IMPROVED METHOD FOR INOCULATION OF MICROORGANISMS

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ABSTRACT

We studied a new method for inoculation of microorganisms to improve homogenization and their growth on plates. We named the method of Technique of Inoculation in test-Tube (TIT). It showed some advantages when compared to pour plate method such as facility and rapidity of preparation, besides easy homogenization. Using TIT, the growth of the microorganisms was reduced to 14 hours. The method was tested for counting total microorganisms in sugarcane juice using image software.

Keywords: Inoculation, Microorganisms, Homogenization, Counting

1. INTRODUCTION

Microbiological analyses are tools used to show the quality of environment, food, drugs and in clinical pathology. The presence of microorganisms may indicate inadequate sanitary conditions in the preparation, handling and storage of foods, besides indicating a potential danger for consumption. It is important to monitor the microorganisms for avoiding nosocomial infections in clinical environments [1].

For counting of microorganisms is more usual to determine the viable number or live cells. Direct plate counting is the method used to count the number of viable cells in a sample. Two procedures are generally used: spread plate and pour plate methods. The first consists for spreading the diluted sample over an agar plate, while in the last, a sample is pipetted into a sterile plate and melted agar is added and mixed with it by rotational movements. In the spread method, the colonies grow up only on the surface. Therefore, the volume used for inoculation is critical, because the agar does not absorb the excess. The volume should be well chosen and lower than 100 μL when using Rodac plates [2].

Pour plate method is more flexible. Colonies grow up throughout the agar, thus a higher range of volume, usually between 100 and 1000 μL can be used. In this case, temperature of melted agar should be controlled, to avoid death of microorganisms. Generally, it is between 40 °C and 45 °C [2].

Direct plate counting presents some disadvantages that may be error causes. Microorganisms that stayed together after cell division provoke overlap and many colonies are counted as one. Some microorganisms need a specific nutritional medium. Therefore, it is not possible to ensure that there is sufficient nutritional value for all the cell types, so a lower counting may be obtained. An uneven distribution of the sample on the agar surface can also cause counting errors, because colonies grow up in located positions on plate [3].

Furthermore, the analysis time is long, once the results are known from 2 to 7 days after the start of the analysis. This is not compatible with perishable products. Another factor that contributes to inaccurate results is a visual counting using the counter of colonies. These errors are due to poor eyesight and to flaws in the quarters [4, 5].

The number of colony forming units (CFU) is only an estimate of the number of living cells, and does not represent just a unique cell, but an array of cells that can be distinguished on plate after growth [6]. Some factors that influence the accuracy of the plating method are technical errors, heterogeneity of the distribution of microorganisms and singular or duplicate plating [7]. Another problem is the size of the colonies. The use of inoculator was suggested to overcome this problem [8].

The purpose of this work was to develop an alternative method for inoculation of sample on plate, which is quicker, more efficient, easily homogenization and requires a smaller quantity of material.

2. MATERIALS AND METHODS

2.1. Reagents and Solution preparations

All reagents were analytical or pharmaceutical grade. The water used in the preparation of the culture media was distilled previously. Plate count agar (PCA) was purchased from Merck and buffered peptone water was obtained from Himedia. The samples of sugarcane juice were acquired in trade local.

For the preparation of buffered peptone water solution, 3.00 g were dissolved into 150 ml of water and sterilized by 15 minutes at 121.8 °C and 1.05 atm in vertical autoclave.

A series of successive dilution of the sugarcane sample from 10^{-1} to 10^{-6} g L^{-1} were prepared by taking 1 ml of the previous dilution to 10 ml volumetric flask. We prepared the 10^{-1} g L^{-1} concentration from stock solution. The stock solution of the

sample was prepared by taking 5.0 ml of sugarcane juice to 50 ml volumetric flask. All volumes were adjusted with sterile buffered peptone water solution.

We prepared PCA in according to protocol described on flask label. About 5.63 g of the culture medium was weighed and dissolved into 250 ml of water. The mixture was stirred up to complete dissolution.

2.2. Technique of Inoculation in test-Tube (TIT)

Exactly, 8.00 ml of PCA solution was transferred to test-tube (16×1 cm) with screw cap using a micropipette. The PCA solutions into test-tube were sterilized in autoclave. After sterilization, the test-tubes were placed into water-bath in laminar flow. We monitored the temperature with digital thermometer at 40 ℃, 45 ℃ or 50 ℃. When PCA medium into test-tube reached the temperature desired, 500 µL of the diluted sample of sugarcane in buffered peptone water solution were added to test-tube. Carefully, the tube was rapidly homogenized with smooth movements and poured on Rodac plate (65×15 mm).

Similar procedure was adopted using petri plate (90×15 mm). However, the volume of PCA solution and sample solution were adjusted proportionally for plate size, 15 ml and 1000 µL, respectively.

The plates were incubated at 35-37 ℃ by 24-48 hs in bacteriological oven (Quimis model Q316M).

2.3. Image Acquisition System

A homemade image acquisition system was built using ordinary materials purchased in store local.

We adapted a digital camera from Canon model PowerShot A550 of 7.1 Megapixel resolution in a support from Toyo model TS-800 used to desoldering of electronic components (SMD-BGA). A tube of 16.5 cm in length and 10 cm in diameter was fully painted with matte black ink. We used light emitter diodes (LED) to make the illumination of the plate and then to obtain the microorganism image. These LEDs were coupled to tube bottom which it was drilled each 3.5 cm. We designed a guide for positioning the plates on base of the support to match exactly with the center of the camera lens.

The images of the plates containing the microorganisms were processed and analyzed using Image Tool software, version 3.0 from University of Texas - Health Science Center [9]. We adopted the ASTM protocol for counting of the microorganisms with image processing software [10].

3. RESULTS AND DISCUSSION

A critical factor using direct plate counting is the homogenization of the sample in the culture medium. To improve the homogenization process, we developed a new methodology for inoculation called Technique of Inoculation in test-Tube (TIT). In this procedure, test-tubes are sterilized in an autoclave with an exact volume of the PCA solution.

After sterilization, an aliquot of the inoculum diluted solution is inserted directly in the medium, whose the temperature was monitored to be maintained constant.

Fig. 1 shows a kinetic study for growth of the colonies at different temperatures. We can see a lower growth of colonies at 40 °C than other two temperatures. This was not expected since temperatures higher than 40 °C could cause death of colonies. We can attribute this anomalous behavior to poor homogenization due to the higher viscosity of the medium in this temperature. Based on these results, we chose to use the inoculation temperature at 45 °C.

We also obtained a faster growth of the colonies. Usually, the incubation time required to stabilize the growth of microorganisms is between 24 and 48 hours using the conventional methods of inoculation. With the TIT procedure, the growth was stabilized in just 14 hours. The loss of sample solution due to transferring from tube to plate was lower than 4%. Considering that the method for counting of microorganisms on plate accepts an error up to 30% [6, 11], we judge this loss negligible.
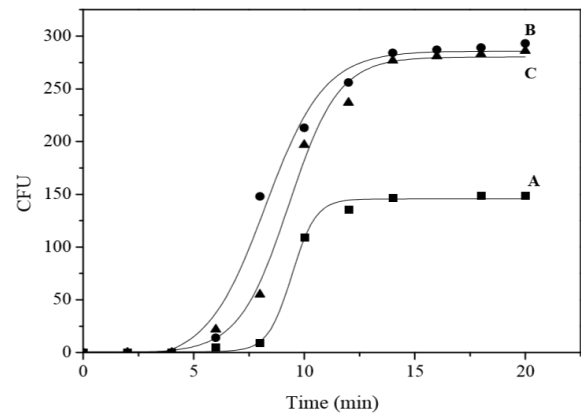

Fig. 1 Kinetic study for microorganism growth on Rodac plates using TIT method at different temperatures. Dilution factor: 10^{-5}. A. 40 ℃; B. 45 ℃; C. 50 ℃
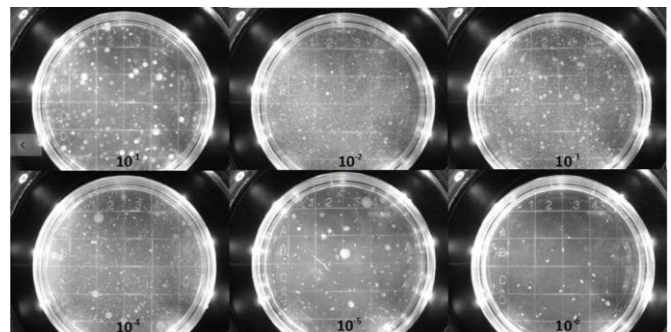

Fig. 2 Photographs for microorganism growth at different dilutions (10^{-1} to 10^{-6}) using TIT method. Incubation time: 14 hours. Temperature of sample inoculation: 45 ℃

When using pour plate method and the temperature of the melted agar is at 40℃, it is difficult to homogenize the medium with the inoculum on the plate in a short period of time, because occurs the agar solidification. The

microorganisms proliferate unevenly on the plate in an inadequate mixture of the medium with the inoculum, forming large colonies even in diluted solutions of inoculum. In addition, it is complicated to homogenize using Rodac plates. Fig. 2 presents the photographs obtained from microorganism growth on Rodac plates using TIT method at different dilutions of inoculum. In the tube, the homogenization is easier and the culture medium plus inoculum can be poured on plate, no more need of rotational movements, which causes a risk of loss of inoculated sample. The advantages of the proposed procedure compared to the pour plate method are shown on Table 1.

Table 1 Comparison between pour plate method and TIT procedure for inoculation of microorganisms.

Characteristics	Pour Plate	TIT
Sterilization volume	undefined	defined
Intensity of preparation	high	low
Inoculation	on plate	in culture medium
Homogenization	hard	easy
Operator experience	high	low

We estimate the number of CFUs in sugarcane juice for different dilutions on the plates (Table 2). The results showed large variability even with a better mixing achieved with the TIT method. In according to ASTM [10], the best countable range for CFU must be from 30 to 300 CFU for pour plate. Hence, the adequate dilution for Rodac plate was 10^{-5} and for Petri plate was 10^{-6} (Table 2). Therefore, the found quantity of microorganisms in sugarcane juice presented an average value of 5.8×10^{8} CFU/ml.

Table 2. Quantity of CFUs determined in sugarcane juice.

Dilution factor	Counting of CFUs/ml sugarcane juice	
	Rodac (33 cm^2)	Petri (64 cm^2)
10^{-6}	1.9×10^{9}	5.9×10^{8}
10^{-5}	5.7×10^{8}	1.9×10^{8}
10^{-4}	TNTC*	TNTC
10^{-3}	TNTC	TNTC
10^{-2}	TNTC	TNTC
10^{-1}	TNTC	TNTC

* *TNTC = Too numerous to count.*

The microorganism counts in sugarcane juice presented a higher value. Although Brazilian National Health Surveillance Agency (ANVISA) does not establish a range for contamination of microorganisms in sugarcane juice, this result shows inadequate hygienic-sanitary conditions. Epidemiological data can be due the cross-contamination during food preparation. The sources of this contamination may be unhygienic handling, the raw material itself, airborne, inadequate cleaning of the utensils such as mechanical press, knives, contact surfaces, clothes, and vendors' hands [12].

4. CONCLUSION

The new Technique of Inoculation in test-Tube (TIT) showed several advantages when compared to pour plate method such as easier inoculation, lower time of incubation, more uniform growth and low overlapping of CFUs.
The obtained results for counting of microorganisms in sugarcane juice presented some variability, but they showed probable inadequate hygienic-sanitary conditions.
Although the TIT method has improved the inoculation on plates for counting of microorganisms may be interesting to establish a reference to CFU size.

5. ACKNOWLEDGEMENTS

6. REFERENCES

1. Bruch MK, and Smith FW, *Appl Microbiol*, 1968; **16(9):** 1427-1428.
2. Madigan MT, Martinko JM, Parker J, Biology of Microorganisms, New Jersey: Prentice Hall, Upper Saddle River; 1997.
3. Claus GW, Understanding Microbes, New York: W. H. Freeman and Company, 1989.
4. Chain VS, Fung DYC, *J Food Protect*, 1991; **64(3):** 208-211.
5. Vasada PC, Chandler RE, Hull RR, *Dairy Food Environ. Sanit.*, 1993; **13(9):** 510-515.
6. Sutton S, *J Valid Technol*, 2011; **17(3):** 42-46.
7. Jongenburger I, Reij MW, Boer EPJ, Gorris LGM, Zwietering MH, *Int J Food Microbiol*, 2010; **143:** 32–40.
8. Khromov-Borisov NN, Saffi J, Henriques JAP, *Tech. Tips Online*, 2001; **6:** 51-57.
9. Wilcox CD, Dove SB, McDavid WD, Greer DB, http://compdent.uthscsa.edu/dig/itdesc.html, accessed in November 03, 2013.
10. ASTM, DS465-93, Standard practice for determining microbial colony counts from waters analyzed by plating methods, 1998.
11. Breed R, Dotterrer WD, *J Bacterial*, 1916; **1:** 321-331.
12. Oliveira ACG, Seixas ASS, Sousa CP, Souza CWO, *Cad Saude Publica*, 2006; **22(5):** 1111-1114.