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Optimization of sugar assimilation test for identification of clinically isolated yeast

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

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CERTIFICATE

This is to certify that the dissertation entitled **"OPTIMIZATION OF SUGAR ASSIMILATION TEST FOR IDENTIFICATION OF CLINICALLY ISOLATED YEAST"** is the bona fide record of research work done by **Ms. AFAF ALANI BINTI CHE OTHMAN** during the period from **August 2003** to **March 2004** under our supervision.

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TABLE OF CONTENTS

LIST OF FIGURE

LIST OF TABLES

ABSTRACT

The study to developed the sugar assimilation test for identification of clinically isolated yeast was done using the swab lawning method and the method of incorporation of suspension on to medium. The result obtained, showed that the swab lawning method was not feasible to be used for identifying clinically isolated yeast based on the sugar assimilation test. However, the method of incorporation of suspension on medium was comparatively better and was carried out by optimizing the test by varying the sugar concentration, medium pH and incubation temperature. Candida spp. were screened using 14 types of sugars. The optimized conditions for identification purpose was 20% (w/v) of sugar concentration, medium pH of 5.6 and the incubation temperature of 30^0C for both *Candida albican* and *Candida tropicalis*. Under these optimized conditions for identification, *Candida albican* did not utilized cellobiose, whereas *Candida tropicalis* utilized cellobiose. But the optimum growth conditions for both species were same. Therefore the method of incorporation of suspension under the optimized conditions may be used in the identification of clinically isolated yeast especially in differentiating *Candida albican* and *Candida tropicalis*.

INTRODUCTION

The incidence of fungal infections has increased over the years due in part to the increase of non-*Candida albicans* species. In some instances, more than one-third of candidal infections are caused by species other than *Candida albicans* and which is of increasing significance, as they tend to be more resistant to antifungal agents. These candidal species also have different susceptibilities to antimycotics. Hence rapid identification and differentiation between *Candida spp.* is important, particularly in systemic candidiasis.

Several yeast species of the genus *Candida*, are endogenous, opportunistic pathogens. They normally reside harmlessly as commensals in human and animal host; but they can take advantage of host debilities and disorders to cause infections of a remarkably wide range of tissues (Odds., 1979). Genus *Candida* is a yeast like fungi that are commonly part of the normal flora of the mouth, skin, intestinal tract, and vagina and are necessary bacterium as part of the normal flora for human health. The problem arises when *Candida* becomes out of balance. *Candida spp.* form moist, pasty, and usually white colonies. Spores are produced along the easily fragmented filaments and become so numerous that the filaments can be entirely obscured. The spores can reproduce themselves by "budding". The species are commonly found in soil and organic debris and can also cause human disease (Willinger *et al.*, 2001)

The name 'candidiasis' was originally used to described infections due to a single yeast species, *Candida albicans*; but evidence suggested that *Candida spp.* other than *Candida*

albicans were also involved. As the science of medical microbiology become more sophisticated, infections by members of *Candida* have been reported from an ever-increasing number of sites. The isolation of yeast of many types from human sources has been recorded. *C. albican, C. guilliermondii, C. krusei, C. parapsilosis, C. pseudotropicalis, C. stellatoidea* and *C. tropicalis* were usually regarded by clinician as the principle medically important *Candida* causing candidiasis (Odds., 1979).

Candida albican

Candida albican is a type of parasitic yeast like fungus that inhabits the intestines, genital tract, mouth, esophagus, and throat. Because of its many and varied symptoms, this disorder is often misdiagnosed. When *C.albicans* overgrows and infects the vagina, the result is a yeast infection, with symptoms of large amount of white, cheesy discharge and intense itching and burning (Collier *et al.*, 1998).

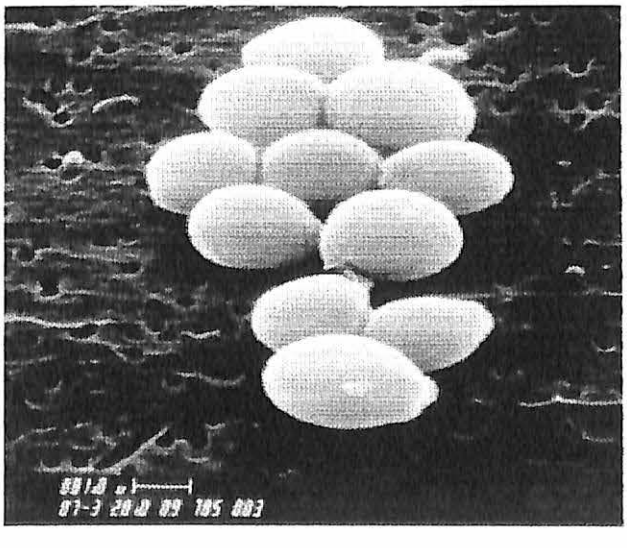

Figure 1: Scanning electron micrograph of the pathogenic yeast *Candida albicans*.

Candida tropicalis

Candida tropicalis is a major cause of septicemia and disseminated candidiasis, especially in patients with lymphoma, leukemia and diabetes. It is the second most frequently encountered medical pathogen, next to *Candida albicans*, and is also found as part of the normal human mucocutaneous flora. Sucrose negative variants of *Candida tropicalis* have also been increasingly sighted in cases of disseminated candidiasis. Environmental isolations have been made from faeces, shrimp, kefir, and soil (Collier *et al.*, 1998).

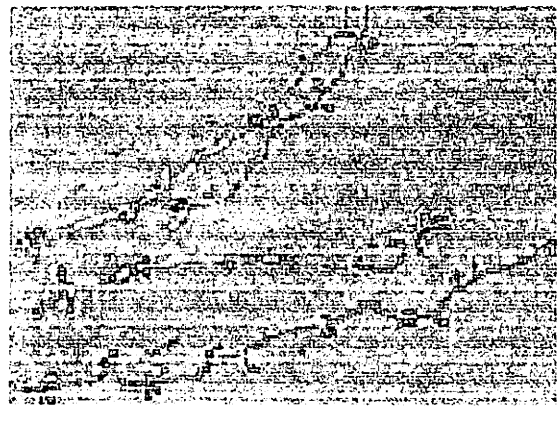

Figure 2 : Scanning of *Candida tropicalis* on Corn meal agar under microscope

Laboratory Identification of *Candida* Species

At present, the identification and differentiation of Candida spp. is based on a variety of tests, including morphologic examination, sugar and nitrate assimilation, biochemical reactions and agglutination with specific antibodies. The morphology characteristics identification includes appearance and color of colonies on primary isolation media such

as Sabaroud and CHROMagar, size and shape of the cells, production of hyphae and/or pseudohyphae, the ability to produce germ tubes and the ability to produce chlamydoconidia (chlamydospores). Whereas, the biochemical characteristics identifications are based on the assimilation of carbohydrates or sugar, assimilation of nitrate and fermentation of sugars (Richardson & Carlson, 2002).

The following identification kit products have found a useful place in the routine laboratory, although the list is not exhaustive such as API ID 32C, API 20C AUX, Fungicrom, Auxacolor, Candifast, and Fungitest. The development of commercial kits have provided the routine laboratory with the opportunity to standardize the identification of *Candida* species. However, for many laboratories, the high cost involved may exclude the use of identification kits (Richardson & Carlson, 2002).

These conventional and non-conventional methods used are somehow accurate but it is slow, expensive and at time labour intensive. Therefore, in this research project, our aim is to produce a more rapid, cheaper and easier method, which can be done in the laboratory by employing several strategies which has not been done or thoroughly studied before.

LITERATURE REVIEW

Recently, there has been an increase in fungal infections in immunocompetent as well as immunocompromised patients. Yeast (particularly *Candida albican*) are the most common fungi isolated from human infections (Judith S. Heelan *et al.*, 1998). The higher incidence and severity of yeast infections has led to an increase in the therapeutic and prophylaxis use of antimycotics. Various yeast species are inherently or potentially resistant to amphotericin B and the new azole agents. Fluconazole is the standard treatment for candidiasis. However, as *Candida glabrata* become less susceptible to fluconazole, and as *Candida krusei* is intrinsically resistance to this drug, infections by these strains may necessitate alternative treatment with amphotericin B and itraconazolen (Bauters *et al.*, 1999).

Among the etiologic agents of yeast infections, *C. albican, C. glabrata, C. krusei* and *C. tropicalis* predominates in many countries, including Malaysia. Hence, given their susceptibilities to antimycotics, rapid differentiation between *Candida* spp. may be important. At present, the identification of *Candida* spp. is based on a variety of tests, including germ tube formation, production of chlamidospores, sugar and nitrate assimilation, biochemical reactions, and agglutination within specific antibodies. All these methods require pure cultures of the isolates and, take 24h to 36 h to complete. The most rapid biochemical test rely on detection of certain key enzymes. The differentiation between *albicans* and non-*albican* spp., in particular, is frequently made according to the presence or absence of N-acetyl-β-D-galactosaminidase. A lot of study have been done to

find the most rapid test, but all the test are costly and cannot be efforded by many routine laboratory (Bauters *et al.*, 1999).

A comparative study using the rapid yeast plus panel with API 20C Yeast System for identification of clinically significant isolates of *Candida* spp. showed the RapID yeast plus system correctly identified 125 yeast isolates, with an accuracy of 94% (125 of 133) (Judith *et al.*, 1998). Excellent correlation was found in the recognition of the three yeasts most commonly isolated from human sources. The test was 99% (105 of 106 isolates) accurate with *C. albican, C. tropicalis* and *C. glabrata.* RapID yeast plus system compares favorably with the API 20C system and provide a simple, accurate alternative to convetional assimilation methods for the rapid identification of the methods for rapid identification of the most commonly encountered isolates of *Candida* species (Heelan *et al.*, 1998).

Researcher from the Institute of Medical Microbiology, University Hospital RWTH Aachen, Gemany have also studied the rapid identification of *Candida glabrata* using a dipstick to detect trehalose-generated glucose. Based upon of *C. glabrata* well known ability to rapidly hydrolize trehalose, they have developed a novel and cost-effective test incubating one yeast colony emulsified in citrate buffer containing 4% trehalose for 3h at 37C. Trehalose-generated glucose is detected with a commercially available dipstick. For evaluation, consecutive clinical isolates and several reference strains of *C. glabrata, C. albican,* and other yeast species with potential ability for utilization of trehalose were tested. Identification of *C. glabrata* was achieved within 3h, with specificity of 99.1%

and a sensitivity of 98.8% when grown on Sabaroud dextrose agar supplemented with 4% glucose (Heidrun Peltroche-Llaesahuanga *et al.*, 1998)

A membran filtration test for rapid presumptive differentiation of four *Candida* employing a rapid enzymatic two steps test was done by Bauter *et al.*, (1999). The first step involved membrane filtration of liquid sample, followed by preincubation of the membrane filter on Sabauroud glucose agar supplemented with ticarcillin-clavulanic acid to yield microcolonies. The second step involved the part of the part of the filter was placed on absorbent pads impregnated with substrate-enzyme complex. The membrane filter in contacted in the assay medium was incubated to allow cleavage of the enzyme-subatrate, resulting in fluorescent microcolonies under wavelength UV light. This approach is able to presumptively differentiate *C. albican, C. glabrata, C. krusei* and *C.tropicalis*. Promising experimental evaluation using spiked blood samples have shown this feasibility of the two-step approach to be used for the rapid detection of candidemia, where sensitivity and speed are crucial.

Fluorophore-assisted carbohydrate electrophoresis (FACE) is a straightforward, sensitive method for determining the presence and relative abundance of individual oligomannosyl residues in *Candida* mannoprotein; the major antigenic determinant located on the outer surface of the yeast cell wall. The single terminal aldehydes of oligomannosyl residues released by hydrolysis were tagged with the charged fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and separated with high resolution on the basis of size by polyacrylamide gel electrophoresis. FACE analysis revealed the major oligomannosides

released by acid hydrolysis and β-elimination of Fehling-precipitated mannan from *Candida albicans*. FACE was also amenable to the analysis of samples obtained by direct hydrolysis of whole yeast cells. Whole-cell acid hydrolysis and whole-cell β-elimination of two isolates each of *C. albicans*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. rugosa*, *C. stellatoidea*, and *C. tropicalis* resulted in oligomannoside gel banding patterns that were species and strain specific for the 16 isolates surveyed. Whereas some bands were specific for an individual isolate or species, there are other bands which were shared by two or three species in various groupings. FACE represents an accessible, sensitive, and quantitative analytical tool enabling the characterization of yeast mannan complexity (Goins and Cutler, 2000)

C. dubliniensis has been reported to lack the ability to utilize xylose (XYL) and α-methyl-D-glucoside (MDG) and to grow poorly or not at all at 45°C, whereas *Candida albicans* isolates utilize XYL and MDG and usually grow well at 45°C. They tested *C. dubliniensis* and *C. albicans* with both the API 20C AUX and Vitek YBC systems to evaluate the ability of the XYL and MDG tests contained within each of these systems to distinguish between the two species. The ability to grow at 45°C was also examined. None of the *C. dubliniensis* isolates grew at 45°C, and 23% *C. albicans* isolates exhibited poor or no growth at 45°C. The XYL and MDG tests contained within the API 20C AUX system were both negative for *C. dubliniensis* isolates and were positive for (XYL) and (MDG) of the *C. albicans* isolates. With the Vitek system, *C. dubliniensis* isolates were XYL and MDG negative. Conversely, *C. albicans* isolates were XYL and MDG positive with the Vitek system. Clinical microbiology laboratories could use lack of growth at 45°C and a negative XYL test with either the API 20C AUX or Vitek yeast

identification system to provide a presumptive identification of *C. dubliniensis*. A negative MDG test result with either system would also be helpful but may misclassify *C. albicans* as *C. dubliniensis*, especially when the API 20C AUX system is used (Gales *et al.*, 1999).

A variety of fungi produce the hydrolytic enzyme β-*N*-acetylhexosaminidase (HexNAcase), which can be readily detected in assays by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as a substrate. A microtiter plate-based HexNAcase assay for distinguishing *Candida albicans* and *Candida dubliniensis* strains from other yeast species has been developed. HexNAcase activity was detected in *C. albicans* strains *C. dubliniensis* strains but not in other *Candida* species. The HexNAcase activity in *C. albicans* and *C. dubliniensis* was strain specific. All except three clinical *C. albicans* isolates among the *C.albicans* strains tested produced enzyme activity within 24 h. These strains did produce enzyme activity, however, after a prolonged incubation period. For two of these atypical strains, genomic DNA at the *C. albicans HEX1* gene locus, which encodes HexNAcase, showed nucleotide differences from the sequence of control strains. Among the other *Candida* species tested, only *C. dubliniensis* had a DNA sequence that hybridized with the *HEX1* probe under low-stringency conditions. The microtiter plate-based assay used in the present study for the detection of HexNAcase activity is a simple, relatively inexpensive method useful for the presumptive identification of *C.albicans* and *C. dubliniensis* (Niimi *et al.*, 2001).

Rapid identification of *Candida* species has become more important because of an increase in infections caused by species other than *Candida albicans*, including species

innately resistant to azole antifungal drugs. Elie *et al.*, (1998), have developed a PCR assay with an enzyme immunoassay (EIA) format to detect amplicons from the five most common *Candida* species using universal fungal primers and species-specific probes directed to the ITS2 region of the gene for rRNA. They designed probes to detect seven additional *Candida* species (*C. guilliermondii*, *C. kefyr*, *C. lambica*, *C. lusitaniae*, *C. pelliculosa*, *C. rugosa*, and *C. zeylanoides*) included in the API 20C sugar assimilation panel, five probes for species not identified by API 20C (*C. haemulonii*, *C. norvegica*, *C. norvegensis*, *C. utilis*, and *C. viswanathii*), and a probe for the newly described species *C. dubliniensis*, creating a panel of 18 *Candida* species probes. The PCR-EIA correctly identified multiple strains of each species tested, including five identified as *C. albicans* by the currently available API 20C database but determined to be *C. dubliniensis* by genotypic and nonroutine phenotypic characteristics. Species identification time was reduced from a mean of 3.5 days by conventional identification methods to 7 h by the PCR-EIA. This method is simple, rapid, and feasible for identifying *Candida* species in clinical laboratories that utilize molecular identification techniques and provides a novel method to differentiate the new species, i.e *C. dubliniensis*, from *C. albicans* (Elie *et al.*, 1998)

OBJECTIVE

1. Study of Candida spp. growth using the methods of swab lawning and incorporation of yeast suspension onto medium.

2. Optimization of sugar assimilation by Candida spp. using varying amount of sugar under different growth conditions.

Scope

Sugar assimilation test is a conventional method for the definitive identification of clinically isolated yeast to species level and is based upon the ability of yeast to utilize sugar by assimilation. Assimilation is the utilization of a carbon source by a yeast in the presence of oxygen. A positive assimilation reaction is usually read as the presence of growth that produce an opacity on the medium and indicates the ability of the isolate to assimilate sugars. In this study, the sugar assimilation test will be optimized by varying the concentration of sugar, temperature and pH of the growth media. It was expected that optimization of the sugar assimilation test can be use as an accurate, simple and cost effective method for the detection and identification of clinically isolated yeast mainly *Candida albicans* and *Candida tropicalis.*

MATERIALS AND METHODS

Microorganism

The pure stock culture of *Candida albican* and *Candida tropicalis* used throughout this study were obtained from Mycology Laboratory, Department of Microbiology and Parasitology, Universiti Sains Malaysia.

Sugars source

The sugar used throughout this study were obtained from a commercial preparation. All sugars are in powder form. First Line Sugar consists of glucose, sucrose,trehalose, maltose galactose cellobios and arabinose. Meanwhile the Second Line Sugar are consists of lactose, melibiose, raffinose, rhamnose, mannitole, inositol and xylose. The sugar concentrations were prepared in 10%, 20% and 30% (w/v).

The prepared sugar solution was filtered sterilized into a universal bottle. By using a sterile Pasteur pipette, one drop of the sugar solution was put on blank paper discs. The paper discs were kept in incubator at 37^0C for 3 days to dry and later prior to use.

Media

The agar media used in this study are carbon free of carbon source. Bacto agar is the media used for the method of incorporation of yeast suspension. Bacto agar with added yeast nitrogen base agar and bromocresol purple as indicator is use for swab lawning method. These agar were prepared at pH 5.6, 7 and 10 for yeast suspension method and pH 5, 7 and 10 for swab lawning method.

Identification based on sugar assimilation by API system

The *Candida albican* and *Candida tropicalis* were identified by API system. This test is based on sugar assimilation test principle. It was done as a reference for this study.

Method of incorporation of suspension on medium

Bacto agar was first prepared (15 ml in each universal bottle) before adding 1ml of *Candida spp.* that have already been concentrate in sterile distilled water at 7 Macfarland. The mixture is then poured and let to solidify. The sugar discs is then put on the agar (5 discs to a plate) and each sugars were labeled by numbering them from 1 to 14.

Swab lawning method

The Bacto agar is poured onto a petridish to solidify. Then 100ul of microorganisms concentrated earlier in sterile distilled water at 2 Macfarland, was spread onto the agar by using a hockey stick. Then the sugar discs were put onto the lawned agar about 5 discs per plate were used.

Optimization on sugar concentration

The optimization on sugar concentration was done using the method of incorporation of suspension on medium and swab lawning method. This method is accomplished in duplicate for each concentration. *Candida albicans* and *Candida tropicalis* were cultured with the concentration of sugars of 10%, 20% and 30% (w/v).

Optimization on medium pH

The culture method is similar to section 3.7, but varies only in the pH. The pH for method of incorporation of suspension on medium were pH 5.6, 7, and 10 and for swab lawning method the pH were 5, 7, and 10.

Optimization on incubation temperature

The optimization on incubation temperature was done by varying the temperature. The temperature used were 24^0C, 30°C and 37^0C. For both method, the test were done in duplicates.

Measurement of growth

The growth diameter were measured around the disc of sugar. The positive reaction will produce a growth zone around the disc indicating utilization of a particular sugars or carbohydrates. Negative reaction will produce clear zone when the sugars or carbohydrates are not utilized by microorganisms or no growth is seen.

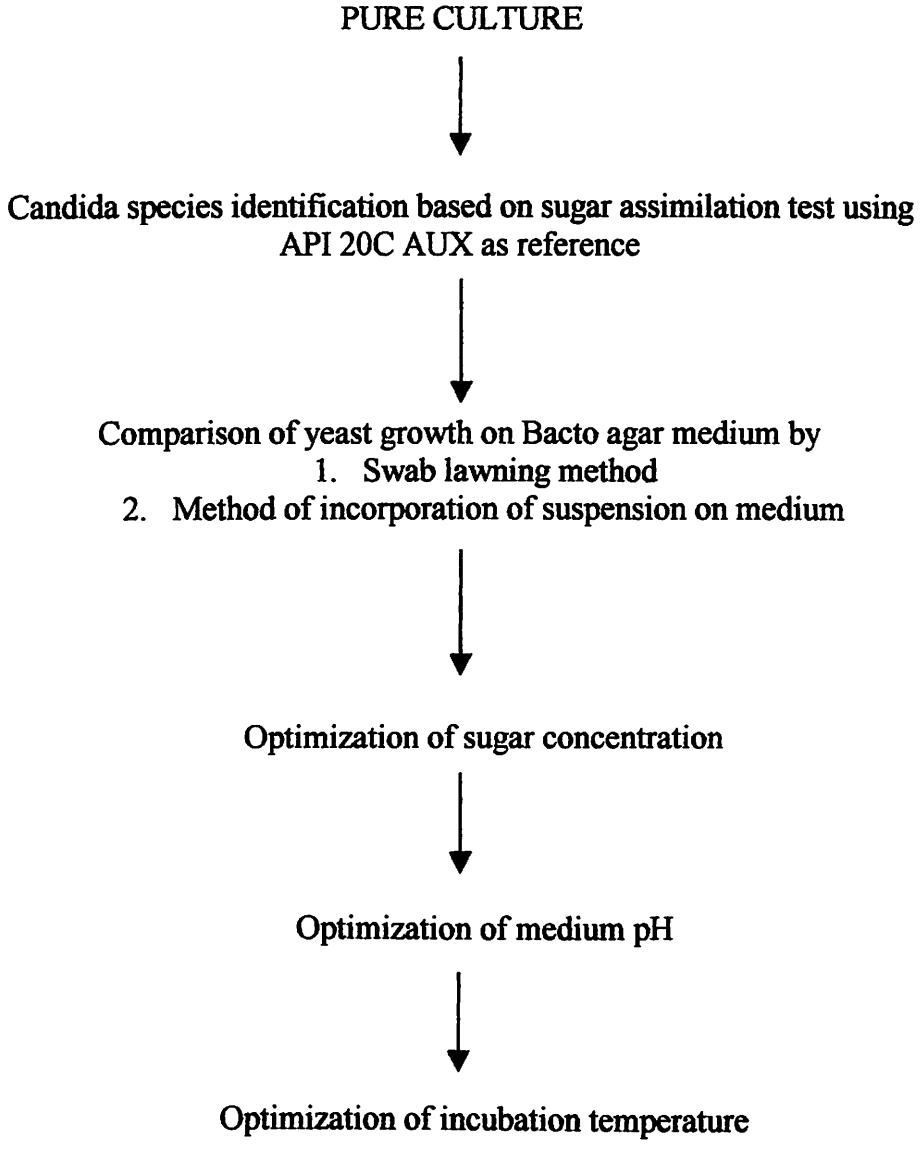

Figure 3 : Overall optimization of sugar assimilation under various growth conditions

RESULTS AND DISCUSSIONS

Optimization of Sugar Concentration

A study on the optimization of sugar concentration was done using the method of incorporation of suspension on medium and swab lawning method. The result from the swab lawning method were not feasible to be used for the identification. There is no growth at all after 24 and 48 hours incubation, but after reincubation for 72 hours later, there is growth all over the plate. This is probably due to the indicator used in the media of this method. May be the bromocresol purple used was not suitable in this study.

The results of sugar assimilation in the method of incorporation of suspension on medium are shown in Table 1, 2 and 3 for *Candida albican* and Table 4, 5 and 6 for *Candida tropicalis*.

In *Candida albican*, showed positive in the first five sugars of first line sugar in all concentrations. The diameter also almost same to one another. But in the second line sugar, the positive results had shown in only Mannitol and Xylose in 10% and 20% (w/v) but negative in 30% (w/v). The diameters of growth for all the positive results also not much different.

In *Candida tropicalis*, the results showed positive in first six sugars in the first line sugar and Mannitol in the second line sugar in all concentrations. It also showed positive in

Xylose in 10% and 20% (w/v) of sugar concentration but no growth in 30% (w/v). The diameter of growth also almost same to one another.

After the observation, we have decided to precede this research only by the method of incorporation of suspension on medium. We also chose the 20% (w/v) of sugar concentration because it obtained the best result then other concentrations and has been used as the standard concentration in Myclogy laboratory in USM.

Table 1 : Optimization of sugar assimilation (10%,w/v) by *Candida albican*

No	Sugar	Sugar concentration, 10% (w/v)			
		Set 1		Set 2	
	1st Line Sugar	**Growth**	**Diameter (cm)**	**Growth**	**Diameter (cm)**
1	Glucose	+	3.2	+	3.0
2	Sucrose	+	3.2	+	3.2
3	Trehalose	+	3.0	+	3.2
4	Maltose	+	2.6	+	2.4
5	Galactose	+	3.3	+	3.2
6	Cellobios	-	-	-	-
7	Arabinose	-	-	-	-
	2nd Line Sugar				
8	Lactose	-	-	-	-
9	Melibiose	-	-	-	-
10	Raffinose	-	-	-	-
11	Rhamnose	-	-	-	-
12	Mannitol	+	3.6	+	3.5
13	Inositol	-	-	-	-
14	Xylose	+	3.8	+	3.5

The optimization of sugar assimilation was done using the method of incorporation of suspension onto medium and sugar concentration of 10% (w/v), pH 5.6 and 24^{0}C.

Table 2 : Optimization of sugar assimilation (20%,w/v) by *Candida albican*

No	Sugar	Sugar concentration, 20% (w/v)			
		Set 1		Set 2	
	1st Line Sugar	**Growth**	**Diameter (cm)**	**Growth**	**Diameter (cm)**
1	Glucose	+	3.2	+	3.2
2	Sucrose	+	3.4	+	3.2
3	Trehalose	+	3.2	+	3.2
4	Maltose	+	2.6	+	2.8
5	Galactose	+	3.5	+	3.6
6	Cellobios	-	-	-	-
7	Arabinose	-	-	-	-
	2nd Line Sugar				
8	Lactose	-	-	-	-
9	Melibiose	-	-	-	-
10	Raffinose	-	-	-	-
11	Rhamnose	-	-	-	-
12	Mannitol	+	3.6	+	3.6
13	Inositol	-	-	-	-
14	Xylose	+	3.8	+	3.6

The optimization of sugar assimilation was done using the method of incorporation of suspension onto medium and sugar concentration of 20% (w/v), pH 5.6 and 24^{0}C.

Table 3 : Optimization of sugar assimilation (30%,w/v) by *Candida albican*

No	Sugar	Sugar concentration, 30% (w/v)			
		Set 1		Set 2	
	1st Line Sugar	**Growth**	**Diameter (cm)**	**Growth**	**Diameter (cm)**
1	Glucose	+	2.8	+	3.0
2	Sucrose	+	3.2	+	3.4
3	Trehalose	+	3.2	+	3.2
4	Maltose	+	3.0	+	2.8
5	Galactose	+	3.4	+	3.2
6	Cellobios	-	-	-	-
7	Arabinose	-	-	-	-
	2nd Line Sugar				
8	Lactose	-	-	-	-
9	Melibiose	-	-	-	-
10	Raffinose	-	-	-	-
11	Rhamnose	-	-	-	-
12	Mannitol	-	-	-	-
13	Inositol	-	-	-	-
14	Xylose	-	-	-	-

The optimization of sugar assimilation was done using the method of incorporation of suspension onto medium and sugar concentration of 30% (w/v), pH 5.6 and 24^{0}C.

Table 4 : Optimization of sugar assimilation (10%,w/v) by *Candida tropicalis*

No	Sugar	Sugar concentration, 10% (w/v)			
		Set 1		Set 2	
	1st Line Sugar	**Growth**	**Diameter (cm)**	**Growth**	**Diameter (cm)**
1	Glucose	+	2.4	+	2.5
2	Sucrose	+	2.4	+	2.6
3	Trehalose	+	2.5	+	2.6
4	Maltose	+	2.3	+	2.3
5	Galactose	+	2.8	+	2.7
6	Cellobios	+	3.2	+	3.1
7	Arabinose	-	-	-	-
	2nd Line Sugar				
8	Lactose	-	-	-	-
9	Melibiose	-	-	-	-
10	Raffinose	-	-	-	-
11	Rhamnose	-	-	-	-
12	Mannitol	+	2.7	+	2.8
13	Inositol	-	-	-	-
14	Xylose	+	2.8	+	2.6

The optimization of sugar assimilation was done using the method of incorporation of suspension onto medium and sugar concentration of 10% (w/v), pH 5.6 and 24^{0}C.

Table 5 : Optimization of sugar assimilation (20%,w/v) by *Candida tropicalis*

No	Sugar	Sugar concentration, 20% (w/v)			
		Set 1		Set 2	
	1st Line Sugar	**Growth**	**Diameter (cm)**	**Growth**	**Diameter (cm)**
1	Glucose	+	3.2	+	3.0
2	Sucrose	+	3.4	+	3.2
3	Trehalose	+	3.0	+	2.8
4	Maltose	+	2.9	+	2.8
5	Galactose	+	3.5	+	3.7
6	Cellobios	+	3.0	+	3.5
7	Arabinose	-	-	-	-
	2nd Line Sugar				
8	Lactose	-	-	-	-
9	Melibiose	-	-	-	-
10	Raffinose	-	-	-	-
11	Rhamnose	-	-	-	-
12	Mannitol	+	3.2	+	3.2
13	Inositol	-	-	-	-
14	Xylose	+	3.4	+	3.1

The optimization of sugar assimilation was done using the method of incorporation of suspension onto medium and sugar concentration of 20% (w/v), pH 5.6 and 24^{0}C.