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***Yeast Killer Fungus (YKF): characterisation of  
a promising antifungal compound against  
Candida species***

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**Thesis submitted for the degree of  
MSc by Research in Microbiology**

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## ■ Abstract

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Fungal infections reduce the quality of life for 1.7 billion individuals worldwide killing approximately 1.6 million people every year. Candidemia is one of the most common fungal infections in humans, established from pathogenic *Candida* species. About 65% of candidemia cases arise from the commensal *C. albicans*. Emergence of resistance to current antifungal drugs is growing concern. The Kent Fungal Group has recently identified an organism named *Yeast Killer Fungus (Resinicium bicolor)*. The novel YKF secretes a compound that demonstrates desirable traits for a promising antifungal agent.

The aim of this Research Project is twofold: firstly, to characterise the antifungal bioactivity of the YKF compound; and secondly, to utilise biochemical approaches in order to identify the YKF compound.

Firstly, a protocol was established to routinely test the antifungal properties of the YKF compound. During this experiment, it was determined that a fresh YKF culture supernatant (s/n) reaches its full potential at 14 days when stored at optimal 20-22°C, room temperature. In comparison, YKF s/n stored in 4-5°C temperature reached maximum antifungal strength after 34 days. Suggesting room temperature YKF antifungal compound deteriorates faster. Results presented in this thesis demonstrate that the YKF s/n inhibits the cellular growth of *C. albicans* and other *Candida* species including the emerging multi-drug resistant *C. auris* pathogen. Importantly, results presented in this thesis strongly indicate that the YKF compound is a fungicidal agent as it is able to kill *C. albicans* cells. This is key as current antifungal drugs are mainly fungistatic, halting microbial growth. Mass spectroscopy analysis

showed that the YKF compound had close extract spectra with tomatidine, a component synthesised by the tomato plant. However, the nature of the YKF compound still remains unknown.

In conclusion, YKF expresses a strong susceptibility towards a broad range of *Candida* species and suitable fungicidal characteristics. Very beneficial for a future antifungal drug in the market.

**Keywords:** YKF, *Candida* species, *C. albicans*, antifungal, promising agent

## ■ Table of content

---

Abbreviations.....	[9]
Acknowledgments.....	[10]
<b>Introduction</b>	
1. Fungal infections: A overview.....	[11-12]
2. <i>Candida</i> species role in fungal infections.....	[12-20]
2.1. The <i>Candida</i> family members.....	[13-14]
2.2. Cellular biology of <i>Candida</i> species.....	[15-20]
2.2.1. Yeast-to-hyphae switch.....	[15-16]
2.2.2. Yeast-to-opaque switch.....	[16-17]
2.3. The clinical manifestation of Candidemia.....	[17-20]
3. Current antifungal drug treatment.....	[21-25]
3.1. Azole class family.....	[21-24]
3.2. Echinocandins class family.....	[21-22]
3.3. Additional antifungal class.....	[22-24]
4. Toxicity in prolonged antifungal therapy.....	[24-25]
5. Antifungal drug resistance: A growing concern.....	[26-29]
5.1. Azole class mechanisms of resistance.....	[26-27]
5.2. Echinocandins class mechanisms of resistance.....	[27-28]

5.3. Drug resistance and toxicity.....	[28-29]
6. Antifungal drugs development and “the good” features.....	[29-31]
7. Introduction of the <i>Yeast Killer Fungus</i> .....	[31]
8. Aim of the thesis.....	[32]
<b>Methods and Materials</b>	
1. YKF growth and cultivation.....	[33]
2. Media conditions.....	[34]
i. Normal YPD liquid media.....	[34]
ii. YPD 1/10 liquid media.....	[34]
iii. Solid media.....	[34]
3. <i>Candida</i> strains and growth conditions.....	[35-36]
i. <i>Candida</i> inoculation.....	[35]
ii. <i>C. albicans</i> and <i>NCA</i> isolates.....	[36]
4. YKF s/n activity and testing.....	[36-38]
i. Disc-diffusion assay.....	[36]
ii. Evolutionary experiment.....	[37-38]
5. Growth assay to determine YKF s/n activity against <i>C. albicans</i> .....	[38-43]
i. <i>C. albicans</i> biological replicates.....	[38-39]
ii. Growth assay: 96 well plate.....	[39-42]
6. Determining YKF compound antifungal property.....	[44-47]
i. The <i>C. albicans</i> inoculations in normal YPD media.....	[44]
ii. YKF s/n and YPD 1/10 testing.....	[44]

iii. Spin and Wash procedure.....	[44-45]
iv. Calculation for <i>C. albicans</i> cells per plate.....	[45-47]
7. Chloroform extraction.....	[47-48]
i. Chloroform solvent addition.....	[47]
ii. Phase separation and resuspension.....	[48]
8. Thin-layer chromatography (TLC).....	[48-50]
i. Silica paper controls.....	[48]
ii. TLC technique.....	[48-49]
iii. The solvents systems used.....	[49-50]

## Results

1. YKF antifungal components inhibit <i>C. albicans</i> .....	[51-52]
2. YKF compound inhibits growth of various <i>Non-Candida Albicans</i> .....	[53-56]
3. The evolutionary antifungal activity of the YKF s/n.....	[56-60]
4. Increasing YKF s/n volume inhibits <i>C. albicans</i> growth.....	[60-63]
5. Antifungal characteristics of the YKF compound.....	[64-66]
6. Biochemical approach of the YKF compound.....	[66-72]

## Discussion

1. Fungal Infections: A Global Problem.....	[73]
2. The <i>Yeast Killer Fungus</i> (YKF) secretes an antifungal compound.....	[73-74]
3. Establishing a protocol to reliably test the YKF compound activity.....	[74-75]
4. <i>Candida</i> genus species are susceptible to the YKF compound.....	[75-76]

5. Fungicidal VS Fungistatic features of the YKF compound.....	[76-77]
6. Biochemical processes and limitations.....	[77-81]
7. Further studies.....	[82-84]
In conclusion.....	[84]
<b>References</b> .....	[85-96]

## ■ List of Figures

---

<i>Figure 1</i> .....	[14]
<i>Figure 2</i> .....	[16]
<i>Figure 3</i> .....	[17]
<i>Figure 4</i> .....	[20]
<i>Figure 5</i> .....	[23]
<i>Figure 6</i> .....	[28]
<i>Figure 7</i> .....	[31]
<i>Figure 8</i> .....	[33]
<i>Figure 9</i> .....	[37]
<i>Figure 10</i> .....	[38]
<i>Figure 11</i> .....	[39]
<i>Figure 12</i> .....	[42]
<i>Figure 13</i> .....	[43]
<i>Figure 14</i> .....	[45]
<i>Figure 15</i> .....	[47]
<i>Figure 16</i> .....	[50]



<i>Figure 17</i> .....	[52]
<i>Figure 18</i> .....	[53]
<i>Figure 19</i> .....	[54]
<i>Figure 20</i> .....	[56]
<i>Figure 21</i> .....	[59]
<i>Figure 22</i> .....	[60]
<i>Figure 23</i> .....	[62]
<i>Figure 24</i> .....	[63]
<i>Figure 25</i> .....	[65]
<i>Figure 26</i> .....	[68]
<i>Figure 27</i> .....	[69]
<i>Figure 28</i> .....	[71]
<i>Figure 29</i> .....	[72]

## ■ List of Tables

---

<i>Table 1</i> .....	[24]
<i>Table 2</i> .....	[34]
<i>Table 3</i> .....	[36]
<i>Table 4</i> .....	[41]
<i>Table 5</i> .....	[65]
<i>Table 6</i> .....	[71]

## ■ Abbreviations

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YKF – Yeast Killer Fungus

IFI – Invasive Fungal Infections

YPD – media made from: yeast extract, peptone, and dextrose

C. albicans – Candida albicans

TLC – Thin-layer Chromatography

NCA – Non-Candida albicans

s/n – supernatant

ZoI – Zone of Inhibition

MS - Mass spectroscopy

MDR – multi-rug resistance

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## ■ Introduction

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### 1. Fungal infections: A overview

Fungi are eukaryotic organisms found ubiquitously worldwide. Fungi exist in nature in various ways in, plant form or as microorganisms parasitizing in mammalians. However, several fungal species are pathogenic very few of those are primary pathogens in humans [1]. As a consequence, fungal pathogens cause sever disease in individuals that are experiencing advance stages of serious illnesses when their immune system is suppressed. In the recent years, fungal related diseases have risen significantly [1-3]. Present estimate indicates that nearly 1.7 billion people are affected, 300 million of those are more serious in nature leading to approximately 1.6 million deaths to occur annually [1-3]. The global health impact consists of high morbidity and mortality of 30-80%, in addition, to the economic implications presiding a multibillion pounds economic liability per year [1-5].

Fungal infections manifest in two pathological forms, superficial and invasive, also commonly referred as systematic, fungal infection. Superficial fungal infections (SFIs) are defined when the pathogenic fungi are located in the stratum corneum, known as the epidermis or, the skin outer layer [6]. These dermatophyte infections are considered as localised infections that regularly occur among all ages, sex, and global dispersal. These fungal infections are easier to treat and cause less discomfort for the patient. Good personal hygiene in concert with antifungal drug therapy is the usual successful treatment for superficial infections [7].

On the other hand, invasive fungal infections (IFIs) are more challenging to treat and are regarded as a critical world health concern. IFIs are considered those yeast

infections that colonise internally, generalised, visceral, and severe life-threatening infections that commonly occur in immune-compromised individuals [8]. IFIs due to antifungal treatment proceed to chronic manifestation internally in humans and as such are consistently regarded as systemic infections [8].

Currently, invasive fungal infections are among the most challenging clinically administered infections in humans. The total mortality for individuals experiencing IFIs is as high as 45% in a global range [9-10]. Invasive fungal infections occur most frequently in immunocompromised individuals, and less frequently in healthy individuals [11]. Accordingly, the success of novel advanced treatments in cancer care, haematopoietic stem cell (HSC) or organ transplant procedures, neonatal or autoimmune disease therapy, HIV/AIDS infection, and surgery, put these categories of individuals at a significantly high risk [12]. In many instances, such treatment therapies can be the leading cause of fungal infection in the first place [11]. Mortality rate for IFIs individuals remains at high-level although several antifungal drugs are largely available [1][3][5]. Such elevated mortality levels often exceed deaths caused by tuberculosis and malaria [5][13-14]. Therefore, pinpointing to the significance of investigating fungal related diseases, their main component and antifungal therapy.

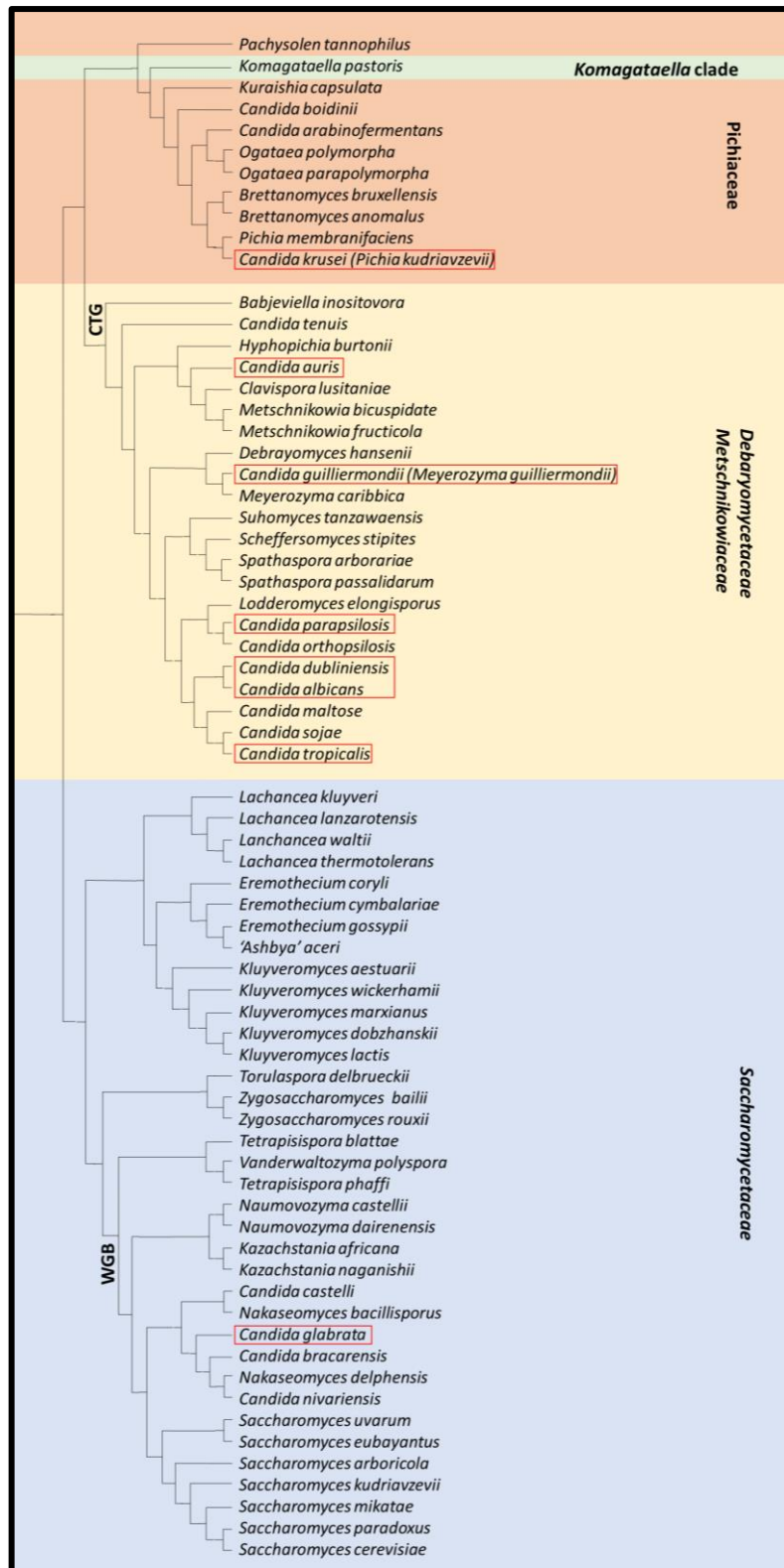
## **2. *Candida* species role in fungal infections**

Mammalian species, and especially humans, are home to one of the most crucial fungal microorganisms to be intensively studied the *Candida* species. These ubiquitous species belong to the polyphyletic fungal order of *Saccharomycotina* [15].

The *Candida* family are generally harmless commensal microorganisms which are predominantly found to colonise the mucous membrane in humans.

## 2.1. The *Candida* family members

*Candida* species are a diverse family of fungal organisms with very distinct evolutionary phylogenesis. *Candida albicans* are the most populous of the *Candida* species which live as a commensal in the human body but turns pathogenic due to genetic or environmental factors. *C. albicans* together with *Candida dubliniensis* are of the same species groups and along together with *C. tropicalis* and *Candida parapsilosis* belong to the same clade called *Candida*. *Candida guilliermondii*, also known as *Meyerozyma guilliermondii*, are the next closest relative as they all belong to the *Debaryomycetaceae* taxonomic family. *C. auris* are the next nearest fungal relatives to the *Debaryomycetaceae* family members [16-17]. These *Candida* species belong to the CTG clade where the CUG codon amino acids leucine to serine are reassigned [18-19]. *C. krusei* (or *Pichia kudriavzevii*), are the most distant relative to *Candida* genus members [16-17]. *C. glabrata* is the furthest fungi species related to any of the organisms belonging to the *Candida* species. *C. glabrata* is a closer fungal relative to the simple yeasting bud *Saccharomyces cerevisiae* (*S. cerevisiae*) and belongs to the WGD (whole-genome duplication) clade [18-19]. *C. krusei* and *C. glabrata* are the only of the *Candida* species that do not belong to CTG clade. The taxonomic illustration of the most clinically relevant *Candida* family is presented below on *Figure 1*.



**Fig. 1. The phylogeny of *Candida* species involved in candidemia.** Squared in red are the *Candida* species used on this thesis. Most of the *Candida*, including *C. albicans* belong to the CTG *Debaryomycetaceae* clade. *C. glabrata* and *C. krusei*, belong to the diverse WGD *Saccharomycetaceae* clade and *Pichiaceae* clade, respectively.

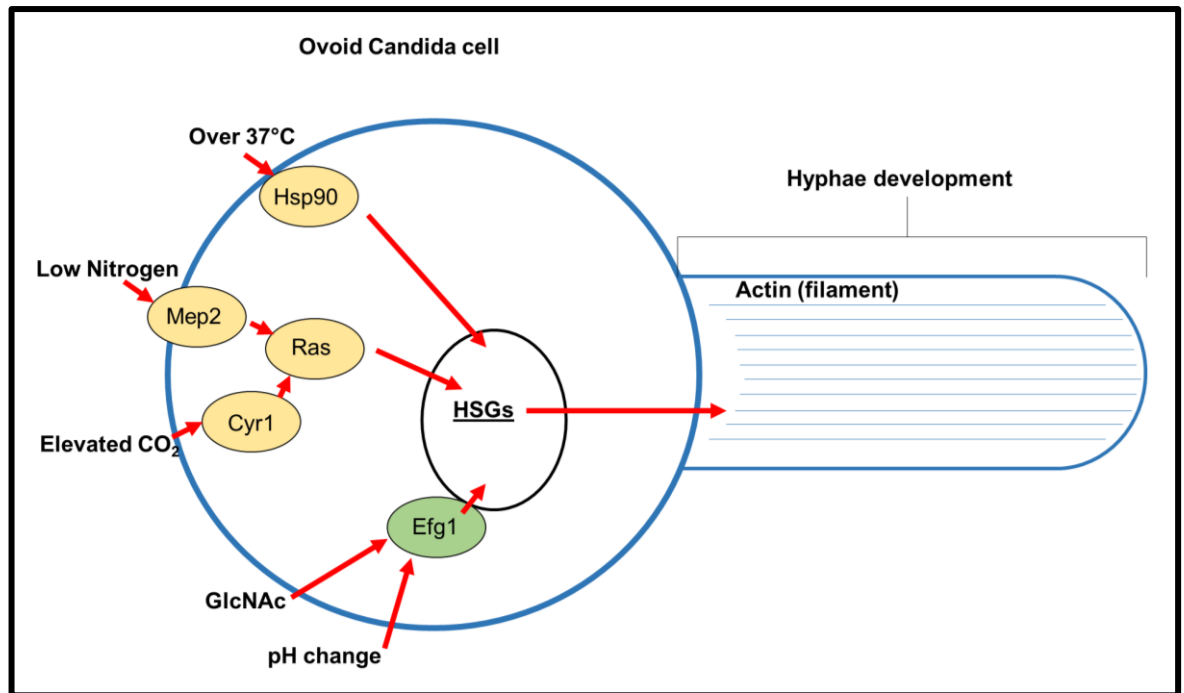
## 2.2. Cellular biology of *Candida* species

*Candida* species are genera of a diverse fungal kingdom. *Candida* are ascomycetous-like fungal species where most family members are anamorphic [20]. *Candida* species, when grown in laboratory environment, display round, relatively large in shape, in a white to light yellow colour [21]. *C. albicans*, named as such from the Latin word *albico* for “whiten” or “becoming white” are the most studied and pervasive fungal pathogen in humans. *C. albicans* undergo a diploid life cycle and express a wide-ranging variety of morphological phenotypes due to their ability to phenotypical switch and transitional switch from yeast bud to other forms [21-22].

### 2.2.1. Yeast-to-hyphae switch

The *C. albicans* and other *Candida* species are grown as round, ovoid, shaped fungal organisms. The morphological yeast-to-hyphae disfiguration arises by various external stimuli as seen in Figure 2. The environmental promoters investigated to cause hyphae development are: a temperature increase of above 37°C, carbon and/or nitrogen starvation, reduced oxygen, elevated levels of carbon dioxide, change in pH, increasing cell density during quorum sensing, and generation of N-acetylglucosamine (GlcNAc) [23-24]. Furthermore, pathogenic *C. albicans* undergo the filamentous hyphal cell change either outside or inside the host bloodstream. This hyphal change is extensively suggested to contribute towards the fungal virulence [25]. *C. tropicalis* and *C. dubliniensis* are also known to undergo the yeast-to-hyphae transformation but on more limited environmental conditions range [26]. Recent studies also suggest the *C. auris* also forms hyphae, however, unlike *C. albicans* and other closely phylogenetic related *Candida*, only do so when evade the host protective barriers [27].



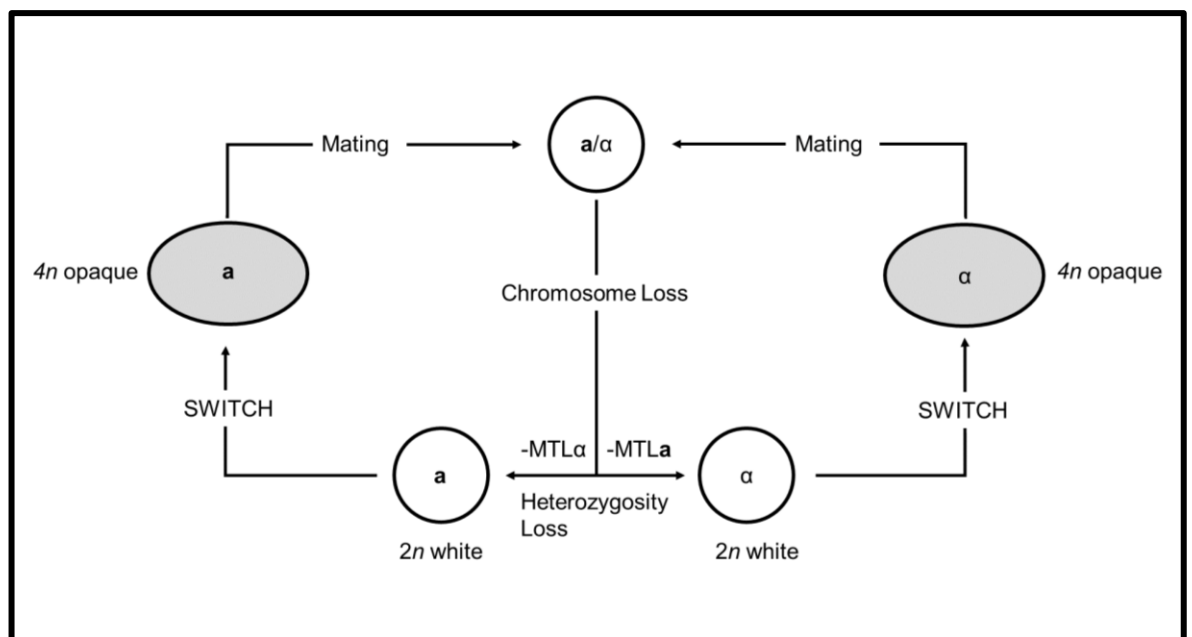


**Fig. 2. A simple schematic representation of the environmental stimuli that lead to the filamentous hyphae phenomena.** The outer factors interfere with cellular mediators which activate transcriptional changes. The alternation cause in turn the development of hyphal morphological properties. However, certain molecular processes are still not fully understood.

### 2.2.2. Yeast-to-opaque switch

The other significant morphological change that happens to *C. albicans* is white-to-opaque switch. The opaque cells adopt a darker colour, growing larger and elongated cells, and express an opaque specific set of genes [28]. The structural switch is suggested to occur primarily as a result of transcriptional regulators, however, the full molecular mechanisms for the switch state are not fully understood [28]. The key component on this process is the *C. albicans* mating cycle. The transcriptional factors encode the Mating Type Like (MTL) locus referred to as, *a/α* [29-30]. This process, demonstrated on Figure 3, occurs when *a/α* cells under homozygosis mating leading to *a/a* and *α/α* cells hence prompting white-to-opaque

switch to arise [31]. MTL-homozygous **a/a** and  $\alpha/\alpha$  cells mate only with those cells that exhibit the opaque phenotype [21][32]. The white-to-opaque switch is directed by the *WOR1* (*White to Opaque Regulator 1*) protein [33]. The phenotypic switches occur spontaneously as a result of environmental, epigenetic, or other additional factors, the yeast bud-to-hypha and the white-to-opaque are the main *C. albicans* switches.



**Fig. 3. The white-to-opaque switch of *C. albicans* operated by the MTL.** The diploid *C. albicans* when mate generate a **a/α** cell tetraploid. To return to the diploid state the cell must undergo loss of chromosome thereby no conventional meiosis does not occurred. The MTL process causes the cell switch and a large darker oval cell is produced. For **a** cell mating with  $\alpha$  cells is possible but both have to be in structural opaque for this to happen.

### 2.3. The clinical manifestation of Candidemia

*Candida* species specifically reside on human gastrointestinal (GI) and genitourinary (GU) tracts [15]. However, as mentioned, in immunocompromised

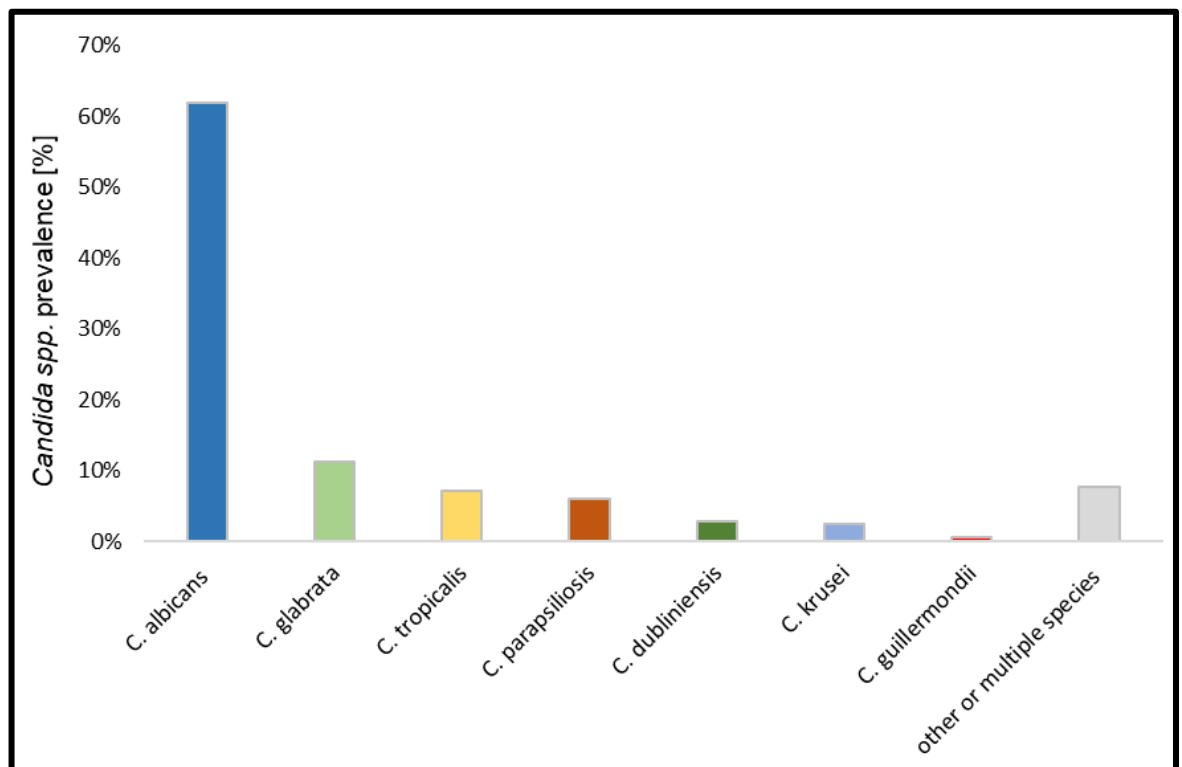
individuals *Candida* species become pathogenic organisms. Patients undergoing intensive care such as, chemotherapy, HSC, or organ transplantation, *Candida* organisms can cause mouth and oesophagus infections. In patients undergoing chemotherapy *Candida* microorganisms are transmitted via the intestinal epithelium membrane thereby heavily affecting the liver and spleen. These organs are both effected as they are connected through portal circulation [34]. HIV infected individuals are also significantly influenced by pathogenic *Candida* organisms, resulting, to circulatory infections [35-36]. In healthy women, pathogenic *Candida* species severely affect the reproductive organs causing *vaginitis* [35-36]. Candidemia, in the early 2000s, was determined to be the fourth most common nosocomial infection in the USA, causing over 10,000 deaths annually as a result of *Candida* species infections [35-39].

Most of candidemia cases, as mentioned, are related to *C. albicans*. The majority of individuals worldwide carry *C. albicans* as harmless commensal microorganism inhabiting the mucosa membrane [40]. The evolutionary process of *C. albicans* gives them the ability to refuge and live in intense conditions within the human microenvironment. They colonise the high pH intestine, benefiting from the anaerobic and high supply nutrient of the GI tract or reside on the low pH vaginal system, profiting from glycogen resources and aerobic environment [41-44]. *C. albicans* rapidly adapt to different locations, mainly to the evolutionary ability to go unnoticed by human immune system macrophages. *C. albicans* avoid interaction with *dectin-1 receptor* on the macrophage using glycoproteins to hide  $\beta$ -1-3-D-glucan in its cell wall [45]. *C. albicans* are one of many other fungi that reside as commensals in the human organism but on a pathogenic form infect and cause challenging diseases.

Furthermore, candidemia in approximately 92% of cases has been caused by members of *Candida* species. *C. albicans* account for most of *Candida* caused candidemia at 65.3%, *C. glabrata* are responsible for 11.3%, *C. tropicalis* for 7.2%, *C. parapsilosis* accounts for 6%, and 2.4% of cases arise because *C. krusei* (Figure 4) [46]. The other most common organisms, *C. guilliermondii* only account for 0.7% of candidemia incidents. A new emerging fungus is *C. auris*, first, reported in 2008 in Pakistan and then named in 2009 by *Sato et al.* from investigations collected on fungal-infected patients in Japan [47][48]. *C. auris*, as a recently recognized fungi, based on various studies suggest prevalence in candidemia is uncertain as it is globally spread with highest number of reported cases in India, the UK, Spain, and the New York State in the US [16]. In comparative studies *C. auris* has shown to be less virulent than *C. albicans* but trends of higher mortality rate [49-53]. In contrast to other *Candida* species, *C. auris* can successfully spread to other nosocomial environments [54]. *C. auris* is able to reside in such environments due to its capability to form biofilms and has proved to be difficult to kill even with high-levels of disinfectant treatment [54-55].

The incidence frequency of *Candida* species isolates differs based on patient characteristics such as, age or ongoing disease, and geographical location. *C. albicans* and *C. parapsilosis* are predominantly observed in younger individuals and their infection prevalence decreases with age. In comparison, *C. glabrata* prevalence in candidemia infected patients increased with older age [56]. Underlying disease is also a factor in for the advancement of candidemia. *C. albicans* are indicated to be the primary cause of candidemia in oncology and haematology related cases, respectively 51% and 33% of precedence [57].

Furthermore, *C. krusei* related infections are expressed in Europe and North America, 3.4% and 3.1% respectively, whereas *C. glabrata* is more common in North America, accounting for 21% of the candidemia infected individuals. *C. tropicalis* is over-represented in Latin America, at 13.2%, and Pacific Asia, at 11.7%, in comparison, *C. guilliermondii* is expressed in 2.2% of the cases in Latin America [4][46]. *Candida* species are a diverse family of yeast that holds responsibility for IFIs at various ages and geographical position.



**Fig. 4, Candidemia infection prevalence per *Candida* species.** *C. albicans* are involved in approximately 65% of all Candidemia related infections. The yeast *C. glabrata* accounts for a considerable prevalence in Candidemia cases at about 11%. Other *Candida* species are less involved in the global occurrence of Candidemia.

### 3. Current antifungal drug treatment

There are numerous antifungal drugs available for fungal infections. Azole and echinocandins are the two main antifungal class of drugs.

#### 3.1. Azole class family

Azoles are the largest family of antifungal drugs. Azole drugs inhibit the sterols biosynthesis enzyme lanosterol  $14\ \alpha$ -demethylase, seen on *Figure 5*, which prevents the biosynthesis of the fungal cell membrane ergosterol [58]. Analogous to the animal cell cholesterol, ergosterol is the largest fungal cell membrane sterol agent. The efficacy of azoles as antifungals relates to the sufficient morphological differences of human cholesterol and fungal ergosterol. Thus, azole antifungal target does not biosynthetically cross-react with the human host cells. The azole family is categorised in three groups the triazoles, like fluconazole; the imidazoles, including ketoconazole; and the itraconazole hydroxylated analogue, posaconazole (*Table 1*) [59]. The azole drugs block  $14\ \alpha$ -lanosterol demethylase a cytochrome P450-dependent enzyme, encoded by *ERG11* gene. In the cell membrane lanosterol is converted to ergosterol by the *ERG11* gene consequently inhibits the growth and replication of the fungi [60].

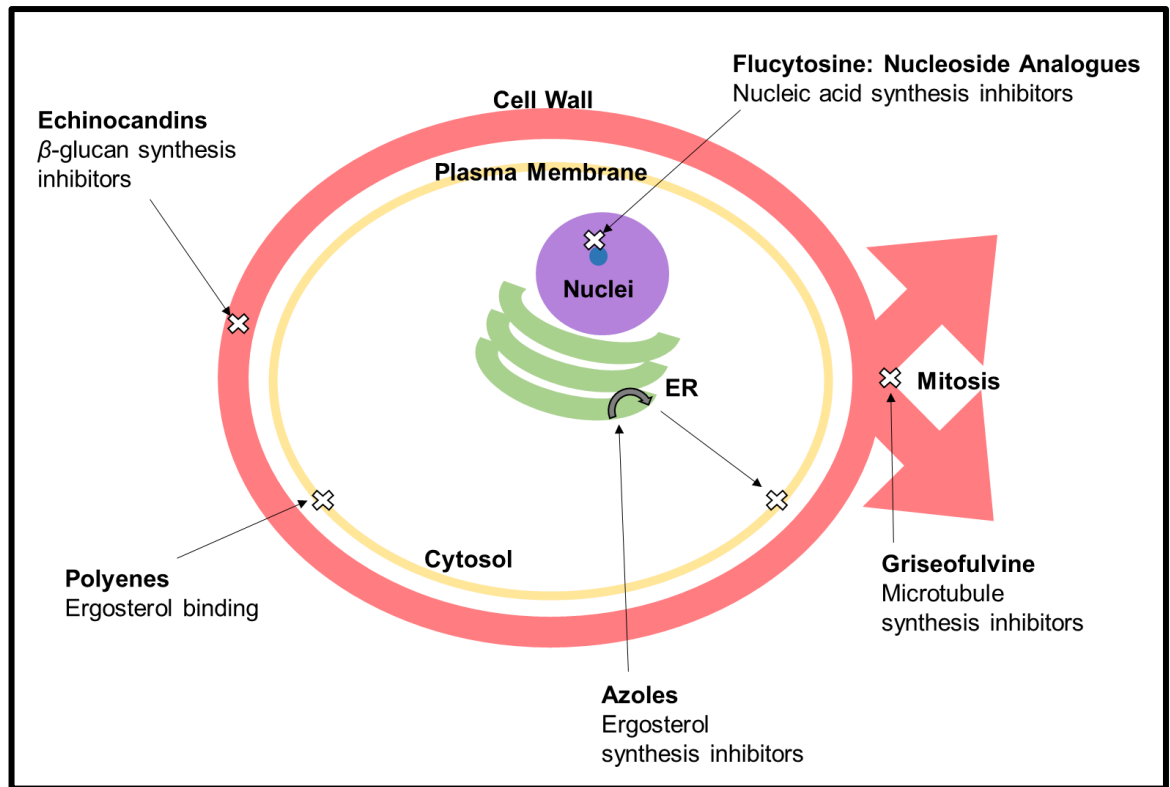
#### 3.2. Echinocandins class family

Echinocandins are the second largest family of antifungal drugs. Echinocandins are the newest class of antifungal drugs available, shown on *Table 1* [61]. Caspofungin, micafungin and anidulafungin were identified in the 1970s however took 30 years to be approved. The caspofungin drug was only approved in 2001 and is the only novel antifungal class of drug to gain approval in the 21<sup>st</sup> century [62]. The membrane-bound glucosyltransferase enzyme,  $\beta$ -(1,3)-*D*-glucan synthase forms

more than half of the cell fungal cell wall components and is the prime structural polysaccharide for glycoproteins, chitins and other cell wall agents attach [63]. Therefore, echinocandins lipopeptidic agents, particularly target fungal wall enzyme  $\beta$ -(1,3)-D-glucan synthase inhibiting its function, presented on *Figure 5*. This prevent the establishment of the crucial cell wall polysaccharide component  $\beta$ -(1,3)-D-glucan, necessary for cell wall integrity and preventing osmotic lysis [64-65]. Echinocandin drugs noncompetitively block  $\beta$ -(1,3)-D-glucan synthase enzyme complex, targeting specifically the *FsK1* subunit, disrupting fungal cell wall structure thereby causing osmotic imbalance and cell death [63].

### **3.3. Additional antifungal class**

Various other antifungal targets are in the market demonstrated on *Figure 5* and *Table 1*. This include Polyenes like nystatin and amphotericin B which bind ergosterol to the cell membrane disrupting the lipidic components leading to development of aqueous pores in the membrane (*Figure 5*). As a result, the cellular permeability is compromised causing cytosolic component leakage and eventually cell lysis [66]. Flucytosine is a pyrimidine nucleoside analogue which inhibits thymidylate synthase disrupting DNA synthesis (*Figure 5*) [67]. The phosphorylated 5-fluorodeoxyuridine monophosphate is integrated into RNA thus also interfering with RNA synthesis [68]. Griseofulvin is a tricyclic spirodiketone which disrupts cellular mitosis (*Figure 5*). This antifungal agent interferes with spindle and generation of cytoplasmic microtubules, hence inhibiting mitotic cellular division [69].



**Fig. 5. The mode of action for various antifungal agents and the primary target.** Azoles target cell membrane by blocking lanosterol *14- $\alpha$ -demethylase* activity. Echinocandins inhibit cellular wall enzyme (*1,3*)- $\beta$ -*D*-glucan synthase activity. Polyenes agents attach to ergosterol components, interfering with the plasma membrane of the fungi. Nucleoside analogues like, flucytosine disrupt fungal DNA and RNA synthesis in the nucleus. Griseofulvine disrupts the mitotic process by inhibiting the synthesis of the microtubules.



Antifungal class	Mode of action	Antifungal drugs	
Azoles	Inhibit the lanosterol $14\text{-}\alpha$ - <i>demethylase</i> mechanism	Triazoles	Fluconazole Itraconazole Voriconazole
		Imidazoles	Ketoconazole Miconazole Clotrimazole Econazole
		Posaconazole	
Echinocandins	Inhibit the activity of $(1,3)\text{-}\beta$ - <i>D-glucan</i> synthase enzyme	Caspofungin Micafungin Anidulafungin	
Polyenes	Interfere with ergosterol binding	Nystatin Amphotericin B	
Nucleoside analogues	Inhibit and disrupt DNA/RNA synthesis	Flucytosine	
Antibiotic	Interfere with $\beta$ - <i>microtubulin</i> during mitosis	Griseofulvin	

**Table 1. The antifungal classes: major molecular targets and the available drugs to date.**

#### 4. Toxicity in prolonged antifungal therapy

The current drugs against fungi related infections have improved significantly over the past few decades. However, these drugs still express high levels of toxicity. In

already immunosuppressed individuals that undergo a systemic antifungal treatment for a long period are subject to a higher risk of toxicity. The side effects related to the high toxicity lead to mild to life-threatening. Itraconazole, an orally managed azole lead in most instances to nausea, GI irritations, and also to a rash which is a common side effect for most antifungal agents. Itraconazole taken with the oligosaccharide cyclodextrin excipient for a long-lasting treatment course of action becomes intolerable for the human organism [70]. In older adults, prolonged therapy of itraconazole results, in a triad of disorders hypokalaemia, hypertension, and oedema [70]. Individuals with history of heart failure and/or blood pressure issues are not recommended prolonged treatment with itraconazole.

Additionally, long-lasting voriconazole therapy causes phototoxic reactions in association with blistering, erythema, and cheilitis [71-72]. Such reactions are not preventable but by discontinuing the treatment most cases are reversible with no additional side effects. Nonetheless, there have been growing concern that prolonged phototoxicity for certain individuals leads to squamous cell carcinoma development [71-73]. Thus, it is strongly recommended that most patients should undergo screening for any signs of skin cancer especially if there are clear manifestation of photodamage reactions [72]. The antifungal agents provide short term effective therapy, whereas long term treatment demonstrate high rates of toxicity that would give rise to life-threatening consequences.

## 5. Antifungal drug resistance: A growing concern

Antifungal drugs are highly effective, however, there is an increasing level of tolerability that *Candida* species and additional pathogenic yeast are expressing towards antifungals agents.

### 5.1. Azole class mechanisms of resistance

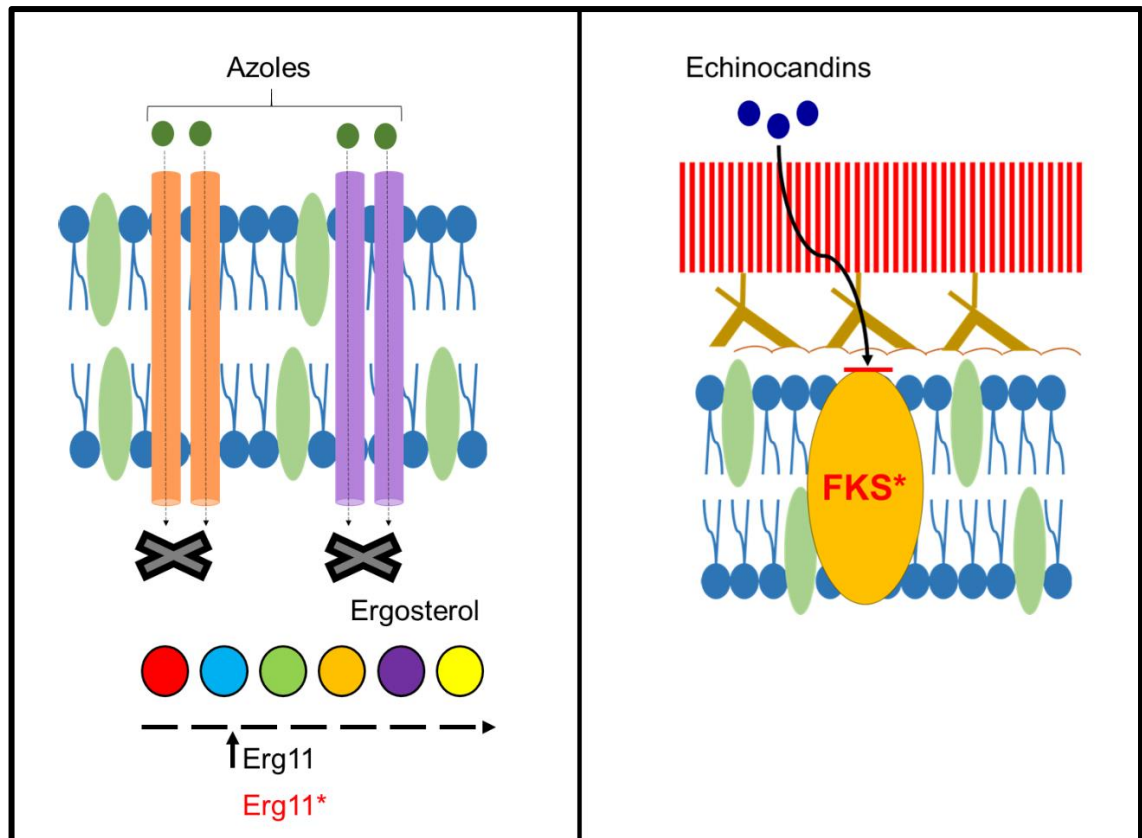
The leading cause in azole resistance are *lanosterol 14  $\alpha$ -demethylase* mutations subsequently resulting in drug-target downregulated affinity or increased generation or both [74]. *ERG11* gene, presented in *Figure 6*, is responsible for encoding *lanosterol 14  $\alpha$ -demethylase* mutations. Fluconazole resistance, as a result of *ERG11* mutations is most commonly associated to *C. krusei* resistance but is also suggested for *C. albicans*, *C. parapsilosis*, *C. tropicalis* where increased resistance to fluconazole are on the rise [75-76]. In *C. albicans* and *C. tropicalis* upregulation of *lanosterol 14  $\alpha$ -demethylase* within the fungal cell, overwhelms azole concentrations hence causing resistance to the antifungal drugs [74]. In addition, upregulation of the efflux pumps is also a factor for the prevalent of azole resistance [74]. Overexpression of *CDR* genes which encode the *ATP-binding cassette* (ABC) efflux pumps, leads in azole resistance in *C. albicans*, *C. glabrata* and *C. krusei* [77]. Upregulation of the *MDR* genes which encode the *major facilitator* (MF) efflux pumps, is associated only with resistance for *C. albicans* and *C. parapsilosis* [76].

Susceptibility to azole drugs alternates from various *Candida* species. Studies have shown that fluconazole and voriconazole have a significantly high susceptibility of 97% and 98.5% for *C. albicans* isolates. For *C. tropicalis* 91% and 90% are susceptible to for *C. parapsilosis* 93 and 97%, for *C. glabrata* 68% and 83%

respectively where susceptible to fluconazole and voriconazole [46]. *C. guilliermondii* which accounts for less than 1% of candidemia cases, demonstrated 74% susceptible for fluconazole and 91% for voriconazole [4][46]. Moreover, the new emergent *Candida* pathogen, *C. auris* appears to exhibit high levels of antifungal resistance to fluconazole and voriconazole agents, 7 and 46% respectively [47].

## **5.2. Echinocandins class mechanisms of resistance**

Yet similar to azoles effectiveness, *Candida* and other fungal species are gradually increasing resistance. Point mutations in *FKS1* and/or *FKS2* genes, seen on Figure 6, are associated with *Candida* species developing echinocandin resistance [78-40]. Nevertheless, the precise resistance pathway is not fully understood. Prolonged exposure arises mutations in *FSK1* gene, this phenomenon has been observed in *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* [81]. As of yet *FSK2* acquired mutations are only detected in *C. glabrata* [82]. The increasing rates of mortality and morbidity associated with fungal, especially *Candida* species infections, occurs as a result of limited antifungal drugs.



**Fig. 6. The antifungal drugs resistance mechanisms.**

On the left is presented the azoles family member drugs that target cell plasma membrane. Mutations in *ERG11* genes lead to overexpression or alternation of the azole target. Therefore, azole drugs do not interact with ergosterols and antifungal resistance occurs.

On the right is presented the Echinocandins member drugs make their way through the gluco- and mannoproteins matrix,  $\beta$ -1,6-*glucan* complex integrated on the outer cellular wall. Mutations on *FKS* genes (mutations showed as (**FKS\***)), however, prevent the echinocandins to target  $\beta$ -(1,3)-*D-glucan synthase*. As a result, antifungal resistance arises.

### 5.3. Drug resistance and toxicity

Various antifungal drugs have various functions therefore, in certain instances more than one drug has to be administered. However, this rises another concern that is drug-drug interactions. Coadministration of certain antifungal drugs, such as any type of triazole with echinocandin drug – caspofungin, risks the possibility of low

concertation in the circulation or inhibiting each other's metabolites causing an increased risk of treatment failure [86]. The development of antifungals is shackled, in comparison to the development of novel antibacterial drugs as, yeast are eukaryotic organisms sharing closely related cellular aspects to humans hence lack broad target for pathogen-specific antifungals [10][84]. The need to discover novel antifungal drugs and the understanding their mechanisms is vital for treating fungal infections. Very few antifungal drugs remedy IFI, many of which are significantly developing resistance. Consequently, there is a growing demand for novel and long lasting affective antifungal drugs.

## **6. Antifungal drugs development and “the good” features**

A novel antifungal drug to enter the consumable mark into a pharmacy shelf requires certain features, demonstrated on *Figure 7*, and rigorous testing for efficacy. Firstly, new agents must have a broad spectrum for targeting various fungal organisms. The ideal antifungal agent should attack and kill development of the fungal species responsible for most fungal infections, including members of the *Candida*, *Aspergillus*, *Cryptococcus*, *Lomentospora* and *Fusarium* species, class family.

Secondly, for the past decades a concern has been toxicity levels associated with antifungal drugs. For instance, the increased toxicity levels associated with amphotericin B have expressed irritations after prolonged usage. Therefore, the new antifungal agents have to express a much-reduced level of toxicity equal to none. The new drugs should be safe for a prolonged course of treatment.

Thirdly, another important aspect of new antifungals is drug-to-drug interaction. The occurrence of drug-to-drug interaction could lead irritation or even organ

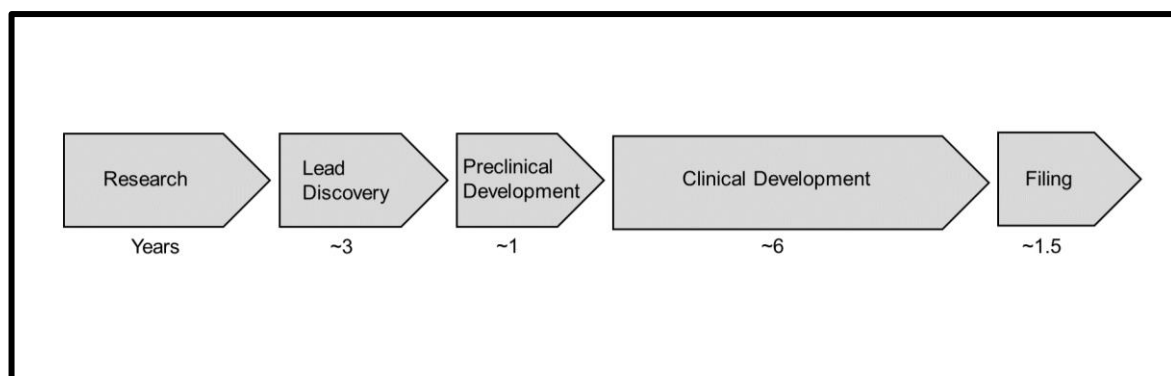
complications. Therefore, new antifungal drugs should have a clear mode of action against fungal infections and such interaction must not occur.

In addition, the current drugs available, even though provide clear relief of symptoms, still remain fungistatic. Although fungistatic drugs simply inhibit the growth for a temporary period of time and do not to provide full clearing of infection. Thus, an ideal antifungal agent would be a fungicidal drug killing and further halting fungal contagious organ spread.

Moreover, a new drug needs to undergo drug discovery screening and development. After initial essential research, compound analysis and assays, and *in vivo* investigation. If deemed safe, a period of drug discovery justification and candidate selection occurs. Then it proceeds into preclinical development and later proceed to clinical trial phases once approval by the governmental authorities is granted for human trails. After being safe and passes a success rate higher than 50%, is approved by the drug administration authorities for mass production.

Sixthly, the new antifungal should be efficient for the consumer. Therefore, oral administration, i.e. pill, is easier for administration rather than intravenously which is uncomfortable and time consuming.

Lastly, novel antifungals have to accessible for every individual worldwide and be cost efficient. Drugs need to be available and at low rate price for purchase not only in the developed world but also in developing and least developed countries.



**Fig. 7, The drug discovery process and the time scale for a drug to go from a molecular laboratory research to be filled ready for consumption.**

## 7. Introduction to the *Yeast Killer Fungus*

Prof Brian Cox within the Kent Fungal Group recently identified a fungus which he fittingly named the *Yeast Killer Fungus* (YKF) due to its ability to release a metabolite that inhibits fungal growth in a *Candida* streaked-plate. This fungus was fittingly named as a “*killer fungus*”, as it secretes a compound which exhibits preferable antifungal properties. Previous studies conducted by Buscaino lab members, have identified YKF as 99.67% genomic identical to *Resiniium bicolor*, a fungus found in decayed wood [85]. Preliminary research showed YKF to have antifungal activity against *C. albicans*, but no antibacterial activity was suggested [85]. This new fungus also exhibits growth inhibition of *C. albicans-fluconazole resistant* strains. Importantly, *in vitro* characterisation using human cell lines suggested no human toxicity present [85]. Additionally, heat stability test showed that YKF compound is resistible to temperatures as high as 120°C [85]. These factors demonstrate the compound secreted by YKF organism, is a promising antifungal component.



## 8. Aim of the thesis

Previous research indicates that the antifungal compound generated by the *Yeast Killer Fungus* has potential for an antifungal agent. This could lead, to pharmacological screening and development of a future drug. During the course of this project the microbiological activity characteristics and biochemical properties of the YKF antifungal compound have been studied.

To determine the microbiological bioactivities of the YKF compound several aspects were investigated. Initially, an evolutionary experiment was conducted to acquire a laboratory protocol for routine use. In addition, the YKF supernatant bioactivity towards the *C. albicans* and various other *NCA*'s, including the infectious agent *C. auris* was investigated. An important aspect of the research was the study of the antifungal characteristic of the YKF compound. It was pivotal to establish if the YKF compound is a fungicidal or fungistatic agent.

Moreover, the secondary aim of the research was the biochemical analysis of the YKF compound. The goal was for the YKF compound to be purified, isolated and identified in order to obtain more knowledge on the antifungal compound and to use its concentration for microbiological investigations.

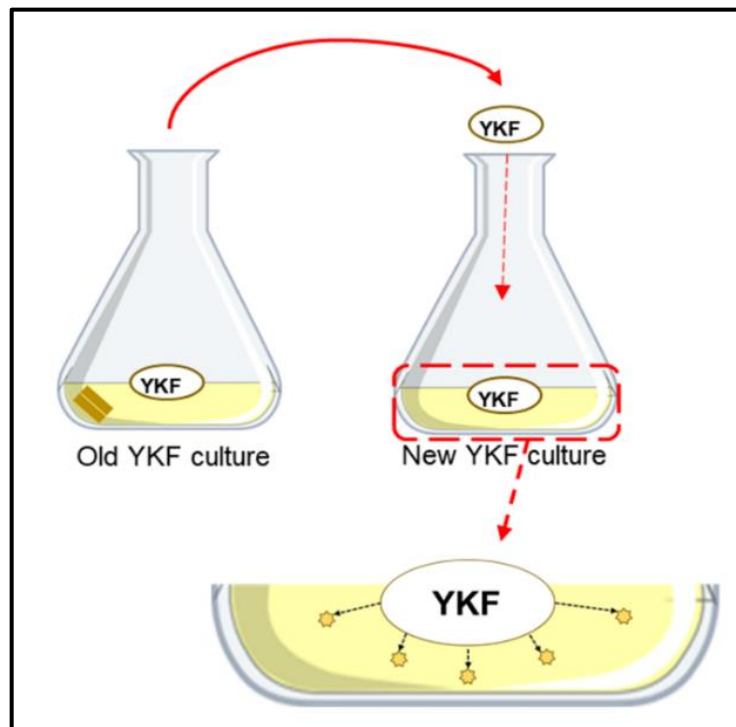
The fungal infections are on the rise the need for new antifungals is to be addressed urgently. Therefore, the yeast killer fungus promises desirable features that could, in the near future, undergo drug discovery procedures and have a novel antifungal drug in the shelves.

## ■ Methods and Materials

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### 1. YKF growth and cultivation

The *Yeast Killer Fungus* (YKF) was stored in Buscaino's lab in the cold room (4-5°C). YKF (puffball-like shape fungal structure) was grown in YPD 1/10 liquid media made from yeast-extract, peptone and dextrose (Table 2). The YKF was also grown in solid media. Using a 1 mL pipetted, the tip of the 1 mL tip was cut to extract the YKF puffball from the original culture to grow it in fresh YPD 1/10 media. The YKF organism in the media releases the antifungal YKF compound, as shown in Figure 8. YKF in the liquid was stored at room temperature.



**Fig. 8. The YKF supernatant.** The YKF organism inoculated in fresh YPD 1/10 liquid media. Once in the media starts to release the antifungal compound generating the YKF supernatant (s/n).

## 2. Media conditions

- i. **Normal YPD liquid media.** To make 200 mL YPD liquid media (also to be referred from this moment onwards as *normal YPD*) 2 mg yeast-extract, 4 mg bacto™ peptone, 4 mg dextrose (glucose) and 200 mL water was used. Various YPD media volumes were used such as 400 mL and 1 L (*Table 2*).
- ii. **YPD 1/10 liquid media.** YPD 1/10 has the same ingredients as normal YPD, however, tenth the amount. Therefore, for 200 mL was used 0.2 mg yeast-extract, 0.4 mg bacto™ peptone, 0.4 mg dextrose (glucose) and 200 mL water (same amount as normal YPD). The same was done for different liquid media volumes, *Table 2*.
- iii. **Solid media.** For plates (solid media), the same amount (mg) of agar was added for normal and tenth YPD. However, at various overall volumes different amount of agar was added as seen in *Table 2*. 20 mL of the agar media was pipetted in plates during the course of the research project.

All liquid and solid media were autoclaved before used in an experiment.

<b>Media</b>		<b>YPD</b>			<b>YPD 1/10</b>		
<i>Liquid</i>	Volume	200 mL	400 mL	1 L	200 mL	400 mL	1 L
	Yeast extract	2 mg	4 mg	10 mg	0.2 mg	0.4 mg	1 mg
	Bacto-peptone	4 mg	8 mg	20 mg	0.4 mg	0.8 mg	2 mg
	Dextrose	4 mg	8 mg	20 mg	0.4 mg	0.8 mg	2 mg
	Water	200 mL	400 mL	1 L	200 mL	400 mL	1 L
<i>Solid</i>	Agar (addition)	4 mg	8 mg	20 mg	4 mg	8 mg	20 mg

**Table 2. Concentrations for normal YPD and YPD1/10<sup>th</sup> in liquid or solid form.**

### 3. *Candida* strains and growth conditions

- i. ***Candida* inoculation.** The *Candida* strains were inoculated in 5 mL normal YPD media in glass tubes and were incubated at 30°C for 17-20 hours (referred on this thesis as *overnight growth*).
- a. Streaked-plate. From the overnight *Candida* inoculates 50 uL was diluted in 200 uL YPD 1/10 media. This 250 uL *Candida* culture was pipetted onto the plate, using a glass cell spreader the culture was spread all over the agar plate (*Petri dish*).
- b. Poured-plate. In a 50 mL falcon tube, overnight *Candida* inoculates 50 uL was diluted in 20 mL YPD 1/10 agar. The falcon was centrifuged for the *Candida* strain to mix well with the agar media and then was poured into a plastic plate.

ii. ***C. albicans* and NCA isolates.**

The <i>Candida</i> strains		Buscaino Lab code	Obtained	
<i>Candida albicans</i>	<i>C. albicans</i>	(AB 55)	Berman's lab	
<i>Non-Candida</i>	<i>C. guilliermondii</i> B-3163	(AB 121)		
<i>Albicans (NCA)</i>	<i>C. tropicalis</i> 660	(AB122)	Myers lab	
	<i>C. krusei</i> 653	(AB 123)		
	<i>C. glabrata</i>	BG2		(AB 124)
		L5		(AB120)
	<i>C. parapsilosis</i>	(AB126)		
	<i>C. dubliniensis</i> *	(AB 262)		
<i>C. auris</i>	DMC 21092	AB 759	Hall Lab  (clinical isolates)	
	JCM 15448	AB 762		
	KSCK 17809	AB 761		
	KCKC 17810	AB 760		

**Table 3. *Candida* stains used and the lab that were obtained from.**

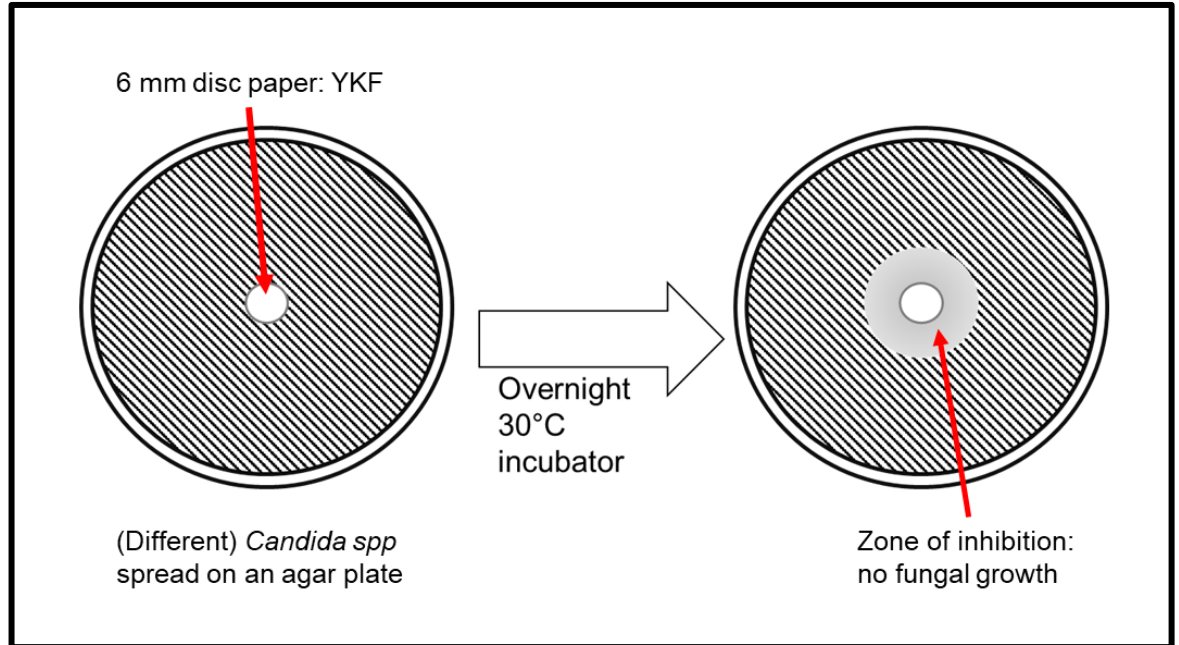
\* *C. dubliniensis* (AB262), strain name CdTLO1-His-FLAG, was also used. This strain containing a Wu284 tagged with a tandem affinity 6x His / 3x FLAG tag. An M2 antibody is used for FLAG.

**4. YKF s/n activity and testing**

- i. **Disc-diffusion assay.** The activity of the YKF was determined using the disc-diffusion assay. In a *Candida* culture streaked-plate, a 6 mm paper disc was placed in the centre. A volume of 40 µL of YKF s/n was pipetted in the disc paper. The plates were incubated overnight at 30° overnight.

The following day, as seen in *Figure 9*, a zone of inhibition (Zoi) is shown.

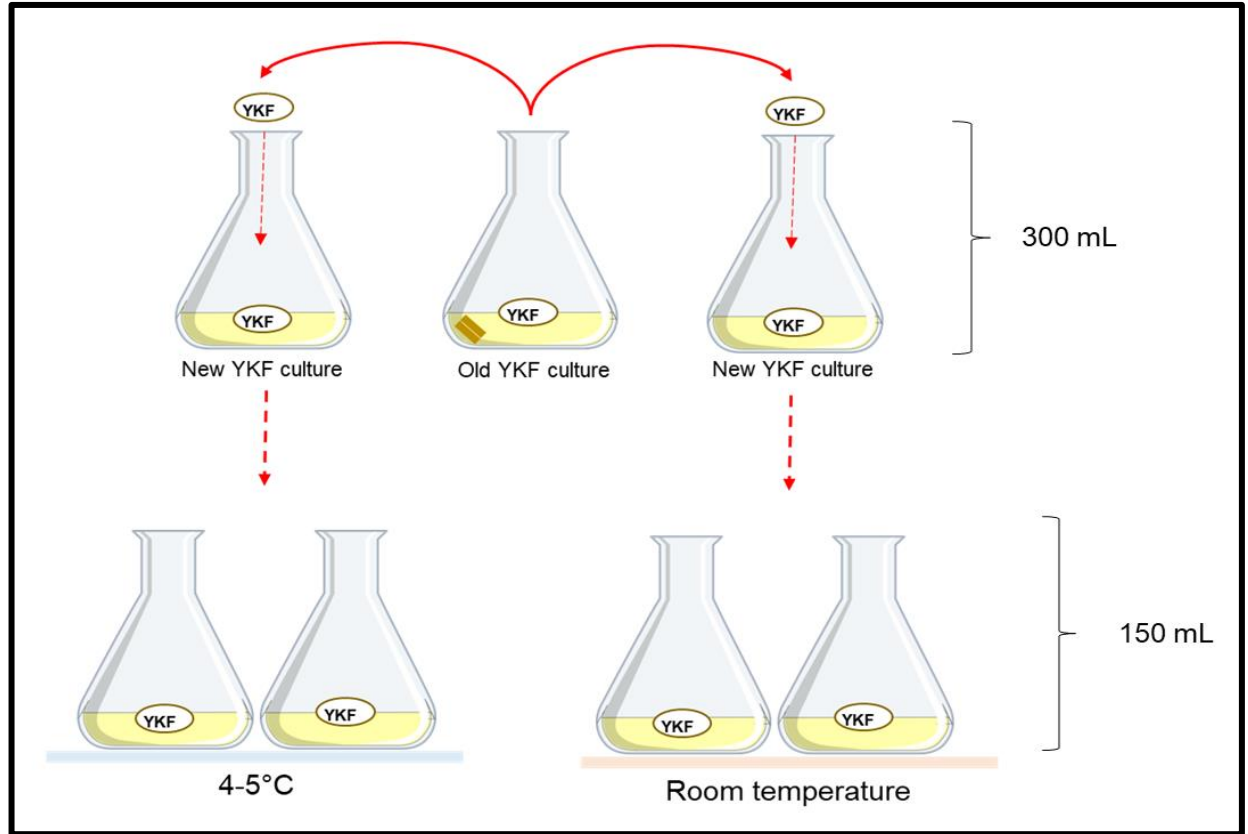
This assay was the used throughout the project to test the activity.



**Fig. 9, Disc-diffusion assay procedure.** YKF s/n added onto the 6 mm disc paper placed on the centre of a streaked-plate. After an overnight inoculation, a halo-like are is shown around the disc paper. This are is referred as the zone of inhibition.

- ii. **Evolutionary experiment.** Two YKF puffballs-like fungi were placed in two 300 mL YPD 1/10 (500 mL) flasks (total volume of 600 mL). The flasks were named 'YKF (R)' and 'YKF (C)'. The YKF supernatant from these cultures showed no Zoi for period of 9 day. Therefore, each flask was separated in two 150 mL volume YKF s/n into smaller flasks, as shown in *Figure 10*. From culture YKF (R) and YKF (C) two subcultures for each were created 'YKF (R. 1)': 'YKF (R. 2)' and 'YKF (C. 1)': 'YKF (C. 2)' respectively. The flasks 'YKF (C. 1)' and 'YKF (C. 2)' were stored in cold temperatures at 4-5° (*Figure 10*). Whereas the flasks 'YKF2 (R. 1)' and 'YKF2 (R. 2)' were kept at 20-22°C, room temperature levels (*Figure 10*).

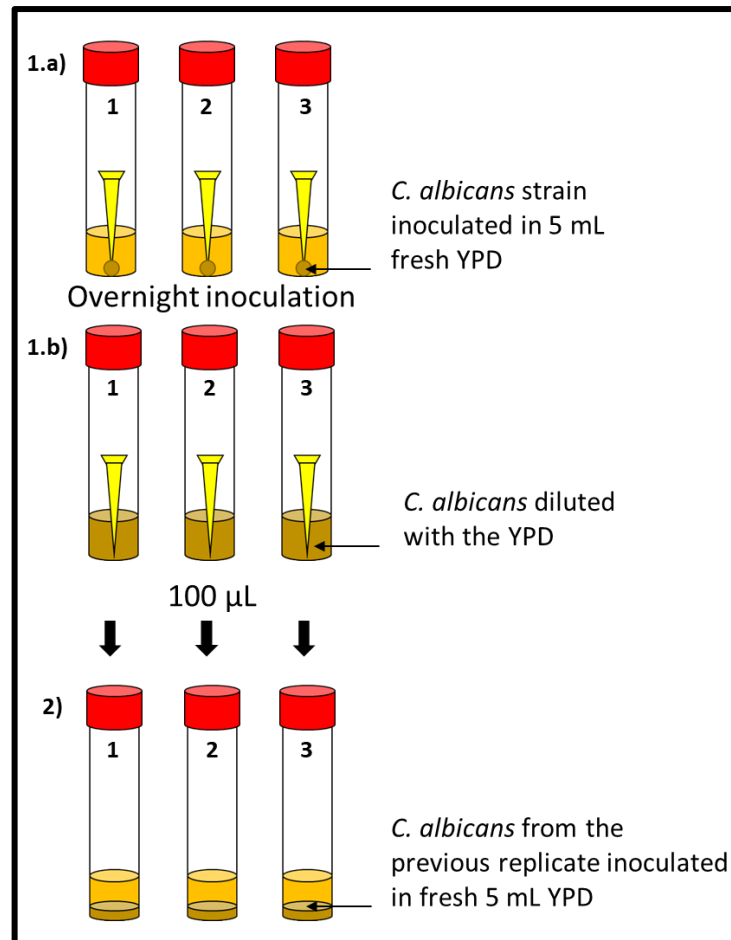
The activity and diameter of the ZOI for all four cultures was measured every two days.



**Fig. 10, The evolutionary experiment procedure.** YKF culture was prepared, two flasks in 150 mL YKF s/n were stored at 4-5° (cold room temperature) and two 150 mL flasks stored at 20-22°C temperature to observe the maximum activity against *C. albicans* for each culture.

## 5. Growth assay to determine YKF s/n activity against *C. albicans*

- i. ***C. albicans* biological replicates.** Three tubes containing *C. albicans* strains were inoculated (section 3.i) for overnight growth in incubator at 30°C. The following day 100 µL of the overnight inoculates was pipetted into 5 mL of normal YPD and placed in the incubator at 30°C to grow overnight. The technical procedure is illustrated below in *Figure 11*.



**Fig. 11, Biological replicates of *C. albicans* for the growth assay.**

1.a) *C. albicans* strains was inoculated in 5 mL of YPD liquid media.

1.b) *C. albicans* grow (over a period of 18-24 hours) in the media reaching stationary phase.

2) 100 µL from each tube was taken and inoculated in fresh YPD liquid media to grow overnight.

- ii. **Growth assay: 96 well plate.** To determine the YKF viability 96 well plate was used. In each well, 100 µL of YKF s/n and *C. albicans* was added. The absorbance ( $OD^{600}$ ) of *C. albicans* culture inoculated the previous day, was measured. For each volume going in the well 7 eppendorf tubes containing YKF s/n and in another one with no YKF s/n just 100 µL of *C. albicans* as a control were prepared. A YKF s/n volume target was set: 0.1 µL, 0.2 µL, 0.5 µL, 1 µL, 5 µL, 10 µL and 20 µL.



- a. **96 well plate preparations.** In each well 90  $\mu\text{L}$  was targeted for YKF s/n and YPD media (YKF: YPD) whereas 10  $\mu\text{L}$  for *C. albicans* overnight culture and YPD media (55: YPD).
- b. **YKF s/n calculations.** In each eppendorf tube, was calculated for a volume of 11 wells and not 9 wells per 8 rows as were needed. This was done in case there was spillage or laboratory error occurred. For example, for 1  $\mu\text{L}$  YKF s/n in an Eppendorf tube 11  $\mu\text{L}$  was mixed with 979  $\mu\text{L}$  YPD media. Meaning, 1  $\mu\text{L}$  YKF s/n with 89  $\mu\text{L}$  YPD media, a total of 90  $\mu\text{L}$  YKF: YPD, illustrated in *Figure 12*. For each YKF s/n and media concentrations the calculations were conducted as demonstrated in *Table 4*. For 0.1  $\mu\text{L}$ , 0.2  $\mu\text{L}$  and 0.5  $\mu\text{L}$  final amount, 1  $\mu\text{L}$ , 2  $\mu\text{L}$  and 5  $\mu\text{L}$  were diluted into 10  $\mu\text{L}$  YPD 1/10 liquid media. Multiplied by 11x, hence: 11  $\mu\text{L}$  YKF s/n  $\rightarrow$  110  $\mu\text{L}$  YPD 1/10 media; 22  $\mu\text{L}$  YKF s/n  $\rightarrow$  110  $\mu\text{L}$  YPD 1/10 media and 55  $\mu\text{L}$  YKF s/n  $\rightarrow$  110  $\mu\text{L}$  YPD 1/10 media.

YKF s/n concentration per well	YKF s/n in eppendorf tube	YPD media in eppendorf tube
0.1 µL	11 µL	979 µL
0.2 µL	22 µL	968 µL
0.5 µL	55 µL	935 µL
1 µL	11 µL	979 µL
5 µL	55 µL	935 µL
10 µL	110 µL	880 µL
20 µL	220 µL	770 µL

**Table 4. The concentration of YKF s/n and YPD media in an eppendorf tube prior to being pipetted onto the 96 well plate.**

c. ***C. albicans* culture calculations.** A series of equations were conducted to determine the amount of *C. albicans* and normal YPD media needed. The absorbance of the *C. albicans* overnight inoculation was measured. This was required for a series of calculations shown below. Then, 50 µL of *C. albicans* culture was diluted in 450 µL of normal YPD as seen in *Figure 12*. This was done for all three technical replicates and was pipetted in the appropriate wells of the 96 well plate as shown in *Figure 13*.

Equations to determine *C. albicans* final concentration per technical:

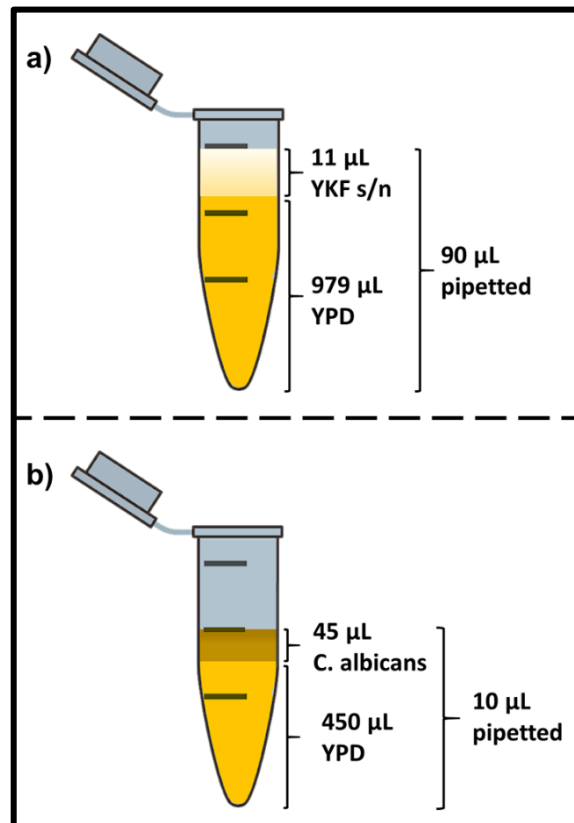
*Concentration in tube* = *OD/dilution*

*Cells/µL tube* = (*concentration in tube* X *cells/µL (30,000)*)/1

*Cells/µL in Eppendorf* = (*cells/µL plate*)/ 0.1 = 600

$Vol. \text{ tube to Eppendorf} = (600 \times 1000) / \text{cells}/\mu\text{L tube}$

$Vol. \text{ media } (\mu\text{L}) \text{ to Eppendorf } 1 = 1000 - Vol. \text{ tube to Eppendorf}$



**Fig. 12, The volume of YKF s/n and *C. albicans* culture diluted into normal YPD before pipetted on the 96 well plate.**

a) 11 µL of YKF s/n diluted in 979 µL YPD. This is an example for 1 µL YKF s/n target, for various concentrations different YK s/n and YPD was used ( *Table 4*). Final volume of tube 1 mL and 90 µL pipetted in the respective well.

b) 45 µL *C. albicans* diluted in 450 µL normal YPD. Reaching final volume 500 µL and 10 µL of this mixture was pipetted onto the corresponding well of each technical triple replicate.

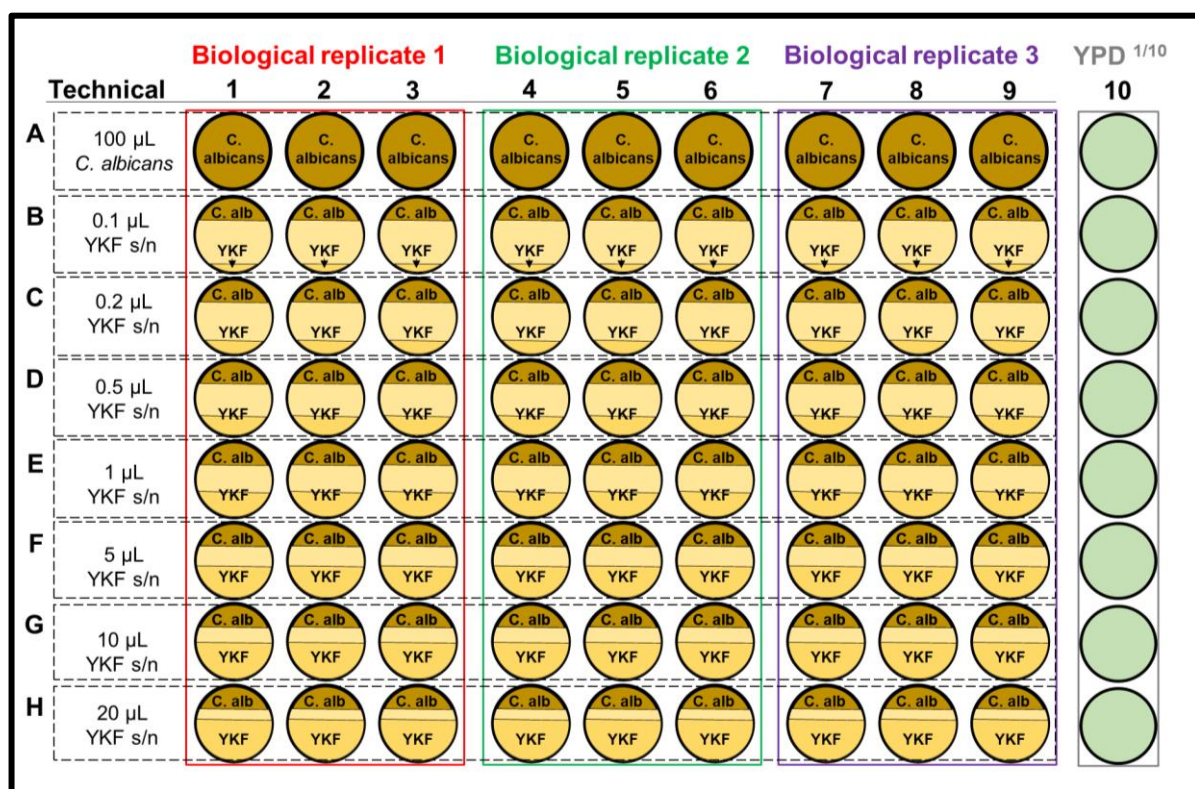
**d. The volumes per well.** For each of the *C. albicans* technical, three replicates were conducted.

**Column:** As demonstrated in *Figure 13*, in a group of three columns *C. albicans* diluted in normal YPD media

from that technical was pipetted. Thus, columns 1-3 technical 1, columns 4-6 technical 2 and columns 7-9 technical 3. Additionally, in column 10, all rows, only 100  $\mu$ L of YPD 1/10 was pipetted as a media control.

**Row:** In the first row (A), columns 1-9, only 100  $\mu$ L of *C. albicans* was pipetted as a control. Additionally, for the rows 2-8 the volume of YKF s/n increased as is shown in

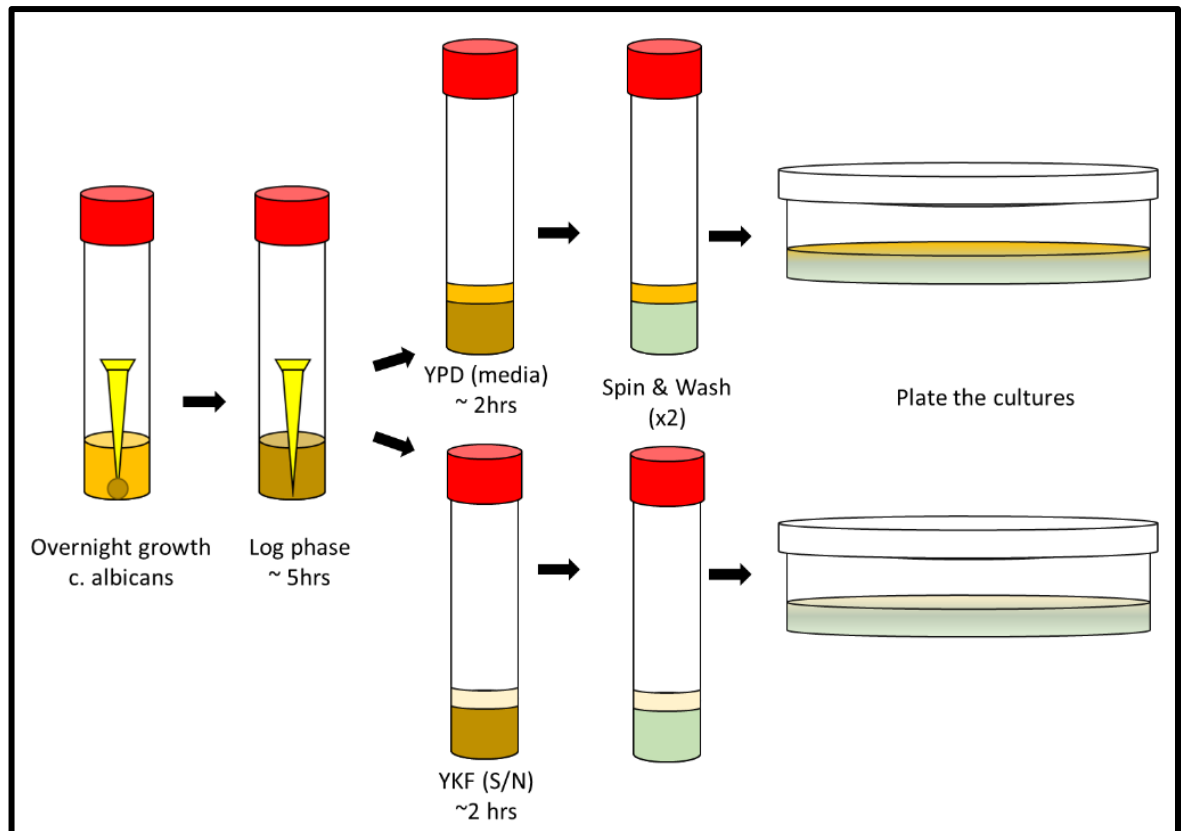
Figure 13.



**Fig. 13, Illustration of a 96 well plate - the volume of YKF s/n and *C. albicans* (*C. alb*) in each well for three biological replicates.** Three technical replicates were conducted. On the first row was pipetted 100  $\mu$ L of *C. albicans* inoculated in normal YPD liquid media. In the rest of the of the wells various amounts of YKF s/n: 0.1  $\mu$ L, 0.2  $\mu$ L, 0.5  $\mu$ L, 1  $\mu$ L, 5  $\mu$ L, 10  $\mu$ L, 20  $\mu$ L, and *C. albicans* (55) were pipetted in each well. The last column was pipetted 100  $\mu$ L of YPD 1/10 liquid media.

## 6. Determining YKF compound antifungal property

- i. **The *C. albicans* inoculations in normal YPD media.** An experiment was established to determine the YKF antifungal trait, as seen below in *Figure 14*. *C. albicans* strain was inoculated overnight in normal YPD at 30°C incubator. The following day the absorbance of the *C. albicans* was measured. To reach an absorbance of approximately OD= 0.3, 150 µL *C. albicans* was inoculated into 5 mL fresh normal YPD. The OD measured was OD= 0.266. The new tube was placed in the incubator at 30°C for about 5 hour 30 minutes to reach lag phase and an OD= 0.6 (approximately).
- ii. **YKF s/n and YPD 1/10 testing.** Afterwards, the OD was measured OD= 0.676. The new *C. albicans* inoculate was separated in two 15 mL falcons, in one falcon 1 µL of YPD 1/10 liquid was added whereas in the other falcon 1 µL of YKF s/n was added. The falcon tubes were incubated at 30°C incubator for 1 hour 30 minutes. The absorbance of each tube was measured,  $OD^{YKF} = 0.412$  and  $OD^{YPD} = 0.508$ .
- iii. **Spin and Wash procedure.** Next, the falcon tubes were placed in the centrifuge at 20°C, 4000 rpm for 5 minutes. The pellet for each tube (YKF and YPD 1/10 tube) was collected and separated from the supernatant. The pellet was washed with fresh YPD1/10<sup>th</sup> liquid media and placed in the centrifuge. This step was repeated another time to wash the pellet from any waste and remove the antifungal YKF s/n.



**Fig. 14, Determining YKF fungistatic and fungicidal characteristics.** *C. albicans* was inoculated overnight. 150  $\mu\text{L}$  was added onto 5 mL YPD liquid media, an  $\text{OD} = 0.3$  (approximately) was measured. After, approximately 5 hours the lag phase *C. albicans* were separated into two falcon tubes. In one tube 1  $\mu\text{L}$  of YPD 1/10 was added whereas in the other tube 1  $\mu\text{L}$  of YKF s/n was added. The tubes were incubated at  $30^\circ\text{C}$  for about 2 hours. Afterwards, the falcon tubes were placed in the centrifuge, spun at 4000 rpm at  $20^\circ\text{C}$  for 5 minutes. Then the pellet was separated from the s/n and washed with fresh YPD 1/10 reaching a total volume of 4 mL. This step was conducted again. Then, at various concentrations (300, 500, and 700 cells per plate) were spread onto the YPD 1/10 agar plates.

iv. **Calculation for *C. albicans* cells per plate.** After, wash and spin step the absorbance was measured again,  $\text{OD}^{\text{YKF}} = 0.394$  and  $\text{OD}^{\text{YPD}} = 0.508$ .

There are  $3 \times 10^7$  cells/mL as a result:

$$\text{OD}^{\text{YKF}} = 0.394 \times (3 \times 10^7 \text{ cells/mL}) = 11'820'000 \text{ cells OR } 1.182 \times 10^7 \text{ cells}$$

$$\text{OD}^{\text{YPD}} = 0.508 \times (3 \times 10^7 \text{ cells/mL}) = 42'600'000 \text{ cells OR } 4.26 \times 10^7 \text{ cells}$$

An end result of 300, 500 and 700 cells per plate was needed (performed five times per each target-cells for the YPD control and YKF s/n culture). Subsequently, an equation was set.

**YKF s/n calculation:**

0)  $300 \text{ cells} \rightarrow 300\text{cells}/200\mu\text{L}$  (amount pipetted) = 1.5 cells/ $\mu\text{L}$

200  $\mu\text{L}$  is the volume that has been used throughout the project to pipette into an agar plate

$1.5 \text{ cells}/\mu\text{L} \times 1000\mu\text{L}/1\text{mL} = \underline{1'500 \text{ cells/mL}}$

1)  $1.182 \times 10^7 \rightarrow 1/100 \text{ dilution} \rightarrow 1.182 \times 10^5$

2)  $C1V1=C2V2$

$1.182 \times 10^5 \times X = 1'500 \text{ cells/mL} \times 1 \text{ mL}$

$X \approx 0.0127 \text{ mL}$  OR 12.7  $\mu\text{L}$   $\rightarrow C. albicans$  culture needed

Therefore, 1 mL – 12.7  $\mu\text{L}$  = 987.3  $\mu\text{L}$   $\rightarrow$  YPD 1/10 liquid media

1 mL is the capacity of an eppendorf tube used for this experiment.

3) 200  $\mu\text{L}$  *C. albicans*: media pipetted onto the YPD 1/10 agar plate

**YPD media calculation:**

0)  $300 \text{ cells} \rightarrow 300\text{cells}/200\mu\text{L}$  (amount pipetted) = 1.5 cells/ $\mu\text{L}$

$1.5 \text{ cells}/\mu\text{L} \times 1000\mu\text{L}/1\text{mL} = \underline{1'500 \text{ cells/mL}}$

1)  $4.26 \times 10^7 \rightarrow 1/100 \text{ dilution} \rightarrow 4.26 \times 10^5$

2)  $C1V1=C2V2$

$4.26 \times 10^5 \times X = 1'500 \text{ cells/mL} \times 1 \text{ mL}$

$X \approx 0.0035 \text{ mL}$  OR 3.5  $\mu\text{L}$   $\rightarrow C. albicans$  culture needed

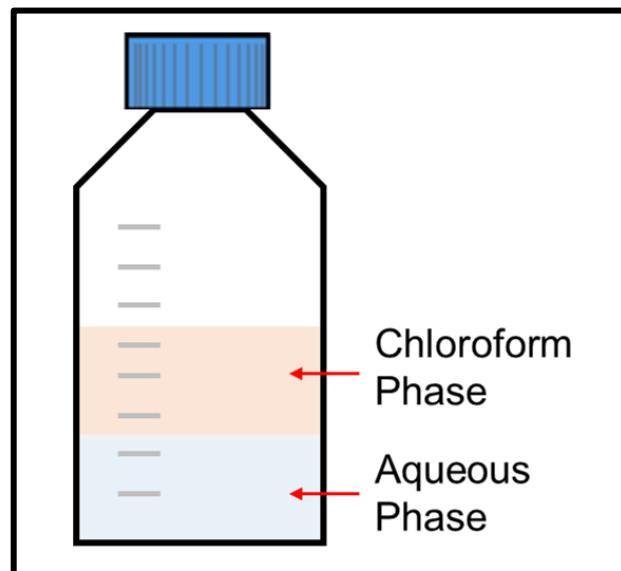
Therefore, 1 mL – 3.5  $\mu\text{L}$  = 996.5  $\mu\text{L}$   $\rightarrow$  YPD 1/10 liquid media

3) 200  $\mu$ L *C. albicans*: media pipetted onto the YPD 1/10 agar plate

The same equation was used for 500 and 700 cells per plate for YKF and YPD 1/10 cultures.

## 7. Chloroform extraction

- i. **Chloroform solvent addition.** 50 mL of YKF s/n was added into a Duran bottle. In the same Duran bottle 50 mL of Chloroform (purchased from fisher scientific) was added. The same procedure was conducted using 50 mL normal YPD and adding 50 mL of chloroform in another separate Duran bottle. The YPD: chloroform was used as a control. The Duran bottles were placed in the roller mixer for an hour.



**Fig. 15, The separation of phases on chloroform extraction.** The YKF supernatant, or YKD 1/10<sup>th</sup> media, are mixed with chloroform a purer sample. Two phases are created, one the aqueous phase with the unnecessary media component and the organic, or chloroform phase with the purified compound.



- ii. **Phase separation and resuspension.** Onto a glass funnel the mixtures were added to collect the chloroform (organic) phase and the supernatant (aqueous phase), as demonstrated in *Figure 15*. Using the disc-diffusion assay the aqueous phase activity was tested whereas the chloroform phase collected were left in the fume cupboard (closed window) to dry completely for 1-2 days. After the drying out, 2 mL of chloroform was added in both YKF and YPD chloroform phases to resuspend.

## 8. Thin-layer chromatography (TLC) procedures

- i. **Silica paper controls.**
  - a. **Silica paper preparations.** In the silica paper 40  $\mu$ L of YKF s/n and YPD 1/10 and the extracted YKF s/n and YPD 1/10, liquid media was pipetted. The silica paper was let to be dry for approximately 5 and 15 minutes for non-extractions and extractions, respectively.
  - b. **Testing silica paper in poured-plate medium.** After a solvent system was chosen (8.iii), the silica paper (for each case) was placed on the bottom of the plate. 10 mL of agar media was mixed with 500  $\mu$ L of *C. albicans* overnight inoculate and placed in a water bath at 50°C whereas, in the meantime, 10 mL of YPD 1/10 agar media was added. The media from the water bath was added onto the rest of YPD agar on the plate. The plates were let placed in the incubator at 30°C. After overnight growth in the incubator the zone of inhibition diameter was measured and analysed.

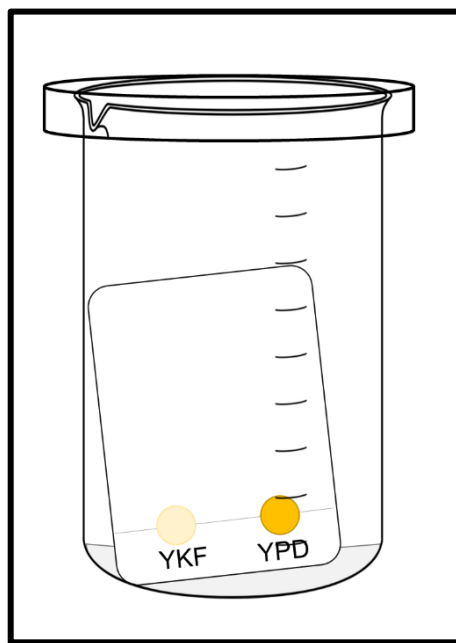
**ii. TLC technique.**

**a. Pre-TLC chamber preparation.** In a silica paper a line was drawn to mark the baseline (point of origin), two dots were also drawn along the line one for the YKF and one for media extractions. 40  $\mu$ L of each extraction was pipetted in the silica paper. The paper was let in the fume cupboard to dry for about 15 minutes. A glass 50 mL beaker was used as a TLC chamber.

**b. TLC chamber and assessment.** The silica paper treated with a solvent system (**8.iii**) was placed into the chamber, as shown in *Figure 16*, and run for approximately 10-20 minutes. Afterwards, the silica paper was placed on the fume cupboard to dry for about 5 minutes and was observed under UV light. Next, potassium permanganate ( $\text{KMnO}_4$ ) was selected as the stain of choice. Potassium permanganate is a general stain that enables to detect functional groups that are sensitive to oxidation like, alkenes and alkynes. The TLC paper was dipped in potassium permanganate ( $\text{KMnO}_4$ ) and dried with a heat gun to observe the separation spots. The area where the spot migrated appeared in a bright yellow on a purple background.

**iii. The solvents systems used.** For treatment of the silica paper to test the activity in a poured-plate and used as a solvent system for the TLC chamber various different solvents were used: DCM: hexane (2.5 mL: 2.5 mL), DCM: 5 % methanol (4.75 mL DCM: 0.25 mL methanol), DCM: 10 % methanol (4.5 mL DCM: 0.5 mL methanol), DCM: 15 % methanol (4.25 mL DCM: 0.45 mL methanol), DCM: 20% methanol (4 mL DCM: 1 mL methanol), DCM: 25 % methanol (3.75 mL DCM: 1.25 mL methanol), ethyl

acetate (2 mL). For testing the bioactivity towards *C. albicans* in a poured-plate the silica papers treated with solvents were placed in the fume cupboard to dry for approximately 15 minutes.



**Fig. 16, Thin-layer chromatography procedure.** The TLC chamber using a beaker. The silica paper, containing the chloroform extraction YKF s/n and YPD 1/10 media placed in the baseline. The polar/nonpolar solvents at the bottom is absorbed by the silica paper to visualise spot separation.

## ■ Results

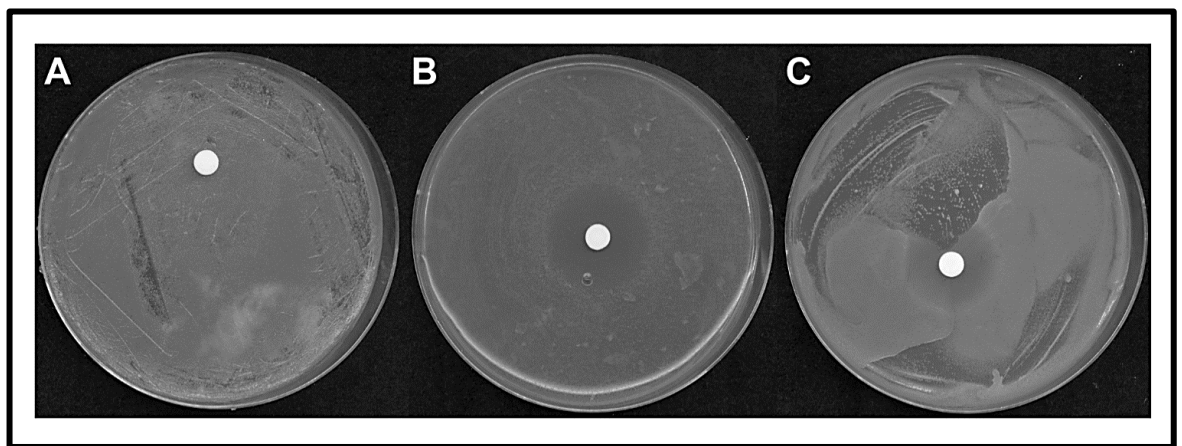
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### 1. YKF antifungal components inhibit *C. albicans*

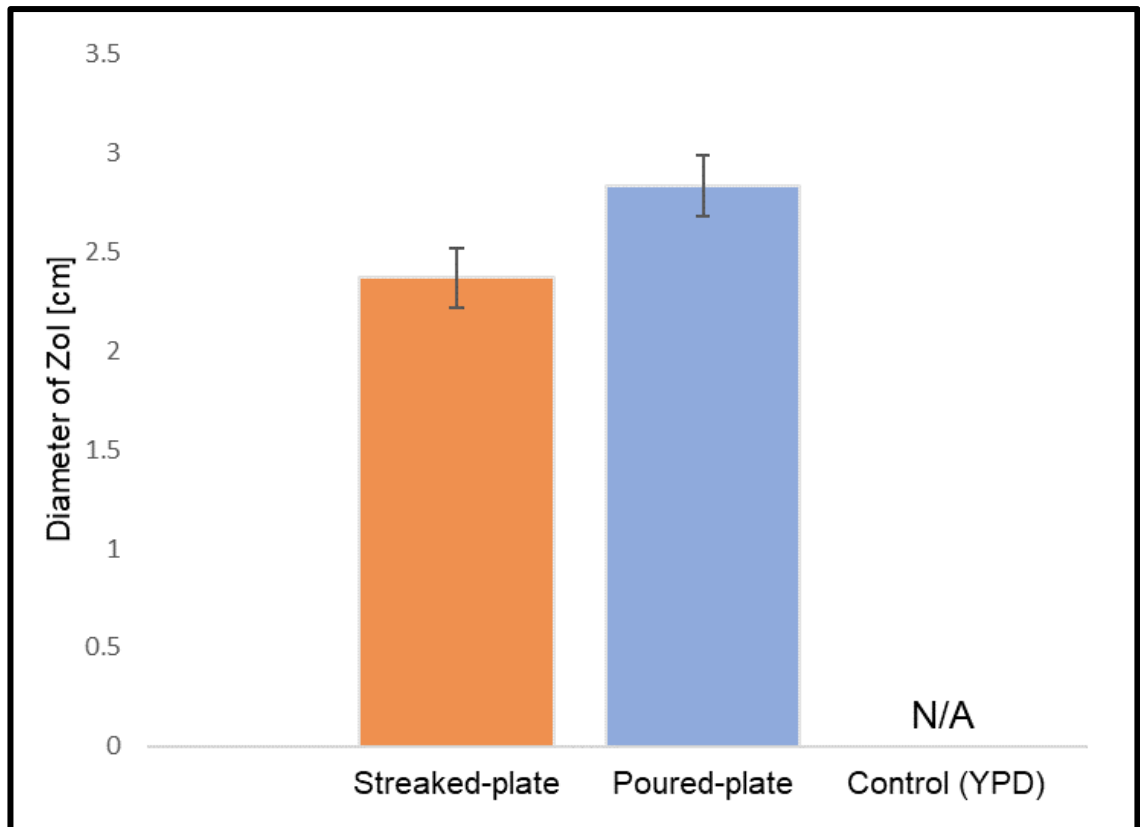
Previous results from the Buscaino lab demonstrated that the *Yeast Killer Fungus* (YKF) produces an unknown compound that displays antifungal qualities. To determine the bioactivity of YKF supernatant towards *C. albicans*, a disc-diffusion assay was performed. Accordingly, fresh YPD 1/10 liquid media was inoculated with a two-month old YKF culture. The flask was stored at room temperature, for a period of two weeks that the YKF to release its antifungal particles into the liquid media. The YKF fungal organisms floating in the YPD 1/10 liquid media start to release the antifungal component in the media generated the YKF supernatant (s/n). The YKF s/n was spotted on an autoclaved disc paper and placed on agar plates seeded with a loan of *C. albicans* cells. As a negative control a disc paper containing only YPD 1/10 media was used.

As shown in *Figure 17*, the YKF antifungal compound, evidently inhibits *C. albicans* growth as a halo-like region, clear of *C. albicans* cells, was created around the disc paper containing the YKF compound whereas for the control there no “halo” was visible. This halo-like area is the known as the *zone of inhibition* (Zoi) as it suppresses the growth activity of microbial species i.e., *C. albicans*. The YKF zone of inhibition for *C. albicans* streaked-plate and poured-plate for each triplicate were on average, approximately 2.4 and 2.7 cm, respectively. *Figure 18* demonstrates the measured diameter of the zones of inhibition for each triplicate. The streaked-plate provides a YKF activity concentrated on the surface of the plate where the fungal colonies are present. The poured-plate showed, that the YKF compound not only halted *C.*

*albicans* for cells grown on the surface, but also, its bioactivity permeates through the solid media inhibiting cells below surface. For either growth media type used the YKF compound antifungal agents inhibit *C. albicans* growth at an approximate similar level. Thereafter, the streaked-plate was used as the growth media method to proceed in additional experiments as an efficient laboratory procedure.



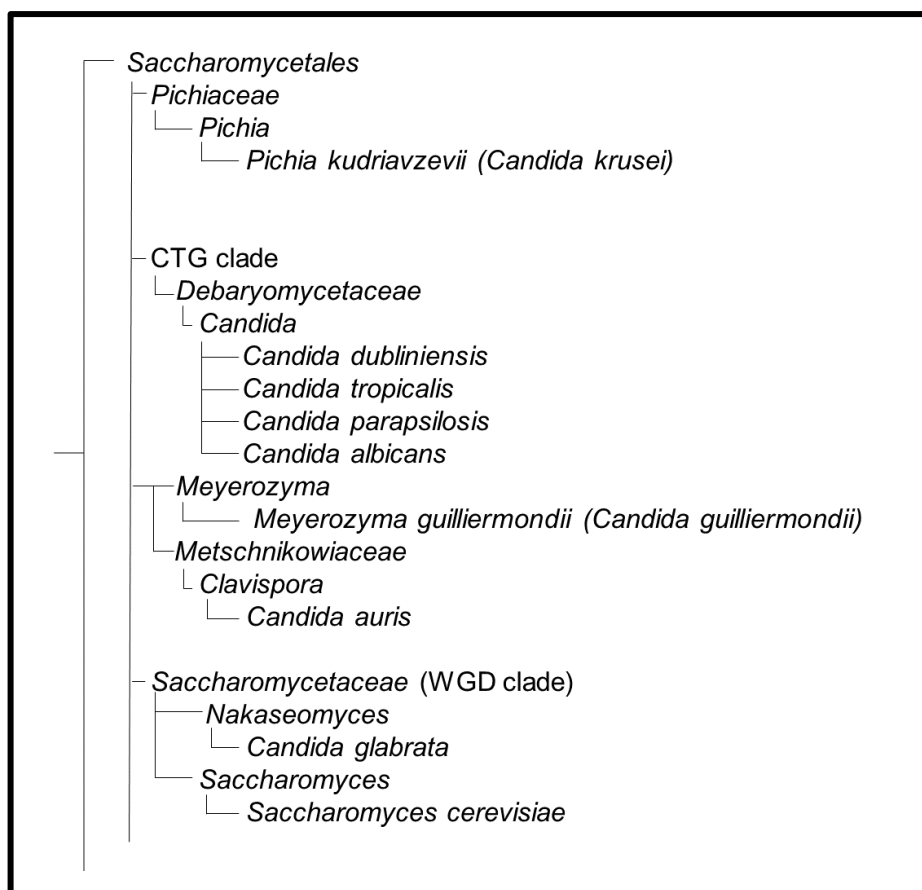
**Fig. 17, Control and YKF activity in *C. albicans* streaked-plate and poured-plates.** (A) normal YPD liquid media pipetted used as a control. (B) YKF Zol in *C. albicans* mixed with agar, poured-plate growth medium. (C) YKF activity in *C. albicans* streaked over an YPD 1/10 agar plate.



**Fig. 18, The diameter of the YKF s/n Zol on *C. albicans* for different agar growth media.** The *C. albicans* streaked onto the 1/10<sup>th</sup> YPD agar plate showed a slight lower Zol. In comparison, the *C. albicans*: 1/10<sup>th</sup> YPD poured-plate media showed a larger “halo” in diameter. The halo-like area around the disc containing YKF, zone of inhibition, was measured for each triplicate. The error bar represents the average standard deviation (SD) of three biological replicates.

## **2. YKF compound inhibits growth of *Non-Candida Albicans***

To assess whether the YKF is a broad spectrum compound its activity was tested towards additional *Candida* species commonly referred as *Non-C. albicans* (NCA). The NCA used for this experiment were the *Candida* species that are largely associated with candidemia and phylogenetically closely related to *C. albicans* such as, *C. guilliermondii*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei*, and four *C. auris* (Figure 19). Additionally, the non CTG clade fungal pathogen, *C. glabrata* was used although phylogenetically closer to *S. cerevisiae*.



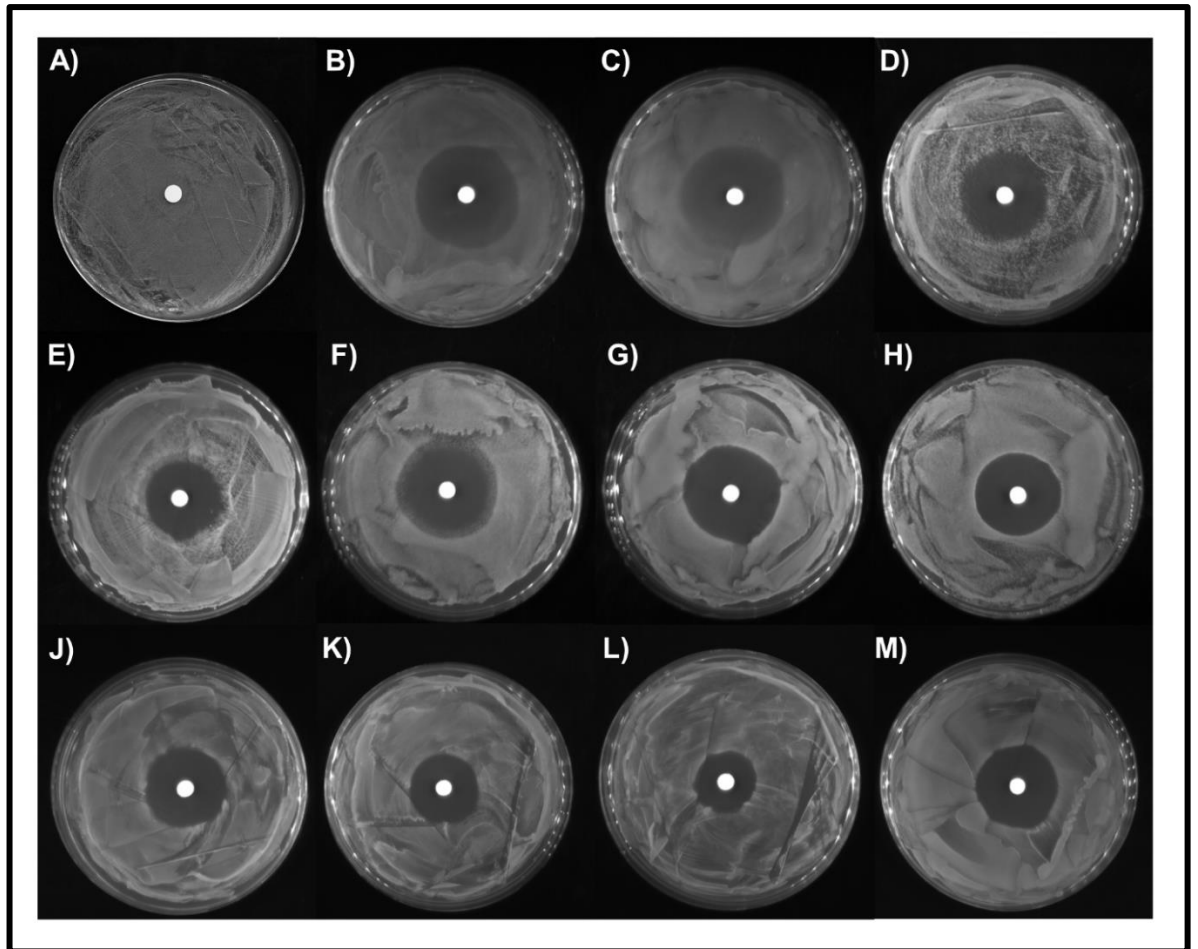
**Fig. 19, Phylogeny of the *C. albicans* and the Non-*C. albicans* strains.** *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis* are members of the *Candida* genus, and share a close ancestry with *C. guilliermondii*. *C. auris*, and *C. krusei* belong to more distant phylum sharing a much further distant ancestor. All belonging to the CTG clade. *C. glabrata*, however, is closer associated with *S. cerevisiae*, belonging to WGD node. This figure represents only the *Candida* species used on this project in comparison to the previous image (Figure 1). *S. cerevisiae* is presented in this image as a comparative yeast between *C. glabrata* and the other *Candida*.

The YKF compound activity was analysed using a disc-diffusion assay. Onto the micro-filter paper YKF s/n was added on an agar streaked-plate with NCA species. YKF compounds inhibit growth of the NCA used as a halo-like area is created around the YKF-injected filter disc in comparison to the YPD negative control that showed no such area (Figure 20/A). It was expected that *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, as these fungi share taxonomic family and genes

with *C. albicans* to display similar reaction to the YKF antifungal components. The diameter of YKF inhibitory activity for both *C. glabrata* isolates was approximately 3.9 cm (*Figure 20/B and 20/C*). The members of the *Candida* genus showed an inhibitory diameter of 3.2 cm for *C. tropicalis* (*Figure 20/D*), 3.8 cm *C. parapsilosis* (*Figure 20/G*) and 3.1 cm for *C. dubliniensis* (*Figure 20/H*). *C. krusei* and *C. guilliermondii* an inhibitory area of approximately 2.7 cm and 3 cm respectively was shown (*Figure 20/E and F*).

Surprisingly the four *C. auris* strains used also demonstrated a reasonable halo-like region. These strains collected from clinical isolates from different countries. *C. auris* strains DMS 21092 and JCM 15448 isolates were collected from Japan, as shown in *Figure 20/J and K*, with a zone of inhibition diameter of approximately 2.6 cm and 2.0 cm respectively. The *C. auris* strains KCKC 17809 and KCKC 17810 collected from South Korean hospital isolates, had a diameter of about 1.7 cm and 2.5 cm, respectively (*Figure 20/L and M*). Although these *C. auris* strains were collected from different hospitals in different countries they presented a relatively good area of inhibition, hinting to the YKF compound antifungal strength towards a diverse family of pathogenic *Candida* species. It was observed similarly to *C. albicans*, YKF had an inhibitory activity towards these *NCA* strains hence creating a halo-like region.





**Fig. 20, YKF activity on *C. albicans* and NCA strains.**

First row: YPD control (**A**) and *Non-C. albicans* (NCA): *C. glabrata* L5 (**B**), *C. glabrata* BG2 (**C**), *C. tropicalis* (**D**)

Second row: *C. krusei* (**E**), *C. guilliermondii* (**F**), *C. parapsilosis* (**G**) and. *C. dubliniensis* (**H**)

Third row: *C. auris* strains, DMS 21092 (**J**), JCM 15448 (**K**), KCKC 17809 (**L**), KCKC 17810 (**M**).

This experiment was conducted in triplicate.

### **3. The evolutionary antifungal activity of the YKF s/n**

Identifying the time period, the YKF s/n reaches the maximum antifungal activity towards *C. albicans* was crucial. Knowing the exact point in time, the YKF s/n reaches full antifungal potential is important to establish a robust protocol that could

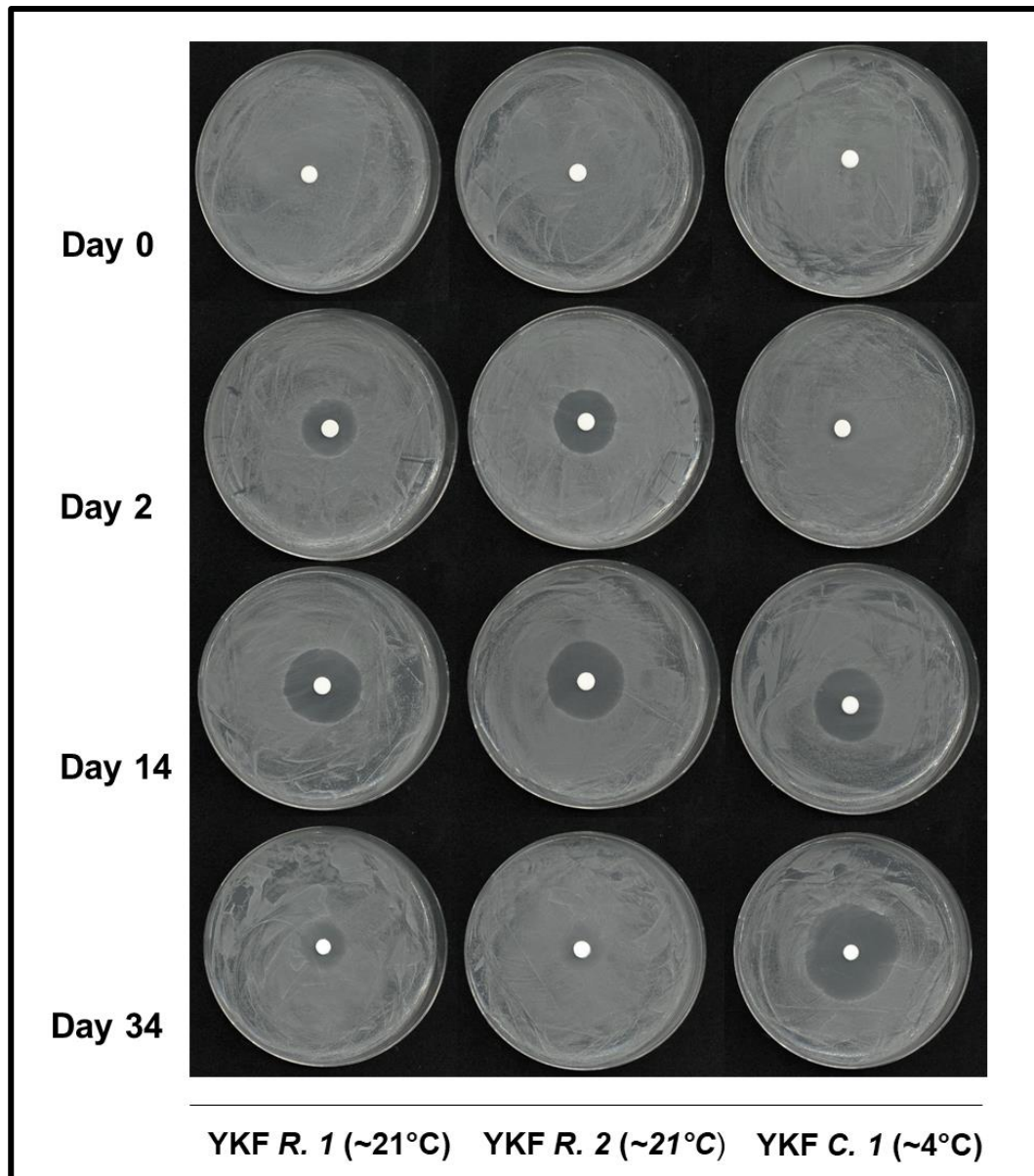
be used for all future experiments. In order to determine the strength of the YKF supernatant activity, an evolutionary experiment was performed. Therefore, two original YKF cultures were separated into four flasks containing YKF grown in YPD 1/10 media. These four cultures were cultured in parallel, two were placed at 4-5°C and two at room temperature. Every two days, for a period of 38 days, YKF samples from each culture was tested and the diameter of the Zol was measured using the disc-diffusion assay.

As expected, at time  $0 (t=0)$ , when YKF fungal organism was added onto YPD media no zone of inhibition was visible. On day 2 a halo-like region was observed for both YKF (*R. 1* and *R. 2*) culture incubated at 20-22°C (*Figure 21*). It was observed that YKF (*R. 1*) and YKF (*R. 2*) showed, every 2 days a relatively similar area of inhibition, both reached maximum strength on the same time and both started to deteriorate on a similar fashion. After two days both demonstrated a Zol of a diameter of roughly 2 cm. These cultures reached maximum strength on day 14. The Zol diameter of YKF (*R. 1*) and YKF (*R. 2*) were 2.5 and 2.6 cm, respectively. In room temperature the supernatant bioactivity starts to decline causing their antifungal strength to weaken.

In comparison, on day 2 the YKF cultures stored at 4°C expressed no zone of inhibition on the *C. albicans*. On day 4, one of the cultures incubated at 4°C started to show a zone of inhibition with a of less than 1 cm. The YKF *C. 1* was unfortunately contaminated. The YKF s/n stored at 4-5°C (YKF *C.2*) showed a slow increase in its antifungal activity strength. Nonetheless, on day 34 it reached its strongest antifungal activity, demonstrated in *Figure 21*, with a Zol diameter of 3.6 cm.

This experiment although conducted using in two parallel cultures was only performed once, with no additional replicates. This could have occurred because YKF cellular division in warmer and colder temperatures differs. As the YKF (C. 1) was contaminated, the data for YKF s/n incubated at 4-5°C should be interpreted with a degree of caution.

The curve and the maximum strength, illustrated in *Figure 22*, in terms of diameter, for the YKF antifungal potency. As seen in *Figure 22*, the antifungal activity strength for all YKF cultures started to decrease thus the experiment was stopped after 38 days. Therefore, the YKF s/n reaches its maximum activity for cold and room temperature, on day 14 and day 34, respectively. This was key to establish the highest antifungal strength at two different temperature settings. Subsequently, all other experimental aims were conducted using the room temperature YKF s/n at the optimal antifungal strength, 14 days after inoculation in fresh media.



**Fig. 21. The evolutionary experiment set to determine the YKF's antifungal strength.**

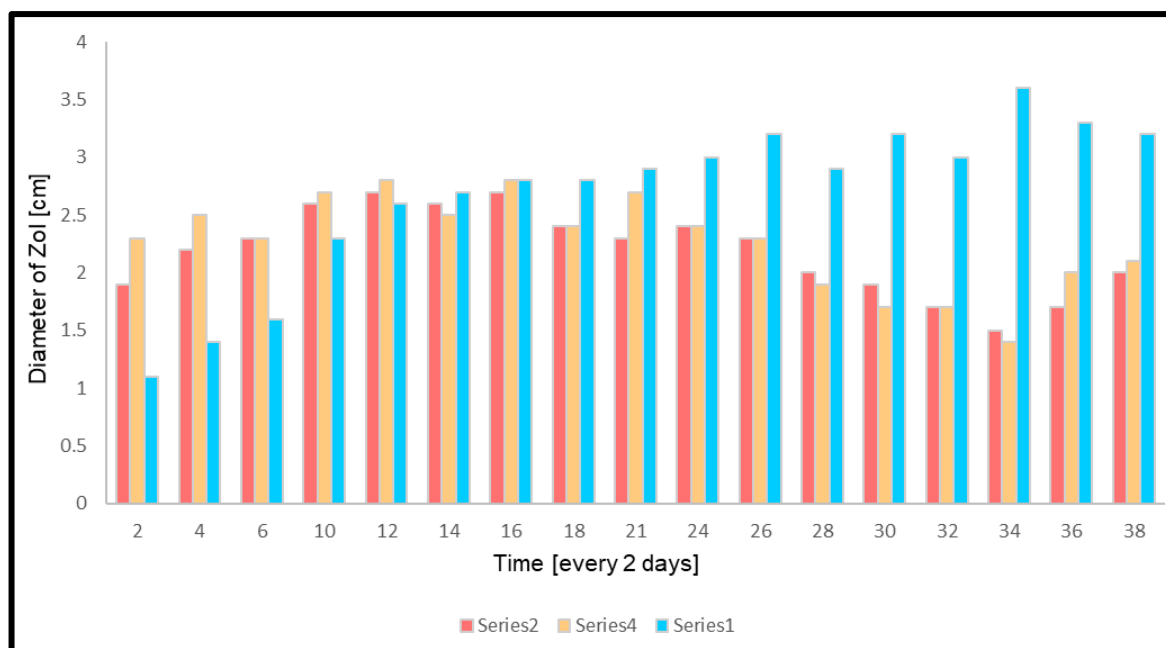
Day 0: the day YKF culture inoculated on the YPD 1/10 media showed no area of inhibition.

Day 2: room temperature YKF cultures (*R. 1* and *R. 2*) shows Zol.

Day 14: YKF *R. 1* and *R. 2* reaches peak antifungal activity. After day 14 both room temperature stored cultures start to degrade.

Day 34: YKF C. 2 (4-5°C) reaches maximum antifungal activity.

Day 38: The evolutionar experiment was concluded. YKF (C. 2) was contaminated hence no data was collected from this biological replicate.



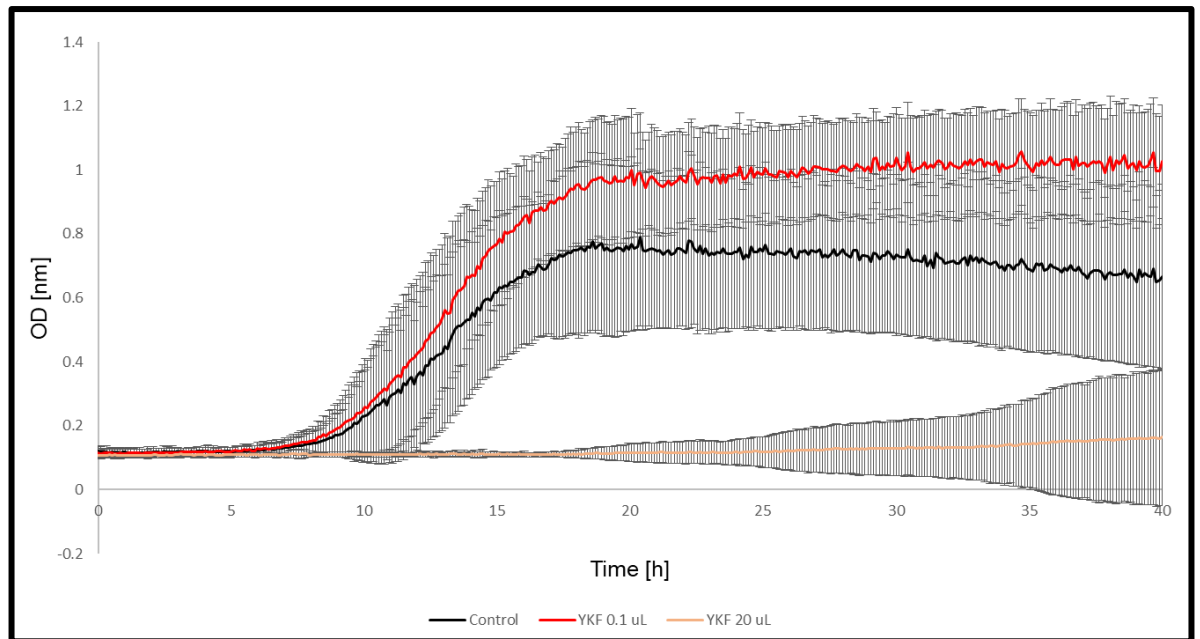
**Fig. 22. A graphical representation of the YKF s/n antifungal strength against *C. albicans*.** The (x) axis represents the time frame and the (y) axis the diameter of the antifungal Zol in centimetres. YKF (R. 1) and YKF (R. 2) reach maximum activity on day 14 whereas YKF (C. 2) reaches its highs antifungal activity on day 34.

#### 4. Increasing YKF s/n volume inhibits *C. albicans* growth

So far it was established that the YKF inhibits the growth of *C. albicans* in a streaked-plate medium. However, to have a better understanding of the YKF interaction with *C. albicans* a growth assay experiment was performed. A 96 well plate was used in order to determine the minimal volume of YKF s/n necessary to inhibit *C. albicans* growth. It is important to note that on these experiments are used different volumes of YKF s/n with an unknown concentration of YKF antifungal compound. Increasing the amount (from 0.1  $\mu$ L to 20  $\mu$ L) of YKF s/n taken from a two-week old culture, was incubated with *C. albicans* liquid culture in a 100  $\mu$ L final volume. A culture containing *C. albicans* with no YKF s/n was used as a control.

This experiment demonstrates that with increasing the YKF s/n volume the *C. albicans* cells do not grow (*Figure 23*). This suggests that the YKF at higher volume inhibits, the growth of *C. albicans*. As observed in *Figure 24/A & B*, the lower amounts used in this experiment, 0.1  $\mu\text{L}$  and 0.2  $\mu\text{L}$  of YKF s/n have no effect in the *C. albicans* growth. Their curve dispersion resembles the curve of the control (**black**). The log phase initiates at approximately 7 hours and reach stationary stage at about 15 hours, remaining stable for the rest of the plate reader measurement. YKF s/n at 0.2  $\mu\text{L}$ , unlike with the rest of the amounts, was run in duplicates and not in triplicate.

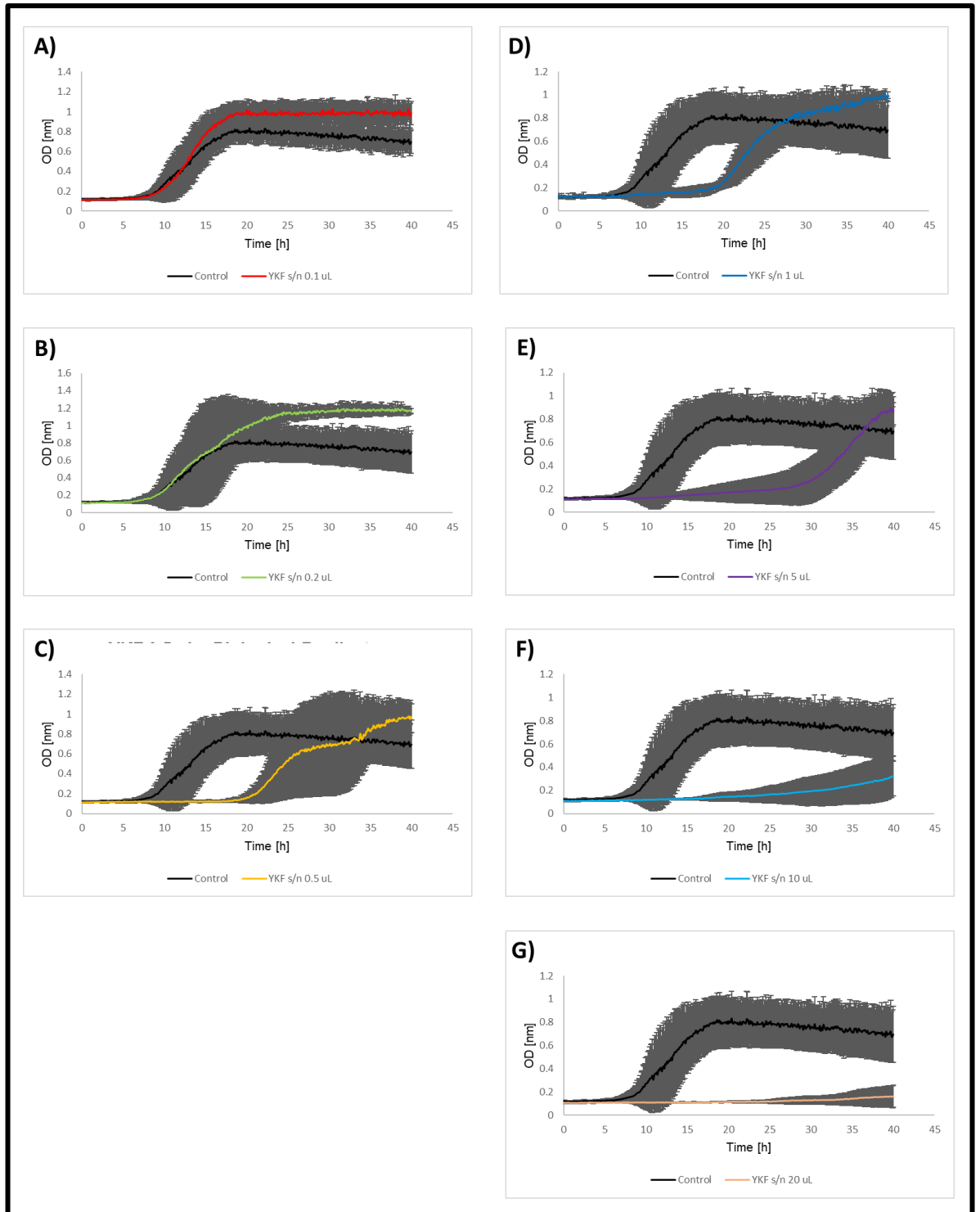
It is visible that at 0.5  $\mu\text{L}$  of YKF s/n, as seen in *Figure 24/C*, the growth of *C. albicans* is inhibited for approximately 20 hours and then steadily begins to increase. As the volume of YKF s/n increases it can be observed that *C. albicans* takes longer to grow. For YKF s/n 0.5  $\mu\text{L}$  and 1  $\mu\text{L}$  the log phase lasts for approximately 17 hours and afterwards begins to steadily increase (*Figure 24/E*). YKF s/n at 5  $\mu\text{L}$  stalls *C. albicans* growth for nearly 30 hours, reaching exponential phase after 30 hours. For YKF 10  $\mu\text{L}$  the log phase of *C. albicans* lasts for nearly 40 hours for as long as it was run in the plate reader. From the data represented in the graph at 40 hours there is a slight increase on the curve signalling *C. albicans* emerge growth (*Figure 24/F*). In comparison, at 20  $\mu\text{L}$  YKF the growth of *C. albicans* is inhibited and as seen in *Figure 24/G*, there is no significant increase on the curve (**light orange**). It appears that *C. albicans* remain in log phase for the entire period the plate reader was run indicating that YKF s/n increase in volume may not only has an inhibition ability but could have a fungicidal characteristic.



**Fig. 23, Graphical representation of the YKF s/n viability against *C. albicans*.** Various volumes of YKF s/n were used to determine its antifungal activity by inhibiting *C. albicans* growth. This graph presents the control run with no YKF (black curve), and 0.1 and 20  $\mu\text{L}$  of YKF s/n (the lowest and highest volumes used). The error bars show the average SD of the three biological replicates.

Figure 24, demonstrates the individual graphs for the control and the biological replicates for each YKF increased volume, illustrated with error bars. Error bars represent the variability and impreciseness of the data, using standard deviation equation in excel. Standard deviation expresses any error or differentiation in the data represented in the graph.

The error bars for the biological replicates of YKF 0.2, 0.5 and 10  $\mu\text{L}$  are higher as the log phase initiates (Figure 24). This could be as such phase, for various technical replicates could have initiated earlier or later in time hence the irregularities in the error bars. Nevertheless, it does not compromise the evidence that by increase the volume of YKF s/n its antifungal effect inhibits the growth of *C. albicans*.



**Fig. 24, Graphical illustration of all biological technicals for the control with all YKF volumes.** The biological replicates of volume increase of YKF interaction with *C. albicans* with the control (black). On the left is shown: 0.1  $\mu\text{L}$  (A), 0.2  $\mu\text{L}$  (B) and 0.5  $\mu\text{L}$  (C) YKF s/n. On the right is shown: 1  $\mu\text{L}$  (D), 5  $\mu\text{L}$  (E), 10  $\mu\text{L}$  (F) and 20  $\mu\text{L}$  (G) YKF s/n volumes tested against *C. albicans* used a growth assay.



## 5. Antifungal characteristics of the YKF compound

A vital concern regarding antifungal agents is the YKF compound characteristic. An ideal antifungal drug would be a fungicidal agent. Therefore, it is essential to determine if the YKF compound expresses fungistatic, ability to inhibit fungal growth, temporarily, or fungicidal, kills fungal microorganisms. Therefore, an experiment was set to determine such YKF compound antifungal traits.

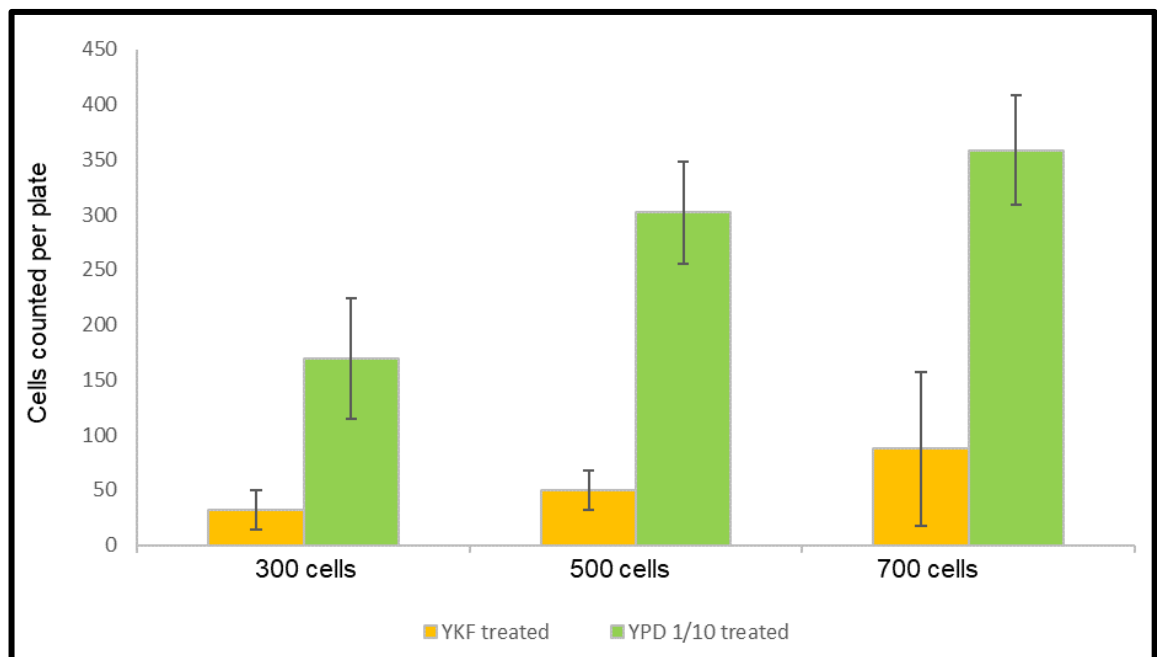
An overnight inoculated *C. albicans* culture was diluted in YPD liquid media and was placed in the incubator. When the culture reached log phase was separated into two falcon tubes (*Figure 14*). In one tube YKF was added and in another falcon YPD 1/10 which was used as a control. The tubes were incubated again at 30°C to reach early lag phase.

Following washes, the cultures with the YKF and YPD 1/10 media were spread onto plates with the aim to pipette 300, 500 and 700 *C. albicans* cells per plate. This experiment was conducted five times for designated amount of *C. albicans* colonies per plate. This was conducted to ascertain the YKF compound antifungal characteristics when the cells amount increases.

The *C. albicans* colonies, incubated over night at 30°C, were counted manually. From the count, it was observed that the *C. albicans* cells treated with YKF, were significantly less in number as the ones treated with YPD 1/10, as seen in *Figure 25*. Calculating the percentage of YKF treated colonies against YPD 1/10 treated colonies, demonstrated in *Table 5*, only 34-39% of *C. albicans* grew. These results strongly suggest that YKF antifungal compound is fungicidal hence killing *C. albicans*.

Colonies pipetted	300			500			700		
	YKF	YPD	YKF/YPD (%)	YKF	YPD	YKF/YPD (%)	YKF	YPD	YKF/YPD (%)
Colonies counted	34	230	15%	73	403	18%	120	319	38%
	66	149	44%	132	375	35%	120	408	29%
	67	145	46%	180	308	58%	123	404	30%
	65	167	39%	155	335	46%	145	448	32%
	46	127	36%	108	247	44%	155	366	42%
Sum	55.6	163.6	34%	129.6	333.6	39%	132.6	389	34%

**Table 5, The cells counted for YKF and YPD 1/10 treated *C. albicans*, the percentage YKF/YPD and the sum for each quintuple.**



**Fig. 25, A graphical representation the *C. albicans* culture treated with YKF s/n and YPD media.** The 300, 500 and 700 colony unites were aimed to be pipetted onto YPD 1/10 agar plates. The bars demonstrate the number of *C. albicans* cells grown. *C. albicans* treated with YKF s/n are shown in yellow whereas the ones treated with YPD 1/10 media (control) shown in green. The error bars represent the sum of the SD of the three technical replicates.

These results demonstrate that approximately 40% of *C. albicans* continue to grow. These could either indicate that YKF compound is not fully fungicidal and could have some fungistatic characteristics, or a larger concentration of the YKF compound would be required. Nonetheless, considering that 1  $\mu$ L YKF kills the vast majority, about 60%, of *C. albicans*, YKF has desirable fungicidal traits. As most *C. albicans* cells are shown to have died out, these results suggest that the YKF compound is a fungicidal agent rather than fungistatic.

## **6. Biochemical approach of the YKF compound**

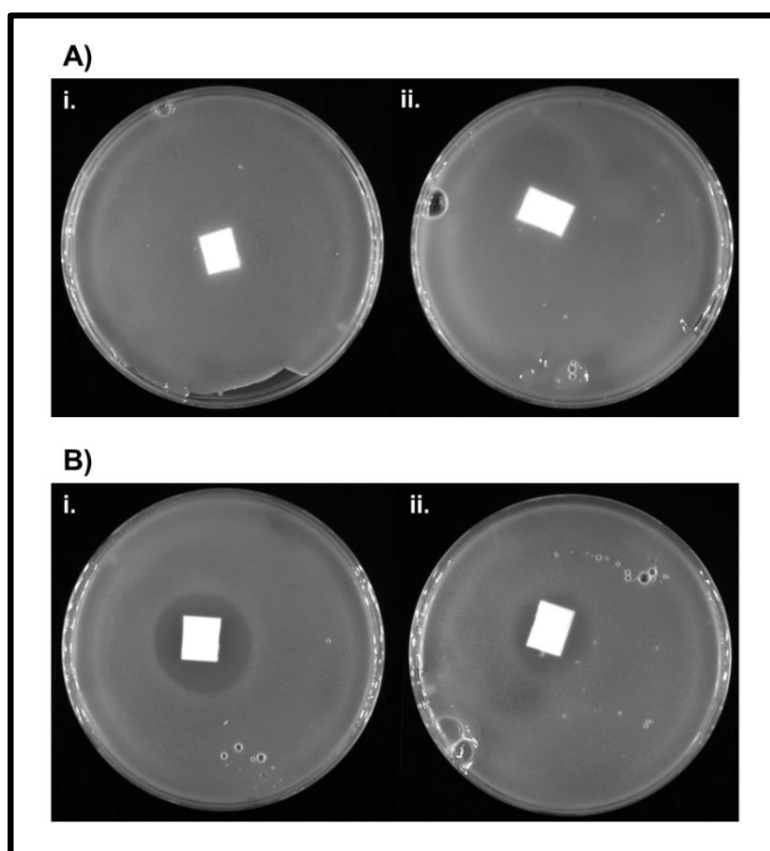
Isolating and identifying the antifungal component of the YKF is a key aspect of the research project. Fractionation techniques were modulated to purify the YKF supernatant and isolate the antifungal agent. The YKF supernatant activity was investigated using silica paper. This was conducted to determine the YKF supernatant is absorbed by the silica paper hence to later proceed with Thin Layer Chromatography (TLC) technique. Onto the silica paper was added YKF supernatant pre-chloroform extraction, contained in the optimal environment, and the organic phase YKF s/n that underwent chloroform extraction. In *Figure 26/B i* and *ii* is demonstrated pre-extraction and extracted chloroform phase YKF s/n. Either expressed a reasonable  $R_f$  of 1.6 cm and 2.5 cm respectively. Pre- and post-chloroform extracted YPD 1/10 liquid media, displayed on *Figure 26 A i* and *ii*, were also conducted as a negative control. For both, YPD media controls no halo-like region was observed.

Additionally, the chloroform phase of the YKF and the YPD media, as a control, were added onto the silica paper. The silica paper was treated with various polar and non-

polar chemicals employed, as a mobile phase for the TLC experiment used to determine the YKF compound. The mobile phase should have the ability to migrate an analyte organic material. The silica paper was treated with lower methanol, respectively, 2%, 5%, and 10% in 5 mL dichloromethane (DCM). After run on a thin-layer chromatography chamber, the silica paper was employed with potassium permanganate to depict the separated spot. As shown on *Figure 27/a, b, c*, decreasing the concentration of the very polar substance demonstrated that for either YKF or YPD particles did not move through the paper but remained on the baseline. This could suggest that the YKF compound is relatively a more polar agent. As a result, the concentration of the more polar methanol was increased to 15%, 20% and 25% in 5 mL of DCM. It was observed, as is seen in *Figure 27/d, e, and f*, that both the YKF and YPD particles moved to the front (top) of the paper.

Furthermore, the silica paper was treated with with polar:non-polar reagents as a solvent system. Dichloromethane (DCM) was employed as a moderately polar solvent was mixed with the non-polar hexane in a 1:1 ratio. Similarly to the other solvents employed DCM:hexane solvent system, demonstrated in *Figure 27/g*, no YKF compound spot was visible. The TLC plate was also treated with ethyl acetate, a very polar solvent shown on *Figure 27/h*. Nevertheless, this additive also did not expressed no spot separation or difference between the YKF agent and the YPD media. The similarity of the YKF supernatant and the control YPD media, as observed in all the silica TLC experiments, is attributed to the fact that the YKF supernatant is composed of the same media components as the control YPD media. Both contain yeast extract, bactopectone and glucose.

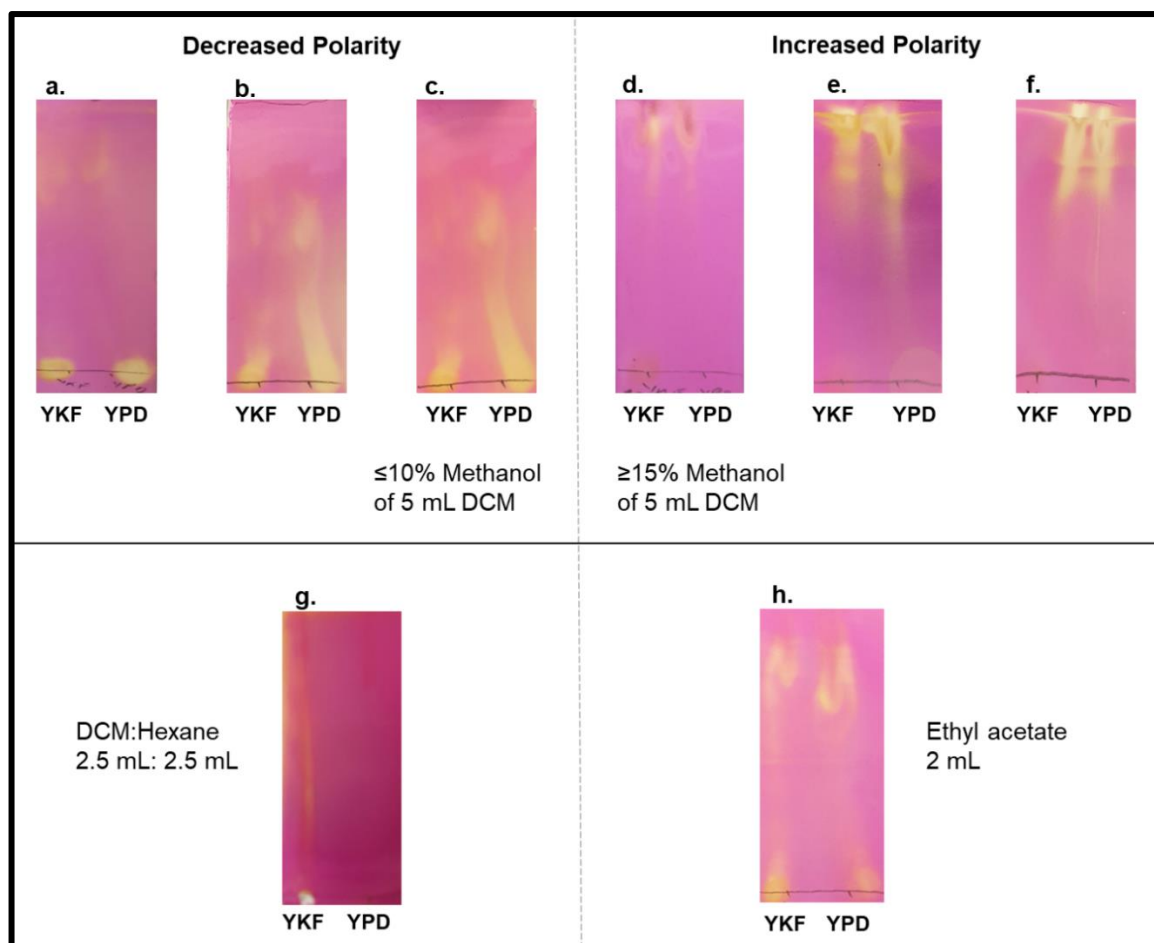
As none of the TLC bioassays revealed any spots, additionally techniques would have been attempted. However, due to time restrictions because of COVID19 pandemic lockdown, this experiment was halted to a stop hence opening the scene to speculative scrutinisation (further interpretation on the following section: **Discussion: Part. 6**).



**Fig. 26, YKF supernatant activity observed using silica paper.**

A) pre- (i.) and post-extraction (ii.) activity of the YPD 1/10 liquid media.

B) pre- (i.) and post-extraction (ii.) activity of the YFK supernatant. The YKF presents the reasonable zone of inhibition before and after undergoing chloroform extraction. The halo-like area for B)i. suggests that the YKF compound is in the chloroform (organic) phase when treated with chloroform.



**Fig. 27, TLC paper treated with methanol and Dichloromethane (DCM).**

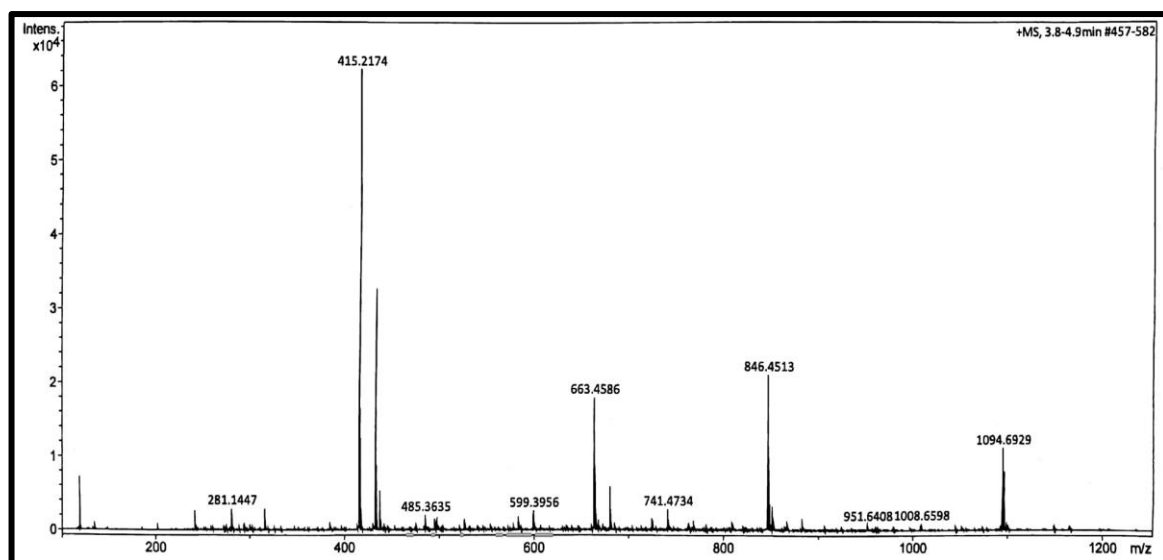
Moderately: very polar solvents: by decreasing the polar levels of methanol (very polar) in 5 mL DCM (moderately polar), 0/2% (a.), 0.5% (b.) and 10% (c.) methanol the YKF compound does not move from the baseline. In comparison, increasing the polarity, 15% (d.), 20% (e.) and 25% (f.) methanol in 5 mL DCM the YKF compound moved to the solvent front (the top).

Moderately polar: non-polar solvents: 2. 5 mL of DCM (non-polar additive) mixed with 2. 5 mL hexane (moderately polar) demonstrated no spot separation (g). There was a bright yellow resembling a line from baseline to the silica paper front for both YKF and YPD media (not quite visible on the picture).

Very polar solvent: using only the very polar 2mL ethyl acetate (h) as a solvent system also showed no spot separation.

A mass spectroscopy of the YKF supernatant was run to analyse the YKF compound composition. The analysis of the chloroform extracted YKF compound

on positive ion mode electrospray ionization mass spectrometry (ESI-MS) displayed a range of ion peaks. Minor peaks are presented at  $m/z$  846.4513, 663.4586, 432.2439, in addition to other lesser peaks, as seen on *Figure 28*. The highest ion peak (base peak) detected was 415.2174  $m/z$  (mass-to-charge ratio value) presenting the most intensively abundant ion fragments. The antifungal compound, however, is not isolated from the TLC experiment, this cluster of molecules is not pure. Therefore, to obtain insight on the molecular structure from the MS analysis, the exact mass data of the highest peak is placed in a mass database, named: *massbank.eu*. This provides a degree of speculative insight to examine the unknown antifungal compound. As demonstrated in *Table 6*, several agents share a closely related exact mass with the base peak of the mass spectrometry. Tomatidine exhibits 50 spectra in comparison to the other components. This data does not suggest that the YKF antifungal compound is tomatidine but could indicate to a certain relation between the two.



**Fig. 28, Mass spectrometry of the non-purified YKF chloroform phase.** The highest peak detected is at  $415.2174\text{ m/z}$  at  $\times 10^4$  relative intensity. This exact mass data was placed in the massbank.eu website shown on *Table 6*.

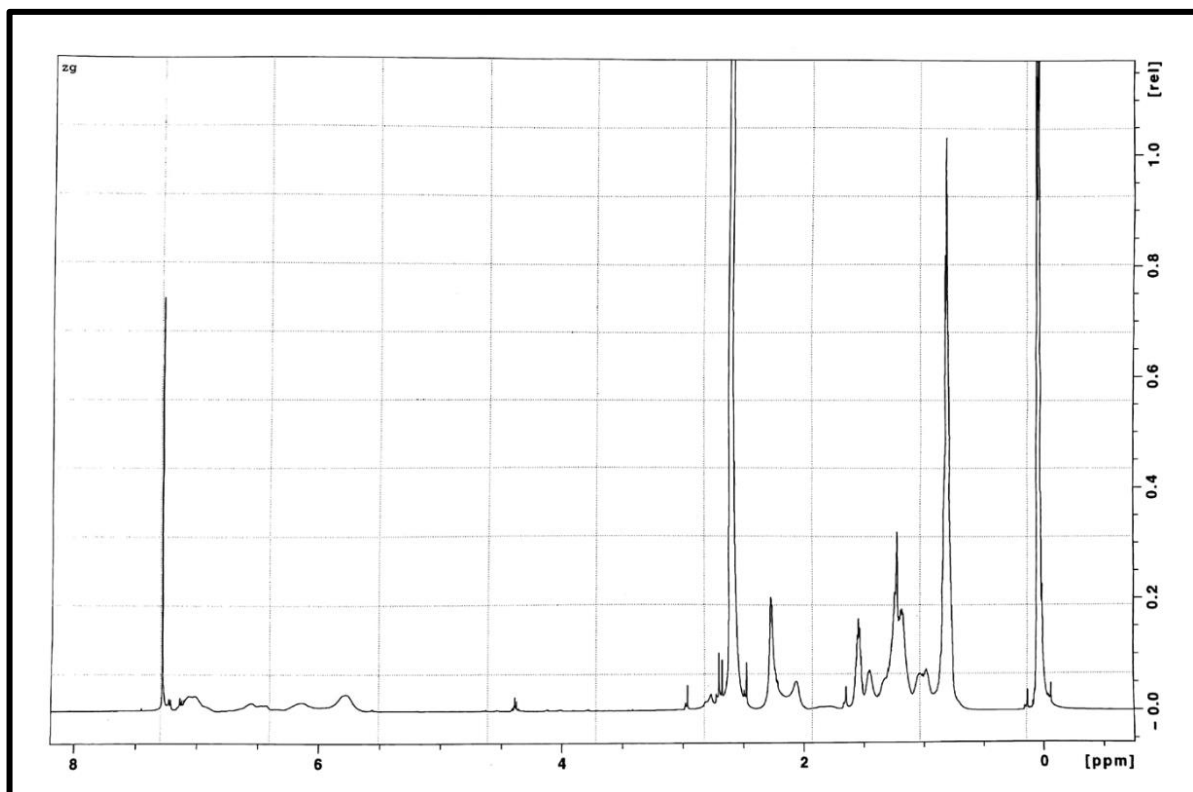
Name	Formula	Exact Mass
Acemetacin 25 spectra	$\text{C}_{21}\text{H}_{18}\text{ClNO}_6$	415.08228
Salmeterol 5 spectra	$\text{C}_{25}\text{H}_{37}\text{NO}_4$	415.27225
Tomatidine 50 spectra	$\text{C}_{27}\text{H}_{45}\text{NO}_2$	415.34500
Zamifenacin 6 spectra	$\text{C}_{27}\text{H}_{29}\text{NO}_3$	415.21469

**Table 6, the data produced form the massbank.eu website presenting the most closely related compound to the data from the mass spectrometry front peak.**

An NMR of the YKF s/n was conducted to observe the chemical properties of the compound. The NMR presents the chemical shift through depicting the protons. The furthest shift observed, *Figure 29*, is at 7.3 ppm (parts per million). This indicates the presence of an aromatic substance with a hydrogen at the phenyl group. The other proton peaks at about 0.9 ppm suggest alkyl (methyl) and smaller peak at 1.5 detects the presence of an alkyl methylene (*Figure 29*). The peak at approximately



2.5 ppm is an indicative to an alkynyl present in the composition (*Figure 29*). The larger peak at about 0 ppm simple suggest background noise and solvents used for NMR preparation. The MS and NMR spectroscopy research were run by Dr Gary Robinson at University of Kent.



**Fig. 29. NMR spectroscopy analysis of the YKF s/n.** The NMR data shows the chemical shift of the protons. The most distant chemical shift at 7.3 ppm could indicated to an aromatic compound. Other nearer to 0 axis implies the presence of alkyls and an alkynyl.

## ■ Discussion

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In this work, microbiological and biochemical approaches were utilized to study the antifungal properties of compound secreted by the Yeast Killer Fungus.

### **1. Fungal Infections: A Global Problem**

Fungal infections account for some of the most difficult human diseases to manage. Fungal-related disease occur in healthy individuals but the most considerably they manifest in severe illness, jeopardizing medical treatment for other serious diseases [1]. Fungal infections led to reduced quality of life for billions of individuals worldwide and the numbers of fungal infections are increasing every year [2]. Candidemia, caused by the *Candida* family yeast are one of the most sever invasive fungal infections [15]. New emerging pathogenic fungi like, the recently characterised organism *C. auris* that is able to evade drug therapy targets are making it harder for affected individuals to receive effective medical treatment [54]. In addition, there is an eminent global concern regarding an increase in fungal drug resistance. Azoles, echinocandins and other antifungal drug therapies do not provide the most effective treatment for fungal infections not only for the serious pathogenic organism *C. auris* but also for the most common fungal pathogen, *C. albicans*. Therefore, the need for development of new, effective and safe antifungal agents is critical.

### **2. The Yeast Killer Fungus (YKF) secretes an antifungal compound**

Previous work conducted from Prof Brian Cox and the Buscaino laboratory (within Kent Fungal Group) has identified a fungus named Yeast Killer Fungus (*Resinicium*

*bicolor*). The YKF fungus secretes in its culture media a compound that expresses antifungal activity.

### **3. Establishing a protocol to reliably test the YKF compound activity**

YKF secretes over time an antifungal compound in its media culture. To date the antifungal activity nature of the YKF compound remains unknown. Therefore, any research aimed at understanding its antifungal properties cannot be conducted as the compound was not purified, concentration remains unknown, but it is conducted using the volume of YKF cell culture media known as supernatant. In this culture media, the concentration of YKF compound in the supernatant is likely variable and it will accumulate over time.

One of the first aims of my thesis research was to establish a robust protocol to test YKF s/n antifungal properties. My priority was to identify the optimal window in order to “harvest” the YKF s/n and test its activity. Therefore, I performed an evolutionary experiment and established that YKF s/n reached maximum activity 14 days post inoculation, when grown in 20-22°C. After 14 days the YKF compound begins to degrade as the antifungal strength of YKF s/n is reduced. On the other hand, the activity of the YKF s/n cultivated in cold room temperatures (4-5°C) takes 34 days to reach full antifungal potential. Even after a few days it continues to retain its antifungal strength to stop the growth of *C. albicans* cells. Under low temperatures the *killer fungus* maintains its morphological and biochemical form, therefore, the process of degradation takes a longer period of time. It was concluded that the ideal time to use the YKF s/n was after a period of 14 days since YKF culture inoculation

in media. This procedure would be used for additional experiments until the YKF compound is purified and concentration is known.

#### **4. *Candida* genus species are susceptible to the YKF compound**

Work presented in this thesis demonstrates that YKF compound is active not only against the *C. albicans* but also against other *Candida* species. Indeed, YKF compound stopped the growth of *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii*. Importantly, YKF compound is also active against the recent emerging pathogen *C. auris*. This is key due to *C. auris* multi-drug resistant (MDR) fungal pathogenic trait. *C. auris* develops resistance for azoles due to point mutations in the lanosterol 14  $\alpha$ -demethylase gene, and upregulation of the ERG11 and the efflux pump genes. Five single-nucleotide polymorphisms (SNPs) in different genome loci and FKS1/2 gene mutations cause *C. auris* resistance for polyenes and echinocandin drugs, respectively. As a result of this mutations *C. auris* is resistant to approximately 60% of azole drugs, 15% of the polyene, amphotericin B, and less than 2% of the caspofungin. Accordingly, caspofungin drugs are currently used as the first-line for *C. auris* infections treatment management [54]. These factors make *C. auris* a dangerous fungus in the human body and difficult to treat. Therefore, YKF compound could provide an antifungal window for the MDR *C. auris* infections therapy and treatment. The four *C. auris* clinical isolates obtained were susceptible to the YKF antifungal compound. This is a landmark as *C. auirs* is difficult to treat and is highly resistant to the current antifungal drugs available.

Unexpectedly further distant organisms such as, *C. krusei* or *C. glabrata* demonstrated susceptibility to the YKF agents. *C. krusei* belongs to the Pichiaceae

family part of the CTG clade whereas, *C. glabrata* along with *Saccharomyces cerevisiae* are categorised members of the *Saccharomycetaceae* family part of the WGD clade. Both strains of *C. glabrata* showed a large area of inhibition thereby exhibit high sensitivity to the antifungal agents of the *killer fungus*.

Consequently, the YKF displays a broad spectrum of antifungal activity towards *C. albicans*, the *Candida* species of the CTG clade, to the pathogenic MDR *C. auris*, and to the WGD clade yeast *C. glabrata*.

## **5. Fungicidal VS Fungistatic features of the YKF compound**

An ideal antifungal drug must kill the human pathogenic fungi in order to prevent invasive fungal infection prolonged treatments and the high levels of toxicity which would be as damaging to human tissues as the fungal infection itself. Unfortunately, the current antifungal drugs in the market are fungistatic. These drugs treat fungal infections by inhibiting pathogenic fungal species growth and development by keeping them at bay. Therefore, work to develop and identify fungicidal agents which kill the infectious fungi is persistent.

Experiments demonstrated in this thesis show that YKF compound is a fungicidal component. The YKF compound showed to kill approximately 60% of the *C. albicans* cells. The ability that YKF s/n did not perform 100% killing could be due to several reasons. The main factor for such performance could be because the concentration of the YKF compound is unknown. The YKF component concentration on the supernatant volume could not have been enough to kill the *C. albicans* cells. Therefore, until the YKF compound is purified and isolated the concentration needed to kill 100% of the *C. albicans* remains unknown and impossible to speculate at this

point. Another factor that could have played a role in not showing a total killing of the *C. albicans* could have been due to degradation of the YKF compound. Although throughout the project YKF s/n used was 2 weeks after inoculation the culture media could have begun degrading faster. Changes in the required optimal room temperature could have caused such property. Nevertheless, the results obtained are very promising for future work once the compound is purified and characterised. This research presents that the *Yeast Killer Fungus* secreted compound exhibits a desirable fungicidal trait for an effective antifungal agent.

## **6. Biochemical processes and limitations**

One of the main goals of this project was to isolate and identify the *Yeast Killer Fungus* compound by utilizing biochemical approaches. Unfortunately, these experiments were not completed due to governmental and university COVID-19 pandemic lockdown rules. The biochemical investigation and additional microbiological aims were promptly stopped. Such limitations and additional research needed are discussed on part 7: '*Further studies*'.

The mass spectroscopy (MS) analysis presented that most spectra, 50 spectra, corresponded to the glycoalkaloid compound tomatidine. This steroidal alkaloid is mostly observed as a plant-derived alkaloid synthesised in tomato leaf hence the name tomatidine. Glycoalkaloids are known to have antimicrobial activity to protect the plant from microbial infections [86]. Tomatidine has been presented to express bacteriostatic effect on certain bacteria such as, *Staphylococcus aureus* SCVs [87]. There have been additional studies that suggest glycoalkaloids, especially

tomatidine antimicrobial activity against the simple yeast *S. cerevisiae*, and importantly to this thesis, towards a few *Candida* species. A study has shown that tomatidine is highly susceptible to *C. albicans* and *C. krusei* but expressed no activity against *C. glabrata* [88]. This, however, as it is demonstrated in this thesis, is not the case for the YKF compound which has demonstrated to kill *Candida* species growth, including *C. glabrata*. Although the YKF compound investigated utilizing MS analysis is not purified and isolated from the supernatant, nevertheless, the antifungal compound could be a derivative of tomatidine, or a closely related chemical component.

Although at this point, we are unable to identify the YKF could be a derivative of tomatidine or there can be more than one compound determining the antifungal properties. The MS analysis also presented 25 and 6 spectra for acemetacin and zamifenacin, respectively. Both these components are synthetic drugs. Therefore, the MS data could indicate that there may be more than one antifungal agent present. Considering the fact that there was no compound separation in the TLC experiment does suggest two or more agents exhibiting antifungal property.

The mixture of DCM with methanol as a solvent system suggested that by increasing the methanol concentration the unknown antifungal compound was observed to migrate to the baseline of the TLC plate. This indicates that the antifungal compound shows to be relatively more of a polar compound. However, to have had a better overview other solvent system should have been employed. The solvents used could have also been a factor that no spot was observed in the TLC silica paper. During the mobile phase, the chemical solvents used were selected as general

solvent system used often in chromatography separation. DCM, methanol, hexane, ethyl acetate could simply not be able to cause any separation of the unknown antifungal YKF compound.

The limitation with biochemical aspect was due to time restrictions. Therefore, other methodology techniques would have been performed to determine and identify the YKF compound.

Chloroform used for the biomolecular extraction procedure could have interacted with the solvent mixture system chosen for the TLC. Dimethyl sulfoxide (DMSO) could have been used as an alternative molecular extraction technique. In addition, other additives that could have been attempted for the solvent system including very polar additives such as, ethanol or isopropanol; or moderately polar solvents like, acetonitrile, diethyl ether, toluene; with non-polar solvents such as, pentane petroleum ether, cyclohexane. As DCM is a general, although moderately polar additive and methanol is stronger polar solvent, either a non-polar compound could be used or a different moderately polar solvent. Nonetheless, a variety of solvent systems mixtures could have provided a different outline of the TLC plate.

Additionally, another reason that spot separation did not occur could have been because of the dye system used. The potassium permanganate ( $\text{KMnO}_4$ ) dye which was used to stain the silica gel paper could have been the issue. Potassium permanganate is a general stain used widely as the first-choice to depict the TLC particle separation. However, there are several other staining reagents which could have presented different data. Potassium permanganate is commonly used as it



detects oxidation sensitive alkenes and alkynes groups. As the NMR presented a spike at approximately 7.5 ppm indicating an aromatic agent hence an iodine dye could be used. Iodine is one of the oldest staining methods used for compound visualization and could have provided the compound isolation needed. Phosphomolybdic acid (PMA) stain, referred as a “universal” stain is another staining method. PMA is good for most functional groups and is sensitive to solutions in low concentrations. Other stains that could be used are morin hydrate and vanillin which are general staining tests, ninhydrin which is excellent for detecting amino acids, dinitrophenylhydrazine (DNP) is mainly used for aldehydes and ketones, Dragendoff’s reagent which provides good results for plant derived alkaloids, cerium sulphate which is also a general stain but is particularly developed alkaloids, and ceric ammonium sulphate specifically effective for vinca alkaloids (aspidospermas). Therefore, the TLC silica paper could have been employed with several excellent staining agents that could have provided a separation spot of the YKF compound.

Additionally, issues could have been the thin layer chromatography technique. Other separation and isolation chromatography techniques could have been used. High performance liquid chromatography (HPLC) could have been used. HPLC is a versatile and currently gaining popularity as a robust analytical technique. Separation occurs by exploiting the compound’s different rate of migration [89]. During this technique detectors have to be chosen for compound identification. UV detectors are highly sensitive thus sensing small particles, or MS could be used to detect ionisation [89]. Moreover, phytochemical screening assay (PSA) is a simple, inexpensive and robust method for plant compound identification. Phytochemicals

are plant derived chemicals regularly described as secondary metabolic particles in plants [89]. Phytochemicals are found in tomato plants, as well as the glycoalkaloid tomatidine. Speculating that YKF compound could be a component secreted by a plant thus the PSA technique could provide additional insight.

The concentration of the YKF supernatant could have been an issue. The inability to show any spots could have be to the fact that for every silica TLC a certain volume, 40 $\mu$ L, was used. The concentration of the YKF compound on the supernatant volume could not have been enough. There may not have been enough YKF molecules concentrated within the sample to be able to separate from the rest of the supernatant or to be depicted in the TLC paper.

Changes in pH could cause the YKF compound in the supernatant to undergo structural alterations. The antifungal particles in the liquid culture media are continuously subject to the outer environmental conditions. Fungal species are known to modulate the pH in order to adjust a better continues living environment for themselves. This could have been as result of its interactions, not only with chloroform but with other solvents. This conditions such as alternations in the optimal pH could have led to its inability to show in a silica TLC. Subsequently, further studies need to be conducted on the biochemical aspect to purify and identify the antifungal component. This is a key phase for future research and potential drug discovery processes.

## 7. Further studies

Further research into the biochemical trait of the YKF compound are required in order to examine the antifungal compound. It is essential for the YKF compound to be purified and isolated. Once such stage occurs, the purified YKF compound to be tested *in vitro* on *C. albicans* and the other *NCA* fungi's used on this project. The characterisation experiment that showed that YKF compound is fungicidal to *C. albicans* should be test for the same *NCA* fungi used on this thesis. Determining if YKF compound is fungicidal or fungistatic would be particularly interesting for WBG clade *C. glabrata* and the pathogenic *C. auris*. Additional antimicrobial studies using various other fungi such as, the opportunistic pathogenic moulds of the *Aspergillus* family, or other species that cause fungal-related deaths, like *Cryptococcus*. These fungi, along with *Candida* species account for approximately 90% of all fungal infections related fatalities [1][90]. *Candida* accounts for most invasive fungal infections, *Aspergillus* is the most common mould infection, and the *Cryptococcus* is the most prevalent fatal fungal-related disease globally. Therefore, the ability that YKF agent could inhibit or even kill the growth of such species would be landmark for antifungal drug research. A broad range of fungal targets is required for all new antifungal drugs hence it would provide an interesting outline for YKF compound spectrum activity.

The YKF natural agent to be tested in combination with other existing antifungal agents. Neither in previous work nor in this project, combinations of YKF s/n with current antifungal drugs, such as fluconazole, has not been conducted, this would provide interesting results as many fungal infections are managed in drug pairs.

Most antifungal drugs are known to interact quite well with one-another to target pathogenic fungi. Interestingly, it has been suggested that tomatidine expresses stronger antifungal results in combination with fluconazole [88][91]. Therefore, YKF compound in combination with fluconazole could also provide stronger antifungal results. However, this interaction should be observed to determine the ability to cause drug-to-drug interaction which could lead to toxicity. Antifungal drugs when administered for long periods of time cause toxicity leading to epithelial cells organ damage. This could be tested *in vitro* but the better understanding of such toxic mechanism would be *in vivo* using model organisms.

Subsequently, an import investigation would be to test *in vivo* the YKF compound antifungal bioactivity towards *Candida* species. The simple model organisms, *C. elegans* or, the fruit fly *Drosophila melanogaster*, would be infected with *C. albicans*, and/or other *Candida* pathogens. Later, to be treated with YKF either in the supernatant form (if not purified) or using the molecular compound (if purified). This would provide a clear spectrum of understanding of the YKF mechanisms *in vivo*. Additionally, mammalian model organism such as, mice would provide a better understanding of the YKF's activity. Testing in laboratory fungal infected mice would deliver the best results for the molecular activity of YKF compound as, mice share 85% genome similarity with humans.

Additionally, as drug resistance is a significant concern for the current antifungal agents this is an aspect worth identifying. Although, it could be difficult to determine resistance, experiments could be designed to observe such phenomenon. An

antifungal target with the abilities to evade the known resistance mode of action or other pathway could be a landmark for antifungal therapy.

As the antifungal agent generated by the YKF was not isolated further work needs to be conducted. A variety of other dyes could have been used. A different dye staining system could provide a separation spot on the TLC. Therefore, if a spot is present in the silica TLC paper, the spot area would be scraped off and extracted, using solvents such as methanol. Later a disc-diffusion assay to be conducted to observe its direct effect on *C. albicans* and other *NCA* species. If a zone of inhibition is present the next course of action would be an MS analysis to determine the molecular agent/s expressing the desirable antifungal properties.

### **In conclusion**

Fungal infections cause difficult managed infections, with some being life-threatening. The increase of fungal resistance to the available limited antifungal drugs, is adding to the global concerns. Luckily, the *Yeast Killer Fungus* provides hope for the future. That is supported by the susceptibility of *C. albicans* and several other *Candida* species to the YKF's antifungal component. Interestingly, even the newly recognized and highly pathogenic *C. auris* is vulnerable to the YKF compound. Additional studies on the biochemical activities of the YKF compound would provide a clear picture to its antifungal properties. Nevertheless, the YKF compound delivers a novel and broad range action, traits ideal for a future antifungal drug on the shelves.

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