CHAPTER 2

MATERIALS AND METHODES

Organism

321 of yeast strains were isolated from clinical specimens from Siriraj Hospital, Srinakarin Hospital, Chulalongkorn Hospital and Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. The isolates were cultured from urine (33.02%), throat and mouth (13.70%), cerebrospinal fluid (10.90%), sputum (10.28%), animal (6.54%), pus (6.54%), bronchial washing and lung (4.98%) vaginal and cervical swab (3.12%). And 24 standard strains were given by Thailand Institute of Scientific and Technological Research; National Institute of Health, Bethesda, Maryland, ; Division of Mycology, Center for Disease Control, Atlanta, and Duke University. 5 genera and 16 species of the 24 standard strains were shown in table 2. These standard strains were used as the control for all of the identification methods. All yeasts were restreaked to make pure cultures and subcultured on Sabouraud dextrose agar for 24-48 hours then stocked the cultures in cold room (4°C).

In order to make the stock culture of yeasts to be active, yeasts were subcultured on Sabouraud dextrose agar for two generations (a generation was 24-48 hours) and the morphological and biochemical tests had been investigated twice.

Identification methods

All isolated yeasts and standard yeast strains were tested for morphological and physiological characteristics by classical methods (I) which took time more than 24 hours,and rapid methods (II) which took time 24 hours or less. And all of the tests were done altogether except for the rapid carbohydrate assimilation test. These methods were compared by using chi-square test.

I. Classical methods

I.1 Carbohydrate assimilation test
auxanographic method (40)

I.2 Carbohydrates fermentation test (40,50)

I.3 Urease test (40)

I.4 Nitrate assimilation test (auxanographic method) (40)

I.5 Growth in Sabouraud broth for surface film production (51)

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II. Rapid methods

II.1 Carbohydrate assimilation test (modified from Wickerham tube method (40,50)

II.2 Germ tube test (40)

II.3 Rapid urease swab test (see appendix)

II.4 Rapid nitrate utilization swab test (see appendix)

II.5 Chlamydoconidia formation test (55)

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II.7 L-DOPA paper strip test (73)

II.8 India ink-preparation (28)

II.9 Growth in acidic pH Sabouraud broth (42)

I. Classical methods

I.1 Carbohydrate assimilation test(Auxanographic Method)

Materials

1. Six tubes of yeast nitrogen base (YNB) agar with indicator
2. Three sterile plates, 150 mm.
3. Fifteen different kinds of carbyhydrate discs (see appendix)
4. Sterile distilled water
5. Forceps
6. Tested strain

Method

1. YNB agar was melted and allowed to cool to 50°C in water bath.
2. The active growth yeast was suspended in steriled distilled water about no. 4-5 McFarland density.

3. Three millilitre of yeast suspension was pipetted in 6 tubes of melted YNB agar and mixed thoroughly.

4. Two tubes of inoculated YNB agar were poured in a plate and allowed the agar to harden.

5. Fifteen different carbohydrate discs were placed on the surface of the harden agar as in the Figure 1.

6. Plates were incubated at room temperature for 24-72 hours.

7. Positive result should be observed by changing of color from violet to yellow and/or the growth of yeast colonies around each kind of carbohydrates discs at 24, 48 and 72 hours.

I.2 Carbohydrate fermentation test

Materials

1. Six tubes of fermentation broth (see appendix) with Durham tube, each containing one of the following carbohydrates: glucose, sucrose, maltose, trehalose, galactose and lactose.

2. Sterile liquid paraffin

3. Sterile distilled water

4. Tested strain

Method

1. The active growth yeast was suspended in sterile distilled water about no. 4-5 McFarland density.

Plate I

Plate II

Plate III

Figure 1 Demonstration of carbohydrate discs in the assimilation for each isolate plates.

Plate I : glucose galactose sorbose sucrose maltose

Plate II : cellobiose trehalose lactose melibiose raffinose

Plate III : xylose soluble starch arabinose inositol dulcitol

2. One milliliter of yeast suspension was pipetted into each tube of six different carbohydrate fermentation broth.

3. Steriled liquid paraffin is overlayed upon the inoculated broth about 1.0 - 1.5 cm. thickness.

4. The tubes were incubated at room temperature and examined for the production of gas in Durham tube for 3,5,7 and 14 days.

I.3 Urease test

Materials

1. Christensen urea agar slant (see appendix).
2. Tested strain

Method

1. The active growth yeast was streaked on Christensen urea agar slant

2. The inoculated slant was incubated at room temperature for 3 days and examine for the change of color from orange to brightly pink as the positive test.

I.4 Nitrate assimilation test (Auxanographic method)

Materials

1. Yeast carbon base agar (see appendix)
2. Potassium nitrate disc and peptone disc. (see appendix)
3. Steriled plate
4. Tested strain

Method

1. The active growth yeast was suspended in steriled distilled water about 0.5 McFarland density.
2. Half of millilitre of yeast suspension was pipetted into a melted (50°C) yeast carbon base agar tube, mixed throughly and poured into a plate, allowed agar to harden.
3. Plate was devided into two parts and place potassium nitrate disc on one side and peptone disc on the other as the control positive growth.
4. Plate was incubated at room temperature for 3 days and examined for the growing zone around each disc indicated a positive result or assimilated.

I.5 Growth on Sabouraud broth for surface film production

Materials

1. A tube of Sabouraud broth (see appendix)
2. Tested strain

Method

1. The active growth yeast was suspended in sterile distilled water about no. 4-5 McFarland density.
2. One drop of yeast suspension was inoculated in Sabouraud broth.
3. Tube of inoculated broth was incubated at room temperature for 3-5 days and examined for the surface film production.

I.6 Temperature tolerance

Materials

1. Three Sabouraud dextrose agar slants (see appendix)
2. Tested strain

Method

1. The active growth of yeast was streaked in 3 tubes of Sabouraud dextrose agar slants.

2. The inoculated tubes were incubated at 25 C, 37 C and 42 C for 5-7 days and examined for growth in 3 tubes.

II. Rapid methods

II.1 Carbohydrate assimilation test [modified from Wickerham tube methcd (8)].

Materials

1. Yeast nitrogen base broth with and without 15 different kinds of carbohydrate . (see appendix)

2. Sterile distilled water

3. Sterile microtiter plate U-type-bottom (see appendix)

4. Automatic pipette

5. Microdropper contains 25 microliters per drop

6. Tested strain

Method

1. The active growth yeast was suspended in sterile distilled water equivalent to McFarland no. 0.5 for Candida,

Trichosporon and Geotrichum spp. and McFarland no. 1 for Cryptococcus and Rhodotorula.

2. Yeast nitrogen base broth with and without carbohydrate was pipetted to microtiter wells as in the diagram 1. The volume was 200 microliters per well.

3. The 25 microliters of yeast suspension was dropped on each microtiter well by using microdropper.

4. Microtiter plate was incubated at room temperature (25-30°C) for 18-24 hours. The positive reaction can be detected by changing color of indicator from purple to yellow or assimilated those carbohydrates (Fig.2, Fig.3).

II.2 Germ tube test

Materials

1. Sterile test tube
2. pooled human serum
3. glass slide and cover slip
4. Tested strain

Method

1. Half of mililitre pooled human serum was pipetted into each sterile test tube.

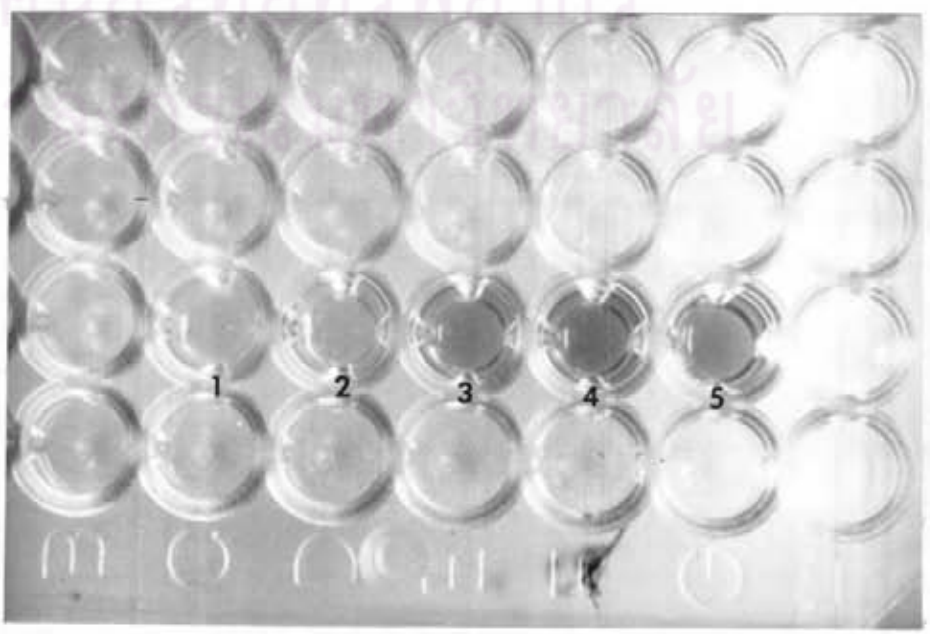
2. A light inoculum of active growth yeast was emulsified in serum so as to make concentration about McFarland no. 1

H	G	F	E	D	C	B	A		
lactose	treholose	cellobiose	maltose	sucrose	sorbose	galactose	glucose	1	Tested strain no. 1
control	dulcitol	inositol	arabi-nose	xylose	soluble starch	raffinose	meli-biose	2	
								3	
lac.	tre.	cel.	mal.	suc.	sor.	gal.	glu.	4	Tested strain no. 2
con.	dul.	ino.	ara.	xyl.	sol.st.	raf.	mel.	5	
								6	
lac.	tre.	cel.	mal.	suc.	sor.	gal.	glu.	7	Tested strain no. 3
con.	dul.	ino.	ara.	xyl.	sol.st.	raf.	mel.	8	
								9	
lac.	tre.	cel.	mal.	suc.	sor.	gal.	glu.	10	Media control
con.	dul.	ino.	ara.	xyl.	sol.st.	raf.	mel.	11	
								12	

Diagram 1. The sequence of carbohydrates in microliter plate.

Figure 2. Rapid carbohydrate assimilation in microtiter plate

Figure 3. Demonstration the positive and negative reaction for rapid carbohydrate assimilation (positive test = No 1,2; negative test = No 3,4,5)



or less.

3. Tube were incubated in 37°C incubater for 2 1/2 hours.

4. After 2 1/2 hrs. incubation time, the suspension was wet mounted and examined for production of germ tube under high air dry objective lens.

II.3 Rapid urease swab test

Materials

1. The urea impregnated cotton-tipped applicator (see appendix)

2. 1% benzalkonium chloride pH 4.86 +/- 0.01

3. Tested strain

Method

1. The cotton tipped applicator was sweeped across the surface of active growth yeast.

2. The inoculated applicator was placed into a test tube containing 3 drops of 1% benzalkonium chloride.

3. The cotton-tipped portion of the applicator was swirled firmly against the bottom of the test tube to embeded the test organism well into the cotton fiber and the tube was pluged with cotton.

4. Tests were incubated in a 45°C water bath and were examined after 5, 10, 15, 20 and 30 minutes for color changed of indicator from pale yellow to purple which indicated a positive urease test (Fig.4).

II.4 Rapid nitrate utilization test

Materials

1. The potassium nitrate impregnated cotton-tipped applicator (see appendix)

2. 0.8% sulfanilic acid in 5 N acetic acid.

3. 0.5% ∝-napthylamine in 5 N acetic acid.

4. Two clean test tubes.

5. Tested strain

Method

1. The cotton tipped applicator was sweeped across the surface of active growth yeast.

2. The inoculated applicator was placed into a test tube and swirled firmly against the bottom of the tube to embeded the test organism well into the cotton fibers and the test tube was pluged with cotton.

3. The test tube was incubated for 10 minutes at 45°C , the swab was removed from the tube and inserted into a second tube containing two drops each of ∝-napthylamine and sulfanilic acid

Figure 4. Demonstration of positive and negative test for rapid urease test

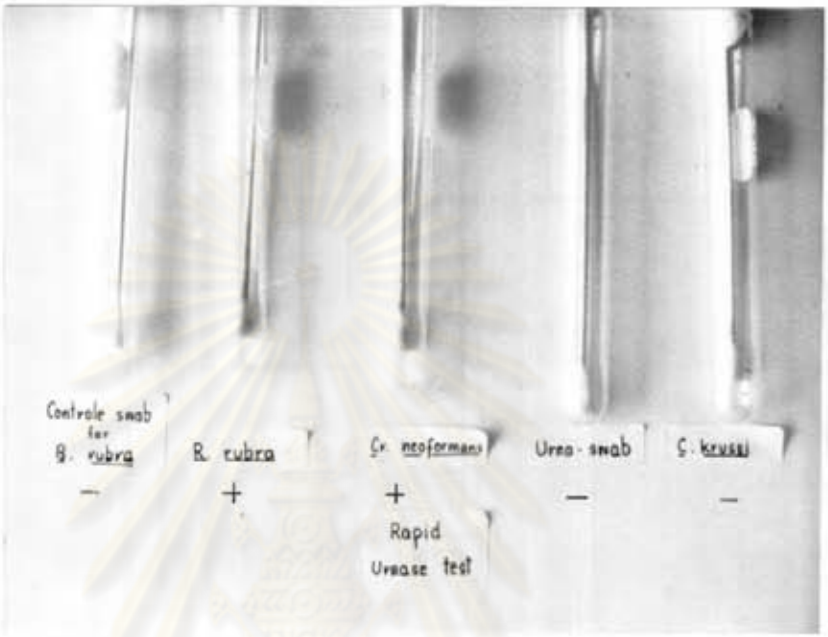
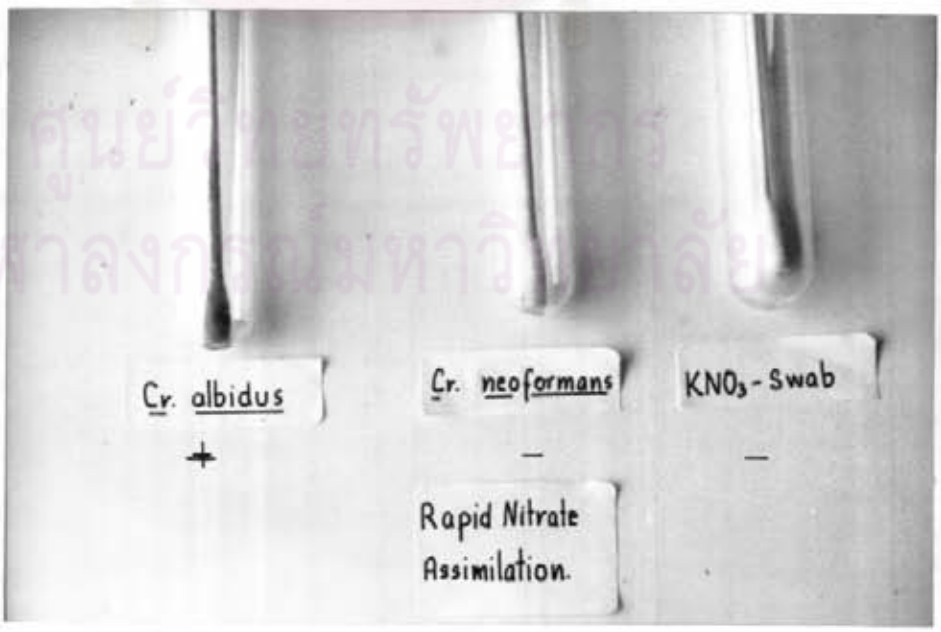


Figure 5. Demonstration of positive and negative reaction for rapid nitrate assimilation test.



reagent and examined for a change in colour from white to bright cherry red which indicated a positive result (Fig. 5).

II.5 Chlamydoconidia formation

Materials

1. Glutinous rice tween 80 agar plate (see appendix)
2. Steriled cover slip
3. Tested strain

Method

1. Glutinous rice tween 80 agar plate was divided into 4 sections.
2. The active growth yeast was streaked in a quarter of the divided plate.
3. Sterile cover slip was placed on the streak.
4. The inoculated plate was incubated in 25°C incubator for 24 hours and examined for chlamydoconidia production under high air dry objective lens.

II.6 Cycloheximide sensitivity

Materials

1. Mycobiotic agar slant which 0.1% actidione was added. (see appendix)

2. Sabouraud dextrose agar slant (see appendix) as a control.

3. Tested strain

Method

1. Light inoculum of active growth yeast was streaked on the Sabouraud dextrose agar first to avoid transfer of cycloheximide to ccntrol slant and then streak a light inoculum on the Mycobiotic agar slant.

2. The inoculated tubes were incubated at room temperature for 24 hours then compared growth in two tubes and reported for sensitivity when showed test tube no or less growth than the control tube.

II.7 L-DOPA paper strip test

Materials

1. L-DOPA paper strip (3,4 β - dihydroxyphenyl alanine) (see appendix)

2. Sterile distilled water

3. Steriled petri dish

4. Tested strain

Method

1. L-DOPA-paper strip was placed on Petri dish and refreshed by drops of sterile distilled water.

2. The active growth yeast was heavy inoculated on the paper and examined for development of dark colour at 15, 30, 45 and 60 minutes.

II.8 India ink-preparation

Materials

1. Glass slide and cover slip

2. 50% of India ink

3. Tested strain

Method

1. One drop of India ink was placed on glass slide.

2. The active yeast culture was smear in India ink.

3. Cover slip was placed on the mixture and examined for encapsulated yeast cells by using low objective lens and confirmed with high air dry objective lens.

II.9 Growth in acidic pH Sabouraud broth

Materials

1. Two tubes of Sabouraud broth pH 1.5 (see appendix)

2. Tested strain

Method

1. The active growth yeast was suspended in sterile water equivalent to McFarland no. 4-5 density.

2. A drop of the yeast suspension was incubated in Sabouraud broth pH 1.5.

3. Tube of inoculated broth was incubated at room temperature for 3 days and examined for the density of growth by comparing with C. albicans as the positive test.