CHAPTER 3

RESULTS

1. Optimization of bacterial culturing time

The number of endospores produced at different time of incubation was shown in Figure 5. Bacterial number reached the highest at 72 h of incubation (10^7 CFU/ml).

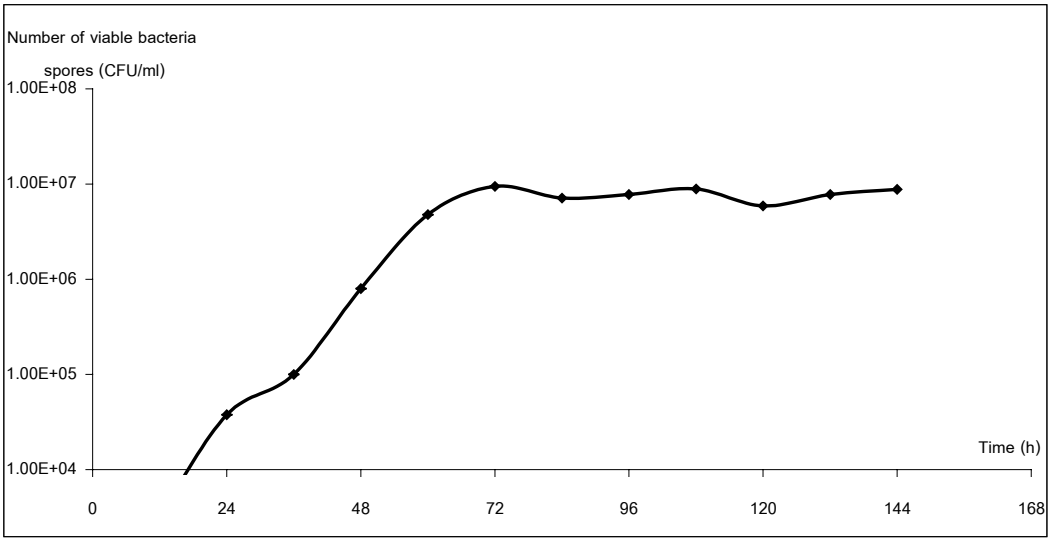

Figure 5. Numbers of viable bacterial endospores at different time of incubation

2. Preparation of *B. megaterium* formulations

Five granule and three tablet formulations containing *B. megaterium* for spray application were successfully prepared. All formulations were white in color, except formulation GS-Alg which contained yellowish sodium alginate. Three granule and four tablet formulations containing *B. megaterium* for broadcast application were also prepared. All formulations are white in color. Various types of formulations were presented in Figure 6.

Figure 6. *B. megaterium* formulations; (a) formulation GS-SCMC, (b) formulation TS-15, (c) formulation GB-19 and (d) formulation TB-39

3. Enumeration of viable bacteria in the formulations

The number of viable bacteria in five granule formulations prepared for spray application (GS) were approximately 10^7 CFU/g (Table 6). In these preparations, the bacterial suspension containing approximately 10^8 CFU/ml was used (30 ml / 100 g of formulation). The number of viable bacteria still remained high after granule production (Table 6).

Table 6. Number of viable bacteria in granules containing *B. megaterium* for spray application

Formulation	Number of viable bacteria (± S.D.) (CFU/g)
Bacterial suspension (ml)	3.75 (±0.96) x 10^{8}*
GS-Alg	7.00 (±1.63) x 10^{7}
GS-HPMC	9.00 (±1.15) x 10^{7}
GS-SCMC	7.25 (±2.63) x 10^{7}
GS-MC	5.50 (±1.29) x 10^{7}
GS-HEC	7.25 (±1.89) x 10^{7}

* Number of bacteria in suspension used for granules preparation

In another study, the number of bacteria in suspension was increased to 10^{10} CFU/ml and used as an active ingredient for two granule and three tablet formulations for spray application, three granule and four tablet formulations for broadcast application (Table 7). Formulation GS-Alg and GS-SCMC contained viable bacteria about 10^{9} CFU/g after using 10^{10} CFU/ml bacterial suspension as the active ingredient (Table 7). In the case of tablets for spray application (TS), the number of bacteria were monitored in both granules before compression and tablets, each granule before compression should contain viable bacteria about 10^{9} CFU/g because bacterial suspension containing viable bacteria about 10^{10} CFU/ml was also used as the active ingredient in all formulations (30 ml in 100 g of each formulation). However, the result showed that the granules before compression contained viable bacteria about 10^{9} CFU/g and the finished products contained viable bacteria about 10^{6} CFU/g. Formulations for broadcast application contained viable bacteria about 10^{8} CFU/g (Table 7). These results indicated that there was a slight decline of bacteria number during the preparation process.

Table 7. Number of viable bacteria in formulations containing *B. megaterium*

Formulation	Number of viable bacteria (± S.D.) (CFU/g)	
	Granules before compression	Tablets
Bacterial suspension (ml)	1.25 (±1.16) x 10^{10}*	1.25 (±1.16) x 10^{10}*
GS-Alg	8.75 (±6.41) x 10^{9}	
GS-SCMC	4.13 (±2.36) x 10^{9}	
TS-5	3.13 (±1.13) x 10^{9}	2.00 (±1.31) x 10^{6}
TS-10	3.38 (±1.41) x 10^{9}	4.00 (±1.69) x 10^{6}
TS-15	3.13 (±1.81) x 10^{9}	6.88 (±1.25) x 10^{6}
GB-19	7.50 (±1.31) x 10^{8}	
GB-29	5.50 (±2.67) x 10^{8}	
GB-39	6.38 (±2.00) x 10^{8}	
TB-19	-	4.00 (±0.79) x 10^{8}
TB-29	-	4.03 (±2.10) x 10^{8}
TB-39	-	5.20 (±2.66) x 10^{8}
TB-49	-	1.90 (±0.92) x 10^{8}

* Number of bacteria in suspension used for preparation

\- The compression process did not include in the formulation process

4. Evaluation of physical properties of *B. megaterium* formulations

4.1 Evaluation of physical properties of bacterial granules for spray application (GS)

4.1.1 Particle size of granules

The particle size of all granule formulations was in the range of 1.405 – 1.421 mm (Table 8) and there was no significant difference among various formulations.

Table 8. Particle size of bacterial granules for spray application (GS)

Formulation	Particle size of granules (± S.D.) (mm)
GS-Alg	1.416 (±0.002)
GS-HPMC	1.413 (±0.003)
GS-SCMC	1.411 (±0.006)
GS-MC	1.416 (±0.004)
GS-HEC	1.420 (±0.001)

4.1.2 Disintergration time of bacterial granules in water

There was much difference in disintegration time among different formulations ($p<0.01$). Formulation GS-Alg and GS-SCMC performed shorter disintegration time than others. Only formulation GS-MC was incompletely dissolved at room temperature (26-30°C). The rank of disintegration time was in following orders: GS-HEC > GS-HPMC > GS-Alg and GS-SCMC.

Table 9. Disintegration time to prepare 1% w/w solution of bacterial granules for spray application (GS)

Formulation	Disintegration time (± S.D.) (min) *
GS-Alg	7.53 (±0.16) c
GS-HPMC	28.41 (±2.38) b
GS-SCMC	9.86 (±0.08) c
GS-MC	- **
GS-HEC	44.71 (±4.42) a

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

** Incomplete dissolved at room temperature (26 - 30°C)

4.1.3 pH of 1% w/w and 5% w/w solution of bacterial granules in water

The pH value of 1% w/w and 5% w/w solution prepared from bacterial granules was range from weak acid to neutral (4.97 – 6.97) as shown in Table 10.

Table 10. The pH value of 1% w/w and 5% w/w solution of bacterial granules for spray application (GS)

Formulation	The pH value (± S.D.)	
	1% w/w solution	5% w/w solution
GS-Alg	6.56 (±0.06)	6.41 (±0.06)
GS-HPMC	5.70 (±0.49)	5.35 (±0.38)
GS-SCMC	6.76 (±0.21)	6.60 (±0.08)
GS-MC	5.69 (±0.36)	5.50 (±0.45)
GS-HEC	6.49 (±0.60)	6.47 (±0.23)

4.1.4 Viscosity of 1% w/w and 5% w/w solution of bacterial granules

Table 11 showed that there was significant difference in viscosity value of 1% w/w and 5% w/w solution among various formulations ($P>0.01$). The viscosity value of 5% w/w solution of formulation GS-HEC was the highest. The rank of viscosity of 5% w/w solution was in following orders: GS-HEC > GS-HPMC and GS-MC > GS- SCMC > GS-Alg. These high viscosity values did not cause any trouble in the spraying process using a hand-held sprayer.

Table 11. The viscosity value at 250 rpm of 1% w/w and 5% w/w solution of bacterial granules for spray application (GS)

Formulation	The viscosity value at 250 rpm (± S.D.) (cps)	
	1% w/w solution*	5% w/w solution*
Water	5.47 (±0.13) d	5.47 (±0.13) d
GS-Alg	10.00 (±0.10) c	20.60 (±0.30) d
GS-HPMC	10.37 (±0.15) b	163.0 (±14.3) b
GS-SCMC	16.13 (±0.15) a	89.30 (±1.55) c
GS-MC	10.30 (±0.10) cb	140.8 (±27.9) b
GS-HEC	10.34 (±0.10) b	272.8 (±24.4) a

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.2 Evaluation of physical properties of bacterial tablets for spray application (TS)

4.2.1 Average weight, thickness, hardness and friability of tablets

The result of average weight, thickness, hardness and friability of three formulations containing different amount of SCMC 1500 was shown in Table 12. It was noticed that the average weight of tablets decreased when increase the amount of SCMC 1500. The thickness of all formulations was in the range of 2.197 – 2.523 mm and the hardness of all formulations was in the range of 52.3 – 74.6 N. The friability of all formulations was less than 1%. There was no significant difference in hardness among each formulation ($P>0.01$).

Table 12. Average weight, thickness, hardness and friability of bacterial tablets for spray application (TS)

Formulation	Average weight* (± %RSD) (mg)	Thickness* (± S.D.) (mm)	Hardness (± S.D.) (N)	Friability* (± S.D.) (%)
TS-5	260.90 (±2.702) a	2.460 (±0.063) a	62.4 (±10.1)	0.839 (±0.109) a
TS-10	252.40 (±2.852) b	2.357 (±0.059) b	66.0 (±10.2)	0.572 (±0.006) b
TS-15	250.61 (±2.243) b	2.347 (±0.150) b	69.2 (±5.40)	0.607 (±0.096) ab

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.2.2 Disintegration time of bacterial tablets

The disintegration time of tablets formulations was presented in Table 13. All tablet formulations performed disintegration within approximately 15 - 30 min. The increasing of disintegration time was found as the ratio of SCMC in tablet formulations increased. Formulation TS-15 showed approximately 2 times longer disintegration time than formulation TS-5.

Table 13. Disintegration time to prepare 1% w/w solution prepared from bacterial tablets for spray application (TS)

Formulation	Disintegration time (± S.D.) (min)*
TS-5	14.17 (±1.02) c
TS-10	23.30 (±1.24) b
TS-15	27.21 (±1.09) a

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.2.3 pH of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets

The pH values of 1% w/w, 5% w/w and 10% w/w solution prepared from bacterial tablet formulations were in the range of 6.69 – 7.69 as shown in Table 14.

Table 14. The pH value of 1% w/w solution prepared from bacterial tablets for spray application (TS)

Formulation	The pH value (± S.D.)		
	1% w/w solution	5% w/w solution	10% w/w solution
TS-5	7.40 (±0.06)	6.84 (±0.15)	7.09 (±0.13)
TS-10	7.17(±0.05)	6.99 (±0.10)	7.40 (±0.13)
TS-15	7.15 (±0.04)	7.11 (±0.08)	7.60 (±0.09)

4.2.4 Viscosity of 1% w/w, 5% w/w and 10% w/w solution of bacterial tablets

The viscosity value of 10% w/w solution of formulation TS-15 in water was the highest value among different formulation (Table 15). The viscosity had increased depending upon the concentration. For the same formulation, the viscosity of 10% w/w solution was the highest and the viscosity of 1% w/w solution was the lowest. Among formulation TS-5, TS-10 and TS-15, the viscosity of formulation TS-15 was the highest and the viscosity of formulation TS-5 was the lowest at the same concentration.

Table 15. The viscosity value at 250 rpm of 1% w/w solution prepared from bacterial tablets for spray application (TS)

Formulation	The viscosity value at 250 rpm (± S.D.) (cps)		
	1% w/w solution*	5% w/w solution*	10% w/w solution*
Water	5.47 (±0.13) b	5.47 (±0.13) d	5.47 (±0.13) d
TS-5	7.32 (±0.22) ab	11.63 (±0.32) c	17.77 (±0.06) c
TS-10	9.03 (±1.48) a	16.90 (±0.79) b	52.77 (±4.24) b
TS-15	9.29 (±0.17) a	36.83 (±1.16) a	120.47 (±3.01) a

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.3 Evaluation of physical properties of bacterial granules for broadcast application (GB)

4.3.1 Particle size of granules

The particle size of granules was in the range of 1.407 – 1.419 mm (Table 16). There were significantly different in particle size ($p<0.01$). The rank of particle size was in following orders: GB-19 > GB-29 > GB-39.

Table 16. Particle size of bacterial granules for broadcast application (GB)

Formulation	Particle size of granules (± S.D.) (mm) *
GB-19	1.418 (±0.001) a
GB-29	1.413 (±0.001) b
GB-39	1.409 (±0.002) c

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.3.2 Density of granules

Bulk density of granules was in the range of 0.39 – 0.59 g/ml and true density of granules was in the range of 0.23 – 0.37 g/ml (Table 17). Both bulk and true density decreased when increased the amount of HVO in formulations.

Table 17. Bulk density and true density of bacterial granules for broadcast application (GB)

Formulation	Bulk density* (± S.D.) (g/ml)	True density* (± S.D.) (g/ml)
GB-19	0.57 (±0.02) a	0.35 (±0.02) a
GB-29	0.46 (±0.02) b	0.31(±0.02) ab
GB-39	0.41 (±0.02) b	0.25 (±0.02) b

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.4 Evaluation of physical properties of bacterial tablets for broadcast application (TB)

4.4.1 Average weight, thickness, hardness and friability of tablets

The average weight of formulation TB-19 and TB-29 was in the range of 58.24 – 60.89 mg. The average weight of formulation TB-39 and TB-49 was 54.81 (±6.86) and 51.98 (±6.31) mg, respectively (Table 18). The thickness of all formulations was in the range of 2.89 – 3.01 mm. The friability of all formulations was in the range of 1.07 – 2.26% (Table 18). There were significantly different in thickness and also friability among various formulations. The hardness of all formulations varied from 13.4 to 36.0 N. It was clearly shown that the hardness of tablets decreased when increased concentration of HVO in formulations. The hardness of formulation TB-19, TB-29, TB-39 and TB-49 were 36.00 N, 25.25 N, 17.10 N and 13.40 N, respectively.

Table 18. Average weight, thickness, hardness and friability of bacterial tablets for broadcast application (TB)

Formulation	Average weight* (± %RSD) (mg)	Thickness* (± S.D.) (mm)	Hardness* (± S.D.) (N)	Friability* (± S.D.) (%)
TB-19	60.89 (±6.23) a	2.96 (±0.15) ab	36.00 (±3.87) a	1.28 (±0.64) ab
TB-29	58.24 (±3.10) a	2.94 (±0.11) ab	25.25 (±3.96) b	1.07 (±0.28) b
TB-39	54.81 (±6.86) b	2.89 (±0.10) b	17.10 (±3.66) c	2.11 (±0.32) ab
TB-49	51.98 (±6.31) c	3.01 (±0.11) a	13.40 (±2.11) d	2.26 (±0.20) a

* Means followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test

4.4.2 Evaluation of floating ability of tablets

The percentage of floating tablets against time is presented in Figure 7. The floating ability of formulation TB-49 was approximately 90-100% after dispersing of tablets up to 2 h. More than 50% of formulation TB-39 remained on the water surface up to 1 h and all tablets were disappeared after 1 h 30 min. On the other hand, formulation TB-19 and TB-29 completely submerged at 20 and 35 min, respectively. The percentages of floating ability of formulation TB-19, TB-29, TB-39 and TB-49 at 15 min were 2.5%, 42.5%, 72.5% and 100%, respectively.

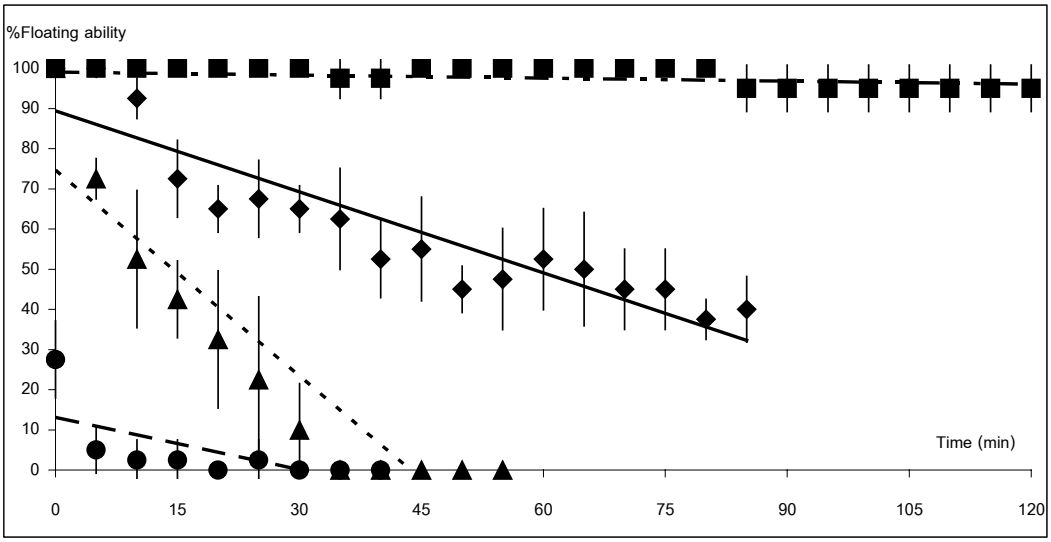

Figure 7. The percentage of floating ability of tablet formulations containing *B. megaterium* for broadcast application (TB): ● formulation TB-19, ▲ formulation TB-29, ◆ formulation TB-39 and ■ formulation TB-49

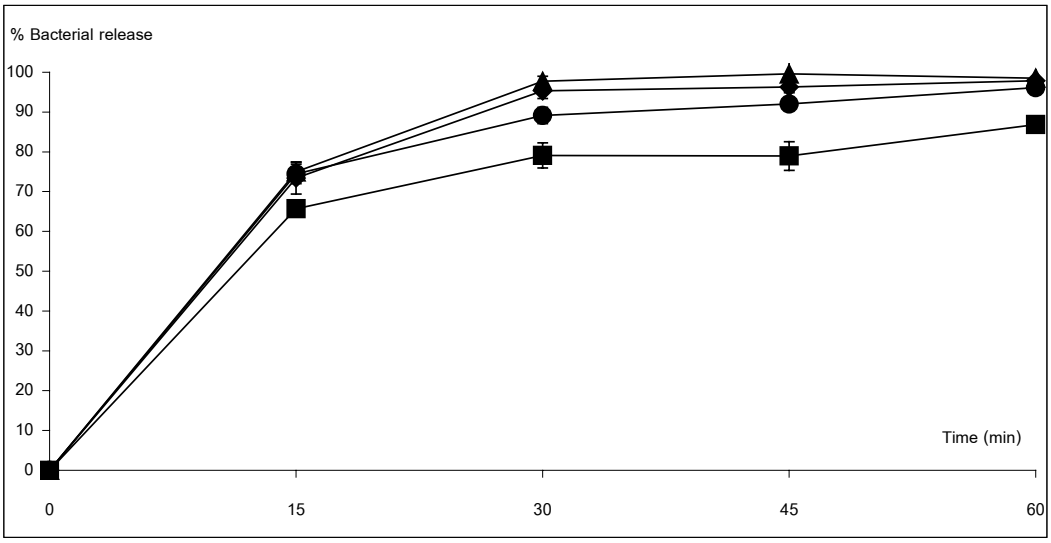

Figure 8. The percentage of bacterial release from tablet formulations containing *B. megaterium* for broadcast application (TB): ● formulation TB-19, ▲ formulation TB-29, ◆ formulation TB-39 and ■ formulation TB-49

4.4.3 Evaluation of bacterial release

The percentage of bacterial release from formulations TB-19, TB-29, TB-39 and TB-49 was 74.49%, 74.80%, 73.23% and 65.65%, respectively, at 15 min after the formulation was applied (Figure 8). Formulation TB-19, TB-29 and TB-39 released the bacteria completely at 60 min, while formulation TB-49 released the bacteria about 87% at 60 min after the formulation was dispersed.

5. Scanning electron microscope (SEM) observation of the selected formulations and bacterial endospores on surface of rice tissues

5.1 SEM observation of the selected formulations

Endospores of *B. megaterium* were observed on the surface of granule and tablet formulations (Figure 9). The shape of endospores were cylindrical or rod. The endospore size was approximate 1-2 μm. The endospores on the surface of formulation GS-Alg, GS-SCMC and TS-15 (Figure 9 (a) – (c)) were clearly

detected. In contrast, the endospores on the surface of formulation GB-19 (Figure 9 (d)) and TB-39 (Figure 9 (e)) could not be clearly observed.

5.2 SEM observation of endospores on surface of rice tissues

The endospores of *B. megaterium* were observed on the surface of leaf sheath after placing into different formulations for spray application (Figure 10). The bacterial endospores were also detected on the surface of leaf blade which possessed hairy texture as shown in Figure 10.

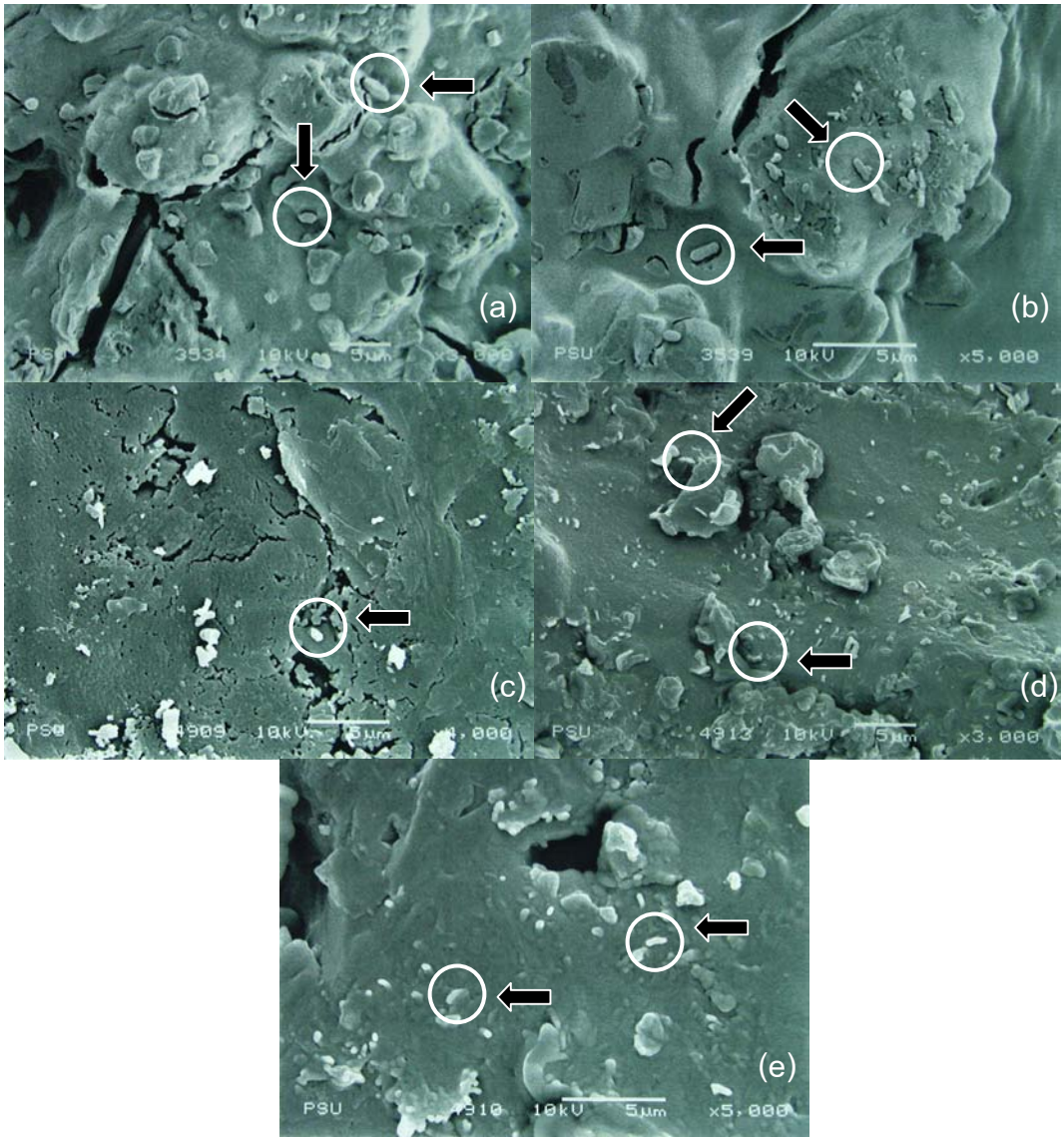

Figure 9. Micrographs of bacterial endospores on the surface of selected formulations: (a) formulation GS-Alg (x3000), (b) formulation GS-SCMC (x5000), (c) formulation TS-15 (x4000), (d) formulation GB-19 (x3000) and (e) formulation TB-39 (x5000)

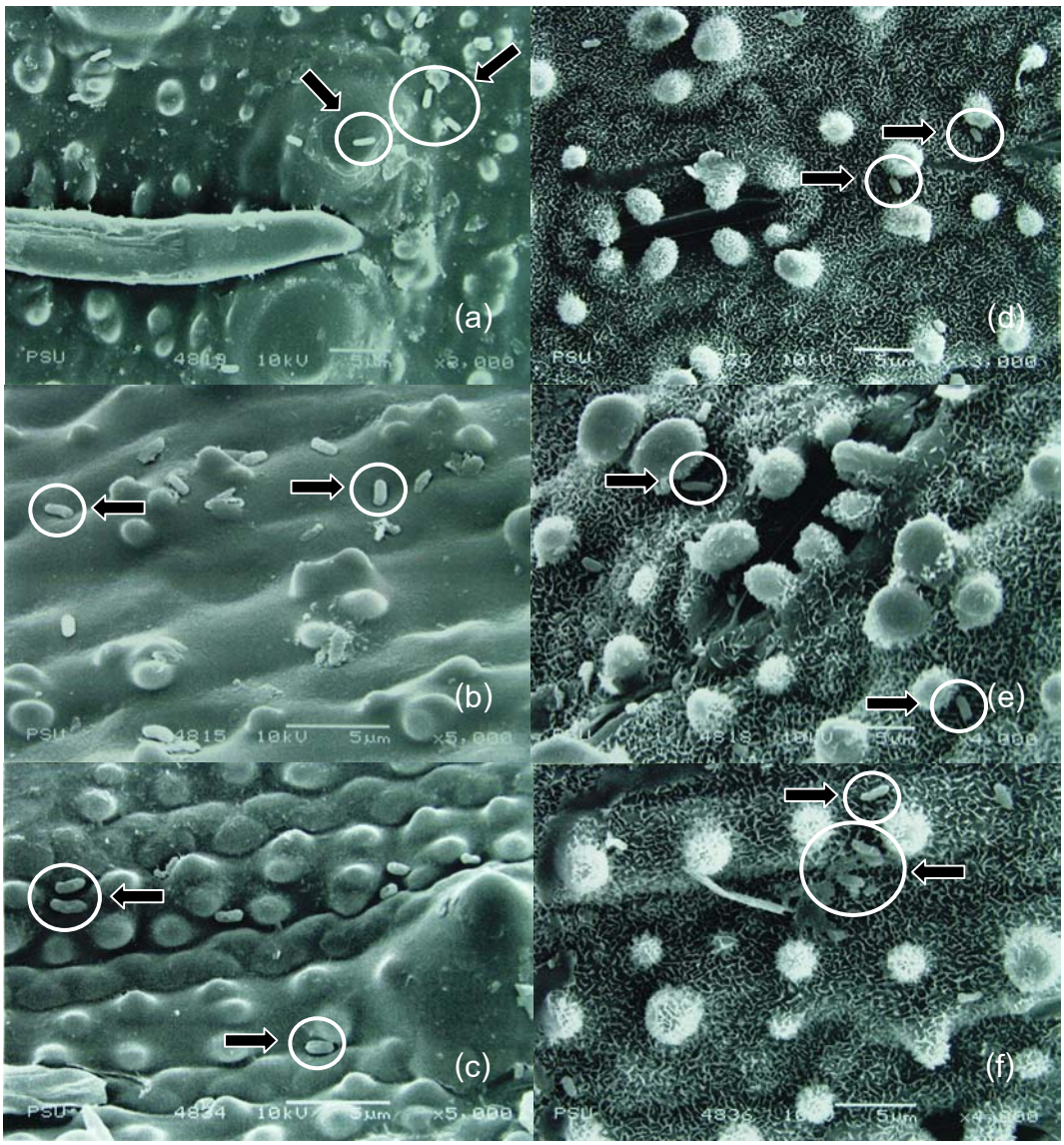

Figure 10. Micrographs of bacterial endospores on the plant surface after placing into the formulations: (a) formulation GS-Alg solution on the leaf sheath (x3000), (b) formulation GS-SCMC solution on the leaf sheath (x5000), (c) formulation TS-15 solution on the leaf sheath (x5000), (d) formulation GS-Alg solution on the leaf blade (x3000), (e) formulation GS-SCMC solution on the leaf blade (x4000) and (f) formulation TS-15 solution on the leaf blade (x 4000)

6. Testing the efficacy of selected granule and tablet formulations containing *B. megaterium* under greenhouse conditions

Biocontrol of rice sheath blight disease was evident in the assessment of % tillers with sheath blight symptoms where treatment with formulation GS-SCMC (granules for spray application) had the lowest % disease incidence (Table 19). Formulation GS-Alg and GS-SCMC were effective in sheath blight protection because there was statistical difference when the percentage of tills with sheath blight symptoms was used as the criterion. When lesion lengths were compared, there was no significant difference in entire length of the lesion rice tiller among different treatments (Table 19). There was also no statistical difference among all treatments when average fresh and dry weight were compared (Table 19). The lesion on each rice tiller which had sheath blight symptoms was shown in Figure 11.

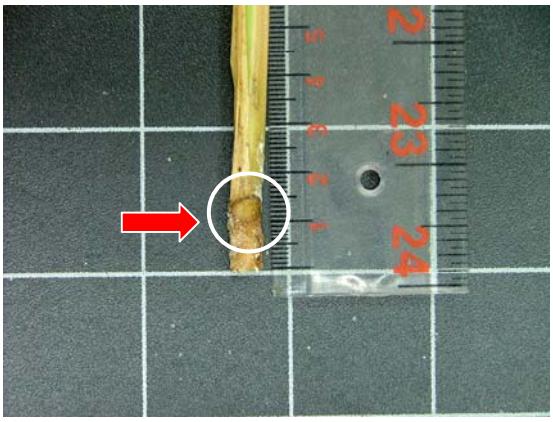

Figure 11. The lesion on each rice tiller which had sheath blight symptoms

7. Evaluation of *B. megaterium* adhesion on plant surface

After spraying the selected formulations for 4, 7 and 14 days, the number of bacteria on leaf sheath and leaf blade were counted using the drop plate method. The number of viable bacteria on the leaf sheath remained high (approximately 10^3 CFU/g of leaf sheath) at 14 days after spraying with 1% w/w formulation GS-SCMC and 10% w/w formulation TS-15. In case of GS-Alg, the number of bacteria did not change at 14 days compared to at 4 days (Table 20). The number of viable bacteria on leaf blade remained high more than 10^2 CFU/g of leaf blade 14 days after spraying with 1% w/w formulation GS-Alg or 1% w/w formulation GS-SCMC, and more than 10^3 CFU/g of leaf blade 14 days after spraying with 10% w/w formulation TS-15 (Table 21). From the data, the number of viable bacteria on plant surface after spraying with the selected formulations was higher than the number of bacterial suspension.

Table 20. Number of viable bacteria on surface of leaf sheath after spraying the selected bacterial formulation for 4, 7 and 14 days

Formulation	Number of viable bacteria (± S.D.) (CFU/g of leaf sheath)		
	4 days *	7 days	14 days
Bacterial suspension	5.67 (±3.44) x 10^3	4.33 (±2.34) x 10^2	2.67 (±2.42) x 10^2
GS-Alg	1.10 (±0.69) x 10^4	9.00 (±5.48) x 10^3	1.07 (±0.56) x 10^4
GS-SCMC	2.67 (±1.63) x 10^4	6.00 (±1.79) x 10^3	8.00 (±7.30) x 10^3
TS-15	5.33 (±5.01) x 10^4	1.37 (±0.59) x 10^4	8.67 (±4.69) x 10^3

* Days after spraying the selected bacterial formulations

Table 21. Number of viable bacteria on surface of leaf blade after spraying the selected bacterial formulation for 4, 7 and 14 days

Formulation	Number of viable bacteria (± S.D.) (CFU/g of leaf blade)		
	4 days *	7 days	14 days
Bacterial suspension	$<10^2$	$<10^2$	$<10^2$
GS-Alg	8.67 (±3.72) x 10^2	1.10 (±0.33) x 10^3	8.00 (±5.80) x 10^2
GS-SCMC	4.67 (±1.03) x 10^3	3.17 (±0.64) x 10^2	3.43 (±1.80) x 10^2
TS-15	1.17 (±1.07) x 10^5	1.87 (±0.30) x 10^3	1.13 (±0.69) x 10^3

* Days after spraying the selected bacterial formulations

8. Testing the inhibition of *R. solani* mycelial growth

The inhibition of *R. solani* mycelial growth was presented in Figure 12. The formulations had ability to inhibit mycelial growth of *R. solani* although the tests were carried out after the formulations storing at room temperature (26 - 30°C) for 3 and 6 months (Table 22). Formulation GS-SCMC inhibited mycelial growth of *R. solani* (96.79%) after storage for 6 months and it performed best in suppressing sheath blight disease in greenhouse conditions. Formulation GS-Alg, TS-15, GB-19 and GB-39 could also suppress sheath blight disease in greenhouse conditions, the %inhibition of mycelial growth were 97.94%, 96.91%, 97.45% and 96.98%, respectively, after storage for 6 months.

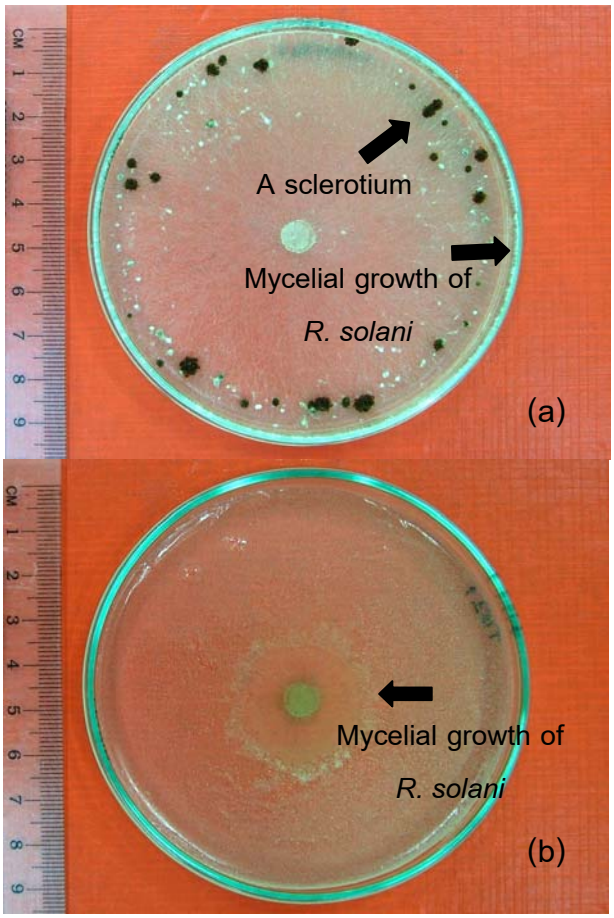

Figure 12. Inhibition of *R. solani* mycelial growth tested on PDA incorporated with 1% w/w solution of the sterile water (as a control treatment) or the selected bacterial formulation; (a) control treatment and (b) formulation TS-15

Table 22. Inhibition of *R. solani* mycelial growth tested on PDA incorporated with 1% w/w solution of the selected bacterial formulations

Formulation	% Mycelial inhibition (± S.D.)	
	3 months	6 months
GS-Alg	98.43 (±0.49)	97.94 (±1.21)
GS-SCMC	97.36 (±0.49)	96.79 (±1.81)
TS-15	96.89 (±1.18)	96.91 (±0.96)
GB-19	98.09 (±0.72)	97.45 (±1.23)
TB-39	97.13 (±0.37)	96.98 (±0.88)
Control	0.00 (±0.00)	0.00 (±0.00)

9. Viability of bacteria in the selected formulations at room temperature

The number of viable bacteria remained high after storage at room temperature for 6 months (Table 23). The number of viable bacteria in formulation GS-Alg and GS-SCMC had declined from 10^9 CFU/g to 10^7 CFU/g and 10^6 CFU/g, respectively. Formulation TS-15 contained viable bacteria about 10^6 CFU/g and the number of viable bacteria in formulation GB-19 and TB-39 was 10^8 CFU/g through 6 month storage.