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around 500 nm is featureless in all the spectra. The monotonic increase absorbance for the poorly solubilized eumelanin was ascribed to scattering effects, which are less effective in soluble natural melanins [2]. Regardless, the origin of the broadband absorption spectrum of melanins paired with the determination of their chemical structure have been the object of scientific debate. A model based on an interplay of geometrical order and disorder of eumelanin aggregate structures has been successfully used to describe the absorption spectra by a first-principles computational investigation [9].

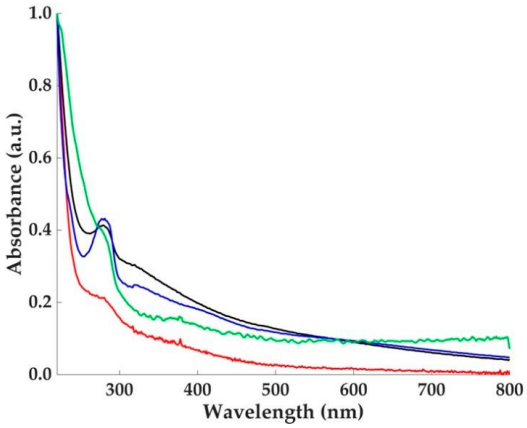

Figure 2. Ultraviolet-visible (UV-Vis) absorption spectra of Sc-Ms1 melanin (red line), melanin-like pigment synthesized by Sc-Ms1 tyrosinase (black line), dopa (blue line), and cysteinyldopa (green line) melanin-like pigments synthesized by Tv laccase. An excess of dopa was used as substrate for the enzymic synthesis. The peak at 274 nm is dependent on unreacted substrate and the presence of protein.

In Figure 3, the FT-IR spectra are reported. All spectra were characterized by a broad absorption band in the region of 3400 cm^{-1} due to the stretching vibrations of –OH and $–NH_2$ groups. Features centered around 2900 cm^{-1} were present in all melanin samples and were assigned to the vibrations of the CH_2 groups. The band at 1600 cm^{-1} was due to the C=O stretching vibration mode. The presence of the C–S stretching vibration peak at 700–600 cm^{-1} is usually used to identify the presence of pheomelanin in natural samples [22,23]. This peak was evident in the cysteinyldopa sample (Figure 3c) and was present with less intensity in the Sc-Ms1 sample (Figure 3a), suggesting the presence of pheomelanin. Furthermore, the IR profiles of these spectra were similar. The IR profile of the spectrum reported in Figure 3b (Sc-Ms1 tyrosinase dopa melanin) is similar to that reported in Figure 3d (Tv laccase dopa-melanin), with traces of a peak around 600 cm^{-1}.

Natural melanin and melanin-like pigments are characterized by a persistent EPR signal due to the presence of exceptionally stable free radicals [6,29,36].

Normally, the melanin EPR spectra are recorded at X-band ($\nu \approx 9$ GHz), but at this frequency, the EPR spectrum of dopa-melanin (eumelanin) is characterized by a single slightly asymmetric line with a g-factor of ca. 2.0032 and a linewidth of 0.4–0.6 mT. This spectrum is ordinarily published to support the evident paramagnetism of the sample, but no distinctive information is usually supplied. Furthermore, the magnetic parameters are strongly dependent on pH variations and hydration conditions [8,37]. In this paper, a multifrequency EPR approach is used to describe the complex pattern of the Sc-Ms1 natural melanin based on the enzymic synthetic dopa and cysteinyldopa melanins.

In Figure 4, the X-band EPR (right side) spectrum of Sc-Ms1 melanin (Figure 4a) is compared with the spectra obtained by the reaction of Sc-Ms1 tyrosinase (Figure 4b) and Tv laccase (Figure 4c) with dopa as the substrate. All the spectra were characterized at this frequency by a single slightly asymmetric line with a linewidth of 0.7, 0.6, and 0.5 mT, respectively (Table 1). All the spectra were recorded under non-saturating conditions.

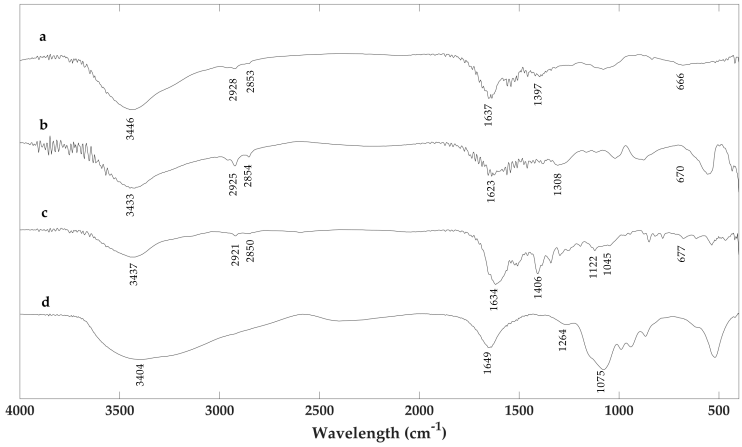

Figure 3. Fourier-transform infrared (FTIR) spectra of (**a**) Sc-Ms1 melanin, (**b**) Sc-Ms1 tyrosinase dopa-melanin, (**c**) Tv laccase cysteinyldopa melanin, and (**d**) Tv laccase dopa-melanin. All spectra were recorded in transmittance mode.

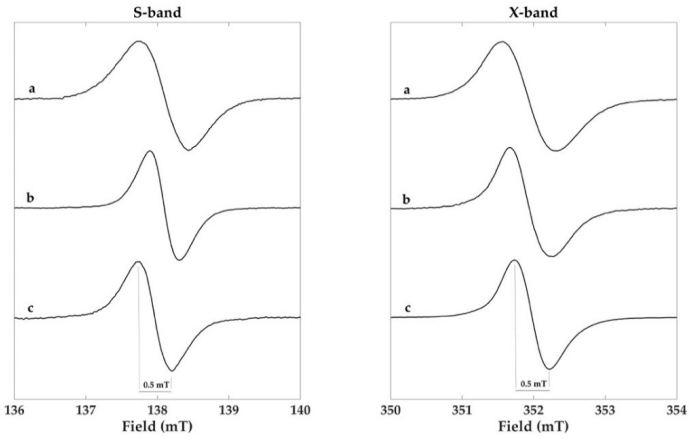

Figure 4. S- (3.9 GHz) and X-band (9.8 GHz) EPR spectra of the (**a**) Sc-Ms1 natural melanin, (**b**) Sc-Ms1 tyrosinase dopa-melanin, and (**c**) Tv laccase dopa-melanin samples. Spectra were recorded at 1.90 mW microwave power at S-band and 1.46 mW at X-band. Spline functions were used for the baseline correction of the S-band spectra.

Table 1. Magnetic parameters for the natural and enzymic synthetic melanin samples.

Sample	A^N_{iso}	A^N_z	$2A^N_z$	g_{iso} *	g_z	$g_x = g_y$	ΔB_{pp} § (mT)
Sc-Ms1 melanin				2.0047			0.7
Sc-Ms1 Tyr. dopa melanin				2.0038			0.6
Tv Lac. dopa melanin				2.0036			0.5
Tv Lac. cysteinyldopa melanin	0.7	1.6	3.2	2.0050	2.0028	2.0060	3.2

* determined from the S-band (3.8 GHz) electron paramagnetic resonance (EPR) spectra; § determined from the X-band (9.8 GHz) EPR spectra. Errors were estimated to g values ±0.0002 and hyperfine splittings ±0.05 mT.

The advantage of using a multifrequency EPR approach is that valuable informations for the sample characterization can be obtained by recording EPR spectra above and below the conventional X-band frequency (ν = 9.8 GHz). The S-band frequency (ν = 3.9 GHz) is used for a precise determination of the g isotropic value (g_{iso}). At low frequencies, the spectral anisotropy is minimized, the EPR spectra are symmetric, and a direct measurement of the g_{iso} can be obtained at the crossover point on the first derivative spectrum [31]. The S-band EPR spectra for the three samples are reported in Figure 4 (left side) and the g_{iso} values in Table 1. In this context, the information derived from the analysis of the signal width (referred hereafter as peak to peak signal amplitude, ΔB_{pp}) and the g values suggest that only in the case of laccase dopa-melanin the data are consistent with the formation of eumelanin (ΔB_{pp} = 0.5 mT and g = 2.0036) with the presence of carbon centered radicals. Furthermore, a certain degree of powder sample hydration cannot be excluded [8].

The signal amplitude and the g value of the X-band EPR spectra for the Sc-Ms1 tyrosinase dopa-melanin and Sc-Ms1 natural melanin were 0.6 and 0.7 mT and 2.0038 and 2.0047, respectively (Table 1), suggesting the presence of more than one radical species, considering that the g value for a pure eumelanin sample was reported to be 2.0032 and 2.0050–2.0055 for pure pheomelanin [8,33].

Q-band EPR experiments are crucial for addressing this point. At 35 GHz, different species can be separated based on their different anisotropies [38,39]. Given this context, cysteinyldopa melanin was synthesized using Tv laccase at neutral pH following the procedure reported in D'Ischia et al. [6] with a dopa:cysteine molar ratio of 1:2. The cysteinyldopa then polymerizes into various benzothiazine derivatives [40]. The X- and Q-band EPR spectra of the cysteinyldopa powder sample are reported in Figure 5 paired with the simulated spectra (red lines). The spectrum at X-band had a broad signal amplitude (3.2 mT) and a high g value (2.0050) and resembled the EPR spectrum of an immobilized nitroxide [6,31]. At this frequency, the EPR spectrum was dominated by the z-component of the ^{14}N hyperfine splitting (Figure 5). The nitrogen coupling constant A_z (1.6 mT) was estimated from the Q-band cysteinyldopa spectrum, as highlighted in Figure 5. The isotropic nitrogen coupling constant (A^N_{iso}) was calculated following the same procedure as the nitroxides, assuming an axial symmetry with the tensor components of A anf g: $A_x = A_y = 0.2\ A_z$ and $g_x = g_y$. The best fit, at both frequencies, was obtained using the same set of magnetic parameters reported in Table 1, changing only the frequency. The partly nitrogen-centered free radicals in cysteinyldopa melanin demonstrate the presence of the semiquinonimine radicals as was previously reported [31,41].

In Figure 6, the Q-band spectra of Sc-Ms1 melanin and Sc-Ms1 tyrosinase melanin (Figure 6a,b, respectively) show a completely different and more complex lineshape compared to the single featureless line obtained at X-band (Figure 4a,b).

The Sc-Ms1 natural melanin showed a complex EPR signal pattern, clearly indicating the presence of more than one radical species, one of which was identified as pheomelanin contribution. Pheomelanins and eumelanins are pigments produced in humans; only few reports address the presence of pheomelanins in bacteria and fungi [21–23,25]. Given this context, the data reported here represent one of the first reports detecting pheomelanins in bacteria. The natural and certainly more complex melanin sample was described based on the enzymic-synthesized eumelanin and cysteinyldopa melanin (Figure 6c,d, respectively) with the Q-band spectral simulation (Figure 6a). The best fit was obtained considering the presence of the two different species and simulating the spectra with the magnetic parameters reported in Table 1. A contribution of 20% eumelanin and 80%

pheomelanin was derived from the simulation. Notably, this is only a gross estimation of the two pigments' contribution based on those synthesized by the use of Tv laccase. Furthermore, cysteinyldopa in the cells was directly synthesized through dopa cysteinylation or by the mediation of glutathione, depending on the presence of cysteine in the bacterial culture [17]. Again, the Sc-Ms1 tyrosinase was inactivated by any amount of cysteine greater than 0.01 mM [13]. The use of a cysteine-rich culture medium for Sc-Ms1 growth might justify the production of pheomelanin. The presence of the two different pigments was also confirmed by the high g value (2.0047). In the literature, a calibration curve reporting the dependence of the amount of pheomelanin on the g_{exp} factor in human red hairs was built [33]. A g value of 2.0046 was reported for a pheomelanin/eumelanin ratio of 59%, whereas a g value of 2.0038 was reported for the Sc-Ms1 tyrosinase dopa-melanin (Figure 6b), which accounts for a pheomelanin/eumelanin melanin ratio of 18%. This can be ascribed to the nature of the tyrosinase that was purified from the culture broth. Conversely, the laccase-derived sample (Figure 6c) did not show any composite signal at high frequency, supporting the assumption that laccase-derived melanin is constituted of purely heterogeneous eumelanin units. Nevertheless, the broad signal (ΔB_{pp} 1.05 mT) recorded at Q-band can account for the presence of more than one radical species. Performing hydration-controlled X-band analysis, was observed that the water content and pH can strongly influence the solid-state EPR signal [8]. A model was proposed in which two coexisting free radical species were present in an eumelanin sample, with the carbon centered (g = 2.0032) and semiquinone free radicals (g = 2.0045), whose intensity increased as pH increased. In our case, the eumelanin sample (Figure 6c) with g = 2.0036 fully agrees with the g value reported in the literature for a powder sample at neutral pH, supporting the hypothesis of carbon center free radicals with a semiquinone free radical contribution, whose formation is due to the comproportionation reaction [8]. Regardless, as Q-band frequency ($\nu \cong 35$ GHz) was insufficient to solve the anisotropies of these two species, higher frequencies might be desirable to determine the different contributions. The cysteinyldopa synthesis was performed using a dopa:cysteine molar ratio of 1:2.

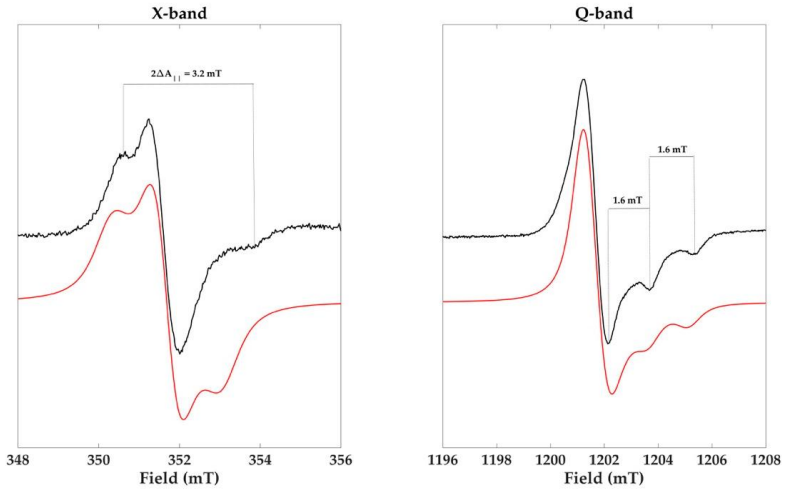

Figure 5. X- (9.9 GHz) and Q-band (33.7 GHz) spectra of the cysteinyldopa melanin paired with their simulated spectra (red line).

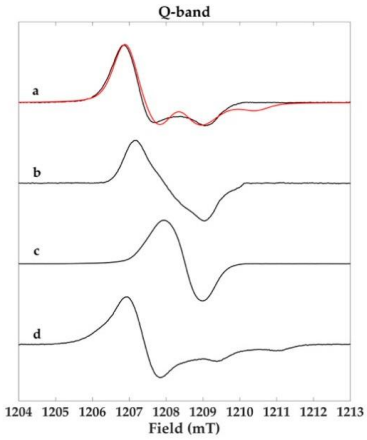

Figure 6. Q-band (33.9 GHz) EPR spectra of (**a**) Sc-Ms1 natural melanin, (**b**) Sc-Ms1 tyrosinase dopa-melanin, (**c**) Tv laccase dopa-melanin, and (**d**) Tv laccase cysteinyldopa melanin samples. Spectra were recorded with 0.06 mW microwave power.

3. Materials and Methods

All chemicals and Tv laccase were obtained from Sigma Aldrich (Milano, Italy) and used without further purification. The Ms1 strain was collected and isolated from Algerian Sahara soil and was chosen for its ability to produce melanin exopigments, both in solid and liquid media. It was molecularly characterized and assigned to *S. cyaneofuscatus* Pridham et al. 1958 species [13].

3.1. Sc-Ms1 Tyrosinase Purification

Sc-Ms1 tyrosinase was produced by growing the strain in MPPM broth (glycerol 10 g/L, glucose 10 g/L, soya flour 10 g/L, casamino acids 5 g/L, yeast extract 5 g/L, 4.0 $CaCO_3$ 4 g/L, bacteriological agar 15 g/L, and 1 mL of trace salts solution (g/100 mL: 1.0 $FeSO_4$, 0.9 $ZnSO_4$, 0.2 $MnSO_4$), pH 7.0) supplemented with 1 mM filter sterilized $CuSO_4$ for 72 h [13]. After cell harvesting by centrifuging, the tyrosinase enzyme was purified from Ms1 strain culture supernatant as previously described [13]. Briefly, proteins were precipitated from the culture supernatant with 65% ammonium sulphate, resuspended in 50 mM potassium phosphate buffer (pH 6.5), and dialyzed at 4 °C for 24 h against the same buffer. After dialysis, the enzyme solution was concentrated by replacing the dialysis buffer with a 20% (*w*/*v*) polyethylene glycol 8000 solution (in 50 mM potassium phosphate buffer, pH 6.5) and incubating at 4 °C for 24 h. The concentrated solution was finally dissolved in 50 mM potassium phosphate buffer and applied to a DEAE Sephadex ^{TM} A-50 (GE Healthcare, Waukesha, WI, USA), using batch technique to separate the enzyme from melanin.

3.2. Melanin Isolation and Purification

Melanin was isolated from the Sc-Ms1 actinobacteria culture broth and purified by precipitation in an acidic environment. HCl 6 N was added to the solution until melanin precipitation. Next, the precipitate was separated from the solution by centrifugation at 15,000× *g* for 10 min at 4 °C, and washed with deionized water until the pH became neutral.