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Using Direct Epifluorescent Microscopic Count for Rapid Enumeration of Viable Yeast and Bacteria in Injured Conditions

CHIN-CHENG HUANG[1]*, SHWU-MEEI LIAU[1], WEI-CHUNG TSAI[1] AND HSI-HUA WANG[2]

[1.] *Food Industry Research and Development Institute, Hsinchu, Taiwan, R.O.C.*

[2.] *Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, R.O.C.*

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ABSTRACT

Traditional method for assessing cell count requires an incubation period of 2~3 days. The direct microscopic count (DMC) method gives rapid enumeration of yeast and bacteria. However, the application is limited, since it is not possible to distinguish accurately between viable and nonviable cell. In this report, several nucleic acid dyes from Molecular Probe Inc. were used to stain the cells for enumeration of live and dead yeast in injured conditions, in which the dye can stain the DNA of nucleus and mitochondria, which then fluoresce yellow under fluorescence microscope, as well as stain the cytoplasm of actively growing cells to fluoresce green, while the cytoplasm of inactive cells fluoresced orange. The direct microscopic count multiplied by the live cell count ratio obtained by this direct epifluoroscent microscopic count (DEMC) method gives the estimated viable yeast count. According to our results, the correlation coefficient (R^2) between the cell count obtained by the DEMC method and that by the standard plate count was 0.91 for yeast and 0.96 for bacteria in frozen and heating conditions, respectively. Requiring only 30 min, this method can also be used for the rapid enumeration of viable bacteria in injured conditions.

Key words: live cell nucleic acid stain, direct epifluorescent microscopic count, direct microscopic count, viable yeast, injured cell, rapid enumeration

INTRODUCTION

The conventional test of food microorganism detection is generally impractical mainly because it requires 2-3 days of incubation, during which the food products may have already been packaged, distributed, sold or even eaten by consumers. A rapid and effective test can save cost and time and has, therefore, been developed one after another.

The direct microscopic count (DMC) method is rapid but unable to distinguish viable cells from dead cells or food particles. This makes DMC-estimated cell counts higher than those of conventional tests[(1)], and much more inaccurate especially when samples are abundant with dead cells. Direct epifluorescent microscopic count (DEMC) method was developed by combining DMC method and fluorescent staining techniques, applied in the enumeration of viable bacteria in raw milk[(2)]. Then it is also useful for viable bacterial counts in ground beef and pork if sample pretreatment is modified and some required enzyme are added[(3,4,5,6,7)]. Direct epifluorescent filter technique (DEFT), a rapid test by taking viable cells with germ-free cotton stick from slaughtered body on the lines in slaughter plant, shared a correlation coefficient of 0.87 with total yeast plate count. Each sample could be performed in 15 min[(8)]. This technique was applied for the control of quality for the hazard analysis critical control points (HACCP) on producing lines. Therefore, many researchers believe that modified DEMC will become the main rapid test in viable cell count if higher accuracy can be achieved[(8)].

Fluorescent dye is the most important factor contributing to successful DEMC. Acridine orange, the first stained dye in yeast, turns into green fluorescence in viable cells and orange fluorescence in dead cells[(9)]. Its later adoption for the detection of yeast contamination in beer revealed of poor correlation between cell activity and fluorescent color, despite the declared ability to differentiate viable and dead cell distinction[(10)]. A sample pre-staining with methylene blue was recommended to reduce the high orange fluorescence of dead cells. Rodrigues and Kroll[(11)] asserted microscopic detection with acridine orange staining could not identify heat-killed yeast from the rest. They developed an overcoming solution by combining two fluorescent dyes, acridine orange and Janus Green B. Polycarbonate membrane was used to filter yeast samples, sequentially incubated 3~5 hr in tryptone soya broth for cell recovery and 6 hr on nutrient agar for final microcolony count by DEFT. Bromocresol purple (BCP) was used by Kurzweilova and Sigler[(12)] as a fluorescent dye to stain dead yeast cells for a rapid test, which BCP associates with dead yeast of plasma membrane damage and therefore is inappropriate for heated or frozen food samples, either. Despite the ability to separate viable and dead cell, polymerase chain reaction (PCR) and DNA probes[(13,14)] are not yet able to quantify the kinds of cells contained in a sample.

* Author for correspondence. Tel: +886-3-5223191 ext. 252; Fax: +886-3-5214016; E-mail: cch31@firdi.org.tw

Recently, Molecular Probes Co. has developed a series of dyes, SYTO live cell nucleic acid stains, having a property to penetrate through cell membrane and binding with nucleic acid for fluorescent excitation and spectra emission. Some studies with SYTO dyes application have been reported, such as both mitotic orientation of cerebral cortex in development[15] and apoptosis of mouse thymus[16] with SYTO 11 and bacteria recognition as living or dead in water by flow cytometer with SYTO 16[17]. In this report, we exploited SYTO dyes to count live and dead yeasts and bacteria in heated or frozen cell samples for the live cell ratio, multiplying the total cell number of DMC to figure out viable cell count. The estimated yeast and bacterial counts were compared with those obtained by the total yeast and bacterial plate count (TYPC and BCPC) for their mutual correlation and evaluated if the 30 min DEMC could be a reliable rapid test in living cell enumeration.

MATERIALS AND METHODS

I. *Yeast Strain, Medium and Growth Conditions*

The pure cultures of *Saccharomyces cerevisiae* BCRC 21494 (= 4126 ATCC = 1200 CBS) and *Bacillus cereus* BCRC 10603 were purchased from the Bioresource Collection and Research Center (BCRC) of Food Industry Research and Development Institute (FIRDI) in Hsinchu, Taiwan. The yeasts were incubated and shaken in YMB medium (yeast malt broth containing yeast extract 3 g/L, malt extract 3 g/L, and peptone 5 g/L) for 3-5 days. *Bacillus cereus* BCRC 10603 was incubated in MYP broth (mannitol yolk polymyxin containing meat extract 1 g/L, peptone 10 g/L, D-mannitol 10 g/L, sodium chloride 10 g/L, and phenol red 0.025 g/L), then centrifuged at 8,000×g for 2 min in Eppendorf tubes. Cell pellets were washed with phosphate buffer saline (PBS).

II. *Direct Microscopic Count (DMC)*

According to Splittstoesser's method[1], 1 mL of homogenized cell suspension was pipetted onto the slide on hemocytometer (Cambridge Instruments Inc, Buffalo, NY, USA) and covered with a cover glass for phase-contrast microscopic observation at 400 × magnification powers. Note the depth of the chamber and the area of grid were 0.1 mm and 1 mm^2, respectively, the volume of sample applied to the counting chamber was 1.0×10^{-4} mL. The quotient of sample volume on the slide (1.0×10^{-4} mL) and magnification power (400) was 2.5×10^{-7} mL, representing the volume of sample covering a unit square. The cell numbers per each unit square from 15 views were summed for average. When cell numbers were lower than 10^4 count/mL, 0.2 μm polycarbonate membrane (Nuclepore PC, Corning Costar, UK) was used to filter cell suspension[11]. Afterwards, the polycarbonate membrane was placed directly under a phase-contrast microscope for counting cell numbers. Therefore,

Direct microscopic count (count/mL) = Average cell number per unit square / (Diluting factor × 2.5×10^{-7} mL)

III. *Direct Epifluorescent Microscopic Count (DEMC)*

Fifty microliter of the homogenized cell suspension mentioned above was taken onto a slide or a polycarbonate membrane used for filtering microorganisms, and stained with a series of SYTO 11~16 live cell nucleic acid stains (Molecular Probes, Inc., Eugene, Oregon, USA). According to the method of Lebaron *et al*.[17], the final concentration of dyes mixed well with the homogenized yeast suspension was 5 μmol/L. The sample-holding slides were held in darkness for 15 min and then placed under 100 × oil immersion objective epiflurorescent microscope (OPTIPHOT-2, Nikon with a 100 W mercury vapor lamp) after removal of cover glass. Viable cells of green fluorescence and dead cells of orange fluorescence were summed up from a total of 15 fields. The quotient of live cell count and total cell count was the ratio of live cell, designated as live yeast count ratio or live bacterial count ratio.

IV. *Estimated Live Yeast Count*

The total cell number from DMC was multiplied by the live yeast count ratio from DEMC mentioned above as estimated live cell count.

V. *Total Yeast Plate Count (TYPC) and Bacillus Cereus Plate Count (BCPC)*

After dilution, 1 mL of the yeast suspension was taken into a culture dish and pour-plated with potato dextrose agar (PDA) containing 40 ppm of streptomycin sulfate for a 3-day incubation. The *Bacillus cereus* count was taken into a culture dish and spread-plated with MYB agar at 37°C for a 2-day incubation. The number of cell colony was counted in CFU/mL.

VI. *Comparison of Cell Counts after Heating and Freezing Treatments*

Yeast and bacterial suspension of 10^4~10^7 CFU/mL in PBS was allotted by 10 mL into test tubes, incubated in water bath of 50, 60 or 70°C for 20 min or frozen at -18 or -40°C for 4 hr. Samples were then cooled down or warmed up to room temperature by running cool or heating water, respectively. If cell numbers were lower than 10^4 CFU/mL, cell suspension was filtered through polycarbonate membrane. Viable cell counts were then taken by DMC or DEMC and compared with TYPC and BCPC. The logarithms of cell counts estimated by TPC and those by DEMC were plotted on the X- and Y-axis, respectively. The SAS 6.12 software was used to perform sign test and signed rank (Wilcoxon) test for the correlation coefficient between two variables X and Y.

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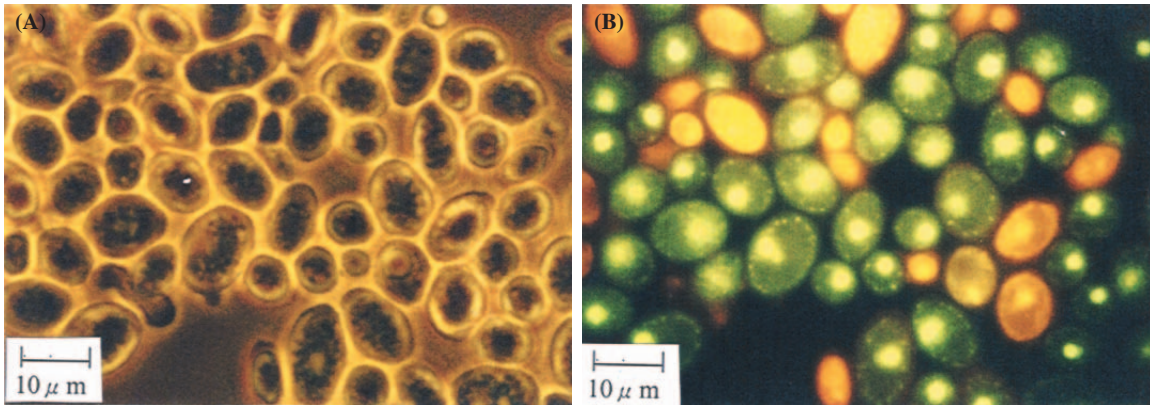

Figure 1. SYTO 11 nucleic acid stain of *Saccharomyces cerevisiae* BCRC 21494 cells, (A) phase contrast microscopic photograph, (B) direct epifluorescent microscopic photograph.

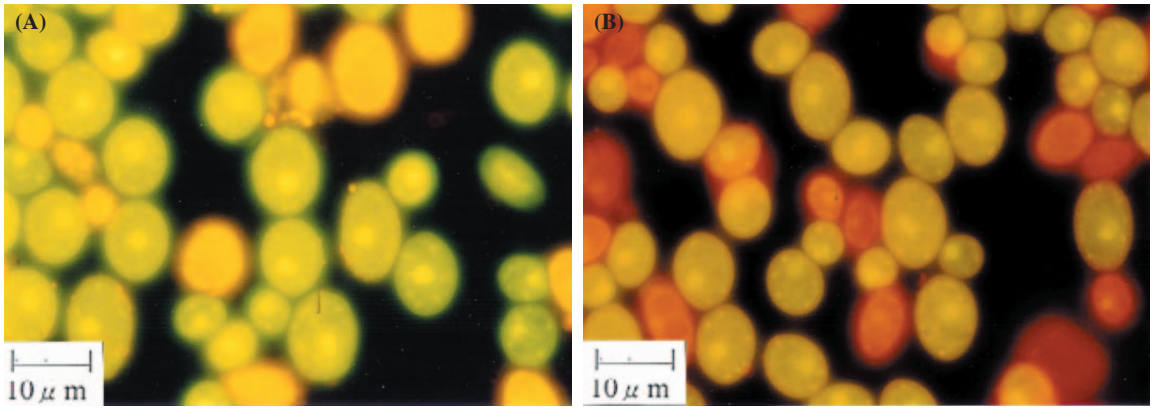

Figure 2. Direct epifluorescent microscopic photograph of *Saccharomyces cerevisiae* BCRC 21494 cells, (A) SYTO13, (B) SYTO15.

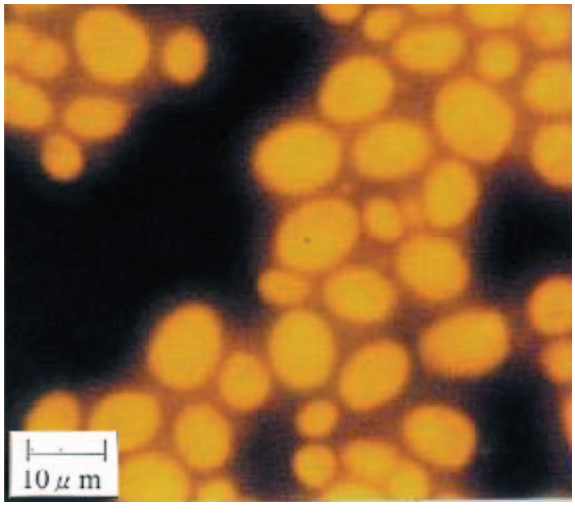

Figure 3. Direct epifluorescent microscopic photograph of *Saccharomyces cerevisiae* BCRC 21494 cells after heated at 70°C for 20 min and stained with SYTO 11 nucleic acid stain.

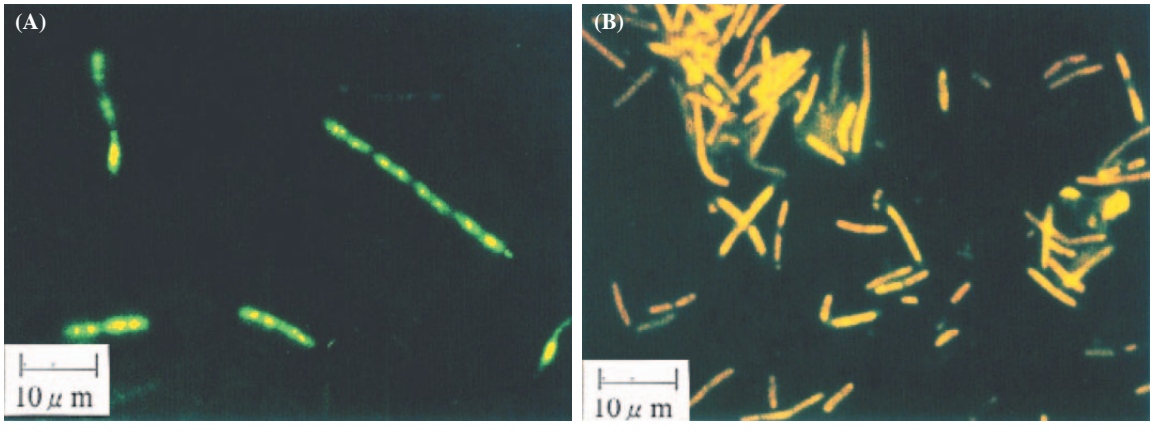

Figure 4. Direct epifluorescent microscopic photograph of *Bacillus cereus* BCRC 10603 after stained with SYTO 11 nucleic acid stain, (A) vegetative cells, (B) after heated at 70°C for 20 min.

RESULTS

I. *Staining of Live and Dead Yeast Cells*

Saccharomyces cerevisiae BCRC 21494 was cultured in YMB and harvested in stationary phase for SYTO dyes staining. Samples stained with SYTO 11, SYTO 13 and SYTO 15 dyes exhibited the highest intensities of fluorescence, especially those stained with SYTO 11 (as shown in Figure 1B). Figure 1A shows an image of phase-contrast microscope showing yeast morphology. The fluorescence intensities of SYTO 13 and SYTO 15 dye staining are shown in Figure 2. SYTO 13 emitted green and yellow fluorescence whereas SYTO 15 produced yellow-green and red fluorescence. The contrast between the two colors yielded by SYTO 13 or SYTO 15 for cell nucleus and cytoplasm was lower than that done by SYTO 11. Among the series of SYTO dyes, SYTO 11 gave the most intensive and clearest fluorescence as shown in Figure 1B.

Two fluorescent colors were visible. For viable cells, cell nucleus and mitochondria near to plasma membrane were obviously yellow fluorescence and cytoplasm was green fluorescence. For dead cells, cytoplasm presented orange fluorescence but cell nucleus and mitochondria produced rather weak yellow fluorescence. It was undestructed or undegraded DNA that the SYTO dye bound with to stain yeast cells yellow fluorescent, while heat-altered DNA in heat-treated yeast cells could not bind with SYTO dye. A photograph of phase contrast microscope of yeast morphology (Figure 1A) shows that little difference between living and dead cells could be observed. After heating at 70˚C for 20 min and then subject to dye staining, yeast and bacterial cells became completely orange fluorescent. Cell nucleus and mitochondria showed the disappearance of yellow fluorescence, as well as the weakness of orange nimbus surrounding the cell external (Figure 3).

Figure 4 shows the staining result of *Bacillus cereus* BCRC 10603 cells, stained with SYTO 11 dye after heated at 70˚C for 20 min. The determination criteria for dead and viable cells were consistent with those for yeast. Cells emitting green fluorescence in cytoplasm and yellow in nuclear area were viable *B. cereus*, while those showing orange fluorescence in both portions were dead *B. cereus*.

II. *Comparison with TYPC in Injured Conditions*

Table 1 compares DEMC, DMC and TYPC in viable cell counts of heated or frozen *Sacchaoromyces cerevisiae* BCRC 21494. The yeast suspensions without heating or freezing treatment were counted simultaneously as control. Controls were at two different densities of *Sacchaoromyces cerevisiae* BCRC 21494 cells receiving no heating or freezing treatment. The DMC-estimated counts were in a range of $2.2\sim2.8 \times 10^7$ cell/mL (high concentrations), $3.9\sim5.4 \times 10^4$ cell/mL (low concentrations) apparently including a great portion as variation attributed to dead cells caused by heating. DEMC could correct the variation of DMC count by a correcting factor, (i.e. the live yeast count ratio in Table 1), which was the ratio of viable yeast cell (green fluorescence) number to the sum of both viable and dead cells. The ratio decreased with the increase of heating temperature (50, 60 and 70˚C), but was not significant of two frozen-treatment (-18 and -40˚C). After multiplying by the correct factor, the counts of DMC became the estimated live yeast counts of DEMC, which were rather close to the total yeast plate count of TYPC. The correlation coefficient was 0.91 (Figure 5).

Table 1. Comparison between direct microscopic count (DMC), direct epifluorescent microscopic count (DEMC) and total yeast plate count (TYPC) of *Saccharomyces cerevisiae* BCRC 21494 cells after heating or freezing treatments at different temperatures

Treatment	DMC* (cell/mL)	DEMC		TYPC*** (CFU/mL)
		Live yeast count ratio (%)	Estimated live yeast** (10^6 cells/mL)	
None (control)	$(31 \pm 12)\cdot10^6$	96 ± 2	$(30 \pm 2)\cdot10^6$	$(17 \pm 2)\cdot10^6$
	$(60 \pm 10)\cdot10^3$	94 ± 3	$(56 \pm 3)\cdot10^3$	$(48 \pm 3)\cdot10^3$
Heating				
50˚C, 20 min	$(28 \pm 8)\cdot10^6$	93 ± 4	$(26 \pm 3)\cdot10^6$	$(14 \pm 4)\cdot10^6$
	$(49 \pm 12)\cdot10^3$	93 ± 5	$(46 \pm 6)\cdot10^3$	$(39 \pm 3)\cdot10^3$
60˚C, 20 min	$(22 \pm 7)\cdot10^6$	4 ± 1	$(88 \pm 1)\cdot10^4$	$(87 \pm 7)\cdot10^3$
	$(54 \pm 15)\cdot10^3$	7 ± 1	$(38 \pm 2)\cdot10^2$	$(88 \pm 8)\cdot10^2$
70˚C, 20 min	$(25 \pm 10)\cdot10^6$	0	0	$(40 \pm 4)\cdot10^1$
	$(39 \pm 10)\cdot10^3$	0	0	0
Freezing				
-18˚C, 4 hr	$(22 \pm 7)\cdot10^6$	83 ± 4	$(18 \pm 3)\cdot10^6$	$(67 \pm 5)\cdot10^5$
	$(31 \pm 5)\cdot10^3$	75 ± 5	$(27 \pm 3)\cdot10^3$	$(18 \pm 3)\cdot10^3$
-40˚C, 4 hr	$(27 \pm 10)\cdot10^6$	85 ± 3	$(23 \pm 3)\cdot10^6$	$(71 \pm 3)\cdot10^5$
	$(39 \pm 4)\cdot10^3$	82 ± 4	$(31 \pm 2)\cdot10^3$	$(25 \pm 5)\cdot10^3$

*Data are means ± standard deviation of 15 views.

**Estimated live yeast count = DMC × live yeast count ratio.

***Data are means ± SD of 3 replicates.

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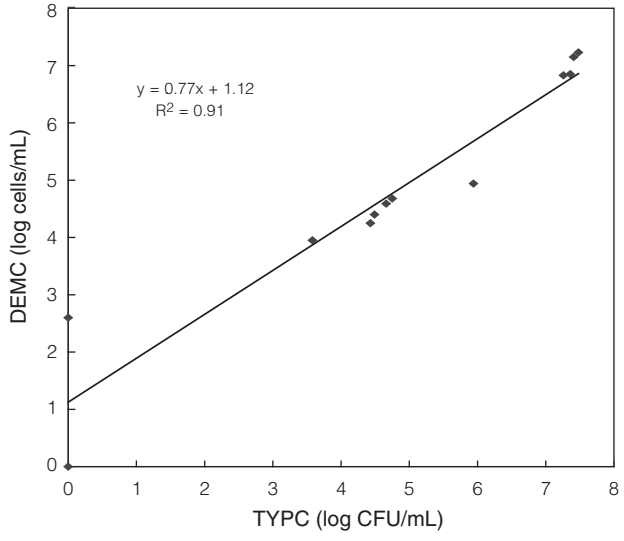

Figure 5. Relationship between the estimated live yeast count (DEMC) and total yeast plate count (TYPC) (also see Table 1).

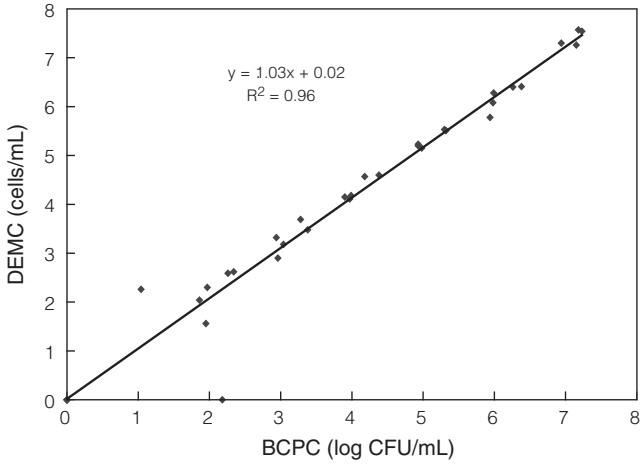

Figure 6. Relationship between the estimated live *Bacillus cereus* count (DEMC) and *Bacillus cereus* plate count (BCPC) (also see Table 2).

The difference between the live yeast counts of DEMC and TYPC (Table 1) became greater when cells underwent 60˚C or 70˚C heating treatment for 20 min. The DEMC counts were approximately 10 times higher than TYPC counts after 60˚C heating treatment, but became less than TYPC counts after 70˚C heating treatment. Since heating or freezing treatment can cause cell death, plus DMC method is not capable of distinguishing viable cells from dead cells, cells either live or dead could be counted by DMC method after heated at 60˚C or 70˚C for 20 min. Therefore, a substantial error would be made in estimation of viable cell count.

Table 2 compares the counting results of *Bacillus cereus* BCRC 10603 estimated by DEMC, DMC and conventional BCPC methods after heating or freezing treatment. Controls were six different concentrations of *Bacillus cereus* cells receiving no heating or freezing treatment because cells on day 2 were in exponential growth phase without any production of endospores. Correction could be carried out for DMC method by using DEMC method with a correcting factor, defined as the ratio of viable cell count to total cell number (i.e. live bacterial count ratio in Table 2). Viable cells were represented by green-fluorescent cells under fluorescence microscope. This ratio would be reduced inversely with increased extent of heating. Corrected value calculated by multiplying DMC count with correcting factor obtained from DEMC method was exactly the live bacterial count estimated by DEMC method, which was very close to total viable plate count with conventional BCPC method. The correlation coefficient (R^2) between the two estimates was 0.96. As shown in Figure 6, live bacterial counts estimated by DEMC were 1-4 folds higher than the values with conventional BCPC (except for groups of maximal bacterial counts with treatment of heating at 60˚C or 70˚C for 20 min). Possible reasons were the same as the deduction for yeast counting mentioned above.

DISCUSSIONS

SYTO 11 brought in the best staining efficiency on yeast cells in this study (Figure 1B). Live yeast nucleus and mitochondria were stained yellow fluorescent and cytoplasm was stained green fluorescent, while dead cells presented orange fluorescence in cytoplasm and weak yellow fluorescence in cell nucleus and mitochondria (Figure 3). After heat treatment, cytoplasm content once leaking away through heat-destructed cell wall displayed orange fluorescence with SYTO dyes, and the nucleic acid with heat-altered conformation in nucleus and mitochondria could not bind with the dyes to exhibit yellow fluorescence.

As how to identify viable and dead cells, SYTO 11 seems to be more advantageous than acridine orange which merely stains cells with different fluorescent color. By introducing the life status-distinctive feature of SYTO dyes, DMC might be improved and considered for a viable cell count method. As shown in Figure 1B, 60 intact yeast cells (47 green fluorescent and 13 orange fluorescent) were observed in the epifluorescent microscope field. The viable cell count ratio of this field was 47/60, which was unavoidably different from those of other fields. We, therefore, reduced the possible variation by using the mean of the ratios in 15 fields.

The differences among SYTO nuclei acid stains were mainly the permeability to cell walls, and membrane, fluorescent intensity, and affinity for nuclei acid. Currently, SYTO dyes have been applied to a wide range of cell staining, including animal cells, yeast and bacteria. Numerous studies have adopted SYTO dyes as the main staining reagents, especially SYTO 13 dye. For instance, *Pseudomonas aeruginosa* grown in waste frying oil with intracellular accumulation of polyhydroxyalkanoates[18], human bone marrow cells counted by flow cytometry[19], healthy and apoptotic cells[20], *Escherichia coli* RNA and DNA[21] were all stained with SYTO 13 dye, and Gram-

positive and Gram-negative bacteria could be distinguished with SYTO 13 dye and hexidium iodide (HI)[22]. On the other hand, SYTO 16 dye has been used to distinguish apoptotic and nonapoptotic mouse thymocytes[16].

Tables 1 and 2 show the comparison of viable yeast and bacterial counts of DMC, DEMC, and cultural method without and after heating or freezing treatments. The DMC method caused a rather large variation because of the inability to distinguish viable and dead cells. The DEMC method could make a correction for DMC by using live yeast count ratio. The estimated live cell counts method shared a correlation coefficient of 0.91 and 0.96 with the counts of TYPC and BCPC. When viable yeasts was over 10^6 count/mL, however, DEMC-estimated live yeast counts were 1~2 times higher than those of TYPC and BCPC. The possible reasons were: (1) one yeast and bacterial cell right before the accomplishment of budding or fission was counted as two by DEMC, but grows into one colony in TYPC, (2) some yeasts form colonies slowly, especially if damaged by physic or chemical treatments[2], (3) the temperature of culture medium in TYPC for pour plate was about 55°C, which might further damage or kill yeasts and bacteria, and resulted in lower estimated counts.

After heated at 50°C for 20 min, 93% of yeast cells (Table 1) and 86-93% of bacterial cells (Table 2) still presented green fluorescence. This result was similar to that by Martinez de Maranon *et al.*[23], in which the yeast activity and cell membrane integrity remained well after heated at

Table 2. Comparison between direct microscopic count (DMC), direct epifluorescent microscopic count (DEMC) and *Bacillus cereus* BCRC 10603 plate count (BCPC) vegetative cells after heating or freezing treatments at different temperatures

Treatment	DMC* (cell/mL)*	DEMC		BCPC*** (CFU/mL)
		Live cell count ratio (%)	Estimated live cells** (10^6 cells/mL)	
None (control)	a. $(37 \pm 10) \cdot 10^6$	a. 96 ± 3	a. $(35 \pm 3) \cdot 10^6$	a. $(17 \pm 2) \cdot 10^6$
	b. $(28 \pm 10) \cdot 10^5$	b. 94 ± 4	b. $(26 \pm 4) \cdot 10^5$	b. $(24 \pm 3) \cdot 10^5$
	c. $(35 \pm 14) \cdot 10^4$	c. 98 ± 2	c. $(34 \pm 3) \cdot 10^4$	c. $(20 \pm 7) \cdot 10^4$
	d. $(42 \pm 20) \cdot 10^3$	d. 94 ± 5	d. $(40 \pm 10) \cdot 10^3$	d. $(24 \pm 4) \cdot 10^3$
	e. $(51 \pm 18) \cdot 10^2$	e. 97 ± 2	e. $(49 \pm 4) \cdot 10^2$	e. $(19 \pm 5) \cdot 10^2$
	f. $(45 \pm 23) \cdot 10^1$	f. 95 ± 3	f. $(42 \pm 7) \cdot 10^1$	f. $(22 \pm 5) \cdot 10^1$
Heating 50°C, 20 min	a. $(19 \pm 4) \cdot 10^6$	a. 93 ± 7	a. $(18 \pm 3) \cdot 10^6$	a. $(14 \pm 4) \cdot 10^6$
	b. $(21 \pm 12) \cdot 10^5$	b. 90 ± 6	b. $(19 \pm 7) \cdot 10^5$	b. $(98 \pm 12) \cdot 10^4$
	c. $(18 \pm 10) \cdot 10^4$	c. 87 ± 8	c. $(16 \pm 8) \cdot 10^4$	c. $(86 \pm 10) \cdot 10^3$
	d. $(15 \pm 9) \cdot 10^3$	d. 89 ± 10	d. $(13 \pm 9) \cdot 10^3$	d. $(93 \pm 9) \cdot 10^2$
	e. $(18 \pm 5) \cdot 10^2$	e. 86 ± 11	e. $(15 \pm 6) \cdot 10^2$	e. $(11 \pm 1) \cdot 10^2$
	f. $(23 \pm 7) \cdot 10^1$	f. 88 ± 10	f. $(20 \pm 7) \cdot 10^1$	f. $(93 \pm 7) \cdot 10^0$
Heating 60°C, 20 min	a. $(15 \pm 8) \cdot 10^6$	a. 4 ± 1	a. $(6 \pm 1) \cdot 10^5$	a. $(87 \pm 7) \cdot 10^4$
	b. $(21 \pm 12) \cdot 10^5$	b. 7 ± 2	b. $(14 \pm 2) \cdot 10^4$	b. $(95 \pm 8) \cdot 10^3$
	c. $(18 \pm 10) \cdot 10^4$	c. 8 ± 2	c. $(14 \pm 2) \cdot 10^3$	c. $(79 \pm 10) \cdot 10^2$
	d. $(15 \pm 9) \cdot 10^3$	d. 5 ± 2	d. $(8 \pm 2) \cdot 10^2$	d. $(92 \pm 12) \cdot 10^1$
	e. $(18 \pm 5) \cdot 10^2$	e. 6 ± 2	e. $(11 \pm 1) \cdot 10^1$	e. $(73 \pm 10) \cdot 10^0$
	f. $(23 \pm 7) \cdot 10^1$	f. 8 ± 2	f. $(18 \pm 1) \cdot 10^0$	f. $(11 \pm 3) \cdot 10^0$
Heating 70°C, 20 min	a. $(27 \pm 8) \cdot 10^6$	0	0	$(40 \pm 4) \cdot 10^1$
	b. $(21 \pm 10) \cdot 10^5$	0	0	0
	c. $(19 \pm 12) \cdot 10^4$	0	0	0
	d. $(23 \pm 5) \cdot 10^3$	0	0	0
	e. $(31 \pm 12) \cdot 10^2$	0	0	0
	f. $(24 \pm 8) \cdot 10^1$	0	0	0
Freezing -18°C, 4 hr	a. $(25 \pm 10) \cdot 10^6$	a. 83 ± 5	a. $(20 \pm 5) \cdot 10^6$	a. $(87 \pm 5) \cdot 10^5$
	b. $(15 \pm 8) \cdot 10^5$	b. 80 ± 3	b. $(12 \pm 2) \cdot 10^5$	b. $(96 \pm 7) \cdot 10^4$
	c. $(21 \pm 5) \cdot 10^4$	c. 82 ± 3	c. $(17 \pm 2) \cdot 10^4$	c. $(85 \pm 5) \cdot 10^3$
	d. $(19 \pm 8) \cdot 10^3$	d. 79 ± 4	d. $(15 \pm 3) \cdot 10^3$	d. $(98 \pm 8) \cdot 10^2$
	e. $(25 \pm 4) \cdot 10^2$	e. 84 ± 2	e. $(21 \pm 1) \cdot 10^2$	e. $(87 \pm 4) \cdot 10^1$
	f. $(45 \pm 8) \cdot 10^1$	f. 81 ± 7	f. $(36 \pm 6) \cdot 10^1$	f. $(89 \pm 6) \cdot 10^0$
Freezing -40°C, 4 hr	a. $(37 \pm 10) \cdot 10^6$	a. 85 ± 4	a.($37 \pm 4) \cdot 10^6$	a. $(15 \pm 2) \cdot 10^6$
	b. $(28 \pm 12) \cdot 10^5$	b. 88 ± 9	b. $(25 \pm 11) \cdot 10^5$	b. $(18 \pm 4) \cdot 10^5$
	c. $(38 \pm 14) \cdot 10^4$	c. 85 ± 6	c. $(32 \pm 8) \cdot 10^4$	c. $(21 \pm 5) \cdot 10^4$
	d. $(42 \pm 8) \cdot 10^3$	d. 87 ± 9	d. $(37 \pm 7) \cdot 10^3$	d. $(15 \pm 4) \cdot 10^3$
	e. $(35 \pm 5) \cdot 10^2$	e. 87 ± 5	e. $(30 \pm 3) \cdot 10^2$	e. $(24 \pm 6) \cdot 10^2$
	f. $(34 \pm 7) \cdot 10^1$	f. 84 ± 7	f. $(29 \pm 5) \cdot 10^1$	f. $(18 \pm 7) \cdot 10^1$

*Data are means ± standard deviation of 15 views.

**Estimated live bacterial count = DMC × live cell count ratio.

***Data are means ± SD of 3 replicates.

50˚C for 1 hr. However, the number of green fluorescence cells dropped to 4% of high yeast count, and 7% of low yeast count (4-8% of the bacterial cells) after heated at 60˚C for 20 min, although most cells were morphologically intact. Eventually, cell integrity was broken and no cell was green fluorescent after heated at 70˚C or 20 min. After freezing treatment at -18 or -40˚C for 4 hr, 75-85% of yeast cell (79-88% of the bacterial cells) still exhibited green fluorescence, and the DEMC-estimated counts were approximately 3 times of the cells still exhibited green fluorescence and the DEMC-estimated live yeast counts were approximately 3 times those of TYPC and BCPC. It might be because the freezing-injured cells required a longer time than 3 days for recovery and incubation in TYPC and BCPC.

As for the reason why DEMC-estimated live yeast counts were about 10 times higher than TYPC-estimated ones after 60˚C heating for 20 min, it might be because the heating injury of yeast cells became worse or lethal during pour plate at 55˚C in TYPC. It was likely that the heating-injured yeast cells required a longer time for recovery and incubation. The vanished DEMC-estimated live yeast counts after 70˚C heating might be attributed to the difficulty of finding approximately 400 viable yeast cells among 250 million cells in 1 mL under epifluorescent microscope. In spite of this difficulty, the DEMC method developed in this study was still valuable because it could offer useful information on the existence and ratio of dead yeast cells in food samples within 30 min starting from sample preparation. It was exactly what the TYPC method could not give.

In addition to rapid detection, the DEMC method adopted in the present study also provided a wide range of advantages, including free of radioactivity, easy operation, direct observation on microbial morphology, and direct determination for contaminant bacteria if found. In the present study, polycarbonate membrane was adopted to concentrate bacterial cells to overcome the detection limit of 10^4 CFU/mL. When food particles are present in practical situation, how to separate microbial cells from food particles remains a challenge.

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