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A new method to determine initial viability of entrapped cells using fluorescent nucleic acid staining

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ABSTRACT

Entrapped bacterial cells are widely used in several biotechnological applications. Cell entrapment procedures are known to affect the viability of bacterial cells. To determine the effect of entrapment procedures on viability of bacterial cells, dissolution of the entrapment matrices using chelating agents or heat is required immediately after the entrapment is completed. Chelating agents and heat applied in the matrix dissolution reduce cell viability and in turn hinder accurate quantification of viable cells. In this study, a method to determine the effect of entrapment procedure on bacterial cell viability which involves entrapping cells directly onto glass slides was developed. The developed method showed less viability reduction than the methods requiring matrix dissolution. The percentage of live cells in the culture before entrapment ranged from 54% to 74%, while the percent of live cells after entrapment determined by the developed method was 39–62%.

1. Introduction

Microbial entrapment has applications in commercial and industrial wastewater treatment (Chen and Lin, 1994), biodegradation of toxic compounds (Siripattanakul et al., 2008), bioremediation, biosorption of heavy metals (Arica et al., 2001; Luan et al., 2006), microbial fuel cells, biohydrogen production (Wang et al., 2010a), fermentation (Najafpour et al., 2004) and metabolite production (Anisha and Prema, 2008). Advantages of entrapped cells include protection from environmental stresses (pH and temperature) and enhancement of biological and physical stability of the microorganisms (Dervakos and Webb, 1991; Charlet et al., 2000). In addition, relative to free cells, higher biomass concentrations and higher activity can be achieved through cell entrapment (Dervakos and Webb, 1991; Anisha and Prema, 2008).

It has been reported that the biotechnological processes carried out by entrapped cells are better than those carried out by corresponding free cells, due to the added protection from negative environmental factors (Yang et al., 1994). However, several studies suggest that cell viability may be reduced during the cell entrapment process (Yang et al., 1994; Chen and Lin, 1994; Chang and Tseng, 1998; Charlet et al., 2000; Li-seng et al., 2007). The effect of entrapment on cell viability likely differs depending on techniques, procedures, and the matrix used for entrapment. Hydrogels are the most commonly used entrapment matrices; they include carrageenan, polyvinyl alcohol (PVA), and alginate.

There are several existing assays for the determination of cell viability. One of the most commonly used methods is the heterotrophic plate count. The heterotrophic plate count assay is a gold standard assay for viability assessment, which depends on the capability of specific microorganisms to proliferate and form colonies on solid media. The assay is relatively simple but takes a significant amount of time (1–5 days). The heterotrophic plate count assay also has limitations for non-culturable, dormant, and inactive cells (Lahtinen et al., 2005). The plate count technique might not be suitable for determining the viability of entrapped cells because the cells must be de-entrapped before the application of the method. The de-entrapment requires the addition of heat or chemicals to dissolve the matrix which could affect the viability of the cells (Wadhawan et al., 2010; Haque and Russell, 1974; Groh et al., 1996). For example, the de-entrapment of phosphorylated-PVA (PPVA) entrapped cells requires the matrix to be heated to more than 70 °C and calcium alginate (CA) matrix de-entrapment requires an addition of acid or chelating agents.

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A number of fluorescence-based assays have been developed to determine free and immobilized (biofilm) cell viability, including the commercially available LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, OR, USA) (Kakimoto et al., 2007; Sankaran et al., 2008; Chae et al., 2009; Lee et al., 2009; Wang et al., 2010b; Sarkar et al., 2010). The principle of this approach is to use fluorescent dyes to differentially label viable and non-viable cells. Viable and non-viable cells are then enumerated using a microscope with an epifluorescence attachment. The assay is sensitive and takes much less time (less than one hour) compared to the heterotrophic plate count. The LIVE/DEAD® assay has been successfully used to differentially label and quantify live and dead bacterial cells for environmental applications (Queric et al., 2004; Biggerstaff et al., 2006). Few studies have used this assay for entrapped cells by dissolving or dissecting the hydrogel (Charlet et al., 2000; Cunningham et al., 2004). This disruption could affect the viability of cells and could result in an underestimation of viability.

The objective of this research was to develop a method to determine the effect of the entrapment procedure on cell viability without matrix de-entrapment. The method development focused on the ability to quantify the viability of cells after the entrapment procedures, which are known to affect the viability of cells (Chang and Tseng, 1998; Charlet et al., 2000; Li-seng et al., 2007). PPVA and CA were chosen as entrapment matrices in this study, since they are widely used in several applications. Both pure and mixed cultures were used in this study as model bacterial cells to examine the broader applicability of the developed method.

2. Methods

2.1. Chemicals

PVA (99.0–99.8% fully hydrolyzed, molecular weight 77,000–79,000, J.T. Baker), sodium alginate (unspecified grade, Pfaltz and Bauer), and chemicals used for cell entrapment were obtained from VWR International Co., PA, USA. The bacterial viability kit used was the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 or L13152 (Molecular Probes, OR, USA).

2.2. Bacterial cultures and growth conditions

Escherichia coli K-12 JM109 (JM109), *Agrobacterium radiobacter* J14a (J14a), an atrazine-degrading mixed culture (ADMC), and a denitrifying mixed bacterial culture (DNMC) were used in this study. These cultures were selected because of their use in other research activities in our laboratory and represented a broad range of activities and origins (Hill and Khan, 2008; Siripattanakul et al., 2008).

JM109 was from Promega Inc. (Catalog number P9751, Madison, WI, USA). The strain was plated onto Luria–Bertani plates (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and incubated overnight at 37 °C. A single colony from the overnight incubation was used to prepare a liquid culture of LB, which was also incubated overnight at 37 °C but with constant shaking to aerate the culture. Ten milliliter of the overnight culture was centrifuged, the supernatant was discarded and the pellet was used for entrapment (described below). It should be noted that this overnight culture contained both live and dead cells. To entrap only dead cells, the same overnight culture was heat killed at 121 °C for 15 min. To obtain different ratios of live:dead cells, different volumes of the overnight culture and the heat killed culture were used to make a 10 ml culture for entrapment. Volumetric ratios of the overnight culture to the heat killed culture used in this study were 1:0, 1:1, and 0:1. These volumetric ratios gave different live and dead cell ratios which were required for the evaluation of the methods. There was no need to know the absolute numbers of live and dead cells in order to check whether the methods would provide sensible results.

J14a was obtained from the National Soil Tilth Laboratory, Ames, IA, USA. ADMC was enriched from atrazine contaminated soil collected from a field site in Oakes, ND, USA, following the procedure of Siripattanakul et al. (2008). J14a and ADMC were cultivated following Siripattanakul et al. (2008).

DNMC was enriched from mixed liquor suspended solids (MLSS) obtained from the Moorhead Wastewater Treatment Facility, Moorhead, MN, USA. The MLSS was acclimated under anoxic conditions with a hydraulic retention time of 1 day for 4 months. DNMC was acclimated and cultivated based on the procedure described by Hill and Khan (2008). The concentration of cells in each culture used in this study was approximately 4–5 log CFU/ml.

2.3. Experimental approach

Typically, the final forms of PPVA and CA entrapped cell matrices are spherical beads. However, fluorescence microscopy based assays require samples which permit penetration of dye and light. A previous approach to assess viability of entrapped bacteria was to first dissolve the bead in what will henceforth be referred to as the spherical bead entrapment (SBE) viability method. However, the conditions required to dissolve the bead could lead to an underestimation of viability (Wadhawan et al., 2010). Therefore, a modified entrapment and sample preparation method was developed for determining the viability and studying the effect of entrapment procedures on cells using the LIVE/DEAD® assay. This novel glass slide entrapment (GSE) viability method involves directly entrapping the bacteria on a slide. Schematics depicting the two methods are presented in Fig. 1.

2.4. Cell entrapment and de-entrapment

2.4.1. SBE method

2.4.1.1. CA entrapment. The procedure of entrapping bacteria in CA was modified from the procedure by van Ginkel et al. (1983). Sodium alginate was dissolved at 80 °C in deionized water (DW) until it formed a homogeneous 2% (w/v) alginate solution. Bacterial pellets were prepared as described above and were homogeneously mixed in the alginate solution. Spherical beads were produced by dropping a mixture of 10 μl of sodium alginate solution and the culture into a 3.5% (w/v) of calcium chloride solution using a 1 ml syringe. The beads were submerged for 60 min.

2.4.1.2. PPVA entrapment. The procedure of entrapping bacteria in PPVA was based on the procedure by Siripattanakul et al. (2008). A 10% (w/v) aqueous PVA solution and the mixture of the culture and the solution were prepared in the same manner as described above for the CA entrapment. A spherical bead was produced by dropping 10 μl of the PVA solution and culture into a saturated solution of boric acid using 1 ml syringe. Finally, the bead was allowed to soak in a phosphate solution (1 M at pH 7) for 60 min for PVA phosphorylation.

2.4.1.3. De-entrapment procedures. To dissolve the CA bead, 100 μl of 0.30 M sodium citrate prepared in 0.15 M sodium chloride solution was added to the bead and incubated at 37 °C for 20 min. For dissolving the PPVA bead, 100 μl of DW was added to the bead and incubated at 70 °C for 15 min. Both CA and PPVA beads were vigorously vortexed every 5 min throughout the incubation.

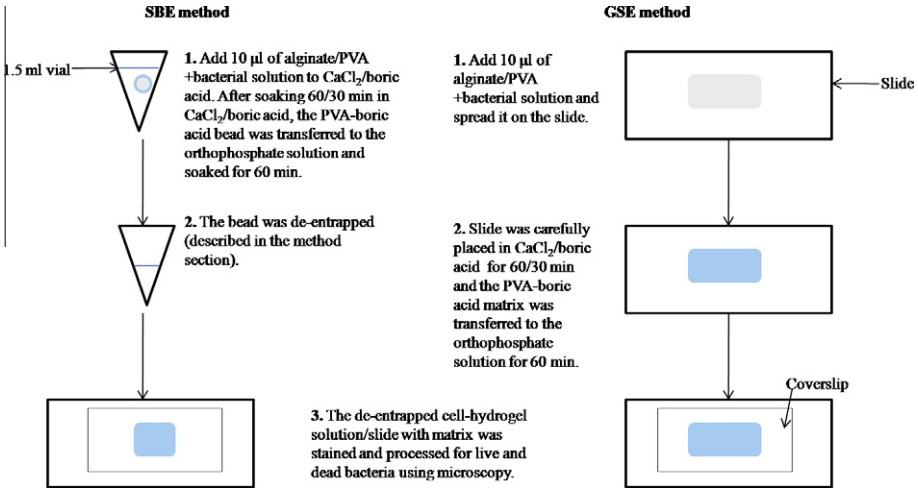

Fig. 1. Flow diagrams explaining the GSE and SBE methods.

2.4.2. GSE method

2.4.2.1. CA entrapment on slide. One drop (~10 µl) of the sodium alginate and bacterial solution (prepared as described in Section 2.4.1.1) was placed on a microscope slide and spread into a thin layer, approximately 500 µm in thickness using a micropipette tip. The microscope slide was submerged in a 3.5% (w/v) $CaCl_2$ solution for 60 min.

2.4.2.2. PPVA entrapment on slide. One drop (~10 µl) of the PVA and bacterial solution (prepared as described in Section 2.4.1.1) was placed on a microscope slide and spread into a thin layer, approximately 500 µm in thickness. The microscope slide was submerged in a saturated boric acid for 30 min for PVA-boron cross-linking. Finally, the slide was allowed to soak in a phosphate solution (1 M at pH 7) for 60 min for PVA phosphorylation.

2.5. LIVE/DEAD® staining and viability determination

The staining method was based on the manufacturer's instructions for the *Bac*Light™ LIVE/DEAD® kit (L7012 or L13152, Molecular Probe, OR, USA). The kit employs two nucleic acid stains, which can be used to differentiate between live and dead cells. SYTO®9 is a green fluorescent dye which freely enters both live and dead cells. Propidium iodide only enters dead cells as it requires a damaged cell membrane for entry. Propidium iodide replaces SYTO®9 in dead cells with the result that live cells fluoresce green and dead cells fluoresce red. The viability of cells was determined by enumerating the live and dead cells using a microscope (BX61™, Olympus, PA, USA) with an epifluorescence attachment. In our study, live and dead cells were enumerated in 20 microscopic fields at 600× magnification. SYTO®9 and propidium iodide excitation wavelengths were 480 and 535 nm respectively. Emission wavelengths were 535 (green) and 610 (red) nm, respectively.

Using the assay described above, the extent to which spectral overlap occurred when using each filter set with SYTO®9 and propidium iodide was determined. The LIVE/DEAD® assay was used to study the effect of the GSE method and the results were compared to the SBE method. This was done only for JM109 with three different ratios of live and dead bacterial cultures (prepared as described in Section 2.2). Finally, the usefulness of the GSE method with the LIVE/DEAD® assay when applied to other entrapped cultures was examined.

2.5.1. Assessment of spectral overlap

The extent of spectral overlap was determined using a heat killed suspension of JM109 or J14a. Heat killed cells were labeled with SYTO®9 alone, propidium iodide alone, and a 1:1 ratio of SYTO®9:propidium iodide, in accordance with the manufacturer's instructions. Each sample of labeled cells was viewed using SYTO®9 and propidium iodide specific filter sets.

2.5.2. Effect of dye ratios on labeling

The kit manufacturer recommends a 1:1 ratio of dyes; however, a different dye ratio may be more effective. The ratio of dyes used in the assay may potentially impact the assessment of cell viability. For example, all live cells may not fluoresce with a 1:1 ratio of SYTO®9 to propidium iodide leading to an underestimation of viability. In this case, the optimal dye ratio may be 2:1. Three different ratios of dye combinations, SYTO®9:propidium iodide of 1:1, 1:2, and 2:1, were tested using JM109 and J14a suspensions.

2.5.3. LIVE/DEAD® staining in the GSE method

The viabilities of four bacterial cultures (JM109, J14a, ADMC, and DNMC) were tested. The viability of JM109 was evaluated using both methods and both entrapment media. J14a and ADMC were entrapped in PPVA, and DNMC was entrapped in CA. They were evaluated using the GSE method. Free cells of all three cultures were also tested for viability comparisons before and after entrapment. Live and dead cells of all samples were counted using the protocol described earlier. The viabilities of JM109, J14a, and ADMC were tested twice to ensure the reproducibility of the procedure.

2.6. Statistical analysis

Results from the experiments were statistically analyzed using the SAS program (Version 9.1, SAS Institute Inc., Cary, NC, USA). The numbers of red (propidium iodide stained) and green (SYTO®9 stained) labeled JM 109 cells served as the dependent variables while the method constituted the independent variable. The data were analyzed by the analysis of variance (ANOVA) and Bonferroni *t*-test at 5% significance level. The use of the Bonferroni *t*-test over the regular *t*-test was to control the experiment wise error rate. ANOVA was used to analyze differences between the cell viability of cultures before and after entrapment for both methods. A follow up with multiple pair wise comparison for the numbers of live or

dead cells provided by each method (including the number of cells before entrapment) was performed.

3. Results and discussion

3.1. Assessment of spectral overlap

The heat killed J14a or JM109 cultures were stained with each dye individually and with the combined dyes to determine the extent of fluorescence overlap between filters. Fig. 2A shows the result of a sample stained only with SYTO®9 and viewed under the green filter. As expected, cells appeared fluorescent green under the green filter, but did not appear under the red filter (data not shown). Similarly, Fig. 2B shows the result of heat killed cells stained only with propidium iodide and viewed under the red filter. The heat killed cells stained with propidium iodide fluoresced under the red filter but not the green filter (data not shown). When both SYTO®9 and propidium iodide were used at a 1:1 ratio, the heat killed J14a cells appeared red under the red filter and were not visible under the green filter (Fig. 2C and D). This demonstrates that there was no spectral overlap between the filters used in our study.

3.2. Effect of dye ratios on labeling

The results of the 2:1 and 1:2 ratios of SYTO®9 to propidium iodide did not differ from that of the 1:1 ratio (data not shown). Therefore, a 1:1 ratio was used for the remainder of the study (free DNMC and ADMC, and entrapped J14a, DNMC, and ADMC).

3.3. Application of the LIVE/DEAD® assay to entrapped cells

3.3.1. Cell staining

There was a concern that the entrapment matrices would interfere with the assay, either by preventing fluorescent dyes and light from penetrating the target cells or by contributing to background autofluorescence. Fig. 3 shows that while some autofluorescence did occur, the bacteria could be easily differentiated from the entrapment matrices, enabling viability determination. For the SBE method, where the matrix was dissolved before staining, no background fluorescence was observed (data not shown). For the GSE method, the staining characteristics of the entrapped bacteria are shown in Fig. 3. Fig. 3A and B (from the same field) show the results of the DNMC entrapped in CA under the green and red filters, respectively. Fig. 3C and D (from the same field) show the ADMC entrapped in PPVA under the green and red filters, respectively. Background fluorescence was observed for both matrices; however, the signal-to-noise ratio was sufficient to identify target bacteria. Washing the stained hydrogel with deionized water might have been helpful in overcoming the background fluorescence. Images similar to those of Fig. 3C and D were obtained for J14a entrapped in PPVA and JM109 entrapped in CA and PPVA (data not shown).

3.3.2. Cell quantification

Different ratios of live and dead cells of JM109 were entrapped and the cell viability was estimated. Table 1 shows percentage of live and dead cells obtained from estimating the viability of JM109 cells (only overnight culture in the matrices) using both methods and both entrapment media. Depending on the standard deviations, the viability of cells determined by the SBE method was less than that determined by the GSE method only for PPVA but not for CA.

Table 2 describes statistical grouping of data for JM 109 cells before and after entrapment in CA and PPVA for both methods. To interpret the results in the table, choose the entrapment media of interest and live or dead cells and read the letters in the column(s) alphabetically. Different letters represent differences between the means of two or more values of the variable, which in this case is the method (including before entrapment). Sharing the same letter indicates no significant difference (p-value >0.05) in mean values of the variables.

Statistically, there was no difference between the number of dead cells before and after entrapment in the CA matrix when

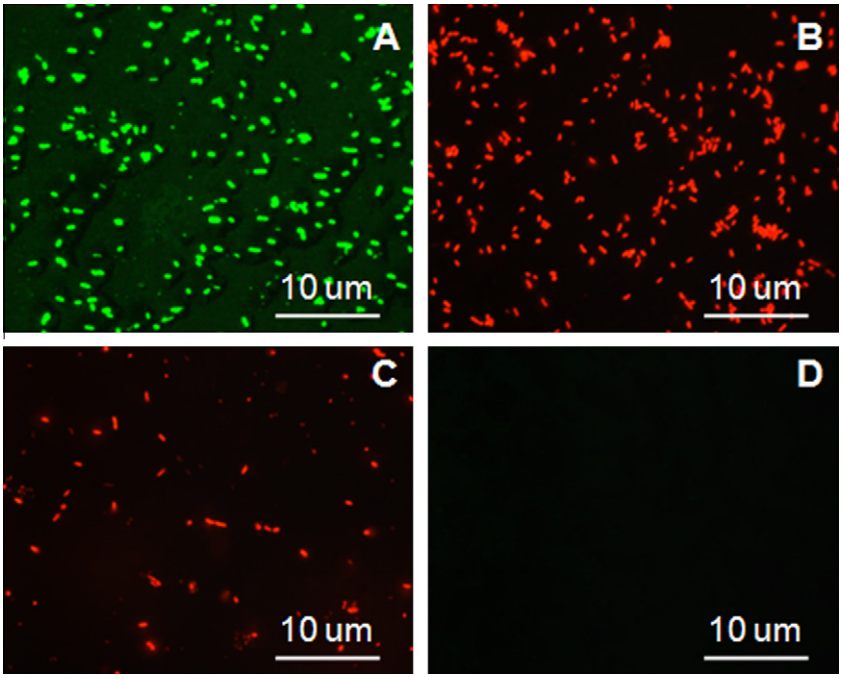

Fig. 2. Images of dead J14a suspension stained with different dyes: (A) only SYTO®9 using the green filter; (B) only propidium iodide using red filter; (C) both SYTO®9 and propidium iodide using the red filter; (D) both SYTO®9 and propidium iodide using the green filter ((C) and (D) are from the same field). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

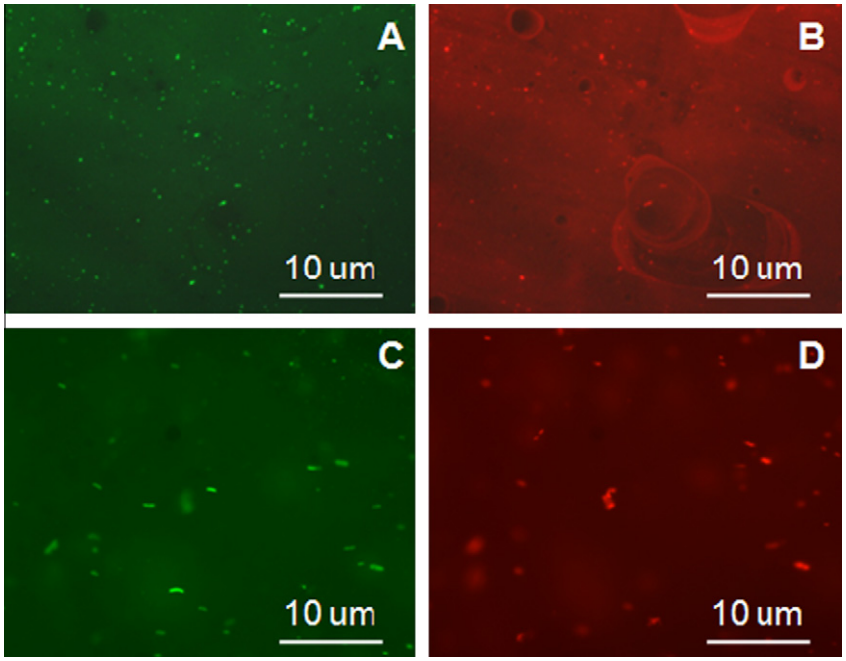

Fig. 3. Images of bacterial cells entrapped in CA and PPVA. (A) Cells in CA matrix using FITC filter; (B) cells in CA matrix using TRITC filter; (C) cells in PPVA matrix using FITC filter; (D) Cells in PPVA matrix using TRITC filter ((A) and (B), and (C) and (D) are from the same fields, respectively).

Table 1
Ratios of live and dead JM109 determined using two different methods.

Matrix	Cell viability	SBE[a]	GSE[a]
CA	Live cells	43 ± 4	51 ± 5
	Dead cells	56 ± 4	48 ± 5
PPVA	Live cells	39 ± 4	50 ± 4
	Dead cells	60 ± 4	49 ± 4

[a] Mean ± standard deviation.

Table 2
Statistical grouping (*t*-test with Bonferroni adjustment) of the results on JM 109.

	CA				PPVA	
	Live		Dead		Live	Dead
Before entrapment		A		A	A	A
SBE	B	A	B		B	A
GSE	B		B	A	B	A

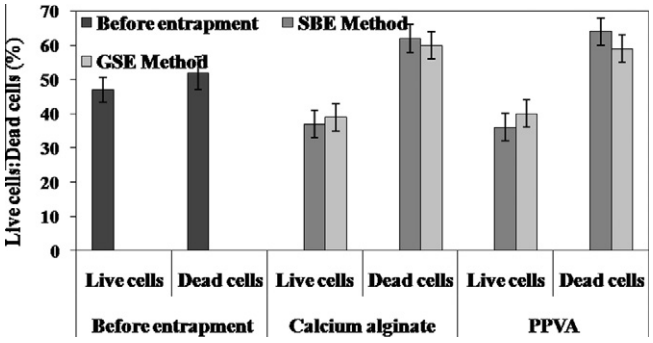

Fig. 4. Effects of the GSE and SBE methods on the percentage of live and dead JM109. Ratio of overnight culture to heat killed culture entrapped as 1:1.

the entrapped cells were enumerated using the GSE method (p-value = 1.00). There was a significant difference between the number of dead cells before and after the CA entrapment when evaluated using the SBE method (p-value = 0.0093). The numbers of live cells entrapped in CA provided by the two methods were not statistically different (p-value = 0.0882). The p-values comparing the numbers of live cells before and after being entrapped in PPVA provided by the two methods are significant (<0.0001 for SBE and 0.0009 for GSE) suggesting that there was a negative effect on cell viability due to the entrapment. The p-values comparing the numbers of the PPVA entrapped cells predicted by the two methods were not significant (1.00 for dead cells and 0.60 for live cells).

To further evaluate and confirm the results, a different ratio of live to dead JM109 cells was used. Fig. 4 also shows reduction in the percentage of live JM109 cells (ratio of overnight culture to the heat killed culture entrapped was 1:1) for both methods and both entrapment media when compared to the free culture. This result was similar to the data reported in Table 1. The GSE method was used to determine the effect of entrapment on the viability of J14a, ADMC, and DNMC. The results are detailed in Table 3. The ratio of live bacterial cells before the entrapment ranged from 54% to 74%, while the ratio of live cells after the entrapment was 39–62%. It should be noted that before entrapment the percentages of live cells were low (54–74%) because the cells were collected at the late-exponential phase. Using higher percentages of live cells (90–100%) would have been just another condition. The fraction of live cells should not affect the procedure and the result.

All the results from both of the SBE and GSE methods indicate that entrapment reduced bacterial viability when compared to free culture. There is a greater reduction in cell viability when using the SBE method (Table 1 and Fig. 4), which could be due to the reduction in cell viability due to the de-entrapment procedures. Both sodium citrate and heat (70 °C) used for CA and PPVA de-entrapment, respectively, are known to reduce cell viability (Wadhawan et al., 2010; Haque and Russell, 1974; Groh et al., 1996).

The SBE method requires the cells to be de-entrapped from the matrix before performing the cell viability assay. The spherical matrix does not allow sufficient dye and light penetration making

Table 3
Ratios of live and dead cells using the GSE method.

Culture	Sample description	Ratio (%)	
		Live cells[a]	Dead cells[a]
JM109	Before entrapment	65 ± 6	35 ± 6
	After entrapping in CA	51 ± 5	48 ± 5
	After entrapping in PPVA	50 ± 4	49 ± 4
J14a	Before entrapping in PPVA	54 ± 5	46 ± 5
	After entrapping in PPVA	39 ± 4	61 ± 4
ADMC	Before entrapping in PPVA	63 ± 5	37 ± 5
	After entrapping in PPVA	41 ± 3	59 ± 3
DNMC	Before entrapping in CA	74	26
	After entrapping in CA	62	38

[a] Mean ± standard deviation.

microscopy inapplicable for entrapped cells. The de-entrapment procedure is an additional step and decreases viability of the entrapped cells. In this study, the incubation time for the matrix de-entrapment using acid and heat (70 °C) is small (15–20 min) as the amount of entrapping media to be dissolved is small (10 μl). Some other applications (discussed in Section 1) require entrapping cells in spherical matrices (beads) which need a large volume of entrapping media ($\geqslant$100 μl) and in turn require a longer de-entrapment incubation time (45 min). Drastically decreases in cell viability were observed with these cases (Wadhawan et al., 2010). Statistically, the GSE method is equal or in some cases better than the SBE method. It also consumes less time than the SBE method and therefore is recommended for determining the effect of entrapment on cell viability. Using scanning electron microscopy to compare the polymeric structures of the entrapment matrices and coatings involved in the SBE and GSE methods is suggested for future work.

4. Conclusions

Methods involving dissolution of the matrix for estimating cell viability can give an underestimated count of live bacteria. A method for applying the commercially available LIVE/DEAD® assay to determine the effect of two entrapment procedures on bacterial cell viability was successfully developed. Based on this method, the entrapment reduced the ratio of live cells by 12–15%. This new method can be used for optimizing entrapment procedures. The advantages of the method are that it is simple and rapid with no need for de-entrapment. The method could potentially be used for other entrapment matrices not tested in this study.

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