Journal of Sustainability Science and Management
Volume 20 Number 2, February 2025: 256-272
eISSN: 2672-7226
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ASSESSMENT OF DECAPSULATION METHOD AND THE EFFECT OF ALGINATE ENCAPSULATION ON *E. Coli* CELL VIABILITY

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http://doi.org/10.46754/jssm.2025.02.004
Received: 29 May 2024 *Revised: 31 July 2024* *Accepted: 10 August 2024* *Published: 15 February 2025*

Abstract: Alginate-encapsulation of bacterial cells is a cell immobilisation technique used in various biotechnology fields due to its numerous benefits. One of its cited benefit is the increased preservation of bacterial cell viability post-encapsulation. However, the process of encapsulation may inherently negatively affect cell viability. This study explores changes in cell viability after subjecting *E. coli* BL21 DE3 cells to alginate encapsulation. Cell viability of free cells is compared with cells that have undergone alginate encapsulation, and cell viability is measured using the resazurin microtitre assay (REMA) and viability-PCR (vPCR). A decapsulation step is designed to reliably assess the viability of the encapsulated cells, which involves an enzymatic approach using alginate lyase in combination with a chemical sequestration approach using sodium citrate. The optimum decapsulation condition is determined to be a two-hour incubation at 37°C in a solution of 50 mM sodium citrate and 500 µg/µL alginate lyase. The study also found that cell viability decreased following alginate encapsulation, with both REMA and vPCR showing a mean reduction of 14.43% and, 31.54%, respectively. This decrease in cell viability is synonymous with other cited studies. However, the magnitude of the decrease varied due to the use of different experimental parameters.

Keywords: Alginate, encapsulation, bacterial cell viability, REMA, vPCR.

Introduction

Alginate encapsulation is a standard method used for cell immobilisation (Smidsrød & Skjåk-Braek, 1990). Cell encapsulation is popular in various domains of biotechnology due to its advantages (Dervakos & Webb, 1991), namely (1) Enabling reuse of the encapsulated biocatalysts, (2) increased durability and survivability of cells, and (3) improved product yields (Vojtísek & Jirků, 1983; Dervakos & Webb, 1991). However, it is up to the applicant to ensure that the benefits of encapsulating cells outweigh the cost, time, effort, and undesirable side effects of it. Encapsulating cells requires resources and introduces an extra step upstream of the respective applications. In addition, encapsulating cells can induce metabolic changes and introduce diffusion limitations (Zur *et al.*, 2016).

Ironically, encapsulating cells may cause a reduction in cell viability. Some cell immobilisation processes themselves may temporarily expose cells to harsh environments. Encapsulation of cells in gellan gum for instance, typically requires cells to be mixed with the gellan gum matrix at temperatures of about 45°C (Moslemy *et al.*, 2002). Mixing cells in the immobilisation matrix may also subject them to shear forces that may reduce cell viability (Chisti, 2001; Kong *et al.*, 2003). Some microcapsule generation methods also employ high voltages such as those used in electro spraying (Bugarski *et al.*, 1994).

While it is generally acknowledged that cell viability is preserved to some degree following the encapsulation process, the extent to which it is maintained may not be the focus of some

research if more meaningful endpoints are suited to the applicant. Achieving better product yields in bioprocessing applications, for example, is often more critical than preserving cell viability. Furthermore, it is possible that cells may recuperate their loss of viability within the encapsulation matrix (Melvik & Dornish, 2004). Nevertheless, in some applications, the initial preservation of cell viability is critical such as those in biosensing (Michelini & Roda, 2012), probiotics (Shokryazdan *et al.*, 2017), and cell transplantation (Beck *et al.*, 2007).

Our study aims to quantify the changes in cell viability caused by encapsulation in calcium alginate. *E. coli* BL21 DE3 was chosen as the model organism for the study. There were a few motivations behind this study, namely the limited number of studies on the relationship between bacterial cell viability and alginate encapsulation. Most studies were also relatively old (> 10 years since publication) and have predominantly been conducted on mammalian cell lines (Table 4). The studies also mainly relied on just a single cell viability assay and did not control for certain parameters. With these precautions in mind, our study aims to circumvent these issues as described further.

One of the expected challenges is that assessing encapsulated cells' viability could yield biased results depending on the method used for determining cell viability. As a precaution, a decapsulation step is encouraged. Decapsulation can also be done using multiple approaches such as the use of chelating agents (Smidsrød & Skjåk-Braek, 1990; de Groot *et al.*, 2003; Li *et al.*, 2006; Sidhu *et al.*, 2012; Li *et al.*, 2019), enzymatic digestion (Lu *et al.*, 2012; Zhang *et al.*, 2017), and mechanical homogenisation (Capela *et al.*, 2007). It is also vital to ensure that the chosen decapsulation method does not negatively affect cell viability.

Different cell viability assays also work on other principles. For this study, the Resazurin Microtitre Assay (REMA) and viability-PCR (vPCR) were chosen to assess the viability of encapsulated cells. REMA measures the metabolic activity of cells, specifically the resazurin reduction activity (Riss *et al.*, 2004), whereas vPCR measures cell viability based on cell membrane integrity (Nogva *et al.*, 2003). The reliance on two cell viability assays based on two different mechanisms could provide more nuance to the observed cell viability changes.

Methodology

Preparation of E. Coli BL21 DE3 Cells Encapsulated in Calcium Alginate

A 4% w/v sodium alginate solution was prepared by mixing sodium alginate powder in distilled water. The solution was then autoclaved for 20 minutes at 121°C for sterilisation and to ensure all the sodium alginate powder had fully dissolved. *E. coli* BL21 DE3 was cultured in Luria-Bertani (LB) Broth at room temperature overnight at 110 rpm on a standard tabletop shaker. Cells were palletised by centrifugation at 10,000 x g for five minutes. The supernatant was decanted and 0.25 volumes of the sterilised 4% w/v sodium alginate solution were added to the cell pellet. The solution was pipetted repeatedly to ensure the cells were mixed until they were homogeneous.

The sodium alginate mixture was added dropwise using a 1,000 µL micropipette to a 0.20 M calcium chloride solution and left to crosslink for 10 minutes to form calcium alginate beads. The beads were harvested, placed on a filter paper, and washed with distilled water three times.

Assessing Decapsulation Efficiencies of Calcium Alginate in Different Solutions

A few decapsulation solutions were tested to determine the optimal composition for decapsulation. The following solutions were prepared with the following compositions: (1) PBS pH 6.3; (2) PBS 6.3 + 55 mM sodium citrate; (3) PBS pH 6.3 + 55 mM sodium citrate + 500 µg/µL alginate lyase; and (4) PBS pH 6.3 + 500 µg/mL alginate lyase. Sodium citrate concentrations were referenced from another study (Li *et al.*, 2019), whereas the concentration

and working pH of 6.3 of alginate lyase were referenced from the manufacturer's website (Sigma-Aldrich).

Decapsulation was conducted by adding three 4% w/v calcium alginate beads containing encapsulated *E. coli* BL21 DE3 cells into 500 µL of the tested decapsulation solution. A positive control was prepared by adding three drops of *E. coli* BL21 DE3 cells suspended in 4% w/v sodium alginate solution to represent a fully decapsulated state. For experiments visualising the decapsulation process, 0.1 volumes of 1% w/v Congo Red was supplemented to the sodium alginate solution. Decapsulation was carried out at 37°C for three hours. 50 µL of the leachate was collected hourly to evaluate the decapsulation progress using REMA.

Optimising Temperature for Preparation of Heat-killed Cells

A simple experiment was set to determine the optimal temperature for preparing heat-killed cells to be used as negative controls, as higher killing temperatures could denature DNA and create false negative results for vPCR. Overnight *E. coli* BL21 DE3 cultures were aliquoted into volumes of 2 mL in microfuge tubes and exposed to temperatures of 50°C, 75°C, and 100°C for 10 minutes in a water bath. Room temperature samples were used as a live cell control. The heat-treated samples were aliquoted into two fractions for REMA and vPCR. These aliquots were used to assess killing and amplification efficiencies, respectively.

Assessing Cell Viability Using REMA

REMA protocol was obtained from the Assay Guidance Manual with some modifications (Riss *et al.*, 2004). Resazurin stock solution was prepared by dissolving resazurin sodium salt (Invitrogen™) in sterile PBS to achieve a stock concentration of 440 uM. This stock solution was used to prepare working solutions for REMA by dissolving in nine volumes of sterile LB broth to achieve a final working concentration of 44 µM resazurin. To perform the assay, 90 µL of this mixture was pipetted into each well of a 96-well microplate (Greiner Bio-One, Cat no. 650161).

The reaction was initiated by adding 10 µL of the test cell culture – or leachate for the decapsulation test – into each well and incubated at 37°C in the dark. Readings were taken using Infinite® M200 PRO multimode plate reader and controlled using the Magellan 7.1 software. In the instrument settings, the fluorometric mode was selected, and the excitation wavelength, λ_{ex} was set to 560 nm while the emission wavelength, λ_{em} was set to 590 nm.

REMA standards were prepared using overnight cell culture and diluted with heat-killed cell cultures to achieve relative viable cell concentrations of 0%, 20%, 40%, 60%, 80%, and 100%. The heat-killed cell cultures were chosen as the diluent instead of regular culture media to maintain similar turbidity across standards. They were prepared by subjecting cells to 10 minutes of killing in a 75°C water bath.

Assessing Cell Viability Using vPCR

Inhibition of DNA Amplification with PMA-photocrosslinking

The protocol for PMA-photocrosslinking was referenced from another study with some modifications (Nocker *et al.*, 2007). The following was conducted in the dark. Lyophilised propidium monoazide (PMA) was purchased from Biotium and dissolved in autoclaved ultrapure water to make a stock concentration of 10 µg/µL, about 25 mM, 1,000 times the working concentration. Next, 2 µL of stock PMA solution was added to 2 mL of overnight cell culture in a 2 mL microfuge tube with a transparent cover. For the dead cell control, 2 µL of stock PMA solution was added to 2 mL of heat-treated cells killed at 75°C for minutes. After adding PMA, all tubes were inverted a few times and left to sit in the dark for 10 minutes.

PMA-photocrosslinking of DNA was initiated by placing all tubes about 15 cm under a 100W LED flood light while ensuring the light source is evenly distributed. A live cell sample and a dead cell sample were covered with

aluminium foil prior to placement to be used as a non-photocrosslinked control. All other test cell samples were left to sit under the light for 20 minutes and samples were inverted a few times every five minutes. All samples were then prepped for DNA extraction.

Preparing PMA-crosslinked Cells for DNA Extraction

The photocrosslinked cells were pelleted in the dark by centrifuging at 10,000 g for five minutes. The supernatant was discarded and replaced with 1 mL of sterile PBS to wash and remove any residual PMA. The cell pellet was inverted a few times while suspended in PBS and centrifuged at 10,000 g for five minutes. The supernatant was discarded, and the cells are now ready for DNA extraction.

DNA Extraction Using CTAB and Phenol:Chloroform Method

The DNA extraction protocol was adapted from another study with some modifications (Wilson, 2001). A lysis buffer was prepared with the following composition: 9.34 mL TE buffer (10 mM Tris-Cl, 1 mM EDTA) pH 8.0, 600 µL 10% SDS, 60 µL Proteinase K. Cell lysis was initiated by adding 600 µL of the lysis buffer to each cell pellet prepared previously. The mixture was gently pipetted until the cell pellet had dissolved and left to incubate at 37°C for one hour.

After incubation, the cell lysate was added with 100 µL of 5 M NaCl and mixed thoroughly, followed by the addition of 80 µL of 10% w/v CTAB in 0.7 M NaCl and incubated at 65°C for 10 minutes. An approximately equal volume (800 µL) of Phenol:Chloroform:Isoamyl alcohol (P:C:I) of ratio 25:24:1 was added to the cell lysate, inverted a few times, then centrifuged at max RCF (~21.1k) on a tabletop centrifuge for 15 minutes.

After centrifuging, 600 µL of the top aqueous layer was transferred to a new tube, avoiding white precipitates at the interface. Similarly, an equal volume of Chloroform:Isoamyl alcohol (C:I) of ratio 24:1 was added to the newly transferred aqueous layer, inverted a few times, and then centrifuged at max RCF again for 15 minutes to remove any residual phenol and impurities. 500 µL of the top aqueous layer was transferred to a new microfuge tube and ready for DNA precipitation.

DNA was precipitated by adding 0.6 volumes (300 µL) of ice-cold isopropanol and mixed by inversion a few times. The mixture was incubated at -20°C overnight to ensure maximal precipitation. The DNA was pelleted by centrifuging at max RCF for 30 minutes at 9°C. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol while gently inverting the tube. The DNA pellet was recollected at the bottom of the tube by centrifuging at max RCF for five minutes. The supernatant was decanted and the tubes were centrifuged at low speed briefly to spin down the remaining ethanol adhered to the wall of the tube. The remaining ethanol was siphoned out using a 10 µL micropipette while avoiding the DNA pellet. The tubes were placed inverted to air dry for another 10 minutes. The DNA pellet was dissolved in 50 µL of TE buffer pH 8.0 for storage and kept in a refrigerator until use.

RNA Removal from DNA Extract

Due to the ability of RNA to interfere with spectrophotometric quantification of DNA, substantial RNA removal was essential to ensure accurate quantification of DNA for downstream qPCR protocols. The RNA removal protocol was referenced from the Joint Genome Institute (Yoshinaga & Dalin, 2016).

RNAse A (QIAGEN) at the concentration of 100 mg/mL (17,500U) was diluted with ultrapure water to a concentration of 10 µg/mL (dilution factor of 10,000). The RNA removal was initiated by adding 2 µL of the diluted RNAse A solution to the previously prepared 50 µL DNA extracts. The tubes were incubated at 37°C for one hour in a heating block.

A solution consisting of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol was added to precipitate the DNA and remove degraded RNA. The tubes were centrifuged at max RCF at -9°C for 30

minutes to pelletise the DNA. The supernatant was decanted, and the DNA pellet was washed with 1 mL of 70% ethanol as described in the DNA extraction method. After washing, the DNA pellet was resuspended in 30 µL of pH 8.0 TE buffer and kept in the refrigerator for at least two hours to ensure homogenous solubilisation of the DNA extract for spectrophotometric quantification.

Quantification and Preparation of DNA Extracts for qPCR

DNA extracts were quantified using DeNovix DS-11 spectrophotometer. A dsDNA quantification mode was selected for DNA quantification. The instrument was zeroed using TE buffer. DNA concentration of DNA extracts was quantified by placing 2 µL of the DNA sample on the pedestal. Each DNA sample was measured twice to ensure that readings were consistent to ensure homogeneity. After measurement completion, 10 µL of each DNA sample was aliquoted into a fresh tube and topped off with TE buffer to a desired DNA template concentration of 10 ng/µL for qPCR.

Thermocycling Conditions and Setup for qPCR

The qPCR reactions were set up in a MicroAmp 96-well plate with reaction volumes of 20 µL. PowerUp™ SYBR™ Green Master Mix from Applied Biosystems was used to prepare the reaction using ultrapure water as the diluent. The primers used were of 0.5 µM working concentration, namely 8F primer (5'-AGAGTTTGATCCTGGCTCAG) and 1492R primer (5'-CGGTTACCTTGT TACGACTT). For the DNA templates, 1 µL of 10 ng/µL DNA extract was used. qPCR standards were prepared from a purified DNA sample of relative concentrations ranging from 10^0 to 10^{-5}.

qPCR was conducted using QuantStudio™ Real-Time PCR system. ROX was selected as the internal dye. Amplification conditions were set up according to the manufacturer's guidelines but modified slightly to adjust for more extended amplicon sizes, similar to another study (Banihashemi *et al.*, 2012).

The settings were: (1) UDG activation: 50°C, two minutes; (2) Dual Lock Polymerase activation: 95°C, two minutes; (3) DNA denaturation: 95°C, 30 seconds; (4) Primer annealing: 55°C, 30 seconds; and (5) Extension: 72°C, 90 seconds. Steps 3 to 5 were cycled a total of 40 times. (6) Holding stage: 72°C, five minutes. Melt curve was generated using the following parameters: (1) 1.6°C ramping to 95°C, 15 seconds hold; (2) 1.6°C ramping down to 60°C, 60 seconds hold; and (3) Dissociation step: 0.15°C ramping up to 95°C, 15 seconds hold.

Results and Discussion

Calcium Alginate Decapsulation

Figure 1 depicts the decapsulation efficacy of 4% w/v calcium alginate beads in different decapsulation solutions when incubated at 37°C. Sodium alginate was pre-stained with Congo Red prior to encapsulation to aid in visualisation.

Figure 2 describes the decapsulation efficacies of various decapsulation solutions but using *E. coli* BL21 DE3 cells encapsulated in 4% w/v calcium alginate.

Based on both Figure 1 and Figure 2, decapsulation was only observed in solutions 2 and 3. However, the decapsulation rate in solution 2 was slower than in solution 3. Solution 3 was fully decapsulated by the second hour, whereas solution 2 required three hours for full decapsulation. Full decapsulation in solution 3 could also be inferred by the second hour from the plateauing of the curve indicated in Figure 2. However, even when fully decapsulated, the amount of cells released is not the same as the free cell counterpart, suggesting that viable cell counts were lost after encapsulation.

Effect of Killing Temperature on vPCR Efficacy

Figure 3 shows that DNA amplification was inhibited by PMA when cells were exposed to temperatures 75°C and 100°C in the presence of light. This indicated that cell membranes were compromised, thus, indicating that virtually all cells were successfully killed. Cells exposed to

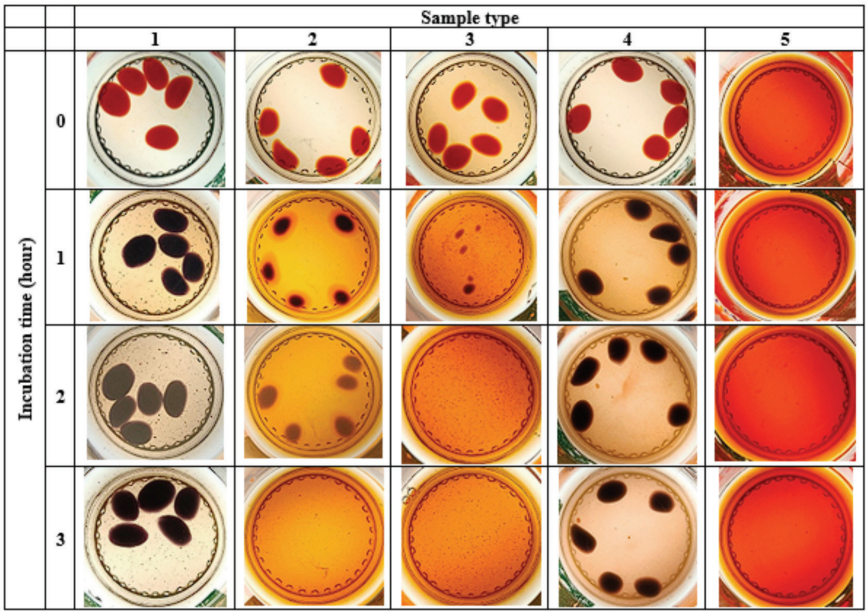


Figure 1: Decapsulation efficacy of 4% w/v calcium alginate beads in different decapsulation solutions incubated at 37°C. Sample type: (1) PBS; (2) PBS + 55 mM sodium citrate; (3) PBS + alginate lyase + 55 mM sodium citrate; (4) PBS + alginate lyase; and (5) positive control consisting of five drops sodium alginate + PBS + alginate lyase + 55 mM sodium citrate

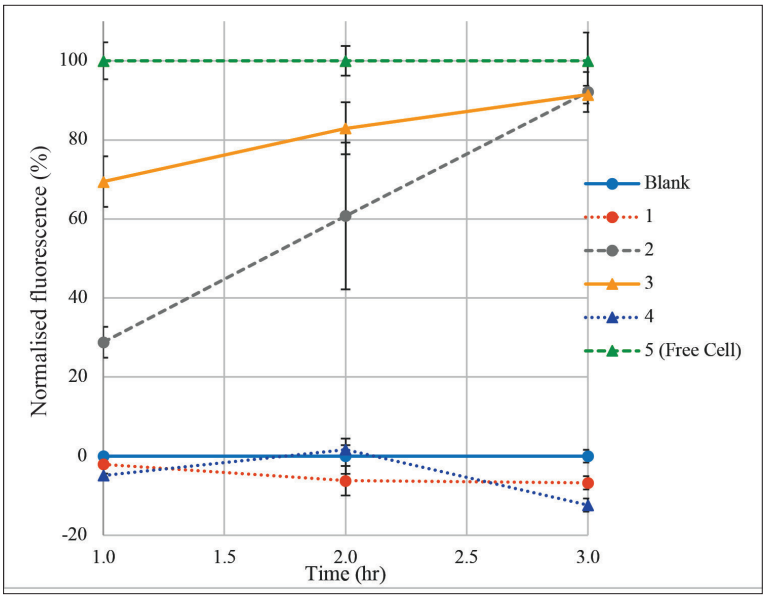


Figure 2: Decapsulation efficacy of *E. coli* BL21 DE3 cells encapsulated in 4% w/v calcium alginate (n = 3) in different decapsulation solutions across time (hours). Decapsulation efficacy was assessed by subjecting the leachate to REMA and the normalised fluorescence (% ± SD) was used to reflect the amount of cells being released. Decapsulation solutions used: (1) PBS; (2) PBS + 55 mM sodium citrate; (3) PBS + alginate lyase + 55 mM sodium citrate; (4) PBS + alginate lyase; and (5) positive control consisting of three drops sodium alginate + PBS + alginate lyase + 55 mM sodium citrate

50°C in the light produced a band with lower intensity, indicating that only a small fraction of cells were killed. However, cells exposed to 100°C in the dark also produced a less intense DNA band. Since PMA was not crosslinked to DNA in this case, the other explanation for the reduced band intensity was likely DNA damage induced by thermal stress (Karni *et al.*, 2013). Therefore, 100°C might not be the ideal temperature for killing cells as it causes false-negative results in vPCR. On a separate note, ambient temperature during photocrosslinking by the 100 W LED light source had no noticeable heating effect on the samples. In contrast, the employment of ice overcooled the samples, which interfered with the photocrosslinking efficiency (data not shown).

Similarly, Figure 4 shows the respective Ct values when the DNA extracts of the cells were subjected to qPCR. The Ct values reflect the DNA band intensity in Figure 3, indicating the least amount of amplification in cells exposed to 75°C and 100°C when photocrosslinked with PMA. DNA amplification was also greatly reduced at exposure to 100°C even without photocrosslinking, further indicating that DNA integrity was partially compromised at a too-high temperature. It was thus determined that the optimal condition for preparing heat-killed cells was 75°C for 10 minutes.

Purity of DNA Extract

Extraction of the gDNA from *E. coli* BL21 DE3 was confirmed via gel electrophoresis indicated by a band above the 10,000 bp mark in Figure 5. RNA was present in gDNA extracts prior to RNAse A treatment as shown in lane 2 and had the highest observed yield spectrometrically. RNA was also present on the gel image in lanes 3 and 4 as indicated by the smears with working RNAse a concentrations of 0 and 1 µg/µL, respectively. Maximal RNA removal was obtained in lanes 5 and 6 whereby working RNAse A concentrations above 10 µg/µL did not improve RNA removal efficacy, as indicated by the DNA yield. Overall, the larger absence of RNA from the lanes correlated with decreased spectrometric DNA yield.

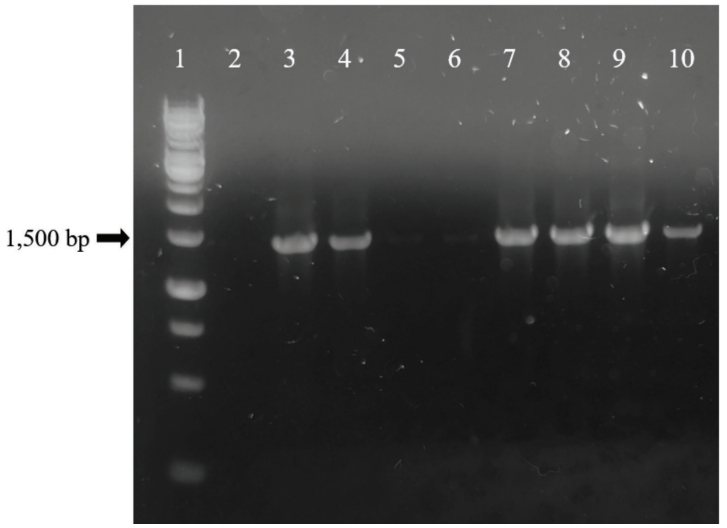


Figure 3: Gel image showing 16S-amplified DNA extracts from cells treated at different temperatures for 10 minutes and exposed to PMA with or without light exposure for 30 minutes: (1) Fermentas DNA ladder SM0311; (2) non-template control; (3 to 6) cells exposed to room temperature, 50°C, 75°C, and 100°C, respectively in the presence of light with PMA; (7 to 10) cells exposed to room temperature, 50°C, 75°C, and 100°C, respectively in the dark with PMA. Gel running conditions: 1% w/v agarose, 135V for 30 minutes in TBE, 2 µL DNA ladder, 3 µL amplified DNA extract

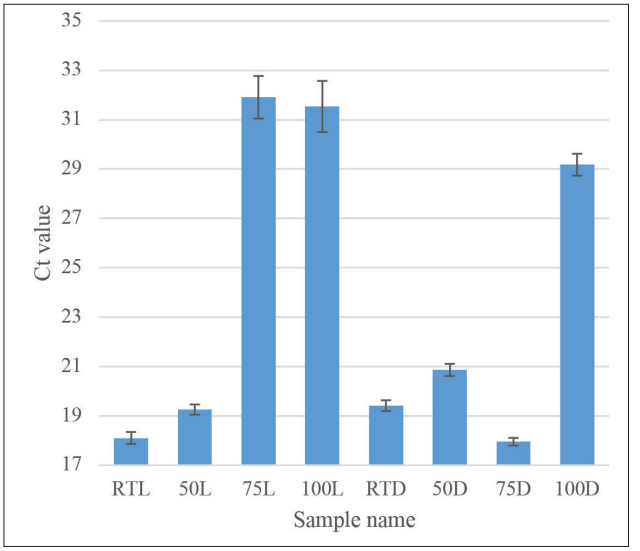
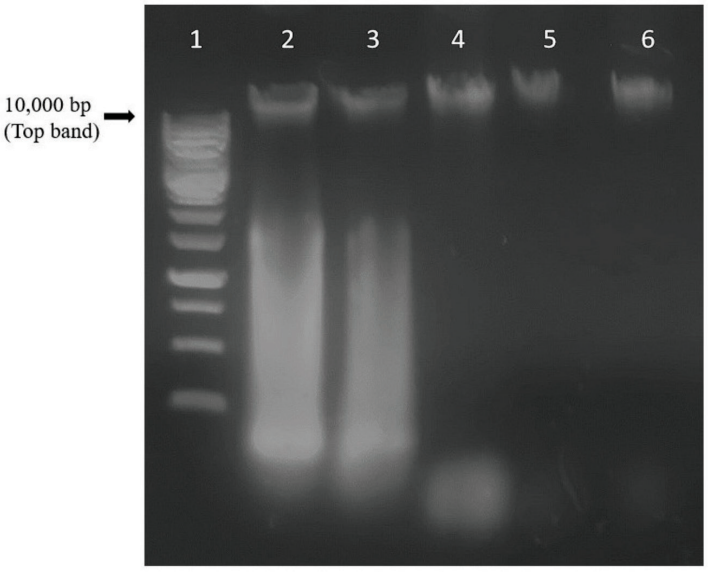


Figure 4: C_t values (± SD) from qPCR of 16S-amplified 20 ng gDNA templates (n = 3) from free *E. coli* BL21 DE3 cells exposed at different temperatures for 10 minutes with or without the presence of light with PMA. Samples are named by exposure temperature (RT, 50°C, 75°C, 100°C), followed by light exposure status (L = Light, D = Dark)



Lane	DNA Yield (ng/µL)
1	DNA ladder #SM0311
2	2413.6
3	2116.3
4	1559.9
5	691.1
6	695.8

Figure 5: Genomic DNA extracts and their respective yields (ng/µL) quantified spectrometrically by DeNovix DS-11 before and after treatment with varying concentrations of RNAse A. (1) 2 µL of DNA ladder (Vivantis #SM0311); (2) gDNA extract before RNAse A treatment; (3 to 6) gDNA extracts after treatment with RNAse A of working concentrations 0, 1, 10, and 100 µg/µL, respectively and reprecipitated

Viability of Free Cells and Encapsulated Cells Using REMA

A standard curve for REMA was constructed using overnight cell culture and diluted with heat-killed cell culture to produce varying relative concentrations. The importance of the standard curve was to ensure linearity, which conforms to the Beer-Lambert Law and to ensure that experimental readings fell within the range set by the standard curve. The standard curve for REMA is shown in Figure 6.

Free cells suspended in 4% w/v sodium alginate and decapsulated AECs were subjected to REMA, their respective raw fluorescence readings are depicted in Figure 7. Free cells were assumed to have 100% cell viability while the blanks were considered to have 0% cell viability. All three trials showed a consistent trend that reported a decrease of encapsulated cell viability. The numerical percentage decrease (% ± SD) is shown in Table 1.

Viability of Free Cells and Encapsulated Cells Using vPCR

A qPCR standard curve was constructed to determine the amplification efficiency as well as the degree of correlation between the C_t values and the amount of DNA template used. A standard curve was constructed as depicted in Figure 8. The amplification efficiency of the qPCR running conditions was only at 88.855% but had a relatively good correlation, indicated by an R^2 value of 0.9696. This meant that a decrease in a Ct value of 1 indicates an 88.855% increase in the gDNA template amount in contrast to a 100% per -1 C_t under ideal circumstances. The amplification efficiency was important to be determined to allow for more accurate translation of C_t values into cell viability quantification.

The DNA extracts from free cells and encapsulated cells were subjected to qPCR and the resulting C_t values are depicted in Figure 9. Free cells had a mean ± SD C_t value of 18.864 ± 0.126 whereas encapsulated cells had a value of 19.460 ± 0.467. When considering the amplification efficiency of 88.855%, it was determined that encapsulated cells had a mean reduction of 31.54% in cell viability compared to free cells. When standard deviations were considered, cell viability's mean reduction ± SD ranged from 6.89 to 49.67%. C_t values of heat-

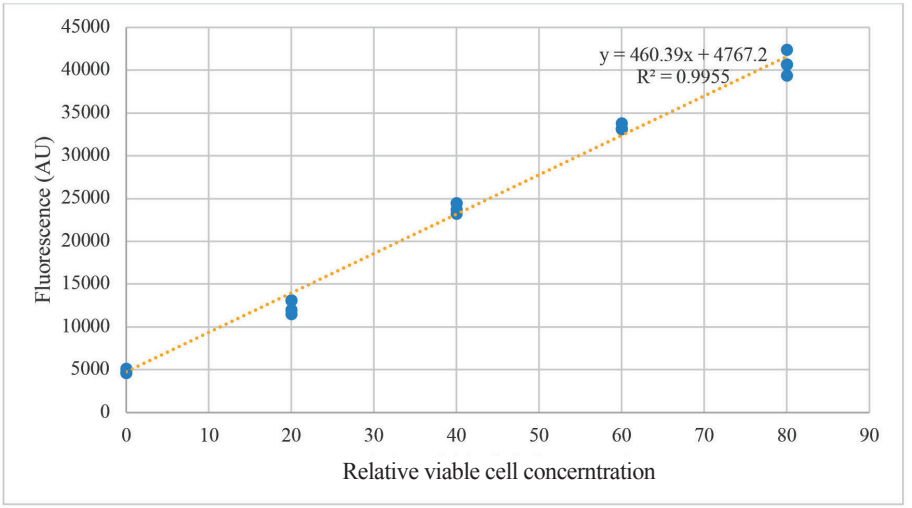


Figure 6: REMA standard curve of consisting of varying relative viable cell concentrations (n = 3) and the respective fluorescence (AU), λ_{ex} = 560 nm, λ_{em} = 590 nm. Dilutions were made by diluting viable cells with heat-treated cell cultures

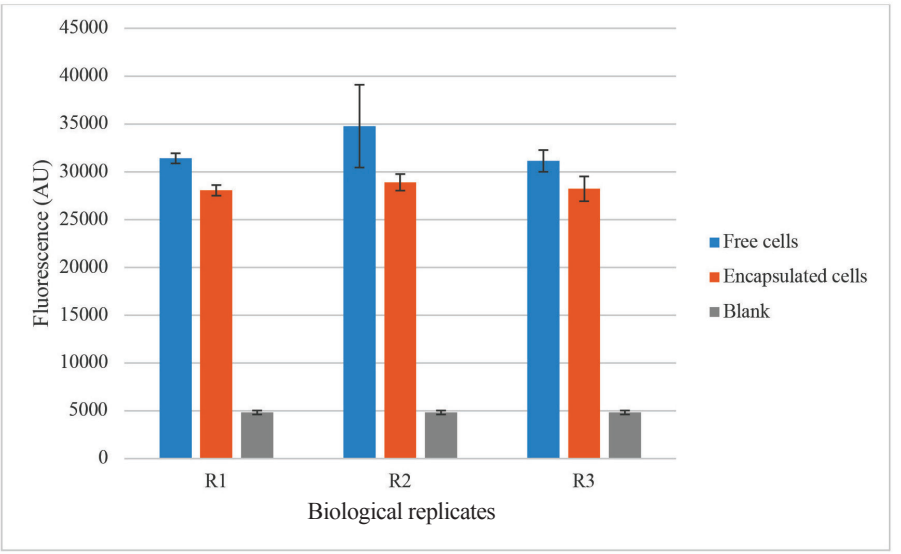


Figure 7: REMA fluorescence (AU ± SD) of cells suspended in sodium alginate vs. decapsulated AECs (n = 3). Three different batches of cells were used for the experiment as three biological replicates. The cells and beads in each biological group were aliquoted into three technical replicate groups

Table 1: Numerical viability loss (% ± SD) of encapsulated cells compared to free cells based on Figure 7

Viability Loss of Encapsulated Cells (%)				
Item	**R1**	**R2**	**R3**	**Average**
Mean	12.58	19.62	11.09	14.43
± SD	3.32	14.93	7.12	6.23

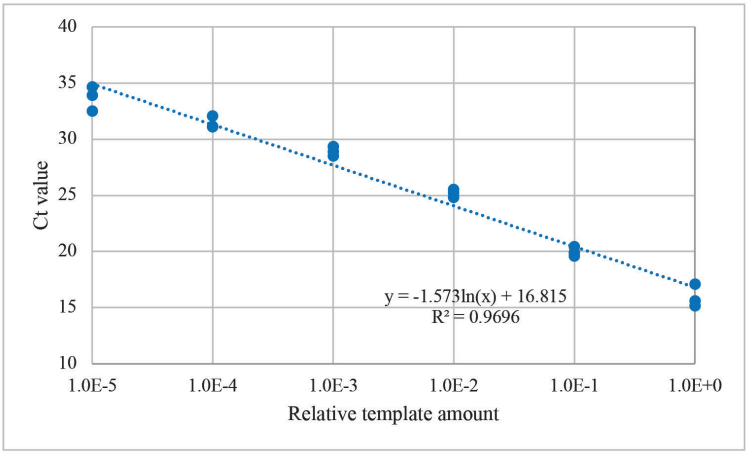


Figure 8: qPCR standard curve of 10-fold serially diluted templates (n = 3). A relative template amount of one represents 209.5 ng of DNA. qPCR efficiency: 88.855%, directly obtained from QuantStudio™ Design and Analysis Software

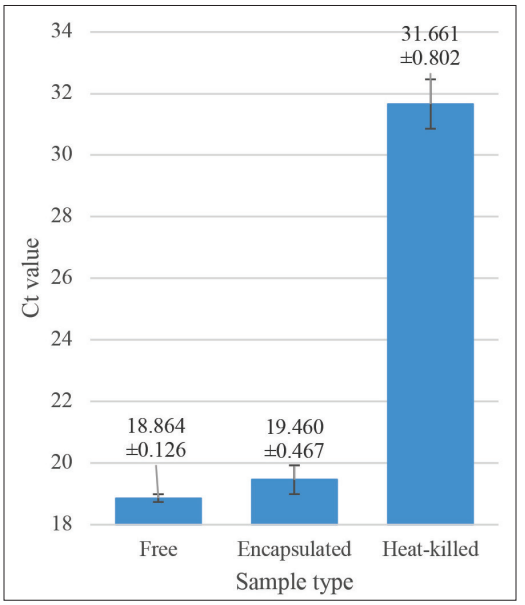


Figure 9: C_t ± SD values (n = 3) of 10 ng DNA extracts from free cells and encapsulated cells obtained from vPCR. Heat-killed cells were killed at 75°C for 10 minutes, photocrosslinked with PMA, and the resulting DNA extract was used as a negative control

killed cells were included to indicate expected C_t values representing 0% cell viability. An overview of results and numerical values are tabulated in Table 2.

Comparison between REMA and vPCR

The obtained results from REMA and vPCR are consolidated below in Table 3. It is observed that REMA and vPCR both consistently showed a decrease in cell viability of encapsulated cells compared to the free cell counterparts. This suggests that the encapsulation of *E. coli* BL21 DE3 cells in calcium alginate result in a decrease in cell viability. However, the magnitude of the decrease differed between the two approaches. Overall, REMA showed a mean loss of 14.43% whereas vPCR showed a mean loss of 31.54%. Results from vPCR had a larger standard deviation compared to REMA. Taking the standard deviations into consideration, the overlapping cell viability loss reported by both methods were within 8.20% to 20.67%.

Comparison of Various Cell Viability Studies

Previous studies have also assessed changes in cell viability following alginate-encapsulation with differences in experimental design. Such differences include: (i) Cell type, (ii) encapsulation parameters, (iii) decapsulation parameters, and (iv) cell viability assay used.

The first study (Kong *et al.*, 2003) found a decrease of 15% to 60% of cell viability following encapsulation in 2% w/w sodium

Table 2: Summary table of C_t values from free cells and encapsulated cells and the translated mean % viability loss ± SD

Item	C_t Free	C_t Encapsulated	C_t Free – Ct Encapsulated	Viability Loss of Encapsulated Cells (%)
Mean	18.864	19.460	-0.596	31.54
SD	0.13	0.47	±0.48	-
Mean – SD	18.738	19.927	-1.189	49.67
Mean + SD	18.990	18.993	-0.003	6.89

Table 3: Summary table comparing the mean ± SD viability loss of encapsulated cells between REMA and vPCR

Item	Assay	
	REMA	vPCR
Mean Viability Loss (%)	14.43	31.54
Min (Mean – SD)	8.20	6.89
Max (Mean + SD)	20.67	49.67

alginate in distilled water or alpha-minimum essential medium crosslinked in 0.1 M $CaCl_2$. The authors attributed the degree of cell viability reduction to the viscosity of the alginates, which correlated to the magnitude of shear forces experienced by the cells. The cells used in the study employed MC3T3 osteoblasts and were decapsulated in a PBS-EDTA solution, the resulting cell viability was quantified using the Trypan Blue Assay. However, our study could not attribute alginate viscosity to cell viability reduction due to our experimental design, mainly our free cell controls were suspended in sodium alginate (without subjecting to crosslinking with Ca^{2+} ions).

The second study (Hoesli *et al.*, 2011) found a decrease of 16 ± 2% and 10 ± 2% to 17 ± 2% in cell viability, following encapsulation in 2% calcium alginate using the external gelation method and 1.5% calcium alginate using internal gelation, respectively. The authors attributed cell viability loss of the internal gelation method with the duration of the acidification step (this step initiates crosslinking with Ca^{2+} ions). Cells employed were mouse insulinoma six cells and decapsulation was carried out using 90% 55 mM citrate, 10 mM HEPES, 90 mM NaCl, and pH 7.4 mixed with 10% culture medium. The authors noted that the decapsulation step had minimal impact on cell viability, retaining > 98% cell viability. Cell viability was measured using the Trypan Blue Assay. Similarly, our study showed a decrease in cell viability of 8.2% to 20.7% using REMA and 6.9% to 49.7% using vPCR using external gelation. However, several differences in experimental design were noted, i.e., the type of cells, the concentration of calcium alginate, and the cell viability assay used.

The third study (Murtas *et al.*, 2005) found a decrease in 6% to 7% in cell viability following encapsulation in 2% sodium alginate + 0.9% NaCl crosslinked in 1.1% w/v (~0.1 M) $CaCl_2$ pH 7.2 for one minute. The authors attributed cell viability loss to cell handling during the encapsulation procedure. The cells used were human hepatocyte HepG2 cells and primary rat hepatocyte cells decapsulated in 10 mM EDTA. Cell viability was measured using the Trypan Blue Assay. The current study also showed a decrease in cell viability but also differed in the cell type, alginate concentration, calcium chloride concentration, and cell viability assay used.

The last study (Mater *et al.*, 1995) claimed that virtually no statistically significant decrease cell viability occurred following encapsulation when optimum decapsulation conditions were met. Bacterial cells were used similarly to our study but using different species, namely *Pseudomonas putida* strains PaW8 and PaW130 encapsulated in 2% w/v calcium alginate. The optimum decapsulation stated by the authors employed the use of 0.05 M Na_2CO_3/0.02 M citric acid and cell viability was measured using plate counting (measured in CFU/mL gel). The authors noted that the degree of cell viability loss was attributed to differ decapsulation solutions and conditions, the magnitude also differed depending on the bacterial strain used. However, our study could not attribute cell viability loss to the decapsulation solution used as both our controls and encapsulated experimental groups were similar.

In general, most studies showed a notable decrease in cell viability caused by alginate-encapsulation regardless of the type of encapsulation method used. However, the magnitude in the decrease still varied among different studies due to differences in experimental setup and objectives. Among the studies scrutinised, even the worst-case scenario still preserved at least 40% of viable cells (Table 4).

Explaining Causes of Decreased Cell Viability

The difference in the magnitude of decreased cell viability following encapsulation by the two differing cell viability assays was likely due to their differing mechanisms of action, as discussed below:

(i) Cell washout

In the encapsulated cell samples, the loss of viable cells following encapsulation is due to

Table 4: Comparisons of findings and experimental design between our study and other studies

Cell Type	Viability Loss	Encapsulation Conditions	Decapsulation Conditions	Assay Type	Reference
E. coli BL21 DE3	8.20% to 20.67% (REMA) 6.89% to 49.67% (vPCR)	4% w/v sodium alginate in 0.20 M $CaCl_2$ for 10 minutes	Two hours in PBS pH 6.3 + 55 mM sodium citrate + 500 µg/mL alginate lyase	REMA vPCR	Our study
MC3T3 osteoblast	15% to 60% depending on alginate viscosity	2% w/w sodium alginate dissolved in distilled water or alpha minimum essential medium in 0.1 M $CaCl_2$	PBS-EDTA	Trypan Blue	(Kong *et al.*, 2003)
Mouse insulinoma 6	16 ± 2% (external gelation) 10 ± 2% to 17 ± 2% (internal gelation)	2% alginate (external gelation) in 110 mM $CaCl_2$ 1.5% alginate (internal gelation)	90% 55 mM citrate, 10 mM HEPES, 90 mM NaCl, pH 7.4 with 10% culture medium	Trypan Blue	(Hoesli *et al.*, 2011)
Human hepatocyte HepG2 Primary rat hepatocyte	6% to 7%	2% w/v sodium alginate + 0.9% NaCl in 1.1% w/v $CaCl_2$ pH 7.2 for one minute	10 mM EDTA	Trypan Blue	(Murtas *et al.*, 2005)
P. putida PaW8 and PaW130	No statistically significant decrease	2% w/v sodium alginate Co-encapsulation in 0.2 M $CaCl_2$	Optimal decapsulation: 0.05 M Na_2CO_3/0.02 M citric acid	Cell counting via plating (CFU/mL gel)	(Mater *et al.*, 1995)

cell washout during crosslinking in calcium chloride solution and the following washing phase. Washout could occur at the surface of calcium alginate beads, whereby cells that were not fully encapsulated within the matrix could be detached. In contrast, the free cell samples were not subjected to washing or placement in the crosslinking solution. Therefore, cell washout could not have happened in the free cell controls, thus, explaining the reduction in viable cell numbers. No relevant studies were found that can be used to estimate the amount of cell washout that could occur. Hence, it is difficult to gauge whether this effect could have significantly skewed the cell viability results of REMA.

However, in vPCR, the reduction in cell viability could not be theoretically attributed to cell washout. Any differences in cell number were corrected by fixing the amount of gDNA template used in vPCR. In our experiments, gDNA templates were standardised for the same experiment. While cell washout could lead to lower DNA yield, the resulting extracts were always more than the amount required for qPCR anyway. Regardless of gDNA yield, the ratio of PMA-crosslinked vs. non-crosslinked gDNA should not be affected by the yield.

(ii) Alteration of membrane permeability by Ca^{2+}ions

Following decapsulation, cells were treated with PMA for photocrosslinking. It is possible that Ca^{2+} ions that were sequestered from encapsulated cells had increased the permeability of cell membrane. Ca^{2+} ions have been known to increase membrane permeability in bacteria for applications such as cell transformation (Bukau *et al.*, 1985). The increase in membrane permeability could have increased inhibition of amplification in vPCR, resulting in a higher C_t value of the encapsulated cells. In hindsight, decapsulated cells could have been pelleted and washed to remove the decapsulating solution prior to photocrosslinking with PMA.

Membrane permeability could also be increased during exposure to Ca^{2+} ions during the crosslinking process and this increased permeability persisted long enough until the photocrosslinking process with PMA. As a result, this could have falsely increased the amount of gDNA crosslinked with PMA, leading to a greater decrease in PCR amplification. Increasing the interval between after-cell encapsulation and PMA photocrosslinking could remediate this issue.

(iii) Compromised cell membranes with lingering active enzymes

Results of this work indicate that vPCR showed a greater mean decrease in cell viability compared with REMA. This result can also indicate that the relative amount of cell membrane damage was greater than that of a decrease in redox metabolism of resazurin in REMA. However, cell viability quantification may be inflated in REMA. The reduction of resazurin in REMA is thought to be mediated by enzymes (O'Brien *et al.*, 2000). Therefore, disruption of the cell membrane may release the intracellular enzymes, resulting in cell death, but the lingering enzymes may still be metabolically active causing false positive readings. Another study has also shown that disrupted cells that released intracellular enzymes could still reduce resazurin (Chen *et al.*, 2018), leading to inflated cell viability readings. This further emphasises the importance of washing after the decapsulation step and before REMA to remediate this confounder.

The nature of the experimental design used in this study also limits the generalisability of its findings. First, alginate encapsulation can be done by tweaking many parameters, from the cell type used, alginate composition, alginate concentration, and encapsulation conditions. Secondly, our study did not assess the impact of the cell viability decrease in the context of practical settings. In many practical applications, cell viability may not directly translate into improving the desired effect and perhaps other beneficial aspects of encapsulation may outweigh the disadvantages. Lastly, the reliability of our findings hinges upon the limitations of the cell viability assays used, which we have addressed in the previous section. Additionally, the cell

viability assay used in this study also differed from other reported studies.

Conclusions

Our findings found that the optimal decapsulation conditions for 4% w/v calcium alginate capsules is two hours with 37°C incubation in a solution of 55 mM sodium citrate and 500 µg/µL alginate lyase. We also discovered that cell viability is reduced following alginate-encapsulation. However, the magnitude of cell viability reduction widely varies depending on a multitude of factors such as cell type and encapsulation conditions. Studies on alginate encapsulation and the effects of this on cell viability have mainly focused on mammalian cells, which have different physical characteristics compared with bacterial cells. Despite this, our findings largely match the trends of those conducted on mammalian cells. Nevertheless, it is highly recommended that more research be conducted on bacterial cells to supplement the current literature.

Acknowledgements

The authors thank Ministry of Higher Education Malaysia for funding this research via the Fundamental Research Grant Scheme FRGS/1/2017/STG05/UNIMAS/03/1. Both authors also acknowledge with gratitude the contribution of Associate Professor Dr. Azham Zulkharnain from Shibaura Institute of Technology, Japan for his critical comments and vast contribution towards the success of this study.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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