**Overproduction of hydroxytyrosol in *Saccharomyces cerevisiae* by
heterologous overexpression of the *Escherichia coli* 4-
hydroxyphenylacetate 3-monooxygenase**

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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.

Keywords: Hydroxytyrosol, *Saccharomyces cerevisiae*, *Escherichia coli*, Polyphenols

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 ~ 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 FADH₂ 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 components (*hpaB* and *hpaC* genes) to overproduce HT in *S. cerevisiae* cultivated in different media (Figure 1). This is an efficient and easy approach to overproduce HT in the main fermentative yeast and it can be the first step for increasing the content of this natural antioxidant in fermented foods and, thus, attracting consumers attention towards fermented beverages.

Material and methods

Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Supplementary Table 1. For cloning *hpaB* and *hpaC* into p426GPD and p425GPD yeast expression vectors, the *hpaB* and *hpaC* open reading frames were PCR amplified from genomic DNA of *E. coli* BL21

(DE3) pLysS and the primers hpaB-F, hpaB-R, hpaC-F and hpaC-R were used to carry out these amplifications (Supplementary Table 2). PCR products and vectors were digested with *Bam*HI and *Xho*I enzymes, gel purified and then ligated to obtain p426GPD-hpaB and/or p425GPD-hpaC plasmids. *E. coli* positive transformants were selected and plasmids sequenced with GPDPro-F and CYC1-R primers (Supplementary Table 2). The resulting recombinant vectors p426GPD-hpaB and p425GPD-hpaC were transformed into *S. cerevisiae* using the LiAc/SS Carrier DNA/PEG method (Gietz, 2014) and selected on selective agar medium according to strain auxotrophic markers and plasmid maintenance needs.

Media and growth conditions

Yeast strains were maintained and grown in YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) or in SC-ura-leu medium [1.7 g/L yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco), 5 g/L ammonium sulfate, 20 g/L glucose, 1.92 g/L drop-out without uracil and leucine (Formedium), supplemented with 16 g/L agar (Pronadisa) for solid media] at 28°C. For overexpression experiments, minimal medium (MM) (1.7 g/L YNB, 0.283 g/L ammonium sulfate, 20 g/L glucose) was used and the amino acid supplementation was performed according to strain auxotrophic markers and requirements of plasmid maintenance. *E. coli* strains were grown at 37 °C in LB media (5 g/L yeast extract, 10 g/L peptone, and 5 g/L NaCl) supplemented with 100 mg/L ampicillin in order to maintain the plasmids.

To test the effect of HpaB and HpaBC complex overexpression on cell growth , 96-well plates were inoculated with cells from diluted overnight saturated cultures to reach an initial OD₆₀₀ = 0.1 in 0.25 mL of fresh SC medium. Likewise, to test a possible toxicity of tyrosol, the same culture conditions were assayed but supplementing SC medium with

different tyrosol concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM). The incubation was set up at 28°C with orbital shaking at 500 rpm and growth readings (OD₆₀₀) were automatically recorded by a SPECTROstar Nano (BMG Labtech) reader every 30 min. Growth parameters were calculated from each condition as was previously described (Bisquert, Muñiz-Calvo, & Guillamón, 2018). All the experiments were carried out in biological triplicates.

To determine tyrosol and HT production, *S. cerevisiae* BY4743 control strain transformed with empty vectors or expressing *hpaB* and/or *hpaC* were inoculated from overnight SC-ura-leu precultures into flat bottom 24 multi-well plates with 1.5 mL fresh medium (MM or SC) and fed with appropriate substrates at an initial OD₆₀₀ of 0.1. Tyrosine and tyrosol were added to a final concentration of 1 mM and the cultures were incubated for 72 h. Final OD₆₀₀ was measured and recorded and then tyrosol and HT levels were determined.

Determination of extracellular tyrosol and hydroxytyrosol concentration by UHPLC-MS/MS

Samples were mixed with 50% methanol, centrifuged at 7500 g for 10 min at 4 °C and filtered through a 0.22 µm nylon filter before UHPLC-MS/MS analysis. Tyrosol and HT were analyzed using an Acquity ultra high performance liquid chromatography (U-HPLC) system (Waters), equipped with a Kinetex XB-C18 100 Å column (2.1 x 100 mm, 1.7 µm; Phenomenex). Buffer A (milliQ water with 1 mM ammonium acetate, pH 5) and buffer B (acetonitrile) were used as a mobile phase. Analytes were eluted at 40 °C with a flow rate of 0.4 mL/min and the injection volume was 5 µL. The gradient program was as follows: 0 – 2.5 min 97:3 % (v/v), 2.5 – 3.5 min, 10:90 % (v/v), 3.6 – 8 min, 97:3 % (v/v). Eluted compounds were detected using an ACQUITY® TQD triple quadrupole mass spectrometer (Waters)

equipped with a Z-spray electrospray ionization source. Spectra were acquired in negative ionization multiple reaction monitoring mode employing an interchannel delay of 0.1 s. The multiple-reaction method transitions were m/z 137 $\rightarrow$ 93 and 137 $\rightarrow$ 106 for tyrosol and m/z 153 $\rightarrow$ 123 for HT.

Results and Discussion

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

Phenolic compounds such as tyrosol and HT exhibit antimicrobial and antifungal activity (Canal, Ozen, & Baysal, 2019; Ghalandari et al., 2018). 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. The maximum growth rate ($\mu_{\max}$) and the area under the growth curve (AUC) were determined for all the different tyrosol concentrations (Figure 2). Tyrosol addition caused no differences in growth parameters across all the concentrations assayed for each strain. According to these results a concentration of 1 mM of tyrosol was established as a supplementation condition. Nevertheless, the growth of the strains overexpressing *hpaB*, *hpaC* or both *hpaB* and *hpaC* was significantly lower than the control strain in a SC medium without tyrosol addition (Figure 2; Supplementary Figure 1). A redox imbalance in these overexpressing strains could cause this growth reduction because both enzymes, HpaB and HpaC, consume the reduced cofactors FADH₂ and NAD(P)H, respectively.

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

We determined the tyrosol and hydroxytyrosol production of the *hpa*-overexpressing and control strains after growth for 72 h in SC or MM medium with or without spiking of 1

mM tyrosine or 1 mM tyrosol (Figures 3 and 4; Supplementary Table 3). Curiously, the titers of both products were higher in MM medium, in spite of a higher OD₆₀₀ was reached in SC than in MM medium (Supplementary Figure 2). The combination of nitrogen limitation (MM) together with the addition of tyrosine resulted in the best condition for tyrosol production (Figure 3; Supplementary Table 3). It has been widely reported that nitrogen limitation strongly promotes the production of aromatic alcohols in different yeast species (Beltran, Esteve-Zarzoso, Rozès, Mas, & Guillamón, 2005; González et al., 2018). 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, Kebaara, Atkin, & Nickerson, 2008; Gori, Knudsen, Nielsen, Arneborg, & Jespersen, 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 HpaB in yeast (Figure 3). However, no difference in tyrosol concentration was observed between both overexpressing cells.

The HT 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 HT. Namely, the control strain was able to produce from 5 to 40 µg/L HT in both media, with a higher production in the presence of tyrosine and, mainly, tyrosol (Figure 4A). Remarkably, the overexpression of *hpaB* increased between 20 and 22-fold the concentration of HT in the growth medium, whereas the overexpression of both genes of the complex increased HT levels between 240 and 316-fold (Figure 4). Despite no differences between both overexpressing strains were observed for tyrosol production (Figure 3), the cells co-expressing both *hpaB* and *hpaC* genes exhibited a higher production of HT (Figure 4). These results suggest that, in the absence of

hpaC, tyrosol can be converted into a product different to HT, decreasing the final HT 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). 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. The effect of tyrosine addition on HT production was always higher in the MM medium, which is the optimal medium for the synthesis of the substrate tyrosol (Figure 4). In any case, the highest levels of HT production (4600 ± 900 $\mu\text{g/L}$) were obtained for the strain overexpressing both *hpaB* and *hpaC* in MM with added tyrosol (Figure 4C).

Conclusions

The synthesis results in the control strain clearly evidences the native capacity of *S. cerevisiae* for hydroxylating tyrosol into HT. However, this so far unknown hydroxylase is not as efficient as the alcohol dehydrogenase (*ADH6*) that converts the p-hydroxyphenylacetaldehyde (4-HPAA) into tyrosol (Figure 1). In this study, we have increased in three orders of magnitude (from $\mu\text{g/L}$ to mg/L) the production of HT in a lab strain of *S. cerevisiae* by simply cloning and heterologously expressing the *hpaB* and *hpaC* genes from *E. coli*. We succeeded in introducing the functional hydroxylase complex HpaBC in *S. cerevisiae* and we also demonstrated the ability of HpaBC to transform tyrosol into HT in yeast. Co-expression of the two components of the complex produced remarkable improvements in HT titers. Yeast cells with *hpaB* and *hpaC* co-expression yielded HT titers between 5 and 10-fold greater than cells overexpressing only *hpaB*, thus evidencing the

196 importance of an oxidoreductase feedback that provides reducing power to improve
197 hydroxylation efficiency.

198 This work lays down the first steps for overproducing HT in yeasts by a metabolic
199 engineering approach. Our findings establish the basis to continue optimizing cultivation
200 media and yeast strains that are able to synthesize HT from glucose, without spiking
201 precursors. Yeast strains capable of overproducing higher alcohols directly from glucose have
202 already been reported (Cordente et al., 2018). A recent study has also shown that certain
203 mutant forms of HpaBC exhibit greater activity and specificity to produce hydroxytyrosol
204 from tyrosol (Chen et al., 2019). Moreover, it has been described the synthesis of tyrosol by
205 hydroxylation of 2-phenylethanol employing a mutant toluene monooxygenases (TMOs) of
206 *Pseudomonas* sp. as biocatalysts (Brouk and Fishman, 2009; Notomista et al., 2011). It would
207 be worthy to study the native hydroxylating activity of the HpaBC complex or to improve its
208 specificity according to the information published by these authors. This information could be
209 used in future metabolic engineering work for the synthesis of HT by yeast, which could be
210 used for multiple purposes taking into account the potent bioactivity of this molecule.

211
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218 **References**

- 219 Álvarez-Fernández, M. A., Fernández-Cruz, E., Cantos-Villar, E., Troncoso, A. M., &
 220 García-Parrilla, M. C. (2018). Determination of hydroxytyrosol produced by
 221 winemaking yeasts during alcoholic fermentation using a validated UHPLC–HRMS
 222 method. *Food Chemistry*, 242, 345–351. <https://doi.org/10.1016/j.foodchem.2017.09.072>
- 223 Angerosa, F., D’Alessandro, N., Konstantinou, P., & Di Giacinto, L. (1995). GC-MS
 224 Evaluation of Phenolic Compounds in Virgin Olive Oil. *Journal of Agricultural and*
 225 *Food Chemistry*, 43, 1802–1807. <https://doi.org/10.1021/jf00055a010>
- 226 Ballou, D. P., Entsch, B., & Cole, L. J. (2005). Dynamics involved in catalysis by single-
 227 component and two-component flavin-dependent aromatic hydroxylases. *Biochemical*
 228 *and Biophysical Research Communications*, 338, 590–598.
 229 <https://doi.org/10.1016/j.bbrc.2005.09.081>
- 230 Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of
 231 the timing of nitrogen additions during synthetic grape must fermentations on
 232 fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food*
 233 *Chemistry*, 53, 996–1002. <https://doi.org/10.1021/jf0487001>
- 234 Bisquert, R., Muñoz-Calvo, S., & Guillamón, J. M. (2018). Protective role of intracellular
 235 Melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*.
 236 *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.00318>
- 237 Bordiga, M., Lorenzo, C., Pardo, F., Salinas, M. R., Travaglia, F., Arlorio, M., ... Garde-
 238 Cerdán, T. (2016). Factors influencing the formation of histaminol, hydroxytyrosol,
 239 tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids
 240 concentration. *Food Chemistry*, 197, 1038–1045.
 241 <https://doi.org/10.1016/j.foodchem.2015.11.112>
- 242 Canal, C., Ozen, B., & Baysal, A. H. (2019). Characterization of antimicrobial activities of
 243 olive phenolics on yeasts using conventional methods and mid-infrared spectroscopy.
 244 *Journal of Food Science and Technology*, 56, 149–158. [https://doi.org/10.1007/s13197-](https://doi.org/10.1007/s13197-018-3468-4)
 245 [018-3468-4](https://doi.org/10.1007/s13197-018-3468-4)
- 246 Chen, W., Yao, J., Meng, J., Han, W., Tao, Y., Chen, Y., ... Tang, S. Y. (2019). Promiscuous
 247 enzymatic activity-aided multiple-pathway network design for metabolic flux
 248 rearrangement in hydroxytyrosol biosynthesis. *Nature Communications*, 10, 1–12.
 249 <https://doi.org/10.1038/s41467-019-08781-2>

250 Chung, D., Kim, S. Y., & Ahn, J. H. (2017). Production of three phenylethanoids, tyrosol,
 251 hydroxytyrosol, and salidroside, using plant genes expressing in *Escherichia coli*.
 252 *Scientific Reports*, 7, 1–8. <https://doi.org/10.1038/s41598-017-02042-2>
 253 Cordente, A. G., Solomon, M., Schulkin, A., Leigh Francis, I., Barker, A., Borneman, A. R.,
 254 & Curtin, C. D. (2018). Novel wine yeast with ARO4 and TYR1 mutations that
 255 overproduce ‘floral’ aroma compounds 2-phenylethanol and 2-phenylethyl acetate.
 256 *Applied Microbiology and Biotechnology*, 102, 5977–5988.
 257 <https://doi.org/10.1007/s00253-018-9054-x>
 258 Di Tommaso, D., Calabrese, R., & Rotilio, D. (1998). Identification and quantitation of
 259 hydroxytyrosol in Italian wines. *HRC Journal of High Resolution Chromatography*, 21,
 260 549–553. [https://doi.org/10.1002/\(SICI\)1521-4168\(19981001\)21:10<549::AID-](https://doi.org/10.1002/(SICI)1521-4168(19981001)21:10<549::AID-JHRC549>3.0.CO;2-Z)
 261 [JHRC549>3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1521-4168(19981001)21:10<549::AID-JHRC549>3.0.CO;2-Z)
 262 Dickinson, J. R., Salgado, L. E. J., & Hewlins, M. J. E. (2003). The catabolism of amino acids
 263 to long chain and complex alcohols in *Saccharomyces cerevisiae*. *Journal of Biological*
 264 *Chemistry*, 278, 8028–8034. <https://doi.org/10.1074/jbc.M211914200>
 265 Ellis, H. R. (2010). The FMN-dependent two-component monooxygenase systems. *Archives*
 266 *of Biochemistry and Biophysics*, 497, 1–12. <https://doi.org/10.1016/j.abb.2010.02.007>
 267 Fernández-Mar, M. I., Mateos, R., García-Parrilla, M. C., Puertas, B., & Cantos-Villar, E.
 268 (2012). Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A
 269 review. *Food Chemistry*, 130, 797–813. <https://doi.org/10.1016/j.foodchem.2011.08.023>
 270 Furuya, T., & Kino, K. (2014). Catalytic activity of the two-component flavin-dependent
 271 monooxygenase from *Pseudomonas aeruginosa* toward cinnamic acid derivatives.
 272 *Applied Microbiology and Biotechnology*, 98, 1145–1154.
 273 <https://doi.org/10.1007/s00253-013-4958-y>
 274 García-García, M. I., Hernández-García, S., Sánchez-Ferrer, Á., & García-Carmona, F.
 275 (2013). Kinetic study of hydroxytyrosol oxidation and its related compounds by red
 276 globe grape polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, 61, 6050–
 277 6055. <https://doi.org/10.1021/jf4009422>
 278 Ghalandari, M., Naghmachi, M., Oliverio, M., Nardi, M., Shirazi, H. R., & Eilami, O. (2018).
 279 Antimicrobial effect of Hydroxytyrosol, Hydroxytyrosol Acetate and Hydroxytyrosol
 280 Oleate on *Staphylococcus Aureus* and *Staphylococcus Epidermidis*. *Electronic Journal of*
 281 *General Medicine*, 15. <https://doi.org/10.29333/ejgm/85686>

282 Ghosh, S., Kebaara, B. W., Atkin, A. L., & Nickerson, K. W. (2008). Regulation of aromatic
 283 alcohol production in *Candida albicans*. *Applied and Environmental Microbiology*, 74,
 284 7211–7218. <https://doi.org/10.1128/AEM.01614-08>

285 Gietz, R. D. (2014). Yeast transformation by the LiAc/SS carrier DNA/PEG method. *Methods*
 286 *in Molecular Biology*, 1163, 33–44. https://doi.org/10.1007/978-1-4939-0799-1_4

287 González, B., Vázquez, J., Morcillo-Parra, M. Á., Mas, A., Torija, M. J., & Beltran, G.
 288 (2018). The production of aromatic alcohols in non-*Saccharomyces* wine yeast is
 289 modulated by nutrient availability. *Food Microbiology*, 74, 64–74.
 290 <https://doi.org/10.1016/j.fm.2018.03.003>

291 Gori, K., Knudsen, P. B., Nielsen, K. F., Arneborg, N., & Jespersen, L. (2011). Alcohol-based
 292 quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces*
 293 *hansenii*. *FEMS Yeast Research*, 11, 643–652. [https://doi.org/10.1111/j.1567-](https://doi.org/10.1111/j.1567-1364.2011.00755.x)
 294 [1364.2011.00755.x](https://doi.org/10.1111/j.1567-1364.2011.00755.x)

295 Guerrini, S., Mangani, S., Romboli, Y., Luti, S., Pazzagli, L., & Granchi, L. (2018). Impact of
 296 *Saccharomyces cerevisiae* Strains on Health-Promoting Compounds in Wine.
 297 *Fermentation*, 4, 26. <https://doi.org/10.3390/fermentation4020026>

298 Hazelwood, L. A., Daran, J. M., Van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008).
 299 The Ehrlich pathway for fusel alcohol production: A century of research on
 300 *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology*, 74,
 301 2259–2266. <https://doi.org/10.1128/AEM.02625-07>

302 Hu, T., He, X. W., Jiang, J. G., & Xu, X. L. (2014). Hydroxytyrosol and its potential
 303 therapeutic effects. *Journal of Agricultural and Food Chemistry*, 62, 1449–1455.
 304 <https://doi.org/10.1021/jf405820v>

305 Jones, J. A., Collins, S. M., Vernacchio, V. R., Lachance, D. M., & Koffas, M. A. G. (2016).
 306 Optimization of naringenin and p-coumaric acid hydroxylation using the native *E. coli*
 307 hydroxylase complex, HpaBC. *Biotechnology Progress*, 32, 21–25.
 308 <https://doi.org/10.1002/btpr.2185>

309 Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J.-C., Lorquin, J., Delattre, M., ...
 310 Labat, M. (2001). Simple phenolic content in olive oil residues as a function of
 311 extraction systems. *Food Chemistry*, 75, 501–507.

312 Li, X., Chen, Z., Wu, Y., Yan, Y., Sun, X., & Yuan, Q. (2018). Establishing an Artificial
 313 Pathway for Efficient Biosynthesis of Hydroxytyrosol. *ACS Synthetic Biology*, 7, 647–

314 654. <https://doi.org/10.1021/acssynbio.7b00385>
 315 Liebgott, P. P., Amouric, A., Comte, A., Tholozan, J. L., & Lorquin, J. (2009).
 316 Hydroxytyrosol from tyrosol using hydroxyphenylacetic acid-induced bacterial cultures
 317 and evidence of the role of 4-HPA 3-hydroxylase. *Research in Microbiology*, 160, 757–
 318 766. <https://doi.org/10.1016/j.resmic.2009.09.015>
 319 Lin, Y., & Yan, Y. (2014). Biosynthesis of caffeic acid in *Escherichia coli* using its
 320 endogenous hydroxylase complex. *Microbial Cell Factories*, 11, 3–11.
 321 <https://doi.org/10.1186/1475-2859-11-42>
 322 Minussi, R. C., Rossi, M., Bologna, L., Cordi, L., Rotilio, D., Pastore, G. M., & Durán, N.
 323 (2003). Phenolic compounds and total antioxidant potential of commercial wines. *Food*
 324 *Chemistry*, 82(3), 409–416. [https://doi.org/10.1016/S0308-8146\(02\)00590-3](https://doi.org/10.1016/S0308-8146(02)00590-3)
 325 Minuti, L., Pellegrino, R. M., & Tesei, I. (2006). Simple extraction method and gas
 326 chromatography-mass spectrometry in the selective ion monitoring mode for the
 327 determination of phenols in wine. *Journal of Chromatography A*, 1114(2), 263–268.
 328 <https://doi.org/10.1016/j.chroma.2006.02.068>
 329 Ooi, B. G., Wanamaker, L. E., Markuszewski, B. M., & Chong, N. S. (2008). Genetic and
 330 enological analysis of selected *Saccharomyces cerevisiae* strains for wine production.
 331 *International Journal of Food Science and Technology*, 43, 1111–1120.
 332 <https://doi.org/10.1111/j.1365-2621.2007.01577.x>
 333 Piñeiro, Z., Cantos-Villar, E., Palma, M., & Puertas, B. (2011). Direct liquid chromatography
 334 method for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines.
 335 *Journal of Agricultural and Food Chemistry*, 59(21), 11683–11689.
 336 <https://doi.org/10.1021/jf202254t>
 337 Romboli, Y., Mangani, S., Buscioni, G., Granchi, L., & Vincenzini, M. (2015). Effect of
 338 *Saccharomyces cerevisiae* and *Candida zeylanoides* on quercetin, vitisin A and
 339 hydroxytyrosol contents in Sangiovese wines. *World Journal of Microbiology and*
 340 *Biotechnology*, 31, 1137–1145. <https://doi.org/10.1007/s11274-015-1863-9>
 341 Xue, Y., Chen, X., Yang, C., Chang, J., Shen, W., & Fan, Y. (2017). Engineering *Escherichia*
 342 *coli* for Enhanced Tyrosol Production. *Journal of Agricultural and Food Chemistry*, 65,
 343 4708–4714. <https://doi.org/10.1021/acs.jafc.7b01369>
 344 Yüksel Aydar, A., Öncü Öner, T., & Elif Fatma, Ü. (2017). Effects of Hydroxytyrosol on
 345 Human Health. *EC Nutrition*, 11, 147–157.

Figures legends

Fig 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 (HT) by the 4-hydroxyphenylacetate-3-hydroxylase (HpaBC) from *E. coli*. It is worth mentioning that yeast is able to endogenously synthesize HT and, therefore, there are one or several unknown enzymes that can lead to HT.

Fig 2. Effect of tyrosol concentration and overexpression of *hpaB* and *hpaC* on yeast growth. The maximum growth rate ($\mu_{\max}$) was determined in SC supplemented with tyrosol ranging from 0 to 5 mM 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.

Fig 3. Tyrosol production from glucose or tyrosine in MM and SC. (A) The yeast strains used were BY4743 overexpressing *hpaB* (green) or both *hpaB* and *hpaC* (orange) from *E. coli* and the control (transformed with empty vectors; blue). Cells were cultured 72 h at 30 °C in MM or SC and the same media supplemented with 1 mM tyrosine. Error bars represent standard deviations calculated from biological triplicates. Tyrosol concentrations were determined from supernatant extracted with methanol and subjected to UHPLC-MS/MS.

Fig. 4 Effect of overexpression of *hpaB* or *hpaB hpaC* in BY4743 on hydroxytyrosol production. Hydroxytyrosol produced by yeast strain BY4743 transformed with the empty vectors p425GPD and p426GPD (blue) (A), with *hpaB* (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.

Fig S1. Effect of tyrosol concentration and overexpression of *hpaB* and *hpaC* on yeast growth. The area under the growth curve (AUC) at 35 h was determined for BY4743 overexpressing *hpaB* (HpaB) or both *hpaB* and *hpaC* (HpaB + HpaC) from *E. coli* and the control (transformed with empty vectors) in SC supplemented with tyrosol ranging from 0 to 5 mM. Error bars represent standard deviations calculated from biological triplicates (A). Simplified kinetic curves for control, *hpaB* and *hpaB hpaC* strains in SC with 0 mM and with the maximum tyrosol concentration were assayed (5 mM) (B).

Fig S2. Growth of *hpaB* and *hpaC* expressing cells in MM and SC media. The OD₆₀₀ was measured after 72 h growth in MM and SC, and the same media supplemented with 1 mM tyrosine or 1 mM tyrosol for three strains. The yeast strains used were BY4743 overexpressing *E. coli hpaB* (green) or both *hpaB* and *hpaC* (orange) from *E. coli* and the control (transformed with empty vectors) (blue) (A). Error bars represent standard deviations calculated from biological triplicates.

Supplementary Tables

Supplementary Table 1. List of yeast strains and plasmids used in this study.

Name	Description	Source
Yeast strains		
BY4743	MATa/ α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0</i>	Euroscarf
Control	BY4743 p426GPD p425GPD	This study
HpaB	BY4743 p426GPD- <i>hpaB</i> p425GPD	This study
HpaB+ HpaC	BY4743 p426GPD- <i>hpaB</i> p425GPD- <i>hpaC</i>	This study
Plasmids		

p425GPD	Multicopy vector (<i>LEU2</i> marker, GPD promoter)	(Mumberg et al., 1995)
p426GPD	Multicopy vector (<i>URA3</i> marker, GPD promoter)	(Mumberg et al., 1995)
p425GPD-hpaC	p425GPD with the <i>hpaC</i> gene from <i>E. coli</i> , Amp ^R	This study
p426GPD-hpaB	p426GPD with the <i>hpaB</i> gene from <i>E. coli</i> , Amp ^R	This study

399

400 **Supplementary Table 2.** List of oligonucleotides used in this study.

Name	Sequence (5'-3')
hpaB-F	AGGTCGTGGGATCCCCATGAAACCAGAAGATTTC
hpaB-R	CCGCTCGAGTTATTTTCAGCAGCTTATCCAGC
hpaC-F	AGGTCGTGGGATCCCCATGCAATTAGATGAACAACGC
hpaC-R	CCGCTCGAGTTAAATCGCAGCTTCCATTTC
GPDPro-F	CGGTAGGTATTGATTGTAATTCTG
CYC1-R	GCGTGAATGTAAGCGTGAC

401

402

Supplementary Table 3. Tyrosol and HT production by Control, HpaB and HpaB + HpaC strains after 72 h growing in MM and SC media with 1 mM tyrosine or 1 mM tyrosol supplementation. Values for detected tyrosol are represented in $\text{mg} \cdot \text{L}^{-1} \pm \text{SD}$, while concentration for HT is represented in $\mu\text{g} \cdot \text{L}^{-1} \pm \text{SD}$.

	Control		HpaB		HpaB + HpaC	
	tyrosol ($\text{mg} \cdot \text{L}^{-1}$)	HT ($\mu\text{g} \cdot \text{L}^{-1}$)	tyrosol ($\text{mg} \cdot \text{L}^{-1}$)	HT ($\mu\text{g} \cdot \text{L}^{-1}$)	tyrosol ($\text{mg} \cdot \text{L}^{-1}$)	HT ($\mu\text{g} \cdot \text{L}^{-1}$)
MM	2.31 ± 0.11	5.4 ± 2.2	0.70 ± 0.07	2.9 ± 1.1	0.96 ± 0.03	33 ± 7
MM + tyrosine	54.3 ± 0.8	19.8 ± 0.8	38.7 ± 0.7	60 ± 7	39 ± 2	1150 ± 50
MM + tyrosol	230 ± 70	38 ± 3	230 ± 90	148 ± 30	138.3 ± 14.3	4600 ± 900
SC	6.0 ± 0.7	2.3 ± 0.5	3.23 ± 0.17	18.9 ± 1.3	4.2 ± 0.6	104 ± 18
SC + tyrosine	17.2 ± 1.1	3.3 ± 1.8	6.97 ± 0.19	29 ± 4	8.5 ± 1.0	128 ± 11
SC + tyrosol	149.3 ± 19.2	12 ± 3	170 ± 80	262 ± 8	160 ± 60	3400 ± 500

407

Gluconeogenesis

Glycolysis

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A **B**

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