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Comparison of Conventional SPC Method with Resource Saving SP-SDS Method for Micro Flora Enumeration

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Abstract

We propose a simple technique for micro flora enumeration from diverse sample with no prior idea of viable count and require less consumable. This technique is termed as Single Plate - Serial Dilution Spotting (SP-SDS). The objective of this research was to compare the accuracy and fidelity of SP-SDS method vs. standard plate count (SPC) method by statistical analysis. For pure culture, serial dilutions were prepared from 0.1 O. D and 20 µl aliquots of six dilutions were applied as 10-15 micro drops in six sectors over agar medium in 9 cm Petri plates. For liquid or colloidal sample, the sample was directly used as stock. For solid samples 10% w/v suspension was used. Dilutions and plating were done as described for pure cultures and incubation was done for 24 hours. Following incubation at least one dilution level yielded 6-60 CFU per sector comparable to SPC method. Method was tested on diverse bacteria, yeast and composite samples like food, milk, marine water, sewage, soil etc. SP-SDS gave better results in comparison with SPC with respect to all pure cultures and composite samples. SP-SDS offered wider application over alternative method SPC.

Keywords

Dilutions, Enumeration, SP-SDS, standard plate count.

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INTRODUCTION

In microbiology, a colony forming unit (CFU) is a unit used to estimate the number of viable bacteria or fungal cells in a sample. Agar standard plate count (SPC) procedures Pour plate and Spread plate are routinely used for quantitative determination of aerobic bacteria [1]. In the Standard plate count procedure, an unknown sample is diluted many folds and each dilution is mixed with liquid agar and pour in a Petri dish in pour plate method and spread on agar medium by spreader in spread plate method. After incubation, that plate which has between 30-300 colonies is used for counting. Since the standard plate count method is both time consuming and costly [2], the Drop plate method can be used to determine the number of viable suspended bacteria. The Drop plate exhibit many positive characteristics. The plating and counting procedure require less

labour than standard plate count method. The plating and counting steps are very convenient and manageable [3]. The Drop plate method is more economical [4]. The SPC method allows growth within the nutrient agar as well as on the surface; however, some relatively heat sensitive microorganisms may be damaged by the melted agar [5]. Enumeration of viable cells is essential step in study of pure culture, environmental sample, water sample, food sample and clinical sample. In surface Spread plate method, the continuous use of spreader causes irrecoverable injury to bacterial cell [6].

MATERIALS AND METHOD

Protocol optimization by pure culture: Pure cultures of bacteria from different phylo-genetic groups varying in Gram reaction and cell characteristics were used towards optimizing the single plate-serial dilution spotting (SP-SDS) technique [7]. The organisms included *Escherichia coli, Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus, Bacillus cereus, pseudomonas aeruginosa* and yeast *Candida albicans. E. coli* was used as the primary candidate for protocol optimization followed by *B. subtilis*. Yeast (*Saccharomyces cerevisiae*) was used in this study employing potato dextrose agar (PDA) [7]. Different composite samples representing public health, food, environmental and agricultural were tested for bacterial and yeast CFU enumeration [7]. Overnight nutrient agar (NA) slant derived (18–24 h) cultures were used in all studies involving pure bacterial cultures. For pure bacterial and yeast cultures, a uniform cell suspension was prepared by dispersing the overnight colony growths from agar slants. After allowing any cell clumps to settle down, the clear upper part was transferred to a fresh tube.

Dilution preparation: The optical density (OD) was determined at 600 nm in a spectrophotometer based on which the 'anchored stock' of 0.1 OD (10^0) was prepared. Decimal serial dilutions (100–1000 µl) of 10^{-1}–10^{-6} were prepared from the 10^0 stock in 1.5 ml tubes with 4–5 repeated flushing and changing of tips. For preparing the stock and serial dilutions, freshly autoclaved and sterilized water was used. For water and clear liquid specimens, the direct sample formed the 10^oanchored stocks was used. Thick and colloidal suspensions such as milk and fruit juice were used directly or after adjusting OD at 600 nm to 1.0 or 10 while for solid specimens (food, soil) a suspension prepared in water with 1.0 g sample per 10 ml formed the 10^0 stock.

Plating by SP-SDS method: To execute SP-SDS, the reverse of the 9-cm Petri-dishes containing surface dry agar media were drawn to six sectors with the marking of first and last dilution sectors for clear identification. Using a calibrated micropipette, 20 µl aliquots from selected six dilutions were applied as 10–12 micro-drops in these demarcated areas. During sample spotting, the same tip was used starting with the lowest dilution. Care was exercised to avoid tip marks on the medium during sample application not to mistake them for CFU. The sterility of the diluent was ensured by spotting 20 µl at the bottom part of the plate. The plates were allowed to be dried and incubated inverted at 28–37 ℃ as required for specific organisms/sample.

CFU enumeration: It was done after 18–48 h with the marking of colonies on the reverse of the plate. The colony development pattern at different dilutions in SP-SDS was recorded as spot growth, too many to count (tmtc) or countable/acceptable (6–60 range). After recording the dilution level yielding acceptable colonies and the CFU per sector, CFU per 100 µl was worked out as n ×_5 (n = colonies in 20 ml sample applied area). The CFU/ml of the 10^0 stock was arrived at as the product of $n \times 5 \times 10^{d+1}$ (d = dilution level yielding the countable colonies) [7].

Standard Plate Count (SPC): It was performed for comparative study from same sample as per the standard procedure.

RESULT AND DISCUSSION

In the initial trial employing *S. aureus* the first three serial dilutions (10^{-1}to10^{-4}) showed spot growth, 10^{-4} displayed too numerous to count (tntc) and 10^{-5} and 10^{-6} yielded well presented colonies in the acceptable range (Fig.1A). The plates applied with the Milk sample exhibited cluster of diverse colony types at 10^{-1}–10^{-2} including some spread growth and at 10^{-3} and 10^{-4} shows countable colonies (Fig. 2B). Thus, at least one dilution in a plate yielded CFU in the acceptable range ensuring the success of the trial at 24 hours of incubation. All the composite samples (Milk sample, Marine water sample, Food sample, Soil sample and Sewage sample) yielded countable colonies by SP-SDS method. Three replications for each sample were employed for the statistical analysis. The Mean value and SD (Standard Deviation) estimated for SP-SDS method in Microsoft Excel 2007.

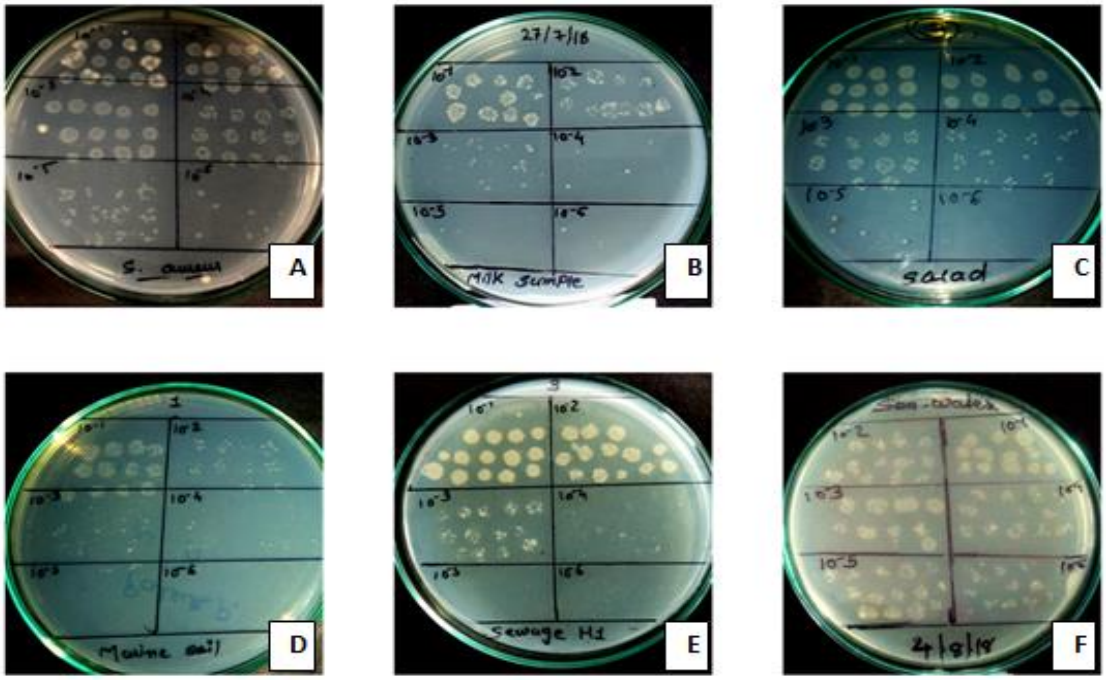

Fig. 1: SP-SDS Results for Different samples on plates, (A) Pure culture (B) Milk Sample (C) Food Sample (D) Soil Sample (E) Sewage Sample (F) Marine water Sample.

TABLE 1: Result of enumeration of CFU/ml of various pure cultures and composite samples by SP-SDS method and SPC method.

Samples	CFU/Ml ± SD By SP-SDS	By SPC
Pure culture		
Escherichia coli	2.20×10^8	4.5×10^6
Candida albicans	8.15×10^6	Tntc/tltc
Bacillus subtilis	2.60×10^8	Tntc/tltc
Milk samples		
Raw milk	3.67×10^6	Tntc/tltc
Raw milk	$1.28 \pm 0.18 \times 10^5$	Tntc/tltc
Raw milk	$33.43 \pm 14.4 \times 10^6$	8.3×10^4
Boiled and 6 h open	$6.3 \pm 6.0 \times 10^5$	Tntc/tltc
Boiled and 12 h refrigerated	$4.76 \pm 3.9 \times 10^5$	6.4×10^3
Marine water samples		
Marine water	6.5×10^4	Tntc/tltc
Marine water	2.8×10^9	5.1×10^7
Marine water	2.4×10^6	Tntc/tltc
Marine water 2 days old	$1.07 \pm 1.1 \times 10^6$	Tntc/tltc
Marine water 1 week old	-	Tntc/tltc
Food samples		
Sweet sample	$1.29 \pm 1.6 \times 10^5$	3.9×10^4
Cut vegetable (potato)	$2.05 \pm 2.5 \times 10^5$	Tntc/tltc
Cut vegetable (onion)	-	Tntc/tltc
Dry yeast	-	Tntc/tltc
Tap water	-	Tntc/tltc
Salad (street food)	$3.55 \pm 2.21 \times 10^8$	2.8×10^5
Green sauce	$9.5 \pm 2.29 \times 10^4$	Tntc/tltc
Red sauce	$1.69 \pm 0.5 \times 10^4$	4.3×10^4

Fruit salad	$1.40 \pm 0.10 \times 10^5$	Tntc/tltc
Antacid liquid(sweet)	-	Tntc/tltc
Soil samples		
Marine soil	$4.0 \pm 0.9 \times 10^6$	1.57×10^4
College campus soil	$2.77 \pm 0.3 \times 10^5$	3.9×10^4
Black garden soil	$2.9 \pm 0.6 \times 10^4$	Tntc/tltc
Red garden soil	$10.25 \pm 1.7 \times 10^4$	3.2×10^6
Soil from watery area	$5.1 \pm 1.09 \times 10^5$	Tntc/tltc
Sewage samples		
Sewage sample 1	$4.3 \pm 1.0 \times 10^6$	Tntc/tltc
Sewage sample 2	$5.6 \pm 0.7 \times 10^6$	1.27×10^4
Sewage sample 3	$1.2 \pm 0.13 \times 10^7$	4.8×10^5
Sewage sample 4	$3.2 \pm 0.7 \times 10^7$	Tntc/tltc
Sewage sample 5	$2.8 \pm 1.57 \times 10^7$	Tntc/tltc
Sewage sample 6	$5.0 \pm 0.8 \times 10^7$	1.3×10^5

CONCLUSION

In SP-SDS six different dilutions of bacterial suspension or composite samples are spotted as micro-drops across 9-cm plate agar surface. It represents a simple, efficient and resource saving technique for bacterial CFU enumeration when there is no clear idea that at which dilution countable colonies could be expected. SP-SDS with three replications suited diverse samples including pure cultures, yeast cultures, milk and food samples, soil and sewage samples and marine water samples giving better result as compare with Standard Plate Count (SPC) method.

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