

BAM: Yeasts, Molds and Mycotoxins

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Bacteriological Analytical Manual

Chapter 18

Yeasts, Molds and Mycotoxins

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The large and diverse group of microscopic foodborne yeasts and molds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile environmental requirements. Although the majority of yeasts and molds are obligate aerobes (require free oxygen for growth), their acid/alkaline requirement for growth is quite broad, ranging from pH 2 to above pH 9. Their temperature range (10-35°C) is also broad, with a few species capable of growth below or above this range. Moisture requirements of foodborne molds are relatively low; most species can grow at a water activity (a_w) of 0.85 or less, although yeasts generally require a higher water activity.

Both yeasts and molds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on food type, organisms involved, and degree of invasion; the contaminated food may be slightly blemished, severely blemished, or completely decomposed, with the actual growth manifested by rot spots of various sizes and colors, unsightly scabs, slime, white cottony mycelium, or highly colored sporulating mold. Abnormal flavors and odors may also be produced. Occasionally, a food appears mold-free but is found upon mycological examination to be contaminated. Contamination of foods by yeasts and molds can result in substantial economic losses to producer, processor, and consumer.

Several foodborne molds, and possibly yeasts, may also be hazardous to human or animal health because of their ability to produce toxic metabolites known as mycotoxins. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though

the generating organisms may not survive food preparation, the preformed toxin may still be present. Certain foodborne molds and yeasts may also elicit allergic reactions or may cause infections. Although most foodborne fungi are not infectious, some species can cause infection, especially in immunocompromised populations, such as the aged and debilitated, HIV-infected individuals, and persons receiving chemotherapy or antibiotic treatment.

The dilution plating and the direct plating methods may be used to detect fungi in foods. The direct plating method is more efficient than the dilution plating method for detecting individual mold species, including most of the toxin producers, but it is less effective in detecting yeasts. It is also used to determine whether the presence of mold is due to external contamination or internal invasion. Methodology for testing the ability of isolates of toxigenic mold species to produce mycotoxins on sterile rice water substrate is included here.

Enumeration of Yeasts and Molds in Food--Dilution Plating Technique

A. Equipment and materials

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate, see Chapter 1 (</food/laboratory-methods/bam-food-samplingpreparation-sample-homogenate>)
2. Equipment for plating samples, see Chapter 3 (</food/laboratory-methods/bam-aerobic-plate-count>)
3. Incubator, 25°C
4. Arnold steam chest
5. pH meter
6. Water bath, 45 ± 1° C

B. Media (</food/laboratory-methods/media-index-bam>) and Reagents (</food/laboratory-methods/reagents-index-bam>)

Media

1. Dichloran rose bengal chloramphenicol (DRBC) agar (M183 (</food/laboratory-methods/bam-media-m183-dichloran-rose-bengal-chloramphenicol-drbc-agar>))
2. Dichloran 18% glycerol (DG18) agar (M184 (</food/laboratory-methods/bam-media-m184-dichloran-18-glycerol-dg18-agar>))
3. Plate count agar (PCA), standard methods (M124 (</food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods>)); add 100 mg chloramphenicol/liter when this medium is used for yeast and mold enumeration. This medium is not efficient when "spreader" molds are present.

4. Malt agar (MA)(M185 (/food/laboratory-methods/bam-media-m185-malt-agar-ma))
5. Malt extract agar (Yeasts and Molds) (MEAYM) (M182 (/food/laboratory-methods/bam-media-m182-malt-extract-agar-yeasts-and-molds-meaym))
6. Potato dextrose agar (PDA), dehydrated; commercially available (M127 (/food/laboratory-methods/bam-media-m127-potato-dextrose-agar))

Antibiotic solutions

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

C. Procedures:

Sample preparation

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Test individual subsamples or composite according to respective Compliance Program for the food under analysis. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10^{-1} dilution, then homogenize in a stomacher for 2 min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10^{-6} should suffice.

Plating and incubation of sample

Spread-plate method. Aseptically pipet 0.1 ml of each dilution on pre-poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method. Use sterile cotton-plugged pipet to place 1.0 ml portions of sample dilution into pre-labeled 15 × 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

From preparation of first sample dilution to pouring or surface-plating of final plate, no more than 20 min (preferably 10 min) should elapse. **Note:** Spread plating of diluted sample is considered better than the pour plate method. When the pour plate technique is used, fungal colonies on the surface grow faster and often obscure those underneath the surface, resulting in less accurate enumeration. Surface plating gives a more uniform growth and makes colony isolation easier. DRBC agar should be used for spread plates only.

Incubate plates in the dark at 25°C. Do not stack plates higher than 3 and do not invert. **Note:** Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mold are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mold and yeast counts (MYC) as less than 1 times the lowest dilution used.

Isolate individual colonies on PDA or MA, if further analysis and species identification is necessary.

Enumeration of Molds in Foods--Direct Plating Technique for Foods That Can Be Handled with Forceps (Dried Beans, Nuts, Whole Spices, Coffee and Cocoa Beans, etc.)

A. Equipment and materials

1. Freezer, -20° C
2. Beakers, sterile, 300 ml
3. Forceps, sterile
4. Arnold steam chest
5. Water bath, 45 ± 1° C
6. Incubator, 25° C

B. Media and Reagents

1. Dichloran rose bengal chloramphenicol (DRBC) agar (M183 (/food/laboratory-methods/bam-media-m183-dichloran-rose-bengal-chloramphenicol-drbc-agar))
2. Dichloran 18% glycerol (DG18) agar (M184 (/food/laboratory-methods/bam-media-m184-dichloran-18-glycerol-dg18-agar))
3. Antibiotic solutions (see previous section)
4. NaOCl (commercial bleach) solution, 10%
5. Sterile distilled water

C. Analysis of non-surface-disinfected (NSD) foods

Sample and media preparation

Before plating, hold sample at -20° C for 72 h to kill mites and insects that might interfere with analysis.

Prepare DRBC agar as described in the appendix. If DRBC is not available, or the water activity of the analyzed sample is less than 0.95, use DG18 agar. Media should be prepared no more than 24 h prior to use.

Plating and incubation of sample

From each sample, transfer about 50 g into a sterile 300 ml beaker. Using 95% ethanol-flamed forceps place intact food items on surface of solidified agar, 5-10 items per plate (depending on size of food item) 50 items total per sample.

Flame forceps between plating of each item. Use several forceps alternately to avoid overheating. Do not plate visibly moldy or otherwise blemished items.

Align 3-5 plates in stacks and identify with sample number plus date of plating. Incubate stacks, undisturbed in the dark at 25°C for 5 days. If there is no growth at 5 days of incubation, re-incubate for another 48 h to allow heat- or chemically-stressed cells and spores enough time to grow.

Reading of plates

Determine occurrence of mold in percentages. If mold emerged from all 50 food items, moldiness is 100%; if from 32 items, moldiness is 64%. Determine percent occurrence of individual mold genera and species in like manner. Experienced analysts may identify *Aspergillus*, *Penicillium* and most other foodborne mold genera directly on medium with low power (10-30X) magnification.

D. Analysis of surface-disinfected (SD) foods

Perform disinfection in clean laboratory sink, not stainless steel, free from any acid residues, with tap water running (precautions against chlorine gas generation). Wear rubber gloves and transfer about 50 g of sample into a sterile 300 ml beaker. Cover with 10% chlorine (commercial bleach) solution for 2 min, while swirling beaker contents gently but constantly in clockwise-counterclockwise motion. Decant 10% chlorine solution and give beaker contents two 1-min rinses with sterile distilled water. Prepare plates; plate sample, incubate, and read plates as in non-surface disinfected direct plating method, above. Compare NSD and SD results from the same sample to determine if moldiness was due mainly to surface contamination or to internal invasion and growth. Isolate individual colonies on PDA or MA.

Fluorescence Microscopy Procedure for Quantitation of Viable and Nonviable Yeasts in Beverages

Methods for counting viable yeasts by plating are described above. A direct microscopic procedure for counting nonviable and viable yeasts in beverages and other liquid samples is presented here. Quantitating yeast cells by microscopy eliminates the need for extended incubation, thus reducing the analytical time required. All yeasts can be counted, and living and dead yeast cells can be differentiated.

- A. Millipore disk filter holders for standard syringes
- B. Millipore filters: AABG, 0.8 μm , black, gridded; 25 mm diameter
- C. Syringes, disposable
- D. Pipets
- E. Forceps
- F. Bibulous paper
- G. Microscope slides and 24 \times 24 mm coverslips
- H. Fluorescence microscope: blue excitation; 10X eyepieces with Howard mold count or other eyepiece grid; 20 \times or 40 \times objective

- **Reagents**

1. Aniline blue; 1% in M/15 K_2HPO_4 (M/15 is equivalent to 11.6 g/L), adjusted to pH 8.9 with K_3PO_4 . A stock solution can be made; age improves fluorescence.
2. NaOH; 25 g in 100 ml water

- **Sample preparation for filterable liquids (e.g. water and grape juice)**

Filter aliquot (usually 10 ml) of sample through Millipore filter (AABG, 0.8 μ m, black, gridded). (Portion size can be increased or decreased, depending on level of contamination). Use Millipore disk filter holder which attaches to standard syringe. Make sure that syringe is accurate. If not, remove plunger, attach syringe to filter holder, and pipette 10 ml into syringe. Press all of sample through filter. Do this with air cushion of about 3 ml between plunger and sample. Keep filter holder vertical to ensure even distribution of sample over filter. Remove filter from filter holder and place on microscope slide; grid should be parallel to edges of slide to facilitate counting.

- **Sample preparation for non-filterable liquids that clog the filter (e.g. orange juice)**

To suppress background interference in fluorescence microscope, mix 4 ml sample with 1 ml sodium hydroxide (25 g in 100 ml water). Shake well and wait 10 min. Place Millipore filter (AABG, 0.8 μ m, black, gridded) on a piece of bibulous paper and spread 0.1 or 0.01 ml (depending on level of contamination) of sample over filter. When filter surface is dry, place filter on microscope slide, keeping grid parallel to edges of slide to facilitate counting.

- **Microscopic counting procedure**

Cover filter with a drop of aniline blue, 1% in M/15 (11.6 g/L) K_2HPO_4 , adjusted to pH 8.9 with K_3PO_4 . Spread aniline blue stain over whole filter with glass rod or coverslip without touching filter itself. Wait about 5 min; then cover filter with 24 \times 24 mm coverslip.

Count yeasts, using fluorescence microscope with blue excitation. Use 10X eyepiece with Howard mold count or other eyepiece grid, and 20X (or 40X) objective. Count 3 squares of eyepiece grid in each field of filter not covered by gasket. Count budding yeasts as 1 cell if daughter cell is obviously smaller than mother cell. If they are approximately equal in size, count them as 2 cells. Count all yeasts located completely within an eyepiece square and all yeasts touching left and lower border of eyepiece square. Do not count yeasts touching right and upper borders.

This method also differentiates dead (heat- or formaldehyde-killed) and living yeast cells. Dead cells show fairly uniform fluorescence, and plasma may be granular. In living cells, the cell wall stains brighter and is more defined than the plasma, which is less prominent and uniformly stained.

- **Calculations to determine number of yeasts per ml**

Determine area of filter covered by 1 square of eyepiece grid, using objective (stage) micrometer. For filtered samples, the working area of the Millipore filter (portion not covered by the gasket) is 380 mm². For nonfiltered samples, it is the entire filter, or 491 mm², since no gasket is used.

No. of yeasts per ml =

$$\frac{\text{No. of yeasts counted}}{\text{No. of eyepiece squares examined}} \times \frac{\text{working area of filter}}{\text{area of one eyepiece square}} \times \frac{1}{\text{volume of liquid}}$$

- **NOTE:** For non-filterable liquids, volume includes only net amount used and not volume of NaOH added (i.e., 80% of total volume applied to filter).
- For background information on the method, including photographs of dead and living yeast cells, **see** Koch et al., ref. 8, below.

Methods for Determining Toxin Production by Molds

A. Equipment and materials

1. Erlenmeyer flasks, 300 ml, wide-mouth
2. Cotton, nonabsorbent
3. Funnels, short-stem glass, 90-100 mm diameter
4. Filter paper, 18 cm diameter, folded (Schleicher & Schuell No. 588)
5. Boiling chips, silicon carbide
6. Fume hood equipped with steam bath; air-flow rate, 100 cubic ft/min
7. Blender, high speed, explosion-proof
8. Thin layer chromatographic apparatus or high-performance liquid chromatograph
9. Incubator, 22-25°C

B. Media and reagents

1. Long or short grain polished rice
2. Chloroform for extraction of aflatoxins, ochratoxins, sterigmatocystin, xanthomegnin, luteoskyrin, patulin, penicillic acid, citrinin, T-2 toxin, zearalenone
3. Methanol for extraction of deoxynivalenol
4. Appropriate mycotoxin standards

5. NaOCl solution, 5%

C. Toxin production

Into 300 ml wide-mouth Erlenmeyer flask, add 50 g rice and 50 ml distilled water. Plug flasks with cotton and autoclave 20 min at 121°C and 15 psi. Aseptically multispore-inoculate separate cooled flasks with individual mold isolates. Incubate inoculated flasks at 22-25°C until entire surface is covered with growth, and mycelium has penetrated to bottom of flask (15-20 days). To each flask, add 150 ml chloroform (150 ml methanol if toxin in question is deoxynivalenol), using short-stem glass funnel inserted alongside unremoved cotton plug (to minimize mold spore dissemination). Heat flask contents in fume hood on steam bath until solvent begins to boil. (Conduct all subsequent steps in fume hood.) With spatula, break up moldy rice cake and transfer flask contents into explosion-proof blender and blend at high speed for 1 min. Filter blender contents through filter paper inserted into short-stem glass funnel. Collect filtrate in 300 ml Erlenmeyer flask. Return rice cakes to blender, add 100 ml unheated solvent and blend 1 min at high speed. Filter as above and combine filtrates. Add boiling chips to flask containing filtrates and evaporate with steam to 20-25 ml. If analysis is not to follow immediately, evaporate to dryness and store flask in the dark. Rinse all glassware, etc., used for extraction in 5% NaOCl solution before soap and water cleansing. Submerge rice cake in 5% NaOCl solution for 72 h before autoclaving and disposal.

D. Toxin analysis

Appropriate mycotoxin standards are required for both qualitative and quantitative analysis of toxin. Use either thin layer chromatography as described in references 16 or 17 or high performance liquid chromatography, as described in reference 15a, to determine mycotoxins extracted from mold cultures. Naturally occurring mycotoxins in foods or feeds can best be determined by methods described in *Official Methods of Analysis* (16).

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Media

Dichloran 18% glycerol (DG18) agar (M184)

Reagent	Quantity
Glucose	10.0 g

Reagent	Quantity
Peptone	5.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ ·7H ₂ O	0.5 g
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	800 ml

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 g glycerol (analytical reagent grade), and sterilize by autoclaving at 121°C for 15 min. Temper medium to 45° C and pour plates under aseptic conditions. The final a_w of this medium is 0.955. DG18 agar is used as a general purpose mold enumeration medium and is preferred when the a_w of the analyzed food is less than 0.95. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and molds must be enumerated, DRBC agar should be used.

Dichloran rose bengal chloramphenicol (DRBC) agar (M183)

Reagent	Quantity
Glucose	10.0 g
Bacteriological peptone	5.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ ·7H ₂ O	0.5 g
Rose bengal (5% aqueous soln., w/v)	0.5 ml
Dichloran (0.2% in ethanol, w/v)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	1.0 liter

Final pH should be 5.6

Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121°C for 15 min. Temper to 45 ± 1° C in a water bath and pour plates.

Notes: DRBC agar is especially useful for analyzing samples containing "spreader" molds (e.g. *Mucor*, *Rhizopus*, etc.), since the added dichloran and rose bengal effectively slow down the growth of fast-growing fungi, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.

Media containing rose bengal are **light-sensitive**; relatively short exposure to light will result in the formation of inhibitory compounds. Keep these media in a dark, cool place until used. DRBC agar should be used for spread plates only.

Malt Agar (MA) (M185)

Reagent	Quantity
Malt extract, powdered	20.0 g
Agar	20.0 g
Distilled water	1.0 liter

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121° C. Temper medium to 45° C and pour plates under aseptic conditions. To prepare slants dispense 5-6 ml of steamed medium (before autoclaving) into each of several 16 × 125 mm screw-cap tubes, loosely cap tubes and sterilize as above. After autoclaving lay tubes in a slanting position and let them cool. This medium is recommended as a general maintenance medium.

Malt Extract Agar (Yeasts and Molds)(MEA) (M182)

Reagent	Quantity
Malt extract, powdered	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	20.0 g
Distilled water	1.0 liter

Mix ingredients, heat to dissolve agar and sterilize at 121° C for 15 min. Temper media to 45° C and pour plates under aseptic conditions. Dehydrated MEA is commercially available, but since more than one MEA formula exists, check for the appropriate composition. This medium is recommended for the identification of *Aspergillus* and *Penicillium*.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
Chapter 18.