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Microbial melanin-like material: A factor beyond influencing the brown color of activated sludge

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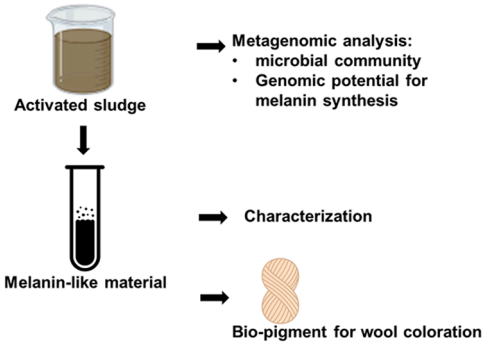
HIGHLIGHTS

- Melanin-like material was successfully isolated from activated sludge.
- Extracted material distincted from commercial humic acid and synthetic melanin.
- Metagenomics indicated widespread pyomelanin synthesis pathway.
- Melanin-like material contributes to activated sludge brown coloration.
- Extracted material showed strong binding on wool fibers.

GRAPHICAL ABSTRACT



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ABSTRACT

Melanin is a group of phenolic-quinone pigments. Natural melanin is nearly ubiquitous; found in all types of living organisms, ranging from mammals to bacteria. However, its presence and biosynthesis genomic potential in activated sludge have not been investigated. To explore this potential, melanin-like material was extracted from activated sludge collected from a municipal wastewater treatment plant. The extracted melanin-like material was characterized through biochemical analyses in comparison to synthetic melanin and humic acids that are commercially available. Metagenomic analysis of microbial community members in activated sludge and detection of tyrosine-derived melanin synthesis genes was performed. Additionally, the potential application of the extracted melanin-like material as a natural pigment was evaluated by testing its ability to color wool yarn. It was found that melanin-like material extracted from activated sludge accounted for around 11% of sludge dry mass. The isolated material displayed intrinsic autofluorescence, strong UV absorption, high oxidative stability, and free radical-rich EPR signal. FTIR analysis indicated a mixed polymer dominated by pyomelanin-like structures with eumelanin features, distinguishing it from synthetic melanin and humic acid. Metagenomic screening of the sludge community revealed widespread genomic potential for pyomelanin monomeric precursors biosynthesis across key functional genera (e.g. genera *Zoogloea, Nitrotoga, Nitrosomonas*, Ca. *Accumulibacter, Azonexus*, Ca. *Competibacter, Propionivibrio*, and *Rhodoferax*). These results suggest that microbial melanin-like material is an overlooked contributor to sludge coloration. Furthermore, the extracted pigment exhibited high affinity and wash fastness on wool fibers, demonstrating its potential for valorization as a sustainable biobased colorant.

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1. Introduction

Melanin is the broad term used to refer to a group of phenolic-quinone pigments derived from natural and synthetic sources (d'Ischia et al., 2015). Natural melanin is nearly ubiquitous; found in all types of living organisms, ranging from mammals to cuttlefish ink, and down to bacteria. Often the resulting pigments are brown or black in color (El-Naggar and El-Ewasy, 2017). Melanins are classified into five groups based on their chemical structure: eumelanin, pheomelanin, neuromelanin, allomelanin and pyomelanin (Guo et al., 2023). Its existence provides organisms with a broad variety of functionalities, for instance, eumelanin and pheomelanin provide pigmentation to the human skin, eyes and hair. They also absorb harmful UV (ultraviolet) rays and protect the cells from sun damage. Bacteria can produce various types of melanin as well, such as pyomelanin, eumelanin and allomelanin (Elsayis et al., 2022). The functions of melanin in bacteria cultures were assigned to be an adaptive response to elevated environmental stress conditions such as light, temperature, oxidative stress, as well as heavy metals (Elsayis et al., 2022).

It is worth pointing out that, melanin and humic acids are both dark brown to black colored polymers commonly found in soil. Humic acids derived from remains of decomposed plant materials such as lignin, tannins, cellulose, and cutins (Hayes and Swift, 2020). Despite that both polymers play important roles in long-term soil fertility by enhancing the retention of ions, biomass, and water (Paim et al., 1990), they are completely different in their chemical compositions and structures. Similar as the soil, sludge in biological wastewater treatment systems, such as the activated sludge, exhibits dark brown to black coloration. This coloration is traditionally attributed mainly to the presence of humic acids. However, considering that bacteria can produce melanin, it is plausible that microbial melanin may be another factor influencing the (dark) brown color of activated sludge.

It should be mentioned that the importance of melanin is beyond coloration of the sludge. Melanin is an important textile pigment because it is a natural, eco-friendly colorant that provides stable brown to black shades. It offers excellent resistance to light and heat while also imparting UV-protective and antioxidant properties to fabrics. These features make melanin valuable for sustainable and functional textile applications (Song et al., 2025; Singh et al., 2025). Currently, melanin is extracted from the ink of cuttlefish or produced synthetically through the chemical oxidation of tyrosine. While the synthetic melanin is widely used, they often fail to fully replicate the structural and functional complexity of natural (biological) melanin. Given the growing interest in biological melanin, the potential presence of melanin in activated sludge represents a promising opportunity. If substantial quantities of melanin can be recovered from activated sludge, it may offer a sustainable, cost-effective, and industrial alternative for large-scale microbial melanin production.

It is known that microorganisms can synthesize several types of melanin, such as eumelanin, allomelanin and pyomelanin, most notably pyomelanin and eumelanin which are tyrosine-derived pigments (Seo and Choi, 2020). Little study has been focused on bacterial melanin synthesis pathways within activated sludge communitiy members. Examining the genomic potential for melanin biosynthesis helps identify which dominant taxa encode these pathways and how melanin may contribute to their stress tolerance and ecological fitness. Understanding these genomic capacities is also important for exploring the feasibility of valorizing the waste sludge, supporting future efforts to extract or biotechnologically produce melanin from activated sludge as a renewable resource.

To explore this potential, melanin-like material was extracted from activated sludge obtained from a municipal wastewater treatment plant. The extracted melanin-like material was characterized through biochemical analyses and compared to synthetic melanin and humic acids that are commercially available. Metagenomic analysis of microbial community members in activated sludge and detection of tyrosine-derived melanin synthesis genes was performed to assess the genomic potential for melanin production. Additionally, the potential application of the extracted melanin-like material as a natural pigment was evaluated by testing its ability to color wool yarn. Findings will shed light on the feasibility of recovering new functional biopolymers from waste sludge.

2. Material and methods

2.1. Melanin-like material extraction from activated sludge

The flocculent sludge was collected from a municipal wastewater treatment plant using the activated sludge process (The Harnaschpolder wastewater treatment plant, the Netherlands), 1000 mL of sludge were put into falcon tubes washed with demi water for three times. The supernatant was carefully removed each time by spinning down the biomass using centrifuge at 1050 g for 15 mins. Afterwards, the pellet was collected and lyophilized. The extraction of melanin-like material from the lyophilized sludge was performed according to Kurian et al. (2024) with modifications. The lyophilized sludge (1 g) was added into 40 ml 7.2 N HCl and heated at 105 °C for 8 h in a sealed boiling flask with nitrogen in the headspace. Subsequently, the suspension was filtered out and the resulting residual fraction was washed with deionized water eight times, followed by acetone five times, and dried at 60 °C overnight (Kurian, 2024).

As there might be human hair in the activated sludge, to exclude the potential interference of melanin from human hair, hair from 5 different people (1 g each) were collected. The melanin was extracted using the same method.

2.2. Characterization of the extracted melanin-like material

2.2.1. Intrinsic fluorescence

Images of the extracted melanin-like compounds were recorded using a Zeiss Axio Imager M2 microscope equipped with the fluorescent illuminator X-Cite XYLIS 720 L. The image was processed and exported in .tif format with the Zeiss microscopy software (ZEN version 3.3).

2.2.2. Solubility and the reaction with oxidizing reagent

To study the solubility of the extracted material in comparison with commercial synthetic melanin and humic acid, 0.1 mg of the aforementioned samples were transferred separately into Eppendorf tubes and kept in room temperature for 5 h. Each tube contained 1 mL of the following solvents: miliQ water, acetone, 1 M HCl, 1 M NaOH, 1 M NH4OH, ethanol, methanol, DMSO, n-butanol, Ethyl acetate, N-hexane, Diethyl ether. The samples were centrifuged for 20 min at room temperature and at 10,000 rpm. The pellet was collected and dried. The weight loss of the pellet was obtained to evaluate whether the samples were insoluble, partly soluble, or fully dissolved.

To investigate the reaction of the extracted material in comparison with commercial synthetic melanin and humic acid with an oxidizing reagent, 0.1 mg of the aforementioned samples were transferred separately into glass tubes containing 1 ml 10% hydrogen peroxide (H_2O_2). After 24 h, 0.5 ml of 1 M NaOH was added into the tubes which showed no decolorization. The color change was monitored for 72 h.

2.2.3. UV–Visible spectroscopic analysis

The UV–vis spectra of the individual samples were measured in a flat bottom, UV light permeable 96-well plate at room temperature using a TECAN Infinite® 200 PRO microplate reader. The dried melanin-like material (5 mg) was dissolved in 0.5 M NaOH solution (0.5 ml), and diluted with miliQ water to the concentration of 500 mg/L. Its UV–vis absorption spectrum was recorded and compared with a commercial synthetic melanin and humic acid that were dissolved in the same basic solution, and diluted with miliQ water to the concentration of 1000 mg/L. All the spectra were recorded in 1 nm steps in the range of 200–800

nm.

2.2.4. FT-IR spectroscopy

The FT-IR spectra of the extracted melanin-like material, the commercial synthetic melanin and humic acid was recorded on a Perkin Elmer FT-IR spectrometer at room temperature, with the wavenumber range from 650 cm^{-1} to 4000 cm^{-1}. Resolution of 1 cm^{-1} and accumulation of 8 scans were applied to each sample.

2.2.5. Electron paramagnetic resonance spectroscopy (EPR)

The extracted melanin-like material, commercial synthetic melanin and humic acid were grounded into fine powder and were investigated using Bruker EMXplus EPR spectrometer at room temperature. Each sample was prepared by transferring the powder in an open-EPR quartz glass tube. All EPR spectra were recorded using the following parameters: microwave frequency 9.780 GHz, microwave power 20 mW or 0.05 mW (radical), a modulation frequency of 100 kHz, and a modulation amplitude of 10 G or 2.5 G (radical). Spectra were scanned from 5–605 mT (wide) or 344–354 mT (radical).

2.3. Genome data collection and melanin biosynthetic pathways identification

The collected flocculent sludge was incubated at 37 °C in a water bath with 20 μg mL^{-1} RNase (Sigma-Aldrich), followed by incubation at 50 °C with 10 μL proteinase K (Sigma-Aldrich). DNA extraction was performed using 800 μL phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich) equilibrated with 10 mM Tris (pH 8), followed by centrifugation (15 min, 17,000 × g), ethanol washing (70%), and resuspension in 100 μL $T_{10}E_{0.1}$ buffer (pH 8). DNA concentration was measured using the dsDNA HS kit on a Qubit 4 fluorometer (Thermo Fisher Scientific). Size selection to remove small DNA fragments was performed using AMPure XP beads (Beckman Coulter), targeting effluent DNA concentration ≥40 ng μL^{-1}. DNA quality was assessed by gel electrophoresis and TapeStation analysis (Agilent 2200). Sequencing was carried out on a Nanopore PromethION P2i using the ligation sequencing kit v14 (SQK-LSK114), with flow cell washing (EXP-WSH004) performed twice during the run to maximize throughput. Additional removal of small and medium DNA fragments was performed using AMPure XP beads to obtain high–molecular-weight DNA, which was eluted in 40 μL $T_{10}E_{0.1}$ buffer (pH 8) and quality-checked again on the Agilent TapeStation.

Libraries were prepared using the ONT ligation sequencing kit (SQK-LSK114) following the manufacturer's protocol and sequenced on a PromethION P2i flow cell (~6000 active pores). Basecalling in duplex mode, demultiplexing, and quality filtering (Phred >10) were performed using Dorado v2. Reads were assembled de novo with myloASM, retaining contigs >500 bp (N50 = 69,346 bp). Taxonomic annotation of the contigs assembled was conducted using Kraken2 and followed by structural annotation using Prokka. As the focus was to identify the tyrosine-dependent melanin synthesis genes, in addition to Prokka annotation and to remove database biases, the presence of conserved protein families associated with tyrosinase (Tyr; PF00264), tyrosine aminotransferase (Tat; PF00155), and 4-hydroxyphenylpyruvate dioxygenase (HppD; PF00682) were also evaluated using HMMER v3. Searches were performed using hmmsearch with default parameters and reporting thresholds recommended by KEGG; when no threshold was provided, an e-value cutoff of 1e-20 and >75% query coverage was applied.

To extend the search of tyrosine-related melanin biosynthesis pathways to functionally dominant microorganisms that are involved in carbon, nitrogen and phosphorus removal in common activated sludge process, the genera *Zoogloea, Nitrotoga, Nitrosomonas, Candidatus Accumulibacter, Azonexus, Competibacter, Propionivibrio*, and *Rhodoferax*, which have previously been identified as key functional members of activated sludge systems were analyzed. Based on the GTDB representative genomes database, a total of 475 genome accession numbers were identified for the targeted genera. To reduce the computational time, for each of these genera, the accession numbers for two GTDB representative genomes were randomly selected, retrieved and utilized for pathway identification (Supplementary Table S2). Of the representative genomes analysed from these genera, the genome quality was ensured by setting criteria of >75% genome completion, <5% genome contamination, and >70% presence of unique single copy genes. The genomic potential for tyrosine-derived melanin synthesis was evaluated by screening for conserved protein families associated with pyomelanin and eumelanin formation - tyrosinase (Tyr, PF00264), tyrosine aminotransferase (Tat; PF00155) and 4-hydroxyphenylpyruvate dioxygenase (HppD; PF00682) using GToTree v1.8.16 and HMMER v3.3.2 with profile-specific e-value cutoffs (Lee, 2019; Eddy, 2011). For each genome, the identified gene counts for each enzyme were normalized to the total single-copy core genes identified in the respective genome. Phylogenetic reconstruction was performed to contextualize the distribution of melanin-associated genes across taxa, with alignments generated using MUSCLE v5.1 and tree inference conducted using FastTreeMP v2.1.11 (Edgar, 2021; Price et al., 2010). The phylogenetic tree was visualized using iToL v7.

2.4. Application: Developing pigment coating on woolen yarn

The extracted melanin-like material (0.2 g) was dissolved in 1 ml 1 M NaOH, then diluted to 5 ml followed by the addition of 1 ml (3%) acetic acid. Afterwards, the woolen yarn was soaked in the melanin solution and heated to 100 °C for 10 min. The yarn was rinsed with water, and air dried at the room temperature.

3. Results

3.1. Characterization of the extracted melanin-like material

3.1.1. Yield and morphology

The yield of melanin-like material extracted from the flocculent sludge was (11.5 ± 2.2) % of the total dry mass of the sludge. It had a dark brown colour (Fig. 1a), and emitted autofluorescence when it was observed under the microscope at both the FITC (excitation peak around 495 nm and an emission peak around 519 nm) and Cy3 (excitation maximum around 550 nm and an emission maximum around 570 nm) channels (Fig. 1). It is interesting to notice that the aggregates which looked pale (i.e. where the white arrow pointed) in the phase contrast picture still emitted a similar intensity of autofluorescence as the darker part. The intrinsic fluorescence of melanin at similar emission range has been reported in literature (Sarna et al., 2022). Different polymerization and oxidation status of melanin influence its intrinsic fluorescence, e.g. more oxidized or aggregated melanin can quench fluorescence (Kayatz et al., 2001).

The yield of melanin from human hair was 1.2–3.7% of the total mass of hair, which was much lower than that of the extracted melanin-like material from the flocculent sludge. In addition, the amount of hair in the sludge is relatively low due to the pretreatment in the municipal wastewater treatment plant to remove hair. Thus, it was reasonable to ignore the contribution of human hair to the extracted melanin-like material.

3.1.2. Solubility and the reaction with oxidizing reagent

The solubility profiles of the three tested samples are summarized in Table 1. Unlike the humic acid sample, which was fully soluble in acetone, 1 M NaOH, 1 M NH_4OH, and 1 M Na_2CO_3, both the synthetic melanin and the melanin-like material extracted from activated sludge were completely insoluble in acetone and only partially soluble in 1 M NaOH, 1 M NH_4OH, and 1MNa_2CO_3. These distinct solubility characteristics clearly demonstrated that the extracted melanin-like material is chemically different from humic acid.

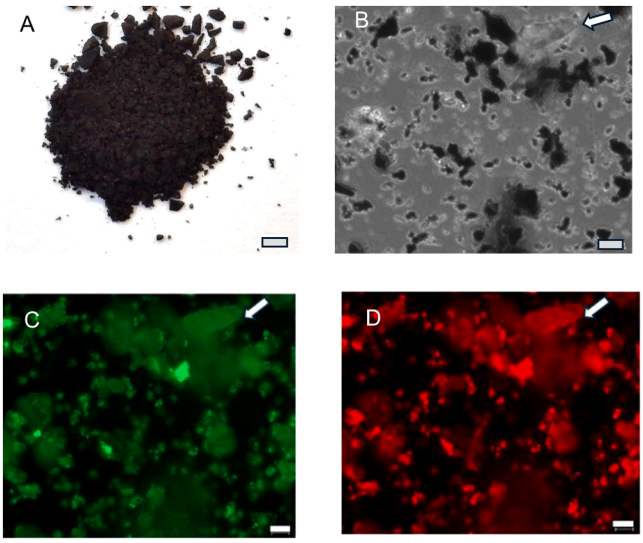

Fig. 1. Morphology of the extracted melanin-like material and its auto-fluorescence. A: the extracted melanin-like material is dark-brown, bar = 5 cm; B: phase contrast microscopy picture; C: microscopy picture at FITC channel (excitation peak around 495 nm and an emission peak around 519 nm); D: microscopy picture at Cy3 channel (excitation maximum around 550 nm and an emission maximum around 570 nm), bar in B, C and D = 20 μm.

Regarding the reaction with H_2O_2 as the oxidizing reagent, it was observed that, after 30 mins, the solid humic acid sample completely dissolved, indicating oxidative degradation by H_2O_2. In contrast, the synthetic melanin and the extracted melanin-like material were still in solid form after 24 h in 10% H_2O_2, implying that they are more resistant to the oxidation of H_2O_2 than the commercial humic acid. After the addition of 1 M NaOH, the synthetic melanin was first dissolved gradually within 12 h, then the solution turned from brown into light yellow. While the extracted melanin-like material was still in solid form after 72 h, with partially decolorized into light yellow suspension. Obviously, the oxidation resistance of the extracted melanin is much stronger than the commercial humic acid and the synthetic melanin.

3.1.3. UV–vis spectroscopy

The dark brown color of the extracted melanin-like material suggested notable UV–vis absorption properties. To investigate this, the absorption spectra of all three samples—extracted melanin-like material, synthetic melanin, and humic acid—dissolved in NaOH solution were recorded (Fig. 2). All samples exhibited strong absorbance in the UV region (200–400 nm), with a gradual decline across the visible spectrum. A prominent absorption peak was observed between 202 and 210 nm for all samples, indicative of the presence of aromatic structures. Notably, despite being tested at half the concentration of the other two samples, the extracted melanin-like material exhibited a higher absorbance in this region. This result strongly suggests that the UV absorption capacity of the extracted melanin-like material surpasses that of both commercial humic acid and synthetic melanin.

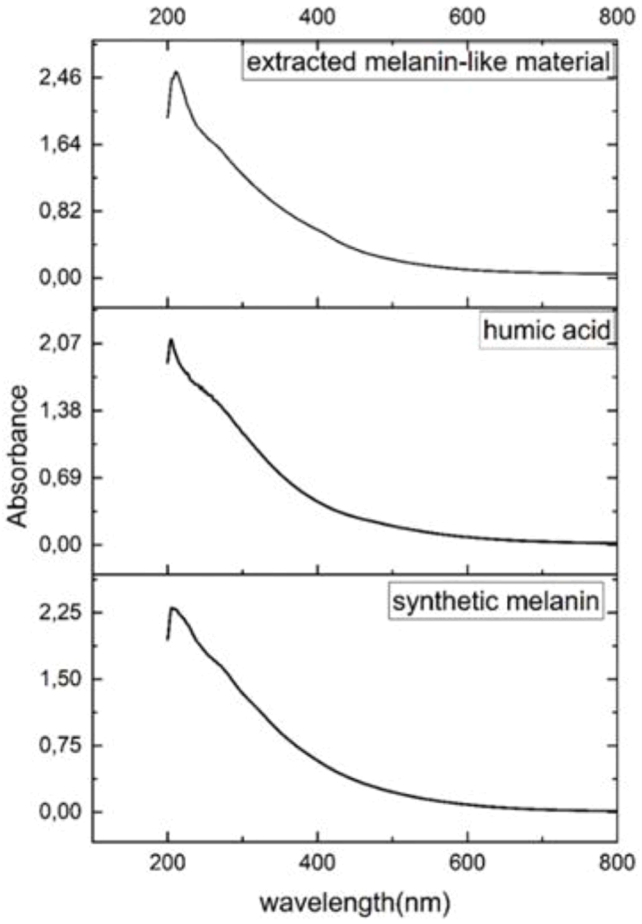

Fig. 2. UV–vis absorption spectra of commercial humic acid, melanin and the melanin-like material extracted from flocculant sludge.

3.1.4. Fourier-Transform infrared spectroscopy (FT-IR)

Fourier-transform infrared (FTIR) spectroscopy was employed to compare the functional groups present in the three samples. All the spectra exhibit a broad absorption band in the range of 3600–2500 cm^{-1}, attributed to the stretching vibrations of phenolic, carboxylic, and aromatic functional groups (Dai et al., 2023) (Fig. 3). The overtone bands between 2300 and 1700 cm^{-1} are characteristic of aromatic structures (Stanciu, 2025). Specifically, the sludge-derived melanin-like material exhibits a relative complex spectrum, with extremely strong aliphatic C–H stretching bands at 2931 and 2852 cm^{-1}, suggesting the abundance of $–CH_2$ groups. An intense carboxylic acid C = O absorption at 1715 cm^{-1} and the aromatic and quinone vibrations at 1619 and 1451 cm^{-1} imply the presence of carboxylated aromatic units. The distinct band at 1082 cm^{-1} is associated with C–O stretching within phenolic rings (Table 2). Thus, the presence of methylene and carboxylic acid groups, phenolic aromatic rings, and quinone-type structures strongly suggests that a substantial portion of this material consists of a pyomelanin-like polymer, likely derived from homogentisic acid monomers (Turick

Table 1
The solubility of commercial humic acid, synthetic melanin and the extracted melanin-like material.

	water	acetone	1M HCl	1M NaOH	1M NH_4OH	1M Na_2CO_3	ethanol	methanol	DMSO	n-butanol	Ethyl acetate	N-hexane	Diethyl ether
Humic acid	-	+	-	+	+	+	-	-	partial	-	-	-	-
Synthetic melanin	-	-	-	partial	partial	partial	-	-	-	-	-	-	-
Extracted melanin-like material	-	-	-	partial	partial	partial	-	-	-	-	-	-	-

- insoluble.
+ soluble.

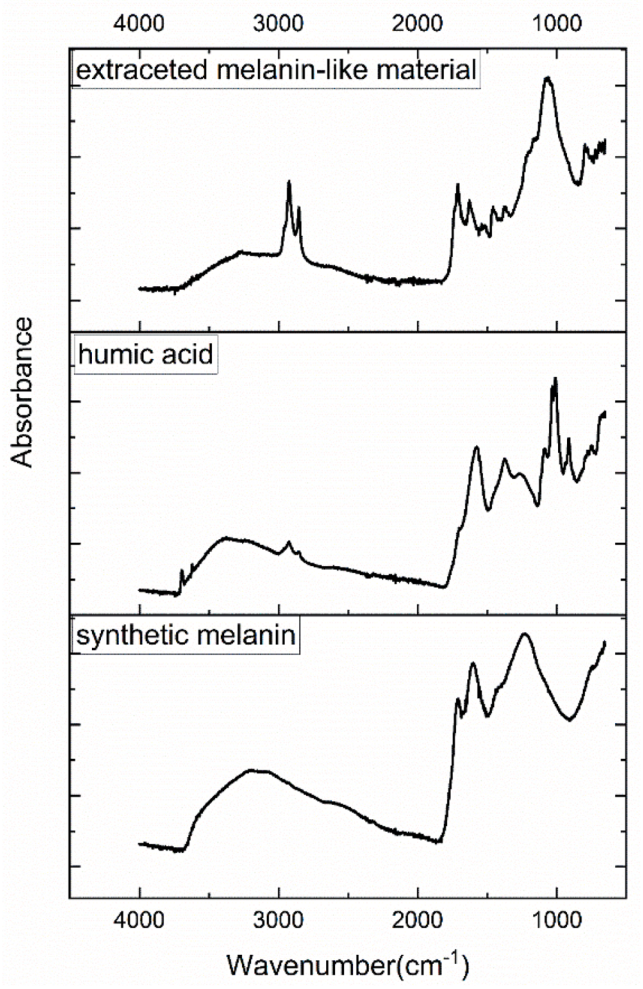

Fig. 3. The FTIR absorption spectra of commercial humic acid, synthetic melanin and the melanin-like material extracted from flocculant sludge.

et al., 2002). In addition, the presence of a weak indole N–H bending band at 1511 cm^{-1} suggests there might be incorporation of DOPA-derived eumelanin components, indicating a mixed melanin-like polymer, as indole-based vibrational features in this region are characteristic of eumelanin formed through the oxidative polymerization of L-DOPA (Bridelli et al., 1999; Ito and Wakamatsu, 2008).

The FTIR spectrum of synthetic melanin displayed features characteristic of DOPA-derived eumelanin, including a broad O–H/N–H stretching band at 3181 cm^{-1} and a carbonyl absorption at 1704 cm^{-1} associated with quinone and carboxylic functionalities. Bands at 1510–1530 cm^{-1} corresponding to N–H bending and at 1350–1380 cm^{-1} assigned to C–N stretching were also observed. The presence of a phenolic C–O stretching band at 1219 cm^{-1}, together with the absence of aliphatic C–H vibrations, further supports the assignment of the material as DOPA-derived eumelanin-like material (Ito and Wakamatsu, 2008).

Table 2
list of the wavenumbers of the bands in the FTIR spectrum of the extracted melanin-like material, commercial humic acid and synthetic melanin.

Sample	Wavenumber (cm^{-1})
Extracted melanin-like material	3263,2931,2852,1715,1619,1511,1451,1367,1082,788
Humic acid	3693,3604,3388, 2919, 2859, 1566,1350,1036,1000,910
Synthetic melanin	3181, 1704, 1528, 1380, 1219

In contrast, the humic acid standard showed distinct spectral features, including sharp free O–H stretching bands at 3693 and 3604 cm^{-1}, aliphatic C–H stretching at 2919 and 2859 cm^{-1}, and dominant carboxylate absorptions at 1566 and 1350 cm^{-1}. Characteristic absorptions from polysaccharide chains (1045–900 cm^{-1}) were detected exclusively in the humic acid spectrum (Miano and Senesi, 1992).

In summary, the spectrum of the extracted melanin-like material exhibited functional groups of both pyomelanin and eumelanin, with stronger feature of pyomelanin-like polymer. This is different from the spectrum of the synthetic melanin that mainly contain DOPA eumelanin, while the commercial humic acid spectrum does not show the functional groups of the typical quinones and semiquinones structures in melanin.

3.1.5. Electron paramagnetic resonance (EPR) spectroscopy

Melanin is known to possess paramagnetic properties due to the presence of quinone and semiquinone moieties within its structure, which give rise to unpaired electrons and diverse paramagnetic centers (Gessler et al., 2014). To investigate this intrinsic property, electron paramagnetic resonance (EPR) spectroscopy was employed to analyze the three samples.

Initially, power saturation behavior of the extracted melanin-like material was assessed by recording EPR spectra at microwave powers ranging from 0.025 to 200 mW. The plot of power-normalized radical EPR signal intensity versus microwave power revealed that the material exhibits saturation at very low power levels, with non-saturating conditions only observed below 0.05 mW. Consequently, a power setting of 0.05 mW (36 dB) was selected for subsequent measurements to ensure accurate detection of free radicals with slow spin relaxation (suggesting limited dipolar interactions with other paramagnetic species).

Under the optimized conditions, EPR spectra for all three samples were recorded and are presented in Fig. 4. All spectra displayed characteristic sharp isotropic signals indicating the presence of stable free radicals. The observed g-values (~2.005) and peak width were consistent across all samples and are characteristic of carbon- and/or oxygen-centered free radicals delocalized over aromatic structures (Zdybel et al., 2024).

Quantitative analysis based on the double integration of the absorption peaks, which reflects the relative spin concentration, revealed that the extracted melanin-like material exhibited a remarkably higher number of unpaired electron spins compared to the other two samples (Table 3, Fig. 4). In particular, the spin concentration in the extracted material was more than three times greater than that of humic acid, highlighting its enhanced paramagnetic character.

3.2. Analysis of microbial community and genomic potential for melanin biosynthesis

Taxonomic classification (read-based) identified approximately 80% of the microbial community members belonged to the phyla Pseudomonadota, Bacteroidota, Chordata, Actinomycetota, Bacillota, and Planctomycetota (supplemental material 1). At the class level, Alpha-, Beta-, Gamma-proteobacteria, Flavobacteria, and Actinomycetes collectively accounted for approximately 50% of the microbial community. To assess melanin biosynthetic potential, the assembled contigs were screened on the basis of structural annotations and also for conserved protein families associated with eumelanin (DOPA-melanin) and pyomelanin formation.

Melanin formation involves the enzymatic generation of reactive monomeric precursors, followed by spontaneous, non templated self polymerization into melanin polymer. As shown in Fig. 5, eumelanin is synthesized via the DOPA pathway, where the enzyme tyrosinase (Tyr) converts L-tyrosine to L-DOPA and then to dopaquinone, which eventually self-polymerizes into eumelanin (Song et al., 2025). Pyomelanin, on the other hand, is a polymer of homogentisic acid (HGA). It is produced during the catabolism of L-tyrosine, when HGA is excreted, it undergoes auto-oxidation and spontaneous self-polymerization to form

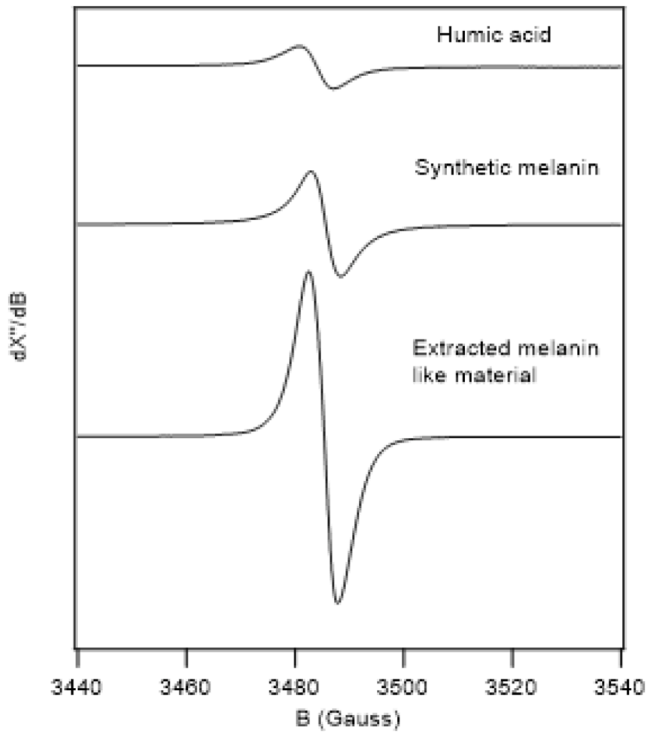

Fig. 4. EPR spectra of commercial humic acid, synthetic melanin and the melanin-like material extracted from flocculant sludge.

Table 3
EPR characteristics of the radical signal, peak-peak width, g-value and double integration of the radical signal under non-saturating conditions (36 dB, 2.5 Gauss MA) of the three samples.

Sample	g_{iso}	Peak-peak width (Gauss)	Double integral
Humic acid	2.005	6.13	239
Synthetic Melanin	2.005	5.4	469*
Extracted melanin-like material	2.005	5.2	850

* tube was not filled enough to cover the cavity.

pyomelanin. The enzymes involved in HGA synthesis are tyrosine aminotransferase (TyrB) and 4-hydroxyphenylpyruvate dioxygenase (HppD) (Boyce et al., 2015). Structural annotations of the contigs with prokka idenitified several coding sequences associated with Tyr, TyrB and HppD genes. Additionally, the targeted detection of protein families related to eumelanin and pyomelanin biosynthesis in the assembled contigs further ascertained that the microbial community identified in the flocculant sludge possesses the genomic potential for eumelanin and pyomelanin production (Supplementary Table S1) .

Furthermore, it is interesting to determine whether eumelanin and pyomelanin synthesis pathways are more conserved or broadly present among microbial community members essential for wastewater treatment processes. To this end, the genes *Tyr, TyrB*, and *HppD* were queried based on identification of PFAMs (Protein Families) in previously identified MAGs assigned to the genera *Zoogloea, Nitrotoga, Nitrosomonas*, Ca. *Accumulibacter, Azonexus*, Ca. *Competibacter, Propionivibrio*, and *Rhodoferax* (Kleikamp et al., 2023). It was found that PFAMs (Protein Families) linked to pyomelanin formation were significantly more abundant than those associated with eumelanin formation. As shown in Fig. 5C, Enzymes *TyrB* and *HppD* were identified in all the genera, while *Tyr* was only identified in one members of the *Nitrosomonas* genera. This suggests that there is a higher genomic potential for pyomelanin biosynthesis than for eumelanin biosynthesis among those genera.

3.3. Application: Dyeing of wool yarn

Optimal dying of wool fibers with the melanin-like material was achieved at a pH range of 3–4. Dyeing white wool yarn with 2 mg of melanin per gram of yarn yielded a light beige coloration, while doubling the amount to 4 mg/g produced a light brown shade (Fig. 6a). The dyed yarns retained their color after being washed in the washing machine with the addition of the detergent for wool fabrics (Fig. 6b), indicating good dye fastness and the stability of the extracted melanin-like pigment on wool fibers. Thus, the extracted melanin-like material is a promising pigment for wool fibers.

4. Discussion

4.1. Melanin-like material present in the flocculent sludge

In the current study, melanin-like material was successfully isolated from flocculent sludge, with a yield of approximately 11% of the total sludge dry mass. The isolated material appeared dark brown in color and precipitated in acidic solutions, as well as in organic solvents. The FTIR spectrum of the material exhibited functional groups of both pyomelanin and eumelanin, with stronger feature of pyomelanin-like polymer. In addition, it also contained stable carbon-centered and/or oxygen-centered free radicals conjugated to aromatic rings, indicating a high degree of radical activity. Compared to humic acid and synthetic melanin, this extracted melanin-like material exhibited higher UV absorption, stronger resistance to oxidation, a greater abundance of quinone and semiquinone structures, and a substantially larger content of free radicals.

The metagenomic analysis of the microbial community associated with the flocculent sludge clearly indicated the presence of the pyomelanin and eumelanin pathway, with a higher genomic potential for pyomelanin biosynthesis than for eumelanin. This is in accordance with the result of the FTIR analysis, which exhibited stronger feature of pyomelanin, and the significant difference between the extracted melanin-like material and the synthetic DOPA-eumelanin.

In bacteria, pyomelanin and eumelanin are synthesized through two distinct pathways originating from L-tyrosine (Seo and Choi, 2020). Pyomelanin derives from tyrosine degradation and HGA auto-oxidation, whereas bacterial eumelanin depends on tyrosinase-mediated DOPA oxidation followed by spontaneous polymerization of DOPA-derived intermediates. Given the chemical diversity of melanin, systematic characterization of their distinct biosynthetic pathways will be essential for elucidating the breadth of melanin-associated metabolic strategies in environmental microorganisms and for guiding future functional and ecological studies.

To summarize, a melanin-like material was successfully extracted from flocculent sludge, exhibiting physicochemical properties consistent with natural melanins reported in the literature. Genomic analyses further identified microbial metabolic pathways potentially involved in its biosynthesis. These findings open new avenues for exploring the origin and function of melanin-like materials within flocculent sludge. Future research should focus on assessing the prevalence of melanin production across different wastewater treatment systems and elucidating the specific metabolic mechanisms responsible for its formation.

4.2. Revisiting the origins of brown coloration in activated sludge: melanin-like pigments versus humic acids

Activated sludge is typically brown in color, a characteristic that has long been attributed to the presence of humic acids formed during the degradation of organic matter. However, the current research suggests that microbial pigments, such as melanin-like polymers, may also contribute significantly to this coloration. Both melanin and humic acids exhibit brown to black appearances due to their aromatic, conjugated, and chemically disordered polymeric structures, which absorb most

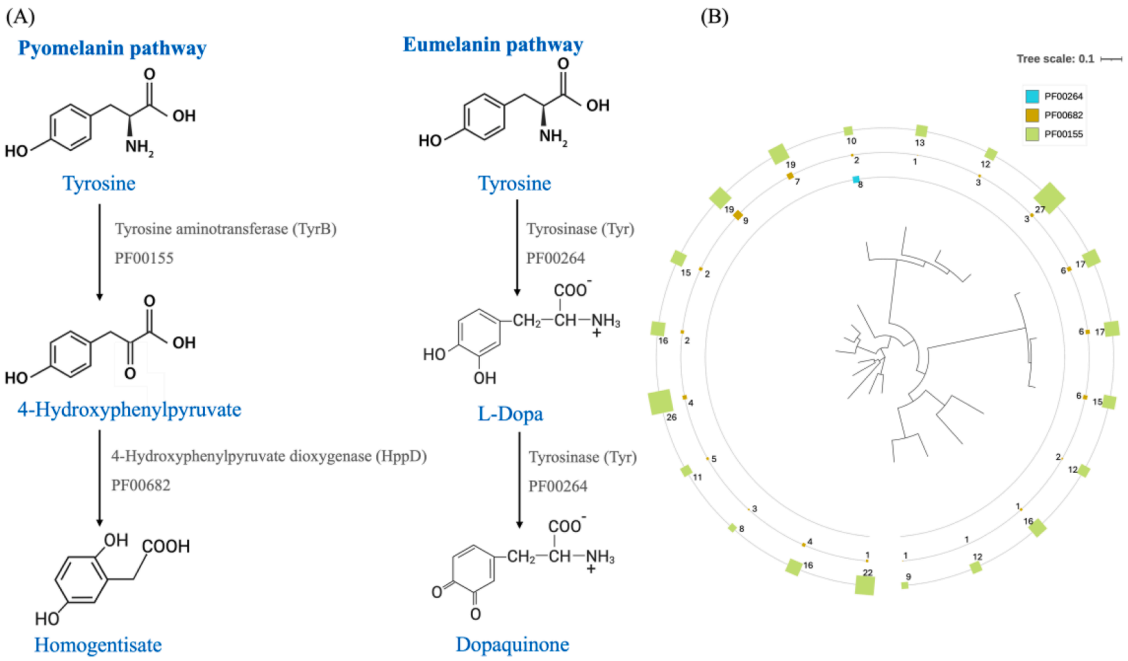

Fig. 5. Biosynthetic potential for pyomelanin and eumelanin formation in functionally dominant activated sludge microorganisms. (A) Schematic representation of pyomelanin and eumelanin biosynthesis pathways, highlighting key metabolites and associated enzymes. Pyomelanin formation proceeds via tyrosine catabolism to homogentisate, mediated by tyrosine aminotransferase (TyrB) and 4-hydroxyphenylpyruvate dioxygenase (HppD), followed by oxidation and auto-polymerization. Eumelanin (DOPA-melanin) biosynthesis is inferred from the presence of tyrosinase (Tyr), which catalyzes the rate-limiting oxidation of tyrosine to DOPA-quinone prior to spontaneous polymerization. (B) Phylogenetic tree of GTDB representative genomes analyzed in this study, with concentric annotation rings indicating the presence and abundance of the enzyme represented as PFAM copy number per genome. From inner to outer rings, annotations correspond to tyrosinase (blue square), tyrosine aminotransferase (green square), and hydroxyphenylpyruvate dioxygenase (brown square). Quantitative abundance data is provided in Supplementary Table S2.

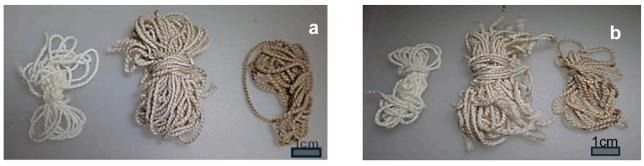

Fig. 6. a: The wool yarn before and after dyeing with the extracted melanin-like material (left: control; middle: 2 mg melanin-like material/g yarn; right: 4 mg melanin-like material/g yarn). b: the dyed wool yarn after being washed in the washing machine with the addition of detergent for wool fabrics (left: control; middle: 2 mg melanin-like material/g yarn; right: 4 mg melanin-like material/g yarn).

incoming visible light and reflect little back to the observer (as indicated by the UV-vis absorption spectra in 3.1). Despite this visual similarity, melanin and humic acids are fundamentally distinct in their origins, structures and functions.

As shown in the above genomic analysis, the precursors of melanin are enzymatically synthesized by microorganisms. Subsequent steps, including oxidation and polymerization, proceed primarily through non-enzymatic auto-oxidation, leading to the formation of the final heterogeneous melanin polymer. They may be cell-bound, or secreted extracellularly (Riley, 1997; Nosanchuk and Casadevall, 2003; Solano, 2017). Humic acid, by contrast, is primarily formed through chemical and microbial decomposition of organic matter in the soil (e.g., plant residues, proteins, lipids) during the humification process. It is not synthesized by a specific organism but results from non-specific microbial degradation and chemical reactions such as oxidation, condensation, and polymerizations (Lehmann and Kleber, 2015).

Regarding the chemical structure, melanin is composed of heterogeneous, redox-active aromatic polymers, typically derived from precursors such as L-DOPA, homogentisic acid, or other precursors. It contains indole, quinone, and semiquinone structures, with a high density of free radicals (Solano, 2017). In contrast, humic acid is a heterogeneous mixture of aromatic and aliphatic components. It lacks the specific indolic or phenolic precursors characteristic of melanin. Thus, it is generally less redox-active than melanin and more structurally diverse, with a less defined polymeric backbone (Lehmann and Kleber, 2015). This was clearly shown by the comparison of the extracted melanin-like material with the commercial humic acid in the current research.

Based on the function of melanin reported in literature, it is reasonable to speculate that, melanin-like material in sludge likely serves a protective function for melanin-producing microorganisms, shielding them from environmental stresses such as UV radiation, oxidative agents, and toxic metals (Nosanchuk and Casadevall, 2003; Solano, 2017). Humic acid, however, acts primarily as a byproduct or end product of organic matter degradation, contributing to the bulk organic fraction of sludge. Unlike melanin-like material, it is not synthesized for protective purposes (Lehmann and Kleber, 2015).

Therefore, although melanin and humic acid are both brown in color, they are fundamentally different: melanin is a microbial pigment with specific biological functions and defined biosynthetic pathways, while humic acid is a decomposition product of complex organic matter, formed through non-specific microbial and chemical processes. The understanding that the brown color in flocculent sludge is mostly due to the presence of humic substances or humic acids is not always correct.

4.3. *Melanin-like material from sludge as a valuable resource*

Melanin has been reported as a valuable biopolymer with a range of functional properties, including UV protection, antioxidant activity, and metal chelation. In addition, melanin has attracted increasing attention as a natural pigment owing to its environmental compatibility and strong affinity for protein-based fibers such as wool and silk (Gessler

et al., 2014). Its complex polyaromatic structure facilitates effective binding to wool fibers, particularly under acidic conditions (Huang et al., 2023). Commercial melanin is typically derived from cuttlefish ink; however, reliance on this source raises ethical concerns and limits availability due to the seasonal nature of cuttlefish harvesting (Liu and Simon, 2003). Although chemical synthesis methods exist, studies have shown that synthetic melanin differs structurally and functionally from naturally derived melanin (Mostert, 2021). These limitations have driven interest in alternative, non-animal and non-synthetic sources of melanin. In this context, the possibility of recovering melanin-like material from sludge presents a promising new resource. One potential application of sludge-derived melanin-like material is its use as a natural colorant for wool fibers. The strong dye fastness and stability of the extracted melanin-like pigment on wool observed in this study highlight its promise as an environmentally friendly biopigment. Given that melanin is a multifunctional polymer exhibiting UV-protective capability, antioxidant activity, and metal-chelating properties, future studies should further explore the potential applications of this sludge-derived melanin-like material in sustainable material design.

5. Conclusions

- A melanin-like material was successfully extracted from activated sludge, comprising ~11% of sludge dry mass. The extracted pigment exhibited strong UV absorption, high oxidative resistance, and abundant stable radicals. It was chemically distinct from commercial humic acids and synthetic melanin. FTIR and EPR data indicated a predominantly pyomelanin-like polymer.
- Metagenomic screening confirmed widespread microbial potential for tyrosine-derived melanin monomeric precursors biosynthesis. Pyomelanin-related genes (tyrosine aminotransferase and 4-hydroxyphenylpyruvate dioxygenase) are broadly distributed among key functional taxa.
- Microbial melanin-like material is an overlooked sludge component that contributes to the brown coloration.
- The pigment's strong affinity and wash fastness on wool indicate its potential as biobased pigment applications.

CRediT authorship contribution statement

Yuemei Lin: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Samarpita Roy:** Writing – review & editing, Writing – original draft, Formal analysis. **Peter-Leon Hagedoorn:** Writing – review & editing, Formal analysis.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2026.126195.

Data availability

No data was used for the research described in the article.

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