# Chapter 10

# Enumerating Yeast in Foods and Water Using the Spread Plating Technique

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## Abstract

Yeasts arouse great interest from the scientific community both for the application in the biotechnology field and for the ability of this microorganism to cause various degrees of food deterioration, being one of the main concerns of food hygiene and food safety. Microbiological examination of foods is predominantly based on culturing techniques to detect and enumerate living microorganisms. Each of these tests follows differentiated procedures, which, in turn, depend on the target microorganism. One of these techniques is the spread plate, which consist of adding a known volume of food sample on a petri dish containing a specific solidified, which will be covered in more detail in this chapter.


**Key words** Standard plate counts, Solid culture medium, Yeast detection, Yeast enumeration, Spoilage yeasts

## 1 Introduction

Yeasts are extremely important when considering biotechnological and industry applications. They are used for a long time in different industrial sectors and contribute to the production of various fermented foods, alcoholic beverages, bread making, dairy products, meat, cereal-based foods, and others. The probiotic activity observed in some yeast is another novel property that is attracting increasing interest [1, 2]. On the other hand, yeasts can also cause various degrees of deterioration and in foods and beverages, with major economic loss [3].

Yeast spoilage is favored in products with low pH, generally 5.5 or lower, products with high sugar (40–70%) or high salt (5–15% NaCl) content and by the presence of organic acids and other easily metabolized carbon sources. Yeast spoilage is often manifested by growth on the surface of products such as cheeses and meats. In addition, they are responsible fermentation of sugars in liquid

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and fluid products, such as fruit juices (concentrated or not), soft drinks, honey, jams and preserves, salad dressings, soy sauce, sugar syrups as well as cider and wines, causing formation of off-flavor compounds, loss of texture, gas production, and package swelling and shrinkage [3, 4]. Thus, the enumeration of yeast is one of the most significant criteria for the control of hygiene and for the prevention of deterioration in food and beverages [5, 6].

The isolation, enumeration, and identification of yeasts from foods and water follow the same principles and steps involved in the characterization of microorganisms, in general. These involve the sequential operations of rinsing or maceration of the sample, dilution of the suspension, enumeration of the yeast cells in suspension by agar plating, purification of isolates, and identification of isolates to either genus, species or strain level [7].

Yeasts in foods can be enumerated using the standard plate count method, and the results are expressed in number of colony-forming units per g or per mL (CFU/g or CFU/mL). Yeast enumeration tests use a variety of culture media, whose formulation varies according to the tests they are intended for. In general, the choice of culture medium that favors the growth of yeasts and inhibits the growth of bacteria and filamentous fungi is of great importance for the stages of isolation and identification [8].

The spread plate technique has advantages in relation of the pour plate technique (*see* Chapter 10), such as flexibility in handling, less harmful temperature effects to microorganisms, and easy to enumerate and to select colonies [9]. In this way, the sample is carefully applied over the solidified nutrient medium and spread using the glass or plastic rod. The use of the spread plate technique in food microbiology is reliable and widely used. In this chapter, an overview of methods for yeast enumeration using the spread plate technique is provided.

## 2 Materials

1. Prepare all solutions (media and reagents) using distilled, deionized, or of equivalent quality water.
2. Storage should be done in flasks made of inert materials, such as neutral glass or polyethylene.
3. Most of the culture media are sterilized by moist heat. However, there are heat-sensitive media components that should be sterilized by filtration.
4. Sterilization time depends on the size of load and containers. Excessive autoclaving time should be avoided to prevent Maillard reaction degradation and breakdown of medium constituents.

5. Add heat labile supplements with aseptic precautions to the cooled medium (45 °C) [10].
6. Equipment and supplies required to perform the spread plate must be controlled carefully to produce accurate yeasts counts.

### 2.1 Basic Equipment for Preparing Culture Media and Enumerating Yeasts [11]

1. Autoclave.
2. Sterile micropipettes (in a sort range of volumes).
3. Sterile pipette tips.
4. Glass or plastic petri dishes, sterile.
5. Bottles, flasks, and tubes, for heating and storage of culture media, and to prepare dilutions.
6. Vortex mixer.
7. Stomacher®.
8. Spreaders, made of glass or plastic, and ethyl alcohol for flame sterilization.
9. Cryovials.
10. Freezer storage boxes.
11. Bunsen burner.
12. Laboratory incubator, with temperature range able to set to the cultivation conditions.
13. pH meter.

### 2.2 Diluents [12] (See Notes 1 and 2)

1. Peptone water (PW) (0.1% m/v): Peptone 1 g, distilled or deionized water 1 L. Dissolve the peptone in the water, adjust pH to 7.0 ± 0.2 and sterilize at 121 °C for 15 min, 1 atm in an autoclave.
2. Butterfield's phosphate buffer (PB) (0.1 M, pH 7.0): Stock solution: Monopotassium phosphate ($KH_2PO_4$) 34 g.
3. Saline Solution (NaCl 0.85%): Sodium chloride (NaCl) 8.5 g, distilled water 1 L.

### 2.3 Culture Media (See Note 3)

Different culture media can be used for the specific yeast count in food and beverage, they can be classified according to Table 1. Their composition, pH that the media must be to be used, and directions to prepare are presented below.

#### 2.3.1 Basal Media

*Malt extract (MEA) agar (g/L)*: Malt extract (20), glucose (20), peptone (1), agar (15). Final pH 5.6 ± 0.2.

*Potato dextrose (PDA) agar (g/L)*: Potato starch or potato extract (from 200 g potato infusion), dextrose (20), agar (15). Final pH 5.6 ± 0.2.

*Yeast extract peptone dextrose (YPD) agar (g/L)*: Yeast extract (10), peptone (20), agar (15). Final pH 7.0 ± 0.2.

**Table 1**
**Different culture media for yeast enumeration in food and beverages**

| Classe | Medium | Purpose | References |
|---|---|---|---|
| *Basal* | Malt extract (MEA) agar<br>Potato dextrose (PDA) agar<br>Peptone yeast extract (YPD) agar<br>Sabouraud-glucose (SGA) agar<br>Tryptone-glucose-yeast extract (TGY) agar<br>Universal medium for yeasts (YM) agar | For the isolation and enumeration of yeasts from foods the use of general-purpose media which allow the recovery of all kinds of yeast | [13, 14, 15, 16] |
| *Acidified* | Acidified media can be made from one of the basal means above | Adjust the pH to 3.5, whose pH, inhibits bacterial growth. Acidification must be done before pouring the agar medium with an appropriate amount of HCl (0.1 N), sulfuric acid (0.1 N), or organic acids 10% (tartaric acid, lactic, citric) may be used as well | [17] |
| *Biostatic agents and antibiotics* | Basal medium plus, chloramphenicol, pimaricin, or chlortetracycline | Is suggested the use of two different antibiotics each in 100 mg/L concentration. Inhibits the growth of Gram-positive and Gram-negative bacteria | [15, 18, 19] |
| | Basal medium supplemented with dyes | Auramine-O (25 μ/m), gentian violet (5 μ/mL) and malachite green (1 μ/m) inhibit completely the growth of various fungal species. | [20] |
| | Dichloran Rose Bengal Chloramphenicol (DRBC) agar | The advantage of this medium is that the spreading growth of mold colonies is restricted. In addition, contain chloramphenicol for the inhibition of bacteria | [21–24] |
| | Oxytetracycline glucose yeast extract (OGYE) agar | Enumeration and cultivation of yeasts and fungi from foods | [25] |
| *Selective* | Dichloran 18% glycerol (DG18) agar | The final concentration of glycerol in the medium, 18%, reduces the value of $a_w$ in the medium from 0.999 to 0.95 and favors the recovery of xerophilic yeasts such as *Zygosaccharomyces rouxii* | [26–28] |
| | Malt extract agar (MEA) with 30% glucose | Used to recover xerophilic yeasts from concentrated products | [15] |
| | Tryptone yeast extract agar (TGY) with 10% glucose | Is most suitable for enumerating *Z. rouxii* in a wide range of reduced aw foods | [14, 29] |
| | Basal medium (MEA or TGY) supplemented with 0.5% acetic acid | Spoilage yeast capable to grow in low acid and/or preservative-containing foods | [30] |
| | MYGP Copper Agar | Used for isolation and cultivation of wild yeasts in the brewing industry | [31] |

(continued)

**Table 1 (continued)**

| Classe | Medium | Purpose | References |
|---|---|---|---|
| *Differential* | Lysine agar | Useful medium to enumerate non-*Saccharomyces* yeasts when they are present in foods or beverages along with *Saccharomyces* species | [32, 33] |
| | Molybdate Agar | Isolation and differentiation of a variety of yeasts | [34, 35] |
| | Wallerstein Laboratory Nutrient Agar (WL) | Allows differentiation between commercial *Saccharomyces cerevisiae* from wild | [36, 37] |
| | CHROMagar Candida | Differentiation between *Candida* species | [38, 39] |
| | *Dekkera/Brettanomyces* Differential Medium (DBDM) agar | Able to efficiently recover *Dekkera/Brettanomyces* sp. from wine-related environments | [40] |

Source: Adapted [45]

*Sabouraud-glucose (SGA) agar (g/L)*: Glucose (20), peptone (1), agar (15). Final pH 7.0 ± 0.2.

*Tryptone-glucose-yeast extract (TGY) agar (g/L)*: Tryptone (5), glucose (100), yeast extract (5), agar (15). Final pH 7.0 ± 0.2.

*Universal medium for yeasts (YM) agar*: Yeast extract (3), malt extract (5), peptone (10), glucose (20), agar (15). Final pH 7.0 ± 0.2.

#### 2.3.2 Acidified Media

Acidified media can be made from one of the basal means above. Acidification must be done before pouring the agar medium with an appropriate amount of HCl (0.1 M), sulfuric acid (0.1 M) or 10% organic acids (tartaric acid, lactic, citric) (*see* **Note 4**).

#### 2.3.3 Biostatic Agents and Antibiotics

Basal medium supplemented with chloramphenicol, chlortetracycline, or pimaricin 100 mg/L.

Basal medium supplemented with dyes Auramine-O Auramine (25 μ/m), gentian violet (5 μ/mL), and malachite green (1 μ/m) (*see* **Note 5**).

*Dichloran Rose Bengal chloramphenicol (DRBC) agar (g/L)*: Glucose (10), bacteriological peptone (5), potassium phosphate monobasic (1), magnesium sulfate heptahydrate (0.5), chloramphenicol (0.1), rose Bengal (5% sol., w/v) (0.5 mL), dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol (1.0 mL), agar (15). Final pH should be 5.6 (*see* **Note 6**).

*Oxytetracycline glucose yeast extract (OGYE) agar (g/L)*: Yeast extract (5), glucose (20), biotin (0.0001), oxytetracycline (0.1), agar (15). Final pH should be 7.0 ± 0.2 (*see* **Note 7**).

#### 2.3.4 Selective Media

*Dichloran 18% glycerol (DG18) agar (g/L)*: Peptone (5), glucose (10), potassium dihydrogen phosphate ($KH_2 PO_4$) (1), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) (0.5), dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol (1.0 mL), chloramphenicol (0.1), agar (15), glycerol (220). Final pH 5.6 ± 0.2 (*see* **Note 8**).

*Malt extract (MEA) agar with 40% glucose (g/L)*: Malt extract (12), yeast extract (3), glucose (400), agar (15). Final pH 5.5 ± 0.2 (*see* **Note 9**).

*Basal medium (MEA or TGY) supplemented with 0.5% acetic acid (g/L)*: Malt extract, powdered (20), glucose (20), peptone (1), agar (15). Final pH 3.8 ± 0.2.

*MYGP copper agar (g/L)*: Malt extract (3), yeast extract (3), glucose (10), peptone (5), agar (15), Tween 80 (10 mL), $CuSO_4$ 1.95% (w/v) aqueous stock solution. Final pH 6.2 ± 0.2.

#### 2.3.5 Differential Media

Chapter 11 shows some examples of differential culture media that allow characterizing different yeast colonies such as Lysine agar, Molybdate Agar, Wallerstein Laboratory Nutrient, and CHROMagar Candida (*see* **Notes 10**, **11**, and **12**).

*Dekkera/Brettanomyces differential medium (DBDM) agar (g/L)*: Yeast nitrogen base 6.7 g, ethanol 6% v/v, cycloheximide 0.01 g, *p*-coumaric acid 0.1 g, bromocresol green 0.022 g, agar 20 g. Distilled water 1 L. Final pH 5.4 ± 0.2.

## 3 Method

### 3.1 Diluents (See Note 13)

#### 3.1.1 Peptone Water (PW) (0.1% m/v)

1. Dissolve the peptone in the water.
2. Adjust pH 7.0 ± 0.2.
3. Sterilize at 121 °C for 15 min.

#### 3.1.2 Butterfield's Phosphate Buffer (PB) (0.1 M, pH 7.0)

1. Stock solution: Dissolve the monopotassium phosphate in 500 mL of water.
2. Adjust the pH to 7.2 with 1 N sodium hydroxide (NaOH) solution (about 175 mL).
3. Dilute the stock solution to 1 L.
4. Sterilize at 121 °C for 15 min.
5. Leave to cool at room temperature.
6. Store in refrigerator.

#### 3.1.3 Saline Solution (NaCl 0.85%)

Sterilize at 121 °C for 15 min.

### 3.2 Culture Media Preparation

#### 3.2.1 Basal Media

*Malt extract (MEA) agar, peptone yeast extract agar (YPD), Sabouraud-glucose agar (SGA), and Universal medium for yeasts (YM) agar*:

1. Mix ingredients steam to dissolve agar.
2. Sterilize for 15 min at 121 °C.

*Potato dextrose (PDA) agar*:

1. Boil 200 g sliced, unpeeled potatoes in 1 L distilled water for 30 min.
2. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
3. Mix in other ingredients and boil to dissolve.
4. Autoclave for 15 min at 121 °C.

*Tryptone-glucose-yeast extract (TGY) agar (g/L)*:

1. Sterilize at 121 °C for 10 min (prolonged heating will cause medium browning).

#### 3.2.2 Acidified Media

1. Adjusting the pH of the culture medium to 3.5 to 4-0 before pouring it into petri dishes.
2. Weigh the agar separately and dissolve it in 200 mL of distilled water.
3. Mix the other reagents in distilled water (350 mL) and adjust the pH.
4. Complete the item 3 volume with water to 800 mL.
5. Sterilize the two portions separately.
6. Join the two portions before pouring into the petri dishes.

#### 3.2.3 Biostatic Agents and Antibiotics

Chloramphenicol is heat stable and can be added with other ingredients before autoclaving, hence its use is more convenient.

Antibiotics

1. It is suggested the use of two different antibiotics, each one in 100 mg/L concentration.
2. Prepare chloramphenicol stock solution: 0.1 g chloramphenicol in 40 mL distilled water.
3. Add this solution to 960 mL medium mixture before autoclaving (*see* **Note 14**).
4. Prepare chlortetracycline stock solution: 0.5 g of the antibiotic in 100 mL distilled water.
5. Sterilize by sterile filtration.

6. Use 10 mL of this solution for each 990 mL of autoclaved, cooled to 40–50 °C medium.
7. Refrigerate in the dark and re-use remaining antibiotic stock solutions for up to a month.
8. Stock solutions should be brought to room temperature before adding to cooled medium [41].

Biostatic Agents

Stock solutions of the dyes are prepared using distilled water. Because of the relatively low solubility of some dyes in water, they can be first dissolved in a minimal amount of 95% ethanol and then made to volume with distilled water.

DRBC Agar

Sterilize by autoclaving at 115 °C for 15 min.

OGYE Agar

1. Sterilize by autoclaving at 115 °C for 15 min.
2. Cool to 50 °C.
3. Aseptically add 0.1 g of the antimicrobial agent, oxytetracycline, to the medium at 50 °C. Mix well.

#### 3.2.4 Selective Media

DG18 Agar

1. Mix the items and steam to dissolve agar.
2. Bring the volume to 1 L with distilled water.
3. Add 220 g glycerol (analytical reagent grade).
4. Sterilize by autoclaving at 121 °C for 15 min.

MEA Agar with 40% Glucose

1. Dissolve malt extract and agar in water by heating in a double saucepan.
2. Reduce heat to avoid caramelization and add glucose.
3. Stir until dissolved.
4. Sterilize by autoclaving at 115 °C for 15 min.

MEA or TGY Supplemented with 0.5% Acetic Acid

1. Sterilize by autoclaving at 115 °C for 15 min.
2. After cooling to 50 °C, add 5 mL glacial acetic acid per liter of medium.
3. Pour into plates immediately since the medium cannot be re-heated.

MYGP Copper Agar

1. Dissolve and mix all the reagents except $CuSO_4$.
2. Sterilize by autoclaving at 115 °C for 15 min.
3. Autoclave separately $CuSO_4$ 1.95% (w/v) aqueous stock solution.
4. Added $CuSO_4$ stock solution to this medium immediately before pouring the plates to give a final $CuSO_4$ concentration of 195 ppm.

#### 3.2.5 Differential Media

DBDM Agar

1. Suspend 20 g agar in 500 mL distilled water.
2. Sterilize by autoclaving at 115 °C for 15 min.
3. All components should be sterilized by membrane filtration.
4. Sterile filter solution through an 0.45-μm absolute membrane into a previously sterilized container.
5. Mix the sterile-filtered nutrients with the sterilized agar just prior to pouring the plates.

### 3.3 Samples Preparation (See Note 15) [42, 43]

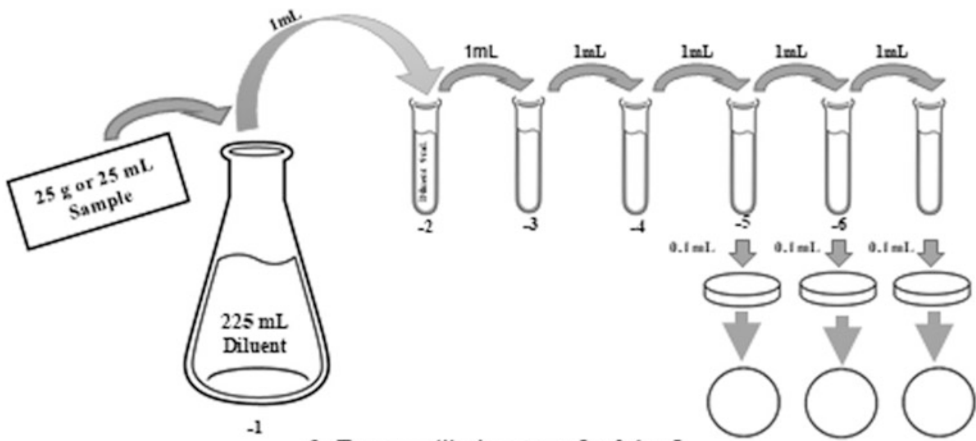
1. Homogenize the sample following the procedures described in Chapter 8 (*see* **Note 16**).
2. Aseptically remove the portions to be used for microbiological analysis.
3. Analytical units for general quantification of yeasts consists of 25 g or 25 mL of the sample, although alternative quantities can be used (10 g or 10 mL) (*see* **Note 17**).
4. To proceed with the analysis, the analytical unit must be diluted and homogenized with a suitable diluent, to allow inoculation into or onto culture media.
5. Liquid foods: transfer the analytical unit directly to tubes or flasks containing the amount of diluent necessary for a 1:10 dilution.
6. Solid or concentrated liquid foods:
   - (a) Transfer the analytical unit to a sterile homogenization flask or bag.
   - (b) Add to the sample the amount of diluent necessary to obtain a 1:10 dilution.
   - (c) Homogenize the analytical unit with the diluent (*see* **Note 18**).

### 3.4 Serial Dilutions

After homogenization, prepare a series of decimal dilutions of the sample based on the estimated concentration of yeast in the sample [12, 43].

1. The primary homogenate of a food sample is generally prepared in a 1:10 ratio ($10^{-1}$), obtained by adding $m$ grams or milliliters of the sample to $9 \times m$ (milliliters) of diluent (Fig. 1).
2. From the initial dilution ($10^{-1}$), make the other desired dilutions.
3. Select dilutions for the spread plate method so that the total number of colonies on the plate will be between 15 and 150 (*see* Subheading 3.6).

**1**. Make a 10-fold dilution series:

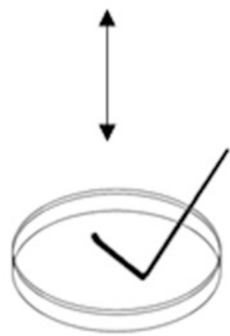



**2**. For one dilution, transfer 0.1 mL os suspension to each plate. After inoculating all replicate plates in one dilution, go to **3**. Repeat for next two dilutions.

**3.** For each plate, sterilize a spreader in a flame after dipping it in etanol. Let the spreader cool briefly. Go to **4**.

**4.** Briefly touch the spreader to the agar of na inoculated plate to cool, away from the inoculum. Then, spread the inoculum by moving the spreader in na arc on the surface of the agar while rotating the plate.

Continue until the inoculum has been absorbed into the other agar. Repeat **3** and **4** for the other replicates. Then, go to **5**.




**5**. Repeat steps 2, 3 and 4 for each dilution. When done, let the agar dry for a few minutes, tape the plates together, and incubate.

**Fig. 1** Spread plate technique procedure for yeast enumeration

### 3.5 Enumeration [43, 44]

The basic procedure in the spreading technique consists of inoculating (0.1 mL) of the homogenized sample (and its dilutions) on to the agar plates already solidified and spread inoculum with a sterile glass or plastic spreader (Drigalski), followed by incubation of the plates until visible growth occurs (Fig. 1) (*see* **Note 19**).

1. Select three or more dilutions of the sample to be inoculated.
2. Using a pipette with a maximum holding capacity of 1 mL (and 0.1 mL graduation markings).
3. Inoculate 0.1 mL of each dilution onto the surface of previously prepared plates.
4. Verify whether the identification of the plate corresponds to the sample and dilution that are being inoculated and whether the plate contains the correct culture medium.
5. Work in a laminar flow cabinet or in the proximity of the flame of a Bunsen burner.
6. Carefully spread the inoculum onto the entire surface of the medium as fast as possible, using glass or plastic spreader (Drigalski), and continue until all excess liquid is absorbed.
7. Utilize a different spreader for each plate or, alternatively, flame sterilize the spreader after each plate, starting with the greatest dilution plate and going to the smallest dilution plates.
8. Let the agar dry for a few minutes, put the plates together.
9. Incubate them upside down. Incubate them inverted.
10. Let plates remain undisturbed until counting.

### 3.6 Incubation (See Notes 20–22) [4]

The incubation temperature for yeast enumeration varies from 25 to 28 °C, when the growth can be observed between 24 h and 120 h. For enumeration of mesophilic yeasts, it is recommended to incubate the plates at 28 °C for 72 h using basal media. The time × temperature relationship of incubation must also be considered according to the origin of the sample. Whether these samples are cold or chilled, colony formation should be evaluated at 15 °C for 7–10 days of incubation. Selective and differential media (Table 1) can delay the appearance of colonies, and growth should then be observed for 48 h.

### 3.7 Counting the Colonies and Calculating the Results [12]

1. At the end of the incubation period, examine plates for uniformity of colonies and lack of contamination.
2. For counting the colonies and calculating the results, select plates with 15–150 colonies (*see* **Note 23**).
3. Calculate the number of cells per g or mL of original culture (CFU/g or CFU/mL) using the Eq. 1.

$$\frac{\text{CFU}}{\text{g}} \text{or} \frac{\text{CFU}}{\text{mL}} = n \times \frac{1}{\text{sample volume}} \times \text{D.F.} \quad (1)$$

where.

*n*: average of colonies in the plate's replicates for the same dilution factor.

*sample volume*: the volume, in mL, taken from the respective dilution to spread plating.

*D.F.*: dilution factor is the reciprocal of the chosen dilution used to count the colonies number.

4. As an example, the number of colonies resulting from a triplicate of a $10^{-6}$ dilution is:

Plate 1: 30 colonies.

Plate 2: 28 colonies.

Plate 3: 32 colonies.

$n = 30$ colonies.

These arose from 0.1 mL of a $10^{-6}$ dilution, so D.F. is $\frac{1}{10^{-6}} = 10^6$.

$$\frac{\text{CFU}}{\text{g}} \text{or} \frac{\text{CFU}}{\text{mL}} = n \times \frac{1}{\text{sample volume}} \times \text{D.F.} = 30 \times \frac{1}{0.1} \times 10^6$$
$$= 3.0 \times 10^7$$

### 3.8 Morphological Characterization [15, 45]

Due to morphological differences between the colonies of yeast species, the following factors should be included in a comprehensive colony description (Fig. 2).

Shape: Circular or irregular.

Size: Large (5 mm), moderate (2–5 mm), or small (2 mm).

Surface: Whether glistening or dull. Concentric, radial tripes, radial valleys, or granulated.

Edges: Plain, filiform, undulate, folded, serrated, lobate, or filamentous.

Side view: Convex, umbonate, crateriform, crateriform folded on center, convex and folded, concave, pseudomycellium, folded and flattened, flat or raised. Figure 3 shows colonies of some yeast species in YPD agar.

### 3.9 Purification and Maintenance of Yeast Culture [15, 45]

After the yeast enumeration process, it is necessary to purify and keep cells in pure culture before and after confirming your identity. Cultures must be pure for sequence analysis, growth tests and morphological examination.

1. For purification, streak the selected colonies onto the surface of suitable medium such as glucose-peptone-yeast extract agar or YM agar.

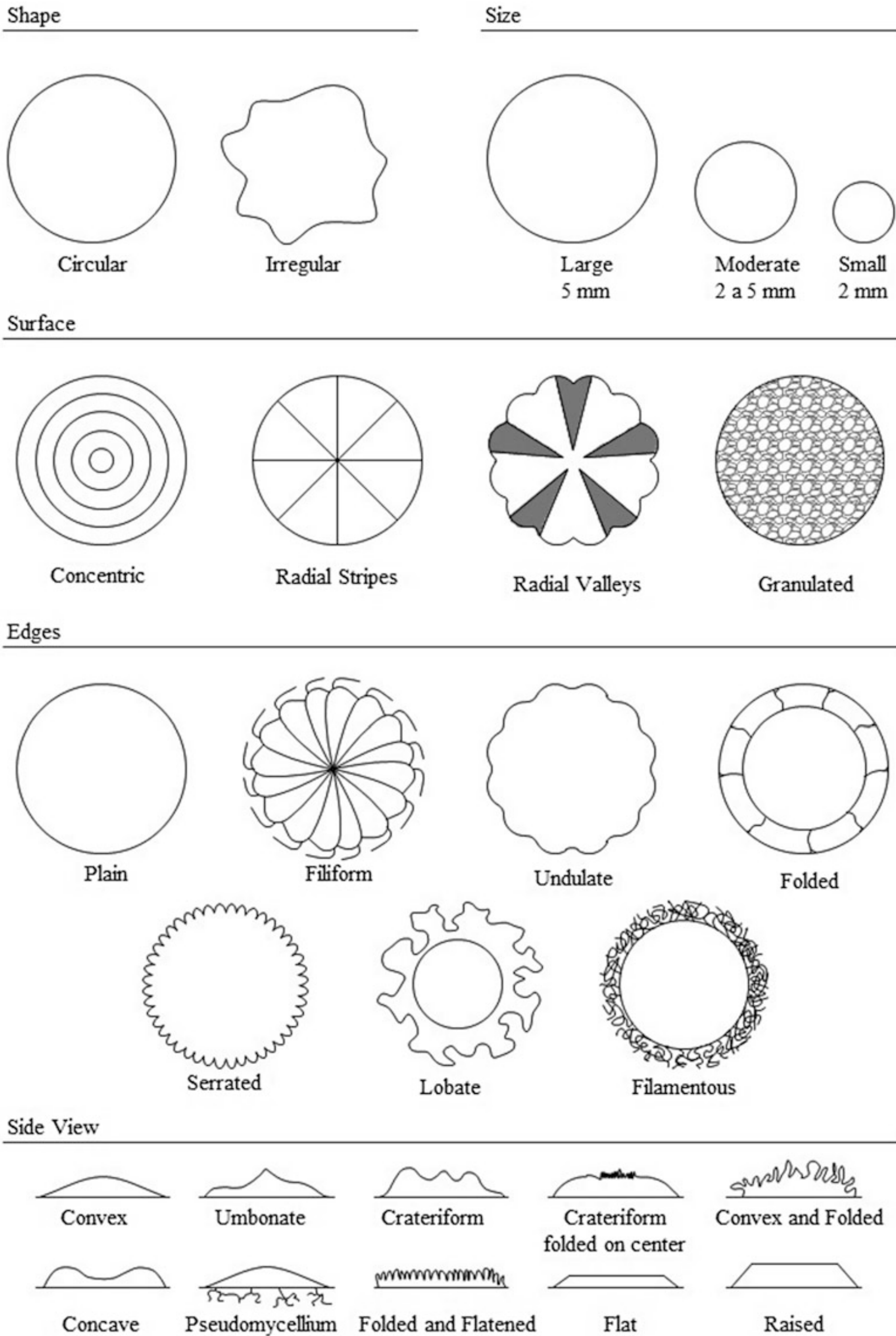



**Fig. 2** Main morphologies of yeast colonies

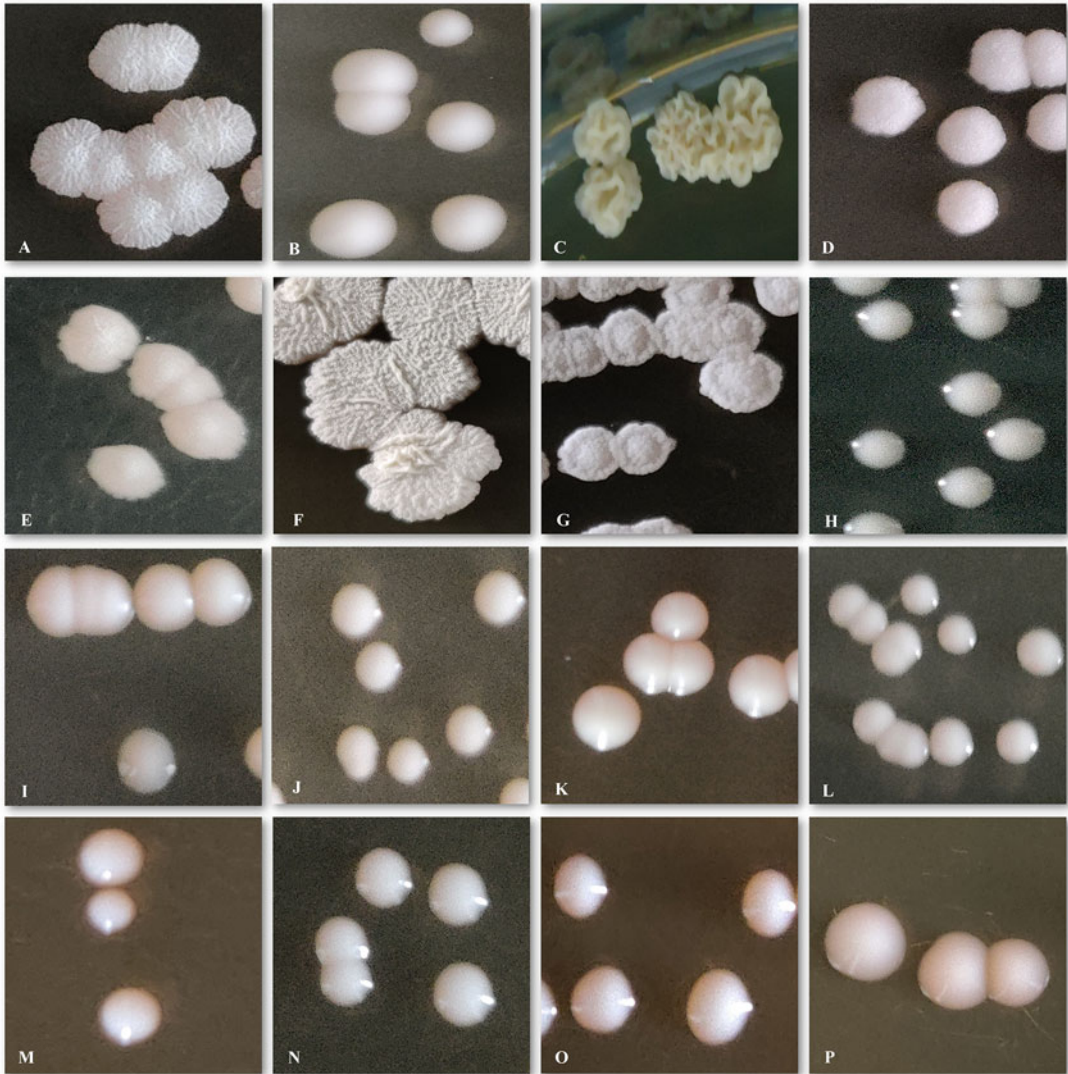



**Fig. 3** Morphology of colonies from different yeasts grown in YPD agar. (**a**) *Wickerhamomyces anomalus*; (**b**) *Kazachstania exigua*; (**c**) *Kodamaea ohmeri*; (**d**) *Yarrowia lipolytica*; (**e**) *Candida rugosa*; (**f**) *Schwanniomyces vanrijiae*; (**g**) *Candida ethanolica*; (**h**) *Pichia guilliermondii*; (**i**) *Saturnispora* sp.; (**j**) *Cyberlindnera saturnus*; (**k**) *Candida tropicalis*; (**l**) *Candida parapsilosis*; (**m**) *Schizosaccharomyces pombe*; (**n**) *Torulaspora delbrueckii*; (**o**) *Saccharomyces cerevisiae*; (**p**) *Hanseniaspora uvarum*

2. Incubate the plates at 28 °C for 24 ± 3 h.
3. Single, well-separated colonies of each form are selected and streaked again; twice is generally sufficient to obtain pure cultures, but it may be necessary to streak colonies several times.

Considering the completed isolation and purification steps, it becomes necessary to store the pure cultures. The maintenance of cultures can be performed in solid medium, liquid medium, freezing, or lyophilization (*see* Chapter 9 for more details).

## 4 Notes

1. It is important to use a diluent containing enough solute to minimize osmotic shock to fungal cells in high-sugar or high-salt foods when serial dilutions are made prior to plating.
2. In some cases, to favor the separation of cell aggregates, the use of surfactant agents such as polysorbate (Tween 80) (0.05% v/v) is recommended.
3. Heat the mixture in suitable containers (borosilicate glass or stainless steel) until ingredients are in solution and the agar is melted completely. The volume of agar and the type of container used should be such that no part of the contents will be more than 2.5 cm from the glass or from the surface of the agar.
4. Like most yeasts, they give strong growth under acid conditions (pH 3.0–7.0), with limiting values being around pH 1.5–2.5 and pH 8.0–8.5, at least for *Saccharomyces cerevisiae*.
5. An appropriate amount of each dye solution is added preferably to MEA before autoclaving. The final dye concentrations in culture media ranged from 0.25 to 5.000 ppm (0.25–5.000 μg/mL) depending on the dye assayed.
6. The advantage of this medium is that the spreading growth of mold colonies is restricted, allowing more accurate colony counts on crowded plates. Plates with this medium must be incubated in the dark to prevent formation of photo-induced inhibitors. However, some yeast and mold strains may be inhibited completely by rose Bengal if the medium is exposed to light. Stock solutions of rose Bengal and dichloran do not require sterilization and are stable for long periods. Caution: Chloramphenicol is toxic; skin contact should be avoided. To examine foods with a water activity greater than 0.95.
7. OGYE agar: The medium loses its bacteriostatic effect if incubated at temperatures greater than 25 °C.
8. Ideal media for foods with a water activity less than or equal to 0.95. In addition to chloramphenicol and dicloran, also contains glycerol, which reduces the water activity of the medium.
9. Some species are osmophilic (e.g., *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus, Zygosaccharomyces rouxii*), diagnostic plating can include use of a high osmotic medium supplemented with 40% glucose. If these media are used, the sample diluent should be high osmotic as well, e.g., 0.1% peptone water with 40% glucose.
10. Lysine agar is a particularly useful medium to enumerate non--*Saccharomyces* yeasts when they are present in foods or beverages along with *Saccharomyces* species. It exploits the fact that

most *Saccharomyces* species cannot utilize lysine as a nitrogen source and will not form colonies on this medium. However, some *Saccharomyces* (e.g., *S. unisporus*, *S. kluyveri*), occasionally found in food ecosystems, can utilize lysine, and grow on this agar [33]. Generally, agar plating media are incubated at 25–30 °C for 2–7 days, after which colonies are examined.

11. Reliable counts for brewers' yeast are obtained with the medium at pH 5.5. Adjustment to pH 6.5 facilitates the counting of bakers' and distillers' yeasts. The time and temperature of incubation will vary according to the materials tested and the organisms sought. Temperatures of 25 °C are used for brewing materials and 30 °C for baker's yeasts.
12. Chromogenic medium must be stored in the dark.
13. Late plating of diluted samples should be avoided. Depending on the diluent used and the yeast in the diluted sample, the number of viable cells can be reduced by up to 30% of the initial population after a period of contact between the sample and the diluent.
14. When both chloramphenicol and chlortetracycline are used, add 20 mL of the above chloramphenicol stock solution to 970 mL medium before autoclaving.
15. The analytical unit is the amount of material withdrawn from a sample to be subjected to one or more tests. The number of analytical units that should be withdrawn and the amount of material of each analytical unit depend on the number and types of tests that will be performed on the same sample.
16. Withdrawing the analytical unit(s), the content of the sample should be well homogenized to ensure that the portion to be removed will be representative for the material as a whole.
17. Larger sample sizes increase reproducibility and lower variance compared with small samples.
18. Homogenization can be achieved by manual agitation, shaking the flask in an inverted position 25 times (concentrated liquids, soluble powders), agitation in a peristaltic homogenizer (Stomacher®) for 1–2 min (soft foods, pasty foods, ground or minced foods, poorly soluble powders) or in a blender (hard foods).
19. Spread plates, rather than pour plates, are recommended for both isolation and enumeration because the increased aeration of surface growth favors recovery and subsequent growth of the yeast cells.
20. In the absence of laboratory incubator, room temperature may be an alternative, however the required time should be extended to 7 days.

21. Incubation at 30 °C for 48–72 h for Lysine agar, WL Agar, molybdate agar, and at 37 °C for 48 h in CHROMagar Candida. Colonies are counted after 12 days in DBDM agar incubation at 25 °C.
22. Psychrophilic species have been isolated from refrigerated condiments, so isolation plates of these food products may need to be incubated at 5–10 °C for several weeks if low-temperature spoilage is occurring.
23. If all plates from dilutions tested show no colonies, report the CFU/g or CFU/mL as <1 times the lowest dilution.

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