1.0 INTRODUCTION

1.1 Prevalence of fungal infection in oral cavity

Candida sp. inhabits various parts of the human system including the epidermis, vagina, gastro-intestinal tract, nails and oral cavity (Williams *et al.*, 2011). The disease caused by *Candida* sp. has become a common disease in the late 19th and 20th century and its prevalence is still increasing worldwide as a result of multiple factors which can facilitate the conversion of its commensal level to the parasitic level (Samaranayake *et al.*, 2009). According to Scardina *et al.* (2007), the risk factors that enhance the severity of a candidal infection can be found widely in patient with impaired salivary gland, drug abusers, high carbohydrate diet, smoking habits and Cushing's syndrome. Candidal infection can occur in almost all human organs. However, it is the systemic infection that can be much more severe and may lead to mortality. According to Leroy *et al.* (2009), the mortality rate due to systemic infection of *Candida* sp. was up to 60% and still increasing. The treatment of candidal infection can be difficult and most of the diagnosis can only be achieved by autopsy. With the current incidence in Europe, there has been increasing reports in candidemia of 5-folds within 10 years (Bassetti *et al.*, 2009). In a most recent study, candidal infection was also associated in oral cancer, burning mouth syndrome, endodontic disease and taste disorder (Williams *et al.*, 2011).

Candida albicans is the main causative agent of oropharyngeal candidiasis. Researchers have however found that non-albicans species also contribute significantly to the development of oral candidosis (Magaldi *et al.* 2001). Cases due to non-albicans species are increasing in number and this has raised great concern to the society.

In Malaysia, based on a survey in 1999, from a total of 1114 yeasts isolates from the University of Malaya Medical Center (UMMC), 1.2% was identified as *Candida krusei* (Ng *et al.*, 1999). A case-control study among 100 healthy subjects in the UMMC by Rasool *et al.* (2005) had shown a predilection for the Chinese infected by *Candida* sp. while the Indian group was found to be the least infected. This is an indication that the dispersion of *Candida* sp. may vary between ethnic groups. This is a strong indication that the habit and background of each different ethnicity might have influence on the dispersion. Candidal infection is still of a major concern although a survey carried out in the Hospital of Kuala Lumpur had shown a decreased in candidal isolation in the last four years (HKL, 2011). Despite this claim however, total isolates of *Candida albicans* was found to increase every year.

1.2 *Candida krusei* in oral infection

Candida krusei has been identified as the fifth medically dominant pathogen after *Candida albicans, Candida glabrata, Candia kefyr* and *Candida parapsilosis* (Samaranayake and Samaranayake, 1994). Together with *Candida glabrata, Candida krusei* were recognized as a common pathogen isolated from the blood stream worldwide (up to 25%) (Quindós *et al.*, 2008). *Candida krusei* was also classified as an important pathogen involved in the escalating serious infections involving immunocompromised patients (Samaranayake, 1997; Singh *et al.*, 2002; Hakki *et al.*, 2006; Pfaller *et al.*, 2008; Quindós *et al.*, 2008). *Candida krusei* was associated with the increased of nosocomial infection within the last two decades and has remained the causative agent of morbidity and mortality in immunocompromised patients (Muñoz *et al.*, 2005).

In the oral cavity, *Candida krusei* is known as one of the pathogenic yeasts associated with oral candidosis. It persists in the form of patchy to confluent, whitish pseudomembranous lesion. The site of infection is often composed of epithelial cells, yeasts and pseudohyphae. Oral candidosis associated with *Candida krusei* has been reported to increase widely in adolescent and children infected with oral cancer. Compared to *Candida glabrata* and *Candida tropicalis* the occurrence of *Candida krusei* in oral cancer patients was found to be higher (Gravina *et al.*, 2007).

Candida krusei is an opportunist with multi-drug resistance and this include towards fluconazole, a drug often prescribed for the treatment of candidal infection (Samaranayake, 1997; Furlaneto-Maia *et al.*, 2008). *Candida krusei* is however still susceptible towards flucytosine voriconazole, echinocandins (caspofungin, anidulafungin and micafungin) and amphotericin B at least until 2004. Later studies then showed a reducing susceptibility incidence towards *Candida krusei* in oral candidosis as a consequence of heterozygous mutation which had altered the sensitivity of the yeast (Pfaller *et al.*, 2004; Pfaller *et al.*, 2008). In addition, treatment with amphotericin B was found to be delayed in *Candida krusei* compared to *Candida albicans* infections. A higher concentration of amphotericin B was also noted at $\geq$ 1 mg/kg of body weight per day in order to eliminate the development of *Candida krusei.* Several reports have also discovered that there is decreasing susceptibility of *Candida krusei* towards caspofungin, anidulafungin and micafungin (Hakki *et al,* 2006).

Data from a surveillance program in 2001 to 2005 in the Asia-Pacific region has reported that, 1.3% of 137,487 isolates of *Candida* sp. from the Asia-Pacific region was found to be *Candida krusei* (Pfaller *et al.*, 2008). Malaysia had the highest number of candidal isolates out of 8 other countries which include Australia, China, India,

Indonesia, South Korea, Taiwan and Thailand. Malaysia was found to have as high as 39.54% candidal isolates and out of this candidal population, 1.3% isolates were *Candida krusei*. A decreasing susceptibility of *Candida krusei* towards voriconazole was also reported compared to *Candida albicans.* Based on a worldwide observation, strains from the American Latin regions had showed the lowest susceptibility towards voriconazole, which contradicts an analysis carried out in the South-East Asean countries that about 75% of *Candida krusei* is still susceptible towards the anti fungal agents (Pfaller *et al.*, 2008).

1.3 Rationale of study

Candida krusei was classified in several studies as an emerging pathogen, especially in immunocompromised patients (Merz *et al.*, 1986; Samaranayake, 1997; Abbas *et al.*, 2000). In neutropenic patients, report has shown that an infection by *Candida krusei* can lead to high mortality especially in leukemia patients who are receiving fluconazole as prophylaxis (Abbas *et al.*, 2000). Many therapies using fluconazole and also caspofungin were failed in reducing the infection (Fleck *et al.*, 2007). This phenomenon has increased the awareness of researchers to focus on the virulence factors of *Candida krusei* in the attempt to reduce the disease caused by the pathogen. One of the most important virulent factors of *Candida* sp. is the ability to switch its phenotypic in order to survive in an unfavourable growth environment (Haynes, 2001). Thus, it is important to validate the ability of *Candida krusei* in phenotypic switching and adherence. The understanding on this two characteristics shed information on the pathogenicity of *Candida krusei* in causing candidal infection. *Candida* sp. is also able to adhere firmly to hard surfaces of dentures. Candidal adherence can occur either on the soft tissue such as the buccal and palatal surfaces or on the hard tissue surface of dentures (Haynes, 2001). The ability to attach to hard

surfaces is actually the key factor in initiating candidal infections. In addition, this research is also targeted at assessing the antifungal activities of commercialized antifungal agents and two plant extracts against *Candida krusei*. *Nigella sativa* and *Piper betle* are two plants frequently used in traditional practices to cure various types of illnesses. A positive antifungal property would enable these plants to be further tested for use as antifungal agent.

1.4 Hypothesis of study

Phenotypic switching affects the biological properties of *Candida krusei.*

1.5 Aims of study

The aims of study are:

1) To determine the phenotypic switching ability of *Candida krusei.*

2) To determine the effect of phenotypic switching on the biological properties of *Candida krusei*.

3) To evaluate the consequences of phenotypic switching on the susceptibility towards commercialized antifungal agents and plant extract (*Nigella sativa* and *Piper betle*).

2.0 LITERATURE REVIEW

2.1 Oral *Candida*

2.1.1 *Candida* as a component of oral ecosystem

Candida sp. was identified as a common member of the oral microflora and was estimated at 40% to 60% of the microbial population in the oral cavity. It can be present either as transient or permanent colonizer in the oral cavity (Mitchell, 2007; Thein *et al.*, 2007). It is also recognised as an opportunistic microorganism that is able of causing oral diseases such as oral candidosis (Marsh and Martin, 2009).

2.1.2 Colonization sites

Candida sp. is identified to colonise several host cell types including epithelial, endothelial and phagocytic cells. In the oral cavity, *Candida* sp. prefers to colonise several surfaces including the buccal and labial mucosa, dorsum or lateral borders of tongue, hard and soft palate regions, tooth surfaces and denture-bearing areas (Cannon *et al.*, 1995; Siar *et al.*, 2003). This colonising ability is contributed by factors including the ability of the oral candidal species to produce specific enzymes such as agglutinin-like proteins and integrin-like protein that lead to the formation of biofilm on oral surfaces. In addition, other factors which influence the colonisation of *Candida* sp. are the reduction of salivary flow, low salivary pH, trauma, carbohydrate-rich diets and epithelial loss (Siar *et al.*, 2003; Marsh, 2006).

2.1.3 Growth requirement and susceptibility

2.1.3.1 Influence of oral fluids

Saliva provides moisture and helps in lubricating the oral cavity. It also promotes the formation of thin film approximately 0.1 mm deep over all external surfaces in the oral cavity. Saliva is produced by the major and minor salivary glands. The major salivary glands consist of paired parotid, submandibular and sublingual glands; whereas, the minor salivary glands are found in the lower lip, tongue, palate, cheeks and pharynx. The chemical composition of secretions from each gland is different; however, the major role of the whole saliva is to maintain the integrity of teeth by clearing off food debris and buffering the potential damaging acids produced by the oral biofilm or dental plaque. Bicarbonate, phosphates and peptides are examples of buffering agent in the saliva which gives normal saliva a mean pH of 6.75 to 7.25 (Marsh and Martin, 2009).

The flow rate of saliva is under the influence of circadian rhythms where the lowest flow often recorded during sleeping. Low flow rate of saliva reduces the protective function of saliva and increases the colonisation and development of microorganisms including *Candida* sp. Salivary composition is also affected by circadian rhythms for example the total concentration of protein in whole saliva during resting time is estimated at 220 mg/100 mL, whereas the total protein in stimulated saliva is estimated at 280 mg/ 10 mL. This different amount of protein may affect the distribution of the normal microflora in the oral cavity as some proteins are known to serve as receptors in the colonisation of microorganisms to the saliva-coated surfaces of the teeth (Marsh and Martin, 2009). Protein and glycoprotein such as mucin in the saliva act as the primary source of nutrients for resident microflora including the

candidal species. In addition to adherence, some proteins are also involved in host's defence mechanism by aggregating exogenous microorganisms, hence, facilitating their clearance from the mouth during swallowing or spitting.

In addition to saliva, the gingival crevicular fluid (GCF) in the oral cavity can also influence the colonisation of oral *Candida* sp. The flow of GCF is slow at healthy sites but increased drastically at areas with gingivitis by 147% and up to 30-fold at areas with advanced periodontal diseases. GCF also has a role in the development of subgingival plaque around and below the gingival margin. Among the host defence components in GCF are includes IgG and neutrophils. GCF also contains higher total protein compared to saliva. Thus, GCF is capable of providing nutrient sources to several commensal microorganisms in the oral cavity (Marsh and Martin, 2009).

2.1.3.2 Influence of nutrients

Candida sp. is a chemoheterotrophic organism that requires carbon and nitrogen for growth. According to Madigan and Martinko (2006), the mutual interaction of carbon and nitrogen is important in the metabolism of microorganisms. Carbohydrates are the most readily utilised form of carbon in both oxidative and non-oxidative way. Thus, the presence of carbohydrates influenced the colonisation of *Candida* sp. in the oral cavity. Certain carbohydrates such as sucrose and glucose have been shown to increase the adhesion potential of *Candida albicans* towards hard and soft surfaces of oral cavity. Glucose is an acid promoters which will lead to the reduction of pH in oral environment with consequence of activation of acid proteinases and phospholipases which then enhances the adherence of *Candida* sp. In addition, the production of mannoprotein surface layer in the glucose presenting environment has been shown to

assist the adherence of *Candida* sp. including *Candida krusei* in the oral cavity (Marsh and Martin, 2009).

Candida sp. has nitrogen content of around 10% of their dry weight (Wai, 2009). The source of nitrogen is usually provided by organic compounds which can be easily found in the oral environment. Nitrogen is also determined as the main stimulatory factor in yeast extract as it encourages bio-stimulation on microbial growth.

2.1.3.3 Influence of body temperature

The optimum growth temperature for candidal species including *Candida krusei* has been shown to range between 30 ºC to 37 ºC (Singh *et al.*, 2002). This range of temperature is considered as the optimum temperature of various pathogenic microorganisms in the oral cavity. Any alteration in the normal body temperature may however influence the competitiveness among the normal microflora which will then enhances the development of opportunistic microorganisms such as *Candida* sp. Nonetheless, many experimental assays were conducted at 37 ºC and this is generally accepted as the standard incubation temperature for candidal species (Marsh and Martin, 2009).

2.1.4 Pathogenic determinants of *Candida*

The virulent factors of each different candidal species are not similar and can be a comparative factor between each different species (Haynes, 2001). Among the important virulent factors of *Candida* sp. include phenotypic switching, adherence to host cells, cell surface hydrophobicity and enzymes production.

2.1.4.1 Phenotypic switching

Phenotypic switching is an important technique of survivorship of *Candida* sp. within a growth environment including the oral cavity. Switching ability promotes *Candida* sp. to adapt in suppressed environment and develop as the dominant host microniches. *Candida* sp. can undergo reversibly high frequency of phenotypic switching which increases and ensures the survivability of the pathogen (Haynes, 2001). The details of this virulence factor will be further discussed in section 2.3.

2.1.4.2 Adherence ability

The adherence ability of *Candida krusei* is an important factor in the initiation of oral candidosis. Adherence can occur either on the hard tissue surfaces such as the teeth and palatal surface or smooth surfaces such as the buccal and lingual surfaces (Samaranayake *et al.*, 1994). Several characteristics of candidal species which contribute to the adherence on these surfaces include the formation of pseudohyphae and extracellular matrix.

A single filament hypha (plural, hyphae) is a long branching filamentous structure of fungus which can be found easily in the developmental phase of *Candida* sp. (Madigan and Martinko, 2006). It is classified as the main mode of vegetative fungal growth and consists of one or more cells which are surrounded by tubular cell walls made of chitin. Hyphae usually grow together to form a compact tufts which are known as mycelium. Hyphae formation is usually referred to the germination of fungi. However, it is also involves in the colonisation of the target host. Pseudohyphae are distinguished from the true hyphae by their method of growth which lacks cytoplasmic connection between the cells. The pseudohyphae of *Candida* sp. are usually found to possess incomplete budding blastoconidia whereby cells remain attached to the mother cells after division. *Candida albicans* and *Candida krusei* has been recognised to develop pseudohyphae which adhere to the monolayer of human epithelial cells (Soll, 1992; Dede and Okungbowa, 2009).

In many cases, extracellular matrix is also produced by oral microorganisms. Extracellular matrix is a network of non-living mass which provides support to cells including the *Candida* sp. The presence of extracellular matrix provides support to cells attachment. This anchorage property assists the colonisation of candidal species to hard tissue surfaces and thus, contributes to the formation of biofilm. When in a biofilm, the resistance of candidal species towards various antifungal agents including amphotericin B might be increased (Hawser *et al.*, 1998).

2.1.4.2.1 Dental biofilms

Dental biofilm is defined as a thin layer comprising of various communities of microorganisms including bacteria, fungus and yeast that are attached on tooth surfaces and on the surface of prosthesis including dental acrylic surfaces and human epithelial cells (Holmes *et al.*, 2002; McCarron *et al.*, 2004). Microorganisms in the biofilm are enclosed in a matrix of extracellular polymeric substance (EPS) (Branchini *et al.*, 1994; Samaranayake *et al.*, 2002). This biofilm provides protection to the microorganisms and facilitates the interaction among themselves with the contribution of biochemical substances such as catalase and superoxidase dismutase (Socransky and Haffajee, 2002; Marsh, 2004; Marsh, 2006). The development of biofilm is dependent on the dietary, salivary and oral environmental factors that interact with the microorganisms within the community of biofilm.

The formation of biofilm has been shown to reduce the susceptibility of microorganism to antimicrobial agents which may then lead to the increase in pathogenicity (Marsh, 2006). This phenomenon is suggested to occur due to the restriction of the antimicrobial agents to penetrate the matrix of the biofilm which then reduces the susceptibility of the target microorganism (Gilbert *et al.*, 2002). In some cases, the resistance of a pathogen in a biofilm can increase to 1000-fold towards an antibiotic (Stewart and Corteston, 2001).

2.1.4.2.2 Development of dental biofilms

The development of dental biofilm involves several stages which are the acquired pellicle formation on the teeth surface; adhesion, reversible and irreversible interactions between the pellicle and the colonising microbes; co-aggregation between microorganisms; and detachment of microbes from the oral surfaces. These sequences of events may eventually form a structural and functional organised microbial community that if allowed to accumulate, may enhance the potential of periodontal disease and dental caries (Marsh, 2004; Wan Nordini Hasnor, 2007).

The acquired pellicle formation is the formation of a thin, acellular layer which works as the receptor of the attachment of the early plaque colonies such as *Streptococcus mitis, Streptococcus oralis* and *Streptococcus sanguinis.* There are two phases involved in the formation of the acquired pellicle which are the adsorption of discrete protein of low molecular weight to the enamel surfaces followed by the adsorption of protein aggregates of high molecular weight (Hannig, 1999).

The adhesion, both the reversible and irreversible adsorption properties of the microorganism is a pioneer stage in the development of dental biofilm. Research has shown that there is a reversible interaction which involves long-range physico-chemical forces between the microbial and acquired pellicle on the oral surfaces (Marsh and Martin, 1999). The net negative charge of the bacterial cell wall will interact with the negative charged glycoprotein on the pellicle through a divalent cation bridge while, the lipophilic adhesin of the microbial cell wall will recognise the hydrophobic receptors on the ephitelial cell (Schonfeld, 1992). The van der Walls and electrostatic repulsion forces which produce a weak area of net attraction facilitates reversible attraction

between the microorganisms and the oral surface area. On the other hand, irreversible interactions involve short range interactions with specific physico-chemical forces between adhesins and the receptors on the surface area of the microbial cell surface and the acquired pellicle, respectively. *Streptococcus oralis* and *Streptococcus gordonii* are two examples of microorganisms involved in irreversible interaction that bind to mucoglycoprotein of the acquired pellicle (Murray, 1992).

Subsequent to the colonisation of the early plaque bacteria to the acquired pellicle, co-aggregation or co-adhesion of other microorganisms will takes place. This is a process of microbial adhesion involving the late colonisers on the early colonizer of dental biofilm. It is a phenomenon of cell-to-cell recognition of genetically distinct partner cell types (Marsh and Martin, 1999). The co-aggregation can be facilitated either through intrageneric such as the interaction between streptococci and among *Actimomyces* (*Streptococcus sanguis* and *Actinomyces* sp.) or intergeneric such as the interaction between *Streptococci* and *Actinomyces* (*Streptococcus* sp. or *Actinomyces* and *Prevotella* sp.). *Candida krusei* has been found to be involved in co-aggreagation with *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius* in the present of sucrose (Kiyora *et al.*, 2000). Protein such as lectins is usually involves in co-aggregation. This carbohydrate-binding protein will attach to the carbohydrate-binding protein receptors of other cells which then contribute to the increased thickness of the dental biofilm.

Once a climax community is achieved in the biofilm, detachment of some microbes may occur in the final process oral biofilm development. The microorganism is released from the matrix of biofilm to the fluid surrounding the biofilm a process

which have been reported to be facilitated by several enzymes such as protease (Hunt *et al.*, 2004), fluid shear stress (Stoodley *et al.*, 2001), multivalent cross-linking cation (Caccavo *et al.*, 1996) and microbial growth status (Jackson *et al.*, 2002). This process of detachment will however help the microorganism to colonise other surfaces in the oral cavity. An example of microorganism involved in the detachment process from the oral biofilm is *Prevotella loescheii* which produces protease that hydrolyse its fimbrae-associated adhesion which is important in its co-aggregation with *Streptococcus oralis* (Cavedon and London, 1993; Marsh and Martin, 1999).

2.1.4.3 Cell surface hydrophobicity

The virulence factor of *Candida krusei* can also be observed from the cell surface hydrophobicity characteristic. This factor is classified as one of the most important adherence mechanisms in the colonisation of the host surface. *Candida krusei* is more hydrophobic compared to other medically important *Candida* sp. (Samaranayake *et al.*, 1993). *Candida krusei* was reported to possess the same hydrophobicity level with *Candida glabrata* and *Candida tropicalis* but is more hydrophobic compared to *Candida albicans* and *Candida parapsilosis.*

2.1.4.4 Enzyme

Hydrolytic enzymes of *Candida* sp. have been reported to contribute to the pathogenicity in causing oral diseases such as oral candidosis. The enzymes include aspartyl proteinase, phospholipases, lipases, phosphomonoesterase and hexosaminidase (Williams *et al.*, 2011). Among these enzymes, aspartyl proteinase has attracted most interest and is widely considered to be central in the development of candidal infection.

Aspartyl proteinase is a hydrolytic enzyme which is secreted by the transcription and translation of sphingolipid activator protein (SAP) gene. This enzyme has the ability to attack host and also contributes as a defence system of yeast. Examples of candidal species possessing this enzyme are *Candida albicans* and *Candida krusei* (Samaranayake, 1994).

Another important hydrolytic enzyme is phospholipase which is identified as an enzyme that attacks the host tissue. This enzyme activity has been observed in many fungal pathogens including *Candida* sp. There are 4 types of phospholipases which are type A, B, C and D. Phospholipase A and C can be found in *Candida albicans*; however, there is no evidence that shows the activity of phospholipase B and D in candidal species (Samaranayake, 1994). Phospholipase A can attack cell membranes and can be easily found on the cell surface especially at the sites of bud formation. Hence, the enzyme activity can be enhanced when the hyphae are in direct contact with the host tissue (Williams *et al.*, 2011).

2.2 *Candida krusei*

Candida krusei is classified as a facultative saprophytic fungus that is infrequently isolated from the human mucosal surface area (Do Carmo-Sousa, 1969; Odds, 1988; Thein *et al.*, 2007). This pathogenic yeast has been detected as an oral commensal and represented between 10% to 15% of yeasts isolated from the oral cavity of human. Since the 1960s, *Candida krusei* has emerged as a pathogen associated with the development of oral candidosis (Samaranayake, 1994).

2.2.1 Taxonomic status

Candida sp. is classified under the family of *Cryptococcaceae* as imperfect fungi. The family of *Cryptococcaceae* includes the genera *Torupsilosis* and *Cryptococcus.* The strain is recognised as the causative agent of thrush which infects the mucosal layer including tongue, lips, gums or palate. The association of this numerous generic and variable species of microorganisms and the lesion formed in the oral cavity is called "thrush fungus" (Odds, 1988).

Candida krusei has been discovered since 1839 by Langenbeck and was firstly isolated from the buccal epithelial layer in a typhus patient. However, it was unclassified as pathogenic to human until 75 years later when Castellani found that the strain was actually a commensal in warm-blooded animals (Castellani, 1912; Samaranayake and Samaranayake, 1994). In general, the yeast morphology of *Candida krusei* comprises of asexual and sexual species. The sexual form was renamed as *Issatchenkia orientalis* whereas the asexual form had remained as *Candida krusei* (Odds and Merson-Davies, 1989).

2.2.2 Biology of *Candida krusei*

Candida krusei is classified as yeast that possesses elongated cell shape with "long grain rice" appearance. The appearance of this cell is shared with *Candida kefyr* or also known as *Candida pseudotropicalis* (Samaranayake, 1997). The measurement of *Candida krusei* is approximately 2.2 to 5.6 x 4.3 to 15.2 µm. It forms spreading colony with matt or rough whitish yellow surface on SDA which in a way enable us to identify it directly from morphological observation (Samaranayake and Mac Farlane,

1990). The ultra structure of *Candida krusei* comprised of a six-layered cell wall with a few intra-cytoplasmic organelles including small vesicles, lipid droplets, ribosomes and glycogen-like particles (Joshi *et al.*, 1975). The multilayered cell wall of *Candida krusei* comprise of an outer irregular coat of flocculent material, an electron dense zone, a granular layer, less granular layer, a thin layer of dense granules and another sparsely granular layer outside the trilaminar cell membrane (Samaranayake and Samaranayake, 1994).

Candida sp. may exist in three morphological forms which are blastospores (yeast-like ovoid cells), filamentous hyphae and chlamydiospores (dormant phase of the microorganism). Chlamydiospores is a thick-walled spherical cell with approximately 10 µm in diameter (Melville and Russells, 1975). In many causes however, *Candida krusei* is usually found in only two basic morphological forms which are the yeast and pseudohyphae forms. Pseudohyphae are important in the adherence of cells to the surfaces of the host (Soll, 1992; Dede and Okungbowa, 2009). Both characteristics may however appear simultaneously thus making it difficult to differentiate between these two basic characteristics.

Candida krusei can grow in an environment with temperature ranging from 35 ºC to 45 ºC (Odds, 1988). A characteristic that gives advantage to *Candida krusei* is that it can grow in vitamin-free media, which is a major contrasting feature from the other clinically important *Candida* sp. It is also reported that from a wide panel of carbohydrates component *Candida krusei* can only ferment glucose (Barnett *et al.*, 1983 and Silverman *et al.*, 1990) which is also displayed by *Candida pintolopesii*. It has been shown that when saliva is supplemented with glucose, a number of short-chain

carboxylic acids such as acetate, pyruvate, succinate, propionate, lactate and formate will be formed. Another feature that adds to the advantage of *Candida krusei* is that it can produce acetoin which can be utilised when the growth environment is exhausted of carbon sources (Lategan *et al.,* 1981).

2.3 Phenotypic switching ability of oral *Candida*

Two mechanisms were postulated to be involved in the ability of *Candida krusei* to survive and adapt in a suppressed environment. First is by undergoing mitotic recombination and second is by carrying out phenotypic switching. A direct consequence of mitotic recombination is the lost of heterozygosity throughout the entire genome. This deletion of genome however affects the viability of *Candida* sp. especially in the multiple changing conditions (Vargas *et al.*, 2004). Phenotypic switching, on the other hand is a phenomenon that occurs as a result of changes in the growth environment. A severely suppressed growth condition may lead to high frequency switching in candidal cells. This adaptation is associated with the alteration of gene expression which eventually may lead to alteration of adhesiveness, susceptibility and the resistance of candidal cells to phagocytosis and polymorphonucleur leukocyte. This mechanism of action does not involves deletion of any candidal genome thus, the heterozygosity of the entire genomic are well maintained (Martin and Marsh, 2009).

Phenotypic switching is identified as one of the important virulent factors in *Candida albicans* (Anderson *et al.*, 1987; Soll, 1992; Jones, *et al.*, 1994; Vargas, *et al.*, 2004) and *Candida glabrata* (Lackhe *et al.*, 2000; Lackhe *et al.*, 2002). The significance of the switching strategy is in a way similar to the human immunity

function whereby it is aimed to counter threats in the host's environment. Until now, there is no report that highlights the ability of *Candida krusei* to undergo phenotypic switching. However, scientist has suggested that phenotypic switching mechanism enhances the survivability of *Candida* sp. by rapidly changing its phenotype as an adaptive response to the suppressed environment (Hellstein *et al.,* 1993).

Phenotypic switching may influence the normal physiological growth of candidal species such as *Candida albicans* (Vargas et al, 2004). Under the smooth white and wrinkled phenotypes, *Candida albicans* has been shown to exhibit faster growing colonies than when it exhibited a heavy myceliated and ring phenotype. In addition, phenotypic switching is also discovered to be able to alter the adhesiveness properties of *Candida* sp. (Kennedy *et al.,* 1988). Furthermore, this virulence attribute may also induce the formation of tube and pseudohyphae in *Candida* sp. which then enhance the adherence capacity of the candidal strains (Lackhe *et al.,* 2002).

2.4 Management of candidal infection

2.4.1 Antimicrobial agents

Antimicrobial agents are described as drugs which selectively help to eliminate microbial pathogens from host cell due to toxicity mechanism. There are three categories of antifungal agents available for the treatment of candidosis. They are polyenes, azoles and the DNA analogue 5-fluorocytosine. Examples of polyenes are nystatin and amphotericin B, while the azoles include miconazole, fluconazole, clotriminazole, ketoconazole and itraconazole. The principal of antifungal agents used

against yeast infections in the oral cavity belongs to the first two categories which are the polyenes and the azoles (Samaranayake & Ferguson, 1994).

2.4.1.1 Disinfectant

2.4.1.1.1 Chlorhexidine

Chlorhexidine (CHX) has antifungal and antibacterial properties. The principle of treatment with CHX is based on the rapid absorption of the chemical component into the surface of the microorganisms which increases the permeability of the cell membrane. As a result, it causes precipitation of the cytoplasmic content which then kills the microorganism directly (Davies, 1973). It is also widely used in the treatment of oral candidosis (Budtz-Jorgansen and Loe, 1972; Kulak *et al.*, 1994). Regular rinsing with chlorhexidine helps in the treatment of this disease (Langslet *et al.* 1974). However, the prolonged usage of CHX may stain the dental hard tissue surfaces brownish.

2.4.1.2 Chemical-Based agents

2.4.1.2.1 Azoles

These antifungal agents have five-member of organic rings containing two or three nitrogen molecules (Anil, 2002). Azoles are categorised as N-1 substituted imidazoles and triazoles. Imidazoles that are usually used clinically includes ketoconazole and miconazole while the triazoles include the itraconazole and fluconazole. Triazoles such as fluconazole are inhibitor of cytochrome p-450 enzyme which is involved in the general synthesis of fungal cell membrane. The principal

action of azoles is through the conversion of 14-α-methylsterol to ergosterol within the fungal membrane as such conversion may lead to the blockage of 14-α-demethylation step in the synthesis of ergosterol. As a result, the composition of ergosterol will deplete whereas the 14-α-methysterol will accumulate and becomes permeable to the intracellular constituents. This target process is known as 14-α-demethylase. Imidazoles on the other hand may interfere with the fungal oxidative enzymes which then lead to lethal accumulation of hydrogen peroxide in the cell (Anil, 2002).

2.4.1.2.1a Fluconazole

Fluconazole is an antifungal agent which is commonly used in the treatment of candidal infection such as oral candidosis. It is a water-soluble chemical compound which can be found in the form of tablet, oral solution and saline-based intravenous solutions (Anil, 2002). In a bulk powder form, it appears as a white crystalline powder with slight solubility water and alcohol. The absorption of fluconazole has been reported to be unaffected by food or gastric acidity (Lim *et al.*, 1991; Debruyne and Ryckelynck, 1993; Zimmermann *et al.*, 1994).

Fluconazole has been useful in the prevention of *Candida albicans*-associated endocarditis and diseases caused by *Candida parapsilosis* and *Candida tropicalis*. However, this antifungal agent was found to be ineffective towards *Candida krusei* and *Candida glabrata* (Van't Wout, 1996; Samaranayake, 1997; Venkateswarlu *et al.*, 1997; Singh *et al.*, 2002). In other species, fluconazole exerts fungistatic affects. This fungistatic azoles is often used for lifetime therapy of AIDS patients. This is of concern as long-term usage may lead to drug resistance especially when switched candidal species are involved (Baily *et al.*, 1994).

2.4.1.2.1b Voriconazole

Voriconazole is a triazole derivative of azoles with a wide range of effectiveness in the treatment of fungi including *Candida* sp., *Aspergillus* sp. and *Cryptococcus neoformans*. In comparison to fluconazole therapy, voriconazole exhibited 1.6 to 160-fold greater inhibition of ergosterol P-450-dependent α-demethylase in *Candida albicans, Candida krusei* and *Aspergillus fumigates* (Sheehan *et al.,* 1999). Voriconazole is more potent in inhibiting the growth of *Candida krusei* compared to fluconazole with 16-fold higher than the treatment on *Candida albicans* (Fukuoka *et al.,* 2003).

2.4.1.2.2 Polyenes

The polyenes are antifungal drugs that target the cell membrane containing ergosterol. Polyenes include nystatin and amphotericin B which are categorised as the amphipathic; having both hydrophobic and hydrophilic sides. It acts as an agent that is able to intercalate into membrane layer and forming channels causing potassium ions to leak out of the cell and destroy the proton gradient of *Candida* sp. (Vanden Bossche *et al.*, 1994).

2.4.1.2.2a Amphotericin B

Amphotericin B is a polyene which posses both fungicidal and fungistatic activities that are broad-spectrum against *Blastomyces dermatidis, Coccidioides imnitis, Crytococcus neuformans, Histoplasma capsulatum, Paracoccidioides brasiliensis, Sporotrichium* sp., *Candida glabrata* and *Candida albicans* except *Candida lusitaniae* which is resistant. This topical antifungal agent is usually used in treating primary oral

candidosis and used as an adjunct to parenteral therapy in secondary candidosis with manifestations of both systemic as well as on oral mucosal surfaces (Samaranayake *et al.*, 2009).

Amphotericin B acts on the sterol on the cell walls of the target cells which then damages the cell walls and releases the ionic content including potassium and glucose. This phenomenon leads to the inhibition of glycolysis which then inhibits the growth of the candidal species (Anil, 2002).

2.4.1.2.2b Nystatin

Nystatin was discovered in 1949, obtained from *Streptomyces noursei* that was found in the soil sample of a farm in Virginia. It is found to be the most established antifungal agent that is effective in the treatment of superficial fungal infection caused by *Candida* sp. Nystatin can damage the cell membranes of yeasts by altering the permeability (Kerridge, 1986). The reaction starts when nystatin binds to the sterol component in the membrane of the yeast and alters the permeability. Nystatin has the ability to treat superficial fungal infection caused by *Candida* sp. It has been shown to exhibit both the fungistatic and fungicidal activities. However, nystatin is a poor therapy for oral candidal infection. It is poorly absorbed by the host and usually it is passed unchanged through the gastrointestinal tract (Anil, 2002).

Furthermore, it is discovered that nystatin can suppress the adhesion of *Candida albicans* to cells of the buccal epithelium (Macura, 1987; Vuddhakul *et al.*, 1988; Abu-el Teen *et al.*, 1989). Nystatin can also inhibit the formation of germ tube which is

known as the virulence factor of selected candidal strains such as *Candida albicans* and *Candida dubliniensis.*

2.4.1.3 Plant-based agents

2.4.1.3.1 Family *Piperaceae*

Piper betle is a plant belonging to the *Piperaceae* which originated from the South East Asia including India, Sri Lanka and Bangladesh. The betel leaf itself is known as Sireh (Malay), Paan (Urdu and Hindi), Vetrilai (Tamil) and Ikmo (Philippines). *Piper betle* plant is an evergreen plant with glossy heart-shaped leaves with white catkin. Usually, the leave is chewed together with betle nut, lime and gambier leaves. The nut gives the reddish colour to the saliva and thus darkens the teeth. Betle leave is believed to be a folk medicine in the treatment of various diseases including bad breath, headache, boils, conjunctivitis, itches, mastitis, mastoiditis and ringworm (Chopra *et al.*, 1956). The essential oil of *Piper betle* was reported to contain antibacterial, antiprotozoan and antifungal properties. Research has shown that the plant may produce bacteriostatic and fungistatic effects against *Salmonella thyphi*, *Escherichia coli* and *Candida albicans* respectively (Indu and Ng, 2002; Guha, 2006).

Piper betle was found to be effective as anti-dermatophyte against *Candida albicans, Microsporum gypseeum* and *Trichosporon beigelii* and phyto-pathogen such as *Sclerotium rolfsii, Alternaria solani* and *Phytophthora infestons* (Rahman *et al.*, 2005). The extract of this plant was also identified as an important antioxidant which scavenged the free radicals and detoxify the organism which then prevented cardiovascular disease, cancers (Gerber *et al.*, 2002; Serafini *et al.*, 2002), Parkinson's

and Alzheimer's diseases (Di Matteo and Esposito, 2003). The leaf extract was identified to inhibit radiation-induced lipid peroxidation. In addition, the extract also increased the activity of superoxide dismutase which indicated the elevation of antioxidant status in Swiss albino mice (Chourhury & Kale, 2002). Research has also proved that the antioxidant component within *Piper betle* leaf was higher than tea (Dasgupta and De, 2004). The active compounds which were identified from the extract include chavicol, chavibetol, allylpyrochatichol, chavibetol acetate and allylpyrochatichol diacetate. Various nutritional compounds has been identified to be present in *Piper betle* extract which include vitamin A, vitamin B, iodine, iron, calcium, potassium, tannin, riboflavin and carbohydrate. Furthermore, the leaf was also said to contain enzymes such as diastase and catalase (Guha and Jain, 1997).

Piper betle crude aqueous extract has been reported to reduce the cell surface hydrophobicity of *Streptococcus sanguis*, *Streptococcus mitis* and *Actinomycetes* sp. (Fathilah *et al.*, 2006). Hydrophobicity is an important mechanism which enhances the adherence of pathogen to saliva-coated teeth surface.

Figure 2.1: The leaves of *Piper betle.*

Figure 2.2: *Piper betle* tree.

2.4.1.3.2 Family *Ranunculaceae*

Nigella sativa is a herbaceous plant which is known as fennel flower plants derived from *Ranunculaceae* or Buttercup family. The maximum height of this plant is about 60 cm with blue flower producing small-caraway black seeds (Khan, 1999; Al-Jabre *et al.*, 2003). The plant is also known as black cumin (English), shonaiz (Persian), krishnajirika (Sanskrit), kalajira (Bangali), kalonji (Urdu and Hindi) and Habbatus-sawda (Arabic). It is a common plant in the Middle East, Eastern Europe, Western and Middle Asia. This plant has been identified as a remedy for many ailments since the ancient times of the Egyptian, Romans and Greeks (Al-Jabre et al., 2003). The medicinal parts of *Nigella sativa* were reported in the book of medicine *Canon fi Tibb* by Avicenna which states that the black seed is a good medicine which acts as expectorant, stimulates body's energy and helps in the recovery from fatigue and dispiritedness. In the Quran, the black seed is known as the cure for any kind of known disease except death (Al-Bukhari, verse 815). Besides medicine, it is also used as a flavour for bread and pickles. Many active ingredients are found from *Nigella sativa* which include thymoquinone, thymol, dithmoquinone, thymohydroquinone, carvacrol, nigellicine, nigellidine, nigellimine-N-oxide and alpha-hedrin (Canonica *et al.*, 1963; Mahfouz and El-Dakhakhny, 1966; El-Alfy *et al.*, 1975; Khan, 1999, Randhawa and Al-Ghamdi, 2002; Al-Jabre *et al.*, 2003).

Thymoquinone and thymohydroquinone present in the extract of *Nigella sativa* were reported to have anti-inflammatory activities. Studies on rat perionatal mast cells have shown that nigellone at low concentrations worked as an active inhibitor of histamine which is produced during cell-antigen segregation. The action was created due to the inhibition of protein kinase-C and the decrease of calcium concentration

which is involved in an inflammation mechanism. These results have suggested that the nigellone can be used as an effective medicine in the prevention of asthma and allergic condition (Zawahry, 1963; Chakravarty, 1993; Khan, 1999). Thymoquinone which is the active component of *Nigella sativa* acted as anti-fungal agent towards *Candida albicans* (Al-Jabre *et al.*, 2003), *Tricophyton rubrum* (Al-Jabre *et al.*, 2005) and *Aspergillus* sp. (Al-Qurashi *et al.*, 2007). Thymohydroquinone is also able to inhibit the growth of Gram positive microorganism such as *Escherichia coli.* Diethyl-ether extract of *Nigella sativa* was found to be effective on *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* It was also discovered to act synergistically with streptomycin and gentamycin (Atta-ur-Rehman *et al.*, 1985; Morsi, 2000).

In the early 1960's, researchers found that the volatile oil of *Nigella sativa* contained antimicrobial component (Toppozoda *et al.*, 1965). The oil was found to inhibit the growth of certain Gram positive bacteria such as *Streptococcus aureus,* Gram negative bacteria such as *Escherichia coli* and fungi such as *Candida albicans.* Furthermore, the fractionation process of the oil which produces the phenolic content was found to increase the effectiveness of the oil up to ten thousand times and is non-toxic to human (Toppozoda *et al.*, 1965).

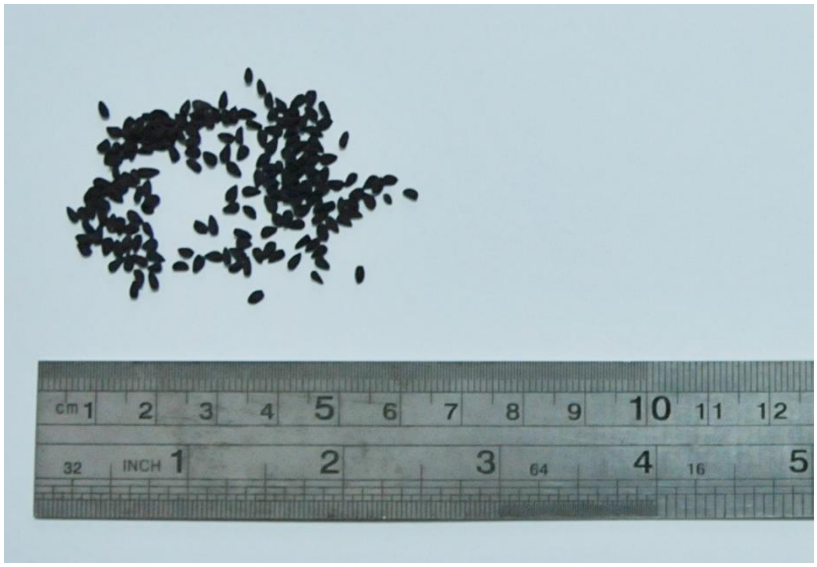


Figure 2.3: The seeds of *Nigella sativa.*

3.0 MATERIALS AND METHODS

3.1 RESEARCH MATERIALS

3.1.1 Chemicals

Decon 90 (Decon, England)

Dibasic sodium phosphate anhydrous powder (Sigma, USA)

Ethanol 95% (John Kollin Corporation, USA)

Germisep (Hovid, Malaysia)

Glutaraldehyde

Glycerol (Merck, Germany)

Monobasic potassium phosphate (Sigma, USA)

Osmium tetraoxide 1%

Phloxine B (Sigma, USA)

Potassium chloride (Sigma, USA)

Savlon (Johnson and Johnson, Malaysia)

Sodium chloride (BDH, England)

3.1.2 Glasswares

Beaker (Bibby, UK)

Conical flask (Pyrex, England)

Glass beads (3 mm diameter) (Merck, Germany)

Glass bottle (Schott, UK)

3.1.3 Consumables

Aluminium foil (Diamond, USA)

Blank disk (Oxoid, UK)

Bunsen burner gas (Campingaz, France)

Microtitre plate (96 wells) (Nunc, Denmark)

Petri dish (Brandon, USA)

Pipette tips (Appendorf, Canada)

3.1.4 Media

Bacto agar powder (BD, USA)

D (+) Glucose (Sigma, USA)

Mueller-Hinton (MH) agar powder (BD, USA)

Peptone powder (BD, USA)

Yeast extract powder (BD, USA)

3.1.5 Antifungal agents

Amphotericin B (250 µg/mL) solution (PAA, Germany)

Chlorhexidine digluconate (20%) (Sigma, USA)

Fluconazole (25 µg) discs (Oxoid, UK)

Nystatin (100 µg) discs (Oxoid, UK)

Voriconazole (1 µg) discs (Oxoid, UK)

3.1.6 Plant specimens

Nigella sativa (Durra, Syria)

Piper betle (Kedah, Malaysia)

3.1.7 Microbial test strain

Candida krusei (ATCC 14243), American Type Culture Collection, USA.

3.1.8 Microbial identification system

API 20 C AUX (BioMériux, France)

BIOLOG YT Micro Plates (BIOLOG, USA)

Mc Farland standards (BD, USA)

3.1.9 Equipments

Analytical balance, Denver XL-1810 (USA)

Analytical balance, Mettler AJ100J and Denver XL-1810 (USA)

Autoclave, HICLA VE HVE-50 (Hirayama, Japan)

Chiller, 4 ºC (Mutiara, Malaysia)

Compact digital camera (Olympus, Japan)

Digital Camera Light Reflection (DSLR) D90 (Nikon, Japan)

Electric drying cabinet, Weifo KD-112 (Weifo, Singapore)

Freezer, -80 ºC, Hetofrig CL410 (Hetofrig, Denmark)

Fume cupboard, Ductless Labcaire 4850 (Labcaire, England)

Haemacytometer (Marienfield, Germany)

Hotplate (Cimarec 3, USA)

Incubator (Memmert, Germany)

Laminar flow unit, ERLA CFM Series (Australia)

Light microscope (Nikon, Japan)

Micropipettors (Appendorf, Canada)

Microwave oven (Panasonic, UK)

Peristaltic pump (Bio-Rad Econo. Pump)

Scanning Electron Microscope (JOEL, Japan)

Spectrophotometer, Shimadzu UV160A (Shimadzu, Japan)

Speed vacuum concentrator, HETO/HS-1-110 (Denmark)

Sputter coater S150B (Edwards, USA)

Stereoscope (Olympus, Japan)

Thermocirculator E3500 (Polaron, UK)

Vortex mixer (Glas-Col, USA)

Water distiller (J Bibby Merit)

Water purifier system (ELGA, UK)

3.2 RESEARCH METHODS

3.2.1 Research outline

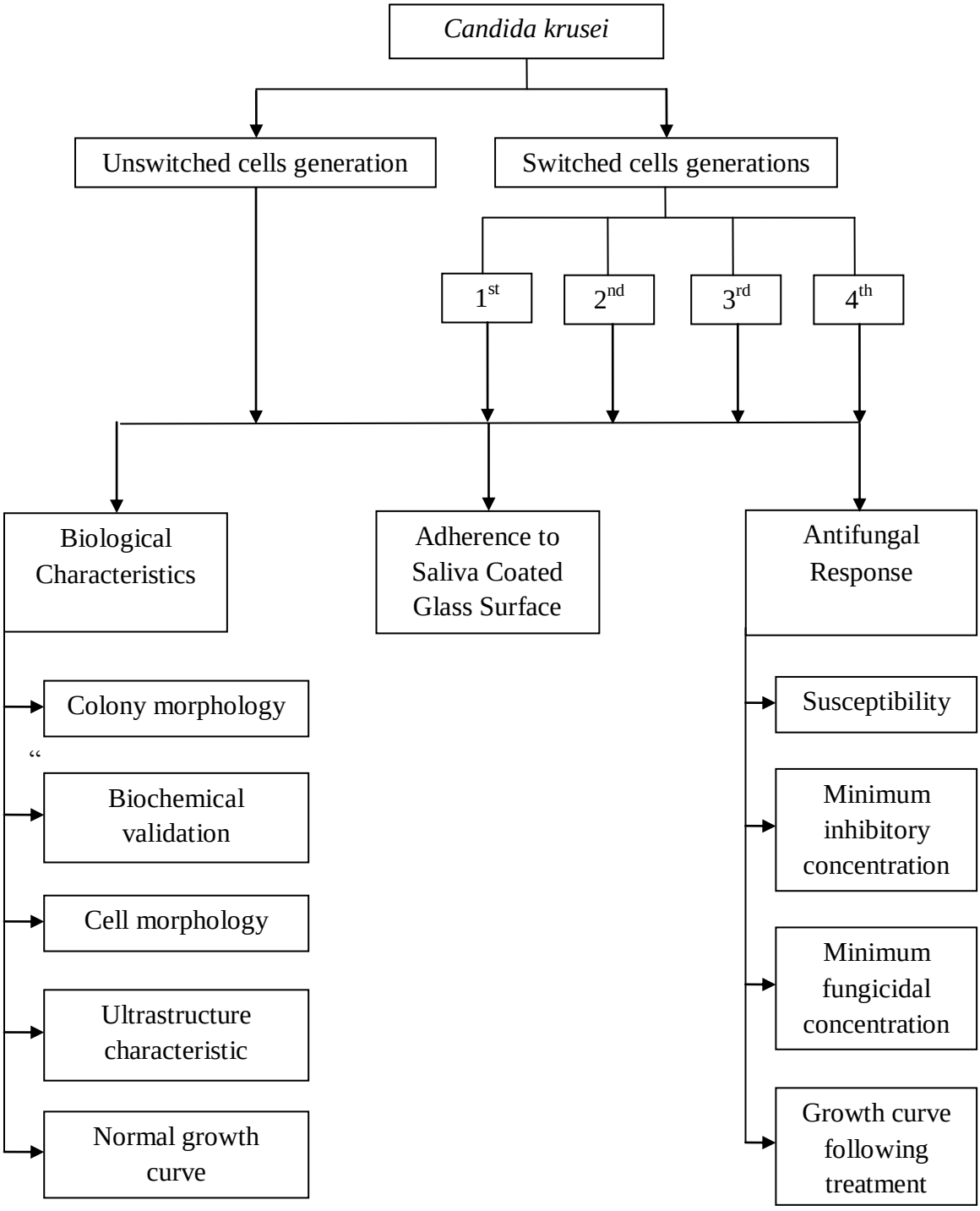


Figure 3.1: Schematic diagram of research methodology.

3.2.2 Preparation of broth media

3.2.2.1 Yeast extract potato dextrose (YEPD) broth

Table 3.1: Compounds required for making YEPD broth.

Materials	g
D (+) Glucose	20
Peptone powder	20
Yeast extract powder	10

All the nutrients above were dissolved in 1 L of distilled water and boiled in a microwave oven. The mixture was sterilized by autoclaving at 121 ºC for 15 minutes at 15 psi. The sterilized YEPD broth was kept in the 4 ºC refrigerator for later used within a time period of a month.

3.2.2.2 YEPD broth supplemented with phloxine B

1 L of YEPD broth was prepared as above and 0.005 g of phloxine B (0.05%) was added to the broth mixture. Phloxine B was mix thoroughly. The supplemented broth was then sterilized by autoclaving at 121 ºC for 15 minutes at 15 psi and kept at 4 ºC for further use in the experiment. The prepared media was best to be used within a month.

3.2.3 Preparation of agar media

3.2.3.1 Yeast extract potato dextrose (YEPD) agar

Table 3.2: Compounds required for making YEPD agar.

Materials	g
D (+) Glucose	20
Peptone powder	20
Yeast extract powder	10
Bacto agar powder	20

All nutrients above were dissolved in 1 L of distilled water and boiled in a microwave oven. The mixture was autoclaved at 121 ºC for 15 minutes at 15 psi. The sterilized media was poured into sterile petri dishes and left to solidified.

Agar slants were also prepared by dispensing approximately 3 mL of the sterilized agar into the sterile universal bottles and left to solidify on a slant bench surface. The solidified YEPD agar plates and slants were stored 4 ºC refrigerator for later use within a period of a month. The agar plates were stored in an inverse direction until the next usage and were best used within a period of a month.

3.2.3.2 YEPD agar supplemented with phloxine B

1 L of YEPD agar suspension was prepared as above and 0.005 g of phloxine B (0.05%) was added to the agar mixture and boiled in a microwave oven. Similar sterilization procedure to section 3.2.2.1 was carried out. The sterilized media was then poured into sterile petri dishes and left to solidified.

Agar slants were also prepared by dispensing approximately 3 mL of the sterilized agar into the sterile universal bottles and left to solidify on a slant bench surface. The solidified YEPD agar plates and slants were kept in the 4 ºC refrigerator for later use within a month. The agar plates were kept in inverse direction until the next usage.

3.2.3.3 Mueller-Hinton (MH) agar

38 g of MH agar powder were dissolved in 1 L of distilled water and boiled in a microwave oven. The mixture was then sterilized following the procedure in section 3.2.2.1. The sterilized medium was then poured in sterile petri dishes and left to solidified. All plates were kept at 4 ºC in an inverse direction and were best used within a period of one month.

3.2.3.4 CHROMagar

47.7 g of CHROMagar powder were dissolved in 1 L of distilled water and boiled in a microwave oven for 5 minutes. The mixture was then sterilized according to the procedure in section 3.2.2.1 and poured in sterile petri dishes and left at room

temperature to solidify. All plates were kept at 4 ºC in an inverse direction. The plates were best used within a period of one month.

3.2.4 Preparation of *Candida krusei* (ATCC 14243) stock culture

With reference to the manufacturers' instruction, an ampoule containing lyophilised cells of *Candida krusei* (ATCC 14243) was added with 0.5 mL of sterile distilled water to rehydrate the dry pellet. Following rehydration, 100 µL of the suspension was then inoculated on to YEPD agar plate and incubated at 37 ºC for 24 hours (Lackhe *et al.*, 2002).

3.2.4.1 Short term storage on agar slants

Several colonies of *Candida krusei* from YEPD agar plate were picked, subcultured on YEPD slants and incubated overnight and stored at 37 ºC. It was then stored at 4 ºC prior to use in the experiment.

3.2.4.2 Long term storage in 20% glycerol

Glycerol stock media is required to maintain cells' viability for long term storage. *Candida krusei* strain from YEPD slant was inoculated into 5 mL of YEPD broth and incubated overnight at 37 ºC. Following incubation, 800 µL of the growth suspension was transferred into sterile microfuge tube which has been added with 200 µL of glycerol. The glycerol stock of *Candida krusei* was then stored at -80 ºC for long storage purposes.

3.2.5 Preparation of *Candida krusei* switched cultures

Cells from the slant culture of unswitched *Candida krusei* was revived in YEPD broth that has been supplemented with 5 mg mL^{-1} of phloxine B dye. This dye was used to create a stress growth environment for the cells (Lackhe *et al.*, 2002). The suspension was then incubated for 4 to 5 hrs at 37 ºC. Following incubation, the turbidity of the growth suspension was spectrophotometrically measured and standardised to an optical absorbance 0.144 at a wavelength of 550 nm (10^6 cells mL^{-1}). The suspension was then serially diluted to give a plate count of approximately 50 cells each. The suspension was then spread evenly on the agar surface and incubated overnight at 37 ºC. The colony forming units (CFU/mL) were then examined. Colonies exhibiting different characteristics from the normal were enumerated and photographed. These colonies were considered as having a phenotypic switched from the unswitched *Candida krusei* and designated as the 1st switched generation. Several cells from the 1st switched generation were again subcultured on to another set of YEPD (supplemented with phloxine B) agar plate and the whole procedure was repeated to produce the 2nd, 3rd and 4th generation of the switched cells. The CFU/mL of each generation of unswitched and switched *Candida krusei* was calculated according to the formula:

$$\text{Total CFU/mL} = \frac{\text{Number of formed colonies}}{\text{Dilution factors x volume used (mL)}}$$

The result was interpreted as the mean of CFU/mL and standard deviation (SD).

3.2.6 Determination of biological characteristics of *Candida krusei*

3.2.6.1 Colony morphology

3.2.6.1.1 YEPD agar

Candida krusei from the slant was inoculated into 0.5 mL of YEPD broth and standardized to an OD of 0.144 at 550 nm. 100 µL of the suspension was then inoculated on to YEPD agar plate and incubated at 37 ºC for 24 hours (Lackhe *et al.*, 2002). The morphology of *Candida krusei* which include the surface appearance, margin, forms and elevation were observed and recorded.

3.2.6.1.2 CHROMagar

Using a sterile wire loop, *Candida krusei* was inoculated on CHROMagar using single colony dilution streaking method. The plate was incubated at 37 ºC for 24 to 72 hours. Following incubation, the colour of the grown colonies were observed and recorded.

3.2.6.2 Biochemical analysis

3.2.6.2.1 API 20 C AUX identification system

5 mL of distilled water was dispensed into each honey-combed wells of API 20 C AUX tray to provide a humid environment. The tray was labeled according to the sample used. A suspension of *Candida krusei* was prepared and standardized at a turbidity of 2 McFarland using sterile saline. 100 µL of the suspension was inoculated onto YEPD agar and incubated at 37 ºC for 24 hours. Another 100 µL of *Candida krusei* suspension was inoculated into C-medium which was supplied by the

manufacturer in the identification system kit. The suspension mixture was homogenized gently to prevent the formation of bubbles. The homogenized suspension was pipetted into each of 20 cupules on the test strip placed in the tray. The tray was put in an incubator at 37 ºC for 48 to 72 hours. After incubation, the strip was examined and the turbidity of each cupule was compared to the control sample. The positive or negative outcomes of all cupules in the strip were compared to the table provided by the manufacturer to confirm the species.

3.2.6.2.2 BIOLOG YT MicroPlates

The methodology was carried out according to the instruction provided by the manufacturer. BIOLOG is an identification system which dependent on the substrate-enzyme interactions of the microbial strain. An overnight cultured of *Candida krusei* was suspended in sterile distilled water. Later, 100 µL of the cell suspension standardized at 47% transmittance was inoculated into each well of the YT MicroPlates. The YT MicroPlates were incubated at 26 ºC for 24, 48 and/or 72 hours. The metabolic patterns were interpreted by Biolog's MicroLog 3 computer software which matched to the library of species database.

3.2.6.3 Cell morphology

Candida krusei was inoculated in YEPD broth and incubated at 37 ºC for three hours. Following this, one to two loopfuls of the suspension was placed on clean glass slide. With circular movement of the loop, the suspension was spread evenly into a thin area with approximately the size of 1 cm^2. The smear was fixed by air-drying. The smear was then gently flooded with crystal violet and left to stand for one minute, after

which the stained was washed with tap water. Later, the smear was flooded with iodine and left for one minute and washed with tap water. 95% of ethyl alcohol was dropped on the smear followed by immediate washing with tap water. A counterstain, safranin was applied and left to stand for 45 seconds. Finally, the smear was then washed with tap water and blot dried with tissue paper. The slide was then examined using a light microscope at 100 x magnification under oil immersion.

3.2.6.4 Ultrastructural characteristic

A colony of *C. krusei* growing on YEPD agar was removed using a cork borer and transferred into a sterile petri dish. The specimen was then fixed by immersing it in glutaraldehyde and Sorensen's phosphate solution (1:1). After an hour, the specimen was washed with Sorensen's phosphate and distilled water (1:1) and then post-fixed with 1% osmium tetraoxide and distilled water (1:1) over a period of 14 hours at 4 ºC. The specimen was then put aside for an additional one hour at 25 ºC under a laminar flow. Following that, the 1% osmium tetraoxide was gently pipetted out and the specimen was again washed with distilled water and put through a series of ascending ethanol concentrations (10%-100%) to dehydrate the specimen. The specimen was then immersed in 100% ethanol twice to ensure maximum elimination of water in the samples. Gradual displacement of ethanol with acetone was then carried out (20 minutes each) using the following ratios of ethanol (EtOH) to acetone (v/v) with 3:1, 1:1 and 1:3.

Following that, the specimen was immersed in 100% acetone for four times, 20 minutes each time, followed by critical point drying (CPD) process using the Polaron E3500. The specimen was then mounted on aluminium stubs and coated with gold

palladium using an Edward's sputter coater (S150B). The specimen was then examined under the scanning electron microscope (SEM) (JEOL SEM) at 2000 x magnification. Samples of the 1st, 2nd, 3rd and 4th switched cells were also similarly prepared for SEM examination.

3.2.6.5 Growth curves

In a sterilized Schott bottle, 50 mL of YEPD broth and 0.5 mL of *Candida krusei* was added and the concentration of the suspension was standardized to 0.144 OD_{550nm} (10^6 cells mL^{-1}). The suspension was then vortexed for 30 second and the initial OD at 550 nm was recorded. The bottle was placed in a shaking water bath at 37 ^oC and the growth of the cells was monitored by recording the OD reading at every one hour interval. The OD reading was converted to CFU/mL and a graph of log_{10}CFU/mL versus incubation time was plotted. The experiment was stopped once the stationary phase was achieved. The protocol was carried out in triplicate and repeated several times to ensure reproducibility (Fathilah, 2004). The procedures were repeated and the growth curves of the 1st, 2nd, 3rd and 4th switched cell generations were also determined.

3.2.7 Determination of biological characteristics of switched *Candida krusei*

The determination of the biological characteristics of switched *Candida krusei* was carried out following procedures in section 3.2.6.1 to 3.2.6.5. Observations with regards to the colony and cell characteristics, the ultrastuctural changes and the growth curves of the switched cells were recorded.

The recovery population of the 1st switched generation of *Candida krusei* was determined from the percentage of the total CFU/mL of switched colony of the 1st switched generation to the total CFU/mL of the unswitched *Candida krusei.* Whereby, the recovery populations of the 2nd to the 4th switched generations of *Candida krusei* were obtained from the percentage of the total CFU/mL of switched colony of *Candida krusei* to the total CFU/mL of the previous switched generation of *Candida krusei.*

3.2.8 The effect of phenotypic switching on adherence to saliva-coated hard surface

3.2.8.1 Preparation of phosphate-buffered saline (PBS) solution

Table 3.3: Chemical ingredients required for the preparation of Phosphate-Buffered Saline (PBS).

Materials	**g**
Sodium chloride	4
Potassium chloride	0.1
Dibasic sodium phosphate anhydrous powder	0.72
Monobasic potassium phosphate	0.12

The chemicals were dissolved in 1 L of distilled water and standardized at pH 7. The solution was then autoclaved at 121 ºC for 15 minutes at 15 psi. The pH were adjusted to pH 7.0 and then stored at 4 ºC.

3.2.8.2 Collection of stimulated whole saliva (SWS)

SWS was collected following method described by Holmes *et al.* (2002) with some modification by Rahim *et al.* (2008). 25 mL of saliva was collected everyday from a single volunteer with healthy oral condition in order to reduce any variation between different individuals. Initially, the subject was required to rinse with distilled water for 10 seconds to ensure the cleanliness of the oral cavity. The volunteer was given a piece of rubber band to chew in order to stimulate salivary flow and SWS was collected in a sterile ice-chilled test tube with the addition of 1,4-dithio-D,L-threitol (DTT) to a concentration of 2.5 mM. The specimen was stirred slowly before centrifugation at 17,000 g for 30 minutes. The supernatant obtained was filtered through 0.2 µm pore low-protein binding filter (Supor® membrane) into a sterile test tube. SWS was then stored at -20 ºC for use in the adherence study.

3.2.8.3 Adherence to saliva-coated hard surface

An artificial mouth model named the Nordini's Artificial Mouth (NAM) model was used in this experiment (Wan Nordini Hasnor, 2007; Rahim *et al.*, 2008). Saliva-coated glass beads of 3 mm diameter were used to mimic the hard tissue surfaces of the tooth in the oral cavity. A modified Pasteur pipette served as a chamber where the glass beads were then placed. The inflow and outflow of media from rubber tubings connected to and from the chamber mimics the inflow and outflow of saliva in the oral cavity. The flow rate of the media was controlled at 0.3 mL min^{-1} using a peristaltic pump (Econo Pump, Bio Rad). Following the inoculation of 20 mL of 10^6 cell mL^{-1} of *C. krusei* suspension, the artificial mouth system was run overnight. The glass beads were asceptically removed and immersed in separate appendorf vials containing 1 mL of PBS. Each of the vials was placed in a sonicator for 60 seconds to dislodge the

adhered cells. The population of the adhered cells was determined by plating 100 µL of the suspension obtained on YEPD agar plates. The total CFU/mL following a 24 hours incubation period at 37°C was determined and recorded. This procedure was followed closely to the steps outlined by Wan Nordini Hasnor (2007). Similar procedure was repeated on the 1st to 4th switched generations of cells.

3.2.9 Antifungal response of *Candida krusei*

3.2.9.1 Preparation of aqueous plant extracts (Fathilah, 2004)

3.2.9.1.1 *Piper betle* aqueous extract

Piper betle leaves were cleaned and the wet weight was taken. The leaves were oven dried at 60 ºC for approximately 24 to 48 hours. The dried leaves were weighed and recorded. 100 g of the leaves were cut into small pieces and put into a conical flask. 2 L of distilled water were added and boiled until the volume was reduced to half. Later, the decoction was filtered into a 500 mL beaker. The filtrate was re-boiled until it was concentrated to a final volume of 100 mL. Finally, the concentrated extract was freeze dried to produce *Piper betle* extract powder and kept in a dry cabinet in 30% relative humidity (RH).

3.2.9.1.2 *Nigella sativa* aqueous extract

100 g of *Nigella sativa* seeds were cleaned and put in 2 L of distilled water. The suspension of the mixture was boiled until the volume reduced to half. Next, filter paper was used to separate debris. The filtrate was transferred into a 500 mL beaker. The suspension was reboiled until it reached a final volume of 100 mL. The suspension

was freeze dried to produce *Nigella sativa* extract powder and kept in dry cabinet at 30% relative humidity (RH).

3.2.9.2 Susceptibility analysis

The susceptibility of *C. krusei* to CHX was determined following two methods; the Kirby-Bauer disc diffusion test and broth dilution methods (Cappucino and Sherman, 2005). According to the standard procedure of the *Clinical and Laboratory Standards* Institute (CLSI), *C. krusei* suspension can be prepared by suspending 1 to 2 colonies of *C. krusei* grown on YEPD agar into 5 mL of 0.85% of sterile saline. The optical density of the cell suspension was then standardised to an OD of 0.144 at 550 nm wavelength. 100 µL of the suspension was pipetted out and evenly swabbed on Mueller-Hinton (MH) agar (BD, USA). Paper discs which have been impregnated with 120 µg (100 µL of 0.12%) CHX were carefully placed on to the swabbed MH agar plate. The diameter of an inhibited growth zone surrounding the discs following an overnight incubation at 37°C was then measured. On the same plate, discs incorporated with 25 µg fluconazole and 1 µg voriconazole were used as the positive and negative control, respectively. The susceptibility of *Candida krusei* to other antifungal agents including amphotericin B (25 µg), nystatin (100 µg), *Piper betle* (1 mg) and *Nigella sativa* (2 mg). The susceptibility of all switched cells towards these agents were also determined and compared to the responses of the unswitched *Candida krusei.*

3.2.9.3 Determination of the minimal inhibitory concentration (MIC)

The MIC of CHX was determined using the broth microdilution method (Cappucino and Sherman, 2005). A sterile 96-well microtiter plate was labelled W1 to W7 horizontally and samples number vertically. Using a sterile pipette, 100 µL of

YEPD broth was added to W2 through W7 while 100 µL of CHX (0.12%) was added into W1 and W2. The plate was slowly agitated to mix the content. Using a sterile pipette, 100 µL of W2 was transferred to W3. Following thorough mixing, 100 µL of W3 was transferred to W4 and the procedure was continued through W6. After mixing, 100 µL from W6 was discarded. W7 that received no CHX and W1 that has no *Candida krusei* served as negative and positive control respectively for the experiment. Lastly, 100 µL of *Candida krusei* suspension was added to W2 through W7 aseptically. W7 that received no CHX served as a positive control. The plate was incubated overnight at 37 ºC. The concentration of CHX in the well that showed no turbidity was taken as the MIC of CHX towards *Candida krusei.* The MIC's of *Candida krusei* in the unswitched and all switched generations to all other agents including amphotericin B, nystatin, *Piper betle* and *Nigella sativa* were also determined.

3.2.9.4 Determination of the minimal fungicidal concentration (MFC)

The minimal fungicidal concentration (MFC) of CHX was determined following the method described by Cappucino and Sherman (2005). MFC is referred as the minimum fungicidal concentration at which 99% to 99.5% *Candida krusei* is killed. This value was determined by inoculating 100 µL from the well of the previous microtiter plate representing MIC on to YEPD agar plates respectively in triplicate. The suspension was spread evenly on the agar surface and incubated for 18 to 24 hours at 37 ºC. Following incubation, the concentration from the plate which showed no growth of *Candida krusei* was considered as the MFC concentration of CHX towards *Candida krusei*. The MFC's of amphotericin B, nystatin, *Piper betle* and *Nigella sativa* were determined following the same procedure.

3.2.9.5 Determination on the effect of CHX, Amphotericin B and *Piper betle* aqueous extract on the growth curve of phenotype-switched *Candida krusei*

The effect of the respective antifungal agents on the growth curves of *Candida krusei* was performed by monitoring the growth of the cells in a growth condition which have been treated with the agents. Five sterilized Schott bottles were labeled with unswitched (U), 1st switched generation (S1), 2nd switched generation (S2), 3rd switched generation (S3) and 4th switched generation (S4) of *Candida krusei* respectively. 40 mL of YEPD broth and 5mL of CHX stock (2 mg/mL) were added to each of the labeled Schott bottle to give a final concentration of 0.2 mg/mL (sub-MIC of CHX). Following this, 5 mL of each generation of *Candida krusei* (10^6 cell mL^{-1}) was then added respectively into each of the Schott bottle according to the indicated labeled on the bottle to give a total volume of 50 mL. The experiment was carried out in triplicates. The suspension was vortexed vigorously for 30 seconds and the initial absorbance was taken at OD_{550nm}. The suspension was then incubated in a shaking water bath at 37 ºC. The changes in OD of the growth suspension were recorded using spectrophotometer at every one hour interval. A graph of increase in cell population against growth time was plotted. The experiment was stopped when the stationary phase was achieved.

Similar protocol was repeated to determine the effect of the agents on the growth process of the 1st to the 4th switched cells with amphotericin B (250 µg/mL) and *Piper betle* (60 mg/mL) by replacing the CHX (2 mg/mL). The final concentration of amphotericin B and *Piper betle* aqueous extract at sub-MIC value of amphotericin B (25 µg/mL) and *Piper betle* (6 mg/mL) were used in the experiment.

The growth rate constant (GR) and the generation time (GT) of *Candida krusei* was finally calculated using the formula below:

$$GR = [(\log_{10}N_{t2} - \log_{10}N_{t1})2.303] / (t_2-t_1);\ t_2>t_1$$

$$GT = (\log_{10}N_{t2} - \log_{10}N_{t1}) / \log_{10}2$$

N_{t1} = initial concentration

N_{t2} = final concentration

t1 = initial time

t2 = final time

4.0 RESULTS

4.1 Biological characteristics of *Candida krusei*

4.1.1 Colony morphology

Sub-culturing of *Candida krusei* on YEPD agar was observed to produce colony with undulate margins, circular forms and umbonate elevation. The surface appearance of the unswitched *Candida krusei* was observed as dry and round surfaces with white to cream colour. The nitrogen depleted growth environment induced by the addition of Phloxine B had caused variations in the colony characteristics (Table 4.1, Figure 4.1). The 1st and 2nd switched generations were observed to have colonies with undulate margin, circular form and umbonate elevation which were similar to the morphological characteristics of the unswitched *Candida krusei*. The surface appearance of *Candida krusei* in the 1st and 2nd generations was observed as wrinkled appearance which was absent in the unswitched *Candida krusei.* The 3rd switched generation showed different colony morphology with heavily wrinkled, myceliated surface appearance, lobate margin, irregular form and umbonate elevation. The 4th switched generation exhibited similar surface appearance and elevation compared to the 3rd switched generation except for the filamentous margin and circular form.

The ability of *Candida krusei* to utilise chromogenic substrate and developing colourised colony was determined by CHROMagar. This study has observed that all colonies of unswitched and switched generations of *Candida krusei* grown on CHROMagar exhibited pink colour colonies with pale border, dry and rough surface appearances, undulate margin, circular form and umbonate elevation.

4.1.2 Recovery population of phenotypic switched *Candida krusei*

Comparative to the unswitched *Candida krusei*, the recovery population in terms of CFU/mL showed that the 3rd switched generation has the highest population recovery of 85.7% followed by the 4th generation at 70.8% and 46.6% for the 1st switched generation. The 2nd switched generation recovered the lowest population of only 36.4% (Table 4.1).

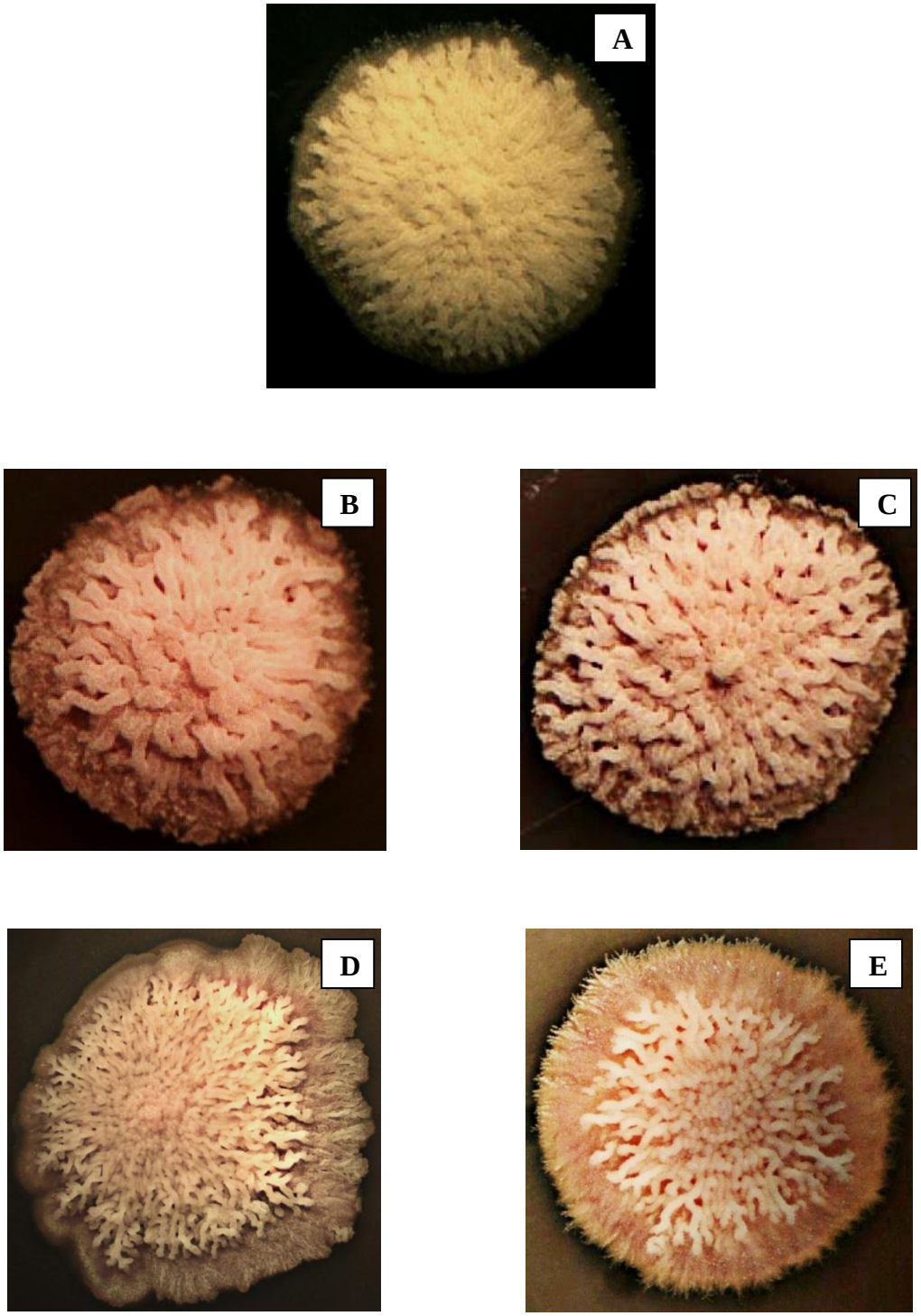


Figure 4.1: Colony morphology of the unswitched and switched *Candida krusei*. (A) Unswitched, (B) 1st switched generation, (C) 2nd switched generation, (D) 3rd switched generation and (E) 4th switched generation.

Table 4.1: The characteristics of growth colonies of the unswitched and all switched generations of *Candida krusei*. Note: The terminologies used were according to Samaranayake *et al.* (1994), Vargas *et al.* (2004) and Cappucino and Sherman (2005).

Growth generation	**Colony characteristics**				**Percentage of Recovered Population (%)**
	Surface Appearance	**Margins**	**Forms**	**Elevation**	
Unswitched	Dry and rough	Undulate	Circular	Umbonate	100.0
1st switched	Dry, rough and wrinkled	Undulate	Circular	Umbonate	46.6
2nd switched	Dry, rough and wrinkled	Undulate	Circular	Umbonate	36.4
3rd switched	Dry, rough, heavily wrinkled and myceliated	Lobate	Irregular	Umbonate	85.7
4th switched	Dry, rough, heavily wrinkled and myceliated	Filamentous	Circular	Umbonate	70.8

4.1.3 Biochemical validation of *Candida krusei*

API 20 C AUX identification system was used to determine on the ability of *Candida krusei* to utilise substrates as a source of carbon. Results obtained indicated that the unswitched and all switched generations of *Candida krusei* were able to ferment only glucose (Figure 4.2). The BIOLOG identification system used in the study was based on the principle of substrate-enzyme interactions. The unswitched and all switched generations of *Candida krusei* were shown to be able to ferment N-acetyl-D-glucosamine and α-D-glucose. Except for the unswitched and 1st generation which also fermented γ-aminobutyric acid (GABA), the 2nd, 3rd and 4th switched generations responded negatively towards the fermentation of γ-aminobutyric acid.

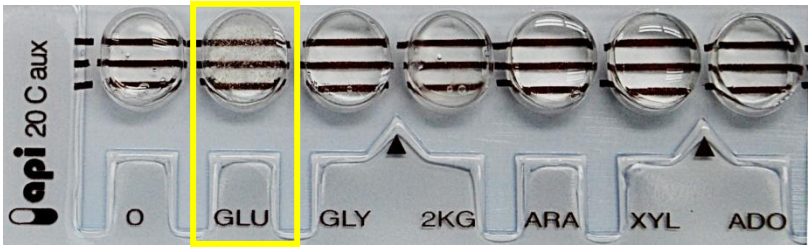


Figure 4.2: Biochemical test using API 20 C AUX. *Candida krusei* was shown to positively fermented glucose as indicated by the hazy lines in the well within the yellow box.

4.1.4 Cell morphology of *Candida krusei*

Examination of prepared slides under 100 x magnification using light microscope following simple staining showed that the cells of the unswitched and switched *Candida krusei* have pseudohyphae (Figure 4.3). Blastoconidia were present and observed as oval to elongated shape with the presence of verticillate branches.

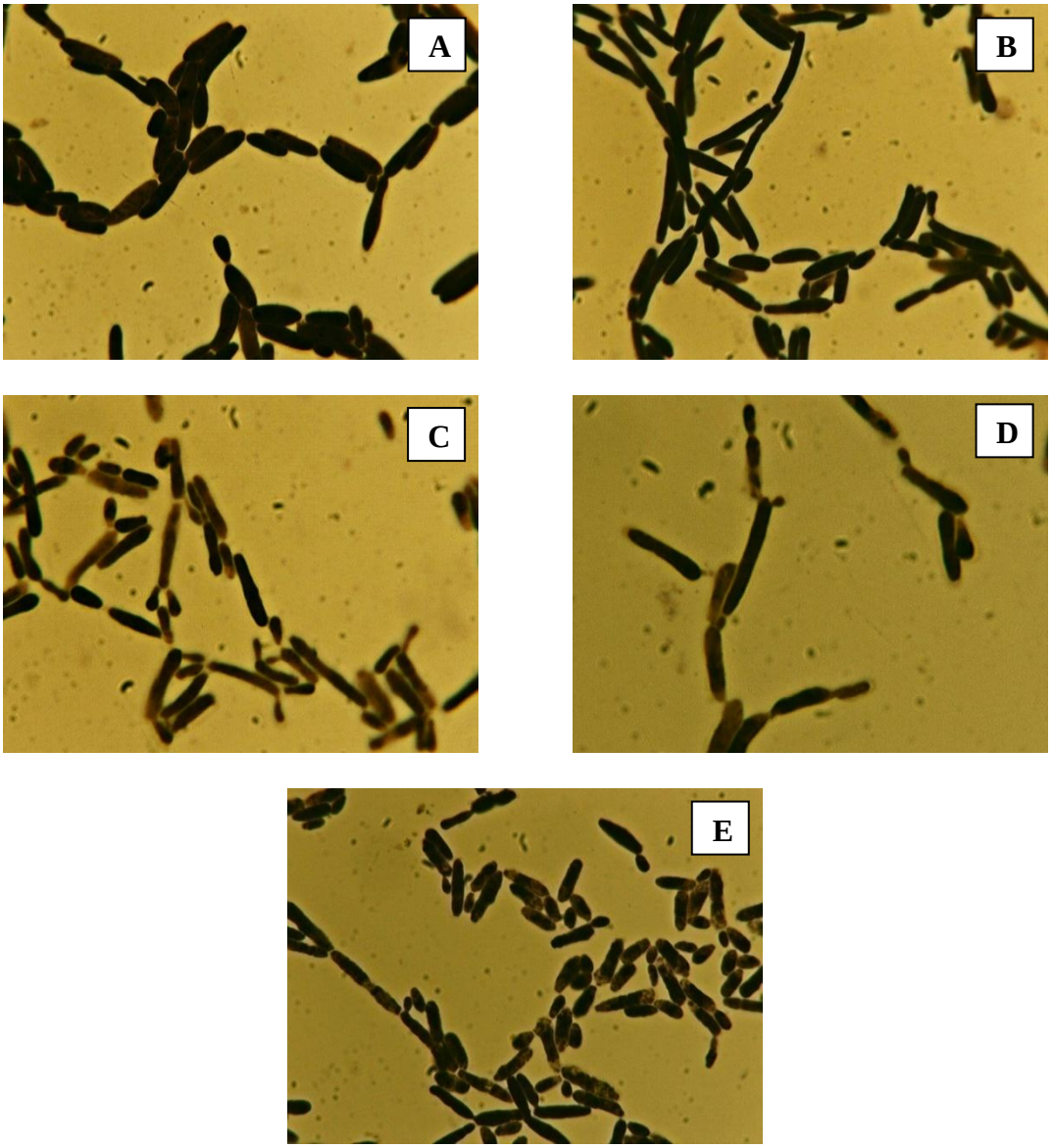


Figure 4.3: Unswitched and switched *Candida krusei* examined at 100 x magnification using a light microscope; (A) unswitched, (B) 1st switched generation, (C) 2nd switched generation, (D) 3rd switched generation and (E) 4th switched generation.

4.1.5 Ultrastructural characteristics of *Candida krusei*

Scanning electron micrographs of the unswitched and all switched generations of *C. krusei* were observed as branched cells with elongated pseudohyphae and elongated to ovoidal blastoconidia and budding off in verticillate branch. The surface appearance of the pseudohyphae was observed as smooth for the unswitched, 1st and 2nd

generations. However, the 3rd and 4th switched generations had exhibited changes on the cell surface showing rough texture instead. In contrast to other switched generations, the 4th generation was observed to exhibit pimpled or punctate appearance on the cell surface (Soll, 1992). The dimension of the 1st switched generation pseudohyphae was found to be approximately 5.0-11.0 x 3.0-4.0 µm whereby the pseudohyphae of the 2nd switched generation was identified to be the most extended compared to other generations with dimension of 5.0-15.0 x 2.0-4.0 µm. The size of pseudohyphae of the 3rd switched generation was determined as approximately 3.0-7.0 x 2.0-3.0 µm. The smallest pseudohypae was observed in the 4th switched generation with size ranging approximately 2.0-6.0 x 2.0-5.0 µm. In addition, the unswitched and the 3rd switched generations were observed to develop extracellular matrix which was absent in the 1st, 2nd and 4th switched generations (Figure 4.4).

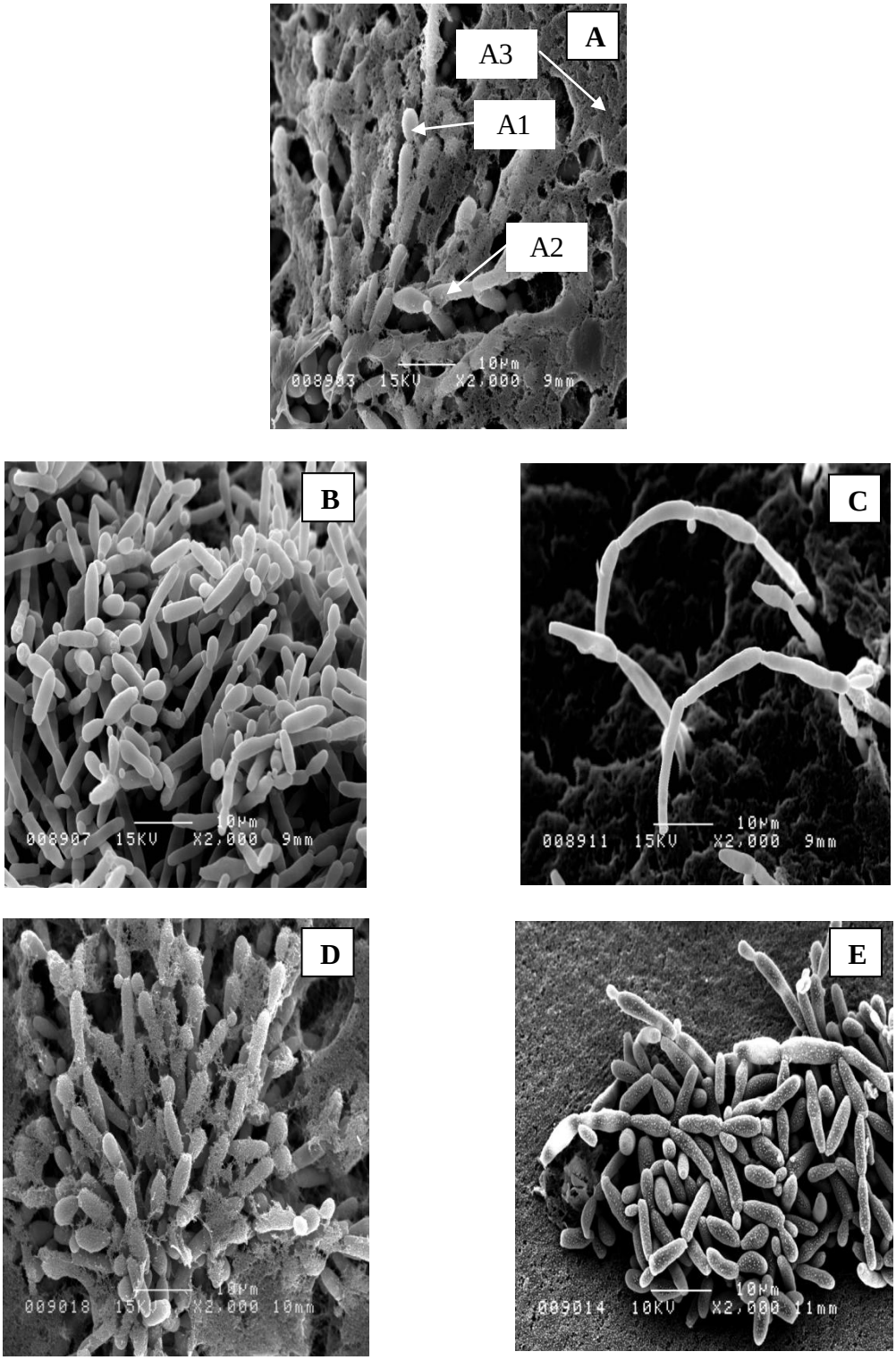


Figure 4.4: SEM micrographs of *Candida krusei* observed for the various growth generations. (A) unswitched, (B) 1st switched generation, (C) 2nd switched generation, (D) 3rd switched generation and (E) 4th switched generation (x 2000). Note: (A1) Blastoconidia (A2) Pseudohyphae (A3) Extracellular matrix.

4.1.6 Growth curves of *Candida krusei*

Figure 4.5 showed the growth curves plotted from the study. Descriptively, the growth curves of the unswitched and all switched generations showed no significant difference between all generations ($p>0.05$). However, slight deviations of growth curves were observed among the generations. The early log phase of the unswitched and all switched generations of *Candida krusei* were determined at three hours and the middle of the log phase were achieved after seven hours of incubation.

The specific growth rate (GR) of *Candida krusei* was found to differ in unswitched and all switched generations (Table 4.4). In the unswitched state, the GR of *Candida krusei* was determined at $0.677 \pm 0.021\ h^{-1}$. A slight decreased in GR was observed in the 1st switched generation to $0.648 \pm 0.131\ h^{-1}$ and determined as the lowest GR among all generations of *Candida krusei*. The 2nd switched generation had showed an increased in the GR with $0.708 \pm 0.021\ h^{-1}$ which was also identified as the highest GR. The GR was observed to decrease in the 3rd ($0.689 \pm 0.132\ h^{-1}$) and 4th switched generation ($0.700 \pm 0.100\ h^{-1}$).

Consequently, the generation time (GT) of all the respective curves also differ in the unswitched and all switched generations (Table 4.4). In the unswitched state, the GT was determined at 3.905 ± 0.031 h. A slight decreased in the GT was observed in the 1st switched generation at 3.740 ± 0.101 h and determined as the lowest GT among generations of *Candida krusei*. The 2nd switched generation showed an increase in GT to 4.085 ± 0.001 h which was also identified as the highest GT. The GT of the 3rd switched generation was 3.976 ± 0.102 h whereby the 4th switched generation was 4.041 ± 0.005 h.

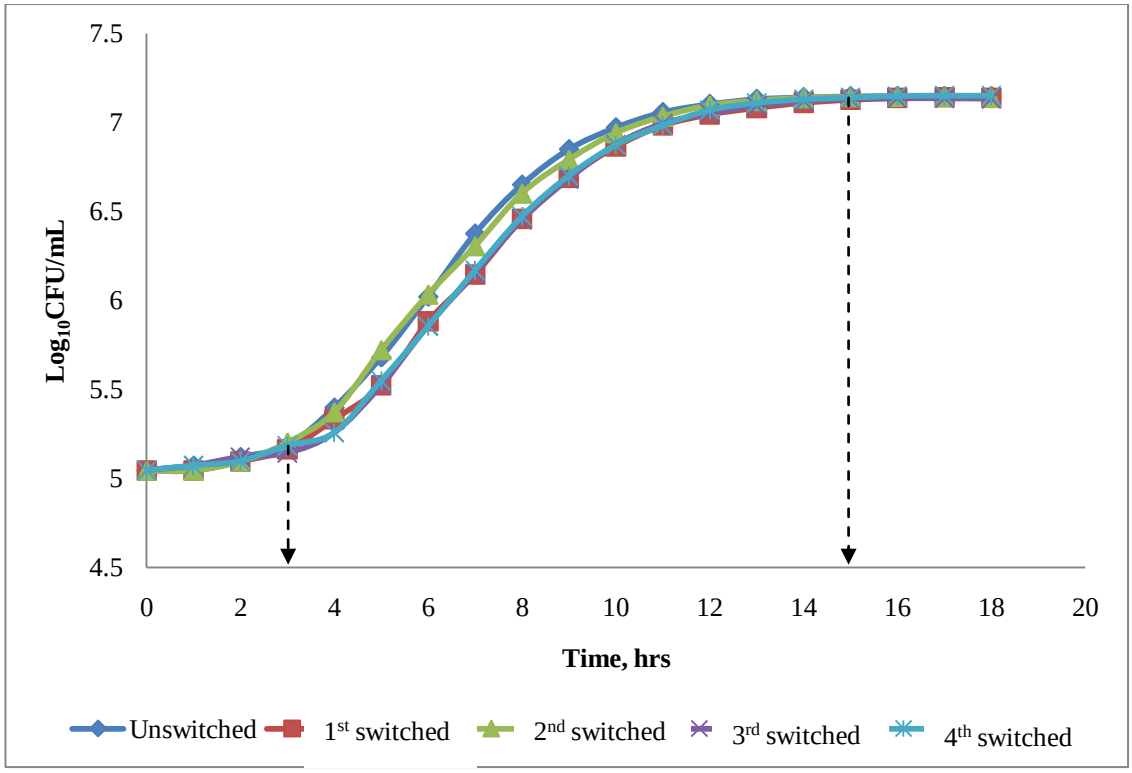


Figure 4.5: The growth curve (GC) of *Candida krusei*. A comparison between unswitched and all switched generations in untreated environment.

4.2 Adherence capacity of *Candida krusei* to saliva-coated glass surfaces

The ability of *Candida krusei* to adhere to the surfaces of saliva-coated glass beads was recorded at (5.62 ± 2.95) x 10^2 CFU/mL. Figure 4.6 showed an increase to (15.29 ± 10.32) x 10^2 CFU/mL in the 1st switched generation compared to the unswitched *Candida krusei*. A drastic increased in adherence capacity in the 2nd switched generation to (154.0 ± 60.2) x 10^2 CFU/mL was observed. However, the adherence capacity was reduced in the 3rd and 4th switched generations of *Candida krusei* to (18.76 ± 7.56) x 10^2 CFU/mL and (9.38 ± 0.37) x 10^2 CFU/mL, respectively.

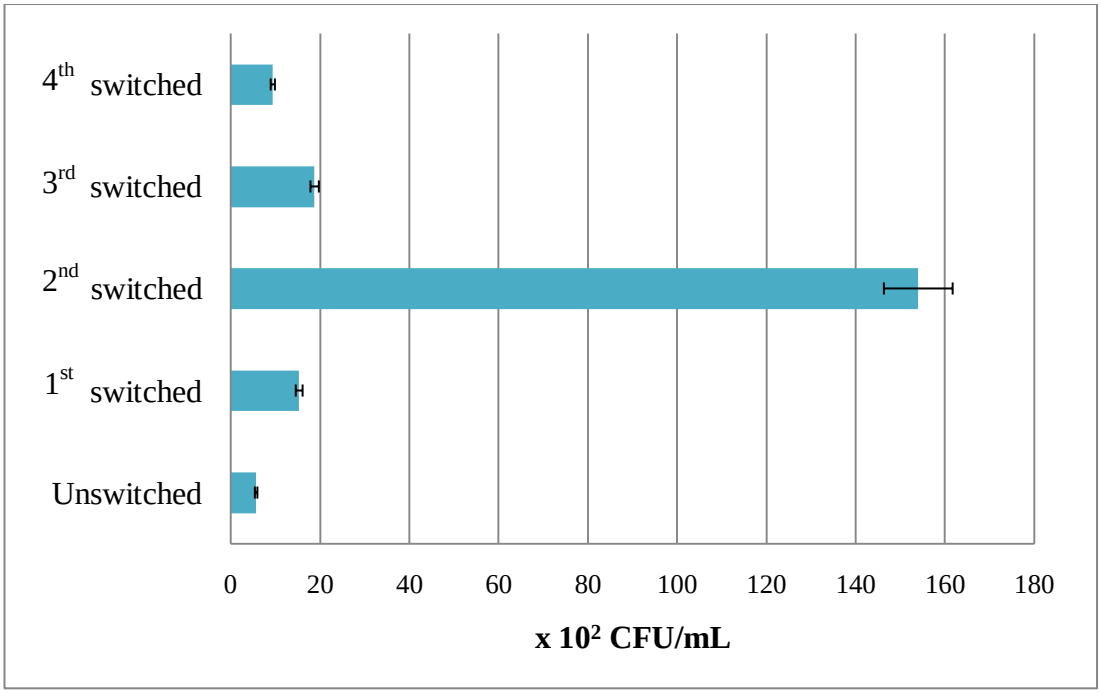


Figure 4.6: The adherence capacity of *Candida krusei* to saliva-coated glass surface. The values were means ± standard deviation (n=9).

4.3 Antifungal responses of *Candida krusei*

4.3.1 Disinfectant

4.3.1.1 Susceptibility towards CHX

The degree of susceptibility towards CHX was found to differ in the unswitched and all switched generations. In the unswitched state, *Candida krusei* was found to be susceptible to CHX with a growth inhibition zone of 3.8 ± 0.1 cm and was the most susceptible towards CHX compared to other generations. The degree of susceptibility was gradually decreased in the 1st and the 2nd switched generations with inhibition zone of 3.5 ± 0.2 cm and 3.0 ± 0.1 cm, respectively (Figure 4.7). In the 3rd and 4th switched generations, susceptibility towards CHX was observed to increase gradually with inhibition zone of 3.4 ± 0.2 cm and 3.5 ± 0.1 cm, respectively. The MIC and MFC of

Candida krusei at each switched generation were determined at 0.4 µg/µL (Table 4.2, Table 4.3).

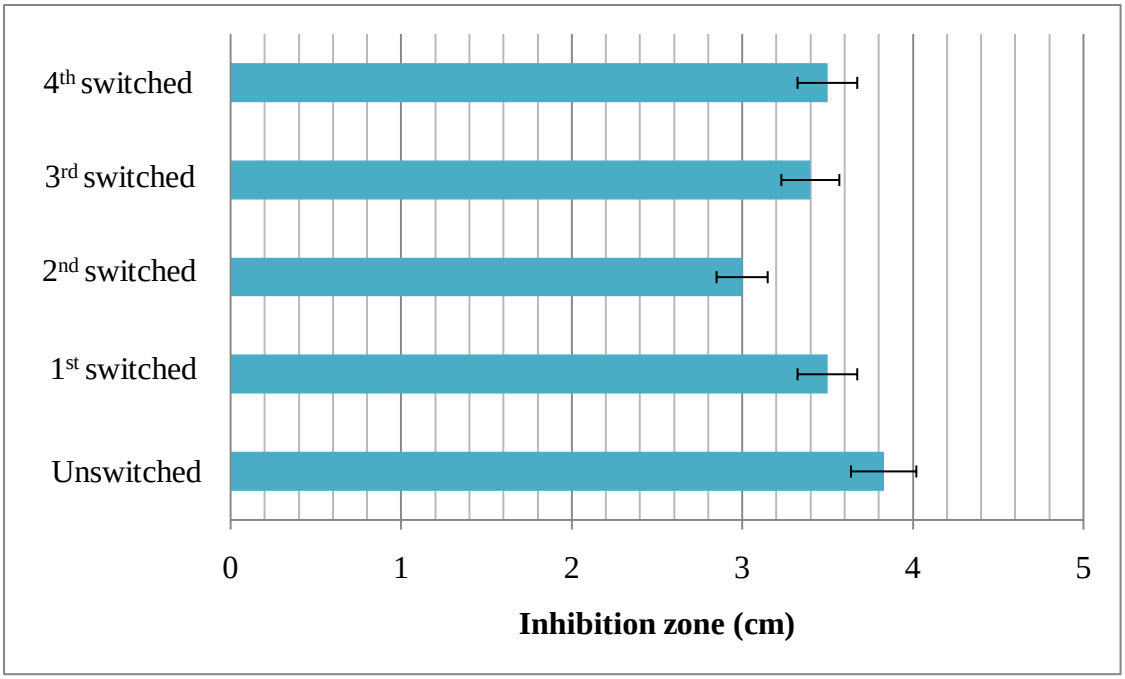


Figure 4.7: The susceptibility of *Candida krusei* in the unswitched and switched forms towards CHX as determined using the disc diffusion method. The values were means ± standard deviation (SD) (n=9).

4.3.2 Chemical-based agents

4.3.2.1 Susceptibility towards amphotericin B

The susceptibility of the unswitched *Candida krusei* towards amphotericin B was recorded to have an inhibition zone diameter of 2.2 ± 0.1 cm. The degree of susceptibility was gradually increased in the 1st, 2nd and 3rd switched generations with inhibition zone of 2.3 ± 0.3 cm, 2.4 ± 0.1 cm and 2.6 ± 0.3 cm, respectively with the 3rd switched generation was found to be the most susceptible towards amphotericin B (Figure 4.8). The susceptibility was determined to decrease in the 4th switched

generations with inhibition zone of 2.4 ± 0.1 cm. The MIC and MFC of *Candida krusei* at each switched generation were determined at 10 µg/mL (Table 4.2, Table 4.3).

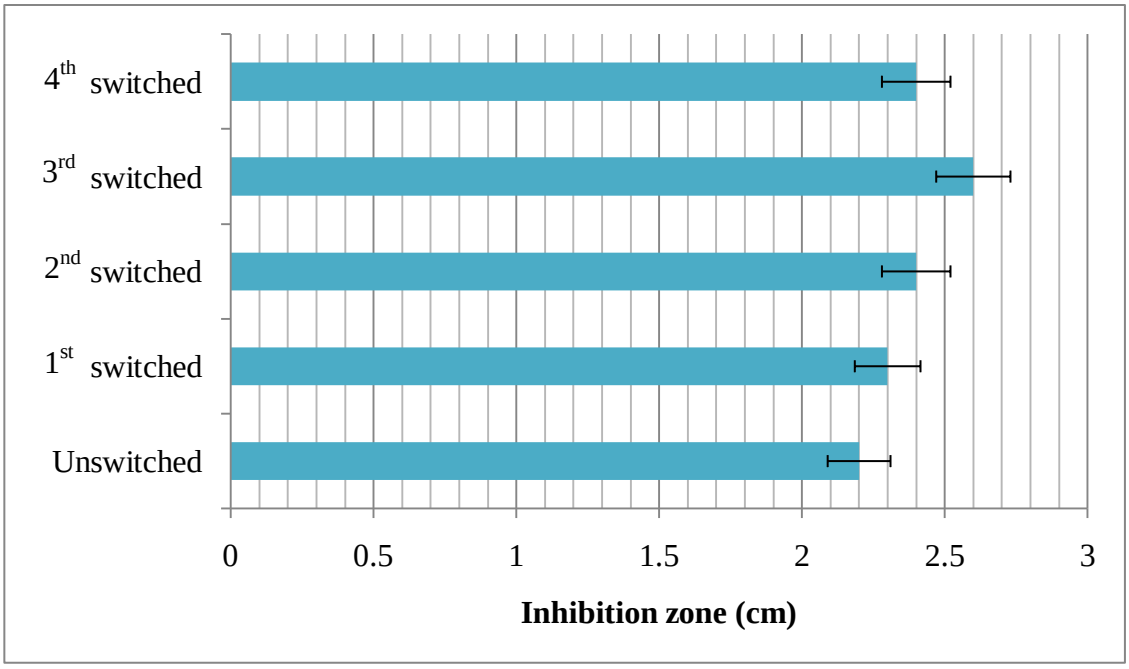


Figure 4.8: The susceptibility of *Candida krusei* in the unswitched and switched forms towards amphotericin B as determined using the disc diffusion method. The values were means ± standard deviation (SD) (n=9).

4.3.2.2 Susceptibility towards nystatin

The susceptibility of unswitched *Candida krusei* towards nystatin was recorded to have an inhibition zone of 2.4 ± 0.1 cm. The degree of susceptibility remained unchanged in the 1st switched generation. However, the susceptibility was decreased in the 2nd switched generation with inhibition zone of 1.9 ± 0.2 cm respectively (Figure 4.9) and identified as the least susceptible towards nystatin. In the 3rd and 4th switched generations, the susceptibility towards nystatin was observed to increase gradually with inhibition zones of 2.3 ± 0.1 cm and 2.6 ± 0.1 cm respectively. The 4th switched generation was determined as the most susceptible among generations of *Candida*

krusei. The MIC and MFC of *Candida krusei* at each switched generation were determined at 50 unit/mL (Table 4.2, Table 4.3).

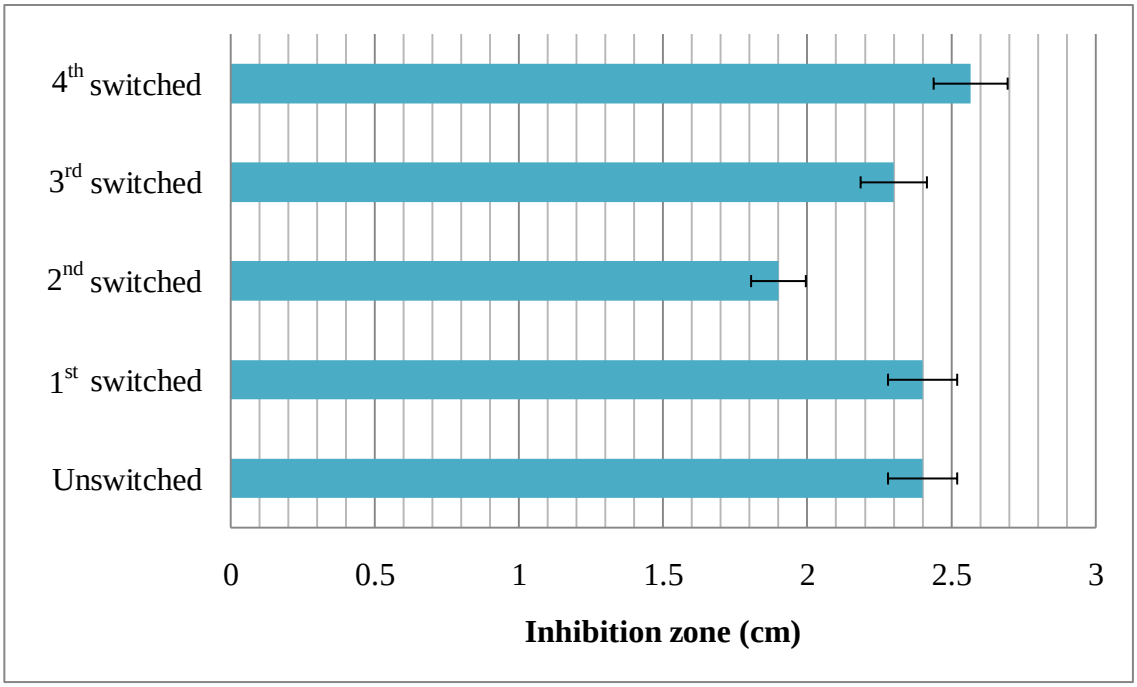


Figure 4.9: The susceptibility of *Candida krusei* in the unswitched and switched forms towards nystatin as determined using the disc diffusion method. The values were means ± standard deviation (SD) (n=9).

4.3.3 Plant-based agents

4.3.3.1 Susceptibility towards *Piper betle* aqueous extract

The degree of susceptibility towards *Piper betle* aqueous extract was found to differ in unswitched and all switched generations. In the unswitched state, *Candida krusei* was found to be susceptible to *Piper betle* aqueous extract with inhibition zone diameter of 2.2 ± 0.1 cm. Differently for the 1st switched generation where the degree of susceptibility was found to increased with inhibition zone diameter of 2.3 ± 0.2 cm (Figure 4.10). The susceptibility of *Candida krusei* towards *Piper betle* aqueous extract was identified to decrease in the 2nd switched generation with 2.1 ± 0.1 cm

inhibited zone. The susceptibility in the 3rd switched generation remains unchanged with inhibition zone of 2.1 ± 0.2 cm. The 4th switched generation was found to have a lowered susceptibility compared to the 2nd and 3rd generations with inhibition zone of 2.0 ± 0.2 cm which was the least susceptible towards *Piper betle* aqueous extract among generations of *Candida krusei*. The MIC and MFC towards *Piper betle* aqueous extract were determined at 12.5 mg/mL for unswitched and all switched generations of *Candida krusei* (Table 4.2, Table 4.3).

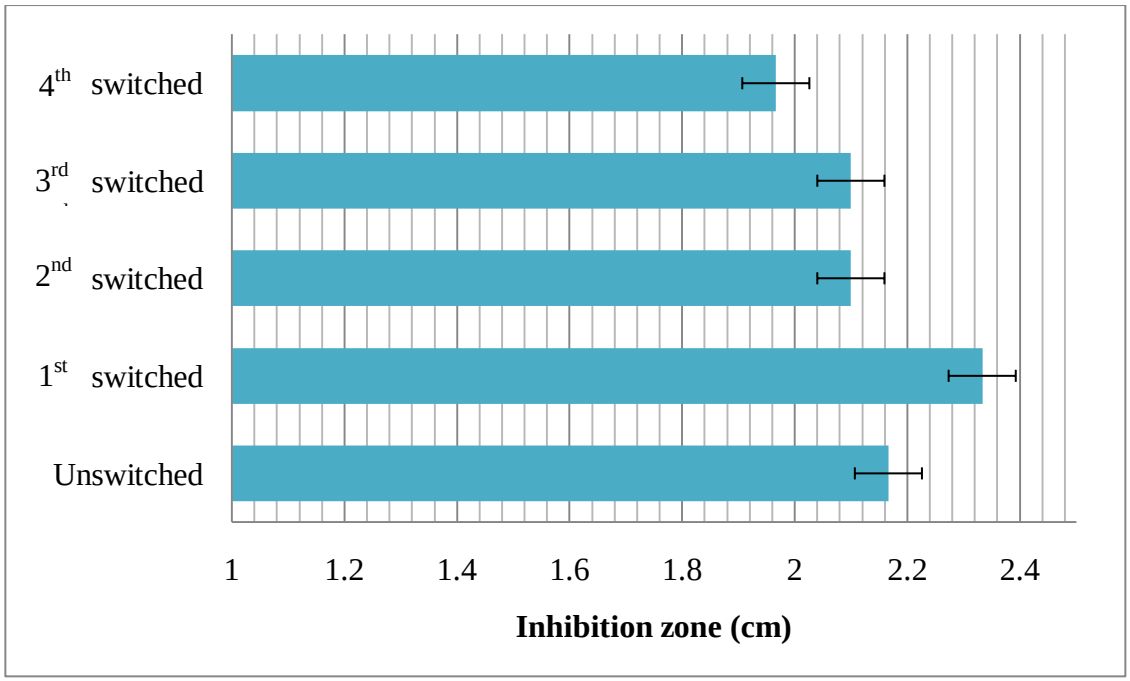


Figure 4.10: The susceptibility of *Candida krusei* in unswitched and switched forms towards *Piper betle* aqueous extract as determined using the disc diffusion method. The values were means ± standard deviation (SD) (n=9).

4.3.3.2 Susceptibility towards *Nigella sativa* aqueous extract

From the analysis, all generations of *Candida krusei* were found to be resistant to *Nigella sativa* aqueous extract (Table 4.2).

Table 4.2: The effect of phenotypic switching on the susceptibility of *Candida krusei* towards CHX, amphotericin B, nystatin, *Piper betle* and *Nigella sativa* aqueous extract. (R) is referred to resistance. The inhibition zones were the mean ± standard deviation (SD) with n=9. Note: Concentration of CHX is dependent to the concentration used in commercialized product. The concentration of amphotericin B and *Piper betle* are dependent to the concentration used in the determination of susceptibility by CLSI whereby the concentration of *Piper betle* is standardize to the concentration used in the determination of susceptibility of *Candida krusei* towards *Nigella sativa.*

Type of antimicrobial agents	**Active ingredients**	**Concentration**	**Growth generations**				
			Unswitched	1st switched	2nd switched	3rd switched	4th switched
			Inhibition zone (cm)				
Disinfectant	CHX	1.2 µg/µL	3.8 ± 0.1	3.5 ± 0.2	3.0 ± 0.1	3.4 ± 0.2	3.5 ± 0.1
Chemical based	Amphotericin B	100 µg/mL	2.2 ± 0.1	2.3 ± 0.3	2.4 ± 0.1	2.6 ± 0.3	2.4 ± 0.1
	Nystatin	250 unit/mL	2.4 ± 0.1	2.4 ± 0.1	1.9 ± 0.2	2.3 ± 0.1	2.6 ± 0.1
Plant based	*Piper betle*	200 mg/mL	2.2 ± 0.1	2.3 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	2.0 ± 0.2
	Nigella sativa	>200 mg/mL	R	R	R	R	R

Table 4.3: The MIC and MFC of CHX, amphotericin B, nystatin, *Piper betle* and *Nigella sativa* aqueous extract towards the unswitched and all switched generations of *Candida krusei* (n=9).

Growth generations	**CHX** (µg/µL)		**Amphotericin B** (µg/mL)		**Nystatin** (unit/mL)		***Piper betle*** (mg/mL)		***Nigella sativa*** (mg/mL)	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Unswitched	0.4	0.4	50	50	10	10	12.5	12.5	>200	>200
1st switched	0.4	0.4	50	50	10	10	12.5	12.5	>200	>200
2nd switched	0.4	0.4	50	50	10	10	12.5	12.5	>200	>200
3rd switched	0.4	0.4	50	50	10	10	12.5	12.5	>200	>200
4th switched	0.4	0.4	50	50	10	10	12.5	12.5	>200	>200

4.4 Growth curves of unswitched and switched generations of *Candida krusei* under treated environment

4.4.1 Disinfectant

4.4.1.1 Chlorhexidine (CHX)

Figure 4.11 showed the various growth curves plotted from the study. The growth curves of the unswitched and all switched generations have showed no significant difference among generations. However, slight deviations of growth curve were observed among the generations. The early log phase of unswitched and all switched generations of *Candida krusei* were determined at one hour incubation whereby the middle log phase were observed at 5.5 hours of incubation.

The specific growth rate (GR) of CHX treated *Candida krusei* was found to differ in unswitched and all switched generations. In the unswitched state, the GR of *Candida krusei* was determined at $3.618 \pm 0.051\ h^{-1}$. A gradual decreased in GR was observed in the 1st and 2nd switched generation with $0.597 \pm 0.029\ h^{-1}$ and $0.339 \pm 0.004\ h^{-1}$ (43.2%) respectively. The 2nd switched generation was determined as the lowest GR among generations of *Candida krusei*. However, the degree of GR was found to increase in the 3rd switched generation with $0.592 \pm 0.022\ h^{-1}$. A slight decreased in GR was determined in the 4th switched generation with $0.566 \pm 0.022\ h^{-1}$.

Consequently, the generation time (GT) of CHX treated *Candida krusei* was also identified to differ in unswitched and all switched generations. In the unswitched state, the GT of *Candida krusei* was determined at 3.566 ± 0.031 h. A slight decreased in GT was observed in the 1st and 2nd switched generation with 3.444 ± 0.035 h and

1.953 ± 0.028 h respectively with the 2nd switched generation was determined as the lowest GT among generations of *Candida krusei*. The degree of GT was found to increase in the 3rd switched generation with 3.414 ± 0.022 h. However, the 4th switched generation was observed to encounter a slight decrease in GT with 3.267 ± 0.025 h.

Table 4.4 and figure 4.14 showed the GR and GT of unswitched and all switched generations of *Candida krusei* were decreased. Among the generations, 2nd switched generations were observed to be the most influence in CHX growth condition with 52.1% reduction in GR. Whereby, the 1st switched generation was observed to be the least influence by 7.9% reduction. These are similar to the GT where the most influence generation was determine at the 2nd switched generation whereas the least influenced was the 1st switched generation with 52.2% and 7.9% GT reduction respectively.

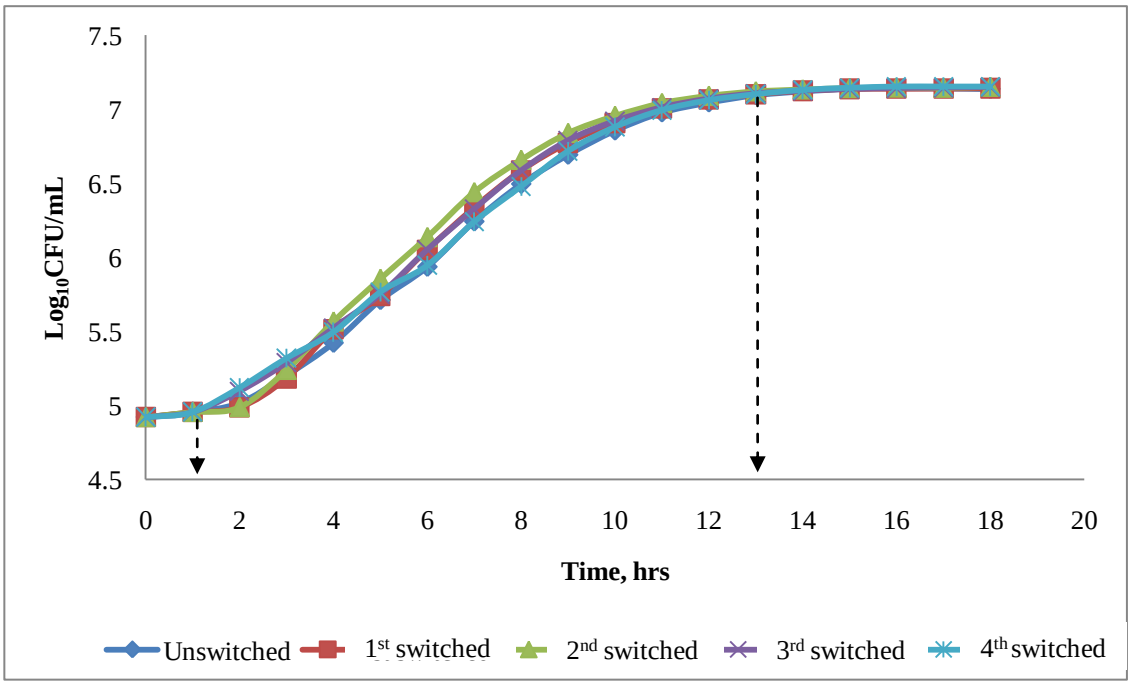


Figure 4.11: The growth curve (GC) of *Candida krusei*. A comparison between unswitched and all switched generations in CHX treated environment.

4.4.2 Chemical-based agent

4.4.2.1 Amphotericin B

Figure 4.12 showed the various growth curves plotted from the study. The growth curves of the unswitched and all switched generations showed no significant difference among all generations ($p>0.05$). Slight changes in the deviation of growth curve were observed among the generations. The early log phase of unswitched and all switched generations of *Candida krusei* were determined at one hour incubation whereby the middle of the log phase were achieved after five hours incubation.

The growth rate (GR) of amphotericin B treated *Candida krusei* was found to differ in unswitched and all switched generations. The GR of *Candida krusei* was determined at $0.585 \pm 0.013\ h^{-1}$ in unswitched stage. An increased in GR was observed in the 1st switched generation with $0.631 \pm 0.014\ h^{-1}$ and determined as the highest among generations. Following that, a decreasing in GR were observed in the 2nd and 3rd switched generations with GR of $0.585 \pm 0.017\ h^{-1}$ and $0.556 \pm 0.021\ h^{-1}$ respectively. However, the 4th switched generation was observed to have an increased in GR with $0.606 \pm 0.010\ h^{-1}$.

Consequently, the generation time (GT) of amphotericin B treated *Candida krusei* was also identified to differ in unswitched and all switched generations. In the unswitched state, the GT of *Candida krusei* was determined at 3.378 ± 0.051 h. An increased in GT was observed in the 1st switched generation with 3.643 ± 0.042 h and determined as the highest GT among the generations. A decreased in GT was identified in the 2nd switched generation to 3.377 ± 0.054 h whereby the lowest GT was observed

in the 3rd switched generation with 3.208 ± 0.073 h. However, the GT was found to increase in the 4th switched generation with 3.498 ± 0.081 h.

Table 4.4 and figure 4.14 showed the GR and GT of unswitched and all switched generations of *Candida krusei* were decreased. Among the generations, 3rd switched generations were observed to be the most influenced in amphotericin B growth condition with 19.3% reduction in GR. Whereby, the 1st switched generation was observed to be the least influence by 2.6% reduction. These are similar to the GT where the most influence generation was determine at the 3rd switched generation whereas the least influenced was the 1st switched generation with 19.3% and 2.6% GT reduction respectively.

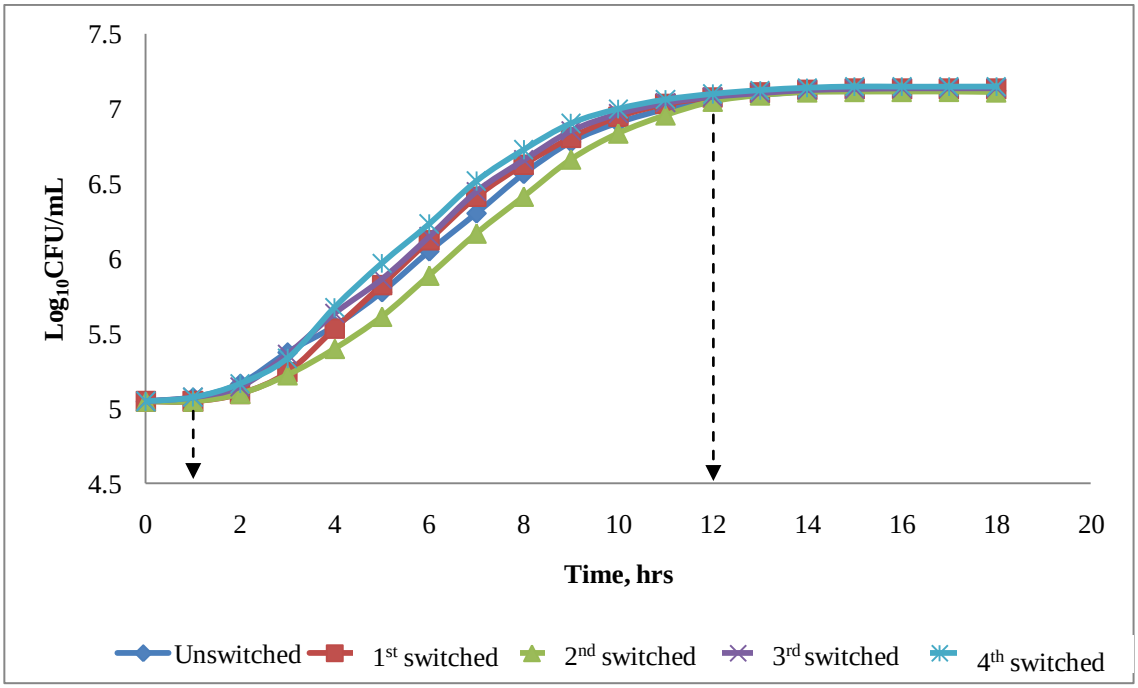


Figure 4.12: The growth curve (GC) of *Candida krusei*. A comparison between unswitched and all switched generations in amphotericin B treated environment.

4.4.3 Plant-based extract

4.4.3.1 *Piper betle* aqueous extract

Figure 4.13 showed the various growth curves plotted from the study. The growth curves of the unswitched and all switched generations has no significant difference among all generations ($p>0.05$). However, slight deviations of growth curve were observed among the generations. The early log phase of unswitched and all switched generations of *Candida krusei* were determined at one hour incubation whereby the middle of the log phase were achieved after seven hours incubation.

The growth rate (GR) of *Piper betle* treated *Candida krusei* was found to differ in unswitched and all switched generations. In the unswitched state, the GR of *Candida krusei* was determined at 0.560 ± 0.044 h^{-1}. A drastic decreased of GR was observed in the 1st switched generation with 0.387 ± 0.053 h^{-1} (30.9%) and was the lowest GR among generations of *Candida krusei* (Figure 4.20). However, the 2nd switched generation has showed an increased in GR with 0.507 ± 0.031 h^{-1} (31%) followed by 3rd and 4th switched generations with GR 0.532 ± 0.032 h^{-1} (4.9%) and 0.586 ± 0.132 h^{-1} (10.2%) respectively. The GR of the 4th switched generation was determined as the highest among unswitched and switched generations of *Candida krusei*.

The generation time (GT) of *Piper betle* treated *Candida krusei* was also identified to differ in unswitched and all switched generations. In the unswitched state, the GT of *Candida krusei* was determined at 3.233 ± 0.321 h. The GT was observed to decreased in the 1st switched generation with 2.235 ± 0.231 h and determined as the lowest GT between generations. The 2nd switched generation showed an increased in

GT with 2.923 ± 0.221 h followed by the 3rd and 4th switched generations with GR 3.069 ± 0.234 h and 3.382 ± 0.312 h respectively. The 4th switched generation was determined as the highest GT among unswitched and all switched generations of *Candida krusei.*

Table 4.4 and figure 4.14 showed the GR and GT of unswitched and all switched generations of *Candida krusei* were decreased. Among the generations, 1st switched generation was observed to be the most influence in *Piper betle* growth condition with 43.4% reduction in GR. Whereby, the 4th switched generation was observed to be the least influenced by 16.3% reduction. These are similar to the GT where the most influenced generation was determine at the 3rd switched generation whereas the least influenced was the 1st switched generation with 40.2% and 16.3% GT reduction respectively.

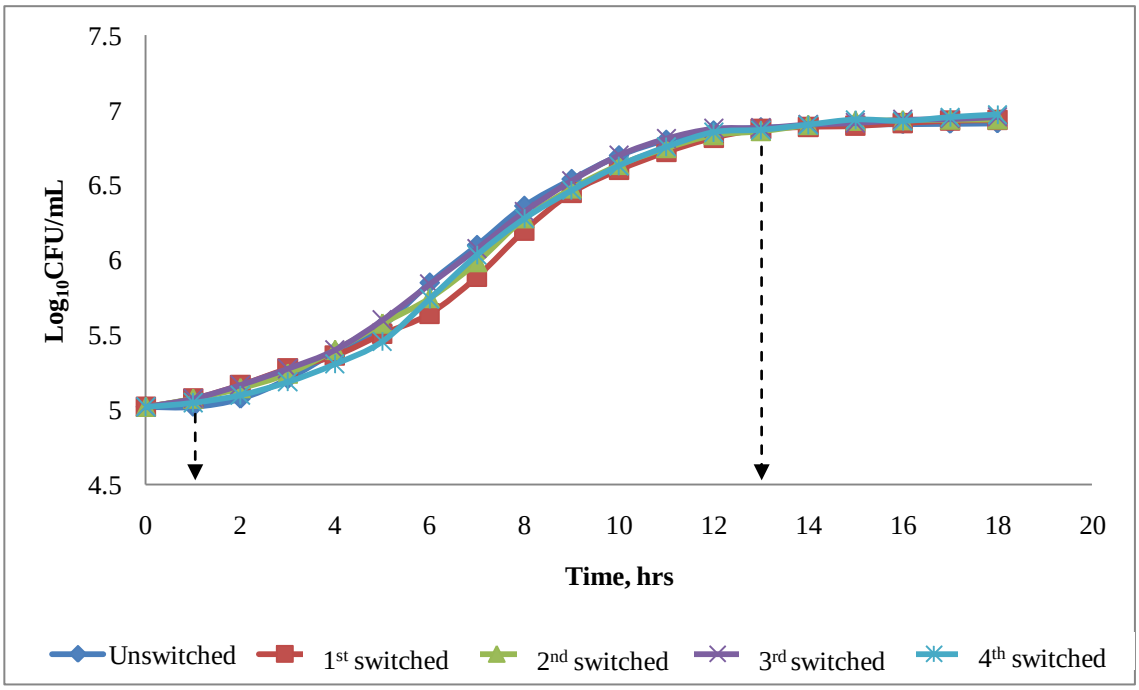
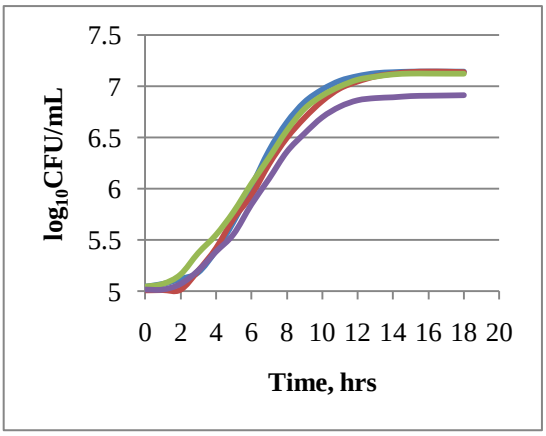
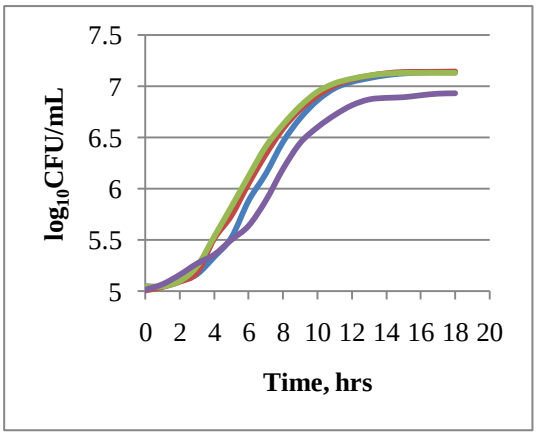


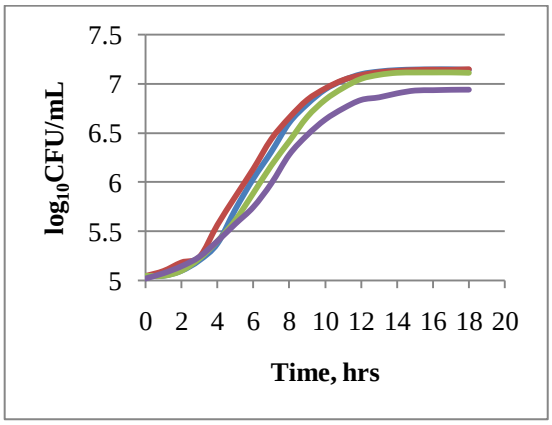
Figure 4.13: The growth curve (GC) of *Candida krusei*. A comparison between unswitched and all switched generations in *Piper betle* aqueous extract treated environment.



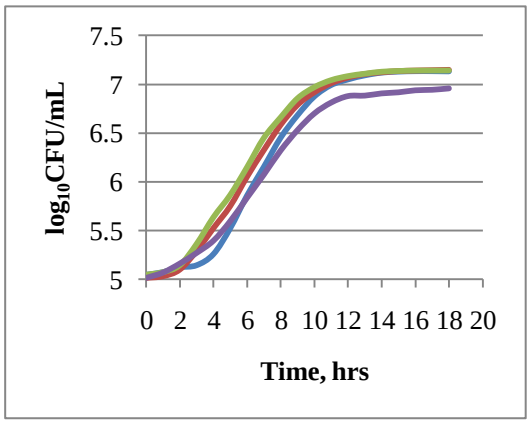
Unswitched



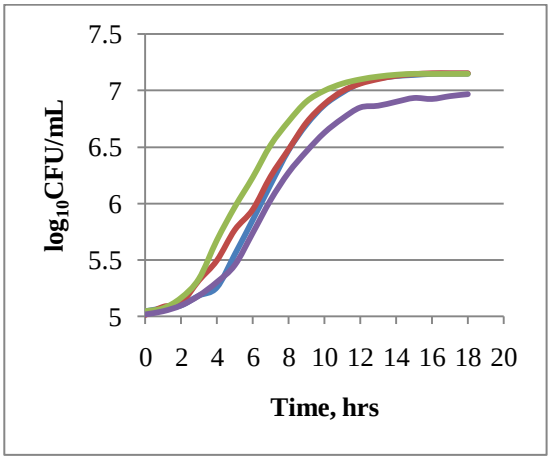
1st switched



2nd switched



3rd switched



4th switched

Figure 4.14: The growth curve (GC) of unswitched and switched *Candida krusei* of untreated (—), CHX (—), amphotericin B (—) and *Piper betle* (—) treated growth environment.

Table 4.4: The changes in the generation times (GT) and specific growth rate (GR) of unswitched and all switched generations of *Candida krusei* when their growth were perturbed with the introduction of CHX, amphotericin B and *Piper betle*. The concentrations used in the study were dependent to the concentration of sub-MIC.

Growth conditions	growth rate (GR) and Generation times (GT)	Growth generations				
		Unswitched	1st switched	2nd switched	3rd switched	4th switched
Untreated	GR (h^{-1})	0.677 ± 0.021	0.648 ± 0.131	0.708 ± 0.021	0.689 ± 0.132	0.700 ± 0.100
	GT (h)	3.905 ± 0.031	3.740 ± 0.101	4.085 ± 0.001	3.976 ± 0.102	4.041 ± 0.005
CHX	GR (h^{-1})	0.618 ± 0.051	0.597 ± 0.029	0.339 ± 0.004	0.592 ± 0.022	0.566 ± 0.022
	GT (h)	3.566 ± 0.031	3.444 ± 0.035	1.953 ± 0.028	3.414 ± 0.022	3.267 ± 0.025
Amphotericin B	GR (h^{-1})	0.585 ± 0.013	0.631 ± 0.014	0.585 ± 0.017	0.556 ± 0.021	0.606 ± 0.010
	GT (h)	3.378 ± 0.051	3.643 ± 0.042	3.377 ± 0.054	3.208 ± 0.073	3.498 ± 0.081
Piper betle	GR (h^{-1})	0.560 ± 0.044	0.387 ± 0.053	0.507 ± 0.031	0.532 ± 0.032	0.586 ± 0.132
	GT (h)	3.233 ± 0.321	2.235 ± 0.231	2.923 ± 0.221	3.069 ± 0.234	3.382 ± 0.312

The values were the mean ± standard deviation (SD) of triplicates from three determinations (n=9).

5.0 DISCUSSION

In this study, phenotypic switching of *Candida krusei* was induced by the addition of phloxine B (tetrabromotetrachlorofluorescein) which acts as switching detecting agent for *Candida* sp. The addition of phloxine B in the media has created a nitrogen suppressed growth condition for *Candida krusei* (Coote, 2001). The chemical compounds in Phloxine B dye have been shown able to facilitate the detection of mutant yeast that lack the capability synthesizing purine and pyramidine bases or amino acids. It has also been reported that phloxine B is not a growth inhibitor, but it promotes death of candidal yeast under nitrogen limitation conditions (Middelhoven et al., 1976). Thus, the application of phloxine B in the growth environment had caused *Candida krusei* cells to switch in order to overcome the killing factors provided by the dye.

Candida krusei in this study exhibited characteristic of cream to whitish colour, dry and rough surface appearance with undulate margin, circular forms and umbonate elevation when cultured on YEPD agar. Samaranayake and Samaranayake (1994) had earlier described the colony morphology of *Candida krusei* as matt, rough surface appearance with cream to whitish colour on SDA. Both observations did not conform to the characteristics given in the ATCC manual which described *Candida krusei* as butyrous surface with entire margin on SDA. Variations in the colony characteristics may be due to the different growth medium used. This may affect the development of colony morphology of *Candida krusei*. YEPD agar is a medium containing rich mineral of zinc and able to suppress the expression of phenotype. Nevertheless, it does not influence the switching system of *Candida* sp. (Odds *et al.*, 1989) and therefore the usage of YEPD in the study did not interfere with the phenotypic switching determination.

The colony morphology in each generation of switched *Candida krusei* was observed different in terms of surface appearance, margin, form and elevation. This finding was similar to the reports on *Candida albicans* and *Candida glabrata* where various colony morphology phenotypes were observed at different growth generations including smooth, myceliated and wrinkled surface appearance (Vargas *et al.*, 2004). In our study, we had observed the transition in the colony morphology characteristics between the 2nd and 3rd switched generations which are similar to the reported findings on *Candida albicans* which showed to exhibit predominant transition between unmyceliated to myceliated colony (Soll *et al.*, 1987). Phenotypic switching phenomenon could also occur after a prolonged incubation (Slutsky *et al.*, 1985; Slutsky *et al.*, 1987) which may enhance the development of different colony morphology of *Candida krusei*. These different switched phenotypes act as a survival strategy of *Candida krusei*, as different phenotype serve a different role in providing adaptability and survivability at different condition (Soll, 1992).

CHROMagar is a chromogenic medium which is widely used in the identification and detection of yeasts including *Candida krusei* (Hospenthal *et al.*, 2006). The superiority to inhibit the growth of bacterial strains was determined to be higher compared to SDA which is generally used in the identification of candidal species (Sivakumar *et al.*, 2009). From the study, the colonies of *Candida krusei* were determined to grow pink in colour with pale border, dry and rough surface appearances, undulate margin, circular form and umbonate elevation which are similar to the finding by Hospenthal *et al.* (2002). The colourization of the colony is due to the reaction of specific enzymes produced by *Candida krusei* towards chromogenic substrates yielding microbial colonies expressing specific pigmentation, hence allowing the confirmation of

the species by the detection of colour and colony morphology of the candidal strains (Sivakumar *et al.*, 2009).

The recovery population determined the sustainability of each switched generation of *Candida krusei* under suppressed growth environment. In our study, the recovery population of the 3rd switched generation was found to be the highest recovered at 85.7% followed by the 4th generation at 70.8%. 1st switched generation was identified to have recovery percentage of 46.6% and the 2nd switched generation was determined to have the least recovery population with only 36.4%. The difference on the recovery population was suggested to occur due to the different phenotype plasticity among switched generations. Similar findings on the various population recovery was reported where different switched generations possessed different percentage recovery population, thus, suggested that different generations represent different survival ability and stability due to the suppressed environment (Lackhe *et al.*, 2000).

From the study, the unswitched and all switched generations of *Candida krusei* were identified to ferment only glucose out of 19 other substrates including glucose, glycerol, 2-keto-D-gluconate, L-arabinosa, D-xylose, adonitol, xylitol, galactose, inostol, sorbitol, α-methyl-D-glucoside, N-acetyle-D-glucosamine, cellobiose, lactose, maltose, sucrose, trehalose, melezitose and raffinose. According to Melville and Russells (1975), *Candida krusei* can ferment dextrose, producing acid and gas. This phenomenon was reported similar with the finding by Samaranayake and Samaranayake (1994) where *Candida krusei* was reported to ferment only glucose out of a large panel of carbohydrates. The unswitched and all switched *Candida krusei* were observed

fermenting N-acetyl-D-glucosamine ($C_8H_{15}NO_6$) as a carbon source. This may suggest that all generations of *Candida krusei* are able to ferment N-acetyl-D-glucosamine which is a derivative of the monosaccharide glucose. *Candida krusei* was also determined as a pathogenic microorganism which is able to grow in vitamin-free media (Odds, 1988). From the study, γ-aminobutyric acid (GABA) was found to be one of the nutrient sources for the unswitched and 1st switched generations of *Candida krusei.* According to Kumar and Punekar (1997), most yeasts and fungi utilise GABA as a source of carbon and nitrogen. This substrate was identified as an important agent which associate to the sporulation and spore metabolism of the yeast. Information on the role of GABA in fungal biology is gradually increasing.

Based on light microscope observation, in general *Candida krusei* forms elongated pseudohyphae with elongated to ovoidal blastoconidia and budding off verticillate branch. These characteristics conform to the description on cellular characteristics of *Candida krusei* by Samaranayake and Samaranayake (1994). *Candida krusei* was also described in the ATCC manual as 'long grain rice' shaped yeast with branched pseudohyphae and elongated blastoconidia.

However, based on SEM micrograph, some variations in the cell morphology characteristics were observed throughout different switched generations, which could occurred due to some environmental constrains during the blastoconidia-hyphae transitions. The transition of smooth to pimpled and punctate morphology in the 3rd to the 4th switched generation of *Candida krusei* observed in our study was similar to the response as the transition of white to opaque cell in *Candida albicans* switched generations. According to Soll (1992), the formation of pimpled and punctate

characteristic observed in the ultrastructure of candidal cells could be an outcome of blastoconidia and pseudohyphae maturity in each level of the switched generations. In addition, the variant colony morphologies have been described in several reports to be dependent on the proportion and distribution of blastoconidia and pseudohyphae. Their presence could have led to the changes in the colony morphology of the switched *Candida krusei* (Vargas *et al.*, 2004).

In the study, 2nd switched generation of *Candida krusei* was identified to be more extended compared to other generation. According to Anderson and Soll (1987), this extension which also occurs among switched *Candida albicans* is due to the distribution of actin granules which is mostly found on the apex of the pseudohyphae and the generations of various characteristics of pseudohyphae were dependent on the pattern of actin granule distribution between growing blastoconidia and pseudohyphae in the candidal strains. It is also suggested that the hyphae-specific genes may be transiently recruited among switched *Candida krusei* as an adaptation to the environmental changes which then led to the different dimension and size of the cell of *Candida krusei*. Thus, hyphae-specific function and hyphae specific gene expression were identified to play an important role in generating unique phenotype at different switched generation of *Candida krusei*.

From the susceptibility tests, all switched generations of *Candida krusei* were found to be susceptible to CHX. According to several researches, CHX affects the plasma membrane of the candidal cell by non-specific binding to the negatively charged protein and phospholipid moieties of the cell wall. This binding will then alter the cellular membrane structure and interfere with the cellular osmotic balance that lead to

the susceptibility of candidal strains (Freitas *et al.*, 2003; Bonacorsi *et al.*, 2004; Veerman *et al.*, 2004) towards CHX. In addition, this study demonstrated that the unswitched and all switched generations of *Candida krusei* were susceptible to amphotericin B. According to Anil (2002) and Williams *et al.* (2011), amphotericin B is grouped as polyene which acts as a broad spectrum of fungicidal and fungistatic. This polyene affects the composition of the sterol on the cell wall of the target cells which then damage the cell walls. The damaging caused potassium ions and glucose to be released out from the cell, disturbing the glycolysis which finally inhibits the growth of the candidal cells.

The unswitched and all switched *Candida krusei* were found to be susceptible to nystatin. This sensitivity occurred due to the mechanism of altering the cell permeability of candidal strains that induce cell porosity (Kerridge, 1986). The interaction between nystatin and ergosterol component within the cell membrane influence the cell permeability due to the lost of cytoplasmic membrane which then lead to the mortality of *Candida krusei* (Williams *et al.*, 2011).

The study has shown that the unswitched and all switched *Candida krusei* were also susceptible to *Piper betle* aqueous extract. *Piper betle* was classified as antifungal agents having the potential of damaging the cell membrane of the candidal species which lead to the lost of the cell viability and leakage of the intracellular constituents (Indu and Ng, 2002; Guha, 2006). The active components such as hydroxichavicol, stearic acids and hydroxyl fatty acids esters has extensively reported as the antibacterial and antifungal agents and widely used in traditional therapeutic (Pauli *et al.*, 2002; Nalina and Rahim, 2007).

In this study, some variations in the degree of susceptibility between the various switched generations displayed the responses of the switched cells to survive and attain overall fitness. In other words, as described by Vargas (2004), when a cell undergo switching, many of its features such as cell physiology, antigenicity of the cell surface, the composition of its basic molecules like protein, lipid and sugar may be altered and stimulated in the attempt to achieve the best adaptability to the environmental constrain.

All generations of *Candida krusei* in our study had shown the ability to adhere to the surfaces of saliva-coated glass beads. This finding was reported by Samaranayake *et al*. (1994) as *Candida krusei* was found to adhere higher on inert surfaces compared to the buccal epithelial cells (BEC). In addition, *Candida krusei* was also reported to exhibit high hydrophobicity ability which encouraged adherence. The hydrophobicity of *Candida krusei* was identified to have 5-fold greater than *Candida albicans* (Samaranayake *et al*., 1994). Nevertheless, our study had found that the adherence ability varied among switched generations of *Candida krusei*. The adherence of all switched *Candida krusei* were found to be higher compared to the unswitched generation. The 2nd switched generation was determined to have the highest adherence ability followed by 3rd, 1st and 4th switched generation. According to Jones *et al.* (1994), phenotype switched can change the ability of *Candida* sp. to attach to a surface. In addition, the type or form of hyphae following phenotypic switch has been found to influence the adherence of candidal cells to inert surfaces (Jones *et al.*, 1994). This might explain the highest adhering ability exhibited by the 2nd switched generation cells (Anderson and Soll, 1987).

From the study, the unswitched and all switched generations of *Candida krusei* showed varying degrees of responses following exposure to antimicrobial agents. The unswitched and all switched generations were observed to influence in antimicrobial presenting growth environment. The reducing in GR and GT in all growth generations indicated that the microbial agents showed efficacy in the treatment of *Candida krusei.* These different responses in the growth activities were determined as an outcome of phenotypic switching. Regulation of the growth activities could be an attempt to maintain the fitness of the cells to survive under adverse conditions (Vargas *et al.*, 2004). According to Cowen *et al.* (2001), an observation on the ability of *Candida albicans* to adapt to the inhibitory concentration in fluconazole treated environment found that the strain was producing different genes expression that involve in drug resistance which then lead to the variability in the generation time (GT) of some isolates that had been generated from one progenitor which suggested similar response occurred in the phenotypic switching of *Candida krusei* presented in this study.

6.0 CONCLUSION

This study has determined the phenotypic switching capability of *Candida krusei* which contributed to the changes in the biological characteristics as well as the adherence capacity towards hard surface. The various in responses among unswitched and switched *Candida krusei* towards CHX, nystatin, amphotericin B and plant extracts (*Nigella sativa* and *Piper betle*) indicated that the phenotypic switching affects the susceptibility of the candidal strains. Thus, it is concluded that the phenotypic switching of *Candida krusei* leads to the pathogenic property in the oral cavity.

7.0 FUTURE STUDIES

1) To determine the biological characteristics of phenotypic switched genetically *Candida krusei*.

2) To determine specific genes which enhance the phenotypic switching properties of *Candida krusei.*

3) To characterize mono-species biofilm (MSB) and dual-species biofilm (DSB) of phenotypic switched *Candida krusei* and non-krusei on denture acrylic surface.

4) To determine the factors affecting biofilm formation of MSB and DSB of phenotypic switched *Candida krusei* and non-krusei.

5) To evaluate the consequences of MSB and DSB of phenotypic switching *Candida krusei* and non-krusei on the susceptibility towards active component of *Piper betle* aqueous extract.

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GEN 02

Anti-adhesion Effect of Chlorhexidine (CHX) against *Candida tropicalis* to Acrylic Denture Plates

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Abstract

Candida tropicalis is one of the oral microorganism species that form the microbial plaque which can accumulate on the fitted surfaces of dentures. Chemical cleaning methods are more effective for daily denture cares because it's usually contains chlorhexidine gluconate which is an antimicrobial agent. The objectives of this study is to determined the anti adhesion effect of chlorhexidine (CHX) against *Candida tropicalis* to acrylic denture plates. Oradex is a commercialized oral rinse that contains 0.12% clorhexidine gluconate as the active compound. The supernatant of saliva was collected and used in the experiment. The denture plates were washed with CHX and sterile distilled water before immersing in saliva. The plates were then tested with undiluted CHX, diluted CHX and distilled water as the control. After that, they were immersed in *Candida tropicalis* suspension, transferred into saline solution and sonicated. The resulting suspensions were then incubated at 37°C for 2 days before calculating the CFU. The plates that were immersed in undiluted CHX had the lowest CFU followed by the diluted CHX. The control plates that were immersed in distilled water had confluent growth indicating the absent of anti adherence capability. The findings suggest that CHX is useful for controlling the adhesion of *C. tropicalis* against acrylic denture plates and thus, oral rinses containing CHX agent can be used to soak the denture plates in order to control microbial adhesion.

Introduction

Microbial plaque that accumulates on the fitted surfaces of dentures is composed of several oral microorganism species, including *Candida tropicalis*. Several denture cleaning methods are used clinically for the reduction of denture plaque, debris and stains and these are generally divided into mechanical and chemical cleaning methods. It has been proven that chemical cleaning methods are more effective and indispensible for daily denture care. There are two types of oral rinse that is medicated and non-medicated oral rinses. Medicated oral rinse usually contains chlorhexidine gluconate. Chlorhexidine is an antimicrobial agent which has a wide spectrum of antibacterial activity encompassing Gram positive and Gram negative bacteria, yeast and other microbes.

Objectives

The study was carried out with the objective of determining the anti-adhesion effect of CHX against *C. tropicalis* to acrylic denture plates.

Materials and Methods

Oral rinse

Commercialized oral rinse used in the experiment is Oradex containing 0.12% chlorhexidine gluconate as the active compound.

Collection of saliva

Volunteers were required to rinse their mouth with distilled water, followed by chewing a piece of rubber band to stimulate the salivary glands to produce excess saliva. The saliva was then collected in an ice chilled tube and was centrifuged at 17,000g for 30 minutes. Supernatant was obtained and used in the experiment.

Disinfection of acrylic resin denture plates

Firstly, the denture plates were soaked in CHX for 20 min to ensure the sterility of the denture plates. Secondly, the denture plates were washed with sterile distilled water thrice and stored in sterile containers containing sterile distilled water.

Saliva coating of acrylic resin denture plates

The sterile denture plates were immersed in saliva prepared as above, for 2 min, after which the saliva was decanted and the denture plates were rinsed with sterile distilled water. The denture plates are now coated with the experimental pellicle from saliva.

Exposure of CHX treated denture plates to Candida tropicalis

The saliva-coated denture plates were divided into 3 test groups. The first group was tested with undiluted CHX containing 0.12% CHX. The second group was tested with 2 times dilution of CHX. And the last group was using only distilled water as the control group. The treated denture plates were immersed in these solutions for 1 minute. Following this exposure, the denture plates were immersed in 2 x 10^7 cfu/ml of *Candida tropicalis* suspension for 2 h at 37°C. The denture plates were then washed 3 times with normal saline. The final wash was retained and sonicated for 15 min, after which the resulting suspensions were plated on SDA plates. The SDA plates were incubated at 37°C for 48 h. Finally, the CFU counts were carried out.

Results and Discussion

Currently in the field of dentistry, there is a need to apply innovative methods and techniques which could assist in reducing and preventing microbial colonization on denture plates. Studies have indicated that denture wearers, especially elderly people had a high *Candida* sp. accumulating on the denture plates which could finally lead to other dental problems (Arita et al., 2005). As reported by Arita et al. (2005), a few studies have been carried out elsewhere to develop a cheap and reliable apparatus that is easy to use.
In our study, we prepared the acrylic resin denture plates by coating with saliva to mimic the actual oral surfaces in the oral environment. These saliva-coated denture plates were then treated with CHX and finally immersed in the 2x10^7 *Candida tropicalis*. The usage of CHX was selected as this antimicrobial agent has been reported in many studies to have good antimicrobial effect against oral microorganisms (Arita *et al.* 2005). The steps finally involved sonication of the denture plates in order to detached the adhered *C. tropicalis* to the treated denture plates. The anti-adhesion effect of chlorhexidine (CHX) against *C. tropicalis* adhering

to acrylic resin denture plates were confirmed by the numbers (CFU/mL) of *C. tropicalis* growth on Sabouraud agar (SDA).

The results showed that when acrylic denture plates were soaked in distilled water, a high number of *C. tropicalis* were able to adhere to the plates, which was shown by a confluent growth of the microbes on SDA. This was possibly due to the absent of anti-adhesion effect in distilled water which could assist in inhibiting the adherence of *C. tropicalis* to the denture plates.

In contrast, when the plates were coated with CHX, the adherence of *C. tropicalis* was reduced tremendously to only 100 CFU/mL of microbes (Figure 1). However, when two times dilution of CHX was used to coat/treat the acrylic denture plates, an increase amount of 300 CFU/ml of *C. tropicalis* were found to be able to adhere to the CHX-treated denture plates. The increased in *C. tropicalis* population when using diluted CHX-treated denture plates indicates that less anti-adhesion effect was exhibited in the experiment.

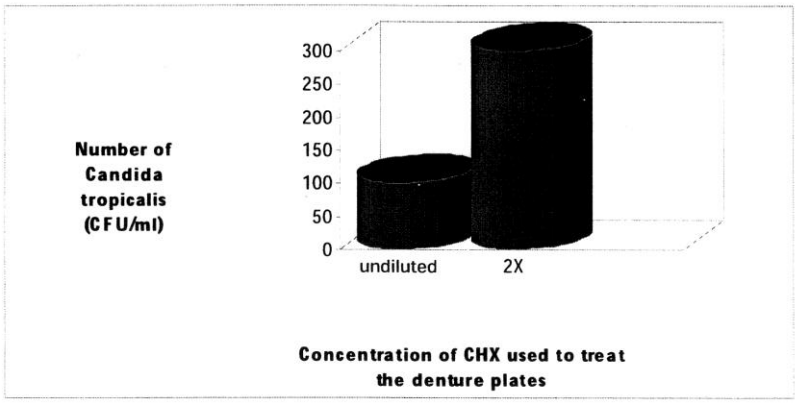

Figure 1 : Anti-adhesion effect of different concentration of CHX against *Candida tropicalis* to acrylic denture plates

Conclusion

The findings suggest that CHX is useful to control adhesion of *C. tropicalis* against acrylic denture plates and thus, oral rinses containing CHX agent can be used to soak the denture plates in order to control microbial adhesion.

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Abstract. The 2nd Thailand International Conference on Oral Biology, TiCOB, Thailand, 2008.

B-16 **Phenotypic switching pattern of *Candida krusei* in nitrogen limited growth environment**

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Objective: To analyze the switching pattern of *C. krusei* in nitrogen limited growth environment. **Methods:** *Candida krusei* colonies were randomly selected and isolated from samples originating from the oral cavities of three different target groups; individuals with healthy oral cavity, periodontal diseased patients and denture wearers. Colonies representative of each group was then grown on yeast extract potato dextrose (YEPD) agar containing 5 mg Phloxine B dye. Following a 5 days incubation period, colonies showing a switch in the colour from the original were counted and considered as having a phenotypic switch in the first generation. These colonies were again subcultured on new media plates. Phenotypic switching of *Candida krusei* was repeated and observed for four generations. **Results:** Phenotypic switching of *Candida krusei* obtained from the oral cavity of denture wearers showed an increased pattern until the third generation (14.80 x 107 cfu/ml) but reduced drastically in the fourth generation. In contrast, phenotypic switching of *Candida krusei* from periodontitis patients more suppressed in the first and second generation, but increased switching was observed in the third generation (9.99 x 107 cfu/ml). However, the ability to phenotypically switched was induced in the fourth generation. The control group of healthy oral cavity showed similar pattern with *Candida krusei* from denture wearers but maximum phenotypic switching was in the 2nd generation with the highest phenotypic switching rate (19.00 x 107 cfu/ml) compared to the other two groups. **Conclusion:** *Candida krusei* was observed to have the ability to switch its phenotypic characteristic when the growth condition is unfavourable. The switching pattern was more active and prominent in *Candida krusei* isolated from healthy oral cavity compared to those that have periodontal problems and wearing dentures.

Keywords: *Candida krusei,* phenotypic switching

Abstract. IADR PAPF/APR, Wuhan, China, 22th to 24th September 2009.

Paper: The-Effect-of Phenotypic-Switching of Candida krusei on its Susceptibility towards Fluconazole-and-Voriconazole (2nd Meeting of IADR Pan Asian Pacific Federation (PAPF) and the 1st Meeting of IADR Asia/Pacific Region (APR) (Sept. 22-24, 2009))

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722 The-Effect-of Phenotypic-Switching of *Candida krusei* on its Susceptibility towards Fluconazole-and-Voriconazole

Location: Grand Ballroom (2nd Floor) (Shangri-La Hotel)

A. HAFIZ, A. FATHILAH, and W. HIMRATUL-AZNITA, Department of Oral Biology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

Objective:

To compare the effect of phenotypic switching of *Candida krusei* on its susceptibility towards fluconazole and voriconazole

Methods:

Candida krusei was revived from ATCC stock and cultured using spreading technique on Yeast Extract Potato Dextrose (YEPD) agar containing 5 mg Phloxine B dye. Following a 5-days incubation period, colonies showing a switch in the colour from the original were counted and considered as having a phenotypic switch in the first generation. These colonies were then subcultured on new media plates. Phenotypic switching of *Candida krusei* was repeated and observed for four generations. Each generation was tested for their response to fluconazole and voriconazole disks on Mueller-Hinton (MH) agar plate and incubated for 24 hours at 37ºC.

Results:

Candida krusei in the first to the third generation was found to be resistant to fluconazole. In contrast, a mild susceptibility was observed in the fourth switched generation. Alternatively, *Candida krusei were sensitive* for all switched generations towards voriconazole. However, the sensitivity level of *Candida krusei* was found to increase relative to the number of switched generations, as observed by the bigger clearance zone in the antifungal disc diffusion experiment.

Conclusion:

The susceptibility of *Candida krusei* towards the antifungal agents fluconazole and voriconazole was found to be greatly affected by its phenotypic status.

See more of: Poster Session III
See more of: Scientific Groups

<< Previous Abstract | Next Abstract >>

Abstract. 3rd Dental Postgraduate Students Seminar 2010, Kuala Lumpur, 29th to 30th June 2010.

Characterization Of The Virulence Factors Of *Candida krusei*

Hafiz, A., A. R. Fathilah, and W.H. Himratul-Aznita

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Objectives: The purpose of this study was to evaluate the consequences of phenotypic switching generation towards adherence; susceptibility towards chlorhexidine (CHX) and growth profile of each of the switched *Candida krusei* generations. **Methods:** Four generations of phenotype switched *Candida krusei* and pure ATCC 14243 *Candida krusei* were subcultured from glycerol stock. Samples were evaluated for the adherence ability by using the NAM model. Disk diffusion test, Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) assay were carried out using CHX. The effect of the CHX on the growth profile of all switched generation of *Candida krusei* was also being carried out according to the standard procedures. **Results:** The adherence test showed the 2nd generation *Candida krusei* had the highest adherence in saliva coated glass beads. Furthermore, the second generation of *Candida krusei* has shown to be the least susceptible towards CHX compared to other generations. Under the influence of CHX, the 2nd generation of *Candida krusei* showed the fastest growth during the log phase and the earliest to reach the stationary phase compared to the other generations. **Conclusion:** The 2nd switched generation was found to be the most virulent stage compared to the other 3 generations.

Abstract. My1Bio Conference 2010, Kuala Lumpur, 30th to 31th October 2010.

Poster 3.15

THE RESPONSE OF PHENOTYPICALLY SWITCHED *CANDIDA KRUSEI* TO THE EXTRACT OF *PIPER BETLE*

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Objective: To study the response of phenotypically switched *Candida krusei* to extract of *Piper betle*. **Methods:** *Candida krusei* (ATCC 14243) was revived from stock culture on YEPD agar containing 5 mg Phloxine B dye. Following a 5-days incubation period, colonies showing a different colony morphology were enumerated and considered as having a phenotypic switch from the original culture. These colonies were considered as the first generation of *Candida krusei* and subcultured on new media plates to observe for subsequent swiching. The phenotypic switch was repeated and observed for four generations. Each generation was tested for antifungal susceptibility towards *Piper betle* aqueous extract by disc diffusion test, minimum inhibitory concentration test (MIC), minimum fungicidal test (MFC) and growth profile. **Results:** Based on disc diffusion test, it was found that all switched generations were susceptible to *Piper betle* extract with an ascending sensitivity from the first to the fourth generation. The growth of each generation was influence by *Piper betle* extract with decreasing in OD% at stationary phase from untreated *Candida krusei* with 16% (first generation), 20% (second generation), 18% (third generation) and 16% (fourth generation). **Conclusion:** The susceptibility and growth profile of each switched generations of *Candida krusei* was found to be greatly affected in the presence of *Piper betle* aqueous extract.

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