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Research Article

Discovery of Melanogenic Bacteria from Anak Krakatau Volcano Soil through Enzymatic, Antioxidant-Based Screening and Its Volatile Metabolic Profiles

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Abstract
Microbial melanin has attracted increasing attention due to its multifunctional properties, particularly its antioxidant activity, making it attractive for biotechnological applications. Volcanic soils represent extreme, metal-rich ecosystems that may harbor melanogenic bacteria with unique metabolic adaptations. This study aimed to isolate and characterize melanin-producing bacteria from the volcanic soil of Mount Anak Krakatau, evaluating their melanogenic capacity, antioxidant activity, and volatile metabolite profiles. Eight bacterial isolates were obtained using tyrosine agar supplemented with $CuSO_4$ to selectively promote tyrosinase-mediated melanogenesis. Three isolates (AK3, AK5, and AK7) exhibited clear melanogenic phenotypes, as indicated by the progressive development of brown to black pigmentation during cultivation. Tyrosinase activity and L-DOPA concentration showed dynamic changes throughout the production period, with isolate AK5 demonstrating the highest tyrosinase activity (4,500 ± 135.27 U/mL) and L-DOPA accumulation (up to 89.82 ± 0.13 µg/mL), reflecting efficient biotransformation of L-tyrosine. The antioxidant activity of free-cell supernatants, evaluated using the ferric reducing antioxidant power (FRAP) and total antioxidant capacity (TAC) assays, yielded substantial values, with AK5 exhibiting the highest performance among the testing isolates and further identified as *Bacillus cereus* AK5 by phylogenetic analysis of 16S rRNA gene sequences. Gas chromatography–mass spectrometry (GC–MS) analysis revealed distinct, isolate-specific volatile organic compound (VOC) profiles, which we hypothesize may reflect adaptive secondary metabolic responses to oxidative stress generated during melanogenesis, though this link requires further experimental validation. To the best of our knowledge and based on the available literature, this study reports the first isolation of melanin-producing bacteria with antioxidant potential from volcanic soil at Mount Anak Krakatau, highlighting this environment as a promising source of melanogenic bacteria for pharmaceutical applications.

Keywords: Anak Krakatau; antioxidant capacity; melanogenesis; tyrosinase activity; volcanic soil

1. INTRODUCTION

Melanin is a complex aromatic biopolymer produced by various organisms, including bacteria. It is known for its diverse biological functions, including protection against radiation, binding of heavy metals, and potent antioxidant activity [1]. In microbial systems, the biological properties of melanin are significantly influenced by the type of microorganism, the biosynthetic pathway, and the environmental conditions to which the microbe adapts. As a result, melanin produced by different microbes can exhibit distinct biological activities, even when derived from similar biosynthetic pathways [2]–[4].

Bacterial melanin biosynthesis is primarily mediated by the enzyme tyrosinase, which catalyzes the oxidation of L-tyrosine to L-DOPA and subsequently to dopaquinone. This compound then polymerizes non-enzymatically to form melanin [5]. The process requires copper(II) ions (Cu^{2+}) as an essential cofactor, making the presence of copper in the environment and culture medium a key factor in determining the efficiency of melanogenesis [6][7]. Numerous studies have shown that $CuSO_4$ supplementation in the medium can enhance melanin production in various bacteria and actinomycetes, including *Streptomyces* and *Bacillus*. This supplementation also increases tolerance to oxidative stress and heavy metals [7]–[13].

Extreme geochemical environments, such as young volcanic soils, present promising ecological niches for studying melanin-producing microbes. Mount Anak Krakatau, which formed after the major 1883 eruption and experienced another significant eruption in 2018, produced new volcaniclastic deposits rich in metals, including iron (Fe) and copper (Cu). Soils derived from

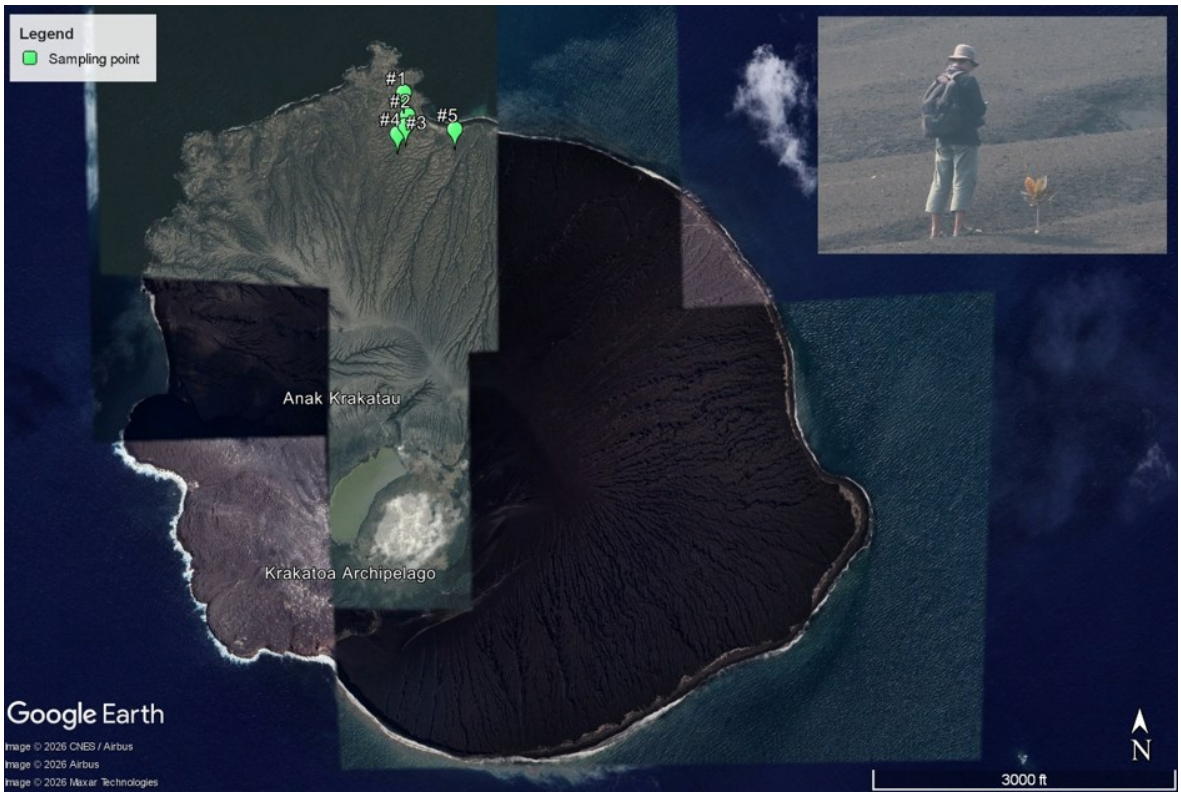

Figure 1. Location of soil sampling at Mount Anak Krakatau

volcanic material are characterized by metal stress and highly oxidative conditions, which ecologically favor the selection of microorganisms with protective mechanisms, such as melanin production [14][15]. However, to date, no reports have specifically investigated melanin-producing bacteria in the soil of Mount Anak Krakatau.

In addition to melanin itself, the bacterial melanogenesis process generates intracellular redox stress through oxidative reactions mediated by tyrosinase. This stress promotes the release of various water-soluble antioxidant metabolites into the medium, including low-molecular-weight phenolic compounds, melanogenesis intermediates such as L-DOPA, and other redox-active metabolites. Consequently, cell-free supernatants often exhibit higher antioxidant activity than melanin purified by acid-base extraction, which tends to lose soluble antioxidant components and undergo structural aggregation [3][16][17]. On the other hand, microbes produce volatile organic compounds (VOCs) as part of their secondary metabolism, which is integrated with oxidative stress responses and environmental adaptation. Bacterial VOCs include alcohols, aldehydes, terpenoids, and aromatic compounds that can act as signaling molecules, membrane protectants, or contributors to the antioxidant activity of culture systems [18]–[20]. We hypothesize that bacteria inhabiting the metal-rich, oxidative volcanic soil of Anak Krakatau employ melanogenesis as a primary defense mechanism, generating both soluble antioxidant metabolites and strain-specific volatile compounds as coordinated adaptive responses.

Based on this background, this study aims to isolate and select melanin-producing bacteria from the soil of Mount Anak Krakatau, evaluate tyrosinase activity, L-DOPA dynamics, and the antioxidant activity of cell-free supernatants during melanin production, and characterize the volatile compound profile. This study is the first to report on melanin-producing bacteria and their antioxidant activity in the soil of Mount Anak Krakatau, providing preliminary insights into the relationship between melanogenesis, antioxidant metabolites, and VOC profiles in bacteria from an extreme volcanic environment.

2. MATERIALS AND METHODS

2.1. Materials

Soil samples from Mount Anak Krakatau (6°

5'33.13 "S 105°25'28.32" E), L-tyrosine (Merck, USA), beef extract (Merck, USA), peptone (Merck, USA), $CuSO_4 \cdot 5H_2O$ (Merck, USA), nutrient broth (HiMedia, IND), acetate buffer, 2,4,6-tris(2-pyridyl) -1,3,5-triazine (TPTZ, Sigma Aldrich, USA), HCl (Merck, USA), $FeCl_3$ (Merck, USA), synthetic L-DOPA (Chem Cruz Biotechnology, Inc, USA), sulfuric acid (Merck, USA), sodium phosphate (Merck, USA), and ammonium molybdate (Merck, USA).

2.2. Methods

2.2.1. Soil Sample Collection

Soil samples were collected from the volcanic deposits of Anak Krakatau Volcano (Sunda Strait, Lampung Province, Indonesia) at various points surrounding recent eruption sites (range at 6° 5'33.13"S 105°25'28.32" E), as shown in Figure 1**.** Samples were taken at a depth of 30 cm from the soil surface and subsequently transferred to the Microbiology Laboratory at the Sumatra Institute of Technology for further processing.

2.2.2. Isolation of Melanin-producing Bacteria

Melanin-producing bacteria were isolated using 10 g of enriched soil in 90 mL of nutrient broth medium supplemented with 0.5% L-tyrosine. The mixture was incubated for 24 h at 30 °C with shaking at 120 rpm. The resulting suspension was diluted from 10^{-1} to 10^{-5}. Aseptically, 0.1 mL of each dilution was transferred to sterile Petri dishes containing modified tyrosine agar medium (composed of 0.5% L-tyrosine, 0.3% beef extract, 0.5% peptone, 2% bacteriological agar, and 0.04% $CuSO_4 \cdot 5H_2O$) [7]. After incubation for 5 days at 30 °C, the growing isolates were purified again on tyrosine agar medium.

2.2.3. Screening Bacteria in the Production of Melanin

Bacterial isolates were taken from 1 mL and inoculated into the Nutrient broth medium as a starter culture. One percent of the starter culture was then inoculated into a modified tyrosine broth production medium, which contained 0.5% L-tyrosine, 0.3% beef extract, 0.5% peptone, and 0.04% $CuSO_4 \cdot 5H_2O$ [7]. The isolates were incubated at 30 °C until they produced a black pigment in the medium. A dark brown color indicated that the isolate had produced melanin pigment [21]. The bacterial isolates that have been successfully isolated are designated with the code AK for Anak Krakatau.

2.2.4. Identification of Selected Isolate based on 16S rRNA

Bacterial genomic DNA was isolated using the

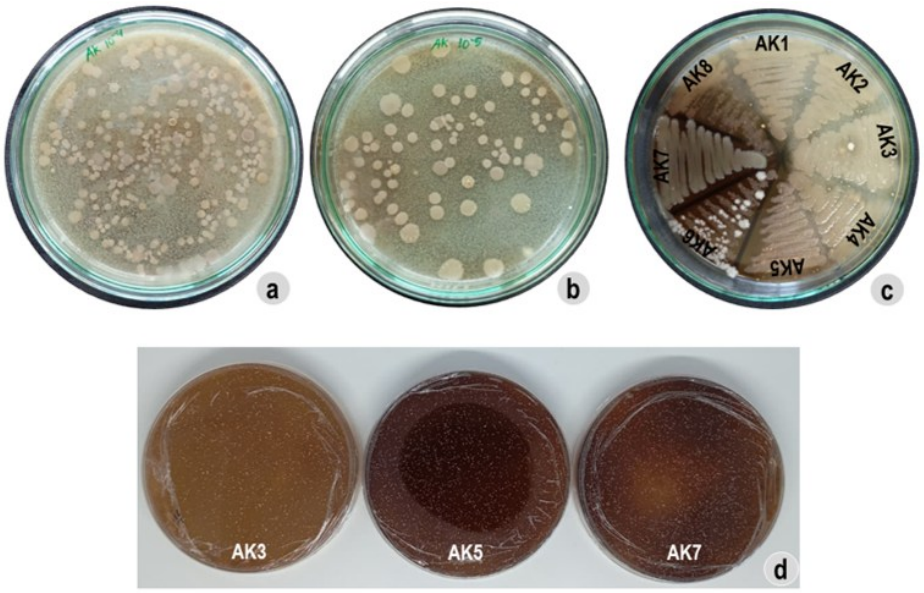

Figure 2. Bacterial colonies at the following stages: (a and b) isolation, (c) purification after 48 hours of incubation, and (d) colonies exhibiting color diffusion on tyrosine agar + $CuSO_4 \cdot 5H_2O$ medium after 120 h of incubation at 30 °C.

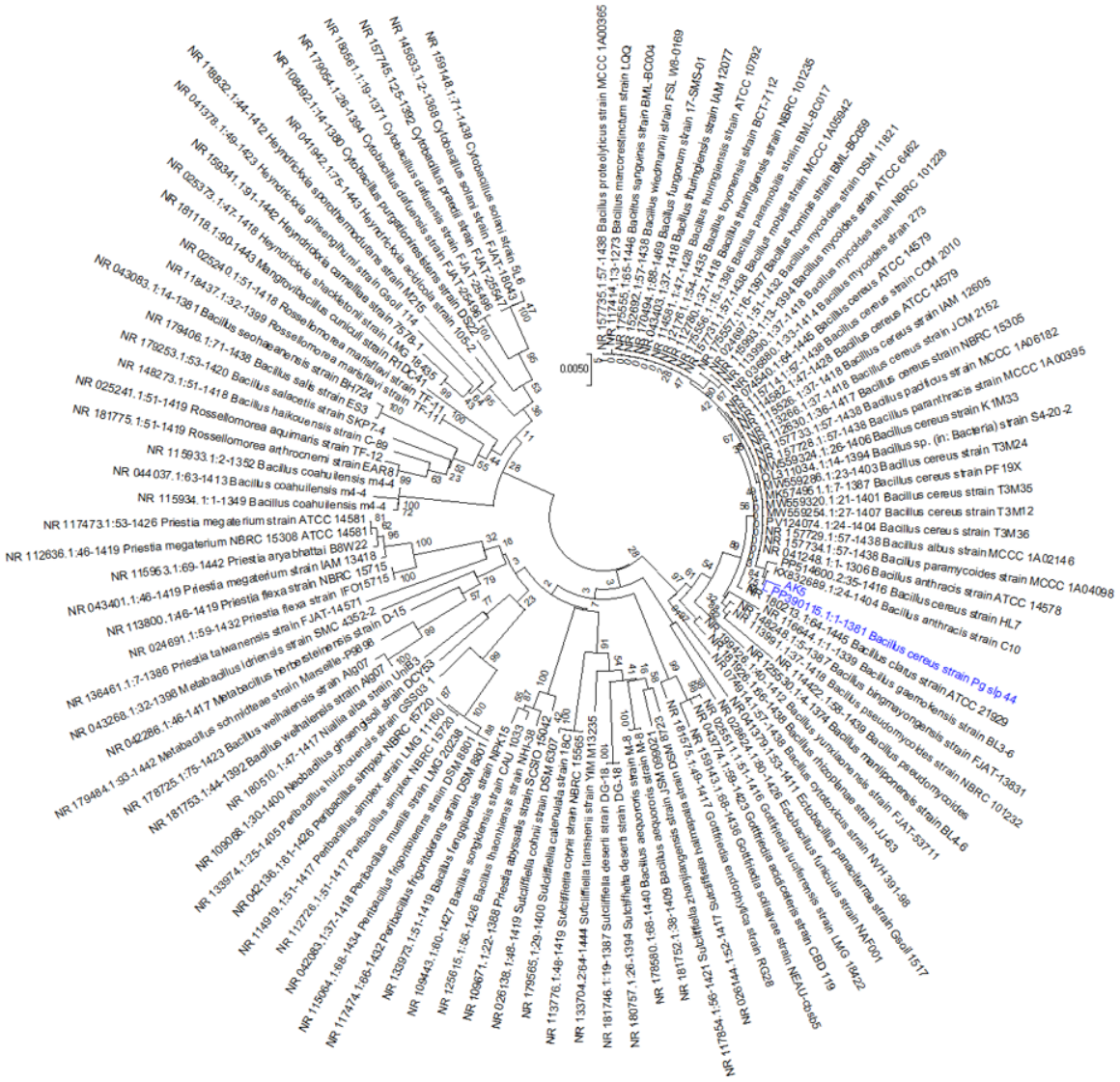

Figure 3. A phylogenetic tree of *Bacillus cereus* AK5 and related species was constructed using the neighbor-joining method with 1,000 bootstrap repetitions. The scale bar represents a genetic distance of 0.005.

Quick-DNA Magbead Plus Kit (Zymo Research, D4082) according to the manufacturer's protocol. Amplification was performed using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- TACGGTTACCTTGTTACGACT-3') (Hou et al. 2018) under the following PCR conditions: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 90 s, followed by a post-elongation step at 72 °C for 10 min. The denaturation through elongation steps was performed for 35 cycles. The composition of the PCR mix for 16S rRNA gene amplification in a 50 µL volume was 25 µL MyTaq HS Red Mix, 2X (Bioline, BIO-25048), 4 µL of primer 27F (10 pmol), 4 µL of primer 1492R (10 pmol), 4 µL of template DNA, and 13 µL of nuclease-free water. The target DNA band size is approximately 1500 bp. The amplification products were then subjected to electrophoresis and sequenced by Genetika Science Indonesia. Analysis was performed at 1st Base, Selangor, Malaysia. The obtained DNA sequencing results were then compared with data available on the National Center for Biotechnology Information (https://ncbi.nlm.niv.gov) website using the BLAST-N program. A phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis (MEGA 11) using the neighbor-

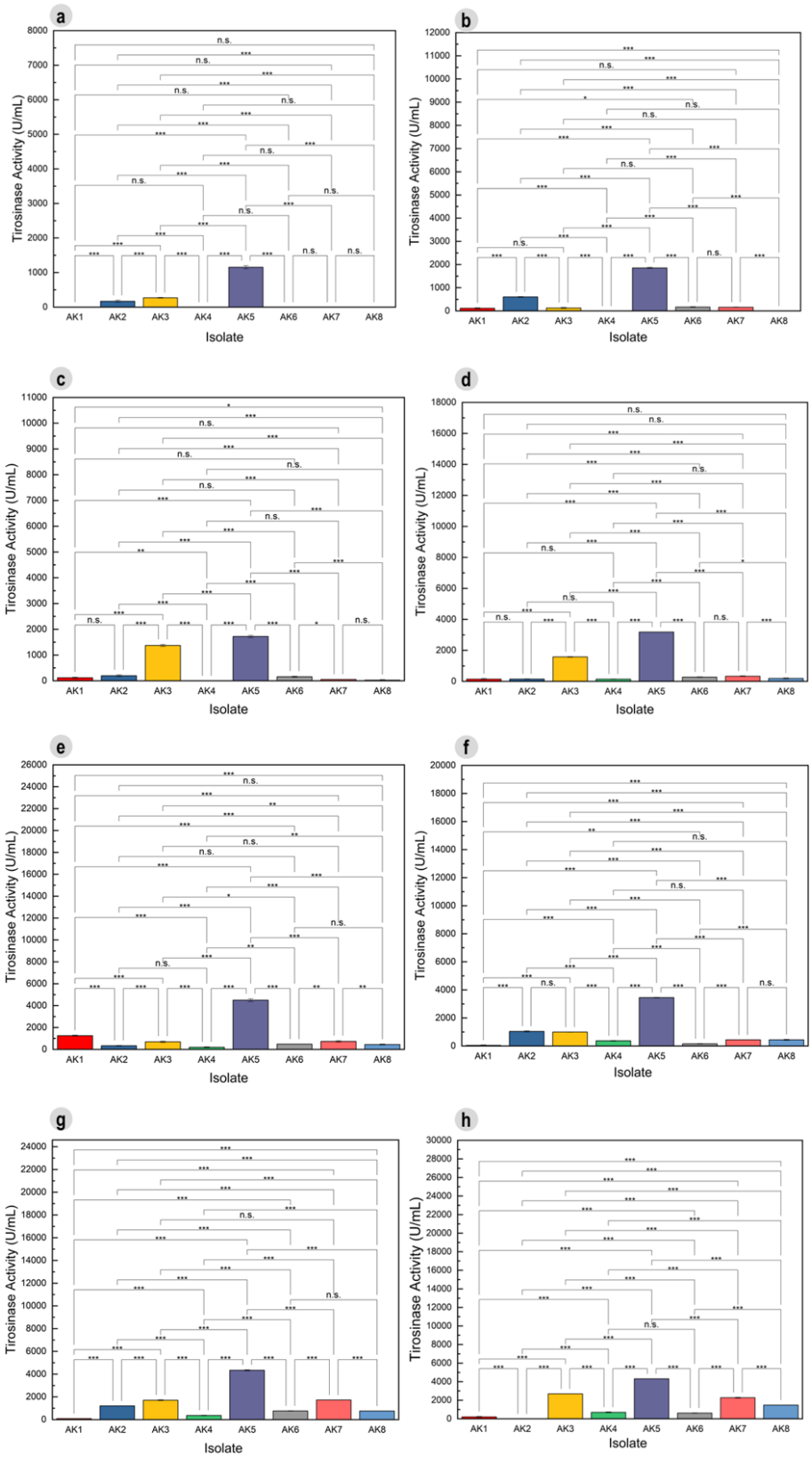

Figure 4. Tyrosinase activity during melanin production from isolates in tyrosine broth supplemented with $CuSO_4 \cdot 5H_2O$ on (a) day one, (b) day two, (c) day three, (d) day four, (e) day five, (f) day six, (g) day seven, and (h) day eight. Error bars represent the standard deviation of three replicates. Mean values with different superscripts indicate different levels of significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) based on Tukey's test.

joining method with 1000 bootstrap replicates.

2.2.5. Testing tyrosinase activity during melanin production

Bacterial isolates were grown in modified tyrosine broth medium and incubated at 30 °C for 8 days. Daily, 2 mL of the culture was harvested. The bacterial culture was centrifuged at approximately 4,000 xg for 30 min using a Thermo ScientificTM HeraeusTM MegafugeTM 8 centrifuge (Germany). The resulting cell-free supernatant was used to measure tyrosinase activity. Tyrosinase activity was assessed by reacting the supernatant with 0.001 M L-tyrosine, 0.5 M phosphate buffer (pH 6.5), and grade water. The absorbance of the reaction mixture was measured at a wavelength of 280 nm using a Thermospectronic Spectrophotometer (Genesys 150 UV-Vis, US) [22]. A reaction mixture without supernatant served as a treatment control. Tyrosinase activity was calculated using Equation (1).

$$\text{Unit of Enzyme/mL} = \frac{\text{Absorbance of sample} - \text{Absorbance of control}}{0.001 \text{ x } 0.1} \quad (1)$$

One unit (U) of tyrosinase activity is defined as the amount of enzyme causing a change in absorbance of 0.001 per minute per mL of cell-free supernatat at 280 nm under the stated assay conditions. The denominator values in the formula (0.001 × 0.1) represent the substrate concentration (0.001 M L-tyrosine) and the volume fraction of supernatant in the reaction mixture (0.1 mL in a total 1 mL reaction), respectively.

2.2.6. Estimation of L-3, 4-dihydroxyphenylalanine (L-DOPA) concentration during melanin production

The concentration of L-DOPA was measured in previously harvested cell-free supernatant. The supernatant was reacted with 0.5 M HCl and 10% nitrite-molybdate reagent, followed by the addition of 1.0 M NaOH. Sterile distilled water was then added to the reaction mixture. The absorbance of the mixture was measured at a wavelength of 530 nm using a Thermospectronic Spectrophotometer (Genesys 150 UV-Vis, US). Synthetic L-DOPA (Chem Cruz Biotechnology, Inc., US) was used as a standard to determine the concentration of L-DOPA in the cell-free supernatants [23].

2.2.7. Determination of antioxidant activity of cell-free supernatants during melanin production

2.2.7.1. Fe-Reducing Power Assay (FRAP)

The antioxidant activity of cell-free supernatants during melanin production was evaluated based on their iron-reducing power using the FRAP method [24]. The cell-free supernatant was reacted with the FRAP reagent, which consisted of 300 mM acetate buffer (pH 3.6), a 10 mM solution of 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3$ in a 10:1:1 (v/v/v) ratio. A FeSO4·7H2O solution served as the standard for calculating the amount of Fe^{2+}-TPTZ formed. Antioxidant strength was measured at a wavelength of 593 nm using a Thermospectronic Spectrophotometer (Genesys 150 UV-Vis, US). The ascorbic acid standard curve was used to quantify the antioxidant strength in reducing Fe^{3+} to Fe^{2+}, with results expressed as ascorbic acid equivalents per gram of melanin (mg AAE/mL).

2.2.7.2. Total antioxidant capacity (TAC) using phosphomolybdenum assay

The total antioxidant capacity of melanin in reducing Mo(VI) to Mo(V) was assessed by reacting the cell-free supernatant with phosphomolybdenum reagent, which contained 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Ascorbic acid was used to create a calibration standard curve. The absorbance of the solution was measured at a wavelength of 695 nm using a Thermospectronic Spectrophotometer (Genesys 150 UV-Vis, US) (Prieto et al 1999) [25]. Total antioxidant capacity was expressed in terms of ascorbic acid equivalents per mL of cell-free supernatant (mg AAE/mL).

2.2.8. Determination of compounds in cell-free supernatants of isolates using Gas Chromatography -Mass Spectrometry (GC-MS) analysis

Volatile and semi-volatile compounds from the extracts were analyzed using an Agilent 7890B gas chromatograph coupled with an Agilent 5977A mass selective detector (MSD, Agilent Technologies, Inc., Santa Clara, CA, USA). Compound separation was achieved on an HP-5ms Ultra Inert capillary column (30 m × 250 µm × 0.25 µm). Helium served as the carrier gas at a constant

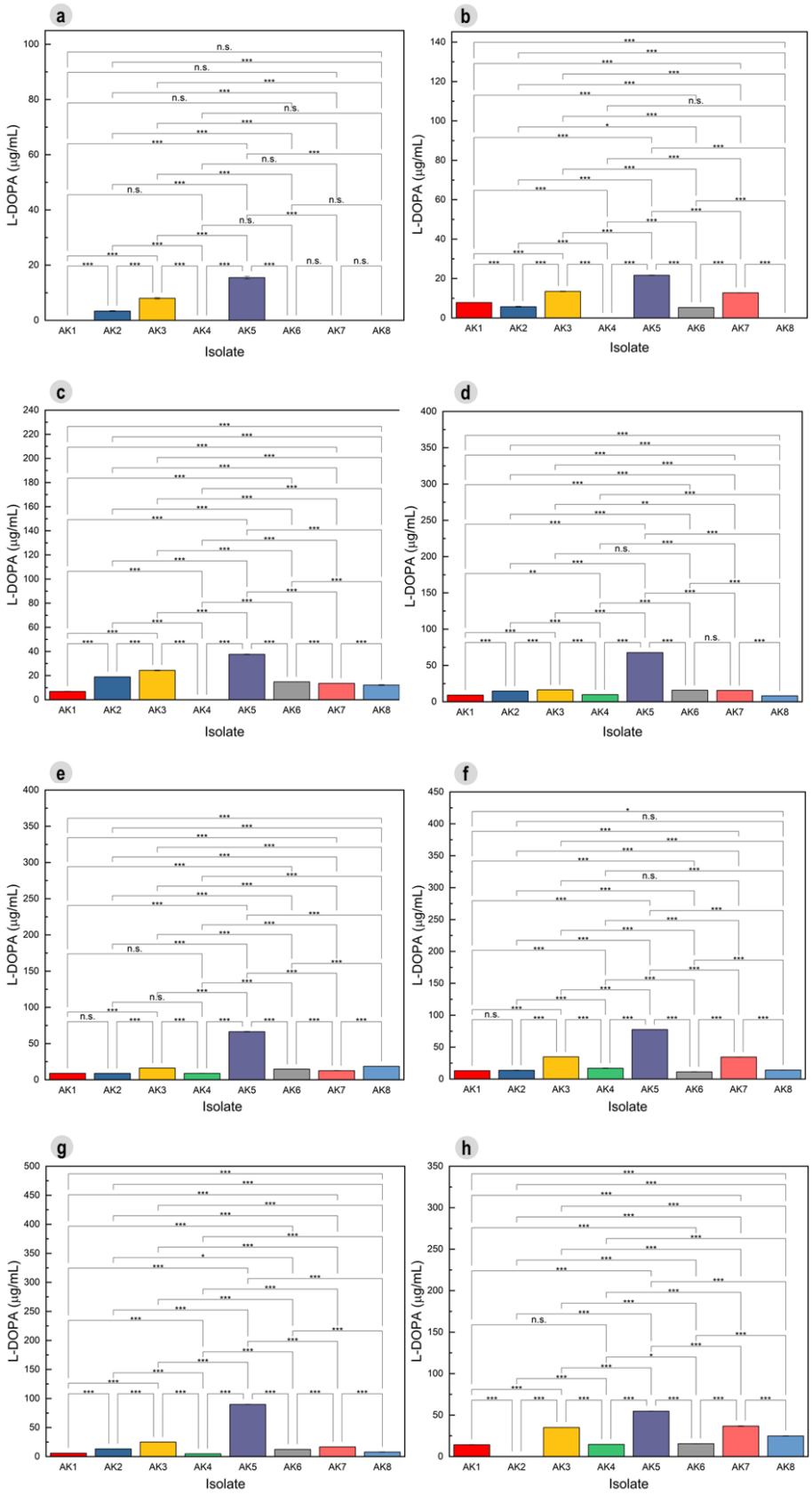

Figure 5. L-DOPA concentration during melanin production from isolates in tyrosine broth medium supplemented with $CuSO_4 \cdot 5H_2O$ on (a) day one, (b) day two, (c) day three, (d) day four, (e) day five, (f) day six, (g) day seven, and (h) day eight. Error bars represent the standard deviation of three replicates. Mean values with different superscripts indicate varying levels of significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) based on Tukey's test.

flow rate of 1 mL/min. Samples were weighed and dissolved in 2 mL of HPLC-grade ethanol, then filtered through a 0.45 µm PTFE microfilter. A total of 1 µL of the prepared sample was injected into the GC inlet at 240 °C in splitless mode. The oven temperature program began at an initial temperature of 40 °C, held for 3 minutes, then increased to 280 °C at a constant rate of 10 °C/min and was maintained for an additional 2 min. The MS operating conditions included an electron ionization energy of 70 eV, a scan range of 30–550 µm, a scan rate of 13.8 spectra/s, and a solvent delay of 3 min. The ionization chamber and transfer line temperatures were set at 200 °C and 250 °C, respectively.

Data processing was conducted using MassHunter Workstation software. Compound identification was based on mass spectrum fragmentation patterns compared to the National Institute of Standards and Technology (NIST) spectral database. Confirmation of identity involved comparing the experimental Kovats Index (KI), calculated from the retention time of the n-alkane standard series (C7–C30) using a linear regression equation, with the literature KI values. The relative quantity of each compound was expressed as a percentage of the area. Prior to sample injection, a solvent blank (HPLC-grade ethanol processed under identical instrument conditions) was analyzed; compounds detected in the blank were excluded from the reported volatile profiles as a standard quality control criterion. It is acknowledged that ethanol extraction preferentially recovers semi-volatile and solvent-soluble compounds; headspace-based techniques such as SPME-GC-MS are recommended for future studies to obtain a more representative volatile profile. An uninoculated medium blank was not analyzed in this study; therefore, some detected compounds (particularly common alkanes and simple aldehydes) may partially originate from medium components rather than bacterial metabolism, and should be interpreted with appropriate caution.

2.3. Data Analysis

Data are presented as mean ± standard deviation from three independent biological replicates. Statistical analysis was conducted to assess the significance between isolates and incubation days for each measurement parameter, utilizing one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Mean values with different superscripts indicate significant differences (*p<0.05; **p<0.01; ***p<0.001) based on the Tukey test. Graphical and statistical analyses were performed using OriginPro 2025b Learning Edition software (Northampton, USA).

3. RESULTS AND DISCUSSIONS

3.1. Isolation and Screening of Melanin-producing Bacteria

Several attempts have been made to obtain microbial strains suitable for melanin production

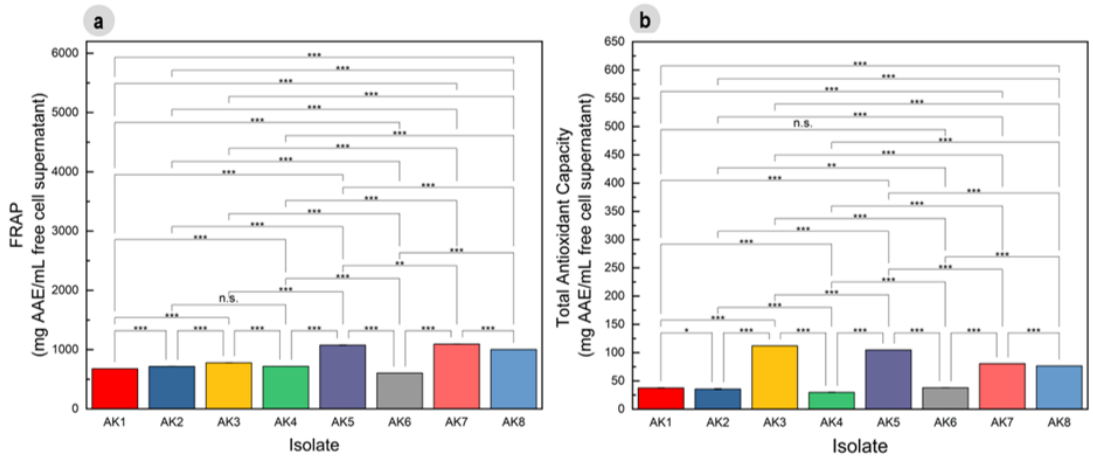

Figure 6. Antioxidant activity of isolates based on (a) Fe^{3+} reduction capacity (FRAP) and (b) Mo(VI) reduction. Mean values with different superscripts indicate varying levels of significance (*p<0.05; **p<0.01; ***p<0.001), as determined by Tukey's test.

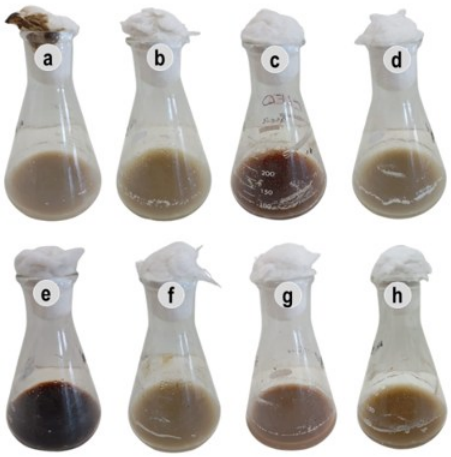

Figure 7. Color of melanin production culture medium isolates: (a) AK1, (b) AK2, (c) AK3, (d) AK4, (e) AK5, (f) AK6, (g) AK7, (h) AK8 in tyrosine broth medium supplemented with $CuSO_4 \cdot 5H_2O$ after 8 days of incubation.

with potential biological activity. The origin and type of microbe significantly influence the biological activity and type of microbial melanin [2]. This study focused exclusively on isolating melanogenic bacteria from the soil of Mount Anak Krakatau. Eight soil isolates from the Anak Krakatau volcano successfully grew on tyrosine agar medium supplemented with $CuSO_4 \cdot 5H_2O$ (Figure 2(b)). These isolates demonstrated the ability to utilize tyrosine as a precursor for melanin formation and $CuSO_4 \cdot 5H_2O$ as a cofactor for the enzyme tyrosinase (Figure 2(a)) [7]. Copper serves as a cofactor that plays a crucial role in essential biological processes, including respiration, defense against oxidative stress, iron homeostasis, and pigment formation (melanization) [26]. The soil isolates from Mount Anak Krakatau exhibited varying colors, predominantly white and brownish-yellow. Three of the eight isolates showed brown halos on tyrosine agar (Figure 2(d)). The formation of colonies or blackish-brown diffusion on the medium is intrinsically linked to melanin synthesis, which is mediated by tyrosinase activity that oxidizes L-tyrosine in the medium [21].

The presence of copper sulfate in the medium influences the microbial community. Microbes that thrive in environments with high metal concentrations, such as copper sulfate, are those that exhibit tolerance to these conditions [27], including melanin-producing species. Several microbes can produce melanin when $CuSO_4$ is added to the growth medium, including *Streptomyces nashvillensis* [28], *S. kathirae* [8], S. *glaucescens* NEAE-H [9], *S. roseochromogenes* ATCC 13400 [12], *Bacillus megaterium* M36 [29], and *Bacillus licheniformis* [10]. The concentration of $CuSO_4$ in the medium is tailored to the geographical conditions of Mount Anak Krakatau. This volcano emerged after the 1883 eruption and is regarded as one of the fastest-growing volcanoes in Indonesia [15]. Anak Krakatau erupted in 2018, resulting in a significant deposition of new volcanic material on the island and its surroundings. The soil derived from this volcanic material contains high levels of SiO_2, Fe_2O_3, and Cu [14]. These conditions indicate that the soil and habitat are under metal stress, which supports the presence of melanin-producing bacteria that accumulate Cu as a cofactor for melanin formation. These conditions suggest, though do not confirm, that the isolates may possess copper-tolerance mechanisms consistent with the metal-rich geochemical environment of Anak Krakatau volcanic soil. Metal tolerance assays and quantitative soil metal analysis are recommended to validate this hypothesis. To the best of our knowledge, this finding represents the first report of melanogenic bacteria from the soil of Mount Anak Krakatau. Among the three melanogenic isolates, based on 16S rRNA gene analysis, isolate AK5 showed 99.20% similarity to *Bacillus cereus* 2490 (E-value 0.00, query coverarge 100%) (Figure 3). The genus *Bacillus* has been widely reported as a producer of melanin, including *Bacillus cereus* [30], *B. subtilis* 4NP-BL

[11], the thermophilic *B. haynesii* [31], and *B. thuringiensis* [32]. The remaining isolates (AK3 and AK7) were not molecularly identified in this study, a limitation acknowledged and to be addressed in future work. In summary, the selective use of $CuSO_4$-supplemented tyrosine agar successfully yielded three melanogenic isolates from Anak Krakatau volcanic soil, with AK5 (identified as B. cereus) emerging as the lead isolate for further characterization.

3.2. Tyrosinase Activity of Isolates during Melanin Production

Screening bacterial isolates is essential for identifying potential melanin-producing strains. Several parameters are necessary for this selection, including tyrosinase activity, L-DOPA concentration, yield, and the time required for melanin production [6]. The tyrosinase activity of the isolates varied with the incubation period (Figure 4). Among the eight isolates tested, isolate AK5 exhibited the highest tyrosinase activity on day 5, with a measurement of 4,500 ± 135.27 U/mL. This was followed by isolates AK7 and AK3, which showed activities of 730 ± 72.11 U/mL and 693.3 ± 66.58 U/mL, respectively.

Based on tyrosinase activity, isolates AK5, AK3, and AK7 possess sufficient levels of the tyrosinase enzyme to function optimally on days 5 and 8 during the biotransformation of L-tyrosine to L-DOPA. The tyrosinase activity of AK5 is higher than that of *S. lasalocidi* NTB 42 (3,530 U/mL) [21] but lower than that reported for *S. glaucescens* NEAH-H (6,089.10 U/mL) [9]. The observed increase in tyrosinase activity during the late incubation phase may be attributed to cell lysis, the release of intracellular enzymes, or active secretion as a late-phase metabolic response; however, in the absence of growth curve data and total extracellular protein quantification, this interpretation remains speculative. Overall, these inter-isolate comparisons at the same time points indicate that AK5 (*B. cereus*) exhibits the highest tyrosinase-mediated biotransformation capacity, with strain-specific differences in peak activity timing (day 5 vs. day 8) suggesting distinct melanogenesis kinetics [28].

3.3. L-DOPA Concentration of Isolates during Melanin Production

The tyrosinase enzyme is involved in the formation of L-DOPA, and its activity fluctuates with tyrosinase levels. The concentration of L-DOPA produced by the isolates ranged from 3.37 to 89.82 µg/mL. The highest concentration was observed in isolate AK5 on days 5 and 7, measuring 89.82 ± 0.13 µg/mL and 77.44 ± 0.15 µg/mL, respectively, and this was significantly different ($p<0.05$) from the other isolates. Isolates AK7 and AK3 recorded the second- and third-highest L-DOPA concentrations at 35.03 ± 0.18 µg/mL and 36.73 ± 0.33 µg/mL, respectively (Figure 5). Overall, the dynamic L-DOPA profiles confirm that

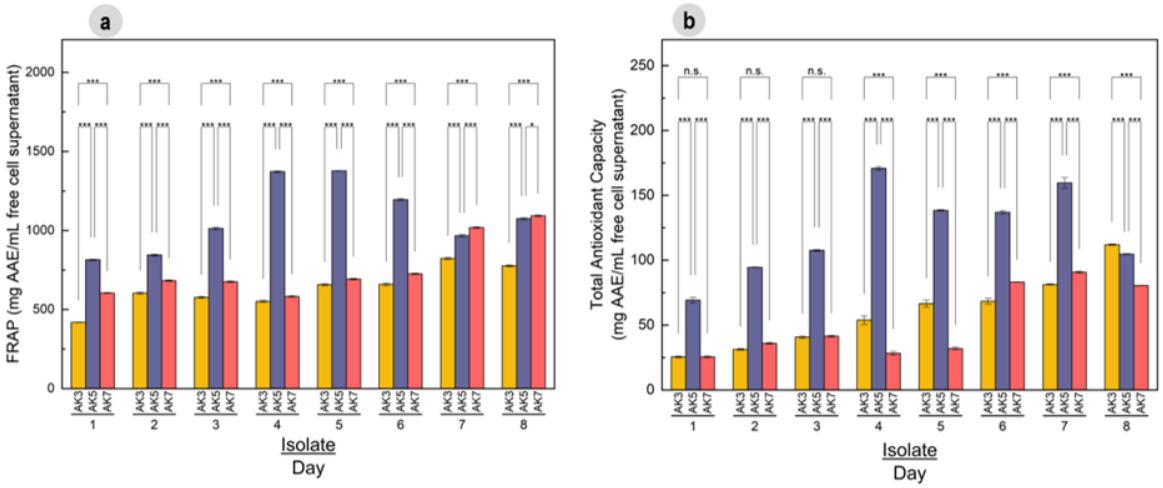

Figure 8. Antioxidant activity of three melanogenic isolates based on (a) Fe^{3+} reduction capacity (FRAP) and (b) Mo(VI) reduction. Mean values with different superscripts indicate varying levels of significance (*p < 0.05; **p < 0.01; ***p < 0.001) based on Tukey's test.

Table 1. Composition of volatile compounds in the cell-free supernatant of AK3 isolate.

No.	RT (min)	Compound	KI Experimental	KI Literature	Area %
1	3.14	(*Z*)-3-Hexenol	861	861	1.49
2	4.27	*n*-Heptanal	899	899	79.83
3	4.95	Tricyclene	930	930	9.92
4	5.91	*n*-Heptanol	966	970	-0.38
5	7.90	*p*-Cymene	1028	1028	3.60
6	8.92	(*E*)-β-Ocimene	1057	1052	3.96
7	10.30	*p*-Mentha-2,4(8)-diene	1091	1090	1.57

isolate AK5 supports the most efficient tyrosinase-driven L-tyrosine biotransformation, with peak accumulation on days 5 and 7, consistent with its elevated tyrosinase activity.

3.4. Antioxidant Activity of Isolates during the Melanin Production Process

The antioxidant potential of cell-free supernatants was evaluated to capture the extracellular redox environment formed during melanogenesis, rather than the activity of purified melanin alone. The antioxidant strength of cell-free supernatants was assessed using the FRAP method, which measures the reduction of Fe(III) ions to Fe (II) or the conversion of ferric tripyridyl triazine (Fe^{3+}-TPTZ) complexes to ferrous tripyridyl triazine (Fe^{2+}-TPTZ) [33]. Antioxidant activity was observed in all cell-free supernatants on day 8 (the final day of production), with comparisons made between isolates at this same time point. The results indicated that all isolates exhibited measurable reducing activity at the final stage of production (day 8), indicating the accumulation of redox-active metabolites in the culture medium [3]. Among the isolates, AK5 demonstrated the highest Fe^{3+} reduction activity (1075.48 ± 5.77 mg AAE/mL in the cell-free supernatant), which was significantly different from that of the other isolates ($p < 0.05$, see Figure 6(a)), whereas AK6 showed the lowest activity (602 ± 3.57 mg AAE/mL). A similar pattern was observed for total antioxidant capacity (TAC), where AK3, AK5, and AK7 exhibited the highest activities on day 8 (Figure 6(b)). To contextualize these values, it is important to emphasize that antioxidant capacities reported in the literature are often derived from purified compounds or standardized extracts, whereas the present study evaluates cell-free supernatants representing a complex extracellular metabolite system [34]. In addition, the melanogenesis pathway itself contributes to antioxidant potential through the continuous formation of intermediate compounds with inherent redox activity [35] Therefore, previously reported values, such as those for *Ascosphaera apis* [36] or melanin from *Innonotus obliquens* [16], are not directly comparable on a quantitative basis due to differences in sample composition, normalization approaches, and assay conditions.

However, these results are closely associated with the melanogenesis process, which inherently generates redox-active intermediates and antioxidant metabolites in the extracellular environment. At the final stage of melanin production, only three isolates (AK3, AK5, and AK7) exhibited a distinct color transition to blackish brown (Figure 7), indicating active melanogenic pathways. Such color progression—from light yellow-orange to dark brown—has been widely recognized as a phenotypic indicator of melanin biosynthesis and extracellular pigment accumulation [28]. Temporal monitoring revealed that antioxidant activity dynamically evolved throughout the production process, with significant differences observed across incubation time points ($p < 0.05$). Notably, isolated AK5 reached its peak Fe^{3+}-reducing capacity on day 5 (1,377.86 ± 1.42 mg AAE/mL, see Figure 8), preceding the final accumulation phase of visible pigment. Similarly, the highest TAC was observed on day 7, suggesting that maximal redox activity occurs during intermediate stages of melanogenesis rather than at its completion.

This pattern supports the concept that antioxidant capacity is strongly linked to the accumulation of soluble redox-active intermediates, such as L-DOPA, dopaquinone, and related phenolic derivatives, which are transiently enriched prior to polymerization into mature melanin [37]–[39]. Multiple types of antioxidant activity measurements were conducted to capture various mechanisms of action, as a single test does not encompass all radical sources or antioxidants in a mixed or complex system, leading to varying contributions to antioxidant activity [40].

The FRAP and TAC values of cell-free supernatants are significantly higher than those reported for purified melanin. Cell-free supernatants are rich in soluble antioxidant metabolites, including pigments, phenolics, alkaloids, polysaccharides, and other redox-active molecules [17]. This study demonstrates the presence of various antioxidant metabolites, as measured by in vitro assays, in these supernatants. The supernatants contain a mixture of low molecular weight phenolic compounds, intermediate metabolites (e.g., L-DOPA, dopaquinone), and other redox-active compounds released during the melanogenesis process, not just melanin polymers [3]. Collectively, these compounds contribute to higher FRAP and TAC values than pure melanin alone.

Melanin produced in culture broth exhibits spectral characteristics that indicate the presence of phenolic residues and other molecular contaminants that interact electronically before purification. In contrast, melanin purified through acid-base precipitation results in the loss of these soluble antioxidants, undergoes structural cross-linking, and forms larger aggregates with diminished electron donor capacity. This suggests that purification processes, such as alkali extraction and acid precipitation, can alter the chemical structure and molecular composition of the initial sample, thereby reducing its electron donation capacity [28][41]. Consequently, the high antioxidant activity observed in the supernatant reflects the synergistic action of pre-polymerized melanin (precursor) and other soluble active redox compounds, rather than solely purified melanin polymers.

Taken together, these findings demonstrate that antioxidant activity during melanin production is a process-dependent phenomenon governed by the dynamic interplay between precursor formation, intermediate transformation, and polymerization. This shifts the focus from melanin as a static end-product to melanogenesis as an active extracellular redox-generating process. Several methodological limitations of this study must be acknowledged: (1) melanin yield was not quantified, preventing direct correlation between pigment concentration and antioxidant capacity; (2) antioxidant activity of uninoculated culture medium was not measured, so a minor contribution from medium components (beef extract, peptone) cannot be excluded; (3) antioxidant data were not normalized per biomass or total protein content, limiting inter-study comparability; and (4) only the most active isolate (AK5) was molecularly identified. These parameters are recommended as priorities for future studies. In summary, among the three melanogenic isolates, AK5 consistently demonstrated the highest antioxidant capacity across both FRAP and TAC assays, with peak activity temporally linked to the active melanogenesis phase.

3.5. Volatile Compound Composition in Samples

The three isolates capable of producing color

Table 2. Composition of volatile compounds in the cell-free supernatant of AK5 isolate.

No.	RT (min)	Compound	KI Experimental	KI Literature	Area %
1	3.20	(*Z*)-3-Hexenol	864	861	0.83
2	4.34	Nonane	901	900	85.29
3	4.94	Artemisia triene	930	928	5.09
4	10.31	*p*-Mentha-2,4(8)-diene	1091	1090	1.21
5	18.32	*p*-Menth-1-en-7-al	1275	1274	3.91
6	20.48	2*E*,4*E*-Decadienal	1321	1314	2.80
7	22.46	Neryl acetate	1366	1365	0.88

Table 3. Composition of volatile compounds in the cell-free supernatant of AK7 isolate.

No.	RT (min)	Compound	KI Experimental	KI Literature	Area %
1	3.21	(*Z*)-3-Hexenol	864	861	0.43
2	4.28	*n*-Heptanal	899	899	4.33
3	4.36	Nonane	903	900	71.34
4	7.86	*p*-Cymene	1027	1025	2.40
5	8.92	Eucalyptol (1,8-cineole)	-	-	3.96
6	14.93	Methylchavicol	1198	1196	0.97
7	15.68	trans-Carveol	1215	1217	1.79
8	17.82	(*E*)-2-Decenal	1264	1263	1.52
9	18.31	*p*-Menth-1-en-7-al	1274	1274	1.66
10	20.48	2*E*,4*E*-Decadienal	1321	1314	2.41
11	20.99	d-Elemene	1333	1339	7.03
12	22.46	Neryl acetate	1366	1365	0.97
13	22.62	Cyclosativene	1370	1369	3.78

changes (melanogenicity) and exhibiting the best antioxidant activity display varying volatile compound compositions. The cell-free supernatant from these isolates contains a mixture of oxygenated monoterpenes, such as eucalyptol and trans-carveol, as well as monoterpene hydrocarbons like p-cymene and beta-ocimene (Table 1). Eucalyptol (1,8-cineole) and trans-carveol, which were detected in the cell-free supernatant, are commonly found in plant volatile profiles and are associated with antioxidant and antimicrobial activities. While comparisons with plant essential oil profiles are informative for structural analogy, it should be noted that plant and bacterial terpenoid biosynthesis differ significantly in pathway regulation and enzyme machinery. These comparisons should therefore be interpreted cautiously and are not intended to imply mechanistic equivalence. Additionally, *p*-cymene, limonene, and eucalyptol are dominant components in several GC-MS analyses of aromatic plants [42].

The presence of (*Z*)-3-hexenol (leaf alcohol) in all samples highlights the characteristic "green" or fresh aroma commonly associated with plant extracts. While this compound is well-known in plant aroma studies, it can also be produced by microbes through alternative metabolic pathways as a byproduct of fatty acid metabolism or lipid peroxidation, serving as a chemical signal. The detection of such volatile alcohols in bacterial cultures indicates that in vitro microbes produce a diverse array of volatile molecules, not just melanin pigments as reported in plant growth-promoting rhizobacteria, including *Pseudomonas koreensis*, *Pseudomonas fluorescens*, *Lysinibacillus sphaericus*, and *Paenibacillus alvei* [20].

Isolate AK5, which showed a significant abundance of the main component at a retention time of 4.34 minutes, was identified as nonane, exhibiting a high dominance of 85.29% (Table 2). Short-chain alkanes, such as nonane, are commonly detected in microbial VOC profiles and environmental extracts. Although they do not consistently demonstrate direct biological activity, such as antioxidant properties, the prevalence of these alkanes may reflect common metabolic patterns in cell membranes and secondary metabolite pathways of bacteria. The nonane group is predominant in many biologically active natural products due to its exceptional characteristics compared to other groups, making it useful in asymmetric catalysis and as a potential anticancer compound [43]. Additionally, another study identified volatile alkanes as metabolite products in various bacterial isolates analyzed by GC-MS, although their role in the context of antioxidants remains poorly understood [44].

The AK7 isolate profile is the most complex, revealing aromatic components such as methylchavicol (estragole) and several

sesquiterpenes, including d-elemene and cyclosativene (Table 3). Methylchavicol is a phenylpropanoid recognized as a bioactive component in the essential oils of medicinal and food plants [45]. The presence of these phenylpropanoid compounds in the volatile profile of bacteria suggests that microbes can produce or modify aromatic metabolic pathways similar to those of plants, potentially contributing to antioxidant activity and chemical communication signals in their environment [46]. Metabolomic studies on bacteria further indicate that bacterial VOCs vary significantly between strains, encompassing alcohols, aldehydes, ketones, acids, amines, and terpenoids, all of which may serve as bioactive candidates or biomarkers for biotechnology applications [20]. Additionally, siloxane compounds (such as cyclotrisiloxane and cyclopentasiloxane) are considered background noise from the stationary phase of the column (column bleed) or the injector septum, and do not originate from the extract sample without a sterile medium extract blank, we cannot exclude the possibility that some detected compounds originate from medium components or environmental contamination. These compounds should therefore be interpreted with caution, and their biological origin requires confirmation in future studies using headspace-SPME with appropriate controls.

Isolate AK3 is predominantly composed of aliphatic aldehydes, particularly *n*-heptanal, which constitutes nearly 80% of its total composition. Additionally, it contains moderate amounts of monoterpene hydrocarbons, such as tricyclene and ocimene (~19%), and lacks significant quantities of alkane or sesquiterpene fractions. This profile differs markedly from the other two samples. In contrast, Isolate AK5 is characterized by very high levels of alkanes, mainly nonane, which account for over 85% of its composition, along with a relatively low content of terpenoid compounds (monoterpenes) at less than 12% of the total. This profile reflects a dominant saturated hydrocarbon fraction (Figure 9). Isolate AK7 also exhibits a dominance of alkanes (10.8%), as well as trace amounts of aldehydes, monoterpenes, and other compounds, including methylchavicol.

The production of VOCs in the cell-free supernatants of melanogenic isolates is consistent with the hypothesis that bacterial melanogenesis and secondary metabolism are interconnected: tyrosinase-mediated oxidation of L-tyrosine generates intracellular oxidative stress, which may activate secondary metabolic pathways including isoprenoid (MEP/DOXP), fatty acid oxidation, and aromatic amino acid pathways producing strain-specific VOCs as adaptive byproducts. This oxidative stress correlates with secondary metabolism and affects the VOC production axis, a working hypothesis that requires validation through genomic, transcriptomic, or enzymatic approaches. The activity of tyrosinase, which oxidizes L-

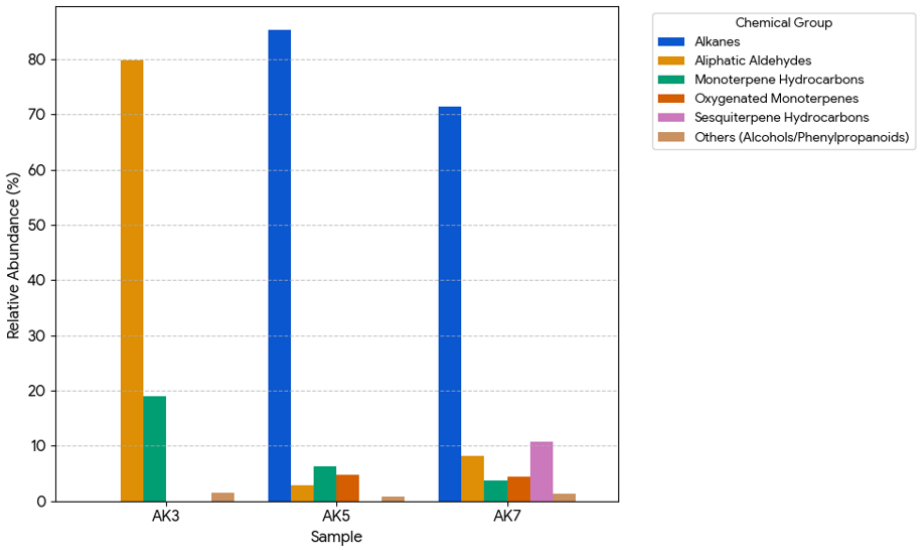

Figure 9. Chemical composition profile of the cell-free supernatant isolates.

tyrosine to L-DOPA and dopaquinone, generates intracellular redox stress. This stress drives the activation of secondary metabolic pathways as a compensatory mechanism to maintain cellular homeostasis. In this context, VOC release serves both as a metabolic byproduct and as a functional molecule that aids in the physiological adaptation of cells to their environment and provides protection against oxidative stress [18][19][47].

We hypothesize that the detected oxygenated and hydrocarbon monoterpenes (e.g., p-cymene, β-ocimene, eucalyptol, and trans-carveol) may be derived from the non-mevalonate isoprenoid pathway (MEP/DOXP), which is known to be responsive to oxidative stress and membrane dynamics. These compounds are reported to contribute to membrane stabilization and ROS protection, potentially acting synergistically with melanin; however, this pathway assignment is based on chemical structural analogy and requires genomic or enzymatic validation [48], [49]. The detection of (*Z*)-3-hexenol in all isolates suggests the activation of a lipid-based VOC pathway associated with the oxidation or peroxidation of unsaturated fatty acids. This compound reflects redox imbalance and has been identified as a physiological indicator of oxidative stress in biological systems. The dominance of nonane in isolate AK5 suggests a relatively simple volatile metabolic profile, likely resulting from the degradation of membrane lipids. This reflects an energy allocation strategy that prioritizes melanin biosynthesis and non-volatile antioxidants [50], [51].

Conversely, the presence of phenylpropanoid and sesquiterpene aromatic compounds in the AK7 isolate suggests a more complex diversification of secondary metabolism that intersects with the metabolism of aromatic amino acids, specifically phenylalanine and tyrosine. This pathway is also directly related to melanogenesis. The shikimate pathway and its aromatic derivatives are known to play a significant role in the production of volatile secondary metabolites in microorganisms; this pathway assignment for AK7 is proposed as a working hypothesis requiring genomic validation [52]–[54]. Overall, the observed VOC profiles are consistent with the working hypothesis that VOC production may serve as an adaptive metabolic response coordinated with melanogenesis in extreme volcanic environments. The relationship between VOC profiles and antioxidant activity described herein is correlative and descriptive, not causal. Experimental validation, for example, through VOC-fraction bioassays or antioxidant activity assays of isolated volatile fractions, is required to substantiate any functional claim. Ethanol extraction preferentially recovers semi-volatile compounds, and an uninoculated medium blank was not analyzed; some detected compounds may partially originate from medium components. Among the three melanogenic isolates, AK7 exhibited the most complex VOC profile, encompassing phenylpropanoids (methylchavicol), sesquiterpenes (d-elemene, cyclosativene), aldehydes, and monoterpenes, suggesting the most diversified secondary metabolic activity. The isolate -specific volatile fingerprints observed here provide a descriptive foundation for future mechanistic studies.

4. CONCLUSIONS

This study provides preliminary evidence that the volcanic soil of Anak Krakatau harbors melanogenic bacteria that produce measurable antioxidant metabolites and exhibit isolate-specific volatile profiles. Among eight isolates screened, AK3, AK5, and AK7 exhibited distinct melanogenic traits, as evidenced by tyrosinase activity, L-DOPA dynamics, and visible pigmentation during cultivation. Isolate AK5, identified as *Bacillus cereus* AK5 through 16S rRNA gene sequencing, demonstrated the highest tyrosinase activity (4,500 ± 135.27 U/mL on day 5) and the strongest antioxidant performance across both FRAP and TAC assays among the melanogenic isolates. GC–MS analysis revealed distinct, isolate-specific VOC profiles; the mechanistic link between these profiles, oxidative stress, and melanogenesis represents a working hypothesis requiring further experimental validation. To the best of our knowledge, this work represents the first report of melanin-producing, antioxidant bacteria isolated from the soil of Anak Krakatau Volcano, establishing this extreme environment as a promising source for the discovery of melanogenic bacteria. Future studies

should focus on the molecular identification of all isolates, genomic characterization of melanin biosynthesis gene clusters, quantitative assessment of melanin yield, and evaluation of the biological activities of purified compounds, to fully realize the biotechnological potential of these melanogenic bacteria from extreme volcanic environments.

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Conflicts of Interest

The authors declare no conflict of interest.

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DECLARATION OF GENERATIVE AI

Not applicable.

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