SURVEY OF NATURAL DISTRIBUTION OF BLACK YEASTS IN MALAYSIA WITH SPECIAL EMPHASIZE ON *Exophiala dermatitidis*

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µm	Micrometer
BG	Botanical Garden
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BP	Bootstrap
BSA	Bovine serum albumin
CAA	Caffeic Acid Ferric Citrate Test Agar
CFU	Colony forming unit
CG	*Cryptococcus gattii*
cm	Centimeter
CMA	Corn meal agar
CN	*Cryptococcus neoformans*
CNGA	*Cryptococcus neoformans* var. *gattii*
CNGR	*Cryptococcus neoformans* var. *grubii*
Co. Ltd	Limited liability company
CTAB	Cetyltrimethylammonium bromide buffer
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
g	Grams
HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
ITS	Internal transcribed spacer
KB	Kepala Batas
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
L-DOPA	L-s,4-dihydroxyphenylalanine
log	Logarithm

M	Molar
MARDI	Malaysian Agricultural Research and Development Institute
MAT	Mating-Type
M-Czapek Dox	Modified-Czapek- Dox broth
MEGA	Molecular Evolution and Genetic Analysis
min	Minutes
mL	Milliliter
ML	Maximum-likelihood
mm	Millimeter
mM	Millimole
mm^3	Millimeter cubic
N	Normality
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
nm	Nanometer
nm^2	Nanometer square
OD	Optical density
OsO_4	Osmium tetraoxide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDA-C	Potato dextrose agar supplemented with chloramphenicol
PDB	Potato dextrose broth
PFA	Potato flakes agar
pH	Logarithmic scale used to specify the acidity and basicity of and aqueous solution
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
R-PDA	Rose Bengal PDA chloramphenicol
rpm	Revolutions per minute
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulfate

SEM	Scanning electron microscope
SSA	Sunflower seed agar
TAE	Tris base, acetic acid and EDTA buffer solution
TEM	Transmission electron microscope
TM	Trademark
Trad-RB	Traditional Rose Bengal
UV	Ultraviolet
V	Volt
VOC	Volatile organic compounds

TINJAUAN BERKENAAN TABURAN SEMULAJADI YIS HITAM DI MALAYSIA DENGAN FOKUS KEPADA *Exophiala dermatitidis*

ABSTRAK

Yis hitam mempunyai ekologi yang luar biasa kerana ia boleh hidup dalam persekitaran yang toksik dan suhu yang melampau. *Exophiala dermatitidis* merupakan sejenis patogen oportunis, yang pernah dipencilkan daripada bilik sauna, kayu landasan kereta api, najis burung frugivorus, dan kelawar, tanah yang dikontaminasi dengan sisa hidrokarbon petrol dan mesin basuh pinggan di seluruh dunia. *Cryptococcus neoformans* pula boleh dipencilkan daripada batang pokok berongga, pokok *Eucalyptus* dan najis burung. Kedua- dua yis ini boleh dipencilkan dari rantau tropika dunia. Namun, laporan tentang taburan semulajadi yis hitam ini di Malaysia masih terhad. Justeru, taburan semulajadi yis hitam ini di Malaysia telah dikaji melalui tinjaun ini. Sebanyak 47 strain *Exophiala* spp. dan 4 strain *Cryptococcus* spp. telah berjaya dipencilkan. Memandangkan *Exophiala* spp. adalah kulat dimorph, ia telah ditumbuhkan di atas dua jenis medium kultur untuk mengetahui pertumbuhan peringkat yis dan hifanya. ditumbuhkan di atas dua jenis medium kultur untuk mengetahui pertumbuhan dimorfnya. Ujian hidrolisis urea, ujian DNAse dan ujian proteinase dijalankan untuk mengetahui sifat fisiologi yis-yis hitam ini. Seterusnya, yis hitam ini dipilih untuk analisis penjujukan DNA berasaskan bentuk sel konidiogenus dan pengeluaran klamidospora. Sebanyak 22 strain *E. dermatitidis* telah dikenal pasti; daripada landasan kereta api (n = 5), batang pokok terbakar (n = 2), tanah (n = 5) dan jalan yang tercemar (n = 5) dengan hidrokarbon, najis burung merpati (n = 2) serta longkang yang menghala ke Sungai Pinang dan lumpur di dalam Sungai

Pinang (n = 3). Satu strain *E. heteromorpha* telah dikenal pasti dan ia dipencilkan dari jalan yang tercemar dengan hidrokarbon manakala 2 strain *C. neoformans* dikenal pasti yang berasal daripada najis burung merpati. Genotip *E. dermatitidis* dikenalpasti melalui analisis filogenetik NJ dan dibahagikan kepada genotip A (n = 2), A2 (n = 7), dan B (n = 10). Kewujudan genotip A4(n = 3) yang baru, disahkan melalui kedudukan yang terasing pada kladogram dalam kedua – dua analysis filogenetik ML dan NJ. Keupayaan yis hitam untuk hidup dalam persekitaran yang tercemar dengan hidrokarbon dikaji dengan membekalkan minyak enjin dan melakarkan lengkuk pertumbuhannya serta memerhatikan ultrastruktur yis hitam ini menerusi TEM. Penemuan struktur yang menyerupai klamidospora dari kultur yang berusia 1 dan 3 bulan memberi informasi bahawa yis hitam ini boleh hidup dalam ekologi yang luar biasa dengan membentuk klamidospora. Kesimpulannya, *E. dermatitidis*, *E. heteromorpha* dan *C. neoformans* dapat dipencilkan daripada habitat semulajadinya selain daripada pesakit. Kebolehan yis hitam ini untuk hidup dalam persekitaran yang tercemar dengan hidrokarbon juga telah berjaya dibuktikan.

SURVEY OF NATURAL DISTRIBUTION OF BLACK YEASTS IN MALAYSIA WITH SPECIAL EMPHASIZE ON *Exophiala dermatitidis*

ABSTRACT

Black yeasts are ecologically remarkable as they can live in extreme, unusual and toxic environments. *Exophiala dermatitidis,* a human opportunistic pathogen was previously reported to be isolated from sauna facilities, oak sleepers of railway ties, faeces of frugivorous birds and bats, land contaminated with waste petrol hydrocarbons and dishwashers worldwide. *Cryptococcus neoformans*, on the other hand, was isolated from tree trunk hollows, *Eucalyptus* trees and faeces of birds. Both of these yeasts were frequently isolated from tropical regions of the world. As there are limited reports of environmental isolation of these black yeasts in Malaysia, their natural distribution in the environment were surveyed in this study. Forty-seven strains of *Exophiala* spp. and four strains of *Cryptococcus* spp. were isolated from various locations in Malaysia. As *Exophiala* spp. are dimorphic fungi, the colonies were grown under different conditions to identify the yeast and hyphal growth of the black fungi. Urea hydrolysis, DNAse test and proteinase activity were also conducted to test their physiological properties. The black yeasts were characterised by the type of conidiogenous cells, and chlamydospores produced and their identity confirmed by sequencing of the ITS region. Overall, 22 strains of *E. dermatitidis* were successfully identified from railway track stones (n = 5), burnt tree bark (n = 2), oil dripped soil sample (n = 5), hot spring biofilm (n = 1), tar road contaminated with petrol hydrocarbon (n = 4), pigeon droppings (n = 2), drain and deep mud of Sungai Pinang (n = 3). A single strain of *E. heteromorpha* was isolated from tar road contaminated with petrol hydrocarbon, whereas two strains of *C. neoformans* were isolated from

pigeon droppings. The genotypes of the isolated *E. dermatitidis* were identified by NJ phylogenetic analysis and grouped into genotype A (n = 2), A2 (n = 7) and B (n = 10). The existence of a new genotype A4 (n = 3) was confirmed by their grouping in a separate cladogram position in both maximum likelihood and neighbour joining phylogenetic analyses. The survival of the black yeast in a hydrocarbon contaminated environment was studied by supplying engine oil and plotting a growth curve. The ultrastructure of the grown yeast in this condition was observed by TEM. The discovery of the chlamydospore-like structures which was recognized from 1 and 3 months-old cultures suggests that the black fungi may survive in harsh environments by forming chlamydospores. In conclusion, the black yeasts, *E. dermatitidis, E. hetermorpha,* and *C. neoformans* were isolated from the natural environments in Malaysia besides human patients. Its survival in the environment contaminated with hydrocarbon was also evidently proven.

CHAPTER 1

INTRODUCTION

Black yeast is a term used to describe a group of fungi that have melanised cell wall and divides by budding to form daughter cells. A matrix of extracellular polymeric substances may encapsulate the daughter cells. These yeasts can also exhibit mycelial growth and generate conidia (Sterflinger, 2006). *Cryptococcus neoformans* (San Felice) Vuillemin, and *Exophiala dermatitidis* (Kano) de Hoog are human pathogenic black yeasts found from the natural environments.

Cryptococcus neoformans from the phylum Basidiomycota are classified into two varieties, *C. neoformans* and *Cryptococcus gattii* Vanbreus & Takashio. The former infects immunocompromised patients whereas the later can be found from an immunocompetent individual (Granados and Castaneda, 2005). Infection with these yeasts begins when the yeast is inhaled into the lung from the surroundings. The conditions that contribute to the pathogenicity of *C. neoformans* depend on the capsule synthesis, melanin production, phospholipase secretion, urease, proteinase production and also growth at 37 °C (Anaissie et al., 2009). *C. neoformans* is mostly associated with pigeon's droppings, whereas *C. gattii* is mainly isolated from trees and decayed wood in hollows of trees such as *Eucalyptus* spp. (Randhawa et al., 2003). *Cryptococcus neoformans* can also be isolated from different locations where pigeons gather and build their nests, in densely populated resident, commercial areas, parks and trees (Abulreesh et al., 2015). Pigeon only helps in the propagation of the fungus in its droppings and to spread the fungus (Johnston et al., 2016).

Exophiala dermatitidis which belongs to the phylum Ascomycota have mucoid colonies and can grow from temperatures 5 °C up to 42 °C (Gumral et al., 2014). It is

an ascomycete anamorphic fungus with pleomorphic nature. Fourteen species of *Exophiala* have been showed to cause disease to humans in the form of subcutaneous, cutaneous, systemic or disseminated infections with *E. dermatitidis* as the most common human opportunist (Gumral et al., 2014). The fungus produces melanin constitutively (Seyedmousavi et al., 2011), besides producing extracellular polysaccharide which is being used as a virulence factor (Sudhadham et al., 2008). According to Sav et al. (2016a), some of the species can be rare opportunists, strictly saprobic but some can be commonly isolated from the human host such as *E. dermatitidis* and *Exophiala phaeomuriformis*.

Exophiala spp. have the capability to survive in environments rich in toxic hydrocarbons such as xylene (Zhao et al., 2010), from steam baths besides fruit surfaces and human faeces. Faeces of frugivorous birds and bats are positive for this black yeast. Besides that, railway ties which are contaminated with oily debris and faeces gave high isolation of this fungus. The spread of this fungus to human dominated environment could be because of the consumption of the wild berries contaminated with the fungal propagule (Sudhadham et al., 2008).

Both these black yeasts have been reported to be isolated from South East Asia mainly from tropical regions (Sudhadham et al., 2008; Tay et al., 2010). However, there are limited reports of isolation of *Cryptococcus* spp. from environments in Malaysia and no report of environmental isolation of *Exophiala* spp. from this country.

Hence, the new knowledge obtained through this study would reveal the unknown ecology and distributions of the pathogenic microorganisms, *Cryptococcus neoformans* and *Exophiala dermatitidis* in the natural environment in Malaysia. Understanding the ecological factors that influence the growth and reproduction of the

black yeast in the environment is necessary to predict the possible routes of infection for these black yeasts.

RESEARCH QUESTIONS:

1. Where are the natural habitats of black yeasts in Malaysia?
2. Is the aromatic hydrocarbon an important substrate which enriches the black yeast growth?
3. How does *E. dermatitidis* survive in elevated temperature and limited nutrient environments?

HYPOTHESES:

1. Railway ties, hot springs, faeces of frugivorous birds and hydrocarbon contaminated areas are preferable environments for *Exophiala* spp. Eucalyptus trees, tree trunk hollow and pigeon droppings are suitable environments for *Cryptococcus* spp.
2. Engine oil can be utilized by *Exophiala* spp. for growth.
3. Thick cell wall and the presence of lipid helps the survival of *Exophiala dermatitidis* in elevated temperature and limited nutrient environments.

OBJECTIVES:

1. To isolate black yeasts from different locations in Malaysia.
2. To determine the requirement of aromatic hydrocarbon for the growth of black yeasts.
3. To examine the difference in the cell wall structure of chlamydospores-like structure with normal yeast cells, emphasizing the difference in organelles and cell wall layers.

CHAPTER 2

LITERATURE REVIEW

2.1 Black yeasts

Black yeast and their filamentous relatives with the most clinically relevant species are located within the order *Chaetothyriales*. Some of the genera within this order are *Exophiala*, *Coniosporium*, *Cladophialophora*, *Cyphellophora*, *Fonsecaea*, *Rhinocladiella* and *Phialophora* (Seyedmousavi et al., 2014).

Among these seven genera, species in the genus *Exophiala* are frequently termed black yeast due to the ability to form budding yeast-like and hyphal forms. The asexual reproduction of the fungi in this genus is by annellidic conidiogenous cells which were formed intercalarily and at the tip of the hyphae. Species in this genus differed according to the temperature. *E. pisciphila*, a psychrophilic waterborne pathogen is pathogenic to fish while *E. mesophila* was present from municipal drinking water. However, *E. dermatitidis,* followed by *E. xenobiotica* and *E. oligosperma* were the most clinically important species in this genus (Revankar and Sutton, 2010).

Cladophialophora species, on the other hand, lacked conidiophores and produces conidia in non-fragile chains. Shield cells and attachment scars were absent from the neurotropic pathogen *C. bantiana* (Revankar and Sutton, 2010). *C. bantiana* is the most common agent of cerebral phaeohyphomycosis (Anaissie et al, 2009).

The next genus in this order is *Coniosporium*. This black fungus has black, velvety colonies on natural substrate and is characterised to have thick-walled, heavily pigmented arthroconidia with meristematic development. *C. epidermidis* is not regularly encountered in the dermatological specimens but can cause superficial infections in humans (Li et al., 2008).

Simple phialides with multiseptated, curved conidia are the characteristics of *Cyphellophora* sp. The thallus is melanized and the phialide opening is either directly on the hyphae or sometimes on flask shape conidiogenous cells. This black yeast-like fungus was reported to cause superficial skin or nail infections in humans (Feng et al., 2012).

Fonsecaea pedrosoi is an agent of chromoblastomycosis. Both *F. pedrosoi* and *F. monophora* formed conidia from swollen denticles which results in secondary and tertiary conidia up to four conidia in chains (Revankar and Sutton, 2010).

The next genus in the order *Chaetothyriales* is *Phialophora. P. verrucosa* is the second most common cause of chromoblastomycosis after *F. pedrosoi* (Anaissie et al., 2009). This species produces their conidia from phialides with conspicuous darkened collarettes which are either funnel or vase shape, for *P. verrucosa* and *P. americana*, respectively.

The final genus in this order is *Rhinocladiella. R. aquaspersa* is an uncommon cause of chromoblastomycosis. The colonies have septated, brown coloured hyphae which give rise to long, erect, unbranched conidiophores. Pale brown, single cell conidia were produced at the apex of the conidiophore (Anaissie et al., 2009).

Some of the species in the seven genera were included in Figure 2.1. *E. dermatitidis* produced oval conidia and tapered conidiogenous cells, terminally and intercalarily at 400× magnification (Nachman et al., 1996) (Figure 2.1A). *Cladophialophora bantiana* had long non-fragile chains of conidia which were melanised (Revankar and Sutton, 2010) (Figure 2.1B). Next, *Coniosporium epidermidis* was observed to have melanised conidial chains (Li et al., 2008) (Figure 2.1C). *Cyphellophora guyanensis* had intercalary conidiogenous cells with collarettes. The conidia were curved and

multiseptated (Feng et al. 2012) (Figure 2.1D). Conidia were formed from swollen denticles of *Fonsecaea monophora* which results up to 4 conidia (Revankar and Sutton, 2010) (Figure 2.1E). Funnel shaped collarettes was observed at the tips of the phialides of *Phialophora verrucosa* (Revankar and Sutton, 2010) (Figure 2.1F). Finally, *Rhinocladiella aquaspersa* was observed forming conidia at the apex of the conidiogenous cells (Badali et al., 2010) (Figure 2.1 G).

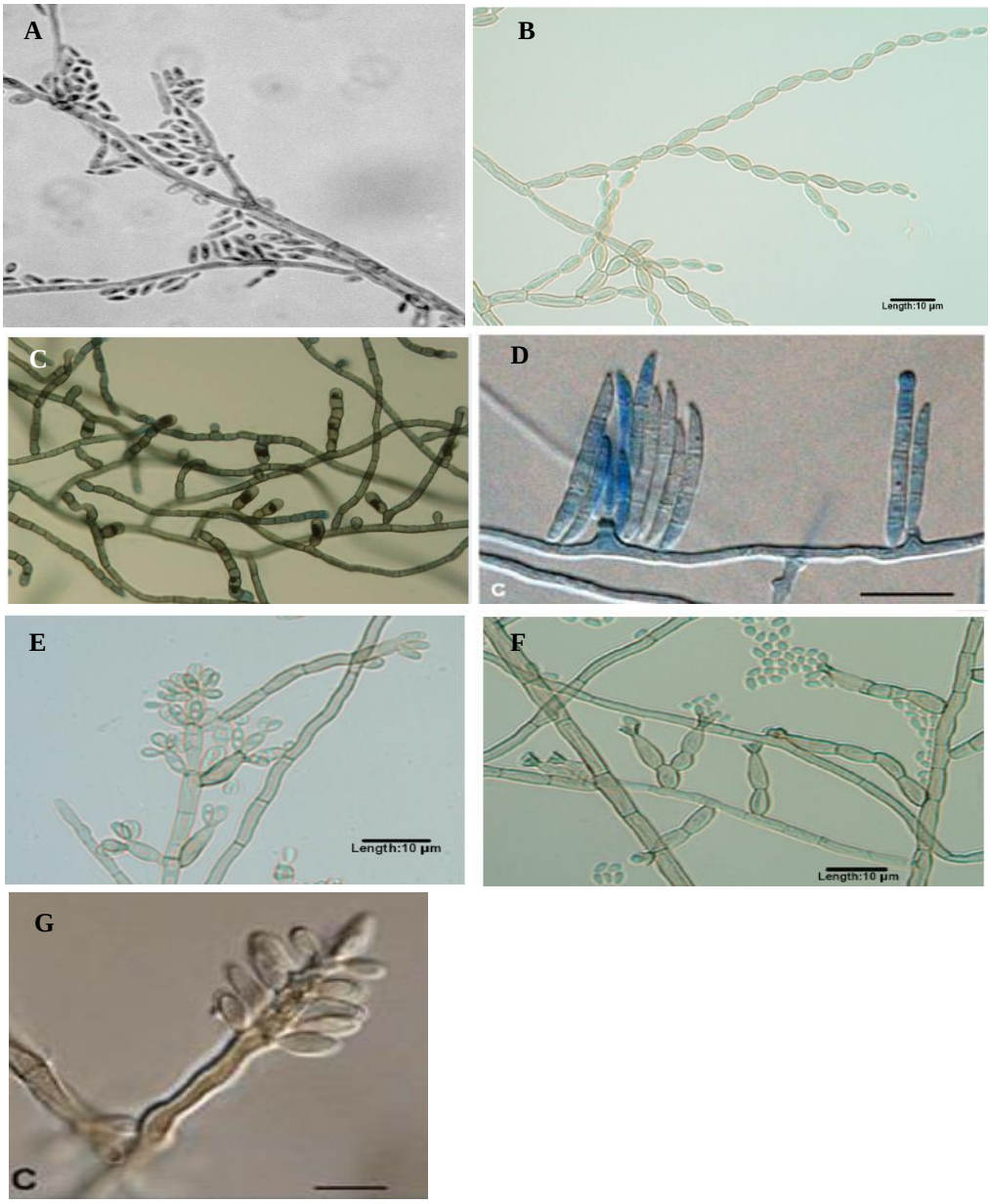

Figure 2.1 Seven clinically important genera in the order *Chaeothyriales.* Some of the species in the seven genera were included in Figure 2.1. *E. dermatitidis* produced oval conidia and tapered conidiogenous cells, terminally and intercalarily at 400× magnification (Nachman et al., 1996) (**A**). Long non-fragile chains of conidia which

were melanised were observed from *Cladophialophora bantiana* (Revankar and Sutton, 2010) (**B**). Melanised conidial chain was present in *Coniosporium epidermidis* (Li et al., 2008) (**C**). Curved, multiseptated conidia were observed from *Cyphellophora guyanensis*. Conidiogenous cells with collarettes were also present. (Feng et al. 2012) (**D**). *Fonsecaea monophora* had conidia which was formed from swollen denticles (Revankar and Sutton, 2010) (**E**). *Phialophora verrucosa* had funnel shaped collarettes at the tips of the phialides (Revankar and Sutton, 2010) (**F**). *Rhinocladiella aquaspersa* formed conidia at the apex of the conidiogenous cells (Badali et al., 2010) Scale bar (10µm) (**G**).

Among these seven genera, *Exophiala* spp. were chosen to isolate from this study, as this was the most common pathogenic black yeast which was clinically important among the seven genera and had many species that were pathogenic.

Cryptococcus neoformans is an encapsulated yeast species that cause infection in humans and animals. Out of the 50 species in this genus, *C. neoformans* and *C. gattii* are considered as human pathogens due to the ability to grow at 37 °C. Although, this genus is not part of the black yeast genera, however, as these species had the ability to produce melanin which deposits in the cell wall, giving a brown colour to the yeast, these species were also considered as black yeasts (Anaissie et al., 2009).

Exophiala spp. and *Cryptococcus* spp. were the two main genera that was selected to isolate from this study. There were several main reasons for selecting these two genera. First, the virulence factors such as the production of capsule, melanin formation, and ability to grow at 37 °C, of both the black yeast were quite similar. Next, both these black yeasts were present from quite similar environmental sources such as wood, and bird droppings besides being reported to be mostly isolated from tropical regions (Anaissie et al., 2009). Thus, both these black yeasts were chosen in this study.

2.2 Ecology of black yeasts

Black yeasts are fungi that have melanised cell wall and forms daughter cells by budding. Mycelial growth and conidia production are also present in yeast cells (Sterflinger, 2006). The black yeasts can live in extreme, toxic and unusual environments (de Hoog et al., 2011). The thick and melanised cell wall protects the black yeast in such harsh environments. *Exophiala dermatitidis* and *Cryptococcus neoformans* are the two - human pathogenic black yeasts that reside in the human-dominated environments (Sterflinger, 2006; Revankar and Sutton, 2010).

2.2.1 Distribution of *Exophiala* spp.

Exophiala spp. are frequently found more in the tropical regions of East and Southeast Asia compared to temperate climates. *Exophiala dermatitidis*, a human pathogenic species was isolated from pineapple and mango fruit surfaces although the isolation rates are lower compared to the presence of this species from floor, wall and seat of steam bath in a study conducted in Thailand (Sudhadham et al., 2008). Turkish steam bath in sauna complexes, sauna halls and water bowl in Finnish sauna were positive for this species too, with higher abundance per culture plate in steam baths compared sauna (Matos et al., 2002) proving that this black yeast prefers some compound present in this nutrient scarce and high temperature environment.

Besides steam bath, one of the common isolations of *Exophiala* spp. is from the railway station. A study conducted at railway stations in several provinces in Thailand reported enormous isolation of *E. dermatitidis* Genotype A and B compared to the other locations besides railway stations. This indicates the blackish debris on the railway ties as one of the preferable niches for this black yeast (Sudhadham et al., 2008). *Exophiala bergeri* and *Exophiala xenobiotica* were also isolated from railway

ties treated with creosote 15 years ago in Southern Brazil which were chosen based on the records of chromoblastomycosis in that region (Vicente et al., 2008). *Exophiala sideris* a novel black yeast was isolated from oak railway ties between the rails in Netherlands after enriching the samples with toluene and xylene (Seyedmousavi et al., 2011). Sampling from the oak railway treated with creosote from Iran resulted in *E. dermatitidis* as the dominant species followed by *E. phaeomuriformis*, *Exophiala heteromorpha* and *E. xenobiotica* (Yazdanparast et al., 2017). However, the species of *Exophiala* differs when high altitude creosote - treated railway ties were examined based on a study conducted in Turkey. The common species were *Exophiala crusticola* followed by *E. phaeomuriformis* and *E. heteromorpha*. These results show that hydrocarbon supports the growth of black yeast in different altitudes (Dogen et al., 2013). This experiment was further justified by the finding of Gumral et al. (2014), which determined the diversity of black yeast by sampling 845 railway ties from 11 Turkish cities representing lowest to the highest altitude. About 11.1% of the samples were positive for black yeast with 75% predominance of thermophile species. They confirmed that the species diversity of black yeast on railway track relies on the type of railway ties and altitude height through their study. A study conducted by Zhao et al. (2010), reported the ability of the black yeast, *E. xenobiotica* to utilise volatile aromatic hydrocarbon such as toluene in the enrichment process after isolating the black yeast from oak railway ties outside the rail. *Exophiala bergeri* was also identified after enrichment of samples with benzene which was initially isolated from oak railway ties between rails in Netherland. The growth of these ascomycetous black yeast can be concluded to be influenced by both the aromatic hydrocarbon present on the oak railway ties and the temperature of the surroundings.

Animal droppings yielded *E. bergeri* according to reports by Zhao et al. (2010). *Exophiala bergeri* was isolated from the guano-rich soil after enrichment with toluene. Besides that, droppings of common myna (*Acridotheres tristis*), rhinoceros hornbill (*Bucerous rhinocerous*) and flying fox (*Pteropus scapulatus*) which were collected in Thailand yielded *E. dermatitidis*. The wild berries that were ingested by these frugivorous animals could be bearing *E. dermatitidis* which might get dispersed via their faeces (Sudhadham et al., 2008).

Black yeasts are also able to survive and grow in man - made environments that are rich in hydrocarbon. Sampling done in a land farming area in Brazil which received a large amount of petrol hydrocarbon yielded *E. xenobiotica*. The hydrocarbon present in the soil might serve as an enrichment factor, thus favouring the growth of the black yeast and suppressing or inhibiting the growth of other competing species (Satow et al., 2008). The same species was also predominantly isolated from car gasoline tanks and was efficient in utilizing toluene as a carbon source (Isola et al., 2013). The ability of this black yeast to metabolize monoaromatic hydrocarbon further confirms the oligotrophic nature of it (Satow et al., 2008).

Another man – made niche which accommodates the black yeast is the dishwashers. *Exophiala dermatitidis* and *E. phaeomuriformis* were the most frequently isolated from dishwashers mainly rubber seals of the door (Zalar et al., 2011). Dishwashers are a favourable ecological niche as it is consistently rich in nutrient and in alkaline condition. The system of washing machines has become preferable to occupy by the black yeast because lower temperatures and less aggressive detergents have been used to save energy nowadays (Zalar et al., 2011). In addition to that, three other mesophilic species were isolated from washing machine soap dispenser too which are *Exophiala equina*, *Exophiala lecanii-corni* and *Exophiala mesophila*. The

detergents used have various complex mixtures of chemicals with different amounts of hydrocarbons such as compounds in fragrance, preservatives and anion surfactants enabling the oligotrophic black fungi to grow well in such environments (Isola et al., 2013).

Surprisingly, sterile environments such as hospital facilities are also contaminated with this black yeast which may cause nosocomial infection. *Exophiala mesophila* was isolated from a dental unit waterline. Their presence is often overlooked as they grow slowly (Porteous et al., 2003). Apart from dental unit waterlines, *Exophiala pisciphila*, *Exophiala cancera* and *E. equina* were isolated from water systems in Brazil from six hemodialysis units. The black fungi were not eliminated from the water treatment process as it was isolated from treated water for dialysis, dialysate and from water inlet of municipal supply. As this black yeast is an oligotroph, it can grow at low density in environment such as drinking water where other saprophytes are absent (Figel et al., 2013). The occurrence of this black yeast as biofilm from domestic water taps is not uncommon, too. From the study conducted in Germany, *E. lecanii-corni* was the most abundant species from biofilm but was not isolated from the water sample from the distribution system. This might be because the conidia of this black yeast are hydrophilic and might get attached to human skin or cleaning utensils which eventually spreads via aerosols resulting in biofilm. This eventually leads to retrograde contamination (Heinrichs et al., 2013).

Another unique isolation of *E. dermatitidis* is from the humidifier (Nishimura and Miyaji, 1982). This shows that the black yeast can survive in the nutrient low environment and high temperatures inside the humidifier. There were also initial reports of isolation from sawdust, wood and bark by Dixon et al. (1980). The humid and poor nutrient environment seems to support the growth of this black yeast.

Moreover, based on a study in Brazil, it was reported that the black yeast can also survive on babassu coconut shells. Lipids, terpenes and other hydrocarbons are present in the babassu coconut, thus providing a special environment for microbial decomposition. *Exophiala alcalophila*, *Exophiala palmae* and *E. spinifera* were isolated strains from babassu coconuts. There were also reports of chromoblastomycosis from that region. However, babassu coconut shells were not source for the disease. This showed that the black yeast might be evolutionarily adapted to higher plants by assimilation of alkylbenzene present in the babassu coconut (Nascimento et al., 2017). Vicente et al. (2008) also reported the isolation of *E. dermatitidis* from *Eucalyptus* wood and *E. xenobiotica* from rotten wood. Soil under coffee tree was positive for *E. bergeri*. This shows that some *Exophiala* spp. can utilize substances present in decaying wood or woody materials for its survival.

Generally, this oligotrophic fungus can be found in moist, hot environments besides isolation from rotten plant materials and hydrocarbon contaminated areas. This black yeast can thrive in microhabitats at a low density where common saprophytes are not present. This might be due to their low competitive ability against other fast-growing microorganisms present at that habitat. However, it also able to reside in the human body causing chromoblastomycosis and neurotropic infections in immunocompetent humans (Vicente et al., 2008).

2.2.2 Distribution of *Cryptococcus* spp.

Cryptococcus spp. on the other hand is a basidiomycete yeast which has a worldwide distribution and is frequently associated with pigeon droppings and *Eucalyptus* tree (Krockenberger et al., 2001).

Ellis and Pfeiffer, (1990) discovered that *Cryptococcus neoformans* var. *gattii* (CNGA) is associated with *Eucalyptus camaldulensis*. The yeast was isolated mostly during the flowering season and from the plant debris such as wood, bark, leaves and accumulated debris collected under the canopies of *E. camaldulensis*. The disease incidence was also high for aboriginal population of the Northern territory, Australia as the *E. camaldulensis* grows along watercourse which plays an important role for the aborigines who live closely associated with the *Eucalyptus* trees. Patients with CNGA infection were mostly from subtropical and tropical regions particularly from southern Asia, central Africa, California and Brazil which evidently corresponds with the distribution of the *Eucalyptus* trees which do not randomly grow in Europe but specified to certain location depending on the climate. A similar relationship between *E. camaldulensis* and CNGA was found between the fungus and *Eucalyptus tereticornis* showing that the global distribution of both the *Eucalyptus* are similar (Pfeiffer and Ellis, 1992)

Pfeiffer and Ellis, (1992) also discovered that three other species which are *Eucalyptus blakelyi*, *Eucalyptus gomphocephala* and *Eucalyptus rudis* as additional host for this variety of *Cryptococcus*. *Eucalyptus camaldulensis* seeds bearing the dormant dikaryotic mycelium of CNGA had been exported from Australia to other countries resulting in a worldwide association of *Eucalyptus* trees with *Cryptococcus* spp. This was further confirmed by the study conducted by Chakrabarti et al. (1997) in India. CNGA was successfully isolated from flowers of *E. camaldulensis* tree whereby the seeds were originally from Australia. The recent combination gave *C. gattii* (CG) as a synonym of CNGA. Gugnani et al. (2005) also reported the isolation of CG and *C. n.* var. *grubii* (CNGR) from flowers and bark of *E. terreticornis* and *E. camaldulensis* respectively from different parts of India. However, the isolation of this

fungus from *Eucalyptus* trees need not be successful every time, as sampling done during other seasons besides flowering season fail to yield CG (Ellis and Pfeiffer, 1990). On the other hand, Bedi et al. (2012) reported that the CNGR and CG can be isolated during all the season in a year from bark, flower, buds and fruits of *E. camaldulensis* and *E. terreticornis*. The isolation rate of CG is however quite low when sampled at other locations besides Australia. Out of 696 samples that were collected by Chakrabarti et al. (1997), only 5 were positive for this variety of *Cryptococcus* and the seeds of the positive trees originally came from Australia proving that not all *Eucalyptus* trees are associated with this yeast.

Apart from that, there are also reports of isolation of *C. neoformans* var. *neoformans* (CNNE) and CNGA from the decaying wood of the hollow tree trunk (Randhawa et al., 2003). However, the isolation from the hollow tree trunk was not specified to one type of tree. This explains that CNNE and CNGA are associated with a specialized ecological niche which results from wood biodegradation. The presence of the laccase enzyme in CNNE and CG further aids the lignin biodegradation process which eventually provides a suitable substrate for the growth of the yeast (Randhawa et al., 2001; Gugnani et al., 2005). This was further confirmed by the study done by Randhawa et al. (2003) which reported the list of trees which harboured CNNE. CNNE was reported to be positive in decayed wood and other plant debris from 22 species from 17 genera of trees from different countries. This emphasized that this yeast is not specific to the plant debris of tree species but is more generalized. Although almost all the environmental findings of this pathogen are from urban areas and some rural areas, Fortes et al. (2001) first reported the isolation of CG from decaying wood in a hollow of a native jungle tree in the wild Amazon rain forest. This further confirms that the

decaying wood provides a suitable habitat for this basidiomycete yeast. As CG is associated with trees, CN, on the other hand, is commonly isolated from bird excreta.

One of the common isolations of CN besides plant material is from pigeon's droppings. The birds are resistant to the disease caused by CN (Casadevall, 2005). Although diverse avian orders such as *Passeriformis*, *Columbiformis*, *Anseriformis*, *Accipitriformis* and *Psittaciformes* are associated with CN, the birds only carry the pathogen externally on their beaks, claws or internally by the feeding on vegetal material or ground feeding contaminated with Cryptococci. Despite ingesting the pathogen, the birds do not develop a systemic infection due to the body temperature of the bird and the ability of the macrophage of the bird to suppress the fungal growth in its body although some yeast might escape the defence mechanism (Johnston et al, 2016). Sites highly contaminated with the bird excreta such as public buildings, monuments and dockside warehouses with great flow of people were positive for *C. neoformans* from a study conducted in Brazil (Ribeiro and Ngamskulrungroj, 2008). The worldwide presence of this yeast from pigeon droppings was further justified by the presence of the same serotype from bird excreta mainly pigeons from Klang valley in Malaysia (Tay et al., 2005).

As mentioned by Gonzalez-Hein et al., (2010), pigeons are not the sole carrier of this yeast. However, the prevalence of CN from other birds besides pigeon is quite low. Approximately only 3.5% of other avian droppings as compared to 11.5% from pigeon droppings were positive for CN. In spite of the frequent isolation of CN from avian droppings, not all samples gave a positive result. Birds kept in captive had a lower prevalence of CN due to the cleaning and disinfection practises performed which eventually makes the bird less susceptible to the cryptococcosis (Lugarini et al., 2008). However, the isolations of CN from dry droppings were more frequent compared to

wet droppings due to the high concentration of ammonia in fresh excrete which makes it alkaline (Lugarini et al., 2008). Abulreesh et al. (2015) also stated that the dry droppings contain fewer bacteria thus reducing the competition rate for nutrients among this fungus and bacteria.

The abundance of creatinine from pigeon dropping seems to be the nitrogen source for the yeast which is not exposed to sunlight enabling their rapid growth. The yeast is also able to be stable up to 2 years or more in pigeon droppings revealing that, pigeon faeces could be the real niche of CN (Gonzalez- Hein et al., 2010). In general, the faeces might get contaminated by CN from the fungal cells distributed by soil or air which gets dispersed by the wind. The rich environment in the excreta supports the growth of the yeast cells eventually. As stated by Lugarini et al. (2008), uninfected pigeon droppings became infected when exposed to the cells of CN.

In brief, *C. neoformans* can be isolated from pigeon and other avian droppings whereas *C. gattii* is mostly associated with *Eucalyptus* trees and decaying wood inside tree trunk hollow. The yeast can cause respiratory and neurological disease in humans when inhaled from an environmental source making it a human pathogen.

2.3 Taxonomy of the black yeasts.

2.3.1 Identification of *Exophiala* spp.

(a) Morphological, macroscopic and microscopic observation

Exophiala species are Ascomycetes, which belongs to the family Herpotrichiellaceae is in the order Chaetothyriales (Isola et al., 2013). These species are characterised by the presence of melanin in the cell wall, yeast-like growth and annellidic conidiogenesis (Seyedmousavi et al., 2011). Although there are more than 40 species in this genus, fourteen have been proven to cause infection for humans (Gumral et al., 2014).

Among the black yeasts, *E. dermatitidis, E. oligosperma, E. phaeomurifomis, E. xenobiotica* and *E. lecanii-corni* can cause systemic infection whereas *E. bergeri, E. spinifera, E. jeanselmei, E. mesophila* and *E. attenuate* mainly induced cutaneous and subcutaneous infection. *E. xenobiotica*, *E. dermatitidis*, *E. oligosperma*, *E. phaeomuriformis* and *E. lecanii-corni* are the most frequently seen clinical species (Zeng et al., 2007).

The main characteristics to differentiate some of the common species morphologically were tabulated in Table 2.1.

Exophiala dermatitidis the most common species (Anaissie et al., 2009) has smooth, dark, pasty colony appearance after 3 days of incubation on SDA, PDA and CMA with yeast like growth. After some time, superficial and submerged hyphae appeared on the periphery of the colonies. Incubating the black yeast in 37 °C for 3 weeks resulted in moist, dark and flat colonies with the centres of the colonies still pasty but slightly elevated and sloped towards the border (Nishimura and Miyaji, 1982). Slide cultures of this black yeast on PDA showed the dimorphic nature of this

organism. Long slender conidiophores, with clusters of oval conidia at the tips of the phialides were formed with aggregates of conidia along the sides of the conidiophores (Nachman et al., 1996). *Exophiala heteromorpha* which was previously identified as *E. jeanselmei* var. *heteromorpha* has minute annelated zones which are in tooth shape (de Hoog et al., 2003).

Studies conducted by Nishimura and Miyaji, (1983) however revealed that the shapes and sizes of the conidia are quite similar among the species but the number of annelids differs between some species of *Exophiala. Exophiala spinifera* was reported to have long annelated tips with 20 annellation compared to only approximately 5 annellation for *E. dermatitidis* and *E. jeanselmei*. However, molecular identification was not conducted in this study to confirm the morphological characteristics.

Table 2.1 Summary of distinguishable characteristics among common clinical *Exophiala* spp.

Characteristics	*E. dermatitidis*	*E. xenobiotica*	*E. phaeomuriformis*	*E. jeanselmei*	*E. spinifera*	*E. lecanii-corni*	*E. oligosperma*
Presence of capsule /EPS	Exopolysaccharide present (Yurlova and de Hoog, 2002)	Absent (de Hoog et al., 2006)	Absent (Yurlova and de Hoog, 2002)	Absent (Yurlova and de Hoog, 2002)	Capsule present (Yurlova and de Hoog, 2002)	Absent (Yurlova and de Hoog, 2002)	Absent (de Hoog et al., 2006)
Presence of special structures	Sclerotic morphology observed in acidic condition (Szaniszlo et al., 1976)	Spherical, subhyaline chlamydospores present (de Hoog et al., 2006)	Yeast- like cells convert into spherical sclerotic cells with several cross walls (Matos et al., 2003)	-	Chlamydospores present (Harris et al., 2009)	Chlamydospores like structures present (de Hoog et al., 1994)	Spherical, subhyaline chlamydospore present (de Hoog et al., 2003)
Annelation/ shape of conidiogenous cells	Cylindrical, bottle or flask shaped conidiogenous cells with evident annelation (Nishimura and Miyaji, 1982). Figure 2.2	Conidiogenous cells lemon shaped or fusiform with flaring irregular annellated zones (de Hoog et al., 2006) Figure 2.3	Annellated zones produced on germinating cells, mature budding cells and hyphae with which are short and inconspicuous (Matos et al., 2003) Figure 2.4	Mature conidiogenous cells rocket shaped, slightly darker than supporting hyphae with regular tapering annellated zones (de Hoog et al.,2003) Figure 2.5	Annellated zones with long clearly visible, frilled hyphae (de Hoog et al., 2003) Figure 2.6	Torulose hyphae forming coherent chains of barrel shaped cells (de Hoog et al., 1994) Figure 2.7	Most of the strains are yeast- like. Few annelidic cells found are stouter than *E. jeanselmei* when it produces rocket shaped conidiogenous cells (de Hoog et al., 2003) Figure 2.8

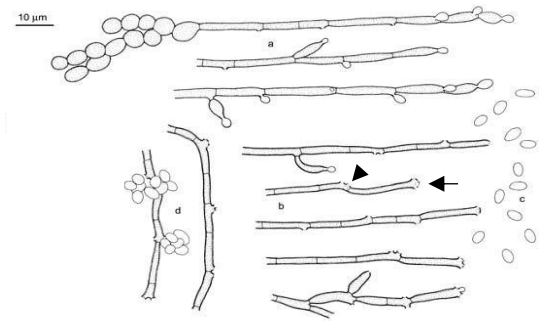

Figure 2.2 *Exophiala dermatitidis* in drawings, (a) Conidia formation from young conidiogenous cells, (b) Mature conidiogenous cells with evident annelation (arrows), (c) conidia, (d) conidia heads (de Hoog et al., 2000a).

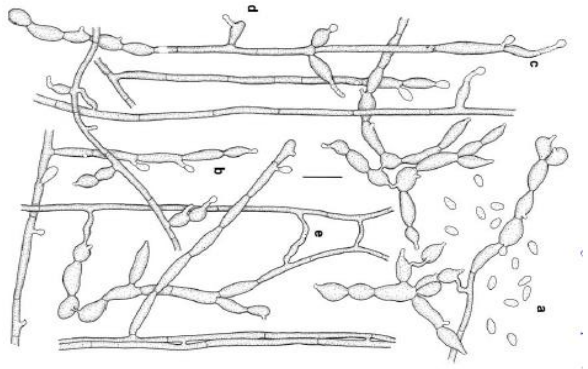

Figure 2.3 *Exophiala xenobiotica* in drawings, (a) Conidia, (b) Conidiophores, (c,d) Conidiogenous cells in lemon shape with irregular annelated zones, (e) anastomoses. Scale bar 10µm (de Hoog et al., 2006).

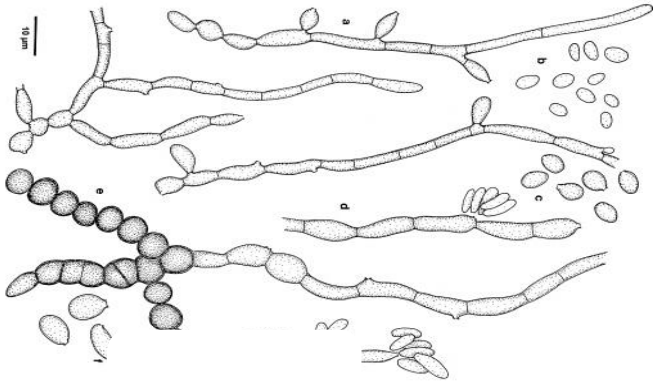

Figure 2.4 *Exophiala phaeomuriformis* in drawings (a) hyphae with non-elongating annelation, (b) budding cells, (c) germinating cells, (d) packets of conidia formed from hyphae with non-elongating annellated zones (Matos et al., 2003).

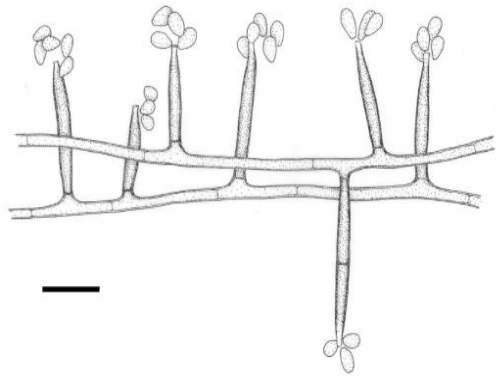

Figure 2.5 *Exophiala jeanselmei* in drawings, shows the conidiogenous cells in rocket form and darker than the supporting hyphae with regular tapering annellated zones. Scale bar 10µm (de Hoog et al., 2003).

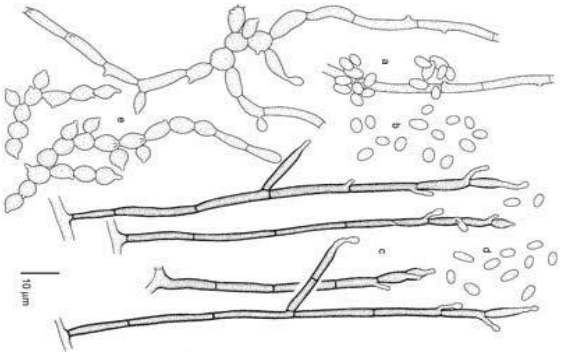

Figure 2.6 *Exophiala spinifera* in drawings, (a,b) hyphae with conidia, (c) annellation zones long on conidiophores, (d) conidia, (e) torulose hyphae (de Hoog et al., 2000a).

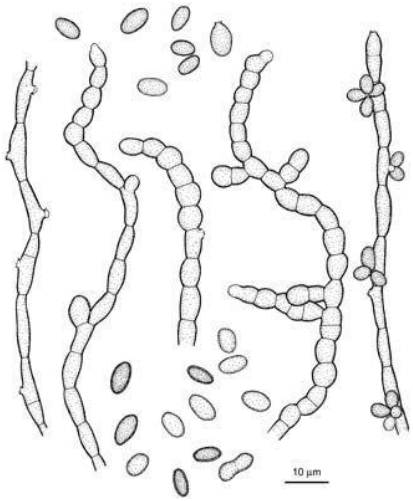

Figure 2.7 *Exophiala lecanii-corni* in drawings, torulose hyphae forming coherent chains of barrel shaped cells (de Hoog et al., 2000a).

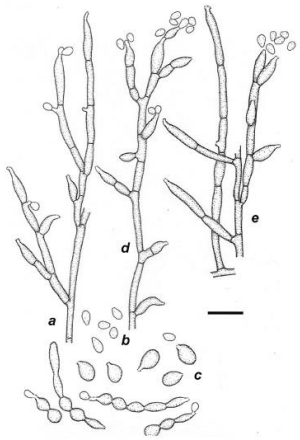

Figure 2.8 *Exophiala oligosperma* in drawings (a, e) shows the rocket shaped conidiogenous cells which are stouter than (d) immature conidiogenous cells of *E. jeanselmei*, (b) shows the conidia, (c) shows the germinating cell. Scale bar 10µm (de Hoog et al., 2003).

Besides that, as mentioned by Sterflinger (2006), the identification of the black yeast species by morphology is quite confusing due to the very fine difference in conidiogenesis. Most of the species in the genus *Exophiala* are difficult to classify and identify due to their passage in complicated life cycles where diagnostic features are variably expressed resulting in very similar microscopic structures among phylogenetically remote species unless sequencing was conducted (Zeng et al., 2007).

Although the predominant morphology of black yeast is a budding yeast cell, due to its polymorphism, the fungi can produce true hyphae, pseudohyphae, moniliform hyphae and sclerotic forms. Both pseudohyphae and moniliform hyphae appear similar under the light microscope due to septal regions constrictions but they differ under the transmission electron microscope based on reports by Wang and Szaniszlo (2007). Pseudohyphae are polarized chains of relatively, normal size of yeast that separates poorly whereas moniliform hyphae do not separate at the septal regions and get elongated until true hyphae with parallel side walls form. Conidia, on the other hand,

are produced from conidiophores that develop from aerial, moniliform and true hyphae (Wang and Szaniszlo, 2007). Sclerotic cells and sclerotic bodies are enlarged, non-polarized and divide by 1 internal transverse septum or multiple intersecting septa (Szaniszlo et al., 1976).

The morphological and structural changes during the conversion from yeast to mold of *E. dermatitidis* was further described by Oujezdsky et al. (1973). The budding yeast with thin walls has abundant mitochondria and ribosomes, several vacuoles and some accumulated storage material. Once the thin yeast transforms into thick-walled yeast, fewer mitochondria and ribosomes were present but the number of storage material increased in the cells. Only the thick-walled yeast with lipid bodies in their cytoplasm converted into hyphal forms by forming moniliform hyphae followed by true hyphae (Figure 2.9). For the yeast to form hyphae-like structures, the yeast must attain spore-like characteristics by having thick walled and accumulation of substrates reserves in the cell (Oujezdsky et al., 1973).

On the other hand, thick-walled multicellular sclerotic bodies of *E. dermatitidis* were observed in chromomycotic lesions either in the macrophages or in present free in the tissue. This structure is commonly seen in vivo than in vitro unless acidic induction is provided for the yeast cells (Szaniszlo et al., 1976).

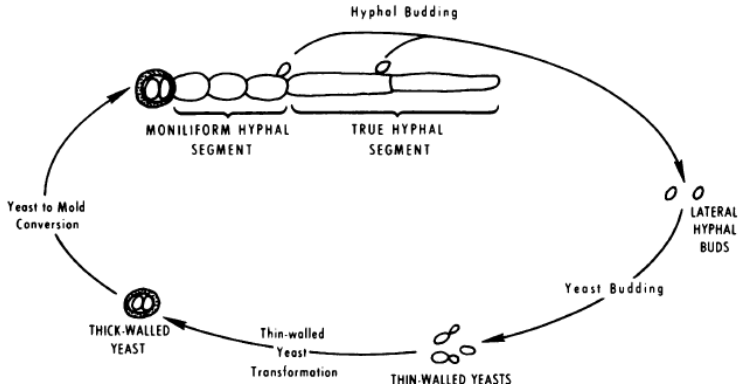

Figure 2.9 The morphological and structural changes during conversion from yeast to mold of *E. dermatitidis* (Oujezdsky et al., 1973).

The sclerotic cell was observed to be phenotypically arrested between the yeast like and hyphae growth stage. Szaniszlo et al. (1976) further described that the yeast cells in the induced acidic medium will have a swollen appearance followed by septation in the cell becoming multicellular and thick walled. Once the sclerotic body that was initially induced from the yeast like form gets transferred to neutral media, the sclerotic bodies give rise to hyphal form which is initiated with moniliform hyphae followed by true hyphae (Figure 2.10).

Besides all these polymorphic structures, *Exophiala* spp. can be identified microscopically by its capsule formation during the yeast phase growth. However, the presence of the capsule varies across the species of *Exophiala.* Yurlova and de Hoog, (2002) described the presence of significant halo around *E. spinifera* yeast cells and hyphal elements whereas, *E. dermatitidis* had irregular exopolysaccharides (EPS) with fibrillar substructures seen under Indian ink staining. Coalescent capsules were evident around *E. dermatitidis* cluster of cells. Other species in this genus produce insignificant EPS or almost none.