PRODUCTION OF POWDERED SELECTED ENDOPHYTIC BACTERIA USING BIOREACTOR AND SPRAY DRYING TECHNIQUE

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ABSTRACT – *Selected **e**ndophytic bacteria strains, including Bacillus subtilis, B. pumilus, Pantoea agglomerans, which produce plant growth hormones, were studied for optimal compatibility using bioreactor and spray dry techniques to establish the most effective powdered bacterial consortium as a biofertilizer in cocoa plants. The selected endophytic bacteria were grown separately in nutrient broth, exhibiting the greatest growth activity at the end of exponential phase, 24 hours into the cultivation period using a bioreactor (Techfors, Infors, Switzerland–30L). The bioreactor was run with 30^{0}C temperature, stirring speed of 150 rpm and aeration at 0.5 vvm respectively. The cultures were mixed into 10% (w/v) sterile skim milk as carrier and were spray-dried at constant inlet temperature of 120 ± 2^{o}C using GEA MOBILE MINOR spray dryer. In conclusion, results obtained from this study has reveal that the selected endophytic bacteria possess the greatest production of powdered product using bioreactor, (Techfors, Infors, Switzerland–30L) fermenter and drying technique using GEA MOBILE MINOR spray dryer with specific parameters*

***Keywords**:* Endophytes, bacteria, bioreactor, spray dry and powder product.

INTRODUCTION

The use of endophytic bacteria as bio-fertilizers has gained significant attention in recent years due to their ability to promote plant growth and reduce the dependence on chemical fertilizers and pesticides (Vale *et al*., 2010). Endophytic bacteria are capable of colonized the internal structures of plant hosts without causing any negative effects or diseases (Khan *et al*., 2014). Instead, they establish a mutually beneficial symbiotic relationship with the plant. Endophytic bacteria produce bioactive substances, including plant growth hormones, which stimulate plant growth and development. This interaction between the plant and endophyte is often advantageous for both parties. The plant provides protection and nutrients to the endophyte, while the endophyte, in return, enhances plant growth through the production of growth-promoting bacteria produce bioactive substances, including plant growth hormones, which stimulate plant growth and development.

In most cases, effective microbial inoculants consist of a single strain. However, the present research focus has turned to the formation of microbial consortiums that they would perform better than single strains (Woo and Pepe, 2018). Whereas a single application may be successful, combination inoculants have the potential to respond to a wider range of environmental circumstances and have a number, of modes of action (Sarma *et al*., 2015). However, significant questions remain unanswered, such as whether single strains or multi-strain combinations are better, and whether strains in a mixture are compatible. The endophytic bacterial consortiums were chosen based on their capacity to activate plant growth hormones individually. The microorganisms were mixed together, in the hopes that the consortium would be more effective in promoting plant growth, despite the fact that antagonistic interactions between the microbes in the combination could reduce the predicted results (Sarma *et al*., 2015).

The industrial process and technology for efficient scale-up production of plant endophytes in bioreactors is currently underdeveloped. There is often a lack of consideration for the specific requirements of endophytes due to their intracellular lifestyles and growth within living, asymptomatic plant tissue (Reinhold-Hurek and Hurek, 2011). While there is a focus on scale-up challenges and optimization in the production process, both upstream and downstream elements need to be considered. This includes addressing the biological

aspects of endophytes, as their cellular properties may fluctuate during the adaptation process to the new fermentation environment, despite the implementation of control systems (Crater and Lievense, 2018).

In addition to bioprocess design considerations, it is important to assess and mitigate the microbial endophyte's growth requirements and adaptive shifts in thermo-dynamic, physico-chemical, and molecular traits. The endophyte must be able to grow outside of the plant host cells and adapt to the conditions inside the bioreactor during each step of scale-up fermentation (Ganeshan *et al*., 2021). Overall, the development of bioreactor design phases for efficient scale-up production of plant endophytes requires a comprehensive understanding of the biological nature of these organisms and their specific growth requirements. Both the technical aspects of bioprocess design and the biological considerations for endophyte adaptation and growth need to be looked-into account to optimize the production process effectively. Spray drying technique is a process that can help protect bacteria strains against harsh environmental conditions, generating a convenient storage and transportation of powder product. However, to produce powder product using spray dry technique, has different process parameter that need to be considered for example drying temperature, carrier material, drying time, flow configuration, strain type and storage condition (Zuidam & Shimoni, 2010).

Therefore, the present study will address research findings on the evolution of selected endophytic bacterial characteristic individually, and to observe the parameter and time growth of selected endophytic bacteria using Bioreactor (Techfors, Infors, Switzerland–30L). The cultures were mixed into 10% (w/v) sterile skim milk as carrier and will go through Gea Mobile Minor Spray Dryer to perform powder products. The experiments, will provide useful information for the development of successful endophytic bacterial plant growth hormones consortia powdered form for long-term usage of cocoa plantation or others agricultural applications in future.

MATERIALS AND METHODS

Bacterial strains and culture medium.

The endophytic bacterial strains *Bacillus subtilis, B. pumilus, Pantoea agglomerans,* which produce plant growth hormones and best compatibility combination study, were previously isolated from tissues of healthy *Theobroma cacao* L. These strains were sourced from the Microbiology Laboratory of Biotechnology Division, Malaysian Cocoa Board. Stock cultures of each strain were preserved at -80°C in nutrient broth containing 15% glycerol (Shin et al., 2007). Working cultures were initiated by transferring from the stock cultures onto nutrient agar (NA) in Petri dishes and then incubating them for 24 hours at 28°C.

Growth of Selected Endophytic Bacterial using Bioreactor

Overnight, individually selected endophytic bacteria were cultured in 250 mL of nutrient broth (NB), then transferred to 2500 mL Erlenmeyer flasks. Subsequently, this culture was used to inoculate a 25 L bioreactor (10% volume) (Techfors, Infors, Switzerland – 30L), which had been sterilized at 121°C for 15 minutes and cooled to 37°C. During the fermentation process, several parameters were closely monitored for their significance, including pH, aeration, and antifoam levels. The fermentation proceeded in batch mode, with dissolved oxygen levels maintained at the required saturation (0.5 vvm) using filtered air. Stirring speed was set at 150 rpm in cascade mode to achieve and sustain a constant pO2 level. Foam production was controlled with a 5% antifoam solution (Sigma Aldrich, Germany), while the cultivation temperature remained constant at 37°C. Throughout the fermentation period, the pH of the medium was kept at 6.8 using a standard pH electrode (Mettler Toledo, USA), with adjustments made through the addition of phosphoric acid and liquid ammonia. pH was continuously monitored using the pH sensor unit. Sampling of optimum growth cultures were made at 24h fermentation, with analysis focusing on optical density at 550 nm.

Preparation of Cultures for Spray Drying

Selected endophytic bacteria from Bioreactor that reach optimum growth of stationary phase (24h) were harvested by centrifugation at 10 000 g for 10 min at 4ºC. Then the harvested cell pellets were mixed into 1000 ml of 10% (w/v) sterile skim milk as carrier which gave an initial number of approximately 10^9 CFU/ml. The sample was spray-dried at a constant air inlet temperature of 120 ± 2ºC using GEA MOBILE MINOR spray dryer. The flow rate of the feed was maintained at 75 ± 2ºC, to achieve the desired air outlet temperature. Spray dried powders were collected and stored in tight sealed container. The experiments were repeated at least three times. Throughout the fermentation process, samples of the culture were collected at intervals of 4, 8, 12, 16, 20, and 24 hours and analyzed for optical density at 550 nm. Viable counts were plated on NA and were converted to log CFU/ml and percentages were calculated.

Observation of particle size of spray drying products.
Visual observation was investigated by the particle size of spray-drying powdered using Scanning Electron Microscope and Energy Dispersive X-ray by sending to Quasi-S Technology Sdn Bhd Malaysia at vary magnification modes.

Statistical Analysis
All data results obtained were analyzed using SPSS versión 13.0 Windows program (SPSS Inc., Chicago, IL) using one-way variance analysis (ANOVA). Duncan tests were performed to find out the significant differences for each data obtained at $p < 0.05$.

RESULTS AND DISCUSSIONS

Batch fermentations were conducted in a 30 L Bioreactor (Techfors; Infors, Switzerland) to investigate the impact of individual selected endophytic bacteria on cell growth in NA medium against time. Biomass production was monitored throughout the cultivation period by measuring OD550nm. It was observed during cultivation that as the bacterial cultures grew, pO2 saturation decreased due to increased oxygen uptake by the culture. To maintain a higher pO2 concentration, the stirrer speed was regulated in cascade mode, while continuous aeration with filtered air was employed. Any fluctuations in pO2 levels, combined with pH changes, prompted intervention from the pO2 controller.

Figure 1 illustrates the impact of the cultivation period on the selected endophyte isolate. Analysis of the supernatants from the selected endophyte isolate revealed a noticeable increase in growth over time. The maximum growth of the isolate was observed at 24 hours of cultivation, with subsequent incubation periods (at 28 and 48 hours) showing a gradual decrease. The relationship between growth rates over time and changes in biomass is evident, particularly during the exponential growth phase, which occurred prominently between 12 and 24 hours.

Generally, the exponential phase of growth for the four endophyte isolates commenced after 4 hours of incubation and concluded before the 24-hour mark. During this phase, bacterial cells actively reproduced via binary fission (Llorens et al., 2010). Following the 24-hour incubation period, all four endophytes entered their stationary growth phase, where the rates of bacterial cell growth and death reached equilibrium. Subsequently, the death rate surpassed the growth rate at 28 hours post-incubation. This could be attributed to various factors such as limited nutrients in the culture medium, the production of secondary metabolites as defense mechanisms against changes in culture conditions (Muhsinin *et al.*, 2016), and the accumulation of waste products.

The experiment demonstrates that individually selected endophytic bacteria exhibit high cell-free growth activity towards the end of the exponential phase, ultimately entering the stationary phase after 24 hours of fermentation in the bioreactor.

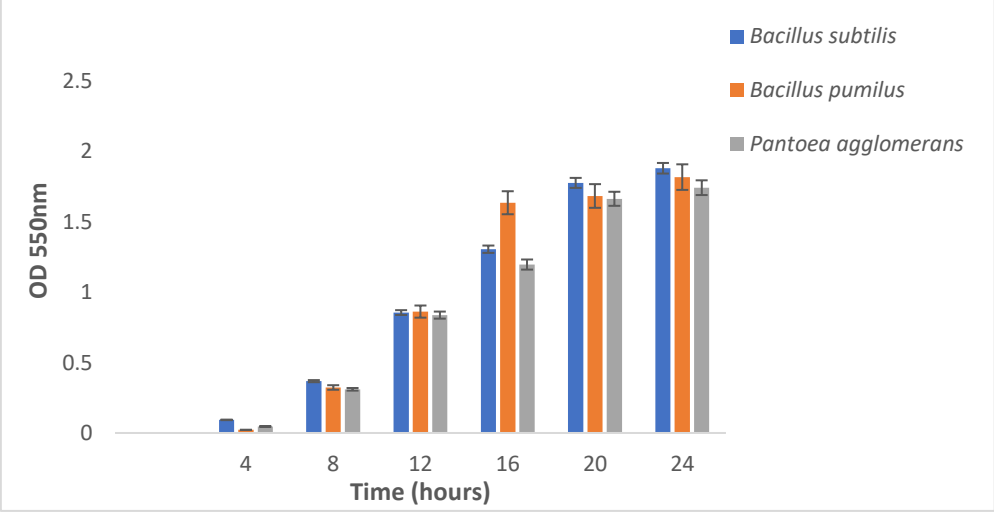

Figure 1. The growth of selected endophytic bacteria of optical density against time (hours).

Observation through SEM (Scanning Electron Microscopy) is aimed at examining the physical properties and particle size of the powder product in more detail. The physical properties of the resulting powder are in the form of complete spheres and spheres that shrink in the middle (Figure 2). It can be observed that there are still large, and complete spherical particles, which remain separate when drying is performed at a low outlet temperature of 65°C. In contrast, the use of high temperatures such as 85°C produces small, shrunken spherical powder particles that tend to stick together. The particle size of the powdered product isolated from the endophytic bacteria B. subtilis LKM-BL varies and is uneven, ranging from 2 µm to 12.5 µm (Figure 3).

As observed in this study, the shrink effect has also been reported in previous research. Wang & Mutukumira (2022) encapsulated *L. reuteri* DPC16 in 10% reconstituted skim milk and 10% Gum arabic, resulting in squashed and wrinkled spherical capsules. Lucas *et al*. (2020) conducted experiments encapsulating curcumin with 1% Gum Arabic, and also observed spherical capsules with concavities and surface deflations at low concentrations of gum arabic. This effect is directly related to the conditions during spray drying, particularly heat penetration and water evaporation from the liquid droplet (Barbosa-Canovas *et al*., 2005). Once the particles come into contact, with the hot air, the droplet surface remains dry, forming a layer around the particle that hinders internal water evaporation (Wei *et al*., 2019). The extreme mechanical resistance of this shell can cause the droplet to undergo rapid inflation by boiling, leading it to either grow or shrink depending on the airflow or encapsulating materials (Moreira et al., 2021). Fang *et al*. (2012) demonstrated that intermediate inlet temperatures are associated with partially collapsed matrices and good mechanical strength and dissolution properties of the resultant powder.

SEM observation by Thummar and Ramani (2016) on Lactobacillus fermentum MTCC 8711 powder showed similar images to those obtained from the study on the endophytic bacteria isolate B. subtilis LKM-BL. The product particles appeared concave spherical when subjected to the spray drying process using 11% skim milk as a carrier agent. A similar effect was observed due to spray drying on the Bifidobacterium PL1 isolate using starch as a carrier agent (O'Riordan *et al*. 2002). According to Pedroza-Islas *et al*. (1999), such shapes occur due to the rapid drying and cooling process during spray drying.

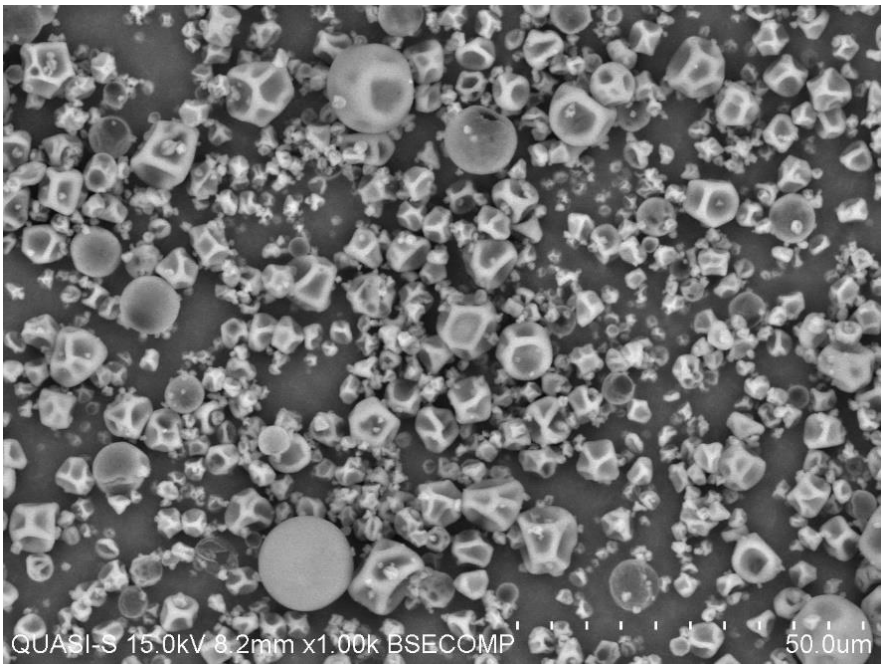

Figure 2. Observation using SEM of the powder product particles isolated from the endophytic bacteria (Magnification 1000X).

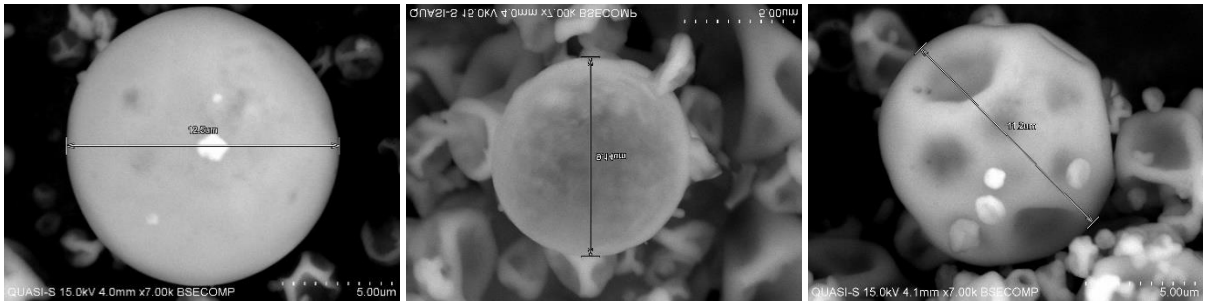

Figure 3. Differential of particles size of powder product after spray dry (Left: 12.5 um, middle: 9.14um, and right: 11.2um)

CONCLUSIONS

Creating an effective consortium formulation necessitates combining selected endophytic bacterial strains in a manner where they can grow in optimal biomass. These experiments offer valuable insights for crafting successful endophytic bacterial consortia from bioreactor to drying technique using spray dry to perform powdered product. The cultures should be able to withstand the harsh conditions of the high heat involved in the spray drying process, with the help of skim milk as protective medium. The results reveal that the selected endophytic bacterial possess the greatest production of powdered product using bioreactor along with spray drying technique for the and aimed at enhancing the management of cocoa plantation fertilizer.

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