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Exploring Antimicrobial Potentials of Melanin from A Black Yeast Strain

Hani Saleh (Corresponding authors)

Botany Department, Faculty of Science, Mansoura University,

Egypt. E-mail: hany.saleh12489@yahoo.com

Ahmed Abdelrazak

Botany Department, Faculty of Science, Mansoura University,

Egypt. E-mail: ahmed_bt@mans.edu.eg

Ashraf E. Elsayed

Botany Department, Faculty of Science, Mansoura University,

Egypt. E-mail: ashraf.badawy@yahoo.com

Yehia Osman

Botany Department, Faculty of Science, Mansoura University,

Egypt. E-mail: lazeikyao@gmail.com

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Abstract

Melanin is a pervasive pigment that synthesized in all living organisms from prokaryotes to eukaryotes by polymerization or hydroxylation of organic compounds. Melanin is important for survival of fungi in extreme conditions, like as high salinity that are typical for hypersaline environment. The ascomycetous black yeast *Hortaea werneckii* was isolated from such environment was identified as a potential source of melanin. Melanin has different applications in many fields like as cosmetics and pharmaceutical industries. In this study,

Hortaea werneckii was isolated from hypersaline Egyptian habitat on enrichment media. Our study was carried out to study the ability of *Hortaea* to produce melanin and optimization of culture conditions that enhance the production. Detection of different genes responsible for production of melanin from the isolate was also studied. The optimum cultivation conditions that led to a maximum melanin yield was found to be; temperature 22^{o}C and pH 6.0 achieving 48.5 mg/L. Also, extracted melanin showed antimicrobial activity against different pathogens like as, *Staphylococcus sp*, *Streptococcus pyogenes*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis, Erwinia carotovora.*

Keywords: Melanin, Black Yeast, Antimicrobial, Central Composite Design

1. Introduction

The *Hortaea werneckii* is the most dominant halophilic fungus described (Plemenitaš and Gunde-Cimerman, 2005). It also named a "black yeast" because of melanin production (Hoog and GuÉho; Göttlich *et al.*, 1995). It was isolated from different saline habitats, such as seawater-related environments, wood obscure in hypersaline waters (Zalar *et al.*, 2005), and from surface layers of microbial mats of salterns (Cantrell *et al.*, 2006). *H. werneckii* represents 85 to 90% of all isolated fungi from environments where salt concentration reached 20% NaCl, and it occasionally was isolated from saline waters with lower concentrations; below 10% NaCl (Gunde-Cimerman *et al.*, 2000; Butinar *et al.*, 2005).

Melanin, the polyphenolic dark-brown to black polymers, is observed in all living organisms from prokaryotes to eukaryotes (Bell and Wheeler, 1986). Fungal melanin existed in the cell wall and is formed by auto-oxidation or enzymatically through tyrosinase and poly-ketidesynthase in 3, 4-dihydroxyphenylalanine (DOPA) and 1,8-dihydroxynaphthalene (DHN), respectively (Kutty, 2009). Melanin enables fungi to cope with extreme environments like as, radiation resistance (Zhdanova *et al.*, 2000), oxidizing agents (Jacobson *et al.*, 1995), extreme temperatures (Rosas and Casadevall, 1997), protection against enzymatic lysis (Butler and Lachance, 1987), osmotic stress (Ravishankar *et al.*, 1995), high concentrations of salts and detergents. Microbial Melanin has a great potential to be used in UV sunscreens, biomedicine, dermo-cosmetics, nanotechnology and materials science (Pombeiro-Sponchiado *et al.*, 2017).

In the present work; the isolated local black yeast strain was found to be a potential high melanin producer. On the shake flask level, the melanin production was enhanced via application of a Central Composite Design matrix. Subsequently, the highly purified melanin antimicrobial activity was estimated against various multi-drug resistance pathogens and it showed activity against *Candida albicans, Klebsiella pneumonia, Staphylococcus aureus than strep* and *Streptococcus pyogenes.*

2. Materials and Methods

2.1 Samples Collection

In November 2013, marine sediment and water samples were collected from solar saltern, located on Gamassa-Baltim road (latitude and longitude are 31.47-31.46), Mediterranean Sea

coast in Egypt. Samples were transported directly to the laboratory in sterilized containers and the black yeasts were isolated by serial dilution method (up to 10^{-7}).

2.2 Media Preparation

Isolation of the black yeast was carried out after growing in liquid medium composed of: 50 ml sea water, 2 g/L yeast extract and the pH was adjusted to 8 as described by (Gunde-Cimerman *et al.*, 2000). A mixture of penicillin G as 1 million unit dissolved in 5 ml sterilized distilled water, while ampicillin and streptomycin were prepared as 1 gm dissolved in 10 ml and they were added to inhibit bacterial contaminants. The inoculated flasks were incubated at 28^{o}C for one week, in a rotary shaker incubator. Once the culture turned black, a 10 µL aliquot was transferred and streaked onto agar plates. Cells and hyphae of the purified isolates were studied under light microscope (40x).

2.3 Isolates Identification

Internal Transcribed Spacer (ITS) sequences were known as the most rapid and accurate tool used for identification of yeast isolates. In order to identify the local isolate, we have to purify its genomic DNA. DNA was extracted from the locally isolated black yeast species by the FastDNA® Spin Kit (Qbiogene Co,), using manufacturer suggested protocol. The DNA concentration, purity and integrity were assessed by measuring the absorbance at 260 nm using Nano Drop spectrophotometer and agarose gel electrophoresis, respectively. Two ITS primers were used in the amplification process: 18ITS1 forward primer (CTTGGTCATTTAGAGGAAGTAA) and 18ITS4 reverse primer (TCCTCCGCTTATTGATATGC) (Elsayed et al., 2016). PCR was performed in a total volume of 50 µl and PCR master mix, containing 5.0 µl 1X buffer (10mM Tris-HCl PH 8.3, 50mM KCl, 2mM $MgCl_2$), 0.8 µl of deoxynucleoside triphosphate (dNTPs) mixture, 2 µl each of ITS primers, 3.0 µl of 50 mM MgCl2, 0.5 µl of Taq DNA polymerase, 1 µl of extracted DNA and distilled water used to complete 50 µl The PCR amplification reaction was achieved by using DNA Thermal cycler according to the following program; first denaturation temperature was 94^{o}C for 5 min, denaturation at 94^{o}C for 45 sec, annealing at 55^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 10 min (Cole *et al.*, 2008).

The PCR product was purified by the Dye Ex 2.0 Spin Kit. The purified products were sequenced using applied biosystems ABI PRISM Big Dye Terminator v1.1 method. DNA sequences were analyzed with Finch TV version 1.4.0, CAP3 software to prepare the contiguous sequence (Contig) and Seaview software version 4.5.3 was used to identify the isolates by creating the evolutionary tree. BLAST and Sequence Match was used to compare the sequence to the published ones in the GenBank database.

2.4 Pigment Extraction

Melanin was extracted and purified from the local black yeasts as per (Gadd, 1982). The whole culture was centrifuged at 10,000 rpm for 10 min, the collected cell pellet was washed with distilled with distilled water and used for melanin extraction. The harvested biomass of the isolate was autoclaved in presence of 1N NaOH (20 min, 121℃, at pressure 1.5 bar),

followed by centrifugation for 10 min at 8,000 rpm and the supernatant containing melanin was collected. Melanin was precipitated by Conc. HCl until pH reached 2.0, collected again by centrifugation at 10,000 rpm for 10 min. The precipitated melanin was then dried in dehumidified atmosphere.

2.5 Detection of Genes Responsible for Melanin Biosynthesis

Polymerase chain reactions (PCR) were performed to detect the presence of all genes encode for enzymes involved in the synthesis of melanin in black yeast. The three enzymes participate in the biosynthesis of DHN melanin are Polyketide synthase, Scytalone dehydratase and HN reductase. Five sets of primers were designed, via primer 3 software (version 0.4.0), depending on the previously published sequences (Table 1). The PCR reactions were carried out in 20 µL reaction volume and included: 4 µL 5X buffer, 1 µl of genomic DNA as a template, 0.5 µl of Taq DNA polymerase, 2 µl of each primer forward and reverse and completed up to 20 µl by distilled water. A 40 cycle PCR amplification included a first denaturation step at 94^{o}C for 3 min, and a final extension step at 72^{o}C for 5 min. Each PCR cycle consisted of a denaturation at 94^{o}C for 30 sec, annealing at 52^{o}C for 30 sec, and an extension at 72^{o}C for 1 min. The gel-electrophoresis was performed at a voltage of 50 V/cm. At final, bands was visualized under long UV light and photographed (Feng *et al.*, 2001).

Table 1: Primers used for detection of genes encode for melanin production enzymes

Target gene	Primers	Nucleotide sequence	Product size (bp)
Poly-Ketide Synthase	PKF-1 F	AACGGCGGCCTATACAGTTC	605
	PKS-1 R	TTCCTCGGCGATGATACTGC	
	PKS-2 F	ACCAGCTCTTTCCAGGAAGC	415
	PKS-2 R	TGCTGACCAGGGTTAGGAGA	
Scytalone Dehydratase	SCD F	CCAGCTTAGCCCGATGATCT	227
	SCD R	TCAAAGGTGATGTTGCCAGC	
Hydroxy naphthalene (HN)-reductase	THN-1 F	ACCCCTAAGCAAACACCCTG	964
	THN-1 R	CTCCGTACACGCAGTCTCAA	
	THN-2 F	GGCTCAGGTAAGCTCAAGCA	360
	THN-2 R	GGTGAAGACACGGTCGAACT	

2.6 Optimization of Cultivation Conditions

Temperature and pH are the most important factors that affect the productivity of most microbial metabolites. Central composite design (CCD) was applied to determine the optimum levels of the two variables using Minitab 16 software. Each variable had five levels, six center points and star points included in CCD matrix to evaluate the curvature. CCD have the ability to supply an consideration of the main effect of every variable individually, quadratic effect and also for interaction between factors (Elrazak et al., 2013). A second-order polynomial model was created for the estimation of the medium composition optimum production for melanin production (Equation 1):

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_{ii} + \Sigma \beta_{ij} X_{ij} \qquad Equ.1$$

Where, β_i, β_{ii} and β_{ij} are the regression coefficients for each factor, square effects and interaction between factors respectively. The tested levels used to generate the CCD matrix are summarized in Table 2.

Table 2: Variables and their levels used for CCD experiment

Variables	Levels				
	-2	-1	0	+1	+2
Temperature (^oC)	10.85	15	25	35	39.14
pH	6.08	6.5	7.5	8.5	8.9

Thirteen experiments were carried out in 250 mL flask containing 50 mL sea water and yeast extract; 2 g/L. Responses were measured in terms of growth (dry weight g/L) and melanin production (mg/L). Validation for CCD experiments were performed based on the generated analysis result(s).

2.7 Antimicrobial Activity of DHN-Melanin in Vitro

The antimicrobial activity of DHN-melanin extracted from *H. werneckii* EGYNAD08 was studied using disc diffusion method against clinical isolates of *Bacillus subtilis, Staphylococcus epidermis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Candida albicans, Erwinia carotovora, Methicillin resistant Staphylococcus aureus (MRSA), Proteus vulgaris, Streptopyogenesis and Enterobacter cloacae*.

Sterilized filter paper discs (6 mm) were immersed in 1 ml of extracted melanin dissolved in DMSO solution and control discs were immersed in DMSO only. All the discs were dried and then placed onto the surface of the tested bacterial and fungal lawns, incubated for 18 to 24 hours at 37^{o}C, and the inhibition zone were recorded (Kumar and Mamidyala, 2011). The result was compared with Streptomycin antibiotic discs in concentration of 10 µg.

3. Results

3.1 Isolation and Identification of a Melanin Producer Yeast

A halotolerant black yeast was isolated from samples collected from salt salterns on Mediterranean Sea coast. The isolate was selected for its noteworthy black color colonies and hence its ability to produce the black pigment "Melanin". Microscopic examinations showed the individual cell have a filamentous growth with double cell pattern with septa and melanized cell wall (Figure 1).

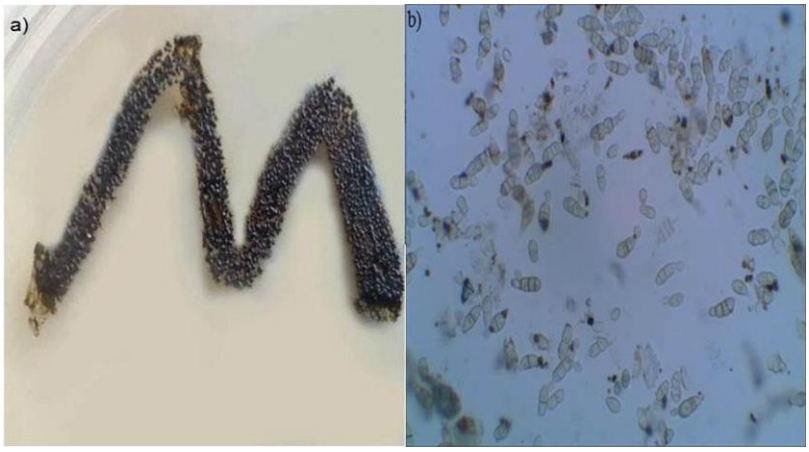

Figure 1: a) growth of black yeast on agar plates, b) microscopic examination

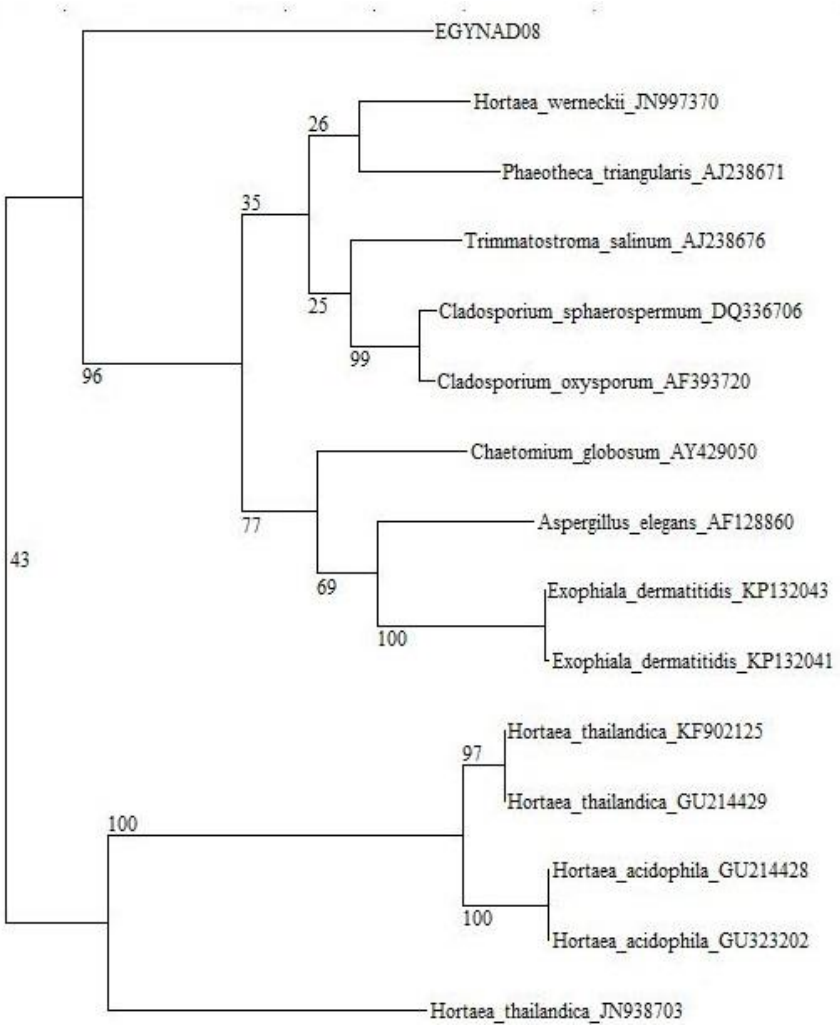

Figure 2: Phylogenetic tree of *Hortaea werneckii* EGYNAD08 based on the analysis of ITS genes sequences showing its phylogenetic position compared to the closely related species of black yeast

The isolate was identified by means of ITSregion and 18S RNA gene sequencing and analysis as Hortaea werneckii. This ITS region sequencing was exposed to a multiple alignment algorithm against the closest published sequences and a phylogenetic tree was generated (Figure 2) using seaview software (version 4.5.3). The closest strain to the isolate under investigation was found to be H. werneckii Hw6 JN997370. The obtained sequence was submitted to the Genbank and was issued an accession number of (KU341734).

3.2 Detection of Genes Responsible for Melanin Biosynthesis

PCR technique was used to determine the genes encode the biosynthesis pathway of DHN-melanin in *H. werneckii* EGYNAD08. The product sizes of the used oligonucleotide primers were almost identical with the published ones for other known melanin genes in different fungal strains. PCR product appeared at 605 bp, 415 bp, 227 bp, 964 bp and 360 bp were obtained and coincides with those characteristic for melanin encoding genes enzymes: Polyetide synthase in *Aspergillus fumigatus* (PKS1), Polyketide synthase of *Wangialla dermatitis* (PKS2), scytalone dehydratase in *Colletotrichum lagenarium* (SCD), HydroxyNapthalene of *Aspergillus fumigatus* (HN1) and HydroxyNapthalene of *Alternaria alternate* (HN2), respectively (Figure 3).

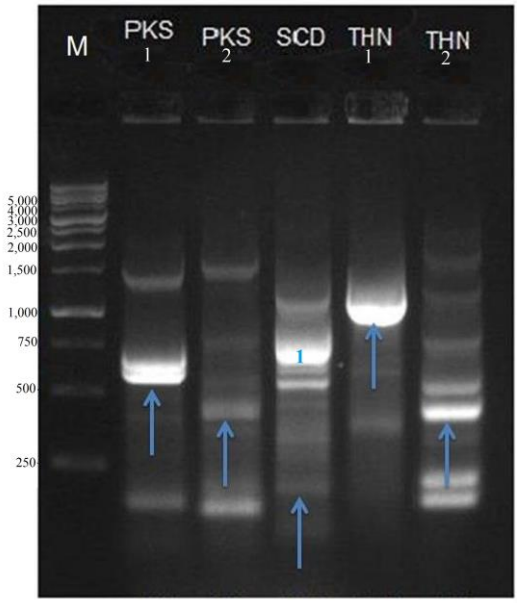

Figure 3: PCR product showing genes encodes melanin produced by *Hortaea werneckii* EGYNAD08 at annealing temperature (Ta=52^{o}C)

The highly amplified DNA fragments represent the PCR products of the genes PKS, SCD and HN encode for the polyketide synthase, scytalone dehydratase and hydroxynaphthalene reductase enzymes in *H. werneckii* (high intensity bands) as pointed to by the arrows. The multiple bands with different molecular sizes and electrophoretic mobility suggested a non-specific binding of the oligonucleotide primers used. These primers were designed as indicated in the materials and methods section against the published sequences for other fungi: *Aspergillus fumigatus*, *Wangialla dermatitis*, *Colletotrichum lagenarium* and *Alternaria*

alternate respectively. The faint PCR product of the enzyme SCD compared to band 1 in the same lane puzzled us.

3.3 Optimization of the Cultivation Conditions

A CCD matrix identified the optimum temperature and pH led to highest yield of melanin. The optimized responses of the experiment were expressed as dry weight of the organism and concentration of melanin (Table 3).

Table 3: CCD Matrix responses for optimization of pH and temperature

Run	Variables		Responses	
	Temperature ^{o}C	pH	Dry weight. g/L	Melanin mg/L
1	0	0	1.4	10.78
2	0	0	1.8	22.56
3	-1	-1	1.3	17.01
4	+1	-1	**0**	**0**
5	+2	0	0.8	0
6	0	0	1.4	10.78
7	-1	+1	1	9.84
8	0	0	1.8	22.56
9	+1	+1	1	0
10	0	+2	**2.8**	23.25
11	-2	0	0	0
12	0	-2	2.5	**37.10**
13	0	0	1.4	10.78

Maximum and minimum values were **Bold** and underlined

From Table 3, it was clear that the lowest dry weight and melanin yield were obtained at run number 4 and the highest were achieved at run number 10 for dry weight (2.8 gm/L) and run number 12 for melanin yield (37.10 mg/L).

The ANOVA of the linear, quadratic effects and the interaction between factors was applied and each term with P-value<0.1 were determined as statistically significant (Table 4). The analysis clearly showed that; the quadratic effect of temperature to be the most significant (at 99% level of confidence) factor affecting the growth and melanin production of the isolate *H. werneckii* EGYNAD08 and followed by the quadratic effect of pH (at 89% level of confidence) on the same calculated responses.

Table 4: Analysis of variance of the CCD experiment for optimization of pH and temperature

Variables	Responses					
	dry weight (g/L)			Melanin (mg/L)		
	Sum of squares	F-value	P-value	Sum of squares	F-value	P-value
A. Temperature	0.003	0.01	0.913	90.16	1.52	0.258
B. pH	0.158	0.58	0.473	89.55	1.51	0.259
A2	4.566	14.45	**0.007**	673.65	11.33	**0.012**
B2	0.952	3.47	**0.105**	191.65	3.22	**0.116**
A*B	0.422	1.54	0.255	12.88	0.22	0.656

P- Significance probability

The significant factors presented as **Bold** and underlined

Second order polynomial models that predicted the dry weight and the concentration of melanin as a function of the experimental variable are shown in equations 2 and 3:

For dry weight,

$$Y1=22.746+0.131*A-6.221*B+0.007A*B-0.370*A2+0.032*B^2 \quad \textit{Equ. 2}$$

For melanin,

$$Y2=316.357+3.238*A-86.562*B+0.179A*B-0.098*A2+5.248*B^2 \quad \textit{Equ. 3}$$

A 3D response plots were generated to show the interaction and the possible optimum levels of each of the tested variables on each of the calculated responses (Figure 4)

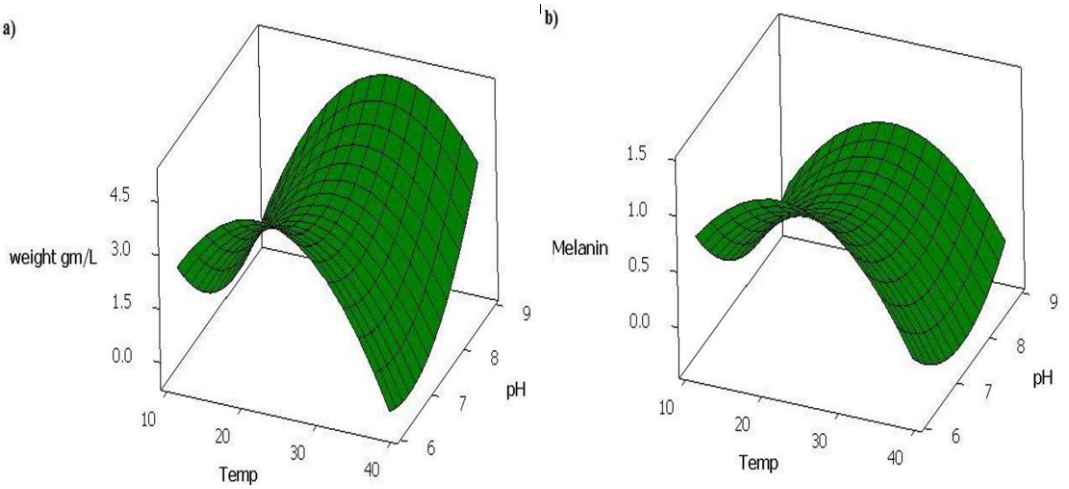

Figure 4: 3D Surface plot showing the effect of interaction between temperature and pH on a) dry weight of *Hortaea werneckii* EGYNAD08 gm/L and b) Melanin mg/L

After mathematical and statistical analysis and by using the generated optimization model; the optimum temperature was found to be 22°C with an initial pH 6.0 that resulted in maximum yield of melanin. The predicted value was 40.73 mg/L of melanin but the melanin concentration achieved was maximized to 48.5 mg/L.

3.4 Antimicrobial Activity of the Extracted Melanin in Vitro

It was evident from the data presented in Table (5) that DHN-melanin has inhibitory effect on some bacteria and fungi used in this test. As clear zone formed around melanin disc was measured by ruler and detected as the response of inhibitory effect of melanin and streptomycin. Melanin has inhibited *Candida albicans* as well as *Streptococcus pyogenes* and *Erwinia carotovora.* According to the guideline of using CLSI any number below 16 mm zone of inhibition is neglected and indicative of resistance, 16-21mm Intermediate sensitivity, 21-34 mm susceptible.

Table 5: Antimicrobial activity of DHN-melanin

Bacterial strains	Inhibition zone diameter (mm)		
	Melanin	Control	Streptomycin antibiotic (10 µg)
Streptococcus pyogenes	18	-	18
Escherichia coli	-	-	-
Pseudomonas aeruginosa	-	-	11
Enterobacter cloacae	-	-	10
Proteus vulgaris	-	-	-
Staphylococcus epidermis	12	-	20
Erwinia carotovora	14	8	18
MRSA	-	-	17
Staphylococcus aureus	12	-	10
Klebsiella pneumonia	9	-	-
Bacillus subtilis	-	-	15
Candida albicans	17	8	13

(-) represent negative effect of melanin on a pathogen

The extracted melanin from *Hortaea werneckii* EGYNAD08 showed more activity than Streptomycin against *Candida albicans, Klebsiella pneumonia, Staphylococcus aureus* and similar to activity against *Streptococcus pyogenes.*

4. Discussion

A halophilic new black yeast strain was isolated and was found to possesses high potentials for production of high amounts of a black pigment; melanin. It exhibited close similarities as those published by (Gunde-Cimerman *et al.*, 2000).

Nucleotide sequences tools, such as the basic local alignment search tool (BLAST) and SeqMatch analysis of the nucleotide sequences of the nuclear ribosomal internal transcribed spacer (ITS) region confirmed the belonging of the local isolate of black yeast to the genus *Hortaea* (Schoch *et al.*, 2012). Phylogenetically, the local strain was found to be closely related to *Hortaea werneckii*.

Moreover, PCR technology and use of specific primers have detected the existence of all enzymes necessary to synthesis melanin by this local isolate. The primers were designed to detect polyketide synthase (PKS), scytalone dehydratase (SCD) and hydroxy naphthalene reductase (HN) within the genome of the local isolate *Hortaea werneckii* KU341732. These three enzymes act in subsequent fashion to synthesize melanin. (Tsai *et al.*, 1999) working on *Aspergillus fumigatus* have reported that PKS converts aceyl-coA or malonyl-coA into 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). In 1996, this compound was found to be reduced into scytalone by HN reductase by *C. lagenarium* (Perpetua *et al.*, 1996). Whereas, SCD catalyzed the dehydration of scytalone into 1,3,8-trihydroxynaphthalene (1,3,8-THN), as reported in the fungus *A. alternate* (Kimura and Tsuge, 1993). Detection of presence of complete set of melanin genes encode for the biosynthesis of melanin was of great importance to understand how the local isolate of *Hortaea werneckii* EGYNAD08 makes melanin and confirmed it as DHN- melanin. This type of melanin is mostly associated to ascomycetes (Bell *et al.*, 1976).

The optimization of the conditions production of DHN- melanin by the local strain such as temperature and pH was targeted since these two factors exerted remarkable effect on growth and productivity of many important compounds by several industrial microbes (Pombeiro-Sponchiado *et al.*, 2017). The best and most recent approaches to attain such optimization are the application of statistic-based methodology (Elrazak *et al.*, 2013; Osman *et al.*, 2016). A prominent approach is the use of the Central Composite Design (CCD) experiment. Usually, the results of the CCD were subjected to multi-way ANOVA to show the quadratic effect of both temperature and pH. These two variables exerted the most significant influence on growth of the local isolate as well as the production of melanin. Further analysis illustrated by the 3D surface plot (Figure 4), showed that maximum production of melanin (37.1 mg/L) was achieved at temperature of 22^{o}C and pH 6.0. This is a four-fold increase compared to the amount produced by the isolated strain prior optimization. Most of the earlier studies have revealed that the optimum pH range of the fungal growth and melanin production is between 3.0 and 10.0 (Gunasekaran and Poorniammal, 2008) as published article by Petrovič et al., indicated that the maximum production of melanin by *Hortaea werneckii* strain B-736, isolated from salterns near Slovenia, was achieved at temperature 27^{o}C and pH 7.0 (Petrovič *et al.*, 1999)

Unlike one factor at a time (OFAT) experiments, experiments that designed statistically have

the ability to test the interaction between factors and the square terms which detect the quadratic effect of the variable. At level of confidence 90 %, when a P-value of any factor is less than 0.1, it could be estimated as statistically significant. General wise; the smaller P-value, the higher the significance of the corresponding variable.

Generally, melanin possesses a number of unique features and qualities to be used in food processing industries, cosmetics, pharmaceutical, and electronic. Moreover, microbial melanin are produced easily on cheap culture medium and resist seasonal variations (Plonka and Grabacka, 2006; d'Ischia et al., 2015). Despite the advancements of technology in identification of different chemical compounds, the chemical structure of melanin is not definitive, which complicate studies on its antimicrobial activities. Structure of molecules gives clue on its biological activities. This important aspect is of great help in exploring the antimicrobial potential of the yeast melanin against a wide array of multidrug resistant microbes (Nosanchuk and Casadevall, 2006). The extracted melanin from the local *Hortaea werneckii* EGYNAD08 showed a 20% increase in activity against *Staphylococcus aureus* which is a remarkable increase against standard antibiotic and successfully inhibited the streptomycin resistant *Klebsiella pneumonia*. This is a promising potentiality for the use of melanin in fighting such invasive microbe. In addition, the obtained melanin showed a fungistatic activity against *Candida albicans*.

5. Conclusion

This study provides a valuable halophilic melanin producer; *Hortaea werneckii* EGYNAD08 as it was isolated from halophilic Egyptian habitat. Genes responsible for melanin biosynthesis was identified as polyketide synthase, scytalone dehydratase and hydroxyl naphthalene reductase. CCD experiment was carried out to optimize cultivation conditions and was identified as 22^{o}C and 6.0 for temperature and pH respectively. As melanin have many aspects in pharmaceutical and medical application, the antimicrobial activity was estimated against various pathogens showing a very promising antimicrobial activity against the proposed multi-drug resistancestrains.

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