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Application of New Rapid Fluorescent Staining Method for Direct Enumeration of Live/Dead Bacterial Cells in Different Food Matrix

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ABSTRACT

Present work was aimed to study the applicability of the most widely used direct epifluorescent filter technique (DEFT) to enumerate viable and non-viable bacteria using SYTO® 9 and propidium iodide (PI) dyes in complex food matrix. Experimental conditions such as dye concentration, incubation temperature, pH, incubation time and use of different resuspension buffer for optimum application of dual stains were examined with pure cultures. The technique was calibrated by comparing fluorescent emission of bacterial cells with that of known concentration of pure bacterial culture suspension. The results of relative viability of Lactobacillus spp. (MTCC 4185) and Salmonella spp. (MTCC 1163) cells observed under microscope correlated well with the number of live cells in the suspension with R^2 *of 0.967 and 0.984 respectively. The total counts obtained by DEFT using dual SYTO® 9 and PI stains were far superior than counts obtained by DEFT using acridine orange and DAPI stains. The DEFT count using dual SYTO® 9 and PI dye and SPC count for viable cell enumeration, showed good agreement, accuracy and acceptable interchangeability.*

Key words: *Lactobacillus, Salmonella, DEFT, live/dead, SYTO® 9, PI.*

INTRODUCTION

Microscopy has always been a prominent technique in the search for rapid direct methods of enumerating microorganisms, one of the first to be used by the food industry. Developments in epifluorescent bacterial direct count between the 1940s and 1980s mainly involved improved cell staining procedures[18]. In this method, a pre-treated sample is filtered and collected over the membrane, where the cells are stained with fluorescent dyes and incident UV light illumination is used to examine the filter surface.

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Direct Epifluorescent Filter Technique (DEFT) offers ease of use, reliability, and low cost. The actual staining and counting in epifluorescent techniques takes less than 0.5-1 h and the sensitivity can be improved with efficient sample pre-treatment steps which slightly increase the total detection time. This makes the simple and versatile filter-based technique the preferred method for enumeration studies[17].

Staining bacteria with Acridine Orange (AO) or 4′, 6-diamidino-2-phenylindole (DAPI) and counting them on black polycarbonate filters using epifluorescent microscopy have been extensively used procedure for direct counting[13,19,38]. Acridine orange counterstaining was successfully applied to estuarine for enumerating particle-associated bacteria[8]. Various specific stains were introduced, such as acriflavine for humic-rich samples[3] and highly dsDNA specific fluorescent stain, SybrGreen I for the enumeration of viruses[9,32,46]. The most common fluorescent stains as indicators of viability are based on either membrane integrity or enzyme activity[24,31,41]. Commercial live/dead viability kit is based on membrane integrity and contains mainly combination of a membrane-permeable green fluorescence dye for live cells and red fluorescent membrane impermeable dye for dead cells. Monomeric cyanines (SYTO dyes, SYBR dyes) or fluorescein derivatives (cFDA, FTGU) are often used as probes for live cells and phenanthridinium dyes, like Propidium Iodide (PI) or homodimeric dyes (TOTO-1, YOYO-1)[47,48] are used for dead cells detection.

Existing epifluorescent techniques are limited only to soil sediments, low viscosity fluid but no standard method has been developed for simultaneous quantification of viable and non-viable cells from complex food matrix in the shortest possible time. The present study was aimed to standardize experimental conditions that contribute to accurate determination of viable and non-viable cells count and comparing the sensitivity of the developed rapid microbial assessment method with that of the conventional techniques.

MATERIALS AND METHODS

Microbial strains

To develop the technique for accurate quantification of viable and non-viable bacterial cells using SYTO® 9 and Propidium Iodide (PI) stain, *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) were chosen as representatives of gram-positive and gram-negative bacteria respectively. Cultures were procured from the microbial type culture collection and gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *Lactobacillus* spp. was cultivated in Lactobacilli MRS broth (peptone 10.0 g/l, beef extract 10.0 g/l, yeast extract 5.0 g/l, glucose 20.0 g/l, sodium acetate 5.0 g/l, triammonium citrate 2.0 g/l, Na_2HPO_4 2.0 g/l, $MnSO_4.4H_2O$ 0.2 g/l, $MnSO_4.7H_2O$ 0.2 g/l and tween-80 0.1 g/l, pH 6.2-6.6, Himedia India) at temperature of 30°C for 24 h. *Salmonella* spp. was cultivated in Nutrient agar (Himedia, India) at 37°C for 24 h. After incubation, culture broth of 10 ml was taken in 15 ml centrifuge tubes and centrifuged for 10 min at 10,000 ×g under refrigerated conditions[33] (Pascaud et al., 2009). The resulting pellet was resuspended in 2 ml of filter sterilized (0.2 µm) water by vigorous pipetting. The culture suspension was diluted and adjusted to 5×10^7 cells/ml with filter sterilized (0.2 µm) water.

Stains preparation for live/dead bacterial count

For differentiation of live/dead microbial cells, two stains were used: SYTO® 9 and Propidium Iodide (PI). SYTO® 9 penetrates all bacterial membranes (intact and injured) and labels bacterial cells green. Propidium Iodide can only penetrate injured bacterial membranes and labels the bacterial cells red while diminishing the green stained by SYTO® 9.

SYTO® 9:- Commercially available 100 µl of SYTO® 9 solution of concentration 5mM in DMSO (Invitrogen molecular probes, Eugene, Oregon, USA) was used for staining live microbial cells. For staining, SYTO® 9 stock solution of 50 µM/ml concentration was prepared by dissolving 20 µl of SYTO® 9 in 2 ml of distilled water. This stock solution was filtered through 0.2 µm plastic syringe filter

(25 mm, Axiva) and stored in dark at -20°C in 1-2 ml plastic microcentrifuge tubes.

Propidium iodide (PI):- Propidium iodide (Invitrogen Molecular Probes, Eugene, Oregon, USA) stock solution of final concentration of 500 µM /ml was prepared by dissolving 5 mg of PI in 15 ml of deionized water. This stock solution was filtered through 0.2 µm plastic syringe filter and stored in 1-2 ml plastic microcentrifuge tubes at 2-6°C.

Slide preparation and enumeration of live/dead bacterial cells

For the analysis of viable/non-viable cells in a suspension, 200 µl of pure culture suspension or treated food sample (appropriately diluted) were stained with 20 µl of SYTO® 9 and 40 µl of PI stains stock solution as prepared above at final concentration of 1 µM and 20 µM respectively, and incubated in dark for 20 min at room temperature. After staining, the sample volume in the vial was made to 1 ml with 0.2 µm filter sterilized water for its even distribution over the filter. Sample was filtered by applying vacuum (KNF Lab Laboport, India) through 0.2 µm pore size pre-wetted black nuclepore track-etch membrane filter (25 mm, Whatman), with the shiny side up, on to the wetted 0.45 µm pore size cellulose acetate (25 mm, Axiva membrane filter) back filter placed on filter tower (Axiva, India) and rinsed twice with 0.2 µm filter sterilized water. Under vacuum the membrane filter was carefully removed, from the underlying backing filter. The filter, sample side up was laid, on to the film of immersion oil on the glass slide. Again put one drop of immersion oil onto the centre of the filter and gently laid a cover slip on the filter. Prepared slides were enumerated using Olympus CX31 RTSF epifluorescence microscope equipped with 30W, 6V halogen lamp with excitation and emission filters (FRAEN RG-3, USA) of 480/530 nm and 535/630 nm for SYTO® 9 and PI respectively. Images were captured using ProgRes® camera and analysed with Jenoptik ProgRes® CapturePro 2.7.7 (Jena, Germany). The numbers of bacteria per ml were estimated from the images of stained cells captured in five different fields and total cell number in each field was about 100–200[27]. Bacterial cell per millilitre will be counted as:-

Bacteria = $(N \times A_t)/(d \times V_f \times A_g)$

Where N is the number of cell counted, A_t is effective area of the filter, A_g is the area of counting grid, V_f is the volume of diluted sample filtered, and d is the dilution factor (V_{final}/V_{sample})[19]. The green fluorescent bacterial cells were enumerated as viable cells and red as non-viable cells.

Optimal protocol for live/dead cell enumeration

The nucleic acids and other media components can bind the SYTO® 9 and propidium iodide dyes in unpredictable ways, resulting in unacceptable variations in staining. So it is very important to optimize the conditions that could affect the staining efficiency. For optimization experiment overnight grown pure bacterial culture of *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) were used. The density of culture suspension was adjusted to approximately 5×10^7 cells/ml, prepared in 0.2 µm filtered sterilized water. Two aliquots (1.5 ml) of each culture suspensions of desired cell density were centrifuged for 10 min at 8000 × g (4°C) and the supernatants were discarded. One of the cell pellets was directly resuspended in the same volume of water (1.5 ml) and used as viable cell suspension. Bacterial cells in the second pellet were killed by treating with 70 % (v/v) ethanol at 60 °C for 1 h[28] (Morono et al., 2004) for testing. After removal of the biocide, dead cells were washed, centrifuged for 10 min at 8000 × g (4°C), and re-suspended in 0.2 µm filtered sterilized water (1.5 ml). Live and dead cell suspension prepared were mixed in the ratio of 1:1 and used for the optimization study. The evaluation of stained culture suspension was based on the qualitative examination (visual inspection of the slides) considering (a) signal intensity (b) staining specificity and (c) background level.

Optimization of stain concentration

It is necessary to adjust the amounts or proportions of the two stains for optimum discrimination between live and dead cells[33]. The *Lactobacillus* spp. and *Salmonella* spp., live and dead cells (ratio 1: 1) mixtures prepared (200 µl) were stained at four different concentrations of SYTO® 9 and PI viz. 1/10, 2/20, 4/40 and 2/40 (µM/µM). Stained samples were incubated for 20 min at room temperature in the dark.

Duration of stain exposure

Time of incubation of stains with sample were also tested, since a short time could lead to lack of sensitivity, leaving poorly or not stained cells, while long incubations could increase the background level. For the study, 200 µl of cell mixtures (5×10^7 cells/ml) of both strains were stained with 40 µl of each SYTO® 9 and PI stain stock solutions and incubated in dark for 10, 20, 30 and 40 min at room temperature.

Optimization of buffer for sample preparation

Two different buffers, namely Phosphate buffer (g/l: $Na_2HPO_4.2H_2O$- 1.78, KH_2PO_4- 0.27, KCl- 0.20, NaCl- 8.01 and pH- 7.4) and 0.1 M sodium phosphate buffer (g/l: $NaH_2PO_4.2H_2O$- 3.1, Na_2HPO_4- 10.9 and pH- 7.4) were used for resuspending and diluting bacterial pellets prior to staining, to check their capability to restore a neutral pH. Deionised water was also used for resuspending and diluting pure bacterial culture pellet (control). Cell mixture (5×10^7 cells/ml) of 200 µl prepared was stained with 40 µl of each SYTO® 9 and PI stains solution and incubated in the dark for 20 min at room temperature.

Effect of pH

SYTO® 9 dye fluorescence is sensitive to pH (Molecular Probes, USA). To determine the effect of pH on staining efficiency, different pH of 5, 6, 7 and 8 filter sterilized water was used to resuspend and dilute pure culture pellets. Two hundred micro litre of suspended pure culture at different pH were stained with 40 µl of each SYTO® 9 and PI stains and incubated in dark for 20 min at room temperature.

Effect of incubation temperature

Different temperature may facilitate the entry of dyes to the microbial cells and affect the efficiency of staining. To determine the effect of temperature, 200 µl of suspended pure cultures were stained with 40 µl of each SYTO® 9 and PI stains solution and incubated at three different temperatures (30°C, 40°C and 50°C) in dark for 20 min. Sensitivity and intensity of dyes were examined using Olympus CX31 RTSF epifluorescence microscope.

Preparation of live/dead microbial cell mixtures

To check the linearity and accuracy of developed technique, optimized conditions were applied on different proportion of live/dead cell suspensions prepared. It is important because it is the ability of the method when used with the given matrix to give results that correlate well with the amount of analyte present in the sample. An increase in analyte should correspond to a linear or proportional increase in the enumeration results[14] with SYTO® 9 and PI. Concentration of approximately 5×10^7 cells/ml of live and dead cells of *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) were mixed in different ratio to give different proportions of live cells (0 %, 25 %, 50 %, 75 %, and 100 % v/v). A pure culture suspension of 200 µl was stained with 40 µl of each SYTO® 9 and PI stains and incubated in dark for 20 min at room temperature Green fluorescence cells were enumerated and divided by total cell number (green and red) to obtain percent proportion of live cell stained (Ratio G/T). Live cells were checked for linear regression by plotting against the known proportion of live cell in the pure culture suspension.

Validation of the developed rapid method for live/dead enumeration

To check the accuracy of the above improved method of live/dead microbial enumeration technique, eight different food samples (raw milk, butter, mixed fruit juice, tamarind sauce, carrot juice, tomato sauce, samosa and kulcha) belonging to four different categories viz. dairy, fruits, vegetables and cereal products were enumerated and compared with the conventional techniques such as the standard plate count for viable bacterial cell and other techniques such as DEFT using acridine orange, DEFT using DAPI stain and haemocytometer method for total cell count. Hobbie *et al*[13]., and Schallenberg *et al*[37]., protocols were followed for quantitation of total bacterial cells with DEFT technique using acridine orange and DAPI stain respectively. Before enumeration different pre-treatments, homogenization for 2 min (5,000 rpm) followed by sonication (2 min) and centrifugation at 8,000 rpm (10 min) for dairy products were applied. The treatments for the food samples other than dairy products were homogenization for 1 min (5,000 rpm) followed by sonication (2 min) and centrifugation at 1,000 rpm for 2 min, while for juices samples, 2 min sonication followed by centrifugation at 1,000 rpm for 2 min were used (Data not shown). Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternate methods on identical samples, the term relative accuracy used here is complementary to the accuracy and trueness as defined in ISO[16].

Standard Plate Count (SPC)

For the enumeration of total aerobic mesophilic micro-organisms by the conventional technique, Plate Count Agar (Hi Media, India) was used according to the technique established by the International organization for standardisation[15]. Each food sample (25 g) was suspended in 250 ml of autoclave sterilized water and analysed using serial dilution technique. The mean number of colonies counted was expressed as colony forming units (CFU)/ g of sample.

Statistical analysis

Samples were tested in triplicate, and the experiments were repeated and the mean values plotted. In order to get a more symmetric distribution, counts were transformed into logarithms. To calculate statistical differences among means, one way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) and Duncan's multiple range (DMRT) test at 5% probability using SPSS 16.0 software. The statistical confidence interval was considered significant at $p \leq 0.05$. The correlation coefficient, slope, and intercept for the results obtained by DEFT standard plate count of food samples were calculated using linear regression methods ($\alpha \leq 0.05$) using MS Excel 7.0 (Microsoft Inc., USA). The vertical y-axis (independent variable) was used for the alternative method and the horizontal x-axis (dependent variable) for the reference method.

RESULTS AND DISCUSSION

Live/dead cell differentiating stain optimization

Optimization of concentrations of SYTO® 9 and PI

Stocks[40] conducted "cell-free" physicochemical measurements with the "Viability Stain, BacLight" and revealed that the staining principle is not that simple and for an interpretation of the staining outcome, the relative concentrations of PI, SYTO® 9 and DNA are of crucial importance and that appropriate control or validation experiments should be performed. For accurate quantitation of live/dead bacterial cells, *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) as representatives of gram-positive and gram-negative bacteria respectively were chosen for the optimization study. Each culture was mixed in equal proportion of live/dead (1: 1) cells and used. It is necessary to adjust the amount or proportion of the two stains for optimum discrimination between live and dead cells[33].

At 1 µM and10 µM concentration of SYTO® 9 and PI respectively, the viable *Lactobacilli* cells stained green and were very few in comparison to dead cell with red fluorescence (Fig 1a), while viable and dead cells of *Salmonella* spp. were stained in equal ratio (Fig 2a). As concentration of SYTO® 9 and PI increased to 2 µM and 20 µM respectively, it was noticed that green and red fluorescence emission was proportional to the proportion of live and dead cells of *Lactobacilli* spp. and *Salmonella* spp. (Fig 1b and Fig 2b). It was observed that with further increase in concentration of SYTO® 9 dye, background fluorescence increased in both cultures (Fig 1 c,d and Fig 2 c,d). The typical shape of the PI stained cells was not perceived in both the strains. This may indicate that the treatment given to bacterial species for dead cell preparation was harsh and led to disintegration of cell morphology. Attempts to enhance the green and red fluorescence signal by increasing the concentrations of both fluorescent stains were not successful as the fluorescence intensity remains constant. Hence, the final concentration of SYTO® 9 and PI used for further optimization study was 2 µM and 20 µM respectively. In contrast, Duffy and Sheridan[10]; Alakomi *et al*[1]., and Maukonen *et al*[25]., used SYTO® 9 and PI concentration of 10 µM and 60 µM respectively for the enumeration studies. However, Pascaud *et al*[33]., observed optimum SYTO® 9 and PI ratio of 6: 60 (µM) for quantitation of mixed microbial communities in soil.

Though *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) are not representative of the entire microbial community, the successful staining of live and dead cells with SYTO® 9 and PI shows the usefulness of these fluorescent stains in DEFT for rapid quantification of microorganisms. The combined usage of SYTO® 9 and PI in a commercially available kit (BacLight™ – Molecular Probes®) was first described in 1996 and was promoted as a rapid and reliable method for the assessment of bacterial viability, that gives quantitative results and can be applied to microplate reader, flow cytometer and microscopes[5,20,43,44].

Effect of different staining conditions on SYTO® 9 /PI stained cells

The evaluation of the stained samples was based on the qualitative examination (visual inspection of the slides) considering (a) signal intensity, (b) staining specificity and (c) background level and results are reported in **Table 1**. For all the conditions tested, *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) in pure culture behaved in a similar manner and are therefore discussed together as below.

Optimization of buffer for sample preparation

Two different buffers, phosphate buffer and sodium phosphate buffer were used for resuspending and diluting bacterial pellets prior to staining, to check their capability to restore a neutral pH. When bacterial pellets were prepared in phosphate buffer, high green background fluorescence was observed. Few bacterial cells fluoresced green when sodium phosphate buffer (NaPBS), was used for resuspending and diluting bacterial culture, while brightly fluorescent green and red cells were observed when water was used for culture preparation. The count obtained was according to the known proportion of live/dead (1: 1) cells in the mixture. Similarly, Corich *et al*[7]., tested three stains (FDA, CTC and BacLight) on whey starter cultures and it was observed that cells were brightly coloured and background fluorescence was almost absent when water was used, while the phosphate-treated samples showed a disturbing level of basal fluorescence.

Duration of stain exposure

To check the effect of incubation time on staining efficiency, SYTO® 9 and PI stain were incubated with a mixture of live/dead (1: 1) cell suspension in the dark for 10, 20, 30 and 40 min at room temperature. After 10 min incubation time of SYTO® 9 /PI with bacterial suspension, only a few cells were fluorescent green and red. With increase in incubation time up to 20 min, the cell fluorescence increased significantly, shape of the cells could be easily observed and the cells stained were according to the proportion of live/dead cell mixture used for the study. Further incubation up to 40 min did not improve the staining efficiency. Alakomi *et al*[1]., and Maukonen *et al*[25]., reported 15 min incubation of SYTO® 9 and PI dye for the enumeration of bacteria in probiotic preparation, non-dairy drinks and pharmaceutical products. However, Duffy and Sheridan[10] employed 10 min incubation of SYTO® 9 and PI dye with processed meat samples.

Table 1: Qualitative assessment of different staining conditions on *Lactobacillus* and *Salmonella* cultures using SYTO® 9 and PI stain

Cultures	Solution used for resuspension			Incubation time (min)				Temperature (°C)			pH			
	Water	NaPBS	PBS	10	20	30	40	30	40	50	5	6	7	8
Lactobacillus	++	±	-	+	++	++	++	++	+	-	+	+	++	±
Salmonella	++	±	-	+	++	++	++	++	+	-	+	+	++	±

(+) poor, (++) good, (±) unacceptable, (-) negative

Effect of pH

Effect of pH on staining efficiency was tested using water used for culture resuspension and dilution at different pH of 5, 6, 7 and 8. At pH 5 and 6, bacterial cells stained were few as compare to live/dead (1:1) proportion with weak green and red fluorescence. At pH 5 and 6, bacterial cells those stained were lesser as compared to the live/dead (1: 1) proportion with weak green and red fluorescence. As pH increased to 7, the green and red fluorescence increased significantly and cells stained were comparable with live/dead (1: 1) proportion. Fluorescence shift from 50: 50 ratio of red and green fluorescent cells to more of red cells and very few green fluorescent cells was observed, when pH was increased to 8. Martin and Lindqvist[23] observed that it is deemed necessary to determine pH effect on staining efficiency, since low pH could decrease staining efficiency of a dye.

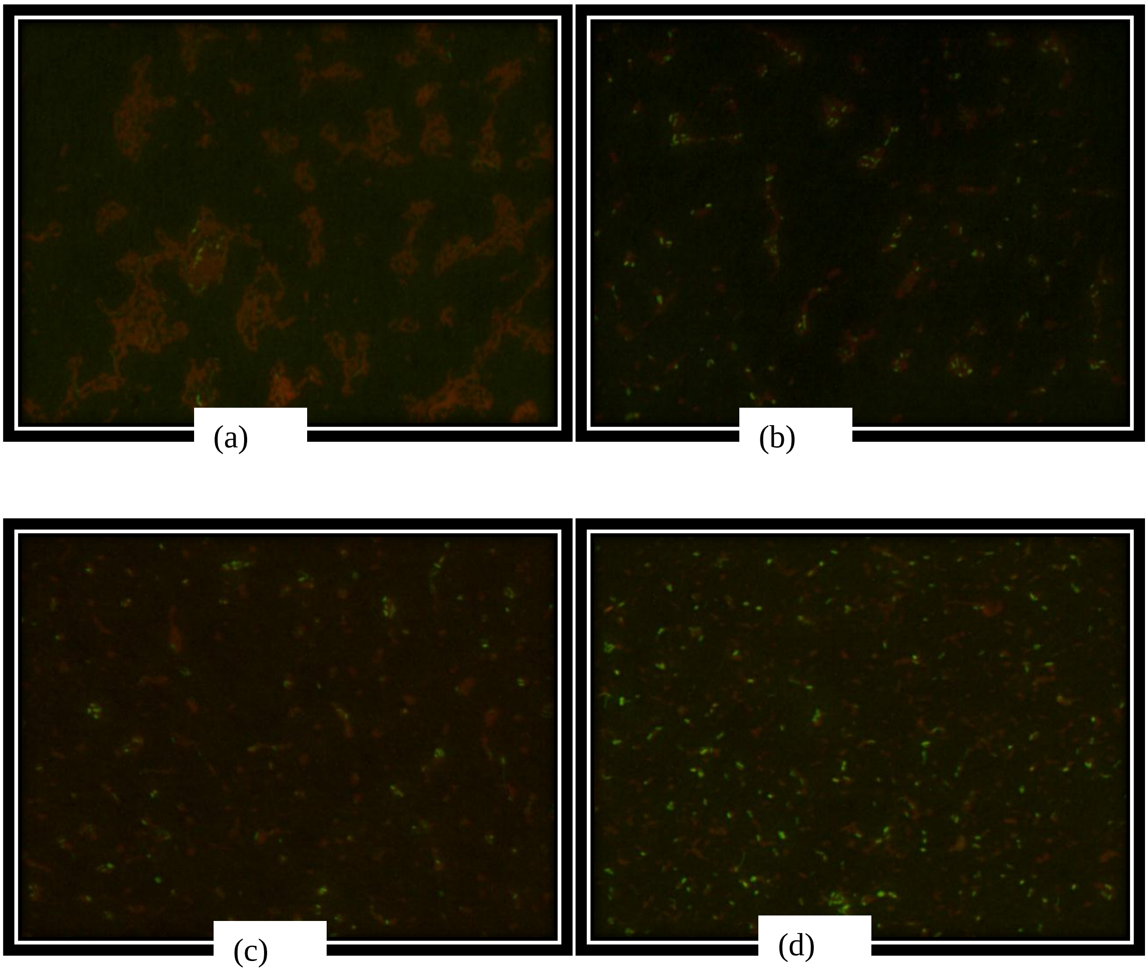


Fig. 1: Optimization of SYTO® 9 and PI concentration in *Lactobacillus* spp. (MTCC 4185) (a) 1 µM and 10 µM (b) 2 µM and 20 µM (c) 2 µM and 40 µM and (d) 4 µM and 40 µM respectively

Effect of incubation temperature

The physiological state of the bacteria may affect both the number of binding sites for the stains and the permeability of the membrane. Boulos *et al*[5]., also suggested that growth rates and temperature of the culture have an impact on the cell staining. Lowering the temperature also has an impact on the permeability of the membrane, since it will decrease the fluidity and permeability of the lipids in the phospholipid bilayer of the outer membrane[45]. Since SYTO® 9 penetrates all cell membranes; its efficiency can be limited either by decreased membrane permeability to this stain or by an insufficient accumulation of the stain to become detectable. The same limitations apply to PI, which only stains nucleic acids in cells with damaged membranes[22]. The effect of incubation temperature could therefore be the dominant factor to explain the decrease in staining efficiency. In our study, bacterial cells stained best at 30 °C temperature (Table 1). The cells permeabilized at 50 °C and the bacterial cell fluorescence changed to increased red fluorescence intensity with weaker green fluorescence intensity with blurred cell appearance.

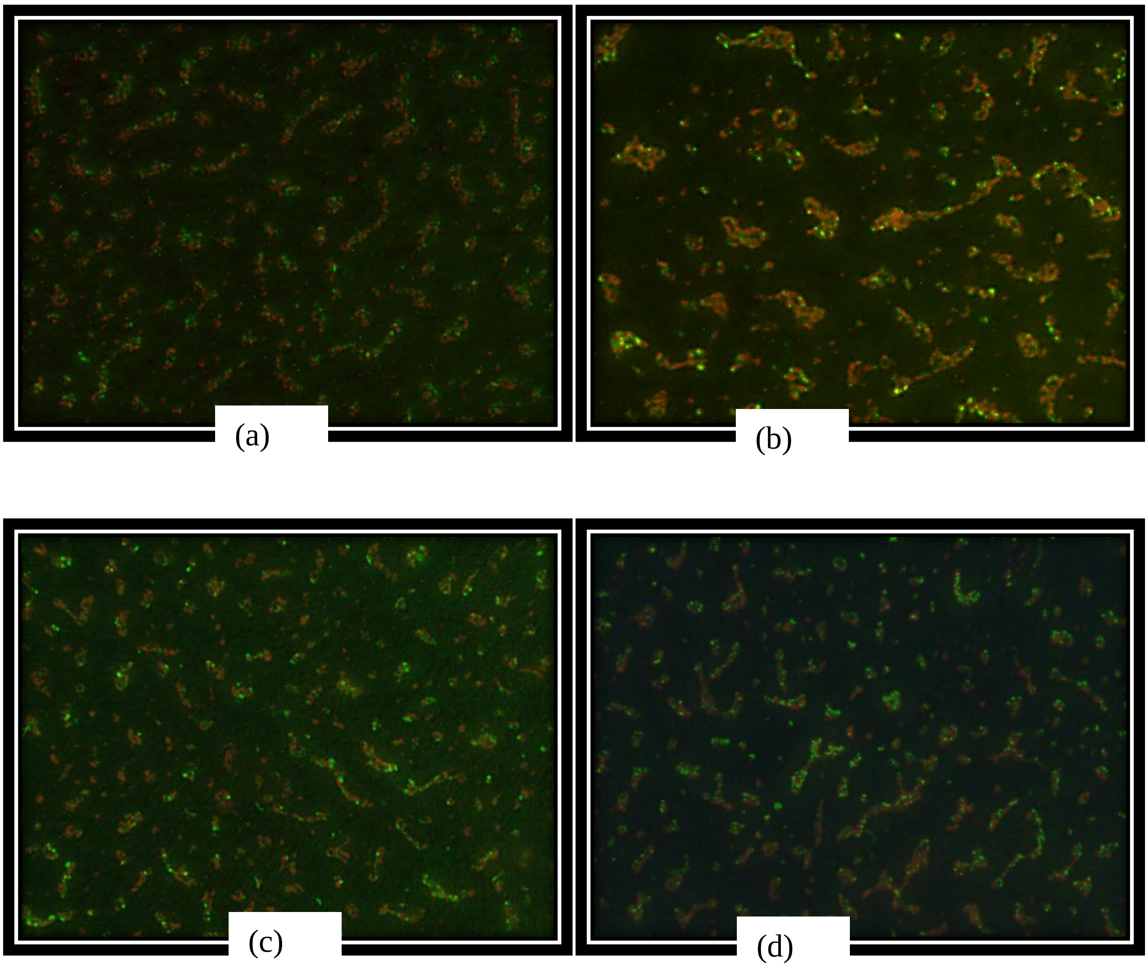


Fig. 2: Optimization of SYTO® 9 and PI concentration in *Salmonella* spp. (MTCC 1163) (a) 1 µM and 10 µM (b) 2 µM and 20 µM (c) 2 µM and 40 µM and (d) 4 µM and 40 µM respectively

Checking the linearity of the developed technique

Linearity and accuracy of the developed technique was examined by comparing known proportion of live cell in the pure culture suspension with percent live cells obtained by staining pure culture with SYTO® 9 and PI dye. Percent live cells (green) can be calculated by the ratio of SYTO® 9 and SYTO® 9 plus PI stained cells (Green: Green+Red or G/T). The results of relative viability of *Lactobacillus* spp. (MTCC 4185) and *Salmonella* spp. (MTCC 1163) cells (Ratio G/T) observed under microscope, correlated well with the number of known live cells in the suspension with R^2 of 0.967 and 0.984 respectively (Fig 3 a and b). The percentage of live bacteria obtained by DEFT technique using SYTO® 9 and PI was 9.78 and 14.65% for *Lactobacillus* spp. and *Salmonella* spp. respectively, which was lower than 25% of the known live cells added. When 50% known live cell mixtures were enumerated using optimized DEFT technique, the live cell count obtained was, 50.2% and 49.23% for *Lactobacillus* spp. and *Salmonella* spp. respectively. Similarly, Netuschil et al[29]., (2014) observed that the mixture of 50% living and 50% dead bacteria does not normally lead to green/red (50/50) fluorescence and vice versa: 50% of green fluorescing bacteria in a sample does not mean that there are 50% vital cells. Therefore, "green/red fluorescence ratios have to be calculated for each proportion of live/dead cells." Excellent results were recorded at zero and 100 % proportion of live cell in the both pure culture suspension as G/T Ratio was same. As in typical for the live/dead stain, the viable *Lactobacilli* spp. and *Salmonella* spp. population demonstrated strong green fluorescence and no red fluorescence, while ethanol treated completely permeabilized population (100% dead) showed strong red fluorescence and no green fluorescence. Thus, the epifluorescent microscopic technique with optimized SYTO® 9 and PI dye concentration gave good estimate of the viability state of bacterial cultures and can be applied in enumeration studies.

In the statistical sense, accuracy of measurement is the closeness of a measured value to its true value[39]. In estimating accuracy of bacterial counts by microscopic methods, obtaining the true value is a challenging task. Chae *et al*[6]., regarded the true value as the best available measure of true concentration and this concept led them to seek the counts obtained under the most favourable conditions as the true estimates of particle abundance under microscope. In practicing the concept, the authors used the greatest counts with highest particle density per field as the true particle counts per field. In the study by Lisle *et al*[21]., the true values were nominally determined by preparing samples with dilutions from a single high-density stock, the cell abundance of which was enumerated with an inter-laboratory standardized growth curve of a bacterial strain. Because the estimation of abundance in the stock and the dilution operations are subject to random errors, when the accuracy is interpreted on a relative scale. According to Chae *et al*[6]., highest particle density may be an incidence of overestimation or close to the upper limit of possible random variation, rather than the mean.

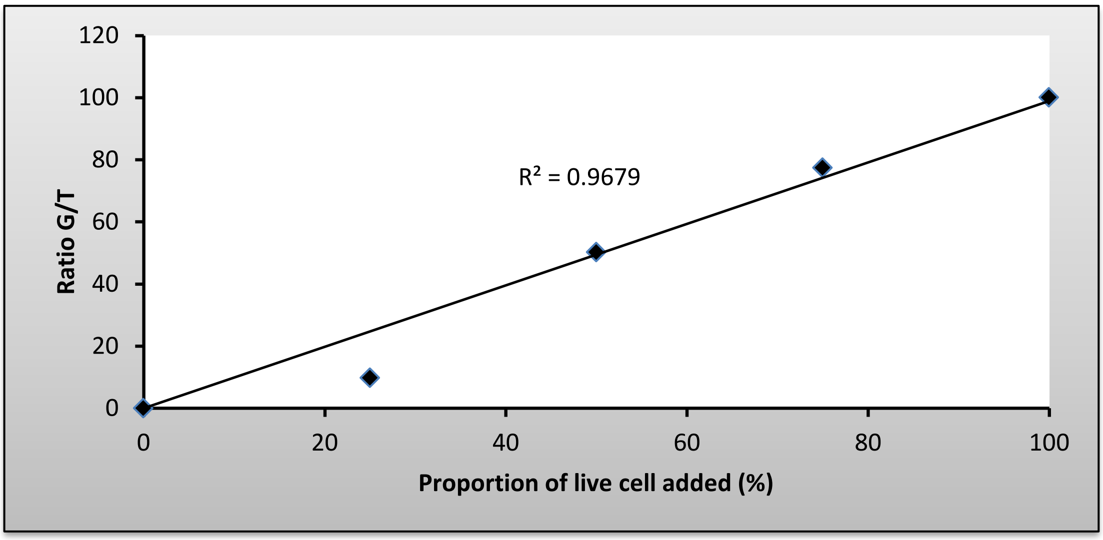
Bacterial enumeration using different dyes and its comparison with the conventional techniques

Bacterial enumeration of the eight food samples (raw milk, butter, mixed fruit juice, tamarind sauce, carrot juice, tomato sauce, samosa and kulcha) was done by DEFT (AO, DAPI, SYTO® 9 and PI stained) and compared with that of conventional techniques such as SPC and haemocytometer counts. The DEFT count using SYTO® 9 and PI dye ($44.73\pm3.5\times10^5$, $197.33\pm21.5\times10^4$ and $667.66\pm13.76\times10^4$ cells/g respectively) was comparable with that by using acridine orange dye ($35\pm1.57\times10^5$, $166\pm5.5\times10^4$ and $643.51\pm15.5\times10^4$ cells/g respectively) in butter, carrot juice and tomato sauce samples respectively, while in raw milk, samosa, mixed fruit juice, kulcha and tamarind sauce samples, higher DEFT count using SYTO® 9 and PI dye was observed (Table 2). The DEFT count using AO and SYTO® 9 and PI dye showed good agreement and accuracy in this study. However, Phe *et al*[34]., reported underestimation of cell count of *Escherichia coli* cells in chlorinated water using BacLight (SYTO® 9 and PI) kit. Similarly, for activated sludge samples, BacLight was, reportedly, not appropriate because of high background fluorescence and nonspecific binding[4].

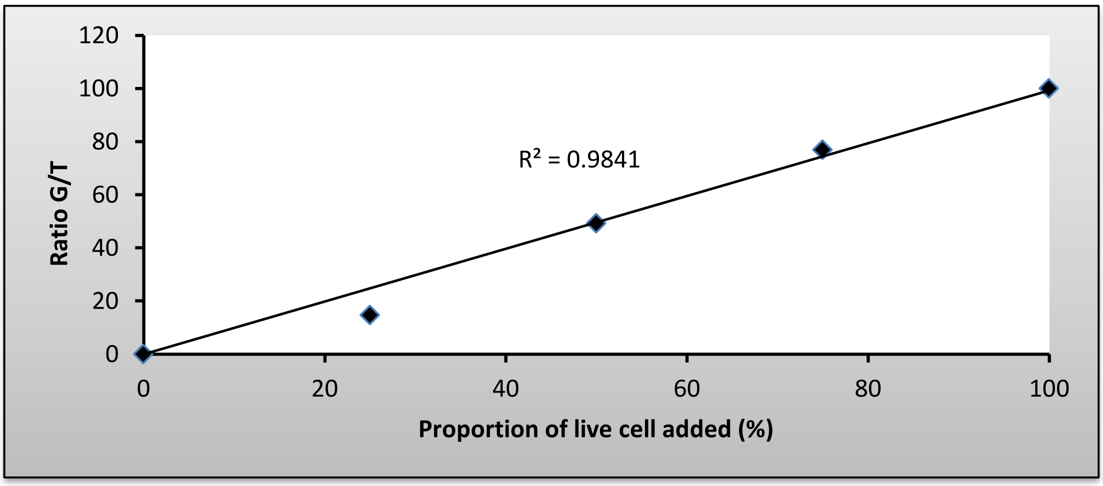
Table 2: Total bacterial densities obtained by different techniques of enumeration

Techniques/ Food Samples		DEFT using AO stain (cells/g)	DEFT using DAPI stain (cells/g)	DEFT using SYTO® 9 and PI stain (cells/g)		SPC (cfu/g)	Haemocytometer (cells/g)
				Viable Cell Count	Total Cell Count		
Dairy products	Raw milk $(\times 10^6)$	453.7^{a}±12.10	294.7^{b} ±10.52	156.03^{c} ±3.87	549.7^{a} ±24.57	132.2^{c} ±0.15	144.9^{c} ±3.61
	Butter $(\times 10^5)$	35.0^{d} ±1.57	3.2^{d} ±0.23	3.34^{d} ±0.10	44.7^{d} ±3.59	1.7^{d} ±0.01	2.10^{d} ±5.24
Fruit products	Mixed fruit juice $(\times 10^3)$	458.3^{a} ±20.15	292.3^{b} ±15.68	367.5^{b} ±12.05	524.7^{a} ±39.68	310.0^{b} ±18.35	257.0^{b} ±3.15
	Tamarind sauce$(\times 10^4)$	502.0^{a} ±34.55	25.8^{d} ±5.56	86.1^{dc} ±3.65	596.6^{a} ±21.92	31.70^{d} ±2.15	42.0^{d} ±7.49
Vegetable products	Carrot juice $(\times 10^4)$	166.0^{c} ±75.58	49.3^{d} ±1.85	64.1^{cd} ±2.64	197.3^{c} ±21.52	54.4^{cd} ±3.86	48.0^{cd} ±3.53
	Tomato sauce$(\times 10^4)$	643.5^{a} ±15.52	287.3^{b} ±4.41	278.3^{b} ±11.28	667.6^{a} ±13.76	230.33^{bc} ±15.22	203.6^{c} ±8.45
Cereal based products	Samosa $(\times 10^4)$	420.3^{b} ±29.58	111.7^{c} ±4.75	41.1^{d} ±3.53	653.7^{a} ±7.40	38.5^{d} ±1.75	7.7^{e} ±0.59
	Kulcha $(\times 10^5)$	320.0^{b} ±14.82	122.3^{c} ±8.53	25.4^{d} ±1.05	357.7^{b} ±2.31	11.3^{d} ±1.75	5.1^{e} ±0.55

* Values are mean ± Standard Deviation (n = 3) with same superscript in each row are not significantly different at $p \leq 0.05$.



(a)



(b)

Fig. 3: Percentage of live bacteria obtained by DEFT using SYTO® 9 and PI (a) *Lactobacillus* spp. (MTCC 4185) (b) *Salmonella* spp. (MTCC 1163)

The DEFT count obtained using DAPI stain in samosa, tamarind sauce, butter, kulcha, carrot juice, tomato sauce, raw milk and mixed fruit juice samples significantly decreased by 98%, 95%, 93%, 81%, 75%, 57%, 46% and 44% respectively, than that of count obtained by using SYTO® 9 and PI dye. Similarly, Seo *et al*[38]., demonstrated that the DAPI staining method produced inaccurate results for both *E. coli* and *Lactobacillus* spp. cultures, while BacLight and AO showed good agreement. Suzuki *et al*[42]., reported that DAPI staining generally resulted in smaller bacterial cell size than the AO staining, since AO binds to both DNA and RNA but DAPI binds only to DNA. Porter and Feig[35] found 16% underestimation of bacterial cells when DAPI dye was used for enumeration study. Newell *et al*[30]., suggested that this pattern was true for seawater than it was for fresh water and recognized that only a subset of AO-stained bacteria could be stained by DAPI because some cells in environments and cultures have low DNA content for various reasons, including starvation and viral lysis. McNamara *et al*[26]., also demonstrated that total bacterial numbers (TBNs) for bacterial cells in starvation-survival mode were underestimated by DAPI staining.

The bacterial count obtained by standard plate technique and haemocytometer method was comparable in all the food samples except samosa and kulcha samples. Viable bacterial count obtained by DEFT using SYTO® 9 and PI dye was significantly comparable with that of SPC count in all the food samples ($p<0.05$). Results showed a good agreement and accuracy of the developed technique, implying an acceptable interchangeability. The samples consisted of not only bacteria but also other particulate matters such as mineral particles, soot and droplets. Those particles were distinguishable from bacterial cells according to their irregular and aggregate morphology and fluorescent color. The bacterial cells were usually spherical and had a size close to or smaller than 1 μm in diameter, which was consistent with the reported size of bacteria in surface soil and sea-water[36]. As suggested by Hara *et al*[11]., and Hara and Zhang[12], mineral particles usually in irregular shape looked yellow by BacLight stain and greenish yellow or white by DAPI stain.

CONCLUSION

This study demonstrates the potential of Direct Epifluorecent Microscopic Technique (DEFT) using SYTO® 9 and PI staining to determine viable and total bacterial numbers after pretreatment in all food samples of four different catogories. The assay takes 45 to 60 min depending on process applied. The total DEFT microscopic analysis time is between 30 s and 2 min, depending on numbers of bacteria in food sample. This time frame compares favourably with current culturing methods, which take 72 h. Thus, it is a reliable, rapid and easy-to-use test and yields both viable and total counts in one step. The preparation are easy to read because of the high degree of contrast between the green color of the viable bacteria and the red color of the dead cells and also produces less background fluorescence.

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