Encapsulation of *Beauveria bassiana*

bcc2660 spores for enhanced growth

and tolerance in laboratory Condition

BY

Mr. Piyapong Suvansanya

ID 4955112

A special project submitted to the School of Biotechnology, Assumption
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ABSTRACT

Beauveria bassiana is an entomopathogenic fungus that is widely used to control insect pests in many countries. The encapsulation of *B. bassiana* spores to enhance growth and its germination were conducted. The initial formula consisted of 2 % (w/v) calcium chloride, 10^{12} spores per ml of *B. bassiana* spores and 2 % (w/v) yeast extract. The optimization of encapsulated formula was done by varying % calcium chloride from 0.5 % to 2 % (w/v) and by varying different nutrients, which were 2 % (w/v) yeast extract and 100 % (v/v) potato dextrose broth. Quality of encapsulation formula developments was determined by observing % rehydration and % sporulation of encapsulated beads. The best formula, 0.75 % (w/v) calcium chloride and potato dextrose broth gave the highest % rehydration (74 %) and % sporulation (100 %). The encapsulated beads were subsequently tested for insect bioassay against third instar of *Phenacoccus manihoti* and third instar larvae of *Spodoptera exigua*. At 10 days, mortalities of *P. manihoti* and *S. exigua* were 18.7 % and 73.3 %. The mortality rate of *S. exigua* was significantly higher than *P. manihoti*. The result suggested that the movement of the insect influenced the infection of encapsulated *B. bassiana.*. I hypothesize that the encapsulation *B. bassiana* can infect *S. exigua* better than *P. manihoti* because *S. exigua* moves around more extensively and *P. manohoti* moves relatively slow in a limited area.

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INTRODUCTION

Cassava production in Thailand

Cassava (*Manihot esculenta* Crantz) is considered as one of the economic crops in Thailand that receives high demand in Thailand and foreign countries. This has caused its price to be as high as 2,900 baht – 3,100 baht per ton. For this reason, the Thai community raised awareness of improvement and development in the production of cassava in order to exceed the demand of the market. In January 2011, it is reported that Thailand has grown cassava 1.25 million hectares and produced 21.9 million ton per years. However, the current production cannot supply the demand of the market (25 million tons) because in areas the production is low and crops are infected by mealy bug. In 2007, it was found that the mealy bug infected 50,000 hectares of cassava farm and in 2010 the infection become severely in large scale. Currently the mealy bugs control mostly relies on chemical pesticide such as carbamates, pyrethroids, fipronil, and organophosphate. However, concerned over the effects of environmental issues related to the use of chemical pesticide such as toxic deposition in crops causing hazardous effects on human health, water contamination has risen to new pest management system called "integrated pest management programs". Biological control plays an important role in the integrated pest management system in terms of an alternative choice for prevention (Lomer et al., 2001). In terms of industrial development, bio pesticide faces many challenges to maximize its effectiveness; spore must be dried to ensure adequate shelf life and spore inactivation in field due to solar radiation.

OBJECTIVES

This experiment aims to encapsulate *B. bassiana* and formulate the composition of an encapsulated agent to enhance germination and sporulation of *B. bassiana*, and increase their efficiency in controlling mealy bugs in laboratory.

LITERATURE REVIEW

General Biology of *Beauveria bassiana*

Beauveria bassiana (*Subdivision* Deuteromycotina: Class Hyphomycete) was first discovered in larval silkworm (*Bombyx mori*) as a white muscardine disease by Agostino Bassi De Loid in 1835 (Tanada and Kaya, 1993). *B. bassiana* is found to be naturally distributed on some plant and in soils worldwide (Feng et al., 1994). Generally, *B. bassiana* has a wide host range of more than 200 species of insects; mostly Lepidoptera and Coleoptera are recorded as a host for *B. bassiana*.

B. bassiana produces three types of spores, being submerged conidia, aerial conidia and blastospore. Aerial conidia are produced on sporogenous hyphae "philaides" on host cadaver or saprophytic life stage. Normally the spores develop in zigzag shape thread like on conidiophores. Blastospores are spores that are produced in the insect hymolymph. This type of spore is constituted of a similar cell wall of hyphae because of the propagation by producing a pore. From the research of Pendland (1993) it was found that the *B. bassiana* that propagated inside the insect tissue shared the same type of carbohydrates and structural component such as chitin which result as a protoplast like form. In addition, aerial conidia contain coating of monoamines known as hydrophobins that may offer environmental protection and increase spore hydrophobicity (Bidochka et al., 1995). The differences between three types of spores were determined by Thomas (1987) in term of survivability, infectivity, surface characteristic and spore morphology. He found that blastospores have the least hydrophobicity, very few carbohydrates on cell surface, low survivability but they are the most virulent in infection. On the other hand, submerged conidia and aerial conidia show a similar result of hydrophobicity, surface contained carbohydrates, virulence. However, aerial conidia have a better survivability than submerged conidia.

Benefit of using *B. bassiana* as insecticide

One of the benefits of *B. bassiana* as bio-pesticide is its cost effectiveness due to its low production costs. *B. bassiana* can also be used for controlling many types of insect pests because it has a broad host range including aphids, whiteflies, mealy bugs, grasshoppers and beetles. Due to its natural occurrence and specification on insect targets, the fungus only affects the insect pests without harming other organisms and environment.

The need for more persistent *B. bassiana* formulation

Normally the infection of *B. bassiana* occurs by spores contacting the insect and germinates. This is by spray or horizontal transmission of the spores from the infected insects. However, there are many factors that could affect the efficiency of *B. bassiana* in the field (Burgess, 1998). Humidity is one of the limiting factors that affect the infection of *B. bassiana* because epizootics cannot be established in arid condition effectively. Therefore, the insect control is limited and relies only on contact with the spray residue or secondary pick up from vegetation. This raised the need for a formulation that enhances spore persistence in the extreme environment. In addition, there are other factors that contribute to spore viability such as culturing condition during spore production, storing condition and timing of spray application. Never the less, it is found by Ignoffo (1992) that ultraviolet radiation from sunlight caused major problems in the field application of bio control.

Ultraviolet

Ultraviolet-radiation (wavelength between 280-320 nm) causes direct mutation on DNA (for example, pyrimidine dimmers, cross- linking with protein) or indirectly causes formation of reactive oxygen-derived free radicals. Furthermore, UV radiation exposure causes a delay in spore germination due to protein damage and defense response of conidia resulting in slower growth rate. From the research, 65 isolates of *B. bassiana*, 23 isolates of

Metarhizium anisopliae, 14 isolates of *Metarhizium flaviride* and 33 isolates of *Paecilomyces fumosoroseus* were compared by Fargues et al. (1996) and found that *M. flavoviride* were the most tolerated to the sunlight followed by *B. bassiana* and *M. anisopliae*, *with P. fumosoroseus*. The oxidative agent was found to be the most significant damage factor for fungal spore germination when exposed to UV. It occurs by forming H_2O_2 molecule and may accumulate into the cell due to partial inactivation of catalase (resulting from heat and available NaCl (Martin, 1992)). Hydrogen peroxide inactivates superoxide dismutase and accumulates superoxide or O_2. Result in combination of hydrogen peroxide with O_2 to produce free radical (HO·). However, Ignoffo and Garcia, (1994) has found that the used of anti oxidant in *Bacillus thurigiensis* and *Baculovirus heliothis* helps reducing the damage from UV- radiation exposure.

Water Stress

Water content is one of the main factors that plays important role in initiating germination in *B. bassiana* spores. The optimal relative humidity for spore germination and hyphal growth was found to be 95 %- 100% (Burgess, 1998). Furthermore, Hart and MacLeod (1955) have found that *B. bassiana* needs a relative humidity above 94 % in order to function effectively.

The effects of water stress are divided into high water content and low water content. High water content occurs during rehydration and freeze thawing with the effect of osmotolerance and imbibitions. Compatible solutions such as carbohydrates and phytols are use to stabilize cell and can accumulate in high concentration without inhibiting or destabilizing enzyme (Crowe et al., 1990). However, high concentration of compatible solution favors the exclusion of the compatible solutes from the surface of the biomolecule causing lesser efficiency in cell metabolisms (thermodynamically disfavorable situation). Biomolecule such as membranes and enzymes are surrounded by layers of water to stabilize biomolecule by hydrogen bonding. Under extreme dryness condition, water stress occurs and caused the removal of bound water from the biomolecule. This resulted in reducing cell stability and caused metabolism to be less functional. From the research, Crowe et al. (1988) has shown that non reducing disaccharides were used to stabilized membranes and enzyme by storing them in glass state in extreme dryness condition. The disaccharide are found on

both side of the phospholipids bilayer and provided cell protection by preventing water loss (Crowe et al., 1986).

B. bassiana infection process

The understanding of infection mechanisms that entomopathogenic fungi use for infecting is necessary in order to aid the development of a new technique or formula involved with enhancing the spore infection. The spore is infected by 5 stages which are 1) adherence to the insect cuticle; 2) germination on the cuticle; 3) formation of infective structures; 4) penetration of the insect cuticle and 5) evasion of the host immune system.

1. Adherence to the insect Cuticle

The adhesion processes of the spores are divided into three stages which are 1) adsorption; 2) consolidation of attachments and 3) germination and penetration through cuticle. The spores of *B. bassiana* have characteristic of hydrophobic which give them ability to adhere to the insect cuticle easily. In addition, the spores are easily suspended in oil and in field application; oil prevent the evaporation of the spore suspension before contacting with the insect (Vimala-Devi and Prasad, 1996). Consolidation involves spore components that help adhesion such as lectins and enzymes. The antigen at the surface correlates with the virulence and insect host specificity (Boucias and Pendland, 1991)

2. Germination of the spores

Humidity is one of the factors related to the effectiveness in spores germination and hyphae growth. Form the research of Burgess (1998), he has found that spore germinated between 95 % r.h. – 100 % r.h.. However, in the real field environment, spores are able to obtain sufficient humidity from insect intersegmental membrane and enable germination

(Burgess, 1998). Furthermore, spore germination also depends on the presences of exogenous nutrients inside the spores and the presences of the specific target host surface on the spore surface. It was found by Lloyd (1999) that *B. bassiana* spore surface is strongly linked to carbohydrate surface profile.

3. Formation infective structure

The penetration of *B. bassiana* occurs by forming a series of infection structures called "appressorium" (St Leger et al., 1994). In addition, the formation involves with the tactile signal from the component and morphology of the insect surface which differ between different insect host and different insect body parts (St Leger et al., 1994).

4. Penetration of insect cuticle

Appressorium is also involved with the production of a variety of enzymes (protease, chitinase and lipase) which help in cuticle degradation and nutrient exploitation (St Leger et al., 1994). Furthermore, the degradation of cuticle helps in the penetration of appressorium and ensures the infection of the fungi.

5. Evasion of host immune system

B. bassiana produces mycotoxin called "beauvericin" after it invades into the hemolymph of the insect to weaken the immune system of the insect and enhance the infection (Roberts, 1981). Reproduction of *B. bassiana* inside the insect shown as blastospores and hyphal bodies are designed to aid in spore dispersal in the insect hemolymph and immune system invasion. Furthermore, the absence of some chemical component such as chitin and cell wall of protoplast like spore (blastospore) help in avoiding phagocytosis of insect host immune system (Pendland et al., 1993).

Used of *B. bassiana* in Thailand

Sprayable formulation of *B. bassiana* has been widely used in Thailand for controlling *Nilaparvata lugens* (Patirupanusara P. et al. 2007), *Frankliniella occidentalis* (Sengonca, C. et al., 2006) and *Coptotermes curvignathus* (Mahitthafongku N. et al. *2010)*. However, the spray formulation relies heavily on surface contact with insect to initiate the infection causes high labor cost in applying. In addition, with problems of solar radiation, the sprayable formula is still ineffective in term of spore survival and germination ability (Burgess, 1998).

Encapsulation formulation

In term of bio-control application, development of formulation plays important role in maximizing effectiveness of infection. There are four main objectives in formulating entomophathogenic fungi consisted of 1.) to stabilized the spores, 2.) to make a user friendly product, 3) to protect bio material from extreme environment and 4.) to minimize the risk of exposure to the applicator (Jackson et al., 1997).

Encapsulation is a process of entrapping spore in form of granule. There have been uses of granulation in many biopesticides to improve their efficacy and tolerance in extreme environment, for example, *M. anisopliae* (Schwarz, 1995); *F. Oxysporum* (Connick, 1998); and *E. neoaphidis* (Shah, 1999). In addition, Knudsen (1990) had encapsulated *B. bassiana* with alginate and found that it caused 3-44 % mortality of aphid after 9-15 days. Addition of wheat bran in encapsulated formula also proliferate the fungi biomass in soil (Lewis et al., 1987). The experiment conducted by Caudwell and Gatehouse (1996), has shown that the encapsulated formula with maize-starch extrusion is effective in terms of UV resistance and infection as a bait (infect on insect surface during ingestion) for *S. gregaria* and *Melanoplus sanguinipes*. The main components of the encapsulation included fungal spores, gelling agent, and nutrients (Schwarz, 1995).

Sodium alginate is a polysaccharide gum extracted from seaweed and is used in food products, cosmetics and agriculture (McNeeley & Pettitt, 1973). Sodium alginate used as

encapsulating agent offer advantages over spray formulation in term of 1.) enhance shelflife, 2.) biodegradability, 3.) ease of preparation, 4.) ease of application, 5.) provide protection in extreme environment, 6.) enhance germination and sporulation and 7.) low cost of production. Diffusion setting is immobilization techniques for sodium alginate and used in many industries for restructuring of foods and encapsulating biomaterial. This is by rapid gelling kinetics at a high speed setting. The droplet of alginate solution form 3D structure and entrapped the active agent when exposed to gelling ion. In order to increase the formulation shelf life, the fresh beads are dried by hot air ventilation to lower the moisture content leaded spore to acquire anabiosis stage and prevent premature germination of the spore (Schwarz, 1995). For field application, encapsulated beads have only one step of preparation which is bead rehydration. This reduces labor cost and provides uncomplicated use for farmer. Rehydrated granules act as spore's carrier and also provide surface for germination and sporulation. The infection of encapsulated *B. bassiana* relies mainly on sporulation in a real field to produce infective aerial conidia to infect target host. Furthermore, formulating encapsulated beads with addition of suitable nutrients and protective agents such as sun screen could be done in order to enhance germination and spore persistence to extreme environment, for example water stress, ultraviolet and heat stress. From the experiment conducted by Burgess (1998), it is shown that the addition of UV blocker with water based formulation increase survival of *B. bassiana* 6.6 times. In addition, when the water droplet evaporates the UV blocker become concentrating and increase its protection to the spores. In addition the nutrient provided for spore germination also give indirect benefits of increasing in fungal bio mass in soil to help aiding in matter decomposition and plant growth (Knudsen et al., 1990). Moreover, Sodium alginate is a non toxic substance that decomposed naturally without causing any effects on environment or user.

METHODOLOGY

Chemical

Sodium alginate and calcium chloride were purchased from Thai food chemical company Ltd. Other media components were purchased from Difco.

Method

1. Preparation of *B. bassiana* conidia

B. bassiana was inoculated onto PDA plates by using spread plate technique and allowed to grow for 10 days at 25 °C. The conidia were detached from the mycelium by scrapping with spatula and transferred to falcon tube containing 10 ml of distilled water. The suspension was filtered through linen cloth in order to separate mycelium and transferred to a sterile tube. The concentration of conidia was determined by counting with a hemocytometer and diluted with distilled water to final concentration of 10^{13} spores/ml.

2. Encapsulation of formula B1,B2 and B3(modified from Knudsen, 1991)

2.1 Preparation of Gelling ions

Four concentrations of gelling ions were prepared 2 % w/v of $CaCl_2$ (B formula), 1 % w/v of $CaCl_2$ (B1 formula), 0.75 % w/v of $CaCl_2$ (B2 formula) and 0.5 w/v of $CaCl_2$ (B3 formula). The gelling ions from all fours formulas were autoclaved at 121°C for 15 minutes. The solutions were cooled in laminar hood for 45 minutes at 25°C before using in encapsulation process.

2.2   Preparation of encapsulated agent

Encapsulated agent were prepared by mixing 10 g(2 % w/v) of yeast extract and 10 g(2 % w/v) of sodium alginate with 450 ml of distilled water in Duran bottle and autoclaved at 121°C for 15 minutes. The solution was cooled in laminar hood for 45 minutes at 25°C before using in encapsulation process.

2.3   Encapsulation process

The encapsulated agent was inoculated with 50 ml of *B. bassiana* (10^{13} spores/ml) and shake gently by vortexing to let the spore disperse in the solution. Forming of alginate beads were done by dropping 100 ml of encapsulated agent by using syringe (size 10 ml) into 200 ml of calcium chloride solution under agitated condition using magnetic stirrer. The beads were filtered through linen cloth in order to separate from calcium chloride solution. The beads were dried by using hot air ventilation at 40 °C ± 2 °C for 4 hours and stored in air tight package at room temperature.

2.4   Quality analysis of beads from formula B, B1, B2, B3

2.4.1   Sporulation of the *B. bassiana* beads from formula B, B1, B2, B3

50 beads were rehydrated in a glass beaker containing 50 ml of distilled water for 20 minutes and placed onto a sterile glass plates for 3 replications. The plates were incubated at 25°C for 3 days and sporulations were observed under stereomicroscope.

2.4.2   % Rehydration of formula B, B1, B2, B3

The % rehydration was observed by recording the weight of the beads after encapsulation (as 100 % hydration), recording the weight of dried beads (0 % hydration), and recording weight of the beads after soaked for 20 minutes (% rehydration) and substituted into the following equation.

$$\% \ rehydration = \frac{Rehydration \ weight - Dried \ weight}{Fresh \ weight - Dried \ weight} \times 100$$

3. Potato dextrose broth nutrient substituent test

3.1 Preparation of Gelling ions

Calcium chloride solution was prepared using formula B2 as described in 2.1.

3.2 Preparation of encapsulated agents

3.2.1 Yeast extract formula based

The encapsulated agent was prepared using the same ingredients as mentioned earlier in 2.2.

3.2.2 Potato dextrose formula based

Potato dextrose broth was prepared by chopping 100 g (20 % w/w) of potato into 1 cm^2 square shapes and boil them in 450 ml of distilled water for 15 minutes with addition of 10 g (2 % w/v) sucrose. The solution was filtered through linen cloth in order to separate the solution from the potato tissue. the encapsulated agent with potato dextrose broth were prepared by mixing 20 % w/w of potato dextrose broth with 10 g(2 % w/v) of sodium alginate and autoclaved at 121°C for 15 minutes. The solution was cooled in laminar hood for 45 minutes at 25°C before using in encapsulation process.

3.3 Encapsulation process of yeast extract and potato dextrose formula

The encapsulation process of yeast extract formula and potato dextrose formula were carried out as described in 2.3.

3.4 Quality analysis of yeast extract and potato formula

3.4.1 Sporulation of the yeast extract and potato formula

The sporulation test of yeast extract formula and potato dextrose formula were carried out as described in 2.4.1.

3.4.2   % Rehydration of potato dextrose formula and yeast extract formula

The % rehydration test of potato dextrose formula and yeast extract formula were carried out as described in 2.4.2.

4.   Adhesion test (modified from Baruch S. Sasha, 1988)

4.1   Preparation of Gelling ions

Calcium chloride solution was prepared using formula B2 as described in 3.1.

4.2   Preparation of encapsulated agents

The encapsulated agent was prepared using potato dextrose formula as described in 4.2.2.

4.3 Encapsulation process for big beads production

The encapsulation process was carried out as described in 2.3.

4.4 Encapsulation process for small beads production

The encapsulation process was carried out as described in 2.3, additionally using needle size 18G for extruding encapsulated agent into calcium chloride.

4.5Adhesion test on glass slide

60 dried beads of big formula and small formula were rehydrated in separated 50 ml of distilled water for 20 minutes and gently place 10 big beads and 10 small beads onto glass slide separately. This was done for 6 replications. The glass slides were turned upside down and the % of the remaining beads were observed after 1 hours, 3 hours and 24 hours.

4.6 Adhesion test on cassava leaf

Cassava leafs were dissected into 5 parts equally.60 dried beads of big formula and small formula were rehydrated in separated 50 ml of distilled water for 20 minutes. 10 big beads and 10 small beads were placed onto the pieces of cassava leaf separately for 6 replications. The leaves were turned upside down and the % of the remaining beads were observed after 1 hour, 3 hours and 24 hours.

5. Infection test on mealy bugs (*Phenacoccus manihoti*)

The infection test was done by cutting tissue papers into 2 cm diameter circle shape and placed them onto a well plate for 48 replications. A pumpkin was cut into 1.5 cm x 1.5 cm square shapes and placed onto each plastic well. 10 of 3 instar mealy bugs were transferred into each well by using sterile brushes. Sporulated beads from formula B2 were subsequently transferred onto the pumpkin in number of 1 bead, 3 beads, 5 beads and control for 12 replications each. % mortality of mealy bug from *B. bassiana* after 2,4,6,8 and 10 days were recorded.

6. Infection test on beet army worm (*Spodoptera exigua*)

The test was performed by wrapping kale leafs (insecticide free) with wet cotton and placed one branch into 20 cm × 30 cm translucent box for 12 replications. 10 of 3 instar beet army worms were transferred into each box by using sterile forceps. Sporulated beads from formula B2 were subsequently transferred onto the pumpkin in number of 1 bead, 3 beads, 5 beads and control for 3 replications each. % mortality of beet army worm from *B. bassiana* after 2,4,6,8 and 10 days were recorded.

RESULT

Encapsulation formula were modified from Knudsen (1991) and named formula B. The optimization of encapsulated formula was done by varying % w/v of calcium chloride and named formula B, B1, B2 and B3.

3.1 Quality analysis of formula B, B1, B2 and B3

The encapsulated beads from formula B, B1, B2 and B3 after encapsulation process were shown in figure 3.3 and The beads produced from formula B3 (0.5 % w/v $CaCl_2$) were fragile and easily disintegrated by physical contact with forceps and glass slit. Figure 3.4 showed % sporulation and % rehydration of the beads formula B, B1, B2 and B3 where B3 presented the highest % rehydration followed by formula B2, Formula B1 and formula B respectively (figure 3.4). Formulas B1, B2, B3 showed significantly higher in % sporulation and % rehydration than formula.
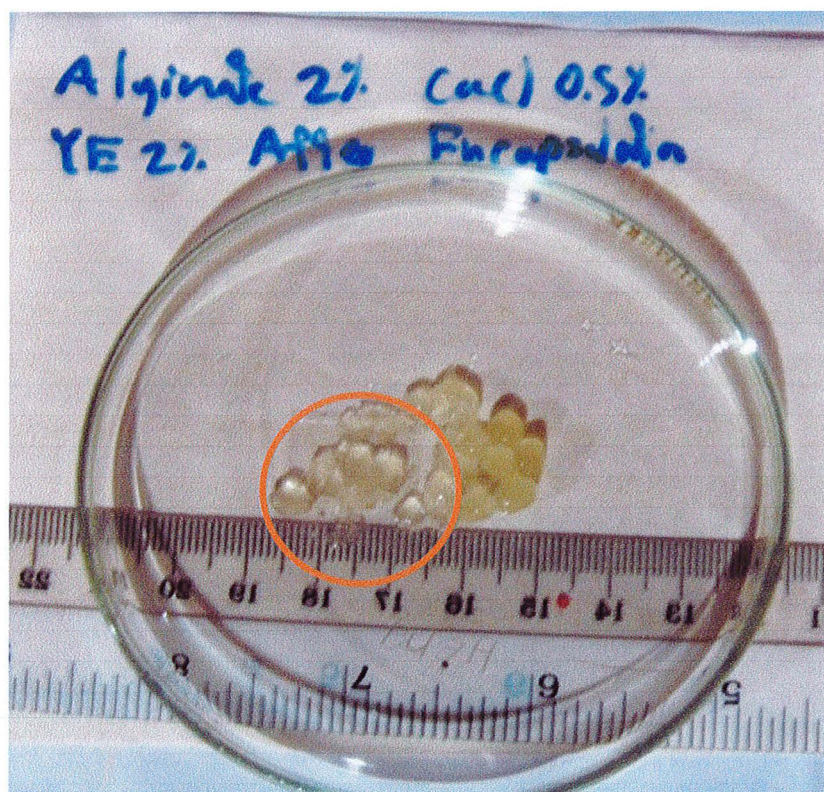
Figure 1. View of encapsulated *B. bassiana* formula B3 after encapsulated showing unstable structure of B3 formula (CaCl$_2$ 0.5 % w/v) .
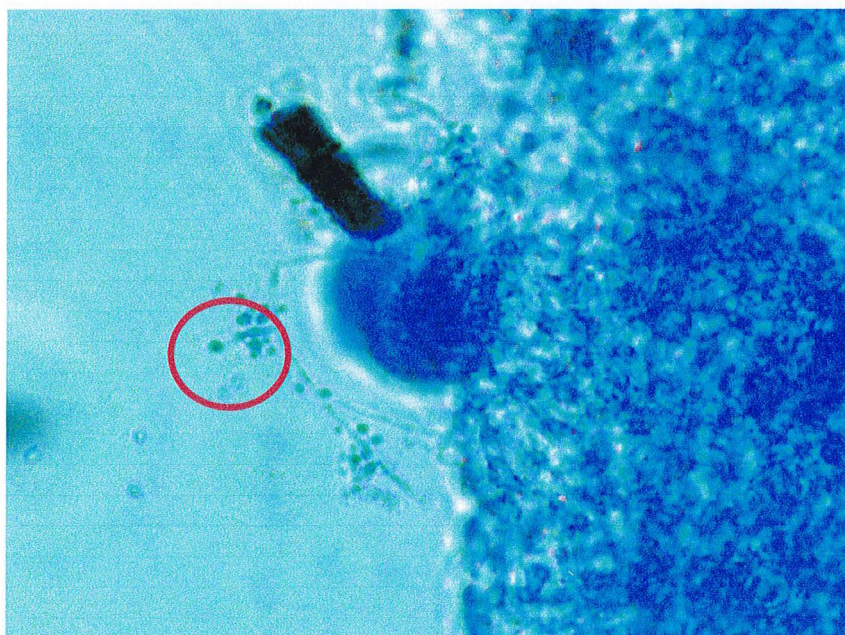
Figure 2. Lactophenol cotton blue staining of encapsulated *B.bassiana* formula B2 shows characteristic of *B.bassiana* spore. Circle indicates zig-zag rachis and oval conidia of *B.bassiana* spore. (x 400 magnifications).
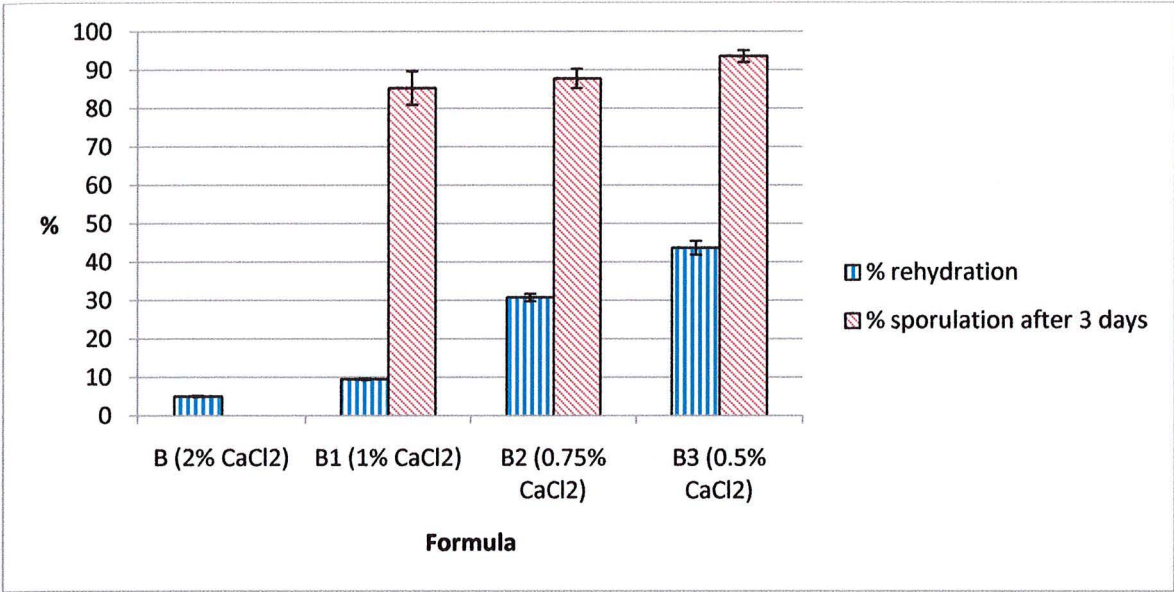
Figure 3. % rehydration and % sporulation of beads from formula B, B1, B2 and B3 (* % rehydration is % weight recovery of the bead after soaked for 20 minutes).

3.2 Quality analysis of potato dextrose formula and yeast extract formula

% sporulation and % rehydration of encapsulation formula with potato dextrose and formula with yeast extract in figure 3.5 showed that the encapsulated beads from PDB and YE had similar % of sporulation but potato dextrose have higher % rehydration. % rehydration of encapsulated bead with potato dextrose formula was significantly higher than % rehydration of encapsulated beads with yeast extract formula. The cost comparison in table 3.1 between the production of encapsulated bead formula with potato dextrose formula and formula with yeast extract has shown that potato dextrose formula cost lesser than yeast extract formula. After rehydrated and incubated after 2 days, the bead with PDB formula showed higher length of mycelium growth compared to yeast extract formula (table 3.2).
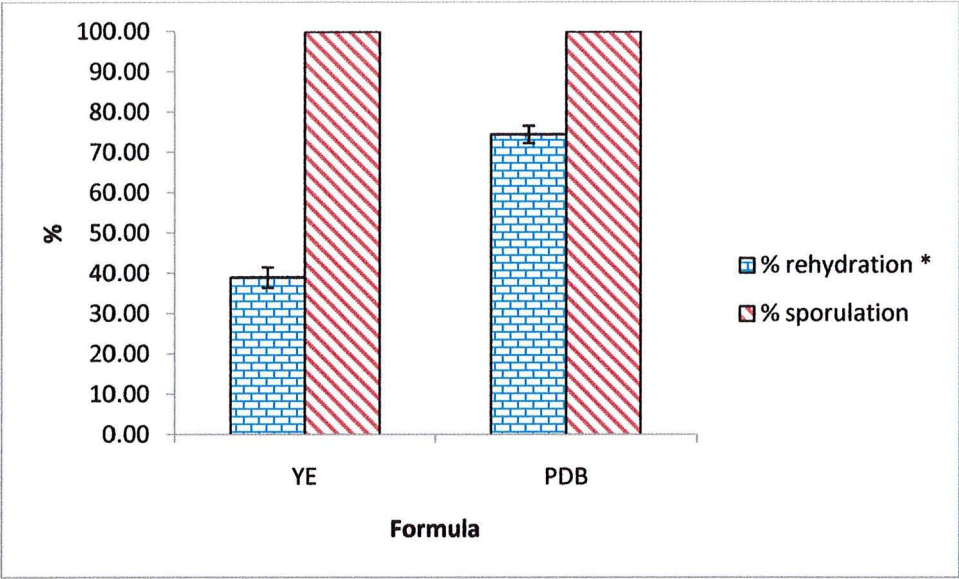
Figure 4. % rehydration and % sporulation of YE formula and PDB formula after rehydrated 20 minutes and incubated at 25°C for 3 days (* % rehydration is % weight recovery of the bead after soaked for 20 minutes).

Table 1. Production cost comparison between PDB formula and YE formula.

Formula (PDB)	Cost of PDB formula	Formula BB (YE)	Cost of YE formula
Potato 200g	10 baht	Yeast Extract 20g	80 baht
Sucrose 20g	0.6 baht	Alginate 20g	29 baht
Alginate 20 g	29 baht	Calcium chloride 7.5 g	0.11 baht
Calcium chloride 7.5 g	0.11 baht		
Total 1000 ml	**Total cost: 39.71 baht**	**Total 1000 ml**	**Total cost: 109.11 baht**
PDB reduce the cost of production by 63.6 %			
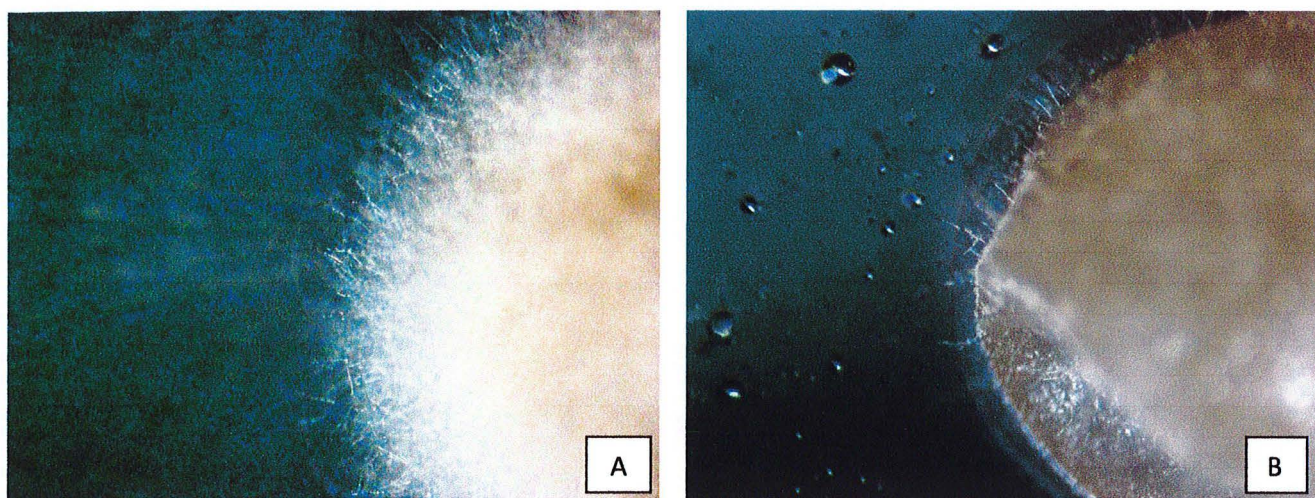
Figure 5.   Stereomicroscopic view of encapsulated beads from PDB formula (A) and YE formula (B) after rehydrated and incubated at 25°C 3 days(45 × magnification).

3.3 Adhesion test

% adhesion on big beads (3.3 mm ± 0.3 mm diameters) and smalls (2.2 mm ± 0.2 diameters) bead was shown in figure 3.6. The big bead and small bead showed 100 % adhesion on glass slide over 24 hours. However, small beads showed higher % adhesion on cassava after 24 hour compared to big beads. The % adhesion between big beads and small beads were significantly different from each other.
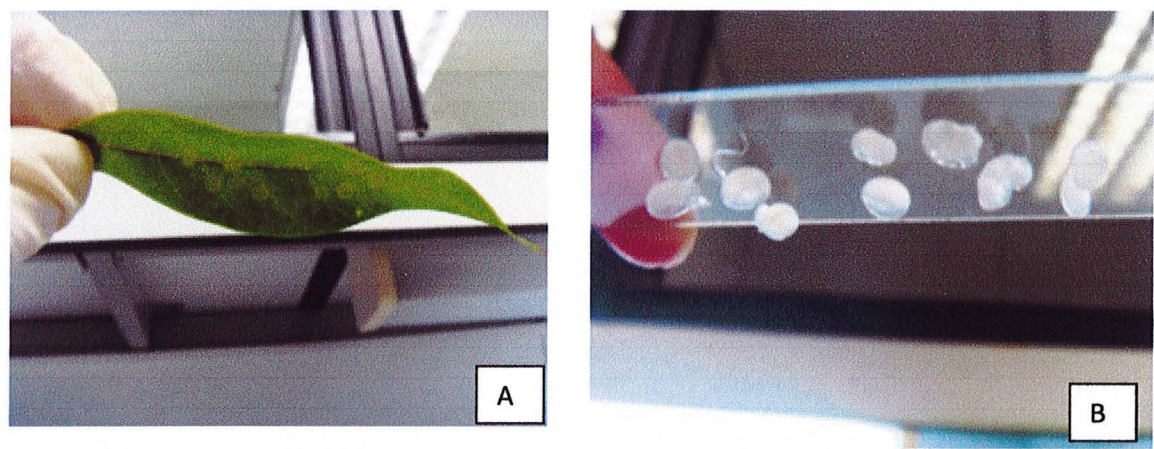
Figure 6. Adhesion test on cassava leaf (A) and glass slide (B).
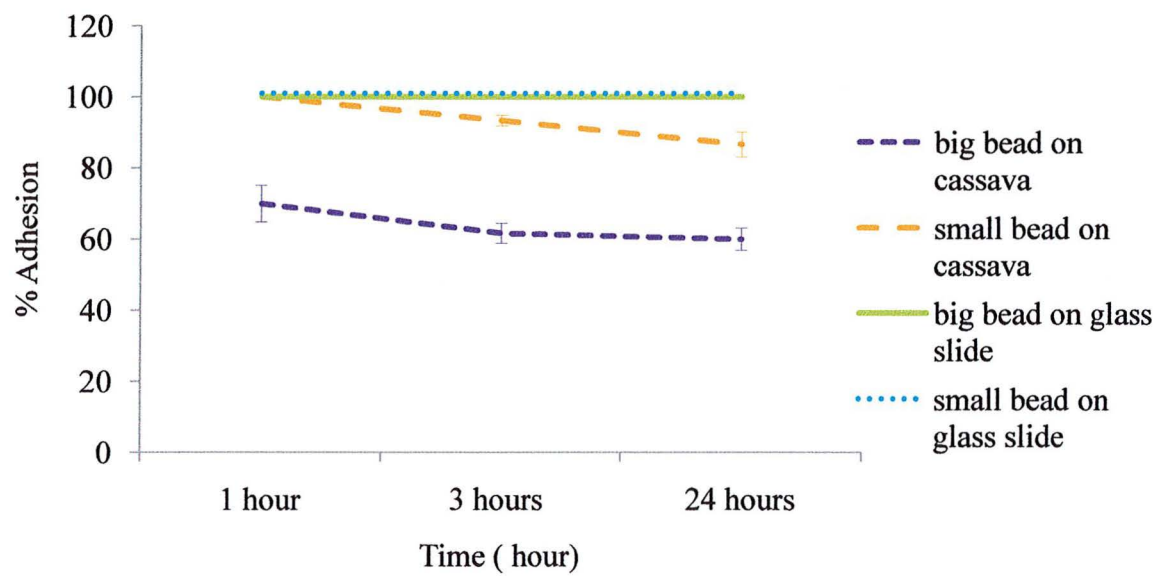
Figure 7. Adhesion of small beads and big beads on glass slide and cassava leafs.

3.4 Infection test on mealy bug (*Phenacoccus Manihot*)

Characteristic of mealy bug infected with *B. bassiana* and morphology of *B. bassiana* spores is shown in figure 3.7. The mortality of mealy bugs from *B. bassiana* after treated with encapsulated *B. bassiana* spores for 2-10 days is shown in figure 3.8. On day 6 the first day mortality occurred, there were no significant differences in % mortality between each treatment but on day 10 there were significantly different between control, and 3 beads, 5 beads. However, the mortality of mealy bugs of each treatment do not reached 20 % after 10 days.
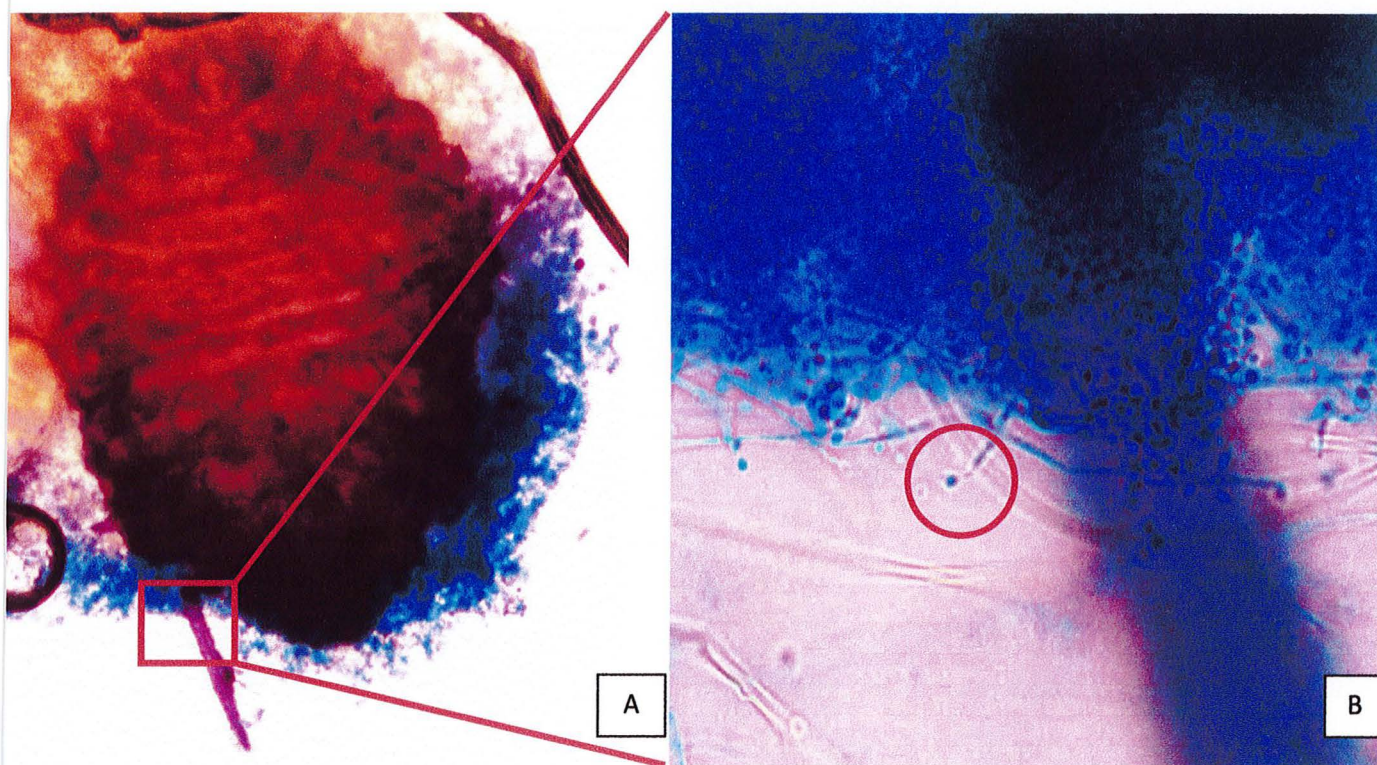
Figure 8. Microscopic view of mealy bug infected with *B. bassiana*. Circle indicate zig-zag rachis and oval conidia of *B.bassiana*. (x 40 magnifications (A), x 400 magnifications (B)).

% Mortality of mealy bug after exposed to encapsulated B. bassiana spores
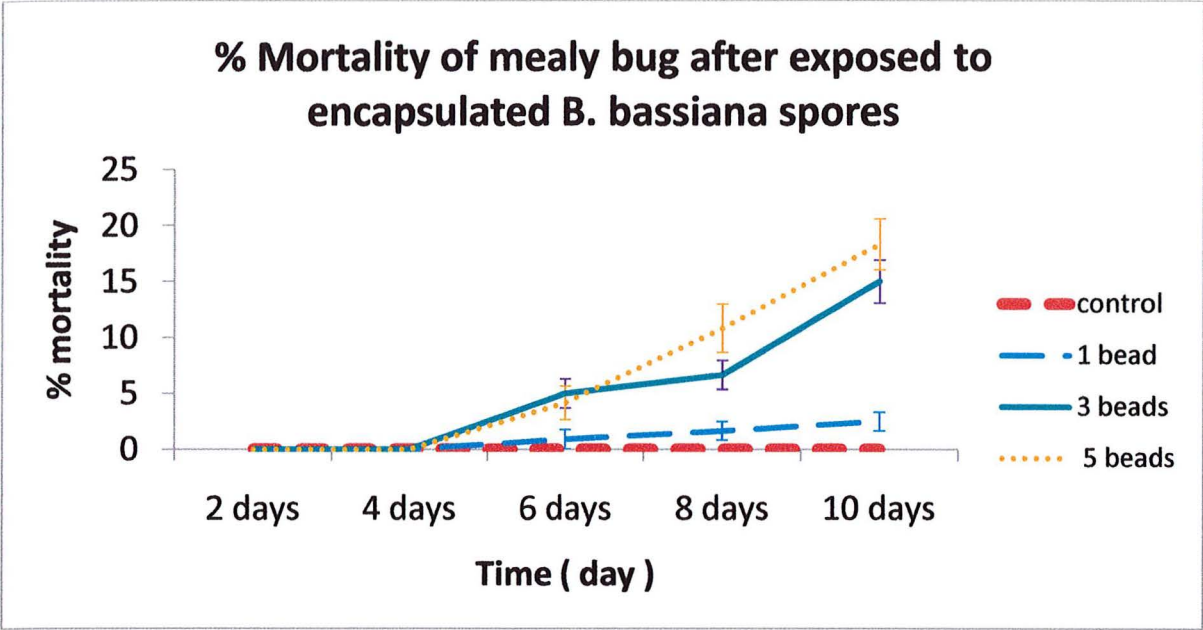
Figure 9. Mean cumulative % mortality of mealy bug after exposed to treatment with control, 1 bead, 3 bead and 5 beads.

3.5 Infection test on beet army worm (*Spodoptera exigua*)

The characteristic of beet army worm infected with *B. bassiana* showed in figure 3.9.The progression of mortality of beet army worm exposed to encapsulate beads showed in figure 3.10. On day 6 the first day mortality occurred, treatment with 5 beads caused significantly greater mortality of beet army worm than treatment with 1 bead and 3 beads. There were significantly differences in % mortality of beet army worm after 10 days between 5 beads and control. The number of bead that caused 50 % mortality in insect after 10 days is 3.4 beads with lower limit of 2.3 beads and upper limit of 5.3 beads.
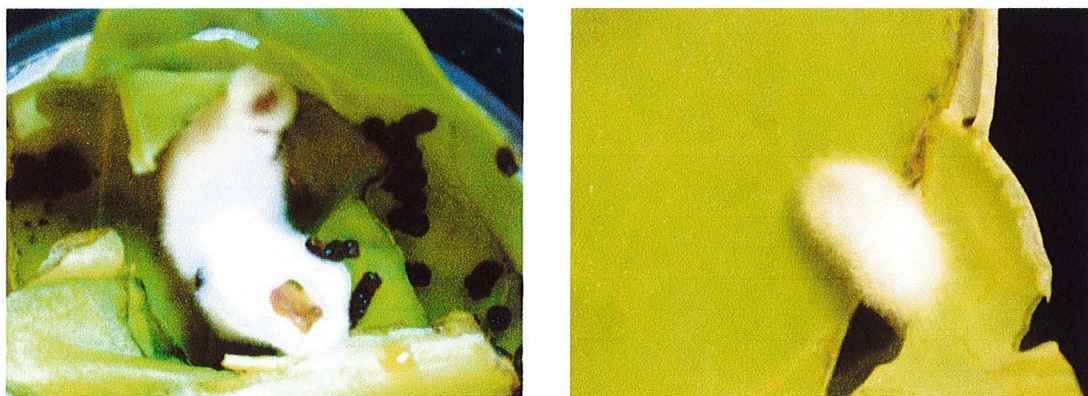
Figure 10.   Stereomicroscopic view of beet army worm infected with *B. bassiana* presented with white mycelium covered the worm body (25× magnification).
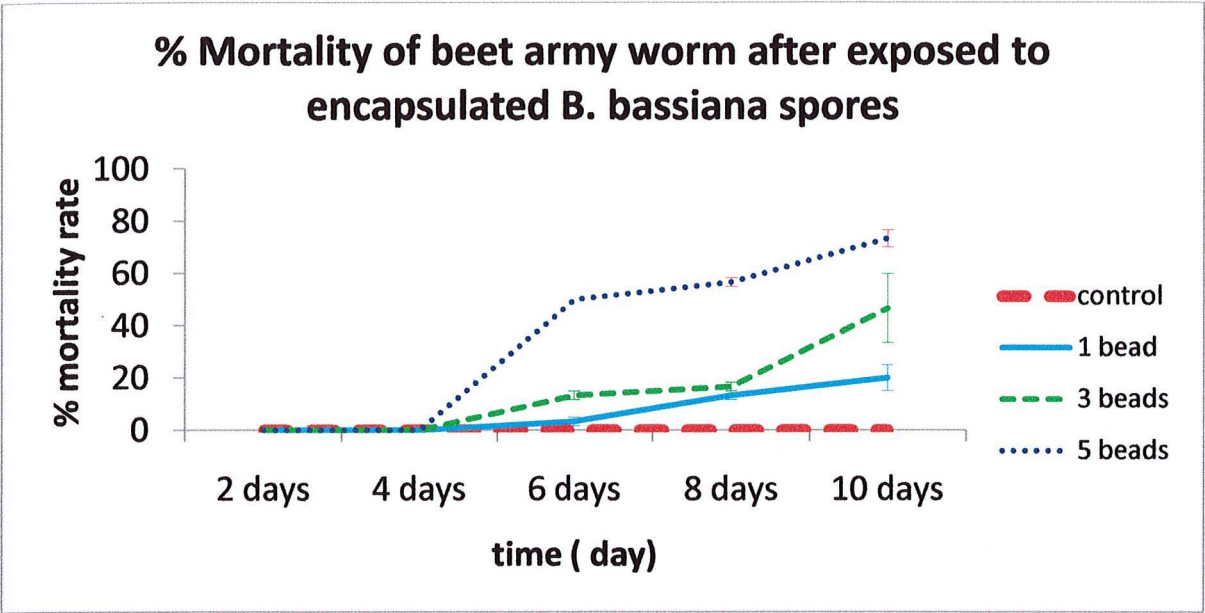
Figure 11. Mean cumulative % mortality of beet army worm after exposed to treatment with control, 1 bead, 3 bead and 5 beads.

DISCUSSION

Formulation of Encapsulated *B. bassiana*

Encapsulation of B, B1, B2, B3 formulas

The formulation of encapsulating agent was modified from Knudsen formula (1991) and named formula B. The formula composed of 2 %w/v sodium alginate, 2 %w/v calcium chloride, 2 % w/v yeast extract and 10 % v/v 10^{13} *B. bassiana* spores per ml. The formula describes the use of sodium alginate to form 3 dimensional structures for entrapping spores. Yeast extract was provided as nutrient for spore germination and mycelium growth. One of the important factors affecting structure of alginate is gelling ion (Martinsen et al., 1989). In the encapsulation process, calcium chloride ions act as binders holding polysaccharides in alginate together and creating a shape gelling zone at the surface of the structure. By altering the concentration of gelling ions, the strength of the bead also changed. Low concentration of calcium ions caused the structure of the gel to become loosely packed and elastic. However, with high concentration of calcium ions, the gel surface becomes strengthened and tightly packed. In order to optimize the efficiency of water retention in encapsulated beads, calcium chloride were varied for formulating formulas B1(1 % w/v of CaCl), B2 (0.75 % w/v of CaCl) and B3(0.5 % w/v of CaCl). Since moisture content is an important factor determining the efficacy for spore germination and mycelium growth, % rehydration of the beads were determined. The rehydration result (table 4, figure 2.) showed that % calcium chloride had reverse relationship with % rehydration. The encapsulation B1, B2 and B3 formula had provided suitable condition for the germination (table 4, figure 2). However with highest % rehydration the spores can germinate and sporulate the best. B3 possessed the highest % rehydration and % sporulation but, figure 1. showed that the beads structures are too fragile and cannot withstand the production process. Likewise formula B2 can provide stable structure that can withstand the production process and also yield highest % rehydration apart from formulas B and B1. This caused Formula B2 to be chosen for further experiment.

Encapsulation of PDB and yeast extract formulas

In term of application, it is necessary for the production of the encapsulated beads to be at low cost and ease able for large scale production (Jackson et al., 1997). In the formulation of B2 formula, yeast extract provide nutrient for the germination and sporulation but however yeast extract is highly expensive and do not support in large scale production. Potato dextrose agar is a media widely used for fungi cultivation and proven to be most effective in culturing *B. bassiana* compare to Water Agar (WA), Corn Meal Agar (CMA) Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Agar with Yeast Extract (YE) (Suasa-ard W. et al. 2008). Since PDA production cost is lower than YE production cost by 63 % (table 1) and was proven to be effective as nutrient for *B. bassiana* growth, PDA was selected to formulate the encapsulated *B. bassiana*. the study of suitable condition for *B.bassiana* had yielding results of PDB formula as the most suitable due to the highest in % rehydration and capability of providing sufficient nutrient for the germination and sporulation (figure 3 and table 6). In addition, figure 4. showed that PDB supplement support longer mycelium growth of *B. bassiana* after 3 days compared to yeast extract formula. Furthermore, from the study of Crowe (1988) non reducing disaccharide can also help protect spores from heat damage during drying process by distributing on both sides of phospholipid bilayers. In this case, PDB formula contain sucrose which helps preserving spores in drying process resulting in functional spores that can germinate and producing mycelium effectively. With the advantages of low production cost and suitable condition for the spore's sporulation, PDB formula was used for the further experiment.

Adhesion test

For a field of application, it is essential that the encapsulated beads can deliver the fungal spores to the insect. Therefore, the adhesion test was performed to test the adhesive ability of encapsulated beads. The test was modified from Sasha B. (1988) by using glass slide and cassava leaf as surface objects. The glass slide represents a hydrophobic surface that determines the adhesive ability of the beads to a plain surface and the cassava leaf represents a natural surface containing the wax layer. All big beads and small beads can adhere to glass

slide after 24 hours (Figure 5. and Table 9). This shows that both of the bead sizes are capable of adhering to the plain object. However, on cassava leaves the small beads could adhere to better than big beads (Figure 5, Table 9). This perhaps because the small beads are lighter, consequently leadings to lesser gravitational force acting on the beads surface. The small beads formula was chosen for further experiment due to its higher adhering efficiency. This can possibly enhance distribution of the fungal spores to target insect pests.

Infection test on mealy bug (*Phenacoccus Manihot*)

Encapsulated beads work as a spore carrier and provide protection and suitable condition for fungal spores to germinate and sporulate before infection on target insect. The infection test was performed in order to test biocontrol efficiency of encapsulated beads. Gulsar (2010) tested the pathogenicity of spray formula of *B.bassiana* against mealy bug and found that it caused 45±3 % mortality rate on second day. However, my result (see Figure 7.) showed that for every treatment the infection started after 6 days and did not exceed 20 % mortality after 10 days. These showed that the spray treatment works more effectively in controlling mealy bugs. This is because a spray allows fungal spores to contact insects more readily but encapsulated *B. bassiana* infected the insect by contacting to insect through their movement. Renard (1998) found that mealy bug search for food by rubbing it's labium onto the plant surface and when they find a preferred spot, they stop and feed the plant crops by sucking the sap through plant phloem. The feeding behavior caused the movement of mealy bug to be lesser. This reduces the chance for mealy bug to expose to *B. bassiana* spores. Therefore, encapsulated formula of *B. bassiana* might not be suitable for controlling mealy bug.

Infection test on beet army worm (*Spodoptera exigua*)

In order to prove the relationship between the movement of insect and the infection capability of encapsulated beads, the test of encapsulated *B. bassiana* was also performed with beet army worm. Beet army worms consume plant parts by devouring which caused

them to move constantly in order to obtain sufficient food. This caused them to expose to encapsulated *B. bassiana* more than mealy bug. Duriya (2005) found that a spray of *B. bassiana* spores caused 50 % to 100 % mortality of beet army worm after 3 days and 4 days. My result (see Figure 9.) showed that the mortality of beet army worm started after 6 days in all treatment and 5 beads caused highest mortality of 36 %. In addition, after 10 days the treatment of 5 beads and 3 beads show significantly higher in % mortality of beet army worm compare to control. This result is quite consistent with my hypothesis of the relationship between movement and the infection of the insect. These showed that spray and encapsulated *B. bassiana* can be used to control beet army worm. However, spray infected beet army worm faster than encapsulated *B. bassiana*. This is because the encapsulated *B. bassiana* required contact from the insect to initiate the infection. The result (Table 17.) showed that the number of bead and % mortality of beet army worm had positive proportional relationship with % mortality. The infection of *B. bassiana* in beet army worm increased with the number of bead due to the exposure of the spores on the beads to beet army worms during food scavenging. Furthermore, from the calculation, it is shown that the number of beads that caused 50 % mortality after 10 days in beet army worm is 3.4 beads with lower limit of 2.3 beads and upper level of 5.3 beads. This experiment showed that beet army worms can be controlled by using encapsulated *B. bassiana* in laboratory condition.

In conclusion, encapsulated beads showed as potential method for improvement of bio-control by *B. bassiana* for highly motile insects. This can also open a new possibility of controlling wider ranges of the insect pests. For example, flea beetle (*Phyllotreta sinuata*), sugarcane borer (*Diatraea saccharalis*) and termite (*Isoptera spp.*). This is because these insects have part of their life circle in soil earth and the condition of soil earth aids the encapsulated beads in term of water retention and heat tolerance leading the infection to occur more effectively. Furthermore, nutrient rich bead can increases fungal biomass in soil and help plant to obtain more nutrients for their growth. Above all, the knowledge of the encapsulated formulation can be further used to develop and maximize the function of *B. bassiana* in order to help controlling the insect pest. For example addition of sun screen for UV resistance (Burgess, 1998).

CONCLUSION

1. In the production of encapsulated beads, the used of low concentration calcium chloride gives encapsulated bead ability to rehydrate more water but if too low it caused the structure of the beads to be fragile and unable to withstand the production process . It was found that 0.75 % w/v calcium chlorides provide stable structure of the beads while enhancing the germination and sporulation of *B. bassiana.*

2. PDB formula reduced the cost of production 63.6 % and can rehydrated water 35 % higher than yeast extract. Furthermore, PDB formula can provide suitable condition for spores germination and sporulation.

3. Small beads smalls (2.2 mm ± 0.2 diameters)can adhere to cassava leaf better than big beads(3.3 mm ± 0.3 mm diameters) by 26 %.

4. Encapsulated beads were not suitable for controlling mealy bugs effectively due to the insect feeding behavior and low movement. The result showed that the encapsulated bead caused lower than 20 % mortality of mealy bug after 10 days.

5. Encapsulated beads were suitable for controlling beet army worm due to the insect rapid movement. It was found that the number of bead that caused 50 % mortality in insect after 10 days is 3.4 beads with lower limit of 2.3 beads and upper limit of 5.3 beads.

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APPENDIXS

Table 2.   Duncan analyses on % rehydration of treatment with formula B, B1, B2 and B3 at 95 % confidence interval.

Duncan

% rehydration	N	Subset for alpha = .05	
		1	2
B	3	7.1747	
B1	3		36.0462
B2	3		47.9658
B3	3		48.6842
Sig.		1.000	.051

Table 3.   Duncan analysis on % sporulation of treatment with formula B, B1, B2 and B3 at 95 % confidence interval.

Duncan

% sporulation	N	Subset for alpha = .05	
		1	2
B	3	.0000	
B2	3		87.3333
B3	3		92.0000
B1	3		94.0000
Sig.		1.000	.361

Table 4.   Mean and standard deviation of % rehydration and % sporulation of formula - B,B1,B2 and B3.

Treatment	% Rehydration	SD	% Sporulation	SD
B	7.2[a]	± 0.3	0.0[a]	± 0.0
B1	36.0[b]	± 10.1	94.0[b]	± 10.4
B2	48.0[b]	± 7.1	87.3[b]	± 12.2
B3	48.7[b]	± 3.8	92.0[b]	± 2.0

Table 5. Pair sample t test on % rehydration of PDB formula and YE formula at 95% confidence interval.

Paired Samples Test

		Paired Differences							
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
					Lower	Upper			
Pair 1	PDB - YE	35.27031	9.13826	5.27598	12.56962	57.97101	6.685	2	.022

Table 6. Pair sample t test on % sporulation of PDB formula and YE formula at 95% confidence interval.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	PDB	100.0000(a)	6	.00000	.00000
	YE	100.0000(a)	6	.00000	.00000

Table 7 . Mean and standard deviation of % rehydration and % sporulation of potato dextrose formula and yeast extract formula.

Treatment	% rehydration	SD	% sporulation	SD
YE	39.98[a]	± 4.30	100.00[a]	± 0.00
PDB	74.39[b]	± 3.70	100.00[a]	± 0.00

Table 8. Pair sample t test on % adhesion of big beads and small beads formula at 95 % confidence interval.

Paired Samples Test

		Paired Differences						
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. (2-tailed)
Pair 1	Big - Small	-2.66667	1.36626	.55777	-4.10047	-1.23286	-4.781	5	.005

Table 9. Mean and standard deviation of % adhesion of big beads and small beads.

Treatment	% adhesion after 24 hours	SD
big beads	60.00[a]	± 10.95
small beads	86.67[a]	± 12.11

Table 10. Duncan analyses on % mortality of mealy bug from *B. bassiana* after 6 days of treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% mortality	N	Subset for alpha = .05	
		1	2
control	12	.0000	
1 bead	12	.1667	.1667
3 beads	12		.4167
5 beads	12		.5000
Sig.		.331	.069

Table 11. Duncan analyses on % mortality of mealy bugs from *B. bassiana* after 8 days of treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% mortality	N	Subset for alpha = .05		
		1	2	3
control	12	.0000		
1 bead	12	.1667		
3 beads	12		.6667	
5 beads	12			1.0833
Sig.		.424	1.000	1.000

Table 12. Duncan analyses on % mortality of mealy bugs from *B. bassiana* after 10 days of treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% sporulation	N	Subset for alpha = .05	
		1	2
control	12	.0000	
1 bead	12	.2500	
3 beads	12		1.5000
5 beads	12		1.8333
Sig.		.265	.139

Table 13. Mean and standard deviation of % mortality of mealy bugs from *B. bassiana* after 10 days treatment with control, 1 bead, 3 beads and 5 beads.

	% mortality after 2 days	SD	% mortality after 4 days	SD	% mortality after 6 days	SD	% mortality after 8 days	SD	% mortality after 10 days	SD
)l	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.17	0.39	0.17	0.39	0.25	0.45
ls	0.00	0.00	0.00	0.00	0.50	0.52	0.67	0.65	1.50	0.80
ls	0.00	0.00	0.00	0.00	0.42	0.51	1.08	0.67	1.83	0.58

Table 14. Duncan analyses on % mortality of beet army worm from *B. bassiana* after 6 days treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% mortality	N	Subset for alpha = .05		
		1	2	3
control	3	.0000		
1 bead	3	.3333		
3 beads	3		1.3333	
5 beads	3			5.0000
Sig.		.347	1.000	1.000

Table 15. Duncan analyses on % mortality of beet army worm from *B. bassiana* after 8 days treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% mortality	N	Subset for alpha = .05		
		1	2	3
control	3	.0000		
1 bead	3		1.3333	
3 beads	3		1.6667	
5 beads	3			5.6667
Sig.		1.000	.438	1.000

Table 16. Duncan analyses on % mortality of beet army worm from *B. bassiana* after 10 days treatment with control, 1 bead, 3 beads and 5 beads at 95 % confidence interval.

Duncan

% sporulation	N	Subset for alpha = .05	
		1	2
control	3	.0000	
1 bead	3	2.0000	
3 beads	3	4.6667	4.6667
5 beads	3		7.3333
Sig.		.062	.233

Table 17. Mean and standard deviation of % mortality of beet army worm from *B. bassiana* after 10 days treatment with control, 1 bead, 3 beads and 5 beads.

treatment	% mortality after 2 days	SD	% mortality after 4 days	SD	% mortality after 6 days	SD	% mortality after 8 days	SD	% mortality after 10 days	SD
control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1 bead	0.00	0.00	0.00	0.00	3.33	5.77	13.33	5.77	20.00	17.32
3 beads	0.00	0.00	0.00	0.00	13.33	5.77	16.67	5.77	46.67	46.19
5 beads	0.00	0.00	0.00	0.00	50.00	0.00	56.67	5.77	73.33	11.55