LAB MANUAL FOR YEAST STUDY

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Updated September 2008

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I. YEAST ISOLATION FROM BEETLE GUT

A. Collection of beetles

Preparation: paper bags, Ziplock bags, pencil, knife, Aspirator, vials, white sheet

Important points:

1. Host information is very important. Keep the host mushroom too.
2. Insects ideally should be put in separate containers (Ziplock bags or plastic vial or box) and put into the same paper bag containing the mushroom host.
3. Take notes for the collection date, place, and host information and put in the paper bag with the insect and mushroom host.
4. Sort out beetles from mushrooms ASAP because they do escape.
5. Keep the beetles for 3 days in a clean container with slightly moistened filter paper so that surface debris is removed.

B. Dissection of Beetles

Put the beetles in a 0.5ml Eppendorf tube with 95% EtOH for 1-2 min. [You need to use a little common sense here because the size of tube and amount of solution varies depending on the size of insect.]

1.
2. Wash with 1ml of 0.7% sterile saline in 1.5ml Eppendorf tube.
3. Plate this saline on an acidified YM agar plate as negative control.
4. Use forceps (or pins) to dissect beetles under a microscope on a sterile slide (or petri plate for larger beetles), and remove gut aseptically from body.
5. Transfer the gut to a fresh 0.5ml Eppendorf tube containing 50-100μl of 0.7% sterile saline.
6. Crush the gut with a pipette tip, and plate all the solution (including gut pieces) on acidified YM agar (or XA agar, see below).
7. Streak the gut solution with a loop, and incubate at room temperature (25˚C).

Fungivorous beetles: Use AYM or YM plates

Xylophagous beetles: Use ½ Xylose Agar (XA, see p. 6)

C. Yeast Isolation

1. Check the gut plates every day (for usually up to 5 days), and observe colonies under a microscope to check yeasts. Make a water mount of a piece of the colony and observe under a compound microscope if necessary. Yeasts and bacteria are distinguishable by their cell sizes.
2. Pick a single colony up, and streak to on a fresh acidified YM (streak three times by crossing the lines on the plate: **single colony isolation**). This will be the first time.
3. Perform single colony isolation at least twice before starting identification and other experiments. The second or further purification is best done on YM agar.

D. Maintenance of Yeast Cultures

1. Purified yeast isolates should be kept at 4˚C on 2% Malt extract slant culture. Add autoclaved mineral oil to well-growing fresh slants. Make sure the oil covers at least 2 cm above the agar. These cultures should be subcultured every 2-3 months.

Guidelines for working with oil-preserved cultures

- USE sterile technique!! This is our only set of working cultures.
- DO NOT get the oil on the parafilm!! If you do, peel off the parafilm and wipe the tube clean before replacing with new parafilm.
- DO NOT take all the cells, use 1μl loops (white, Greiner Bio-one) if possible. This is our only set so share the cells with everyone.

2. Prepare frozen form ASAP after purification, and keep at -80˚C.

General Frozen form: 3 ml of liquid culture (2days old, in YM broth) + 1ml of 60% glycerol in water (sterilized by autoclave). The final concentration of glycerol should be 10-15%.

Making frozen form from a slant culture: Prepare a fresh slant culture (YM or 2% Malt extract). Add 5 ml of 10% glycerol to test tube and wash off the cells with disposable pipette to homogenize. Transfer mixture to vials or straw.

frozen vials here for lab use in -80C.

1) Prepare YM slant agar (or any other medium required for the best growth of the organisms you want to preserve).

2) Prepare autoclaved 10-15% glycerol. (Adjust with sterile water. Aim for 10% but 10-25% is OK. Store 10% glycerol stocks as 7-10 ml in screw cap test tubes, and discard the remaining solution after using it to prevent any possible contamination.)

3) Culture for 5-7 days or until enough cells are grown up.

4) Add 5ml of 10% glycerol directly onto the slant culture. Homogenize the cells in glycerol with a pipette.

5) Dispense 0.3-0.5ml of the cell mixture into each cryo-vial and put in -80C immediately. Make at least 10 vials, saving 3 for seed vials and the remaining 7 for research purposes. Record the numbers of vials, and make replacements before using up 'research' vials. One vial should be used after 2-3 months to check the viability and purity.

Straw method:

- Seal one end of a straw with a heat sealer. Autoclave.
- Inoculate 1.5ml of YM broth with a little bit of viable cells. Incubate for 3 days or until culture grows at a logistic phase.
- Add 0.5ml of 60% autoclaved glycerol into the YM culture. Mix well.
- Dispense 0.5ml of the broth into each straw, making a total of 3 straws.
- Seal carefully with the heat sealer. Label with the appropriate isolate number.
- Keep at -80˚C.

Frozen Form sets:

SET1, SET2, SET3 = all identical replicates, kept in separate incubators in different localities. BOXES = each box has 3 SETs. CELL = each box has 100 cells, and each cells contain 3 different isolates.

Suggestions for maintaining frozen forms:

1. Make at least 10 vials; keep 3 for 'seed' and use the rest for research purposes.
2. Accurate maintaining numbers and location of frozen vials: The maintenance of frozen vials takes training and organization. It is an essential step in our work. S/he should keep a careful record each time; and the vials should be replenished before they become is depleted.

E. General Media For Yeast Study

1. YM broth (general medium for liquid culture)

a. In 1000ml distilled water, add

- Yeast Extract……..3g
- Malt Extract…….3g
- Bacto Peptone……5g
- Glucose (dextrose)……10g

b. Autoclave at 121˚C for 15 min.

2. YM agar (general medium for agar plate culture)

a. Add 2% of agar in YM broth, and autoclave at 121˚C for 15 min.

b. Cool at room temperature or in water bath with mixing with spin bar and pour into Petri dishes.

Note: Approximate number of plates that can be poured with 500 ml:

20 plates for big plates (100x15 mm), 40 for small plates (60x15 mm)

3. Acidified YM agar (medium for yeast isolation)

a. Autoclave YM agar medium, and cool it down to about 65˚C (if you can touch the bottle with your hand, it's ready).

b. Add 0.7 ml of HCl to 1000ml of YM agar, and mix with spin bar.

c. Pour into Petri dishes.

4. 2% Malt slant agar (medium for stock culture)

a. Dissolve 2% (20g) of Malt Extract and add 2% (20g) agar in 1000ml of distilled water.

b. Heat the media with microwave to dissolve agar completely, and dispense 3-5 ml of the medium in each test tube (16 X 125 mm) with a syringe dispenser.

d. Autoclave at 121˚C for 15 min. Slant the racks on a wall after autoclaving.

***** Repeatedly force water through syringe dispenser soon after using so that the device is not damaged.**

F. Specialized Media For Yeast Study

1. Diluted V8 Agar:

1. Mix together V8 Juice and equal volume of dH_2O.
2. Adjust to pH 5.5 with NaOH and filter. Keep stock solution in freezer.
2. Dilute to 1:19 with dH_2O. Add 2% agar and autoclave at 121˚C for 15 min.

2. ½ CM (1/2 Corn meal agar)

1. Mix 8.5g Corn meal agar and 8-10g agar in 1L water. Autoclave at 121˚C for 15 min.

3. ½ XA (1/2 Xylose agar)

1. Mix 1L of ½X Yeast Nitrogen Base, 5g xylose, and 2% agar. Autoclave at 121˚C for 15 min.

G. Staining Yeasts and Ascospores

Vegetative structures: Use Gram stain. Single stain or counter staining works well.

Spore structures: Use Carbol Fuschin Acid Fast Method. This stain is good for penetrating the thick wall of certain spores such as *Metschnikowia* or *Spathaspora*.

Carbol Fuschin:

0.3g basic fuschin
10ml 95% EtOH
100ml 5% phenol

Mix and shake well.

Acid Fast staining:

Fix cells on slide. Add a piece of paper towel and flood with carbol fuschin. Heat until steam begins to form and continue heating for 1 minute. Wash the slide with water and destain briefly with acid-alcohol (95% EtOH + 3% HCl). Counter stain with Methylene Blue.

II. TESTS AND MEDIA FOR YEAST IDENTIFICATION

A. Observation of Yeast Morphology

1. Media

- YM broth
- YM agar
- Dalmau plate - cornmeal agar (DPC). See *The Yeast - a Taxonomic Study*

2. Steps

a. Inoculate cells on YM agar plate by making one line on center of plate and 2 dots on both sides of the line. Seal all plates with Parafilm. (You need to figure out the numbers of isolates which you can observe in a day, say 10-15. Do not make more plates than you can observe on time.)

b. After incubating 2-3 days at 25˚C, take cells from YM agar culture and inoculate to YM broth and DPC. For DPC, inoculate the same as YM agar above. Place a heat-sterilized cover glass on a dot or the edge of the line.
c. Incubate all the cultures at 25˚C for up to 2 weeks

3. Observations (See *The Yeasts* for more information) – Use Morphology Worksheet (p. 33)

a. **YM broth**: observe after 7 days, under a compound microscope (400X)
    1. Vegetative cell shape: see p. 81 of *The yeasts.*
    2. Cell size (*μ*m): measure the length and width of at least 20 individual cells under a compound microscope.
    3. Note any specific shape: single, in pair, cluster, pseudo or true hyphae.
b. **YM agar**: observe after 7-10 days by eyes or sometimes under a microscope.
    1. Note shape of colony: color, texture, surface (see p. 81 of *The Yeasts*)
    2. Note: true hyphae, pseudohyphae, ascus, and any specific structures.
c. **DPC**: observe after 7-10 days by eyes and microscope through the cover glass of the cover plate.
    1. Note cell shapes, shape of colony, hyphae or pseudohyphae

Note: Need to observe the cultures on time. Do not incubate too long!

a. Take a note in detail and draw if you cannot describe. Also note the dates of inoculation and observation.
b. Be careful of cross contamination.
c. Keep YM agar cultures after observation for DBB color test.

B. Assimilation of Carbon Compounds

1X solution is ~50mM concentration of C source

Carbon compounds to be tested:

C1 D-Glucose	C2 D-Galactose	C3 L-Sorbose	C4 D-Glucosamine
C5 D-Ribose	C6 D-Xylose	C7 L-Arabinose	C8 D-Arabinose
C9 L-Rhamnose	C10 Sucrose	C11 Maltose	C12 Trehalose
C13 a-Methyl-D-glucoside	C14 Cellobiose	C15 Salicin	C16 Arbutin
C17 Melibiose	C18 Lactose	C19 Raffinose	C20 Melezitose
C21 Inulin	C22 Soluble starch	C23 Glycerol	C24 Erythritol
C25 Ribitol	C26 Xylitol	C27 L-Arabinitol	C28 D-Glucitol
C29 D-Mannitol	C30 Galactitol	C31 myo-Inositol	C32 D-Glucono-1,5-lactone

C33 2-Keto-D-gluconate (small 10 ml tube)	*C34 5-Keto-D-gluconate	C35 D-Gluconate	C36 D-Glucuronate
*C37 D-Galacturonic acid	C38 DL-Lactate	C39 Succinate	C40 Citrate
C41 Methanol	C42 Ethanol	C43 Propane 1,2 diol	C44 Butane 2, 3 diol
C45 Quinic acid	C46 D-Glucarate	*C47 D-Galactonate	N - No Carbon source

*Rarely tested

1. Preparation of media (for 10X stocked media and negative control)

a. Add 4.5 ml of deionized water (dH_2O) into each 16 x 125mm test tube for ascomycetous yeasts and autoclave.

For basidiomycetous yeasts use the following instead of water.

- dH_2O 1000 ml
- Bacto yeast extract (Difco) 50 mg
- Bacto casamino acid (Difco) 50 mg

Autoclave at 121˚C for 15 min.

b. Make 10X stock solutions by mixing the following:

10X stock solution

- Yeast nitrogen base 6.7 g (see p. 9)
- Carbon compound 5.0 g (or ~ 5.0 ml)
- dH_2O 100 ml

Filter sterilize solution. **Do not autoclave!**

Store at –20˚C.

pH should be adjusted to pH 5.0 for C38, C39, C40.

For **negative control** (10X), no carbon compound should be added.

Yeast Nitrogen Base (10X):

- 5g Ammonium sulfate
- 10ml (100X) vitamins (see p. 17)
- 10ml (100X) amino acids (see p. 17)
- 10ml (100X) trace elements (see p. 17)
- 10ml (100X) of each salts (see p. 17)

Salts: Potassium phosphate (mono), Potassium phosphate (di), Magnesium sulfate, Sodium chloride, Calcium chloride.

Fill up to 100ml with dH_2O.

c. Add 0.5 ml of 10X stock solutions aseptically into prepared water tubes with

a dispenser or pipette.

d. Prepare 50ml sterilized centrifuge tubes for cell suspensions. Add autoclaved water (0.1ml X (No. of tests + 10)) into the tubes.

2. Preparation of 1X carbon test solution (C21, C22, C30, C33, C34, C42)

a. Mix the media (1X) as follows:

Base solution

- Bacto yeast nitrogen base 6.7 g
- Bacto yeast extract 50 mg (only for basidio-)
- Bacto casamino acids 50 mg (only for basidio-)
- DW 1000 ml

for C21, C22, C30, C33 (1X);

- Carbon compounds 750 mg
- Base solution 150 ml

for C42 (1X);

- Carbon compounds 4.5 ml
- Base solution 150 ml

for C34 (1X);

- Carbon compounds 450 mg
- Base solution 150 ml

b. After filtration, 5 ml of the media are pipetted into autoclaved empty tubes aseptically. These are ready to be inoculated.

3. More Carbon Assimilation Media

a. For C22 (soluble starch, 1X)

Autoclave instead of using filter for sterilization!

b. For C41 (Methanol, 1X)

- Filter 10ml of Methanol with a syringe filter, and keep in 50ml tube at 4˚C.
- Add 1 drop of filtered Methanol with sterilized pipette (dropper) to a tube containing 5ml of Carbon Negative Mix (1X).

c. For O1, O2 (Cyclohexamide)

1. Prepare C1 (D-glucose) medium.
2. Prepare Cyclohexamide solution

O1 stock solution:

Cyclohexamide 50mg

EtOH 10ml

O2 stock solution:

Cyclohexamide 500mg

EtOH 10ml

3. Dissolve and filter with a syringe filter. Keep in 50ml tubes at 4˚C.
4. Add 100μl of solution into C1 medium. Do not autoclave.

d. For T2, T3, T4, T5 (Temperature tests)

1. Prepare C1 (D-glucose) medium
2. Inoculate and incubate at the following temperatures

T2 = 30°C T3 = 35°C T4 = 37 °C T5 = 40°C

4. Inoculation and Observation

a. Prepare precultures on YM agar and incubate at 25˚C for 2-4 days.
b. Take fresh colonies from precultures and make a light suspension (about 1+) with 10ml of autoclaved water in 50ml tubes.
c. Inoculate 0.1ml of the suspensions into the test media, and incubate at 25˚C up to 3 weeks. Observe the cultures on 7 and 21 days. See *The Yeasts* on how to check the culture. Use **Assimilation Worksheet** provided at the end of this manual (p. 35)
d. Save a positive, +++ culture (C1) for starch formation test (p. 20)
e. Checking Criteria: Using a black-lined card (see *The Yeasts*)

\+ lines are clear, edges are sharp and not blurry.
++ lines are just beginning to become blurry to almost invisible.
+++ lines are invisible.

Checking Criteria: Using a black-lined card (see *The Yeast*)

\+ lines are clear, edges are sharp and not blurry.
++ lines are just beginning to become blurry to almost invisible.
+++ lines are invisible.

C. Fermentation Test

Preparation: Durham tubes (12 X 150 (or 13 X 100)) mm test tubes contains small (6 X 50 mm) inverted tubes), caps.

Compounds to be tested

F1 D-Glucose	F2 D-Galactose	F3 Maltose	F4 a-Methyl-D-glucoside
F5 Sucrose	F6 a, a-Trehalose	F7 Melibiose	F8 Lactose
F9 Cellobiose	F10 Melezitose	F11 Raffinose	F12 Inulin
F13 Starch	F14 D-Xylose	F15 Negative control	

1. Preparation of media

a. Mix the following

- Sugar (C source) 2 g (for **Raffinose** [F11] use 4 g)
- Yeast extract 1 g
- DW 100 ml

b. Dispense 6ml of media into tubes.

c. Autoclave at 121˚C for 15 min. (After autoclaving, the Durham tubes should be full of medium and no air bubble.)

Note: No sugar should be added for F15.

2. Inoculation

a. Prepare precultures on YM agar and incubate for 2-4 days.

b. Take fresh colonies from precultures and make light suspensions (about 1+) in sterile 50ml tubes.

c. Inoculate 0.1 ml of the suspensions into the test media with an Eppendorf dispenser, and incubate at 25˚C up to 3 weeks. Shake periodically.

3. Observation

a. Observe the insertion of gas every 7 days.

Checking criteria:

w = very small bubble

\+ = relatively small bubble

++ = < 1/2 of tube

+++ = > 1/2 of tube

D. Assimilation of Nitrogen Compounds

Preparation: Test tubes, caps or cotton plugs, plastic dropping pipettes (sterilized), Petri dishes.

1. Yeast Carbon Base broth

Yeast Carbon Base	1X, 100ml	2X, 100ml
Glucose	1g	2g
Vitamins (10X)	10ml	20ml

Amino acids (100X)	1ml	2ml
Trace Elements (100X)	1ml	2ml
Salts (100X)	1ml each	2ml each

Salts: Potassium phosphate (monobasic), Potassium phosphate (dibasic), Magnesium sulfate, Sodium chloride, Calcium chloride ($2H_2O$ or $6H_2O$).

Adjust with dH_2O to 100ml total.

HOW MUCH 2X CARBON BASE DO YOU NEED?

For nitrogen test media

250ml x 12 (N0-NP) = 3L

For starvation culture

2.5ml x (20 + 5 extra) = 62.5ml for 20 isolates

2. Test Media, 500ml each (N0-NP) for 20 plates

1. Dissolve each Nitrogen source in separate bottles with 250ml of **2X** yeast carbon broth

N-0 Negative control	no nitrogen
N-1 Potassium nitrate	0.15 g
N-2 Sodium nitrite	0.21 g
N-3 Ethylamine-HCl	0.13 g
N-4 L-Lysine-HCl	0.33 g
N-5 Cadaverine-HCl	0.27 g
N-6 Creatine	0.20 g
N-7 Creatinine	0.17 g
N-8 D-Glucosamine	0.33 g
N-9 Imidazole	0.10 g
N-10 D-Tryptophan	0.32 g
N-P Ammonium sulfate	0.20 g

2. Adjust pH to 5.5-6.5.
3. Add 10g of agar in each solution and add 250ml of dH_2O.
4. Autoclave at 120˚C for 15-20 minutes and pour into Petri plates (100 x 15mm).

5. Inoculation

Part I

a. Prepare preculture on YM agar for 2-4 days.

b. Prepare **light suspension** of cells in Yeast carbon base starvation broth. (**Do not add too much cells!)** Incubate at 25˚C for 5-7 days to consume the nitrogen compounds carried from the preculture medium and pools of the cells.

Part II

c. Inoculate a drop of suspension onto agar plates (multi-point inoculation) using sterilized plastic dropping pipettes. Each plate can hold 4 isolates.

d. Allow the plates to dry before sealing and moving them to the incubator.

6. Observation

a. Incubate at 25˚C for up to 2 weeks.

Observations: 3, 7, 14 days. Use Assimilation Worksheet to record data

Checking Criteria:

1. Standards:

   Positive control (N-P) is always +++

   Negative control (N-0) is always –

2. Decide if the treatment is +, ++, +++, or – by comparing the both the colony size and cell density with both N-0 and N-P plates.

\+ very little growth, just a little stronger than N-0.

++ much stronger than N-0 but still not as strong as NP, although it may be close.

+++ as strong as N-P or stronger growth.

E. Vitamin Requirement Test

Preparation: Test tubes (16 X 125 mm), caps or cotton plugs, pure water, plastic pipettes (sterilized).

Vitamin stock solutions: Prepare each basal solution and mix according to the table below

After *cold filter sterilization*, keep in the freezer (-20˚C).

[Note that the vitamin basal solutions used for basidiomycete yeasts are slightly different from that for ascomycete yeasts. Check phylogenenetic position of the yeast you plan to test.

1. Basal solution

• Riboflavin	4 mg/100ml of 0.04N Acetic acid	40 μg/ml
• Thiamin-HCl	2 mg/100ml of 0.04N Acetic acid	20 μg/ml
• PABA	2 mg/100ml of 0.04N Acetic acid	20 μg/ml
• Pyridoxine-HCl	2 mg/100ml of 0.04N Acetic acid	20 μg/ml
• Ca-Panthothenate	10 mg/100ml of 50% Ethanol in dH_2O	100 μg/ml

• Niacine	10 mg/100ml of 50% Ethanol in dH_2O	100 μg/ml
• Biotin	5 mg/100ml of 50% Ethanol in dH_2O	50 μg/ml
• Folic acid	10 mg/100ml of 20% Ethanol in 0.01N NaOH	100 μg/ml
• Inositol	200 mg/100ml of H_2O	2000 μg/ml

2. Vitamin Mixture Table (10X) for 150ml final volume

Medium name (V)		1	2	3	4	5	6	7	8	9	10	11	Final Conc.
		-	-	-	-	-	-	-	-	-	-	+	in 1L (1x)
Missing Vitamin(s)		all	Ino	Pant	Biot	Thia	B&T	Pyri	P&T	Nia	PABA	none	
Biotin	0.6ml	X	+	+	X	+	X	+	+	+	+	+	20 ug
Ca-Pant	30.0ml	X	+	X	+	+	+	+	+	+	+	+	2000 ug
Folic acid	0.03ml	X	+	+	+	+	+	+	+	+	+	+	2 ug
Inositol	7.5ml	X	X	+	+	+	+	+	+	+	+	+	10000 ug
Niacin	6.0ml	X	+	+	+	+	+	+	+	X	+	+	400 ug
PABA	15.0ml	X	+	+	+	+	+	+	+	+	X	+	200 ug
Pyrid-HCl	30.0ml	X	+	+	+	+	+	X	X	+	+	+	400 ug
Riboflavin	7.5ml	X	+	+	+	+	+	+	+	+	+	+	200 ug
Thiamin-HCl	30.0ml	X	+	+	+	X	X	+	X	+	+	+	400 ug
dH_2O(ml)			31.5	60	31	60	61	60	90	36	45	30	
Total						150 ml each							

+ = add the vitamin solution X = do not add the vitamin solution

3-1. Medium Preparation - Part 1

1-1. Negative control & basal medium for **ascomycetous yeasts**

a. Mix the base solution

Base solution

- Vitamin free base (Difco) 16.7 g
- dH_2O 1000 ml

Or make **vitamin free base** and **negative control** (1X) by mixing the following.

- Ammonium sulfate 5 g
- Glucose (dextrose) 10 g
- Amino acids (10X) 100 ml (100X for carbon base)
- Trace elements (100X) 10 ml
- Salts 10 ml each 100X stock solutions

  Salts: Potassium phosphate (mono), Potassium phosphate (di)

  Magnesium sulfate, Sodium chloride, Calcium chloride (p. 17)
- Fill water up to 1000 ml

b. Dispense the base solution (4.5ml per tube) and autoclave at 121˚C for 15 min.

1-2. Negative control medium & basal medium for **basidiomycetous yeasts**

a. Mix the base solution.

Base solution

- Glucose 10.0 g
- Vitamin free casamino acids (Difco) 5.0 g
- KH_2PO_4 1.0 g
- $MgSO_4$-$7H_2O$ 0.5 g
- NaCl 0.1 g
- $CaCl_2$-$2H_2O$ 0.1 g
- DW 1000 ml

The pH of the base solution should be 5.5.

b. Dispense the base solution (4.5ml per tube) and autoclave at 121˚C for 15 min.

3-2. Medium Preparation – Part 2

a. Prepare the base solution as above (4.5ml in each tube).

b. Add 0.5 ml of **Vitamin stock** solution (10X) aseptically.

c. Incubate for 3 days to confirm the sterility.

4. Inoculation

a. Prepare preculture on YM agar for 2-4 days.

b. Prepare **light suspension** of cells in vitamin free base medium.

c. Incubate for 5-7 days to consume the vitamins carried from the preculture medium.

d. Inoculate a drop of suspension in the medium prepared in 3-2 (p. 15).

e. Incubate at 25˚C for up to 2 weeks (observe at 3, 7, 14 days).

5. Observation

Check 3 days, 7 days, and 14 days.

1. Standards:

   ++ for Positive control

   – for Negative control

2. Checking Criteria: Using a black-lined card (see *The Yeasts*).

   – if similar in turbidity as Negative control

   \+ if more turbid than negative control but not close to positive control

   ++ if very close to or stronger than positive control

F. What You Need To Prepare for Testing 20 Isolates

Preparation: Test tubes (16 X 125 mm), caps or cotton plugs, syringe dispenser

Media:

1) Fermentation media

   15 tests x (20 isolates) + 2 extra = 330 small tubes

2) Carbon, T2,T3,T4,T5,O1,O2

   [Carbon (45 tests [including 1 test small tube] + 1 Negative) + Temp (4 tests) + Osmotic (2 tests)]

   x 20 isolates = 1040 tubes [including 20 test small tubes]

3) Vitamin media

   11 tests x (20 isolates + 1 extra) = 231 tubes

4) Nitrogen Media

   (12 tests x 20 isolates)/4 isolates per plate = 60 plates

Before Tests:

1) Prepare fresh cultures on YM agar (3-4 days incubated at 25°C)

2) Starvation cultures (only for Vitamin and Nitrogen media)

G. Base Solutions

1) Vitamin (10X) Be careful of the concentration of each stock solution!

- Biotin (0.01g/10ml stock) 0.2ml (200μg)
- Calcium Pantothenate (0.1g/10ml stock) 2ml (20μg)
- Folic acid (0.01/10ml stock) 0.02ml (20μg)
- Inositol (1g/10ml stock) 1ml (100mg)
- Niacin (0.1g/10ml stock) 0.4ml (4mg)

- PABA (0.1g/10ml stock) dissolve in EtOH 0.2ml (2mg)
- Pyridoxine HCl (0.1g/10ml stock) 0.4ml (4mg)
- Riboflavin (0.1g/10ml stock) 0.2ml (2mg)
- Thiamine HCl (0.1g/10ml stock) 0.4ml (4mg)
- dH_2O Fill up to 1000ml (1L)

2) Amino Acids (100X for Carbon Base, 10X for other bases)

- L-Histidine (0.1g/10ml stock) 1ml (10mg)
- DL-Methionine (0.1g/10ml stock) 2ml (20mg)
- DL-Tryptophane (0.1g/10ml stock) + HCl 2ml (20mg)
- dH_2O Fill up to 100ml

3) Trace Elements (100X)

- Boric acid (0.01g/ml stock) 0.5ml (5mg) (500μl)
- Copper sulfate (0.1g/ml stock) 4μl (0.4mg) (4μl)
- Potassium iodide (0.1g/ml stock) 0.01ml (1mg) (10μl)
- *Ferric chloride (0.1g/ml stock) 0.02ml (2mg) (20μl)
- Manganese sulfate (0.1g/ml stock) 0.04ml (4mg) (40μl)
- *Sodium molybdate (0.1g/ml stock) 0.02ml (2mg) (20μl)
- Zinc sulfate(0.1g/ml stock) 0.04ml (4mg) (40μl)
- dH_2O Fill up to 100ml

* Ferric chloride and Sodium molybdate will form a precipitate if mixed together one right after another.

4) Salts (100X): Make each solution separately.

- Potassium phosphate (monobasic) 0.85g/10ml
- Potassium phosphate (dibasic) 0.15g/10ml
- Magnesium sulfate 0.5g/10ml
- Sodium chloride 0.1g/10ml
- Calcium chloride 0.1g/10ml

H. Gelatin liquefaction test

1. Test medium (20% gelatin medium)

   In 1000ml distilled water, add:

   - gelatin 200g
   - glucose(dextrose) 5g
   - yeast nitrogen base 6.7g

2. Dispense 2.5ml into each test tube (13x100 mm), and autoclave at 121˚C for 15 min.
3. Inoculate fresh culture (2-3 day old), and incubate at 25˚C.
4. Check regularly for up to 3 wks for sign of liquefaction.

– no sign of liquefaction

\+ strong liquefaction. Well liquified gelatin around the colony.

+/w weak liquefaction. Weak, but certain signs of liquification.

I. Test for Hydrolysis of Urea

1. Test medium (make only the amount needed every time, like 2.5ml X 20 isolates = 60ml + 10ml extra)

Mix the following

- Difco Urea R broth 38.7g
- dH_2O 1000ml

2. Filter sterilize (use filter unit (up to 150ml) if the amount is above 100 ml. For small amount, use syringe filter. Do not boil or autoclave the broth.
3. Dispense 0.5 ml into autoclaved empty test tubes (12mm) aseptically.
4. Add a loopful of cells from 2-3 day old cultures is suspended in the broth, and incubated at 37˚C.
5. Check every 30 min for up to 2 hours for a change of the color to bright pink or red. Check again after 4hr from inoculation.

Note: Many + isolates produce the color change within 30 min, most do so within 2hr and all within 4hr.

Checking Criteria:

– No color change.

\+ Obvious color change to bright pink or red.

J. Test for Tolerance of 1% of Acetic Acid

1. Test medium

In 100 ml dH_2O water, add

- glucose(dextrose) 10g
- tryptone 1g
- yeast extract 1g
- agar 2g

2. Autoclave at 121˚C for 15 min.
3. Cool down around 55-65˚C, and add 1 ml of glacial acetic acid.
4. Make plates (60 X 15 mm). Draw two cross lines on the bottom of plate, and label isolate #.
5. Inoculate 4 isolates on 4 different spaces on the plate, and incubate at 25˚C.
6. Check every week up to 4 weeks for the development of colonies. Use Assimilation Worksheet (p. 35).

Note: Do not inoculate too many cells for clear decision.

Checking Criteria: – no sign of colony + well formed colony on the plate

K. Test for High Osmotic Pressure

1. Test media, 100ml total volume
   - 50% glucose agar (50% glucose in 1% yeast extract, add 2% agar)
   - 60% glucose agar (60% glucose in 1% yeast extract, add 2% agar)
   - 10% NaCl agar (10% NaCl in 1% yeast extract, add 2% agar)
   - 16% NaCl agar (16% NaCl in 1% yeast extract, add 2% agar)
   - 1% YE agar - Negative Control (1% yeast extract, add 2% agar)

   Fill up to 100ml with dH_2O after adding agar.

   *For glucose media, **shake** every 20 seconds while dissolving sugar by microwave!
2. Autoclave at 110˚C for 10 min for glucose media (at 121˚C for 15 min for NaCl media).
3. Pour plates (60X15 mm). Draw two cross lines on the bottom of plate, and label isolate #.
4. Inoculate 4 isolates on the 4 spaces on the plate and incubate at 25˚C.
5. Check every week up to 4 weeks for the development of colonies. Use Assimilation Worksheet (p. 35).

Note: Do not inoculate too much cells for clear decision. Overheating leads to browning. Any media showing this must be discarded. Seal each plate with parafilm to prevent he evaporation during incubation.

Checking Criteria: – no sign of colony + well formed colony on the plate

L. Diazonium Blue B (DBB) Color Test

1. Culture the yeast on a YM agar plate for 5-7 days, and then incubate at 55-60˚C for 16 hours. Cool the plates down to room temperature before testing.
2. Prepare DBB reagent. Make the amount needed every time. Do not use old stock solution. Keep it in ice bath or refrigerator and use before it turns dark yellow (within about 30 min.)

Diazonium Blue B salt (Fast Blue salt B)	15 mg
Chilled 0.25 M Tris buffer (pH 7.0)	15 ml

3. Drop one or two drops of chilled DBB reagent onto the surface of each colony.

Checking Criteria: Use Assimilation Worksheet (p. 35).

– no color change

\+ dark-red to violet red color within 1-2 min at room temp.

M. Formation of Extracellular Amyloid Compound (starch formation)

1. Prepare Lugol's iodine solution

Iodine	1g
Potassium iodide	2g
DW	300 ml

2. Prepare a culture in a medium containing 1% glucose or use the culture for glucose assimilation test (C1 or T2) after checking the 3 wk result.
3. Add one or two drops of Lugol's iodine solution into the culture, and mix.

Checking Criteria: Use Assimilation Worksheet (p. 35).

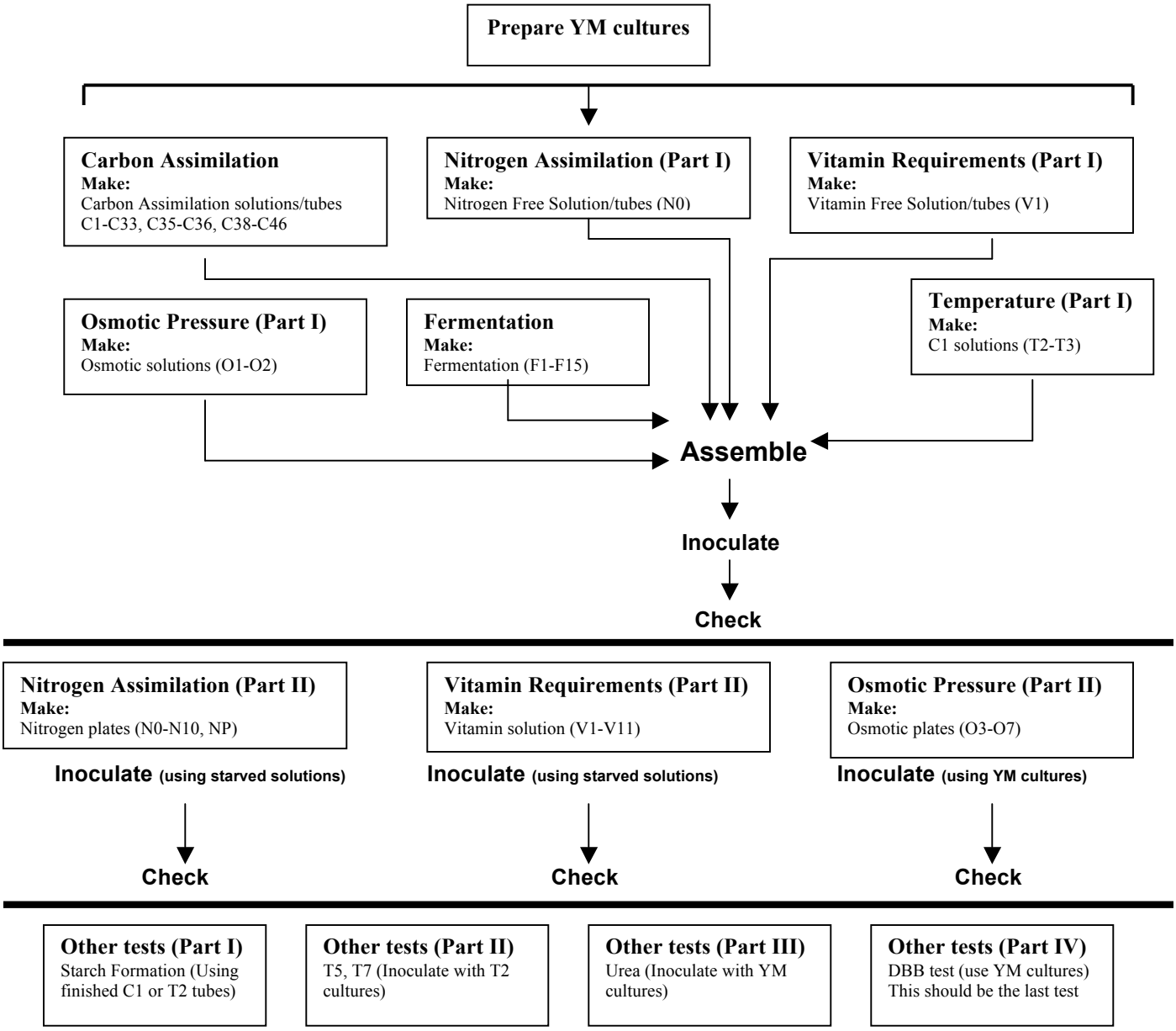
Use soluble starch culture (C22) as a positive control.

– no green or dark blue color change

\+ change the color to the range of green to dark blue

N. Assimilation Flow Chart

Check Materials/Equipment
Large test tubes (16 x 125 mm)
Small test tubes (13 x 100 mm)
Durham tubes
Eppendorf syringes
Dropping pipettes
Dispensing pipettes
50 ml Eppendorf extraction tubes
Microfilters (Nalgene)
Chemicals
Small/Large plates



III. MOLECULAR BIOLOGICAL METHODS FOR YEAST STUDY

A. DNA Extraction From Yeast Cells

Preparation: PCR water (autoclaved filter sterilized water), Lysis buffer (50 mM Tris-HCl [pH 7.2], 50 mM EDTA, 3% SDS), Phenol-Chloroform, plastic pestle and mortar

1. Collect yeast cells from agar culture with loop or by centrifugation of liquid culture, and put in 1.5 ml eppendorf tube (about 0.1g).
2. Rinse the cells with 300μl of lysis buffer or saline, vortex, and centrifuge. Discard buffer.
3. Add 3μl of 2-mercaptoethanol and 50μl of lysis buffer.
4. Grind the cells with a plastic pestle for at least 1 min.
5. Add 250μl of lysis buffer (total 300 μl).
6. Incubate at 65˚C for 30 min.
7. Add 300 μl of Phenol-Chloroform, and vortex for at least 30 seconds.
8. Spin down, and transfer upper layer to a fresh tube.
9. Repeat 7-8 until the solution is clear (at least twice).
10. Add 500 μl of isopropanol and 20μl 3M sodium acetate into the tube containing upper solution, and mix gently by shaking it up side down.
11. Spin down, and discard solution (do not lose the white precipitation).
12. Rinse the pellet with 700μl 70% EtOH, incubate at room temp. for 5 minutes and spin down. Discard the EtOH.
13. Rinse the pellet with 700μl 100% EtOH, and spin down. Discard the EtOH.
14. Dry the pellet at room temperature for 15 min, and dissolve by adding 25μl of PCR water.

Check DNA concentration: 1μl sample + 4 μl dye by 1% agarose gel electrophoresis. Mix 12 μl of **SYBR** Safe in 100ml 1X TAE buffer (or whatever buffer is being used to run the sample) after the run to stain the DNA for 45 minutes. SEE PRECAUTIONS

1% agarose gel: 0.7-1g agarose / 100ml of 1X TAE buffer (Do not use water!)

50X TAE buffer: 121g Tris + 28.6ml glacial acetic acid + 50ml 0.5M EDTA, fill up to 500ml with DW. Dilute 50 times to make 1X.

10X loading buffer: 20% Ficoll 400, 0.1M EDTA (pH 8), 1% SDS, 0.25% Bromophenol blue.

or

Mix 5g glycerol + 1ml 0.5M EDTA (pH 8.0) + 0.5ml 10% SDS. Fill up to 10ml with dH_2O Autoclave. Add 0.17ml 0.25% Bromophenol blue.

B. PCR (Polymerase Chain Reaction) Method

1. Turn on the PCR machine to allow warming up.
2. Dilute extracted DNA to 1/10-1/100 with PCR water (final concentration 1 ng-1μg/μl).

3. Add the following solutions in 0.2 ml tube or 0.5 ml tube.

   Reaction mix (in 25 μl sol.)

• 10X reaction buffer	2.5 μl
• 10 mM dNTP	.5 μl
• primer #1 (10 μM)	.5 μl
• primer #2 (10 μM)	.5 μl
• diluted DNA template	1.0 μl
• Taq DNA polymerase (5U/μl)	0.0125 μl
• PCR water	20.375 μl

   If you have more than 3 samples, make premix solution of buffer, dNTP, primers, and Taq polymerase.

4. Add 2 drops of mineral oil into tube if you use 0.5 ml tube. Do not add oil for 0.2 ml tube.
5. Spin down 10 seconds.
6. Place tubes into the holes of plate.
7. Start PCR cycle. The PCR cycles were saved as certain numbers or file names.

   An example of PCR parameter

   a. 95˚C 5 min
   b. 95˚C 1 min
   c. 55˚C 1 min — b-d 35 cycles
   d. 72˚C 1 min
   e. 72˚C 10 min
   f. Keep 4˚C

8. Check the product by 1% agarose electrophoresis
   a. Mix 3 μl of product with 3 μl of 2X loading buffer (dye)
   b. Run at 70-80 V for 30-60 min in 1x TAE buffer
   c. Stain the gel in EtBr solution (5μl EtBr/100ml water) for 15-20 minutes

PCR Primer sets:

SSU: NS1-SS3E or NS1-NS8

ITS: ITS4-ITS5

LSU: LS1-LR3 or LS1-LR5.

C. PCR by Heating Method: Useful for 26S (LSU) and ITS amplification

1. Collect a loopful of cells and mix into 100μl of saline or PCR water in a 0.5ml Eppendorf tube.
2. Heat at 99°C for 5 minutes.

3. Spin down for 20 seconds.
4. Use 2μl of solution for PCR.

** note this DNA extraction is only useful when prepared freshly (approximately one day) and cannot be stored in the freezer.

D. Purification of PCR Product: UltraClean purification protocol for 45 of PCR product

1. Add 65 μl of UltraSalt to 1.5ml tube.
2. Transfer all PCR products to 1.5ml tubes with UltraSalt.
3. Vortex UltraBind (matrix) until homogenized. Add 3 μl to prepared tube.
4. Incubate for 5 minutes at room temperature. Mix several times (3-5) by inverting tubes during incubation.
5. Centrifuge for 5 seconds. Remove supernatant.
6. Add 0.5ml of UltraWash. Vortex for 5-10 seconds. Centrifuge for 5-10 seconds. Discard solution.
7. Repeat step 6. Centrifuge again briefly and pipe out all remaining solution.
8. Add 10-15 of PCR water. Vortex.
9. Incubate at room temperature up to 5 minutes. Centrifuge for 1 minute.
10. Remove the supernatant immediately and transfer to new properly labeled tubes.
11. Seal the UltraSalt and UltraBind (matrix) with Parafilm.

ExoSAP Purification Protocol (for smaller sample –can be added directly to small tubes)

1. To purify PCR samples using ExoSAP solution mix the following:

For 15-20 μl PCR products, use

0.5 μl Exonuclease I

0.5 μl SAP

1.0 μl water

2. Place mixture in a thermocycler for an hour (“PCR clean up” programmed)

Thermocycler cycles:

1. 37C for 45 min
2. 80C for 15 min
3. 10C for forever

3. The purified PCR products can be used directly for sequencing with the 0.5 μl BigDye protocol. You can reduce your PCR reactions to 20-25 μl reactions instead of the 50 μl as done previously and use 2-5 μl for the gel, then do your ExoSAP, and you can skip the gel after purifying because we know the products are there still!

Order enzymes from Fermentas (800.340.9026)

Exonuclease I: #EN0581

Shrimp Alkaline Phosphatase: #EF0511

E. Preparation of Sequencing Sample

Preparation: 3M Sodium Acetate (pH 5.2, filter sterilized), 100% and 70% EtOH (use autoclaved water)

Note: The reduced volume for sequence samples outlined below works well most of the time for sequencing short PCR products (e.g., D1/D2 region or ITS, but the reduced amounts are not always good for SSU.) For longer sequences you may need to use the following mixture:

Template DNA: total 4 μl (purified PCR product + PCR water)
BigDye termination mix: 4 μl
Primer (25 ng/μl or 5 μM): 2 μl

Total 10 μl [no buffer is necessary]

1. Turn the PCR machine on. It needs 5-10 min to warm up.
2. Put purified PCR product (usually 1 μl) into 0.2 ml tube. Make total DNA template volume of 2.5 μl by adding PCR water, but you will need to compare the result of agarose electrophoresis for PCR to decide how much water to be added.
3. Make premix in a 0.5 ml tube (n = sample number + 0.1)

BigDye termination mix	0.5 μl	x	n
Primer (25 ng/μl or 5 μM)	1.0 μl	x	n
BigDye Buffer	1.0 μl	x	n

   [If the number of samples are < 3, add each solution separately in each 0.2 ml tube.]
4. Dispense 2.5 μl of premix to each tube containing PCR product and spin down for 10 sec.
5. Run the reaction according to the following parameters
   a. 96˚C 10 sec
   b. 50˚C 5 sec
   c. 60˚C 4 min
   25 cycle of a-c and keep at 15 ˚C
6. Start the cycle, and put the samples when the block temperature is higher than 80˚C.
7. After the reaction, spin down the samples for 10 sec.
8. Add 5 μl of PCR water to each tube. (Total sample volume should be 10 μl).

Purification:

Ethanol Precipitation Method

1. Prepare the same numbers of 0.5 ml tubes as samples, and add 2μl of 3M Sodium Acetate and 50μl of 100% EtOH.

2. Transfer each sequencing samples to the 0.5 ml tubes.
3. Vortex and incubate at room temp. for 10 min. And spin down for 20 min.
4. Discard EtOH with pipette. Do not touch the bottom of the tube with tip. You may leave small amount of EtOH in the tube.
5. Add 250 *µ*l of 70% EtOH in each tube, and spin down for 10 min.
6. Discard EtOH with pipette. Do not touch the bottom of the tube with tip. You may leave a small amount of EtOH in the tube.
7. Put the tubes in PCR machine at 50˚C to dry up. Rehydrate the samples with 15*µ*l of HI-DI. Keep at 4˚C.

SEPHADEX CLEANING PROTOCOL (generally replaced by ethanol purication)
For Cycle Sequencing Reactions

Amersham Biosciences
http://www5.amershambiosciences.com/aptrix/upp01077.nsf/content/na_homepage

Item	**Item number**	**Amount**	**Price**	**Uses**
Sephadex G-50 fine	**17-0042-01**	**100 g**	**$164.00**	**~230 samples**
AutoSeq G50 columns	**27-5340-01**	**50 columns**	**$152.00**	**unlimited**

[when using NEW G50 columns (unused ones)]

1. Add 5 µl of PCR water to each sample to make the total volume to 10 µl.
2. Resuspend the resin in the new column by voltexing gently.
3. Loosen the cap one-fourth turn and snap off the bottom closure
4. Place the column in a 1.5 ml screw-cap microcentrifuge tube (*collection tube*) for support.
5. Prespin the column for I min at 3000 rpm (2000X g).
6. Discard the solution in the support tube, and place the column in a new autoclaved 1.5 ml tube. (Do not leave the column too long after this step. Use immediately before drying the resin.)
7. Slowly apply the sample to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin. Do not allow any of the sample to flow around the side of the bed.
8. Spin the column for I min at 3000 rpm. The purified sample is collected in the bottom of the supported tube. DO NOT DISCARD!
9. Transfer the purified sample to 0.5 ml tube, and keep in freezer.
10. Wash the column and collection tubes with DW carefully and rinsed thoroughly for reuse, and columns can be soaked in DW.

[when using USED G50 columns]

1. Boil filters about 2-5 minutes to insure water will flow through.
2. Hydrate Sephadex at least 15 minutes before use.

   0.0625 g Sephadex / 1 ml ddH_2O

1.56 g Sephadex / 25 ml ddH_2O

3. Label 1.5 ml tubes with sample numbers.(Columns do not need to be labeled.)
4. Add 600-700 µl of hydrated Sephadex to each spin column.
5. Be sure to swirl Sephadex mixture often during use to keep it in suspension.
6. Spin columns in collection tubes for 2-3 minutes at 3000 rpm.
7. Discard liquid from collection tubes.
8. Transfer Sephadex columns to the 1.5 ml labeled tubes. (Once this step is completed, you must hurry or Sephadex will dry out.)
9. Slowly apply the sample to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin. Do not allow any of the sample to flow around the side of the bed.
10. Spin the column for I min at 3000 rpm. The purified sample is collected in the bottom of the supported tube. DO NOT DISCARD!
11. Transfer the purified sample to 0.5 ml tube, and keep in freezer.
12. Store filters in purified water.
13. Wash the column and collection tubes with DW carefully and rinsed thoroughly for reuse, and columns can be soaked in DW.

Purification Drying:
[when using G50 columns]

1. Dry samples with Vacuum centrifuge found in Dr. Urbatsch's lab then rehydrate the samples with 15µl HI-DI.

[when using Ethanol purification]

1. Dry in lab thermal cycler. Rehydrate the samples with 10µl HI-DI.

F. Cloning Directly from Gut Contents

Preparation: TOPO TA CLONING KIT FOR SEQUENCING (INVITROGEN LIFE TECHNOLOGIES)

1. LB plates (2-3 for each transformation) containing 50-100 µg/mL ampicillin or 50 µg/mL kanamycin
2. 42 °C water bath
3. 37 °C shaking and non-shaking incubators

LB plates (pH 7.0)

- 1% tryptone
- 0.5% yeast extract
- 1% NaCl
- 1.5% agar

Autoclave for 20 minutes at 15 psi. Allow temperature decreased to 55 °C and add Ampicillin. Pour plates and store at 4 °C)

1. Prepare PCR sample from gut contents
    a. Dissect beetle under microscope.
    b. Put the gut in a tube containing saline solution.
    c. Crush the gut with a plastic pestle
    d. Put the tube in boiling water for 5 min.
    e. Vortex and dilute 10 -100X
    f. Use 1ul for PCR.

2. Cloning Reactions:
    a. Water bath equilibrated to 42 °C
    b. Warm the vial of SOC medium to room temperature
    c. Warm LB Ampicillin plates at 37 °C from 30 min.
    d. Thaw ON ICE 1 vial of One Shot cells for each transformation

    Mix the following reagents:
    - Fresh PCR product: 0.5-4 μL
    - Salt solution: 1 μL
    - Sterile distilled water: add to 5 μL, and then add
    - TOPO vector: 1 μL

    Total: 6 uL

    Mix gently and room temperature for 5 min. Then put on ice.

3. Chemical transformation
    a. Add 2 μL of the TOPO cloning reaction above into a vial of One Shot competent cells and mix gently. DO NOT MIX BY PIPETTING UP AND DOWN.
    b. Incubate on ice for 5-30 min.Heat-shock the cells for 30 seconds at 42 °C WITHOUT shaking. Immediately transfer the vials to ice.
    c. Add 250 μL of room temperature SOC medium.
    d. Cap tightly and 200 rpm shake the vials horizontally at 37 °C for 1 hour.
    e. Spread 10-50 μL from each transformation on a prewarmed selective plate and incubate overnight at 37 °C.

4. Analysis of clones using PCR

a. Take colonies and transfer to LB + ampicillin plates (each plate can inoculate up to 12 colonies). Overnight incubate at 37 °C.
b. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and Taq polymerase. Use a 25 μL reaction volume for each colony.
c. Pick colony cultures (about 0.5 μL) and resuspend them individually in the PCR cocktail.
d. PCR for 30-35 cycles.
e. Visualize by agarose gel electrophoresis, or perform restriction analysis:

5. Restriction analysis of PCR products

Each well or microtube:

10 U/μL Hae III (1U)	0.1 μL
10x buffer 2	0.8 μL
purified water	2.1 μL
PCR product	5.0 μL
Total	8.0 μL

incubate at 37 °C for1-2 hours.

Visualize by 1% agarose gel electrophoresis, or perform restriction analysis.

IV. QUANTITATIVE METHODS

Dilution Plating (after <http://www.waksmanfoundation.org/labs/mbl/microbes.html>)

Dilution plating is a technique used to approximate the number of colony-forming units (CFUs) present in a sample. This technique can be used to try to get an idea of what yeasts and fungi are in the substrate (basidiomata or wood) tested. In order to estimate the number of fungi present, you will need to dilute a sample of known weight or volume by a defined amount using serial dilutions to obtain "countable numbers." Countable numbers are about 50-200 CFUs per plate, and you will have to experiment to determine a suitable dilution.

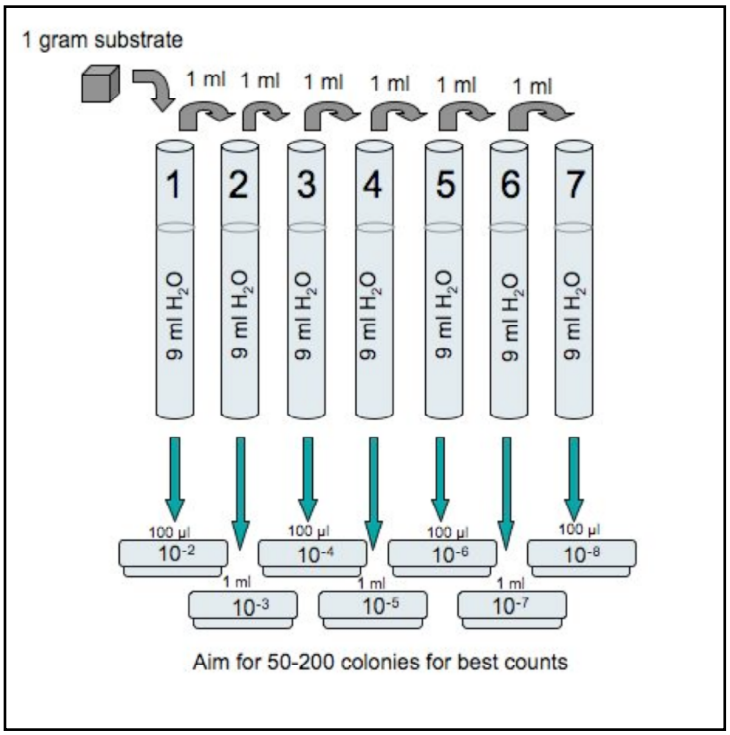
Preparation: Sterile pulverizing implement, pipetter, 7 sterile pipette tips, 7 sterile tubes each containing 9mm sterile H_2O, 7 plates appropriate for yeast growth (YMA), Sharpie marker for labeling tubes and plates

Procedure: Weigh out one gram of your sample, pulverize the sample as much as possible (aseptically), and place it into vial # 1. Shake the vial vigorously approximately 100 times.

Use a pipetter with a sterile tip to place 1 ml of the mixture from vial 1 into vial two. You may have to let the larger pieces of your sample settle to the bottom first.

Using a new pipette tip, take 1 ml of liquid from vial 2 and place it in vial 3. Take a new pipette tip and transfer 1 ml of liquid from vial 3 into vial 4. Transfer 1 ml of liquid from vial 4 into vial 5. Continue this series of transfers, using a new pipette tip each time, until you have transferred 1 ml into each of the vials.

Use the dilutions that you made to plate samples from each of the tubes (1-7). Label the bottom of each plate with the numbers 1-7. Shake and then use a pipetter with a sterile tip to take a 100 *µ*l sample from each vial and place it onto the appropriately labeled plate.



Incubate the plate top down for 2-3 days. [Plate 1 = 10^{-2} dilution, plate 2 = 10^{-3} dilution, plate 3 = 10^{-4} dilution, plate 4 = 10^{-5} dilution, plate 5 = 10^{-6} dilution, plate 6 = 10^{-7} dilution, plate 7 = 10^{-8} dilution.]

COUNTING THE PLATES

After the plates have incubated and colonies have grown, pick a plate that has about **50-200 colonies** on it. Count the exact number of colonies on the plate (include small colonies, but count large colonies that are composed of spreading hyphae only once). Each colony is assumed to have arisen from a single cell.

Note the number of the tube from which you made the count. You should add this number of zeros to the end of the number you wrote down. Add one more zero to your total number. [**Each of these zeros

represents the factor of ten by which you diluted your original sample. Placing one gram in 9 ml represents one factor of ten dilution. Each successive transfer is another factor of ten dilution.This technique is not perfect, and you will only be able to get a minimum estimate of the number of many yeasts present.

V. PHOTOGRAPHING YEASTS

1. Prepare cultures as for morphology cultures after 7 days. YM broth and CM are used.

2. Prepare slides:
   a. Clean slides
   b. Glass cover slips

3. YM broth: Shake the tube lightly to mix the cells and place a very small amount of cultured broth onto the slide. Take pictures of budding cells and hyphae if present. If the cells are moving, you have added too much liquid. Remaking the slide is a must.

4. CM agar: Push the cover slip that was on the Damoult plate away to expose the filamentous areas beneath. Cut a small piece of agar with the hyphae and place onto a drop of 60% glycerol on a slide. Cover with the cover glass and very gently push it down. Do not crush the agar! Fill in the glass-cover slip gap with more glycerol. Take pictures of pseudohyphae, true hyphae, spores, and any other strange structures.

5. Microscope:
   a. Nikon
   b. Oil lenses (Usually 60X by 1, 1.25, 1.5X objective lenses)

6. Microscope Settings: MORE TO COME

VI. MAILING YEAST CULTURES

1. To NRRL (Peoria): Use the departmental label with purple strip on top. **Stamp "First Class."** Stamps are found in the departmental office. Place in department's US Postal Service "out box" in Room 208.

2. To CBS in The Netherlands or other Foreign Countries: Use the departmental label as above. Stamp "Air Mail."

3. For foreign countries also use the US Customs form from the post office (be sure to use both green side and white side). Fill in required information. For the check boxes, check "Other." For description use "Non-pathogenic yeast cultures." For weight use the lab scale and convert grams to ounces. The rest should be self-explanatory. Place in department's US Postal Service "out box" in Room 208.

4. In cases of emergency (need culture collection numbers fast or when the cultures might die from heat or cold, FexEx can be used. We usually would use 2-day or overnight second delivery. Please check about this.

VII. LABORATORY PRECAUTIONS

- Be familiar with all the LSU health and safety protocols; be certain you understand how to dispose of chemicals used in the lab: http://appl003.lsu.edu/PubSafety/oes.nsf/$Content/Safety+Documents+&+Information?OpenDocument
- All **cultures** should be treated with caution and care. We do not knowingly work with yeasts pathogenic to mammals, but some cultures that grow at 37°C are potentially harmful.
- **Living cultures** should be autoclaved before discarding. Although orange hazard bags may be used for autoclaving, the orange bag must be placed in a black garbage bag before discarding it.
- **Broken glass** should be placed in the broken glass container only. When the broken glass container is full, it should be boxed and taped shut and discarded in the trash.
- **SYBR Safe** and other DNA stains should be handled very carefully (USE GLOVES) and discarded in the appropriate manner approved by chemical safety. These chemicals may alter genetic material.

[XVI, 1–47]

1. Shapes based on the sphere and ellipsoids[1]

	axis aa	*axis* bb	*length:breadth*[3]
1 by 1	globose (spherical)[2]	(spherical)	1·0–1·05
1 to 2	subglobose (prolate spheroidal)	(oblate spheroidal)	1·05–1·15
2 to 3	broadly ellipsoidal (subprolate)	(suboblate)	1·15–1·3
3 to 4	ellipsoidal (prolate)	(oblate)	1·3–1·6; elongate
4 by 4	oval (perprolate)	(peroblate)	
5 by 5	fusiform		

[1]This figure is adapted from Payak (*Mycopathologia* **16**: 72, 1962).
[2]The terms in parentheses are those used by Erdtman (*An Introduction to pollen analysis*, 1954).
[3]These ratios are those of Bas (*Persoonia* **5**: 321, 1969).

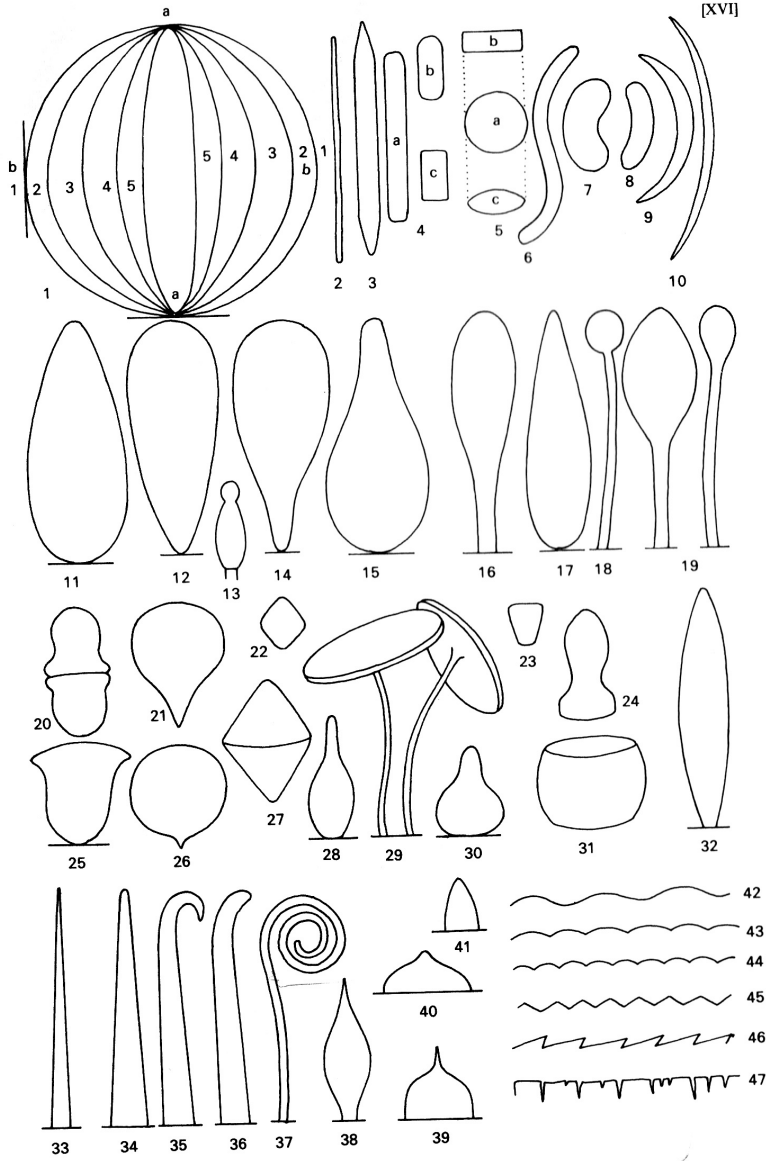
2. Filiform.
3. Acerose.
4. Cylindrical. (Restricted by Bas (1969) to cylinders with a length:breadth of 2·0–3·0).
   a. Bacilliform (1: *b* > 3·0); *b*, *c*. oblong; *b*. apices rounded (obtuse); *c*. apices truncate.
5. *a*. Discoid or lenticular in surface view.
   b. Discoid in side view.
   c. Lenticular in side view.

6. Sigmoid.
7. Reniform (Fabiform).
8. Allantoid.
9. Lunate (Crescentic).
10. Falcate.
11. Ovoid.
12. Obovoid.
13. Lecythiform.
14. Pyriform.
15. Obpyriform.
16. Clavate.
17. Obclavate.
18. Capitate.
19. Spathulate.
20. Bicampanulate.
21. Turbinate.
22. Quadrangular (Rhomboidal).
23. Cuneiform.
24. Dolabriform.
25. Campanulate.
26. Napiform.
27. Biconic.
28. Lageniform.
29. Peltate.
30. Ampulliform.
31. Doliiform.
32. Cymbiform (Navicular).
33. Acicular.
34. Subulate.
35. Hamate (Uncinate).
36. Corniform.
37. Circinate.
38. Ventricose.

Apices
39. Mucronate.
40. Papillate.
41. Acute.

Edges
42. Sinuate.
43. Crenate.
44. Crenulate.
45. Dentate.
46. Serrate.
47. Laciniate.

See also Systematics Association Committee for Descriptive Terminology [*Taxon* **9**: 245, 1960 (list of works); **11**: 145, 1962 (terminology of simple symmetrical plane shapes; chart)].



Form 1

Morphology test

Isolate number:

1. YM broth

Date of inoculation: **Date of observation:**

a. Cell size (-) x (-) check at least 20 cells

b. Cell shape (circle the shapes and mark the most popular one)

sphere and ellipsoid (globose, subglobose, ellipsoidal, oval, fusiform)

fusiform cylindrical ovoid others ( )

c. Asexual reproduction

budding fission arthrospores

d. Other morphology

clusters chain pseudohyphae (yes no) true hyphae (yes no)

others ( )

2. YM agar

Date of inoculation: **Date of observation:**

Colony color: texture:

surface:

others ( )

3. DPC

Date of inoculation: **Date of observation:**

Colony color: margin:

pseudohyphae (yes no) true hyphae (yes no)

others ( )

Draw the shape on the back if you cannot describe.

FORM 2. THIS IS FOR EXAMPLE ONLY. USE ASSIMILATION SHEETS PRINTED FROM EXCEL FILE.

Assimilation Test Work Sheet																	
		Isolate No:							Isolate No:								
		3 d	1wk	2wk	3wk	4wk	Final		3 d	1wk	2wk	3wk	4wk	Final			
F1	D-Glucose							F1									
F2	D-Galactose							F2									
F3	Maltose							F3									
F4	α-Methyl-D-glucoside							F4									
F5	Sucrose							F5									
F6	α, α-Trehalose							F6									
F7	Melibiose							F7									
F8	Lactose							F8									
F9	Cellobiose							F9									
F10	Melezitose							F10									
F11	Raffinose							F11									
F12	Inulin							F12									
F13	Starch							F13									
F14	D-Xylose							F14									
F15	Negative fermentaion con.							F15									
C1	D-Glucose							C1									
C2	D-Galactose							C2									
C3	L-Sorbose							C3									
C4	D-Glucosamine							C4									
C5	D-Ribose							C5									
C6	D-Xylose							C6									
C7	L-Arabinose							C7									
C8	D-Arabinose							C8									
C9	L-Rhamnose							C9									
C10	Sucrose							C10									
C11	Maltose							C11									
C12	Trehalose							C12									
C13	α-Methyl-D-glucoside							C13									
C14	Cellobiose							C14									
C15	Salicin							C15									
C16	Arbutin							C16									
C17	Melibiose							C17									
C18	Lactose							C18									
C19	Raffinose							C19									
C20	Melezitose							C20									

MYCOLOGY LAB, LSU

C21	Inulin							C21									
C22	Soluble starch							C22									
C23	Glycerol							C23									
C24	Erythritol							C24									
C25	Ribitol							C25									
C26	Xylitol							C26									
C27	L-Arabinitol							C27									
C28	D-Glucitol							C28									
C29	D-Mannitol							C29									
C30	Galactitol							C30									
C31	myo-Inositol							C31									
C32	D-Glucono-1,5-lactone							C32									
C33	2-Keto-D-gluconate							C33									
C34	5-Keto-D-gluconate							C34									
C35	D-Gluconate							C35									
C36	D-Glucuronate							C36									
C37	D-Galacturonic acid							C37									
C38	DL-Lactate							C38									
C39	Succinate							C39									
C40	Citrate							C40									
C41	Methanol							C41									
C42	Ethanol							C42									
C43	Propane 1,2 diol							C43									
C44	Butane 2, 3 diol							C44									
C45	Quinic acid							C45									
C46	D-Glucarate							C46									
C47	D-Galactonate							C47									
T1	Growth at 25C		+		+		+	T1		+		+		+			
T2	Growth at 30C							T2									
T3	Growth at 35C							T3									
T4	Growth at 37C							T4									
T5	Growth at 40C							T5									
T6	Growth at 42C							T6									
T7	Growth at 45C							T7									
O1	0.01% Cycloheximide							O1									
O2	0.1% Cycloheximide							O2									
O3	1% Acetic acid							O3									
O4	50% D-Glucose							O4									
O5	60% D-Glucose							O5									
O6	10% NaCl							O6									
O7	16% NaCl							O7									
N0	Negative cont. (N-)							N0									
N1	Nitrate (Potassium)							N1									
N2	Nitrite (Sodium)							N2									

N3	Ethylamine							N3							
N4	L-Lysine							N4							
N5	Cadaverine							N5							
N6	Creatine							N6							
N7	Creatinine							N7							
N8	D-Glucosamine							N8							
N9	Imidazole							N9							
N10	D-Tryptophan							N10							
NP	Positive cont. (N+)							NP							
V1	w/o vitamins							V1							
V2	w/o myo-Inositol							V2							
V3	w/o Pantothenate							V3							
V4	w/o Biotin							V4							
V5	w/o Thiamin							V5							
V6	w/o Biotin & Thiamin							V6							
V7	w/o Pyridoxine							V7							
V8	w/o Pyrid. & Thiam.							V8							
V9	w/o Niacin							V9							
V10	w/o PABA							V10							
V11	Positive cont. (vitamin)							V11							
M1	Starch formation							M1							
M2	Acetic acid production							M2							
M3	Urea hydrolysis							M3							
M4	DBB reaction							M4							