Production and properties of non-cytotoxic pyomelanin by laccase and comparison to bacterial and synthetic pigments

Faustine Lorquin1,2, Fabio Ziarelli3, Agnès Amouric1, Carole Di Giorgio2, Maxime Robin2, Philippe Piccerelle2 & Jean Lorquin1*

Pyomelanin is a polymer of homogentisic acid synthesized by microorganisms. This work aimed to develop a production process and evaluate the quality of the pigment. Three procedures have been elaborated and optimized, (1) an HGA-Mn2+ chemical autoxidation (PyoCHEM yield 0.317 g/g substrate), (2) an induced bacterial culture of Halomonas titanicae through the 4-hydroxyphenylacetic acid-1-hydroxylase route (PyoBACT, 0.55 g/L), and (3) a process using a recombinant laccase extract with the highest level produced (PyoENZ, 1.25 g/g substrate) and all the criteria for a large-scale prototype. The chemical structures had been investigated by 13C solid-state NMR (CP-MAS) and FTIR. Car–Car bindings predominated in the three polymers, Car–O–Car (ether) linkages being absent, proposing mainly C3–C6 (α-bindings) and C4–C6 (β-bindings) configurations. This work highlighted a biological decarboxylation by the laccase or bacterial oxidase(s), leading to the partly formation of gentisyl alcohol and gentisaldehyde that are integral parts of the polymer. By comparison, PyoENZ exhibited an Mw of 5,400 Da, was hyperthermostable, non-cytotoxic even after irradiation, scavenged ROS induced by keratinocytes, and had a highly DPPH-antioxidant and Fe3+-reducing activity. As a representative pigment of living cells and an available standard, PyoENZ might also be useful for applications in extreme conditions and skin protection.

Abbreviations

DPPH  1,1-Diphenyl-2-picrylhydrazyl
FTIR  Fourier Transform InfraRed spectroscopy
GC–MS  Gas chromatography coupled to mass spectrometry
GPC/SEC  Gel permeation/size exclusion chromatography
L-Dopa  L-3,4-dihydroxyphenylalanine
OECD  Organisation for Economic Co-operation and Development
rMt  Recombinant laccase of Myceliophthora thermophile
PEG/PEO  Polyethylene glycol/polyethylene oxide
RP-HPLC-DAD  Reverse-phase HPLC coupled to a diode array detector
TMS  Trimethylsilyl

Pyomelanin is a natural polymer of homogentisic acid (HGA, 2,5-dihydroxyphenylacetic acid) synthesized through the L-tyrosine pathway by bacteria, fungi, mammals, and plants, and belongs to the heterogeneous group of allomelanins1. In living cells, the HGA 1,2-dioxygenase disruption or deletion leads to pyomelanin accumulation2 (Fig. 1S). The first property of the pigment is to protect microorganisms from UV light limiting free radicals and ROS generation3. The excitation of L-Dopa melanin by UV light produces cell-damaging ROS4, whereas the formation of ROS by light has never been reported with pyomelanin. Therefore, pyomelanin increases resistance to light, for instance in Legionella5. Its antioxidant role has also been demonstrated in...
also diminishes the oxidizing stress of the host microorganism, by its high tolerance to H$_2$O$_2$, as demonstrated in *Ralstonia solanacearum* and clinical isolates of *Pseudomonas aeruginosa* from infected patients. Moreover, pyomelanin is an antibacterial and antifungal agent against microbial outside attacks, especially pathogenic, reduces biofouling, chelates heavy metals, and contributes to microbial pathogenesis as it is associated with virulence in a broad range of pathogenic fungi and bacteria. Pyomelanin potentially reduces soluble FeIII in FeII, being essential in many bacteria such as *Legionella pneumophila*, that ensure homeostasis by an appropriate Fe$^{2+}$/Fe$^{3+}$ ratio for their survival. Consequently, in vivo or in vitro, pyomelanin may serve as a terminal electron acceptor, electron shuttle, or conduit for electrons, a complementary iron acquisition to the siderophore role. Several ways for pyomelanin synthesis may be explored. In microorganisms, two distinct pathways led to HGA formation, through a 4-HPD dioxygenase (4-HPPP; EC 1.13.11.27; route 1), the most described from many bacteria mostly pathogen, and via a 4-HPA-1-hydroxylase (4-HPAH-1; EC 1.14.13.18; route 2) (Fig. 1S). To date, there are very little data on microbial pyomelanin production and yield reminds weak (max. 0.35 g/L), comparatively to the eumelanin pigment produced at 28.8 g/L by a tyrosinase overexpression in a recombinant *Streptomyces kathirae*. The construction of a recombinant 4-HPD enzyme is not an option because the substrate 4-HP is expensive. Recently, as opposed to the plant enzyme-based assay, an optimized high-throughput screening assay using human 4-HPPP was constructed using the *E. coli* strain C43 (DE3) supplemented with L-Tyr in the culture medium, a useful tool to find new inhibitors against alkaptonuria disease. However, no pyomelanin yield was reported. On the other hand, the 4-HPAH-1 enzyme responsible for HGA synthesis in *Delftia acidovorans* and *Azotobacter evansii* had been partially characterized. In 2008, the sequence of the *D. acidovorans* enzyme had been given by several genomic approaches. The enzyme contains two components, *hpaH* which codes for a flavoprotein NAD(H)-dependent oxidase that transforms 4-HPA in a non-identified metabolite called *Z*, the second *hpaC* catalyzes the conversion of *Z* in HGA. *hpaH* and *hpaC* were cloned together, however, no HGA synthesis occurred from these constructions. To date, HGA is very expensive, at about $800/gram. Several methods of chemical synthesis have been developed but the majority not were cloned together, however, no HGA synthesis occurred from these constructions. To date, HGA is very expensive, at about $800/gram. Several methods of chemical synthesis have been developed but the majority not were cloned together, however, no HGA synthesis occurred from these constructions.

### Results

#### Development of an enzymatic process (PyoENZ).

To produce high quantities of pyomelanin, appropriate chemical synthesis of HGA on the one hand, and the use of laccase on the other. From this optimized enzymatic process, the resulting pigment (PyoENZ) and its chemical structure and properties had been examined. For comparison, bacterial pyomelanin (PyoBACT) from an induced *Halomonas* culture, the chemical process, was also extensively characterized. Furthermore, the mechanism of HGA polymerization was re-evaluated with a lot of care.

#### Production of pyomelanin (PyoBACT) by an induced wild Halomonas strain.

A bacterial strain able to compete with the enzymatic process (PyoENZ) in terms of production yield, was sought. The strategy consisted to select a *Halomonas* species among a large collection, like our previous studies. Phenolic compounds in the medium were identified and controlled along with the growth of the induced cultures, the strains preferentially utilized the aromatic over glucoses. These halophile bacteria easily grow and have been shown to produce dihydroxy phenols from 4-hydroxyphenylacetic acid (4-HPA), such as HGA in *H. olivaria*, *H. venusta*, *H alkalihiphila*, and 3,4-dihydroxyphenylacetic acid (3,4-DHPA) in *H. alkaliantartica*, *H. neptunia*, *H. sulfaedris* (this work), and *H. sp*. HTB24 (this work), through routes 2b and 3, respectively (Fig. 1S). *H. titanicae* is a γ-proteobacterium isolated on the Titanic wreck, its genome now entirely available, and has been selected for the most intense brown-black color from 5 mM 4-HPA supplemented cultures measured by the *A$_{400nm}$* (this work). The suspected presence of pyomelanin was first confirmed by identification of HGA (λ$_{max}$ 290 nm) only in the exponential phase, but not 3,4-DHPA or other dihydroxyphenylacetic derived compounds, by RP-HPLC-DAD and GC-MS.
of the TMS-derived metabolites, showing a matched fragmentation spectrum with that of the HGA standard. The strain could not grow in the presence of L-Tyr and was unable to metabolize 2-HPA or 3-HPA, hence suggesting that a 4-HPA-5-hydroxylase or a 4-HPA-6-hydroxylase were not implied, respectively. We concluded that *H. titanicae* was able to produce pyomelanin by direct conversion of 4-HPA to HGA through a 4-HPA-1-hydroxylase (4-HPAH-1, route 2b, Fig. 1S). Following this, pyomelanin production has been optimized. Because 5 mM 4-HPA was rapidly consumed in 2 days and served as an inducer of 4-HPAH enzymes, successive additions of well-defined amounts of 4-HPA at different culture times were carried out by following an experimental design procedure (see Methods). Finally, pyomelanin was overproduced in a 500 mL medium by adding 5 mM 4-HPA at starting, then 10 mM after 3 days, in a total culture time of 6 days. In these conditions, *H. titanicae* was able to furnish 0.55 ± 0.09 g PyoBACT per Liter of culture, a mean of three independent experiments. Relative to the total amount of 4-HPA added, the recalculated yield was 0.241 ± 0.04 g PyoBACT per g of 4-HPA.

**Production by chemical autoxidation (PyoCHEM).** While pyomelanin issued from the HGA autoxidation has been commonly used, the reaction has never been optimized to date. The common precursor 2,5-DMPA could also be synthesized from 2,5-dimethoxyacetophenone by a Willgerodt-Kindler reaction type. Step 4 is the final HCl precipitation followed by washing and drying. Doubling the amount of HBr (step 1) led to incomplete demethylation and the extra formation of 2,5-dihydroxyphenylacetalddehyde (~6%) identified from the HPLC-DAD spectrum (λ<sub>max</sub> 292 nm) and the EI-MS profile (molecular ion [M + 2TMS] at m/z 294, characteristic fragments [M – CHO] at m/z 265, and [M – CH₂CHO] at m/z 251), similarly to the NIST data bank and previous data. In step 2, the addition of sulfite (Na₂SO₃) was unnecessary because the solution was immediately buffered to 6.8 and the polymerization by the laccase followed (step 3). The alkaline opening of the lactone was essential, indeed the rMt laccase was unable to open the lactone nor demethylate 2,5-DMPA at pH 6.8, even after several days of agitation. BQA, 1,4-benzoquinone acetic acid; gentisaldehyde, 2,5-dihydroxybenzaldehyde; gentisyl alcohol, 2,5-dihydroxybenzyl alcohol; 2,5-DMPA, 2,5-dimethoxyphenylacetic acid; 2,5- DMAPO, 2,5-dimethoxyacetophenone.

![Figure 1.](image1.png)
Structural data of the three pyomelanin. Better than 1H-NMR, solid-state 13C-NMR analyses of polymers can provide not only structural features through the resonances of the monomers but also the types of bindings. Pyomelanin issued from the three developed processes, PyoENZ, PyoBACT, and PyoCHEM were analyzed by solid-state 13C CP-MAS at their optimal signal resolution in conjunction with FTIR experiments.

Solid-state 13C CP-MAS NMR. Spectra were cumulated in Fig. 2, and chemical shifts summarized in Table 1 along with those of pure HGA analyzed in the same conditions. The three spectra exhibited common, typical, and prominent signals in slightly varied positions, at δ 172–173.4 ppm that corresponded to the unprotonated carbon in C-O/C = O of the carboxylic group, then at 149.3–149.4 of the unprotonated carbon (suggested C5) of the ring bearing the -OH group, with shoulders at 143.4 for the three pyomelanin (suggested C2), and at 118–119 ppm provided by the ethylenic and protonated carbons of the ring (-CH = C-) together in broadband. Less high signals at 33.0–34.8 ppm observed on the three structures represented the saturated aliphatic carbons (-CH2-) of the acetic acid moiety. From the three 13C solid-state NMR spectra, the main differences are in the region around δ 45–78 ppm, precisely at 52.5 and 67.9 (larger) ppm in PyoENZ, and 52.5 ppm alone in PyoBACT, whereas these two shifts are absent in PyoCHEM (Fig. 2). They suggested secondary reactions during the biological BQA polymerization that did not occur during autoxidation of HGA in abiotic and alkaline conditions. By comparison to standard molecules analyzed in parallel, the δ 52.5 ppm shift corresponds to the bi-protonated carbon of the ethanolic moiety (-CH2-O-) from 2,5-dihydroxybenzyl alcohol (gentisyl alcohol). Besides gentisyl alcohol, we also noted minor peaks at δ 190.5 and 191 ppm in PyoBACT and PyoENZ, respectively, absent in PyoCHEM, and ascribed to an aldehyde group of the end-product 2,5-dihydroxybenzaldehyde (gentisaldehyde) (Table 1). Gentisyl alcohol and gentisaldehyde resulted from a decarboxylation reaction extensively detailed in Fig. 1. With a lot of precautions because solid-state NMR was a semi-quantitative tool, the relative level of the decarboxylation products in the polymers was deduced from areas of the corresponding peaks (Fig. 2), and approximately evaluated at 11–13% (gentisyl alcohol 9–10% + gentisaldehyde 2–3%), these two compounds could not be identified by FTIR. Besides, low signals visualized at δ 17.2–23.1 (PyoENZ) and 17.4–23.9 ppm (PyoBACT) were attributed to lipid residues provided by the enzyme extract and the culture medium, respectively. The broad signal at δ 67.9 ppm present in PyoENZ only had been assigned to the hydroxylated 13C of a saccharide moiety (>C-OH) also brought by the laccase extract, whereas it was absent in PyoBACT, probably because the H. titanicae medium was not supplemented with glucose. Importantly, the area ratio of the 170/118 ppm resonances for each pyomelanin...
(Fig. 2, data framed) indicates a correct -CH₂-COOH substitution for PyoENZ and PyoBACT with a value of 1/6, whereas a loss of the carboxylic moiety on PyoCHEM structure (ratio 1/10) has occurred. More information on the polymer assembly was necessary to elucidate the mechanism of polymerization and the types of linkage between the rings, i.e. Car-Car (aryl carbon) or/and Car-O-Car (aryl ether) linkages.

**FTIR analyses.** PyoBACT and PyoENZ exhibited very similar FTIR and C-NMR spectra, hence the study was focused on the PyoENZ and PyoCHEM absorptions (Fig. 5S) noting that the spectrum of PyoCHEM was better resolved in reason to its less high $M_w$ (2,300 Da, Table 2). The peaks at the following wavenumbers and their corresponding structures included the bands for PyoENZ and PyoCHEM, respectively at (i) 3401 and 3278 cm⁻¹ (broad) indicative of the –OH stretch of polymeric structures; (ii) two smaller bands for each compound at 2960 (PyoENZ) and much more intense at 2925–2927 cm⁻¹ (PyoCHEM), which corresponded to stretching vibrations of the aliphatic Car–H groups; (iii) 1711 and 1720 cm⁻¹ quite resolved here and ascribed to carbonyl stretching (C=O) of the -COOH group, these bands were however absent on other microbial pyomelanin; (iv) 1623 and 1656 cm⁻¹ absorptions that were described as typical for aromatic C=C conjugated with C=O groups (quinones), with a stronger response in PyoCHEM; (v) 1384 and 1385 cm⁻¹ (both minor) would be assigned to the O–H bond of the hydroxyl groups attached to the ring; and (vi) strong bands at 1197 (PyoENZ) and 1222 cm⁻¹ (PyoCHEM) of the phenolic-OH links.

**Presence of N in PyoENZ And PyoBACT.** Especially, a reaction of substitution on the C₄ position of the BQA ring by primary and secondary amines had been reported, such substitutions might occur in biological systems. Here, the volume of the laccase extract added for the PyoENZ synthesis seemed insignificant, hence it remained difficult to look for amide or amine bonds from polymers, especially when they are minor. These C-N absorptions were generally encountered at δ 155–180 ppm (amides formed from the carboxylic moiety) and 135–145 ppm (aromatic amines) in C-NMR, mainly at 3000–3500 cm⁻¹ (N-H stretching vibrations of aromatic nitrogens).

| Compound  | Protonated carbons | Unprotonated carbons |
|-----------|--------------------|---------------------|
|           | δ (ppm) | Assignation  | δ (ppm) | Assignation  | References |
| PyoENZ    |         |             |         |             |             |
| 17.2      | –CH₃   | (lipid residue) | 122 sh  | Car₁⁻ (suggested) |
| 23.1      | –CH₂-R | (lipid residue) | 143.4 sh | Car₂–OH |
| 34.3      | –CH₂– (acetic group) | 149.3 |         |             |             |
| 52.5 br   | –CH₂–OH (gentisyl alcohol) | 172.0 | –COOH (carboxylic C) |         |             |
| 67.9 br   | Saccharide moiety (>C–OH) |         |         |             |             |
| 119.0     | –C₆H₃=C₆             | 173.0 | –COOH (carboxylic C) |         |             |
| 191.0 w   | –CHO (aldehyde group on C₆) |         |         |             |             |
| PyoBACT   |         |             |         |             |             |
| 17.4      | –CH₃   | (lipid residue) | 124 br  | Car₁⁻ (suggested) |
| 23.9      | –CH₂-R | (lipid residue) | 143.4 sh | Car₂–OH |
| 34.8      | –CH₂– (acetic group) | 149.2 |         |             |             |
| 52.5 br   | –CH₂–OH (gentisyl alcohol) | 152 sh | Undetermined |         |             |
| 118.1     | –C₆H₃=C₆             | 173.4 | –COOH (carboxylic C) |         |             |
| 190.5 w   | –CHO (aldehyde group on C₆) |         |         |             |             |
| PyoCHEM   |         |             |         |             |             |
| 33.0      | –CH₂– (acetic group) | 143.4 sh | Car₂–OH |         |             |
| 110.0     | Undetermined | 149.4 |         |             |             |
| 118.1     | –C₆H₃=C₆             | 173.4 | –COOH (carboxylic C) |         |             |
| Standards|         |             |         |             |             |
| HGA       | 33.9    | –CH₂– (acetic group) | 122.3 | Car₁⁻ | Data similar to the literature,
|           | 116.5   | Car₄=H | 146.7 | Car₁⁻–OH |
|           | 117.0   | Car₅=H | 148.3 | Car₂–OH |
|           | 120.9   | Car₆=H | 180.9 | –COOH (carboxylic C) |
| Gentisyl alcohol | 55.1 | –CH₂–OH | 125.9 | Car₁=CH₂OH | Data quite similar to those of Molbase site (www.molbase.com) |
|           | 115.9   | –CH₂–OH | 147.1 | Car₂=OH |
|           |         |         | 154.4 | Car₃=OH |
| Gentisaldehyde | 119.2 | –C₆H₃=C₆ (Car₄=H)* | 125.9 | Car₁ (–CHO attachment) | Data quite similar to those of Molbase site (www.molbase.com) |
|           | 200.6   | –CHO (aldehyde group on C₆) | 149.0 | Car₂–OH |             |
|           |         |         | 155.6 | Car₃–OH |             |

Table 1. Summary of the $^{13}$C CP-MS solid-state NMR chemical shifts and their corresponding assignation for the three pyomelanin pigments, and comparison with standards. Car, aromatic carbon; sh, shoulder; br, broad; w, weak. *Unresolved; # well resolved.
amines) in FTIR, thus drowned in those of the major functional groups. Faced with this inability to detect traces of nitrogenous derivatives by NMR and FTIR, elemental analyses of the three pyomelanin were carried out and showed the presence of N in PyoENZ (2.75%) and PyoBACT (3.65%), as expected none in PyoCHEM, and higher in the indole-based melanin MelSYNTH (6.34%) and MelSEPIA (6.31%) (Table 2). The presence of N in PyoENZ and PyoBACT is due to amino acids and amines linked on C4 of the HGA rings and provided by the rich laccase extract and the components of the H. titanicae culture medium, respectively.

Table 2. Summarized chemical and biological properties of pyomelanin and commercial melanin. Since the A400 nm value of solubilized pyomelanin depends on the size of the pigment, a spectrophotometric quantification by surrogate melanin for calibration will not be correct. Weighing precisely the final purified pigment remains the only valuable technique for the quantification of pyomelanin as well as other melanin.

| Production yield | PyoENZ | PyoCHEM | PyoBACT | MelSYNTH | MelSEPIA |
|------------------|--------|---------|---------|----------|----------|
| MW (g/mol)       | 5400   | 2300    | 5700    | 2000     | ND³      |
| Mw (g/mol)       | 360    | 350     | 470     | 250      | -        |
| Ml (g/mol)       | 3600   | 2500    | 4600    | 1600     | -        |
| D (Mw/Ml)        | 15.3   | 6.64    | 11.9    | 7.9      | -        |
| Monomer composition | HGA, BQA, GA, GALD³ | HGA, BQA³ | HGA, BQA, GA, GALD³ | DHI, DHICA³ | DHI, DHICA³ |

Elemental analysis (%):

- C: 49.15 ± 0.08, 54.61 ± 0.11, 40.58 ± 0.03, 49.69 ± 0.10, 34.10 ± 0.03
- H: 3.40 ± 0.02, 2.59 ± 0.10, 3.15 ± 0.03, 2.83 ± 0.05, 3.23 ± 0.02
- N: 2.75 ± 0.02, 0, 3.65 ± 0.05, 6.34 ± 0.03, 6.31 ± 0.08
- O: 44.70, 42.80, 52.62, 41.14, 56.36
- C/O: 1.10, 1.27, 0.77, 1.20, 0.6
- C/N: 18 – 11 11 8 5.4

Chemical formula:

- PyoENZ: C221H140N11O15 (5362 g/mol)
- PyoCHEM: C105H59O6 (2295 g/mol)
- PyoBACT: C193H179N15O18 (5697 g/mol)
- MelSYNTH: C83H57N9O5 (1995 g/mol)
- MelSEPIA: C55H35N4O23 (3750 g/mol)

Solubility:

- NaOH 0.05 N: ≤ 10 mg/mL
- DMSO: ≤ 0.5 mg/mL
- Other solvents:
  - Insoluble
  - Insoluble
  - Insoluble
  - Insoluble

DPPH-antioxidant activity (IC50 µg/mL): 27.5

ROS scavenging activity (IC50 µg/mL): 82.2 ± 5.5

UV spectrum range: 200–700 nm

Fe³⁺-reducing activity:

- % Related to MelSYNTH: 96, 95, 54, 100, 34
- In ng Fe³⁺/h/µg³: 5.30, 5.24, 2.98, 5.52, 1.94

Stability to T°C:

- ≤ 80 °C, 72 h: ≤ 80 °C, 72 h: ≤ 80 °C, 72 h: ND: ND

Cytotoxicity (≤ 500 µg/mL):

- No, No, No, No, No

Phototoxicity (PIF):

- No (<2), No (<2), No (<2), No (<2), No (<2)

Linkage determination. Interestingly, the FTIR spectra showed absorption at 1534 cm⁻¹ strongly present in PyoCHEM (Fig. 5S, red) and absent in PyoENZ (blue) and PyoBACT. This resonance did not correspond to amides and was rather ascribed to aromatic C₅=H. From this remarkable difference, it has been established that PyoENZ
contains much less Car-H free, which means much more Car Car linkages than PyoCHEM. As an important finding from the three pyomelanin 13C NMR spectra, Car O Car (aryl ether) linkages were absent (Fig. 2), the related signal generally resonates at around δ 160–167 ppm14,43. Hence, the three HGA polymers were assembled by Car Car linkages only.

Alkaline-H2O oxidation assays. This treatment has also been tried on the three pyomelanin and the commercial melanin41 (Table 2). While hydrolyzed MelSYNTH and MelSEPIA melanin led to the two expected degradation products similarly to the literature1,41,42, pyrrole-2,3-dicarboxylic acid (PDCA, an indicator of DHICA-derived units) and pyrrole-2,5,5-tricarboxylic acid (PTCA, of DHICA-derived units), any compound has been detected from the three hydrolyzed pyomelanin by LC(DAD)-MS analyses (see Table 2). Thus, pyomelanin could not be hydrolyzed by such peroxide treatment, even after doubling or lowering the peroxide concentration. A pyrosis-GC-MS coupling method had been developed to analyze pyomelanin from Penicillium chrysogenum44 but reported too much heterogeneity to obtain uniform results between samples. This method utilizes heat to break the polymer into smaller fragments, such as 4-methoxybenzene acetic acid, 4-methoxybenzene propa-noic acid, and other minor phenolic compounds, but not HGA. Unfortunately, this technique failed in PyoENZ, PyoBACT, and PyoCHEM with any identifiable compound.

Physicochemical properties (summarized in Table 2). All pigments (3 pyomelanin, 2 commercial L-Dopa melanin MelSYNTH and MelSEPIA) are insoluble in neutral or acidic water as well as many usual organic solvents, entirely soluble in alkaline media such as NaOH (0.05 N minimal conc.). Exceptionally, all the pigments are soluble in DMSO at a concentration that does not exceed 0.5 mg/mL after 24 h agitation. Solubility in H2O was not improved after 2 days at 80 °C. As solid form and/or solubilized in alkaline solutions, PyoENZ, PyoBACT, and PyoCHEM were stable until 80 °C for 3 days (max. tested) with no degradation products detected by RP-HPLC, and GC–MS analyses, near size modification by GPC/SEC. Molecular weights (Mw) of the three HGA-pigments were successfully determined and have been found at 5,400 Da (dispersity 15.3) for PyoENZ, Ɖ 11.9) for PyoBACT, and a less high Mw at 2,300 Da (5,700 Da (Fig. 6S, Table 2), explaining why PyoCHEM and MelSYNTH were more rapidly solubilized in DMSO than the others. These Mw data were very close to those resulting from the elemental analyses (Table 2), indicating that these pigments were sufficiently purified by successive water and ethanol-washings.

Antiradical properties. The scavenging ROS activity was studied for PyoENZ, comparatively to the standards MelSEPIA and MelSYNTH. UVA induces damage by directly transferring energy or indirectly through ROS generated as primary and secondary radiolytic products44. Therefore, the protection by melanin pigment against UVA may be due to their ability to scavenging ROS in the cells. To prove this, a fluorescein-derived compound (DCFH-DA) was used to detect the generation and change of ROS in UVA-visible irradiated keratinocyte cells. UV A may be due to their ability to scavenging ROS in the cells. To prove this, a fluorescein-derived compound (DCFH-DA) was used to detect the generation and change of ROS in UVA-visible irradiated keratinocyte cells. Indeed, keratinocytes are a source of ROS that may affect neighboring skin cells, such as melanocytes, and influence the process of melanogenesis or contribute to the progression of vitiliginous lesions. Fluorescence measurements showed that PyoENZ effectively scavenged ROS generated by UVA-visible light in the test system with an IC50 of 82.2 ± 5.6 μg/mL, while IC50 of MelSYNTH 284.1 ± 12.3 μg/mL was higher and that of MelSEPIA very far (Table 3). Thus, the amount of ROS in the cells decreased as the concentration of PyoENZ increased, much more efficiently than the concurrent pigment MelSYNTH.

The DPPH-antioxidant activity was rarely reported due to the insolubility of the pyomelanin in organic solvents, and because the stable DPPH reagent reacts at slightly alkaline pH values. The assays were carried out on the three HGA-pigments, along with the two standards (MelSEPIA, MelSYNTH) and common antiradical agents such as Trolox, ascorbic acid, and propyl gallate, all prepared in DMSO. Figure 7S-A and 7S-B indicated that PyoENZ, PyoCHEM, and PyoBACT were stable until 80 °C for 3 days (max. tested) with no degradation products similarly to the literature41,42, pyrrole-2,3-dicarboxylic acid (PDCA, an indicator of DHICA-derived units) and pyrrole-2,5,5-tricarboxylic acid (PTCA, of DHICA-derived units), any compound has been detected from the three hydrolyzed pyomelanin by LC(DAD)-MS analyses (see Table 2). Thus, pyomelanin could not be hydrolyzed by such peroxide treatment, even after doubling or lowering the peroxide concentration. A pyrosis-GC-MS coupling method had been developed to analyze pyomelanin from Penicillium chrysogenum44 but reported too much heterogeneity to obtain uniform results between samples. This method utilizes heat to break the polymer into smaller fragments, such as 4-methoxybenzene acetic acid, 4-methoxybenzene propa-noic acid, and other minor phenolic compounds, but not HGA. Unfortunately, this technique failed in PyoENZ, PyoBACT, and PyoCHEM with any identifiable compound.

Electron-transfer efficacy. By an adapted ferrozine assay, PyoENZ, PyoCHEM, and MelSYNTH exhibited equivalent and highest Fe3+-reducing activity among the five polymers tested (Fig. 3). From these data, the equivalent Fe3+-reducing activity of PyoENZ and PyoCHEM could not be explained, while PyoENZ and PyoCHEM have a different Mw (Table 2), thus none the same number of -OH and carboxylic groups, and even if gentisyl alcohol and gentisaldehyde (at ~ 11 to 13%) are present in PyoENZ structure only. Comparatively to MelSYNTH (100%), the reducing activity in decreasing order was PyoENZ (96), PyoCHEM (95), and to a less extent PyoBACT (54) and MelSEPIA (34). Because PyoENZ has the best production yield and is dedicated to potent applications, its Fe3+-reducing activity was evaluated at 1.73 μM per hour related to 50 μg of pigment, i.e. 5.30 ng Fe3+/h/μg.
strategies by optimized production of HGA autoxidation (PyoCHEM), induced bacterial culture (PyoBACT), and was observed as compared to the non-treated cells, thus formally postulating the absence of toxic effect on skin for the assessment of the toxic hazard of cosmetic ingredients. No reduction of the metabolic activity of the cells cytotes are the most abundant cells of the epithelial layer of the skin and are used as a part of the 3D skin model from pigment prepared in alkaline solutions at dilutions which in no way modified the pH of the assay. Keratinocytes, cytotoxicity toward human keratinocytes has been evaluated by the vital dye NR penetration technique, fluorescence (Fluo) was expressed as a percentage as compared to the irradiated and non-irradiated control. Cells were decanted, washed twice by a 25 mM PBS buffer at pH 7.4, then loaded with 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) to a final concentration of 20 μM, and incubated in dark at 37 °C for 30 min. ROS were then measured by fluorescence intensity of dichlorofluorescein (DCF) at an excitation wavelength of 499 nm and an emission wavelength of 521 nm, in an Infinite M200 Pro fluorescence reader (TECAN, Swiss) equipped with a 1-cm quartz cell. Irradiation was carried out with the Suntest CPS + solar simulator (xenon arc lamp 1100 W, with filters to restrict light transmission below 290 nm and near IR), at a dose of UVA-visible light of 138 kJ/m², the irradiance was at 765 W/m² and irradiation time 3 min. The temperature of the samples was maintained at 4 °C using a water-cooling system linked to the irradiation chamber. Inhibition of DCFH-DA fluorescence (Fluo) was expressed as a percentage as compared to the irradiated and non-irradiated control (without pyomelanin or melanin); ROS release (%) = 100 × (Fluo+irr control − Fluo-irr control)/(Fluo+irr control − Fluo-irr control). ROS release was detected by fluorometric measurement and inhibition of the DCFH-DA reagent. PyoENZ has been obtained at the highest level, 1.25 g per g 2,5-DMPA, a yield > 1 g/g due to compounds linked and brought by the concentrated enzyme extract. This procedure meets all the criteria to design a large-scale prototype, high-efficient, cheapest, with mild conditions, and without sterility constraints that are essential in the case of microbial cultures. HGA-lactone was easily prepared from 2,5-DMPA, or even from 2,5-dimethoxyacetophenone to reduce the costs by an additional reaction of Willgerodt-Kindler. Despite the great number of extensive works on pyomelanin-producing microorganisms, to date there have been only three reported quantifications of the pigment, first with the wild yeast Yarrowia lipolytica that furnished 0.035 g/L of culture, second 0.173 g/L culture of the Shewanella algae BrY strain supplemented by 2 g of L-Tyr/L, and third 0.35 g/L by random mutagenesis of Pseudomonas putida. In this work, an induced culture of H. titanicae was shown to convert 4-HPA to HGA by a 4-HPA-1-hydroxylase (4-HPAH-1) at the best microbial yield to date, 0.55 g/L culture, a feature confirmed by the presence in its genome of the related ipaH/C genes (unpublished). Such bioconversion generally occurred with less energy consumption, and for these reasons more efficiently. It seems reasonable to assume that the bacterial (PyoBACT) and the chemical (PyoCHEM) processes will never be able to compete with the laccase process (PyoENZ) in terms of production, except maybe by developing a recombinant overproducing microorganism. From these results,

| Concentration (µg/mL) | Fluorescence intensity | ROS release (%) | IC50 (µg/mL) |
|-----------------------|-----------------------|----------------|--------------|
| **PyoENZ**            |                       |                |              |
| 50                    | 331.6 ± 3.3           | 65.92 ± 2.64   | 82.2 ± 5.6   |
| 100                   | 244.3 ± 7.7           | 35.05 ± 1.71   |              |
| 250                   | 187.0 ± 6.6           | 14.86 ± 2.16   |              |
| 500                   | 164.0 ± 4.0           | 6.70 ± 2.28    |              |
| **MelSYNTH**          |                       |                |              |
| 100                   | 412.6 ± 2.8           | 94.51 ± 2.61   | 284.1 ± 12.3 |
| 250                   | 373.3 ± 11.0          | 80.67 ± 4.90   |              |
| 500                   | 326.0 ± 12.2          | 63.97 ± 5.13   |              |
| **MelSEPIA**          |                       |                |              |
| 100                   | 414.6 ± 5.3           | 95.17 ± 1.70   | > 500        |
| 250                   | 393.3 ± 4.9           | 87.70 ± 3.57   |              |
| 500                   | 334.6 ± 19.9          | 66.92 ± 5.87   |              |

Table 3. Effect of PyoENZ on scavenging ROS generated by UVA irradiation on keratinocyte cells. The ROS release was detected by fluorometric measurement and inhibition of the DCFH-DA reagent. PyoENZ antiradical activity was compared to that of the melanin standards. Determinations resulted from triplicate assays and IC50 values were calculated by the Phototox v2.0 software (ZEBET, Germany). Experimentally, keratinocyte cells (see phototoxicity protocol for preparation) were UVA-irradiated in presence of graded concentration (50–500 µg/mL) of PyoENZ and standard melanin (MelSYNTH, MelSEPIA), or not (control). Cells were decanted, washed twice by a 25 mM PBS buffer at pH 7.4, then loaded with 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) to a final concentration of 20 μM, and incubated in dark at 37 °C for 30 min. ROS were then measured by fluorescence intensity of dichlorofluorescein (DCF) at an excitation wavelength of 499 nm and an emission wavelength of 521 nm, in an Infinite M200 Pro fluorescence reader (TECAN, Swiss) equipped with a 1-cm quartz cell. Irradiation was carried out with the Suntest CPS + solar simulator (xenon arc lamp 1100 W, with filters to restrict light transmission below 290 nm and near IR), at a dose of UVA-visible light of 138 kJ/m², the irradiance was at 765 W/m² and irradiation time 3 min. The temperature of the samples was maintained at 4 °C using a water-cooling system linked to the irradiation chamber. Inhibition of DCFH-DA fluorescence (Fluo) was expressed as a percentage as compared to the irradiated and non-irradiated control (without pyomelanin or melanin); ROS release (%) = 100 × (Fluo+irr control − Fluo-irr control)/(Fluo+irr control − Fluo-irr control). IC50 (µg/mL) values were calculated by the Phototox v2.0 software.

**Cytotoxicity.** For applications with pyomelanin as an ingredient for cosmetics or pharmaceutical preparations, cytotoxicity toward human keratinocytes has been evaluated by the vital dye NR penetration technique, from pigment prepared in alkaline solutions at dilutions which in no way modified the pH of the assay. Keratinocytes are the most abundant cells of the epithelial layer of the skin and are used as a part of the 3D skin model for the assessment of the toxic hazard of cosmetic ingredients. No reduction of the metabolic activity of the cells was observed as compared to the non-treated cells, thus formally postulating the absence of toxic effect on skin cell metabolic activity for PyoENZ, PyoBACT, PyoCHEM, MelSYNTH, and MelSEPIA until 500 µg/mL (Table 2). Furthermore, using the normalized OECD protocol commonly used for cosmology product evaluation, the three pyomelanin and the two standard melamins were found non-phototoxic (PIF < 2) (Table 2).

**Discussion**

The laccase process is the most efficient provider of pyomelanin. Comparison of three realistic strategies by optimized production of HGA autoxidation (PyoCHEM), induced bacterial culture (PyoBACT), and for the first time using a recombinant laccase (PyoENZ) was undertaken. PyoENZ has been obtained at the highest level, 1.25 g per g 2,5-DMPA, a yield > 1 g/g due to compounds linked and brought by the concentrated enzyme extract. This procedure meets all the criteria to design a large-scale prototype, high-efficient, cheapest, with mild conditions, and without sterility constraints that are essential in the case of microbial cultures. HGA-lactone was easily prepared from 2,5-DMPA, or even from 2,5-dimethoxyacetophenone to reduce the costs by an additional reaction of Willgerodt-Kindler. Despite the great number of extensive works on pyomelanin-producing microorganisms, to date there have been only three reported quantifications of the pigment, first with the wild yeast Yarrowia lipolytica that furnished 0.035 g/L of culture, second 0.173 g/L culture of the Shewanella algae BrY strain supplemented by 2 g of L-Tyr/L, and third 0.35 g/L by random mutagenesis of Pseudomonas putida. In this work, an induced culture of H. titanicae was shown to convert 4-HPA to HGA by a 4-HPA-1-hydroxylase (4-HPAH-1) at the best microbial yield to date, 0.55 g/L culture, a feature confirmed by the presence in its genome of the related ipaH/C genes (unpublished). Such bioconversion generally occurred with less energy consumption, and for these reasons more efficiently. It seems reasonable to assume that the bacterial (PyoBACT) and the chemical (PyoCHEM) processes will never be able to compete with the laccase process (PyoENZ) in terms of production, except maybe by developing a recombinant overproducing microorganism. From these results,
Pycnoporus cinnabarinus fied recombinant Trametes versicolor evaluated that the enzyme furnished ~ twofold less pyomelanin than rMt, while the puri-

increases after UF (PEG/PEO standards). Turick et al. showed two main suggested assembly modes, C4-C6 (α-bindings) and C3-C6 (β-bindings), giving preference to

to synthesize pyomelanin from 4-HPA and the property of the pigment to reduce Fe3+, raise the question of the survival of the bacterium at 4,000 m depth by maintaining a Fe3+/Fe2+ ratio.

A few remarks are worth noting about the oxidation of HGA. Besides the biological implications of metal-catalyzed oxidations, true autoxidation of biomolecules does not occur in biological systems, instead, this autoxi-
dation is the result of transition metals bound to these biomolecules. By analyzing the PyoCHEM structure, surprisingly the 13C solid-state NMR spectrum revealed an unexplained loss (~40%) of carboxylic moiety during the alkaline Mn2+-autoxidation of HGA without observable by-products of this degradation and hence contrib-
tutes to the low pyomelanin yield. To date, the in vitro polymerization of HGA by a laccase has never been studied before. Here, the rMt laccase had been found to efficiently catalyze the HGA polymerization in terms of yield, and still confirmed the involvement of these oxidases in biological environments. It should be noted that pyomelanin-forming bacteria generally grow at pH 6–7, while the autoxidation is optimal at pH 8–9, one more element in favor of the laccase(s) action in living cells. The rMt enzyme supplied as a rich and concentrated extract is largely available and one of the cheapest in the market. Partial purification by ultrafiltration of the rMt extract would be an additional stage unnecessary. Indeed Aljawish et al. showed that, if the brown color decreases after UF (~90%), it eliminates only 2.5-fold of total proteins and the specific activity of the UF-enzyme increased by only 2.1-fold. Other laccases had also been assayed in parallel. At their optimal parameters, we

Figure 3. Fe3+ reduction by pyomelanin issued from the three processes (PyoENZ, PyoBACT, PyoCHEM), and comparison to the commercial melanin (MelSEP, MelSYNTH). HGA and ascorbic acid were used as positive controls. The Fe3+-reducing activity was ada from other methods. Briefly, 400 µM ferrozine and 120 µM anhydrous FeCl3 (final conc.) were extemporaneously mixed in a Tris-HCl 25 mM buffer pH 7.5 (solution A). Then, 5 µL (50 µg) of the melanin stock solutions, each at 10 mg/mL in NaOH 0.05 N, was added in 1 mL of A in closed glass tubes. Blanks were prepared identically without melanin. Mixtures were incubated for 12 h at room temperature and the developed color was measured at 562 nm. As positive controls, 10 µL (44 µg) of ascorbic acid 25 mM solution, and 20 µL (50 µg) of HGA 15 mM, which have defined reduction activity, were also mixed with 1 mL A, incubated, and measured identically. To quantify the activity, a standard curve was generated with known concentrations of ferrous sulfate complexed with ferrozine (solution A), a stock solution of FeSO4·7H2O 3 mM in milliQ-H2O used. After correction, the concentration of Fe2+ varied from 0.92 to 13.8 µg, i.e., 6 to 90 µM, and a typical standard equation was: A562 nm = 0.026 x [Fe2+ (µM)] + 0.0093. All values were expressed as a mean of three independent experiments, SD < 5% not given to lighten the figure. These experiments suggest that the reducing capacity of HGA is lost after polymerization, and ascorbic acid remains the stronger reducing agent which is however degraded on time.

Biological pyomelanin is a Cα-Cα assembly polymer that contains two decarboxylation-issued products. Because alkaline-H2O, hydrolyses and pyrolysis experiments failed, the chemical structure of the three pyomelanin was determined by 13C solid-state NMR, and partly confirmed by FTIR analyses. Like the hydroquinone polymerization, Cα-Cα bindings between the rings predominated in PyoENZ, PyoBACT, and PyoCHEM, a finding deduced from the absence of Cα-O-Cα (ether linkages) resonance in the NMR spectra of these polymers. The reactions that govern the polymerization of HGA by the rMt laccase were proposed in Fig. 4A and showed two main suggested assembly modes, Cα-Cα (α-bindings) and Cα-Cβ (β-bindings), giving preference to the Cα-Cα mode because of less subject to steric effects. Based on the NMR data, it was not possible to differentiate between the eight possibilities (Fig. 4A). The mechanisms of polymerization through radical reactions have also been proposed in Fig. 4B, considering the high reactivity of the primary phenoxy radicals in favor of ary|
Figure 4. The proposed mechanism of HGA polymerization by the laccase or in abiotic conditions, giving the most probable structure in (A) and the most active radical reactions detailed in (B). These two figures show the configurations of the Cα-Cα links present in the three pyomelanin structures, whereas Cα-O-Cα (ether) links are absent as demonstrated by 13C solid-state NMR (see text). In these structures, gentisyl alcohol (major) and gentisaldehyde (minor) issued from the decarboxylation mechanism (laccase process, bacteria) are supposed to be incorporated into the polymer in the same manner as HGA radicals at locations of the chain that could not be determined at this time.
The pyomelanin PyoENZ for multiple applications. In addition to a DPPH-antioxidant activity equivalent to ascorbic acid, a high thermostability over time, a non-degradability in cells, PyoENZ efficiently scavenges ROS from irradiated human keratinocytes much better than the concurrent MelSYNTH (Table 3). Comparatively and with a similar technique, 400 µg/mL of L-Dopa melanin isolated from Pseudomonas maltophilia has been reported to almost scavenge ROS totally from UVA-induced fibroblast cells. Human eumelanin and phaeomelanin photo-oxidize ROS meanwhile they photoconsume O₂ and are protective against skin cancer. Nevertheless, they photochemically generate melanin degradation products that are responsible for sunlight-induced melanoma formation by inducing cyclobutane-pyridine dimers (CPDs) from DNA. PyoENZ contained both products, but in reverse order of level, gentisyl alcohol (major) and gentisaldehyde (weak compound). No decarboxylation was observed during the abiotic and alkaline synthesis of PyoCHEM.

Conclusions
Pyomelanin issued from the three processes has different properties, giving a large priority to PyoENZ that can now be produced in interesting yield and at low cost. The pigment efficiently scavenges ROS, exhibits high DPPH-antioxidant activity, is non-degradable, photostable, non-toxic, and can be stocked indefinitely without any precaution. As a representative pigment of microbial pyomelanin, PyoENZ becomes an available standard for laboratories, might be used for applications that require extreme conditions, as an electron-transfer agent, why not for energy storage, and exploited for skin protection, assuming it cannot penetrate the blood skin vessels.

Methods
Chemicals and enzymes. Solvents of mass spectrometry grade were supplied by BIOSOLVE (Dieuze, France), media for human keratinocytes, and mouse fibroblasts cultures from DUTSCHER (Brumath, France), main chemicals including standard melanin from SIGMA. Natural melanin (MelSEP, reference M2649) consists of purified eumelanin from the ink of Sepia officinalis. Synthetic melanin (MelSYNTH, M8631) is an L-Dopa melanin obtained from L-Tyr in presence of H₂O₂. HGA and HGA-lactone were used as standards for HPLC and the GC-mass data bank. The Aspergillus sp. laccase (SAE0050) consisted of a highly concentrated and brown miscible-water solution (density 1.15 g/mL, stored at 4 °C) obtained by submerged fermentation of the recombinant Myceliophthora thermophila laccase expressed in Aspergillus oryzae. This enzyme originally furnished by Novozym under reference 51,003, was re-named here ‘laccase rM’. Pure laccases from Pycnoporus cinnabarinus and Trametes versicolor (SIGMA, 38429) were also used.

Halomonas strain selected and growth conditions. The strain Halomonas titanicae was provided from the DSMZ collection (Germany), isolated and taxonomically characterized in 2010, and compared in our laboratory among a large collection of Halomonas spp. The strain was grown in a shaker (130 rpm) at 30 °C in a basal medium containing (in g/L), yeast extract 1.0, NaCl 20, KH₂PO₄ 0.6, NH₄Cl 1.0, MgCl₂·6 H₂O 10, CaCl₂·2 H₂O 0.1. The pH was adjusted to 7.0 with a 4 N NaOH solution. Aliquots (25 and 500 mL) were dispensed into flasks and sterilized by autoclaving at 120 °C for 20 min. Ca- and Mg-chloride stock solutions were sterilized separately, and the accurate volumes were added to the medium. L-Tyr and 4-hydroxyphenylacetic acid (4-HPA) stock solutions (250 mM in milliQ-H₂O, neutralized, heated moderately, stabilized by 0.2 µm pore size filtration) were added before inoculation at 5 mM final concentration. The strain was pre-cultivated twice for 2 days each, in 25 mL of the same saline basal medium containing either L-Tyr or 4-HPA. The pre-culture served as inoculum at 10% (v/v) for the culture in 500 mL volume that was agitated at 150 rpm, until the A₆00nm no longer changed. To overproduce pyomelanin, 500 mL cultures supplemented by repeated addition of 4-HPA amounts were carried out using the experimental design AZURAD software (a company of Marseille). The 8 experiments resulted from the defined parameters, the response Yi (mass of pyomelanin per Liter of culture), and the 6 entry parameters, Xᵢ for the 4-HPA concentrations added (2, 5, and 10 mM), and X₂ for the time of
supplementation (at 0, 2 or 3 days of growth). An experimental domain of cubic form was chosen and a second-degree polynomial model applied.

**Process for the production of pyomelanin (Pyo\textsubscript{ENZ}) by the rmT laccase.** The first part of the procedure consisted of an adapted HGA synthesis\textsuperscript{25}. The second part is the polymerization step by the rmT laccase. The starting compound 2,5-dimethoxyphenylacetic acid (2,5-DMPA) 5 g was solubilized in 40 mL of 48% HBr and refluxed gently for 4.5 h in a 100 mL-bicol flask provided with a refrigerant maintained at 10 °C. The resulting deeply red solution was evaporated to dryness in vacuo, the residue (3.80 g, 99.7% yield, 99.8% purity) identified as HGA-lactone following its UV spectrum (λ\textsubscript{max} 232, 289 nm, bands slightly lower than that of HGA), elution in RP-HPLC (retention time 4.2 min), and GC–MS analyses of the TMS-derived compound (rt 16.3 min), similarly to the standard. In the second step of the procedure and typically, 1.0 g of HGA-lactone was dissolved by agitation in 130 mL hot milliQ-H\textsubscript{2}O (70 °C), stayed 3–5 min and few drops of NaOH 2 N added until pH 9.3 (pH-meter) to hydrolyze the lactone into HGA (in HPLC-DAD, rt 2.7 min, λ\textsubscript{max} 290 nm), complete ring-opening was ensured by analysis of a 5 μL sampling diluted 10× in MeOH. Immediately after, 35 mL of Na-phosphate buffer 0.3 M pH 6.8 were added, the concentration of HGA and buffer at this stage was 40 mM and 65 mM, respectively. Once the temperature of the solution has reached 30–40 °C, 3–4 mM of concentrated laccase rmT were added (2250–3000 U in total), the enzyme activity was 750 U/mL (SD < 5%) as determined by the syringaldazine assay (see Fig. 2S, Additional information). Then the mixture was agitated at 130 rpm in dark at 30 °C for 48 h. The formed brown-black pigment was further precipitated by adding 34 mL of HCl 37% (2 N final concentration), agitated for 2 min, and stayed for 24 h, at ambient temperature in dark. The precipitated pyomelanin was centrifuged, washed with milliQ-H\textsubscript{2}O and ethanol, dried, and weighed as previously for Pyo\textsubscript{BACT} and Pyo\textsubscript{CHEM} (Fig. 1, step 4). The yield of the process was determined as a mean of three independent preparations from 2,5-DMPA.

**Bacterial (Pyo\textsubscript{BACT}) and chemical (Pyo\textsubscript{CHEM}) pyomelanin preparation.** Bacterial cultures at the stationary phase were centrifuged (8,500 g, 30 min), pyomelanin (Pyo\textsubscript{BACT}) in the supernatant was precipitated by the addition of 2 N HCl (final conc.), and the solution left to rest for 24 h, at room temperature in the dark. After centrifugation (8,500 g, 20 min), the brown-black pellet was washed successively with milliQ-H\textsubscript{2}O (3×) and ethanol (1×), centrifuged, dried at 70 °C for 2 days, weighed, and powdered as fine particles before storage in glass vials at room temperature. Chemical pyomelanin (Pyo\textsubscript{CHEM}) was prepared from 2,5-DMPA (1 g, 5.1 mmol) that was demethylated by HBr (reflux) and giving HGA-lactone by rotative evaporation. The lactone was then dissolved in hot H\textsubscript{2}O identical to Pyo\textsubscript{ENZ} preparation (see previously), and the aqueous HGA (0.84 g, 98.1% yield, evaluated by HPLC with pure HGA as calibrant) formed by alkaline treatment at pH 9.3. Autoxidation of HGA was then continued in presence of 5 mM MnCl\textsubscript{2} 4H\textsubscript{2}O, the solution agitated in dark for 3 days with a barrel at 30 °C, the pigment precipitated with 6 N HCl (final conc.), left to sedent for 12 h, and centrifuged (8,500 g, 20 min). The light-brown colored supernatant indicated the presence of many oxidized compounds that could not precipitate by increasing the acid until 10 N. After washing and drying, the pellet, which was weighed and stored as for the Pyo\textsubscript{BACT} and Pyo\textsubscript{ENZ} pigments. To determine the optimal (HGA)/(Mn\textsuperscript{2+}) ratio, concentrations of HGA (1–300 mM) and MnCl\textsubscript{2} (0.5–20 mM) were assayed in the same manner, the black-brown solutions diluted 50× in NaOH 0.1 N and absorbance read at 400 nm (A\textsubscript{400 nm}).

**Homogentisic acid and gentisyl alcohol syntheses.** Alkaline hydrolysis of 1.0 g of HGA-lactone in hot 130 mL H\textsubscript{2}O was immediately acidified until pH 5 with drops of HCl 37%, followed by the addition of 6.5 mL of a saturated NaCl solution (5% v/v final conc.). Then HGA was extracted 3× in a funnel with AcOEt, the whole organic phase washed 1× with milliQ-H\textsubscript{2}O, clarified with solid Na\textsubscript{2}SO\textsubscript{4}, filtered, and evaporated to dryness. To eliminate residual BQA, the dried HGA was dissolved in 0.1% HCOOH and applied on a glass column (20 mL Luer-lock tip syringe mounted with a vacuum flask and a pump) containing 10 cm\textsuperscript{2} of Lichroprep RP\textsubscript{18} (from SIGMA) previously conditioned with MeOH and acidified H\textsubscript{2}O. After washing by 2 vol. of acidified H\textsubscript{2}O, HGA was eluted by a mixture of MeOH-acidified H\textsubscript{2}O (1:9, v/v), evaporated to dryness, and resulted in a 99.9% purity light grey HGA (yields ~ 70 wt%), as determined by RP-HPLC and GC-MS analyses, indicating that recrystallization was not necessary. As standard for NMR analyses, pure gentisyl alcohol was synthesized from gentisaldehyde by NaBH\textsubscript{4}-reduction in tetrahydrofuran (yield 49%)\textsuperscript{91}, purity confirmed by GC-MS and \textsuperscript{1}H-NMR in d\textsubscript{6}-DMSO.

**Cytotoxicity.** The viability of cells exposed to melanin was expressed as the concentration-dependent reduction of the vital dye Neutral Red (NR) uptake in intracellular lysosomes. Assays were carried out with the three prepared pyomelanin and the two melanin standards (Table 2), all prepared at 10 mg/mL in NaOH 0.05 N (stock solution). Human epidermal keratinocytes neonatal cells were maintained in a complete keratinocyte serum-free medium (Panserin 412, from DUTSCHER) supplemented with bovine pituitary extract (30 µg/mL), recombinant epidermic growth factor (rEGF; 0.2 ng/mL), and an antibiotic cocktail of 10 U/mL penicillin-100 µg/mL streptomycin. Precultures were seeded into 96-well plates (0.2 mL per well) at 1.10\textsuperscript{5} cells/mL concentration. After incubation at 37 °C (5% CO\textsubscript{2}) for 24 h until semi-confluent, the medium was decanted, replaced by 200 µL of
complete medium containing the melanin (8 concentrations, 0–500 µg/mL), and cells were incubated again for 24 h. After removing the medium, cells were washed, placed into the NR medium (50 µg/mL NR in the complete medium), and incubated for 3 h (37 °C, 5% CO2). The medium was removed, cells were washed three times with 0.2 mL of HBSS (Hank’s Balanced Salt Solution, from DUTSCHER) to eliminate the excess dye, and 50 µL per well of a destaining solution (50% ethanol, 1% acetic acid, 48% milliQ H2O) was added. The plates were shaken for 15 min at room temperature in the dark. The membrane damage degree, i.e., the increase of released NR, was determined by the A450 nm in an Infinite M200 Pro (TECAN, Swiss) reader. The results obtained for wells treated with the pigment were compared to those of untreated (100% viability) and converted to percentage values. Cell viability was calculated as Viability (%) = [A450 (test well) − A450 (blank)] / [A450 (negative control) − A450 (blank)]. The concentration of the pigment causing a 50% release of NR as compared to the control culture (IC50, in µg/mL) was calculated by non-linear regression analysis using the Phototox v2.0 software (ZEBET, Germany).

**Phototoxicity.** The in vitro and normalized 3T3 NRU assay (OECD number 432) was used. Balb/c 3T3 mouse fibroblasts (3T3-L1, ATCC CL-173, from US type Culture Collection) were grown in DMEM supplemented with L-glutamine 4 mM and 10% of inactivated calf serum, seeded into two 96-well plates (0.1 mL per well) at 1.104 cells/mL concentration, and incubated (37 °C, 5% CO2) for 24 h until semi-confluent. The medium was decanted and replaced by 100 µL of HBSS (see before) containing the appropriate pigment concentrations (8 concentrations, 0–500 µg/mL), then cells were incubated (37 °C, 5% CO2) in the dark for 60 min. From the two plates prepared for each series of pigment concentrations and the controls, one was selected, generally at random, for the determination of cytotoxicity without irradiation (− Irr), and the other for the determination of phototoxicity with irradiation (+ Irr). For each of these sets, an experiment was done (in HBSS) and positive control (chlorpromazine final concentrations from 1 to 100 µg/mL (− Irr) and 0.01 to 1 µg/mL (+ Irr), diluted in ethanol) were performed. The percentages of cell viability were calculated as previously (cytotoxicity). Irradiation was performed with a solar simulator Suntest CPS+ (ATLAS MATERIAL TESTING TECHNOLOGY BV, Lochem, Netherlands) device equipped with a xenon arc lamp (1100 W), a glass filter restricting transmission of light below 290 nm, and a near IR-blocking filter. The irradiance by the xenon lamp was maintained with light energy of 550 W/m² during 1 h (i.e. 200 J/cm² UV-visible irradiation). Changes in the polymer structure were monitored by UV-visible spectroscopy from 200 to 700 nm and GPC/SEC (see Fig. 6S), comparatively to non-irradiated samples.

**Metabolites identification, pyomelanin monitoring.** Phenolic compounds along the three processes were identified by RP-HPLC-DAD and GC-MS according to our previous works33–35. To control the pigment formation during the bacterial culture and for optimization of the processes, the black-brown solution was determined by the A540 nm in an Infinite M200 Pro (TECAN, Swiss) reader. The results obtained for wells treated with the pigment were compared to those of untreated (100% viability) and converted to percentage values. Cell viability was calculated as Viability (%) = [IC50 (− Irr) / IC50 (+ Irr)] was expressed to finalize the results. Based on validation studies (OECD 432 guideline), a test substance exhibiting a PIF < 2 predicts no phototoxicity, 2 < PIF < 5 a probable, and PIF > 5 a phototoxicity.

**Photostability of PyoEME in solution.** It was evaluated on 4 mL glass-closed tubes containing 3 mL each of PyoEME solution at 0.05, 0.1, and 0.5 mg/mL NaOH 0.05 N. The tubes were placed horizontally and irradiated in the Suntest CPS+ solar simulator, respecting the ICH Q1B guidelines (European Medicines Agency). A strong irradiance by the xenon lamp was maintained with light energy of 550 W/m² during 1 h (i.e. 200 J/cm² UV-visible irradiation). Changes in the polymer structure were monitored by UV-visible spectroscopy from 200 to 700 nm and GPC/SEC (see Fig. 6S), comparatively to non-irradiated samples.

**References**

1. Solano, F. Melanins: Skin pigments and much more-types, structural models, biological functions, and formation routes. New J. Sci. https://doi.org/10.1128/AEM.02077-09 (2009).
2. Schmaler-Ripcke, J. et al. Production of pyomelanin, a second type of melanin, via the tyrosine degradation pathway in Aspergillus fumigatus. Appl. Environ. Microbiol. 75, 493–503. https://doi.org/10.1128/AEM.02077-08 (2009).
3. Türc, C. E., Knox, A. S., Becnel, J. M., Ekehukwu, A. A., Milliken, C. E. Properties and function of pyomelanin. In Biopolymers, Magdy El-Nasr eds, IntechOpen, pp. 449–472. https://doi.org/10.5772/10273 (2010).
4. Korytowski, W., Pilas, B., Sarna, T. & Kalyanaraman, B. Photoinduced generation of hydrogen peroxide and hydroxyl radicals in melanins. Photochem. Photobiol. 45, 185–190. https://doi.org/10.1111/j.1751-1097.1997.tb01362.x (1987).
5. Steinert, M., Engellhard, H., Flügel, M., Wintermeyer, E. & Hacker, J. The LLY protein protects Legionella pneumophila from light but does not directly influence its intracellular survival in Hartmannella vermiformis. Appl. Environ. Microbiol. 61, 2428–2430. https://doi.org/10.1128/ae.61.6.2428-2430.1995 (1995).
6. Bole, B. R. & Singh, P. K. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proc. Natl. Acad. Sci. USA 105, 12503–12508. https://doi.org/10.1073/pnas.0801499105 (2008).
7. Keith, K. E., Killip, L., He, P. Q., Moran, G. R. & Valvano, M. A. Burkholderia cenocepacia C5424 produces a pigment with antioxidiant properties using a homogentisate intermediate. J. Bacteriol. 189, 9057–9065. https://doi.org/10.1128/JB.00436-07 (2007).
8. Ahmad, S. et al. Identification of a gene involved in the negative regulation of pyomelanin production in Ralstonia solanacearum. J. Microbiol. Biotechnol. 27, 1692–1700. https://doi.org/10.4041/jmb.1705.105049 (2017).
9. Rodríguez-Rojas, A. et al. Inactivation of the hmg gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology 155, 1050–1057. https://doi.org/10.1099/mic.0.024745-0 (2009).
21. Neuckermans, J., Mertens, A., De Win, D., Schwaneberg, U. & De Kock, J. A robust bacterial assay for high-throughput screening.

19. Nikodinovic-Runic, J., Martin, L. B., Babu, R., Blau, W. & O’Connor, K. E. Characterization of melanin-overproducing transposon mutant of Legionella pneumophila confers ferric reductase activity.

33. Liebgott, P. P., Labat, M., Casalot, L., Amouric, A. & Lorquin, J. Bioconversion of tyrosol into hydroxytyrosol and 3,4-dihydroxyphenylacetic acid by Rhodococcus opacus PD63.2, 1–7. https://dx.doi.org/10.1093/fmmv/041 (2005).

22. Maccarrone, M., Catani, M. V., Iraci, S., Melino, G. & Finazzi-Agrò, A. A survey of reactive oxygen species and their role in dermotology. J. Eur. Acad. Dermatol. Venereol. 29(10), 185–202. https://dx.doi.org/10.1016/j.jved.2003.09.019 (2004).

17. Chatfield, C. H. & Cianciotto, N. P. The secreted pyomelanin pigment of Pseudomonas aeruginosa confers ferric reductase activity.

37. Singh, D., Kumar, J. & Kumar, A. Isolation of pyomelanin from bacteria and evidence showing its synthesis by 4-hydroxyphenylpyruvate dioxygenase inhibitors.

34. Liebgott, P. P., Labat, M., Amouric, A., Tholozan, J. L. & Lorquin, J. Tyrosol degradation to hydrous ferric oxide is mediated by cell-associate melanin. FEMS Microbiol. Lett. 2020, 1–10. https://dx.doi.org/10.1093/fmmv/041 (2005).

25. Nair, V. S., Srinivasan, B., Dhawan, B. & Srinivasan, P. The role of 4-hydroxyphenylpyruvate dioxygenase in enhancement of solid-phase electron transfer by Shewanella oneidensis MR-1. FEMS Microbiol. Ecol. 67, 233–245. https://dx.doi.org/10.1007/s00128-006-9307-x (2009).

10. Abdul-Hussien, Z. R. & Atia, S. S. Antimicrobial effect of pyomelanin extracted from Pseudomonas aeruginosa. Int. J. Environ. Res. 7, ID: 6829. https://www.journaldir.com/sites/default/files/js-6829.pdf (2017).

11. Zeng, Z. et al. Pyomelanin from Pseudalteromonas lipolytica reduces biofouling. Microbiol. Biotechnol. 10, 1718–1731. https://dx.doi.org/10.1093/1751-7915.12773 (2017).

12. Turick, C. E., Knox, A. S., Leverette, C. L. & Kritzas, Y. G. In-situ uranium immobilization by microbial metabolites. J. Environ. Radiat. 99, 890–899. https://dx.doi.org/10.1016/j.jenvrad.2007.11.020 (2008).

36. David, C., Daro, A., Szalai, E., Atarhouch, T. & Mergeay, M. Formation of polymeric pigments in the presence of bacteria and evidence on the role of 4-HPA 3-hydroxylase.

13. Liebgott, P. P., Amouric, A., Brochier-Armanet, C. & Lorquin, J. Halomonas olivaria sp. Nov., a moderately halophilic bacterium isolated from olive processing effluents. J. Membr. Sci. 560, 605–613. https://dx.doi.org/10.1016/j.memsci.2013.09.019 (2014).

11. Liebgott, P. P., Amouric, A., Sánchez-Atam, A. & Solano, F. Characterization of the melanogenic system in Vibrio cholerae, ATCC 14035. Pigment Cell Res. 8, 147–153. https://dx.doi.org/10.1111/j.1040-7462.1995.tb00656.x (1995).

16. Turick, C. E., Tisa, L. S. & Caccavo, F. Jr. Electron transfer from Shewanella Algae BRy to hydrous ferric oxide is mediated by cell-associate melanin. FEMS Microbiol. Lett. 220, 99–104. https://dx.doi.org/10.1016/S0378-1097(03)00096-X (2003).

27. Maccarrone, M., Catani, M. V., Iraci, S., Melino, G. & Finazzi-Agrò, A. A survey of reactive oxygen species and their role in dermatology. J. Eur. Acad. Dermatol. Venereol. 28(10), 185–202. https://dx.doi.org/10.1016/j.jved.2003.09.019 (2004).

14. Ito, S. et al. Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: Application to chemical analysis of human hair melanins. Pigment Cell Melanoma Res. 24, 605–613. https://dx.doi.org/10.1016/j.pccm.2010.07.003 (2017).

28. Ruzafa, C., Sanchez-Amat, A. & Solano, F. Characterization of the melanogenic system in Vibrio cholerae, ATCC 14035. Pigment Cell Res. 8, 147–153. https://dx.doi.org/10.1111/j.1040-7462.1995.tb00656.x (1995).

29. David, C., Daro, A., Szalai, E., Atharhouch, T. & Mergey, M. Formation of polymeric pigments in the presence of bacteria and evidence showing its synthesis by 4-hydroxyphenylpyruvate dioxygenase acid by Alcaligenes eutrophus CH34 and mutants. Eur. Polym. J. 32, 669–679. https://dx.doi.org/10.1016/0014-3057(95)00207-3 (1996).

30. Fang, W., Fernandez, E. K. K., Roberts, D. W., Bidochka, M. J. & Leger, R. J. A laccase exclusively expressed by Metarhizium anisopliae strain from olive processing effluents. J. Appl. Microbiol. 105, 1084–1095. https://dx.doi.org/10.1111/j.1574-6941.2008.00670.x (2009).

31. Singh, D., Kumar, J. & Kumar, A. Isolation of pyomelanin from bacteria and evidence showing its synthesis by 4-hydroxyphenylpyruvate dioxygenase enzyme encoded by HPPD gene. Int. J. Syst. Evol. Microbiol. 64, 46–54. https://dx.doi.org/10.1099/ijs.0.049007-0 (2014).
46. Narayanan, S., Kurian, N. K. & Bhat, S. G. Ultra-small pyromelanin nanogranules abiotically derived from bacteria-secreted homogentisic acid show potential applications in inflammation and cancer. *BioNanoScience* **10**, 191–203. https://doi.org/10.1007/s12668-019-00689-x (2019).

47. Braconi, D. et al. Proteomic and redox-proteomic evaluation of homogentisic acid and ascorbic acid effects on human articular chondrocytes. *J. Cell. Biochem.* **111**, 922–932. https://doi.org/10.1002/jcb.22780 (2010).

48. Ben Tahar, I., Kus-Łukiewicz, M., Lara, Y., Jawaux, E. & Fickers, P. Characterization of a non-toxic pyromelanin pigment produced by the yeast *Yarrowia lipolytica*. *Biotechnol. Prog.* **36**, e2912. https://doi.org/10.1002/bptp.2912 (2019).

49. Yao, Z. Y. & Qi, J. H. Comparison of antioxidant activities of melanin fractions from chestnut shell. *Molecules* **21**, 487–487. https://doi.org/10.3390/molecules21040487 (2016).

50. Matsuda, S. et al. Disruption of DNA damage response by propyl gallate and 9-a-monoacridine. *Toxicol. Sci.* **151**, 224–235. https://doi.org/10.1093/toxsci/kfw039 (2016).

51. Ito, N. et al. Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogenesis. *Fed. Chem. Toxicol.* **24**, 1071–1082. https://doi.org/10.1016/S0278-6915(86)90291-7 (1986).

52. Bigelis, R. & Black, K. A. Biotransformation of L-tyrosine and L-phenylalanine to 2,5-dihydroxyphenylacetic acid. Patent EU 0343330 A2. https://patents.google.com/patent/EP0343330A2/ (1989).

53. Miller, D. M., Kus-Liskiewicz, M., Lara, Y., Javaux, E. & Fickers, P. Characterization of a non-toxic pyomelanin pigment produced by the yeast *Yarrowia lipolytica*. *Biotechnol. Prog.* **36**, e2912. https://doi.org/10.1002/bptp.2912 (2019).

54. Aljawišh, A. et al. Laccase-catalysed oxidation of ferulic acid and ethyl ferulate in aqueous medium: a green procedure for the synthesis of new compounds. *Food Chem.* **145**, 1046–1054. https://doi.org/10.1016/j.foodchem.2013.07.119 (2014).

55. Geng, J. et al. Photoprotection of bacterial-derived melanin against ultraviolet A-induced cell death and its potential application as an active sunscreen. *J. Eur. Acad. Dermatol. Venereol.* **22**, 852–858. https://doi.org/10.1111/j.1468-3083.2007.02574.x (2008).

56. Szewczyk, G. et al. Aerobic photoreactivity of synthetic eumelanins and pheomelanins: generation of singlet oxygen and superoxide anion. *Pigment Cell Melanoma Res.* **29**, 669–678. https://doi.org/10.1111/jpcm.12514 (2016).

57. Premi, S. et al. Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure. *Science* **20**, 842–847. https://doi.org/10.1126/science.1256022 (2015).

58. Mostert, A. B. et al. Role of semiconductivity and ion transport in the electrical conduction of melanin. *Proc. Natl. Acad. Sci. USA* **109**, 8943–8947. https://doi.org/10.1073/pnas.111948109 (2012).

59. Lomasclo, A. et al. Overproduction of laccase by a monokaryotic strain of *Pseudomonas cinnabinarius* using ethanol as inducer. *J. Appl. Microbiol.* **98**, 618–624. https://doi.org/10.1111/j.1365-2672.2003.01879.x (2003).

60. Claeyis-Bruno, M. et al. Methodological approaches for histamine quantification using derivatization by chloroethylnitrosourea and ELISA measurement, part II: optimisation of derivated histamine detection with coated plates using optimal design. *Chemosom. Appl. Lab. Syst.* **80**, 176–185. https://doi.org/10.1016/j.chemom.2005.06.006 (2006).

61. Foss, R. J., Nadolski, G. T. & Lockwood, S. F. Synthesis of carotenoid analogs or derivatives with improved antioxidant characteristics. US Patent US 2009/0099061 A1. https://patents.google.com/patent/US20090099061A1/ (2009).

62. Song, T. et al. Gallium (III) nitrate inhibits pathogenic *Vibrio splendidus* interfering with the iron uptake pathway. *J. Microbiol. Biotechnol.* **29**, 973–983. https://doi.org/10.4014/jmb.1903.03008 (2019).

63. Saya, V. M., Galkina, B. N., Hong, M. Y., Yang, P. C. & Huang, G. S. A novel melanin-like pigment derived from black tea leaves with immuno-stimulating activity. *Food Res. Int.* **43**, 337–343. https://doi.org/10.1016/j.foodres.2009.11.001 (2010).

64. Bal, D., Kraska-Dziadecka, A., Gradowska, W. & Gryff-Keller, A. Investigation of a wide spectrum of inherited metabolic disorders by 13C NMR spectroscopy. *Acta Biochimica Polonica* **55**, 107–118. http://www.actabp.pl/pdf/1_2008/107.pdf (2008).

Acknowledgements

We thank Morgane Miодini for technical assistance in the optimization of the laccase process, Jean-Valère Nau- bron for FTIR spectra, Magalie Claeyis-Bruno from the Institut des Sciences Moléculaires (Ism2, Marseille) for her help in the Azurad software operation, Gregory Excoffier for the elemental analyses, and Marion Rollet from the Institut de Chimie Radicaleaire (ICR, Marseille) for the determination of the molecular weights.

Author contributions

J.L. initiated this study, designed the experiments, analyzed the data, supervised the research, and reviewed the manuscript. F.L. designed and executed the experiments, collected and analyzed the data, wrote the manuscript, and prepared the figures. E.Z. executed the NMR spectra and corrected the concerned part of the text. A.A. studied with J.L. the *H. titanicae* genes, proposed this strain as a good candidate for producing pyomelanin, and helped in the culture experiments. C. Di G. managed the cytotoxicity, phototoxicity and ROS experiments with F.L. M.R. synthesized gentisyl alcohol and HGA. P.P. participated in the strategy and entirely supervised the financial aspects of this work. All authors have read and approved the final manuscript.

Funding

FL works were supported in part by an ANRT-CIFRE (Association Nationale Recherche Technologie-Conventions Industrielles de Formation par la Recherche, France) fellowship.

Competing interests

The authors declare no competing interests.

Additional information

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-87328-2.

**Correspondence** and requests for materials should be addressed to J.L.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Open Access  This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021