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Screening and selection of Melanin production in *Cryptococcus Neoformans* and its phenotypic expression under different temperature ranges

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Abstract

Cryptococcus neoformans is a pathogenic fungus that produces melanin pigmentation, which is a major virulence factor that contributes to its pathogenicity. C. neoformans strains with varying levels of melanin production were screened and selected to evaluate their growth and melanin production under different temperature ranges. This was carried out in order to ascertain the optimal conditions for melanin production and understand the effects of temperature on the phenotypic expression of C. neoformans. Out of the 150 isolates that were tested for melanin production, 10 strains of C. neoformans were isolated from three market locations in Uselu, New Benin and Egor in Benin City, Nigeria, which produced dark brown melanin pigmentation on Niger seed Agar medium. The intensity of melanin production under various temperature ranges (25 ℃, 42 ℃ and 37 ℃) in C. neoformans strains was also evaluated. The results showed varying intensity of very low (+), low (++), high (+++) and very high (+++++) melanin production. The optimal temperature for melanin production varied among C. neoformans strains, with some strains producing high levels of melanin at various temperatures of 42 ℃, 37 ℃ and 25 ℃. This research was able to demonstrate the ability of C. neoformans to produce melanin, revealing that C. neoformans strains exhibits thermotolerance and varying levels of melanin production, there is no correlation between temperature and melanin and that melanin production is not solely dependent on environmental factors but also dependent on genes.

Keywords: *Melanin, Thermotolerance, Phenotype, Growth, Virulence*

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1. INTRODUCTION

Cryptococcus neoformans is a pathogenic fungus that produces melanin, and this pigment is a key virulence component that contributes to the fungal pathogenicity and survival within a host (Almeida *et al.,* 2015). Melanin helps *C. neoformans* evade the host's immune system and survive in harsh environments (Casadevall *et al.,* 2000). Melanin production in *C. neoformans* is a multifaceted process influenced by both environmental stressors and nutrient availability (Baker *et al.,* 2022; Watkins *et al.,* 2017). This fungus, a common human pathogen, utilizes melanin as a protective shield against various hostile conditions and host defense mechanisms (Esher *et al.,* 2018). Specifically, nutrient starvation, exposure to reactive oxygen and nitrogen species, temperature extremes, and even certain antifungal drugs can trigger melanin production (Momin and Webb, 2021). Recent research on *C. neoformans* has revealed that melanin production is controlled by both the melanin biosynthesis pathway and the cAMP signaling pathway, where genes like LAC1 and LAC2 encoding laccases are necessary for melanin synthesis (Lee *et al.,* 2019).

Despite the significance of melanin production in *C. neoformans,* there is limited understanding of the optimal conditions for melanin production and its phenotypic expression in different strains. This knowledge gap poses a limitation to the development of effective strategies to combat *C. neoformans* infections (García-Rodas *et al.,* 2015).

This study aims to investigate the following research question: How do different strains of *C. neoformans* vary in their melanin production capabilities, and how does temperature influence the phenotypic expression of melanin?

2. MATERIALS AND METHOD

Sample Collection

Pigeon droppings were collected from three market locations, namely, Uselu, New Benin and Egor in Benin City, Edo State, with five (5) samples collected from each market. The samples were gathered using a sterilized spatula and stored in properly labeled McCartney bottles to indicate their respective collection sites for ease of reference. The labeled samples were then transported to the Department of Plant Biology and Biotechnology laboratory for the experiment.

Media used for Selection and Preparation of isolates

The following were prepared and used in this research: Urea Agar Base, for preliminary selection; Niger Seed Agar for selection of melanin producing *C. neoformans*; Yeast Peptone Dextrose Agar (or Broth) (YPDA or YPDB) for routine culturing and subculturing. Approximately 1 g of pigeon droppings was added to 500 μl YPDB in eppendorf. The mixture was gently shaken to ensure the inoculum dissolved properly, then followed by incubation using Gallenhamp incubator at 37℃ for 24 hours. After incubation, 100 μl was spread evenly on Yeast Peptone Dextrose Agar plates. The plates were placed in a Gallenhamp incubator and incubated at 37℃ for 48 hours. Single colony isolates were picked and stored in YPDB and kept in the refrigerator at +4℃ for screening and selection. Aseptic conditions were maintained throughout the laboratory exercise.

Urease test assay

Urease test was the preliminary test for the isolation of *C. neoformans*. Urease activity was carried out as described by Junior *et al*. (2013) and adapted by Oghomwenakhin and Eboigbe, (2019). Then after, 20% urea solution was added to an already prepared Urea Agar Base medium. McCartney bottles containing the urea medium in slant form were inoculated with a drop of isolates which was previously kept at +4℃ in the refrigerator. This was followed by incubation at 25 ℃ or room temperature. Observation was made for color change from yellow to pink after one hour, until 24 hours of incubation.

Screening for Melanin production

The isolates that tested positive for urease test were further screened using niger seed agar medium. Niger seed agar was used as a selective medium for the confirmation of *C. neoformans* in this research. The isolates were confirmed by their ability to produce melanin (dark brown pigmentation) after incubation for a minimum of 48 hours at 37℃. The Niger seed medium was prepared following method of Pham *et al.* (2014). Afterwards, 50 g of Niger seed (*Guizotia abyssinica*) was mashed and boiled for 30 minutes. After boiling and sieving, the infusion containing 1 g glucose orthophosphate and 15 g agar was used to prepare 1 litre of the medium. The medium was then sterilized and poured into Petri plates. The isolates that tested positive for the urease test were then used to inoculate the niger seed agar medium plate using serial dilution and spreading method. The colonies that turned dark brown were selected as *C. neoformans* (Randhawa, 2006)

Phenotypic Expression of melanin and colony production under various temperature ranges

In order to check the optimum condition for the growth and production of melanin, the screened isolates of *C. neoformans*, were incubated in fresh niger seed agar medium plate under three different temperature ranges; 25℃, 37℃ and 42℃ , within 24hrs for colony count (as growth parameter) and further left for 7 days for melanin intensity measurement

Statistical Analysis

To determine if there was any significant difference in the growth of *C. neoformans* strains at the various temperature ranges, the data were subjected to one-way analysis of variance (ANOVA).

3. RESULT AND DISCUSSION

From this work, collections from three (3) areas in Benin City were sampled in order to isolate *C. neoformans* that produces melanin. The preliminary results revealed that out of 150 isolates that were screened, 30 tested positive in Egor, 29 tested positive in Uselu and New Benin respectively. Further test shows that showed that the following 10 strains tested positive for melanin production: NB24, NB17, NB40, NB59, NB16, NB15, NB57, NB5, NB18 and US29. Plate 1 shows the production of melanin from one of the isolated strain.

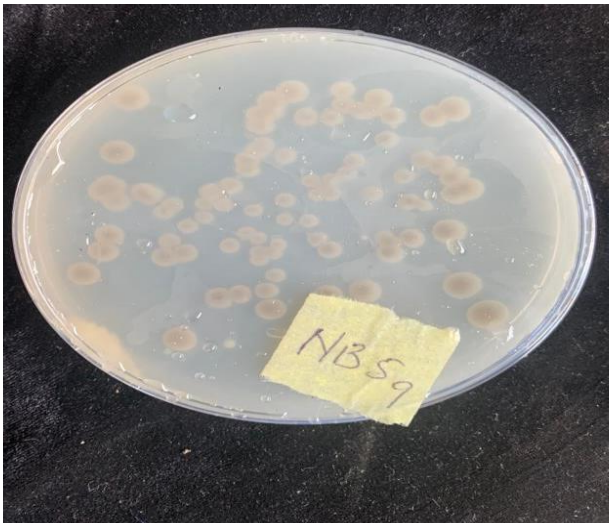

Figure 1: *C. neoformans NB59 strain with visible dark brown pigmentation of melanin.*

The growths of the various strains were examined at 42 ℃, 37 ℃ and 25 ℃, respectively. The result shows that most strains exhibited increased growth at 42 ℃ (Table 1). Accordingly, NB5 and NB18 exhibited exceptionally high growth in terms of colony count at 42 ℃. Most strains showed reduced growth at 37 ℃ compared to 42 ℃. Strain US29 shows relatively low growth across all temperatures.

Table 1: *Growth of C. neoformans strains under different temperature range.*

STRAINS	No. of Colonies 25º C	No. of Colonies 37º C	No. of Colonies 42º C
NB_{24}	164	83	562
NB_{17}	324	206	492
NB_{40}	216	137	728
US_{29}	127	32	374
NB_{57}	219	134	511
NB_{59}	201	108	447
NB_{5}	225	62	774
NB_{16}	283	253	535
NB_{15}	202	68	416
NB_{18}	167	163	749

Following the growth results under various temperature ranges, the condition for optimal production of melanin was also checked. The result is as shown in Table 2. For example, very high (++++) melanin production was observed in strains NB24 at 42 ℃ and 25 ℃, NB15 at 37 ℃ and 25 ℃ including NB57 at 37 ℃. Strains also showed very low melanin (+) NB18 at 25℃, 37℃ and 42℃, US29 at 42℃.

Table 2: *The intensity of melanin production under various temperature ranges in C. neoformans strains.*

Temperature Range			
Strain	25 ℃	37 ℃	42 ℃
NB24	++++	+	++++
NB17	+++++	++	++
NB40	++	++	+++
NB59	+++	++	+++
NB16	++	+++	+++
NB15	++	++++	+++

NB57	++	++++	+++
NB5	+	++++	++
NB18	+	++	+
US29	++	+	+

Key (Melanin Intensity): + = very low, ++ =Low, +++ = High, ++++ and above **= Very High**

Discussion

The ability to produce melanin is considered a virulence factor in *C. neoformans* which contributes to its pathogenicity and survival within host. In this research we have screened for melanin producing *C. neoformans* with the goal to determine the optimum condition by which this fungus produces melanin. The results showed that from the 150 isolates tested for melanin production, 10 strains of *C. neoformans* were isolated from environmental samples in three market areas in Uselu, New Benin and Egor in Benin City capable of producing dark brown melanin pigmentation on Niger seed Agar medium.

In order to evaluate the level of intensity of melanin production as phenotypic expression in *C. neoformans*, we further observed the growth of the fungus under various temperature range at 42℃, 37℃ and 25℃. It was clear from the results that temperature is a critical factor in the optimal growth of *C. neoformans*. Most strains exhibited increased growth (no of colonies) at 42℃ suggesting that *C. neoformans* is thermotolerant (Bloom *et al.,* 2019; Oghomwenakhin and Eboigbe, 2019). This may give the reason why temperature is considered as virulence factor in the fungus (Pettit *et al.,* 2010; Kraus, *et al.,* 2004). Different strains displayed distinct growth patterns across Temperatures, however, 42℃ appeared to be the optimal temperature for most strains with significantly higher colony counts. NB5 and NB18 exhibited exceptionally high growth at 42 ℃, suggesting strong thermotolerance trait considered to be expressed as a house keeping gene vital in metabolism.

The optimal temperature for melanin production varied among *C. neoformans* strains, some strains produced high levels of melanin at the optimal temperature of 42℃ and outside the optimal temperature range of 37℃ and 25℃. The ability for *C. neoformans* strains to survive and grow at different temperature range may contribute to its ability to survive within host body temperature. However, attempting to compare melanin production with temperature, the result showed no obvious. Although, some strains showed high growth but little to no melanin production in high temperature, this suggests that growth and melanin production are different processes influenced by distinct factors (Brilhante *et al.,* 2017, Kumari *et al.,* 2024).

The result showed varying intensity of very low (+), low (++), high (+++) and very high (+++++) melanin production under different temperatures of 25 ℃, 42 ℃ and 37 ℃. The study was unable to underpin a relationship between melanin production and temperature because of the irregularities of melanin production in the different *C. neoformans* strains at different temperatures. This is consistent with previous studies, showing that melanin production is not entirely dependent on environmental factors such as temperature and nutrient medium, but inherently dependent on genes (Momin and Webb, 2021; Samarasinghe *et al.,* 2018).

4. CONCLUSION

This study demonstrates the ability of *C. neoformans* to produce melanin. The research also revealed that *C. neoformans* strains exhibit thermotolerance and varying levels of melanin production with no direct relationship between temperature and melanin production. This shows the complex interactions between environmental factors and *C. neoformans* pathogenicity. Further research is needed to elucidate the molecular mechanisms underlying melanin production and its role in *C. neoformans* virulence.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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