11

Fungal Isolation and Enumeration in Foods

Dante Javier Bueno, Julio Oscar Silva, and Guillermo Oliver

1. Introduction

Humans have now been growing and storing enough food for a long enough time that some rapidly evolving organisms, such as fungi, are moving into niches created by the exploitation of certain plants as food ***(1)***.

Food is expected to be nutritious. The most important of the physicochemical conditions that affects fungal growth is related to the biological state of the food. Living foods, particularly fresh fruits, vegetables, and also grains and nuts before harvest, possess powerful defense mechanisms against microbial invasion. When the specific microorganisms overcome defense mechanisms, the spoilage of a living food starts. Other factors to consider are water activity, hydrogen ion concentration, temperature, gas tension, consistency, nutrient status, specific solute effect, and preservation ***(1)***.

The consequences of mold contamination of foods are diverse ***(2)***: unsightly appearance, chemical (removal or change of most of the constituents) and nutritional value changes, modification of organoleptic quality, difficulties in preservation, occupational hazards (mycoses, allergies), and toxicoses (mycotoxicoses).

It is possible to recognize a succession of three distinct mycoflora during the storage of cereals ***(3)***, but they can also be mixed:

1. Field fungi growing and established before harvesting (*Alternaria*, *Fusarium*, *Helminthosporium*, *Cladosporium*).
2. Storage fungi taking over and dominanting in the silo (*Aspergillus* and *Penicillium*).
3. Advanced decay fungi (*Papulospora*, *Sordaria*, *Fusarium graminearum*, and members of the order Mucorales).

Some kind of foods are pelleted during the manufacturing process. This reduces fungal contamination, because the spores are relatively susceptible to heat; however, it does not eliminate all viable spores ***(4,5)***. Even if fungal spores could be destroyed during this process, a quick contamination can occur later ***(5)***. Therefore, Suárez ***(6)*** concluded that the pelleting process plays a minor role in fungal control.

From: *Methods in Molecular Biology, vol. 268: Public Health Microbiology: Methods and Protocols*
Edited by: J. F. T. Spencer and A. L. Ragout de Spencer © Humana Press Inc., Totowa, NJ

In this chapter, we describe the methods currently used to isolate and enumerate fungi from different foods. You can consult workshops from the International Commission on Food Mycology (ICFM) or books such as *Fungi and Food Spoilage* by J. I. Pitt and A. D. Hocking ***(1)***, for more details.

2. Materials

2.1. Growth Media

1. Dichloran rose bengal chloramphenicol agar (DRBC): 10 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 15 g/L agar, 25 mg/L rose bengal (0.5 mL of a 5% w/v solution in water), 2 mg/L dichloran (1 mL of a 0.2% solution in ethanol), 100 mg/L chloramphenicol, pH 5.5–5.8. Sterilize by autoclaving at 121°C for 15 min. In the dark, the medium is stable for at least 1 mo at 1–4°C. Substances such as dichloran and rose bengal ***(7)*** inhibit the spread of mold colonies.
2. Potato dextrose agar (PDA): 400 g/L potatoes, 15 g/L glucose, 20 g/L agar, pH 6.0–6.2.
   a. Wash and cut the potatoes and then put them in 1000 mL of water.
   b. Steam or boil for 30–45 min.
   c. Filter the potato broth through cotton, and add water to recover the initial volume.
   d. Melt the agar in 500 mL of potato broth and add the glucose to the other 500 mL.
   e. Mix and sterilize by autoclaving at 121°C for 15 min and add antibiotic solution.
3. Czapek-Dox agar: 30g/L sucrose, 3 g/L $NaNO_3$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 0.01 g/L $Fe_2(SO_4)_3$, 1g/L K_2HPO_4, 13 g/L agar; pH 7.3 ± 0.1. Sterilize by autoclaving at 121°C for 15 min and add antibiotic solution.
4. Malt extract agar (MEA): 20 g/L malt extract, 1 g/L peptone, 20 g/L glucose, 20 g/L agar, pH 5.6. Sterilize by autoclaving at 121°C for 15 min and add antibiotic solution.
5. Tap water agar (TWA): add 15 g/L agar to 1 L of tap water. Sterilize by autoclaving at 121°C for 15 min and add antibiotic solution. This can provide a substrate for growth and sporulation of plant pathogenic fungi such as *Fusarium*, *Drechslera*, *Bipolaris*, and some other dematicaeous Hyphomycetes.

2.2. Solutions

1. Stock solution of succinate/palmitate of chloramphenicol.
   a. Dissolve 1 g of succinate/palmitate of chloramphenicol in 5 mL of sterile distilled water or physiological solution.
   b. Transfer this solution to another recipient containing 95 mL of sterile distilled water or physiological solution. Use 1 mL of the stock solution for each 100 mL of steilized medium.
   c. Media containing chloramphenicol are easier to prepare, are not affected by autoclaving, and have greater long-term stability.
2. Peptone water solution (0.1%): dissolve 1 g of peptone in 1000 mL of distilled water and sterilize by autoclaving at 121°C for 15 min.

3. Methods

3.1 Direct Plating

Direct plating is the preferred method for detecting, enumerating, and isolating fungi from particulate foods such as grains and nuts. The results of this analysis are expressed as percentage infection of particles. This technique provides no direct indica-

tion of the extent of fungal invasion in individual particles. However, it is reasonable to assume that a high percentage of infection is correlated with extensive invasion in the particles ***(1)***. For solid food, for which sampling involves cutting pieces of food, direct plating is essentially a qualitative technique ***(8)***.

1. With surface desinfection (internal mycoflora): This process provides an effective measurement of inherent mycological quality. The process removes the inevitable surface contamination arising from dust and other sources.
   a. Immerse particles in a chlorine solution (0.4%) for 2 min. Stir, and then drain the chlorine (*see* **Note 1**).
   b. Rinse the particles twice with sterile distilled water.
   c. Leave the particles to dry in a laminar flow cabinet.
   d. Plate 6–20 particles per plate onto solidified agar depending on the particle size.
   e. Incubate plates upright for 5–7 d at 25–28°C (*see* **Notes 2** and **3**).
   f. Count the numbers of infected particles, and express results as a percentage (number of particles infected/number of total particles). Differential counting of various genera is often possible.
   g. Isolate different strains (*see* **Note 4**).
2. Without surface desinfection ***(1,9)***: especially when surface contaminants become part of the downstream mycoflora (external mycoflora) or pelleted food for animals (total mycoflora, **Fig. 1**).
   a. Plate 6–20 particles per plate onto solidified agar, depending on particle size.
   b. Incubate plates upright for 5–7 d at 25–28°C (*see* **Notes 2** and **3**).
   c. Count the numbers of infected particles, and express the results as a percentage (number of particles infected/number of total particles). Differential counting of various genera is often possible.
   d. Isolate different strains (*see* **Note 4**).

3.2. Dilution Plating

Dilution plating is the appropriate method for mycological analysis of liquid or powdered foods. It is also suitable for grains intended for flour manufacture and in other situations in which total fungal contamination is relevant. It is usually possible to enumerate plates with up to 150 colonies, but if a high proportion of rapidly growing fungi are present, the maximum number will be lower. The minimum number may be 10–15 colonies. For yeast, enumeration is easier. In the absence of filamentous fungi, from 30 to 300 colonies per plate can be counted ***(1)***.

1. Stomach or blending the materials for 2 or 1 min, respectively (*see* **Notes 5**, **6**, and **7**).
2. Make serial dilutions in peptone water until the ration is 1:10,000.
3. Inoculate 0.1 mL of the suspension of different dilutions onto solidified agar and spread it with a sterile bent glass rod (*see* **Note 8**).
4. Incubate plates upright for 5–7 d at 25–28°C (*see* **Notes 2** and **3**).
5. Count the numbers of viable spores, and express results as viable count per gram of sample (colony-forming unit/g).
6. Isolate different strains (*see* **Note 4**).

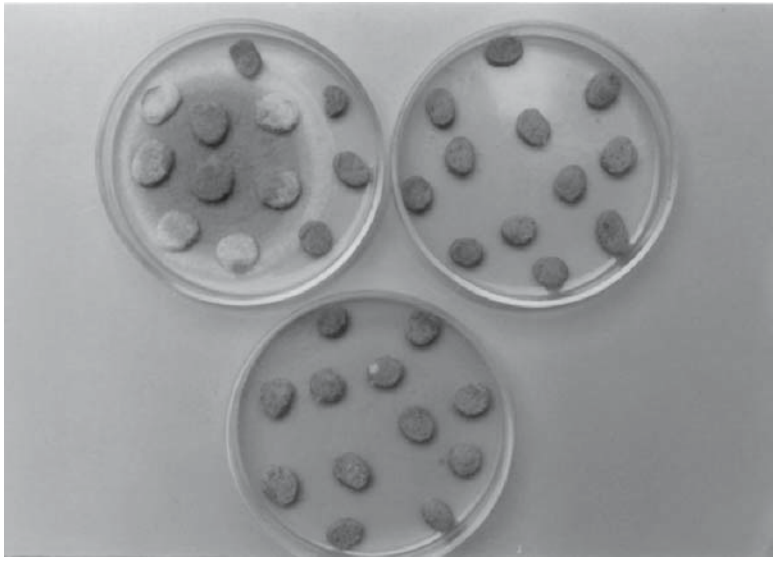

Fig. 1. Direct plating technique without surface desinfection of the kernel in pelleted food for animals.

4. Notes

1. For commodities such as peanuts or maize, in which high levels of *Aspergillus flavus* or *Penicillium* species may be present, Pitt and Hocking ***(1)*** recomend inmersing the particles in 70% ethanol for 2 min followed by 2 min in 0.4% clorine solution.
2. Some common fungi can shed large number of spores during handling, which in an inverted dish will be transferred to the lid. Reinversion of the Petri dish for inspection or removal of the lid may liberate spores into the air or onto solidified agar and cause important contamination problems.
3. In tropical regions, incubation at 30°C is recommended as a more realistic temperature for enumerating fungi from commodities stored at ambient temperatures. In cool regions such as Europe, 22°C has been recommended as the optimal incubation temperature ***(1)***.
4. Streaking techniques are commonly used for yeast isolation, similar to bacterial purification. For filamentous fungi, isolation depends on picking a small sample of hyphae or spores and placing this sample as a point inoculum on an appropriate medium.
5. Stomaching is recomended for dispersing and separating fungi from finely divided materials such as flour and spices, and for soft foods such as cheeses and meats.
6. Harder or particulate foods such as grains, nuts, or dried foods like dried vegetable should be soaked (30–60 min) before stomaching.
7. Blending is recomended for extremely hard particles.
8. Sterilize the rod by flaming it with ethanol before use.

References

1. Pitt, J. I. and Hocking, A. D. (1997) *Fungi and Food Spoilage*. Blackie Academic and Professional, London.
2. Moreau, C. 1979. Moulds, toxins and food. John Wiley & Sons, New York.
3. Christensen, C. M. and Kaufmann, H. H. (1965) Deterioration of stored grains by fungi. *Annu. Rev. Phytopathol.* **III,** 69–84.
4. Abarca, M. L., Bragulat, M. R., Castellá, G, and Cabañes, F. J. (1994) Mycoflora and aflatoxin-producing strains in animal mixed feeds. *J. Food Proteins* **57,** 248–256.
5. Wyatt, R. (1990) Importancia de los hongos en la salud aviar. *Avicultura Profesional* **8,** 48–50.
6. Suárez, O. (1999) Manejo de los granos en las fábricas de alimentos. *Industria Avícola* **10,** 18–21.
7. King, A. D., Hocking, A. D., and Pitt, J. I. (1979) Dichoran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Env. Microbiol.* **37,** 959–964.
8. Pitt J. I. (1989) Food mycology—an emerging discipline. *J. Appl. Bacteriol.* **67,** 1S–9S.
9. Bueno, D. J., Silva, J. O., and Oliver, G. (2001) Mycoflora in commercial pet foods. *J. Food Protection* **64,** 741–743.