Isolation and identification of *Candida albicans* to produce in house helicase for PCR

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Abstract. *Candida albicans* is a dimorphic fungus that can grow in a wide range of temperature. In such case, this microorganism has the potential to produce enzymes that able to function at elevated temperature. These enzymes are also essential in the field of molecular biology and recombinant technologies. Therefore, the enzymes produced by *Candida albicans* could be applied in the polymerase chain reaction (PCR). The PCR is the most widely used in DNA amplification. In this study, *Candida* spp. were successfully isolated and collected from Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. Different culture media were used to identify the morphology of colony. Based on the colonies growth on chromogenic agar, *Candida* sp. was identified. Microscopic examination (light and scanning microscopy) was carried out to identify the morphology of the isolate. A presumptive identification of germ tube test was performed to find out the dimorphic and pathogenicity characteristic of isolate. The formation of germ tubes from the isolate showed positive result of *Candida albicans*. A commercial Analytical Profile Index (API) Candida identification kit was used in this study as a phenotypic identification of *Candida* sp. The result of API Candida was confirmed that the isolate was the *Candida albicans*. *Candida albicans* was successfully isolated and identified phenotypically in this study for future in house helicase production.
Keywords: *Candida albicans*, local isolate, helicase production

Introduction

In recent years, the incidences of fungal infections caused by the opportunistic fungal pathogens have increased tremendously, even though we are advance in health care and therapeutic methods. Moreover, most of the opportunistic fungal pathogens are the antifungal drug-resistant, resulting in high rate of fatality due to fungal infections. Of the causative agents, yeasts such as *Candida* species have been claimed as opportunistic fungal pathogens. Indeed, *Candida* species are the most common human commensal that able to cause a broad spectrum of disease in hosts (Williams and Lewis, 2000, Kim and Sudbery, 2011). In such case, *Candida albicans* has become one of the opportunistic fungal pathogen that colonizes mucosal surface of the oral and vaginal cavities in host (Ramage *et al.*, 2001).

Candida albicans can be isolated from persons with deficiency in immune system such as human immunodeficiency virus (HIV) patients (Meurman *et al.*, 2007). In nature, *Candida albicans* is a diploid and dimorphic fungus. *Candida albicans* has the ability to grow in two different ways; blastospore phase (reproduce by budding or yeast-like form) and hyphal phase (Scully *et al.*, 1994). This yeast exhibits different morphological forms under different environmental conditions. Therefore, the phenotypic transition of *Candida albicans* can be induced by several external factors such as pH or temperature as well as different chemical compounds like *N*-acetyl-D-glucosamine, amino acids, and serum, etc. The ability of *Candida albicans* to transit between yeast-like and hyphal form has been implicated in its pathogenicity (Molero *et al.*, 1998). As a result, *Candida albicans* has been implied in molecular biology and pathogenicity studies.

Nowadays, *Candida* infections can be detected easily and accurately in conjunction with rapid methods in isolation and identification of *Candida* species in patients. With regards of this, clinicians and scientists are able to isolate and identify the *Candida* species from clinical samples in diagnostic laboratory based on the phenotypic and genotypic properties of *Candida* species (Sullivan and Coleman, 1998). *Candida albicans* is a microorganism that able to grow in a wide range of temperature. Due to this reason, it has the ability to carry out different biological processes such as ATP hydrolysis reaction at elevated temperature. The enzymes produced by this microorganism could be applied in recombinant DNA technology. Thus, the purpose of this study is to study the phenotypic properties of a local clinical isolate of *Candida albicans* in hospital, Malaysia in order to produce in house helicase for PCR in future studies.

Materials and Methods

Isolation and identification of microorganisms

The clinical samples of *Candida* sp. were collected from Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. The clinical strains were isolated from patients that have been infected with *Candida* sp. The isolated strains of *Candida* sp. were subcultured onto sterile Sabouraud's dextrose agar (Difco, USA) and incubated at 37°C for 18 hours. The well-grown cultures were then kept at 4°C in a refrigerator for further identification. A chromogenic culture medium, ChromID Candida (bioMérieux, France) was used for rapid direct identification of *Candida* sp. The isolated strains of *Candida* sp. were subcultured onto the ChromID Candida plates and incubated at 37°C for 24 hours. The identification of *Candida* sp. was determined via the colour appearance of colonies formed on the ChromID Candida plates.

Microscopy examination and morphological studies

A loopful of isolated *Candida* sp. colonies was used for Gram staining and examined under light microscope. The spread plate method was performed in this study. 100.0 µl of the *Candida* sp. cells (1.0×10^6 cells/ml) was spread onto a sterile Sabouraud's dextrose agar (Difco, USA). The inoculated plate was incubated at 37°C for 18 hours and the sample was processed through agar fix-drying method. Then, the processed sample was viewed under scanning electron microscope. Besides that, a germ tube test was performed in this study. In germ tube test, a loopful of isolated *Candida* sp. colonies was inoculated into 1.0 mL of fetal bovine serum (Gibco, USA). The inoculated culture was incubated at 37°C for 3 hours. After 3 hours incubation, a wet mount was prepared and examined by phase contrast microscopy to identify the germ tubes formation of the isolated *Candida* sp.

Biochemical test

A commercial Analytical Profile Index (API) yeast identification kit, namely API Candida (bioMérieux, France) was used to conduct the biochemical test on the isolated *Candida* sp. The API Candida strip consists of 12 biochemical tests. Yeast suspension was prepared as inoculum for the test. The prepared yeast suspension was distributed into each tubes of the test. One of the strips was used as negative control where sterile distilled water was used as inoculum. The biochemical tests of API Candida strip are shown in Table 1.

Results and Discussion

At present, infections caused by *Candida* sp. are growing gradually; hence early diagnosis and prompt antifungal therapy are essential. In such reason, various methods of presumptive identification of *Candida* sp. are developed and available in current clinical microbiology methods. In this study, the isolated strains of *Candida* sp. were subcultured onto Sabouraud's dextrose agar. Kumar *et al.* (2011) mentioned that Sabouraud's dextrose agar is commonly used for the isolation of *Candida* sp. Based on the appearance of colonies on Sabouraud's dextrose agar, the colonies were cream-white with a soft consistency; and also the surfaces and margins of the colonies were round with convex and smooth. The fresh and young colonies from Sabouraud's dextrose agar were then subcultured onto ChromID Candida for further identification. After 24 hours of incubation at 37°C, the colonies had grown well and appeared in smooth with blue colour; hence *Candida albicans* was identified as stated in the manufacturer's instructions. The ChromID Candida allows the rapid and specific identification of *Candida albicans* due to specific hydrolysis of a hexosaminidase chromogenic substrate, which resulting in formation of blue colour colonies (Baumgartner *et al.*, 1996, Eraso *et al.*, 2006). Williams and Lewis (2000) reported that ChromID Candida has proven excellent for presumptive identification of *Candida albicans*. As a result, chromogenic agar such as ChromID Candida was selected in this study to use as primary isolation with the combination of Sabouraud's dextrose agar (Schoofs *et al.*, 1997, Sullivan and Coleman, 1998, Raju and Rajappa, 2011).

Gram staining is a diagnostic method that usually used to diagnose bacterial infections. However, this method has been widely used for preliminary identification of yeast cells. Hence, Gram staining is commonly accepted as a presumptive diagnosis of *Candida albicans*. The results of Gram staining are based on the chemical and physical properties of microorganism cell walls. *Candida albicans* is frequently described as a dimorphic fungus that exists in blastopore and hyphal forms (Scully *et al.*, 1994, Sudbery *et al.*, 2004). In Figure 1a, Gram staining of smears shows the Gram-positive yeast cells (dark blue/purple colour) and some of the yeast cells were

budded under light microscope (400x). The cell walls of yeast cells contain a thick peptidoglycan layer that encircles the cells. Due to this reason, the yeast cells are able to retain crystal violet stain in Gram staining. In fact, *Candida albicans* appears as distinctly yeast-like at the early stage of infection, whereas there are gradually more pseudohyphae and hyphae cells at the later stages (Sudbery *et al.*, 2004). As *Candida albicans* is Gram-variable, this makes it confusable with other harmless yeast cells. Therefore, more comprehensive diagnosis methods are necessary to fully confirm the diagnosis.

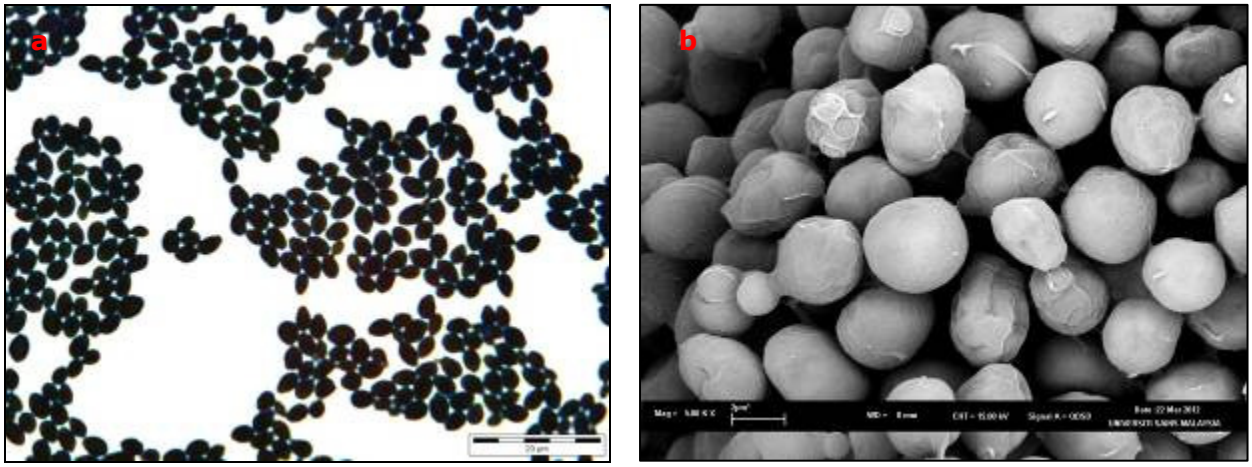

Figure 1. (a) Gram staining of *Candida* sp. under light microscope (400x).
(b) SEM micrograph of isolated *Candida* sp. (5,000x).

The morphology of isolated yeast cells was further identified via observation under scanning electron microscope (SEM). SEM is a technology that very useful for three-dimensional imaging and enables to produce images of the surface of microorganisms (Madigan *et al.*, 2009). According to the protocol in this study, the isolated yeast cells (1.0×10^6 cells/ml) were cultured onto Sabouraud's dextrose agar and incubated at 37°C for 18 hours. After 18 hours incubation, the yeast culture was processed via agar fix-drying method prior to view under SEM. The size of inoculum is an important factor in the study of the morphology of yeast cells under SEM. High inoculum size might be triggered the formation of thick and sticky mucus on the yeast cell surface. In such case, it is very difficult for us to distinguish the morphology of yeast cells even though the sample is views under SEM. The morphology of yeast cells were in ovoid or spherical-shaped with budding as shown in Figure 1b. Hence, microscopic examination via SEM is an essential step to identify the morphology of yeast cells precisely compare to staining method.

A germ tube test was carried out in this study to differentiate *Candida albicans* from *Candida* spp. This germ tube test is widely known as presumptive test for identification of *Candida albicans*. Figure 2 shows the formation of germ tubes from isolated *Candida* sp. in fetal bovine serum at 37°C for 3 hours. Under certain environmental conditions, the yeast cell produces a germ tube which is the initial morphological form of hyphae. Therefore, in the presence of inducer such as serum, a hyphal form of germ tube is elongated from the round mother cell of *Candida albicans* within 3 hours at 37°C (Sudbery, 2001, Sudbery *et al.*, 2004). After 3 hours incubation, a wet mount was prepared and examined by phase contrast microscopy at 400x. Based on the results, number of germ tube formed was more than five (n>5), thus no false-positive results in this test. Hereby, the isolated *Candida* sp. can be identified as *Candida albicans*.

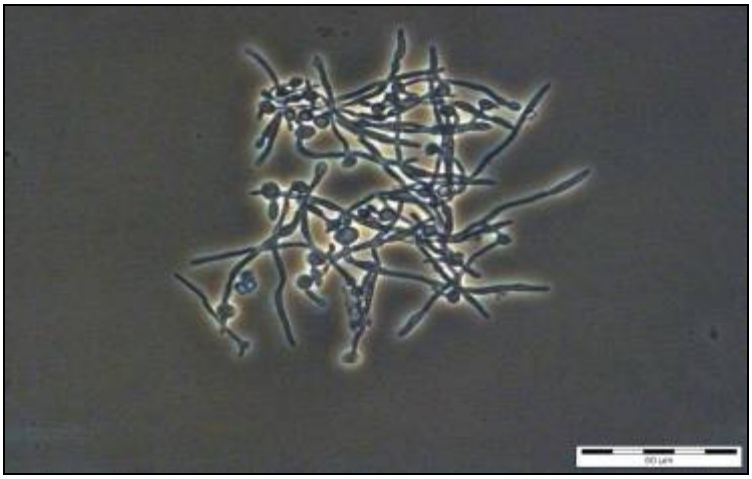

Figure 2. Germ tube test of isolated *Candida* sp. (400x).

The production of germ tubes by yeast cells of *Candida albicans* in serum is a useful rapid test for identification since it gives results within 3-4 hours. However, further biochemical identification is needed for confirmation.

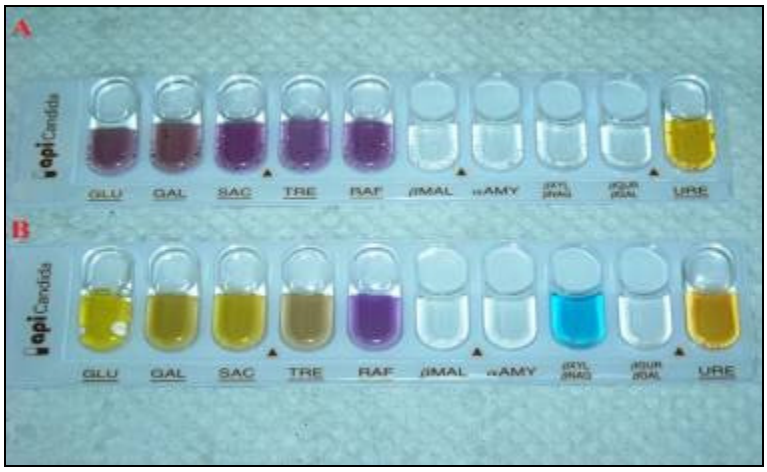

Figure 3. A commercial API Candida system. (A) Negative control. (B) Positive results.

Table 1. The list of biochemical tests in API Candida system.

*Tests	Active Ingredients	Qty (mg/well)	Reactions	Results	
				Negative	Positive
GLU	D-glucose	1.4	Acidification (Glucose)	violet / grey-violet	yellow green / grey
GAL	D-galactose	1.4	Acidification (Galactose)		
SAC	D-saccharose	1.4	Acidification (Saccharose)		
TRE	D-trehalose	1.4	Acidification (Trehalose)		
RAF	D-raffinose	1.4	Acidification (Raffinose)		
βMAL	4-nitrophenyl-βD-maltopyranoside	0.08	β-maltosidase	colourless	pale yellow / bright yellow
αAMY	2-chloro-4-nitrophenyl-αD-maltotrioside	0.168	α-amylase	colourless	pale yellow / bright yellow
βXYL	4-nitrophenyl-βD-xylopyranoside	0.095	β-xylosidase	colourless / very pale yellow/ blue / green	pale yellow / bright yellow

βGUR	4-nitrophenyl-βD-glucuronide	0.063	β-glucuRonidase	colourless / blue/green	pale yellow / bright yellow
URE	urea	1.68	Urease	yellow-pale orange	red
βNAG	5-bromo-4-chloro-3-indoxyl-N-acetyl-βD-glucosaminide	0.09	N-acetyl-β-glucosaminidase	colourless / yellow	blue / green
βGAL	5-bromo-4-chloro-3-indolyl-βD-galactopyranoside	0.0815	β-galactosidase	colourless / yellow	blue / green

*GLU, GAL, SAC, TRE, RAF and URE tests were covered with mineral oil for anaerobic incubation

A biochemical identification of isolated *Candida* sp. was performed in order to confirm the species of isolated microorganism. There was a series of biochemical tests in API Candida test strip. This API Candida system is specific designed for *Candida* sp. identification. The API Candida system has proven that able to produce good results without extra tests in the identification of majority of isolated yeasts, especially *Candida* sp. in clinical microbiology laboratories compared to ID 32C test system (Fricker-Hidalgo *et al.*, 1996). Thus, the physiological characteristics of *Candida* sp. can be determined easily and rapidly via this API Candida system (Verweij *et al.*, 1999). The reading of API Candida system was based on the changes in turbidity and colour in each well of a test strip. The numerical codes obtained from the test results were used to identify the test microorganism with database provided by the manufacturer. Then, the numerical codes were confirmed using the api*web*™ identification software (bioMérieux, France). Figure 3A shows the negative control of API Candida test strip, in which no inoculum was added in each well. Based on the results, we can be said that the isolated *Candida* sp. was *Candida albicans*, according to the numerical codes in API Candida system as shown in Figure 3B. Total of 12 biochemical tests were performed in API Candida system (see Table 1). The positive results (Figure 3B) showed the changes of colour in GLU, GAL, SAC, TRE and βNAG. These positive results indicated that the isolated yeast cells have acidification reaction with glucose, galactose, saccharose and trehalose under anaerobic condition, except raffinose. Besides that, the isolated yeasts cells also showed positive results in reaction of *N*-Acetyl-β-glucosaminidase (βNAG). *Candida albicans* produces a hydrolytic enzyme, known as *N*-Acetyl-β-glucosaminidase or chitobiase. This enzyme acts on the chitin oligomers *N,N′*-diacetylchitobiose and *N,N,N′′*-triacetylchitotriose (Niimi *et al.*, 2001, Anees *et al.*, 2010). In addition, 5-bromo-4-chloro-3-indoxyl-N-acetyl-βD-glucosaminide is known as *N*-Acetyl-β-glucosaminidase histochemical substrate that releases an insoluble blue chromophore after enzymatic action.

Conclusions

In this study, several isolation and identification methods were carried out. These methods included the identification via conventional techniques and the used of commercial identification kits. Clinical samples of *Candida* sp. were isolated and identified as well as recognized according to the phenotypic characteristics of *Candida* sp. Despite of only a presumptive identification, we can deduce that the isolated *Candida* sp. was *Candida albicans*. However, deeper studies such as genome analysis or genotypic characterization of the *Candida* sp. were suggested in future work in order to provide more information for future in house helicase production.

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