SCREENING AND SELECTION OF MELANIN-PRODUCING STRAINS AND ANALYSIS OF GROWTH PATTERNS UNDER TEMPERATURE VARIATIONS, NUTRIENT STARVATION, AND FLUCONAZOLE EXPOSURE AMONG *CRYPTOCOCCUS NEOFORMANS*

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FEBRUARY, 2025

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A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY, FACULTY OF LIFE SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (HONOURS) DEGREE (B.Sc.) IN PLANT BIOLOGY AND BIOTECHNOLOGY

FEBRUARY, 2025

CERTIFICATION

We certify that this research work was carried out by **Fejiro Success OBORODE** of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

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DEDICATION

This project is dedicated to God Almighty for his guidance, direction, and strength and also to my mom Mrs.Clara Oborode.

ACKNOWLEDGEMENTS

First and foremost, I am deeply grateful to Almighty God for His guidance, protection, and provision throughout this project. My heartfelt appreciation goes to my project supervisor, Dr. L. Ebiogbe, for his support, encouragement, and constructive guidance, which were crucial to the success of this work. I also want to thank Prof. E. D. Vwioko, the Head of the Department of Plant Biology and Biotechnology, for his leadership and for creating an environment conducive to learning and research. To the academic and non-academic staff of the department, I truly appreciate your support and assistance during this project. I would also like to thank my project colleagues, Igbinosun Uwayemwen, Jesuoghae Osamagbe, Uwubanmwen Josephine, and Osemwegie Nancy, for their teamwork, dedication, and cooperation throughout the project. I am especially grateful to my friends, Isioma, Angela, Precious, Dora, Stephy Princess, Favour, Daniel, Dumebi, Emma, Gideon, and Erica for their encouragement and companionship, which made this experience memorable. Lastly, my deepest thanks go to my parents and siblings for their unwavering love, prayers, and sacrifices. Your constant support has been my greatest motivation. Thank you all for your contributions to the success of this project.

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ABSTRACT

Cryptococcus neoformans is a capsular fungal pathogen that causes life-threatening opportunistic infections, particularly in immunocompromised individuals. This research involved isolating, screening, and growing *C. neoformans* from samples collected from pigeon droppings in Uselu, Egor, and New Benin markets, Benin City, Edo State. One gram of pigeon droppings was added to Yeast Peptone Dextrose Broth (YPD) for preliminary culturing and incubated at 25°C for 24 hours. The mixture was then plated onto Yeast Peptone Dextrose Agar (YPDA) plates, incubated for five days, and sub-cultured to obtain contamination-free single colonies. Identification of the organism involved a urease test followed by a Niger seed agar test. The Niger seed agar test detected melanin-producing strains through pigmentation, while the urease test confirmed its presence through a yellow-to-pink color change. Out of 75 samples, 63 tested positive for *C. neoformans*, signifying its environmental prevalence, 21 from this 63 tested positive for melanin production. Growth pattern analysis showed optimum growth at 37°C, aligning with its pathogenicity in humans, and normal growth at 25°C. Under starvation conditions, colony formation was visibly inhibited, indicating its dependence on environmental nutrients for establishment. Susceptibility tests with fluconazole showed inhibited growth, confirming its efficacy as an antifungal agent. This study provides information on the environmental distribution, melanin production, and adaptability of *C. neoformans* in variable growth environments. The results suggest the organism's ecological ubiquity, the role of melanin as a virulence factor, and its ability to thrive in environments with high nutrient availability and favorable temperatures. These findings are significant in understanding fungal survival and the management of cryptococcal infections.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Cryptococcus neoformans is a free-living, encapsulated fungal organism capable of causing opportunistic infections in both plant and animal hosts (Springer *et al.,* 2017). *Cryptococcus neoformans* is widely distributed in the environment and inhabits various ecological niches, particularly in soil and bird droppings **(**Lin, and Heitman, 2006). According to Coelho *et al.,* (2014)*, C. neoformans* seldom causes isllness in individuals with a healthy immune system, as the infection is typically resolved or remains asymptomatically dormant. However, *C. neoformans* poses a serious threat to those with compromised immunity resulting from disease or medical treatment, especially AIDS patients, and is estimated to cause 180,000 deaths annually **(**Rajasingham *et al.,* 2017). Cryptococcal infection begins when desiccated fungal cells or spores are inhaled and settle in the lungs, potentially leading to cryptococcal pneumonia in immunocompromised individuals (May *et al.,* 2016), The primary infection can advance through extrapulmonary spread, as yeast cells carried in the bloodstream can cross the blood-brain barrier and settle in brain tissue (Chrétien *et al.,* 2002; Chang *et al.,* 2004).

Despite ongoing efforts to create an effective vaccine (Datta and Pirofski, 2006), there is currently no dependable method to prevent *C. neoformans* infections. Even the latest treatment approaches, which use a combination of antifungal drugs, only reduce 10-week mortality rates to 24% (Iyer *et al.,* 2021). Therefore, gaining a better understanding of the factors that enhance fungal virulence is crucial in our fight against this pathogenic organism. One of the adaptations that protect *C. neoformans* from extreme environmental conditions and during host infection is

the accumulation of a melanin layer in its cell wall. Melanins are a diverse array of pigments, typically black or dark brown, produced through the oxidative polymerization of phenolic or indolic compounds (Eisenman and Casadevall, 2012). They are known for their remarkable resistance, structural complexity, and unique physical properties, including the capacity to absorb a broad range of UV light. Melanin pigments serve various roles in nature, including camouflage, thermal regulation, and protection against radiation (Hill, 1992). In pathogenic fungi, melanin is associated with heightened virulence (Casadevall *et al.,* 2000) and resistance to drugs (Nosanchuk and Casadevall, 2006). In *C. neoformans*, melanin is produced from phenolic compounds in small vesicles known as melanosomes, which are then transported and deposited within the cell wall (Eisenman *et al*., 2009). The melanin particles aggregate into larger granules that intertwine with the components of the cell wall, leading to increased thickness over time (Eisenman *et al.,* 2005). During infection, the presence of melanin helps protect *C. neoformans* from being engulfed and eliminated by host macrophages (Wang *et al.,* 1995; Blasi *et al.,* 1995; Liu *et al.,* 1999) and enhances the severity of infection by promoting neurotropism (Polacheck *et al.,* 1982; Lee *et al.,* 1996). Since melanization is one of the two main virulence factors for *C. neoformans*, targeting this process, possibly by inhibiting the laccase enzyme that facilitates melanin synthesis, has been identified as a promising therapeutic strategy (Coelho and Casadevall, 2016).

1.2 CYPTOCOCCUS NEOFORMANS OVERVIEW

Cryptococcus neoformans is an encapsulated fungal pathogen capable of causing severe infections, particularly in immunocompromised individuals. It primarily exists in soil contaminated by bird droppings and produces melanin, a major virulence factor that enhances its resistance to environmental stressors and host immune responses. This fungus can disseminate

from the lungs to the central nervous system, leading to life-threatening cryptococcal meningitis. Despite antifungal treatments, high mortality rates persist. Research highlights melanin's critical role in pathogenesis and its potential as a therapeutic target (Eisenman *et al.,* 2012; Nosanchuk and Casadevall, 2006)

1.2.1 Scientific classification of *Cryptococcus neoformans*

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Hymenogastraceae

Genus: *Cryptococcus*

Species: *Cryptococcus neoformans*

1.3 HISTORY OF THE ETIOLOGIC AGENTS AND CRYPTOCOCCOSIS

Cryptococcus neoformans was first isolated from peach juice by Sanfelice in 1894 in Italy and was named *Saccharomyces neoformans* Sanfelice 1894). In the same year, Busse provided the first description of a case of cryptococcosis (Busse 1894) and isolated a yeast culture from a sarcoma-like lesion in the infected young woman's tibia. Busse called the fungus Saccharomyces, while naming the disease Saccharomycosis hominis (Busse 1895). Around the same time, Curtis studied a yeast-like fungus isolated from a tumor in a patient's hip, and, noting its difference from the cultures of both Busse and Sanfelice, described the fungus as Saccharomyces subcutaneous tumefaciens n. sp. (Curtis 1986). Curtis' strain was later determined to be the first

clinical isolate of *C. gattii* (Kwon-Chung *et al*. 2002). Because the Busse and Sanfelice's strains lacked the sugar fermentation and ascospore formation that are the hallmarks of the genus Saccharomyces, Vuillemin reclassified the yeasts as Cryptococcus hominis and *C. neoformans,* respectively, in 1901 (Vuillemin 1901). The characteristic neurotropism of *C. neoformans* was first recognized in 1914 by Verse´ (1914), and 2 years later by Stoddard and Cutler (1916). However, Stoddard and Cutler called the etiologic agent Torula histolytica and the disease "torulosis" by misinterpreting the fungal capsule as evidence of fungal histolytic action in the host tissue (Kwon-Chung and Bennett 1992). Confusion about the identity of the cryptococcosis agent persisted until Benham performed comprehensive studies with clinical Cryptococcus strains and concluded that all of the strains from human infections belonged to one species with two varieties based on serological differences (Benham 1935, 1950). She proposed to replace "torulosis/torula meningitis" with cryptococcosis and to conserve the fungal name *C. neoformans* (Benham 1950). Cryptococcal antigenic heterogeneity was confirmed in 1950 by Evans who identified three serotypes: A, B, and C (Evans 1950). A fourth serotype, D, was discovered in 1968 (Wilson *et al*. 1968). The environmental source *of C. neoformans* was unknown until Emmons isolated *C. neoformans* from soil collected in Virginia in 1951, reporting that the pathogen was abundant in pigeon nests and droppings (Emmons 1951, 1955). It took nearly 40 more years to discover the environmental source of *C. gattii* as trees when Ellis reported isolation of serotype B strains from Eucalyptus camaldulensisin Australia in 1990 (Ellis and Pfeiffer 1990). The laboratory diagnosis of *C. neoformans* was drastically simplified by the early 1960s when Seeliger and Staib discovered that *C. neoformans* could be distinguished from other white clinical yeasts by their urease activity (Seeliger 1956) and melanin formation (Staib 1962b) besides the presence of capsule. By the mid-1970s, the complete life cycles of *C. neoformans*

and *C. gattii* became known when heterothallic sexuality was discovered in both species (Kwon-Chung 1975, 1976b). The discovery of a heterothallic life cycle ushered Cryptococcus into the modern era, facilitating the development of the tools for classical genetic analysis and providing evidence that virulence factors such as capsule and melanin formation followed Mendelian inheritance (Kwon-Chung *et al*. 1982c). These discoveries were timely, as cryptococcosis had been considered a rare disease until immunosuppressive therapy came into wide use starting in the 1970s. The importance of understanding the pathogen became even more pressing as an unprecedented rate of increase in cryptococcosis began in the early 1980s, with AIDS becoming the leading risk factor of the disease. Building on these critical early advances and the need to develop a deeper understanding to combat this emergent threat, the Cryptococcus community has rapidly expanded into a robust and highly collaborative field of international researchers who have established a broad array of molecular techniques (Edman and Kwon-Chung 1990; Toffaletti *et al*. 1993; Lodge *et al*. 1994) and genomic sequences (Loftus *et al*. 2005; Kronstad *et al*. 2011) with which to develop a deeper understanding of these deadly pathogens.

1.3.1 Ecology of *Cryptococcus neoformans*

Although *C. neoformans* was first isolated from peach juice, the most important saprophytic sources globally are weathered droppings from pigeon (Columba livia) and soil (Emmons 1955). The fungus is not the normal flora of soil; samples positive for *C. neoformans* have mostly been from areas frequented by pigeons, chickens, turkeys, or occasionally other avian species (Emmons 1955; Ajello 1958). The species has also been recovered from the guano of a range of bird species including canaries, parrots, munia birds, and budgerigars (Staib 1962a; Swinne-Desgain 1975; Bauwens *et al*. 1986). Although the ecological relationship *between C. neoformans* and avian species has been globally consistent, the precise link between birds and

the cryptococcal natural habitat has yet to be defined. Pigeons are rarely infected because of their high body temperature (41˚C–42˚C) exceeding the growth temperature ranges of *C. neoformans,* but isolation from crops, beaks, or feet suggests that the feed they ingest could be contaminated with the fungus (Littman and Borok 1968; Khan *et al*. 1977). Recently, arboreal sources of *C. neoformans* have been increasingly reported in various parts of the world. Isolation of *C. neoformans* from the bark, treetrunk hollows, and decaying wood of more than 36 arboreal genera have been reported (Mitchell *et al*. 2011). The vast majority of environmental strains, regardless of the geographical source, belong to serotype A molecular type VNI. Serotype D molecular type VNIV strains from the environment are mostly from central Europe (Mitchell *et al*. 2011). Another important ecologic factor of *C. neoformans* may be its interaction with other organisms in soil such as various bacteria, amoebas, mites, sow bugs, and worms (Nielson *et al*. 1978; Ruiz *et al*. 1981; Steenbergen *et al*. 2001). In fact, Castellani (1931) first reported isolation of Acanthamoeba castellani as a contaminant of Cryptococcus cultures. The interaction between *C. neoformans* and soil amoeba is viewed as an important factor for the evolution of *C. neoformans* as a successful facultative intracellular pathogen. . *C. neoformans* can survive in amoebae and the fungus can utilize the same pathogenic strategy in human macrophages, which in some respect provides a similar environment. It has been proposed that such predation in the environmental niche has selected for the cryptococcal virulence traits that contribute to pathogenesis in human hosts (Steenbergen *et al*. 2001; Casadevall and Pirofski 2007).

1.3.2 Epidemiology

Cryptococcosis caused by *C. neoformans* occurs worldwide, and most patients with severe infections have a known condition that weakens their immune system (Perfect 2010; Sorrell *et al.*, 2011). HIV infection is the leading risk factor for cryptococcal meningoencephalitis globally,

meaning the spread of cryptococcosis aligns with the AIDS pandemic. Consequently, sub-Saharan Africa, with over 25 million people living with AIDS, has the highest number of cases (Park *et al.,* 2009, 2011). The disease impacts nearly 1 million people each year, causing around 700,000 deaths (Park *et al.* 2009). Other key risk factors include long-term corticosteroid use, organ transplants, cancer, diabetes, sarcoidosis, and idiopathic CD4 lymphocytopenia (Casadevall and Perfect 1998; Perfect 2010). Although most cryptococcosis patients are immunocompromised, *C. neoformans* can also infect people who appear healthy. For *C. gattii* infections, the proportion of cases in apparently healthy patients is notably higher (Chen *et al*. 2000, 2008; Sorrell *et al.* 2011). Some of these patients may have unidentified immune system defects not detected by routine clinical tests. Recent findings have identified GM-CSF-neutralizing autoantibodies in the plasma of seemingly healthy patients with *C. gattii* infection, suggesting that further immunological testing could reveal hidden risks (Saijo *et al.,* 2014). The *C. neoformans* molecular type VNI is the most common cause of cryptococcosis globally, accounting for 63% of cases, followed by VNII and VNIII (6% each) and VNIV (5%) (Meyer *et al.*, 2011). Most clinical isolates are of the MATα mating type, except in regions like Botswana, where MATa strains can make up 10% of cases (Litvintseva *et al.,* 2003). Evidence from sub-Saharan Africa shows sexual recombination between genetically distinct subgroups of *C. neoformans* serotype A strains (Litvintseva *et al.,* 2003). In contrast, *C. gattii* accounts for less than 20% of global cryptococcosis cases, compared to 80% for *C. neoformans*. The risk factors for *C. gattii* infections remain unclear. Among *C. gattii* strains, VGI is the most prevalent (9%), followed by VGII (7%), VGIII (3%), and VGIV (1%) (Meyer *et al*. 2011). Earlier studies noted *C. gattii* as more common in tropical and subtropical regions, but recent research shows its expansion to temperate areas (Kwon-Chung and Bennett 1984, 1992). Outbreaks on Vancouver

Island, Canada (Hoang *et al.,* 2004; MacDougall *et al.,* 2007), and in the Pacific Northwest of the United States (MacDougall *et al.,* 2007; Byrnes and Heitman 2009; Byrnes *et al.,* 2010) highlight this shift. The outbreak strains VGIIa and VGIIc were found to be more virulent in mice compared to other molecular types (Kidd *et al.,* 2004; Fraser *et al.,* 2005a; Byrnes *et al.,* 2010; D'Souza *et al.,* 2011). Factors such as the importation of Eucalyptus trees from Australia, increased global travel, and climate change may have contributed to the spread of *C. gattii* to new regions.

1.4 MELANIN PRODUCTION

Melanin is produced by a wide variety of fungal species and the pigment deposited in the cell wall is known to play an important protective role against environmental stress (Nosanchuk and Casadevall 2006). Unlike the black fungi commonly found in soil or on plants, which synthesize dihydroxynaphthalene (DHN) melanin constitutively*, C. neoformans* produces melanin only in the presence of substrates such as 3,4-dihydroxyphenylalanine (DOPA) and other di/polyphenolic compounds (Chasakes 1975; Polacheck *et al.,* 1982). The early observations, using melanin-lacking mutants isolated by UV irradiation, suggesting the importance of melanin as a virulence factor (Kwon-Chung *et al.,* 1982c; Rhodes *et al.,* 1982) was confirmed by the use of LAC1 gene-deletion mutants (Salas *et al.,* 1996). *C. neoformans* contains two laccase genes, LAC1 and LAC2, in the genome but only LAC1 is expressed significantly under most conditions and virulence is reduced only when the LAC1 gene is deleted (Zhu and Williamson 2004; Pukkila-Worley *et al.,* 2005). .The cryptococcal laccase, a member of the multicopper oxidases is localized in the cell walls (Zhu *et al*. 2001; Waterman *et al*. 2007b) and its transport to the cell wall is Sec6 dependent (Panepinto *et al.,* 2009). Melanization of cryptococci require numerous additional genes such as the copper transporter Ccc2, the copper chaperone Atx1, the chitin

synthase Chs3, the transcriptional coactivator Mbf1, the chromatin remodeling enzyme Snf5 (Walton *et al.,* 2005), the transcription factor Rim101, and its regulatory gene Rim20 (Liu *et al*. 2008). As in the case of capsule formation, melanization is regulated by number of different pathways (Liu *et al.*, 2008). How does melanin provide a survival advantage in the host? There have been numerous studies regarding the role of cryptococcal melanin in protection from phagocytosis (Wang *et al*. 1995; Liu *et al.* 1999), killing by host cells (Blasi *et al*. 1995; Wang *et al*. 1995), oxidants (Wang and Casadevall 1994; Blasi *et al.* 1995; Jacobson and Hong 1997), and microbicidal peptides (Doering *et al.* 1999). In addition, melanin is reported to protect cryptococci from antifungal agents including amphotericin B (Ikeda *et al*. 2003;Martinez and Casadevall 2006), caspofungin (van Duin *et al*. 2002; Martinez and Casadevall 2006), and azoles (van Duin *et al.* 2002, 2004; Ikeda *et al.* 2003)

1.4.1 The structure of *Cryptococcus neoformans* melanin-like pigment

Structural studies of melanins are exceedingly difficult because they are insoluble in most solvents, and their amorphous and heterogeneous nature precludes X-ray crystallographic analysis. To date no structure for a naturally occurring melanin has been solved. The problem may be beyond current technology considering that melanins formed in the environment or during infection may be the result of polymerization of various substrates resulting in an extremely heterogenous structure (Williamson, 1997). The *C. neoforman,* derived black pigment produced in vitro by growth in media with L-DOPA has been analysed by electron-spin-resonance spectroscopy (Wang,1995) and elemental analysis (Wang, 1996; Rosas *et al.,* 2000). The electron-spinresonance spectroscopy revealed that the pigment was a stable, free radical with spectroscopic features that defined it as a melanin (Wang *et al.,* 1995). Elemental quantitative analysis of *C. neoformans* melanin 'ghosts' synthesized after growth in L-DOPA

shows a C:N:O ratio of 16:2:5, which is significantly different from the ratio of 9:1:2 found in synthetic DOPA-melanin (Rosas *et al.,* 2000) This difference in elemental composition is unlikely to result from the isolation process itself, as synthetic DOPA-melanin showed no difference in C:N:O ratio when processed through the same extraction procedure (Rosas *et al.,* 2000). Instead, it is possible that melanin ghosts contain other substances incorporated into the melanin polymer by the polymerization process, which could alter the C:N:O ratio from the one that would have resulted from the polymerization of L-DOPA alone to form a pure DOPA-melanin (Rosas *et al.,* 2000). Alternatively, it is conceivable that there are biochemical differences in the polymerization reaction carried out by this fungus that result in a polymer with a different elemental composition.

1.4.2 The role of melanization and the capsule in environmental survival

As *C. neoformans* is a free-living fungus that does not require parasitism in an animal host for survival, it is probable that the ability to synthesize melanin or produce a capsule was positively selected for by its environmental niche. *C. neoformans* cells in pigeon excreta is melanized (Rosas *et al.,* 2001; Nosanchuk *et al.,* 1999) and it is likely that melanin is important for survival in the environment. In this regard, melanin has been shown to protect *C. neoformans* against ultraviolet light (Wang *et al.,* 1994), hot and cold temperatures (Rosas *et al.,* 1997), the fungicidal effects of Ag+ (Garcia-Rivera *et al.,*2001) and cell wall-degrading enzymes such as those that may be produced by fungal predators (Rosas *et al.,* 2001).

The capsular polysaccharides may also have a role in environmental survival, as soil amoebae are less able to phagocytose capsular strains (Steenbergen *et al.,* 2001), and melanin production by acapsular cells affords protection against amoebae (Steenbergen *et al.,* 2001). Hence, it seems

that both melanin and the capsule have the capacity to serve as a versatile armour-like barrier against a variety of environmental stresses.

1.5 JUSTIFICATION OF STUDY

This study on screening and selection of melanin producing strains of *Cryptococcus neoformans* and analyzing its growth pattern under varying temperature, nutrient limitation and fluconazole is justified by the need to understand fungal virulence factor, enhance fungal resistance to stress and antifungal treatments. Identifying melanin producing strains and assessing their growth patterns under different conditions will provide insights into fungal resilience, inform diagnostic tool development, and support improved strategies for managing cryptococcal infections effectively.

1.6 AIM AND OBJECTIVES

The aim of this study was to screen and select melanin-producing strands of *Cryptococcus neoformans* and analyse the growth pattern of *Cryptococcus neoformans* using Temperature Range, Nutrient Limitation, and Fluconazole.

The objective of this research were:

a) To isolate and culture *Cryptococcus neoformans* from environmental sources.

b) To optimize screening methods for detecting melanin production in isolated strains

c) To identify genetic or environmental factors influencing melanin production in *Cryptococcus neoformans***.**

d) To analyse the growth patterns of *Cryptococcus neoformans* under varying conditions, including different temperature ranges, nutrient starvation, and antifungal treatments (e.g., fluconazole), to assess their effects on growth and melanin production

CHAPTER TWO

MATERIALS AND METHODS

2.1.1 Source of Cryptococcus sample

Pigeon droppings were collected from three locations: Uselu, New Benin, and Egor in Benin City, Edo State. The samples were gathered using a sterilized spatula and stored in properly labeled McCartney bottles to indicate their respective collection sites for easy identification. The labeled samples were then transported to the Department of Plant Biology and Biotechnology laboratory for the experiment.

2.1.2 Preparation of media

Yeast Peptone Dextrose Agar (YPDA) was used to culture the initial samples and to maintain the verified isolates. To prepare the YPDA medium, 2.5 g of yeast extract, 5 g of peptone, 5 g of dextrose, and 5 g of agar were dissolved in 250 ml of distilled water.

For the urease test, Urea Base Agar was prepared by dissolving 6 g of urease base in 250 ml of distilled water and mixing it thoroughly with 5 g of urea dissolved in 12.5 ml of water.

The presence of *Cryptococcus neoformans* was confirmed using a selective medium prepared from Niger seed extract.

2.1.3 Preparation of isolates

Approximately 1 g of pigeon droppings was added to 500µl of Yeast Peptone Dextrose Broth (YPD) in McCartney bottles. The mixture was gently shaken to ensure the inoculum dissolved properly, then incubated at 25°C for 24 hours. After incubation, about 100µl of the YPD broth

was spread evenly onto Yeast Peptone Dextrose Agar (YPDA) plates. The plates were placed in a Gallenhamp cooled incubator and incubated at 25°C for 5 days.

2.1.4 Sub-culturing of the isolates

The isolates from the initial culture were further sub-cultured on Yeast Peptone Dextrose Agar (YPDA) to obtain contamination-free single colonies. This step was carried out to eliminate other organisms present in the samples collected from the three locations. The purified isolates were then cultured on Niger seed medium, a selective medium designed for the identification of *Cryptococcus neoformans*.

2.1.5 Urease test assay

Urease activity was carried out as described by Junior *et al.* (2013). A 20% urea solution was added to an already prepared Urea Base Agar medium. McCartney bottles containing the urease medium in slant form were inoculated with *Cryptococcus neoformans* and incubated at 25°C for 48 hours. Observations were made for color changes. The initial color of the medium was yellow before incubation. A color change from yellow to pink after 24 hours of incubation indicated increased enzyme activity, where urea is hydrolyzed to ammonia and carbonate (Casadevall and Steenbergen, 2003).

2.1.6 Niger Seed Agar test

Niger seed agar was used as a selective medium for the confirmation of *Cryptococcus neoformans* in this experiment. The isolates were confirmed by their ability to produce melanin (dark pigmentation) after incubation for a minimum of 48 hours at 37°C. To prepare the medium, 25g of Niger seed (*Guizotia abyssinica*) was mashed and boiled for 30 minutes. After boiling, the seeds were added to the infusion, which also contained 10g of glucose orthophosphate, and

15g of agar. The solution was thoroughly mixed and diluted to 500ml with distilled water. The medium was then sterilized and poured into Petri dishes, where it solidified. Serial dilution was carried out, and the inoculum was applied to the plates. The plates were incubated to allow growth and melanin production, which was used as an indicator of *C. neoformans* presence. The ability of the isolates to grow at this temperature (37°C) was also used as a criterion for selection.

2.2.6 Growth condition tests

After identifying the melanin-producing *Cryptococcus neoformans,* a fresh batch of Yeast Peptone Dextrose Agar (YPDA) was prepared, poured into 10 plates, and allowed to solidify. The *Cryptococcus neoformans* inoculum was then applied to all 10 plates. Five plates were incubated at 25°C, while the remaining five were incubated at 37°C for 24 hours. Another nutrient medium, Yeast Peptone Agar (YPA), was prepared without the addition of dextrose and poured into 5 plates. Once solidified, the plates were inoculated with *Cryptococcus neoformans* and incubated at room temperature for 24 hours. Lastly, YPDA was prepared with the addition of an antifungal Fluconazole and poured into 5 plates. After solidifying, these plates were inoculated with *Cryptococcus neoformans* and incubated at room temperature for 24 hours. The test was conducted to assess the growth and adaptability of *Cryptococcus neoformans* under different growth conditions, including temperature variation, nutrient limitation, and antifungal exposure, to better understand its pathogenic potential and drug resistance.

CHAPTER THREE

RESULTS

In this work, three locations in Benin City, Edo State was sampled for the occurrence of *Cryptococcus neoformans*, which were Uselu, New Benin, and Egor. 5 Samples were obtained from each location, making a total of 15 samples.

Table 3.1 Shows the result of environmental isolates that were positive and negative. for *Cryptococcus neoformans* after the isolation process was carried out and sub-cultured on the growth medium for further confirmation. The environmental isolates are NEB1, NEB2, NEB3, and NEB4. NEB5, EGR1, EGR2, EGR3, EGR4, ERG5, USM1, USM2, USM3, USM4, USM5. The positive sign and negative sign show the presence or absence of *C. neoformans,* respectively.

Table 3.1: Environmental isolate that were Positive and Negative for *C. neoformas*

Site of collection	Sample/isolate	Test result
New Benin Market	NEB1	+
New Benin Market	NEB2	+
New Benin Market	NEB3	+
New Benin Market	NEB4	_
New Benin Market	NEB5	_
Uselu Market	USM1	+
Uselu Market	USM2	+
Uselu Market	USM3	_
Uselu Market	USM4	+
Uselu Market	USM5	+
Egor market	EGR1	+
Egor market	EGR2	+
Egor market	EGR3	+
Egor market	EGR4	+
Egor market	EGR5	+

+ : positive; - : negative

Table 3.2 Presents the results of the urease test conducted on *C. neoformans* isolated from Uselu Market. Twenty slant tubes containing urease medium were inoculated with *C. neoformans* and incubated at room temperature for 48 hours. A color change from yellow to pink confirmed the presence of *C. neoformans.* The urease test was employed as both a screening and confirmation method for the organism. Out of the 25 tubes tested, 19 yielded positive results, while 6 showed negative results.

Table 3.2: Urease Test of *C.neoformans* Isolated from pigeon droppings in Uselu Market

Samples	Positive	Negative
USM1a		-
USM1b		-
USM1c	+	
USM1d	+	
USM1e	+	
USM2a	+	
USM2b	+	
USM2c		-
USM2d	+	
USM2e	+	
USM3a	+	
USM3b	+	
USM3c	+	
USM3d	+	
USM3e	+	
USM4a	+	
USM4b	+	
USM4c		-
USM4d	+	
USM4e	+	
USM5a	+	

USM5b	+	
USM5c		-
USM5d		-
USM5e	+	

USM1: Uselu Market Sample 1; USM2: Uselu Market Sample 2; USM3: Uselu Market sample 3; USM4: Uselu Market sample 4; USM5: Uselu Market sample 5.

Table 3.3 Provides the urease test results for *C. neoformans* isolated from Egor Market. Twenty slant tubes containing urease medium were inoculated with the organism and incubated at room temperature for 48 hours. A color change from yellow to pink confirmed the presence of *C. neoformans.* The urease test was utilized as both a screening and confirmation method. Among the 25 tubes tested, 21 produced positive results, while 4 were negative.

.

Table 3.3: Urease Test of *C.neoformans* Isolated from pigeon droppings in Egor

Samples	Positive	Negative
EGR1a	+	
EGR1b	+	
EGR1c	+	
EGR1d	+	
EGR1e	+	
EGR2a		-
EGR2b	+	
EGR2c	+	
EGR2d	+	
EGR2e	+	
EGR3a	+	
EGR3b	+	
EGR3c	+	
EGR3d	+	
EGR3e		-

EGR4a	+	
EGR4b	+	
EGR4c	+	
EGR4d		-
EGR4e	+	
EGR5a		-
EGR5b	+	
EGR5c	+	
EGR5d	+	
EGR5e	+	

EGR1: Egor Sample 1; EGR2: Egor Sample 2; EGR3: Egor Sample 3 ; EGR4: Egor Sample 4; EGR5: Egor Sample 5.

Table 3.4 Summarizes the urease test results for *C. neoformans* isolated from New Benin Market. Twenty urease medium slant tubes were inoculated with the organism and incubated at room temperature for 48 hours. A color change from yellow to pink indicated the presence of C. neoformans. This test served as both a screening and confirmation method. Of the 25 tubes tested, 23 were positive, while 2 were negative.

Table 3.4: Urease Test of *C.neoformans* Isolated from pigeon droppings in New Benin Market

Samples	Positive	Negative
NEB1a	+	
NEB1b	+	
NEB1c	+	
NEB1d	+	
NEB1e	+	
NEB2a		-
NEB2b	+	
NEB2c	+	
NEB2d	+	
NEB2e	+	
NEB3a	+	
NEB3b		-
NEB3c	+	
NEB3d	+	
NEB3e	+	
NEB4a	+	

NEB4b	+
NEB4c	+
NEB4d	+
NEB4e	+
NEB5a	+
NEB5b	+
NEB5c	+
NEB5d	+
NEB5e	+

NEB1 :New benin sample 1 ; NEB2: New benin sample 2; NEB3: New benin sample 3; NEB3 : New benin sample 3; NEB4: New benin sample 4 ; NEB: New benin sample 5.

Plate 3.1: Urease Activity in *Cryptococcus neoformans*

A:Yellow-colored urease medium before inoculation and incubation at 25°C.; B: Color change to pink, indicating a positive urease test after inoculation and incubation.

Out of the 63 urease-positive *Cryptococcus neoformans* isolates, 21 (33.3%) produced melanin on Niger Seed Agar, whereas 42 (66.7%) did not. The presence of dark brown pigmentation in the positive samples demonstrated their ability to produce melanin, a crucial virulence component of *C. neoformans.*

Table 3.5: Melanin Production in *Cryptococcus neoformans* Using Niger Seed Agar Test

Urease-Positive Sample	Melanin Production (Dark Brown, +)	No Melanin Production (Whitish, -)
Sample 1		-
Sample 2		-
Sample 3	+	
Sample 4		-
Sample 5	+	
Sample 6		-
Sample 7		-
Sample 8		-
Sample 9	+	
Sample 10		-
Sample 11		-
Sample 12	+	
Sample 13		-
Sample 14	+	
Sample 15	+	
Sample 16		-
Sample 17		-
Sample 18		-
Sample 19	+	
Sample 20		-

Sample 21	+	
Sample 22		-
Sample 23		-
Sample 24		-
Sample 25		-
Sample 26	+	
Sample 27		-
Sample 28		-
Sample 29	+	
Sample 30	+	
Sample 31		-
Sample 32	+	
Sample 33		-
Sample 34		-
Sample 35		-
Sample 36	+	
Sample 37		-
Sample 38	+	
Sample 39		-
Sample 40	+	
Sample 41		-
Sample 42		-
Sample 43		-

Sample 44		-
Sample 45		-
Sample 46	+	
Sample 47		-
Sample 48		-
Sample 49	+	
Sample 50		-
Sample 51	+	
Sample 52		-
Sample 53		-
Sample 54		-
Sample 55		-
Sample 56	+	
Sample 57		-
Sample 58	+	
Sample 59		-
Sample 60		-
Sample 61	+	
Sample 62		-
Sample 63		-

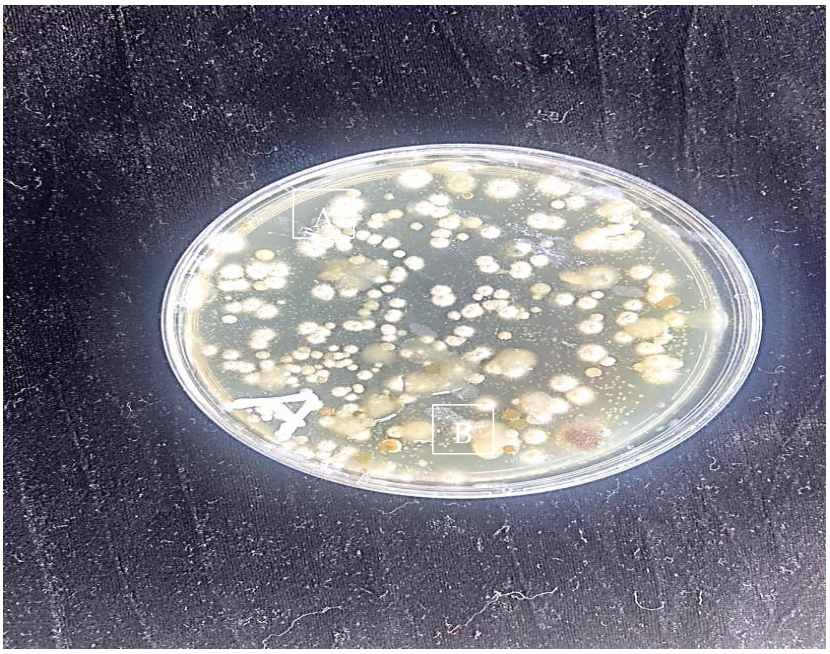

Plate 3.2: Melanin Production on Niger Seed Agar for *Cryptococcus neoformans* Confirmation

A: Non-melanin producing colony (whitish)

B: Melanin producing colony (brown)

The growth of *Cryptococcus neoformans* was assessed under different conditions, including temperature variations, Nutrient starvation, and antifungal treatment, to determine their effects on colony formation. Table 3.5 Presents the colony counts observed across five distinct plates (A, B, C, D, E) for each condition, as well as the average number of colonies (A.NOC) for each treatment. From the table it shows that At 25°C, *Cryptococcus neoformans* showed strong growth, particularly on Plate C (625 colonies), with an average of 220 colonies. Growth was even more pronounced at 37°C, where Plate C had 811 colonies and the average increased to 247.2. However, under Nutrient starvation (N.D.), colony formation dropped significantly, with an average of only 82 colonies, indicating limited growth due to Nutrient starvation. Exposure to fluconazole treatment caused a decline in colonies, averaging just 35.4. This shows fluconazole effectiveness in inhibiting growth.

Table 3.6: Analysis of *Cryptococcus neoformans* growth under varying growth conditions

G.C	NOC.A	NOC.B	NOC.C	NOC.D	NOC.E	A.NOC
25ºC	139	86	625	248	2	220.0
37ºC	52	152	811	208	13	247.2
N.S	118	45	107	92	138	82.0
A.F	138	16	12	5	6	35.4

G.C: Growth Condition; NOC.A: Number of colony in plate A ; NOC.B : Number of colony in plate B ; NOC.C : Number of colony in plate C ; NOC.D : Number of colony in plate D ; NOC.E : Number of colony in plate E ; A.NOC : Average number of colony ; N.S : Nutrient starvation; A.F : Antifungal (Fluconazole)

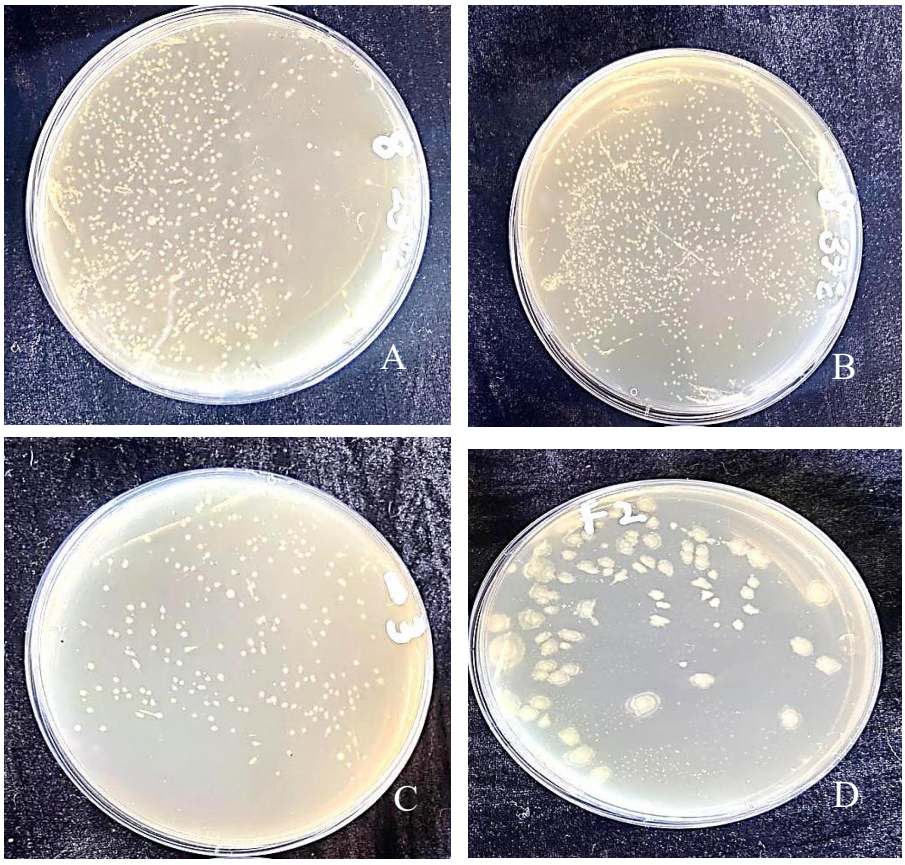

Plate 3.3: Growth analysis of *Cryptococcus neoformans* Under Different Environmental and Treatment Conditions

A: Growth at 25°C; B: Growth at 37°C; C: Growth under nutrient starvation; D: Growth under fluconazole treatment

DISCUSSION

According to Okagaki *et al.* (2010), *Cryptococcus neoformans* is a spherical, encapsulated, non-myceliated, non-fermenting fungal cell. It is harmful to both people and animals.

Immunocompromised people are the main victims of deadly meningitis caused by *C. neoformans* (Cogliati, 2012).According to Chakrabarti *et al.* (1997), the main environmental sources of *C. neoformans* are soil tainted with pigeon droppings, eucalyptus trees, and decaying wood that forms hollows in living trees. The organism can live independently as well as in conjunction with a range of different hosts (Olszewski *et al.,* 2010). In this study, the isolation and identification of *Cryptococcus neoformans* strains from environmental sources, particularly pigeon droppings in market places in Benin City, was successfully accomplished. The use of both urease tests and Niger seed agar was effective for confirming the presence of *C. neoformans* across various environmental samples. The majority of isolates from the Uselu, Egor, and New Benin markets were positive for *Cryptococcus neoformans* using the urease test. According to Casadevall and Steenbergen (2003), the urease enzyme is a key virulence factor in various pathogenic pathogens, including *Cryptococcus neoformans* and *Cryptococcus gattii*. This confirms the widespread presence of *C. neoformans* in the environment, especially in areas associated with pigeons, which are known carriers of the pathogen (Cassadevall *et al.,* 1996). The results from the urease test, where a significant portion of the isolates showed positive results, further supported the effectiveness of this test as a screening tool for *C. neoformans*. Specifically, the samples from New Benin Market displayed the highest positive rates, with 23 out of 25 tubes showing color changes indicating urease activity. Similarly, high rates of positivity were observed in Uselu and Egor markets, underscoring the environmental persistence of *C. neoformans* and its potential risk

to public health. This finding highlights the importance of routine monitoring of such pathogens, particularly in areas where pigeons congregate.

Further analysis of *C. neoformans* growth patterns under varying environmental conditions provided valuable knowledge into its resilience and adaptability. The growth conditions included exposure to different temperatures, nutrient starvation, and antifungal treatments. At a temperature of 25°C, *C. neoformans* exhibited strong colony formation, with the highest growth observed at 37°C, which is the optimal temperature for human pathogens. This result suggests that *C. neoformans* is well adapted to thrive in a variety of environmental conditions but particularly favors warmer temperatures (Pettit *et al.,* 2010). In contrast, growth under nutrient starvation conditions showed a significant decrease in colony formation, with a marked reduction in the average number of colonies observed on the plates. This finding suggests that nutrient availability plays a crucial role in supporting the growth of *C. neoformans*, emphasizing the organism's reliance on environmental nutrients for proliferation. Additionally, exposure to fluconazole, an antifungal agent, showed inhibited growth, with colony counts dropping drastically to an average of 35.4 colonies. This illustrates the antifungal activity of fluconazole and the susceptibility of *C. neoformans* to this treatment, reinforcing the importance of antifungal agents in controlling cryptococcal infections (Carrillo-Muñoz *et al.,* 1997). However, the observed decrease in growth under fluconazole treatment also suggests potential challenges in managing resistant strains of *C. neoformans*, especially in immunocompromised individuals.

CONCLUSION

The results of this study suggest that *C. neoformans* is a highly resilient organism, capable of adapting to various environmental conditions. The successful isolation and confirmation of the pathogen from environmental sources emphasize its widespread presence in urban areas, which could pose a potential health risk to the population. The growth analysis conducted under different conditions, including temperature fluctuations, nutrient limitations, and antifungal treatment, provides valuable insights into the organism's growth patterns and resistance mechanisms. These findings are significant in understanding fungal survival and the management of cryptococcal infections, highlighting the importance of ongoing surveillance and the development of more effective antifungal treatments to combat *C. neoformans* in both clinical and environmental contexts.

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