Brazilian Journal of Microbiology (2024) 55:2227–2237
https://doi.org/10.1007/s42770-024-01433-8

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Physicochemical characterization of the brown pigment produced by *Azospirillum brasilense* HM053 using tryptophan as precursor

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Received: 24 October 2023 / Accepted: 18 June 2024 / Published online: 2 July 2024

Abstract

Microorganisms are known to be a promising source of biopigments because they are easy to obtain, can be produced on a commercial scale, and are environmentally friendly. Therefore, the aim of this work was to characterize a brown pigment (BP) produced by HM053 in NFbHPN-lactate medium. The BP was extracted from the pellet (BPP) or supernatant (BPS), in the presence (BPPTrp, BPSTrp) or absence (BPPw, BPSw) of tryptophan (Trp). The UV-vis results were similar among all BP samples and compared with commercial melanin used as a standard, and the maximum absorption was observed around 200–220 nm. FTIR spectra showed that BP and commercial melanin had slight differences, with a small band between 3000–2840 cm^{-1}, related to C-H in the CH_2 and CH_3 aliphatic groups, which is not observed in the commercial melanin. Between BPP and BPS showed a different structure with bands in the region 1230–1070 cm^{-1} related to groups C-O. The thermogravimetric curves for BPSw and BPSTrp showed similar behavior, with 4 stages of mass loss. The similarity between BPPw and BPPTrp with 2 stages of mass loss was also observed. Scanning electron microscopy results showed morphological differences between BPP and BPS, where BPP had a physical structure more homogeneous and a regular flat surface, while the BPS physical structure did not seem homogeneous and the surface was uneven with some spherical structures as commercial melanin.

Keywords Brown pigment · Melanin · *Azospirillum brasilense* · Biotechnology · Biosources

Introduction

Microbial pigment contributes to pathogenicity by protecting the microorganism from host defense activity. The pigment is also used to protect them from adverse environmental conditions such as ultraviolet radiation and toxic heavy metals to oxidative stress [1–3]. Microorganisms are known to be a promising source of biopigments due to their stility, availability, cost-effectiveness, labor, yield, and ease of extraction and production on a commercial scale compared to other biosources such as animal and plant sources [4] and [5]. In addition, it is a safer product, and easily degradable in the environment, which makes it environmentally friendly. Among the biopigments, melanin has been highlighted because it plays many self-protective roles as well as scavenging phenolic compounds, blocking UV radiation, absorbing free radicals, chelating toxic iron, and protecting against environmental stress and can be found in almost all living organisms [3]. Also, in biotechnology melanin has been applied in cosmetics as a photoprotector,

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Responsible Editor: Eleni Gomes.

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pharmacology industry, environmental bioremediation, and used in organic semiconductors and bioelectronics [6, 7] and [3].

Melanin is characterized as a dark polyphenolic pigment that is normally synthesized by the oxidative degradation of tyrosine catalyzed by the enzyme tyrosinase (EC 1.14.18.1) and then polymerized to an insoluble granular substance [8] and [9]. However, an alternative pathway for pigment production using tryptophan instead of tyrosine as a biosynthetic precursor has been demonstrated in fungi and bacteria [10–13]. Tryptophan has been described as a precursor for indole pigments in microorganisms [12, 13].

Azospirillum sp is a plant growth-promoting bacteria (PGPB) known to be used as an inoculant, contributing to more sustainable agriculture. This genus has been extensively studied for its ability to fix atmospheric nitrogen through nitrogenase enzyme and synthesis of phytohormones, such as IAA using tryptophan as a precursor [14–18] The synthesis of brown pigment, melanin pigment, has already been reported for *Azospirillum brasilense* Sp7 (ATCC 29,145) and *Azospirillum lipoferum* [19] and [20]. For *A. lipoferum* laccase activity was observed and correlated with the production of a dark brown pigment [21]. In *A. brasilense* the brown pigment has been implicated in protecting the nitrogenase enzyme from oxidative damage [22]. While *Rhizobium* bacteria use melanogenic enzymes for the development of the symbiosis in the root nodules of plants [23].

The *Azospirillum brasilense* strain HM053 is a spontaneous mutant of *A. brasilense* FP2 strain (Sp7 ATCC 29,145, Sm^R, Nal^R) with ethylenediamine resistance (Nif^C, EDAR, Nal^R, Sm^R) [24]. This spontaneous mutant can constitutively fix nitrogen, even in the presence of high ammonium concentrations, and excretes NH_4 derived from nitrogen fixation [25]. HM053 has a point mutation, substitution of a proline residue by leucine at position 347 of the glutamine synthetase enzyme (gene *glnA*) [26, 27], and tests in wheat showed that it is a PGPB with high potential as a biofertilizer [27].

Then, the aim of this work was to perform the physicochemical characterization of the brown pigment composition produced by *Azospirillum brasilense* HM053 and to show that tryptophan is the biosynthetic precursor for brown pigment synthesis instead of tyrosine.

Materials and methods

Bacterial growth conditions and growth curve

The strain HM053 of *A. brasilense* was grown at 30 °C in 50 mL of NFbHPN-lactate medium [24] with 20 mmol L^{-1} of NH_4Cl under shaking at 140 rpm for 72 h. Streptomycin and nalidixic acid were added to the medium at a final concentration of 80 µg mL^{-1} and 10 µg mL^{-1}, respectively. Strain HM053 was grown under three conditions: (1) NFbHPN-lactate medium; (2) NFbHPN-lactate medium plus 5 mM of tryptophan; and (3) NFbHPN-lactate medium plus 5 mM of tyrosine. In the NFbHPN-lactate agar medium, the darker colonies were selected prior to inoculation into the liquid medium, as shown in Fig. 1. Under these conditions, the growth curve at 600 nm wavelength was performed at 24, 48, and 72 h of bacterial growth using a UV-Vis spectrophotometer (Biospectro SP-220). A 1 mL cuvette with a 1 cm optical path was used for all UV-Vis spectrophotometer analyses.

Quantification of indole compounds

The colorimetric Salkowski's reaction [28] was used for the determination of indole-3-acetic acid (IAA) produced by *A. brasilense*. The concentrations were calculated using commercial indole-3-acetic acid (Sigma-Aldrich) as a standard in different concentrations: 1, 2, 5, 10, 15, and 20 µg mL^{-1}.

Brown pigment purification and solubility

For the brown pigment (BP) purification, the culture was grown under the conditions described above and then centrifuged at 6,000 rpm for 10 min to separate cells and supernatant. The brown pigment of the cell pellet (BPP) was purified three times with 5% trichloroacetic acid, washed twice with ether-ethanol (1:1 v/v) and once with absolute ether to remove impurities, and centrifuged (5 min at 5,000 rpm) between these steps. The residue was then dissolved in 0.1 mol L^{-1} sodium carbonate by heating a water bath at 100 °C for 10 min. The solution was then centrifuged (10 min at 5,000 rpm) to remove insoluble material and was dried at 45 °C overnight. This protocol has been previously reported by [29–31]. For the brown pigment from the supernatant (BPS), the samples were not centrifuged between the steps with trichloroacetic acid, ether-ethanol, and absolute ether steps; the added solution was removed by pipetting after washing.

To simplify the sample naming, in the presence of tryptophan, we refer to BP samples as BPPTrp or BPSTrp, from cell pellet or supernatant, respectively. In the absence of tryptophan or tyrosine, we simply refer to them as BPPw or BPSw. The BP was soluble in NaOH, 3 mol L^{-1}, but showed no solubility in organic solvents (hexane, ethyl ether, dichloromethane, and DMSO) as well as in water.

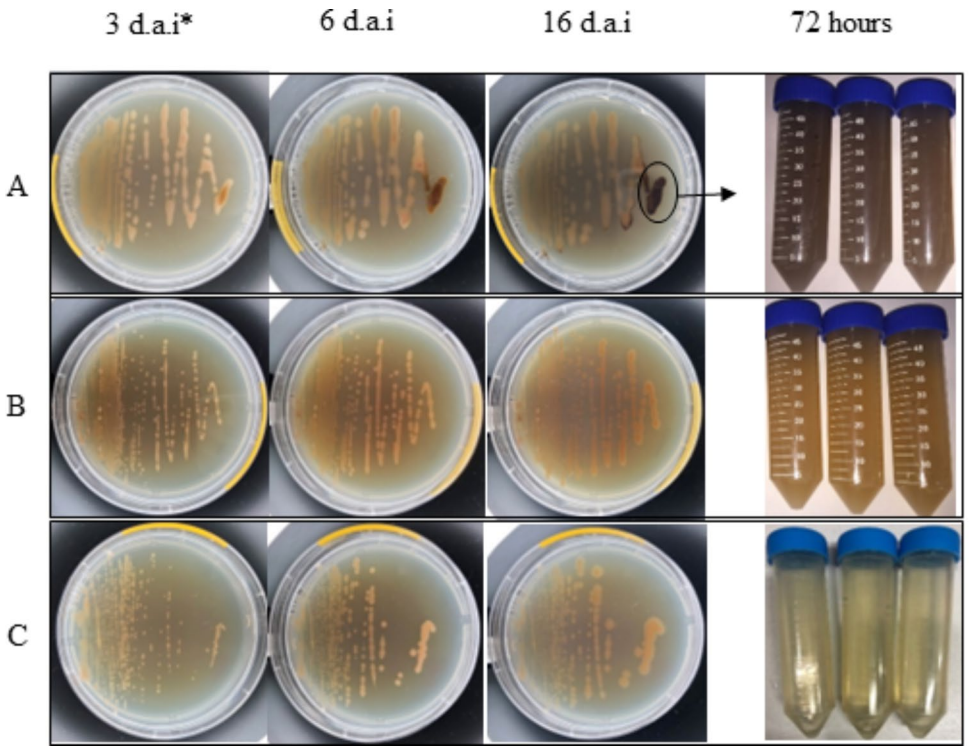

Fig. 1 Strain HM053 in NFbHPN-lactate medium growth at 30 °C in 3 different conditions: **(a)** NFbHPN-lactate medium; **(b)** NFbHPN-lactate medium with tryptophan; and **(c)** NFbHPN-lactate medium with tyrosine. The black arrow indicates the selected darker area used for growth in a liquid medium under agitation (140 rpm at 30° C) for 72 h. *d.a.i: days after inoculation

HPLC analysis

Cell growth was monitored by spectroscopic analysis as a function of tryptophan consumption analyzed by HPLC. Cell growth was measured in a UV-Vis spectrophotometer (Biospectro SP-220) at 600 nm. The HPLC experiments to monitor tryptophan consumption were performed on a Varian equipped with a C-18 column (Luna, Phenomenex, 250 × 4.6 mm i.d., 5 µm particle size). The flow rate of the solution was 0.8 mL min^{-1} and a gradient elution from 5 to 88% B over 30 min, back to 5% B in 32 min, and 5% B for another 5 min (solvent A = water and solvent B = MeCN). UV detection was set at 250 nm. The calibration curve of tryptophan was constructed at concentrations of 1, 2, 3, 4, and 5 mmol L^{-1}, with $R^2 = 0.9917$.

Characterization of BP by UV-Vis and FTIR spectroscopy

The samples used for UV-Vis analyses were prepared at a concentration of 0.02 mg mL^{-1} and a PerkinElmer Lambda 25 spectrophotometer was used; the spectra were collected in the range of 200–600 nm. FTIR spectra were obtained in a Perkin Elmer Fourier Transform IR spectroscopy equipment using KBr pellets in the range of 400–4000 cm^{-1}, recorded by 14 scans of accumulations. The UV-Vis and FTIR spectra were compared with synthetic melanin from Sigma-Aldrich as a standard.

Scanning electron microscopy (SEM)

The BPPTrp, BPSTrp, BPPw, and BPSw samples were dried at 60 °C and placed adhered to a carbon surface for microscopic analysis using a Hitachi TM3030Plus Tabletop microscope. PowerPoint Office version 2019 was used to edit the SEM images.

Thermogravimetric analysis

A Perkin Elmer Thermogravimetric analyzer was used to obtain TGA/DTG curves of the BPP and BPS. The sample masses were 5.491 and 6.953 mg for BPSTrp and BPSw, respectively. On the other hand, for BPPTrp and BPPw, they were 3.119 and 2.999 mg, respectively. The samples were analyzed using a heating range of 30 °C to 900 °C at a rate of 10 °C min^{-1} in a nitrogen atmosphere at a flow rate of 20 mL min^{-1}. UV-Vis, HPLC, FTIR, and TGA/DTG graphics were generated using Origin8 8.0 software version 2.0.0.19.

Results

Brown pigment (BP) production

We have shown that *A. brasilense* strain HM053 is capable of producing a BP after 72 h of inoculation in the NFbHPN-lactate medium. HM053 produced a dark brown pigment, but only in some parts of the agar medium, and

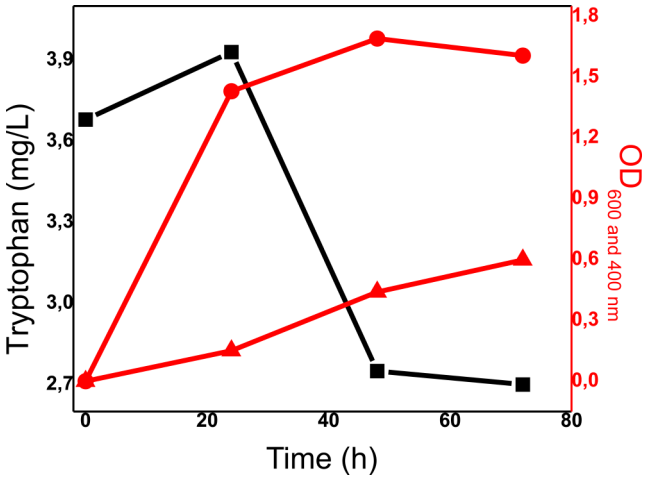

Fig. 2 Growth, tryptophan consumption, and brown pigment production by *Azospirillum brasilense* HM053 under NFbHPN-lactate medium supplemented with tryptophan. Values are the mean ± standard deviation of three biological replicates.: Tryptophan consumption over time;: *Azospirillum brasilense* HM053 growth curve at 600 nm;: *A. brasilense* HM053 brown pigment production at 400 nm

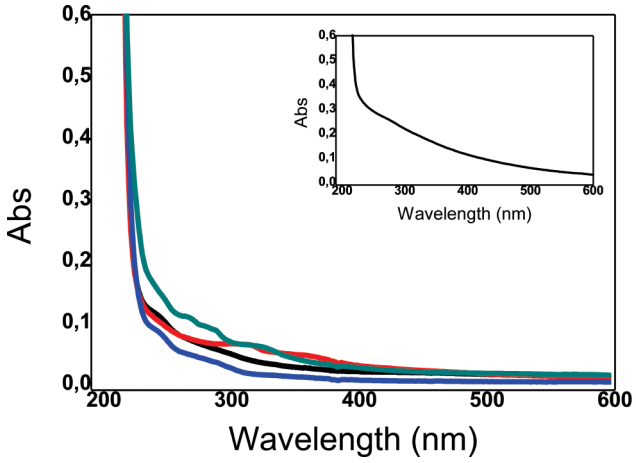

Fig. 3 Absorption spectra in the UV-Vis region to BPSTrp (–) and BPSw (–); BPPTrp (–) and BPPw (–). In the insert is commercial melanin as standard, 0.02 mg mL^{-1} in NaOH

the pigment color was intensified as the culture aged, from 3 to 16 days after inoculation (d.a.i) (Fig. 1A). Therefore, we selected the darkest brown colonies on the plates before inoculation in NFbHPN-Lactate medium liquid (Fig. 1A). In this condition, strain HM053 showed more variation in the ability to produce pigment, sometimes the medium did not show the black color, even growing in the same conditions (data not shown). Otherwise, in the presence of tryptophan, HM053 produced a light brown pigment that was more stable and homogeneous in the agar or liquid medium (Fig. 1B). Growth in the medium with tyrosine did not produce any brown pigment (Fig. 1C), so in the next analyses we considered 2 conditions: (1) NFbHPN-lactate medium supplemented with tryptophan; and (2) medium without any additional amino acid source.

Monitoring of BP

The data obtained by HPLC analysis showed that tryptophan is consumed along the bacterial growth time while light BP is produced, suggesting that tryptophan may be a precursor of light BP. According to Fig. 2, it is observed that tryptophan decreases with bacterial growth and pigment production increases, as observed by the optical density (OD) at 600 and 400 nm, respectively. Pigment production was monitored at 400 nm for comparison with literature data for the assessment of melanin pigment [30].

Spectroscopy characterization

UV-VIS analysis

Absorption UV-Vis spectroscopic analyses of BPPw, BPPTrp, BPSw, and BPSTrp were performed in the range of 200 to 600 nm. The maximum absorption spectra were around 220 nm for all pigments with bands of lower intensity in the range of 250–400 nm, as shown in Fig. 3.

To compare the UV-Vis spectra of BPSTrp, BPSw, BPPTrp, and BPPw, commercial melanin was used as a standard. The commercial melanin showed an intense absorption band with a maximum of around 200 nm and another band of lower intensity from 250 to 350 nm (Fig. 3, insert), as observed for the BP samples. Melanin is composed of different monomers that may interact differently causing the absorption bands to overlap, which may explain the behavior of the UV-Vis spectrum (Fig. 3).

FTIR spectroscopy analysis

The BPP and BPS were characterized by FTIR spectroscopy technique (Fig. 4) and compared with commercial melanin (Fig. 4A). A broad band is observed in the 3600–3000 cm^{-1} region for all samples. In this region, the stretching vibrations are attributed to N-H and O-H groups present in the indolic and pyrrolic systems. Figure 4A shows the FTIR spectra of commercial melanin. From this result, it was shown that BP and commercial melanin have slight differences between them, which is expected since BP is a natural pigment and other is synthetic.

Comparison of BPSw and BPSTrp spectra showed similarities between them (Fig. 4B), as well as for BPPw and BPPTrp (Fig. 4C). However, when BPS and BPP are

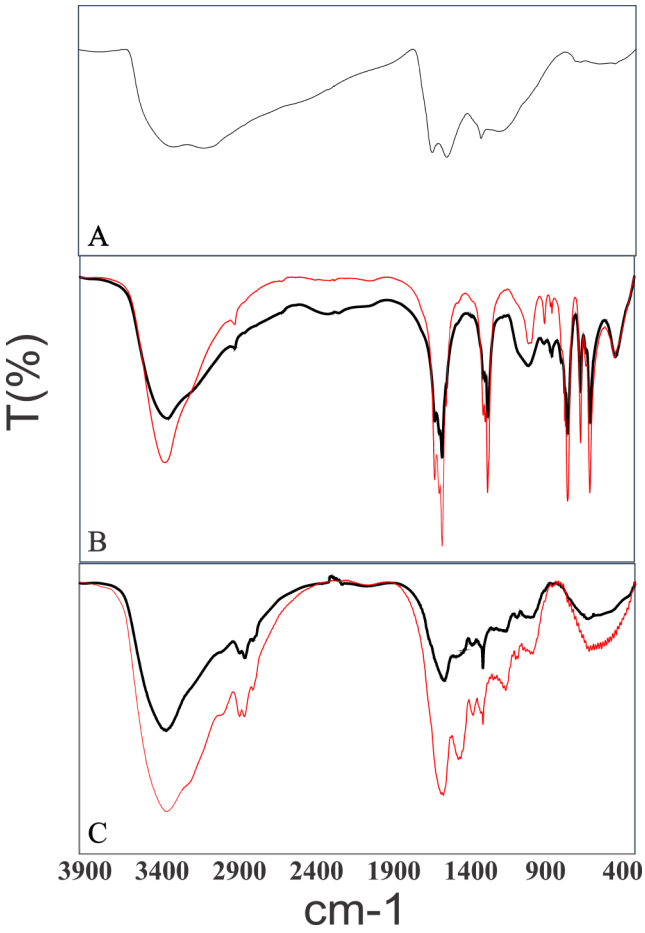

Fig. 4 FTIR spectrum for (**A**) Commercial melanin; (**B**) BPSTrp (–) and BPSw (–); (**C**) BPPTrp (–) and BPPw (–)

compared the FTIR differences between them are highlighted (Fig. 4B and C). In Fig. 4B and C, for the FTIR spectra of the pigment, a small band was observed between 3000–2840 cm^{-1}, related to C-H in the CH_2 and CH_3 aliphatic groups, which is not observed in the commercial melanin due to the broadening of the band at 3400 cm^{-1} (Fig. 4A).

In the region between 1715 and 1650 cm^{-1}, bands related to C = O of ketone, carboxylic acids, and esters stretching are observed. In addition, in the 1625 cm^{-1} region, the stretching vibrations responsible for C = C groups related to aromatic systems are also observed. The O-H group present in the phenolic and carboxylic functions are shown in 1400–1300 cm^{-1}, also observed in the commercial melanin.

The stretching vibrations in the range of 1230–1070 cm^{-1} related to the C-O groups show that the BPS has a different structure than BPP. Groups related to the aromatic ring and CH_2 polymer are observed in the range of 980–690 cm^{-1}.

Thermogravimetric analysis

The thermogravimetric curves between BPSw and BPSTrp show similar behavior with four stages of mass loss (Fig. 5). The first stage is characterized by a loss of 3% with respect to the moisture in the sample around 100 °C. The second and third stages are characterized by 37 and 30% mass loss related to the organic fraction around 190 and 220 °C, respectively. Finally, the last stage occurs where the mass loss is practically constant in the range of 220-800ºC (Fig. 5). This last stage is related to the stable part of the polymer, which can be related to a polymeric and inorganic part of the sample.

BPPw and BPPTrp also showed similar thermogravimetric curves between them but with some different stages of stability and decomposition (Fig. 6). Two stages of mass loss are observed, and the first stage of decomposition is observed at about 200 °C with a mass loss of 10%, and a second stage at about 380 °C with 50% of the mass loss, also has a stable part in the range of 380–900 °C (Fig. 6).

Scanning electron microscopy (SEM)

The SEM results showed morphological differences between BPP and BPS (Fig. 7), complementing the differences observed by chemical analysis (FTRI and TGA/DTG) (Figs. 4 and 5, and 6). BPP has a physical structure more homogeneous and on a regular flat surface (Fig. 7A), while the physical structure of BPS does not seem homogeneous and the surface is uneven with some spherical structures (Fig. 7B). On the other hand, the morphological structure was similar when compared between to the pellet samples (BPPw and BPPTrp), as well as when compared between the supernatant samples (BPSw and BPSTrp) (Fig. 7).

Indole-3-acetic acid production

The Salkowski's method was used for the determination of IAA. Through this analysis, IAA production was found only when the *A. brasilsene* strain HM053 was grown in an NFbHPN-lactate medium with tryptophan (Table 1). Also, the IAA production increased approximately 27-fold from 48 to 72 h (Table 1) Curiously, light BP production used the same growth condition as for IAA production at 72 h (Table 1).

Discussion

We reported that *A. brasilense* HM053 is able to produce dark BP in NFbHPN-lactate medium, and light BP in the same medium supplemented with tryptophan. In bacteria,

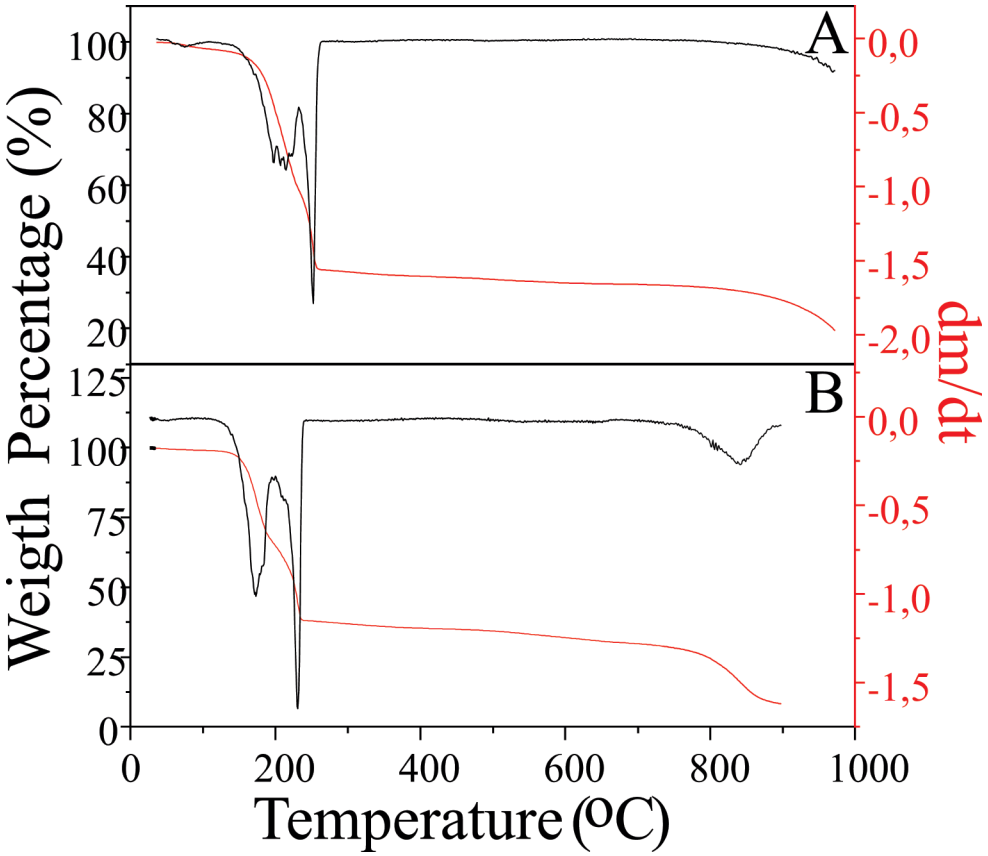

Fig. 5 Thermogravimetric curve (TGA/DTG) for BPSTry (**A**) and BPSw (**B**), in a nitrogen atmosphere at a flow rate of 10 °C min^{-1}

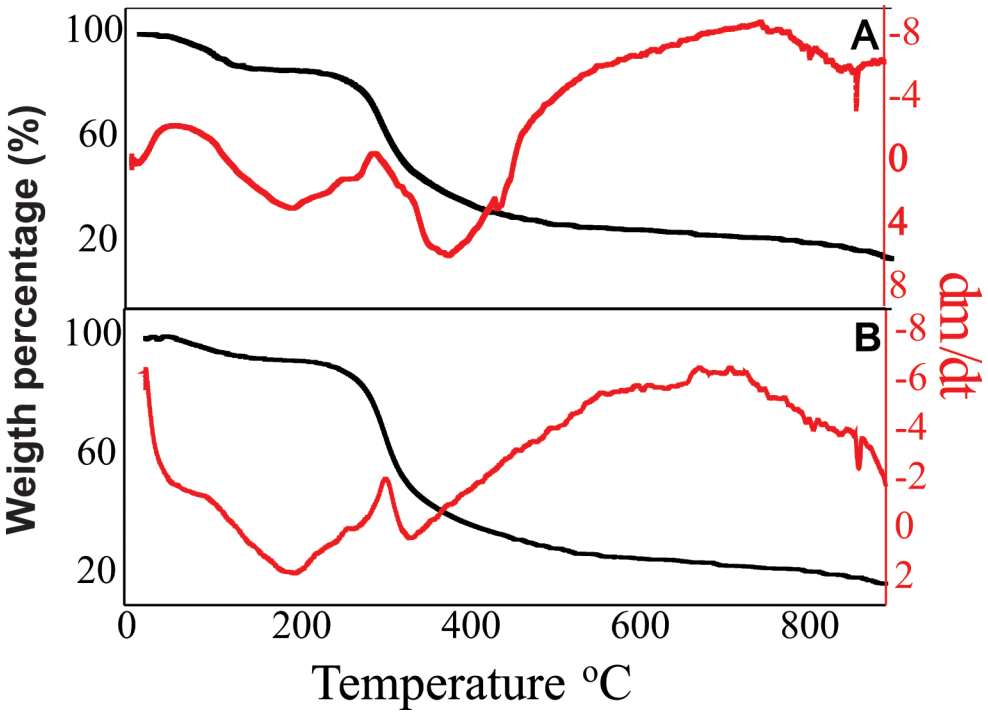

Fig. 6 Thermogravimetric curve (TGA/DTG) of BPPTrp (**A**) and BPPw (**B**) in a nitrogen atmosphere at a flow rate of 10 °C min^{-1}

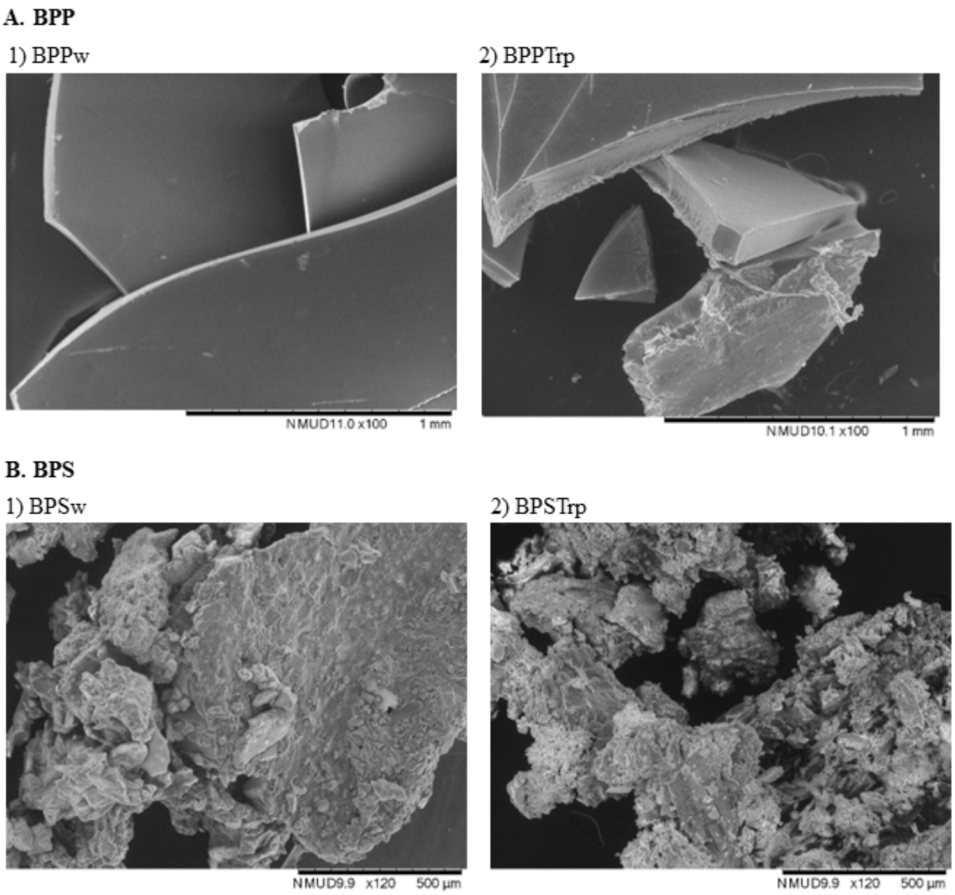

Fig. 7 Scanning electron microscopy (SEM) of BP produced by *Azospirillum brasilense* strain HM053. **A**. BP extracted from pellet (BPP) and **B.** from supernatant (BPS) after growth in liquid NFbHPN-lactate medium: (1) without tryptophan (2) with tryptophan

Table 1 Indole-3-acetic acid (IAA) production by *A. brasilense* HM053 at 72 h after inoculation

Growth conditions	IAA production (µg mL^{1-})	
	48 h	72 h
HM053 grown in NFbHPN-lactate medium	0.00	0.00
HM053 in NFbHPN-lactate medium with Trp	2.05 ± 0.01	54.55 ± 0.02
HM053 in NFbHPN-lactate medium with Tyr	0.00	0.00

Trp: Tryptophan; Tyr: Tyrosine

melanin is produced under adverse conditions such as nutrient starvation, UV radiation, desiccation, osmotic stress, and competition in the rhizosphere [2]. Under these conditions, *A. brasilense* has the ability to encyst cells, consisting of a central body filled with poly-β-hydroxybutyrate (PHB) granules, which confers a survival advantage to this bacterium, especially in an aging culture. Melanin has been shown to surround these encysting cells and to be secreted into the medium in an aging culture [19]. These data support our results showing the ability of *A. brasilense* to produce dark BP over time in NFbHPN-lactate medium on aged culture (limiting nutrition source).

For *A. brasilense* Sp7 strain, the brown pigment produced depends on the medium composition and growth conditions. In NFbHP-lactate agar medium supplemented with low levels of carbon source (8 mM of fructose or malate) and a minimal amount of nitrogen (0.5 mM KNO_3), pigment production is induced under limiting conditions. When amino acid sources, such as tyrosine, cysteine, and/or phenylalanine were added, the amount of pigment formed was not improved in various media tested [19], as shown in our results for tyrosine, which did not induce or improve pigment production. Otherwise, tyrosine is known to be a precursor of melanin for other microorganisms [2, 20, 30].

From UV-Vis results all BP samples and commercial melanin, had the same behavior with the maximum absorption spectra around 220 nm. Has been reported a broad band, ranging from 280 to 400 nm, and a maximum peak at 200 nm, with a continuous decrease in the absorbance from UV-Vis wavelength, which is characteristic of melanin pigment [32, 33], similar to our results. These results are also observed in sepia melanin, which was obtained from *Sepia Officialis* [34].

To complement the chemical analysis, FTIR spectroscopy and TGA thermogram analysis were performed. Our FTIR spectral results were similar to the FTIR spectral

profile found for the melanin of *Rubrivivax benzoatilyticus*, which was associated with the presence of groups such as: O-H, C-H, C = C, C-O, C = O, the presence of nitrogen and the absence of sulfur content (present in indole systems and found in indole-type melanin) [13, 33]. These authors correlated their IR results with pyomelanin [33] and eumelanin [13].

Usually, the TGA thermograms for natural melanin showed three stages (the first stage around 70 °C, the second around 200–360 °C, and third around 800–1000 °C) of thermal degradation [35, 36]. Here we found 4 stages for BPS (Fig. 5), which was more similar compared to the literature, and 2 stages for BPP (Fig. 6) with TGA/DTG thermograms more stable, showing a slightly different behavior.

Chemical characterization of melanin can be a challenging task because the pigment is highly heterogeneous, insoluble in organic solvents, hydrophobic, and resistant to chemical degradation. These physicochemical properties limit the number of analytical approaches that can identify and characterize the pigment. In addition, published data are relatively inconsistent because differences in extraction and purification, sample preparation, or data processing make it difficult to compare results. On the other hand, applying the same procedures to different melanin sources, regardless of their cellular localization, biosynthetic pathways, or possible interferences, may not yield equivalent results [37]. For these reasons, the number of publications on melanin characterization is relatively small.

Furthermore, BPP and BPS were insoluble in water or organic solvents (hexane, ethyl ether, dichloromethane, and DMSO) and soluble in 3 mol L^{-1} NaOH under heating. Similar physicochemical properties were reported for melanin isolated from the bacterium *Rubrivivax benzoatilyticus* [13]. It was observed that BPP was more difficult to solubilize than BPS, perhaps due to the particular physical properties of each sample. SEM analysis of melanin showed similar spherical/ball-like structures, confirming our results for BPS [13, 33]. Otherwise, BPP showed a variety of structures that differed from previous results reported in the literature.

The genus *Azospirillum* is able to produce plant hormones, such as IAA, which it can support as PGPB [17, 38]. *A. brasilense* has multiple IAA biosynthetic pathways, been tryptophan-dependent or not. For HM053, IAA production was similar to that of *A. brasilene* strain Sp7 (50 µg mL^{-1} of IAA) [38]. Tryptophan has been reported as a substrate for auxin synthesis by various bacteria via the indole-3-acetamide pathway, which converts tryptophan by tryptophan 2-monooxygenase to indole-3-acetamide and then by indole3-acetamide hydrolase to indole-3-acetic acid (IAA). The intermediate indoleacetamide can be identified by Salkowski's chemical assay, as we have done (Table 1). In *Azospirillum brasilense* and *A. lipoferum*, the indoleacetamide pathway is similar to the indolepyruvic acid pathway [17]. Other studies support the possibility of multiple IAA biosynthesis pathways in *Azospirillum brasilense* [38, 39] with the indolepyruvic acid pathway being the most common [40, 41]. This is a controversial issue because in according to [17], the indoleacetamide pathway is the most prevalent.

Tryptophan has also been implicated in pigment production in various microorganisms, such as fungi [12, 42] and bacteria [10, 13]. From our results, the BP produced by *A. brasilense* HM053 has some similar chemical properties to those reported in the literature [13, 33, 35–37], which we may suggest that the BP is a melanin-like.

Melanin is a multifunctional natural pigment widely produced by organisms from all domains of life, from bacteria to mammals, with specific physicochemical properties, such as a highly negative charge, high molecular weight, and hydrophobicity [37, 43]. Most bacterial melanins (eumelanin, pheomelanin, pyomelamin, and neuromelanin) are formed *via* the DOPA pathway from aromatic amino acids, such as tyrosine, or from other catecholamine substrates, such as dopamine and norepinephrine. In contrast, DHN melanin is synthesized via the DHN pathway from malonyl-coenzyme A or acetyl-CoA as precursors. These pathways are facilitated by different sets of enzymes, i.e. tyrosinase/laccase for the DOPA pathway and polyketide synthase for the DHN pathway [2, 3]. In general, melanin synthesized by the DHN pathway is endogenous and tightly bound to the inner side of the cell wall, making extraction of melanin extremely difficult [44]. On the other hand, melanin synthesized extracellularly is easier to extract [3], as is the case for *A. brasilense* strain HM053, making it a potential biosource for melanin.

Microbial melanin is bioavailable, biocompatible, and biodegradable, making it a promising candidate for many industrial applications. For example, in the cosmetic and pharmaceutical industries, melanin is a natural "sunscreen" that absorbs the broad band of the UV visible light spectrum and can also be used for hair dyeing. It is also a powerful antioxidant, scavenging or quenching molecules on superoxide anions and singlet oxygen species generating a positive physiological response and inducing the immune system [3, 45, 46]. Melanin protection has been tested against radiation and increased the survival of mice by 100% [47]. In environmental applications, melanin acts as a chelator of heavy metals in water systems [48], and melanin nanostructures exhibit broad-spectrum antimicrobial activity against foodborne pathogens with potential applications in the health and food industries [49, 50]. Melanin has also been used as a component for organic electronic devices due

to its hydration-dependent semiconductor-like behavior [51, 52].

In animal models, tryptophan has been proposed as a precursor for melanin synthesis (named Trp-melanin), but its biosynthetic pathway has not been identified [53–55]. Recently, Trp-melanin synthesis by the bacterium *Rubrivivax benzoatilyticus* JA2 was reported, with similar physicochemical properties when compared to BP from *A. brasilense* strain HM053 [13], suggesting that this Trp-melanin is not synthesized from the canonical tyrosine-based melanin biosynthetic process, and that laccase probably plays a role in the oxidative metabolism of Trp-melanin synthesis. These authors did not find a laccase gene in *R. benzoatilyticus* JA2, but suggested a laccase-like enzyme, as has been reported in melanin synthesis in a few bacteria [56, 57]. Thus, these authors proposed the tryptophan as a precursor that may be converted to 5-hydroxytryptophan to an unknown hydroxyindole derivative and then polymerized to Trp-melanin. Similar results have been reported by [58].

Conclusion

A. brasilense strain HM053 is able to produce dark and light BP in the NFbHPN-lactate medium but does not use tyrosine as a precursor. Thus, the catalytic activity of tyrosinase does not play a central role in brown pigment synthesis by *A. brasilense* HM053, as is usually the case for another organism. From HPLC analysis, we suggest that tryptophan is a precursor for the synthesis of light BP. In the absence of tryptophan, a dark BP was produced in some parts of the plate in the aging culture. However, we do not know the mechanism to induce the dark BP, sometimes, even in aging culture (nutrient starvation), the medium does not express the dark color. This suggests a metabolic diversity for pigment synthesis in *A. brasilense* that should be better understood. In general, all BP samples had similar chemical profiles, except for some differences that were observed between BPP and BPS from TGA analysis and scanning electron microscopy. From the physicochemical results, such as UV-vis analysis, IR spectra, and TGA, we can strongly suggest that the BP is melanin-like, making *A. brasilense* HM053 a promising source of melanin with a high potential application. However, further work on the biosynthetic pathway of Trp-melanin-like should be carried out for a better understanding of its synthesis, such as the development of studies for its biotechnological application.

Acknowledgements We dedicate this work to the memory of Professor Dra. Claudia C. G. Martin-Didonet, who was in charge of this project and all the research lines at the Laboratory of Microbial Biochemistry at the Universidade Estadual de Goiás– CET, where we carried out this search. We thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the financial support, the Universidade Estadual de Goiás– CET and the postgraduate program in Sciences Applied to Healthcare Products and Molecular Sciences.

Author contributions All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Karina Freire d'Eça Nogueira Santos and Marilene Silva Oliveira. The first draft of the manuscript was written by the corresponding author, Karina Freire d'Eça Nogueira Santos. All authors commented on previous versions of the manuscript, such as Enderson Petrônio de Brito Ferreira and Alliny das Graças Amaral. All authors read and approved the final manuscript.

Funding This study was supported by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) (n° 448297/2014-0) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), that provided the PNPD/CAPES fellowships to the authors Karina Freire d'Eça Nogueira Santos (n °88882.315237/2019-01) and Marilene Silva Oliveira (n° 817164/2015).

Data availability The datasets analyzed during the current study are available from the corresponding author on request.

Declarations

Competing interests The authors declare that there are no conflicts of interest among the authors regarding. The publication of this manuscript. The authors have no financial or non-financial interests to disclose.

References

1. Brunke S, Seider K, Almeida RS et al (2010) Candida Glabrata tryptophan-based pigment production via the Ehrlich pathway. Mol Microbiol 76:25–47. https://doi.org/10.1111/j.1365-2958.2010.07052.x
2. Pavan ME, López NI, Pettinari MJ (2020) Melanin biosynthesis in bacteria, regulation and production perspectives. Appl Microbiol Biotechnol 104:1357–1370. https://doi.org/10.1007/s00253-019-10245-y
3. Tran-Ly AN, Reyes C, Schwarze FWMR, Ribera J (2020) Microbial production of melanin and its various applications. World J Microbiol Biotechnol 36:1–9. https://doi.org/10.1007/s11274-020-02941-z
4. Joshi VK, Attri D, Bala A, Bhushan S (2003) Microbial pigments. Indian J Biotechnol 2:362–369
5. Tuli H, Chaudhary P, Beniwal V (2015) Microbial pigments as natural color sources: current trends and future perspectives. 52:4669–4678. https://doi.org/10.1007/s13197-014-1601-6
6. Saxena D, Ben-Dov E, Manasherob R et al (2002) A UV tolerant mutant of Bacillus thuringiensis subsp. kurstaki producing melanin. Curr Microbiol 44:25–30. https://doi.org/10.1007/s00284-001-0069-6
7. Guo J, Rao Z, Yang T et al (2014) High-level production of melanin by a novel isolate of Streptomyces kathirae. 357:85–91. https://doi.org/10.1111/1574-6968.12497
8. Lerch K, Ettlincer L (1972) Purification and characterization of a tyrosinase from Streptomyces gzaucescens. Eur J Biochem 31:427–437. https://doi.org/10.1111/j.1432-1033.1972.tb02549.x
9. Ikeda K, Suzuki K, Yoshioka H et al (1998) Construction of a new cloning vector utilizing a cryptic plasmid and the highly expressed melanin-synthesizing gene operon from Streptomyces

Castaneoglobisporus. FEMS Microbiol Lett 168:195–199. https://doi.org/10.1111/j.1574-6968.1998.tb13273.x
10. Duerre JA, Buckley PJ (1965) Pigment production from tryptophan by an Achromobacter species. J Bacteriol 90:1686–1691. https://doi.org/10.1128/jb.90.6.1686-1691.1965
11. Alkhalaf LM, Ryan KS (2015) Biosynthetic manipulation of tryptophan in bacteria: pathways and mechanisms. Chem Biol 22:317–328. https://doi.org/10.1016/j.chembiol.2015.02.005
12. Preuss J, Hort W, Lang S et al (2013) Characterization of tryptophan aminotransferase 1 of Malassezia Furfur, the key enzyme in the production of indolic compounds by M. Furfur. Exp Dermatol 22:736–741. https://doi.org/10.1111/exd.12260
13. Ahmad S, Mohammed M, Mekala LP, Chintalapati S (2020) Tryptophan, a non-canonical melanin precursor: new L-tryptophan based melanin production by Rubrivivax benzoatilyticus JA2. Sci Rep 1–12. https://doi.org/10.1038/s41598-020-65803-6
14. Okon Y, Heytler PG, Hardy RWF (1983) N 2 fixation by Azospirillum brasilense and its incorporation into host Setaria italica. Appl Environ Microbiol 46:694–697. https://doi.org/10.1128/aem.46.3.694-697.1983
15. Dobereiner J, Pedrosa FO, Thomas DB (1988) Nitrogen-fixing Bacteria in nonleguminous crop plants. Q Rev Biol 63:338–339. https://doi.org/10.1086/415968
16. Dobbelaere S, Croonenborghs A, Thys A et al (1993) Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. Plant Soil 212:155–164. https://doi.org/10.1023/A:1004658000815
17. Bar T, Okon Y (1993) Tryptophan conversion to indole-3-acetic acid via indole-3-acetamide in Azospirillum brasilense Sp7. Can J Microbiol 39:81–86. https://doi.org/10.1139/m93-011
18. Okon Y (1994) Azospirillum/plant associations. CRC, Boca Raton
19. Sadasivan L, Neyra CA (1987) Cyst production and brown pigment formation in aging cultures of Azospirillum brasilense ATCC 29145. J Bacteriol 169:1670–1677. https://doi.org/10.1128/jb.169.4.1670-1677.1987
20. Givaudan A, Effosse A, Bally R (1918) (Developments in Plant and Soil Sciences 48) A. Quispel (auth.), M. Polsinelli, R. Materassi, M. Vincenzini (eds.)-Nitrogen Fixation_ Proceedings of the Fifth International Symposium on Nitrogen.pdf
21. Givaudan A, Effosse A, Faure D et al (1993) Polyphenol oxidase in Azospirillum lipoferum isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of Azospirillum lipoferum. FEMS Microbiol Lett 108:205–210. https://doi.org/10.1111/j.1574-6968.1993.tb06100.x
22. Eskew DL, Focht DD, Ting IP (1977) Nitrogen fixation, denitrification, and Pleomorphic Growth in a highly pigmented Spirillum Lipoferum. 34:582–585. https://doi.org/10.1128/aem.34.5.582-585.1977
23. Hawkins FKL, Johnston AWB (1988) Transcription of a Rhizobium ieguminosarum biovar phaseoli gene needed for melanin synthesis is activated by nifA of Rhizobium and Klebsiella pneumoniae. Mol Microbiol 2:331–337. https://doi.org/10.1111/j.1365-2958.1988.tb00036.x
24. Pedrosa F, Yates MG (1984) Regulation of nitrogen fixation (nif) genes of Azospirillum brasilense by nifA and Ntr (gln) type gene products. FEMS Microbiol Lett 23:95–101. https://doi.org/10.1111/j.1574-6968.1984.tb01042.x
25. Machado HB, Funayama S, Rigo LU, Pedrosa FO (1991) Excretion of ammonium by Azospirillum brasilense mutants resistant to ethylenediamine. Can J Microbiol 37:549–553. https://doi.org/10.1139/m91-092
26. Ghenov F, Gerhardt ECM, Huergo LF et al (2022) Characterization of glutamine synthetase from the ammonium-excreting strain HM053 of Azospirillum brasilense. Brazilian J Biol 82:1–8. https://doi.org/10.1590/1519-6984.235927
27. Santos KFDN, Moure VR, Hauer V et al (2017) Wheat colonization by an Azospirillum brasilense ammonium-excreting strain reveals upregulation of nitrogenase and superior plant growth promotion. Plant Soil 415:245–255. https://doi.org/10.1007/s11104-016-3140-6
28. Tang YW, Bonner J (1947) The enzymatic inactivation of indoleacetic acid. I. Some characteristics oS the enzyme contained in pea seedlings. Arch Biochem 13:11–25
29. Whittaker J (1963) Changes in melanogenesis during the dedifferentiation of chick retinal pigment cells in cell culture. Dev Biol 8:99–127. https://doi.org/10.1016/0012-1606(63)90028-9
30. Shivprasad S, Page WJ (1989) Catechol formation and melanization by na + -Dependent Azotobacter chroococcum: a protective mechanism for Aeroadaptation? Appl Environ Microbiol 55:1811–1817. https://doi.org/10.1128/aem.55.7.1811-1817.1989
31. Banerjee A, Supakar S, Banerjee R (2014) Melanin from the nitrogen-fixing bacterium Azotobacter chroococcum: a spectroscopic characterization. PLoS ONE 9:1–7. https://doi.org/10.1371/journal.pone.0084574
32. Meredith P, Sarna T (2006) The physical and chemical properties of eumelanin. Pigment Cell Res 19:572–594. https://doi.org/10.1111/j.1600-0749.2006.00345.x
33. Mekala LP, Mohammed M, Chinthalapati S, Chinthalapati VR (2019) Pyomelanin production: insights into the incomplete aerobic L-phenylalanine catabolism of a photosynthetic bacterium, Rubrivivax benzoatilyticus JA2. Int J Biol Macromol 126:755–764. https://doi.org/10.1016/j.ijbiomac.2018.12.142
34. Magarelli M, Passamonti P, Renieri C (2010) Purification, characterization and analysis of sepia melanin from commercial sepia ink (Sepia Officinalis). Rev CES Med Vet y Zootec 5:18–28. https://doi.org/10.21615/1424
35. Wang LF, Rhim JW (2019) Isolation and characterization of melanin from black garlic and sepia ink. Lwt 99:17–23. https://doi.org/10.1016/j.lwt.2018.09.033
36. Gómez-Marín AM, Sánchez CI (2010) Thermal and mass spectroscopic characterization of a sulphur-containing bacterial melanin from Bacillus subtilis. J Non Cryst Solids 356:1576–1580. https://doi.org/10.1016/j.jnoncrysol.2010.05.054
37. Pralea IE, Moldovan RC, Petrache AM et al (2019) From extraction to advanced analytical methods: the challenges of melanin analysis. Int J Mol Sci 20:1–37. https://doi.org/10.3390/ijms20163943
38. Hartmann a., Singh M, Klingmüller W (1983) Mutants Excreting High amounts of Indoleacetic Acid. Can J Microbiol 29:916–923. https://doi.org/10.1139/m83-147
39. Prinsen E (1993) Azospirillum brasilense Indole-3-Acetic acid biosynthesis: evidence for a Non-tryptophan Dependent Pathway. Mol Plant-Microbe Interact 6:609. https://doi.org/10.1094/MPMI-6-609
40. Barbieri P, Zanelli T, Galli E, Zanetti G (1986) Wheat inoculation with Azospirillum brasilense Sp6 and some mutants altered in nitrogen fixation and indole-3-acetic acid production. FEMS Microbiol Lett 36:87–90. https://doi.org/10.1111/j.1574-6968.1986.tb01672.x
41. Vande Broek A, Lambrecht M, Eggermont K, Vanderleyden J (1999) Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in Azospirillum brasilense. J Bacteriol 181:1338–1342. https://doi.org/10.1128/jb.181.4.1338-1342.1999
42. Chaskes S, Cammer M, Nieves E, Casadevall A (2014) Pigment production on l-tryptophan medium by cryptococcus gattii and cryptococcus neoformans. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0091901
43. Prota G (1992) Melanins and Melanogenesis. Academic P., San Diego

44. Toledo A, Franco ME, Yanil L, Al S et (2017) Melanins in fungi: types, localization and putative biological roles. Physiol Mol Plant Pathol 99:2–6. https://doi.org/10.1016/j.pmpp.2017.04.004
45. Tada M, Kohno M, Niwano Y (2010) Scavenging or quenching effect of melanin on Superoxide Anion and Singlet Oxygen. J Clin Biochem Nutr 46:224–228. https://doi.org/10.3164/jcbn.09-84
46. Pugh ND, Tamta H, Balachandran P et al (2008) The majority of in vitro macrophage activation exhibited by extracts of some immune enhancing botanicals is due to bacterial lipoproteins and lipopolysaccharides. Int Immunopharmacol 8:1023–1032. https://doi.org/10.1016/j.intimp.2008.03.007
47. Kunwar A, Adhikary B, Jayakumar S et al (2012) Melanin, a promising radioprotector: mechanisms of actions in a mice model. Toxicol Appl Pharmacol 264:202–211. https://doi.org/10.1016/j.taap.2012.08.002
48. Tran-Ly AN, Ribera J, Schwarze FWMR et al (2020) Fungal melanin-based electrospun membranes for heavy metal detoxification of water. Sustain Mater Technol 23:e00146. https://doi.org/10.1016/j.susmat.2019.e00146
49. Kiran GS, Dhasayan A, Lipton AN et al (2014) Melanin-templated rapid synthesis of silver nanostructures. J Nanobiotechnol 12:18. https://doi.org/10.1186/1477-3155-12-18
50. Patil S, Sistla S, Bapat V, Jadhav J (2018) Melanin-mediated synthesis of silver nanoparticles and their Affinity towards tyrosinase. Appl Biochem Microbiol 54:163–172. https://doi.org/10.1134/S0003683818020096
51. Kim YJ, Wu W, Chun S-E et al (2013) Biologically derived melanin electrodes in aqueous sodium-ion energy storage devices. Proc Natl Acad Sci 110:20912–20917. https://doi.org/10.1073/pnas.1314345110
52. Bothma J, De Boor J, Divakar U, Al E (2008) Device-quality electrically conducting melanin thin films. Adv Mater Adv Mater 20:3539–3542. https://doi.org/10.1002/adma.200703141
53. Lambrus BG, Cochet-Escartin O, Gao J et al (2015) Tryptophan hydroxylase is required for eye melanogenesis in the planarian schmidtea mediterranea. PLoS ONE 10:7–9. https://doi.org/10.1371/journal.pone.0127074
54. Allegri G, Vogliardi S, Bertazzo A et al (2003) Involvement of 5-hydroxytryptophan in melanogenesis. Adv Exp Med Biol 527:723–730. https://doi.org/10.1007/978-1-4615-0135-0_85. PMID: 15206795
55. Vogliardi S, Allegri G, Bertazzo A et al (2002) An investigation on the role of 5-hydroxytryptophan in the biosynthesis of melanins. J Mass Spectrom 37:1292–1296. https://doi.org/10.1002/jms.383
56. Castro-Sowinski S, Martinez-Drets G, Okon Y (2002) Laccase activity in melanin-producing strains of Sinorhizobium meliloti. FEMS Microbiol Lett 209:119–125. https://doi.org/10.1111/j.1574-6968.2002.tb11119.x
57. Lucas-Elío P, Solano F, Sanchez-Amat A (2002) Regulation of polyphenol oxidase activities and melanin synthesis in Marinomonas mediterranea: identification of ppoS, a gene encoding a sensor histidine kinase. Microbiology 148:2457–2466. https://doi.org/10.1099/00221287-148-8-2457
58. Vèkey JT, Somogyi A, Bertazzo A et al (1992) Studies on structure characterization of tryptophan melanin: comparison between filament and curie-point pyrolysis gas chromatography/mass spectrometry. J Mass Spectromerty 27:1216–1219. https://doi.org/10.1002/oms.1210271111