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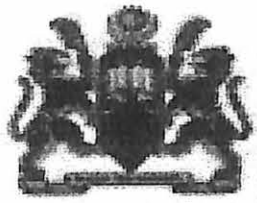

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OPTIMIZATION OF CORNMEAL AGAR CULTURE FOR YEAST MICROSCOPIC MORPHOLOGY IDENTIFICATION TEST

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Health Sciences (Biomedicine)

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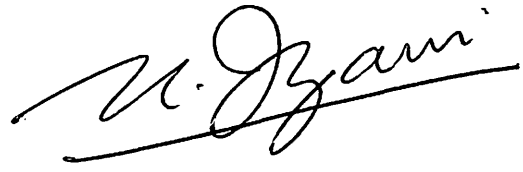
2004

CERTIFICATE

This is to certify that the dissertation entitled

"Optimization of Cornmeal Agar Culture For Yeast Microscopic Morphology Identification Test"

is the bona fide record of research work done by Nor Aida Kasma Binti Mohd Noor during the period from August 2003 to March 2004 under my supervision.

Signature of Supervisor : 

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Date : 15/04/2004

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Nor Aida Kasma Binti Mohd Noor

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ABSTRACT

Optimization of Cornmeal-Tween 80 (CMT) agar culture for yeast microscopic morphology identification test was done at varying pH and incubation temperature. A series of experiment were undertaken to optimize the medium by morphology identification of *Candida albicans, Candida glabrata* and *Candida tropicalis*. The result obtained from this study showed that the optimum pH is 5 for *Candida albicans* and *Candida tropicalis*, whereas pH 5 to 7 for *Candida glabrata*. The optimum temperature is 24°C to 30°C. From this study, the selective feature for identification of *Candida albicans* is the formation of clamydospore at 30°C and pH 5. The species is different form another species when the culture was inoculated at pH 10 at 37°C and 45°C. There was no growth observed under these conditions. For *Candida tropicalis*, the special selective feature is that the species can survive at incubation temperature of 45°C compared to other species. Selective feature for *Candida glabrata* was not observed under the optimized growth conditions.

INTRODUCTION

Yeasts are important to humans for many reasons. They cause infections, both major and minor, but they play a very important part in food industries. Within the field of medicine, the important yeasts are fungi that are primarily unicellular. These eukaryotic cells produce diseases in humans and animals or they contribute to the infection. Yeasts reproduce primary by budding; when the buds remain attached, they form chains called pseudohyphae. Fewer than 30 of the recognized species of yeast fall into the category of 'yeasts of medical importance'.

Yeasts are a significant part of the normal flora of humans, particularly on the skin and mucous membranes. Most infections are endogenous. When the host defenses are weakened in some way, the yeast of the normal flora, unchecked by natural immunity, cause disease. The infection typically starts as a localized lesion on mucous membrane and disseminates. The severity of the infection depends on how the defenses were weakened and how long the condition exists.

Most yeast is an opportunist rather than pathogenic; because they lack offensive properties such as the ability to penetrate skin, they must wait for their chance to cause infection. The severity of the infection is governed more by the underlying condition of the patient and patient's response to the challenge than by the pathogenic characteristics of the yeast.

Candida spp.

Candida is yeast like and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. Candida is a member of normal flora of skin, mouth, vagina, and stool. As well as being a pathogen and a colonizer, it is found in the environment, particularly on leaves, flowers, water, and soil. While most of the *Candida spp.* are mitosporic, some have known teleomorphic state and produce sexual spores.

Candida infections include inter-triginous candidiasis, paronychia, onychomycosis, vulvovaginitis, thrush, pulmonary infections, eye infections, endocarditis, meningitis, fungemia and disseminated infections. The genus Candida includes around 154 species (www.doctorfungus.org/mycoses/human /candida.htm).

While *Candida albicans* is the most common cause of candidiasis, other species also cause infection. Other important *Candida's* are *Candida tropicalis, Candida parapsilosis, Candida krusei, Candida guilliermondii, Candida glabrata (Torulopsis),* and *Candida kefyr*. Some species are regularly associated with a specific clinical disease. Candidiasis may be the primary condition, or it may develop secondary to another condition.

Macroscopic colony morphology

Colonies of most *Candida spp.* are white or cream colored. The texture are usually pasty and somewhat dry; but some *Candida spp.* produce mucoid colonies. A feathery filamentous fringe may be develop in the agar around colonies, especially if the cultures were old.

Microscopic morphology

Hyphae, pseudohyphae, and individual blastoconidia can all be present in yeast colonies; sometimes in the same colony. Pseudohyphae are chains of blastoconidia that have remained attached, forming a structure that resembles a string of sausages. No septa develop in pseudohyphae, but there is a constriction where the 'daughter' blastoconidium emerges from the 'mother'. Blastoconidia are unicellular round or oval forms. The daughters may remain attached to the mother cell but usually the blastoconidia are realeased, leaving a scar on the mother at the point of attachment. Daughter cell eventually become mother cells in turn. The clamydospore produced by *Candida albicans* are, in fact, thick walled vesicles that are simply swollen cells.

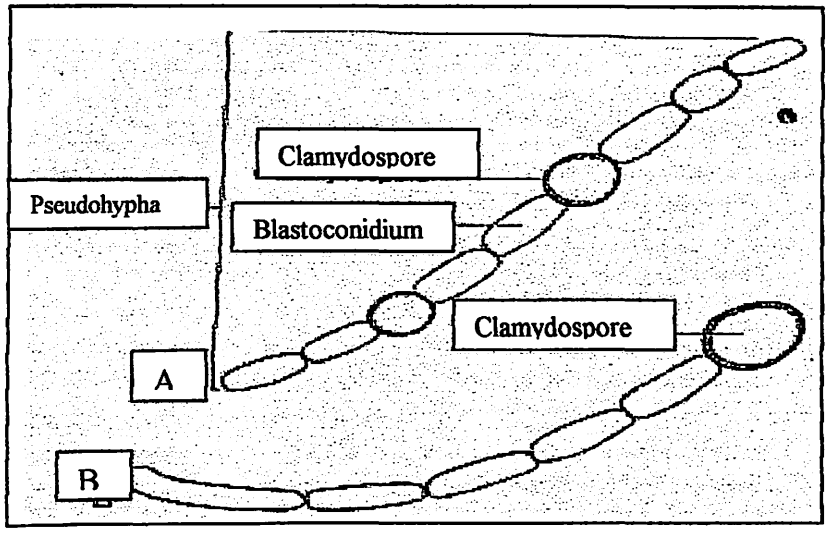

Figure 1: Chlamydospores are large, thick-walled vesicles with dense protoplasm that help fungus survive under harsh conditions. They may be intercalary. (A), e.g., between blastoconidia of the pseudohyphae, or terminal on the pseudohyphae (B).

Candida albicans

Pathogenicity: Most common cause of candidiasis, an acute, subacute, or chronic infection involving any part of the body. This organism is isolated from normal skin, normal mouth and vaginal mucous membranes and normal stools.

Rate of growth: Rapid, mature in 3 days.

Colony morphology: Cream colored, pasty, smooth.

Microscopic morphology: On cornmeal-Tween 80 agar, at 25°C for 72h, pseudohyphae (and some true hyphae) with clusters of round blastoconidia at the septa and large, thick walled terminal chlamydospores. Formation of chlamydospores is inhibited at 30°C-

37°C. Gives a positive reaction to the germ tube test. Blastoconidia of *Candida spp.* is measure approximately 3-7 x 3-14um (Davise, 1987).

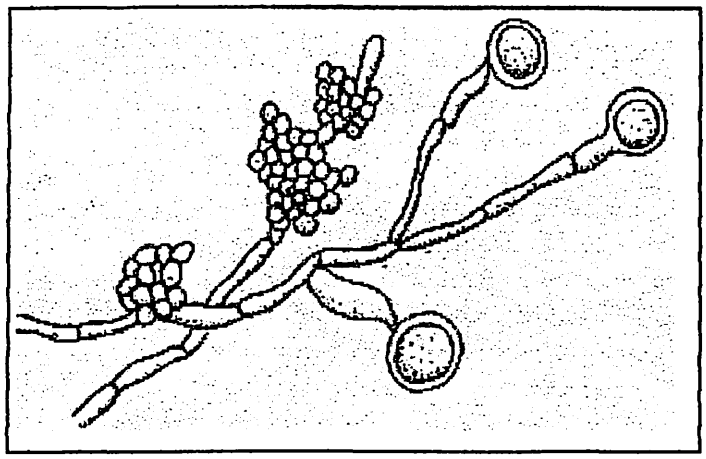

Figure 2: Microscopic morfology of *Candida albicans* on CMT agar

Candida glabrata

Pathogenicity: Causes torulopsosis, an infection occurring usually in the bloodstream or urinary tract and sometimes in the lungs. The organism is also found in healthy individuals and appears to cause infection only in particularly susceptible person.

Rate of growth: Rapid; mature in 3 days.

Colony morphology: Small, yeaslike colonies: pasty, smooth, white to cream colored.

Microscopic morphology: On cornmeal-Tween 80 agar at 25°C for 72 h, only small (2-3 x 4-5 um), round to oval, single terminal budding, nonencapsulated yeast cells are seen, no pseudohyphae are formed (Davise, 1987).

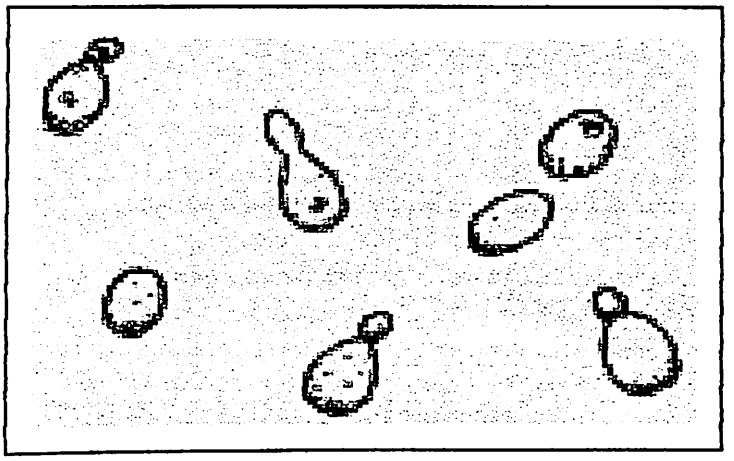

Figure 3: Microscopic morphology of *Candida glabrata* on CMT agar

Candida tropicalis

Pathogenicity: As true as many species of Candida and other yeasts, this fungus is known to cause infection, especially in patients:

1. With a breakdown in the body's immune system
2. On prolonged treatment with antibiotics, corticosteroids, or cytotoxic drugs
3. With diabetes mellitus
4. Known to be drug addicts.

It is also found without evidence of disease.

Rate of growth: Rapid; mature in 3 days.

Colony morphology: Creamy with mycelial fringe.

Microscopic morphology: On cornmeal-Tween 80 agar at 25°C for 72h, forms blastoconidia singly or in very small groups all along the pseudohyphae. True hyphae may also be present. A few teardrop shaped clamydospores may be produced

occasionally. An organism that closely resembles *C. tropicalis* has been isolated from clinical specimens and given the name *Candida paratropicalis*. It physiologically differs only slightly from the sucrose-negative form of *C. tropicalis* and is not considered to be separate species by some mycologists (Davise, 1987).

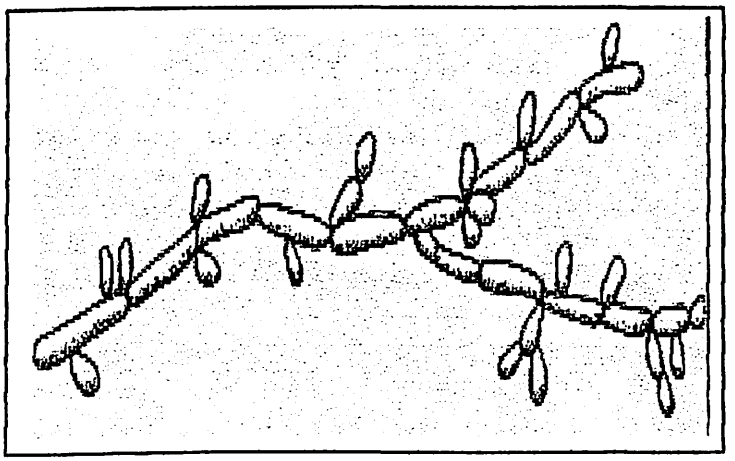

Figure 4: Microscopic morphology of *Candida tropicalis* on CMT agar

In this study, it was expected that varying the growth pH and growth temperature would show the optimization of Cornmeal-Tween 80 agar culture for Candida microscopic morphology identification test.

LITERATURE REVIEW

Identification to the species level of yeasts isolated from clinical specimens is often problematic for diagnostic laboratories, but it has become increasingly necessary. Greater numbers of immunosuppressed patients, a widening range of recognized pathogens, and the discovery of resistance to antifungal drugs mean that the common practice of identification or exclusion of *Candida albicans* alone is no longer adequate.

A number of researchers have found Cornmeal agar (CMA) to be effective for the presumptive identification of *Candida albicans*, *Candida tropicalis*, and T*richosporon.* Pfaller et al. (1996), also found it to be reliable for the presumptive identification of *Candida glabrata*, although others did not concur with this. Ann et al. (1999), found that *Candida glabrata* could not be distinguished by its appearance on CMA plates alone, having an appearance similar to those of *Candida parapsilosis, Saccharomyces cerevisiae, P. wickerhamii, Cryptococcus neoformans,* and *Candida guilliermondii.*

However, Cornmeal-Tween 80 agar (CMT) plates allow identification, except for rare isolates of *Candida famata*, a yeasts that form pink glossy colonies on CMA but that have small yeast cells and no pseudomycelium on CMT can presumptively be identified as *Candida glabrata* (Ann et al., 1999).

CMA is used for stimulating the production of chlamydospores, pseudohyphae and blastoconidium by most strains of *Candida spp.* and for cultivating another pathogenic

yeast. It is valuable for morphologic differentiation of many yeast like organisms. It suppresses vegetative growth of many fungi while stimulating sporulation. Cornmeal medium is usually used for the identification of microscopic morphology with the slide culture method.

Another characteristic of *Candida albican* is germ tube (GT) formation. The observation of germ tube production as a method for the presumptive identification of *Candida albicans* isolates from clinical samples has been used successfully for many years. Recent investigations have reported that more than 5% of *Candida albicans* strains may be GT negative (Crist et al., 1996), while false-positive results can occur with other species of the genus.

Therefore, observing germ tube in CMA medium did not seem rational. However, the use of Cornmeal broth made it possible to induce high rates of GT formation (Sachiko, 1998), which led to the development of a rapid clinical mycological method for identifying *Candida spp.* A research done by Institute of Medical Care Technology (Sachiko, 1998), using cornmeal broth as the medium for GT formation, simple and rapid practical conditions were evaluated to obtain high rates of GT formation by establishing an optimal concentration of inoculating fungal solution or an optimal concentration of cornmeal broth. Under optimal condition, the rate of identifying *Candida albicans* is 100%, demonstrating that GT detection with the non-slip glass incubation (NSSI) method is a rapid and reliable identification method.

Besides using CMA and CMT medium, reference procedures that use biochemical, morphological, and temperature studies are used to identifying the *Candida spp.* These methods are often not practicable for the clinical laboratory because they are labor-intensive and run over several weeks. Packaged kit systems are widely used, but they are expensive and are limited by the sizes of their databases, while automated systems have many of the same limitations.

With the favorable evaluation of CHROMagar Candida (CMA; CHROMagar Company, Paris, France), Ann et al. (1999), have attempted to devise a simple, rapid scheme for the routine identification of clinically important yeasts and also to investigate whether it is possible to extend the range of usefulness of the medium. They used colony appearance on CMA in combination with morphology on corn meal-Tween 80 agar and compared their identifications with the results obtained with the API 20C AUX or API 32C system. The accuracy of identification and the turnaround time were equivalent for each method, and the cultural method was less expensive.

Venitia et al., 2002, used a new chromogenic agar medium (Candida diagnostic agar [CDA]) for differentiation of *Candida spp.* This medium is based on Sabouraud dextrose agar and contains of glucose, mycological peptone, agar along with a novel chromogenic glucosaminidase substrate and ammonium. They reported that colonies of *Candida tropicalis* and *Candida kefyr* were uniformly pink, and colonies of other *Candida spp.*, including *Candida glabrata* and *Candida parapsilosis*, were white. Several recently developed alternative diagnostic tests, such as chromogenic agar media or

commercial rapid identification kits, are considered too expensive for routine use and are seemingly characterized by often inconsistent results (P. Buzzini and A. Martini, 2000).

Another method of optimization is by varying incubation temperature and pH medium. Incubation procedures were compared and the non-slip slide glass incubation (NSSI) method was found to be more reliable than the tube incubation method or slid-covered slide glass incubation method. The GT formation was completed in 1 to 2 hour in the NSSI method and clear-cut GTs were observed under optical microscopy (Sachiko, 1998).

Optimal filamentation occurs near neutral pH and is much reduced at pH below 6.0. The yeast form is exclusively present at pH 4.0. In conjunction with neutral pH, filamentation is favored by an elevated temperature of around 37°C and is largely absent below 34°C (Buffo et al., 1984). The glucosaminidase substrate in CDA was hydrolyzed by *Candida albicans* and *Candida dubliniensis*, yielding white colonies with deep-red spots on a yellow transparent background after 24 to 48 h of incubation at 37°C (Venitia et al., 2002). *Candida albicans* dominantly develop germ tubes in a dilute cornmeal broth at 37°C (Sachiko, 1998).

The inhibitory effect of low pH on yeast growth is compounded by the presence of organic acids in the medium (Thomas et al., 2001). They reported that the inhibition of yeast growth by acetic acid and lactic acid is a function of the pH and the buffering capacity of the medium and of the total amount of the organic acid added.

OBJECTIVE OF THE STUDY

Therefore, the objective of this study is to:

1. Optimized the Cornmeal-Tween 80 agar medium for detection and identification of *Candida spp.*
2. Use of microscopic morphology features as criteria in differentiating *Candida spp.*
3. Optimization of growth condition in view of changes in morphology for the detection and identification of *Candida spp.*

Scope

The main purpose of this study was to optimized Cornmeal-Tween 80 agar for detection of *Candida spp.* using microscopic morphology identification test. The main medium used was Cornmeal-Tween 80 agar (CMT). The species of Candida were *Candida albicans, Candida glabrata* and *Candida tropicalis.*

The optimizations on the growth pH and on the incubation temperature will be done. The first step is to prepare the medium at varying pH (5, 7, and 10). The *Candida spp.* are inoculated in CMT agar used slide culture method.

The Candida will be cultured at different pH medium to differentiate which pH is suitable for observing morphologies characteristic. Each inoculate are incubated at different temperatures (24°C, 30°C, 37°C and 45°C). From these, the optimum temperature for *Candida spp.* growth can be obtains.

By varying the pH and incubation temperature, the optimization of growth condition in view of changes in morphology for the detection and identification of *Candida spp.* can be observed.

Beside microscopic identification, macroscopic identification test to identify growth, colony morphology and coloration of *Candida spp.* will also be done. Two different mediums are use in this method to make a comparison between these mediums. The mediums are Sabaroud dextrose agar (SDA) and CMT. The optimizations on growth pH and incubation temperature will be done similar to the microscopic identification.

MATERIALS AND METHODS

Yeast culture

The *Candida spp.* used throughout this study was obtained from Mycology Laboratory, Department Of Microbiology, USM Health Campus. In this study only three species are used namely *Candida albican, Candida glabrata* and *Candida tropicalis.*

Growth medium

Cornmeal agar is the main growth medium. Tween 80 was added to the medium as an inducer. Cornmeal-Tween 80 (CMT) agar enhanced production of characteristic microscopic structures such as chlamydospores. CMT agar can be inoculated from the primary culture to encourage development of chlamydospores and to see the relationships among hyphae, pseudohyphae, and the other structure. For macroscopic identification, Sabaroud Dextrose agar (SDA) was used.

Growth condition

The optimization of growth was done with varying pH medium and temperature. The pH used are 4, 7 and 10 and temperatures are 24°C, 30°C, 37°C and 45°C. CMT and SDA were prepared with different pH. For adjustment of pH, 0.1M HCL and 0.1M NaOH were used. The pH of most culture media is near neutral.

The mediums were prepared with different pH (5, 7 and 10). Every species of Candida was inoculated and incubated on different growth temperature (24°C, 30°C, 37°C and 45°C). The tests were done in duplicates. For every pH medium, the incubation temperatures are different. For every species, there are 24 slide cultures prepared. When the temperature is fixed, the pH medium are varies.

Identification method

Macroscopic examination of cultures

For the macroscopic examination, SDA and CMT agar was used. The cultures were prepared in duplicates for every species. After initial inoculation and incubation (in different temperatures), media will be examined after one day and the third day for growth of Candida spp. Rapid growers will appear by the first or second day the culture plates were checked, whereas slow-growing fungi may not

be evident for 2-3 weeks or longer. When mature growth has developed on SDA and CMT, the texture and surface color of the colony was carefully noted. The colour of the reverse of the colony was also recorded along with any pigment that diffuses into the medium. To prevent dehydration of the plates, a pan of water was placed at the bottom of the incubator.

Microscopic examination of growth

It is best to examine a fungus microscopically when the culture first begins to grow and form conidia or spores. In this study, microscopy examinations were done at day one and day three of incubation. In many instances the manner of conidiation or sporulation, which is so important for identification purpose, is obscured in old culture. For the microscopic morphology identification, the formation of clamydospores, blastoconidia and pseudohyphae will be reported.

There are several methods for microscopically examining a fungus culture. The methods are tease mount, cellophane tape mount and slide culture. In this study, we used the slide culture method, in which Candida spp. are inoculated onto a small piece of Cornmeal-Tween 80 agar medium placed on a slide glass for culturing (Figure 5).

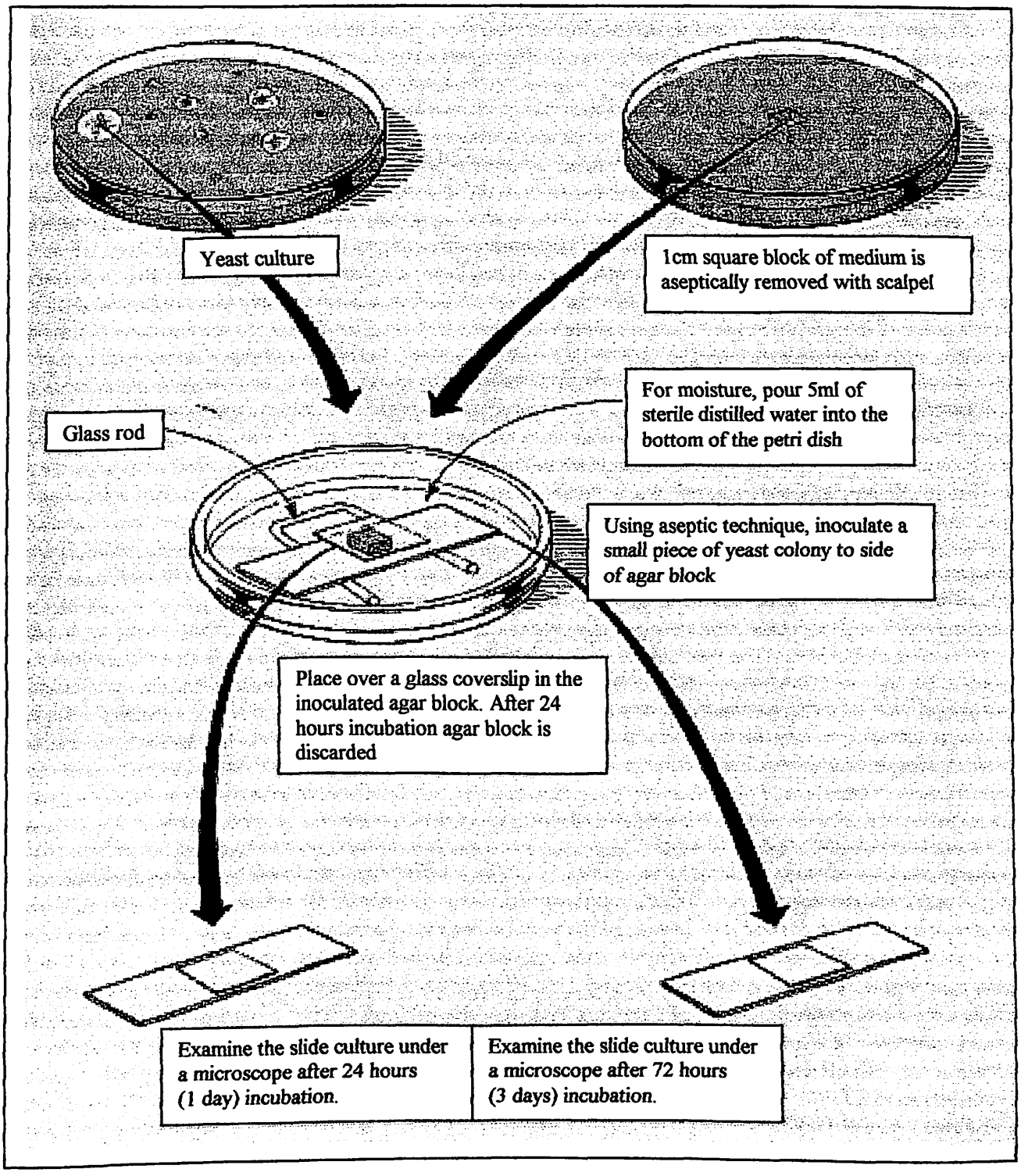

Figure 5: Slide culture method

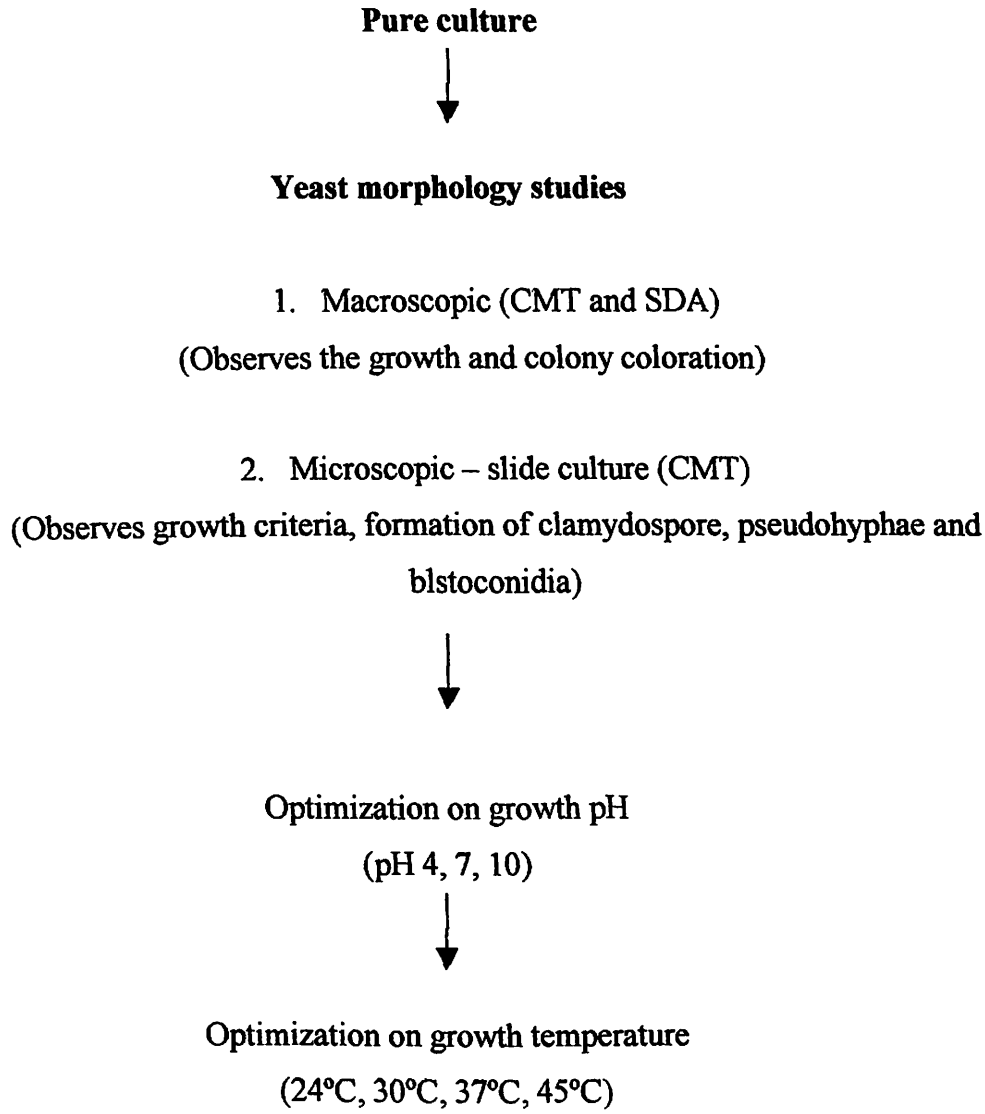

Figure 6: Flow chart of procedure optimization of Cornmeal-Tween 80 agar for *Candida spp.* morphology identification test.

RESULT AND DISCUSSION

Optimization of Cornmeal Tween-80 agar culture for morphology identification test of *Candida spp.*

A series of experiment were undertaken to optimize the medium by morphology identification of *Candida albicans, Candida glabrata* and *Candida tropicalis.*

Effects on varying incubation temperature

Temperature is one of the most important factors influencing the activity of bacterial enzymes. Enzymes have minimal, optimal, and maximal temperatures. At the optimum temperature the enzymatic reactions progress at maximum speed. In this study we will attempt to measure the effects of various temperatures on growth rate was done.

Candida species are grown routinely at 37°C or occasionally at 30°C, with optimum temperatures between 25°C and 30°C. When cultures were incubated at 24°C and 30°C, all the three species studied showed growth. *Candida tropicalis* was seen growing at all temperatures. At pH medium 10, there was no growth by *Candida albicans* at 30°C, 37°C and 45°C but profuse growth was seen with *Candida tropicalis*. At 37°C, the *Candida glabrata* growth was inhibited. For the morphology identification the most suitable incubation temperature appeared to be 24°C and 30°C.

Effects on varying pH of medium

Maximal growth for all *Candida spp.* was observed when CMT with a pH 5 and 7 was used. The pH of unmodified CMT medium is 5.6 (within the range). The results showed poor growth of *Candida albicans* and *Candida tropicalis* at pH medium 10. However profuse growth was observed for *Candida tropicalis* when grown at pH 10.

The hydrogen ion concentration of an organism's environment exerts the greatest influence on its growth. The concentration of hydrogen ion limits the activity of enzymes with which an organism is able to synthesize new protoplasm. If the composition of the medium, incubation temperature or osmotic pressure is varied, the hydrogen ion requirements become different and this will somehow affect the growth and thus the morphology.

Effects of incubation time

For the microscopic identification test, the optimal incubation period is 24 hour. During this period, the growth of *Candida spp.* is optimal and easy to identify. When the culture is over incubated (2 or 3 days), the growth of *Candida spp.* is so profuse that the identification is not accurate.

Formation of pseudohyphae

Yeast reproduced primarily by budding; when the buds remain attached, they form chains called pseudohyphae (Figure 7). In slide culture preparation, *Candida albicans* and *Candida tropicalis* demonstrates pseudohyphae. There is no formation of psudohyphae in *Candida glabrata.* The pseudohyphae are classified in three characteristics (short, long and branches).

For *Candida albicans*, pseudohyphae is formed when pH medium is not too alkaline but no pseudohyphae is formed at pH 10. Short pseudohyphae is form at temperatures 24°C and 45°C. Long and branches pseudohyphae are form at 30°C and 37°C. This is because the optimal temperature for growth is at 25°C to 30°C. The psedohyphae formed when the organism is exposed to environmental factors that inhibit the division of the cell wall while allowing growth to proceed as normal.

There are long and branches pseudohyphaes in *Candida tropicalis* at almost all condition.

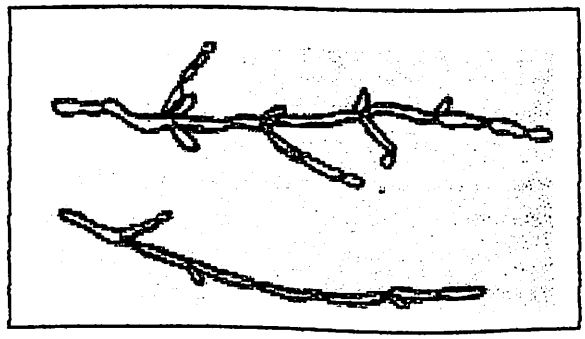

Figure 7: Yeast pseudohyphae

Formation of blastoconidia

Blastoconidia are unicellular round or oval in forms. It will appear in single, double, or in small clusters. For *Candida albicans*, the blastoconidia are form in cluster or small cluster on temperature of 37°C and 45°C. There is no growth at pH 10. No formation of blastoconidia was observed at temperature 24°C and 30°C.

Most of the slide culture preparation for *Candida tropicalis* produced ovoid or elongated blastoconidia. They typically occur singly along the pseudohyphae and at junction of psuedohyphae. There showed rare double or cluster blastoconidia. No formation of blastoconidia was observed in *Candida glabrata.*

Formation of clamydospore

Clamydospore is a resting stage, formed when a cell swells up and developed a thick resistant wall as shown in Figure 8. Chlamydospores are large, thick-walled vesicles with dense protoplasm that help fungi survive under harsh conditions. The clamydospore occur only in *Candida albicans.* These are special characteristic which differentiate *Candida albicans* from other *Candida spp.* The clamydospores are only formed at 24°C and 30°C.

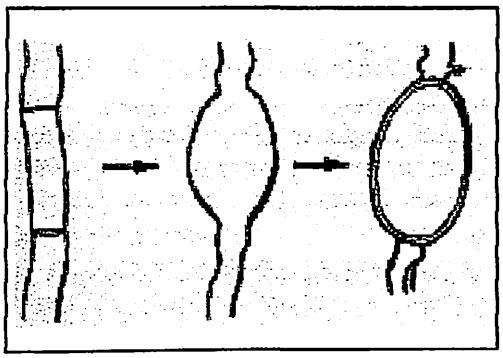

Figure 8: Formation of clamydospore