METABOLIC ADAPTATION OF *CANDIDA ALBICANS* BIOFILMS

BY

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LIST OF ABBREVATIONS

ABCD	Amphotericin B colloidal dispersion
ABLC	Amphotericin B lipid complex
AgNPs	Silver nanoparticles
ALS	Agglutinin like sequence
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
CYB	Lactate dehydrogenase
CAT	Catalase
cfu/ mL	Colony forming unit
CHD-FA	Carbohydrate derived fulvic acid
CNS	Central nervous system
CO_2	Carbon dioxide
CSLM	Confocal laser scanning microscope
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECM	Extracellular matrix
eDNA	Complementary deoxyribonucleic acid
EDTA	Ethylenediaminetetraacatate
EP1	Intracellular exopolysaccharide
EPS	Extrapolymeric substance
GRXS	Glutaredoxins
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HAART	Highly active antiretroviral treatment

HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
HSE	Heat shock element
Hsf	Heat shock factor
Hsps	Heat shock proteins
ICL	Isocitrate lyase
IEPS	Extracellular polysaccharides
IPS	Intracellular polysaccharide
KOAc	Potassium acetate
L-Amp	Liposomal Amphotericin B
LI	Lipases
JEN	Lactate permeases
M	Molar
MAP	Mitogen activated pathway
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MOPs	3-(N-morpholino) propane-sulphonic acid
mRNA	Messenger ribonucleic acid
MSL	Malate synthase
MTL	Mating type locus
MTT	3-(4, 5-dimethylthuazol-2-yl)-2,5 diphenyltetrazoliumbromide
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinuelecotide
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
nm	Nanometre
nM	Nanomolar
NO	Nitric oxide
OD	Optical density

PBS	Phosphate buffered saline
PCK	Phosphoenolpyruvate carboxylkinase
PDC	Pyruvate dehydrogenase
PL	Phospholipases
PYC	Pyruvate carboxylase
qRT-PCR	Quantitative reverse transcriptase real polymerase chain reaction
QSMs	Quorum sensing molecules
RHE	Reconstituted human epithelium
RNase	Ribonuclease
RPMI 1640	Roswell park memorial institute medium
ROS	Reactive oxygen species
rpm	Rotation per minute
SAP	Secreted aspartyl proteases
SEM	Scanning electron microscope
SEPS	Soluble extracellular polysaccharide
sHSPs	Small heat shock proteins
Sods	Superoxide dimutases
TAE	Tris-acetate/EDTA
TCA	Tricarboxylic acid
TRP	Thioredoxim
um	Micrometre
WO-1	White Opaque strain
YPD	Yeast peptone dextrose
YPL	Yeast peptone lactate

LIST OF SYMBOLS

α	Alpha
β	Beta
µg	Microgram
>	Greater than
%	Percentage
≥	Greater or equals to
µL	Microliltre
^{0}C	Degree Celsius
v/v	Volume per volume
w/v	Weight per volume
mL	Millilitre
mm	Millimetre
mM	Millimolar
mg	Milligram
mg/mL	Milligram per millimetre

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PENYESUAIAN METABOLIK *CANDIDA ALBICANS* BIOFILM

ABSTRAK

Biofilm kulat mempunyai kepentingan klinikal yang tinggi. Salah satu yang ketara adalah biofilm *Candida albican* yang berupaya untuk memulakan jangkitan baru atau jangkitan semula kira-kira 40% daripada jangkitan kandidiasis yang disebarkan. Ciri utama biofilm yang membezakannya daripada sel –sel plankton adalah toleransi yang tinggi terhadap rawatan serta sistem imun. Laporan menunujukkan bahawa pertumbuhan sesil oleh sel bebas terapung mempunyai daya ketahanan beberapa kali ganda terhadap ujian nilai perencatan minimum atau MIC. Daya ketahanan yang tinggi itu adalah disebabkan oleh cir-ciri yang terdapat pada *C. albican* termasuk pengumpulan bahan extrapolymericn (EPS), penyesuaian fenotip serta fleksibiliti metabolic dan lain-lain. Secara amnya, kajian ini melibatkan ujian ke atas peranan fisiologi metabolisme terutamanya laluan enzim glyoxylate isocitrate lyase atau ICL ke atas pembentukan biofilm, daya ketahanan**,** morfologi, dan komponen dinding sel. Keputusan yang menarik telah diperolehi yang mana biofilm yang terbentuk daripada strain mutasi ICL1 heterozigot dan homozigus menunjukkan tahap toleransi yang sama tinggi dengan strain kawalan (reference strain). Sifat enzim ICL dimorphism telah terjejas sebagaimana yang berlaku dalam strain mutasi. Keupayaan pemencilan oleh beta-1,3-glucan yang merupakan komponen utama karbohidrat dalam extracellular matrix atau ECM tidak menunjukkan sebarang tindak kesan balas. Penyingkiran enzim memberi kesan terhadap transkripsi protin dalam *FKS1*, *ERG11* dan *CDR2*. *FKS1 dan ERG11* telah menunjukkan peningkatan di setiap peringkat pertumbuhan manakala *CDR2* telah menunjukkan peningkatan pada fasa awal pertumbuhan. Akan tetapi, ekspresi telah menurun berbanding strain

kawalan. Oleh itu, laluan glyoxylate tidak boleh bertindak sebagai penentu kerintangan biofilm tetapi perlu untuk hidup. Kajian ini dapat dijadikan asas untuk mengkaji interaksi secara tunggal atau sinergi ke atas ubat yang berpotensi sebagai antikulat.

METABOLIC ADAPTATION OF *CANDIDA ALBICANS* BIOFILMS

ABSTRACT

Fungal biofilms have high clinical importance. A notable one is *Candida albicans* biofilm, whose importance is attributed to its ability to institute new and reoccurring infections, accounting for about 40% of disseminated candidiasis. The major characteristic of biofilms which differentiates it from planktonic cells is its high tolerance to treatments and the immune system. Sessile growths have been reported to be several folds resistant to the minimum inhibitory concentration (MIC) of free floating cells drug treatment, and consequently frustrating the efficacy of drugs. The high resistivity is attributed to distinctive properties including accumulation of extrapolymeric substance (EPS), phenotypic adaptation switch and metabolic flexibility among others. In this study, the influential roles of metabolism physiology, particularly glyoxylate pathway Isocitrate lyase (ICL) enzyme on biofilm formation, resistivity, morphology, and cell wall components were evaluated. It was interesting to find that, heterozygous and homozygous *ICL1* mutant strains formed biofilm and exhibited a high tolerance level similar to the reference strain. The enzyme ICL impaired dimorphism trait as observed in mutant strains. Furthermore, sequestrating ability of beta-1, 3-glucan, a major carbohydrate component of the extracellular matrix (ECM) was not impaired. Deletion of ICL conferred a considerable effect on glucan synthase pathway *FKS1*, gene encoding 14-α-demethylase enzyme necessary for lanosterol conversion to ergosterol *ERG11* and efflux pump gene *CDR2* transcription. *FKS1 and ERG11* were up regulated throughout the developmental stages; *CDR2* was up regulated at the early phase. However, expression was down regulated compared to the reference

strain. Therefore, Glyoxylate pathway is not a specific determinant of biofilm resistivity, but essential for its survival. This study serves as a foundation for future experimental investigation of sole and synergistic alternative pathways interactions for potential drug targets.

CHAPTER 1 INTRODUCTION

Fungal pathogens have gained wide attention in the last century due to high rate of infections caused by them (Calderone, 2002). Infection happens predominantly in immunocompromised individuals with lower cluster of CD4 lymphocyte count, cancer and surgical patients (Jabra-Rick *et al.,* 2004). *C. albicans* is a major systemic and most commonly isolated fungal pathogen, accounting for about 80% of clinical fungal infections (Douglas, 2002). In spite of advancement in the management of hospital acquired infections, *C. albicans* infections rank fourth most commonly isolated nosocomial blood stream pathogen (Ramage *et al*., 2005; Douglas, 2002; Pfaller *et al.,* 1998). Fungal infections fatality rate is about 40%, with *C. albicans* responsible for about 80-90% of cases (Sardi *et al.,* 2013; Ramage *et al.,* 2012; Brown *et al.,* 2007). Microbes exist in nature mostly as attached aggregated communities (Ballie & Douglas, 2000). Biofilm is an irreversible assemblage and attachment of microorganisms on surfaces protected in a self secreted polymeric substance (Ramage *et al.,* 2012; Ballie & Douglas, 2000). Human fungal pathogens including *Candida* species, *Aspergillus* and *Cryptococcus* produce biofilms and institute infections such as urinary tract infection (Finkel & Mitchell, 2011; Douglas, 2003).

A characteristic attribute of biofilm is resistance to antimicrobials controlled by multifactorial and interconnected factors. Implicated mechanisms includes β-1,3-glucan, extracellular matrix (ECM), efflux pump mediated resistance, cell density, over expression of drug target genes and metabolism plasticity (Bink *et al.,* 2012; Ramage *et al.,* 2012; Al-fattani & Douglas, 2006). *C. albicans* is metabolically

flexible, i.e. the type of nutrient available determines the absorption pathway (Askew *et al.,* 2009). The absence of fermentable carbon signals alternative pathways particularly β-oxidation, glyoxylate and gluconeogenesis cycles (Askew *et al.,* 2009).

1.1 Significance of Study

The gene conferring resistance, ATP dependent transporters are highly expressed in planktonic cells and early phase of biofilm, thereby decreasing susceptibility (Murkejee *et al.,* 2003). However, their absentia function in mature biofilm resistance shows that other molecular mechanisms and genes are involved (Ramage *et al.,* 2001). Expression of *ERG11* when challenged with fluconazole may signal point mutation and increased copy of 14-α-sterol demethylase via upregulation of the encoding gene (Sanglard, 2002). Additionally studies have proposed that the utilization of alternative pathways may have an indirect role on biofilm resistance by providing hexoses which are monomer building blocks for synthesis of β-1,3-glucan in the matrix (Nett *et al.,* 2009; Nobile *et al.,* 2009). Biofilm matrix comprises of β-1, 3-glucan, which not only contribute to adhesion but also protects sessile cells from antifungal agents through drug sequestration (Nett *et al.,* 2007). Experimental studies showed that glucan synthesis by glucan synthase is dependent on the expression of glucan synthase *FKS1* gene. Production machinery is signalled when glucan concentration is low; in turn triggering glucan delivery enzymes to act and deliver glucan to the matrix in a controlled pathway (Taff *et al.,* 2012; Nett *et al.,* 2011; Nett *et al*., 2007). This study aims to further define the contribution of metabolism to *Candida* biofilm resistance, providing insight for development of drug targeting isocitrate lyase enzyme as a potential strategy for generating antifungal drugs effective against *Candida* biofilm infections.

1.2 Study Objective

High resistance exhibition is a peculiar trait of biofilms. *C. albicans* like bacterial biofilms is 1000X tolerant to treatment therapy unlike their free floating cells (Ballie & Douglas, 2000). Metabolic flexibility is an attribute extensively explored for survival, allowing for the use of fermentable and non- fermentable carbon sources. Glyoxylate pathway is an anabolic cycle that converts acetyl-CoA to succinate and malate for the synthesis of carbohydrates though gluconeogenesis cycle. It is the most preferred nutrient absorption route because the process is faster through decarboxylation step bypass and lower energy consumption compared to TCA pathway (Lorenz *et al.,* 2004).

Thus, a question arises whether their biofilm exploit other range of carbon when glucose is absent, and if the alternative carbons have significant role as contributing factor to their high tolerance of antifungal therapy in humans. Relatively, H_2O soluble polysaccharide β-1, 3 -glucan is a major component of the extracellular matrix suggested to contribute to resistivity of biofilms. Glucan concentration at each formation phase were quantified and correlated to energy synthesis to examine if absence of glyoxylate affects hexoses buildup.

To further ascertain the role of glyoxylate pathway in biofilm formation and antifungal drug resistance, ability of heterozygous and homozygous knockout strains of *C. albicans* (*Ca*ICL1/*icl1* and *Caicl1/icl1*) lacking ICL to form biofilm and build resistance to antifungal agents was studied. In addition to observe if a correlation exist between cell carbohydrates and matrix glucan concentration during the developmental stages. Transcriptional regulation of biofilm associated genes for the mutant strains were also studied under biofilm-forming conditions.

Specific objectives of study;

i. To study the functional role of *Candida albicans* glyoxylate ICL enzyme by comparing the ability of constructed heterozygous knockout *(Ca*ICL1/*icl1),* homozygous knockout (*Caicl1/icl1)* and wildtype strains to form biofilm and express their resistance phenotype.

ii. To examine single, double knockout and the wildtype strains cell wall and the ECM properties; specifically hexoses and glucan synthesis.

iii. To study transcriptional regulation of biofilm resistance associated genes *FKS1*, *ERG11*, and *CDR2* in the strains.

CHAPTER 2 LITERATURE REVIEW

2.1 *Candida albicans* Infections

C. albicans and related species; *Candida glabrata, Candida tropicalis, Candida krusei* and *Candida parapillosis* are commensal organisms, residing as benign flora of niches including the gastrointestinal tract, buccal cavity, vagina and mucosal surfaces of the body. They make up about 50% of microbiota population in humans (Odds, 1988). *C. albicans* is a unicellular oval shaped diploid fungus that grows as yeast cell and filamentous hyphae (Ramage *et al.,* 2012; Bendel *et al.,* 2003; Douglas, 2002). The proliferation and the increasing frequency of pathogenic opportunistic *Candida* infections are to a certain extent favoured by the use of antibiotics, use of oral contraceptives, poor oral hygiene and hormonal imbalance, which offsets population normalcy balance (Ruhnke, 2006; Douglas, 2002). About 50% of women aged over 25 years experience vulvovaginal candidiasis once or more in their life time. Moreover, food with high starch level may contribute to chronic infections (Ciara & Dorothy, 2005). Being an opportunist pathogen, risk factors influencing superficial, disseminated and invasive candidiasis have been identified. These factors include immunosuppression, low birth weight, broad spectrum antibiotics, parenteral nutrition, and haemolysis among others (Calderone & Clancy, 2012; Pappas *et al.,* 2004). Infections caused by *C. albicans* can be superficial or invasive. Invasive candidasis is predominantly endogenous in origin, but transmission from person to person can also occur; known as exogenous transmission (Pfaller, 1996), occurring mostly in stem cell and liver transplant, and intensive care patients.

Trans-establishment can be exogenous through health care professionals and contaminated health care materials, and endogenous transmission is encouraged by weak immune system (Pfaller, 1996). This gives rise to colonization opportunities to inhabitants (Soll, 2001; Cole *et al.,* 1996), and thus causing oropharyngeal candidiasis: characterized by white patches on the mucosa surface of the mouth; colonization of the respiratory tracts due to haematogenous spread of colonies content of oropharyngeal origin; diaper rush in kids; burning and itching of the inflammatory region in women, nail and skin folds (Colombo & Guimaraes, 2003; Ruhnke, 2002).

Chronic mucocutaneous candidiasis is an important superficial *Candida* infections involving colonization of both mucosal and skin surfaces by *Candida* species (Bhowate & Dubey, 2004). It develops mostly in patients with impaired T-cell function, causing poor cell mediated immunity, characterized by continual candidal infection of skin, scalp and nails, (Bhowate & Dubey, 2004; De Moraes-Vasconcelos D *et al.,* 2001). Disseminated or systemic candidiasis is a bloodstream infection also known as candidemia. It occurs as a result of breakage in the epithelial tissues mostly in immuocompromised individuals. It encompasses myocarditis, hepatosplenic abscesses and central nervous system (CNS) infections, with possibility of leading to death. Also, HIV patients suffered from oral thrush before the advent of the HAART (Highly Active Anti-retroviral Treatment) therapy, which includes a protease inhibitor that also inhibits an important virulence attribute of *C. albicans* secreted aspartyl protease (Munro & Hube, 2002).

2.2 Treatment Therapy

The choice of treatment of fungal infections depends on the immunological status of patient, the site of infection, type of infection, and infecting *Candida* organism (Pappas *et al.,* 2004). The most commonly used clinically approved antifungal drugs include the azoles (ketoconazole, voriconazole, fluconazole), polyenes (amphotericin B) and echinocandins (micafungin, caspofungin and anidulafungin) (Pappas *et al.,* 2004). Combination of drugs is mostly encouraged to fight drug resistance which develops during treatment process (Shinde *et al.,* 2012). Polyenes; particularly amphotericin B, is commonly used in combination with flucytosine, because *C. albicans* can develop tolerance when treated with flucytosine alone (Shinde *et al.,* 2012). Until 1950s, amphotericin B was the standard for treatment, typically used to treat systemic infections due to its high fungicidal activity, and mostly administered intravenously (Brajtbutg *et al.,* 1990). Amphotericin B increases cell wall permeability by binding to ergosterol, and in turn limits the function of the ergosterol (Brajtbutg *et al.,* 1990). It is however unfortunate that the clinical utility of amphotericin B is restricted because it causes nephrotoxicity and hypokalemia in patients (Deray, 2002; Ghannoum & Rice, 1999). Meanwhile, resistance has also been reported in some species, although in a less frequent way (Ellis, 2002). Furthermore, the adverse effect of the drug has reduced due to the development of lipid formulations, but not eliminated (ABLC, ABCD, L- Amp) (Odds *et al.,* 2003). Azoles are mostly utilized as frontline defence and treatment, due to its bioavailability, water solubility and true fungistatic activity with fewer side effects. It can be administered orally and intravenously (Nailis *et al.,* 2010). Fluconazole halts sterol biosynthesis, by binding to 14-α demethylase enzyme encoded by *ERG11,* thereby obstructing lanosterol conversion to ergosterol causing

buildup of toxic sterol intermediates (Ghannoum & Rice, 1999). However, long term usage results in development of resistance as reportedly seen in *C. albicans* (Nailis *et al.,* 2010; Sanglard & Odds, 2002; Franz *et al.,* 1998).

Voriconazole has a broader spectrum activity than fluconazole (Hazen *et al.,* 2003). It exhibits significant clinical efficacy against acute invasive aspergillosis and fluconazole resistant infections (Hazen *et al.,* 2003). However, a drawback of voriconazole is late oral administration, typically after intravenous application for dose maintenance in the blood (Hazen *et al.,* 2003). Also, miconazole is an effective therapeutic broad spectrum that exerts fungistatic activity on wide range of *Candida* species and dermatophytes, enhanced by reactive oxygen species (ROS) production and by hydrogen peroxide degrading enzymes inhibition: for example peroxidase and catalase (Vandernbosch *et al.,* 2010). The availability of ROS increases susceptibility of cells and potency of the drug (Francois *et al.,* 2006; Sreedhara Swamy *et al.,* 1974). The advent of echinocandins notably caspofungin, was a major advancement in fungal infection treatment. It is water soluble and constitutes prophylactic and fungicidal actions against fungal infections (Shao *et al.*, 2007). It is the only class of fungal cell wall inhibitors approved for clinical use (Shao *et al.*, 2007), and currently used for the treatment of invasive candidiasis. Casopfungin targets the β- 1, 3- glucan synthesis (Odd *et al.,* 2003). However, echinocandins agents have been associated with liver toxicity (Hoehamer *et al.,* 2010). Despite therapeutic treatment developments, *C. albicans* has became tolerant through phenotypic variations; forming complexes identified as biofilms, which prevent the diffusion of the drugs into the microenvironment; also by shielding and protecting the cells from the fungicidal behaviour of the antimicrobials (Walker *et al.,* 2008; Ramage *et al.,* 2005).

2.3 Virulence Traits of *C. albicans*

Understanding the virulence factors associated with *Candida* infection is paramount to knowing the mechanisms used by *C albicans* to cause infections, to avoid the immune system and exhibit resistance to drugs. *C. albicans* has extensive mechanisms facilitating its successful invasion, evasion, disease progression and establishment leading to clinical problems (Kim & Sudbery, 2011; Calderone & Fonzi, 2001). Infection establishment by *Candida* treads a pattern proceeding with adherence to surfaces, replication of cells, production and accumulation of hydrolytic enzymes that damage tissues, and penetration of epithelial surfaces (Calderone, 2002; Coller & Kavanagh, 2000). These virulence attributes aids it pathogenicity, underlay its transition from commensal resident to opportunistic pathogen (Kim & Sudbery, 2011). *Candida* species colonizes and cause disease at distinct sites with unique physiological environment (Calderone & Fonzi, 2001). It also disrupts certain properties possessed by the potential host, which may promote the manifestation of disease through imbalance of physiological processes and homeostasis (Calderone, 2002). Oral candidiasis is encouraged by hypoxia which in turn activates the expression of stress adaptation genes (Yun- Liang Yang, 2003). These include morphogenesis, secretion of hydrolytic enzymes, and expression of adhesin molecules phenotypic switching and biofilm formation (Naglik *et al.*, 2004; Calderone, 2002).

2.3.1 Adhesins and Invasins

Adherence of *C. albicans* to surfaces is an essential step for successful colonization (Coller & Kavanagh, 2000). Adherence is mediated by ligand receptor interactions, electrostatic charges and chemical bonds (Coller &

Kavanagh, 2000). Expressed cell wall molecules such as invasins and adhesins often seen as extracellular proteins on host cells promote *C. albicans* pathogenicity by enabling its attachment and proliferation, (Calderone & Braun, 1991). Agglutinin-like sequences *(ALS*) family genes encode glycosyl phosphatidylinositols (GPI) surface protein known to facilitate fusion to host cells epithelial and endoepithelial surfaces (Liu & Filler, 2011). Als3 is the most well characterized protein from ALS family multifunctional proteins, acting as an adhesin and invasin. Als3 mediates host cell damage and uptake of *C. albicans* through active penetration and endocytosis (Liu & Filler, 2011; Wachler *et al.,* 2011; Hoyer *et al.,* 2008). Active penetration often requires viable hyphae for successful penetration, through yet to be elucidated mechanisms, unlike endocytosis invasion that requires either through dead or living hypahe, influenced by Als3 and Ssa1 through clathrin dependent process (Wachler *et al.,* 2011; Phan *et al.,* 2007). They express different phenotypic features during infection depending on environment's susceptibility to invasion and progression (Liu & Filler, 2011; Hoyer, *et al.,* 2008).

Hwp1, a hyphae specific surface glycoprotein, is another relevant adhesin which links hyphae to the host cell though transglutaminases-mediated mechanisms (Sundstrom, 1999). Other proteins have been implied to exert indirect function on adhesion such as MNT1 and PMT1 mannosyltransferases for cell wall synthesis of mannan (Martinez-Lopez *et al.,* 2006).

2.3.2 Morphogenesis

Polymorphism trait refers to morphological changes in the cell shape. Majorly unicellular yeast cells, filamentous growth forms, and the pseudohyphae (Whiteway & Bachewich, 2007; Saville *et al.*, 2003). The transformation of yeast cell into hyphae is an adaptation mechanism to environmental stress (Whiteway & Bachewich, 2007; Casadevall & Pirofski, 2003). Its dissemination into the bloodstream is favoured by yeast form of growth for easy dispersal (Han *et al.*, 2011). It metamorphosis to hyphae serves as an escape route from the immune system; because the phagocytes may find it difficult to abolish high population of filaments fused on infected tissues (Jayatilake *et al.*, 2006; Calderone & Fonzi, 2001).When engulfed by phagocytes, they grow in filaments, thereby penetrating the wall of the phagocytic cell and killing the cell in the escape process (Jayatilake *et al.*, 2006; Calderone & Fonzi, 2001).

A range of acknowledged features induces morphological transition in *C. albicans* (Mayer *et al.*, 2013). For example, high alkaline pH medium and temperature $>37^0$C promotes hyphae development; likewise, presence of N-acetylglucosamine, CO_2, nitrogen starvation, and glucose and muramyl dipeptides induces filamentous forms (Mayer *et al.*, 2013; Sudbery, 2011; Xu *et al.*, 2008). Molecules mediating cell to cell communications (QSMs) regulate morphogenesis, in correlation with the cell density; tyrosol activates yeast filamentous growth at low cell density (Han *et al.*, 2011). Interestingly, hyphae morphogenesis influence non associated hyphae virulence factors such as adhesions and hydrolytic enzymes (Han *et al.*, 2011).

2.3.3 Hydrolytic Enzymes

Of importance is proteinases, secreted aspartyl proteases (SAP) and phospholipases contributing to virulence through digestion of the host cell membrane proteins, lipids and surface molecules for invasion and extracellular proteolytic activity (Silva *et al.,* 2011; Naglik *et al.,* 2003). Secreted aspartyl proteases *(*SAP) family comprises of 10 members (Albrecht *et al.,* 2006). Meanwhile, SAP1 have been reported to be indispensable for virulence through exertion of damage to the reconstituted human epithelium (RHE) (Naglik *et al.,* 2003). PLB (PLB1-5) are part of phospholipases (PL) family of four members, classified based on specific ester bond cleavage during hydrolysis of glycerophospholipids (Silva *et al.,* 2011; Theiss *et al.,* 2006). They contribute to host cell membrane disruption and degradation during invasion (Silva *et al.,* 2011; Theiss *et al.,* 2006). Extracellular enzymes lipases (LI) comprises of 10 members (LIP1-10) **(**Leidich *et al.,* 1998). They exert lipolytic properties by enabling digestion of fatty acids as carbon source (Hube *et al.,* 2000). Also, the enzymes interact synergistically with other enzymes and proteins to promote adhesion, penetration and attack on the immune system (Gacser *et al.,* 2007).

2.3.4 Phenotypic Switching

Phenotypic switching is a reversible high frequency of white opaque transition (Huang, 2012). It is a process of cellular morphological change, antigenic variation and tissue affinity; distinguishable by colony morphology, virulence, metabolism, mating ability and specific gene expression (Huang, 2012; Slutsky *et al.,* 1987). Transition is mediated by mating type like (*MTL)*

locus, which is also responsible for sexual replication process, through homozygous a and α loci complexes (Miller & Johnson, 2002). Homozygous complexes enable switching and vice versa (Zordan *et al.,* 2006; Miller & Johnson, 2002). White cells are more virulent, mediating colonization and immune system scrutiny escape (Porman *et al.,* 2012). Most strains of *C. albicans* strains isolated from patients with vaginitis displayed relatively spontaneous high frequencies of phenotypic switching suggesting a role for switching in virulence (Calderone & Fonzi, 2001). WO-1 phenotype is the best studied switching type, reflected by cell shape, colour, ability to form hyphae and carbon requirement characteristics (Sahni *et al.,* 2009). Switching of cells plays a vital role in establishment of invasive infections (Lan *et al.,* 2002). Opaque cells requires alternative assimilation processes, which may improve it virulence in superficial infections (Huang, 2012), while white cells preference for glucose facilitates and maintains disease development in systemic infections (Lan *et al.,* 2002). Furthermore, a suggested correlation with metabolism reinstates the fact that adaptation to various niches is ensured through variety of mechanisms (Lan *et al.,* 2002; Miller & Johnson, 2002; Kvaal *et al.,* 1999).

2.3.5 Stress Responses

Environmental stress responses are adaptive processes that contribute to *C. albicans* survival in the environment. They do so by conferring protection in opposition to host induced stresses (Enjalbert *et al.,* 2003). Most studied stresses are oxidative, heat shock and osmotic stress and nutrient depletion. Depending on the environmental signals, genes involved in stress responses are differentially expressed to meet the need for successful

infection establishment (Brown *et al.,* 2012). These signals include nitrosative stress, osmotic stress, oxidative stress, low and high temperature (Brown *et al.,* 2012). During phagocytosis, *C. albicans* counteract nitrosative stress caused by RNS and nitric oxide (NO) exerted though the engulfing phagocyte by upregulating Yhb1 flavohemolyglobin protein, that detoxifies NO and RNS. Deletion of *YHB1* gene in *C. albicans* slows evasion. This may be due to its ability to encourage and promote yeast to hyphal growth (Hromatka *et al.,* 2005). During osmotic stress, the cell is signalled through MAPK (mitogen activated protein kinase pathway) activated by Hog1 protein kinase, to develop counter reactions (Monge *et al.,* 2006; Smith *et al.,* 2004). MAPK pathway is also involved in other cellular processes including morphogenesis, cell wall formation, respiratory mechanisms and stress responses (Smith *et al.,* 2004). Osmotic stress is opposed by increased expression of glycerol biosynthesis enzymes GPD2 (glycerol-3-phosphate dehydrogenase), and GPP1 (glycerol-3-phosphatase) for glycerol accumulation. A response regulated by Hog1 transcription factor (Mayer *et al.,* 2013; Enjabert *et al.,* 2003).

Oxidative stress response is activated when cells are exposed to abnormal intensity of yeast generated ROS through usual metabolic processes or environment oxidants such as hydrogen peroxide (H_2O_2), potassium superoxide (KO_2) and hydroxyl radicals (OH^-) and neutrophils hypocholorous acid. Thus, initiating DNA, proteins, and lipids degradation that leads to cell lysis (Tosello *et al.,* 2007). *C. albicans* uses the activity of antioxidant detoxifying enzymes particularly, superoxide dimutases (SODS), glutaredoxins (GRXS), thioredoxim (TRX) and catalases (CAT), regulated by

Cap1 transcription factor *(*Tosello *et al.*, 2007; Enjalbert *et al.*, 2006; Alonso-Monge *et al.*, 2003; Estruch, 2000).

Heat shock stress (HSS) results from contact with environmental stressors such as toxins, nutrient starvation, hypoxia, nitrogen deficiency and high temperature (Burnie *et al.*, 2006). These stressors triggers heat shock proteins (Hsps) and small heat shock proteins (sHsps), a process regulated by evolutionary conserved heat shock transcription factor (Hsf1) through binding of specific sequences in their promoters (heat shock elements, HSE). They prevent protein aggregation, denaturation and unfolding (Sorger & Pelham, 1987). In addition, elevated level of Hsps may help morphological shift to hyphae development, a phenomenon common with stress in *C. albicans*. Eleven essential Hsps are reported to be associated with HSS response in *C. albicans* (Nicholls *et al.*, 2009). They have been illustrated to affect antifungal drug resistance particularly Hsp70 and Hsp90. Hsp21 is important to maintain trehalose intracellular levels (Nicholls *et al.*, 2009). Furthermore, Hsp90, an invasin protein that plays a role during HSS has been shown to induce biofilm formation and resistance (Robbins *et al.*, 2011).

2.3.6 Biofilm Formation

Another major virulence factor that also relates to the background of this study is biofilm formation. *Candida* species possess the ability to attach to host tissues and medical device surfaces, attracting and replicating more cells, building up extracellular matrix (ECM) that protects the community from immune attack and antifungal agents (Chandra *et al.*, 2008). It thereafter

disperses to invade the blood stream and cause infection (Chandra *et al.,* 2008; Pierce *et al.,* 2008; Hawser & Douglas, 1994).

2.4 Metabolism

2.4.1 Cell Carbon Metabolism

C. albicans is a facultative anaerobe capable of carbon assimilation through cellular respiration and fermentation, contrasting its homologue *S. cerevisiae* that is solely dependent on fermentation (Lorenz *et al.,* 2004). Maintenance of cellular reactions involves biochemical reactions that permit the breakdown of molecules for cell growth (Karin & Ben, 2010). The uptake of 6 carbon compounds e.g. Glucose is through glycolytic pathway that takes place in the cytoplasm converting glucose molecules into pyruvate and energy in form of two adenosine triphosphate (ATP) and two nicotinamide adenine dinuclecotide (NADH) as shown in figure 2.1 (Askew *et al.,* 2009). In the absence of fermentative carbon sources, *C. albicans* use non-fermentative carbon sources through three paramount pathways namely glyoxylate, fatty acid β-oxidation and gluconeogenesis that favours virulence (Lorenz & Fink, 2001).

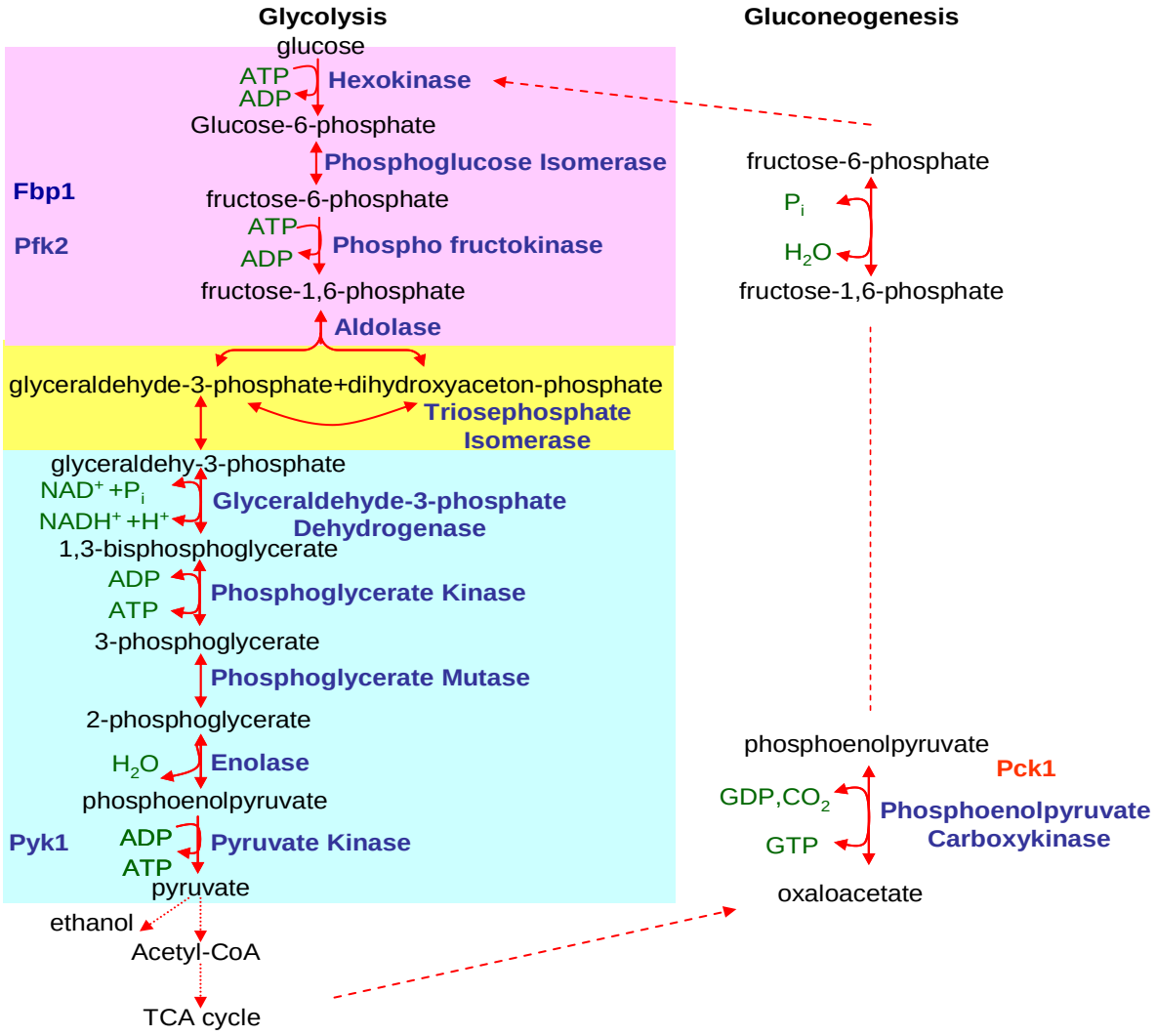

Figure 2.1. Glycolysis and gluconeogenesis pathway. Glycolytic and gluconeogenesis cycles make energy available for life sustenance in microorganisms and higher living forms. ATP, NADH and pyruvate are the end product of carbohydrates, derived through catabolic glycolytic pathway. Glycolysis pathway is divided into three steps indicated by pink, yellow (irreversible reaction) and green areas. Gluconeogenesis is the reversible reaction of glycolysis. It synthesises glucose from non-carbohydrate sources such as glycerol, lactate oxaloacetate. Glucose-6-phosphatase converts fructose-6-phosphate to glucose. Enzymes catalysing each step are written in blue and red, and the synthesized products marked in black. Adapted from Berg *et al.*, (2002).

Metabolic mechanism implored by *C. albicans* is dependent on the available nutrient in the environment (Barelle *et al.*, 2006; Fradin *et al.*, 2005; Garcia- Sanchez *et al.*, 2005). Pathogens are metabolically dynamic, mediating their survival in diverse ecological niches, by imploring diauxic shift (Rodaki *et al.*, 2009). Studies showed that 16 metabolic pathways involved in carbon catabolite metabolism (CCM) are significantly up-regulated during morphological change to filamentous growth, stimulated by nutritional stress in order to maintain cellular functions (Han *et al.*, 2011). When engulfed by macrophages, they make rapid shift from preferred to available non-preferred carbon sources (Prigneau *et al.*, 2003). They assimilate available carbon sources through anabolic pathways (Prigneau *et al.*, 2003). Studies have also shown that during early phase of infection and phagocytosis, glycolytic genes are down regulated and alternative pathways: glyoxylate, β-oxidation; amino acid biosynthesis genes are over expressed (Rubin- Bejerano *et al.*, 2003; Lorenz & Fink, 2001). This was confirmed by northern blotting showing the repression of M1g1 regulator, a glycolytic transcriptional regulator, hyphal and alternative pathway repressor (Gurvitz *et al.*, 2006).

2.4.2 Gluconeogenesis

Gluconeogenesis pathway is an intermediate cycle for generating hexoses from non carbohydrate molecules for glycolysis (Barelle *et al.*, 2006; Lorenz *et al*., 2004). Deletion of *PCK1* gene confers a significant virulence decrease in mouse model of systemic infection (Fradin *et al.*, 2005). Phosphoenolpyruvate is condensed by phosphoenolpyruvate carboxylkinase enzyme (PCK); the reaction continues until fructose-1, 6-biphosphate is converted to glucose.

2.4.3 Beta Oxidation Pathway

This pathway mediates the synthesis of acetyl CoA from fatty acids, a reaction localized to the mitochondria and peroxisome (Perkarska *et al.*, 2006). Fatty acids are transported by carnitine acetyltransferase enzymes (Hiltunen *et al.*, 2003). It is an adaptation pathway essential for survival in hostile environments (Lorenz *et al*., 2004). Upon macrophage engulfment, metabolism is switched to permit lipid utilization, signalled by expression of FOX2 enzyme (Lorenz *et al*., 2004). Recently, it was reported not to be fully significant for virulence as shown in a *FOX2* mutant strain, which exhibited virulence capability (Piekarska *et al.*, 2006; Lorenz *et al*., 2004). However, it may have a complimentary role in the virulence of *C. albicans* (Piekarska *et al.*, 2006; Lorenz *et al*., 2004). Energy production is facilitated through metabolism of fatty compounds such as oleate to acetyl-CoA (C_2), a precursor for the glyoxylate pathway for haemostasis maintenance and pathogenicity (Prigneau *et al.*, 2003). Four catalytic enzymes involved are acyl CoA dehydrogenase, enoyl- CoA hydratase, 3-hydroxyl acyl CoA dehydrogenase and beta- ketoacyl-CoA thiolase (Prigneau *et al.*, 2003). A process repeated until 2 carbon compound e.g. Acetyl- CoA is generated for glyoxylate and TCA cycle as shown in figure 2.2 (Strijbis & Distel, 2010).

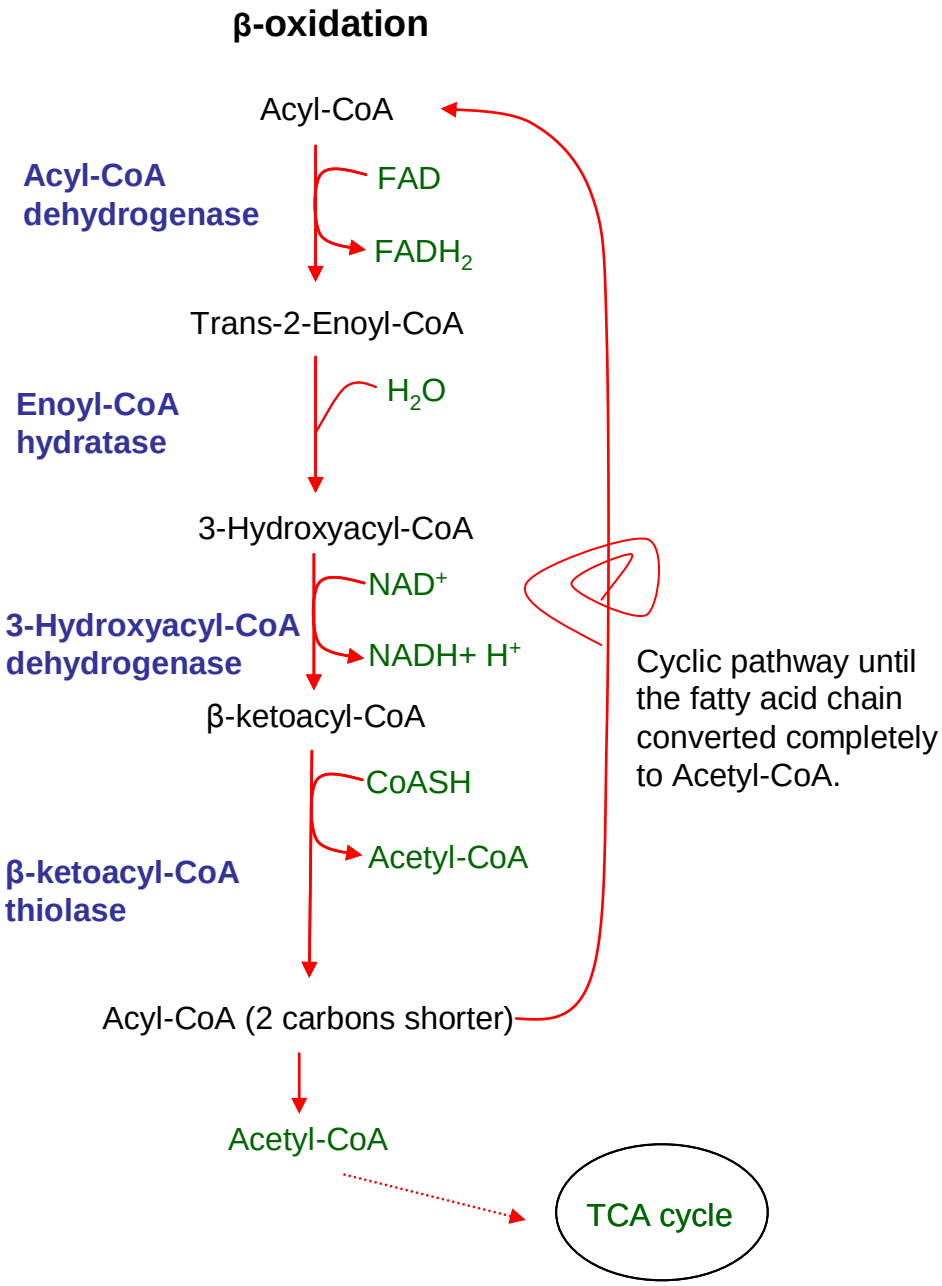

Figure 2.2. Fatty acid βeta-oxidation. The cycle comprises of four distinct steps namely oxidation, hydration, oxidation and cleavage. The reacting enzymes are marked in blue. Adapted from Nelson and Cox, 2005.

2.4.4 Tricarboxylic acid (TCA) and Glyoxylate Pathway

Tricarboxylic acid (TCA) pathway also known as citric acid cycle utilizes acetyl CoA synthesized from pyruvate and fatty acids, and oxaloacetate as a precursor substrate for citric acid synthesis, catalysed by citrate synthase enzyme (Soares- Silva *et al.,* 2004). Glyoxylate cycle is a shunt of TCA. It utilizes non carbohydrate sources during phagocytosis and environmental stress, which provides the potential to generate glucose from two-carbon molecules, imperative for full virulence in *C. albicans* (Lorenz *et al.*, 2004; Lorenz & Fink, 2001). This pathway as shown in figure 2.3 is mediated by two major enzymes, namely isocitrate lyase (ICL) that catalyses isocitrate intermediate to glyoxylate, and malate synthase (MLS) which cleaves glyoxylate to malate, and subsequently converted to phosphoenolpyruvate (C_4) (Barelle *et al.*, 2004; Lorenz & Fink, 2001). Glyoxylate cycle is predicted to be vital pointer for nutrient deprivation. Earlier reports showed that deletion of *ICL1* and *MSL1* genes following macrophage engulfment increases it susceptibility to immune defences (Fradin *et al.*, 2005; Lorenz & Fink, 2001).

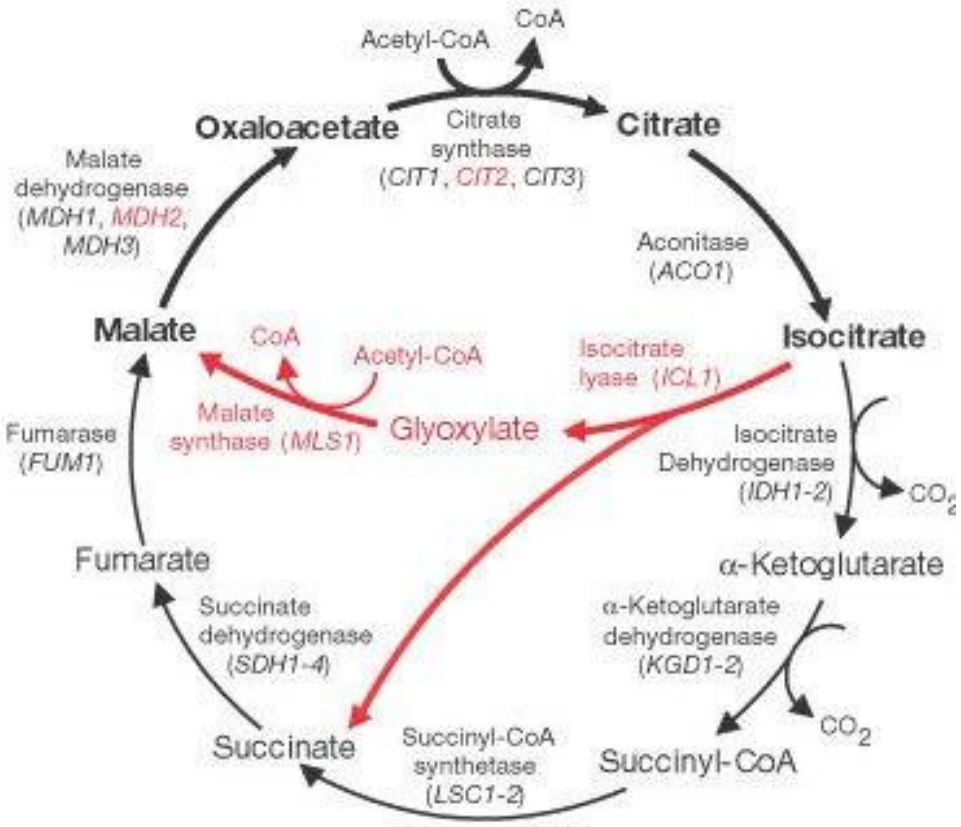

Figure 2.3. **Depiction of enzymatic steps in the TCA and glyoxylate cycle**. Glyoxylate cycle is a shunt of TCA; shorter and less energy consuming by bypassing decarboxylation step. The oxaloacetate (C_4) synthesized enters into the gluconeogenesis pathway. Specific steps and enzymes of glyoxylate cycle are shown in red. Adapted from (Lorenz & Fink, 2001).

Evidently, glucose availability represses transcriptional expression of non-preferred pathways encoding genes (Piekarska *et al.,* 2008). Doblin et al. 2012 study using quantitative reverse transcriptase real polymerase chain reaction (qRT-PCR), demonstrated that the pathogenic yeast can take in relevant concentrations of two carbon compounds such as oleate and glucose simultaneously. This property was credited to the lack of post translational regulation mechanism and ubiquitination. Apart from glyoxylate, gluconeogenic and β-oxidation pathways, little is known about other pathways that may be solely or synergistically involved. Another important thing is that despite the crucial role of alternative pathways for survival, glycolytic genes activation is necessary for disease progression. That is non preferred sources might be required for adaptation during immunity defence, but not for disease advancement, thereby affirming their collective role (Piekarska *et al.,* 2008).

2.5 Microbial Biofilms

Biofilms was first described in the 17th century by Van Leeuwenhoek while examining a plague on a tooth surface. They create distinct growth from planktonic cell growth characterized by interwoven assemblage of cells (Douglas *et al.,* 2003; Donlan & Costerton, 2002). Formation of biofilm can be described as a defence mechanism, and a collective effort to avoid and combat the immune cells. Living cells of different species of microorganisms form complex clumps, living in close proximity on biotic and abiotic hydrated surfaces, such as bypass grafts, ocular lenses etc., and disseminating to cause septicaemia (Douglas *et al.,* 2008). These active cells changes from free floaters to stickers on surfaces through rapid change of adaptive phenotypes. They are often times irreversibly attached through the use of pili. In most clinical scenarios, biofilm causing diseases are predominately bacteria

and polymicrobial communities (Douglas, 2003). Recognition of specific and non specific attachment sites, nutritional cues, and exposure to antimicrobial stimulates organism's response in the form of biofilm development (Finkel & Mitchell, 2011; Ballie & Douglas, 2000). High rate of urinary catheter and central line blood stream infections have close association with indwelling devices, including pacemakers. Urinary catheters now frequent with more than 10 million recipients each year, which encourages microbial biofilm growth (Taff *et al.*, 2012).

2.6 *C. albicans* Biofilm

Fungal biofilms associated infections are becoming more prominent especially in immunosuppressed patients (Ghannoun & Rice, 1999). Unlike bacterial biofilm that has been intensively studied with considerable amount of information on their properties. However in recent years, more studies have focused on fungal biofilms to give insight about the complex developmental process as it have been implicated in substantial proportion of human infections (Nett *et al.*, 2007; Ballie & Douglas, 2000). The most studied yeast pathogen is *C. albicans.* The etiologic cause of invasive candidiasis when defences are repressed (Lionakis & Netea, 2013). *C. albicans* is the most common yeast pathogen associated with implanted medical device implants such as central catheters, intracardiac prosthetic valves (Taff *et al.*, 2012). It causes life threatening conditions such as urinary tract infection, and endocarditis, with mortality rate as high as 30% (Finkel & Mitchell, 2011; Nett *et al.*, 2010). *C. albicans* biofilm studies are expected to generate data that will serve as a reference foundational background for other fungi. Several experimental and discovery studies have generated lots of information and working definitions for biofilm (Ramage *et al.*, 2005; Hawser & Douglas, 1994).