PRELIMINARY STUDY OF THE CHARACTERISTICS AND GROWTH PATTERNS OF SELECTED ENDOPHYTIC BACTERIA USING BIOREACTOR FOR THE CONSORTIUM PRODUCTION OF BIOFERTILIZER

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ABSTRACT – *Selected **e**ndophytic bacteria characteristic and growth studies using bioreactor were conducted to establish the most effective endophytic bacterial consortium to produce powder plant growth hormone as a biofertilizer for cocoa plants. Four selected endophytic bacterial isolates with potential to be developed as biofertilizer, that has been isolated within the tissues of healthy Theobroma cacao L. plants were identified as Bacillus amyloliquefaciens, B. pumilus, B. subtilis, and Pantoea agglomerans based on previous 16S ribosomal DNA sequences. The strains already tested for their mutual compatibility are to be developed as mixtures of endophytic bacterial consortia biofertilizer. In conclusion, results obtained from this study has reveal that the selected endophytic bacteria possess the greatest growth activity at the end of exponential phase at 24 hours of cultivation period in a culture medium individually using a bioreactor.*

***Key words**:* Endophytes, Bacteria, Characteristic, Bioreactor, and Consortium.

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 togethers in the hopes that the consortium would be more effective in encouraging plant growth, despite the facts 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.

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) in order to provide useful information for the development of successful endophytic bacterial plant growth hormones consortia for long-term cocoa plantation or others agricultural applications in future.

MATERIALS AND METHODS

Bacterial strains and culture medium.

The cultures of four endophytic bacterial strains producing plant growth hormones have been isolated previously within tissues of healthy *Theobroma cacao L.* were obtained from the Microbiology Laboratory of Biotechnology Division, Malaysian Cocoa Board. Stock culture of each strain were maintained at -80ºC in nutrient broth with 15% glycerol (Shin *et al*., 2007). The working cultures were established by transferring from stock cultures onto nutrient agar (NA) in Petri dishes and incubated for 24 h at 28ºC.

In Vitro Characterization of Plant Growth Promoting Traits of selected endophytes

Detection of Indole acetic acid (IAA)

IAA was detected according to the method of Wang et al, (2020). Bacterial isolates were grown in NB supplemented with tryptophan (500 mg/L) and incubated at 28ºC for 24, 48, and 72 h on rotary shaker. Cultures were centrifuged at 12,000 g for 10 min. A 1 ml volume of supernatant and an equal volume of Salkowski reagent (50ml of 35% Perchloric acid, 1ml of 0.5 M $FeCI_3$ solution) was added and optical density of 535 nm (OD535) of solution was measured after 30 min of reaction in dark. Development of pink color indicated IAA production. The different concentration of IAA (0, 20, 40, 60, 80, 100, 120, 140, and 180 mg/L) was prepared. OD535 of each concentration of IAA solution was determined and the standard curve was drawn. Then the IAA content of bacterial culture medium was calculated based on standard curve.

Detection of Catalase activity

A drop of 24 h-old bacterial colony was placed on a clean glass slide and 3% hydrogen peroxide was added before mixing by using a sterile toothpick. Effervescence indicated catalase activity.

Detection of Ammonia production

All the bacterial isolates were tested for ammonia production as described by Cappuccino and Sherman (1992). Overnight bacterial cultures were inoculated in 10 mL peptone broth and incubated at 30 ± 0.1 °C for 48 h in a refrigerated incubator shaker. After the incubation period, 0.5 mL of Nessler's reagent was added and development of faint yellow to dark brown colour was observed and recorded as an indicator of ammonia production.

Detection of Protease activity

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. digest of casein 5.0, Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30 C and zone of clearance around the colony indicating the enzymatic degradation of protease (Chaiharn *et al*., 2008).

Growth Pattern of Selected Endophytic Bacterial using Bioreactor

Overnight selected endophytic bacterial individually was prepared in 250 mL NB, further transferred, in 2500 mL Erlenmeyer flask. The culture was later used to inoculate into a 25 L bioreactor (10%), (Techfors, Infors, Switzerland–30L) fermenter previously sterilized at 121 °C for 15 min and cooled to 37 °C, respectively. During fermentation process some parameters were observed due to their importance; the change in pH, aeration, and antifoam. The fermentation was run in batch mode with dissolved oxygen level maintained at required saturation (0.5 vvm) by using filtered air and with stirring speed 150 rpm in cascade mode in order to achieve and keep pO2 level constant. The production of foam was hindered by using 5 % antifoam solution (Sigma Aldrich, Germany) and the cultivation temperature was set at 37 °C. During fermentation, the pH of the media was maintained at 6.8 using a standard pH electrode (Mettler Toledo, USA) by the addition of phosphoric acid and liquid ammonia and monitored using the pH sensor unit. During fermentation, sampling of the culture (4,8,12,16, 20, 24, 28, 32, 36, 40, 44, and 48 h) in the medium was performed and analysed for optical density at 550 nm.

RESULTS AND DISCUSSIONS

Characterization of selected microbial was done for plant growth hormone activities such as indole-3-acetic acid (IAA), ammonia production, catalase, and protease production. All the bacterial isolates showed producing of plant growth hormone (Table 1). After 24 hours, *Pantoea agglomerans* produced high IAA (0.323) followed by *Bacillus amyloliquefaciens* with 0.062, *B. subtilis* with 0.052, *B. pumilus* with no IAA activity detected. After 48 hours, *Pantoea agglomerans* showed highest production of IAA with with 0.971, followed by *B. pumilus* and lastly *Bacillus amyloliquefaciens* with 0.350. After 72 hours, *B. subtilis* produced higher IAA with optical density of 0.553 and *B. pumilus* showed the lowest IAA production with 0.087. Plant growth hormone, IAA also known as auxin is one of the most common phytohormones responsible for plants' growth, cell division, and tissue differentiation (Singh *et al.*, 2022). Therefore, selected endophytic bacterial showing plant growth hormone activities and can be used as plant inoculants to enhance the growth of plants.

Table 1. Plant growth hormone activities of selected endophytic bacteria on indole-3-acetic acid (IAA) at differences time (Optical density at 535 nm)

Selected endophytic bacterial	24 hours	48 hours	72 hours
Bacillus amyloliquefaciens	0.062	0.350	0.495
Pantoea agglomerans	0.323	0.971	0.532
B. subtilis	0.052	0.688	0.553
B. pumilus	-	0.807	0.087

Bacterial strains with catalase activity are highly resistant to environmental, mechanical, and chemical stresses (Kumar *et al*., 2012). Based on Table 2, that showed no selected endophytic isolates showed positive reaction on catalase, but all bacterial isolates showed positive reaction on ammonia production and three bacterial isolates showed positive reaction on protease production. The production of ammonia is useful for plant as directly or indirectly, helps influence plant growth (Geetha *et al*., 2014). Production of hydrolytic enzyme such as protease is recognized as the prominent functional traits for indirect plant growth promotion, and this enzyme essential during the colonization and migration of endophytes through the degradation of cell walls (Mishra *et al.*, 2020). Although beneficial bacteria spread and enter the inner plant tissue using the same entry mechanisms

as the bacterial pathogens, the host develops strategies to allow several bacterial genera.

Table 2. Detection of catalase, ammonia, and protease production selected endophytic bacteria.

Bacterial Isolates	Catalase	Ammonia	Protease
Bacillus amyloliquefaciens	×	√	×
Pantoea agglomerans	×	√	√
B. subtilis	×	√	√
B. pumilus	×	√	√

x- negative activities and √- positive activities

Batch fermentations in 30 L Bioreactor (Techfors; Infors, Switzerland) (Figure 1) was performed to examine the effects on cell growth of selected endophytic bacteria individually in NA medium against time. Bio-mass production was monitored during cultivation at OD550nm. During culturation, it was also observed that as the bacterial cultures grows pO2 saturation decreases; this was due to the increase in O2 uptake by the culture. To maintain higher pO2 concentration, the stirrer speed was maintained in cascade mode, with the continuously aeration using filter air. Any change in pO2 level combined with a pH change triggers the pO2 controller.

Figure 1. The growth of selected endophytic bacteria using 30 L Bioreactor (Techfors; Infors, Switzerland).

The effect of cultivation period of the selected endophyte isolate is illustrated in Figure 2. Selected endophyte isolate supernatants showed that the growth was relatively increased against time. The maximal growth of isolate was recorded at 24 h of cultivation, and a further incubation period (28 and 48 h) showed a gradually decreased. The relationship between growth rates in time and

change in biomass can easily be noticed as the exponential growth phase between 12 and 24 hours is the most remarkable.

In general, exponential phase of the four endophytes isolate started at the 4 hours incubation and ended before 24 hours incubation period. At this phase, bacterial cells begin to actively reproduce by binary fission (Llorens *et al.*, 2010). After 24 h incubation period, all the four endophytes were found to have entered their stationary phase of growth where the bacterial cell growth and death rates have reached an equilibrium. Eventually, the death rate exceeds the growth rate at 28 h after the incubation period. This might have due to limited nutrients in the culture medium, production of secondary metabolites as a defence against changes of culture conditions (Muhsinin *et al.*, 2016), and accumulation of waste products. The experiment showed that selected endophytic bacteria individually produced high cell-free growth activity during the end of exponential phase, and entering stationary phase at 24 hours fermentation using bioreactor.

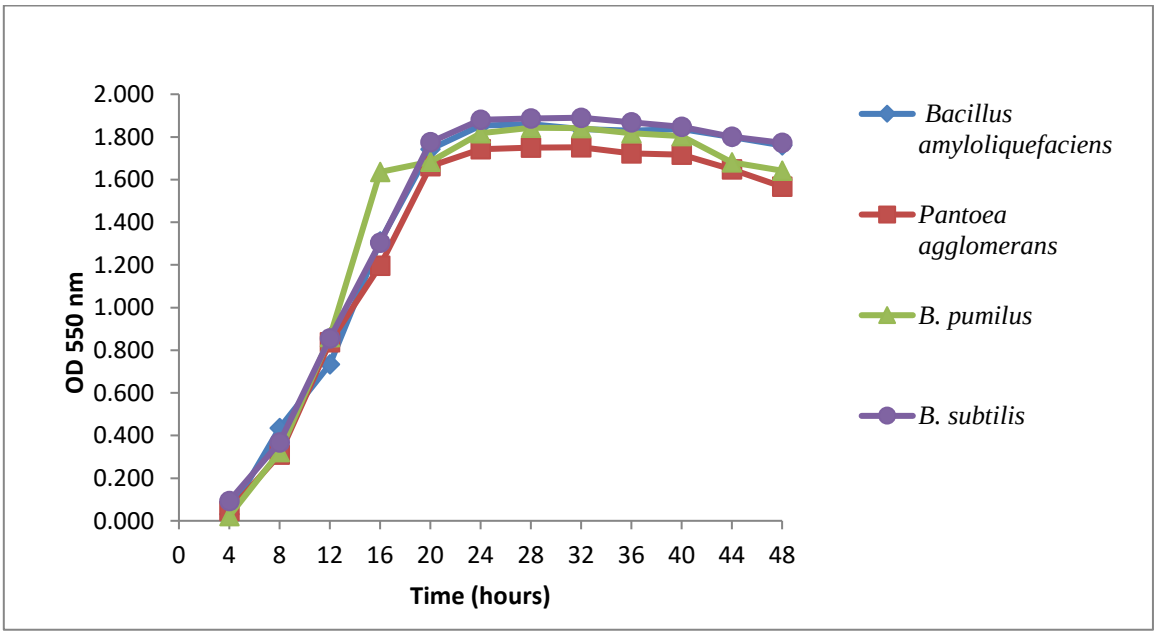

Figure 2. The growth of selected endophytic bacteria of cell-free supernatant against times.

CONCLUSIONS

The characteristics of selected endophytic bacterial species individually assayed showed multiple abilities to have plant growth hormone of IAA, ammonia, and protease activities. The results also obtained from this study has reveal that the selected endophytic bacteria individually possess the greatest growth activity at the end of the exponential phase after 24 hours of bioreactor cultivation period in a culture medium. The isolates can be further formulated into biofertilizer consortium. The preparation of a successful consortium formulation requires the mixture of endophytic bacterial strains in the consortium to grow in the presence of each other without incompatibility or antagonistic activities. The experiments may provide useful information for the development of successful endophytic bacterial consortia for the management of cocoa plantation fertilizer.

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