Chapter 9

Standardizing Suspension of Yeast for Inoculation in Food Fermentations

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Abstract

The standardization of the inoculum is essential for the success of all fermentative process and depends mainly on maximizing the activity of the microorganism to improve the productivity and final yield. Different methods can be used to estimate and standardize the inoculum, such as the growth curve method, in which the combination of absorbance measurements and viable cell count or dry weight analyses reflect the microbial population in the sample. Another method is the measurement of cell density, in which the McFarland equivalence turbidity standards are compared with the inoculum turbidimetry with a standard scale that estimates the inoculum concentration. This chapter describes methods of standardization and preservation of the microbial inoculum.

Key words Inoculum, Standardization, Fermentation process, Growth curve, McFarland

1 Introduction

The use of yeasts in the production of food and beverages improved over the years. Since the twentieth century, the utilization of yeast has become a standard practice in industrial fermentation [1]. The inoculum is defined as a suspension of microorganisms sufficiently concentrate that is added to start the fermentation itself. In industrial fermentation applications, the quality and quantity of inoculum play an essential role in achieving the fermentation rate and final product yield. For most fermentations, the inoculum volume, taking into account a freshly prepared cell culture, is typically between 3% and 10% of the total volume of the substrate to be fermented [2].

The inoculum preparation starts standardizing cell suspension and, once the desired cell concentration is reached, the inoculum must be added to the bioreactor. The inoculum concentration

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depends on the desired metabolites produced by yeast, the available concentration of nutrients for growth, growth conditions (temperature, pH, oxygen availability), and what is the expectation of yield and productivity [3].

Prior knowledge of the growth rate of the microorganism is essential for different fermentation processes. In general, before inoculation, it is necessary to know the microorganism growth curve, which is characterized by three main phases: the lag, exponential, and stationary phases [4]. These phases can be determined through its growth curve that allows estimating the density at which point a cell culture should be before being transferred to a new medium. Therefore, the build of a growth curve is a method used to standardize the inoculum for food, which can be performed by combining methods that indirectly quantify cell concentration, such as measuring absorbance, and methods with direct quantification, such as viable cell counting and dry weight [5, 6].

The standardization of yeast suspension for inoculation in food can be performed by cell density as well. McFarland Equivalence Turbidity Standards can be used to approximate the concentration of cells in a suspension visually. For visual comparison, the turbidity of the pattern and the yeast suspension must have the same dispersion in the light, so that the approximate yeast population in the cell suspension is calculated [7]. In specific fermentative processes, the inoculum standardization can be carried out with the help of the Neubauer chamber, counting the number of yeast cells per milliliter (*see* Chapter 11).

Yeast cell cultures are generally easy to store, maintain, and cultivate, resulting in inoculum with a large number of cells. Microorganisms used in industrial processes must be adequately preserved as a pure culture. Through different techniques, it is possible to maintain all the characteristics of the microbial cell and, thus, whenever new production is started, the quality of the final product is also kept. Therefore, inoculum preservation methods are essential for laboratories, industrial applications, and biotechnology and related areas [8].

This chapter includes methods for standardizing yeast cell suspension for inoculation in foods, such as growth curve employing cell absorbance [9] and cell dry weight [10, 11], McFarland equivalence turbidity [12], and methods for inoculum preservation, such as deep freezing at −70 °C [13, 14], lyophilization [15, 16], and refrigerated storage [17].

2 Materials

2.1 Growth Curve

1. Sterile Erlenmeyer.
2. Pipettes.
3. Shaking incubator.

4. Sterile tubes.
5. Laboratory incubator with the temperature set at the temperature specified by the test to be performed.
6. pH meter.

2.1.1 Culture Media

YEPD (yeast extract peptone dextrose) Broth (g/L): Yeast extract (10), peptone (20), glucose (20).

Malt Extract Broth (g/L): Malt extract (17), mycological peptone (3), final pH 5.4 ± 0.2.

Sabouraud Dextrose Broth (g/L): Mycological peptone (10), dextrose (20), final pH 5.6 ± 0.2.

Potato Dextrose Broth (g/L): Potatoes, infusion from (200), dextrose (20), final pH 5.1 ± 0.2.

2.1.2 Spectrophotometer

1. Necessary equipment and appropriate techniques for preparation of sample and dilution (*see* Chapter 8).
2. Equipment for plating samples (*see* Chapter 10).
3. Spectrophotometer.
4. Pipettes.
5. Cuvette.

2.1.3 Dry Weight

1. Centrifuge.
2. Vacuum oven at 60 °C.
3. Desiccator.
4. Analytical balance.
5. Sterile tubes.

2.2 McFarland Equivalence Turbidity

1. Equipment for plating samples (*see* Chapter 10).
2. Loop sterilization device.
3. Inoculating loop, swabs, or transfer pipettes.
4. Sterile tube.
5. Saline or broth.
6. Vortex mixer.
7. Light source.

2.3 Preservation of Inocula

1. Eppendorf or similar tubes.
2. Glycerol (80%).
3. Freezer at −70 °C.

2.3.1 Freezer Freezing at −70 °C

2.3.2 Lyophilization

1. Skimmed milk (or another cryoprotectant).
2. Ampoules borosilicate glass.
3. Sterile Pasteur pipettes.
4. Blowtorch.
5. Freezer at −70 °C.
6. Freeze dryer.

2.3.3 Refrigerated Storage

1. Culture media with agar (add 20 g/L).
2. Sterile tubes.
3. Inoculating loop.
4. Mineral oil.
5. Sterile distilled water.

3 Methods

3.1 Growth Curve

1. Prepare the broth for yeast growth (300 mL) (*see* **Note 1**).
2. Inoculate 5 mL of broth media in sterile tubes.
3. Add stock yeast cultures (*see* **Note 2**).
4. Place in a shaking incubator.
5. Incubate overnight at 30 °C.
6. Add the 200 mL of broth media in a sterile Erlenmeyer.
7. Inoculate 1% (v/v) of yeast pre-inoculum (*see* **Note 3**).
8. Incubate at 30 °C.

3.1.1 Spectrophotometer

1. Collected, aseptically in laminar flow cabinet, 2 mL of culture broth for absorbance readings and plating; at pre-established time intervals (*see* **Note 4**).
2. Place 1 mL of the culture broth in the Cuvette (*see* **Note 5**).
3. Perform absorbance readings (Abs) on a spectrophotometer (*see* **Note 6**).
4. Make the respective dilutions of the yeast suspension (*see* Chapter 8) (*see* **Note 7**).
5. Perform plating using the spreading technique (*see* Chapter 10).
6. The readings and plating will be taken until the yeast growth reaches the stationary phase (*see* **Note 8**).
7. Incubation of 30 °C.
8. Perform logarithmic transformations for values of viable cell count (*see* **Note 9**).

9. Make a linear regression curve, plot log CFU/mL versus time (h).
10. From the graph, identify the exponential phase of growth. Using two-time points within the exponential phase of growth and corresponding cell numbers.
11. Calculate the mean generation time from the equation:

$$X = 2^n \times X_0$$

where.
X_o = initial concentration of cells.
X = concentration of cells after time t.
n = number of generations.

12. Standardize the inoculum according to the need.

3.1.2 Dry Weight

1. Collected, aseptically in laminar flow cabinet, 10 mL of culture broth for absorbance readings and dry weight analyses; at pre-established time intervals (*see* **Note 10**).
2. Centrifuge yeast suspension at 10,000 × *g* for 15 min.
3. Discard the supernatant (*see* **Note 11**).
4. Dry the cells in a vacuum oven at 60 °C for 24 h (*see* **Note 12**).
5. Place in a desiccator for 30 min.
6. Weigh on an analytical scale.
7. Construct a calibration curve, plotting the absorbance values representing the corresponding cell density and dry weight in a graph, obtaining a linear regression equation (*see* **Note 13**), this equation was used to estimate the cell mass to be used as an inoculum.

3.2 McFarland Equivalence Turbidity

1. Plating the inoculum using the spreading technique (*see* Chapter 10).
2. Incubation for 24 h at 30 °C.
3. Sterilize the inoculating loop (*see* **Note 14**).
4. Prepare the inoculum by suspending five distinct colonies, ±1 mm in diameter in 5 mL of sterile distilled water (*see* **Note 15**).
5. Evenly suspend the inoculum on a vortex mixer for 15 s.
6. Invert the McFarland Equivalence Turbidity Standard gently to suspend the polystyrene microparticles entirely.
7. Visually compare the turbidity of an actively growing broth culture or a yeast suspension prepared from an 18–24 h culture to the appropriate McFarland Standard.

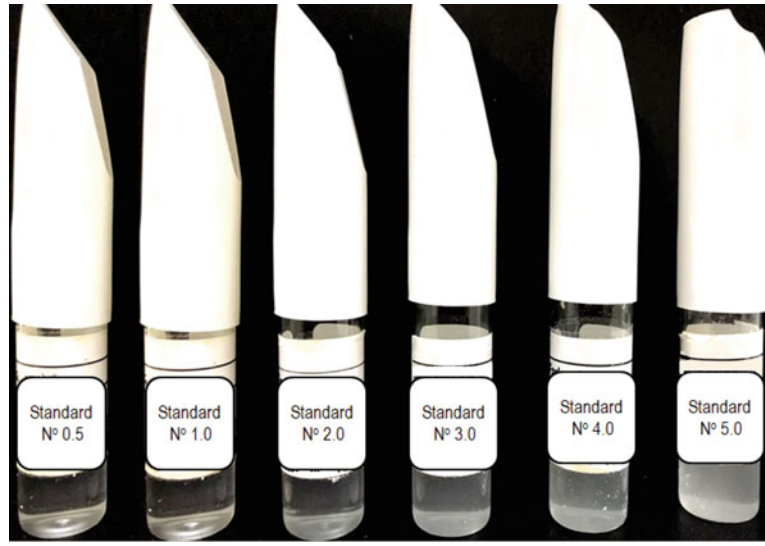

Fig. 1 Standard McFarland scale. Standard No. 0.5: Approximate Cell Density 1.5×10^8/mL; No. 1.0: Approximately 3.0×10^8/mL; No. 2.0: Approximately 6.0×10^8/mL; No. 3.0: Approximately 9.0×10^8/mL; No. 4.0: Approximately 1.2×10^9/mL; No. 5.0: Approximately 1.5×10^9/mL

8. For visual comparison, use adequate light or read the tubes against the white card with contrasting black lines (*see* **Note 16**).
9. Equal obliteration or distortion indicates a turbidity match (Fig. 1).
10. After standardization, use the yeast suspension (*see* Subheading 4) to inoculate in the food (*see* **Note 17**).

3.3 Preservation of Inoculum

3.3.1 Freezer Freezing at −70 °C

1. Cultivate samples in culture broth (*see* Subheading 2.1.1) (*see* **Note 1**).
2. Incubate with shaking at 30 °C overnight.
3. Add 0.8 mL of the yeast cultures in Eppendorf or similar tubes (*see* **Note 18**).
4. Add 0.2 mL of 80% glycerol (*see* **Note 19**).
5. Place the tubes in a freezer at −70 °C (*see* **Note 20**).

3.3.2 Lyophilization

1. Cultivate samples in medium broth (*see* Subheading 2.1.1) (*see* **Note 1**).
2. Incubate with shaking at 30 °C for 48 h.
3. Mix equal volumes of the inoculum in culture medium and skimmed milk (*see* **Note 21**).
4. Using sterile Pasteur pipettes, inoculate about six drops (0.2 mL) of the suspension into the ampoules.

5. Constrict the ampoule with the aid of a torch, to facilitate vacuum closure after freeze-drying.
6. Freeze samples at −70 °C freezer overnight.
7. Lyophilize the samples in the freeze dryer for 6 h (*see* **Note 22**).
8. Vacuum seal with a blowtorch.
9. Remove the ampoules from the freeze dryer and store (*see* **Note 23**).

3.3.3 Refrigerated Storage

1. Pour the culture media agar into sterile petri dishes and slanted tubes.
2. With inoculating loop, spread the sample in the slanted tubes and petri dishes.
3. Incubation at 30 °C for 48 h.
4. For maintenance on agar, store at refrigeration temperature (4–8 °C).
5. For maintenance in mineral oil, submerge the slant agar surface in mineral oil, store at refrigeration temperature (4–8 °C).
6. For storage in distilled water, with the inoculation loop, remove 10 colonies of yeasts from petri dishes and suspend in sterile distilled water, seal, and store (*see* **Note 24**).

4 Notes

1. For all the media mentioned in Subheading 2.1.1, add the components to sterile distilled or deionized water and autoclave 15 min at 121 °C, adjust the pH. If you are going to use another media check how to make and sterilize.
2. The yeasts can be freeze-dried, frozen, dried, or another way (*see* Subheading 3.3). Therefore, the inoculated quantity can be in g or mL. In this stage, the inoculated quantity is not standardized; the reactivation of the inoculum to be viable in the construction of the growth curve is important. Be careful about the possibility of using inoculum of improper age.
3. The initial concentration of inoculum should be similar to the study of the growth of yeasts in culture medium with the reality that occurs in food, which has a low initial microbial load.
4. Sample collection intervals depend on the concentration of the inoculum, usually in the first hours, in which the microorganism is in the lag phase of growth, samples are taken over a longer time, as the absorption increases gradually and the microorganism enters the log phase, samples are taken in short periods (15–30 min, for example), to have several points for the construction of the growth curve.

5. The glass cuvette and quartz cuvette are indicated for the analysis of liquid samples, and the glass cuvettes are indicated for when working in a visible region, while the quartz cuvettes are for the ultraviolet region with wavelength below 340 nm. Disposable plastic cuvettes are often used in rapid tests where speed is more important than high accuracy, but with the inconvenience of being used only once.
6. The wavelength depends on the color of the culture medium broth used, usually for media yellow use 580–595 nm, orange use 595–650 nm, generally for yeast suspensions use 540, 600, or 640 nm.
7. Dilutions are essential to be able to correlate the count of cell numbers with the absorbance, at the beginning of the growth curve, few dilutions are necessary; however, when yeast enters the exponential phase, the number of dilutions must always increase, to obtain plates with the number of countable colonies.
8. The stationary phase is reached when the absorbance readings on the spectrophotometer start to have constant values, with small variations.
9. Logarithmic transformations are done to mitigate the variation between the analyzed data and equalize the differences between the data.
10. Relatively large samples are needed for the measurements to be meaningful. This means that it is not possible to follow the growth of a microbial population from its initial masses, being necessary that the mass reaches a critical level.
11. Washing the cells before drying can cause loss of material.
12. The cell suspension pellet, free of supernatant, should be placed in a container of known weight, and weighing should be done until there is no change in weight (constant).
13. The yeast suspension tubes should be of similar diameter as the McFarland Equivalence Turbidity Standard.
14. Sterilize the entire wire tip by passing it at an angle through the flame of a gas burner until the entire length of the wire becomes orange from the heat to ensure absolute sterilization, including the shaft to remove any dust or possible contaminants. Cool the wire tip in the petri dish lid before obtaining the inoculum or touch the middle edge of the dish to avoid killing the cells and spreading the culture.
15. Touch only a single growth area with the inoculation wire to obtain the inoculum. Never drag the loop or needle across the surface and be careful not to dig into the solid medium.
16. Using instruments which use alternative light sources, such as scattered light, has not been validated.

17. The minimum inoculum concentration must be sufficient for the satisfactory development of the fermentation process of the target food.
18. Use screw-capped tubes with a volume of 1.5 mL or more, which are suitable to stock yeast in triplicate.
19. The final concentration of the glycerol obtained is 16% [13]. The proportion of glycerol and culture medium can also change. For example, you can add 50% yeast suspension and 50% glycerol, in this case, the glycerol solution concentration must be changed.
20. At every 6 months interval, the samples can be subculture to verify cell viability. For this, a scrape of the middle surface, still frozen, can be removed and transferred to a plate with culture medium agar, incubating it at 30 °C for 48 h.
21. Use 20% skimmed milk as a cryoprotectant, substances that protect cell structures during the period of freezing, thawing, and dehydration. Other cryoprotectant can be used such as glucose, trehalose, chitosan, etc.
22. Immediately after removing from the freezer, the ampoules must be placed in the freeze dryer in operation. The samples must not have a drop in temperature or thaw.
23. It can be stored in cardboard boxes at room temperature. Every 6 months, revitalize each sample in culture broth to check viability.
24. Storage in distilled water can be done at room temperature. For cell viability tests, the inoculum must be transferred to new culture medium every 3 months for the method of storage in distilled water and mineral oil, and every 30 days for maintenance on agar.

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