I. **PRINCIPLE**:

A. Yeasts are a heterogenous group of fungi that superficially appear to be homogeneous. Yeasts grow in a conspicuous unicellular form that reproduces by fission, budding, or a combination of both. True yeasts reproduce sexually, developing ascospores or basidiospores under favorable conditions. The majority of ascomycetous and basidiomycetous yeasts isolated by the lab go unrecognized because most of them are heterothallic. In most instances, only one of the mating types is isolated and therefore no asci or basidia are produced.

B. Yeast-like fungi (imperfect yeasts) reproduce only by asexual means. The identification of these fungi is based upon a combination of morphological and biochemical criteria. Morphology is primarily used to establish the genera, whereas biochemical assimilations are used to differentiate the various species.

C. Principal Criteria and Tests for Identifying Yeasts

1. Culture characteristics - Colony color, shape, texture
2. Asexual structures

    a. Shape and size of cells
    b. Bipolar, fission, multipolar or unipolar "budding"
    c. Absence or presence of arthroconidia, ballistoconidia, blastoconidia, clamp connections, endoconidia, germ tubes, hyphae, pseudohyphae, or sporangia and sporgangiospores.

3. Sexual structures - Arrangement, cell wall ornamentation, number, shape and size of ascospores or basidiospores
4. Physiological studies

    a. Assimilation
    b. Cycloheximide resistance
    c. Fermentation
    d. Nitrogen utilization
    e. Urea hydrolysis
    f. Temperature studies

II. **ISOLATION TECHNIQUES FOR MIXED CULTURES**:

All initially isolated yeasts may be contaminated or in mixed culture. Direct mounts and subsequent streaking for colony isolation are necessary to confirm the purity of each yeast isolate. Pure cultures are essential for assimilation procedures. Streaking subcultures for spatial isolation is adequate in most cases. The following additional techniques can be used to purify yeasts:

A. Bacterial contamination

1. Colony isolation on SAB agar

    a. Suspend a small portion of the yeast to be decontaminated in sterile distilled water.
    b. Streak a loopful of the suspension for colony isolation onto a plate of SAB agar.

II. **ISOLATION TECHNIQUES FOR MIXED CULTURES**: (cont'd)

c. Incubate at 30°C for 48 hours.
d. Examine for isolated colonies. Verify purity with a direct exam.
e. If colonies are not pure, further steps are necessary (Step 2).

2. Colony isolation on SAB agar plus chloramphenicol SAB plus penicillin and streptomycin, or BHI plus 10% blood plus gentamicin and chloramphenicol.

a. Suspend a small portion of the yeast to be decontaminated in sterile distilled water.
b. Streak a loopful of the suspension for colony isolation onto one of the above media.
c. Incubate at 30°C for 48 hours. Check for purity. If colonies are not pure, further steps are necessary (Step 3).

3. Acidification of SAB broth

a. Suspend a small portion of the yeast to be decontaminated in sterile distilled water.
b. To each of 4 tubes containing 10 ml of SAB broth, add 1 drop of 1 N HC1 to the first tube, 2 drops to the second tube, 3 drops to the third tube, and 4 drops in the fourth tube.
c. Add 0.5 ml of the contaminated yeast suspension to each tube.
d. Incubate at 30°C for 24 hours.
e. Subculture a loopful of each broth to SAB agar plates. Streak for isolation.
f. Incubate the SAB plates at 30°C for 48 hours.
g. Check purity. If still not pure, consult the supervisor.

B. Mixed Yeasts

1. Suspend a portion of each suspected colony type in a tube of sterile distilled water.
2. Streak a loopful of the suspension onto a SAB agar plate.
3. Incubate the SAB plate at 30°C for 48 hours.
4. Check for purity. If the yeast is not pure, it must be restreaked. Note that some strains have both rough and smooth colony types in pure culture.

C. Mould Contamination

1. Colony isolation on yeast malt (YM) agar.

a. Suspend a small portion of the yeast-mould colony in sterile water.
b. Streak a loopful of the suspension for colony isolation onto a plate of YM agar.
c. Incubate the YM plate at 30°C for 4-6 days.
d. Check for purity. If not pure, further steps are necessary (Step 2).

2. Colony isolation in yeast malt broth.

a. Transfer a small portion of the yeast-mould isolate to a tube containing 10 ml of YM broth.
b. Incubate the YM broth at 30°C for 48 hours. Remove a small portion of the sediment with a sterile capillary pipette by slipping the pipette along the edge of the tube to the bottom without disturbing the mycelial pellicle.

II. **ISOLATION TECHNIQUES FOR MIXED CULTURES**: (cont'd)

c. Streak the sediment for colony isolation onto a plate of YM agar.
d. Incubate at 30°C for 4-7 days and then prepare a direct mount. If the yeast is not pure, further steps are necessary (Step 3).

3. Colony isolation in shake culture.

a. Transfer a small portion of the yeast-mould isolate to a 250 ml Erlenmeyer flask containing approximately 100 ml of YM broth.
b. Place the flask on a rotary shaker and incubate at 30°C for 4-6 days.
c. Carefully remove a small amount of the sediment with a sterile capillary pipette. Ensure that the balls of mycelium are not removed by accident.
d. Streak the sediment for colony isolation onto a plate of YM agar.
e. Incubate at 30°C for 4-7 days and then prepare a direct mount using a small portion of each yeast colony to be identified. If the yeast is not pure, consult the supervisor.

III. **IDENTIFICATION SCHEME**:

To identify yeasts, first examine the colony color, shape and texture. If the colony is black to brown in color or moist mycelial in texture, prepare a direct mount. Using the flow chart, determine the genus based on microscopic morphology. If the colony is pink to red, streak the colony out for isolation. After incubation, check for the presence of satellite colonies. Examine microscopically for forcibly discharged conidia. Using the flow chart, determine the genus based on morphology. Assimilations will be necessary to determine the species of *Rhodotorula*. If the colony is white or cream color, perform a germ tube test. If the germ tube test is positive, the yeast can be identified as *Candida albicans*. If germ tubes are absent, confirm the purity of the yeast isolate and inoculate an Vitek YBC or an API20C strip and a Dalmau plate. No identifications will be performed on yeast recovered from respiratory sites except if the yeast resembles *Cryptococcus neoformans* or if the identification is requested by the physician to the director. For patient care, all germ tube negative yeast from respiratory sites will be reported as yeast not *Candida albicans* or *Cryptococcus* sp. Most of the commonly recovered yeasts can be identified to the species level using the morphology on corn meal agar and assimilation results. (See Dalmau morphology flow chart). Rarely, it is hydrolysis, cycloheximide resistance or ascospore induction. These procedures are available to aid in yeast identification. Problem identifications should be brought to the attention of the supervisor. Identifications using a commercial method will be carried out by using materials from the Clinical Microbiology Laboratory which has been ecked for QC. Reimbursement of the material used will be made to them by the MMRC.

IV. **IDENTIFICATION PROCEDURES**:

A. Direct Mounts - Direct mounts are made in order to study yeast morphology microscopically and to determine purity of the isolates.

B. Lactophenol Mount

1. Place a small drop of lactophenol (LP) on a clean glass microscope slide.
2. Remove a small portion of the yeast colony and place it into the drop of LP and suspend the cells.
3. Place a clean cover glass over the suspension and observe microscopically.
4. Seal edges of the cover glass with fingernail polish to temporarily preserve the mount.

YEAST MORPHOLOGY FLOW CHART

Brown to black colony			Direct Mount		Yeast only	*Phaeococcomyces*
					Yeast and hyphae	*Aureobasidium* *Exophiala* *Wangiella*
Pink to red colony	Satellite colonies	Present	Forcibly discharged conidia present			*Sporobolomyces*
		Absent			Purify	*Rhodotorula*
White colony			Germ Tube Test	Positive		*Candida albicans*
				Negative	Purify	Dalmau plate YBC-Vitek
Moist mycelial colony			Direct Mount	Arthroconidia		*Geotrichum* *Trichosporon*
				Other types of conidia		hypohomycete

* Young colonies of *Cryptococcus neoformans* are often indistringuishable from young colonies of *Candida*. Therefore, technologists performing the germ tube test must be alert to yeasts that have a microscopic morphology suggestive of *Cr. neoformans*.

DALMAU MORPHOLOGY FLOW CHART

<table>
<tr><td colspan="6">Ascospores present</td><td>Hansenula
Pichia
Saccharomyces</td></tr>
<tr><td colspan="6">Ballistoconidia present</td><td>Sporobolomyces and similar yeasts</td></tr>
<tr><td colspan="6">Basidiospores present</td><td>Filobasidiella
Filobasidium
Leucosporidium</td></tr>
<tr><td rowspan="3">Hyphae, pseudohyphae, or both present</td><td rowspan="3">chlamydospores</td><td colspan="3">Present</td><td></td><td>Cadida albicans</td></tr>
<tr><td rowspan="2">Absent</td><td rowspan="2">arthroconidia</td><td rowspan="2">Present</td><td rowspan="2">blastoconidia</td><td>Present</td><td>Trichosporon</td></tr>
<tr><td>Absent</td><td>Geotrichum</td></tr>
<tr><td colspan="3">Absent</td><td colspan="2"></td><td>Candida
Saccharomyces</td></tr>
<tr><td colspan="6">Sporangia present</td><td>Prototheca
Sarcinosporon
Fissuricella or similar fungi</td></tr>
<tr><td colspan="6">Yeast only</td><td>Cryptococcus
Rhodotorula
Torulopsis
Candida guilliermondii or an ascomycetous yeast not producing ascospores, i.e., Saccharomyces</td></tr>
</table>

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

C. Germ Tube Test

1. The germ tube test provides a simple, reliable and economical procedure for the presumptive identification of Candida albicans. About 95% of the clinical isolates produce germ tubes when incubated in serum at 35ºC for 2.5-3 hours. A germ tube represent the initiation of a hypha directly from the yeast cell. They have parallel walls at their point of origin. Germ tube formation is influenced by the medium, inoculum size and temperature of incubation. Fresh normal pooled human sera or a commercially available germ tube solution (Remel Lenexa kansa) are to be used as the medium for the test. The inoculum should result in a very faintly turbid serum suspension. Over-inoculation will inhibit the development of germ tubes. Incubate in at 35ºC-37ºC for 2.5-3 hours.
2. A germ tube test is to be set up daily on all cultures positive for yeast from sterile sites, results to be reported in the computer as soon as available as either *Candida albicans* or yeast not *Candida albicans* or *Cryptococcus* identification to follow.
3. The germ tube test is also used as a preliminary screen on yeast isolated in the laboratory to rule out the presence of *Cryptococcus neoformans* in respratory or sterile site specimens. If a yeast is encountered with the morphology of *Cr. neoformans* when screening with the germ tube, the supervisor should be notified immediately, a note should be made in the worksheets and computer, and if proper, physicians should be notified.
4. Since the time needed for the final identification of a yeast after the germ tube test may take 2-4 days for the germ tube negative yeast and because on occasions, the physician will need a presumptive identification for proper therapy (i.e. urinary infections by T. glabrata versus Candida sp.), a presumptive identification is to be given from the morphology of the yeast in the germ tube test and or colony morphology.
5. Procedure

    a. Label 12 x 75 mm test tubes.
    b. Using a Pasteur pipette, dispense 3 drops of fresh pooled human serum into the tubes. Serum can be obtained from the Serology lab.
    c. With a sterile wooden applicator stick, lightly touch a yeast colony and place the stick into the serum.
    d. Suspend the yeast in the serum. Discard the stick in a discard container.
    e. Incubate the test at 35ºC for 2.5-3 hours.
    f. Place a drop of the suspension on a clean microscope slide.
    g. Place a clean cover glass over the suspension and then examine it with a microscope using the low power objective. Use the high power objective to confirm the presence or absence of germ tubes.*
    h. Read controls and record results.
    i. If time is not allowed to read and record the test results add a drop of 10% formalin/formaldehyde to each tube, seal the top with parafilm, and store at 2-4° in the refrigerator.

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

* Young colonies of *Cryptococcus neoformans* are often indistinguishable from young colonies of *Candida sp*., therefore technologists performing germ tube tests must be alert to yeasts that have a microscopic morphology suggestive of *Cr. neoformans.* Mature cells of *Cr. neoformans* are spherical, 3-4 um in diameter, with a pointed bud scar. Detached buds are smaller, and oval in shape. Yeasts suspected of being *Cr. neoformans* must be evaluated as soon as possible with an India Ink mount and supervisor notified.

D. Commerical Yeast Identification Systems

1. Principle

a. Two commerically available yeast identification systems are described: API 20C Yeast Identification System (API Analytab Products, Plainview, NY) and the Biomerieux Vitek System (Hazelwood, MO). These two yeast identification systems are easy to use. The API 20C requires less preparation of reagents. The Vitek system is an automated system.
b. The two systems are based on modifications of the classic auxanographic technique of carbohydrate assimilation. When an organism is able to assimilate a particular carbohydrate, in the cupules of reconstituted substrates (API), or, accompanied by a color change, in the wells containing the substrate of Vitek, the systems must be supplemented with morphological studies, and both systems should have germ tube tests done in conjunction with them as a means of obtaining a more-complete profile of the yeast cells being identified.
c. Yeast identification determines the identity of an etiological agent causing disease and provides access to the information published in the literature regarding diagnosis, patient management, and prognosis. Yeast identifications require isolates in pure culture.
d. The Vitek YBC will be used routinely in this laboratory because it is semi-automated and technologist time is not involved in the set up or interpretation of the test. The API 20C is used as a back-up for the YBC.

2. Specimen - A pure culture of 24- to 48-hour-old yeast cells growing on Sabouraud glucose (SAB) or other nonselective agar is required.

3. Reagents

a. API 20C Yeast Identification System - In addition to the kit, the following supplies are needed.

(1) Sterile wooden applicator sticks
(2) Sterile Pasteur pipettes (5 ml)
(3) Squeeze bottle
(4) Incubator (30°C)
(5) Water bath (48 to 50°C)
(6) SAB agar plates

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

b. Vitek Yeast Identification System - In addition to the kit, the following supplies or equipment is needed.

(1) Sterile wooden applicator sticks
(2) Sterile tubes containing 1.8 ml of 0.45 to 0.5% saline
(3) Colorimeter (Vitek colorimeter, product no. 52-1210)
(4) Filling stand (Vitek product 52-0700)
(5) Vitek System Senior, which includes the following:

(a) Filler-sealer module
(b) Reader incubator unit
(c) Printer
(d) Computer (with a minimum of an R4.01, 1989 updated data base)
(e) Data terminal

(6) Fine-tip markers
(7) Incubator (30°C)
(8) Sabouraud dextrose agar plates

4. Quality Control - Include known isolates of *Torulopsis glabrata*, *Candida albicans*, and *Cryptococcus laurentii.* For specific details refer to the QC Section.
5. Procedure

a. API 20C Yeast Identification System

(1) Melt the basal medium in the ampoules by placing them in an autoclave for 2 minutes or in a boiling water bath (do not prolong boiling; ampoule may explode).
(2) Place the ampoules in a water bath at 48 to 50°C, and allowthem to cool.
(3) Prepare an incubation tray. Use a squeeze bottle to dispense 20 ml of water into the tray, and then place the strip into the incubation tray.
(4) Open the ampoules according to the manufacturer's instructions, and inoculate the molten medium with an applicator stickthat has touched one or two colonies (>2 mm diameter). Adjust to a density just below 1+ on a Wickerham card.
(5) Inoculate the strip (20 cupules; approximately 0.2 ml each) by using a Pateur pipette and following the manufacturer's directions, and then place thelid on the tray.
(6) Incubate the trays at 28 to 30°C for 72 hours. Read and record the results after 24, 48, and 72 hours of incubation.

b. Vitek Yeast Identification System

(1) Use one to three colonies to prepare the yeast suspension in the 1.8 ml saline tubes. Adjust the suspension to a McFarland no. 2 standard by using the Vitek colorimeter (46 to 56% transmittance, 450 nm filter).
(2) After labeling the yeast cards with a marker, place the card in the filling stand with a transfer tube that is in the yeast suspension.
(3) Inoculate the cards via the filling module.
(4) Seal the cards via the sealer module and incubate at 30°C for either 24 or 58 hours, depending on the readings provided by the instrument.

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

6. Results

a. API 20C Yeast Identification System

(1) Although API is a widely used commerical yeast identification system, it does not include rhamnose (for *C.lusitaniae*) and urea.
(2) Germ tube tests andmorphological studies should be included.
(3) API yeast profiles sometimes give one to three different yeast identifications for an individual isolate. Supplemental tests may then be required.
(4) It takes 3 days to obtain final results.

b. Vitek Yeast Identification System

(1) Heavy encapsulated yeasts and isolates with extensive mycelial growth are sometimes difficult to suspend.
(2) Morphological studies using a Dalmau plate or additional tests are required in order to confirm the identification of some isolates or when some strains react similarly in the test system.
(3) Most of the results are obtained after 24 hours and a few isolates (26%) may require additional incubation.

E. Nitrate Utilization

Yeasts have the ability to use ammonium sulfate, asparagine, peptone, and urea aerobically as sole sources of nitrogen if adequate vitamins are provided. In contrast, aliphatic amines, potassium nitrate, sodium nitrate, and some amino acids are utilized selectively by different yeasts. In general, if a yeast can utilize nitrate, it can also use nitrate as a nitrogen source. In the auxanographic method, yeast carbon base medium is seeded with a yeast suspension. Potassium nitrate impregnated discs are applied to the surface. Growth around the disc is a positive reaction.

1. Procedure

a. Prepare a yeast suspension from a 24- to 48-hour old colony in sterile distilled water equal to a MacFarland number 1 standard.
b. Melt a tube containing 15 ml of yeast carbon base medium for each yeast to be tested. Melted tubes can be cooled in a 50ºC water bath.
c. Add 1.0 ml of the yeast inoculum to the tube of yeast carbon base medium. Mix.
d. Pour the medium in a 15 x 100 mm sterile Petri dish and allow the agar to harden at room temperature.
e. Aseptically place discs containing KNO_3 and peptone on the medium surface. Label the plates if the discs look alike.
f. Incubate the plate with its inoculated surface up at 30ºC.
g. Check for growth around the peptone disc. If growth is absent, the test is not valid and must be repeated.
h. Record results of growth around the KNO_3 disc. Growth is a positive result.

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

2. Quality Control - Control isolates will be used each time a set of nitrate utilization tests are run.

   a. Positive (growth around KNO_3 disc) - *Rhodotorula glutinis*
   b. Negative - *Candida albicans*

NOTE: This procedure is not routinely performed and is only used for rare situations when the API or Vitex cannot identify a yeast.

F. Urea Hydrolysis - microtiter

The rapid urea hydrolysis test in microtiter wells is used to screen isolates for *Cryptococcus neoformans*. Under these conditions, *C. neoformans* will rapidly hydrolyze urea, which results in a pink to red color.

1. Procedure

   a. Reconstitute each vial of Difco Urea R broth with 3 ml of sterile distilled water on the day that it is to be used.
   b. Dispense 3-4 drops into each well to be used in a micro- titer plate.
   c. Transfer a heavy inoculum of each yeast colony to a well containing urea broth. A subculture or reincubation of the isolation plate may be necessary if there is insufficient growth of the colony. Isolation of the yeast will be necessary if the culture is contaminated with bacteria. Colonies to be tested should be no older than 7 days.
   d. Seal wells with scotch tape and incubate for 4 hours at 37°C.

2. Quality Control

   a. Positive (pink to red color) - *Cryptococcus neoformans*
   b. Negative - *Cryptococcus albidus*
   c. Uninoculated

G. Ascospore Induction and Detection

One step in indentifying a yeast involves determining whether or not the isolate has the ability to form ascospores. Some yeasts will readily form ascospores on primary isolation medium, whereas others require special media. The ability to form ascospores varies from isolate to isolate and may be lost in old laboratory strains. If only one mating type of a heterothallic yeast is present, no ascospores will be formed. Ascospore media contain small amounts of carbohydrates; this restricts vegetative growth while enhancing ascospore formation.

1. Procedure

   a. Inoculate the yeast to yeast malt agar for enrichment. Incubate 2-3 days.
   b. Inoculate the yeast from the yeast malt agar to a V-8 juice agar slant. Incubate aerobically at 20-25°C.

IV. **IDENTIFICATION PROCEDURES**: (cont'd)

c. Most freshly isolated strains begin forming ascospores in 1-2 days. Older stock cultures usually require a longer period of time.
d. Examine the culture in 3-5 days and weekly thereafter for 3 weeks. Prepare wet mounts of the yeast in distilled water.
e. Examine the wet mounts using the oil immersion lense. Ascospore form, surface topography, size, color, brims, number of ascospores per ascus, and the presence or absence of inclusion bodies are characteristics used in part to identify the various species.
f. If ascospores cannot be readily seen in a wet mount, perform an acid-fast stain. The Kinyoun stain is recommended. Ascopsores are acid-fast.

2. Quality Control

Each time a yeast is inoculated to a V-8 juice agar slant, *Saccharomyces cerevisiae* will be inoculated concurrently as a control. When performing the Kinyoun stain, use the same *S. cerevisiae* as a positive control for the staining procedure.

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