Proceedings of the 10th International Conference on Agriculture, Vol.8, Issue 1, 2023, pp.12-23

ISSN 2682-7018 online
DOI: https://doi.org/10.17501/26827018.2023.8102

THE IMPACT OF PHYSICAL PARAMETERS DURING THE FERMENTATION PROCESS AND CRITICAL MOISTURE CONTENT DURING PRODUCT STORAGE

Bohari NH*, Mohd Fishal EM and Abd Razak IB

Crop Protection and Biosolution Department, FGV R&D Sdn Bhd, Bandar Enstek, Negeri Sembilan, Malaysia

Abstract: Trichoderma sp. is a well-known biocontrol agent of Ganoderma basal stem rot (BSR) disease in oil palms. A lot of commercial products containing the beneficial microbe are available around the world. PalmaShield, containing Trichoderma asperellum (M103), is one of the commercialized and marketed products in Malaysia by FGV Agri Services Sdn. Bhd. M103 has already been proven to suppress Ganoderma growth in in-vitro studies by causing more than 85% inhibition of radial growth and has reduced more than 50% of BSR disease infections at the nursery stage. The journey to mass produces the M103 requires optimization of the fermentation process and maintaining the stability of the final product. For the fermentation process, the physical parameters were tested. M103 can grow well in submerged fermentation with an initial pH of 3, an incubation temperature between 28 and 30°C, a shaker speed of 150 rpm, and an inoculum size of 2 to 3% v/v. The culture broth from the fermentation process is mixed with clay for the development of powder formulations. During the final stage, continuous contamination of the final product in storage would be challenging. The moisture content of the final product was found to be the critical parameter to be controlled to eliminate the risk of bacterial contamination and maintain the viability of M103. Therefore, several drying techniques were studied, and product stability was monitored for 1 year. These findings provide important parameters to be controlled during the production process of Trichoderma's product in powder form.

Keywords: contamination, fermentation, powder, Trichoderma

Introduction

The oil palm industry is important as palm oil is globally used in the food, detergent, cosmetic, and biofuel industries (AISE, 2020; MPOC, 2023). The widespread use of palm oil further increases its demand and global production (The Star, 2023). Indonesia and Malaysia are the leading exporters of palm oil at the moment (Shahbandeh, 2023). However, as the main producer and exporter of palm oil, there are several challenges that need to be faced in this industry (Maluin et al., 2020). One of the challenges that significantly threatens oil palm cultivation is the basal stem rot (BSR) disease caused by *Ganoderma boninense* (Rebitanim et al., 2020). *G. boninense* is a white rot, wood-decaying fungi that can cause 43% economic loss in the oil palm plantation within 6 months of infection (Siddiqui et al., 2021; Khoo & Chong, 2023). Due to this highest threat of *Ganoderma* infection, a lot of studies have been conducted to manage and control the disease. One of the popular and sustainable approaches is using biocontrol agents of *Trichoderma*-based products (Virdiana et al., 2019; Sukariawan et al., 2021).

*Corresponding Author's Email: *idksdariyawanse@gmail.com*

The interest in using *Trichoderma*-based products is not new, and many commercial products containing this beneficial fungus have already been marketed worldwide in various formulations (Raimi et al., 2021; Guzmán-Guzmán et al., 2023). *Trichoderma asperellum* (M103) has been proven to combat *G. boninense* PER71 and has been commercialized and marketed in Malaysia as a PalmaShield product (Mohd Fishal et al., 2022; The Petri Dish, 2019). This M103 is mass-produced using submerged fermentation and is formulated in powder form. A previous study by Suthar et al., (2017), reported that fermentation is an important process where beneficial microbes can be produced on a large scale for field applications. In fermentation, both growth medium and physical parameters are crucial to be controlled to achieve higher microbial biomass, metabolites, or enzyme production (Zhang et al., 2020). Once fermentation parameters have been established, the formulation, packaging, and shelf life of the products must be considered (Teixidó et al., 2022). A suitable and stable formulation is important for commercialization, as the viability of beneficial microbes will affect the effectiveness of the product during application (Cumagun, 2014).

Since this beneficial microbe of M103 has been discovered and proven to be effective in controlling BSR disease, it is important to ensure that this M103 is successfully delivered to the agricultural industry. With the aim of developing a stable and effective product, this study was conducted to increase biomass production during the fermentation process and extend the shelf life of the powder formulation through the drying process. In addition, the risk of contamination during product storage must also be eliminated.

Materials and Methods

Microorganism and preparation of standardized inoculum

The *Trichoderma asperellum* M103 (Figure 1) was obtained from the Microbial Culture Collection of Beneficial Microbes Laboratory, FGV R&D Sdn. Bhd, Malaysia. The isolate was grown on potato dextrose agar (PDA) plate and incubated for 5-6 days at 28°C. One full-grown culture plate was added with 25 mL of sterile reverse osmosis (RO) water and slowly scraped with L-shape glass rod. The suspension was filtered using 2-layer sterile gauze to separate the mycelium debris and collected in a sterile bottle. The spore suspension was counted using a Haemocytometer-based counting method and the concentration was standardized at 10^8 spores mL^{-1}. The spore suspension (1%) was used as an inoculum seed.

Figure 1: Trichoderma asperellum (M103)

Microorganism and preparation of standardized inoculum

Potato Dextrose Broth (PDB) was used as the liquid growth medium for the fermentation process. 100 mL of PDB in 250 mL of Erlenmeyer flask were inoculated with standardized inoculum in each experiment. The mycelium biomass of M103 developed after 3 days of incubation was used as an indicator for the growth performance of M103.

Screening of essential physical parameters of the fermentation process

To screen the favorable physical parameters during the fermentation process, the effect of each parameter was studied in a certain range as stated in Table 1.

Table 1: Physical parameters of the fermentation process

No.	Physical Parameter	Study Range
1	Initial pH of growth medium	pH 1-10
2	Incubation temperature (°C)	23, 24, 25, 28, 30, 35, 36, 37
3	Shaker speed (rpm)	80, 100, 130, 150, 180, 200
4	Inoculum Size (%)	0.1, 1.0, 2.0, 3.0, 4.0, 5.0

Mycelium dry weight measurement

The mycelium biomass of M103 was harvested from a total volume of fermentation culture (100 mL per flask) and filtered through Whatman filter paper (No. 1). The biomass was washed with RO water to remove residual broth or growth medium. The washed biomass was oven dried at 65°C for 48 hours (about 2 days) and the weight was determined. The average values were obtained from three replicates.

Preparation of M103 in powder formulation

The PDB medium (30 L) in a glass vessel was inoculated with 2 % of 3-days old of M103 culture broth. The fermentation process was conducted at room temperature (28 - 30°C) and 800 rpm stirrer speed. After 3 days of incubation, the M103 mycelium biomass was removed from the glass vessel and formulated by mixing it with clay powder.

Screening of different drying techniques

The powder formulated M103 was divided into 4 portions to screen the suitable drying techniques. 4 treatments of different drying techniques are stated in Table 2 and Figure 2. The drying process was conducted for 6 days at a room temperature of 28 - 30°C. The moisture level of the formulated M103 was analyzed daily by using a moisture analyzer (MA 160, Sartorius). The moisture pan was filled with formulated M103 (5 g) from each treatment and heated at 105°C until a constant weight was achieved.

Table 2: Different drying techniques of powder formulated M103

No.	Treatment	Drying process for 6 days
1	Without drying	Directly packed into final packaging (pail) without drying process
2	Polyethylene (PE) tarpaulin	Spread on PE tarpaulin
3	Oven air-dried	Dried using an oven (without heating)

4	Polypropylene (PP) tray	Dried using tray

Figure 2: Powder formulated M103 dried using different techniques

Shelf life and stability of the powder formulated M103

The formulated M103 from each drying technique was stored in the final packaging (pail) after 6 days of the drying process. The viability of M103 and moisture content of the powder formulation was monitored and performed at two months intervals up to 1 year of storage. The temperature observed during storage is between 23 to 33°C.

Viability of M103 measurement

The viability of M103 is the spores viable in the powder-formulated product. The viable spore of M103 was calculated using the following formula through serial dilution of the product. The results are expressed as colony forming unit per gram (CFU g^{-1}):

$$CFU g^{-1} = \frac{average\ number\ of\ colonies\ x\ volume\ of\ diluent\ (mL)}{volume\ of\ sample\ plated\ (mL)\ x\ dilution\ factor\ x\ sample\ (g)}$$

Statistical analysis

The study was conducted in a completely randomized design (CRD). The comparison on mycelium production by varying different parameter were performed using a simple analysis of variance (ANOVA) and the significant data were determined using Fisher's least significant difference *(LSD test)* at $P < 0.05$. Analyses were conducted using Minitab Statistical Software (version 18, Minitab Inc., United States).

Results and Discussions

Essential physical parameters of the fermentation process

Fermentation is an important process for the cultivation of beneficial microbes. Generally, there are two important parameters to be controlled in the fermentation process which are the growth medium (nutrient) and physical parameters. This study focuses on the impact of several physical parameters on the mycelium production of *Trichoderma asperellum* (M103). The physical parameters such as incubation temperature, initial pH of the growth medium, inoculum size, and shaker speed were studied.

Different initial pH of the growth medium was adjusted in the range of acidic to alkali while maintaining the incubation temperature at 28°C and shaker speed at 130 rpm. The total mycelium produced was harvested after 3 days of the fermentation process. pH 1 - 10 were studied and the impact on mycelium production was illustrated in Figure 3. M103 was able to be cultivated in a wide range of pH, with optimum pH of 3. This finding study shows that *Trichoderma sp.* favours acidic pH rather than alkaline pH (Singh et al., 2014; Kareem et al., 2020).

The influence of the incubation temperature of M103 was studied in the range of 23°C to 37°C. Referring to Figure 4, M103 is stable to be cultivated at temperatures of 23°C to 30°C with optimum production of mycelium at temperatures of 28°C to 30°C. However, M103 was unable to grow and not performed well at temperatures above 35°C. A similar pattern to the growth of the *Trichoderma* strains T154 and T214 which shows greater growth rate at temperature of 35°C (Carro-Huerga et al., 2021. Figure 5 shows that different inoculum size also influences mycelium production. M103 show an increase in mycelium production when inoculated with 2 to 3 % of inoculum.

Shaking speed or agitation speed also plays an important role in the fermentation process. As the study was conducted using an Erlenmeyer flask, the term shaking speed is preferable. The shaking speed is related to provide better-dissolved oxygen concentrations in the fermentation medium (Muhamad et al., 2021). According to Figure 6, the optimum shaking speed is required for increasing mycelium production. The mycelium produced will be lower if the shaking speed is too slow or too fast. For M103, the optimum shaking speed is between 130 to 180 rpm. *Trichoderma virens* UKMP-1M also produce higher biomass at 180 rpm (Hamzah et al., 2012).

According to Figure 3 to Figure 6, all the physical parameters significantly affected the mycelium production of M103. The optimum range of each physical parameter obtained from this study provides a promising reference for future optimization study using statistical approach.

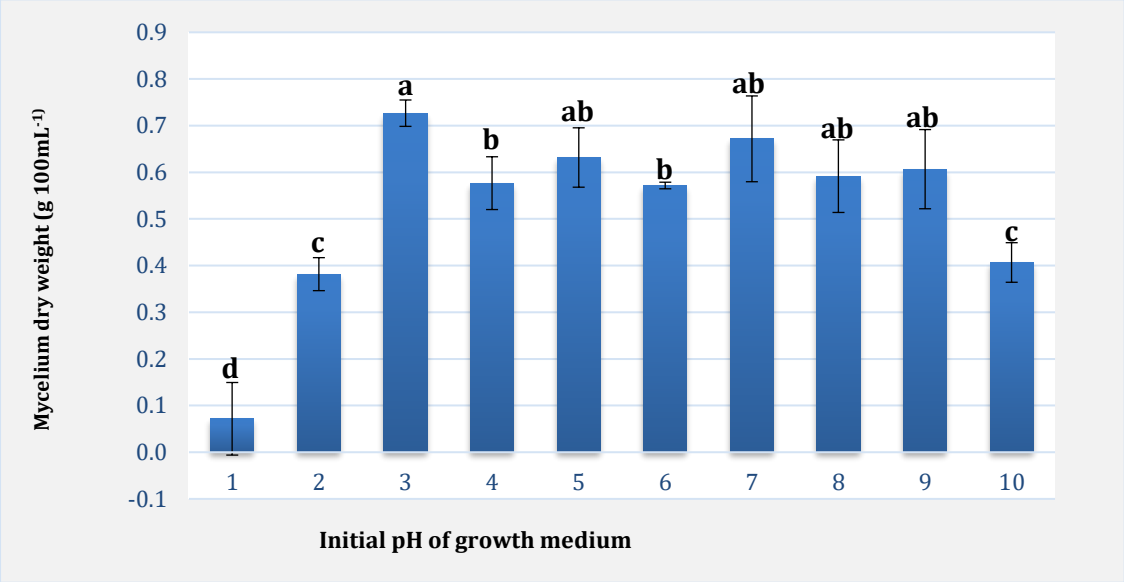


Figure 3: Effect of different initial pH of growth medium on mycelium production after 3 days incubation at 28°C and 130 rpm. Vertical bars represent the mean values ± SE. Different letter(s) indicate significant difference between treatments by Fisher LSD test at P<0.05

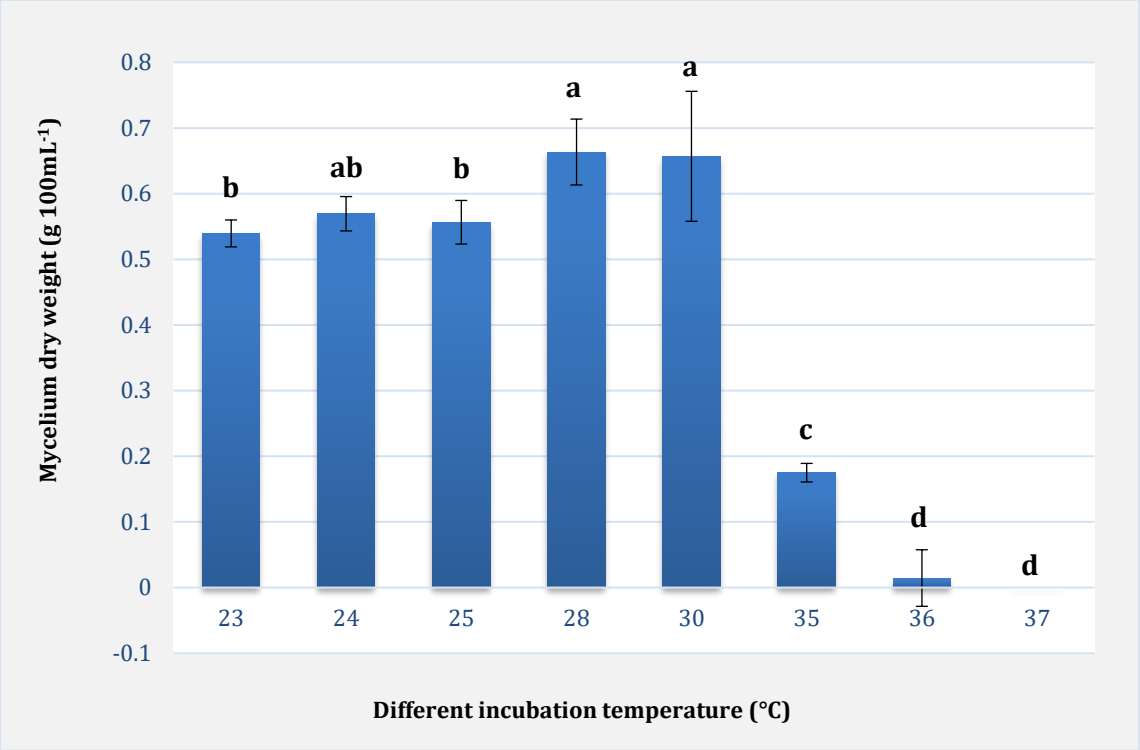


Figure 4: Effect of different incubation temperatures on mycelium production after 3 days incubation. Vertical bars represent the mean values ± SE. Different letter(s) indicate significant difference between treatments by Fisher LSD test at P<0.05

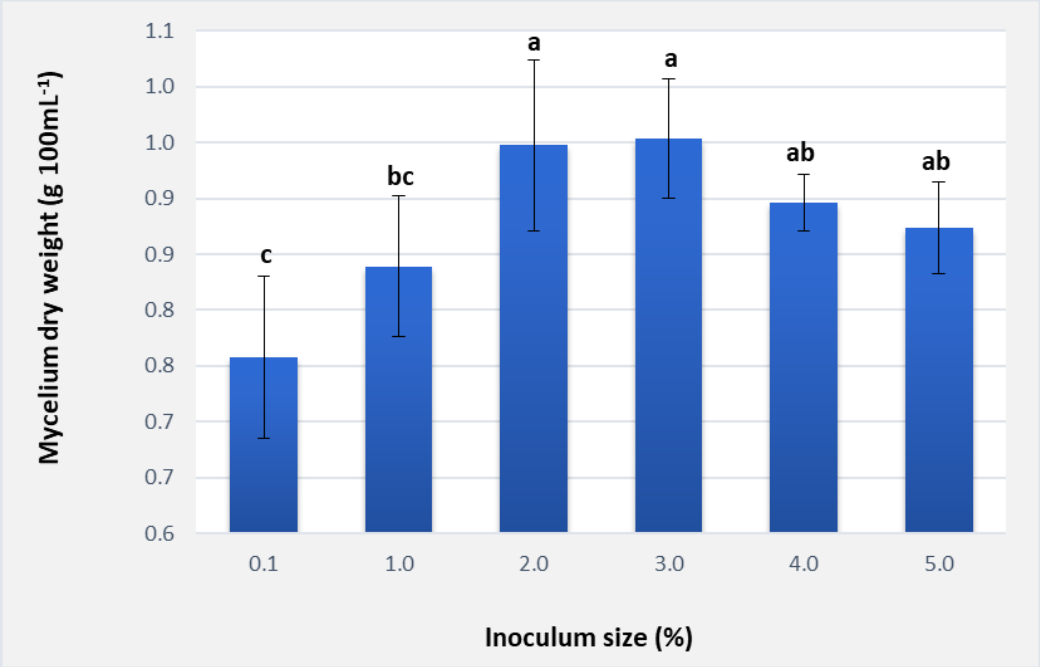


Figure 5: Effect of different inoculum sizes on mycelium production after 3 days of incubation at 28°C and 130 rpm. Values are the mean of three replicates. Vertical bars represent the mean values ± SE. Different letter(s) indicate significant difference between treatments by Fisher LSD test at P<0.05.

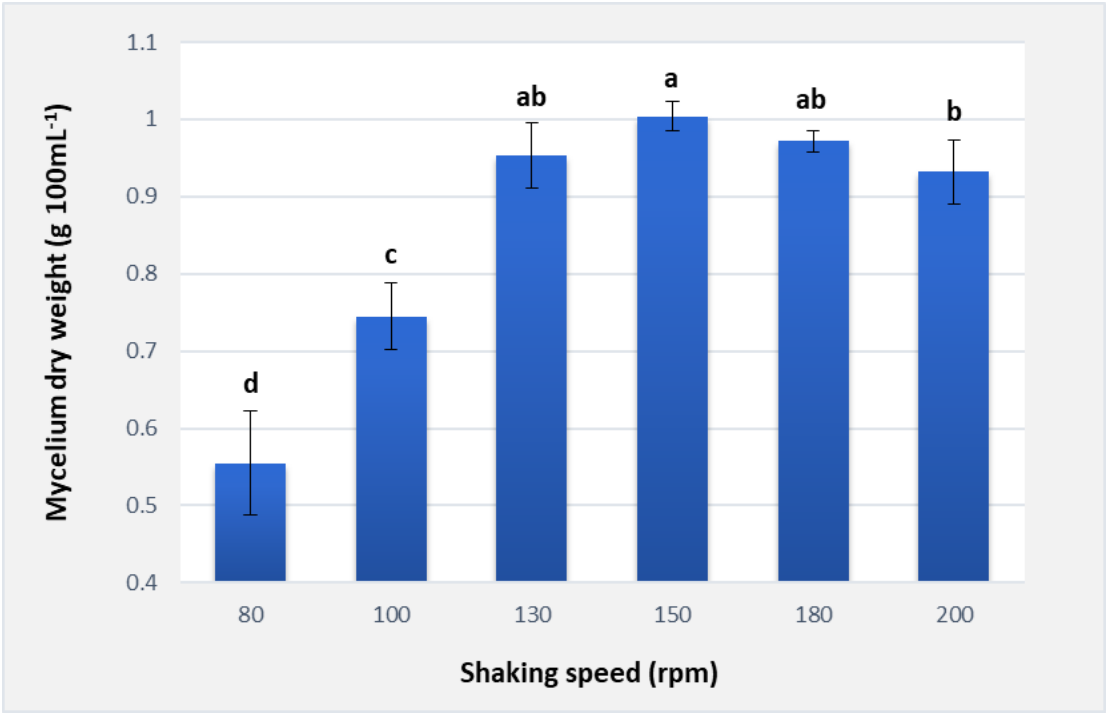


Figure 6: Effect of different shaking speed on mycelium production after 3 days incubation at 28°C. Values are mean of three replicates. Vertical bars represent the mean values ± SE. Different letter(s) indicate significant difference between treatments by Fisher LSD test at P<0.05.

Moisture content and shelf life of powder formulated M103

In developing a *Trichoderma*-based product, a lot of parameters should be considered. One of the important parameters is the suitable formulation. It is crucial to develop a suitable formulation to guarantee the delivery of viable active ingredients of *Trichoderma* to the field. In this study, M103 was formulated in powder form. At the initial stage of production, it was difficult to maintain the viability of M103 during the storage period. The reduction of M103 viability and bacteria contamination occurred between storage periods. Due to this critical issue, the improvement in the production process was conducted by the addition of the drying process. The drying process is important as dried powder formulations could prevent spoilage by microbial contamination and promote longer shelf life of the microbes (Jin and Custis, 2011; Ishak et al., 2021).

The drying process is related to the moisture content of the product. Different drying techniques require different drying periods to achieve certain moisture content. Figure 7 shows the viability of M103 and moisture content in the product dried using different techniques for 6 days. The drying process by oven air-dried techniques shows drastically decrease in moisture content from 13.36 % to 0.84% within 6 days. Without the drying process, the viability of M103 in the product only increases from 4.17 x 10^4 to 6.24 x 10^6 CFU g^{-1} product. Compared with the product that has been dried using different techniques, the viability of M103 in the product increased from 10^4 to 10^7 CFU g^{-1}. Based on this study, drying process was significantly reducing the moisture content of the product and increasing the viability of M103.

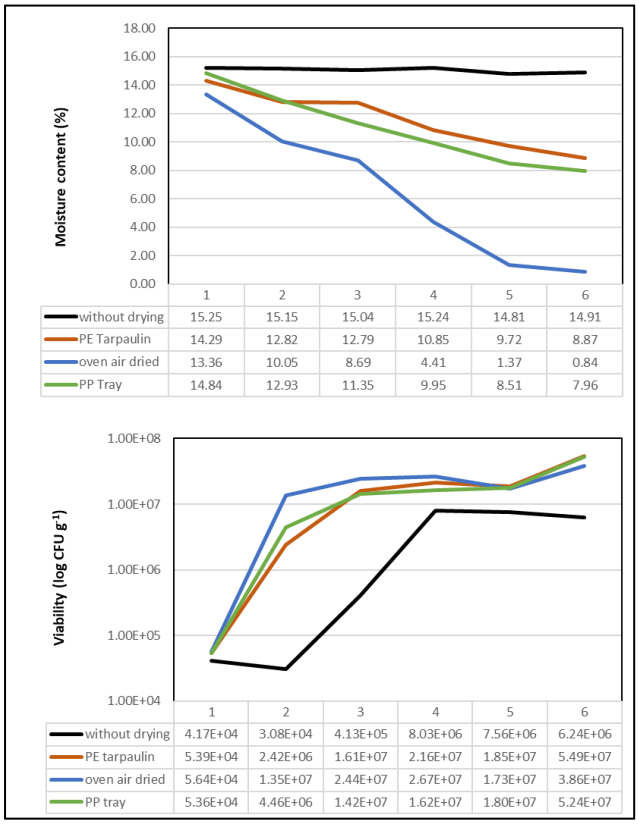
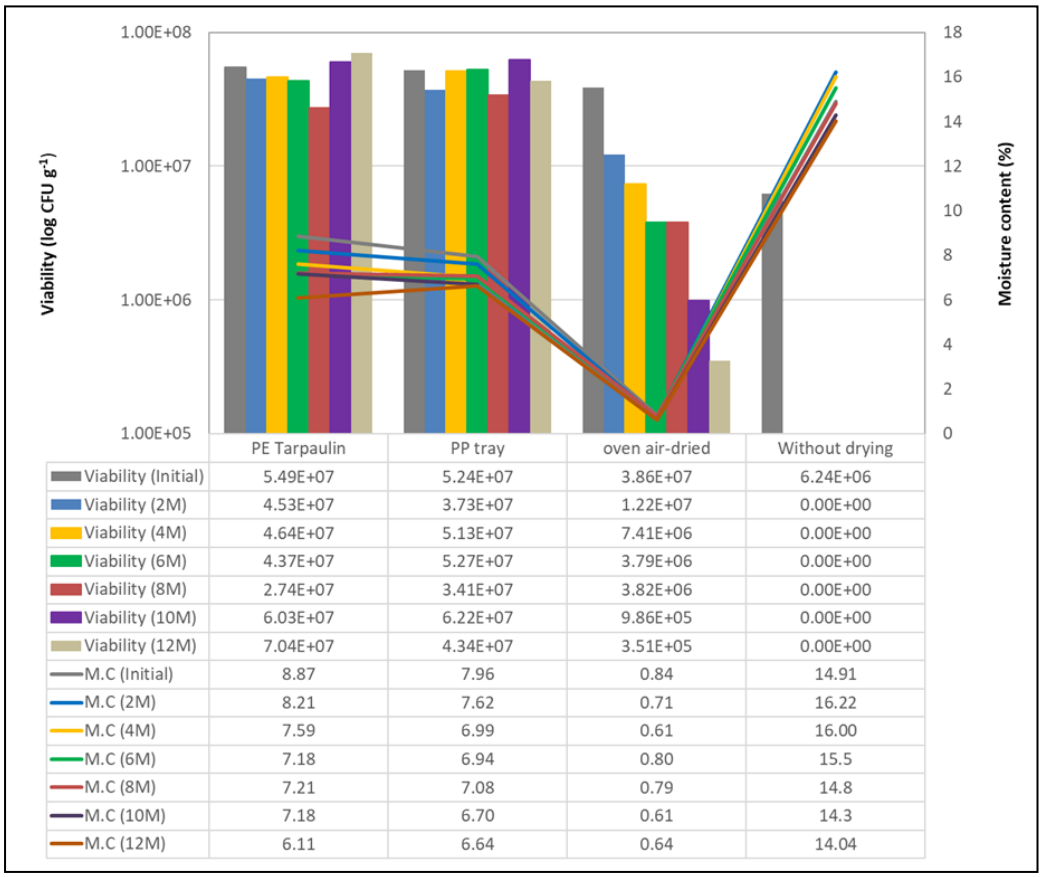


Figure 7: Moisture content and viability of M103 in powder formulated M103 using different drying techniques from Day 1 to Day 6

The dried formulated M103 from different drying techniques were packed in pail and continuously monitored for 1 year of storage. Referring to Figure 8, the product packed in pail without drying process was contaminated with bacteria within 2 months of storage. The higher moisture content cause higher risk of bacterial contamination (Rodríguez-León et al., 1999). Decreasing trends of M103 viability was observed in the product dried using oven air-dried techniques. After 12 months of storage, viability decreased to 3.51 x 10^5 CFU g^{-1}. These results indicate that moisture content that is too high (14-16%) and too low (below 1%) is not suitable for M103 formulated powder.

While for products dried using PE tarpaulin and PP tray, the viability of this product is stable and maintained at a concentration of 10^7 CFU g^{-1} for up to 12 months of storage. Hence, the initial moisture content of the powder-formulated product before packaging was found to be in the range of 6 % to 9 % to maintain the viability of M103. Similar with the previous study, the powder formulations of *Trichoderma* using talc were dried to 8% moisture to maintain their shelf life for 3 to 4 months (Kumar et al., 2014; Ramanujam et al., 2010). The oven air-dried technique can still be used during the drying process, but the drying period should be shortened according to the moisture content of the product. According to Figure 7, the product using oven air-dried technique only took 3 days to reach the acceptable moisture content range of 8.69 %. In addition to using different drying processes, all the data depicted in Figure 8 shows how important it is to monitor and control the moisture content of the powder-formulated product. Optimum moisture content is essential for the viability of M103 as a key step to becoming a good biocontrol agent.



	PE Tarpaulin	PP tray	oven air-dried	Without drying
Viability (Initial)	5.49E+07	5.24E+07	3.86E+07	6.24E+06
Viability (2M)	4.53E+07	3.73E+07	1.22E+07	0.00E+00
Viability (4M)	4.64E+07	5.13E+07	7.41E+06	0.00E+00
Viability (6M)	4.37E+07	5.27E+07	3.79E+06	0.00E+00
Viability (8M)	2.74E+07	3.41E+07	3.82E+06	0.00E+00
Viability (10M)	6.03E+07	6.22E+07	9.86E+05	0.00E+00
Viability (12M)	7.04E+07	4.34E+07	3.51E+05	0.00E+00
M.C (Initial)	8.87	7.96	0.84	14.91
M.C (2M)	8.21	7.62	0.71	16.22
M.C (4M)	7.59	6.99	0.61	16.00
M.C (6M)	7.18	6.94	0.80	15.5
M.C (8M)	7.21	7.08	0.79	14.8
M.C (10M)	7.18	6.70	0.61	14.3
M.C (12M)	6.11	6.64	0.64	14.04

Figure 8: Moisture content and viability of M103 in powder formulated M103 dried using different technique and stored for 12 months

Conclusion

This study has highlighted important parameters that need to be considered in the two main processes of *Trichoderma*-based product development. Appropriate physical parameters significantly affect the fermentation process and the development of higher mycelial biomass. However, this study was limited by using one factor at a time without interaction with all the physical parameters. The preliminary results obtained from this study will be used as a reference for future studies using a statistical approach. Whereas for product storage, moisture content becomes a critical parameter that needs to be controlled for successful and effective product development. Drying process must be implemented to control the moisture content of the powder formulated product before storage. In the coming years, various drying technologies developed will provide better opportunity of drying process and increase the productivity of *Trichoderma*-based products. The effectiveness and continuous supply of *Trichoderm*a-based products is important for sustainable agriculture, especially in the oil palm sector which also contributes to global food security.

Acknowledgements

This study was completed as part of research funded and supported by FGV Agri Services Sdn Bhd and FGV R&D Sdn Bhd.

Declaration of Interest Statement

The authors declare no conflict of interest.

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