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Optimization of *Bacillus licheniformis* MAL tyrosinase: *in vitro* anticancer activity for brown and black eumelanin

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ABSTRACT

The influence of tyrosinase in catalyzes/stimulates the eumelanin production was studied. Accordingly, bacterial sp. was isolated and identified as *Bacillus licheniformis* based on 16S **rRNA**. It could grow and gave monophenolase and diphenolase productivity in medium contained tyrosin and Cu^{2+} only. The tyrosinase enzymes were optimized by studying different environmental and nutritional factors. The maximum monophenolase and diphenolase productivity were obtained at 60 °C, pH9, Cu^{2+}(0.01g), liver extract (1 g/L) and the oxygen level fixed at 20%. Also, the mannose as a carbon source increased the monophenolase production 6.2 times. For the first time, two types of eumelanin were extracted by hydrochloric acid treatment. The black and brown eumelanin weighed (0.1 g/100 mL and 0.7 g/100 mL respectively) and characterized by using FTIR and UV/Vis spectroscopy techniques. Their morphological structure and its elemental composition were characterized by SEM and EDAX respectively. The black melanin showed promising anticancer activity towards HEPG-2 and HCT-116 cell lines with IC_{50} values (6.15, 5.54 μg) compared to Doxorubicin (4.05, 4.45 μg) respectively.

1. Introduction

Tyrosinases or polyphenol oxidase (monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1) had interesting properties for biotechnological purposes. It is known as copper-containing enzymes that are widely distributed in nature [1]. These enzymes called type 3 copper proteins which have a diamagnetic spin-coupled copper pair in the active centers and great potential for production of commercially valuable diphenols from monophenols [2, 3]. Tyrosinases are known as a bifunctional enzyme that catalyzes the o-hydroxylation of monophenols and subsequent oxidation of o-diphenols to quinones [1, 4]. Bacterial tyrosinase enzyme takes attention in several biotechnological applications due to their ability to oxidize both phenolic molecules and phenolic groups associated with protein-, i.e. the side chain of L-tyrosine.). Similarly, Kanteev et al. [5]; McMahon et al. [6] reported that *Bacillus megaterium* and *Pseudomonas putida* F6 are good tyrosinase producers. Accordingly, production and characterization of tyrosinase take attention in the last few years [7].

Tyrosinase represented a clear role in wound healing and the first defense of immune response of plants, sponges and many invertebrates [8]. Tyrosinase enzyme was applied in different pharmaceutical applications such as cosmetics and food industries as an important catalytic enzyme [9]. The wellknown function is the melanin yielded from L-tyrosine via the L-dihydroxyphenylalanine (L-dopa) [8]. Also, the bioremediation applications in contaminated soil and wastewater considered one of the most famous tyrosinase application which containing phenolic compound [10] and dyes [11, 12].

Tyrosinase considered the key enzyme responsible for melanin yielded. It contains copper and catalyzes two reactions in the melanin biosynthesis. The melanin is macromolecules which non-enzymatically produced by the polymerization of reactive quinones [1, 8]. Tyrosinases canalized two different oxygen-dependent reactions that occur subsequently: *monophenols*-hydroxylation for yielding o-diphenols (cresolase activity) and the consecutively oxidation of o-diphenols to *ortho*-quinone (catecholase activity) [13].

The elemental composition is highly reactive and could tan proteins by the covalent bonds or auto-oxidize, producing the brown pigments. In the human skin, melanin is an effective agent in light absorption; the pigment is able to disperse over 99.9% of absorbed UV radiation. Owing to this property, melanin has an important role in the skin cells protection against the UV radiation and accordingly, it reduced the cancer risk. Many studies revealed a lower incidence of skin cancer in individuals with more concentrated melanin, i.e. individuals with darker skin character. Conversely; the relationship between skin pigmentation and

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photoprotection is still being investigated. There are many hindering factors for exact characterization of melanin. Melanin is insoluble in a broad range of solvents. Generally, melanin has low purity and the composition of melanin is varied and led to the heterogeneity of their structural features. In addition, the determination of the ratio of the various units found in melanin is not yet available. The molecular structure and organization of melanin are complicated and not completely known [14, 15]. A great number of investigations on melanin have been done, but untill now it is still not well understood. An accurate definition of melanin biopolymer does not exist as its structure is not well known untill now [16, 17, 18].

Three types of melanins are known such as eumelanin, pheomelanin and allomelanin. Eumelanins are defined as black to brown color pigments yielded by oxidative polymerization of L-tyrosine (and/or phenylalanine) to L-DOPA, which is further changed into dopachrome and then to melanin [19]. *Bacillus licheniformis* has an important role in many applied biotechnologies e.g. prevent infection of *Vibrio parahae molyticus* [20, 21].

In this study, the monophenolase and the diphenolase activities were produced from the petroleum pipe isolate. Enzymes optimization was achieved through environmental and nutritional factors. For the first time black and brown eumelanins was extracted from the *Bacillus licheniformis* tyrosinase and their structure were characterized. The anticancer activity of black and brown eumelanin was studied against MCF-7, HEPG-2, and HCT-116 cell lines.

2. Materials and methods

2.1. Bacterial isolate

2.1.1. Origin of the samples

The sample was collected from rock sequence (The middle green layer had H_2S odor lies between two red layers) at petroleum pipes in Maracopara, France. *B. licheniformis* bacterial strain was isolated at N_2/CO_2 (80-20%) at 1% O_2 using Roll-tube techniques for anaerobic bacteria and nutrient agar medium.

2.2. Microorganism identification

2.2.1. Molecular identification by 16S rRNA sequencing (DNA extraction)

A single colony of the isolate was picked and inoculated into LB broth at 37 °C for 24 h, the genomic DNA was extracted from the grown culture using ABT DNA mini extraction kit (Applied biotechnology Co., Egypt) according to manufacturer's instructions and stored at − 20 °C until required.

2.2.2. Amplification and sequencing of the 16S rRNA gene

The amplification of the 16S rRNA gene was done by PCR instrument (model. TechneTC312) using the universal primers 27F GAGTTTGATCCTGGCTCAG, 1492R GGTTACCTTGTTACGACTT [22]. The PCR condition was as follows: initial cycle at 95 °C for 5 min followed by 35 cycles (denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 90 seconds). Then the amplified product was checked by electrophoresis on 1% agarose gel. The unpurified PCR product was submitted for Sol Gent Co. Ltd SouthKorea for purification and sequencing. The sequence results were analyzed for homologies with the microorganisms available in the NCBI database by blast search and the phylogenetic tree was created by (Genius biometrics program). The aligned sequence of this amplified region of the 16S rRNA segment from the tyrosinase isolate was submitted to Gen bank under accession NO MH978895 and named MAL.

2.3. Production of tyrosinase enzyme by Bacillus licheniformis

Hungate tube had nutrient agar with freshly prepared *Bacillus licheniformis is* was scratched and cultivated in 100 mL of closed glass bottles contained 40 mL of TC medium contained (g/L): L-tyrosine 1 and $CuSO_4$0.01. Then 3 mL of inoculums was transferred into the TC and the conditions were 48 h at 37 °C, pH7and static condition and secondly onto specific broth medium (SAM), contained (g L^{-1}): glucose, 1.0; yeast extract, 0.1; KH_2PO_4,0.3; $MgCl_2$, 0.5; NaCl, 10; $CaCl_2$, 0.1; NH_4Cl, 1.0; $BaCl_2$, 10.0; $CuSO_4$, 0.02; tyrosine, 1.0. The operating conditions were maintained as mentioned above. The SAM was used as the basal medium for the optimization studies.

2.4. Tyrosinase activity and protein determination

The monophenolase and diphenolase activities of tyrosinase were determined spectrophotometrically by using 3-methyl-2-benzothiazolinone hydrazone (MBTH), [23]. Monophenolase activity corresponds to the oxidation rate of 1.25 mM of L-tyrosine in sodium phosphate buffer 50 $mmol^{-1}$, pH 6.8) at 30 °C (standard conditions), in the presence of MBTH (5 mM) and L-DOPA with a ratio L-DOPA/L-tyrosine = 0.057 [24]. Diphenolase activity was corresponding here to the oxidation of 4.2 mM of L-DOPA in the sodium phosphate buffer 50 mM, pH 6.8) at 30 °C (standard conditions), followed at 484 nm (extinction coefficient: 22 300 M^{-1} cm^{-1}). One unit of activity is defined as the quantity of the enzyme that produced 1 μmol MBTH-adduct per min. The experiments were performed in triplicate and the standard deviation was lower than 10% from the mean value.

2.5. Tyrosinase optimization

The effect of different parameters to optimize the tyrosinase production conditions were achieved in glass bottles (100 mL) close with fixed rubber stoppers and filled with 40 mL culture medium, incubated at static conditions and the enzyme assay was done at the optimum conditions. Firstly, the effect of different oxygen concentrations (5, 10, 15, 20, and 21%) was adjusted for each bottle contained the (SAM) medium. Where, two massflows, one for N_2 (0–500 mL min^{-1}) and one for O_2 (0–200 mL min-1), were connected to the bottle. The total flux was 400 mL min-1. The settings for N_2 and O_2 flows were calculated in order to reach the desired O_2 concentrations in the headspace (in volume percentage). Secondly, the effect of different incubation temperature at (30–65 °C) was studied where each bottle was incubated at the desired temperature for 48 h. The pH ranged from (3–9) were tested, where each bottle contained the SAM medium were adjusted at the desired pH and incubated at the optimum conditions. The effect of different concentrations of Cu^{2+} was tested. Each concentration started from 0.0125 up to 0.02 mg was added separately to the SAM medium. The effect of different carbon sources was studied. The glucose (control) in SAM was replaced by mannose, maltose, fructose, galactose or raffinose as carbon sources (1 g/L). Finally, the influence of different nitrogen sources was studied. The yeast extract was replaced by different nitrogen sources such as peptone, tryptone, malt extract, beef extract and liver extract (1 g/L).

2.6. Cell culture

Four human cell lines, HCT-116 (colon carcinoma cell line), Hep-G2 (liver carcinoma cell line), MCF-7 (human breast carcinoma cell line) and HFB-4 (normal human melanocytes) were purchased from the American Type Culture Collection (Rockville, Maryland, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS with 100 U/mL penicillin and 100 U/mL streptomycin. The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

2.7. Melanin extraction

The experiment was performed according to the modified method of Gadd [25]. Bacterial strain was grown on a modified medium at 37 °C for a period of one week. The brown colored of bacterial culture was washed with 1 M NaOH and was autoclaved at 120 °C for 20 min. The pigmented NaOH sample was centrifuged at 5,000 rpm for 5 min and the supernatant was collected. The alkaline pigment extract was acidified to pH 2 by

using concentrated HCl black melanin precipitation and obtained through centrifugation. Excess of HCl was added to precipitate the brown melanin using centrifugation at 10000 rpm for 10 min. The black and brown melanin precipitates were washed with distilled water, dried overnight and their weight was determined. The purified black and brown powder hygroscopic, that should be kept refrigerated at -20 °C to avoid photo-chemical and photo-physical alterations.

2.8. Determination of molecular weight of black and brown melanin

Black and brown melanin were prepared at different concentrations and the flow time of equal volumes for each concentration at 30 °C was determined in a U-shaped Ubbelhode-type viscometer. The flow time of the same volume of DMSO was also determined as control. Thus, specific viscosity/C ($_{sp}$) was estimated. A plot of black and brown melanin concentration (C) against $_{sp}$/c (intrinsic viscosity) (C) therefore gives a straight line, the intercept with Y ordinate gave the value of (η). This was a lone viscometrically method for polymer molecular weight determination as reported by Mark-Houwink-Kuhn-Sakurada (MHKS) equations [26].

2.9. Ultraviolet-visible spectroscopy (UV-Vis)

The black and brown extracted melanin solutions were prepared by dissolving in DMSO 0.5 mg/mL. The UV–Vis spectra measurements were recorded (200–500 nm) using UV-2401 (PC)S, UV-Vis recording spectrophotometer (Shimadzu, Japan).

2.10. FT-IR spectroscopy

FT-IR (Bruker Vectra 22) Spectrometer equipped with a Dura Sample IR II™ detector used for characterization and identification of black and brown melanin powder extract with a spectral resolution of 4 cm^{-1} with 400–4000 cm^{-1}.

2.11. Scanning electron microscopy (SEM)

The surface morphology of brown and black melanin was examined using scanning electron microscopy (JEOL 5410) microscope with an accelerating voltage conducted at 10 kV. The black and brown melanin samples were gold coated using a Hitachi coating unit IB-2 coater under a high vacuum, 0.1 Torr, high voltage, 1.2 kV and 50 mA.

2.12. Energy dispersive X-Ray spectroscopy

EDAX (or EDS) is an x-ray spectroscopic method for determining elemental compositions (qualitative and quantitative analysis). It can be used with/during imaging with SEM. When done with an SEM instrument, the signal can be acquired from a spot, an area, a line profile or a 2D map.

2.13. Anticancer assay

The cells were seeded in a 96-well plate at a density of 10^4 cells/well for 48 h at 37 °C under 5% CO_2. After incubation, the cells were treated with phthalimide analogs in a range of concentration (6.25–200 μM) and incubated for 24h then the medium was discarded. Fix with 10% trichloroacetic acid (TCA) 150 μL/well for 1 h at 4 °C. Wash by water 3 times (TCA reduce SRB protein binding). Wells were stained by SRB 70 μL/well for 10 min at room temperature with 0.4%. Wash with acetic acid 1% to remove the unbound dye. The plates will be 24 h in air dried. The dye will be solubilized with 50 μL/well of 10 m Mtris base (pH 7.4) for 5 min. on a shaker at 1600 rpm. The optical density (OD) of each well was measured at 570 nm with an ELISA microplate reader (EXL 800 USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of the untreated sample) X 100 and the IC_{50} values were calculated using sigmoidal concentration-response curve fitting models (Sigma Plot software).

2.14. Statistical analysis

Data analysis was carried out with MICROSOFT EXCEL (2007). All data are presented as means ± standard error of means. All enzyme optimization experiments were independently replicated 2 times (n = 2), with 2 measurements per replicate. The mean of the repeated measurements yielded the value for each replicate.

3. Results and discussion

3.1. Tyrosinases production

The tyrosinase enzyme is a fundamental enzyme principally used in melanin production and in the different pharmaceutical industry. Till this moment there are scarce publications on bacterial tyrosinases and there is not enough information concerning the structure and characterization of the yielded melanin [15]. Within this context, one bacterium was isolated from the surface of petroleum pipes Maracopara, France at 1% O_2. This isolate could grow aerobically and recorded a promising monophenolase and diphenolase activities in the presence of tyrosine and Cu^{2+} only (1.3 U/mL, 15 U/mL for monophenolase and diphenolase respectively). This result suggested that the microorganism could gain energy through the tyrosine oxidation. The bacterial isolate was identified based on 16S rRNA as *Bacillus licheniformis* with 99.9 % similarity (Fig. 1.). The studying of tyrosinase activities in bacteria has only recently been reported [7, 27].

3.2. Tyrosinases optimization

The study started with the optimization of tyrosinase production through different factors. The activity of tyrosinases from *Bacillus licheniformis* depends on the cultivation conditions which include environmental and nutritional factors. Our study focused on the effect of aeration, different pH values and incubation temperature as environmental factors and carbon, nitrogen sources and copper concentration as nutritional factors. The results in Table 1 showed that the highest diphenolase activity was obtained at 20% O_2 (29 U/mL) and the activity decrease about 17% in the open air and when the O_2 was limited to 5%

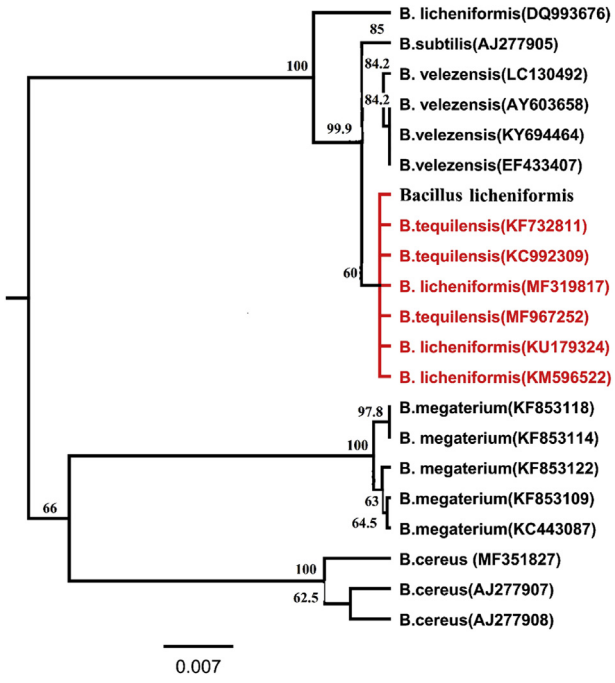

Fig. 1. Phylogenetic tree of *Bacillus licheniformis*.

Table 1
Effect of different parameters on production of *Bacillus licheniformis* monophenolase and diphenolase.

The influence different parameters on tyrosinase and diphenolase productivity							
Oxygen conc. (%)	5	10	15	20	Open air	-	-
Daiphenolase (U/mL)	0	14.14 ± 0.02	16.7 ± 0.03	29 ± 0.00	24.1 ± 0.02	-	-
Monophenolase (U/mL)	0	0.44 ± 0.02	1.18 ± 0.01	2.7 ± 0.04	3 ± 0.01		
Temp. (°C)	30*	40	45	*50	55	60	65
Daiphenolase (U/mL)	17 ± 0.01	28.57 ± 0.05	229 ± 0.09	32.71 ± 0.04	35 ± 0.03	39.29 ± 0.03	35.42 ± 0.02
Monophenolase (U/mL)	1.42 ± 0.90	2.70 ± 0.00	3.05 ± 0.01	4.28 ± 0.02	5.57 ± 0.02	8.10 ± 0.01	8.22 ± 0.00
pHs	5	6	7	8	9	10	-
Daiphenolase (U/mL)	35.7 ± 0.03	37.28 ± 0.05	39.28 ± 0.04	45.7 ± 0.04	46 ± 0.02	44.57 ± 0.02	-
Monophenolase (U/mL)	51.5 ± 0.90	60 ± 0.07	68 ± 0.03	72 ± 0.03	77 ± 0.09	76 ± 0.06	-
Cu^{2+}(mg)	1.25*	2.5	5	10	15	20	-
Daiphenolase (U/mL)	45.71 ± 0.05	107.42 ± 0.03	147.86 ± 0.03	188 ± 0.90	116 ± 06	100 ± 0.07	
Monophenolase (U/mL)	11 ± 0.04	22 ± 0.04	27.57 ± 0.07	33.57 ± 0.06	26.42 ± 0.06	14.30 ± 0.05	
N. sources (1 g/L)	Peptone	Tryptone	Yeast extract	Malt	Beef extract	Liver extract	C* (without)
Daiphenolase activity	217 ± 0.90	203 ± 1	207.60 ± 0.66	189 ± 2.0	227 ± 0.90	229 ± 0.80	189 ± 0.980
Monophenolase (U/mL)	56.57 ± 0.09	37.71 ± 0.04	33 ± 0.02	51 ± 0.02	33 ± 0.04	58.57 ± 0.01	34.42 ± 0.02
C. sources (1 g/L)	Mannose	Maltose	Fructose	Glucose	Galactose	Raffinose	C*(without)
Daiphenolase (U/mL)	247 ± 0.90	253 ± 0.90	181 ± 1.50	258.6 ± 0.70	262 ± 0.90	251.70 ± 1.40	230 ± 0.80
Monophenolase (U/mL)	362 ± 0.50	224 ± 0.70	77 ± 0.70	399 ± 1.00	87.57 ± 0.70	125 ± 0.70	59 ± 0.80

C* = Control.

the enzyme activity lost completely. The monophenolase activity achieved its optimum activity at 20% and open-air oxygen (2.7, 3 U/mL). This result was agreed with Muller et al. [28] who concluded that oxygen played an important role in increasing the tyrosinase activity. The recorded data in Table 1 showed that the tyrosinase activity was greatly affected by the incubation temperature, where the enzyme productivity was maximum at 60–65 °C for the monophenolase production (8.10–8.22 U/mL) and 60 °C for the diphenolase (44.57 U/mL) but at low temperature (30 °C) the activity decreased to 1.42 U/mL and 17 U/mL for monphenolase and diphenolase, respectively. On contrary, to this paper, McMahon et al. [6]; Selinheimoet al. [29] reported that the optimum temperature for tyrosinase activity obtained from *Pseudomonas putida* and *Trichoderma reesei* showed maximum activity at 30 °C and the maximum activity of *Bacillus megaterium* and *Lentinula boryana* tyrosinases was at 40 °C [30, 31]. Mostly the optimum pH of the tyrosinase depended mainly on its origin. In this study, the maximum *Bacillus licheniformis* monphenolase and diphenolase activities was obtained at pH9 (46 and 77 U/mL respectively). This result coincided with the tyrosinase from *T. reesei* which has a basic pH optimum of 9 [2].

The most important factors which have a great influence on tyrosinase production is the nutritional factors which included C, N factors and copper concentrations in the medium. The first nutritional factor is copper, tyrosinase enzyme is copper-dependent enzyme since the tyrosinase structure consists mainly of copper which recorded as an essential element to tyrosinase enzyme activity and it must be added to the tyrosinase production medium [8]. The result in (Table 1) proved that the tyrosinase activity was increased by the increase of Cu^{2+} concentration until 10 mg/L which gives rise to maximum tyrosinase activity (EA = 33.57, 188 U/mL) for monophenolase and diphenolase respectively. This result agrees with Zou et al. [32] who motioned that the addition of 0.13 g/L had great influence in the optimization of *Auricularia auricula* tyrosinase. Barceloux [33] stated that copper is an essential micronutrient found in trace amounts in nature. In cells, it is associated with the active site of numerous enzymes, such as cytochrome c oxidase, tyrosinase, laccase, dopamine b-hydroxylase, lysyl oxidase and Cu/Zn superoxide dismutase (SOD) [34].

Among all tested nitrogen sources, liver extracts played a significant role in increasing the enzyme productivity for the monophenolase and diphenolase productivity respectively (58.57 and 229U/mL). Tyrosinase production is triggered by nitrogen depletion [35] but some nitrogen sources do not affect the enzyme activity [36]. The brown color for *Streptomyces* isolates was yielded on peptone-yeast extract iron agar [37].

Most of the tested carbon sources had a good effect on tyrosinase productivity, especially mannose and glucose. The data shown in Table 1 clearly showed that the best sole carbon source for monophenolase was glucose followed by mannose as 399 U/mL, 362 mL, respectively. This result agreed with Raju et al. [38]. On the other hand, the best sole carbon source for diphenolase was galactose followed by maltose as 262 U/mL and 253 U/mL, respectively. This result was similar to Galhaup et al. [39] who mentioned that the disaccharides or high molecular weight carbon substrates have been found to be the best supporters for the enzyme production. The previous result was much better than Valipour and Arikan [7]. They mentioned that after optimization, the production of *Bacillus sp.* MV29 tyrosinase 0.7I U/mL was obtained. On the other hand, the highest *Auricularia auricula* tyrosinase activity was 17.22 U/mL [32].

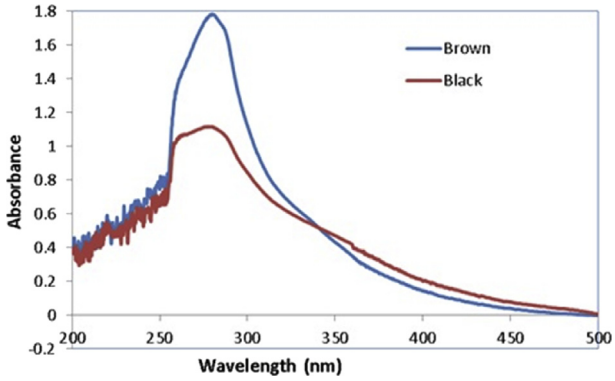

Fig. 2. UV-Vis absorption spectra of extracted black and brown eumelanin.

3.3. Extraction of melanin

The black and brown melanin pigment was precipitated by HCl and yielded 0.1 g/100 mL and 0.7 g/100 mL, respectively. The yield exceeded the melanin pigment extracted from *Klebsiella* sp. GSK (130 mg/L) [40]. The molecular weight for black and brown was estimated to be 51 kDa and 35.5 kDa, respectively. The two melanins recorded higher molecular weight than the Chinese black tea melanin extracted from apricot with molecular mass 8 and 14.8 kDa [41] showed lower molecular weight than *Lentinula edodes* melanin extracted ranged from 72 and 105 kDa [42]. Characterization of both melanin was confirmed by a spectroscopic analysis which included:

3.4. UV–visible light absorption spectrum

UV/Vis spectroscopy analysis is routinely used for determining a high degree of the conjugated system. UV-Vis absorption spectra of the black and brown melanin obtained from *Bacillus licheniformis* showed in Fig. 2.

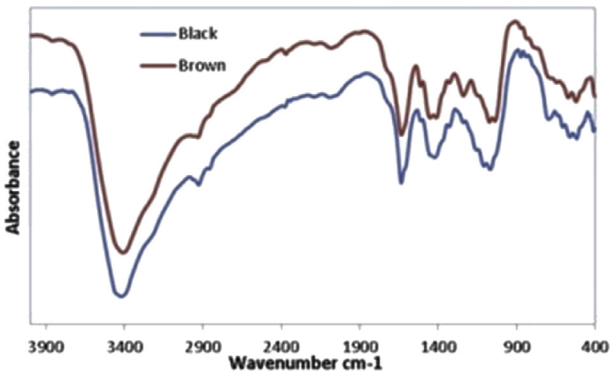

Fig. 3. FTIR spectra of extracted black and brown eumelanin.

Maximum absorbance has been observed of both melanin types in the UV region. It was cleared that its optical density decreased gradually with an increase of wavelength. This data agreed with Riley [43]. On contrary other reports disagreed with this data on the melanin isolated from the fruiting body of *A. auricular* [44]. Black and brown melanin exhibit in the region from 260 to 310 nm and null absorption in the visible light region. However, the wavelengths of the melanin absorption spectrum in the UV range represented the decreasing line in melanin molecule extracted from a plateau in the range of 270–280 nm, due to the presence of the aromatic amino acid in melanin molecule. In addition, the absorption peaks at 260 and 280 nm were observed, suggested that the melanin molecules might contain other protein, nucleic acid and lipids [45]. Brown melanin showed a high maximum wavelength absorbance in compare to the black melanin because of the difference of pH between solutions and melanin type causing a minor change of the natural melanin structure. Brown melanin showed higher molar absorption units (hyperchromic effect), at the same concentration than black melanin. Generally, both brown and black melanin have a significant feature which was observed in the intensive absorption over a wide wavelength range by high conjugate regions.

3.5. FTIR analysis

FTIR spectrum showed some narrow and sharp peaks which could be produced by some fragments and created by the destruction in the step of acid precipitation. FT/IR analysis of black and brown melanin was performed as shown in Fig. 3. Both types of melanin have broad absorption at 3300–3500 cm^{-1}corresponding to the stretching vibration of the –OH

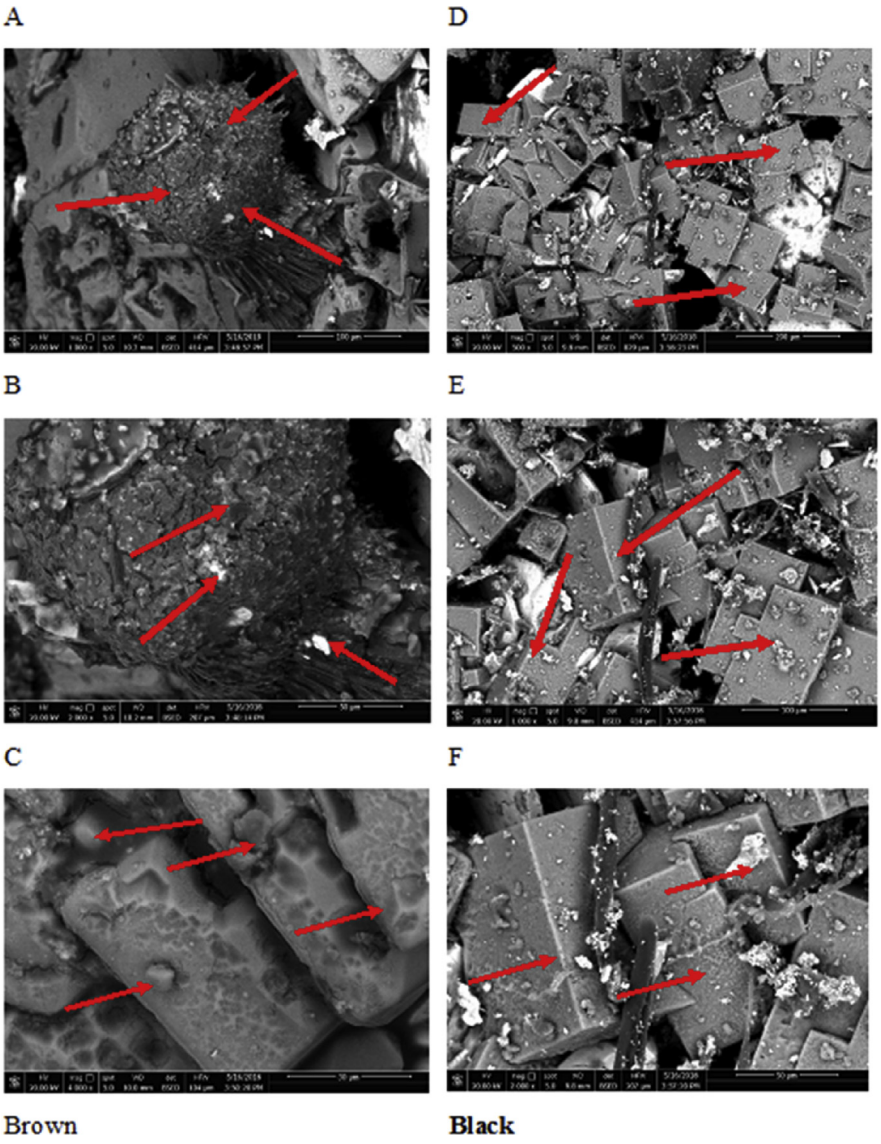

Fig. 4. SEM of extracted black and brown eumelanin.

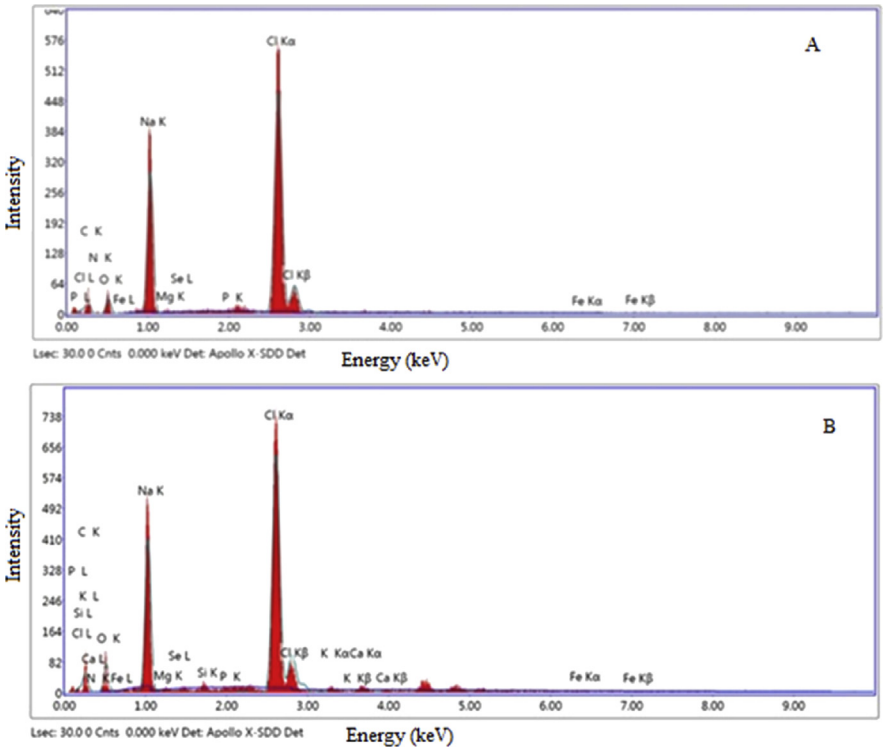

Fig. 5. EDAX elemental analysis of (A) brown and (B) black eumelanin.

and –NH_2 groups. However, black melanin has a sharper absorption band than brown melanin. Black and brown melanin also exhibited absorption at 2930 cm^{-1}corresponding to stretching vibrations of C–H (from aromatic rings) and C–H (from aliphatic compounds). The characteristic band at 1630 cm^{-1}, caused by the oscillation of coupled bonds of C=C, $–COO^-$and C=O types. The IR spectra of black melanin showed a more intense spectral line around 1600 cm^{-1} compared to brown melanin, which showed a less extended peak. The band at 1430 $cm^{-1}$was attributed to methylene group vibration. At 1150 cm^{-1} absorbance caused by the oscillation of C–O groups of carbohydrates, alcohols, and phenols, which were compatible to absorption peaks for fungal melanin. Peaks around 1100 cm^{-1} are related to a water-soluble carbohydrate moiety (chitin-melanin glucan complex) [46]. The IR spectrum of brown and black melanin obtained was comparable to the black tea melanin [47], and *Nyctanthes arbor-tristis* fruits [48]. All previous results suggested that the brown melanin has lower molecular-mass and less moisture than black melanin.

3.6. SEM

SEM gave an idea about the morphological structure of melanin samples. The images showed surface morphologies of brown and black crystals took at various magnifications. The images present large crystals with a rough surface covered by several holes. The results in Fig. 4 pointed to the difference of morphological structure between brown and black melanin at the same magnification scale. At 100 μm magnification scale, the brown melanin appeared aggregates of no definite shape with different sizes that are grouped together (Fig. 4A). Reducing the magnification scale to 50μm showed small aggregates (Fig. 4B). Further increasing of the magnification at 30 μm scale showed the rectangular crystals clearly (Fig. 4C). Black melanin appeared as small cubic-shaped crystals at 200μm scale (Fig. 4D). The increase in magnification from 100μm to 50μm showed the cubic-shaped crystals clearer and larger (Fig. 4E and F). On the contrary, the commercial sepia melanin appeared to be formed by aggregates of spherical particles with different sizes [49].

3.7. EDAX analysis

EDAX provide innovative minerals characterization systems for micro- and nano-elemental analysis (Fig. 5). EDAX elemental analysis showed that brown melanin contained mainly from C, N, O, Na, Mg, P, Cl, Fe and Se elements. However, black melanin contained from C, N, O, Na, Mg, Si, P, Cl, K and Ca elements with different ratios as listed in Table 2. There is no doubt that all these elements are very essential in human biological activity and may be responsible for melanin activation. Elemental composition demonstrated that the melanin produced classified as eumelanin, not phaeomelanin due to the absence of sulfur. Ye et al. [50] reported that the phaeomelanin from *Lachnum* YM404 has high S content (14.83). In addition, eumelanin consists of Na, Mg, P, Cl, Fe, Se, K and Ca elements were higher compared to phaeomelanin. Furthermore, based on the above results, it was concluded that the *B. lichenoformis* melanin mainly consists of pure brown and black eumelanin. Mbonyiryivuze et al. [49] reported that the elements of commercial sepia melanin were only C, O, Na, Mg, Cl, S, and Ca. Also, the major compositions of sepia melanin are C, O, Na, Cl, while the minor was Mg, Ca, K, S and N.

3.8. Cytotoxicity test

The purified melanin pigment was tested against three different cancer cell line including mammary gland breast cancer cell line (MCF-7), human hepatocellular carcinoma cell line (HepG2) and colon carcinoma cell line (HCT116) using SRB assay. IC_{50} values are reported in Table 3. The melanin sample showed activity towards HEPG-2, HCT-116 and MCF-7 cell line with IC_{50} values of 6.15 μg, 5.54 μg, and 7.91 μg respectively in compared to Doxorubicin (4.05 μg). The IC_{50}values of brown melanin were less efficient towards HCT-116 cell line (8.75 μg/mL) in compared to the black melanin (5.54 μg). Arun et al [18] mentioned that the in vitro inhibition of cell proliferation in HEP 2 carcinoma cell line recorded 53% at 60μg melanin. Also, Kurian, et al. [51] said that MTT assay appeared that BTCZ31 melanin prevented the growth of L929 cell line, the cytotoxic concentration of melanin was

Table 2
EDAX elemental weight % of brown and black eumelanin.

Elements	Weight %		Atomic %		Net Int.		Error %	
	Brown melanin	Black melanin	Brown melanin	Black melanin	Brown melanin	Black melanin	Brown melanin	Black melanin
C K	32.36	35.46	49.2	51.39	13.98	27.91	15.86	13.87
N K	3.67	1.4	4.79	1.74	1.33	0.82	64.95	86.06
O K	11.04	17.04	12.6	18.53	14.51	37.84	16.24	13.66
NaK	24.08	21.86	19.13	16.56	163.06	223.3	8.34	8.34
MgK	0.33	0.36	0.25	0.26	2.51	4.35	68.69	29.65
SiK	-	0.44	-	0.27	-	9.06	-	22.47
P K	0.48	0.36	0.28	0.2	5.95	7.27	31.88	31.13
ClK	25.45	21.39	13.11	10.5	332.19	450.77	3.15	2.87
FeK	0.36	-	0.12	-	1.69	-	65.16	-
SeK	2.23	-	0.52	-	1.52	-	66.56	-
KK	-	0.29	-	0.13	-	4.71	-	61.52
CaK	-	0.46	-	0.2	-	6.63	-	59.51

Table 3
In vitro anticancer activity for *Bacillus subtilis* melanin by SRB assay ($IC_{50} \pm SE$ μg/mL).

Compounds	IC_{50} μg/mL.			
	MCF-7	HEPG-2	HCT-116	HFB4
Black melanin	7.91 ± 0.94	6.15 ± 0.74	5.54 ± 0.25	46.6838
Brown melanin	10.07 ± 01.33	9.74 ± 0.89	8.75 ± 1	98.5724
Dox.	2.96	4.05	4.45	5.00

105.4 μg/mL (IC_{50}). Morphological changes on abnormal cell showed smaller, shrank, round and had angles.

The cytotoxicity of two melanin types was tested on the HFB4 (normal cells). The cytotoxicity of the black melanin and brown melanin at 100 μg/mL concentration recorded 87 % and 57% respectively. While at the minimum concentration 6.25 μg/mL the cytotoxicity of black melanin and brown melanin was 18 % and 8% respectively and in between, at 50 μg/mL the cytotoxicity of black melanin was 34 % however the cytotoxicity of brown melanin was 18%. The results recorded that IC_{50} μg/mL for black and brown melanin's were 46.6838 and 98.5724 μg/mL respectively. Also, the brown melanin recorded high safety in comparing to the black form. In this finding, it was reported that the efficient cytotoxic activity of melanin against three tested cancer cell lines and the low cytotoxicity at low concentration against HFB4 normal non-cancerous cells showed that melanin pigment could be used as potential natural anticancer [52].

4. Conclusion

This research focused on tyrosinase optimization and melanin extraction from the petroleum pipes isolate called *B. licheniformis*. For first time, two types of black and brown eumelanin were extracted and characterized by UV spectra, FT-IR, SEM, and EDAX analysis. The anticancer and the cytotoxicity activities were determined for the two melanin types. The potential cytotoxic activity of melanins against different cancer cell line and low cytotoxicity against normal non-cancerous cells recommended melanin pigment to be applied as new natural anticancer.

Declarations

Author contribution statement

Al Shimaa Gamal Shalaby: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Tamer I. M. Ragab: Performed the experiments; Analyzed and interpreted the data.

Mohamed M. I. Helal: Analyzed and interpreted the data, Wrote the paper.

Mona A. Esawy: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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