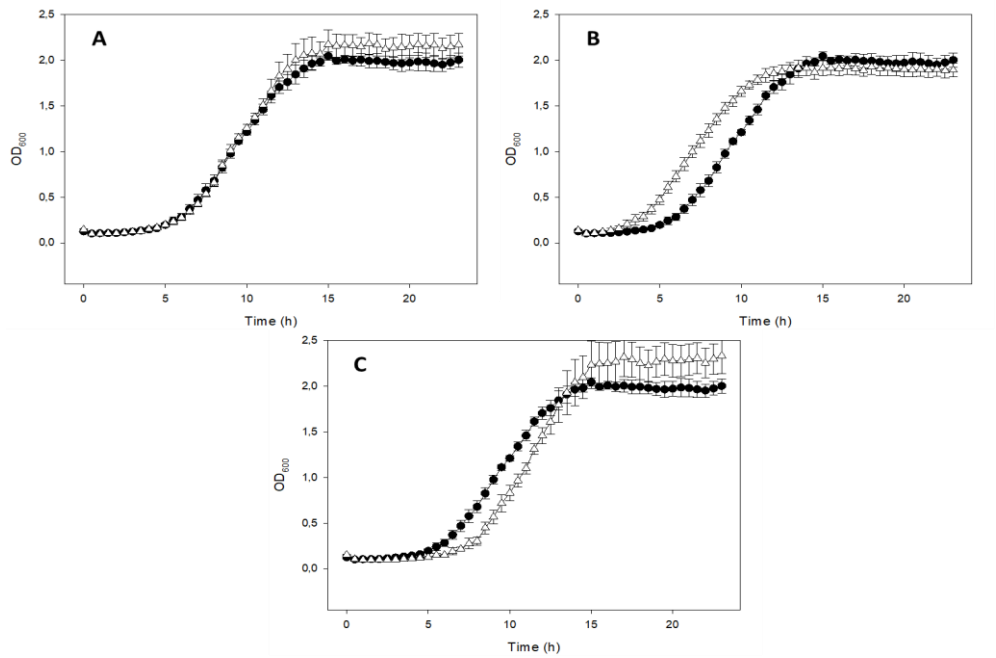

Figure 2.2 Effect of melatonin on yeast cell growth. Cell growth curve for the control cells (black circles) and melatonin-treated cells (white triangles) with concentrations of 0.05 (**A**), 0.1 (**B**), and 20 mM (**C**).

As the 0.1 mM melatonin-treated cells showed improved growth, we compared their intracellular ROS levels against the control cells. As is depicted in the Figure 2.3, a significant decrease of ROS in the melatonin-charged cells during exponential growth was observed. This results are in agreement with previously obtained (Vázquez et al. 2018).

Thus, the first remarkable effect of the presence of intracellular melatonin was the modulation of the lag phase, which depended on the treatment dose, showing a shortened lag phase for cells treated with a concentration of 0.1 mM and prior to stress treatments. We chose this melatonin dose for further testing. It is difficult to consider explaining such advanced growth by using this molecule as a nutrient because its intracellular concentration is very low but we may think of melatonin as an inducer molecule that promotes growth. Melatonin as a trigger for an early start-up of cell growth poses interesting questions about its role as a signaling molecule involved in growth modulation in a population density-dependent manner. Although melatonin has not been described as a quorum-sensing molecule in yeast, its presence can directly or indirectly modulate the yeast metabolism at different levels. Therefore, further insights into cell-to-cell communication and melatonin membrane receptors are needed. In fact other molecules that derive from tryptophan metabolism, such as tryptophol and other aromatic alcohols, have been reported to act as quorum-sensing molecules (Chen and Fink 2006; González et al. 2017, 2018a).

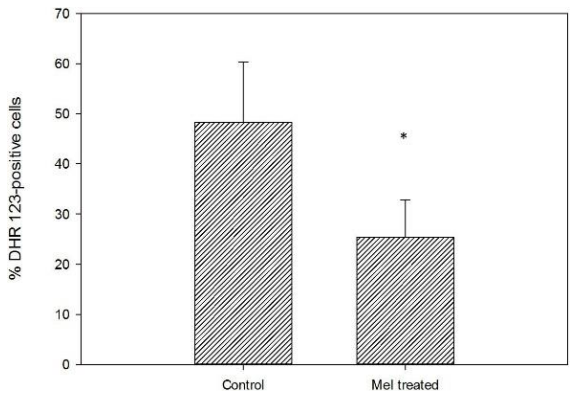

Figure 2.3 Melatonin effect on intracellular reactive oxygen species concentration in yeast. Intracellular ROS concentration in the control and melatonin-treated cells with a dose of 0.1 mM, represented as the percentage of cells showing positive DHR 123 staining ($*P < 0.05$).

2.2.2. Protective role of melatonin against oxidative stress and UV radiation

To evaluate the possible role of melatonin as an antioxidant agent in *S. cerevisiae*, the melatonin-treated and untreated cells were subjected to oxidative stress shock by H_2O_2. After this oxidative agent was present for 1 h, cells were plated to determine the mortality percentage after oxidative stress shock. This oxidative stress resulted in the non-viability of approximately half the population in the control cells (not enriched in melatonin).

However, the intracellular presence of this molecule raised viability to 87%, which clearly indicates the amelioration of oxidative damage by this molecule being present (Figure 2.4A).

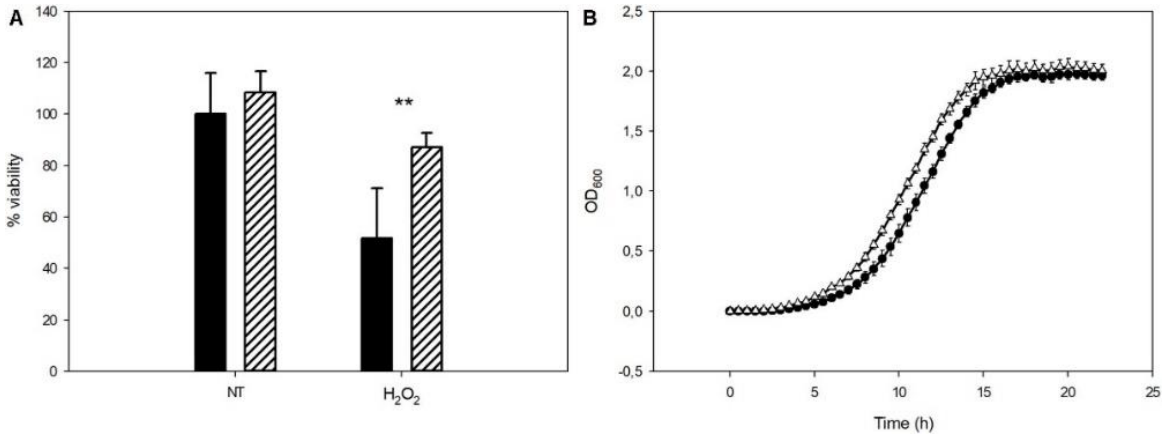

Figure 2.4 Effect of melatonin and oxidative stress in yeast on the viability and growth of yeast. (A) Viability of yeast cells before (NT) and after stress (H_2O_2) for the control and melatonin-treated cells (solid black and stripes, respectively). (B) Effect of H_2O_2 on the control (solid circles) and melatonin-treated cells (empty triangles) growth. Asterisks denote significance for control compared to melatonin-treated cells for each condition ($**P < 0.01$).

The same H_2O_2-challenged cells were also grown in SC to determine the impact of this stressor on yeast growth. Growth curves were used to determine the AUC of both the melatonin-treated and untreated cells (Figure 2.4B). The melatonin-treated cells had a significantly higher AUC than the control cells. Similarly to the above-mentioned growth of the unstressed cells, the main difference between both growth curves relied on a shorter lag phase in the melatonin-treated than in the untreated ones.

As far as we know, melatonin has not been connected with UV protection for yeast. To explore this possibility, the melatonin-treated and untreated cells were irradiated with UVC light. According to the literature (Birrell et al., 2001), the dose used for viability assays provokes loss of viability above 50%. The viability and AUC of the UV-stressed cells were also determined as they were in oxidative stress shock. The viability of the untreated cells lowered to 21% when challenged with UVC light (248.5 J/m2), while the viability of the melatonin-treated cells was significantly higher (62%) (Figures 2.5 and 2.6A). The growth curve of the UV-stressed cells also showed a higher AUC for the melatonin-treated cells compared to the stressed control (Figure 2.6B).

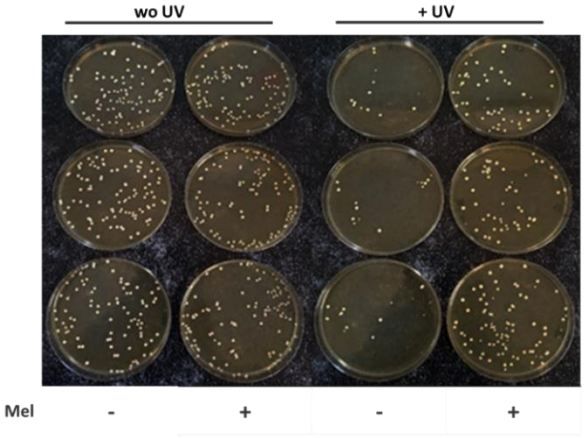

Figure 2.5 Protective effect of melatonin against UV radiation in *S. cerevisiae*. Cells treated with melatonin (Mel +) and non-treated (Mel -) were irradiated with 0 J/m2 (wo UV) or with 248.5 J/m2 UVC (254 nm) (+UV). Cells were washed after irradiation and plated on solid media and allowed to grow at 30ºC at least 3 days. For more details see the material and methods section. Each column consists of biological triplicates.

As far as we know, this is the first report to show a significant protection of intracellular melatonin in yeast against UV irradiation, with a marked reduction in cell mortality after exposure to radiation. ROS supression on UV-irradiated leukocytes due to melatonin have been reported and melatonin also showed protective effect on UV stressed melanocytes, which lends more support to this function of melatonin (Fischer et al. 2002; Janjetovic et al. 2017; Skobowiat et al. 2018).

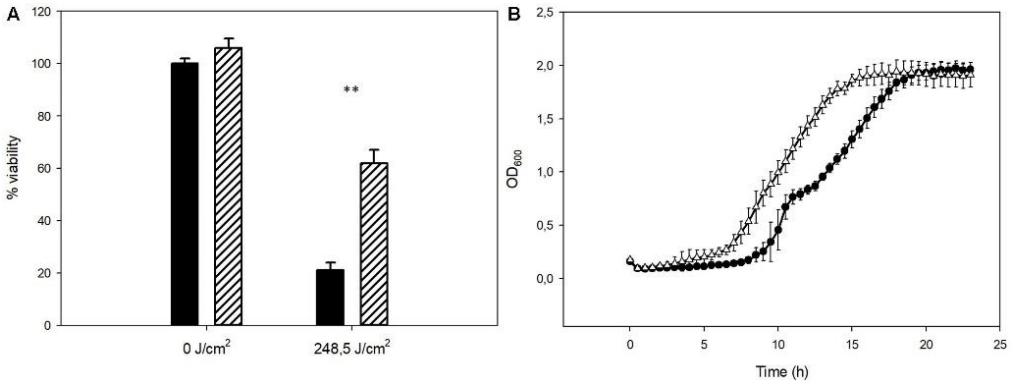

Figure 2.6 Effect of melatonin against UV radiation on the viability and growth of yeast. (A) Viability of yeast cells before (NT) and after UV radiation (248.5 J/cm2) for the control and melatonin-treated cells (solid black and stripes, respectively). (B) Effect of UV radiation on the control (circles) and melatonin-treated cells (triangles) growth. Asterisks denote significance for control compared to melatonin-treated cells for each condition (∗∗P < 0.01).

Melatonin's protective effect may occur due to several mechanisms. Melatonin can directly act as a direct scavenger to detoxify reactive oxygen (Reiter et al. 2001; Anisimov et al. 2006), but can indirectly reduce oxidative stress by increasing the activities of antioxidative defense systems by stimulating the synthesis of other important intracellular antioxidants, such as glutathione (Antolín et al. 1996; Rodriguez et al. 2004), by increasing the efficiency of the mitochondrial electron transport chain (Martín et al. 2000; León et al. 2005; López et al. 2009), and interacting synergistically with other antioxidants (Gitto et al. 2001; López-Burillo et al. 2003a). Thus, one point to look at in-depth in the near future is whether this protective role is the consequence of only direct antioxidant and photoprotectant properties, or if it is mainly a signaling molecule that triggers a molecular and physiological response to cope with these stress situations and to regulate cellular growth. To this end, we compared the transcriptional activity in Mel-treated and untreated cells in some key genes related to the response to oxidative stress and UV radiation.

2.2.3. Transcriptional response to oxidative stress and UV radiation in the melatonin-enriched cells

Several studies has been shown that melatonin possess genomic actions, regulating the expression of several genes which influences both antioxidant

enzyme activity and cellular mRNA levels for these enzymes (Rodriguez et al. 2004). Therefore, we wanted to determine if the transcriptional activity of genes related to the response to oxidative stress and UV damage was modified by intracellular melatonin. Quantitative polymerase chain reaction (qPCR) was used for the transcriptional analysis of those genes involved in antioxidant defense. As melatonin strongly affects mitochondrial activity (Zhang and Zhang, 2014; Reiter et al., 2016), we selected four cytosolic genes (*SOD1*, *TRX2*, *GPX2*, and *CTT1*) and their counterparts, mitochondrial genes (*SOD2*, *TRX3*, *GPX3*, and *CTA1*), to analyze how melatonin modulated their early transcriptional response against stress (Figures 2.7 and 2.8).

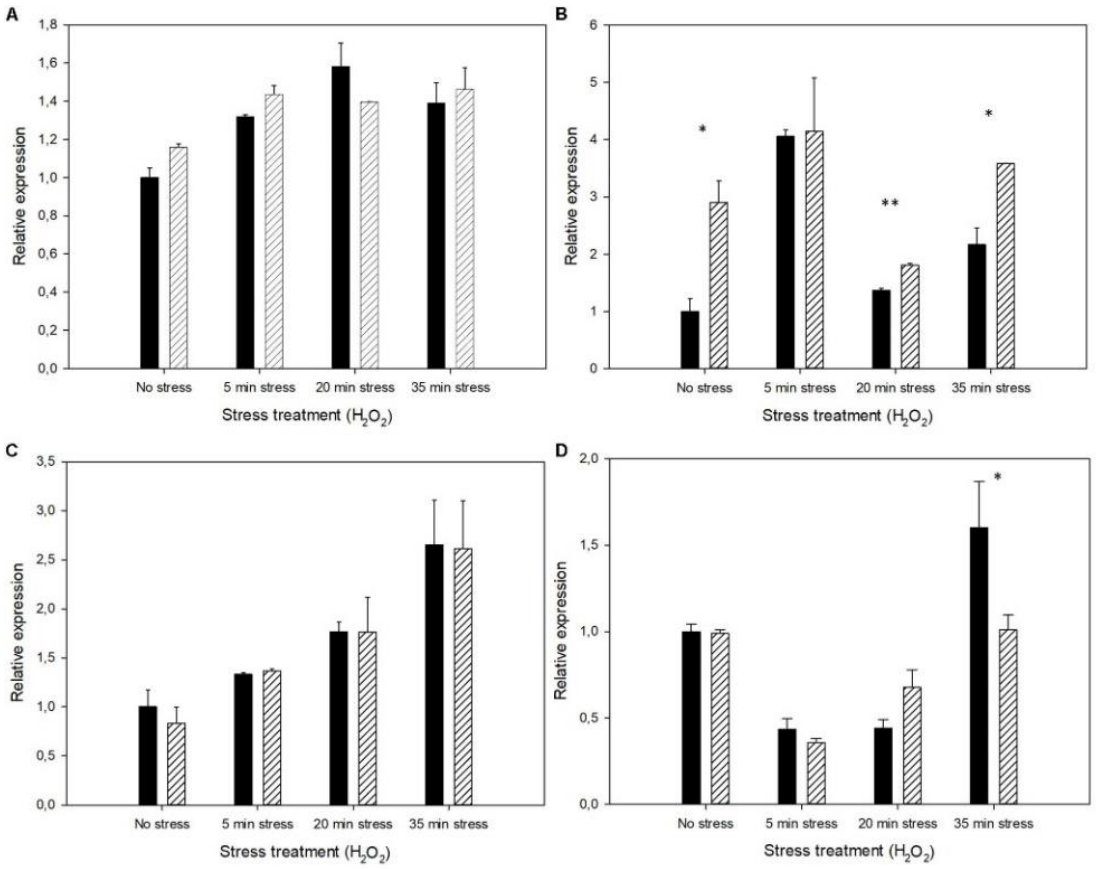

Figure 2.7 Effect of melatonin and H_2O_2 on expression of genes encoding cytosolic enzymes involved in the response to oxidative stress. Expression profiles of the untreated cells (solid black) compared to the melatonin-treated cells (stripes bars) showing the relative amount of the transcripts of the antioxidant genes with cytosolic activity (A) *SOD1*, (B) *TRX2*, (C) *GPX2*, and (D) *CTT1* under oxidative stress conditions (H_2O_2). Changes in gene activity are shown in relation to the expression of the untreated cells before stress exposure (set as value 1). Asterisks denote significance for control compared to melatonin-treated cells for each time-point (∗P < 0.05; ∗∗P < 0.01).

The first remarkable result was that the uptake of melatonin in the exponentially growing cells, before the stress treatment, had already modified transcriptional activity by up-regulating cytosolic gene *TRX2* and down-regulating mitochondrial genes *SOD2*, *GPX3*, and *CTA1* (Figures 2.7 and 2.8). The comparison of the transcriptional activity in melatonin-treated and untreated cells before stress exposure may indicate the signaling role of melatonin. Recently, the interaction of melatonin with glycolytic proteins in both *Saccharomyces* and non-*Saccharomyces* has been described (Morcillo-Parra et al. 2019b, 2020b), which may reinforce the possible signaling role of melatonin in yeast.

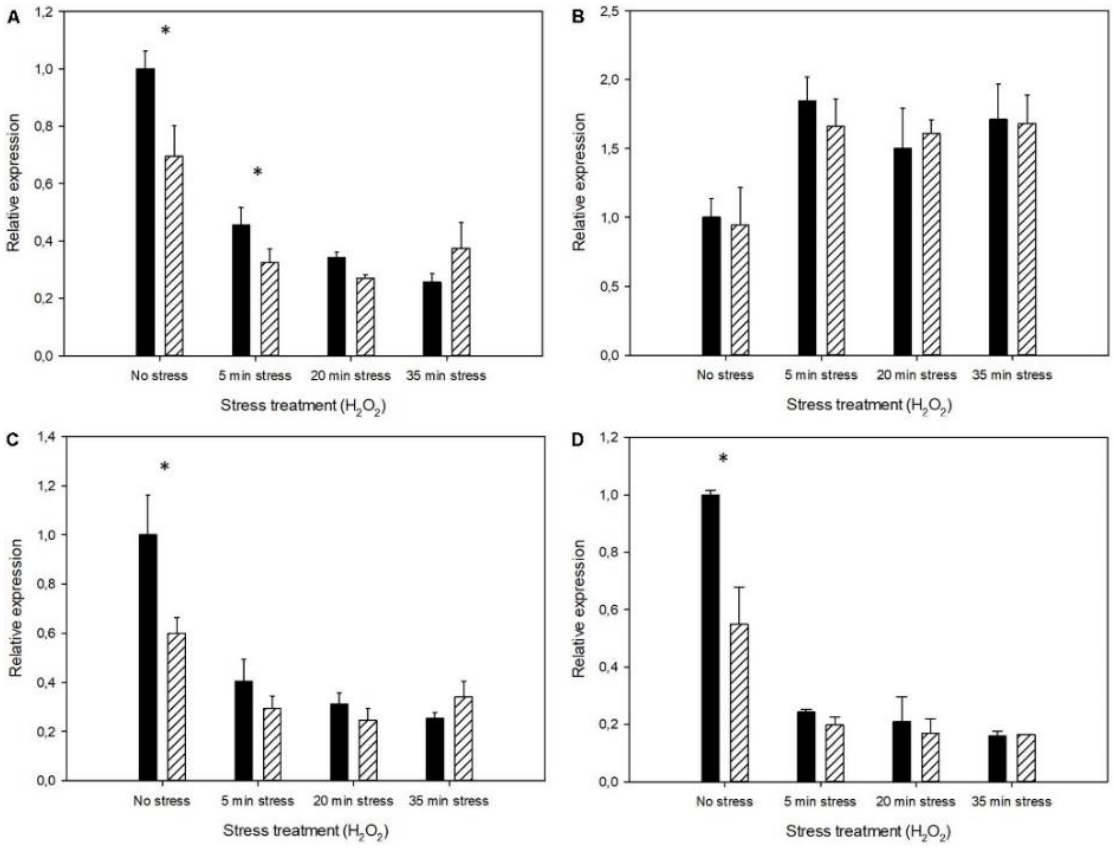

Figure 2.8 Effect of melatonin and H_2O_2 on expression of genes encoding mitochondrial enzymes involved in the response to oxidative stress. Expression profiles of the untreated cells (solid black) in comparison to the melatonin-treated cells (stripes bars) showing the relative amount of the transcripts of the antioxidant genes with mitochondrial activity (A) *SOD2*, (B) *TRX3*, (C) *GPX3*, and (D) *CTA1* under oxidative stress conditions (H_2O_2). Changes in gene activity are shown in relation to the expression of the untreated cells before stress exposure (set as value 1). Asterisks denote significance for control compared to melatonin-treated cells for each time-point ($*P < 0.05$).

On the other hand, and contrarily to what was expected, oxidative shock did not always result in the immediate induction of these antioxidant genes. In fact, most mitochondrial genes showed down-regulation immediately after oxidative stress (only *TRX3* was up-regulated) (Figure 2.8). Regarding cytosolic genes (Figure 2.7) *SOD1*, *TRX2*, and *GPX2* showed higher transcript levels after the H_2O_2 incubation, whereas *CTT1* was also transiently down-regulated after stress, followed by an immediate increase in transcripts. The melatonin-treated cells showed a similar regulation trend as the untreated cells, but this up- or down-regulation was more buffered, with less marked changes in the relative expression. This smaller impact of oxidative shock on the melatonin-charged cells can be explained because, as mentioned above, these genes were already activated by melatonin uptake prior to the stress treatment.

Thus, intracellular melatonin seemed to somehow prepare the transcriptional machinery to provide a quick response to oxidative stress. It is important to note that the presence of intracellular melatonin is not constant over time, since melatonin can be metabolized into different metabolites, as discussed in the previous chapter. The mainly early down-regulation of the genes associated with mitochondrial function (except for *TRX3*) has also been observed as a transient effect in early stages of the stress response to other oxidants like cumene hydroperoxide, while other cytosolic antioxidant genes are up-regulated at the same time (Sha et al. 2013). Vazquez et al. (2017) reported the induction of many antioxidant genes as an effect of the presence of melatonin in the medium, and obtained higher transcript levels after 16 h of exposure to melatonin. In our case, we studied the short transcriptional response of the key genes involved in oxidative and UV protection after cell exposure to these stresses. However, we observed no early induction of all the protective genes that we studied after stress exposure. Further studies into the gene expressions that cover a wider time range are likely to reveal the global up-regulation of redox and ROS-removing enzymes, as previously described (Gasch et al. 2000; Causton et al. 2001; Vázquez et al. 2017).

We also analyzed the gene expression levels of *RAD18* and *RAD52*, two sensitive genes to UV radiation that are involved in the repair of ionizing-radiation-induced DNA damage in *S. cerevisiae* (Bailly et al., 1997; Symington, 2002). As with the antioxidant genes, the melatonin-treated cells showed differences in gene expression before being challenged with UV exposure (Figure 2.9).

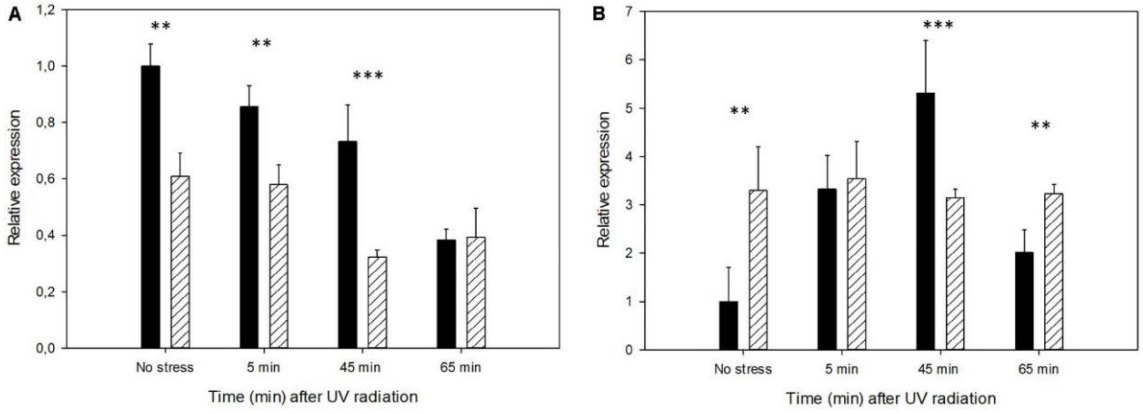

Figure 2.9 Expression profiles of the untreated cells (control) in comparison to the melatonin-treated cells (solid black and stripes, respectively) showing the relative amount of the transcripts of the DNA-repairing genes (**A**) *RAD18* and (**B**) *RAD52* after UV irradiation. Changes in gene activity are shown in relation to the expression of the untreated cells before stress exposure (set as value 1). Asterisks denote significance for control compared to melatonin-treated cells for each time-point (∗∗P < 0.01; ∗∗∗P < 0.001).

For *RAD52* gene we observed a threefold induction by only melatonin uptake. Exposure to UV radiation caused this gene to be induced in control cells, being 45 minutes after irradiation the time point with the highest expression. Nevertheless, when the cells were treated with melatonin minimal changes were observed across all time points keeping the expression levels as before UV irradiation. In contrast, *RAD18* was down-regulated with only melatonin absorption. Furthermore, the trend after exposure to UV radiation and within the time frame studied for control cells was a decrease in expression. In melatonin-treated cells, expression was similar at all times, with a slight decrease at 45 min, and which ended up equalizing with control cells at 65 min. Therefore, similar to some antioxidant defense genes, the intracellular presence of melatonin modified transcriptional activity, predisposing cells to UV stress.

2.3. Conclusions

The results obtained in this chapter supports the role of melatonin as an antioxidant molecule in yeast, as previously described, and provides new evidences for its ability to confer yeast cells protection against oxidative stress. Besides, it is described for the first time in yeast the role of melatonin in protection against UV radiation by lowering mortality and improving growth performance after stress. Among the possible mechanisms of action of melatonin, we proved its ability to modify the gene expression of antioxidant and DNA-repairing genes before and after the stress conditions. The transcriptional response of studied genes revealed that melatonin itself provokes changes in expression (*TRX2*, *SOD2*, *GPX2*, *CTA1, RAD52* and *RAD18*), which seems to prepare cells against upcoming stress. Nevertheless, more insight into further gene expression profiles, plasma membrane transporters of melatonin and endogenous synthesis conditions may shed some light on melatonin's biological importance, and reveal other features of this bioactive compound in yeast.

CHAPTER 3

Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites.

This chapter has been published in: Journal of Pineal Research (2019) 66:e12554 (https://doi.org/10.1111/jpi.12554)

3.1. Introduction

Sprenger et al. (1999) conducted the first study about melatonin synthesis in yeast. In this work, the authors reported that baker's yeast was able to synthesize melatonin from tryptophan, serotonin, *N*-acetylserotonin and 5-methoxytryptamine as potential precursors. Since then, very few studies have been carried out on this topic since most research has focused on demonstrating the presence of melatonin or biosynthesis by yeasts in a fermentative context (Rodriguez-Naranjo et al. 2011b; Garcia-Moreno et al. 2013; Fernández-Pachón et al. 2014; Vigentini et al. 2015; Fernández-Cruz et al. 2016). Moreover, the biosynthetic pathway of melatonin and other indolic compounds is completely unknown in yeasts. The only gene described and characterized as being involved in melatonin production is *PAA1*, a polyamine acetyltransferase identified as the homolog of aralkylamine *N*-acetyltransferase (AANAT) (Ganguly et al. 2001), while the remaining genes and enzymes of the route are unknown to date.

Another issue to find out in the coming future is the physiological sense of melatonin synthesis in yeasts. As for its properties, it is clear that melatonin has a multiple effect on different organisms, such as the regulation of circadian rhythms and seasonal reproduction, immune system modulator, inhibitor of tumor growth and protection against UV light, among others (Elsner et al. 2004; Chowdhury et al. 2008; Tan et al. 2010; Kleszczyński et al. 2011; Reiter, RJ.; Tan, DX; Rosales-Corral, S; Manchester 2013). Melatonin's antioxidant action is well established, and it can act as a radical scavenger, by regulating antioxidant enzymes or contributing to the homeostasis of mitochondria (Martín et al. 2000; Rodriguez et al. 2004; Hardeland 2005; Dar et al. 2015). Recently, three independent works have also proved the role of melatonin as an antioxidant and as UV protector in *S. cerevisiae* (Vázquez et al. 2017, 2018; Bisquert et al. 2018). Melatonin is also hypothesized to act as a growth signal molecule (Rodriguez-Naranjo et al. 2012), but the role of produced melatonin is not very well known.

The work performed in this chapter aims to provide new empirical data to unveil melatonin production in *S. cerevisiae*. To this end, we carried out several pulses with the intermediates of the pathway (tryptophan, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, tryptamine, and 5-methoxytryptamine) in cells in

different growth stages. We evaluated the bioconversion of several indolic compounds related with melatonin in exponentially growing yeast cells using yeast minimal media (SD) in the batch mode and synthetic must (SM) in a continuous culture. We also performed the experiment in arrested cells in salt medium (SLT) by following, but extending, the study conducted by Sprenger et al. (1999). The intracellular content of cells and extracellular media was analyzed by ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

3.2. Results and discussion

It is known that melatonin is produced by yeasts during alcoholic fermentation and this production depends on the medium, mainly the tryptophan and sugar concentrations, and also the growth phase (Rodriguez-Naranjo et al. 2011b; Rodriguez-naranjo et al. 2013; Fernández-Pachón et al. 2014; Meng et al. 2017; Fernández-Cruz et al. 2018), among other conditions. However, the biosynthetic pathway of melatonin and other indolic compounds is completely unknown in yeasts. Conversely, this metabolic route has been extensively studied and well established in other organisms.

Although differences in the intermediates and branches of the route have been found in different organisms (Tan et al. 2016), there is a high degree of conservation in the enzymatic reactions which leads to the synthesis of melatonin. To unveil the putative biosynthetic route and the order of the enzymatic steps in yeasts, we studied the production of indolic compounds, both intra- and extracellularly, after pulses of different intermediates of the route in different growth systems: exponential growth conditions in SD and continuous culture with SM. Chemostat cultures help to accurately control many variables, such as the specific growth rate, temperature and pH, among others. This steady state is only disrupted by the pulses of different intermediates, to produce an over-flow of its metabolic conversion.

3.2.1. Pulses of melatonin intermediates to yeast cells: a general overview

Tryptophan, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, tryptamine, and 5-methoxytryptamine were pulsed to the cells grown in SD (the batch culture) or SM (the continuous culture) and to the cells incubated in a nonproliferative medium SLT (arrested cells). After 15 minutes of the pulses, the intracellular and extracellular metabolites were analyzed by UHPLC/HRMS. As many data were collected, and to provide a better understanding, we represented the increases in the different indolic compounds on a heatmap (Figure 3.1), however all the concentrations can be found in the Table 3.1.

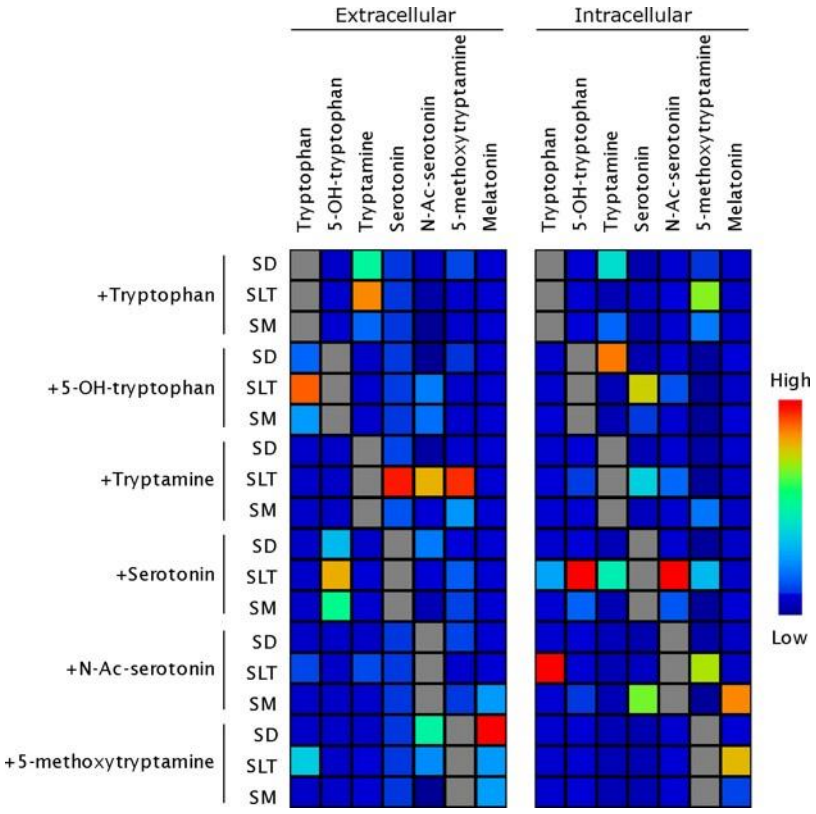

Figure 3.1 Effect of the addition of different potential melatonin precursors (rows) on the increase in its related products (columns) extra- and intracellularly in *S. cerevisiae*. Differences in concentrations were normalized and the increases in the compound used in each pulse are not considered (gray boxes). Defined synthetic (SD), salt medium (SLT), and synthetic must (SM)

Although different growth systems provide considerable similarities in the different indole bioconversions after each pulse, we also detected differences in

the production of the indolic metabolites among growth media, which could be explained by either a higher permeability of cells to the pulsed compounds or a different bioconversion rate in the various growth states. Most of the produced metabolites were detected either intracellularly or extracellularly, but a direct correlation was not always found between the presence in the medium and in the cellular compartment. This could also be due to the fact that under specific conditions, certain compounds are released to the medium or are rapidly converted into other metabolites (Mueller et al. 2001). Previous publications have also reported the rapid turnover of melatonin, as well as other 5-methoxylated-related indoles, as a result of different mechanisms, such as photooxidation (Poeggeler and Hardeland 1994), hydroxylation by free radicals (Tan et al. 2002), or metabolization by different enzymes (Tan et al. 2015), which can also contribute to these differences observed among the various growth systems and cellular states.

The conversion of tryptophan into serotonin involves two reactions: hydroxylation and decarboxylation. However, the order of these reactions described for distinct organisms differs. On the one hand, tryptophan is hydroxylated to 5-hydroxytryptophan, by the tryptophan hydroxylase (TPH) enzyme, and is then decarboxylated, by aromatic L-amino acid decarboxylase (AADC), which gives rise to serotonin. On the other hand, and alternative way, AADC can decarboxylate tryptophan into tryptamine, and then tryptamine is hydroxylated by tryptamine 5-hydroxylase (T5H), to result in serotonin. The second part of the biosynthetic route consists in the conversion of serotonin into melatonin. Once again, this conversion can also occur in two different ways and the enzymes described as being involved are AANAT or acetylserotonin *O*-methyltransferase (ASMT)/caffeic acid *O*- methyltransferase (COMT). If serotonin is acetylated by AANAT, it produces *N*-acetylserotonin, which can then be *O*-methylated by ASMT/COMT to produce melatonin. However, serotonin can first be *O*-methylated and then the resulting 5-methoxytryptamine is acetylated by AANAT to generate melatonin.

3.2.1.1. Hydroxylation activity

As explained above, there are two hydroxylation activities involved in the synthesis of serotonin. The hydroxylation of tryptophan to 5-hydroxytryptophan is carried out by the TPH enzyme. This reaction is known as the first step in the classic melatonin biosynthetic pathway described for animals. Conversely, in plants, hydroxylation is a second step in the biosynthetic pathway and occurs by T5H converting tryptamine into serotonin. In order to determine which hydroxylation reaction preferentially occurs in *S. cerevisiae*, we used tryptophan and tryptamine as substrates in feeding experiments. When tryptophan was added as a precursor, we were not able to detect 5-hydroxytryptophan either intra- or extracellularly for any of the growth systems (Figure 3.1).

Nonetheless, when we supplemented the media with tryptamine, we mostly detected serotonin both extra- and intracellularly in all the growth systems (Table 3.1 and Figure 3.2A). We highlight the vast amount of intracellular serotonin observed in SLT (more than 300 ng/mL), which was by far the highest concentration detected for any analyzed conditions. In light of these results, the conversion of tryptamine into serotonin seemed the preferential hydroxylation reaction, whereas tryptophan hydroxylation must be a very scarce or a null enzymatic reaction in *S. cerevisiae*.

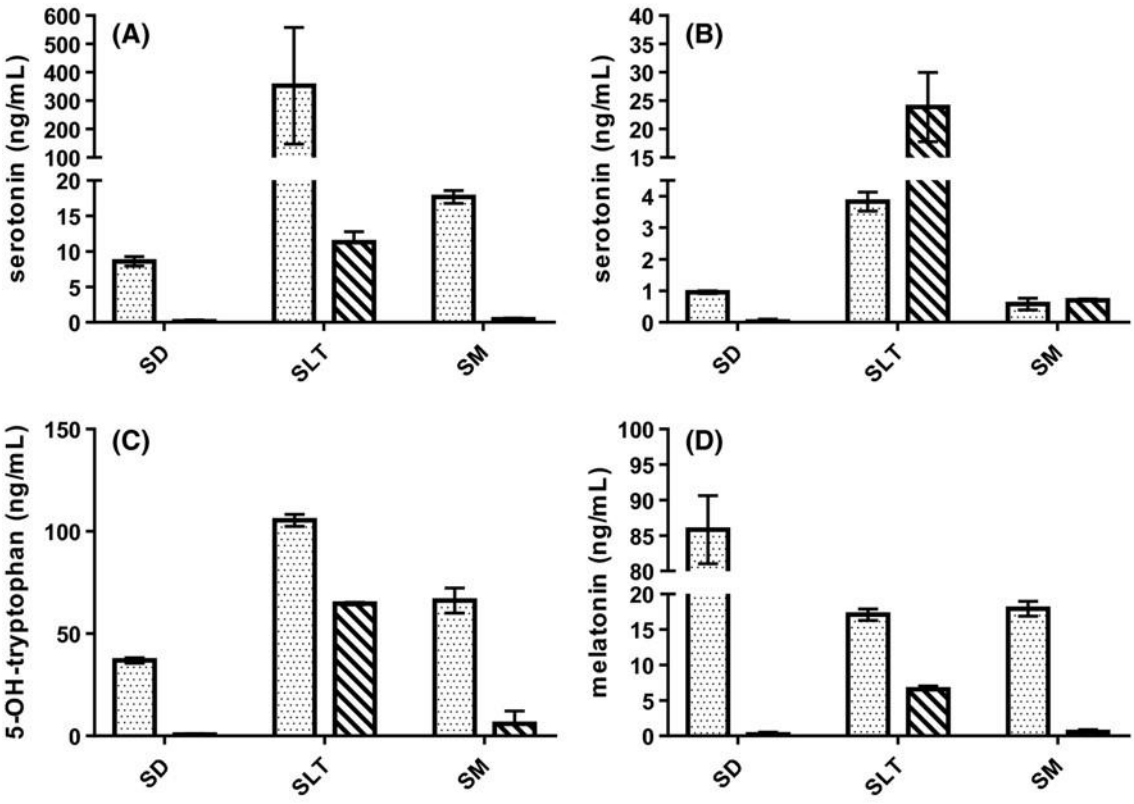

Figure 3.2 Metabolite production in yeast after addition of exogenous indolic compounds. Tryptamine (**A**), 5-hydroxytryptophan (**B**), serotonin (**C**), and 5-methoxytryptamine (**D**) in the extra- and intracellular production (dotted and stripped bars, respectively). Different growth systems are defined synthetic (SD), salt medium (SLT), and synthetic must (SM). The error bars represent standard deviation.

Aromatic amino acid hydroxylases (AAAH) are nonheme iron family of enzymes in which TPH is encompassed together with phenylalanine and tyrosine hydroxylase (PAH and TH) (Fitzpatrick 2000). Moreover, AAAH are pterin-dependent enzymes that require the presence of special cofactors, such as tetrahydrobiopterin or tetrahydromonapterin (Ehrenworth et al. 2015), although there is an exception for a TPH from dinoflagellate *Gonyaulax polyedra*, which does not require tetrahydropterin cofactors (Burkhardt and Hardeland 1996). In fact, the heterologous expression of AAAH in *S. cerevisiae* has involved the engineering of the biosynthetic and recycling pathways for these essential cofactors in order to avoid continuous and expensive exogenous additions (Ehrenworth et al. 2015; Germann et al. 2016; Zhang et al. 2016). Notwithstanding, in these works some native fungi genes, such as dihydrofolate

reductase (*DFR1*) and GTP cyclohydrolase I (*FOL2*), involved in tetrahydrofolate and folic acid biosynthesis, have been shown to play a positive role in pterin-based cofactors regeneration (Germann et al. 2016; Zhang et al. 2016).

Despite our data pointing out the lack of genes and enzymes for the biosynthesis of 5-hydroxytryptophan from tryptophan, we observed the transformation of this compound into other metabolites when it is present in the medium. The 5-hydroxytryptophan pulse resulted mainly in the synthesis of tryptophan, but also in that of tryptamine, serotonin, and *N*-acetylserotonin. This compound has been detected in both grape must (Rosazza et al. 1973; Fernández-Cruz et al. 2018), and in a synthetic laboratory medium (Fernández-Cruz et al. 2017). Thus, the presence of 5-hydroxytryptophan in natural or synthetic media could be used by yeasts to produce different indolic metabolites. In our case, the different media that we used (SLT, SD, and SM) were modified and did not contain amino acids as a nitrogen source. Thus, we avoided the presence of tryptophan or 5-hydroxytryptophan, which could interfere with bioconversion assay.

Further evidence for the hydroxylation of tryptamine to serotonin in *S. cerevisiae* relies on the direct correlation between an effective shikimate pathway for the synthesis of tryptophan and the presence of tryptamine-hydroxylation activity, as described in many organisms (Tan et al. 2016). The shikimate pathway is the central core of the aromatic amino acid biosynthetic pathway in bacteria, fungi, and plants (Braus 1991), but is not operative in animals.

3.2.1.2. Decarboxylation step

A lyase enzyme that belongs to the group of AADC is responsible for carrying out the two possible alternative decarboxylation reactions involved in the synthesis of serotonin: either the conversion of tryptophan into tryptamine or 5-hydroxytryptophan into serotonin. We detected both metabolites after the pulse of tryptophan and the pulse of 5- hydroxytryptophan, respectively, and with differences in the concentration and location depending on the growth medium (Figure 3.1 and Table 3.1). Tryptamine was detected mainly extracellularly after the pulses in SD and SLT. As mentioned above, the decarboxylation of tryptophan into the biogenic amine is the first step in most organisms with an

active shikimate pathway (Tan et al. 2016). Our data also support this reaction as being the most probabilistic step in the synthesis of melatonin in *S. cerevisiae*. Yet despite being less abundant, the presence of 5- hydroxytryptophan has also been evidenced in natural media (Rosazza et al. 1973; Fernández-Cruz et al. 2018). In these cases, 5- hydroxytryptophan can also be decarboxylated to directly produce serotonin. In fact, we detected intracellular and extracellular serotonin after the 5- hydroxytryptophan pulse, with good accumulation in the cells incubated in SLT (Figure 3.2B).

However, question marks appear with these results: Which enzyme carries out this decarboxylation? Are both decarboxylation activities performed by the same decarboxylase? In *S. cerevisiae*, the decarboxylation of the alpha-ketoacid from tryptophan (indolpyruvate) could be performed by any of the different broad substrate specificity decarboxylases *PDC1*, *PDC5*, *PDC6*, and *ARO10* (Dickinson et al. 2003; Vuralhan et al. 2003). Yet despite the presence of several decarboxylases in *S. cerevisiae*, none has been described with tryptophan decarboxylase activity, but they have been reported in other fungi (Niedens et al. 2013; Kalb et al. 2016).

The mammalian AADC enzyme (DDC) catalyzes the decarboxylation of different types of aromatic amino acids, whereas several decarboxylases with different activity and substrate specificity have been described in plants (Torrens-Spence et al. 2014). The tryptophan decarboxylase (TDC) of plants can use tryptophan and 5-hydroxytryptophan as substrates (Noé et al. 1984; De Luca et al. 1989). This TDC of plants can be functional in *S. cerevisiae* because serotonin was produced, at the expense of the 5- hydroxytryptophan levels (Park et al. 2008b).

One interesting result, which has not been reported to date, was the significant 5- hydroxytryptophan production that took place when serotonin was used as a precursor under all the conditions, mainly extracellularly (Figure 3.2C). This production involved a reversible reaction of the decarboxylation step, that is, the carboxylation of serotonin into 5-hydroxytryptophan. This way that 5-hydroxytryptophan formation occurs has not been taken into account to date. Nonetheless, more studies are needed to understand the mechanism and the physiological relevance of this 5-hydroxytryptophan production.

3.2.1.3. *N*-acetylation step

In the second part of the melatonin pathway, *N*-acetylation can occupy the first step with the conversion of serotonin into *N*-acetylserotonin or with an alternative step with the acetylation of 5-methoxytryptamine into melatonin. Both *N*-acetylations are led by *AANAT*, and homologous yeast gene *PAA1* has been described (Ganguly et al. 2001). Once again, we observed the production of both metabolites *N*-acetylserotonin and melatonin under most of the conditions tested after the respective pulses (Figure 3.1 and Table 3.1). The highest *N*-acetylserotonin concentration was observed intracellularly in the cells incubated in SLT, but the most striking result was the high melatonin concentration detected in the growth medium after the 5-methoxytryptamine pulses (Figure 3.2D). According to our experience, feeding cells with 5-methoxytryptamine was the most reproducible and effective method to synthesize melatonin. The fact that melatonin in *S. cerevisiae* can be preferentially synthesized from 5-methoxytryptamine, and at higher concentrations, instead of from *N*-acetylserotonin, supports the alternative pathway hypothesized by Tan et al. (2016), and highlights that the last enzyme in this pathway must be *N*-acetyl transferase instead of *O*-methyltransferase. This hypothesis has also been supported by the results obtained by Ganguly et al, (2001) in which the characterization of enzyme encoded by *PAA1* was able to acetylate 5-methoxytryptamine to melatonin. Interestingly, in that work tryptamine was the second amine preferably acetylated by *PAA1* (Ganguly et al. 2001). This finding is important since *N*-acetyltryptamine has not been considered as a key metabolite involved in the melatonin synthesis pathway. Additional studies on this finding reveal further information about the importance of the *N*-acetyltryptamine in the synthesis of melatonin.

3.2.1.4. *O*-methylation step

Axelrod and Weissbach (1960) described enzyme ASMT as being responsible for performing the conversion of *N*- acetylserotonin into melatonin at the expense of S-adenosyl-L-methionine (SAM). This enzyme, along with other enzymes that possess *N*-acetylserotonin *O*-methyltransferase activity, such as COMT, has been regarded as the last enzyme of the conventional melatonin biosynthesis

pathway. This activity in plants is the penultimate one as it has been found that both recombinant *Arabidopsis thaliana* ASMT and COMT are able to use serotonin as a substrate to produce 5-methoxytryotamine (Lee et al. 2014; Byeon et al. 2016). To elucidate what could take place in *S. cerevisiae*, we carried out pulses with both substrates serotonin and *N*-acetylserotonin. However, we cannot rule out any of these metabolic steps because we detected the production of both metabolites under different growth conditions and at distinct locations. For *N*-acetylserotonin used as a substrate, we observed melatonin production in both intra- and extracellular compartments during growth in SM, but with wide variability. As regards serotonin as a substrate, we observed an increase in 5-methoxytryptamine under extracellular SLT and SM conditions and only under intracellular conditions for SLT (Figure 3.1 and Table 3.1). Likewise, the key point in future research is to discover which enzyme in yeasts is able to methylate both substrates.

3.2.1.5. Catabolism

Regarding melatonin catabolism, hydroxylation, demethylation, deacetylation, and deoxygenation reactions can lead to the synthesis of different metabolites, such as *N*1-acetyl- *N*2-formyl-5-methoxykynuramine (AFMK), *N*1-acetyl-5-methoxykynuramine (AMK), 2-hydroxymelatonin, or 6-hydroxymelatonin in distinct organisms (Krotzky and Hardeland 2008; Hardeland 2017b) (Figure 3.3; Figure 3.5).

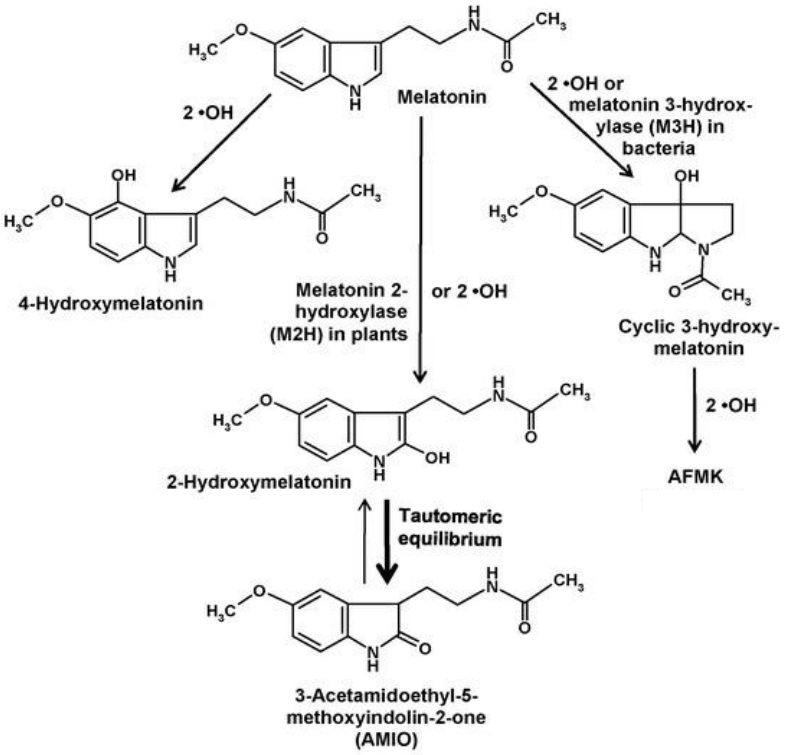

Figure 3.3 Hydroxylation reactions at ring atoms 2, 3, and 4 of melatonin. *N*1-acetyl-*N*2-formyl-5-methoxykynuramine (AFMK) (Hardeland, 2017)

Subsequent studies on yeast melatonin metabolism should include the catabolites downstream of melatonin synthesis, to further enrich the comprehension of this metabolic pathway. Tan et al. (2007) reported that some of these melatonin-catabolites can reach up to 1000-fold melatonin levels. Thus, melatonin should be considered as an intermediate rather than a final product in the synthesis of indolic compounds in yeasts. In fact, the catalytic efficiency (V_{max}/K_m) of melatonin 2-hydroxylase (M2H) from plants, which converts melatonin into 2-hydroxymelatonin, is 2000-fold higher than that of ASMT (Byeon and Back 2015; Byeon et al. 2015).

The conversion of melatonin into *N*-acetylserotonin by *O*-demethylation has been described for humans and implies subforms of cytochrome P(450) (Young et al. 1985; Hardeland 2017b). To find out if this reaction also occurs in yeast, we performed a pulse of melatonin to a wine *S. cerevisiae* strain incubated in SLT

medium. Interestingly, we observed the production of large amounts of *N*-acetylserotonin and 5-methoxytryptamine in the extracellular fraction (Figure 3.4).

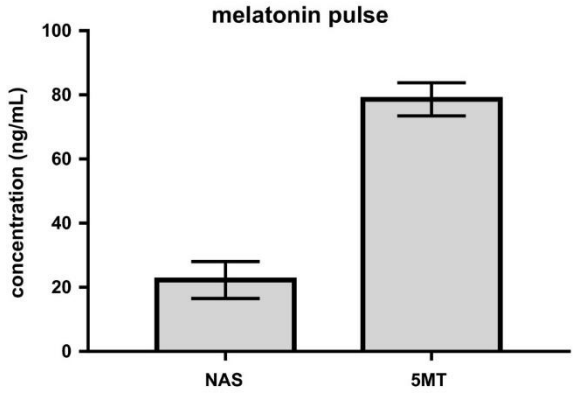

Figure 3.4 Melatonin catabolites produced extracellularly after 1 mM exogenous melatonin addition to arrested yeast cells in salt medium. *N*-acetylserotonin (NAS), 5-methoxytryptamine (5MT). Error bars represent standard deviation.

To date, the *O*-demethylation of melatonin into *N*-acetylserotonin had not been reported in *S. cerevisiae*. The catabolic deacetylation pathway leading to 5-methoxytryptamine formation was previously observed in *S. cerevisiae* (Sprenger et al. 1999) (Figure 3.5).

This pathway it is really important in dinoflagellates and also exists in the vertebrates (Hardeland 2015). In dinoflagellates the enzyme responsible for melatonin deacetylation is aryl acylamidase which is induced by is induced by high levels of melatonin (Hardeland et al. 2007).

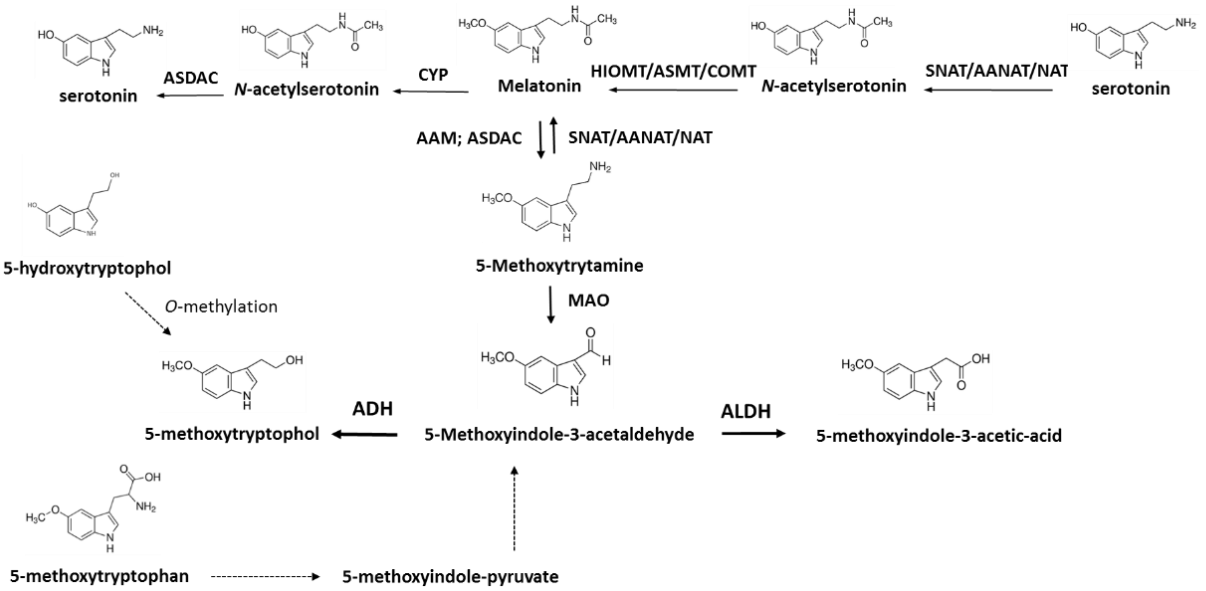

Figure 3.5 Role of 5-methoxytryptamine in melatonin metabolism and melatonin catabolism pathways in other organisms.

Another reverse melatonin related pathway was recently discovered in plants, by which *N*-acetylserotonin is converted into serotonin by *N*-acetylserotonin deacetylase (ASDAC) rather than into melatonin (Lee et al. 2020) (Figure 3.5). The gene with ASDAC activity has been found and it is a histone deacetylase gene (*HDAC10*) which exhibited activity toward *N*-acetylserotonin, *N*-acetyltyramine, *N*- acetyltryptamine, and melatonin, being the highest activity for *N*-acetylserotonin (Lee et al. 2018).

In humans, melatonin is metabolized principally to 6-hydroxymelatonin, which is further conjugated with sulfate and excreted in urine, whereas melatonin *O*-demethylation represents a minor reaction (Ma et al. 2005). In our case, 5-methoxytryptamine formation was greater than that of *N*-acetylserotonin, but both catabolites were found in significant amounts in the growth medium. In future experiments, we should broaden the metabolites analyzed to other melatonin catabolism products such as 6-hydroxymelatonin.

3.2.2. Melatonin synthesis in yeast: a putative biosynthetic pathway

Based on the results obtained from the different bioconversion experiments, the putative melatonin biosynthetic pathway is proposed in Figure 3.6. 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. Later this tryptamine is used as a precursor for serotonin synthesis by hydroxylation.

Yet despite us not detecting the 5-hydroxytryptophan formation from tryptophan, *S. cerevisiae* is able to transform this compound into serotonin, and its presence in the medium can serve to produce different indolic compounds. Interestingly, we also observed the 5-hydroxytryptophan production in larger amounts in a reversible reaction from serotonin. This serotonin carboxylation has not yet been reported and it is worth studying in forthcoming experiments to gain insights into not only the physiological and metabolic conditions under which this transformation happens in yeasts, but also into the putative genes and enzymes involved in it. From serotonin, the two converted products were *N*-acetylserotonin (*N*-acetylation) and 5MT (*O*-methylation), with the latter being the preferred substrate to produce melatonin. Finally, the yeast was able to convert melatonin into its two precursors, *N*-acetylserotonin and 5-methoxytryptamine, by *N*-deacetylation or *O*-demethylation activities, respectively, in reversible steps to most described melatonin pathways.

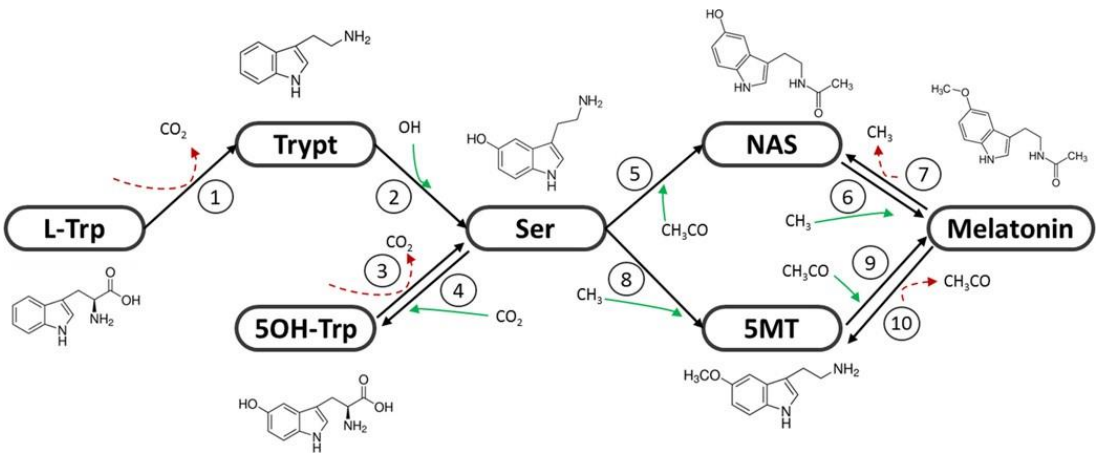

Figure 3.6 The hypothesized melatonin biosynthetic pathway in yeast. L-Trp (tryptophan), Tryp (tryptamine), 5OH-Trp (5- hydroxytryptophan), Ser (serotonin), NAS (*N*-acetylserotonin), 5MT (5-methoxytryptamine). Enzymatic steps are shown in numbers: 1 (tryptophan decarboxylation), 2 (tryptamine hydroxylation), 3 (5-hydroxytryptophan decarboxylation), 4 (5-hydroxytryptophan carboxylation), 5 (serotonin *N*-acetyltation), 6 (*N*-acetylserotonin *O*-methylation), 7 (N-acetylserotonin O-demethylation), 8 (serotonin *O*-methylation), 9 (5-methoxytriptamine *N*-acetylation), 10 (melatonin deacetylation)

3.3. Conclusions

In the present chapter, we provide new empirical data on the production of melatonin and other indole-related compounds in the yeast *S. cerevisiae*. Based on the results obtained from the different bioconverted indolic compounds tested (tryptophan, 5-hydroxytryptophan, tryptamine, serotonin, *N*-acetylserotonin, 5-methoxytryptamine, and melatonin), we have hypothesized a melatonin synthesis pathway in yeast. Our results showed that serotonin, in yeast, was prevalently formed via tryptophan decarboxylation, and followed by tryptamine hydroxylation as occurs in plants. Melatonin production from serotonin can be accomplished by serotonin *N*-acetylation, followed by *O*-methylation or, in turn, by *N*-acetylation of 5-methoxytryptamine. Accordingly, the classic pathway of melatonin synthesis in vertebrates does not seems prevalent in yeast. As important observation, bottlenecks in the pathway still exist as supplementation of a substrate did not lead to the synthesis of all downstream metabolites. Additionally, when melatonin was added to the cells, both, *N*-acetylserotonin and 5-methoxytryptamine were produced revealing the importance of melatonin catabolism. Thus, melatonin itself should not be considered as a final metabolite, but rather as an intermediate.

Table 3.1. Intracellular and extracellular indolic compounds detected after the pulses of different intermediates of the melatonin route in different growth systems.

Growth media	Source	Pulse	Tryptophan	5-OH-tryptophan	Tryptamine	Serotonin	N-Ac-serotonin	5-methoxytryptamine	Melatonin
SD	E	w/o	0.2674 ± 0.1941	0.0254 ± 0.0359	0.4043 ± 0.5342	0.0837 ± 0.0027	0.1317 ± 0.0599	0.1944 ± 0.1936	0.0693 ± 0.0083
SD	E	Tryptophan	22647.5084 ± 120.7965	0.0509 ± 0.0550	2.0203 ± 0.0154	0.0247 ± 0.0234	0.4372 ± 0.0049	0.3704 ± 0.0527	0.0148 ± 0.0150
SD	E	5-hydroxytryptophan	79.7982 ± 1.4358	14543.4494 ± 146.2267	0.0477 ± 0.0543	1.0397 ± 0.0389	0.0207 ± 0.0240	0.2757 ± 0.1134	0.0300 ± 0.0141
SD	E	Tryptamine	0.8681 ± 0.5288	0.1171 ± 0.0324	13307.44 ± 449.3404	8.6904 ± 0.6549	0.2129 ± 0.0569	0.2029 ± 0.0241	0.0300 ± 0.0049
SD	E	Serotonin	1.2826 ± 0.7311	36.9021 ± 1.2747	0.0641 ± 0.0039	16955.0487 ± 432.8983	1.1657 ± 0.2085	0.2554 ± 0.1921	0.0586 ± 0.0190
SD	E	*N*-acetylserotonin	1.0836 ± 0.0187	0.0256 ± 0.0362	0.1020 ± 0.0187	0.6205 ± 0.1445	37319.7851 ± 561.5968	0.3521 ± 0.0028	0.0000 ± 0.0000
SD	E	5-methoxytryptamine	2.0843 ± 0.1933	0.0573 ± 0.0088	0.1059 ± 0.0146	0.2303 ± 0.1220	2.6075 ± 0.9223	42274.437 ± 1508.9679	85.8813 ± 4.7885
SD	I	w/o	74.5135 ± 3.4763	0.0551 ± 0.0076	0.0891 ± 0.1229	0.1745 ± 0.0442	5.3070 ± 1.8632	0.0899 ± 0.0552	0.1043 ± 0.0786
SD	I	Tryptophan	11959.2169 ± 336.8456	0.1363 ± 0.1300	1.1449 ± 1.3576	0.0251 ± 0.0297	0.2163 ± 0.0059	0.3713 ± 0.3251	0.2048 ± 0.2724
SD	I	5-hydroxytryptophan	57.2145 ± 7.0238	5303.4599 ± 616.3556	2.4362 ± 0.1242	0.2043 ± 0.0272	0.0000 ± 0.0000	0.1035 ± 0.0179	0.3894 ± 0.0054
SD	I	Tryptamine	47.4219 ± 1.0914	0.1124 ± 0.0784	55.7071 ± 9.0436	0.3405 ± 0.1206	0.0037 ± 0.0018	0.1307 ± 0.0349	0.2105 ± 0.1397
SD	I	Serotonin	62.5730 ± 6.7399	0.7249 ± 0.3309	0.0966 ± 0.0078	122.0935 ± 32.245	2.1048 ± 0.1749	0.0746 ± 0.0374	0.0105 ± 0.0049
SD	I	*N*-acetylserotonin	74.3907 ± 14.8681	0.2242 ± 0.0069	0.1073 ± 0.0855	0.1094 ± 0.0136	15.4426 ± 2.8555	0.1459 ± 0.0542	0.0370 ± 0.0238
SD	I	5-methoxytryptamine	64.1768 ± 4.5060	0.0616 ± 0.0048	0.0612 ± 0.0796	0.0000 ± 0.0000	0.3932 ± 0.0606	240.6263 ± 24.5659	0.3366 ± 0.2203
SLT	E	w/o	1054.9180 ± 68.4860	0.5537 ± 0.0303	0.1043 ± 0.1296	0.0427 ± 0.0603	0.0874 ± 0.0368	1.0125 ± 0.2041	0.6478 ± 0.0518
SLT	E	Tryptophan	34027.8124 ± 629.6247	1.6358 ± 0.2279	2.9763 ± 0.0371	0.0000 ± 0.0000	0.1961 ± 0.0095	0.7951 ± 0.1269	0.6269 ± 0.1599
SLT	E	5-hydroxytryptophan	1668.3005 ± 69.1821	28216.1233 ± 905.2998	0.1231 ± 0.0117	3.8754 ± 0.2389	1.1355 ± 0.1681	0.5414 ± 0.0424	0.0000 ± 0.0000
SLT	E	Tryptamine	938.0967 ± 33.9517	0.0137 ± 0.0049	16352.4395 ± 136.1437	352.6379 ± 205.1763	4.0657 ± 0.5506	3.9708 ± 2.6940	0.0000 ± 0.0000
SLT	E	Serotonin	1000.8418 ± 10.2509	105.9437 ± 2.8974	0.1722 ± 0.0886	26457.218 ± 71.5183	0.4966 ± 0.1147	1.2994 ± 0.2817	0.6807 ± 0.1437
SLT	E	*N*-acetylserotonin	1101.0848 ± 47.4869	0.0388 ± 0.0009	0.3274 ± 0.2792	1.5679 ± 1.4752	29507.7116 ± 4993.3714	0.6443 ± 0.0959	0.0000 ± 0.0000
SLT	E	5-methoxytryptamine	1279.5899 ± 23.6798	0.2609 ± 0.0256	0.1827 ± 0.0005	0.0881 ± 0.0303	1.2525 ± 0.5609	30077.8073 ± 149.742	17.7405 ± 0.7654
SLT	I	w/o	2490.6281 ± 519.0069	0.0000 ± 0.0000	0.2563 ± 0.0313	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0198 ± 0.0279	0.1097 ± 0.0530
SLT	I	Tryptophan	35973.3598 ± 3351.2393	0.0000 ± 0.0000	0.0284 ± 0.0151	0.7345 ± 0.7692	0.1781 ± 0.0082	2.2086 ± 0.2324	0.1378 ± 0.0230
SLT	I	5-hydroxytryptophan	2302.9020 ± 64.3623	831.5195 ± 282.9551	0.0000 ± 0.0000	23.8890 ± 6.0981	0.7085 ± 0.1945	0.0000 ± 0.0000	0.0180 ± 0.0000
SLT	I	Tryptamine	2993.1536 ± 62.9527	1.7425 ± 2.4642	19003.837 ± 388.055	11.2836 ± 1.5078	1.1322 ± 0.0668	0.0000 ± 0.0000	0.0000 ± 0.0000
SLT	I	Serotonin	9619.9673 ± 3081.2890	64.6768 ± 0.6196	1.4838 ± 1.6560	17055.8667 ± 1859.0488	10.5000 ± 0.7071	1.0439 ± 0.3084	0.1406 ± 0.0057
SLT	I	*N*-acetylserotonin	35032.0411 ± 3132.6794	0.0000 ± 0.0000	0.0354 ± 0.0004	0.7160 ± 1.0126	281.0747 ± 40.4686	2.3216 ± 0.9038	0.1148 ± 0.0558
SLT	I	5-methoxytryptamine	1693.6094 ± 38.7502	0.0000 ± 0.0000	0.1442 ± 0.0877	0.1401 ± 0.0826	0.0366 ± 0.0061	3373.9563 ± 121.4355	6.6868 ± 0.4310
SM	E	w/o	0.7654 ± 0.5502	0.0243 ± 0.0421	0.0237 ± 0.0383	0.0384 ± 0.0556	0.4106 ± 0.4674	0.2705 ± 0.2740	0.0662 ± 0.0542
SM	E	Tryptophan	24897.4552 ± 9065.2042	1.4387 ± 1.9264	0.4067 ± 0.5751	0.0587 ± 0.0829	0.2650 ± 0.1853	0.0000 ± 0.0000	0.0598 ± 0.0845

SM	E	5-hydroxytryptophan	148.5039 ± 87.4412	35058.6258 ± 18321.8693	0.0460 ± 0.0650	0.6162 ± 0.1302	1.3499 ± 1.8663	0.0928 ± 0.1312	0.1462 ± 0.1542
SM	E	Tryptamine	0.7325 ± 0.0455	0.0107 ± 0.0024	4806.1775 ± 166.7591	17.7168 ± 0.8374	0.8200 ± 0.1531	0.8953 ± 0.0363	0.0697 ± 0.0056
SM	E	Serotonin	0.4388 ± 0.0349	66.2582 ± 6.0893	0.0610 ± 0.0019	7605.9533 ± 233.9142	0.5950 ± 0.1383	0.4166 ± 0.0676	0.0632 ± 0.0257
SM	E	*N*-acetylserotonin	0.5534 ± 0.2883	0.1108 ± 0.0079	0.0306 ± 0.0432	0.1712 ± 0.1097	15611.3433 ± 1926.9269	0.3829 ± 0.5414	17.1135 ± 24.2021
SM	E	5-methoxytryptamine	0.3812 ± 0.2804	0.0248 ± 0.0351	0.0755 ± 0.0083	0.0890 ± 0.0209	0.2134 ± 0.2017	32364.6409 ± 1503.9003	17.9945 ± 1.0043
SM	I	w/o	166.2032 ± 4.6980	0.0007 ± 0.0010	0.2422 ± 0.2391	0.0239 ± 0.0176	0.0253 ± 0.0325	0.1549 ± 0.0953	0.0158 ± 0.0181
SM	I	Tryptophan	44584.4186 ± 42896.4949	0.7940 ± 1.1229	0.6218 ± 0.8793	0.1727 ± 0.2442	0.0000 ± 0.0000	0.8079 ± 1.1425	0.1274 ± 0.1333
SM	I	5-hydroxytryptophan	489.2274 ± 394.4147	42457.06 ± 28784.8354	0.0460 ± 0.0650	2.3447 ± 2.3142	0.0151 ± 0.0214	0.0928 ± 0.1312	0.2709 ± 0.3306
SM	I	Tryptamine	231.6399 ± 15.5569	0.2444 ± 0.0533	31.8801 ± 1.3526	0.4828 ± 0.1208	0.0038 ± 0.0012	0.7779 ± 0.5653	0.0079 ± 0.0085
SM	I	Serotonin	238.3308 ± 63.4051	5.8774 ± 6.2779	0.0348 ± 0.0491	1065.2192 ± 1281.1118	0.8135 ± 0.9479	0.1184 ± 0.1674	0.2225 ± 0.2163
SM	I	*N*-acetylserotonin	307.5429 ± 126.141	1.5787 ± 2.1388	0.0044 ± 0.0062	21.0041 ± 29.5258	49.5579 ± 40.6246	0.0000 ± 0.0000	7.1477 ± 10.0949
SM	I	5-methoxytryptamine	134.9343 ± 4.1042	0.0356 ± 0.0503	0.0036 ± 0.0051	0.0755 ± 0.0992	0.0674 ± 0.0926	511.6347 ± 61.7613	0.5714 ± 0.3041

Batch exponential growth (SD), continuous culture (SM) and arrested-cells (SLT); I: Intracellular; E: Extracellular; w/o: without

CHAPTER 4

Overexpression of selected yeast genes as potential orthologs involved in melatonin biosynthesis. Role of yeast polyamine acetyltransferase (*PAA1*) in melatonin production.

4.1. 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 (Tan et al. 2016). For melatonin synthesis, the majority of studies have been performed in vertebrates, particularly in mammals (hamster, rat, mice), and more recently in plants (Axelrod and Weissbach 1960; Byeon et al. 2014a; Tan et al. 2015).

As explained in previous chapters, there are several key differences in the melatonin synthetic pathway in plants and animals. In the first part, in which serotonin is synthesized from tryptophan, the main differences are as follows: (i) the synthetic capacity of melatonin in plants is not limited by tryptophan availability in their environments as plants can synthesize tryptophan through the shikimic acid pathway; (ii) for serotonin biosynthesis in plants, tryptophan is first decarboxylated and then hydroxylated, while this order is reversed in animals. In the second part of the route, by which melatonin is formed from serotonin, the following differences have been described: (i) the origins of plant and animal serotonin *N*-acetyltransferases (SNATs) are completely different and share no homology. Many SNATs and acetylserotonin *O*-methyltransferase (ASMT) homologs are present in plants, which is not the case in animals, especially mammals; (ii) in plants, caffeic acid 3-*O*-methyltransferase (COMT) may be the dominant enzyme in *N*-acetylserotonin methylation instead of ASMT, and there are no reports that COMT exists in animals; (iii) the subcellular location for melatonin synthesis differs between both. Chloroplasts and mitochondria are both involved in melatonin synthesis in plants. Under normal conditions, chloroplasts are dominant sites that produce melatonin in plants. If normal processes are blocked, for example the transcriptional suppression of T5H, the dominant melatonin synthetic pathway would shift from the chloroplast to the mitochondrion (Roseboom et al. 1998; Park et al. 2013; Byeon et al. 2014b, a; Lee et al. 2014; Tan et al. 2014b, 2015).

The enzymes found to be involved in this melatonin biosynthetic pathway from both plants or animals include: tryptophan hydroxylase (TPH), aromatic-L-amino-

acid decarboxylase (AADC/DDC/TDC), tryptamine hydroxylase (T5H), SNAT, and ASMT/ COMT.

The nomenclature of several of these enzymes should be clarified. For example, aralkylamine *N*-Acetyltransferase (AANAT), which has also been previously called SNAT and *N*-acetyltransferase (NAT). They are not just synonyms, but differ in substrate specificity (Tan et al. 2016). AANAT/SNAT and NAT can all catalyze the acetylation of indolamines at different rates depending on substrate specificity and their concentrations. However, NAT is not selective for indoles, whereas AANAT/SNAT is more selective for arylalkylamines, including indole-ethylamines (e.g., tryptamine) (Tan et al. 2016). Currently, only one homolog of SNAT has been identified in mammals, whereas several homologues of SNAT have been discovered in vertebrates other than mammals. For instance, at least three SNAT homologs have been identified in fish (Tan et al. 2015). Moreover, *O*-methylating enzymes are ASMT and COMT, but can also be known as hydroxyindole-*O*-methyltransferase (HIOMT).

Although the ability to synthesize melatonin has been determined in microorganisms like bacteria, microalgae and yeasts (Manchester et al. 1995; Poeggeler and Hardeland 2003; Rodriguez-Naranjo et al. 2012; Rodriguez-naranjo et al. 2013), very few attempts have been made to identify melatonin synthetic pathways in microorganisms, and currently very little is known about the processes involved. Based on the results obtained from the bioconversion experiments in Chapter 3, a putative melatonin biosynthetic pathway for yeast has been put forward. In this proposed route, serotonin would be formed predominantly by decarboxylation of tryptophan, followed by hydroxylation of tryptamine, which mainly happens in plants. Thereafter, melatonin production from serotonin can be accomplished by *N*-acetylation, followed by *O*-methylation or *O*-methylation and, in turn, by *N*-acetylation.

Despite the obtained results, definitive biochemical and molecular evidence is still lacking to understand the complete metabolic pathway. The search for genes homologous to those described in vertebrates and plants represents a key point to improve the synthesis of these molecules during the fermentation process.

Thus, the biosynthetic pathway of melatonin in yeast remains to be identified with certainty.

To date, no reports have been found or characterized for the enzymes responsible for melatonin synthesis and the genes that encode the enzymes in any yeast with only one exception: gene *PAA1*, a polyamine acetyltransferase, has been proposed as a homolog of *AANAT* of vertebrates, which converts serotonin into *N*-acetylserotonin (Ganguly et al. 2001). This gene has been cloned and characterized *in vitro*. The kinetic studies of *PAA1* have revealed that the K_ms with 5-methoxytryptamine and serotonin are 2.7±0.6 and 5.1±0.4 mM, respectively, and enzyme activities 14.5 and 3.0 pmol/pmol enzyme/h, respectively. Hence the calculated catalytic efficiency of the *PAA1* reaction with 5-methoxytryptamine is approximately 7-fold greater than that with serotonin, which indicates that the predominant substrate is 5-methoxytryptamine instead of serotonin (Ganguly et al. 2001; Tan et al. 2016).

Based on the results of Chapter 3, we aimed to identify the genes that may encode for melatonin biosynthetic steps in *S. cerevisiae*. To this end, we searched the *S. cerevisiae* proteins with the highest percentages of homology to the proteins involved in the synthesis of each intermediate in the biosynthetic pathway for both vertebrates and plants by an *in silico* analysis. The putative genes selected as candidates were cloned and overexpressed in both *E. coli* and *S. cerevisiae* to determine if they possessed the catalytic activity of the reference enzyme. Additionally, to clarify the role of *PAA1* in melatonin biosynthesis in yeast, we tested the differences in the mutant, the wild type and the overexpressing *PAA1* strains by measuring the acetylated amine products by UHPLC-MS/MS.

4.2. Results and discussion

4.2.1. *In Silico* analysis and identification of potential genes involved in melatonin biosynthetic pathway in *S. cerevisiae* genome

As previously mentioned, several enzymes involved in melatonin metabolism from both vertebrates and plants have been cloned and characterized (Coon et al. 1995; Ichihara et al. 2001; Isorna et al. 2011; Park et al. 2012; Byeon et al.

2014b, 2016; Byeon and Back 2015; Lee et al. 2018). We used the sequences from these organisms to identify the putative orthologs in the yeast genome for TPH, T5H, AADC/TDC/DDC, AANAT, SNAT, ASMT/HIOMT and COMT. The identification of the putative *S. cerevisiae* orthologs was performed with the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990), more specifically BLASTp, which searches protein databases using a protein query combined with the domain enhanced lookup time accelerated BLAST (DELTA-BLAST) algorithm to yield better homology detection (Boratyn et al. 2012). From the *in silico* screening of the available genome yeast sequences, the potential orthologs of the melatonin biosynthesis genes from plants or vertebrates are shown in Table 4.1. The BLAST results are sorted by the expected value (E-value) by default. A low E-value indicates a few hits, but high quality ones. The term query coverage is the percent of the query length included in the aligned segments. The similarity between two sequences can be expressed as a percent sequence identity, regardless of the position in the alignment, while identity is restricted to the same residues at the same positions in the alignment of both sequences.

For TPH, no orthologous sequences were found when using *Mus musculus* or *Homo sapiens TPH1* or *TPH2* as a query. The previous results obtained in the bioconversion experiments in Chapter 3 already showed that *S. cerevisiae* was unable to produce 5-hydroxytryptophan from tryptophan, which denotes lack of TPH activity. Furthermore, the domain required for the union of essential cofactors for aromatic amino acid hydroxylating enzymes (AAAH), such as tetrahydrobiopterin or tetrahydromonapterin, was also absent. In previous metabolic engineering works in which AAAH was overexpressed in yeasts, the authors had to insert the necessary pathways for the *de novo* biosynthesis and recycling of these cofactors to gain TPH activity (Germann et al. 2016; Zhang et al. 2016). However, an alternative hydroxylase activity should be expected in the biosynthetic pathway of *S. cerevisiae* because melatonin is produced from tryptophan as a substrate. One possible alternative is the hydroxylation step of the tryptamine to be converted into serotonin (T5H), as occurs in plants. For T5H activity, *Oryza sativa* CYP71P1 (*GenBank: AK071599*) was used as a query. The hits obtained by the BLAST search were three genes, *ERG11*, *ERG5* and *DIT2,*

with 3×10^{-98}, 2×10^{-66} and 1×10^{-61} E-values, respectively. Those genes encode for the three unique cytochromes P450 (CYPs) present in *S. cerevisiae* (Park et al. 2008a). More specifically, CYP51 (encoded by *ERG11*), which is a sterol 14α-demethylase, undertaking the C14-de-methylation of lanosterol (Karst and Lacroute 1977), and CYP61 (encoded by *ERG5)*, which is a C-22 sterol desaturase (Skaggs et al. 1996), are both involved in the ergosterol biosynthetic pathway (Sokolov et al. 2019), whereas CYP56 (encoded by *DIT2*) is a dityrosine hydroxylase involved in yeast sporulation (Briza et al. 1990, 1994). These CYPs are localized in the endoplasmic reticulum via an *N*-terminal anchor and are associated with a separate NADPH-cytochrome P450 reductase (CPR). Query cover and similarity were 90-95% and 31-33% for the three alignments respectively, whereas the identity of the three sequences was 16-18% (Table 4.1).

To discover possible AADC activity in *S. cerevisiae,* we used the TDC/DDC sequences from *O. sativa* (*GenBank: AK069031*)*, Catharanthus roseus* (*GenBank: P17770*)*, H. sapiens* (*GenBank:1644), M. musculus (GenBank: AF071068.1)* and *Bos Taurus (GenBank: BT026145.1). S. cerevisiae* genes *DPL1* and *GAD1* were selected as candidates with 3×10^{-91} and 6×10^{-86} E-values, respectively*. DPL1* is described as a dihydrosphingosine phosphate lyase that regulates intracellular levels of sphingolipid long-chain base phosphates (LCBPs) and degrades phosphorylated long-chain bases, but prefer C16 dihydrosphingosine-l-phosphate as a substrate (Saba et al. 1997). *GAD1* is a glutamate decarboxylase that converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism (Coleman et al. 2001). The results in query cover, identity and similarity terms were 90-95%, 15% and 29% respectively.

Regarding serotonin *N*-acetyltransferase activity, we used different sequences from either *AANAT* or *SNAT* as a query. When the AANAT protein sequence from vertebrates was used, *PAA1* was one of the obtained results and its E-value was 1×10^{-19}. This result was expected as *PAA1* has been previously characterized as a homolog from vertebrate AANAT. The query cover for the alignment was 70%, with an identity percentage of 18% and 33% similarity (Table 4.1).

Finally, for *N*-acetylserotonin *O*-methyltransferase activity, two *ASMT/HIOMT* candidate genes were selected in yeast based on BLAST in *H. sapiens*

ASMT/HIOMT (ENA: AAA58582.1) and *O. sativa* and *Arabidopsis thaliana COMT (ENA: BAF22945.1 and AED96460.1, respectively)*. *ERG6* and *CRG1* were the *S. cerevisiae* genes selected as possible candidates for this activity with 9×10^{-16} and 6×10^{-16} E-values, respectively. *ERG6* showed 45% query cover and 17% identity with *B. taurus ASMT*, whereas *CRG1* yielded 18% identity an 46% query cover (Table 4.1). Both genes encode for methyltransferase enzymes. *ERG6* encodes for delta(24)-sterol C-methyltransferase, which is involved in ergosterol biosynthesis (Gaber et al. 1989), whereas *CRG1* encodes for an S-adenosylmethionine-dependent methyltransferase implicated in lipid homeostasis and also mediates resistance to a drug cantharidin (Lissina et al. 2011). From these results obtained by a BLAST analysis, the genes with the best score for subsequent overexpressions in *E. coli* and *S. cerevisiae* organisms were chosen. Although no high identity values were obtained, positive similarities at the function domain level were observed for the selected candidates. However, as the protein structure is more conserved during evolution than the protein sequence, analyzing the structure to establish orthology is recommended when the protein of interest is in the twilight zone (20-35% sequence identity), which will be considered for future searches.

4.2.2. Bacterial and yeast overexpression of selected *S. cerevisiae* genes

Whole-cell biotransformation consists in the chemical modification of a compound carried out by a living organism (unlike using purified enzyme preparation) (Zöllner et al. 2010). By this system, the activity of a certain enzyme can be measured directly from the supernatant of the growth medium by taking advantage of the cofactors generated by the cell, which avoids having to add exogenously them (Lin and Tao 2017). Additionally, the presence and protective nature of the cell envelope or compartments help to stabilize enzymes, and may allow enzymes to be applied under severe reaction conditions (Lin et al. 2013). Both *E. coli* and *S. cerevisiae* are the most frequently used host organisms for the overexpression of heterologous proteins largely because of their well-studied genetic background, which is a mature and powerful genetic toolset for metabolic engineering. *E. coli* can produce high expression levels of recombinant proteins, and yeasts combine the simplicity of a unicellular organism with the ability to

perform most of the post-translational modifications required for a biologically active recombinant protein (Vieira Gomes et al. 2018).

To determine the activity of the genes selected from the BLAST analysis, plasmids containing the different genes were constructed and transformed into *E. coli* and *S. cerevisiae*. For *E. coli*, an IPTG induction system and GST-tagging were implemented in the heterologous system by using the pGEX-5X-1 vector (GE Healthcare Life Sciences). For *S. cerevisiae*, a constitutive overexpressing system was followed through the 2μ derived vectors with GPD1 as the promoter (Mumberg et al. 1995). The different strains were cultivated as detailed in the Material and Methods section, and the metabolites of interest were measured from the extracellular fraction. Following the methodology described in Chapter 3, enzyme activity was determined by spiking culture media with the substrate and following the production of the metabolite as a consequence of the candidate gene overexpression.

As a quality control of the overexpressing system, the gene responsible for the activity of interest in other organisms was also overexpressed in both organisms. For T5H, AADC, SNAT and ASMT activity, we used rice (*O. sativa*) CYP71A1, human (*H. sapiens*) *DDC*, bovine (*B. taurus*) *AANAT* and human (*H. sapiens*) *ASMT* as positive controls, respectively. For rice CYP71A1, in the work by Park et al. (2011), the authors found that only when the *N*-terminal deletion of 37 amino acids was combined with GST tagging did functional T5H activity in *E. coli* occur. Thus, we used this version of the protein in our T5H control.

Table 4.1. Identification of putative orthologs in yeast of enzymes involved in the biosynthesis of melatonin in plants and animals by basic local alignment search tool.

Enzyme activity	**Putative ortholog in yeast**	**Description**	**E-value***	**Query cover****	**Identity Percentage*****	**Similarity Percentage*****
Trytptophan hydroxylase	-	-	-	-	-	-
Aromatic amino acid decarboxylase	*DPL1*	Dihydrosphingosine phosphate lyase	3×10^{-91}	92%	15% (69/472)	29% (139/472)
	GAD1	Glutamate decarboxylase	6×10^{-86}	90-95%	15% (75/518)	29% (151/518)
Tryptamine hydroxylase	*DIT2*	N-formyltyrosine oxidase	1×10^{-61}	91%	16% (79/489)	31% (153/489)
	ERG5	C-22 sterol desaturase	2×10^{-66}	95%	16% (84/511)	32% (165/511)
	ERG11	Lanosterol 14-alpha-demethylase	3×10^{-98}	90%	18% (87/490)	33% (162/490)

Serotonin *N*-acetyltrasnferase	*PAA1*	Polyamine acetyltransferase	1×10^{-19}	70%	18% (27/152)	33% (51/152)
N-acetylserotonin *O*-methyltransferase	*ERG6*	Delta(24)-sterol C-methyltransferase	9×10^{-16}	45%	17% (28/164)	29% (49/164)
	CRG1	S-AdoMet-dependent methyltransferase	6×10^{-16}	46%	18% (30/166)	29% (49/166)

Note: Numbers in parentheses are the numbers of identical or similar residues divided by the length of the region used for the calculations.

* The expected value (E-value) is a parameter that describes the number of hits "expected" to be seen by chance when searching a database of a particular size.

** Query cover is the percentage of query length included in the aligned segments

*** Identity indicates the extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an alignment, whereas similarity refers to the extent to which nucleotide or protein sequences are related (including the percent sequence identity and/or percent positive substitutions

4.2.2.1. Activity of the candidate genes in *E. coli*

4.2.2.1.1. Decarboxylation step

A lyase enzyme that belongs to the AADC group may be responsible for carrying out the decarboxylation of tryptophan into tryptamine or 5-hydroxytryptophan into serotonin in the melatonin biosynthetic pathway. The *in silico* analysis results suggested that *DPL1* or *GAD1* could be potential candidates to perform any of these reactions involved in serotonin synthesis. To test this hypothesis, we overexpressed *DPL1* and *GAD1* in *E. coli*.

However, neither *DPL1* nor *GAD1* overexpression resulted in the increase detection of tryptamine or serotonin compared to the control strain. As expected, the *H. sapiens DDC* overexpression yielded about 50 $ng \cdot mL^{-1}$ of tryptamine and 400 $ng \cdot mL^{-1}$ of serotonin after grown in the presence of 1 mM of tryptophan or 5-hydroxytryptophan, respectively (Figure 4.1). Preference for the decarboxylation of 5-hydroxytryptophan to serotonin, compared to the conversion of tryptophan into tryptamine shown by *H. sapiens* DDC, is consistent with its role in serotonin synthesis in the classic melatonin biosynthetic pathway described for animals.

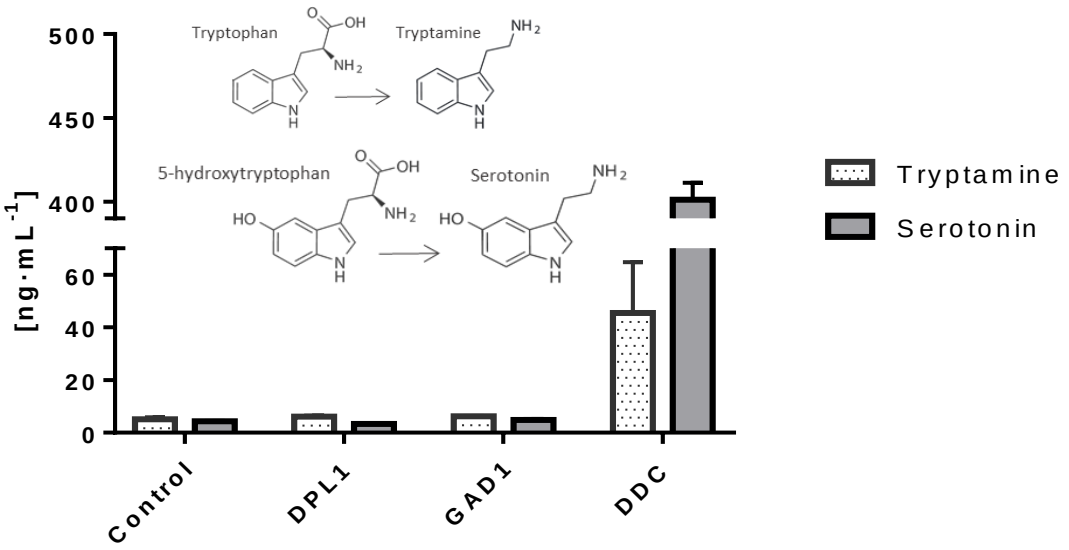

Figure 4.1 Effect of the overexpression of *DPL1*, *GAD1* and *DDC* in tryptamine and serotonin production in *E. coli*. Rosetta cells were transformed with empty vectors pGEX-5X-1 (control), pGEX-5X-1 DPL1 (DPL1), pGEX-5X-1 GAD1 (GAD1) and pGEX-5X-1 DDC (DDC). Tryptamine and serotonin were determined after culturing the different strains for 24 h in 2xTY with 1% glucose supplemented with 1 mM tryptophan or 5-hydroxytryptophan, respectively. Cultivation conditions and expression induction are detailed in the Materials and Methods. Tryptamine and serotonin were determined from the supernatant extracted with ethanol by HPLC-MS/MS.

4.2.2.1.2. Hydroxylation step

There are two hydroxylation activities involved in the synthesis of serotonin in melatonin's biosynthetic pathways. The first reaction in the vertebrate pathway consists in the hydroxylation of tryptophan to 5-hydroxytryptophan, which is carried out by the TPH enzyme. However, it is shown in Chapter 3 of this thesis that this hydroxylation reaction must be residual or null in *S. cerevisiae* as we were unable to detect the conversion of tryptophan into 5-hydroxytryptophan intra- or extracellularly for any of the growth systems during the bioconversion experiments. Furthermore, the *in silico* search for the orthologous genes from the *TPH* sequence of the different organisms did not yield any results for both the hypothetical protein and the synthesis of the essential cofactor required by it, which supports our hypothesis.

Thus by taking our results into account, we focused on the possible candidate to perform the hydroxylation reaction of tryptamine into serotonin. From the BLAST

search using plant *T5H*, we obtained *ERG5*, *ERG11* and *DIT2* as possible candidates. As previously mentioned, we overexpressed these genes together with a *N*-truncated and GST-tagged version of T5H as native T5H cannot be functionally expressed in *E. coli* (Park et al. 2011). None of the selected *Saccharomyces* genes showed tryptamine hydroxylation activity when overexpressed in *E. coli* in a medium enriched with 1 mM of tryptamine. Conversely, rice T5H led to the production of large amounts of serotonin, which exceeded 1000 ng·mL^{-1} after 50 h of T5H overexpression in the same medium.

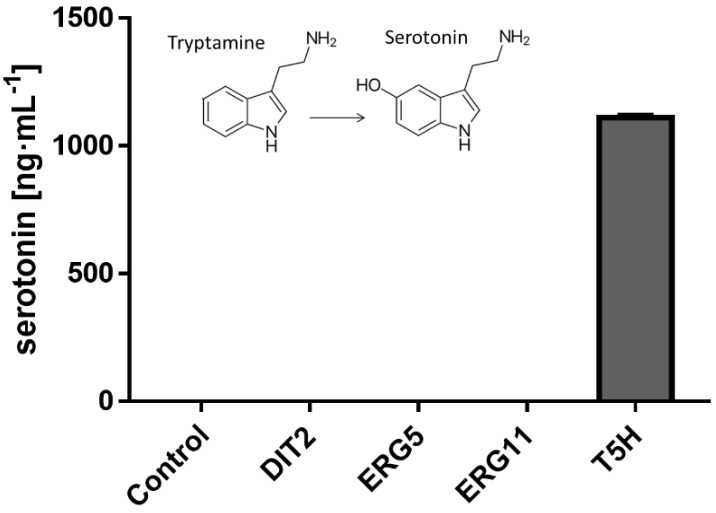

Figure 4.2 Effect of the overexpression of *DIT2*, *ERG5*, *ERG11* and T5H in tryptamine hydroxylation in *E. coli*. Rosetta cells were transformed with empty vectors pGEX-5X-1 (control), pGEX-5X-1 DIT2 (*DIT2*), pGEX-5X-1 ERG5 (*ERG5*), pGEX-5X-1 ERG11 (*ERG11*) and pGEX-5X-1 T5H (T5H). Serotonin was determined after culturing the different strains for 24 h in 2xTy with 1% glucose supplemented with 1 mM tryptamine. Cultivation conditions and expression induction are detailed in the Materials and Methods. Serotonin was determined from the supernatant extracted with ethanol by HPLC-MS/MS.

We used low IPTG concentration (0.25 mM), since the expression rate of target protein is much lower, and more chaperones can be available to assist recombinant protein folding and prevent p-bodies formation (Utekal et al. 2014). The lack of catalytic activity observed in these genes may be due to their non functional expression as previously described for several membrane-bound CYPs in *E. coli* (Park et al. 2011). For this reason, we constructed a series of

truncates at the N-terminal end for *ERG11* fused with GST as *ERG11* was the gene with the best results in the *in silico* search. Several authors have shown that the solubility and functionality of these membrane-bound protein types improve in these truncated versions (Park et al. 2011; Ichinose and Wariishi 2013; Zelasko et al. 2013; Hausjell et al. 2018). We also supplemented the medium with 0.5 mM of 5-aminolevulinic acid, which is a heme precursor that has sometimes been shown to be beneficial for increasing the expression of active P450s (Quehl et al. 2016; Hausjell et al. 2018). However, these strategies did not lead to tryptamine hydroxylase activity to be detected after any of the several modifications made (data not shown). Future works could consider further potential optimization to overcome the problems related with the expression of P450s in *E. coli*. For instance, the co-expression of auxiliary proteins or cultivation-parameter tuning has been reviewed (Hausjell et al. 2018). Moreover, the use of microsomal fractions for activity assays by combining microsomal preparations of the different CYPs from *S. cerevisiae,* together with the CPR, could be interesting approximations to bear in mind.

4.2.2.1.3. *O*-methylation step

The conversion of *N*-acetylserotonin into melatonin is the last step in the classic melatonin biosynthetic pathway. *ERG6* and *CRG1* were the candidate genes to carry out this activity in *S. cerevisiae* as possible orthologs to ASMT, which is the enzyme responsible for *N*-acetylserotonin *O*-methyltransferase activity in vertebrates.

We overexpressed *ERG6* and *CRG1* from *S. cerevisiae* and *ASMT* from *H. sapiens* in *E. coli,* and we measured the melatonin concentration after 50 h of 1 mM *N*-acetylserotonin supplementation in the medium. As shown in Figure 4.3, the overexpression of *ERG6* or *CRG1* did not produce melatonin detection in the medium. Only *ASMT* overexpression yielded a large amount of melatonin in the medium, which ranged from 200 to 2000 $ng \cdot mL^{-1}$.

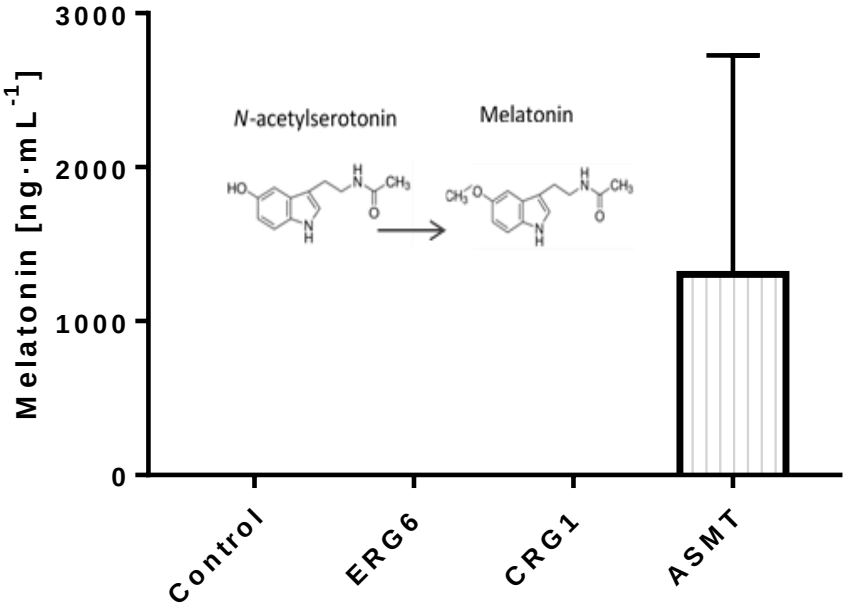

Figure 4.3 Effect of the overexpression of *ERG6*, *CRG1* and ASMT in N-acetylserotonin *O*-methylation in *E. coli*. Rosetta cells was transformed with empty vectors pGEX-5X-1 (control), pGEX-5X-1 ERG6 (ERG6), pGEX-5X-1 CRG1 (CRG11) and pGEX-5X-1 ASMT (ASMT). Melatonin was determined after culturing the different strains for 24 h in 2xTY with 1% glucose supplemented with 1 mM *N*-acetylserotonin. Cultivation conditions and expression induction are detailed in the Materials and Methods. Melatonin was determined from the supernatant extracted with ethanol by HPLC-MS/MS.

4.2.2.1.4. *N*-acetylation step

Two different *N*-acetylation reactions can occur from serotonin to melatonin: the conversion of serotonin into *N*-acetylserotonin or, alternatively, the acetylation of 5-methoxytryptamine into melatonin. Unlike the other genes in the melatonin biosynthetic pathway, gene *PAA1* from *S. cerevisiae* has been already described as SNAT/AANAT homolog by Ganguly et al. (2001), which was why we did not perform any search of orthologs for this activity and we simply validated the activity of the referenced gene *PAA1*.

As expected, *PAA1* showed SNAT activity (Figures 4.4 and 4.5). In fact *PAA1* overexpression in this system led to 966±260, 48±15 and 515±93 ng/mL of N-acetyltryptamine, *N*-acetylserotonin and melatonin after incubating in the

presence of 1 mM of tryptamine, serotonin and 5-methoxytryptamine, respectively. These results slightly differ from those previously obtained by Ganguly et al. (2001), in which the preferred substrate for *PAA1* was 5-methoxytryptamine, followed by tryptamine. Our results revealed a marked preference by tryptamine and its conversion into *N*-acetyltryptamine. It was noteworthy that the latter compound has not yet been related to melatonin synthesis. In any case, the titers of acetylated products by *PAA1* were much less abundant that the product obtained by the transformation with the bovine AANAT gene (positive control).

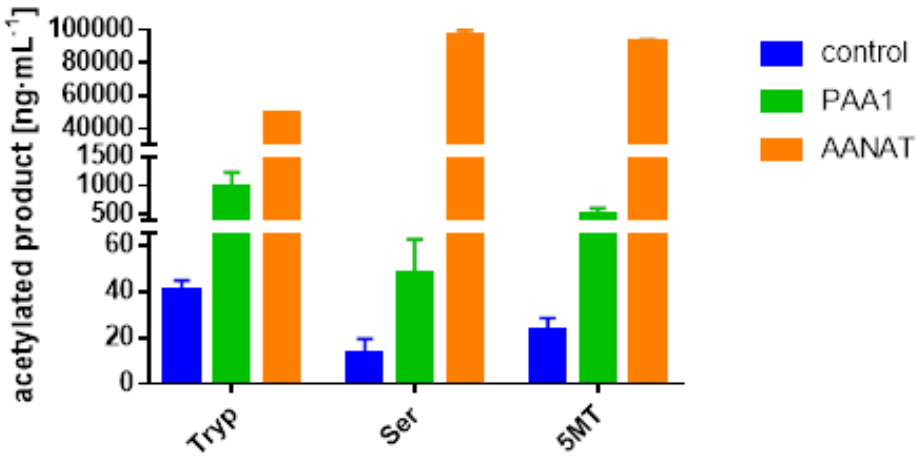

Figure 4.4. Effect of the overexpression of *PAA1* and *AANAT* in *E. coli* on the acetylation of different substrates. The extracellular medium fractions were obtained after culturing the different strains for 24 h in 2xTY with IPTG and 1 mM tryptamine, serotonin or 5-metoxitriptamine treatment and centrifuging the culture medium. The acetylated product levels were subjected to HPLC-MS/MS. Data are the means±standard deviations of triplicate experiments. For more details on the experiment (IPTG induction, growth, HPLC-MS/MS analysis, etc.), see the Material and Methods section.

Interestingly enough, when 1 mM of tryptamine was added to the *E. coli* harboring the empty pGEX-5X-1 vector (control strain), and the same plasmid containing *PAA1* or *AANAT* (PAA1 and AANAT strains, respectively), we observed serotonin production for both the control and PAA1 strains (Figure 4.5). For the AANAT overexpressing strain, we did not detect serotonin, but 989±43 ng/mL of *N*-acetylserotonin was observed. This indicates that *E. coli* was able to natively produce serotonin from tryptamine. With the AANAT strain, it would seem that

this strain was capable of converting practically all the tryptamine added in *N*-acetyltryptamine and the serotonin natively produced in *N*-acetylserotonin. Furthermore, 78±132 ng/mL of melatonin were produced by the AANAT strain with 230 ng/mL in one of the analyzed clones. More studies are needed to gain a more in-depth understanding of how this melatonin was produced in the AANAT strain, but one possibility is that 5-methoxytryptamine could be originated and AANAT would perform the acetylation needed to produce melatonin.

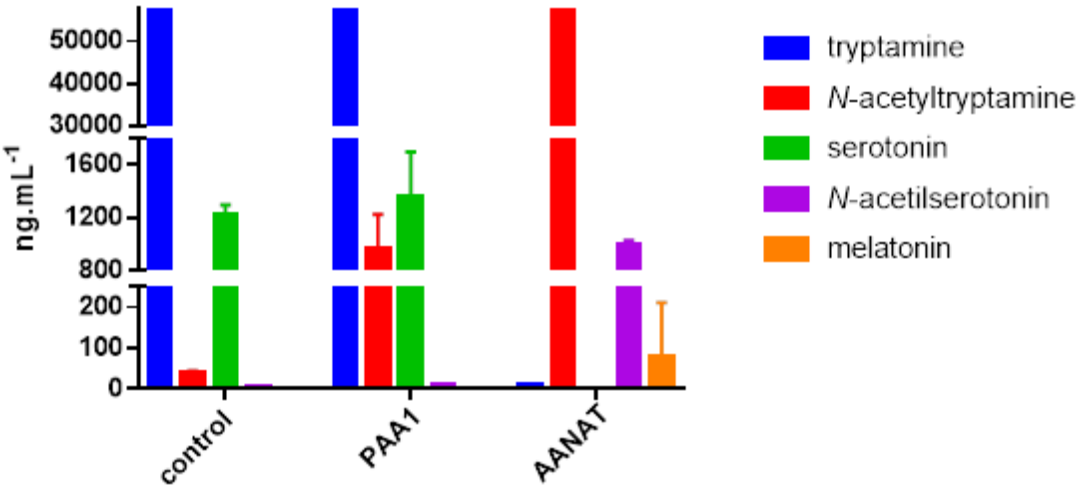

Figure 4.5 Heterologous expression of *PAA1* and *AANAT* in *E. coli* on the production of tryptamine derivatives. Production of tryptamine, *N*-acetyltryptamine, serotonin, *N*-acetylserotonin and melatonin in the control *E. coli* harboring an empty pGEX-5X-1 vector and the recombinant *E. coli* harboring pGEX-5x-1-PAA1 or pGEX-5x-1-AANAT. The extracellular medium fractions were obtained after culturing the different strains for 24 h in 2xTy with IPTG and 1 mM tryptamine and after centrifuging the culture medium. Tryptamine derivative levels were subjected to HPLC-MS/MS. Data are the means±standard deviations of triplicate experiments. For more details on the experiment (IPTG induction, growth, HPLC-MS/MS analysis, etc.), see the Material and Methods section.

4.2.2.2. Activity of the candidate genes in *S. cerevisiae*

As in *E. coli*, the same genes were overexpressed in the plasmid of strong expression p426GPD in *S. cerevisiae* strain BY4743 to assess their putative enzymatic activities related to the melatonin biosynthetic pathway. The same substrates were added to the culture medium, and the transformation activity of each overexpressed gene was assessed by analyzing the product yield in the supernatant.

4.2.2.2.1. Decarboxylation step

Similarly, to the *E. coli* system, *DPL1* and *GAD1* overexpression did not significantly increase tryptamine production compared to the control strain (BY4743 harboring the p426GPD empty vector) (Figure 4.6). Conversely, the overexpression of the positive control *H. sapiens DDC* yielded 37-fold more tryptamine than the other overexpressing strains (5-8 ng·mL^{-1} *vs*. 240 ng·mL^{-1}) (Figure 4.6).

Regarding serotonin production from 5-hydroxytryptophan, no serotonin was detected for any of the overexpressing strains, except for the positive control *DDC,* which produced about 600 ng·mL^{-1}. Interestingly, the preference of DDC for 5-hydroxytryptophan decarboxylation into serotonin, in comparison to tryptophan decarboxylation into tryptamine, was observed in the *S. cerevisiae* or *E. coli* overexpressing systems.

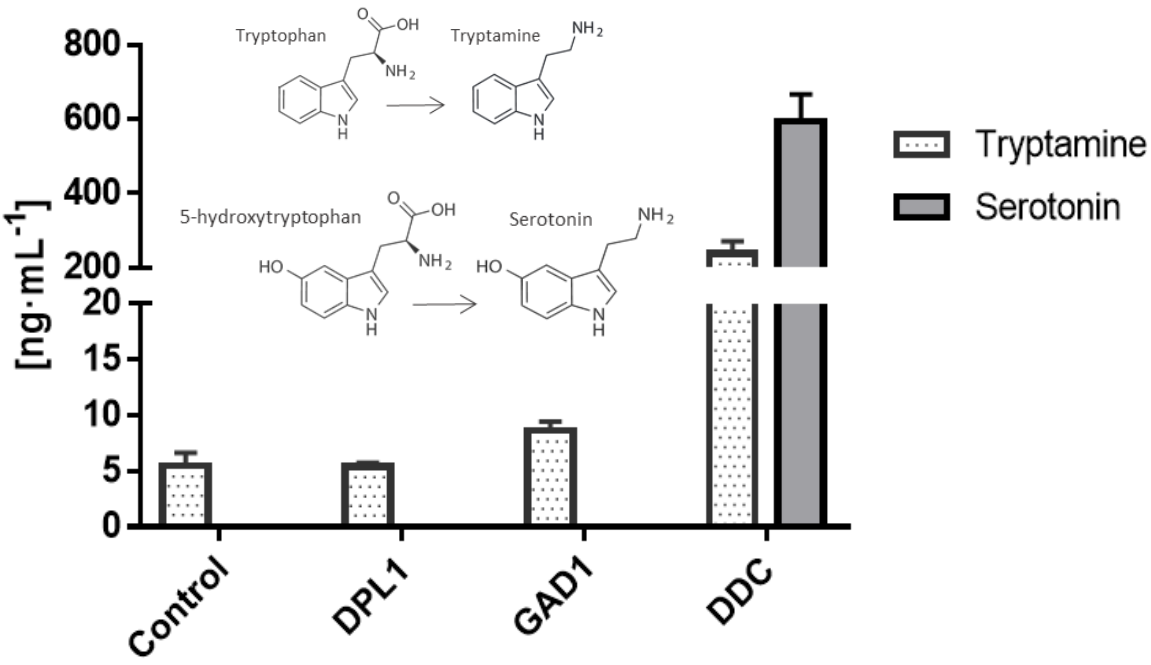

Figure 4.6 Effect of the overexpression of *DPL1*, *GAD1* and *DDC* in tryptamine and serotonin detection in *S. cerevisiae*. BY4743 was transformed with empty vectors p426GPD (control), p426GPD DPL1 (*DPL1*), p426GPD GAD1 (*GAD1*) and p426GPD DDC (DDC). Tryptamine and serotonin were determined after culturing the different strains for 50 h in SC-ura supplemented with 1 mM tryptophan or 5-hydroxytryptophan, respectively. Tryptamine and serotonin were determined from the supernatant extracted with ethanol by HPLC-MS/MS.

4.2.2.2.2. Hydroxylation step

Unfortunately, the *DIT2*, *ERG5* and *ERG11* overexpression in *S. cerevisiae* did not show any tryptamine hydroxylase activity. Surprisingly, neither did rice *T5H* overexpression indicate any transformation activity of tryptamine into serotonin (data not shown). This was surprising because we previously observed marked conversion into *E. coli* (Figure 4.2). Conversely, we observed the native conversion of tryptamine into serotonin by yeast in the bioconversion experiments of Chapter 3. These contradictory results with both experiments could be due to differences in the growth conditions and sampling times between them.

The lack of functionality observed in the control gene *T5H* could be due to the host system or the construction type used, which consists of fusing GST together with the deletion of 37 amino acids at the *N*-terminal end. This construction was the only one to display tryptamine hydroxylase activity in *E. coli* (Park et al. 2011), but this truncated version of the gene might not work in *S. cerevisiae*. In a previous study, it was concluded that the approach consisting in the use of fusion partners to generate chimeric constructs and directed mutagenesis is useful for bacterial hosts but not always effective for yeast (Öberg et al. 2011). Unfortunately, we were unable to amplify T5H without GST fusion. Thus in the near future, we will attempt to use a chemically synthesized gene and codon optimized for its overexpressing use in *S. cerevisiae*.

4.2.2.2.3. *O*-methylation step

Both *ERG6* and *CRG1* encode the methyltransferase enzymes involved in ergosterol synthesis and lipid homeostasis, respectively. To test whether any of them could transform *N*-acetylserotonin into melatonin, we overexpressed those genes and we measured melatonin levels after 50 h of growth in a medium enriched with 1 mM *N*-acetylserotonin. However, the strain overexpressing the control gene *ASMT* was the only one to show significantly more melatonin production than the control strain (Figure 4.7) (820 $ng \cdot mL^{-1}$). Therefore, we were unable to assign the capability to methylate *N*-acetylserotonin to enzymes *ERG6* and *CRG1*. A new search for alternative methyltransferases will be done to identify new candidates.

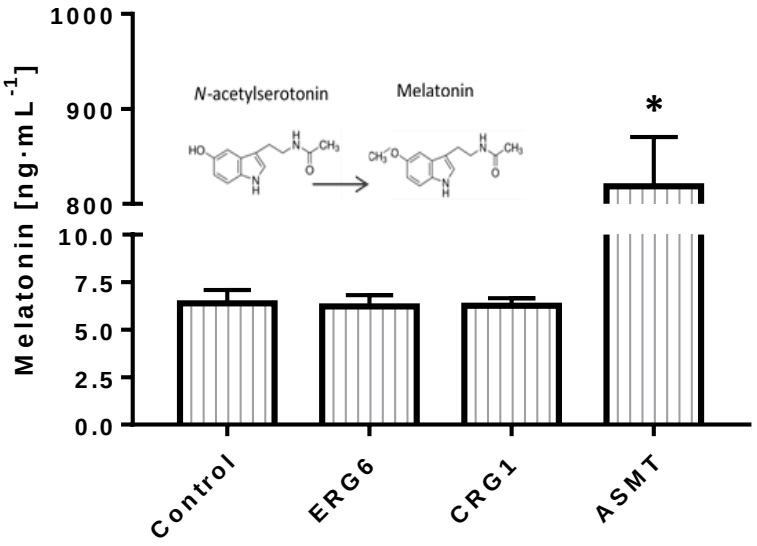

Figure 4.7 Effect of the overexpression of *ERG6*, *CRG1* and *ASMT* in melatonin detection in *S. cerevisiae*. BY4743 was transformed with empty vectors p426GPD (control), p426GPD ERG6 (ERG6), p426GPD CRG1 (CRG1) and p426GPD ASMT (AMST). Melatonin was determined after culturing the different strains for 50 h in SC-ura supplemented with 1 mM *N*-acetylserotonin. Melatonin was determined from the supernatant extracted with ethanol by HPLC-MS/MS.

4.2.2.2.4. *N*-acetylation step

When we overexpressed *PAA1* in yeast in a medium supplemented with 1 mM 5-methoxytryptamine, as this precursor has been described as the best amine substrate for *PAA1* (Ganguly et al. 2001), as shown in Figure 4.8, no differences in melatonin concentration were detected between the control strain (transformed with the empty vector) and the *PAA1* overexpressing strain. Conversely, vertebrate *AANAT* overexpression produced a 25-fold higher melatonin concentration than the control and PAA1 strains.

This was an unexpected result because the acetyltransferase activity of this enzyme toward this substrate has been previously characterized *in vitro* (Ganguly et al. 2001). Furthermore, the acetylation activity of *PAA1* toward tryptamine, serotonin and 5-methoxytryptamine was also observed in our single substrate conversion experiment in *E. coli* (Figures 4.4 and 4.5). Therefore, in order to clarify the role of *PAA1 in vivo*, we continued to examine in-depth the validation

of *PAA1* in *S. cerevisiae* with different experiments, as we indicate in the next section.

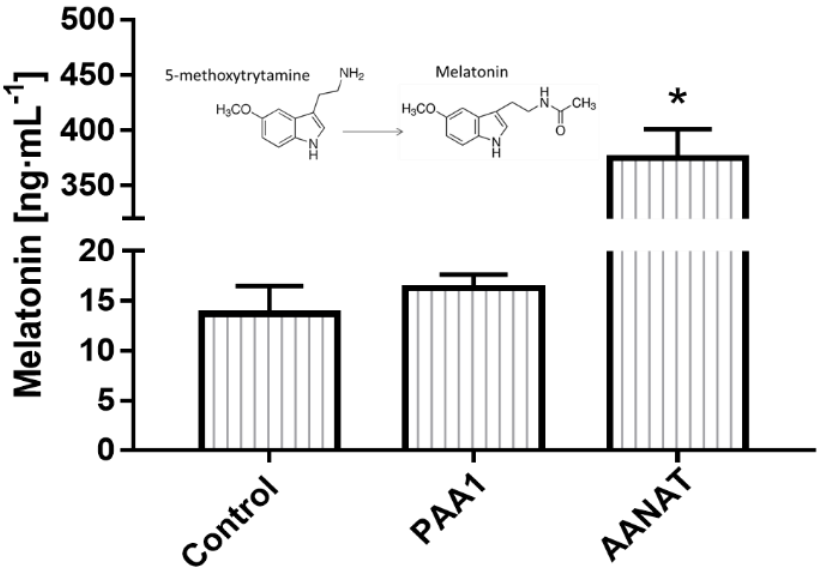

Figure 4.8 Effect of the overexpression of *PAA1* and *AANAT* in melatonin detection in *S. cerevisiae*. BY4743 was transformed with empty vectors p426GPD (control), p426GPD PAA1 (PAA1) and p426GPD AANAT (AANAT). Melatonin was determined after culturing the different strains for 50 h in SC-ura supplemented with 1 mM 5-methoxytryptamine. Melatonin was determined from the supernatant extracted with ethanol by HPLC-MS/MS.

4.2.3. Validation of PAA1 acetyltransferase activity on polyamines and arylalkylamines

The non acetyltransferase activity observed for *PAA1* after its overexpression in *S. cerevisiae* made us rethink its possible involvement in melatonin synthesis. Liu et al. (2005) suggested that polyamines are the true substrate for *PAA1 in vivo*. These authors explained the *in vitro* activity observed by Ganguly et al. (2001) given the similarity of the ethyl amine group in the arylalkylamines with the propyl and butylamines present in polyamines. Non canonical metabolic activity in *S. cerevisiae* in the formation of metabolic side products under unbalanced physiological conditions has also been described, such as cellular stress situations (Piedrafita et al., 2015), which may explain this *PAA1* activity in arylalkylamines with an *in vitro* system.

Therefore, we decided to complement the experiment by including two other amines, tryptamine and serotonin. Thus, we determined if there were any differences in the acetylation of tryptamine, serotonin or 5-methoxytryptamine between the knockout of *PAA1* (ΔΔpaa1) belonging to the EUROSCARF collection of the wild-type BY4743 (control strain) and the over-expressing strain constructed by us in the same genetic background (Figure 4.9).

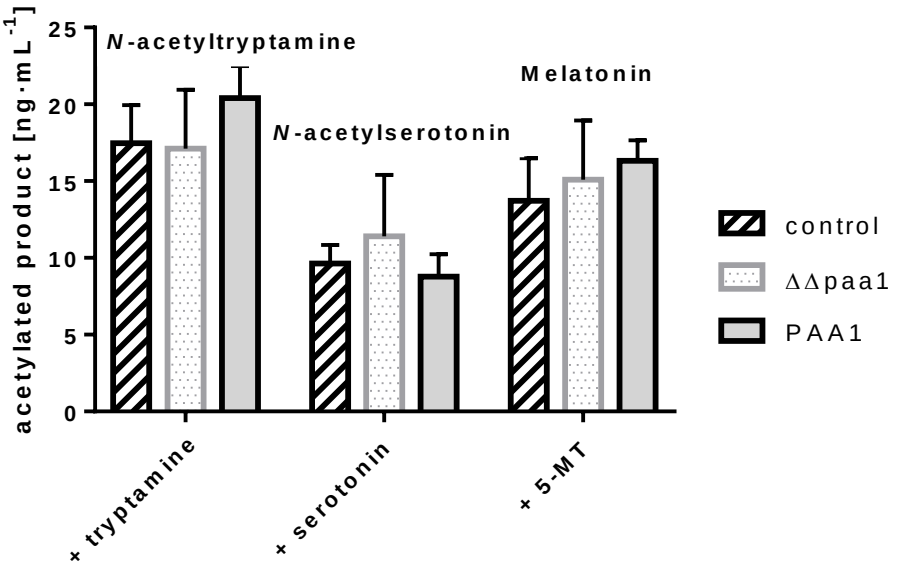

Figure 4.9 Effect of *PAA1* on acetylation of different arylalkylamines in *S. cerevisiae*. BY4743 harboring p426GPD (control), BY4743 strain knockout for PAA1 transformed with p426GPG (ΔΔpaa1) and BY4743 overexpressing PAA1 with p426GPD (PAA1). Strains were grown in SC-ura, as described in material and methods. The media was supplemented with 1 mM tryptamine, serotonin or 5-methoxytryptamine (5-MT) for the detection of the acetylated products *N*-acetyltryptamine, N-acetylserotonin and melatonin respectively. After 50 h of the supplementation, the extracellular fraction was obtained by centrifugation of the culture medium and it was subjected to HPLC-MS/MS. Data are means ± standard deviations of triplicate experiments.

Although we detected the acetylated products for all the used substrates, no significant differences were found between strains. It should be noted that melatonin was still produced by *PAA1*-deficient strains when 1 mM of 5-methoxytryptamine was added exogenously to the medium, which indicates that *PAA1* does not seem involved, or is not at least an exclusive enzyme, in melatonin synthesis. Furthermore, our results showed that tryptamine seemed to be the favorite substrate for *PAA1*, which is contradictory to the results of Ganguly

et al. (2001), who reported preference acetylation activity with 5-methoxytryptamine.

In order to assure if the induction of *PAA1* in the over-expressing strain had been performed, we determined the gene transcriptional activity of both this strain and the wild type by qPCR (Figure 4.10). *PAA1* was found to be expressed over 270 time-fold compared to the control strain, which indicates that *PAA1* overexpression had been correctly achieved.

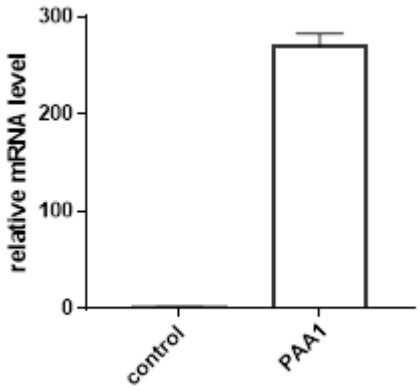

Figure 4.10. *PAA1* was effectively overexpressed in BY4743 p426GPD PAA1 starin. *PAA1* gene expression in BY4743 control strain (p426GPD empty vector) and BY4743 with p426GPD PAA1. Changes in *PAA1* gene expression are shown in relation to the control wild type strain during exponential growth in SC-ura medium (set as value 1). The relative amount of *PAA1* mRNA was determined by qPCR, normalizing against *ACT1* levels.

As we evidenced that *PAA1* overexpression was not connected with the increase in acetylation activity toward the different aralkylamines, we wondered if the problem could lie in the induction of this gene not being translated into functional proteins. Liu et al. (2005) previously reported that *PAA1* overexpression caused partial growth inhibition in a medium without pantothenate, but not in a rich medium. To this end, we performed a drop test in a SC-ura medium with and without pantothenate to indirectly verify if we had functionally overexpressed *PAA1*. Besides overexpressing the *PAA1* strain, we also used the wild-type strain BY4343 transformed with the empty vector p426GPD and the mutant strain for *FMS1* (ΔΔ*fms1*-BY4743), a strain that cannot grow in medium without pantothenate because it is necessary for β-alanine and pantothenic acid biosynthesis (White et al. 2001). The growth defect observed by *PAA1*

overexpression is caused by a drop in the availability of coenzyme A (CoA), which is consumed as a consequence of the marked acetylation activity of polyamines, mainly spermine, by *PAA1* (White et al. 2001; Liu et al. 2005) (Figure 4.11).

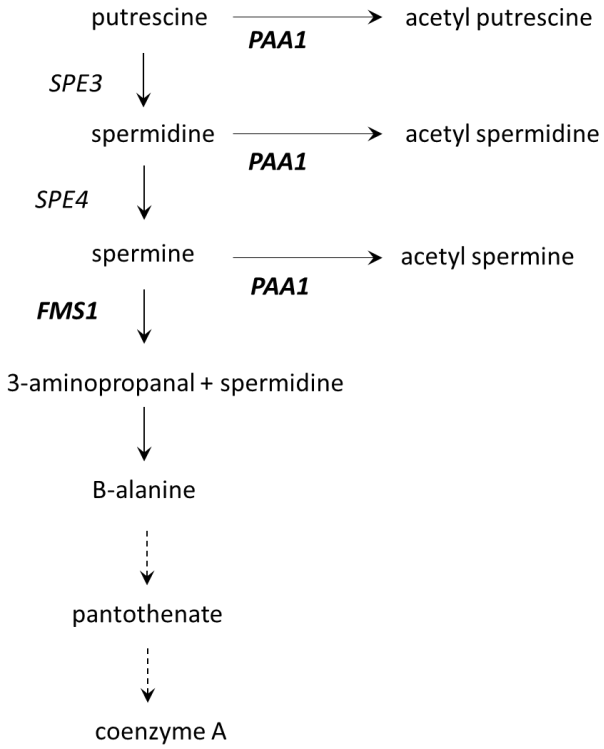

Figure 4.11 Schematic representation of the biosynthetic pathway for the synthesis of coenzyme A in yeast.

The drop tests showed a growth defect when overexpressing *PAA1* and a total inhibition in the ΔΔ*fms1* strain, as previously reported (Liu et al. 2005), which implies that *PAA1* was not only overexpressed, but also translated, into functional proteins (Figure 4.12).

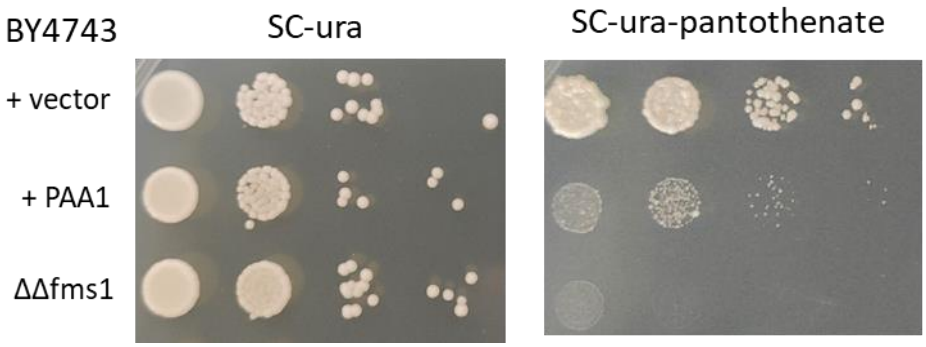

Figure 4.12 *PAA1* overexpression causes growth inhibition in a pantothenate-free medium. The BY4743 cells transformed with plasmids p426GPD-PAA1 (PAA1), p426GPD (empty vector), and the BY4743 ΔΔfms1 cells transformed with p426GPD (ΔΔfms1), were grown on SC-ura overnight. Growth tests were carried out in solid medium, as described in the Material and Methods, and in the SC-ura media with and without pantothenate. They were allowed to grow at 30ºC for at least 3 days.

CoA is the common acyl carrier required for the conversion of pyruvate into Acetyl-CoA, which is an essential cofactor for the acetylation of various molecules like histones, proteins and small molecules. *PAA1* overexpression is assumed to consume more Acetyl-CoA during the acetylation of its substrates and, in parallel, lead to low CoA levels.

This result prompted us to deduce that the Acetyl-CoA that dropped in the *PAA1* overexpressing strain could possibly explain the lack of acetylation activity with the arylalkylamines as substrates. Therefore, we decided to determine if the external addition of Acetyl-CoA would have any effect on the transformation of 5-methoxytryptamine into melatonin by *PAA1*. We supplemented the medium with 0.3 mM of Acetyl-CoA and measured the 5-methoxytryptamine conversion into melatonin in the BY4743 wild type, *PAA1* overexpressing, *B. taurus AANAT* overexpressing and ΔΔ*paa1* strains. However, the addition of acetyl-CoA did not increase the acetylation of 5-methoxytryptamine in any case, including bovine *AANAT* (Figure 4.13). This finding indicates that lack of a cofactor is not limiting for the melatonin production by these enzymes.

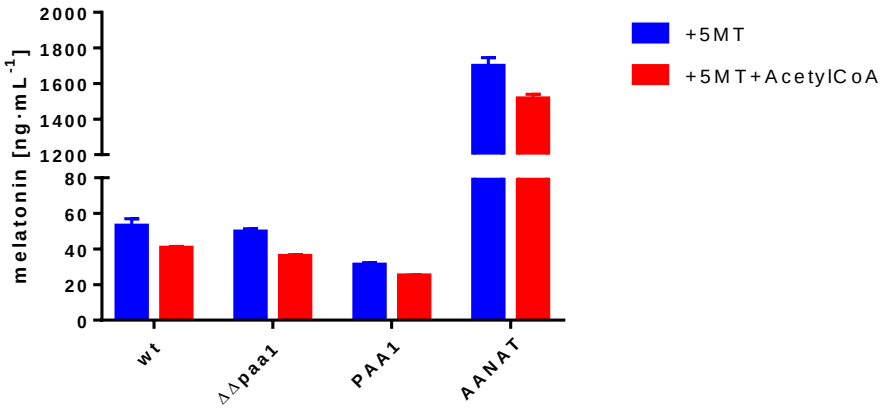

Figure 4.13 Effect of Acetyl-CoA on 5-methoxytryptamine acetylation in different yeast strains. Cells from the wild-type (wt), PAA1-deficient (ΔΔpaa1), PAA1 overexpressing (PAA1) and Bos taurus AANAT overexpressing (AANAT) strains were cultured 72 h at 30°C in SC-ura with 1 mM of 5-methoxytryptamine (blue) and the same medium supplemented with 0.3 mM of Acetyl-CoA (red). The melatonin concentration was determined from the supernatant diluted 50% with ethanol and analyzed by UHPLC-MS/MS.

Thus, in view of these results, we suggest that there may be other *N*-acetyltransferases in *S. cerevisiae* capable of acetylating serotonin and 5-methoxytryptamine, which would be responsible for the acetylation of these substrates in a strain defective for *PAA1.*

In alphaproteobacteria, *N*-acetylating enzymes with some homology to AANAT may be involved in the *N*-acetylation of 5-methoxytryptamine, whereas in both plants and cyanobacteria, SNAT lacks substantial homology to AANAT and, thus, must classified as a paralog (Hardeland 2015). Some SNAT homologs can respond to photoperiodic changes, while others may be up-regulated by oxidative stress. In addition, some homologs of SNAT have very little to do with melatonin production, but can be related to the detoxification of toxic amines (Falcon et al. 2014). Enzyme *PAA1* showed fair similarity to vertebrate AANAT, but lacks the regulatory N- and C-terminal flanking regions conserved in all vertebrate AANATs (Ganguly et al. 2001). We constructed a phylogenetic tree to see the relation of *S. cerevisiae* SNAT, *PAA1,* with the SNATs in other species. The results revealed that evolutionarily *PAA1* is more closely related to the SNATs in vertebrates rather than to the lineage of plants (Figure 4.14).

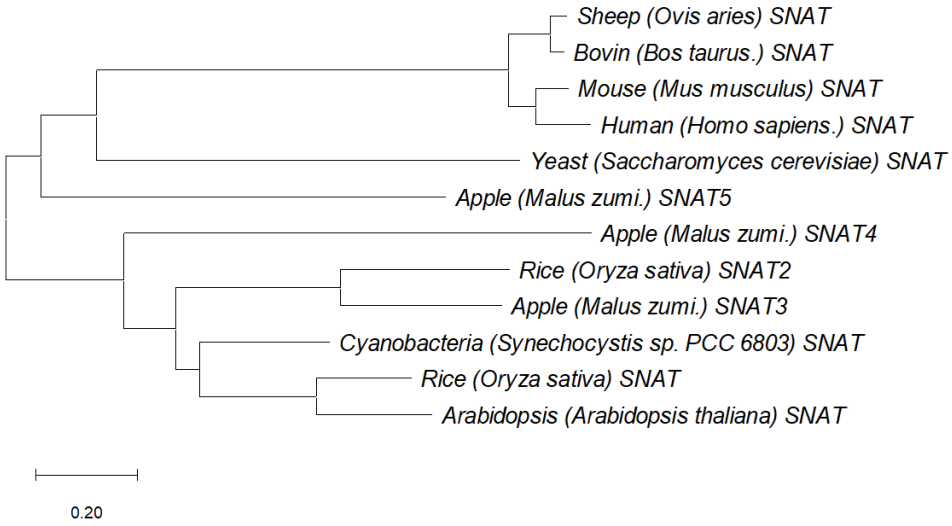

Figure 4.14 Phylogenetic tree of *S. cerevisiae* SNAT (*PAA1*). The phylogenetic tree was constructed by the neighbor-joining method and a bootstrap test with 1000 iterations with the MEGA5.2 software. The GenBank accession numbers are NP_442603 (cyanobacteria, Synechocystis sp. PCC 6803, SNAT), AK059369 (rice, *O. sativa*, SNAT1), AK068156 (rice, *O. sativa*, SNAT2) ABD19662 (Arabidopsis, *A. thaliana*, SNAT), NP_001009461 (sheep, *O. aries*, SNAT), NM_009591 (mouse, *M. musculus*, SNAT), KJ156532 (apple, *M. zumi*, SNAT3), KJ156533 (apple, *M. zumi*, SNAT4), KJ156534 (apple, *M. zumi*, SNAT5).

Thus, in summary, *PAA1* has been previously considered to be a SNAT/AANAT homolog. However, our results show that *PAA1* is, at least, not the exclusive enzyme for the acetylation of serotonin, tryptamine and 5-methoxytryptamine in *S. cerevisiae*. When we performed the BLAST searches for the AANAT candidates, we obtained other hits by employing different SNAT sequences as queries. For example, *GNA1*, which encodes an evolutionarily conserved glucosamine-6-phosphate acetyltransferase, was obtained with an E-value of 9×10^{-11}, 73% of query cover, 16% identity and 36% similarity when *B. taurus AANAT* was used as a query. We also included an SNAT5 isoform from *M. zumi* as a BLAST search query because it this isoform has been reported to be closer to animal SNATs than other SNAT isoforms from this species (Wang et al., 2017) (Figure 4.14). When a BLAST search was done with amino acid sequence SNAT5 from *M. zumi*, *MAK3* from *S. cerevisiae* was obtained with as the best hit with an E- value of 3×10^{-18}, query cover was 85%, and with almost 20% identity and 35% similarity. The *MAK3* product comprises a catalytic subunit of the NatC type *N*-

terminal acetyltransferase. These new searches with the combination of a more in-depth analysis of the protein structure are promising candidates to find out alternative AANAT activity in *S. cerevisiae*.

4.3. Conclusions

In this chapter, we aimed to identify the possible melatonin biosynthetic genes in *S. cerevisiae*. No candidate genes were found for TPH activity in yeast. However, for TDC, two genes were selected (*DPL1* and *GAD1*), three for T5H (*DIT2*, *ERG5* and *ERG11*), one for SNAT (*PAA1*) and two for ASMT (*ERG6* and *CRG1*) by an *in silico* analysis using BLASp. Unfortunately, all the candidate genes as potential orthologs involved in the synthesis of overexpressed melatonin did not display greater activity than the wild type on the different substrates in *S. cerevisiae,* but much less transformation capacity than the strains harboring the positive control genes of other organisms.

Only *PAA1* displayed activity toward serotonin, tryptamine, and 5-methoxytryptamine, but only if it was overexpressed in *E. coli*. Lack of *in vivo* acetylation activity for *PAA1* in yeast was due to neither a limitation of cofactor Acetyl-CoA nor a non functional translated protein. In fact, the melatonin production from 5-methoxytryptamine in the *PAA1* mutant, the wild type or the *PAA1* overexpressed strains did not significantly differ in the concentration of the acetylated products. According to our results, *PAA1* may not be the exclusive enzyme in the acetylation of arylalkylamines, such as serotonin, tryptamine and 5-methoxytryptamine, in *S. cerevisiae*. The presence of other *N*-acetyltransferases in yeast capable of acetylating serotonin and 5-methoxytryptamine may be responsible for the acetylation of these substrates in a mutant strain for *PAA1*. Nevertheless, more insight into the protein structure homology could help us to search and detect more plausible genes involved in melatonin production in *S. cerevisiae*.

CHAPTER 5

Metabolic engineering of *Saccharomyces cerevisiae* to enhance hydroxytyrosol production and other metabolites related to shikimate metabolism

The information of this chapter has been partially published in:

Food Chemistry 308 (2020)

https://doi.org/10.1016/j.foodchem.2019.125646

In addition, as a result of this work, we have submitted this overproducing strain to the Spanish Patent and Trademark Office with application number 202031186 and it can be found in Annex I of this thesis.

5.1. Introduction

Hydroxytyrosol is a polyphenol considered as a potent natural antioxidant present in leaves and fruits of olive, extra-virgin olive oil and in olive oil industrial by-products and wastewaters (Angerosa et al. 1995; Lesage-Meessen et al. 2001). Furthermore, in both white and red wines, hydroxytyrosol has also been detected in concentrations ranging from 0.28 to 9.6 mg/L (Di Tommaso et al. 1998; Minussi et al. 2003; Minuti et al. 2006; Piñeiro et al. 2011; Bordiga et al. 2016). Moreover, hydroxytyrosol is a molecule with great interest due to its multiple bioactivities and human health benefits (Fernández-Mar et al. 2012; Hu et al. 2014; Yüksel Aydar et al. 2017). The presence of hydroxytyrosol in wine has been related to different sources including the hydroxylation of tyrosol by the endogenous polyphenol oxidase from grapes (García-García et al. 2013). However, some studies directly relate hydroxytyrosol to the metabolism of yeasts during alcoholic fermentation (Romboli et al. 2015; Bordiga et al. 2016; Álvarez-Fernández et al. 2018; González et al. 2018b; Guerrini et al. 2018; Rebollo-Romero et al. 2020).

In yeast, the higher aromatic alcohol tyrosol is synthesized from tyrosine through the well-established Ehrlich pathway. Briefly, amino acids are first transaminated into α-keto acids followed by decarboxylation in aldehydes, and finally reduced to the fusel alcohol (Dickinson et al. 2003; Hazelwood et al. 2008; Ooi et al. 2008).

Various strategies have used yeast genes to overproduce tyrosol and hydroxytyrosol in *E. coli*. For instance, the heterologous co-expression of yeast *ARO8* and *ARO10* genes led to an important accumulation of tyrosol when tyrosine was added to the medium (Xue et al. 2017a; Xu et al. 2020). Furthermore, the co-expression of yeast *ARO10* and *ADH6*, and the overexpression of the native aromatic hydroxylase complex HpaBC produced important amounts of hydroxytyrosol in *E. coli* (Chung et al. 2017; Li et al. 2018). HpaBC encodes the 4-hydroxyphenylacetate 3-hydroxylase (HPAH), a member of the two-component flavin-dependent monooxygenase family. The large subunit flavin-dependent monooxygenase is encoded by *hpaB*, whereas *hpaC* encodes for the NAD(P)H:flavin oxidoreductase subunit, which supplies $FADH_2$ to HpaB (Ballou et al. 2005; Ellis 2010). The broad substrate specificity of HpaBC from different bacterial species has enabled its utilization for the hydroxylation of

multiple substrates including tyrosol, naringenin, *p*-coumaric acid, afzelechin, caffeic acid, 3-(4-hydroxyphenyl) propanoic acid, ferulic acid and coniferaldehyde (Liebgott et al. 2009; Furuya and Kino 2014; Lin and Yan 2014; Jones et al. 2016; Chen et al. 2019).

In this study, we have firstly cloned the *E. coli* hydroxylase HpaBC complex components (*hpaB* and *hpaC* genes) to overproduce hydroxytyrosol in *S. cerevisiae* cultivated in different media. After determining the effectiveness of HpaBC in converting tyrosol to hydroxytyrosol, we integrated HpaBC in multiple copies in the genome of *S. cerevisiae*. Afterwards, we increased productivity of hydroxytyrosol by redirecting the metabolic flux towards the synthesis of tyrosol in order to avoid the exogenous tyrosol or tyrosine supplementation. The combination of the selected modifications in our engineered strain combined with optimization of the culture conditions, resulted in elevated levels of hydroxytyrosol from glucose. So far there are only a few reports of biosynthesizing tyrosol in *S. cerevisiae* (Jiang et al. 2018; Torrens-Spence et al. 2018; Guo et al. 2019). However, to the best of our knowledge, this is the first report on heterologous production of hydroxytyrosol from glucose in yeast.

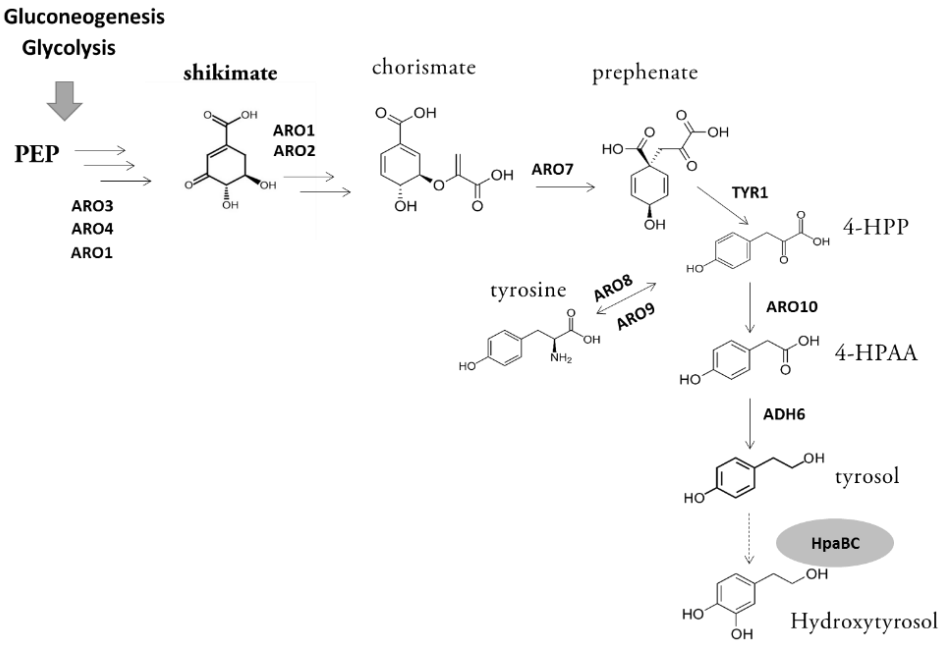

Figure 5.1 Formation of hydroxytyrosol in yeast from its precursor tyrosol in the presence of heterologous HpaBC from *E. coli*. The aromatic amino acid tyrosine is formed from the central carbon metabolism through the seven-step metabolic pathway known as "shikimate pathway". Briefly, prephenate is converted by the yeast prephenate dehydratase (*TYR1*) into 4-hydroxyphenylpyruvate (4-HPP). The aromatic aminotransferases *ARO8* and *ARO9* convert 4-HPP into tyrosine, but these enzymes can also convert tyrosine into 4-HPP through the first transamination-step of the catabolic degradation route known as the Ehrlich pathway. After the decarboxylation of 4-HPP in 4-hydroxyphenylacetaldehyde (4-HPAA), the reduction of 4-HPAA results in the higher alcohol tyrosol through the last step of the Ehrlich pathway. Finally, tyrosol is converted into hydroxytyrosol by the 4-hydroxyphenylacetate-3-hydroxylase (HpaBC) from *E. coli*. It is worth mentioning that yeast is able to endogenously synthesize hydroxytyrosol and, therefore, there are one or several unknown enzymes that can lead to hydroxytyrosol.

5.2. Results and discussion

5.2.1. Effect of *hpaB* and *hpaC* overexpression on yeast growth

The growth of the strains overexpressing *hpaB*, *hpaC* or both *hpaB* and *hpaC* was significantly lower than the control strain in a SC medium (Figure 5.2). On the other hand, since we fed our media with tyrosol, we evaluated the growth kinetics of the control and overexpressing strains in a SC medium supplemented with different tyrosol concentrations ranging from 0 to 5 mM (Figure 5.3). This was due to phenolic compounds such as tyrosol and hydroxytyrosol previously exhibited antimicrobial and antifungal activity (Ghalandari et al. 2018; Canal et al.

2019). The maximum growth rate (μ_{max}) and the area under the growth curve (AUC) were determined for all the different tyrosol concentrations. Tyrosol addition caused no differences in growth parameters across all the concentrations assayed for each strain. According to these results a concentration of 1mM of tyrosol was established as a supplementation condition (Figure 5.3).

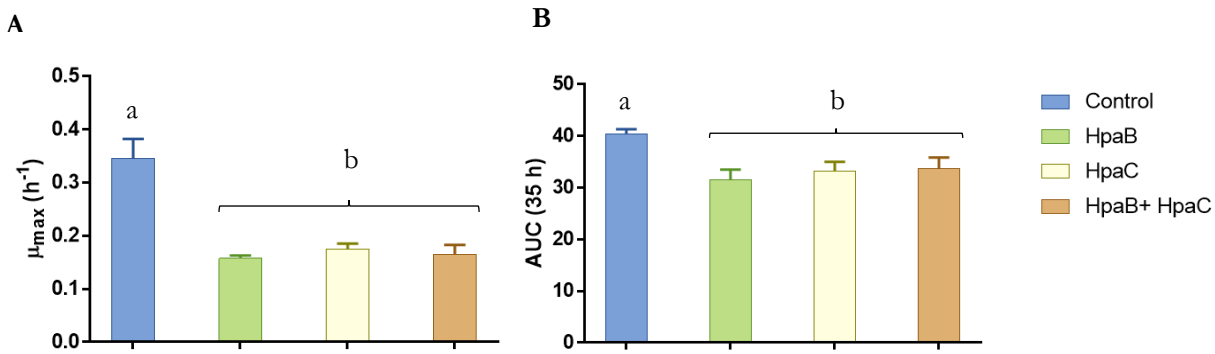

Figure 5.2 Effect of overexpression of *hpaB* and *hpaC* in yeast growth. The maximun growth rate (μ_{max}) (**A**) and the area under the curve (AUC) at 35 h (**B**) were determined for BY4743 overexpressing *hpaB* (green), *hpaC* (yellow) or both *hpaB* and *hpaC* (orange) from *E. coli* and the control (transformed with empty vectors) (blue) in SC. Error bars represent standard deviations calculated from biological triplicates. The values under the same letter are not significantly different according to the Tukey HSD test (α = 0.01).

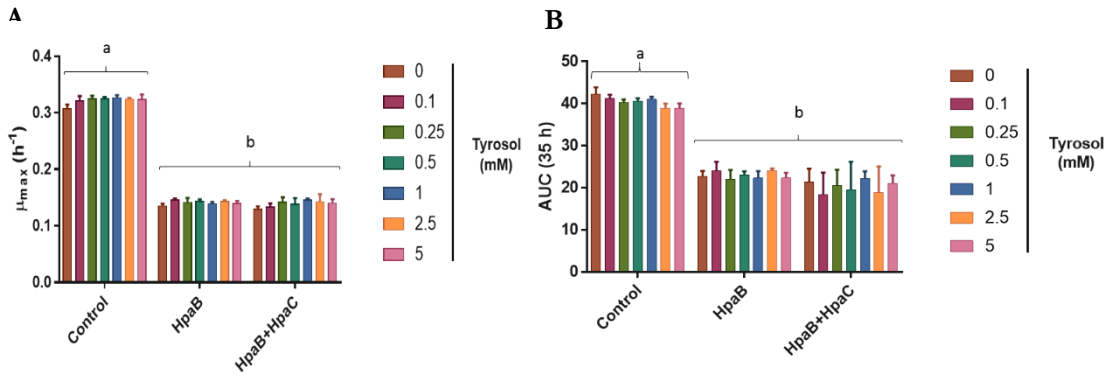

Figure 5.3 Effect of tyrosol concentration and overexpression of hpaB and hpaC on yeast growth. The maximun growth rate (μ_{max}) (**A**) and the area under the curve (AUC) at 35 h (**B**) were determined in SC supplemented with tyrosol ranging from 0 to 5mM for BY4743 overexpressing E. coli hpaB or both hpaB and hpaC, and the control (transformed with empty vectors). Error bars represent standard deviations calculated from biological triplicates.

We hypothesized that the growth defect must be due to a redox imbalance in these strains that overexpressing could cause this growth reduction because both enzymes, HpaB and HpaC, consume the reduced cofactors FADH2 and NADH, respectively. A decrease in biomass production caused by a decreased on NADH or NADPH content has previously been reported in yeast (Heux et al. 2006; Vemuri et al. 2007; Suástegui et al. 2017). To test this hypothesis, we ventured to increase the cytosolic NADH by using knockout mutant strains for *ADH3*, *NDE1* and *NDE2,* which are NADH-oxidating enzymes. *ADH3* encodes for mitochondrial alcohol dehydrogenase isozyme III which is involved in the reoxidation of mitochondrial NADH under anaerobic conditions and ethanol production (Bakker et al. 2000, 2001). In a ΔΔadh3 strain, the excess of intramitochondrial NADH is transferred to the cytosol through an ethanol-acetaldehyde shuttle in order to restore the redox balance (van Dijken and Scheffers 1986). *NDE1* and *NDE2* encode for mitochondrial external NADH dehydrogenases which provide cytosolic NADH to the mitochondrial respiratory chain (Luttik et al. 1998; Bakker et al. 2000). We evaluated the growth kinetics of these mutant strains in comparison with the wild type, all of them overexpressing *hpaB* + *hpaC*.

As it is depicted in Figure 5.4, the strains ΔΔadh3, ΔΔnde1 and ΔΔnde2, which overexpressed *hpaB* and *hpaC*, showed a better growth than the BY4743 HpaB +HpaC strain. Our hypothesis about the redox imbalance as consequence of the overexpression of the genes of the complex HpaBC was right. We will take into consideration this result to improve the yield of hydroxytyrosol.

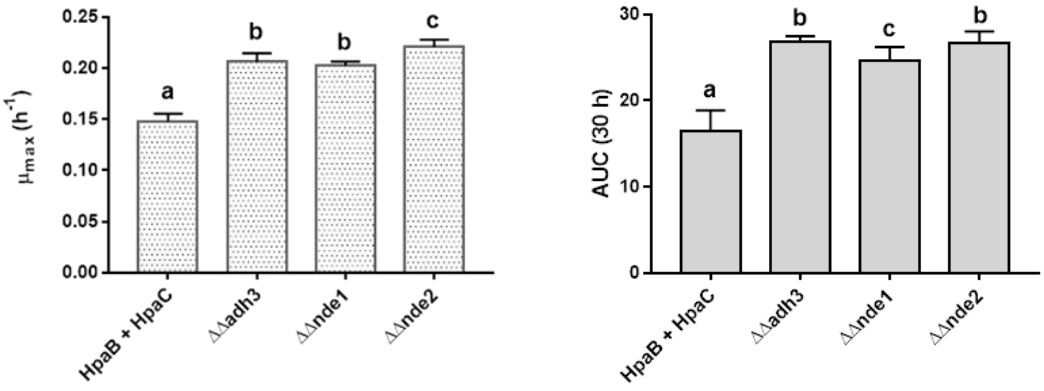

Figure 5.4 Yeast deletion mutants for *ADH3*, *NDE1* or *NDE2* showed better growth, when overexpressing hpaB and hpaC, than the BY4743 HpaB +HpaC strain. The maximum growth rate (μ_{max}) and the area under the curve (AUC) at 30 h were determined for BY4743 (control) and BY4743 knock-out mutants for *ADH3* (ΔΔadh3), *NDE1*(ΔΔnde1) and *NDE2* (ΔΔnde2). All these strains were transformed with p426GPD-hpaB and p425GPD-hpaC. Growth was conducted in SC-ura-leu at 28 ºC as detailed in materials and methods. Error bars represent standard deviations calculated from biological triplicates. The values under the same letter are not significantly different according to two-tailed student's t-test. (α = 0.05).

5.2.2. Tyrosol and hydroxytyrosol production in strains overexpressing *hpaB* and *hpaC*

We determined the tyrosol and hydroxytyrosol production of the *hpaB and hpaC*-overexpressing and control strains after growth for 72 h in SC or MM medium, with or without spiking of 1mM tyrosine or 1mM tyrosol (Figures 5.5 and 5.6; Table 5.1).

Curiously, the titers of both products were higher in MM medium, in spite of a higher OD_{600} was reached in SC than in MM medium (Figure 5.5B). The combination of nitrogen limitation (MM) together with the addition of tyrosine resulted in the best condition for tyrosol production. It has been widely reported that nitrogen limitation strongly promotes the production of aromatic alcohols in different yeast species (Beltran et al. 2005; González et al. 2018b). Furthermore, the increase in precursors, in the form of aromatic amino acids, also resulted in a general increase of the synthesis of these compounds via the Ehrlich pathway (Ghosh et al. 2008; Gori et al. 2011). The strains expressing *hpaB* or both *hpaB* and *hpaC* displayed lower levels of tyrosol than the control strain in both media, which is consistent with tyrosol being a substrate for HpaBC in yeast (Figure 5.5).

However, no difference in tyrosol concentration was observed between both overexpressing cells.

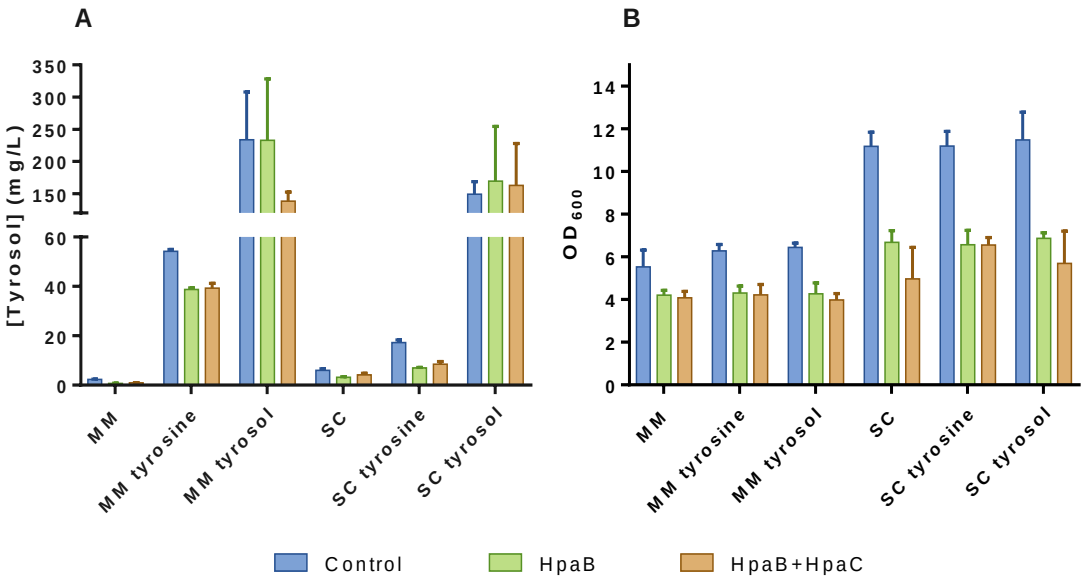

Figure 5.5 Tyrosol production (A) and growth (B) of *hpaB* and *hpaC* expressing cells in MM and SC. The yeast strains used were BY4743 overexpressing *hpaB* (green) or both *hpaB* and *hpaC* (orange) and the control strain BY4743 transformed with the empty vectors (blue). Cells were cultured 72 h at 30 °C in MM or SC and the same media supplemented with tyrosine or tyrosol 1mM. Error bars represent standard deviations calculated from biological triplicates. Tyrosol concentrations were determined from supernatant extracted with methanol and subjected to UHPLC-MS/MS.

Table 5.1 Tyrosol and hydroxytyrosol (HT) production by control (BY4743 + p425GPD +p426GPD), *hpaB* (BY4743 + p425GPD +p426GPDhpaB) and *hpaB hpaC* (BY4743 + p425GPDhpaC +p426GPDhpaB) strains after 72 h growing in MM and SC media with 1 mM tyrosine or tyrosol supplementation. Values for detected tyrosol are represented in mg/L ± SD, while concentration for HT is represented in µg/L ± SD.

	control		hpaB		hpaB hpaC	
	tyrosol (mg/L)	HT (µg/L)	tyrosol (mg/L)	HT (µg/L)	tyrosol (mg/L)	HT (µg/L)
MM	2.31 ± 0.11	5.43 ± 2.21	0.7 ± 0.07	2.85 ± 1.06	0.96 ± 0.03	33.39 ± 6.82
MM + tyrosine	54.25 ± 0.76	19.75 ± 0.77	38.72 ± 0.65	59.37 ± 6.5	39.25 ± 2.03	1150.95 ± 53.97
MM + tyrosol	-	38.09 ± 3.23	-	147.98 ± 24.82	-	4576.59 ± 852.77
SC	5.95 ± 0.73	2.28 ± 0.47	3.23 ± 0.17	18.91 ± 1.26	4.17 ± 0.59	103.55 ± 17.7
SC + tyrosine	17.2 ± 1.08	3.33 ± 1.81	6.97 ± 0.19	28.99 ± 4.04	8.53 ± 1.04	127.54 ± 11.01
SC + tyrosol	-	12.32 ± 2.8	-	262.06 ± 7.56	-	3433.97 ± 466.63

The hydroxytyrosol titers of the control strain ranged within the µg/L scale whereas tyrosol production reached mg/L. However, the overexpression of the genes of the flavin-dependent monooxygenase family significantly improved the production of hydroxytyrosol. Namely, the control strain was able to produce from 5 to 40 µg/L hydroxytyrosol in both media, with a higher production in the presence of tyrosine and, mainly, tyrosol (Figure 5.6A). Remarkably, the overexpression of *hpaB* increased between 20 and 22-fold the concentration of hydroxytyrosol in the growth medium, whereas the overexpression of both genes of the complex increased hydroxytyrosol levels between 240 and 316-fold (Figure 5.6). Despite no differences between both overexpressing strains were observed for tyrosol production (Figure 5.5), the cells co-expressing both *hpaB* and *hpaC* genes exhibited a higher production of hydroxytyrosol (Figure 5.6). These results suggest that, in the absence of *hpaC*, tyrosol can be converted into a product different to hydroxytyrosol, decreasing the final hydroxytyrosol yield. This is consistent with previous studies, which reported that the complex HpaBC exhibits a broad substrate spectrum, including chloro- and methyl-aromatic compounds (Prieto et al. 1993; Lin and Yan 2014).

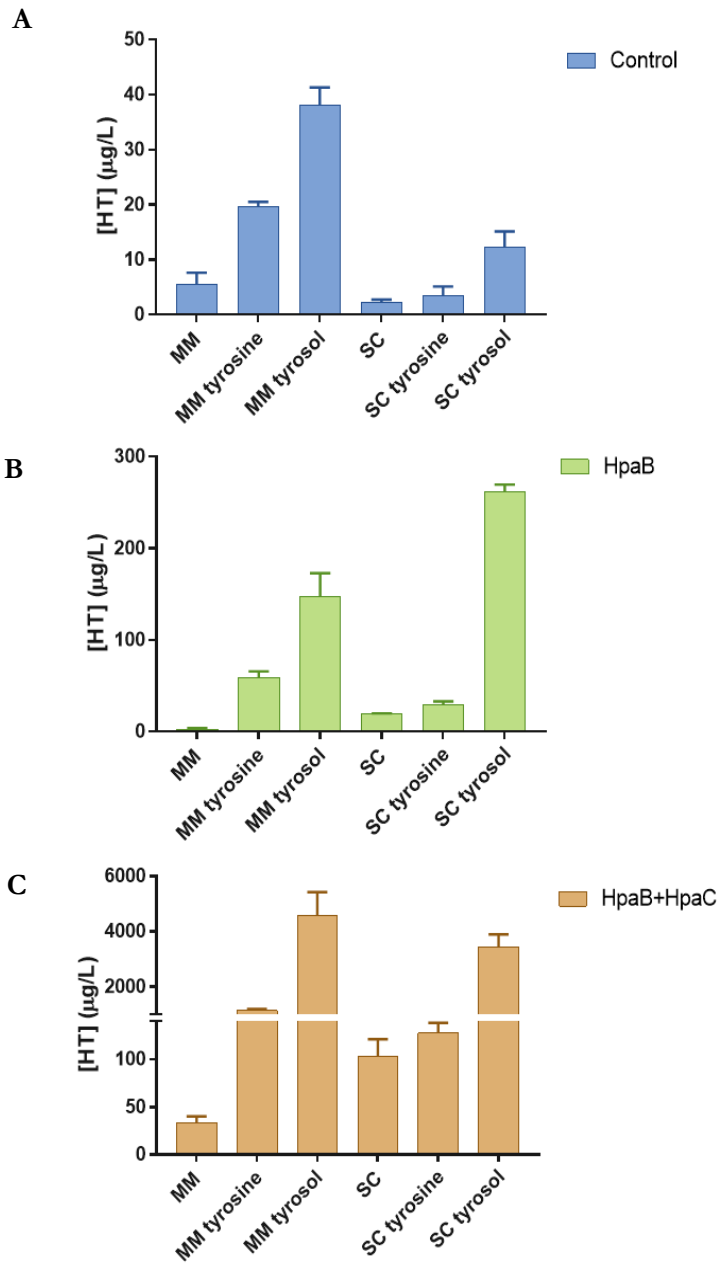

Figure 5.6 Effect of overexpression of *hpaB* or *hpaB* and *hpaC* in BY4743 on hydroxytyrosol production. Hydroxytyrosol produced by yeast strain BY4743 transformed with the empty vectors p425GPD and p426GPD (blue) (**A**), with h*paB* (green) (**B**), and *hpaB hpaC* (orange) (**C**) from *E. coli* under different media. Cells were cultured 72 h at 30 °C in MM or SC and the same media supplemented with tyrosine or tyrosol 1 mM. Hydroxytyrosol concentration was determined from supernatant extracted with methanol, and analyzed by UHPLC-MS/MS

Interestingly enough, the overexpression of both *hpaB* and *hpaC* genes in a medium without any added precursors was able to produce levels of HT similar to those achieved by the control strain in a medium with a high concentration of

tyrosol. In any case, the highest levels of hydroxytyrosol production (4600 ± 900 µg/L) were obtained for the strain overexpressing both *hpaB* and *hpaC* in MM with added tyrosol (Figure 5.6C). After this proof of concept, we decided to integrate both genes into the yeast genome, and we used the integration plasmid pCfB2988 from the EasyCloneMulty series (Maury et al. 2016). This vector contains a USER cassette to insert two genes using a bidirectional promoter and a target sequence for multiple integrations at sites sharing homology with Ty1Cons2 in *S. cerevisiae* genome. After integration of pCf2988 containing HpaBC into BY4743, we evaluated the hydroxytyrosol production of 24 different strains in SC media with 1 mM of tyrosol (Figure 5.7).

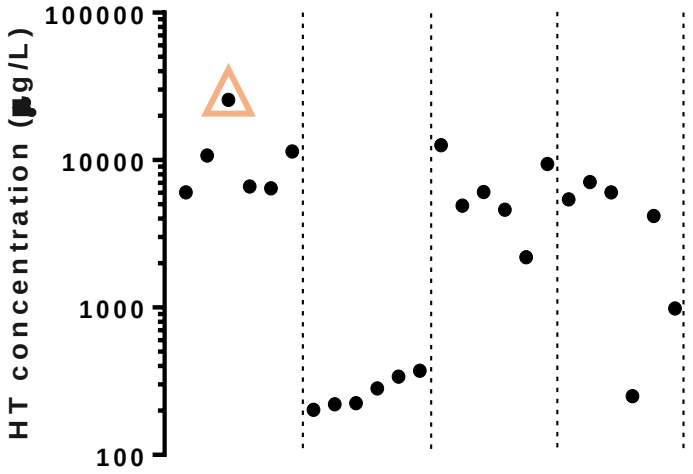

Figure 5.7 Production of hydroxytyrosol (HT), in SC medium with tyrosol, by different BY4743 strains harboring hpaBC integrated in several copies. The most productive strain was selected for further studies and is indicated with a triangle

Interestingly, although all the colonies increased the production of hydroxytyrosol, a clear difference in production was observed in the different colonies transformed with the integrative HpaBC cassette. This can be explained by the fact that each colony could have integrated a different copy number of the HpaBC complex. These findings established the basis for continuing to optimize yeast strains that could synthesize hydroxytyrosol from glucose, without having to add precursors to the medium. This is very interesting, due to glucose is ~600, ~6250 and ~5000000 times-cheaper than tyrosine, tyrosol and hydroxytyrosol,

respectively. Therefore, we selected the most productive colony (BY4743 HpaBC) (Figure 5.7), to explore additional modifications in order to increase the hydroxytyrosol synthesis from glucose.

5.2.3. Engineering a tyrosol and hydroxytyrosol-overproducing yeast strain

5.2.3.1. Effect of gene-knockouts of competitive pathways for tyrosol synthesis

Given the potential for HpaBC to hydroxylate tyrosol, we first decided to increase the flux towards this endogenous precursor for hydroxytyrosol synthesis. To this end, we evaluated the elimination of *TRP2*, *PHA2* or *ABZ1* since they are involved in chorismate metabolism, which is the last common substrate for all the AAA synthesis (Figure 5.8). *TRP2* encodes an anthranilate synthase which catalyzes the initial step of tryptophan biosynthesis, *ABZ1* encodes a para-aminobenzoate synthase involved in the synthesis of *p*-aminobenzoic acid from chorismate but also has been related with 2-phenylethanol production, and *PHA2* encodes prephenate dehydratase which consumes prephenate in phenylalanine biosynthesis pathway. We thought that by eliminating enzymes that uses this common substrate, the flow of chorismate towards the synthesis of tyrosine and tyrosol would be increased. However, when we evaluated the tyrosol levels in the different yeast strains derived from the single knockouts for the *ABZ1*, *TRP2* or *PHA2* genes in MM medium, we did not observe any significant improvement in tyrosol production in *TRP2* knockout strain or even a decrease was observed for *ABZ1* and *PHA2* (Figure 5.8). Thus, the single deletion of those genes was not an optimal strategy in order to increase the tyrosol production in yeast.

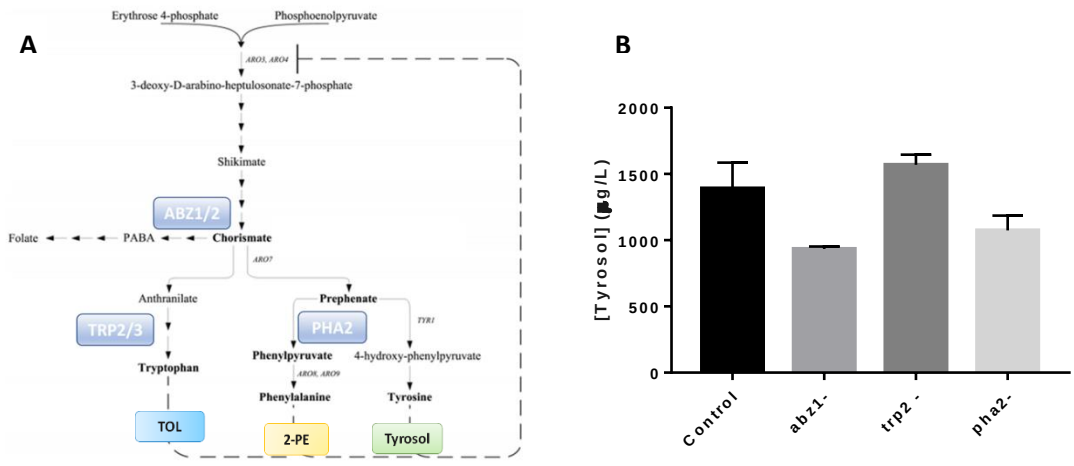

Figure 5.8 Effect of the knockout *ABZ1*, *TRP2* or *PHA2* on tyrosol production in BY4743 background growing in MM. Overview of the shikimate pathway *S. cerevisiae* (A) (Figure adapted from Winter et al., 2014). Tyrosol levels produced by BY4743 wild type strain (control), BY4743 mutant for *ABZ1* (abz1-), BY4743 mutant for *TRP2* (trp2-) and BY4743 mutant for *PHA2* (pha2-) were determined at 72 h (B). Tyrosol concentration was determined from supernatant extracted with methanol and subjected to UHPLC-MS/MS.

5.2.3.2. Modification of aromatic amino acid metabolism

5.2.3.2.1. Effect of *ARO10* on tyrosol and hydroxytorosol production

Tyrosol in *S. cerevisiae* is naturally produced from the catabolism of amino acids via Ehrlich pathway and the phenylpyruvate decarboxylase *ARO10* catalyzes the entrance reaction into this pathway through the conversion of 4-hydroxyphenylpyruvate (4HPP) to 4- hydroxyphenylacetaldehyde (4HPAA) (Figure 5.1). The overexpression of *ARO10* in *E. coli* has led to a high *de novo* production of tyrosol in this microorganism (Xue et al. 2017a; Xu et al. 2020). Therefore, we decided to overexpress *ARO10* in BY4743 and see the effect on tyrosol production. As is shown in the Figure 5.9, *ARO10* overexpression increased 5-fold the tyrosol levels respectively in SC media.

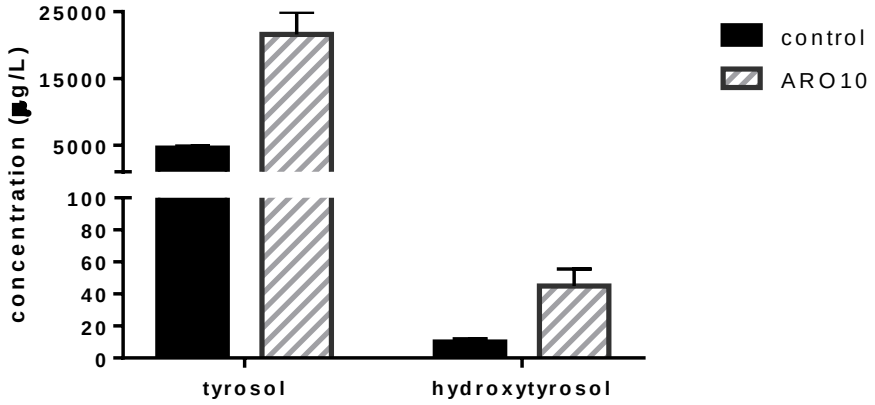

Figure 5.9 Effect of *ARO10* overexpression on tyrosol and hydroxytyrosol production. BY4743 was transformed with the empty vector p426GPD (control) and p426GPD ARO10 (ARO10) and grown in SC-ura medium at 30 °C for 72h. Tyrosol (solid bars) and hydroxytyrosol (stripped bars) were determined from supernatant extracted with methanol and subjected to UHPLC-MS/MS.

This is in agreement with the previous results in which the substitution of the *ARO10* promoter, by one of stronger expression, led to an increase in the production of higher alcohols (Trenchard et al. 2015; Hassing et al. 2019). We also wanted to determine if *ARO10* had an effect on increasing not only on tyrosol but also hydroxytyrosol levels. The overexpression of *ARO10* produced 45 µg/L of hydroxytyrosol from glucose in SC-ura medium (Figure 5.9), which were similar than the hydroxytyrosol produced by the BY4743 in MM with tyrosol supplementation (Figure 5.6A). This is a relevant result because it evidenced that the increase in tyrosol represented an enhanced activity of the native hydroxylase of *S. cerevisiae*, which also produced an increase in hydroxytyrosol.

Logically, the next step consisted in overexpressing *ARO10* in the strain harboring the HpaBC. Thus, we introduced the 2µ plasmid harboring p425GPD::*ARO10* into the BY4743 HpaBC (hpaBC + *ARO10*) strain and evaluated the hydroxytyrosol production from glucose in MM and SC (Figure 5.10). Hydroxytyrosol titers produced from glucose by strain hpaBC + *ARO10* reached mg/L whereas the strain harboring hpaBC with the empty vector only reached µg/L, showing the clear effect of *ARO10* overexpression in hydroxytyrosol production. Remarkably, hpaBC + *ARO10* produced ~45-times

more hydroxytyrosol than the wild type BY4743 overexpressing *ARO10* (Figure 5.9). These results, highlighted the synergic effect between the HpaBC and *ARO10*.

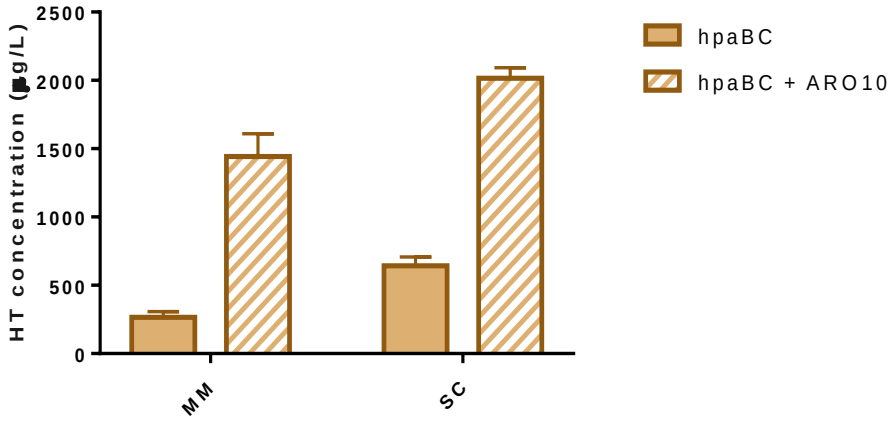

Figure 5.10 Effect of overexpression of *ARO10 on* hydroxytyrosol (HT) production in BY4743 HpaBC strain. BY4743 HpaBC transformed with empty p425GPD plasmid (solid bars) or with p425GPD*ARO10* (stripped bars) were cultured for 72 h at 30 °C in MM or SC-leu. Hydroxytyrosol concentration was determined from supernatant extracted with methanol, and analyzed by UHPLC-MS/MS.

5.2.3.2.2. Effect of individual ARO genes overexpression, and their feedback resistant derivatives forms, on hydroxytorosol and tyrosol production

After checking the potential *ARO10* to increase the synthesis of tyrosol and hydroxytyrosol, we wanted to examine other modifications that could increase the endogenous pool of hydroxytyrosol precursors. The first step in amino acid biosynthesis is the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) via the shikimate pathway. This step is performed by one of the two DAHP synthase isozymes, *ARO3* and *ARO4* (Figure 5.1). *ARO3* is allosterically inhibited by phenylalanine whereas *ARO4* by tyrosine (Braus 1991). The chorismate mutase, encoded by *ARO7*, has been identified as a second reaction subjected to allosteric regulation, so that it is inhibited by tyrosine and stimulated by tryptophan (Schmidheini et al. 1989). *ARO7* catalyzes the conversion of

chorismate to prephenate (Figure 5.1), the last precursor common to both phenylalanine and tyrosine.

Previous works described that modified *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO7*^{G141S} results in feedback inhibition-insensitive enzymes (Schmidheini et al. 1989; Fukuda et al. 1991; Luttik et al. 2008; Reifenrath and Boles 2018). Thus, different engineered strains in order to improve the titers of the intermediates of tyrosine and phenylalanine pathway as well as the shikimate and Ehrlich pathway intermediates have included the expression of these variants (Luttik et al. 2008; Curran et al. 2013; Trenchard et al. 2015; Reifenrath and Boles 2018; Reifenrath et al. 2018; Hassing et al. 2019).

Therefore, we next examined whether the single overexpression of *ARO3*, *ARO4*, *ARO7* and its feedback resistant derivatives (*ARO3*^{K222L}, *ARO*^{4K229L} and *ARO7*^{G141S}) in the 2µ plasmid p423GPD had any effect on increasing the pool of hydroxytyrosol precursor, tyrosol, and on hydroxytyrosol itself. These constructions were directly carried out in the BY4743 HpaBC because it was evidenced the synergistic effect between these genes of the AAA metabolism and the hydroxylase complex HpaBC for hydroxytyrosol production. As shown Figure 5.11, the single overexpression of all the genes resulted in an increase in tyrosol levels for SD medium, being *ARO3*, *ARO4*, *ARO10*, *ARO3*^{K222L} and *ARO4*^{K229L} overexpression which had the most pronounced impact (~20 to ~110-fold increase). In SC medium, the fold-increase on tyrosol was much lower (~1.5 to ~3.35) and only *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO10* showed a slight increase in tyrosol, compared to the control strain, while both *ARO7* and *ARO7*^{G141S} produced a significant decrease (Table 5.2).

Table 5.2 Tyrosol and hydroxytyrosol (HT) production by control (BY4743 HpaBC + p423GPD) and BY4743 HpaBC containing the same plasmid but with one of the following genes: *ARO3*, *ARO4*, *ARO7*, *ARO10*, *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO7*^{G141S} after 72 h growing in SD and SC media. Values for detected tyrosol are represented in mg/L ± SD, while concentration for HT is represented in µg/L ± SD.

	SD		SC	
	Tyrosol (mg/L)	HT (µg/L)	Tyrosol (mg/L)	HT (µg/L)
control	0.75 ± 0.42	14.84 ± 4.79	10.99 ± 0.48	25.22 ± 1.18
ARO3	24.82 ± 0.30	591.52 ± 267.49	11.51 ± 1.69	1975.93 ± 1384.29
ARO4	11.32 ± 0.09	577.05 ± 45.63	9.63 ± 0.19	1341.17 ± 1156.43
ARO7	0.64 ± 0.01	32.82 ± 4.06	0.93 ± 0.09	176.44 ± 35.19
ARO10	24.27 ± 0.52	554.83 ± 76.39	36.87 ± 1.33	2016.36 ± 76.99
ARO3K22L	26.85 ± 0.99	1344.05 ± 152.11	16.84 ± 0.17	2697.58 ± 478.92
ARO4K229L	57.24 ± 2.15	2078.13 ± 274.09	27.30 ± 1.77	6006.30 ± 912.46
ARO7G141S	1.05 ± 0.14	30.47 ± 2.60	1.49 ± 0.20	373.09 ± 32.48

Regarding hydroxytyrosol levels, the overexpression of all the genes (*ARO3*, *ARO4*, *ARO7*, *ARO10, ARO3*^{K222L}, *ARO4*^{K229L} and *ARO7*^{G141S}) in the BY4743 HpaBC resulted in a substantial improvement in hydroxytyrosol production compared to the control strain in both media. The higher amount of hydroxytyrosol titers (about 6 mg/L) was achieved by overexpressing the tyrosine feedback resistant *ARO4*^{K229L}, resulting in an increase of 240-times of hydroxytyrosol in SC medium (Table 5.2, Figure 5.11).

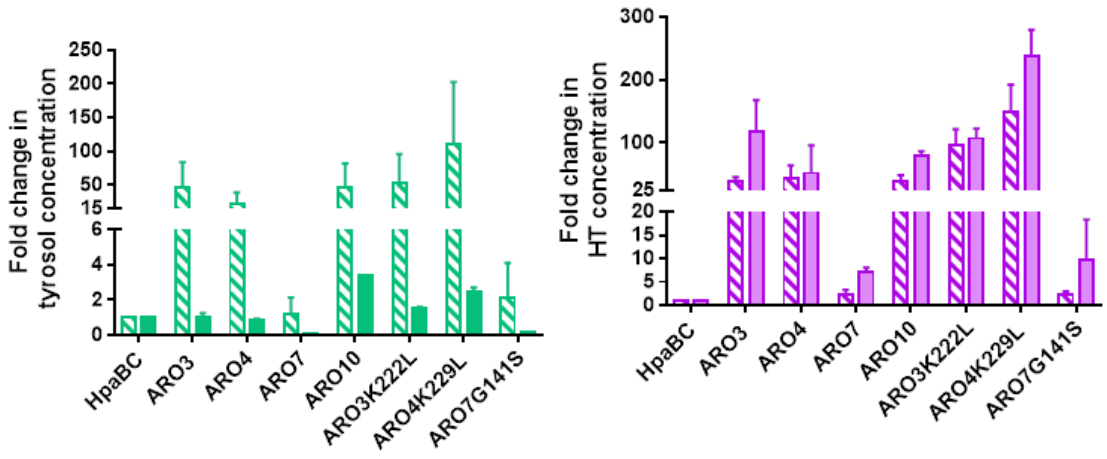

Figure 5.11 Effect of single overexpression of several genes involved in amino acid metabolism on tyrosol and hydroxytyrosol production. BY4743 HpaBC was transformed with empty p423GPD plasmid (control) or with p423GPD containing one of the following genes: *ARO3*, *ARO4*, *ARO7*, *ARO10*, *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO7*$^{G141S.}$ Each strain harboring one plasmid was cultured for 72 h at 30 °C in SD +leu (stripped bars) or SC-his (solid bars). Strains were cultured for 72 h at 30 °C in SC-his. Tyrosol and hydroxytyrosol were determined from supernatant extracted with methanol, and analyzed by UHPLC-MS/MS. Measured concentrations were normalized to metabolites produced by control strain and error bars representing standard deviations calculated from biological triplicates of one cultivation.

The huge improvement on hydroxytyrosol levels produced by *ARO4*^{K229L} overexpression, suggest that carbon flux increases to the aromatic amino acid pathway by releasing the feedback inhibition. In previous works, mutations in *ARO4* are frequently used in order to increase carbon flux into the aromatic amino acid biosynthetic pathway for the overproduction of valuable secondary metabolites, including tyrosol (Luttik et al. 2008; Curran et al. 2013; Gold et al. 2015; Jiang et al. 2018; Hassing et al. 2019). The highest subsequent hydroxytyrosol levels were obtained by the overexpression of *ARO3* followed by its alleviated feedback resistant form *ARO3*^{K229L} (116 and 107-fold-change, respectively). Interestingly, although overexpression of *ARO7* and *ARO7*^{G141S} led to a decrease in tyrosol production in SC, it did produce a 7 to 10-fold increase in hydroxytyrosol in the same medium (Figure 5.11). Nevertheless, a great positive correlation between tyrosol levels and hydroxytyrosol was observed (r=0.92; data not shown).

5.2.3.2.3. Effect of combined overexpression of ARO genes on hydroxytyrosol and tyrosol production

A possible additive effect in the production of hydroxytyrosol might result from the combination of overexpressing some of the previous genes. The combined overexpression of up to four ARO genes, together with the overexpression of the complex HpaBC, was carried out in the BY4741 strain. The availability of a high number of auxotrophies in the reference strain forced us to change from the BY4743 to its haploid version that harbors four auxotrophies (Δ*ura3*, Δ*leu2*, Δ*his3*, Δ*met15*). Furthermore, we additionally deleted *TRP1* gene in BY4741 in order to generate a new auxotrophy (see more details in Material and Methods). This strain was transformed with the pCfB2988 cassette containing the HpaBC and screened in the presence of tyrosol for select the clone with higher tyrosol hydroxylation activity as we did before for BY4743 HpaBC. After clone selection, we removed the auxotrophic marker by using the Cre/loxP-mediated marker removal procedure system (Gueldener et al. 2002) and overexpressed the different combinations of *ARO3*, *ARO4*, *ARO7*, *ARO10*, $ARO3^{K229L}$, $ARO4^{K229L}$ and $ARO7^{G141S}$ by using different 2µ plasmids as is detailed in materials and methods. For these new constructed strains, besides tyrosol, we also measured the other higher alcohols (tryptophol and 2-phenylethanol) in order to understand better the carbon flux direction. Figure 5.12 shows the production of the three higher alcohols produced by the different engineered strains.

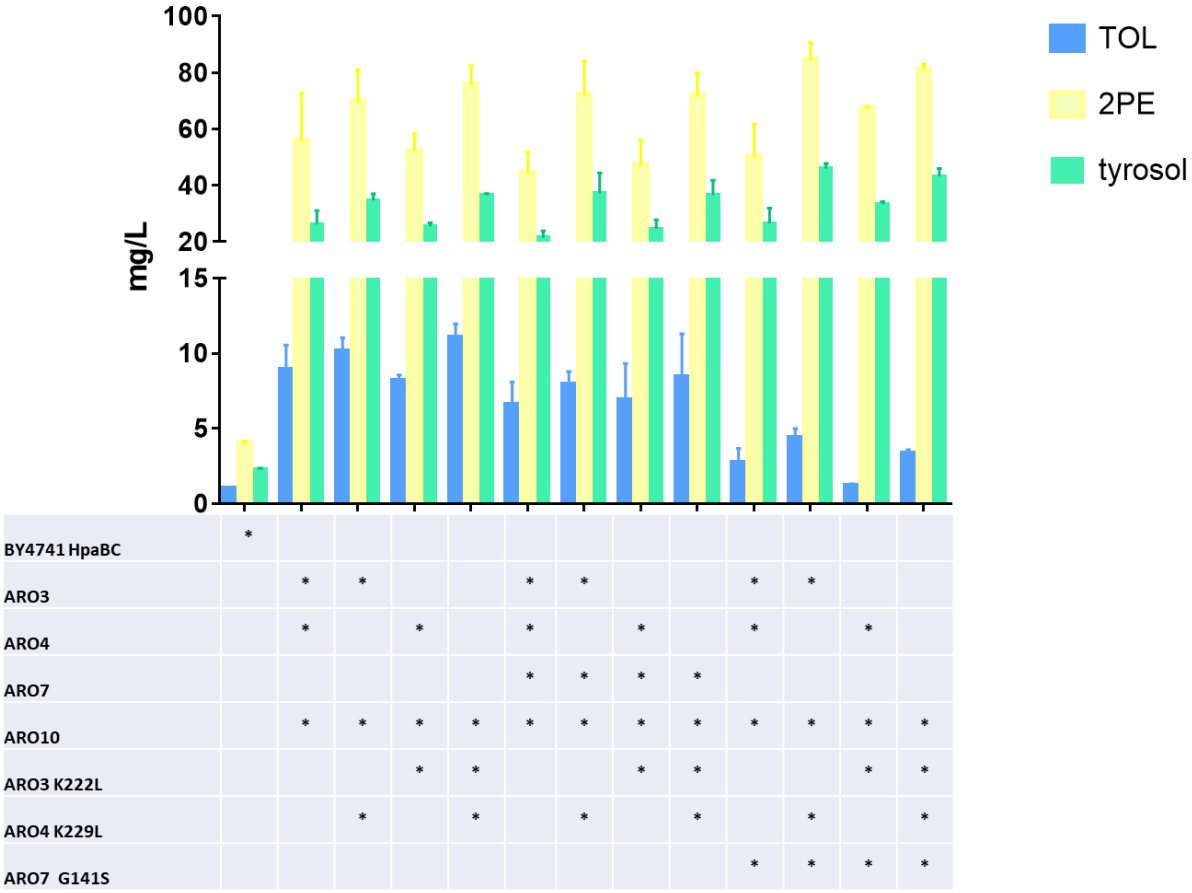

Figure 5.12 Effect of the combination overexpression of several genes involved in aromatic amino acid metabolism on higher alcohols production. BY4741 HpaBC was transformed with different plasmids containing each a *S. cerevisiae* gene for its overexpression (*ARO3*, *ARO4*, *ARO7*, *ARO10* and feedback resistant derivatives $ARO3^{K222L}$, $ARO4^{K229L}$ and $ARO7^{G141S}$). These plasmids were combined as is shown by an asterisk in one column in the table. The different strains were cultured for 72 h at 30 °C in SC-his. For more details about each strain see materials and methods section. Tryptophol (TOL), 2-phenylethanol (2PE) and tyrosol concentration were determined from supernatant extracted with methanol, and analyzed by HPLC-PDA**.**

Remarkably, all the overexpression combinations showed a huge increase in higher alcohols, compared with the control strain. Among the three different higher alcohols, 2-phenylethanol was the detected with greater concentration in all the engineered strains, followed by tyrosol and finally tryptophol (Figure 5.12). We detected up to 90 mg/L of 2-phenylthanol in one clone of the strain that co-overexpressed *ARO3*, $ARO4^{K229L}$, *ARO10* and $ARO7^{G141S}$. These concentrations are similar to those obtained by Shen et al. (2016), who achieved 96 mg/L of 2-phenylethanol by overexpression of the *ARO10* and *ADH1* genes together with the deletion of the *ARO8* gene in BY4741. Regarding tyrosol levels, about 20 ~

45 mg/L were obtained by our engineered strains (Figure 5.12). Interestingly, the engineered strain which lead the highest 2-phenylethanol levels (*ARO3*, *ARO4*^{K229L}*, ARO10* and *ARO7*^{G141S}*)* was the same that produced higher tyrosol concentrations, in agreement with the high correlation previously observed between tyrosol and 2-phenylethanol (r=0.98; data not shown). Interestingly, the single overexpression of both *ARO7* wild type allele or the feedback-insensitive form *ARO7*^{G141S} alone produced a huge decrease in tyrosol levels (Figure 5.11), but, when this allele, specially the *ARO7*^{G141S}, was co-overexpressed with other modifications, including *ARO3*/*ARO3*^{K222L}, *ARO4*^{K229L} and *ARO10*, tyrosol production increased by about 20-fold compared with the control strain (Figure 5.12).

Regarding to tryptophol, the levels detected ranged from 1 to 11 mg/L, being the strain that co-overexpressed *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO10* the best producer. Remarkably, when the modified strains overexpressed *ARO7*^{G141S}, together with other modifications, a significant decrease on tryptophol levels were observed (Figure 5.12). For example, when we introduced *ARO7*^{G141S} into the higher tryptophol producer strain (*ARO3*^{K222L}, *ARO4*^{K229L} and *ARO10*) the tryptophol levels dropped from 11.05 ± 0.95 to 3.33 ± 0.27 mg/L. A similar, but milder effect was observed when co-overexpressed *ARO7* with other combinations (Figure 5.12). This result makes sense since the tryptophol pathway is competing with the synthesis pathways of tyrosol and 2-phenylethanol at the chorismate node. Therefore, when *ARO7* is overexpressed, chorismate is used preferentially for the production of prephenate and its derivatives (2-phenylethanol and tyrosol), which results in a lower availability of chorismate for the synthesis of the precursor of tryptophol, anthranilate. The overexpression of the insensitive-feedback allele *ARO7*^{G141S} enhanced even more this metabolic flux towards the tyrosol and 2-phenylethanol synthesis.

Moreover, when we examined the shikimic acid production, all the modified strains showed an increase in shikimic levels ranging from 1100 to 4500 µg/L (Figure 5.13). The higher producer strain was the same as for tyrosol and 2-phenylethanol production (*ARO3*, *ARO4*^{K229L}*, ARO10* and *ARO7*^{G141S}*).*

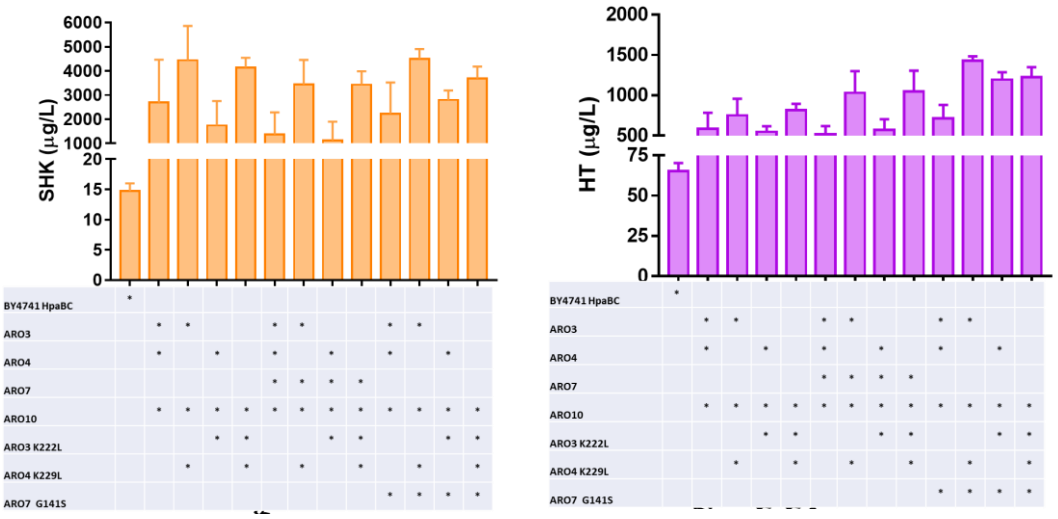

Figure 5.13 Effect of the combination of several overexpressed genes involved in aromatic amino acid metabolism on shikimic acid and hydroxytyrosol production. BY4741 HpaBC was transformed with different plasmids containing each a *S. cerevisiae* gene for its overexpression (*ARO3*, *ARO4*, *ARO7*, *ARO10* and feedback resistant derivatives *ARO3K222L*, *ARO4K229L* and *ARO7G141S).* These plasmids were combined as is shown by an asterisk in one column in the table. The different strains were cultured for 72 h at 30 °C in SD+Met. For more details about each strain or growth conditions see materials and methods section. Shikimic acid (SHK) and hydroxytyrosol (HT) concentration were determined from supernatant extracted with methanol, and analyzed by UHPLC-MS/MS.

Table 5.3 Tyrosol and hydroxytyrosol (HT) production by control (BY4741 HpaBC) and the same strain overexpressing *ARO3*, *ARO4*, *ARO7*, *ARO10*, *ARO3*^{K222L}, *ARO4*^{K229L} and *ARO7*^{G141S} in different combinations, after 72 h growing in SD+Met medium. Values for detected tyrosol are represented in mg/L± SD, while concentration for HT is represented in µg/L± SD.

	Tyrosol (mg/L)	HT (µg/L)
BY4741 HpaBC	2.27 ± 0.10	65.39 ± 4.76
ARO3 ARO4 ARO10	26.16 ± 4.98	585.33 ±198.90
ARO3 ARO4*ARO10	34.73 ± 2.37	754.04 ±202.81
ARO3*ARO4 ARO10	25.46 ± 1.23	547.64 ± 71.25
ARO3*ARO4*ARO10	36.58 ± 0.57	818.16 ±77.11
ARO3 ARO4 ARO10 ARO7	21.41 ± 2.47	518.85 ± 102.21
ARO3 ARO4*ARO10 ARO7	37.22 ± 7.25	1030.77 ± 269.70
ARO3*ARO4 ARO10 ARO7	24.61 ± 3.19	570.86 ± 134.17
ARO3*ARO4*ARO10 ARO7	36.62 ±5.30	1050.33 ± 255.10
ARO3 ARO4 ARO10 ARO7*	26.58 ± 5.43	716.86 ± 165.73
ARO3 ARO4*ARO10 ARO7*	45.95 ± 1.91	1428.13 ± 57.75
ARO3*ARO4 ARO10 ARO7*	33.54 ± 0.78	1195.31 ±92.74
ARO3*ARO4*ARO10 ARO7*	43.18 ± 2.88	1226.08 ±123.54

Asterisk indicates overexpression of the mutant variant of the gene (e.g. ARO3 * to indicate *ARO3*^{K222L})

Regarding hydroxytyrosol production, the combination of *ARO3*, *ARO4*^{K229L}, *ARO10* and *ARO7*^{G141S} overexpression in BY4741 HpaBC strain was the best producing strain, which produced about 1.5 mg/L of hydroxytyrosol (Figure 5.13, Table 5.3).

Nonetheless, these strains harboring several combinations of overexpressed genes did not overcome in titers of hydroxytyrosol to the previous construction in the diploid BY4743 HpaBC that only overexpressed *ARO4*^{K229L} (Figure 5.11 and Table 5.2). Variations in the production levels of aromatic compounds, such as shikimic acid, between BY4743 and BY4741, genetically modified in the same way, have been previously reported (Suástegui et al. 2016). The authors demonstrated that differences in the production of aromatic compounds were highly dependent on the ability of each strain to self-adjust the copy number of episomal vector. The capacity of auto-adjust plasmid copy number in accordance to the burden of excessive enzyme expression has been demonstrated previously (Carquet et al. 2015). However, since the levels of alcohols produced between our both strains were similar, the difference in the number of integrated copies of the HpaBC complex could explain the differences in the observed hydroxytyrosol levels. Therefore, we decided to select the diploid BY4743 HpaBC

ARO4^{K229L} as the best producing hydroxytyrosol strain for further optimization experiments. We aimed to assess the best growth conditions of this strain for hydroxytyrosol production.

5.2.3.3. Modification of culture conditions to improve the hydroxytyrosol production: glucose concentration and sampling time.

Previously we studied the effect of the addition of tyrosine and tyrosol in the medium on the production of tyrosol and hydroxytyrosol (Figures 5.5 and 5.6; and Table 5.1). Since we wanted to achieve sugar-based hydroxytyrosol biosynthesis in yeast, we investigated whether the increase in precursor glucose would lead to an increase in the production of hydroxytyrosol levels. We therefore analyzed the hydroxytyrosol production by BY4743 HpaBC *ARO4*^{K229L} (our selected engineered strain for hydroxytyrosol biosynthesis) and BY4743 HpaBC as a control, in SD medium with 160g/L in a time-lapse of 319 h (Figure 5.14). Surprisingly, the effect of glucose was remarkably important on hydroxytyrosol titers. By increasing the glucose concentration to 160 g/L, the hydroxytyrosol titers increased to 370 mg/L in the HpaBC *ARO4*^{K229L} overexpressing strain. This was more than a 100-fold increase compared to the same medium with 20 g/L of glucose. Interestingly, hydroxytyrosol was accumulated and its concentration increased until it reached 295 h, while at 319 h it began to decrease. On the contrary, we did not see this trend in SD with 20 g/L of initial glucose, in which the amounts remained constant from 120 to 197 h. For this reason, we do not continue sampling longer in this medium. Likewise, the higher alcohols accordingly showed a great increase with 160 g/L of glucose. The titers of tyrosol, 2-phenylthanol and tryptophol increased 2.4, 5.6 and 5.4 for HpaBC *ARO4*^{K229L} strain at 197 h (Figure 5.14). Nonetheless, we would like to remark that, for the first time, we got more hydroxytyrosol production than its substrate tyrosol for the HpaBC *ARO4*^{K229L} strain in 160 g/L of glucose.

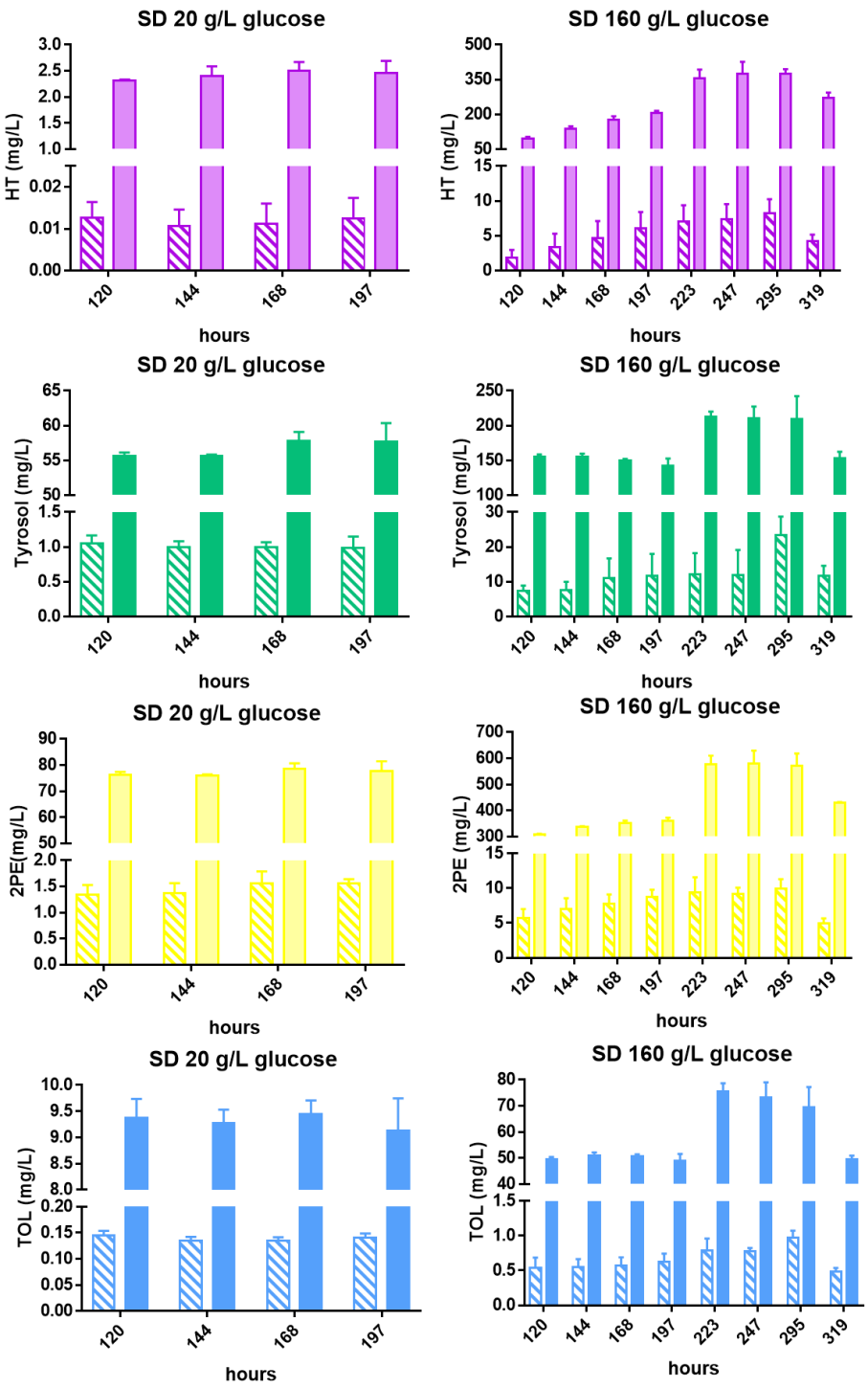

Figure 5.14 Time profile of higher alcohols and hydroxytyrosol titers production. BY4743 HpaBC (stripped bars) and the same strain but overexpressing *ARO4*^{K229L} (solid bars) were cultured in 250 mL flaks with 50 mL of SD with 20 or 160g/L of glucose. The culture was allowed to grow until 319 h at 30 °C with orbital shaking at 150 rpm. For more details about each strain or growth conditions see materials and methods section. Error bars representing standard deviations calculated from biological triplicates of one cultivation. Hydroxytyrosol HT), Tryptophol (TOL), 2-phenylethanol (2PE) and tyrosol concentration were determined at different time points from supernatant extracted with methanol, and analyzed by HPLC-PDA.

This significant increase in hydroxytyrosol and the AAA higher alcohols can be partially explained by a higher growth in the high glucose concentration medium (Figure 5.15). However, the increases observed in these metabolites are much higher than the increase in biomass production, therefore, this higher yield must rely on an enhanced metabolic flux in the AAA biosynthesis pathway as consequence of a higher concentration of substrate.

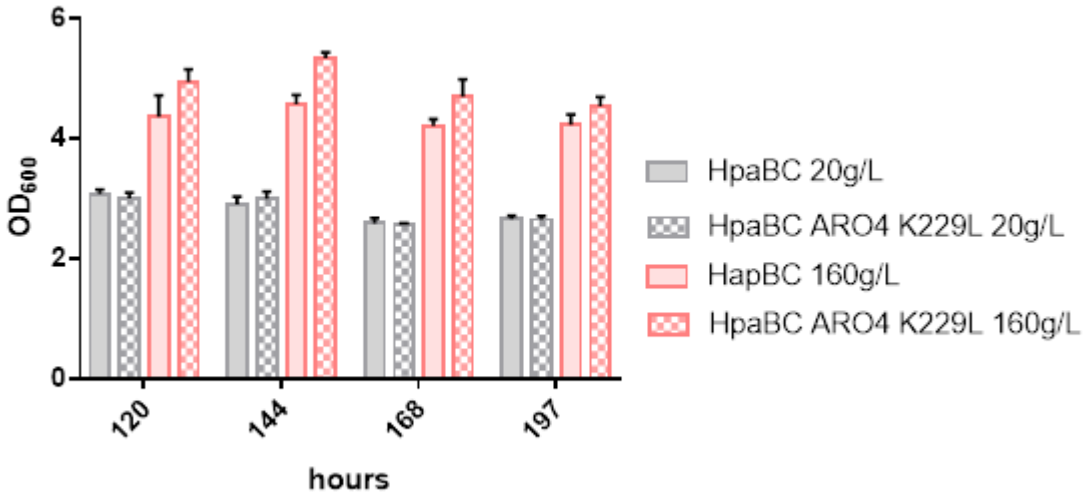

Figure 5.15 Yeast growth does not explain the hydroxytyrosol production from glucose by ARO4^{K229L} overexpressing strain. BY4743 HpaBC strain (solid bars) and BY4743 HpaBC overexpressing *ARO4^{K229L}* (pattern bars) were cultured in 250 mL flaks with 50 mL of SD with 20 or 160g/L of glucose (grey and pink color bars, respectively). The culture was allowed to grow until 197 h at 30 °C with orbital shaking at 150 rpm, and OD_{600} was recorded at different time points. Error bars representing standard deviations calculated from biological triplicates of one cultivation.

Finally, we decided to compare the improvement in the production of hydroxytyrosol, tyrosol, 2-phenylethanol and tryptopol produced by our different constructed strains in the best cultivation conditions. Therefore, we included the wild-type strain BY4743 transformed with empty vectors as a control together with the BY4743 HpaB + HpaC (in 2 µ plasmids), the BY4743 HpaBC (integrated) and our best producer strain, BY4743 HpaBC ARO4K229L. We took samples at 120 and 240 h of growth in SD medium with 160 g/L of glucose, and we normalized the metabolites produced by the different strains with the metabolites produced by the control strain. Generally, the largest increase observed for the production of various metabolites was for HpaBC ARO4K229L strain as we expected (Figure 5.16, Table 5.4). The highest increase determined for this strain was with the

production of hydroxytyrosol, which exceeded the production by more than 230,000 times compared to the control strain (Figure 5.16).

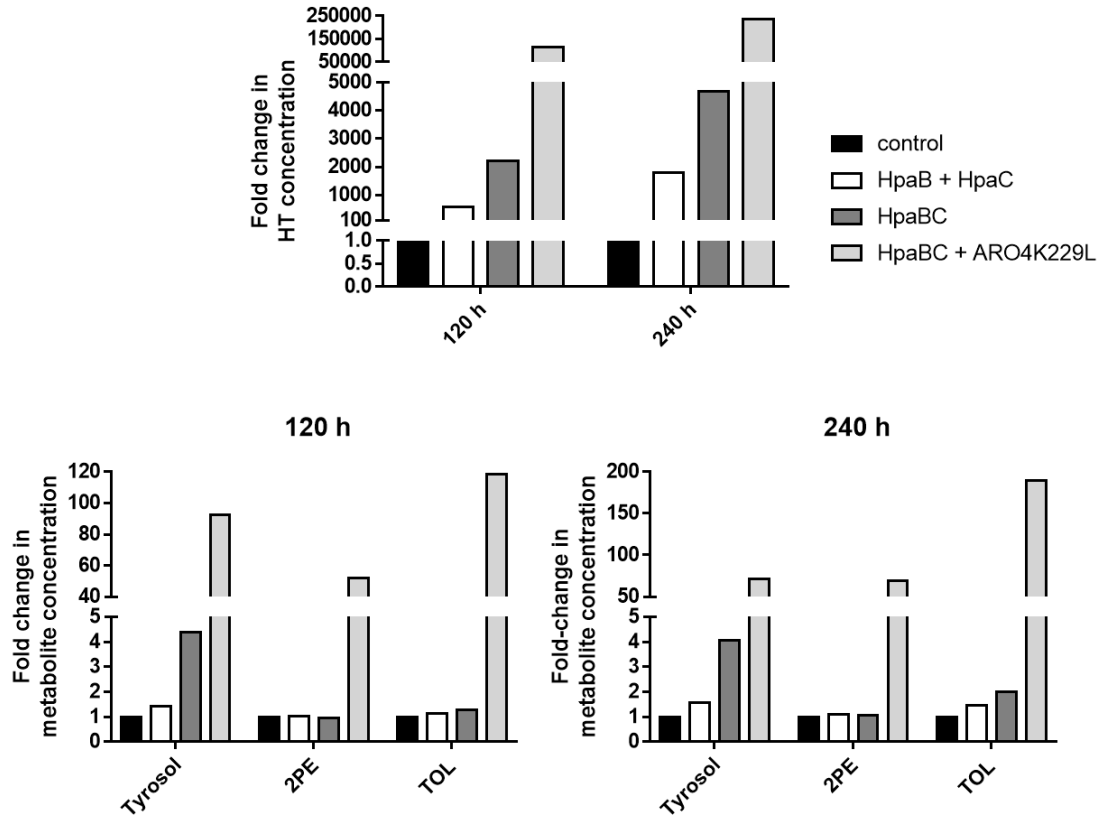

Figure 5.16 Improvement in the production of hydroxytyrosol and higher alcohols by yeast strains with various genomic modifications. BY4743 control strain (transnformed with empty vectors), BY4743 HpaB + HpaC, BY4743 HpaBC and BY4743 HpaBC ARO4K229L were cultured in SD medium with 160 g/L of glucose. Metabolites were measured from the supernatant extracted with methanol at 120 and 240 h, and analyzed by HPLC-PDA. Measured concentrations were normalized to metabolites produced by control strain.

The HpaBC was the second strain that showed the highest increase, although not for all metabolites and in lower magnitude orders than HpaBC ARO4K229L (Figure 5.16). Curiously, the HpaBC strain also significantly increased the production of tyrosol and hydroxytyrosol but not of the other AAA higher alcohols, tryptophol and 2-phenyl ethanol, suggesting that the complex HpaBC only impacts on the tyrosine biosynthetic pathway. Moreover, these results reinforce the idea that deregulation of *ARO4* is essential to increase fluxes towards the production of aromatic 'fusel' metabolites as previously reported (Luttik et al. 2008).

Table 5.4 Hydroxytyrosol (HT), tyrosol, 2-phenylethanol (2PE) and tryptophol (TOL) titers produced by BY4743 control strain (transnformed with empty vectors), BY4743 HpaB + HpaC, BY4743 HpaBC and BY4743 HpaBC ARO4K229L, after 120 and 240 h growing in SD with 200 g/L of glucose. Values are represented in mg/L± SD.

	120 h				240 h			
	HT	Tyrosol	2PE	TOL	HT	Tyrosol	2PE	TOL
control	0.001 ± 0.001	1.677 ± 0.104	5.948 ± 0.021	0.416 ± 0.002	0.002 ± 0.000	2.948 ± 0.139	8.437 ± 0.124	0.388 ± 0.083
HpaB +HpaC	0.473 ± 0.149	2.366 ± 0.339	6.023 ± 1.833	0.468 ± 0.112	2.850 ± 0.875	4.588 ± 0.915	9.219 ± 1.860	0.572 ± 0.083
HpaBC	2.402 ± 0.794	7.350 ± 1.609	5.706 ± 1.300	0.533 ± 0.151	7.363 ± 2.186	12.014 ± 7.131	9.140 ± 0.957	0.755 ± 0.052
HpaBC ARO4K229L	95.119 ± 8.388	155.576 ± 3.226	308.121 ± 2.609	49.387 ± 1.011	374.484 ± 52.042	210.704 ± 16.677	579.669 ± 50.043	73.353 ± 5.671

5.2.4. Further strategies for improving production titers of hydroxytyrosol

In this study, we have obtained an engineered *S. cerevisiae* strain that produce the higher concentration of hydroxytyrosol, and its related higher alcohols, from glucose ever reported so far, providing promising basis for future improvements. Besides the different constructions explained in the previous sections, which driven to the overproducer strain, we also assessed other strategies that are worth to mention, in spite of the fact that they did not finally improve the hydroxytyrosol synthesis. Firstly, we explored the replacement of *GPD* promoter for *hpaB* and *hpaC* overexpression by two promoters (*HXT7* and *RTN2*) of induction during the stationary phase. They were selected for their weak expression during cultivation with excess of glucose and their induction in glucose limiting conditions. *HXT7* is highly expressed at transcriptional level under low hexose conditions but they are strongly repressed at high glucose concentrations (Ye et al. 2001; Lai et al. 2007), and has been used previously for controlling the production phase of 3-hydroxypropionic acid (Maury et al. 2018). *RTN2* is activated upon the diauxic shift and it has been previously characterized as stronger promoter for the production of foreign proteins (Kim et al. 2006). When we used these promoters for controlling *hpaB* and *hpaC* overexpression in plasmids, we observed a better growth, however, this approach did not lead to any measurable improvement on hydroxytyrosol production (data not shown). Thus, we decided to continue with constitutive strong promoters. However, glucose-dependent promoters have been shown different dynamic expression control in batch and fed-batch cultivations (Maury et al. 2018). Since we performed only batch cultivations, further optimization of culturing conditions such as fed-batch in a bio-reactor could be interesting strategies for decoupling the production and growth phases and increase hydroxytyrosol levels.

As noted, the tyrosol hydroxylation to hydroxytyrosol performed by the complex HpaBC is one of the main bottleneck in our engineered strain since accumulated tyrosol occurred. In a recent work, it was found that three amino acid substitutions in HpaB (*S210T*/*A211L*/*Q212E*) produced ~19-fold higher activity on tyrosol than the wild-type enzyme (Chen et al. 2019). Thus, we decided to overexpress this mutant form (A10) and screened its activity in hydroxytyrosol formation. However,

when we compared tyrosol hydroxylation between wild type and A10 in SC supplemented with 1 mM tyrosol, we observed ~ 5-fold lower activity for the mutant enzyme A10 (data not shown) than for the native alleles. Therefore, we decided to go on with the with the wild-type allele for our overproducer strain.

Furthermore, we investigated if the supplementation of medium growth with ascorbic acid could decrease oxidation of hydroxytyrosol since it has been shown to be beneficial for hydroxytyrosol production in *E. coli* (Li et al. 2017). However, we did not observe any significant improvement on hydroxytyrosol production between with or without ascorbic acid addition (data not shown).

However, we also consider that we have not reached the maximum potential production of HT and further metabolically engineering strategies can be applied in the near future to improve its yield. One strategy could be to increase the flux of necessary precursors into the shikimate pathway. According to previous studies, the overexpression of the transketolase (*TKL1*) and the deletion of the glucose-6-phosphate dehydrogenase (*ZWF1*) increase the entrance of the glycolytic intermediates, glucose-6-phosphate and fructose-6-phosphate into the pentose phosphate pathway, which turns out in the increase of E4P (an essential precursor for the shikimate pathway).

The heterologous overexpression of the shikimate kinase II from *E. coli* (encoded by *aroL*) has been used to boost the carbon flux toward chorismate synthesis and this effort successfully eliminated the formation of shikimate and improved 2-phenylethanol (Hassing et al. 2019) and *p*-coumaric acid production (Rodriguez et al. 2015).

Although upstream modifications of the aromatic amino acid biosynthesis may be interesting, due to the fact that the overexpression of the deregulated DAHP synthase revealed the accumulation of intermediates such as higher alcohols, we think that improvements in downstream steps could result more effective for our HpaBC *ARO4*^{K229L} overexpressing strain. Concerning 2-phenylethanol, it was the higher alcohol produced in greater amount compared to the rest, reaching over 550 mg/L (Figure 5.14). This means that prephenate (the last common substrate for the phenylalanine and tyrosine branches) is being more directed to 2-

phenylethanol than tyrosol synthesis. Despite the fact that the deletion of *PHA2* in the BY4743 wild type strain did not have a statistically significant ($p > 0.05$) effect on tyrosol levels (Figure 5.7), we think that the downregulation of this gene, by replacing its native promoter by one of weaker activity, might be of interest in our best hydroxytyrosol producer strain to improve hydroxytyrosol yields. Since *PHA2* is competing with *TYR1* to prephenate, future investigations for improving the flux of prephenate through tyrosol formation could include overexpression of *TYR1* and/or the downregulation of *PHA2* expression by its native promotor-replacement. The main advantage of using weaker promoters to regulate *PHA2* expression would be that the strain would not be auxotrophic for phenylalanine. *TYR1* overexpression has been identified as the bottleneck for tyrosine production and its overexpression resulted successful to increase tyrosine levels (Mao et al. 2017).

Another future strategy could aim the increase of NADH pool for improving growth of our selected strains. As mentioned above, the growth defect observed by *hpaB* and *hpaC* overexpression is ameliorated by the deletion of genes which are responsible for the decrease on NADH cytosolic levels (Figure 5.4).

Finally, since yeast strain background, even among those belonging to the same species, can play a major role in ensuring optimal titers, yields, and productivities (Strucko et al. 2015; Suástegui et al. 2016), further transfer into other strains of the key modifications in hydroxytyrosol production is strongly recommended.

5.3. Conclusions

In this chapter we report the successfully introduction of the HpaBC hydroxylase complex from *E. coli* in *S. cerevisiae* and we demonstrate the ability of this complex to transform tyrosol into hydroxytyrosol in yeast. Further, we metabolically engineered *S. cerevisiae* for high-level production of hydroxytyrosol from glucose, by integrating HpaBC complex into the genome and overexpressing several AAA pathway-related genes. Single overexpression of all the genes (*ARO3*, *ARO4*, *ARO7*, *ARO10*, $ARO3^{K222L}$, $ARO4^{K229L}$ and $ARO7^{G141S}$) did have a statistically significant effect on hydroxytyrosol production. However, the overexpression of $ARO4^{K229L}$ showed the greatest effect. By additional

optimization of the medium and culture conditions, the strain harboring HpaBC and overexpressing *ARO4*^{K229L} produced the highest titer of hydroxytyrosol, reaching 374.5 mg/L in shake flask experiments. The reported results are the highest hydroxytyrosol titer in *S. cerevisiae* to date. This work lays down the first steps for overproducing hydroxytyrosol in yeasts form glucose by a metabolic engineering approach and for further developing a yeast cell factory for hydroxytyrosol production. Due to the high price of hydroxytyrosol in the market (approximately 8000 ~18500 €/g), this constructed strain could be a very useful biotechnological tool for producing this molecule. Hydroxytyrosol is a very interesting molecule for the food, pharmaceutical and cosmetic industries. In fact, we have patented this overproducer strain (patent number P202031186; Annex I of this thesis) for the future exploitation in the case that a company of these sectors would be interested.

GENERAL DISCUSSION

The improvement of analytical techniques has allowed the scientific community to identify molecules present in the wine that are in lower concentration. This fact turns the wine into a complex mixture of more than hundreds of molecules present in different concentrations (Nisbet et al. 2014). Although a part of these molecules may have a plant origin and its content can vary as a consequence of enological practices such as fermentation temperature, grape cultivar, oak-wood aging, duration of wine aging, etc… (Vitalini et al. 2011; Fernández-Cruz et al. 2018), the metabolism of microorganisms plays an important role in both modifications and *de novo* production of different metabolites during the AF process (Guerrini et al. 2018). Thus, during AF, wines are enriched in molecules characterized by specific chemical and biological properties, which leads to a greater number of bioactive compounds than those of the corresponding grapes (Guerrini et al., 2018; Rodriguez-Naranjo et al., 2011; Romboli et al., 2015).

Recently, phenol and indole derivatives compounds from yeast catabolism of AAA, such as serotonin, melatonin or hydroxytyrosol, are receiving considerable attention due to their activity and effects on human health (Fernández-Mar et al. 2012; Aredes-Fernandez et al. 2016; Varoni et al. 2018; Gallardo-Fernández et al. 2019). Since the presence of melatonin in wine was described and the increase in its levels was related to the activity of yeasts, research on this topic has increased the interest of different research groups. Both *Saccharomyces* and non-*Saccharomyces* yeast have been shown to have the ability to produce AAA-derived molecules during alcoholic fermentation (Fernández-Cruz et al. 2017; Álvarez-Fernández et al. 2018; Fernandez-Cruz et al. 2019), and an increase in melatonin production has been described when both types of yeast were co-inoculated with respect to the single one (Valera et al. 2019), highlighting the importance of the metabolic interaction between yeast on the production of this kind of molecules. Additionally, *O. oeni* has been shown, in a recent work, to be capable of producing melatonin during MLF, although yeast was described as a more efficient producer (Fracassetti et al. 2020). Therefore, the metabolic interactions that can occur between yeast-yeast and yeast-LAB during winemaking cannot be overlooked. Furthermore, some of the metabolites derived from AAA have already been identified that can act as quorum sensing

molecules; *e.g.* tryptophol and other aromatic alcohols (Chen and Fink 2006; González et al. 2018a).

Some works highlight a key role of fermentation kinetics in the production and accumulation of these molecules in wine. For example, Romboli et al. (2015) found that by aerating the must during AF, *S. cerevisiae* significantly improved its fermentation vigor but led to lower levels of tyrosol and hydroxytyrosol. Regarding melatonin, recent studies describe that melatonin can bind glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase, enolase and pyruvate kinase, and it seems that this binding only occurs in yeasts with high fermentative capacity (*Saccharomyces*, *Starmerella*, *Torulaspora*) but not in other yeasts that show a predominance of oxidative metabolism (*Hanseniaspora*, *Metschnikowia*). Other studies suggested that melatonin production by yeasts is directly related with growth phase (Vigentini et al. 2015; Fernández-Cruz et al. 2016) but, there appears to exist a significant strain dependent effect on melatonin production (Vigentini et al. 2015). So, still further research is required to ascertain the combination of inoculation strain and inoculation strategy that could optimize the concentration of bioactive compounds. Furthermore, the metabolic biosynthetic pathway and the physiological functions of melatonin in yeast are not fully understood. The study of these two issues is a key point to improve the synthesis of these molecules during the fermentation process. Therefore, this thesis aimed to gather deeper understanding of the biochemical bases involved in the synthesis of molecules derived from AAA in *S. cerevisiae* and to understand it better from a molecular point of view. To this end, the development of rapid detection methods of melatonin in terms of reliability, easy-to-use and quickness, as alternative to HPLC that allows the realization of extensive screenings with a large number of strains and fermentation conditions, can be a useful tool. Thus, the first aim of this thesis was to set up and optimize an electrochemical method for yeast samples.

Detection method for melatonin in yeast samples

The study on the presence of melatonin in fermented products or in yeast derived samples have been performed with several methods as it is described in the introduction of the present thesis. Among the different methods, HPLC-MS/MS allows to follow the behavior of several metabolites during the fermentation process, in parallel for both intracellular and extracellular media, with higher resolution and a lower LOD than other methodologies. However, these methods also require special and expensive facilities and, depending on the platform, "harsh" sample pretreatment, all of which makes it difficult to carry out extensive screening tests.

In this context, we adapted a solid-state electrochemical technique for the detection of melatonin and other related metabolites directly from dried cells (avoiding intracellular content extraction), to improve the detection and monitoring melatonin production in yeast samples in a reliable and sensitive, and more easy and economical way compared with HPLC methods.

The voltammetric methodology adapted was applied first to the metabolites of interest: melatonin, serotonin, 5-hydroxytryptophan, *N*-acetylserotonin, tryptophol, 3-indoleacetic acid, and tryptophan. Once the corresponding signals of each compound have been characterized, we proceed to the determination of melatonin directly on yeast cells. Comparison among melatonin detection by our voltammetry methodology and HPLC, which required and additional intracellular extraction, was performed and a good agreement between the results were obtained by both methodologies. This methodology has been successfully applied to other organisms, such as plants, for testing the antioxidative capacity of samples or obtaining electrochemistry-based chemotaxonomy information (Doménech-Carbó et al. 2015c; Ortiz-Miranda et al. 2016). Also identification of fungal species based on the record of the voltammetric response due to metabolites containing phenolic units has been achieved by VIMP directly on fungal colonies (Mateo et al. 2017).

Other rapid methods based on fluorescence have been used for melatonin determination. Enzyme-linked immunosorbent assays (ELISA) in grape skin,

wine and beer samples was employed (Iriti et al. 2006; Maldonado et al. 2009; Rodriguez-Naranjo et al. 2011a). However, accordingly with several studies the main disadvantages of the ELISA test are non-linearity, false positive and false negative results, and the relatively high price of the commercial kits (Horwitz 1982; Hernández-Ruiz and Arnao 2008; Paredes et al. 2009; Rodriguez-Naranjo et al. 2011a). For this reason, we are developing a competitive indirect ELISA with new antibodies (in collaboration with Dr. Antonio Abad from IATA-CSIC) suitable for the detection of melatonin in fermented beverages. Preliminary assays with this method showed good specificity and sensitivity, and LOD and LOQ values similar to LC-MS / MS.

Morcillo-Parra et al. (2019) optimized a novel method based on BLA cell-based bioassay using the mammalian melatonin receptor MTNR-1B. This method allowed melatonin quantification with higher sensitivity than HPLC-MS/MS and no cross reaction was observed between melatonin and its precursors. However, in complex matrices, such as grape must or wine, sample extraction prior to melatonin detection was needed mainly because the culture cells were affected by ethanol or high sugar concentrations. A new method based on the other mammalian melatonin receptor MTNR1A has been employed using yeast cells as biosensor (Shaw et al. 2019). The use of yeast cells instead of cell cultures overcomes the problems related to detection in complex matrices, allowing it to be a continuous detection system since there is no need for extraction of the sample prior to detection. However, the sensitivity of this system must be improved for its applicability in samples derived from alcoholic fermentation in which melatonin presence is low. So, suitability of the method for detection and quantification of melatonin is strongly subjected to sample's features and it should be chosen accordingly to the intended application.

In summary, the electrochemical method presented here is fast, cheap and easy to monitor melatonin production directly in yeast cells. However, our voltammetric technique is rather semi-quantitative and, therefore, it should be considered in this context complementary to chromatographic techniques. Even so, our voltammetric method could be very useful for initial screenings with a large

number of samples before HPLC analysis, which significantly reduces the cost of the analysis.

Role of melatonin in yeast

The connection between AAA metabolism in yeast and the presence of bioactive compounds during the fermentation is well established. However, the melatonin biosynthesis pattern is not well understood and a clear condition that resulted in consistent melatonin production has not been yet identified. In fact, inconsistences in melatonin production has been observed in different studies (Fernández-Cruz et al. 2017; Morcillo-Parra 2019; Motlhalamme 2020). The knowledge of the effect of melatonin in yeast could shed some light to the conditions that trigger melatonin production.

Melatonin's physiological effects have been widely studied in a variety of organisms in animal and plant kingdoms. Although the role of melatonin in yeast is still not clear, some recent works have connected melatonin as antioxidant in response to oxidative stress damage by hydrogen peroxide in *S. cerevisiae* (Vázquez et al. 2017; Sunyer-Figueres et al. 2020).

To assess the possible role of melatonin in yeast, we performed intracellular enrichment of melatonin by incubating the cells during 30 min in the presence of different doses of this molecule. The intracellular melatonin enriched levels after this treatment highlighted the capacity of yeast to take up this indole. However, the intake mechanism is still unknown and it could be conducted by passive diffusion or by the active transport facilitated by any specific permease. In mammalians cells melatonin can easily cross physiological barriers due to its amphiphilic nature (Reiter and Tan 2003), while members of the SLC2/GLUT family glucose transporters have also shown be involved in melatonin uptake in mice cells (Hevia et al. 2015). The enrichment of the cells in melatonin decreased the latency growth phase and the ROS formation, but not at all the assayed concentrations. Thus, it seems that the antioxidant effect of this molecule is concentration-dependent, being able to show a pro-oxidant effect at high levels. A few studies, using several tumor and non-tumor culture cells, reported that, at

pharmacological concentrations, melatonin can act as a conditional pro-oxidant and stimulates ROS production (Reviewed in Zhang & Zhang, (2014)). This happens in the study of Vazquez et al. (2017), in which the presence of melatonin in the culture medium, without the presence of any oxidant agent, turned out in a slightly accumulation of ROS, which was later associated with increased catalase activity (Vázquez et al. 2018). The different concentration of intracellular melatonin in our work and that of Vázquez et al. (2017) could explain the observed difference on the effect of melatonin itself (prior stress) on ROS formation.

The improved growth observed in the melatonin-enriched cells cannot be explained owing to this molecule is used as a nutrient by yeast, considering that its intracellular concentration was very low. Therefore, we think that melatonin may act as an inducing molecule that promotes growth. Melatonin as a trigger for an early start-up of cell growth raises interesting questions about its role as a signaling molecule involved in modulating growth in a population density dependent manner. This possibility has been recently studied by Valera et al., (2019). They found that melatonin supplementation had no impact on the biomass of yeast but, caused changes in fermentation kinetics, viability and species distribution during fermentation. Specifically, when melatonin was added to the must, the fermentations performed by mixed *Saccharomyces* and non-*Saccharomyces* cultures were faster than for the single culture of both species. Although the observed changes were dependent on the levels of nitrogen in the media and the composition of the inoculum, they also observed that non-*Saccharomyces* species persisted until the end of fermentation when melatonin was available in low-nitrogen conditions. Thus, the presence of this molecule seems to somehow determine the microbial interactions between species during the alcoholic fermentation.

Intracellular melatonin was able to protect against oxidative and UV stresses and improve yeast growth after both stressors in *S. cerevisiae*. To the best of our knowledge, this is the first study utilizing UV radiation as oxidative stress inducer to investigate the physiological response of melatonin in *S. cerevisiae*. Our results showed a significant protection of intracellular melatonin in yeast against UV

irradiation. In keratinocytes or leukocytes, melatonin and its derivatives (including AFMK and *N*-acetylserotonin) has been related to counteract the UV radiation-induced damage and oxidative stress (Elsner et al. 2004; Fischer et al. 2006; Skobowiat et al. 2018). It would be of great interest to analyze in more detail the mechanism by which melatonin can exert this protective role. Interestingly, a protective role of serotonin against ionizing radiation was related for several *S. cerevisiae* species (Goncharenko et al. 1978). Also, serotonin synthesis from tryptophan or 5-hydroxytryptophan precursors were shown to exert photoprotection in *Candida guilliermondii* (Fraiking et al. 1981) and an increase in serotonin synthesis was observed after irradiation to near UV (334 nm) (Goncharenko et al. 1980). The study of the synthesis of melatonin after different stress exposure could help to reveal conditions that trigger its synthesis. In plants, UV light, cadmium, cold and drought-induced significantly increased the production of melatonin (Hardeland, 2016; Lee et al., 2017; Zhang et al., 2015). Mothalame (2020) evaluated the melatonin production on yeast after different stressor addition in the culture medium. Specifically, sublethal concentrations of H_2O_2, $ZnCl_2$, $CuSO_4$ and NaCl were added to YPD, both after inoculation and during exponential phase growth. The results obtained by these authors suggested that yeast synthesized melatonin as a defense mechanism. These indications raise the possibility that yeasts found in hostile or natural environments, in which they have to fight against stressors, could retain or present a greater melatonin-producing capacity.

Therefore, the melatonin levels in yeast after exposure to different stressors need further investigation and may help explain the observed inconsistencies in the levels of melatonin observed for several authors (Mothalame, 2020; Morcillo-Parra et al., 2020).

Comparison of transcriptional activity in untreated and melatonin-treated cells could be an interesting way to reveal if the exhibited protection observed in our conditions is due to the direct antioxidant and photoprotective properties of the molecule itself, or whether it is mainly a signaling molecule that triggers a molecular and physiological response to cope with these stress situations and to activate cellular growth. Our results evidenced that transcriptional activity of the

genes involved in response to oxidative stress was modified by intracellular melatonin without any stressor. When these cells were subjected to the stress situation, induced by H_2O_2 or UVC light, the changes in gene activity in terms of more marked transcriptional changes were more buffered than in non-enriched cells. These results seem to indicate that melatonin could prepare the cells to better endure stress situations by early modifying the mRNA levels of these antioxidant and photoprotective genes, in agreement with Vazquez et al., (2017). These authors suggested that the presence of melatonin, which generated non-toxic levels of ROS, could play a role as a cell signaling molecule to pre-adapt and prepare cells for subsequent lethal exposure. Similarly, transcriptomic analysis conducted by Motlhalamme (2020) revealed that the melatonin supplementation buffered the genomic response to oxidative stress. Thus, melatonin have been demonstrated to modulate gene expression of genes involved in detoxification of ROS (Zhang and Zhang 2014) and, in yeast, this molecule has been related with the overexpression of genes involved in sulphur metabolism (*MET28*, *MET1*, *MET2*, *MET14*, *MET16* and *MET17*) (Motlhalamme 2020).

Likewise, our results showed that among all the genes analyzed after stress exposure, we observed no early induction and mainly a down-regulation of the genes that coded for mitochondrial enzymes, with the exception of *TRX3*. This early down-regulation of the genes associated with mitochondrial function has also been observed as a transient effect in early stages of the stress response to other oxidants like cumene hydroperoxide, while other cytosolic antioxidant genes are up-regulated at the same time (Sha et al. 2013). Further studies into the gene expressions that cover a wider time range are likely to reveal the global up-regulation of redox and ROS-removing enzymes, as previously described (Gasch et al. 2000; Causton et al. 2001; Vázquez et al. 2017). Recently, Sunyer-Figueres et al. (2020) reported a transcriptomic analysis of the role of supplemented melatonin at the transcriptional level in *S. cerevisiae*, in the presence and absence of oxidative stress. In this study, the authors found that melatonin modulated the expression of many genes, mainly downregulating the expression of mitochondrial genes in the absence of oxidative stress while upregulating them under oxidative stress. However, we observed a down-

regulation of mitochondrial genes after stress exposure although we studied a shorter transcriptional response after stress exposure than these authors. Further, they also observed the upregulation of cytosolic genes after stress exposure which is clearly in agreement with our observations. The transient down-regulation of mitochondrial genes in early stages of the stress response while other cytosolic antioxidant genes are up-regulated at the same time has been reported previously (Sha et al., 2013).

To conclude this point, the protection exerted by melatonin against oxidative stress was investigated in terms of lowering mortality and improving growth performance after stress, which supports the role of melatonin as an antioxidant molecule. Melatonin was found to protect yeast cell against H_2O_2 and, for the first time, UV radiation. We have demonstrated the ability of melatonin to modify the gene expression of antioxidant and DNA-repairing genes under stress conditions but, melatonin itself (prior any stress) provokes changes in expression, which seems to prepare cells against upcoming stress. However, other gene expression profiles, plasma membrane transporters of melatonin and endogenous synthesis conditions needs to be investigated further and could reveal other features of this bioactive compound in yeast and shed some light on biological importance of melatonin in yeasts.

Melatonin synthesis in *S. cerevisiae*

The biosynthetic pathway of the synthesis of melatonin from tryptophan is still unknown in yeast. In animals and specially in plants, more than one pathway can be involved in the conversion of tryptophan to this indoleamine and the genes or enzymes involved are well characterized. Therefore, several metabolites are connected with melatonin biosynthetic pathways such as tryptophan, 5-hydroxytryptophan, tryptamine, serotonin, *N*-acetylserotonin and 5-methoxytryptamine. Considering all these aspects, our first objective was to follow the bioconversion pathway of tryptophan into melatonin in yeast by pulsing pathway intermediates in cells at different growth stages and analyzing intracellular and extracellular samples for the presence of all the indolic compounds (Chapter 3). Besides, we aimed to identify and to study possible ortholog genes in yeast involved in melatonin synthesis (Chapter 4).

From the bioconversion experiments conducted in Chapter 3, we found that the highest amount of serotonin quantified was after tryptamine supplementation. Tryptamine was formed mainly from tryptophan but also, to a lesser extent, from 5-hydroxytryptophan. On the other hand, 5-hydroxytryptophan supplementation resulted in the synthesis of serotonin and *N*-acetylserotonin. However, the hydroxylation of tryptophan to 5-hydroxytryptophan does not seem to occurs in yeasts, despite their ability to transform this molecule into serotonin when it is present in the medium. In agreement with that, Germann et al., (2016), had to heterologously insert the enzyme responsible for hydroxylation of tryptophan into 5-hydroxytryptophan in yeast. Furthermore, the authors had to insert the biosynthetic and regenerative pathway of BH4, the main cofactor needed for the hydroxylase activity, since the genes involved are not present in yeast. Additionally, a reversible steps conversion from serotonin into 5-hydroxytryptophan were observed in our experiments. With these observations, we hypothesized that the first part of the melatonin synthesis pathway in yeast differs from the description for mammals since the first step would consist in the decarboxylation of tryptophan to tryptamine and not to its hydroxylation. Subsequently, tryptamine would be converted to serotonin by hydroxylation.

From serotonin precursor, *N*-acetylserotonin and 5-methoxytryptamine were both possible melatonin intermediates. Although the detected *N*-acetylserotonin levels were higher, 5-methoxytryptamine appeared to be the preferred substrate for producing melatonin. Finally, we detected *N*-acetylserotonin as a melatonin catabolite in yeast for the first time, and 5-methoxytryptamine was also produced from melatonin. Both catabolites were present in significant amounts in the growth medium, which highlight the importance of consider melatonin as an intermediate rather than a final product in the synthesis of indolic compounds in yeasts.

The melatonin production that we observed for all the conditions tested from 5-methoxytryptamine as a substrate, together with the production of tryptamine from tryptophan, strengthen the hypothesis that the predominant biosynthetic pathway in yeast could be more similar to that of plants than mammals (Tan et al. 2016). Also, higher tryptamine formation from tryptophan in several yeast

strains belonging to *Saccharomyces* and non-*Saccharomyces* has been reported by Fernandez-Cruz et al., (2019). Furthermore, the high levels of 5-methoxytryptamine accumulation observed in the pioneering study by Sprenger et al. (1999) adds additional support to this idea. Another fact that supported this hypothesis was that Ganguly et al. (2001) reported a greater catalytic efficacy of the acetyltransferase *PAA1* towards 5-methoxytryptamine than for serotonin (Ganguly et al. 2001). This led to the suggestion that *PAA1* could be the final enzyme in the melatonin biosynthetic pathway using 5-methoxytryptamine as the substrate.

We further performed an *in silico* analysis, by using BLAST, with the protein sequences of plant and animal enzymes involved in melatonin pathway, to select *S. cerevisiae* genes as possible orthologs. Among the genes obtained, *PAA1* was selected as the possible ortholog of *AANAT*, as was previously reported by Ganguly et al. (2001). However, we observed that the absence or overexpression of this enzyme in yeast did not produce significant differences in the production of melatonin from the 5-methoxytryptamine. Conversely, when *PAA1* was overexpressed in *E. coli*, it did show activity towards the different arylalkylamines (tryptamine, serotonin and 5-methoxytryptamine). Liu et al. (2005) already indicated that arylalkylamines were unlikely *in vivo* subtrates of *PAA1*, and that the activity previously described through *in vitro* characterization was probably due to the similarity of the ethyl amine moiety present on arylalkylamines to the propyl and butyl amines present on polyamines. Thus, they indicated that polyamines and more specifically spermine, was the actual substrate for *PAA1*. Taking in account these and our results, we conclude that *PAA1* seems not to be involved, or at least not an exclusive enzyme, in the synthesis of melatonin, but that there must be other *N*-acetyltransferases in yeasts capable of acetylating 5-methoxytryptamine and serotonin. Phylogenetic analysis revealed that *PAA1* had closer relationship with the SNATs in vertebrates, rather than to the lineage of plants. However, apple SNAT isoform, SNAT5 showed a closer relationship with the animals SNATs (Wang et al. 2017) and with yeast SNAT than that of plants. This SNAT isoform is localized in mitochondria (Wang et al. 2017), and it can be considered for further searches of putative SNATs on yeast.

Of the rest of the selected yeast genes *in silico*, none showed the expected activity (aromatic hydroxylation, decarboxylation, *O*-methylation) in the overexpression tests in *E. coli* or *S. cerevisiae*. Ganguly et al. (2001) suggested that melatonin synthesis in yeast may reflect the opportunistic action of metabolic enzymes unrelated to broad functions. We also think that the production of melatonin by yeast may not be done by dedicated biosynthetic melatonin enzymes, but by substrate-promiscuous enzymes that are involved in this process in certain circumstances. Regarding to the method used for selection of putative orthologs, inferring functional similarity based solely on significant local similarity is less reliable that inferences based om global similarity and conserved active site residues (Pearson 2013). Additional searches for possible candidate orthologs based on structural homology and protein-docking models with different substrates could help identify other possible acetyl transferases as well as the other melatonin-involved enzymes in yeast. This is specially recommended when the pairwise sequence identity between two proteins is 25 ~ 30% identity, what is known as the “twilight zone” (Rost 1999).

On the other hand, we realized that important bottlenecks existed since the supplementation of a particular metabolite as a substrate did not lead to the synthesis of all subsequent metabolites. Further studies including time-sampling points more frequently would be needed in order to clarify the conversion fate of the different metabolites. Nevertheless, this strategy meets practical limitations which can be addressed by using continuous metabolite monitoring strategies like *in vivo* fluorescent biosensor systems. Also, fluctuations inherent to population-level measurements may mask bioconversion of metabolites that occur at different times or different paces between individuals. Morcillo-Parra et al. (2020) partially overcame fluctuations between sampling points by synchronizing cell phases prior to sampling. However, variability in melatonin production was still observed to some extent despite synchronizing almost the total of cells in the culture. Such variability was suggested to occur due to rapid appearance and disappearance of melatonin in the culture media. In this context, new analytical techniques with a sensitivity at the single-cell level and the ability to perform quantitative analyses will be needed. Single-cell metabolite analysis using the microarrays for mass spectrometry (MAMS) platform has been used

with *S. cerevisiae* cells and worked to unravel metabolite-metabolite correlations (Ibáneza et al. 2013).

We cloned and overexpressed genes involved in the various steps of the plant or animal melatonin synthesis pathway and used them as a positive reference control in order to test the putative ortholog yeast genes. Specifically, human *DDC*, rice *T5H*, human *ASMT* and bovine *AANAT* were overexpressed as a positive control in both *E. coli* and *S. cerevisiae*.

By single overexpression of these heterologous genes, we realized the host importance for heterologous protein overexpression. This observation has been highlighted in several works (Bernaudat et al. 2011; Vieira Gomes et al. 2018). Our data revealed differences on the activity for the different genes. For example, the *DDC* overexpressed in *S. cerevisiae* showed much greater activity to their substrates than its overexpression in *E. coli*. In contrast, the heterologous expression of *AANAT* in *E. coli* far outweighed its activity relative to its overexpression in *S. cerevisiae*.

Finally, it is important to highlight that melatonin does not have to be considered a final metabolite since different catabolic pathways have been reported in different organisms (Krotzky and Hardeland 2008; Hardeland 2017c), including yeast (Sprenger, 1999). We observed in our experiments the conversion of melatonin into *N*-acetylserotonin by O-demethylation, which had not been reported in *S. cerevisiae*. Therefore, subsequent studies on the metabolism of yeast melatonin should include catabolites downstream of melatonin synthesis such as 6-hydroxymelatonin, 2-hydroxymelatonin, AFMK, AMK, etc., for a better understanding of this metabolic pathway.

To sum up, we proposed a putative melatonin biosynthetic pathway in yeast based on the bioconversion of several intermediates of the pathway in different cellular growth phases. The data generated suggests that yeast may utilize more than one pathway to synthesis melatonin and indicates, for the first time, that melatonin synthesis in yeast could be more similar to plants than vertebrates. We found that 5-methoxytryptamine pulse resulted in melatonin consistent production and that *PAA1* is not an exclusive enzyme in the synthesis of melatonin from this

substrate. Additionally, we think that it is probable that there are not specific biosynthetic enzymes for melatonin in *S. cerevisiae* and that the synthesis of this molecule could be due to the action of nonspecific enzymes with broad substrate specificity.

Production of other bioactive compounds derived from aromatic amino acid metabolism in yeast

Tyrosol is produced by yeast during AF in large quantities from the catabolism of aromatic amino acid tyrosine. On the other hand, hydroxytyrosol is a high-value-added compound, formed by tyrosol hydroxylation, which has been recently connected with yeast metabolism (Romboli et al. 2015; Álvarez-Fernández et al. 2018; Rebollo-Romero et al. 2020).

Both compounds are considered bioactive compounds (Fernández-Mar et al. 2012), with antioxidant, antimicrobial, free-radical scavenging, cardioprotective and neuroprotective activities (Thirunavukkarasu et al. 2008; Fernández-Mar et al. 2012; Hornedo-Ortega et al. 2018a). Conversely to melatonin, the pattern of hydroxytyrosol synthesis during AF is more reproducible and consistent (Rebollo-Romero et al. 2020).

There are several methods for obtaining hydroxytyrosol, as is reviewed in the introduction of the present thesis, including chemical synthesis and biotechnological approaches. Since chemical synthesis is not suitable for large-scale industrial production due to expensive substrate, harsh conditions, complicated steps or low yield (Li et al. 2019), biotechnology offers an interesting alternative to produce hydroxytyrosol. Whole cell biosynthesis of hydroxytyrosol have been achieved with different bacterial microorganisms (Liebgott et al. 2007; Bouallagui and Sayadi 2018; Hassing et al. 2019; Li et al. 2019; Yao et al. 2020).

S. cerevisiae can be an interesting candidate for hydroxytyrosol overproduction since it produced endogenous tyrosol and through rational metabolic engineering, tyrosine and tyrosol yields from glucose have been significant improved (Gold et al. 2015; Gottardi et al. 2017; Averesch and Krömer 2018; Guo et al. 2019). With this premise, we seek to produce high levels of hydroxytyrosol in *S. cerevisiae* (Chapter 5). We successfully engineered a yeast strain capable

of producing huge levels of hydroxytyrosol from tyrosol and tyrosine provided in the culture media by overexpressing *hpaB* and *hpaC* genes from *E. coli*.

Remarkably, the control BY4743 strain was natively able to produce extracellular hydroxytyrosol in these conditions, evidencing the ability of *S. cerevisiae* to hydroxylate tyrosol into hydroxytyrosol. However, the unknown hydroxylase which converts tyrosol into hydroxytyrosol was not as efficient as the alcohol dehydrogenase (ADH6) that converts *p*-hydroxyphenylacetaldehyde to tyrosol. A key point for the improvement of natively hydroxytyrosol production will be to find out the gene encoding this hydroxylase activity. Thus, this is an important task for future works.

Tyrosine and tyrosol were used for hydroxytyrosol production, being tyrosol the preferred starting material, however the price of tyrosol and tyrosine is around 6250 and 600 times higher than that of glucose, respectively. Therefore, glucose is a more appropriate source to increase the endogenous levels of tyrosine and tyrosol, via the central yeast metabolic pathway, and finally hydroxytyrosol production. Thus, we next proceeded to engineer a tyrosol-overproducing yeast strain in order to overproduce hydroxytyrosol from glucose.

Since the use of plasmids that require maintenance of the selective pressure is not desired, we proceed to integrate the *hpaB* and *hpaC* genes into the *S. cerevisiae* by using an integrative vector that causes multiple integration at sites sharing homology with Ty1. Yeast Ty retrotransposon sites are a family of transposable elements that are dispersed throughout the genome in high numbers (Cameron et al. 1979; Kazazian 2004). The strain selected with multiple integrations of HpaBC in the Ty sites showed better tyrosol hydroxylation capacity than that the strain overexpressing plasmids.

After obtaining this HpaBC overexpressing strain, we were focused on the tyrosol increase, the preferred substrate for hydroxytyrosol synthesis. In previous studies *ARO10* from *S. cerevisiae* was overexpressed to increase tyrosol levels in *E. coli* (Chung et al. 2017; Xue et al. 2017b). Moreover, overexpression of genes of the shikimate pathway including *ARO3, ARO4* and *ARO7*; and the elimination of the feedback inhibition by overexpressing the feedback insensitive forms *ARO3*^{K222L},

ARO4^{K229L} and *ARO7*^{G141S} has been previously implemented for the successfully increase in the production of various compounds deriving from the aromatic amino acid biosynthetic pathway (Curran et al. 2013; McKenna et al. 2014; Deloache et al. 2015; Williams et al. 2015; Gold et al. 2015; Rodriguez et al. 2015; Suástegui et al. 2017; Gottardi et al. 2017; Mao et al. 2017; Jiang et al. 2018; Reifenrath and Boles 2018; Hassing et al. 2019).

Thus, we analyzed the effect of the individual overexpression of either the wild-type or feedback insensitive alleles of *ARO3*, *ARO4* and *ARO7* in our HpaBC integrated strain. Among all the modifications carried out, the overexpression of the tyrosine insensitive *ARO4* allele (*ARO4*^{K229L}) was by far the greatest producer. This strain has shown the highest amount of hydroxytyrosol produced from glucose by using a metabolically engineered yeast and establish the basis for its upgrading in the future. However, we consider that the potential of hydroxytyrosol yield by this strain has not been reached yet and further modification can be implemented to improve this production. Growth-limiting issues, as the ones observed as consequence of HpaBC overexpression, could be solved by decoupling the growth phase from the production phase (David et al. 2016; Maury et al. 2018). The optimization of culturing conditions and the study of the best production system, such as the use of fed-batch in a bio-reactor, could be interesting strategies for decoupling the production and growth phases and to increase hydroxytyrosol levels.

Concluding remarks and future perspectives

In this thesis work, the main goal was to study the molecular and physiological mechanisms involved in the production of bioactive compounds derived from the aromatic amino acid metabolism. We have mainly focused in the production of melatonin and hydroxytyrosol, two metabolites derived from the metabolism of tryptophan and tyrosine in *S. cerevisiae*. It is important to note that melatonin synthesis by yeast was framed in a context of poor bibliographic background on the subject. Despite the increase in the number of research groups starting to study the melatonin production by yeast in the recent years, the biosynthetic pathway and its physiological role remains unclear. Thus, we wanted to shed light on both processes. In this way by different experiments, we demonstrated for the

first time a protective role of intracellular melatonin against UV light radiation. We also support the role of melatonin as an antioxidant molecule in yeast, being capable of modulating the expression of related genes in the antioxidant response. Even so, in the near future it should be examined in more detail whether this protective function is the consequence of the direct antioxidant and photoprotective properties of the molecule, or whether it is due to its signaling action triggering a molecular and physiological response to cope with these situations of stress and to regulate cell growth.

Furthermore, we realized that the biosynthetic melatonin pathway was more similar with plants than with animals and we hypothesized the possible metabolic pathway that occurs in *S. cerevisiae*. Also, we found that melatonin in yeast was reproducibly synthesized from spiked 5-methoxytryptamine in the culture medium. However, physiological conditions that stimulate consistent melatonin production in yeast remain unanswered. In addition, there appear to be bottlenecks or regulation based on metabolite concentration, as supplementation with a metabolite as a precursor did not always lead to subsequent metabolite synthesis and, in some cases, even a reverse step in the pathway was observed.

It is important to note that in future studies we should focus on melatonin catabolites in our analyses, which could help explain the pattern of rapid synthesis and disappearance of this molecule and to identify the predominant catabolic pathways present in S*. cerevisiae.*

Unfortunately, the identification of putative orthologous genes involved in the synthesis of melatonin in yeast, by *in silico* searches, was not successful since none of the genes selected by sequence similarity was found to exert the expected activity. New searches should be carried out but based on models of structural homology and protein-docking with different substrates to identify the most important protein domains involved in the activity of interest. This approach could help to identify other possible enzymes involved in melatonin synthesis in yeast. Once identified, the selected genes can be tested using mutant knockout and overexpressing strains, and ultimately enzyme purification and *in vitro* characterization. Nonetheless we cannot rule out that melatonin could be a by-

product of unrelated metabolic activities produced by the opportunistic action of metabolic enzymes with broad functions.

With regard to hydroxytyrosol, to date, none physiological role has been ascribed to the synthesis of hydroxytyrosol in yeast. We have designed a yeast strain to overproduce hydroxytyrosol, a high-added-value phenolic compound, from glucose. This overproducing strain opens future approaches to study the possible role of hydroxytyrosol in yeast by phenotyping this strain against different types of stresses (oxidative, osmotic and temperature), as well as its capability in controlling bacterial populations, or its capacity as a potent antioxidant in wine that could help to reduce SO_2 levels during winemaking.

The possibility of carrying out extensive screenings of different strains could identify those that are more endogenously overproducers. The selection of these strains and the use of techniques that are not considered GMOs, such as adaptive evolution or artificial hybridization, are interesting strategies to explore in the future for obtaining non-GMO strains, which can be used in the production of fermentation beverages with a higher added-value by the presence of bioactive molecules.

Finally, we must bear in mind that the bioactive molecules detected in wine may have a non-exclusive origin from yeasts and may also be due to the metabolic cooperation between yeast and bacteria. Thus, the microbial interactions between these organisms could be responsible for the final metabolic and analytical profile of the wine. So, in the future, the study and identification of the molecular and microbial interactions, which determine the final dynamics of the microbial population and how these interactions affect the final metabolic profile, could be a tool for the design of a strain/species consortium of yeast and bacteria, with the highest conversion rate of different metabolites into bioactive compounds in fermented beverages or products.

CONCLUSIONS

The main conclusions drawn from the results obtained in this thesis are:

1. Melatonin can be measured directly from yeast cells by the adapted electrochemical method, avoiding intracellular extraction, which allows monitoring the melatonin biosynthesis of different yeast strains.

2. *S. cerevisiae* can uptake melatonin from the medium in a concentration-dependent manner and its presence can modify:

    a) the growth performance mainly by modulation of the lag phase

    b) the intracellular levels of ROS

    c) the transcriptional activity of the genes involved in response to oxidative stress and UV damage

3. Melatonin exerts protection against UV radiation by lowering mortality and improving growth performance after stress in yeast.

4. Melatonin confers yeast cells protection against oxidative stress by preparing cells to better endure stress situations through early modifying the mRNA levels of antioxidant and photoprotective genes.

5. Serotonin, in yeast, was prevalently formed via tryptophan decarboxylation, followed by tryptamine hydroxylation whereas melatonin production from serotonin can be accomplished by serotonin *N*-acetylation, followed by *N*-acetylserotonin *O*-methylation or, in turn, by *N*-acetylation of 5-methoxytryptamine. The classical pathway for vertebrate melatonin synthesis does not seems to be prevalent in yeast.

6. Melatonin is produced consistently by the addition of 5-methoxytryptamine in the medium. On the contrary, tryptophan, 5-hydroxytryptophan, serotonin and *N*-acetylserotonin additions did not always led to production of melatonin in a reproducible way.

7. Gene overexpression systems in *E. coli* and *S. cerevisiae* resulted in a suitable method to test candidate genes' function *in vivo* despite we were not able to identify any gene directly related with the melatonin biosynthesis pathway.

8. *PAA1,* is not an exclusive enzyme in the acetylation of arylalkylamines, including serotonin, tryptamine and 5-methoxytryptamine, in *S. cerevisiae*. There must be other *N*-acetyltransferases in yeast capable of acetylating serotonin and 5-methoxytryptamine.

9. *S. cerevisiae* has the native capacity to hydroxylate endogenous tyrosol into hydroxytyrosol. However, the gene encoding this activity is still unknown in this yeast.

10. The combination of the co-overexpression of HpaBC complex from *E. coli* and the yeast $ARO4^{K229L}$ allele in *S. cerevisiae* produced a high impact on hydroxytyrosol production.

11. The metabolically engineered strain for hydroxytyrosol production represents a landmark in the use of *S. cerevisiae* as a cell factory for the production of high added-value metabolites.

RESUMEN EN ESPAÑOL

Saccharomyces cerevisiae, comúnmente conocida como la levadura del vino o la cerveza, es un organismo eucariota unicelular que pertenece al reino de los hongos y al filo Ascomycota (debido a la formación de ascosporas durante la esporulación) y que se reproduce principalmente asexualmente por gemación.

Dada su arquitectura eucariota, la levadura tiene varios atractivos como organismo experimental. Por un lado, se utiliza como organismo modelo para obtener conocimientos adicionales sobre las células humanas. Esto puede ser posible debido al alto grado de conservación de muchos procesos celulares clave entre la levadura y nuestras células (Nielsen 2019). En cuanto a los genes de levadura, casi la mitad (47%) de ellos pueden ser reemplazados con éxito por sus ortólogos humanos (Kachroo et al. 2015). La autofagia, la translocación y secreción de proteínas, así como degradación de proteínas asociadas con el retículo endoplásmico, funciones celulares mediadas por actina, choque térmico y plegamiento de proteínas y funciones de chaperona son algunos ejemplos de procesos realmente adecuados para su estudio en levaduras (Nielsen 2019; Akram et al. 2020). Por otro lado, *S. cerevisiae* puede ser útil para descubrir efectos diana de compuestos específicos necesarios para el descubrimiento y desarrollo de fármacos. Aunque *S. cerevisiae* no es un patógeno amenazante, se ha utilizado como modelo para la patogénesis fúngica, así como un valioso banco de pruebas para desarrollar tratamientos (Goldstein y McCusker 2001; Parsons et al. 2006; Hanson 2018).

También *S. cerevisiae* es una opción popular para ser utilizada como "factoría celular". Esto se debe a su rápido crecimiento, que contribuye a su rentabilidad y facilidad de uso, junto con una gran cantidad de investigaciones previas sobre su fisiología y metabolismo. También su excelente maleabilidad genética se ve facilitada en gran medida por una preferencia conveniente por la recombinación homóloga (HR) sobre la no homologa o de unión de extremos no homólogos (NHEJ) para la reparación de rotura bicatenaria (DSB), que ha permitido la inserción específica de material genético externo en el sitio deseado y diferentes ediciones genómicas (Nielsen et al.2013; Hanson 2018). Además, en comparación con *E. coli*, *S. cerevisiae* tiene la designación generalmente reconocida como segura (GRAS) de la Administración de Alimentos y Medicamentos de los EE. UU. (FDA) y es microorganismo robusto y libre de

endotoxinas (Guo et al.2019). Todas estas ventajas han llevado al uso de levaduras para la producción de multitud de compuestos como alcoholes de segunda generación, químicos aromáticos, enzimas, ácidos grasos, aminoácidos y compuestos derivados, entre otros, utilizando materias primas renovables en la fermentación (Hansen et al. 2009; Nandy y Srivastava 2018; Yu et al.2018; Cordente et al.2019; Hu et al.2019; Levisson et al.2019; Nielsen 2019).

Por otra parte, quizás una de las utilidades industriales más importantes de esta levadura es la producción de cerveza, pan y vino a través de la fermentación alcohólica. Durante la fermentación alcohólica los azúcares de la masa o el mosto (esencialmente glucosa, fructosa, sacarosa o maltosa) son metabolizados para generar dióxido de carbono y etanol. Una característica fisiológica fundamental de *S. cerevisiae* es su capacidad para fermentar azúcares tanto en condiciones anaeróbicas como aeróbicas (Piškur et al. 2006). En condiciones aeróbicas, la respiración es posible con O_2 como aceptor de electrones final, pero *S. cerevisiae* es capaz de realizar la fermentación alcohólica hasta que el azúcar se agota del medio. Este comportamiento se conoce como el efecto Crabtree (Crabtree 1928; De Deken 1966) y las levaduras que expresan este rasgo se denominan levaduras Crabtree-positivas. Debido a este efecto*, S. cerevisiae* producirá etanol en condiciones aerobias y con una gran concentración de glucosa externa en lugar de producir biomasa mediante el ciclo de Krebs, proceso que ocurre mayoritariamente en las levaduras Crabtree-negativas. Se cree que este fenómeno se desarrolló como mecanismo competitivo con el fin de inhibir el crecimiento de otros microorganismos debido a la toxicidad del etanol producido.

En cuanto a la composición del vino, las concentraciones medias de los principales componentes 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). Por tanto, el etanol, junto con otros metabolitos de la glucólisis, como el glicerol y los ácidos orgánicos, son cuantitativamente los principales componentes del vino que se producen durante la fermentación alcohólica. Sin embargo, la mejora de las técnicas analíticas ha permitido a la comunidad científica identificar moléculas en concentraciones más

bajas. De hecho, el vino debe considerarse más bien como una mezcla compleja de más de cientos de moléculas presentes en diferentes concentraciones (Nisbet et al. 2014). Parte de estas moléculas pueden proceder de uva, pero sin lugar a duda la actividad de los microorganismos durante la fermentación del mosto resulta relevante en la aportación y/o conversión de las moléculas que forman parte del vino final. Por tanto, el vino es una bebida fermentada que, a pesar de la presencia de etanol, un metabolito tóxico para la salud, contiene metabolitos secundarios, algunos de ellos en pequeñas concentraciones, que pueden determinar la calidad, la estabilidad y la bioactividad final de este producto.

En vinos, la investigación sobre compuestos bioactivos y promovedores de la salud durante años se ha centrado principalmente en los polifenoles, presentes de forma natural en las uvas. Principalmente los flavonoides (como antocianinas y flavan-3-oles) y no flavonoides (principalmente estilbenoides y ácidos fenólicos), han sido los más estudiado debido a su excelente potencial antioxidante (Zamora 2009; Iriti and Varoni 2014). Sin embargo, algunos estudios recientes se han centrado en los compuestos producidos a partir del metabolismo de los aminoácidos aromáticos. Entre algunas de estas moléculas bioactivas destacan las de carácter indólico, derivadas del metabolismo del triptófano, como la melatonina, la serotonina, la *N*-acetilserotonina o el ácido 3-indolacético (Rodriguez-Naranjo et al., 2011a; Fernández-Cruz et al., 2016), así como los llamados alcoholes superiores derivados de aminoácidos aromáticos, como el triptófol, el 2-fenil etanol, el tirosol y el hidroxitirosol. La presencia de estos compuestos en los vinos se ha relacionado con el metabolismo de la levadura durante la fermentación alcohólica (Rodríguez-Naranjo et al., 2011b; Álvarez-Fernández et al., 2019a) y se ha visto que puede contribuir tanto a la calidad y estabilidad del vino final (Cordente et al., 2019) como a la salud del consumidor (Vilela 2019).

La melatonina originalmente fue considerada como un producto único de la glándula pineal de vertebrados, sin embargo, ha sido identificada su presencia en una amplia gama de invertebrados, plantas, bacterias y hongos en las últimas dos décadas.

La síntesis de melatonina por *S. cerevisiae* en condiciones de laboratorio fue demostrada por primera vez por Sprenger et al. (1999). El hecho de que *S.*

cerevisiae sea el principal responsable de la fermentación alcohólica podría implicar un papel principal de la levadura en la síntesis de melatonina en condiciones de fermentación alcohólica. Esta hipótesis fue demostrada de manera inequívoca por Rodríguez-Naranjo et al. (2011). Estos autores realizaron el proceso de vinificación y monitorearon la síntesis de melatonina durante la fermentación alcohólica y observaron la producción de melatonina a partir de mostos que inicialmente carecían de esta molécula, antes de convertirse en vino. Posteriormente, evaluaron la capacidad de producción de melatonina en un medio sintético y observaron que la fase de crecimiento de la levadura y la composición del medio, especialmente las concentraciones de triptófano y azúcares reductores, afectaban la síntesis de esta molécula de diferente manera dependiendo de la cepa de levadura (Rodríguez-Naranjo et al. 2012). En otro estudio muy reciente, se evaluaron distintos parámetros de fermentación (incluyendo la concentración de azúcar y nitrógeno, la temperatura de fermentación o la población inicial) en cuanto a su efecto en la síntesis de melatonina (Morcillo-Parra et al. 2020a). Lo que los autores vieron fue que el contenido de azúcar y la baja temperatura (12ºC) fueron los parámetros que incidieron en el perfil de producción de melatonina intracelular, siendo la baja temperatura la condición que condujo a la mayor producción. En la última década, diferentes estudios se han centrado en demostrar la producción de melatonina tanto por *Saccharomyces* como por no *Saccharomyces* en diferentes medios (Vigentini et al.2015; Fernández-Cruz et al. 2016, 2017, 2018, 2019; Fernández-Cruz 2018; Morcillo-Parra, 2019; Motlhalamme, 2020).

Sin embargo, todavía se desconoce mucha información con respecto a la síntesis de estas moléculas bioactivas por la levadura porque es un tema de estudio muy reciente. En este contexto, el objetivo principal de este trabajo de tesis ha sido **estudiar los mecanismos moleculares y fisiológicos implicados en la producción de compuestos bioactivos derivados del metabolismo de los aminoácidos aromáticos, principalmente compuestos derivados del triptófano como la melatonina y la serotonina, en *S. cerevisiae*.** Para ello, este objetivo general se dividió en los siguientes objetivos específicos:

Objetivo 1: Adaptar y establecer una técnica sencilla, rápida y de bajo coste para detectar la presencia de melatonina en muestras intracelulares de *S. cerevisiae.*

Dado que la melatonina, así como los compuestos directamente relacionados en las levaduras, son sensibles a los cambios de temperatura y a los agentes oxidantes, las concentraciones de estas moléculas pueden variar durante su determinación. El método de análisis de melatonina validado es la cromatografía líquida de alta resolución acoplada a la espectrometría de masas (HPLC-MS/MS). Sin embargo, se requieren costosos procesos de limpieza de la muestra (como la extracción en fase sólida), así como la disponibilidad del equipo, lo que encarece el análisis. Establecimos una técnica rápida y simple basada en un método electroanalítico para monitorear rápidamente la melatonina y los metabolitos derivados del triptófano relacionados (como el ácido 3-indolacético y el triptofol) usando la variación de las respuestas electroquímicas de extractos tomados directamente de células de levadura secas (y evitando extracción de contenido intracelular). Este método se basa en el hecho de que estos compuestos indólicos se oxidan electroquímicamente, lo que impulsa su determinación mediante técnicas voltamétricas. Una vez adaptada la técnica, obtuvimos las respuestas de voltamperometría en varias cepas industriales de *Saccharomyces* enriquecidas con triptófano (QA23, BMV58, AROMA WHITE). Para la cepa vínica QA23, también se monitorizó la producción de melatonina a partir de los sustratos 5-hidroxitriptófano y serotonina. Finalmente, comparamos la síntesis de estos compuestos en dos cepas mutantes con deleción en dos genes clave del metabolismo del triptófano (ARO10 y TRP1) en relación con su cepa silvestre BY4743, cuando se añadió triptófano. Finalmente, se compararon las variaciones en la intensidad relativa de los picos del ánodo correspondientes a la oxidación de las moléculas indólicas con el método HPLC-MS/MS.

Objetivo 2: Determinar el efecto de la melatonina sobre la levadura *S. cerevisiae* como antioxidante y protector UV.

En humanos, la melatonina es una hormona multifuncional que modula distintos procesos fisiológicos, como los ritmos circadianos y las funciones reproductivas, y que también actúa como molécula antioxidante. Un gran número de estudios han demostrado que la melatonina juega un papel adicional en el envejecimiento

celular y en enfermedades relacionadas con la edad, antiinflamatorias e inmunorreguladoras, así como anticancerígeno para algunos tipos de cáncer (Mediavilla et al.2011; Escrivá et al.2016; Fathizadeh et al.2019). Recientemente, se ha sugerido que la melatonina puede ser una terapia complementaria o incluso regular para infecciones virales mortales como SARS, MERS, COVID-19, Ébola y gripe aviar) debido a la capacidad de controlar la respuesta inmune innata y reducir la inflamación derivada de la infección (Tan et al. 2014a; Anderson et al. 2015; Bahrampour Juybari et al. 2020; Reiter et al. 2020; Tan y Hardeland 2020).

De forma similar en plantas, se han descrito múltiples roles reguladores fisiológicos para la melatonina. Además, en muchas especies de plantas, la melatonina se ha visto que confiere una mayor tolerancia durante sus respuestas a estímulos ambientales como la sal, la sequía, las temperaturas extremas, los metales pesados o el daño de los rayos UV.

El posible efecto que ejerce la melatonina en levaduras se estudió en la cepa de laboratorio BY4743 en presencia y ausencia de H_2O_2 y radiación UV (ambos descritos como generadores de especies reactivas del oxígeno (ROS) (Farrugia et al. 2012) . Para ello, primero evaluamos distintas concentraciones de melatonina (0.05, 0.1 y 20 mM) y su captación intracelular, para establecer una dosis adecuada para ensayos posteriores. Nuestros resultados mostraron una captación dependiente de dosis, y seleccionamos 0.1 mM (equivalente a 66.23 ng/10^8 células) debido a que fue la dosis que ejerció tanto una mejora en el crecimiento, principalmente debido a un acortamiento de la fase de latencia, como una disminución de los niveles de ROS intracelulares. Por el contrario, los cultivos tratados con 0.05 mM no mostraron diferencias para estos parámetros y 20 mM incluso produjo una disminución del crecimiento, sugiriendo que a concentraciones elevadas la melatonina puede llegar a ejercer un efecto prooxidante.

A continuación, se determinó el crecimiento y la viabilidad de la levadura después de la exposición a los diferentes estreses (H_2O_2 y UV), tanto en células enriquecidas con melatonina como en las no enriquecidas (control). Nuestros resultados mostraron que la melatonina intracelular ejerció protección frente a ambos estreses, reduciendo marcadamente la mortalidad celular después de la

exposición a la radiación. Finalmente, se realizaron análisis transcripcionales por PCR en tiempo real (qPCR) para genes, citosólicos y mitocondriales, involucrados en la defensa antioxidante para las células tratadas con melatonina, y tratadas y sin tratar con H_2O_2 y luz UV. El primer efecto notable que observamos fue que la presencia de melatonina intracelular modificó la actividad transcripcional de los genes de respuesta a estrés sin haber un estrés presente. Cuando las células enriquecidas en melatonina se sometieron a la situación de estrés, los cambios transcripcionales mostraron una mayor amortiguación que en las células sin melatonina. Estos resultados parecen indicar que de alguna forma la melatonina prepara a las células para soportar mejor las situaciones de estrés mediante la modificación temprana de los niveles de ARNm de estos genes antioxidantes y fotoprotectores. Resultados similares fueron descritos por Vázquez et al. (2017), en los que la presencia de melatonina externa en el medio indujo la expresión de varios genes antioxidantes. Sin embargo, no observamos una inducción temprana después de la exposición al estrés de todos los genes protectores que estudiamos. De hecho, observamos una disminución de expresión de los genes que codifican las enzimas mitocondriales, con la excepción del gen *TRX3*, mientras que la expresión de otros genes antioxidantes citosólicos es inducida al mismo tiempo. Por otra parte, se ha observado como un efecto transitorio en las primeras etapas de la respuesta al estrés para otros oxidantes como el hidroperóxido de cumeno (Sha et al., 2013). Por último, dado que hemos estudiado la respuesta transcripcional temprana, probablemente, estudios adicionales sobre las expresiones de genes que cubran un rango de tiempo más amplio revelen la inducción global de las enzimas redox y de eliminación de ROS.

Objetivo 3: Desvelar la vía biosintética de la melatonina en *S. cerevisiae*

El primer trabajo relacionado con la biosíntesis de melatonina en levaduras, llevado a cabo por Sprenger et al. (1999) demostró la capacidad de producir melatonina a partir del triptófano, así como otros sustratos potenciales (también presentes en las vías de la melatonina de animales y plantas), en células cultivadas en un medio de ayuno. Tras este trabajo, ningún estudio posterior se ha centrado en estudiar la ruta de síntesis de melatonina en levadura. Nuestro objetivo fue por tanto proporcionar nuevos datos empíricos sobre la producción

de melatonina en levaduras siguiendo, pero ampliando, este trabajo anterior para aclarar los pasos involucrados en la vía biosintética de la melatonina. Para ello, se evaluó los productos generados a partir de diferentes sustratos de la ruta (L-triptófano, 5-hidroxitriptófano, serotonina, *N*-acetilserotonina, triptamina y 5-metoxitriptamina), en la cepa de levadura vínica QA23. Este estudio se realizó utilizando distintos medios y diferentes fases de crecimiento. Más concretamente se analizó la bioconversión de sustrato utilizándose un medio salino (SLT) (en el que las células se encuentran en una fase de ayuno), un medio pobre (SD) (en el que las células se encuentran en fase de crecimiento exponencial) y por último en un mosto sintético con una concentración de nitrógeno de 100 mg/L procedente del NH_4Cl (SM) (en el que las células fueron crecidas en cultivo continuo). En todos los casos, tras 15 minutos tras la adición del compuesto indólico de interés como sustrato se tomó muestra del contenido intracelular y extracelular y se determinaron los metabolitos por HPLC-MS/MS.

La ruta de síntesis de melatonina descrita para vertebrados y plantas podemos dividirla en dos partes, siendo la primera la que transcurre en la producción de serotonina desde el triptófano, y la segunda en la conversión de la serotonina en melatonina.

En cuanto a los resultados más relevantes obtenidos de estos experimentos, lo primero que vimos es que, la conversión de triptófano a serotonina, parece no ocurrir como en vertebrados sino como ocurre en plantas. En vertebrados, el primer paso de la ruta de síntesis de melatonina empieza con la hidroxilación del triptófano en 5-hidroxitriptófano por la enzima triptófano hidroxilasa (TPH; EC 1.14.16.4). Sin embargo, en plantas, el triptófano suele ser descarboxilado a triptamina por la enzima triptófano descarboxilasa (AADC; EC 4.1.1.28) en la primera reacción. Cuando añadimos triptófano como precursor, no fuimos capaces de detectar 5-hidroxitriptófano intra- o extracelularmente en ninguno de los medios o condiciones de crecimiento. Sin embargo, sí que detectamos triptamina sobre todo extracelularmente en los medios SD y SLT.

El segundo paso para la síntesis de serotonina en vertebrados consiste en la descarboxilación del 5-hidroxitriptófano en serotonina por la AADC, mientras que en plantas la triptamina es hidroxilada a serotonina por la triptamina hidroxilasa (T5H). En nuestros ensayos fuimos capaces de detectar ambas conversiones

por parte de *S. cerevisiae* de forma más pronunciada en medio SLT. Además, vimos reversibilidad en la conversión de 5-hidroxitriptófano en serotonina, el cual no había sido descrito hasta la fecha. También cabe destacar que la conversión de triptamina en serotonina condujo a la producción adicional de metabolitos posteriores de la ruta como *N*-acetilserotonina y 5-metoxitriptamina.

En la segunda parte de la ruta de síntesis de melatonina, en vertebrados, la serotonina es preferiblemente acetilada en *N*-acetilserotonina por la serotonina *N*-acetiltransferasa (SNAT; EC 2.3.1.87) y posteriormente convertida a melatonina tras la O-metilación llevada a cabo por la *N*-Acetilserotonina *O*-metiltransferasa (ASMT). Sin embargo, en plantas puede ocurrir estas mismas actividades o bien la formación de 5-metoxitriptamina a partir de serotonina, tras la actuación *O*-metiltransferasa de las enzimas *O*-metiltransferasa del ácido cafeico (COMT) o la ASMT y la conversión de la misma en melatonina a través de la SNAT de plantas. Para dilucidar lo que sucede en *S. cerevisiae*, realizamos pulsos con los sustratos serotonina, 5-metoxitriptamina y *N*-acetilserotonina. Nuestros resultados mostraron un aumento de 5-metoxitriptamina en condiciones extracelulares de SLT y SM y solo en condiciones intracelulares para SLT cuando suplementamos los medios con serotonina. Además, la adición de 5-metoxitriptamina como sustrato siempre condujo a la producción de melatonina. Sin embargo, no podemos descartar ninguno de los pasos metabólicos que conllevan a la síntesis de melatonina desde serotonina porque detectamos la producción de melatonina a partir de ambos sustratos (5-metoxitriptamina y *N*-acetilserotonina) en las diferentes condiciones de crecimiento.

Cabe comentar que la mayoría de los metabolitos producidos se detectaron intracelularmente o extracelularmente, pero no siempre se encontró una correlación directa entre la presencia en el medio y en el compartimento celular. Esto podría deberse al hecho de que, en condiciones específicas, ciertos compuestos se liberan al medio o se convierten rápidamente en otros metabolitos.

Todos los resultados de los experimentos de bioconversión realizados nos permitieron reconstruir y plantear una posible ruta de síntesis de melatonina desde el aminoácido inicial triptófano. Con respecto a los genes y enzimas

implicados, se sabe muy poco sobre los genes implicados en la síntesis de melatonina en levaduras. El único gen que se ha propuesto como homólogo de la SNAT de los vertebrados es el *PAA1*. Sin embargo, el papel de *PAA1* en la vía biosintética de la melatonina todavía debe dilucidarse. Trabajos previos indican la posibilidad de que las arilalquilaminas (como la triptamina, la serototonina o la 5-metoxitriptamina) no sean sustratos reales *in vivo* de *PAA1* en *S. cerevisiae*, siendo las aminas biógenas espermina, espermidina o putrescina sus sustratos naturales. En un intento por arrojar luz sobre el papel de *PAA1* en la síntesis de melatonina en levadura, evaluamos las diferencias en los niveles de acetilación para diferentes arilalquilaminas en la cepa de laboratorio BY4743 silvestre, la BY4743 mutante para *PAA1 y* la BY4743 sobreexpresante de *PAA1*. Nuestros resultados no mostraron diferencias en la producción de los distintos productos acetilados e incluso detectamos producción de melatonina por la cepa mutante de *PAA1.* La presencia de otras SNATs en levaduras, capaces de acetilar serotonina y 5-metoxitriptamina, pueden ser las responsables de la acetilación de estos sustratos en la cepa mutante para *PAA1.* Estos resultados sugieren que esta enzima, al menos, no es exclusiva para la síntesis de melatonina.

Con el fin de identificar los genes que responden a la síntesis de melatonina en levaduras, realizamos un análisis BLAST utilizando las secuencias de proteínas de vertebrados y plantas. Los genes de levadura seleccionados como supuestos ortólogos, así como un gen de animales o plantas utilizado como control positivo del sistema, se sobreexpresaron tanto en *S. cerevisiae* como de forma heteróloga en *E. coli.* Ninguno de los genes identificados como posibles ortólogos mostró actividad esperada al sobreexpresarse en ninguno de los dos huespédes, con la excepción de *PAA1* que si mostró curiosamente actividad SNAT en *E. coli* pero no en *S. cerevisiae*. En cuanto a los controles utilizados a partir de los genes de otros organismos, todos mostraron la actividad esperadas con la excepción de T5H en *S. cerevisiae,* probablemente debido al tipo de construcción empleada.

Objetivo 4: Sobreproducción de moléculas bioactivas en *S. cerevisiae* mediante ingeniería genética

El hidroxitirosol se considera uno de los antioxidantes más potentes de la naturaleza. La mayor parte del hidroxitirosol actualmente disponible en el mercado proviene de extractos de hoja de olivo o de residuos de la industria del olivo (alpechín). Sin embargo, el hidroxitirosol extraído en muchas ocasiones resulta con baja pureza. Por el contrario, el hidroxitirosol puro alcanza precios de mercado muy elevados debido a la baja concentración en sus fuentes naturales, los bajos rendimientos de extracción y la dificultad de sintetizar químicamente el hidroxitirosol. Por tanto, es de gran interés producir este compuesto mediante enfoques biotecnológicos, utilizando microorganismos para obtener rendimientos que puedan ser viables para la producción industrial. Como se ha descrito previamente, la levadura *S. cerevisiae* presenta una serie de ventajas respecto a otros microorganismos que la hacen una candidata idónea para convertirla como factoría celular de distintos compuestos.

Por todo ello, decidimos generar una cepa de levadura capaz de producir elevados niveles de hidroxitiriosol. En primer lugar, clonamos dos genes que forman parte del complejo de hidroxilasa HpaBC de *E. coli* (*hpaB* y *hpaC*) para producir un aumento del hidroxitirosol en diferentes medios. La mayor cantidad de hidroxitirosol obtenida tras sobreexpresar dichos genes fue de 4.6 mg/L a las 72 h de crecimiento en un medio mínimo, pero con la adición exógena de 1 mM de tirosol.

Debido a que el tirosol es producido de forma endógena por *S. cerevisiae* a través de la ruta de Ehrlich, y tras determinar la efectividad de HpaBC para convertir tirosol en hidroxitirosol, integramos HpaBC en múltiples copias en el genoma de *S. cerevisiae* y, posteriormente, aumentamos la productividad del hidroxitirosol redirigiendo el flujo metabólico hacia la síntesis de tirosol endógeno. Las modificaciones que probamos consistieron en la sobreexpresión de los genes ARO, (*ARO3*, *ARO4*, *ARO7* y *ARO10*), así como las versiones desreguladas insensibles a la represión catabólica por triptófano, fenilalanina y tirosina, ($ARO3^{K222L}$, $ARO4^{K229L}$ y $ARO7^{G141S}$). La combinación de la integración en el genoma de HpaBC junto con la sobreexpresión de $ARO4^{K229L}$ y la optimización de las condiciones de cultivo, dio como resultado niveles elevados de hidroxitirosol desde glucosa, llegándose a superar los 370 mg/L de

hidroxitirosol. Además de hidroxitirosol, se produjeron también elevados niveles de los alcoholes superiores tirosol, triptofol y 2 feniletanol

Dado que la glucosa es aproximadamente entre 600 y 6000 veces más económica que la tirosina y el tirosol, respectivamente, y al elevado precio del hidroxitirosol puro en el mercado (8000 ~18500 €/g, Sigma), esta cepa podría ser una herramienta biotecnológica muy útil para producir esta molécula de forma segura, simple y disminuyendo los costes. Por ello, se ha depositado ante la Oficina Española de Patentes y Marcas la solicitud Patente de referencia con el número de referencia 202031186.

BIBLIOGRAPHY

Abdo H, Catacchio CR, Ventura M, D'Addabbo P, Calabrese FM, Laurent J, David-Vaizant V, Alexandre H, Guilloux-Bénatier M, Rousseaux S (2020) Colonization of wild *Saccharomyces cerevisiae* strains in a new winery. Beverages 6:9. https://doi.org/10.3390/beverages6010009

Achmon Y, Fishman A (2015) The antioxidant hydroxytyrosol: biotechnological production challenges and opportunities. Appl Microbiol Biotechnol 99:1119–1130. https://doi.org/10.1007/s00253-014-6310-6

Akram Z, Ahmed I, Mack H, Kaur R, Silva RC, Castilho BA, Friant S, Sattlegger E, Munn AL (2020) Yeast as a model to understand actin-mediated cellular functions in mammals—Illustrated with four actin cytoskeleton proteins. Cells 9:672. https://doi.org/10.3390/cells9030672

Alani E, Cao L, Kleckner N (1987) A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116:541–545

Alexandre H, Costello PJ, Remize F, Guzzo J, Guilloux-Benatier M (2004) *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: Current knowledge and perspectives. Int J Food Microbiol 93:141–154. https://doi.org/10.1016/j.ijfoodmicro.2003.10.013

Allouche N, Damak M, Ellouz R, Sayadi S (2004) *Pseudomonas aeruginosa* for synthesis of the antioxidant hydroxytyrosol via conversion of tyrosol. Appl. Environ. Microbiol 70:2105–2109. https://doi.org/10.1128/AEM.70.4.2105

Allouche N, Sayadi S (2005) Synthesis of hydroxytyrosol, 2-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid by differential conversion of tyrosol isomers using *Serratia marcescens* strain. J Agric Food Chem 53:6525–6530. https://doi.org/10.1021/jf050972w

Alonso-del-Real J, Contreras-Ruiz A, Castiglioni GL, Barrio E, Querol M (2017) The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited aeration, inoculum proportions, and sequential inoculation. Front Microbiol 8:2807. https://doi.org/10.3389/fmicb.2017.02087

Alonso-del-Real J, Pérez-Torrado R, Querol A, Barrio E (2019) Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization. Environ Microbiol 21:1627–1644. https://doi.org/10.1111/1462-2920.14536

Alsammar H, Delneri D (2020) An update on the diversity, ecology and biogeography of the *Saccharomyces* genus. FEMS Yeast Res 20: foaa013. https://doi.org/10.1093/femsyr/foaa013

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Álvarez-Diduk R, Galano A, Tan DX, Reiter RJ (2015) *N*-acetylserotonin and 6-hydroxymelatonin against oxidative stress: implications for the overall protection exerted by melatonin. J Phys Chem B 119:8535–8543. https://doi.org/10.1021/acs.jpcb.5b04920

Álvarez-Fernández MA, Fernández-Cruz E, Cantos-Villar E, Troncoso AM, García-Parrilla, MC (2018) Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC–HRMS method. Food Chem 242:345–351. https://doi.org/10.1016/j.foodchem.2017.09.072

Álvarez-Fernández MA, Fernández-Cruz E, Valero E, Troncoso AM, García-Parrilla MC (2019) Efficiency of three intracellular extraction methods in the determination of metabolites related to tryptophan and tyrosine in winemaking yeast's metabolism by LC-HRMS. Food Chem 297: 124924. https://doi.org/10.1016/j.foodchem.2019.05.198

Ancín-Azpilicueta C, González-Marco A, Jiménez-Moreno N (2008) Current knowledge about the presence of amines in wine. Crit Rev Food Sci Nutr 48:257–275

Anderson G, Maes M, Markus RP, Rodriguez M (2015) Ebola virus: melatonin as a readily available treatment option. J Med Virol 87:537–543. https://doi.org/10.1002/jmv

Angerosa F, d'Alessandro N, Konstantinou P, Di Giacinto L (1995) GC-MS evaluation of phenolic compounds in virgin olive oil. J Agric Food Chem 43:1802–1807. https://doi.org/10.1021/jf00055a010

Anisimov VN, Popovich IG, Zabezhinski MA, Anisimov SV, Vesnushkin GM, Vinogradova IA (2006) Melatonin as antioxidant, geroprotector and anticarcinogen. Biochim Biophys Acta - Bioenerg 1757:573–589. https://doi.org/10.1016/j.bbabio.2006.03.012

Anli RE, Bayram M (2009) Biogenic amines in wines. Food Rev Int 25:86–102. https://doi.org/10.1080/87559120802458552

Antolín I, Obst B, Burkhardt S, Hardeland R (1997) Antioxidative protection in a high-melatonin organism: The dinoflagellate *Gonyaulax polyedra* is rescued from lethal oxidative stress by strongly elevated, but physiologically possible concentrations of melatonin. J Pineal Res 23:182–190. https://doi.org/10.1111/j.1600-079X.1997.tb00353.x

Antolín I, Rodríguez C, Sáinz RM, Mayo JC, Uría H, Kotler ML, Rodríguez-Colunga MJ, Tolivia D, Menéndez-Peláez A (1996) Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J 10:882–890. https://doi.org/10.1096/fasebj.10.8.8666165

Aredes-Fernández PA, Rodriguez-Vaquero MJ, Raquel Apud G, Gilda Stivala M (2016) Bioactive compounds in wine. Recent advances and perspectives. Nova science publishers, New York

Arnao MB., Hernández-Ruiz J (2007) Melatonin in plants: more studies are necessary. Plant Signal Behav 2:5, 381–382. https://doi.org/10.4161/psb.2.5.4260

Arnao MB, Hernández-Ruiz J (2015) Functions of melatonin in plants: a review. J Pineal Res 59:133–150. https://doi.org/10.1111/jpi.12253

Arnao MB, Hernández-Ruiz J (2018) Phytomelatonin, natural melatonin from plants as a novel dietary supplement: Sources, activities and world market. J Funct Foods 48:37–42. https://doi.org/10.1016/j.jff.2018.06.023

Arnao MB, Hernández-Ruiz J (2020) Is phytomelatonin a new plant hormone? Agronomy 10:95. https://doi.org/10.3390/agronomy10010095

Arnao MB, Hernández-Ruiz J (2009) Assessment of different sample processing procedures applied to the determination of melatonin in plants. Phytochem Anal 20:14–18. https://doi.org/10.1002/pca.1083

Auesukaree C (2017) Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng 124:133–142. https://doi.org/10.1016/j.jbiosc.2017.03.009

Avbelj M, Zupan J, Kranjc L, Raspor P (2015) Quorum-Sensing kinetics in *Saccharomyces cerevisiae*: A symphony of ARO genes and aromatic alcohols. J Agric Food Chem 63:8544–8550. https://doi.org/10.1021/acs.jafc.5b03400

Averesch NJH, Krömer JO (2018) Metabolic engineering of the shikimate pathway for production of aromatics and derived compounds-Present and future strain construction strategies. Front Bioeng Biotechnol 6:32. https://doi.org/10.3389/fbioe.2018.00032

Axelrod J, Weissbach H (1960) Enzymatic O-Methylation of N-Acetylserotonin to Melatonin. Science (80-) 131:1312. https://doi.org/doi: 10.1126/science.131.3409.1312.

Back K, Tan DX, Reiter RJ (2016) Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. J Pineal Res 61:426–437. https://doi.org/10.1111/jpi.12364

Bagheri B, Bauer FF, Setati ME (2015) The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. South African J Enol Vitic 36:243–251

Bahrampour Juybari K, Pourhanifeh MH, Hosseinzadeh A, Hemati K, Mehrzadi S (2020) Melatonin potentials against viral infections including COVID-19: Current evidence and new finding. Diabetes Metab Syndr 14:337–339

Bakker BM, Bro C, Kotter P, Luttik MA, van Dijken JP, Pronk JT (2000) The mitochondrial alcohol dehydrogenase *ADH3p* is involved in a redox shuttle in *Saccharomyces cerevisiae*. J Bacteriol 182:4730–4737. https://doi.org/10.1128/JB.182.17.4730-4737.2000

Bakker BM, Overkamp KM, Van Maris AJA, Kötter P, Luttik MA, van Dijken JP, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37. https://doi.org/10.1016/S0168-6445(00)00039-5

Ballester-Tomás L, Randez-Gil F, Pérez-Torrado R, Prieto JA (2015) Redox engineering by ectopic expression of glutamate dehydrogenase genes links NADPH availability and NADH oxidation with cold growth in *Saccharomyces cerevisiae*. Microb Cell Fact 14:1–11. https://doi.org/10.1186/s12934-015-0289-2

Ballou DP, Entsch B, Cole LJ (2005) Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases. Biochem Biophys Res Commun 338:590–598. https://doi.org/10.1016/j.bbrc.2005.09.081

Balzer I, Hardeland R (1991a) Photoperiodism and effects of indoleamines in a unicellular alga, *Gonyaulax polyedra*. Science 253:795–797. https://doi.org/10.1126/science.1876838

Balzer J, Hardeland R (1991b) Stimulation of bioluminescence by 5-methoxylated indoleamines in the dinoflagellate*, Gonyaulax polyedra*. Camp Biochem Physiol 98:395–397

Banach AA, Ooi BG (2014) Enhancing the yields of phenolic compounds during fermentation using *Saccharomyces cerevisiae* strain 96581. Food Nutr Sci 5:2063–2070. https://doi.org/10.4236/fns.2014.521218

Bao Z, Xiao H, Liang J, Zhang L, Xiong X, Sun N, Si T, Zhao H (2015) Homology-Integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. ACS Synth Biol 4:585–594. https://doi.org/10.1021/sb500255k

Barbosa C, Mendes-Faia A, Lage P, Mira NP, Mendes-Ferreira A (2015) Genomic expression program of *Saccharomyces cerevisiae* along a mixed-culture wine fermentation with *Hanseniaspora guilliermondii*. Microb Cell Fact 14:1–17. https://doi.org/10.1186/s12934-015-0318-1

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

Barcenilla J, Estrella I, Gómez-Cordovés C, Hernández T, Hernández L(1989) The influence of yeasts on certain non-volatile components of wine. Food Chem 31:177–187. https://doi.org/10.1016/0308-8146(89)90056-3

Bartowsky EJ (2017) *Oenococcus oeni* and the genomic era. FEMS Microbiol Rev 41:S84–S94. https://doi.org/10.1093/femsre/fux034

Beitollahi H, Ghofrani Ivari S, Alizadeh R, Hosseinzadeh R (2015) Preparation, characterization and electrochemical application of ZnO-CuO nanoplates for voltammetric determination of captopril and tryptophan using modified carbon paste electrode. Electroanalysis 27:1742–1749. https://doi.org/10.1002/elan.201500016

Belda I, Ruiz J, Esteban-Fernández A, Navascués E, Marquina D, Santos A, Moreno-Arribas V (2017) Microbial contribution to wine aroma and its intended use for wine quality improvement. Molecules 22:1-29. https://doi.org/10.3390/molecules22020189

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

Belloch C, Orlic S, Barrio E, Querol A (2008) Fermentative stress adaptation of hybrids within the *Saccharomyces* sensu stricto complex. Int J Food Microbiol 122:188–195. https://doi.org/10.1016/j.ijfoodmicro.2007.11.083

Bellut K, Arendt EK (2019) Chance and challenge: Non-*Saccharomyces* yeasts in nonalcoholic and low alcohol beer brewing–A review. J Am Soc Brew Chem 77:77–91

Beltagi AM, Khashaba PY, Ghoneim MM (2003) Determination of melatonin hormone in bulk form, tablets and human serum by square-wave cathodic adsorptive stripping voltammetry. Electroanalysis 15:1121–1128

Beltran G, Esteve-Zarzoso B, Rozès N, Mas A, Guillamón JM (2005) Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. J Agric Food Chem 53:996–1002. https://doi.org/10.1021/jf0487001

Berbegal C, Spano G, Fragasso M, Grieco F, Russo P, Capozzi V (2018) Starter cultures as biocontrol strategy to prevent *Brettanomyces bruxellensis* proliferation in wine. Appl Microbiol Biotechnol 102:569–576. https://doi.org/10.1007/s00253-017-8666-x

Bernaudat F, Frelet-Barrand A, Pochon N, Dementin S, Hivin P, Boutigny S, Rioux JB, Salvi D, Seigneurin-Berny D, Richaud P, Joyard J, Pignol D, Sabaty M, Desnos T, Pebay-Peyroula E, Darrouzet E, Vernet T, Rolland N (2011) Heterologous expression of membrane proteins: Choosing the appropriate host. PLoS One 6: e29191. https://doi.org/10.1371/journal.pone.0029191

Best SA, Midgley JM, Huang W, Watson DG (1993) The determination of 5-hydroxytryptamine, related indolealkylamines and 5-hydroxyindoleacetic acid in the bovine eye by gas chromatography-negative ion chemical ionization mass spectrometry. J Pharm Biomed Anal 11:323–333. https://doi.org/10.1016/0731-7085(93)80024-U

Betteridge A, Grbin P, Jiranek V (2015) Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. Trends Biotechnol 33:547–553. https://doi.org/10.1016/j.tibtech.2015.06.008

Bhattacharjee A (2018) Phytomelatonin: a comprehensive literature review and recent advance on medicinal meadow. Int J Hydrol 2:396–403. https://doi.org/10.15406/ijh.2018.02.00102

Bianco C, Imperlini E, Calogero R, Senatore B, Amoresano A, Carpentieri A, Pucci P, Defez R (2006) Indole-3-acetic acid improves *Escherichia coli*'s defences to stress. Arch Microbiol 185:373–382. https://doi.org/10.1007/s00203-006-0103-y

Bisquert R, Muñiz-Calvo S, Guillamón JM (2018) protective role of intracellular melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*. Front Microbiol 9:1–11. 10.3389/fmicb.2018.00318

Blount BA, Weenink T, Vasylechko S, Ellis T (2012) Rational diversification of a promoter providing fine-tuned expression and orthogonal regulation for synthetic biology. PLoS One 7:1–11. https://doi.org/10.1371/journal.pone.0033279

Bonnefont-Rousselot D, Collin F (2010) Melatonin: Action as antioxidant and potential applications in human disease and aging. Toxicology 278:55–67. https://doi.org/10.1016/j.tox.2010.04.008

Boratyn GM, Schäffer AA, Agarwala R, Altschul SF, Lipman DJ, Madden TL (2012) Domain enhanced lookup time accelerated BLAST. Biol Direct 7:1–14. https://doi.org/10.1186/1745-6150-7-12

Bordiga M, Lorenzo C, Pardo F, Salinas MR, Travaglia F, Arlorio M, Coïsson JD, Garde-Cerdán T (2016) Factors influencing the formation of histaminol, hydroxytyrosol, tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids concentration. Food Chem 197:1038–1045. https://doi.org/10.1016/j.foodchem.2015.11.112

Borneman AR, Chambers PJ, Pretorius IS (2007) Yeast systems biology: modelling the winemaker's art. Trends Biotechnol 25:349–355. https://doi.org/10.1016/j.tibtech.2007.05.006

Borneman AR, Pretorius IS (2015) Genomic Insights into the *Saccharomyces* sensu stricto complex. Genetics 199:281–291. https://doi.org/10.1534/genetics.114.173633

Boronat A, Martínez-Huélamo M, Cobos A, de la Torre R (2018) Wine and olive oil phenolic compounds interaction in humans. Diseases 6:76. https://doi.org/10.3390/diseases6030076

Boselli E, Minardi M, Giomo A, Frega NG (2006) Phenolic composition and quality of white d.o.c. wines from Marche (Italy). Anal Chim Acta 563:93–100. https://doi.org/10.1016/j.aca.2005.10.024

Botstein D, Fink GR (2011) Yeast: An experimental organism for 21st century biology. Genetics 189: 695-704. 10.1534/genetics.111.130765

Bouallagui Z, Sayadi S (2006) Production of high hydroxytyrosol yields via tyrosol conversion by *Pseudomonas aeruginosa* immobilized resting cells. J Agric Food Chem 54:9906–9911. https://doi.org/10.1021/jf062145g

Bouallagui Z, Sayadi S (2018) Bioconversion of *p*-Tyrosol into hydroxytyrosol under bench-scale fermentation. Biomed Res 9: 2018:7390751. doi: 10.1155/2018/7390751

Boyton PJ, Greig D (2014) The ecology and evolution of non-domesticated *Saccharomyces* species. Yeast 31:4499–462. https://doi.org/10.1002/yea

Branco P, Francisco D, Chambon C, Hébraud M, Arneborg N, Almeida MG, Caldeira J, Albergaria H (2014) Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl Microbiol Biotechnol 98:843–853. https://doi.org/10.1007/s00253-013-5411-y

Braus GH (1991) Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. Microbiol Rev 55:349–370. https://doi.org/10.1080/00222931003764089

Bravo-Ferrada BM, Hollmann A, Delfederico L, La Hens DV, Caballero A, Semorile L (2013) Patagonian red wines: Selection of *Lactobacillus plantarum* isolates as potential starter cultures for malolactic fermentation. World J Microbiol Biotechnol 29:1537–1549. https://doi.org/10.1007/s11274-013-1337-x

Britton J, Davis R, O'Connor KE (2019) Chemical, physical and biotechnological approaches to the production of the potent antioxidant hydroxytyrosol. Appl Microbiol Biotechnol 103:5957–5974. https://doi.org/10.1007/s00253-019-09914-9

Briza P, Breitenbach M, Ellinger A, Segall J (1990) Isolation of two developmentally regulated genes involved in spore wall maturation in *Saccharomyces cerevisiae*. Genes Dev 4:1775–1789. https://doi.org/10.1101/gad.4.10.1775

Briza P, Eckerstorfer M, Breitenbach M (1994) The sporulation-specific enzymes encoded by the *DIT1* and *DIT2* genes catalyze a two-step reaction leading to a soluble LL-dityrosine-containing precursor of the yeast spore wall. Proc Natl Acad Sci U S A 91:4524–4528. https://doi.org/10.1073/pnas.91.10.4524

Brooks SJ, Doyle EM, O'Connor KE (2006) Tyrosol to hydroxytyrosol biotransformation by immobilised cell extracts of *Pseudomonas putida* F6. Enzyme Microb Technol 39:191–196. https://doi.org/10.1016/j.enzmictec.2005.10.025

Brouk M, Fishman A (2009) Protein engineering of toluene monooxygenases for synthesis of hydroxytyrosol. Food Chem 116:114–121. https://doi.org/10.1016/j.foodchem.2009.02.020

Bunnik EM, Le Roch KG (2013) An introduction to functional genomics and systems biology. Adv Wound Care 2:490–498. https://doi.org/10.1089/wound.2012.0379

Burkhardt S, Hardeland R (1996) Circadian rhythmicity of tryptophan hydroxylase in the marine dinoflagellate *Gonyaulax polyedra* stein and the role of tryptophan hydroxylation in bioluminescence. Comp Biochem Physiol - B Biochem Mol Biol 115:411–416. https://doi.org/10.1016/S0305-0491(96)00156-3

Byeon Y, Back K (2015) Molecular cloning of melatonin 2-hydroxylase responsible for 2-hydroxymelatonin production in rice (*Oryza sativa*). J Pineal Res 58:343–351. https://doi.org/10.1111/jpi.12220

Byeon Y, Lee HJ, Lee HY, Back K (2016) Cloning and functional characterization of the *Arabidopsis N*-acetylserotonin *O*-methyltransferase responsible for melatonin synthesis. J Pineal Res 60:65–73

Byeon Y, Lee HY, Lee K, Park S, Back K (2014a) Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT. J Pineal Res 56:107–114. https://doi.org/10.1111/jpi.12103

Byeon Y, Lee HY, Lee K, Back K (2014b) Caffeic acid *O*-methyltransferase is involved in the synthesis of melatonin by methylating *N*-acetylserotonin in *Arabidopsis*. J Pineal Res 57:219–227. https://doi.org/10.1111/jpi.12160

Byeon Y, Tan DX, Reiter RJ, Back K (2015) Predominance of 2-hydroxymelatonin over melatonin in plants. J Pineal Res 59:448–454. https://doi.org/10.1111/jpi.12274

Cai P, Gao J, Zhou Y (2019) CRISPR-mediated genome editing in non-conventional yeasts for biotechnological applications. Microb Cell Fact 18:1–12. https://doi.org/10.1186/s12934-019-1112-2

Cameron DE, Bashor CJ, Collins JJ (2014) A brief history of synthetic biology. Nat Rev Microbiol 12:381–390. https://doi.org/10.1038/nrmicro3239

Cameron JR, Loh EY, Davis RW (1979) Evidence for transposition of dispersed repetitive DNA families in yeast. Cell 16:739–751. https://doi.org/10.1016/0092-8674(79)90090-4

Canal C, Ozen B, Baysal AH (2019) Characterization of antimicrobial activities of olive phenolics on yeasts using conventional methods and mid-infrared spectroscopy. J Food Sci Technol 56:149–158. https://doi.org/10.1007/s13197-018-3468-4

Canton B, Labno A, Endy D (2008) Refinement and standardization of synthetic biological parts and devices. Nat Biotechnol 26:787–793. https://doi.org/10.1038/nbt1413

Caro CA, Lillo L, Valenzuela FJ, Cabello G, Lang E, Gallegos-Vallejos D, Castillo C (2016) Oxidation of melatonin on a glassy carbon electrode modified with metallic glucosamines. Synthesis and characterization. J Solid State Electrochem 20:993–1000. https://doi.org/10.1007/s10008-015-3066-6

Carquet M, Pompon D, Truan G (2015) Transcription interference and ORF nature strongly affect promoter strength in a reconstituted metabolic pathway. Front Bioeng Biotechnol 3:1–9. https://doi.org/10.3389/fbioe.2015.00021

Causton HC, Ren B, Sang Seok Koh, Harbison CT, Kanin E, Jennings EG, Tong IL, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12:323–337. https://doi.org/10.1091/mbc.12.2.323

Chamkha M, Cathala B, Cheynier V, Douillard R (2003) Phenolic composition of champagnes from chardonnay and pinot noir vintages. J Agric Food Chem 51:3179–3184. https://doi.org/10.1021/jf021105j

Chee MK, Haase SB (2012) New and redesigned pRS plasmid shuttle vectors for genetic manipulation of *Saccharomyces cerevisiae*. G3 Genes, Genomes, Genet 2:515–526. https://doi.org/10.1534/g3.111.001917

Chen H, Fink GR (2006) Feedback control of morphogenesis in fungi by aromatic alcohols. Genes Dev 20:1150–1161. https://doi.org/10.1101/gad.1411806

Chen W, Yao J, Meng J, Han W, Tao Y, Chen Y, Guo Y, Shi G, He Y, Jin JM, Tang SY (2019) Promiscuous enzymatic activity-aided multiple-pathway network design for metabolic flux rearrangement in hydroxytyrosol biosynthesis. Nat Commun 10:1–12. https://doi.org/10.1038/s41467-019-08781-2

Choi HJ, Kim YH (2018) Simultaneous and sequential integration by cre/loxp site-specific recombination in *Saccharomyces cerevisiae*. J Microbiol Biotechnol 28:826–830. https://doi.org/10.4014/jmb.1802.02004

Chowdhury I, Sengupta A, Maitra SK (2008) Melatonin: Fifty years of scientific journey from the discovery in bovine pineal gland to delineation of functions in human. Indian J Biochem Biophys 45:289–304

Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110:119–122. doi: 10.1016/0378-1119(92)90454-w

Chung D, Kim SY, Ahn JH (2017) Production of three phenylethanoids, tyrosol, hydroxytyrosol, and salidroside, using plant genes expressing in *Escherichia coli*. Sci Rep 7:1–8. https://doi.org/10.1038/s41598-017-02042-2

Ciani M, Comitini F (2015) Yeast interactions in multi-starter wine fermentation. Curr Opin Food Sci 1:1–6. https://doi.org/10.1016/j.cofs.2014.07.001

Coleman ST, Fang TK, Rovinsky SA, Turano FJ, Moye-Rowley WS (2001) Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. J Biol Chem 276:244–250. https://doi.org/10.1074/jbc.M007103200

Comitini F, Gobbi M, Domizio P, Romani C, Lencioni L, Mannazzu I, Ciani M (2011) Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol 28:873–882. https://doi.org/10.1016/j.fm.2010.12.001

Contreras A, Hidalgo C, Henschke PA, Chambers PJ, Curtin C, Varela C (2014) Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. Appl Environ Microbiol 80:1670–1678. https://doi.org/10.1128/AEM.03780-13

Coon SL, Roseboom PH, Baler R, Weller JL, Namboodiri MAA, Koonin EV, Klein DC (1995) Pineal serotonin *N*-acetyltransferase: Expression cloning and molecular analysis. Science 270:1681–1683. https://doi.org/10.1126/science.270.5242.1681

Cordente AG, Curtin CD, Varela C, Pretorius IS (2012) Flavour-active wine yeasts. Appl Microbiol Biotechnol 96:601–618. https://doi.org/10.1007/s00253-012-4370-z

Cordente AG, Schmidt S, Beltran G, Torija MJ, Curtin CD (2019) Harnessing yeast metabolism of aromatic amino acids for fermented beverage bioflavouring and bioproduction. Appl Microbiol Biotechnol 103:4325–4336. https://doi.org/10.1007/s00253-019-09840-w

Corujo-Antuña JL, Abad-Villar EM, Fernández-Abedul MT, Costa-García A (2003) Voltammetric and flow amperometric methods for the determination of melatonin in pharmaceuticals. J Pharm Biomed Anal 31:421–429. DOI: 10.1016/s0731-7085(02)00349-7

Covas MI, Miró-Casas E, Fitó M, Farré-Albadalejo M, Gimeno E, Marrugat J, de la Torre R (2003) Bioavailability of tyrosol, an antioxidant phenolic compound present in wine and olive oil, in humans. Drugs Exp Clin Res 29:203–206

Crabtree HG (1928) The carbohydrate metabolism of certain pathological overgrowths. Biochem J 22:1289–1298. DOI: 10.1042/bj0221289

Crépin L, Truong NM, Bloem A, Sanchez I, Dequin S, Camarasa C (2017) Management of multiple nitrogen sources during wine fermentation by *Saccharomyces cerevisiae*. Appl Environ Microbiol 83:1–21. https://doi.org/10.1128/AEM.02617-16

Crespi F, Ratti E, Trist DG (1994) Melatonin, a hormone monitorable *in vivo* by voltammetry? Analyst 119:2193–2197. https://doi.org/10.1039/AN9941902193

Cubillos FA, Brice C, Molinet J, Tisné S, Abarca V, Tapia SM, Oporto C, García V, Liti G, Martínez C (2017) Identification of nitrogen consumption genetic variants in yeast through QTL mapping and bulk segregant RNA-Seq analyses. G3 Genes, Genomes, Genet 7:1693–1705. https://doi.org/10.1534/g3.117.042127

Curiel JA, Morales P, Gonzalez R, Tronchoni J (2017) Different non-*Saccharomyces* yeast species stimulate nutrient consumption in *S. cerevisiae* mixed cultures. Front Microbiol 8:1–9

Curran KA, Leavitt JM, Karim AS, Alper HS (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. Metab Eng 15:55–66. https://doi.org/10.1016/j.ymben.2012.10.003

Curran KA, Morse NJ, Markham KA, Wagman AM, Gupta A, Alper HS (2015) Short synthetic terminators for improved heterologous gene expression in yeast. ACS Synth Biol 4:824–832. https://doi.org/10.1021/sb5003357.

Da Silva NA, Srikrishnan S (2012) Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. FEMS Yeast Res 12:197–214. https://doi.org/10.1111/j.1567-1364.2011.00769.x

Dar TA, Zargar MA, Dar KB, Anees S, Masood A, Ganie SA, Bhat AH (2015) Melatonin: A potential anti-oxidant therapeutic agent for mitochondrial dysfunctions and related disorders. Rejuvenation Res 19:21–40. https://doi.org/10.1089/rej.2015.1704

Dashko S, Zhou N, Compagno C, Piškur J (2014) Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Res 14:826–832. https://doi.org/10.1111/1567-1364.12161

David F, Nielsen J, Siewers V (2016) Flux control at the malonyl-coa node through hierarchical dynamic pathway regulation in *Saccharomyces cerevisiae*. ACS Synth Biol 5:224–233. https://doi.org/10.1021/acssynbio.5b00161

Davis I, Liu A (2015) What is the tryptophan kynurenine pathway and why is it important to neurotherapeutics? Expert Rev Neurother 15:719–721. https://doi.org/10.1586/14737175.2015.1049999

De Carvalho CC (2017) Whole cell biocatalysts: essential workers from Nature to the industry. Microb Biotechnol 10:250–263. https://doi.org/10.1111/1751-7915.12363

De Deken RH (1966) The Crabtree effect: A regulatory system in yeast. J gen Microbiol 44:149–156

De La Torre R, Covas MI, Pujadas MA, Fitó M, Farré M (2006) Is dopamine behind the health benefits of red wine? Eur J Nutr 45:307–310. https://doi.org/10.1007/s00394-006-0596-9

De Luca V, Marineau C, Brisson N (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: comparison with animal dopa decarboxylases. Proc Natl Acad Sci U S A 86:2582–2586. DOI: 10.1073/pnas.86.8.2582

Delneri D, Tomlin GC, Wixon JL, Hutter A, Sefton M, Louis EJ, Oliver SG (2000) Exploring redundancy in the yeast genome: An improved strategy for use of the cre-loxP system. Gene 252:127–135. https://doi.org/10.1016/S0378-1119(00)00217-1

Deloache WC, Russ ZN, Narcross L, Gonzales AM, Martin VJJ, Dueber JE (2015) An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. Nat Chem Biol 11:465–471. https://doi.org/10.1038/nchembio.1816

Di Tommaso D, Calabrese R, Rotilio D (1998) Identification and quantitation of hydroxytyrosol in Italian wines. HRC J High Resolut Chromatogr 21:549–553. https://doi.org/10.1002/(SICI)1521-4168(19981001)21:10<549::AID-JHRC549>3.0.CO;2-Z

Dickinson JR, Salgado LEJ, Hewlins MJE (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J Biol Chem 278:8028–8034. https://doi.org/10.1074/jbc.M211914200

Doménech-Carbó A, Cebrián-Torrejón G, Lopes-Souto A, Martins de Moraes M, Jorge-Kato M, Fechine-Tavares J, Barbosa-Filho JM (2015a) Electrochemical ecology: VIMP monitoring of plant defense against external stressors. RSC Adv 5:61006–61011. https://doi.org/10.1039/c5ra11336a

Doménech-Carbó A, Domínguez I, Hernández-muñoz P, Gavara R (2015b) Electrochemical tomato (*Solanum lycopersicum* L.) characterisation using contact probe in situ voltammetry. Food Chem 172:318–325. DOI: 10.1016/j.foodchem.2014.09.066

Doménech-Carbó A, Gavara R, Hernández-Muñoz P, Domínguez I (2015c) Contact probe voltammetry for in situ monitoring of the reactivity of phenolic tomato (*Solanum lycopersicum* L.) compounds with ROS. Talanta 144:1207–1215. https://doi.org/10.1016/j.talanta.2015.07.092

Doménech-Carbó A, Ibars AM, Prieto-Mossi J, Estrelles E, Scholz F, Cebrián-Torrejón G, Martinin M (2015d) Electrochemistry-based chemotaxonomy in plants using the voltammetry of microparticles methodology. New J Chem 39:7421–7428. https://doi.org/10.1039/c5nj01233c

Doménech-Carbó A, Labuda J, Scholz F (2013) Electroanalytical chemistry for the analysis of solids: Characterization and classification (IUPAC technical report). Pure Appl Chem 85:609–631. https://doi.org/10.1351/PAC-REP-11-11-13

Doménech A, Doménech-Carbó MT, Pasies T, Bouzas MC (2011) Application of modified tafel analysis to the identification of corrosion products on archaeological metals using voltammetry of microparticles. Electroanalysis 23:2803–2812. https://doi.org/10.1002/elan.201100577

Domínguez I, Doménech-Carbó A (2015) Screening and authentication of tea varieties based on microextraction-assisted voltammetry of microparticles. Sensors Actuators B Chem 210:491–499. https://doi.org/10.1016/j.snb.2015.01.009

Duina AA, Miller ME, Keeney JB (2014) Budding yeast for budding geneticists: A primer on the *Saccharomyces cerevisiae* model system. Genetics 197:33–48. https://doi.org/10.1534/genetics.114.163188

Dujon BA, Louis EJ (2017) Genome diversity and evolution in the budding yeasts (*Saccharomycotina*). Genetics 206:717–750. https://doi.org/10.1534/genetics.116.199216

Duportet X, Aggio RBM, Carneiro S, Villas-Bôas SG (2012) The biological interpretation of metabolomic data can be misled by the extraction method used. Metabolomics 8:410–421. https://doi.org/10.1007/s11306-011-0324-1

Eelderink-Chen Z, Mazzotta G, Sturre M, Bosman J, Roenneberg T, Merrow M (2010) A circadian clock in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 107:2043–2047. https://doi.org/Doi10.1073/Pnas.0907902107

EFSA 2010 (2010) Scientific opinion on the substantiation of health claims related to melatonin and subjective feelings of jet lag (ID1953), and reduction of sleep onset latency, and improvement of sleep quality (ID 1953) pursuant to Article 13(1) of Regulation (EC) No 19. EFSA J 8:1–14. https://doi.org/10.2903/j.efsa.2010.1467

EFSA 2011 (2011) Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), mainte. EFSA J 9:1–25. https://doi.org/10.2903/j.efsa.2011.2033

EFSA 2017 (2017) Safety of hydroxytyrosol as a novel food pursuant to Regulation (EC) No 258/97. EFSA J. https://doi.org/10.2903/j.efsa.2017.4728

Ehrenworth AM, Sarria S, Peralta-Yahya P (2015) Pterin-dependent mono-oxidation for the microbial synthesis of a modified monoterpene indole alkaloid. ACS Synth Biol 4:1295–1307. https://doi.org/10.1021/acssynbio.5b00025

Ehrlich F (1907) Über die bedingungen der fuselölbildung und über ihren zusammenhang mit dem eiweissaufbau der hefe. Ber Dtsch Chem Ges 40:1027–1047. https://doi.org/10.1002/cber.190704001156

El-Dalatony MM, Saha S, Govindwar SP, Abou-Shanab RAI, Jeon BH (2019) Biological conversion of amino acids to higher alcohols. Trends Biotechnol 37:855–869. https://doi.org/10.1016/j.tibtech.2019.01.011

Ellis HR (2010) The FMN-dependent two-component monooxygenase systems. Arch Biochem Biophys 497:1–12. https://doi.org/10.1016/j.abb.2010.02.007

Fischer TW, Scholz G, Knöll B, Hipler UC, Elsner P. Melatonin suppresses reactive oxygen species in UV-irradiated leukocytes more than vitamin C and trolox. Skin Pharmacol Appl Skin Physiol. 2002 Sep-Oct;15(5):367-73. doi: 10.1159/000064543. PMID: 12239433. (añadir en el text)

Fischer TW, Scholz G, Knöll B, Hipler UC, Elsner P (2004) Melatonin suppresses reactive oxygen species induced by UV irradiation in leukocytes. J Pineal Res 37:107–112. https://doi.org/10.1111/j.1600-079x.2004.00142.x

Enache TA, Oliveira-brett AM (2011) Pathways of Electrochemical Oxidation of Indolic Compounds. Electroanalysis 23:1337–1344

Engin A, Engin AB (2015) Tryptophan metabolism: Implications for biological processes, health and disease.Humana Press, Totowa, Nueva Jersey.

Engler C, Kandzia R, Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS One 3:e3647.

Erland LAE, Chattopadhyay A, Jones AMP, Saxena PK (2016) Melatonin in plants and plant culture systems: Variability, stability and efficient quantification. Front Plant Sci 7:1721. https://doi.org/10.3389/fpls.2016.01721

Escrivá L, Manyes L, Barberà M, Martínez-Torres D, Guiseppe M (2016) Determination of melatonin in *Acyrthosiphon pisum* aphids by liquid chromatography-tandem mass spectrometry. J Insect Physiol 86:48–53. https://doi.org/10.1016/j.jinsphys.2016.01.003

Esteve-Zarzoso B, Hierro N, Mas A, Guillamón JM (2010) A new simplified AFLP method for wine yeast strain typing. LWT - Food Sci Technol 43:1480–1484

Falcon J, Coon SL, Besseau L, Cazamea-Catalan D, Fuentes M, Magnanou E, Paulin CH, Boeuf G, Sauzet S, Jorgensen EH, Mazan S, Wolf YI, Koonin EV, Steinbach PJ, Hyodo S, Klein DC (2014) Drastic neofunctionalization associated with evolution of the timezyme AANAT 500 Mya. Proc Natl Acad Sci 111:314–319. https://doi.org/10.1073/pnas.1312634110

Farrugia G, Balzan R, Madeo F, Breitenbach M (2012) Oxidative stress and programmed cell death in yeast. Front Oncol 2:64. https://doi.org/10.3389/fonc.2012.00064

Fathizadeh H, Mirzaei H, Asemi Z (2019) Melatonin: An anti-tumor agent for osteosarcoma. Cancer Cell Int 19:1–8. https://doi.org/10.1186/s12935-019-1044-2

Federico JVG, Hernández IG, Silva MF, Cerutti S (2016) Analytical trends for the determination of melatonin and precursors in plants. In: Ravishankar G., Ramakrishna A (eds) Serotonin and melatonin: Their functional role in plants, food, phytomedicine, and human health, 1st editio. Boca Ratón, pp 31–46

Fernández-Cruz E, Álvarez-Fernández MA, Valero E, Troncoso AM, García-Parrilla MC (2017) Melatonin and derived L-tryptophan metabolites produced during alcoholic fermentation by different wine yeast strains. Food Chem 217:431–437. DOI: 10.1016/j.foodchem.2016.08.020

Fernández-Cruz E, Álvarez-Fernández MA, Valero E, Troncoso AM, García-Parrilla MC (2016) Validation of an analytical method to determine melatonin and compounds related to l-tryptophan metabolism using UHPLC/HRMS. Food Anal Methods 9:3327–3336. https://doi.org/10.1007/s12161-016-0529-z

Fernández-Cruz E, Cerezo AB, Cantos-Villar E, Troncoso AM, García-Parrilla MC (2018) Time course of L-tryptophan metabolites when fermenting natural grape musts: effect of inoculation treatments and cultivar on the occurrence of melatonin and related indolic compounds. Aust J Grape Wine Res 25:92-100. https://doi.org/10.1111/ajgw.12369

Fernández-Cruz E, González B, Muñiz-Calvo S, Morcillo-Parra MA, Bisquert R, Troncoso AM, García-Parrilla MC, Torija MJ, Guillamón JM (2019) Intracellular biosynthesis of melatonin and other indolic compounds in *Saccharomyces* and non-*Saccharomyces* wine yeasts. Eur Food Res Technol 245:1553–1560. https://doi.org/10.1007/s00217-019-03257-5

Fernández-Mar MI, Mateos R, García-Parrilla MC, Puertas B, Cantos-Villar E (2012) Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. Food Chem 130:797–813. https://doi.org/10.1016/j.foodchem.2011.08.023

Fernández-Pachón MS, Medina S, Herrero-Martín G, Cerrillo I, Berná G, Escudero-Lõpez B, Ferreres F, Martín F, García-Parrilla MC, Gil-Izquierdo A (2014) Alcoholic fermentation induces melatonin synthesis in orange juice. J Pineal Res 56:31–38. DOI:10.1111/jpi.12093

Ferreira IM, Guido LF (2018) Impact of wort amino acids on beer flavour: A review. Fermentation 4:1–13. https://doi.org/10.3390/fermentation4020023

Fischer TW, Zbytek B, Sayre RM, Apostolov EO, Basnakian AG, Sweatman TW, Wortsman J, Elsner P, Slominski, A (2006) Melatonin increases survival of HaCaT keratinocytes by

suppressing UV-induced apoptosis. J Pineal Res 40:18–26. https://doi.org/10.1111/j.1600-079X.2005.00273.x

Fitzpatrick PF (2000) The aromatic amino acid hydroxylases. In: Purich DL (ed) Advances in enzymology and related areas of molecular biology. John Wiley & Sons, Inc, pp 235–94

Fleet GH (2008) Wine yeasts for the future. FEMS Yeast Res 8:979–995. https://doi.org/10.1111/j.1567-1364.2008.00427.x

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

Fracassetti D, Francesco Lo Faro A, Moiola S, Orioli M,Tirelli A, Iriti M, Vigentini I, Foschino R (2020) Production of melatonin and other tryptophan derivatives by *Oenococcus oeni* under winery and laboratory scale. Food Microbiol 86:103265. https://doi.org/10.1016/j.fm.2019.103265

Fracassetti D, Vigentini I, Lo Faro AFF, De Nisi P, Foschino R, Tirelli A,Orioli M, Iriti M (2019) Assessment of tryptophan, tryptophan ethylester, and melatonin derivatives in red wine by SPE-HPLC-FL and SPE-HPLC-MS methods. Foods 8:99. https://doi.org/10.3390/foods8030099

Fraiking GY, Strakhovskaya MG, Rubin LB (1981) Involvement of near-UV-induced synthesis of serotonin in photoprotection and potentiation of far-UV lethality in the yeast *Candida guillermondii*. Photochem Photobiol 33:33–37. DOI: 10.1111/j.1751-1097.1981.tb05501.x

Fu S (2016) Adulterants in urine drug testing. Adv Clin Chem 76:123–163. https://doi.org/10.1016/bs.acc.2016.05.003

Fu SF, Wei JY, Chen HW, Liu YY, Lu HY, Chou JY (2015) Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms. Plant Signal Behav 10: e1048052. https://doi.org/10.1080/15592324.2015.1048052

Fuhrberg B, Hardeland R, Poeggeler B, Behrmann G (1997) Dramatic rises of melatonin and 5-methoxytryptamine in *Gonyaulax* exposed to decreased temperature. Biol Rhythm Res 28:144–150. https://doi.org/10.1076/brhm.28.1.144.12978

Fukuda K, Watanabe M, Asano K, Ouchi K (1991) A mutated *AR04* gene for feedback-resistant DAHP synthase which causes both o-DL-phenylalanine resistabce and beta-phenetyl-alcohol overproduction in *Saccharomyces cerevisiae*. Curr Genet 20:453-456. DOI: 10.1007/BF00334771

Furuya T, Kino K (2014) Catalytic activity of the two-component flavin-dependent monooxygenase from *Pseudomonas aeruginosa* toward cinnamic acid derivatives. Appl Microbiol Biotechnol 98:1145–1154. https://doi.org/10.1007/s00253-013-4958-y

Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M (1989) The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol 9:3447–3456. https://doi.org/10.1128/mcb.9.8.3447

Galano A, Reiter RJ (2018) Melatonin and its metabolites *vs* oxidative stress: From individual actions to collective protection. J Pineal Res 65:1–33. https://doi.org/10.1111/jpi.12514

Galano A, Tan DX, Reiter RJ (2011) Melatonin as a natural ally against oxidative stress: A physicochemical examination. J Pineal Res 51:1–16. https://doi.org/10.1111/j.1600-079X.2011.00916.x

Galdieri L, Mehrotra S, Yu S, Vancura A (2010) Transcriptional regulation in yeast during diauxic shift and stationary phase. OMICS 14:629–638. https://doi.org/10.1089/omi.2010.0069

Gallardo-Fernández M, Hornedo-Ortega R, Alonso-bellido IM, Rodríguez-Gómez JA, Troncoso AM, García-Parrilla MC, Venero JL, Espinosa-Oliva AM, de Pablos RM (2020) Hydroxytyrosol

decreases LPS- and α -Synuclein-induced microglial activation *in vitro.* Antioxidants 9:36. https://doi.org/10.3390/antiox9010036

Gallardo-Fernández M, Hornedo-Ortega R, Cerezo AB, Troncoso AM, García-Parrilla MC (2019) Melatonin, protocatechuic acid and hydroxytyrosol effects on vitagenes system against alpha-synuclein toxicity. Food Chem Toxicol 134:110817. https://doi.org/10.1016/j.fct.2019.110817

Gallone B, Steensels J, Mertens S, Dzialo MC, Gordon JL, Wauters R, Theßeling FA, Bellinazzo F, Saels V, Herrera-Malaver B, Prahl T, White C, Hutzler M, Meußdoerffer F, Malcorps P, Souffriau B, Daenen L, Baele G, Maere S, Verstrepen KJ (2019) Interspecific hybridization facilitates niche adaptation in beer yeast. Nat Ecol Evol. https://doi.org/10.1038/s41559-019-0997-9

Ganguly S, Mummaneni P, Steinbach PJ, Klein DC, Coon SL (2001) Characterization of the *Saccharomyces cerevisiae* homolog of the melatonin rhythm enzyme arylalkylamine *N*-acetyltransferase (EC 2.3.1.87). J Biol Chem 276:47239–47247. https://doi.org/10.1074/jbc.M107222200

García-García MI, Hernández-García S, Sánchez-Ferrer Á, García-Carmona F (2013) Kinetic study of hydroxytyrosol oxidation and its related compounds by red globe grape polyphenol oxidase. J Agric Food Chem 61:6050–6055. https://doi.org/10.1021/jf4009422

García-Granados R, Lerma-Escalera JA, Morones-Ramírez JR (2019) Metabolic engineering and synthetic biology: Synergies, future, and challenges. Front Bioeng Biotechnol 7:1–4. https://doi.org/10.3389/fbioe.2019.00036

Garcia-Moreno H, Calvo JR, Maldonado MD (2013) High levels of melatonin generated during the brewing process. J Pineal Res 55:26–30. https://doi.org/10.1111/jpi.12005

Garcia-Parrilla MC, Cantos E, Troncoso AM (2009) Analysis of melatonin in foods. J Food Compos Anal 22:177–183. https://doi.org/10.1016/j.jfca.2008.09.009

García-Ríos E, Guillén A, De La Cerda R, Pérez-Través L, Querol A, Guillamón JM (2019) Improving the cryotolerance of wine yeast by interspecific hybridization in the genus *Saccharomyces*. Front Microbiol 10:1–12. https://doi.org/10.3389/fmicb.2018.03232

García JJ, Lõpez-Pingarrõn L, Almeida-Souza P, Tres A, Escudero P, García-Gil FA, Tan DX, Reiter RJ, Ramírez JM, Bernal-Pérez, M (2014) Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: A review. J Pineal Res 56:225–237. https://doi.org/10.1111/jpi.12128

Gardana C, Iriti M, Stuknytė M, De Noni I, Simonetti P (2014) 'Melatonin isomer' in wine is not an isomer of the melatonin but tryptophan-ethylester. J Pineal Res 57:435–441. https://doi.org/10.1111/jpi.12183

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

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell. 11:4241-57. doi: 10.1091/mbc.11.12.4241.

Germann SM, Baallal Jacobsen SA, Schneider K, Harrison SJ, Jensen NB, Chen X, Stahlhut SG, Borodina I, Luo H, Zhu J, Maury J, Forster J (2016) Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*. Biotechnol J 11:717–724. https://doi.org/10.1002/biot.201500143

Geu-Flores F, Nour-Eldin HH, Nielsen MT, Halkier BA (2007) USER fusion: A rapid and efficient method for simultaneous fusion and cloning of multiple PCR products. Nucleic Acids Res 35:0–5. https://doi.org/10.1093/nar/gkm106

Ghalandari M, Naghmachi M, Oliverio M, Nardi M, Shirazi HRG, Eilami O (2018) Antimicrobial effect of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate on *Staphylococcus aureus* and *Staphylococcus epidermidis*. Electron J Gen Med 15:em46.https://doi.org/10.29333/ejgm/85686.

Ghosh S, Kebaara BW, Atkin AL, Nickerson KW (2008) Regulation of aromatic alcohol production in *Candida albicans*. Appl Environ Microbiol 74:7211–7218. https://doi.org/10.1128/AEM.01614-08

Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD, Smart KA, (2007) Yeast responses to stresses associated with industrial brewery handling. FEMS 31: 535-569. 10.1111/j.1574-6976.2007.00076.x

Gibson BR, Storgårds E, Krogerus K, Vidgren V (2013) Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species *Saccharomyces eubayanus*. Yeast 30:255–266. https://doi.org/10.1002/yea.2960

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318

Gientka I, Duszkiewicz-Reinhard W (2009) Shikimate pathway in yeast cells: Enzymes, functioning, regulation - A review. Polish J Food Nutr Sci 59:113–118

Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350:87–96. https://doi.org/10.1016/S0076-6879(02)50957-5

Gitto E, Tan D-X, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, Fulia F, Barberi I (2001) Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrrioxamine (desferoxamine) in rat liver homogenates. J Pharm Pharmacol 53:1393–1401. https://doi.org/10.1211/0022357011777747

Gnügge R, Liphardt T, Rudolf F (2016) A shuttle vector series for precise genetic engineering of *Saccharomyces cerevisiae*. Yeast 33:83–98. https://doi.org/10.1002/yea

Gnügge R, Rudolf F (2017) *Saccharomyces cerevisiae* shuttle vectors. Yeast 34:205–221. https://doi.org/10.1002/yea

Gobert A, Tourdot-Maréchal R, Morge C, Sparrow C, Liu Y, Quintanilla-Casas B, Vichi S, Alexandre H (2017) Non-*Saccharomyces* yeasts nitrogen source preferences: Impact on sequential fermentation and wine volatile compounds profile. Front Microbiol 8:1–13. https://doi.org/10.3389/fmicb.2017.02175

Gobert A, Tourdot-Maréchal R, Sparrow C, Morge C, Alexandre H (2019) Influence of nitrogen status in wine alcoholic fermentation. Food Microbiol 83:71–85. https://doi.org/10.1016/j.fm.2019.04.008

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. Science 274:546–567. https://doi.org/10.1126/science.274.5287.546

Gold ND, Gowen CM, Lussier FX, Cautha SC, Mahadevan R, Martin VJJ (2015) Metabolic engineering of a tyrosine-overproducing yeast platform using targeted metabolomics. Microb Cell Fact 14:1–16. https://doi.org/10.1186/s12934-015-0252-2

Goldstein AL, McCusker JH (2001) Development of *Saccharomyces cerevisiae* as a model pathogen: A system for the genetic identification of gene products required for survival in the mammalian host environment. Genetics 159:499–513.

Gómez-Acebo E (2015) GRAS Notice (GRN) No. 600 for Seprox hydroxytyrosol. https://www.fda.gov/media/96937/download

Gómez FJV, Raba J, Cerutti S, Silva MF (2012) Monitoring melatonin and its isomer in Vitis vinifera cv. Malbec by UHPLC-MS/MS from grape to bottle. J Pineal Res 52:349–355. https://doi.org/10.1111/j.1600-079X.2011.00949.x

Goncharenko EN, Gorskaia TG, Gudz TI, Zolotareva LT, Kovaleva ZI (1978) Role of endogenous substances in creating a background of enhanced radioresistance. Report 11. The nature of differences in radioresistance yeast cells (experiments with different strains of *Saccharomyces cerevisiae*). Radiobiol 18:199–122

Goncharenko EN, Gorskaya TG, Kaplya SA, Kovaleva ZA, Rubin LB, Fraikin GY (1980) Mechanisms of photoprotection of yeast cells against ionizing radiation. Radiobiol 20:266–268.

González B, Mas A, Beltran G, Cullen PJ, Torija MJ (2017) Role of mitochondrial retrograde pathway in regulating ethanol-inducible filamentous growth in yeast. Front Physiol 8:1–20. https://doi.org/10.3389/fphys.2017.00148

González B, Vázquez J, Cullen PJ, Mas A, Beltran G, Torija MJ (2018a) Aromatic Amino acid-derived compounds induce morphological changes and modulate the cell growth of wine yeast species. Front Microbiol 9:1–16. doi: 10.3389/fmicb.2018.00670

González B, Vázquez J, Morcillo-Parra MÁ, Mas A, Torija MJ, Beltran G (2018b) The production of aromatic alcohols in non-*Saccharomyces* wine yeast is modulated by nutrient availability. Food Microbiol 74:64–74. https://doi.org/10.1016/j.fm.2018.03.003

González SS, Barrio E, Querol A (2008) Molecular characterization of new natural hybrids of *Saccharomyces cerevisiae* and *S. kudriavzevii* in brewing. Appl Environ Microbiol 74:2314–2320. https://doi.org/10.1128/AEM.01867-07

Gori K, Knudsen PB, Nielsen KF, Arneborg N, Jespersen L (2011) Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*. FEMS Yeast Res 11:643–652. https://doi.org/10.1111/j.1567-1364.2011.00755.x

Gottardi M, Reifenrath M, Boles E, Tripp J (2017) Pathway engineering for the production of heterologous aromatic chemicals and their derivatives in *Saccharomyces cerevisiae*: bioconversion from glucose. FEMS Yeast Res 17:1–11. https://doi.org/10.1093/femsyr/fox035

Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res 30:e23. https://doi.org/10.1093/nar/30.6.e23

Guerrini S, Mangani S, Romboli Y, Luti S, Pazzagli L, Granchi L (2018) Impact of *Saccharomyces cerevisiae* strains on health-promoting compounds in wine. Fermentation 4:1–14. https://doi.org/10.3390/fermentation4020026

Guirimand G, Kulagina N, Papon N, Hasunuma T, Courdavault V (2020) Innovative tools and strategies for optimizing yeast cell factories. Trends Biotechnol S0167-7799(20)30232-8. doi: 10.1016/j.tibtech.2020.08.010

Güldener U, Heck S, Fiedler T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24:2519–2524. doi: 10.1093/nar/24.13.2519

Guo W, Huang Q, Liu H, Hou S, Niu S, Jiang Y, Bao X, Shen Y, Fang X (2019) Rational engineering of chorismate-related pathways in *Saccharomyces cerevisiae* for improving tyrosol production. Front Bioeng Biotechnol 7:1–7. https://doi.org/10.3389/fbioe.2019.00152

Gutiérrez A, Beltran G, Warringer J, Guillamón JM (2013) Genetic basis of variations in nitrogen source utilization in four wine commercial yeast strains. PLoS One 8:1–13. https://doi.org/10.1371/journal.pone.0067166

Hall BG, Acar H, Nandipati A, Barlow M (2014) Growth rates made easy. Mol Biol Evol 31:232–238. https://doi.org/10.1093/molbev/mst187

Hansen EH, Møller BL, Kock GR, Bünner CM, Kristensen C, Jensen OR, Okkels FT, Olsen CE, Motawia MS, Hansen J (2009) *De novo* biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe)* and baker's yeast (*Saccharomyces cerevisiae*). Appl Environ Microbiol 75:2765–2774. https://doi.org/10.1128/AEM.02681-08

Hanson PK (2018) *Saccharomyces cerevisiae*: A unicellular model genetic organism of enduring importance. Curr Protoc Essent Lab Tech 16:1–15. https://doi.org/10.1002/cpet.21

Hanson SJ, Wolfe KH (2017) An evolutionary perspective on yeats mating-type switching. Genetics 206:9–32. https://doi.org/10.1007/978-94-009-3391-0_4

Hardeland R (1993) The presence and function of melatonin and structurally related indoleamines in a dinoflagellate, and a hypothesis on the evolutionary significance of these tryptophan metabolites in unicellulars. Experientia 49:614–622. https://doi.org/10.1007/BF01923941

Hardeland R (2005) Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. Endocrine 27:119–130. DOI: 10.1385/endo:27:2:119

Hardeland R (2007) Melatonin and 5-methoxytryptamine in non-metazoans. Reprod Nutr Dev 39:399–408. https://doi.org/10.1051/rnd:19990311

Hardeland R (2010) Melatonin metabolism in the central nervous system. Curr Neuropharmacol 8:168–181. https://doi.org/10.2174/157015910792246244

Hardeland R (2015) Melatonin in plants and other phototrophs: Advances and gaps concerning the diversity of functions. J Exp Bot 66:627–646. https://doi.org/10.1093/jxb/eru386

Hardeland R (2016) Melatonin in plants - Diversity of levels and multiplicity of functions. Front Plant Sci 7:198. doi: 10.3389/fpls.2016.00198

Hardeland R (2017a) Focus on the melatonin metabolite *N*1-Acetyl-5-methoxykynuramine (AMK). Biomed J Sci Tech Res 1:1–5. https://doi.org/10.26717/BJSTR.2017.01.000318

Hardeland R (2017b) Taxon- and site-specific melatonin catabolism. Molecules 22:e2015. https://doi.org/10.3390/molecules22112015

Hardeland R, Balzer I, Poeggeler B, Fuhrberg B, Una H, Behrmann G, Wolf R, Meyer TJ, Reiter RJ (1995) On the primary functions of melatonin in evolution: Mediation of photoperiodic signals in a unicell, photooxidation, and scavenging of free radicals. J Pineal Res 18:104–111. https://doi.org/10.1111/j.1600-079X.1995.tb00147.x

Hardeland R, Pandi-Perumal S, Poeggeler B (2007) Melatonin in plants–focus on a vertebrate night hormone with cytoprotective properties. Funct Plant Sci Biotechnol 1:32–45

Hartley J, Temple G, Brasch M (2000) DNA cloning using *in vitro* site-specific recombination. Genome Res 10:1788–1795. doi: 10.1101/gr.143000

Hassing EJ, de Groot PA, Marquenie VR, Pronk JT, Daran JM (2019a) Connecting central carbon and aromatic amino acid metabolisms to improve *de novo* 2-phenylethanol production in

Saccharomyces cerevisiae. Metab Eng 56:165–180. https://doi.org/10.1016/j.ymben.2019.09.011

Hausjell J, Halbwirth H, Spadiut O (2018) Recombinant production of eukaryotic cytochrome P450s in microbial cell factories. Biosci Rep 38:1–13. https://doi.org/10.1042/BSR20171290

Hazelwood LA, Daran JM, Van Maris AJA, Pronk JT, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74:2259–2266. https://doi.org/10.1128/AEM.02625-07

Heravi KM, Lange J, Watzlawick H, Kalinowski J, Altenbuchner J (2015) Transcriptional regulation of the vanillate utilization genes (vanABK operon) of *Corynebacterium glutamicum* by VanR, a PadR-like repressor. J Bacteriol 197:959–972. https://doi.org/10.1128/JB.02431-14

Hernández-Orte P, Cacho JF, Ferreira V (2002) Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J Agric Food Chem 50:2891–2899. https://doi.org/10.1021/jf011395o

Hernández-Ruiz J, Arnao MB (2008) Distribution of melatonin in different zones of lupin and barley plants at different ages in the presence and absence of light. J Agric Food Chem 56:10567–10573. https://doi.org/10.1021/jf8022063

Heux S, Cachon R, Dequin S (2006) Cofactor engineering in *Saccharomyces cerevisiae*: Expression of a H2O-forming NADH oxidase and impact on redox metabolism. Metab Eng 8:303–314. https://doi.org/10.1016/j.ymben.2005.12.003

Hevia D, González-Menénez P, Quiros-González I, Miar A, Rodríguez-García A, Tan DX, Reiter RJ, Mayo JC, Sainz RM (2015) Melatonin uptake through glucose transporters: A new target for melatonin inhibition of cancer. J Pineal Res 58:234–250. https://doi.org/10.1111/jpi.12210

Hirata F, Hayaishi O, Tokuyama T, Senoh S (1974) *In vitro* and *in vivo* formation of two new metabolites of melatonin. J Biol Chem 249:1311–1313.

Hornedo-Ortega R, Cerezo AB, de Pablos RM, Krisa S (2018a) Phenolic compounds characteristic of the mediterranean diet in mitigating microglia-mediated neuroinflammation. Front Cell Neurosci 12:1–20. https://doi.org/10.3389/fncel.2018.00373

Hornedo-Ortega R, Cerezo AB, Troncoso AM, Garcia-Parrilla MC, Mas A (2016) Melatonin and other tryptophan metabolites produced by yeasts: Implications in cardiovascular and neurodegenerative diseases. Front Microbiol 6:1–7. https://doi.org/10.3389/fmicb.2015.01565

Hornedo-Ortega R, Cerezo AB, Troncoso AM, Garcia-Parrilla MC (2018b) Protective effects of hydroxytyrosol against α-synuclein toxicity on PC12 cells and fibril formation. Food Chem Toxicol 120:41–49. https://doi.org/10.1016/j.fct.2018.06.059

Hornedo-Ortega R, Da Costa G, Cerezo AB, Troncoso AM, Tristan R, Garcia-Parrilla MC (2018c) *In vitro* effects of serotonin, melatonin, and other related indole compounds on amyloid-β kinetics and neuroprotection. Mol Nutr Food Res 62:1–12. https://doi.org/10.1002/mnfr.201700383

Horstman JA, Wrona MZ, Dryhurst G (2002) Further insights into the reaction of melatonin with hydroxyl radical. Bioorg Chem 30:371–382. https://doi.org/10.1016/S0045-2068(02)00511-4

Horwitz W (1982) Evaluation of analytical methods used for regulation of foods and drugs. Anal Chem 54:67–76. https://doi.org/10.1021/ac00238a002

Howell KS, Cozzolino D, Bartowsky EJ, Fleet GH, Henschke PA (2006) Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. FEMS Yeast Res 6:91–101. https://doi.org/10.1111/j.1567-1364.2005.00010.x

Hu T, He XW, Jiang JG, Xu XL (2014) Hydroxytyrosol and its potential therapeutic effects. J Agric Food Chem 62:1449–1455. https://doi.org/10.1021/jf405820v

Hu Y, Zhu Z, Nielsen J, Siewers V (2019) Engineering *Saccharomyces cerevisiae* cells for production of fatty acid-derived biofuels and chemicals. Open Biol 9: 190049. https://doi.org/10.1098/rsob.190049

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003) Global analysis of protein localization in budding yeast. Nature 425:686–691. https://doi.org/10.1038/nature02026

Ibáneza AJ, Fagerer SR, Schmidt AM, Urban PL, Jefimovs K, Geiger P, Dechant R, Heinemann M, Zenobi R (2013) Mass spectrometry-based metabolomics of single yeast cells. Proc Natl Acad Sci U S A 110:8790–8794. https://doi.org/10.1073/pnas.1209302110

Ichihara N, Okada M, Takeda M (2001) Characterization and purification of polymorphic arylalkylamine *N*-acetyltransferase from the American cockroach, *Periplaneta americana*. Insect Biochem Mol Biol 32:15–22. https://doi.org/10.1016/S0965-1748(01)00075-3

Ichinose H, Wariishi H (2013) High-level heterologous expression of fungal cytochrome P450s in *Escherichia coli*. Biochem Biophys Res Commun 438:289–294. https://doi.org/10.1016/j.bbrc.2013.07.057

Iriti M (2009) Melatonin in grape, not just a myth, maybe a panacea. J Pineal Res 46:353. https://doi.org/10.1111/j.1600-079X.2008.00616.x

Iriti M, Rossoni M, Faoro F (2006) Melatonin content in grape: myth or panacea? J Sci Food Agric 86:1432–1438. https://doi.org/10.1002/jsfa.2537

Iriti M, Varoni EM (2014) Cardioprotective effects of moderate red wine consumption: Polyphenols vs. ethanol. J Appl Biomed 12:193–202. https://doi.org/10.1016/j.jab.2014.09.003

Iriti M, Varoni EM (2015) Melatonin in Mediterranean diet, a new perspective. J Sci Food Agric 95:2355–2359. https://doi.org/10.1002/jsfa.7051

Iriti M, Varoni EM, Vitalini S (2010) Melatonin in traditional Mediterranean diets. J Pineal Res 49:101–105. https://doi.org/10.1111/j.1600-079X.2010.00777.x

Ishtar Snoek IS, Yde Steensma H (2007) Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. Yeast 24:1–10. DOI:10.1002/yea.1430

Isorna E, Aliaga-Guerrero M, M'Rabet A El, Servili A, Falcõn J, Muñoz-Cueto JA (2011) Identification of two arylalkylamine *N*-acetyltranferase 1 genes with different developmental expression profiles in the flatfish *Solea senegalensis*. J Pineal Res 51:434–444. https://doi.org/10.1111/j.1600-079X.2011.00907.x

Jang SW, Liu X, Pradoldej S, Tosini G, Chang Q, Iuvone PM, Ye K (2010) *N*-acetylserotonin activates TrkB receptor in a circadian rhythm. Proc Natl Acad Sci U S A 107:3876–3881. https://doi.org/10.1073/pnas.0912531107

Janjetovic Z, Jarrett SG, Lee EF, Duprey C, Reiter RJ, Slominski AT (2017) Melatonin and its metabolites protect human melanocytes against UVB-induced damage: Involvement of NRF2-mediated pathways. Sci Rep 7:1274. https://doi.org/10.1038/s41598-017-01305-2

Jensen NB, Strucko T, Kildegaard KR, David F, Maury J, Mortensen UH, Forster J, Nielsen J, Borodina I (2014) EasyClone: Method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. FEMS Yeast Res 14:238–248. https://doi.org/10.1111/1567-1364.12118

Jessop-Fabre MM, Jakočiūnas T, Stovicek V, Dai Z, Jensen MK, Keasling JD, Borodina I (2016) EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9. Biotechnol J 11:1110–1117. https://doi.org/10.1002/biot.201600147

Jiang J, Yin H, Wang S, Zhuang Y, Liu S, Liu T, Ma Y (2018a) Metabolic engineering of *Saccharomyces cerevisiae* for high-level production of salidroside from glucose. J Agric Food Chem 66:4431–4438. https://doi.org/10.1021/acs.jafc.8b01272

Jolly NP, Varela C, Pretorius IS (2014) Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215–237. https://doi.org/10.1111/1567-1364.12111

Jones JA, Collins SM, Vernacchio VR, Lachance DM, Koffas MAG (2016) Optimization of naringenin and p-coumaric acid hydroxylation using the native *E. coli* hydroxylase complex, HpaBC. Biotechnol Prog 32:21–25. https://doi.org/10.1002/btpr.2185

Juhnevica-Radenkova K, Moreno DA, Ikase L, Drudze I, Radenkovs V (2020) Naturally occurring melatonin: Sources and possible ways of its biosynthesis. Compr Rev Food Sci F 19: 4008-4030. https://doi.org/10.1111/1541-4337.12639

Kachroo AH, Laurent JM, Yellman CM, Meyer AG, Wilke CO, Marcotte EM (2015) Systematic humanization of yeast genes reveals conserved functions and genetic modularity. Science 348:921–925. https://doi.org/10.1126/science.aaa0769

Kalb D, Gressler J, Hoffmeister D (2016) Active-Site engineering expands the substrate profile of the basidiomycete l-tryptophan decarboxylase CsTDC. ChemBioChem 17:132–136. DOI: 10.1002/cbic.201500438

Karadag A, Ozcelik B, Saner S (2009) Review of methods to determine antioxidant capacities. Food Anal Methods 2:41–60. https://doi.org/10.1007/s12161-008-9067-7

Karkovic Markovic A, Toric J, Barbaric M, Jakobušic B (2019) Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. Molecules 24:2001. doi: 10.3390/molecules24102001

Karst F, Lacroute F (1977) Ergosterol biosynthesis in *Saccharomyces cerevisiae*. Mutants deficient in the early steps of the pathway. Molec gen Genet 154:269–277. https://doi.org/10.1039/C19680001067

Katz Ezov T, Chang SL, Frenkel Z, Segré AV, Bahalul M, Murray AW, Leu JY, Horol A, Kashi Y (2010) Heterothallism in *Saccharomyces cerevisiae* isolates from nature: Effect of HO locus on the mode of reproduction. Mol Ecol 19:121–131. https://doi.org/10.1111/j.1365-294X.2009.04436.x

Kazazian HH (2004) Mobile elements: Drivers of genome evolution. Science 303:1626–1632. https://doi.org/10.1126/science.1089670

Kim D, Hayles J, Wood V, Park H, Won M, Yoo H, Duhig T, Nam M, Palmer G, Han S, Jeffery L, Baek S, Lee H, Shim YS, Lee M, Kim L, Heo K, Noh EJ, Lee A, Jang Y, Chung K, Choi S, Park J, Park Y, Him HM, Park S, Kang E, Kim HB, Kang H, Kim K, Song K, Song KB, Nurse P, Hoe K (2010) Analysis of a genome-wide set of gene deletions in the fission yeast *Schizosaccharomyces pombe*. Nat Biotechnol 28:617–623. https://doi.org/10.1038/nbt.1628.Analysis

Kim JJ, Sangwoo K, Jeon CO, Yun J, Lee HS, Ro HS (2006) Screening of yeast diauxic promoters for production of foreign proteins. J Microbiol Biotechnol 16:1459–1463.

Kim JS (2016) Genome editing comes of age. Nat Protoc 11:1573–1578. https://doi.org/10.1038/nprot.2016.104

Kleszczyński K, Hardkop LH, Fischer TW (2011) Differential effects of melatonin as a broad range UV-damage preventive dermato-endocrine regulator. Dermatoendocrinol 3:27–31. doi: 10.4161/derm.3.1.14842

Kneen MM, Stan R, Yep A, Tyler RP, Saehuan C, McLeish MJ (2011) Characterization of a thiamin diphosphate-dependent phenylpyruvate decarboxylase from *Saccharomyces cerevisiae*. FEBS J 278:1842–1853. https://doi.org/10.1111/j.1742-4658.2011.08103.x

Kocadağli T, Yilmaz C, Gökmen V (2014) Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. Food Chem 153:151–156. https://doi.org/10.1016/j.foodchem.2013.12.036

Kollmann MT, Locher M, Hirche F, Eder K, Meyer HHD, Bruckmaier RM (2008) Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows. Domest Anim Endocrinol 34:14–24. 10.1016/j.domaniend.2006.09.005

Komorsky-Lovrić Š, Lovrić M (1995) Kinetic measurements of a surface confined redox reaction. Anal Chim Acta 305:248–255. https://doi.org/10.1016/0003-2670(94)00455-U

Komorsky-Lovrić Š, Lovrić M, Bond AM (1992) Comparison of the square-wave stripping voltammetry of lead and mercury following their electrochemical or abrasive deposition onto a paraffin impregnated graphite electrode. Anal Chim Acta 258:299–305. https://doi.org/10.1016/0003-2670(92)85105-F

Krotzky M, Hardeland R (2008) Metabolism of the melatonin metabolite *N*1-acetyl-*N*2-formyl-5-methoxykynuramine in *Saccharomyces cerevisiae*. Cytologia 73:123–128. https://doi.org/10.1508/cytologia.73.123

Kurtzman CP (2003) Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. FEMS Yeast Res 4:233–245. https://doi.org/10.1016/S1567-1356(03)00175-2

Lai B, Plan MR, Averesch NJH, Yu S, Kracke F, Lekieffre N, Bydder S, Hodson MP, Winter G, Krömer JO (2017) Quantitative analysis of aromatics for synthetic biology using liquid chromatography. Biotechnol J 12(1) https://doi.org/10.1002/biot.201600269

Lai MT, Liu DYT, Hseu TH (2007) Cell growth restoration and high level protein expression by the promoter of hexose transporter, *HXT7*, from *Saccharomyces cerevisiae*. Biotechnol Lett 29:1287–1292. https://doi.org/10.1007/s10529-007-9397-3

Lairón-Peris M, Pérez-Través L, Muñiz-Calvo S, Guillamón JM, Heras JM, Barrio E, Querol A (2020) Differential contribution of the parental genomes to a *S. cerevisiae* × *S. uvarum* hybrid, inferred by phenomic, genomic, and transcriptomic analyses, at different industrial stress conditions. Front Bioeng Biotechnol 8:1–20. https://doi.org/10.3389/fbioe.2020.00129

Langdon QK, Peris D, Baker EP, Opulente DA, Nguyen HV, Bond U, Gonçalves P, Sampaio JP, Libkind D, Hittinger CT (2019) Fermentation innovation through complex hybridization of wild and domesticated yeasts. Nat Ecol Evol. https://doi.org/10.1038/s41559-019-0998-8

Lass-Flörl C, Fuchs D, Ledochowski M, Speth C, Dierich MP, Würzner R (2003) Antifungal properties of 5-hydroxytryptamine (serotonin) against *Candida* species *in vitro*. J Med Microbiol 52:169–171. https://doi.org/10.1099/jmm.0.04987-0

Lass-Flörl C, Wiedauer B, Mayr A, Kirchmair M, Jenewein I, Ledochowski M (2002) Antifungal properties of 5-hydroxytryptamine (serotonin) against *Aspergillus* sp. *in vitro*. Int J Med Microbiol 291:655–657

Leavitt JM, Tong A, Tong J, Pattie J, Alper HS (2016) Coordinated transcription factor and promoter engineering to establish strong expression elements in *Saccharomyces cerevisiae*. Biotechnol J 11:866–876. https://doi.org/10.1002/biot.201600029

Lee HY, Byeon Y, Lee K, Lee HJ, Back K (2014) Cloning of *Arabidopsis* serotonin *N*-acetyltransferase and its role with caffeic acid *O*-methyltransferase in the biosynthesis of melatonin *in vitro* despite their different subcellular localizations. Nutr Clin Pract 57:418–426. https://doi.org/10.1111/jpi.12181

Lee K, Choi G-H, Back K (2017) Cadmium-induced melatonin synthesis in rice requires light, hydrogen peroxide, and nitric oxide: Key regulatory roles for tryptophan decarboxylase and caffeic acid *O*-methyltransferase. J Pineal Res e12441. https://doi.org/10.1111/jpi.12441

Lee K, Hwang OJ, Back K (2020) Rice *N*-acetylserotonin deacetylase regulates melatonin levels in transgenic rice. Melatonin Res 3:32–42. https://doi.org/10.32794/mr11250046

Lee K, Lee HY, Back K (2018) Rice histone deacetylase 10 and *Arabidopsis* histone deacetylase 14 genes encode *N*-acetylserotonin deacetylase, which catalyzes conversion of *N*-acetylserotonin into serotonin, a reverse reaction for melatonin biosynthesis in plants. J Pineal Res 64:e12460. https://doi.org/doi: 10.1111/jpi.12460

Lee K, Zawadzka A, Czarnocki Z, Reite RJ, Back K (2016) Molecular cloning of melatonin 3-hydroxylase and its production of cyclic 3-hydroxymelatonin in rice (*Oryza sativa*). J Pineal Res 61:470–478. https://doi.org/10.1111/jpi.12361

Lee ME, DeLoache WC, Cervantes B, Dueber JE (2015) A highly characterized yeast toolkit for modular, multipart assembly. ACS Synth Biol 4:975–986. https://doi.org/10.1021/sb500366v

León J, Acuña-Castroviejo D, Escames G, Tan DX, Reiter RJ (2005) Melatonin mitigates mitochondrial malfunction. J Pineal Res 38:1–9. https://doi.org/10.1111/j.1600-079X.2004.00181.x

Leone AM, Silman RE, Hill BT, Whelan RDH, Shellard SA (1988) Growth inhibitory effects of melatonin and its metabolites against ovarian tumour cell lines *in vitro*. In: Gupta D, Attanasio A, Reiter RJ (eds) The pineal gland and cancer, brain research promotion. Tubingen, W. Germany., pp 273–281

Lerner AB, Case JD, Heinzelman R V. (1959) Structure of melatonin. J Am Chem Soc 81:6084–6085. https://doi.org/10.1021/ja01531a060

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W (1958) Isoloation of melatonin, the pineal gland factor that lightens melanocytes. J Am Chem Soc 80:2587. https://doi.org/10.1021/ja01543a060

Lesage-Meessen L, Navarro D, Maunier S, Sigoillot JC, Lorquin J, Delattre M, Simon JL, Asther M, Labat M (2001) Simple phenolic content in olive oil residues as a function of extraction systems. Food Chem 75:501–507. https://doi.org/10.1016/S0308-8146(01)00227-8

Levisson M, Araya-Cloutier C, De Bruijn WJC, Van der Heide M, Salvadr-López JM, Daran JM, Vincken JP, Beekwilder J (2019) Toward developing a yeast cell factory for the production of prenylated flavonoids. J Agric Food Chem 67:13478–13486. https://doi.org/10.1021/acs.jafc.9b01367

Li C, Jia P, Bai Y, Fan TP, Zheng X, Cai Y (2019) Efficient synthesis of hydroxytyrosol from l-3,4-dihydroxyphenylalanine using engineered *Escherichia coli* whole cells. J Agric Food Chem 67:6867–6873. https://doi.org/10.1021/acs.jafc.9b01856

Li M, Yang Z, Hao J, Shan L, Dong J (2008) Determination of tyrosol, 2-phenethyl alcohol, and tryptophol in beer by high-performance liquid chromatography. J Am Soc Brew Chem 66:249. https://doi.org/10.1094/ASBCJ-2008-0914-01

Li X, Chen Z, Wu Y, Yan Y, Sun X, Yuan Q (2018) Establishing an artificial pathway for efficient biosynthesis of hydroxytyrosol. ACS Synth Biol 7:647–654. https://doi.org/10.1021/acssynbio.7b00385

Liebgott PP, Amouric A, Comte A, Tholozan JL, Lorquin J (2009) Hydroxytyrosol from tyrosol using hydroxyphenylacetic acid-induced bacterial cultures and evidence of the role of 4-HPA 3-hydroxylase. Res Microbiol 160:757–766. https://doi.org/10.1016/j.resmic.2009.09.015

Liebgott PP, Labat M, Casalot L, Amouric A, Lorquin J (2007) Bioconversion of tyrosol into hydroxytyrosol and 3,4-dihydroxyphenylacetic acid under hypersaline conditions by the new *Halomonas sp*. strain HTB24. FEMS Microbiol Lett 276:26–33. https://doi.org/10.1111/j.1574-6968.2007.00896.x

Lin B, Tao Y (2017) Whole-cell biocatalysts by design. Microb Cell Fact 16:1–12. https://doi.org/10.1186/s12934-017-0724-7

Lin BX, Zhang ZJ, Liu WF, Dong ZY, Tao Y (2013) Enhanced production of *N*-acetyl-*d*-neuraminic acid by multi-approach whole-cell biocatalyst. Appl Microbiol Biotechnol 97:4775–4784. https://doi.org/10.1007/s00253-013-4754-8

Lin Y, Yan Y (2014) Biosynthesis of caffeic acid in *Escherichia coli* using its endogenous hydroxylase complex. Microb Cell Fact 11:3–11. https://doi.org/10.1186/1475-2859-11-42

Lisanti MT, Blaiotta G, Nioi C, Moio L (2019) Alternative methods to SO2 for microbiological stabilization of wine. Compr Rev Food Sci Food Saf 18:455–479. https://doi.org/10.1111/1541-4337.12422

Lissina E, Young B, Urbanus ML, Guan XL, Lowenson J, Hoon S, Baryshnikova A, Riezman I, Michaut M, Riezman H, Cowen LE, Wenk MR, Clarke SG, Giaever G, Nislow C (2011) A systems biology approach reveals the role of a novel methyltransferase in response to chemical stress and lipid homeostasis. PLoS Genet 7: e1002332. https://doi.org/10.1371/journal.pgen.1002332

Lissoni P (2006) Modulation of anticancer cytokines IL-2 and IL-12 by melatonin and the other pineal indoles 5-Methoxytryptamine and 5-Methoxytryptophol in the treatment of human neoplasms. Ann N Y Acad Sci 917:560–567. https://doi.org/10.1111/j.1749-6632.2000.tb05421.x

Lissoni P, Bucovec R, Bonfanti A, Giani L, Mandelli A, Roselli MG, Rovelli F, Fumagalli L (2001) Thrombopoietic properties of 5-methoxytryptamine plus melatonin versus melatonin alone in the treatment of cancer-related thrombocytopenia. J Pineal Res 30:123–126. https://doi.org/10.1034/j.1600-079X.2001.300208.x

Lissoni P, Malugani F, Bukovec R, Bordin V, Perego M, Mengo S, Ardizzoia A, Tancini G, Gerardo OS (2003) Reduction of cisplatin-induced anemia by the pineal indole 5-methoxytryptamine in metastatic lung cancer patients. Neurochem Int 24:83–85.

Lissoni P, Rovelli F, Frassineti A, Fumagalli L, Malysheva O, Conti A, Maestroni G (2000) Oncostatic activity of pineal neuroendocrine treatment with the pineal indoles melatonin and 5-methoxytryptamine in untreatable metastatic cancer patients progressing on melatonin alone. Neuroendocrinol Lett 21:319–323.

Lissoni P, Rovelli F, Giani L, Gavazzeni C, Grazia Roselli M, Mainini E, Mazzi C (1998) Endocrine effects of two pineal hormones other than melatonin in healthy volunteers: 5-methoxytryptophol and 5-methoxytryptamine. Recenti Prog Med 89:183—185

Liu B, Sutton A, Sternglanz R (2005) A yeast polyamine acetyltransferase. J Biol Chem 280:16659–16664. https://doi.org/10.1074/jbc.M414008200

Liu H, Chen Y, Liu Y, Yang Z (2013) A sensitive sensor for determination of l-tryptophan based on gold nanoparticles/poly(alizarin red S)-modified glassy carbon electrode. J Solid State Electrochem 17:2623–2631. https://doi.org/10.1007/s10008-013-2152-x

Liu JJ, Kong II, Zhang GC, Jayakody LN, Kim H, Xia PF, Kwak S, Sung BH, Sohn JH, Walukiewicz HE, Rao CV, Jin YS (2016) Metabolic engineering of a probiotic *Saccharomyces boulardii*. Appl Environ Microbiol 82:2280–2287. https://doi.org/10.1128/AEM.00057-16

Liu N, Liang Y, Bin J, Zhang Z, Huang J, Shu RX, Yang K (2014) Classification of green and black teas by PCA and SVM analysis of cyclic voltammetric signals from metallic oxide-modified electrode. Food Anal Methods 7:472–480. https://doi.org/10.1007/s12161-013-9649-x

Liu RH (2013) Dietary bioactive compounds and their health implications. J Food Sci 78:A18-25. https://doi.org/10.1111/1750-3841.12101

Liu Y, Rousseaux S, Tourdot-Maréchal R, Tourdot-Maréchal R, Sadoudi M, Gougeon R, Schmitt-Kopplin P (2017) Wine microbiome: A dynamic world of microbial interactions. Crit Rev Food Sci Nutr 57:856–873. https://doi.org/10.1080/10408398.2014.983591

Ljungdahl PO (2009) Amino-acid-induced signalling via the SPS-sensing pathway in yeast. Biochem Soc Trans 37:242–247. https://doi.org/10.1042/BST0370242

Ljungdahl PO, Daignan-Fornier B (2012) Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics 190:885–929. https://doi.org/10.1534/genetics.111.133306

Lleixà J, Martín V, Giorello F, Portillo MC, Carrau F, Gemma B, Mas A (2019) Analysis of the NCR mechanisms in *Hanseniaspora vineae* and *Saccharomyces cerevisiae* during winemaking. Front Genet 10:1–9. https://doi.org/10.3389/fgene.2018.00747

Lleixà J, Martín V, Portillo MC, Carrau F, Gemma B, Mas A (2016) Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. Front Microbiol 7:1–12. https://doi.org/10.3389/fmicb.2016.00338

Lonvaud-Funel A (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek. 76:317–331. https://doi.org/10.1023/A:1002088931106

Lonvaud-Funel A (2001) Biogenic amines in wines: Role of lactic acid bacteria. FEMS Microbiol Lett 199:9–13. https://doi.org/10.1016/S0378-1097(01)00157-4

Lopes TS, Klootwijk J, Veenstra AE, van der Aar PC, van Heerikhuizen H, Raué HA, Planta RJ (1989) High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high-level expression. Gene 79:199–206. https://doi.org/10.1016/0378-1119(89)90202-3

López-Burillo S, Tan DX, Mayo JC, Sainz RM, Manchester LC, Reiter RJ (2003a) Melatonin, xanthurenic acid, resveratrol, EGCG, vitamin C and a-lipoic acid differentially reduce oxidative DNA damage induced by Fenton reagents: a study of their individual and synergistic actions. J Pineal Res 34:269–277. doi: 10.1034/j.1600-079x.2003.00041.x.

López-Burillo S, Tan DX, Rodriguez-Gallego V, Manchester LC, Mayo JC, Sainz RM, Reiter RJ (2003b) Melatonin and its derivatives cyclic 3-hydroxymelatonin, *N*1-acetyl-*N*2-formyl-5-methoxykynuramine and 6-methoxymelatonin reduce oxidative DNA damage induced by Fenton reagents. J Pineal Res 34:178–184. https://doi.org/10.1034/j.1600-079X.2003.00025.x

López-Malo M, García-Rios E, Chiva R, Guillamón JM, Martí-Raga M (2014) Effect of deletion and overexpression of tryptophan metabolism genes on growth and fermentation capacity at low temperature in wine yeast. Biotechnol Prog 30:776–783

López A, García JA, Escames G, Venegas C, Ortiz F, López LC, Acuña-Castroviejo D (2009) Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. J Pineal Res 46:188–198. https://doi.org/10.1111/j.1600-079X.2008.00647.x

Lovrić M, Komorsky-Lovric Š (1988) Square-wave voltammetry of an adsorbed reactant. J Electroanal Chem 248:239–253. https://doi.org/10.1016/0022-0728(88)85089-7

Lovrić M, Komorsky-Lovrić Š, Bond AM (1991) Theory of square-wave stripping voltammetry and chronoamperometry of immobilized reactants. J Electroanal Chem 319:1–18

Luttik MAH, Overkamp KM, Kötter P, de Vries S, van Dijken JP, Pronk JT (1998) The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J Biol Chem 273:24529–24534. https://doi.org/10.1074/jbc.273.38.24529

Luttik MAH, Vuralhan Z, Suir E, Braus GH, Pronk JT, Daran JM (2008) Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: Quantification of metabolic impact. Metab Eng 10:141–153. https://doi.org/10.1016/j.ymben.2008.02.002

Ma X, Idle JR, Krausz KW, Gonzalez FJ (2005) Metabolism of melatonin by human cytochromes P450. Drug Metab Dispos 33:489–494

Madhani HD (2007) From a to alpha: yeast as a model for cellular differentiation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Magasanik B (1992) Regulation of nitrogen utilization. In: Jones EW , Pringle JR P, Broach JR (eds) The molecular and cellular biology of the yeast *Saccharomyces*: Gene Expression. Cold Spring Harbor Laboratory Press, NY, pp 283–317

Maier R (2009) Bacterial Growth. In: Pepper I, Gerba C, Gentry T, Maier R (eds) Environmental Microbiology, 2nd Editio. pp 37–54

Maldonado MD, Moreno H, Calvo JR (2009) Melatonin present in beer contributes to increase the levels of melatonin and antioxidant capacity of the human serum. Clin Nutr 28:188–191. DOI:https://doi.org/10.1016/j.clnu.2009.02.001

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. Science 339:823−826. DOI: 10.1126/science.1232033

Manchester LC, Coto-Montes A, Boga JA, Andersen LPH, Zhou Z, Galano A, Vriend J, Tan DX, Reiter RJ (2015) Melatonin: An ancient molecule that makes oxygen metabolically tolerable. J Pineal Res 59:403–419. https://doi.org/10.1111/jpi.12267

Manchester LC, Poeggeler B, Alvares FL, Ogden GB, Reiter RJ (1995) Melatonin immunoreactivity in the photosynthetic prokaryote *Rhodospirillum rubrum*: implications for an ancient antioxidant system. Cell Mol Biol Res 41:391—395

Manfroi L, Silva PHA, Rizzon LA, Sabaini PS, Glória MBA (2009) Influence of alcoholic and malolactic starter cultures on bioactive amines in Merlot wines. Food Chem 116:208–213. https://doi.org/10.1016/j.foodchem.2009.02.034

Mao J, Liu Q, Song X, Wang H, Feng H, Xu H, Qiao M (2017) Combinatorial analysis of enzymatic bottlenecks of l-tyrosine pathway by p-coumaric acid production in *Saccharomyces cerevisiae*. Biotechnol Lett 39:977–982. https://doi.org/10.1007/s10529-017-2322-5

Marhuenda J, Medina S, Martínez-Hernández P, Arina S, Zafrilla P, Mulero J, Genieser HG, Ferreres F, Gil-Izquierdo A (2016) Melatonin and hydroxytyrosol-rich wines influence the generation of DNA oxidation catabolites linked to mutagenesis after the ingestion of three types of wine by healthy volunteers. Food Funct 7:4781–4796. https://doi.org/10.1039/c6fo01246a

Marhuenda J, Medina S, Martínez-Hernández P, Arina S, Zafrilla P, Mulero J, Oger C, Galano JM, Durand t, Ferreres F, Gil-Izquierdo A (2017) Melatonin and hydroxytyrosol protect against

oxidative stress related to the central nervous system after the ingestion of three types of wine by healthy volunteers. Food Funct 8:64–74. https://doi.org/10.1039/c6fo01328g

Marini AM, Soussi-Boudekou S, Vissers S, Andre B (1997) A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol Cell Biol 17:4282–4293. DOI: 10.1128/mcb.17.8.4282

Markoski MM, Garavaglia J, Oliveira A, Olivaes J, Marcadenti A (2016) Molecular properties of red wine compounds and cardiometabolic benefits. Nutr Metab Insights 9:51–57. https://doi.org/10.4137/NMI.S32909

Marsit S, Dequin S (2015) Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. FEMS Yeast Res 15:1–12. https://doi.org/10.1093/femsyr/fov067

Martín M, Macías M, Escames G, Reiter RJ, Agapito MT, Ortiz GG, Acuña-Castroviejo D (2000) Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red *in vivo*. J Pineal Res 28:242–8

Maruca A, Catalano R, Bagetta D, Mesiti F, Ambrosio FA, Romeo I, Moraca F, Rocca R, Ortuso F, Artese A, Costa G, Alcaro S, Lupia A (2019) The mediterranean diet as source of bioactive compounds with multi-targeting anti-cancer profile. Eur J Med Chem 181:111579. https://doi.org/10.1016/j.ejmech.2019.111579

Mas A, Guillamon JM, Torija MJ, Beltran G, Cerezo AB, Troncoso AM, García-Parrilla MC (2014) Bioactive compounds derived from the yeast metabolism of aromatic amino acids during alcoholic fermentation. Biomed Res Int 2014:1-7. https://doi.org/10.1155/2014/898045

Maslov L, Jeromel A, Herjavec S, Korenika AMJ, Mihaljević M, Plavša T (2011) Indole-3-acetic acid and tryptophan in Istrian Malvasia grapes and wine. J Food, Agric Environ 9:29–33

Mateo EM, Gómez JV, Montoya N, Mateo-Castro R, Gimeno-Adelantado JV, Jiménez M, Doménech-Carbó A (2017) Electrochemical identification of toxigenic fungal species using solid-state voltammetry strategies. Food Chem 267:91-100. https://doi.org/10.1016/j.foodchem.2017.02.033

Maury J, Germann SM, Baallal Jacobsen SA, Jensen NB, Kildegaard KR, Herrgàrd MJ, Schneider K, Koza A, Forster J, Nielsen J, Borodina I (2016) EasyCloneMulti: A set of vectors for simultaneous and multiple genomic integrations in *Saccharomyces cerevisiae*. PLoS One 11:1–22. https://doi.org/10.1371/journal.pone.0150394

Maury J, Kannan S, Jensen NB, Öberg FK, Kildegaard KR, Forster J, Nielsen J, Workman CT, Borodina I (2018) Glucose-dependent promoters for dynamic regulation of metabolic pathways. Front Bioeng Biotechnol 6:1–12. https://doi.org/10.3389/fbioe.2018.00063

McKenna R, Thompson B, Pugh S, Nielsen DR (2014) Rational and combinatorial approaches toengineering styrene production by *Saccharomyces cerevisiae*. Microb Cell Fact 13:1–12. https://doi.org/10.1186/s12934-014-0123-2

Mediavilla MD, Sanchez-Barcelo EJ, Tan DX, Manchester L, Reiter RJ (2011) Basic mechanisms involved in the anti-cancer effects of melatonin. Curr Med Chem 17:4462–4481. https://doi.org/10.2174/092986710794183015

Mell JC, Burgess SM (2003) Yeast as a model genetic organism. eLS doi: 10.1038/npg.els.0000821

Mena P, Gil-Izquierdo Á, Moreno DA, Martí N, García-Viguera C (2012) Assessment of the melatonin production in pomegranate wines. LWT - Food Sci Technol 47:13–18. https://doi.org/10.1016/j.lwt.2012.01.009

Meng JF, Shi TC, Song S, Zhang ZW, Fang YL (2017) Melatonin in grapes and grape-related foodstuffs: A review. Food Chem 231:185–191. https://doi.org/10.1016/j.foodchem.2017.03.137

Mercolini L, Mandrioli R, Raggi MA (2012) Content of melatonin and other antioxidants in grape-related foodstuffs: Measurement using a MEPS-HPLC-F method. J Pineal Res 53:21–28. https://doi.org/10.1111/j.1600-079X.2011.00967.x

Mercolini L, Saracino MA, Bugamelli F, Ferranti A, Malaguti M, Hrelia S, Raggi MA (2008) HPLC-F analysis of melatonin and resveratrol isomers in wine using an SPE procedure. J Sep Sci 31:1007–1014

Milanovic V, Ciani M, Oro L, Comitini F (2012) *Starmerella bombicola* influences the metabolism of *Saccharomyces cerevisiae* at pyruvate decarboxylase and alcohol dehydrogenase level during mixed wine fermentation. Microb Cell Fact 11:10–13. https://doi.org/10.1186/1475-2859-11-18

Minussi RC, Rossi M, Bologna L, Cordi L, Rotilio D, Pastore GM, Durán N (2003) Phenolic compounds and total antioxidant potential of commercial wines. Food Chem 82:409–416. https://doi.org/10.1016/S0308-8146(02)00590-3

Minuti L, Pellegrino RM, Tesei I (2006) Simple extraction method and gas chromatography-mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine. J Chromatogr A 1114:263–268. https://doi.org/10.1016/j.chroma.2006.02.068

Mitsui R, Yamada R, Ogino H (2019) CRISPR system in the yeast *Saccharomyces cerevisiae* and its application in the bioproduction of useful chemicals. World J Microbiol Biotechnol 35:1–9. https://doi.org/10.1007/s11274-019-2688-8

Money NP (2018) The rise of yeast. How the sugar fungus shaped civilization. Oxford University Press, New York, USA

Morcillo-Parra MÁ (2019) Melatonin metabolism in yeast cells during alcoholic fermentation. Universitat Rovira i Virgili. PhD Thesis.

Morcillo-Parra MÁ, Beltran G, Mas A, Torija MJ (2020a) Effect of several nutrients and environmental conditions on intracellular melatonin synthesis in *Saccharomyces cerevisiae*. Microorganisms. https://doi.org/10.3390/microorganisms8060853

Morcillo-Parra MÁ, Beltran G, Mas A, Torija MJ (2019a) Determination of melatonin by a whole cell bioassay in fermented beverages. Sci Rep 9:3–10. https://doi.org/10.1038/s41598-019-45645-7

Morcillo-Parra MÁ, González B, Beltran G, Mas A, Torija MJ (2020b) Melatonin and glycolytic protein interactions are related to yeast fermentative capacity. Food Microbiol 87:103398. https://doi.org/10.1016/j.fm.2019.103398

Morcillo-Parra MÁ, Valera MJ, Beltran G, Mas A (2019b) Glycolytic proteins interact with intracellular melatonin in *Saccharomyces cerevisiae*. Front Microbiol 10: 2424. https://doi.org/10.3389/fmicb.2019.02424

Mortimer RK, Johnston JR (1986) Genealogy of principal strains of the yeast genetic stock center. Genetics 113:35–43.

Motlhalamme T (2020) Characterization of melatonin production and physiological functions in yeast. Stellenbosch University. PhD Thesis.

Mueller U, Hardeland R, Poeggler BFuhrberd B, Burkhardt, S (2001) Pathways of melatonin catabolism in the dinoflagellate *Gonyaulax polyedra*. Biol Rhythm Res 32:455-469.

Mülazimoğlu IE, Mülazimoğlu AD (2012) Investigation of sensitivity against different flavonoid derivatives of aminophenyl-modified glassy carbon sensor electrode and antioxidant activities. Food Anal Methods 5:1419–1426. https://doi.org/10.1007/s12161-012-9393-7

Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122. https://doi.org/10.1016/0378-1119(95)00037-7

Muriana FJG, Montserrat-De La Paz S, Lucas R, Bermúdez B, Jaramillo S, Morales JC, Abio R, López S (2017) Tyrosol and its metabolites as antioxidative and anti-inflammatory molecules in human endothelial cells. Food Funct 8:2905–2914. https://doi.org/10.1039/c7fo00641a

Muszyńska B, Ekiert H, Kwiecień I, Mašlanka A, Zodi R, Beerhues L (2014) Comparative analysis of therapeutically important indole compounds in *in vitro* cultures of *Hypericum perforatum* cultivars by HPLC and TLC analysis coupled with densitometric detection. Nat Prod Commun 9:1437–1440. https://doi.org/10.1177/1934578x1400901009

Muszyńska B, Sułkowska-Ziaja K (2012) Analysis of indole compounds in edible *Basidiomycota* species after thermal processing. Food Chem 132:455–459. https://doi.org/10.1016/j.foodchem.2011.11.021

Nandy SK, Srivastava RK (2018) A review on sustainable yeast biotechnological processes and applications. Microbiol Res 207:83–90. https://doi.org/10.1016/j.micres.2017.11.013

Naseeb S, James SA, Alsammar H, Michaels CJ, Gini B, Nueno-Palop C, Bond CJ, McGhie H, Roberts IN, Delneri D (2017*) Saccharomyces jurei* sp. nov., isolation and genetic identification of a novel yeast species from *Quercus robur.* Int J Syst Evol Microbiol 67, 2046–2052.

Nasmyth KA (1982) Molecular Genetics of yeast mating type. Annu Rev Genet 16:439–500. https://doi.org/10.1146/annurev.ge.16.120182.002255

Neiman AM (2011) Sporulation in the budding yeast *Saccharomyces cerevisiae*. Genetics 189:737–765. https://doi.org/10.1534/genetics.111.127126

Niedens BR, Parker SR, Stierle DB, Stierle AA (2013) First fungal aromatic L-amino acid decarboxylase from a paclitaxel-producing *Penicillium raistrickii*. Mycologia 91:619–626. https://doi.org/10.1111/crj.12516

Nielsen J (2017) Systems biology of metabolism. Annu Rev Biochem 86:245–275. https://doi.org/10.1146/annurev-biochem-061516-044757

Nielsen J (2019) Yeast systems biology: Model organism and cell factory. Biotechnol J 1800421. https://doi.org/10.1002/biot.201800421

Nielsen J, Keasling JD (2011) Synergies between synthetic biology and metabolic engineering. Nat Biotechnol 29:693–695. https://doi.org/10.1038/nbt.1937

Nielsen J, Larsson C, van Maris A, Pronk J (2013) Metabolic engineering of yeast for production of fuels and chemicals. Curr Opin Biotechnol 24:398–404. https://doi.org/10.1016/j.copbio.2013.03.023

Nisbet MA, Tobias HJ, Brenna JT, Sacks GL, Mansfiel AK (2014) Quantifying the contribution of grape hexoses to wine volatiles by high-precision [$U^{13}C$]-glucose tracer studies. J Agric Food Chem 62:6820–6827. https://doi.org/10.1021/jf500947x

Noé W, Mollenschott C, Berlin J (1984) Tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures: purification, molecular and kinetic data of the homogenous protein. Plant Mol Biol 3:281–288

Nora LC, Westmann CA, Guazzaroni M-E, Siddaiah C, Gupta VK, Silva-Rocha R (2019) Recent advances in plasmid-based tools for establishing novel microbial chassis. Biotechnol Adv 37:107433. https://doi.org/10.1016/j.biotechadv.2019.107433

Nour-Eldin HH, Hansen BG, Nørholm MHH, Jensen JK, Halkier BA (2006) Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. Nucleic Acids Res 34:e122. https://doi.org/10.1093/nar/gkl635

Öberg F, Sjöhamn J, Conner MT, Bill RM, Hedfalk K (2011) Improving recombinant eukaryotic membrane protein yields in *Pichia pastoris*: The importance of codon optimization and clone selection. Mol Membr Biol 28:398–411. https://doi.org/10.3109/09687688.2011.602219

Oladi E, Mohamadi M, Shamspur T, Mostafavi A (2014) Spectrofluorimetric determination of melatonin in kernels of four different *Pistacia varieties* after ultrasound-assisted solid-liquid extraction. Spectrochim Acta - Part A Mol Biomol Spectrosc 132:326–329. https://doi.org/10.1016/j.saa.2014.05.010

Olivier B (2015) Serotonin: A never-ending story. Eur J Pharmacol 753:2–18. https://doi.org/10.1016/j.ejphar.2014.10.031

Ooi BG, Wanamaker LE, Markuszewski BM, Chong NS (2008) Genetic and enological analysis of selected *Saccharomyces cerevisiae* strains for wine production. Int J Food Sci Technol 43:1111–1120. https://doi.org/10.1111/j.1365-2621.2007.01577.x

Ortiz-Miranda AS, König P, Kahlert H, Scholz F, Osete-Cortina L, Doménech-Carbó MT, Doménech-Carbó A (2016) Voltammetric analysis of *Pinus* needles with physiological, phylogenetic, and forensic applications. Anal Bioanal Chem 408:4943–4952. https://doi.org/10.1007/s00216-016-9588-7

Owsiak A, Bartosz G, Bilinski T (2010) Oxidative stress during aging of the yeast in a stationary culture and its attenuation by antioxidants. Cell Biol Int 34:731–736. https://doi.org/10.1042/CBI20100134

Oxenkrug G, Requintina P, Bachurin S (2001) Antioxidant and antiaging activity of *N*-acetylserotonin and melatonin in the *in vivo* models. Ann N Y Acad Sci 939:190–9. DOI: 10.1111/j.1749-6632.2001.tb03626.x

Padilla B, Gil J V., Manzanares P (2016) Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Front Microbiol 7:1–20. https://doi.org/10.3389/fmicb.2016.00411

Palamand SR, Grigsby JH (1974) Stale flavors in beer: identification of o-aminoacetophenone and ethyl nicotinate in beer. Brew Dig 49:58–59.

Pan X, Zhu L, Lu H, Wang D, Lu Q, Yan H (2015) Melatonin attenuates oxidative damage induced by acrylamide *in vitro* and *in vivo*. Oxid Med Cell Longev 2015:1-12. https://doi.org/10.1155/2015/703709

Pape C, Lüning K (2006) Quantification of melatonin in phototrophic organisms. J Pineal Res 41:157–165.

Paredes SD, Korkmaz A, Manchester LC, Tan DX, Reiter RJ (2009) Phytomelatonin: A review. J Exp Bot 60:57–69. https://doi.org/10.1093/jxb/ern284.

Parekh RN, Shaw MR, Dane Wittrup K (1996) An integrating vector for tunable, high copy, stable integration into the dispersed Ty δ sites of *Saccharomyces cerevisiae*. Biotechnol Prog 12:16–21. https://doi.org/10.1021/bp9500627

Park J, Lee S, Choi J, Ahn K, Park B, Park J, Kang S, Lee YH (2008a) Fungal cytochrome P450 database. BMC Genomics 9:402. https://doi.org/10.1186/1471-2164-9-402

Park M, Kang K, Park S, Back K (2008b) Conversion of 5-hydroxytryptophan into serotonin by tryptophan decarboxylase in plants, *Escherichia coli*, and yeast. Biosci Biotechnol Biochem 72:2456–2458. https://doi.org/10.1271/bbb.80220

Park S, Byeon Y, Back K (2013) Transcriptional suppression of tryptamine 5-hydroxylase, a terminal serotonin biosynthetic gene, induces melatonin biosynthesis in rice (*Oryza sativa* L.). J Pineal Res 55:131–137. https://doi.org/10.1111/jpi.12053

Park S, Byeon Y, Lee K, Kang K, Back K (2012) Molecular cloning of rice serotonin *N*-acetyltransferase, the penultimate gene in plant melatonin biosynthesis. J Pineal Res 55:7–13. https://doi.org/10.1111/jpi.12011

Park S, Kang K, Lee SW, Ahn MJ, Bae JM, Back K (2011) Production of serotonin by dual expression of tryptophan decarboxylase and tryptamine 5-hydroxylase in *Escherichia coli*. Appl Microbiol Biotechnol 89:1387–1394

Paroni R, Dei Cas M, Rizzo J, Ghidoni R, Montagna MT, Rubino FM, Iriti M (2019) Bioactive phytochemicals of tree nuts. Determination of the melatonin and sphingolipid content in almonds and pistachios. J Food Compos Anal 82:103227. https://doi.org/10.1016/j.jfca.2019.05.010

Parsons AB, López A, Givoni IE, Williams DE, Gray CA, Porter J, Chua G, Sopko R, Brost RL, Ho CH, Wang J, Ketela T, Brenner C, Brill JA, Fernández GE, Lorenz TC, Payne GS, Ishihara S, Ohya Y, Andrews B, Hughes TR, Frey BJ, Graham TR, Andersen RJ, Boone C (2006) Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. Cell 126:611–625. https://doi.org/10.1016/j.cell.2006.06.040

Pasteur L (1859) Nouveaux faits concernant la fermentation alcoolique. Comptes Rendus Chim 48:640-642.

Pearson WR (2013) An introduction to sequence similarity ("homology") searching. Curr Protoc Bioinforma 42:3.1.1-3.1.8. https://doi.org/10.1002/0471250953.bi0301s42.An

Pelagio-Flores R, López-Bucio J (2016) Serotonin and melatonin in plant growth and development. In: Ravishankar GA, Ramafrishna A (eds) Serotonin and Melatonin 97–110.

Peng C, Andersen B, Arshid S, Larsen MR, Albergaria H, Lametsch R, Arneborg N (2019) Proteomics insights into the responses of *Saccharomyces cerevisiae* during mixed-culture alcoholic fermentation with *Lachancea thermotolerans*. FEMS Microbiol Ecol 95:1–16. https://doi.org/10.1093/femsec/fiz126

Peng C, Viana T, Petersen MA, Larsen FH, Arneborg N, (2018) Metabolic footprint analysis of metabolites that discriminate single and mixed yeast cultures at two key time-points during mixed culture alcoholic fermentations. Metabolomics 14:0. https://doi.org/10.1007/s11306-018-1391-3

Pérez-Coello MS, Díaz-Maroto MC (2009) Volatile compounds and wine aging. In: Polo C, Moreno-Arribas MV (eds) Wine Chemistry and Biochemistry.Springer, New York, NY. https://doi.org/10.1007/978-0-387-74118-5_16

Pérez-González A, Castañeda-Arriaga R, Álvarez-Idaboy JR, Reiter RJ, Galano A (2018) Melatonin and its metabolites as chemical agents capable of directly repairing oxidized DNA. J Pineal Res e12539. https://doi.org/10.1111/jpi.12539

Pérez-Torrado R, Rantsiou K, Perrone B, Navarro-Tapia E, Querol A, Cocolin L (2017) Ecological interactions among *Saccharomyces cerevisiae* strains: Insight into the dominance phenomenon. Sci Rep 7:1–10. https://doi.org/10.1038/srep43603

Peris D, Belloch C, Lopandić K, Álvarez-Pérez JM, Querol A, Barrio E (2012) The molecular characterization of new types of *Saccharomyces cerevisiae* x *S. kudriavzevii* hybrid yeasts unveils a high genetic diversity. 29:81–91. https://doi.org/10.1002/yea

Peris D, Sylvester K, Libkind D, Gonçalves P, Sampaio JP, Alexander WG, Hittinger CT (2014) Population structure and reticulate evolution of Saccharomyces eubayanus and its lager-brewing hybrids. Mol Ecol 23:2031–2045. https://doi.org/10.1111/mec.12702

Pfeiffer T, Morley A (2014) An evolutionary perspective on the Crabtree effect. Front Mol Biosci 1:1–6. https://doi.org/10.3389/fmolb.2014.00017

Piekarska I, Rytka J, Rempola B (2010) Regulation of sporulation in the yeast *Saccharomyces cerevisiae*. Acta Biochim Pol 57:241–250

Piñeiro Z, Cantos-Villar E, Palma M, Puertas B (2011) Direct liquid chromatography method for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines. J Agric Food Chem 59:11683–11689. https://doi.org/10.1021/jf202254t

Piškur J, Rozpedowska E, Polakova S, Merico A, Compagno C (2006) How did *Saccharomyces* evolve to become a good brewer? Trends Genet 22:183–186. https://doi.org/10.1016/j.tig.2006.02.002

Poeggeler B, Hardeland R (2003) Non-vertebrate melatonin. J Pineal Res 34:233–241

Poeggeler B, Hardeland R (1994) Detection and quantification of melatonin in a dinoflagellate, *Gonyaulax polyedra*: Solutions to the problem of methoxyindole destruction in non-vertebrate material. J Pineal Res 17:1–10. https://doi.org/10.1111/j.1600-079X.1994.tb00106.x

Pretorius I (2016) Conducting wine symphonics with the aid of yeast genomics. Beverages 2:36. https://doi.org/10.3390/beverages2040036

Pretorius IS (2000) Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16:675-729.

Pretorius IS (2017) Synthetic genome engineering forging new frontiers for wine yeast. Crit Rev Biotechnol 37:112–136. https://doi.org/10.1080/07388551.2016.1214945

Pretorius IS, Boeke JD (2018) Yeast 2.0-connecting the dots in the construction of the world's first functional synthetic eukaryotic genome. FEMS Yeast Res 18:1–15. https://doi.org/10.1093/femsyr/foy032

Pretorius IS, van der Westhuizen T, Augustyn OP. (1999) Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. A Review. South African J Enol Vitic 20:61–70

Prieto MA, Perez-Aranda A, Garcia JL (1993) Characterization of an *Escherichia coli* aromatic hydroxylase with a broad substrate range. J Bacteriol 175:2162–2167. https://doi.org/10.1128/jb.175.7.2162-2167.1993

Proestos C, Bakogiannis A, Psarianos C, Koutinas AA, Kanellaki M, Komaitis M (2005) High performance liquid chromatography analysis of phenolic substances in Greek wines. Food Control 16:319–323. https://doi.org/10.1016/j.foodcont.2004.03.011

Pronk JT (2002) Auxotrophic yeast strains in fundamental and applied research. Appl Environ Microbiol 68:2095–2100. https://doi.org/10.1128/AEM.68.5.2095

Prusty R, Grisafi P, Fink GR (2004) The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proc Natl Acad Sci 101:4153–4157. https://doi.org/10.1073/pnas.0400659101

Qu W, Wang F, Hu S, Cui D (2005) Electrocatalytic properties and voltammetric determination of melatonin at a nanostructured film electrode. Microchim Acta 150:109–114. https://doi.org/10.1007/s00604-005-0328-5

Quehl P, Hollender J, Schüürmann J, Brossette T, Maas R, Jose J (2016) Co-expression of active human cytochrome P450 1A2 and cytochrome P450 reductase on the cell surface of *Escherichia coli.* Microb Cell Fact 15:26. https://doi.org/10.1186/s12934-016-0427-5

Querol A, Bond U (2009) The complex and dynamic genomes of industrial yeasts: Minireview. FEMS Microbiol Lett 293:1–10. https://doi.org/10.1111/j.1574-6968.2008.01480.x

Quirós M, Rojas V, Gonzalez R, Morales P (2014) Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. Int J Food Microbiol 181:85–91. https://doi.org/10.1016/j.ijfoodmicro.2014.04.024

Radi A, Bekhiet GE (1998) Voltammetry of melatonin at carbon electrodes and determination in capsules. Bioelectrochemistry Bioenerg 45:275–279

Raes J, Bork P (2008) Molecular eco-systems biology: Towards an understanding of community function. Nat Rev Microbiol 6:693–699. https://doi.org/10.1038/nrmicro1935

Rantasalo A, Landowski CP, Kuivanen J, Korppoo A, Reuter L, Koivistoinen O, Valkonen M, Penttilä M, Jäntti J, Mojzita D (2018) A universal gene expression system for fungi. Nucleic Acids Res 46: e111. https://doi.org/10.1093/nar/gky558

Rantsiou K, Dolci P, Giacosa S, Torchio F, Tofalo R, Torriani S, Suzzi G, Rolle L, Cocolina L (2012) *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl Environ Microbiol 78:1987–1994. https://doi.org/10.1128/AEM.06768-11

Raposo R, Ruiz-Moreno MJ, Garde-Cerdán T, Puertas B, Moreno-Rojas JM, Gonzalo-Diago A, Guerrero RF, Ortiz V, Cantos-Villar E (2015) Effect of hydroxytyrosol on quality of sulfur dioxide-free red wine. Food Chem 192:25–33. https://doi.org/10.1016/j.foodchem.2015.06.085

Raposo R, Ruiz-Moreno MJ, Garde-Cerdán T, Puertas B, Moreno-Rojas JM, Zafrilla P, Gonzalo-Diago A, Guerrero RF, Cantos-Villar E (2016) Replacement of sulfur dioxide by hydroxytyrosol in white wine: Influence on both quality parameters and sensory. LWT - Food Sci Technol 65:214–221. https://doi.org/10.1016/j.lwt.2015.08.005

Raschmanová H, Weninger A, Glieder A, Kovar K, Vogl T (2018) Implementing CRISPR-Cas technologies in conventional and non-conventional yeasts: Current state and future prospects. Biotechnol Adv 36:641–665. https://doi.org/10.1016/j.biotechadv.2018.01.006

Rebollo-Romero I, Fernández-cruz E, Carrasco-galán F, Valero E, Cantos-Villar E, Cerezo AB, Troncoso AM, Garcia-Parrilla MC (2020) Factors influencing the production of the antioxidant hydroxytyrosol during alcoholic fermentation: Yeast strain, initial tyrosine concentration and initial must. LWT - Food Sci Technol 130:109631. https://doi.org/10.1016/j.lwt.2020.109631

Reifenrath M, Bauer M, Oreb M, Boles E (2018) Bacterial bifunctional chorismate mutase-prephenate dehydratase *PheA* increases flux into the yeast phenylalanine pathway and improves mandelic acid production. Metab Eng Commun 7:e00079 . https://doi.org/10.1016/j.mec.2018.e00079

Reifenrath M, Boles E (2018) Engineering of hydroxymandelate synthases and the aromatic amino acid pathway enables de novo biosynthesis of mandelic and 4-hydroxymandelic acid with *Saccharomyces cerevisiae*. Metab Eng 45:246–254. https://doi.org/10.1016/j.ymben.2018.01.001

Reinmuth WH (1962) Theory of diffusion limited charge-transfer processes in electroarialytical techniques. Methods 34:1446–1454

Reinmuth WH (1960) Inversible systems in stationary electrode polarography. Anal Chem 32:1891–1982

Reiter, RJ.; Tan, DX; Rosales-Corral, S; Manchester L (2013) The universal nature, unequal distribution and antioxidant funtions of melatonin and its derivatives. Mini-Reviews Med Chem 13:373–384

Reiter JR, Tan DX, Qi W, Manchester LC, Karbownik M, Calvo JR (2000) Pharmacology and physiology of melatonin in the reduction of oxidative stress *in vivo*. NeuroSignals 9:160–171. https://doi.org/10.1159/000014636

Reiter RJ, Ma Q, Sharma R (2020) Treatment of Ebola and other infectious diseases: melatonin " goes viral ." Melatonin Res 3:43–57. https://doi.org/10.32794/mr11250047

Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B (2017) Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. Cell Mol Life Sci. https://doi.org/10.1007/s00018-017-2609-7

Reiter RJ, Tan DX, Manchester LC, Qi W (2001) Biochemical reactivity of melatonin with reactive a review of the evidence. Cell Biochem Biophys 34:237–256

Reiter RJ, Tan DX (2003) Melatonin: A novel protective agent against oxidative injury of the ischemic/reperfused heart. Cardiovasc Res 58:10–19. https://doi.org/10.1016/S0008-6363(02)00827-1

Reiter RJ, Tan DX, Manchester LC, Terron MP, Flores LJ, Koppisepi S (2007) Medical implications of melatonin: receptor-mediated and receptor-independent actions. Adv Med Sci 52:11–28.

Reiter RJ, Tan DX, Rosales-Corral S, Galano A, Zhou XJ, Xu B (2018) Mitochondria: Central organelles for melatonins antioxidant and anti-aging actions. Molecules 23:1–25. https://doi.org/10.3390/molecules23020509

Renaud S, Lorgeril M De (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339:1523–1526. https://doi.org/10.1016/0140-6736(92)91277-F

Restuccia D, Loizzo MR, Spizzirri UG (2018) Accumulation of biogenic amines in wine: Role of alcoholic and malolactic fermentation. Fermentation 4:1–18. https://doi.org/10.3390/fermentation4010006

Ribéreau-Gayon P, Dubourdieu D, Donèche B, Lonvaud A (2006) Biochemistry of alcoholic fermentation and metabolic pathways of wine yeasts. In: Ribéreau-Gayon P, Dubourdieu D, Donèche B, Lonvaud A (eds) Handbook of Enology: The microbiology of wine and vinifications. John Wiley & Sons, Ltd, Chichester, UK, pp 53-77. https://doi.org/10.1002/0470010363.ch2

Robles-Almazan M, Pulido-Moran M, Moreno-Fernandez J, Ramírez-Tortosa C, Rodríguez-García C, Quiles JL, Ramírez-Tortosa MC (2018) Hydroxytyrosol: Bioavailability, toxicity, and clinical applications. Food Res Int 105:654–667. https://doi.org/10.1016/j.foodres.2017.11.053

Rodríguez-Gutiérrez G, Wood S, Fernández-Bolaños Guzmán J, Duthie GG, De Roos B (2011) Determination of 3,4-dihydroxyphenylglycol, hydroxytyrosol and tyrosol purified from olive oil by-products with HPLC in animal plasma and tissues. Food Chem 126:1948–1952. https://doi.org/10.1016/j.foodchem.2010.12.044

Rodríguez-Morató J, Robledo P, Tanner JA, Boronat A, Pérez-Mañá C, Oliver Chen CY, Tyndale RF, de la Torre R (2017) CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol. Food Chem 217:716–725. https://doi.org/10.1016/j.foodchem.2016.09.026

Rodriguez-Naranjo MI, Gil-izquierdo A, Troncoso AM, Cantos-Villar E, Garcia-Parrilla MC (2011a) Melatonin is synthesised by yeast during alcoholic fermentation in wines. Food Chem 126:1608–1613. DOI: 10.1016/j.foodchem.2010.12.038

Rodriguez-Naranjo MI, Gil-Izquierdo A, Troncoso AM, Cantos-Villar E, Garcia-Parrilla MC (2011b) Melatonin: A new bioactive compound in wine. J Food Compos Anal 24:603–608

Rodriguez-naranjo MI, Ordóñez JL, Callejón RM, Cantos-Villar E, Garcia-Parrilla MC (2013) Melatonin is formed during winemaking at safe levels of biogenic amines. Food Chem Toxicol 57:140–146

Rodriguez-Naranjo MI, Torija MJ, Mas A, Cantos-Villar E, Garcia-Parrilla MC (2012) Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions. J Pineal Res 53:219–224. https://doi.org/10.1111/j.1600-079X.2012.00990.x

Rodriguez A, Kildegaard KR, Li M, Borodina I, Nielsen J (2015) Establishment of a yeast platform strain for production of *p*-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. Metab Eng 31:181-188. https://doi.org/10.1016/j.ymben.2015.08.003

Rodriguez C, Mayo JC, Sainz RM, Antolín I, Herrera F, Martín V, Reiter RJ (2004) Regulation of antioxidant enzymes: A significant role for melatonin. J Pineal Res 36:1–9. https://doi.org/10.1046/j.1600-079X.2003.00092.x

Røkke G, Korvald E, Pahr J, Oyås O, Lale R (2014) biobrick assembly standards and techniques and associated software tools. Methods Mol Biol 1116:1-24. doi: 10.1007/978-1-62703-764-8_1.

Rollero S, Bloem A, Ortiz-Julien A, Camarasa C, Divol B (2018) Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. Front Microbiol 9:1–12. https://doi.org/10.3389/fmicb.2018.00196

Romboli Y, Mangani S, Buscioni G, Granchi L, Vincenzini M (2015) Effect of *Saccharomyces cerevisiae* and *Candida zemplinina* on quercetin, vitisin A and hydroxytyrosol contents in Sangiovese wines. World J Microbiol Biotechnol 31:1137–1145. https://doi.org/10.1007/s11274-015-1863-9

Romboli Y, Mangani S, Buscioni G, Vincenzini M (2013) Variability of tyrosol, hydroxytyrosol and tryptophol contents in Sangiovese wines produced by a single strain of *Saccharomyces cerevisiae*. In: Enoforum 2013. https://www.infowine.com/intranet/libretti/libretto11581-01-1.pdf

Rosazza JP, Juhl R, Davis P (1973) Tryptophol formation by *Zygosaccharomyces priorianus*. Appl Microbiol 26:98–105. https://doi.org/10.1093/library/s1-1.1.313

Roseboom PH, Namboodiri M a. A, Zimonjic DB, Popescu NC, Rodriguez I, Gastel JA, Klein DC (1998) Natural melatonin "knockdown" in C57BL/6J mice: Rare mechanism truncates serotonin *N*-acetyltransferase. Mol Brain Res 63:189–197. https://doi.org/10.1016/S0169-328X(98)00273-3

Rossouw D, Bagheri B, Setati ME, Bauer FF (2015) Co-flocculation of yeast species, a new mechanism to govern population dynamics in microbial ecosystems. PLoS One 10:1–17. https://doi.org/10.1371/journal.pone.0136249

Rossouw D, Du Toit M, Bauer FF (2012) The impact of co-inoculation with *Oenococcus oeni* on the trancriptome of *Saccharomyces cerevisiae* and on the flavour-active metabolite profiles during fermentation in synthetic must. Food Microbiol 29:121–131. https://doi.org/10.1016/j.fm.2011.09.006

Rost B (1999) Twilight zone of protein sequence alignments. Protein Eng 12:85–94. https://doi.org/10.1093/protein/12.2.85

Ruiz-Moreno MJ, Raposo R, Moreno-Rojas JM, Zafrilla P, Cayuela JM, Mulero J, Puertas B, Guerrero RF, Piñeiro Z, Giron F (2015) Efficacy of olive oil mill extract in replacing sulfur dioxide in wine model. LWT - Food Sci Technol 61:117–123. https://doi.org/10.1016/j.lwt.2014.11.024

Ryan OW, Skerker JM, Maurer MJ, Li X, Tsai JC, Poddar S, Lee ME, DeLoache W, Dueber JE, Arkin AP, Cate JHD (2014) Selection of chromosomal DNA libraries using a multiplex CRISPR system. Elife 3:1–15. https://doi.org/10.7554/eLife.03703

Saba JD, Nara F, Bielawska A, Garrett S, Hannun YA (1997) The *BST1* gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. J Biol Chem 272:26087–26090. https://doi.org/10.1074/jbc.272.42.26087

Sabel A, Bredefeld S, Schlander M, Claus H (2017) Wine phenolic compounds: Antimicrobial properties against yeasts, lactic acid and acetic acid bacteria. Beverages 3:1–14. https://doi.org/10.3390/beverages3030029

Saber AL (2010) Novel potentiometric sensors for determination of melatonin and oxomemazine in biological samples and in pharmaceutical formulations. Electroanalysis 22:2997–3002

Sadoudi M, Tourdot-Maréchal R, Rousseaux S, Steyer D, Gallardo-Chacón JJ, Ballester J, Vichi S, Guérin-Schneider R, Caixach J, Alexandre H (2012) Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol 32:243–253. https://doi.org/10.1016/j.fm.2012.06.006

Safavi A, Momeni S (2010) Electrocatalytic oxidation of tryptophan at gold nanoparticle-modified carbon ionic liquid electrode. Electroanalysis 22:2848–2855

Salinas F, Rojas V, Delgado V, López J, Agosin E, Larrondo LF (2018) Fungal light-oxygen-voltage domains for optogenetic control of gene expression and flocculation in yeast. MBio 9:1–14. https://doi.org/10.1128/mBio.00626-18

Salinas F, Rojas V, Delgado V, Agosin E, Larrondo LF (2017) Optogenetic switches for light-controlled gene expression in yeast. Appl Microbiol Biotechnol 101:2629–2640. https://doi.org/10.1007/s00253-017-8178-8

Salvadó Z, Chiva R, Rozès N, Cordero-Otero R, Guillamón JM (2012) Functional analysis to identify genes in wine yeast adaptation to low-temperature fermentation. J Appl Microbiol 113:76–88. https://doi.org/10.1111/j.1365-2672.2012.05308.x

Santos MC, Nunes C, Saraiva JA, Coimbra MA (2012a) Chemical and physical methodologies for the replacement/reduction of sulfur dioxide use during winemaking: Review of their potentialities and limitations. Eur Food Res Technol 234:1–12. https://doi.org/10.1007/s00217-011-1614-6

Santos MM, Piccirillo C, Castro PML, Kalogerakis N, Pintado ME (2012b) Bioconversion of oleuropein to hydroxytyrosol by lactic acid bacteria. World J Microbiol Biotechnol 28:2435–2440. https://doi.org/10.1007/s11274-012-1036-z

Satoh Y, Tajima K, Munekata M, Keasling JD, Lee TS (2012) Engineering of l-tyrosine oxidation in *Escherichia coli* and microbial production of hydroxytyrosol. Metab Eng 14:603–610. https://doi.org/10.1016/j.ymben.2012.08.002

Schmid-Burgk JL, Xie Z, Benenson Y (2013) Hierarchical ligation-independent assembly of PCR Fragments. In: Valla S, Lale R (eds) DNA cloning and assembly methods:Methods in molecular biology 1116. Springer protocols, pp 49–54

Schmidheini T, Sperisen P, Paravicini G, Hutter R, Braus G (1989) A single point mutation results in a constitutively activated and feedback-resistant chorismate mutase of *Saccharomyces cerevisiae*. J Bacteriol 171:1245–1253. DOI: 10.1128/jb.171.3.1245-1253.1989

Semkiv M V., Dmytruk K V., Sibirny AA (2016) Development of a system for multicopy gene integration in *Saccharomyces cerevisiae*. J Microbiol Methods 120:44–49. https://doi.org/10.1016/j.mimet.2015.10.023

Sentheshanmuganathan S, Elsden SR (1958) The mechanism of the formation of tyrosol by *Saccharomyces cerevisiae*. Biochem J 69:210–8. https://doi.org/10.1042/bj0690210

Sha W, Martins AM, Laubenbacher R, Mendes P, Shulaev V (2013) The genome-wide early temporal response of *Saccharomyces cerevisiae* to oxidative stress induced by cumene hydroperoxide. PLoS One 8:1–15. https://doi.org/10.1371/journal.pone.0074939

Shaw WM, Yamauchi H, Mead J, Gowers GOF, Bell DJ, Öling D, Larsson N, Wigglesworth M, Ladds G, Ellis T (2019) Engineering a model cell for rational tuning of GPCR signaling. Cell 177:782-796.e27. https://doi.org/10.1016/j.cell.2019.02.023

Shellard SA, Whelan R, Hill BT (1989) Growth inhibitory and cytotoxic effects of melatonin and its metabolites on human tumour cell lines *in vitro*. Br J Cancer 60:288–290. https://doi.org/10.1038/bjc.1989.272

Shen L, Nishimura Y, Matsuda F, Ishii J, Kondo A (2016) Overexpressing enzymes of the Ehrlich pathway and deleting genes of the competing pathway in *Saccharomyces cerevisiae* for increasing 2-phenylethanol production from glucose. J Biosci Bioeng 122:34–39. https://doi.org/10.1016/j.jbiosc.2015.12.022

Siddiqui MS, Choksi A, Smolke CD (2014) A system for multi-locus chromosomal integration and transformation-free selection marker rescue. FEMS Yeast Res 14:1171–1185. https://doi.org/10.1038/jid.2014.371

Siewers V (2014) An overview on selection marker genes for transformation of *Saccharomyces cerevisiae*. In: Mapelli V (ed) Yeast metabolic engineering: Methods and protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 3–16

Sikorski RC, Philip H (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces ceratisiae*. Genetics 122:19–27. https://doi.org/10.1007/978-3-540-72584-8_65

Silva LR, Andrade PB, Valentão P, Seabra RM, Trujillo ME, Velázquez E (2005) Analysis of non-coloured phenolics in red wine: Effect of *Dekkera bruxellensis* yeast. Food Chem 89:185–189. https://doi.org/10.1016/j.foodchem.2004.02.019

Sinah N, Williams CA, Piper RC, Shields SB (2012) A set of dual promoter vectors for high throughput cloning, screening, and protein expression in eukaryotic and prokaryotic systems from a single plasmid. BMC Biotechnol 12:1. https://doi.org/10.1186/1472-6750-12-54

Singh M V., Weil PA (2002) A method for plasmid purification directly from yeast. Anal Biochem 307:13–17. https://doi.org/10.1016/S0003-2697(02)00018-0

Skaggs BA, Alexander JF, Pierson CA, Schweitzer KS, Chun KT, Koegel C, Barbuch R, Bard M (1996) Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. Gene 169:105–109. https://doi.org/10.1016/0378-1119(95)00770-9

Skobowiat C, Brożyna AA, Janjetovic Z, Jeayeng S, Oak ASW, Kim TK, Panich U, Reiter RJ, Slominski AT (2018) Melatonin and its derivatives counteract the ultraviolet B radiation-induced damage in human and porcine skin *ex vivo*. J Pineal Res 65:1–16. https://doi.org/10.1111/jpi.12501

Soejima H, Tsuge K, Yoshimura T, Sawada K, Kitagaki H (2012) Breeding of a high tyrosol-producing sake yeast by isolation of an ethanol-resistant mutant from a *trp3* mutant. J Inst Brew 118:264–268. https://doi.org/10.1002/jib.46

Sokolov SS, Trushina NI, Severin FF, Knorre DA (2019) Ergosterol turnover in yeast: An interplay between biosynthesis and transport. Biochem 84:346–357. https://doi.org/10.1134/S0006297919040023

Sompol P, Liu X, Baba K, Paul KN, Tosini G, Iuvone PM, Ye K (2011) *N*-acetylserotonin promotes hippocampal neuroprogenitor cell proliferation in sleep-deprived mice. Proc Natl Acad Sci USA 108:8844–8849. https://doi.org/10.1073/pnas.1105114108

Spencer JFT, Spencer DM (1983) Genetic improvement of industrial yeast. Annu Rev Microbiol 37:121–142. doi: 10.1146/annurev.mi.37.100183.001005.

Sprenger J, Hardeland R (1999) Melatonin and 5-methoxytryptamine in yeast: Requirement of precursors. In: Hardeland R (ed) Studies on antioxidants and their metabolites. Cuvillier Verlag, Göttingen, Germany, pp 191–198

Sprenger J, Hardeland R, Fuhrberg B, Han SZ (1999) Melatonin and other 5-methoxylated indole in yeast: presence in hight concentrations and dependence on tryptophan availability. Cytologia 64:209–213. https://doi.org/https://doi.org/10.1508/cytologia.64.209

Stege PW, Sombra LL, Messina G, Martinez LD, Silva MF (2010) Determination of melatonin in wine and plant extracts by capillary electrochromatography with immobilized carboxylic multi-walled carbon nanotubes as stationary phase. Electrophoresis 31:2242–2248. https://doi.org/10.1002/elps.200900782

Stephanopoulos G (2012) Synthetic biology and metabolic engineering. ACS Synth Biol 1:514–525. https://doi.org/10.1021/sb300094q

Storici F, Coglievina M, Bruschi C V. (1999) A 2-μm DNA-based marker recycling system for multiple gene disruption in the yeast *Saccharomyces cerevisiae*. Yeast 15:271–283. https://doi.org/10.1002/(SICI)1097-0061(19990315)15:4<271::AID-YEA371>3.0.CO;2-U

Stovicek V, Borja GM, Forster J, Borodina I (2015) EasyClone 2.0: expanded toolkit of integrative vectors for stable gene expression in industrial *Saccharomyces cerevisiae* strains. J Ind Microbiol Biotechnol 42:1519–1531. https://doi.org/10.1007/s10295-015-1684-8

Stovicek V, Holkenbrink C, Borodina I (2017) CRISPR/Cas system for yeast genome engineering: advances and applications. FEMS Yeast Res 17:1–16. https://doi.org/10.1093/femsyr/fox030

Strakhovskaia M, Serdalina A, Fraĭkin Gi (1983) Effect of the photo-induced synthesis of serotonin on the photoreactivation of *Saccharomyces cerevisiae* yeasts. Nauchnye Doki Vyss Shkoly Biol Nauk 3:25–28

Strucko T, Buron LD, Jarczynska ZD, Nødvig CS, Mølgaard L, Halkier BA, Mortensen UH (2017) CASCADE, a platform for controlled gene amplification for high, tunable and selection-free gene expression in yeast. Sci Rep 7: 41431. https://doi.org/10.1038/srep41431

Strucko T, Magdenoska O, Mortensen UH (2015) Benchmarking two commonly used *Saccharomyces cerevisiae* strains for heterologous vanillin-β-glucoside production. Metab Eng Commun 2:99–108. https://doi.org/10.1016/j.meteno.2015.09.001

Styger G, Prior B, Bauer FF (2011) Wine flavor and aroma. J Ind Microbiol Biotechnol 38:1145–1159. https://doi.org/10.1007/s10295-011-1018-4

Su Y, Gamero A, Rodríguez ME, Lopes CA, Querol A, Guillamón JM (2019) Interspecific hybridisation among diverse *Saccharomyces* species: A combined biotechnological solution for low-temperature and nitrogen-limited wine fermentations. Int J Food Microbiol 310:108331. https://doi.org/10.1016/j.ijfoodmicro.2019.108331

Su Y, Seguinot P, Sanchez I, Ortiz-Julien A, Heras JM, Querol A, Camarasa C, Guillamón JM (2020) Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions. Food Microbiol 85:103287. https://doi.org/10.1016/j.fm.2019.103287

Suástegui M, Guo W, Feng X, Shao Z (2016) Investigating strain dependency in the production of aromatic compounds in *Saccharomyces cerevisiae*. Biotechnol Bioeng 113:2676–2685. https://doi.org/10.1002/bit.26037

Suástegui M, Yu Ng C, Chowdhury A, Sun W, Cao M, House E, Maranas CD, Shao Z (2017) Multilevel engineering of the upstream module of aromatic amino acid biosynthesis in *Saccharomyces cerevisiae* for high production of polymer and drug precursors. Metab Eng 42:134–144. https://doi.org/10.1016/j.ymben.2017.06.008

Sunyer-Figueres M, Vázquez J, Mas A, Torija MJ, Beltran G (2020) Transcriptomic insights into the effect of melatonin in *Saccharomyces cerevisiae* in the presence and absence of oxidative stress. Antioxidants 9:1–26. https://doi.org/10.3390/antiox9100947

Sze SF, Ng TB, Liu WK (1993) Antiproliferative effect of pineal indoles on cultured tumor cell lines. J Pineal Res 14:27–33. https://doi.org/10.1111/j.1600-079X.1993.tb00481.x

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar Sl (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. https://doi.org/10.1093/molbev/msr121

Tan D, Hardeland R (2020) Potential utility of melatonin in deadly infectious diseases related to the overreaction of innate immune response and destructive inflammation: focus on COVID-19. Melat Res 3:120–143. https://doi.org/10.32794/mr11250052

Tan D, Reiter R, Manchester L, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R (2002) Chemical and physical properties and potential mechanisms: Melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2:181–197. https://doi.org/10.2174/1568026023394443

Tan DX, Hardeland R, Back K, Manchester LC, Alatorre-Jimenez MA, Reiter RJ (2016) On the significance of an alternate pathway of melatonin synthesis via 5-methoxytryptamine: comparisons across species. J Pineal Res 61:27–40. doi: 10.1111/jpi.12336

Tan DX, Hardeland R, Manchester LC, Paredes SD, Korkmaz A, Sainz RM, Mayo JC, Fuentes-Broto L, Reiter RJ (2010) The changing biological roles of melatonin during evolution: From an antioxidant to signals of darkness, sexual selection and fitness. Biol Rev 85:607–623. doi: 10.1111/j.1469-185X.2009.00118.x.

Tan DX, Hardeland R, Manchester LC, Rosales-Corral S, Coto-Montes A, Boga JA, Reiter RJ (2012) Emergence of naturally occurring melatonin isomers and their proposed nomenclature. J Pineal Res 53:113–121. https://doi.org/10.1111/j.1600-079X.2012.00979.x

Tan DX, Korkmaz A, Reiter RJ, Manchester LC (2014a) Ebola virus disease: Potential use of melatonin as a treatment. J Pineal Res 57:381–384. https://doi.org/10.1111/jpi.12186

Tan DX, Manchester LC, Burkhardt S, Sain RM, Mayo JC, Kohen R, Shohami E, huo YS, Hardeland R, Reiter RJ (2001) *N*1-acetyl-*N*2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite, functions as a potent antioxidant. FASEB J 15:2294–2296. https://doi.org/10.1096/fj.01-0309fje

Tan DX, Manchester LC, Esteban-Zubero E, Zhou Z, Reiter RJ (2015) Melatonin as a potent and inducible endogenous antioxidant: Synthesis and metabolism. Molecules 20:18886–18906. https://doi.org/10.3390/molecules201018886

Tan DX, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ (2013) Mitochondria and chloroplasts as the original sites of melatonin synthesis: A hypothesis related to melatonin's primary function and evolution in eukaryotes. J Pineal Res 54:127–138. https://doi.org/10.1111/jpi.12026

Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ (2007) One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 42:28–42. https://doi.org/10.1111/j.1600-079X.2006.00407.x

Tan DX, Zheng X, Kong J, Manchester LC, Hardeland R, Kim SJ, Xu X, Reiter RJ (2014b) Fundamental issues related to the origin of melatonin and melatonin isomers during evolution: relation to their biological functions. Int J Mol Sci 15:15858–15890. https://doi.org/10.3390/ijms150915858

Ter Schure EG, Van Riel NAW, Verrips CT (2000) The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 24:67–83. https://doi.org/10.1016/S0168-6445(99)00030-3

Thirunavukkarasu M, Penumathsa SV, Samuel SM, Akita Y, Zhan L, Bertelli AA, Maulik G, Maulik N (2008) White wine induced cardioprotection against ischemia-reperfusion injury is mediated by life extending Akt/FOXO3a/NFkappaB survival pathway. J Agric Food Chem 56:6733–6739. DOI: 10.1021/jf801473v

Torrens-Spence MP, Lazear M, Von Guggenberg R, Ding H, Li J (2014) Investigation of a substrate-specifying residue within *Papaver somniferum* and *Catharanthus roseus* aromatic amino acid decarboxylases. Phytochemistry 106:37–43. DOI: 10.1016/j.phytochem.2014.07.007

Torrens-Spence MP, Pluskal T, Li F, Carballo V, Weng JK (2018) Complete pathway elucidation and heterologous reconstitution of *Rhodiola Salidroside* biosynthesis. 11:205–217. https://doi.org/10.1016/j.molp.2017.12.007

Trantas E, Navakoudis E, Pavlidis T, Nikou T, Halabalaki M, Skaltsounis L, Ververidis F (2019) Correction: Dual pathway for metabolic engineering of *Escherichia coli* to produce the highly valuable hydroxytyrosol. PLoS ONE 14: e0212243. https://doi.org/10.1371/journal.pone.0226760

Trenchard I-J, Siddiqui M, Thodey K, Smolke CD (2015) *De novo* production of the key branch point benzylisoquinoline alkaloid reticuline in yeast. Metab Eng 31:74–83. https://doi.org/10.1016/j.ymben.2015.06.010

Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S, La Guardia M (2005) The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr Res Rev 18:98–112. https://doi.org/10.1079/nrr200495

Tudela R, Ribas-agust A, Buxaderas S, Riu-Aumatell M, Castellari M, López-Tamames E (2016) Ultrahigh-Performance Liquid Chromatography (UHPLC) − Tandem Mass Spectrometry (MS/MS) quantification of nine target indoles in sparkling wines. J Agric Food Chem 64:4772–4776. https://doi.org/10.1021/acs.jafc.6b01254

Urata Y, Honma S, Goto S, Todoroki S, Iida T, Cho S, Honma K, Kondo T (1999) Melatonin induces γ-glutamylcysteine synthetase mediated by activator protein-1 in human vascular endothelial cells. Free Radic Biol Med 27:838–847. https://doi.org/10.1016/S0891-5849(99)00131-8

Valera MJ, Morcillo-Parra MÁ, Zagórska I, Mas A, Beltran G, Torija MJ (2019) Effects of melatonin and tryptophol addition on fermentations carried out by *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast species under different nitrogen conditions. Int J Food Microbiol 289:174–181. https://doi.org/10.1016/j.ijfoodmicro.2018.09.013

Vally H, Misso NLA, Madan V (2009) Clinical effects of sulphite additives. Clin Exp Allergy 39:1643–1651. https://doi.org/10.1111/j.1365-2222.2009.03362.x

Vally H, Thompson PJ (2003) Allergic and asthmatic reactions to alcoholic drinks. Addict Biol 8:3–11. https://doi.org/10.1080/1355621031000069828

Van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Lett 32:199–224. https://doi.org/10.1016/0378-1097(86)90291-0

Van Tassel D, Roberts N, Lewy A, O Neill SD (2001) Melatonin in plant organs. J Pineal Res 31:8–15. doi: 10.1034/j.1600-079x.2001.310102.x.

Vanegas KG, Lehka BJ, Mortensen UH (2017) SWITCH: A dynamic CRISPR tool for genome engineering and metabolic pathway control for cell factory construction in *Saccharomyces cerevisiae*. Microb Cell Fact 16:1–12. https://doi.org/10.1186/s12934-017-0632-x

Varela C, Borneman AR (2017) Yeasts found in vineyards and wineries. Yeast 34:111–128. https://doi.org/10.1002/yea.3219

Varela C, Pizarro F, Agosin E (2004) Biomass content governs fermentation rate in nitrogen-deficient wine musts. Appl Environ Microbiol 70:3392–3400. https://doi.org/10.1128/AEM.70.6.3392-3400.2004

Varoni EM, Paroni R, Antognetti J, Lodi G, Sardella A, Carrassi A, Iriti M (2018) Effect of red wine intake on serum and salivary melatonin levels: A randomized, placebo-controlled clinical trial. Molecules 23:1–10. https://doi.org/10.3390/molecules23102474

Vázquez J, González B, Sempere V, Mas A, Torija MJ, Beltran G (2017) Melatonin reduces oxidative stress damage induced by hydrogen peroxide in *Saccharomyces cerevisiae*. Front Microbiol 8:1–14. doi: 10.3389/fmicb.2017.01066. eCollection 2017

Vázquez J, Grillitsch K, Daum G, Mas A, Torija MJ, Beltran G (2018) Melatonin minimizes the impact of oxidative stress induced by hydrogen peroxide in *Saccharomyces* and Non-conventional yeast. Front Microbiol 9:1–12. doi: 10.3389/fmicb.2018.01933

Vemuri GN, Eiteman MA, McEwen JE, Olsson L, Nielsen J (2007) Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 104:2402–2407. https://doi.org/10.1073/pnas.0607469104

Viana F, Gil J V., Genovés S, Vallés S, Manzanares P (2008) Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol 25:778–785. https://doi.org/10.1016/j.fm.2008.04.015

Vieira Gomes A, Souza Carmo T, Silva Carvalho L, Mendonça Bahia F, Parachin NS (2018) Comparison of yeasts as hosts for recombinant protein production. Microorganisms 6:38. https://doi.org/10.3390/microorganisms6020038

Vigentini I, Gardana C, Fracassetti D, Gabrielli M, Foschino R, Simonetti P, Tirelli A, Iriti M (2015) Yeast contribution to melatonin, melatonin isomers and tryptophan ethyl ester during alcoholic fermentation of grape musts. J Pineal Res 58:388–396

Vigentini I, González R, Tronchoni J (2019) Genetic improvement of wine yeasts. In: Romano P, Ciani M, Fleet GH (eds) Yeasts in the production of wine. Springer Verlag, New York, pp 315–342

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

Vilela A (2019) The importance of yeasts on fermentation quality and human health-promoting compounds. Fermentation 5:46. https://doi.org/10.3390/fermentation5020046

Visioli F, Galli C, Bornet F, Mattei A, Patelli R, Galli G, Caruso D (2000) Olive oil phenolics are dose-dependently absorbed in humans. FEBS Lett 468:159–160. https://doi.org/10.1016/S0014-5793(00)01216-3

Vitalini S, Cas MD, Rubino FM, Vigentini I, Foschino R, Iriti M, Paroni R (2020) LC-MS/MS-based profiling of tryptophan-related metabolites in healthy plant foods. Molecules 25:311. https://doi.org/10.3390/molecules25020311

Vitalini S, Gardana C, Simonetti P, Fico G, Iriti M (2013) Melatonin, melatonin isomers and stilbenes in Italian traditional grape products and their antiradical capacity. J Pineal Res 54:322–333. https://doi.org/10.1111/jpi.12028

Vitalini S, Gardana C, Zanzotto A, Fico G, Faoro F, Simonetti P, Iriti M (2011) From vineyard to glass: Agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. J Pineal Res 51:278–285. https://doi.org/10.1111/j.1600-079X.2011.00887.x

Vuralhan Z, Morais MA, Tai SL, Piper MD, Pronk JT (2003) Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:4534–4541. https://doi.org/10.1128/AEM.69.8.4534-4541.2003

Wang C, Jicheng Z, Liu XY, Norrie G, Zhdanova I, Ben-Shushan A, Ford I (2015) Effect of exogenous melatonin on ethanol fermentation and antioxidant activity of *Saccharomyces cerevisiae*. Mod Food Sci Technol 31:102–108. https://doi.org/10.13982/j.mfst.1673-9078.2015.4.017

Wang C, Yin LY, Shi XY, Xiao H, Kang K, Liu XY, Zhan JC, Huang WD (2016) Effect of cultivar, temperature, and environmental conditions on the dynamic change of melatonin in mulberry fruit development and wine fermentation. J Food Sci 81:958–967. https://doi.org/10.1111/1750-3841.13263

Wang L, Feng C, Zheng X, Guo Y, Zhou F, Shan D, Liu X, Kong J (2017) Plant mitochondria synthesize melatonin and enhance the tolerance of plants to drought stress. J Pineal Res 1–11. https://doi.org/10.1111/jpi.12429

Wang YQ, Ye DQ, Zhu BQ, Wu GF, Duan CQ (2014) Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. Food Chem 163:6–15. https://doi.org/10.1016/j.foodchem.2014.04.064

Wei Z, Pan S, Wang W, Yan H (2012) Study of melatonin sensor based on molecular imprinting technique and electropolymerization membrane. Chinese J Anal Chem 40:1219–1224

Weidman SW, Kaiser ET (1966) The mechanism of the periodate oxidation of aromatic systems. J Am Chem Soc 88:5820–5827. https://doi.org/10.1021/ja00976a024

White WH, Gunyuzlu PL, Toyn JH (2001) *Saccharomyces cerevisiae* is capable of de novo pantothenic acid biosynthesis involving a novel pathway of β-alanine production from spermine. J Biol Chem 276:10794–10800. https://doi.org/10.1074/jbc.M009804200

Wiame JM, Grenson M, Ars HN (1985) Nitrogen catabolite repression in yeasts and filamentous fungi. Adv Microb Physiol 26:1–88. https://doi.org/10.1016/S0065-2911(08)60394-X

Williams TC, Averesch NJH, Winter G, Plan MR, Vickers CE, Nielsen LK, Krömer JO (2015) Quorum-sensing linked RNA interference for dynamic metabolic pathway control in *Saccharomyces cerevisiae*. Metab Eng 29:124–134. https://doi.org/10.1016/j.ymben.2015.03.008

Winters M, Arneborg N, Appels R, Howell K (2019) Can community-based signalling behaviour in *Saccharomyces cerevisiae* be called quorum sensing? A critical review of the literature. FEMS Yeast Res 19:1–11. https://doi.org/10.1093/femsyr/foz046

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung

C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Véronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285:901–906. https://doi.org/10.1126/science.285.5429.901

Wrona MZ, Dryhurst G (1987) Oxidation chemistry of 5-Hydroxytryptamine. Mechanism and products formed at micromolar concentrations. J Org Chem 52:2817–2825. https://doi.org/10.1021/jo00389a032

Wrona MZ, Lemordant D, Lin L, Blank CL, Dryhurst G (1986) Oxidation of 5-hydroxytryptamine and 5,7-dihydroxytryptamine. A new oxidation pathway and formation of a novel neurotoxin. J Med Chem 29:499–505. doi: 10.1021/jm00154a013

Xiao-ping W, Lan Z, Wen-Rong L, Jiang-Ping D, hong-Qing C, Guo-Nan C (2002) Study on the electrochemical behavior of melatonin with an activated electrode. Electroanalysis 14:1654–1660. https://doi.org/10.1002/elan.200290007

Xu W, Yang C, Xia Y, Zhang L (2020) High-Level production of tyrosol with non-induced recombinant *Escherichia coli* by metabolic engineering. J Agric Food Chem 68:4616-4623 https://doi.org/10.1021/acs.jafc.9b07610

Xue Y, Chen X, Yang C, Chang J, Shen W, Fan Y (2017a) Engineering *Eschericha coli* for enhanced tyrosol production. J Agric Food Chem 65:4708–4714. https://doi.org/10.1021/acs.jafc.7b01369

Yang J, Zhang D, Wang L, Long N, Zhang M, Zhang L (2015) An electrochemical method for high sensitive detection of thiabendazole and its interaction with human serum albumin. Food Anal Methods 8:507–514. https://doi.org/10.1007/s12161-014-9919-2

Yang S, Zheng X, Xu Y, Zhou X (2002) Rapid determination of serum melatonin by ESI-MS-MS with direct sample injection. J Pharm Biomed Anal 30:781–790. https://doi.org/10.1016/S0731-7085(02)00387-4

Yao J, He Y, Su N, Bharath SR, Tao Y, Jin JM, Chen W, Song H, Tang SY (2020) Developing a highly efficient hydroxytyrosol whole-cell catalyst by de-bottlenecking rate-limiting steps. Nat Commun 11:1–12. https://doi.org/10.1038/s41467-020-14918-5

Ye L, Berden JA, van Dam K, Kruckeberg AL (2001) Expression and activity of the *Hxt7* high-affinity hexose transporter of *Saccharomyces cerevisiae*. Yeast 18:1257–1267. https://doi.org/10.1002/yea.771

Yin B, Li T, Li Z, Dang T, He P (2016) Determination of melatonin and its metabolites in biological fluids and eggs using high-performance liquid chromatography with fluorescence and quadrupole-orbitrap high-resolution mass spectrometry. Food Anal Methods 9:1142–1149. https://doi.org/10.1007/s12161-015-0288-2

Young IM, Leone RM, Francis P, Stovell P, Silman RE (1985) Melatonin Is metabolized to *N*-acetylserotonin and 6-Hydroxymelatonin in man. J Clin Endocrinol Metab 60:114–119. https://doi.org/10.1210/jcem-60-1-114

Yu R, Nielsen J (2019) Big data in yeast systems biology. FEMS Yeast Res 19:1–10. https://doi.org/10.1093/femsyr/foz070

Yu T, Zhou YJ, Huang M, Liu Q, Pereira R, David F, Nielsen J(2018) Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. Cell 174:1549-1558.e14. https://doi.org/10.1016/j.cell.2018.07.013

Yüksel Aydar A, Öncü Öner T, Elif Fatma Ü (2017) Effects of Hydroxytyrosol on Human Health. EC Nutr 11:147–157.

Zamora F (2009) Biochemistry of alcoholic fermentation. In: Moreno-Arribas M.V., Polo M.C. (eds) Wine Chemistry and Biochemistry. Springer, New York, pp 3-26. https://doi.org/10.1007/978-0-387-74118-5_1

Zampol MA, Barros MH (2018) Melatonin improves survival and respiratory activity of yeast cells challenged by alpha-synuclein and menadione. Yeast 35:281–290. https://doi.org/10.1002/yea.3296

Zelasko S, Palaria A, Das A (2013) Optimizations to achieve high-level expression of cytochrome P450 proteins using *Escherichia coli* expression systems. Protein Expr Purif 92:77–87. https://doi.org/10.1016/j.pep.2013.07.017

Zhang HM, Zhang Y (2014) Melatonin: A well-documented antioxidant with conditional pro-oxidant actions. J Pineal Res 57:131–146

Zhang J, Wu C, Sheng J, Feng X (2016) Molecular basis of 5-hydroxytryptophan synthesis in *Saccharomyces cerevisiae*. Mol BioSyst 12:1432–1435

Zhang N, Sun Q, Zhang H, Cao Y, Weeda S, Ren S, Guo YD (2015) Roles of melatonin in abiotic stress resistance in plants. J Exp Bot 66:647–656. https://doi.org/10.1093/jxb/eru336

Zhang ZL, Chen J, Xu Q, Rao C, Qiao C (2012) Efficient synthesis of hydroxytyrosol from 3,4-dihydroxybenzaldehyde. Synth Commun 42:794–798. https://doi.org/10.1080/00397911.2010.531369

Zhao D, Yu Y, Shen Y, Liu Q; Zhao Z, Sharma R, Reiter R (2019) Melatonin synthesis and function: Evolutionary history in animals and plants. Front Endocrinol 10:1–16. https://doi.org/10.3389/fendo.2019.00249

Ziyatdinova G, Salikhova I, Skorobogatova N, Chibisova M, Budnikov H (2015) New electrochemistry-based approaches to brandy quality evaluation using antioxidant parameters. Food Anal Methods 8:1794–1803. https://doi.org/10.1007/s12161-014-0059-5

Zöllner A, Buchheit D, Maurer HH, Maurer HH, Peters FT, Bureik M (2010) Production of human phase 1 and 2 metabolites by whole-cell biotransformation with recombinant microbes. Bioanalysis 2:1277–1290

Zupan J, Avbelj M, Butinar B, Kosel J, Šergan M, Raspor P (2013) Monitoring of quorum-sensing molecules during minifermentation studies in wine yeast. J Agric Food Chem 61:2496–2505. https://doi.org/10.1021/jf3051363

ANNEX I: PUBLICATIONS

Food Anal. Methods
DOI 10.1007/s12161-016-0699-8

Detecting and Monitoring the Production of Melatonin and Other Related Indole Compounds in Different *Saccharomyces* Strains by Solid-State Electrochemical Techniques

Sara Muñiz-Calvo[1] · **José Manuel Guillamón**[1] · **Irene Domínguez**[2] · **Antonio Doménech-Carbó**[2]

Received: 29 July 2016 / Accepted: 14 October 2016

Abstract Recently, melatonin (Mel) has been detected in wine, and as its quantity increases during alcoholic fermentation, yeasts are primarily responsible for its production during the process. L-tryptophan (L-Trp) is an indolic molecule that plays an important role as a precursor of a large amount of tryptamine molecules, including Mel. We propose a novel method for detecting Mel and other derived indole compounds directly on yeast cells based on the voltammetry of immobilized particles methodology. This will abridge and facilitate the detection of these highly conserved and electroactive molecules. Tafel and modified Tafel analyses have been used to distinguish among Mel, tryptophol, and indole-3-acetic acid. We obtained different voltammetry responses in various *Saccharomyces* strains treated with L-Trp, 5-hydroxytryptophan, and serotonin and without them. The different responses allowed us to assess the production of indole compounds from L-Trp and other precursors. The voltammetric changes in mutant strains with deleted genes of tryptophan metabolism were also tested.

Electronic supplementary material The online version of this article (doi:10.1007/s12161-016-0699-8) contains supplementary material, which is available to authorized users.

✉ José Manuel Guillamón
guillamon@iata.csic.es

✉ Antonio Doménech-Carbó
antonio.domenech@uv.es

[1] Food Biotechnology Department, Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Av. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[2] Analytical Chemistry Department, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain

Published online: 28 October 2016

Keywords Indolic compounds · Melatonin · *Saccharomyces* · Voltammetry · Yeast

Introduction

Originally, melatonin (Mel) was seen as a unique product of the pineal gland of vertebrates and was called a neurohormone. However, in the last two decades, it has been identified within a wide range of invertebrates, plants, bacteria, and fungi. Therefore, today, Mel is considered a ubiquitous molecule present in most living organisms (Tan et al. 2015). In particular, this indolamine has been significantly detected in many food plants, and consequently, it can now be considered a dietary component, even if its daily intake is very difficult to estimate (Iriti et al. 2010; Iriti and Varoni 2015) Among food plants, grape vine (*Vitis vinifera*) represents a source of Mel that deserves being paid special attention because of wine production, an alcoholic beverage of economic relevance, with the presence of molecules with putative bioactivity.

Mel has been recently detected in wine, and despite the contribution of grapes to the final concentration of Mel and other indolamines, significant increases in these molecules in the fermentation step of the wine-making process (Vigentini et al. 2015) indicate the pivotal role of yeasts in this production. A pioneering paper demonstrated that *Saccharomyces cerevisiae* synthesizes Mel and metabolizes it to other 5-methoxylated indoles (5-methoxytryptamine and 5-methoxytryptophol) (Sprenger et al. 1999). More recently, Rodriguez-Naranjo et al. (2012) proved the synthesis of Mel in several wine yeast strains under conditions that mimicked grape must fermentation. These authors concluded that Mel synthesis largely depends on the growth phase of yeasts and the concentration of L-tryptophan (L-Trp) and sugars in the growth medium.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 February 2018
doi: 10.3389/fmicb.2018.00318

Protective Role of Intracellular Melatonin Against Oxidative Stress and UV Radiation in *Saccharomyces cerevisiae*

*Ricardo Bisquert, Sara Muñiz-Calvo and José M. Guillamón**

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Valencia, Spain

Melatonin (Mel) is considered a potent natural antioxidant molecule given its free-radical scavenging ability. Its origin is traced back to the origin of aerobic life as early defense against oxidative stress and radiation. More complex signaling functions have been attributed to Mel as a result of evolution in different biological kingdoms, which comprise gene expression modulation, enzyme activity, and mitochondrial homeostasis regulation processes, among others. Since Mel production has been recently reported in wine yeast, we tested the protective effect of Mel on *Saccharomyces cerevisiae* against oxidative stress and UV light. As the optimal conditions for *S. cerevisiae* to synthesize Mel are still unknown, we developed an intracellular Mel-charging method to test its effect against stresses. To assess Mel's ability to protect *S. cerevisiae* from both stresses, we ran growth tests in liquid media and viability assays by colony count after Mel treatment, followed by stress. We also analyzed gene expression by qPCR on a selection of genes involved in stress protection in response to Mel treatment under oxidative stress and UV radiation. The viability in the Mel-treated cells after H_2O_2 stress was up to 35% greater than for the untreated controls, while stress amelioration reached 40% for UVC light (254 nm). Mel-treated cells showed a significant shortened lag phase compared to the control cells under the stress and normal growth conditions. The gene expression analysis showed that Mel significantly modulated gene expression in the unstressed cells in the exponential growth phase, and also during various stress treatments.

Keywords: melatonin, *Saccharomyces cerevisiae*, UV radiation, oxidative stress, gene expression

OPEN ACCESS

Edited by:
Joaquin Bautista-Gallego, Instituto de la Grasa (CSIC), Spain

Reviewed by:
Atte Von Wright, University of Eastern Finland, Joensuu, Finland
Kwok-Ming Yao, University of Hong Kong, Hong Kong

****Correspondence:***
José M. Guillamón
guillamon@iata.csic.es

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 December 2017*
Accepted: *09 February 2018*
Published: *28 February 2018*

Citation:
Bisquert R, Muñiz-Calvo S and Guillamón JM (2018) Protective Role of Intracellular Melatonin Against Oxidative Stress and UV Radiation in Saccharomyces cerevisiae. Front. Microbiol. 9:318. doi: 10.3389/fmicb.2018.00318

INTRODUCTION

Melatonin (Mel, *N*-acetyl-5-methoxytryptamine) is an indole-amine that is considered ubiquitous in most living organisms. Mel antioxidant properties have been widely studied in higher organisms such as mammals or plants. However, its antioxidant capacity does not seem to be limited to its ability to scavenge free radicals. It has been also reported that its presence is capable of stimulating the synthesis of other important intracellular antioxidants, such as glutathione (Rodriguez et al., 2004), inducing antioxidant enzymes by suppressing pro-oxidant enzymes, and improving the mitochondrial function and thereby reducing free radical formation (Acuña Castroviejo et al., 2011;

Received: 16 November 2018 | Revised: 4 January 2019 | Accepted: 4 January 2019

DOI: 10.1111/jpi.12554

ORIGINAL ARTICLE

WILEY Journal of Pineal Research

Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites

Sara Muñiz-Calvo[1] | **Ricardo Bisquert**[1] | **Edwin Fernández-Cruz**[2] | **María C. García-Parrilla**[2] | **José M. Guillamón**[1]

[1]Food Biotechnology Department, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Paterna, Valencia, Spain

[2]Department of Food Science and Nutrition, Facultad de Farmacia, University of Seville, Seville, Spain

Correspondence
José M. Guillamón, Food Biotechnology Department, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Paterna, Valencia, Spain.
Email: guillamon@iata.csic.es

Funding information
Generalitat Valenciana, Grant/Award Number: PROMETEOII/2014/042; Ministry of Economy, Industry and Competitiveness, Spanish government, Grant/Award Number: AGL2013-47300-C3-3-R, AGL 2013-47300-C3-2-R, AGL2016-77505-C3-1-R and AGL 2016-77505-C3-2-r

Abstract

Melatonin (Mel), originally considered a neurohormone, has been detected in beverages and food-fermented products in which yeast metabolism is highly important. This indolamine is synthesized from serotonin, with L-tryptophan being the initial substrate of both. Regarding Mel metabolism, the biosynthetic pathway in mammals consists in four-step reactions. However, six genes are implicated in the synthesis of Mel in plants, which suggest the presence of many pathways. The aim of this study was to provide new empirical data on the production of Mel and other indole-related compounds in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). To this end, we performed the addition of the pathway intermediates in *S. cerevisiae* cells in different growth stages (exponential and arrested cells) to follow the bioconversion and new indolic compound production from them. The different bioconverted indolic compounds tested (L-tryptophan, 5-hydroxytryptophan, tryptamine, serotonin, N-acetylserotonin, 5-methoxytryptamine, and Mel) were analyzed by UHPLC-MS/MS from the extra- and intracellular contents. Our results showed that serotonin, in yeast, was prevalently formed via tryptophan decarboxylation, followed by tryptamine hydroxylation as in plants. Mel production from serotonin can be achieved by either N-acetylation, followed by O-methylation or O-methylation, in turn followed by N-acetylation. Accordingly, the classic pathway of Mel synthesis in vertebrates does not seems prevalent in yeast.

KEYWORDS

bioconversion, indole biosynthesis pathway, indolic compounds, melatonin metabolism, substrate pulses, yeast

1 | INTRODUCTION

Lerner et al[1] in 1958 described melatonin (Mel) to be a neurohormone in the bovine pineal gland. It was subsequently demonstrated that this indoleamine was also present in multiple extrapineal tissues.[2,3] In the last two decades, Mel has been well accepted as a ubiquitous molecule present in many organisms belonging to different biological kingdoms.[4-6]

In 1999, Sprenger et al[7] conducted the first study about Mel synthesis in yeast. In this work, the authors reported that baker's yeast was able to synthesize Mel from L-tryptophan (L-Trp), serotonin (Ser), N-acetylserotonin (NAS), and 5-methoxytryptamine (5MT) as potential precursors. Since then, very few studies have been carried out on this topic since most research has focused on demonstrating the presence of Mel or biosynthesis by yeasts in a fermentative context.[8-12] Moreover, the biosynthetic pathway of

Food Chemistry 308 (2020) 125646

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Overproduction of hydroxytyrosol in *Saccharomyces cerevisiae* by heterologous overexpression of the *Escherichia coli* 4-hydroxyphenylacetate 3-monooxygenase

Sara Muñiz-Calvo, Ricardo Bisquert, Sergi Puig, José Manuel Guillamón*

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), C. Catedrático Agustín Escardino 7, E-46980 Paterna, Valencia, Spain

ARTICLE INFO

Keywords:
Hydroxytyrosol
Saccharomyces cerevisiae
Escherichia coli
Polyphenols

ABSTRACT

Hydroxytyrosol (HT), which is a polyphenol with a high antioxidant power and many associated health benefits, has been found in wines. Wine yeasts are capable of producing high amounts of the higher alcohol tyrosol, which is the precursor for HT synthesis. We have improved the ability of *Saccharomyces cerevisiae* to produce HT by heterologously expressing the HpaBC enzyme complex of *Escherichia coli*, which hydroxylates tyrosol into HT. By overexpressing the *hpaB* and *hpaC* genes, we achieved HT titers of 1.15 ± 0.05 mg/L and 4.6 ± 0.9 mg/L in a minimal medium in which either 1 mM tyrosine or 1 mM tyrosol were respectively added. This work demonstrates that the overexpression of HpaBC in yeast is a promising tool to overproduce HT at the expense of endogenous tyrosol through central carbon catabolism flux redirection to tyrosine catabolism.

1. Introduction

Hydroxytyrosol (HT) is a polyphenol considered as a potent natural antioxidant present in leaves and fruits of olive, extra-virgin olive oil and in olive oil industrial by-products and wastewaters (Angerosa, D'Alessandro, Konstantinou, & Di Giacinto, 1995; Lesage-Meessen et al., 2001). Moreover, HT is a molecule with great interest due to its multiple bioactivities and human health benefits (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Hu, He, Jiang, & Xu, 2014; Yüksel Aydar, Öncü Öner, & Elif Fatma, 2017). Furthermore, in both white and red wines, HT has also been detected in concentrations ranging from 0.28 to 9.6 mg/L (Bordiga et al., 2016; Di Tommaso, Calabrese, & Rotilio, 1998; Minussi et al., 2003; Minuti, Pellegrino, & Tesei, 2006; Piñeiro, Cantos-Villar, Palma, & Puertas, 2011). The presence of HT in wine has been related to different sources including the hydroxylation of tyrosol by the endogenous polyphenol oxidase from grapes (García-García, Hernández-García, Sánchez-Ferrer, & García-Carmona, 2013). However, some studies directly relate HT to the metabolism of yeasts during alcoholic fermentation (Álvarez-Fernández, Fernández-Cruz, Cantos-Villar, Troncoso, & García-Parrilla, 2018; Bordiga et al., 2016; González et al., 2018; Guerrini et al., 2018; Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015). In yeast, the higher aromatic alcohol tyrosol is synthesized from tyrosine through the well-established Ehrlich pathway. Briefly, amino acids are first transaminated into α-keto acids followed by decarboxylation in aldehydes, and finally reduced to the fusel alcohol (Dickinson, Salgado, & Hewlins, 2003; Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008; Ooi, Wanamaker, Markuszewski, & Chong, 2008).

Various strategies have used yeast genes to overproduce tyrosol and HT in *E. coli*. For instance, the heterologous co-expression of yeast *ARO8* and *ARO10* genes led to an important accumulation of tyrosol when tyrosine was added to the medium (Xue et al., 2017). Furthermore, the co-expression of yeast *ARO10* and *ADH6*, and the overexpression of the native aromatic hydroxylase complex HpaBC produced important amounts of HT in *E. coli* (Chung, Kim, & Ahn, 2017; Li et al., 2018). *HpaBC* encodes the 4-hydroxyphenylacetate 3-hydroxylase, a member of the two-component flavin-dependent monooxygenase family. The large subunit flavin-dependent monooxygenase is encoded by *hpaB*, whereas *hpaC* encodes for the NAD(P)H:flavin oxidoreductase subunit, which supplies $FADH_2$ to HpaB (Ballou, Entsch, & Cole, 2005; Ellis, 2010). The broad substrate specificity of HpaBC from different bacterial species has enabled its utilization for the hydroxylation of multiple substrates including tyrosol, naringenin, p-coumaric acid, afzelechin, caffeic acid, 3-(4-hydroxyphenyl) propanoic acid, ferulic acid and coniferaldehyde (Furuya & Kino, 2014; Jones, Collins, Vernacchio, Lachance, & Koffas, 2016; Liebgott, Amouric, Comte, Tholozan, & Lorquin, 2009; Lin & Yan, 2014).

In this study, we have used the *E. coli* hydroxylase HpaBC complex

* Corresponding author.
E-mail address: guillamon@iata.csic.es (J.M. Guillamón).

https://doi.org/10.1016/j.foodchem.2019.125646
Received 15 July 2019; Received in revised form 1 October 2019; Accepted 3 October 2019
Available online 15 October 2019
0308-8146/

Melatonin Research (Melatonin Res.) *http://www.melatonin-research.net*

Review

Melatonin in yeast and fermented beverages: analytical tools for detection, physiological role and biosynthesis

Sara Muñiz-Calvo[1], Ricardo Bisquert[1], José Manuel Guillamón[1*]

[1]Food Biotechnology Department. Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Avda. Agustín Escardino, 7, 46980 Paterna, Valencia, Spain
* Correspondance: guillamon@iata.csic.es, Tel: +34 96 390 00 22

Running Title: Melatonin in yeast

Received: February 24, 2020; Accepted: March 29, 2020

ABSTRACT

The recently established relation between the metabolism of aromatic amino acids of yeast and the production of different bioactive molecules during fermentation opens up new and interesting research topics. Among these molecules, melatonin has drawn researchers' attention in the last decade given its potential benefits for human health. This review summarizes melatonin production in fermented beverages, and conventional and current methods for detecting melatonin in yeast-derived samples. In addition, the role of melatonin in yeast is discussed and the biosynthetic pathway of melatonin is presented in *Saccharomyces cerevisiae*.

Keywords: *Saccharomyces cerevisiae*, fermentation, bioactive, antioxidant, indoleamine serotonin, tryptophan, melatonin.

1. INTRODUCTION

The development of precise analytical tools allows us to detect most food components, even those at very low concentrations, and with much interest shown in their potential bioactivity. Apart from tryptophan being a proteinogenic amino acid, it is a key precursor of the different relevant molecules deriving from its metabolism, such as indole-3-acetic acid (IAA), serotonin, N-acetyl serotonin and melatonin. For example, IAA is known as the major auxin-type phytohormone that regulates many cellular processes and plant development. However, it has been shown that it can act as a signaling molecule in microorganisms, and it exerts tolerance to various toxic compounds and stress conditions in *E.* coli (1), which means it is capable of regulating gene expression. Moreover, the exogenous administration of IAA in *Saccharomyces cerevisiae* leads to the conversion of vegetative cells into their filamentous form (2). Other structurally related molecules like indole derivate molecules, including tryptamine and melatonin, are synthesized through the metabolism of tryptophan in plants, but also by many microorganisms, including bacteria and yeast (3). Although a large proportion of compounds deriving from tryptophan in food is of plant origin, their presence in fermented products can also be related to the microorganisms involved in the fermentation process which, through their metabolism, increase the levels of such molecules of

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P202031186	
Fecha de recepción:	26 noviembre 2020, 14:24 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	ES1641.1569	
Solicitante:	Consejo Superior de Investigaciones Científicas (CSIC)	
Número de solicitantes:	1	
País:	ES	
Título:	SACCHAROMYCES CEREVISIAE RECOMBINANTE PARA LA PRODUCCIÓN DE HIDROXITIROSOL	
Documentos enviados:	Descripcion.pdf (28 p.) Reivindicaciones-1.pdf (3 p.) Dibujos-1.pdf (5 p.) Resumen-1.pdf (1 p.) OLF-ARCHIVE.zip FEERCPT-1.pdf (1 p.) FEERCPT-2.pdf (1 p.) SEQLPDF.pdf (33 p.) SEQLTXT.txt	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	C=ES,O=PONS PATENTES Y MARCAS INTERNACIONAL SL,2.5.4.97=#0C0F56415445532D423834393231373039,CN=50534279J ANGEL PONS (R: B84921709),SN=PONS ARIÑO,givenName=ANGEL,serialNumber=IDCES-50534279J,description=Reg:28065 /Hoja:M-421468 /Tomo:23494 /Folio:199 / Fecha:22/02/2012 /Inscripción:9	