

## Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*

M. Helan Soundra Rani, T. Ramesh, J. Subramanian, \*M. Kalaiselvam

CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, Tamilnadu, India.

### ABSTRACT

Melanin is nearly a ubiquitous pigment synthesized by living organisms in the course of hydroxylation and polymerization of organic compounds. Melanins have immense application potentials in the field of agriculture, cosmetics and pharmaceutical industries (photoprotection and mosquitocidal activity isolated from *Streptomyces*). In this study, the halophilic black yeast, *Hortaea werneckii* produced a diffusible dark pigment on potato dextrose agar. This work was designed to study the ability of the yeast for the production, characterization and optimization of the melanin pigment with different nutrient sources. Characterization of melanin was analyzed by UV spectroscopy, FT IR, SEM and antibacterial potential of melanin was also studied. The favorable condition for the high yield of melanin was found to be glucose as carbon source and peptone as nitrogen source with the optimum parameters like temperature 30°C, salinity 15‰, pH 7.0, incubation period of 168 hrs and the rice bran as a cheaper substrate, which produced 5.60g/L of melanin. It also showed inhibitory activity against potential pathogens and activity was observed in *Salmonella typhi* (17mm) and *Vibrio parahaemolyticus* (15mm). It was concluded that the melanin of *Hortaea werneckii* isolated from solar salterns possess a high antibacterial activity and could act as a suitable source of new antimicrobial natural products.

**Keywords:** Halophilic fungi, black yeast, *Hortaea werneckii*, melanin, antibacterial activity.

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### \*Address for correspondence:

**M. Kalaiselvam**

CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, Tamilnadu, India.

E-mail: kalaifms@gmail.com

### INTRODUCTION

Melanin is a well-known and universal pigment in living organisms. It brings many benefits to human beings and especially plays a key role to protect internal tissues from the harmful effects of ultraviolet rays [1- 4]. The most common form of biological melanin is a complex polymer of either or both of 5, 6-indolequinone and 5, 6-dihydroxyindole carboxylic acid. Melanin pigments are negatively charged hydrophobic [5], and high-molecular-weight compounds. It is used commercially as a component of photo productive creams for anti-melanoma therapy and also reported to possess immunopharmacological properties [6]. Recent studies have shown that melanins are highly immunogenic [7] and have anti-inflammatory properties [8, 9]. It has been

shown to protect micro-organisms against UV-radiation [10-12], microbial lysis [13-16], oxidants killing by alveolar macrophages [17, 18] and defense responses of host plants and animals against fungal infection [19-21]. Melanin synthesis in fungi is associated with virulence in a number of human pathogenic fungi, such as *Cryptococcus neoformans* [22-24] and *Sporothrix schenckii* [25, 26]. *Hortaea werneckii*, melanized halophilic black yeast like fungus capable of causing Tinea nigra in humans [27]. This fungus has been reported from several environments around the world and it has shown a preference to hypersaline habitats but not used for the melanin production. They confer certain advantages to fungi such as increasing their survival potential in some

environments and enhancing their virulence [28]. The strain had the highest frequency of isolation and grew at the maximum NaCl concentration (25%) tested (optimum at 10%) and had an optimal growth at 29°C [29]. Cultures of *Hortaea werneckii* produced more hyphae and became more melanized at 30°C. Ultra structural studies of *Hortaea werneckii*, cell walls showed the organization of melanin granules is dependent on the concentration of salt in the medium [30, 31]. The membrane fluidity decreased with the rise in NaCl concentration, while in halophilic yeast, such as *Hortaea werneckii* the fluidity is maintained over increasing salt levels and reduces the porosity. Several types of melanin have been described in bacteria, fungi and animals; eumelanins, phaeomelanins, allomelanins and pyomelanins. Eumelanins are formed from Quinines and free radicals. Fungal melanins are complex pigments which are produced by two different synthetic pathways, known as the DHN (1,8-dihydroxynaphthalene) and L-DOPA (L-3,4-dihydroxyphenylalanine) pathways, depending on the species [32]. The DHN pathway of melanin biosynthesis is very common in the fungi kingdom [33]. When compared to terrestrial fungi the marine fungi are expected to produce novel and potentially active metabolites and also the scarcity of reports regarding melanin from marine habitats especially hypersaline environments leads us to undertake the study.

## MATERIALS AND METHODS

### Isolation and Identification:

Solar salterns water samples were collected from Marakkanam near Pondicherry, India. Samples were capped and brought to the laboratory. The samples were serially diluted (up to 10<sup>-6</sup> dilutions) by adding 1ml of water sample in 9ml of distilled water. About 1.0ml of diluted samples were plated on potato dextrose agar (1M NaCl concentration) media by pour plate technique and added 150mg l<sup>-1</sup> chloramphenicol to avoid bacterial contamination by then kept in light chamber at 30°C about 7-15 days. The isolated fungal strains were identified by standard mycological manual [34].

### Extraction and purification of melanin:

Due to the black appearance of the colony, the yeast was subjected for melanin extraction. The method of Gadd [35] was used for extracting the melanin pigment. Disc (15mm diam) were cut from 15 days old colonies, boiled for 5min in 5ml distilled water and centrifuged. The pigment was extracted by autoclaving with 3ml of 1M NaOH (20mins, 120°C). The alkaline pigment extract was acidified to pH 2 with concentrated HCl to precipitate the melanin. The precipitate was washed 3 times in distilled water and dried overnight at 20°C in a dehumidified atmosphere before further analysis.

### UV and Fourier- Transform infrared (FT IR) Spectrophotometer:

The pigment was subjected in 3 ml of sodium borate buffer (pH 8.0) and its UV-visible spectrum was measured in Perkin Elmer. For the IR investigation, the purified pigment was ground with infra red quality KBr (1:10), pressed into discs under vacuum using SHIMADZO spectrophotometer [36]. The spectral width was 4000-5000cm<sup>-1</sup> and the spectral resolution was recorded.

### Optimization of melanin:

To optimize the culture conditions for melanin production, the strain was inoculated at different conditions such as temperatures (25, 30, 35, 40 and 45°C), salinity (10‰, 15‰ and 20‰), pH range (6, 7, 8 and 9) and incubation periods (24, 48, 96, 120, 144, 168 and 192 hrs) and also tested various carbon sources (dextrose, fructose, sucrose and glucose), nitrogen sources (yeast extract, peptone and corn steep) and cheaper substrates (rice bran, wheat bran and coconut cake). The inoculums were maintained in rotatory shaker. All the experiments were carried out in 1000ml conical flasks containing 100ml of production medium.

### Antibacterial activity of melanin pigment:

Antibacterial activity was tested by well diffusion method. Pathogens like *Salmonella typhi*, *Klebsiella pneumoniae* and *Vibrio parahaemolyticus* were swabbed on a Muller- Hinton agar and 10µl of pigment extract was placed in the well and incubated at 37°C for 24hrs.

### Scanning Electron Microscopy:

The melanin sample were fixed in 2% (v/v) glutaraldehyde (pH 7.4) prepared on a phosphate buffer and cell fixation and initial contrasting was performed with 2% water solution of OsO<sub>4</sub> for 2hrs. After washing in distilled water, dehydration was carried out in a series of ethanol solutions and twice in acetone. The samples were dried in the grid and the samples were observed under a Scanning Electron Microscope.

### RESULTS

In this study, hypomyces black yeast *Hortaea werneckii* was chosen for melanin study (Fig. 1a, 1b).

#### UV-Vis Absorption spectra:

The nature of the pigment was confirmed by spectral properties. Its UV spectrum was typical of the absorption of melanin. Observation of melanin in the UV absorption spectrum exhibited absorption peak of maxima at 235nm-300nm which was shown in the (Figure 2).

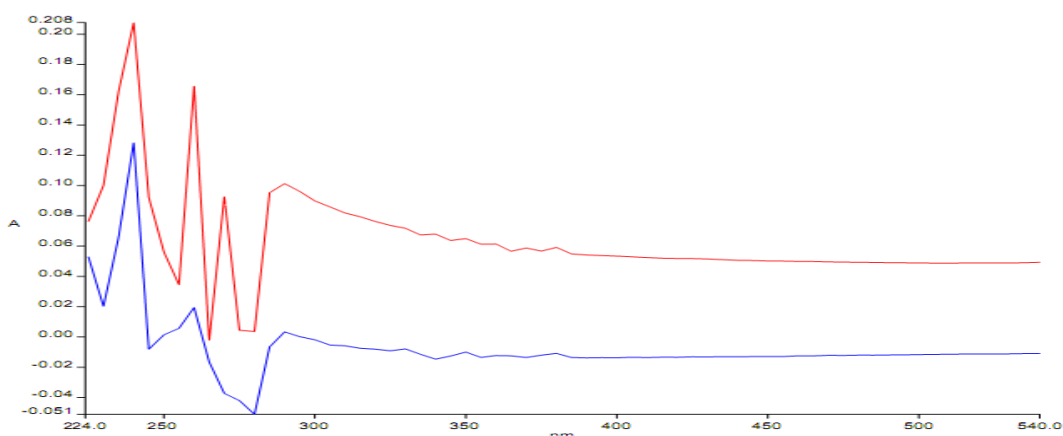


Figure 2: Absorption spectrum (UV) of melanin pigment by black yeast *H. werneckii*

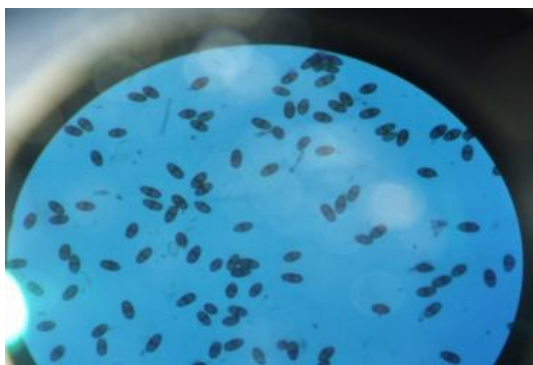


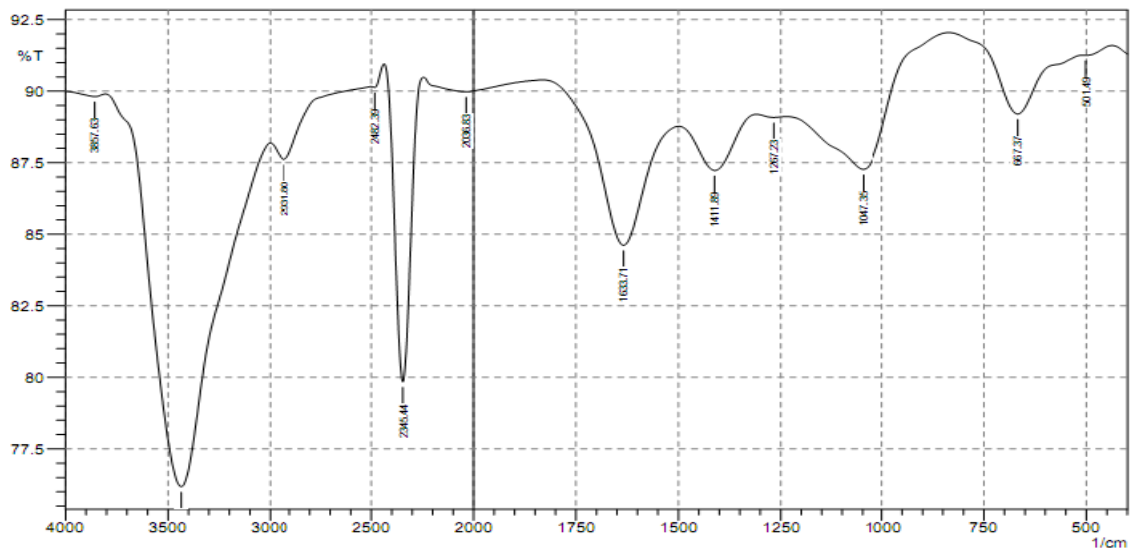
Fig. 1a: Microscopy observation of *Hortaea werneckii*.



Fig. 1b: Colony morphology of black yeast

#### Fourier Transform infrared (FT IR) Spectrophotometer:

The IR spectrum of the pigment was also characterized as fungal melanin (Fig.3). The chemical properties of the resultant dark pigment were determined including its elemental composition with infrared (IR) spectrum. In this study, IR spectrum of melanin shows peaks near 3,435cm<sup>-1</sup> was ascribed to the amino second group (NH). The peak at 2,931cm<sup>-1</sup> was assigned to the C-H stretch bond related to methane group. The peak at 1,633cm<sup>-1</sup> was the amino group with NH<sub>2</sub> stretching. The peak at 1,267cm<sup>-1</sup> relates to the anhydride group (C-O) in synthetic melanin and all extracted fungal pigment. This may be a strain related phenomenon, or C/N ratio might have resulted from impurities which were difficult to remove from melanin. Based on this finding, we suspect that the resulted pigment may be Eumelanin not any other types which lacks nitrogen.

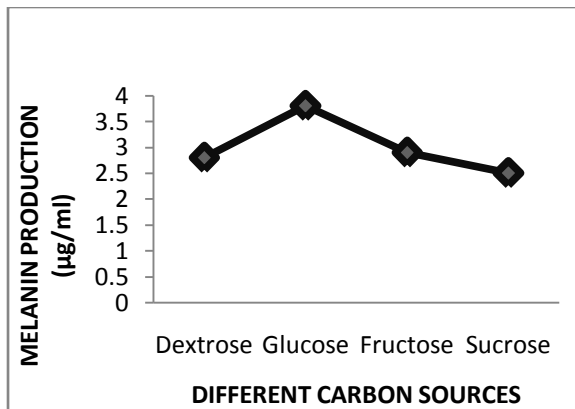


**Fig 3: Infra-red spectrum (FT-IR) of melanin pigment of Black yeast *Hortaea werneckii***

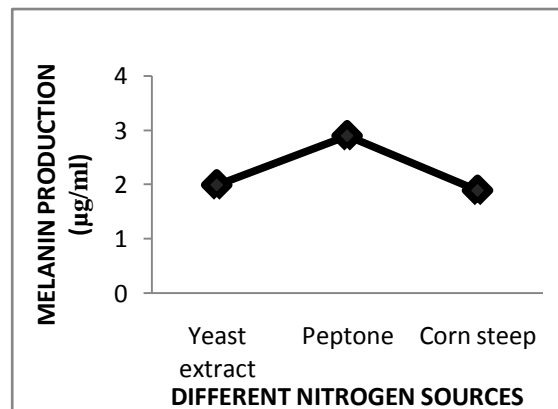
**Optimization of melanin:**

The optimized conditions for melanin production were analyzed at different parameters and high quantity of melanin was produced in the presence of glucose (Fig.4), peptone (Fig.5), rice bran (Fig.6) in

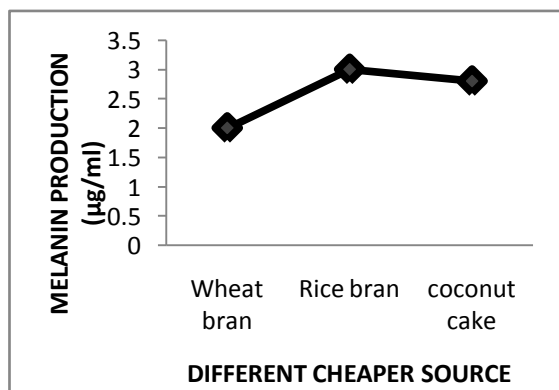
15‰ salinity (Fig.7), at 30°C (Fig.8), pH range 7.0 (Fig.9) and incubation periods (Fig.10). 5.60g/L of crude melanin was extracted under optimum condition.



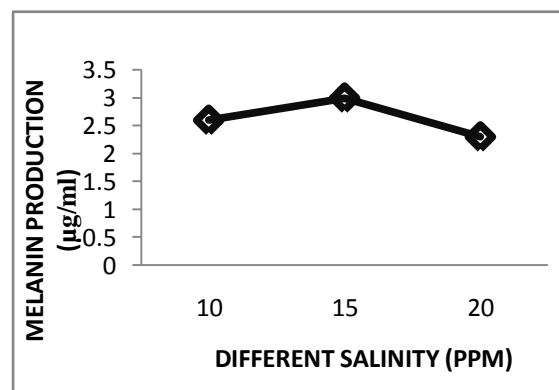
**Fig 4: Effects of different carbon sources melanin production**



**Fig 5: Effects of different nitrogen sources on melanin production**



**Fig 6: Effects of different cheaper sources**



**Fig 7: Effects of different salinity on melanin production**

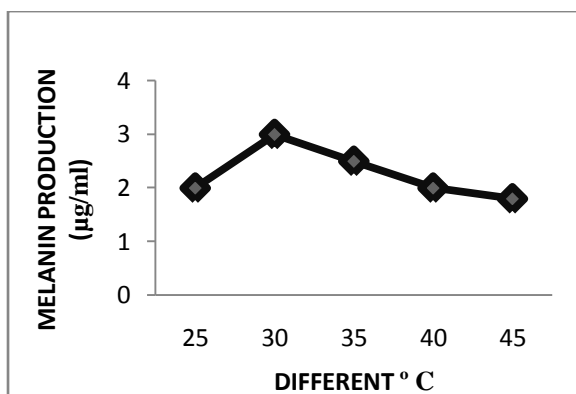


Fig 8: Effects of different temperatures on melanin production

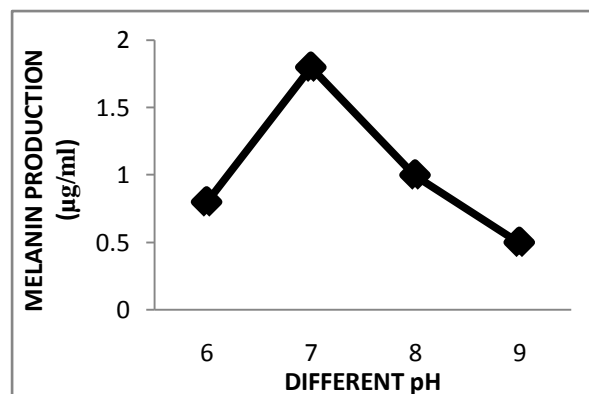


Fig 9: Effects of different pH on melanin production

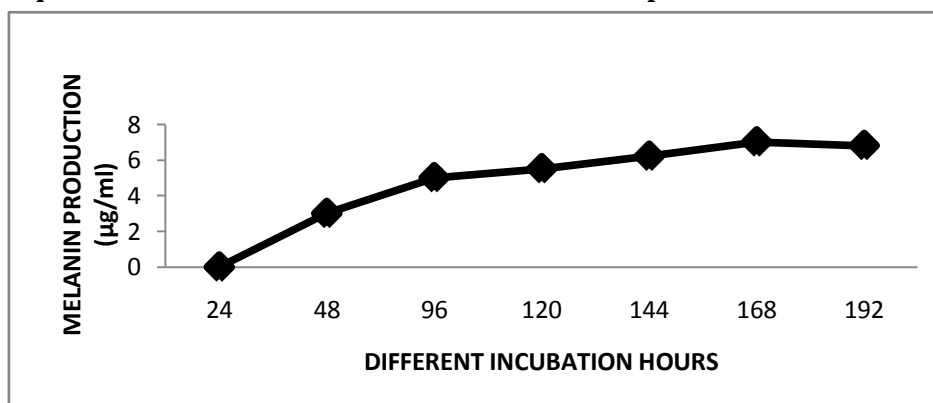


Fig 10: Effects of different incubation periods on melanin production

**Antibacterial activity of melanin pigment:**

The melanin pigment had antibacterial activity against all tested pathogens and the maximum zone of clearance against

*Salmonella typhi* (17mm), *Vibrio parahaemolyticus* (15mm) and *Klebsiella pneumonia* (11mm) were observed which was shown in the Fig. 11.

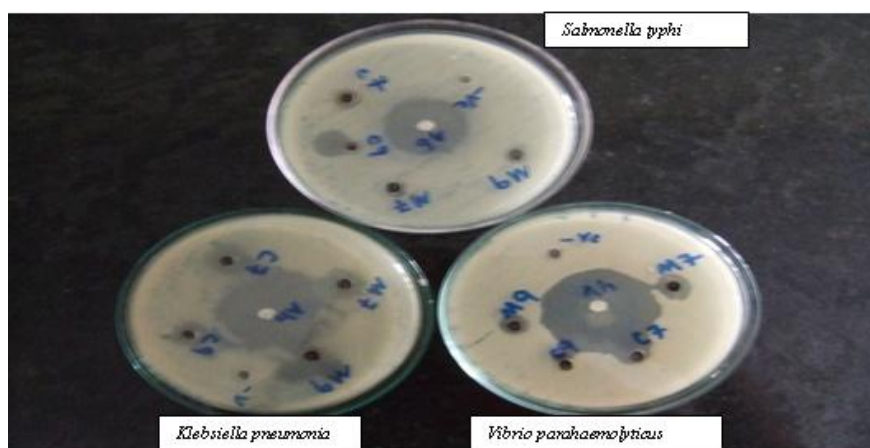
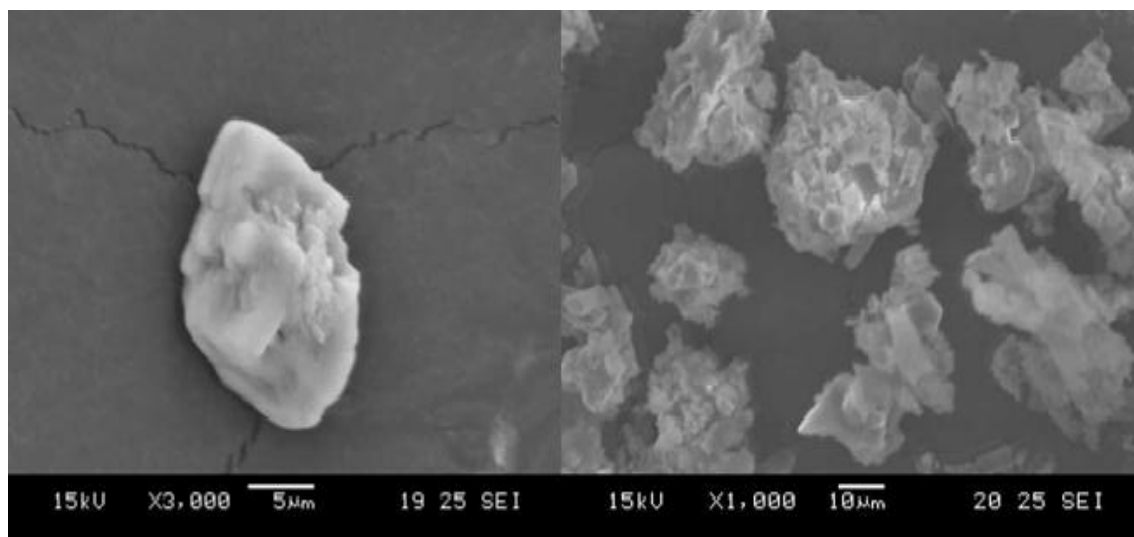


Fig 11: Antibacterial activity of Melanin pigment against bacterial pathogens

**Scanning Electron Microscopy:**

The suspensions of black particulate material at a magnification of 3,000x revealed structures resembling yeast cells

and 1,000x of SEM photograph shown the particulate materials which was shown in the Figure12.



**Fig 12: SEM magnification of Melanin particles in 3000x and 1000x**

### DISCUSSION

In this study, the black yeast *Hortaea werneckii* produced 5.60g/L of melanin pigment were synthesized at optimized conditions. Glucose was observed as best carbon source for the melanin production when compared to other sources. Similarly, the same results were reported as glucose, the best carbon source for yielding high quantity (7.22g/L) of melanin by *Yarrowia lipolytica* [37]. Starch was the effective carbon sources for *Streptomyces* sp. followed by glycerol and fructose [38, 39]. In case of nitrogen source, peptone results in hyper secretion of pigment when compared to yeast extract and corn steep, which is accompanied by the previous reports (40). However, it was feasible to combine yeast extract and peptone into a single medium to increase production and the pigment production [40]. When compared to other substrates rice bran showed more effect on the production and extracted high quantity of pigment. The maximum pigment production was observed at 30°C, pH 7.0 and salinity of 15‰, where the optimum pH and temperature was found to be pH 7.0 at 32°C. The maximum amount (20.76g/L) of pigments synthesized by the fungus *Aspergillus carbonicus* at 15<sup>th</sup>-25<sup>th</sup> day's incubation period [41]. In this study, 5.60g/L of crude melanin was extracted at 6<sup>th</sup> day.

Halophilic black yeast potentially produced active secondary metabolites which possess

high antibacterial activity. Melanin pigment showed significant antibacterial activity against three pathogenic organisms. From the results, it is evident that the melanin extract showed strong antibacterial activity against *Salmonella typhi* (17mm), *Vibrio parahaemolyticus* (15mm) and *Klebsiella pneumonia* (11mm). The purified pigment showed 20mm zone of clearance against *Escherichia coli*. Similarly, the same results were reported and the zone of inhibition against the *Escherichia coli* was 20mm [42]. Melanin extracts showed the strong activity against *Escherichia coli* (18mm), *Lactobacillus vulgaris* (19mm), followed by *Staphylococcus aureus* (18mm), *Proteus mirabilis* (15mm), *Vibrio cholera* (17mm), *Salmonella typhi* (12mm), *Salmonella paratyphi* (11mm) and *Klebsiella oxytoca* (9mm) [43].

In this study, the absorption spectrum of black yeast showed the absorption maxima at 235-300nm which were highly correlates with the absorption spectrum of *Aspergillus carbonicus*. It exhibited absorption range at 280-310nm which may be the reason of high intensity light absorption is typical of dark color pigment. It was absorbed strongly in the UV region and progressively less as the wavelength increased. This is due to the presence of much complex conjugated structure in the melanin molecule [44]. The similar reason that all spectra showed a strong UV absorption in the 200-300nm region that can be attributed to the  $\pi \Rightarrow \pi^*$  and  $n \Rightarrow \pi^*$  of

the amino, carboxylic and aromatic moieties. The presence of oxygen containing groups may contribute to the dark color of pigments [45].

Likewise, IR spectrum of melanin from black yeast shows peaks near  $3,435\text{cm}^{-1}$  was ascribed to the amino second group (NH). The peak at  $2,931\text{cm}^{-1}$  was assigned to the C-H stretch bond related to methane group. The peak at  $1,633\text{cm}^{-1}$  was the amino group with  $\text{NH}_2$  stretching. The peak at  $1,267\text{cm}^{-1}$  relates to the anhydride group (C-O) in synthetic melanin and all extracted microbial pigment, and the similar results were found with the peaks near  $3,381\text{cm}^{-1}$  was ascribed to the OH bond [46]. The peak at  $2,925\text{cm}^{-1}$  was assigned to the C-H stretch bond related to methane group. The peak at  $1,633\text{cm}^{-1}$  was the amino group with  $\text{NH}_2$  stretching and the melanin bright spectral absorption lines of  $2,925\text{cm}^{-1}$  to  $2,938\text{cm}^{-1}$  peaks related to hydroxyl group (OH) peak at  $3,344$  to  $3,436\text{cm}^{-1}$  relates to the amino second group (NH) and peaks at  $1,243\text{cm}^{-1}$  to  $1,305\text{cm}^{-1}$  relates to the anhydride group (C-O) in synthetic melanin and all extracted microbial pigment. Also, there were  $2,925\text{cm}^{-1}$  to  $2,938\text{cm}^{-1}$  peaks relating to the methane group (CH)  $1,628\text{cm}^{-1}$  to  $1,651\text{cm}^{-1}$  peaks relating to the amino group (NH) in the pigment extracted from *Kluyveromyces marxianus* and *Streptomyces chibaensis* [47]. Oxidation of OH group to carboxyl group enhances the color intensity because of the appearance of C-O double bonds. This provides an explanation for the correlation between extinction coefficients and contents of COOH groups in melanin. Thus the melanin with a high content of COOH groups is characterized by the greatest value of the extinction coefficient and this is due to the presence of many complex conjugated structures in the melanin molecule.

#### CONCLUSION

It is inferred from this study, that the *Hortaea werneckii* strain have the potential for melanin production. Synthesized melanin showed the promising antibacterial activity against life threatening bacterial pathogens of *Vibrio parahaemolyticus*, *Klebsiella pneumoniae* and *Salmonella typhi*. Rice bran acts as the cheapest source for increased production of

melanin when compared to other. Melanin produced from *Hortaea werneckii* can act as the cheap and efficient source for medicinal purpose and also in cosmetology.

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