RESEARCH ARTICLE

Effect of phenotypic switching on the biological properties and susceptibility to chlorhexidine in *Candida krusei* ATCC 14243

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Abstract

Phenotypic switching is characterized as a virulence factor of *Candida* spp. This study was carried out to evaluate the phenotypic switching ability of *C. krusei* ATCC 14243 and to determine its effect on the biological properties, adherence capacity and susceptibility towards chlorhexidine digluconate (CHX). To induce switched generations *C. krusei* was cultured under nitrogen-depleted growth conditions by adding phloxine B. These phenotypically switched colonies were designated as the 1st generation. Subsequent sub-culturing was performed to produce the 2nd, 3rd and 4th switched generations. The recovery of the 3rd generation was the highest at 85.7% while that of the 4th generation was lower at 70.8%, and the recovery of the 1st and 2nd generations gradually reduced to 46.6% and 36.4%, respectively. All generations of *C. krusei* were susceptible towards CHX. The unswitched *C. krusei* was the most susceptible but the least adherent to coated hard surfaces. The 2nd generation was the least susceptible, but with the highest adherent ability. The minimum inhibition concentration and minimal fungicidal concentration of *C. krusei* of all generations were determined at 0.4 mg mL^{-1}. These observations suggest that the switching activity of *C. krusei* induces changes to its biological properties and susceptibility towards CHX.

Introduction

Candida krusei is one of the causative agents of oral candidiasis after *C. albicans*, *C. tropicalis* and *C. glabrata* (Scully *et al.*, 1994). *Candida krusei* has the dimensions 2.2–5.6 × 4.3–15.2 μm (Samaranayake & Samaranayake, 1994). In general, the colony is whitish to cream in colour with rough surface and appearance, and butyrous with entire margin on Sabouraud's dextrose agar (SDA). Microscopically, the cell has ovoid-ellipsoidal morphology with pseudohyphae formation. In terms of reproduction, *C. krusei* is known to breed either sexually or asexually (Kurtzman *et al.*, 1980; Barnett *et al.*, 1983; Odds, 1988). Infection cases involving *C. krusei* were found to increase dramatically, especially among immuno-compromised patients, drugs abusers, patients with human immunodeficiency virus (HIV), infants, as well as in elderly people (Samaranayake & Samaranayake, 1994; Imbert *et al.*, 2003). *Candida krusei* was also found to be sensitive towards common antifungal agents such as amphotericin B, but resistant towards fluconazole (Samaranayake, 1997; Abbas *et al.*, 2000; Pelletier *et al.*, 2005).

Phenotypic switching has been identified as one of the important virulent factors of *Candida* spp. Phenotypically switched cells have been isolated in several cases including those from individuals with HIV (Vargas *et al.*, 2004). Under the switched condition, *Candida* spp. can rapidly adapt to different growth environment (Odds & Merson-Davies, 1989; Odds, 1994). This switching property can be manipulated through ultra-violet radiation and alteration in its growth requirements (Middelhoven *et al.*, 1976; Haynes, 2001). Studies on phenotypic switching have been focused on *C. glabrata* and *C. albicans* (Anderson & Soll, 1987; Jones *et al.*, 1994; Lackhe *et al.*, 2000, 2002; Vargas *et al.*, 2004), and until today, information with regard to *C. krusei* is still scarce.

The objectives of this research were to characterize the phenotype of four generations of switched *C. krusei* cells and to observe the effect of the switching on the biological properties and susceptibility towards chlorhexidine

digluconate (CHX). In this study, biological properties referred to cell and colony morphology as well as adherence activity.

Materials and methods

Preparation of stock culture

Candida krusei ATCC 14243 obtained from the American Type Culture Collection (ATCC) was rehydrated with 0.5 mL of sterile distilled water. 100 μL of the suspension was then inoculated on to yeast extract peptone dextrose (YEPD) agar. Following incubation at 37 °C for 24 h, the colonies were picked and subcultured on YEPD slants. The slants were kept refrigerated at 4 °C for use in the experiments. 20% glycerol stocks of the cells were also prepared and kept at −80 °C for long term storage (Lackhe *et al.*, 2002).

Preparation of switched cultures

A loopful of *C. krusei* obtained from the YEPD agar slant stock was inoculated in YEPD broth (BD) that had been supplemented with 5 mg mL^{-1} of phloxine B dye. This dye was used to create a stressed growth environment for the cells (Lackhe *et al.*, 2002). The suspension was then incubated for 4–5 h at 37 °C. Following incubation, the turbidity of the growth suspension was spectrophotometrically measured and standardized to an optical density (OD) of 0.144 at a wavelength of 550 nm (10^6 cells mL^{-1}). The suspension was then serially diluted and cultured on YEPD agar at 25 °C over a period of 5 days. This temperature was suitable for phenotypic switching protocols (Lackhe *et al.*, 2002; Vargas *et al.*, 2004). A plate with approximately 50 CFU was examined for switched colonies. Colonies displaying morphological variations from the unswitched colonies were selected to represent the 1st switched generation of *C. krusei*. The cells from the 1st switched generation were again subcultured onto another set of YEPD (supplemented with phloxine B) agar plates and the whole procedure was repeated to produce the 2nd, 3rd and 4th generations of the switched cells.

Determination of the recovery population of *C. krusei*

The recovery population was defined as the percentage of the total CFU recovered after phloxine B treatment at a particular switched generation to the number of CFU recovered from the immediate previous switched generation. In other words, the recovery of the 1st switched generation was calculated as the percentage of the CFU enumerated from the 1st switched generation to the number of CFU enumerated from the unswitched *C. krusei*. The recovery population of subsequent switched generations was calculated in a similar manner.

Determination of cell morphology

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 buffer (1 : 1). After an hour, the specimen was washed with Sorensen's phosphate and distilled water (1 : 1) and then postfixed with 1% osmium tetraoxide and distilled water (1 : 1) over a period of 14 h at 4 °C. The specimen was then put aside for an additional one hour at 37 °C 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 min each) using the following ratios of ethanol to acetone (v/v) with 3 : 1, 1 : 1 and 1 : 3.

Following that, the specimen was immersed in 100% acetone four times, 20 min each time, followed by critical point drying process using the Polaron E3500. The specimen was then mounted on aluminium stubs and coated with gold using an Edward's sputter coater (S150B). The specimen was then examined under the scanning electron microscope (JEOL JSM6400) at 10–15 kV. Samples of the 1st, 2nd, 3rd and 4th switched cells were also similarly prepared for SEM examination.

Determination of the susceptibility of *C. krusei* to CHX

The susceptibility of *C. krusei* to CHX was determined following the Kirby-Bauer disc diffusion test (Cappucino & Sherman, 2005). According to the standard procedure of the Clinical and Laboratory Standards Institute (CLSI), *C. krusei* suspension was prepared by suspending one or two colonies of *C. krusei* grown on YEPD agar into 5 mL of 0.85% of sterile saline. The cell suspension was then standardized to an OD of 0.144 at 550 nm wavelength. One hundred microlitres of the suspension was pipetted out and evenly swabbed on Mueller–Hinton (MH) agar (BD). Paper discs which had been impregnated with 120 μg (100 μL of 0.12%) CHX were carefully placed onto the swabbed MH agar plate. The diameter of an inhibited growth zone surrounding the discs following

24 h incubation at 37 °C was then measured. This procedure was repeated on all the switched generations.

The broth dilution method of Cappucino & Sherman (2005) was followed to determine the minimum inhibition concentration (MIC) and minimal fungicidal concentration (MFC) of CHX towards *C. krusei*. A sterile 96-well microtitre plate was labelled W1–W7 horizontally and numbered vertically. Using 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 a thorough mixing, 100 μL of W3 was transferred to W4 and the procedure was continued through until W6. After mixing, 100 μL from W6 was discarded. Lastly, 100 μL of *C. krusei* suspension was aseptically added to W2 through W7. W7 that received no CHX and W1 did not contain *C. krusei* served as the respective negative and positive control for the experiment. The plate was incubated for 24 h at 37 °C. The determination of MFC was carried out by inoculating 100 μL of suspension at the MIC together with the next two concentrations that did not show any turbidity. The suspension was spread evenly on YEPD agar surface and incubated for 24 h at 37 °C. Following incubation, the concentration of the suspension, which showed no growth of *C. krusei*, was considered as the MFC concentration of CHX towards *C. krusei*.

Determination of the adherence ability of *C. krusei* on saliva-coated hard surface

An artificial mouth 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 simulate the hard tissue surfaces of the tooth in the oral cavity. A modified Pasteur pipette serving as a chamber for the glass beads was then placed. The inflow and outflow of media from rubber tubings connected to and from the chamber mimic 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). For the preparation of unswitched inoculum, several colonies from the unswitched plate were suspended in sterile YEPD broth and standardized to 10^6 cells mL^{-1}. Subsequently, for the preparation of the 1st switched generation inoculum, several colonies from the switched plate displaying morphological variations designated as 1st switched generation were suspended in sterile YEPD broth and standardized to similar cell concentration. The inoculums of subsequent switched generations were prepared in a similar manner. Following inoculation, the artificial mouth system was run for 24 h with temperature maintained at 37 °C. After 24 h, the glass beads were aseptically removed and immersed in separate Eppendorf vials containing 1 mL of PBS. Each of the vials was placed in a sonicator for 60 s 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 cells following a 24 h incubation period at 37 °C was determined and recorded. This procedure was followed closely to the steps outlined by Wan Nordini Hasnor (2007). A similar procedure was repeated on the 1st to 4th switched generations of cells to determine the adherence ability of the cells under the switched conditions.

Statistical analysis

All data were analysed using SPSS version 16.0.

Results

The colony morphology of *C. krusei*

The nitrogen-depleted growth environment caused by the addition of phloxine B had caused variations in the colony characteristics (Fig. 1, Table 1). Sectoring was not observed in the original unswitched, 1st and 2nd switched generations. All were umbonate and exhibited similar col-

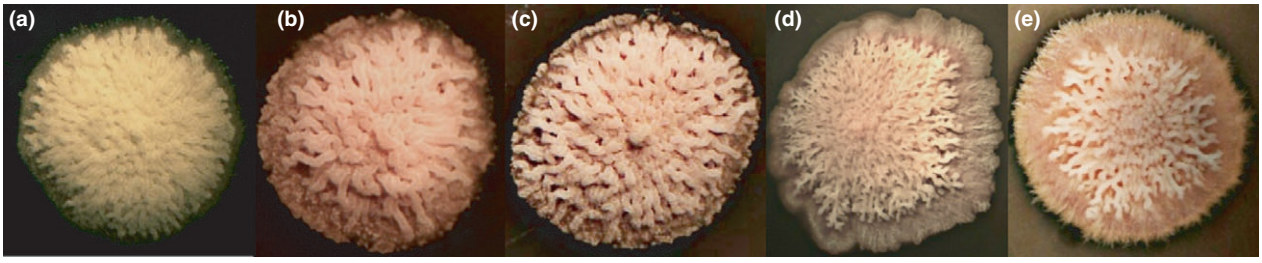

Fig. 1. Colony morphology of *Candida krusei* (ATCC 14243) under the unswitched and switched state. (a) Unswitched, (b) 1st switched generation, (c) 2nd switched generation, (d) 3rd switched generation, and (e) 4th switched generation.

Table 1. The characteristics of growth colonies of the unswitched and switched generations of *Candida krusei*

Growth generation	Colony characteristics				Recovery 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 heavily myceliated	Lobate	Irregular	Umbonate	85.7
4th switched	Dry, rough, heavily wrinkled and heavily myceliated	Filamentous	Circular	Umbonate	70.8

ony morphology with undulate margin and circular form. The surface of *C. krusei* in the 1st and 2nd generations had a wrinkled appearance, which was absent in unswitched *C. krusei*. The 3rd switched generation showed different colony morphology with dry, rough, heavily wrinkled, heavily myceliated surface appearance, lobate margin, irregular forms and umbonate elevation. The 4th switched generation exhibited similar surface appearance and elevation to the 3rd switched generation except for the filamentous margin and circular forms.

The recovery population of phenotypic switched *C. krusei*

Compared with the unswitched *C. krusei*, the recovery population in terms of cells mL^{-1} showed that the 3rd switched generation had the highest population recovery of 85.7%, followed by the 4th generation at 70.8% and the 1st switched generation at 46.6%. The 2nd switched generation showed the least recovery at only 36.4% (Table 1).

The cell characteristics of *C. krusei*

Scanning electron micrographs showed the unswitched and switched generations of *C. krusei* as branched cells with elongated pseudohyphae, elongated to ovoidal blastoconidia, or budding-off in verticillate branch. The surface of the pseudohyphae was smooth for the unswitched, 1st and 2nd generations. However, the 3rd and 4th switched generation exhibited the cell surface with a rough texture. In contrast to other switched generations, the 4th generation exhibited pimpled or punctate appearance on the cell surface (Fig. 2). The pseudohyphae of the 2nd switched generation was more extended compared with other generations with dimensions of 5.0–15.0 × 2.0–4.0 μm, whereas the smallest was in the 4th switched generation with size ranging 2.0–6.0 × 2.0–5.0 μm. The dimension of pseudohyphae from the 1st switched generation was 5.0–11.0 × 3.0–4.0 μm, while in the 3rd switched generation, the size was 3.0–7.0 × 2.0–3.0 μm (Fig. 2).

Susceptibility of *C. krusei* towards CHX

The degree of susceptibility towards CHX differs in the unswitched and all switched generations (Fig. 3). *Candida krusei* was found to be most susceptible to CHX under the unswitched state with a growth inhibition zone of 3.8 ± 0.1 cm. The degree of susceptibility was shown to decrease significantly in the 1st and 2nd switched generations with inhibition zones of 3.47 ± 0.2 and 3.0 ± 0.1 cm, respectively ($P < 0.05$). In the 3rd and 4th switched generations, susceptibility towards CHX increased slightly with inhibition zones of 3.43 ± 0.2 and 3.5 ± 0.1 cm, respectively. The MIC and MFC of *C. krusei* was 0.4 mg mL^{-1} and remained unchanged despite the switched conditions.

Adherence capacity of *C. krusei* to saliva-coated glass surface

The ability of *C. krusei* to adhere to the surface of saliva-coated glass beads was recorded at (5.62 ± 2.95) × 10^2 cells mL^{-1} (Fig. 4). An increase in (15.29 ± 10.32) × 10^2 cells mL^{-1} was observed in the 1st switched generation compared with the unswitched *C. krusei*. The increase was, however, not significant. In contrast, a significant increase in the adherence capacity was observed in the 2nd switched generation at (154.0 ± 60.2) × 10^2 cells mL^{-1} ($P < 0.05$). The adherence capacity, however, decreased significantly in the 3rd and 4th switched generations of *C. krusei* with (18.76 ± 7.56) × 10^2 cells mL^{-1} and (9.38 ± 0.37) × 10^2 cells mL^{-1} respectively ($P < 0.05$).

Discussion

The study focused on monitoring the switching pattern of *C. krusei* cultured under nitrogen-depleted growth conditions. In the study reported by Laffey & Butler (2005) and Middelhoven *et al.* (1976), colonies formed under similar nitrogen-stressed conditions were considered to have undergone phenotypic switching. Our target was towards identifying the biological properties that

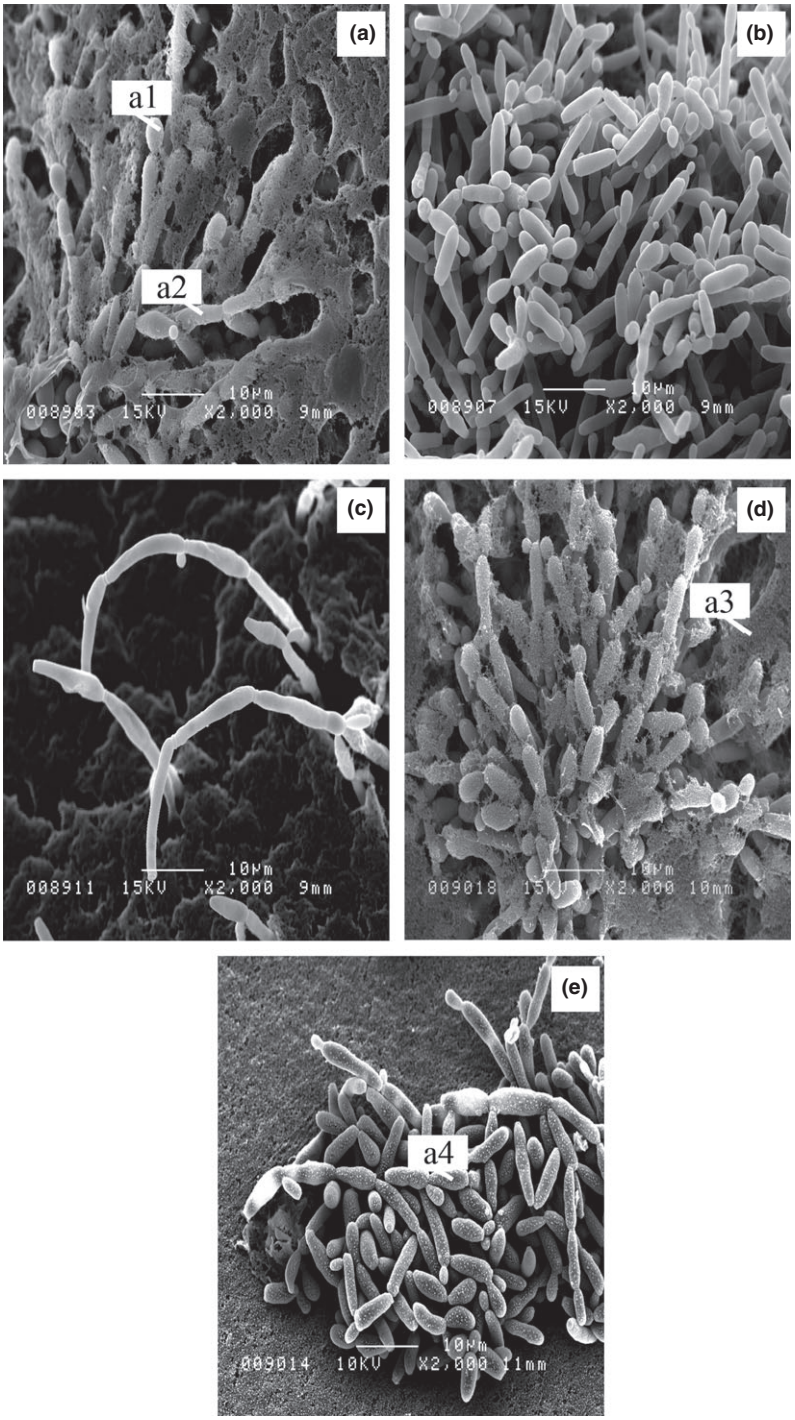

Fig. 2. Scanning electron micrographs of *Candida krusei* for the various growth generations. (a) unswitched, (b) 1st switched generation, (c) 2nd switched generation, (d) 3rd switched generation, (e) 4th switched generation. (a1) Blastoconidia (a2) Pseudohyphae (a3) Extracellular cell (a4) Pimpled/punctate cell.

were affected by the switching activity caused by the stressed growth condition. Therefore, recovery count and other aspects of biological activities were concentrated upon rather than the determination of switching frequency and reversibility.

Phenotypic switching of *C. krusei* is induced by the addition of phloxine B (tetrabromotetrachlorofluoresce-

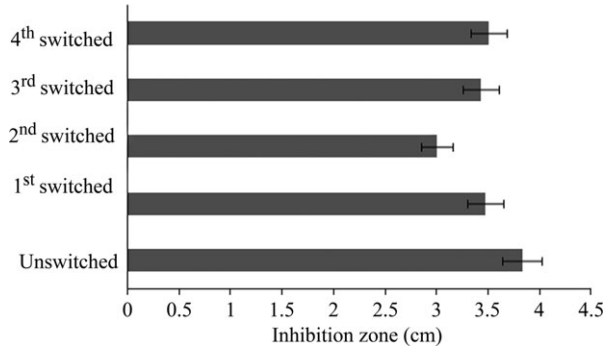

Fig. 3. The susceptibility of *Candida krusei* in the unswitched and switched forms towards CHX as determined using the disc diffusion method. The values were means ± SD of triplicate tests (n = 9).

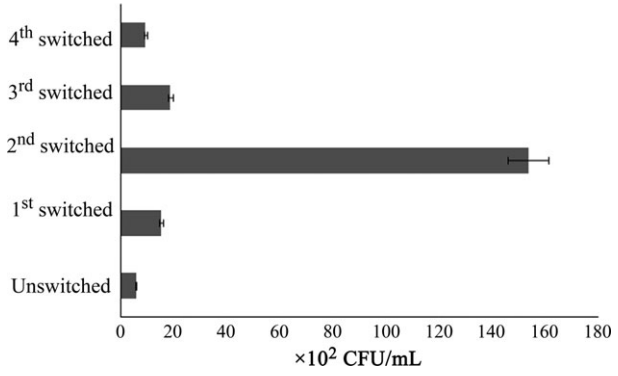

Fig. 4. The adherence capacity of *Candida krusei* to saliva-coated glass surface. The values were means ± SD (n = 9).

in), which acts as a switching detecting agent for *Candida* spp. The addition of phloxine B in the media has created a nitrogen-suppressed growth condition for *C. krusei* (Coote, 2001). The chemical compounds in phloxine B dye have been shown to facilitate the detection of mutant yeast that lack the capability of 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). The application of phloxine B in the growth environment causes *C. krusei* cells to switch to overcome the killing factors provided by the dye (Alby & Bennett, 2009).

Phenotypic switching of *C. krusei* was carried out using serially diluted suspension of *C. krusei* and cultured on YEPD phloxine B agar at 25 °C over a period of 5 days. This temperature is suitable in phenotypic switching protocols to prevent dehydration of the YEPD phloxine B agar (Lackhe *et al.*, 2002; Vargas *et al.*, 2004).

Candida krusei (ATCC 14243) in this study exhibited colony characteristics of cream to whitish colour, dry and rough surface appearance with undulate margin, circular forms and umbonate elevation when cultured on YEPD agar. Samaranayake & Samaranayake (1994) had earlier described the colony morphology of *C. krusei* as matt, rough surface appearance with cream to whitish colour on SDA. Both observations do not conform to the characteristics given in the ATCC manual, which described *C. krusei* as butyrous surface with entire margin on SDA. Variations in the colony characteristic may be due to the different growth medium used. This may affect the development of colony morphology of *C. krusei*. YEPD agar is a medium that is rich in zinc and able to suppress the expression of certain phenotypes. Nevertheless, it does not influence the switching system of *Candida* spp. (Odds & Merson-Davies, 1989). Therefore, the use of YEPD in this study did not interfere with the phenotypic switching determination.

The colony morphology in each generation of switched *C. krusei* is different in terms of surface appearance, margin, form and elevation. This observation was similar to the studies on *C. albicans*, *C. parapsilosis* and *C. glabrata* in which various colony morphology phenotypes were observed at different growth generations including smooth, myceliated and wrinkled surface appearance (Vargas *et al.*, 2004; Laffey & Butler, 2005). In this study, the transition in the colony morphology characteristics between the 2nd and 3rd switched generations is similar to the reported findings on *C. albicans* with predominant transition between unmyceliated and myceliated colony (Soll *et al.*, 1987). Phenotypic switching phenomenon could also occur after a prolonged incubation, (Slutsky *et al.*, 1985, 1987) which may enhance the development of different colony morphology of *C. krusei*. These different switched phenotypes act as a survival strategy of *C. krusei*, as different phenotypes serve different roles in providing adaptability and survivability in different conditions (Soll, 1992).

The recovery population determined the sustainability of each switched generation of *C. krusei* under suppressed growth environment. The recovery population of the 3rd switched generation was the highest, followed by the 4th switched generation. The difference in the recovery populations is probably due to the different phenotype plasticity among switched generations. Similar findings on the various population recovery have been reported where different switched generations possessed different percentage recovery population, and this suggests that different generations represent different survival ability and stability in a suppressed environment (Lackhe *et al.*, 2000).

Based on scanning electron micrographs, in general, the ultrastructure of *C. krusei* ATCC 14243 conforms to the description on the cell characteristics of *C. krusei* by Samaranayake & Samaranayake (1994). *Candida krusei* is

also described in the ATCC manual as 'long grain rice' shaped yeast with branched pseudohyphae and elongated blastoconidia. However, some variations in the cell morphology characteristics were observed throughout different switched generations, probably due to environmental constraints during the blastoconidia–hyphae transitions. The transition of smooth to pimpled and punctate morphology in the 3rd to the 4th switched generations of *C. krusei* is similar to the transition of white to opaque cell in *C. albicans* switched generations. According to Soll (1992), the formation of pimpled and punctate characteristics 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 *C. krusei* (Pomes *et al.*, 1985; Vargas *et al.*, 2004).

The 2nd switched generation of *C. krusei* was more extended compared with other generations. According to Anderson & Soll (1986), this extension, which also occurs among switched *C. albicans*, is due to the distribution of actin granules, which is mostly found on the apex of the pseudohyphae, and the generation of various characteristics of pseudohyphae is 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 *C. krusei* as an adaptation to the environmental changes, which led to the different dimension and size of the cell of *C. krusei*. Thus, hyphae-specific function and hyphae-specific gene expression play an important role in generating unique phenotype at different switched generation of *C. krusei*.

All switched generations of *C. krusei* were susceptible to CHX. CHX affects the plasma membrane of the candidal cell through nonspecific binding to the negatively charged protein and phospholipid moieties. This binding alters the cellular membrane structure and interferes with the cellular osmotic balance that lead to the susceptibility of *Candida* (Freitas *et al.*, 2003; Bonacorsi *et al.*, 2004; Veerman *et al.*, 2004; Pusateri *et al.*, 2009) towards CHX. Variations in the degree of susceptibility between the various switched generations display the responses of the switched cells to survive and attain overall fitness. When a cell undergoes 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 constraint (Vargas *et al.*, 2004).

Adherence is another feature affected by the switching of the phenotype. All generations of *C. krusei* showed the ability to adhere to the surface of saliva-coated glass beads. A similar observation was reported by Samaranayake & Samaranayake (1994) where *C. krusei* was found to adhere more on inert surfaces compared with the buccal epithelial cells. In addition, *C. krusei* exhibits high hydrophobicity, which encourages adherence. The hydrophobicity of *C. krusei* is fivefold greater than *C. albicans* (Samaranayake & Samaranayake, 1994). The adherence ability varies among switched generations of *C. krusei* and the adherence of all switched *C. krusei* is higher compared with the unswitched generation. The 2nd switched generation has the highest adherence ability followed by 3rd, 1st and 4th switched generation. Phenotype switch can change the ability of *Candida* spp. to attach to a surface (Jones *et al.*, 1994; Jain & Fries, 2009). The type or form of hyphae following phenotypic switch influences the adherence of candidal cells to inert surfaces. This might explain the highest adhering ability exhibited by the 2nd switched generation cells (Anderson & Soll, 1987).

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