



Isotopic labelling-based analysis elucidates biosynthesis pathways in *Saccharomyces cerevisiae* for Melatonin, Serotonin and Hydroxytyrosol formation

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ABSTRACT

Yeasts can synthesise bioactive compounds such as Melatonin (MEL), Serotonin (SER) and Hydroxytyrosol (HT). Deciphering the mechanisms involved in their formation can lead to exploit this fact to increase the bioactive potential of fermented beverages. Quantitative analysis using labelled compounds, 15-N2 L-tryptophan and 13-C tyrosine, allowed tracking the formation of the above-mentioned bioactive compounds during the alcoholic fermentation of synthetic must by two different *Saccharomyces cerevisiae* strains. Labelled and unlabelled MEL, SER and HT were undoubtedly identified and quantified by High Resolution Mass Spectrometry (HRMS). Our results prove that there are at least two pathways involved in MEL biosynthesis by yeast. One starts with tryptophan as precursor being known for the vertebrates' pathway. Additionally, MEL is produced from SER which in turn is consistent with the plants' biosynthesis pathway. Concerning HT, it can be formed both from labelled tyrosine and from intermediates of the Erlich pathway.

1. Introduction

Melatonin (MEL), *N*-acetyl-5-methoxytryptamine, is a neurohormone involved in the regulation of circadian rhythms in humans. It is widespread present in different kingdoms such as fungi, plants, and vertebrates (Szafrńska & Posmyk, 2017).

Its presence in foods has attracted much interest for its recognized bioactive properties (antioxidant, neuroprotective, anti-inflammatory and cardiovascular properties) (R. J. Reiter et al., 2000; Russel J. Reiter et al., 2010; Sanchez-Barcelo et al., 2012) and has been analysed in different foods and beverages. Indeed, it has been detected in several foodstuffs including, among others, tomatoes, strawberries (Stürtz et al., 2011), cherry (Zhao et al., 2013), apple (Lei et al., 2013), walnuts (Russel Reiter et al., 2005) and pistachios (Oladi et al., 2014) as well as fermented foods such as wine (Rodríguez-Naranjo et al., 2011), fermented orange beverages (Fernández-Pachón et al., 2014) and beer (Edwin Fernández-Cruz et al., 2020). The role of yeast to explain the presence of MEL in fermented products was initially highlighted when

this bioactive compound was detected in a fermented grape must in which MEL was initially absent (M. Isabel Rodríguez-Naranjo et al., 2011). These findings could be confirmed when MEL was determined after the alcoholic fermentation of a synthetic must, pinpointing the synthesis of MEL by *Saccharomyces* (María Isabel Rodríguez-Naranjo et al., 2012). Yeasts are well known for transforming nitrogen sources into metabolites that have an impact in sensorial properties of wines (Mas et al., 2014; Bell & Henschke, 2005). In addition, these studies show that yeasts are at the same time capable of synthesising bioactive compounds, opening new possibilities of research.

Consequently, the interest of unravelling the biosynthetic pathway involved in MEL synthesis has increased not only to favour the concentration of bioactives in foods but also to understand the significance of MEL for yeast and its biological role. Recently, Morcillo-Parra and their co-workers, (Morcillo-Parra et al., 2019) proved that MEL bounds to glycolytic enzymes reinforcing the possible role of MEL as a signal molecule, likely related to fermentation metabolism. This signalling role may explain the fact that MEL appears and disappears during the

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fermentation process (Fernandez-Cruz et al., 2019) following a zig zag pattern likely associated to the binding to different proteins (Morcillo-Parra et al., 2019). So far, the biosynthetic pathway described in mammals (Ruddick et al., 2006), involves four steps reactions considering the amino acid L-tryptophan (L-TRP) as the initial precursor, which is subsequently transformed into 5-hydroxytryptophan (5-OH-TRP), SER, N-acetylserotonin (NAS) and 5-methoxytryptamine (5-MT-TRYPT) as displayed in Fig. 1A. These metabolites were determined in the intracellular compartment of *Saccharomyces* and *non-Saccharomyces* wine yeasts during alcoholic fermentation of synthetic musts (Álvarez-Fernández et al., 2019).

On the other hand, Muñiz Calvo and their colleagues performed experiments adding intermediates (L-Trp, 5-OH-TRP, SER, NAS, tryptamine (TRYPT), and 5-MT) in *S. cerevisiae* cells in different grown stages as pulses to assess the bioconversion into new indolic compounds (Muñiz-Calvo et al., 2019). Their results showed that the

decarboxylation of L-TRP forming TRYPT could be an initial step. Secondly, a hydroxylation of TRYPT could form SER. This alternative route in yeasts is different from those described in vertebrates in which 5-OH-TRP is the first step (Hardeland et al., 1993). Fig. 1A represents the proposed pathway. This research was carried out with several pulses with the intermediates of the pathway (L-TRP, 5-OH-TRP, SER, NAS, TRYPT, and 5-MT- TRYPT) applied to cells in different growth stages and conditions, thus it might differ from a natural fermentation media in which these intermediates are present at the concentrations that yeasts synthesise them (Hardeland et al., 1993).

In addition to MEL, another relevant bioactive, hydroxytyrosol (HT), has been very recently detected in the intracellular compartment of yeast (Álvarez-Fernández et al., 2018a). In fact, HT is present in wine (Di Tommaso et al., 1998) but at lower concentrations than in extra virgin olive oil, (Mateos et al., 2001) the major dietetic source. HT is indeed a polyphenol typically found in olives, and also present in olive mill

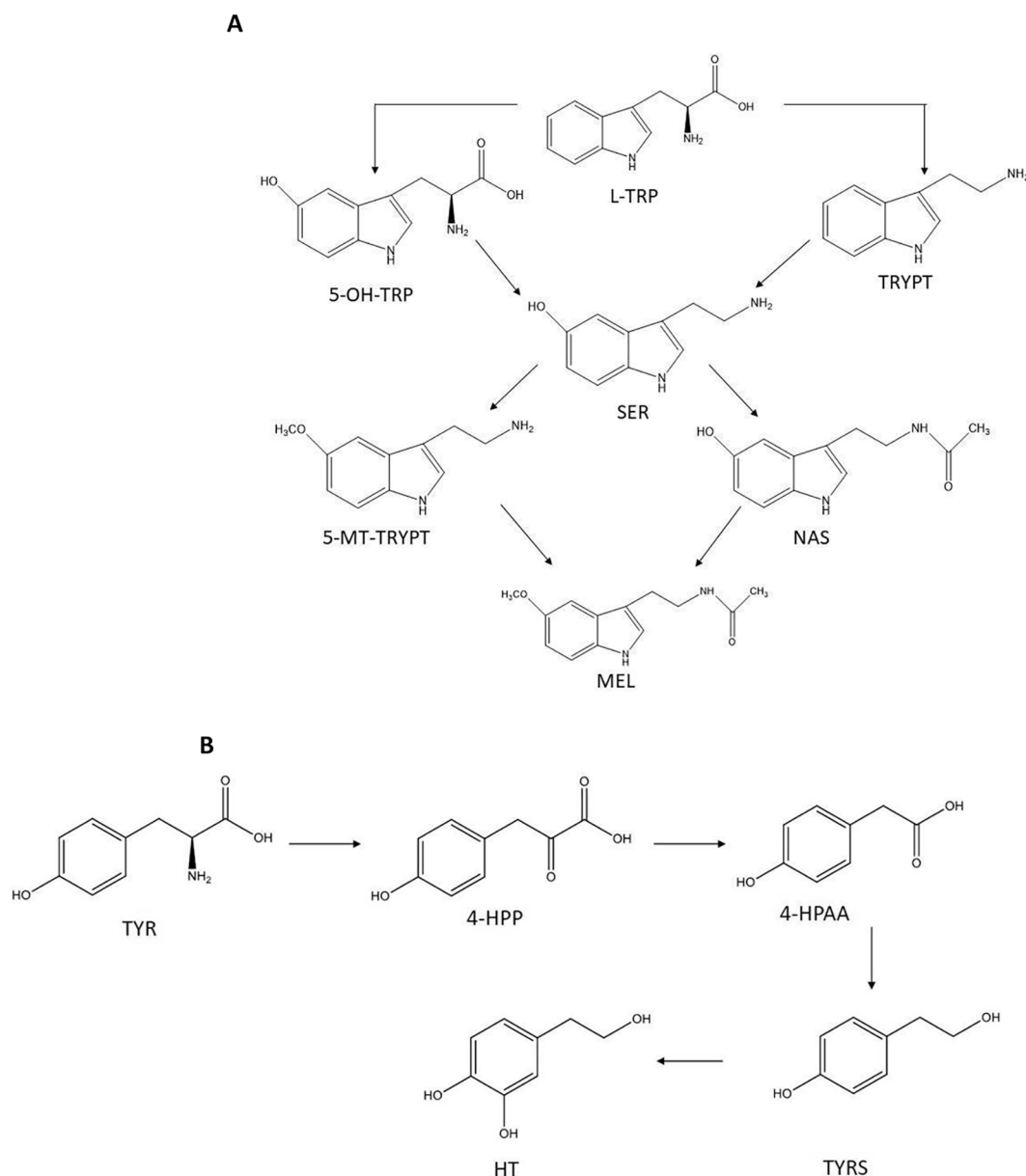


Fig. 1. Illustration of the biosynthetic pathway of **A.** melatonin from tryptophan and **B.** hydroxytyrosol from tyrosine. MEL: melatonin, L-TRP: tryptophan, 5-OH-TRP: 5-hydroxytryptophan, SER: serotonin, NAS: N- acetylserotonin which are the predominant intermediates in mammalian model whereas additional or alternative steps including TRYPT: tryptamine and 5-MT-TRYPT: 5- methoxytryptamine are reported for yeast and plant models. HT: hydroxytyrosol, TYR: tyrosine, 4- HPP: 4-hydroxyphenylpyruvate, 4-HPAA: 4- hydroxyphenylacetic acid, TYRS: Tyrosol.

wastewater. In nature, HT in olives is found in the form of its elenolic acid ester, oleuropein (Vissers et al., 2002). Conversely, HT in wines may derive, partly at least, from the hydroxylation of tyrosol (TYRS) which in turn is formed by the Ehrlich pathway as shown in Fig. 1B.

All these findings proved that yeasts, in particular *Saccharomyces* but also certain *non-Saccharomyces*, do synthesize bioactive compounds with impact in human health. To exploit this data and improve the bioactive composition of fermented products, it is necessary to gain knowledge about the pathways and steps leading to their formation and accumulation.

A classical experimental design to unravel biosynthetic pathways is the use of labelled compounds that permit more easily to follow the new-formed molecules. This approach has been successfully applied to monitor the formation of aromatic compounds and a better understanding of amino acid metabolism in yeasts (Rollero et al., 2017). Therefore, the aim of this paper is to decipher the metabolic pathways related to the synthesis of bioactive compounds in yeast, namely MEL, HT and SER. For this purpose, we performed alcoholic fermentation experiments with synthetic must that includes labelled L-TRP or TYR as potential precursors of MEL and HT respectively. This strategy allows to follow the bioactive compounds during different days of the fermentation process.

2. Material and methods

2.1. Reagents and materials

All standards used in this study (L-Tryptophan- $^{15}\text{N}_2$ ($^{15}\text{N}_2$ L-TRP) (Fig. 2A), L-TRP, 5-OH-TRP, SER, NAS, MEL, TRYPT, Tryptophol (TOL), L-Tyrosine-(phenyl-4- ^{13}C) (^{13}C TYR) (Fig. 2B), Tyrosine (TYR), 4-hydroxyphenylacetic acid (4-HPAA), TYRS and HT) were purchased from Sigma Aldrich (St.Louis, MO, USA). HPLC grade methanol was supplied from Merck (Darmstadt, Germany) and formic acid was provided by Prolabo® (Obregon, Mexico).

2.2. Yeast strains

The experiments on alcoholic fermentation of synthetic musts were performed with two different commercial wine yeast strains: *S. cerevisiae* Lalvin YSEO QA23 (Lallemand; QA) and *S. cerevisiae* Red Fruit (Enartis; RF). They were selected based on our previous experience in which QA23 proved to synthesize MEL and other indole compounds (Fernández-Cruz et al., 2017) while Red Fruit produces HT (Álvarez-Fernández et al., 2018b). All the strains were provided as active dried yeast and were rehydrated for 30 min at 37 °C and plated on yeast extract peptone dextrose (YPD) agar (2% peptone, 2% glucose, 1% yeast extract and 2% agar). Then, they were incubated at 28 °C in an oven for 2 days. Afterwards, the pre-culture was prepared in 150 mL of YPD broth (2% peptone, 2% glucose and 1% yeast extract) and shaken at 150 rpm, 28 °C, overnight, before inoculation.

2.3. Alcoholic fermentation

The experiments of alcoholic fermentations were carried out using a

synthetic must (SM) at pH 3.5., prepared based on literature (Riou et al., 1997) with some modifications. The must containing isotopically labelled TYR was prepared at a concentration of 10 mg/L of labelled TYR and 260 mg/L of $(\text{NH}_4)_2\text{SO}_4$. In the case of the must that contains labelled L-TRP, its concentration was 13.4 mg/L and 260 mg/L of $(\text{NH}_4)_2\text{SO}_4$. The concentration of glucose and fructose as a carbon source is 100 g/L each and the amounts of malic and citric acid were 5 and 0.5 g/L, respectively. The concentration for the remaining compounds was maintained unaltered (Riou et al., 1997). Mineral salts, sugars and organic acids were the first to be added. Subsequently, the solution was shaken, and the pH of the medium was adjusted to 3.5, with NaOH. Red Fruit was inoculated in the labelled TYR medium, while QA23 was inoculated in the labelled L-TRP medium.

Each Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell/mL and capped with taps equipped with a capillary to release carbon dioxide. During fermentations, all the flasks were stored in an orbital incubator at 28 °C. The flasks were weighed daily before and after sampling. Cell growth and evolution were assessed by measuring the optical density (DO) at 600 nm every sampling day (Fig. 3 3.1–3.6).

To obtain the most reliable results and capture the random biological variation, six biological replicates of each fermentation were considered.

2.4. Extracellular metabolites extraction.

A sample volume corresponding to 10^9 cells of each Erlenmeyer flask was taken for ten days in the case of fermentation with Red Fruit yeast and for seven days in the case of fermentations with QA23 yeast. The number of cells was counted taking 1 mL of each Erlenmeyer and using the Neubauer chamber and the DO value. Immediately, the samples were centrifuged, at 4500 rpm for 3 min at 4 °C, in order to separate the cells from extracellular contents. The extracellular samples were collected and stored at – 80 °C until the analysis.

All extracellular extracts were cleaned up as reported by Fernández-Cruz (E. Fernández-Cruz et al., 2017) with some modifications. C18 SPE cartridges (1 g, Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milli Q water. For cleaning extracellular samples an aliquot of 2 mL of extracellular sample was loaded, followed by a washing step with 2 mL of a 10% v/v methanol/water solution. The analytes were eluted with 1 mL of methanol. Methanol was then evaporated until dryness by using a vacuum concentrator (HyperVAC-LITE, GYOZEN, Korea) at 30 °C and 2000 rpm for 8 h. Each sample was reconstituted with 100 µL of methanol/0.1% formic acid and immediately analysed.

2.5. UHPLC-HRMS instrumental analysis

The instrument of analysis consisted of a UHPLC Vanquish coupled to a Q-Exactive Plus mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (all from Thermo Fisher Scientific, San Jose, CA). The mass analyser was calibrated before analysis using Pierce® ESI Negative and Positive Ion Calibration Solutions (Thermo Fisher Scientific). Chromatographic separation was carried out on an Agilent Zorbax SB-C18 column (100 mm × 2.1 mm × 1.8 µm) at 40 °C

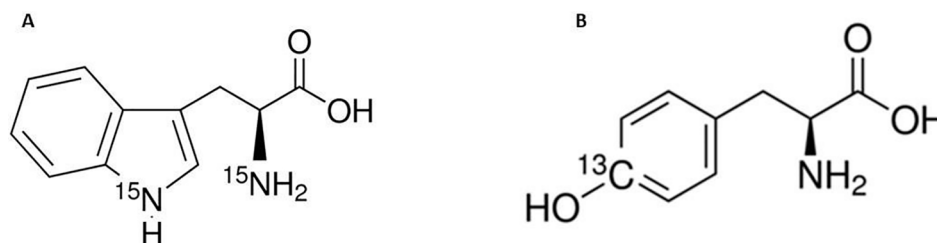
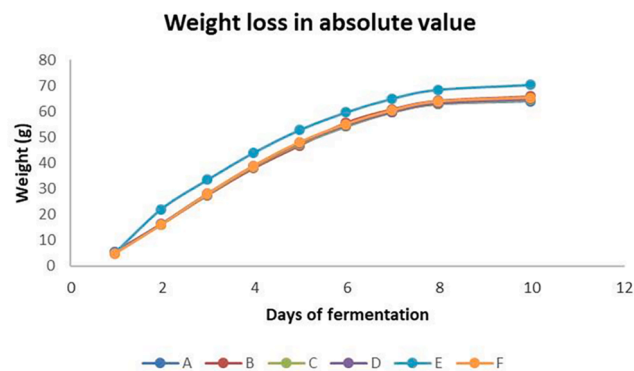
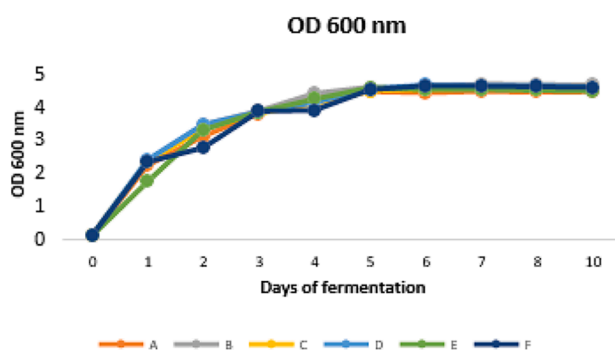


Fig. 2. Structure of A: L-Tryptophan- $^{15}\text{N}_2$ and B: L-Tyrosine-(phenyl-4- ^{13}C).

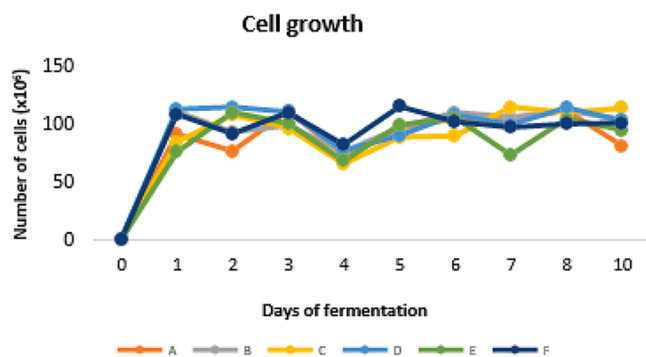
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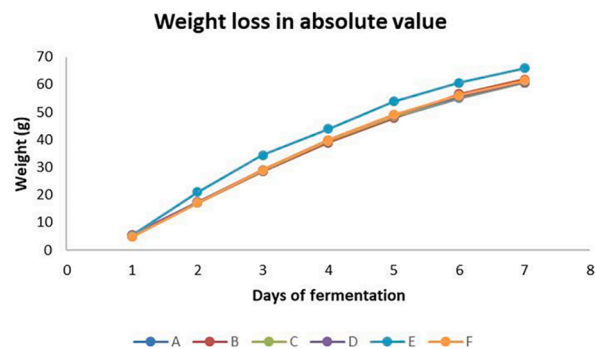
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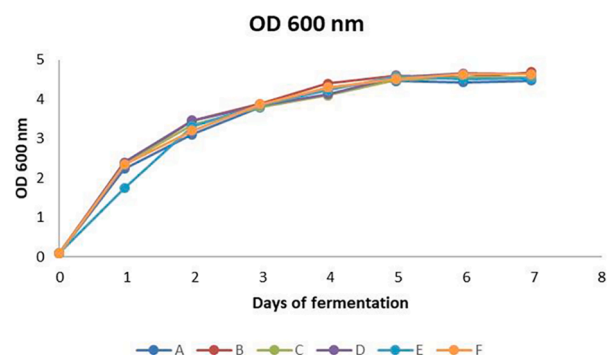
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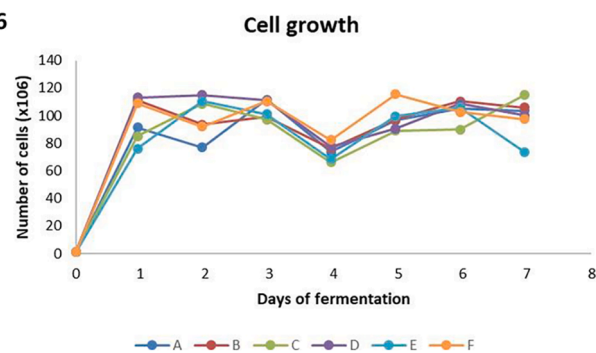


Fig. 3. Monitoring of the evolution of both fermentations: 3.1, 3.2, 3.3 → 13-C TYR as precursor of HT during the ten days of alcoholic fermentation by the yeast *S. cerevisiae* Red Fruit; 3.4, 3.5, 3.6 → 15-N₂ L-TRP as precursor of MEL during the seven days of alcoholic fermentation by the yeast *S. cerevisiae* Lalvin YSEO QA23. by weight, OD and cell growth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with a binary solvent system composed by acidified milliQ water (0.1% formic acid, solvent A) and acidified LC-MS acetonitrile (0.1% formic acid, solvent B). A gradient elution was programmed as follows: 0–5 min: 1% B; 5–10 min: 1%–40% B; 11–13 min: 95% B; 14–16 min: 1% B. For the calibration curve, ten solutions containing the 14 analytes (¹⁵N₂ L-TRP, L-TRP, 5-OH-TRP, SER, NAS, MEL, TRP, TOL, ¹³C TYR, TYR, 4-HPAA, TYR and HT) were prepared in a range of 0.0003–50 mg/L. Injection volume was 1 μL and the flow was set at 0.4 mL/min. The

temperature of the column was maintained at 40 °C.

The main parameters of the HRMS source were: Spray voltage (4000 V), Sheath gas (60 a.u.); Auxiliary Gas (20 a.u.), Capillary temperature and probe heater temperature (200 °C), S-lens RF level (50). A FullScan mode in the range *m/z* 100–400 was selected for the acquisition at a resolution of 70,000 (FWHM). The automatic gain control target was set at 3.10⁶ ions and the maximum injection time was set at 200 ms. Subsequent data analysis was performed by Xcalibur Software

(version 3.0.63), to confirm compound identification and quantify the different analytes, using the molecular formula and exact mass of the indole compounds, both of which are available in public databases, such as PubChem and ChemSpider. For quantification, extracted ion chromatograms for each labelled and unlabelled metabolite were obtained at a scan with of 0.01 uma around the exact mass of the compound.

3. Results

In order to monitor the evolution of the fermentations, some parameters were recorded. Before and after sampling, the flasks were weighed daily as displayed in Figs. 3.1 and 3.4 showing a decrease during fermentation as expected. Optical density (OD) measured at 600 nm (Figs. 3.2 and 3.5) and Cell growth (Figs. 3.2 and 3.6) determined thanks to a Neubauer's chamber increase at day 1 and keep constant till the end of the fermentation.

Four compounds deriving from L-TRP (5-OH-TRP, Ser, NAS, and MEL) were followed during the seven days of alcoholic fermentation and unequivocally identified through their retention time, exact mass and matching their mass spectra with that of the commercial standard. Accurate mass determination allowed us to unequivocally distinguish labelled and unlabelled compounds standard (Table 1A). Quantitation was performed using external calibration curves with 10 points, which were freshly prepared at every analytical session.

Tables 2A and 2B and Fig. 4A and 4B display the concentration of the compounds derived from each aromatic amino acid (L-TRP and TYR respectively) determined at each day during the fermentation experiment. Results are the average of the six biological replicates analysed in duplicate. As expected, labelled compounds deriving from $^{15}\text{-N}_2$ L-TRP included in the must (Table 2A) were detected in the samples taken during fermentation. Indeed, compounds believed to be intermediates in the synthesis of melatonin are detected following a time sequences as follows; day 0 $^{15}\text{-N}_2$ L-TRP, day 1, labelled 5-OH-TRP, day 2, labelled SER and labelled NAS and finally at day 3, labelled MEL. This timeline fits perfectly with the pathway described for the synthesis of melatonin in

Table 1

Parameters of labelled and unlabelled compounds. Ionization mode (+/-), exact mass (g/mol) and retention times (min). A: Compounds deriving from L-Trp. B: Compounds deriving from TYR.

A.			
Compound	Ionizationmode	Exact mass(g/mol)	Retention time (min)
L-TRP	+	205.23261	7.53
5-OH-TRP	+	221.23524	2.43
SER	+	177.22476	2.01
NAS	+	219.25634	8.89
MEL	+	233.28446	10.55
TRP	+	161.22213	7.75
TOL	+	162.20291	10.54
$^{15}\text{-N}_2$ L-TRP	+	207.23252	7.65
$^{15}\text{-N}_2$ 5-OH-TRP	+	223.23474	2.52
$^{15}\text{-N}_2$ SER	+	179.22557	2.03
$^{15}\text{-N}_2$ NAS	+	221.25476	8.90
$^{15}\text{-N}_2$ MEL	+	235.28693	10.57
$^{15}\text{-N}_2$ TRP	+	163.22334	7.80
$^{15}\text{-N}_2$ TOL	+	164.20877	10.57
B.			
Compound	Ionizationmode	Exact mass(g/mol)	Retention time(min)
TYR	-	180.19214	1.34
4-HPAA	-	151.15576	8.65
TYRS	-	137.16631	3.37
HT	-	153.16144	6.24
^{13}C TYR	-	181.19472	1.37
^{13}C 4-HPAA	-	152.15693	8.68
^{13}C TYRS	-	138.16146	3.39
^{13}C HT	-	154.16239	6.26

mammals.(Ruddick et al., 2006; Reiter, 1991) At the initial point (day 0), just $^{15}\text{-N}_2$ L-TRP is present, and it was consumed promptly. Consequently, its concentration sharply decreases during the first three days of fermentation in line with other works described in the literature (E. Fernández-Cruz et al., 2017). Indeed, $^{15}\text{-N}_2$ L-TRP has a maximum concentration at day 0 (9 mg/L); at day 1 its concentration is 0.3 mg/L, at day 2 of fermentation there is only left, 0.005 mg/L and at day 3 all the $^{15}\text{-N}_2$ L-TRP has been consumed.

However, the graph shows that unlabelled L-TRP (not present at day 0) appears on Day 1 and day 2 at a concentration of 0.01 and 0.002 mg/L, respectively. This L-TRP which has not been added to the fermentation medium, agrees with the fact that yeast is involved in a *novo* tryptophan formation which can be used to synthesise MEL thereafter. The order of appearance of non-labelled compounds is as follows: nonlabelled L-TRP, non-labelled 5-OH-Trp, non-labelled SER (day 1); non-labelled NAS (day 2), non-labelled MEL (day 4). These results evidenced that an important percentage of the intracellular tryptophan is synthesised from the shikimate pathway, using chorismate as carbon skeleton and ammonium (or other amino acid) as provider of nitrogen, by transaminase reactions. Therefore, the formation of MEL, does not depend entirely on the original L-TRP content of the must. Consequently, results prove that there exists at least another alternative pathway for the formation of MEL, which does not depend on the original L-TRP content of the must. Consequently, the results from our experimental design clearly show that L-TRP in must is a precursor for the synthesis of MEL and SER in yeasts but it is not the only one.

It is worth to mention that unlabelled compounds L-TRP, 5-OH-TRP and SER are all determined at day 1. Interestingly, it takes longer time for labelled metabolites to be produced. For instance, it takes 48 h for labelled SER to be formed (it is measured at day 2). Considering that the not labelled L-TRP appears at day 1 it seems likely that another pathway for SER synthesis might exist. In the case of 5-OH-TRP, it is present in the medium from day one to day five at concentrations ranging between 0.005 and 0.009 mg/L. SER is already present on day 1 and is consumed on day 6. Moreover, the concentrations are higher than that of the labelled SER ranging from 0.013 mg/L on day 1 to 0.0005 mg/L on day 6. Similarly, to $^{15}\text{-N}_2$ NAS, unlabelled NAS appears on day 2 and is consumed on day 7. Finally, MEL is detected on day 4 reaching a maximum concentration on day 5 of 0.002 mg/L.

Unlabelled TRYP could not be detected on days 2–5, TOL it is maintained on days 1–5 at a concentration ranging from 0.6 to 0.8 mg/L and decreases from day 6 reaching a concentration of 0.03 mg/L on day 7.

Regarding experiments conducted with ^{13}C TYR, three compounds derived from TYR (4-HPAA, TYRS and HT) were assessed during the nine days of alcoholic fermentation and unequivocally identified with commercial pure standards (Table 1B). In Table 2B the concentrations of the different labelled and unlabelled compounds are displayed. ^{13}C TYR is present on day zero (9 mg/L) and is completely consumed since it is not detected neither on day one nor in any of the following days. However, unlabelled TYR is not detected in any of the 10 days of fermentation.

Fig. 4B shows the concentrations of the labelled and unlabelled 4-HPAA, TYRS and HT throughout the 9 days of fermentation and values are consistent with the route previously described in the literature.(Hazelwood et al., 2008) Labelled 4-HPAA appears the first day at a concentration of 0.5 mg/L and is consumed on the eighth day reaching a maximum concentration on day 3 of 7.8 mg/L. In agreement with the maximum concentration of 4-HPAA on the third day, the maximum TYRS concentration is found on day five at 3.5 mg/L. Finally, HT concentrations throughout the 9 days of fermentation remain practically constant, its concentration being around 0.018 mg/L.

We must highlight the presence of unlabelled compounds derived from TYR. Since the only source of TYR in the medium is the labelled one, these unlabelled compounds should then come from another alternative pathway. Noteworthy, the unlabelled HT concentrations are

Table 2
Concentrations (mg/L) \pm SD of the **A:** L-Trp derived metabolites (labeled (L) and not isotopically labeled (NL) during the seven days of alcoholic fermentation by the yeast *S. cerevisiae* Lalvin YSEO QA23. **B:** TYR derived metabolites (L and NL) during the ten days of alcoholic fermentation by the yeast *S. cerevisiae* Red Fruit. ND: Not detected.

A.												
Days offermentation	Concentration \pm SD (mg/L)											
	L-TRP		5-OH-TRP		SER		NASS		MEL		TRYPT	TOL
	L	NL	L	NL	L	NL	L	NL	L	NL	NL	NL
0	9.35 \pm 0.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0053 \pm 0.0008	0.31 \pm 0.005
1	0.14 \pm 0.02	0.011 \pm 0.002	0.007 \pm 0.001	0.009 \pm 0.002	ND	0.013 \pm 0.0001	ND	ND	ND	ND	0.0045 \pm 0.0004	0.63 \pm 0.02
2	0.015 \pm 0.001	0.0022 \pm 0.0001	ND	0.0028 \pm 0.0001	0.0025 \pm 0.0001	0.016 \pm 0.0006	0.00053 \pm 0.00005	0.00078 \pm 0.0001	ND	ND	ND	0.70 \pm 0.08
3	0.0005 \pm 0.0001	ND	0.0035 \pm 0.002	0.0033 \pm 0.001	0.003 \pm 0.0002	0.014 \pm 0.0005	0.00064 \pm 0.0001	0.0012 \pm 0.0002	0.00022 \pm 0.00002	ND	ND	0.68 \pm 0.00002
4	ND	ND	0.0037 \pm 0.001	0.0055 \pm 0.0005	0.005 \pm 0.0004	0.012 \pm 0.0005	0.00034 \pm 0.0005	0.0017 \pm 0.0005	0.00023 \pm 0.00005	0.0014 \pm 0.0001	ND	0.77 \pm 0.05
5	ND	ND	ND	0.0059 \pm 0.0007	0.0053 \pm 0.0008	0.036 \pm 0.002	0.0040 \pm 0.0006	0.0019 \pm 0.0006	ND	0.0023 \pm 0.0008	ND	0.84 \pm 0.04
6	ND	ND	ND	ND	ND	0.00019 \pm 0.0008	9.27E ⁻⁵ \pm 0.00001	0.00053 \pm 0.0001	0.00046 \pm 0.00008	0.0011 \pm 0.0007	0.007 \pm 0.0004	0.13 \pm 0.008
7	ND	ND	ND	ND	ND	ND	3.59E ⁻⁵ \pm 0.00005	0.00012 \pm 0.00008	0.00082 \pm 0.00005	0.00083 \pm 0.0001	0.005 \pm 0.0007	0.032 \pm 0.006

B.								
Days offermentation	Concentration \pm SD (mg/L)							
	TYR	4-HPAA		TYRS		HT		
	L	L	NL	L	NL	L	L	NL
0	9.28 \pm 1.2	ND	ND	ND	ND	ND	ND	ND
1	ND	0.48 \pm 0.005	0.38 \pm 0.02	0.24 \pm 0.05	0.81 \pm 0.04	0.0003 \pm 0.0001	0.0122 \pm 0.005	
2	ND	2.84 \pm 1.0	1.70 \pm 0.03	1.023 \pm 0.6	1.45 \pm 0.5	0.0019 \pm 0.0008	0.0103 \pm 0.006	
3	ND	7.82 \pm 2.5	0.24 \pm 0.01	0.86 \pm 0.3	0.73 \pm 0.06	0.0019 \pm 0.0006	0.0132 \pm 0.007	
4	ND	4.44 \pm 1.2	4.65 \pm 1.8	2.60 \pm 0.1	1.10 \pm 0.4	0.00185 \pm 0.0004	0.0133 \pm 0.002	
5	ND	3.04 \pm 1.5	3.23 \pm 1	3.50 \pm 1.6	0.90 \pm 0.1	0.00183 \pm 0.0005	0.0136 \pm 0.008	
6	ND	1.79 \pm 0.001	1.23 \pm 0.02	2.56 \pm 0.2	0.93 \pm 0.08	0.00156 \pm 0.002	0.0114 \pm 0.002	
7	ND	1.52 \pm 0.2	0.98 \pm 0.07	2.92 \pm 1.4	0.90 \pm 0.06	0.001848 \pm 0.001	0.0128 \pm 0.004	
8	ND	1.93 \pm 0.3	1.49 \pm 0.2	3.11 \pm 1	0.94 \pm 0.04	0.00178 \pm 0.004	0.0131 \pm 0.008	
10	ND	ND	ND	1.92 \pm 0.5	0.64 \pm 0.03	0.00135 \pm 0.0006	0.00103 \pm 0.0006	

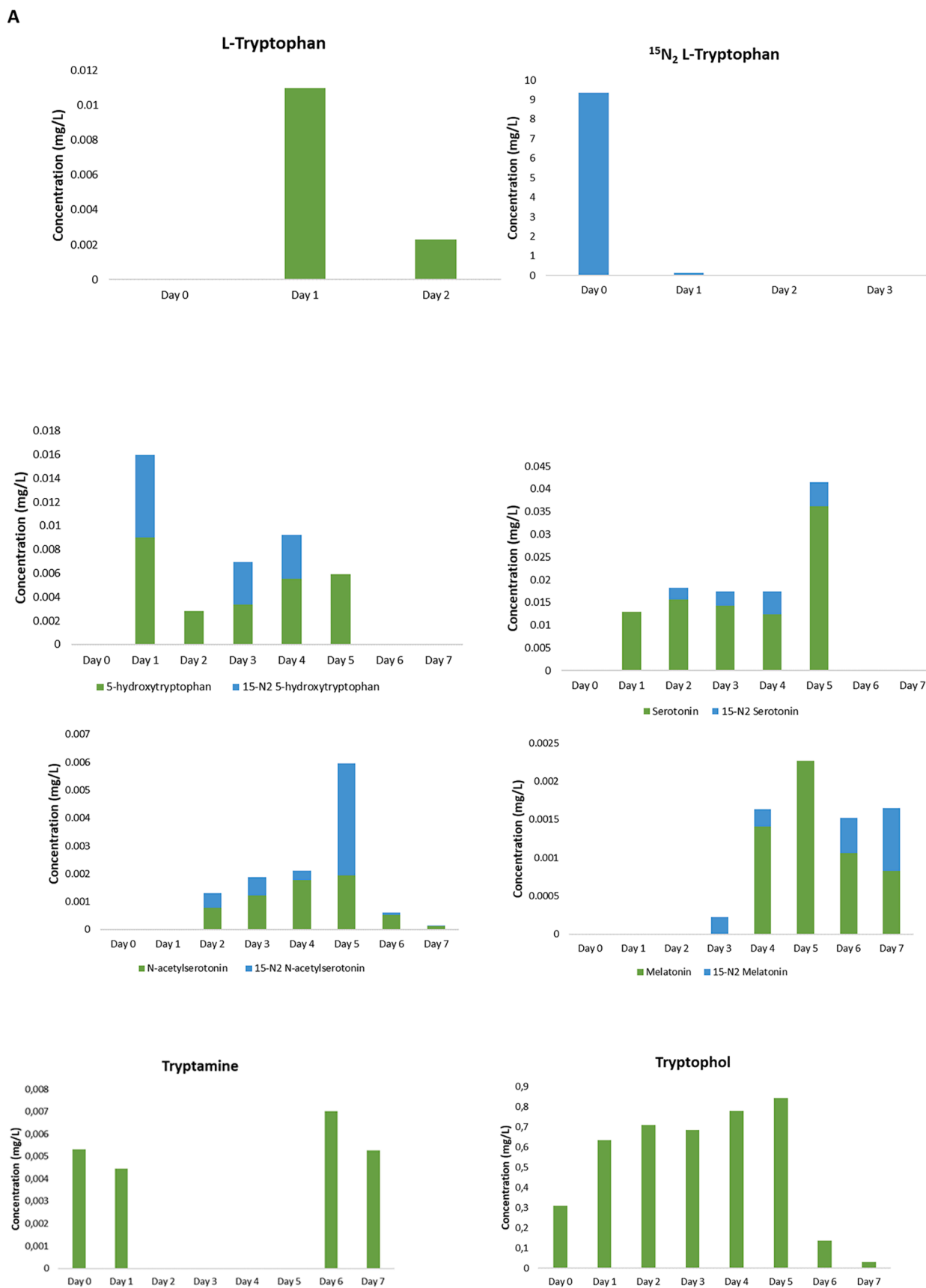


Fig. 4. Concentrations of the: **A.** L-Trp derived metabolites (labeled and not isotopically labeled) during the seven days of alcoholic fermentation by the yeast *S. cerevisiae* Lalvin YSEO QA23; **B.** TYR derived metabolites (labeled and not isotopically labeled) during the ten days of alcoholic fermentation by the yeast *S. cerevisiae* Red Fruit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

B

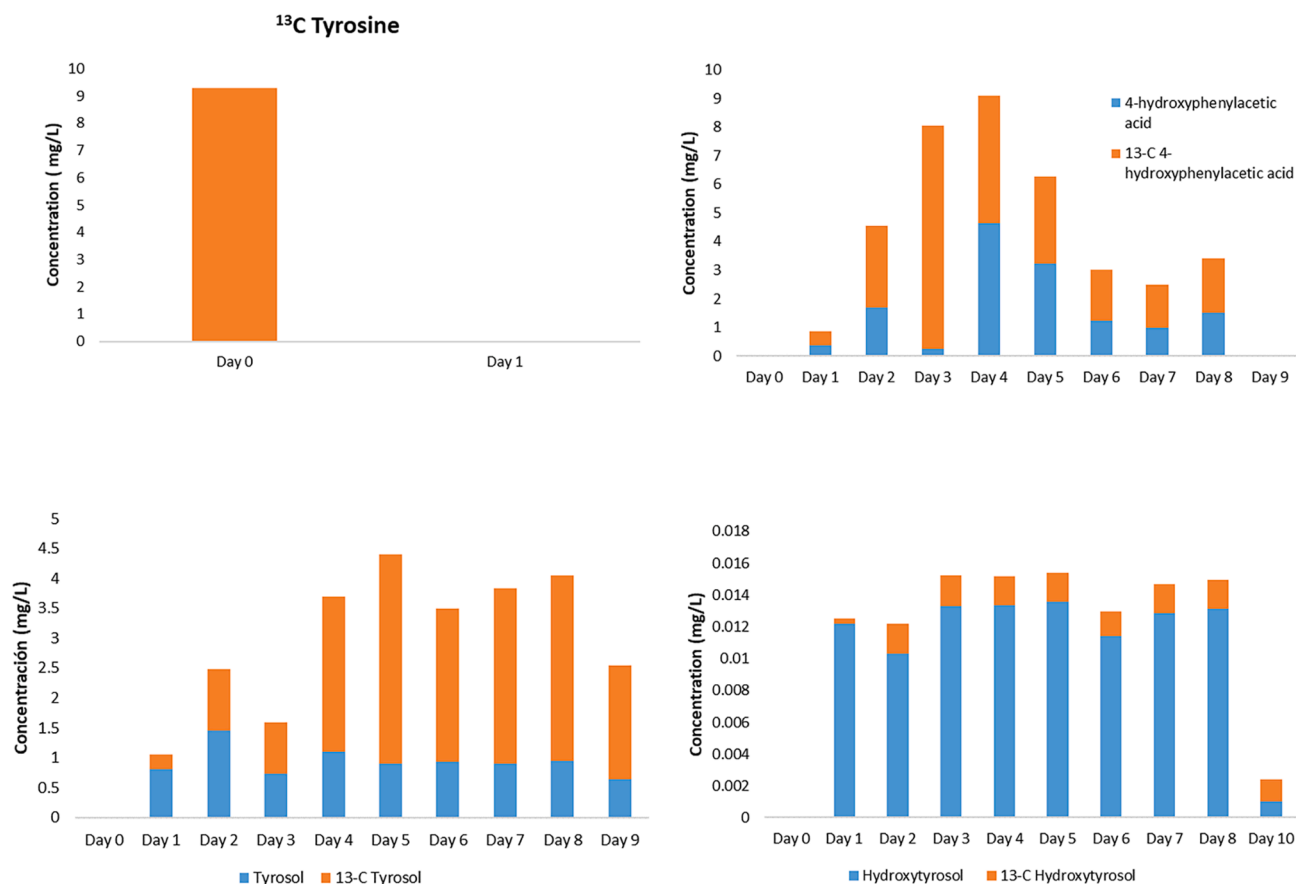


Fig. 4. (continued).

around ten times higher than those of labelled HT (Table 2). These results evidence that the shikimate pathway is more important in the formation of tyrosol than the Ehrlich pathway. That is, sugar metabolism is the main precursor of the aromatic higher alcohol synthesis instead of amino acid catabolism.

4. Discussion

Sprenger and co-workers (Sprenger et al., 1999) reported for the first time that MEL was present in *S. cerevisiae*. Having a circadian rhythm regulator role in vertebrates, the question was to understand its function in yeast, but their data did not support the hypothesis of a regulatory function. Indeed, the role of MEL in yeast and other microorganisms seems far from being understood. Although the presence of circadian rhythms in yeast has been reported (Eelderink-Chen et al., 2010). Furthermore, the response in yeast seems to be induced by temperature changes only after several generations, apparently related to the primary nitrogen metabolism, particularly, to the expression of transporter genes of some nitrogen compounds (MEP2, transporter on ammonium and GAP1, general amino acid transporter) (Eelderink-Chen et al., 2010).

To better understand these functions, it is essential to unravel the possible synthetic pathways for this compound. Sprenger et al.³¹ showed that when starving yeast cells were exposed to the precursor L-TRP (1 mM), MEL did increase (25.3 ng/mg protein). Furthermore, the supplementation with either SER (1 mM), NAS (1 mM) or 5-methoxytryptamine (0.05–0.1 mM) resulted in an increase of MEL (18.0, 43.1 and 90–300 ng/mg protein, respectively). Therefore, they showed that the precursors of the normal biosynthetic pathway described for

vertebrates from L-TRP and subsequent formation of 5-OH-TRP, SER and NAS also led to the formation of MEL. In our experimental design, just the addition of labelled L-TRP is considered without the addition of any other precursor. In turn, we can track labelled compounds to verify the proposed pathway during alcoholic fermentation of a synthetic must (Sprenger et al., 1999) (María Isabel Rodríguez-Naranjo et al., 2012). Moreover, the compounds already described in the vertebrate's pathway are in turn detected in our experiment following a consistent sequence.

In plants, the biosynthetic pathway of MEL begins with L-TRP bioconversion to TRYPT by the enzyme L-TRP decarboxylase (Tan & Reiter, 2020). Then, the subsequent hydroxylation reaction is catalysed by tryptamine-hydroxylase to 5-hydroxytryptamine (Fig. 1A). Our results, however, cannot support this pathway in yeast as the TRYPT is present exclusively in the non labelled form thus not originated from the initial L-TRP. If non labelled MEL is thereafter formed from this non labelled TRYPT remains to be confirmed. According to our results, the only TRYPT detected was unlabelled thus involving another different pathway than that one starting with the original L-TRP present in must. In previous works, feeding yeast with a high amount of TRYPT resulted in SER synthesis (Muñiz-Calvo et al., 2019). Consequently, it is possible that this unlabelled TRYPT is involved in the formation of unlabelled derivatives detected as SER. In fact, the concentration of unlabelled SER fits well with the sum of the concentrations of its two unlabelled precursors 5-OH-Trp and TRYPT.

In summary, it is clear that L-TRP is a precursor of MEL and the rest of derivatives above described. However, from our results it can be deduced that the initial L-TRP in the must is not the solely precursor of MEL for *S. cerevisiae*, thus implying that they may use more than one

pathway for MEL biosynthesis (Dei Cas et al., 2021) and that they are working simultaneously during alcoholic fermentation.

The results obtained in the fermentation carried out with ¹³C TYR and the *S. cerevisiae* Red Fruit yeast verify that HT is formed from TYR by the Ehrlich pathway (Hazelwood et al., 2008) (Fig. 4B). TYR turns into 4-hydroxyphenylpyruvate (4-HPP) through the first transamination-step of the catabolic degradation cascade known as the Ehrlich pathway. After the decarboxylation of 4-HPP in 4-hydroxyphenylacetic acid (4-HPAA), the reduction of 4-HPAA results in the higher alcohol TYRS through the last step of the Ehrlich pathway. Finally, TYRS is converted into HT. Fig. 4B displays the compounds present in the HT synthesis pathway appearing throughout the nine days of fermentation following a logical order.

Many of the fusel alcohols are thought to be formed during alcoholic fermentation by one of two pathways related to amino acid metabolism: (a) catabolism of grape amino acids, characterized by deamination of amino acids to α -keto acids followed by decarboxylation to an aldehyde and, finally, reduction to a fusel alcohol (the Ehrlich pathway) (Hazelwood et al., 2008) or (b) production of α -keto acids during amino acid biosynthesis from sugars, which may then be degraded to fusel alcohols. (Ugliano et al., 2009) Consistently, our results show that both pathways lead to the formation of HT (Fig. 4B). The first one includes labelled compounds from TYRS. The second, the synthesis from α -keto acids. Indeed, non labelled TYR is being detected. Strains capacity of over-producing higher alcohols directly from glucose has already been reported (Cordente et al., 2018).

In addition, in previous works we have used different concentrations of TYR (10 and 60 mg/L) in the fermentation medium without obtaining notable differences in the amount of HT (Rebollo-Romero et al., 2020), which confirms that most of the HT that is formed does not come from the initial TYR must content.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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