Survivability of *P. oxalicum* T3.3 bioformulation on carrier materials and storage temperature

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Abstract*—Good bioformulation play crucial roles in the successfully of commercialize biological control products. The development of bioformulation is necessary to improve product stability, delivery and bioactivity. The aim of this study is to assess the shelf life of P. oxalicum T3.3 conidia in the different ratio(1:1,1:2,2:1) of Biochar:Biocompost (BcBp) and Peat:Vermiculite (PtVm) and temperature (4ºC and room temperature) for 6 months. The results showed that P. oxalicum T3.3 was able to sustain highest viable cell (CFU) at 4ºC storage temperature. BcBp and PtVm have the highest cell viability at ratio 2:1 and 1:1, respectively. Both BcBp and PtVm showed potential carriers for the development of biofungicide for agriculture purposes.*

Keywords— Biochar, Formulation, Peat, Penicillium, Shelf life.

I. INTRODUCTION

Fungi have been a great spotlight as a biological control agent and large scale of work has been borne in order to find fungi with a good potential as a fungicides (Lopez, 1998). Some species of fungi have the abilities to secrete active substances that can be lethal to certain life form (Santamarina *et al*., 2002). Most fungal showed antagonistic effect to certain bacteria or fungi that cause plant disease. *Penicillium* spp. is one of the potential biological agent that capable to control certain pathogenic fungal species by inducing resistance in plant and by its metabolites (Narayanasamy, 2013). Besides, *Penicillium* spp. has been reported to have ability to suppress *Fusarium* and *Verticillium* wilt of tomato plant (Whipps, 2001). However, the use of fresh cells of potential biological control agent (BCA) have been widely studies in controlling plant disease (Mahdy *et al*., 1987; Larsen & Knechel, 1997; Cal & Melgarejo, 2000 and Shojaee *et al*., 2014). Even though it is effective and suitable for research purposes but it is not practical to be used by farmers. The inconsistency of the fresh cells of BCA makes it impossible for commercialization and longer time storage.

Formulated biological control products must be able to retain their similar productivity to the fresh cells (Janisiewicz & Jeffers, 1997). The major concern of the commercialize products are the stability of the shelf life of biological control in the market. Baker & Henis (1990) highlight that a commercialize product must be able to retain their stability for at least one year and must be stored at room temperature. Apart from that, the formulated product must be easy to handle and have stability over a range of -5 to 35 ºC (Junaid *et al*., 2013). Formulation of the biological control is the mixture of active ingredients such as conidia with the inert material such as surfactant in order to modify the physical characteristics to more desirable form (Junaid *et al*., 2013). Thus, the aim of this project is to assess the shelf-life of *P. oxalicum* T3.3 conidia in different ratio of carrier and storage temperature. The present study will increase knowledge on the development of commercialize biological control products for agriculture purpose.

II. MATERIAL AND METHOD

2.1 Microorganism

P. oxalicum T3.3 used in this study was previously isolated from dragon fruit plant by Suhaila (2014). *P. oxalicum* T3.3 was grown on Potato Dextrose Agar (PDA) plate for 7 days at 30ºC.

2.2 Fermentation media

Mixture of peat, vermiculite and soya bean (PVS) (1:1:1, wt/wt/wt) were used as basal medium. PVS (12 g) with 10 mL of distilled water was prepared in 250 mL flask. Then, the mixtures were sterilized by autoclaving in autoclave at 121ºC for 15 min. The mixture was allowed to be cooled until it reaches room temperature before inoculation process.

2.3 Preparation of spore suspension

One disc of *P. oxalicum* T3.3 grown on PDA from 7 days culture was inoculated into PVS media and incubated at 28 ºC for 8 days. Afterwards, spores suspension was harvested and adjusted to 10^9 CFU/mL and the spore's suspension was used for the preparation of the bioformulation.

2.4 Preparation of carrier materials

Inert carriers used for bioformulation were biochar, biocompost, peat moss and vermiculite. The carrier were divided into two mixture (biochar and compost; peat and vermiculite). Grounded carriers were further screened under 1 mm sieve to get fine powder form for inoculation. The fine powder were then oven- dried at 70 ºC for 48 hours and placed in an airtight polyethylene bags for sterilization in autoclaved at 121 ºC for 20 minutes in consecutive days before used for fungi inoculation.

2.5 Development of bioformulation at different storage temperature

Two combination of sterile powder bioformulation with the same ratio of carrier were prepared. Each mixture bioformulation; Peat:Vermiculite (PtVm) and Biochar:Biocompost (BcBp), 100 g each were mixed with 1 g of carbomethylcellulase (CMC) as additives. The mixture were inoculated under sterile condition with 40 mL of inoculum suspension after autoclaved at 121 ºC for 20 min (Vidhyasekaran & Muthamilan, 1995). The concentration of inoculum at initial was adjusted to 10^9 CFU/mL. Bioformulations were then stored at 4ºC and room temperature for 6 months.

2.6 Development of bioformulation at different ratio

Carriers were divided into two mixture; Peat:Vermiculite (PtVm) and Biochar:Biocompost (BcBp). Each carrier was mixed with different ratio at 1:1, 2:1 and 1:2 with the total weight of 100 g. The mixture were inoculated under sterile condition with 40 mL of inoculum suspension after autoclaved at 121 ºC for 20 minutes (Vidhyasekaran & Muthamilan, 1995). The concentration of inoculum at initial was adjusted to 10^9 CFU/mL. Bioformulations were then stored for 6 months.

2.7 Viability assessment of shelf life of bioformulation

The bioformulation were stored at different temperature at 4 ºC and room temperature and assessment were carried out for 6 months by monitoring the viability of the antagonistic fungi in the formulation. One gram of the sample was drawn from each formulation periodically at 0, 4, 8, 12, 16, 20 and 24 weeks of storage time and was mixed with 9 mL of sterile saline water. From this, serial dilution was made. One mL sterile saline water was added to three replicate tubes per treatment and spore concentration was adjusted about 1 x 10^7 spores/mL. Suspensions were plated onto three replicate petrifilm (1 mL per petrifilm) and were incubated at 30 ºC for 72 h. The numbers of germinated spores were counted in each petrifilm (3M) and colony forming unit was calculated. The viability lost was determined by comparing with the initial concentration, CFU/mL.

$$\text{CFU of sample} = \frac{\text{Average colonies x Dilution factor}}{\text{Sample size}}$$

III. RESULTS AND DISCUSSION

There were significant differences in survival of *P. oxalicum* T3.3 among the carriers and storage temperatures at initial day to 180 days. Formulation of BcBp and PtVm were able to retain the viable cells of log 5 and 4 CFU/mL for 180 days, respectively. At 4 ºC, viable cells of BcBp carrier was decreased significantly throughout the assessment. During 180 days of storage time, the viable cells were recorded with the value of log 5.43±0.13 CFU/mL (Figure 1). At room temperature, the numbers of viable cells in BcBp formulation sharply decreased at 30 to 60 days and then significantly decrease toward the end of assessment. The CFU unit recorded for BcBp at room temperature at the end of experiment was log 3.67±0.56 lower than at 4ºC. The results showed that BcBp was able to retained higher CFU at 4 ºC.

Fig. 2 shows that the viable cells in PtVm bioformulation were significantly declined throughout the experiment for both storage temperatures. At room temperature, viable cells were dropped from log 7.80±1.13 CFU/mL to log 5.50± 0.71 CFU/mL during 60 to 90 days storage time. Viable cell were decreased significantly from 120 to 180 days of storage with log 3 CFU/mL at the end of experiment. As for 4 ºC, the number of viable cell were constant from 90 to 120 days and start to decreased slowly until the end of assessment. The number of viable cells detected in 4 ºC was log 4.46±0.15 CFU/mL which was higher than at room temperature after 180 days. Statistically, this study suggested that there were significant differences in CFU count among the bioformulations at the end of the experiment with $P \leq 0.05$.

Fig. 3 and Fig. 4 show that there were no insignificant differences in viability cell at the end of experiment. BcBp at ratio 2:1 showed the highest cell viability with log 5.63±0.47 CFU/mL whereas 1:1 ratio at log 5.43±0.13 CFU/mL and followed closely by 1:2 with log 5.40±0.09 CFU/mL. PtVm also do not have a significant difference between ratios after 180 days. Ratio 2:1 gave the lowest viable cell with log 4.49±0.20 CFU/mL while 1:1 has the highest viable cell with log 4.79±0.71 CFU/mL and ratio 1:2 was recorded with log 4.69±0.36 CFU/mL. This study showed that different in ratio of carriers do not affect significantly on the viable cell at the end of assessment. Statistically, this study suggested that there were significant differences in CFU count among the bioformulations with $P \leq 0.05$.

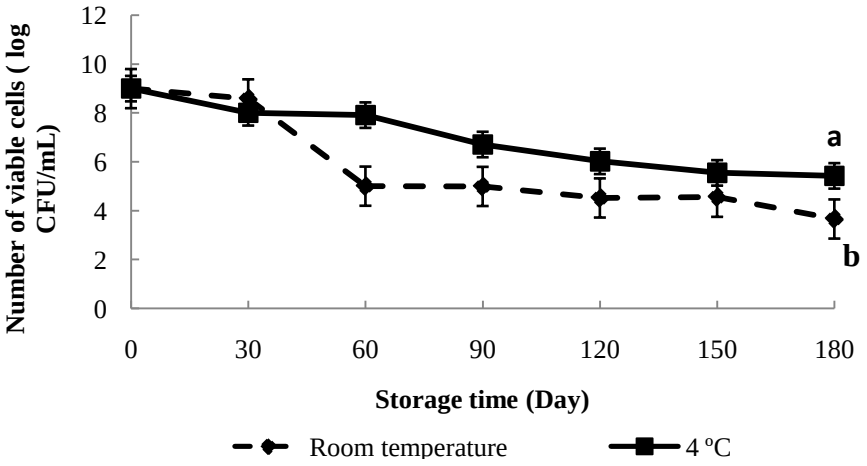

FIGURE 1: Survival of *P. Oxalicum* T3.3 in the Mixture of Biochar+Biocompost (BcBp) Bioformulation at Different Temperature.

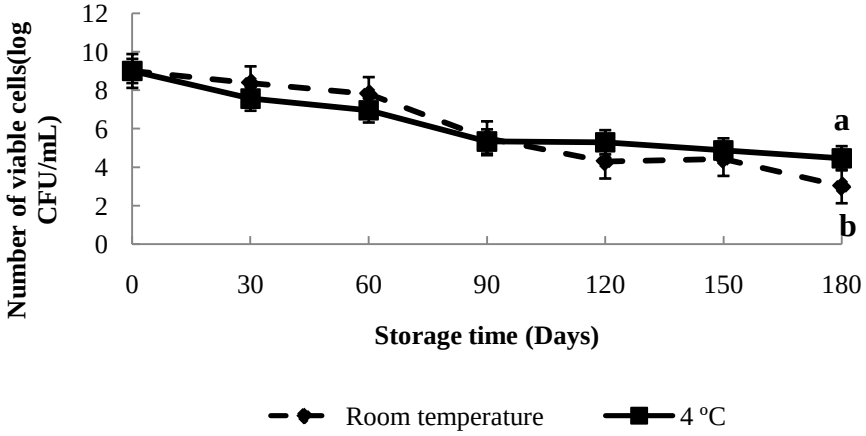

FIGURE 2: Survival of *P. Oxalicum* T3.3 in the Mixture of Peat+Vermiculite (PtVm) Bioformulation at Different Temperature.

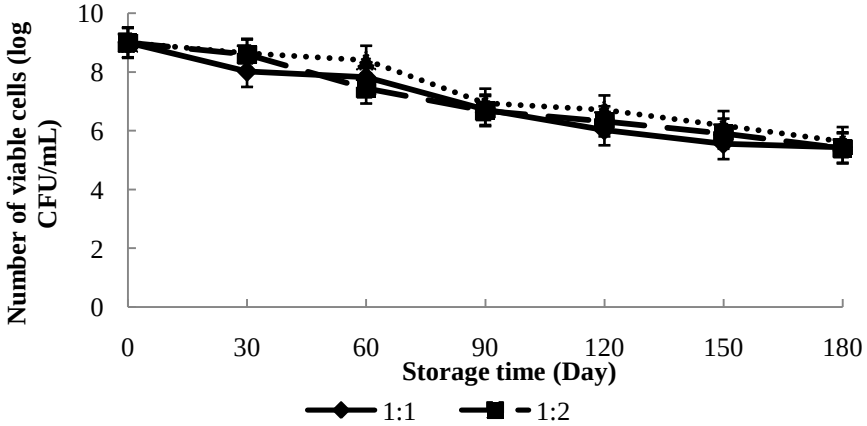

FIGURE 3: Survival of *P. Oxalicum* T3.3 in the Biochar+Biocompost (BcBp) Formulation at Different Ratio.

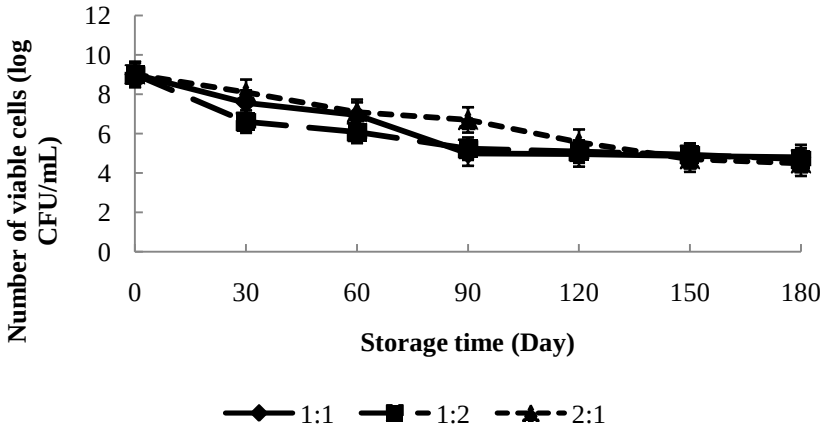

FIGURE 4: Survival of *P. Oxalicum* T3.3 in the Peat+Vermiculite (PtVm) Formulation at Different Ratio.

The initial viable cells in this study were higher than recorded by Pimenta *et al* (2008). *P. oxalicum* T3.3 populations were dropped at 30 days because of the lack of nutrient and moisture of the carriers. This is because when the fungi were transitioning from logarithmic to stationary phase, the P_{total}, N_{total}, K_{total} and the moisture content decreased due to the storage condition and microbial activities (Tate, 2000). During 120 days, death rate was slowly decreased until 180 days storage time. In this study, the survival of *P. oxalicum* T3.3 in PtVm and BcBp were less than those reported by other researches due to different types of carrier used (Bazilah *et al*., 2011; Singh *et al*., 2014).

The viability of cells in both formulation were higher at 4 ºC instead of room temperature because of the moisture availability surrounding the cells (Bazilah *et al*., 2011). Many of beneficial microbes have longer shelf-life when stored at lower temperature (Balume *et al*., 2015; Phiromtan *et al*., 2013). Low temperature also reduced water loss in the formulation and preserves the efficiency of fungi. Apart from that, high storage temperature help microbial growth which causes the microbial to produce more wastes (Bazilah *et al*., 2011). It also has been proved by Soe & De Costa (2012) where powder based formulation were able to maintain high cell viability over long time at 4 ºC and room temperature compared to spore suspension.

The results indicated that BcBp was the most suitable carrier for production *P. oxalicum* T3.3. The mixture of biochar and biocompost (BcBp) showed able to survive the viable cell of the inoculum after 180 days. Both of the carrier have microporous structure which is good to be used as a carrier (Somarathne *et al*., 2013). Biocompost and biochar itself have a beneficial nutrient which provided a good microbial habitat and help to prolong their survival rate. Apart from that, it promote the growth of plants by the interaction of plant microbial (Warnock *et al*., 2007). BcBp has high surface area because of their porous structures and it help to attract and absorb water. Thus, nutrients that good for microbial growth such as phosphorus and nitrogen also retained (Somarathne *et al*., 2013).

Peat and vermiculite (PtVm) were also considered as a good carrier since both of the carriers can be used as microbial inoculant alternative with each other. The mixture of both carriers help in improved microbial growth, promote seed germination when it used as seed treatments, and improved plant growth and yield. Nehra & Choudhary (2015) claimed that the used of peat as carrier was significantly reduces threat that resulting from contamination. Vermiculite was a good carrier because of the multilamellate structure that provided good aeration and space for microbial proliferation. Apart from that, it has good sticking properties which help to amend with peat as carrier and it also help the number of viable cells does not change significantly when stored at room temperature (Daza *et al*., 2000). BcBp performed well in preserving the *P. oxalicum* T3.3 population. Besides that, traditional carrier, PtVm also able retained a good number of viable cells even though it has lower number of viable cells than BcBp. Statistically; both formulations show a significant result at the end of assessment.

IV. CONCLUSION

In conclusion, both bioformulation were able to retained high CFU number during storage temperature 4 ºC compared to room temperature for 180 days. In comparison, BcBp bioformulation has higher cell viability with log 5.43±0.13 CFU/mL whereas PtVm bioformulation with log 4.46±0.15 CFU/mL. Bioformulation was able to preserve high cell population because of the presence of moisture surrounding the cells which help to reduce water loss in the formulation. Apart from that,

the both bioformulation also were tested for their effect on the different carrier ratio and study found that BcBp were able to maintain high CFU with log 5.63±0.47 CFU/mL at 2:1 while ratio 1:1 of PtVm recorded high CFU with 5.43±0.13 CFU/mL. A good carrier choice plays an important role in preserving cell viability. Present study showed that mixture of biochar and biocompost as good carriers for microbial habitat compared to peat and vermiculite.

ACKNOWLEDGEMENTS

The authors would like to thank the University Putra Malaysia under Putra Grant (Vote no.9485200) for financial support.

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