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Optimizing production of a biopesticide protectant by black yeast

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Abstract

Natural protection of the *Bacillus thuringiensis*-based biopesticides from inactivation by the UV component of the cosmic rays constitutes a big challenge to environmentalists, health concerned groups, and industry. Melanin pigment produced by a variety of microbes has the capabilities of protecting these types of biopesticides. A black melanin produced by a locally isolated strain of the yeast *Hortaea werneckii* EGYNDA08 possesses the qualities of a sun protectant agent. This UV bio-protectant increased the killing potency of a locally isolated *B. thuringiensis* subsp. *aegypti* (*Bt-C18*)-based biopesticide ninefold upon feeding the first instar larvae of the cotton leaf worm, *Spodoptera littrolis*. This black melanin was extracted, characterized, and exposed to different optimization process for the purpose of enhancing its productivity. The optimization process employed medium engineering techniques to generate a suitable cheap production medium not only at bench-scale level but also at the bioreactor level. These optimization techniques have led to increase the melanin produced by the local isolate of *Hortaea werneckii* EGYNDA08 up to 228 mg/l compared to 8 mg/l prior to optimization. This study concluded that black yeast melanin could be used at a wide range as a potential green alternative for the conventional chemically based sunscreens that currently used to protect biopesticides from inactivation by the cosmic rays.

Keywords: *Bacillus thuringiensis*, Melanin, Black yeast, Biopesticide protectant

Background

Melanin refers to natural dark brown to black high molecular weight pigment which is usually complexed with carbohydrates and proteins. It produced by many living organisms as a polymer of oxidized tyrosine. There are three main types of melanin: eumelanin, pheomelanin, and allomelanin. Fungal melanin existed in the cell wall or as extracellular polymers formed by auto-oxidation of phenolic compounds or enzymatically using enzymes such as tyrosinase or polyketide synthase for 3,4-dihydroxyphenylalanine (DOPA) or 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis respectively (Kutty 2009). The phenolic compounds that the fungal melanin is derived from include, tyrosine via DOPA in some groups of fungi and acetyl CoA via DHN melanin in ascomycetes and related asexual fungi and γ-glutaminyl-3,4-dihydroxy-benzene or catechol in basidiomycetes. The main melanin producers among the fungus world include *Cryptococcus neoformans*, *Aspergillus niger*, and *Penicillium marneffei* in addition to black yeast such as *Wangialla dermatitis* and *Hortaea werneckii* (Youngchim et al. 2004).

Melanin was reported to have specific role in the capacity of fungi to withstand extreme environments including radiation resistance (Zhdanova et al. 2000), protection against enzymatic lysis (Butler and Lachance 1987), oxidizing agents (Jacobson et al. 1995), extreme temperatures (Rosas and Casadevall 1997), osmotic stress (Ravishankar et al. 1995), and high concentrations of salts and detergents and the high heat (Zalar et al. 2011).

Distinctive properties of melanin suggest wide environmental and medical applications. Fungal melanin has gained more interest for their potential in many fields of biomedicine, dermocosmetics, nanotechnology, and materials science. Radiation was used in medical treatment like radio-immunotherapy or therapy by external beam that can effect on bone marrow and this was considered as a harmful side effect for using radiation; many experiments estimate that melanin was successfully used to protect bone

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marrow from such side effect (Schweitzer et al. 2010). In cosmetic industry, melanin gained great interests due to its ability to protect skin against the serious effects of UV radiation (Solano 2014). Some microorganisms can exhibit a common strategy in response to UV radiation including the synthesis and accumulation of UV light absorbing secondary metabolites that act as sunscreens (Garcia-Pichel and Castenholz 1991; Garcia-Pichel and Castenholz 1993); melanin was known as the best secondary metabolites that act as sunscreens Kollias et al. 1991). Microbial melanin pigment, especially from marine species, is an attractive option for researchers and industries because it is safer, easily degradable, and eco-friendly and does not cause harmful effects (Pombeiro-Sponchiado et al. 2017).

In the present work, a local black yeast strain namely *Hortaea werneckii* EGYNDA08 was previously isolated from halophilic Egyptian habitat and holds high potentiality for melanin production. The produced melanin was characterized, and its yield was enhanced via placket-Burman matrix and subsequently a central composite design algorithm. The produced melanin was found to be efficient in protecting *Bacillus thuringensis*-based biopesticide from deactivation by solar light.

Materials and methods

Strains and growth conditions

Hortaea werneckii EGYNDA08 (Elsayed et al. 2016) was previously isolated from Egyptian local habitats. The strain's colonies were found to be dark brown in color indicating the astronomical potentiality to produce melanin pigment. The used strain was isolated from water samples and collected from solar salter on the Mediterranean Sea coast in Egypt, exactly from Gamasa shore on Gamasa-Baltim road (latitude and longitude are 31.47 and 31.46 respectively) in November 2013. The isolate was grown on liquid minimal media prepared for cultivation of black yeast (Gunde-Cimerman et al. 2000).

Pigment extraction

Melanin "the black pigment" was subjected for extraction and purification from black yeasts as per Gadd (1982). After a week of growth, the culture was centrifuged at 10,000 rpm for 10 min, supernatant was discarded and pellets were washed by distilled water and collected to be used for the extraction. Melanin was extracted by autoclaving the yeast harvested biomass with 1 N NaOH (20 min, 121 °C, at pressure 1.5 bar). The treated biomass was centrifuged for 10 min at 8000 rpm, and the developed supernatant contained the desired melanin pigment. Subsequently, melanin was precipitated by adding concentrated HCl until pH 2 and then centrifuged at 10,000 rpm for 10 min. Melanin was finally dried in dehumidified atmosphere.

Characterization of melanin pigment

The extracted pigment from *H. werneckii* EGYNDA08 was characterized by certain chemical and physical tests including the following:

- Chemical properties are used to define the extracted pigment as melanin was declared methods described by Thomas (1955). The solubility of the extracted pigment was examined in different common solvents such as distilled deionized water, HCl, NaOH, ethanol, methanol, chloroform, acetone, and benzene. Bleaching test was performed by various oxidizing agents like H_2O_2 and $KMnO_4$ and also by reducing agents like H_2S, besides resistance of degradation by concentrated acids. Precipitation of the pigment was examined by 1% $FeCl_3$.
- The extracted pigment was subjected to spectrophotometric analysis that was performed using UV-visible spectrophotometer in a broad range of wavelength of 200–800 nm using (SHIMADZU UV-160, Tokyo, Japan). Pigment was diluted 1:10 times using 1 N sodium hydroxide. Authentic melanin standard (Sigma Aldrich; reference M8631) was used as reference.
- Extracted pigment was recorded on IR spectrometer (Nicolet iS10, USA), and spectra were collected at the wave number region of 400–4000 cm using KBr pellets acquired by pressing under vacuum uniformly prepared mixtures of pigment sample and spectrometry grade KBr. The result recorded was compared against authentic melanin standard (Sigma Aldrich, reference: M8631).
- Nuclear magnetic resonance (NMR) spectroscopy was recorded to the extracted pigment using a model (JEOL NMR ECA-500). The pigment was suspended in dimethyl sulfoxide (DMSO-d6). The proton NMR spectrum of the sample was obtained at 400 MHz using a model (JEOL NMR ECA-500). The chemical shift scale was in parts per million (ppm).

Effect of different sodium chloride concentrations on the growth of the isolate and melanin production

Salinity test was performed to explore the effect of different sodium chloride concentrations on the production of melanin and growth by the isolate under investigation. Cells were grown on potato dextrose agar (PDA) medium which was supplied by different NaCl concentrations 0, 2.5, 5, 10, 15, 20, and 25%. Fifty milliliters of liquid media inoculated by black yeast in 250 mL Erlenmeyer flask at 22 °C for a week in a rotary shaker at 170 rpm. After growth and the appearance of black color, the resulted melanin was extracted and estimated.

Screening of different medium components by Plackett-Burman (PB) design

A PB screening experiment was performed to screen different carbon sources, nitrogen sources, and metal ions to test and compare their effect on the ability of the isolated strain to produce melanin.

A set of 12 experiments, with 10 variables and one dummy, was performed as generated by the software package and shown in Table 2 and each run had its unique composition. The screened medium components include four different carbon sources (sucrose, fructose, glycerol, and sugar cane molasses (SCM)), three different nitrogen sources (corn steep liquor, malt extract, and ram horn) in addition to three different metal ions (NaCl, $MgSO_4$, and K_2HPO_4). The dummy variable was used to estimate the standard error of the experiments.

Each variable was investigated at a maximum (high) "+" level and a minimum (low) "−" level. Presence/absence principle was used to test the variables, as absence of variable was detected by low level which always considered as 0 and the maximum level was considered as 1 g/l for carbon and nitrogen sources. But in case of metal ions, the minimum value was represented as half of the reported value; for NaCl, $MgSO_4$, and K_2HPO_4, the minimum value was 0, 0.1, and 1 g/l respectively and maximum was 10, 0.5, and 2 g/l respectively.

Experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of sea water and medium components according to every run; flasks were incubated in a 170 rpm rotary shaker at 22 °C and pH 6 for 1 week. Responses were measured in terms of growth and productivity of melanin (mg/l).

Comparison of the significance of each factor and its main effect on the growth of isolate *H. werneckii* EGYNDA08 and production of melanin is the main target of the screening experiment (Montgomery 2008).

Sugar cane molasses, corn steep liquor, and ram horn were waste materials obtained from agriculture and animal waste products, and they were tested as potential cheap components for the melanin medium production. Ram horn hydrolysis was performed as reported by Kurbanoglu and Kurbanoglu (2004).

Optimizing central composite design (CCD) for medium composition

The most significant variables resulted from PB design subsequently optimized via applying a central composite design algorithm to identify the optimum level of each of the tested variable. Applying a CCD experiment allowed capturing the main effect of each variable, quadretic effect, and interaction between them. The potential components of production medium were sugar cane molasses, corn steep liquor, malt extract, and glycerol. Both carbon sources, sugar cane molasses and glycerol, were tested in a range between 0 up to 10 g/l, with 5 g/l representing the center point. In addition to the tested carbons sources, two different nitrogen sources were also tested in a range between 0 and 5 g/l, with 2.5 g/l representing the tested center point.

A set of 31 experiments was performed as shown in Table 3, in 250 ml Erlenmeyer flask containing 50 ml of sea water and tested media combination prepared according to the design The initial pH was 6.0 for all flasks, and they were incubated at a 170 rpm in rotary shaker incubator for a week.

The calculated responses were dry weight (g/l) for the growth and melanin productivity (mg/l). 3D surface plot was generated to facilitate understanding interaction between factors and estimate the optimum concentration of each component.

A second-order polynomial model was created for the estimation of the medium composition optimum production for melanin production (Eq. 1):

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X_{ii} + \Sigma\, \beta_{ij} X_{ij} \quad (1)$$

where β_i is the regression coefficients for individual factor effect, β_{ii} is the regression coefficients for square effects of factor, and β_{ij} is the regression coefficients for interaction between factors. The matrix applies and the analysis of variance (ANOVA) was carried out by Minitab 16 software. Validation experiments for CCD experiment were performed based on analysis result(s).

Bioreactor batch cultivation

A batch run was performed using bioreactor with a working volume of 400 ml. The main parts of the bioreactor set up are shown in Fig. 1. It was equipped with main bioreactor vessel (three-neck wolf flask) with total volume of 1 l, magnetic bar to supply agitation to the culture, air pump that ended by porous sparger to provide the culture with air (sterilized by disc filter capsule), cold light emitting source (Volpi, Intralux; 4100 EURO device), and external feed bottle that feed the vessel with seed culture. The cold light source was applied to study the effect of light and did not emit any heat to the system to avoid any extra heating generated due to light exposure.

The batch fermentation was stirred at 100 rpm, and incubation temperature was 22 °C. Air flow was abled all over the batch run to provide aeration (1 l/min). Seed culture was prepared with the same nutrient concentration resulted from the previously carried out CCD experiment and was found to be 40 ml sea water, yeast extract 2 g/l, NaCl 10 g/l, K_2HPO_4 1 g/l, sugar cane molasses 10 g/l, corn steep liquor 3.8 g/l, and malt extract 2.5 g/l. Samples were pulled at time intervals 24 h and

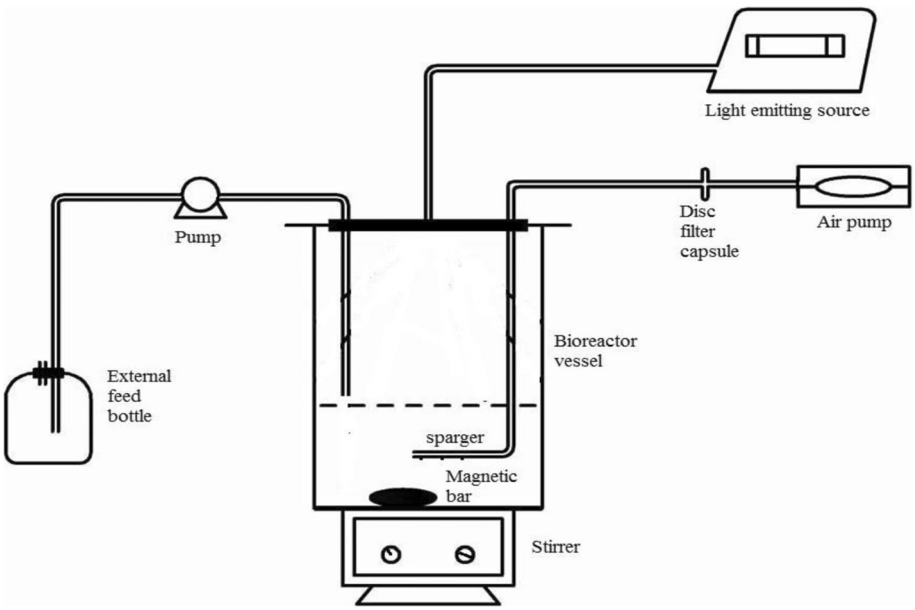

Fig. 1 Batch bioreactor set up

analyzed for growth (dry weight g/l) and melanin yield (mg/L).

Two-level fractional design was applied to study the effect of light on *H. werneckii* EGYNDA08 growth and production of melanin at the bioreactor scale. In terms of light effect, two variables were tested including brightness and intensity with two levels and one center point (The brightness levels were 0, 50, and 100 Lumin, while the intensity levels were 60, 80, and 100 Lux). The whole system and the media were sterilized prior to the start of the experiment, and the air was filtered via a sterile filter membrane to prevent any post contaminations.

UV protection of *B. thuringiensis* subsp. *aegypti*-based biopesticides

B. thuringiensis subsp. *aegypti* (*Bt-C18*) was one of Prof. Osman's collection, Agriculture Genetic Engineering Research Institute (AGERI), Agriculture Research Center, Giza, Egypt. This strain was deposited in the American Type Culture Collection (ATCC) since October 8, 1999, under the patent deposit designation number 55922. The bacterium was grown in T3 broth at 30 °C to sporulation stage. The harvested cellular pellet of the culture containing mixture of spores and crystal toxin were used throughout the study. Crude *Bt-C18* spore-crystal complex was mixed with equal concentration of the black yeast melanin; these concentrations were 5, 10, and 15 ppm. Each of the mixtures was spread, as a thin film, on a glass plate (20 × 20 cm) and left exposed to the direct sun (in a mid-August day, 2017) from 9 am to 3 pm, to get maximum exposure to UV. A non-melanin crude *Bt-C18* spore-crystal complex (control) was exposed to the direct sun in the same manner. Second instar larvae of *S. littoralis* were fed on an artificial diet supplied with either 5, 10, or 15 ppm of the sun-exposed Bt spore-crystal complexes with or without melanin (Osman et al. 2014). Mortality of *S. littoralis* larvae were scored and compared to control insects.

Statistical analysis

All the one-way ANOVA was performed using Origin 8 statistical package, while all the multi-way ANOVA was performed using Minitab 16 statistical software package (Elrazak et al. 2013).

Results and discussion

Microbial melanin, with its unique features, constitutes a group of most interested pigments. It has recently gained global attention by pharmaceutical, cosmetics, electronic, and food processing industries d'Ischia et al. 2015). Microbial pigments gained their popularities from being more viable alternatives than pigments produced by animals or plants. These have no seasonal constraints and do not compete for farming land; moreover, the production of microorganisms is easily produced on cheap culture media (Kumar et al. 2015; Akilandeswari and Pradeep 2016). Additionally, melanin pigments were found to be easily degradable, safer for products, and more cost-effective than synthetic one.

In this research; *H. werneckii* EGYNDA08 was isolated from local Egyptian marine (salt marshes) habitat and was selected based on its dark black appearance, which reflected its capability to produce valuable melanin. This isolate was characterized at the classical and molecular

levels, and it is the black melanin that was also chemically and physically characterized.

Characterization of the melanin pigment

The dark pigment extracted from the black yeast, *H. werneckii* EGYNDA08, was chemically characterized (Table 1). The data showed that the extracted pigment was soluble in sodium hydroxide (alkaline solvent) but not soluble in water or organic solvents (chloroform, ethanol, and acetone). It was precepitated using concentrated hydrochloric acid (HCl), declorized by hydrogen peroxide (H_2O_2), and exhibited positive reaction in presence of ferric chloride ($FeCl_3$).

The extracted black melanin pigment was also characterized using UV, FTIR, and NMR spectral analysis. The UV-visible absorbance (200–800 nm) spectrum of the extracted pigment and melanin standard (Sigma Aldrich) exhibited a strong absorbance in the UV region, and a characteristic peak was detected at 231 nm (Fig. 2a) for extracted melanin from *H. wernekii* EGYNDA08, which was highly similar to melanin standard peak at 236, suggesting the presence of phenol groups.

The FTIR spectrum of melanin extracted from *H. wernekii* EGYNDA08 (Fig. 2b) showed the peaks near 3448 cm^{-1}, 2928 cm^{-1}, 1633 cm^{-1}, and 1264 cm^{-1} referred to the amino group (NH), the C–H stretch bond related to methane group, the amino group with ($NH_{2)}$ stretching, and the anhydride group (C–O) in the extracted fungal pigment, respectively. The result was calibrated against melanin standard purchased from Sigma Aldrich.

The NMR analysis was performed for the extracted pigment to detect the structural composition. The proton NMR spectrum was estimated for the melanin sample that extracted from the isolate under study. The peaks that observed in the spectra were shown in (Fig. 2c) and was agreed with the characterization of DHN melanin in Kutty et al. 2013.

Table 1 Chemical characterization of extracted melanin from *Hortaea werneckii*

Test	Result
Color	Black
Solubility in H_2O	–
Solubility in NaOH	+
Solubility in organic solvents (chloroform, ethanol, and acetone)	–
Precipitation with HCl	+
Decolorization by oxidants agents (H_2O_2)	+
Precipitation for polyphenols with $FeCl_3$	+

Positive (+) and negative (–) response

All chemical and physical characterizations have proved that the black pigment from the local isolate of *H. wernekii* EGYNDA08 was identical to the standard melanin purchased from Sigma Aldrich, with a catalog number M8631.

Effect of salinity on microbial melanin production

The isolate under investigation was halotolerant black yeast and can survive in a wide range of salinity. Effect of different concentration of sodium chloride was estimated on dry weight of growth and production of melanin by *H. werneckii* EGYNDA08. Dry weight and melanin production decreased by increasing the concentration of sodium chloride. Dry weight was 18.6, 17.5, 15.7, 10, 8.9, 3.1 and 1.5 g/l for 0, 2.5, 5, 10, 15, 20, and 25% of NaCl respectively and 124, 120, 104, 80, 74, 32, 8 mg/l for melanin concentration.

H. werneckii is considered as the most extremely known salt-tolerant eukaryote, and it is resembled to *Debaryomyces hansenii* yeast species that can tolerate NaCl concentration up to 25%. In this work, *H. werneckii* EGYNDA08 was found to be able to tolerate salt concentration up to 25%, in nearly saturated salt concentration, although it showed a slow growth behavior. The isolate was also found to be able to grow over a wide range of NaCl concentrations and even in complete absence of salt. Melanin concentration decreased by increasing salinity because the growth medium affects melanization of *H. werneckii*; these results are in line with the results achieved by Gunde-Cimerman et al. (2000) and Kogej et al. (2004).

Melanin production optimization

Different medium components include carbon, nitrogen sources, and metal ions that commonly have a main role in a bio-reaction as they are directly linked with biomass and metabolite production and were screened for their significant effect on melanin production by *H. werneckii* EGYNDA08.

Screening of different medium components using PB

A screening PB experiment was carried out to detect the most significant medium components that could be subsequently optimized by applying central composite design to indicate the exact optimum combination. PB experiments with 11 variables were tested and reported that the obtained dry weight ranged from 0.8 (run 10) to 6.4 g/l (run8) and melanin production ranged from 8.14 (run 10) to 117.83 mg/l (run 4) (Table 2).

The statistical analysis of the responses was performed using Minitab 16 software to detect the most significant factors that effect on growth and melanin production at $\alpha = 0.1$ with level of confidence 90%. Factors that had a P value $\leq \alpha$ were considered to be significant.

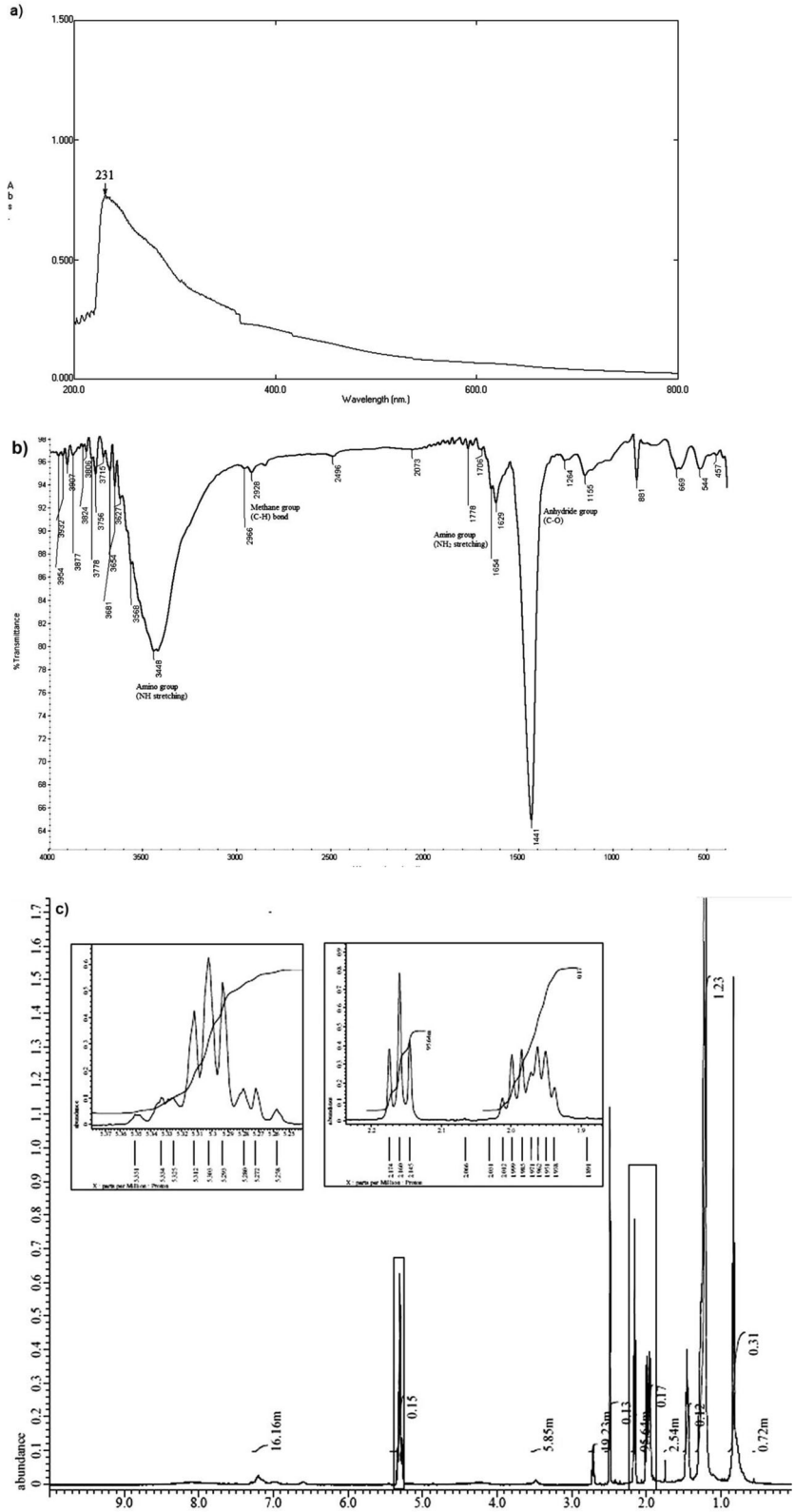

Fig. 2 Physical characterization of melanin extracted from *Hortaea werneckii* EGYNDA08. **a** UV-visible absorbance. **b** Infrared spectrum (FT-IR). **c** NMR analysis

Table 2 The matrix and responses for the Plackett-Burman design for the tested isolate to explore the significant effect of different medium components on the melanin productivity

Run	Variables											Responses	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Dry weight (g/l)	Melanin (mg/l)
1	−	−	−	+	+	+	−	+	+	−	+	3.4	32.59
2	−	−	+	+	+	−	+	+	−	+	−	5.2	87.74
3	−	+	−	−	−	+	+	+	−	+	+	3.8	37.6
4	+	+	−	+	−	−	−	+	+	+	−	4	*117.83*
5	−	+	+	−	+	−	−	−	+	+	+	3.5	75.21
6	+	−	+	+	−	+	−	−	−	+	+	4.9	68.94
7	+	−	+	−	−	−	+	+	+	−	+	3.6	66.43
8	+	−	−	−	+	+	+	−	+	+	−	*6.4*	110.31
9	+	+	+	−	+	+	−	+	−	−	−	3.8	57.66
10	−	−	−	−	−	−	−	−	−	−	−	*0.8*	*8.14*
11	−	+	+	+	−	+	+	−	+	−	−	3.3	62.67
12	+	+	−	+	+	−	+	−	−	−	+	4.6	116.57

Maximum and minimum values were given as italics where X_1 symbolizes sucrose, X_2 symbolizes corn steep liquor, X_3 symbolizes $MgSO_4$, X_4 symbolizes fructose, X_5 symbolize malt extract, X_6 symbolizes K_2HPO_4, X_7 symbolizes glycerol, X_8 symbolizes ram horn, X_9 symbolizes NaCL, X_{10} symbolizes sugar cane molasses, and X_{11} symbolizes dummy

Four variables were found having the most significant effects were chosen for central composite design to detect the optimum level of each one; these variables were sugar cane molasses, corn steep liquor, malt extract, and glycerol. These variables were significant and represented in the design as waste products collected from the local market and were expected to reduce the overall production cost.

The production cost is considered as the main drawback that faces any biotechnological process; optimization is considered as the most effective elucidation for this problem (Sangkharak and Prasertsan 2007). The statistical optimization approaches were proven to be a successful technique for investigation of multiple variables of a process because it make optimization process more easy with fewer trials of experiments (Bajaj et al. 2009; El Naggar et al. 2015).

Central composite design to optimize the production medium composition

Sugar cane molasses, corn steep liquor, malt extract, and glycerol were chosen as the potential cheap production medium components. CCD experiment was performed to indicate the optimum medium composition as previously mentioned. The matrix and responses of the design were shown in Table 3.

The maximum amount in growth (dry weight) was observed at run number 14 while the maximum melanin productivity was obtained at run number 16. Analysis of variance of the linear, quadretic effect, and the interaction among factors were shown in Table 4 where factors with P value < 0.1 were considered as significant. The most significant factors that affected melanin productivity were the main effect of sugar cane molasses, glycerol, and corn step liquor, and quadretic effect of sugar cane molasses, malt extract, and glycerol.

ANOVA resulted from the main effect of each factor, the quadratic effect and interaction among them were shown in Table 4. The P value was estimated that the main effect of malt extract and glycerol were highly significant for the isolate growth, while sugar cane molasses, glycerol, and corn steep liquor were highly significant for melanin production. Also, the quadratic effect of corn steep liquor showed a significant effect on growth and production of melanin; on the other hand, the quadratic effect of sugar cane molasses, malt extract, and glycerol had a significant effect on melanin production. In addition, the interaction among malt extract and glycerol and between malt extract and corn steep liquor had a significant effect for isolate growth. Depending on the resulted mathematical model, the optimum combination of factors for maximum melanin productivity was evaluated as 10 g/l sugar cane molasses, 3.8 g/l corn steep liquor, and 2.5 g/l malt extract. The maximum productivity obtained under these optimum conditions was 210 mg/l.

Effect of light on melanin production

The growth and melanin productivity within batch bioreactor was obtained, and the result was calculated after 96 h from incubation of the reactor. The two-level FFD experiments were generated by Minitab 16 software; two variables were tested including brightness and intensity of the cold light source with two levels and one center point resulting in five runs (Table 5). Responses were measured for each of the performed run for *H.werneckii*

Table 3 Matrix and responses of CCD for carbon and nitrogen sources optimization

Run	Variables				Responses	
	SCM	Malt extract	Glycerol	CSL	Dry weight (g/l)	Melanin yield (mg/l)
1	2	2	− 2	2	3	90.88
2	0	0	0	0	3.95	55.93
3	0	− 1	0	0	5	29.30
4	1	0	0	0	3.4	66.35
5	2	2	− 2	− 2	3.5	77.01
6	2	− 2	2	− 2	3.2	59.77
7	0	0	0	− 1	*0*	*0*
8	− 2	2	2	2	6	54.84
9	− 2	2	− 2	− 2	3.5	35.09
10	0	0	1	0	3.4	73.87
11	− 2	− 2	2	2	2.2	61.18
12	0	0	0	0	3.95	55.93
13	− 1	0	0	0	3.5	78.34
14	2	2	2	− 2	*10*	39.17
15	− 2	2	− 2	2	2.5	68.0
16	2	− 2	− 2	2	4	*97.14*
17	0	0	0	1	3.4	82.10
18	2	− 2	2	2	4	77.56
19	0	0	0	0	3.95	55.93
20	− 2	2	2	− 2	5	17.39
21	0	1	0	0	3.1	49.90
22	− 2	− 2	− 2	− 2	1.4	56.48
23	0	0	0	0	3.95	55.93
24	2	− 2	− 2	− 2	3.3	62.67
25	2	2	2	2	3.1	76.30
26	− 2	− 2	2	− 2	2.2	37.60
27	− 2	− 2	− 2	2	3	63.22
28	0	0	0	0	3.95	55.93
29	0	0	0	0	3.95	55.93
30	0	0	0	0	3.95	55.93
31	0	0	− 1	0	6.1	71.60

Maximum and minimum yields were displayed as italics

EGYNDA08 including growth optical density (600 nm), dry weight (g/l), pH variability, and melanin yield (mg/l).

The main effect plot (Fig. 3a, b) was generated to detect the optimum values of brightness and intensity on the calculated responses. As shown in Table 5, the optimum levels of brightness and intensity were 50% lumin and 80% lux respectively. Within the bioreactor environment, in addition to the lighting conditions, the amount of melanin produced was found to be 228 mg/l representing approximately five-fold increase compared to the amount produced prior to optimization. A validation experiment was carried out, within bioreactor, under the proposed light conditions, and samples were withdrawn over time for the purpose of monitoring the growth and the yield of melanin under the achieved optimum conditions.

The growth was measured as optical density, dry weight, and pH of medium were measured under both light and dark conditions (Fig. 4).

As shown in Fig. 4, light conditions had a significant role on the growth of *H. werneckii* EGYNDA08 and melanin yield as the dry weight was increased up to 7.4 g/l and melanin yield was 228 mg/l in compared to 6.2 g/l and 160 mg/l for dry weight and melanin yield in dark conditions.

Table 4 ANOVA for CCD experiment at confidence level 90%

Variable	Responses					
	dry weight (g/l)			Melanin (mg/l)		
	Sum of squares	*F* value	*P* value	Sum of squares	*F* value	*P* value
A:sugar cane molasses	4.060	2.10	0.167	1927	9.68	*0.007*
B:malt extract	8.843	4.57	*0.048*	118.9	0.60	0.451
C:glycerol	5.838	3.01	*0.102*	939.3	4.72	*0.045*
D:corn steep liquor	0.305	0.16	0.697	3834.2	19.26	*0.000*
A^2	0.001	0.00	0.967	295	3.57	*0.077*
B^2	0.373	0.44	0.518	944.1	3.59	*0.076*
C^2	1.364	2.19	0.158	417.3	3.75	*0.071*
D^2	8.477	4.38	*0.053*	593.5	2.98	*0.103*
A*B	0.600	0.31	0.585	53.9	0.27	0.610
A*C	0.140	0.07	0.791	33.4	0.17	0.688
A*D	3.515	1.81	0.197	0.4	0.00	0.964
B*C	8.555	4.42	*0.052*	99.4	0.50	0.490
B*D	6.890	3.56	*0.078*	94	0.47	0.502
C*D	2.175	1.12	0.305	48.9	0.25	0.627

Significant factors were in italics

The production of melanin was enhanced under light conditions where the pigment was known to absorb light in a wide range of wavelengths, including the UV region, and the intensity of absorption decreases slowly when wavelengths increase (Meredith and Sarna 2006; Gessler et al. 2014). Melanin has the ability to absorb light energy and convert photon energy into heat energy that promotes growth (Riesz et al. 2006). Scaling up the process from the shake flask level to bioreactor level showed an increase in melanin productivity up to 228 mg/l. The maximum yield of melanin was achieved after 96 h from inoculating the reactor.

UV protection of *B. thuringiensis* subsp. *aegypti* (*Bt-C18*) toxins

B. thuringiensis is one of the most effective biological control agents used in agriculture bio-control though the main barrier facing its vigorous application is that most of the *B. thuringiensis* formulation is unstable under field conditions, due to the ultraviolet radiation in sunlight (Sansinenea et al. 2015).

In this experiment, the insecticidal activity of *B.thuringiensis* subsp. *aegypti* (*Bt-C18*) was investigated for controlling the population of cotton worm *S. littroalis*. The result of bioassay revealed that DHN melanin extracted from *H. werneckii* EGYNDA08 aids *Bt-C18* to survive the direct exposure to light conditions in comparison to the dark conditions and the effect of light on *Bt-C18* only without melanin (Table 6).

As shown in Table 6, the addition of melanin to *Bt-C18* had protected it against the inactivation of sunlight and played a powerful role preventing the harmful effect of UV radiation. Trials R1, R2, and R3 had shown that the mortality of cotton worm was increased in the presence of melanin in contrast with the presence of *Bt-C18* only. Although melanin was added in minimum

Table 5 The matrix and calculated responses for two-level FFD

Run	Variables		Responses			
	Brightness	Intensity	Growth of the isolate			Melanin yield (mg/l)
			O.D (600 nm)	Dry weight (g/l)	Final pH	
1	1	− 1	9.07	4.5	5.7	150
2	− 1	1	7.83	5.5	6.2	100
3	0	0	*19.66*	*10*	*7.7*	*220*
4	1	1	13.57	5	5.7	160
5	− 1	− 1	4.13	4.2	5.6	90

The highest response were estimated and in italics

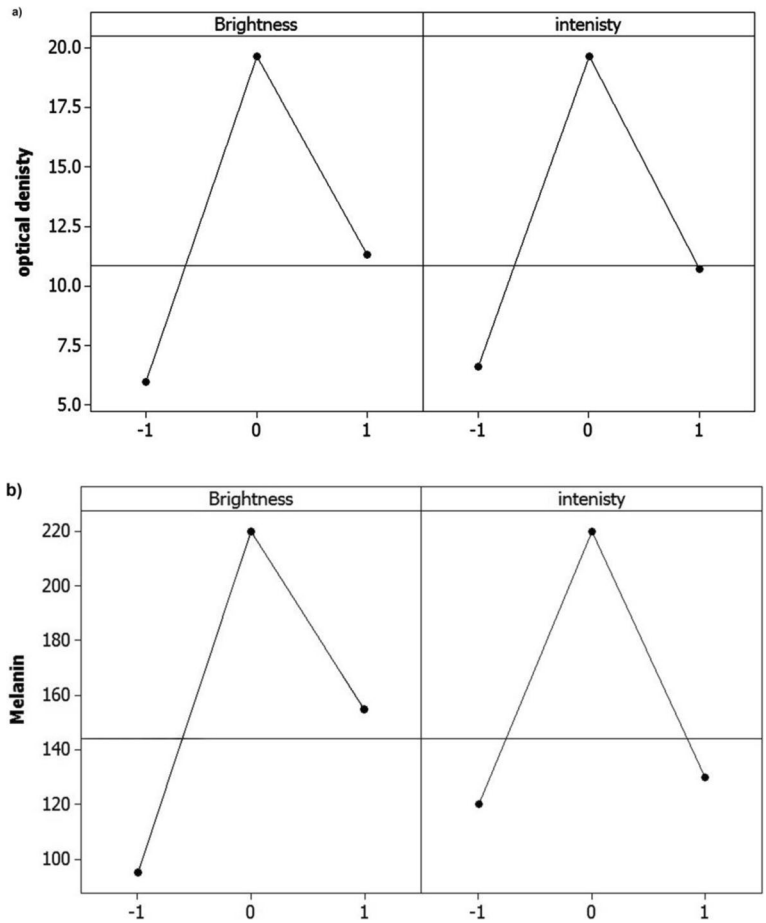

Fig. 3 Main effect plots showing optimum brightness and intensity affected on **a** optical density and **b** production of melanin by *Hortaea werneckii* EGYNDA08

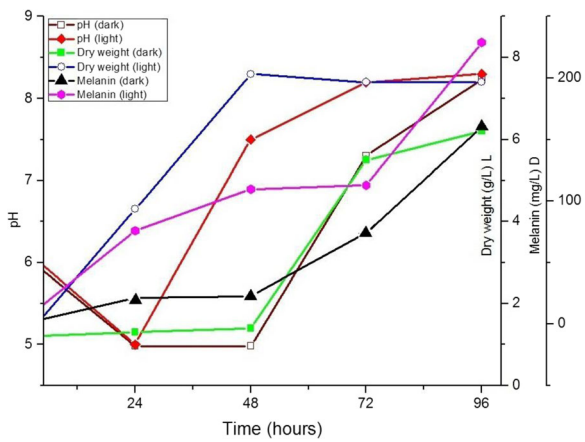

Fig. 4 Time trajectory showing the growth, melanin yield, and pH variation for the tested microbe *Hortaea werneckii* EGYNDA08 under both light and dark conditions

concentration (5 ppm), but it showed a great effect that increased by increasing melanin concentration until it showed higher effect than *Bt-C18* in dark conditions.

The insecticidal activity of *B. thuringiensis* strain *Bt-C18* was assayed with and without melanin in different concentrations (5, 10, and 15 ppm) after light exposure and in dark conditions on *S. littrolis*. DHN melanin extracted from *H. werneckii* EGYNDA08 was supplied to *Bt* formulations and subjected to light to detect the effect of melanin on protection of bioinsecticide.

Exposure of *Bt-C18* into light and dark conditions without melanin indicated that light conditions showed lethal effect on *Bt-C18* and its insecticidal potential against *S. littoralis* was decreased in contrast with its role in dark conditions; this result confirmed that sunlight have a negative effect on lethal of *Bt* (Brar et al. 2006). On the other hand, when DHN melanin was supplied to *Bt-C18*, the effect of bioinsecticide was maximized and the number of pathogenic larvae of *S. littoralis* was decreased. Melanin in minimum concentration (5 ppm) had a major effect in protection of bioinsecticide from light and UV and gave it

Table 6 UV protection of bioinsecticide by DHN melanin

Control insects			Test									
			Bt-C18 in dark		*Bt-C18* exposed to sunlight							
					Bt–C18		*Bt-C18* + melanin (5 ppm)		Bt-C18 + melanin (10 ppm)		Bt-C18 + melanin (15 ppm)	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
R1	0 ± 0	10 ± 0	6.6 ± 1.15	3.3 ± 1.15	5.6 ± 0.57	4.3 ± 0.57	7 ± 1	3 ± 1	8.3 ± 0.57	1.6 ± 0.57	8 ± 0	2 ± 0
R2	1.33 ± 0.57	8.6 ± 0.57	6.6 ± 1.15	3.3 ± 1.15	5.6 ± 0.57	4.3 ± 0.57	8 ± 0	2 ± 0	8.6 ± 0.57	1.3 ± 0.57	7.3 ± 1.15	2.6 ± 1.15
R3	1 ± 0	9 ± 0	7.3 ± 1.15	2.6 ± 1.15	6.6 ± 1.15	3.3 ± 1.15	7.3 ± 0.57	2.6 ± 0.57	9 ± 1	1 ± 0	9 ± 0	1 ± 0

the ability to perform its function as bioinsecticide; when melanin concentration increased to 10 and 15 ppm, its role was maximized and toxicity of *Bt-C18* was increased. Melanin was proven to be a potential natural bioprotectant for *Bt* formulas which is in line with the work performed by Sansinenea and Ortiz (2015).

Further work is needed to understand the real mechanisms behind the responses of the yeast to the different nutritional, physical and light/darkness conditions on growth, and melanin production.

Conclusions

A black yeast strain was isolated from halophilic habitat in northern Egypt, and it was identified as *Hortaea werneckii* EGYNDA08. Its melanin proved to be a strong radioprotectant to *B. thuringiensis* (*Bt-C18*)-based biopesticides. This supports the notion that microbial melanin can reasonably replace chemical radioprotectants making the pest control via *B. thuringiensis* (*Bt-C18*)-based biopesticide a greener process.

The growth and productivity of this unicellular fungus was optimized to produce the black melanin using cheap media components via two-level factorial design. The most potential components were subsequently subjected to response surface methodology which maximized the productivity of melanin. Surprisingly, light (brightness and intensity) and darkness duration resulted in increasing melanin productivity up to 228 mg/l under light conditions.

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Authors' contributions

All authors contributed equally and they all read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

The agreement of publication was taken, and as a corresponding author, I confirm that.

Competing interests

The authors declare that they have no competing interests.

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References

Akilandeswari P, Pradeep B (2016) Exploration of industrially important pigments from soil fungi. Appl Microbiol Biotechnol 100:1631–1643

Bajaj IB, Lele SS, Singhal R (2009) A statistical approach to optimization of fermentative production of poly (γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324. Bioresour Technol 100:826–832

Brar SK, Verma M, Tyagi R, Valéro J (2006) Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. Process Biochem 41: 323–342

Butler MJ, Lachance M-A (1987) Inhibition of melanin synthesis in the black yeast *Phaeococcomyces* sp. by growth on low pH ascorbate medium: production of spheroplasts from "albinized" cells. Can J Microbiol 33:184–187

d'Ischia M et al (2015) Melanins and melanogenesis: from pigment cells to human health and technological applications. Pigment Cell Melanoma Res 28:520–544

El Naggar AM, El Sayed HA, Elsalamony RA, Elrazak AA (2015) Biomass to fuel gas conversion through a low pyrolysis temperature induced by gamma radiation: an experimental and simulative study. RSC Adv 5:77897–77905

Elrazak AA, Ward AC, Glassey J (2013) Response surface methodology for optimising the culture conditions for eicosapentaenoic acid production by marine bacteria. J Ind Microbiol Biotechnol 40(5):477–487

Elsayed A, Mowafy AM, Soliman HM, Gebreil A, Magdy NI (2016) Characterization of new strains of *Hortaea werneckii* isolated from salt marshes of Egypt. Egypt J Basic Applied Sci 3:350–356

Gadd G (1982) Effects of media composition and light on colony differentiation and melanin synthesis in *Microdochium bolleyi*. Trans Br Mycol Soc 78:115–122

Garcia-Pichel F, Castenholz RW (1991) Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. J Phycol 27: 395–409

Garcia-Pichel F, Castenholz RW (1993) Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. Appl Environ Microbiol 59:163–169

Gessler N, Egorova A, Belozerskaya T (2014) Melanin pigments of fungi under extreme environmental conditions (review). Appl Biochem Microbiol 50:105–113

Gunde-Cimerman N, Zalar P, de Hoog S, Plemenitaš A (2000) Hypersaline waters in salterns–natural ecological niches for halophilic black yeasts. FEMS Microbiol Ecol 32:235–240

Jacobson ES, Hove E, Emery HS (1995) Antioxidant function of melanin in black fungi. Infect Immun 63:4944–4945

Kogej T, Wheeler MH, Rižner TL, Gunde-Cimerman N (2004) Evidence for 1, 8-dihydroxynaphthalene melanin in three halophilic black yeasts grown under saline and non-saline conditions. FEMS Microbiol Lett 232:203–209
Kollias N, Sayre RM, Zeise L, Chedekel M (1991) New trends in photobiology (invited review). J Photochem Photobiol A: Chem 9:135ą160
Kumar A, Vishwakarma HS, Singh J, Dwivedi S, Kumar M (2015) Microbial pigments: production and their applications in various industries. Int J Pharmaceutical Chem Biol Sci 5:203–212
Kurbanoglu EB, Kurbanoglu NI (2004) Ram horn peptone as a source of citric acid production by *Aspergillus niger*, with a process. J Ind Microbiol Biotechnol 31: 289–294
Kutty S, Lawman D, Singh I, Philip R (2013) Black yeasts from the slope sediments of Bay of Bengal: phylogenetic and functional characterization. Mycosphere 4:346–361
Kutty SN (2009) Marine yeasts from the slope sediments of Arabian Sea and Bay of Bengal PhD thesis. In: Cochin University of Science & Technology
Meredith P, Sarna T (2006) The physical and chemical properties of eumelanin. Pigment Cell Res 19:572–594
Montgomery DC (2008) Applications of design of experiments in engineering. Qual Reliab Engng Int 24:501–2. https://doi.org/10.1002/qre.969.
Osman YA, El-Morsi AA, Elwakil MA, Omer FH (2014) Five distinctive phages from an Egyptian industrial strain of *Bacillus thuringiensis* subsp. *aegypti*. J Environ Sci Technol 7(1):67
Pombeiro-Sponchiado SR, Sousa GS, Andrade JC, Lisboa HF, Gonçalves RC (2017) Production of melanin pigment by fungi and its biotechnological applications. In: Melanin. InTech
Ravishankar J, Muruganandam V, Suryanarayanan T (1995) Isolation and characterization of melanin from a marine fungus. Bot Mar 38:413–416
Riesz J, Gilmore J, Meredith P (2006) Quantitative scattering of melanin solutions. Biophys J 90:4137–4144
Rosas ÁL, Casadevall A (1997) Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. FEMS Microbiol Lett 153:265–272
Sangkharak K, Prasertsan P (2007) Optimization of polyhydroxybutyrate production from a wild type and two mutant strains of Rhodobacter sphaeroides using statistical method. J Biotechnol 132:331–340
Sansinenea E, Ortiz A (2015) Melanin: a photoprotection for *Bacillus thuringiensis* based biopesticides. Biotechnol Lett 37(3):483–490
Sansinenea E, Salazar F, Ramirez M, Ortiz A (2015) An Ultra-Violet Tolerant Wild-Type Strain of Melanin-Producing Bacillus thuringiensis. J Microbiol 8(7): e20910. https://doi.org/10.5812/jjm.20910v2.
Schweitzer AD et al (2010) Melanin-covered nanoparticles for protection of bone marrow during radiation therapy of cancer. Int J Radiat Oncol Biol Phys 78: 1494–1502
Solano F (2014) Melanins: Skin Pigments and Much More—Types, Structural Models, Biological Functions, and Formation Routes. New J Sci 28:498276. https://doi.org/10.1155/2014/498276.
Paech K, Tracey MV (2013) Modern Methods of Plant Analysis/Moderne Methoden der Pflanzenanalyse. Springer Sci Business Media 2: https://doi.org/10.1007/978-3-642-64961-5.
Youngchim S, Morris-Jones R, Hay RJ, Hamilton AJ (2004) Production of melanin by *Aspergillus fumigatus*. J Med Microbiol 53:175–181
Zalar P, Novak M, De Hoog G, Gunde-Cimerman N (2011) Dishwashers–a man-made ecological niche accommodating human opportunistic fungal pathogens. Fungal Biol 115:997–1007
Zhdanova NN, Zakharchenko VA, Vember VV, Nakonechnaya LT (2000) Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. Mycol Res 104:1421–1426