การผลิตกล้าเชื้อด้วยเทคนิคอินฟิวชั่นเพื่อใช้เป็นต้นแบบในการผลิตหัวเชื้อแบรดีไรโซเบียมในระดับกึ่งอุตสาหกรรม

Infusion Starter Culture-Based as a Model for Pilot Scale of Bradyrhizobial Inoculant Production

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บทคัดย่อ

แบรดีไรโซเบียมเป็นแบคทีเรียที่มีประโยชน์ต่อพืชตระกูลถั่วเมื่ออาศัยอยู่ร่วมกันภายใต้สภาวะพึ่งพาซึ่งกันและกัน โดยอาศัยกระบวนการตรึงไนโตรเจนของเชื้อแบรดีไรโซเบียมที่เป็นที่รู้จักกันโดยทั่วไป สำหรับงานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาเทคนิคอย่างง่ายที่สามารถใช้กล้าเชื้อในปริมาณน้อยเพื่อการผลิตหัวเชื้อแบรดีไรโซเบียมที่มีคุณภาพสูงในรูปแบบต่าง ๆ โดยในงานวิจัยนี้เริ่มต้นจากการเตรียมกล้าเชื้อ *Bradyrhizobium diazoefficiens* USDA110 โดยการเจือจางเชื้อด้วยวิธีทำมือเพื่อให้ได้เชื้อที่ความเข้มข้น 10 ถึง 10^6 โคโลนีต่อหน่วย ก่อนนำไปปลูกเชื้อเพื่อผลิตหัวเชื้อในรูปแบบต่าง ๆ ผลการทดลองพบว่า ระดับการเจือจางเชื้อที่มีจำนวนเซลล์ต่ำที่สุดเมื่อนำไปใช้เป็นกล้าเชื้อ ยังคงตรวจพบการเจริญของเชื้อได้ต่อไป โดยเมื่อใช้กล้าเชื้อจำนวน 10 โคโลนีต่อหน่วยสำหรับการปลูกเชื้อพบว่าเชื้อสามารถเจริญต่อได้มากกว่า 10^8 โคโลนีต่อหน่วย เมื่อบ่มเชื้อที่ระยะเวลา 14, 21 และ 14 วัน ในหัวเชื้อรูปแบบเหลว รูปแบบผสมในวัสดุพาหะ (พีท) และรูปแบบผสมเม็ดวุ้น ตามลำดับ ดังนั้นจำนวนเชื้อที่ความเข้มข้น 10 โคโลนีต่อหน่วย จึงนำมาใช้ในการทดสอบการผลิตหัวเชื้อด้วยเทคนิคอินฟิวชั่น โดยนำ syringe pump ต่อเข้ากับ peristaltic pump เพื่อทำให้ได้กล้าเชื้อจำนวน 10 เซลล์ต่อหน่วย ก่อนฉีดเข้าไปเพื่อผลิตหัวเชื้อรูปแบบต่าง ๆ แบบกึ่งอัตโนมัติ เพื่อเป็นต้นแบบในการผลิตหัวเชื้อในระดับกึ่งอุตสาหกรรม โดยผลการทดลองพบว่า หัวเชื้อ แบรดีไรโซเบียมทุกรูปแบบที่ผลิตด้วยเทคนิคนี้ มีจำนวนเชื้อเพิ่มขึ้นและมีอายุการเก็บรักษาได้นานอย่างน้อย 3 เดือน ดังนั้นงานวิจัยนี้จึงเป็นงานแรกที่แสดงให้เห็นว่า การใช้กล้าเชื้อที่เจือจางในระดับที่มีจำนวนเซลล์น้อย สามารถใช้ในการผลิตหัวเชื้อได้ จึงทำให้ไม่จำเป็นต้องเตรียมกล้าเชื้อในปริมาณมากในการผลิต ดังนั้นกระบวนการนี้จึงสามารถนำไปประยุกต์ใช้กับการผลิตหัวเชื้อแบรดีไรโซเบียมที่มีคุณภาพสูง-ต้นทุนต่ำ ในระดับกึ่งอุตสาหกรรมได้ต่อไป

ABSTRACT

Bradyrhizobium is bacteria that benefits to legume plant under symbiosis condition. This process is widely recognized as biological nitrogen fixation. The objective of this study was to develop a simple technique with a low number of starter cell culture preparation for producing various types of high-quality bradyrhizobial inoculant. In this study, the starter culture of Bradyrhizobium diazoefficiens USDA110 was manually prepared to obtain the final cell concentrations starting from 10 to 106 CFU/unit. The lowest amount of cell that could be able to grow and increase the cell number in different types of inoculant was observed. It was found that the number of cells starting at the concentration of 10 CFU/unit could be able to grow and increase to more than 108 CFU/unit at 14, 21, and 14 days after inoculation (dai) in liquid-, peat-, and encapsulation-inoculants, respectively. Therefore, final cell suspension at the concentration of 10 CFU/unit was used in this study for inoculant production. The syringe- and peristaltic-pumps were incorporated with a model for pilot scale to create the micro-injection of starter culture at 10 cells/unit on different inoculant types. The results showed that all types of inoculants produced by this technique could maintain the shelf-life of inoculants for at least 3 months. This is the first demonstration of the diluent with a very low amount of starter culture. Thus, this process could be applied further for high-quality bradyrhizobial inoculant on large scale with low cost production.

คำสำคัญ: แบรดีไรโซเบียม หัวเชื้อ กล้าเชื้อที่เตรียมด้วยเทคนิคอินฟิวชั่น ระดับกึ่งอุตสาหกรรม

Keywords: Bradyrhizobium, Inoculants, Infusion starter, Pilot scale

INTRODUCTION

Bradyrhizobia are involved in the symbiotic with legumes by the formation and colonization of root nodules. The bacteria produced nitrogenase enzyme for fixing nitrogen to ammonium located nodules and make it available N for the plant. The process called biological N_2-fixation (Dart et al., 1969; González-López et al., 2005). Therefore, Bradyrhizobium is one of biofertilizers that able to fix nitrogen higher than 200 kg/hectares (Denton et al., 2017). In addition, bradyrhizobial inoculant was low cost and can reduce the chemical fertilizer utilization. Further, biofertilizer can help to conserve soil, community of microorganisms, and support sustainable agriculture.

Conventional industrial rhizobium inoculant production required a large amount of moneys due to, many types equipment are required to set up in the process as well as many skills full manpower are also needed to control several steps of inoculant production. For commercial scale, a large capacity for medium sterilization, a unit for starter preparation, an aerating system, as well as the requirement of fermenter are the main operating units that create the cost of production (Somasegaran and Hoben, 2012). However, fermenter is expensive and it is not easy to operate. A high risk of contamination could be occurred with the non-experienced producer. This situation is difficult for new investors to produce inoculant. Thus, an appropriate technology for rhizobial inoculant production is required to produce high quality rhizobial inoculants although in the pilot scale.

In this study, the principle of dilution technique was applied to develop a new production system to reduce the cost of production. The dilution technique was used to dilute high concentration of starter culture for injection into carrier. Thus, production could be operated without using a large fermenter. The dilution of liquid rhizobium starter at 100-fold to liquid

inoculant and 1,000-fold for peat inoculant could increase the production capacity of legume inoculant (Somasegaran and Hoben, 2012; Somasegaran, 1985; Tittabutr et al., 2007). Moreover, it has been reported that the starter culture at initial concentration of 10^6 CFU/g could be used to produce immobilized bead inoculant and later the cells number was increased up to 10^9 CFU/g (Bashan, 1986). Nonetheless, the dilution of starter culture more than 1,000 folds has never been reported. Therefore, it was interesting to investigate whether higher level of dilution or less amount of starter culture could be used for rhizobial inoculant production. In this study, the micro scale dilution technique of starter culture was performed by incorporation of syringe- and peristaltic pumps to conduct the diluent of starter culture by mixing with sterile water and injected directly to test the production of three different inoculant types, including liquid-, peat-, and encapsulation-inoculants of soybean bradyrhizobium. Therefore, the goal of this research was to invent an appropriate technique that could be applied further to produce high quality bradyrhizobial inoculants in large scale with low cost and high quality.

MATERIALS AND METHODS

1. Microorganism culture condition

Bradyrhizobuim diazoefficiens USDA110 was used throughout as a model in this experiment. *B. diazoefficiens* USDA110 was obtained from Department of Agriculture (DOA), Bangkok, Thailand. *B. diazoefficiens* USDA110 was grown in YEM-based medium (containing (g/l) 10.0 mannitol; 0.5 K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl; 0.5 yeast extract and pH 6.8) by shaking on rotary shaker with 180 rpm at room temperature (28-30ºC) for 6 days (cell growth at late log phase). Then, the culture was adjusted OD_{600} equal to 1.0 or 10^9 cells/ml before used as starter culture.

2. Preparation different types of inoculant and carriers

Liquid-based inoculant: YEM broth was added with 2% polyvinylpyrrolidone (PVP) (w/v) (Tittabutr et al., 2007) and adjusted the pH to 6.8 before packing the volume of 99 ml into 120 ml-plastic bottles. Peat-based inoculant: Peat was established a particle size 80-100 mesh. Then, peat was neutralized with $CaCO_3$ to obtain the final pH at 7.0. Additionally, the dried peat was added with 25% (v/w) of YEM broth, mixed well prior to pack 90 g in a low-density polypropylene bag. Encapsulated cell-based inoculant: The YEM broth added with 2% (w/v) sodium alginate and 0.75% (w/v) skim milk without Ca (Shcherbakova et al., 2018). Then, the pH was adjusted to 6.8. The solution was transferred into 1-liter flask. All of carriers were sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes and the peat inoculant 20 minutes for three time.

3. Preparation of starter culture and growth condition to investigate the lowest cell concentration for different types of inoculant production

The starter culture of USDA110 was manually diluted to be 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 cells/ml using 0.85% NaCl solution. Then, each diluted starter culture was inoculated into different types of inoculant as following. Liquid-based inoculant: 1 ml of each diluted starter culture was inoculated into 99 ml of sterilized YEM in the plastic bottle as described above to obtain the final concentrations of starter cell as 10, 10^2, 10^3, 10^4, 10^5, and 10^6 CFU/ml in the plastic bottle. The liquid inoculant was incubated at room temperature (28-30ºC) for 28 days without shaking. Peat-based inoculant: 1 ml of each diluted starter culture and 9 ml sterile water were injected into sterilized 90 g peat carrier as described above and mixed well by hands kneading to obtain the final concentrations of starter cell as 10, 10^2, 10^3, 10^4, 10^5,

and 10^6 CFU/g. The final moisture of peat-based inoculant was adjusted around 40% (w/w). Peat-based inoculant was incubated at room temperature (28-30ºC) for 28 days. Encapsulated cell-based inoculant: 1 ml of each diluted starter culture was inoculated into 99 ml of sterilized alginate encapsulated-based solution in the flask as described above. The final concentrations of starter cell as 10, 10^2, 10^3, 10^4, 10^5, and 10^6 CFU/ml and mixed well by shaking at 180 rpm for 30 min at room temperature. Then, the bacterial cell suspension was slowly dropped continuously using a syringe with needle size 18Gí1" (Nipro, Thailand) into the 250 ml flask containing 100 ml of sterilized 0.1 M $CaCl_2$ solution. The flask was interval shaken by hands during dropping to avoid the agglomeration of beads and left the solution at room temperature for 30 min (Shcherbakova et al., 2018). Then, beads were rinsed 4 times with sterilized normal saline and the wet beads were transferred into fresh YEM broth to continue culturing for 7 days by shaking at 180 rpm, 28-30ºC. At 7 day after inoculation (dai), the wet beads were rinsed again with sterilized normal saline before packaging into a low-density polypropylene bag (100 g/bag) and kept at room temperature until 28 dai. The number of cells from each type of inoculant was determined at 0, 7, 14, 21, and 28 dai by serial dilution and total plate count on YEM-Congo Red medium. The lowest cell number of starter culture that could grow and increase the number of cells to 10^8 cells/unit was selected for inoculant production in next step.

4. Dilution of starter culture with the application of syringe- and peristaltic-pumps for inoculant production

Liquid-based inoculant: Step one: the syringe pump (No.1) was operated to inject 1 ml of starter culture USDA110 at 10^9 CFU/ml into 1 liter bottle containing 999 ml of sterilized normal saline solution to obtain the final cell number at 10^6 CFU/ml. Step two: the syringe pump (No.2) and the peristaltic pump (No.3) were operated to inject 10 µl (10^6 CFU/ml) of bacterial culture and 990 µl of sterilized water, respectively as a mixture before injecting into 125 ml bottle containing 99 ml of sterilized YEM broth with 2% PVP (Figure 1). Peat-based inoculant: Step one: the syringe pump (No.1) was operated to inject 0.1 ml of starter culture USDA110 at 10^9 CFU/ml into 1 liter bottle containing 999.9 ml of sterilized normal saline solution to obtain the final cell number at 10^5 CFU/ml. Step two: the syringe pump (No.2) and the peristaltic pump (No.3) were operated to inject 0.1 ml (10^5 CFU/ml) of the bacterial culture and the 9.9 ml of sterilized water, respectively as a mixture of 10 ml before injecting into 90 g of sterilized peat packed in a low-density polypropylene bag total 100 g (Figure 1).

Encapsulated cell-based inoculant: Step one: the syringe pump (No.1) was operated to inject 0.1 ml of starter culture USDA110 at 10^9 CFU/ml into 1 liter bottle containing 999.9 ml of sterilized normal saline solution to obtain the final cell number at 10^5 CFU/ml. Step two: The diluted bacterial suspension from the 1st step was flowed through the syringe pump (No.2) by adjusting the flow rate at 10 µl/min to mix with sterilized alginate encapsulated-based solution which continuously flow at 1 liter through the peristaltic pump (No.3). The mixture of bacterial suspension was slowly dropped continuously into the sterilized 0.1 M $CaCl_2$ solution and the beads were transferred into fresh YEM broth and continue culturing for 7 days as described above. Then, the beads were packed in a low-density polypropylene bag (Figure 1). The final population numbers of bacterial cell in all inoculants were theoretically expected to be 10 CFU/unit. In this experiment, the cell numbers in all types of inoculant were determined at 0 and 30 dai as mention above.

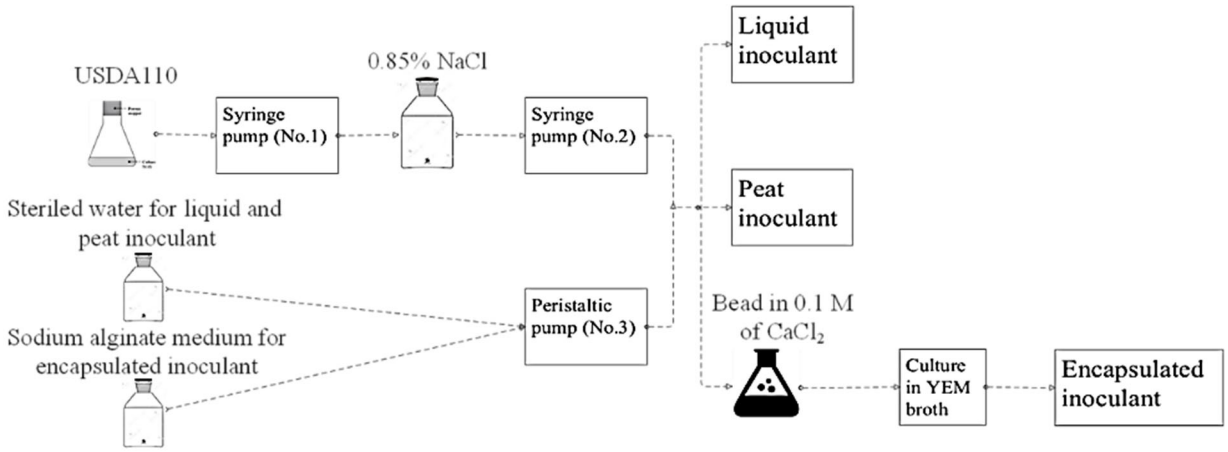

Figure 1. Model of inoculant production from pump by diluted starter into liquid, peat and encapsulated inoculants.

5. Investigation of inoculant shelf-life when store at room temperature

All types of inoculant were produced again as described above and then kept at room temperature 28±lºC for 3 months. The number of a living cells in the inoculant was determined at 0, 30, 60, and 90 dai by most probably number (MPN) with plant (soybean: *Glycine max*) infection method (Somasegaran, 1985). The inoculants were diluted with 10-fold dilution series using normal saline as diluent. Each 1 ml of diluted inoculants was inoculated into a seedling of soybean germinated in the pouch by aseptic technique. Soybean growth conditions in the lightroom were a 16-h-day/ 8-h-night cycle at 28ºC/23ºC. The plants were watered with N-free nutrient solution during the experiment at 3 weeks. The experiments were set up with 5 replicates for each treatment. The data were collected as the number of plants nodulated by USDA110.

6. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and the significance of mean values were tested at a 5% significance level by Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

1. Minimal cell number of starter culture could be used for producing inoculant

To determine a lowest cell number of starter culture that could be used for inoculant production, six levels of cell concentration starting from 10 to 10^6 CFU/unit were used to produce liquid-, peat-, and encapsulated cell-based inoculants. The inoculants were done significantly decreased according to different number of starter cultures. The result showed that bradyrhizobial cells at all starting concentrations could grow well in the bottle although without shaking (Figure 2a). This probably due to in the bottles containing bacterial suspension had air space 25 ml, the remained oxygen in the bottle might be properly supplied for bradyrhizobium growth. This was in accordance to the report from (Shakhawat and Mårtensson, 2007) who suggested that bradyrhizobium could grow under microaerophilic condition at less than 0.01 atm. Higher cell numbers of starter culture resulted in increasing the bacterial cell number more than that of low concentration starter number. Inoculant with starter cultures at 10 and 10^2 CFU/ml were contained cell number around 10^7 CFU/ml at 7 dai, while inoculant with starter cultures at 10^4, 10^5, and 10^6

CFU/ml were contained the cell number only 10^8 CFU/ml at 7 dai. Interestingly, there were no significant differences of cell number among all treatments at 14 dai. The cell number of all treatments were more than 10^8 CFU/ml and remained at this level until at 28 dai. This finding confirmed that the low cost of inoculant could be possible. *B. japonicum* USDA110 using of starter culture 10^5 CFU/ml could grow the cell population number more than 10^8 CFU/ml in additives YEM-broth (Tittabutr et al., 2007). Mohamed et al. (2019) reported that supplementation of the polymer additives could support cell growth of Rhizobial strain USDA 3100 as high as 10^9 CFU/ml in liquid inoculant.

In case of peat-based inoculant, the number of cells in peat increased slower than that of liquid inoculant. This might be due to nutrient-, and oxygen limiting condition during storage making thicken cell wall and altered protein production (Dart et al., 1969; Feng et al., 2002). Although high concentrations of starter culture were used, the number of cell increased less than 10^8 CFU/g at 7 and 14 dai (Figure 2b). However, the cell number in peat reached more than 10^8 CFU/g at 21 dai. There were no significant differences of bacterial cell number in peat produced from high or low concentration of starter cultures. Therefore, starter culture at 10 CFU/g could also be used for peat-based inoculant production. It has been reported that starter cultures of *B. japonicum* and *Rhizobium phaseoli* were diluted 10^6, 10^7, or 10^8 cells/ml that were injected to sterile peat the cells could grow as 10^9 CFU/g at 7 dai (Somasegaran, 1985).

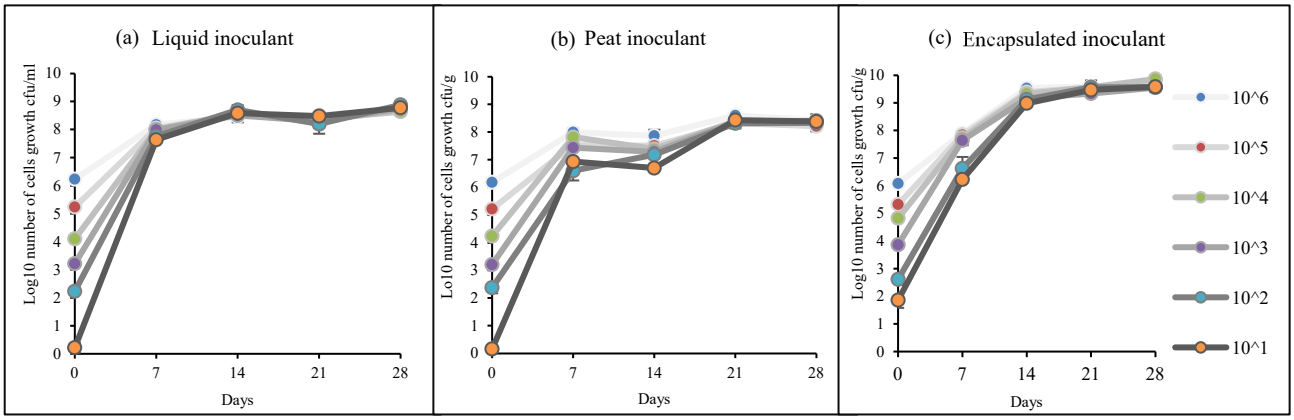

Figure 2. (a) The cell number of *B. diazoefficiens* USDA110 in liquid inoculant; (b) peat-based inoculant; (c) Encapsulated inoculant manually produced from different concentrations of starter culture.

For alginate encapsulation cell-based inoculant, the process of inoculant production required a step of further culturing the encapsulated cells in the liquid medium for 7 days after inoculation to form beads. It was found that using concentration of starter cultures of 10^3 to 10^6 CFU/g could reach the number of cell more than 10^7 CFU/g in encapsulation cell-based inoculant at 7 dai. The numbers of cell all were significantly higher than that of using low concentration of starter culture at 10 and 10^2 CFU/g (Figure 2c). At 14 dai, the number of cell in all treatments except from the starter culture at 10 CFU/g reached more than 10^9 CFU/g which higher than the number of cells in liquid- and peat-based inoculants. However, the number of cell in encapsulated inoculant produced from 10 CFU/g starter culture reached more than 10^9 CFU/g at 21 dai and remained at this high number of cells at 28 dai. The encapsulated beads might have large area surfaces that

can compact high cells density in bead form and results in increase the number of cell more than 10^9 CFU/g. The immobilized *Azospirillum brasilense* starter culture was concentrated cell as 10^{11} CFU/g in beads that was grown in fresh TYG medium for increasing cell as 10^{12} CFU/g (Bashan et al., 2002). The advantages of using alginate microspheres were the controlled release of bacteria, slow biodegradation, and extend shelf life. However, 10-42% of immobilized *Mesorhizobium ciceri* ST-282 cell was leakage after 24 h (Shcherbakova et al., 2018). Based on these data, it was confident that the starter culture at concentration of 10 CFU/unit could be used for producing with these three types of inoculant production.

2. Application of syringe- and peristaltic-pumps for inoculant production with low concentration of starter culture

The syringe- and peristaltic-pumps were incorporated into the system in order to reduce the volume and cell concentration of starter culture for inoculant production (Figure 3a). The first syringe pump was operated to feed small volume of starter culture into diluent. Then, the second syringe pump fed the diluted starter culture to mix with more diluent feeding from peristaltic pump which control the injection of very low concentration of cell into package. Based on this operation, the starter culture was diluted to 10^7 folds from initial concentration. The initial cell concentration was expected to be 10 CFU/unit in the inoculant. However, the concentrations of initial cells were 8.66×10^1, 6.66×10^1, and 5×10^1 CFU/unit in liquid-, peat-, and alginate encapsulated cell-based inoculants, respectively (Figure 3a). Then, the concentration of cell increased after incubated at room temperature to 4.51×10^8, 5.6×10^8, and 2.24×10^9 CFU/unit in liquid-, peat-, and alginate encapsulated cell-based inoculants, respectively at 30 dai. The number of cells from all methods meet the standard of commercial inoculant of biofertilizer (Malusá and Vassilev, 2014). Therefore, this operating process could be used for large scale inoculant production without preparation of large volume of starter culture. However, the process of incubation for 14 to 21 days is required to increase the number of cells. In case of encapsulated cell-based inoculant, there were many steps of operation. The peristaltic pump was required to control the dropping speed of cell into $CaCl_2$ solution to form the beads. Thus, the encapsulated bead inoculant in this process is appropriate with a laboratory scale. Previously, the dilution of starter culture at 10^3-fold has been used to inoculate into sterile peat bags using injection pump for a small-scale commercial inoculant production (Kannaiyan, 2002). The dilution technique economically reduced the fermenter scale and also other accessories machines needed for growing rhizobia (Somasegaran, 1985). A standard pilot-scale fermenter establishing for the production of *Rhizobium* biofertilizer was reported to invest at least 5,000 USD (Sethi and Adhikary, 2012). Therefore, the large investment in fermenter could be reduced. Based on the operating process in this study, the low volume of starter culture could effectively increase the cell number in inoculant similar to that of using large scale fermenter.

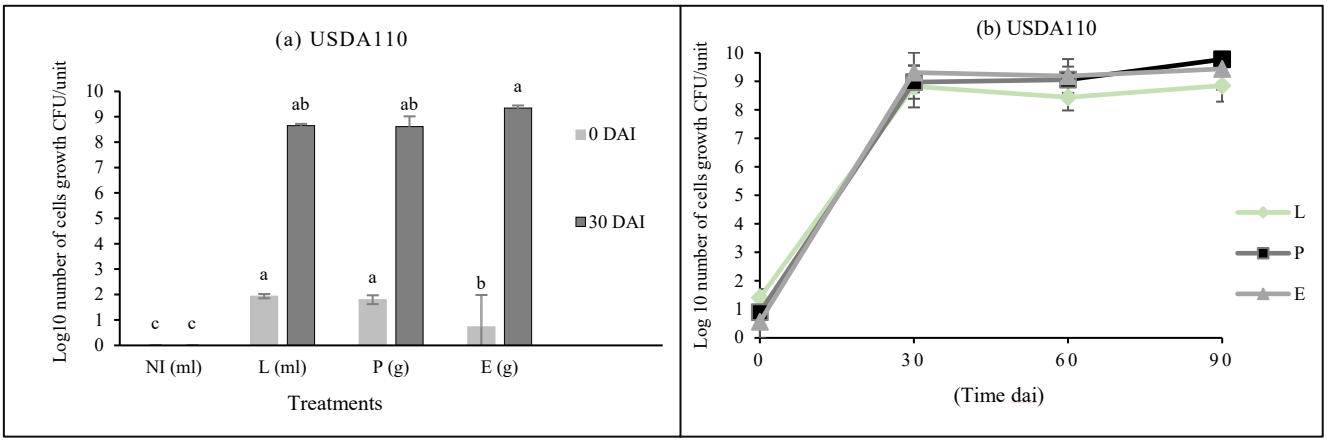

Figure 3. (a) The *B. diazoefficiencs* USDA110 inoculant production by pumps, (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)); (b) The cell survival number of *B. diazoefficiencs* USDA110 inoculants in liquid (L), peat (P), and encapsulated bead (E) after storage 0, 30, 60 and 90 days. Significance at $p \leq 0.05$ is indicated by mean standard error bars.

3. Shelf-life of B. diazoefficiens USDA110 inoculant

The survival of bradyrhizobium in each type of inoculant was demonstrated in Figure 3b. The number of survival cell was more than 10^8 CFU/unit in liquid- and peat-based inoculants when determined by plant infection-MPN technique. Interestingly, the number of survival cell in alginate encapsulated cell-based inoculant was higher than 10^9 CFU/g at 3 months storage. Saiprasad (Saiprasad, 2001) reported that sodium alginate was the most accepted hydro-gel, low toxicity, low cost, quick gelation, and biocompatibility characteristics which is suitable for cell encapsulation. However, it has been reported that the alginate granules cells of *B. japonicum* M8 at initial concentration of 10^9 CFU/g were reduced at 3 months of storage (Shcherbakova et al., 2018).

In peat-based inoculant, cells were grown up to 10^8 CFU/g after stored at 30 dai. Then, cells were increased from 10^8 to 10^9 CFU/g at 60 dai and maintained the survival cells at 10^9 CFU/g at 90 DAI (Figure 3b). Although the nutrients in peat carrier was slowly degraded to support rhizobial cells growth, it is enough to promote cells increasing from 10 CFU/g to 10^9 CFU/g in the carriers during storage time. It has been reported that the initial cells start from 10^{10} CFU/g in peat could maintain cell densities until 120 days (Albareda et al., 2008). Einarsson et al. (1993) postulated that peat contained small nutrient-supplemented of pumice that supports bacteria growth. Moreover, rhizobial cell could be immobilized in peat carrier and the low moisture content in peat affected cells survival (Žvagiņa, 2015). The suitable moisture content in peat for maintaining the shelf-life of *Bradyrhizobium* is 30-50%, and when the moisture increased up to 50%, fungi contamination was observed (Temprano, 2002). Although, the lower moisture content in peat reduces the contaminant in rhizobium inoculant at 1 CFU of contaminants per 3.28×10^3 CFU/g of rhizobia, non-steriled peat which contain high contaminants had cells number at 10^8 CFU/g in peat inoculant after 12 weeks at 26°C (Roughley and Vincent, 1967). Moreover, the optimization temperature that could maintain of rhizobium inoculants was better at 4°C in comparison to 28°C (Daza et al., 2000).

The lowest survival of cell was found in liquid-based inoculant. Although the liquid inoculant

containing PVP, it could protect cells survival at 10^8 CFU/ml for 3 months when stored at room temperature (Figure 3b). However, liquid inoculant is easy to prepare and contain high nutrient (Albareda et al., 2008). The *B. japonicum* in G6+PVP medium could maintain 10^9 CFU/ml period storage at 25ºC for 180 dai (Singleton et al., 2002), and the *B. japonicum* USDA3100 was cultured in YEM-broth+2% PVP that prolonged shelf-life as 10^8 CFU/ml during storage at 25-35ºC for 60 dai (Mohamed et al., 2019).

CONCLUSIONS

A large scale of bradyrhizobium inoculants could be produced using a small amount of starter culture. The varied small amount of starter culture from 10 to 10^6 CFU/unit could grow and increase the cell population number up to 10^8 CFU/unit in liquid-, peat-, and encapsulation-inoculants. Moreover, the 10 CFU/unit of starter culture can be used in the pilot-scale for three types of bradyrhizobial inoculants. The syringe- and peristaltic pump were incorporated into the system of micro-injection starter culture at 10 CFU/unit could produce liquid-, peat-, and encapsulated inoculant. Additionally, the pump pilot-scale model that could be operated on a large scale of liquid-, and peat inoculant, but were not appropriate for a large scale of encapsulated inoculant, for the reason that the pumps were slowly dropping of sodium alginate solution to form the bead that could be produced small scale of encapsulated inoculant. The shelf-life of most inoculants product by pump model could maintained the cell number of inoculants at least for 3 months with the cell population number more than 10^8 CFU/unit. This is the first demonstration of the diluent with a very low amount of starter culture as 10 CFU/unit that could grow and increase cell number at 10^8 CFU/unit in different types of inoculant. Base on this process, 1 ml of starter culture could able to inoculate into 100,000 packages of liquid-, peat-, and encapsulated-inoculant. Thus, this process could be applied further for the simple and low-cost high-quality inoculant production.

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