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Pseudomonas aeruginosa encapsulated with calcium carbonate microshells for potential biocontrol of the *Ganoderma boninense*

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Abstract−The endophytic bacterium, *Pseudomonas aeruginosa*, was successfully encapsulated into calcium carbonate microshells and coated with sodium alginate in combination with two other materials: skim milk and empty fruit bunch (EFB). The presence of bacteria cells was confirmed by a strand-like structure, a biofilm through morphology and elemental analysis. The survivability of microencapsulated bacteria was found to last for 17 months when they were maintained in a storage condition of 4 °C. Different coating materials used exhibited significant differences in the *P. aeruginosa* survival during the storage time. Their bioactivity against *Ganoderma boninense* resulted in a percentage inhibition radial growth (PIRG) value of more than 70%, which is better than its counterparts, the free *Pseudomonas* cells. With promising viability results of $\times 10^6$ CFU/mL after three-month storage, the results demonstrate that skim milk-coated alginate might be good protection for *P. aeruginosa* that could sustain the viable bacteria at the target site. This is toward a greener, biological control-based plant protection for *Ganoderma* diseases in the oil palm planting industry.

Keywords: *Pseudomonas aeruginosa*, Survival, Calcium Carbonate, Microencapsulation, Granules

INTRODUCTION

The demand for oil and fat in the world has increased over the years, inducing the expansion of oil palm plantation areas. The bracket fungus, *Ganoderma boninense*, has become one of the major threats to oil palm production, especially in Southeast Asia. This serious disease leads the palm oil becoming infertile, lose weight in fruit bunches and die within less than three years [1]. In combating this disease, several chemical formulations have been formulated after some modifications were made from cultural practices [2,3] and thorough studies have been conducted against basal stem rot diseases (BSR) caused by *Ganoderma boninense* either in laboratory testing or field application [4,5]. However, until now the results have been unsatisfactory due to chemical handling and its exposure during manufacturing.

Recently, the knowledge of microbial strains has been explored and the development of biological formulations as an alternative control is emerging. It has been experimentally demonstrated that endophytic bacteria such as *Burkholderia* and *Pseudomonas* species [6], *Trichoderma* and *Hendersonia* fungus are capable to suppress the *Ganoderma* incidence by 30-80% [7,8]. Most of the existing approaches appear to deal with pure microbial isolates as an antagonist to the *Ganoderma* pathogen. As far as we know, the utilization of biological control in the field is limited due to its handling and shelf life.

Encapsulation of microorganisms may offer advantages to preserving the integrity of the microbial cells by maintaining their viability and microbe functionality, which helps them maintain their effectiveness during application [9]. In recent decades, a report on endophytic fungus, *Hendersonia*, in combination with alginate and nutrient carrier (dhal powder) has given a promising result towards *Ganoderma* disease treatment, as the formulation is still effective after a long time storage [10]. An early observation reported over two decades ago showed a potential for long-term storage of *Pseudomonas aeruginosa* to survive until about four years; unfortunately, a definitive link between the survivability and bacteria vitality during storage was inconclusive [11].

$CaCO_3$ microparticles have attracted interest as they have tremendous advantages over other biomimetic materials. This inorganic microparticle is known in nature due to its stability, nontoxicity, and capability as a binding medium in soil for ground reinforcement [12]. Furthermore, the preparation of $CaCO_3$ is safe and ecofriendly with easy raw material availability and cost-effectiveness. Its high porosity, good bioavailability and slow degradability characteristics have made it beneficial in the agricultural sector as controlled release formulation or pesticide removal absorbent [13-15].

Therefore, here we explore the survival of *Pseudomonas* bacteria strain as a biocontrol, encapsulated in calcium carbonate ($CaCO_3$)

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microshells, using the encapsulation technique and investigate their behavior towards the targeted pathogen. *Pseudomonas aeruginosa* is *a* bacterium that was chosen in this work as it has an antagonistic effect against *Ganoderma boninense* and has been proven to have the highest percentage of inhibition compared to the other ten different potential species [16].

MATERIALS AND METHOD

1. Materials

Calcium chloride ($CaCO_3$, molecular weight 110.98), sodium carbonate (Na_2CO_3, molecular weight 105.98) and alginic acid $(C_6H_8O_6)_n$ were purchased from Sigma Aldrich and used as received. Sucrose was obtained from Merck, skim milk was obtained from Millipore and distilled water was used in all of the experiments. The bacterial culture medium used in the study such as LB broth, Miller (Luria-Bertani), potato dextrose agar and bacto-agar were supplied by Difco.

2. Bacterial Culture Conditions:

Pseudomonas aeruginosa bacteria were taken from a glycerol stock. After thawing, a 10 μL aliquot of bacteria was streaked on the plate and incubated at 37 °C, overnight. The bacteria were inoculated into a flask containing Luria-Bertani broth with shaking under aerobic conditions at 150 rpm and 28 °C. The bacterial culture was harvested during the exponential growth phase (16 hours), and underwent a centrifugation process at 5,000 rpm for 15 minutes at 4 °C.

3. Encapsulation of Bacteria into Calcium Carbonate Micro Shells

The encapsulation of *Pseudomonas* bacteria into calcium carbonate was carried out according to the previous method [17,18], with some modifications to suit our particular application. Initially, the bacterial cells (approximately 100 mg mL^{-1}) were soaked in 11.7% sucrose before being resuspended in 0.33 M aqueous $CaCl_2$ for 20 minutes under stirring at room temperature. Then, an equal volume of 0.33 M Na_2CO_3 solution was added to a beaker containing bacterial cells and $CaCl_2$, and the mixture was stirred vigorously for 5 min. The samples were collected immediately to avoid any recrystallization and underwent a centrifugation process at 10,000 rpm for 5 minutes. The pellet obtained was denoted as microencapsulated bacteria and then was dried under laminar flow, overnight before being stored in a chiller at 4 °C.

4. Characterization of Microencapsulated Bacteria

The crystallinity of the microencapsulated bacteria was determined using powder X-Ray diffraction (Rigaku SmartLab, Japan). The data was collected at a range between 2-60 degrees at a scan rate of 4 deg/min. Nicolet iS10 Fourier Transform - InfraRed Spectrometer was used to identify the presence of functional groups in the microencapsulation with a wavenumber of 500-4,000 cm^{-1}. The thermal stability of samples was studied on alumina crucibles with a Mettler Toledo instrument model TGA-DSC HT 3 in the range of 25-1,000 °C, a heating rate of 10 °C/min and under nitrogen flow of about 50 mL/min. The microencapsulation sample was examined with a field emission scanning electron microscope (FESEM) using a NOVA NanoSEM 230 for morphology identification. To load a sample in FESEM, the sample was stuck to aluminum stubs with a double-sided carbon sticker. Energy dispersive X-ray (EDX), Max20 was used to determination of the elemental composition of the samples.

5. Coating of Microencapsulated Bacteria with Alginate

The microencapsulated bacteria were coated with alginate by following the granulation protocol of endophytic microbe [10]. The amount of 1 g of alginic acid (final concentration 1% w/v) was dissolved in absolute alcohol. The microencapsulated bacteria (at wet weight 20% w/v) were added to the alginate solution before adding skim milk, empty fruit bunch (EFB) and distilled water in producing the final 100% mixture. The mixture was stirred homogeneously using IKA® Ultra Turrax homogenizer. The mixture then was dropped into a 2 M $CaCl_2$ solution in obtaining granules. The coated granules then were rinsed with sterile distilled water, dried under laminar flow, overnight and kept in a chiller.

6. Survival of Bacteria after Encapsulation and Coating Process

The viability of *Pseudomonas* cells in their encapsulation form and coated granule was checked using spread plate techniques. Series dilution of microencapsulated bacteria or coated granules was prepared between the range, 10^0 to 10^8. 100 μL of each dilution was spread evenly on sterilized Luria Bertani (LB) agar plates using a glass spreader and incubated at 37 °C. After 18 hours, the number of bacterial colonies was counted using Eq. (1) below [19];

$$\frac{(\text{Average count}) \times (\text{dilution plated})}{(\text{mL plated})} = \text{CFU/mL} \quad (1)$$

The cell viability of microencapsulated bacteria was checked monthly until no colony was seen while for coated granules, the reading was taken up to 3 months (based on the expected duration for storage before application).

7. In Vitro Activity of Coated Granular Formulations Against *Ganoderma boninense*

The coated granules were tested for their antagonistic activity against *Ganoderma* pathogen via dual culture assay [16]. A 5 mm inoculum plug was punched using a sterile cork borer from the seven-day-old potato dextrose agar (PDA) culture of *G. boninense* and plugged in the middle of the PDA plate containing tested granules. Coated granules weighed 0.5 g and were placed three cm away from the *G. boninense* plug for each side. A PDA plate with only *G. boninense* served as a control. All the test antagonistic pairings were incubated at 28±2 °C. The mycelial growth of *Ganoderma* fungus was measured after seven days of incubation and the percentage inhibition of radial growth (PIRG) was evaluated as given in Eq. (2) [20];

$$\text{Percentage of inhibition (\%)} = (M_1 - M_2)/M_1 \times 100 \quad (2)$$

where M_1 refers to the radial growth of Ganoderma fungus in a control plate, and M_2 refers to the radial growth of Ganoderma fungus in the dual culture plate.

8. Statistical Analysis

Cell viability and dual culture assay experiments were analyzed with GraphPad Prism Version 6 software (California, USA). Statistical analyses were performed by applying a significance level of 0.05. Significant differences between samples were determined by Tukey's test, and all results are expressed as the mean and standard deviation for three independent tests.

RESULTS AND DISCUSSION

The encapsulation of *P. aeruginosa* was performed via the biomineralization process. The *Pseudomonas* cells, as ureolytic bacteria, were introduced into a supersaturated Ca^{2+} and CO_3^{2-} mixture, allowing the crystallization to facilitate over the bacterial cell, which served as nucleation sites. This may be explained by the interaction of Ca^{2+} ions with the negatively charged bacteria cell wall, inducing deposition [21]. The presence of biofilm extracellular polymeric substances (EPS) in *Pseudomonas* bacteria also enhanced biomineralization by providing the ions for $CaCO_3$ crystals, thus creating an alkaline environment for the support growth of calcite. This phenomenon highly encouraged precipitation as the mineral started to develop at biofilm surfaces [22,23]. It was also reported that the involvement of sucrose in the $CaCO_3$ during the synthesis can increase the vaterite content and enhance the biomineralization process [24]. Sucrose was injected into the mixture along the encapsulation process, to maximize the initial bacterial count in the microencapsulation system [25].

1. X-Ray Diffraction

The crystallinity of *Pseudomonas* bacteria, pure $CaCO_3$ and microencapsulated system were examined and displayed in Fig. 1. After parametric optimizations involving different conditions of bacterial culture, the ratio of each component in the mixture and storage parameter, the microencapsulated bacteria prepared at optimized conditions is presented in Fig. 1(c). During the synthesis of the microencapsulated bacteria, amorphous calcium carbonate (ACC) was converted to $CaCO_3$ in two stages. The first stage involved the dehydration of ACC to the vaterite phase and then the vaterite was transformed into the calcite phase through dissolution and reprecipitation mechanisms. The non-uniformed structure of microencapsulated bacteria may be due to the reaction of sucrose and bacteria on the vaterite phase (metastable phase) during the mineralization process as discussed previously [26].

The presence of a broad peak below 10° for *P. aeruginosa* (Fig. 1(a)) may be related to the amorphous state in bacteria, then followed by reflection peaks at 21.4, 25.1, 27.5, 29.9, 33.2, 40, 44, and 50.4° (★). The microencapsulation pattern (Fig. 1(c)), also showed identical peaks (★) with some changes in the intensity, indicating the bacteria's presence in the system. Pure $CaCO_3$ (Fig. 1(b)) displayed high crystallinity and the obvious angle of 29.5° (■) represents a calcite peak [27].

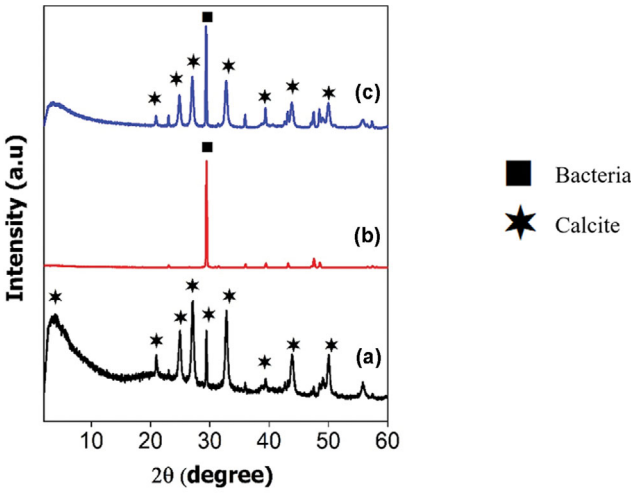

Fig. 1. X-Ray diffraction patterns of *P. aeruginosa* bacteria (a), pure calcium carbonate (b) and the microencapsulated bacteria (c).

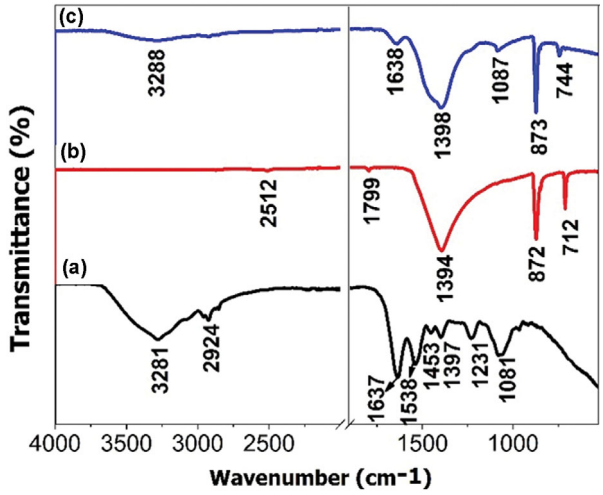

Fig. 2. The FTIR spectra of the bacteria, *P. aeruginosa* (a), pure calcium carbonate (b) and microencapsulated *P. aeruginosa* into calcium carbonate (c).

2. Fourier Transform Infrared

The FTIR spectra of *P. aeruginosa*, pure calcium carbonate and microencapsulated bacteria are shown in Fig. 2. *P. aeruginosa* (Fig. 2(a)) shows an absorption band at 3,281 cm^{-1}, corresponding to the OH stretching vibration of the hydroxyl group and amine groups of the protein. A narrow peak at 2,924 cm^{-1} indicates asymmetrical C-H stretching that is present in the cell wall of the bacteria. An absorption band at 1,637 cm^{-1} can be attributed to the C=O stretching of carboxylic acid and ester groups from lipid and fatty acids. The band at 1,538 cm^{-1} may represent the NH stretching of the amide bond due to the protein-peptide bond. The bands observed at 1,453 and 1,397 cm^{-1} are due to the CH_3 and CH_2 asymmetric and symmetric deformation, while the PO^{-2} asymmetric stretching is shown at 1,231 cm^{-1}. The broadband at 1,081 cm^{-1} is characteristic of the C-O-C bending vibration [28].

For the pure calcium carbonate (Fig. 2(b)), five bands were observed; two small humps at 2,512, 1,799 cm^{-1} and a strong band at 1,394 cm^{-1} which are highly related to the O-C-O stretching mode of carbonate [29], bands at 872 and 712 cm^{-1} are the characteristics of the out of plane bending vibrations [30]. These intense peaks are all due to the calcite phase [31].

After encapsulation of the bacteria in the $CaCO_3$ (Fig. 2(c)), it was noted the bands were shifted to higher wavenumbers, indicating the interaction of bacteria and material had occurred. The presence of bands at 3,288, 1,638 and 1,087 cm^{-1} revealed the functional groups of protein in the bacteria structure. Bands observed at 1,398, 873 and 744 cm^{-1}, are related to the characteristic bands in the calcite or calcium carbonate [32].

3. Thermal Study

Fig. 3 displays the weight loss (TGA) and derivative weight loss

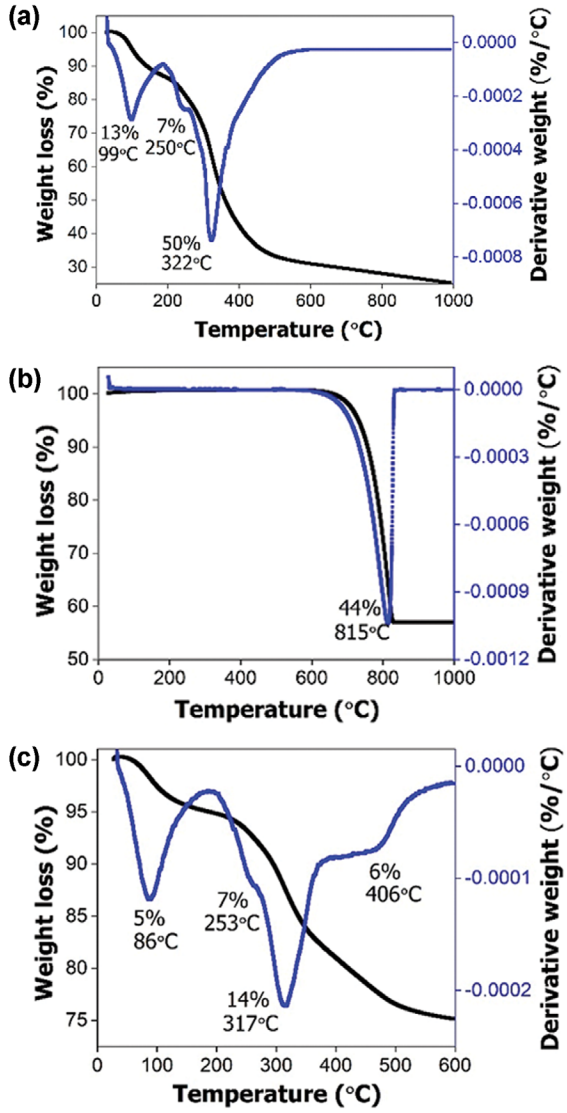

Fig. 3. TGA-DTG thermogram of the bacteria, *P. aeruginosa* (a), pure calcium carbonate (b) and the bacteria-microencapsulated compound (c).

(DTG) of the *Pseudomonas* bacteria, $CaCO_3$ and the microencapsulated bacteria. The thermal degradation of *Pseudomonas* (Fig. 3(a)) contains three phases of weight loss: 99, 250 and 322 °C with weight loss of 13, 7 and 50%, respectively. A weight loss below 150 °C is usually related to the loss of residual water molecules. The second and third weight loss may be attributed to the pyrolysis of organic components of the bacteria, such as protein and lipids [33]. For pure $CaCO_3$ (Fig. 3(b)), there is only one stage of weight loss that lies at 815 °C, which corresponds to the combustion of $CaCO_3$ itself [34].

The thermal decomposition process of bacteria-encapsulated $CaCO_3$ microshells (Fig. 3(c)) involves three stages at 86, 253, 317 and 406 °C. The first peak with a weight loss of 5% is accounted for due to water loss, while the second peak (7%) represents the degradation of sucrose by forming a black aerated solid [32,33]. Both the second (14%) and third (6%) peaks revealed the degradation of biopolymers, the organic components of the bacteria. Even though the thermal degradation of $CaCO_3$ was not observed, it is highly possible that since the bacteria were encapsulated into $CaCO_3$, their degradation event might happen simultaneously with the bacteria.

Table 1. Energy-dispersive X-ray spectroscopy (EDX) data of *P. aeruginosa*, $CaCO_3$ and microencapsulated bacteria

Sample	Element	Weight %	Atomic %
Pseudomonas aeruginosa	C K	17.22	55.47
	O K	9.91	23.96
	Na K	0.75	1.27
	Cl K	2.81	3.06
	K K	3.14	3.11
	Pt M	66.17	13.12
$CaCO_3$	C K	18.91	39.59
	O K	25.11	39.43
	Ca K	27.55	17.30
	Pt M	28.43	3.66
Microencapsulated bacteria	C K	22.97	40.08
	O K	34.29	44.91
	Ca K	25.11	13.13
	Pt M	17.63	1.89

4. Microscopic Analysis

Biofilm is a sticky and sugary substance secreted by certain microorganisms, especially bacteria, when they adhere to a surface or each other. Bacterial biofilms contain proteins (fibrinous), polysaccharides (alginate) and eDNA. The bacteria use the biofilm in order to reproduce strands, which allows them to develop more and form complex communities. The adaptation of bacteria to the environment through biofilm networks also makes the bacteria more resistant to any attacks [37].

In obtaining detailed information on biofilm, FESEM-EDX measurements were performed on the samples. Measurements were conducted on pure *P. aeruginosa*, calcium carbonate without bacteria and calcium carbonate with encapsulated bacteria under 25,000x and 100,000x magnifications, as shown in Fig. 4.

The FESEM micrographs of *P. aeruginosa* show an undulating surface (Fig. 4(b)), which may resemble the extracellular polymeric substances (EPS) characteristics [38]. Recent research has reported that the granule-like structure is due to $CaCO_3$ (Fig. 4(d)), which may be related to rhombohedral calcite polymorphism [39]. Fig. 4(f) shows the strand-like structures observed around the calcium carbonate, which is due to the film of the *Pseudomonas*. The presence of bacterial biofilm on $CaCO_3$ was supported by the FTIR spectrum shown in Fig. 2(c), as the presence of bands belonging to protein has been identified.

EDX analysis disclosed the chemical composition of *Pseudomonas* bacteria (Fig. 5(a)), showing the presence of oxygen, carbon, chlorine, sodium and potassium. The microencapsulated bacteria (Fig. 5(c)) shows several adhered cells, confirming the presence of biofilm, where the EDX shows that the atomic percentage of carbon and oxygen increases by about 5% compared to pure $CaCO_3$ (Fig. 5(b)).

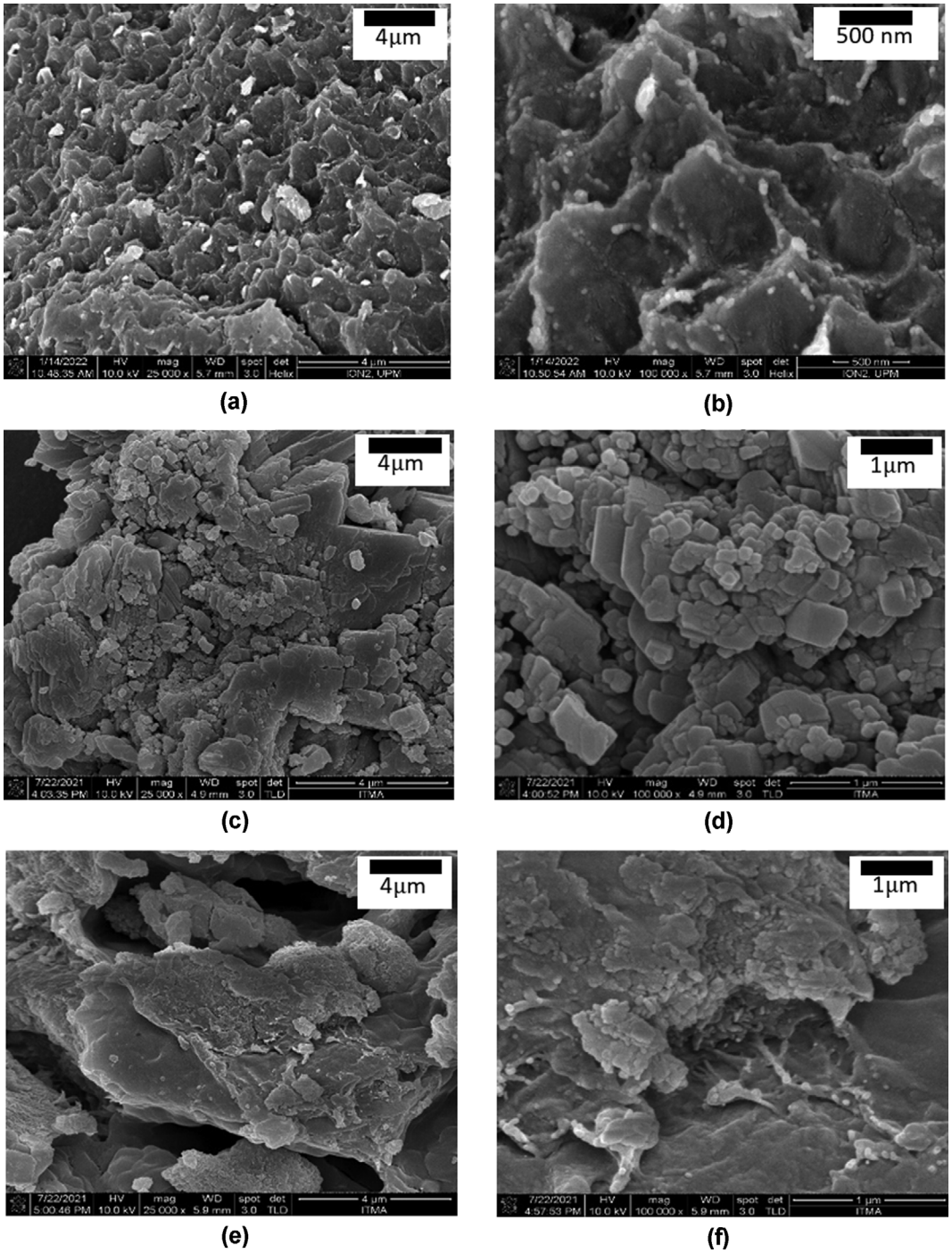

Fig. 4. Field emission scanning electron micrographs of *P. aeruginosa* bacteria (a), (b) pure calcium carbonate (c), (d) and microencapsulated bacteria (e), (f). (a), (c) and (e) are under 25,000x magnification while (b), (d) and (f) are under 100,000x magnification.

5. The Survival of Microencapsulated Bacteria after Immobilization in $CaCO_3$ and Coating

The survival of encapsulated *P. aeruginosa* was tested on different storage temperatures, chiller (4 °C) and room temperature (28 °C). However, the bacterial cell count dropped drastically to zero within three months when stored at room temperature. Therefore, the viability experiment was focused only on the chiller condition, which lasts over a year, as presented in Fig. 6.

The initial cell count of *P. aeruginosa* before encapsulation was found to be 6.0×10^8 CFU mL^{-1}. With the optimum growth condition of *P. aeruginosa* at 150 rpm and 28 °C for 16 hours, the loading capacity of bacteria after the encapsulation process was 5.5×10^8 CFU/mL, which was reduced by 11% from the initial count cell. This count cell was achieved after the insertion of sucrose separately during the synthesis of $CaCO_3$. After the microencapsulation process, the result demonstrated decreasing trend in cell survival

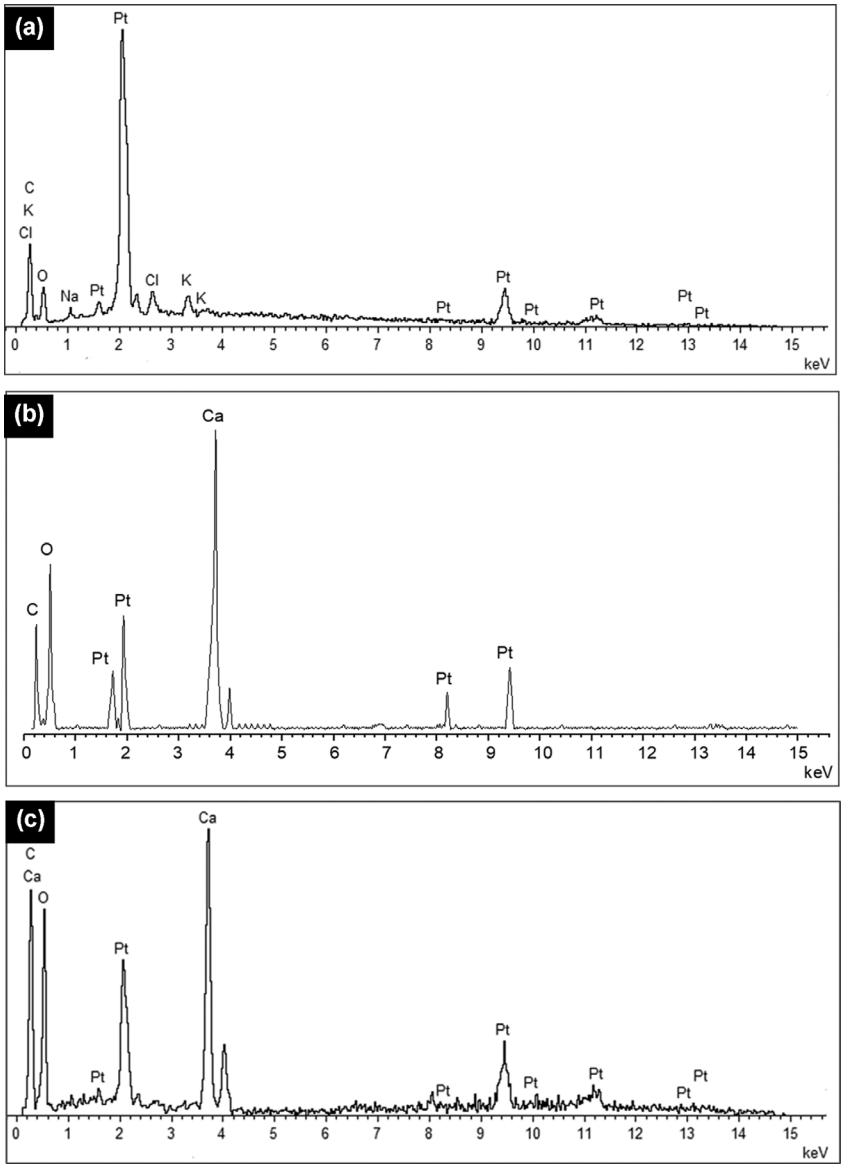

Fig. 5. Energy dispersive x-ray analysis (EDX) of *P. aeruginosa* (a), pure calcium carbonate (b) and calcium carbonate-microencapsulated *P. aeruginosa* bacteria (c).

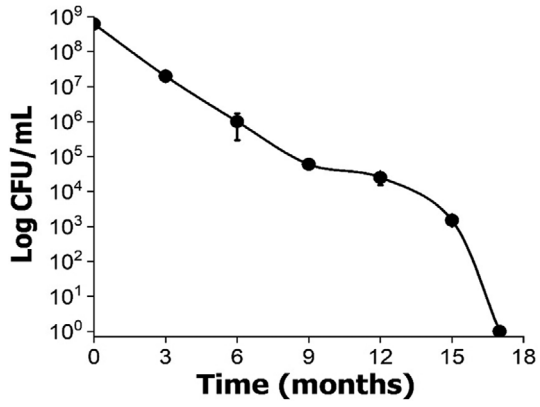

Fig. 6. The viability of *P. aeruginosa* cells encapsulated in $CaCO_3$ and stored at 4 °C over a year. Each point indicates the mean of three plate counts from three samples.

during the storage time.

The population of encapsulated cells shows a decline rate between 94-97%, after 3 to 9 months with viability values of 2.0×10^7, 1.0×10^6 and 6.0×10^4 CFU mL^{-1}, respectively. During the intervals of 9 to 12 months, the viability rate was only reduced by 58%, with a maintained cell count of $\times10^4$ CFU mL^{-1}. After a year, the survival of cells declined to 1.0×10^3 CFU mL^{-1} and lost its viability at a 17-month interval. Although the observation has shown that the encapsulation method affected the bacteria's survival, however, encapsulation can provide good protection to the bacterial cells. This is similar to previous findings [40] that suggested that the reduction of bacteria cellular viability over time can be related to the drying process due to the water removal that has occurred.

The granulation method is one of the ways to produce more stable and easier-to-store formulations. The microencapsulated bacteria were coated with alginate, empty fruit bunch (EFB), or skim milk

Table 2. Ratio of skim milk to empty fruit bunch (EFB) used for the coated granular formulations

Formulation	Skim milk (%)	EFB (%)
G1	50	50
G2	0	100
G3	100	0

powder as a binder, and the composition used in each formulation is described in Table 2. Three different coated granules were stored at 4 °C conditions and the bacterial readings were recorded as triplicate for three months as illustrated in Fig. 7.

Based on Fig. 7, it was revealed that the survival cell in the coated granule with skim milk (G3) was statistically significant among them. This study also shows that a combination of skim milk and alginate (G3) provides the best coating agent, followed by EFB-alginate (G2) and skim milk-EFB-alginate (G1). The viability of *Pseudomonas* cells in the granular formulation of G3 is always significantly higher throughout the months and managed to sustain the viability at 5×10^6 CFU mL^{-1} after three months of storage, as presented in Table 3. This is attributed to the skim milk composition, such as lactose and whey protein, which act as a barrier to

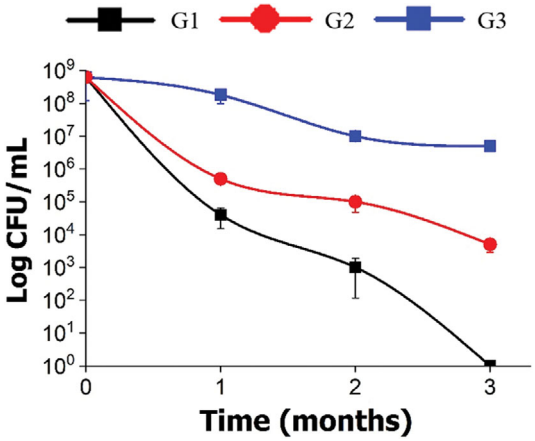

Fig. 7. The viability trend of bacteria in different coated granules at 4 °C within 3 months.

the accessibility of external substances resulting in better cell stability during storage [41]. The lowest *Pseudomonas* count cells were shown in the granular formulation of G1 with only 1×10^1 CFU mL^{-1}. The overall result indicated that the selection of coating and per-

Table 3. Data count of *P. aeruginosa* colonies in different coated granules

Formulations	Cell viability (CFU/mL)			
	Storage duration (months)			
	0	1	2	3
G1	$6\times10^8\pm1.10^a$	$4\times10^4\pm2.50^c$	$1\times10^3\pm8.84^c$	$1\times10^1\pm0.00^c$
G2	$6\times10^8\pm0.44^a$	$5\times10^5\pm1.33^b$	$1\times10^5\pm5.22^b$	$5\times10^3\pm2.10^b$
G3	$6\times10^8\pm1.30^a$	$2\times10^8\pm8.13^a$	$1\times10^7\pm3.12^a$	$5\times10^6\pm1.36^a$

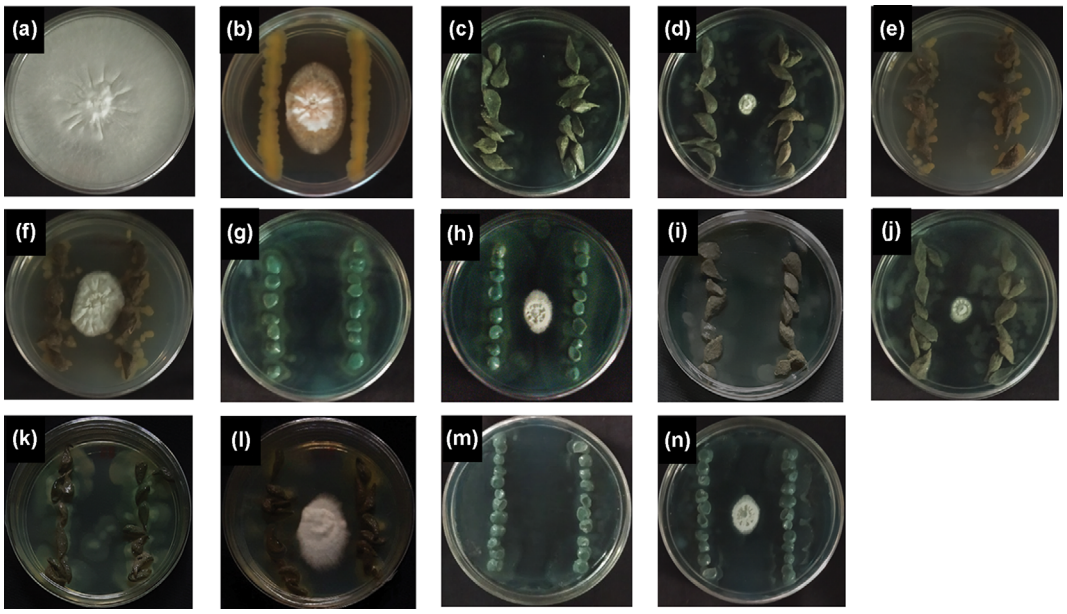

Fig. 8. Dual culture test of *G. boninense,* control (a) *P. Aeruginosa* bacteria (b), G1 control (c), G1 (d), G2 control (e), G2 (f), G3 control (g), G3 (h) coated granules at the initial week and inhibition zone of G1 control (i), G1 (j), G2 control (k), G2 (l), G3 control (m) and G3 (n) coated granule after 3-month storage.

Table 4. Decay rate and correlation coefficient (R^2) value of the *Pseudomonas* viability based on various granular formulations

Formulations	G1	G2	G3
Correlation coefficient (R^2)	0.7687	0.8877	0.7714
The decay rate, k	-2.0×10^4	-2.5×10^5	$-9.0-10^7$

centage ratio of coating used is crucial in obtaining the optimum conditions for bacterial cells.

The correlation of coated granules' viability with time has been studied and the parameters such as the decay constant, k and the coefficient value, R^2 are shown in Table 4. The finding is expected, since the G3-coated granules show the lowest decay rate with a value of -9.0×10^7, nicely matching the slowest viability reduction as shown by the data in Table 3. G1 granules appeared to have the highest decay rate as the bacterial viability dropped drastically during the storage time.

6. Efficacy of Coated Granular Formulation on *Ganoderma boninense* Mycelia Growth

The inhibition effect of coated granules on a targeted pathogen, *G. boninense* using a dual-culture assay, is illustrated in Fig. 8. The study presents *G. boninense* (negative control), *P. aeruginosa* isolate, granules without the tested pathogen (positive control) and the coated granular formulations against the tested pathogen. To study the behavior of bacterial activity in the granular formulations, the inhibition activity was tested again after three months. The inhibition values (%) of each sample are summarized in Table 5.

Experimental results revealed the inhibitory effect of granular formulations on pathogen was better compared to *P. aeruginosa* with an inhibition range value of 81-95% compared to 65%, respectively. The statistical analysis indicated the inhibition rates of all three coated granules against *G. boninense* were significantly different. At an earlier month, the inhibition value of G1 granules was the highest, followed by G3 and, lastly, G2 coated granules.

After three months of storage, all inhibition readings showed a slightly decreasing trend. Comparing the initial and the final months, a significant difference was observed for G1 and G2 coated granules; however for G3 granules, the inhibition value remained unchanged with no statistical difference.

The inhibition rate of the formulations toward the target pathogen decreased after storage time as a result of the reduction of viable bacteria in the coated granules. Even though the viability decreased drastically in a certain formulation such as G1 granules, the percentage inhibition was still higher, with a value of 75-89%. This inhibitory result can be reliable evidence that *Pseudomonas* cells were successfully preserved during storage.

Table 5. Percentage of PIRG value of coated granules in different formulations

Formulations	PIRG value (%)	
	Storage duration	
	Initial week	After 3 months
G1	$94.74\pm0.00^{A}_{a}$	$89.47\pm0.00^{A}_{b}$
G2	$81.58\pm1.32^{C}_{a}$	$75.00\pm2.63^{B}_{b}$
G3	$88.59\pm1.32^{B}_{a}$	$88.16\pm1.52^{A}_{a}$

Notes: Different uppercase letters indicate significant differences among the three different samples ($p<0.05$). Different lowercase letters indicate significant differences between the two intervals ($p<0.05$).

These findings are in line with those found in the literature, where the release of *P. aeruginosa* from $CaCO_3$ microparticles can be possible in three ways: through diffusion, carrier dissolution or recrystallization of metastable phase such as vaterite and aragonite to the stable form, calcite. As the $CaCO_3$ microparticles are known to be pH-dependent carriers, this pH parameter would be critical for the release mechanism. This carrier is highly sensitive to reduced pH where the target site, *Ganoderma boninense* pathogen has an acidic environment with a value pH of 5.5. At this acidic condition, free protons tend to react with CO_3^{2-} to form HCO_3^-, degrading the $CaCO_3$ crystals and effectively releasing the *P. aeruginosa* bacteria [42].

CONCLUSION

This study has shown that calcium carbonate microshell is a useful long-term carrier for the bacteria, *Pseudomonas aeruginosa*, which can survive well for 17 months in storage at 4 °C. The viability of *Pseudomonas* decreases over time after the microencapsulation process, which may be related to the drying involved in the process. The encapsulated-coating method demonstrated the integrity and functionality of bacteria were successfully preserved as only a small significant difference was observed in their inhibition against *Ganoderma boninense*. Among the three coating formulations designed in this study, skim milk was found to provide the best preservation coating method for *Pseudomonas* cells. In addition to the superior physicochemical properties of $CaCO_3$ for soil application, the coated bacteria cells could be beneficial to the environment and have a high potential for future commercialization in the agricultural sector. This is toward a greener, biological control-based plant protection for the oil palm planting industry.

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