Supplementary information

Production of non-cytotoxic pyomelanin by a laccase: properties and chemical structure compared to bacterial and synthetic pigments

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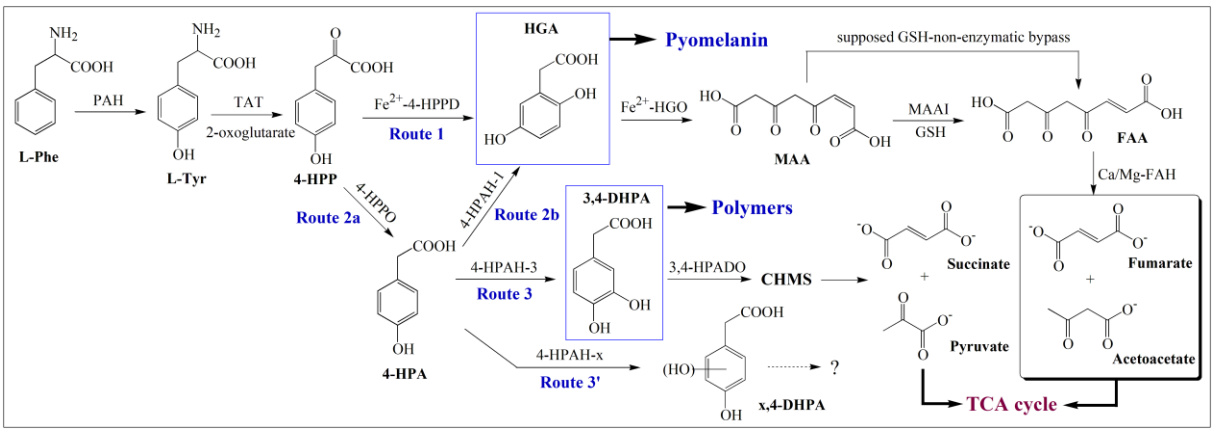


Figure 1S. L-tyrosine (L-Tyr) pathway in microorganisms and degradation of homogentisic acid (HGA). The pathway is firstly initiated by the common transamination of L-Tyr in 4-hydroxyphenylpyruvate (4-HPP) by an L-Tyr transaminase (TAT; EC 2.6.1.5) in presence of 2-oxoglutarate. From there, two distinct routes 1 and 2 led to HGA formation. In the most described route 1, 4-HPP is converted to HGA by a 4-hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27), a nonheme and Fe^{2+}-dependant enzyme. In the less studied route 2, 4-HPP is converted into 4-hydroxyphenylacetic acid (4-HPA) by a 4-hydroxyphenylpyruvate oxidase (HPPO, EC 1.2.3.13). From this, a 4-HPA-1-monooxygenase (4-HPAH-1, EC 1.14.13.18), a key NAD(P)H-dependent enzyme, hydroxylates the ring on C_1 followed by a shift of the acetic group on C_2 and leading to the HGA formation[22]. HGA is further converted into 4-maleylacetoacetic acid (MAA) by a HGA 1,2-dioxygenase (HGO, or HGA oxidase, EC 1.13.11.5) which opens the ring through an *ortho*-cleavage (C_1-C_2 cleavage). Then MAA is enzymatically isomerized in fumarylacetoacetate, this last finally transformed in fumarate and acetoacetate that breeds the Krebs cycle[34]. CHMS, 5-carboxymethyl-2-hydroxymuconic semialdehyde acid; DHPA, dihydroxyphenylacetic acid; FAA, fumarylacetoacetate; FAH, fumarylacetoacetate hydrolase (EC 3.7.1.2); 3,4-HPADO, 3,4-dihydroxyphenylacetate dioxygenase (EC 1.13.11.15); 4-HPAH-x, 4-dydroxyphenylacetic-x-hydroxylase (or monooxygenase, x=1-6); MAAI, maleylacetoacetate isomerase (EC 5.2.1.2); PAH, phenylalanine-4-hydroxylase (EC 1.14.16.1).

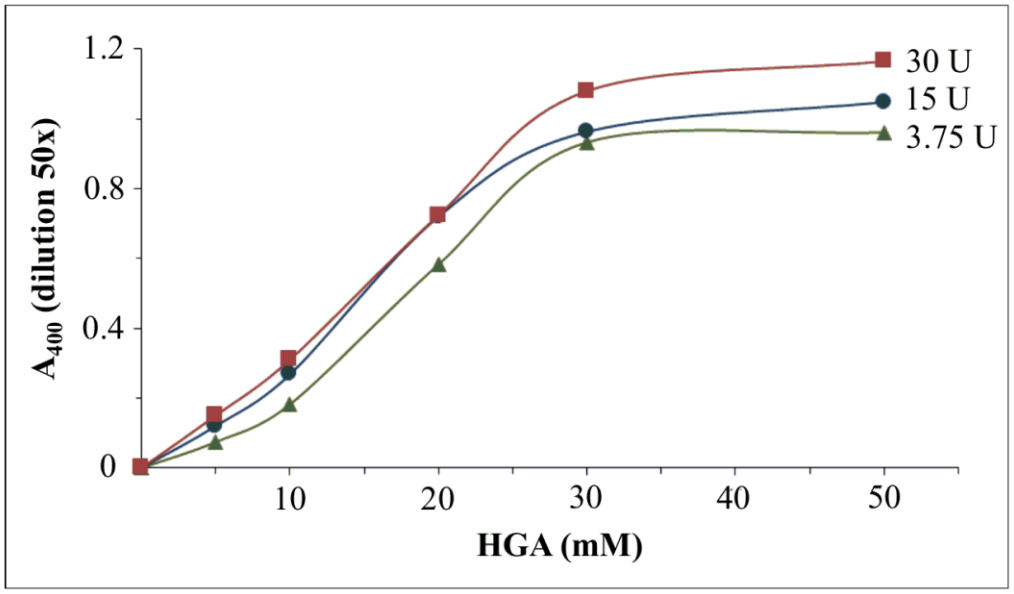


Figure 2S. Determination of the maximal HGA concentration for the pyomelanin synthesis by the rMt laccase at different activities. Conditions: reaction volume 1 mL at pH 6.8. SD ≤ 5% of the mean. The rMt laccase activity determination was adapted from the Sigma technical sheet. A 216 µM syringaldazine stock solution was prepared extemporaneously in MeOH and diluted 10x. The reaction was performed in a total volume of 1.5 mL and contained 1.1 mL of a 100 mM Na-phosphate buffer at pH 6.8 (73 mM final concentration), 250 µL of the laccase rMt, and the reaction started by the addition of 150 µL of the syringaldazine solution (2.16 µM final conc.). The absorbance at 530 nm (A_{530} sample) was read after 10 min incubation in dark at 30°C and compared to a blank performed in absence of laccase (A_{530} reference). Enzyme activity was expressed in Units/mL by using the formula: (A_{530} sample - A_{530} reference)/0.001 x 0.25 x 10, where 0.001 corresponds to the $A_{530\ nm}$ variation per laccase unit at pH 6.8 at 30°C, 10 is the dilution factor of the substrate. The activity was determined in triplicate and standard deviations less than 5% of the mean.

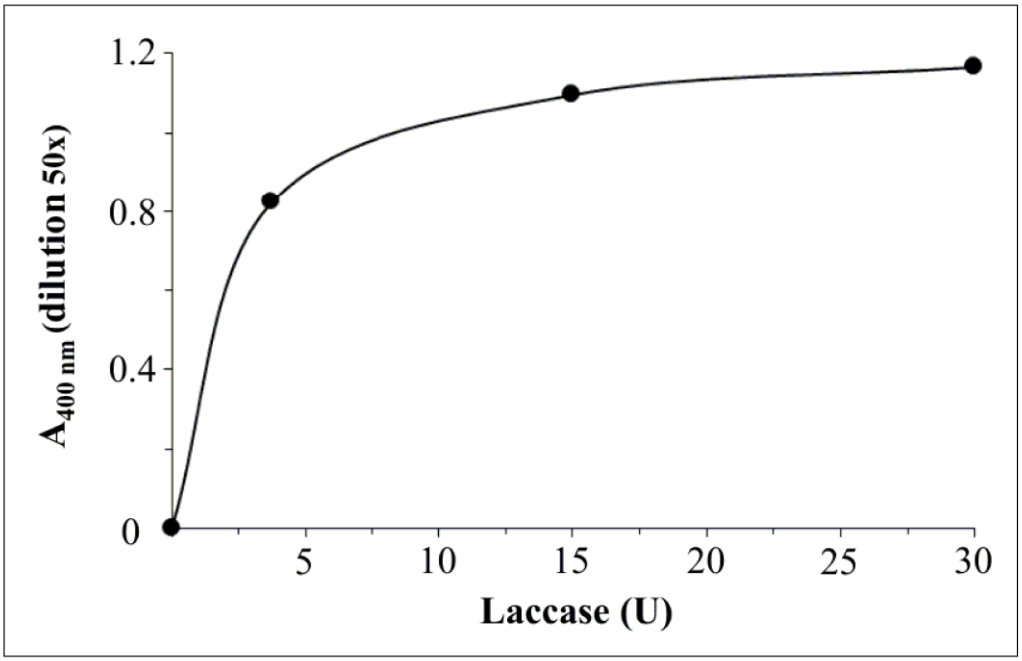


Figure 3S. Determination of the maximal rMt laccase activity for HGA polymerization. Conditions: reaction volume 1 mL, pH 6.8, HGA 40 mM, enzyme activity of the stock solution 750 U/mL (syringaldazine assay, see Fig. 2S). SD ≤ 5% of the mean.

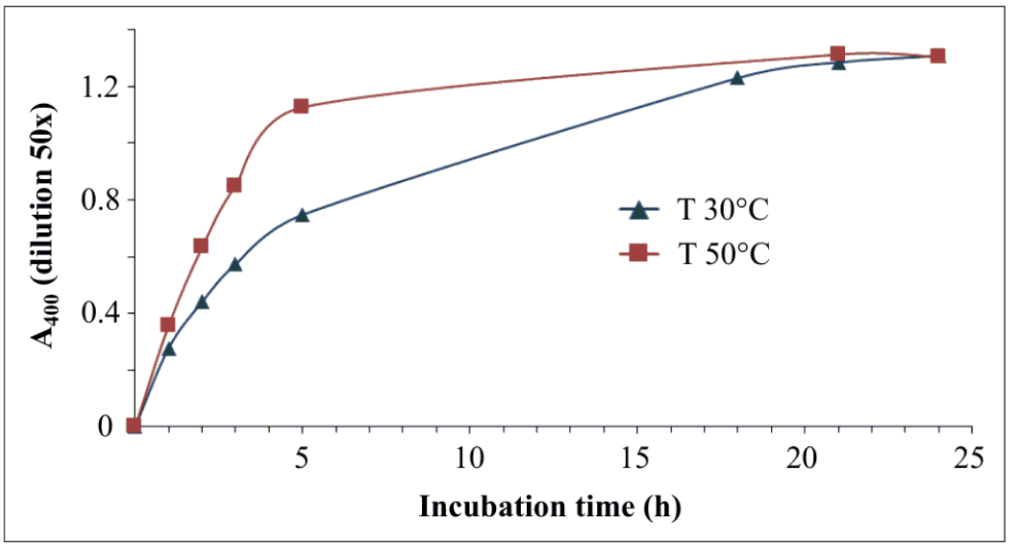


Figure 4S. Determination of the optimal incubation time and temperature on the polymerization of HGA. Conditions: reaction volume 1 mL, pH 6.8, HGA 40 mM, laccase activity 22 U per assay (syringaldazine assay, see Fig. 2S). SD ≤ 5% of the mean.

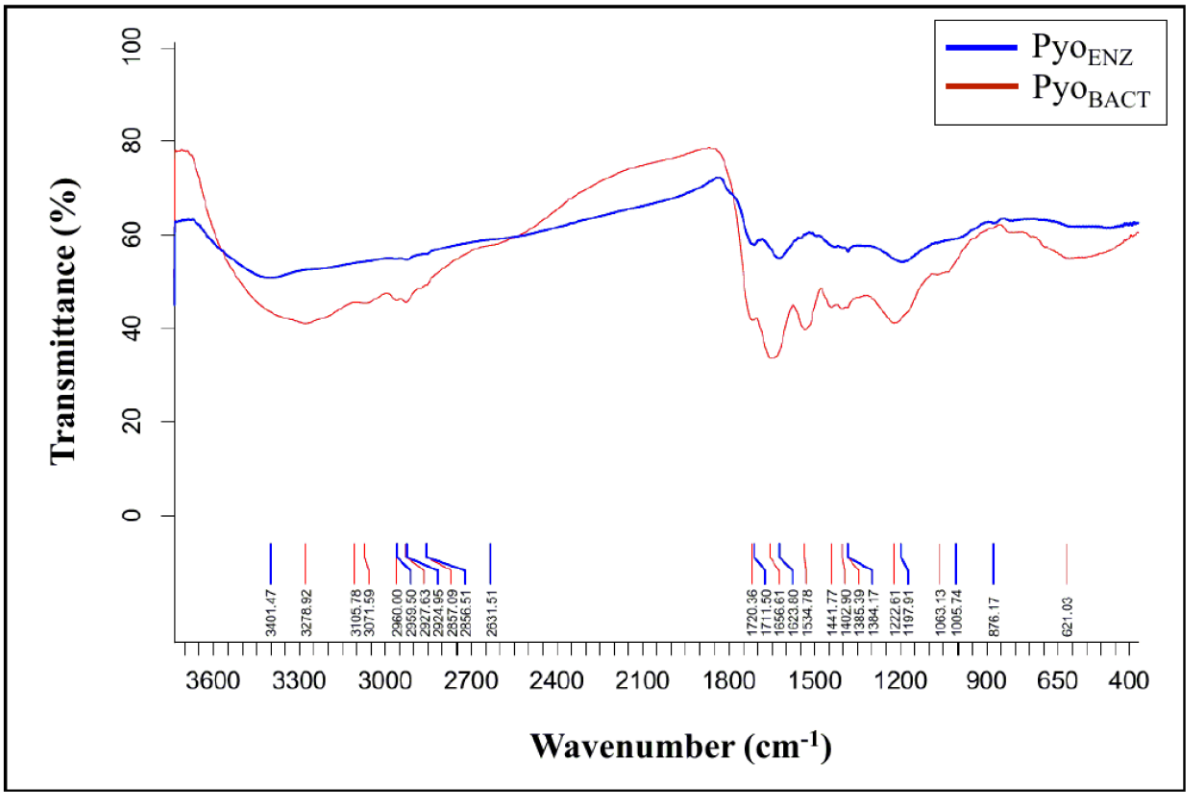


Figure 5S. Fourier-transform infrared (FTIR) spectra of pigments Pyo_{ENZ} (blue line) and Pyo_{CHEM} (red line). Spectra were recorded in the range of wavenumbers (cm^{-1}) of 500-4,000 at a resolution of 4 cm^{-1}, on a Bruker Vertex 70 FT-IR spectrometer (Bruker Optik GmbH, Germany). Transmittance (T%) is expressed in terms of arbitrary units. Samples were prepared as KBr pellets. One mg of pyomelanin was ground to homogeneity in a mortar together with 200 mg dry KBr and immediately pressed to a pellet (10 mm diameter) with 7.5 tons of pressure.

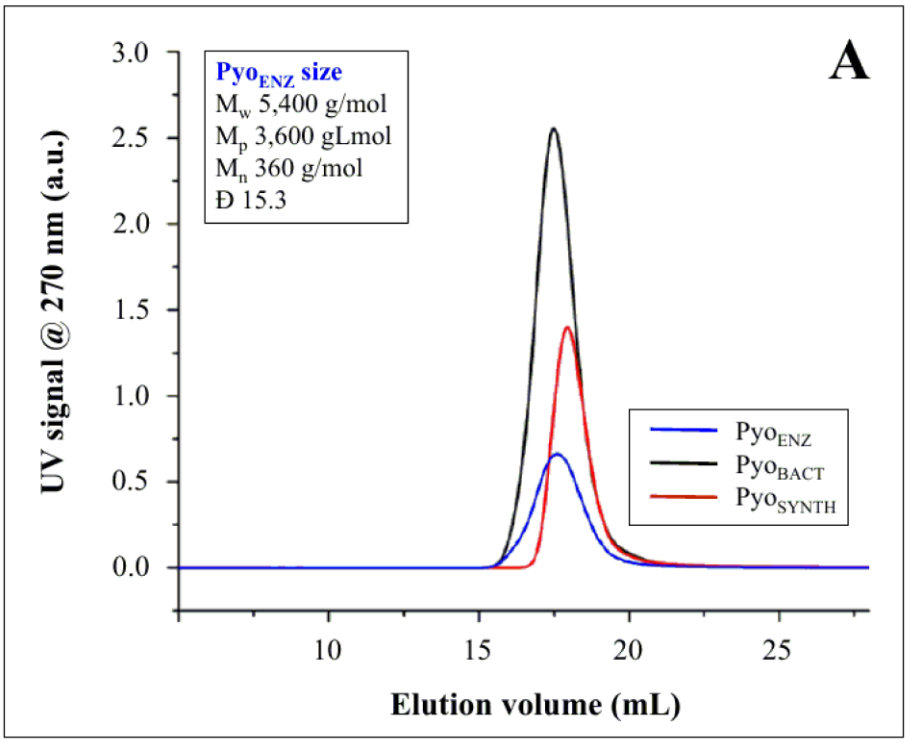
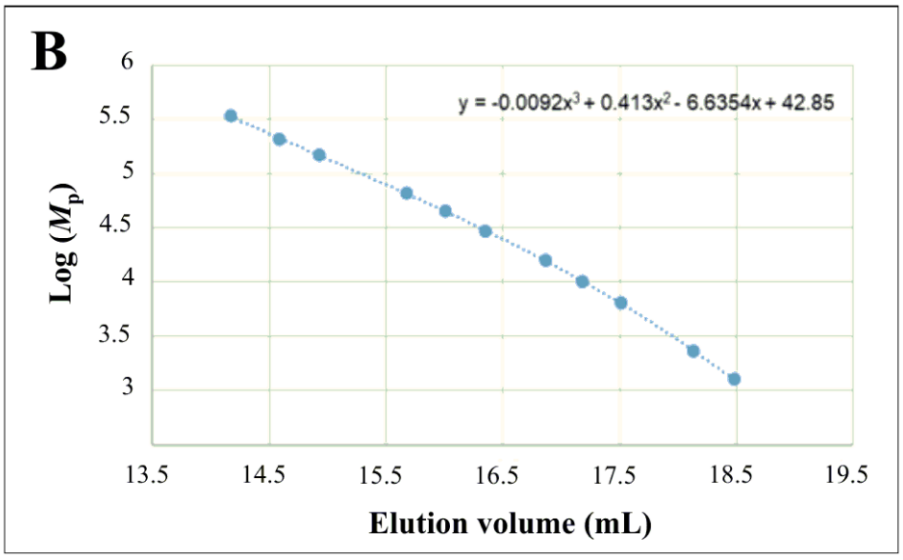
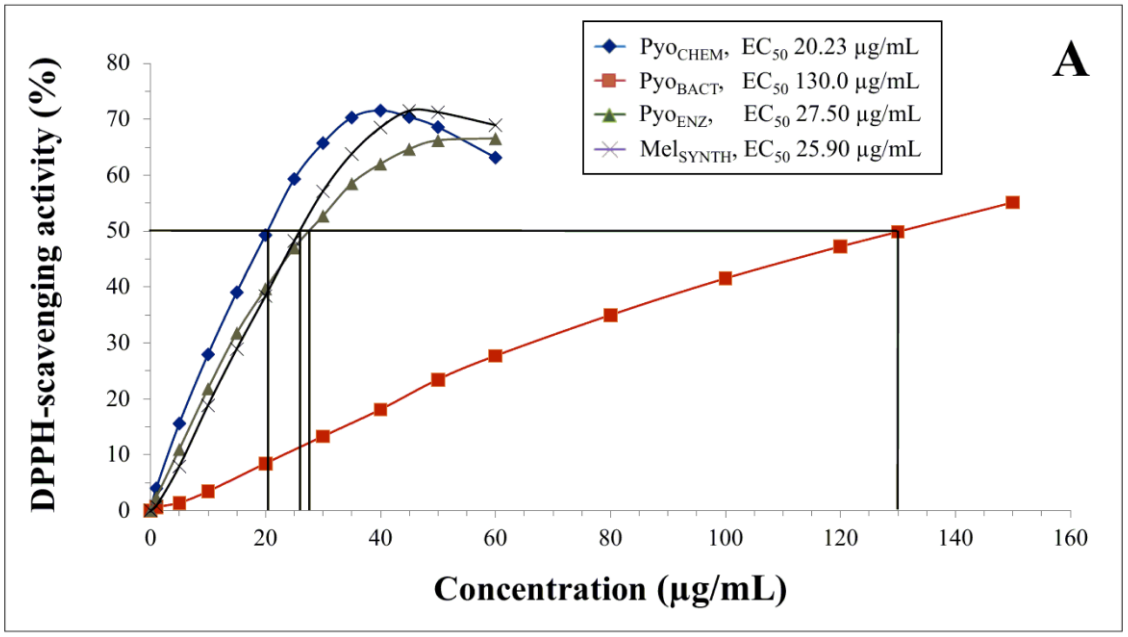


Figure 6S. (**A**) Size exclusion chromatography (SEC) elution profile of the three pyomelanin, Pyo$_{ENZ}$, Pyo$_{BACT}$, and Pyo$_{CHEM}$; (**B**) Calibration curve built using Na-polystyrene sulfonate standards eluted in the same conditions as A. The determination was assessed on an HPLC Waters system composed of a 515 pump, two detectors on line, a 2487-UV set at 270 nm, a 2410-RI, and a 717plus autosampler, all controlled by an Empower GPC-upgraded software. The columns and the RI detector were maintained at 40°C. Separations were achieved on two MCX columns (300 x 8.0 mm, the first at 10^3 and the second at 10^5 Å pore size) from PSS (Perfect Separation Solution, Germany), and protected by a guard column (50 x 8 mm, 5 µm). About 2.5 mg of dried melanin was dissolved in 1 mL of NaOH 0.1 N containing 3 µL ethylene glycol (flow marker), from which 20 µL were injected for the isocratic separation using NaOH 0.1 N as eluent at a flow rate of 1 mL/min. Data were acquired by the software to display M_w (molecular weight), M_n (number average molecular weight), M_p (molecular weight of the highest peak), and Đ (dispersity, M_w/M_n) for each eluted polymer. The system was previously calibrated by Na-polystyrene sulfonate standards (from PSS, M_p 1,260 Da to 340 kDa) eluted in the same manner. A typical equation where v is the elution volume (in mL) is given in **B**. If the solubility of pyomelanin is not ensured, for instance with dimethylformamide (DMF)-LiCl 100 mM for sample preparation and eluent, the pigment forms high M_w agglomerates (~40-50 kDa) on aqueous or organic GPC/SEC supports and false the result.





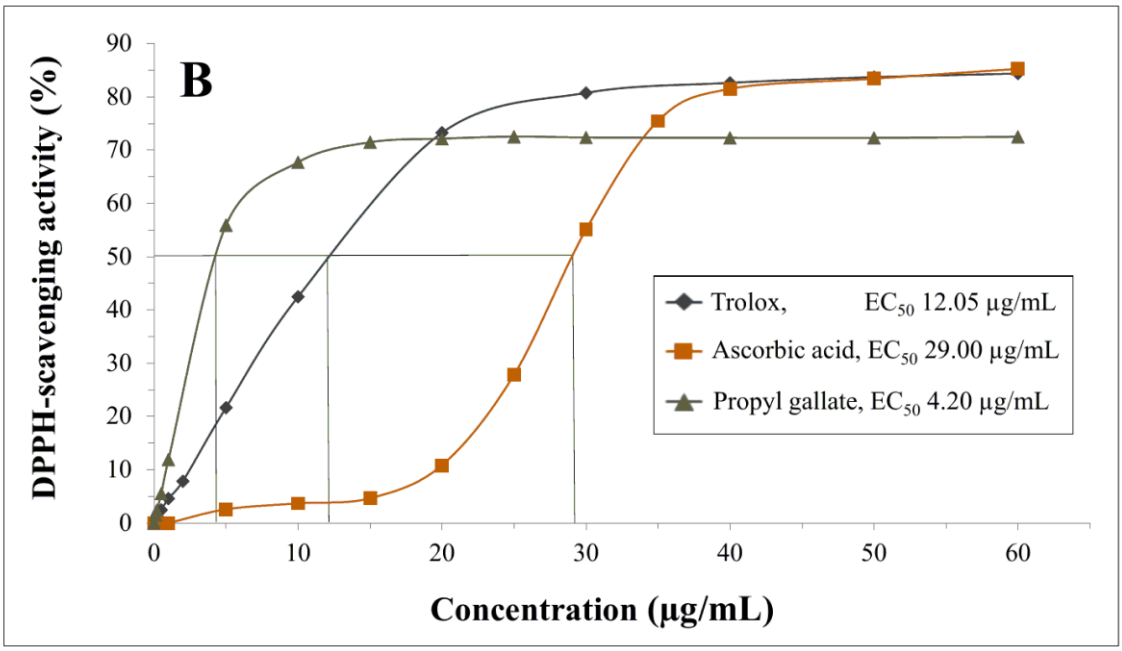


Figure 7S. DPPH free radicals scavenging effect of the purified pyomelanin expressed by the EC_{50} (in µg/mL), comparatively to the commercial melanin Mel_{SYNTH} (**A**), and to standard antioxidants (**B**). All melanin was prepared at 0.5 mg/mL in DMSO by agitation for 24 h, at ambient temperature, with slight sonication (15 s, low power) at two-time intervals. Several concentrations of DPPH were tested to suitably determine the activity. The most convenient assay consisted of mixing 0.6 mL of DPPH 350 µM (in MeOH) with 0.3 mL of pigment in DMSO. The mixture was shaken vigorously, left to stand in the dark for 30 min at room temperature, and $A_{517\ nm}$ read against a blank of DPPH-DMSO (2:1 v/v). Antiradical standards such as Trolox (1 mg/mL stock solution), ascorbic acid (1 mg/mL), and propyl gallate (3,4,5-trihydroxybenzoic acid propyl ester; 0.25 mg/mL), all in DMSO, were used as positive controls and assayed at the range 0.5-60 µg each. The radical scavenging activity (RSA) was determined as the decrease in the $A_{517\ nm}$ and calculated using RSA (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. RSA, and finally expressed as the EC_{50} value which represents the effective concentration of the test compound at which 50% of the DPPH radicals were scavenged. All assays were performed in triplicate, EC_{50} determined by the Prism 7.0 software.