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ORIGINAL ARTICLE

Rapid fluorometric quantification of bacterial cells using Redsafe nucleic acid stain

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ABSTRACT

Background and Objectives: Numerous procedures in biology and medicine require the counting of cells. Direct enumeration of Colony Forming Units (CFUs) is time-consuming and dreary accurate cell counting on plates with high numbers of CFUs is error prone. In this study we report a new indirect cell counting method that was developed based on the use of Redsafe fluorometric assay. The usefulness of Redsafe, a nucleic acid stain, in liquid medium is based on the binding of the fluorescent dye to DNA.
Materials and Methods: Redsafe fluorometric assay was evaluated in comparison with MTT colorimetric assay as a colourimetric assay for enumeration of bacterial cells.
Results: Obtained results showed that fluorometric assay threshold for LB grown *E. coli* is 6×10^4 CFU/ml. Redsafe fluorescent assay can be used as a rapid and inexpensive method for bacterial enumeration and quantification with increased sensitivity.
Conclusion: The sensitivity of the Redsafe fluorometric assay for detection and enumeration of bacterial cells was 2-log-unit more than that was observed for the MTT assay.

Keywords: Bacterial enumeration, Redsafe, MTT assay, fluorometric assay

INTRODUCTION

Many biological researches depend on accurate determination of bacterial quantities (1-3). Commonly, enumeration of Colony Forming Units (CFUs) that is performed with culture and plating out several serial dilutions onto culture plates is time-consuming and dreary (4). More importantly, accurate cell counting on plates with high numbers of CFUs is error prone since it requires a high level of attention and so, often only parts of a plate are analyzed to estimate the whole plate count. Therefore, rapid methods for quantification and enumeration of microorganisms are important. Also, the number of rapid tests for the estimation of the microbiological quality of foods has increased in recent years. Many tests have been proposed, including methods based on antibody recognition, a wide range of biochemical and enzymatic assays, phage probes, membrane filtration and impedance (5-7). Although, some of these methods have low accuracy for mixed bacterial populations, are expensive, and many are still too slow to provide useful result. Hence, detection and enumeration of microorganisms are very important, especially when

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investigating refrigerated food. Despite the high number of rapid methods that have been developed, the methods are generally unsatisfactory for the dairy industry as many of them are inaccurate or complex, and many are still too slow to provide useful results to the manufacturer. Furthermore, a number of the rapid methods available within the dairy industry are used to detect microorganisms rather than enumerate them. Enumeration, rather than detection, is a very important requirement when investigating food quality.

Redsafe, a safe nucleic acid stain (8-10), is developed to replace the Ethidium Bromide which is a carcinogenic fluorescent dye (11, 12). Data from the Ames-test supports that Redsafe is non carcinogenic and it can be disposed as any other non-carcinogenic fluorescent dye (13). Until now, the Redsafe fluorometric assay was not evaluated for the enumeration of *E. coli* in liquid medium.

Hence, in attempt to set up a novel and rapid method for enumeration of *E. coli*, we aimed to assess the ability of the Redsafe in order to establish a fluorometric assay and also to compare it with traditional MTT assay which is based on dehydrogenase system of cells (14, 15). The present paper reports the adaptation of the Redsafe fluorometric assay for the estimation of the microbiological quality and quantity in liquid medium.

MATERIALS AND METHODS

Bacterial growth and samples. *E. coli* origami strain was added to culture and was grown overnight in Luria-Bertani (LB) broth (1% bacto tryptone, 0.5% yeast extract, 1% NaCl) at 37 °C on a shaker at 180 rpm. *E. coli* cell growth was determined by measuring the OD of the broth at 590 nm. Then, three samples consisting of untreated, washed, and boiled bacteria were prepared. The overnight grown bacteria in LB broth was directly used as untreated bacteria. Then to wash bacteria, a 1.5 ml sample was removed from the overnight grown bacteria and placed into centrifuge tube and centrifuged at 5000g for 10 min at 4 °C. The pellet was washed with 1.5 ml of PBS for three times, resuspended in 1.5 ml of PBS and used as washed bacteria. Preparation of boiled bacterial sample was similar to washed bacteria and then followed by addition of a boiling step for 10 min in boiling water.

Determination of bacterial CFU. The number of colony forming unit (CFU) of *E. coli* cells was determined by spread plating of log 10 serial dilutions of samples, consisting of untreated and washed bacteria, on LB-agar and incubated for approximately 20 hours at 37 °C. Numbers of CFU per milliliter were calculated by multiplying the number of colonies by the dilution factor and the volume of the tested sample.

Redsafe fluorescent staining protocol. The fluorometric system is a modification of previously published by Kowalski (7), which was originally devised as a differential fluorescence system for detecting nicked circular DNA versus intact one (5). Three samples consisting of untreated, washed, and boiled bacteria were used in this regard. A total of 80µ of 1:3000 diluted Redsafe (iNtRON, South Korea) in DW was added to 3 ml of diluted bacteria in a fluorimeter cuvette. The fluorescence of the resulting solution was read just after mixing in spectrofluorometer at an excitation wavelength of 270 nm and emission detection at 537 nm to measure the emissions from the Redsafe stained cells in suspension.

MTT assay. The MTT assay was performed as described by Mosmann (16), with some modifications. Untreated and washed bacteria were used as bacterial samples in this assay. The bacterial serial dilutions (1:2) were made using PBS and two hundred microliters of each suspension was transferred to corresponded labeled tubes. Then, 20 µl of a stock solution of MTT (Sigma, UK) at 5 mg/ml in PBS was added to each tube and incubated for 20 min at 37 °C. The tubes were centrifuged at 10000×g for 1 min and the supernatants were discarded. Finally, the formazan crystals were solubilized using 100 µl of DMSO and pipetted into 96-well micro plates. Absorbance was measured with a microplate reader (Eon BioTek, USA) at wavelengths of 550 nm.

RESULTS

Fluorometric method for enumeration of *E. coli*. To develop the Redsafe fluorometric assay for enumeration of bacterial cells, serial diluted samples with defined CFU/ml were used. As illustrated in Fig. 1, fluorometric assay lower threshold for untreated LB grown *E. coli* was 5×10^5 CFU/ml and higher

threshold was 7×10^5 CFU/ml. In order to eliminate possible quenching effectors of culture medium, fluorometric assay was performed with PBS washed bacteria. After washing samples with PBS, lower and higher threshold reached to 6×10^4 and 1×10^5 CFU/ml respectively which was accompanied with increase in sensitivity by almost 7 fold. These data indicated that sensitivity of fluorometric assay is increased by washing of bacteria. Since the method described here is based on the ability of Redsafe to bind to DNA, binding of Redsafe to release bacterial DNA (boiled sample) was performed in comparison with DNA of intact bacterial cells. The results indicated that the fluorometric reading for intact bacteria and released DNA are the same (Fig. 1).

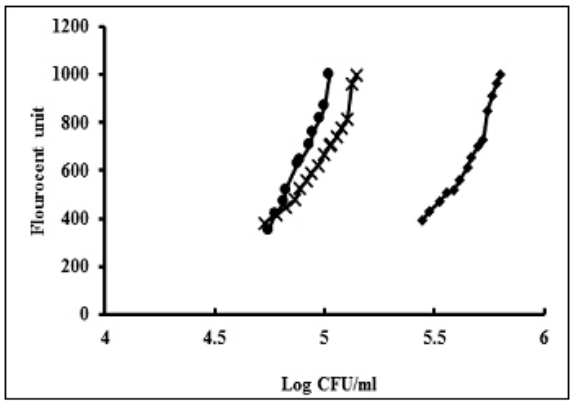

Fig. 1. Redsafe fluorometric assay. The Redsafe fluorometric assay was performed on Untreated ( ), washed ( ) and boiled ( ) bacteria samples. Lower and higher thresholds were $5x10^5$ CFU/ml and $7x10^5$ CFU/ml for untreated bacteria while for washed and boiled bacteria received to $6x10^4$ and $1x10^5$ CFU/ml.

MTT assay for enumeration of *E. coli.* In order to perform the MTT assay, serial dilutions of untreated LB grown *E. coli* with defined CFU/ml were used. Fig. 2 indicates that the lower threshold of this colorimetric assay is 5×10^7 CFU/ml. The higher threshold is about 5×10^8 CFU/ml as the linearity of the assay is lost at higher concentrations. In order to eliminate the possible effects of released reductases from dead cells the MTT assay was performed with washed bacterial cells as well. The results indicate that centrifugation has not effect on sensitivity of MTT assay (Fig. 2).

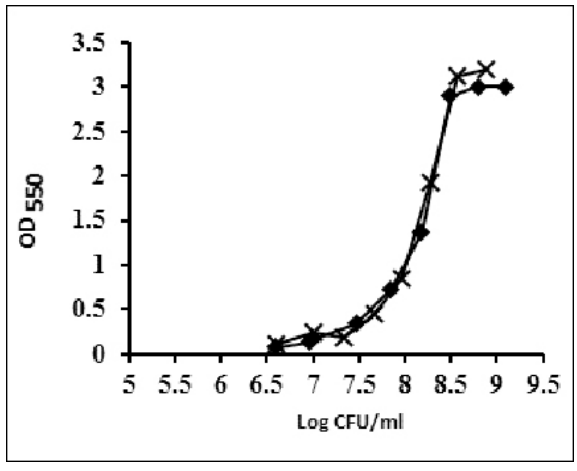

Fig. 2. MTT assay: The MTT assay was performed on Untreated bacteria ( ) and washed bacteria ( ). Lower and higher thresholds of this colorimetric assay were $5x10^7$CFU/ml and $5x10^8$ CFU/ml, respectively.

DISCUSSION

Enumeration and detection of bacterial cells is very important requirement for evaluating food quality. Several indirect methods have been yet proposed so far as rapid method for enumeration of cells (16-20). The use of Redsafe, a newer florescent dye, has been proposed for assessment of nucleic acids but it has never been reported as a fluorometric dye for enumeration of bacteria. This study, for the first time, presents a simple protocol for rapid enumeration of *E. coli* cells by the Redsafe fluorometric assay. The results showed that linear relationship between the emission of the Redsafe dye and the bacterial concentration was stronger when the concentration of *E. coli* cells ranged between 8×10^4 and 1×10^5 CFU/ml. Notably, our results showed that washing of bacterial cells can result in increase in sensitivity by almost 7 fold, probably due to elimination of quenching effectors in LB medium. We also found no augmentation in the Redsafe fluorometric assay measurements following boiling of bacterial cells. Therefore, it can be deduced that Redsafe can conveniently access to DNA of intact bacterial cells and released bacterial DNA.

MTT reduction assay for enumeration of bacterial cells has been reported in previous studies (18, 19, 21). In consistence with other studies, this work showed that the linear relationship between the bacterial concentration and the absorbance of the resultant formazan dye was stronger when the concentra-

tion of *E. coli* ranged between 5×10^7 and 5×10^8 CFU/ml (18, 19, 21). These results showed that required amount of bacteria for Redsafe fluorometric assay is 2-log unit less than that needed for tradition MTT assay. Since Redsafe binds to bacterial cell DNA, Redsafe fluorescent assay can be used to count all type of bacterial cells but MTT assay cannot be applied on bacterial cells without NAD(P)H-dependent oxidoreductases and dehydrogenases (22). While MTT assay only count the living cells Redsafe fluorometric assay counts the dead and live cells which can be helpful in quality assessment of heated and refrigerated food. So, enumeration and detection of bacterial cells is very important requirement when assaying of food quality.

In conclusion, the sensitivity of the Redsafe assay was 6×10^4 CFU/ mL. This sensitivity limit complies with the level of detection required to satisfy regulations in many countries. Results obtained from the present study clearly indicates that the Redsafe fluorescent assay can be used as a rapid and inexpensive method for bacterial quantification and enumeration. This method has the additional advantage that it can be performed in a 96-well microplate format for assessment of large numbers of samples. The sensitivity and the adaptability of the Redsafe fluorometric assay make it an efficient high throughput screening tool for epidemiological and diagnostic investigations, and rapid microbial analysis in food and drink industries.

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