# Chapter 8

# Preparing Yeast Suspension Through Serial Dilution for Enumeration


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## Abstract

Yeasts are essential to obtain a variety of fermented foods and beverages, including bread, cheese, wine, and others. This group of microorganisms may also be associated with food spoilage. To better understand and control the food quality, yeast enumeration is an important step. However, yeasts may be present in a high population, which is challenging to perform direct counting from samples. Thus, serial dilution should be performed before yeast enumeration. This method consists of a consecutive ten times dilution of food sample to reach a value of colonies that can be easily counted in the plates.



**Key words** Foodborne yeasts, Food associated yeasts, Diluent solutions, Ten times dilution, Yeast enumeration


## 1 Introduction

The large and diverse group of food-associated yeasts includes several hundred species. They are an essential agent acting favorably in fermented foods and beverages. However, these microorganisms can attack many foods due to their relatively varied environmental requirements. Although most yeasts are aerobes, they are tolerant of a wide range of pH, ranging from pH 2 to above pH 9. Furthermore, their growth temperature range (10–35 °C) is also vast. Only some species can grow below or above this range. Regarding moisture, foodborne molds require relatively low values. In general, most species can grow at a water activity (aw) of 0.85 or less, although yeasts generally require a higher water activity.

Yeasts may grow in food and cause deterioration and decomposition. They can spoilage any type of food and crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow in processed foods and food mixtures. The dilution plating method is commonly used to detect spoilage yeasts in foods [1–4].


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Quantitative assessment of microorganisms can be challenging due to their abundance, exponential proliferation capacity, species diversity within a population, and specific physiological needs. Further, the four-phase nature in which bacteria and yeast replicate (lag, log, stationary, and death) increases this challenge. An accurate estimation of the microbial population is essential for successful identification, isolation, cultivation, and characterization [1–3, 5]. Hence, microbiologists have employed serial dilution and various plating methods for over a century to quantify bacterial reliably and yeast load in clinical, industrial, pharmaceutical, and academic laboratory environments. This methodology was first described in 1883 by the German scientist and physician Robert Koch, who published his work on infectious disease-causing agents. Known as the father of modern bacteriology, Kock and the techniques first described by him have become a standard for microbial enumeration and cultivation worldwide [6].

The isolation, enumeration, and identification of yeasts from foods and beverages follow the same principles and strategies that are used for yeasts in general. These involve sequential operations of the sample's rinsing or maceration, dilution, and enumeration of yeast cells. Estimation of yeast population may be performed by agar plating, most probable number, membrane filtration, microscopic, or electronic methods. Then, the isolates are purified and identified to genus, species, or strain level [1, 4].

Procedures of homogenization for yeast isolation may consist of manually shaking or mixing the sample with a known volume of diluent using a blender, orbital shaker, or peristaltic agitator (Stomacher), after grinding if necessary. Diluents commonly used comprise distilled water, saline, phosphate buffer, and the most common 0.1% (w/v) peptone water. Contact time ranges from less than 1 to several minutes (generally 5–10 min). Based on the assumption that the separation of yeast cells from natural habitats requires much rougher treatment of samples, a series of ecological surveys were conducted on the yeast population of different fruits. The overall results indicate that pre-isolation treatments based on vigorous shaking, percolation with an excess of water, and the sonication of samples, allowed the recovery of a higher number of colonies forming units and species [1]. Furthermore, as Fleet [7] stated, "the assumption that maceration is an ecologically sound prelude to microbiological analysis requires more rigorous scrutiny, especially since it is already known that extracts of vegetables, herbs, and spices are toxic to some microorganisms."

Sterile peptone water (0.1% w/v) is the recommended diluent for preparing samples to be plated on general-purpose enumeration media [8]. However, given the diversity of intrinsic food characteristics and yeast biodiversity, there is no ideal diluent. It is always necessary to consider the nature of the food, and the species sought to select the diluent. Furthermore, it should not be forgotten that

the most crucial objective is to recover spoilage yeasts. Although it is necessary to standardize this operation, it is recognized by the International Commission on Food Mycology that specific protocols are not yet available, either concerning the type of food or sample contact time [1], which makes it difficult to compare results from different laboratories. According to Fleet [7], based on an international collaborative study under the auspices of the abovementioned Commission, it is possible to conclude that apart from diluent composition and timing between dilution and plating, other factors such as the stage of the cell life cycle, cell stress before dilution, degree of cell clumping and aggregation, shear forces during shaking, presence of contaminating metal ions, pH, and temperature could all have an impact on the survival of the yeast cells during a dilution.

## 2 Materials

1. Prepare the diluents: Peptone water 0.1% (w/v), or saline solution (0.85% NaCl w/v), or peptone salt solution (0.85% NaCl w/v and 0.1% w/v) (*see* **Note 1**).
2. Food sample.
3. Instruments for homogenization such as wrist-action shaker, peristaltic agitator (e.g., Stomacher), magnetic stirrer, orbital shaker, or a blender (*see* **Note 2**).
4. Prepare sterilized tubes containing 9 mL of diluent solution and pipettes for serial dilution (*see* **Note 3**).

## 3 Methods

1. Dilute the food sample 10× in diluent solution (e.g., saline solution or peptone water or peptone salt solution) (*see* **Note 1**): 25 g (solid sample) or 25 mL (liquid sample) and add to the flask containing 225 mL of diluent solution (*see* **Note 4**). Consider this step as the first dilution ($10^{-1}$).
2. Homogenize the samples for 1–5 min by using, e.g., wrist-action shaker, peristaltic agitator, magnetic stirrer, orbital shaker, or a blender (*see* **Note 2**).
3. Transfer aseptically 1 mL from the food sample ($10^{-1}$) into a tube containing 9 mL of diluent solution. In this tube, the sample is diluted 100× ($10^{-2}$) (*see* **Note 3**).
4. Homogenize suspension in the tube and, aseptically, transfer 1 mL of the homogenized sample to a novel tube containing 9 mL of diluent solution. This tube contains sample diluted at 1000× ($10^{-3}$).

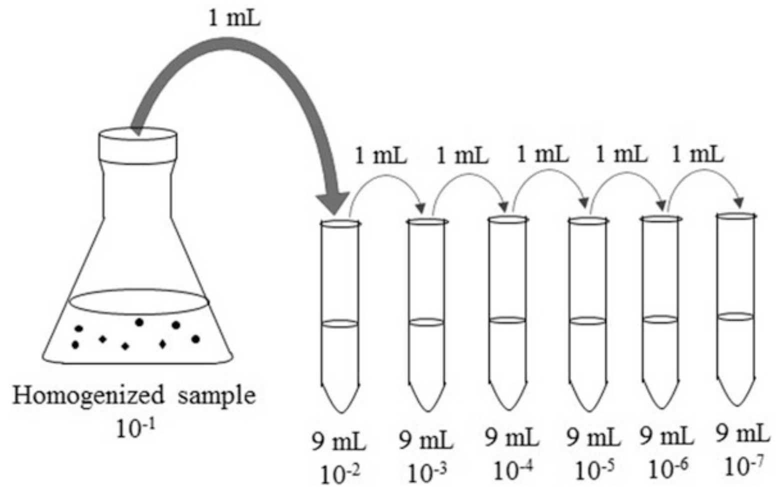


**Fig. 1** Diagram demonstrating serial dilution of food samples for yeast enumeration

5. Repeat **step 2** many times as necessary, depending on the food sample and yeast population present in the samples. Generally, samples diluted until $10^{-7}$ are satisfactory. Figure 1 illustrates the serial dilution preparation.
6. Diluted samples are ready to be spread onto the plates for enumeration of yeast populations. Chapter 10 describes the different methods for yeast enumerations in foods.

## 4 Notes

1. Several diluents may be employed for the enumeration of yeasts in foods. Generally, distilled water is not recommended due to osmotic shock effects. Saline solution (0.85% w/v NaCl), peptone water 0.1% (w/v), or a combination of peptone salt solution 0.85% NaCl (w/v) and 0.1% (w/v) are commonly recommended for analysis of foods. Sterile milk may be used as diluents in the analysis of dairy products. Diluents with a lower water activity (by adding 20–30% of glucose, or 18–26% of glycerol) may be employed to enumerate osmotolerant yeast, such as *Zygosaccharomyces rouxii*. Surfactants such as Tween 80 (0.01–0.05%) may be included in the diluent to enhance cell clumps separation. Furthermore, commercial standard diluents are available and may be used.
2. Low-tech methods of homogenization have also been reported as the use of sterile gloves to hand-squeeze aseptically collected grapes for *S. cerevisiae* isolation [9].

3. Ten dilutions may also be applied using different volume-scale such as adding 100 μL of samples into microtubes containing 900 μL of diluent solution.
4. In case of keeping samples to be further analyzed, it is essential to add 20% glycerol in the diluent and store the samples at −20 °C to maintain microbiota viability.

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